EFFECTOR CD4$^+$ T LYMPHOCYTES
IN THE PRODROME OF POLYARTHRITIS

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Declaration

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Abstract

The inoculation of a single dose of Complete Freund's Adjuvant (CFA) in Dark Agouti rats induces a polyarthritis that resembles rheumatoid arthritis (RA) in humans. The specific role of T cells in the pathogenesis of RA is controversial but a considerable body of evidence suggests that the joint destruction is mediated by T cells within the synovium, which may activate other cells such as macrophages and fibroblasts that are more directly implicated in articular damage. To gain a further understanding of the mechanisms involved in the development of T cell-mediated polyarthritis, the activation and phenotype of CD4$^+$ T cells during the prodrome of adjuvant-induced arthritis (AA) has been investigated in rats.

Research in the Arthritis Research Laboratory has demonstrated previously that activated CD4$^+$ T cells in the thoracic duct (TD) lymph of rats during the late prodromal phase of AA (9 days after inoculation) have the capacity to enter both normal and inflamed joints after the adoptive transfer to syngeneic recipients. Furthermore, these activated CD4$^+$ T cells can transfer disease to naive recipient rats. The arthritogenic population is contained within a subset of CD4$^+$ T cells that expresses CD25, MHC class II, CD134 and CD71.

The delay in onset of AA after inoculation with adjuvant (9-10 days) or adoptive transfer of arthritogenic TD lymphocytes (4-6 days) suggests that progressive differentiation or selection of effector cells is required before disease can be expressed. This study has charted the emergence of activated and arthritogenic CD4$^+$ T cells in the inguinal and popliteal lymph nodes draining the site of inoculation, in the TD lymph and in the joints of the hind paws. In the lymph nodes and TD lymph, the proportion of CD4$^+$ T cells expressing activation markers such as CD25, MHC Class II, CD134 and CD71 and adhesion molecules such as ICAM-1 increased within 3 days following inoculation of CFA and these levels remained elevated throughout the early stages of AA. Arthritogenic lymphocytes were present in the TD lymph by the fourth day after inoculation of CFA. Interestingly, the disease transferred to recipients by lymphocytes from a donor on the fourth day post-inoculation followed similar kinetics to that transferred by TD lymphocytes harvested from donors at later time points. Very few CD4$^+$ T cells were detected in the hind paws during the prodrome of AA, whereas a dramatic influx was observed by day 9 and even more so at day 12 post-inoculation, when joint inflammation

was usually moderate to severe. These CD4$^+$ T cells in the inflamed hind paws had an effector phenotype.

A method was developed for detecting the cytokine production by individual T lymphocytes under conditions that reflected the cytokine production by these cells *in vivo*. This technique revealed that CD4$^+$ T cells from arthritic rats produced more interferon (IFN)-γ than interleukin (IL)-4, suggesting that this disease was mediated by T helper type –1 cells. CD4$^+$ T cells from inflamed joints were prolific producers of IFN-γ, suggesting that this pro-inflammatory cytokine may have played a crucial role in disease pathology. However, when a monoclonal antibody was used to block IFN-γ produced by either transferred arthritogenic lymphocytes or host cells in the active disease, the arthritis was markedly exacerbated, indicating that this cytokine also has down-regulatory effects at an important stage during the development of the inflammatory response.

Publications

Abstracts and conference presentations

Poster presentation by M. Brasted at the 27th Annual Conference of Australasian Society for Immunology, Perth, 1997.

M. Brasted, L. G. Cleland and G. Mayrhofer

"Effect of dose of killed *M. tuberculosis* on induction of polyarthritis in female Dark Agouti rats"

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"Cytokine production and surface phenotype of $CD4^+$ T cells during the pathogenesis of adjuvant-induced arthritis"

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M. Brasted, L. G. Cleland and G. Mayrhofer

"Cytokine production and surface phenotype of CD4+ T cells during the pathogenesis of adjuvant-induced arthritis"

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"Effector CD4+ T cells in a rat model of polyarthritis"

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"*In vivo* administration of monoclonal antibody against interferon-γ exacerbates adoptively-transferred arthritis"

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"The role of T cells in experimentally-induced polyarthritis"

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"Monoclonal antibody DB-1 against interferon-gamma exacerbates adoptively-transferred arthritis"

Scientific Publications

M. Brasted, S. J. Wing, L. D. J. Spargo, L. G. Cleland and G. Mayrhofer. "Generation of arthritogenic T lymphocytes in experimentally-induced polyarthritis". In preparation.

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Abbreviations

AA	Adjuvant-induced arthritis
APC	Antigen presenting cell
CD	Cluster of differentiation
CFA	Complete Freund's Adjuvant
CHO	Chinese Hamster Ovary
CIA	Collagen-induced arthritis
Con A	Concanavalin A
DA	Dark Agouti
DC	Dendritic cells
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
FITC	Fluorescein isothiocyanate
HEV	High endothelial venule
HSP	Heat shock protein
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
LFA	Leukocyte function antigen
LN	Lymph node
mAb	Monoclonal antibody
MHC	Major histocompatibility
MT	*Mycobacterium tuberculosis*
NO	Nitric oxide
PBL	Peripheral blood leukocytes
PCR	Polymerase chain reaction
PMA	Phorbol myristate acetate
PSGL-1	P-selectin glycoprotein ligand 1
RA	Rheumatoid arthritis
rIFN-γ	Recombinant IFN-γ
RNA	Ribonucleic acid
RT	Reverse transcription
SF	Synovial fluid
ST	Synovial tissue
TCR	T cell receptor
TD	Thoracic duct
TDL	Thoracic duct lymph
TGF	Transforming growth factor
Th	T helper
TNF	Tumour necrosis factor
w/v	Weight per volume

CHAPTER 3: THE DETECTION OF INTRACELLULAR CYTOKINES BY FLOW CYTOMETRY

CHAPTER 5: ACTIVATED CELLS IN THE EFFERENT LYMPH OF RATS IN THE PRODROME OF POLYARTHRITIS, WITH PARTICULAR REFERENCE TO CD4$^+$ T LYMPHOCYTES

CHAPTER 6: PHENOTYPE OF CD4$^+$ T LYMPHOCYTES ISOLATED FROM THE HIND PAWS OF RATS DURING THE PRODROME OF ADJUVANT-INDUCED ARTHRITIS

CHAPTER 1

INTRODUCTION

1.1 The immune system
1.1.1 Introduction

The immune system is comprised of both cellular and molecular mechanisms that enable organisms to respond to invasion by foreign organisms and tissue injury. These responses can be considered generally to be a consequence of either innate or acquired immunity, or a combination of the two. Innate immunity includes physical and physiological barriers, phagocytic cells in the blood and tissues, natural killer cells and non-specific soluble factors in the blood and other bodily fluids. Acquired immunity is the result of defence mechanisms that mediate responses in an antigen specific manner. In part, acquired or specific immunity utilises components of innate immunity in a more focussed way.

The tissues in which lymphocytes, the cells that mediate specific immunity, develop are the primary lymphoid organs. Primary lymphoid organs are generative in the sense that they are the sites where lymphocytes develop from undifferentiated precursors and express antigen-specific receptors (reviewed in Ikuta et al., 1992). Bone marrow, the thymus, the Bursa of Fabricius in birds and foetal liver are considered to be primary lymphoid organs. Secondary lymphoid organs are the sites where mature lymphocytes respond to foreign antigens. The secondary lymphoid organs include the spleen, lymph nodes and mucosa-associated lymphoid tissues. The secondary lymphoid tissues are interconnected by the ability of lymphocytes to recirculate, using the blood and lymphatics (reviewed in Trnka and Cahill, 1980).

1.1.2 Autoimmunity

Autoimmunity is an immune response against self (autologous) antigens. Inappropriate immune responses against autoantigens can result in tissue damage and autoimmune disease. There are thought to be multiple mechanisms responsible for the loss of self-tolerance and the onset of autoimmunity (reviewed in Heath et al., 1997).

In simple terms, the development of a protective immune system is thought to involve 3 important stages: the discarding of useless (non-reactive) lymphocytes, the retention of useful lymphocytes (positive selection) and the destruction of autoreactive lymphocytes (negative selection) (von Boehmer et al., 1989). During the development of lymphocytes into mature B or T cells, there is rearrangement and expression of antigen receptor genes. For B lymphocytes, these selective events occur in the bone marrow, while in the case of

T lymphocytes, selection occurs in the thymus. Negative selection of autoreactive T cells is, therefore, dependent on the presentation of autoantigens in the thymus (reviewed in Hanahan, 1998). The elimination of developing lymphocytes when they encounter self-antigen results in 'central tolerance'. For those self-antigens that are not represented during lymphocyte maturation, other mechanisms must be responsible for acting on mature lymphocytes to maintain self tolerance and these are referred to as 'peripheral tolerance'. The details of how autoreactive T cells are regulated in extrathymic tissues is not well understood (reviewed in Heath et al., 1997; Van Parijs and Abbas, 1998).

It is clear that autoreactive T lymphocytes can escape deletion and that they remain unresponsive in healthy individuals. However, these potentially autoreactive cells can become activated and initiate autoimmune diseases. The activation of otherwise unresponsive autoreactive T lymphocytes could occur via several mechanisms, such as: the loss of suppressive elements (such as regulatory T lymphocytes), the exposure of previously concealed antigens (tissue injury is an example of how this may occur) or the expansion of autoreactive T cells in response to infectious agents that express antigenic epitopes that cross-react (molecular mimicry) with self-antigens (Elson et al., 1995). Matzinger (1994) has hypothesised that T cells (including those with specificity for autoantigens) will not respond to antigens presented by antigen presenting cells (APC) unless the APCs are responding to "danger" signals such as interleukin (IL) –1, which they communicate to the T cells by cognate interactions or secreted-messengers. Such danger signals may arise through tissue injury or the products of invasive microorganisms.

There is substantial evidence that the maintenance of tolerance is an active process (reviewed in Fowell et al., 1991; Van Parijs and Abbas, 1998). Immunosuppression induced by cyclosporin A administered for a week to newborn mice causes autoimmune diseases such as thyroiditis, gastritis, insulitis, adrenalitis and oophoritis, which are mediated at least in part by antibodies against specific antigens in multiple organs. The organ destruction can be prevented by the transfer of splenic T cells from normal syngeneic donor mice (Sakaguchi and Sakaguchi, 1989). Thymectomy of 3 day old mice also results in autoimmunity against multiple organs, which can be inhibited by the transfer of CD4^{+} T cells from age matched euthymic mice (reviewed in Bonomo et al., 1995). Fowell and colleagues have provided evidence to suggest that prevention of

autoimmunity occurs via a process of active tolerance and that there is a balance between autoreactive cells and antagonists that prevent their actions. It is thought that this balance is lost in autoimmune diseases (Fowell et al., 1991). Regulatory T cells are generated spontaneously during normal development and they can be activated in response to antigen presentation in particular microenvironments such as the lymphoid compartments of the gut (Mason and Powrie, 1998). Autoimmune diseases can involve B and/or T lymphocytes with specificity for autoantigens, although loss of tolerance in the T cell compartment is sufficient to allow expression of both B cell and T cell-mediated autoimmune disease. Many of the current forms of therapy for autoimmune diseases are based on systemic immunosuppression and as a consequence have detrimental side effects such as an inability to respond to infectious agents.

Autoimmune diseases are thought to be a consequence of both genetic and environmental factors. The genetic factors that contribute to autoimmunity are complex and probably polygenic, although in mice autoimmune disease can be induced by defects of single genes, particularly those involved in homeostasis of the immune system such as CTLA-4 (reviewed in Van Parijs and Abbas, 1998). Although population and family studies have shown that genetic factors are the most significant influence contributing to predisposition to autoimmune disease, there are considerable discordance rates between monozygotic twins, indicating that environmental factors are also involved (reviewed in Theofilopoulis, 1995).

There is evidence to suggest that some autoimmune diseases are triggered by bacterial or viral infections. For example, psoriasis can be triggered by infection with group A β-haemolytic streptococci. It is thought that the disease is perpetuated by CD4$^+$ T cells that react with both streptococcal M protein and an antigen expressed by skin cells (possibly keratin) (Valdimarsson et al., 1995). In addition to the expansion of autoreactive T cells that cross-react with bacterial or viral epitopes, infectious agents may trigger autoimmunity by other mechanisms. Infection, activating the innate immune system, induces the production of many inflammatory mediators. Some of these may activate autoreactive T cells that may have been inactive as a consequence of anergy or active suppression (O'Garra and Murphy, 1993). The chronic inflammation that can result from bacterial or viral infections may create an environment that leads to the inappropriate

presentation of self-antigens by activated dendritic cells to auto-specific T cells (Di Rosa and Barnaba, 1998). This is supported by the observation that recognition of antigen by T cells *in vivo* in the presence of inflammation caused by activated macrophages induces T cell proliferation and continued responsiveness to antigen whereas exposure in the absence of inflammation, induces anergy or deletion of these T cells (reviewed in Mueller and Jenkins, 1997).

The cytokine (see below Section 1.4) milieu during an immune response is likely to be a crucial factor in determining whether autoimmune disease ensues. The cytokine networks involved in autoimmunity (as well as other immune responses) are complex and differ according to relative amounts of the cytokines present. In T cell-mediated autoimmunity, interleukin (IL)-4 is considered generally to be protective whereas interferon (IFN)-γ is thought to promote the disease process (Falcone and Sarvetnick, 1999) although IL-4 can be involved in triggering autoimmune diabetes (Falcone et al., 2001). However, the cytokine networks involved in autoimmunity are not straightforward. For example, the development of $HgCl_2$-induced autoimmunity, which features autoantibody production and gammaglobulinaemia, is dependent on IFN-γ rather than IL-4. This is surprising since IL-4 is important for differentiation and isotype switching of B cells (Kono et al., 1998).

The understanding of autoimmune diseases has been advanced by the use of animal models of certain diseases, such as experimental autoimmune encephalomyelitis as a model of multiple sclerosis. There are several animal models of rheumatoid arthritis (RA) that are discussed in Section 1.8.

1.2 Lymphocytes

1.2.1 Introduction

Lymphocytes are mononuclear immune cells that mediate specific responses to antigens. Precursor cells that arise in the bone marrow develop into mature, naïve T lymphocytes in the thymus or mature, naïve B lymphocytes in the bone marrow. If these naïve lymphocytes interact with their cognate antigen in the appropriate context in a secondary lymphoid organ, they undergo activation, proliferation and differentiation that equips them with either memory or effector functions. The activation and differentiation of T lymphocytes is discussed below in Section 1.2.2. B lymphocytes can recognise epitopes

on native antigens via the B cell receptor (surface immunoglobulin [Ig]). T lymphocytes express a structurally similar antigen receptor, the T cell receptor (TCR), which allows the recognition of processed antigenic peptides in the context of major histocompatibility complex (MHC) molecules on the surface of antigen presenting cell (APC)s. The interaction between the TCR and MHC is stabilised by CD4 or CD8, which bind to MHC following engagement by the TCR on the surface of the T cell (reviewed in Davis et al., 1998). These molecules also recruit the receptor tyrosine kinase lck. Efficient signalling via the TCR also requires the invariant chains CD3 γ, δ and ϵ and the ζ:ζ or the ζ:η dimers and CD45 (reviewed in Janeway, 1992).

Mature T cells can be divided into subsets based on the expression of either the α/β or γ/δ TCR. Most γ/δ T cells express CD8, whereas mature α/β T cells can be divided further into subsets that express either CD4 or CD8. CD4 binds to a non-polymorphic region of MHC class II and thus CD4$^+$ T lymphocytes are restricted to recognising antigenic peptides presented only by MHC class II molecules and likewise, CD8$^+$ cells are MHC class I restricted (reviewed in Swain, 1983). CD4$^+$ T cells are often referred to as "helper T cells" based on the ability of activated CD4$^+$ T cells to provide help to B cells to undergo differentiation into antibody-secreting cells. CD4 plays an important role in the formation of the immunological synapse and intracellular signalling (Vidal et al., 1999). CD8$^+$ T cells are often called "cytolytic T cells" or "cytotoxic T cells", in reference to their capacity to induce lysis of target cells by the intra-cytoplasmic deposition of effector molecules such as perforin. However, these terms are generalisations and there is some overlap in functions between the subsets. For example, in some disease states CD4$^+$ T cells produce perforin and have cytotoxic activity and CD8$^+$ T cells are recognised as having immunoregulatory roles in addition to cytotoxic function. T cells can be divided into subsets according to the expression of surface molecules other than those mentioned above and the production of cytokines and these subsets are discussed later.

1.2.2 T lymphocyte activation

Naïve T lymphocytes require 3 coordinated stimuli to become activated functionally: (a) recognition of their cognate antigenic peptide in context of MHC molecules, (b) co-stimulatory signals and (c) an appropriate milieu of cytokines (reviewed in Matzinger, 1994; Curtsinger et al., 1999). Upon activation, T lymphocytes undergo a series of

differentiation and maturation events that allow the cells to exert effector functions. The effector function of the activated T lymphocytes is determined by the nature of the stimulatory components. The recently- activated T lymphocytes enter the G_1 stage of the cell cycle and become 1.5 to 3 times larger than a resting cell. Such cells are termed "lymphoblasts" and have other characteristic features including a high content of RNA (and numerous polyribosomes), relatively little organised endoplasmic reticulum and vigorous motility (Delorme et al., 1969). Lymphoblasts progress into the S phase of the cell cycle and divide.

Dendritic cell (DC)s are APCs with the specialist function of initiating T cell responses. Immature DCs residing in the sites of antigen entry are able to take up antigen but are unable to activate naïve T cells until they have undergone maturation and have migrated to secondary lymphoid organs (Romani et al., 1989; Streilin and Grammer, 1989; reviewed in Lane and Brocker, 1999). Chemotactic cytokines (chemokines) are induced at sites of injury and attract immature DCs. In contrast, mature DCs are rendered insensitive to such chemokines by the down-regulation of particular chemokine receptors (Dieu-Nosjean et al., 1999) and are sensitive to a different range of chemokines produced in secondary lymphoid tissues (Cyster, 1999). DCs produce chemokines in the secondary lymphoid organs that direct the movement of T cells and B cells to facilitate their interactions and can be considered to govern the immune response to a newly-encountered antigen (reviewed in Hart, 1997; Lane and Brocker, 1999).

Naïve T lymphocytes interact with DCs in the T cell-rich areas of secondary lymphoid tissues (reviewed in Jenkins et al., 2001). While the lifespan of newly-arrived DCs in lymph nodes is approximately 48 hours, antigen specific $CD4^+$ T lymphocytes proliferate and their greatest numbers are observed 4 days after injection of antigen (Ingulli et al., 1997). Exposure of naïve T cells to APCs for longer than 2 days *in vitro* results in a decrease in the expansion of the effector cells and an increase in T cell death. The expansion of the effector cells is thought to be driven by interleukin (IL) –2 and is independent of constant exposure to antigen (Jelley-Gibbs et al., 2000). Kelso and colleagues showed that when mice were immunised with keyhole limpet haemocyanin (KLH), clonogenic KLH-specific $CD4^+$ T cells became apparent in the draining lymph nodes after 3 to 4 days. It seems reasonable to presume that during this time there was

recruitment of their naïve precursors, activation by professional APCs and clonal expansion of the KLH-specific CD4$^+$ T cells (Kelso et al., 1994).

Following activation by DCs, the CD4$^+$ T lymphocytes migrate to the margins of B cell follicles, where they are able to interact with B cells and provide help for a humoral immune response (Garside et al., 1998). However, other activated T cells leave the lymph nodes and are found in the efferent lymph (Hall and Morris, 1963).

An important feature of the immune system is long-lived memory. Upon exposure to antigen that has been encountered previously, B and T cells will respond more rapidly and with increased affinity compared to the initial encounter. This is a consequence of clonal expansion during priming, selection of higher affinity clones and heightened sensitivity of the memory cells to antigens (reviewed in Mackay, 1993a; Sprent, 1997). Naïve cells have more stringent requirements for co-stimulation than antigen-experienced CD4$^+$ T lymphocytes (Croft et al., 1994; Dubey et al., 1995) but they do not differ in their capacity to proliferate. They differ in their ability to produce cytokines (Constant et al., 1994). Furthermore, antigen-experienced T lymphocytes can be activated in response to B cells, whereas naïve T lymphocytes are rendered tolerant in response to antigen presentation by B cells and they can only be activated by professional APCs, such as DCs (Fuchs and Matzinger, 1992).

DCs provide the co-stimulation that is necessary for the initiation of a T cell response. The co-stimulatory pathway involving CD28 and the B7 family of proteins is relatively well characterised and it plays a key role in regulating T cells responses. T cells express CD28, which binds to either B7-1 (CD80) or B7-2 (CD86) on APCs, which provide co-stimulation. However, there are other co-stimulatory molecules such as cytotoxic T lymphocyte antigen 4 (CTLA-4, CD152), inducible co-stimulator (ICOS), Leukocyte function antigen (LFA)-1 and several members of the tumour necrosis factor (TNF) receptor (R) family (CD137, CD134 and CD27) and for this reason, T cell-mediated responses can be independent of CD28 (reviewed in Watts & DeBenedette, 1999). CTLA-4 is a member of the CD28 family and it binds to the B7 molecules with higher affinity than CD28. However, unlike CD28, the ligation of CTLA-4 provides a negative signal and it is thought to be important in down-regulating T cell responses (reviewed in Oosterwegel et al., 1999). Intercellular adhesion molecule (ICAM)-1 (CD54) and B7-1

can individually co-stimulate naïve T cells and are synergistic in combination (Dubey et al., 1995).

The transferrin receptor (CD71) is a type II membrane glycoprotein that is the receptor for transferrin, the major iron carrying protein. CD71 exists as a homodimer that associates non-covalently with the TCR ζ chain in T cells, where it is thought to play a role in signal transduction (Salmeron et al., 1995). CD71 is expressed at low levels on resting leukocytes but it is up-regulated after activation and plays a critical role in cell proliferation (Testa et al., 1993). The expression of CD71 on lymphocytes is considered to be a marker of the early stages of activation.

1.2.3 Lymphocyte recirculation

The continuous recirculation of lymphocytes from blood to lymph provides a mechanism for increasing the likelihood that naïve cells will encounter their cognate antigen, thus enabling the immune system to maintain a large repertoire for diverse antigens (reviewed in Duijvestijn and Hamann, 1989). This continuous recirculation and migration of lymphocytes through blood, tissues and lymph is important for implementing immune surveillance and is also essential for the dissemination of memory and effector cells (those which are antigen-experienced) to sites of antigen exposure. Activated T lymphocytes migrate from the blood into sites of inflammation and some return to lymphoid tissue via the afferent lymphatic ducts. Lymphocytes leave lymph nodes via efferent lymphatics, which in most cases drain ultimately into the thoracic duct (TD) and thence into the blood via the superior vena cava. This process allows lymphocytes to return to the circulation after residing in lymph nodes. In the case of the spleen, lymphocytes enter directly from the blood, enter the periarteriolar lymphoid sheaths and then re-enter the blood (reviewed in Duijvestijn and Hamann, 1989).

The recirculating lymphocytes in the central efferent lymph can be collected by TD cannulation, a technique that involves inserting a plastic tube retrograde into the TD and passing it to the exterior so that the lymph can be collected. In the case of rats, the animals are restrained in Bollman cages to minimise their movement. Approximately 175ml of lymph can be collected over a 24 hour period from rats provided with isotonic saline to drink (Bollman et al., 1948; Rheinhardt, 1956).

Gowans and Knight (1964) radio-labelled lymphocytes from the TD lymph of rats and transferred these cells via the blood to syngeneic recipients. They observed that the labelled small lymphocytes could be detected in the lymph nodes within 15 minutes and they were detected in the TD lymph within 2-4 hours. These observations, along with the indirect evidence that the output of lymphocytes was diminished during extended drainage of the TD lymph, suggested that there is a pool of recirculating lymphocytes, particularly small cells, that recirculate from the lymph to the blood to secondary lymphoid organs and then return to the lymph (Gowans and Knight, 1964). The cycle is thought to take up to approximately 30 hours (Frost et al., 1975). Gowans and Knight (1964) observed that TD drainage depleted all lymph nodes of lymphocytes, rather than just those draining directly into the TD duct, indicating an integrated network of lymphocyte re-circulation. Careful observation of sections of lymph nodes allowed these researchers to note that small lymphocyte entered lymph nodes via post-capillary venules that were "remarkable for the height of their endothelium" (Gowans and Knight, 1964). These are now referred to as high endothelial venules (HEVs). The spleen does not contain HEVs and lymphocytes can enter the periarteriolar lymphatic sheaths unimpeded via small penicillar arterioles that end in the parenchyma (reviewed in Duijvestijn and Hamann, 1989).

1.2.4 Lymphocyte migration and homing

It has been suggested that immunoblasts do not recirculate like naïve cells (Gowans and Knight, 1964; Hall et al., 1977). Upon recognition of a naïve cell's cognate antigen in context of appropriate antigen presentation within a lymph node, almost immediately the lymphocyte is retained in that site for a day or more, during which time the cell proliferates and differentiates rapidly. Following antigenic stimulation there is an acute, transient fall in the output of lymphocytes from the lymph node draining the site of inoculation (Hall and Morris, 1965a). The initial retention of lymphocytes is non-specific. The decrease in cell output following antigenic stimulation is variable but a rebound in cell output occurs (Cahill et al., 1976). Within 3 days, there is an increase in the output of total cells and this is followed by the appearance of lymphoblasts in the efferent lymph from the draining lymph node and from those down stream along the lymphatic chain. Activated lymphocytes leave the lymph nodes in which they were stimulated and

disseminate such that they are active at other sites and are responsible for the propagation of the immune response (Hall et al., 1967).

Large lymphocytes from the TD lymph were shown by Gowans and Knight (1964) to have a limited capacity to recirculate. These cells and those from mesenteric lymph nodes accumulate preferentially in gut-associated lymphoid tissue, whereas those from peripheral lymph nodes localise preferentially in peripheral lymphoid tissue (Griscelli et al., 1969). In addition, activated / large lymphocytes are more likely to accumulate in lymph nodes that are stimulated with antigen (Griscelli et al., 1969) or inflamed sites (Asherson and Allwood, 1972) than are naïve cells. Small T lymphocytes also show biases in their patterns of recirculation (Cahill et al., 1977) and it has been demonstrated that $CD4^+$ T lymphocytes from gut or peripheral lymph nodes return preferentially to their site of origin ie. the gut or peripheral lymph nodes respectively (Washington et al., 1994).

Multiple factors are involved in determining the selective entry into tissues ("homing") of cells, including their site of activation in the peripheral or mucosal lymphoid tissues. Lymphocytes from the mesenteric lymph nodes are more likely to return to mesenteric lymph nodes or Peyer's patches than lymphocytes from peripheral lymph nodes, which are more likely to return to peripheral lymphoid tissue (Griscelli et al., 1969). Similarly, lymphocytes collected from the efferent lymph of peripheral lymph nodes show a greater propensity to return to the peripheral lymph nodes whereas lymphocytes collected from the efferent lymph of the gut are found to return to the mucosal tissues (Cahill et al., 1977; Hall et al., 1977). The capacity for cells to have tissue-specific migratory pathways is dependent on the surface molecules that are expressed by the migratory cells and the appropriate ligands on the blood vessels of the tissues. There is evidence to suggest that these migratory patterns of memory T cells are influenced more strongly by the activation signals that they receive than the site of activation, for example those that are biased toward the production of IFN-γ migrate preferentially to peripheral lymphoid tissues (Premier et al., 1996).

The fate of lymphocytes following antigenic-stimulation is variable. For example, cells may die or they may become memory cells or effector cells. There are still many unanswered questions regarding the fate and life span of lymphocytes following

activation. However, small memory cells do recirculate and express combinations of adhesion molecules that influence their probabilities for recruitment to specific tissue compartments (reviewed in Butcher, 1986). The continued expression of adhesion molecules at elevated levels by memory cells suggested that these cells recirculate primarily through non-lymphoid tissues and drain into the lymphoid tissue via the afferent lymphatics (reviewed in Mackay, 1993b). This suggestion was supported by observations such as that some memory T cells recirculate through the skin and other non-lymphoid tissues and this traffic increases markedly through inflamed tissue. In contrast to memory cells, naïve cells appear to enter non-lymphoid tissues very inefficiently (reviewed in Picker and Butcher, 1992). However, the concept of tissue-specific accumulation being a consequence solely of homing has been challenged by evidence that preferential proliferation, preferential retention and reduced cell death may account for differences in the accumulation of lymphocytes originating in each of the compartments. In addition, the different microenvironments of the lymphoid tissues may provide different survival factors to the lymphocytes following activation that could account for the increased presence of memory cells in tissues compared with naïve cells (Westermann and Pabst, 1996; Westermann and Bode, 1999). It has been suggested that memory T cells may enter the blood following their generation and not return to the site of their generation to any appreciable extent (Dutton et al., 1998). Memory T cells increase in number in the blood and spleen as animals age and these cells may recirculate in the blood without entering lymph nodes or crossing normal endothelium in the absence of an immune response (Dutton et al., 1998).

Recently it has been shown that the migratory behaviour of lymphocytes is governed not only by the adhesion molecules that they express but also by their response to chemokines. Chemokines are a superfamily of low molecular weight cytokines with chemotactic properties. In addition, certain chemokines can activate leukocytes whereas others can suppress T cell responses (Bromley et al., 2000). Many of the properties of chemokines have been determined by *in vitro* studies, although *in vivo* blocking studies and the use of genetically manipulated animals has provided information on the actions of many of these molecules (reviewed in Sacca et al., 1997). The physiological concentration of a chemokine has a profound influence on its action(s). For example, migration of some sub-populations of T cells is inhibited by high concentrations of the

chemokine stromal cell derived factor −1 (SDF-1), whereas this chemokine is chemoattractive at lower concentrations (Poznansky et al., 2000).

In addition to mediating leukocyte chemotaxis and diapedesis, chemokines are also involved in the process by which leukocytes, moving at high speeds under shear forces, adhere firmly to the endothelium (reviewed in Campbell et al., 1999). The expression of chemokines on the apical surface of endothelial cells can promote integrin-mediated cell arrest and diapedesis of lymphocytes independently of a chemotactic gradient (Cinamon et al., 2001). Some chemokines, which are produced by endothelial cells, can increase the attachment of lymphocytes to vascular endothelium in a tissue specific way. This increased attachment favours migration by lymphocytes into the tissue. For example, thymus-derived chemotactic agent-4 (TCA-4) is a chemokine that activates LFA-1-mediated adhesion to HEVs, an important step in the extravasation of lymphocytes. TCA-4 is produced constitutively by HEVs, while its receptor is expressed by lymphocytes, DCs and mesangial cells (Stein et al., 2000). The expression of chemokine receptors can explain, at least in part, the tissue-specific homing of lymphocytes. For example, "intestinal memory T cells" ($\alpha_4\beta_7^{hi}$) express little or none of the CCR4 chemokine receptor, while "skin memory T cells" ($\alpha_4\beta_7^-$) express high levels of CCR4. The interactions between "skin homing T cells" and vascular endothelium in the skin are promoted by thymus and activation-regulated chemokine (TARC), a chemokine recognised by CCR4, the expression of which is induced in endothelial cells of inflamed skin (reviewed in Campbell et al., 1999).

1.3 Communication between T lymphocytes and other cells.
Part A: Surface molecules
1.3.1 Introduction

The recirculation and migration of naïve and antigen-experienced (memory/effector) cells into inflamed tissues is controlled in part by adhesive interactions with vascular endothelium. Naïve and antigen-experienced $CD4^+$ T cells have different patterns of recruitment and recirculation and this is attributable to differences in expression of adhesion molecules (reviewed in Bradley and Watson, 1996). The importance of other factors such as chemokines and differences in survival and proliferation of the cells within tissues has been discussed above.

Activated CD4$^+$ T cells have an increased capacity for adherence to and migration through, inflamed endothelium relative to cells that are naive. Lymphocyte extravasation is a multi-step process mediated by the interaction of adhesion molecules with their ligands on the surface of vascular endothelium. The steps are sequential but overlapping. The first step is primary adhesion (termed "tethering") which is followed by activation-dependent firm adhesion and the final step, transmigration (reviewed in Springer, 1994).

The adhesion of leukocytes to inflamed endothelium can be regulated by numerous mechanisms such as increased surface expression of the various adhesion molecules involved, increased avidity of the receptors for their ligands and the transient activation of reversible pathways involved in the interaction between the leukocytes and the endothelium. Many of these surface molecules have dual roles. In addition to allowing adherence to other cells or to extracellular matrix, they can also activate intracellular signals upon engagement.

1.3.2 Selectins

The selectin family of cell surface molecules consists of three structurally related integral membrane glycoproteins that have a C-type lectin domain that enables low affinity binding to specific oligosaccharide sequences of cellular mucins. The role of selectins is restricted to regulating the interaction between leukocytes and platelets and/or endothelium during inflammation (they are thought to be crucial in the tethering/rolling step of extravasation) and normal recirculation and homing (reviewed in Springer, 1990; Tedder et al., 1995).

Upon activation, endothelial cells express endothelial cell (E)- and platelet (P)- selectins (CD62E and CD62P respectively) and there is evidence that these molecules have complementary but overlapping roles (Tedder et al., 1995). All members of the selectin family bind to P-selectin glycoprotein ligand 1 (PSGL-1, CD162), although CD62P does so with highest affinity. However, CD62P does not bind to all cells expressing PSGL-1 regardless of the level of expression. Importantly, CD62P binds to activated but not resting T cells, although activation is not associated with change in levels of expression of PSGL-1. The difference in affinity for PSGL-1 is thought to be a consequence of differences in glycosylation and/or tyrosine sulphation that occur on activated cells

(Vachino et al., 1995). The expression of a high-affinity ligand for CD62P by CD4$^+$ T cells is thought to be induced by interleukin (IL)-12 (Xie et al., 1999; Lim et al., 1999) and it is also associated with the production of interferon (IFN)-γ (Austrup et al., 1997). As described in more detail in Section 1.6.2, these events may provide a nexus between the migratory behaviour of activated T cells and their cytokine-mediated effector functions.

Most haematopoietic cells, depending on their stage of differentiation, express leukocyte (L)-selectin (CD62L). Naïve T cells express CD62L but it is lost after activation as the result of proteolytic cleavage (Hamann et al., 1988b; Tedder et al., 1995). In addition to the common ligands of selectins already mentioned, L-selectin also binds to certain glycoforms of CD34, GlyCAM-1 and mucosal addressin (Mad) CAM. The cleaved CD62L is thought to be biologically active in the soluble form, as are P- and E- selectin and this may provide a mechanism by which lymphocyte attachment to endothelium can be inhibited (reviewed in Gearing and Newman, 1993).

CD62L is in part responsible (along with CD62E and CD62P) for mediating leukocyte rolling on the vascular endothelium, an early event in leukocyte recruitment at sites of inflammation. However, because CD62L is down-regulated on activated T cells, CD62L has its most important function in the trans-endothelial migration of naïve lymphocytes through HEVs in peripheral lymph nodes and Peyer's patches. CD62L is expressed by leukocytes other than lymphocytes but it is only the latter that enter lymph nodes via HEVs. It is thought that exclusivity of entry via HEVs may be conferred to lymphocytes by the expression of other molecules, such as the $\alpha_4\beta_1$ or $\alpha_5\beta_1$ integrins, CD44, LFA-1 and/or CD31 (reviewed in Mackay, 1993b). In Peyer's patches, the role of L-selectin can be assumed by $\alpha_4\beta_7$, which is expressed by cells that exhibit preferential recruitment to mucosal tissues (reviewed in Picker and Butcher, 1992). T cells in the lymph draining inflamed tissues are mostly CD62L⁻ (Mackay et al., 1992) and cytokine production by CD4$^+$ T cells during primary immune responses is associated with CD62L⁻ cells (Bradley et al., 1991). Expression of CD62L is generally considered a marker for naive T cells but memory cells can revert to a CD62L$^+$ phenotype (Jung et al., 1988).

1.3.3 Integrins

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The integrins are heterodimeric cell surface proteins that are involved in the specific adhesion of cells to each other or to extracellular matrix. These molecules are crucial for the firm adhesion required for leukocyte extravasation. The adhesiveness of integrins can be regulated rapidly, providing a mechanism for the attachment and de-attachment of the trailing edge of cells on the vascular endothelium (reviewed in Springer, 1994). An integrin heterodimer consists of an α and a β chain, where both chains traverse the lipid bi-layer. Sixteen α and 8 β chains have been described and in many but not all combinations. There are similarities in the sequences of both the α and β chains (reviewed in Springer, 1990).

Integrin α_4 subunit (CD49d, VLA-4 α subunit) can mediate rolling and adhesion in inflamed post-capillary venules (Johnson et al., 1996). The association of the α_4 chain with β_1 chain (CD29) forms a heterodimer (formerly known as VLA-4) that is thought to mediate the migration of activated lymphocytes to sites of inflammation by binding to vascular (V)CAM-1, which is up-regulated by activated endothelial cells. The association of the α_4 chain with β_7 chain forms a heterodimer which shares ligands with the α_4/β_1 heterodimer. While the α_4/β_7 integrin can bind both MadCAM-1 and VCAM-1, the α_4/β_1 heterodimer binds only to VCAM-1. The expression of the α_4/β_1 heterodimer by lymphocytes is thought to enable their migration into inflamed synovium (van Dinther-Janssen et al., 1991) and other inflamed sites (Issekutz, 1991). $\alpha_4\beta_7$ is thought to be a gut-homing receptor and important in lymphocyte recirculation via mucosal HEVs (Hamann et al., 1994) but is also important in migration of activated T cells into inflamed tissues. Unlike α_4/β_1, the α_4/β_7 heterodimer can cause rolling of lymphocytes in the presence of MadCAM-1, as well as the process of tethering (reviewed in Bradley and Watson, 1996).

It has been shown that the administration of antibodies against α_4 can block the entry of spleen cells into Peyer's patches but not into peripheral lymph nodes (Issekutz, 1991). In addition, the intravenous administration of anti-α_4 integrin, monoclonal antibody (mAb) TA-2, to rats at days 1, 9 and 17 after induction of adjuvant-induced arthritis (AA) inhibits leukocyte adhesion in the mesenteric venules by 94% and partially inhibits rolling by 57%. The severity of arthritis in rats treated with TA-2 is reduced (Seiffge, 1996). The inhibition of monocyte accumulation in inflamed joints of rats with AA was achieved only by a combination of mAbs against LFA-1, Mac-1 and VLA-4 (Issekutz and Issekutz,

1995). Other researchers have also shown that the administration of antibodies against α_4 can be protective in AA (Barbadillo et al., 1995).

The other member of the β_7 integrin subfamily is $\alpha_E\beta_7$, which mediates adhesion of intraepithelial lymphocytes (IEL) to the intestinal epithelium (Cepek et al., 1994). The interaction between α_E/β_7 integrin on T cells and its ligand, E-cadherin on enterocytes, is involved in the recruitment/retention of T cells into the gut (reviewed in Agace et al., 2000; Schon et al., 1999). IELs are thought to prevent inflammation of the mucosa and mice with a disruption in the gene encoding β_7, develop hyperplasia of the gut probably as a consequence of the failure of IEL to be mobilised (reviewed in Agace et al., 2000; Wagner et al., 1996).

LFA-1 is an integrin consisting of a heterodimer of CD11a (integrin α L subunit) and CD18 (integrin $\beta2$ subunit) that binds to CD54, CD102 (ICAM-2) and CD50 (ICAM-3). Upon activation, there is a transient up-regulation of the avidity of LFA-1 for its ligands (reviewed in Dustin and Springer, 1991). LFA-1 is important in the adhesion/arrest step of extravasation and plays a general role in extravasation via HEVs and in the migration of leukocytes into inflamed tissues (reviewed in Picker and Butcher, 1992; Bradley and Watson, 1996). Mice that are deficient in LFA-1 have fewer T lymphocytes in their peripheral lymph nodes and more in their spleens, compared with wild type mice. Examination of LFA$^{-/-}$ mice has demonstrated that LFA-1 is important in the migration of lymphocytes into peripheral lymph nodes, although the $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrins can compensate partially for this function (Berlin-Rufenach et al., 1999). Administration of anti-LFA-1 antibody to rats with established AA inhibits the accumulation of polymorphonuclear leukocytes by up to 50% in the talar and metatarsal joints. However, like anti-Mac-1, anti-LFA-1 has no effect on T cell migration into the joints, suggesting that this process is independent of either LFA-1 or Mac-1 (Issekutz and Issekutz, 1991; Issekutz et al., 1996).

1.3.4 Intercellular adhesion molecule (ICAM)-1 (CD54)

CD54 is a highly glycosylated protein of the Ig superfamily which is expressed on the surface of a variety of haematopoietic cells, as well as by non-haematopoietic cells such as vascular endothelium. Expression is generally up-regulated upon activation (Springer,

1990; Dustin & Springer, 1991) and a soluble form has been detected in blood (Gearing & Newman, 1993). In rats, resting T cells do not express detectable levels of CD54. CD54 is important in the extravasation of leukocytes via HEVs and into inflamed tissues because of its role in the adhesion/arrest step of extravasation

CD54 is a ligand for the integrins LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and p150,95 (CD11c/CD18) (de Fourgerolles et al., 1995). In addition, CD54 binds to the extracellular matrix glycosaminoglycan hyaluronan (McCourt et al., 1994) and to fibrogen (Languino et al., 1993). Fibrogen can act as a "bridge" between cells that express a receptor for fibrogen, such as CD54 or Mac-1 (Languino et al., 1993). The expression of CD54 on antigen-presenting cells (APC) is thought to contribute to T cell:APC interactions. A rapid increase in the affinity of LFA-1 for CD54 is triggered by cross-linking of the TCR on a resting T cell (reviewed in Dustin and Springer, 1991). CD54 can provide co-stimulatory signals during T cell activation (Dubey et al., 1995) and is thought to play an important role in the formation of the immunological synapse (reviewed in Wedderburn and Diandra, 2000; Dustin and Cooper, 2000).

In the synovium of patients with RA, CD54 is expressed by synovial cells, macrophages and endothelial cells. *In vivo* administration of mAb 1A29 (anti-CD54) in Lewis rats leads to a strong (although incomplete) suppression of actively-induced AA and adoptively-transferred AA. Administration of anti-CD54 has been shown to prevent both the development of arthritogenic cells (capable of transferring the disease adoptively) and the establishment of adoptively-transferred disease (Iigo et al., 1991). These findings indicate that CD54 is involved in both the induction and effector phases of AA.

1.3.5 MHC class II

MHC Class II molecules (RT1-B and –D in the rat) are heterodimers of non-covalently linked α and β chains that are highly polymorphic. APCs use MHC class II molecules to present exogenously derived antigen to $CD4^+$ T cells. As described in Section 1.2.1, T cells recognise antigenic peptide fragments in the context of MHC molecules and in low affinity interactions, this relies on the reaction being stabilised by the non-covalent bonding of CD4 to MHC class II molecules. Signalling through MHC Class II molecules

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provides an additional means of communication between the T cell and APC (Scholl & Geha, 1994).

In the rat, MHC class II molecules are expressed on DCs, B cells, monocytes, macrophages, myeloid and erythroid precursors and some epithelial cells. In addition, rat T cells can synthesise and express MHC class II molecules following activation (reviewed in Pichler and Wyss-Coray, 1994). T cells have been shown to present peptides that can bind to the cell surface (reviewed in Pichler and Wyss-Coray, 1994) as well as taking up, processing and presenting soluble antigen if the T cells are in a state of activation such that they are synthesising MHC molecules (Barnaba et al., 1994). However, there is evidence that T cells are better able to present antigenic peptides than complex, whole antigens possibly because the internalisation of the TCR may favour uptake of peptide antigens (Broeren et al., 1995).

The microenvironment associated with joint destruction is such that peptidic autoantigens could be present as products of tissue degradation by enzymes such as matrix metalloproteinases. If this is the case, antigen presentation by T cells could be involved in the disease. The expression of B7 on T cells implies that T cells are capable of providing co-stimulation and this has been supported experimentally (Barnaba et al., 1994). However, presentation of antigen by T cells leads to initial signs of activation followed by anergy (Taams et al., 1999). It is thought that mechanisms other than a lack of co-stimulation are involved in inducing this anergy (Pichler and Wyss-Coray, 1994). It is possible that antigen presentation between T cells in inflamed sites may down-regulate the inflammatory reaction by inducing anergy in reactive T cells. Alternatively, it could up-regulate the inflammatory reaction by inducing anergy in suppressive T cells.

Since the function of MHC class II molecules on T cells is not completely understood, the presence of these molecules is used as an indicator of activation, without implying that the T cells are involved in the presentation of autoantigens. However, it is possible that presentation of autoantigen does occur and it may be of pathological importance.

1.3.6 OX40 Antigen (CD134)

OX40Ag (CD134) is a membrane glycoprotein that is a member of the TNF-R superfamily, along with CD40 and nerve growth factor receptor (Mallet et al., 1990). In

the rat, CD134 is only found on activated CD4$^+$ cells and maximum expression occurs 24 hours after stimulation *in vitro* and in the spleen 3 days after primary immunisation (Stuber & Stober 1996).

To induce constitutive expression of the ligand of CD134 by DCs in B cell areas of lymph nodes, transgenic mice were generated in which the gene encoding CD134-ligand was under the control of the CD11c promoter (Brocker et al., 1999). These mice have an accumulation of CD4$^+$ T cells in the B cell follicles. This suggests that CD134 is involved in either initiating migration of CD4$^+$ T cells to B cell follicles and/or their retention there. It is thought that ligation of CD134 induces the expression of Blr-1, a receptor for a chemokine produced in B cell follicles, and that this is likely to explain the attraction of T cells into the area (Flynn et al., 1998). Once in the B cell follicle, CD134 is involved indirectly in providing B cell help, as it has been demonstrated in mice that ligation of CD134 on activated CD4$^+$ T cells promotes production of IL-4 and inhibits production of IFN-γ (Flynn et al., 1998). Cross-linking of CD134 on T cells enhances proliferation and cytokine secretion, while CD134 is thought to be involved directly in B cell help since cross-linking of its ligand on B cells enhances activation and transcription of Ig genes. Since B cells express CD40 constitutively and activated T cells express CD40-ligand, if this interaction alone stimulated B cells there would be a risk that activated T cells could stimulate bystander B cells in an uncontrolled manner (Stuber and Strober, 1996). The interaction between CD134 and its ligand on B cells may, therefore, provide an important mechanism to ensure that activated T cells stimulate only activated B cells that express CD134-ligand.

1.3.7 Phagocytic glycoprotein 1 (CD44)

CD44 (Phagocytic glycoprotein 1 [Pgp-1], Lymphocyte homing receptor) is a family of molecules that are generated by alternative splicing of multiple exons and by post-translational modifications. The standard form (without variable exons) is expressed widely on cells of both haematopoietic and non-haematopoietic origin. The receptor function of CD44 is tightly regulated. Upon stimulation with mitogens or IFN-γ, expression of CD44 is increased on activated and memory/effector T cells. However, this up-regulation of CD44 expression can be transient and the cell surface domain of CD44

can be shed rapidly by protease cleavage (reviewed in Lesley et al., 1993; Mackay et al., 1994).

CD44 is a cell adhesion molecule thought to be involved in cell migration, homing and lymphopoiesis. CD44 binds to fibronectin, laminin, collagen I and hyaluronan, which is thought to contribute to the adhesion of leukocytes to endothelial cells, stromal cells and extracellular matrix (reviewed in Lesley et al., 1993). The expression of CD44 by T cells has been associated with a memory phenotype (Budd et al., 1987). As one of its functions, CD44 may be a mediator of rolling and extravasation of memory T cells into sites of inflammation (DeGrendele et al., 1996; Brennan et al., 1999).

1.3.8 Leukocyte common antigen (CD45)

CD45 (Leukocyte common antigen) is a family of cell surface glycoproteins that are heavily glycosylated. The family members are expressed as isoforms that are generated by alternative splicing of 3 exons of the CD45 gene. All cells of haematopoietic origin express CD45 and the isoforms are expressed differentially on subsets of leukocytes.

The high and low molecular weight (MW) isoforms are used often to differentiate naïve and memory $CD4^+$ T cells, since naïve cells express the high MW isoform ($CD45RC^+$ in rat) and switch to the low MW isoform ($CD45RC^-$) upon antigenic stimulation (Luqman & Bottomly, 1992). However, antigen-experienced $CD45RC^-$ cells have been demonstrated to revert to a $CD45RC^+$ phenotype, indicating that this molecule is not always a reliable marker of naiveté (Bunce and Bell, 1997).

Differential expression of isoforms of CD45 by $CD4^+$ T cells has been associated with regulation of autoimmunity. The injection of $CD45RC^{high}$ $CD4^+$ T cells into T cell - deficient rats induces a wasting disease in multiple organs, which can be inhibited by the co-transfer of $CD45RC^{low}$ $CD4^+$ T cells (Powrie and Mason, 1990).

1.4 Communication between T lymphocytes and other cells.
Part B: Cytokines
1.4.1 Introduction

Cytokines are soluble proteins or glycoproteins that act as chemical communicators between cells. These messengers regulate the production, activation and complex

interactions of both immune and non-immune cells (reviewed in Kelso, 1993). There is not a standardised system of nomenclature for cytokines and often names have been derived from different historical approaches. From a functional perspective, cytokines can be divided into families such as TNF- related molecules, the interferons, the chemokines, the interleukin(IL)s and other hematopoietins. However, families of cytokines can be distinguished by structural features including: Haematopoietins (4 α-helical bundles), TNF family (jelly roll motif), Cysteine knot, Chemokines (triple-stranded, anti-parallel β-sheet) and TGF-β (reviewed in Callard & Gearing, 1994).

A brief introduction to several cytokines that play critical roles in CD4$^+$ T cell-mediated responses is given in the following sub-sections and their roles are discussed further in subsequent sections.

1.4.2 Interferon (IFN)- γ

IFN-γ (also known as type II interferon) is encoded by a single copy gene which generates a 1.2kb mRNA strand (Derynck et al., 1982) and a 166 residue polypeptide that includes a hydrophobic signal sequence of 23 residues, which is cleaved before secretion of the cytokine (Rinderknecht et al., 1984). IFN-γ is biologically active in the form of a noncovalent homodimer of 34kDa. IFN-γ is produced by NK cells, CD4$^+$ T cells and CD8$^+$ T cells. There are also reports of IFN-γ-production by macrophages/DCs (Zhou and Tedder, 1995) and surprisingly, neutrophils in the endometrium are reported to be capable of producing IFN-γ (Yeaman et al., 1998).

IFN-γ was first discovered as an inhibitor of virus replication and now it is recognised as having actions on various aspects of the immune response. IFN-γ has numerous effects on B cells, including negative effects on early B cell populations (IFN-γ production in the bone marrow of transgenic mice obliterates all pre-B and B cells), anti-apoptotic effects on mature B cells and antagonistic effects on IgE synthesis induced by IL-4 (reviewed in Young and Hardy, 1995). This cytokine has suppressive effects on the development of early haematopoietic progenitor cells when acting alone but has stimulatory effects when acting in concert with other agents such as IL-3 and stem cell factor. IFN-γ is a potent activator of macrophages (Pace et al., 1983), inducing the production of agents such as nitric oxide (NO) and it plays an important role in monocyte/macrophage differentiation.

In addition, IFN-γ induces the expression of high-affinity IgG Fc receptors on neutrophils, thus enhancing the phagocytic and cytotoxic action of these cells (reviewed in Young and Hardy, 1995). IFN-γ also increases expression of MHC molecules by a variety of cell-types, including macrophages, endothelial cells and some epithelium (King and Jones, 1983; reviewed in Boehm et al., 1997). Mice with disrupted IFN-γ genes (Dalton et al., 1993) or IFN-γ receptor genes (Huang et al., 1993) have increased susceptibility to intracellular pathogens and viruses. Tissue-specific expression of IFN-γ in IFN-γ-transgenic mice results in severe tissue destruction (Young & Hardy, 1995).

The influence of IFN-γ on the migration of TD lymphocytes has been investigated by returning cells labelled with FITC intravenously to rats with TD fistulae and monitoring the emergence of labelled cells in the lymph (Westermann et al., 1993). These experiments demonstrated that intravenous administration of IFN-γ led to a reduced recovery of T cells in the lymph. Cessation of administration of IFN-γ resulted in an increase in the percentage of recovered T cells to levels seen in control (non-IFN-γ treated) rats. Incubation of T cells with IFN-γ before returning them to cannulated rats did not affect recovery rates in lymph, suggesting that IFN-γ does not act directly on T cells but rather that the systemic administration of the cytokine leads to increased immigration into and decreased emigration out of tissues, probably by the induction of adhesion molecules such as CD54 on connective tissue cells (Westermann et al., 1993). Analysis of the expression of CD45RC on CD4⁺ T cells recovered in the TD lymph from cannulated rats indicated that intravenous administration of IFN-γ reduces the recovery of "naive" CD45RC⁺ CD4⁺ T cells but does not effect "memory" CD45RC⁻ CD4⁺ T cells (Westermann et al., 1994). This observation suggests that the effects of IFN-γ on T cell recirculation occurs within the lymph nodes, rather than in peripheral tissues.

The actions of IFN-γ that relate specifically to CD4⁺ T cell-mediated responses are discussed in Section 1.6.

1.4.3 Interleukin (IL)-4

IL-4 (or B Cell stimulating factor 1, BSF-1) is a 20kD cytokine produced mainly by CD4⁺ T cells but also natural killer (NK) cells and activated mast cells. It was first recognised as a B cell growth and differentiation factor but is also a growth factor for activated T cells

and enhances differentiation of Th2 cells. IL-4 acts on B cells to enhance IgG1 and IgE production and to up-regulate expression of MHC class II on B cells and macrophages. The receptor for IL-4 consists of a heterodimeric complex of variable composition (CD124/CD132 or CD124/IL-13Rα chain) and is expressed on mature B and T lymphocytes, haematopoietic precursors, fibroblasts, epithelial and endothelial cells.

IL-4, the hallmark of a Th2 response, is produced in relatively low quantities by murine naive T cells in response to *in vitro* stimuli such as culture in anti-CD3-coated trays. Mainly large- and intermediate- sized cells are found to produce IL-4 under these conditions (Le Gros et al., 1990), implying that theses cells have undergone *in vivo* stimulation before harvest and that the production of IL-4 is dependent on IL-2. However, if exogenous IL-4 is added with anti-CD3 and IL-2, small T cells can produce IL-4 within 2 days (Le Gros et al., 1990).

1.4.4 Interleukin-10

IL-10 is an 18kD polypeptide (originally termed "cytokine synthesis inhibitory factor") that is produced by $CD4^+$ and $CD8^+$ T cells, monocytes, macrophages, activated B cells and keratinocytes. IL-10 has anti-inflammatory properties and mice with a targeted disruption of the IL-10 gene have retarded growth, anaemia and spontaneously develop colitis in the presence of intestinal flora (Kuhn et al., 1993). The expression of MHC class II by APCs is down-regulated by IL-10 and this cytokine has other anti-inflammatory properties such as inhibiting synthesis of cytokines IL-1, IL-6, GM-CSF, TNF-α, IFN-α, IL-8 and IL-12 by activated T cells, NK cells, monocytes and macrophages (reviewed in Koulis and Robinson, 2000; Moore et al., 1993). IL-10 co-stimulates proliferation and differentiation of human B cells, stimulates IL-2 and IL-4 responsiveness of T cells *in vitro* and synergises with TGF-β to stimulate IgA production (reviewed in Moore et al., 1993).

Production of CC and CXC chemokines by monocytes is inhibited by IL-10, thus inhibiting the production of inducible chemokines involved in the recruitment of monocytes, DCs, neutrophils and T cells (reviewed in Moore et al., 2001). IL-10 has been shown to be a chemotactic factor for $CD8^+$ T cells but has inhibitory effects on $CD4^+$ T cell migration in response to IL-8 (Jinquan et al., 1993).

The receptor for IL-10 has been identified and is composed of at least two subunits. It is expressed mainly by cells of haematopoietic origin, including T and B lymphocytes, NK cells, monocytes and macrophages (reviewed in Moore et al., 2001). The IL-10 receptor is usually expressed at low levels and is down-regulated on T cells following activation. By contrast, IL-10 receptor is up-regulated on monocytes following activation (reviewed in Moore et al., 2001).

1.4.5 Interleukin-12

Two covalently linked glycosylated chains, p35 and p40, form the IL-12 heterodimer. IL-12 is produced predominantly by activated monocytes and DCs. However, IL-12 is also produced by other cells types, such as activated B cells and keratinocytes. IL-12 enhances proliferation, cytolytic activity and IFN-γ production by NK cells and T cells (reviewed in Gately et al., 1998). IL-12 induces the development of Th1 cells *in vitro* and *in vivo* and it is a potent co-factor for stimulating growth, IFN-γ synthesis and cell adhesion of already differentiated Th1 cells (Seder et al., 1993; Bradley et al., 1995; Adorini et al., 1996).

1.4.6 Transforming Growth Factor (TGF)-β

The transforming growth factor (TGF)-β family of proteins are involved in a range of functions such as promoting differentiation and morphogenesis and inhibiting proliferation in both immune and non-immune processes. TGF -β1, -β2 and -β3 are biologically active in a homodimeric structure that is cleaved from an inactive precursor molecule (reviewed in Massague, 1990). CD105 (endoglin) is expressed by endothelial cells and is a high affinity receptor for the TGF-β1 and TGF-β3 isoforms but not the TGF-β2 isoform.

TGF-β is produced by every leukocyte lineage and exerts both autocrine and paracrine effects. The β1, β2 and β3 isoforms of TGF are each under the control of a unique promoter and have a distinct pattern of expression. The influence of TGF-β on an immune response is dependent on the context in which it is present (reviewed in Letterio & Roberts, 1998).

Approximately two thirds of mice with a targeted disruption of the TGF-β1 gene die *in utero* and those that survive appear normal at birth and develop normally until 2 weeks of age, after which they develop a wasting syndrome and die within 1-2 weeks thereafter (Kulkarni et al., 1993). The uncontrolled leukocyte activation and massive inflammatory lesions seen in the heart, lungs and other organs in TGF-β1 deficient mice indicates that this cytokine plays important roles in homeostatic regulation of the immune system and self-tolerance (Kulkarni et al., 1993; Van Parijs and Abbas, 1998).

TGF-β is found in abundance in the synovial tissue of rodents with experimentally-induced polyarthritis (Mussener et al., 1997a; Mussener et al., 1997b). Blockade of TGF-β by the local administration of anti-TGF-β antibody in the joints of rats during the induction of streptococcal wall-induced arthritis suppresses joint inflammation (Wahl et al., 1993). However, the effects of TGF-β can seem paradoxical. Local administration of TGF-β into the hind paws of rats immunised with collagen accelerates the onset and increases severity of collagen-induced arthritis (CIA) (Cooper et al., 1992), while the systemic administration of TGF-β suppresses CIA (Thorbecke et al., 1992).

1.4.7 Tumour Necrosis Factor (TNF)- α

TNF- α is produced by many cell types including activated monocytes, macrophages and lymphocytes. Activated macrophages produce TNF-α in greater abundance than other cell types and this production of TNF-α is amplified by other cytokines, particularly IFN-γ (reviewed in Vassalli, 1992). TNF-α is pleiotropic and is synergistic in combination with other cytokines. In addition to being an important mediator of inflammatory responses, TNF- α is also known to regulate growth and differentiation of many cell types (reviewed in Callard & Gearing, 1994).

TNF-α binds to two receptors (TNF-RI [CD120a] and TNF-R II [CD120b]) with relatively high affinity. Most cell types express both of these receptors at a low level, the former constitutively while the latter is inducible (Vandenabeele et al., 1995).

TNF-α is thought to play multiple roles in immunopathological reactions, such as inducing or enhancing the local attraction of macrophages, polymorphonuclear cells

and/or lymphocytes, the activation of cells and involvement in cytokine cascades (reviewed in Vassalli, 1992). The typical systemic effects associated with generalised inflammatory reactions can, in part, be attributable to TNF-α. The systemic administration of a low dose of TNF-α induces transient fever as a result of its direct action on the hypothalamus whereas systemic administration of a high dose of TNF-α reproduces most of the effects of endotoxic shock (reviewed in Vassalli, 1992). TNF-α induces the expression of VCAM-1 by endothelial cells and enhances the expression of other adhesion molecules such as ICAM-1 (induced by IFN-γ) indicating an important role of TNF-α in leukocyte margination (reviewed in Vassalli, 1992). In addition, TNF-α induces chemokine production in tissues that is necessary for inflammation and leukocyte infiltration (reviewed in Sedgwick et al., 2000).

1.4.8 Interleukin-2

IL-2 is produced mainly by CD4$^+$ T cells and it induces activation and proliferation via both autocrine and paracrine mechanisms (reviewed in Barclay et al., 1997). The receptor for IL-2, (CD25 [α chain], CD122 [β chain], CD132 [γc chain]), exists in 3 alternative forms: a high affinity form composed of a heterotrimer of the 3 chains; an intermediate affinity form composed of a heterodimer of CD122 and CD132; and a low affinity receptor composed of a single chain CD25. The IL-2R is expressed on activated lymphocytes and monocytes and the high affinity form is involved in signal transduction.

1.5 Detection of cytokines

1.5.1 Introduction

Cytokines can be investigated at the level of protein, functional activity and mRNA transcripts of the genes that encode them. Each approach has advantages and disadvantages. Bioassays are limited to detecting biologically active cytokines and they are susceptible to the effects of other cytokines and to the presence of inhibitors. Reverse transcription – polymerase chain reaction (RT-PCR) is a highly sensitive assay for the presence of transcripts encoding cytokines but it has the potential to over estimate cytokine production since it quantifies mRNA rather than protein. Enzyme linked immunosorbent assay (ELISA)s are highly specific for cytokine protein but they are used to detect secreted protein, rather than cytokine activity. Furthermore, they do not detect intracellular or surface bound cytokine and they do not provide information about

numbers of cytokine-secreting cells (Sander et al., 1991). However, the enzyme linked immunospot (ELISpot) assay can provide this information. The detection of intracellular cytokines using flow cytometry allows enumeration of cytokine-producing cells but it is dependent on short term culture of the cells *in vitro* prior to labelling with antibody (Schauer et al., 1996).

1.5.2 Bioassays and ELISAs

Antibodies that have specificity for cytokines can be used in quantitative ELISAs. Bioassays can be useful for the detection of cytokine activity and neutralising anti-cytokine antibodies are useful to confirm specificity and also to block the activities of unwanted cytokines. Both of these assays are performed usually on serum samples or supernatants from cultured cells and they measure the combined cytokine production of a population rather than individual cells.

An ELISpot assay involves coating wells with an anti- cytokine capture antibody, incubating cells in the wells and then detecting secreted cytokine that is bound to the capture antibody using a secondary anti-cytokine antibody-enzyme conjugate. The "spots" can be counted and expressed as a proportion of the number of cells originally plated.

As in the case of traditional ELISAs and bioassays, to link cytokine secretion to a particular subset of cells requires that the subset is first isolated, before performing the ELISpot assay. If this additional information is required, the assay can be expensive and time consuming. However, unlike ELISA and bioassays, the ELISpot assay does allow detection of cytokine secretion at the single cell level. However, it does not distinguish biologically active from inactive protein.

1.5.3 Immunofluorescence detection of intracytoplasmic cytokines

Detection of cytokines using immunofluorescence was first reported in 1986 by Laskay and colleagues, in a study describing the detection of IFN-γ in human peripheral blood cells. These researchers used 4% paraformaldehyde as a fixative followed by cold acetone to further fix the cells and make them permeable. This allowed detection of the fixed intracellular cytokine by the use of specific antibodies. The labelled cells were observed using a fluorescence microscope. Specificity was demonstrated by abolition of

fluorescence by pre-incubating the anti- IFN-γ with IFN-γ and the staining was localised to the Golgi by dual fluorochrome immunofluorescence. The co-localisation of IFN-γ and the Golgi suggested that the cytokine is glycosylated before secretion (Laskay et al., 1986). In 1987, this same group published a report describing two-colour staining to characterise the phenotype of the IFN-γ-producing cells (Sandvig et al., 1987). Dual fluorochrome analysis of IFN-γ and a range of cell markers (CD2, CD3, CD4, CD8, CD5, CD25, CD16, or CD11b) showed that IFN-γ was produced by both CD3$^+$ and CD3$^-$ cells. Problems were encountered with aggregation of the cells after treatment with acetone or with NP-40 detergent.

Sander and colleagues found that detection of IFN-γ by flow cytometry was dependent on using a fixative and a detergent that did not cause clumping of the cells or destruction of the antigens of interest. As well as minimising cell-clumping and destruction of surface and intracellular antigens, the fixative and detergent should also preserve cell morphology (Sander et al., 1991) in order to allow identification of lymphocytes based on their forward- and side-scattering properties. The technique of intracellular staining of IFN-γ in individual lymphocytes for fluorescence microscopy (Laskay et al., 1986) was soon adapted to flow cytometry, allowing the examination of large number of individual cells (Andersson et al., 1988). Andersson's research group made thorough investigations of the technical aspects of detecting cytokines by immunofluorescent staining. They reported that aldehydes were better for preserving the cellular morphology than coagulative fixatives such as acetone and alcohols. However, aldehyde-fixed cells required an additional permeabilisation step. The early studies used acetone to permeabilise the cells after fixation with 4% paraformaldehyde (Sandvig et al., 1987). However, it was soon recognised that the use of a 0.1% solution of saponin to permeabilise the fixed cells had the advantage of permeabilising the cells in a reversible way (Sander et al., 1991).

Saponins are plant glycosides that have a high affinity for cholesterol and they are thought to intercalate in the membrane in the place of cholesterol to form ring-like structures in cholesterol-rich membranes. The formation of pores in cell membranes by saponin is a reversible process and saponin must be present constantly during antibody incubations and washes (Sander et al., 1991). Conversely, washing cells in saponin-free solutions restores the integrity of the cell membrane. Although saponin induces slight

modifications in the light scattering properties of cells, it is not thought to alter the representation of antigens on the surface of cells (Jacob et al., 1991).

The intracellular localisation of IFN-γ within the Golgi apparatus of lymphocytes established that the cytokine had been produced by the cells, rather than being absorbed from exogenous sources (Laskay et al., 1986; Sander et al., 1991). To induce an accumulation of cytokines (and other proteins) at the Golgi, Jung and co-workers used the carboxylic ionophore, monensin, to interrupt the intracellular transport process, which led to accumulation of cytokines in the Golgi complex (Jung et al., 1993). Monensin is a metabolite of *Streptomyces cinnamonensis* that binds Na^+ and K^+ ions. It perturbs the intracellular transport of newly-synthesised secretory proteins, proteoglycans and glycoproteins within the Golgi. Monensin may alter the physiology of the cisternae of the Golgi (reviewed in Tartakoff, 1983). Jung and co-workers also found that *in vitro* stimulation was necessary to detect the production of cytokines and that inclusion of monensin during the stimulation increased the fluorescent signal markedly (Jung et al., 1993). Another reagent used to cause an accumulation of normally secreted proteins in cells is Brefeldin A, a macrocyclic lactone produced by a variety of fungi. Although the mechanism by which Brefeldin A acts on cells is not known, it has been shown that newly synthesised proteins are retained in the rough endoplasmic reticulum of cells treated with Brefeldin A, while Golgi stacks are not apparent in these cells (reviewed in Klausner and Donaldson, 1992). Brefeldin A has been used in place of monensin to accumulate cytokine within cells for labelling (Picker et al., 1995; Ferrick et al., 1995).

For detection of intracellular cytokine, Sander and colleagues have found that it is important to use an antibody that is monoclonal, since polyclonal antibodies cause a level of background staining over which it is difficult to detect a specific signal. The secondary antibody must be affinity purified and absorbed to prevent cross reactivity with B cells. Also, the monoclonal antibody must be titrated to optimise the detection signal, because pro-zonal and dilution effects can lead to weak staining. They found that the optimal concentration for most antibodies is 0.5-10µg/ml. (unpublished results cited in Sander et al., 1991).

Andersson and colleagues compared detection of IFN-γ in human blood MNCs by flow cytometry and confocal microscopy. They found that the two methods enumerated similar

percentages of positive cells. They describe the flow cytometer as being advantageous over the confocal microscope because the former has "a standardised control, is less labour consuming and is observer independent" (Andersson et al., 1988).

1.5.4 Reverse Transcription-Polymerase Chain Reaction (RT-PCR) detection of cytokine mRNA

Messenger (m)RNA transcripts are relatively unstable, very sensitive to enzymatic degradation and can be technically difficult to detect. By reverse transcribing mRNA, a complementary (c) DNA strand is generated that is more stable, less sensitive to degradation and can be bound by the DNA polymerase used in the PCR. A reverse transcriptase enzyme executes reverse transcription.

PCR is a method of amplifying small quantities of DNA template. The technique is reliable and it generates enough material for subsequent analyses. A typical reaction requires target DNA, a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), a reaction buffer and magnesium. The reaction undergoes a cyclic series of set temperatures for specified periods of time. Each cycle of amplification theoretically doubles the amount of DNA and the amount of DNA produced thereby increases exponentially.

The advantages of examining cytokine production using RT-PCR is that the method is highly sensitive, a wide range of cytokines can be examined and it can be made very specific by the precise size of the amplified product. However, the detection of mRNA does not prove the expression or secretion of a functional cytokine (Mosmann & Fong, 1989: Howell et al., 1991).

PCR can be used to quantify the amount of starting cDNA (Connolly et al., 1995) but the methods are laborious and time consuming. Alternatively, PCR can be semi-quantitative, comparing amplified products of interest with amplified cDNAs from transcripts of non-inducible, constitutively expressed "housekeeping" genes (such as GAPDH or β-actin) and expressing results as relative values.

1.6 Subsets of CD4$^+$ T cells

1.6.1 Introduction

The first description of subsets of helper T cells was reported by Mosmann and Coffman who observed that in murine T cell clones, the pattern of secreted cytokines was such that the clones usually secreted either IL-2, IFN-γ and TNF-β (Th1 clones) or IL-4, IL-5, IL-6 and IL-10 (Th2 clones) (Mosmann et al., 1986; Mosmann and Coffman, 1989). Similar observations have been made when examining human Th cells, although the production of IL-2, IL-6 and IL-10 is not as tightly restricted to their respective subsets compared with murine cells (reviewed in Mosmann and Sad, 1996). Th3 cells are helper T cells that produce large quantities of TGF-β (reviewed in Mosmann and Sad, 1996).

The cytokine profiles observed in T cell clones led to the consideration of the biases of cytokine profiles of T helper cells participating in immune responses. The validity of the prototypic generalisations to freshly isolated, normal murine CD4$^+$ T cells has been investigated by Swain and her colleagues. These researchers isolated and enriched CD4$^+$ T cells from the spleens and peritoneal exudates of normal mice and assayed for secreted cytokines in response either to Concanavalin (Con)A, PMA and ionomycin or combinations of these stimulants. Swain et al showed that following stimulation, freshly isolated cells produce IL-2, IL-3, GM-CSF and IFN-γ but not IL-4 or IL-5. The latter are only produced in response to priming and re-stimulation (usually over 4-7 days) (Swain et al., 1988). The bias towards the production of either Th1 or Th2 cytokines is thought to be apparent in both effector and memory cells upon re-stimulation (Swain et al., 1990a; Kelso et al., 1994; Swain, 1994). However, polarisation towards the production of either Th1 or Th2 cytokines is not an inevitable consequence of T cell activation and naive T cells can be activated and expanded in a non-polarised state and retain the capacity to polarise towards the production of Th1 or Th2 cytokine upon re-stimulation (reviewed in Lanzavecchia and Sallusto, 2000).

Th2 cells are thought to promote humoral immunity, because IL-4 is required for optimal proliferation of B cells and also enhances isotype switching to IgG1 and IgE (reviewed in Mosmann and Coffman, 1989). Th1 cells can also provide B cell help, although the nature of the help differs and these cells induce class switching to IgG2a (Mosmann et al., 1986; reviewed in Mosmann and Coffman, 1989). The ability of Th1 cells to provide B cell help is thought to be restricted by the cytolytic effects of the Th1 cells (Del Prete et al., 1991).

The promotion by Th2 cells of IgE production suggests that these cells have a regulatory role in IgE-mediated disorders, such as type I hypersensitivity (reviewed in Street and Mosmann, 1991; Wierenga et al., 1991).

Delayed-type hypersensitivity (DTH) is an immune response mediated by Th1 cells via release of IFN-γ (reviewed in Mosmann and Coffman, 1989; Street and Mosmann, 1991). IFN-γ is a potent stimulator of macrophages (Pace et al., 1983) and release of inflammatory mediators (cytokines, nitric oxide, free radicals and proteolytic enzymes) from these cells underlies the inflammation seen in DTH reactions. Through similar mechanisms, Th1 cells are thought to be responsible for the pathology of some autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE), against which Th2 cells are thought to be protective (Kuchroo et al., 1995; Das et al., 1997). The role of Th1 cells in arthritis is discussed in Section 1.7.

The rigidity of the distinction between Th1 and Th2 subsets has been questioned. Kelso and her colleagues examined a large panel of T cell clones (Kelso and Gough, 1988) and freshly isolated cells during a "Th2 response" (Kelso et al., 1995) and found that there was a random association between pairs of cytokines being produced by individual cells. Others have made similar observations in T cell clones derived from KLH-immunised mice (Firestein et al., 1989). Firestein and co-workers explained their results by concluding that a third subset of helper T cells exists, referred to as "Th0" cells. Th0 cells are a subset of CD4$^+$ T cells that are characterised by the production of both Th1 and Th2 cytokines after stimulation. Examination of the co-expression of individual cytokines by CD4$^+$ T cells following primary, secondary and tertiary activation has revealed that the Th0 phenotype is a more accurate description of a population of cells that produce both Th1 and Th2 cytokines, since there is considerable heterogeneity in the co-expression of different cytokines within the population (Bucy et al., 1995). The production of both Th1 and Th2 cytokines by Th0 cells has suggested to some that these cells may represent an intermediate step in the pathway of differentiation towards Th1 or Th2 phenotypes (Mosmann and Sad, 1996). In support of this suggestion, it has been demonstrated that antigen-primed non-polarised T cells have a similar migratory capacity to naive cells, further suggesting that these cells are at an intermediate stage between naive and effector cells (Iezza et al., 2001).

It is not in dispute that IFN-γ is important for DTH reactions (Issekutz et al., 1988) or that certain stimuli such as infestation with helminthic parasites are strong inducers of IL-4 production (reviewed in Mosmann and Coffman, 1989 and Finkelman et al., 1997; Svetic et al., 1993). It is more accurate, therefore, to state that certain immune reactions show a bias towards production of Th1 or Th2 cytokines, rather than that these responses generate Th1 or Th2 cells exclusively (which produce only IFN-γ and TNF-β or IL-4 and IL-5). However, in many Th1-biased responses the IFN-γ is produced mainly by CD4$^+$ T cells and for the sake of simplicity, such cells will be referred to as "Th1 cells" although some may also produce IL-4 or other Th2-associated cytokines. Likewise, CD4$^+$ T cells associated with Th2-biased immune responses will be generalised to "Th2 cells", although some might produce Th1 cytokines in addition to IL-4.

Th1 and Th2 cells may have different migratory patterns and the migratory patterns may be determined by the repertoire of chemokine receptors that the cells express (reviewed in Sallusto et al., 1998). It has been suggested that Th1 cells are more likely to express CXCR3 and CCR5, whereas Th2 cells are more likely to express CCR3 (Loetsher et al., 1998; Sallusto et al., 1997). However, the maintenance of this dichotomy in the expression of chemokine receptors by the Th subsets *in vivo* is controversial (Campbell and Butcher, 2000). The factors which induce the production of particular cytokines by T cells are likely to also induce the expression of particular chemokine receptors that effect the function of those T cells. It is perhaps an over-simplification of a complex interaction to interpret the coincident expression of certain combinations of cytokines and surface receptors as "markers" of functional subsets. The expression of surface molecules and the production of soluble mediators should perhaps be viewed in terms of their own contributions to an immune response, rather than as markers that imply the expression of a range of other functions.

Austrup et al (1997) have demonstrated that Th1 or Th2 populations, that were generated *in vitro* by stimulating CD4$^+$ T cells from mouse lymph nodes, had different capacities for binding the adhesion molecules P-selectin and E-selectin. In light of this observation, it was not surprising that the two subsets were observed to also have different capacities to immigrate into sites of inflammation. Th1 cells entered sites of DTH reactions more

efficiently than Th2 cells, which were better able to enter inflammatory sites of allergic reactions (Austrup et al., 1997). The migration of Th1 cells into DTH lesions in skin was reduced by both anti-P-selectin and anti-E-selectin antibodies and it was almost abolished by the use of the two antibodies in combination. It appears, therefore, that the difference in expression of P-selectin ligand(s) could prove to be a useful surface marker for distinguishing Th1 cells from Th2 cells. This would have the advantage that viable Th1 cells could be selected for functional studies *in vitro* and *in vivo*. In particular, the recirculation and migration of Th1 cells could be examined in disease models.

Finally, expression of Fas-ligand by Th1 and Th2 clones of mouse T cells suggests that this molecule is expressed differentially by the two subsets. Th1 clones were found to express high levels of Fas-L, whereas Th2 clones express low levels (Ramsdell et al., 1994). Furthermore, it was shown that the expression of Fas-L correlates with the different susceptibilities of the two groups to activation-induced cell death (AICD) and the ability to induce Fas-L mediated cytotoxicity. Th1 clones underwent AICD following activation with anti-TCR plus IL-7, whereas the Th2 clones did not. Th1 clones were also shown to induce Fas-L mediated cytolytic activity, whereas Th2 clones do not have this activity (Ramsdell et al., 1994).

The expression of different adhesion molecules (Austrup et al., 1997) and other surface molecules (Ramsdell et al., 1994; Loetsher et al., 1998; Sallusto et al., 1997; Campbell and Butcher, 2000) by Th1 and Th2 cells indicates that these populations have functional differences that are not related simply to cytokine production.

1.6.2 Induction of T helper subsets

After stimulation, naïve cells produce IL-2 in greater abundance than other cytokines. Additional signals are thought to determine whether this population of Th0 cells develops further towards either a Th1 bias or a Th2 bias. These signals include both cytokines and interactions with cell surface molecules on different subsets of APCs (reviewed in Seder and Paul, 1994).

The differentiation of CD4$^+$ cells towards a Th1 or Th2 bias is influenced by the cytokine microenvironment in which the cells are activated. IL-12 is a potent inducer of IFN-γ

production by CD4$^+$ T cells (Seder et al., 1993; reviewed in Gately et al., 1998) and development of Th1 cells is optimal in the presence of both IL-12 and IFN-γ (Schmitt et al., 1994). DCs are thought to be the crucial source of IL-12 that initiates the bias of Th1 populations in autoimmune diseases. Once committed, Th2 cells are resistant to the effects of IL-12, via down-regulation of the β2 subunit of the IL-12 receptor, which is required by the receptor for signalling (Szabo et al., 1997; Rogge et al., 1997).

IFN-γ acts on naïve cells to promote IL-12 responsiveness. However, factors other than IL-12, such as TNF-α and IL-1, are also required for the development of a Th1 response. It has been demonstrated that IFN-α can replace in part the requirement for IFN-γ, which helps to explain how Th1 cells can develop without an initial population of activated Th1 cells producing IFN-γ (Wenner et al., 1996).

It has been reported that the generation of Th2 cells requires the presence of IL-4 during the initial period of stimulation (Swain et al., 1990b; Le Gros et al., 1990; Noble et al., 1993). The requirement of IL-4 for the generation of IL-4-producing cells raises the question as to the source of the initial IL-4. In addition to other cell types that produce IL-4, such as mast cells, basophils and NK-T cells, it has been reported that naïve cells can produce small amounts of IL-4 immediately after stimulation and that this may be sufficient to kick-start the generation of Th2 cells by autocrine action (Demeure et al., 1995; Croft and Swain, 1995). The interaction between heterogenous subsets of CD4$^+$ T cells, including memory cells, is also thought to be important in the generation of helper T cells (Gollob and Coffman, 1994).

The gene encoding IL-13 is closely linked to that encoding IL-4 (Minty et al., 1993). IL-13 has similar functions to IL-4, but it is produced by a broader range of cells (Jung et al., 1996). IL-4 is thought to be important in promoting the development of a Th2-biased response, although Th2 cells can develop *in vitro* during IL-4 blockade suggesting that IL-4-independent pathways also exist. Th2 responses are inhibited by IL-12, although this is IFN-γ-dependent (reviewed in Pearce and Reiner, 1995). The proliferation of Th2 but not Th1 cells is inhibited by IFN-γ. The resistance of Th1 cells to the inhibitory effects of IFN-γ is due to down-regulation of the β chain of the IFN-γ receptor complex in these cells (Pernis et al., 1995).

The type of antigen that evokes the immune response is also thought to be a determining factor in the type of response that is elicited. The ability of APCs to take up the antigen is dependent on the type of antigen and the site in which it is encountered. Different antigenic peptide ligands of TCRs are thought to provide different activation signals, associated with particular patterns of phosphorylation. Weak interactions between the TCR and peptide/MHC complex are thought to promote the generation of IL-4-producing cells, whereas strong interactions promote IFN-γ-producing cells. In addition, the dose of antigen also influences the immune response (reviewed in Constant and Bottomly, 1997). There is evidence that independent Th1 and Th2 responses against different antigens can occur concurrently (Ismail and Bretscher, 1999), suggesting that the regulatory events in generating Th1 and Th2 effector cells are confined to small focal microenvironments.

An important factor influencing the direction of Th cell differentiation is the type and lineage of DC that provide the initial stimulus. Human myeloid DCs (DC1), which arise from peripheral blood monocytes have been shown to induce Th1 differentiation, whereas lymphoid DC (DC2), which are derived from CD4$^+$ CD3$^-$ CD11c$^-$ plasmacytoid cells appear to induce Th2 differentiation (Rissoan et al., 1999). Both these types of DC have the capacity to induce strong proliferation of allogeneic naive CD4$^+$ T cells but differ in the co-stimulatory signals that they provide to the T cells (Rissoan et al., 1999). It has been suggested that migratory myeloid derived DC may be more involved in guiding the activation of T cells in secondary lymphoid organs whereas lymphoid derived DC are better able to induce tolerance (reviewed in Banchereau and Steinman, 1998; Lane and Brocker, 1999).

The site of antigen exposure is another important determining factor in the immune reaction to that antigen. T cells activated in mucosal lymphoid tissues exhibit a bias towards Th2-cytokine production whereas those activated in peripheral lymph nodes that do not drain mucosal tissues exhibit a bias towards Th1-cytokine production (Daynes et al., 1990; Tonkonogy and Swain, 1993).

The bias towards the production of Th1 or Th2 cytokines during an immune response is thought to be mediated in part by the neuroendocrine system. The *in vitro* activation of

naïve T cells in the presence of the glucocorticoid dexamethasone promotes the production of Th2 cytokines (Ramirez et al., 1996) whereas dehydroepiandrosterone promotes the generation of IL-2 producing cells (Daynes et al., 1990). It has been demonstrated that corticosteroid treatment of asthma, which is thought to be mediated by Th2 cells, diminishes the number of bronchial lavage cells that express mRNA transcripts of IL-4 and IL-5 and increases the number of those that express IFN-γ mRNA transcripts (Robinson, 2000). Further highlighting the importance of corticosteroids on immune response are studies in rats that have demonstrated that adrenalectomised animals do not recover from EAE, whereas intact animals do and that treating adrenalectomised animals with corticosteroids induces the recovery from this disease (reviewed in Ramirez et al., 1996). However, the relationship between the neuroendocrine system and immune system remains poorly understood and it is apparent that the influence of corticosteroids on the production of Th1/Th2 cytokines varies according to the nature of the immune response (Macphee et al., 2000).

Gett and Hodgkin have used the incorporation of the fluorescent dye 5- (and 6-) carboxyfluoroscein diacetate succinimidyl ester (CFSE) to measure numbers of cell divisions following activation *in vitro* and compared these with the production of particular cytokines. Cell sorting was used to select murine CD4$^+$ T cells defined as naive on the basis of the phenotype CD62^{hi}, CD44^{lo}. These cells were stimulated with anti-CD3 antibodies, in the presence or absence of additional cytokines (particularly IL-4). They found the cytokines produced were dependent on the number of cell divisions, rather than the duration of each division, the time before entering the first division or from the time of activation. Production of IL-2, -3, -4, -5, -10 or IFN-γ was not detected before the fourth division by either ELISA of culture supernatant or staining of intracellular cytokine. The frequency of T cells producing IL-2 peaked at divisions 4 -5 and decreased with subsequent divisions, whereas cells producing IL-3, IL-4, IL-10 and IFN-γ increased in frequency with each subsequent division after the third. IL-5 and -10 were detectable at divisions 4 through to 7 but increased dramatically by division 8 (detection of CFSE above autofluorescence becomes limiting beyond division 8). Late production of IL-10 could be a mechanism for down-regulating an immune response. For example, IFN-γ production is down-regulated by IL-10 (Gett and Hodgkin, 1998).

As mentioned previously, the ligation of CD28 or CTLA-4 on the surface of T cells to B7 molecules on the surface of APCs provides an co-stimulatory signal during T cell activation (reviewed in Oosterwegel et al., 1999). In addition, the differentiation of CD4$^+$ T helper cells to the two main helper subsets from precursor cells has been demonstrated to be activated differentially by two co-stimulatory molecules, B7-1 and B7-2 (Kuchroo et al., 1995). T cells from B7-1 (CD80) deficient TCR transgenic mice produce more IL-4 than IFN-γ upon activation with their cognate antigen. This suggests that CD80 is involved in providing signals that regulate IL-4 production (Schweitzer and Sharpe, 1999). *In vitro* studies demonstrated that differentiation of naive CD4$^+$ T cells from mice could be biased to Th1 (increased production of IFN-γ and decreased IL-4) by the addition of anti-B7-2 antibodies, while addition of anti-B7-1 antibodies created a bias towards a Th2 response (decreased IFN-γ and increased IL-4) by anti-B7-1 (Kuchroo et al., 1995). The antibodies did not inhibit the activation of T cells but they influenced the cytokine profile produced by the cells, ie. the cells in both groups were capable of the same proliferative responses. In the EAE model, incidence of disease was decreased by blockade of B7-1 and increased by anti-B7-2, while the protective effect of the anti-B7-1 could be abrogated by treating the mice with anti-IL-4 (Kuchroo et al., 1995). However, in another Th1-mediated disease, diabetes in non-obese diabetes (NOD) mice, others have found that disease is ameliorated by treatment with anti-B7-1 antibodies and exacerbated by the use of anti-B7-2 antibodies (Lenschow et al., 1995). These studies highlight the complexity of the stimulatory network that determines the Th-bias of immune responses.

1.7 Rheumatoid arthritis

1.7.1 Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting 1-2% of the population. Peripheral synovial joints become infiltrated by blood-borne cells, mainly T cells, macrophages and plasma cells and this leads to swelling, inflammation and eventually joint failure as a result of the destruction of articular cartilage and subchondral bone. Although the aetiology of the disease is not understood, a complex interplay of genetic and environmental factors are thought to be the responsible for the immuno-pathogenesis of RA (reviewed in Sewell and Trentham, 1993). The onset of disease usually features the development of symptoms over several months although the onset can be abrupt. Less than 50% of patients can continue working after 10 years from the

onset of disease, which is associated with considerable morbidity and mortality (reviewed in Brooks, 1993).

1.7.2 Pathological features of RA

During RA, the synovium, which normally is only a thin membrane with a lining layer of a few cells in thickness, becomes thickened through oedema, hyperplasia of the lining layer and infiltration with inflammatory cells. Peri-vascular lymphoid aggregates form in the deeper layers. T lymphocytes and macrophages are predominant; but plasma cells, DCs, activated fibroblasts and endothelial cells are also abundant. HEV-like post-capillary venules are present in the synovial tissue, reflecting the entry of lymphocytes into the tissue. Invasive granulation tissue (or pannus) forms and is comprised of macrophages, fibroblasts and new vessels. The pannus arises from the inflamed synovium and invades articular cartilage and bone especially at the osseocartilaginous junction (reviewed in Lee and Manolios, 1999). Patients with RA often have follicular hyperplasia in their lymph nodes, which are infiltrated by polymorphonuclear cells (Nosanchuk and Schnitzer, 1969).

The specific role of autoreactive T cells to the pathogenesis of RA is disputed. However, it is generally recognised that the eventual destruction of the articular cartilage and the subchondral bone is a result of molecular mediators produced by activated cells within the synovium, cartilage and adjacent bone and the cytokine and enzyme products of these activated cells (Brennan, 1996). The presence of autoantibodies, mainly IgM, with specificity for IgG (referred to as rheumatoid factor) is a diagnostic feature of RA and is thought to be involved in the pathology of the disease (reviewed in Sewell and Trentham, 1993). In addition, T cells with reactivity for self-Ig molecules have been isolated from the synovial tissue of patients with RA (van Schooten et al., 1994).

1.7.3 Evidence for involvement of T lymphocytes in the pathogenesis of RA

There is some controversy regarding the significance of T cells in the pathogenesis of RA (reviewed in Firestein and Zvaifler, 1990). However, there is substantial evidence to suggest that T cells are important (reviewed in Mueller and Jenkins, 1997). In an editorial in *Arthritis and Rheumatism*, Panayi, Lanchbury and Kingsley describe the events in the immuno-pathogenesis of RA, using the analogy of an orchestra in which the score, is

written by the APCs, conducted by T lymphocytes and played by macrophages, endothelial cells, B lymphocytes and synoviocytes (Panayi et al., 1992).

The argument of Panayi and colleagues for the importance of T cells in initiating and maintaining chronic synovitis is based on the following points: (a) The up-regulation of HLA-DR, VLA-1, CD25, Cdw60 and CD69 and the down-regulation of CD3, CD2 and LFA-1 on T cells in RA joints is evidence that the T cells are activated, (b) The peri-vascular accumulation of lymphocytes, predominantly CD4$^+$ T cells. The production of IgA and IgG by the plasma cells in this area is presumptive evidence that these CD4$^+$ T cells are activated and able to provide help to the B cells for isotype switching, (c) The clear genetic linkage between RA and certain alleles in the major histocompatibility complex is most easily explained by the essential role of MHC class II molecules in the presentation of antigen to CD4$^+$ T cells (Panayi et al., 1992).

Most T cells in the synovial tissue of patients with RA display a memory helper phenotype (that is CD4$^+$ CD45RO$^+$ CD62L^{low}). The population of synovial T cells is enriched for those that express CD54, CD44, $\alpha_4\beta_7$ (reviewed in Szekanecz et al., 1996) and the very late antigen family of integrins (Garcia-Vicuna et al., 1992). The expression of IL-2 receptor by T cells in synovial fluid (SF) (Iannone et al., 1994; Pitzalis et al., 1987) and synovial tissue (Thomas et al., 1992) is rare. However, there is anecdotal evidence that CD25 may have a role in the pathogenesis of RA in that treatment of patients with antibodies against CD25 can ameliorate the disease (Kyle et al., 1989).

Iannone and co-workers (Iannone et al., 1994) used double labelling to examine the surface marker combinations on the T cells from rheumatoid joints. T cells from SF were found to express the activation markers CD69, HLA-DR and VLA-1 but very few expressed the IL-2 receptor. Cush and Lipsky (1988) observed a similar pattern of expression of surface molecules by T cells in synovial tissue (Cush and Lipsky, 1988). This pattern of expression of surface markers is different to that seen when T cells are activated *in vitro*, where they express sequentially CD69, CD25 (IL-2R), HLA-DR and finally VLA-1. Iannone and colleagues suggested that the SF T cells are a heterogeneous population, consisting of sub-populations that express different activation markers. They also suggested that the previously activated T cells may be recruited preferentially and

that endothelial contact during migration may induce the expression of CD69 and HLA-DR (Iannone et al., 1994).

The pathogenicity of isolated mononuclear cells (MNC, primarily T cells) obtained from rheumatoid synovium has been investigated by injecting the cells intra-articularly into the hind limbs of SCID mice (Mima et al., 1995). Although none of the mice went on to develop clinical signs of disease, histological examination revealed that some mice demonstrated synovial hyperplasia similar to that seen in RA patients in the early stages of disease. The development of hyperplasia in the mice could be prevented by the administration of anti-human CD3 antibodies, indicating the involvement of donor T cells. Since not all of the RA patients had cells that induced hyperplasia in the recipient mice, the researchers investigated patient features potentially associated with the effects of the injected cells. Radiological stages of the disease, C- reactive protein, rheumatoid factor levels and disease duration were analysed and only the latter showed linkage with ability to induce synovial hyperplasia in SCID mice. The mean duration of disease in patients whose infiltrating MNCs induced hyperplasia in mice was shorter than the duration of disease in the group of patients whose cells did not induce hyperplasia. Interestingly, immunohistological studies indicated that the proliferating cells in the hyperplastic synovium were derived from the mice not from the human donors, despite evidence that human DNA was present. These researchers concluded 'that pathogenic T cells are present in the SF or tissue of the affected joints at the early stages of the disease and they might contribute to the initiation of the disease' (Mima et al., 1995).

$CD4^+$ cells that are deficient in the expression of the co-stimulatory molecule CD28 are found in greater abundance in the blood and synovial tissue of patients with RA than in healthy controls (Schmidt et al., 1996). These $CD4^+CD28^-$ cells in RA patients are also unusual in that they express perforin. Perforin is a molecule that is produced by cytotoxic cells, suggesting that these cells may have a cytotoxic function. The $CD4^+CD28^-$ cells are thought to be T cells because they express the α/β TCR. Another unusual feature of these cells is that they do not express CD40L, suggesting that they may not be able to provide help to B cells (Namekawa et al., 1998). The $CD24^+CD28^-$ T cells in synovium were found to accumulate in the transitional zones and in peri-vascular infiltrates surrounding follicular aggregates. The presence of these cells in the synovium correlated with the

expansion of the CD4$^+$CD28$^-$ subset in peripheral blood of rheumatoid patients (Namekawa et al., 1998). A correlation has been observed between patients with increased numbers of CD4$^+$CD28$^-$ cells in blood and the presence of extra-articular lesions. There was no correlation with disease duration, history of anti-rheumatic treatment or severity of joint destruction (Martens et al., 1997).

Cush and colleagues have demonstrated that T cells isolated from the synovial tissue and fluid of patients with RA have a greater capacity to migrate through endothelial monolayers *in vitro* when compared with peripheral blood T cells. T cells with a naïve phenotype adhere to endothelial monolayers poorly and have a very limited ability to migrate through them (Cush et al., 1992). The findings therefore indicate that synovial T cells are in general in a greater state of activation than their peripheral blood counterparts.

The chemokines interferon-γ-inducible protein of 10kDa (IP-10) and monokine induced by interferon-γ (Mig) bind to CXCR3 and they are induced by IFN-γ. All or most T cells in inflamed synovium from patients with RA expressed the chemokine receptor CXCR3 and approximately 80% expressed CCR5. The latter is a receptor for regulated upon activation and normal T-cell expressed (RANTES), macrophage inflammatory protein (MIP)-1α and MIP-1β (Qin et al., 1998; Patel et al., 2001). The chemokines IP-10, Mig and MIP-1β have been shown to be expressed in elevated levels in the SF of patients with RA when compared with that from osteoarthritic joints. The levels of mRNA transcripts of these cytokines are elevated in the synovial tissue of patients with RA compared with peripheral blood cells from patients with RA or healthy individuals. These observations suggest that CXC chemokines may be involved in the recruitment of T cells into synovial tissue in RA (Patel et al., 2001; Hosaka et al., 1994).

The intimate physical association between CD4$^+$ T cells and synovial cells such as macrophages has long been recognised (Kurosaka and Ziff, 1983). It is thought that the joint destruction in RA is mediated by the actions of T cells on resident and/or other inflammatory cells (reviewed in Panayi et al., 1992; Yamamura et al., 2001). The administration of antibodies against molecules such as CD54, which reduces intercellular communication between T cells and synovial cells, has produced promising therapeutic effects in clinical trials (Kavanaugh et al., 1994). However, blockade of CD54

interactions may also influence the entry of leukocytes into the tissue in addition to blocking T cell-APC interactions.

In addition to producing pro-inflammatory cytokines (discussed in Section 1.7.4) and pathogenic molecules such as perforin that may contribute to synovial inflammation and tissue destruction, T cells are also thought to be involved in regulating bone loss and joint destruction that is associated with arthritis (Kong et al., 1999). It has been demonstrated that engagement of receptor activator of NF kappa B (RANK), which is expressed on chondrocytes, osteoclast precursors and mature osteoclasts, by RANK ligand (RANK-L) on activated T cells induces osteoclastogenesis and subsequent bone loss (Kong et al., 1999). Synovial T cells from patients with RA produce excessive levels of RANK-L compared with T cells from patients with other arthropathies such as gout (Kotake et al., 2001). These observations provide evidence for an important role for T cells in the tissue damage that is the hallmark of rheumatoid arthritis.

It has been demonstrated that direct cell contact between activated T cells and synoviocytes induces the latter to produce prostaglandin E_2 and interstitial collagenase (MMP-1). However, the inhibitor of MMP-1, tissue inhibitor of metalloproteinase 1 (TIMP), is not produced (Burger et al., 1998). This suggests an indirect mechanism by which synovial T cells could mediate tissue destruction. Degradation of the extracellular matrix components would be favoured by the imbalance between MMP-1 and TIMP, while prostaglandin E_2 is thought to be involved in resorption of the mineral compartment of bone (Burger et al., 1998).

The target antigen(s) of T cells in RA have not been identified. However, several candidates have been proposed. T cell clones with specificity for collagen type II have been derived from the synovial tissue of patients with RA and mononuclear cells with reactivity to collagen types II and III have also been identified in the peripheral blood of rheumatoid patients (Londei et al., 1989; Trentham et al., 1978b). Others have suggested that auto-reactivity occurs as a consequence of molecular mimicry between self-antigens (not excluding collagen peptides) and exogenous antigens such as bacterial antigens. The presentation of such peptides is likely to be intimately associated with MHC haplotypes and the association between RA and certain MHC alleles has long been recognised (Albani and Carson, 1996). Interestingly, there are numerous examples where bacterial

infection result in arthritis and bias towards a Th1 response has been observed. These include Yersinia-induced reactive arthritis (Schlaak et al., 1992), Lyme arthritis (Yssel et al., 1991) and chlamydial reactive arthritis (Simon et al., 1993).

1.7.4 Cytokines In RA

The reports on the patterns of cytokine production by T cells in the synovial tissue of rheumatoid synovium have not contributed much in a positive way. This is the case especially for T cell-derived cytokines. The earlier studies relied on the detection of cytokines by antibodies and these were usually not successful in detecting lymphokines, which are produced in smaller amounts than the relatively abundant monokines.

RT-PCR and *in situ* hybridisation have been used to compare message for T cell cytokines in synovial tissue from patients with RA and reactive arthritis (Simon et al., 1994). Simon and co-workers hypothesised that the T cell cytokines would differ in the two diseases, which have similar synovial pathology but differ in respect to aetiology. Using RT-PCR, they found that the ratio of IFN-γ: IL-4 positive samples was 8:2 in rheumatoid ST compared to 6:5 in the synovium from reactive arthritis, suggesting a stronger Th1 bias in the synovium in patients with RA. The use of immunohistochemistry in combination with *in situ* hybridisation demonstrated that approximately 1 in 300 CD3 T cells contained IFN-γ mRNA and that 1 in 1000 were positive for IL-4 mRNA. There was a similarity in the frequency and location of IFN-γ mRNA positive cells in the two diseases but reactive arthritis showed a greater frequency of cells that were positive for IL-4 mRNA. Although the proportions of lymphokine-producing T cells were low, the authors compared these results to the frequencies of cytokine-producing T cells in two other diseases. In multiple sclerosis, the frequency of T cells responding to myelin-oligodendrocyte glycoprotein in the cerebrospinal fluid is 1:450 (Sun et al., 1991), while in the lesions of Leishmaniasis IFN-γ-secreting T cells have a frequency of 1:1000-1:100 (Cooper et al. 1989). The seemingly low frequency of IFN-γ and IL-4 producing cells in rheumatoid ST is in fact similar to the frequencies in these diseases which also exhibit a T cell infiltrate and a Th1 bias (Simon et al., 1994). Others have also observed an imbalance of Th1 over Th2 cytokines in RA (Miltenburg et al., 1992; Dolhain et al., 1996; Bucht et al., 1996; reviewed in Romagnani, 1994).

The signals controlling T cell activation in rheumatoid synovial tissue are understood poorly. While IL-2 is a potent activator of T cells, it is virtually absent from rheumatoid synovium (Warren et al., 1991; Howell et al., 1991). The recruitment and activation of T cells in RA may be mediated by IL-15. This cytokine shares a number of actions with IL-2 but it is not produced by T cells (reviewed in Carson, 1997). IL-15 is found in the SF of patients with RA in greater amounts than in SF from OA patients and it has been shown to induce proliferation of T cells isolated from rheumatoid SF (McInnes et al., 1996). IL-15 induces the production of TNF-α by synovial T cells, but perhaps of more relevance to RA, it stimulates T cells to initiate interactions with macrophages that induce TNF-α production by these cells (McInnes et al., 1997). IL-15 may play additional roles in regulating the action of T cells in RA, since it exerts anti-apoptotic effects on activated CD4$^+$ T cells and promotes the maintenance of a quiescent state (Dooms et al., 1998).

As mentioned in Section 1.4.5, IL-12 is a potent inducer of IFN-γ production and is central in establishing a Th1 bias in the immune response. IL-12 has been detected in greater abundance in rheumatoid synovium than in OA synovium (Bucht et al., 1996; Morita et al., 1998). The administration of IL-12 to mice with collagen-induced arthritis (CIA), or to mice with other Th1-mediated diseases such as EAE and diabetes, can increase the severity of the disease, accelerate its onset and extend its duration (reviewed in Trembleau et al., 1995b; reviewed in Trembleau et al., 1995a). IL-12 treatment of NOD mice dramatically increases the production of IFN-γ by T cells that infiltrate the pancreatic islets and decreases the production of IL-4, providing further evidence that IL-12 has important immunomodulatory properties in promoting Th1 responses and suppressing Th2 responses (reviewed in Trembleau et al., 1995a).

The balance between the production of Th1 and Th2 cytokines is likely to be crucial in determining the outcome in RA (reviewed in Miossec et al., 1996), although it is unlikely to be a simple matter of Th1 cytokines being solely responsible for the disease. Although the pro-inflammatory properties of IFN-γ are well known, this cytokine is thought also to be involved in disease resolution (Browning, 1987). Furthermore, Th2 cytokines promote humoral immunity and the production of rheumatoid factor is also associated with RA pathology, indicating that Th2 cytokines may be involved in disease pathogenesis.

A number of other cytokines have effects that either counter the production of Th1 cytokines or inhibit their actions. One of these is IL-13, which decreases production of inflammatory cytokines such as IL-1β and TNF-α, is found consistently in RA synovium (Isomaki et al., 1996). *In vitro* studies demonstrated that exogenous IL-13 has effects on macrophages that are similar to IL-4 and inhibits the production of pro-inflammatory cytokines (Isomaki et al., 1996). In contrast to IL-13, efforts to detect IL-4 production in T cell clones isolated from rheumatoid synovium (Miltenburg et al., 1992), in freshly isolated cells that were stimulated *in vitro* (Dolhain et al., 1996), in *ex vivo* synovial explants (Miossec et al., 1992) and in SF by ELISA (Miossec et al., 1990) have indicated that this cytokine is scarce or absent from the synovial tissue and fluid of patients with RA.

IL-10 is another cytokine that has the potential to inhibit Th1 responses and to promote antibody production. IL-10 has been detected in rheumatoid synovium (Katsikis et al., 1994) and T cell clones isolated from rheumatoid synovium have been shown to produce IL-10 upon stimulation *in vitro* (Cohen et al., 1995). IL-10 can inhibit cytokine production by rheumatoid synovial cells and it can also inhibit up-regulation of CD54 in response to IFN-γ (Kawakami et al., 1997).

TGF-β is another cytokine that has been found in the synovial tissue and SF of patients with RA (Miossec et al., 1990; Bucht et al., 1996) and has the potential to down-regulate inflammation (Taketazu et al., 1994; Ulfgren et al., 1995). TGF-β is a pleiotropic cytokine with important immunoregulatory actions and it is likely that this cytokine plays a role in both disease pathology, for example by promoting the recruitment of inflammatory cells, and recovery by its actions such as inhibiting the proliferation and differentiation of lymphocytes (Bucht et al., 1996).

Firestein et al (1990) described the investigation of cytokine gene transcription using *in situ* hybridisation. The presence of mRNA encoding cytokines was examined in SF cells, enzymatically dispersed cells from synovial tissue (ST) and frozen sections of ST. Transcripts encoding IL-1β, IL-6, TNF-α, TGF-β1, GM-CSF and IFN-γ were expressed at lower levels in cells from the SF than in those isolated from ST. The majority of the cells containing IL-1β and TNF-α transcripts were macrophages. These workers were

unable to detect significant numbers of T cells containing cytokine mRNA, suggesting that the majority of cells in synovium producing the above mentioned cytokines were macrophage-like or fibroblast-like cells. Firestein and Zvaifler (1990) used these data in their editorial in Arthritis *and Rheumatism* to challenge the importance of T cells in established RA. They also claimed that if T cells played an important role in RA, they would proliferate in the joint, most would have markers of activation and they would be clonally restricted. This interpretation was challenged in an editorial by Panayi et al (1992) (see Section 1.7.3).

Cells types other than lymphocytes also contribute to the pathology of RA. For example, rheumatoid synovial macrophages are prolific producers of inflammatory mediators such as granulocyte and macrophage colony stimulating factor (GM-CSF), TNF-α, IL-1, IL-6 and IL-8 (reviewed in Feldmann et al., 1996). The abundance of these cytokines, which are potentially pro-inflammatory, has suggested that they contribute to the pathology of the joint inflammation and they have been targets for therapeutic action (reviewed in Feldmann et al., 1996). However, it is difficult to determine which of these cytokines have a primary role in triggering the production of the molecules that are responsible for the joint destruction and which are part of the ensuing inflammatory cascade.

One of the most promising targets for the treatment of RA has been TNF-α, which is produced in abundance in the ST of patients with RA but not in healthy joints (reviewed in Feldmann et al., 1996; Jorgensen et al., 1999). This cytokine has numerous inflammatory effects, such as stimulating the production of prostaglandins and matrix metalloproteinases and inducing the production of other cytokines such as IL-1, IL-6, IL-8 and MIP-1α by synovial fibroblasts. The potency of TNF-α in the pathogenesis of arthritis is illustrated spectacularly in TNF-α transgenic mice, which develop arthritis spontaneously by 4 weeks of age (reviewed in Jorgensen et al., 1999). Although there have been reports that the levels of TNF-α in the SF of patients with RA is relatively low (Hopkins and Meager, 1988), the serum levels of TNF-α are correlated positively with severity of disease (reviewed in Koch et al., 1995; Feldmann et al., 1996), which suggests that TNF-α has systemic activity in RA. It has been shown recently that the levels of TNF-α protein in the lymph draining foot joints of patients with RA are elevated compared with that in lymph of healthy individuals (Olszewski et al., 2001), indicating

that this cytokine is produced locally in the inflamed joints but its activity extends to the local lymph nodes.

Another important inflammatory mediator produced by synovial tissue macrophages and fibroblasts is IL-1 (reviewed in Koch et al., 1995) which appears to be an important contributor to the pathology of early and late RA. IL-1 has been shown to promote activation of synoviocytes and synovial lymphocytes (Hopkins et al., 1988) and it can act locally to stimulate the production of other inflammatory mediators such as matrix metalloproteinases (reviewed in Koch et al., 1995). In addition, IL-1 has the systemic effects of inducing fever and appetite suppression. The blockade of IL-1 in animal models of arthritis has indicated that this cytokine is a promising target for therapy of RA. The production of IL-1 receptor antagonist (IL-1ra) can be induced in monocytes under certain culture conditions *in vitro* and this factor has been detected in rheumatoid SF (reviewed in Arend and Dayer, 1990). IL-1ra has been used therapeutically in clinical trials for the treatment of RA, with some limited success (reviewed in Jorgensen et al., 1999). The efficacy of IL-1ra may be limited by its low affinity for IL-1.

GM-CSF is produced constitutively by synovial tissue macrophages and rheumatoid synovial fibroblasts produce GM-CSF when stimulated by TNF-α and/or IL-1. GM-CSF also induces the expression of MHC class II molecules by monocytes and macrophages and it is an activating factor for these cells (reviewed in Koch et al., 1995). Cells in the pannus, that attack articular cartilage and periarticular bone in RA, produce this cytokine (Chu et al., 1992). GM-CSF can induce the production of TNF-α and IL-1 by monocytes and macrophages and for this reason is likely to play a role in the amplification of joint inflammation.

1.8 Rat models of rheumatoid arthritis

1.8.1 Induction of Adjuvant-induced arthritis (AA)

Adjuvant-induced arthritis (AA) follows the injection of heat-killed *Mycobacteria tuberculosis* in an oily vehicle in certain strains of rats. The usual site of injection is subcutaneously into the hind paws or at the base of the tail. Maximum disease induction can be achieved by direct injection of CFA into the inguinal lymph nodes (reviewed in Taurog et al., 1988) and higher incidence and greater severity of arthritis is observed in rats inoculated via the intracutaneous route compared with subcutaneous injection

(Pearson, 1956). In contrast, injection of CFA into the peritoneal cavity does not induce arthritis (Pearson, 1956).

Some Gram-positive bacteria can also be used to induce arthritis but like *M.tuberculosis*, all require incorporation in an oily vehicle (reviewed in Taurog et al., 1988). Whitehouse and co-workers reported that the arthritogenicity of oils has particular criteria, namely: unchained alkanes require 12 or more carbons; phenylalkanes require 9 or more carbons in the chain; cyclisation usually reduced the arthritogenicity; and some branched hydrocarbons and animal hydrocarbons (zoo-alkanes) were arthritogenic. More than 12 carbons in the fatty acid of esters was the prime requirement for arthritogenicity, which was reduced with desaturation and/or the presence of a hydroxyl group. These researchers described over 100 'adjuvants' but consistently found that in the Wistar rat, the arthritogenicity of the oils was dependent on the presence of the bacteria (Whitehouse et al., 1974). Interestingly, arthritis can be induced in Dark Agouti (DA) rats by the injection of oil alone (Kleinau et al., 1991) and a mild arthritis can be induced in DA rats by percutaneous administration of oil (Kleinau et al., 1994).

1.8.2 Collagen-induced arthritis (CIA)

Type II collagen is the major matrix protein in hyaline cartilage. Trentham and colleagues reported an experimental model of arthritis in rats that was induced by the injection of heterologous collagen type II in CFA (Trentham et al., 1977). This polyarthritis is accompanied by both cellular and humoral immunity to type II collagen. Rats injected with collagen type I in CFA or collagen type II without adjuvant do not develop arthritis (Trentham et al., 1977). Polyarthritis can be induced with chick or bovine type II collagen there are conflicting reports as to whether native rat collagen type II induces CIA in rats (Trentham et al., 1977; Staines et al., 1981). Genetic factors are thought to influence reactivity with collagen and the induction of CIA with homologous collagen (Griffiths and DeWitt, 1984).

Immunity to collagen has been demonstrated also in AA (Trentham et al., 1980) but CIA, unlike AA, can be transferred to naive rats by serum and by affinity purified anti-collagen type II antibody (Stuart et al., 1982). This observation indirectly implicates B cells and antibody in the pathogenesis of CIA. Spleen and lymph node cells from animals inoculated with collagen type II can transfer disease to naïve recipients (Trentham et al.,

1978a) and there is evidence that transfer of T cells is sufficient to transfer CIA to naive recipients (Brahn and Trentham, 1989). CIA can be induced in mice (Wooley et al., 1981), where T cells have also been shown to transfer the disease adoptively (Holmdahl et al., 1985). Together, the results suggest that CIA may, therefore, be a consequence of a pathogenic $CD4^+$ T cell-mediated response that is characterised by delayed-type hypersensitivity to collagen type II and anti- collagen type II antibodies (Thompson et al., 1993). It is noteworthy that cellular sensitivity to collagen has been identified in T cells from patients with RA (Londei et al., 1989, Trentham et al., 1978b) and antibody is also thought to play a role in the pathogenesis of CIA.

1.8.3 Strain difference and MHC linkage in susceptibility to AA

The factors that determine susceptibility to AA are not well understood. The random bred rat strains Wistar, Sprague-Dawley, Long-Evans, Holtzman and Lobund are susceptible to AA. The inbred rat strains Lewis, Dark Agouti, PVG, Wistar Furth, Brown Norway and WKA are also susceptible to arthritogenic doses of CFA. However, environmental factors are also involved. For example, Fisher F344 rats are susceptible to the induction of AA if kept in germ-free conditions but are resistant when housed in conventional conditions (Kohashi et al., 1979).

Buffalo strain rats, which develop only a mild arthritis in response to CFA, exhibit increased severity of disease when treated with low doses of cyclophosphamide, suggesting that suppressor T cells may be involved in conferring resistance to AA (Bersani-Amado et al., 1989). However, treatment with cyclophosphamide or cyclosporin A of Fisher strain rats, which are resistant to the induction of AA when housed in conventional conditions (Kohashi et al., 1979), is unsuccessful in rendering these rats susceptible to AA (van de Landerijt et al., 1993). These findings suggest that resistance to AA involves multiple mechanisms.

In an attempt to identify genetic factors that are involved in susceptibility, Battisto et al (1982) examined the heritability of susceptibility to AA and found that it was controlled by an autosomal dominant gene locus, *Ar*, that is linked 'loosely' to the $RT1^a$ haplotype of the MHC locus. However, other susceptible strains have other haplotypes indicating that although susceptibility to AA may be influenced by the MHC encoded genes, like CIA

(Griffiths and DeWitt, 1984), there are clearly other genetically determined factors involved.

1.8.4 Pathological features of AA

AA shares the following features with rheumatoid arthritis: acute symmetrical arthritis in the peripheral joints followed by progressive and destructive joint disease, arthritis with acute relapses, extra-articular manifestations including, eye lesions, acute and subacute synovitis with a mononuclear cell response, invasion of bone and joint space by a pannus, bursitis and tendinitis, fibrous and bony ankylosis. The most striking difference between the RA and AA is that necrobiotic rheumatoid nodules are a feature only in RA (Pearson, 1963).

Pearson (1956) described the induction of arthritis in Lewis rats by the injection of CFA into the footpad. Arthritis occurred between days 10 to 14 later and it commenced with individual "edematous swellings along the tarsal or carpal bones, on the heel, or over one or several of the small digital joints". These swellings were sometimes transient. Within another two days, the original areas and others that had developed more recently had become inflamed periarticular swellings. There was a predilection for the joints of the hind paws and multiple joints were usually involved. These included the ankle, tarsus, carpus and the interphalangeal joints. Several days later, segmental radial swellings developed that encircled the tail adjacent to the intervertebral discs of the caudal vertebrae and sometimes with erythematous nodules (Pearson, 1963). Pearson's histological examination of the joints and the periarticular tissues in AA revealed five pathological features observed in the early days or weeks of the disease. Firstly, he observed that there was a phase of acute synovitis, with moderate joint effusions. Secondly, a periarthritis was apparent that featured oedema of the sub-synovial tissue, with massive infiltration of mononuclear cells and few polymorphonuclear cells and intense proliferation of fibroblasts. Thirdly, there was peritendinitis and bursitis. Fourthly, the subchondral bone and tendons were invaded by 'connective tissue' and pannus extended over the surface of articular cartilage from the synovial margins. Finally, osteoid formation was observed on the bone adjacent to joints, in parallel with osteoblastic proliferation (Pearson, 1963). During extended periods after induction of disease, from days 20 to 300 after onset, further pathological features were observed. Fibrous adhesions occurred between some articular surfaces and there was dense fibrous thickening of the joint capsular structure,

limiting the mobility of the joints. The synovial villi continued to proliferate and a "low grade inflammatory reaction" was observed in and around some joints. Rare lymphoid aggregates were found in periarticular bone. Bony bridges connected adjacent vertebrae of the tail and some bony ankylosis was observed. There was evidence of continued activity of a pathological process in the joints of some animals for nearly a year after the initial signs of joint inflammation indicating that AA is variably persistent and relapsing disease, both clinically and histologically (Pearson, 1963).

One of the criticisms of AA as a model for RA is that the ankylosis of bones in the tail suggests that the pathogenesis may be related more closely to ankylosing spondylitis than to RA. However, it should be noted that RA does affect the spine, in particular the joints in the neck, which form the most mobile part of the vertebral column. Ankylosis of joints also occurs in juvenile-onset RA, suggesting that ankylosis may be a pathological process that occurs particularly between small and/or immature bones in humans. Also, because humans do not have tails, the fact that the joints of the tails of rats are affected in AA cannot be compared directly to homologous bones in humans.

Baumgartner et al (1974) described AA as having three distinguishable phases: the pre-arthritic, arthritic and osteogenic phases. Days one to four are characterised by acute local inflammation at the site of injection of the adjuvant (eg. tail base) and systemic effects (a reduction in hepatic albumin synthesis); days seven to twelve feature acute inflammation; days twelve to twenty-eight is the phase of chronic inflammation with periarthritis, and the onset of splenomegaly. Days 21 onwards show residual systemic inflammation and the appearance of osteogenic activity. After day 35, the spleen returns to normal size and there is minimal inflammation of the joints, although there is permanent articular deformity.

1.8.5 Pathogenesis of AA

The lack of improvement in arthritic animals treated with broad spectrum antibiotics, the failure to culture organisms from arthritic joints or tissues, and the heightened susceptibility of 'germ-free rats' to AA indicates that 'an infection of the usual type' is not implicated in this disease (Pearson, 1963). Pearson suggested that AA is a consequence of "the operation of hypersensitivity mechanisms". This was based on several observations, such as an association between the severity of the inflammatory

reaction at the local injection site and the subsequent development of arthritis; inhibition of the disease by immunosuppression by corticosteroids or irradiation and the observation of an 'immune tolerance-like' phenomenon in rats that are exposed to CFA as immature animals (Pearson, 1963). Inhibiting lymphocyte proliferation by treatment with Cyclosporin-A is effective in preventing the development of adjuvant arthritis (Del-Pozo et al., 1990).

T cells are necessary for the development of AA. AA cannot be induced in athymic rats (Kohashi et al., 1981) and can be prevented or suppressed by depleting rats of T cells using a monoclonal antibody against the α/β TCR (Yoshino et al., 1990; Yoshino and Cleland, 1992; Holmdahl et al., 1992). A similar pattern is observed in oil-induced arthritis (OIA) (Holmdahl et al., 1992). The effect of anti-α/β TCR antibody on both AA and OIA is seen within 24 hours of administration of the antibody; and the results from these studies suggest that T cells are crucial for both the induction and maintenance of AA and OIA. Administration of anti-CD2 has also been shown to ameliorate established AA in Lewis rats (Hoffmann et al., 1997) but this molecule is expressed by NK cells also and thus does not implicate T cells exclusively.

The *in vivo* administration of antibodies against specific T cell subsets have further narrowed the field of cells responsible for the induction and maintenance of AA. Administration of mAb OX35, which depletes $CD4^+$ T cells *in vivo*, leads to prevention of AA (Billingham et al., 1990b), as does administration of the non-depleting anti-CD4 antibody, mAb W3/25 (Pelegri et al., 1995). The treatment of established AA with mAb W3/25 leads to an amelioration of the disease but within days of ceasing treatment, the severity of arthritis increases to match that of untreated rats (Pelegri et al., 1996b). Administration of mAb OX8 does not alter the severity of the disease, or its onset and duration, suggesting that AA is not dependent on $CD8^+$ T cells (Larson et al., 1985; Pelegri et al., 1995). Pelegri et al (1996a) described an exacerbation of AA in Lewis rats treated with anti-γ/δTCR at certain stages of the disease, indicating a protective role of these cells. However, the lack of effect of mAb OX8 (anti-CD8) in an earlier study by these researchers (Pelegri et al., 1995) is puzzling because most γ/δ-T cells in rats express CD8.

Cells from the lymph nodes, spleen or TD lymph of rats that have AA can transfer the disease to naïve recipients. However, in some cases the cells require *in vitro* stimulation or the recipients must be irradiated for the transfer of disease to be successful (Waksman and Wennersten, 1963; Pearson and Wood, 1964; Whitehouse et al., 1969; Quagliata and Phillips-Quagliata, 1972; Taurog et al., 1983). In the Arthritis Research Laboratory, it has been shown recently that TD lymphocytes from DA rats that are in the prodromal phase of AA can transfer the disease adoptively without requirement for either *in vitro* stimulation of the cells or immunosuppresssion of the recipients. The arthritogenic population in the TD lymph is contained within the CD4$^+$ T cell subset. It has been shown further that CD4$^+$ T cells expressing CD25, MHC class II, CD71 and/or CD134 are necessary and sufficient for the adoptive transfer of arthritis (Spargo et al., 2001). The tracking of donor cells in the adoptive transfer of AA has indicated that T cells (mainly CD4$^+$) localise in the synovium of recipient rats (DeJoy et al., 1990). However, a contribution from host-derived cells may also be necessary to manifest AA by adoptive transfer. Con A - stimulated T cells from the lymph nodes and spleens of rats in the prodrome of AA fail to induce arthritis in nude rats (Van de Langerijt et al., 1994). However, reconstitution of the nude rats with naïve normal syngeneic spleen cells enables the development of arthitis, indicating that a host component is required for the development of AA.

In other autoimmune diseases in rodents the number of autoantigen-specific cells is an important factor in determining the outcome of adoptive transfer. The non-obese diabetic (NOD) mice develop a T cell-mediated insulitis spontaneously at 4 weeks of age and this progresses to irreversible destruction of β cells and clinical diabetes by 14-18 weeks of age. A CD4$^+$ T cell clone that recognises a peptide of the heat shock protein (HSP) can produce hyperglycemia and insulitis when 5×10^6 cells are administered to pre-diabetic NOD mice or to histocompatible congenic mice with no genetic predisposition to diabetes. However, there is a paradoxical effect in that the administration of large numbers (5×10^7) of cells from the diabetogenic T clone does not induce hyperglycemia and in contrast, the cells prevent the onset of diabetes in NOD recipients. This observation that a large number of effector T cells is incapable of inducing autoimmune disease (Segel et al., 1995) highlights the complexity of the mechanisms involved in T cell-

mediated autoimmunity and suggests that the larger dose of cells initiates regulatory mechanisms that are not active in animals receiving fewer cells.

1.8.6 Antigenic targets of arthritogenic cells in AA

Although inoculation with CFA induces arthritis in rats, the nature of the specific antigen(s) that triggers the immune response that leads to joint inflammation is not known. If this target antigen(s) was known, it would allow identification of the arthritogenic cells, in terms of function and phenotype. Currently, the only method of examining the arthritogenicity of T cells is observing whether they are able to transfer the disease to naïve recipients. It has been suggested that cross-reactive antigens of *M.tuberculosis* trigger autoimmunity through molecular mimicry. Collagen has been suggested as another candidate arthritogen, because it may be presented immunogenically during the inflammatory reaction that occurs at the site of CFA inoculation.

Mycobacterial antigens are taken up and processed efficiently by DCs and mycobacteria stimulate the maturation and differentiation of these cells (reviewed in Demangel and Britton, 2000). The potent action of mycobacteria on DCs would be expected to promote the rapid initiation of a $CD4^+$ T cell-mediated response in the lymph nodes draining a site of inoculation of CFA and lead to their influx, proliferation and differentiation (Dresser et al., 1970; Taub et al., 1970).

In an attempt to understand the mechanisms involved in inducing arthritis using mycobacterial antigens, Van Eden et al (1983) generated T lymphocyte clones from rats with AA and looked for reactivity to joint antigens. The clone A2 was shown to induce arthritis when transferred to irradiated, naïve recipients and could protect against subsequent induction of AA when transferred to non-irradiated recipients. A sub-line of clone A2, known as A2b, showed reactivity to mycobacteria but unlike A2 (Van Eden et al., 1985), it did not react to collagen type II but it retained its arthritogenic capacity (Holoshitz,et al., 1984). These clones were shown to have specificity for the 65kD heat shock protein (HSP) of mycobacteria (Van Eden et al., 1988). Immunisation with recombinant 65kD HSP is able to confer protection against AA (van Eden et al., 1988) but not CIA (Billingham et al., 1990a), suggesting that the pathogenesis of the two diseases are under the control of different antigens and mechanisms (Hunt et al., 1993). Mycobacterial 70kD HSP has also been shown to modulate AA, indicating that the

modulatory effects are not restricted to the hsp65 series (Kingston et al., 1996; Tanaka, et al., 1999).

Oral tolerance to mycobacterial antigens by the administration of 65 kDa HSP reduced the severity and delayed the onset of AA (Haque et al., 1996). More effective oral tolerance that protects against AA and adoptively-transferred arthritis has been observed following the gastric administration of collagen type II (Zhang et al., 1990). Oral tolerization using collagen showed initial promise as a therapy for RA (Trentham et al., 1993).

Responses to collagen (types I and II) by T cells from rats with AA have been examined. Blood mononuclear cells from rats with AA proliferate at a greater rate in response to types I and II collagen and denatured type II collagen than those from normal rats, indicating that the arthritic rats have immunity to collagen of these types. Trentham et al (1980) considered their findings to support the hypothesis that an "injection of an adjuvant preparation in rats can trigger the expansion of lymphocyte clones which exhibit autoreactivity to collagen". The T cell response observed in the initial stages of AA may have been generated following macrophages processing collagen that is exposed early in the course of the disease. Zhang et al (1990) acknowledge that the mechanism for AA remains undefined but suggest two alternative mechanisms which would explain the role of collagen in AA. Either a pathogenic immunity to type II collagen develops in AA, or there are cross-reactive epitopes of type II collagen and mycobacteria. However, it is possible that reactivity to mycobacterial antigens induces joint damage and that this leads sequentially to a pathogenic immune response to type II collagen.

1.8.7 Cytokines in the pathogenesis of arthritis in animal models of RA

Investigations of the cytokines involved in animals models of RA have emphasised those involved in cartilage and bone destruction or those involved in recruitment of leukocytes into the synovium. Most of the studies have focussed on TNF-α and IL-1 (reviewed in Brennan, 1994).

There is substantial evidence that implicates TNF-α and IL-1 in the pathogenesis of arthritis (reviewed in Brennan, 1994). TNF-α and IL-1 have been detected in the synovial

tissue of mice with CIA, particularly in the zones of pannus formation and joint erosion (Marinova-Mutafchieva et al., 1997). Administration of IL-1 to rats and mice during the inductive phase of CIA accelerates the onset of joint inflammation (Hom et al., 1991) and blockade of IL-1 either during early or full-blown CIA ameliorates arthritis. Blockade of TNF-α during the early stages of CIA ameliorates disease but administration of anti-TNF-α antibodies to mice with established CIA does not reduce joint inflammation (Joosten et al., 1996). Administration of FR133605, a chemical inhibitor of IL-1 and TNF-α production, during the induction of AA suppresses joint inflammation and destruction (Yamamoto et al., 1997). Other drugs that target TNF-α have also had success in suppressing arthritis in animal models, indicating the importance of TNF-α in the pathogenesis of arthritis (Ross et al., 1997).

Studies that have examined the production of cytokines by T cells in animal models of RA have demonstrated a bias towards the production of Th1 cytokines, particularly during the onset of the disease (Mauri et al., 1996). Injection of IFN-γ into the hind paws of mice during the prodrome of CIA increases the severity of joint inflammation and suppresses the antibody response to collagen type II (Mauritz et al., 1988). Promoting a Th2 response by the administration of IL-4 reduced paw oedema in rats during the development of AA (Bober et al., 2000) and IL-10 has also shown potential as a therapeutic agent for treating arthritis (Bober et al., 2000; Setoguchi et al., 2000)

The role of IFN-γ in the development of AA is complex and it has been studied by the use *in vivo* of a neutralising antibody against IFN-γ (mAb DB-1) and recombinant (r) IFN-γ. There is evidence to suggest that IFN-γ plays a pro-inflammatory role during the early inductive phase of AA. Prophylactic treatment (starting at day −2 before induction) of animals with mAb DB-1 (5mg /rat i.p.) inhibits inflammation and delays joint destruction (Wiesenberg et al., 1989). Others (Jacob et al., 1989) have also demonstrated that a single dose of mAb DB-1 (1mg /rat i.p.) 24 hours before induction of arthritis leads to suppression of disease. However, IFN-γ may play multiple roles at different stages of the early inductive phase of arthritis, since a single dose of rIFN-γ given intraperitoneally, 24 hours before the injection of CFA exacerbates the arthritis whereas delivery of the rIFN-γ 24 hours after the inoculation of CFA leads to a suppression of disease (Jacob et al., 1989).

Interestingly, the treatment of AA with anti-IFN-γ that commences during the stage of clinical signs of arthritis at day 12 post-inoculation, exacerbates the disease (Jacob et al., 1989). When treatment with anti-IFN-γ commences on day 8 after inoculation of CFA, the early oedema of paws is suppressed in the period between days 12 and 14 post-inoculation but it then increases relative to untreated controls in the period after day 20 (Wiesenberg et al., 1989). Wiesenberg and colleagues argued that the induction of adjuvant arthritis was 'an IFN-γ specific immunological phenomenon' but they were unable to reconcile this statement with the paradoxical exacerbation of the disease when blockade of IFN-γ commenced later and they suggested that 'although unlikely, it is also possible that IFN-γ is required first for the induction and then for the self-limitation of the arthritic process'. The suggestion that IFN-γ has a role in down-regulating the joint inflammation during the later stages of AA has been supported by the results of Nakajima et al (1991), who demonstrated that treatment of arthritic rats with rIFN-γ suppresses the secondary phase of swelling from day 18 onwards but does not affect the earlier phase.

The aforementioned studies (Jacob et al., 1989; Wiesenberg et al., 1989; Nakajima et al., 1991) indicate that IFN-γ can have both pro- and anti-inflammatory effects on joint inflammation in AA at the different stages of this disease. There are no published studies to date that separate the effects of IFN-γ on the inductive and effector stages of AA.

The disease-promoting effects of IFN-γ are well documented, for example IFN-γ is a potent stimulator of macrophages (Pace et al., 1983). Many of the products of stimulated macrophages have inflammatory properties, although the effects of these products may be determined by their quantity. The production of nitric oxide (NO) by macrophages is induced by IFN-γ, which up-regulates the production of inducible nitric oxide synthase (iNOS). There is evidence to suggest that NO plays a role in the pathogenesis of many diseases, for example, reactive NO intermediates have been correlated positively with severity of EAE (Lin et al., 1993), while inhibitors of iNOS delay disease onset (Cross et al., 1994). NO is thought to be involved in joint inflammation in AA since administration of iNOS inhibitor to rats during the induction of AA lowers the severity of AA, by suppressing swelling and joint destruction (Stefanovic-Racic et al., 1994).

Although it is apparent that NO is involved in the pathogenesis of many inflammatory diseases, there is evidence that NO may also be involved in the healing process. Studies in EAE have indicated that NO is critical for the recovery from EAE in rats (O'Brien et al., 1999) and that IFN-γ is implicated in the production of NO that is necessary for the down-regulation of EAE in mice (Willenborg et al., 1999). Furthermore, inhibition of iNOS during streptococcal cell wall- induced arthritis increased the erosion of joints and failed to inhibit synovial lesions, although there was some suppression of the acute arthritis (McCartney-Francis et al., 2001). A mechanism by which NO may promote disease recovery is via the inhibition of IFN-γ production by T cells and thereby indirectly promoting the bias of cytokine production towards that typical of Th2 cells (Roozendaal et al., 1999). It is interesting to note that NOS inhibitors can have opposite effects on inflammation, depending on the route of administration (Paul-Clark et al., 2001). Similarly, IFN-γ that is produced locally may have disease-promoting properties whereas IFN-γ that is released systemically might have an over-riding effect that is protective, either by inhibiting the proliferation of certain MNCs, the induction of suppressor cells or by other mechanisms such as the induction of NO.

As is the case in AA, IFN-γ plays multiple roles in CIA. Injection of rIFN-γ into the joints of mice during the development of CIA accelerates the disease onset and increases the severity of joint inflammation (Mauritz et al., 1988), suggesting that IFN-γ can play a role in triggering joint inflammation. However, there is evidence that the actions of IFN-γ are not necessary for the development of this disease. For example, CIA can be induced in mice that are deficient in IFN-γR, where it has an earlier onset and increased severity compared with the disease in wild type litter mates (Manoury-Schwartz et al., 1997; Vermiere et al., 1997). Interestingly, the omission of mycobacteria from the inoculation of collagen to induce CIA prevents the development of CIA in IFN-γR null mice and reduces the susceptibility of wild type mice such that arthritis developed in ~25% of wild type mice compared with ~65% using the standard inoculating dose (Matthys et al., 1999). In light of these results and the observation that Mac-1$^+$ cells are expanded and activated in response to mycobacteria and that this process is curbed by IFN-γ, Matthys et al (1999) have suggested that mycobacteria in CFA stimulates myelopoiesis and possibly the expansion of Mac-1$^+$ cells that act as effector cells and that IFN-γ normally restrains the expansion of these cells (Matthys et al., 1999). In the case of CIA induced without

mycobacteria, it would appear that IFN-γ is required for development of arthritis, perhaps because it is necessary to up-regulate both humoral and cellular immunity to collagen type II whereas when mycobacteria are included in the inoculating dose they provide sufficient stimulus to activate the immune response to collagen in the absence of IFN-γ.

1.9 Hypotheses and Aims

The aims of this project were to investigate the following hypotheses:

1. The delay in onset of AA after inoculation of CFA (9-10 days) or adoptive transfer of arthritogenic thoracic duct lymphocytes (4-6 days) suggests that progressive differentiation or selection of effector cells is required before disease can be expressed.

2. The expression of activation markers and the production of cytokines by CD4$^+$ T cells will be dependent on the time after inoculation of CFA (actively-induced disease) and the location of the cells.

3. The CD4$^+$ T cells, which initiate arthritis, produce Th1 cytokines such as IFN-γ.

4. Treatment of rats with neutralising anti-IFN-γ antibodies will suppress the generation of arthritogenic cells and the induction of adoptively-transferred arthritis.

The following aims investigated these hypotheses:

1. Development of a methodology for detecting the cytokine production by individual T lymphocytes, which reflects the potential of these cells to produce cytokine *in vivo*.

2. Examination of activation markers and adhesion molecules expressed by T cells from rats at different stages during the development of AA, using flow cytometry.

The following sites were of particular interest:

(a) Inguinal lymph nodes – these nodes drain the site of inoculation and it was hypothesised that this would be the location in which the inciting antigens were first presented to T cells.

(b) Thoracic duct (TD) lymph - it is thought that arthritogenic T cells travel from the lymph nodes, via the efferent lymphatics into the blood and then to the joints where they initiate arthritis.

(c) Hind paws - the most commonly affected joints in AA.

(d) Popliteal lymph nodes - these nodes drain the hind paws and may receive APCs that carry synovial target autoantigens.

3. Examination of the profile of cytokines produced by T cells from TDL, inguinal lymph nodes, popliteal lymph nodes and synovial digests from the hind paws of rats at different stages of AA.

4. Examination of the effects of administering anti-IFN-γ antibodies to donors of arthritogenic lymphocytes or to recipients, either before or after the transfer of arthritogenic T cells, on the development of arthritis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Female Dark Agouti (DA) rats aged 6 weeks were obtained from the Gilles Plain Animal Resource Centre, South Australia. Hooded Wistar rats were obtained from the Animal Resource Centre, Perth. Animals were housed in the Basement Animal House of the Institute of Medical and Veterinary Science or Medical School Animal House, University of Adelaide. Food and water were available *ad libitum*. Anaesthetia was induced using a chamber attached to a Midget 3 Anaesthetic apparatus (CIG Medishield-Ramsay, SA, Australia), using either an Ohmeda Fluotec 3 (BOC Health Care, UK) to dispense Fluothane (Zeneca Ltd, Cheshire, UK) or an Ohmeda Isotec 3 to dispense Isoflurane (David Bull Laboratories, Vic, Australia) and maintained using a mask attached to the same apparatus. Animals were anaesthetized during arthritic scoring to allow thorough examination of paws and for injection of adjuvant or cells.

2.2 Induction and monitoring of arthritis in rats

2.2.1 Induction of adjuvant-induced arthritis (AA)

Complete Freund's Adjuvant (CFA) was prepared by grinding 10mg of heat killed *Mycobacterium tuberculosis* H37 RA (Difco, cat. no. 3114-33-8) using a mortar and pestle until the powder was translucent in appearance (approximately 30 minutes of grinding). Ten mls of Incomplete Freund's Adjuvant (Difco, Cat. no. 0639-60-6) was added slowly whilst continuing to grind, until the mixture was uniform in colour and consistency. The resulting CFA was stored in 1ml aliquots at either 4°C or -20°C. Arthritis was induced in 7-8 week old rats by subcutaneous injection of 100µl of CFA at the base of the tail, using a 23 gauge needle attached to a 1ml syringe. The weight and well-being of the rats were assessed daily.

2.2.2 Adoptive transfer of arthritis

Arthritis was transferred adoptively to naive 7 week old rats by intravenous injection (into the tail vein) of thoracic duct (TD) lymphocytes obtained from donors 9 days after inoculation of CFA (see Section 2.2.1). The TDs of the donors were cannulated on the morning of the ninth day after inoculation (or as otherwise stated) and lymph was collected overnight into heparinized PBS (see Section 2.3.3). Typically, donor cells were pooled and TD lymphocytes equivalent to the overnight output from a single donor were

resuspended in approximately 1ml of RPMI 1640/2% FCS before injection into recipient rats. The weight and well-being of the rats were monitored daily.

2.2.3 Measuring the severity of arthritis

Hind- and fore-paws of rats were monitored daily for the presence of articular and soft tissue swelling and scored on a system based on that described by Waksman et al., (1960), such that:

> 0 = no signs of inflammation
>
> 1 = a single focus of inflammation
>
> 2 = more than one focus of inflammation
>
> 3 = more than one focus of inflammation but also including generalised swelling
>
> 4 = swelling of the entire paw.

The maximum score that can be given for a single rat is 16 (ie. a score of 4 for each of the 4 paws).

2.2.4 Skeletal radiography

Rats were X-rayed using a mammograph in the Radiology Department of the Royal Adelaide Hospital.

2.2.5 Infestation of rats with *Nippostrongylus brasiliensis*

N.Brasiliensis larvae were obtained from cultures of faeces from *N.Brasiliensis*-infested rats. After washing in saline, the larvae were counted using a grid with the aid of a dissection microscope. After resuspension at a concentration of 3000-4000 larvae per ml of saline, rats were inoculated subcutaneously in the dorsum of the neck with 1500-2000 larvae. Infested rats were housed in isolation from other rats to prevent cross-infection with *N.brasiliensis*.

2.3 Preparation of cell suspensions

2.3.1 Preparation of cells from lymph nodes

Lymph nodes (either popliteal or inguinal as stated) were removed from euthanased animals using blunt dissection with forceps and placed in a small volume of PBS (see Section 2.4.1) containing 1% (v/v) fetal calf serum (FCS) (MultiSer™, Cytosystems, Castle Hill, NSW, Australia) which had been previously heat inactivated by incubating at

56°C for 45 minutes. The tissue was chopped finely with scissors and transferred to a loose fitting glass homogeniser. Following gentle homogenisation, the suspension was filtered through cotton wool and centrifuged at 200g for 10 minutes in a Beckman GPR centrifuge (Beckman, Palo Alto, CA, USA). The resulting cell pellets were washed twice in PBS containing 1% FCS and then resuspended in this solution. Cell number and viability was ascertained using a haemocytometer and Trypan blue dye exclusion (10µl of cell suspension was mixed thoroughly with 10µl Trypan Blue solution 0.4%, Sigma Chemical Co., USA, and then 10µl of the mixture was placed under the haemocytometer cover-slip by capillary action).

2.3.2 Preparation of cells from hind paws

An encircling incision was made in the skin around the circumference of the upper thigh. A further incision was made through the skin on the anterior of the leg, from the middle toe to join the first incision at the top of the leg. The skin was pulled from the leg and released from the entire paw, using a scalpel to release the skin from the heel and sole regions. Muscles and tendons were incised around the entire circumference of the tibia immediately distal to the musculotendinous junction of the muscles of the posterior compartment. The muscles were scraped from the bone using a scalpel, such that the tibia was free from muscle from the point of division above the ankle to above the knee. The tendons and ligaments attached to the knee were cut and the knee was dislocated carefully without damage to the tibia thus avoiding release of bone marrow during subsequent steps. A scalpel was used to divide the soft tissues between the metatarsal bones. The pair of skinned paws from one rat (including soft tissues and the tibiae) was placed into a 20ml polystyrene tube (Sarsedt, SA, Aust.) containing 9ml of RPMI 1640. One millilitre of Collagenase CLS (52kU/ml dissolved in RPMI 1640) (Worthington Biochemical Corp., NJ, USA) and 100µl of hyaluronidase HSEP (10kU/ml dissolved in PBS) (Worthington Biochemical Corp., NJ, USA) was added and the tube was then incubated at 37°C for 45-60 minutes with agitation. Liquid from the digested hind paws was filtered through a cotton wool column. Undigested pieces of soft tissue were transferred to a loose fitting glass homogeniser. Following gentle homogenisation, the suspension was filtered through cotton wool and pooled with the first filtrate. The resulting single cell suspension was centrifuged at 200g for 10 minutes and then washed thrice in RPMI 1640 + 2% FCS. Cell viability was calculated using a haemocytometer and Trypan blue dye exclusion.

2.3.3 Collection of thoracic duct lymphocytes

Cannulae were constructed using 40-50cm lengths of polyethylene tubing (0.5mm internal diameter and 1.0mm outside diameter) (Dural Plastics & Engineering, Dural, N.S.W., Australia, Cat. No. SP 37). The tubing was shaped into a U-bend at one end by the application of heat. A bevel was cut at the end of the short arm to enable ease of insertion into the duct.

Prior to surgery, the animal was anaesthetised and the abdomen shaved on its ventral and left hand side. A subcostal incision was made on the left hand side from the lateral border of the rectus muscle to the mid-axillary line, approximately 0.5cm below and parallel to the rib cage. Cotton buds were then employed to displace the left kidney and surrounding fat tissue to expose the aorta. Surrounding tissues were then displaced medially and held in place by cotton gauze soaked in saline, supported by a retractor. The thoracic duct was then dissected away from the aorta and a length of surgical silk placed around the duct and tied loosely. The cannula was passed through a 12G needle inserted through the dorsal body wall adjacent to the tie on the thoracic duct. After removal of the needle, the cannula was filled with 1u/ml heparin (Sigma, St Louis, MO, USA) in PBS and clamped at the distal end. The cannula was anchored loosely to the back wall of the animal at the point of exit with a silk tie through a strand of muscle.

The thoracic duct was then engorged by applying pressure below the diaphragm and a small cut made part way through the duct just anterior to the silk thread. After insertion of the cannula, the silk tie around the duct was secured firmly and the cannula secured in place to the dorsal abdominal wall by the second silk tie. After removal of the retractors and gauze, the organs were returned to their original position and the incision was closed with two layers of continuous silk sutures. The animal was then placed in a Bollman cage and provided with food and isotonic saline to drink.

Thoracic duct lymph was collected overnight into 5ml of sterile PBS containing 25u/ml heparin (Sigma, USA) in a 200ml tissue culture flask (Nunc, Denmark). The collection was performed at room temperature. After resuspension of the cells, the lymph was filtered through cotton wool and centrifuged at 200g for 10 minutes. The lymphocytes

were washed twice with PBS/2%FCS and a viable cell count performed using a haemocytometer and Trypan blue dye exclusion.

2.4 Media and tissue culture procedures

All cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air (IR AUTOFLOW CO_2 water-jacketed Incubator, Nuaire).

2.4.1 Balanced salt solutions

Dulbecco's Solution A or Phosphate buffered saline (PBS), pH 7.4, contained 0.14M NaCl, 3mM KCl, 8mM $Na_2HPO_4.12H_2O$ and 1mM KH_2PO_4 (either BDH AnalaR$^{®}$ from Merck, Kilsyth, Victoria, Australia or 'Univar' from Ajax Chemicals, Auburn, NSW, Australia) dissolved in Milli-Q purified water (Millipore Corporation, USA). PBS was prepared as a 20x concentrate: 160g NaCl, 4g KCl, 58g $Na_2HPO_4.12H_2O$ and 4g KH_2PO_4 were dissolved in 900 ml of Milli-Q water. The pH was adjusted with 1M HCl (BDH AnalaR$^{®}$, Merck, Australia) and the volume made up to 1L. The solution was diluted 1/20 in Milli-Q water prior to use.

Dulbecco's solution A+B (DPBS) was prepared by diluting 50ml of PBS 20x concentrate in 750ml of MQ water and adding 100ml MQ water containing 0.1g $CaCl_2$ (Univar Ajax Chemicals, NSW, Aust), 100ml MQ water containing 0.1g $MgCl_2$ (AnalaR, BDH, Aust) and 1g of D-glucose (AnalaR BDH Chemicals, Aust).

2.4.2 Preparation of culture medium

Roswell Park Memorial Institute (RPMI-1640) culture medium was used in all cell culture work, except when culturing CHO cells (see Section 2.4.2). RPMI 1640 was prepared by dissolving a sachet of RPMI-1640 powder (Gibco BRL, Gathersburg, MD, USA) and 2g $NaHCO_3$ ('Univar', Ajax Chemicals, Australia) in approximately 900ml Milli-Q purified water (Millipore Corporation, USA). Sterile stock solutions of N-2-Hydroxyethylpiperazine N'-2-ethanesulphonic acid (HEPES) (Boehringer Mannheim, Germany) pH 7.2, penicillin (Sigma, St Louis, MO, USA) and streptomycin sulphate (Sigma, USA) were added to give final concentrations of 15mM, 67µg/ml and 100µg/ml

respectively. After an adjustment of the pH to 7.4 with 1M HCl, the volume was made up to 1 litre and the medium was sterilised using a Sterivex GS 0.22μm filter unit (Millipore, USA).

Prior to use medium was supplemented with 2mM glutamine (BDH AnalaR®, Merck, Australia) and 10% v/v heat inactivated FCS (MultiSer™, Cytosystems, Australia). After 7 days of storage medium was re-supplemented with glutamine (2mM final concentration).

2.4.3 Culture of CHO cells

Chinese Hamster Ovary (CHO) cells transfected with multiple copies of either the gene encoding rat interleukin 4 (IL-4) or that encoding rat IL-2 were a kind gift from DR. A. N. Barclay MRC Cellular Immunology Unit, University of Oxford, UK. The gene insertion is flanked with a methionine sulphaximine resistance gene and a glutamine synthetase gene and hence the transfectants containing the construct could be selected for by the inclusion of methionine sulphaximine and exclusion of glutamine from the culture medium.

CHO-IL-4 cells were cultured in Glasgow Modified Eagle's Medium (GMEM) (Advanced Protein Products Ltd, UK). GMEM does not contain glutamine. Prior to use, medium was supplemented with 25μM methionine sulphaximine (Sigma, USA) and 10% v/v heat inactivated FCS (MultiSer™, Cytosystems, Australia). Penicillin (Sigma, St Louis, MO, USA) and streptomycin sulphate (Sigma, USA) were added to give final concentrations of 67μg/ml and 100μg/ml respectively.

Non-transfected CHO cells were used as control cells. These cells were cultured in GMEM supplemented with glutamine (or in RPMI 1640) plus 10% v/v heat inactivated FCS.

2.4.4 Harvesting CHO cells from tissue culture flasks

Adherent CHO cell cultures were washed with sterile PBS and then flooded with approximately 3 ml of trypsin-versene solution (CSL Ltd, Aust.) and incubated for approximately 5 minutes in a 37°C incubator. The flask was then tapped gently to

dislodge any cells that remained attached to the flask. The trypsin was neutralised by the addition of 7ml of PBS, the cell suspension was centrifuged at 200g for 10 minutes and the cell pellet was washed thrice in PBS/2% FCS (cells that were re-cultured were washed thrice in culture medium rather than PBS/FCS). Cell viability was assessed using a haemocytometer and Trypan blue dye exclusion.

2.5 Antibodies

2.5.1 Primary antibodies and fusion proteins

Mouse anti-rat monoclonal antibodies (mAbs) used in flow cytometric and immunohistochemical studies are listed in Table 2.1. Purified P-selectin-IgG fusion protein (PharMingen, USA, Cat # 28111A) was used to detect the P-Selectin ligand (PSGL-1) in the procedure described in 2.6.1. The fusion protein incorporates the signal sequence, the lectin domain, the EGF-like repeat and the first two complement-binding domains of mouse P-selectin fused to the Fc region (hinge, C1 and C2) of human IgG1 (Hahne et al., 1993).

The following phycoerythrin (PE)-conjugated mouse anti-rat mAbs were purchased from PharMingen (San Diego, Ca, USA) and used in 2-colour flow analyses: R73-PE (anti-rat α/β TCR), OX35-PE (anti-rat CD4), OX38-PE (anti-rat CD4), OX33-PE (anti-rat CD45RA) and OX8-PE (anti-rat CD8α). PE-conjugated mAb 107.3 (anti-TNP) was used as a negative control.

In order to block non-specific binding through Fc receptors, primary antibodies were always used in the presence of 10% normal rat serum (NRS), which had been heat inactivated at 56°C for 45 minutes.

2.5.2 Conjugated secondary antibodies

Secondary antibodies without reactivity for rat Ig, were used to detect mouse mAbs. A FITC-conjugate of affinity-purified goat anti-mouse Ig (PharMingen, Cat No. 12064D) and a PE-conjugate of F(ab')2 goat anti-mouse IgG (Rockland, Gilbertsville, PA, USA, Cat. No. 710-1831) were used for indirect immunofluorescent labelling of cells for flow cytometry and immunofluorescent staining of cytospins. FITC-Sheep anti-human Ig (H &

Table 2.1 Monoclonal antibodies used for staining cells by the indirect technique

TABLE 2.1: Mouse anti-rat monoclonal antibodies for use in flow cytometric and immunofluorescence analysis of samples.

Antibody	Specificity	Distribution on leukocytes	Form	Conc.	Isotype	Source /Reference
W3/25	CD4	Subset of T cells, thymocytes, mφ, DC	S/N	neat	IgG1	Hybridoma/White et al., 1978
OX8	CD8α-chain	subsets of T cells, thymocytes, NK cells	S/N	neat	IgG1	Hybridoma/Brideau et al., 1980
341	CD8β-chain	subsets of T cells, thymocytes,	S/N	neat	IgG1	Hybridoma/Torres-Nagel et al., 1992
WT-1	CD11a (α chain of LFA-1)	majority of leukocytes	S/N	neat	IgG2a	Hybridoma/Tamatani et al., 1991
WT-5	CD11b (α chain, Mac-1)	NK cells, mφ, DC, granulocytes	S/N	neat	IgA	Hybridoma/Tamatani et al., 1993
OX39	CD25 (IL-2R α chain)	Activated T cells, activated B cells	S/N	neat	IgG1	Hybridoma/Paterson et al., 1987
OX22	CD45RC	CD8$^+$ T cells, NK cells, subset of CD4$^+$ T cells	S/N	neat	IgG1	Hybridoma/Spickett, et al., 1983
1A29	CD54 (ICAM-1)	B cells, subset of T cells	S/N	neat	IgG1	Hybridoma/Tamatani et al., 1990
OX26	CD71 (transferrin R)	Dividing/proliferating cells	S/N	neat	IgG2a	Hybridoma/Jefferies et al., 1985
OX40	CD134 (OX40Ag)	Activated CD4$^+$ T cells	S/N	neat	IgG2b	Hybridoma/Paterson et al., 1987
OX62	Integrin α$_{E2}$ chain	DC, IEL, dendritic epidermal T cells	S/N	neat	IgG1	Hybridoma/Brenan et al., 1992
10/78	CD161 (NKR-P1)	NK cells, subset of T cells, mφ, DC,	S/N	neat	IgG1	Hybridoma/Kraus et al., 1996
OX6	MHC class II	B cells, mφ, DC, activated T cells	S/N	neat	IgG1	Hybridoma/McMaster and Williams, 1979
R73	α/βTCR	α/β Tcells	S/N	neat	IgG1	Hybridoma/Hunig et al., 1989
V65	γ/δ TCR	γ/δ T cells	S/N	neat	IgG1	Hybridoma/Kühnlein P, 1994
OX81	Interleukin-4	Produced by CD4$^+$ T cells, NK cells and activated mast cells	S/N	neat	IgG1	Hybridoma/Ramirez et al., 1996
UA002	unknown	B cells, mφ, DC, activated T cells	S/N	neat	IgG1	Hybridoma/G.Mayrhofer (unpublished)
1B5	Giardia surface antigen	Giardia (negative control)	S/N	neat	IgG1	Hybridoma/G.Mayrhofer (unpublished)
OX85	CD62L (L-selectin)	B cells, subset of T cells, neutrophils	S/N	neat	IgG1	Hybridoma/Seddon et al., 1996.
DB-1	Interferon(IFN) γ	Produced by CD4$^+$ T cells, CD8$^+$ T cells and NK cells	Purified	2.5μg/ml	IgG1	Serotec/Van der Meide et al., 1986
MRα4-1	CD49d (Integrin α$_4$ chain)	T cells, B cells, thymocytes, mast cells, mφ	Purified	0.5mg/ml	IgG2a	PharMingen/Yasuda et al., 1995

S/N = supernatant of hybridoma cells, Ag = antigen, mφ = macrophages

L) (Silenus, Amrad, Vic, Australia) and PE-goat anti-Human IgG Fcγ fragment (Jackson Immunoresearch Laboratory Inc, cat. No. 109-116-098) were used for the detection of P-selectin-IgG fusion protein (PharMingen, USA, Cat # 28111A).

Affinity purified F(ab')$_2$ sheep-anti-mouse Ig conjugated to horse radish peroxidase (Amersham Life Science, Buckinghamshire, England, Cat No. NA 9310) was used in the indirect immunoperoxidase technique for immunohistochemistry. This antibody shows no cross-reactivity for rat Ig.

All antibodies were used in the presence of 10% NRS to block any residual reactivity for rat Ig.

2.5.3 Monoclonal antibody DB-1 (anti-IFN-γ) for *in vivo* administration

DB-1 is a mouse IgG1 monoclonal antibody that binds rat IFN-γ with high affinity (Van der Meide et al., 1986). Six milligrams of DB-1 (BPRC Lot#3-15) was reconstituted in 5ml of water and further diluted in saline to allow 0.8mg/0.5ml to be administered *in vivo* as an intraperitoneal (i.p.) injection.

2.5.4 Preparation of polyclonal IgG from normal mouse serum

A stock solution of ammonium sulphate was prepared by adding 100g of $(NH_4)_2SO_4$ crystals per 100ml of distilled water after which the solution was stirred for 1-2 days using a magnetic stirrer at room temperature. The pH of the saturated solution was adjusted to 7.0 before storage at 4°C.

To precipitate gamma globulin from normal mouse serum, 20ml of serum was placed in a beaker on a magnetic stirrer and 10ml of saturated $(NH_4)_2SO_4$ solution was added slowly (drop wise). This solution was stirred for at least 2 hours at 4°C and then sedimented by centrifugation at 10,000g (Beckman Model J2-21M Induction Drive Ultracentrifuge, Beckman, USA) for 10 minutes. The pellet was resuspended in 5ml of PBS to which 2.5ml of saturated $(NH_4)_2SO_4$ solution was added slowly. After stirring for at least 2 hours at 4°C the precipitate was centrifuged at 10,000g for 10 minutes. The pellet was resuspended in 10ml of PBS and transferred to a dialysis tube for dialysis over a period of 24 hours involving 3 changes of 2 litres of PBS. The dialyzed material was filtered

through a 0.2μm filter (Minisart 0.2μm filter, Sartorius, Germany) and the concentration of protein was determined spectrophotometrically by absorbance at 280nm (DMS 100S UV visible spectrophotometer, Varian). The solution was diluted in PBS to give a final concentration of 1.6mg/ml. An injection of 0.5ml of this solution of polyclonal IgG (0.8mg) was administered intraperitoneally.

2.6 Labelling cells for flow cytometric analyses

2.6.1 Labelling cells with monoclonal antibodies (mAbs) by the indirect technique

All labelling steps were performed at on ice. Cells were dispensed in aliquots of 1-1.5 $x10^6$ cells per FACS tube (Falcon, Becton Dickinson Labware, New Jersey, USA, Cat. No. 2008) and centrifuged for 10 minutes at 200g. Pellets were resuspended in either 50μl of neat culture supernatant or diluted purified mAb (prepared as per Table 2.1), containing 0.01% (w/v) sodium azide ('Labchem', Ajax Chemicals, Australia) (PBS/FCS/Az) and 10% (v/v) NRS. The cells were then incubated on ice for 45-60 minutes. They were then washed twice in 2ml aliquots of PBS/FCS/Az and resuspended in 50μl of secondary antibody (1/100 dilution of either of the secondary antibodies described in 2.5.2) containing 10% NRS. After incubation for 45 minutes, the cells were washed as described above, resuspended and fixed in approximately 750μl of "FACSFix" (10ml formalin, 20g glucose, 0.2g NaN_3 1L PBS). The fixed cells were then stored at 4°C in the dark before being analysed (typically on the same day or within 24 hours).

2.6.2 Labelling cells for dual fluorochrome analysis

The first stage of dual fluorochrome labelling is identical to the indirect labelling procedure described in Section 2.6.1. At completion of this stage, the cells were washed twice in PBS/FCS/Az as described and then resuspended in 5μl of neat heat inactivated normal mouse serum (NMS) for 10 minutes on ice to block any free valances of the bound anti-mouse immunoglobulin conjugate. Without washing, 50μl of PBS/FCS/Az containing 0.2-0.3 μg/ml of the conjugated mAbs (PharMingen) was added to the resuspended cells, which were then incubated for 45-60 minutes on ice. The cells were then washed twice in PBS/FCS/Az and fixed by resuspension in approximately 750μl of

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"FACSFix". The fixed cells were stored at 4°C in the dark prior to analysis (typically on the same day or within 24 hours).

2.6.3 Detection of intracellular cytokines after stimulation of lymphocytes *in vitro*

IFN-γ and IL-4 protein production by lymphocytes was determined using a method modified from Assenmacher (1994). Lymphocytes obtained from LN (Section 2.3.1), TDL (Section 2.2.5) or prepared by enzymatic digestion of hind paws (Section 2.3.2) were cultured in RPMI 1640 (Gibco BRL) supplemented with 10% FCS (MultiSer™, Cytosystems) and 2mM L-glutamine (BDH) (prepared as in Section 2.4.1) for 6 hours (or as otherwise stated) at 37°C in the presence of 10µg/ml Brefeldin A, 100µM calcium ionophore A23187 (ionomycin) and 50ng/ml phorbol myristateacetate (PMA) (all of which were purchased from Sigma, USA). Equal numbers of cells were also cultured in the absence of PMA plus ionomycin. In studies on CHO cells transfected permanently with a construct containing the rat gene encoding IL-4, the cells were incubated in the presence of 10µg/ml Brefeldin A but not PMA or ionomycin. In studies examining the production of RNA during stimulation *in vitro* (Section 3.2.5.2), actinomycin D or cycloheximide were added to the culture medium at a final concentration of 5µg/ml and 20µg/ml respectively, as stated. Cells were then harvested, washed twice in DPBS and the resulting cell pellets were resuspended in 1ml of DPBS and fixed by the addition of 1ml of 10% buffered formalin. After fixation for 6 minutes, the cells were washed three times in 1%FCS/DPBS, counted and stored overnight at 4°C. They were then aliquoted at 1×10^6 cells/tube and incubated with primary antibody (mAb OX81 [anti-IL-4], mAb DB-1 [anti-IFN-γ] or mAb 1B5[isotype control]) containing 10% NRS and 0.1% w/v saponin (Sigma) for 45-60 minutes. After two washes with DPBS/1% FCS/0.1% saponin the cells were incubated with FITC-conjugated goat anti-mouse Ig (PharMingen) in the presence of 10% NRS and 0.1% saponin for 45 minutes. After a further two washes in DPBS/1% FCS/0.1% saponin, followed by one wash in DPBS/1%FCS, the cell pellet was resuspended in 5µl of neat NMS and incubated for 10 minutes on ice prior to direct staining with PE-conjugated antibodies (40µl of a 1/50 dilution of anti-TNP, anti-CD4 or anti-α/β TCR) for 45 minutes. The cells were then washed twice in DPBS/1% FCS and resuspended in 500µl of PBS for immediate analysis.

2.7 Flow cytometry

Labelled cells were analysed using a FACScan Flow cytometer (Becton Dickinson, San Jose, Ca.) equipped with CellQuest software (version and 3.1f). Lymphocyte populations were gated on the basis of their forward (FSC) and side scatter (SSC) characteristics and events were collected from a gate containing all such cells and separately from gates limited to either small or large lymphocytes. The gates used to distinguish small and large lymphocytes were drawn arbitrarily on the basis of the light scatter characteristics of TDL and lymph node mononuclear cell preparations from normal rats. When examining LN or TDL cells at least 10,000 events were collected from the total lymphocyte gate and at least 5,000 events were collected from the 'lymphoblast' gate. In the case of cells obtained from enzymatic digestion of non-arthritic hind-paws, 100,000 total events and 1,000 events in the "lymphocyte gate" were collected typically, depending on the cell populations present. Typically, between 50,000 and 80,000 total events were collected when examining cells obtained from arthritic hind paws because lymphocytes represent a greater proportion of the total population of cells in these digests than in digests from non-arthritic hind paws.

2.7.1 One-colour flow cytometry

The negative control was used to optimise instrument settings and background fluorescence levels. Gates were positioned using the forward scatter (FSC) versus side scatter (SSC) dot plot, corresponding to the lymphocyte populations. Background fluorescence was defined as that part of the negative peak, which contained 98-99% of the recorded events falling in the lymphocyte gate.

2.7.2 Two-colour flow cytometry

The setting up procedure involved the use of two negative control antibodies, representative of the two fluorescence channels used (FL-1: FITC signal and FL2: PE signal). Gates were set as described above and background fluorescence levels for both channels adjusted with the aid of a FL-1 versus FL2 dot plot. Compensation was made for the overlap of the FL-1 signal into the FL2 channel using a brightly stained (FITC) positive control sample and the minimal overlap of the FL2 signal into the FL-1 channel was compensated for using a brightly stained (PE) positive control sample.

2.8 Analysis of RNA production

2.8.1 Labelling newly-synthesised RNA with tritiated uridine

Cells were cultured in triplicate in 96-well trays *in vitro* using a similar protocol to that described in Section 2.6.2, although at a final volume of 250µl and with the addition of [5,6-^{3}H] Uridine (Amersham Biotech Cat. TRK 410) at a final concentration of 4mCi/ml.

2.8.2 Measuring incorporation of tritiated uridine by scintillation counting

Cells were harvested on to glass fibre disks (Skatron Instruments Cat. 11721) using a Skatron semi-automatic cell harvester. These glass fibre disks were then placed in Optiphase HiSafe liquid scintillation cocktail (Wallac, Finland). A β counter was used to count the scintillation generated by each of the glass fibre disks containing the harvested cells and the incorporated radioactivity was measured as counts per minute.

2.9 Preparation of tissue samples for histology and immunohistochemistry

2.9.1 Slow decalcification of paws for frozen sections

A technique has been developed in the Arthritis Research Laboratory (L. D. J. Spargo, unpublished) to prepare whole paws for frozen sectioning, based on a method described by Jonsson et al., (1986). Paws were removed from dead rats by cutting the lower tibia, using bone cutters. The skin was scored with a scalpel on both the anterior and posterior surfaces and the paws were then fixed in 2 changes cold 96% ethanol at 4°C, while mixed by rotation for 24 hours (all subsequent processing was performed at 4°C and on a rotor). The paws were then washed in 2 changes of cold 0.1M Tris (Tris [hydroxymethyl]aminomethane, Sigma, USA) in PBS, pH 6.95 over a period of 24 hours. Decalcification of the paws was achieved by immersing the fixed paws in Tris-EDTA-PVP pH 6.95 (0.1M Tris, 10%w/v ethylenediaminetetra-acetic acid (EDTA) (Sigma, USA), 7.5% w/v polyvinylpyrrolidone, ICN Biomedicals Inc, USA) for approximately 2 weeks, with changes of Tris-EDTA-PVP twice per week. The process of decalcification was monitored and confirmed by x-ray photography. When complete decalcification of all bones had occurred, the paws underwent three steps of infiltration. Firstly, with a 20% (w/v) sucrose solution (AnalaR, BDH Chemicals, Aust); secondly, with a solution

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containing equal parts of sucrose solution and OCT, containing 2.5% Dimethyl sulphoxide (DMSO) (AnalaR BDH Chemicals, Aust.) and finally, with neat OCT containing 2.5% DMSO. Each infiltration step was performed for a period of 24 hours. Paws were then bisected using a scalpel and embedded in OCT as described in Section 2.9.2.

2.9.2 Preparation tissue blocks for sections

Fresh tissue samples were embedded in OCT compound (Tissue-Tek, Miles Inc., Elkhart, IN, USA) and paws that had been decalcified slowly (see Section 2.9.1) were embedded in OCT compound containing 2.5% DMSO. The tissue in OCT or OCT containing 2.5% DMSO, was snap frozen in iso-Pentane ('Univar', Ajax Chemical Company, Auburn, NSW, Australia) chilled over liquid nitrogen. All frozen tissue samples were stored at -70°C until required.

2.9.3 Rapid decalcification of hind paws for paraffin embedding

Paws were removed from dead rats using bone-cutters and scissors and the skin was scored with a scalpel on both the anterior and posterior sides. The process of fixation and rapid decalcification was performed at room temperature in 20ml tubes (Sarsedt, SA, Aust.) with agitation on a shaker. The paws were fixed overnight in 10% formalin solution and then washed overnight in PBS. Paws were then immersed overnight in HNO_3 solution (1%w/v Na_2EDTA, 10% v/v concentrated HNO_3). The process of decalcification was monitored and confirmed using x-ray photography. When decalcification of all bones was completed, the paws were embedded in paraffin (Paraplast tissue embedding medium, Oxford Labware, Sherwood Medical, MO, USA) and sections cut using a Leitz 1512.

2.9.4 Preparation of cytospins

Cytospins of CHO cell suspensions were prepared by loading 1×10^5 cells in 100µl of RPMI/50%FCS into the cell chambers of a Cytospin machine (Shandon Southern). The cells were collected onto subbed glass slides. The Cytospin smears were allowed to dry in air before fixing and staining (see Section 2.10.3).

2.10 Indirect immunoperoxidase staining of cells and tissue sections for immunofluorescence and immunohistochemistry

2.10.1 Indirect immunoperoxidase technique

Tissue sections were cut at 5μm from frozen tissue blocks using a JUNG CM3000 cryostat (Leica, Germany) and collected onto glass slides. After air-drying, the sections were either used immediately or stored at −70°C until required. The slides were allowed to equilibrate to room temperature (if they had been stored) and a circle was drawn around the section with a PAP PEN (Zymed, San Francisco, CA, USA). They were then fixed in 95% ethanol for 10 minutes, washed 3 times in ice-cold PBS over 6 minutes and then incubated for 1 hour at 4°C in a humid chamber with 50μl of the designated mAbs (see Table 2.1) containing 10% NRS. After washing three times in ice-cold PBS (2 minutes each wash), the sections were incubated for 1 hour at 4°C in a humid chamber with 50μl of the affinity purified F(ab')$_2$ sheep-anti-mouse Ig conjugated to horse radish peroxidase (diluted 1/20) (Amersham Life Science). The slides were then washed again 3 times in ice-cold PBS and then taken to room temperature for incubation with 0.7mg/ml diaminobenzidine (DAB), 0.7mg/ml hydrogen peroxide and 0.06M Tris buffer (Sigma Fast 3,3' − diaminobenzidine tablet sets, Sigma Chemical Company, USA). The sections were then counterstained with haematoxylin prior to microscopic examination.

2.10.2 Counterstaining with Haematoxylin

Slides were counterstained by immersion in Gill's haematoxylin for 1 minute, rinsed briefly in PBS and then washed in tap water. For dehydration, the slides were placed in a 70% ethanol bath for 2 minutes and then transferred to two absolute ethanol baths for a period of two minutes for each bath. Lastly, the slides were moved through three xylene ('Unilab', Ajax Chemical Company, Auburn, NSW, Australia) baths, with each clearing step lasting 2 minutes. Slides were mounted using D.P.X neutral mounting medium (Ajax Chemical Company, Auburn, NSW, Australia) and viewed under an Olympus BH-2 light microscope (Olympus, Hatagaya, Shibuya-Ku, Tokyo, Japan). Photomicrographs were taken using Kodak Ektachrome 64T slide film (Eastman Kodak Company, USA).

2.10.3 Indirect immunofluorescent staining of cytospins

This procedure is similar to that described for the indirect immunoperoxidase technique (Section 2.10.1), although cytospins were fixed in formalin and then washed in DPBS/1% FCS/0.1% saponin. Also both the primary and secondary antibodies contained 0.1% saponin. In place of the sheep anti-mouse horseradish peroxidase conjugate (used in the above technique), 50μl of FITC-goat anti-mouse Ig (PharMingen) was added to the sections at a dilution of 1/50. This incubation was carried out in the dark to prevent bleaching of the fluorochromes. Stained sections were mounted in 86% glycerol containing 1% propyl-gallate (Sigma, Cat. No. P-3130). Sections were stored in the dark and then analysed using confocal microscopy (see Section 2.10.5).

2.10.4 Staining tissue sections with haematoxylin and eosin

Paraffin sections were de-waxed by immersed in xylene (twice, each for a duration of 4 minutes) and then hydrated via two washes each in absolute, 70% and 50% ethanol before being washed in tap water. Cryostat sections were fixed for 10 minutes in cold 96% ethanol and then hydrated as described for paraffin sections. The hydrated sections were then stained with Gill's haematoxylin for a period of 2 minutes (for OCT embedded sections) or 15minutes (for paraffin embedded sections). The slides were then washed in tap water and immersed in Scott's water (3.5g NaHCO3, 20g MgSO4, 0.05g sodium azide in 1 litre of Milli-Q water) for 2 minutes. After washing in tap water, the slides were stained with eosin for a period of 30 seconds, after which time they were washed and dehydrated via two washes in absolute ethanol before being immersed in xylene. The slides were mounted using DPX neutral mounting medium and viewed under an Olympus BH-2 light microscope. Photomicrographs were taken using Ektachrome 64T slide film (Eastman Kodak Company, USA).

2.10.5 Confocal microscopy

Confocal microscopy was performed using a MRC 600 confocal microscope (Bio-Rad Microscience, Cambridge, MA, USA). The FITC-label was excited at 488nm and viewed through a 500nm long pass filter. Images collected during these experiments were analysed using the Confocal Assistant™ (Version 4.02© 1994-1996, Todd Clark Brelge) software and the images were stored digitally and coloured artificially.

2.11 Preparation and analysis of mRNA

2.11.1 Preparation of solutions

TE (10mM Tris-HCl [Sigma, USA] pH 7.4 and 0.1mM ethylenediaminetetra-acetic acid (EDTA) [Sigma, USA]) was prepared at pH 8 in Milli-Q water and stored at room temperature.

TAE (40mM Tris-acetate (Sigma, USA) and 1mM EDTA (Sigma, USA) in Milli-Q water) was prepared as a 50x stock consisting of 242g Tris base, 57.1ml glacial acetic acid (BDH, Merck, Australia), and 100ml 0.5M EDTA pH 8. The solution was autoclaved and diluted to 1 x in Milli-Q water prior to use.

10x DNA loading buffer was prepared as a solution of 15% (w/v) Ficoll 400 (Pharmacia Biotech, Sweden), 0.3% (w/v) Bromophenol blue (Progen, Australia) and 0.1 mg/ml RNaseA (Boehringer-Mannheim, Germany). The solution was stored at 4°C and diluted 1/10 for use.

6x DNA loading buffer was prepared by dissolving 0.35% Orange G (Sigma, USA, cat. No. O-3756) in 30% w/v sucrose solution. The solution was stored at 4°C and diluted 1/6 for use.

Solution D ("Denaturing Solution" from Chapt. 4.2.6, contributed by Robert E. Kingston in *Current Protocols in Molecular Biology*, 1997 John Wiley & Sons, Inc) was prepared by adding 1.76ml of 0.75M sodium citrate (pH 7.0), 2.64ml of 10% w/v N-lauroylsacosine (Sigma, USA) and 25g of guanidine thiocyanate (Sigma, USA) to 29.3ml of water and stirring at 65°C until dissolved. The volume was made up to 50ml with water

and autoclaved. To this was added 350µl of 2-mercaptoethanol. Solution D was stored at 4°C for 3 months.

2.11.2 RNase-free conditions and buffer preparations

General laboratory glassware was filled with a solution of 0.1% (v/v) diethyl pyrocarbonate (DEPC; Sigma, USA), a potent inhibitor of RNases, and allowed to stand at 37°C for 2 hours, rinsed several times with sterile water and autoclaved for 30 minutes. The autoclave treatment removes traces of DEPC that might otherwise modify purine residues in RNA by carboxymethylation.

All solutions were prepared in RNase-free glassware, using 0.1% (v/v) DEPC-treated and autoclaved water. Alternatively, where possible, solutions were prepared and treated with 0.1% (v/v) DEPC for a minimum of 12 hours at 37°C, prior to autoclaving as above.

Sterile disposable plastic-ware was considered to be RNase-free and was used for the precipitation and storage of RNA without pretreatment.

2.11.3 Isolation of RNA using RNeasy® columns

Single cell suspensions containing 1 x 10^7 TD lymphocytes were centrifuged at 200g for 10 minutes and the cell pellet was resuspended in 500µl of Solution D in 1ml reaction tubes. The contents of the tube were snap frozen by immersing the tube in liquid nitrogen and stored at -70°C until required.

Total RNA extractions were performed using RNeasy® minispin columns (Qiagen, Cat. No.74104) in accordance with manufacturers instructions, with the exception that Solution D was used instead of "Buffer RLT".

The cell/Solution D lysate mixture was thawed and then 350µl was mixed well with 350µl of 70% ethanol. This 700µl of sample was applied to a RNeasy® mini-spin column within a 2 ml collection tube. After centrifugation at 8000g for 15 seconds, the effluent was discarded and the column was washed by the addition of 350µl of "Buffer RW1"

followed by centrifugation at 8000g for 15 seconds. In order to digest any residual DNA that may have not been removed in the washing steps, 80μl of RNase-free DNase I (27.2 Kunitz units) (Qiagen, 79254) was added to the sample in the column for 15-20 minutes. The column was then washed with 350μl of "Buffer RW1" and centrifuged at 8000g for 15 seconds. A final washing step consisted of adding 500μl of "Buffer RPE" to the column and centrifuging for 2 minutes at 14,000rpm. RNA on the column was then eluted into a fresh collection tube by pipetting 35μl of water onto the column and centrifuging for 1 minute at 8000g.

The integrity of the RNA was examined by performing agarose gel electrophoresis (as described in Section 2.11.8) to ensure that the 16S and 28S ribosomes bands were defined and not smeared (smearing indicates degradation of RNA).

The RNA was then quantified by taking absorbance readings at 260nm and 280nm with a spectrophotometer (Ultraspec$^{®}$ Plus, Pharmacia Biotech, Sweden). The A_{260}/A_{280} ratios of the RNA samples were typically in the range of 1.6-2.0 indicating good purity. The concentration of RNA was calculated using the formula:

RNA concentration (μg/μl) = (A_{260} x dilution factor x 40)/1000

(assuming an A_{260} value of 1 = 40μg/ml RNA)

RNA samples were stored at -70°C.

2.11.4 Isolation of RNA using RNAzol™ B

Total mRNA was extracted from cell suspensions using the RNAzol™ B method (Biotecx Laboratories, Houston, TX, USA). Single cell suspensions containing 1 x 10^7 TD lymphocytes were centrifuged at 200g for 10 minutes and the cell pellet resuspended in 500μl of RNAzol™ B in 1ml reaction tubes. The contents of the tube were snap frozen by immersing the tube in liquid nitrogen.

The RNA was extracted by the addition of 1/10 volume of chloroform, vortexed for 1 minute and placed on ice for 5 minutes. The samples were then centrifuged at 4°C for 15 minute at 13,000 rpm and the upper aqueous layer removed carefully and transferred to a fresh 1.5ml reaction tube. The total RNA was precipitated by the addition of an equal

volume of isopropanol and following a quick vortex, the tube was placed on ice for 15 minutes. The RNA was pelleted by centrifugation at 4°C for 15 minute at 13,000 rpm. The supernatant was decanted carefully in one movement. The pellet was then washed in 70% ethanol and after removal of the ethanol, allowed to dry in air for approximately 30 minutes. After drying, the pellet was resuspended thoroughly in 20μl of DEPC-treated water and the quantity assessed as described in 2.11.2. The integrity of the RNA was assessed by electrophoresis of 2μl of the RNA sample on a 2% agarose gel as described for DNA in section 2.11.9. RNA samples were stored at -70°C.

2.11.5 Reverse Transcription (RT)

A mixture of approximately 2μg of RNA in 30μl of DEPC-treated water and 6μl of 10μM Oligo dT (Geneworks, SA, Aust.) was heated at 70°C for 10 minutes. The RNA/primer suspension was allowed to cool on ice. The following cocktail was added to each reaction tube: 12μl of First Strand buffer, 6μl of 0.1M DTT (both from Gibco BRL, Gaithersburg, MD, USA, Cat No. 18064/104), 3μl of 10mM dNTPs (Pharmacia Biotech, Sweden). The tubes were then heated to 42°C for 2 minutes. To each tube was added 3μl of Superscript II reverse transcriptase at 200units/μl (Gibco BRL, Gaithersburg, MD, USA, Cat No. 18064/104). After incubating at 42°C for 50 minutes, the reverse transcriptase was inactivated by incubating the mix for 15 minutes at 70°C and the mixture was then cooled to 4°C. The resultant complementary DNA (cDNA) was stored at -20°C until required.

2.11.6 Preparation of genomic DNA

Genomic DNA was prepared from rat spleens essentially as outlined in Current Protocols in Molecular Biology (1990). The spleen was removed and frozen immediately in liquid nitrogen. The frozen tissue was then crushed into a fine powder using a pre-chilled mortar and pestle. The powder was suspended in 1.2 ml of digestion buffer (100mM NaCl; 10mM Tris-HCl, pH 8; 25mM EDTA, pH 8; 0.5% SDS [Sigma, USA]; 0.1 mg/ml proteinase K [Sigma, USA.]) per 100mg of tissue. This suspension was incubated with shaking at 50°C for 12 hours. To extract the nucleic acids, an equal volume of phenol/chloroform/iso-amyl (25:24:1) was added and the mixture was then centrifuged for 10 minutes at 1700g in a Beckman J2-21M centrifuge (Beckman, Palo Alto, CA, USA). The aqueous layer was transferred to a new tube and the DNA precipitated by the

addition of one half volume of 7.5M ammonium acetate (BDH, AnalaR®, Merck, Australia) and 2 volumes of ice-cold absolute ethanol (BDH, AnalaR®, Merck, Australia). Following centrifugation at 1700g for 2 minutes (as described above), the pellet was rinsed with 70% ethanol. The ethanol was decanted carefully and the DNA pellet allowed to dry in air. The pellet was dissolved in 500μl of TE buffer (see Section 2.10.1) and the concentration of DNA was determined by measuring the absorption at 260nm in a spectrophotometer (Ultraspec® Plus, Pharmacia Biotech, Sweden) assuming that an $A_{260}1=50\mu g/ml$ of DNA. Purified DNA was stored at -20°C

2.11.7 Oligonucleotide primers

Primers to amplify cytokine mRNA sequences were chosen based on published sequences. These primers are listed in Table 2.2. Primer Designer software (version 2.0; Scientific and Educational Software) was utilised to check the chosen sequences for complementarity, secondary structure, and to determine the melting temperatures (Tm°C) of the oligonucleotides. The concentration of oligonucleotides was determined by spectrophotometry A_{260} 1 = 33μg/ml, and the molarity determined by use of the following formula:

$$\text{Concentration of oligo(M)} = x\ \mu g \times 10^{-6} / [\text{MW of oligonucleotide} / 1\times10^{-3}]$$

2.11.8 Polymerase Chain Reaction (PCR)

Standard PCR reactions used 3-5μl of cDNA (from the reverse transcription of ~2μg of RNA) per 25μl reaction. The amplification reaction contained a 1/166 dilution of the DNA polymerase AmpliTaq Gold™ 5U/μl, a 1/10 dilution of GeneAmp® 10X PCR buffer II, a 1/20 dilution of 25mM $MgCl_2$ Solution (all part of AmpliTaq Gold™ kit purchased from Perkin Elmer), a 1/5 dilution each of forward and reverse primers at 5μM and the volume was increased to 25μl with MQ water. The cDNA was amplified using a PTC-100™ Programmable Thermal Controller (MJ Research, Inc., USA). The cDNA was denatured by heating at 95°C for 10 minutes, prior to 40 cycles consisting of: 95°C for 30 seconds, 58°C for 1 minute and 72°C for 1 minute. These cycles were followed by a final

Table 2.2 Oligonucleotide primers used for the amplification of target cytokine cDNA sequences

Target sequence	5' (forward) primer	3' (reverse) primer	Expected product size	Reference
IL-2	AGC TGT TGC TGG ACT TAC AGG	CAG AAA TTC CAC CAC AGT TGC	311bp	McKnight et al., 1989
IL-4	GAA CCA GGT CAC AGA AAA AAG	CTG CAA GTA TTT CCC TCG TAG G	313bp gDNA Product size: >4kb	McKnight et al., 1991
IL-10	GAC AAT AAC TGC ACC CAC TTC C	ATC ATT CTT CAC CTG CTC C	357 bp	Goodman et al., 1992
IFN-γ	CAC GAA AAT ACT TGA GAG CC	TCT CTA CCC CAG AAT CAG CAC C	297bp Expected gDNA Product size: >2.4 kb	Dijkema et al., 1985
TNF-α	TAC TGA ACT TCG GGG TGA TCG	CCT TGT CCC TTG AAG AGA ACC	292bp Expected gDNA Product size: ~1.3kb	Estler et al., 1992
TGF-β	CTA CTA CGC CAA AGA AGT CAC C	CTG ATC CCA TTG ATT TCC ACG	414bp	Qian et al., 1990
β-actin	CTG GAG AAG AGC TAT GAG C	AGG ATA GAG CCA CCA ATC C	330bp Expected gDNA Product size: 542 bp	Nudel et al., 1983

extension at 72°C for 10 minutes. RT-PCR products were analysed by agarose gel electrophoresis as described below.

2.11.9 Agarose gel electrophoresis and staining of DNA fragments

Following PCR amplification of cDNA a portion of the sample was mixed with a 1/10 dilution of 10x loading buffer or a 1/6 dilution of 6x Orange G. Electrophoresis of digested DNA was carried out on 1% (w/v) agarose (Progen, Australia) gels prepared and run in TAE (see Section 2.10.1) at 100V in a horizontal gel apparatus. The gels were stained in either ethidium bromide ($2\mu g/ml$ in distilled water Sigma, USA) for 5 minutes followed by destaining in H_2O for 10 minutes whilst being gently rocked or with SYBR® Gold nucleic Acid Gel Stain (Molecular Probes, Cat. No. S-11494) for 10-30 minutes. DNA fragments stained with ethidium bromide were visualised by illumination with 254nm UV light and photographed either by using Polaroid 667 film or by the use of a Tracktel GDS-2 gel documentation system (Vision Systems, The Levels, SA, Australia). DNA fragments stained with SYBR® Gold were visualised using a Molecular Imager FX (Biorad, USA) and the images were analysed using Quantity One Quantitation Software version 4 (Biorad, USA).

2.11.10 Measurement of the size of DNA fragments

The sizes of restriction fragments were calculated by comparing their relative mobilities in agarose with those of DNA molecules of known sizes (ie DNA molecular weight markers). The DNA molecular weight markers used contained a ladder that had 100bp steps between 100bp and 1000bp (Geneworks, Adelaide,Cat. No. DMW-100L).

CHAPTER 3

THE DETECTION OF
INTRACELLULAR CYTOKINES BY FLOW
CYTOMETRY

3.1 Introduction

Flow cytometric detection of intracellular cytokines has been used to identify the cytokines produced by CD4$^+$ T cells and for examining the co-expression of cytokines by individual cells (Assenmacher et al., 1994; Ferrick et al., 1995; Openshaw et al., 1995; Elson et al., 1995). The technique has the advantage over ELISA, RT-PCR and bio-assays that other characteristics of the cell producing the cytokine can be identified simultaneously with the detection of the cytokine. Other techniques give information on cytokine production by mixed populations of cells or they require sorting of the cells prior to the assay, if information is required on the nature of the cytokine-producing cells.

In order to investigate the cytokines produced by CD4$^+$ T cells from rats with adjuvant-induced arthritis (AA), it was necessary to optimise existing methods for examining intracellular cytokines to the antibodies available to detect rat cytokines. Monoclonal antibodies (mAb) were available for detection of only a limited number of rat cytokines but fortunately these included mAbs against the hallmark cytokines for the Th1 and Th2 subsets of T helper cells (IFN-γ and IL-4 respectively).

In order to assess performance of these antibodies in the detection of intracellular cytokine by flow cytometry, experimental models were chosen in which there is a known bias towards either a Th1 or Th2 response. The pathology of AA suggests a delayed-type hypersensitivity mechanism mediated by Th1 cells and there is evidence that IFN-γ is involved in the induction of AA (Wiesenberg et al., 1989; Jacob et al., 1989). These observations support the use of AA as a model for Th1-mediated response. AA is induced by the inoculation of rats with CFA and in this chapter, the term "CFA-challenged rats" refers to rats that are in the prodrome of the disease. *Nippostrongylus brasiliensis* infestation was chosen for the Th2 mediated response, since it has been shown that lymphocytes from infested animals produce IL-4 in response to stimulation with Concanavalin (Con)A *in vitro* (Street and Mosmann, 1991).

Upon stimulation, naïve CD4$^+$ T lymphocytes produce primarily IL-2. After priming these cells produce other cytokines and under certain conditions, develop into sub-populations that secrete mainly IFN-γ (Th1) or mainly IL-4 (Th2) (Seder and Paul, 1994). Freshly isolated murine and rat CD4$^+$ T lymphocytes produce limited amounts of Th1 and Th2

cytokines upon *in vitro* stimulation with polyclonal activators such as phorbol myristate acetate (PMA) and Con A. However, the production of IL-4 by CD4$^+$ T lymphocytes requires extended periods of stimulation and the addition of exogenous IL-4 (Swain et al., 1990b; Noble et al., 1993) and IL-2 (Le Gros et al., 1990). Polyclonal activation of CD4$^+$ T cells in the absence of IL-4 generates a population of cells that produce mainly IFN-γ, production of which is enhanced markedly by the presence of IL-12 (Seder and Paul, 1994).

The detection of molecules, such as some cytokines (e.g. IL-4) is at the lower end of the sensitivity range of the flow cytometer and requires special precautions. To minimise loss of fluorescent signal due to bleaching, fixed cells were stored at 4°C in the dark for a maximum period of 16 hours before analysis. Others have reported that fixed and stained cells can be stored in the dark for 1 day (IL-4) or 3 days (IFN-γ and IL-2) before analysis (Jung et al., 1993).

The objective of these studies was to investigate the *minimum* conditions of short-term stimulation needed to detect intracellular cytokines in CD4$^+$ T cells by flow cytometry. The rationale was that the profile of cytokines produced by the lymphocytes should reflect the state of differentiation of the cells *in vivo*, rather than their differentiation *in vitro* in response to stimulation.

3.2 Results

3.2.1 Monoclonal antibody MRC-OX81 can be used for indirect immunofluorescence staining of intracellular IL-4

Chinese Hamster Ovary (CHO) cell lines transfected with rat IL-2 or IL-4 cDNA sequences subcloned into the pEE6.MCMV-GS expression vector (Ramirez et al., 1996) were a kind gift from Dr. A. N. Barclay, Sir William Dunn School of Pathology, University of Oxford. CHO cells transfected with IL-4 cDNA ("CHO-IL-4 cells") were used to investigate the suitability of the mAb OX81 (anti-IL-4) for the detection of intracellular IL-4 by indirect immunofluorescence.

3.2.1.1 Monoclonal antibody MRC OX81 can be used for indirect immunofluorescence staining of IL-4 produced by transfected CHO cells

CHO-IL-4 cells were made permeable with saponin (see Section 2.6.3) and labelled indirectly with mAb OX81 (diluted 1 in 2 with RPMI) as the primary antibody and a PE-conjugated secondary anti-mouse Ig antibody (red line, Figure 3.2.1.1 A). The pre-incubation of neat culture supernatant containing mAb OX81 with an equal volume of culture supernatant from CHO-IL-4 cells inhibited the labelling of CHO-IL-4 cells completely (red line, Figure 3.2.1.1 B). The irrelevant isotype-matched mouse mAb 1B5 stained only ~1% of CHO-IL-4 cells within the equivalent range of fluorescence intensity (thin black line, Figure 3.2.1.1 A and B). When CHO-IL-2 cells were made permeable with saponin and labelled indirectly with mAb OX81 (red line, Figure 3.2.1.1 C), the level of fluorescence was similar to that observed in CHO-IL-2 cells labelled with a negative control antibody, 1B5 (thin black line, Figure 3.2.1.1 C). This indicates that mAb OX81 has specificity for a molecule that is produced and secreted by CHO-IL-4 cells but not by CHO-IL-2 cells, suggesting that mAb OX81 is specific for IL-4 and suitable for the detection of intracellular IL-4 by indirect immunofluorescence.

3.2.1.2 Cells must be made permeable to detect intracellular IL-4

It was found that the amount of cytokine detected could be greatly increased by first culturing the cells with the agent Brefeldin A (discussed later). CHO-IL-4 cells were cultured for four hours in the presence of Brefeldin A before fixation and indirect labelling for confocal microscopy. The fluorescence of CHO-IL-4 cells incubated with the negative control mAb 1B5 is illustrated in Figure 3.2.1.2 A and B. This provided the background level above which the fluorescence of cells labelled positively could be compared. CHO-IL-4 cells were not fluorescent above background levels when incubated with mAb OX81 (anti-IL-4) (Figure 3.2.1.2 C) unless they were made permeable by treatment with 0.1% saponin prior to, and during, incubation with the antibody (Figure 3.2.1.2 D). Cells made permeable with saponin were not labelled with mAb 1B5 (Figure 3.2.1.2 A).

The IL-4 detected in CHO-IL-4 cells by indirect labeling with mAb OX81 can be seen in intracellular compartments that are probably Golgi stacks (Figure 3.2.1.2 E). Following culture of the cells in the presence of Brefeldin A, the staining is distributed diffusely throughout the cytoplasm (Figure 3.2.1.2 F).

Figure 3.2.1.1 Monoclonal antibody OX81 has specificity for CHO cells transfected with IL-4. CHO cells transfected with multiple copies of either the gene encoding rat IL-4 or the gene encoding rat IL-2 were permeabilised with saponin and labelled indirectly with either mAb 1B5 (negative control; PE) or mAb OX81 (anti-IL-4; PE). The fluorescence of cells labelled with mAb OX81 is illustrated with the red line, whereas the black line represents the background fluorescence profile of cells stained with mAb 1B5. The percentage of cells expressing IL-4 is indicated above the histogram marker in each panel. Panel A illustrates CHO-IL-4 cells labelled with mAb OX81, whereas panel B shows the inhibition of labelling of these cells that occurred when mAb OX81 was pre-incubated with culture supernatant from CHO-IL-4 cells. The fluorescence profile of CHO-IL-2 cells labelled with mAb OX81 is represented in panel C.
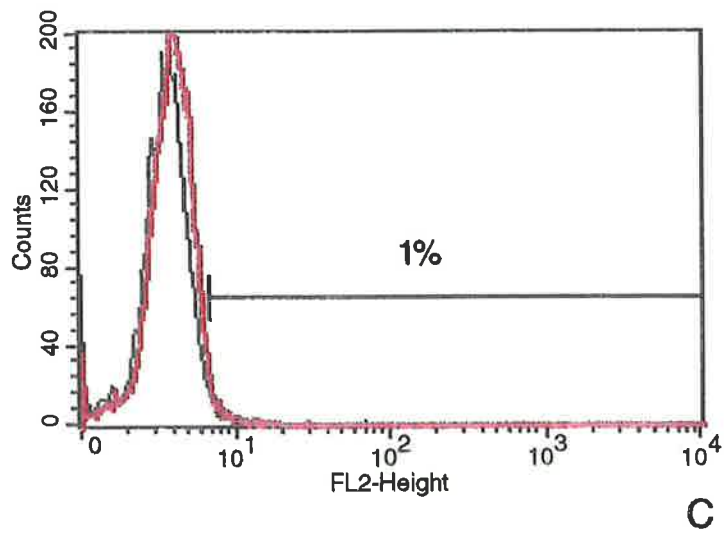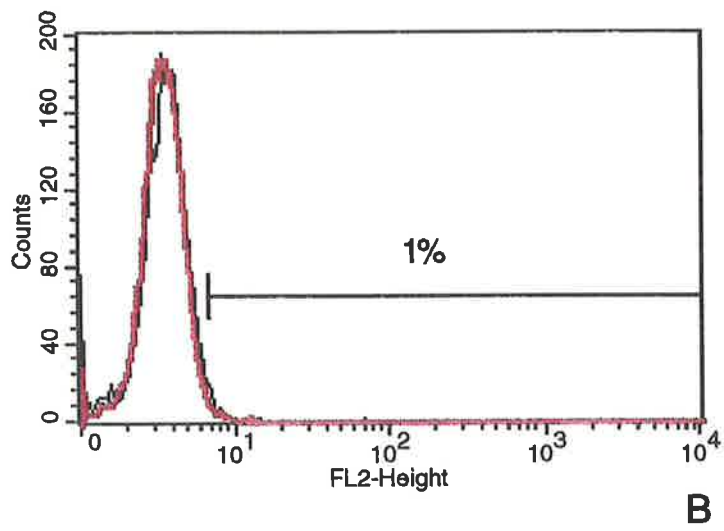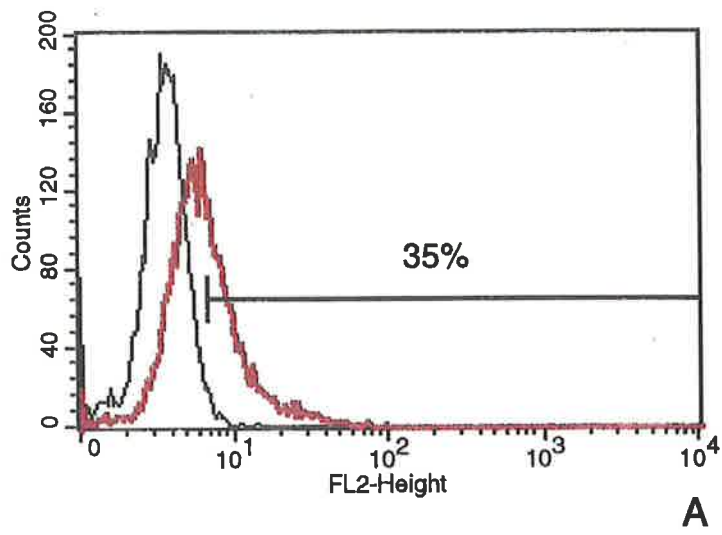
Figure 3.2.1.2 Monoclonal antibody OX81 stains intracellular IL-4 in permeabilised cells. CHO cells transfected with multiple copies of the gene encoding rat IL-4 were cultured for four hours in the presence of Brefeldin A (A, B, C, D, and F) or medium alone (E) and then placed on slides by cytospin. They were then labelled with either mAb 1B5 (anti-*Giardia* surface antigen [A and B]) or mAb OX81 (anti-rat IL-4 [C to F]) using the indirect method. The cells shown in B, D, E and F were washed in buffer containing 0.1% saponin, whereas the cells shown in A and C were washed in saponin-free buffer. The labelling method used to stain IL-4 produced by the cells represented in B, D, E and F included saponin in all antibody incubations. After staining, the preparations were mounted using an aqueous mounting medium containing 1% propyl gallate to minimise bleaching of the fluorochrome. Original magnification 30x (A-D) or 150x (E and F). The cells were examined using a confocal microscope as described in Section 2.9.5.

A 1B5

B Saponin + 1B5

C OX81

D Saponin + OX81

E Untreated

F Brefeldin A
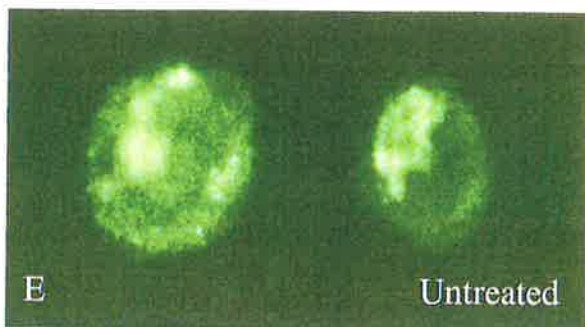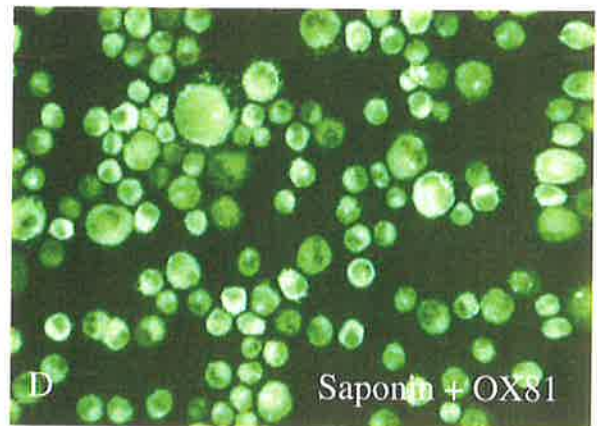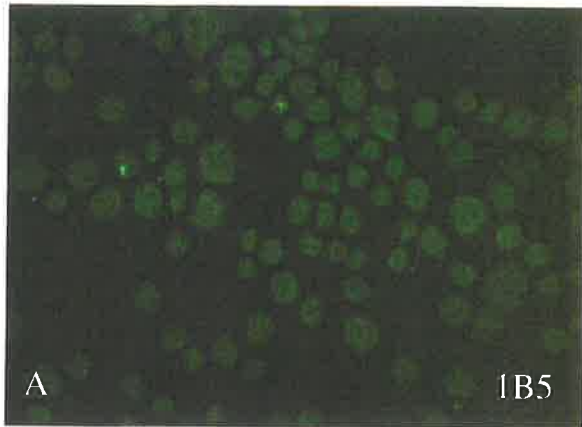
3.2.2 Flow cytometric detection of cytokines within lymphocytes stimulated *in vitro*

The detection of intracellular cytokines by flow cytometry has been reported to be dependent on a period of *in vitro* stimulation of the cells before fixation (Schauer et al., 1996). Lymphocytes from normal rats produce small but detectable levels of cytokines following stimulation *in vitro*. It was necessary, therefore, to define conditions that would distinguish between lymphocytes already committed to cytokine production by stimulation *in vivo* and the production of cytokines by resting lymphocytes that have been activated *in vitro*.

To elicit a Type-2 response, with production of IL-4, TD lymphocytes from rats that had been infested with the helminthic parasite *Nippostrongylus brasiliensis* were examined. *N.brasiliensis* is a nematode parasite that migrates through the vasculature to the lungs and then via the respiratory tract to the gut. It is a potent inducer of Type-2 cytokine responses (reviewed in Finkelman et al., 1997).

Thoracic duct (TD) lymphocytes from a rat in the prodromal phase of AA ("CFA-challenged") were included to represent cells containing a population that was expected to exhibit a Th1-bias. The mouse mAb DB-1 has specificity for rat IFN-γ and has strong neutralising activity of both rat and mouse IFN-γ (Van der Meide et al., 1986). Monoclonal antibody DB-1 was investigated for its suitability as a reagent for detecting intracellular IFN-γ by flow cytometry. Subsequent to these studies, others have reported the suitability of this mAb for detecting IFN-γ by flow cytometry (Bernard et al., 1998). TD lymphocytes from a normal rat were included to determine the baseline production of IL-4 and IFN-γ from normal lymphocytes stimulated *in vitro*.

The TD lymphocytes were stimulated *in vitro* for a period of 24 hours, with Brefeldin A added (10μg/ml) (Openshaw et al., 1995) for the final 4 hours before fixation. The stimulatory conditions tested were 50ng/ml phorbol myristate acetate (PMA) (Openshaw et al., 1995) plus ionomycin A23187 (100μM); Concanavalin A (Con A); or supernatant from CHO-IL-4 cells (IL-4). The concentration of Con A used (5μg/ml) was the optimal concentration for inducing lymphocyte proliferation, determined in a preliminary experiment using ^{3}H-thymidine incorporation to measure proliferation (data not shown).

The concentration of CHO-IL-4 supernatant (10%) was within the range that induced an upregulation of MHC class II on B lymphocytes, as determined by flow cytometry (data not shown).

3.2.2.1 The light scattering properties of stimulated thoracic duct lymphocytes

Typical light scattering properties of stimulated TD lymphocytes are illustrated in Figure 3.2.2.1 A. The lymphocytes were gated (as shown) on the basis of their forward scatter (FSC) and side scatter (SSC) of light. Events with a FSC of less than ~100 (e.g. red blood cells) were excluded from analysis. Within the gate, two clusters of events are apparent, indicating populations of large and small lymphocytes. When examining the fluorescence of indirectly labelled cells in comparison to cell size, a horizontal marker was assigned to separate large and small cells (small and large cells are represented in the lower and upper quadrants, respectively) (Figure 3.2.2.1 B). A vertical divider was positioned such that less than 1% of cells labelled with mAb 1B5 were represented in the right quadrants. This provided the level of background above which the fluorescence of positively labelled cells was defined.

3.2.2.2 The indirect immunofluorescence staining of IL-4 produced by lymphocytes

In the absence of *in vitro* stimuli during a 24-hour culture period, TD lymphocytes from a normal (Figure 3.2.2.2 A), a *N. brasiliensis*-infested (E), or a CFA-challenged (I) rat did not produce detectable levels of IL-4. Stimulation with either PMA plus ionomycin (F) or Con A (G) induced the production of detectable levels of IL-4 by a sub-population of TD lymphocytes from *N. brasiliensis*-infested rats. The proportions of cells producing detectable levels of IL-4 were similar, regardless of whether the cells were stimulated with PMA (Figure 3.2.2.2 F) or Con A (Figure 3.2.2.2G). Only a small increase in the proportion of IL-4 producing cell was observed after stimulating TD lymphocytes from normal (B and C) or CFA-challenged (J and K) either with Con A or PMA plus ionomycin.

TD lymphocytes from all rats were labelled with mAb OX81 following culture in IL-4 conditioned medium for 24 hours. Approximately 23% of TD lymphocytes from a normal rat (D), 12% of TD lymphocytes from a *N. brasiliensis*-infested rat (H) and 16% of TD lymphocytes from a CFA-challenged rat (L) were labelled following culture in IL-4 conditioned medium for 24 hours.

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Figure 3.2.2.1 The light scattering properties of stimulated thoracic duct lymphocytes. Thoracic duct lymphocytes collected from a rat 9 days after inoculation of CFA were cultured for 24 hours in the presence of medium only, PMA plus ionomycin, Concanavalin A or supernatant from CHO-IL-4 cells. In each case Brefeldin A was added for the final 4 hours of the culture period. After fixation the cells were made permeable by treatment with saponin and labelled indirectly with mAb 1B5 (negative control; FITC). The light scattering properties of these cells is represented in panel A, and events lying within the gate were classified arbitrarily as lymphocytes. The size of the cells, which determines the forward scatter of light (FSC), is indicated on the x axis, and the internal complexity of the cells, which determines the side scatter of light (SSC) is represented on the y axis. In panel B, the fluorescence of cells labelled with mAb 1B5 (FITC) is displayed on the x axis, while the forward scatter of the cells is represented on the y axis. The quadrants were assigned such that larger lymphocytes would be depicted in the upper quadrants and smaller lymphocytes in the lower quadrants. The vertical divider was positioned such that a total of less than 1% of events were displayed in the right quadrants. The percentage of the gated events in each quadrant and the mean fluorescence of these events are shown as a table in the upper right quadrant.
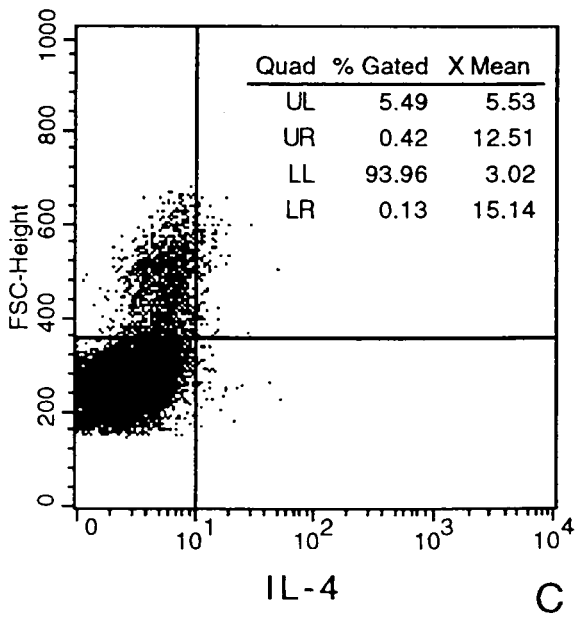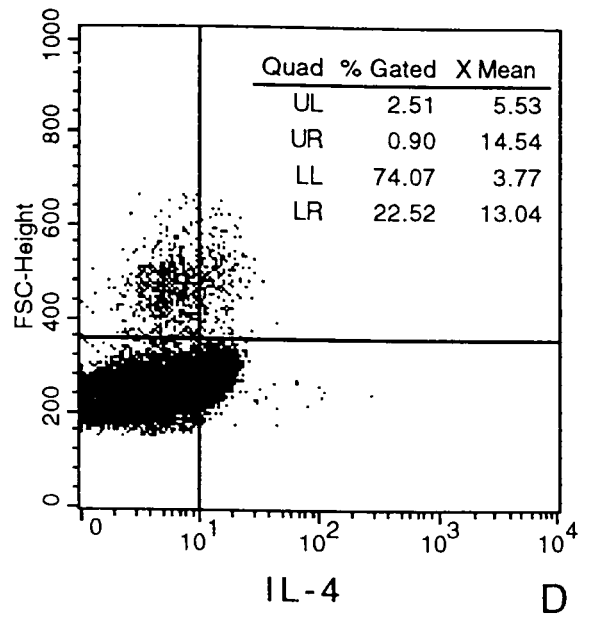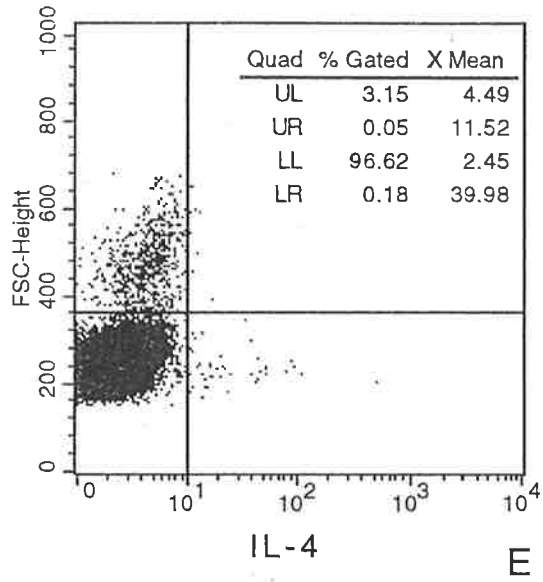
A

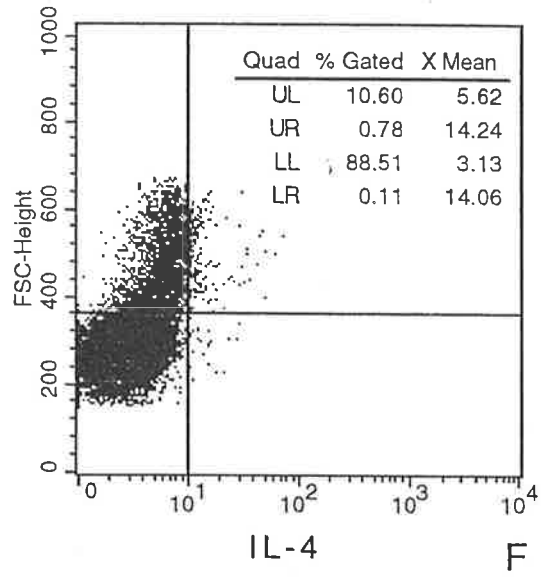| Quad	% Gated	X Mean
UL	6.97	4.71
UR	0.15	12.74
LL	92.66	2.73
LR	0.22	22.63

1B5
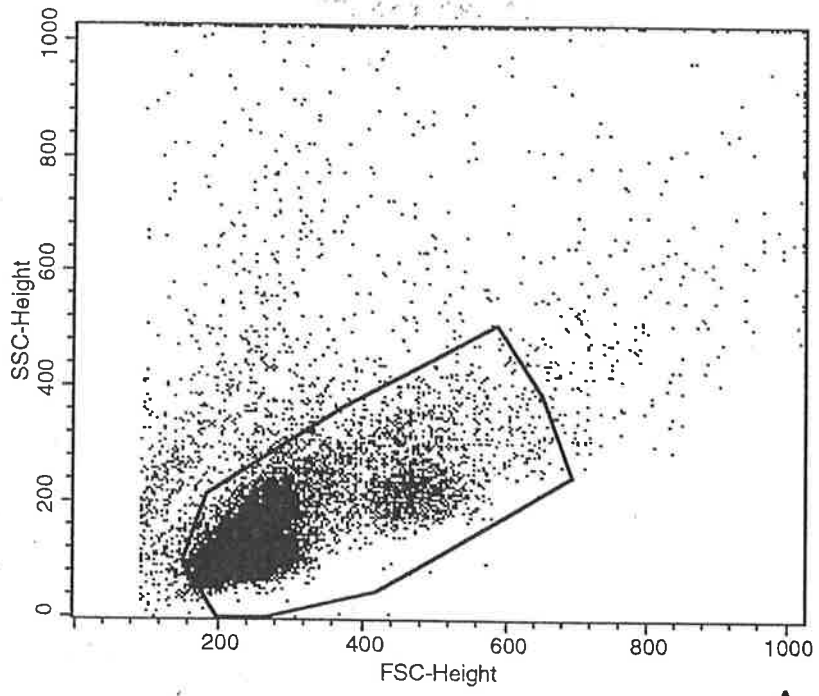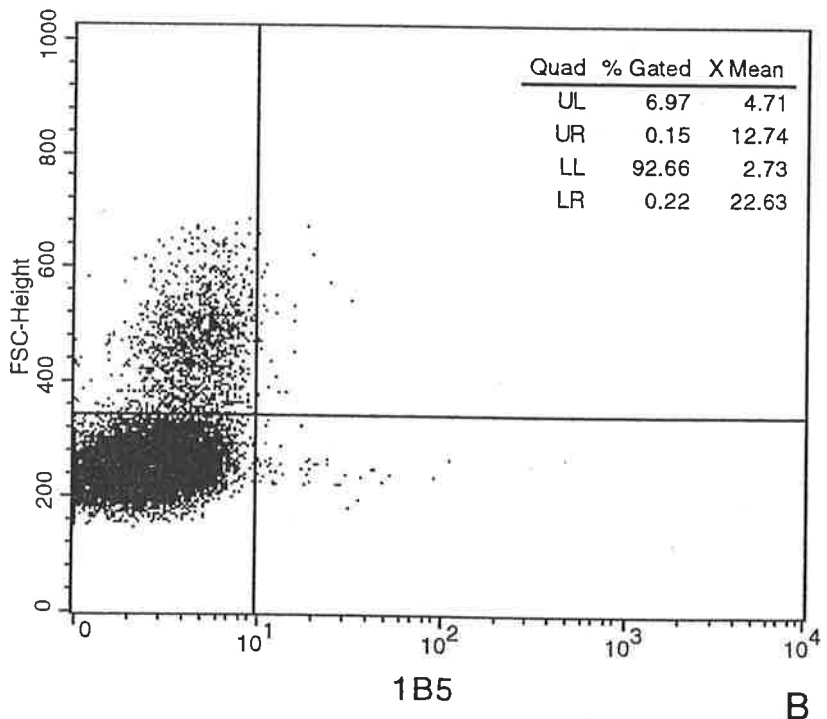
B

3.2.2.2 The indirect immunofluorescence staining of IL-4 produced by lymphocytes. Thoracic duct lymphocytes from either a normal (panels A-D), a *N. brasiliensis* infested (panels E-H) or a CFA-challenged (panels I-L) DA rat were cultured for 24 hours in the presence of medium only (panels A, E and I), PMA plus ionomycin (panels B, F and J), Concanavalin A (panels C, G and K) or culture medium from CHO-IL-4 cells (panels D, H and L). Brefeldin A was present for the final 4 hours of culture before fixation. The cells were made permeable by treatment with saponin and labelled indirectly with either mAb 1B5 (negative control; FITC) or mAb OX81 (anti-IL-4; FITC). The size of the cells, which determines the forward scatter of light (FSC), is indicated on the y axis, and the fluorescence of cells is displayed on the x axis. The quadrant markers were determined as described in Figure 3.2.2.1, whereby the vertical divider was positioned such that a total of less than 1% of events from the flow cytometric analysis of cells labelled indirectly with mAb 1B5 and FITC were displayed in the right quadrants (Figure 3.2.2.1 B). The percentage of the gated events in each quadrant and the mean fluorescence of these events are shown as a table in the upper right quadrant.

none

Quad	% Gated	X Mean
UL	3.09	4.73
UR	0.05	14.62
LL	96.65	2.64
LR	0.21	23.36

IL-4

A

PMA

Quad	% Gated	X Mean
UL	5.91	5.48
UR	0.17	11.88
LL	93.84	3.20
LR	0.08	26.83

IL-4

B

Concavalin A

Quad	% Gated	X Mean
UL	5.49	5.53
UR	0.42	12.51
LL	93.96	3.02
LR	0.13	15.14

IL-4

C

IL-4

Quad	% Gated	X Mean
UL	2.51	5.53
UR	0.90	14.54
LL	74.07	3.77
LR	22.52	13.04

IL-4

D

none

Quad	% Gated	X Mean
UL	3.15	4.49
UR	0.05	11.52
LL	96.62	2.45
LR	0.18	39.98

FSC-Height

IL-4

E

PMA

Quad	% Gated	X Mean
UL	10.60	5.62
UR	0.78	14.24
LL	88.51	3.13
LR	0.11	14.06

FSC-Height

IL-4

F

Concavalin A

Quad	% Gated	X Mean
UL	7.89	5.42
UR	0.94	25.50
LL	90.90	2.80
LR	0.27	30.83

FSC-Height

IL-4

G

IL-4

Quad	% Gated	X Mean
UL	4.76	5.20
UR	1.02	13.96
LL	82.80	3.31
LR	11.41	12.94

FSC-Height

IL-4

H

none

Quad	% Gated	X Mean
UL	3.47	4.94
UR	0.06	14.21
LL	96.33	2.68
LR	0.14	36.67

IL-4   I

PMA

Quad	% Gated	X Mean
UL	7.60	6.02
UR	0.08	17.98
LL	92.27	3.33
LR	0.05	18.59

IL-4

Concavalin A

Quad	% Gated	X Mean
UL	5.24	6.09
UR	0.18	13.48
LL	94.45	3.06
LR	0.13	16.91

IL-4   K

IL-4

Quad	% Gated	X Mean
UL	2.80	5.80
UR	0.74	17.29
LL	81.17	3.89
LR	15.29	14.81

IL-4   L

3.2.2.3 The indirect immunofluorescence staining of IFN-γ produced by lymphocytes

Few TD lymphocytes (~1%) from a normal rat produced detectable levels of IFN-γ following 24 hours of stimulation with either PMA plus ionomycin or with Con A. The proportion that produced detectable levels of IFN-γ was slightly lower following stimulation with PMA plus ionomycin compared with Con A (0.8% [Figure 3.2.2.3 B] and 1.1% [Figure 3.2.2.3 C], respectively). However, this frequency of positive fluorescence was greater than that seen when normal TD lymphocytes were cultured in the absence of stimuli (A) or in the presence of IL-4 conditioned medium (D) and then stained with mAb DB-1 against IFN-γ, or when the cells were stained with mAb 1B5 (Figure 3.2.2.1 B). Similarly, TD lymphocytes from *N.brasiliensis*-infested rats or CFA-challenged rats did not produce detectable levels of IFN-γ in the absence of stimulation (E and I) nor following culture in IL-4 conditioned medium (H and L).

Stimulation with either PMA plus ionomycin or Con A induced the production of IFN-γ by TD lymphocytes from both *N. brasiliensis*-infested and CFA-challenged rats (Figures 3.2.2.3 F, G, J and K). PMA plus ionomycin induced the production of IFN-γ in more TD lymphocytes (~5.7% of cells from *N. brasiliensis*-infested rats [F] and ~6.5% of cells from CFA-challenged rats [J]) than Con A stimulation (4.3% [G] and 4.5% [K] respectively). Therefore, PMA plus ionomycin was chosen as the method of stimulating lymphocytes to produce cytokines for detection by flow cytometry in all subsequent work.

3.2.3 Kinetics of the production of IL-4 and IFN-γ by thoracic duct lymphocytes

To establish a standard protocol for the stimulation of lymphocytes for the detection of intracellular cytokines by flow cytometry, it was necessary to examine the kinetics of cytokine production and to determine the minimum duration of stimulation *in vitro* required for the induction of detectable levels of intracellular cytokine.

3.2.3.1 Time course of cytokine production by stimulated lymphocytes

TD lymphocytes from normal, *N.brasiliensis*-infested or CFA-challenged rats were stimulated *in vitro* with PMA plus ionomycin and harvested at approximately four hourly intervals for fixation. Brefeldin A was present for the final four hours of each incubation before fixation, with the exceptions of the 2 hour time point (present for two hours only)
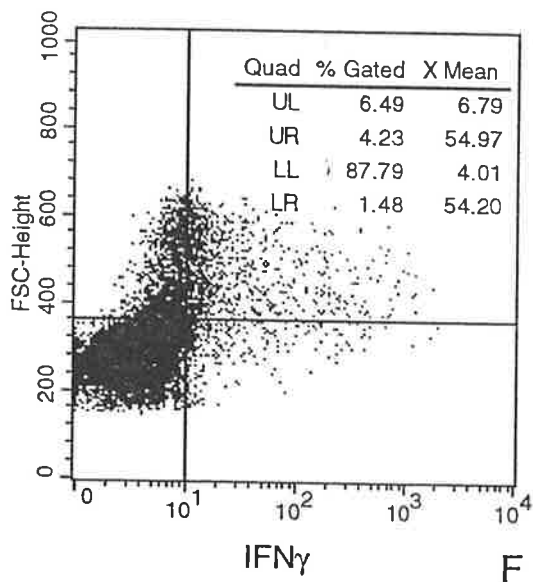
3.2.2.3 The indirect immunofluorescence staining of IFN-γ produced by lymphocytes.

Thoracic duct lymphocytes harvested from either a normal (panels A-D), a *N. brasiliensis* infested (panels E-H) or a CFA-challenged (panels I-L) DA rat were cultured for 24 hours in the presence of medium only (panels A, E and I), PMA plus ionomycin (panels B, F and J), Concanavalin A (panels C, G and K) or culture medium from CHO-IL-4 cells (panels D, H and L). Brefeldin A was present for the final 4 hours of culture before fixation. The cells were made permeable by treatment with saponin and labelled indirectly with either mAb 1B5 (negative control; FITC) (not shown here, see Figure 3.2.2.1) or mAb DB-1 (anti-IFN-γ; FITC). The size of the cells, which determines the forward scatter of light (FSC), is indicated on the y axis, and the fluorescence of cells is displayed on the x axis. The quadrant markers were determined as described in Figure 3.2.2.1, whereby the vertical divider was positioned such that a total of less than 1% of events from the flow cytometric analysis of cells labelled indirectly with 1B5 and FITC were displayed in the right quadrants (Figure 3.2.2.1 B). The percentage of the gated events in each quadrant and the mean fluorescence of these events are shown as a table in the upper right quadrant.

none

Quad	% Gated	X Mean
UL	2.87	4.73
UR	0.06	13.72
LL	96.94	2.69
LR	0.12	50.80

FSC-Height

IFNγ

A

PMA

Quad	% Gated	X Mean
UL	5.73	5.75
UR	0.57	31.88
LL	93.45	3.40
LR	0.24	235.61

FSC-Height

IFNγ

B

Concavalin A

Quad	% Gated	X Mean
UL	5.43	5.84
UR	0.78	16.86
LL	93.43	3.27
LR	0.35	119.23

FSC-Height

IFNγ

C

IL-4

Quad	% Gated	X Mean
UL	2.65	4.98
UR	0.14	20.90
LL	97.00	2.86
LR	0.20	23.84

FSC-Height

IFNγ

D

Quad	% Gated	X Mean
UL	2.97	4.73
UR	0.08	16.88
LL	96.77	2.56
LR	0.18	17.48

FSC-Height

IFNγ   E

PMA

Quad	% Gated	X Mean
UL	6.49	6.79
UR	4.23	54.97
LL	87.79	4.01
LR	1.48	54.20

FSC-Height

IFNγ   F

Concavalin A

Quad	% Gated	X Mean
UL	5.77	6.57
UR	2.61	46.75
LL	89.90	3.48
LR	1.71	44.69

FSC-Height

IFNγ   G

IL-4

Quad	% Gated	X Mean
UL	4.27	5.01
UR	0.11	21.37
LL	95.44	2.73
LR	0.18	21.59

FSC-Height

IFNγ   H

none

Quad	% Gated	X Mean
UL	2.82	5.39
UR	0.02	16.39
LL	97.01	2.80
LR	0.16	269.57

IFNγ

I

PMA

Quad	% Gated	X Mean
UL	3.77	7.76
UR	3.69	97.77
LL	89.74	4.37
LR	2.79	64.84

IFNγ

J

Concavalin A

Quad	% Gated	X Mean
UL	4.06	7.59
UR	1.92	58.84
LL	91.43	4.04
LR	2.60	81.96

IFNγ

K

IL-4

Quad	% Gated	X Mean
UL	3.11	5.45
UR	0.05	19.28
LL	96.64	2.92
LR	0.20	26.68

IFNγ

L
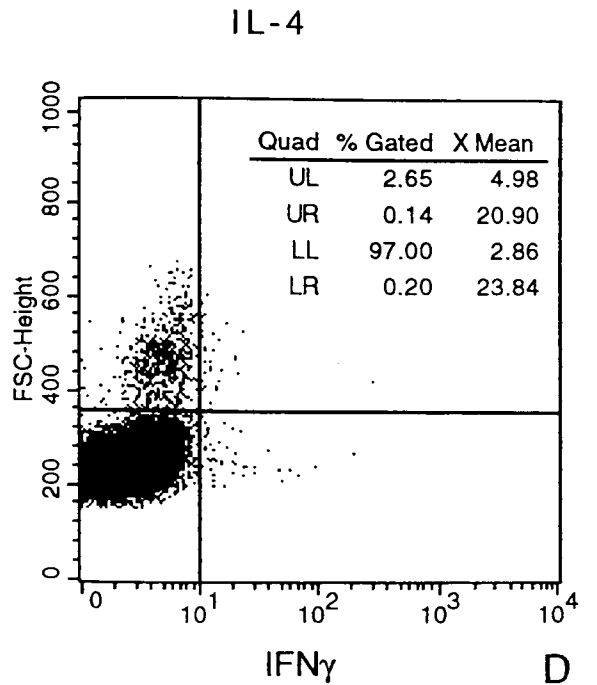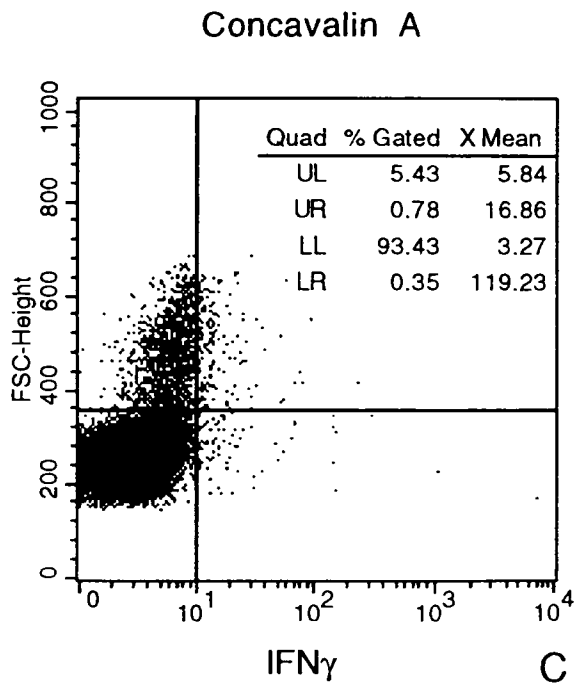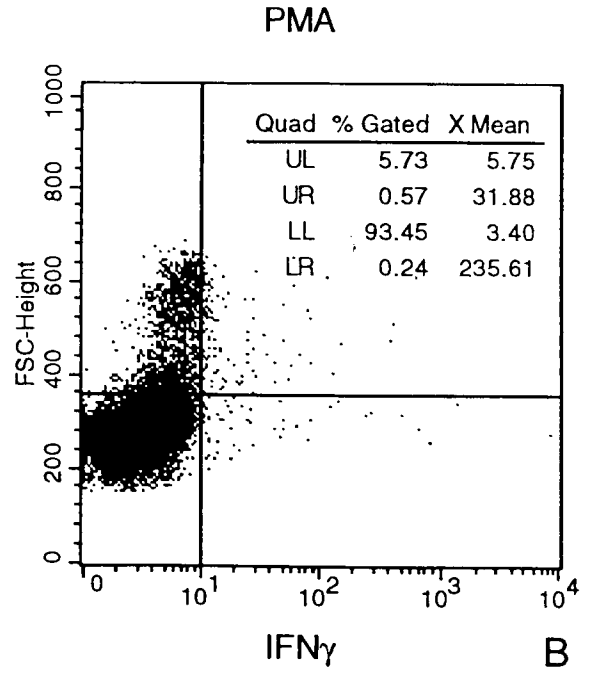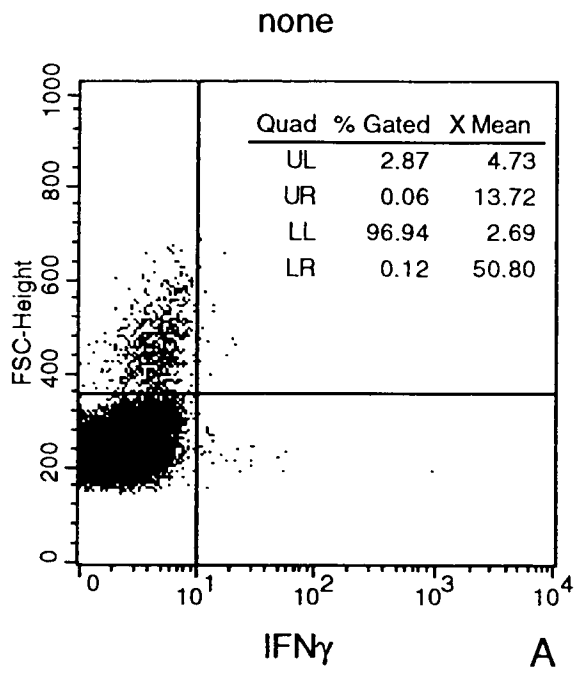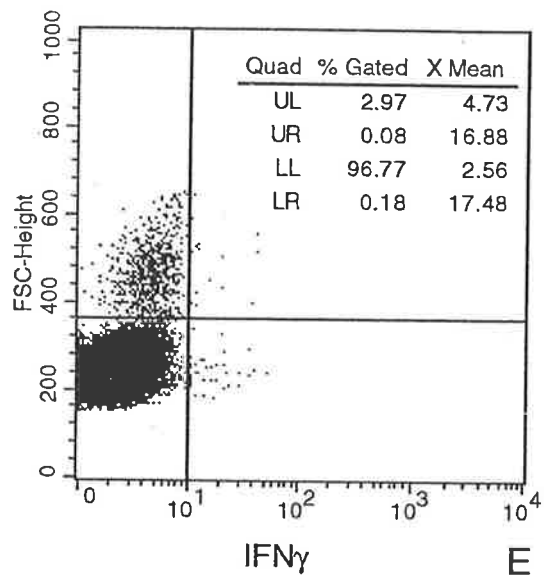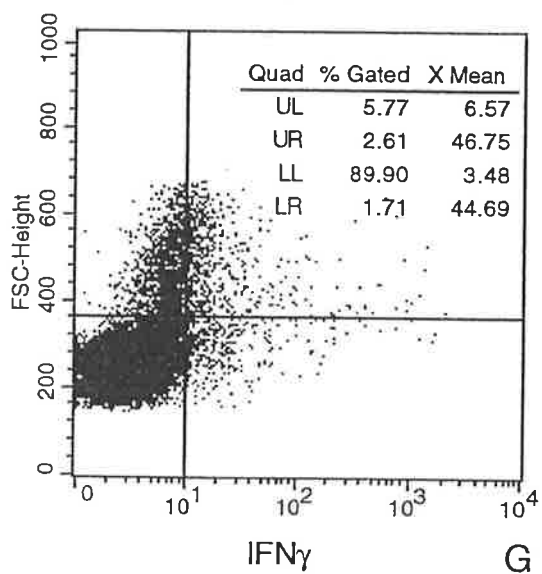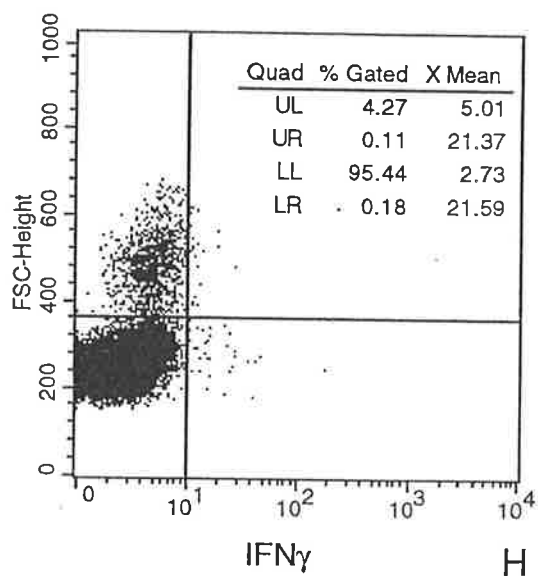
and the 21 hour time point (present for five hours). Fixed cells were made permeable with saponin and labelled indirectly with mAb 1B5 (negative control), mAb OX81 (anti-rat IL-4) or mAb DB-1 (anti-rat IFN-γ) mAb.

Stimulation with PMA plus ionomycin induced few TD lymphocytes from either normal or CFA-challenged rats to produce IL-4. However, TD lymphocytes from *N.brasiliensis*-infested rats produced detectable levels IL-4, with the greatest proportion of IL-4 producing lymphocytes observed between 4 and 8 hours of stimulation (Figure 3.2.3.1 A).

Some TD lymphocytes from all rats produced detectable levels of IFN-γ (Figure 3.2.3.1 B). In this experiment, the proportion of TD lymphocytes from *N.brasiliensis*-infested rats that produced IFN-γ was higher than in cells obtained from CFA-challenged rats. The proportion of IFN-γ producing TD lymphocytes was at its highest between 4 and 16 hours of stimulation (Figure 3.2.3.1 B). Only small numbers of cells expressing intracellular IFN-γ or IL-4 were detected in unstimulated lymphocytes from any of the rats (data not shown).

These studies indicated that TD lymphocytes from rats responding to an *in vivo* stimulus would produce IL-4 (*N.brasiliensis*-infested donor only) or IFN-γ (CFA-challenged or *N.brasiliensis*-infested donors) within 6 hours when stimulated *in vitro* with PMA plus ionomycin. If this period was extended to 16-24 hours, the proportion of cytokine-producing cells did not increase markedly, with the possible exception of IFN-γ production by cells from CFA-challenged donors. The kinetics of IFN-γ production is similar to that observed by others when examining T cell lines (Bernard et al., 1998). Based on these results and the results of others (Prussin and Metcalfe, 1995), six hours of stimulation *in vitro* with PMA plus ionomycin was chosen for the detection of intracellular cytokines by flow cytometry.

3.2.4 Phenotype of cytokine-producing cells

A powerful advantage of detecting intracellular cytokines by flow cytometry is that individual cells producing the cytokine can be further characterized by dual fluorochrome labelling. TD lymphocytes from a Hooded Wistar rat infested with *N.brasiliensis* were stimulated for 6 hours in the presence of PMA plus ionomycin and Brefeldin A (Figure

Figure 3.2.3.1 The kinetics of cytokine production by stimulated lymphocytes.
Thoracic duct lymphocytes were harvested from either a normal (triangles), a *N. brasiliensis* infested (circles) or a CFA-challenged (squares) DA rat. The lymphocytes were cultured for up to 21 hours in the presence of PMA plus ionomycin. Brefeldin A was present for the final 4 hours of culture before fixation and storage until staining. The production of IL-4 (A) and IFN-γ (B) was determined flow cytometric analysis. This was achieved by making the cells permeable with saponin and then labelling indirectly with either mAb OX81 (anti-IL-4; FITC) or mAb DB-1 (anti-IFN-γ; FITC) to determine the percentage of cells with fluorescence greater than that detected in cells labelled with the mAb1B5 (negative control; FITC – data not shown).
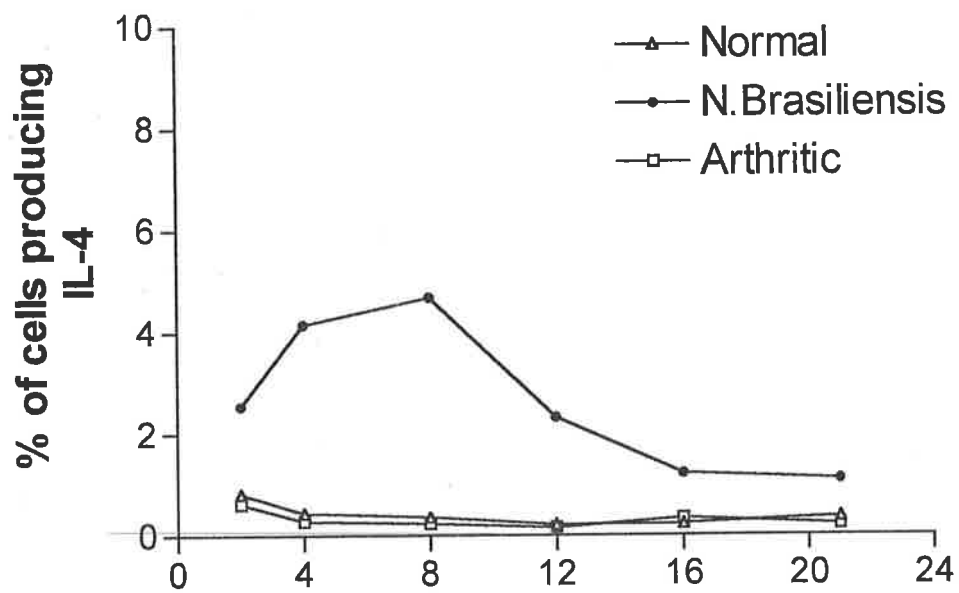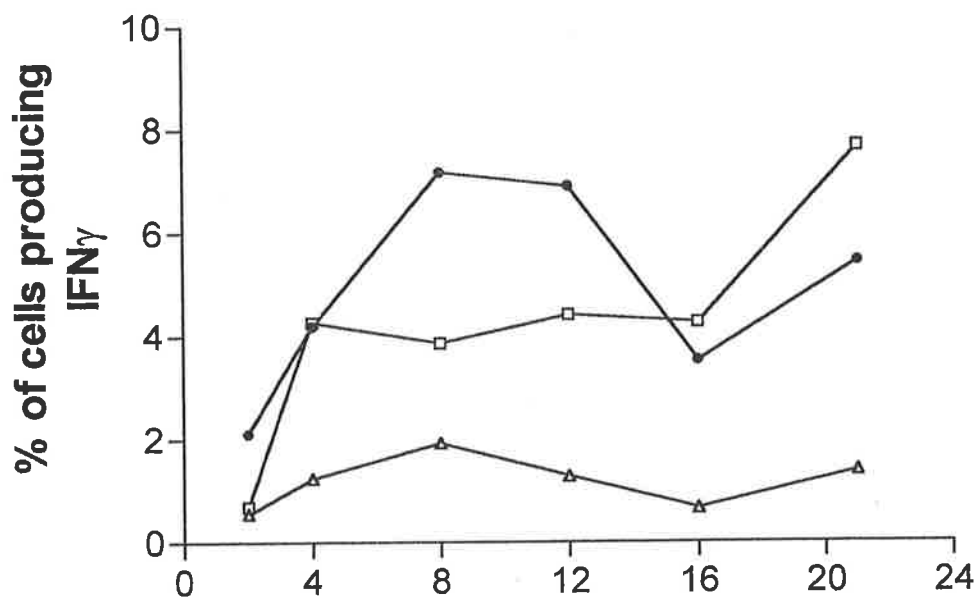
3.2.4.1 B-F, 3.2.4.2 B-F) or in the presence of Brefeldin A only (Figure 3.2.4.1 A, 3.2.4.2 A). These cells were fixed and stained with PE-conjugated mAbs as described elsewhere (section 2.6.3) to identify surface antigen markers on the cells. Table 3.1 summarises the results obtained from examining the production of IL-4 and IFN-γ by subsets of TD lymphocytes (the individual dotplots are displayed in Figure 3.2.4.1-3).

3.2.4.1 Phenotype of IL-4-producing cells

The cells that produce IL-4 are predominantly T cells (Figure 3.2.4.1 B) and of the CD4$^+$ subset (C) rather than the CD8$^+$ subset (D). The proportion of stimulated TD lymphocytes that are CD4$^+$ IL-4$^+$ is similar to the proportion of TD lymphocytes that are CD8$^-$ IL-4$^+$(D); CD45RA$^-$ IL-4$^+$(E) or MHC class II$^-$ IL-4$^+$ (F). This indicates that after 6 hours of stimulation *in vitro*, most of the IL-4 producing cells are CD4$^+$ T cells. Other known producers of IL-4 are either absent from the TD lymph (e.g. mast cells), or present in low numbers (e.g. NK cells, see Section 1.5.3). As seen previously, there was no IL-4 production above background levels in the absence of stimulation (A).

3.2.4.2 Phenotype of IFN-γ-producing cells

The TD lymphocytes that produce IFN-γ are predominantly α/β T cells (Figure 3.2.4.2 B), with more CD4$^+$ T cells producing IFN-γ (C) than CD8$^+$ cells (D). The sum of the proportion of CD4$^+$ IFN-γ^+ and CD8$^+$ IFN-γ^+ is approximately equal to the proportion of stimulated TD lymphocytes that are α/β TCR$^+$ IFN-γ^+ . A very small number of CD45RA$^+$ cells stained for IFN-γ, predominantly at low intensity (E) which suggests that a small population of B cells produce IFN-γ or have IFN-γ bound to IFN-γ receptors on their cell surface. A small number of MHC class II$^+$ IFN-γ^+ were detected (F). Since MHC class II is expressed by activated CD4$^+$ T cells, the horizontal divider was set to include only MHC class II^{bright} cells. Activated T cells express MHC class II at a lower level than either B cells or dendritic cells. Dendritic cells are rare in normal TD lymph and because the events shown on the dotplots represent cells that were gated to include lymphocytes (so called "lymphocyte gate", similar to the gate shown in Figure 3.2.2.1 A), it is unlikely that dendritic cells contributed to the cells in this gate. Although there is some evidence that dendritic cells can produce IFN-γ (Zhou and Tedder, 1995) the small population MHC class II$^+$ IFN-γ^+ cells are more likely to be activated T cells.

3.2.4.1 Phenotype of IL-4-producing cells. Thoracic duct lymphocytes from a *N.brasiliensis*-infested Hooded Wistar rat were stimulated for 6 hours with PMA plus ionomycin in the presence of Brefeldin A (B-F), or cultured in the presence of Brefeldin A only (A). Fixed cells were made permeable with saponin and were first labelled indirectly with either mAb OX81 (anti-IL-4; FITC) or mAb 1B5 (negative control; FITC [not shown]), followed by direct labelling with either mAb OX38 (anti-CD4; PE, [A-C]), mAb R73 (anti-α/β TCR;PE, [B]), mAb OX8 (anti-CD8α; PE [D]), OX33 (anti-CD45RA; PE [E]) or mAb OX6 (anti-MHC class II; PE [F]). The quadrant markers were determined as described in Figure 3.2.2.1, whereby the vertical divider was positioned such that a total of less than 1% of events from the flow cytometric analysis of cells labelled with mAb 1B5 were displayed in the right quadrants (data not shown). The percentage of the gated events in each of the right hand side quadrants is shown.

3.2.4.2 Phenotype of IFN-γ-producing cells. Thoracic duct lymphocytes from a *N.brasiliensis*-infested Hooded Wistar rat were stimulated for 6 hours with PMA plus ionomycin in the presence of Brefeldin A (B-F), or cultured in the presence of Brefeldin A only (A). Fixed cells were made permeable with saponin and were first labelled indirectly with either mAb DB-1 (anti-IFN-γ; FITC) or mAb 1B5 (negative control; FITC [not shown]), followed by direct labelling with either mAb OX38 (anti-CD4; PE, [A-C]), mAb R73 (anti-α/β TCR;PE, [B]), mAb OX8 (anti-CD8α; PE [D]), mAb OX33 (anti-CD45RA; PE [E]) or mAb OX6 (anti-MHC class II; PE [F]). The quadrant markers were determined as described in Figure 3.2.2.1, whereby the vertical divider was positioned such that a total of less than 1% of events from the flow cytometric analysis of cells labelled with mAb 1B5 were displayed in the right quadrants (data not shown). The percentage of the gated events in each of the right hand side quadrants is shown.
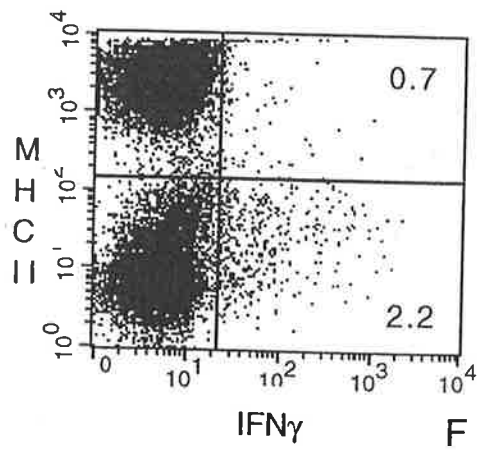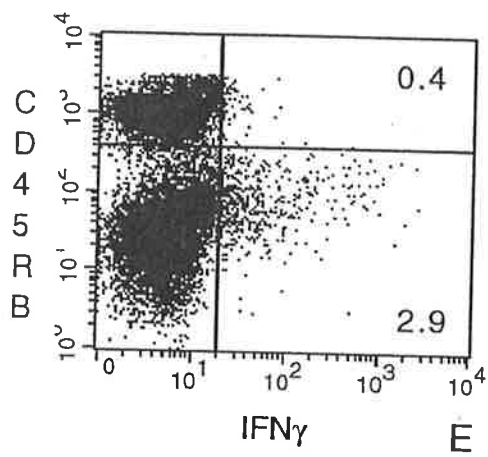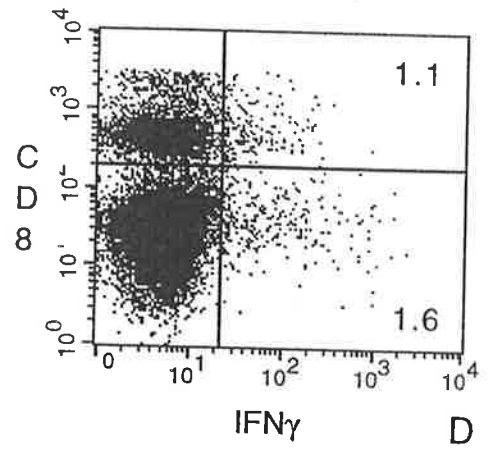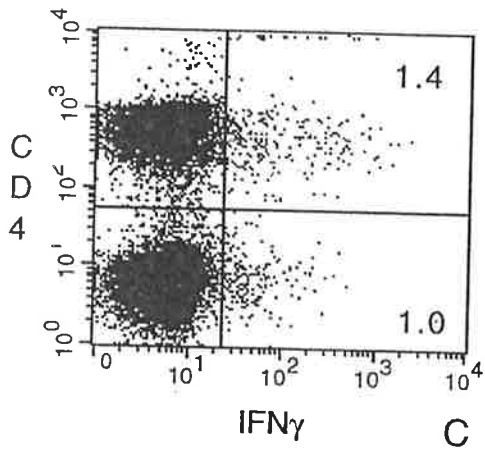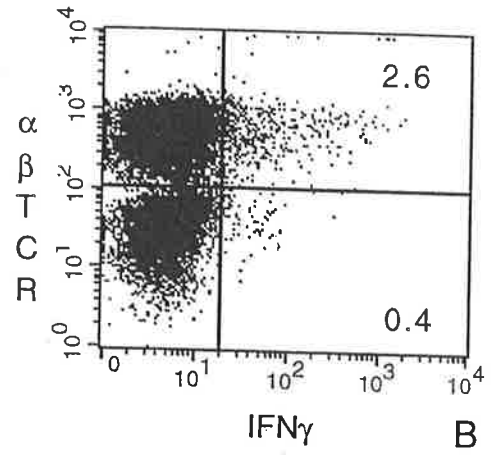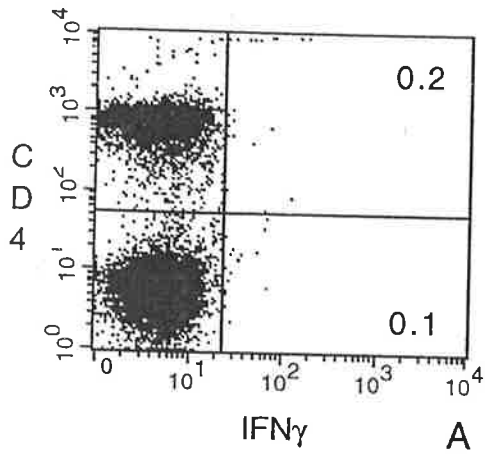
Table 3.1 Summary of the proportions of cells staining for intracellular IL-4 and IFN-γ among subsets of TD lymphocytes from a *N.brasiliensis* infested Hooded Wistar rat. Cells were stimulated *in vitro* for 6 hours by PMA plus ionomycin in the presence of Brefeldin A prior to staining.

Surface marker	Cell type	% of cells stained surface for a marker and IL-4	% of cells stained surface for a marker and IFN-γ
α/β TCR	α/β T cells	3.1	2.6
CD4	CD4$^+$ T cells	2.4	1.4
CD8	CD8$^+$ T cells	0.4	1.1
CD45RA	B cells	0.3	0.4
MHC class II	B cells, DC, some activated T cells	0.3	0.7

The production of IFN-γ by T cells but not B cells is consistent with the results of many published studies (see Section 1.5.2). TD lymphocytes cultured in the presence of Brefeldin A but without stimulation by PMA plus ionomycin do not produce levels of IFN-γ that are detectable by this technique (Figure 3.2.4.2 A).

3.2.5 Detection of intracellular cytokines by flow cytometry is indicative of capacity of the lymphocytes to produce these cytokines *in vivo*

Actinomycin D and cycloheximide (inhibitors of transcription and translation respectively) were used to examine whether production of detectable levels of cytokine by lymphocytes in response to stimulation requires synthesis of RNA.

3.2.5.1 Actinomycin D inhibits the production of RNA by stimulated lymphocytes

A preliminary investigation was undertaken to confirm that the addition of Actinomycin D to wells containing lymphocytes that are in the process of being stimulated with PMA plus ionomycin inhibits RNA synthesis. As shown in Figure 3.2.5.1, the addition of Actinomycin D reduced the incorporation of ^{3}H uridine (measured as counts per minute) compared with the incorporation by cells stimulated in the absence of Actinomycin D. This indicates that at the dose used, Actinomycin D efficiently inhibits RNA synthesis in lymphocytes. Thus, any new protein made following stimulation in the presence of Actinomycin D must be translated from mRNA present in the cell before stimulation.

3.2.5.2 The production of intracellular cytokines is inhibited by cycloheximide and reduced by Actinomycin D

Actinomycin D and cycloheximide were included during the *in vitro* stimulation of TD lymphocytes. The lymphocytes were collected from either a CFA-challenged DA rat (Figure 3.2.5.2 A-D) or a *N.brasiliensis* infested Hooded Wistar rat (Figure 3.2.5.2 E-L) and stimulated for 6 hours with PMA plus ionomycin in the presence of Brefeldin A. The indirect labelling of IFN-γ and IL-4 indicated that both of these cytokines were produced by the TD lymphocytes from the *N.brasiliensis*-infested rat (Figure 3.2.5.2 F and J), whereas lymphocytes from the CFA-challenged DA rat produced IFN-γ (B) but no IL-4 (data not shown). Very few unstimulated cells from either rat produced detectable levels of either cytokine (A, E and I). The inclusion of Actinomycin D during stimulation reduced the proportion of IFN-γ-producing TD lymphocytes from the CFA-challenged rat

3.2.5.1 Actinomycin D inhibits the production of RNA by stimulated lymphocytes. Thoracic duct lymphocytes from a *N. brasiliensis* infested Hooded Wistar rat were stimulated with PMA plus ionomycin for 6 hours in medium alone, or in the presence of 5μg/ml Actinomycin D. Cells were cultured in the presence of 4mCi/ml of $[5,6]^3$H-uridine before harvest onto glass fibre disks to measure isotope incorporation by scintillation counting (see Section 2.8). The counts per minute (CPM) and bars represent mean and standard deviation obtained from triplicate samples.
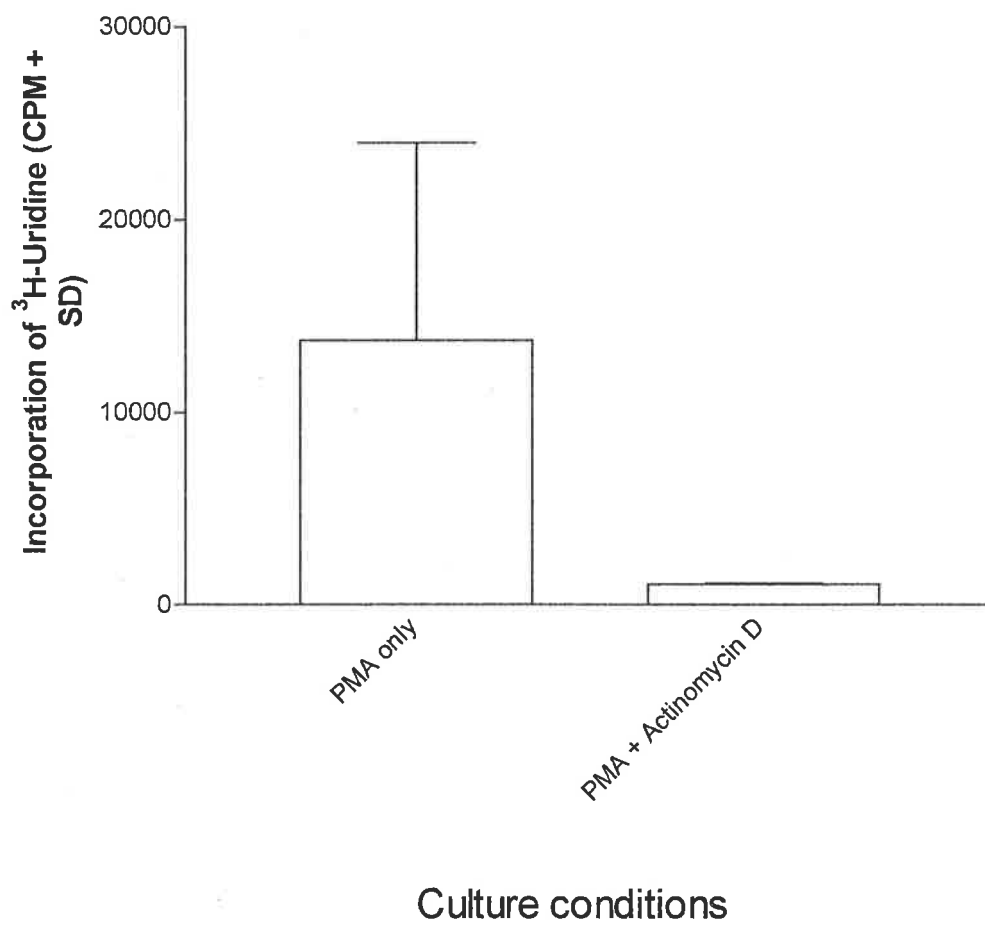
3.2.5.2 The detection of intracellular cytokines by flow cytometry is inhibited by cycloheximide and reduced by actinomycin D. Thoracic duct lymphocytes harvested from either a CFA-challenged DA rat (A-D) or a *N. brasiliensis* infested Hooded Wistar rat (E-L) were stimulated for 6 hours with PMA plus ionomycin in the presence of Brefeldin A. Cells stimulated in the presence of Brefeldin A without the addition of other agents are represented in panels A, B, E, F, I and J. Actinomycin D (5µg/ml) was present during the stimulation of the cells represented in panels C, G and K; and Cycloheximide (20µg/ml) was present during the stimulation of the cells represented in panels D, H and L. The production of IFN-γ and IL-4 was determined by flow cytometric analysis of cells that were made permeable with saponin and then labelling indirectly with either mAb 1B5 (negative control; FITC) (A, E and I), mAb DB-1 (anti-IFN-γ; FITC) (B-H) or mAb OX81 (anti-IL-4; FITC) (J-L). Forward scatter of light (FSC) is indicated on the y axis and the fluorescence of cells following indirect labelling is displayed on the x axis. The quadrant markers were determined as described in Figure 3.2.2.1, whereby the vertical divider was positioned such that a total of less than 1% of events from the flow cytometric analysis of cells labelled indirectly with 1B5 and FITC were displayed in the right quadrants (Figure 3.2.2.1 B). The percentage of the gated events in each quadrant and the mean fluorescence of these events are shown as a table in the upper right quadrant.

Brefeldin A only

Quad	% Gated	X Mean
UL	4.61	5.84
UR	0.06	41.75
LL	95.23	3.19
LR	0.10	31.86

FSC-Height

1B5

A

Brefeldin A only

Quad	% Gated	X Mean
UL	1.98	8.97
UR	2.10	192.85
LL	88.42	5.82
LR	7.49	117.70

FSC-Height

IFNγ

B

Brefeldin A and Actinomycin D

Quad	% Gated	X Mean
UL	4.71	8.09
UR	1.39	105.51
LL	87.44	5.23
LR	6.46	132.81

FSC-Height

IFNγ

C

Brefeldin A and Cycloheximide

Quad	% Gated	X Mean
UL	2.19	8.32
UR	0.34	37.44
LL	95.62	4.86
LR	1.84	37.92

FSC-Height

IFNγ

D

Brefeldin A only

Quad	% Gated	X Mean
UL	9.91	6.20
UR	0.34	24.27
LL	89.53	3.26
LR	0.23	36.53

1B5

E

Brefeldin A only

Quad	% Gated	X Mean
UL	1.14	6.68
UR	3.34	200.25
LL	84.38	4.06
LR	11.14	146.00

IFNγ

F

Brefeldin A and Actinomycin D

Quad	% Gated	X Mean
UL	3.41	6.10
UR	1.57	21.71
LL	92.63	3.34
LR	2.40	27.10

IFNγ

G

Brefeldin A and Cycloheximide

Quad	% Gated	X Mean
UL	2.29	6.40
UR	1.24	15.46
LL	95.17	3.37
LR	1.31	16.30

IFNγ

H

Brefeldin A only

Quad	% Gated	X Mean
UL	2.75	6.90
UR	0.52	16.32
LL	96.08	3.41
LR	0.65	23.67

IB5

I

Brefeldin A only

Quad	% Gated	X Mean
UL	1.80	6.78
UR	1.28	33.54
LL	93.02	3.25
LR	3.90	34.03

IL-4

J

Brefeldin A and Actinomycin D

Quad	% Gated	X Mean
UL	2.92	6.14
UR	0.32	16.25
LL	96.38	3.09
LR	0.37	37.66

IL-4

K

Brefeldin A and Cycloheximide

Quad	% Gated	X Mean
UL	1.99	6.82
UR	0.37	19.33
LL	97.14	3.23
LR	0.49	39.33

IL-4

L

slightly, from 9.59% (B) to 7.85% (C). A more striking reduction in the presence of Actinomycin D was seen when analysing lymphocytes from the *N.brasiliensis*-infested rat. The proportion of IFN-γ producing cells was reduced from 14.48% (F) to 3.97% (G). Nevertheless, the proportion of IFN-γ producing cells is still higher than the proportion in unstimulated lymphocytes (0.57% [E]). The proportion of IL-4 producing cells was greatly reduced by the inclusion of Actinomycin D during stimulation (K), suggesting some RNA synthesis is required for the production of IL-4 in response to stimulation. These results indicate the production of IFN-γ protein can still occur while transcription is inhibited by Actinomycin D but that IL-4 production in response to stimulation is dependent on transcription.

The addition of cycloheximide during the stimulation of TD lymphocytes inhibited the induction of detectable levels of IFN-γ strongly (D and H). A similar finding was made when examining IL-4 production (L). This indicates that protein synthesis is necessary for the accumulation of detectable amounts of either cytokine. This was expected, because neither cytokine could be detected in freshly isolated cells. Similarly, the presence of Brefeldin A during stimulation was necessary for the detection of intracellular cytokines, presumably because inhibition of secretion is necessary for cytokine accumulation to be sufficient for detection by flow cytometry (not shown).

3.2.6 Monensin is not suitable for use to induce an accumulation of cytokines within cells *in vivo*

An attempt was made to establish a protocol in which cytokine production by cells *in vivo* could be detected, thus obviating the need for *in vitro* re-stimulation of lymphocytes. It was reasoned that blocking of cytokine secretion *in vivo* might allow accumulation of intracellular cytokine in cells already engaged in cytokine secretion. Since Brefeldin A is not suitable for *in vivo* use, another Golgi-disrupting agent, monensin, was investigated for short-term use *in vivo*. It was hypothesised that monensin would cause the accumulation of protein in cells *in situ*, allowing their immediate harvest, fixation and labelling to demonstrate those that were actively producing cytokines *in vivo*.

It was necessary first to establish the suitability of monensin for the detection of intracellular cytokines. Lymph nodes cells from CFA-challenged rats (13 days post

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inoculation of CFA) were stimulated *in vitro* with PMA plus ionomycin for a period of six hours before fixation. Either Brefeldin A or monensin was present during the stimulation period. The fixed lymphocytes were made permeable with saponin and labelled indirectly with mAb 1B5 (negative control; FITC) or mAb DB-1 (anti-rat IFN-γ; FITC). Control cells were incubated with mAb 1B5 to establish the level of background binding of isotype-matched immunoglobulin (Figure 3.2.6.1 A). Few lymphocytes produced detectable levels of IFN-γ when stimulated in the absence of either Brefeldin A or monensin (B). Large cells appeared fewer following incubations with either Brefeldin A or monensin (C and D), suggesting these agents may be toxic for activated lymphocytes.

The inclusion of Brefeldin A in the stimulation conditions increased the proportion of cells containing detectable IFN-γ from ~1.9% to ~ 4% (Figure 3.2.6.1 C). Similarly, addition of monensin increased the proportion of cells producing IFN-γ to ~5.1% (D), indicating that monensin, like Brefeldin A, enhances the detection of intracellular cytokines. The presence of >2% of cells that produced IFN-γ in the absence of stimulation (B) is unusual and may reflect the use of lymph node cells in place of TD lymphocytes.

To examine the suitability of monensin as an agent to use *in vivo*, arthritic rats (see Section 2.2) were injected intraperitoneally with either monensin or vehicle (saline) alone. The LD_{50} of monensin is reported to be ~30mg/kg when administered intraperitoneally to mice (Gumila et al., 1997) or 29mg/kg administered orally to rats (personal communication, Dr. A. O'Connor, Sigma). Cells prepared from the inguinal and popliteal lymph nodes were either fixed immediately (monensin-treated rats) or stimulated *in vitro* in the presence of monensin for six hours (control rats) before fixation. Cells were made permeable with saponin and then labelled indirectly with either mAb DB-1 (anti-IFN-γ; FITC) or mAb 1B5 (negative control; FITC). The data represented in Figure 3.2.6.2, panels A-D include only those cells that were gated electronically in the lymphocyte gate (see Figure 3.2.2.1 A). The non-specific staining of stimulated lymph node cells from a control rat by mAb 1B5 is shown in Figure 3.2.6.2 A.

Lymph node cells from control arthritic rats contained more IFN-γ producing cells (7.36%) following stimulation *in vitro* in the presence of monensin (Figure 3.2.6.2 B) than cells that were not stimulated (0.37%) (C). The proportion of cells producing IFN-γ in

Figure 3.2.6.1 Monensin is suitable for *in vitro* use for the detection of intracellular cytokines. Pooled popliteal and inguinal lymph node cells from arthritic rats (13 days post-inoculation of CFA) were stimulated for six hours in the presence of PMA plus ionomycin and either Brefeldin A (C) or monensin (5µg/ml) (D). Cells that were stimulated with PMA plus ionomycin with neither Brefeldin A nor monensin are represented in panel B. The production of IFN-γ was determined by flow cytometric analysis of cells that were made permeable with saponin and then labelling indirectly with either mAb 1B5 (negative control; FITC) (A) or mAb DB-1 (anti-IFN-γ; FITC) (B-D). Forward scatter of light (FSC) is indicated on the y axis and the fluorescence of cells following indirect labelling is displayed on the x axis. The quadrant markers were determined as described in Figure 3.2.2.1, whereby the vertical divider was positioned such that a total of less than 1% of events from the flow cytometric analysis of cells labelled indirectly with 1B5 and FITC were displayed in the right quadrants (Figure 3.2.2.1 B). The percentage of the gated events in each quadrant and the mean fluorescence of these events are shown as a table in the upper right quadrant.
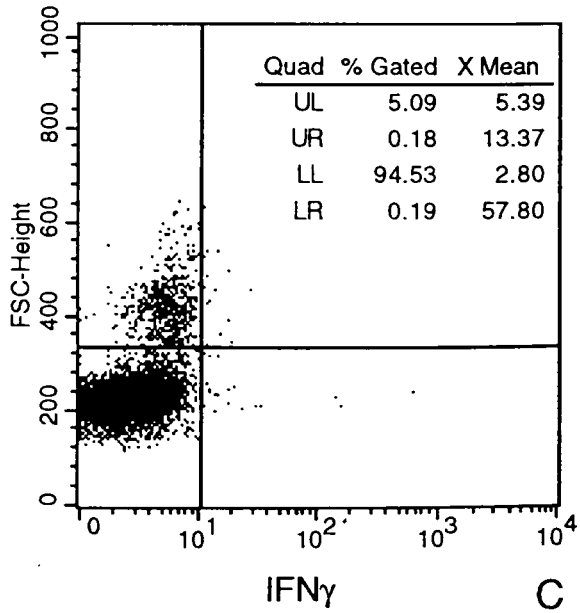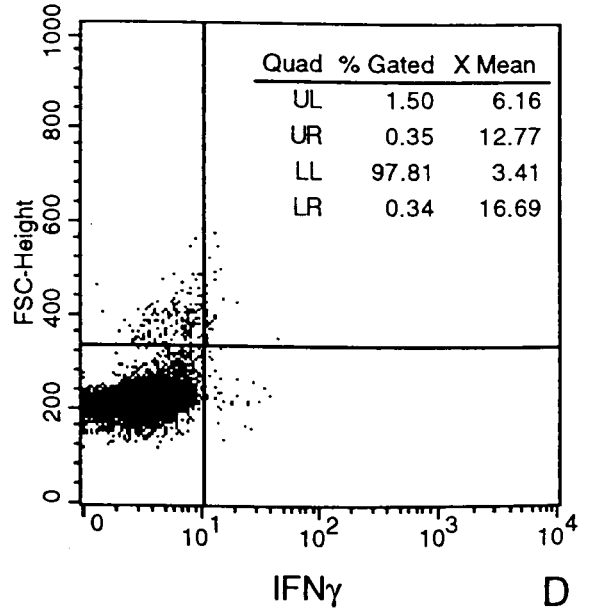
A

FSC-Height

Quad	% Gated	X Mean
UL	3.02	7.77
UR	0.09	57.61
LL	96.69	4.19
LR	0.20	27.37

1B5

A

none

FSC-Height

Quad	% Gated	X Mean
UL	4.37	9.15
UR	0.50	36.54
LL	93.25	4.81
LR	1.87	34.14

IFNγ

B

Brefeldin A

FSC-Height

Quad	% Gated	X Mean
UL	1.02	10.03
UR	0.71	87.10
LL	95.03	6.25
LR	3.24	143.86

IFNγ

C

Monensin

FSC-Height

Quad	% Gated	X Mean
UL	0.86	11.67
UR	1.22	58.81
LL	94.02	7.29
LR	3.91	67.50

IFNγ

D

Figure 3.2.6.2 Monensin is not suitable for use *in vivo* to detect intracellular cytokines. Arthritic rats (13 days post-inoculation of CFA) were injected i.p. with either 2.5mg/kg body weight of monensin or an equivalent volume of saline. They were killed 5 hours later. Pooled popliteal and inguinal lymph node cells from saline treated rats were cultured in the presence of PMA plus ionomycin and monensin (5μg/ml) for six hours in culture medium alone (C) or in the presence of PMA plus ionomycin (A and B). Pooled popliteal and inguinal lymph node cells prepared from monensin-treated rats were fixed immediately before labelling (D). The production of IFN-γ was determined by flow cytometric analysis of cells that were made permeable with saponin and then labelling indirectly with either mAb 1B5 (negative control; FITC) (A) or mAb DB-1 (anti-IFN-γ; FITC) (B-D). Forward scatter of light (FSC) is indicated on the y axis and the fluorescence of cells following indirect labelling is displayed on the x axis. The quadrant markers were determined as described in Figure 3.2.2.1, whereby the vertical divider was positioned such that a total of less than 1% of events from the flow cytometric analysis of cells labelled indirectly with 1B5 and FITC were displayed in the right quadrants (Figure 3.2.2.1 B). The percentage of the gated events in each quadrant and the mean fluorescence of these events are shown as a table in the upper right quadrant.

Stimulated + monensin *in vitro*
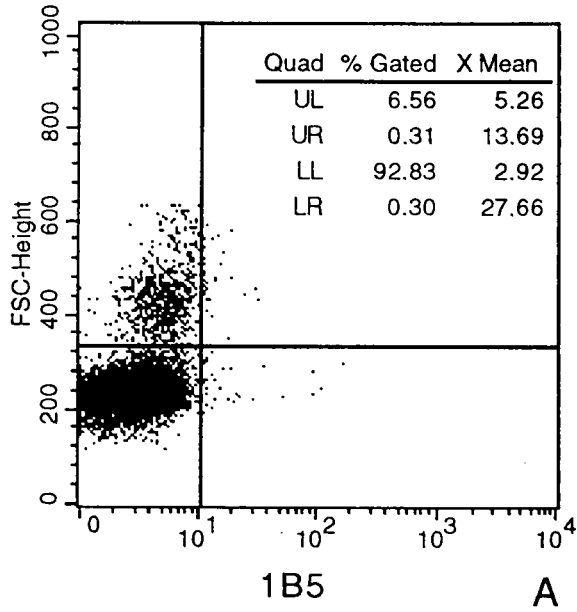
Quad	% Gated	X Mean
UL	6.56	5.26
UR	0.31	13.69
LL	92.83	2.92
LR	0.30	27.66

FSC-Height

1B5

A

Stimulated + monensin *in vitro*

Quad	% Gated	X Mean
UL	4.46	7.13
UR	2.41	56.68
LL	88.18	4.20
LR	4.95	108.52

FSC-Height

IFNγ

B

Unstimulated + monensin *in vitro*

Quad	% Gated	X Mean
UL	5.09	5.39
UR	0.18	13.37
LL	94.53	2.80
LR	0.19	57.80

FSC-Height

IFNγ

C

Monensin *in vivo*

Quad	% Gated	X Mean
UL	1.50	6.16
UR	0.35	12.77
LL	97.81	3.41
LR	0.34	16.69

FSC-Height

IFNγ

D
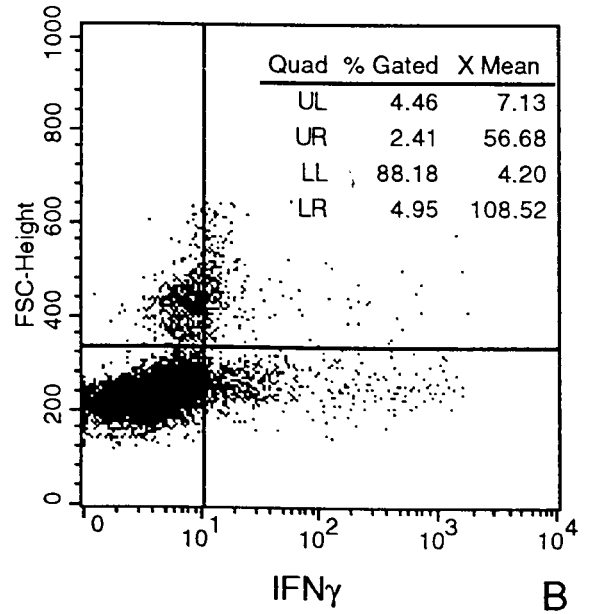
freshly-isolated lymph node cells from a rat treated with 2.5mg/kg body weight monensin, and killed 5 hours later is indistinguishable (D) from the background levels of non-specific staining with mAb 1B5 (A) and of IFN-γ producing cells in unstimulated cultures (C). The treatment of with higher doses of monensin (up to 10mg/kg body weight) was toxic for arthritic rats. Nevertheless, no intracellular cytokines were detected within lymphocytes prepared from rats that survived higher doses of the drug or were killed sooner after administration of higher doses.

3.3 Discussion

3.3.1 MRC-mAb OX81 can be used for indirect immunofluorescence staining of intracellular IL-4

Immunofluorescence staining of CHO cells transfected with multiple copies of the gene encoding rat IL-4 by the indirect method using mAb OX81 indicated that the antibody has specificity for IL-4 under the conditions used to detect the intracellular cytokine. Staining was inhibited by pre-incubation of mAb OX81 with supernatant from CHO-IL-4 cell cultures but not by culture medium alone nor culture medium from cell cultures of CHO-IL-2 cells (data not shown). Furthermore, CHO-cells transfected with multiple copies of the gene encoding IL-2 were not stained by mAb OX81. Others have already demonstrated that mAb OX81 has specificity for IL-4 by the inhibition of an IL-4 bio-assay (Ramirez et al., 1996).

The histogram shown in Figure 3.2.1.1 A represents the fluorescence of permeabilized CHO-IL-4 cells stained indirectly with mAb OX81. The fluorescence intensity of the cells is relatively low because hybridoma supernatant of mAb OX81 was used at a dilution of 1:2 in this experiment and because the only IL-4 in these cells was the small pool of cytokine en route to secretion. Brefeldin A disrupts the function of the Golgi apparatus and causes accumulation of secreted protein within cells, inducing swelling of the Golgi cisternae, which are transformed into long tubular processes (Klausner and Donaldson, 1992). Brefeldin A treated cells did not have discrete Golgi stacks and staining with mAb OX81 indicated that the cytokine was distributed throughout the cytoplasm. The accumulation of protein within the cells that occurs following treatment with Brefeldin A greatly enhances the signal intensity produced after immunofluorescence staining.

3.3.2 Immunofluorescence staining of intracellular cytokines for flow cytometry detects cytokine production by *in vitro* stimulated lymphocytes

The method developed using CHO-IL-4 cells was applied successfully to detect IL-4 production by lymphocytes obtained from a *N.brasiliensis*-infested rat and stimulated *in vitro*. Very few cells obtained from a normal rat or a CFA-challenged rat produced IL-4. The detection of IL-4 production by lymphocytes from the *N.brasiliensis*-infested rat suggest that the cells from this animal were already biased towards the production of certain cytokines (Th2) by the infestation and that stimulation *in vitro* did not alter this bias or induce IL-4 production in cells from un-infested animals.

In an attempt to increase IL-4 production, lymphocytes were cultured in the presence of IL-4 containing supernatant from CHO-IL-4 cells. *In vitro* production of IL-4 by lymphocytes is thought to require the presence of exogenous IL-4 (Swain et al., 1988). However, the addition of IL-4 to cultures of lymphocytes resulted in a large proportion of the population (between 11% [Figure 3.2.2.2 H] and 23% [Figure 3.2.2.2 D]) being labelled by mAb OX81. This raised the possibility that IL-4 detected by mAb OX81 may have bound to the surface of the cells, rather than produced by the cells. This suggestion was supported by the observation that cells cultured in the presence of IL-4 were stained with similar intensity, regardless of whether they were cultured in the presence or absence of Brefeldin A (data not shown).

In contrast to IL-4, intracellular IFN-γ was detected in stimulated TD lymphocytes obtained from both *N.brasiliensis*-infested rats and CFA-challenged rats. Stimulation was required to induce detectable levels of IFN-γ and very few cells that were fixed immediately after collection from the rats contained sufficient IFN-γ to be detected by this technique (data not shown). Lymphocytes from a normal rat produced detectable levels of IFN-γ after stimulation *in vitro* (Figure 3.2.2.3 B and C), although IFN-γ-producing cells were less abundant in stimulated TD lymphocytes from this rat than in cells from the *N.brasiliensis*-infested or the CFA-challenged rats.

As a stimulus, PMA plus ionomycin induced a greater proportion of IFN-γ-producing cells than stimulation with the mitogen, Con A (Figure 3.2.2.3 F-G and J-K). However, the two stimuli induced similar proportions of IL-4-producing cells (Figure 3.2.2.2 F-G).

Others have reported that another mitogenic stimulus, phytohaemaglutinin (PHA), was grossly inferior to PMA in its ability to induce the production of cytokines by human T lymphocytes (North et al., 1996). PMA plus ionomycin was chosen as the stimulus for cytokine production in all subsequent experiments.

Among the stimulated lymphocytes (gated as shown in Figure 3.2.2.1), it is apparent that larger cells contained a greater proportion of both IL-4 (Figure 3.2.2.2 F) and IFN-γ (Figure 3.2.2.3 F and J) producing cells, compared with smaller cells. It seems likely that larger, activated lymphocytes may be the major producers of these cytokines *in vivo*.

3.3.3 Kinetics of production of IL-4 and IFN-γ by thoracic duct lymphocytes after stimulation in vitro with PMA plus ionomycin

The kinetics of cytokine production, in response to stimulation with PMA, was examined to determine standard conditions for subsequent studies into the production of cytokines by $CD4^+$ T cells during the prodrome of AA. A particular consideration was to minimise the duration of the *in vitro* stimulation, so that the cells that produced cytokine within the period of stimulation were cells that could be termed recently activated effector cells.

Investigation of the production of cytokines during a 21 hour period of stimulation indicated that between 4 and 8 hours of stimulation with PMA plus ionomycin are sufficient to induce the production of IL-4 and IFN-γ (Figure 3.2.3.1 A and B). The cytokines produced during this early period after activation appears to reflect the known bias in systemic T-helper response to infestation with *N.brasiliensis* in mice (Street and Mosmann, 1991). TD lymphocytes from *N.brasiliensis*-infested rats producing substantially more IL-4 than lymphocytes from normal or CFA-challenged rats. Interestingly, a considerable proportion of TD lymphocytes from *N. brasiliensis*-infested rats produced IFN-γ. This phenomenon has been observed repeatedly (data not shown) and although it is beyond the scope of this thesis to investigate further, it would be of interest to know whether the IL-4 producing population is distinct from the IFN-γ producing population. *In vitro* stimulation of TD lymphocytes from normal rats resulted in a very slight increase in intracellular IFN-γ, which was substantially less than seen with cells from rats that had been infested with *N.brasiliensis* or challenged with CFA *in vivo*.

Thus the majority of the cytokine detected is attributable to the effects of *in vivo* activation, albeit amplified by *in vitro* stimulation.

3.3.4 Phenotype of cytokine producing cells

Dual fluorochrome staining was used to identify the phenotype of individual cells producing IL-4 and IFN-γ. TD lymphocytes from a *N.brasiliensis*-infested Hooded Wistar rat, stimulated for 6 hours *in vitro* with PMA plus ionomycin in the presence of Brefeldin A, produced IFN-γ and IL-4. Most of the IL-4 producing cells were shown by dual colour analysis to be CD4^{+} α/βTCR^{+} T cells. The Hooded Wistar strain was used because after infestation with *N.brasiliensis*, it appears to produce a more prevalence of IL-4-producing cells than the DA strain (data not shown). Further investigations into the bias of immune responses between these two strains is beyond the scope of this thesis. However, it notable that DA rats produce less IL-4 in response to *N.brasiliensis* infestation (suggesting a weaker Th2-mediated response) and this strain (unlike many others) has 100% incidence of severe arthritis in response to inoculation of CFA. This may indicate that immune responses in DA rats have a Th1 bias and this could be a significant predisposing factor in the development of AA.

In the case of IFN-γ, some of the cytokine producing cells were CD8^{+} T cells (Figure 3.2.4.2 D). It seems unlikely that other cell types analysed in this study produced IFN-γ. The detection of low intensity IFN-γ staining in a very small percentage of CD45RA^{+} cells is of doubtful significance. Other candidate cell types for the production of IFN-γ are NK cells, but these have a low frequency in TD lymph.

3.3.5 Detection of intracellular cytokines by flow cytometry is indicative of capacity of the lymphocytes to produce these cytokines *in vivo*

The addition of Actinomycin D was shown to inhibit the uptake of tritiated uridine by TD lymphocytes during a 6 hour stimulation with PMA plus ionomycn (Figure 3.2.4.1). However, the drug did not prevent the detection of IFN-γ-producing cells (Figure 3.2.5.2 C and G). With the caveat that transcription of the gene encoding IFN-γ is not unusually resistant to the effect of Actinomycin D, it appears that translation alone was sufficient to allow production of enough IFN-γ to be detected by flow cytometry. This indicates that IFN-γ mRNA was present in at least some cells before they were activated *in vitro* and

that transcription had been initiated *in vivo*. However, Actinomycin D did reduce the proportion of IFN-γ secreting cells, especially in TD lymphocytes obtained from a *N.brasiliensis* infested donor, suggesting that the stimulation conditions did lead to some de novo synthesis of mRNA encoding this cytokine. In contrast, detection of IL-4-producing cells was severely inhibited by Actinomycin D (Figure 3.2.5.2 K), indicating that production of this cytokine in response to stimulation is dependent on transcription. Notwithstanding, very little cytokine production in response to *in vitro* stimulation was detected in TD lymphocytes without prior *in vivo* activation.

Addition of cycloheximide prevented the detection of both IFN-γ- and IL-4- producing cells (Figures 3.2.5.2 D, H and L), indicating, as expected, that protein synthesis is necessary to produce detectable levels of intracellular IFN-γ and IL-4.

3.3.6 Monensin is not suitable for use to induce an accumulation of cytokines within cells *in vivo*

The failure to detect intracellular cytokines in freshly isolated cells demanded that cells be stimulated *in vitro* in the presence of either Brefeldin A or monensin to achieve synthesis and accumulation of cytokine protein at detectable levels. However, this raises the possibility that the cytokine production observed under these conditions is not an accurate reflection of the activities (or potential activities) of these cells *in vivo*. An attempt was therefore made to induce the accumulation of cytokine protein within the cells prior to their harvest by the *in vivo* administration of monensin. This pilot study did not achieve its aim and it was not pursued.

The effects of monensin on cell metabolism (principally slowing intracellular transport of newly synthesised secretory proteins) are reversible and incomplete (Tartakoff, 1983) and control of tissue levels of the drug is difficult. The main problem encountered in this study was the toxicity of the compound. At doses that were tolerated by the rats, there was no evidence of cytokine accumulation. It may be suitable for *in vivo* use when administered locally, such as by direct injection into the hind paws of arthritic rats prior to harvesting cells by enzymatic digestion of the soft tissues. Under these conditions, the cells that are exposed to the drug may be effector cells that are undergoing stimulation by antigen presented by local antigen presenting cells. The prospect of detecting cytokines

which are produced whilst the cells are *in situ* is appealing in light of the demonstration that *in vitro* stimulation of cells induces both transcription and translation. In retrospect, the choice of lymph node cells as the cells used to assess the *in vivo* affects of monensin was probably not optimal and cells from a site of effector cell activity may have been better.

3.3.7 General discussion

The aim of these studies was to investigate the detection of cytokines by flow cytometry and to determine the *minimum requirements* for stimulation that are necessary to detect intracellular cytokines produced $CD4^+$ T cells.

The standard stimulatory conditions chosen for later studies of cytokine production by lymphocytes from rats during the prodrome of AA was a 6 hour stimulation with PMA plus ionomycin in the presence of Brefeldin A.

CHAPTER 4

RESPONSE IN THE LOCAL LYMPH NODES DURING THE PRODROME OF ADJUVANT-INDUCED ARTHRITIS, WITH PARTICULAR REFERENCE TO CD4$^+$ T LYMPHOCYTES

4.1 Introduction

The specific role of T cells in the pathogenesis of RA is controversial but a considerable body of evidence suggests that the joint destruction is mediated by activated T cells within the synovium and in particular, by the cytokine products of these cells. Cytokines produced by T cells may activate other cells such as macrophages and fibroblasts and it is probably products from these cells that are responsible for the articular damage. To investigate the pathogenesis of RA, a number of animal models are available although probably none mimic RA perfectly. A model used by the Arthritis Research Laboratory (ARL) is adjuvant-induced arthritis (AA) in the rat, which has a number of clinical and immunological features in common with RA.

Adjuvant arthritis (AA) is induced in Dark Agouti (DA) rats by inoculation of Complete Freund's Adjuvant (CFA) at the base of the tail. The prodromal phase of AA is approximately 9 days in duration and clinical signs of joint swelling become apparent thereafter. The maximum severity of arthritis is observed around 16 days after inoculation of CFA and AA is a partially remitting and recurrent disease (Pearson, 1963; Stanescu et al., 1987).

It is thought that the inoculation of CFA leads to the presentation of inciting antigens (self-antigens and/or cross-reactive mycobacterial antigens) to T cells in the lymph nodes (LN) draining the site of inoculation. These activated T cells, which might be expanded subsequently in response to synovial autoantigens, enter the circulation via the efferent lymphatics and they are distributed to the joints, where they initiate inflammation through the production of cytokines and other inflammatory mediators.

Research in the ARL has demonstrated that activated CD4$^+$ T cells found in the thoracic duct (TD) lymph of rats during the late prodromal phase of the disease (9 days post-inoculation of CFA) have the capacity to enter both normal and inflamed joints after adoptive transfer to syngeneic recipients. Furthermore, these activated CD4$^+$ T cells can transfer the disease to naive recipient rats. The arthritogenic population is contained within a subset of CD4$^+$ T cells that express CD25, MHC class II, CD134 and CD71 (Spargo et al., 2001).

Effector CD4$^+$ T cells can be divided into functional subsets (Th1 and Th2), according to the cytokines that they produce. However, there are few surface markers whose expression correlates with the subsets defined by cytokine production. Therefore, it has been necessary to examine the cytokines produced by CD4$^+$ T cells in order to determine the functional phenotype.

Although AA is used commonly as a model for rheumatoid arthritis, the mechanisms controlling AA are understood poorly. Inoculation of CFA might lead to a typical delayed-type hypersensitivity response, whereby molecular mimicry between mycobacterial antigens and synovial antigens results in the focussing of an inflammatory response in synovial tissues. Alternatively, the inoculation of CFA may lead to a localized immune response that exposes self-antigens, against which autoreactive lymphocytes are activated and expanded.

It was hypothesised that during the 9 days between inoculation of CFA and visible signs of joint swelling, there is an expansion of autoreactive CD4$^+$ T cells that express both surface and secreted molecules that allow the entry of the cells into target tissues and the execution of their effector functions. The specific antigen(s) against which these autoreactive cells are directed is unknown. It was hypothesised that the inoculation of CFA leads to the presentation of inciting antigens (self-antigens and/or cross reactive mycobacterial antigens) to T cells in the local lymph nodes that drain the site of inoculation. These activated T cells, which might be expanded subsequently in response to synovial autoantigens, reach the joints via the TD lymph and the blood circulation.

If this hypothesis is correct, up-regulation of the expression of activation markers on CD4$^+$ T cells would be observed first in the inguinal lymph nodes (draining the site of inoculation) and then subsequently in the popliteal lymph nodes (and in other nodes draining joints). These events would precede the onset of the visible signs of joint swelling because logically, acquisition of arthritogenicity should precede the initiation of joint inflammation. It is predicted that transfer of either inguinal or popliteal lymph node cells, harvested at the end of the prodromal phase of AA, should transfer disease adoptively to naïve rats, thus demonstrating the arthritogenic capacity of the cells.

The up-regulation of expression of ICAM-1 (CD54) (Springer, 1994), transferrin Rc (CD71), IL-2Rc (CD25) (Salmeron et al., 1995), integrin α_4 subunit (CD49d) (reviewed in Springer, 1994), OX40 antigen (CD134) (Paterson et al., 1987; Mallett et al., 1990), down-regulation of expression of CD62L (Tedder et al., 1995) and loss of the high molecular weight isoform of CD45 (Luqman & Bottomly, 1992) on CD4$^+$ T cells are events associated with antigen stimulation. In this chapter, the expression of these molecules by CD4$^+$ T cells from selected lymph nodes was examined using monoclonal antibodies (mAb)s and flow cytometry. Other molecules that serve as indicators of the functional capabilities of T cells, such as CD44 and LFA-1, were examined also using this technique. As discussed in Chapter 3, the detection of cytokines using flow cytometry relies on *in vitro* stimulation of cells before fixation and labelling. The availability of mAbs suitable for this technique allowed the detection of intracellular IL-4 and IFN-γ. These cytokines are useful in distinguishing Th1 (IFN-γ) from Th2 (IL-4) cells.

This chapter describes the events occurring in lymph nodes during the prodromal phase and early inflammatory phase of AA. The inguinal and popliteal lymph nodes were chosen because they drain the site of CFA inoculation and the hind paws respectively. Although joint swelling does not become apparent until 9-10 days after inoculation of CFA, these lymph nodes are enlarged within 3 days and remained enlarged for the observed course of the disease. The arthritogenicity of cells isolated from inguinal and popliteal lymph nodes was examined by transferring the cells into naïve recipient rats. The surface antigen phenotype of the CD4$^+$ T cells was assessed by flow cytometry. The experimental design was such that the phenotype of the lymph node cells at each time point was examined on two separate occasions. Since the analyses of cells from individual animals were performed progressively over several months, the settings of the flow cytometer were standardised and only minor adjustments were made each day in order to ensure accurate compensation between the signals in fluorescence channels 1 and 2 of the instrument.

4.2 Results

4.2.1 The macroscopic features of AA

The physical changes that occur following inoculation of rats with CFA have been well characterised (Pearson, 1956). Rats show evidence of a systemic disease with features

such as anorexia, cachexia and lethargy. Although joint inflammation became macroscopically visible around 9 days after inoculation of CFA, the signs of systemic illness are apparent earlier (Phillipe et al., 1997).

4.2.1.1 Comparison of body weight of rats during the prodrome of AA

A typical course of AA is shown in Figure 4.2.1.1 A (N = 5). Joint inflammation becomes apparent around 9 days after inoculation of CFA and increases in severity for another 5 days until rats are euthanased at day 14 for ethical reasons. The rats were weighed before harvesting the lymph nodes and the hind paws (described in Chapter 6) and as might be expected, those that were inoculated with CFA had lower average body weight than saline-injected controls. The mean weight loss occurred in the first 3 days after inoculation of CFA and continued throughout the 12 days of the experimental period (Figure 4.2.1.1 B). The incidence of AA is essentially 100% in DA rats. It can be predicted confidently that the animals killed during the prodromal period would all have gone on to develop arthritis.

4.2.1.2 Weight of the inguinal and popliteal lymph nodes during the prodrome of AA

Since AA is a systemic disease, it seemed unlikely that any secondary lymphoid organs would be unaffected by the challenge with CFA. Consequently, the lymph nodes chosen for comparison were those taken from saline-injected rats. Inoculation of 100μl of CFA into the base of the tail induced an increase in the weight of the inguinal lymph nodes within 3 days (Figure 4.2.1.2 A). The average weight of these nodes continued to increase at least until day 9 post-inoculation and decreased by day 12. At all time-points investigated, the average weight of the inguinal lymph nodes from CFA-challenged rats was higher than the weight of those from saline-injected controls.

The popliteal lymph nodes from normal rats are very small. However, following inoculation of CFA, there was a striking increase in the weight of these lymph nodes. They remained heavier than those from control rats until at least 12 days after inoculation (Figure 4.2.1.2 B).

4.2.2 T cell sub-populations in cells isolated from the inguinal and popliteal lymph nodes during the prodrome of AA

Figure 4.2.1.1 Severity of arthritis and body weight of rats during the prodrome of AA. Rats were inoculated with CFA by subcutaneous injection into the base of the tail. The time course of AA is shown in Figure A, in which arthritic scores were assigned to each rat in a group (N = 5) which was monitored from disease induction to death at day 14. Arthritic scores were determined as described in Section 2.2.3. The data shown in Figure B were derived from two different experiments in which groups of rats were killed 3, 6, 9 or 12 days after inoculation of CFA and weighed. Rats inoculated with saline were included as a control group. The bars indicate the mean body weight for each group, in which data were combined from the two separate experiments. The total group sizes were as follow: saline-inoculated (N = 20), day 3 post-inoculation of CFA (N = 36), day 6 post-inoculation of CFA (N = 28), day 9 post-inoculation of CFA (N = 26) and day 12 post-inoculation of CFA (20).

A

B
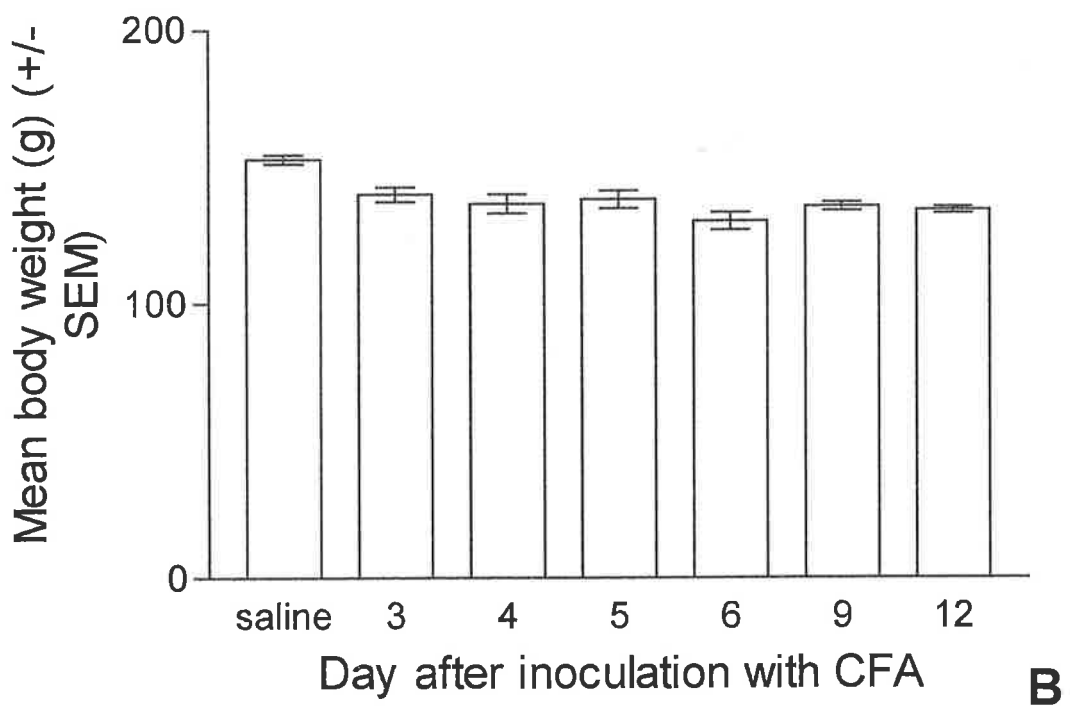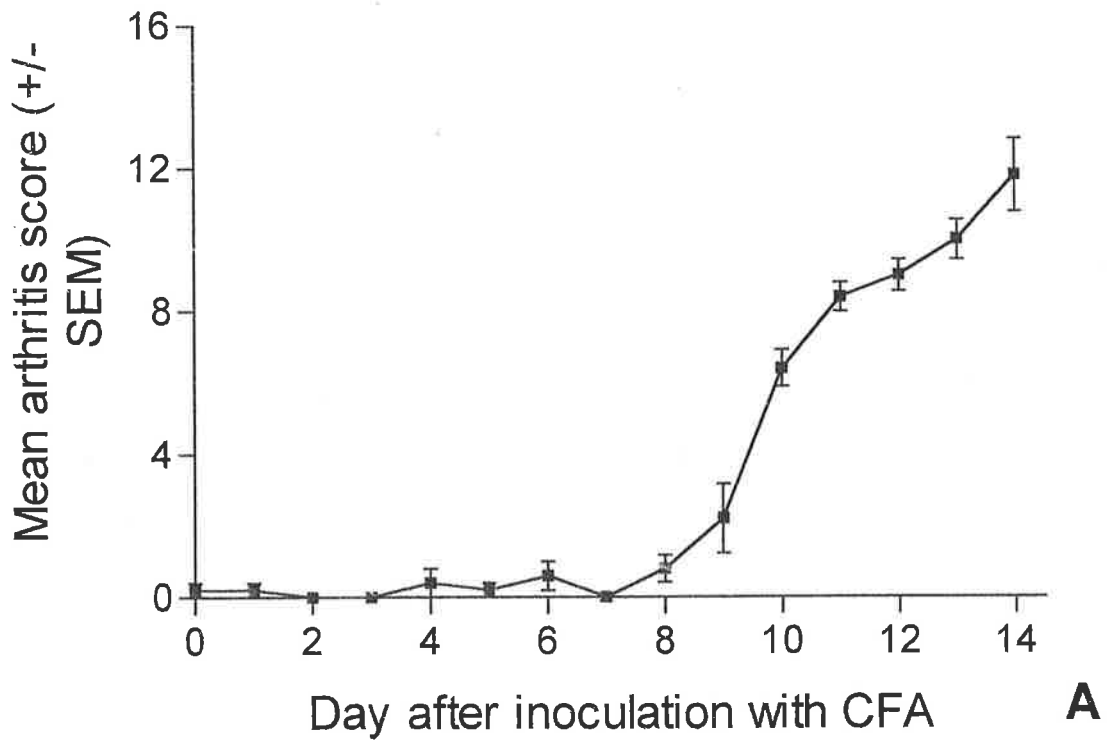
Figure 4.2.1.2 Weight change of lymph nodes during the prodrome of AA. The popliteal and inguinal lymph nodes were removed from rats using blunt dissection with forceps. The pooled inguinal lymph nodes (A) and popliteal lymph nodes (B) from each rat were weighed. The group sizes were as follow: saline-inoculated (N = 20), day 3 post-inoculation of CFA (N = 36), day 6 post-inoculation of CFA (N = 28), day 9 post-inoculation of CFA (N = 26) and day 12 post-inoculation of CFA (20).

A

B
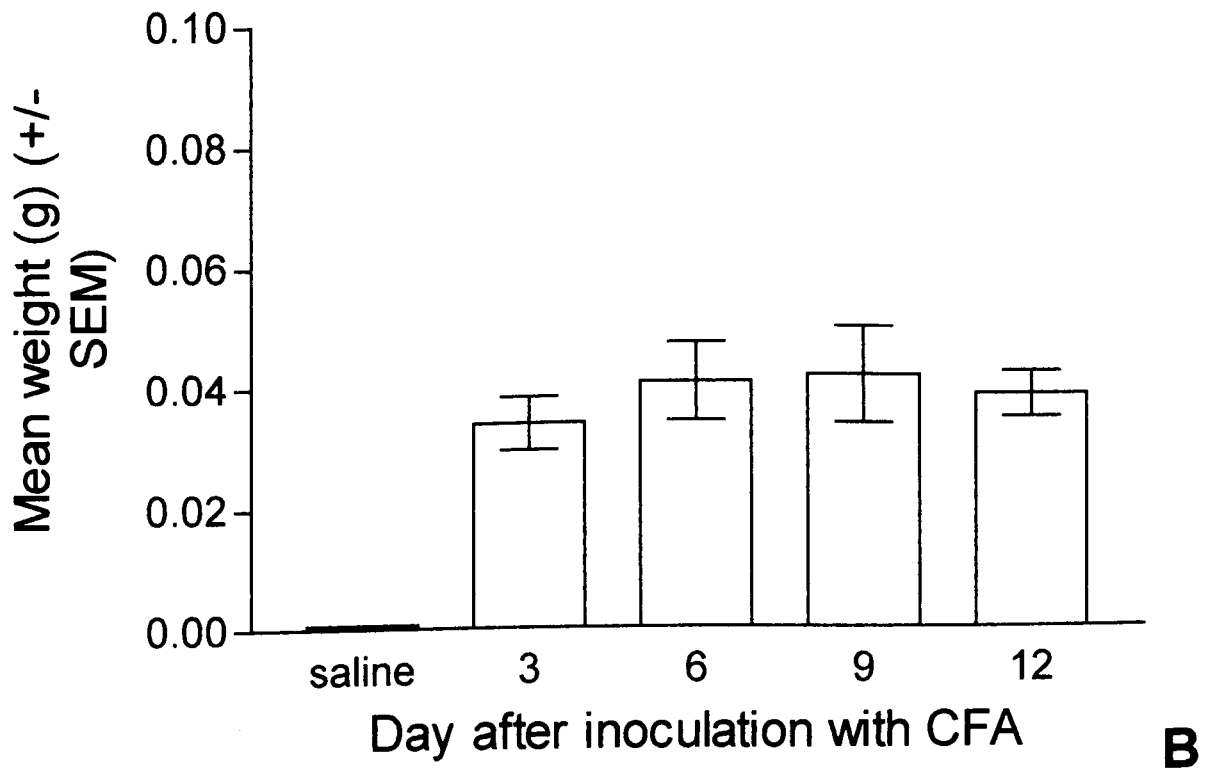
Single cell suspensions were made from lymph nodes obtained from rats described in the legend of Figure 4.2.1.1 B. They were prepared as described in Section 2.3.1. The expression of cell surface antigens was examined using flow cytometric analysis. It has been shown previously that activated CD4$^+$ T cells in the TD lymph from rats during the prodrome of AA have arthritogenic capacity. The focus of this study was, therefore, the CD4$^+$ T cell population in the respective lymph nodes.

4.2.2.1 CD4$^+$ T cells

Dual fluorochrome staining was used to examine the co-expression of various surface molecules by CD4$^+$ T cells. Large cells that expressed low levels of CD4, such as activated macrophages, were eliminated from analysis by electronic gating using the flow cytometry software. The proportions of CD4$^+$ T lymphocytes in the cells prepared from the inguinal and popliteal lymph nodes are shown in Figure 4.2.2.1.

In the inguinal lymph nodes, the percentage of CD4$^+$ T cells decreased by the third day following inoculation of CFA and remained at this lower level until the sixth day. The proportion of CD4$^+$ T cells increased thereafter and by the twelfth day, the levels were similar to those seen in the controls (Figure 4.2.2.1 A and C).

The proportion of CD4$^+$ T cells decreased by the third day in the popliteal lymph nodes. However, at the other time points, the proportion of CD4$^+$ T cells was similar in the popliteal lymph nodes of the CFA-challenged rats and those inoculated with saline (Figure 4.2.2.1 B and D).

4.2.2.2 CD8$^+$ T cells

In both the inguinal and popliteal lymph nodes, the proportion of lymphocytes that expressed the CD8$\alpha\beta$ complex (classical CD8$^+$ T cells, represented in the upper right quadrants of the scatter plots in Figure 4.2.2.2 A and B) increased slightly during the prodrome of AA and remained at this level after the onset of visible joint swelling at days 9-12 after inoculation of CFA (Figure 4.2.2.2 C and D).

4.2.2.3 γ/δ T cells

Figure 4.2.2.1 CD4$^+$ T cell populations in cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Inguinal (A, C) and popliteal (B, D) lymph node cells from saline- or CFA- inoculated rats were first stained indirectly with mAb W3/25 (anti-CD4; FITC), followed by direct labelling with mAb R73 (anti-α/β TCR; PE). Representative two colour plots of inguinal lymph node cells (A) and popliteal lymph node cells (B) isolated from a rat at 6 days post-inoculation of CFA are shown. The proportions of inguinal lymph node cells (C) and popliteal lymph node cells (D) that expressed CD4 at different times after inoculation with CFA are shown graphically, using data generated by flow cytometric analysis. Each triangle indicates the proportion of CD4$^+$ T cells among cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.

A

B

C

D

% of gated cells that are CD4^{bright}

Day after inoculation with CFA
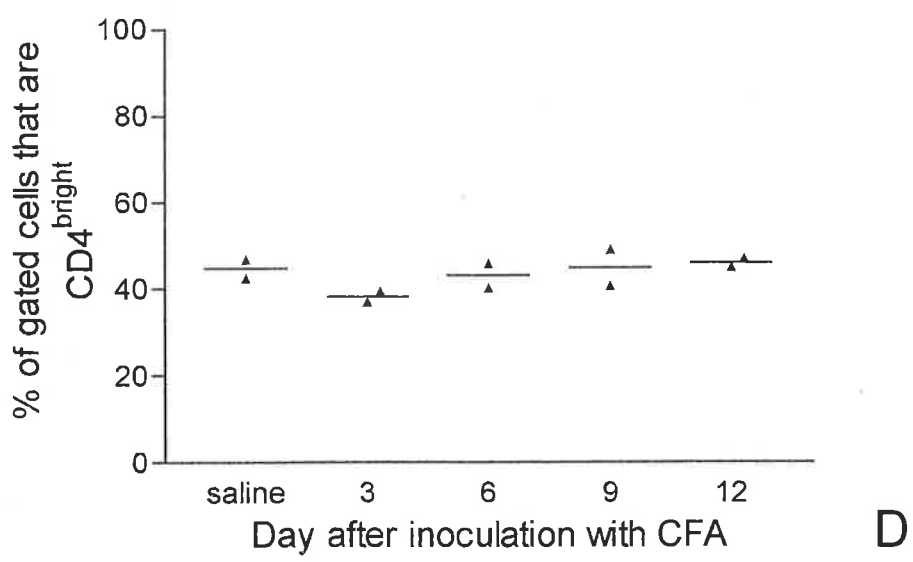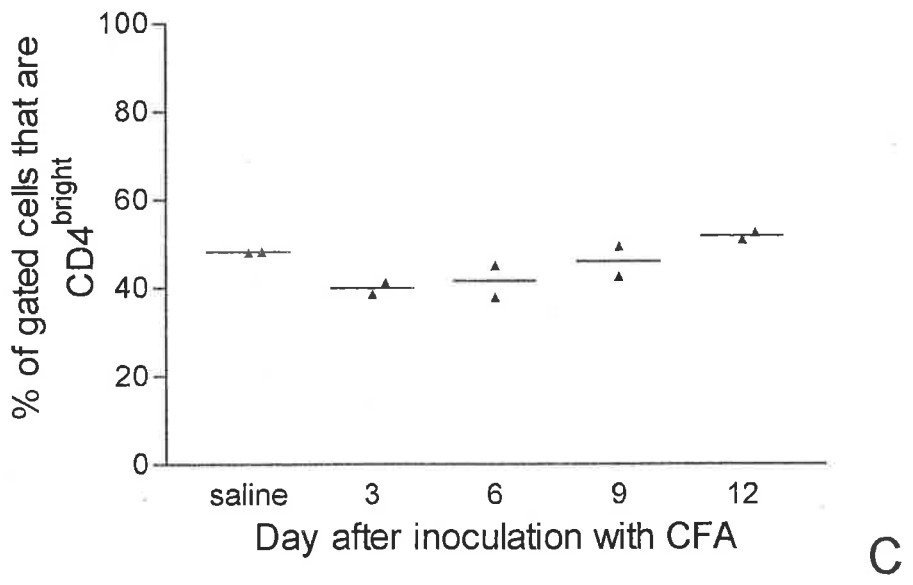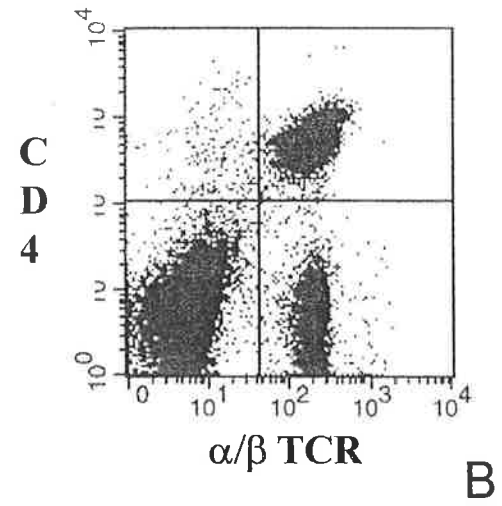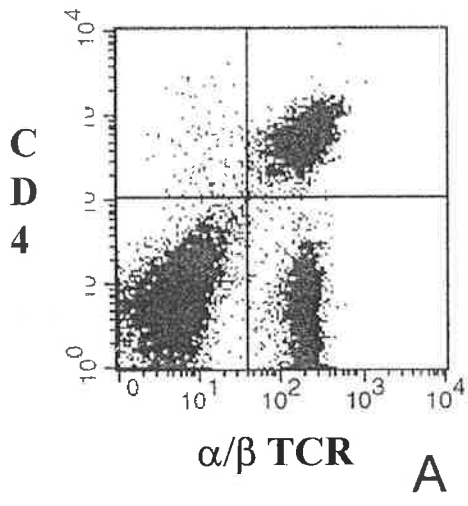
Figure 4.2.2.2 CD8$^+$ T cell populations in cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Inguinal (A, C) and popliteal (B, D) lymph node cells from saline- or CFA- inoculated rats were first stained indirectly with mAb 341 (anti-CD8β; FITC), followed by direct labelling with mAb OX8 (anti-CD8α; PE). Representative two colour plots of inguinal lymph node cells (A) and popliteal lymph node cells (B) isolated from a rat 6 days after inoculation of CFA are shown. The proportions of inguinal lymph node cells (C) and popliteal lymph node cells (D) that expressed both CD8α and CD8β at different times after inoculation with CFA are shown graphically, using data generated by flow cytometric analysis. Each triangle indicates the proportion of CD8$^+$ T cells among cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.
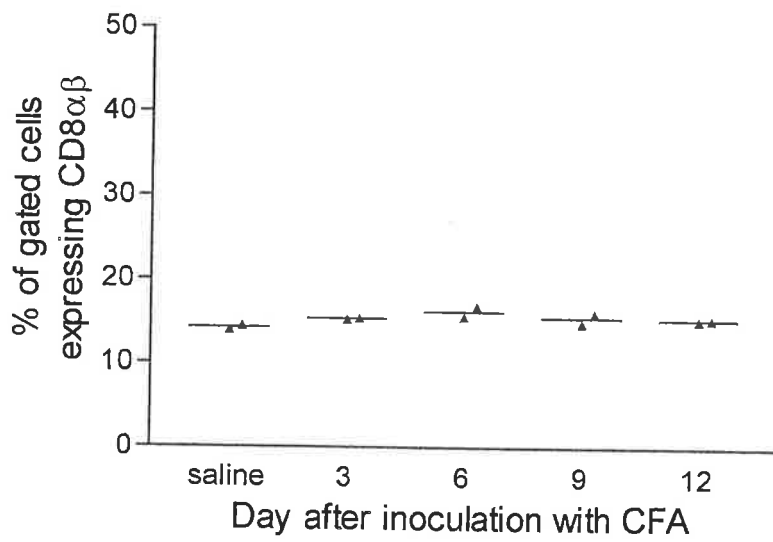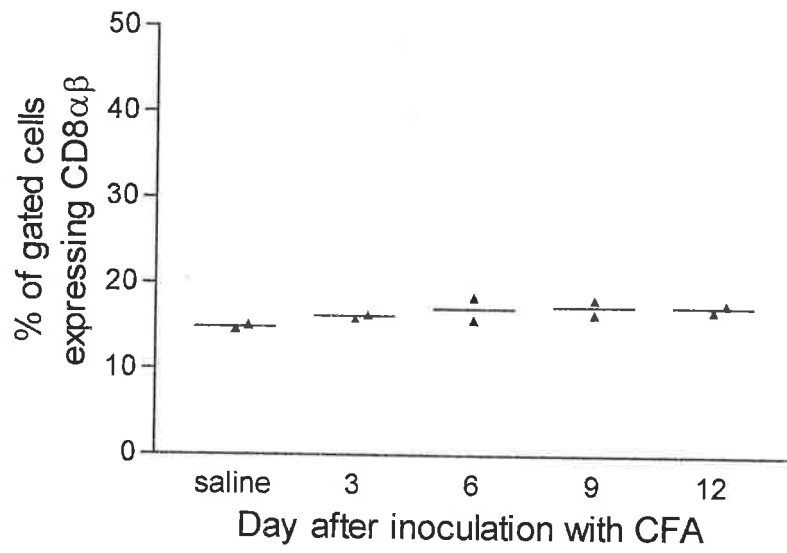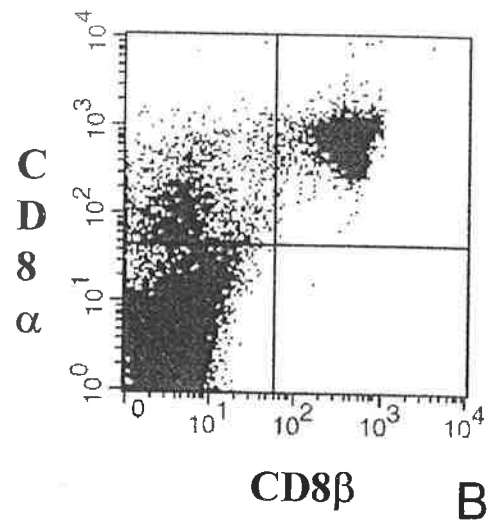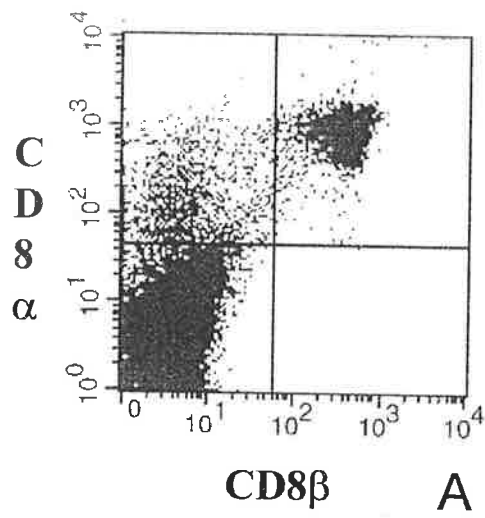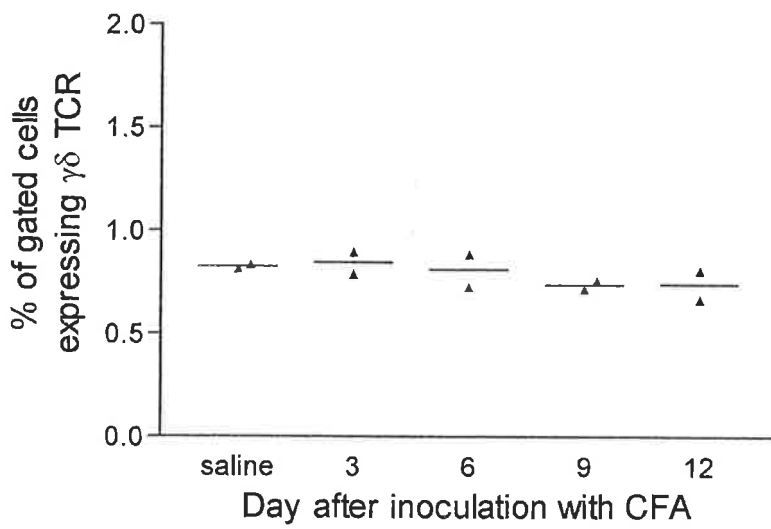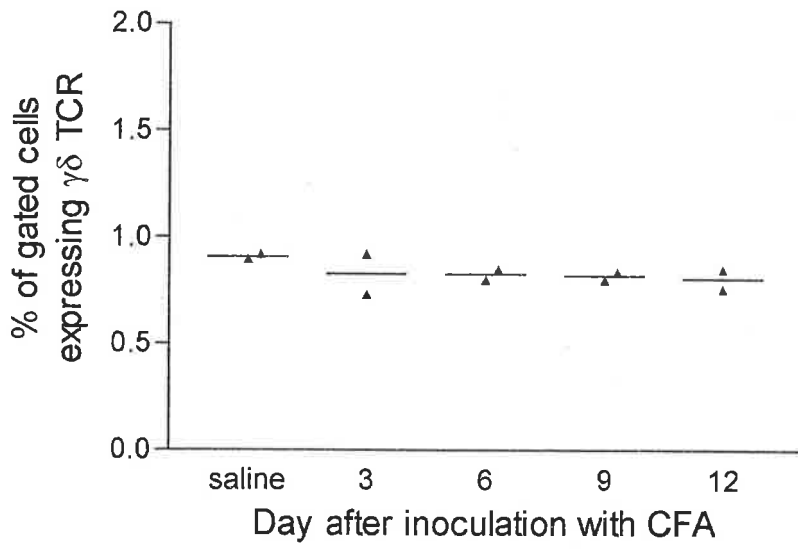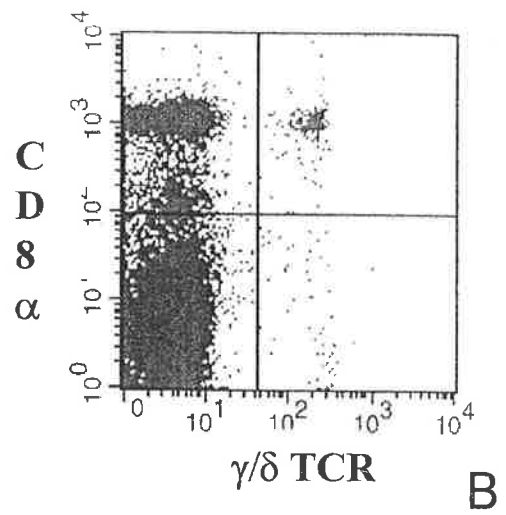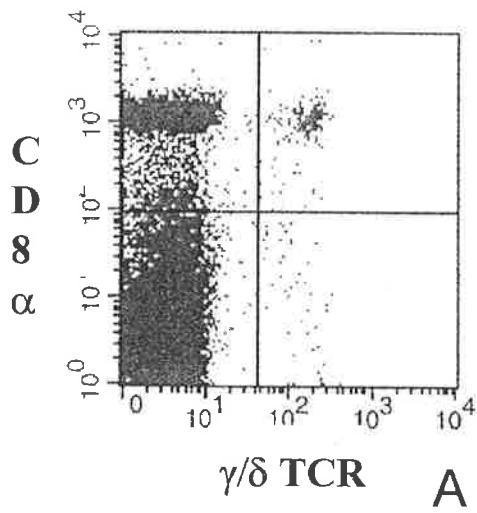
γ/δ T cells represent less than 1% of all lymphocytes isolated from the inguinal and popliteal lymph nodes of normal rats (represented in the upper right quadrants of two colour plots shown in Figures 4.2.2.3 A and B). During the prodrome and early clinical stages of AA, there was a small decrease in the proportion of lymphocytes that expressed the γδTCR in the inguinal and popliteal lymph nodes (Figure 4.2.2.3 A and D).

4.2.2.4 Non-α/β T cells

Following the inoculation of rats with CFA, there was an increase in the proportion of cells in the "lymphocyte" gate that did not express the α/βTCR (γ/δ T cells, B cells, macrophages, NK cells, dendritic cells, etc) in both groups of lymph nodes (Figure 4.2.2.4). By the end of the experimental period (12 days), the proportion of α/βTCR$^-$ T cells had returned to levels similar to those seen in control rats.

4.2.3 Expression of activation markers by CD4$^+$ T cells isolated from the inguinal and popliteal lymph nodes during the prodrome of AA

The expression of surface molecules such as MHC class II, CD25, CD71 and CD134 by CD4$^+$ T cells can be used as an indicator of activation. It has been shown in this laboratory that depletion of CD4$^+$ T cells that express the aforementioned markers removes the cells in TD lymph that can transfer AA adoptively (Spargo et al., 2001). The expression of these molecules (and others) by CD4$^+$ T cells was examined in the inguinal and popliteal lymph nodes during the prodromal period.

4.2.3.1 MHC class II

Within 3 days after inoculation of CFA, the proportion of CD4$^+$ T lymphocytes that expressed MHC class II increased from approximately 10% in both the inguinal and popliteal lymph nodes to approximately 15% and 17% respectively (Figure 4.2.3.1 C and D). The proportion of CD4$^+$ MHC class II $^+$ T cells was greatest on day 9 post-inoculation of CFA (22% and 21% of inguinal and popliteal lymph node CD4$^+$ T lymphocytes, respectively). When the clinical signs of joint inflammation were visible at day 12 post-inoculation, MHC class II$^+$ cells constituted a smaller proportion of the CD4$^+$ T cells in the lymph nodes.

4.2.3.2 IL-2 receptor α chain (CD25)

Figure 4.2.2.3 γ/δ T cell populations in cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Inguinal (A, C) and popliteal (B, D) lymph node cells from saline- or CFA- inoculated rats were first stained indirectly with mAb V65 (anti-γ/δ TCR; FITC), followed by direct labelling with mAb OX8 (anti-CD8α; PE). Representative two colour plots of inguinal lymph node cells (A) and popliteal lymph node cells (B) isolated from a rat 6 days after inoculation of CFA are shown. The proportions of inguinal lymph node cells (C) and popliteal lymph node cells (D) that expressed γ/δ TCR at different times after inoculation with CFA are shown graphically, using data generated by flow cytometric analysis. Each triangle indicates the proportion of γ/δ T cells among cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.

Figure 4.2.2.4 Non–α/β T cell populations in cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Lymph node cells from saline- or CFA- inoculated rats were first stained with indirect labelling with mAb W3/25 (anti-CD4; FITC), followed by direct labelling with mAb R73 (anti-α/β TCR; PE). The proportions of inguinal lymph node cells (A) and popliteal lymph node cells (B) that did not express α/β TCR at different times after inoculation with CFA are shown graphically, using data generated by flow cytometric analysis (Figure 4.2.2.1). Each triangle indicates the proportion of non-α/β T cells among cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.

A

B
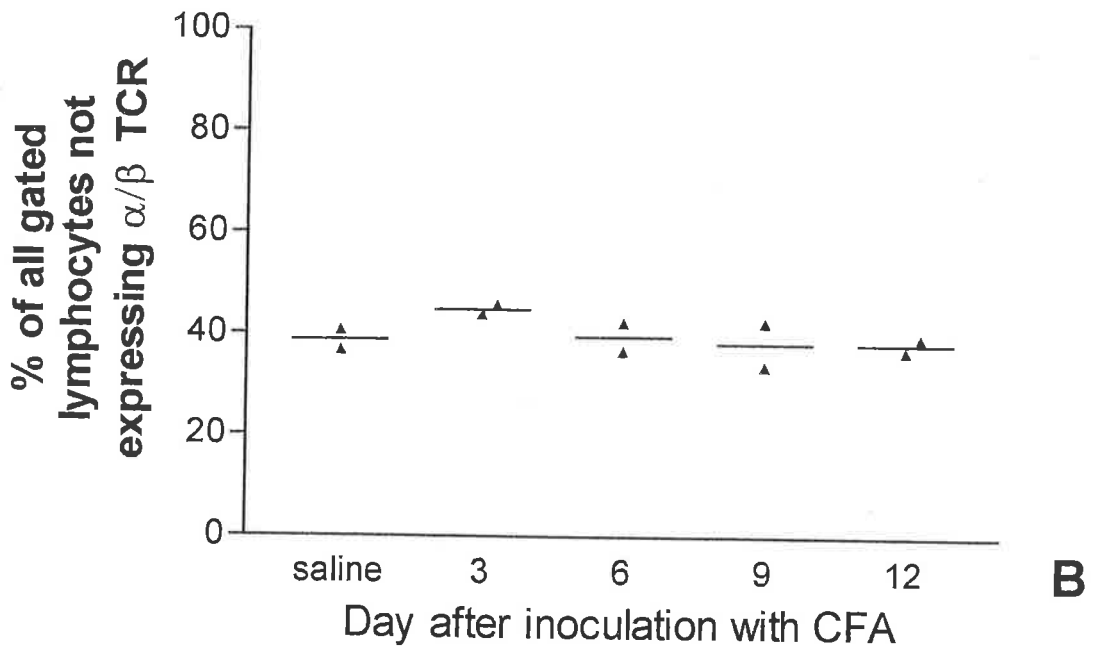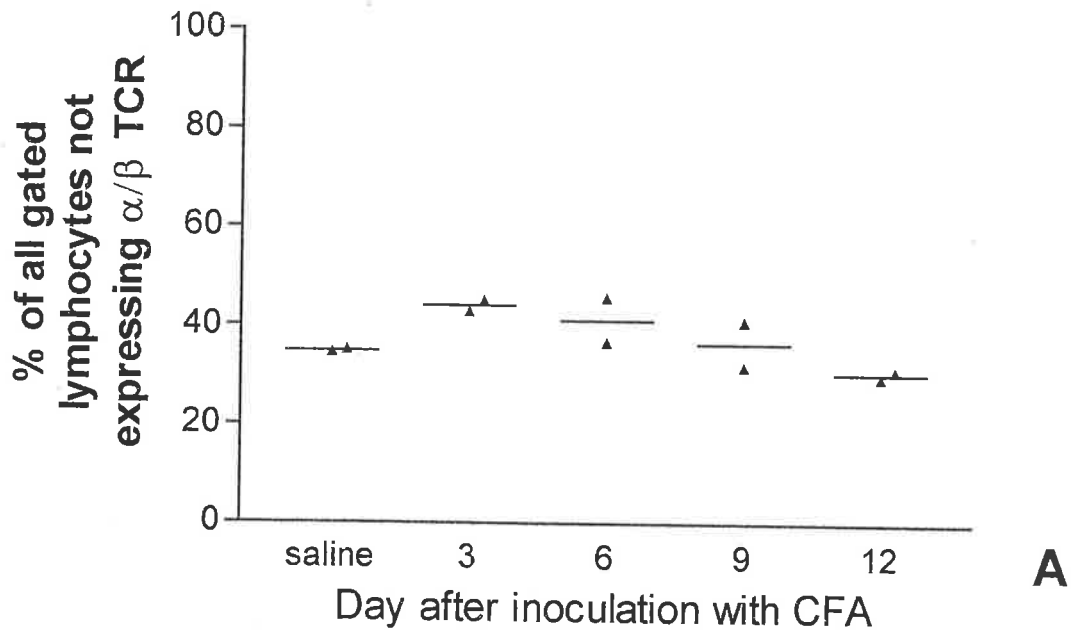
Figure 4.2.3.1 Expression of MHC class II by CD4$^+$ T cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Inguinal (A, C) and popliteal (B, D) lymph node cells from saline- or CFA- inoculated rats were first stained indirectly with mAb OX6 (anti-MHC class II; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative two colour plots of inguinal lymph node cells (A) and popliteal lymph node cells (B) isolated from a rat 6 days after inoculation of CFA are shown. The proportion of inguinal (C) and popliteal (D) lymph node CD4$^+$ T lymphocytes that expressed MHC class II at different times after inoculation with CFA are shown graphically, using data generated by flow cytometric analysis. Each triangle indicates the proportion of MHC class II$^+$ cells among CD4$^+$ T cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.
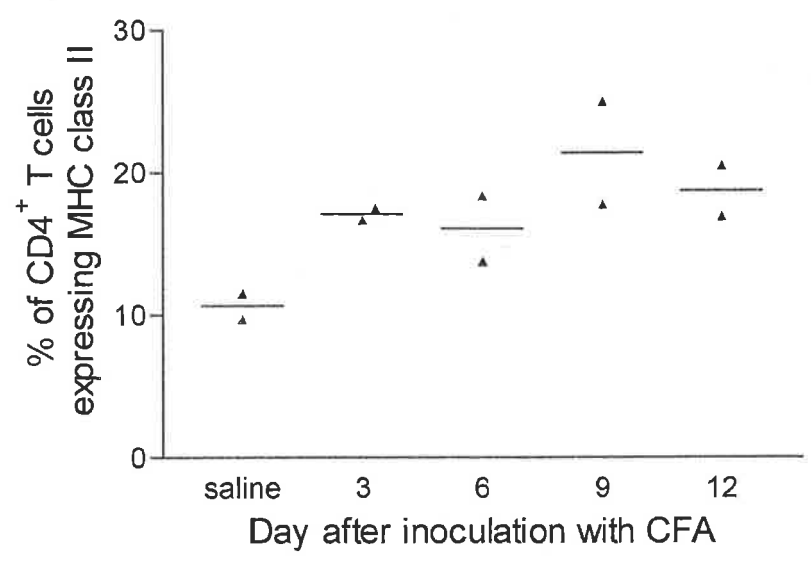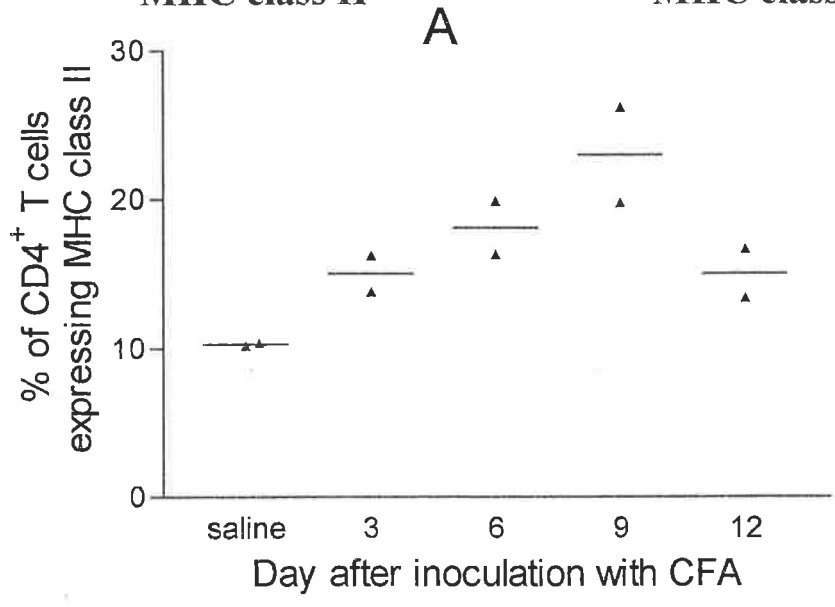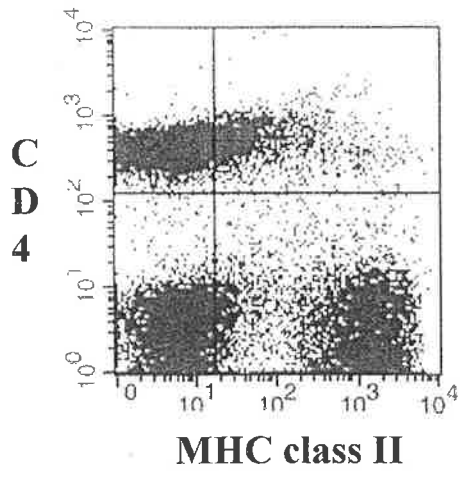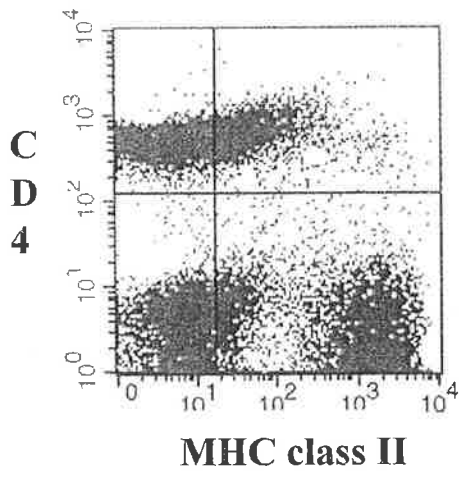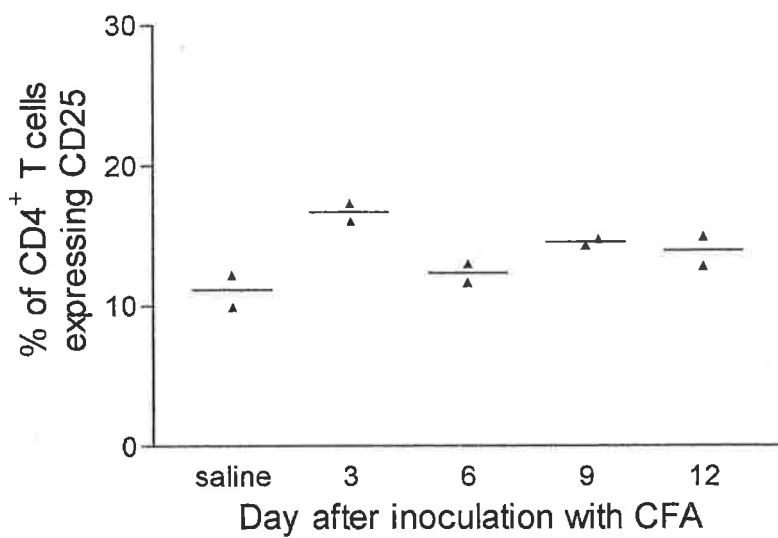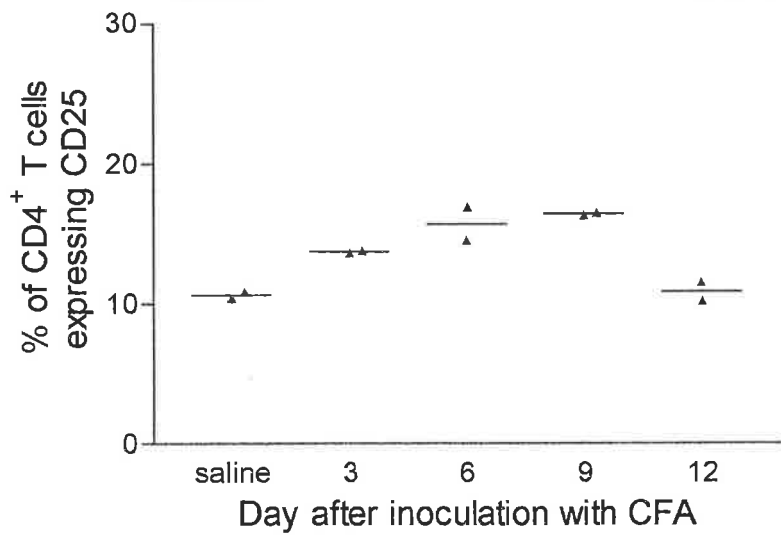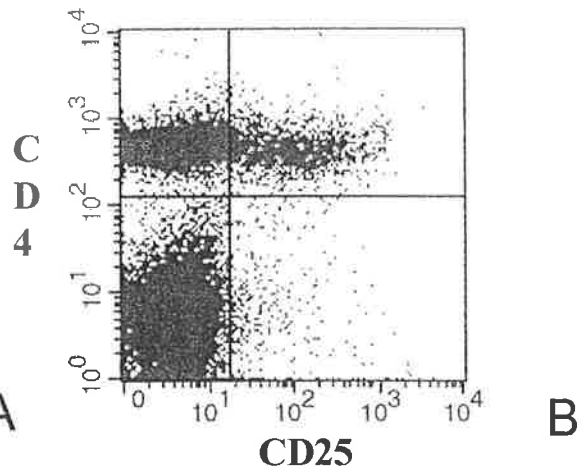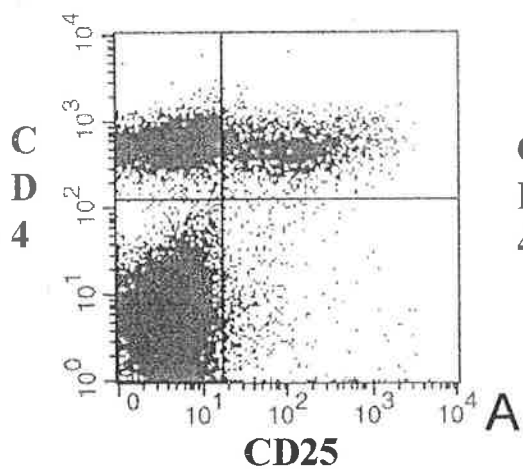
Figure A, B, C, D

In both the inguinal and popliteal lymph nodes, the proportion of CD4$^+$ T cells t
expressed the IL-2 receptor α chain (CD25) increased by the third day after inoculation
CFA (Figure 4.2.3.2 C and D). In the inguinal lymph nodes, the proportion of CD4$^+$
cells expressing CD25 remained elevated on days 6 and 9 post-inoculation but decreas
by day 12 to a level comparable to that seen in the control group. In the popliteal lym
nodes, the percentage of CD4$^+$ T cells that expressed CD25 peaked on day 3 (
approximately 17%). The proportion of CD25$^+$ CD4$^+$ T cells fluctuated but remain(
elevated at approximately 14% at days 9 and 12 after inoculation of CFA (Figure 4.2.3
D).

4.2.3.3 Transferrin receptor (CD71)

Inoculation with CFA induced an increase in the proportion of inguinal lymph node CD4
T cells that expressed transferrin receptor (CD71). This sub-population increased fro(
approximately 1.5% to 7% by day 3, with a peak on day 6 of 10.5%. The proportion (
CD71$^+$ CD4$^+$ T cells remained higher at day 12 post-inoculation than in cells from contr(
rats (Figure 4.2.3.3 A). In the popliteal lymph nodes, the greatest proportion of CD4$^+$ (
cells that expressed CD71 was observed on day 3, at which time approximately 9.5% o
CD4$^+$ T cells expressed the molecule (Figure 4.2.3.3 D). The proportion of CD4$^+$ T cell(
expressing CD71 declined at later time points but at all times remained higher than i(
cells obtained from rats inoculated with saline.

4.2.3.4 CD134 (OX40 antigen)

CD134 (OX40 antigen) was expressed by a greater proportion of CD4$^+$ T cells in both the
inguinal and popliteal lymph nodes (Figure 4.2.3.4 C and D) during the prodrome of AA
than in lymph nodes from rats injected with saline.

In the inguinal lymph nodes, the proportion of CD4$^+$ T cells that expressed CD134
increased from approximately 3.5% (saline-injected rats) to approximately 9.5% at 3 days
after inoculation of CFA and approximately 16% at days 6 and 9 post-inoculation (Figure
4.2.3.4 C). At day 12, when joint inflammation was visible, the proportion of CD134$^+$
CD4$^+$ T cells was lower (approximately 11%) but greater than in the saline-injected
controls.

Figure 4.2.3.2 Expression of IL-2 receptor by CD4$^+$ T cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Inguinal (A, C) and popliteal (B, D) lymph node cells from saline- or CFA- inoculated rats were first stained indirectly with mAb OX39 (anti-IL-2Rα [CD25]; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative two colour plots of inguinal lymph node cells (A) and popliteal lymph node cells (B) isolated from a rat 6 days after inoculation of CFA are shown. The proportion of inguinal (C) and popliteal (D) lymph node CD4$^+$ T lymphocytes that expressed CD25 at different times after inoculation with CFA are shown graphically, using data generated by flow cytometric analysis. Each triangle indicates the proportion of CD25$^+$ cells among CD4$^+$ T cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.

Figure with four panels (A, B, C, D).

Panel A: Flow cytometry dot plot, CD4 (y-axis, 10^0 to 10^4) versus CD25 (x-axis, 0 to 10^4), labeled A.

Panel B: Flow cytometry dot plot, CD4 (y-axis, 10^0 to 10^4) versus CD25 (x-axis, 0 to 10^4), labeled B.

Panel C: Scatter plot, % of CD4$^+$ T cells expressing CD25 (y-axis, 0 to 30) versus Day after inoculation with CFA (x-axis: saline, 3, 6, 9, 12), labeled C.

Panel D: Scatter plot, % of CD4$^+$ T cells expressing CD25 (y-axis, 0 to 30) versus Day after inoculation with CFA (x-axis: saline, 3, 6, 9, 12), labeled D.

Figure 4.2.3.3 Expression of Transferrin receptor by CD4$^+$ T cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Inguinal (A, C) and popliteal (B, D) lymph node cells from saline- or CFA- inoculated rats were first stained indirectly with mAb OX26 (anti-transferrin R [CD71]; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative two colour plots of inguinal lymph node cells (A) and popliteal lymph node cells (B) isolated from a rat 6 days after inoculation of CFA are shown. The proportions of inguinal (C) and popliteal (D) lymph node CD4$^+$ T lymphocytes that expressed CD71 at different times after inoculation with CFA are shown graphically, using data generated by flow cytometric analysis. Each triangle indicates the proportion of CD71$^+$ cells among CD4$^+$ T cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.

Figure A, B, C, D

A

B

CD71

CD71

C

D

Day after inoculation with CFA
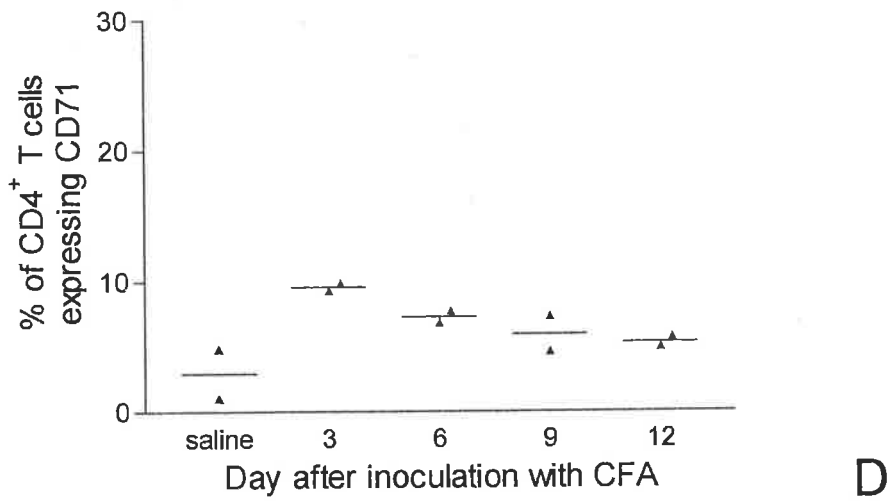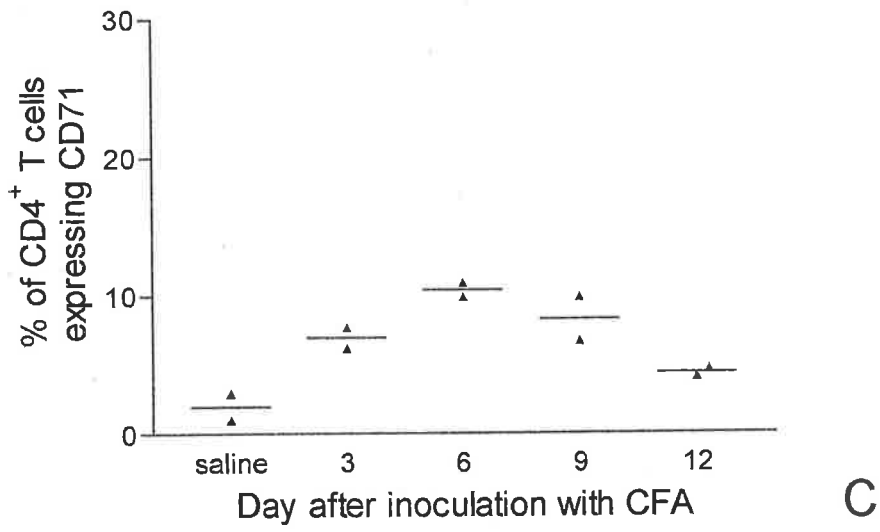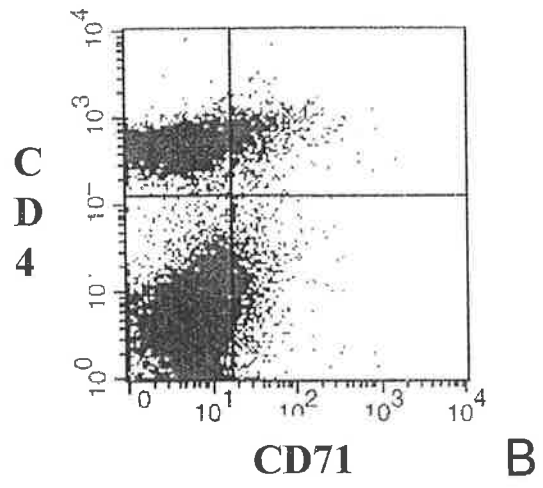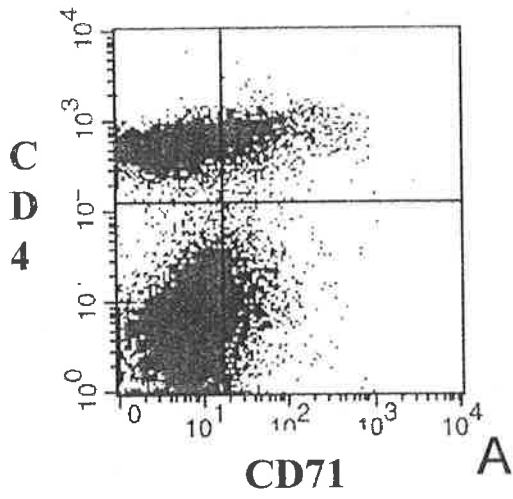
Figure 4.2.3.4 Expression of CD134 (OX40 antigen) by CD4$^+$ T cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Inguinal (A, C) and popliteal (B, D) lymph node cells from saline- or CFA- inoculated rats were first stained indirectly with mAb OX40 (anti-CD134; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative two colour plots of inguinal lymph node cells (A) and popliteal lymph node cells (B) isolated from a rat 6 days after inoculation of CFA are shown. The proportions of inguinal (C) and popliteal (D) lymph node CD4$^+$ T lymphocytes that expressed CD134 at different times after inoculation with CFA are shown graphically, using data generated by flow cytometric analysis. Each triangle indicates the proportion of CD134$^+$ cells among CD4$^+$ T cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.
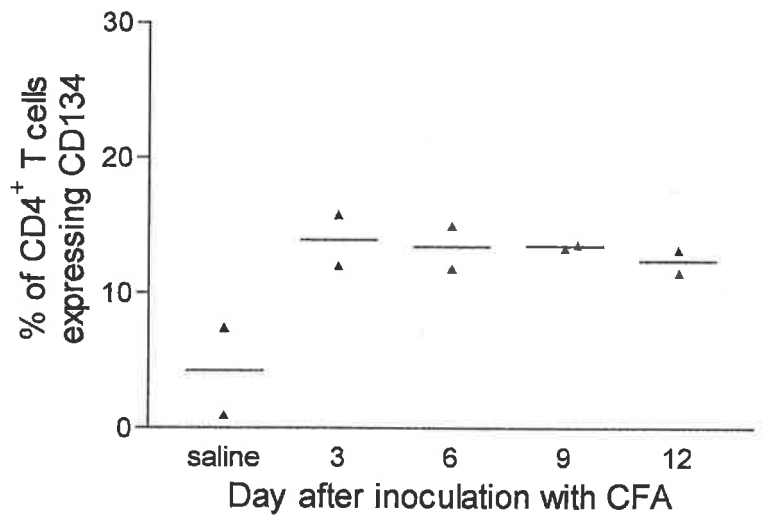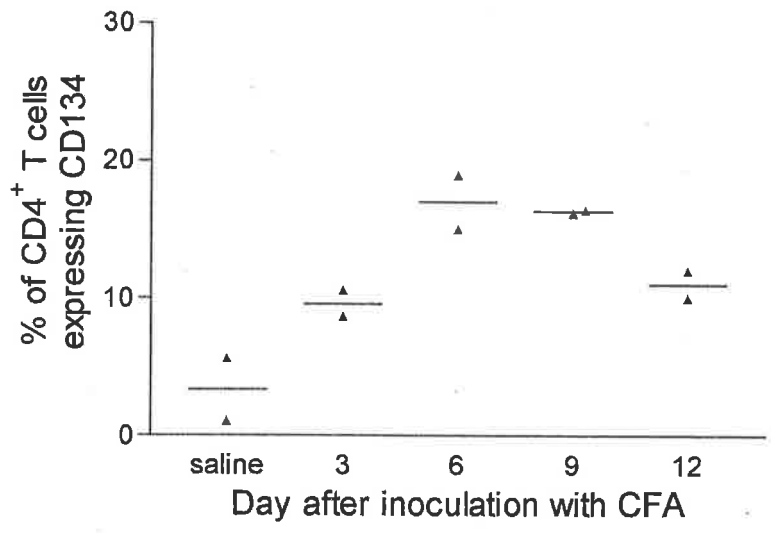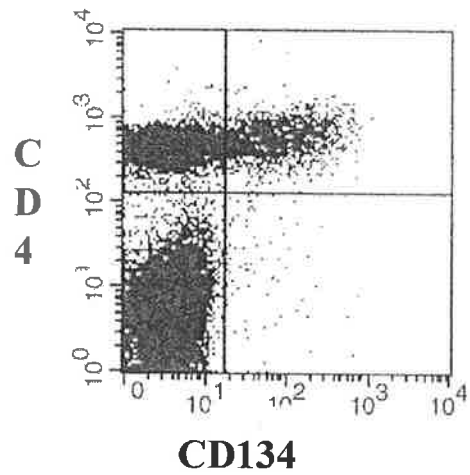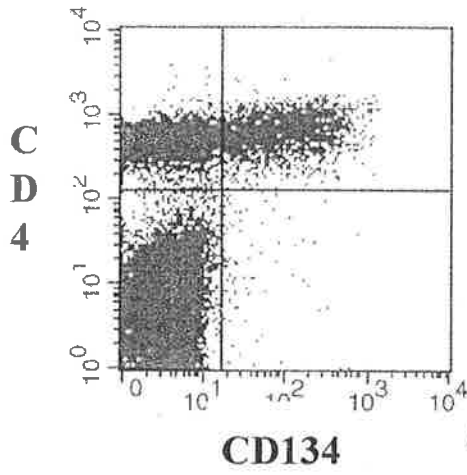
The proportion CD4$^+$ T cells in the popliteal lymph nodes that expressed CD134 rose to approximately 12% by day 3 post-inoculation and remained relatively constant (between 11.5% and 15.5%) throughout the prodrome and early clinical phase of the disease (Figure 4.2.3.4 D).

4.2.3.5 UA002 antigen

The antigenic specificity of mAb UA002 (generated in the Department of Microbiology and Immunology, University of Adelaide) has not yet been determined. This mAb may have specificity for CD39 as it binds to an antigen that is expressed by T cells that have been activated with Concanavalin A, B lymphocytes, macrophages and dendritic cells.

The UA002 antigen was expressed by approximately 5.5% of CD4$^+$ T cells from the inguinal and popliteal lymph nodes of rats inoculated with saline (Figure 4.2.3.5 C and D). In the inguinal lymph nodes, the proportion of CD4$^+$ T cells expressing UA002 antigen increased following inoculation of CFA. These cells were at the greatest frequency on day 9 (15.5%) post-inoculation and the frequency declined by day 12 (Figure 4.2.3.5 C). CD4$^+$ T cells expressing UA002 antigen were approximately twice as abundant in the popliteal lymph nodes following inoculation of CFA compared with those from saline-inoculated rats (Figure 4.2.3.5 D). The frequency of these cells remained relatively unchanged throughout the prodrome and early clinical stage of AA (Figure 4.2.3.5 D).

4.2.3.6.1 CD45RC

The expression of a high molecular weight isoform of CD45 (CD45RC in the rat) has been associated with cells that are of a "naïve" phenotype (Luqman and Bottomly, 1992). However, the proportion of CD4$^+$ T cells that expressed CD45RC at medium to high levels did not appear to vary substantially in the inguinal or popliteal lymph nodes of rats following inoculation of CFA (Figure 4.2.3.6.1 C and D). Most significantly, the proportions of CD45RC$^+$ CD4$^+$ T cells did not decline in either lymph node.

4.2.3.6.2 Level of expression of CD45RC

The relative levels of expression of CD45RC on the CD45RC$^+$ CD4$^+$ T cells was compared using the mean fluorescence intensity of the signal generated from the cells labelled indirectly with mAb OX22. In both the inguinal and popliteal lymph nodes, there
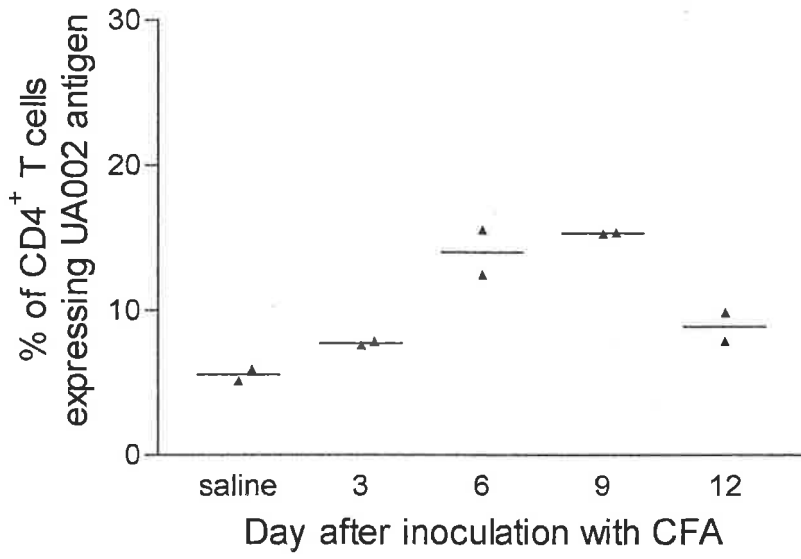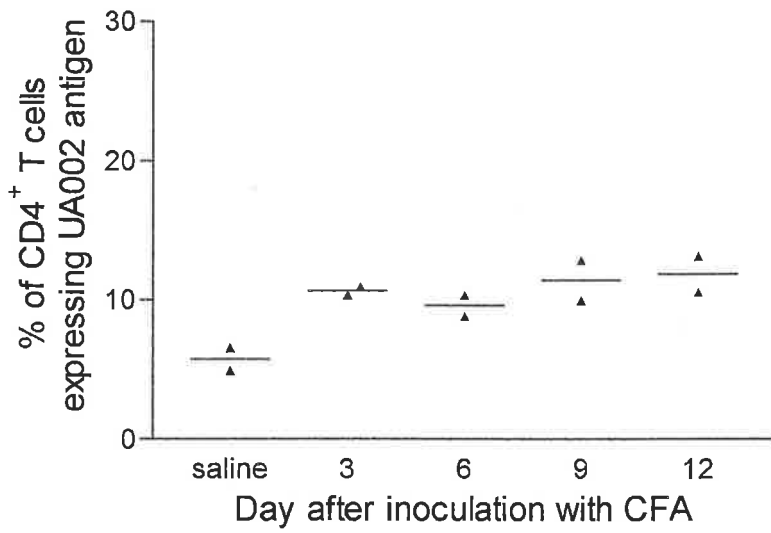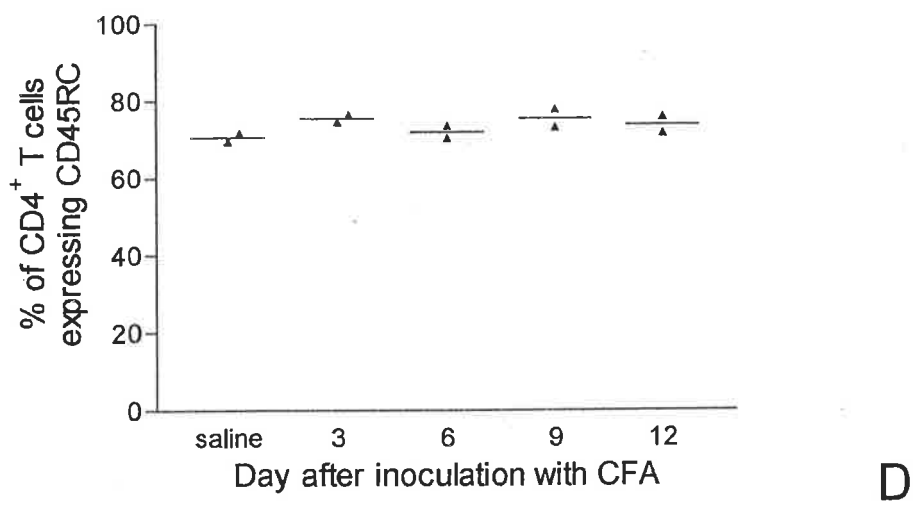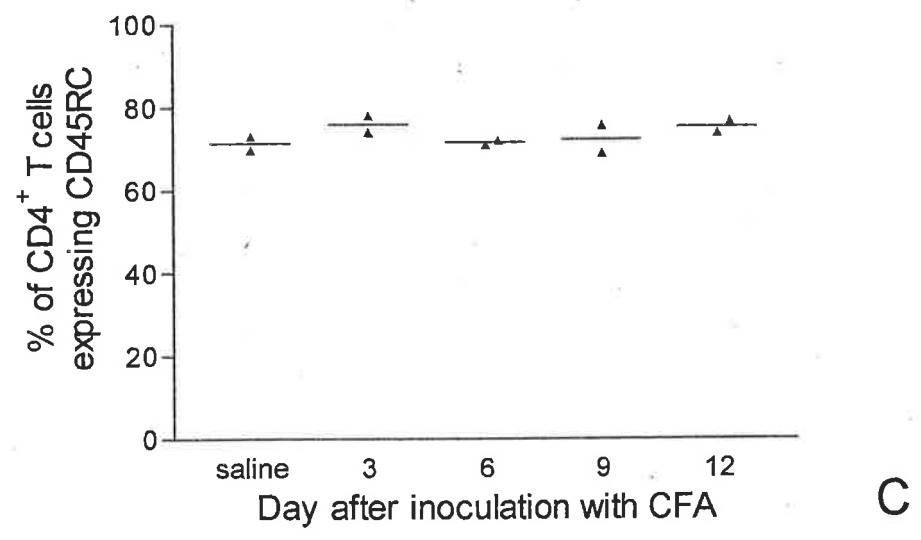
Figure 4.2.3.5 Expression of UA002 antigen by CD4$^+$ T cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Inguinal (A, C) and popliteal (B, D) lymph node cells from saline- or CFA- inoculated rats were first stained indirectly with mAb UA002 (anti-UA002 antigen; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative two colour plots of inguinal lymph node cells (A) and popliteal lymph node cells (B) isolated from a rat 6 days after inoculation of CFA are shown. The proportions of inguinal (C) and popliteal (D) lymph node CD4$^+$ T lymphocytes that expressed UA002 antigen at different times after inoculation with CFA are shown graphically, using data generated by flow cytometric analysis. Each triangle indicates the proportion of UA002 antigen$^+$ cells among CD4$^+$ T cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.

A

B

C

D

CD4 vs **UA002 antigen** (panels A and B)
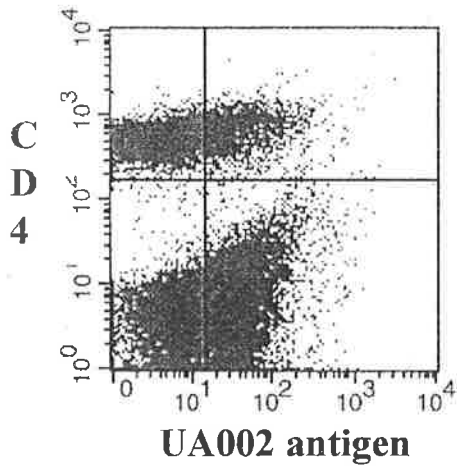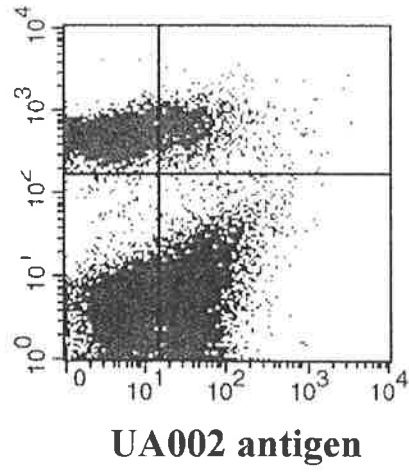
% of CD4⁺ T cells expressing UA002 antigen vs Day after inoculation with CFA (panels C and D)
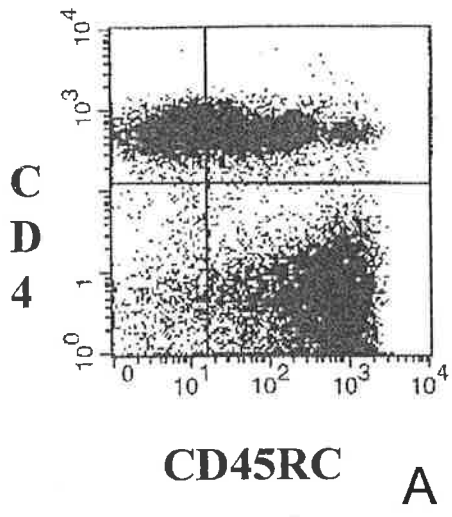
Figure 4.2.3.6.1 Expression of CD45RC by CD4$^+$ T cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Inguinal (A, C) and popliteal (B, D) lymph node cells from saline- or CFA- inoculated rats were first stained indirectly with mAb OX22 (anti- CD45RC; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative two colour plots of inguinal lymph node cells (A) and popliteal lymph node cells (B) isolated from a rat 6 days after inoculation of CFA are shown. The proportions of inguinal (C) and popliteal (D) lymph node CD4$^+$ T lymphocytes that expressed CD45RC at different times after inoculation with CFA are shown graphically, using data generated by flow cytometric analysis. Each triangle indicates the proportion of CD45RC$^+$ cells among CD4$^+$ T cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.
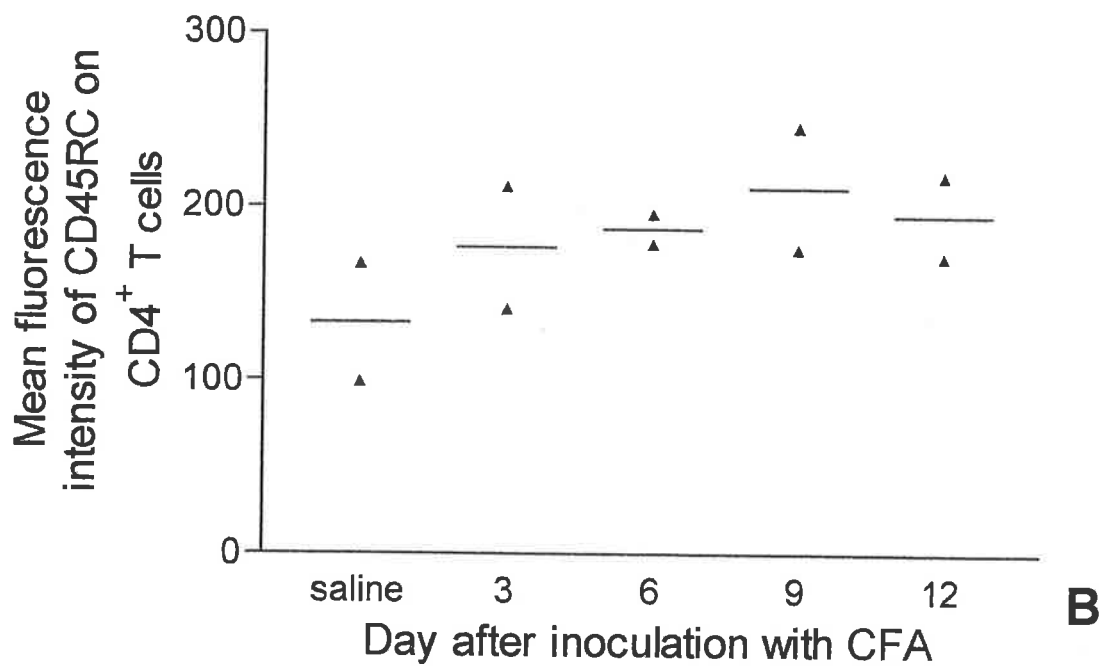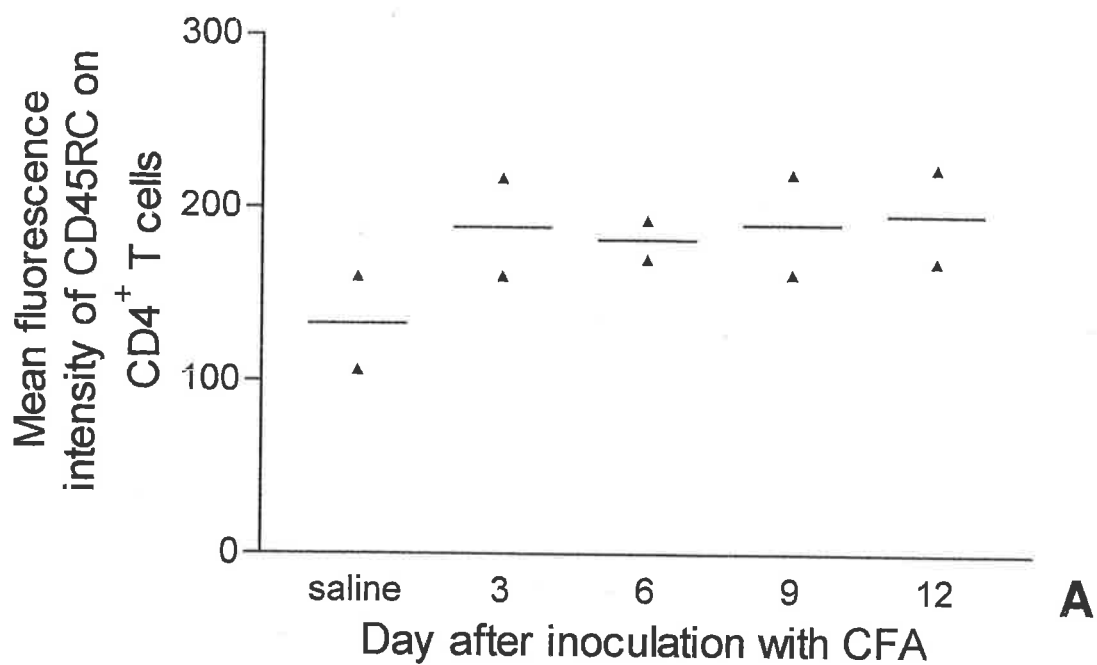
A

B

C

D

CD4

CD45RC

% of CD4$^+$ T cells expressing CD45RC

Day after inoculation with CFA

saline   3   6   9   12
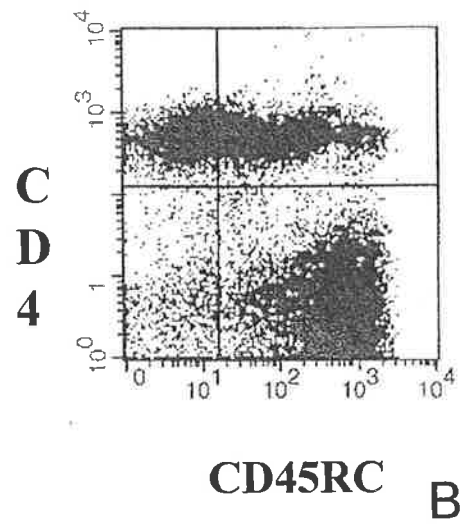
was a trend towards an increased frequency of CD4$^+$ T cells expressing higher levels of CD45RC following inoculation of the rats with CFA (Figure 4.2.3.6.2 A and B). However, there was considerable variation in the results obtained from the two pools of cells (each from ten rats).

4.2.4 Expression of adhesion molecules by CD4$^+$ T cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA

The migration of cells through blood vessels into tissues is initiated by the rolling and tethering of the cells to the vessel wall, which is facilitated initially by interactions involving the selectin family of molecules. The extravasation of these migratory cells requires firm adhesion, which is mediated by the engagement of surface integrins and their ligands (Springer, 1994). The molecules that mediate the adhesion of cells to other cells or to extracellular matrix are known collectively as "adhesion molecules". The acquisition of particular adhesion molecules during activation in the lymph nodes can determine the subsequent migratory preferences of the cells. In addition to providing cells with distinct migratory patterns, these molecules are also thought to be involved in intercellular communication and in retention of the cells in particular extravascular compartments.

4.2.4.1 L-selectin (CD62L)

L-selectin (CD62L) is involved in the tethering step that is a precursor to adhesion and transendothelial migration of naive lymphocytes through high endothelial venules (HEV) in peripheral lymph nodes and Peyer's Patches (Tedder et al., 1995).

The large variation in the proportion of inguinal lymph node CD4$^+$ T cells that expressed CD62L between the two pools of cells prepared from separate groups of control rats suggests that an error may have occurred during the labelling of one of them. With the exception of the single outlying point, the proportion of CD4$^+$ T cells expressing CD62L in the inguinal lymph nodes did not alter markedly following inoculation of CFA, remaining at approximately 10% of CD4$^+$ T cells throughout (Figure 4.2.4.1 C). The frequency of CD4$^+$ CD62L$^+$ T cells in the popliteal lymph nodes decreased on day 3 relative to the same lymph nodes from saline-challenged rats (Figure 4.2.4.1 D). However, there was considerable variation between groups at individual time points following challenge with CFA (Figure 4.2.4.1 D).

Figure 4.2.3.6.2 Level of expression of CD45RC by CD4$^+$ T cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Lymph node cells from saline- or CFA- inoculated rats were first stained indirectly with mAb OX22 (anti- CD45RC; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). The mean fluorescence of CD4$^+$ T cells labelled with mAb OX22 by the indirect immunofluorescence technique is shown for inguinal (A) and popliteal (B) lymph node cells. Flow cytometry was performed on cells obtained 3, 6, 9 or 12 days after inoculation of CFA and those obtained from saline-challenged rats. Each triangle indicates the mean fluorescence intensity of staining with OX22 of CD4$^+$ T cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.

A

Mean fluorescence intensity of CD45RC on CD4⁺ T cells (y-axis, 0 to 300)

Day after inoculation with CFA (x-axis: saline, 3, 6, 9, 12)

B

Mean fluorescence intensity of CD45RC on CD4⁺ T cells (y-axis, 0 to 300)

Day after inoculation with CFA (x-axis: saline, 3, 6, 9, 12)

Figure 4.2.4.1 Expression of L-selectin by CD4$^+$ T cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Inguinal (A, C) and popliteal (B, D) lymph node cells from saline- or CFA- inoculated rats were first stained indirectly with mAb OX85 (anti-L-selectin [CD62L]; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative two colour plots of inguinal lymph node cells (A) and popliteal lymph node cells (B) isolated from a rat 6 days after inoculation of CFA are shown. The proportions of inguinal (C) and popliteal (D) lymph node CD4$^+$ T lymphocytes that expressed CD62L at different times after inoculation with CFA are shown graphically, using data generated by flow cytometric analysis. Each triangle indicates the proportion of CD62L$^+$ cells among CD4$^+$ T cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.
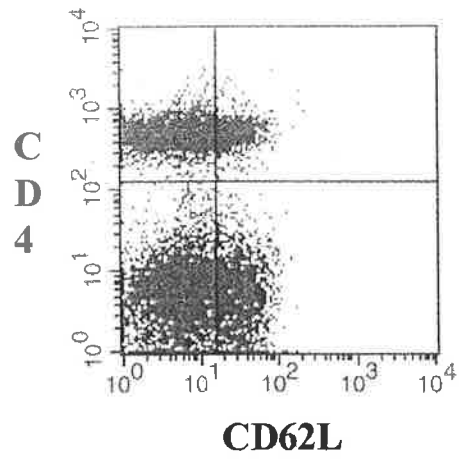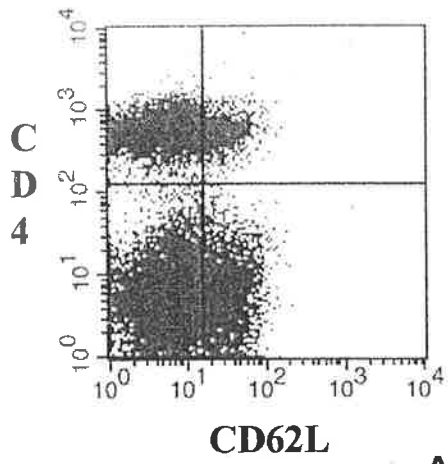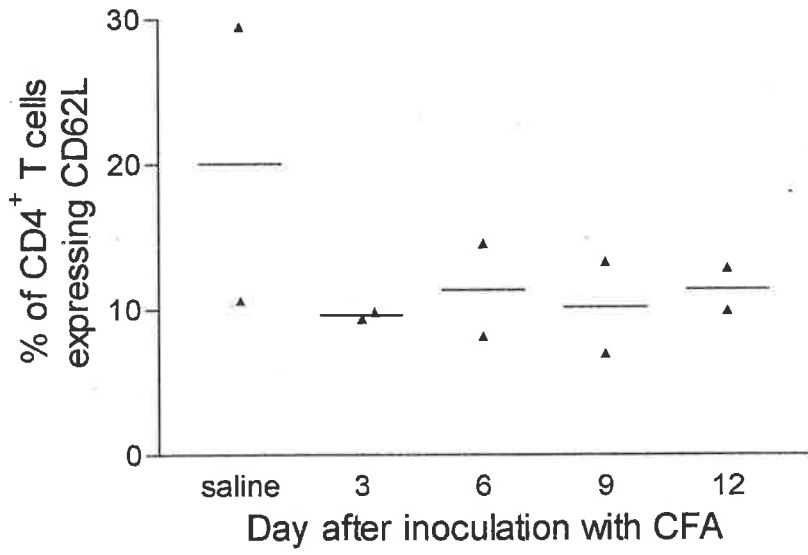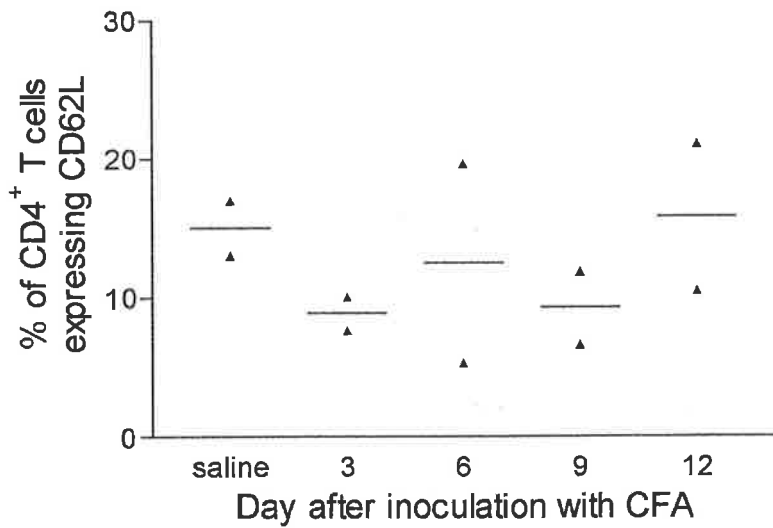
4.2.4.2 ICAM-1 (CD54)

Approximately 8-8.5% of CD4$^+$ T cells in the inguinal and popliteal lymph nodes from saline-challenged rats expressed ICAM-1 (CD54, Figure 4.2.4.2 C and D). Within 3 days after challenge with CFA, the frequency of CD4$^+$ CD54$^+$ T cells increased to approximately 11.5% and 16% in the inguinal and popliteal lymph nodes, respectively. The frequency of these cells continued to increase in the inguinal lymph nodes, reaching approximately 21.5% at day 9 post-inoculation of CFA and then decreasing to 14.5% by day 12 (Figure 4.2.4.2 C). A different pattern was observed in the popliteal lymph nodes in which the proportion of CD4$^+$ T cells that expressed CD54 reached a plateau by day 3 post-inoculation (between 15% and 20%, Figure 4.2.4.2 D).

4.2.4.3 CD11a/CD18 (LFA-1)

Leukocyte function antigen (LFA)-1 (CD11a [integrin α_L subunit] / CD18 [integrin β_2 subunit] heterodimer) was expressed by all CD4$^+$ T cells (representative plots shown in Figure 4.2.4.3 A and B). The relative level of expression was examined by comparing the mean fluorescence intensity of the signal generated by the labelling of the cells with mAb WT-1 by the indirect immunofluorescence technique. The expression of LFA-1 by CD4$^+$ T cells in pools of inguinal and popliteal lymph nodes prepared from a group of 10 saline-injected rats was used for reference. The cells from normal animals had a mean fluorescence intensity of 46 and 48 arbitrary units in the inguinal and popliteal lymph nodes, respectively (Figure 4.2.4.3 C and D). The mean fluorescence intensity of LFA-1 on CD4$^+$ T cells from the inguinal lymph nodes increased within 3 days of challenge with CFA and continued to increase until day 9 (approximately 70.5 units). By the twelfth day after inoculation of CFA, the mean fluorescence of CD4$^+$ T cells had decreased to approximately 47 units (Figure 4.2.4.3 C). Less variation was observed when examining the mean fluorescence of CD4$^+$ T cells from the popliteal lymph nodes of CFA-challenged rats. After an initial increase in the first 3 days after inoculation of CFA, the mean fluorescence of the CD4$^+$ T cells labelled by mAb WT-1 remained on average between 56 and 58 units (Figure 4.2.4.3 D).

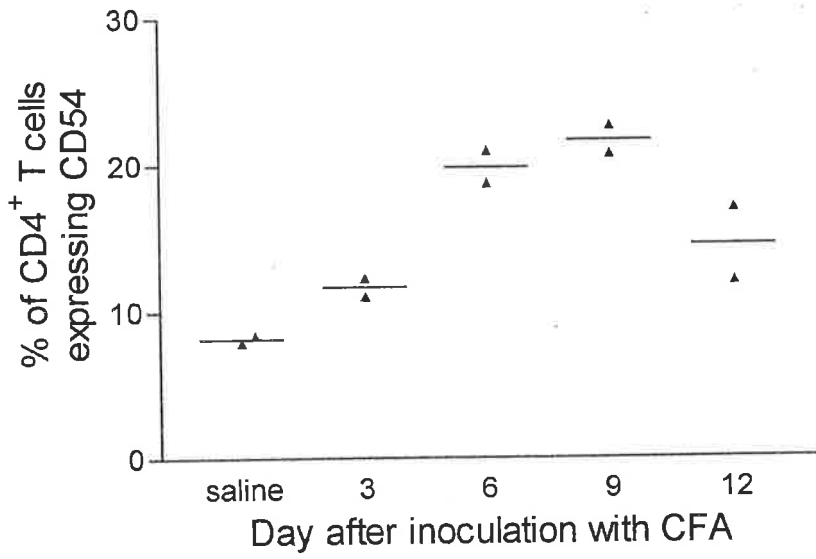### 4.2.4.4 Phagocytic glycoprotein 1 (CD44)

Figure 4.2.4.2 Expression of ICAM-1 by CD4$^+$ T cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Inguinal (A, C) and popliteal (B, D) lymph node cells from saline- or CFA- inoculated rats were first stained indirectly with mAb 1A29 (anti-ICAM-1 [CD54]; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative two colour plots of inguinal lymph node cells (A) and popliteal lymph node cells (B) isolated from a rat 6 days after inoculation of CFA are shown. The proportions of inguinal (C) and popliteal (D) lymph node CD4$^+$ T lymphocytes that expressed CD54 at different times after inoculation of CFA are shown graphically, using data generated by flow cytometric analysis. Each triangle indicates the proportion of CD54$^+$ cells among CD4$^+$ T cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.
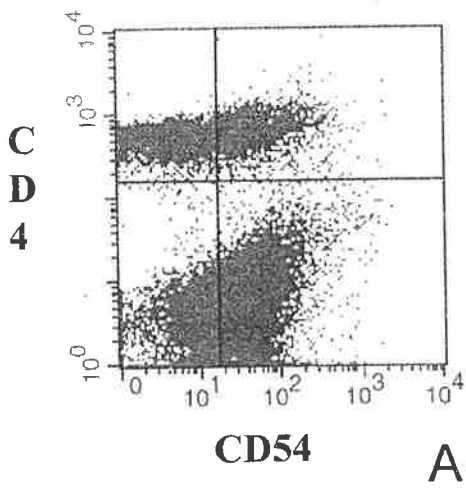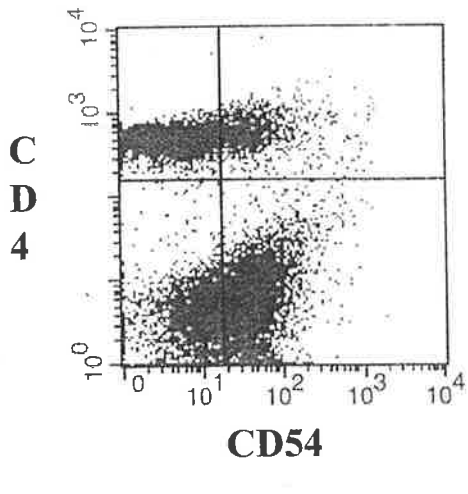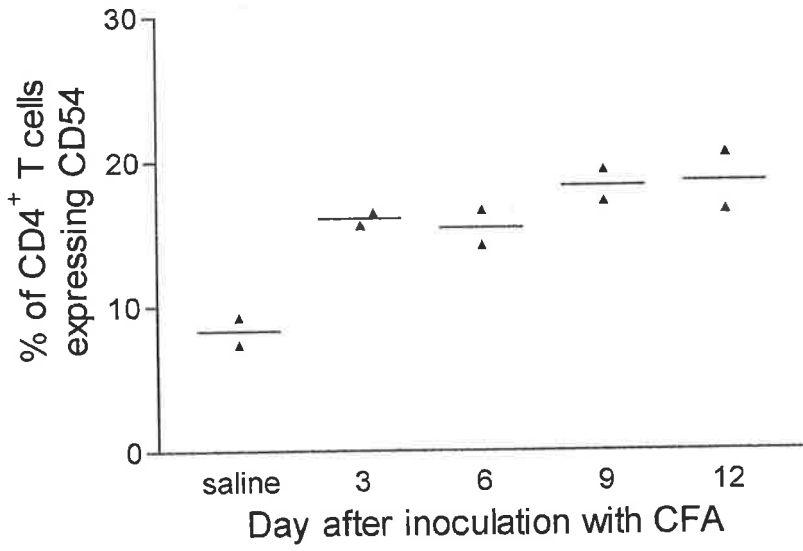
Figure 4.2.4.3 Level of expression of LFA-1 by CD4$^+$ T cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Inguinal (A, C) and popliteal (B, D) lymph node cells from saline- or CFA- inoculated rats were first stained indirectly with mAb WT-1 (anti-LFA-1; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). The mean fluorescence of CD4$^+$ T cells labelled with mAb WT-1 by the indirect immunofluorescence technique is shown for inguinal (A) and popliteal (B) lymph node cells. Flow cytometry was performed on cells obtained 3, 6, 9 or 12 days after inoculation of CFA and those obtained from saline-challenged rats. Each triangle indicates the mean fluorescence intensity of staining with WT-1 of CD4$^+$ T cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.

A

B

C

D
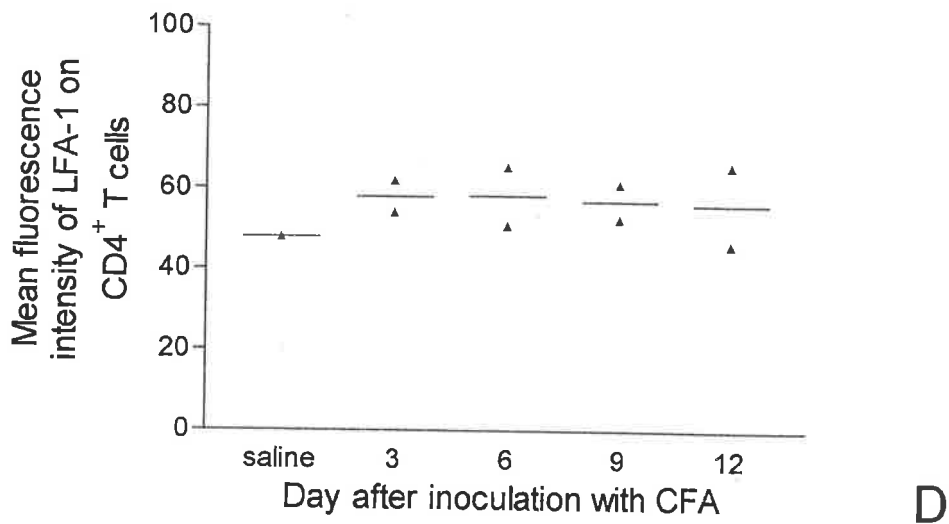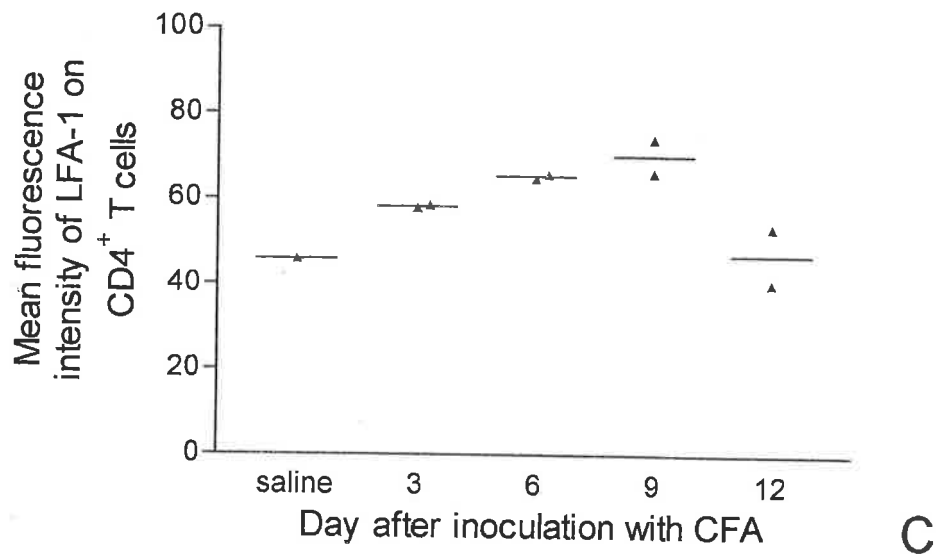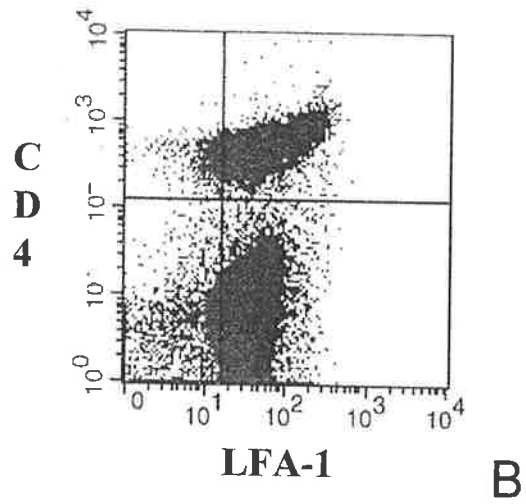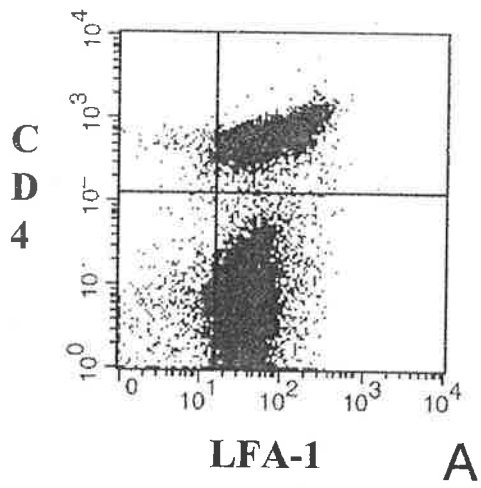
CD44 was expressed by all inguinal and popliteal lymph node CD4$^+$ T cells (representative plots of cells from day 6 rats are shown in Figure 4.2.4.4 A and B). The relative levels of expression by the cells from groups of rats was compared, using the mean fluorescence intensity of the signal generated by flow cytometry from cells labelled indirectly with mAb OX50 (anti-CD44). A small increase in fluorescence intensity of the CD4$^+$ T cells from both the inguinal and popliteal lymph nodes was observed following inoculation of CFA (Figure 4.2.4.4 C and D). This increase was sustained throughout the observation period in cells from the popliteal lymph nodes. However, in cells from the inguinal lymph nodes, the mean fluorescence declined in one of the pools examined at day 12 post-inoculation (Figure 4.2.4.4 D).

4.2.5 Production of cytokines by T cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA

The production of cytokines by lymphocytes from the inguinal and popliteal lymph nodes was examined using flow cytometric analysis of intracellular cytokines. The use of dual fluorochrome labelling allowed the identification of the individual cells producing the cytokine. However, in order to detect intracellular cytokine, freshly isolated cells require a period of *in vitro* stimulation to allow (further) translation and accumulation of cytokines in the intracellular compartments of the cells (see Chapter 3). Lymph node cells were stimulated for 6 hours with phorbol myristate acetate (PMA) plus ionomycin in the presence of Brefeldin A (which disrupts Golgi function and the transport of proteins for secretion). After stimulation, the cells were fixed and made permeable before labelling indirectly with mAbs (see Section 2.6.3). This technique reveals the potential of cells to produce each cytokine rather than giving an absolute frequency of cells that were producing cytokines at time that the cells were harvested. In this part of the project, the cells were labelled with mAb R73 to identify T cells expressing the α/β TCR.

4.2.5.1 IFN-γ

In animals receiving saline, approximately 3.5% of α/β T cells in the inguinal lymph node and 4.5% in the popliteal lymph nodes produced IFN-γ following 6 hours of stimulation *in vitro* (Figure 4.2.5.1 C and D). By day 3 after CFA inoculation, the frequency of IFN-γ-producing α/β T cells increased to 4.5% in the inguinal lymph nodes and 7% in the popliteal lymph nodes. The proportion of α/β T cells that produced IFN-γ in the inguinal

Figure 4.2.4.4 Level of expression of CD44 by CD4$^+$ T cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Inguinal (A, C) and popliteal (B, D) lymph node cells from saline- or CFA- inoculated rats were first stained with indirect labelling with mAb OX50 (anti-CD44; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). The mean fluorescence of CD4$^+$ T cells labelled with mAb OX50 by the indirect immunofluorescence technique is shown for inguinal (A) and popliteal (B) lymph node cells. Flow cytometry was performed on cells obtained 3, 6, 9 or 12 days after inoculation of CFA and cells obtained from saline-challenged rats. Each triangle indicates the mean fluorescence intensity of staining with OX50 of CD4$^+$ T cells among cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.
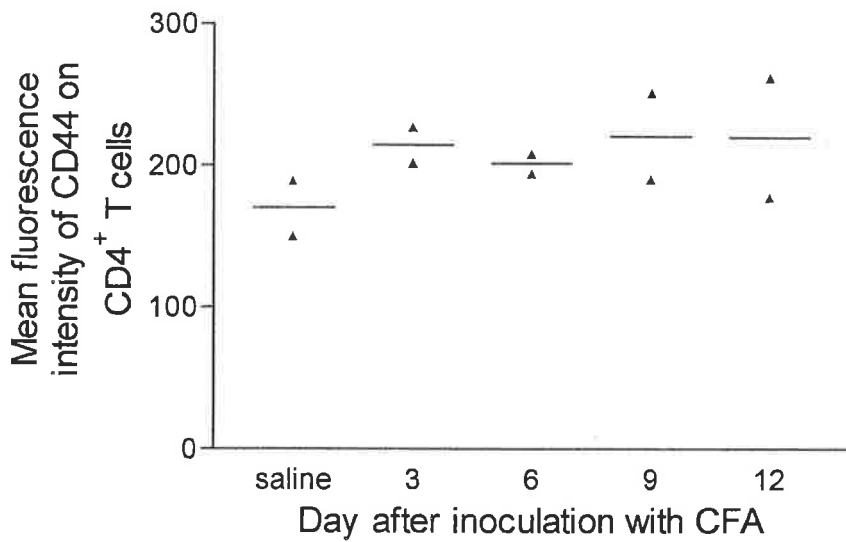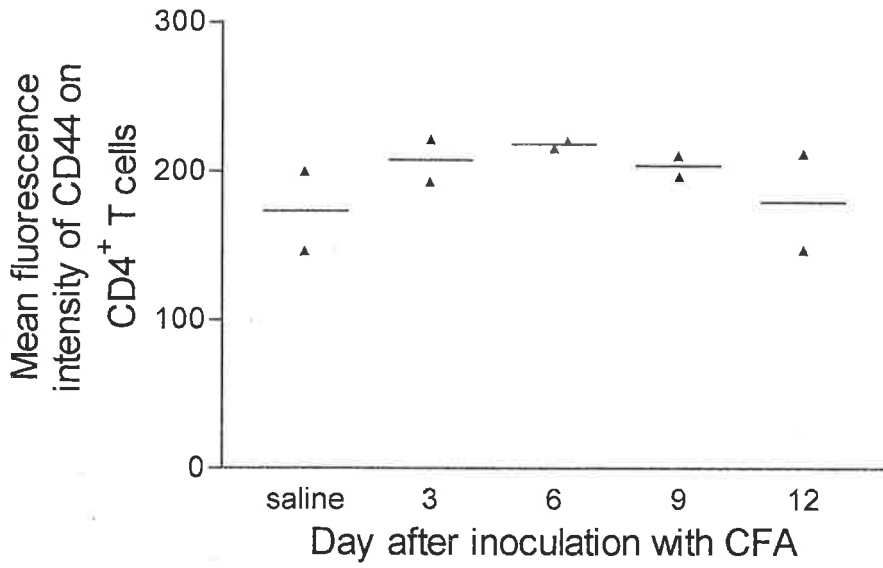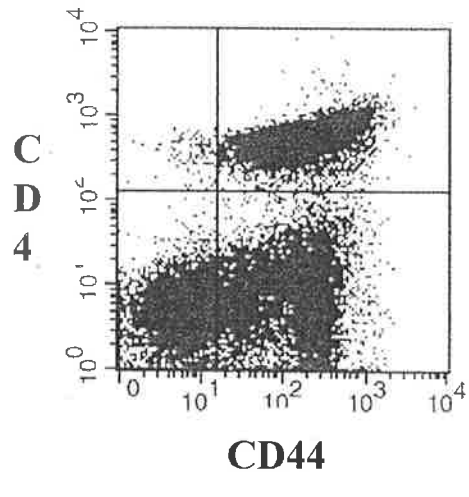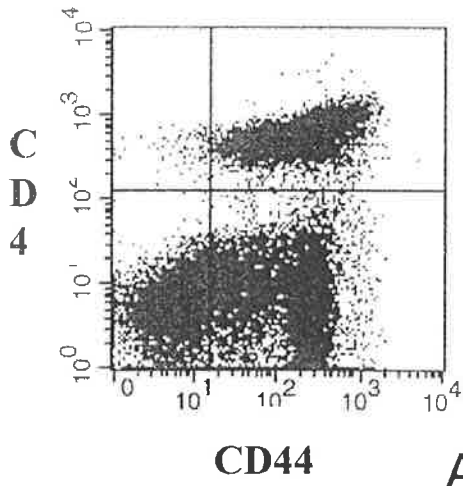
Figure 4.2.5.1 Production of IFN-γ by *in vitro* stimulated T cells obtained from the inguinal and popliteal lymph nodes of rats during the prodrome of AA.
Inguinal (A, C) and popliteal (B, D) lymph node cells from CFA- or saline-challenged rats were stimulated *in vitro* with PMA plus ionomycin in the presence of Brefeldin A for 6 hours before fixing (as described in 2.6.3). Fixed cells were made permeable with saponin and were first stained indirectly with mAb DB-1 (anti-IFN-γ; FITC), followed by direct labeling with mAb R73 (anti-α/β TCR; PE). Representative plots are shown of data obtained by two colour analysis of inguinal (A) and popliteal (B) lymph node cells harvested from rats 6 days after inoculation of CFA. Each triangle indicates the proportion of DB-1 staining α/β T cells among cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.
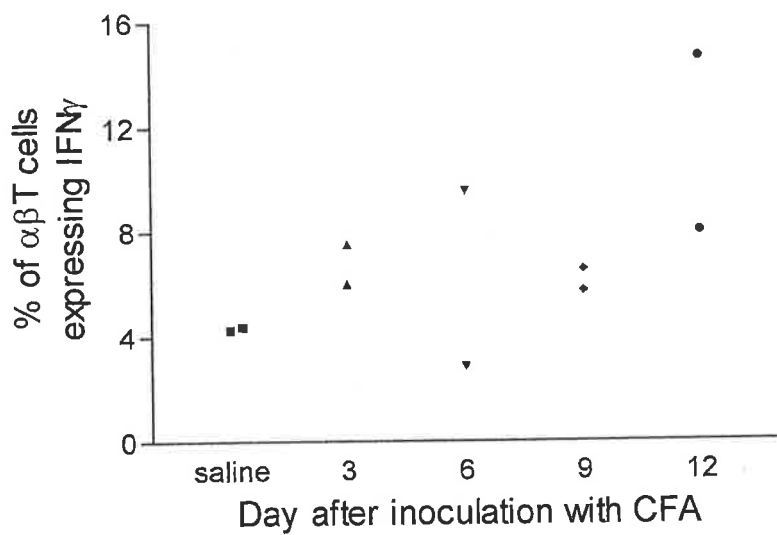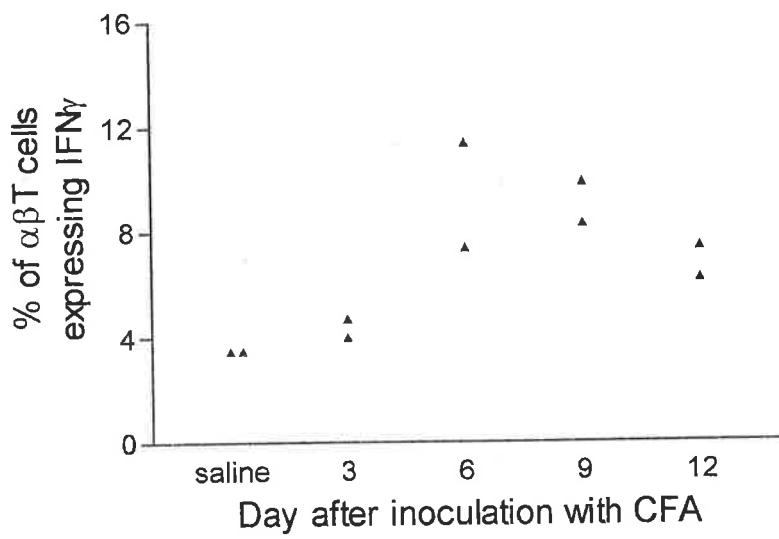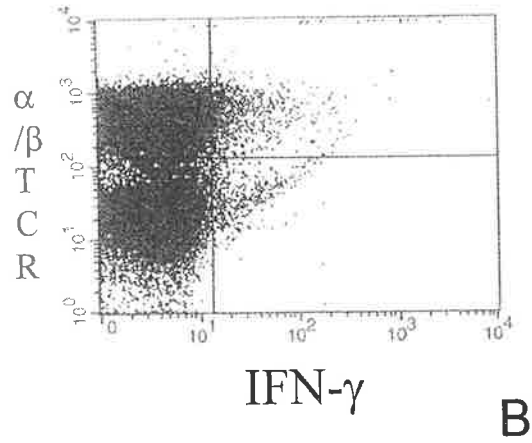
A

B

C

% of αβT cells expressing IFNγ

Day after inoculation with CFA

saline 3 6 9 12

D

% of αβT cells expressing IFNγ

Day after inoculation with CFA

saline 3 6 9 12
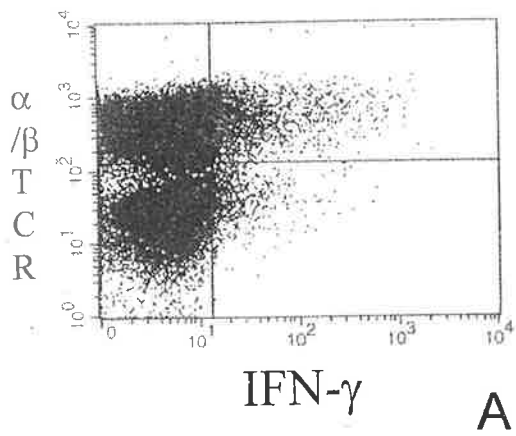
lymph nodes decreased 6-9 days after inoculation but remained higher than in control rats (Figure 4.2.5.1 C). In the popliteal lymph nodes the proportion of IFN-γ-producing α/β T cells was variable between pools but there was a clear trend towards increased frequency of these cells throughout the period following challenge with CFA (Figure 4.2.5.1 D). In rats challenged with CFA 12 days earlier, 9-15% of the α/β T cells produced IFN-γ after *in vitro* stimulation (Figure 4.2.5.1 D).

4.2.5.2 IL-4

On average, less than 1% of α/β T cells from the inguinal and popliteal lymph nodes of saline-injected rats produced IL-4 following stimulation *in vitro* (Figure 4.2.5.2 C and D). This value is within the range of error for the technique. At no time during the period of observation following inoculation of CFA did the proportion of α/β T cells that produced IL-4 exceed 4% in any of the lymph node pools (either inguinal or popliteal) (Figures 4.2.5.2 C and D). In most pools, IL-4-producing α/β T cells were present at frequencies similar to those in control rats.

4.2.6 Arthritogenicity of cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA

To investigate whether arthritogenic cells had been generated in the draining lymph nodes following inoculation of CFA, cell suspensions were prepared from the inguinal and popliteal lymph nodes and injected intravenously into naïve syngeneic recipients (5 or 6 per group of rats receiving inguinal lymph node cells and 1 to 3 per group of rats receiving popliteal lymph node cells). Lymph nodes were harvested from donor rats, either 3, 6 or 9 days after inoculation of CFA. A suspension of 2×10^8 pooled inguinal or popliteal lymph node cells was delivered by slow intravenous injection into each recipient rat. This number is in excess of the number of TD lymphocytes from CFA-challenged rats that is required to transfer of arthritis. For comparison, two recipients received 2×10^8 TD lymphocytes from donors inoculated with CFA nine days before cannulation. These control rats developed a typical course of adoptively-transferred arthritis (Figure 4.2.6.1 A, "D9 TDL control").

Inguinal lymph node cells harvested from rats challenged with CFA 9 days earlier, transferred arthritis to all naïve recipient rats (Figure 4.2.6.1 A). These animals developed

Figure 4.2.5.2 Production of IL-4 by *in vitro* stimulated T cells obtained isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA. Inguinal (A, C) and popliteal (B, D) lymph node cells from saline- or CFA- inoculated rats were stimulated *in vitro* with PMA plus ionomycin in the presence of Brefeldin A for 6 hours before fixing. Fixed cells were made permeable with saponin and were first stained with indirect labelling with mAb OX81 (anti-IL-4; FITC), followed by direct labeling with mAb R73 (anti-α/βTCR;PE). Representative plots are shown of data obtained by two colour analysis of inguinal (A) and popliteal (B) lymph node cells harvested from rats 6 days after inoculation of CFA. Each triangle indicates the proportion of OX81 staining α/β T cells among cells isolated from a pool of lymph nodes harvested from at least 10 rats per group. The horizontal bar indicates the mean calculated from the results of the two groups of rats at each time.
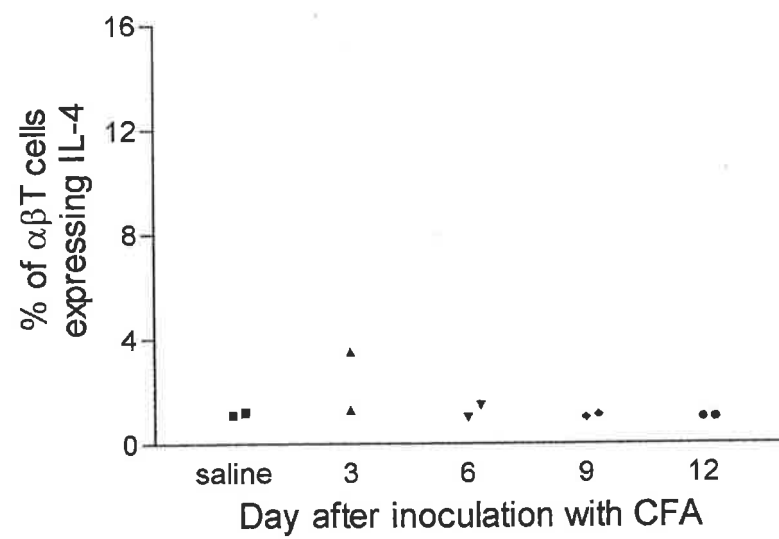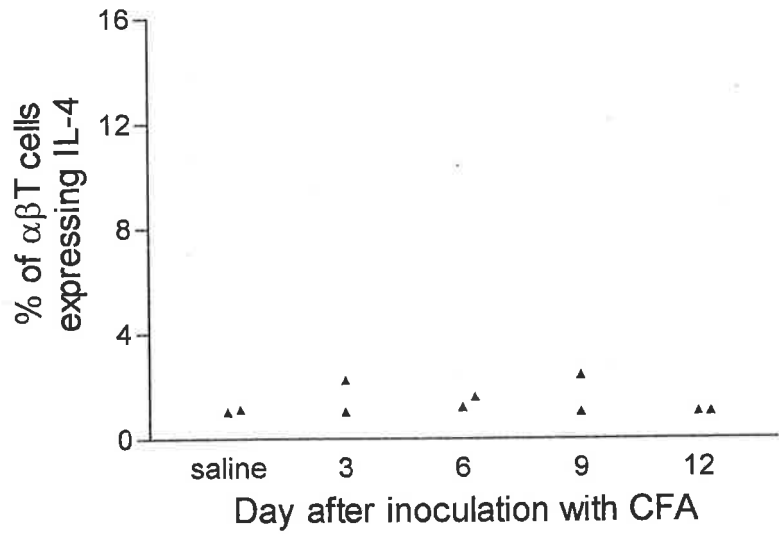
α/β TCR vs IL-4 (A)

α/β TCR vs IL-4 (B)

% of αβT cells expressing IL-4 vs Day after inoculation with CFA (saline, 3, 6, 9, 12) (C)

% of αβT cells expressing IL-4 vs Day after inoculation with CFA (saline, 3, 6, 9, 12) (D)
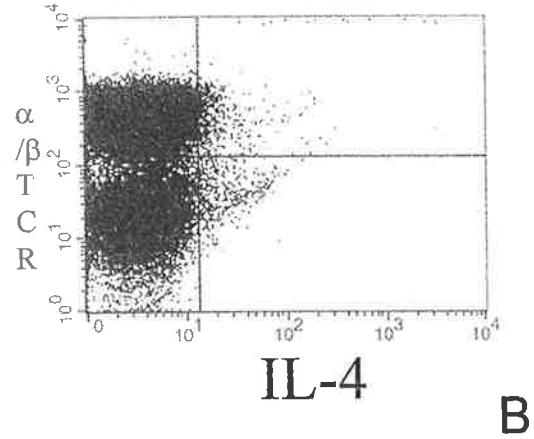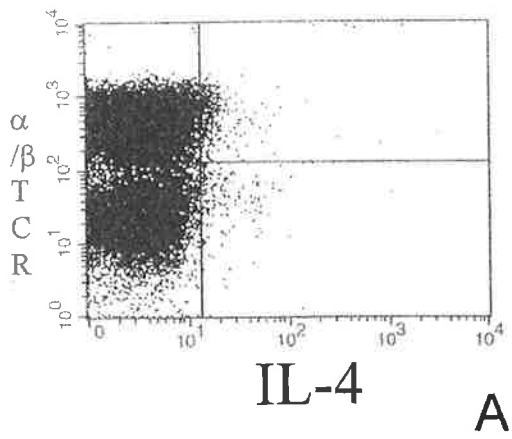
Figure 4.2.6.1 Arthritogenicity of inguinal and popliteal lymph node cells obtained from rats during the prodrome of AA. Cell suspensions were prepared from inguinal (A) or popliteal (B) lymph nodes harvested from donor rats either 3, 6 or 9 days post-inoculation of CFA (open squares, donors at day 3 post-inoculation; triangles, donors at day 6 post-inoculation; circles, donors at day 9 post-inoculation; asterisk, TDL donor at day 9 post-inoculation). A suspension of 2×10^8 cells were injected intravenously into naïve syngeneic recipient rats. Either 5 or 6 recipients were used in the case of inguinal lymph node cells. In the case of popliteal lymph node cells, donor cells obtained 3, 6, and 9 days after inoculation of CFA were injected into 3, 2 and 1 recipients respectively. Scores were obtained by assessing the extent of arthritis in all paws of recipient rats and the data represent the mean arthritic scores +/- SEM of arthritic rats in each group on each successive day after transfer. Days between inoculation of donor rats with CFA and removal of their lymph nodes is indicated in the legend.
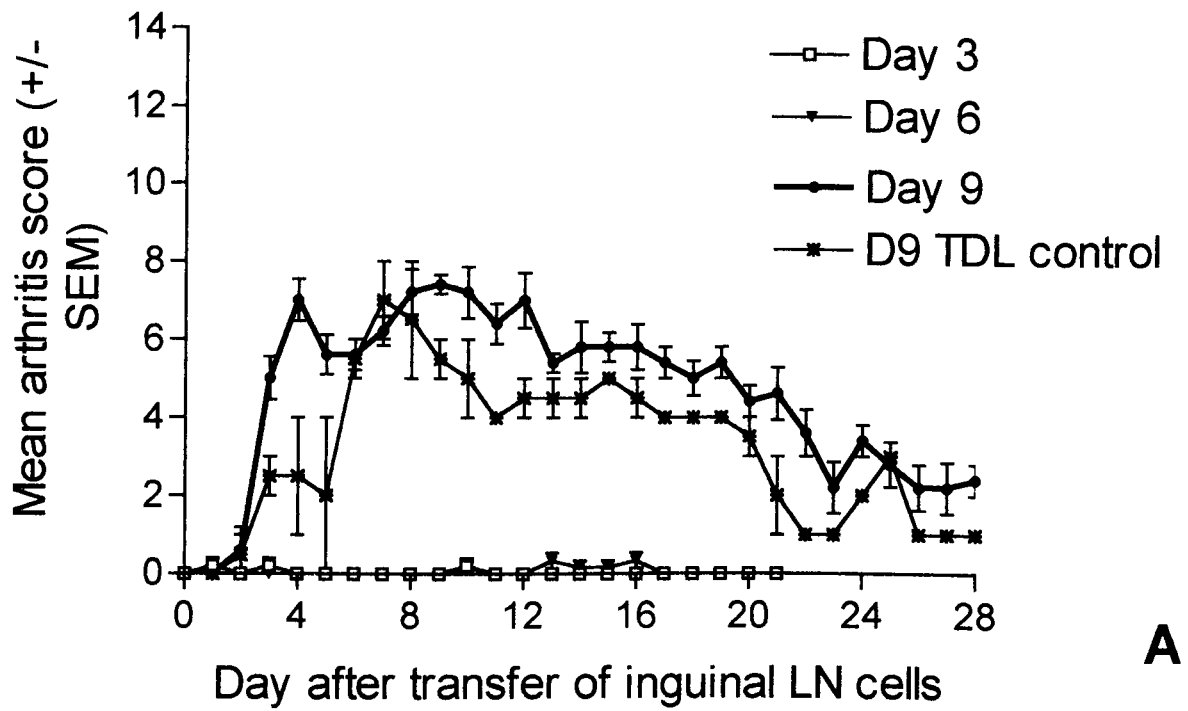
A
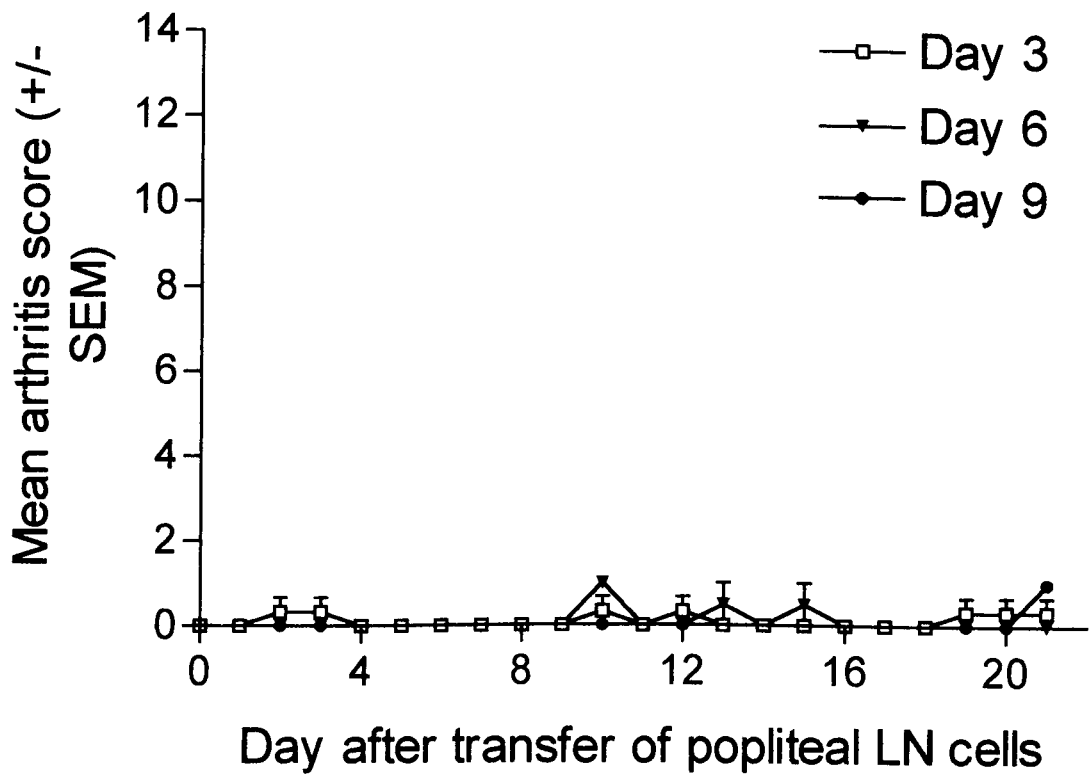
B

arthritis more rapidly that the recipients of TD lymphocytes from arthritic donors and with slightly greater severity. Inguinal lymph node cells harvested from rats at earlier times after inoculation (days 3 and 6) did not transfer arthritis to naïve recipients (Figure 4.2.6.1B). The small size of popliteal lymph nodes limited the yield of lymphocytes and the number of recipients of these cells (day 3, N = 3; day 6, N = 2, day 9, N = 1). At no stage after inoculation did popliteal lymph node cells transfer arthritis to the recipients.

4.3 Discussion

4.3.1 The macroscopic features of AA

The mean body weight of rats decreased during the prodrome of AA (Figure 4.2.1.1 B). The data represent the mean weight of each cohort of rats at the time of sacrifice prior to collection of lymph nodes. The early weight loss shows that although the rats do not show clinical signs of joint inflammation during the prodromal period, they have experienced systemic effects of the inoculation of CFA. With this evidence and past experience that essentially all DA rats develop AA after receiving CFA, it is reasonable to expect that animals in all of the groups would have progressed to develop AA had they not been killed. Others have shown that injection of CFA induces a reduction in the nocturnal mobility of rats throughout the prodrome of AA and a rapid but transient increase in body temperature from approximately day 8 (Phillipe et al., 1997). The effect of CFA on body weight is underestimated in Figure 4.2.1.1 B because under normal circumstances, these juvenile animals would exhibit progressive weight gain.

A feature of the immune response to an antigenic challenge is enlargement of the draining lymph nodes. CFA injected alone produces significant lymphadenopathy (Taub et al., 1970). Enlargement of the inguinal lymph nodes preceded visible joint inflammation (day 9 post-inoculation [Figure 4.2.1.1 A]), by at least 6 days (Figure 4.2.1.2). Although distal to the site of inoculation, the popliteal lymph nodes also were enlarged by the third day following injection of CFA and they remained enlarged until at least the twelfth day (Figure 4.2.1.2). Others have observed that doubling of the number of lymphocytes in a draining node occurs within two days of immunization, which it is not associated with proliferation. Proliferation commences by the end of the third day (Luther et al., 1997).

4.3.2 Comparison of T cell populations in the inguinal and popliteal lymph nodes during the prodrome of AA

By the third day of the prodrome, there was a small decrease in the proportion of lymphocytes in the inguinal and popliteal lymph nodes that expressed CD4 (Figure 4.2.2.1 C and D). In the same period (Figure 4.2.1.2), the size of these lymph nodes increased. If proliferation is not prominent until the end of this period (Luther et al., 1997) the decrease in the proportion of CD4$^+$ T cells is probably the result of an increase in the relative proportions of other lymphocyte subsets. In both the inguinal and popliteal lymph nodes there was a slight increase in the proportion of CD8$^+$ T cells following inoculation of CFA (Figure 4.2.2.2 C and D) and there was also an increase in the proportion of α/βTCR$^-$ lymphocytes (Figure 4.2.2.4). Nevertheless, the increase in lymph node weight was disproportionally large relative to the reduction in proportion of CD4$^+$ T cells, suggesting that all subsets had increased. In this early period, the increase probably results from retention of recirculating cells (Hall and Morris, 1965). Others have made similar observations and reported that during AA the proportion of CD4$^+$ cells decreases in the popliteal lymph nodes and that this is accompanied by an increase in the proportion of B cells (Haynes et al., 1996).

Lymphocytes expressing the γ/δ TCR represent only a small proportion of all lymphocytes in the inguinal and popliteal lymph nodes following inoculation of CFA (Figure 4.2.2.3 C and D). This proportion decreased slightly during the prodrome of AA. Expansion of γ/δ T cell numbers has been reported in the lesions of leprosy (Modlin et al., 1989) and in response to mycobacterial antigens (Kabelitz et al., 1990). However, others have shown that depletion of γ/δ T cells by the use of specific antibodies *in vivo* does not ameliorate or suppress AA (Pelegri et al., 1996a). The findings do not support involvement of γ/δ T cells in the pathogenesis of AA.

4.3.3 Comparison of surface molecules expressed by CD4$^+$ T cells isolated from the inguinal and popliteal lymph nodes during the prodrome of AA

The rapid production of activated T cells in lymph nodes and their emergence in the TD lymph (see Chapter 5) indicate that expansion of arthritogenic populations and/or their maturation is an early event in the development of AA. In addition to events in lymph nodes draining the injection site, there may be a phase of lymphocyte recruitment to lymph nodes draining synovial tissues, stimulation there by joint-derived arthritogen,

followed by re-release into the lymph. The appearance in the TD lymph of CD4$^+$ T cells capable of adoptive transfer of disease is examined in the next chapter.

Both effector and memory cells could be important in the pathogenesis of polyarthritis. This study focuses on effector CD4$^+$ T cells but the presence of T cells expressing activation markers and increased levels of certain adhesion molecules probably indicates that memory cells are generated as well as effector cells.

Changes in the ratios of the major lymphocyte subsets in the lymph nodes can be due either to changes in the relative rates of accumulation of cells from the recirculating pool and/or to differences in the rate of cell division between the subsets. The same considerations also apply to changes in proportions of cells expressing molecules such as CD45RC, CD62L, LFA-1 and CD44 because substantial proportions of the resting lymphocytes in the recirculating pool express these molecules. In contrast, molecules such as MHC class II, CD25, CD71, CD134, UA002 antigen and CD54 are expressed by CD4$^+$ T cells that are either actively dividing or recently activated. Examination of the latter molecules is, therefore, an indirect way of monitoring lymphocyte activation and cell division within stimulated lymph nodes.

The inguinal lymph nodes, which are in the direct drainage of the site of CFA inoculation, contained increased proportions of CD4$^+$ T cells expressing all of the above activation markers within 3 days of inoculation. The presence of an increased proportion of CD71$^+$ cells suggests that some CD4$^+$ T cells had entered the cell cycle by this time. CD71$^+$ CD4$^+$ T cells continued to increase in proportion until day 6 post-inoculation and then declined. Expression of CD134, thought to be a marker of early activation (Stuber and Strober 1996), followed similar kinetics. Increase in the proportion of CD4$^+$ T cells that expressed MHC class II, CD25, UA002 antigen and CD54 continued until days 6-9 post-inoculation by then declined. Taken together, the results suggest that activation and division of CD4$^+$ T cells begins within 3 days of inoculation of CFA, that the stimulatory effect begins to wane as early as 6 days after inoculation, that the continued increase in the proportion of cells expressing markers exemplified by MHC class II and CD54 may reflect maturation of the cells generated in the lymph node and that the decline in the proportions of these cells in the period between day 6 and day 9 after inoculation is due to migration of these cells more rapidly than they are being produced. These changes were not accompanied by

a decrease in the mean fluorescence of CD4$^+$ T cells expressing CD45RC or CD62L (ie. the presence of an increased proportion of cells in transition towards a memory phenotype). The latter observations suggest that accumulation of naïve CD4$^+$ T cells from the recirculating pool has masked any increase in the absolute numbers of CD45RC$^-$ or CD62L$^-$ cells that may been generated. However, the increase in the proportions of CD4$^+$ T cells that express higher levels of LFA-1 and CD44 is consistent with up-regulation of these molecules on the newly-activated population.

Surprisingly, in view of the more peripheral location of the popliteal lymph nodes relative to the inoculation site, increased proportions of cells expressing activation markers were detected in these lymph nodes as early as in the inguinal lymph nodes. The proportion of CD71$^+$ cells declined slowly after 3 days post-inoculation but remained elevated for the entire period of observation. Similarly, the proportion of CD134$^+$ cells remained elevated and the proportions of cells expressing MHC class II, CD25, UA002 antigen and CD54 showed no tendency to decline in the period 9-12 days post-inoculation. It appears, paradoxically, that antigenic stimulation continues longer in lymph nodes that are not exposed directly to the effects of CFA than in lymph nodes that are in the direct drainage of the inoculation site. Early activation in these lymph nodes and the sustained nature of the activity suggests that the adjuvant inoculation induces a systemic effect that leads to activation of lymph nodes and/or that the presentation of an antigen occurs in the popliteal lymph nodes. Although distant dissemination of antigens from the adjuvant depot cannot be excluded, it is tempting to speculate that the antigen responsible for the activity in the popliteal lymph nodes may have arisen from the joints that they drain.

4.3.4 Production of cytokines by T cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA

Mr. Ashley Connolly, in the Arthritis Research Laboratory, used a quantitative reverse transcription – polymerase chain reaction (RT-PCR) technique to examine the kinetics of expression of mRNA encoding cytokines in the inguinal and popliteal lymph nodes of rats inoculated with CFA (Connolly, 1998). The numbers of copies of mRNA encoding IFN-γ, TGFβ and TNFα was increased in the inguinal lymph nodes within 3 days following inoculation of CFA and levels remained above those detected in the control group (non-injected rats). The copy number of mRNA encoding either IFN-γ or TNFα in the samples

isolated from the popliteal lymph nodes of rats inoculated with CFA either 3, 6, 9 or 12 days before harvesting the tissue was much lower than that detected in the inguinal lymph node samples. There were increased copies of mRNA transcripts encoding TGFβ in the popliteal lymph nodes relative to the popliteal lymph nodes of non-injected rats on days 9 and 12 post-inoculation.

The proportion of α/β T cells from inguinal and popliteal lymph nodes saline-inoculated rats that produced IFN-γ protein after 6 hours of stimulation *in vitro* with PMA plus ionomycin was 3.5% and 4.5%, respectively (Figure 4.2.5.1 C and D). Following the inoculation of rats with CFA, an increase in the frequency of α/β T cells producing IFN-γ was observed. The rise in IFN-γ-producing α/β T cells in the inguinal lymph nodes occurred during the prodromal period and preceded the earliest signs of arthritis (day 9) by at least 3 days. The rise in the proportion of T cells producing IFN-γ in the popliteal lymph nodes was observed as greatest at day 12 post-inoculation, when arthritis was established in the hind paws.

The proportions of α/β T cells that produced IL-4 (Figure 4.2.5.2 C and D) were small. These observations were in accordance with those of others, who have found mRNA transcripts encoding IL-4 to be scarce or absent in the popliteal lymph nodes of rats with AA (Schmidt-Weber et al., 1999). This suggests that the immune response that occurs in the inguinal and popliteal lymph nodes is biased towards a Type-1 response.

It is not surprising that the immune response in the lymph nodes draining the site of inoculation of CFA was biased so strongly towards IFN-γ production and away from IL-4 production. Dendritic cells, which are specialised for the activation of naïve T cells, respond to challenge with mycobacteria (either live or heat-killed) with the production of IL-12, which drives the immune response towards production of Type-1 cytokines (reviewed in Demangel and Britton, 2000).

4.3.5 Arthritogenicity of cells isolated from the inguinal and popliteal lymph nodes of rats during the prodrome of AA

Inguinal lymph node cells harvested from rats inoculated 9 days earlier were able to adoptively transfer arthritis to naïve recipient rats (Figure 4.2.2 A). Other researchers

have found that in order to transfer arthritis, lymph node and spleen cells from CFA-challenged rats require stimulation *in vitro* (reviewed in Taurog et al., 1988) and/or irradiation of the recipients (Taurog et al., 1983a; Waksman & Wennersten, 1963). The most orthodox interpretation of the results obtained herein by adoptive transfer is that the mechanism of disease induction in the recipients involves the activities of arthritogenic T cells. However, it is also possible that the cells responsible are antigen-loaded antigen presenting cells (APC [eg. dendritic cells]) or that significant amounts of mycobacterial antigens were transferred along with the cells. The time of onset of the disease in the adoptively-transferred arthritis is more rapid than in actively-induced AA. If the transferred disease is due to contaminating mycobacterial antigens or to APCs bearing arthritogen, then the primary response to this material would have to be accelerated considerably compared with the response to inoculation of CFA. An extensive literature search has been unsuccessful in revealing publications that demonstrate the transfer of AA by APC and attempts to achieve this in the Arthritis Research Laboratory (A. Wright, Honours thesis, 1999) have been unsuccessful. In contrast, it has been demonstrated that AA can be adoptively-transferred with activated CD4^{+} T cells (Spargo et al., 2001). Arthritis can be induced in DA rats with oil alone (oil-induced arthritis, OIA) in the absence of mycobacteria (Kleinau et al., 1991) and this disease can be transferred adoptively (Svelander et al., 1997). This suggests that although mycobacterial antigens may render adjuvants more arthritogenic, they are not essential for either actively-induced or adoptively-transferred arthritis.

Despite evidence of activation among the CD4^{+} T cells harvested from inguinal lymph nodes at 3 days and 6 days post-inoculation, the cells did not transfer arthritis. If arthritogenic T cells were present at these times, they were not in appropriate number to transfer the disease. However, the sizes of the recipient groups were small because of the relatively small numbers of donors lymphocytes that can be harvested. The disadvantage of this is that if the incidence of adoptively-transferred arthritis was less than say 20% at the cell dose used (ie. 1 out of 5 recipients developing arthritis), then arthritogenic cells from the inguinal lymph nodes might not be detected.

Popliteal lymph nodes yield even fewer cells than inguinal lymph nodes and for this reason, the recipient groups were smaller (5 donors were required to produce 2×10^8 lymphocytes for transfer to a single recipient). Furthermore, one recipient of cells

harvested from donors 9 days after inoculation with CFA suffered an anaesthetic accident. It is not possible, therefore, to be unequivocal that popliteal lymph nodes do not contain detectable numbers of arthritogenic T cells. Nevertheless, the presence of activated CD4+ T cells from as early as day 3 post-inoculation in the popliteal lymph node cells did not assure the transfer of disease. Further studies could include an examination of whether *in vitro* activation of the popliteal lymphocyte population, with a mitogen, such as Concanavalin A, can reveal the presence of latent arthritogenic T cells in these lymph nodes. It would also be interesting to examine whether arthritogenic activity emerges in the popliteal lymph nodes at later times (eg. 12 days) after inoculation of CFA, when production of IFN-γ is greater.

4.3.6 General discussion

During a primary immune response, as few as 3 days are required *in vivo* to generate effector CD4+ T cells that mediate helper activity (Bradley et al., 1991). The development of effector CD4+ T cells that display a cytokine response of the distinctive Th1 or Th2 types requires an exposure to antigen presenting cells of not more than 2 days duration (Jelley-Gibbs, et al 1991). As early as the first day after immunisation, T cells are observed in the T cell areas of the draining lymph node in small clusters, presumably around dendritic cells. On the second day after immunisation, T cells are located at the edge of the B cell follicles, interacting with B cells (Garside et al., 1998). By 3 days after immunisation, activated lymphoblasts are observed more frequently in the TD lymph of rats (Delorme et al., 1969).

Within 3 days following inoculation of CFA, the proportion of CD4+ T cells expressing the activation markers MHC class II, CD25 and CD134, as well as the adhesion molecule CD54, had increased in both the inguinal and popliteal lymph nodes and in most cases remained elevated until day 12. This suggests that the CD4+ T cells expressing activation markers and adhesion molecules are a changing population. Following activation and maturation, they may exit the node to be replaced by other activated cells. The latter could arise by continued division of members of the original cohort or they could arise from new waves of antigen-specific resting T cells that arrive from the circulating pool. Interestingly, the pattern of expression of CD71 suggests that cell division reaches a peak by 6 days post-inoculation. A reduction in the proportions of CD4+ T cells that expressed

most of the activation markers was observed at day 12 post-inoculation of CFA, during moderate to severe arthritis, possibly reflecting the diversion of activated cells from the circulating pool because of recruitment and retention in the inflamed joints.

The results are consistent with the hypothesis that inoculation of CFA leads to the stimulation of cells in the draining lymph nodes, which generates a population of activated CD4$^+$ T lymphocytes that are arthritogenic. However, an arthritogenic population was not detected in the inguinal lymph nodes from donor rats until 9 days after inoculation of CFA. It is possible that the process of maturation commences in the lymph node, but it is completed elsewhere. By day 9 post-inoculation, cells in the inguinal lymph node are arthritogenic. Precisely which aspects of their phenotype allows them to exert arthritogenicity is of great interest. Possibilities include expression of adhesion molecules that allow recruitment from synovial blood vessels or the expression of pro-inflammatory cytokines such as IFN-γ that promote synovial inflammation.

Interestingly, an increase in the expression of activation markers and production of IFN-γ by CD4$^+$ T cells was seen in popliteal lymph nodes of rats as early as 3 days post-inoculation of CFA, preceding the onset of inflammation in the joints that they drain by at least 6 days. The popliteal lymph nodes do not directly drain the site of inoculation but in most cases exhibited only slightly smaller proportions of activated CD4$^+$ T cells compared to those from the draining (inguinal) lymph nodes. It is possible that the emergence activated CD4$^+$ T cells in the inguinal lymph nodes preceded that which occurred in the popliteal lymph nodes and this could be examined by harvesting cells at times earlier than 3 days after inoculation of CFA. It is likely that at least some of the activated cells in the popliteal lymph nodes were stimulated originally in the inguinal lymph nodes and were present in the popliteal lymph nodes as a consequence of the recirculation patterns of these cells.

Lymphocytes that have been activated by antigen in the lymph nodes disseminate via the lymph (Hall and Morris 1965). The following Chapter describes lymphocytes that were harvested by drainage of the thoracic duct lymph from rats during the prodrome of AA.

CHAPTER 5

ACTIVATED CELLS IN THE EFFERENT LYMPH OF RATS IN THE PRODROME OF POLYARTHRITIS, WITH PARTICULAR REFERENCE TO CD4$^+$ T LYMPHOCYTES

5.1 Introduction

Rheumatoid arthritis (RA) is a chronic and remitting disease in which cellular infiltration of the synovium, mainly with mononuclear cells, is a prominent feature. Typically, joints are involved symmetrically, including the hands and feet. This pattern of the disease, together with other systemic manifestations, points to a process of dissemination in the pathogenesis of this disease. There is substantial evidence that RA is mediated, at least in part, by T lymphocytes. Activated memory CD4+ T cells (Thomas et al., 1992) and their products (Buchan et al., 1988) have been detected in the synovial tissue of patients with RA. The products of effector T cells in the synovial tissues may activate other cell types, such as macrophages and fibroblasts, which in turn contribute to the articular damage that is characteristic of RA. Thoracic duct drainage of patients, which depletes migratory lymphocytes, has been shown to ameliorate RA (Paulus et al., 1979).

The Arthritis Research Laboratory (ARL) has a particular interest in the role of T cells in polyarthritis. Adjuvant-induced arthritis (AA) in the rat, which is mediated primarily (although not exclusively) by CD4+ T cells (Billingham et al., 1990b; Pelegri et al., 1996b) was chosen as the model in which to examine the pathogenesis of polyarthritis. Although no animal model can mimic a human disease perfectly, AA has important similarities to RA and it has been used in the ARL to investigate the emergence of arthritogenic cells in the thoracic duct (TD) lymph and the dissemination of activated lymphocytes to the joints via the circulation (Spargo et al., 1996).

It is hypothesized that inoculation with CFA leads to the presentation of inciting antigens (self-antigens and/or cross reactive mycobacterial antigens) to T lymphocytes in the lymph nodes draining the site of inoculation. During the nine days between inoculation of CFA and the visible signs of joint swelling, it is envisaged that there is an expansion of autoreactive CD4+ T lymphocytes. These cells are induced to express activation markers, including adhesion molecules. The activated T lymphocytes enter the circulation via the efferent lymphatics and they are then distributed to the joints, where they initiate inflammation through the production of cytokines and other inflammatory mediators.

Chapter 5: Activated cells in the efferent lymph of rats in the prodrome of polyarthritis, with particular reference of CD4$^+$ T lymphocytes

The work of Mr. Llewellyn Spargo in the ARL has shown that the thoracic duct lymph from DA strain rats in the late prodromal phase of AA (9 days after inoculation with CFA) contains arthritogenic CD4$^+$ T cells that can transfer arthritis adoptively to normal rats. A subpopulation of these cells that expresses MHC class II, OX40Ag (CD134), transferrin receptor (CD71) and interleukin (IL)-2R α chain (CD25), are necessary and sufficient for successful transfer of the disease (Spargo et al., 2001). Importantly, in DA rats successful adoptive transfer does not require prior *in vitro* stimulation with a mitogen such as Concanavalin (Con) A, indicating that these cells are fully competent to act as effector cells. Mr. Spargo has demonstrated that TD lymphoblasts from pre-arthritic donors accumulate in tissues of the paws of normal and arthritic rats respectively to a greater extent than those from normal donors (Spargo et al., 1996). This indicates that TD lymphoblasts express a repertoire of surface molecules that enhances their entry into the synovial tissues of both normal and inflamed joints.

Mr. Spargo collaborated with Mr. Ashley Connolly to examine the expression of mRNA transcripts from genes encoding cytokines in activated and resting CD4$^+$ T cells in the TD lymph of normal and arthritic rats (Connolly, 1998). Compared with TD lymphocytes from normal donors, TD lymphocytes from arthritic donors were found by quantitative RT-PCR to express more copies of mRNA encoding interferon (IFN)-γ, but similar numbers of mRNA transcripts encoding TNF-α and transforming growth factor (TGF)-β. When Immuno-magnetic beads were used to separate cells according to the expression of MHC class II, CD25, CD71 and CD134, it was found that the number of copies of mRNA transcripts encoding IFN-γ, TNF-α and TGF-β was significantly higher in the activated CD4$^+$ T cells from arthritic donors than in the corresponding subset from normal donors. The number of copies of mRNA encoding IFN-γ in activated CD4$^+$ T cells from arthritic donors was approximately one thousand times that detected in the activated population of CD4$^+$ T cells found in normal rats, while transcripts encoding TGF-β were present in amounts approximately 80-fold greater than in the cells from normal rats. Few transcripts encoding IL-4 or IL-2 were detected in the TD lymphocytes from either normal or arthritic donors (Connolly, 1998).

Chapter 5: Activated cells in the efferent lymph of rats in the prodrome of polyarthritis, with particular reference of CD4$^+$ T lymphocytes

At the time the studies described in this chapter were undertaken, the only monoclonal antibody (mAb) available for flow cytometric detection of intracellular rat cytokines were DB-1 (anti-IFN-γ) and OX81 (anti-IL-4). IFN-γ and IL-4 are important representatives of the cytokines produced by Th1 and Th2 cells respectively. These antibodies were used to investigate cytokine production by CD4$^+$ TD lymphocytes in rats during the induction of AA. As described in Chapter 3, the duration of *in vitro* stimulation prior to cytokine analysis was relatively short (6 hours), in order that the production of cytokines in this period reflected the activities of cells that had been activated *in vivo*. However, a disadvantage of this method is that only small amounts of cytokines are produced in this time, relative to the production of cytokines by cells that have been hyper-stimulated for extended periods of time *in vitro*. In the case of IL-4 production, this can make the detection of cytokine-producing cells difficult. For this reason, RT-PCR was used to detect mRNA encoding the cytokines, as this technique is very sensitive and it does not require *in vitro* stimulation of the cells. Furthermore, it was possible to examine the transcriptional activities of genes encoding cytokines for which antibodies were not available to analyse the protein by flow cytometry. The RT-PCR technique described in this study is semi-quantitative. It was used to indicate proportionate expression of cytokines, rather than to determine the number of copies of mRNA encoding each cytokine. It is important to note that mRNA transcripts encoding cytokines can be present in lymphocytes, without the production of protein. Therefore, the presence of mRNA is not evidence for production of cytokine, because there is evidence that cytokine production is controlled both transcriptionally and translationally (reviewed in Kelso, 1993). This was supported by the studies described in Chapter 3, where Actinomycin D prevented production of IL-4 by stimulated lymphocytes but only inhibited production of IFN-γ partially.

The work described in this chapter focused on charting the appearance of arthritogenic lymphocytes in TD lymph throughout the prodrome (days 3-9) and the early clinical stage (day 12) of AA. The phenotype of the activated T cells was examined. Groups of at least five rats were injected with CFA either 3, 4, 5, 6, 9 or 12 days before TD cannulation (see Section 2.3.3) and five rats were injected with saline to serve as controls. TD lymphocytes were collected over a period of 16-20 hours from the time of establishment of lymph flow. Hence

"day 3" refers to lymph collected over the duration of the third day and early morning of the fourth day after inoculation with CFA. The TD lymphocytes collected from a single donor were transferred to a single naive syngeneic recipient by intravenous injection, after removal of small aliquots for analysis. The recipients of the adoptively-transferred cells were monitored daily for weight and clinical signs of arthritis (see Section 2.2.2). The experiment was very labour intensive and for this reason, the rats were inoculated and cannulated in staggered fashion so that each batch contained one or more members from each of the experimental groups. This avoided batch-to-batch variation as a confounding influence on the results.

From the TD lymphocytes collected from each donor, approximately 2.4×10^7 cells were removed for labelling to detect expression of cell surface markers (see Section 2.6.2). Dual colour immunofluorescence was used to define CD4$^+$ T cells and respective expression of the following surface molecules MHC class II, CD25, CD71, CD134, CD54, CD62L, functional P-selectin ligand, UA002 antigen, LFA-1, CD44, CD45RC and the integrins of the α_{E2} and α_4 families. For these analyses, the settings of the flow cytometer were standardised and only minor adjustments were made each day in order to ensure accurate compensation between the fluorescent signals in fluorescence channels 1 and 2 of the instrument. The cells were also labelled to assess the relative proportions of α/β and γ/δ T cells in the TD lymph. From a further aliquot of 1×10^7 cells, RNA was extracted for RT-PCR analysis of mRNA encoding IL-2, IL-4, IL-10, TNF-α, TGF-β1 and IFN-γ (see Sections 2.10.3-10). A third aliquot of 2×10^7 cells was cultured in the presence of PMA plus ionomycin and Brefeldin A and these cells were used for flow cytometric analysis of intracytoplasmic IL-4 and IFN-γ (see Section 2.6.3).

5.2 Results

5.2.1 Arthritogenicity of TD lymphocytes obtained from rats during the development of AA

The target antigen of arthritogenic T cells in AA is unknown. The only method available with which to determine the arthritogenic capacity of lymphocytes is to transfer the cells from primed donors into naïve syngeneic recipients and then monitor the recipients for the

development of joint inflammation. To examine the production of arthritogenic effector cells during the induction of AA, rats were inoculated with CFA and their thoracic duct cannulated 3, 4, 5, 6, 9 or 12 days later. The lymphocytes were harvested over a 16-20 hour period and after removal of a sample for phenotypic analysis, the remainder from each animal was injected intravenously into a syngeneic naive recipient. This strategy allowed the analysis of changes in the phenotype of CD4⁺ T cells throughout the early stages of AA and correlations between these changes with the arthritogenic capacity of the cells. As controls, TD lymphocytes were collected from rats that had been injected with saline (one each at days 0, 3, 6, 9 or 12). The incidence of arthritis in recipients is shown in Figure 5.2.1.1.A. TD lymphocytes harvested from donor rats inoculated with saline (controls) or with CFA 3 days prior to cannulation, did not transfer arthritis to naïve recipient rats and the joint scores recorded in recipients of cells from these donor groups are not shown.

TD lymphocytes harvested from three of the five rats inoculated with CFA 4 days before cannulation transferred disease (Figure 5.2.1.1 A and B). A higher incidence of arthritis was observed in rats that received cells from donors immunized 5 days (four out of five recipients developed arthritis, Figure 5.2.1.1 C) or 6 days (five out of seven recipients developed arthritis, Figure 5.2.1.1 D) before TD cannulation. However, only at 9 days after immunization did cells from all of the donors transfer arthritis adoptively and this coincided with the most severe arthritis observed in the recipient groups. The majority of rats receiving cells from day 12 donors developed arthritis, which was of moderate severity (Figure 5.2.1.1 F). The onset of clinical signs of joint swelling was usually apparent by five days after transfer of TD cells, although small inflammatory lesions were observed as early as two days after the transfer of the cells (Figure 5.2.1.1 B-F).
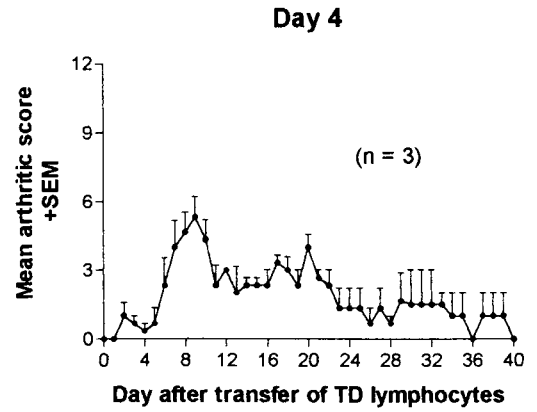
These results indicate that arthritogenic cells begin to migrate into the circulation within five days following inoculation with CFA and that they are present during the rest of the prodrome, which ends approximately at day 9. The potency of TD lymphocytes to transfer disease appears to diminish after the onset of clinical disease in the period between 9 and 12 days after inoculation with CFA.

Figure 5.2.1.1 Arthritogenicity of TD lymphocytes harvested during the prodrome and early clinical phase of AA. TD lymphocytes were harvested from donor rats, either 3, 4, 5, 6, 9 or 12 days after inoculation of CFA. Donor rats inoculated with saline were included as controls. TD lymphocytes from individual donors were injected intravenously into naïve syngeneic recipient rats. Scores were obtained by assessing the extent of joint inflammation in all paws of the recipient rats. The values shown in graphs B - F represent the mean arthritic scores + SEM of arthritic rats in each group of recipients on successive days after transfer. It should be noted the graphs display mean arthritic scores only from rats that were arthritic (the number of arthritic rats is shown in parenthesis) and do not include scores from rats that did not develop arthritis. Recipients of TD lymphocytes from donors that were inoculated with saline (controls) or with cells from donors cannulated 3 days after inoculation of CFA did not develop disease.
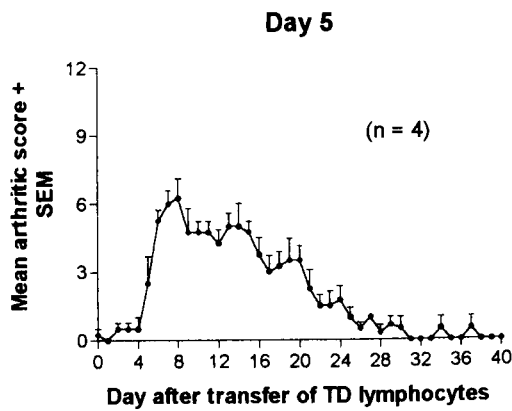
A. The incidence of arthritis in recipients of TD lymphocytes.

B. Development of adoptively-transferred arthritis in recipients of TD lymphocytes from donor rats inoculated with CFA 4 days prior to TD cannulation.

C. Development of adoptively-transferred arthritis in recipients of TD lymphocytes from donor rats inoculated with CFA 5 days prior to TD cannulation.

D. Development of adoptively-transferred arthritis in recipients of TD lymphocytes from donor rats inoculated with CFA 6 days prior to TD cannulation.

E. Development of adoptively-transferred arthritis in recipients of TD lymphocytes from donor rats inoculated with CFA 9 days prior to TD cannulation.

F. Development of adoptively-transferred arthritis in recipients of TD lymphocytes from donor rats inoculated with CFA 12 days prior to TD cannulation.

Day of cannulation of donor after inoculation with CFA	Incidence
saline	0/5
Day 3	0/5
Day 4	3/5
Day 5	4/5
Day 6	5/7
Day 9	5/5
Day 12	5/6

A

Day 4

(n = 3)

Mean arthritic score +SEM

Day after transfer of TD lymphocytes

B

Day 5

(n = 4)

Mean arthritic score + SEM

Day after transfer of TD lymphocytes

C

Day 6

(n = 5)

Mean arthritic score + SEM

Day after transfer of TD lymphocytes

D

Day 9

(n = 5)

Mean arthritic score +/- SEM

Day after transfer of TD lymphocytes

E

Day 12

(n = 5)

Mean arthritic score + SEM

Day after transfer of TD lymphocytes
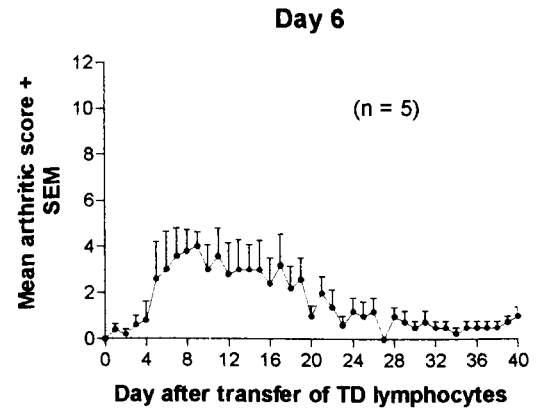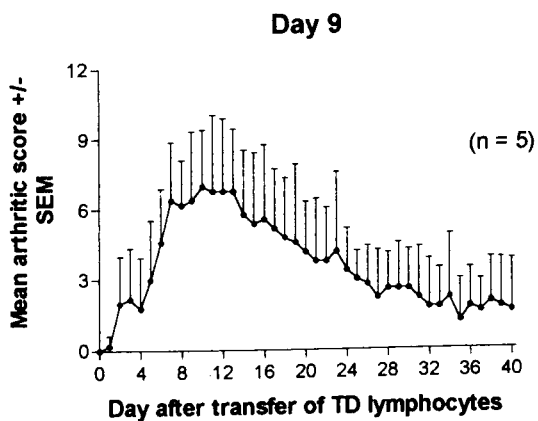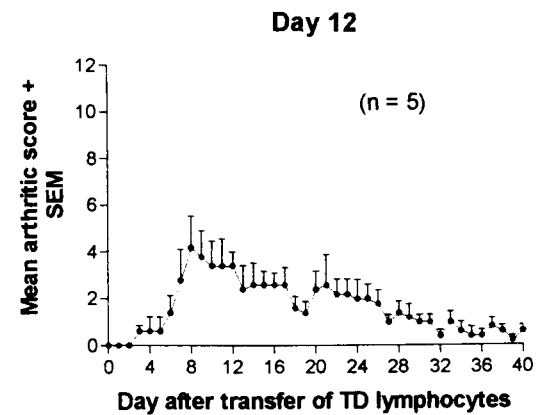
F

5.2.2 T cell subpopulations in the TD lymph during the prodrome of AA

5.2.2.1 Total output and physical characteristics of TD lymphocytes

Flow cytometry was used to examine the surface antigen phenotype of TD lymphocytes isolated at different times after inoculation with CFA. There was no significant difference in the total output of cells per 16 hour collection between groups of rats inoculated with saline or CFA (Figure 5.2.2.1 A). Electronic gating was used to analyse cells according to their physical characteristics (Figure 5.2.2.1 B), where forward scatter (FSC) reflects cell size and side scatter (SSC) is determined by cell complexity. The gates shown in Figure 5.2.2.1 B indicate the boundaries used for analysis of cells described as "small", "large" or "all" in other figures in this chapter. The proportion of cells that are large, an indicator of recent activation, increased significantly following inoculation with CFA (Figure 5.2.2.1 C) and remained elevated until at least 12 days after inoculation.

5.2.2.2 CD4$^+$ T cells

The proportion of CD4$^+$ T cells in the TD lymph increased following inoculation with CFA. Of the small lymphocytes (Figure 5.2.2.2 A and D), the proportion of CD4$^+$ T cells in TD lymph was significantly greater at 4 and 5 days after inoculation with CFA compared with control rats that received saline but the difference was small. In contrast, the proportion of CD4$^+$ large lymphocytes (Figure 5.2.2.2 B and E) was greater than in controls at all times except day 12 after CFA inoculation. When examining the entire population of TD lymphocytes (Figure 5.2.2.2 C and F), CD4$^+$ T cells represent a greater proportion of the total population on days 3, 4 and 5 after inoculation with CFA and return to levels comparable to the control group at later times.

5.2.2.3 CD8$^+$ T cells

The proportion of TD lymphocytes that express the CD8α/β complex (classical CD8$^+$ T cells, represented in the upper right quadrants of the scatter plots in Figure 5.2.2.3 A-C) remained relatively constant following inoculation with CFA (Figure 5.2.2.3). The only significant difference observed was 12 days after inoculation with CFA, when the proportion of large CD8$^+$ T cells was greater than observed at the other times and in TD lymphocytes from the saline injected control rats (Figure 5.2.2.3 B and E).

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Figure 5.2.2.1 Total output and physical characteristics of cells collected from overnight drainage of thoracic duct lymph during the prodrome of adjuvant arthritis. The total number of cells yielded from an overnight collection of TD lymph was assessed using a haemocytometer. The viability of the TD lymphocytes was confirmed using a Trypan-blue exclusion assay. The total number of cells harvested from each rat is represented by a circle and the mean of the group is depicted by a horizontal bar (A). The proportion of the entire population that had "large" morphology was estimated by setting arbitrary gates electronically to distinguish "small" and "large" cells (B). The gate encompassing "all" lymphocytes is shown on a representative scatter plot of the TD lymphocytes obtained from a rat 6 days after inoculation of CFA (B). The proportion of the all TD lymphocytes falling within the large cell gate is shown in (C). The squares represent the proportion of "large" cells from individual rats and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.
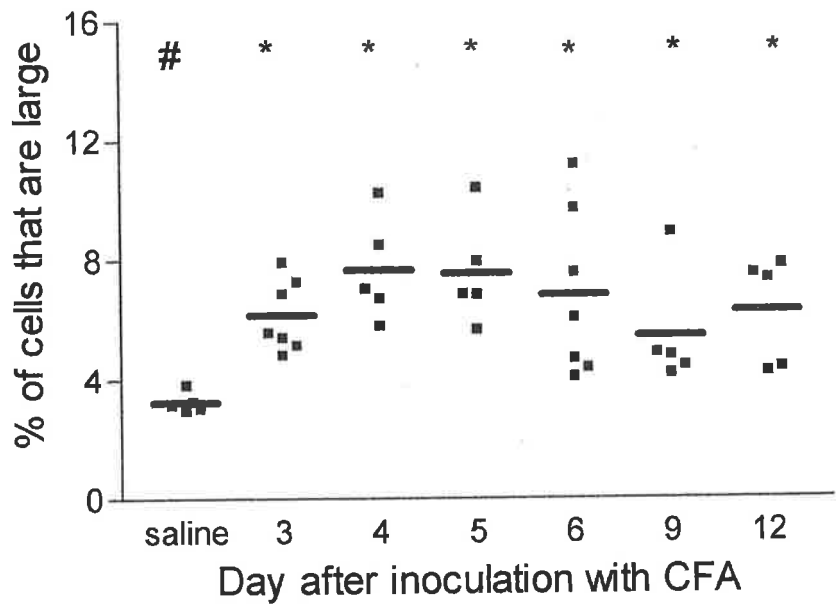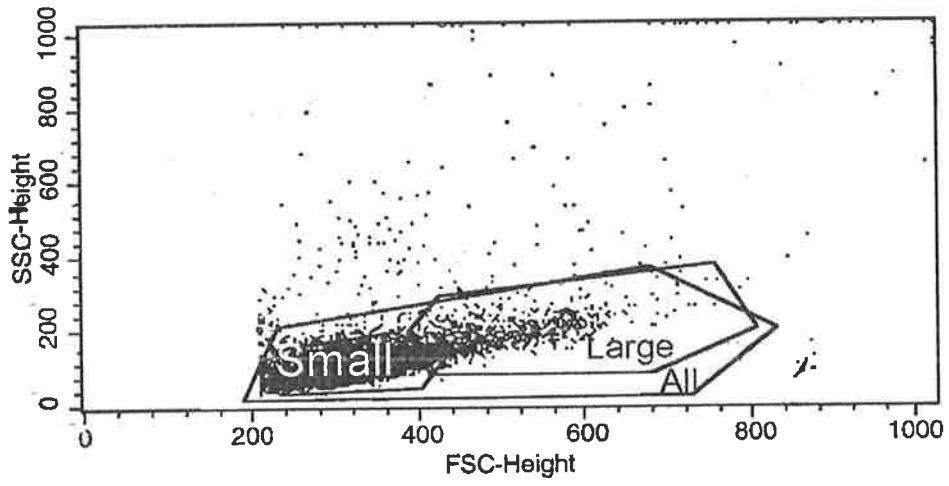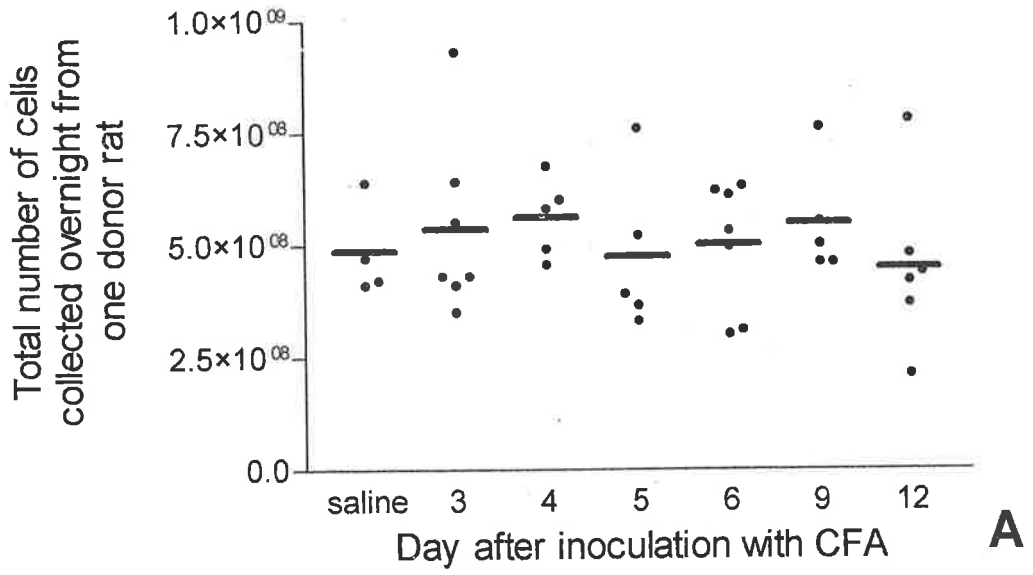
A — Total number of cells collected overnight from one donor rat vs. Day after inoculation with CFA (saline, 3, 4, 5, 6, 9, 12)

B — SSC-Height vs. FSC-Height; gates labeled Small, Large, All

C — % of cells that are large vs. Day after inoculation with CFA (saline, 3, 4, 5, 6, 9, 12); # and * markers

Figure 5.2.2.2 The CD4$^+$ T cell population in thoracic duct lymph during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb W3/25 (anti-CD4; FITC), followed by direct labelling with mAb R73 (anti-α/β TCR; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The proportions of "small" (D), "large" (E) or all (F) TD lymphocytes that expressed CD4 at different times after inoculation of CFA were calculated using data generated by flow cytometric analysis. In each case, a triangle indicates the value obtained from analysing TD cells from an individual rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.
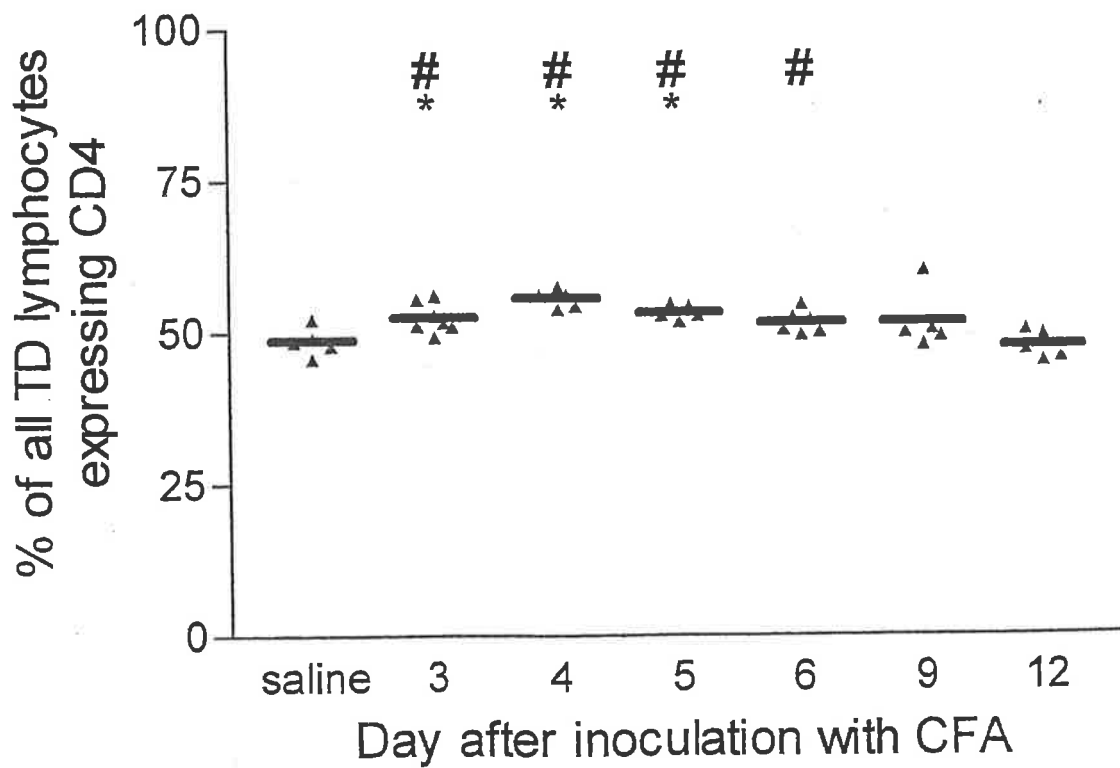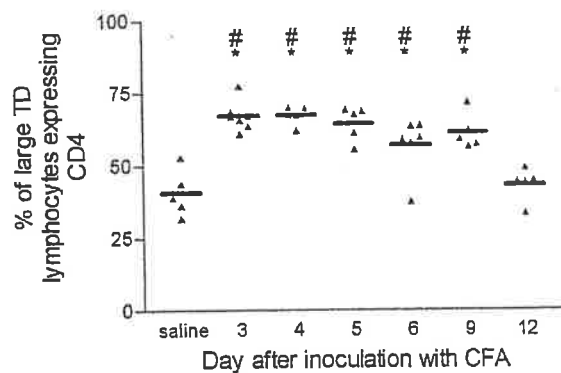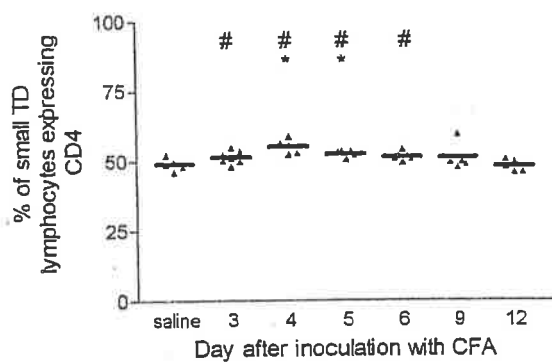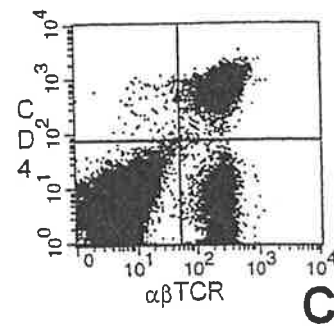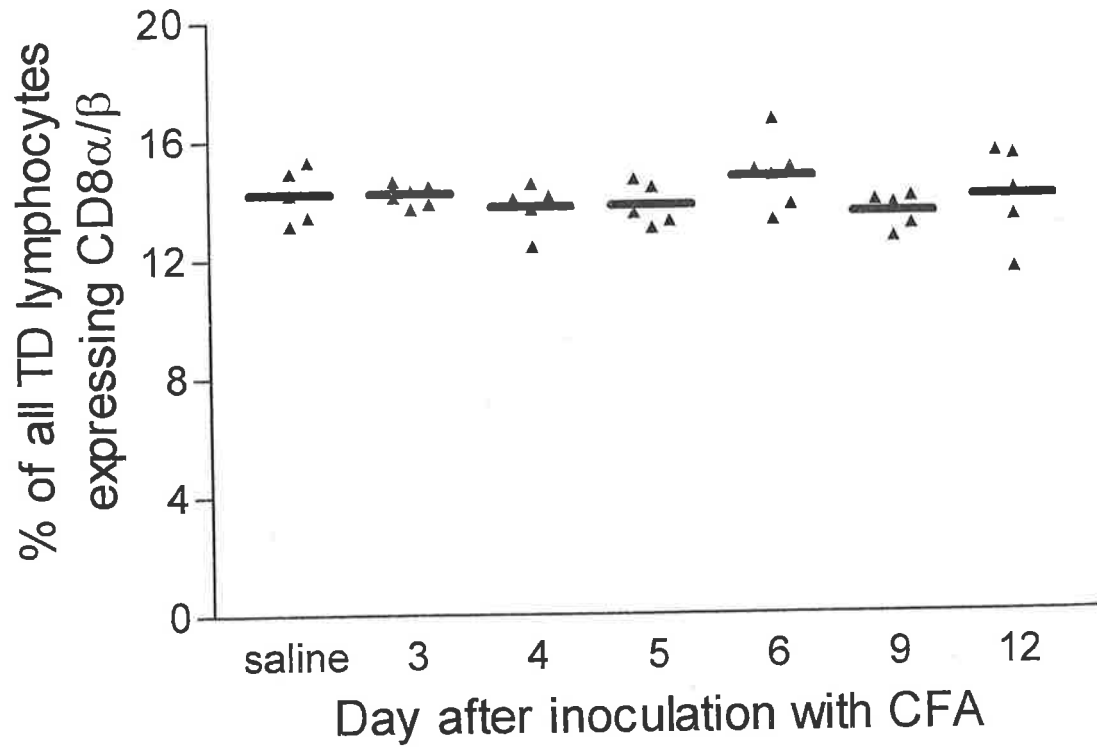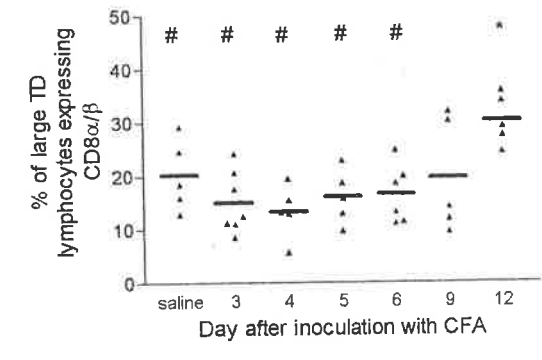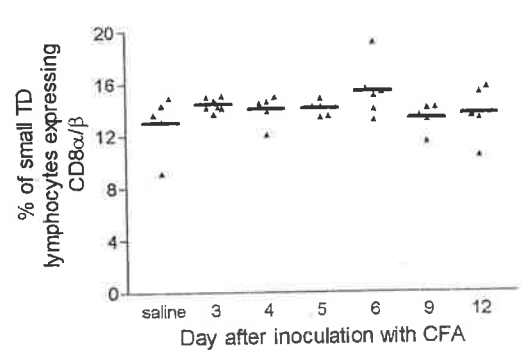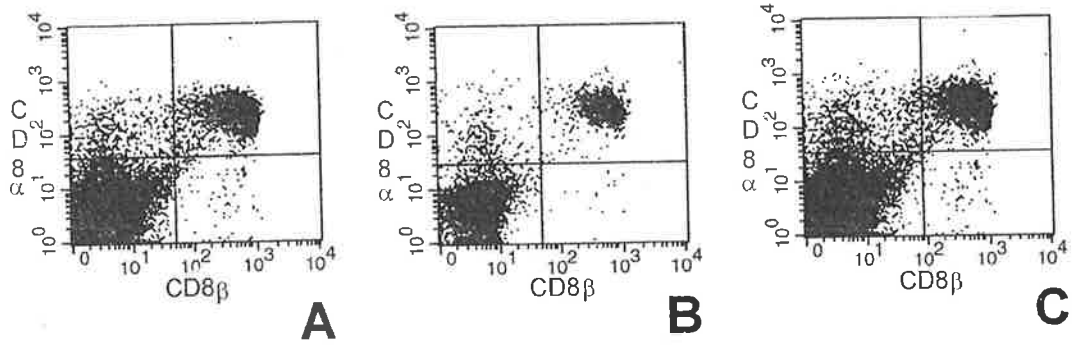
Figure 5.2.2.3 The CD8⁺ T cell population in thoracic duct lymph during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb 341 (anti-CD8β; FITC), followed by direct labelling with mAb OX8 (anti-CD8α; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The proportions of "small" (D), "large" (E) or all (F) TD lymphocytes that expressed both CD8α and CD8β at different times after inoculation of CFA were calculated using data generated by flow cytometric analysis. In each case, a triangle indicates the value obtained from analysing TD cells from an individual rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.

5.2.2.4 γ/δ T cells

T cells expressing γ/δTCR (represented in the upper right quadrants of scatter plots shown in Figures 5.2.2.4 A-C) were a minority population of approximately 1% of TD lymphocytes from control and CFA inoculated donors. Most or all of these cells expressed the CD8α chain. A small increase in the proportion of small cells expressing γ/δTCR (Figure 5.2.2.4 A and D) was seen at day 3 after inoculation of CFA. No significant differences were observed in the proportion of γ/δT cells amongst the large TD lymphocytes following inoculation with CFA (Figure 5.2.2.4 B and E). However, at 12 days after inoculation with CFA, the TD lymph contained fewer γ/δT cells as a proportion of total TD lymphocytes than was observed at other times post-inoculation, except at day 5 (Figure 5.2.2.4 F).

5.2.2.5 Non-α/β T cells

The increase in the proportion of CD4$^+$ T cells in the TD lymph following inoculation with CFA (Figure 5.2.2.2) was accompanied by a corresponding decrease in cells that did not express α/βTCR (γ/δ T cells, B cells) (Figure 5.2.2.5).
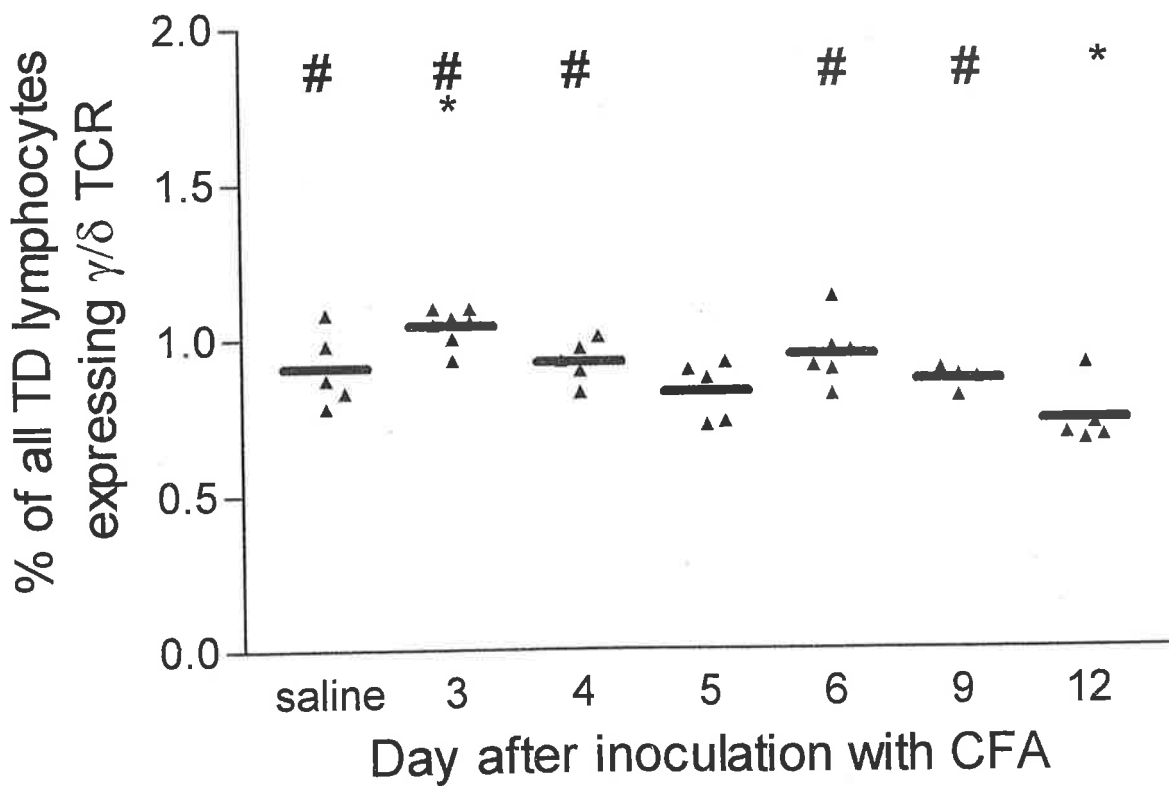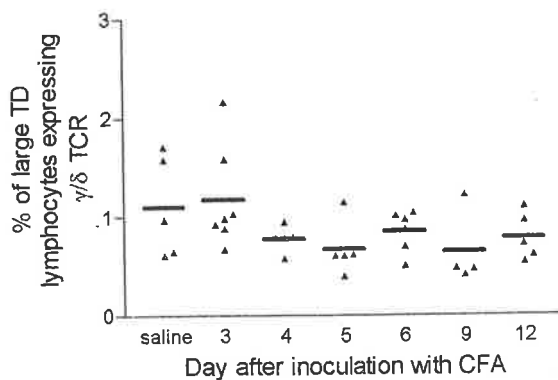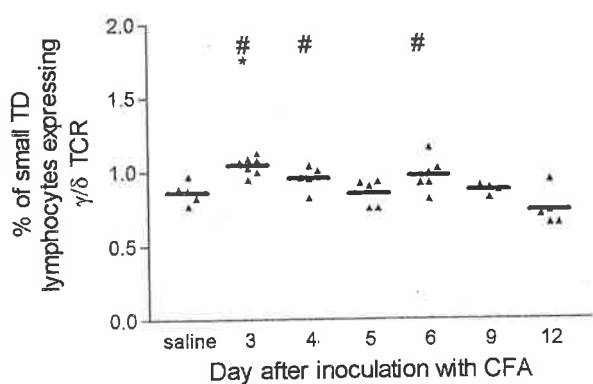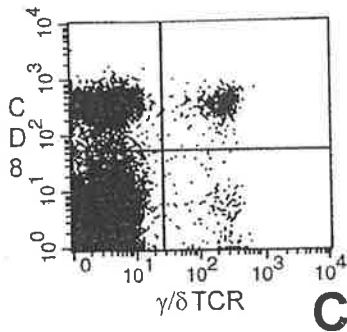
5.2.3 Expression of activation markers by CD4$^+$ T cells in the TD lymph during the prodrome of AA

It has been demonstrated previously that arthritogenic cells in the TD lymph collected from rats 9 days after inoculation are contained within the population of CD4$^+$ T cells that also expresses combinations of MHC class II, CD25, CD71 and CD134 (Spargo et al., 2001). Dual fluorochrome flow cytometric analysis was used, therefore, to examine the expression of these markers of activation by CD4$^+$ T cells throughout the prodrome of AA. In addition to examining markers of activation, molecules that could be important in facilitating the entry of cells into tissues were also investigated (see Section 5.2.4).

5.2.3.1 MHC class II

An increase in the mean proportion of CD4$^+$ T cells expressing the activation marker MHC class II (Figure 5.2.3.1 F) was observed in the TD lymph within three days after inoculation of CFA. Approximately 8% of CD4$^+$ T cells in TD lymph from control rats expressed MHC

Figure 5.2.2.4 The γ/δT cell population in thoracic duct lymph during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats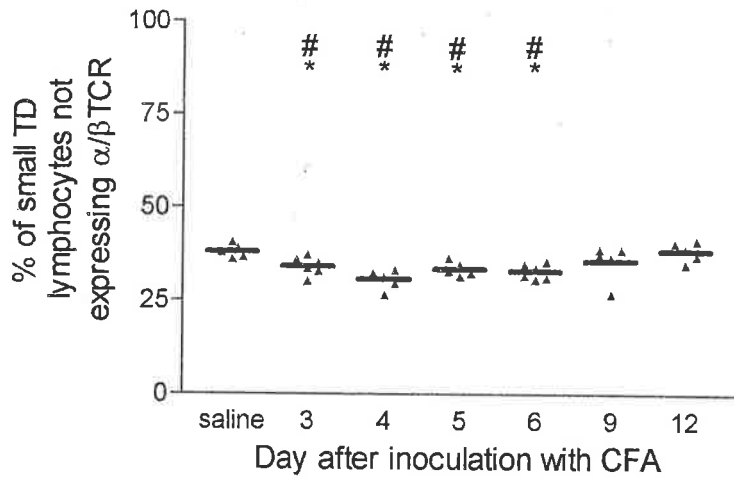 were first labelled indirectly with mAb V65 (anti-γ/δ TCR; FITC), followed by direct labelling with mAb OX8 (anti-CD8α; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The proportions of "small" (D), "large" (E) or all (F) TD lymphocytes that expressed γ/δ TCR at different times after inoculation of CFA were calculated using data generated by flow cytometric analysis. In each case, a triangle indicates the value obtained from analysing TD cells from an individual rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.

A — CD8 / γ/δ TCR

B — CD8 / γ/δ TCR

C — CD8 / γ/δ TCR

D — % of small TD lymphocytes expressing γ/δ TCR vs. Day after inoculation with CFA

E — % of large TD lymphocytes expressing γ/δ TCR vs. Day after inoculation with CFA

F — % of all TD lymphocytes expressing γ/δ TCR vs. Day after inoculation with CFA

Figure 5.2.2.5 The non–α/β T cell population in thoracic duct lymph during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb W3/25 (anti-CD4; FITC), followed by direct labelling with mAb R73 (anti-$\alpha\beta$ TCR; PE). The proportions of "small" (A), "large" (B) and all (C) TD lymphocytes that did not express the α/β TCR at different times after inoculation of CFA were calculated using data generated by flow cytometric analysis. In each case, a triangle indicates the value obtained from analysing TD cells from an individual rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.
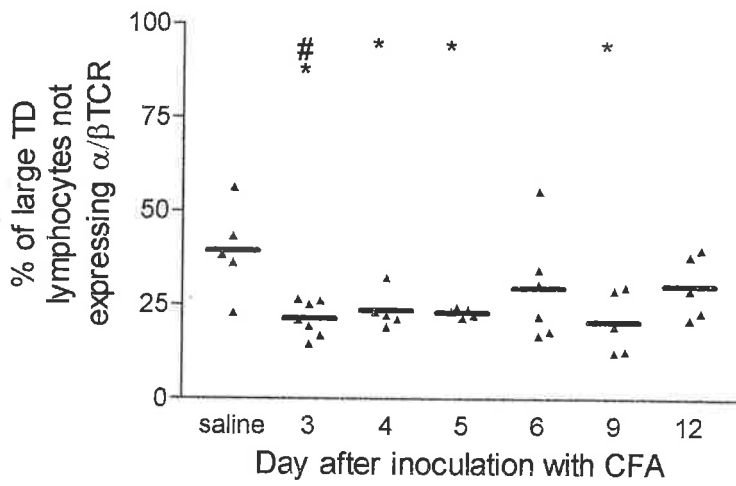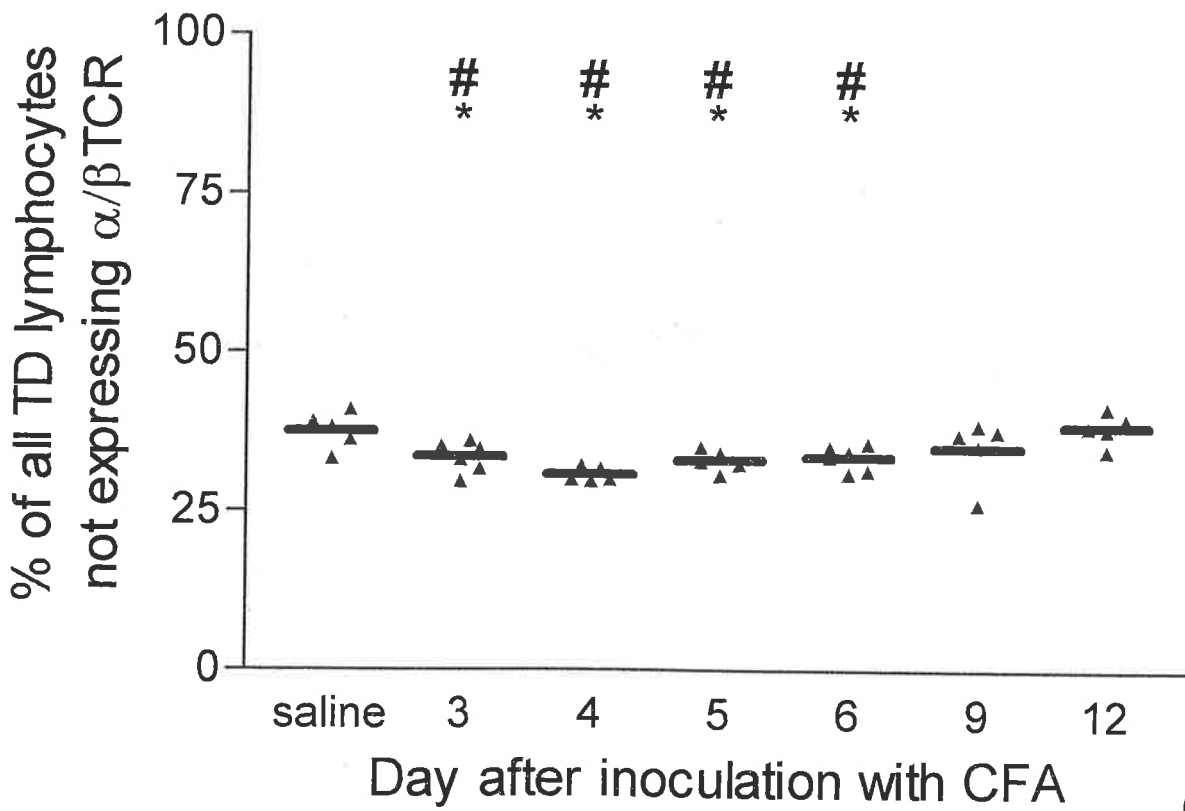
A

B

C

% of small TD lymphocytes not expressing α/βTCR

% of large TD lymphocytes not expressing α/βTCR

% of all TD lymphocytes not expressing α/βTCR

Day after inoculation with CFA

Figure 5.2.3.1 Expression of MHC class II by CD4$^+$ T cells during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb OX6 (anti-MHC class II; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The proportion of "small" (D), "large" (E) and all (F) TD lymph CD4$^+$ T lymphocytes that expressed MHC class II at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.
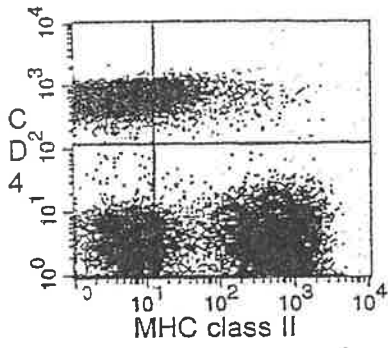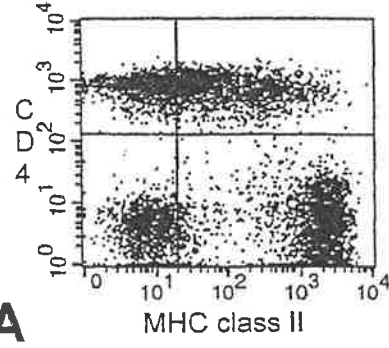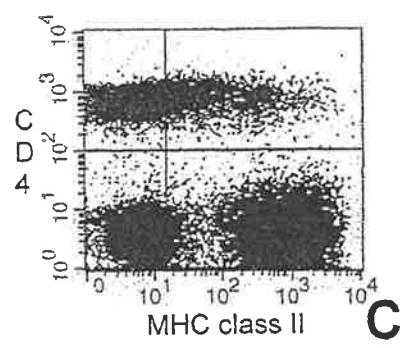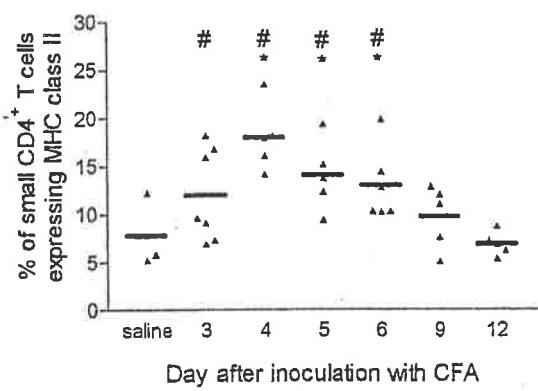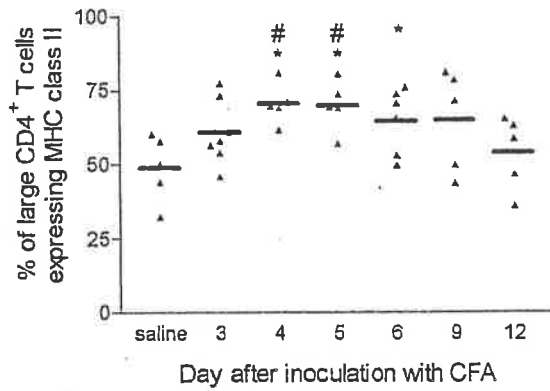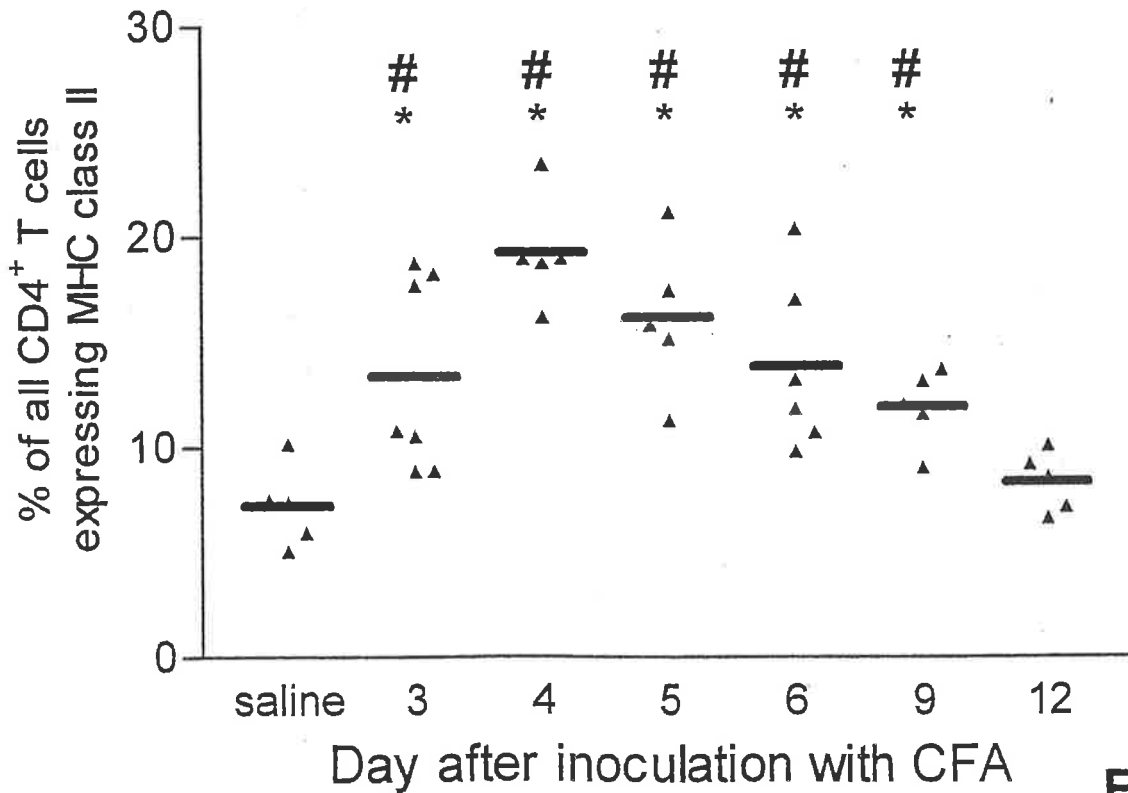
class II, whereas 15% expressed MHC class II in TD lymph collected three days after inoculation with CFA. However, the proportion of MHC class II$^+$ cells in the CD4$^+$ subset ranged from approximately 10% to 20% at this time point. The proportions of CD4$^+$ T cells expressing MHC class II increased further to approximately 21% at day 4, then decreased. However, this subset of CD4$^+$ cells remained elevated in comparison with the control group until 12 days after inoculation with CFA. The proportion of large CD4$^+$ T cells expressing MHC class II on days 4, 5 and 6 exceeded that of the controls but not on day 9 (Figure 5.2.3.1 E). However, the mean value on day 9 was slightly greater than day 6 with failure to achieve significance, possibly attributable to greater variance on day 9. When it is considered that the total output of large lymphocytes is increased after inoculation with CFA, the output of MHC class II$^+$ CD4$^+$ T cells was, clearly, greater at 9 days post-inoculation than in control rats (not shown). Some of the CD4$^+$ T cells included in the small cell gate expressed MHC class II (Figure 5.2.3.1 D).

5.2.3.2 IL-2 receptor α chain (CD25)

When the proportions of CD4$^+$ T cells that expressed the IL-2R α chain (CD25) were examined, a trend was observed that was similar to the MHC class II$^+$ subset. By day 3 after inoculation of CFA, the proportion of CD4$^+$ T cells expressing CD25 had increased from ~5% to ~12%, and these levels remained higher than those observed in either the control group or the day 12 post-inoculation group (Figure 5.2.3.2 F). CD25$^+$ cells were present in greater proportions in the large CD4$^+$ T cells but some CD25$^+$ cells were also observed in the small cell gate.

5.2.3.3 Transferrin receptor (CD71)

The expression of the transferrin receptor (CD71) is associated with cells that have entered the cell cycle (Salmeron et al., 1995). It can, therefore, be considered a marker of activation. Increased proportions of CD4$^+$ T cells expressing CD71 were detected in the TD lymph of rats during the prodrome of AA (Figure 5.2.3.3 F). Between 2% and 4% of CD4$^+$ T cells in the TD lymph from normal rats expressed CD71. Within three days following inoculation with CFA, this proportion increased to approximately 11%. The greatest proportion of CD4$^+$ T lymphocytes expressing CD71 was observed at 3 days after inoculation and although the

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Figure 5.2.3.2 Expression of IL-2 receptor by CD4$^+$ T cells during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb OX39 (anti-IL-2Rα [CD25]; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The proportion of "small" (D), "large" (E) and all (F) TD lymph CD4$^+$ T lymphocytes that expressed CD25 at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.

Figure 5.2.3.3 Expression of Transferrin receptor by CD4$^+$ T cells during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb OX26 (anti-transferrin R (CD71); FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The proportion of "small" (D), "large" (E) and all (F) TD lymph CD4$^+$ T lymphocytes that expressed transferrin R at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.

proportion decreased at later times, it remained significantly different from the control group at all time points examined. The highest proportion of cells expressing CD71 was seen in the large cell fraction but up to 7% of cells in the small cell gate expressed the molecule.

5.2.3.4 OX40 antigen (CD134)

OX40 antigen (CD134) is thought to be a marker of recent activation (Stuber and Strober 1996) and it is expressed only on activated CD4^{+} T cells (Paterson et al., 1987; Mallett et al., 1990). CD134 was expressed by between 2-4% of all CD4^{+} T cells in the TD lymph from control rats (Figure 5.2.3.4 F). Following inoculation with CFA, the proportion increased to 6-12% within three days, and remained elevated until at least day 12. As observed with other activation markers, the greatest proportion of CD134^{+} cells was found in the large cell fraction but again, some cells gated as small also expressed the molecule.

5.2.3.5 UA002 antigen

A similar pattern was observed when examining the percentage of CD4^{+} T cells that express a molecule recognised by mAb UA002, an antibody produced in the Department of Microbiology and Immunology, University of Adelaide. The antigenic specificity of this mAb UA002 has not yet been determined. However, it detects an antigen that is expressed by T cells that have been activated with Concanavalin A, B lymphocytes, macrophages and dendritic cells (possibly CD39). The proportion of TD lymph CD4^{+} T cells that expressed the UA002 antigen during the early stages of AA is shown in Figure 5.2.3.5. UA002 antigen was expressed by a greater proportion of CD4^{+} T cells in the TD lymph of rats inoculated with CFA compared to saline-challenged rats. When examining the CD4^{+} T cell population as a whole, the greatest proportion of CD4^{+} UA002 antigen^{+} cells were observed between days 3 and 5 post-inoculation of CFA and the frequency of these cells decreased thereafter.

5.2.3.6.1.1 CD45RC

The expression of a high molecular weight isoform of CD45 (CD45RC in the rat) by CD4^{+} T cells correlated with a "naïve" phenotype (Luqman and Bottomly, 1992). However, so-called "memory" cells that are CD45RC^{-} have been observed to revert to a CD45RC^{+} "naïve" phenotype (Bunce and Bell, 1997). There was no significant difference, relative to controls,
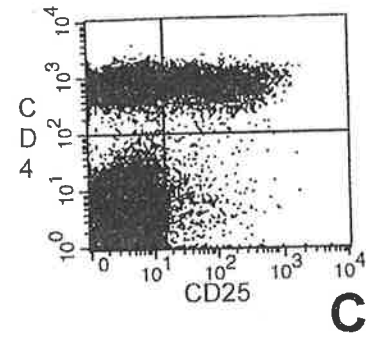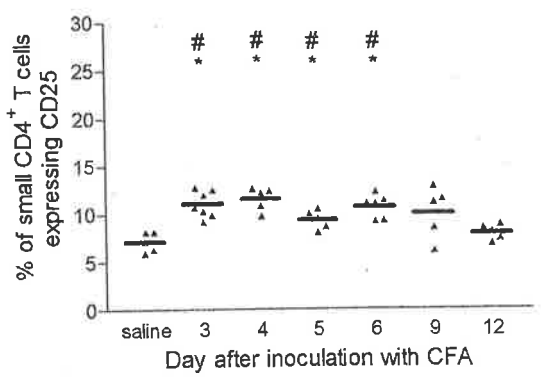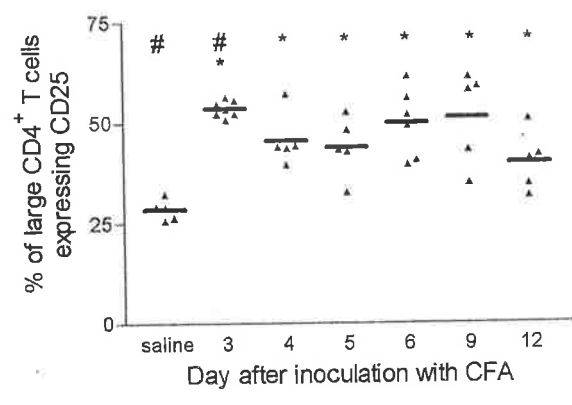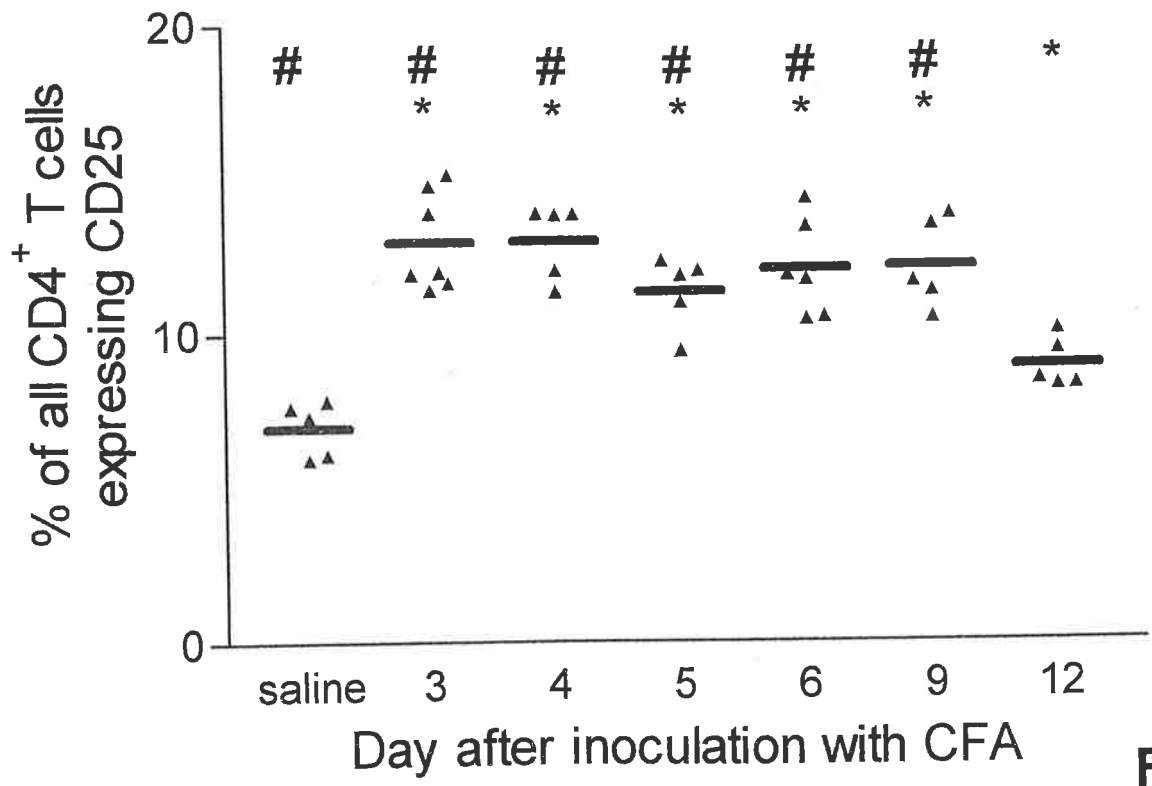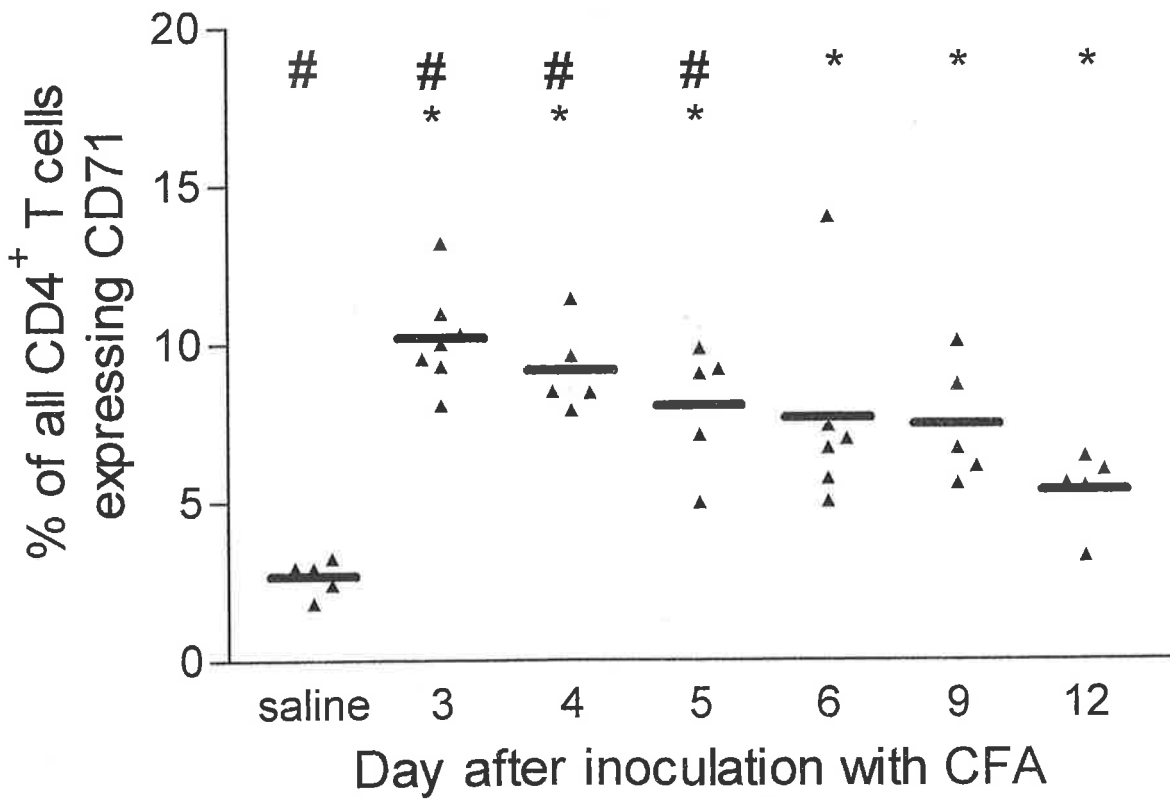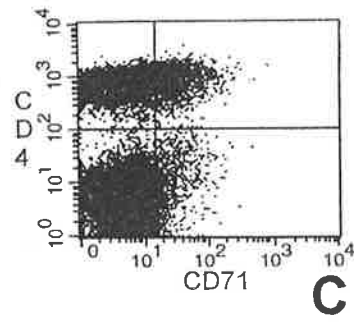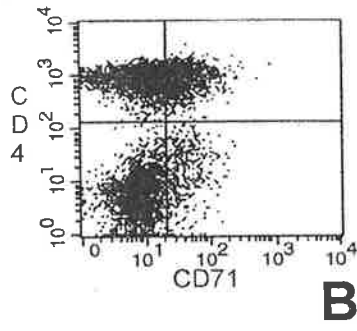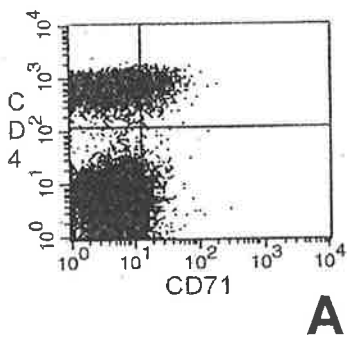
Figure 5.2.3.4 Expression of OX40 antigen by CD4$^+$ T cells during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb OX40 (anti-OX40 antigen [CD134]; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The proportion of "small" (D), "large" (E) and all (F) TD lymph CD4$^+$ T lymphocytes that expressed CD134 at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.

A   B   C

D   E

F

Figure 5.2.3.5 Expression of UA002 antigen by CD4$^+$ T cells during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb UA002 (anti-UA002Ag; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The proportion of "small" (D), "large" (E) and all (F) TD lymph CD4$^+$ T lymphocytes that expressed UA002Ag at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.
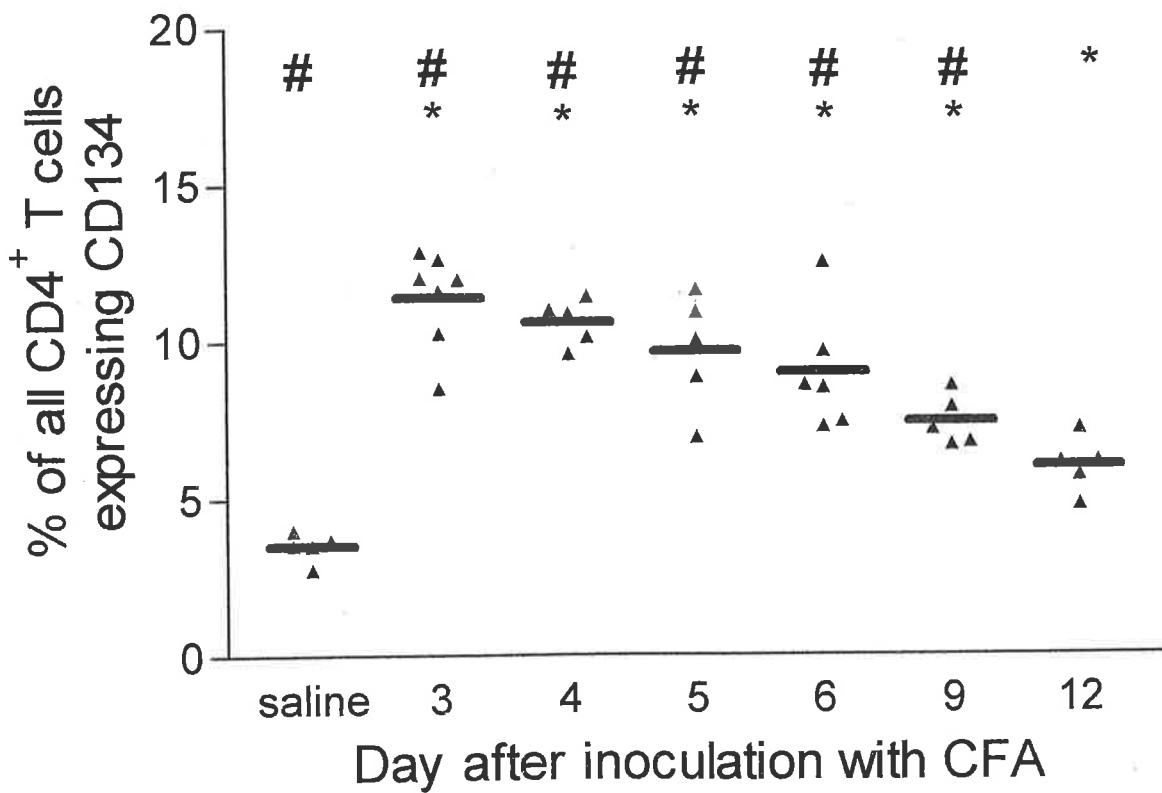
in the percentages of TD lymph CD4$^+$ T cells expressing CD45RC following inoculation with CFA (Figure 5.2.3.6.1 F). However, when examining the large cells separately, inoculation with CFA was followed by a decrease in the proportion of large CD4$^+$ T cells expressing CD45RC as compared to cells from the control group (Figure 5.2.3.6.1 E).

5.2.3.6.2 Level of expression of CD45RC

In large CD4$^+$ T cells, the mean fluorescence of the FITC signal generated by staining CD45RC with mAb OX22 did not change significantly after inoculation with CFA (Figure 5.2.3.6.2 B), indicating that these cells expressed levels of CD45RC similar to their counterparts from normal donors. Surprisingly, the mean fluorescence increased among small CD4$^+$ T cells by day 4 compared with small CD4$^+$ T cells in TD lymph from normal rats and persisted throughout the period of observation (Figure 5.2.3.6.2 A). This was reflected by the mean fluorescence of all CD4$^+$ T cells (Figure 5.2.3.6.2 C). Together with the data in Figure 5.2.3.6.1 D, this suggests that during the prodrome of AA there is preferential retention of CD45RC$^-$ cells and CD45RC^{lo} cells, with continued recirculation of CD45RC$^+$ cells.

5.2.4 Comparison of the expression of adhesion molecules by CD4$^+$ T cells isolated from the TD lymph of rats during the prodrome of AA

The extravasation of circulating cells into tissues involves the presence of surface molecules that facilitate this process. The molecules that are engaged in the adhesion of cells to other cells or to extracellular matrix are known collectively as "adhesion molecules". The initial step involved in extravasation is rolling and tethering, which is promoted by the interaction of the selectin family of molecules with their ligands (Springer, 1994). The second (but overlapping) step is adhesion, which is mediated in part by the interaction between integrins and their ligands. The expression of particular members of the integrin family can determine the migratory preference of cells. Most T cells isolated from the synovial tissue of patients with RA have increased expression of ICAM-1, CD44, $\alpha_4\beta_7$ (reviewed in Szekanecz et al., 1996) and the very late antigen (VLA) family (β1) integrins (Garcia-Vicuna et al., 1992).

In discussing the changes in adhesion molecules expressed by CD4$^+$ T cells in TD lymph during the prodrome of AA, it is worth noting that the division of the cells into "small" and

Figure 5.2.3.6.1 Expression of CD45RC by CD4$^+$ T cells during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb OX22 (anti- CD45RC; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The proportion of "small" (D), "large" (E) and all (F) TD lymph CD4$^+$ T lymphocytes that expressed CD45RC at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.
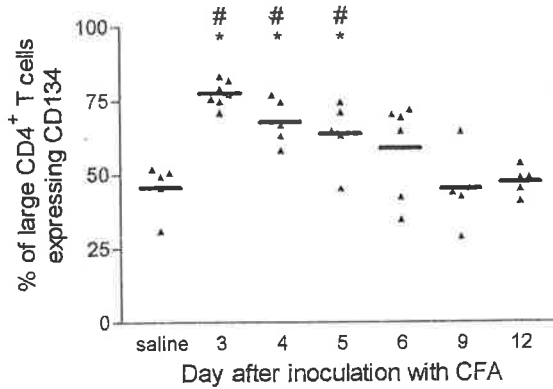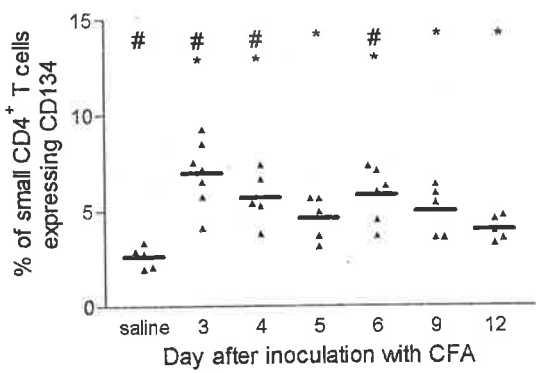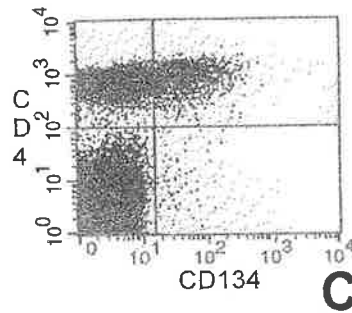
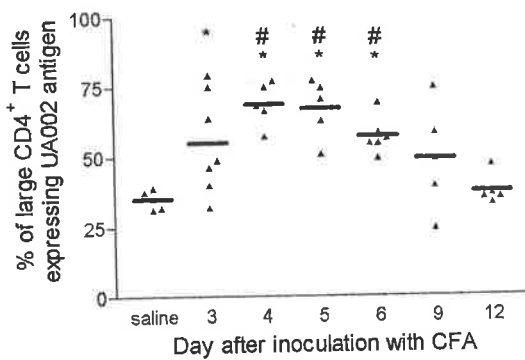Panel A, B, C: CD4 versus CD45RC flow cytometry dot plots.

D

E

F

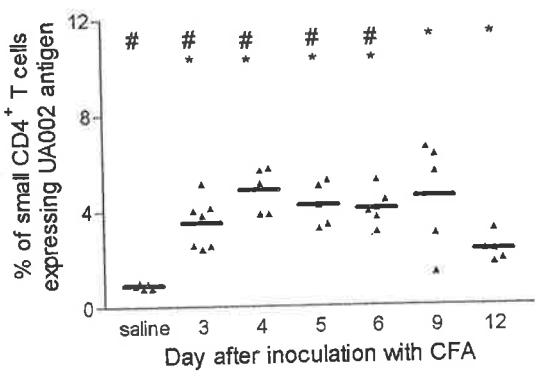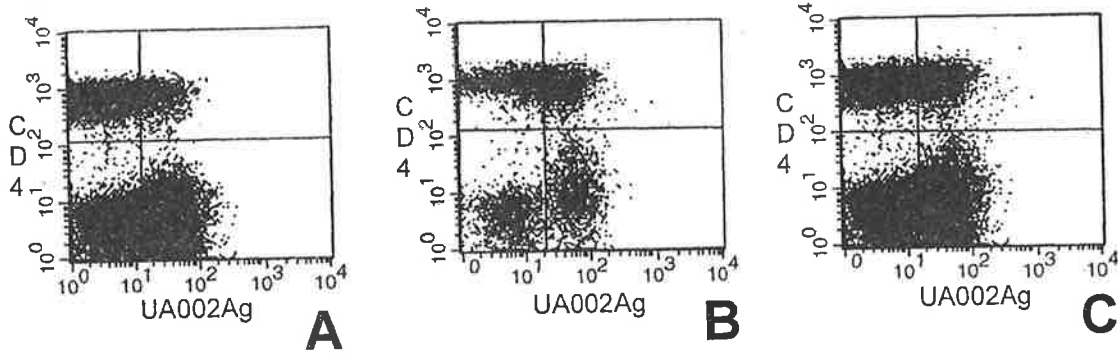**Figure 5.2.3.6.2 Level of expression of CD45RC by CD4$^+$ T cells during the prodrome of adjuvant arthritis**. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb OX22 (anti- CD45RC; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). The mean value of the fluorescent signal generated by the FITC-labelled antibody binding to CD45RC of "small" (D), "large" (E) and all (F) TD lymph CD4$^+$ T lymphocytes at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.

A

B

C
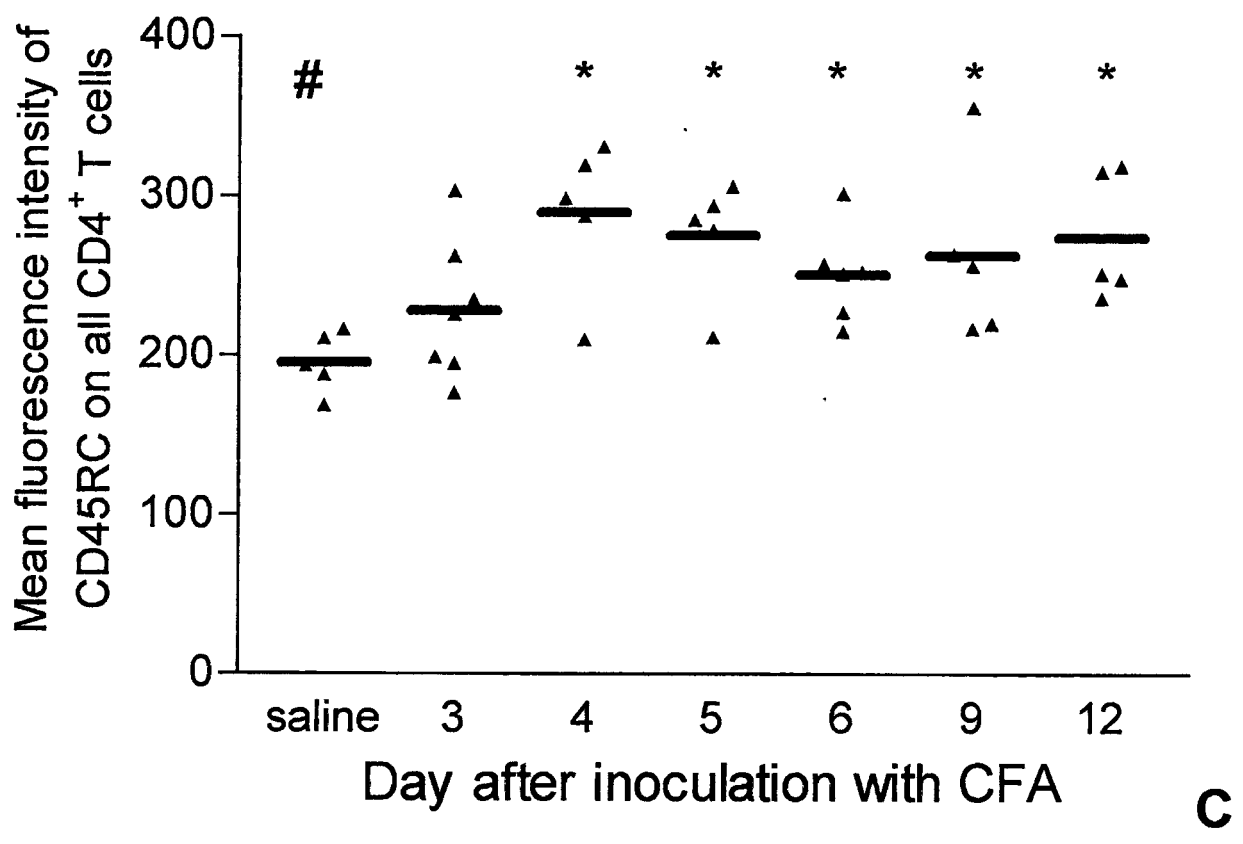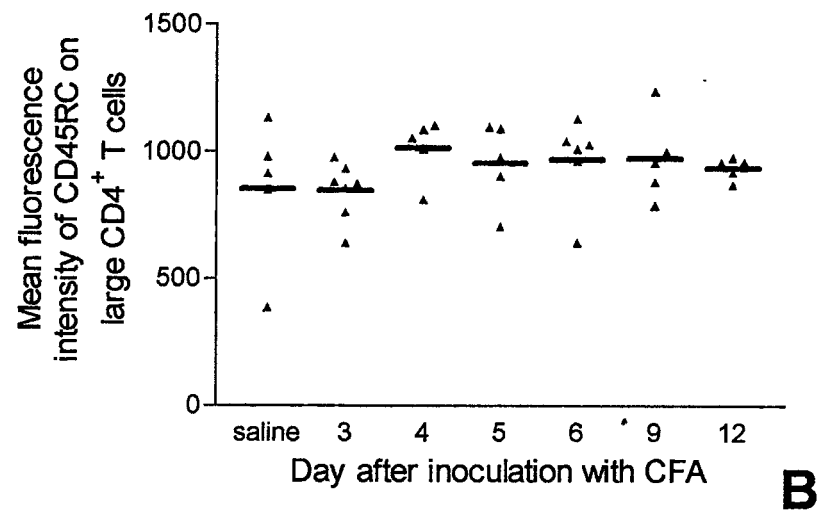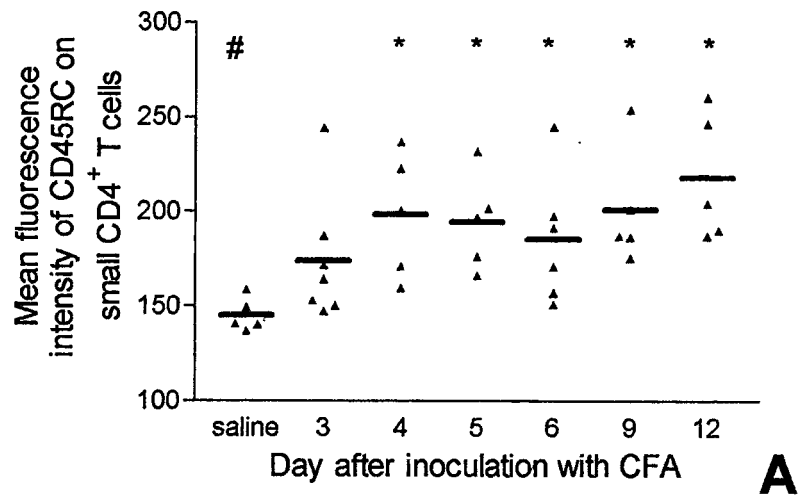
"large" is arbitrary. Expression of activation markers such as MHC class II and CD25 was not confined to cells in the "large" gate. Therefore, while changes in adhesion molecules expressed by "large" cells reflects the phenotype of recently divided lymphocytes, it is not the case that all "small" cells are resting lymphocytes. In the interpretation of changes affecting "small" cells, some changes may be due to the entry of newly-formed cells into the TD lymph but large effects are likely to be due to transient changes in the relative rates of recirculation of different subpopulations within the recirculating pool of small lymphocytes.

5.2.4.1 L-Selectin (CD62L)

L-Selectin (CD62L) is important for the tethering of lymphocytes to endothelium. In particular, it is important for the transendothelial migration of naive lymphocytes through high endothelial venules (HEV) in peripheral lymph nodes and to a lesser degree, in Peyer's patches (Tedder et al., 1995). The proportion of TD lymph CD4$^+$ T cells expressing L-Selectin was increased after inoculation with CFA, and this increase reached significance on days 3, 6 and 12 post-inoculation (Figure 5.2.4.1 F). The same pattern was seen in the small lymphocyte population, suggesting preferential recirculation of naive CD4$^+$ T cells during the prodrome and early phase of AA. This conclusion is supported by the observations on expression of CD45RC (Section 5.2.3.6.2).

The early rise in the proportion of CD62L$^+$ large CD4$^+$ T cells after inoculation of CFA suggests the activation of a new cohort of cells and that these cells down regulate CD62L as they mature. It is interesting that the decline in expression of CD62L, commencing on day 4 post-inoculation, coincides with the earliest appearance of arthritogenic T cells in the TD lymph. The proportion of CD62L$^+$ large cells fell below that in normal donors on day 9 post-inoculation.

5.2.4.2 P-Selectin ligand (CD162)

Although many lymphocytes express a ligand for P-selectin in a non-functional form, activated T cells express a functional form that is thought to differ in glycosylation and/or tyrosine sulphation that allows binding to P-selectin (Vachino et al., 1995). Austrup and co-workers (1997) examined the expression of a functional ligand for P-selectin on T cell lines
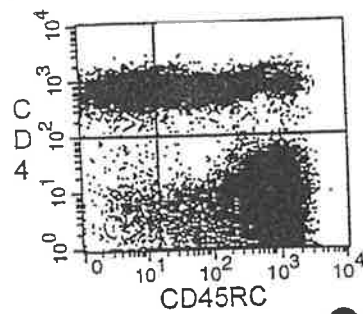
130

Figure 5.2.4.1 Expression of L-selectin by CD4$^+$ T cells during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb OX85 (anti-L-selectin (CD62L); FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The proportion of "small" (D), "large" (E) and all (F) TD lymph CD4$^+$ T lymphocytes that expressed CD62L at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.
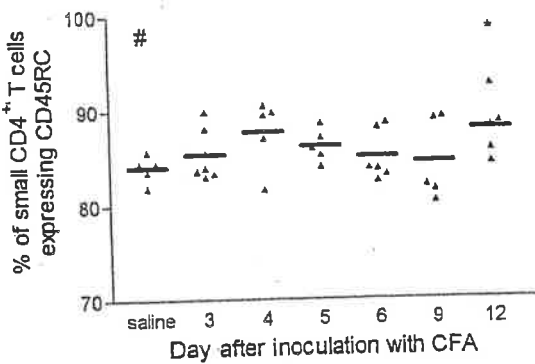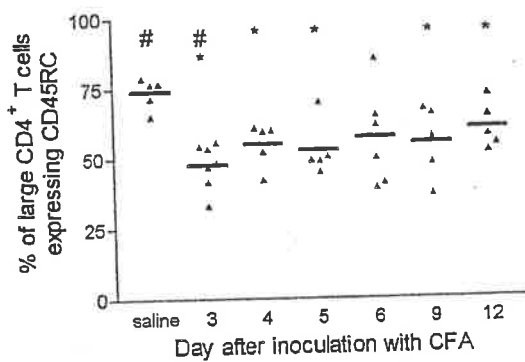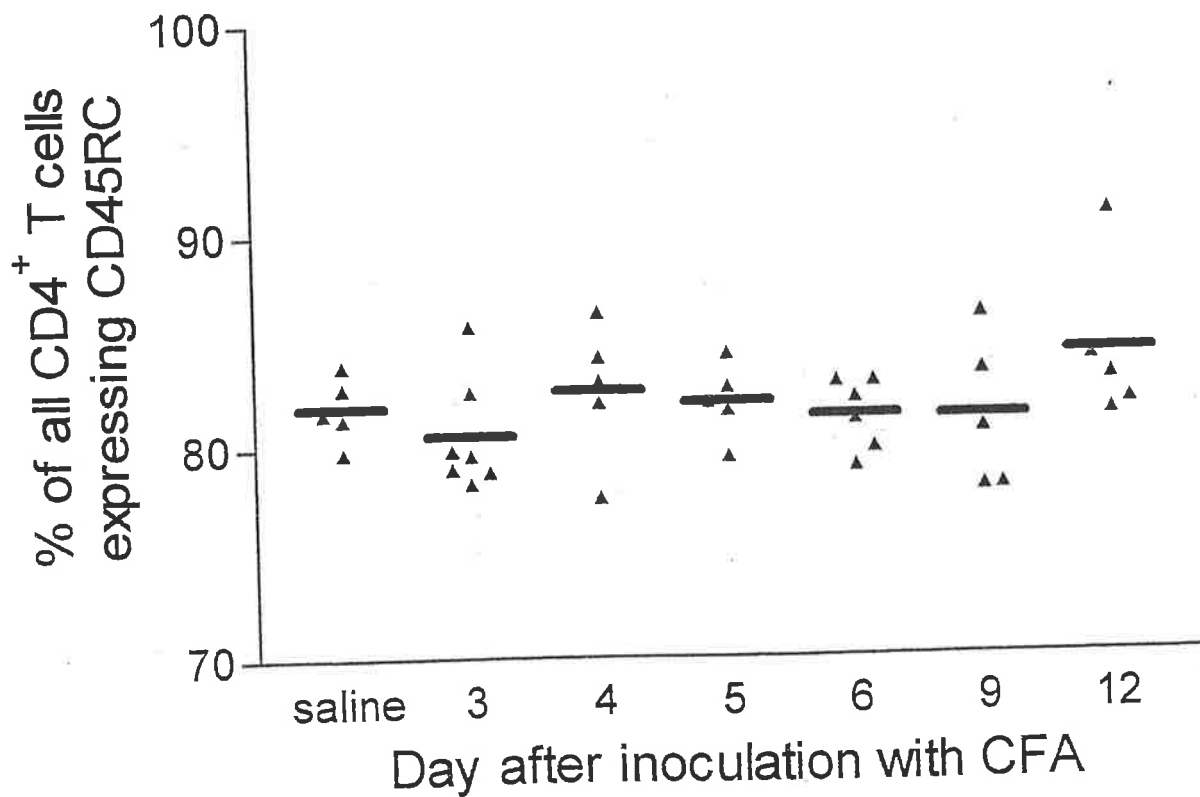
that had been stimulated *in vitro*. They found that functional P-selectin ligand (CD162) was present, almost exclusively, on IFN-γ-producing T cells whereas expression was absent on IL-4-producing cells. The IFN-γ-producing cells were found to migrate into inflamed tissues under going a DTH reaction more effectively than IL-4-producing cells and the migration could be inhibited by the blockade of P-selectin. In addition to its potential value as a surface marker for Th1 cells, the expression of CD162 may provide cells with the capacity to enter inflamed tissue since endothelial cells up-regulate the expression of P-selectin upon activation (Tedder et al., 1995).

To investigate changes in the expression of functional CD162 on circulating CD4$^+$ T cells during the development of AA, TD lymphocytes were labelled indirectly with CD62P:human Ig Fc fusion protein and an anti-human Ig:FITC secondary antibody. A PE-conjugated anti-CD4 antibody was used to identify CD4$^+$ T cells (Figure 5.2.4.2 A-C). The CD62P fusion protein was only available to study the cells obtained from groups of rats cannulated on days 4 or 5 after inoculation and 1 rat at day 6. There was no significant change in expression of functional CD162 by small CD4$^+$ T cells. However, there was a significant rise in the proportion of CD162-expressing large CD4$^+$ T cells by day 4 post-inoculation. It appears that the decline in expression of CD62L by the recently activated cells coincides with up-regulation of the ligand for CD62P.

5.2.4.3 α_{E2} integrin

An increase in the proportion of CD4$^+$ T cells expressing α_{E2} integrin (Figure 5.2.4.3 D and F) was observed after inoculation of CFA. Approximately 2% of CD4$^+$ T cells isolated from the TD lymph of control rats expressed α_{E2} integrin. Within three days after challenge with CFA, the proportion of CD4$^+$ T cells expressing this molecule had increased to ~5% and remained elevated until at least day 12. This increase was significant statistically at all times after inoculation. Interestingly, the proportion of large CD4$^+$ T cells that express α_{E2} integrin was lower at days 6, 9 and 12 after inoculation with CFA compared with control rats and with donors 3-5 days post-inoculation (Figure 5.2.4.3 E). This suggests the α_{E2}^+ small CD4$^+$ T cells recirculate preferentially during the prodrome and early clinical phase of AA, while the

Figure 5.2.4.2 Expression of functional P-selectin ligand by CD4$^+$ T cells during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with P-selectin:Ig fusion protein (FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The proportion of "small" (D), "large" (E) and all (F) TD lymph CD4$^+$ T lymphocytes that expressed functional ligand for P-selectin at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean value of the group.

A

B

C

Functional P-sel ligand (×3)
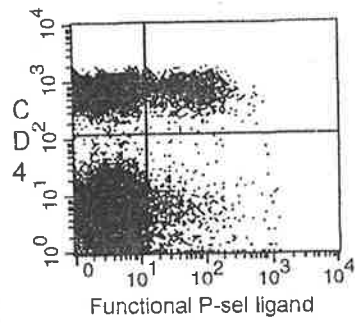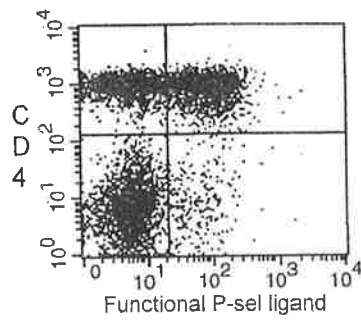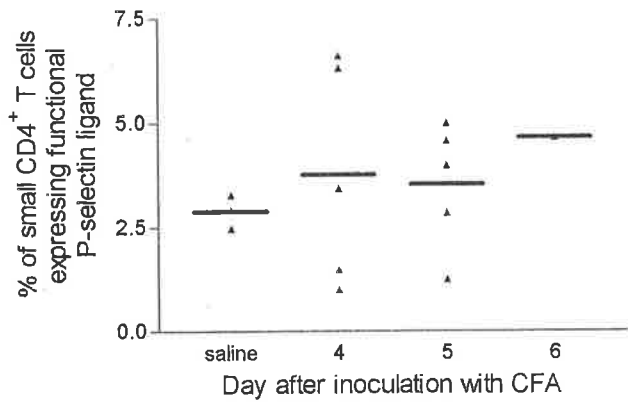
D
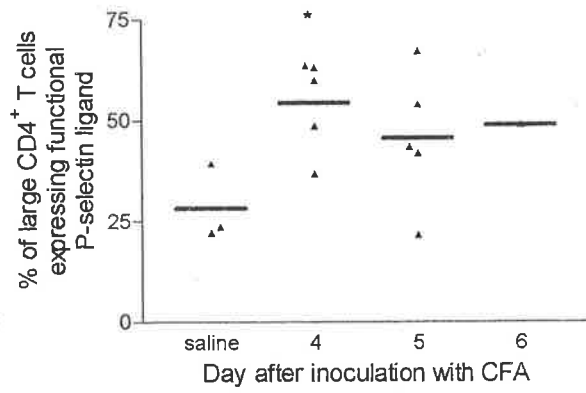
E
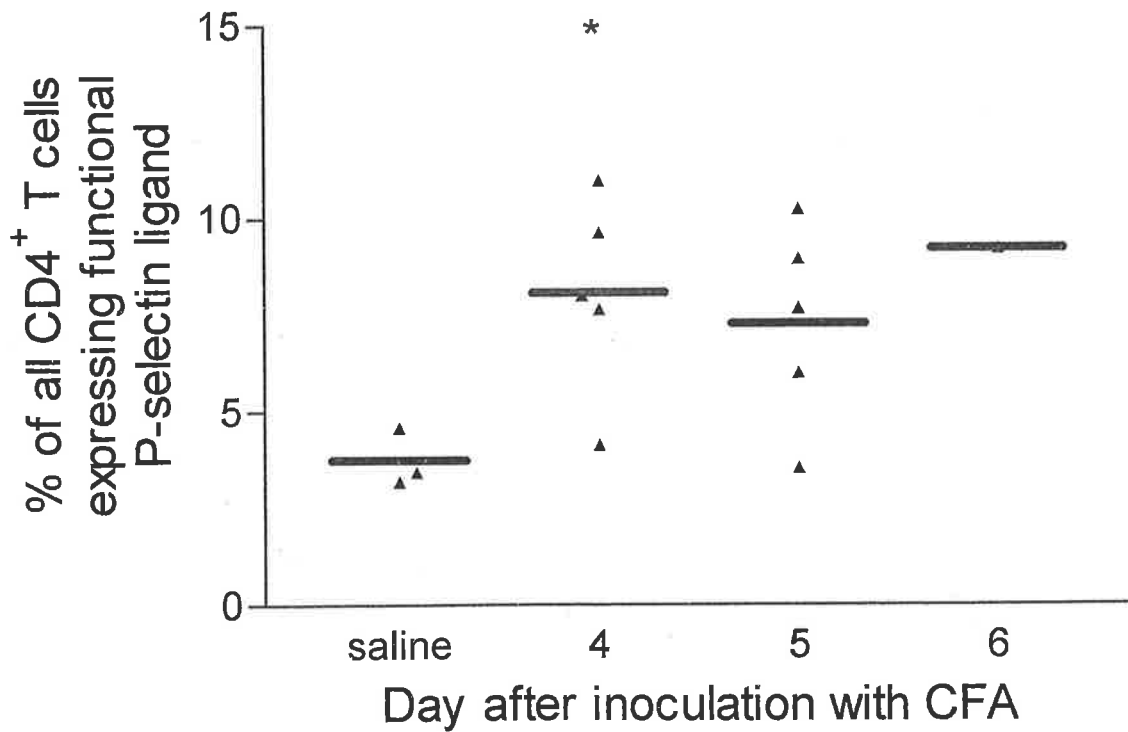
F

Figure 5.2.4.3 Expression of α_{E2} integrin by CD4$^+$ T cells during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb OX62 (anti-α_{E2} integrin; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The proportion of "small" (D), "large" (E) and all (F) TD lymph CD4$^+$ T lymphocytes that expressed α_{E2} integrin at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.

Figure with panels A, B, C (flow cytometry dot plots: CD4 vs alpha E integrin), panels D, E, and F (scatter plots).

Panel D:
% of small CD4+ T cells expressing αE2 integrin vs Day after inoculation with CFA

Panel E:
% of large CD4+ T cells expressing αE2 integrin vs Day after inoculation with CFA

Panel F:
% of all CD4+ T cells expressing αE2 integrin vs Day after inoculation with CFA

α_{E2}^+ large CD4$^+$ T cells found in TD lymph of control rats is diluted with a population of newly formed α_{E2}^- cells.

5.2.4.4 α_4 integrin (CD49d)

The frequency of CD4$^+$ T cells in TD lymph that expressed the α_4 integrin chain (CD49d) increased following inoculation of the rats with CFA (Figure 5.2.4.4B). This increase was significant on days 4 and 5 after inoculation. The mean proportion of CD4$^+$ T cells expressing α_4 integrin in TD lymph from control rats was approximately 20%, although in this group there was one much higher value and for most rats the proportion was less than 10%. There was also considerable rat to rat variation in the expression of α_4 integrin at all time points following inoculation with CFA (Figure 5.2.4.4 D-F). It should be noted that this antibody would detect both the $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins and that differential expression of these two molecules could vary during the course of AA.

5.2.4.5 ICAM-1 (CD54)

Less than 5% of small and approximately 60% of large CD4$^+$ T cells from the TD lymph of normal rats expressed ICAM-1 (CD54; Figure 5.2.4.5 D and E). The proportion of CD4$^+$ T cells (small and large) that express CD54 increased within 3 days after inoculation with CFA, and remained elevated throughout the prodromal period (Figures 5.2.4.5 D-F). There was a fall in the proportion of large CD4$^+$ cells that expressed CD54 between 9 days and 12 days after inoculation (Figure 5.2.4.5 E) and this was also reflected in the total CD4$^+$ population (Figure 5.2.4.5 F). This corresponds with the period of falling arthritogenicity of TD lymphocytes (Figure 5.2.1.1 A) and of decrease in the proportions of cells that express the activation markers MHC class II (Figure 5.2.3.1), CD25 (Figure 5.2.3.2), CD134 (Figure 5.2.3.4) and UA002 antigen (Figure 5.2.3.5).

5.2.4.6 LFA-1 (CD11a/CD18)

LFA-1, through its interaction with ICAM-1 on vascular endothelium, is involved in the adhesion/arrest step of the extravasation process by which leukocytes enter inflamed tissues. LFA-1 was expressed by essentially all CD4$^+$ T cells isolated from the TD lymph of normal rats and of rats during the prodrome of AA (representative plots of TD lymphocytes from a

Figure 5.2.4.4 Expression of α_4 integrin by CD4$^+$ T cells during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb MRα4-1 (anti-α_4 integrin [CD49d]; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The proportion of "small" (D), "large" (E) and all (F) TD lymph CD4$^+$ T lymphocytes that expressed CD49d at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.
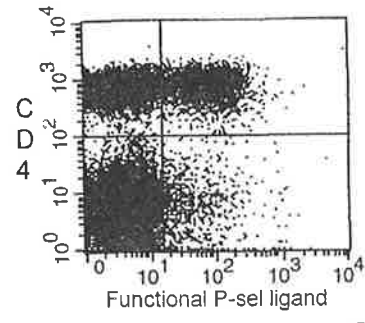
Figure 5.2.4.5 Expression of ICAM-1 by CD4$^+$ T cells during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb 1A29 (anti-ICAM-1 [CD54]; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The proportion of "small" (D), "large" (E) and all (F) TD lymph CD4$^+$ T lymphocytes that expressed CD54 at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean 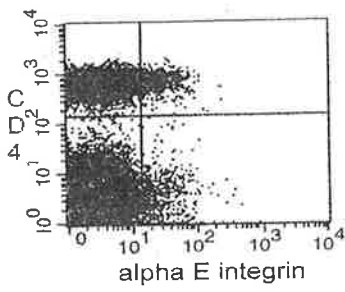value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.

A

B

C

D

E

F

rat cannulated 6 days after inoculation with CFA are shown in Figure 5.2.4.6 A-C). The level of expression (indicated by mean fluorescence intensity of cells labeled indirectly using antibodies against LFA-1) did not appear to change significantly at any time following challenge with CFA (Figure 5.2.4.6 D-F), although there was a trend towards reduced expression by large CD4$^+$ T cells.

5.2.4.7 Phagocytic glycoprotein 1 (CD44)

All TD lymph CD4$^+$ T cells expressed Phagocytic glycoprotein 1 (CD44) (representative plots of TD lymphocytes from a rat cannulated 6 days after inoculation with CFA are shown in Figure 5.2.4.7 A-C). The level of expression, as indicated by mean fluorescence intensity of cells labelled with mAb OX50, was increased between days 3 and 6 following inoculation with CFA (Figure 5.2.4.7 F).

5.2.5 Production of cytokines by thoracic duct cells during the prodrome of AA

Some of the effector functions of CD4$^+$ T cells are performed by cytokines. Two methods were employed to examine cytokine production. In the first, intracellular labelling of cytokines for flow cytometric analysis was used to examine specifically the cytokines produced by CD4$^+$ T cells (see Chapter 3). However, this technique requires a period of *in vitro* stimulation in order to accumulate cytokines in the intracellular compartments of freshly isolated cells (Schauer et al., 1996). To detect transcriptional activity of genes encoding cytokines, without further stimulation, messenger RNA was extracted from the freshly isolated TD lymphocytes and this was reverse transcribed to generate templates for the PCR amplification of specific transcripts. It could be inferred that transcriptional activity detected in this way had occurred *in vivo*.

In order to detect intracellular cytokine by flow cytometry, the TD lymphocytes were stimulated *in vitro* for 6 hours with phorbol myristate acetate (PMA) plus the calcium ionophore A23187 (ionomycin), in the presence of Brefeldin A. Brefeldin A disrupts Golgi function and the transport of proteins for secretion. After stimulation, the cells were fixed, made permeable and labelled with antibodies (see Section 2.6.3). Since the cells had been
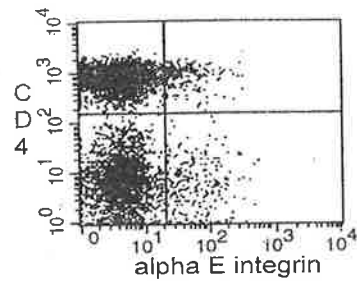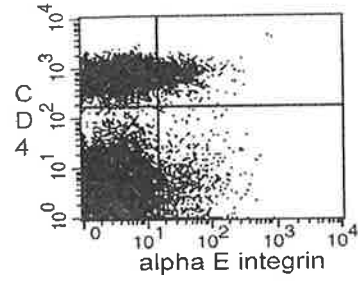
Figure 5.2.4.6 Level of expression of LFA-1 by CD4$^+$ T cells during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb WT1 (anti-LFA-1; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The mean value of the fluorescent signal generated by the FITC-labelled antibody binding to LFA-1 of "small" (D), "large" (E) and all (F) TD lymph CD4$^+$ T lymphocytes at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.
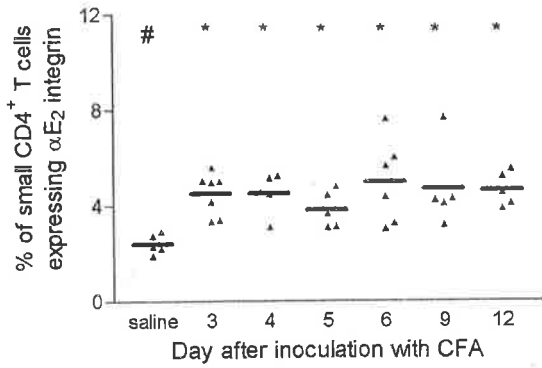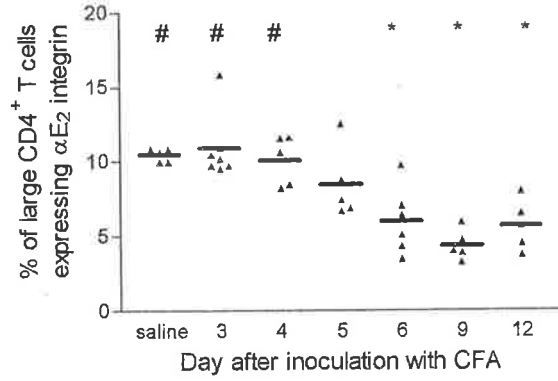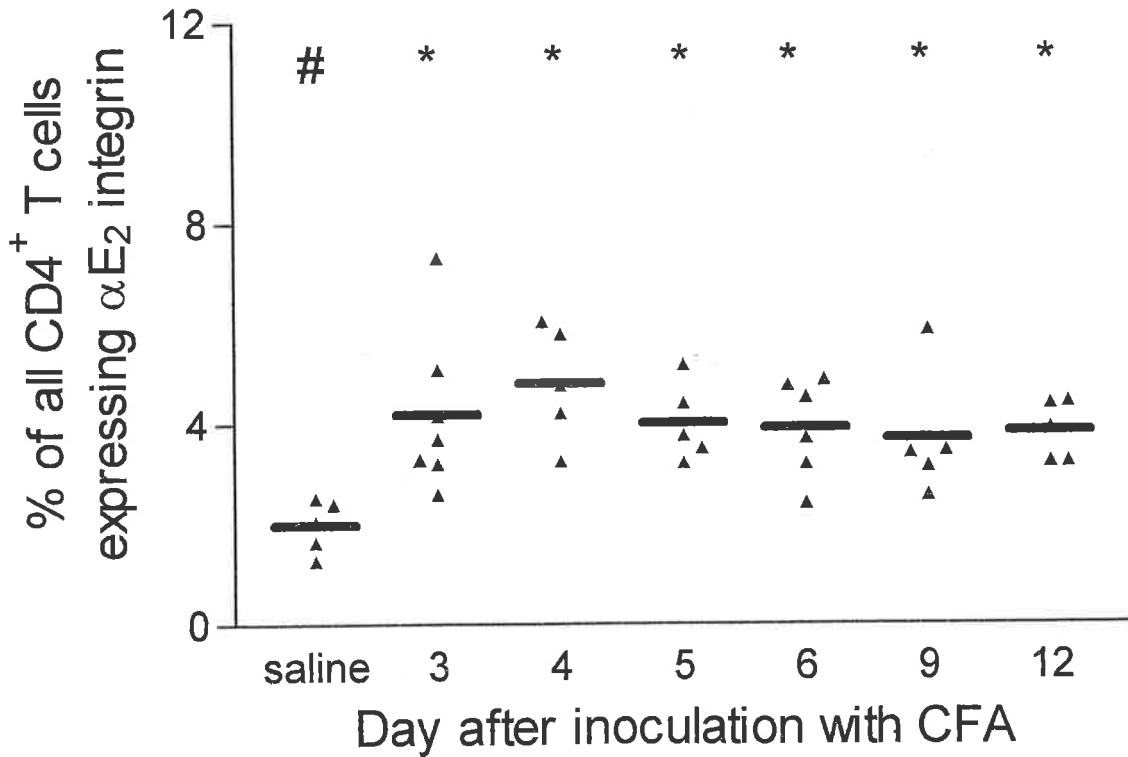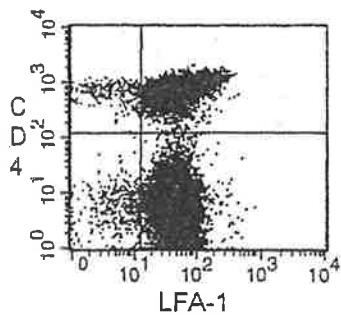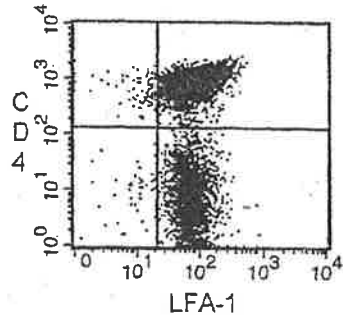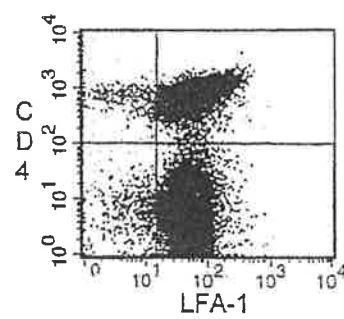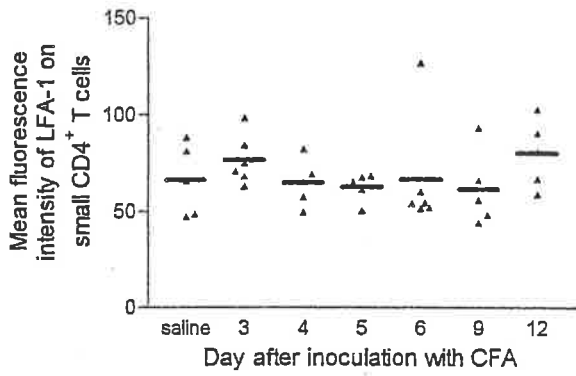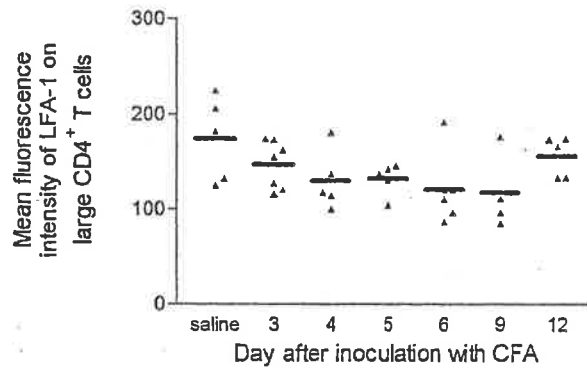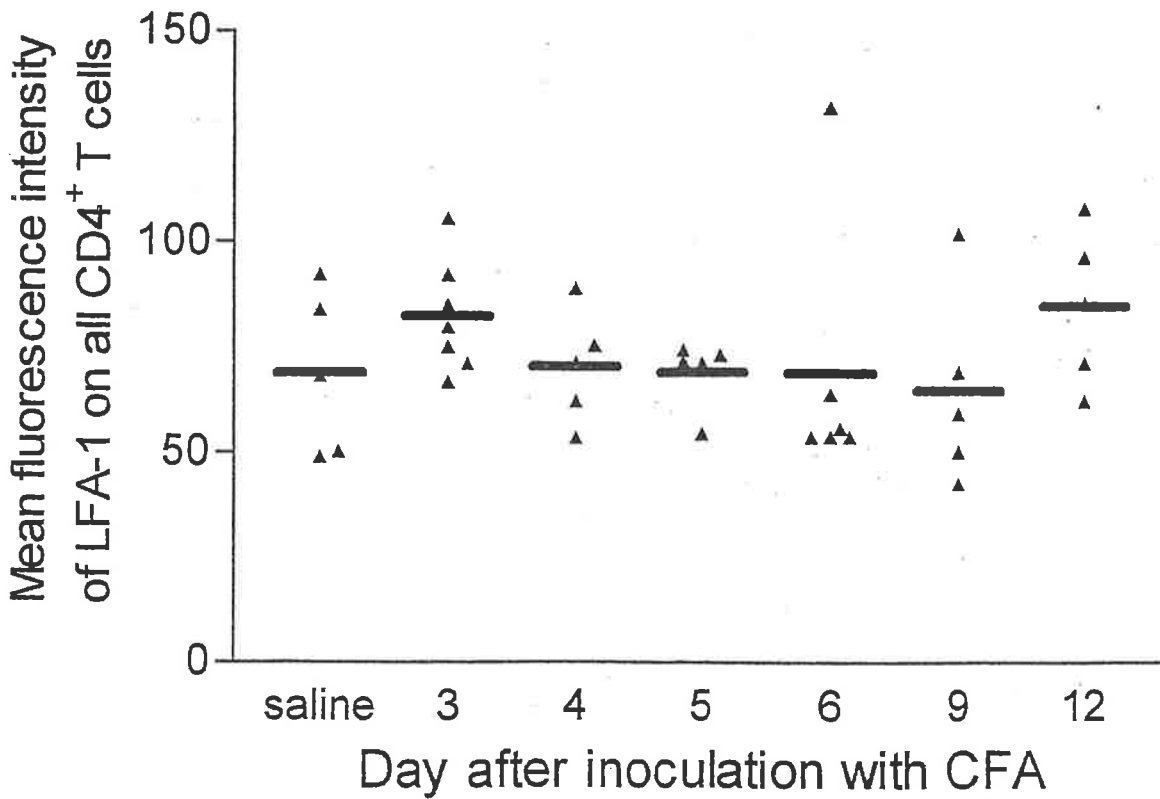
Figure 5.2.4.7 Level of expression of CD44 by CD4$^+$ T cells during the prodrome of adjuvant arthritis. Thoracic duct lymphocytes from CFA inoculated rats were first labelled indirectly with mAb OX50 (anti-CD44; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The mean value of the fluorescent signal generated by the FITC-labelled antibody binding to CD44 of "small" (D), "large" (E) and all (F) TD lymph CD4$^+$ T lymphocytes at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.

A

B

C

D

E

F

Mean fluorescence intensity of CD44 on small CD4$^+$ T cells

Day after inoculation with CFA

Mean fluorescence intensity of CD44 on large CD4$^+$ T cells

Day after inoculation with CFA

Mean fluorescence intensity of CD44 on all CD4$^+$ T cells

Day after inoculation with CFA

CD44

CD4

saline   3   4   5   6   9   12

stimulated *in vitro*, the technique reveals the potential of cells to produce cytokine, rather than the activity of the cells in cytokine production at the time of collection from the donors.

It is a general characteristic of T cell-derived cytokines that they are more difficult to detect than those produced by some other cell types such as macrophages. RT-PCR is a very sensitive method for the detection of specific mRNA transcripts. Although in some situations, there is a correlation between the kinetics of mRNA expression and the amounts of cytokine produced *in vitro*, this may not be the case *in vivo* and there is evidence that production of some cytokines (e.g. TNF-α) is controlled at the translational or post-translational levels (reviewed in Kelso, 1993). Data presented in Chapter 3 indicate that in the case of IFN-γ production by rat lymphocytes, mRNA transcripts are present in freshly isolated cells but the detection of intracellular cytokine requires a period of activation *in vitro*.

By the use of Oligo-dT sequence as a primer in the reverse transcription (RT) reaction, the same batch of cDNA could be used to amplify specific cDNA encoding each of a number of cytokines. A PCR reaction using primers that prime amplification of β-actin cDNA was included as a quality control for the cDNA preparation and also as a reference with which to standardize mRNA levels from sample to sample. Furthermore, the primers used to amplify β-actin cDNA would also prime for the amplification of the genomic (g) sequence. Since the products amplified from gDNA and cDNA differ in size, contamination of the mRNA preparation with DNA would be detected. As a negative control, a reaction that did not contain cDNA was included to ensure that contamination of the reagents had not occurred. The PCR amplification of cDNA can reach a maximum when the reaction is limited by the availability of substrates. To assess whether this endpoint had been reached and to compare levels of specific cytokine mRNA semi-quantitatively between samples, three 10-fold dilutions of the cDNA samples used as template in amplification reactions that contained the specific primers that would prime amplification of the cDNA encoding each of the desired cytokines (Ramirez et al., 1996).

5.2.5.1 Production of IFN-γ protein by T cells

The proportion of IFN-γ producing CD4$^+$ T cells in TD lymph was increased above control levels within three days after inoculation of CFA. The greatest proportion of CD4$^+$ T cells producing IFN-γ was found on day 9 after inoculation (Figure 5.2.5.1 G-I). Most, although not all, IFN-γ-producing cells were CD4$^+$ T cells (Figure 5.2.5.1 A-C). However, the IFN-γ-producing cells were α/βTCR$^+$, suggesting that the CD4$^-$ cells producing IFN-γ were in fact CD8$^+$ T cells. Following a peak at 9 days post-inoculation, IFN-γ producing cells declined significantly by day 12. Most of the IFN-γ producing cells were found within the large cell gate but some were included in the small cell gate.

5.2.5.2 Production of IL-4 protein by T cells

TD lymph CD4$^+$ T cells did not produce detectable levels of IL-4 at any of the time points investigated (Figure 5.2.5.2 A-F), although as demonstrated in Chapter 3, the technique of intracellular staining for flow cytometry is capable of detecting intracellular IL-4 in activated T cells. These results suggest that the immune response induced by CFA inoculation does not release into the circulation CD4$^+$ T cells that are primed for secretion of type 2 cytokines during the prodrome of AA.

5.2.5.3 Detection of transcripts encoding cytokines

The activation of cytokine genes in TD lymphocytes harvested from rats following inoculation with CFA was examined using RT-PCR. Thirty-five cycles of amplification were performed with each set of oligonucleotide primers, to maximise the detection of low abundance cDNA species. Messenger RNA encoding each of the cytokines examined (indicated by a product size in accordance with the predicted size) were detected in all samples from each of the experimental or control groups (representatives shown in Figure 5.2.5.3). However, only small amounts of amplification products were obtained using primers designed to detect transcripts encoding IL-4 (Figure 5.2.5.3 E). In contrast, cDNA prepared by reverse transcription of RNA extracted from TD lymphocytes obtained from a *N. brasiliensis*-infested rat (Nb infested) yielded a clear product when amplified in the presence of the same set of oligonucleotide primers. The low abundance of mRNA encoding IL-4 in the samples prepared from TD lymphocytes from control and CFA-challenged rats is in

Figure 5.2.5.1 Production of IFN-γ by CD4$^+$ T cells obtained during the prodrome of adjuvant arthritis after *in vitro* stimulation. Thoracic duct lymphocytes from CFA inoculated rats were stimulated *in vitro* with PMA plus ionomycin in the presence of Brefeldin A for 6 hours (see Section 2.6.3). Fixed cells were made permeable with saponin and were first labelled indirectly with mAb DB-1 (anti-IFN-γ; FITC), followed by direct labelling with either mAb OX38 (anti-CD4; PE, [A-C]) or mAb R73 (anti-α/β TCR; PE, [D-F]). Representative scatter plots are shown of "small" (A, D), "large" (B, E) and "all" (C, F) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The numbers on the scatter plots (A-F) indicate the proportion of gated events in each quadrant. The proportion of "small" (G), "large" (H) and all (I) TD lymph CD4$^+$ T lymphocytes that expressed IFN-γ at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean value of the group. The symbols * and # indicate groups in which the mean is different from that of the saline-injected group (*) and Day 12 (#) group respectively, with a confidence of >95%, analysed using an unpaired t test.

A B C

D E F

G
% of small CD4⁺ cells expressing IFNγ

H
% of large CD4⁺ cells expressing IFNγ

I
% of all CD4⁺ cells expressing IFNγ

Day after inoculation with CFA
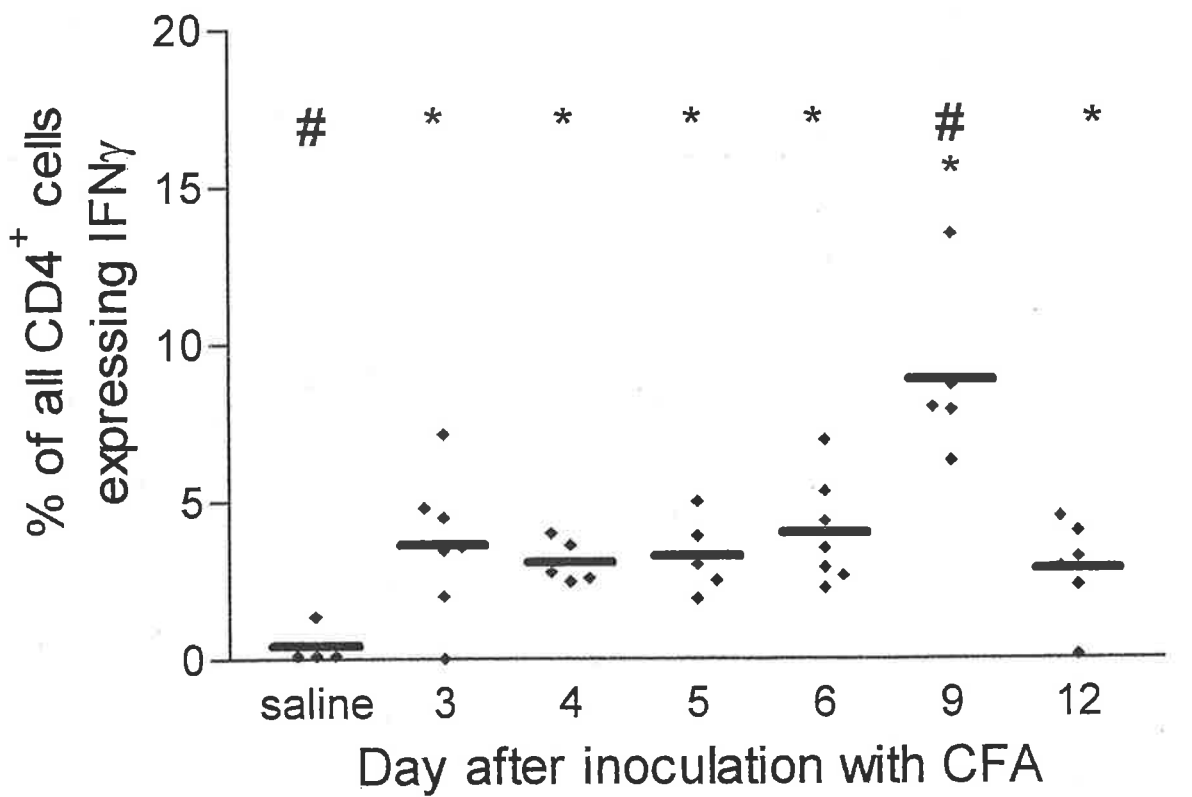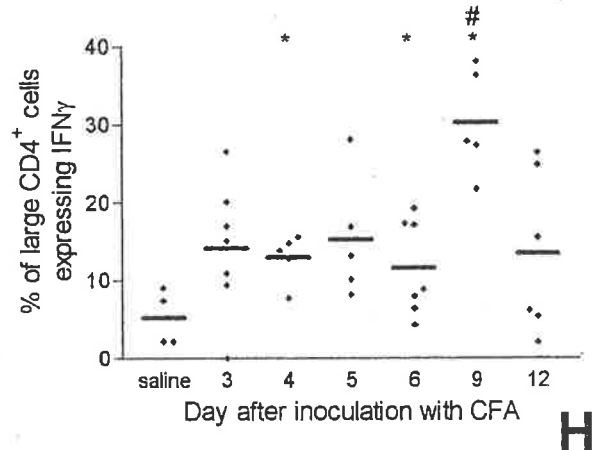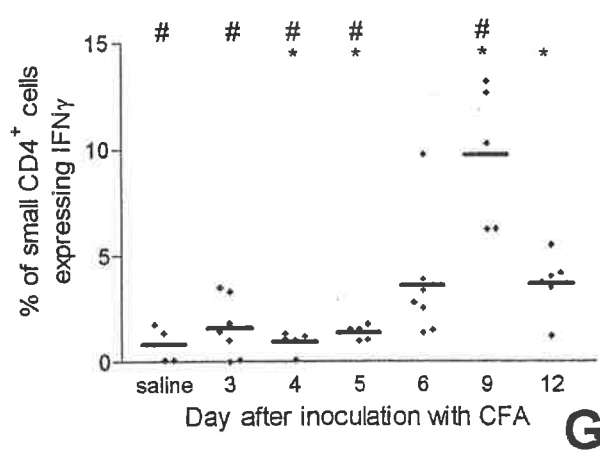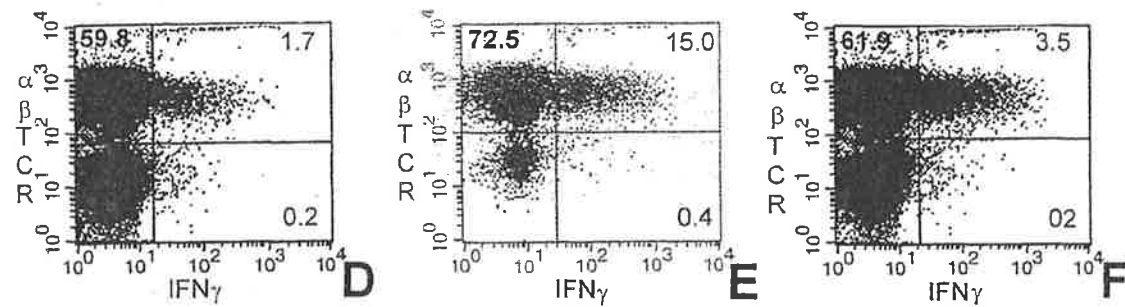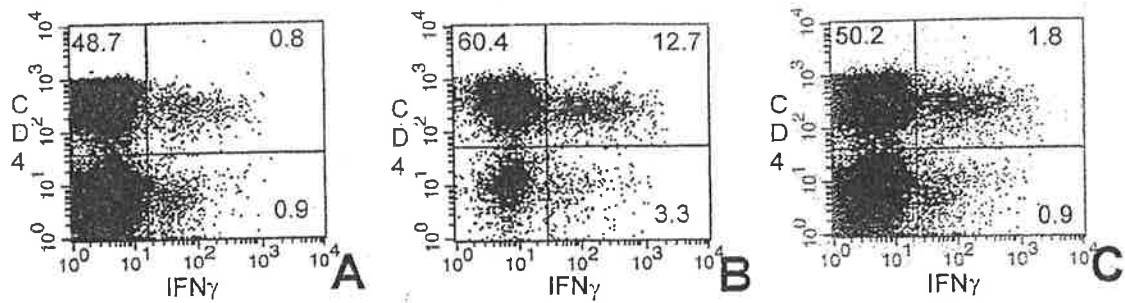
Figure 5.2.5.2 Production of IL-4 by CD4$^+$ T cells obtained during the prodrome of adjuvant arthritis after *in vitro* stimulation. Thoracic duct lymphocytes from CFA inoculated rats were stimulated *in vitro* with PMA plus ionomycin in the presence of Brefeldin A for 6 hours (see Section 2.6.3). Fixed cells were made permeable with saponin and were first labelled indirectly with mAb OX81 (anti-IL-4; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of "small" (A), "large" (B) and "all" (C) TD lymphocytes from a rat cannulated 6 days after inoculation of CFA. The proportion of "small" (D), "large" (E) and all (F) TD lymph CD4$^+$ T lymphocytes that expressed IL-4 at different times after inoculation of CFA was measured by flow cytometry. Each triangle indicates the value obtained from analysing TD lymph CD4$^+$ T cells from a single rat and the horizontal line indicates the mean value of the group. No group has mean value that is different from that of the saline-injected group or Day 12 group, with a confidence of >95%, analysed using an unpaired t test.
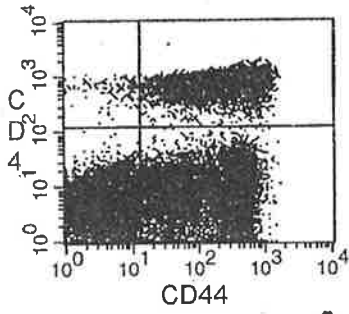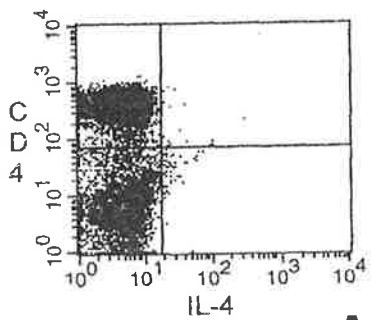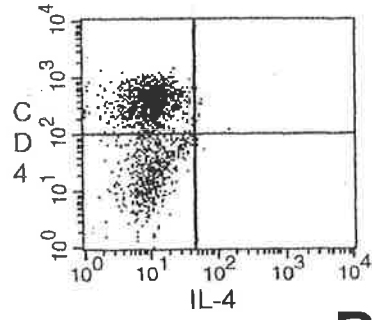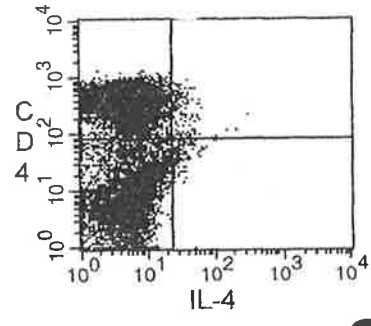
A B C
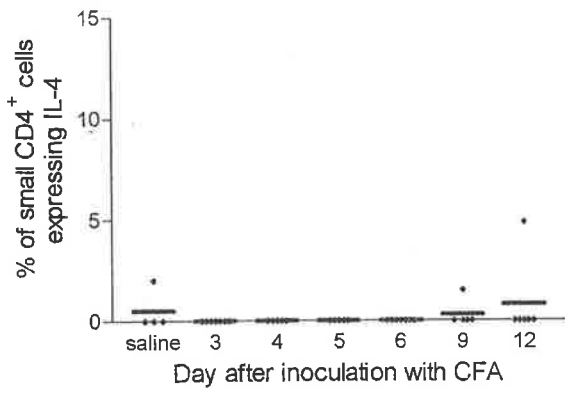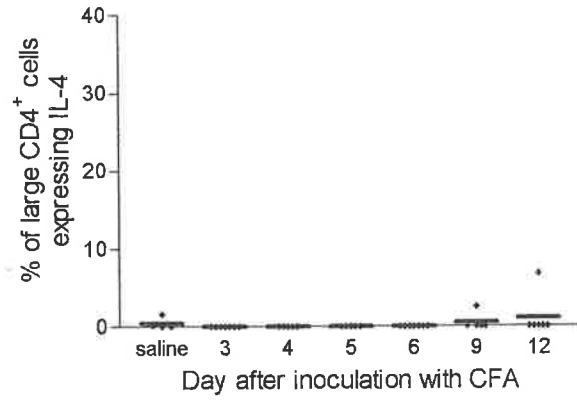
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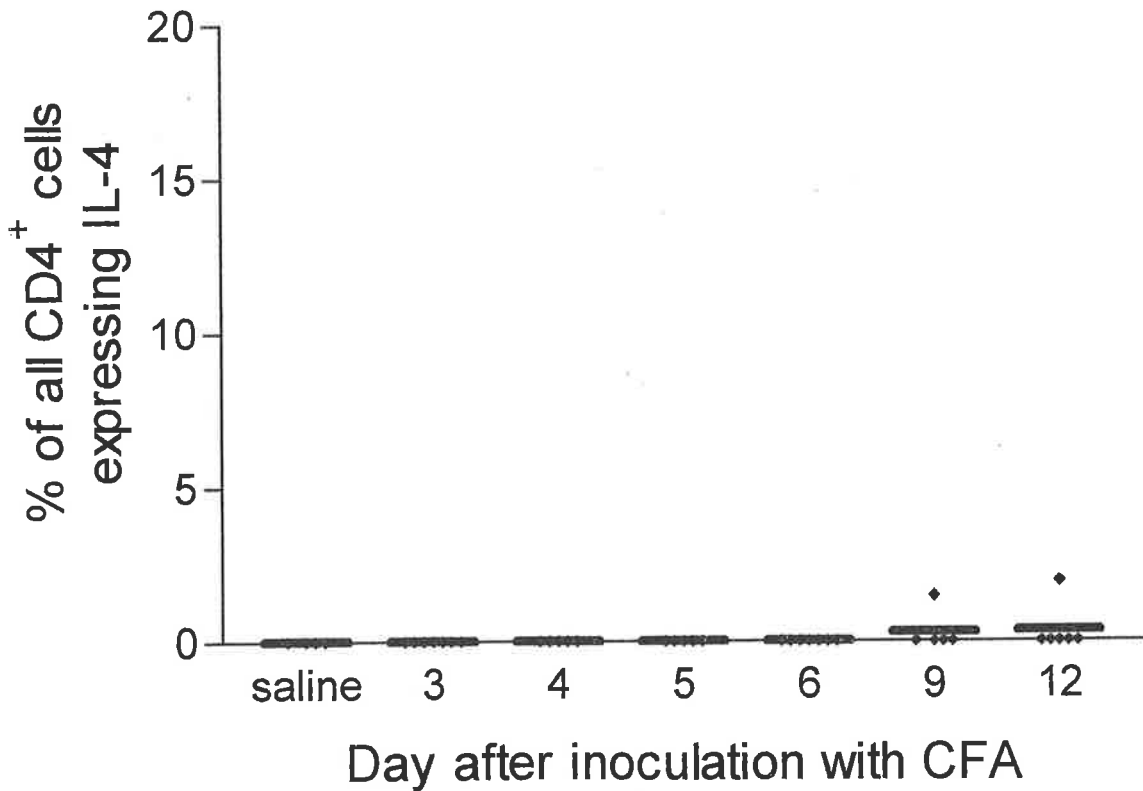
F

Figure 5.2.5.3 Detection of mRNA encoding cytokines produced by TD lymphocytes obtained during the prodrome of adjuvant arthritis. RNA, extracted from TD lymphocytes, was reversed transcribed to generate cDNA. Ten-fold dilutions of the cDNA were prepared and primers specific for cDNA encoding IFN-γ (C), IL-10 (D), IL-4 (E), TNFα (F), TGFβ (G) and IL-2 (H) were used in polymerase chain reactions to amplify relevant cDNA. Primers specific for β-actin cDNA (A and B) were used as internal controls. Amplification reactions were of a final volume of 25μl and contained either 2μl (2), 0.2μl (0.2) or 0.02μl (0.02) of cDNA, as indicated above the lane. In the case of IL-4 (E), a reaction with 2μl of cDNA prepared from the TD lymphocytes from a rat infested with *N.brasiliensis* (Nb infested, E) was used as a positive control for the PCR amplification of IL-4 cDNA. Amplification products were separated on a 1% agarose gel and visualised by SYBR Gold staining. Reactions consisted of 35 cycles of amplification, with the exception of the amplification of β-actin cDNA, which consisted of 20 cycles routinely. However, the gel shown in A contains the product of 35 cycles of amplification of β-actin cDNA, which was performed to detect possible genomic (g) DNA contamination. The top arrow indicates the predicted size (542bp) of product for the amplification of gDNA and the lower arrow indicates the predicted size (330bp) of product for the amplification of cDNA. The molecular markers of a 100bp ladder (M) shown in A were used to estimate the sizes of the amplification products.

control | Day 3 | Day 6 | Day 9 | Day 12
M | 2 | 0.2 | 0.02 | 2 | 0.2 | 0.02 | 2 | 0.2 | 0.02 | 2 | 0.2 | 0.02 | 2 | 0.2 | 0.02 | M

A

control | Day 3 | Day 6 | Day 9 | Day 12
2 | 0.2 | 0.02 | 2 | 0.2 | 0.02 | 2 | 0.2 | 0.02 | 2 | 0.2 | 0.02 | 2 | 0.2 | 0.02

B

C

D

control | Day 3 | Day 6 | Day 9 | Day 12 | Nb infested
2 | 2 | 2 | 2 | 2 | 2

E

control | Day 3 | Day 6 | Day 9 | Day 12
2 | 0.2 | 0.02 | 2 | 0.2 | 0.02 | 2 | 0.2 | 0.02 | 2 | 0.2 | 0.02 | 2 | 0.2 | 0.02

F

G

control | Day 3 | Day 6 | Day 9 | Day 12
2 | 0.2 | 2 | 0.2 | 2 | 0.2 | 2 | 0.2 | 2 | 0.2
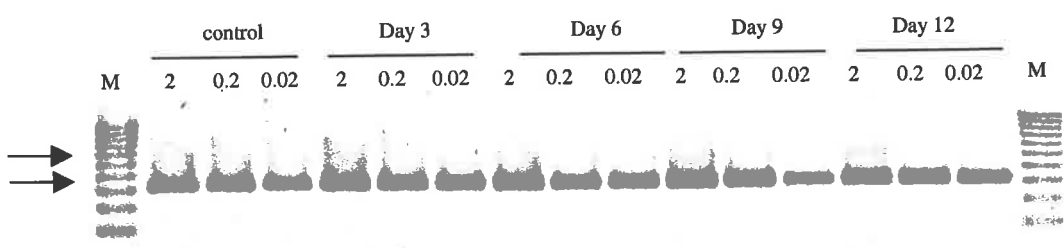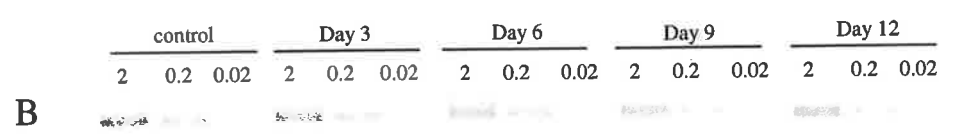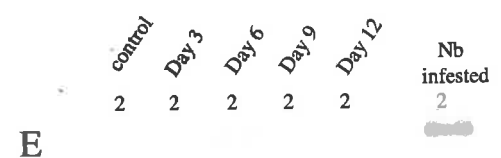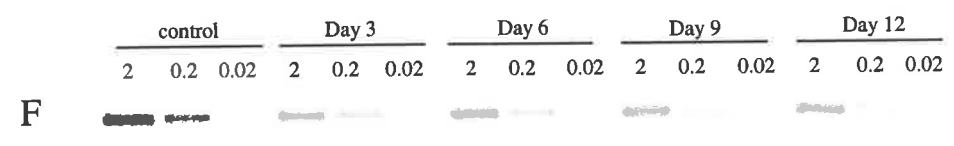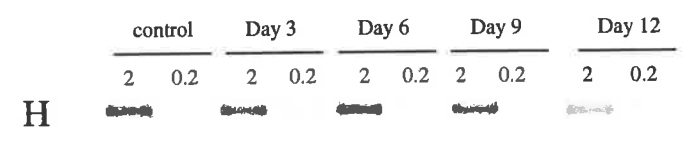
H

accordance with the lack of IL-4 protein detected by flow cytometric analysis in cells from these donors (Figure 5.2.5.2).

5.2.5.4 Semi-quantitative analysis of expression of mRNA encoding cytokines

The levels of mRNA encoding cytokine isolated from freshly isolated unselected TD cells were not different significantly between CFA-challenged and control rats (Figure 5.2.5.3). There was a trend towards higher levels of transcripts encoding IFN-γ at day 9 after inoculation with CFA but this was not significant statistically (Figure 5.2.5.4 B). It may be that re-titration with smaller dilution steps would reveal a difference that is less than ten-fold. Transcripts encoding IL-10 were detected in all samples (Figures 5.2.5.3 D), although changes of ten-fold or greater were not observed following inoculation with CFA (Figure 5.2.5.4 D). Similarly, amplification of cDNA using primers specific for transcripts encoding β-actin (Figure 5.2.5.4 A), IL-4 (Figure 5.2.5.4 C), TNF-α (Figure 5.2.5.4 E), TGF-β (Figure 5.2.5.4 F) and IL-2 (Figure 5.2.5.4 G) did not indicate differences at any time point following inoculation with CFA.

5.3 Discussion

5.3.1 Arthritogenicity of TD lymphocytes obtained from rats inoculated with CFA at different time points prior to cannulation of the thoracic duct

It has been shown previously that CD4$^+$ T cells, harvested from the TD lymph of DA donor rats inoculated with CFA 9 days prior to cannulation, can transfer arthritis to naive syngeneic recipients. In the study described herein, the kinetics of the emergence of arthritogenic cells in the efferent lymph has been examined by collecting TD lymph from rats throughout the prodromal period and during the commencement of clinical disease.

The TD lymphocytes obtained from donors 3 days after inoculation were not able to transfer arthritis to naïve rats (Figure 5.2.1.1 A), despite an increase in the output of activated CD4$^+$ T cells at that time. This allows several possibilities: that the cells departing the lymph nodes had not acquired a mature effector capacity at this stage; that if arthritogenic cells were present, the frequency of those cells was too low to induce disease when transferred to naive recipients; or that the lymph did not contain cells of the correct specificity at this time. It was

Figure 5.2.5.4 Detection of mRNA encoding cytokines produced by TD lymphocytes obtained during the prodrome of adjuvant arthritis. RNA, extracted from TD lymphocytes, was reversed transcribed to generate cDNA. Ten-fold dilutions of the cDNA were prepared and primers specific for cDNA encoding IFNγ (B), IL-4 (C), IL-10 (D), TNFα (E), TGFβ (F) and IL-2 (G) were used in polymerase chain reactions to amplify relevant cDNA. Primers specific for β-actin cDNA (A) were used as internal controls. Amplification reactions were of a final volume of 25μl and contained either 2μl (2), 0.2μl (0.2) or 0.02μl (0.02) of cDNA and the lowest starting concentration of cDNA that generated visible bands in each sample was used to compare the band intensity between samples (usually 0.2μl). Reactions consisted of 35 cycles of amplification, with the exception of the amplification of β-actin cDNA, which consisted of 20 cycles routinely. Amplification products were separated on a 1% agarose gel and visualised by SYBR Gold staining. DNA fragments stained with SYBR® Gold were visualised using a Molecular Imager FX (Biorad, USA) and the images were analysed using Quantity One Quantitation Software version 4 (Biorad, USA). The fluorescence emitted from bands of DNA amplified from samples of cDNA from rats challenged were compared to samples from saline-injected rats (control) and expressed as relative units compared to a value of 1 for the control in each reaction. cDNA was prepared from 3 rats at each time point and representative gels are shown in Figure 5.2.5.3.
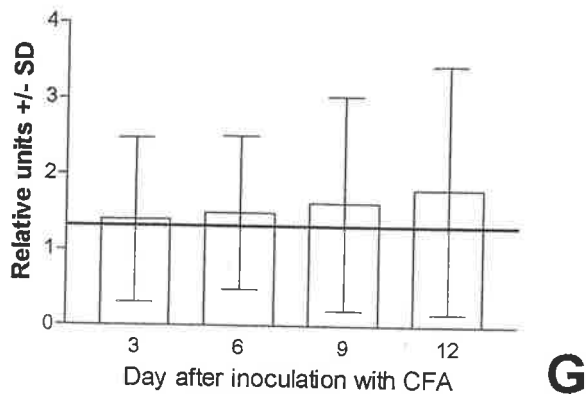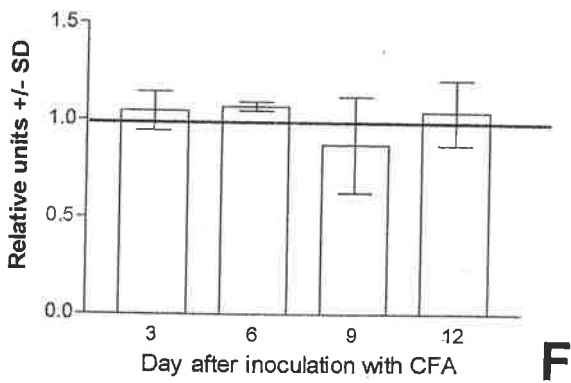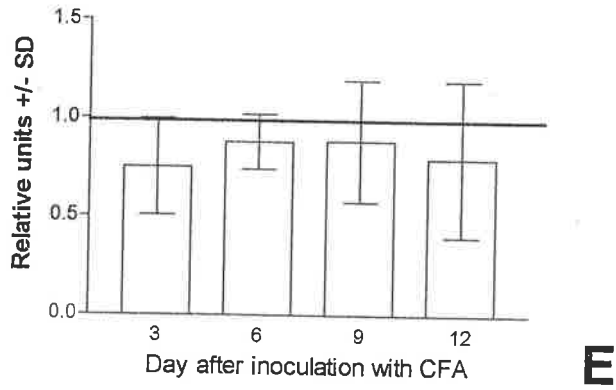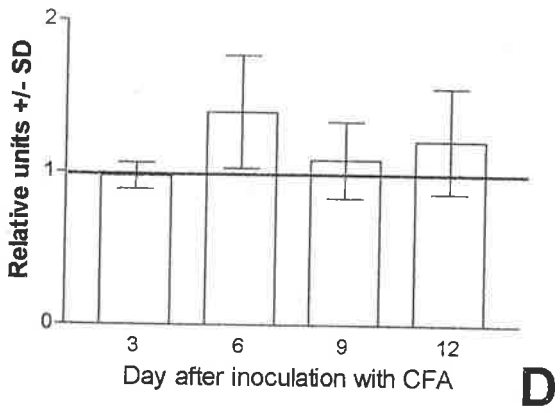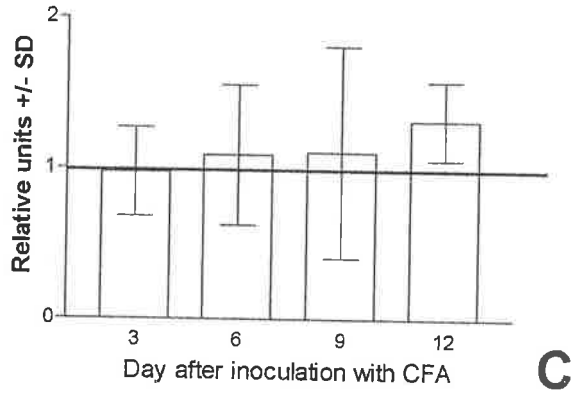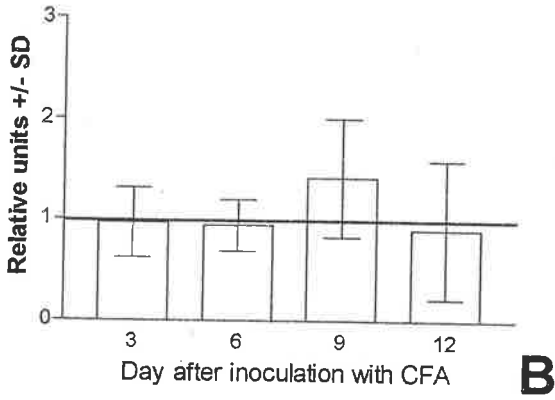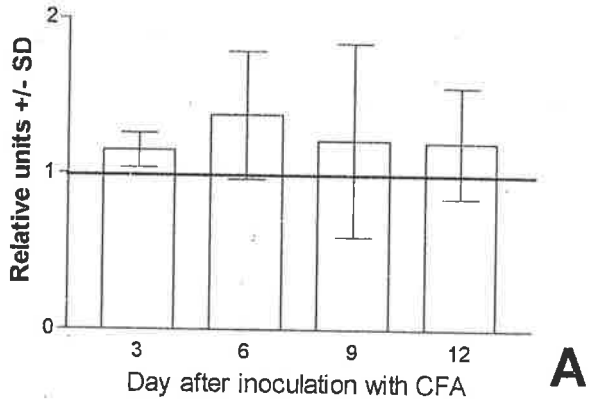
interesting, therefore, that although there was an increase in large CD4$^+$ T cells that express a number of activation markers at this stage (MHC class II, CD25, CD71 and OX40 antigen), these cells retained some phenotypic characteristics of naive cells, such as expression of CD62L. TD lymphocytes collected from rats 4 days after inoculation with CFA were arthritogenic, although cells from only 3 out of the 5 donors were successful in transferring arthritis (Figure 5.2.1.1 B). This indicates that arthritogenic cells first enter the efferent lymph in the period 3 to 4 days after inoculation with CFA. A similar pattern of arthritogenicity was observed when TD lymphocytes were obtained from donor rats at 5 and 6 days after CFA inoculation (Figure 5.2.1.1 C and D). All donors of TD lymphocytes inoculated with CFA 9 days prior to cannulation were able to transfer disease (Figure 5.2.1.1 A and E). The appearance of arthritogenicity appears to correlate with the appearance of greater numbers of CD62L$^-$ large lymphocytes in TD lymph and a greater proportion that express CD62P-ligand and α_4 integrin. These phenotypic changes could result in enhanced entry of cells with arthritogenic potential into synovial tissues.

Although the incidence of transferred arthritis appeared to differ depending on the time between inoculation with CFA and collection of TD lymph, the course of the disease in the recipients was not strikingly different. Joint swelling became apparent usually within 5 days after the transfer of TD cells and the peak of severity was observed usually 8 days after the transfer. Although, the most severe disease was seen in the recipients of TD lymphocytes from donors inoculated 9 days earlier, the range of severity seen in the recipients of TD lymphocytes from donors at other stages during the prodrome overlapped with that observed in recipients of day 9 TD lymphocytes. This suggests that the activation of naive cells and production of arthritogenic effector cells requires a minimum of 3-4 days from the time of administration of CFA. This time would be occupied by the uptake, processing and presentation of mycobacterial/autoantigens by APCs, presentation of these antigens to the naive cells and differentiation of the naive cells and acquisition of arthritogenic capacity. It appears that when released into efferent lymph, the arthritogenic cells have similar maturity whether released early (4 days) or late (9 days) after inoculation of adjuvant – as judged by the rate of onset of adoptively-transferred arthritis. This suggests that numbers of arthritogenic cells may be limiting, such that only at 9 days after inoculation are there

sufficient numbers present to ensure that TD lymphocytes from all donors are effective in transferring disease.

Adoptively-transferred arthritis has a prodrome of approximately 5 days after transfer of arthritogenic lymphocytes, whereas actively-induced arthritis has a prodrome of 9 days after inoculation with CFA. It is interesting to note that arthritogenic cells are detected in the TD lymph within four days after inoculation with CFA and that these cells initiate arthritis in recipient rats within 5 days of transfer. If the minimum duration of the inductive phase is four days and the cells require five days to accumulate in synovium and initiate inflammation, the experimental observations on the appearance of arthritogenic cells in TD lymph and the onset of adoptive disease (4 day inductive phase + 5 day effector phase = 9 days) provides a satisfactory description of the prodromal phase of AA. However, the course of AA is such that the greatest severity of joint swelling is observed at 16 days after inoculation with CFA (Pearson, 1956; reviewed in Taurog et al., 1988). If the period of time required between the mobilisation of arthritogenic effector cells and development of clinical signs of joint swelling is five days, then it might be predicted that the greatest frequency or potency of arthritogenic cells in the TD lymph would be observed before the eleventh day after inoculation with CFA (ie. five days earlier than day 16). It is interesting to note that by 12 days post-inoculation, TD lymphocytes were less able to transfer arthritis than those harvested 9 days post-inoculation, suggesting that the greatest exodus of arthritogenic cells from the lymph nodes into the efferent lymph had occurred before day 12. It is worth considering that inflammation established by the earlier cohorts of arthritogenic cells might enhance the recruitment of cells from later cohorts (Spargo et al., 1996) and possibly accelerate the development of their disease-promoting functions *in situ* in the synovium. The latter calculations are, therefore, minimum estimates of the latency associated with the activities of cells produced during the late prodromal phase of the disease. The persistence of joint inflammation beyond day 14-16 post-inoculation (Pearson, 1956) could reflect a continued lower level of traffic of arthritogenic T cells via TD lymph, or alternatively, the continued activity of T cells already recruited into the synovium.

5.3.2 Comparison of T cell populations in the TD lymph during the prodrome of AA

The total output of TD lymphocytes did not change following inoculation of CFA (Figure 5.2.2.1 A). However, the composition of the lymphocytes did change. More large cells were observed (Figure 5.2.2.1 C) and there was an increase in the proportion of large CD4$^+$ T cells in TD lymph observed 3-9 days after challenge with CFA (Figure 5.2.2.2 E). This could indicate a faster rate of release of CD4$^+$ T cells from lymph nodes following the period of "hold-up" that follows immunisation (Hall and Morris, 1965) but this is believed to apply mainly to small recirculating lymphocytes. Alternatively, the increase in large cells probably indicates release of recently-divided CD4$^+$ T cells generated in regional lymph nodes in response to the adjuvant.

Other researchers have suggested that CD8$^+$ T cells do not play either an inductive (Spargo et al, unpublished) or a regulatory role in AA (Pelegri et al., 1996b). However, the increase in the proportion of large CD8$^+$ T cells that was observed on day 12 post-inoculation (Figure 5.2.2.3 E) coincides with a decrease in arthritogenicity (Figure 5.2.1 A and F), which may indicate the generation of CD8$^+$ suppressor/immunoregulatory cells that have been mobilised by this time point. It may be hypothesised that removal of these cells from TD lymphocytes collected from a donor at day 12 post-inoculation would restore the arthritogenicity of these cells that is usually decreased in comparison to cells from a day 9 donor.

T cells that express the γ/δ TCR represent a small sub-population in TD lymph (around 1% of all TD lymphocytes). There is no evidence that γ/δ T cells are involved in the induction or effector phases of AA (Pelegri et al., 1996a) The proportions of CD8$^+$ T cells and γ/δ T cells did not change significantly during the prodromal period (Figure 5.2.2.3 F and 5.2.2.4 F), suggesting that the increase in proportion of CD4$^+$ T cells must be accompanied by a reciprocal decrease in the proportion of B cells in the period 3-6 days after inoculation with CFA. Although B cells were not analysed, the proportion of TD lymphocytes that did not express α/βTCR (primarily B cells plus a small proportion of γ/δ T cells), decreased in proportion to the increase in CD4$^+$ T cells between days 3 and 6 (Figure 5.2.2.5 C).

5.3.3 Expression of activation markers by CD4$^+$ T cells in the TD lymph during the prodrome of AA

It has been demonstrated previously in this laboratory that the arthritogenic population within the TD lymphocytes from arthritic donor rats is contained within a subset of CD4$^+$ T cells that expresses MHC class II. CD25, CD71 and CD134 (Spargo et al., unpublished). The aim of this study was to chart the emergence of activated CD4$^+$ T lymphocytes in the TD lymph.

Within three days after inoculation of CFA, there was an increase in the population of CD4$^+$ T cells that exhibited a phenotype indicating activation (Figure 5.2.2.1 C). These results are in accordance with the expectations of a typical immune response to an antigenic challenge, which induces an increase in the proportion of lymphoblasts in the efferent lymph within 3-4 days. The proportion of efferent lymph lymphocytes that have the morphological features of blasts continues to increase until 5 days after antigenic challenge and decreases thereafter (Hall and Morris, 1965a; Delorme et al., 1969).

5.3.3.1 MHC class II, CD25 (IL-2 receptor α chain), CD71 (Transferrin receptor), CD134 (OX40 antigen) and UA002 antigen

In accordance with the physical characteristics of the cells, the proportion of CD4$^+$ T cells that expressed the early activation markers CD71 and CD134 had increased by day 3 after inoculation and remained elevated (although decreasing) compared with the control group throughout the prodromal period of AA (Figure 5.2.3.3 and 5.2.3.4). The expression of these molecules is consistent with recent activation and division of these cells (Salmeron et al., 1995; Paterson et al., 1987). The expression of CD25 (Figure 5.2.3.2) and the UA002 antigen by CD4$^+$ T cells followed a similar pattern (Figure 5.2.3.5). Increase in the proportion of CD4$^+$ T cells that express MHC class II occurred more slowly and reached a maximum at day 4 post-inoculation (Figure 5.2.3.1). The functional significance of these activation-induced molecules with respect to the pathogenesis of AA is unclear. The inhibition of IL-2 production by agents such as Cyclosporin A can ameliorate AA (Del-Pozo et al., 1990) and Spargo et al., (2001) have demonstrated that depletion of either MHC class II$^+$ or CD25$^+$ cells reduces the arthritogenic capacity of TD lymphocytes in adoptive transfer. Blockade of the IL-2 receptor with antibodies *in vivo* has been shown to inhibit adoptive transfer of AA but it did not inhibit actively-induced AA (Ferguson et al., 1988). This suggests that IL-2 may be important during the effector phase of AA, possibly in maintenance of the cells in the

synovium. The results suggest that expression of CD25 and CD134 is not sufficient to ensure arthritogenicity, because a large proportion of the large CD4+ T cells collected 3 days after inoculation of CFA express these molecules.

These results suggest that there is an ongoing exodus of recently activated CD4+ T cells from lymph nodes into the efferent lymph of rats challenged with CFA. This commences by day 3 and it continues at least until the establishment of joint inflammation (consistently observed by day 12). It is not possible to estimate the proportions of these activated cells that are specific for exogenous or endogenous arthritogens. However, there are interesting correlations with arthritogenicity. Firstly, Spargo et al, (2001) have shown that depletion of MHC class II+ cells from the CD4+ population in TD lymph removes arthritogenicity. It is notable, therefore, that appearance of arthritogenicity following inoculation with CFA correlates with the peak of MHC class II+ CD4+ T cells in TD lymph 4 days after inoculation. It is also noteworthy that the decrease in arthritogenicity of TD lymphocytes obtained from donors 12 days after inoculation of CFA correlates with a decrease in the number of circulating activated CD4+ T cells at this time.

5.3.3.2 CD45RC

The proportion of total and small CD4+ T cells in TD lymph that express CD45RC did not appear to change significantly during the days following inoculation of the rats with CFA (Figure 5.2.3.6.1 F). However, a statistically significant decrease in the proportion expressing CD45RC was observed in large CD4+ T cells, commencing on day 3 after inoculation (Figure 5.2.3.6.1 E). There was also an increase in the level of expression of CD45RC on mainly small CD4+ T cells, which commenced on day 4 and remained elevated thereafter (Figure 5.2.3.6.2 C). Because CD45RC is a marker of naïve CD4+ T cells, these findings suggest that recirculation of small lymphocytes through lymph nodes is disturbed by immunized with CFA and that cells with the highest levels of CD45RC are released earlier from the "hold-up" in lymph nodes than the cells that express lower levels of CD45RC. In the case of activated CD4+ T cells, the release of a population of CD45RC^{lo} or CD45RC$^-$ cells would be expected to follow antigen challenge (Figure 5.2.3.6.1 F). It should be noted that CD45RC is not a definitive marker of naive CD4+ T cells (Bunce and Bell, 1997) and so-called "memory" cells

that are CD45RC$^-$ have been shown to revert to a CD45RC$^+$ "naive" phenotype under some circumstances. Furthermore, expression of CD45RC by CD4$^+$ T lymphocytes in the rat has been associated with a Th1 memory phenotype (reviewed in Fowell et al., 1991). Preliminary sorting into CD45RC^{hi} and CD45RC^{lo} subpopulations would be necessary in order to correlate the production of IFN-γ following *in vitro* stimulation.

5.3.4 Expression of adhesion molecules by CD4$^+$ T cells in the TD lymph during the prodrome of AA

The migration of TD lymphocytes from arthritic donors into paws (both normal and arthritic) in rats was first demonstrated by Kelly and Harvey in 1978. Later, Spargo and co-workers quantified this and demonstrated that TD lymphoblasts from arthritic donors migrated into hind paws of both normal and arthritic recipients in greater numbers respectively than those from normal donors (Spargo et al., 1996). Both of these studies reported an increase in the accumulation of TD lymphocytes in arthritic paws compared with normal paws, regardless of the source of TD lymphocytes. Those studies suggested that TD lymphocytes from CFA-inoculated rats might express the necessary surface adhesion molecules to permit their entry into synovial tissues and that in the presence of synovitis, there is an increase in the expression of adhesion molecules on the endothelium of synovial blood vessels and that this facilitates the entry of the lymphocytes.

Recent studies of lymphocyte migration have suggested that memory lymphocytes migrate selectively through non-lymphoid tissues whereas naïve lymphocytes migrate selectively through the HEV in lymphoid tissues (reviewed MacKay, 1993). Based on this model, it is reasonable to hypothesise that during the selective migration of activated lymphocytes through a non-lymphoid tissue (synovium) they encounter cells which present synovial or other arthritogenic antigens, thus initiating inflammation. However, another contributing factor to the differences observed in accumulation of naïve and memory lymphocytes in tissues is the duration of their retention within these tissues (Westermann and Pabst, 1996). Different mechanisms appear to regulate the migration of naïve and memory cells from blood to lymph and these include both the route of initial extravasation and the retention of the extravasated cells in the tissues (Westermann et al., 1994).

Naïve T lymphocytes express relatively high levels of L-selectin (CD62L) and low levels of the adhesion molecules P-Selectin ligand, CD44, CD54, LFA-1 and the $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins. Activation reduces the level of expression of CD62L and increases the levels of expression of these other adhesion molecules. The level of expression of each is determined by the stimulus (Sprent, 1997). The lymphoid setting in which a naïve lymphocyte first encounters antigen appears to determine the pattern of surface molecules expressed and this in turn determines its predilections for subsequent traffic (Washington et al., 1994; reviewed in Springer, 1994). This mechanism appears to confer a functional economy and utility on the cells. Effector cells are recruited preferentially to areas where they are most useful, e.g. lymphocytes activated in the mucosal lymphoid tissues return to mucosal sites. There is growing evidence that expression of certain adhesion molecules may be part of a more complicated functional phenotype that includes the cytokines produced by the cells (Meeusen et al., 1996) but reliable and exclusive surface markers that correlate with cytokine production (Th1/Th2) phenotypes have not been established.

5.3.4.1 CD62L (L-selectin)

CD62L (L-selectin) is expressed by naïve lymphocytes and expression is reduced after activation (Tedder et al., 1995). However, the range of expression of CD62L is broad and it is difficult to assign a particular level of expression which distinguishes naive cells from those that have been exposed to antigenic stimulation (Westermann and Pabst, 1996). There was a trend towards increased proportions of CD62L⁺CD4⁺ T lymphocytes in the TD lymph in the period after inoculation of CFA (Figure 5.2.4.1 F). The increase in the proportion of cells expressing markers of activation (eg. CD25, MHC class II, etc) indicates that the proportion of activated CD4⁺ T cells increased in the efferent lymph of the rats during the prodromal stages of AA. The proportion of CD62L⁺ cells in TD lymph at a given time after inoculation will be influenced both by the release of activated cells from draining lymph nodes and by the proportions of naive and memory lymphocytes that are entering efferent lymph from both resting and stimulated lymph nodes. The latter may be influenced by the effect of inflammation on the node (Hall and Morris, 1965), the proportions of naïve and memory T cells that are recruited and on the relative transit times of the two populations through the

lymph nodes. As in the case of cells expressing CD45RC, the contribution of activated cells to the total traffic of CD4$^+$ T cells is relatively small and easily masked by subtle changes in the proportions of naïve and memory resting CD4$^+$ T cells. In the case of small lymphocytes, the most likely explanation for the trend towards greater proportions of CD62L$^+$ cells in TD lymph after inoculation is that the CD62L$^-$ cells are retained preferentially either in inflamed lymph nodes or at other sites of tissue inflammation. In the case of large lymphocytes, it appears that those produced early after inoculation express CD62L but those produced later express lower levels of the molecule (the proportion of large CD4$^+$ cells remains elevated above control levels until day 9 post-inoculation [Figure 5.2.2.2]). Therefore, although the proportions of large CD4$^+$ T cells in TD lymph remains at similar levels during the prodrome of AA, the functional capabilities of the cells changes with respect to expression of this important molecule.

5.3.4.2 P-Selectin ligand (CD162)

The expression of a functional P-selectin (CD62P) ligand (P-Selectin glycoprotein 1, PSGL-1, CD162) has been associated with a Th1 phenotype and the interaction between CD62P and its ligand is thought to be crucial for the migration of effector CD4$^+$ T cells into inflamed synovial tissue (Austrup et al., 1997). Activated endothelial cells express CD62P (Tedder et al., 1995), which binds to CD162 expressed on activated T cells (Vachino et al., 1995).

Both CD62P and CD62L can act as adhesion molecules that promote rolling behavior of leucocytes that is a precursor to tethering and diapedesis (Tedder et al., 1995). Differential expression of CD62L and CD162 by T cells is a mechanism that permits naive and memory/activated T cells to exhibit distinct pattern of migration through lymph nodes and inflamed tissues respectively.

The expression of functional CD162 by CD4$^+$ T lymphocytes isolated from the TD lymph was examined using a CD62P:human Ig fusion protein. The late availability of this reagent prevented the examination of all rats in all groups. However, it was apparent that the expression of the functional CD162 by large CD4$^+$ T cells increased following inoculation of CFA (Figure 5.2.4.2 F). It would be interesting to examine expression of this molecule more

thoroughly because it appears to be important in the migration of activated cells into inflamed synovium as shown in the collagen-induced arthritis model in mice (Austrup et al., 1997). It appears that the proportion of CD4$^+$ T cells is elevated in TD lymph at a time after inoculation with CFA (day 4) when the proportion of CD62L$^+$ large lymphocytes is in decline (Figure 5.2.4.1). A complementary rise in expression of functional CD162 and a decrease in expression of CD62L may equip the cells produced during the later stages of the prodrome for migration into tissues including synovium. It would be of particular interest to examine the arthritogenicity of CD4$^+$ T cells that express this ligand. Immuno-magnetic beads could be used either to isolate these cells for transfer into naïve recipients, or alternatively to deplete the CD162$^+$ cells from arthritogenic TD lymphocytes. These strategies would determine respectively whether CD162 expressing cells were sufficient or necessary for the transfer of polyarthritis by TD cells from donor rats in the prodrome of AA.

5.3.4.3 α_{E2} integrin

The proportion of CD4$^+$ T lymphocytes in TD lymph that expressed α_{E2} integrin increased from approximately 2% in control rats to between 4 and 6% in CFA-inoculated rats (Figure 5.2.4.3 F). The expression of the $\alpha_{E2}\beta_7$ integrin by T cells in rats is restricted largely to intraepithelial lymphocytes and those found in the lamina propria of the gut (Brenan and Rees, 1997). It is difficult to assess whether the increase in proportion of α_{E2}^+CD4$^+$ T cells in the TD lymph from rats during the prodrome of AA is a consequence of the systemic effects of CFA, which include gut inflammation, or a result of changes in the ratio of gut-derived and peripheral lymph-derived lymphocytes as a result of inflammation in peripheral tissues. The decline in the proportion of α_{E2}^+ large CD4$^+$ T cells during the prodromal period suggests that these cells have been diluted with an increase in the output of α_{E2}^- cells by the stimulated peripheral lymph nodes. This is of some interest, because it has been reported that the inflammatory infiltrate in rheumatoid synovium contains $\alpha_E\beta_7^+$ T cells (Elewaut et al., 1998). The rise in proportion of α_{E2}^+ small lymphocytes may reflect continued normal recirculation of lymphocytes through the gut-associated lymphoid tissues during the prodromal period of AA, together with some slowing of the recirculation of the majority of α_{E2}^- population through peripheral tissues.

5.3.4.4 CD49d (α_4 integrin)

The expression of the CD49d (VLA-4 α subunit, α_4 integrin) is associated generally with activated and memory cells, and the $\alpha_4\beta_1$ and $\alpha_4\beta_7$ heterodimers are considered important for homing to inflamed peripheral tissues and mucosal tissues respectively (Springer, 1994). CD49d can mediate rolling and adhesion in inflamed post-capillary venules (Johnston et al., 1996). The intravenous administration of a monoclonal antibody against CD49d to rats during the induction and early clinical stages of AA inhibited leukocyte adhesion and partially inhibited rolling in the mesenteric venules, as well as reducing the severity of AA (Seiffge, 1996). The mean proportion of CD4$^+$ TD lymphocytes expressing CD49d increased following inoculation with CFA, although the only times at which a statistically significant difference was observed between CFA-inoculated donors and controls was 4 and 5 days after inoculation (Figure 5.2.4.4 F). Increases in the proportion of CD49d$^+$ small and large lymphocytes contribute to these changes. The expression of CD49d is, therefore, another change in the functional capacity of CD4$^+$ T cells that may facilitate migration into synovium and contribute to arthritogenicity of the cells during the latter part of the prodromal period of AA. It is notable that marked variance in CD49d expression was observed in all groups studied. The reason for this is not clear although lability of expression in implied.

5.3.4.5 CD54 (ICAM-1)

ICAM-1 (CD54) expression is up-regulated upon activation (Springer, 1990; Dustin & Springer, 1991). It is a ligand for LFA-1 (lymphocyte function-associated antigen-1, CD11a/CD18) (Staunton et al., 1990), Mac-1 (CD11b/CD18), p150,95 (CD11c/CD18) and hyaluronan (McCourt et al., 1994). The binding of CD54 on endothelium to its ligand(s) on leukocytes contributes to the adhesion step of extravasation by which leukocytes enter inflamed tissues and migrate through HEVs. Furthermore, the expression of CD54 on activated CD4$^+$ T cells might be important for stabilising antigen presentation and receipt of stimulatory signals from APCs and interaction with cells that express LFA-1 and other β_2 integrins. The *in vivo* administration of 1A29 (anti-CD54) in Lewis rats can strongly suppress both the inductive and effector phases of AA (Iigo et al., 1991), indicating an important role for CD54 in the pathogenesis of the disease.

The proportion of CD54$^+$CD4$^+$ T cells had increased by 3 days after inoculation of CFA and this change affected both small and large lymphocytes (Figure 5.2.4.5). Because very few small CD4$^+$ T cells in control rats expressed CD54 and the change was approximately 4-fold, it seems likely that the increase in CD54$^+$ small lymphocytes in the rats inoculation with CFA was due to increased output of newly-formed small cells. Certainly, this coincided with a substantial increase in the proportion of CD54$^+$ large cells in the CFA-inoculated animals. This change was already maximal at 3 days post-inoculation (i.e. before demonstrable arthritogenicity in the TD lymphocytes), suggesting that up-regulation of CD54 alone is not sufficient to allow activated CD4$^+$ T cells from inoculated donors to express arthritogenicity.

As in the case of the activation markers CD25, CD71 and UA002 antigen, there was a decline in the proportion of CD54$^+$CD4$^+$ T cells 12 days after inoculation. As mentioned previously, this coincides with a time where there is a concurrent decrease in the arthritogenicity of the TD lymphocytes from inoculated donors. It may be that CD54 is necessary or synergistic with other adhesion molecules but not sufficient, for the expression of arthritogenicity by activated CD4$^+$ T cells.

5.3.4.6 LFA-1 (CD11a/CD18)

Leukocyte function antigen (LFA)-1 (CD11a [integrin α_L subunit] / CD18 [integrin β_2 subunit] heterodimer) can bind to ICAM-1, -2 and –3 (Barclay et al., 1997) and it undergoes a transient up-regulation of avidity for its ligands upon activation. The absence of LFA-1 can be overcome by other integrins and the blockade of LFA-1 by the use of mAb does not completely inhibit the migration of monocytes into arthritic joints. However, studies on combined blockade with mAb against Mac-1 and VLA-4 suggest that the LFA-1/ICAM interaction plays a synergistic role with these other adhesion molecules (Issekutz and Issekutz, 1995).

No significant change was observed in the level of expression of LFA-1 by CD4$^+$ T cells in TD lymph after inoculation of CFA (Figure 5.2.4.6). While measurement of avidity of LFA-1 for CD54 was beyond the scope of this study, it appears that the level of expression of the molecule is not an essential factor in determining arthritogenicity of CD4$^+$ T cells.

5.3.4.7 Phagocytic glycoprotein 1 (CD44)

Phagocytic glycoprotein 1 (CD44) binds hyaluronan and is involved in interaction of extravasated cells with extracellular matrix at sites of inflammation (DeGrendele et al., 1996). The expression of CD44 on lymphocytes is up-regulated upon activation (Budd et al., 1987) and it is thought to be involved in both adhesion and signalling (Lesley et al., 1993).

Within three days after inoculation, there was an increase in the mean fluorescence of cells labeled indirectly with mAb OX50 (anti-CD44; FITC) (Figure 5.2.4.7 F). This reached statistical significance from days 3-6 after inoculation when large and small cells were considered together. Functionally, increased expression of CD44 could contribute to enhanced retention of activated cells in tissues after extravasation from the circulation.

5.3.5 Production of cytokines by thoracic duct T lymphocytes during the prodrome of AA

The production of cytokines by T lymphocytes provides clues to the nature of co-stimulatory signals that they have received during activation and the effector functions of the cells. An understanding of the effector mechanisms expressed by arthritogenic CD4$^+$ T lymphocytes and their role in the pathogenesis of synovitis may suggest targets for intervention in the treatment of arthritis.

In this study, the expression of cytokine mRNA was examined using RT-PCR, while the production of IFN-γ and IL-4 was examined using flow cytometry. The former technique has the advantages of detecting transcription of genes encoding cytokine *in vivo* (without requiring that the cells receive *ex vivo* stimulation) and of not being limited by the availability of specific antibodies for the detection of cytokine protein. However, the expression of mRNA does not always correlate with protein production and caution must be exercised when making conclusions based on the detection of mRNA only. Staining of intracellular cytokines for flow cytometric analysis has the advantage that the cell producing the cytokine can be identified and its surface antigen phenotype examined simultaneously by the use of mAbs. This method allows, therefore, the production of cytokines to be examined

at the single cell level, in contrast to RT-PCR, which examines mRNA extracted from an entire population of TD lymphocytes.

IFN-γ is produced primarily by antigen-experienced T cells and by activated NK cells. The expression of IFN-γ mRNA transcripts has also been detected in some other cell types, such as some B cell lines. However, the level of expression of the gene encoding IFN-γ in these other cell types may reflect a basal level of expression and may be of little patho-physiological significance. The IFN-γ gene seems to be controlled at several levels, including transcriptional activation, DNA methylation, control of mRNA stability and possibly through other post-transcriptional mechanisms (Young & Hardy, 1995). The production of IFN-γ is usually indicative of an immune response initiated in the presence of IL-12 and mediated by Th1-cells (Romagnani, 1994; Trembleau et al., 1995a). Such responses are responsible for delayed-type hypersensitivity reactions, where the ultimate effector cells are the macrophages. The production of IL-4 by activated T cells is associated with induction in the presence of IL-4 and the activities of Th2 cells and is seen in immune responses associated with allergic and atopic disorders (Mosmann and Coffman, 1989; Romagnani, 1994).

Using flow cytometric analysis, it was found that inoculation of rats with CFA led to an increase in the proportion of CD4$^+$ T cells that produce IFN-γ. The proportion of small cells labelled by specific anti-IFN-γ antibody was below the reagent background (mAb 1B5, negative control) when TD lymphocytes from normal donors were stimulated *in vitro* with PMA plus ionomycin (Figure 5.2.5.1 G). Compared with this background, specific labelling of intracellular IFN-γ was detected in a significant proportion of cells at all time points after inoculation with CFA (Figure 5.2.5.1 I). The greatest proportion of CD4$^+$ T cells producing IFN-γ was detected in the TD lymph 9 days after inoculation with CFA (Figure 5.2.5.1 G-I). Cells producing IFN-γ were found in both the small and large cell gates, again suggesting that some activated/recently-produced cells are present in the population of "small" lymphocytes. A smaller proportion of CD8$^+$ (α/β TCR$^+$CD4$^-$) T cells was also found to produce IFN-γ. Work in this laboratory (Spargo et al., 2001) suggests that the latter are not necessary to transfer AA adoptively.

Day 9 after inoculation is the end of the prodromal phase of the disease, with clinical signs of joint inflammation becoming apparent in some animals. Interestingly, it is also at this time that TD lymphocytes are best able to transfer arthritis to naïve recipients (Figure 5.2.1.1 A and E). This correlation between the production of IFN-γ and the arthritogenicity of TD cells suggests that the CD4$^+$ T cells that initiate inflammation in the peripheral joints may be the cells that produce IFN-γ. It is of interest, therefore, that CD4$^+$ T cells that have been activated *in vitro* under conditions that favour the production of IFN-γ are better able to enter inflamed joints than those produced under conditions that favour production of IL-4 (Austrup et al., 1997). Furthermore, there is evidence that *in vivo* administration of antibodies that neutralise IFN-γ can prevent the induction of AA (Wiesenberg et al., 1989, Jacob et al., 1989). This further supports the suggestion that IFN-γ is important in the pathogenesis of AA.

IFN-γ is reported to be a major mediator of T cell recruitment into sites of DTH (Issekutz et al., 1988) and it is thought to promote movement of lymphocytes from the circulation into sites of inflammation. Other studies *in vivo* have indicated that IFN-γ leads to retention of intravenously injected TD lymphocytes in tissues (Westermann et al., 1994). The production of IFN-γ by migratory CD4$^+$ T lymphocytes may be important also for the induction or up-regulation of adhesion molecules such as CD54 and VCAM-1 by endothelial cells, thus aiding extravasation of further cells that promote or perpetuate inflammation. Finally, IFN-γ is a potent activator of macrophages (Pace et al., 1983), leading to the production and release of a variety of mediators of inflammation.

The production of IL-4 by CD4$^+$ T cells was not detected by flow cytometric analysis (5.2.5.2 A-F) and RT-PCR demonstrated fewer IL-4 mRNA transcripts than any other cytokine examined (Figure 5.2.5.3 E). The technique for detection of IL-4 was shown experimentally to be sufficiently sensitive to detect IL-4 message generated within a Th2 mediated response, because intracellular IL-4 and mRNA transcripts encoding IL-4 were detected readily in TD lymphocytes from rats infested with *N.brasiliensis* (Chapter 3, Figures 3.2.4.1 and 5.2.5.3 E,

respectively). The relative abundance of IFN-γ and scarcity of IL-4 suggests that inoculation with CFA leads to stimulatory signals that generate a Th1-mediated immune response.

RT-PCR detected the expression of IL-10 mRNA in all samples analyzed and the level of transcription did not appear to be affected by challenge of the donor rats with CFA (Figure 5.2.5.4 D). Although not as tightly restricted to the Th2 responses as IL-4, the production of IL-10 is usually associated with Th2-mediated immune responses and it is considered to be antagonistic of Th1 responses (Moore et al., 1993). IL-10 inhibits the migration of CD4⁺ T cells (Jinquan et al., 1993) and inhibits synthesis of cytokines and other functions of macrophages (Moore et al., 1993). Production of IL-10 might be expected to inhibit the migration of arthritogenic cells into synovial tissue and to have a dampening effect of production of inflammation by arthritogenic T cells. IL-10 production by migratory CD4⁺ T cells does not correlate with development of arthritogenicity but this cytokine could exert a regulatory role on disease expression. In this regard, correlations between strains of rats with regard to IL-10 expression by migratory T cells and proneness to AA may be of interest.

RT-PCR was used to examine the expression of mRNA by TD lymphocytes throughout the early stages of AA. The titration of cDNA in the PCR reaction was used to make the technique semi-quantitative. In the case of IFN-γ, the results suggest a trend whereby TD lymphocytes from CFA-challenged donor rats had higher levels of IFN-γ-specific mRNA transcripts than cells from normal donors (Figure 5.2.5.4 B), although the difference was not significant statistically. The RNA extraction was performed on unseparated TD lymphocytes, which contained cells other than CD4⁺ T cells. Because only a small proportion of CD4⁻ cells produce IFN-γ (Figure 5.2.5.1 A-F), mRNA from non-producing cells dilutes the IFN-γ-specific mRNA and reduces the sensitivity of the assay. If the total mRNA varies between samples, this dilution can also affect the relative titre of the specific message. However, the trend towards increased IFN-γ-specific mRNA during the late prodrome of AA is supported by the observations by Connolly, who used quantitative RT-PCR and saw increased levels of IFN-γ mRNA transcripts expressed by CD4⁺ T cells in the TD lymph from donors 9 days after inoculation with CFA compared with normal donors (Connolly, 1998). Increases in the levels of cytokine mRNA are usually associated with an increase in the proportion of cells

producing the cytokine, rather than changes in the amount of mRNA synthesised by individual cells (Gauchat et al., 1991).

TNF-α has been shown to play an important role in the pathogenesis of RA and blockade of this cytokine can ameliorate symptoms of the disease (reviewed in Jorgensen et al., 1999). This cytokine is produced by many cell types including T cells, B cells, mast cells, NK cells and macrophages. The expression of mRNA encoding TNF-α by monocytes and T cells is up-regulated upon stimulation, although there is some evidence of regulation of the production of TNF-α protein at the translational level (reviewed in Kelso, 1993). RT-PCR was used to detect the expression of TNF-α mRNA transcripts in unsorted TD lymphocytes of rats following challenge with CFA. Although TNF-α transcripts were detected (Figure 5.2.5.3 F), no significant differences were observed when RNA from TD lymphocytes collected from CFA-challenged rats was compared with RNA from TD lymphocytes from normal rats (Figure 5.2.5.4 E).

Messenger RNA encoding TGF-β1 was present in relative abundance in TD lymphocytes and the level did not seem to be up-regulated upon challenge of the rats with CFA (Figure 5.2.5.3 G and 5.2.5.4 F). However, consistency in the expression of mRNA may not correlate with consistency in the production and secretion of the TGF-β protein. This cytokine is biologically active as a homodimeric protein, which is generated by the formation of two identical precursor molecules (Massague, 1990). Although it has been demonstrated that in some immune reactions the expression of TGF-β mRNA is up-regulated, this does not always occur. For example, the stimulation of blood monocytes with LPS induces significant increases in the secretion of TGF-β without changing the levels of mRNA (reviewed Letterio and Roberts, 1998). The production of bio-active TGF-β is controlled primarily by regulating the secretion and activation of latent forms of TGF-β.

Connolly has demonstrated that CD4$^+$ T cells from the TD lymph of arthritic rats have increased levels of TGF-β mRNA compared with those isolated from normal rats (Connolly, 1998). The difference from the study presented here may be that Connolly examined purified

CD4$^+$ T cells from TD lymph. Since TGF-β is produced also by cells other than CD4$^+$ T lymphocytes, an increase in the levels of TGF-β mRNA by CD4$^+$ T cells may not have been detectable above the background levels of the entire population of TD lymphocytes used in this study of unselected TD cells.

TGF-β has been demonstrated to have multiple roles in animal models of RA. Local administration of TGF-β 1 into the footpads of rats with CIA accelerated the onset of disease (Fava et al., 1991), whereas systemic administration of TGF-β had a protective effect (Kuruvilla et al., 1990). TGF-β has been demonstrated in inflamed synovial tissue of DA rats in both the CIA and OIA models of polyarthritis (Mussener et al., 1997a) and expression of TGF-β mRNA has been demonstrated in the synovial tissues of DA rats with AA (Connolly, 1998). Because of the potentially important immunomodulatory effects of TGF-β in inflammation, it is important that further work is done to identify the cells in TD lymph that produce TGF-β in response to immunisation with CFA. This can be achieved when suitable antibody reagents become available that allow detection of intracellular TGF-β by flow cytometry. Another approach may be to examine the production of the enzyme furin. TGF-β up-regulates the expression of furin, which converts the TGF-β precursor polypeptide into mature, bio-active TGF-β (Blanchette et al., 1997). It may be of interest to examine the expression of furin mRNA in TD lymphocytes as an indirect measure of TGF-β processing by these cells.

The proportion of CD4$^+$ T cells expressing CD25 was observed to increase following inoculation with CFA (Figure 5.2.3.1 F) and this should be accompanied by increased sensitivity of these cells to the action of IL-2. IL-2 stimulates the proliferation of T cells and has both autocrine and paracrine actions. The expression of mRNA encoding IL-2 by TD lymphocytes was examined by RT-PCR. IL-2 mRNA was detected in all TD lymphocyte samples (Figure 5.2.5.3 H) and the level of expression did not appear to increase during the early stages of AA (Figure 5.2.5.4 G). It is likely that the main producers of IL-2 were the CD4$^+$ subset of T cells.

The relationship between the expression of IL-2 mRNA and protein has been the subject of a number of reports. Some *in vitro* studies have indicated that there is a linear relationship between the expression of IL-2 mRNA and the secretion of IL-2 (Gauchat et al., 1991), whereas other studies have demonstrated the presence of IL-2 mRNA, without translation into protein (Howell et al., 1991). Because the primary site of T cell proliferation is the secondary lymphoid tissue, it was perhaps not surprising that only low levels of IL-2 mRNA expression were detected in circulating TD lymphocytes. TD lymphocytes were collected over a ~ 16 hour period. Because cytokine mRNA has a relatively short half life (thought to be a consequence of the AU-rich sequences in the 3' untranslated region), it is unlikely that the presence of IL-2 mRNA was a remnant of transcription that had occurred before the cells left the lymph node. The findings suggest that some activated T cells in TD lymph can recommence secretion of IL-2 rapidly and this could follow contact with APCs carrying cognate antigen in the peripheral tissue.

5.3.6 General discussion

Inoculation of rats with CFA resulted in up-regulation of activation markers and certain adhesion molecules on CD4$^+$ TD lymphocytes. These cells could be equipped for emigration into synovial tissue and interaction with local APCs and with other important inflammatory cells such as macrophages. A predominance of IFN-γ production over IL-4 was apparent in CFA-inoculated rats. This supports suggestions that the pathogenesis of AA and RA may involve a Th1-like immune response concentrated in the synovium (Dolhain et al., 1996).

No strict pattern was observed between the proportion of CD4$^+$ T cells expressing the activation markers MHC class II, CD25, CD71 and CD134 and arthritogenicity. Similar proportions of activated cells were observed in the CD4$^+$ TD cells obtained on day 3 (non-arthritogenic) and day 9 (arthritogenic) after inoculation. Spargo et al (2001) have demonstrated that the arthritogenic population of CD4$^+$ T cells in TD lymph from arthritic donors is contained within a population that expresses the aforementioned activation markers. Lack of arthritogenicity of TD lymphocytes at day 3 post-inoculation could reflect an early generation of less mature cells that are nevertheless arthritogen specific. Some evidence was found that more mature cells predominate later, with reduction in the

proportion that expressed CD62L and increases in the proportions expressing CD162, CD54 and CD49d. The results indicate that CD54 is up-regulated early, along with the activation molecules known to identify arthritogenic cells. This may be important, because CD54 can facilitate extravasation and inter-cellular communication. Furthermore, synergy between CD54/LFA-1 interactions and interactions between other adhesion molecules may be necessary for activated CD4$^+$ T cells to exhibit arthritogenicity.

The delay in appearance of arthritogenic cells until day 4 after inoculation and the observation that cells from some rats but not others transfer the disease (the exception being day 9, when cells from all rats achieved successful transfers) suggests that multiple factors determine arthritogenicity. In adoptive transfer, it has been shown that the number of cells transferred is important (Spargo et al., 2001). The delay implicates "maturation" of cells towards an effector phenotype. Expression of adhesion molecules and cytokines are both likely to be important. Conditions for successful transfer may thus involve multiple components, none of which in itself is sufficient or even necessary, but various combinations of factors may imbue populations of cells with the capacity to transfer disease. Another factor may be selective expansion of arthritogen-specific CD4$^+$ T cells by iterative exposure to immunodominant mycobacterial antigens or self-antigens presented by APCs within synovial tissues.

The presence of activated CD4$^+$ T cells in the TD lymph of rats during the prodrome of AA suggests that these cells have emigrated from the stimulated lymph nodes and that in the normal course of events, they would enter the circulation via the thoracic duct. Passage of these cells through the circulation would allow their eventual dissemination to the joints, where they could initiate inflammation. The next chapter describes the phenotype of CD4$^+$ T cells isolated from the hind paws of rats and their appearance during the prodrome of AA.

CHAPTER 6

PHENOTYPE OF CD4+ T LYMPHOCYTES ISOLATED FROM THE HIND PAWS OF RATS DURING THE PRODROME OF ADJUVANT-INDUCED ARTHRITIS

Chapter 6: Phenotype of CD4$^+$ T lymphocytes isolated from the hind paws of rats during the prodrome of adjuvant-induced arthritis

6.1 Introduction

In chronic, destructive rheumatoid arthritis (RA), the synovial membrane is infiltrated by CD4$^+$ T cells, granulocytes and macrophages. As discussed in earlier chapters, in adjuvant-induced arthritis (AA) there are activated CD4$^+$ T cells in the inguinal and popliteal lymph nodes as early as 3 days after inoculation of CFA. Similarly, there are increased proportions of activated CD4$^+$ T cells in the efferent lymph at this time and arthritogenic cells are detectable in TD lymph collected from donor rats by the fourth day after inoculation. The presence of these activated and arthritogenic cells in the efferent lymph of rats during the prodrome of AA suggests that these cells migrate into the synovial tissue of joints and initiate inflammation. Mr. Llewellyn Spargo in the Arthritis Research Laboratory has demonstrated previously that the arthritogenic cells in the TD lymph during the prodrome of AA are activated CD4$^+$ T cells. This chapter examines the emergence and phenotype of CD4$^+$ T cells in the hind paws of rats during the prodrome of AA.

T cells in the synovium of patients with active RA are a heterogeneous population but are predominantly activated CD4$^+$ T cells. Most express CD45RO and HLA-DR, have up-regulated expression of VLA-1 and express less CD2 or LFA-1 than peripheral blood T cells (Thomas et al., 1992). The ratio of CD4$^+$ to CD8$^+$ T cells in synovium is higher that in peripheral blood and the CD4$^+$ T cells express reduced levels of CD3 and CD4 (Cush and Lipsky, 1988). Cush and Lipsky (1988) observed that the proportion of HLA-DR-expressing T cells isolated from synovium from 7 patients with RA ranged from 19% to 52%, but very few of the cells expressed IL-2 receptor (CD25).

To examine whether a similar population of activated CD4$^+$ T cells was present in the synovial tissues of rats with AA, a technique was developed that allowed isolation of dispersed cell suspensions from normal or arthritic rats. The hind paws were examined because the joints in them are affected most severely in AA. This technique allowed the surface phenotypes of T cells to be examined by flow cytometry.

Prior to day 9 after inoculation of CFA (i.e. control, day 3 and day 6), T cells accounted for approximately 0.1% of all cells harvested. This increased to approximately 0.5% at day 9 (when clinical signs of disease appear in some rats) and 2.0% at day 12 (when the arthritis is established). The CD4$^+$ T cells from control hind paws and those obtained from

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rats before the onset of disease were mostly MHC class II$^-$, IL-2R$^-$ and CD134$^-$. Approximately half expressed CD54 and/or CD45RC. Most of the CD4$^+$ cells obtained from animals 9 days after inoculation expressed an activated phenotype with expression of CD54, MHC class II and CD25. Furthermore, more than 70% were CD45RC$^-$, suggesting that the majority were activated, memory T cells. The proportion (and number) of CD45RC$^-$ cells increased by day 12, when less than 10% expressed CD45RC. The phenotype of the CD4$^+$ T cells in the arthritic paws is similar to that observed in the synovium of humans with RA, with the exception of the high proportion of CD25$^+$ cells. The relative absence of T cells from the synovial tissues before the initiation of AA, their accumulation during the prodrome and their activated phenotype in established arthritis is consistent with the hypothesis that T cells are effector cells involved in the pathogenesis of arthritis.

6.2 Results

6.2.1 Analysis of the change in weight and cellular composition of the hind paws from rats during the prodrome and early clinical phase of AA

The hind paws of DA rats contain the joints affected most commonly during AA. Joint swelling becomes visible around day 9 after inoculation of CFA and is moderate to severe by day 12. The changes in the lymphocyte populations in the soft tissues of hind paws from rats during the prodrome of AA was examined by preparing a single cell suspension as described in Section 2.3.2. It is important to note that the paws were prepared by dislocating the tibia at the knee, allowing removal of the soft tissue of the calf without breaking bones. This method allowed the entire hind paw to be separated from the rat whilst minimising the risk of contamination by bone marrow cells.

6.2.1.1 Weight of the hind paws from rats during the prodrome of AA

The hind paws from rats were skinned and muscles proximal to the bone were removed and each pair of paws was weighed. The mean mass of the hind paws of saline-challenged rats was greater than that of rats that had been challenged with CFA 3 or 6 days before being killed (Figure 6.2.1.1). This followed the decrease in the mean body weight of each rat following inoculation with CFA (discussed in 4.2.2.1). However, at days 9 and 12, the average weight of the hind paw pair had increased as a consequence of inflammation of the soft tissues, in particular the synovium.

Figure 6.2.1.1 Weights of the hind paws of rats during the prodrome of AA. The hind paws (distal to the knee, inclusive of the tibia and fibula) were removed from rats inoculated previously with CFA and the skin and large muscles removed before being weighed as a pair from each rat. Rats inoculated with saline were included as a control group. The bars indicate the mean weight for a pair of hind paws in each group, in which data was combined from the two separate experiments. The group size at each time after inoculation of CFA was 20 rats.
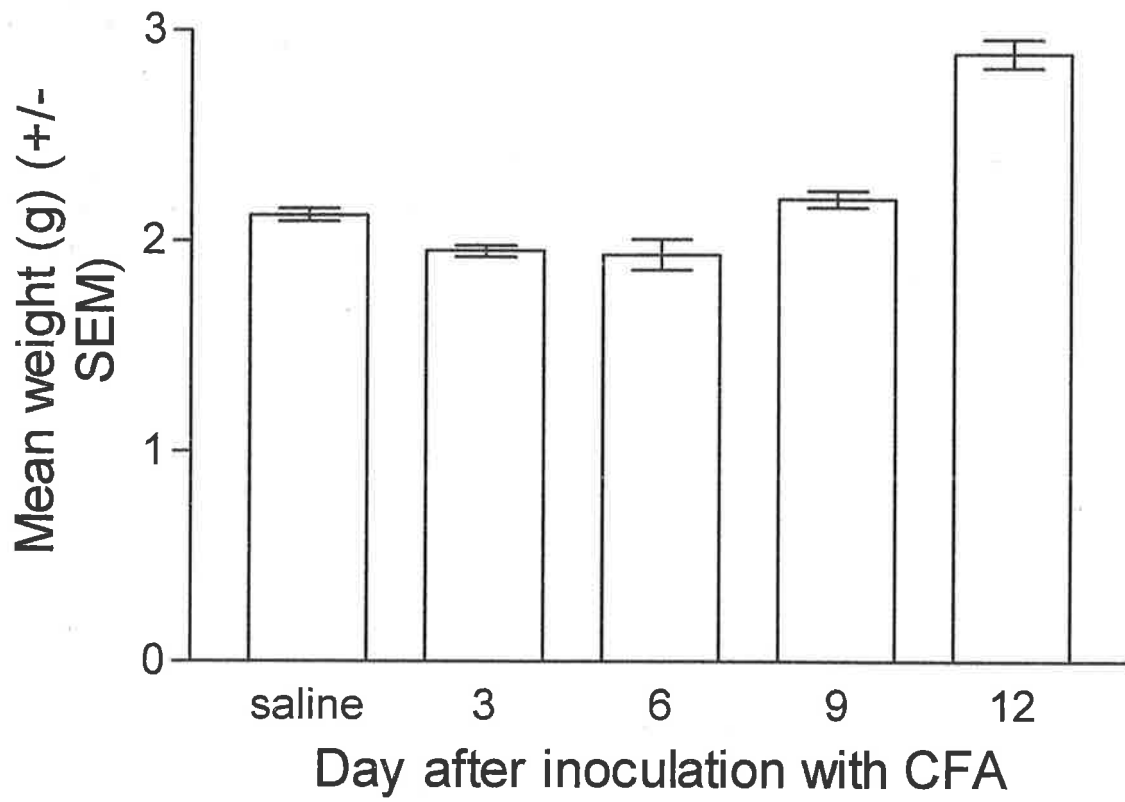
6.2.1.2 Preparation of hind paws for enzymatic digestion

The limbs were skinned and disarticulated at the knees. The tibia and fibulae were cleaned of soft tissues (a representative hind paw is shown in Figure 6.2.1.2 A) and the soft tissues of the ankle and feet were digested in collagenase and hyaluronidase (See Section 2.3.2; based a published technique described by DeJoy, et al (1988) and optimised to digest synovial fragments by Mrs. E. Farmer in our laboratory). This process of digestion yielded a single cell suspension that allowed the cells to be analysed using flow cytometry. Figure 6.2.1.2 B illustrates a pair of hind paws in a 20ml tube before digestion (left) and a pair of hind paws after digestion in a 20ml tube (right). Total cell yields were estimated using a haemocytometer and the relative proportions of lymphocyte subsets were established using flow cytometry.

6.2.1.3 Light scattering properties of cells yielded from enzymatic digestion of the hind paws from rats during the prodrome of AA

The physical characteristics of the cells liberated from the soft tissues of the hind paws using enzymatic digestion, were analysed using flow cytometry. In the shown in Figure 6.2.1.3, each cell is represented as an event, according to cell size (forward scatter, FSC, on the x axis) and complexity (side scatter, SSC, on the y axis). Cells and debris that had a forward scatter value less than a red blood cell (approximately 100 with the instrument settings used) were excluded from analysis. The distribution of events on the scatter plots changed during the prodrome of AA. At each of the time points, a large proportion of the events were clustered in the lower left hand corner of the plot and they represent small, simple events, attributable to red blood cells and debris from the enzymatic digestion.

The distribution of events seen in preparations from the hind paws from rats challenged with saline (control) and those at day 3 post-inoculation was similar. At day 6 post-inoculation, a population of cells with light scattering properties corresponding to granulocytes is apparent and this population is more obvious in the plots prepared from cells obtained 9 and 12 days post-inoculation.

The cluster of events with forward scatter values between 200 and 400 and side scatter values of less than 200 represents primarily lymphocytes (a gate of this population is shown in Figure 6.2.1.1 A). This cluster is denser in the plots representing samples of cells obtained 9 and 12 days after inoculation, compared with samples from normal hind

Figure 6.2.1.2 Isolation of cells by enzymatic digestion of the hind paws from rats during the prodrome of AA. The lower legs (distal to the knee, inclusive of the tibia and fibula) were removed from rats inoculated previously with CFA or saline. Muscle was removed from the tibia and fibula. A representative hind paw, prepared for digestion, is shown in A. A cell suspension was prepared by enzymatic digestion of the soft tissues associated with the isolated hind paws (as described in 2.3.2). The tube on the left of photograph B contains two hind paws prior to digestion and the tube on the right shows the cell suspension released by digestion
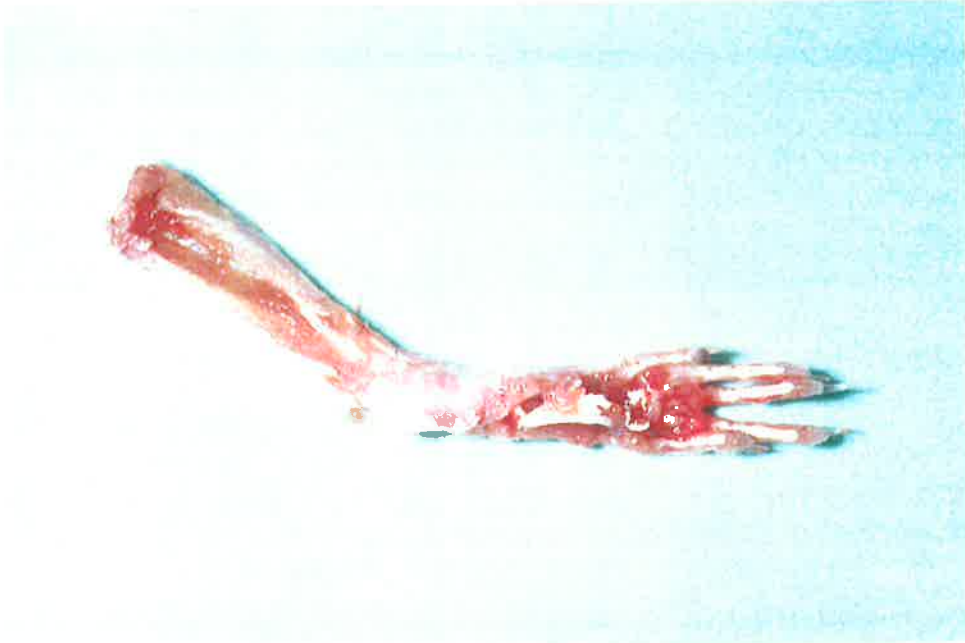
A

B

Figure 6.2.1.3 The light scattering properties of cells obtained by enzymatic digestion of the hind paws of rats during the prodrome of AA. A cell suspension was obtained by enzymatic digestion of the soft tissues from the hind paws of rats inoculated with either CFA or saline. The physical characteristics of the cells were examined using flow cytometry. The scatter plots represent cells from the hind paws of rats injected with saline (A) or those at 3 (B), 6 (C), 9 (D) or 12 (E) days post-inoculation of CFA. The size of the cells, which determines the forward scatter of light (FSC), is indicated on the x axis, and the internal complexity of the cells, which determines the side scatter of light (SSC) is represented on the y axis. Events representing particles larger than or equivalent in size to red blood cells (> FSC 100) were gated electronically for display. The lighter grey area at the bottom left of each plot represents the highest concentration of events. The events in this area correspond to red cells and larger pieces of debris produced during enzymatic digestion.
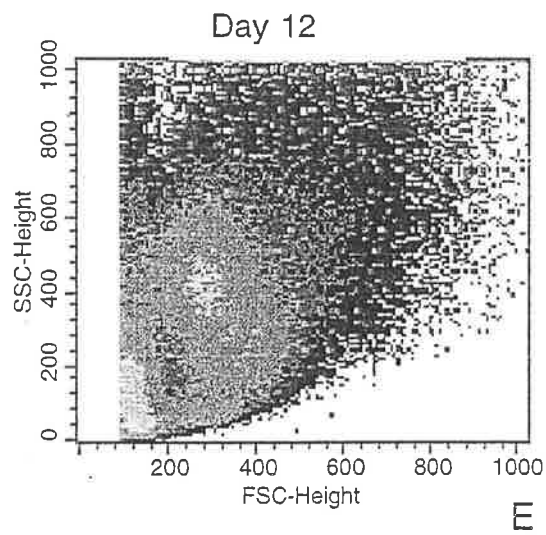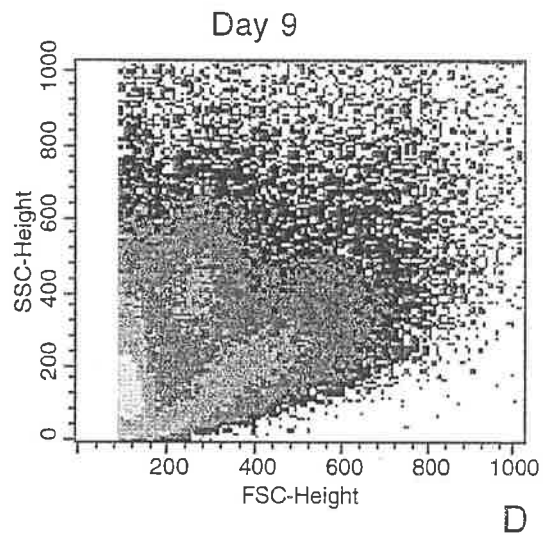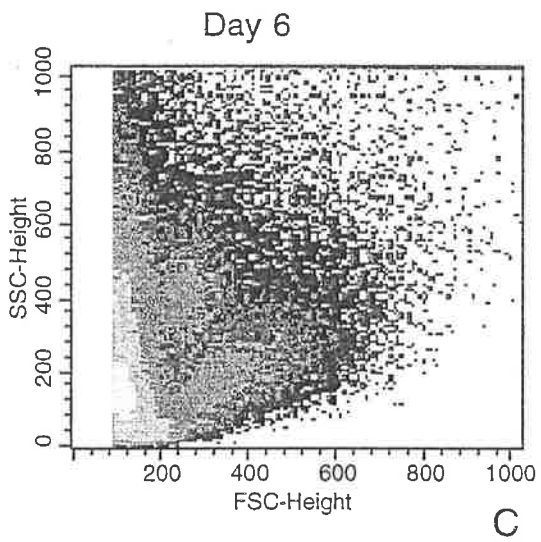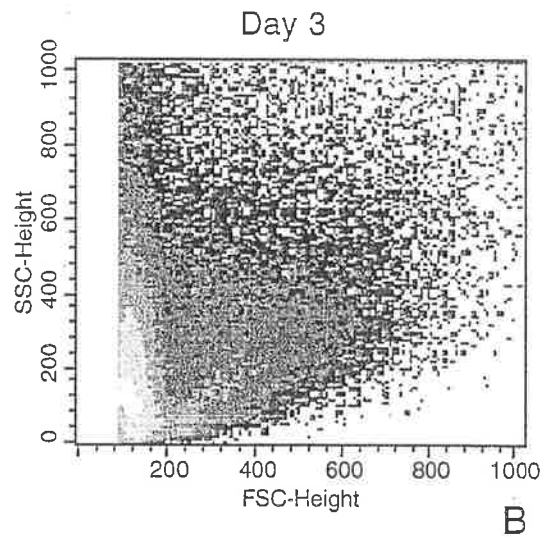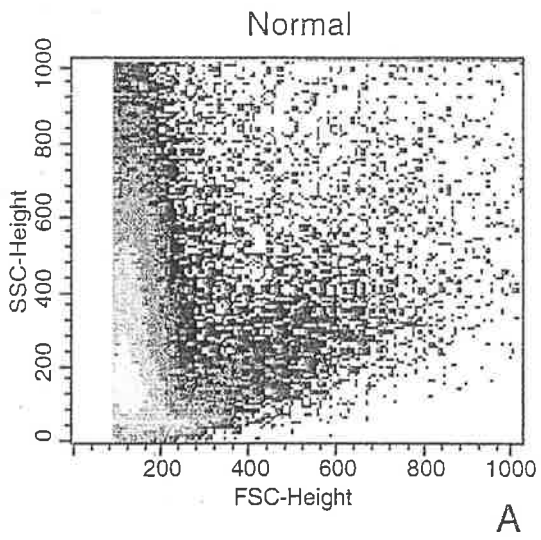
Normal

Day 3

Day 6

Day 9

Day 12

A

B

C

D

E

paws or those taken from rats at earlier stages during the prodrome of AA. This suggests that there are more lymphocytes in the paws at the end of the prodrome and during the early clinical stages of AA than in normal hind paws or during the early prodrome.

6.2.2 Comparison of T cell sub-populations in cells isolated from the hind paws during the prodrome of AA

The sub-populations of lymphocytes isolated from the hind paws during the prodrome of AA were examined using dual colour flow cytometry.

6.2.2.1 Analysis parameters

Electronic gating was used to select for events in the scatter plots that represent cells with the physical characteristics expected of lymphocytes. Staining of cells with mAbs to detect expression of CD4 and Mac-1 was performed to assist with the setting of gates and thresholds for analysis. Figure 6.2.2.1 A shows a scatter plot which includes a gate that was set to encompass both small and large lymphocytes. Figures 6.2.2.1 B to F show the fluorescence profiles of events gated within this area, comparing expression of CD4 (y axis) and Mac-1 (x axis). The horizontal divider on these plots was set to include the CD4^{high}Mac-1$^-$ cells (CD4$^+$ T cells) and exclude CD4$^{low/-}$Mac-1^{high} cells (macrophages). There is little or no difference in the proportion of CD4$^+$ T cells in the samples from the hind paws of normal rats or from rats 3 or 6 days after inoculation of CFA (1.0%, 1.4% and 1.2%, respectively; Figure 6.2.2.1 B, C and D). The proportion of CD4$^+$ T cells increased strikingly to 6.8% and 39.0% in the samples obtained from rats 9 and 12 days after inoculation (Figure 6.2.2.1 E and F). The importance of defining a lymphocyte gate is illustrated in Figure 6.2.2.1 G to L. When all events are included, it is clear that debris and dead cells stain non-specifically with both antibodies. There is also a population of CD4^{high} Mac-1^{high} events, which are α/β TCR$^-$ (not shown). The nature of these events, which are excluded from the lymphocyte gate, was not explored further. They may be either macrophages or dendritic cells, in which larger cell size and/or autofluorescence contributes to the signal obtained in fluorescence 2 channel.

6.2.2.2 CD4$^+$ T cells

The proportion of gated cells (see Section 6.2.2.1) that are CD4$^+$ T cells was evaluated using dual colour staining for CD4 and α/β TCR expression (Figure 6.2.2.2 A and B).

Figure 6.2.2.1 Parameters used to define a "lymphocyte gate" during flow cytometric analysis of cells isolated from hind paws of rats during the prodrome of AA. A cell suspension was obtained by enzymatic digestion of the soft tissues from the hind paws of rats inoculated with either CFA or saline. These cells were first stained indirectly with mAb WT-5 (anti-Mac-1; FITC), followed by direct labelling with mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of all (A, G) or gated (all others) events from rats inoculated either with saline (A, B, G, H) or CFA 3 (C, I), 6 (D, J), 9 (E, K) or 12 days (F, L) before harvesting the hind paws. The size of the cells, which determines the forward scatter of light (FSC), is indicated on the x axis, and the internal complexity of the cells, which determines the side scatter of light (SSC) is represented on the y axis. The gates shown in A and G indicate those used to electronically segregate those cells displayed in panels B-F and H-L respectively.

Day 12

Day 3

Day 6

Day 9

Day 12

Normal

Day 12

Day 3

Day 9

Normal

Day 6

Day 12
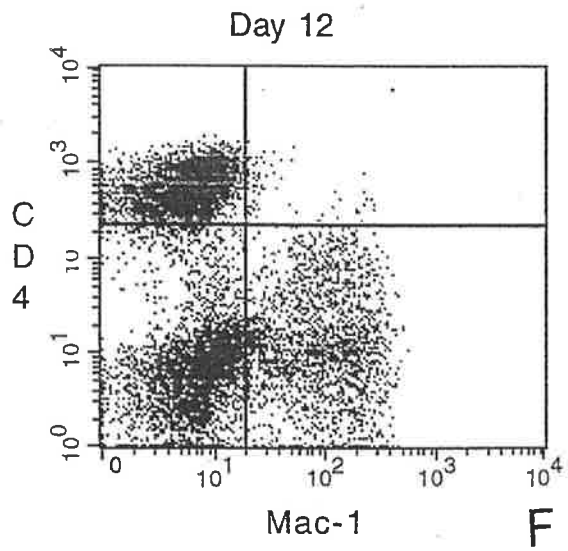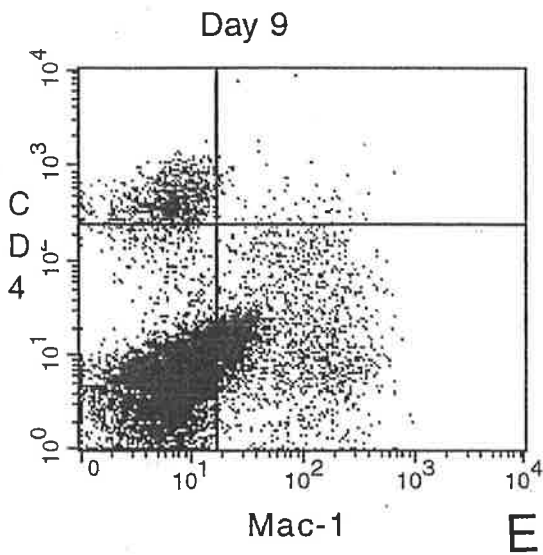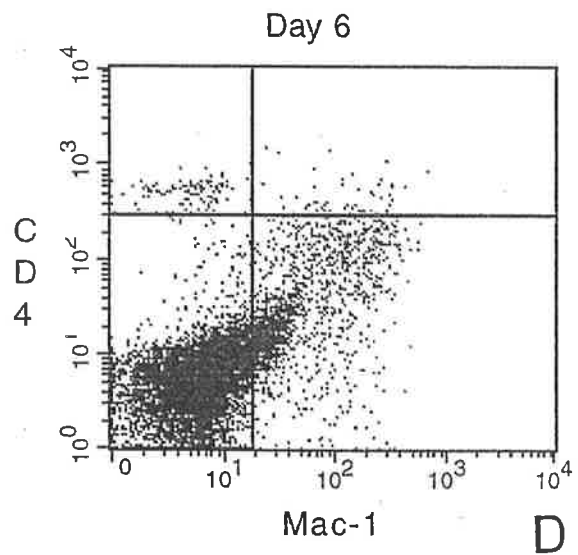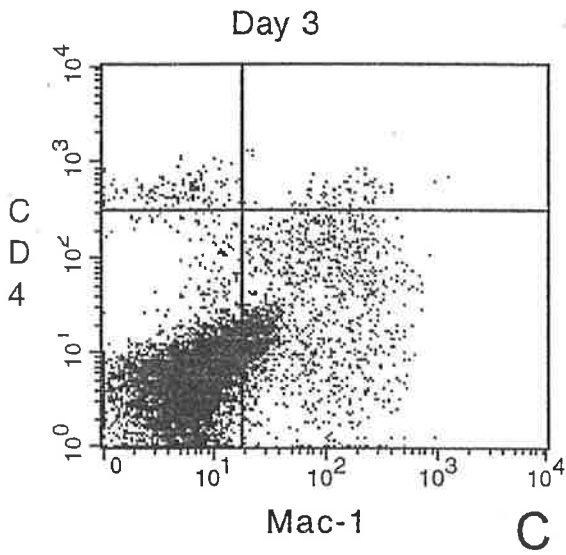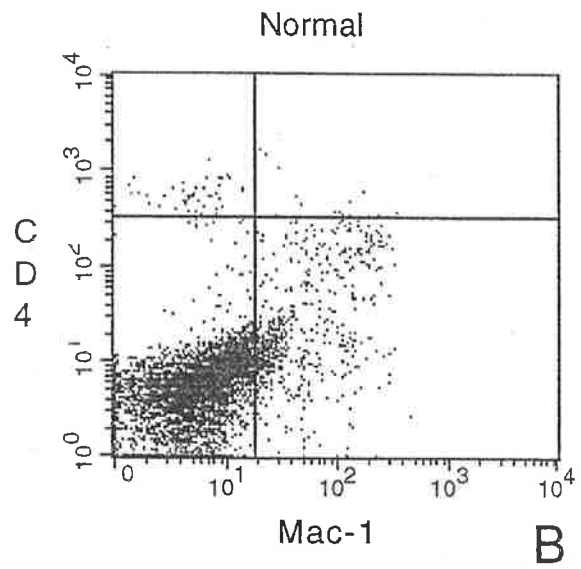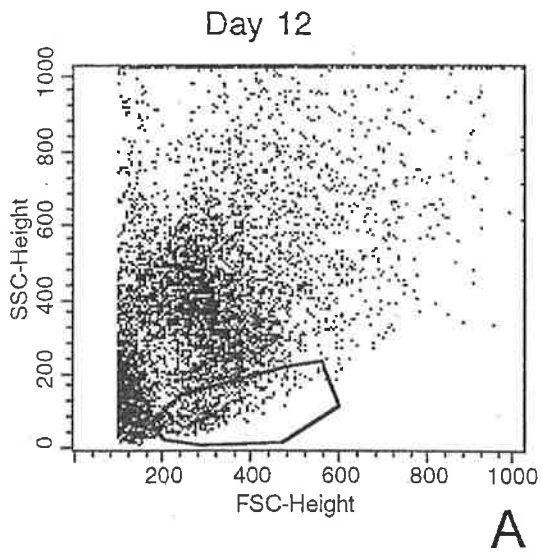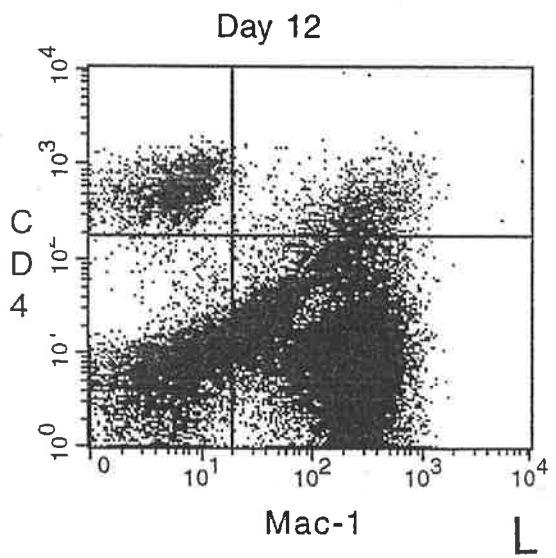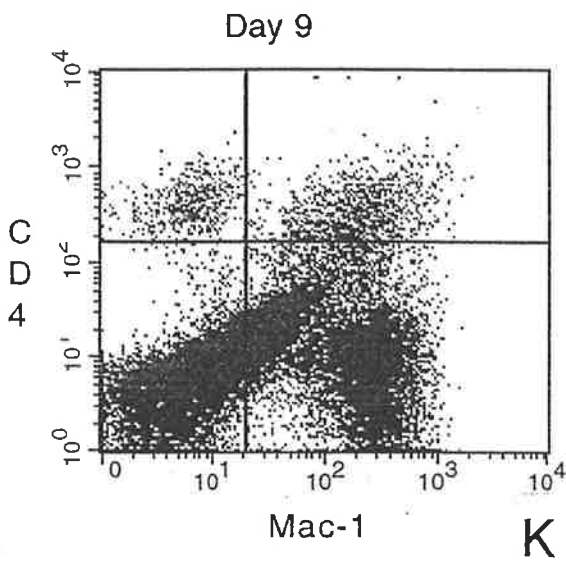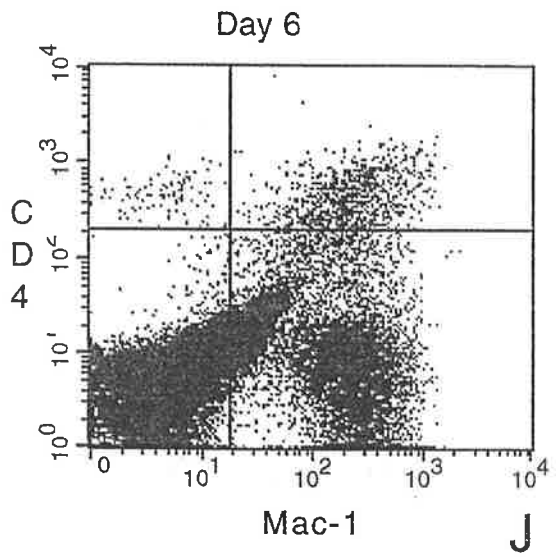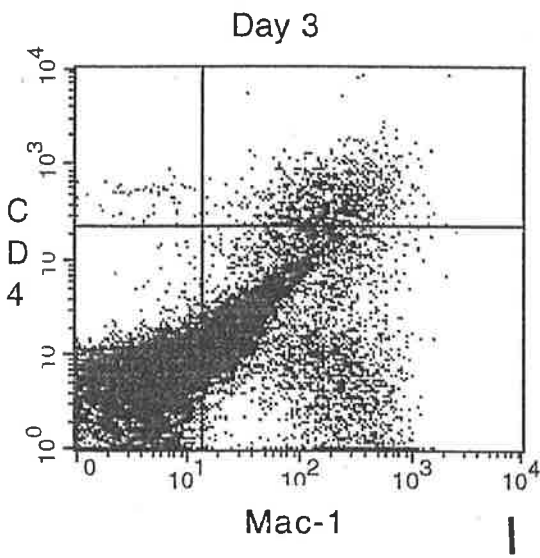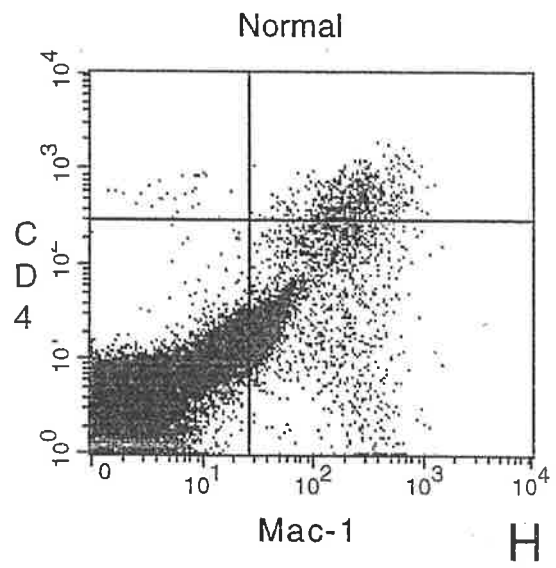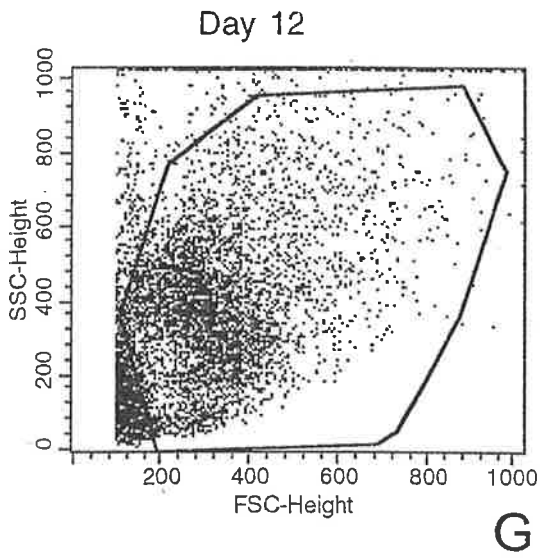
Figure 6.2.2.2 CD4$^+$ T cells from the hind paws of rats in the prodrome of AA. A cell suspension was obtained by enzymatic digestion of the soft tissues from the hind paws of rats inoculated with either CFA or saline. These cells were first stained indirectly with mAb W3/25 (anti-CD4; FITC) and then directly using mAb R73 (anti-α/β TCR; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The proportion of gated events that expressed CD4 and α/β TCR at each time point was calculated using data obtained by flow cytometric analysis (C). Each triangle indicates the proportion of CD4$^+$ T cells in the lymphocyte gate when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.
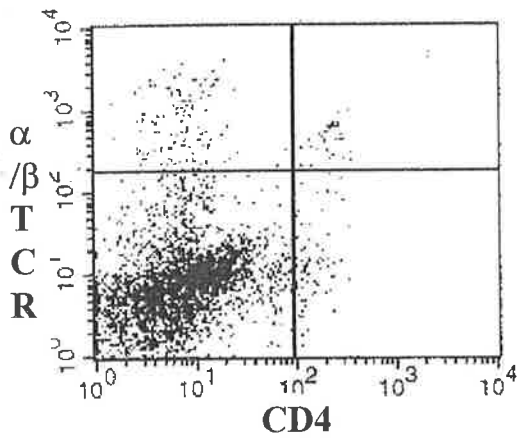
A
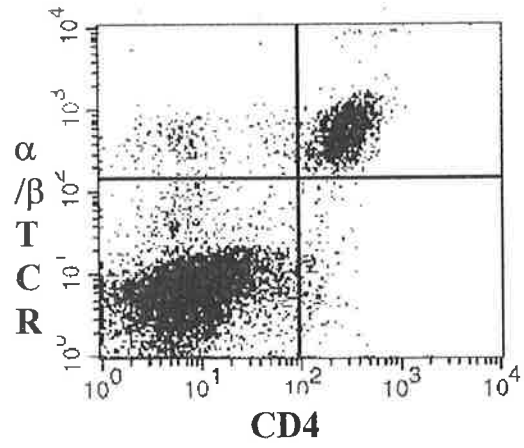
B
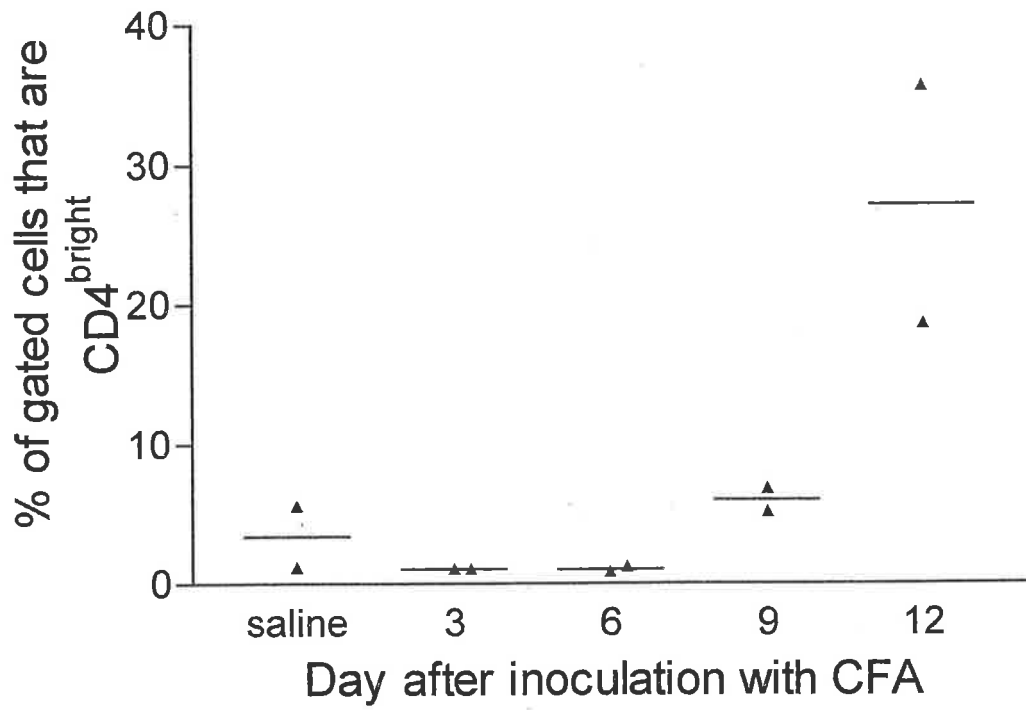
C

CD4$^+$ T cells constitute approximately 1% of events in the lymphocyte gate when examining cells from digested hind paws of control rats and rats in the prodromal period (3 and 6 days post-inoculation) of AA (Figure 6.2.2.2 C). There was a striking increase in the proportion of CD4$^+$ T cells in the lymphocyte gate when preparations were obtained from the hind paws of rats at the onset of visible joint inflammation (approximately 27%, day 12 post-inoculation). In the upper left quadrants of the plots shown in Figure 6.2.2.2 A and B, there are events which are likely to include CD8$^+$ α/β TCR$^+$ cells as well as some other unidentified cells, which may have been stained non-specifically.

6.2.2.3 CD8$^+$ T cells

CD8$^+$ T cells are found at a lower frequency than CD4$^+$ T cells in hind paw preparations. In preparations from control paws, CD8$^+$ T cells represented approximately 1% of the lymphocyte gate (Figure 6.2.2.3 C). On day 12 after inoculation of CFA, approximately 1% of gated events represent cells that express both the α and β chains of CD8 (classical CD8$^+$ T cells; Figure 6.2.2.3 C). The events in the upper left hand quadrant of Figure 6.2.2.3 B represent cells that express CD8α but not CD8β, presumably γ/δ T cells, which can express a CD8 α/α homodimer. In addition, the relative abundance of events in this quadrant suggests that cells of unknown identity may have been labelled non-specifically. At days 9 and 12 post-inoculation, these cells were approximately 30 times less frequent than CD4$^+$ T cells. The relative paucity of CD8$^+$ T cells in the synovial tissues from patients with RA has been observed by others (Kurosaka et al., 1983; Cush and Lipsky, 1988).

6.2.2.4 γ/δ T cells

When analysing cells obtained from the hind paws of normal rats or from rats in the prodrome of AA (Figure 6.2.2.4 C), γ/δ T cells usually represented less than 1% of gated events. There was no consistent change in the proportion of γ/δ T cells during the development of AA.

6.2.3 Expression of activation markers by CD4$^+$ T cells during the prodrome of AA

The expression of activation markers by CD4$^+$ T cells was examined using dual colour flow cytometry. As discussed above, there is a scarcity of CD4$^+$ T cells in the hind paws of normal rats and those from rats in the early prodrome of AA (3 and 6 days after

Figure 6.2.2.3 CD8$^+$ T cells from the hind paws of rats in the prodrome of AA. Cells obtained by the enzymatic digestion of the soft tissues of hind paws were first stained indirectly with mAb 341 (anti-CD8β; FITC) and then directly using mAb OX8 (anti-CD8α; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The proportion of gated events that expressed both CD8α and CD8β at each time point was calculated using data obtained by flow cytometric analysis (C). Each triangle indicates the proportion of CD8$^+$ T cells in the lymphocyte gate when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.

A

B

C

Figure 6.2.2.4 γ/δT cells from the hind paws of rats in the prodrome of AA. Cells obtained by the enzymatic digestion of the soft tissues of hind paws were first stained indirectly with mAb V65 (anti-γ/δ TCR; FITC) and then directly using mAb OX8 (anti-CD8α; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The proportion of gated events that expressed γ/δ TCR at different times after inoculation of CFA was calculated using data generated by flow cytometric analysis (C). Each triangle indicates the proportion of γ/δ T cells in the lymphocyte gate when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.

A

B

C

inoculation with CFA), reducing the accuracy of the analysis at these time points. One hundred thousand events were usually collected from each sample and since in samples from normal rats and rats in the early prodrome CD4$^+$ T cells represented less than 0.1% of the total events, fewer than 100 CD4$^+$ events were analysed for the expression of each activation marker. However, CD4$^+$ T cells were found in greater abundance at days 9 and 12 after inoculation of CFA. As a consequence, there was less variation between samples at these later time points.

6.2.3.1 MHC class II

The expression of MHC class II by CD4$^+$ T cells is associated with an activated phenotype. It was of interest that of the CD4$^+$ T cells found in hind paws of normal rats, between 8% and 24% of these cells expressed MHC class II (Figure 6.2.3.1 C). This indicates that under normal conditions, the population of tissue migratory CD4$^+$ T cells contains recently activated cells. Similar frequencies of MHC class II$^+$ cells were found in the CD4$^+$ T cell populations obtained from the hind paws of rats on days 3 and 6 after inoculation with CFA (approximately 28% and 20%, respectively; Figure 6.2.3.1 C), indicating that there is not a change in the proportion of activated CD4$^+$ T cells during the early prodromal period of AA.

Approximately half of the CD4$^+$ T cells from the hind paws of rats inoculated with CFA 9 days earlier expressed MHC class II (Figure 6.2.3.1 C). This proportion decreased to approximately 35% by day 12 post-inoculation. Interestingly, the proportion of CD4$^+$ T cells that expresses MHC class II during the clinical phase of AA is within the range of expression of HLA-DR (19%-52%) observed by Cush and Lipsky when examining the phenotype of CD4$^+$ T cells in the synovium of patients with RA (Cush and Lipsky, 1988). A similar decrease in the proportion of CD4$^+$ T cells that expressed MHC class II was observed between 9 and 12 days post-inoculation in the TD lymph (see Figure 5.2.3.1 D-F).

6.2.3.2 IL-2 receptor α chain (CD25)

Approximately 13% and 11% of CD4$^+$ T cells obtained from the hind paws of saline-injected rats or from rats 3 days after inoculation of CFA respectively expressed CD25, the IL-2 receptor α chain (Figure 6.2.3.2 C). This proportion increased to approximately

Figure 6.2.3.1 Expression of MHC class II by CD4$^+$ T cells from the hind paws of rats in the prodrome of AA. Cells obtained by the enzymatic digestion of the soft tissues of hind paws were first stained indirectly with mAb OX6 (anti-MHC class II; FITC) and then directly using mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The proportion of CD4$^+$ T cells in the lymphocyte gate that expressed MHC class II at different times after inoculation of CFA was calculated using data generated by flow cytometric analysis (C). Each triangle indicates the proportion of CD4$^+$ T cells in the lymphocyte gate that expressed MHC class II when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.

CD4 / MHC class II

A

B

% of CD4$^+$ T cells expressing MHC class II

Day after inoculation with CFA

saline   3   6   9   12
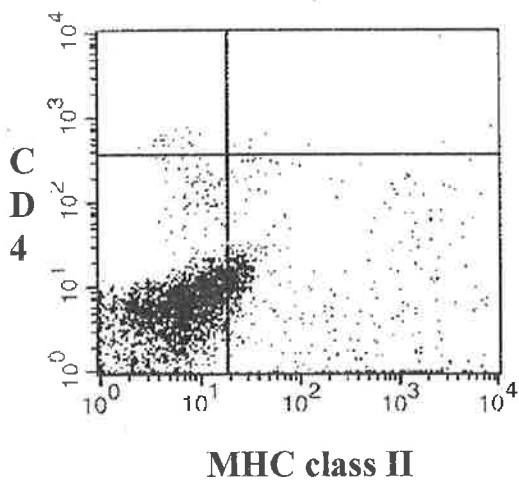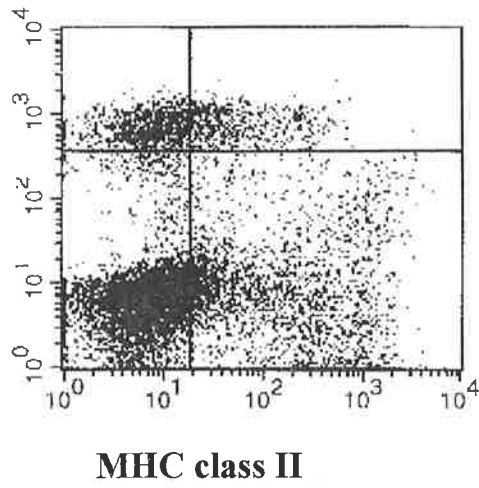
C

Figure 6.2.3.2 Expression of IL-2 receptor by CD4$^+$ T cells from the hind paws of rats in the prodrome of AA. Cells obtained by the enzymatic digestion of the soft tissues of hind paws were first stained indirectly with mAb OX39 (anti-IL-2Rα [CD25]; FITC) and then directly using mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The proportion of CD4$^+$ T cells in the lymphocyte gate that expressed CD25 at different times after inoculation of CFA was calculated using data generated by flow cytometric analysis (C). Each triangle indicates the proportion of CD4$^+$ T cells in the lymphocyte gate that expressed CD25 when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.

A

B

C

Figure axes (Panel C):
% of CD4⁺ T cells expressing CD25 (y-axis, 0 to 100)
Day after inoculation with CFA (x-axis: saline, 3, 6, 9, 12)

27% of the CD4$^+$ T cells obtained from the hind paws of rats 6 days after inoculation of CFA.

At the onset of visible signs of joint inflammation (day 9 after inoculation), approximately 58% of CD4$^+$ T cells in the hind paws expressed CD25. Increased disease severity was observed by day 12 post-inoculation and at this time point, approximately 75% of the CD4$^+$ T cells in the hind paws expressed CD25 (Figure 6.2.3.2 C). This increase in the proportion of CD4$^+$ T cells that expressed CD25 at 12 days post-inoculation compared with 9 days post-inoculation is the opposite of that observed in the TD lymph. A greater proportion of CD4$^+$ T cells isolated from the TD lymph at 9 days post-inoculation expressed CD25 compared to those isolated at 12 days post-inoculation (see Figure 5.2.3.2 D-F). This may reflect either the local activation/re-activation of cells that had arrived recently from the TD lymph via the blood or the selective recruitment of CD25$^+$ CD4$^+$ T cells from the blood.

6.2.3.3 Transferrin receptor (CD71)

The expression of transferrin receptor (CD71) is an indication of cells that are actively dividing (Salmeron et al., 1995). CD71 is expressed by between 1% and 10% of CD4$^+$ T cells in the hind paws of normal rats. Less than 20% of CD4$^+$ T cells from the hind paws from rats in the early prodrome of AA expressed CD71 (days 3 and 6 post-inoculation), whereas at the end of the prodrome (day 9 post-inoculation), approximately 30% of the CD4$^+$ T cells expressed CD71 (Figure 6.2.3.3 C). Interestingly, fewer CD4$^+$ T cells from the hind paws from rats with established arthritis (day 12 post-inoculation) expressed CD71. Approximately 8.5% of CD4$^+$ T cells from the hind paws expressed CD71 at this later time, when disease is moderate to severe.

6.2.3.4 OX40 antigen (CD134)

OX40 antigen (CD134) is expressed on activated CD4$^+$ T cells (Paterson et al., 1987; Mallett et al., 1990). A few CD4$^+$ T cells from the hind paws of normal rats (approximately 2%) expressed this molecule. The frequency of CD4$^+$ T cells that expressed CD134 was approximately 7% at day 3 post-inoculation and varied from 5-7% throughout the development of AA (Figure 6.2.3.4 C).

6.2.3.5 UA002 antigen

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**Figure 6.2.3.3 Expression of Transferrin receptor by CD4$^+$ T cells from the hind paws of rats in the prodrome of AA.** Cells obtained by the enzymatic digestion of the soft tissues of hind paws were first stained indirectly with mAb OX26 (anti-transferrin R (CD71); FITC) and then directly using mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The proportion of CD4$^+$ T cells in the lymphocyte gate that expressed CD71 at different times after inoculation of CFA was calculated using data generated by flow cytometric analysis (C). Each triangle indicates the proportion of CD4$^+$ T cells in the lymphocyte gate that expressed CD71 when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.

A

B

C

% of CD4+ T cells expressing CD71

Day after inoculation with CFA
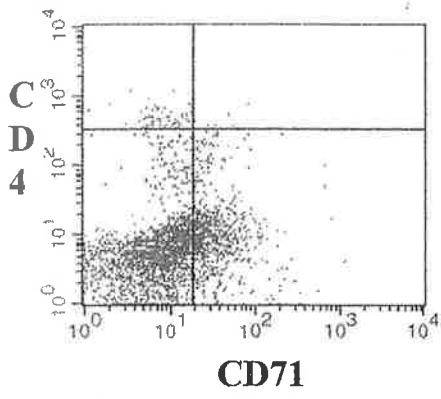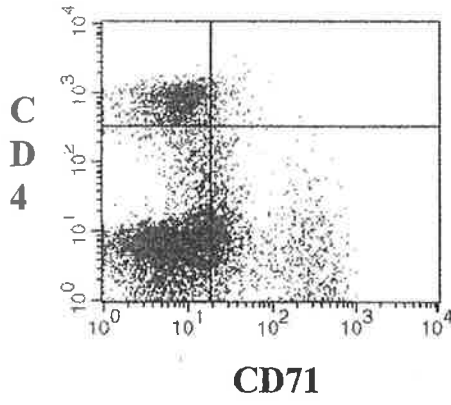
Figure 6.2.3.4 Expression of OX40 antigen by CD4$^+$ T cells from the hind paws of rats in the prodrome of AA. Cells obtained by the enzymatic digestion of the soft tissues of hind paws were first stained indirectly with mAb OX40 (anti-CD134; FITC) and then directly using mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The proportion of CD4$^+$ T cells in the lymphocyte gate that expressed CD134 at different times after inoculation of CFA was calculated using data generated by flow cytometric analysis (C). Each triangle indicates the proportion of CD4$^+$ T cells in the lymphocyte gate that expressed CD134 when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.

A

B

C

% of CD4$^+$ T cells expressing CD134

100

75

50

25

0

saline   3   6   9   12

Day after inoculation with CFA
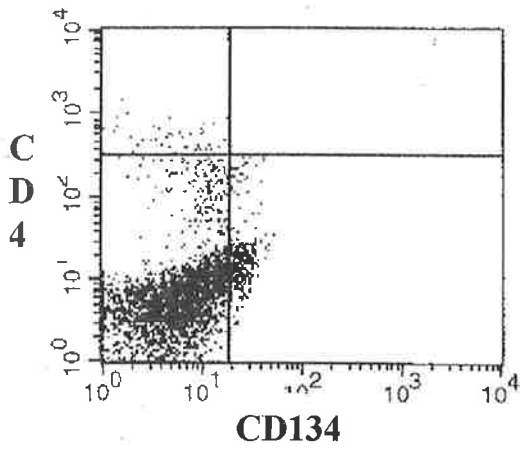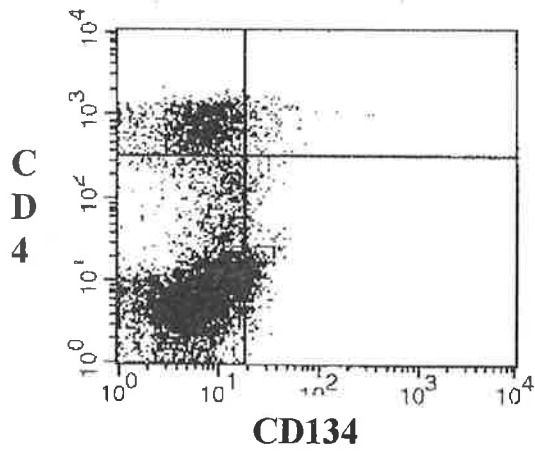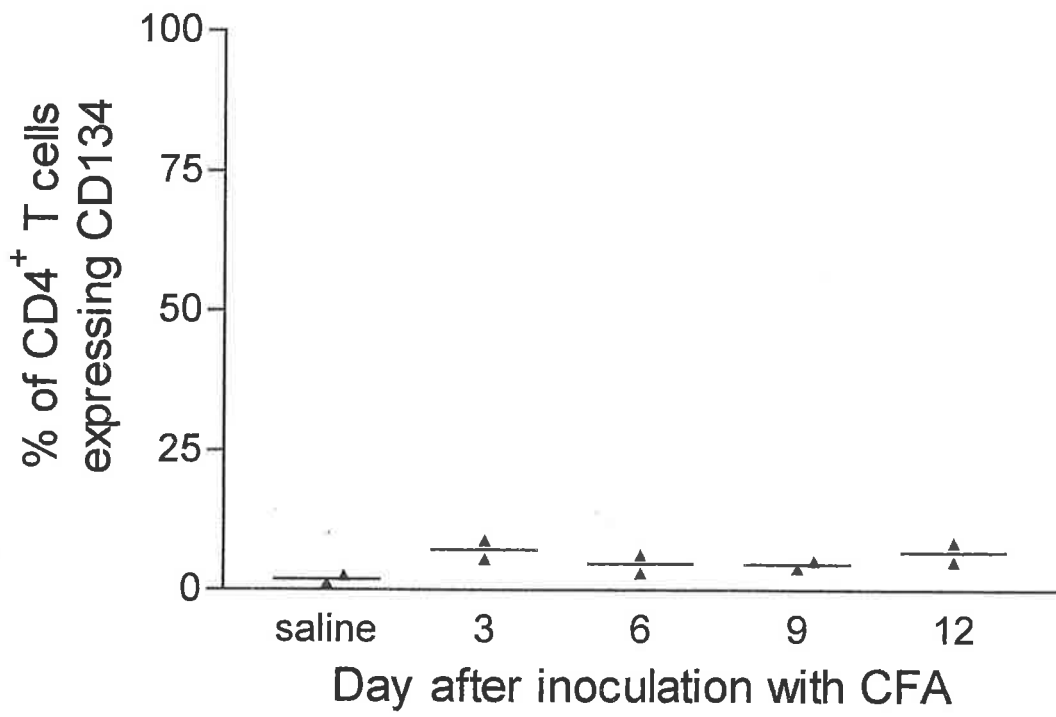
As mentioned in the previously (see Sections 4.2.3.5 and 5.2.3.5), the UA002 antigen is expressed by T cells activated by stimulation *in vitro*. For reasons that are not clear, a large difference in the proportion of UA002 antigen$^+$ CD4$^+$ T cells was observed between two pools of cells obtained from the hind paws of normal donors. Approximately 40% of the CD4$^+$ T cells from the hind paws of rats 3, 6 or 9 days after inoculation of CFA expressed this molecule (Figure 6.2.3.5 C). In cells prepared from the hind paws of rats with moderate to severe arthritis (day 12 after inoculation of CFA), the proportion of the CD4$^+$ T cells that expressed UA002 antigen had fallen to 25%.

6.2.3.6 CD45RC

The expression of a high molecular weight isoform of CD45 (CD45RC in the rat) is associated with naivete (Luqman and Bottomly, 1992). Between 40% and 70% of CD4$^+$ T cells from the hind paws of normal rats and rats in the early prodrome of AA (days 3 and 6) expressed CD45RC. The proportion of CD4$^+$ T cells that expressed CD45RC was much lower (approximately 15%) in the hind paws from rats challenged with CFA 9 days earlier. In animals with moderate to severe arthritis (12 days after inoculation with CFA), very few CD4$^+$ T cells expressed CD45RC (Figure 6.2.3.6 C). This indicates that although "naïve" CD4$^+$ T cells are present in the hind paws of normal rats and those in the prodrome of AA, such cells are rare in established disease.

6.2.4 Expression of adhesion molecules by CD4$^+$ T cells obtained from the hind paws during the prodrome of AA

As described in Section 6.2.2.2, CD4$^+$ T cells represent a minor population in the total cells digested from the soft tissues of the hind paws of healthy (saline-injected) rats and from rats in the early prodrome of AA. As a consequence, even when 100 000 cells were analysed, only approximately 100 were CD4$^+$ T cells. As a consequence, there is considerable variation between groups at these early time points. At days 9 and 12 post-inoculation, there were substantial numbers of CD4$^+$ T cells present and the results at these time points are more reliable statistically. Leukocyte function antigen (LFA)-1 (CD11a [integrin α_L subunit] / CD18 [integrin β_2 subunit] heterodimer) was expressed by all CD4$^+$ T cells (data not shown).

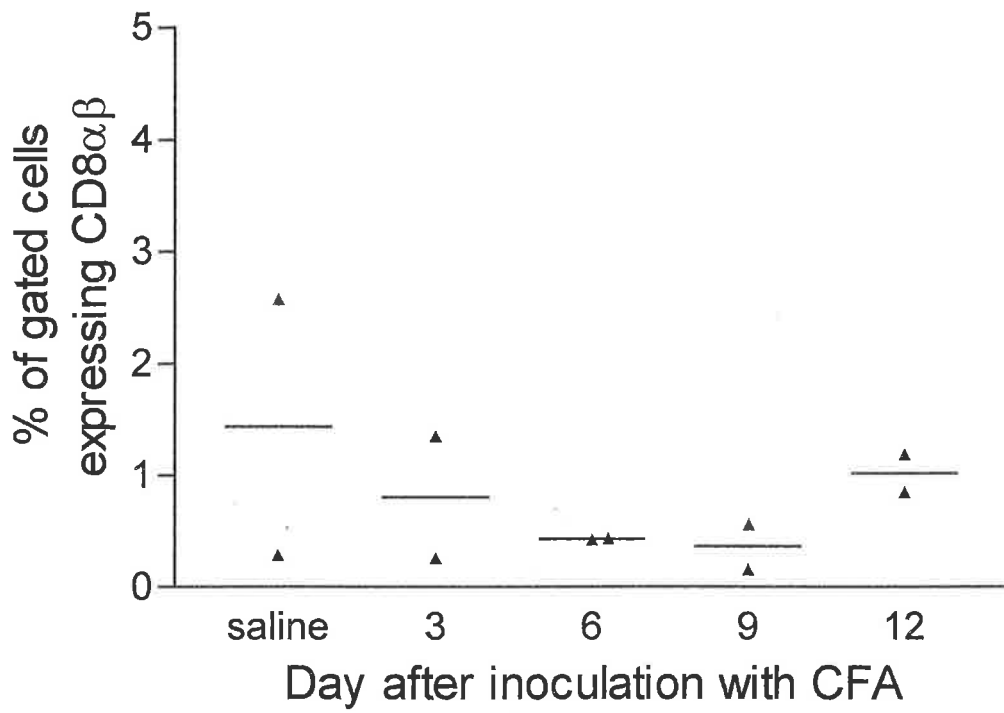### 6.2.4.1 L-selectin (CD62L)

Figure 6.2.3.5 Expression of UA002 antigen by CD4$^+$ T cells from the hind paws of rats in the prodrome of AA. Cells obtained by the enzymatic digestion of the soft tissues of hind paws were first stained indirectly with mAb UA002 (anti-UA002 antigen; FITC) and then directly using mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The proportion of CD4$^+$ T cells in the lymphocyte gate that expressed UA002 antigen at different times after inoculation of CFA was calculated using data generated by flow cytometric analysis (C). Each triangle indicates the proportion of CD4$^+$ T cells in the lymphocyte gate that expressed UA002 antigen when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.
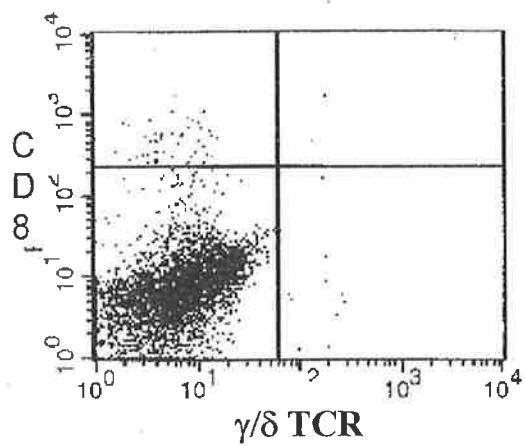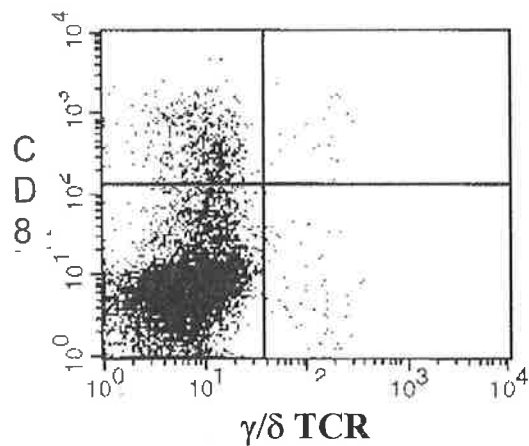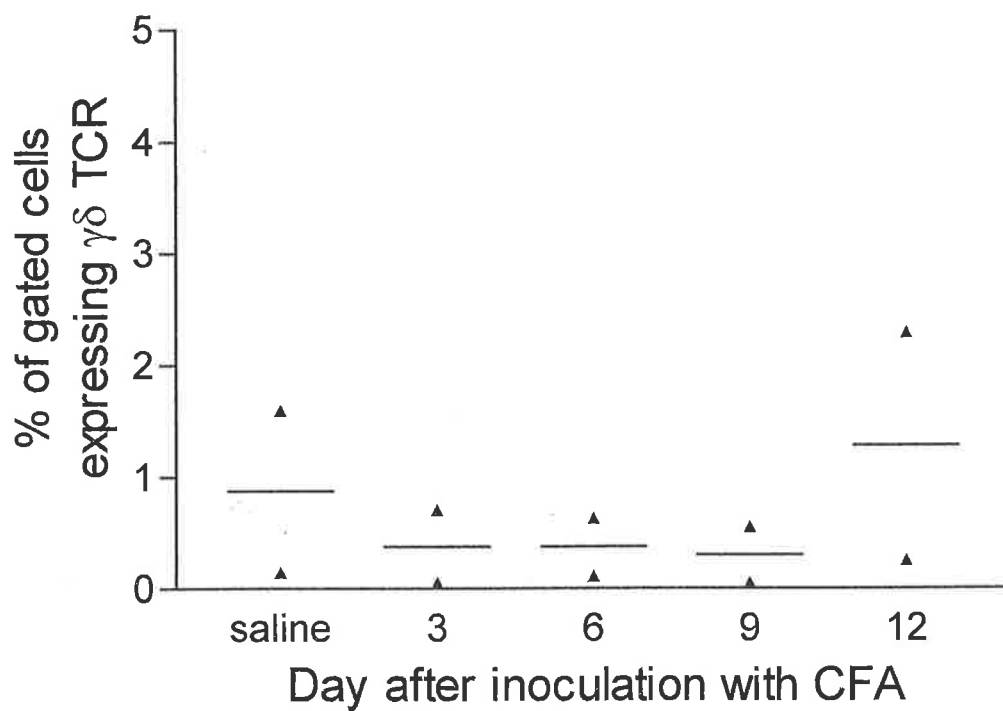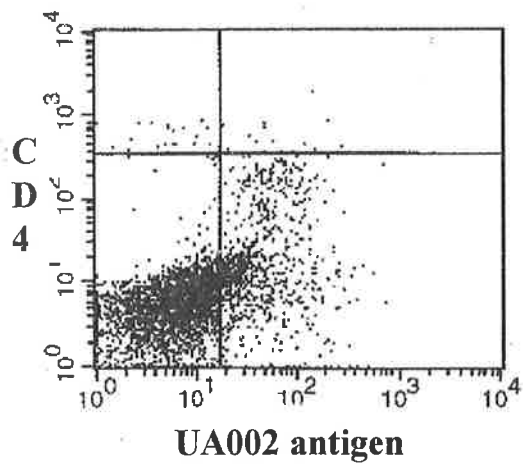
A
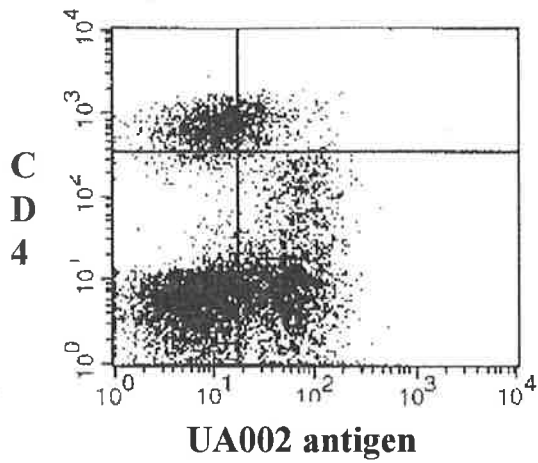
B
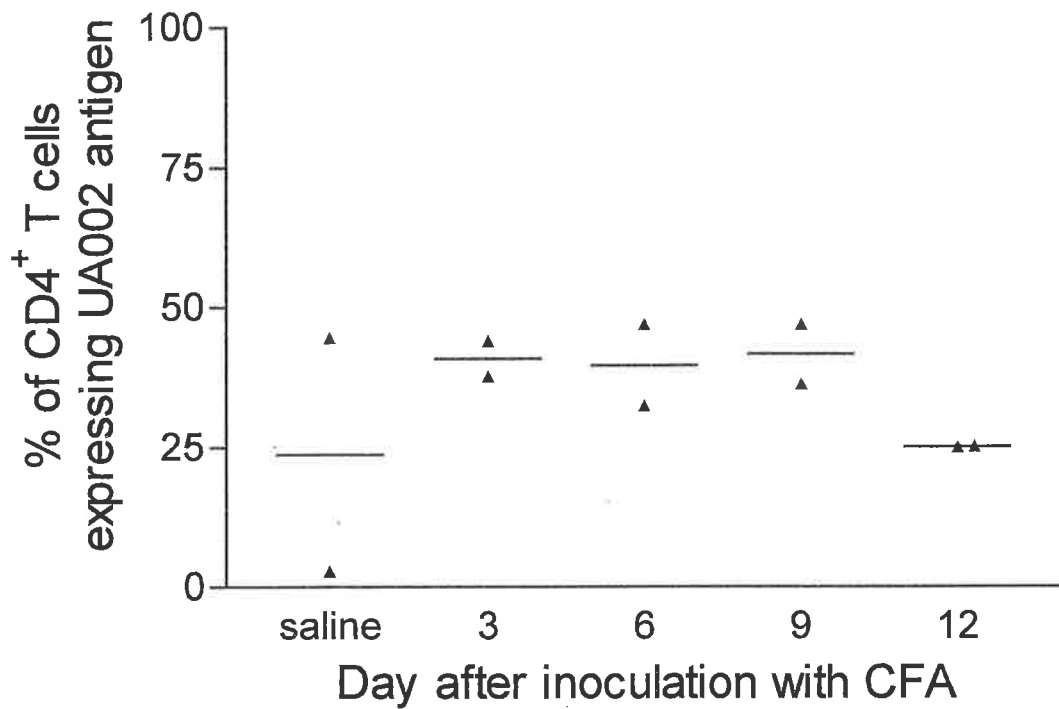
C

Figure 6.2.3.6 Expression of CD45RC by CD4⁺ T cells from the hind paws of rats in the prodrome of AA. Cells obtained by the enzymatic digestion of the soft tissues of hind paws were first stained indirectly with mAb OX22 (anti- CD45RC; FITC) and then directly using mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The proportion of CD4⁺ T cells in the lymphocyte gate that expressed CD45RC at different times after inoculation of CFA was calculated using data generated by flow cytometric analysis (C). Each triangle indicates the proportion of CD4⁺ T cells in the lymphocyte gate that expressed CD45RC when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.

A

B

C

% of CD4$^+$ T cells expressing CD45RC

Day after inoculation with CFA
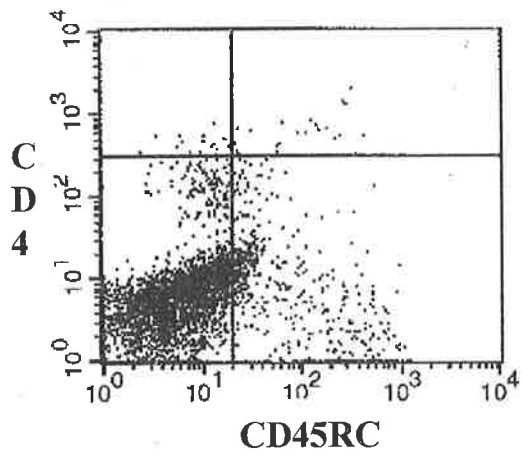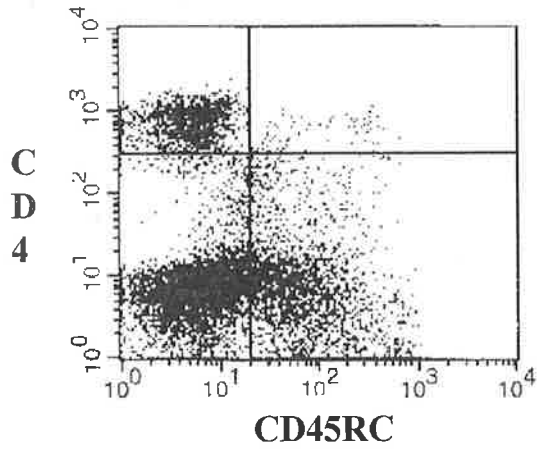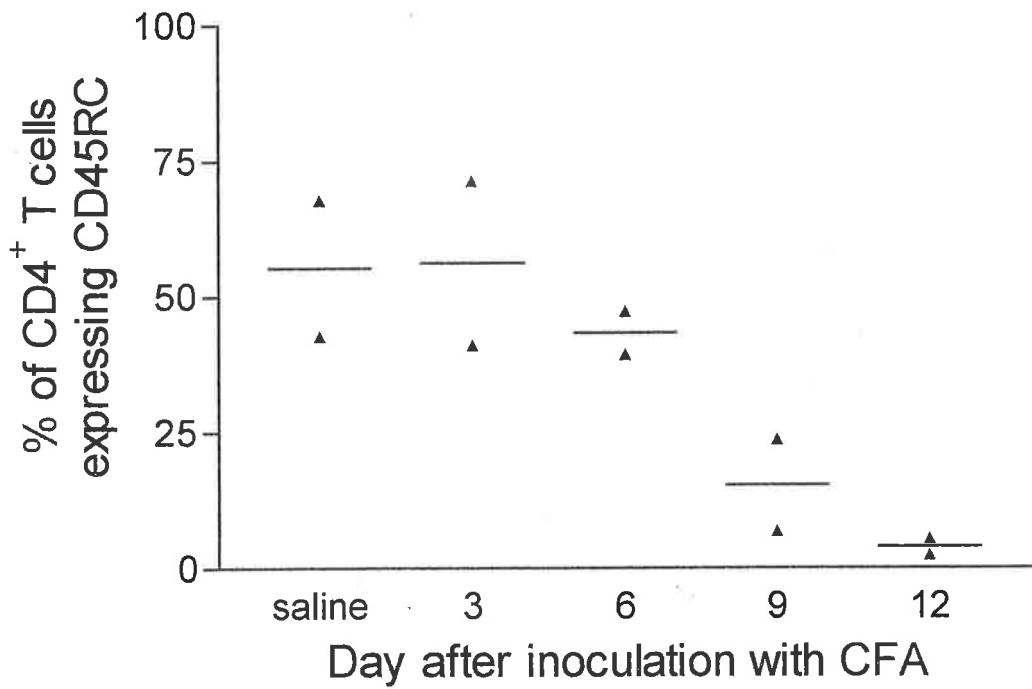
The expression of L-selectin (CD62L) is associated with naïve CD4$^+$ T cells and expression is lost after activation (Bradley et al., 1991). In the hind paws of saline-inoculated rats and those at days 3-6 post-inoculation of CFA, between 3 and 15% of CD4$^+$ T cells expressed CD62L (Figure 6.2.4.1 C). At the onset of clinical signs of joint inflammation (day 9 post-inoculation) and in established arthritis (day 12), very few CD4$^+$ T cells in the hind paws expressed CD62L (Figure 6.2.4.1 A-C). At day 9 post-inoculation, approximately 2.5% of CD4$^+$ T cells were CD62L$^+$, while at day 12, only 1% expressed this molecule. At all times, the expression of CD62L was at low levels (B), compared with that seen on CD4$^+$ T cells from the TD lymph (Figure 5.2.4.1 A-C).

6.2.4.2 α_{E2} integrin

The proportion of CD4$^+$ T cells that expressed α_{E2} integrin varied between 2 and 10% in the cell preparations from the hind paws of both saline-challenged and CFA-challenged rats. There was no clear variation associated with either the prodromal period or the period of active disease (Figure 6.2.4.2).

6.2.4.3 α_4 integrin (CD49d)

Substantial variation was observed when examining the expression of α_4 integrin (CD49d) by CD4$^+$ T cells isolated from the hind paws of rats inoculated with either saline or CFA (Figure 6.2.4.3 C). It is clear that many CD4$^+$ T cells expressed CD49d at low levels (B) making the estimation of the proportion of labelled cells very sensitive to the level of fluorescence set as background. Nevertheless, it is clear that in some animals, the proportion of CD4$^+$ T cells expressing α_4 integrin increased during the course of AA, in particular during the period of clinical disease (days 9-12 post-inoculation).

6.2.4.4 ICAM-1 (CD54)

Very few circulating T cells in rats express detectable levels of ICAM-1 (CD54). In the hind paws from normal rats, approximately 50% of the CD4$^+$ T expressed CD54. During the prodromal phase (days 3 and 6 post-inoculation), it was a consistent finding that more than 50% of the CD4$^+$ T cells from the hind paws expressed the molecule. At the onset of arthritis (day 9 post-inoculation), approximately 78% of CD4$^+$ T cells expressed CD54 and a similar proportion was observed in rats with moderate arthritis (day 12 post-inoculation) (Figure 6.2.4.4 A-C).
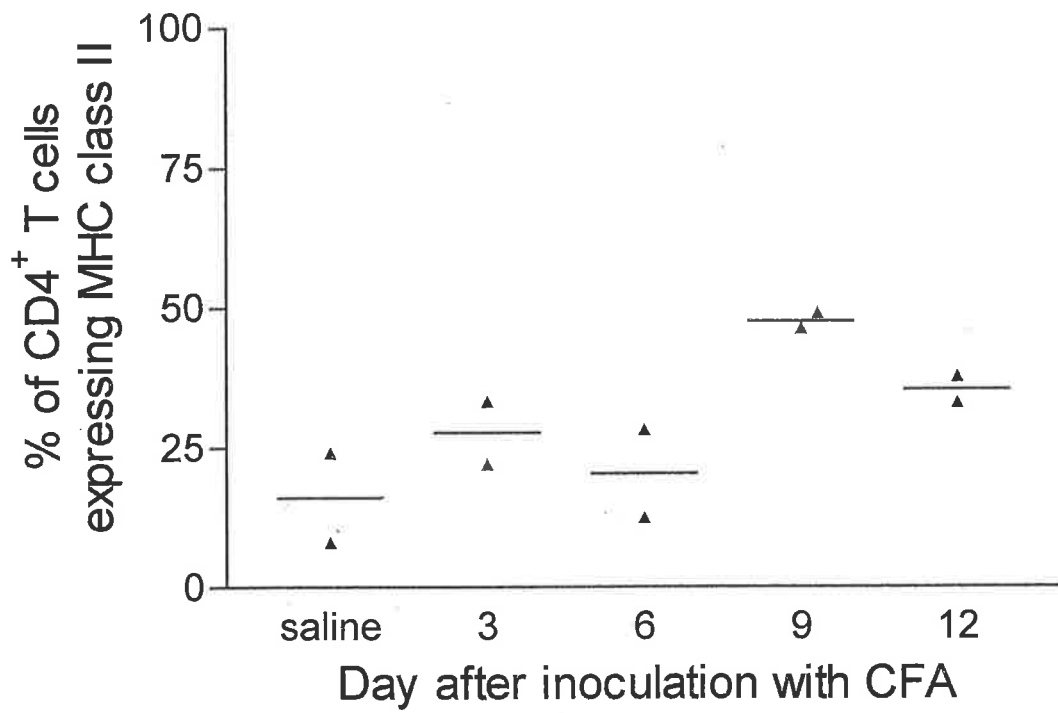
Figure 6.2.4.1 Expression of L-selectin by CD4$^+$ T cells from the hind paws of rats in the prodrome of AA. Cells obtained by the enzymatic digestion of the soft tissues of hind paws were first stained indirectly with OX85 (anti-L-selectin [CD62L]; FITC) and then directly using mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The proportion of CD4$^+$ T cells in the lymphocyte gate that expressed CD62L at different times after inoculation of CFA was calculated using data generated by flow cytometric analysis (C). Each triangle indicates the proportion of CD4$^+$ T cells in the lymphocyte gate that expressed CD62L when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.
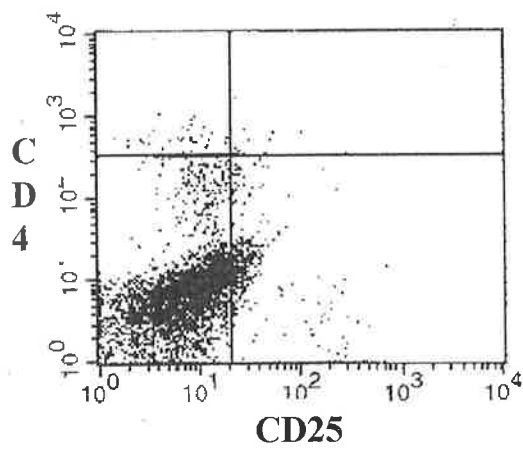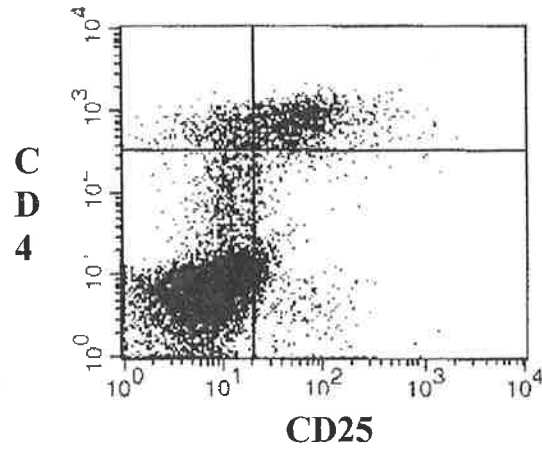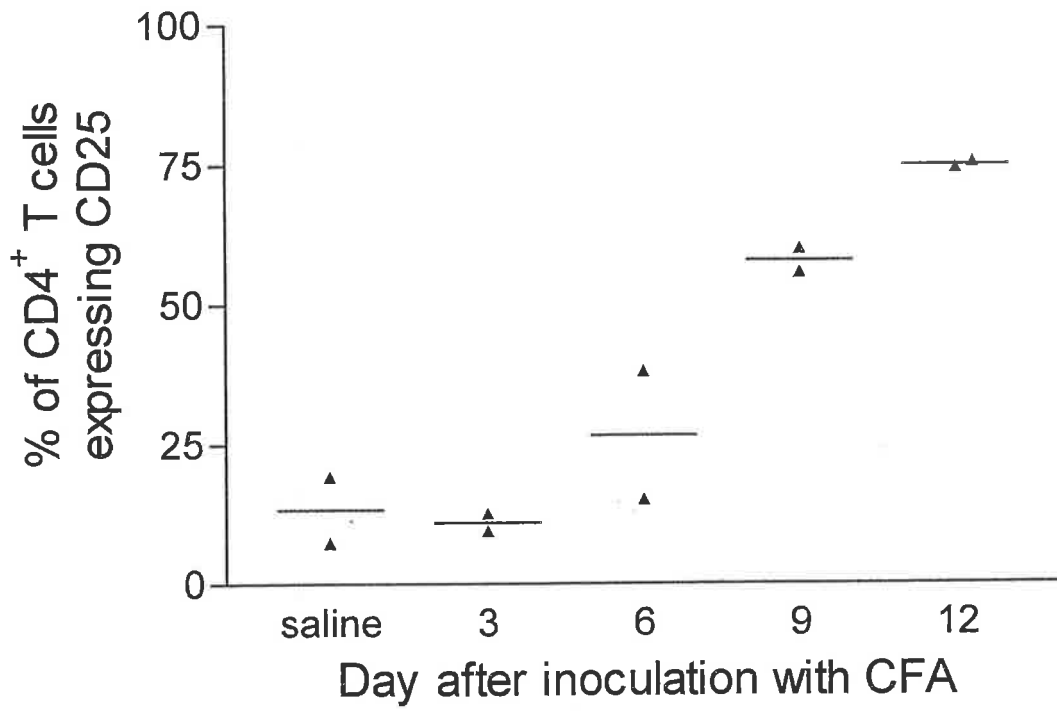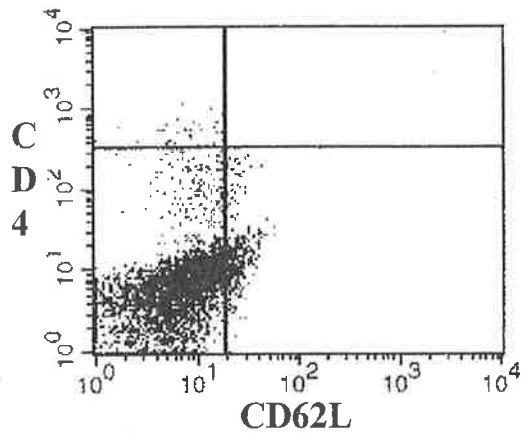
A
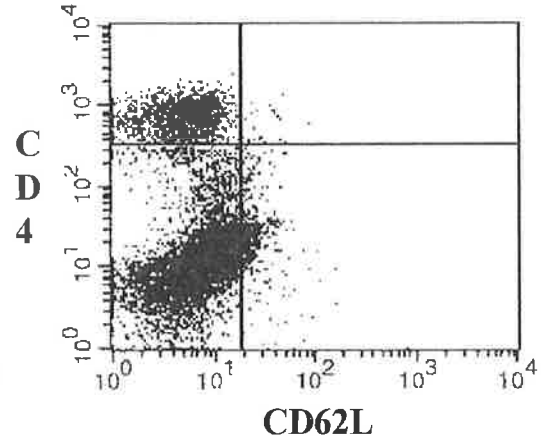
B
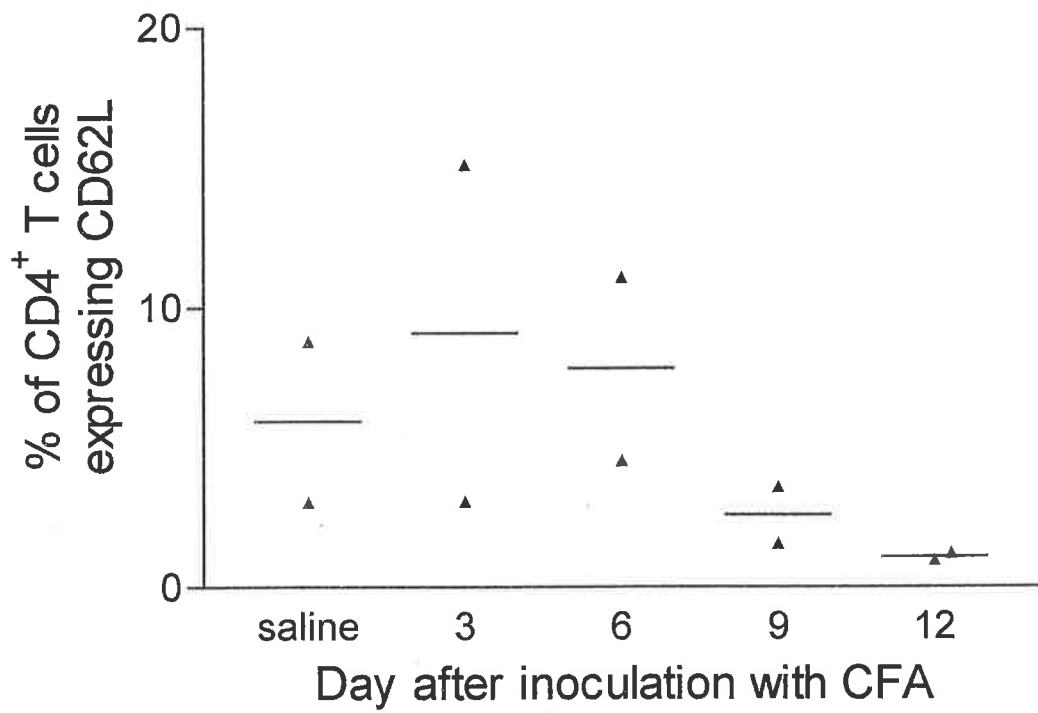
C

Figure 6.2.4.2 Expression of α_{E2} integrin by CD4$^+$ T cells from the hind paws of rats in the prodrome of AA. Cells obtained by the enzymatic digestion of the soft tissues of hind paws were first stained indirectly with mAb OX62 (anti-α_{E2} integrin; FITC) and then directly using mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The proportion of CD4$^+$ T cells in the lymphocyte gate that expressed α_{E2} integrin at different times after inoculation of CFA was calculated using data generated by flow cytometric analysis (C). Each triangle indicates the proportion of CD4$^+$ T cells in the lymphocyte gate that expressed α_{E2} integrin when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.
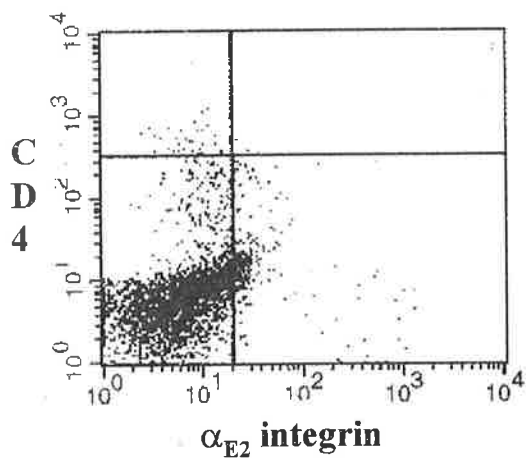
A
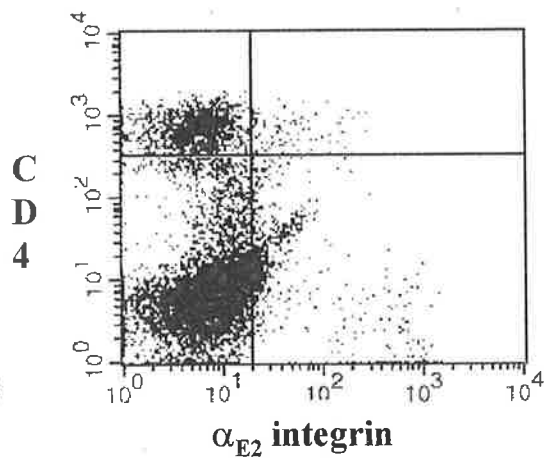
B
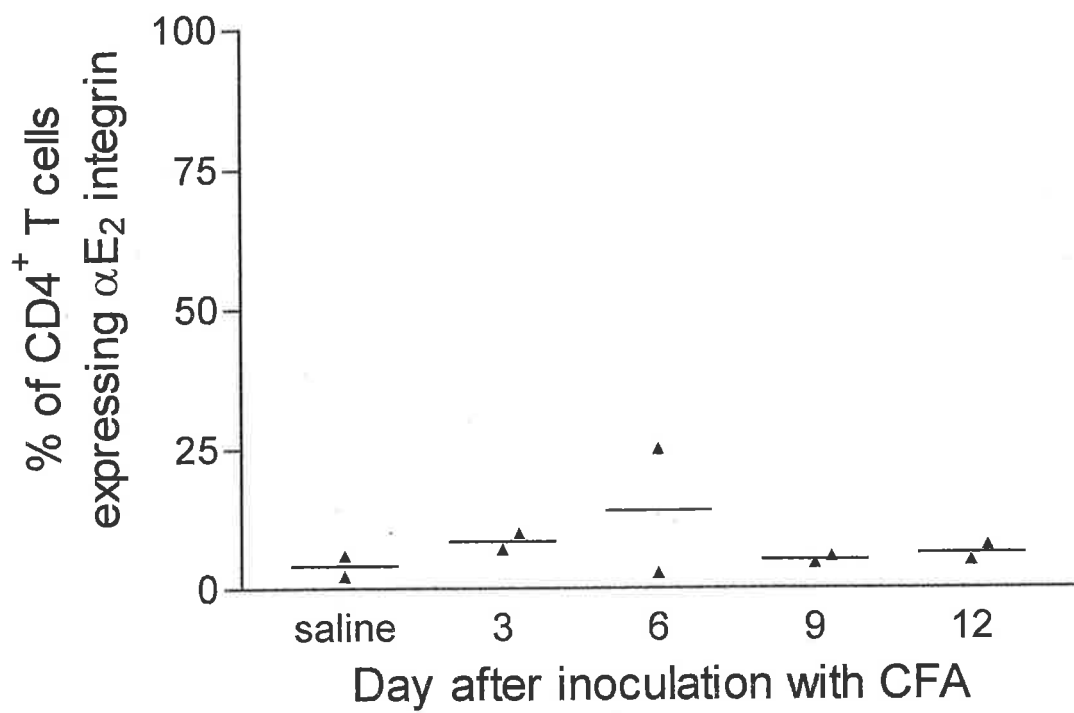
C

Figure 6.2.4.3 Expression of α4 integrin by CD4$^+$ T cells from the hind paws of rats in the prodrome of AA. Cells obtained by the enzymatic digestion of the soft tissues of hind paws were first stained indirectly with mAb MRα4-1 (anti-α4 integrin [CD49d]; FITC) and then directly using mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The proportion of CD4$^+$ T cells in the lymphocyte gate that expressed CD49d at different times after inoculation of CFA was calculated using data generated by flow cytometric analysis (C). Each triangle indicates the proportion of CD4$^+$ T cells in the lymphocyte gate that expressed CD49d when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.
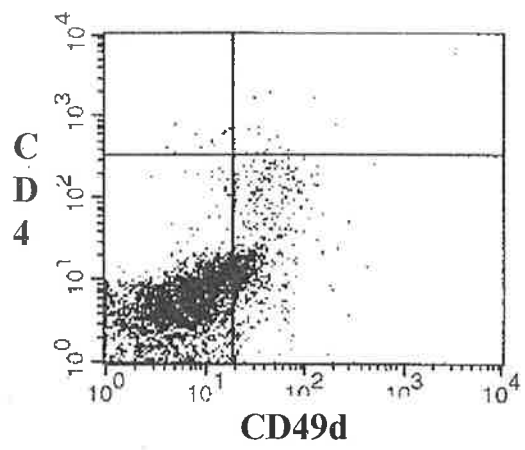
A
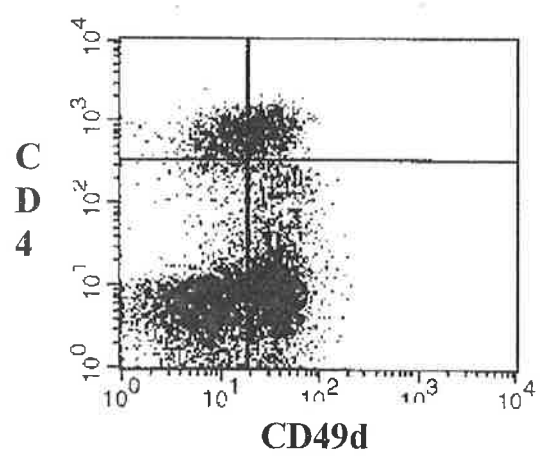
B
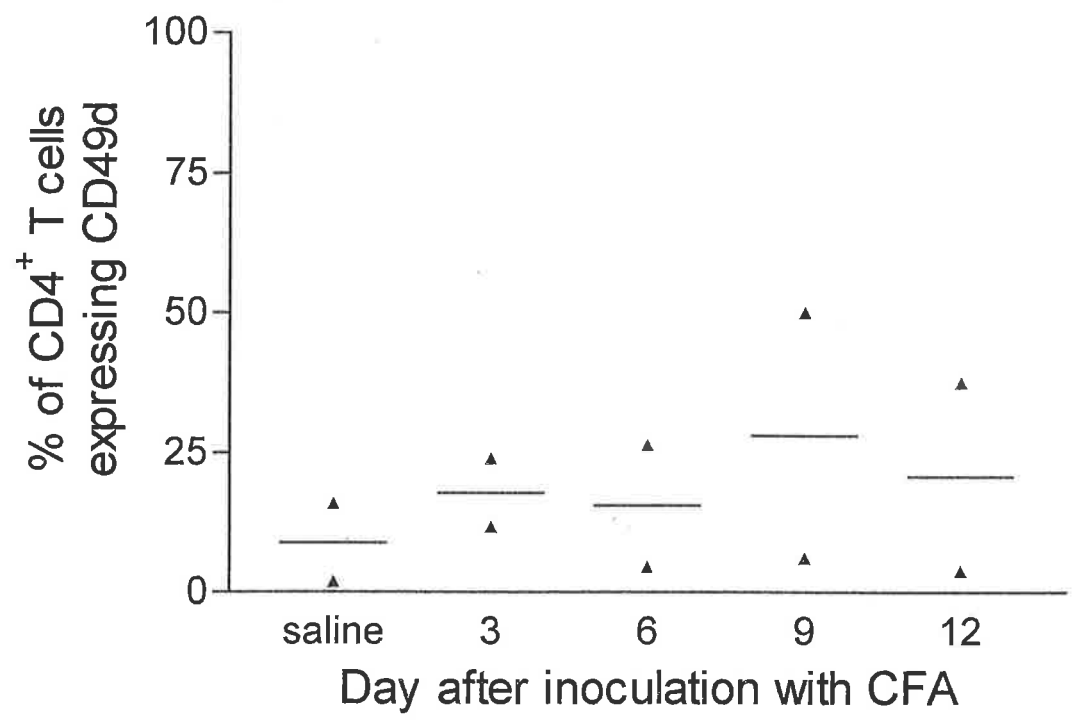
C

Figure 6.2.4.4 Expression of ICAM-1 by CD4$^+$ T cells from the hind paws of rats in the prodrome of AA. Cells obtained by the enzymatic digestion of the soft tissues of hind paws were first stained indirectly with mAb 1A29 (anti-ICAM-1 [CD54]; FITC) and then directly using mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The proportion of CD4$^+$ T cells in the lymphocyte gate that expressed CD54 at different times after inoculation of CFA was calculated using data generated by flow cytometric analysis (C). Each triangle indicates the proportion of CD4$^+$ T cells in the lymphocyte gate that expressed CD54 when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.
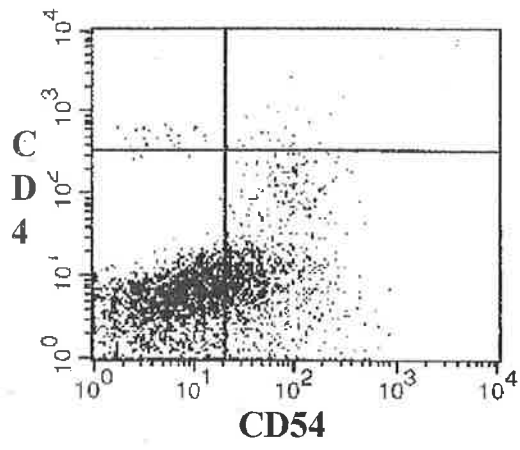
A
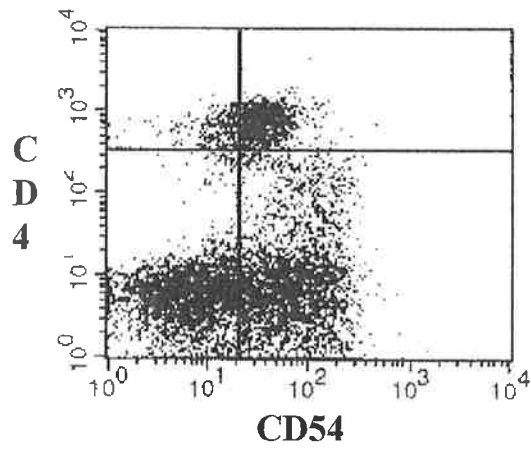
B
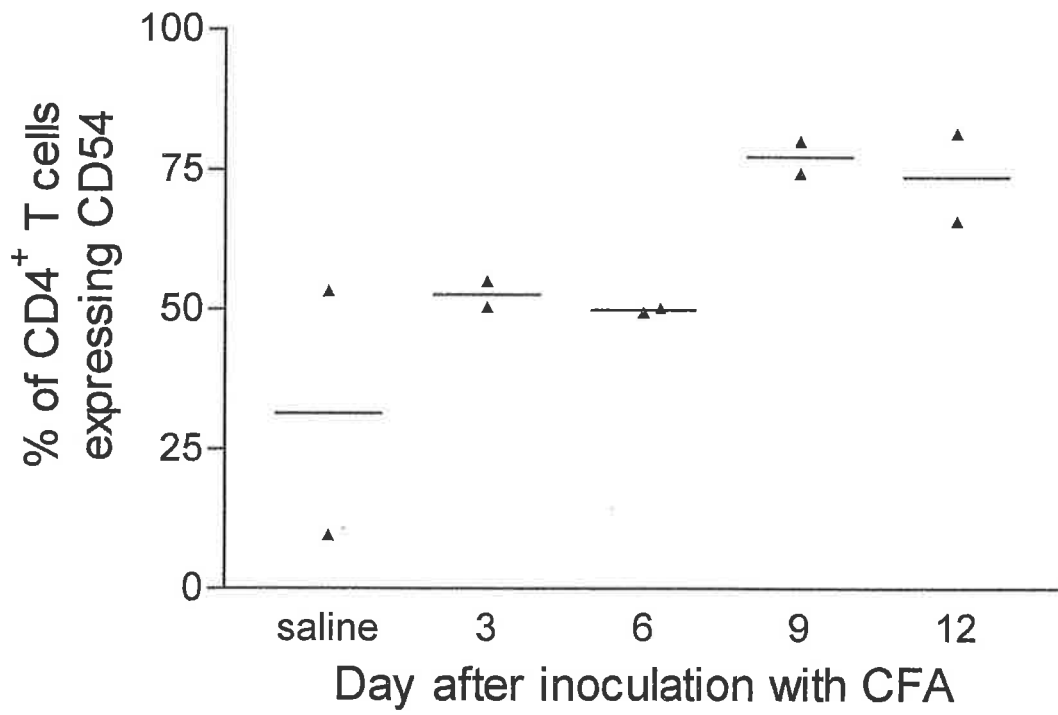
C

6.2.4.5 Phagocytic glycoprotein 1 (CD44)

Phagocytic glycoprotein (CD44) was expressed by all CD4$^+$ T cells isolated from the hind paws from rats challenged with either saline or CFA. The level of expression of CD44 was relatively constant throughout the course of early arthritis. However, CD4$^+$ T cells isolated from paws 9 days post-inoculation of CFA expressed markedly lower levels of CD44 than the cells isolated at other time points (Figure 6.2.4.5 A and C). At day 12 post-inoculation, CD44 was expressed at levels similar to those observed at days 3 and 6 post-inoculation of CFA and in saline-challenged rats (Figure 6.2.4.5 B and C). The lower mean fluorescence intensity of the cells recovered at day 9 post-inoculation may signal the influx of a new population of CD4$^+$ T cells at this time.

6.2.5 Production of cytokines by T cells obtained from the hind paws during the prodrome of AA

Most studies of the cytokines present in the synovium of patients with RA have used RT-PCR or *in situ* hybridization to examine specific mRNA transcripts. Early studies focussed on cytokines produced in abundance by macrophage-like and fibroblast-like cells, such as tumour necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1 and IL-6, although the presence of T cell-derived lymphokines has also been examined (reviewed in Koch et al., 1995; Feldmann et al., 1996).

In this study, flow cytometry was used to examine intracellular IFN-γ and IL-4 protein in α/β T cells stimulated *in vitro* for 6 hours with PMA plus ionomycin in the presence of Brefeldin A, as described in Chapter 3.

6.2.5.1 IFN-γ

During the early prodrome of AA (days 3 and 6 post-inoculation of CFA), IFN-γ-producing α/βT cells were not detected following stimulation *in vitro* with PMA plus ionomycin in the presence of Brefeldin A (Figure 6.2.5.1 C). Approximately 3% of the α/β T cells isolated from the hind paws of saline-challenged rats produced detectable amounts of IFN-γ but these cells had low fluorescence intensity, indicating that the IFN-γ protein was present in low amount.
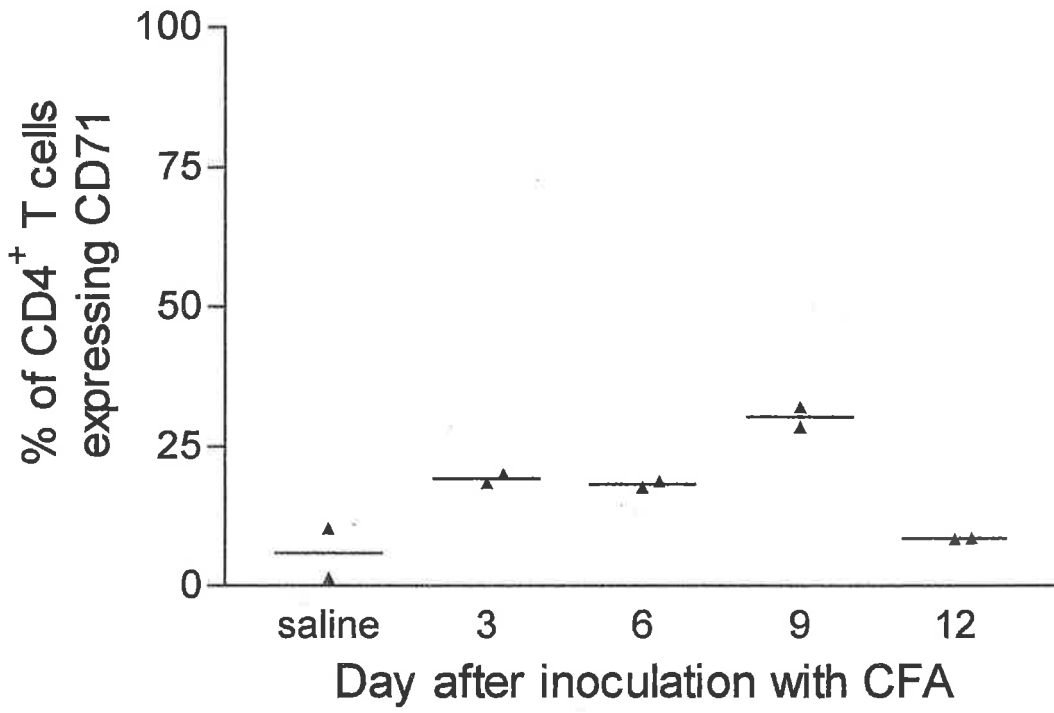
Figure 6.2.4.5 Level of expression of CD44 by CD4$^+$ T cells from the hind paws of rats in the prodrome of AA. Cells obtained by the enzymatic digestion of the soft tissues of hind paws were first stained indirectly with mAb OX50 (anti-CD44; FITC) and then directly using mAb OX38 (anti-CD4; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The mean value of the fluorescent signal generated by the FITC-labelled OX50 binding to CD4$^+$ T lymphocytes at different times after inoculation of CFA was calculated using data generated by flow cytometric analysis (C). Each triangle indicates the mean value of the fluorescent signal generated by the FITC-labelled OX50 binding to CD4$^+$ T lymphocytes when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.
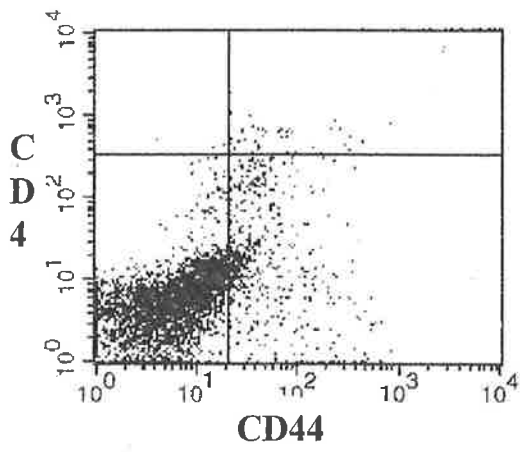
A
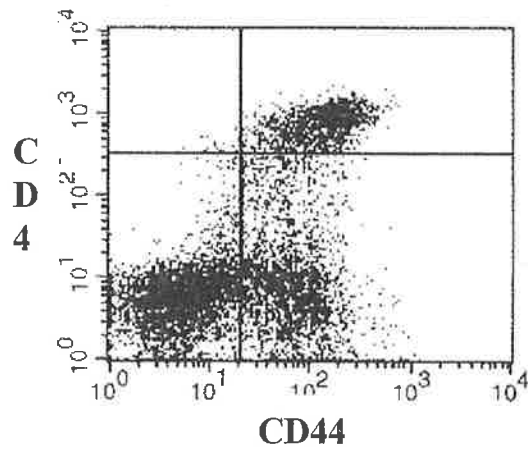
B
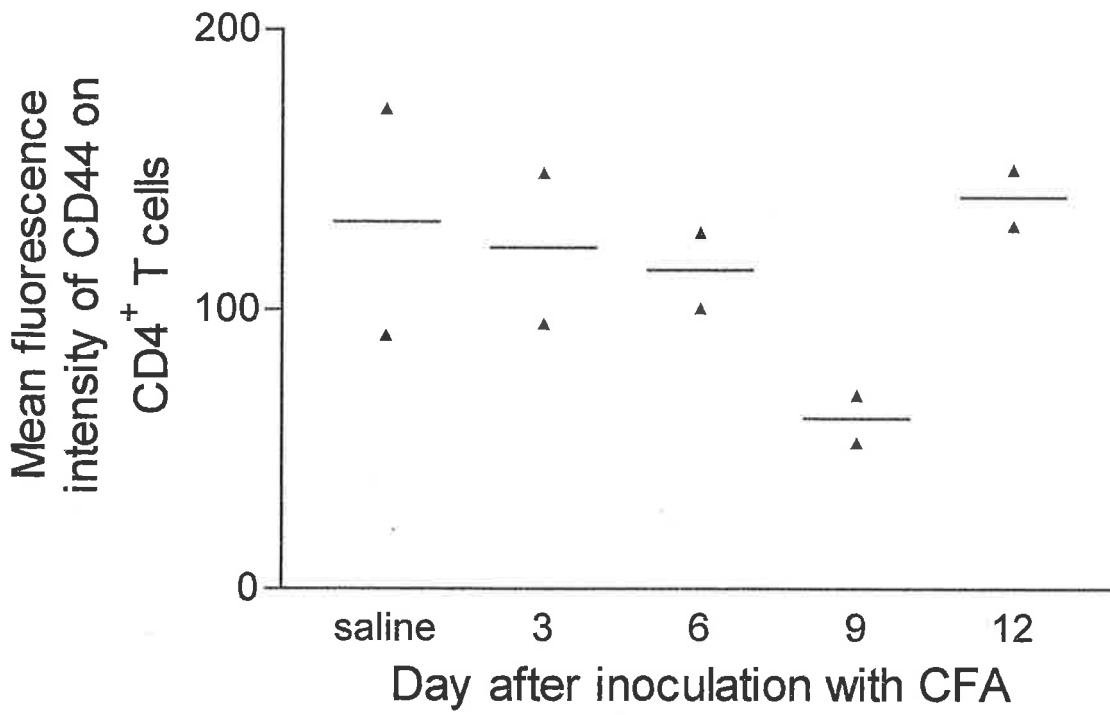
C

Figure 6.2.5.1 Production of IFN-γ by *in vitro* stimulated T cells from the hind paws of rats in the prodrome of AA. Cells obtained by the enzymatic digestion of the soft tissues of hind paws were stimulated *in vitro* with PMA and calcium ionophore A23187 in the presence of Brefeldin A for 6 hours before fixing (as described in 2.6.3). Fixed cells were made permeable with saponin and were first stained indirectly with mAb DB-1 (anti-IFN-γ; FITC) and then directly using mAb R73 (anti-α/β TCR; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The proportion of α/β T cells in the lymphocyte gate that expressed IFN-γ at different times after inoculation of CFA was calculated using data generated by flow cytometric analysis (C). Each triangle indicates the proportion of α/β T cells in the lymphocyte gate that expressed IFN-γ when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.

A

B

C

% of αβT cells expressing IFNγ

Day after inoculation with CFA

In contrast to the scarcity of IFN-γ^+ α/β T cells in the soft tissues of the hind paws from saline-inoculated rats and rats inoculated with CFA 3 or 6 days earlier, there was an abundance of these cells in the hind paws from rats at the onset of visible joint inflammation (day 9 post-inoculation) and in established arthritis (day 12) (Figure 6.2.5.1 A-C). Approximately 44% of α/β T cells from rats at day 9 post-inoculation and 55% of α/β T cells from rats at day 12 post-inoculation produced IFN-γ after 6 hours of stimulation in vitro (Figure 6.2.5.1 B and C). Many of these IFN-γ^+ α/β T cells had relatively intense fluorescence, indicating that these cells contain large amounts of IFN-γ.

6.2.5.2 IL-4

Very few α/β T cells produced detectable levels of IL-4 throughout the prodrome and early clinical phase of AA (Figure 6.2.5.2). A maximum of 1.1% of α/β T cells contained detectable IL-4 (day 3 post-inoculation of CFA) and the low fluorescence intensity of these cells suggests that they contained very low levels of intracellular IL-4 protein. Most samples examined did not contain any cells that produced detectable levels of IL-4, suggesting that this cytokine was not produced in the synovial tissue from the hind paws of rats during either the prodrome or the early clinical phase of AA.

6.3 Discussion

6.3.1 Macroscopic and cellular composition of the hind paws during the prodrome of AA

The weight of the skinned hind paw pairs from individual rats increased at the onset of joint inflammation, indicating swelling of the soft tissues. The physical characteristics of the cells isolated from the hind paws were analysed by flow cytometry. The scatter plots of these cells indicated that the cellular composition of the single cell suspensions, from both saline-injected and CFA-inoculated rats, were heterogeneous and contained an extensive range of cell sizes and of cytoplasmic complexities.

A population of cells with physical characteristics consistent with those of granulocytes increased in frequency 6 days after inoculation of the rats with CFA and was most numerous on days 9 and 12 after inoculation (Figure 6.2.1.2). This suggests that granulocytes infiltrate the synovial tissue early in the prodrome of AA, before the onset of

**Figure 6.2.5.2 Production of IL-4 by *in vitro* stimulated T cells from the hind paws of rats in the prodrome of AA.** Cells obtained by the enzymatic digestion of the soft tissues of hind paws were stimulated *in vitro* with PMA and calcium ionophore A23187 in the presence of Brefeldin A for 6 hours before fixing (as described in 2.6.3). Fixed cells were made permeable with saponin and were first stained indirectly with mAb OX81 (anti-IL-4; FITC) and then directly using mAb R73 (anti-α/β TCR; PE). Representative scatter plots are shown of gated events in preparations from rats inoculated with saline (A) or with CFA 12 days earlier (B). The proportion of α/β T cells in the lymphocyte gate that expressed IL-4 at different times after inoculation of CFA was calculated using data generated by flow cytometric analysis (C). Each triangle indicates the proportion of α/β T cells in the lymphocyte gate that expressed IL-4 when pooled cells from a group of 10 rats were analysed. The horizontal bar indicates the mean calculated from the results of two groups of rats at each time.

A

B

C

% of αβ T cells expressing IL-4

Day after inoculation with CFA
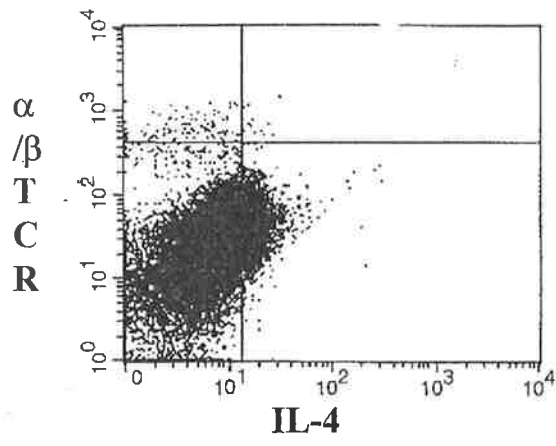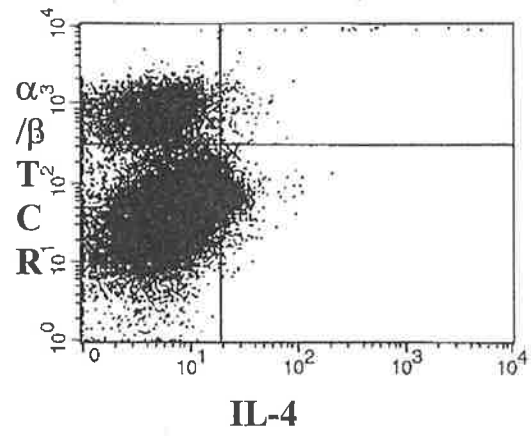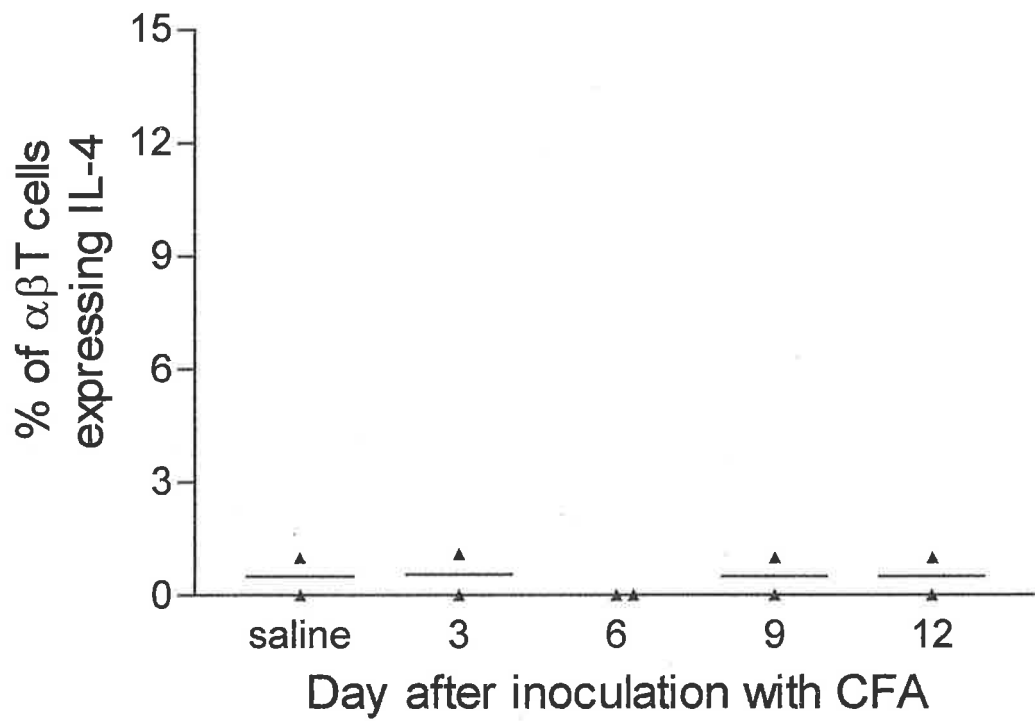
clinically detectable inflammation. The using a mAb against a granulocyte marker, such as CD15, could be used to confirm that these cells were granulocytes. These findings are consistent with the histological features of AA, where infiltration by polymorphonuclear cells into the connective tissue of joints is one of the earliest events in the pathogenesis of AA (Pearson, 1956).

6.3.2 Comparison of T cell sub-populations in the hind paws during the prodrome of AA

Examination of the scatter plots of cells from digested hind paws indicated that cells with the physical characteristics of lymphocytes increased in the hind paws at days 9 and 12 post-inoculation with CFA. However, verification that the cells were indeed lymphocytes and identification of lymphocyte subsets required the use of specific mAbs against lymphocyte cell surface markers. An electronic gate was set, based on the physical characteristics of lymphocytes obtained from lymph nodes and the proportion of events within this gate that expressed CD4 at high levels (CD4$^+$ T cells) was measured. CD4$^+$ T cells represented only a minor population of cells in the hind paws from control rats and rats at days 3 and 6 post-inoculation of CFA. At the end of the prodrome of AA (day 9 post-inoculation), a small but substantial population of CD4$^+$ T cells was present in the hind paws. This population represented a greater proportion of the gated events at day 12 post-inoculation, when arthritis was moderate to severe.

Others have reported that α/β T cells are scarce in the synovium of rats with AA (Pelegri et al., 1995) and that γ/δ T cells are absent from the synovium of both healthy and arthritic rats (Carol et al., 2000). The use of flow cytometry to examine a large number of cells liberated from the hind paws by enzymatic digestion revealed that although γ/δ T cells were rare (less than 0.01% of the total population examined), they were present. However, their frequency did not appear to change following inoculation of CFA.

The scarcity of CD4$^+$ T cells in the hind paws at 3 and 6 days post-inoculation suggests that either arthritogenic CD4$^+$ T cells have not yet entered the synovium or that if present, the few pioneer arthritogenic cells are undetectable above background levels. The increase in CD4$^+$ T cells at day 9 post-inoculation indicates that these cells have either entered the synovium towards the end of the prodrome of the disease or that there has been local

division of immigrant cells. The numbers of CD4$^+$ T cells had increased further by day 12 post-inoculation, suggesting that the CD4$^+$ T cells population present at day 9 had expanded, or that there was an ongoing influx of effector cells. It is important to remember that joint inflammation in AA is usually most severe around day 16 post-inoculation of CFA. The synovitis in rats at day 12 post-inoculation is, therefore, in the phase of exacerbation rather than stabilisation or amelioration. The increase of CD4$^+$ T cells at day 12 compared to the earlier time points may reflect an expanding population of these cells, with an increase in severity of joint inflammation. Whether such expansion is a consequence of cell proliferation and/or the recruitment of cells from the periphery has yet to be determined. In the case of RA, it has been suggested that pre-activated T cells are recruited continuously into joints during the course of the disease (Iannone et al., 1994). In addition, once inflammation was initiated in AA, activated cells would have been recruited to the area regardless of antigen-specificity (Asherson and Allwood, 1972). It is noteworthy that the potency of TD lymphocytes from arthritic donors to transfer the disease to naïve recipients declines between days 9 and 12 after inoculation. This may indicate that the numbers of CD4$^+$ T cells in the synovium is sustained by cell division but to conclude this would require further studies using tritiated thymidine or BudR to label dividing cells.

When the hind paws were prepared for enzymatic digestion, care was taken to avoid contamination from bone marrow. The possibility that the T cells present in the hind paw digests could be intravascular contaminants has been investigated by Ms. Sarah Wing in the Arthritis Research Laboratory. Ms. Wing demonstrated that T cells are present in both normal and arthritic hind paws, regardless of whether the limbs were perfused with saline to flush-out peripheral blood cells before removal.

6.3.3 Comparison of the expression of activation markers by CD4$^+$ T cells in the hind paws during the prodrome of AA

Examining the expression of activation markers on synovial CD4$^+$ T cells isolated from the hind paws from rats with AA revealed heterogeneity within this population and substantial evidence that most, if not all, of these cells had a phenotype consistent with mature effector cells.

6.3.3.1 MHC class II

The function of MHC class II expressed by CD4$^+$ T cells is poorly understood. The lack of antigen specific receptors to assist in the uptake and processing of complex antigens has been used as evidence that T cells do not use MHC class II molecules to present exogenously derived antigens. However, the expression of B7 by activated T cells suggests that they could provide co-stimulatory signals (Barnaba et al., 1994). It is possible that the T cell presentation of peptide fragments, generated by other cells or by destruction of the joint by proteolytic enzymes such as matrix metalloproteinases, could be involved in the perpetuation of arthritis. Nevertheless, it is clear that rat T cells express MHC class II molecules when they are activated.

MHC class II was expressed by a substantial proportion of CD4$^+$ T cells in the hind paws from rats during the early prodrome of AA (between 8 and 28%). This increased to approximately 50% at day 9 post-inoculation and remained at approximately 35% the hind paws of rats with established arthritis. It is not clear whether the MHC class II$^+$ CD4$^+$ T cells present at day 9 are diluted by newly arrived CD4$^+$ T cells that are MHC class II$^-$ or whether the CD4$^+$ T cell population present at day 9 expands with concurrent loss of MHC class II expression by some of the cells.

6.3.3.2 IL-2 receptor α chain (CD25)

It was interesting to observe that the majority of CD4$^+$ T cells from the hind paws from rats with early or established arthritis (days 9 and 12 post-inoculation, respectively) expressed CD25. This is a contrast to synovial T cells in RA, most of which are IL-2R$^-$ (Cush et al., 1988; Iannone et al., 1994). The expression of CD25 suggests that these cells are responsive to IL-2 and have a capacity to proliferate. The lack of expression of IL-2R on CD4$^+$ T cells in RA may reflect the phenotype of these cells in chronic arthritis, rather than during the onset of acute synovitis that is characteristic of AA. It would be interesting to know the phenotype of CD4$^+$ T cells in the synovium of patients with early arthritis. The remitting and relapsing nature of RA makes it difficult to compare with AA.

Minimal amounts of IL-2 (mRNA transcripts or protein) can be detected in the synovial tissue of patients with RA (Chen et al., 1993; Warren, et a, 1991; Howell et al., 1991). It is thought that IL-15, which has IL-2-like activity, has a more significant role that IL-2 in the activation and proliferation of synovial T cells in RA (McInnes et al., 1996). Only

moderate increases in the levels of transcription of the gene encoding IL-2 have been observed in the synovial tissues of rats with AA (Schmidt-Weber et al., 1999).

The differences in the expression of CD25 by CD4$^+$ T cells from rheumatoid synovium compared with those from the joints of rats with AA may reflect differences between the two diseases. Treatment of rats during the inductive phase of AA with a mAb that blocks IL-2R protects rats from the development of actively-induced arthritis but not the adoptively-transferred disease. This suggests that IL-2 is important during the inductive phase of AA but not during the effector phase (Ferguson et al., 1988). This observation suggests that the expression of CD25 by CD4$^+$ T cells in the hind paws from rats at day 12 post-inoculation of CFA simply reflects their activation state rather than being of pathological significance. However, as discussed earlier, it is important to examine whether there is significant local proliferation of CD4$^+$ T cells during the pathogenesis of synovial inflammation in AA.

6.3.3.3 Transferrin receptor (CD71)

Interestingly, the proportion of CD4$^+$ T cells that expressed CD71 (approximately 30%) in the hind paws from rats with the early signs of arthritis (day 9 post-inoculation) was greater than in hind paws with more severe arthritis (day 12 post-inoculation). The expression of CD71 is associated with dividing cells (Salmeron et al., 1995) and the reduction in the proportion of CD4$^+$ T cells that expressed CD71 at day 12 suggests that fewer cells were dividing at this time. These results suggest that during the initiation and onset of joint inflammation there is proliferation of CD4$^+$ T cells, which slows after the disease is established. This is an interesting comparison to RA, in which the synovium is almost devoid of dividing cells (Panayi et al., 1992).

Interestingly, CD4$^+$ T cells that are exposed to IL-15 in the absence of appropriate TCR engagement exit the cell cycle once they have reached G_0/G_1. These cells down-regulate the expression of CD71 and CD25 and remain in an activated but quiescent state (Dooms et al., 1998). Such cells proliferate strongly in response to IL-15 if their TCR is subsequently engaged and hence can be considered long-lived effector cells (Dooms et al., 1998). The population of CD71$^-$ CD4$^+$ T cells present in the hind paws from rats with AA may be such cells.

6.3.3.4 OX40 antigen (CD134)

CD134 was expressed by a small proportion of CD4$^+$ T cells (approximately 5%) in the hind paws from rats during the prodrome and early clinical phase of AA. CD134 expression is associated with recent activation (Stuber & Strober 1996) and the lack of expression by most CD4$^+$ T cells in the hind paws from rats may indicate that the majority of these cells are in the late phase of post-activation maturation. These cells may be non-dividing effector cells.

6.3.3.5 UA002 antigen

A substantial proportion of the CD4$^+$ T cells isolated from the hind paws from rats during the prodrome of AA expressed the UA002 antigen. The molecule recognised by mAb UA002 has yet to be characterised but it is expressed by B lymphocytes, macrophages, dendritic cells and T cells that have been activated with Concanavalin A. Approximately 25% of CD4$^+$ T cells from the hind paws from rats with joint inflammation (day 12 post-inoculation) expressed UA002 antigen, indicating a population of activated cells. The kinetics of UA002 antigen expression has not been examined but the heterogeneity in the CD4$^+$ T cell population may reflect differences in the time between cells being activated in peripheral lymph nodes and their entry into the synovial tissues. The majority of large CD4$^+$ T cells in the TD lymph of rats during the prodrome of AA expressed UA002 antigen (up to 75%, Figure 5.2.3.5). It is not possible to say (based on the result here) whether these cells were arthritogenic but if all or some of them were, then it is possible that expression of UA002 antigen may have been down-regulated soon after entering the synovial tissue.

6.3.3.6 CD45RC

Very few CD4$^+$ T cells in the hind paws from rats with moderate to severe arthritis at day 12 post-inoculation of CFA expressed CD45RC, which suggests that most of these cells have an effector or memory phenotype (Luqman and Bottomly, 1992). This finding is interesting because although memory cells can revert to CD45RC$^+$, naïve cells are not CD45RC$^-$. This indicates that almost all CD4$^+$ T cells in the hind paws from rats with clinical signs of arthritis are antigen-experienced. The heterogeneity of the expression of the other surface molecules examined suggests that there are different populations of activated CD4$^+$ T cells that may have different functional roles. Alternatively, the differences in the expression of activation and adhesion molecules may simply reflect the

differences in probability of expressing each molecule in response to the antigenic stimulus (Hasbold et al., 1999).

Others have reported that autoreactive and IFN-γ-producing CD4$^+$ T cells are contained within the CD45RC^{high} compartment (Fowell et al., 1991). However, a large proportion of the CD45RC$^-$ cells from arthritic hind paws produced IFN-γ after 6 hours of stimulation *in vitro* and very few CD45RC^{high} cells were present.

6.3.4 Comparison of the expression of adhesion molecules by CD4$^+$ T cells in the hind paws during the prodrome of AA

6.3.4.1 L-Selectin (CD62L)

CD62L is expressed only at minimal levels by CD4$^+$ T cells in the synovial tissue of patients with RA (Cush et al., 1992). Similarly, virtually all CD4$^+$ T cells from arthritic hind paws were CD62L$^-$ (Figure 6.2.4.1). During the prodrome of AA, a small proportion (usually less than 10%) of CD4$^+$ T cells in the hind paws expressed low levels of CD62L. CD62L is down-regulated after T cells are activated, indicating that the cells in the synovium of rats with AA have mainly memory or effector function.

6.3.4.2 α$_{E2}$ integrin

A small but distinct population of α$_{E2}$ integrin$^+$ CD4$^+$ T cells were present in the hind paws of both saline-inoculated and CFA-inoculated rats (Figure 6.2.4.2). Expression of α$_{E2}$ integrin by CD4$^+$ T cells is associated with those found in a mucosal microenvironment. Elewaut and colleagues (1998) found that around 8% of T cell lines derived from the synovial tissue of patients with RA expressed α$_E$β$_7$. It seems unlikely that these cells are involved in the pathogenesis of AA, because their numbers changed little during the pathogenesis of the disease. Furthermore, it was shown by Spargo et al (1996) that induction of AA in donor rats did not affect the recruitment of mesenteric lymphoblasts into inflamed or normal synovium.

6.3.4.3 α$_4$ integrin (CD49d)

The α$_4$β$_7$ and α$_4$β$_1$ integrins are thought to be important in the rolling/adhesion step of diapedesis into mucosal and inflamed sites via their interaction with MAdCAM-1,

VCAM-1 and Fibronectin ($\alpha_4\beta_1$ only) expressed on vascular endothelium and connective tissue (reviewed in Bradley and Watson, 1996). Elewaut and colleagues (1998) found that approximately 24% of T cell lines derived from the synovial tissue of patients with RA had the phenotype $\alpha_4\beta_7^+$CD4$^+$ (Elewaut et al., 1998). Others have shown that synovial T cells bind to fibronectin, a ligand of $\alpha_4\beta_1$ integrin (Panayi et al., 1992). There was considerable variation between samples in the proportion of CD4$^+$ T cells that expressed CD49d, although such cells were present in all samples. Interestingly, others have shown that blockade of CD49d by the administration of a specific mAb markedly reduced the severity of joint inflammation in AA, while also inhibiting leukocyte rolling and adhesion to venules (Seiffge et al., 1996) and migration of T lymphocytes into joints (Issekutz et al., 1996).

6.3.4.4 ICAM-1 (CD54)

As in the synovial tissue of patients with RA (Cush et al., 1992), most CD4$^+$ T cells in the hind paws from rats with AA expressed CD54. The highest proportion (approximately 75%) of CD54$^+$ CD4$^+$ T cells was seen at the end of the prodrome, at days 9 and 12 post-inoculation of CFA (Figure 6.2.4.4). In at least one of the samples at each earlier time point (including saline-injected controls), 50% of the CD4$^+$ T cells expressed CD54.

Others have demonstrated that blockade of CD54 by the use of mAb 1A29 *in vivo* inhibits both the inductive and effector phases of AA (Iigo et al., 1991). These researchers suggested that CD54 is likely to be important in both the interaction between APCs and T cells and in the migration of effector cells into the inflammatory lesions. However, it is important to note that the blockade of CD54 could influence a variety of other cells that express either CD54 or LFA-1.

6.3.4.5 Phagocytic glycoprotein 1 (CD44)

The expression of CD44 by CD4$^+$ T cells appeared to be down-regulated on day 9 post-inoculation of CFA, compared with cells isolated from rats at the other times examined (Figure 6.2.3.6). The significance of this is unknown, although it could represent an influx of CD44^{low} cells at this time. Interaction with hyaluronic acid in the extracellular matrix could be an important factor in retaining activated T cells in the synovium.

6.3.5 Production of cytokines by CD4$^+$ T cells in the hind paws during the prodrome of AA

The production of cytokines by α/β T cells isolated from the hind paws of rats during the prodrome and early clinical phase of AA was examined using flow cytometry. The isolated cells were stimulated *in vitro* for 6 hours with PMA plus ionomycin in the presence of Brefeldin A. It is likely that this technique underestimates the proportion of cells that could produce cytokines *in vivo* and instead provides a "snap shot" of what proportion of cells produce detectable cytokine after 6 hours of stimulation. The technique allows a comparison to be made between cell populations that have received similar treatment.

There was an abundance of α/β T cells that produced IFN-γ and very few that produced IL-4. This indicates that the α/β T cells present in inflamed synovial tissue were biased strongly towards a Type-1 response. The near absence of IL-4 protein in the synovial tissue α/β T cells isolated from rats with AA supports studies by others. Schmidt-Weber, et al (1999) examined the expression of mRNA transcripts by genes encoding numerous cytokines and could not detect the presence of IL-4 transcripts in synovial tissue samples taken from Lewis rats at 6, 13 or 20 days after inoculation of CFA.

A bias towards a Th1 response has been demonstrated in the synovial tissue of patients with RA (Simon, et al. 1994; Bucht et al., 1996) and blockade of IFN-γ has demonstrated that this cytokine plays an important role in AA (Wiesenberg et al., 1989; Jacob et al., 1989). Synovial macrophages (including synoviocytes) isolated from rats 10 days (and later) after inoculation with CFA have a more activated phenotype than those isolated at day 7 or from normal rats (Johnson et al., 1986). This activation could be explained by production of IFN-γ by local effector CD4$^+$ T cells, as IFN-γ is a potent stimulator of macrophages (Pace et al., 1983). Others have reported that populations of activated macrophage-like cells expressing high levels of MHC class II molecules are present in rheumatoid synovium (Klareskog et al., 1982).

6.3.6 General discussion

There is some controversy regarding the theory that T cells orchestrate the local inflammatory response in RA (Firestein and Zvaifler, 1990). However, the results

described in this chapter support the general hypothesis that the effector phase of arthritis is mediated by CD4$^+$ T cells.

A generalisation can be made that most of the CD4$^+$ T cells present in the synovium of rats with established arthritis had the phenotype CD45RC$^-$ CD62L$^-$ CD71$^-$ CD25$^+$ CD134$^-$ CD54$^+$ α_{E2}integrin$^-$. In addition, there are possibly overlapping or discrete populations with the phenotype MHC class II$^+$, CD49d$^+$ or UA002 antigen$^+$, although it is possible that there is little or no overlap between the expression of these individual molecules. Complete elucidation of the subsets will require the use of conjugated mAbs and multi-fluorochrome analysis. To this could be added analysis of the surface antigen phenotype of the IFN-γ-producing cells.

It is interesting to consider the heterogeneity of CD4$^+$ T cells present in the synovium of arthritic rats. It has been proposed that in rheumatoid synovium, most of the T cells are recruited non-specifically (Panayi et al., 1992). It is also likely that non-arthritogenic CD4$^+$ T cells are recruited non-specifically into the inflamed synovium in AA and that such cells may express markers that are shared with arthritogenic cells. However, if the generation of arthritogenic cells is considered in light of the theory of acquired immunity proposed by P.D.Hodgkin et al (Hasbold et al., 1999), in which probabalistic, quantitative theories are used to explain cell behaviour, then the view may be taken there will not be a single phenotype that describes arthritogenic CD4$^+$ T cells. Rather, in the generation of arthritogenic cells, each one will have a certain probability of expressing a particular surface marker. This contrasts with a deterministic system, in which a cell can only be arthritogenic if it expresses a precise combination of surface markers and the inoculation of CFA leads to the generation of a unique phenotype which has arthritogenic capacity. With the results presented here, it is impossible to conclude, for example, that the cells expressing MHC class II are arthritogenic and those that are MHC class II$^-$ are non-specifically recruited cells, or vice versa. It is possible that the arthritogenic population is heterogeneous and expresses different combinations of surface markers. Each combination may allow the cells to undertake adhesion and diapedesis (adhesion molecules), receive activating signals (activation markers) and respond by release of pro-

inflammatory cytokines (eg. IFN-γ), although the molecules that perform these functions may be different in each sub-population.

It is important to note that arthritogenicity is a property of the CD4$^+$ population of T cells. Depleting CD4$^+$ T cells from rats prevents the development of AA (Billingham et al., 1990b), while anti-CD4 treatment of rats with established AA ameliorates the disease (Pelegri et al., 1996b) and that CD4$^+$ T cells can transfer the disease adoptively (Spargo et al, 2001). These results indicate that CD4$^+$ T cells are crucial in both the inductive and effector phases of AA.

In summary, the results presented in this chapter demonstrate that at the site of inflammation in AA, there is a relative abundance of CD4$^+$ T cells that are effector cells. The expression of surface molecules by these CD4$^+$ T cells, indicates that these cells are equipped for migration and adhesive interactions with cells in the synovial tissue. In addition, a larger proportion of the CD4$^+$ T cells produced IFN-γ and in greater quantities than those isolated from the lymph nodes (see Section 4.2.5.1) or TD lymph (see Section 5.2.5.1), suggesting a greater potential for producing this pro-inflammatory cytokine in the synovium. The effect of IFN-γ produced by arthritogenic cells was examined indirectly using a mAb with specificity for IFN-γ and these studies are described in the next Chapter.

CHAPTER 7

MODULATION OF ADOPTIVELY-TRANSFERRED ARTHRTIS USING A MONOCLONAL ANTIBODY AGAINST INTERFERON-GAMMA

Chapter 7: Modulation of adoptively-transferred arthritis using a monoclonal antibody
against interferon-gamma

7.1 Introduction

Adjuvant arthritis (AA) is a T cell-mediated polyarthritis and has been used as an animal model of rheumatoid arthritis (RA) in humans. AA has been shown to be mediated by T cells (Kohashi et al., 1981; Stanescu et al., 1987; Yoshino et al., 1990) possibly through the expression of inflammatory mediators such as interferon gamma (IFN-γ). IFN-γ mediates inflammation via mechanisms such as up-regulated production of inducible nitric oxide synthase (iNOS), interleukin (IL)-1 and tumour necrosis factor (TNF)α by macrophages and induction of MHC class II expressed by synoviocytes (Doherty, 1995; Mauritz et al., 1988). Endothelial cells are activated by IFN-γ, leading to expression of MHC molecules and adhesion molecules such as ICAM-1 (reviewed in Boehm et al., 1997). Neutralisation of IFN-γ by the administration of monoclonal antibodies (mAb) at the time of inoculation with either CFA (in the case of AA) or collagen type II (in the case of collagen-induced arthritis [CIA]) suppresses disease, suggesting that IFN-γ is an important pro-inflammatory molecule in the pathogenesis of both these diseases. However, the suppressive effect of neutralising mAb against IFN-γ is dependent on the timing, dose and route of administration of the antibodies. It has been reported that the neutralisation of IFN-γ in rats, by the *in vivo* administration of the mAb DB-1 (Van der Meide et al., 1986) suppresses the development of AA. The administration of mAb DB-1 before (day -1 or -2) or after (days 4, 6 & 8) inoculation with CFA, resulted in arthritis of reduced severity compared with that seen in controls, indicating the importance of IFN-γ in the initiation and development of AA (Jacob et al., 1989; Wiesenberg et al., 1989). Jacob et al, reported that a single dose of mAb DB-1 administered intraperitoneally 24 hours before inoculation with CFA suppressed all but minor signs of arthritis. Administration of mAb DB-1 at the end of the prodromal phase of disease increased the severity of arthritis. This suggests that IFN-γ may be important in down-regulating inflammation, directly or indirectly, via one or more of approximately 200 genes under the control of IFN-γ (Boehm et al., 1997) that may be important in the process of regulating inflammation during the recovery phase of AA.

The role of IFN-γ in the pathogenesis of CIA has been examined using IFN-γR knockout (KO) mice with apparently conflicting results. Some workers have reported that mice with an

IFN-γ-R null mutation have reduced susceptibility to CIA (Kageyama et al., 1998), whereas others have reported that it leads to increased susceptibility, increased severity and accelerated time of onset of the disease (Vermeire et al., 1997; Manoury-Schwartz et al., 1997). Interpretation of the effects of IFN-γR-deficiency in CIA are further complicated by the results of Mattys and co-workers, who demonstrated that inactivation of this gene prevents the development of CIA when mycobacteria are not included in the inoculating dose (Matthys et al., 1999). These reports highlight the complexity of this disease and support the suggestion that joint inflammation can occur via more than one (and possibly many) pathway. These researchers also questioned the wisdom of including mycobacterium in the induction of arthritis, since the systemic response to mycobacterial antigens might bias and deviate the arthritogenic response.

A large proportion of CD4$^+$ T cells isolated from the hind paws of rats with AA were found to produce IFN-γ (see Chapter 6), which led us to speculate that IFN-γ is a molecule that is important in the effector function of the CD4$^+$ T cells that are involved in the pathogenesis of polyarthritis. Investigating adoptively-transferred arthritis allows the separation of the inductive and effector phases of arthritis. Furthermore, the influence of IFN-γ in the effector phase of the arthritis can be examined in the absence of the systemic effects of the response to mycobacteria and adjuvant. Adoptive transfer of washed TD lymphocytes reduces the possibility that mycobacterial antigens are transferred passively to the naive recipient rats.

As shown in Chapter 5, thoracic duct (TD) lymphocytes harvested from TD lymph at the end of the prodromal phase (day 9) of AA can transfer the disease to naïve recipients. Others in this laboratory have shown that this arthritogenic capacity is contained within a population of activated CD4$^+$ cells that are both necessary and sufficient to transfer disease (Spargo et al., 2001). The production of IFN-γ by CD4$^+$ cells isolated from the TD lymph was examined and found to follow a similar temporal pattern as arthritogenicity (i.e. the ability to transfer disease) (see Chapter 5). CD4$^+$ T cells collected from donors rats 9 days after inoculation of CFA produce significantly more of the pro-inflammatory cytokine interferon-γ (IFN-γ) after

in vitro stimulation with PMA plus ionomycin than $CD4^+$ T cells obtained from rats either 3 or 6 days after inoculation of CFA.

The observed coincidence between arthritogenicity and the production of IFN-γ by TD lymphocytes and the production of IFN-γ by $CD4^+$ T cells isolated from inflamed hind paws (see Section 6.2.5.1) suggested that IFN-γ may be involved in the effector functions of arthritogenic T cells. These observations and the observations of others (Jacob et al., 1989; Wiesenberg et al., 1989) led to the formulation of the two main hypotheses that are explored in this chapter.

Firstly, it was hypothesised that neutralisation of IFN-γ would inhibit the development of Th1-biased arthritogenic cells that are able to transfer arthritis to naïve recipient rats. This hypothesis was explored by treating donor rats with a single dose of mAb DB-1 shortly prior to inoculation of CFA. Nine days after inoculation, TD lymphocytes were collected for transfer to naïve recipient rats.

Secondly, it was hypothesised that blockade of IFN-γ in rats that were recipients of arthritogenic TD lymphocytes would prevent the development of adoptively-transferred arthritis. This second hypothesis was explored by treating recipient rats with mAb DB-1 before and during or after the transfer of arthritogenic doses of TD lymphocytes from a pre-arthritic donor rat. The adoptive transfer of arthritis provided an opportunity to examine the effector phase of the disease independently of the inductive phase, and in the absence of mycobacterial antigens.

7.2 Results

7.2.1 TD lymphocytes harvested from donor rats treated with either control antibody or mAb DB-1 are arthritogenic

To investigate the effect of IFN-γ blockade on the generation of arthritogenic lymphocytes, donor rats were injected intraperitoneally with either 0.8mg of mAb DB-1 (anti-IFN-γ) or 0.8mg of control polyclonal IgG prepared from normal mouse serum (see Section 2.5.4), 24

hours before inoculation with CFA. Nine days after inoculation with CFA, the thoracic ducts of these donor rats were cannulated and the lymphocytes collected overnight were washed and injected into naïve recipient rats. Each recipient rat received the TD lymphocytes collected overnight from one donor rat (ie. 1 donor to 1 recipient).

When compared with control antibody the treatment of donor rats with a single dose of 0.8mg of mAb DB-1 did not prevent the development of arthritogenic cells. The arthritis scores of recipients of TD lymphocytes from rats treated with control IgG or with mAb DB-1 are shown in Figure 7.2.1 A and B respectively. It is apparent that TD lymphocytes from both groups of donors transferred arthritis with similar efficiency to naïve rats. The amount of mAb DB-1 used and the timing of administration was based on the results reported by Jacob, et al (1989), who found that a similar dose suppressed significantly the development of AA in Lewis rats.

The mass of each recipient was measured daily to give an indication of the general "well being" of the rats. These results are displayed in Figure 7.2.1 and it is apparent that rats in both groups lost weight at the onset of clinical signs of arthritis, followed by weight gain in the ensuing period.

7.2.2 Blockade of IFN-γ exacerbates adoptively-transferred arthritis

To investigate the effect of IFN-γ blockade on the development of adoptively-transferred arthritis, rats were treated with either mAb DB-1 or control IgG, commencing before the development of clinical disease and continuing during the development of the adoptively-transferred arthritis. Arthritogenic cells were generated by obtaining the TD lymph from eleven donor rats (inoculated 9 days earlier with CFA). The lymphocytes from this lymph were pooled and 3.4×10^8 cells were injected into each recipient rat. In this way, each recipient rat in the two groups received an equal dose of arthritogenic cells. The recipient rats were treated with either 0.8mg of control polyclonal mouse IgG or 0.8mg of mAb DB-1 (anti-IFN-γ) on days −1, 0, 2, 4, 6 and 8 relative to the transfer of arthritogenic TD lymphocytes on day 0.

Figure 7.2.1 TD lymphocytes harvested from donor rats treated with either control IgG or mAb DB-1 are arthritogenic. The development of adoptively-transferred arthritis in recipients of thoracic duct (TD) lymphocytes collected from donor rats inoculated with CFA nine days before cannulation of the TD. The donors were treated with either control polyclonal mouse IgG (A) or mAb DB-1 (IgG1 anti-IFN-γ) (B) on day -1 relative to inoculation of CFA. Recipients were monitored daily for the occurrence of clinical signs of arthritis (bold line, left axis) and changes in weight (thin line, right axis). TD lymphocytes were injected intravenously into recipient rats on day 0. Each recipient rat received the overall output of TD lymphocytes from one donor (average of 5.4 x 10^8 cells). N = 6.
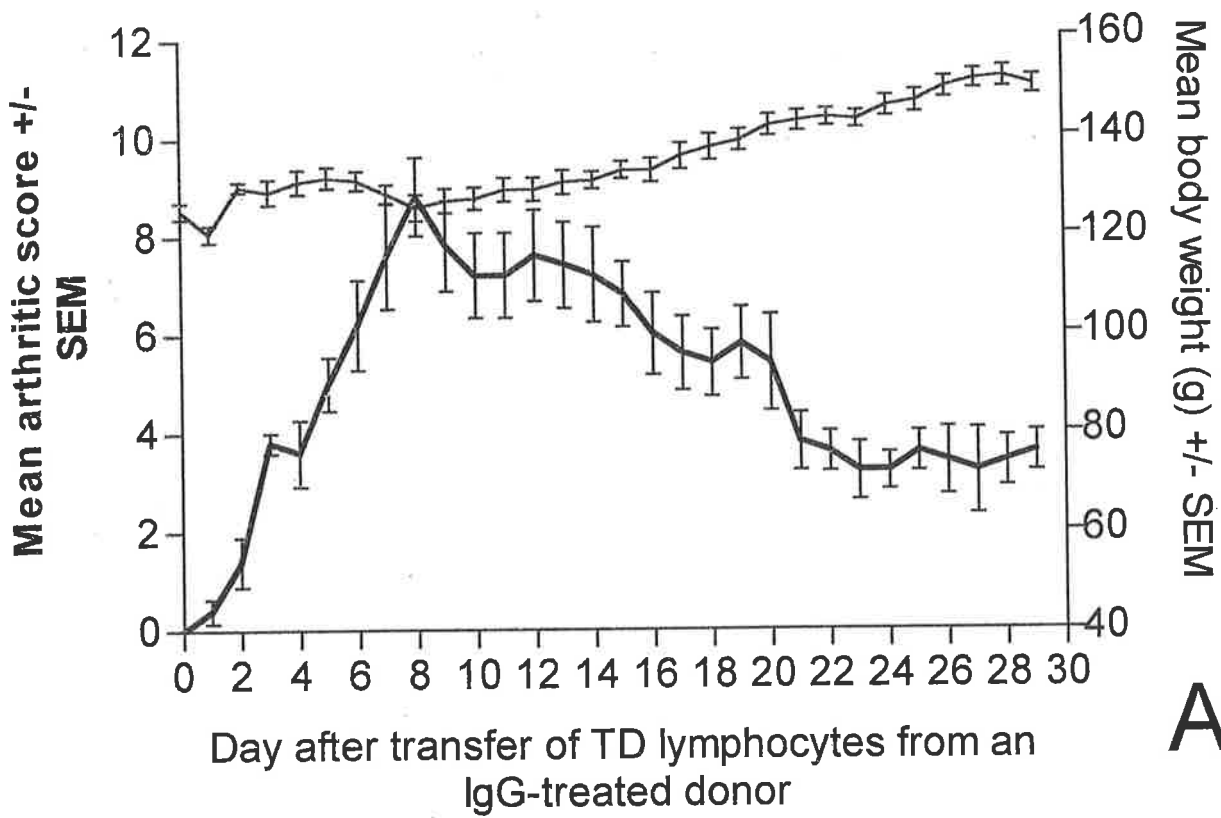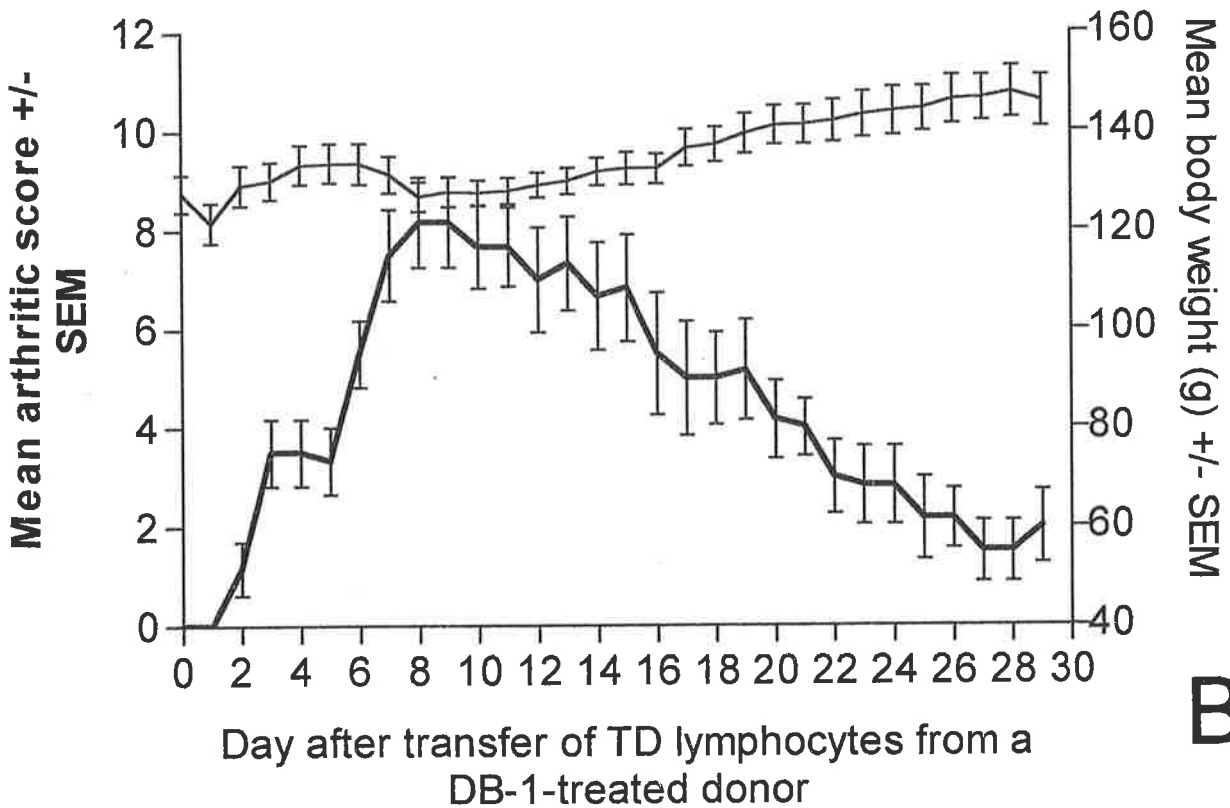
Mean arthritic score +/- SEM

Mean body weight (g) +/- SEM

Day after transfer of TD lymphocytes from an
IgG-treated donor

A

Mean arthritic score +/- SEM

Mean body weight (g) +/- SEM

Day after transfer of TD lymphocytes from a
DB-1-treated donor

B

As shown in Figure 7.2.2.1, recipients that were treated with control antibody (A) developed a relatively mild arthritis (compared with that described in Chapter 5, Figure 5.2.1 E). Rats that were treated with mAb DB-1 developed arthritis of greatly increased severity (Figure 7.2.2.1 B). The disease in the rats treated with mAb DB-1 was monophasic, with accelerated onset of significant inflammation compared with the often biphasic inflammation observed usually the course of adoptively-transferred arthritis. These rats had clinical signs of arthritis as early as 3 days after transfer of arthritogenic lymphocytes and this progressed without remission until the animals were euthanased on ethical grounds at day 14 after transfer. The arthritis of the mAb DB-1 treated rats was somewhat unusual compared with that seen in the normal course of adoptively-transferred arthritis. In particular, there was gross oedema, even in the front paws, which are usually affected less severely than the hind paws. The photographs in Figure 7.2.2.2, showing hind paws from rats treated with either control IgG (A and C) or mAb DB-1 (B and D), were taken on the sixth (A and B) and ninth (C and D) day after the transfer of arthritogenic lymphocytes. The gross oedema around the ankle of the rat treated with mAb DB-1 is apparent, whereas only mild arthritis was observed in the control IgG-treated rats.

The rats in the IgG-control treated group developed only relatively mild arthritis, although it was within the range of severity that has been observed following adoptive transfer of arthritogenic cells. This suggested that treatment with mAb DB-1 exacerbated mild disease into disease comparable to the most severe levels of actively-induced AA. The possibility that the administration of polyclonal IgG had therapeutic action on adoptively-transferred arthritis was addressed in a subsequent experiment (see below). Rats in the normal IgG-treated group increased in weight during the course of disease (Figure 7.2.2.1 A) but at a slower rate than that which would be expected from healthy rats of similar age. Rats with severe adoptively-transferred arthritis had retarded weight gain, reflecting their general poor health (B).

Indirect immunohistochemical staining of fresh-frozen sections of synovial tissue isolated from the affected hind paws was attempted but it failed because the level of non-specific

Figure 7.2.2.1 The effect of IFN-γ blockade on the development of adoptively-transferred arthritis. Rats were injected intraperitoneally with either 0.8mg of control polyclonal mouse IgG (A) or 0.8mg of mAb DB-1 (anti-IFN-γ) (B) on days -1, 0, 2, 4, 6 and 8. TD lymphocytes from pre-arthritic donor rats (inoculated with CFA nine days before TD cannulation) were injected intravenously into recipients on day 0. Each recipient received 1/12th of thoracic duct lymphocytes pooled from 11 donor rats (3.4×10^8 cells/recipient). Recipients were monitored daily for the occurrence of clinical signs of arthritis (bold line, left axis) and changes in weight (thin line, right axis). N = 6.

Mean arthritic score +/- SEM

Mean body weight (g) +/- SEM

Day after transfer of arthritogenic TD lymphocytes

A

Mean arthritic score +/- SEM

Mean body weight (g) +/- SEM

Day after transfer of arthritogenic TD lymphocytes

B

Figure 7.2.2.2 Blockade of IFN-γ increases the severity of joint swelling in the hind paws of rats with adoptively-transferred arthritis. TD lymphocytes from donor rats in the prodromal phase of AA were injected into recipient rats that were treated with either control polyclonal mouse IgG (A and C) or mAb DB-1 (anti-IFN-γ) (B and D) on days –1, 0, 2, 4, 6, and 8. The clinical signs of arthritis were monitored daily (Figure 7.2.2.1) and the hind paws of representative rats from each group were photographed at six (A and B) and nine (C and D) days after the transfer of arthritogenic cells on day 0.

staining was high (data not shown). Presumably, this was a consequence of the presence of mouse Ig in the tissues following the administration of mAb DB-1 or control mouse IgG.

Figure 7.2.2.3 shows photomicrographs of paraffin sections of the heel in the region where the Achilles tendon inserts into the calcaneum. Synovitis and tendonitis were apparent in both the IgG-treated rats (A) and rats treated with mAb DB-1 (B). Massive oedema was apparent in the soft tissues of the rats treated with mAb DB-1 and Figure 7.2.2.3 C shows the low density of cells in the area between the Achilles tendon, calcaneum and tibia. Tendonitis of a flexor tendon in a front paw of a rat treated with mAb DB-1 is shown in Figure 7.2.2.4 A.

Evidence of synovial hyperplasia was observed less frequently in sections of hind paws from the IgG-treated rats than in the mAb DB-1 treated rats. However, a mononuclear infiltration of the synovium of the joints of the hind paws of IgG-treated rats was observed. Figure 7.2.2.4 B shows a synovial villus projection into the joint space between the tibia and talus bones. Examination of this area at a higher magnification (C) indicated that the predominant cell type present had mononuclear morphology. Examination of this area of the hind paw of a rat treated with mAb DB-1 also featured synovial hyperplasia (Figure 7.2.2.5 A, B and C). The synovium that lines the bone featured on the left-hand side of the figure 7.2.2.5 A appears to be invasive. The other region of synovium in this figure forms a villus-like projection into the joint space. An interesting feature of the hind paws of rats in the group that was treated with mAb DB-1 was the abundance of fibrin accumulations in the joint spaces that were associated with the synovial inflammation (B and C). Cells were distributed sparsely within these accumulations and they appeared to be mononuclear cells (C). Similar fibrin accumulations were not apparent in the rats treated with IgG, where joint inflammation was observed less frequently and was of a lesser magnitude.

An interesting observation made by Wiesenberg and co-workers (Wiesenberg et al., 1989) was that although treatment of rats with mAb DB-1 exacerbated the late stages of AA, this treatment inhibited joint destruction as revealed by X-ray analysis. To examine the effect of IFN-γ blockade on joint destruction in adoptively-transferred arthritis, rats treated with either

Figure 7.2.2.3 Adoptively-transferred arthritis is associated with a mononuclear cell synovial infiltrate and blockade of IFN-γ increases the oedema in the hind paw. Paraffin sections of decalcified hind paws from rats with adoptively-transferred arthritis were stained with haematoxylin and eosin (see Sections 2.9.3 and 2.10.4). Photomicrographs were taken of a similar region of the hind paw from representative rats with adoptively-transferred arthritis, treated with either control polyclonal mouse IgG (A) or mAb DB-1 (anti-IFN-γ) (B and C). The bone (b) is the calcaneum and the tendon (t) is the Achille's tendon. The area shown in C is outlined in B by a thin black line. Original magnification 41.25X (A), 16.5X (B) and 82.5X (C).

b

t

A

b

t

B

b

t
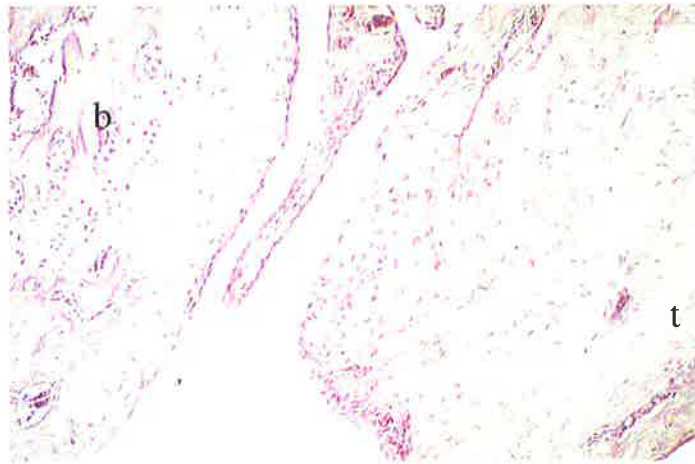
C

Figure 7.2.2.4 Adoptively-transferred arthritis is associated with tendonitis and synovitis. Paraffin sections of decalcified paws from rats with adoptively-transferred arthritis were stained with haematoxylin and eosin (see Sections 2.9.3 and 2.10.4). Sections from the fore and hind paws of representative rats with adoptively-transferred arthritis treated with either mAb DB-1 (anti-IFN-γ) (A) or control polyclonal mouse IgG (B and C) were viewed under a light microscope and photographed. A flexor tendon of a fore paw is illustrated in A, and the joint of the tibia and the talus is shown in B and C. The area shown in C is outlined in B by a thin black line. b = bone, t = tendon, s = synovium, js = joint space. Original magnification 16.5X (A), 41.25X (B) and 82.5X (C).

t

b

js

s
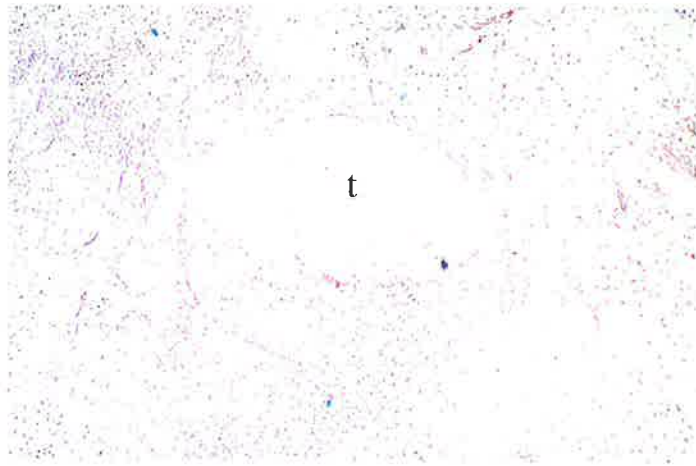
A
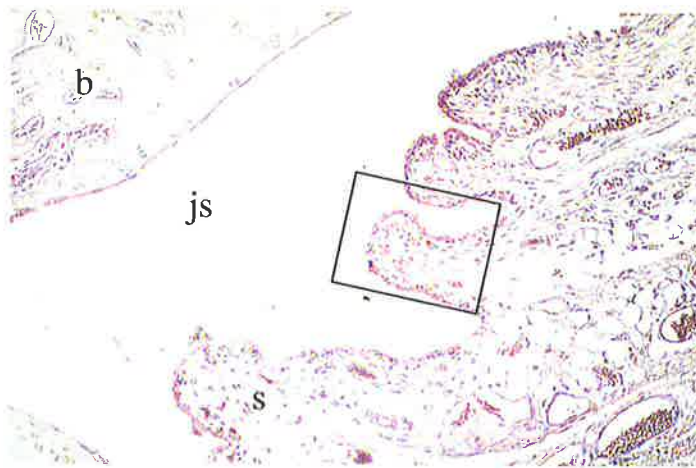
B
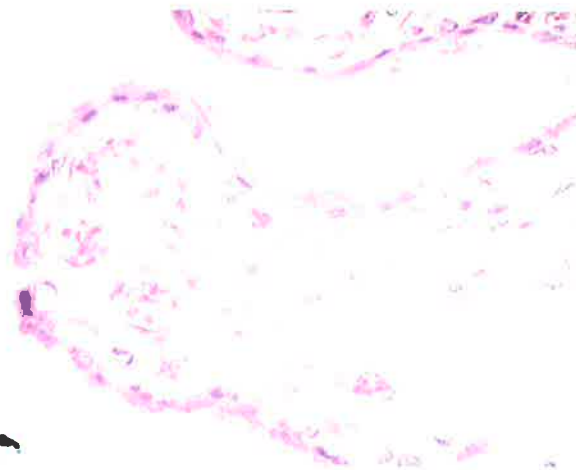
C

Figure 7.2.2.5 Blockade of IFN-γ increases the frequency of fibrin accumulations associated with synovial hyperplasia in the hind paws of rats with adoptively-transferred arthritis. Photomicrographs of paraffin sections of decalcified hind paws from rats with adoptively-transferred arthritis were stained with haematoxylin and eosin. The joint between the tibia and the talus bone of a hind paw of a representative rat with adoptively-transferred arthritis treated with mAb DB-1 (anti-IFN-γ) was viewed under a light microscope and photographed. The area shown in C is outlined in B by a thin black line. b = bone, s = synovium, f = fibrin accumulation. Original magnification 41.25X (A), 41.25X (B) and 165X (C).
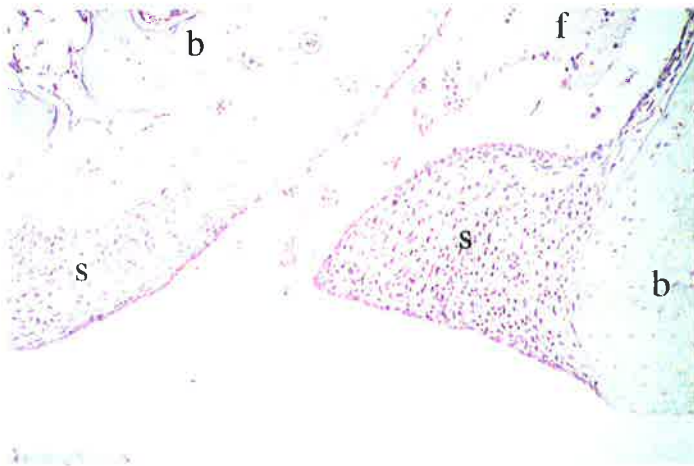
A
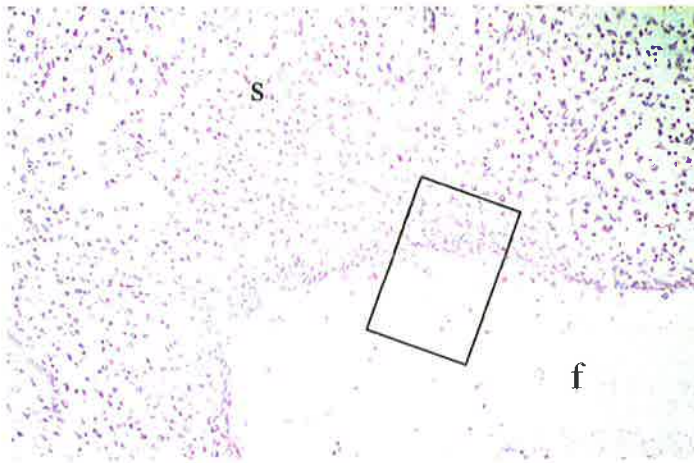
B
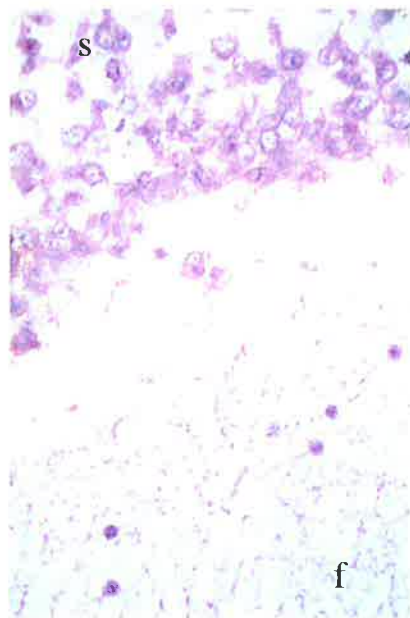
C

mAb DB-1 or control IgG were X-ray immediately after being euthanased. X-rays of representative rats were examined blind by an expert in the field and no differences were observed between mAb DB-1-treated, IgG-treated or normal rats (data not shown).

7.2.3 The exacerbation of adoptively-transferred arthritis by the blockade of IFN-γ is dependent on the timing of the administration of the antibody against IFN-γ

The observation that the blockade of IFN-γ at the time of transfer of arthritogenic lymphocytes exacerbated adoptively-transferred arthritis led to the hypothesis that the mechanisms by which IFN-γ acts may depend on the phase of arthritis. This hypothesis was investigated by delaying the treatment of rats that were recipients of arthritogenic lymphocytes until the predicted onset of the sustained phase of the clinical signs of joint inflammation. Rats were injected intraperitoneally with mAb DB-1 on days 4, 5, 7 and 9, relative to the transfer of arthritogenic TD lymphocytes on day 0. This group was compared to rats that were treated "early" with mAb DB-1 at the time of initiating adoptively-transferred arthritis (on days −1, 0, 2 and 4). A control group of rats was treated with polyclonal mouse IgG on days −1, 0, 2, 4, 6, and 8. In light of the observation of a mild arthritis in the IgG-treated rats shown in Figure 7.2.2.1 B, an additional control group of saline-treated rats was also included. Arthritogenic lymphocytes were obtained from 13 donor rats inoculated with CFA 9 days prior to cannulation of the TD. The TD lymphocytes from an overnight collection of lymph from all donor rats were pooled and 4.3×10^8 cells were injected into the recipient rats in each group, such that each rat received an equal dose of arthritogenic cells. Rats in all groups were injected on days −1, 2, 4, 5, 6, 7, 8 and 9 with either saline or antibody, according to the above schedule, such that each rat received an equal number of injections. When animals in the first two groups were not receiving antibody or control IgG, they received a similar volume of saline.

The adoptively-transferred arthritis in the saline-treated group (Figure 7.2.3.1 A) was not significantly different from that observed in the control IgG-treated group (B). This indicates that the mild arthritis in observed in rats treated with normal polyclonal IgG (described in Section 7.2.2) was not due to a therapeutic effect of administration of polyclonal immunoglobulin.

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Figure 7.2.3.1 Polyclonal mouse IgG does not suppress the development or severity of adoptively-transferred arthritis. Rats were injected intraperitoneally with 500μl of either saline (A) or saline containing 0.8mg of control polyclonal mouse IgG (B) on days -1, 0, 2, 4, 6 and 8. TD lymphocytes from pre-arthritic donor rats (inoculated with CFA nine days before cannulation) were injected intravenously into recipient rats on day 0. Each recipient rat received 1/16th of cells pooled from 13 donor rats (4.3 x10^8 cells/recipient). Recipients were monitored daily for the occurrence of clinical signs of arthritis (bold line, left axis) and changes in weight (thin line, right axis). N= 4.

Mean arthritic score +/- SEM

Mean body weight (g) +/- SEM

Day after transfer of arthritogenic TD lymphocytes

A

Mean arthritic score +/- SEM

Mean body weight (g) +/- SEM

Day after transfer of arthritogenic TD lymphocytes

B
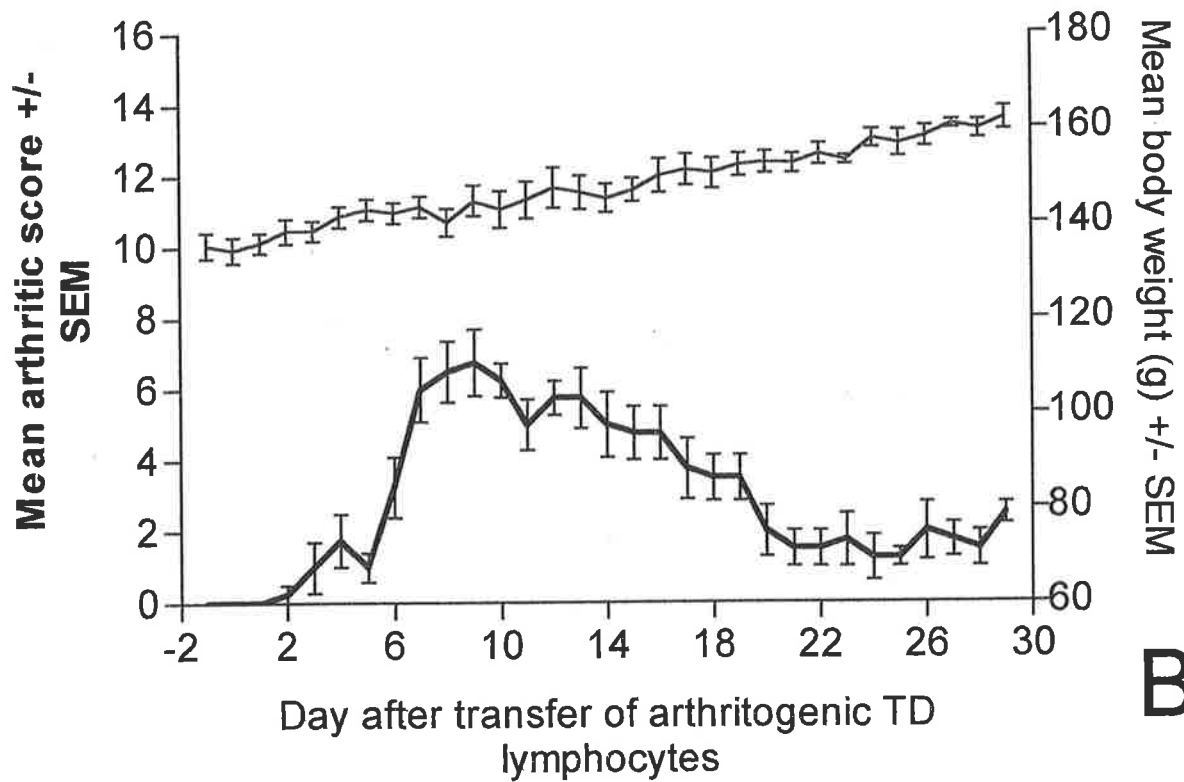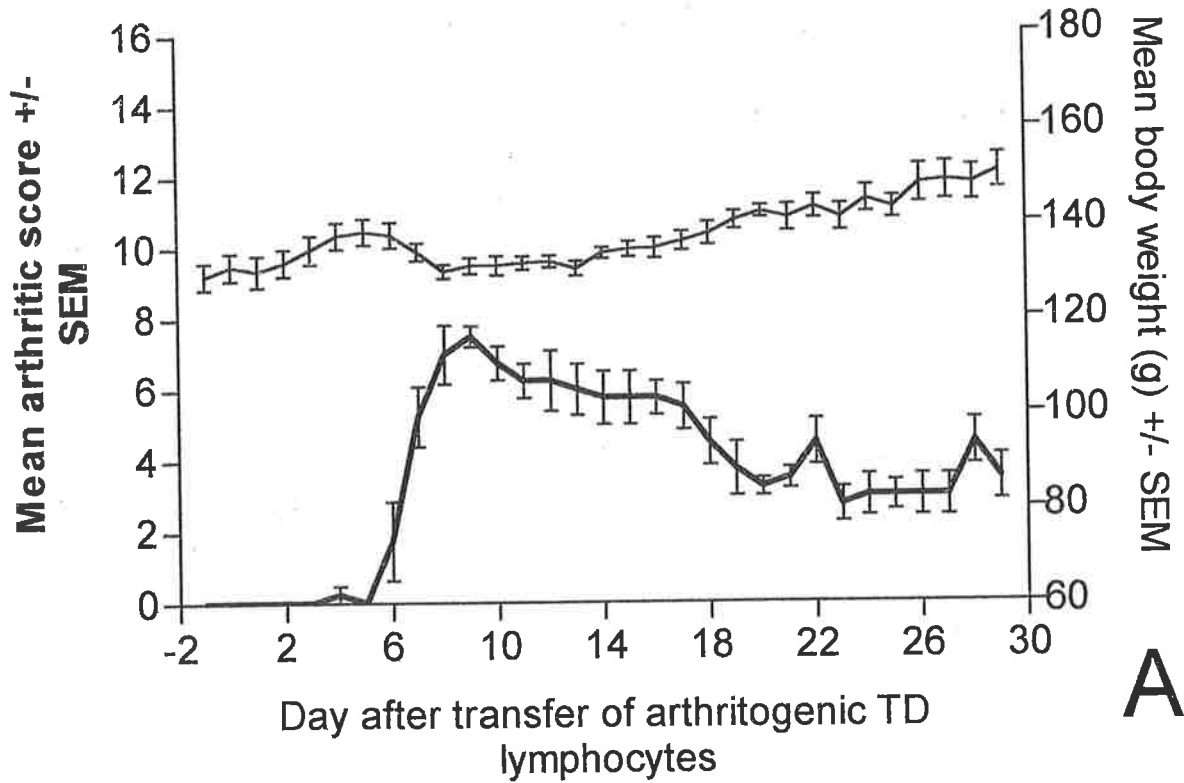
As shown in Figure 7.2.3.2, "early" treatment of recipients with mAb DB-1 (A) exacerbated the disease (Figure 7.2.3.1 A and B) and produced kinetics similar to the earlier protocol (Figure 7.2.2.1 B) in which "early" treatment was continued into the period of established disease (day 8). The rats were euthanased at day 14 because of ethical considerations but at this time there had been no remission in the inflammation. Commencing the treatment with mAb DB-1 "late", that is, on the fourth day after transfer of cells, did not alter the onset or severity of the disease significantly (Figure 7.2.3.2 B) compared with the control groups. The "late" treatment with mAb DB-1 appeared to delay the commencement of resolution of the arthritis until around day 15, whereas rats in the control groups were observed to improve from around day 9.

The pattern of weight fluctuations of the rats differed between the four groups in this experiment. The group of rats that commenced treatment with mAb DB-1 at day 4 ("late" treatment) lost weight from the onset of arthritis (around day 5) and did not begin to regain weight until after day 15, coinciding with an improvement in clinical signs of arthritis. Rats treated with normal polyclonal IgG continued to gain weight throughout the course of disease, while those in the saline-treated group lost weight with the commencement of arthritis but began to regain weight around day 9. The rats treated "early" with mAb DB-1 continued to lose weight from the onset of clinical signs of disease until they were euthanased at day 14. The pattern of weight in each of the experiments illustrated in Figures 7.2.1 A, 7.2.1 B and 7.2.3.1 A indicates a correlation between the commencement of resolution of the arthritis and the commencement of weight gain. This pattern is maintained in the group of rats treated with mAb DB-1 "late" (Figure 7.2.3.1 B), with the weight gain delayed by the same period as the delay in improvement of the inflammation. Although there is a suggestion that treatment with polyclonal mouse IgG prevented the fall in growth trajectory seen in other rats with adoptively-transferred arthritis, further experiments would be necessary to demonstrate a therapeutic effect of this treatment.

7.3 Discussion

Figure 7.2.3.2 Blockade of IFN-γ at the time of transfer of arthritogenic thoracic duct lymphocytes exacerbates adoptively-transferred arthritis. Rats were injected intraperitoneally with 500μl of saline containing 0.8mg of mAb DB-1 (anti-IFN-γ) on either days –1, 0, 2, and 4 (A) or days 4, 5, 7 and 9 (B), relative to the transfer of arthritogenic lymphocytes on day 0. The arthritogenic lymphocytes were obtained by the cannulation of the thoracic ducts of 13 rats that had been challenged with CFA 9 days earlier. These TD lymphocytes were pooled and divided between 16 recipients such that each recipient rat was injected intravenously with an equal arthritogenic dose of 4.3 x10^8 cells. The control groups are shown in figures 7.2.3.2 A and B. Recipients were monitored daily for the occurrence of clinical signs of arthritis (bold line, left axis) and changes in weight (thin line, right axis). N= 4.
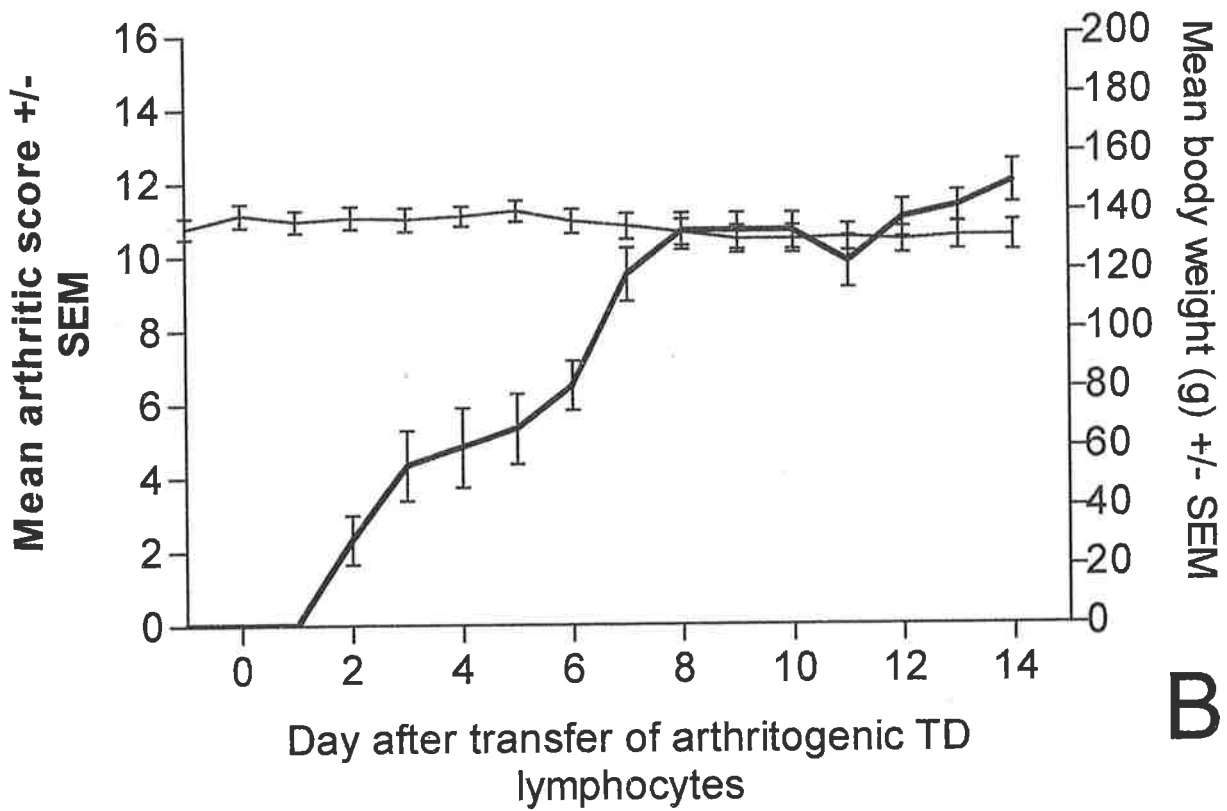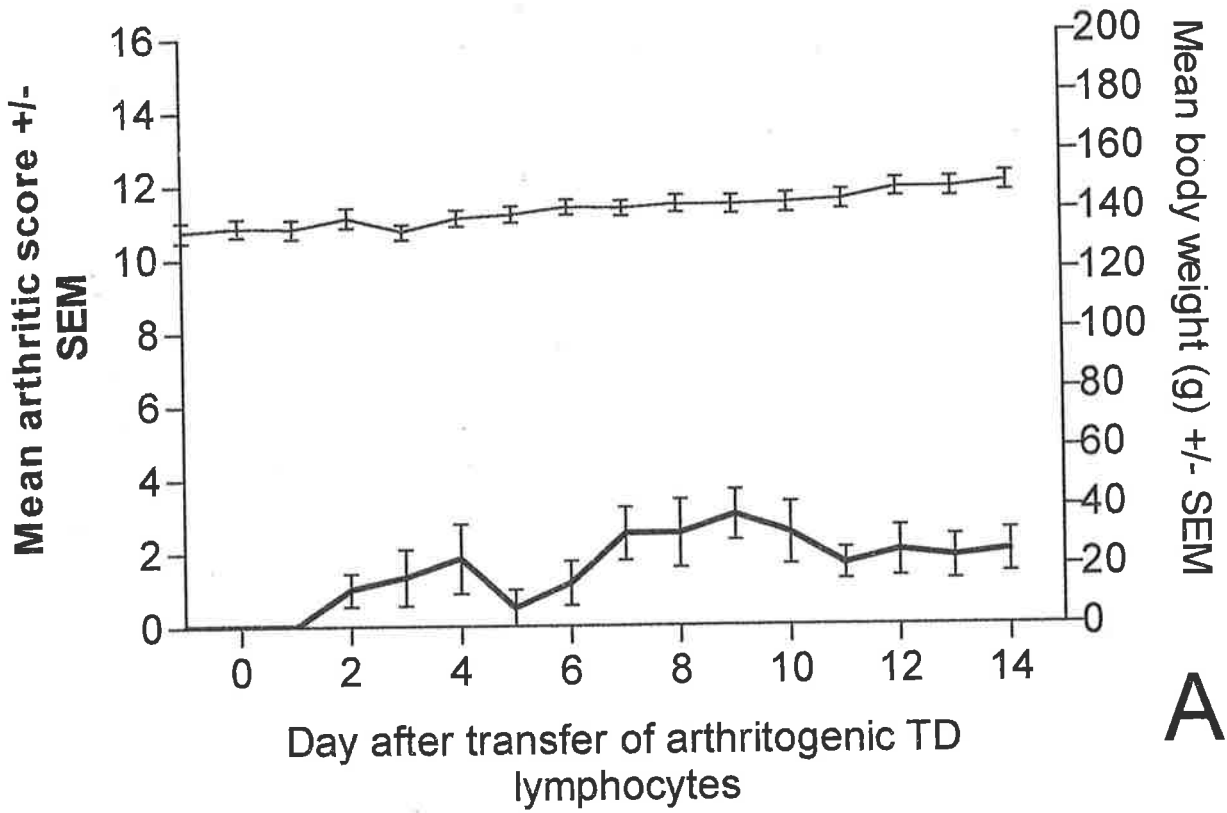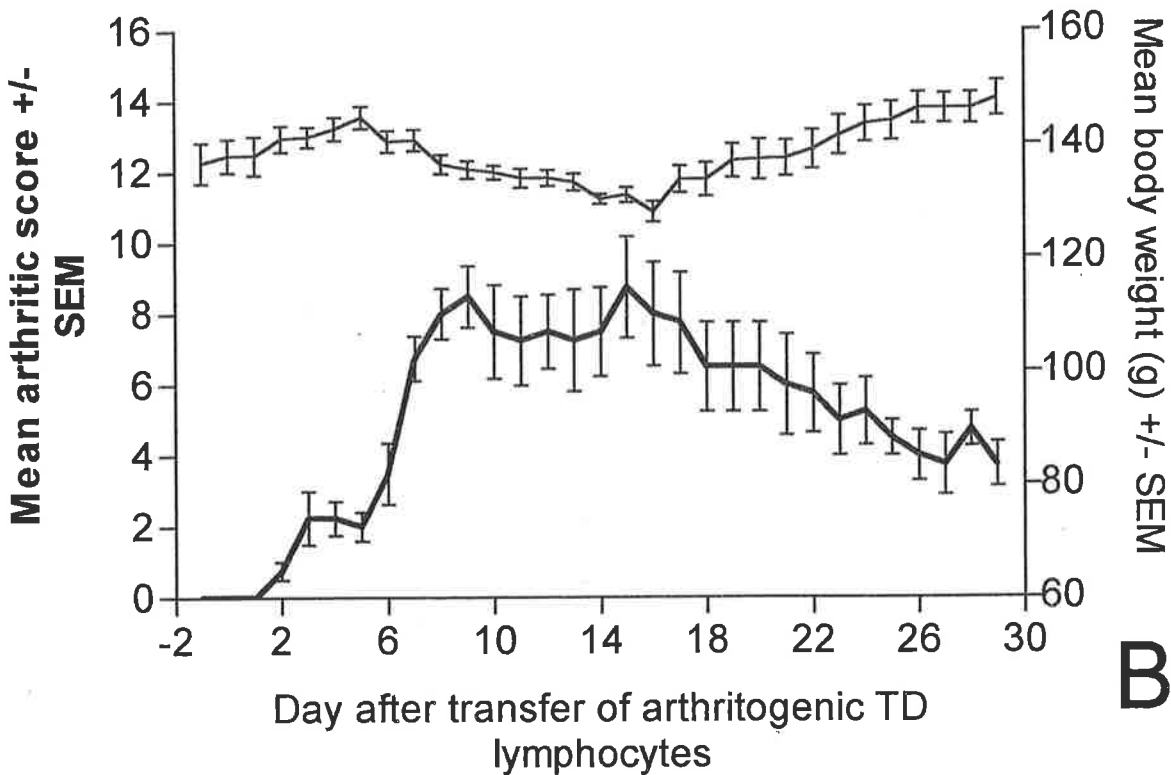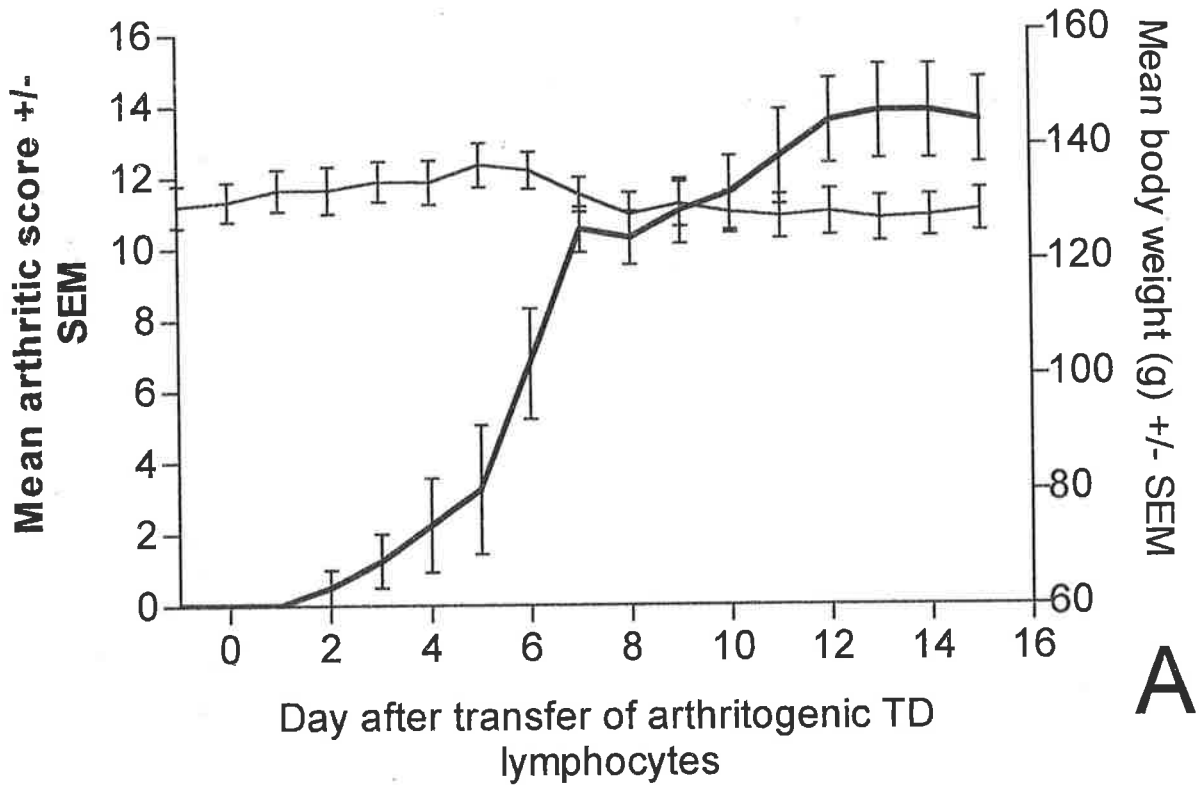
7.3.1 A single dose of 0.8mg of anti-IFN-γ antibody is not sufficient to inhibit the generation of arthritogenic TD lymphocytes

Others have demonstrated that a single dose of 1mg of mAb DB-1, administered 24 hours before inoculation of CFA, is sufficient to suppress the development of AA in Lewis rats (Jacob et al., 1989). These researchers analysed their results using the Mann-Whitney rank test but do not show the range of variance between individuals within a group.

A smaller dose of mAb DB-1 was administered to each rat in the present experiment, since it was assumed that female DA rats of 7 weeks would be approximately 80% of the size of Lewis rats of 10-14 weeks old (used by Jacob, et al). The administration of a single dose of 0.8mg of mAb DB-1 was not sufficient to prevent the generation of TD lymphocytes with the ability to transfer arthritis to naïve recipient rats (Figure 7.2.1). It seems likely that sufficient antibody persisted after the single injection administered by Jacob et al (1989) to neutralize the effects of IFN-γ during the late phase of the prodromal period of the actively-induced disease. The limited availability of mAb DB-1 prevented reproduction of the experiment of Jacob et al, in which the effect of treating DA rats with a single dose of mAb DB-1, 24 hours before the inoculation of CFA was tested in actively-induced disease. If 0.8mg of mAb DB-1 is sufficient to suppress the development of AA, then the results obtained here suggest that arthritogenic cells can be generated in the absence of IFN-γ.

The failure of IFN-γ blockade to suppress the generation of arthritogenic lymphocytes suggests that IFN-γ is not essential in this process. The importance of IL-12 in the generation of CD4$^+$ T cells that secrete Th1 cytokines, rather than IFN-γ, would support this finding. However, it must be questioned whether the dose of mAb DB-1 used was sufficient to neutralise all IFN-γ and in particular, whether the antibody had access to the microenvironment in which activation of T cells occurred. If communication between cells that involves IFN-γ requires directed secretion at sites of cell-cell contact, 'privileged' space may not be accessible to circulating antibody. Nevertheless, this limitation would be expected to apply also to the experiments of Jacob et al (1989).

7.3.2 Blockade of IFN-γ exacerbates adoptively-transferred arthritis

As shown in Chapter 5, IFN-γ-producing CD4^{+} T cells were more abundant in the TD lymph from rats 9 days after inoculation of CFA than at any other time after inoculation. Therefore, it was hypothesised that treatment of the recipients of TD lymphocytes collected from donors at this stage of the prodrome with mAb DB-1 (to neutralise IFN-γ) would prevent or ameliorate the disease. It was hypothesised that the blockade of IFN-γ would inhibit the activation of cells that mediate inflammation, such as macrophages and antigen presenting cells, which have been implicated in the initiation and perpetuation of joint inflammation (Klareskog, et al. 1982; Johnson et al., 1986). In addition, the neutralisation of IFN-γ could reduce the activation of endothelium and other cells which express chemokines and adhesion molecules and as a result, reduce the migration and recruitment of T cells into both lymphoid and synovial tissues (Issekutz et al., 1988; Westermann et al., 1993).

To examine whether blockade of IFN-γ would abrogate the effector function of arthritogenic CD4^{+} T lymphocytes, recipients of arthritogenic TD lymphocytes were treated with mAb DB-1 immediately preceding adoptive transfer and for 8 days after the transfer of arthritogenic lymphocytes. Surprisingly, the blockade of IFN-γ exacerbated adoptively-transferred arthritis. As shown in Figures 6.2.2.1 and 6.2.2.2, rats that were treated with mAb DB-1 developed a more severe arthritis than those treated with control polyclonal IgG. The severity of arthritis in the mAb DB-1 treated group was such that the rats had to be euthanased at day 14 for ethical reasons. The weights of the rats from the two groups are shown also in Figure 7.2.2.1. The rats in the mAb DB-1-treated group lost weight at the onset of joint inflammation, indicating poorer health and/or greater incapacity than the IgG-control treated group.

The onset of disease in the rats treated with mAb DB-1 was accelerated compared with that observed usually during the course of adoptively-transferred arthritis. These rats had significant joint scores by day 3, followed by progressive inflammation. The usual pattern in adoptively transferred disease is biphasic, with early minor inflammatory lesions between days 2-5, followed by the onset of progressive inflammation from days 5-6 after transfer. The rats in the IgG-control treated group followed this pattern. They developed an arthritis that

was at the mild end of the spectrum of disease seen following adoptive transfer of arthritogenic cells. There is little doubt that treatment with mAb DB-1 exacerbated the mild disease seen in the control animals. However, it is also possible that the mild course of the disease in the control animals was due to a therapeutic effect from the polyclonal mouse IgG similar to that produced in some inflammatory diseases of humans by administration of normal gamma globulin. The latter explanation seemed unlikely because the disease in the mAb DB-1 treated rats had qualitative difference (marked oedema) from that usually seen in adoptively-transferred arthritis. These alternatives were explored in a later experiment, which included controls for the therapeutic effects of polyclonal IgG.

Histological examination of paraffin sections of decalcified hind paws revealed massive inflammation of essentially all joints and soft tissues in the hind paws of the rats treated with mAb DB-1 (Figures 7.2.2.3 B and C, 7.2.2.4 A and 7.2.2.5). Inflammation of the synovium was observed less frequently in the IgG-treated rats (Figures 7.2.2.3 A and 7.2.2.4 B and C) compared with the mAb DB-1 treated rats but the features of the infiltrate were similar. It consisted of mononuclear cells, with polymorphonuclear cells observed less frequently. Fibrin accumulation was apparent in some joint spaces in the mAb DB-1-treated group and tendonitis was also present (Figures 7.2.2.5 and 7.2.2.4 A, respectively). Some histological evidence of invasion of the cartilage was observed (Figure 7.2.2.5A) but X-ray analysis failed to reveal any bone erosion. Weisenberg et al (1989) observed that administration of mAb DB-1 to rats with actively-induced AA inhibited joint destruction. The present results support this finding but the early evidence of cartilage erosion evident histologically suggests that bone erosion may have followed if the rats had been allowed to survive beyond the acute phase of the adoptively-transferred disease.

7.3.3 The exacerbation of adoptively-transferred arthritis by the blockade of IFN-γ is dependent on the timing of the administration of the anti-IFN-γ antibody

The administration of anti-IFN-γ mAb at the time of transfer of arthritogenic TD lymphocytes increased the severity of the adoptively-transferred arthritis. However, delaying the administration of anti-IFN-γ until 4 days after the transfer of arthritogenic lymphocytes did not increase the severity of the polyarthritis in the recipient rats. This suggests that IFN-γ

produced by arthritogenic lymphocytes, in addition to any pro-inflammatory effects, also has a role in down-regulating inflammation. This effect, which could be indirect, appears to be most important during the early stages of the adoptively-transferred disease. This suggests that the effector phase of inflammation in AA has an IFN-γ -independent pathway and that once established, the inflammation is relatively unaffected by late administration of neutralising antibody. However, at this stage there was some evidence of delayed resolution of the disease after blockade of IFN-γ.

A possible mechanism by which IFN-γ acts to down-regulate an inflammatory response is via the induction of nitric oxide (NO) production by macrophages. The expression of inducible nitric oxide synthase (iNOS), which had been detected in monocytes from patients with rheumatoid arthritis, is induced by IFN-γ and suppressed by TGF-β (MacMicking et al., 1997). NO has cytotoxic activity and the blockade of NO synthase suppresses the development of AA (Stefanovic-Racic et al., 1994). However, when present in elevated levels, NO can act as an immune suppressant by inhibiting cytokine production and T cell expansion, in addition to down-regulating its own synthesis. Thus, in addition to its cytotoxic role, NO is thought to have important immunoregulatory properties (Liew, 1995). A similar phenomenon has been reported to occur in experimental allergic encephalomyelitis (EAE), in which it has been demonstrated that IFN-γ is critical in the control of the recovery phase. There is substantial evidence that IFN-γ plays a down-regulatory role in EAE, via the induction of NO production by macrophages, which can reduce inflammation and promote tissue remodelling when it is present in elevated levels (Willenborg et al., 1999; O'Brien et al., 1999). Research at the Trudeau Institute provided evidence to suggest in EAE (Chu et al., 2000) and mycobacterial infections (Dalton et al., 2000) IFN-γ may indirectly, via the induction of NO production, induce apoptosis and inhibit proliferation of activated CD4+ T cells and thus induce disease remission.

The results presented in this chapter indicate that a similar mechanism may be active in adoptively-transferred arthritis. The blockade of IFN-γ would inhibit the induction of NO production and thus eliminate a down-regulatory mechanism that would otherwise be active.

It seems unlikely that NO is singularly responsible for the resolution of adoptively-transferred arthritis, because when IFN-γ blockade commenced at the onset of clinical signs of joint inflammation (day 4), the disease was not exacerbated. However, as discussed above, the resolution of the arthritis in these rats was delayed compared with the control groups, although this was not striking. If IFN-γ is the essential molecule involved in disease resolution, more marked differences might have been expected, since blockade of IFN-γ would eliminate one of the most potent inducers of iNOS. To investigate further the immunoregulatory role of NO, iNOS inhibitors such as N-methyl-L-arginine (L-NMA) could be used to examine more directly the role of NO in adoptively-transferred arthritis. If NO is responsible for the resolution of disease, treatment with L-NMA would be expected to extend the course of joint inflammation. However, studies using NOS inhibitors should be planned carefully in light of recent studies that have demonstrated that the route of administration of NOS inhibitors can have profoundly different effects on inflammation (Paul-Clark et al., 2001).

IFN-γ inhibits the production of IL-8, a chemotactic and activating factor for many leukocytes (including neutrophils, T cells and monocytes) which is produced in abundance in the synovium of patients with RA (reviewed in Baggiolini et al., 1994). Although IL-8 is a human molecule, it is likely that the rat homologue CINC, would be similarly down-regulated by IFN-γ and reduce the recruitment of inflammatory cells into the inflamed synovium. The blockade of IFN-γ with mAb DB-1 in adoptively-transferred arthritis may have released such inhibition and led to the exacerbation of adoptively-transferred arthritis.

Others have shown that the systemic administration of rIFN-γ to rats throughout the course of AA does not affect the early stages of disease. However, the secondary phase of joint swelling was suppressed, providing further evidence for the role of IFN-γ in disease resolution (Nakajima et al., 1991). Interestingly, Jacob, et al (1989) found that administration of rIFN-γ from days −2 to 24 increased the severity of arthritis in the early phase of the disease, whereas treatment between days 12 and 24 did not effect the disease course. However, the large experimental variation between groups of control animals in this study

makes interpretation of the results difficult. In addition, Jacob and co-workers administered the rIFN-γ systemically via intraperitoneal injection. It is possible that a different response may have been seen if the IFN-γ was administered locally into the joints.

In studies of collagen-induced arthritis (CIA), conflicting results have been obtained in IFN-γ-R knock-out mice. However, there is substantial evidence that IFN-γ has a role in the initiation of CIA. The local administration of rIFN-γ into the right hind- and fore-paws of mice during the induction of CIA accelerates the onset of CIA and increases the infiltration of $CD4^+$ lymphocytes into the treated paws (Mauritz et al., 1988). A possible mechanism for this effect is the induction by IFN-γ of IL-12 production by macrophages and other cells. IL-12 enhances the production of other Th1-associated cytokines such as IL-2 and TNF-α and inhibits the development of Th2 cells. The administration of anti-IL-12 antibodies during the induction of CIA in mice reduced the severity of arthritis and in *ex vivo* studies on cells from mice, the production of IFN-γ by lymph node cells and TNF-α by synovial cells (Malfait et al., 1998). The development of CIA in IFN-γ-R deficient mice can be prevented by the administration of antibodies against IL-12 (Matthys et al., 1998) and the systemic administration of rIL-12 increases the production of IFN-γ in the hind paws of mice and exacerbated CIA (Parks et al., 1998). The mutual stimulation between $CD4^+$ T cells and dendritic cells/macrophages/synoviocytes through the production of IFN-γ (produced by T cells) and IL-12 (produced by dendritic cells and macrophages) is likely to be involved in the initiation and perpetuation of arthritis.

7.3.4 General comments

Caution must be exercised when interpreting *in vivo* studies using monoclonal antibodies, since some monoclonal antibodies, particularly those of low affinity, have been reported to extend the survival and *in vivo* activity of their cognate cytokine. Since mAb DB-1 is an IgG1 mouse monoclonal antibody that exhibits high affinity binding for rat IFN-γ and efficiently neutralises the anti-viral activity of IFN-γ (Van der Meide et al, 1986), it is unlikely that this occurs with mAb DB-1. Another caution is that the molecular complex formed between mAb DB-1 and IFN-γ could have exacerbated the inflammatory response at

sites with abundant IFN-γ (such as in the hind paws). If this occurred, an exacerbating effect was observed only when the antibody was administered before the transfer of arthritogenic cells but not when it was administered four days later. If the observed exacerbation of disease was induced solely by the formation of immunoreactive mAb DB-1:IFN-γ complexes, then this immunoreactivity occurs only in the early stages of adoptively-transferred arthritis, or it was only pro-inflammatory at this stage. Neither possibility seems very likely.

Others have used mAb DB-1 experimentally in rats in parallel with rIFN-γ and found that the agents induce opposite effects i.e. the administration of mAb DB-1 early in the course of AA suppresses disease whereas administration of rIFN-γ at the same time point exacerbates the arthritis (Jacob et al., 1989; Huang et al., 1999). This suggests that the primary effect of mAb DB-1 is the neutralisation of IFN-γ, rather than the formation of non-specific immune-reactive complexes that exacerbate the disease. Wiesenberg et al, treated rats on alternate days (from day –2 to 20) with high doses of mAb DB-1 (3-5mg) and found that the development of arthritis was delayed and reduced in severity, but not prevented. They suggested that these high doses may have induced anti-idiotypic antibodies, which in turn may have activated macrophages if they were deposited in the joints (Wiesenberg et al., 1989).

Another factor for consideration in the interpretation of studies using mAb DB-1 is that the exchange of IFN-γ from T lymphocytes to other cells may involve intimate cell-cell contact that is not accessible to the systemic administration of anti-IFN-γ antibodies. Under these conditions, the systemic effects of a cytokine might be blocked, whereas the influence of a cytokine delivered to a cell by direct contact might not be affected. Of particular concern is the T cell-APC interaction, making the interpretation of the lack of effect of mAb DB-1 on the generation of arthritogenic CD4$^+$ T cells uncertain.

These complications are not encountered when using cytokine/cytokine receptor gene knock out animals, such as those used to elucidate the role of IFN-γ in collagen-induced arthritis (CIA). However, these studies also should be interpreted with caution, because in these

animals the immune system has developed in the absence of the effect of an important cytokine. The influence of IFN-γ (as well as other cytokines) on lymphocyte education, stimulation of naive lymphocytes and generation of effector function is incompletely understood. Failure to consider these effects on immunological development may lead to misleading conclusions regarding the mechanisms involved in the development of arthritis and other autoimmune diseases. The use of a neutralising monoclonal antibody to IFN-γ facilitates the examination of the influence of this cytokine without the complexities associated with gene knock out animals. In the near future, conditional gene knockouts will provide valuable alternative ways of terminating the influence of a cytokine in a controlled fashion.

Others have shown that neutralisation of IFN-γ during the inductive phase of AA leads to suppression of disease, whereas delaying treatment until the end of the prodromal phase of disease and the beginning of the effector phase exacerbates disease. However, these studies were limited to examining adjuvant-challenged rats and so may be complicated by the systemic response to mycobacterial antigens, which may be irrelevant to the arthritogenic response. The present studies have shown that in adoptively-transferred arthritis, the neutralisation of IFN-γ in the effector phase of disease increases the severity of polyarthritis. The results presented here suggest that IFN-γ has regulatory properties in the effector phase of polyarthritis, which reduce inflammation via mechanisms that may not be involved in the inductive phase of the disease.

Other researchers have shown that neutralisation of IFN-γ has suppressive effects during the inductive phase of AA (Jacob et al., 1989). This suggests that IFN-γ is important for the initiation of joint inflammation rather than for the perpetuation of disease. A role for IFN-γ in the generation of arthritogenic effector cells has not been demonstrated in the present study. The results presented in this chapter indicate that IFN-γ plays an important immunoregulatory role in the effector phase of AA. There have been some promising clinical studies on the use of IFN-γ in the treatment of rheumatoid arthritis. A popular explanation for this is that IFN-γ inhibits the production of IL-4, which in turn inhibits IL-4-enhanced B cell

proliferation and the production of pathogenic antibodies (Browning, 1987). However, the induction of iNOS and NO production may be a more critical mediator of down-regulating the inflammatory response. In light of the results presented here, it could be reasoned that IFN-γ might be of therapeutic value in the treatment of established, but not early, arthritis. However, the results also sound a note of caution. IFN-γ blockade clearly has the capacity to aggravate disease markedly, in a manner, which presumably reflects the dominance of inflammatory pathways at the time of administration. However, the nature of this dominance may not be easy to predict in the individual patient, in whom, unlike rats with experimentally-induced disease, neither a discrete inciting event nor a predictable time course of events can be apprehended. There is a risk that under some conditions, the pro-inflammatory effects of IFN-γ may predominate and in such case, IFN-γ would exacerbate the disease while anti-IFN-γ would be therapeutic.

CHAPTER 8

DISCUSSION

8.1. Introduction

Adjuvant-induced arthritis (AA), which is observed in DA rats following inoculation of CFA, resembles in many aspects the human disease rheumatoid arthritis (RA). It is possible but not proven that the disease is due to an anti-mycobacterial response, in which CD4^{+} T cells are generated that cross-react with self-antigens present in joint tissue. If the immune response was directed primarily against mycobacterial antigens and the mycobacterial-specific CD4^{+} T cells were expanded in response to mycobacterial antigens rather than self-antigens, then it might be expected that the disease would be limited by the release/supply of mycobacterial antigens. The administration of mycobacteria in oil forms a depot of antigen that is released slowly. Rats with AA exhibit ear swelling in response to PPD, indicating that there is indeed a DTH reaction to mycobacterial antigens following immunisation with CFA (not shown) and T cell clones with specificity for mycobacterial antigens have been shown to induce arthritis in naïve recipients when transferred adoptively (Van Eden et al., 1985). Administration of mycobacteria by gavage can inhibit the induction of AA (Haque et al., 1996) and treat established AA (Cobelens et al., 2000), presumably by inducing either oral tolerance to mycobacterial antigens or immune deviation, further highlighting the importance of mycobacterial epitopes as targets in AA.

Alternatively, the subcutaneous injection of CFA could cause tissue damage and activation of local dendritic cell (DC)s, leading to the effective presentation of autoantigens and the activation of autoantigen-specific T cells. In this case, the reaction would be directed primarily against self, with mycobacteria acting purely as an adjuvant capable of activating macrophages and DCs, which in turn activate naïve auto-reactive T cells. There is some evidence that supports this suggestion. Arthritis can be induced in DA rats in the absence of mycobacteria, by inoculation of oil alone (incomplete Freund's adjuvant) (Kleinau et al., 1991). This disease is milder, possibly as a consequence of the reduced adjuvant activity of IFA compared with CFA. Furthermore, AA is a chronic disease that can last for at least a year (Pearson, 1956). If the immune response was targeted primarily against mycobacterial antigens, then the chronic remitting and relapsing aspect of the disease would require the persistence of the mycobacterial antigens for at least this period.

A third possibility is that the inoculation of CFA activates macrophages and DCs systemically and leads to the inappropriate presentation of synovial antigens to T cells and that there is cross-reactivity between synovial antigens and mycobacterial antigens. Such T cells might be expanded concurrently with the local response to mycobacterial antigens in the lymph nodes that drain the site of inoculation. However, the disease can be transferred adoptively by CD4$^+$ T cells alone, indicating that at least in the effector phase, local macrophage/DC activation in synovium is not necessary for the development of arthritis.

8.2 Generation of arthritogenic cells in rats following inoculation of CFA

Despite the anonymity of the target antigen, it is evident that T cells with an arthritogenic capacity are generated in DA rats in response to inoculation of CFA. Activated CD4$^+$ T cells were present in the inguinal lymph nodes, which drain the site of inoculation, within 3 days after inoculation of CFA. A large proportion of the CD4$^+$ T cells present in the inguinal and popliteal lymph nodes remained activated throughout the prodrome and early clinical phase of AA (see Section 8.3). These results are consistent with the hypothesis that inoculation of CFA leads to the stimulation of cells in the regional lymph nodes and that this generates a population of activated CD4$^+$ T lymphocytes. The questions of whether any of these cells were arthritogenic and the kinetics of the appearance of arthritogenic cells in the lymph nodes and in TD lymph, were addressed by adoptive transfer to naïve syngeneic recipients.

Spargo et al (1996) demonstrated the presence of a population of joint-seeking lymphoblasts in the TD lymph from donor rats 9 days after the injection of CFA. This population was shown to arise in peripheral lymphoid tissue rather than in the drainage of the mesenteric lymphatics. More recently (Spargo et al., 2001), it has been found that TD lymphocytes at this time contain a population of arthritogenic cells that can reliably produce arthritis in syngeneic recipients with a dose of 5 x 10^7 purified CD4$^+$ T cells. The arthritogenic population was contained within a sub-population that expressed MHC class II, CD25, CD71 and CD134 and arthritis could be transferred by as few as 1 x 10^6 CD4$^+$ T cells that were selected for these markers (Spargo et al., 2001). However, the timing of the appearance of this arthritogenic population was not determined. This population of activated effector CD4$^+$ T cells is probably responsible for the dissemination of the disease in its characteristic polyarticular distribution.

The work in Chapter 5 shows that arthritogenic cells appear in TD lymph as early as 4 days after inoculation of CFA. The appearance of these cells corresponds well with reports that following immunisation, activated lymphocytes exit the responding nodes, commencing approximately 3-4 days after inoculation (Delorme et al., 1969). It is likely that these arthritogenic cells were released from the lymph nodes draining the site of CFA inoculation, which include the inguinal lymph nodes. The dose of cells transferred in these experiments corresponded to the overnight collection from a single donor. Based on the data of Spargo et al (2001), this suggests that as early as 4 days after inoculation, in excess of 1×10^6 arthritogenic $CD4^+$ T cells were released into the central lymph over a period of approximately 18 hours. The arthritogenicity of the TD lymphocytes, as measured by the incidence of arthritis in the recipients and the severity of the resulting disease, increased to a maximum at day 9 after inoculation of the donor and then appeared to decline between days 9 and 12 after inoculation. These findings confirm the potency of TD lymphocytes from arthritic donors at day 9 post-inoculation, as described by Spargo et al (2001).

Cells isolated from the inguinal lymph nodes of rats at day 9 post-inoculation contained an arthritogenic population. A dose of 2×10^7 cells harvested from the lymph nodes rats at day 9 post-inoculation induced arthritis of greater severity and with accelerated onset compared with the same number of TD lymphocytes collected from rats at day 9 post-inoculation (Figure 4.2.6.1A). However, arthritogenic cells were not detected in the lymph nodes of rats with AA before day 9 post-inoculation, whereas, as mentioned earlier, such effector cells were present in the TD lymph as early as 4 days post-inoculation.

It is interesting to consider the delay until day 9 post-inoculation in the appearance of arthritogenic cells in the inguinal lymph nodes. At days 6 and 9 after inoculation of CFA, a greater proportion of the $CD4^+$ T cells in inguinal lymph nodes were activated (based on the expression of markers such as MHC class II) compared with those from the TD lymph (see Figures 4.2.3.1 C and 5.2.3.1 F). This suggests that in the TD lymph, a greater proportion of the activated $CD4^+$ T cells are arthritogenic, compared to cells in the inguinal lymph nodes. It is possible that at day 6 post-inoculation, the cells prepared from the inguinal lymph nodes did not contain enough cells of sufficient maturity for their effector function to be detected by adoptive transfer. While 2×10^8 lymph node cells were

transferred, this was approximately half the number of TD lymphocytes that were transferred from donors at day 6 post-inoculation, thus preventing a direct comparison with the arthritogenicity of TD cells.

The proportion of CD4$^+$ T cells in the inguinal lymph nodes that expressed the activation markers MHC class II, CD25 and UA002 antigen was greatest at day 9 post-inoculation whereas in the TD lymph, cells expressing these markers peaked 3 - 4 days post-inoculation. This may indicate that arthritogenic cells exit the lymph nodes quickly and that there is a build up of these cells only later in the response. Alternatively, the cells that have an arthritogenic capacity at day 9 post-inoculation in the inguinal lymph nodes may be functionally different from those that are released into the lymph. Perhaps those that are retained in the lymph node are in the process of developing into memory cells and they may not be sufficiently mature to have an effector function until after day 6. Memory cells respond rapidly to antigen and produce a range of cytokines (reviewed in Dutton et al., 1998) and this may explain the accelerated onset of arthritis seen after transfer of inguinal lymph node cells relative to the disease caused by the same number of TD lymphocytes (Figure 4.2.6.1).

The failure to find arthritogenic cells in the popliteal lymph nodes suggests either that arthritogenic cells are not generated at this site or that if they are, the cells are either not retained, they do not reach arthritogenic maturity within 9 days post-inoculation or they differentiate into non-pathogenic effector cells. It can be calculated that of the 2 x 10^8 popliteal lymph node cells that were transferred from donors at day 9 post-inoculation of CFA to naive recipients, approximately 9 x 10^7 (45%) were CD4$^+$ T cells and 1.9 x 10^7 of these were activated (21% of CD4$^+$ T cells expressed MHC class II). Considering that as few as 1 x 10^6 activated CD4$^+$ T cells from the TD lymph of rats at the same stage in the prodrome of AA (day 9 post-inoculation of CFA) can transfer arthritis (Spargo et al., 2001), the lack of arthritogenicity of popliteal lymph node cells cannot be attributed simply to a lack of activated CD4$^+$ T cells. However, because the number of cells that can be obtained from the popliteal lymph nodes of a rat is small, a transfer was attempted in only one rat. Thus, unless the incidence of arthritis transferred by popliteal lymph node cells was 100%, it is possible that the arthritogenic potential of cells from this source may have been missed. To investigate the properties of cells from the popliteal lymph node further, it would be necessary to use a larger pool of donors and more recipients.

Furthermore, it would be interesting to obtain cells from rats inoculated 12 days after inoculation with CFA, to test whether development of arthritogenic cells occurs later at this site, which is remote from the site of inoculation. Finally, it would be instructive to examine whether activated cells in these lymph nodes exert a regulatory (suppressive) effect when co-transferred with arthritogenic cells. Lying downstream of the affected hind paws, the popliteal lymph nodes could be the site of an immunomodulatory response later in the disease process.

The incidence of adoptively-transferred arthritis is low when donor cells are harvested from the TD lymph at days 4, 5 or 6 post-inoculation, yet the onset in those animals that develop disease is within the range that is seen in recipients of cells harvested 9 days after inoculation with CFA (ie. within approximately 5 days after transfer). This suggests that the minimum period for transformation of naive cells into arthritogenic effector cells is 4 days. During this period, there must be uptake and processing of antigen by APCs, presentation of the arthritogen to naïve T cells, a period of clonal expansion and differentiation of the activated T cells and release of effector cells into TD lymph. On the other hand, the period from injection of arthritogenic cells to the onset of clinical arthritis is approximately a further 5 days, irrespective of the stage of the donor beyond the first few days of the prodrome. If the success of adoptive transfer with TD cells was not due to the presence of arthritogenic T cells but rather to the adoptive transfer of small numbers of arthritogen-laden APC, the minimum period for activation of naïve host T cells and the onset of clinical arthritis would be 9 days. The possible involvement of DCs has been excluded previously by Spargo et al (2001), who demonstrated adoptive transfer of AA with positively selected α/β TCR$^+$ cells from TD lymph and in addition, found no reduction in arthritogenicity of cells from the TD lymph following depletion of macrophages and DCs. Together with the argument based on kinetics, this makes an obligatory requirement for antigen-laden APC in the adoptive transfer of AA unlikely.

It is interesting that the duration of the arthritis produced by TD lymphocytes collected either 4 days or 9 days after inoculation of CFA was similar. It seems likely that the rapidly increasing inflammation observed during the development of actively induced AA reflects the recruitment of cells released into lymph from 4 days after inoculation of CFA. This traffic of activated cells increases as greater numbers of arthritogenic cells are

released from stimulated lymph nodes into the lymph, along with efficient recruitment of activated cells into the already inflamed synovium (Spargo et al., 1996).

Currently, the only method available to assess the arthritogenicity of T cell lines or clones is to transfer the cells to naïve syngeneic rats and assess their ability to induce arthritis. It would be interesting to examine the reactivity of the different $CD4^+$ T cell sub-populations isolated from the TD lymph of rats during the prodrome of AA to mycobacterial antigens and to DCs isolated from either the synovium or the draining lymph nodes of arthritogenic joints. This could be correlated with the arthritogenicity of the cells, both before and after *in vitro* stimulation. Furthermore, it would be interesting to examine the kinetics of the release of mycobacteria- and/or synovial antigen- reactive $CD4^+$ T cells relative to the stage of disease. If examined over an extended period, it might be possible to establish whether there is a constant 'trickle' of these cells into the efferent lymph and that such cells contribute to the relapsing and remitting nature of the disease.

However, studies such as those described above would not provide information on the proportion of the activated cells that are specific for exogenous or endogenous arthritogens. As in RA, identification of the arthritogen is vital. This could be achieved by identifying antigenic fractions in protein extracts from inflamed synovial tissue using gel electrophoresis (Abou-Zeid et al., 1987). These could then be used to stimulate the phenotypically-different subsets of $CD4^+$ T cells isolated from the TD lymph of rats in the prodrome of AA. Once a candidate antigen was identified and epitopes were mapped, MHC and peptide tetramers could be prepared to quantify antigen specific T cells (eg. in the TD lymph of rats during the various stages of AA). It is interesting to note that there is evidence that AA is not mediated by monoclonotypic T cells (van Tienhoven et al., 2000), suggesting that in the hunt for the target antigen of arthritogenic T cells, multiple arthritogens may be identified. The arthritogenic T cell clone A2b reacts strongly to mycobacterial antigens (Holoshitz et al., 1984; van Eden et al., 1988) and cartilage proteoglycans (van Eden et al., 1985) but not to collagen type II (Holoshitz et al., 1984). In AA, cellular reactivity with collagen type II by blood mononuclear cells has been demonstrated by others (Trentham et al., 1980). It has been demonstrated in RA also that synovial T cells react with collagen type II (Londei et al., 1989), self-immunoglobulin complexes (van Schooten et al., 1994) and with the endoplasmic reticulum molecular

chaperone BiP, indicating that there are multiple antigenic targets for self-reactive T cells in this disease.

Once a specific antigen has been identified which induces the proliferation of T cells from arthritic rats, it will be important to examine the function of the reacting cells. The advantage of the experimental model is that adoptive transfer can be used to determine whether the reactive cells are arthritogenic. It will be important to determine the function of these cells, as others have demonstrated that mycobacterial antigens contain different epitopes that can induce the development of either arthritogenic (Holoshitz et al., 1983; van Eden et al., 1985) or protective immunoregulatory cells (Kingston et al., 1996; Tanaka et al., 1999; Paul et al., 2000).

8.3 Expression of surface markers by CD4$^+$ T cells during the prodrome of polyarthritis

The microenvironment in which naïve T lymphocytes first encounter antigen is thought to have a strong influence on the trafficking behaviour and function of the cells (Washington et al., 1994; reviewed in Springer, 1994; Meeusen et al., 1996). Naïve T lymphocytes express low levels of the adhesion molecules P-Selectin ligand, CD44, CD54, LFA-1 and the $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, while expressing relatively high levels of CD62L. After activation, the level of expression of CD62L is decreased, while other adhesion molecules (such as those mentioned above) are expressed at higher levels. The level of expression of individual adhesion molecules and of other activation markers is determined by the stimulus (Sprent, 1997).

The inguinal lymph nodes were chosen for study because they are in the direct drainage of the site of CFA inoculation. In contrast, the popliteal lymph nodes are up stream from this site and collect lymph from the lower hind limbs. It would be predicted that proliferation of cells in the inguinal lymph nodes would have commenced by the end of the third day post-inoculation (Luther et al., 1997) and that this would have been preceded by a period of retention of recirculating cells in all lymph nodes draining the inoculation site (Hall and Morris, 1965).

In both the inguinal and popliteal lymph nodes, activation of CD4$^+$ T cells preceded clinical signs of arthritis by at least 6 days following inoculation of CFA. There were increased proportions of CD4$^+$ T cells that expressed the activation markers MHC class II, CD25, CD71, CD134, UA002 antigen and the adhesion molecule CD54 (see Figures 4.2.3.1-5) within 3 days of inoculation. Activated CD4$^+$ T cells also expressed increased levels of LFA-1, CD45RC and CD44 (see Figures 4.2.4.3, 4.2.3.7 and 4.2.4.4). These changes in phenotype indicate an early response to the inoculation of CFA, with cells entering the cell cycle (as indicated by the expression of CD71 [Salmeron et al., 1995]) and differentiating into effector/memory cells. The proportion of CD4$^+$ T cells that expressed CD71 and CD134 (a marker of early activation) decreased beyond day 6 post-inoculation, suggesting that the stimulatory effect of CFA inoculation begins to weaken as early as 6 days after inoculation. The continued increase in the proportion of cells expressing other markers, exemplified by MHC class II and CD54 (see Figure 4.2.4.2), until at least day 9 post-inoculation may reflect maturation of the cells generated in the lymph node. The decrease in the proportion of activated CD4$^+$ T cells in the lymph nodes beyond day 9 post-inoculation suggests that at this stage, newly-activated cells exit the lymph nodes more rapidly than they are formed.

An increase in the proportion of CD4$^+$ T cells that expressed activation markers MHC class II, CD25, CD71, CD134 and UA002 antigen (see Figures 5.2.3.1-5) was also observed in the TD lymph of rats within 3 days following inoculation of CFA. There were no obvious patterns linking the proportion of CD4$^+$ T cells expressing these activation markers with arthritogenicity. Similar or larger proportions of CD4$^+$ T cells in TD lymph expressed activation markers in donors at day 3 post-inoculation (non-arthritogenic) and at day 9 post-inoculation (arthritogenic) than at the other times studied (see Figures 5.2.3.1-5 and 5.2.1). The proportion of CD4$^+$ T lymphocytes that expressed MHC class II, CD25, CD134, UA002 antigen and CD54 decreased from day 9 to day 12 post-inoculation, in parallel with the decline in arthritogenicity that was observed during this period.

The proportion of CD4$^+$ T cells that expressed CD62L, considered to be a non-exclusive marker of naivete (Jung et al., 1988), also increased by 3 days after inoculation of CFA. However, there were no significant differences between the proportions of CD62L$^+$ CD4$^+$ T cells in TD lymph from normal rats and from rats at days 4, 5 and 9 post-inoculation

(Figure 5.2.4.1). The proportion of CD4$^+$ T cells that expressed CD62L was greater in cells obtained by drainage of the TD, compared to those harvested from the inguinal and popliteal lymph nodes (Figures 5.2.4.1, 4.2.4.1). Thus naive cells represent a greater proportion of CD4$^+$ T cells in the TD lymph compared to the inguinal and popliteal lymph nodes of rats during the prodrome of AA suggesting that there may be selective retention of memory cells in secondary lymphoid tissues during this period. There was little variation in the proportion of TD lymph CD4$^+$ T cells that expressed CD45RC, another marker associated with naivete (Luqman & Bottomly, 1992) throughout the prodrome of AA (Figure 5.2.3.6). However, it was interesting to observe that there was a decrease in the proportion of large CD4$^+$ T cells in TD lymph that expressed CD45RC, commencing on day 3 post-inoculation (Figure 5.2.3.6.1), perhaps indicating a qualitative change in the population of lymphoblasts in the efferent lymph during this period. In the same period, there was an increase in the proportion of lymphoblasts in the efferent lymph, which continued to increase until 5 days after antigenic challenge and decreased thereafter. This lymphoblast response resembles that described in lymph after antigen challenge in other systems (Hall and Morris, 1965; Delorme et al., 1969). Expression of the early activation markers CD71 and CD134 decreased after day 3 post-inoculation, although throughout the prodrome they were expressed by a greater proportion of CD4$^+$ T cells than in control rats.

The expression of functional P-selectin ligand (PSLG-1, CD162) by CD4$^+$ T cells has been associated with CD4$^+$ T cells that produce Th1 type cytokines (Austrup et al., 1997). The expression of this ligand by TD lymph CD4$^+$ T cells was examined and was found to be expressed by a greater proportion of cells during the prodrome of AA compared with cells from normal control rats. This reagent was not available until late in the project and this limited the number of rats that could be examined. It would be interesting to examine the expression of PSLG-1 by TD lymphocytes throughout the prodrome of AA, using adequate group sizes at each time point. P-selectin conjugated magnetic beads could be used to isolate CD4$^+$ T cells that express functional PSLG-1, in order to test their arthritogenicity in adoptive transfer.

These results suggest that CD4$^+$ T cells that are activated in the regional lymph nodes that drain the inoculation site drain into the efferent lymph as early as 3 days following inoculation of CFA. This process continues until at least day 12 post-inoculation, perhaps

supplemented by lymphoblasts arising in lymph nodes draining the affected paws. TD lymphoblasts from arthritic donors can migrate into the paws of both normal and arthritic recipients (Kelly and Harvey, 1978; Spargo et al., 1996), indicating they express surface adhesion molecules that are necessary to facilitate their entry into synovial tissues. The T cells may spend variable times in the synovial tissues, where they could be exposed to synovial antigens presented by synovial APCs, such as synoviocytes or dendritic cells. There is evidence in humans that T cells do pass through the synovial tissue. Paulus and co-workers (1977) found that some radioactively-labelled cells, injected directly into the knee joint of patients with RA, remained detectable in the knee for at least 25 days. Some of the injected cells left the knee. These cells were detectable in the TD lymph before they could be detected in either the liver or the spleen, suggesting that they left the synovium via the lymphatics rather than via the blood. These researchers also found that patients injected with autologous TD lymphocytes experienced flare-ups when injected with live cells (but not dead cells), indicating that they can exert effector activity in the synovial tissue.

The majority of $CD4^+$ T cells isolated from the hind paws after arthritis was established did not express either CD134 or CD71 (see Figures 6.2.3.3-4), suggesting that they were effector cells that were no longer dividing. Most of the $CD4^+$ T cells present in the synovium of rats with established arthritis expressed the phenotype $CD45RC^-$ $CD62L^-$ $CD71^-$ $CD25^+$ $CD134^-$ $CD54^+$ $\alpha_{E2}integrin^-$ (see Figures 6.2.3.6, 6.2.4.1, 6.2.3.3, 6.2.3.2, 6.2.4.4 and 6.2.4.2), suggesting that they were mature effector cells. Many also expressed MHC class II, CD49d and UA002 antigen (see Figures 6.2.3.1, 6.2.4.3 and 6.2.3.5). In the future it will be interesting to examine the functions of these cells, such as their susceptibility to antigen induced cell death, requirement for growth factors for survival, signalling requirements for cytokine production and their proliferative potential. A technical challenge for these studies will be isolating these cells in sufficient numbers, since they represent only a small proportion of the total population (Figure 6.2.2.1 and 6.2.2.2).

Interesting correlations have been observed between the expression of MHC class II molecules and the arthritogenicity of $CD4^+$ T cells. Depletion of MHC class II^+ cells from the $CD4^+$ population in TD lymph removes arthritogenicity (Spargo et al., 2001).

Interestingly, the results presented in Chapter 5 show that arthritogenic cells were present at day 4 post-inoculation of CFA, at which time the proportion of $CD4^+$ T cells that expressed MHC class II was maximal (see Figure 5.2.3.1). Although there is no evidence that the expression of MHC class II molecules by TD lymph $CD4^+$ T cells contributes to the arthritogenic function of the cells, it may be a hallmark that the cells have completed the minimum differentiation necessary to express arthritogenicity. It is unlikely that there is a single "arthritogenic phenotype" and it is more likely that there is a spectrum of phenotypes capable of arthritogenicity. In addition to specificity for synovial antigen, there must be minimum requirements, especially for surface molecules that allow entry of the cells into sites such as synovium and other extra-articular microenvironments. These could include adhesion molecules and chemokine receptors that direct cell migration and communication of the migrated cells with other cell types, such as synoviocytes or dendritic cells. There may be over-lap and redundancy of both membrane bound and secreted molecules that are involved in these functions. Analysis for co-expression of surface molecules, combined with high speed cell sorting and transfer to naive recipients may be used in the future to define more narrowly "arthritogenic phenotypes". Such studies may reveal combinations of molecules that could be targeted therapeutically to control the actions of arthritogenic T cells in RA.

It is probable that effector T cells, in addition to those that are arthritogenic, are generated in response to inoculation with CFA. Within the population of activated $CD4^+$ cells, there may be some that have specificity for irrelevant antigens and some directed against tolerogenic epitopes of mycobacterial and/or self-antigens. It would be interesting to investigate whether immunoregulatory cells with suppressive activity are generated. Although there is evidence that $CD8^+$ T cells do not play an important role either in the induction (Spargo et al., 2001) or regulation of AA (Pelegri et al., 1996b), it was interesting to observe that the proportion of large $CD8^+$ T cells in the TD lymph increased on day 12 post-inoculation (Figure 5.2.2.3 E). This rise in the proportion of large $CD8^+$ T cells coincided with a decrease in the arthritogenicity of the TD lymphocytes (Figure 5.2.1 A and F). While this may be coincidental, it could indicate that there is mobilisation of a population of $CD8^+$ immunoregulatory cells in the later phase of AA. It would be interesting to investigate whether the arthritogenicity of TD lymphocytes obtained from donors inoculated 12 days earlier with CFA would be augmented by depletion of $CD8^+$ T

cells or whether CD8$^+$ T cells purified from such lymph would suppress the arthritogenicity of purified CD4$^+$ T cells.

Another population of cells that are of interest in RA are the γ/δ T lymphocytes (Bodman-Smith et al., 2000). Little or no change was observed in the proportion of γ/δ T lymphocytes present in the TD lymph, or in the inguinal or popliteal lymph nodes, during the prodrome of AA (see Figures 5.2.2.4 and 4.2.2.3). The results presented in Chapter 6 indicate that γ/δ T cells were rare in the soft tissues of the arthritic hind paws (less than 0.01% of the total population examined, see Figure 6.2.2.4) and others have not detected them in the synovium of either healthy or arthritic rats (Carol et al., 2000). These observations are consistent with the observed lack of effect of depletion of γ/δ T cells on the course of AA (Pelegri et al., 1996a).

One of the manifestations of RA is gut inflammation (Porzio et al., 1997). Although the effect of AA on the gut has not been investigated, it was interesting to observe that there was a small increase in the proportion of CD4$^+$ T lymphocytes that expressed α$_{E2}$ integrin in the TD lymph during the prodrome of AA. The proportion rose from approximately 2% in control rats to between 4 and 6% in inoculated rats (Figure 5.2.4.3 F). There was also an increase in the proportion of CD4$^+$ TD lymphocytes that express α$_4$ integrin (CD49d) (Figure 5.2.4.4 F). Both α$_{E2}$ integrin (α$_E$β$_7$) and CD49d are important in promoting traffic of lymphocytes into mucosal sites (Brenan and Rees, 1997; Hamann et al., 1994). It is possible that these small sub-populations of cells may be responsible for the development of gut inflammation. The presence of these cells in the TD lymph and the increase in their frequency during the prodrome of AA, may reflect the activation of memory cells that were primed originally in the gut by homologous bacterial antigens that cross-react with those of *M.tuberculosis*.

8.4 Production of cytokines by CD4$^+$ T cells during the prodrome of polyarthritis

The production of cytokines by CD4$^+$ T cells from rats during the prodrome of AA was examined using flow cytometry, while reverse-transcription polymerase chain reaction (RT-PCR) was used to detect and measure transcripts encoding cytokines. The conditions chosen to stimulate cytokine production by lymphocytes was a 6 hour stimulation period with PMA plus ionomycin, in the presence of Brefeldin A. *In vitro* stimulation under

these conditions allowed the detection of IL-4 by TD lymphocytes from *N.brasiliensis*-infested rats but not by TD lymphocytes from either normal and CFA-inoculated rats. The same conditions induced greater IFN-γ production by TD lymphocytes from *N.brasiliensis*-infested rats and CFA-inoculated rats, compared with normal rats (Figure 3.2.3.1). This protocol for stimulation induces the production of detectable levels of cytokines and this appears to reflect the potential of those cells to produce the cytokines *in vivo*. The lack of detectable intracellular IL-4 after the cells were stimulated in the presence of actinomycin D, which inhibits transcription, indicates that production of this cytokine during the stimulation is dependent on transcription (Figure 3.2.5.2 J and K). In contrast, the proportion of IFN-γ-producing cells present following stimulation *in vitro* was only reduced slightly in the presence of actinomycin D, indicating that mRNA transcripts encoding IFN-γ were present before commencing the stimulation *in vitro* (Figure 3.2.5.2B, C, F and G). Others (Elson et al., 1995; Openshaw et al., 1995; Prussin and Metcalfe, 1995) have used similar protocols to detect the production of cytokines by T lymphocytes.

The technique of detecting intracellular cytokines by flow cytometric analysis allowed assessment of the proportion of α/β TCR$^+$ or CD4$^+$ T-lymphocytes that produced IFN-γ after 6 hours of stimulation *in vitro*. Less than 5% of α/β T cells from the inguinal and popliteal lymph nodes and less than 2% of CD4$^+$ T lymphocytes from the TD lymph of saline-injected control rats produced IFN-γ following 6 hours of stimulation *in vitro* (see Figures 4.2.5.1 and 5.2.5.1). The proportion of α/β T lymphocytes that produced IFN-γ increased during the period following inoculation of CFA. It was maximal in the inguinal lymph nodes between days 6 and 9 post-inoculation and in the popliteal lymph nodes on day 12 post-inoculation (see Figures 4.2.5.1). In the TD lymph, the greatest proportion of CD4$^+$ T cells that produced IFN-γ was detected at 9 day post-inoculation (see Figure 5.2.5.2) and a small proportion of CD8$^+$ (α/β TCR$^+$CD4$^-$) T cells was also found to produce this cytokine. Gett and Hodgkin (1998) have demonstrated that following *in vitro* stimulation of naive T cells, the production of IL-2, -3, -4, -5, -10 and/or IFN-γ (detected as either mRNA transcripts or protein) is not measurable before the fourth cycle of cell division. The observation that TD lymph CD4$^+$ T cells produce IFN-γ within 3 days after inoculation with CFA suggests that these cells may have undergone at least four cycles of division since their initial activation. It was interesting to observe that the greatest

proportion of TD lymph CD4$^+$ T lymphocytes that produced detectable amounts of IFN-γ protein was present at day 9 post-inoculation (Figure 5.2.5.2). This coincided with the time when the greatest arthritogenicity was observed in TD lymphocytes. The production of IFN-γ by α/β T cells harvested from inflamed synovium was striking (see Figure 6.2.5.1). A large proportion of the α/β T cells from the hind paws of rats at day 12 post-inoculation produced IFN-γ and the fluorescence intensity of these cells suggested that the cytokine was relatively abundant (see Figure 6.2.5.1 B). The relatively high production of IFN-γ by T lymphocytes in the hind paws compared to the lymph nodes and TD lymph may reflect the fact that the synovium is the site of effector activity, while the lymph nodes are the sites of induction and the cells in TD lymph are in transit to the target tissue. It also suggests that the cells are stimulated after arrival in the synovium, although it cannot be excluded that there is selection during recruitment for cells with the greatest potential to secrete IFN-γ. Evidence from *in vivo* studies has indicated that IFNγ leads to retention of intravenously injected thoracic duct lymphocytes in tissues (Westermann et al., 1993). The production of IFN-γ by TD lymphocytes may, therefore, effect their migratory capacity by promoting the expression of molecules such as CD54 by connective tissue cells. This led to the hypothesis that production of IFN-γ by TD lymphocytes was important in the initiation of joint inflammation. This is discussed in the following section.

In contrast to the production of IFN-γ, very few cells with the potential to produce IL-4 were detected in either the lymph nodes (Figure 4.2.5.2), TD lymph (Figure 5.2.5.2) or the hind paws (Figure 6.2.5.2) of rats during the prodrome or early clinical phase of AA. This does not appear to be due to the insensitivity of the flow cytometric assay, because the technique was sufficiently sensitive to detect IL-4 produced by TD lymphocytes from rats infested with *N.brasiliensis* (see Figures 3.2.4.1 and 5.2.5.4 C). Although mRNA transcripts of the gene encoding IL-4 were also detected in the cells from *N.brasiliensis*-infested rats, they were not detected in most samples of RNA isolated from TD lymphocytes of rats inoculated with CFA (see Figure 5.2.5.3 C). These findings support the flow cytometric observations (see Figure 5.2.5.2). Others have also failed to detect mRNA transcripts of the gene encoding IL-4 in the popliteal lymph nodes of rats with AA (Schmidt-Weber et al., 1999). As in RA (Simon et al., 1994; Bucht et al., 1996), the relative abundance of IFN-γ and scarcity of IL-4 in AA indicates a bias towards the

production of Th1 type cytokines. The bias towards IFN-γ production is likely to have been promoted by IL-12 produced by DCs at the site of inoculation with CFA. DCs are specialised for the activation of naïve T cells and they produce IL-12 in response to challenge with mycobacteria (either live or heat-killed) (reviewed in Demangel and Britton, 2000).

T cells isolated from the hind paws of arthritic rats revealed a CD4$^+$ population characterised by an activated phenotype and production of IFN-γ, consistent with effector function in the inflammatory process. Others have shown that synovial macrophages (including synoviocytes) are activated before and during the onset of clinical arthritis (Johnson et al., 1986; Lopez-Bote et al., 1988). IFN-γ is a potent stimulator of macrophages (Pace et al., 1983). This activation could, therefore, be explained, at least in part, by IFN-γ produced by recently-arrived, activated CD4$^+$ T lymphocytes. Similar populations of activated macrophage-like cells have been observed in rheumatoid synovial samples, where they express high levels of MHC class II molecules, possibly in response to IFN-γ. Essentially none of the T cells isolated from the synovium of arthritic rats produced IL-4 (Figure 6.2.5.2 C).

The cytokines IFN-γ and IL-4 were chosen for particular attention because they are typical products of Th1 and Th2 cells respectively (reviewed in Mossman and Coffman, 1989). The lack of availability of antibodies to study other cytokines of interest by flow cytometry mandated the use of another technique. RT-PCR was used to examine the production of mRNA transcripts from genes that encode other cytokines. This technique was made semi-quantitative by performing serial 10-fold dilutions of a standard quantity of cDNA template and comparing the relative quantities of amplified material produced from each sample by the PCR. This technique did not reveal any significant differences in the levels of mRNA transcripts encoding either IL-2, IL-4, IL-10, TGF-β, TNF-α or IFN-γ in TD lymphocytes throughout the prodrome period (Figure 5.2.5.4). This observation does not exclude changes in the expression of these cytokines during the prodrome of AA, but these are likely to be less than 10-fold. Although there have been no published reports that describe mRNA transcripts encoding cytokines in TD lymphocytes from rats during the prodrome of AA, it has been shown in this laboratory that activated CD4$^+$ T cells in

the TD lymph during the late prodromal period (day 9 post-inoculation) produce more TGF-β than activated CD4$^+$ T cells from normal rats (Connolly, 1998).

It would be of interest to examine the expression of cytokines by TD lymphocytes from rats during the prodrome of AA by real time PCR, using a light-cycler amplification machine. Of particular interest are cytokines that may have an immunoregulatory role, such as TGF-β and IL-10. These cytokines could have a function in tissue remodelling and disease remission. Although there is substantial evidence that pro-inflammatory cytokines such as TNF-α are involved in promoting arthritis, less is known about those that are involved in disease remission. It would, therefore, be of interest to examine the kinetics of the production of these cytokines and to correlate them with the development of clinical signs of the disease, the time at which they are produced and whether immunoregulatory cells are generated in parallel with arthritogenic cells. Such information would be useful in designing therapies for the treatment of RA. It may be possible in the future to manipulate the immune system to promote the ongoing development of immunoregulatory cells and to discourage the development of pro-inflammatory arthritogenic cells.

8.5 Effects of IFN-γ blockade on the effector phase of polyarthritis

The observations of Austrup et al (1997) suggest that CD4$^+$ T cells that produce IFN-γ may be able to enter inflamed joints more readily than those that produce IL-4. There is evidence from other researchers that the administration of antibodies to neutralise IFN-γ can prevent the induction of AA (Jacob et al., 1989). These observations suggest that IFN-γ is important in the pathogenesis of AA. IFN-γ is reported to be a major mediator of T cell recruitment into sites of DTH (Issekutz et al., 1988) and it is thought to promote movement of lymphocytes out of the blood at sites of inflammation. IFN-γ can also promote tissue destruction. For example, tissue-specific expression of a transgene encoding IFN-γ, under the control an insulin promoter, led to the destruction of pancreatic islet cells in mice and the development of insulin-dependent diabetes mellitus (Sarvetnick et al., 1988).

The production of IFN-γ by CD4$^+$ T cells present in the TD lymph and the inflamed hind paws of rats with AA led to the hypothesis that IFN-γ was an important effector molecule

in the pathogenesis of polyarthritis. This hypothesis was investigated using the adoptive-transfer of arthritogenic TD cells, thus allowing separation of the efferent effector phase of AA from the afferent induction phase. The action of IFN-γ was blocked by the administration of DB-1, a mouse mAb against rat IFN-γ. Monoclonal antibody DB-1 was administered either before or after the transfer of arthritogenic doses of TD lymphocytes harvested from donors at day 9 post-inoculation.

Contrary to the hypothesis, blockade of IFN-γ from the time of lymphocyte transfer exacerbated adoptively-transferred arthritis. Recipients of arthritogenic TD lymphocytes that were treated with mAb DB-1 developed a more severe arthritis than those treated with control polyclonal IgG (Figure 6.2.2.1-2). Interestingly, this effect was dependent on the time of administration of the antibody. Delaying the administration of anti-IFN-γ until 4 days after the transfer of arthritogenic lymphocytes did not exacerbate the arthritis in the recipient rats, although there was some delay in the resolution of the disease (Figure 6.2.3.2). These results indicate that IFN-γ is not mandatory for the joint inflammation that follows adoptive transfer of arthritogenic T cells and that IFN-γ may play a role in down-regulating inflammation. This effect may be more pronounced in the early stages of the inflammatory response.

The pro-inflammatory effects of IFN-γ, such as inducing the expression of MHC molecules by macrophages and endothelial cells (King and Jones, 1983; reviewed in Boehm et al., 1997) and enhancing the phagocytic and cytotoxic action of neutrophils (reviewed in Young and Hardy, 1995), are well characterised. On the other hand, the anti-inflammatory effects of IFN-γ are poorly understood (reviewed in Kolb and Kolb-Bachofen, 1998). Recently it has become evident that nitric oxide (NO), which is produced by macrophages in response to IFN-γ (reviewed in MacMicking et al., 1997), is crucial for remission from EAE (O'Brien et al., 1999) and that IFN-γ is necessary for this pathway of down-regulation (Willenborg et al., 1999). The mechanisms by which NO down-regulates inflammatory reactions are not well understood but NO is thought to inhibit the production of IFN-γ by T cells and to promote the bias of cytokine production towards that typical of Th2 cells. This may involve indirect mechanisms, such as induction of macrophages to produce the IL-12 $(p40)_2$ homodimer, which antagonises the actions of IL-12 (reviewed in Kolb and Kolb-Bachofen, 1998; Roozendaal et al., 1999).

NO is thought also to regulate lymphocyte migration, as it induces the down-regulation of selectins, VCAM and ICAM-1 by endothelium. This action could reduce the extravasation of leukocytes into the inflamed tissue (reviewed in Kolb and Kolb-Bachofen, 1998). Blocking IFN-γ by the use of mAb DB-1 may have reduced NO production by macrophages, such that the inflammatory effects of NO outweighed its potential as an anti-inflammatory agent.

In addition to promoting the production of NO, IFN-γ can also act on myelopoietic cells to restrain their expansion (Matthys et al., 1999). Removing this restriction by blocking IFN-γ would allow the expansion of mononuclear cells, which are likely to dominate in the later phase of joint inflammation. Thus IFN-γ that is released systemically could have an over-riding effect that is protective. These and other possibilities could be explored by examining the production of IFN-γ by CD4$^+$ T cells in the hind paws during the course of adoptively-transferred arthritis.

Monoclonal antibody DB-1, administered before the inoculation of CFA, did not inhibit the development of arthritogenic TD lymphocytes (Figure 6.2.1). This result was unexpected, because in the studies of Jacob et al (1989), administration of mAb DB-1 prevented the development of actively-induced AA. However, Jacob et al (1989) had considerable variation in the severity of disease between cohorts of control rats, with maximum severity ranging from mean arthritic scores of approximately 3 to approximately 7. In addition, the data were not presented with error bars making its interpretation difficult. Although the dose of mAb DB-1 was based on that used by Jacob et al (1989), the limited availability of the antibody prevented supplementary studies to examine whether the dose used was sufficient to inhibit the development of actively-induced AA in DA rats or whether an increased dose would inhibit the generation of arthritogenic T cells. Therefore, further studies are needed before it can be concluded that IFN-γ is not required for the generation of arthritogenic T cells.

8.6 Conclusion and general comments

The results presented in this thesis indicate that in response to inoculation of CFA, female DA rats generate effector CD4$^+$ T lymphocytes in the lymph nodes draining the site of inoculation as well as in distal nodes that drain the articular tissues. Activated

lymphocytes are present in the TD lymph within 3 days and arthritogenic cells are present as early as 4 days post-inoculation. A protocol of flow cytometric analysis of intracellular cytokines was established so that the production of IFN-γ and IL-4 could be examined. It was found that CD4⁺ T cells obtained from the TD lymph and α/β T cells from the lymph nodes and hind paws of rats during the prodrome of AA were biased strongly towards the production of IFN-γ, whereas few produced IL-4. This indicates that these CD4⁺ T cells were biased towards a Th1 phenotype. Interestingly, the blockade of IFN-γ by a neutralising mAb exacerbated adoptively-transferred arthritis, suggesting that the production of IFN-γ by TD lymph CD4⁺ T cells may have an unexpected anti-inflammatory role in the early stages of the pathogenesis of synovial inflammation associated with this disease. Enzymatic digestion of the soft tissues of the hind paws from rats revealed that during the clinical stage of AA, CD4⁺ T cells are present that have an effector phenotype and produce an abundance of IFN-γ following stimulation *in vitro*.

The kinetics of production of arthritogenic cells after inoculation of CFA resembles those that have been described for the production of effector cells during the primary response to a range of injected non-replicating antigens. It is tempting to speculate that the presentation of mycobacterial antigens leads to the activation and expansion of latent auto-reactive cells that recognise both mycobacterial and synovial antigens. However, the kinetics of the disease and the appearance in lymph, over a period of 9 days, of cells with the greatest arthritogenic capacity suggests that this is an over-simplification. The depot of antigen that is generated by administering the mycobacteria in oil complicates this issue. The greater arthritogenic capacity of TD lymphocytes from donors at 9 days after inoculation of CFA could be a consequence of local expansion of reactive cells in response to prolonged exposure to mycobacterial antigens presented by APCs in the lymph nodes draining the site of inoculation, a shift in the immune response from mycobacterial to cross-reactive self-antigen or to a period of maturation of cells generated against either into more efficient effector cells. It is interesting to note that AA is remitting, with episodes of joint inflammation occurring for at least one year after the induction of the disease (Pearson, 1956). It is tempting to interpret this as indicating that there is re-activation of memory T cells via the presentation of synovial autoantigens, although it is conceivable that mycobacterial antigens could persist for long periods of time in the draining lymph nodes.

The adoptive transfer of arthritis provided an opportunity to examine polyarthritis in the absence of either mycobacterial antigens or the systemic effects of inoculation of CFA. Initiation of cytokine production by $CD4^+$ T cells *in vitro* requires a period of activation. It seems reasonable to assume that effector functions of arthritogenic cells *in vivo* will also require re-activation within the synovium. Whether this occurs via interaction with cognate antigen presented by local APCs or whether it occurs via a TCR/CD3 independent pathway is an important question. If activation is antigen-dependent, it implies that arthritogenic cells recognise auto-antigen presented locally by APCs. Efforts to identify a foreign antigen, such as an infectious agent, in the pathogenesis of RA have produced equivocal results. In SPF rats, an endogenous arthritogen is more likely to be a self-protein (ie. a true auto-antigen), although it could be a component of the normal gut flora.

It would be interesting to study adoptively-transferred arthritis in terms of the host and donor components and to examine the extent to which naïve cells of host origin get drawn into the later stages of the disease process. A genetic marker such as the CD45 allele $RT7^b$ or a vital dye such as Carboxyfluorescein diacetate succinimidyl ester (CFSE) could be used to track donor cells in naïve recipients. CFSE has the added benefit of allowing assessment of cell division in the donor cells. Preliminary studies using the $RT7^b$ marker in our laboratory (Spargo et al., unpublished data) have indicated that donor cells constitute approximately half of all T cells in the synovial tissue of arthritic rats 10 days after cell transfer. It is not known whether the host-derived cells are activated locally or whether they are recruited non-specifically as bystanders. The phenotype of donor and host cells requires examination by multi-fluorochrome flow cytometric analysis. High speed cell sorting and comparison of the reactivity of the activated T cells of donor and host origin with synovial APCs in autologous mixed lymphocyte reactions (MLR) would provide useful information about the potential autoreactivity of the two populations. Cells derived from such cultures, after further expansion by cycles of growth in IL-2 and re-stimulation, could be tested for arthritogenicity by adoptive transfer. Clones of arthritogenic T cells, derived from these cultures, would be an invaluable resource with which to study the pathogenesis of synovial inflammation and to identify synovial arthritogens. A major strength of an animal model of RA, such as that described in this

study, is that arthritogenicity can be tested by adoptive transfer and that the effector function of arthritogenic T cells can be studied *in vivo*.

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Amendments to 'Effector CD4$^+$ T lymphocytes in the prodrome of polyarthritis'.
Melissa Brasted, July 2002

The work presented in Chapter 5, Section 5.2.1 was done in collaboration with Mr. L.D.J. Spargo, of the Arthritis Research Laboratory. He was responsible for the adoptive transfers and assessment of arthritogenicity, while I was responsible for all studies on the surface antigen and cytokine phenotypes of cells in this chapter. Llew Spargo also contributed valuable advice and technical help throughout the project.

Response to the report by Examiner 1.
My contribution to each results chapter:

Chapter 3:
Culture, harvest, staining of CHO cells, flow cytometry, confocal microscopy and analysis were done by me. CHO cells were a kind gift from Dr. A. N. Barclay, MRC Cellular Immunology Unit, University of Oxford, UK. Paul Sincock assisted in showing me how to set up the confocal microscopy and analysis of these data. Thoracic duct cannulations were performed by Sarah Wing and Graham Mayrhofer. Stimulation, harvest, staining and analysis of thoracic duct lymphocytes were carried out by me. Intraperitoneal and subcutaneous injections, preparation of parasites and harvest of tissue from rats was performed by me, as was the analysis of the cells from these animals. Christine Daly and John Mackrill assisted in demonstrating how to isolate *N.Brasiliensis* from faecal cultures. Craig Murphy taught me how to set the flow cytometer to analyse multiple fluorochromes simultaneously.

Chapter 4:
Sarah Wing performed the thoracic duct cannulations and intravenous injections. Subcutaneous injections, harvest of tissues, preparation of single cell suspensions, stimulation and staining of cells and flow cytometric analysis were done by me. I assessed the arthritic scores but assistance was provided on occasion by Sarah Wing, Judy Bulau, Llew Spargo, Michael Fusco and Eleanor Brasted. Sarah Wing and Judy Bulau provided assistance with weighing tissues and cell counts.

Chapter 5:
Sarah Wing performed the thoracic duct cannulations and intravenous injections. Llew Spargo contributed equally to the assessment of arthritic scores and analysis of these data. Subcutaneous injections, stimulation and staining of cells, flow cytometric analysis, RNA extractions, reverse transcriptions, polymerase chain reactions and analysis were done by me. Ashley Connolly and Kerrilyn Diener demonstrated how to perform RNA extractions, RNA analysis (gels to check integrity and absorbance readings to determine relative quantities), reverse transcriptions and polymerase chain reactions. Assistance with assessment of arthritic scores was provided on occasion by Sarah Wing, Judy Bulau, Llew Spargo, Michael Fusco and Eleanor Brasted. On days when more than 100 samples were double-labelled for flow cytometric analysis, Sarah Wing and Judy Bulau provided assistance with filtering and washing thoracic duct lymphocytes and cell counts.

Chapter 6:

Subcutaneous injections, harvest of tissues, preparation of cells, stimulation and staining of cells, flow cytometric analysis were performed by me. Sarah Wing and Judy Bulau provided assistance with weighing tissues and cell counts. The technique of digesting synovial tissue to isolate single cells was established in the Arthritis Research Laboratory by Elizabeth Farmer. This work by Mrs. Farmer was a significant contribution to the work presented in this chapter because it allowed me to isolate cells from the hind paws of rats with only minor amendments to her protocol.

Chapter 7:
Sarah Wing performed the thoracic duct cannulations and intravenous injections. The work presented in this chapter would not have been possible without the expert surgical skills of Ms. Wing in being able to cannulate 14 rats on a single day. DB-1 was a kind gift from Dr. P. Van der Meide, Primate Centre TNO, Rijswijk, The Netherlands. Immunoglobulins for *in vivo* use and intraperitoneal injections were prepared by me. Preparation of tissues and histological analyses were done by me. Robyn Dollman X-rayed the rats and paws (data not shown). Sectioning of frozen tissue was done by Sarah Wing (data not shown). Prof. Les Cleland assessed the erosion of joints of rats (data not shown). I assessed the arthritic scores but assistance was provided on occasion by Sarah Wing, Judy Bulau, Llew Spargo, Michael Fusco and Eleanor Brasted.

p39, 3rd paragraph: 'The presence of autoantibodies with specificity for IgG is a diagnostic feature of RA' should read 'The presence of rheumatoid factor is a diagnostic feature of RA'.

Figure 5.2.2.1. The label on the gate for 'small' cells has been deliberately printed in white against the cells in black in an attempt to make this label evident. Including the 3 gates used on a single scatter plot demonstrates how the population was sorted for analysis on the basis of the light scattering properties of individual cells.

p152 Kelso 1993 is quoted as providing 'some' evidence for post-translational regulation of TNF. There is substantial literature indicating that the production of TNF-α is controlled at both the transcriptional and post-transcriptional level (reviewed in Anderson, P. 'Post-transcriptional regulation of tumour necrosis factor α production' *Ann. Rheum. Dis.* 2000; **59 (suppl. I)**:i3-i5).

p5, paragraph 1: '..recruit the receptor tyrosine kinase Ick should read '..recruit the receptor tyrosine kinase lck'.

p97, paragraph 2: 'The hooded Wistar strain was used because ..... it appears to produce a more prevalence of IL-4 producing' should read 'The hooded Wistar strain was used because ..... it appears to produce a greater prevalence of IL-4 producing'.

p97, paragraph 2: 'However, it notable that DA rats' should read 'However, it is notable that DA rats'.

p99, paragraph 2: '... necessary to detect intracellular cytokines produced CD4^{+} T cells' should read '... necessary to detect intracellular cytokines produced by CD4^{+} T cells'.

p114, paragraph 4: 'continued until days 6-9 post inoculation by then declined' should read 'continued until days 6-9 post inoculation and then declined'.

p116, paragraph 2: 'The proportion of α/β T cells from inguinal and popliteal lymph nodes saline inoculated rats' should read 'The proportion of α/β T cells from inguinal and popliteal lymph nodes from saline inoculated rats'.

p119, paragraph 3: 'it is possible that the emergence activated CD4⁺ T cells' should read 'it is possible that the emergence of activated CD4⁺ T cells'.

p167, paragraph 1: 'The using a mAb...' should read 'The use of a mAb...'.

p169, paragraph 4: 'has a more significant role that...' should read 'has a more significant role than..'.

Response to the report by Examiner 2.
Figure 3.2.4.1 and 3.2.4.2 "CD45RB" should read "CD45RA".

Figure 6.2.2.3 legend 'Representative scatter plots are shown of gated events in preparations' should read 'Representative scatter plots are shown of events from the lymphocyte gate in preparations'.

Figure 7.2.2.1 legend reads 'Rats were injected intraperitoneally with either 0.8mg of control....' should read 'Recipient rats were injected intraperitoneally with either 0.8mg of control....'.

Figure 4.2.6.1 should include a footnote:
The clinical scores represented in Figure 4.2.6.1 A for adoptively-transferred arthritis are typical for this model. Adoptively-transferred arthritis is less severe and has an accelerated onset compared with actively-induced disease. Both types of arthritis have close to 100% incidence, but the severity of adoptively-transferred arthritis is more variable than actively-induced disease.

Figure 7.2.3.2 reads 'The control groups are shown in figures 7.2.3.2 A and B' should read 'The control groups are shown in figures 7.2.3.1 A and B'.

p123-4 Section 5.2.1 It is stated that the target antigen of arthritogenic T cells in AA is unknown. I acknowledge that it is possible that the antigens required for actively-induced arthritis and the development of arthritogenic T cells might be different from those required for the development of joint destruction. The latter could involve immunoglobulin, a product of B lymphocytes, as occurs in the model described in Korganow, A.-S., et al., 'From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins' *Immunity* 1999, **10**:451-461. However, unlike the model described by Korganow, et al., in our model, arthritis can be transferred by T cells only (ie. excluding B cells) and joint swelling is apparent within 4-5 days after transfer. The production of autoantibodies by host B cells would require at least this long to develop and if they were causal, the production of autoantibodies against joint antigens would be expected to precede visible joint swelling by at least a day. Thus B cells do not appear to be involved in initiating joint inflammation. It is conceivable that they may be involved in later destructive changes seen in affected

joints. Autoantibodies could thus play a role in the perpetuation and/or relapses of arthritis but appear to have little if any part in the prodrome of disease. The prodrome has been the focus of this thesis.

p140-141, Section 5.3.3.1

In the past 10 to 15 years, there has been research suggesting that down-regulation of autoimmune responses can be mediated by T cells which are contained within a population of $CD4^+ CD25^+$ cells that are termed 'regulatory T cells' (reviewed in Mason, D. 'T-cell-mediated control of autoimmunity' *Arthritis. Res.* 2001 **3**:133-135). Approximately 75% of the $CD4^+$ T cells in the synovial tissues of rats at day 12 post-inoculation express CD25 and it is possible that at least some of these cells are regulatory T cells. Since the severity of joint inflammation continues to increase for at least another 4 days after this time point, it suggests that if some or all of these cells have regulatory functions, that they do not have sufficient potency at this time to regulate the effects of the arthritogenic cells. More importantly, it has been shown in this laboratory (Spargo et al, 2001) that depletion of $CD25^+$ cells from the $CD4^+$ T cells in the thoracic duct lymph from arthritic donors depletes most of the arthritogenic activity. It appears, therefore, that CD25 is one of several molecules expressed by arthritogenic $CD4^+$ T cells activated by CFA and that the presence of CD25 is, as might be expected, a marker of activation as well as a marker expressed by some regulatory T cells.