The regulation of chemokine receptor expression upon T lymphocyte activation

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Amendments

1. In Figure 4.20, the X-axis of each graph should read 'CD4'. The Y-axes should be labelled as follows: [A] and [D], CCR5; [B] and [E], CXCR3; [C] and [F], CXCR4.
2. In the following figure and table legends, 'PMBC' should read 'PBMC': Table 4.1 (p143), Table 4.2 (p144), Figures 3.1 – 3.34 and Figures 4.5 – 4.18.
3. On p122, 'principle' should read 'principal'.
4. Insert the following sentence into Section 2.2.1 (p52): For each experiment, blood donors were selected randomly from a pool of approximately 15 volunteers. Each experiment was conducted using PBMCs from a range of volunteers, such that significant observations could be differentiated from natural variation within the population.

Abstract

Following immune challenge, naïve T cells are activated in the secondary lymphoid tissue but then need to move to the site of antigen deposition in the periphery in order to mediate their effector functions. A comparison of chemokine receptor expression patterns on naïve versus effector/memory T cells in peripheral blood suggests that, during activation, T cells reduce their expression of receptors for homeostatic chemokines (those that are produced constitutively within lymphoid tissues) and increase their expression of receptors for inflammatory chemokines (those that are produced in peripheral tissues under conditions of inflammation). This putative switch in chemokine receptor expression would likely play a critical role in allowing effector/memory T cells access to peripheral tissues. In order to test whether such a switch in chemokine receptor expression occurs during T cell activation, human peripheral blood lymphocytes were activated in a mixed leukocyte reaction (MLR) and the expression patterns of eight different chemokine receptors assessed on $CD4^+$ and $CD8^+$ T cells over a 12 day time-course. Within the $CD4^+$ T cell subset, a significantly greater proportion were shown to express CCR5, CCR6 and CXCR3 in allogeneic cultures compared to syngeneic controls, while conversely, a lesser proportion expressed CXCR4. With regard to $CD8^+$ T cells, CXCR4 was lost on a significant fraction of cells in allogeneic but not syngeneic cultures. However, there was no alteration in the percentage of $CD8^+$ T cells expressing of any of the other receptors examined.

On the basis of the results from this screening approach, subsequent experiments focussed on characterising the expression and function of CCR5, CCR6, CXCR3 and CXCR4 on $CD4^+$ T cells during allogeneic activation. It was shown that the

modulation of these receptors was closely correlated with the acquisition of an activated/memory phenotype. The changes in expression of CXCR3 and CXCR4 occurred in close parallel with the cell division process, while in contrast, the up-regulation of CCR5 and CCR6 expression was more dependent on time in culture. In MLR cultures extended for up to 24 days, it was shown that the pattern of chemokine receptor expression acquired within the first 9 days was retained, suggesting that these changes were not limited to a state of acute activation. RT-PCR studies revealed that the increased expression of CCR5, CCR6 and CXCR3 on the cell surface was associated with an increase in mRNA levels, while in contrast, the down-regulation of CXCR4 protein was not mirrored by a corresponding decrease in CXCR4 transcripts. The possibility that CXCR4 down-regulation was effected via internalisation of surface protein was accordingly explored, and while intracellular stores of CXCR4 were not found, both CCR6 and CXCR3 could be detected intracellularly. However, similar to CXCR4, CCR5 was detected only on the cell surface. The increased expression of CCR5 and CXCR3 in allogeneic cultures was associated with an enhanced chemotactic response to the cognate ligands, and the down-regulation of CXCR4 expression accordingly reduced chemotactic responsiveness toward SDF-1/CXCL12. However, the up-regulation of CCR6 did not result in enhanced migration toward MIP-3α/CCL20, and studies using a CCR6 antagonist failed to reveal a role for this receptor in the modulation of activation-induced apoptosis. Analysis of two models of the murine MLR revealed that the up-regulation of CCR5 and CXCR3 was conserved across the human-murine species barrier, while in contrast, the down-regulation of CXCR4 expression was not detected in the murine MLR. It was difficult to analyse the expression of CCR6 on murine T cells, due to the lack of a suitable antibody; however, RT-PCR analysis did not detect any up-regulation of this receptor on allo-activated murine T cells.

In order to extend these findings to an *in vivo* setting, a murine model of allogeneic activation in response to foreign DCs was developed, characterised, and used to study the regulation of chemokine receptor expression on $CD4^+$ T cells *in vivo*. Initial analyses revealed a dramatic increase in LN cellularity following immunisation, due in part to local cellular proliferation and in part to an influx of cells from the circulation. This influx resulted in a large increase in the absolute numbers of $CCR5^+$, $CXCR3^+$ and $CXCR4^+$ $CD4^+$ T cells. However, analysis of the relative proportions of these cell populations suggested that the entry of $CD4^+$ T cells expressing CCR5 or CXCR3 was more efficient than the entry of CXCR4-expressing cells. Within the population of locally-dividing $CD4^+$ T cells, approximately half began expressing CCR5 and CXCR3, which represented a dramatic up-regulation of these receptors in comparison to the non-dividing population. There was also a less striking increase in the percentage of $CD4^+$ T cells expressing CXCR4 in the divided compared to the non-divided population. Analysis of the site of immunisation revealed that allogeneic DCs were retained within the tissue for at least 8 days, and that this was associated with a modest accumulation of $CD4^+$ T cells. In preliminary studies, a number of these infiltrating $CD4^+$ T cells were shown to express CCR5 and CXCR3, supporting the notion that the acquisition of these receptors upon activation may contribute to the ability of T cells to migrate to peripheral sites of immunisation.

Statement of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution, and to the best of my knowledge, contains no material previously published or written by another person, except where due reference has been made.

I consent to this copy of my thesis, when deposited in the University Library, being made available for photocopying and loan.

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Publications arising from this study

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Ebert, L.M., and S.R. McColl. 2001. Up-regulation of CCR5 and CCR6 on distinct sub-populations of antigen-activated $CD4^+$ T lymphocytes. *J Immunol 168:65-72.*

Ebert, L.M., and S. R. McColl. Inflammatory chemokine receptor up-regulation on murine $CD4^+$ T cells activated by allogeneic dendritic cells in vivo. *Manuscript in preparation.*

List of abbreviations

APC	antigen presenting cell
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
CFSE	carboxyfluoroscein succinimidyl ester
CTL	cytotoxic T lymphocyte
DARC	Duffy Antigen for Chemokine Receptors
DC	dendritic cell
DEPC	diethylpyrocarbonate
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DTH	delayed-type hypersensitivity
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminotetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EMA	ethidium monoazide bromide
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter

HBSS	Hank's balanced salt solution
HEV	high endothelial venule
HIV	human immunodeficiency virus
IFN	interferon
Ig	immunoglobulin
IL-	interleukin-
i.p.	intraperitoneal
LN	lymph node
LPS	lipopolysaccharide
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MI	migration index
MLR	mixed leukocyte reaction
NK	natural killer
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	paraformaldehyde
PHA	phytohemagglutinin
PPD	p-phenylenediamine

RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
s.c.	sub-cutaneous
SSC	side scatter
TAE	Tris/acetic acid/EDTA
TCR	T cell receptor
Th1	T helper cell type 1
Th2	T helper cell type 2
TNF-	tumour necrosis factor-

CHAPTER 1

INTRODUCTION

1.1 The recirculation and directed migration of T cells

1.1.1 The role of T cells in immunity

The immune system is a complex collection of tissues, cells and molecules that work together to protect the host from pathogens and tumours (1, 2). Cells of the innate immune system, such as neutrophils and macrophages, control the vast majority of challenges. When this first line of defence is penetrated, an adaptive immune response, mediated by T lymphocytes (T cells) and B lymphocytes (B cells) is initiated. T cells play a central role in both the cell-mediated and humoral (antibody-mediated) components of the adaptive immune response, as well as the perpetuation of the pro-inflammatory innate immune response.

T cells can mediate a direct protective effect by killing virus-infected or cancerous host cells (1, 2). Additionally, cytokines secreted by activated T cells, and molecules expressed on the T cell surface, provide signals required for the full activation of B cells, and the subsequent humoral immune response. Furthermore, T cell-derived cytokines and chemokines can enhance the activity, and promote the accumulation, of non-specific inflammatory cells, thereby enhancing and prolonging the inflammatory response. Thus, the efficient and appropriate activation of T cells is at the centre of a multi-faceted immune response capable of effectively eliminating invaders.

1.1.2 The presentation of antigen to naïve T cells

Most antigens enter the body at epithelial sites such as the gut and skin; however, these areas are so large that they cannot possibly be effectively patrolled directly by lymphocytes. Rather, antigen is captured at peripheral sites by resident dendritic cells

(DCs), which then transport the antigen to regional secondary lymphoid tissue following the receipt of inflammatory signals, such as interleukin (IL-) 1 and tumour necrosis factor (TNF)- α (3, 4). Upon reaching the secondary lymphoid tissue, DCs localise in T cell areas and present processed antigen, bound to major histocompatibility complex (MHC) molecules, to T cells. Following an encounter with antigen-loaded DCs, T cells proliferate and differentiate into effector populations with appropriately polarised helper or cytotoxic functions. Subsequently, the activated T cells relocate to the site of antigen deposition in the peripheral tissues and initiate a local immune response. Finally, following clearance of the infection, a population of memory cells remains to provide a faster and more effective response in the event of secondary challenge (1, 2, 5).

1.1.3 The importance of controlled T cell trafficking in adaptive immunity

In order to undertake this complex series of events, and thereby initiate an effective immune response, T cells must be flexible in their migratory patterns (5, 6). Initially, continuous recirculation through the secondary lymphoid organs maximises the chances of finding cognate antigen. However, following encounter with specific antigen presented by DCs, T cells must immediately halt recirculation in order to begin a program of proliferation and differentiation in the nurturing environment of the secondary lymphoid tissue. Moreover, during this time, subtle microenvironmental re-positioning is likely to occur, enabling activated T cells to interact with nearby B cells and thereby provide help to the humoral arm of the immune response. Finally, activated T cells must leave the lymphoid circulation and relocate to the appropriate site in peripheral tissue where the initial antigen challenge occurred. Similarly, the population of memory cells that remains following clearance of the antigen must retain

the ability to circulate through peripheral tissues, in order to provide effective surveillance in the event of re-infection.

1.1.4 Differential trafficking of naïve and effector/memory T cells

Central to these complex patterns of migration is the differential ability of naïve and effector/memory T cells to gain access to peripheral versus lymphoid tissues (6-8) (Fig 1.1). Naïve T cells do not readily enter peripheral tissues, and instead traffic continuously between the blood and lymphatic circulation systems. Thus, from the bloodstream, naïve T cells enter secondary lymphoid tissues such as the lymph nodes (LNs) and Peyer's patches by passing through high endothelial venules (HEVs), which are regions of endothelium specialised for the passage of naïve lymphocytes. If a DC presenting cognate antigen is not found, the naïve T cells then leave the lymphoid tissue via the efferent lymphatics and thereby enter the general lymphoid circulation, eventually returning to the bloodstream via the thoracic duct.

Effector and memory T cells, on the other hand, have a reduced ability to pass through HEVs. Rather, they preferentially leave the bloodstream by passing through endothelium within peripheral tissues, thus enabling them to survey these sites for antigen. If antigen is not present, they exit the peripheral tissues by draining into the afferent lymphatics and subsequently enter secondary lymphoid tissues via this route. They then continue the recirculation pathway by entering the efferent lymphatic vessels and returning to the bloodstream via the thoracic duct, as for naïve T cells.

1.1.5 Differential trafficking properties of effector T cell sub-populations

The entry of effector/memory T cells into peripheral tissues is non-random. First, there is considerable evidence that memory T cells show a preference for returning to the region in which they were first activated. For example, T cells activated in the mucosal lymphoid tissue appear to demonstrate preferential homing to the intestines, while T cells activated in skin-draining LNs show a propensity to home to the skin (6, 9). Second, T cells of the T-helper 1 (Th1) and T-helper 2 (Th2) subsets also display differential migratory properties (10). Th1 cells promote immune responses directed toward intracellular pathogens, through the secretion of cytokines such as interferon-(IFN-) γ, IL-2 and TNF-β; these cytokines collectively promote cytotoxic T lymphocyte (CTL) activity, macrophage activation and the production of immunoglobulins that bind Fc receptors and fix complement. Conversely, Th2 cells promote immune responses toward extracellular pathogens via the secretion of cytokines such as IL-4, IL-5 and IL-13, which together promote strong humoral immune responses and class-switching toward immunoglobulins of the IgE isotype, as well as inducing eosinophil and mast cell accumulation and activation (11-13). Thus, considering their divergent effector functions, it is important that Th1 and Th2 cells can specifically migrate to those sites in the periphery where they are likely to be most useful. Accordingly, there is now evidence that these two subsets display divergent migratory properties *in vivo*, as Th1 cells show greatly enhanced homing to inflamed sites in the periphery, such as rheumatoid joints and sites of delayed type hypersensitivity (DTH), compared to Th2 cells (14, 15).

1.1.6 Molecular mechanisms of T cell extravasation

The recruitment of T cells through vascular endothelium into either lymphoid or peripheral tissues requires the sequential participation of a number of adhesion molecules (selectins, integrins and their respective counter-receptors) and soluble molecules (cytokines and chemokines) (6, 16, 17), as illustrated in Fig 1.2. The initial interaction of T cells with endothelial cells occurs primarily through the binding of selectins to a carbohydrate determinant on their cognate counter-receptors. This interaction enables the low-affinity tethering and rolling of lymphocytes along the luminal surface of the endothelium. Importantly, the expression of P- and E- selectins on endothelial cells is rapidly increased following exposure to inflammatory cytokines, ensuring that the efficient extravasation of large numbers of T cells is restricted to those areas in the periphery where an inflammatory response is occurring.

The selectin-mediated rolling of T cells allows them to slow down long enough such that chemokines, which are present in the blood vessel lumen, can interact with their cognate receptors on T cells, triggering the activation of integrin molecules on the T cell surface. Activated integrins subsequently bind to counter-receptors present on endothelial cells, leading to the firm adhesion of T cells to the endothelial surface. This firm adhesion step is then followed by the transmigration of T cells through inter-endothelial junctions, subsequent to chemokine-directed movement through the tissue.

The involvement of a number of molecular families, each with many members, provides the process of T cell extravasation with great combinatorial diversity (6, 16, 17), thereby enabling naïve, effector and memory T cells, and their subsets, to display the differential migratory patterns discussed previously. For example, the ability of

naïve T cells to enter lymph nodes via HEVs is, to a large extent, dependent on the expression of L-selectin (18). Whereas naïve T cells uniformly express high levels of this molecule, memory T cells show reduced levels of L-selectin expression (19), a difference that is likely to play a large part in explaining the diminished capacity of memory T cells to enter lymph nodes directly from the bloodstream. Conversely, effector/memory – but not naïve – T cells efficiently bind to E- and P- selectin expressed by endothelial cells in peripheral tissues (15, 20), thereby ensuring that previously activated cells are able to roll along endothelium in the periphery with far greater efficiency than naïve T cells. The preferential homing of other T cell subsets can also be explained, to a certain extent, by differential expression or activation of adhesion molecules (6).

It is important to note, however, that the multi-step nature of the T cell extravasation process provides potential control checkpoints at each and every step, and thus the expression of the appropriate adhesion molecules or counter-receptors is not necessarily sufficient to enable a T cell to leave the bloodstream at a particular site. An additional and essential checkpoint is the ability of the T cell to respond to an appropriate set of chemokines, as chemokines are thought to be required for both the triggering of firm adhesion and subsequent directed migration through the tissue. The regulation of chemokine receptor expression, which will play a major role in determining responsiveness to chemokines, is thus far incompletely understood.

1.2 Overview of the chemokine system

1.2.1 Introduction to the chemokine superfamily

Chemokines are low molecular weight proteins that function as extracellular messengers for the immune system (21-23). They are almost exclusively secreted molecules, although at least two examples of membrane-bound chemokines are known. Their major collective biological activity appears to be that of chemotaxis of leukocytes, although various members of the family can mediate a variety of other functions. As a group, chemokines demonstrate chemotactic activity on the complete range of leukocytes, including T cells, B cells, DCs, natural killer (NK) cells, monocytes/macrophages, neutrophils, basophils, eosinophils and mast cells. Additional functions within the immune system include the regulation of adhesion molecule expression and activity, modulation of the production of other chemokines and cytokines, control of haematopoiesis, functional activation of leukocytes (including eosinophils and monocytes/macrophages), histamine release from basophils, enhanced cytolytic activity of NK cells, neutrophil degranulation and possibly lymphocyte co-stimulation. Additionally, some chemokines appear to play a critical role in processes outside of the immune system, such as development and angiogenesis.

1.2.2 Chemokines: classification and target cell specificity

Thus far, over 45 chemokines have been identified, and more continue to be cloned every year. Based on a characteristic cysteine-containing motif in the predicted primary amino acid structure, chemokines can be classified into two major (CC and CXC) and two minor (C and CX_3C) sub-families (Fig 1.3 and Table 1.1). Members of

the CC sub-family possess a four-cysteine motif in which the two N-terminal cysteines are directly adjacent to one another, while members of the CXC sub-family have an intervening amino acid between these first two cysteines. The C sub-family thus far consists of only two closely-related members, which possess only two of the four cysteines normally found within the motif. Only one member of the CX_3C sub-family has been identified (fractalkine), which is characterised by three amino acids between the first two cysteines. A new nomenclature system has recently been proposed, whereby each chemokine is named according to its sub-family classification (CC, CXC, C or CX_3C) and given a number according to the chromosomal location of its gene (24). For example, the chemokine previously known as I-309 is now known as CCL1 (CC ligand 1). As this nomenclature is still in the process of gaining widespread use, both the old and new nomenclature will be used subsequently throughout this text.

The organisation of chemokines into structurally-based subfamilies correlates moderately well with their biological function (21, 23). In general, members of the C, CC and CX_3C sub-families are chemotactic for monocytes and lymphocyte subsets, with some members also active on DCs and granulocytes other than neutrophils. The activity of CXC chemokines, on the other hand, depends largely on the presence or absence of the ELR (glutamine-leucine-asparagine) motif. Thus, ELR-containing CXC chemokines are generally chemotactic for neutrophils, while lymphocytes are the major targets of non-ELR chemokines.

It is important to note, however, that the classification of chemokine function according to the cysteine-containing and ELR motifs is highly generalised, and there is considerable variation within the sub-families with regard to target cell specificity. For example, not all members of the CC sub-family are chemotactic for monocytes (25,

26), while recent data indicate that the ability to chemoattract neutrophils is not restricted entirely to the ELR-containing CXC chemokines, as was previously believed (27). In particular, although a large number of chemokines are chemotactic for lymphocytes, there is considerable diversity in the ability of these chemokines to attract various lymphocyte subsets (23, 28). Chemokines that are active on T cells are not always active on B cells, and vice versa. Furthermore, within the groups of chemokines that are active on T cells or B cells, some are chemotactic for naïve cells while others are chemotactic only for effector/memory cells. Often there is even further subset restriction, with only particular subsets of activated T cells, for example those of a $CD4^+$ Th1 phenotype (29), or with a skin-homing preference (25), being responsive to a particular chemokine. The ability of chemokines to attract both specific and overlapping subsets of leukocytes is likely to provide the immune system with precision, but at the same time a degree of redundancy which may be required to ensure robustness of such an important biological system (30, 31).

1.2.3 Regulation of chemokine production

1.2.3.1 Inflammatory and homeostatic chemokines

Chemokines are produced by a wide variety of cell types, of both haematopoietic and non-haematopoietic origin (31). Broadly speaking, most chemokines can be loosely categorised into two groups, depending on the manner in which they are produced (32-34) (Table 1.2). The majority of chemokines are classified as inflammatory (also referred to as inducible), as their level of expression is increased in response to inflammatory stimuli. It is likely that these chemokines are responsible for the recruitment of specialised effector cell populations, such as activated T cells, monocytes and granulocytes, to sites of inflammation. In contrast, a smaller number of

chemokines are classified as homeostatic (also referred to as constitutive or lymphoid); these chemokines are expressed at readily detectable levels in the absence of inflammation, particularly within secondary lymphoid tissues. It is thought that this group of chemokines is collectively responsible for the directed trafficking of lymphocyte sub-populations under conditions of homeostasis.

While it is likely that some inflammatory chemokines are produced only in peripheral tissues and that their production is strictly dependent on the presence of inflammation, it is now becoming clear that many others show more complex patterns of regulation. Thus, some inflammatory chemokines are expressed constitutively at a low level in certain peripheral tissues, but are up-regulated at these sites in response to inflammation. For example, MIP-3α/CCL20 is expressed constitutively at a low level in the intestinal epithelium, but the level of expression is greatly increased in response to LPS administration (35). Similarly, some chemokines are constitutively expressed in some peripheral tissues, but expressed in others only upon the induction of an inflammatory response. For example, Mig/CXCL9 and IP-10/CXCL10 are constitutively expressed in the colon (36), but are induced in the skin only under inflammatory conditions (37-39). Finally, a number of chemokines are up-regulated at peripheral sites of inflammation, thereby classifying them as inflammatory, but in addition are constitutively expressed in secondary lymphoid tissues. For example, while eotaxin expression is dramatically induced in the lung under inflammatory conditions such as ovalbumin-induced eosinophilia (40), it is also constitutively expressed in secondary lymphoid tissues such as the thymus and LNs (41). Such heterogeneity within the inflammatory chemokine grouping potentially extends the role of these chemokines beyond that of the directed recruitment of effector cells to sites of inflammation. However, further study is required before the members of the

inflammatory chemokine group can be definitively sub-divided according to these divergent patterns of expression.

1.2.3.2 Patterns of homeostatic chemokine expression

Homeostatic chemokines are generally produced in primary and secondary lymphoid tissues such as bone marrow, thymus, spleen and LNs (34, 42-44), although the expression of MEC/CCL28 has thus far only been demonstrated at sites associated with epithelial mucosa (45). SDF-1/CXCL12 is also unusual, in that it shows a much broader pattern of expression throughout the body than other homeostatic chemokines (46, 47), which may be related to its important functions outside of the immune system (48). Often, the expression of homeostatic chemokines is microenvironment-specific, such that production may be limited to T cell or B cell zones, and in most cases is thought to be relatively stable, as no evidence of regulation by inflammatory cytokines has been observed.

1.2.3.3 Patterns of inflammatory chemokine expression

Examples of increased inflammatory chemokine expression in response to human pathological conditions, or experimentally induced pathology in animals, are extensive in the literature. The range of human pathologies that have been associated with altered chemokine gene or protein expression include multiple sclerosis, transplant rejection, inflammatory bowel disease, asthma, rheumatoid arthritis, glomerulonephritis and vascular disease (21, 49, 50). With regard to animal studies, altered patterns of chemokine expression have been observed during the rejection of various foreign grafted tissues, in rodent models of airway hypersensitivity and arthritis, and in the

rodent model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), to name a few (49-51).

The patterns of inflammatory chemokines produced in a particular tissue are highly dependent on the nature of the immune response occurring there. In particular, a local environment dominated by either Th1 or Th2 cytokines has been suggested to influence the production of a number of chemokines. For example, the production of chemokines such as RANTES/CCL5, MIP-1α/CCL3 and MIP-1β/CCL4 has been associated with a type 1 immune response (52, 53), while the production of MCP-1/CCL2, MDC/CCL22 and eotaxin/CCL11 is often equated with a type 2 immune response (54-57). Of note, these differential expression patterns may be due, at least in part, to differential production of chemokines by Th1 and Th2 effector cells themselves, as these populations have been shown to secrete distinct subsets of chemokines *in vitro* (58).

1.2.4 Chemokine receptors

All chemokines exert their effects via ligation of seven transmembrane domain cell surface receptors (23, 59, 60) (Fig 1.4). These receptors are coupled to heterotrimeric G proteins, which in turn link the receptor to various downstream intracellular signalling pathways. Chemokine receptors are closely related in structure and signal transduction coupling mechanisms to other seven transmembrane domain receptors from a wide range of biological systems, including the cardiovascular system (β-adrenergic receptors) and the central nervous system (receptors for vision, olfaction and taste). However, one notable feature of chemokine receptors is the remarkable degree of promiscuity with regard to ligand binding. Although some chemokine

receptors bind to only one chemokine, others are able to bind as many as seven different chemokine ligands, often with varying affinity and sometimes with varied signalling outcomes.

Generally, chemokine receptors bind members of only one chemokine sub-family, although exceptions do exist. For example, murine SLC/CCL21 (a CC chemokine) binds to and activates CXCR3 (61), while Mig/CXCL9, IP-10/CXCL10 and I-TAC/CXCL11 (all CXC chemokines) bind CCR3, but act as antagonists rather than agonists (62). Thus far, eleven receptors for CC chemokines, six receptors for CXC chemokines and one receptor each for the C and CX_3C chemokines have been identified (Table 1.3). In addition, the Duffy Antigen Receptor for Chemokines (DARC) is an apparently non-functional receptor expressed on erythrocytes and endothelial cells, which binds chemokines from both the CXC and CC sub-families.

An important feature of chemokine receptor signalling is receptor internalisation in response to ligand binding (60). The biological outcome of receptor internalisation is a process known as desensitisation, whereby the cell becomes refractory to further stimulation with the same ligand, or other ligands that bind to the same receptor. This phenomenon is common among G-protein coupled receptors, and may have important biological consequences; for example, by allowing a cell to leave areas of high chemokine production, or to progress to the next step in a migratory pathway that is sequentially controlled by other chemokines (63).

In addition to their role in initiating a biological response to chemokines, chemokine receptors are also gateways for the entry of at least two important human pathogens into target cells. First, chemokine receptors act as co-receptors, in conjunction with

CD4, for the cellular entry of human immunodeficiency virus (HIV) and accordingly, chemokine receptors are currently considered excellent potential targets for the treatment of HIV (64, 65). CCR5 and CXCR4 are the most widely utilised HIV co-receptors, and as such, HIV isolates are now classified as R5 or X4, depending on their co-receptor usage. However, a number of other chemokine receptors are also able to act as HIV co-receptors under certain circumstances. Second, the non-functional chemokine receptor DARC is the surface receptor used *Plasmodium vivax* (a causative agent of malaria), to gain entry into erythrocytes (66).

1.3 Chemokines and chemokine receptors in T cell biology

1.3.1 Chemokines, chemokine receptors and T cells: in vitro *studies*

1.3.1.1 Chemotaxis

Most of our current understanding of the role of chemokines in T cell biology comes from *in vitro* studies. Chemotaxis assays have identified at least 25 chemokines capable of attracting certain types of T cells *in vitro*; these are listed in Table 1.4. The only chemokines identified thus far with the ability to recruit naïve T cells are SDF-1/CXCL12, ELC/CCL19, SLC/CCL21 and DC-CK1/CCL18 (26, 67-70). The remainder of those listed are active only on effector/memory T cell sub-populations, with very little or no activity on naïve cells. Some of these, such as MCP-1/CCL2, are active on a broad range of effector/memory T cells, with little preference for $CD4^+$ or $CD8^+$ cells (71), or Th1 or Th2 subsets (29). Accordingly, the level of expression of the MCP-1 receptor CCR2 is similar in populations of $CD4^+$ and $CD8^+$ (72) and Th1 and Th2 (73) cells. In contrast, other chemokines demonstrate preferential recruitment

of particular T cell subsets. For example, RANTES/CCL5, MIP-1β/CCL4 and IP-10/CXCL10 demonstrate a higher degree of activity on Th1 cells compared to Th2 cells (29, 73, 74). On the other hand, some chemokines such as TARC/CCL17, MDC/CCL22 and eotaxin/CCL11 demonstrate preferential recruitment of Th2 cells (73-77).

1.3.1.2 Adhesion

In addition to stimulating the directional migration of T cells, *in vitro* studies have identified a number of chemokines that also have the capacity to enhance T cell adhesiveness under conditions that mimic the shear stresses of blood flow. SDF-1/CXCL12, ELC/CCL19 and SLC/CCL21, three of the chemokines with the rare ability to chemoattract T cells of a naïve phenotype, also stimulate the rapid firm adhesion of naïve T cells to endothelial cells or endothelial cell adhesion molecules (78, 79). The chemokines Mig/CXCL9, IP-10/CXCL10, TARC/CCL17 and MIP-3α/CCL20 have similar effects on T cell adhesion, but are only active on effector/memory T cell populations, consistent with their chemotaxis profiles (78, 80, 81). The ability of these chemokines to increase the adhesion of T cells to endothelial cells suggests an important role for chemokines in the induction of T cell firm adhesion prior to transmigration through the endothelial barrier. Indeed, preventing the function of CCR6, the receptor for MIP-3α/CCL20, results in a reduction of up to 75% of the migration of skin-homing T cells through dermal microvascular endothelium (82). On the other hand, some chemokines such as MCP-1/CCL2, RANTES/CCL5 and MIP-1β/CCL4 enhance the binding of T cells to components of the extracellular matrix, such as fibronectin, but not to endothelial adhesion molecules (83), implicating

these chemokines in the movement of T cells through the tissue following transmigration.

1.3.1.3 T cell activation and differentiation

In addition to chemotaxis and induction of adhesion, further functions for chemokines in the control of T cell behaviour have also been proposed. A number of *in vitro* studies have suggested that some chemokines may play a co-stimulatory role during the T cell activation process. Of particular interest is a study demonstrating the activation of two divergent signalling pathways in T cells following the interaction of RANTES/CCL5 with its receptor (84). One is mediated by heterotrimeric G-proteins, typical of chemokine receptors, and induces chemotaxis. The other is mediated by protein tyrosine kinases, signalling molecules that are more commonly associated with T cell receptor (TCR) and cytokine signalling, and initiates a range of cellular responses including IL-2 receptor expression. SDF-1/CXCL12 has also been shown to have a co-stimulatory effect on $CD4^+$ T cells (85), while lymphotactin/XCL1 has divergent effects on $CD4^+$ and $CD8^+$ T cell activation, acting as an inhibitor of the former and a co-stimulator of the latter (86).

Following activation and clonal expansion, T cells must acquire the appropriate effector functions in order to initiate an effective local immune response. It is likely that chemokines play a role here also. For example, RANTES has been shown to enhance the cytotoxic potential of CTLs, via the up-regulation of Fas ligand on $CD8^+$ T cells (87). Further, there is now considerable evidence that some chemokines can contribute to the polarisation of T cells into Th1 or Th2 effector cell sub-populations

(88). MIP-1α/CCL3 and MCP-1/CCL2, for example, appear to enhance the development of Th1 and Th2 responses, respectively (55, 89-91).

1.3.2 Differential roles of homeostatic and inflammatory chemokines in the control of T cell migration

Chemokines demonstrate remarkable diversity in their ability to attract particular subsets of T cells. Most notable is the differential activity of chemokines on naïve versus effector/memory T cells. As discussed above and summarised in Table 1.4, the majority of chemokines show virtually no activity on naïve T cells, and yet are able to attract effector or memory T cell populations. On the other hand, a small number of chemokines are able to induce the chemotaxis of naïve T cells, but may show reduced activity on effector T cells. In general, chemokines that are active on naïve T cells are of the homeostatic type, produced constitutively at various sites throughout the body, particularly within secondary lymphoid tissues. On the other hand, those chemokines that attract only effector/memory T cells are generally of the inflammatory type, demonstrating significant up-regulation of production under inflammatory conditions.

These observations suggest that some homeostatic chemokines, through their ability to recruit and induce the adhesion of naïve T cells, play a role in the basal trafficking of T cells under conditions of homeostasis. Conversely, inflammatory chemokines, which are able to attract and stimulate effector/memory T cells to adhere to vascular endothelium, are thought to be important in the specific recruitment of effector T cells to sites of inflammation in the periphery (32-34). These concepts, however, are very novel and the degree to which the proposed scenarios actually play out *in vivo* remains to be determined.

1.3.3 *The chemokine system and control of T cell migration* in vivo

1.3.3.1 Overview

The *in vitro* studies discussed so far imply that chemokines have the potential to play a critical role in a number of aspects of T cell biology. However, the true contribution of chemokines to the control of T cell behavior *in vivo* is not well understood. The genetic deletion of chemokine and chemokine receptor genes, and the use of agonists, antagonists or neutralising antibodies to block chemokine or chemokine receptor function, has provided some insight into the role of these molecules on a whole-animal level. Such manipulations result in quite varied outcomes, as illustrated in Tables 1.5 and 1.6, which summarise the phenotypes of chemokine ligand and receptor knock-out mice, respectively.

1.3.3.2 Homeostatic chemokines and their receptors: role in T cell migration in vivo

The chemokines ELC/CCL19 and SLC/CCL21, along with their shared receptor CCR7, together define a homeostatic chemokine/receptor axis that appears, on the basis of *in vitro* studies, to be important in the homing of naïve T cells to secondary lymphoid tissue, and *in vivo* studies conducted thus far appear to support this. Intravital microscopy, which allows the study of activities within the blood vessels of living animals, has shown that SLC/CCL21 is necessary for integrin activation and firm adhesion of T cells rolling on the HEVs of LNs (92, 93). The importance of this chemokine/receptor axis in regulating LN architecture is evidenced by studies in $CCR7^{-/-}$ mice, or in mice homozygous for a naturally occurring mutation known as *plt*, which results in defective production of SLC/CCL21 and ELC/CCL19 (94, 95). Both of these mouse strains are characterised by disrupted lymphoid architecture and altered T cell immunity (96, 97). Moreover, ectopic expression of SLC/CCL21 in islet β cells

has been shown to be sufficient to induce lymphoid neogenesis (98), thereby further highlighting the importance of this chemokine in maintaining the structure and function of secondary lymphoid tissue microenvironments.

Unlike CCR7 and its ligands, the homeostatic chemokine receptor CXCR4 and its ligand SDF-1/CXCL12 do not play an apparent role in the homing of T cells to LNs or Peyer's patches, as inhibiting CXCR4 function by desensitising with the ligand does not prevent T cells from adhering to HEVs *in vivo* (92, 93). However, transgenic over-expression of CXCR4 on $CD4^+$ T cells results in abnormal patterns of $CD4^+$ T cell homing to the bone marrow, suggesting that that one potential function for this receptor may be the control of cellular movement between the blood and bone marrow (99).

Similar to the lymphoid neogenesis observed in response to ectopic expression of SLC/CCL21, the expression of BLC/CXCL13 in islet β cells also results in the development of structured lymphoid tissue, thereby suggesting a role for this chemokine and its unique receptor, CXCR5, in the control of microenvironmental positioning of lymphocytes (100). In this case, however, the formation of organised T cell zones is likely to be an indirect effect, because the ability of this chemokine to induce the formation of well-structured lymphoid tissue is greatly reduced in B cell-deficient mice. This observation suggests that the recruitment and organisation of T cells is secondary to the recruitment and organisation of B cells in these mice.

1.3.3.3 Inflammatory chemokines and their receptors: role in T cell migration in vivo

With regard to the inflammatory arm of the chemokine system, a limited number of chemokines have been implicated in the T cell recruitment process during the generation of pathological immune responses in animals. For example, blocking the function of MCP-1/CCL2 with a neutralising antibody was shown to greatly reduce the accumulation of T cells in the lung during *Cryptococcus neoformans* infection (101) or at a site of DTH activity in the rat (102). Similarly, the recruitment of polarised Th2 effector cells to a site of allergic airways disease could be reduced following the administration of neutralising antibodies to MDC/CCL22 or eotaxin/CCL11 (56). Furthermore, the administration of antibodies to TARC was shown to diminish the accumulation of T cells in the liver during bacteria-induced hepatic failure (103). Genetic deletion studies have also revealed potential roles for a number of inflammatory chemokines and their receptors in T cell-mediated immune responses (see Tables 1.5 and 1.6). However, in most instances, a direct effect of gene deletion on T cell migration has not been demonstrated. Thus, while the literature to date provides evidence that some inflammatory chemokines are required for effector T cell recruitment *in vivo*, many chemokines have not been investigated, and the chemokine receptors on T cells that mediate these effects have not been clearly identified.

1.3.3.4 Novel functions for chemokines and their receptors identified by in vivo *studies*

In vivo studies have also revealed some novel functions for chemokines that were not initially apparent on the basis of *in vitro* analyses. Of note, a role for the inflammatory chemokine MIP-2/CXCL2 in tolerance induction has been suggested, following the observation that antibodies to this chemokine prevented the migration of tolerance-inducing NK T cells to the spleen (104). Thus, inflammatory chemokines may be

involved in the down-regulation of immune responses as well as the promotion of inflammation through effector T cell recruitment. Furthermore, the homeostatic chemokine SDF-1/CXCL12 has been demonstrated to be important in the recruitment of T cells to the lung during allergic airways disease (105), thereby potentially extending the putative role of this chemokine beyond that of basal T cell trafficking under conditions of homeostasis.

1.3.4 Distinct patterns of chemokine receptor expression on T cell subsets

1.3.4.1 Overview

The expression of an appropriate chemokine receptor on the surface of a T cell is a critical factor in determining which chemokines that T cell is able to respond to. Accordingly, the patterns of chemokine receptors expressed by T cells are likely to have a major impact on their migratory properties. Differential patterns of chemokine receptor expression have been documented on a wide variety of functional T cell subsets.

1.3.4.2 Chemokine receptors expressed on naïve T cells

The naïve T cell subset expresses receptors for homeostatic chemokines but not inflammatory chemokines, as may be expected from the observation that naïve T cells only respond to homeostatic chemokines in *in vitro* chemotaxis assays. CCR7 (a receptor for ELC/CCL19 and SLC/CCL21) and CXCR4 (the receptor for SDF-1/CXCL12) are expressed at high levels on the naïve T cell population (74, 106-109). The receptor for DC-CK1/CCL18 is yet to be identified, but given the specific

attraction of naïve T cells by this chemokine, it is expected that the cognate receptor will demonstrate preferential expression on the naïve T cell population.

1.3.4.3 Chemokine receptors expressed on effector/memory T cells

Generally speaking, effector/memory T cells tend to express a broader range of chemokine receptors than do naïve T cells, and also differ in the types of chemokine receptors expressed. In contrast to the naïve T cell population, effector/memory T cells express a number of inflammatory chemokine receptors, thereby accounting for the ability of these cells to respond to inflammatory chemokines. Furthermore, some studies have suggested that the homeostatic chemokine receptors CCR7 and CXCR4 demonstrate heterogeneous and/or reduced levels of expression on memory T cells, compared to their uniformly high levels of expression on naïve T cells (74, 107-110).

Of note, diverse functional subsets of effector/memory T cells tend to express a divergent range of inflammatory chemokine receptors. This phenomenon is best illustrated by studies of receptor expression on Th1 and Th2 effector populations. In keeping with the preferential activity of some chemokines on Th1 versus Th2 cells discussed already, a number of studies have demonstrated differential expression of the corresponding chemokine receptors on these effector T cell subsets. For example, Th1 cells have been shown to express higher levels of CCR5 and CXCR3 compared to Th2 cells (73, 74, 111). Conversely, Th2 cells appear to express higher levels of CCR3, CCR4 and CCR8 (73, 74, 76, 77, 112).

In addition to the well-recognised Th1 and Th2 subsets, it is clear that effector T cells can be divided into many other functional subsets, and some of these also appear to be

characterised by unique patterns of chemokine receptor expression. For example, populations of CLA^+ memory T cells that preferentially home to the skin express high levels of CCR6 (113) and CCR4 (75, 80). In contrast, populations of gut-resident and gut-homing T cells preferentially express CCR5 and CXCR3 (114), while populations of T cells isolated from the lung express a combination of these receptors, being characterised by high levels of CCR5 expression and moderate levels of expression of CCR4, CCR6, CXCR3 and CXCR4 (115). Furthermore, sites of some inflammatory pathologies are enriched in T cells expressing particular patterns of chemokine receptors. For example, joints affected by rheumatoid arthritis are enriched in T cells expressing CCR5, CXCR3, CXCR4 and CXCR6 (111, 116-118). In contrast, skin-homing T cells obtained from psoriatic skin express higher levels of CCR6 compared to skin-homing T cells obtained from healthy skin.

1.3.4.4 Definition of novel T cell subsets based on differential chemokine receptor expression patterns

Of particular importance, two chemokine receptors have recently been shown to define novel functional subsets of T cells. First, expression of the chemokine receptor CCR7 has been proposed to divide memory T cells into two functional subsets. The $CCR7^-$ population is thought to migrate to sites of peripheral inflammation to mediate effector function, while the $CCR7^+$ population is likely to home to LNs in order to aid in the rapid initiation of a secondary response (108, 109). These two populations of memory T cells are referred to as effector and central memory T cells, respectively. Second, the expression of CXCR5 also appears to define a novel T cell population, referred to as follicular B helper T cells (T_{FH}) (119, 120), or germinal centre Th cells (GC-Th) when co-expressed with CD57 (121). These cells are localised in the B cell areas of

secondary lymphoid tissues and are uniquely adapted to provide help to B cells for antibody production.

It is therefore clear that T cell subsets express unique patterns of chemokine receptors on their surface. What is not well understood, however, is the mechanisms by which the various functional subsets of T cells come to acquire such a divergent range of chemokine receptors. Such an understanding can only come from analyses of chemokine receptor expression during the process of T cell activation, or the acquisition of a defined effector function.

1.3.5 The regulation of chemokine receptor expression on T cells

1.3.5.1 Regulation by cytokines

Given that Th1 and Th2 cells express differential patterns of chemokine receptors, and that the development of the Th1/Th2 phenotype is highly dependent on cytokines, it is perhaps no surprise that cytokines appear to play a pivotal role in the control of chemokine receptor expression on T cells. Mitogenic activation of naïve T cells in the presence of IL-12 and neutralising antibodies to IL-4 results in the development of a Th1-polarised phenotype and the up-regulation of CCR5 and CXCR3 (73, 111). Conversely, activation in the presence of IL-4 and neutralising antibodies to IL-12 results in the generation of Th2-polarised cells and the acquisition of CCR3, CCR4 and CCR8 (73, 74, 76, 77, 112). Thus, IL-4 and IL-12 clearly play important roles in determining the patterns of chemokine receptors expressed by mitogen-activated T cells. Interestingly, however, treatment of peripheral blood T cells with IL-12 in the absence of mitogenic stimulation has little effect on the expression of CCR5 (122),

thus highlighting the importance of TCR stimulation in the acquisition of chemokine receptors by naïve T cells, as will be discussed in more detail to follow.

On the other hand, at least two cytokines are known to modulate chemokine receptor expression in the absence of TCR triggering. The most well-studied of these is IL-2, which up-regulates the expression of CCR1, CCR2, CCR5, CCR7 and CXCR6 *in vitro* (122-127). Further, IL-2 and IL-4 act in a synergistic manner to enhance the expression of CCR3 (128), and IL-2 is able to up-regulate CCR5 expression *in vivo*, as observed in studies of HIV-positive patients receiving IL-2 therapy (129). The effect of IL-2 on CCR6, however, is unclear, due to conflicting reports in the literature (130, 131). IL-15, which shares many biological activities with IL-2, also has the ability to up-regulate a number of CC chemokine receptors on resting peripheral blood T cells (123, 132).

1.3.5.2 Regulation by TCR stimulation

Cytokines clearly play a role in the regulation of chemokine receptor expression, but, as mentioned above, the majority of cytokines are likely to function in coordination with TCR stimulation to alter receptor expression. Of particular importance is the observation that the homeostatic chemokine receptors CCR7 and CXCR4 are expressed uniformly by naïve T cells but show heterogeneous and/or reduced levels of expression on effector/memory T cells (74, 107-110). In contrast, inflammatory chemokine receptors are undetectable on naïve T cells, but are expressed on various subsets of effector/memory T cells (33, 34, 133). These patterns suggest that, at some stage during the T cell activation process, there is a coordinated switch in the nature of the chemokine receptors expressed by T cells, such that inflammatory chemokine

receptors are gained and the expression of some homeostatic chemokine receptors becomes more restricted.

Recent studies have provided some limited support for this concept. In terms of the up-regulation of inflammatory chemokine receptors upon T cell activation, stimulation of peripheral blood T cells with mitogenic lectins or with anti-CD3 (with or without anti-CD28 mediated co-stimulation) has been shown to result in the increased expression of CXCR3, but not of a range of other inflammatory chemokine receptors, including CCR1, CCR2, CCR3, CCR5 and CCR6 (72, 118, 130, 134). In fact, expression of CCR6 has been shown in one study to decrease in response to mitogenic stimulation (131). These results are surprising, considering that memory T cells and T cells isolated from sites of inflammation are known to express these receptors, as discussed previously.

It is likely, however, that the artificial stimulation conditions used in these studies were not optimal for inflammatory chemokine receptor up-regulation. The nature of the co-stimulation signal appears to be critical, as co-stimulation through CD28 and CTLA-4 have very different effects on CCR5 expression, with CD28 co-stimulation alone promoting CCR5 down-regulation but a combination of CD28 and CTLA-4 co-stimulation promoting CCR5 up-regulation (135). Furthermore, the expression of CXCR6 is increased on T cells following stimulation with antigen-loaded DC (117). Thus, it is possible that the up-regulation of many other inflammatory chemokine receptors may only be observed under T cell activation conditions that closely mimic those encountered *in vivo*; namely, interaction with DCs.

The concept of altered homeostatic chemokine receptor expression upon T cell activation has similarly received limited support in the literature. While some studies have demonstrated the down-regulation of CXCR4 upon T cell activation (106, 136, 137), others have concluded, using similar techniques, that CXCR4 is in fact up-regulated upon T cell activation (107, 135, 138). Following mitogenic stimulation, CCR7 appears to undergo a transient period of increased expression that peaks at around day 3 (119, 125), which may coincide with the retention of proliferating T cells in the secondary lymphoid tissue. The longer-term effects of T cell activation on CCR7 expression are, however, not known, and the development of distinct populations of $CCR7^+$ and $CCR7^-$ memory T cells, which have been proposed to exist in the peripheral circulation, remains to be experimentally demonstrated.

Of note, the studies discussed up to this point have, in most cases, analysed receptor expression on either purified naïve T cell subsets, or freshly isolated peripheral blood mononuclear cells (PBMCs), a large proportion of which are also naïve. Quite different results are seen when effector/memory T cells, which already express a range of inflammatory chemokine receptors, are specifically analysed (139-141). The activation of effector/memory T cells with anti-CD3 or anti-CD3 plus anti-CD28 results in the down-regulation of a range of inflammatory chemokine receptors, including CCR2, CCR3, CCR5, CCR6 and CXCR3, although others such as CCR4 and CCR8 are up-regulated. Homeostatic chemokine receptors show divergent responses, with CCR7 being up-regulated and CXCR4 being down-regulated. It is thought that the loss of many inflammatory chemokine receptors and increased expression of CCR7 may promote the migration of memory T cells to the T cell areas of secondary lymphoid tissues, where CCR7 ligands are produced constitutively, thereby facilitating the efficient generation of a secondary immune response.

Thus, *in vitro* studies have not refuted the concept of a switch in chemokine receptor expression upon T cell activation, and studies of pathological conditions provide indirect evidence that such a switch occurs. It is, however, clear that further studies are required, particularly utilising antigen-presenting cell (APC)-based models of T cell activation *in vitro*, as well as whole-animal studies. Thus far, animal studies along these lines are scarce in the literature, attributable at least in part to the paucity of reagents available to study chemokine receptors in rodents. Of note, however, is the finding that $CD8^+$ T cells activated *in vivo* by viral infection respond less efficiently to the homeostatic chemokines ELC/CCL19 and SLC/CCL21 than their non-activated counterparts, have barely detectable levels of CCR7 mRNA, and are accordingly excluded from the T cell areas of the spleen (110).

1.4 The research project

The central hypothesis of this research project is as follows:

"During the activation of naïve T cells, there is a coordinated switch in the nature of the chemokine receptors expressed, such that inflammatory chemokine receptors are gained and the expression of homeostatic chemokine receptors becomes more restricted. This switch will allow activated/memory T cells to adopt alternative migratory patterns that allow access to relevant sites in peripheral tissues."

In order to test this hypothesis, the following aims will be addressed:

1. To identify chemokine receptors that demonstrate altered levels of expression on T cells following interaction with APCs in *in vitro* culture;

2. To perform a detailed analysis of the expression patterns of these chemokine receptors on T cells activated *in vitro*;

3. To analyse the expression of these chemokine receptors on T cells in secondary lymphoid tissue during the induction of an immune response *in vivo*;

4. To assess the impact that changes in chemokine receptor expression have on the migratory properties of T cells.

Table 1.1: Nomenculature and sub family classification of chemokines[1]

Systematic name	Alternative names	
	Human	Mouse
CC sub-family		
CCL1	I-309	TCA-3
CCL2	MCP-1	JE
CCL3	MIP-1α	MIP-1α
CCL4	MIP-1β	MIP-1β
CCL5	RANTES	RANTES
CCL6	*not identified in human*	C10
CCL7	MCP-3	MARC
CCL8	MCP-2	MCP-2
CCL9/10	*not identified in human*	CCF18 / MIP-1γ
CCL11	eotaxin	eotaxin
CCL12	*not identified in human*	MCP-5
CCL13	MCP-4	*not identified in mouse*
CCL14	HCC-1	*not identified in mouse*
CCL15	HCC-2 / MIP-1δ	*not identified in mouse*
CCL16	HCC-4	LCC-1
CCL17	TARC	TARC
CCL18	DC-CK1 / PARC	*not identified in mouse*
CCL19	ELC / MIP-3β / exodus-3	MIP-3β / ELC / exodus-3
CCL20	MIP-3α / LARC / exodus-1	MIP-3α / LARC / exodus-1
CCL21	SLC / 6Ckine / exodus-2	SLC / 6Ckine / exodus-2 / TCA-4
CCL22	MDC / STCP-1	ABCD-1
CCL23	MPIF-1	*not identified in mouse*
CCL24	eotaxin-2 / MPIF-2	*not identified in mouse*
CCL25	TECK	TECK
CCL26	eotaxin-3	*not identified in mouse*
CCL27	CTACK / ILC	CTACK / Eskine
CCL28	MEC	none
CXC sub-family		
ELR-containing		
CXCL1	GROα / MGSAα	GRO / KC (?)
CXCL2	GROβ / MGSAβ / MIP-2α	GRO / KC (?)
CXCL3	GROγ / MGSAγ / MIP-2β	GRO / KC (?)
CXCL5	ENA-78	LIX
CXCL6	GCP-2	CKα-3
CXCL7	NAP-2	*not identified in mouse*
CXCL8	IL-8	*not identified in mouse*
non-ELR		
CXCL4	PF4	PF4
CXCL9	Mig	Mig
CXCL10	IP-10	IP-10
CXCL11	I-TAC	I-TAC
CXCL12	SDF-1α/β	SDF-1
CXCL13	BLC / BCA-1	BLC / BCA-1
CXCL14	BRAK / bolekine	BRAK
CXCL15	*not identified in human*	lungkine
CXCL16	none	none
C sub-family		
XCL1	lymphotactin / SCM-1α	lymphotactin
XCL2	SCM-1β	*not identified in mouse*
CX_3C sub-family		
CX3CL1	fractalkine	neurotactin

[1] Adapted from Zlotnik & Yoshie (24). Only the more common alternative names are shown; the first in the list is the one that will be used subsequently throughout the text.

Table 1.2: Functional classification of chemokines into inflammatory and homeostatic groupings[1]

Inflammatory chemokines	Homeostatic chemokines
CC chemokines	
I-309 / CCL1	DC-CK1 / CCL18
MCP-1 / CCL2	ELC / CCL19
MIP-1α / CCL3	SLC / CCL21
MIP-1β / CCL4	TECK / CCL25
RANTES / CCL5	MEC / CCL28
MCP-3 / CCL7	
MCP-2 / CCL8	
eotaxin-1 / CCL11	
MCP-5 / CCL12	
MCP-4 / CCL13	
TARC / CCL17	
MIP-3α / CCL20	
MDC / CCL22	
eotaxin-2 / CCL24	
eotaxin-3 / CCL26	
CTACK / CCL27	
CXC chemokines	
GROα / CXCL1	SDF-1 / CXCL12
GROβ / CXCL2	BLC / CXCL13
GROγ / CXCL3	
ENA-78 / CXCL5	
GCP-2 / CXCL6	
NAP-2 / CXCL7	
IL-8 / CXCL8	
Mig / CXCL9	
IP-10 / CXCL10	
I-TAC / CXCL11	
CXCL16	
C and CX3C chemokines	
lymphoctactin / XCL1	
fractalkine / CX3CL1	

[1] Adapted from Sallusto *et al* (33) and Moser & Loetscher (34). Chemokines for which expression patterns have not been characterised are not included.

Table 1.3: Chemokine receptors and their ligand-binding profiles[1]

Receptor	Ligand(s)
CCR1	MIP-1α, RANTES, MCP-2, MCP-3, HCC-4
CCR2	MCP-1, MCP-2, MCP-3, MCP-4
CCR3	eotaxin, eotaxin-2, RANTES, MCP-2, MCP-3, MCP-4, MEC
CCR4	TARC, MDC
CCR5	MIP-1α, MIP-1β, RANTES
CCR6	MIP-3α
CCR7	ELC, SLC
CCR8	I-309, HCC-4
CCR9	TECK
CCR10	CTACK, MEC
CCR11	ELC, SLC, TECK
CXCR1	IL-8, GCP-2
CXCR2	IL-8, GCP-2, GRO-α, GRO-β, GRO-γ, ENA-78, NAP-2
CXCR3	Mig, IP-10, I-TAC
CXCR4	SDF-1
CXCR5	BLC
CXCR6	CXCL16
XCR1	lymphotactin, SCM-1β
CX3CR1	fractalkine
DARC	IL-8, GRO-α, MIP-1α, MIP-1β, RANTES

[1]Adapted from Gale & McColl (23). All identified human chemokine receptors, and their major ligands, are shown. Ligands that show only limited affinity for the receptor, or do not induce a significant agonistic response, are not shown

.

Table 1.4: T cell-active chemokines: subset-dependent chemotaxis [1]

Chemokine	T cell chemotaxis profile
Inflammatory chemokines	
I-309 / CCL1	effector/memory (Th2 preference)
MCP-1 / CCL2	effector/memory
MIP-1α / CCL3	effector/memory
MIP-1β / CCL4	effector/memory (Th1 preference)
RANTES / CCL5	effector/memory (Th1 preference)
MCP-3 / CCL7	effector/memory
MCP-2 / CCL8	effector/memory
eotaxin / CCL11	effector/memory (Th2 preference)
TARC / CCL17	effector/memory (Th2 and CLA+ preference)
MIP-3α / CCL20	effector/memory
MDC / CCL22	effector/memory (Th2 and CLA+ preference)
TECK / CCL25	effector/memory, thymocytes
CTACK / CCL27	effector/memory (CLA+ preference)
mig / CXCL9	effector/memory (Th1 preference)
IP-10 / CXCL10	effector/memory (Th1 preference)
I-TAC / CXCL11	effector/memory (Th1 preference)
CXCL16	effector/memory
lymphotactin / XCL1	effector/memory, thymocytes
fractalkine / CX3CL1	effector/memory
Homeostatic chemokines	
DC-CK1 / CCL18	naïve
ELC / CCL19	naïve, effector/memory
SLC / CCL21	naïve, effector/memory
TECK / CCL25	effector/memory, thymocytes
MEC / CCL28	effector/memory (CLA+ preference)
SDF-1 / CXCL12	naïve, effector/memory, thymocytes
BLC / CXCL13	effector/memory

[1] Adapted from Moser & Loetscher (34). All chemokines listed have been demonstrated to be chemotactic for human T cells in *in vitro* assays. The differential responsiveness of various subsets has either been demonstrated in chemotaxis assays, or inferred through subset-restricted expression of the relevant receptors.

Table 1.5: Phenotypes of chemokine ligand knock-out mice[1]

Mouse	Summary of phenotype	Reference
CCL2 / MCP-1	• reduced recruitment of monocytes/macrophages to peritoneum and sites of DTH • defect in Th2-associated immune responses, and reduced lymphocyte production of Th2 cytokines	(91) (142)
CCL3 / MIP-1α	• reduced autoimmune response to Coxsackie-virus • reduced clearance of influenza virus	(143)
CCL11 / eotaxin	• lower numbers of circulating eosinophils • early reduction in eosinophil recruitment to the lung and cornea in models of eosinophil-associated inflammation	(144)
CXCL10 / IP-10	• cardiac allografts derived from CXCL10 $^{-/-}$ mice show increased survival in wild-type recipients	(145)
CXCL12 / SDF-1	• embryonic lethal • defective haematopoiesis in bone marrow and liver • abnormal cardiac development	(48)
CX_3CL1 / fractalkine	• lower numbers of circulating F4/80+ cells	(146)

[1] A summary of the differences between each genetically deficient mouse and its wild-type counterpart is given. In general, where numerous studies have used a particular knock-out mouse, only the basic characterisation of the phenotype is described. Negative results, whereby the knock-out animal was not significantly different to the wild-type, are not described.

Table 1.6: Phenotypes of chemokine receptor knock-out mice[1]

Mouse	Summary of phenotype	Reference
CCR1	• defective myelopoiesis under conditions of homeostasis and LPS challenge • reduced immunity to *Aspergillus fumigatus* • reduced SEA-induced granuloma formation, associated with increased IFN-γ secretion	(147)
CCR2	• reduced recruitment of macrophages to peritoneal cavity • decrease in size and macrophage content of *Mycobacterium bovis* PPD-induced granulomas • altered IL-2 and IFN-γ production by lymphocytes	(148)
CCR4	• resistance to high and low dose models of endotoxemia • reduced numbers of macrophages in LPS-stimulated peritoneal cavity	(149)
CCR5	• altered cytokine production by macrophages and T cells • reduced ability to clear *Listeria monocytogenes* infection • enhance resistance to endotoxemia • increased contact sensitivity response to FITC • increased IgG1 response to soluble protein antigens	(150)
CCR6	• absence of $CD11b^+$ $CD11c^+$ dendritic cell sub-population within intestinal sub-epithelial dome • increased numbers of lymphocytes in intestinal epithelium • reduced intestinal humoral immune response to KLH and rotavirus • enhanced contact sensitivity response to DNFB • reduced DTH response to allogeneic splenocytes	(151) (152)
CCR7	• altered secondary lymphoid tissue architecture • altered frequencies of immunoglobulin isotypes • disrupted migration of DCs, T cells and B cells to secondary lymphoid tissue • impaired T cell and humoral immune responses	(97)
CCR8	• reduced eosinophil content in SEA-induced granulomas • Th2 cytokine production defect • reduced numbers of pulmonary and circulating eosinophils during allergic airways inflammation	(153)
CXCR2	• enlarged lymph nodes and spleen due to increase in myelopoeisis and increased B cell numbers • defective neutrophil migration to peritoneum following thioglycollate injection	(154)
CXCR3	• decreased MLR response • increased acceptance of cardiac allografts	(155)
CXCR4	• embryonic lethal • defective haematopoiesis in bone marrow and liver • abnormal cardiac and cerebellum development	(156)
CXCR5	• absence of inguinal lymph nodes and Peyer's patches • disrupted primary and secondary follicle architecture in spleen • increased B cell numbers in blood and spleen • inability of transferred $CXCR5^{-/-}$ B cells to home to follicles	(157)
CX3CR1	• increased cardiac allograft survival in the presence of cyclosporin	(158)

[1] A summary of the differences between each genetically deficient mouse and its wild-type counterpart is given. In general, where numerous studies have used a particular knock-out mouse, only the basic characterisation of the phenotype is described. Negative results, in which the knock-out animal was not significantly different to the wild-type, are not described.

Figure 1.1 - Recirculation pathways of naïve and effector/memory T cells. Naïve T cells enter secondary lymphoid tissue directly from the bloodstream, via the HEVs (purple arrows). Effector/memory T cells can enter peripheral tissues (such as the skin, as shown), by passing through the endothelial wall of arterioles, and then drain via the afferent lymphatics into secondary lymphoid tissue (green arrows). Both subsets leave the secondary lymphoid tissue through the efferent lymphatic vessels and subsequently return to the bloodstream via the thoracic duct (blue arrows). Note that this diagram is intended only to illustrate general trends in T cell migratory capabilities; it is clear that some memory T cells retain the ability to enter secondary lymphoid tissue via the HEV. Adapted from Dailey (7).

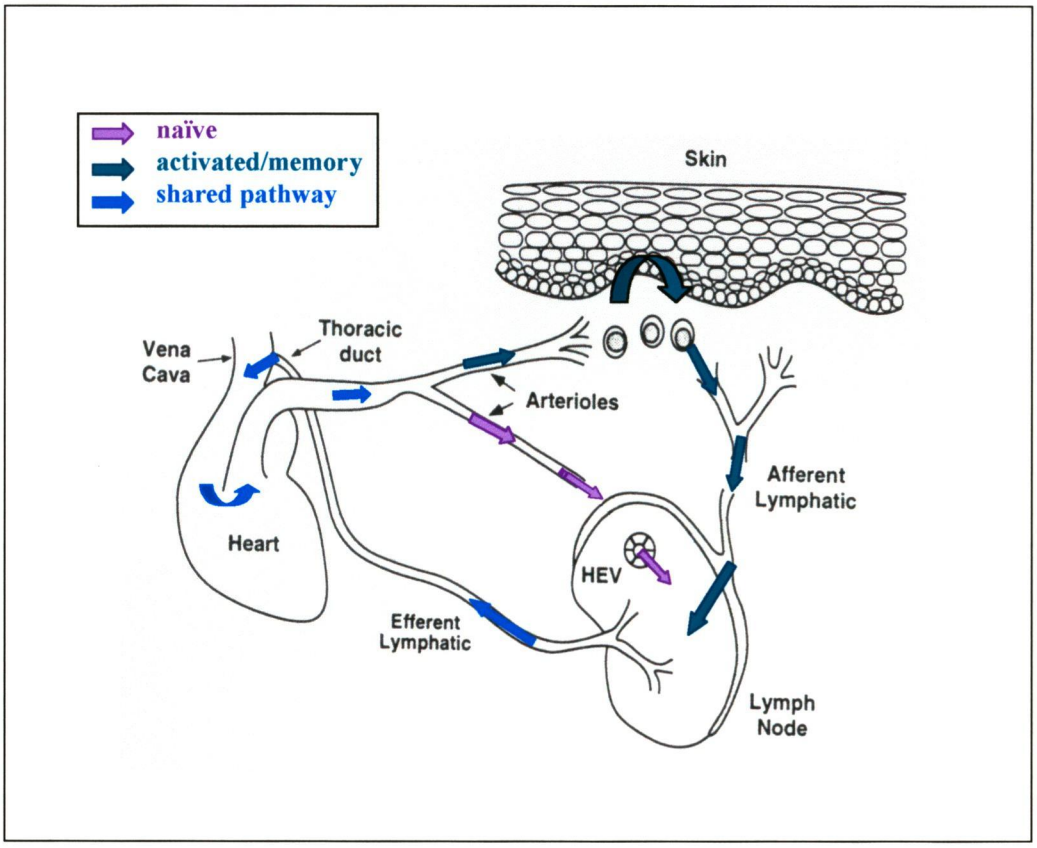

naïve
activated/memory
shared pathway
Skin
Vena Cava
Thoracic duct
Arterioles
Afferent Lymphatic
Heart
HEV
Efferent Lymphatic
Lymph Node

Figure 1.2 – The molecular mechanisms of T cell extravasation. T cells flowing through the blood vessel lumen interact transiently with endothelial cells via the binding of selectins (and, to a lesser extent, α4 integrins) to their counter-receptors on endothelial cells. These interactions initially involve a loose tethering, mediated by clustered adhesion molecules on the tips of microvilli, which is followed by a more sustained rolling motion, which does not require adhesion molecule clustering. Rolling T cells are slowed down sufficiently to enable interactions of chemoattractant molecules (particularly chemokines) with their 7-transmembrane domain receptors on the T cell surface, leading to the activation of T cell-expressed integrins. The subsequent binding of activated integrins on T cells to their counter-receptors on endothelial cells allows for T cell arrest (firm adhesion), which is followed by diapedesis through inter-endothelial junctions and chemokine-guided movement through the tissues. From von Andrian & Mackay (6).

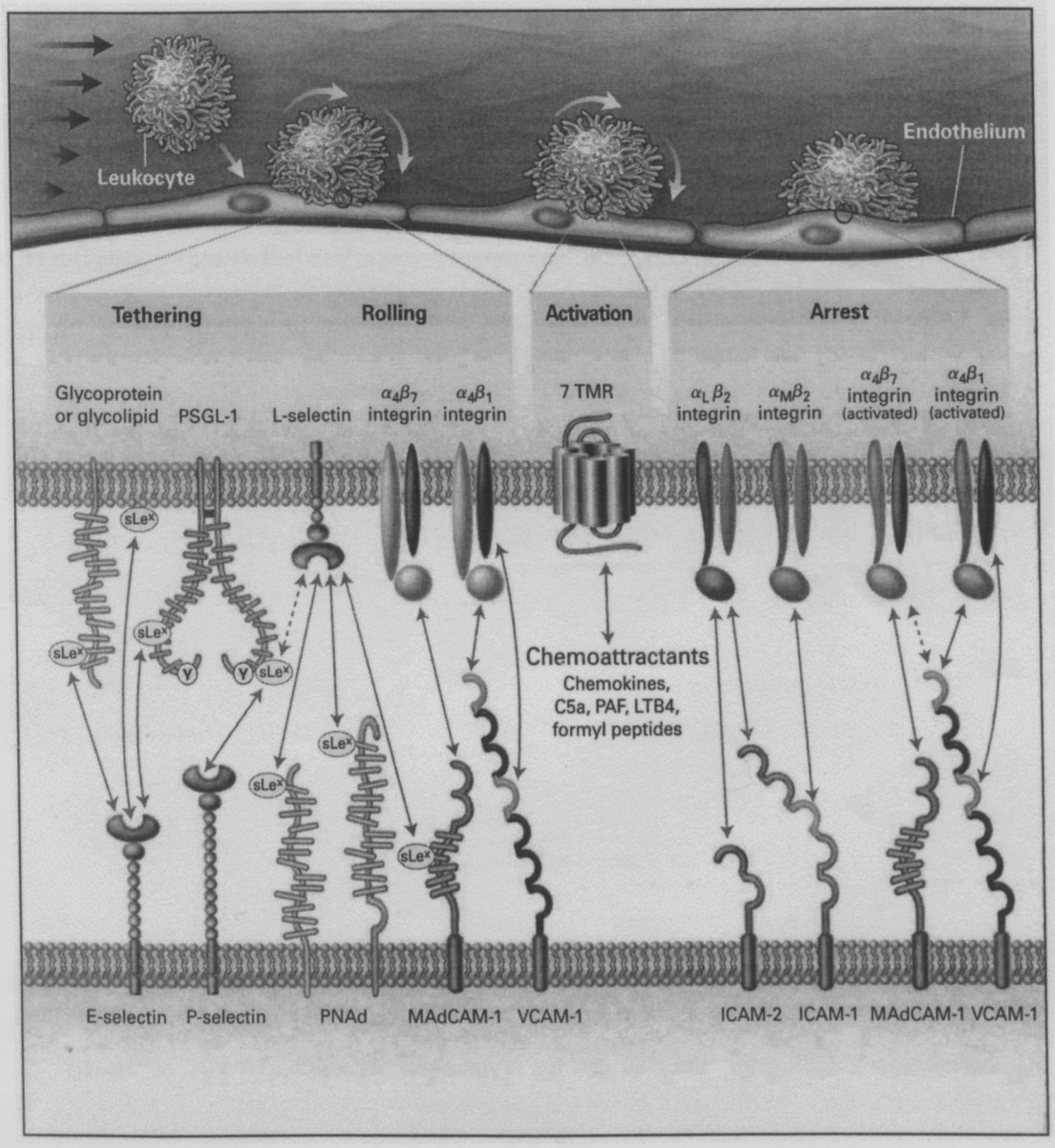

Leukocyte
Endothelium
Tethering
Rolling
Activation
Arrest
Glycoprotein or glycolipid
PSGL-1
L-selectin
$\alpha_4\beta_7$ integrin
$\alpha_4\beta_1$ integrin
7 TMR
$\alpha_L\beta_2$ integrin
$\alpha_M\beta_2$ integrin
$\alpha_4\beta_7$ integrin (activated)
$\alpha_4\beta_1$ integrin (activated)
sLe^x
Y
Chemoattractants
Chemokines, C5a, PAF, LTB4, formyl peptides
E-selectin
P-selectin
PNAd
MAdCAM-1
VCAM-1
ICAM-2
ICAM-1
MAdCAM-1
VCAM-1

Figure 1.3 – The classification of chemokines according to primary amino acid sequence. Chemokines can be classified into two major (CXC / α and CC / β) and two minor (C / γ and CX3C / δ) sub-families, on the basis of a cysteine-containing motif. Members of the CXC sub-family have an intervening amino acid between the first two cysteines, and can be further sub-divided according to the presence or absence of the glutamine-leucine-asparagine (ELR) motif. This sub-family also contains one member with an extended, membrane-bound structure (CXCL16). Members of the CC sub-family are characterised by the juxtaposition of the first two cysteines, while the C sub-family members are missing two of the four cysteines normally found within the motif. The sole member of the CX3C sub-family has three intervening amino acids between the first two cysteines, and an extended, membrane-bound structure. Adapted from Gale & McColl (23).

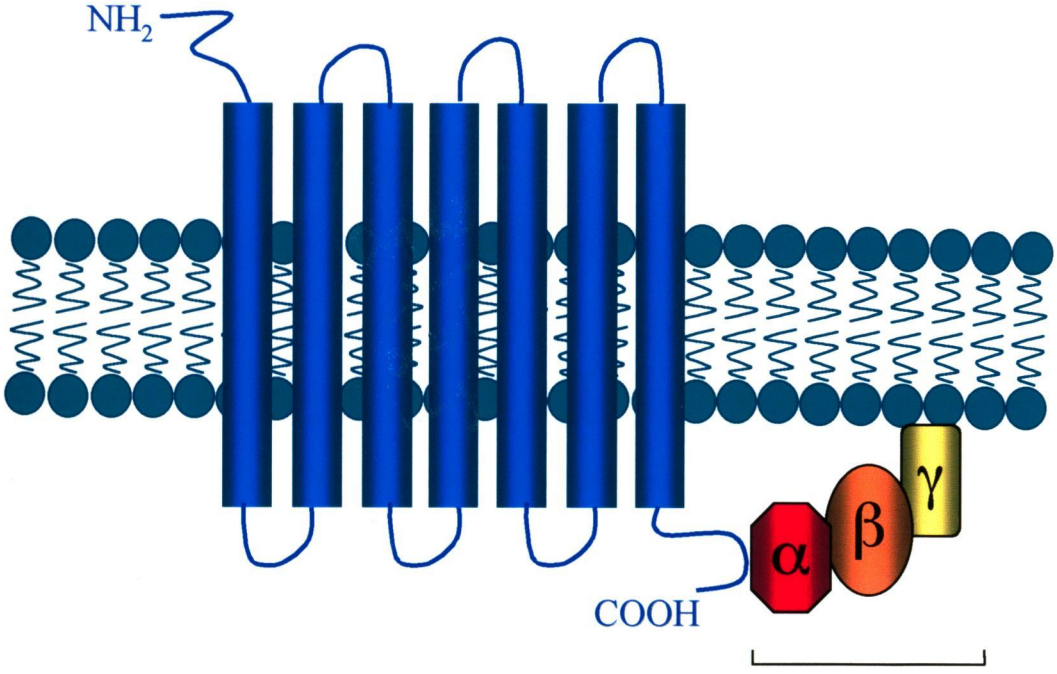
CXC (α) sub-family

CC (β) sub-family

C (γ) sub-family

CX_3C (δ) sub-family

Figure 1.4 – Basic structure of chemokine receptors. Chemokine receptors are characterised by seven transmembrane domains, three extracellular loops and three intracellular loops. The N-terminal region is positioned outside of the cell, while the relatively short C-terminal tail is located intracellularly. Heterotrimeric G-proteins couple the receptor to intracellular second messenger systems, which mediate the various biological functions that result from ligand binding.



heterotrimeric G protein

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals, Solutions and Buffers

2.1.1 General chemicals

The following chemicals were obtained from Sigma Australia (Castle Hill, NSW): dimethylsulphoxide (DMSO), Saponin, polyoxyethylene sorbitan monolaurate (Tween-20), p-phenylenediamine (PPD), β-Mercaptoethanol and diethylpyrocarbonate (DEPC).

The following chemicals were obtained from BDH Chemicals (Kilsyth, Vic): sodium chloride (NaCl), disodium hydrogen orthophosphate (Na_2HPO_4), potassium dihydrogen orthophosphate (KH_2PO_4), D-glucose, ethylenediaminotetra-acetic acid (EDTA), calcium chloride ($CaCl_2$), paraformaldehyde (PFA), dimethylformamide (DMF), sodium hydroxide (NaOH), methanol, ethanol, chloroform, propan-2-ol (isopropanol), glacial acetic acid and hydrochloric acid (HCl).

The following chemicals were obtained from Ajax Chemicals (Auburn, NSW): potassium chloride (KCl), magnesium chloride ($MgCl_2$), sodium azide (NaN_3) sodium carbonate (Na_2CO_3), sodium hydrogen carbonate ($NaHCO_3$), glycerol and acetone.

Tris hydrochloride (Tris-HCl) was obtained from ICN Biomedicals Australasia (Seven Hills, NSW).

The sources of specialised reagents are stated at the relevant place in the text.

2.1.2 Hank's Balanced Salt Solution (HBSS)

The following reagents were dissolved in Milli-Q water to generate 10X stocks: 80g/L NaCl, 4g/L KCl, 0.32g/L Na_2HPO_4, 0.6g/L KH_2PO_4 and 10g/L D-glucose and the solution sterilised by autoclaving. When required for use, the solution was diluted to 1X in Milli-Q water and HEPES buffer (pH 7.4) added to a final concentration of 0.01M.

2.1.3 Dextran solution

Dextran (Amersham Pharmacia Biotech Australia, Castle Hill, NSW) was dissolved to 2% (w/v) in HBSS by gentle stirring at RT, and sterilised by filtration through a 0.2μM membrane (Nalgene, Rochester, NY, USA).

2.1.4 Phosphate buffered saline (PBS)

The following reagents were dissolved in Milli-Q water: 8g/L NaCl, 0.2g/L KCl, 1.15g/L Na_2HPO_4, 0.2g/L KH_2PO_4 and the solution sterilised by autoclaving.

2.1.5 PBS/EDTA

EDTA was dissolved to 10mM (3.72 g/L) in PBS and the solution sterilised by autoclaving.

2.1.6 FITC-labelling buffer

The following reagents were dissolved in Milli-Q water: 8.6g/L Na_2CO_3 and 17.2g/L $NaHCO_3$. The pH was then adjusted to 9.5.

2.1.7 Biotin labelling buffer

The following reagents were dissolved in Milli-Q water: 8.4g/L $NaHCO_3$ and 5.84g/L NaCl. The pH was then adjusted to 8.4.

2.1.8 ELISA coating buffer

$NaHCO_3$ was dissolved in Milli-Q water to a concentration of 0.1M, the pH was adjusted to 9.6 and the solution sterilised by autoclaving.

2.1.9 PBS/Tween

Tween-20 (polyoxyethylene sorbitan monolaurate) was added to PBS to a final concentration of 0.2% (w/v) and the solution mixed thoroughly.

2.1.10 Mowiol mounting medium

Mowiol mounting medium base was purchased from Calbiochem (La Jolla, CA, USA) and prepared as follows: 2.4g of Mowiol was mixed with 6g of glycerol, 6ml of Milli-Q water and 12ml of 0.2M Tris-HCl (pH 8.5). The mixture was gently stirred at room temperature (RT) for 4 hours, incubated at RT for 2 hours without mixing, and then incubated in a 50°C water bath for 10 minutes. Any undissolved powder was then pelleted by centrifuging for 15 minutes at 5000 *x g* and aliquots of the supernatant stored at –20°C. When required, an aliquot was thawed and PPD was added as an anti-fade agent (a saturated solution of PPD was prepared in absolute ethanol and this solution diluted 1:50 into the mounting medium immediately prior to use).

2.1.11 Human staining buffer for flow cytometry

PBS was mixed with 2% heat-inactivated human AB serum and 0.04% NaN_3 (both w/v) and stored at 4°C.

2.1.12 Mouse staining buffer for flow cytometry

PBS was mixed with 1% BSA and 0.04% NaN_3 (both w/v) and stored at 4°C.

2.1.13 Protein-free staining buffer for flow cytometry

PBS was mixed with 0.04% NaN_3 (w/v) and stored at 4°C.

2.1.14 DNase solution for BrdU labelling

Stock solutions of DNase were prepared by dissolving DNase I (Sigma) to 5000 U/ml in a solution of 4.2mM $MgCl_2$ + 0.15mM NaCl in Milli-Q water (pH 5.0). Aliquots of the stock solution were stored at –20°C. When required, an aliquot of stock solution was thawed and diluted to 50 units/ml in 4.2mM $MgCl_2$ + 0.15mM NaCl.

2.1.15 Annexin binding buffer

The following reagents were dissolved in Milli-Q water: 8.78g/L NaCl, 0.38g/L KCl, 0.2g/L $MgCl_2$, 2.0g/L $CaCl_2$ and the solution sterilised by autoclaving. HEPES (pH 7.4) was then added to a final concentration of 10mM.

2.1.16 DEPC-treated water

DEPC was diluted to 0.1% (vol/vol) in Milli-Q water, incubated overnight at RT and then autoclaved.

2.1.17 Tris/acetic acid/EDTA (TAE)

A 20X stock solution was prepared by dissolving 96.8 g/L Tris and 14.88 g/L EDTA in Milli-Q water, followed by the addition of 22.8 ml/L of glacial acetic acid. The solution was autoclaved and diluted to 1X working concentration in Milli-Q water when required for use.

2.2 Primary cell isolation

2.2.1 Isolation of PBMCs from humans

Venous blood was collected from healthy volunteers into lithium-heparin vacuum tubes (Greiner Labortechnik, Frickenhausen, Germany) and the erythrocytes removed by sedimentation in a ½ volume of dextran solution for 30 minutes. Following sedimentation, the upper fraction was transferred to a clean tube and underlaid with a ½ volume of Ficol-Paque (Amersham Pharmacia Biotech). Tubes were centrifuged for 30 minutes at 1500rpm (no brake) and the mononuclear cells collected from the interface and washed in 50ml of HBSS.

2.2.2 Preparation of single cell suspensions from mouse lymphoid organs

2.2.2.1 Splenocytes

Mice were sacrificed by carbon dioxide asphyxiation and spleens removed by blunt dissection. Spleens were placed in a small volume of RPMI-5%FCS (see Section 2.2.3) in a 24-well polystyrene tray (BD Falcon™, BD Biosciences, Franklin Lakes, NJ, USA) and cut into small pieces using scissors, followed by gentle homogenisation with a glass plunger. The resulting homogenate was then filtered through a column of

cotton wool packed loosely into a pasteur pipette, to remove clumps of structural tissue.

2.2.2.2 LN cells and thymocytes

Mice were sacrificed by carbon dioxide asphyxiation and LNs and/or thymi removed by blunt dissection. Organs were placed individually onto nylon cell strainers resting in a 35-mm tissue culture dish (both from BD Falcon™) containing ~1ml of RPMI-5%. The capsule was cut with scissors and lymphocytes released into the dish by gentle homogenisation with the plunger of a 3ml syringe.

2.3 Cell culture

2.3.1 Culture media

2.3.1.1 Serum

Foetal calf serum (FCS) was obtained from Trace Scientific (Noble Park, VIC) and was heat-inactivated by incubation at 55°C for 1 hour. Human AB serum was obtained from the Red Cross (Adelaide, SA); serum from 6 different donors was pooled and then heat-inactivated at 55°C for 1 hour.

2.3.1.2 IMDM-5%FCS

The culture medium referred to throughout the text as IMDM-5%FCS was as follows: IMDM (Iscove's modified Dulbecco's medium; Life Technologies, Melbourne, VIC) containing 50μM β-mercaptoethanol, 2mM L-glutamine, penicillin + gentamycin supplement (both from the Infectious Diseases Laboratories Media Production Unit,

Institute for Medical and Veterinary Science (IMVS), Adelaide, SA) and 5% heat-inactivated FCS.

2.3.1.3 RPMI-10%huAB

The culture medium referred to throughout the text as RPMI-10%huAB was as follows: RPMI supplemented with 10mM HEPES, 2mM L-glutamine, penicillin + gentamycin and 10% heat-inactivated pooled human AB serum. The serum was obtained from the Red Cross, while all other reagents were obtained from the Infectious Diseases Laboratories Media Production Unit, IMVS.

2.3.1.4 RPMI-5%FCS

The culture medium referred to throughout the text as RPMI-5%FCS was as follows: RPMI supplemented with 10mM HEPES, 2mM L-glutamine, penicillin + gentamycin supplement (all obtained from the Infectious Diseases Laboratories Media Production Unit, IMVS), 50μM β-mercaptoethanol and 5% heat-inactivated FCS.

2.3.1.5 RPMI-BSA

The culture medium used for Transwell chemotaxis assays was RPMI containing 0.5% (w/v) bovine serum albumin (BSA; Sigma).

2.3.2 Maintenance of the tsDC cell line

The tsDC cell line was generously provided by Dr Brigitta Stockinger, Division of Molecular Immunology, National Institute for Medical Research, London. Cells were routinely grown at 33°C in a humidified atmosphere with 5% CO_2. The culture

medium was IMDM-5%. The cells grow as a heterogeneous population, ranging from non-adherent to moderately adherent. To remove cells from flasks for passaging or use in experiments, the medium containing floating cells was transferred to a centrifuge tube, the flasks rinsed with PBS and this liquid added to the centrifuge tube. Sufficient PBS/EDTA was added to cover the bottom of the flasks and the flasks incubated for 10 minutes at 33°C, followed by vigorous tapping to dislodge any remaining adherent cells. These detached cells were then pooled with the floating cells collected earlier. In general, cells were passaged approximately 1:3 once per week. In order to stop expression of the viral antigen used to immortalize these cells and thereby induce them to differentiate, the culture temperature was increased to the non-permissive temperature of 39°C for 24 hours.

2.3.3 Human mixed leukocyte reaction (MLR)

PBMC were resuspended to 1.5 x 10^6 viable cells/ml in RPMI-10%huAB. Stimulator cells were prepared from each donor by γ-irradiation (3000 rad; model IBL 437; Cis Bio international, Gif sur Yvette, France), and then mixed in a 1:1 ratio with non-irradiated responder cells to give either allogeneic or syngeneic cultures. Cells were aliquoted into 96-well trays or 24-well trays, in a 200μl or 2ml volume, respectively and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 3-12 days.

2.3.4 Murine MLR

Responder splenocytes were prepared from BALB/c mice and resuspended to 2 x 10^6 viable cells/ml in RPMI-5%. Two alternative sources of stimulator cells were used. The first was splenocytes from [BALB/c x CBA] F1 mice, which were resuspended to 4 x 10^6 viable cells/ml RPMI-5%. The second was tsDC cells, which had been

differentiated at the non-permissive temperature of 39°C for 24 hours previously (see Section 2.3.2). The cells were detached from the culture flask, washed and resuspended to 1 x 10^5 viable cells/ml in RPMI-5%. Responder cells were subsequently mixed with an equal volume of the appropriate stimulator cell population and aliquoted into 96-well trays or 24-well trays, in a 200µl or 2ml volume, respectively. Cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 5 days (for detection of proliferation) or 7 days (for analysis of chemokine receptor expression).

2.4 In vitro assays of cell function

2.4.1 Viable cell counts

Cells were routinely enumerated by diluting in 0.8% trypan blue (Sigma), followed by counting on a haemocytometer (Improved Neubauer, Weber, UK). At least 100 cells were counted per sample.

2.4.2 Analysis of cellular proliferation by incorporation of ^{3}H-thymidine

In order to quantify proliferation of cells in 96-well trays, 1µCi of [*methyl*-^{3}H]-Thymidine (25 Ci/mmol, containing 10% ethanol; Amersham Pharmacia Biotech) was added to each well in a 50µl volume and 18 hours later, the cells harvested onto glass fibre filter mats (Skatron Instruments, Molecular Devices, Sunnyvale, CA, USA) using a Skatron semi-automatic cell harvester. Disks were transferred to plastic vials and ~1ml of OptiPhase Hi-Safe scintillation fluid added (Wallac Scintillation Products, Turku, Finland). Tritium incorporation was then determined by scintillation counting

in a Beckman LS 6000TA liquid scintillation system (Beckman Coulter, Fullerton, CA, USA).

2.4.3 Analysis of cell division by carboxyfluorescein diacetate succinimidyl ester (CFSE) dye dilution

Cell division in the human MLR was assessed fluorometrically by labelling cells with CFSE prior to culture. PBMC were resuspended to 2 x 10^7 cells/ml in HBSS containing 0.1% human AB serum, and CFSE (Molecular Probes, Eugene, OR) was added to a final concentration of 10μM. After incubation at 37°C for 10 minutes, the staining reaction was quenched by the addition of a large volume of complete medium (RPMI-10% huAB) for 5 minutes, followed by 2 washes in the same medium. After the required time in culture, cells were analysed by flow cytometry (in conjunction with surface phenotyping) and cell division defined as a progressive two-fold loss in CFSE fluorescence intensity.

2.4.4 Assessment of apoptosis/cell death using Annexin-V and ethidium monoazide bromide (EMA)

Cell were labelled with EMA (Molecular Probes) by adding 5μl of a 50μg/ml solution to 50μl of cells at 4 x 10^6 cells/ml in a round-bottomed polystyrene tube (BD Falcon™ #352008). Samples were incubated on ice for 15 minutes, ~20 cm from a fluorescent light source, enabling cross-linking of the dye to the DNA. Cells were then washed and resuspended to 4 x 10^6 ceils/ml in annexin binding buffer, and 2μl of undiluted annexin-V:fluorescein conjugate (Boehringer Mannheim, Roche Diagnostics, Indianapolis, IN, USA) added per sample. Following a 20-minute incubation at RT, samples were washed with 3ml of annexin binding buffer and fixed in 200μl of PFA

(1% w/v solution in annexin binding buffer). Cells were then analysed by flow cytometry. Apoptotic cells were defined as positive for annexin-V but negative for EMA, dead cells were defined as positive for both annexin-V and EMA, and viable cells were defined as negative for both annexin-V and EMA.

2.4.5 Cytotoxicity assay

The target cells for this assay were tsDC cells grown at 33°C. Cells were detached as described in Section 2.3.2, resuspended in PBS and labelled with the fluorescent dye Calcein (2μM final concentration; Molecular Probes) in a 37°C water bath for 40 minutes. Following two washes in PBS, sub-confluent monolayers of labelled target cells were generated in 96-well trays by aliquoting 5 x 10^4 viable tsDC cells into each well in a 100μl volume. The cells were then allowed to adhere for at least 5 hours, after which time the medium was aspirated and 200μl of lymphocytes in RPMI-5%FCS were added. In preliminary experiments, a range of effector cell concentrations were tested and on the basis of these studies, subsequent experiments used 5 x 10^5 cells/well. The assay was incubated for 48 hours at 37°C in a humidified atmosphere with 5% CO_2, then the culture medium aspirated and lymphocytes and target cell debris washed away by immersing the trays in four consecutive baths of PBS. Subsequently, 100μl of PBS were added to each well and the degree of target cell monolayer remaining in each well was quantified by measuring fluorescence emission at 520nm on a Molecular Imager FX with Quantity One software (Biorad Laboratories, Hercules, CA, USA). The percent cytotoxicity was calculated using the following formula:

$$100 \times (1 - [(Em_{520} - \text{background}) / (\text{non-sensitised } Em_{520} - \text{background})])$$

where Em_{520} was the reading for test wells, the *background* value was the reading for wells that had lymphocytes but no target cells, and *non-sensitised* Em_{520} was the reading for wells containing tsDC targets and LN cells obtained from a non-sensitised mouse. The use of the non-sensitised LN cells as a background control was found to be critical, as the presence of lymphocytes appeared to non-specifically enhance the survival of the target cells. Thus, the integrity of the tsDC monolayer was higher when the cells were co-incubated with LN cells, compared to incubation of tsDCs alone. Hence, normalisation of the data was required to counteract this effect. The non-sensitised LN cells were pooled from popliteal, brachial, axillary, inguinal and cervical LNs.

2.4.6 Transwell® chemotaxis assay

Cells were resuspended to 5 x 10^6 viable cells/ml in RPMI-BSA. Synthetic chemokines (a kind gift of Prof. I. Clark-Lewis, Biomedical Research Center, University of British Columbia, Vancouver, Canada) were diluted to the required concentration in RPMI-BSA and 600µl added to the lower chambers of a Transwell® plate (6.5mm diameter filter, 5µm pore size; Corning, NY, USA). After adding 100µl of cells to the upper chambers, the assay was incubated for 3 hours at 37°C and cells were collected from the lower chamber after extensive PBS washing of the filter underside with a plastic transfer pipette. In most experiments, the total number of cells in the lower chamber was quantified by duplicate haemocytometer counts (see Section 2.4.1) and this value divided by the number of input cells to calculate percentage migration. To correct for any variations in spontaneous migration, the data is expressed

as migration index (MI), calculated by dividing the percentage migration value obtained in the presence of chemokine by the percentage migration value for negative controls (no chemokine).

For the dose-response studies using MIP-3α/CCL20, cells were first fluorescently labelled by incubating with Calcein (Molecular Probes; 40nM final concentration in RPMI-BSA) for 30 minutes at 37°C, followed by three washes in RPMI-BSA. Labelled cells were used in Transwell assays as described above and then the cells in the lower chamber quantified by transferring to a 96-well microtitre tray and measuring fluorescent emission on the Molecular Imager FX using Quantity One software. Following quantification, cells were labelled with anti-CD4 and analysed by flow cytometry, as described in Section 2.5.6. The percentage migration of $CD4^+$ T cells was calculated by multiplying each Em_{520} value by the percentage of cells positive for CD4 in that sample, then inserting the values into the following formula:

$$(Y - Y_{min})/Y_{max})$$

where Y_{min} was the value obtained in the absence of chemokine, Y_{max} was the value obtained for 100µl of cells added directly to the lower chamber of the Transwell and Y was the value obtained from the test sample.

2.5 Immunostaining of cells and tissue sections

2.5.1 Preparation of footpad tissue sections

To prepare footpad tissue for immunohistological staining, mice were sacrificed by carbon dioxide asphyxiation and the footpad tissue removed by slicing the soft tissue

away from the metatarsal bones using a scalpel and forceps. Samples were immersed in a plastic mould containing Tissue-Tek® OCT embedding medium (Sakura Finetek, Torrance, CA, USA) and frozen using the Gentle Jane snap-freezing system (Instrumedics Inc., Hackensack, NJ, USA). Blocks were stored at –70°C until sectioning. Six micron cryostat sections were cut from embedded tissue using a BRIGHT cryostat (Huntingdon, UK), air-dried onto microscope slides, fixed for 7 minutes in 60% acetone/40% methanol, briefly air-dried and then frozen at -20°C with desiccant.

2.5.2 Preparation of cytospin smears

Cells were resuspended to 1 x 10^6 viable cells/ml, a 100µl aliquot was layered over 50µl of FCS and the mixture spun onto glass slides at 800rpm for 8 minutes using a Shandon Cytospin (Pittsburgh, PA, USA). Slides were allowed to air-dry before being frozen with desiccant at -20°C.

2.5.3 Antibodies and detection reagents

2.5.3.1 Antibodies to human molecules

Antibodies to CD3 (clone OKT3), CD4 (clone OKT4) and CD8 (clone OKT8) were a kind gift of Dr R. Roy (Centre de Recherche en Rheumatologie et Immunologie, Universte Laval, Quebec, Canada). Where required, these antibodies were labelled with fluorescein or biotin, as described below. Anti-CCR5 (clone 2D7) was purchased from Pharmingen (San Diego, CA, USA) in both an unlabelled and a phycoerythrin (PE)-conjugated format. Anti-CCR1 (clone 53504.111), anti-CCR3 (clone 61828.111), anti-CCR6 (clone 53103.111), anti-CXCR3 (clone 49801.111), anti-CXCR4 (clone

44716.111) and anti-CXCR5 (clone 51505.111) were purchased from R&D Systems (Minneapolis, MN, USA). The antibodies against CCR1, CXCR4 and CXCR5 were purchased in a biotinylated form, the antibodies against CCR3 and CXCR3 were labelled with biotin (as described below), while anti-CCR6 was used in either a PE-conjugated or an unconjugated format, depending on the experiment. PE-conjugated antibodies to CD45RA (clone F8-11-13) and CD45RO (clone UCHL1) were obtained from Serotec (Oxford, UK), and PE-conjugated anti-CD25 (clone M-A251) and anti-CD69 (clone FN50) were obtained from Pharmingen. Hybridoma cell lines producing mouse isotype control immunoglobulins were a kind gift of Prof. H. Zola (Child Health Research Institute, Adelaide, SA). Immunoglobulin-containing supernatant was collected from these cells lines using standard tissue culture techniques and the IgG purified by Protein G:sepharose affinity chromatography, according to the manufacturer's protocol (Amersham Pharmacia Biotech). Where appropriate, these antibodies were labelled with fluorescein or biotin, as described below. The PE-conjugated negative control was obtained from Serotec (clone OX34).

2.5.3.2 Antibodies to murine proteins

Goat polyclonal antibodies reactive against murine CCR5, CXCR3 and CXCR4, as well as control goat IgG, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-CD4 conjugated to fluorescein (clone GK1.5) or PE (clone H129.19), and anti-CD8 conjugated biotin (clone 53-5.8) were all purchased from Pharmingen.

2.5.3.3 Detection reagents

Secondary antibodies for flow cytometry (anti-mouse:PE, anti-mouse:biotin and anti-goat:biotin) were obtained from Rockland Immunochemicals (Gilbertsville, PA, USA)

Streptavidin conjugated to fluorescein, PE or PECy5 was obtained from Rockland Immunochemicals for the majority of studies; however, for some studies a streptavidin:PECy5 conjugate was obtained from Caltag Laboratories (Burlingame, CA, USA) due to the Rockland product being discontinued. Anti-goat secondary antibody conjugated to Alexa 594 and streptavidin conjugated to Alexa 350 (for fluorescence microscopy) were obtained from Molecular Probes.

2.5.3.4 Labelling antibodies with fluorescein or biotin

For labelling with fluorescein, the antibody was dialysed (using ¼ inch dialysis tubing, molecular weight exclusion limit 12,000 – 14,000 daltons, Life Technologies) against 2 x 1L changes of FITC-labelling buffer and the antibody concentration subsequently adjusted to 1 – 4 mg/ml. Fluorescein isothiocyanate (FITC; Sigma) was dissolved to 1mg/ml in anhydrous DMSO and added drop-wise to the antibody, at the ratio of 75µl of FITC solution per 1mg of antibody. The coupling reaction was allowed to proceed at RT for 2 hours in the dark, with gentle mixing. Unbound FITC was subsequently removed by dialysis against 2 x 1L changes of PBS.

The concentration of labelled antibody was determined by measuring absorbance at 280 and 492nm and performing the following calculation:

$$\text{mg/ml} = A_{280} - \frac{(A_{492} \times 0.35)}{1.4}$$

The fluorochrome:protein ratio was subsequently determined using the following calculations:

$$\text{fluorochrome:protein ratio} = \frac{\text{moles of FITC}}{\text{moles of antibody}}, \text{ where...}$$

$$\text{moles antibody} = \frac{\text{mg/ml labelled antibody}}{1.5 \times 10^5}$$

$$\text{moles FITC} = \frac{A_{492}}{0.69 \times 10^5}$$

For labelling with biotin, the antibody was dialysed against 2 x 1L changes of biotin labelling buffer and the antibody concentration subsequently adjusted to 1 – 4 mg/ml. Aminohexanoyl-Biotin-N-Hydroxysuccinimide ester (Zymed Laboratories, San Francisco, CA, USA) was dissolved in anhydrous DMF to 10mg/ml and added to the antibody at the ratio of 10µl of biotin solution per 1mg of antibody. The reaction was allowed to proceed for 2 hours at RT with gentle mixing. Unbound biotin was subsequently removed by extensive dialysis against 2 x 1L changes of PBS.

The success of the biotinylation reaction was determined using a one-site enzyme-linked immunosorbent assay (ELISA). Labelled antibody was diluted in coating buffer, added to the wells of a 96-well Costar® high-binding assay plate (Corning, #3590) and incubated overnight at 4°C. Wells were washed twice with PBS/Tween and non-specific binding sites blocked by incubation with PBS + 3% BSA (Sigma) at 37°C for 2 hours. After washing the wells once with PBS/Tween, streptavidin-horseradish peroxidase conjugate (Rockland) was added and the plate incubated for 30 minutes at RT. The wells were washed four times with PBS/Tween and the peroxidase reaction developed by the addition of 200µl/well *Fast™* OPD substrate (Sigma). When appropriate, the reaction was terminated by the addition of 50µl of 3M HCl and absorbance readings taken at 485nm on a Biolumin™-960 96-well plate reader, using Xperiment™ software (Molecular Dynamics, Melbourne, VIC).

2.5.4 Immunofluorescence staining of tissue sections (murine)

Sections were isolated using a hydrophobic pen (Pap Pen, Zymed Laboratories), and subsequently rehydrated in PBS for 5 minutes. Non-specific binding was then blocked by covering samples with Powerblock® (InnoGenex, San Ramon, CA) and incubating at RT for 1 hour. After rinsing off the blocking agent in PBS, 50µl of primary antibody mix (the appropriate goat polyclonal antibody plus biotinylated anti-CD4) was added to the samples. After overnight incubation at 4°C, the sections were washed with 2 changes of PBS (2 minutes each), followed by the addition of a secondary antibody/streptavidin mix. This mix consisted of anti-goat secondary antibody conjugated to Alexa 594, plus streptavidin conjugated to Alexa 350. Any potential cross-reactivity of the anti-goat secondary for rat and mouse immunoglobulins was prevented by pre-blocking the secondary antibody with 400µg/ml of rat gamma-globulin and 200µg/ml of mouse gamma-globulin (Rockland), in the presence of 2% normal mouse serum. Following a 1 hour incubation at 4°C, the sections were washed through 3 changes of PBS (2 minutes each) and coverslipped with Mowiol mounting medium + PPD. All incubations were carried out in a humid chamber. Following staining, slides were viewed and photographed using an Olympus Provis AX reflected light fluorescence microscope (Melville, NY, USA) equipped with a Photometrics CE 200A digital camera (Auckland, NZ), using V for Windows software (Photometrics; version 3.5r) for image capture.

2.5.5 Immunofluorescence staining of permeabilised cytospin preparations (human)

Frozen cytospin preparations (Section 2.5.2) were equilibrated to RT and the relevant area on the slide isolated using a hydrophobic pen. Samples were fixed with PFA (4%

in PBS) for 20 minutes at 4°C, and then washed in ice-cold PBS. Cells were then permeabilised by incubation in 0.1% saponin in PBS for 15 minutes at 4°C, washed, and non-specific binding was blocked by incubating in PBS containing 2% human AB serum and 200μg/ml purified human IgG (Sigma) for at least 15 minutes. The blocking reagent was aspirated and the primary antibody was added. Following a one-hour incubation at 4°C, the slides were washed, streptavidin:fluorescein added and the slides incubated for a further one hour. Following extensive washing, slides were coverslipped with Mowiol + PPD. All incubations were performed in the presence of 0.02% saponin to maintain complete permeabilisation and 2% human serum to reduce background binding, and were carried out in a humid chamber. Following staining, slides were viewed and photographed as described in Section 2.5.4.

2.5.6 Labelling human cells for flow cytometry

Cells were resuspended to 4 x 10^6 viable cells/ml in human staining buffer. Fc receptors were blocked by incubating for 30 minutes at room temperature with 50μg of human IgG (Sigma) per 10^6 cells, then 50μl of cells aliquoted into round-bottomed polystyrene tubes (BD Falcon™ #352008). For experiments in which one primary antibody was unlabelled, cells were mixed with saturating concentrations of the unlabelled primary antibody, incubated for 30 minutes at room temperature and washed once with 3ml of human staining buffer (ie, 3ml of buffer was added to the tube, cells were pelleted by centrifuging for 7 minutes at 350 *x g*, the supernatant was poured off and the cells resuspended in the remaining liquid), prior to the addition of biotin- or PE-conjugated anti-mouse detection antibody. Following a 30-minute incubation on ice, cells were washed and free binding sites on the detection antibody were blocked by incubation with 20μg of mouse gamma-globulin (Rockland) per tube

for 20 minutes at RT. Fluorescein- and/or PE-conjugated primary antibodies, as well as fluorochrome-conjugated streptavidin if required, were then added and incubated for 30 minutes on ice. Cells were washed with 3ml of human staining buffer followed by 3ml of protein-free staining buffer and fixed in 200µl of PFA (1% in PBS).

A slightly different approach was adopted when all primary antibodies were directly labelled with fluorochromes or biotin; cells were mixed with all of the required primary antibodies, incubated at RT for 30 minutes, washed, incubated with fluorochrome-conjugated streptavidin, then washed and fixed as described above.

2.5.7 Detection of intracellular chemokine receptor expression by human cells using flow cytometry

Cells were incubated in PFA (4% in PBS) for 1 hour on ice, then washed with human staining buffer and divided into two aliquots; the first was left untreated, while the second was incubated for 12 minutes in staining buffer containing 0.1% saponin. The cells were then washed with staining buffer and processed for flow cytometry as described above, with the exception that the permeabilised samples of cells were maintained in staining buffer containing 0.02% saponin, to ensure complete membrane permeabilization throughout the procedure.

2.5.8 Labelling murine cells for flow cytometry

2.5.8.1 Standard protocol

Cells were resuspended to 4 x 10^6 viable cells/ml in mouse staining buffer. Fc receptors were blocked by incubating for 30 minutes at RT with 50µg of murine

gamma-globulin (Rockland) per million cells, then 50µl of cells aliquoted into round-bottomed polystyrene tubes (BD Falcon™ #352008). Cells were mixed with saturating concentrations (as determined by titration experiments) of the goat primary antibody, incubated for 30 minutes at RT and washed once with 3ml of mouse staining buffer prior to the addition of biotin-conjugated anti-goat detection antibody. Following a 30-minute incubation on ice, cells were washed with mouse staining buffer and free binding sites on the detection antibody were blocked by incubation with 20µg of goat IgG (Laboratory Animal Services, Adelaide, SA) per tube for 20 minutes at RT. Fluorochrome-conjugated primary antibodies, as well as fluorochrome-conjugated streptavidin, were then added and incubated for 30 minutes on ice. Cells were washed with 3ml of mouse staining buffer followed by 3ml of protein-free staining buffer and fixed in 200µl of PFA (1% in PBS).

2.5.8.2 Modified protocol for the detection of 5-bromo-2-deoxyuridine (BrdU) incorporation

Cells were prepared, blocked with gamma-globulin and labelled for surface antigens, as for the standard protocol (Section 2.5.9.1). After washing in PBS, the cell pellets were resuspended in 500µl of ice-cold NaCl (0.15M). While gently vortexing, 1.2ml of ice-cold 95% (vol/vol) ethanol were added in a drop-wise manner, and the cells incubated on ice for 30 minutes. To wash, 2ml of PBS were added and the cells pelleted by centrifuging at 450 *x g*. Cell pellets were resuspended in 500µl of PFA/Tween (1% PFA + 0.01% Tween in PBS), incubated at room temperature for 30 minutes and subsequently pelleted by centrifugation. In order to generate single-stranded DNA (a prerequisite for the detection of incorporated BrdU), cells were incubated in 1ml of DNase solution at 37°C for 30 minutes, and then washed in 2ml of

PBS. After decanting the supernatant, cell pellets were resuspended in the remaining volume, and 10µl of fluorescein-conjugated anti-BrdU (Becton Dickinson Immunocytometry, San Jose, CA) was added. After incubation at RT for 30 minutes, the cells were washed in 2ml of PBS and resuspended in 200µl of PBS, prior to flow cytometric analysis.

2.5.9 Flow cytometric analysis

Labelled cells were analysed on a Becton Dickinson FACScan and data analysed using CellQuest Pro software (BD Biosciences). For all analyses, lymphocytes (or, in some experiments, monocytes) were gated using forward and side angle light scatter characteristics. Positive events were defined on the basis of quadrant or histogram markers, which were set according to the level of background staining observed using isotype-matched control antibodies. All percentage values presented in the data have been corrected for background staining, by subtracting the percentage of events defined as positive by the markers in relevant control samples (generally <1% for monoclonal antibodies, <5% for polyclonal antibodies).

2.6 Murine *in vivo* assays

2.6.1 Animals

Specific-pathogen free mice were obtained from the Central Animal House of the University of Adelaide, and housed under conventional conditions at the University, where they were provided with food and water *ad libitum*. The strains used were

BALB/c and the F1 progeny obtained from BALB/c x CBA breedings. All mice were female, and used at 6 - 10 weeks of age.

2.6.2 Induction of a local immune response to allogeneic DCs (tsDC)

Confluent flasks of the tsDC cell line were differentiated at 39°C for 24 hours, then detached from the surface of the flask as described in Section 2.3.2. Cells were washed and resuspended to 2 x 10^7 viable cells/ml in endotoxin-free PBS (Infectious Diseases Laboratories Media Production Unit, IMVS). Using a 0.5ml insulin syringe with 29-gauge needle (Becton Dickinson), 25µl of cells were injected sub-cutaneously (s.c.) per footpad. Control footpads received 25µl of endotoxin-free PBS. Each footpad/popliteal LN was treated as an independent event, on the basis of preliminary studies indicating that immunisation in one footpad did not induce a proliferative response in the contralateral popliteal LN (not shown), and on the basis of previous studies (for example, ref (159)).

If required, cells were labelled with CFSE prior to injection as follows: after detaching from flasks, cells were resuspended at 2 x 10^7 cells/ml in PBS containing 0.1% FCS, and CFSE was added to a final concentration of 10µM. After incubation at 37°C for 10 minutes, the staining reaction was quenched by the addition of a large volume of complete medium for 5 minutes, followed by 2 washes in endotoxin-free PBS.

2.6.3 Detection of cellular proliferation by BrdU incorporation

BrdU (Sigma) was dissolved to 4mg/ml in endotoxin-free PBS. On the day prior to experimental end-point, mice received two intraperitoneal (i.p.) injections of 0.2ml each, 8 hours apart.

2.7 Analysis of chemokine receptor expression by reverse-transcriptase polymerase chain reaction (RT-PCR)

2.7.1 RNA extraction from cell pellets

Following culture in the MLR, cells were pelleted, resuspended in 1ml of Trizol (Life Technologies) and stored at –70°C until required. To extract RNA, the cell/Trizol mixture was thawed and then incubated at RT for 5 minutes prior to the addition of 200µl of chloroform. After vigorous shaking, the mixture was incubated for a further 2-3 minutes at RT. Centrifugation at 12,000 *x g* for 15 minutes at 4°C generated two phases. The upper aqueous phase was transferred to a clean reaction tube and the extraction of remaining RNA from the lower phase was repeated with 400µl of DEPC-treated water. RNA from both extractions was precipitated separately by the addition of 500µl of isopropanol, followed by a 10-minute incubation at RT. The precipitate was then centrifuged at 12,000 *x g* for 10 minutes at 4°C. The pellet was washed in 70% ethanol and air-dried for approximately 15 minutes. RNA was dissolved in 20µl of DEPC-treated water and incubated at 55°C for 10 minutes to ensure complete resuspension. RNA purity was determined by measuring optical density at 260nm and 280nm, and calculated using the following formula:

$$[\text{purity} = OD_{260}/OD_{280}].$$

The concentration of RNA was calculated using the following formula:

[concentration (μg/μl) = OD_{260} x dilution factor x 0.04].

2.7.2 DNase I treatment of RNA

RNA was treated with DNase I to remove any contaminating chromosomal DNA. Each reaction was set up as follows, using RNase-free DNase I and the associated buffers as provided (Promega, Madison, WI, USA). RNA (5μg) was diluted to a final volume of 17μl in DEPC-treated water, to which 2μl of 10X reaction buffer and 1μl of enzyme were added. Following incubation at 37°C for 1 hour, the reaction was terminated by the addition of 2μl of 10X stop buffer and heat-inactivation at 65°C for 20 minutes.

2.7.3 Reverse-transcription

Generation of first strand cDNA from RNA was conducted using Superscript II reverse-transcriptase and the associated buffer and dithiothreitol (DTT) reagent as provided (Life Technologies). Each reaction was set up as follows: 1μl of oligo-dT15 primers (500μg/ml; Promega) was combined with 2.5μg of total RNA and the volume adjusted to 12μl with DEPC-treated water. The mixture was heated to 70°C for 10 minutes, then immediately cooled to 4°C and the following reagents added: 4μl of first strand buffer, 2μl of DTT and 1μl of 10mM dNTP mix (10mM each dATP, dTTP, dCTP, dGTP diluted in DEPC-treated water; Amersham Pharmacia Biotech). The contents of the tube were mixed gently, centrifuged briefly and incubated for 2 minutes at 42°C. Finally, 1μl of Superscript II (200 U/μL) was added and the reaction allowed

to proceed at 42°C for 50 minutes before being terminated by heating to 70°C for 15 minutes. The cDNA products were stored at –20°C until further use.

2.7.4 Oligonucleotide primers

Primers were synthesised and purified by reverse-phase chromatography at Geneworks (Adelaide, SA). Primers were received in a lyophilised form, diluted in sterile Milli-Q water and stored at –20°C.

Primer sequences designed to amplify human targets were as follows: *CCR5*: 5'-TGA CAT CTA CCT GCT CAA CC-3' and 5'-CCT GTG CCT CTT CTT CTC AT-3'; *CCR6*: 5'-TCA TCT GCC TTG TTG TGT GG-3' and 5'-TGT CGT TAT CTG CGG TCT CA-3'. *CXCR3*: 5'- ACC TAG CTG TAG CAG ACA CG -3' and 5'- CAT AGC AGT AGG CCA TGA CC -3'; *CXCR4*: 5'- CCA CCA TCT ACT CCA TCA TC -3' and 5'- GGT TCA GAC AAC AGT GGA AG -3'.

Primers designed to amplify murine CCR6 were as follows: 5' – GAC TGA CGT CTA CCT GTT GA – 3' and 5' – GGC TCT GAG ACA GAC CTG TA – 3'.

The same GAPDH primers were used to detect housekeeping gene expression in both human and murine samples. The sequences of these primers were as follows: 5'-TCC TTG GAG GCC ATG TAG GCC AT-3' and 5'-TGA TGA CAT CAA GAA GGT GGT GAA G-3'.

2.7.5 Amplification of target sequences using PCR

PCR reactions were set up using Amplitaq Gold polymerase (Perkin Elmer Life Sciences, Boston, MA, USA) and the supplied buffer and $MgCl_2$. The following 25µl reaction was set up for each template with each primer set: 1.25µl of cDNA template was added to a 0.2ml reaction tube and heat-denatured at 95°C for 10 minutes. The temperature was then reduced to 4°C and the following reagents added: 5µl of each oligonucleotide primer at 5pmol/µl, 2.5µl of 10X reaction buffer, 1.25µl of 25mM $MgCl_2$, 0.5µl of 10mM dNTP mix (10mM each dATP, dTTP, dCTP, dGTP diluted in sterile Milli-Q water; Perkin Elmer), 9.25µl of sterile Milli-Q water and 0.5µl of Amplitaq Gold polymerase (Perkin Elmer). Reactions were cycled in a hot-bonnet thermal cycler as follows: (Step 1) 95°C 10 minutes; (Step 2) 95°C 30 seconds; (Step 3) 55°C 1 minute; (Step 4) 72°C 1 minute, with steps 2-4 repeated for the required number of cycles.

2.7.6 Analysis of PCR products by agarose gel electrophoresis

Two percent (w/v) agarose gels were prepared by boiling agarose in TAE. Gels were run in TAE at ~100 mA in a horizontal gel apparatus. Following electrophoresis, gels were stained with SYBR-gold (diluted to 1X concentration in TAE; Molecular Probes) for 15-30 minutes and visualised/analysed using the Molecular Imager FX and Quantity One software package (Biorad Laboratories, Hercules. CA). The band intensity values for each receptor were expressed as a ratio relative to band intensity for the GAPDH PCR product amplified from the same template.

2.8 Statistical tests

Statistical tests were performed using GraphPad InStat software (San Diego, CA, USA). The two-tailed, unpaired Student's t-test was used for most calculations. However, when comparing two data sets in which the standard errors were significantly different, the alternate (Welch's) t-test was used. For all analyses, p values of less than 0.05 were considered significant.

Summary - Chapter 3

- Following allogeneic activation in an MLR, the proportion of $CD4^+$ T cells expressing CCR5, CCR6 and CXCR3 increased significantly.
- In parallel, the proportion expressing CXCR4 decreased significantly.
- The proportion of $CD4^+$ T cells expressing CCR1, CCR2, CCR3 or CXCR5 was not significantly modulated upon allogeneic activation.
- Similar to the $CD4^+$ population, allo-activated $CD8^+$ T cells also lost the expression of CXCR4.
- However, in contrast to the $CD4^+$ cells, activated $CD8^+$ T cells did not acquire the expression of any new chemokine receptors.
- In all cases, the modulation of chemokine receptor expression was a relatively late event, peaking at a time-point at which cell division had largely ceased.

CHAPTER 3

IDENTIFICATION OF CHEMOKINE RECEPTORS THAT DEMONSTRATE ALTERED PATTERNS OF EXPRESSION ON T CELLS ACTIVATED IN THE MIXED LEUKOCYTE REACTION (MLR)

3.1 Introduction

At the time this project began, the regulation of chemokine receptor expression on T cells upon interaction with APC had not been explored. Furthermore, the only commercially available reagents with which to study chemokine receptors at the protein level were an incomplete range of antibodies to the human receptors, with no antibodies available to study the corresponding murine receptors. Although analyses of a wider range of receptors would have been made possible by studying mRNA levels, these approaches were avoided at this stage for a number of reasons. First, due to the readiness with which chemokine receptors are internalised (60), it is clear that the expression of receptor mRNA does not necessarily correspond to the expression of receptor protein on the cell surface, which is required for responsiveness to chemokine ligands. Second, it was possible that subtle changes in the level of gene expression, or changes on only a minor subset of cells, would not be detected. Third, this approach would not yield any information about the T cell subset/s undergoing the observed changes in chemokine receptor expression.

As it was not known which receptors would be modulated on T cells following antigenic stimulation, nor the time-frame within which this would occur, the first series of experiments consisted of time-course analyses using all commercially available antibodies reactive against human chemokine receptors that are likely to be expressed on T cells (CCR1, CCR2, CCR3, CCR5, CCR6, CXCR3, CXCR4 and CXCR5). Anti-chemokine receptor antibodies were tested in dual-parameter flow cytometric analysis in conjunction with antibodies to CD4 and CD8, to enable any effects seen to be ascribed to one or both of these functional T cell subsets. Thus, it was hoped that this 'screening' approach would yield preliminary data indicating which chemokine

receptors were modulated on either $CD4^+$ or $CD8^+$ T cells during interaction with APCs, thereby providing a focus for subsequent, more detailed, analyses.

As discussed previously, the way in which chemokine receptors are regulated on naïve T cells is likely to differ significantly from the way in which they are regulated on memory T cells, as the latter already express a range of inflammatory chemokine receptors and are therefore potentially already programmed to access peripheral sites. Thus, it was important that the model of T cell activation used in these studies resulted in the activation of naïve T cells, as the hypothesis of this project specifically pertains to the regulation of chemokine receptors on T cells upon their first encounter with antigen. Furthermore, it was important that the experimental model selected involved the presentation of antigen to T cells by APCs, as APC-independent mitogenic stimulation of T cells does not seem to affect the expression of the vast majority of chemokine receptors, as discussed in Chapter 1. Accordingly, the mixed leukocyte reaction (MLR) was selected for all *in vitro* studies.

The MLR is a culture system that involves mixing primary T cells with APCs from a genetically dissimilar individual. T cells respond to foreign (allogeneic) MHC-peptide complexes displayed on the surface of APCs, resulting in T cell activation, proliferation and acquisition of effector function, as for any other antigen-specific response. The unique feature of alloreactivity is the high frequency of alloreactive T cells occurring in the absence of specific immunisation. Thus, whereas the percentage of circulating T cells capable of responding to a nominal peptide antigen is in the range $10^{-6} - 10^{-5}$, the frequency of T cells capable of recognising alloantigen is 100 – 1000 times higher (160-162). Consequently, a readily measurable primary T cell response can be mounted *in vitro* using peripheral T cells from a non-immunised individual,

thus enabling the activation of naïve T cells to be studied. While memory T cells can also respond in an MLR (163), and thus a portion of the response measured is likely to be due to secondary activation of memory T cells, there is now considerable evidence that the majority of antigen presentation within an MLR can be ascribed to DC (4, 164-166). Thus, considering that DC are thought to be the only APCs capable of efficiently activating naïve T cells (4, 167), it is likely that the MLR quite closely resembles a primary immune response.

3.2 Results

3.2.1 Time-course of cellular proliferation

Initially, incorporation of [^{3}H]-thymidine was used to measure cellular proliferation at each of the time-points at which chemokine receptor expression was to be tested. This would allow any observed changes in chemokine receptor expression to be correlated with the timing of the proliferative response. MLR cultures were established by mixing peripheral blood mononuclear cells (PBMC) from one donor with irradiated PBMC from either the same donor (syngeneic; control) or an unrelated donor (allogeneic). Cultures were incubated for the indicated periods of time, with [^{3}H]-thymidine being added for the last 18 hours. As shown in Figure 3.1, syngeneic cultures did not mount a detectable proliferative response at any time-point, as expected. Allogeneic cultures had not incorporated detectable amounts of [^{3}H]-thymidine by day 3, but by day 6 a large proliferative response could be detected. The level of proliferation declined significantly between days 6 and 9, and was barely detectable by day 12.

3.2.2 Phenotype of proliferating cells

In order to determine which subsets of lymphocytes were proliferating in the MLR, cells were labelled with the fluorescent tracker dye CFSE prior to the initiation of culture. Cell division is associated with a reduction in CFSE fluorescence intensity, thus enabling divided and non-divided cells to be identified by flow cytometry, and the expression of cell surface molecules to be correlated with the division status of individual cells (168, 169).

The histogram [A] in Figure 3.2 illustrates the CFSE fluorescence intensity of lymphocytes from syngeneic cultures, which would not be expected to have divided. Accordingly, a single, uniform peak of fluorescence intensity can be seen, with less than 2% of cells displaying a reduced level of CFSE fluorescence intensity at the day 9 time-point. In comparison, lymphocytes from allogeneic cultures (Fig 3.2 [B]) display a clearly bimodal distribution of CFSE fluorescence intensity at day 9, indicating the presence of a population of divided cells containing reduced levels of the CFSE dye.

In order to determine the phenotype of the population of divided cells detected in allogeneic cultures, CFSE-labelled cells were collected after 9 days of culture in the MLR, stained with antibodies to CD3, CD4 or CD8 and analysed by dual-parameter flow cytometry (Fig 3.3). These experiments demonstrate that the majority (89.1 $\pm$ 3.6%) of divided cells were $CD3^+$, indicating that T cells are the major population undergoing proliferation in the MLR (Fig 3.3 [A] and [D]). Although not specifically examined, it is likely that the remainder of cells that divided in the MLR were NK cells, as monocytes and B cells were no longer detectable in allogeneic cultures by the day 9 time-point (not shown), and small numbers of NK cells have been shown previously to undergo proliferation in the MLR (170).

Staining with antibodies to CD4 and CD8 indicated that both populations underwent proliferation in the MLR (Fig 3.3 [B] – [C]), with an average of 51.5 ± 12 % and 43.9 ± 11.4 % of divided cells staining positive for CD4 or CD8, respectively. The relative contributions of the $CD4^+$ and $CD8^+$ T cell populations to cell division were variable, as although most experiments (including the one illustrated in Fig 3.3 [A] - [C]) were characterised by the preferential proliferation of $CD4^+$ T cells, in other experiments there were a greater number of $CD8^+$ T cells in the divided population (see Fig 3.3 [D]). Thus, although both $CD4^+$ and $CD8^+$ T cells divided in every MLR examined, the relative contribution of the two T cell subsets to the proliferative response varied somewhat, highlighting the variability inherent in an outbred human population.

3.2.3 Expression of CCR1

As illustrated in Figure 3.4, CCR1 was not detected on $CD4^+$ T cells at the initiation of culture (day 0). Furthermore, this receptor remained undetectable on $CD4^+$ T cells at each of the subsequent time-points examined (days 3, 6, 9 and 12), regardless of whether cells were taken from allogeneic or syngeneic cultures. Very similar results were obtained when $CD8^+$ T cells were examined; again, CCR1 was not detected at any time-point in either allogeneic or syngeneic cultures (Fig 3.5). For simplicity, only the days 0 and 9 time-points are shown in these figures.

Although the anti-CCR1 antibody was obtained from a prominent commercial supplier and was stored and used as recommended, the possibility remained that the antibody was for some reason non-functional. To demonstrate that the antibody was functional in flow cytometry assays, the level of staining with this antibody was assessed on the monocyte population of freshly isolated PBMC, in parallel with assessment of staining

on the lymphocyte population, by using differential gating during flow cytometric analysis. Figure 3.6 illustrates that, as expected, the fluorescence intensity of lymphocytes stained with the anti-CCR1 antibody was no different to that of lymphocytes stained with an isotype-matched control Ig. In contrast, however, the addition of anti-CCR1 resulted in a notable shift in fluorescence intensity within the monocyte population, demonstrating that this antibody was indeed functional.

3.2.4 Expression of CCR2

At the initiation of culture, CCR2 was expressed on a small proportion (< 15%) of both $CD4^+$ and $CD8^+$ T cells. However, by day 3 of culture, the level of expression of CCR2 had become undetectable, or barely detectable, on $CD4^+$ and $CD8^+$ T cells from both allogeneic and syngeneic cultures. Moreover, this lack of expression was maintained throughout the culture period, up to day 12. These patterns of expression are illustrated in the time-course analyses presented in Figure 3.7 ($CD4^+$ T cells) and Figure 3.9 ($CD8^+$ T cells), and in the representative density plots of day 0 and day 9 data in Figure 3.8 ($CD4^+$ T cells) and Figure 3.10 ($CD8^+$ T cells).

3.2.5 Expression of CCR3

Similar to the observations with CCR1, CCR3 was not also detected on either $CD4^+$ or $CD8^+$ T cells neither at the beginning of culture, nor at any other time during the 12-day culture period. The lack of staining for CCR3 is illustrated for days 0 and 9 in Figure 3.11 ($CD4^+$ T cells) and Figure 3.12 ($CD8^+$ T cells). The anti-CCR3 antibody was a monoclonal antibody obtained from a well-regarded commercial supplier, as for anti-CCR1. However, a biotinylated form of the antibody, which was required for the type of flow cytometric analysis performed, was not commercially available and

accordingly, the antibody was biotinylated using aminohexanoyl-Biotin-N-Hydroxysuccinimide ester, as described in Chapter 2. To ensure that the biotinylation reaction was successful, the labelled antibody was coated onto the wells of a microtitre tray, and streptavidin-horseradish peroxidase conjugate used to detect active molecules of biotin bound to the antibody. Unlabelled anti-CCR3 was included as a negative control. The results of this experiment are presented in Figure 3.13, and demonstrate that the biotinylation reaction was successful. Furthermore, biotinylated anti-CCR3 was shown to label a small proportion of peripheral blood monocytes (Fig 3.14), indicating that the antibody was functional in flow cytometry.

3.2.6 Expression of CCR5

At the beginning of culture, a small proportion (5 - 10%) of $CD4^+$ T cells expressed detectable levels of CCR5. In syngeneic cultures, the percentage of $CD4^+$ T cells positive for CCR5 was maintained at similar levels throughout the culture period. In allogeneic cultures, however, CCR5 was progressively acquired by $CD4^+$ T cells (Fig 3.15 [A]). The increase in CCR5 expression was first detectable at day 6 and increased dramatically by day 9, followed by a slight decrease at day 12. The percentage of $CD4^+$ T cells expressing CCR5 was significantly different between allogeneic and syngeneic cultures at day 9 ($p < 0.05$).

In addition to the increased proportion of $CD4^+$ T cells expressing detectable levels of CCR5, there was also an increase in the amount of CCR5 expressed by these cells, as measured by mean fluorescence intensity (MFI; Figure 3.15 [B]). Thus, the intensity of staining for CCR5 within the $CD4^+$ $CCR5^+$ population was significantly higher for allogeneic compared to syngeneic cultures at both days 6 and 9 ($p < 0.05$ and $p < 0.01$,

respectively). The increased level of CCR5 expression in allogeneic cultures, as well as the increased proportion of CCR5-expressing cells, is also illustrated in the representative density plots presented in Figure 3.16.

In contrast to the up-regulation of CCR5 observed on allo-activated $CD4^+$ T cells, no modulation of this receptor was detected within the $CD8^+$ T cell population, either at the level of the percentage of cells positive or the intensity of CCR5 expression. The time-course analyses (Fig 3.17) demonstrate that CCR5 was expressed on a small proportion of $CD8^+$ T cells at the beginning of culture (10 – 20%; Fig 3.17 [A]), and remained expressed on a similar proportion of cells throughout the culture period, with the exception of a slight decrease at day 3 in both allogeneic and syngeneic cultures, which then recovered over subsequent time-points. However, at no time was the percentage of $CD8^+$ $CCR5^+$ T cells significantly different between allogeneic and syngeneic cultures.

The level of CCR5 expression was also similar amongst $CD8^+$ T cells from allogeneic and syngeneic cultures. Although the intensity of staining for CCR5 appeared to be consistently higher on $CD8^+$ T cells from allogeneic compared to syngeneic cultures over days 9 – 12 (Fig 3.17 [B]), this difference was not statistically significant ($p > 0.05$). The lack of CCR5 up-regulation is also illustrated in the representative density plots presented in Figure 3.18, which demonstrate the similar flow cytometric profiles obtained for syngeneic and allogeneic cultures at day 9, compared to that obtained at day 0.

3.2.7 Expression of CCR6

At the initiation of culture, around 20% of $CD4^+$ T cells expressed detectable levels of CCR6. In syngeneic cultures, there was a very gradual decline in the percentage of CCR6-expressing $CD4^+$ T cells over time, such that the percentage of $CCR6^+$ $CD4^+$ T cells had reduced to a mean value of 12.7 % by day 12 ($p < 0.005$; Fig 3.19 [A]). In allogeneic cultures, a similar gradual decline in CCR6 expression was evident over days 3 – 6. However, this trend was dramatically reversed over days 9 – 12, such that by day 12 the percentage of $CD4^+$ T cells expressing CCR6 was approximately twice that of syngeneic cultures at the same time-point. At both days 9 and 12, the percentage of $CD4^+$ T cells expressing CCR6 was significantly higher in allogeneic compared to syngeneic cultures ($p < 0.01$ and $p < 0.05$, respectively). Furthermore, as noted for CCR5, the up-regulation of CCR6 occurred not only through an increase in the percentage of cells expressing CCR6, but also at the level of intensity of CCR6 expression (Fig 3.19 [B]). Thus, the level of CCR6 expression was significantly higher for $CCR6^+$ $CD4^+$ T cells from allogeneic compared to syngeneic cultures at days 9 and 12 ($p < 0.005$ for both). The enhanced level of CCR6 expression in allogeneic cultures, as well as the numerical increase in CCR6-expressing $CD4^+$ T cells, is additionally illustrated in the density plots presented in Figure 3.20.

Compared to $CD4^+$ T cells, the expression of CCR6 at the beginning of culture was slightly more restricted within the $CD8^+$ T cell population, with an average of 12.5% of $CD8^+$ T cells (in syngeneic cultures) expressing this receptor. During culture, under both syngeneic and allogeneic conditions, the percentage of $CD8^+$ T cells expressing CCR6 was remarkably stable (Fig 3.21 [A]), and at no time was there a statistically

significant difference between allogeneic and syngeneic cultures with regard to the percentage of $CCR6^+$ $CD8^+$ T cells present.

In terms of the intensity of CCR6 expression on the $CD8^+$ T cell population, there was no significant difference between allogeneic and syngeneic cultures until the day 12 time-point, at which time there was a statistically significant increase in the intensity of staining with the anti-CCR6 antibody under allogeneic conditions (Figure 3.21 [B]; $p < 0.05$). The density plots in Figure 3.22 also illustrate the increased level of CCR6 expression by $CD8^+$ T cells in allogeneic cultures at day 12 but not at day 9, as well as demonstrating the lack of increase in the percentage of CCR6-bearing $CD8^+$ T cells upon allogeneic activation.

3.2.8 Expression of CXCR3

With regard to $CD4^+$ T cells, Figure 3.23 demonstrates that CXCR3 was expressed on an average of 34.4% of cells at day 0. In syngeneic cultures, this level of expression was not significantly altered at any time during the culture period. In allogeneic cultures, however, there was a clear increase in the percentage of $CXCR3^+$ cells present over days 9 - 12, with an average of 57.9% positive by day 12. The difference between allogeneic and syngeneic cultures was statistically significant at day 9 ($p < 0.05$), and is illustrated in the density plots presented in Figure 3.24. The observed numerical increase in CXCR3-expressing cells was not, however, mirrored by a significant increase in the intensity of CXCR3 expression. Thus, although the density plots in Figure 3.24 suggest a slightly increased level of staining for CXCR3 on $CD4^+$ T cells in allogeneic compared to syngeneic cultures at day 9, there was no statistically

significant difference in the intensity of CXCR3 expression at any time-point (Fig 3.23 [B]).

In contrast to the $CD4^+$ T cell population, the proportion of $CD8^+$ T cells expressing CXCR3 was relatively constant throughout the culture period (Fig 3.25 [A]). The percentage of $CXCR3^+$ $CD8^+$ T cells was quite high at the initiation of culture (mean = 71.4% in syngeneic cultures) and was similar by the end of the culture period in both allogeneic and syngeneic cultures. At no time during the culture period was the percentage of $CD8^+$ T cells positive for CXCR3 significantly different between allogeneic and syngeneic culture conditions. Moreover, Figure 3.25 [B] indicates that there was no significant alteration in the intensity of CXCR3 expression on the $CXCR3^+$ $CD8^+$ T cell population. The lack of CXCR3 up-regulation, both at the level of the percentage of cells positive and the intensity of expression, is also illustrated in the density plots in Figure 3.26.

3.2.9 Expression of CXCR4

At the initiation of culture, CXCR4 was expressed on around 65% of $CD4^+$ T cells in both allogeneic and syngeneic samples. However, after 3 days in culture, the percentage of $CD4^+$ $CXCR4^+$ T cells had increased to 100% under both culture conditions (Fig 3.27 [A]). This uniform expression of CXCR4 was maintained on $CD4^+$ T cells in syngeneic culture until at least day 12, while in contrast, $CD4^+$ T cells in allogeneic cultures demonstrated a progressive loss of CXCR4 expression, which was first detectable at day 6 and increased dramatically by day 9 (Figure 3.27 [A]). Furthermore, the density plots presented in Figure 3.28 suggest that many of the $CD4^+$ T cells that retained detectable levels of CXCR4 expression nevertheless expressed

lower levels of this receptor, in comparison to the CXCR4-expressing cells from syngeneic cultures. However, due to the extent of variation in staining intensity for CXCR4 noted throughout these experiments, any observed differences in the level of staining for CXCR4 were not statistically significant for the pooled data (Fig 3.27 [B]).

In contrast to all other chemokine receptors investigated thus far, the patterns of regulation of CXCR4 on $CD8^+$ T cells were virtually identical to those observed on $CD4^+$ T cells. Thus, CXCR4 was expressed on 30 - 60% of $CD8^+$ T cells at day 0, but became expressed by 100% of cells by day 3 (Fig 3.29). In syngeneic cultures, the uniform expression of CXCR4 persisted throughout the culture period, while in contrast, allogeneic cultures demonstrated a progressive decrease in the proportion of $CXCR4^+$ $CD8^+$cells over days 6 – 12. Also, those $CD8^+$ T cells that retained detectable expression of CXCR4 appeared to express lower levels of this receptor compared to $CD8^+$ T cells from syngeneic cultures (Fig 3.30), although the pooled data demonstrated no statistically significant difference due to the high degree of sample-to-sample variance (Fig 3.29 [B]).

3.2.10 Expression of CXCR5

CXCR5 was expressed by a small proportion of $CD4^+$ T cells at the day 0 time-point (mean 4.5% in syngeneic cultures). In neither allogeneic nor syngeneic cultures did the percentage of $CD4^+$ T cells expressing CXCR5 alter appreciably over the 12-day time-course, and at all time-points tested, there was no significant difference between the percentage of cells positive for CXCR5 in allogeneic compared to syngeneic cultures (Fig 3.31 [A]). Moreover, within the sub-population of $CD4^+$ T cells that expressed detectable levels of CXCR5, the intensity of expression was similar in both allogeneic

and syngeneic cultures throughout the time-course (Fig 3.31 [B]). The conclusion that CXCR5 was not up-regulated in response to allogeneic activation, in terms of either the percentage of cells positive or the level of expression, is also supported by the density plots presented in Figure 3.32.

With regard to $CD8^+$ T cells, the expression of CXCR5 at the beginning of culture was barely detectable, with only 0.2 – 1% of $CD8^+$ T cells staining positive for this receptor. Moreover, the percentage of $CXCR5^+$ $CD8^+$ T cells was not significantly altered over subsequent time-points in either allogeneic or syngeneic cultures, such that by day 12, there were still less than 2% of cells positive (Fig 3.33). The lack of CXCR5 expression by the vast majority of $CD8^+$ T cells is also illustrated in the density plots presented in Figure 3.34. With regard to the intensity of staining for CXCR5, Figure 3.33 [B] demonstrates that the level of CXCR5 expression was relatively stable for both allogeneic and syngeneic cultures over the days 0 – 9 time period. There, was, however, a statistically significant increase in the MFI of staining for CXCR5 on $CD8^+$ T cells in allogeneic cultures compared to syngeneic controls at the day 12 time-point.

3.3 Discussion

3.3.1 Heterogeneity in expression of CD4 and CD8

The flow cytometry data (density plots) presented throughout these studies illustrate that the expression patterns of CD4 and CD8 on PBMC were not uniform. Within the populations of cells that expressed detectable levels of these markers, distinct sub-

populations could be identified that expressed low, intermediate or high levels of the marker. Moreover, in some cases, the expression of chemokine receptors correlated with the divergent patterns of CD4 or CD8 expression.

At day 0, the expression of CD4 was relatively uniform, with the exception of a minor sub-population of cells expressing low levels of this marker. Despite gating on the lymphocyte population during flow cytometric analysis using forward scatter and side scatter properties, it is likely that this population of cells represents either monocytes or DCs that are present in the lymphocyte gate, as these cell types are known to express low levels of CD4 (171). Accordingly, as the intention of these experiments was to analyse T cells, quadrant markers were set to exclude this population, such that these cells did not contribute to the numerical values obtained.

In comparison to the observations made at the initiation of culture, a distinct pattern of heterogeneous CD4 expression was observed at the day 9 time-point. In allogeneic, but not syngeneic, cultures, two discrete sub-populations of $CD4^+$ T cells were evident. One sub-population in allogeneic cultures expressed similar levels of CD4 to those observed at day 0, while the other expressed higher levels of CD4. In syngeneic cultures, however, the expression of CD4 was uniform, and the levels were similar to those expressed on the day 0 cells. Of particular note, the expression of a number of chemokine receptors (CCR5, CCR6, CXCR3 and CXCR4) differed greatly between these two populations of $CD4^+$ T cells. This phenomenon will be covered in more detail in Chapter 4.

Heterogeneous patterns of CD8 expression were also detected, at both the day 0 and day 9 time-points. At day 0, a range of CD8 staining intensities were observed.

Furthermore, sub-populations of T cells expressing heterogeneous levels of CD8 could also be detected at day 9, in both allogeneic and syngeneic cultures. It is interesting to note that the expression of some chemokine receptors varied considerably amongst these sub-populations of $CD8^+$ T cells. For example, in both allogeneic and syngeneic cultures, the sub-populations expressing either low or high levels of CD8 were characterised by low levels of CCR6 expression, while the sub-population expressing intermediate levels of CD8 was characterised by high levels of CCR6 expression (see Figs 3.22 [B] and [C]). Furthermore, the sub-population of T cells expressing high levels of CD8 in allogeneic cultures was characterised by enhanced expression of CXCR3 (Fig 3.26) and reduced expression of CXCR4 (Fig 3.30).

While the differential expression of chemokine receptors on $CD8^+$ T cell sub-populations is interesting, the identity of sub-populations expressing distinct levels of CD8 is unclear. For example, a sub-population of T cells, possibly of extrathymic origin, that expresses reduced levels of CD8 has been detected in small numbers in the peripheral blood of healthy individuals, and shown to be significantly expanded in certain pathological conditions (172). Similarly, apoptotic $CD8^+$ T cells that have undergone antigenic stimulation also appear to express reduced levels of CD8 (173, 174). Furthermore, there is some evidence that CD8 may be up-regulated following T cell activation by mitogenic lectin stimulation (175), which may explain the appearance of a small sub-population of T cells expressing moderately elevated levels of CD8 in the MLR. Note, however, that the appearance of T cells expressing elevated levels of CD8 was not as consistent or striking as the appearance of T cells expressing elevated levels of CD4. Finally, the possibility remains that a small proportion of the $CD8^+$ cells detected are not T cells, as NK cells are also known to express the CD8 marker (176).

Thus, considering that the identities of the various sub-populations of $CD8^+$ T cells are unknown, the quadrant markers for all analyses have been set to include all cells expressing detectable levels of CD8. Had significant modulation of chemokine receptor expression been detected on the total $CD8^+$ T cell population, it was intended that more detailed studies would be carried out to more definitively identify the various $CD8^+$ T cell populations. However, as CXCR4 was the only receptor to be specifically modulated on $CD8^+$ T cells, this issue was not pursued further.

3.3.2 CCR1, CCR2, CCR3 and CXCR5 are not up-regulated on $CD4^+$ or $CD8^+$ T cells at any time-point

The absence of detectable CCR1 on either $CD4^+$ or $CD8^+$ T cells at any time-point was surprising, given that previous studies have detected CCR1 on a large proportion of peripheral blood T cells (72, 134, 177). In support of the present findings, however, is the observation that freshly isolated memory T cells from human blood do not migrate to RANTES, a CCR1 ligand, and do not express detectable CCR1 transcripts (127). Further, the antibody used in this study was demonstrated to be functional by means of a positive reaction on monocytes. Of note, a panel of antibodies reactive against different epitopes of CCR5 have been shown to produce different levels of staining in flow cytometric analysis, an observation that has been proposed to indicate multiple conformational states of this receptor (178). Accordingly, given that different antibodies were used in previous studies compared with the present, a similar scenario with regard to CCR1 may explain the discordant results.

The observation that sub-populations of $CD4^+$ and $CD8^+$ T cells expressed CCR2 at the beginning of culture was not surprising, as resting memory T cells in peripheral blood are known to express this receptor (72, 134). However, the striking down-

regulation observed in both allogeneic and syngeneic cultures was unexpected, and has not been previously observed. Given the non-specific nature of this down-regulation, which was comparable in allogeneic and syngeneic cultures, it is likely that the loss of CCR2 is related in some way to the physiological changes encountered by PBMC when introduced into *in vitro* culture.

CCR3 was not detected on either $CD4^+$ or $CD8^+$ T cells at any time during the culture period. In this context, it is interesting to note that a number of previous studies have demonstrated that the expression of CCR3 on $CD4^+$ T cells is limited to effector/memory cells of a Th2 phenotype (73, 74, 77, 141). The MLR is a prototypic Th1 response, characterised by the production of large amounts of IFN-γ and IL-2 but limited amounts of IL-4 and IL-10 (179-181). It is therefore not surprising that CCR3, a Th2-associated chemokine receptor, was not detected during the development of a Th1-biased immune response such as the MLR. A similar argument may also be applied to explain the lack of CCR2 up-regulation. Although the preferential expression of CCR2 on the Th2 subset has not yet been demonstrated, a number of studies have reported the ability of MCP-1, a CCR2 ligand, to enhance Th2 subset differentiation (55, 89, 90, 142). Thus, it may be important that T cells activated in the MLR do not express CCR2, which may disrupt the Th1 polarisation process.

CXCR5 was not up-regulated on $CD4^+$ T cells during the MLR, either in terms of the percentage of cells expressing detectable levels of the receptor, nor the intensity of expression. Similarly, there was no increase in the proportion of $CD8^+$ T cells that expressed CXCR5 in allogeneic compared to syngeneic cultures at any time-point. In contrast, there was a small increase in the level of CXCR5 expressed by $CD8^+$ T cells in allogeneic cultures at day 12. However, despite the statistical significance of this

observation, it is unclear what – if any – the biological significance is, given that this change was limited to a modest increase in the level of CXCR5 expressed by a very minor proportion of cells.

CXCR5 is expressed by a sub-population of memory T cells in peripheral blood (119, 182) and may therefore be expected to be gained by T cells during activation. However, recent studies have shown that CXCR5-expressing T cells (119, 120), or at least a sub-population thereof (121), are uniquely adapted to providing B cell help, and it is thus conceivable that the up-regulation of this receptor was not appropriate in the context of the MLR, which has no humoral component. Similarly, CXCR5 is not up-regulated by anti-CD3 plus anti-CD28 stimulation (119), which is also a B cell-independent model of T cell activation. Thus, it is possible that a B cell-expressed molecule is required for the induction of CXCR5 on activated T cells. OX40L, an activation molecule expressed on stimulated B cells, is a likely candidate, as murine T cells have been shown to up-regulate expression of CXCR5 following ligation of the OX40L counter-receptor, OX40 (183). Thus, the up-regulation of CXCR5 by activated B cells could play an important role in retaining helper T cells in the B cell area of secondary lymphoid tissue, where the CXCR5 ligand BLC/CXCL13 is constitutively expressed (184).

3.3.3 CCR5, CCR6 and CXCR3 are up-regulated on $CD4^+$ T cells in allogeneic but not syngeneic cultures

The up-regulation of CCR5 and CCR6 upon allogeneic activation of $CD4^+$ T cells is in contrast to the findings of a number of other studies that have failed to detect increased expression of these receptors in response to T cell activation with mitogenic lectins (107, 131), anti-CD3 (72, 130) or anti-CD3 plus anti-CD28 (122, 134). The observed

up-regulation is, however, consistent with the fact that circulating memory T cells and long-term activated T cell clones express these receptors (72, 107, 123, 126, 130, 134), and CCR5 expression has been detected on clusters of cells in the LN that are morphologically consistent with T cell blasts (126). Moreover, up-regulation of CCR5 has been demonstrated using an alternative model of T cell activation, as will be discussed in more detail to follow.

It is likely that the activation of T cells by APCs, as occurs in the MLR, provides T cells with various signals in addition to stimulation through the TCR that are required for the induction of novel patterns of chemokine receptor expression. If these signals are absent or inappropriate, as may be the case with lectin, anti-CD3 or anti-CD3 plus anti-CD28 stimulation, up-regulation of chemokine receptor expression will not be observed. In support of this concept, HIV infection studies have demonstrated that the infection of $CD4^+$ T cells with R5 virus isolates is increased following activation by APCs, yet decreased following stimulation with anti-CD3 plus anti-CD28 (135, 185). These findings suggest that the up-regulation of CCR5, which is required for the entry of R5 viruses, only occurs following T cell interaction with APCs, and not in response to APC-independent *in vitro* stimulation.

The signals required for the up-regulation of CCR5 and CCR6 could include physiologically relevant co-stimulatory pathways as well as immunomodulatory cytokines. With regard to co-stimulatory pathways, it has been demonstrated that the activation of $CD4^+$ T cells with immunobeads coated with anti-CD3 plus varying amounts of co-stimulatory molecules has differential effects on CCR5 expression (135). Co-stimulation through the CD28 pathway alone results in T cell activation and proliferation but the down-regulation of CCR5 to undetectable levels. In contrast,

simultaneous co-stimulation through the CD28 and CTLA-4 pathways results in both T cell activation/proliferation and a significant up-regulation of CCR5 expression. Based on these findings, it is conceivable that T cells encounter a set of co-stimulatory molecules during the MLR that together initiate the relevant intracellular signalling pathways that are required for the acquisition of chemokine receptors such as CCR5 and CCR6. The importance of CTLA-4 in CCR5 up-regulation is, however, somewhat unexpected, as this molecule is more often associated with the down-regulation of the immune response (186). Thus, a simplistic interpretation of this data is that CCR5 plays a role in immune suppression, a concept that is supported by the recent finding that CCR5 ligands can induce tumour-infiltrating T cells to undergo apoptosis (187). However, much remains to be understood about the role of CTLA-4 *in vivo*, and it is feasible that the expression of CCR5 is a result of CTLA-4-induced immune modulation, rather than suppression. Of relevance here is data suggesting that CTLA-4 may deviate the immune response to a Th1 bias (186), in keeping with the preferential expression of CCR5 on Th1 cells (73, 111).

With regard to the role of cytokines in the up-regulation of CCR5 and CCR6, IL-2 and IL-12 are both likely candidates. Although T cell activation using anti-CD3 alone does not have a significant effect on CCR5 or CCR6 expression (72, 130), the combination of anti-CD3 plus IL-2 induces a dramatic up-regulation of CCR5 expression levels (118, 122, 126). Thus, it is possible that the lack of CCR5 up-regulation in response to anti-CD3 treatment is due to insufficient production of IL-2 in the cultures. Furthermore, the Th1-inducing cytokine IL-12 has the ability to up-regulate CCR5 expression on both human and murine T cells when used in combination with anti-CD3 (122, 188), in keeping with the observed Th1 association of this receptor (73, 111).

The up-regulation of CXCR3 observed in the MLR is in keeping with previous reports of CXCR3 up-regulation following CD3 cross-linking (72, 118), suggesting that the control of this receptor is directly linked to stimulation of the TCR complex, rather than being dependent on the nature of the antigen or co-stimulatory signals provided by the APC, as postulated for CCR5 and CCR6. In this context, however, it is noteworthy that CXCR3 has been shown in some studies to be expressed at lower levels on Th2 cells compared to Th1 cells (73, 74). Thus, it is conceivable that the acquisition of CXCR3 is a default pathway, activated whenever stimulation through the TCR is detected, and Th2-inducing cytokines can disrupt this pathway to some degree, thereby leading to lower expression on Th2 cells. In the case of the MLR, which is characterised by a Th1 phenotype (179-181), it is likely that these cytokines would not be produced in sufficient amounts to mediate CXCR3 down-regulation.

3.3.4 CXCR4 is specifically down-regulated on $CD4^+$ and $CD8^+$ T cells in allogeneic cultures

With regard to CXCR4 expression on T cells, previous studies have demonstrated differing expression patterns following peripheral blood T cell activation using anti-CD3 or phytohemagglutinin (PHA), with expression being increased (107, 138), decreased (106, 136) or remaining relatively unchanged (72). The present study provides evidence, using a biologically relevant model of a primary immune response, that CXCR4 is down-regulated following activation by APCs. Furthermore, this is the first report of CXCR4 down-regulation on $CD8^+$ T cells, as previous studies have either not identified the T cell subset undergoing CXCR4 down-regulation, or have examined only the $CD4^+$ subset.

A possible explanation for the earlier reports of CXCR4 up-regulation in response to T cell activation is the observation that CXCR4 surface expression is increased in culture without stimulation. This non-specific up-regulation of CXCR4 on T cells upon introduction to culture has been observed in many other studies (106, 136, 137, 189), although the biological mechanism responsible for this effect is still unclear. Importantly, however, previous studies which concluded that CXCR4 was up-regulated in response to PHA or anti-CD3 plus anti-CD28 treatments did not include parallel cultures without stimulation (107, 138), thus raising the possibility that the observed increased expression of CXCR4 was not stimulus-specific.

In support of the findings presented here, the activation of antigen-specific memory Th2 cells from the peripheral blood of allergic individuals similarly results in the down-regulation of CXCR4 on the surface of T cells (137). Considering the documented Th1 bias of the MLR (179-181), the combined results of the present study and that previous study (137) suggest that the down-regulation of CXCR4 in response to antigen-induced activation occurs similarly in naïve and memory populations, and in both Th1 and Th2 environments, and may therefore be directly linked to stimulation through the TCR, as proposed for CXCR3.

3.3.5 Lack of chemokine receptor up-regulation on $CD8^+$ T cells

Although CCR5, CCR6 and CXCR3 were significantly up-regulated on allo-activated $CD4^+$ T cells, none of these receptors were acquired by $CD8^+$ T cells following allogeneic activation. Thus, there was no significant increase in the percentage of $CCR5^+$ $CD8^+$, $CCR6^+$ $CD8^+$ or $CXCR3^+$ $CD8^+$ T cells in allogeneic compared to syngeneic cultures at any time-point tested. There was, however, a modest increase in

the intensity of CCR6 expression on a sub-population of $CD8^+$ T cells in allogeneic cultures at the day 12 time-point. This observation suggests that, although CCR6 could not be newly acquired by allo-activated $CD8^+$ T cells, cells that already expressed this receptor were able to increase the level at which it was expressed. As CCR6 expression on circulating T cells has been shown to be restricted to the memory population (130), it is likely that the observed up-regulation of CCR6 expression on $CD8^+$ T cells occurred at a result of secondary activation of memory T cells.

On the basis of the CFSE studies, it can be concluded that the inability of $CD8^+$ T cells to acquire novel patterns of inflammatory chemokine receptor expression is not due to a lack of activation, as this population clearly did divide in the MLR. As $CD4^+$ and $CD8^+$ T cells appear to be subject to the same molecular requirements for extravasation into peripheral tissue (6), it is likely that both subsets require the expression of appropriate inflammatory chemokine receptors in order to access peripheral sites of inflammation. However, it is also conceivable that the receptors involved will be different, thereby allowing the efficient recruitment of the appropriate T cell subset. A number of the chemokine receptors listed in Table 1.3 (Chapter 1) were not tested in this series of experiments, as they had not been identified at the time these studies were performed, or specific antibodies were not commercially available. Thus, one or more of these receptors, such as CCR10, CCR11 or CXCR6, may be up-regulated on $CD8^+$ T cells during the MLR. Furthermore, it is likely that more chemokine receptors will be identified and characterised in the next few years, and the possibility remains that these may also be up-regulated on the $CD8^+$ T cell population.

3.3.6 Temporal distinction between proliferation and modulation of chemokine receptor expression

The proliferation time-course studies indicated that cell division peaked at around the day 6 time-point, and had largely declined by day 9. This is in distinct contrast to the observed changes in chemokine receptor expression, as significant alterations in the percentage of cells expressing CCR5, CCR6, CXCR3 or CXCR4 were first noted at the day 9 time-point. The observation that chemokine receptor expression was not altered at day 3, before cell division had begun, suggests that the regulation of these receptors is coupled to the cell division process. The relationship between regulation of chemokine receptor expression and cell division will be explored in more detail in Chapter 4.

3.3.7 Conclusions

The aim of this series of experiments was to identify chemokine receptors that demonstrated altered patterns of expression on $CD4^+$ or $CD8^+$ T cells following activation by APCs in the MLR. Out of eight chemokine receptors tested, four were shown to be acquired or lost by allo-activated $CD4^+$ T cells, while only one was lost by activated $CD8^+$ T cells and no new receptors were gained. Thus, within the $CD4^+$ T cell subset, a significantly greater proportion were shown to express CCR5, CCR6 and CXCR3 in allogeneic cultures compared to syngeneic controls, while conversely, a lesser proportion expressed CXCR4. With regard to $CD8^+$ T cells, CXCR4 was lost on a significant fraction of cells in allogeneic but not syngeneic cultures, although no new chemokine receptors became expressed. While two receptors (CCR6 and CXCR5) did demonstrate a modest increase in the level of expression following allogeneic activation, this was likely to be due to the secondary activation of memory T cells, and does not provide evidence for the acquisition of these receptors by naïve T cells. In all

cases, the modulation of chemokine receptor expression was a relatively late event, peaking at a time-point at which cell division had largely ceased.

Figure 3.1. *Time-course of proliferation in the MLR.* PMBC were purified from two unrelated donors and cultured in 96 well trays under syngeneic or allogeneic conditions for the indicated period of time. Eighteen hours prior to harvest, 1μCi of [^{3}H]-thymidine was added to each well, and incorporation determined by scintillation counting. Values represent mean $\pm$ SEM ($n \geq 4$ at each time-point).

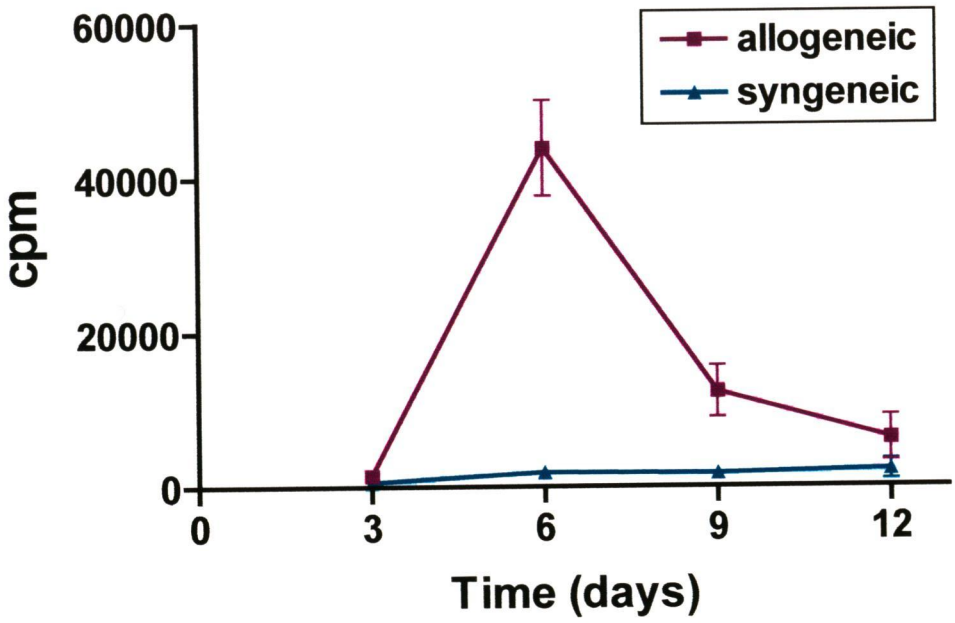
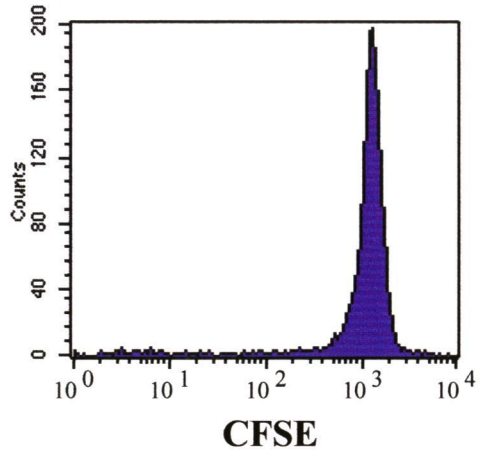

60000
40000
20000
0
cpm
0
3
6
9
12
Time (days)
allogeneic
syngeneic

Figure 3.2. *Comparison of cell division in allogeneic and syngeneic day 9 cultures using CFSE.* PMBC were purified from two unrelated donors and mixed to generate a syngeneic [A] or allogeneic [B] MLR. Cells were labelled with CFSE, cultured for 9 days and subsequently analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. Data shown are representative of at least 3 experiments.

[A] *syngeneic*



[B] *allogeneic*

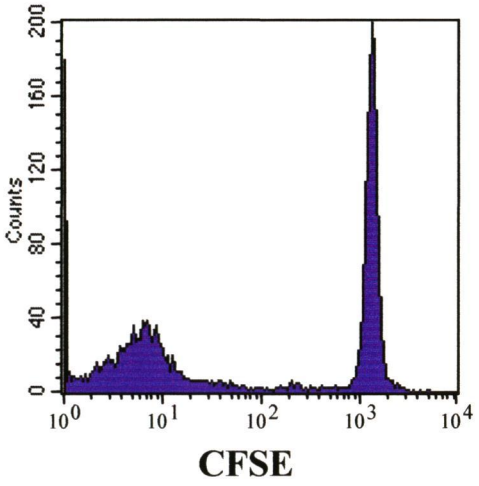


Figure 3.3. *Cell division within the $CD3^+$, $CD4^+$ and $CD8^+$ lymphocyte sub-populations during the MLR.* PMBC were purified from two unrelated donors and mixed to generate an MLR. Cells were labelled with CFSE, cultured for 9 days, then labelled with antibodies to CD3 [A], CD4 [B] or CD8 [C] and analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. Density plots from representative experiments are shown in [A] – [C], while the values obtained for 6 separate experiments are shown in [D].

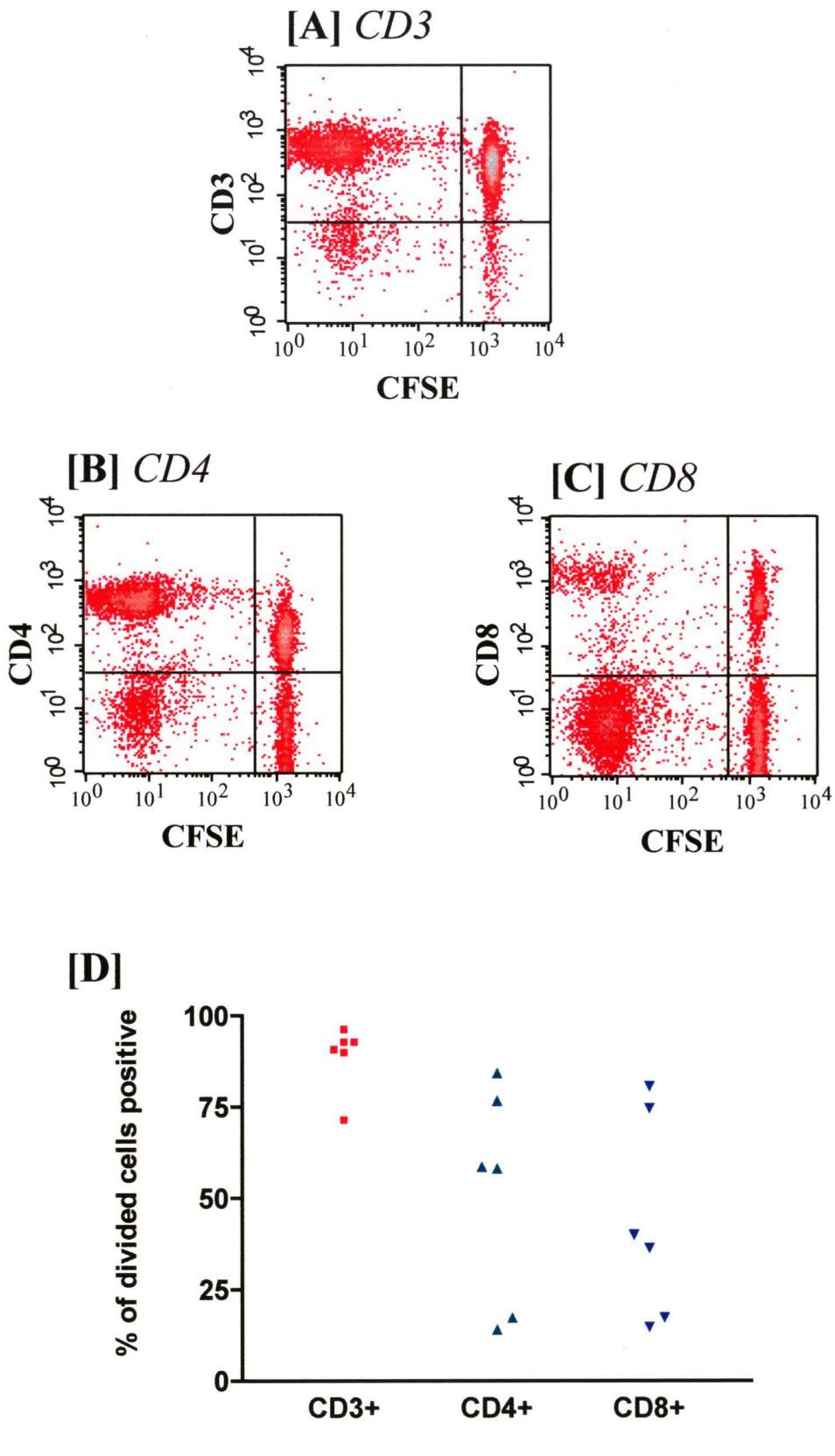
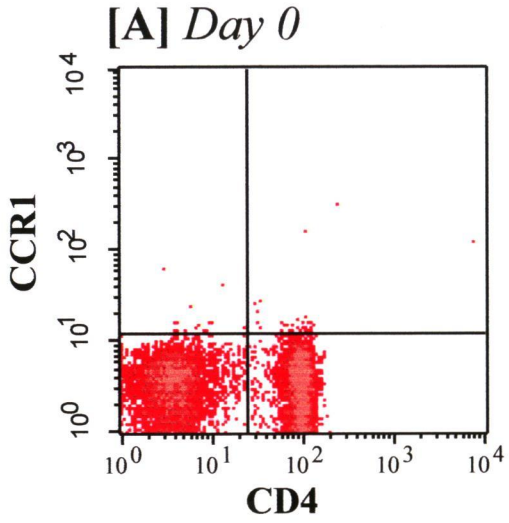
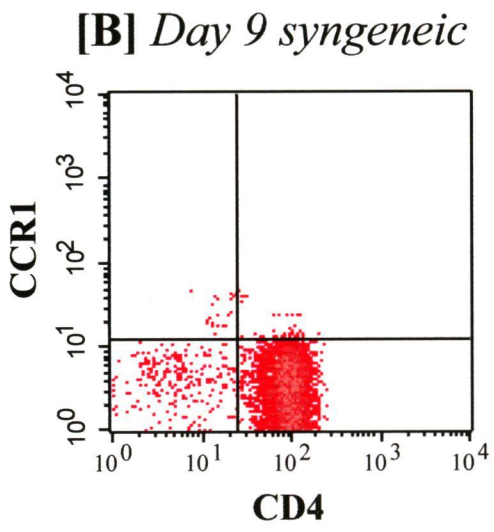

[A] CD3
CD3
CFSE
[B] CD4
CD4
CFSE
[C] CD8
CD8
CFSE
[D]
% of divided cells positive
100
75
50
25
0
CD3+
CD4+
CD8+

Figure 3.4. *Expression of CCR1 on $CD4^+$ T cells in allogeneic and syngeneic MLR cultures.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 days of culture under syngeneic [B] or allogeneic [C] conditions, cells were labelled with antibodies to CCR1 and CD4 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 3 experiments.


[A] Day 0
CCR1
CD4
10^0
10^1
10^2
10^3
10^4


[B] Day 9 syngeneic
CCR1
CD4
10^0
10^1
10^2
10^3
10^4

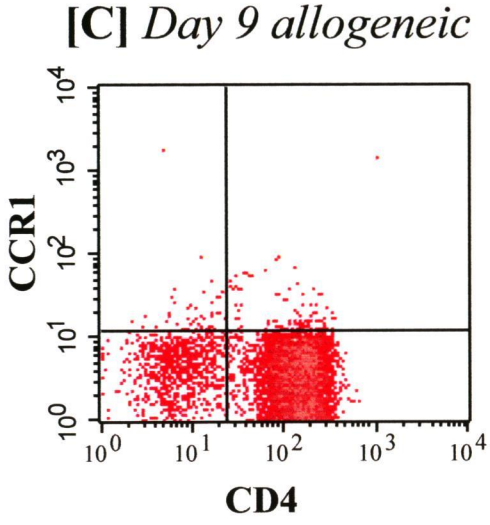
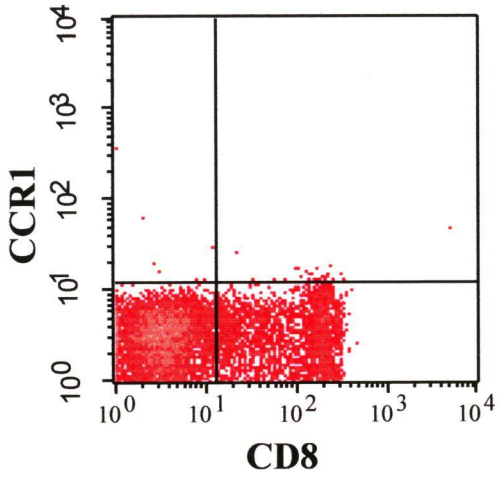
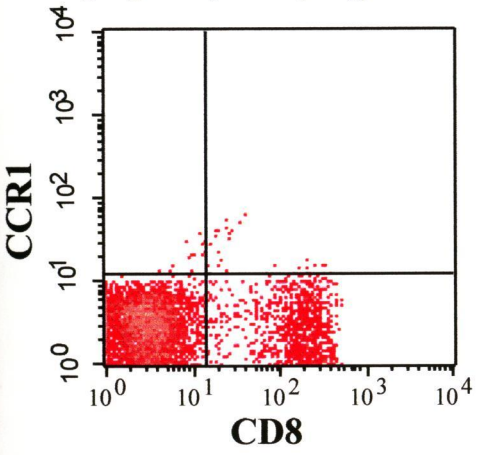

[C] Day 9 allogeneic
CCR1
CD4
10^0
10^1
10^2
10^3
10^4

Figure 3.5. *Expression of CCR1 on $CD8^+$ T cells in allogeneic and syngeneic MLR cultures.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 days of culture under syngeneic [B] or allogeneic [C] conditions, cells were labelled with antibodies to CCR1 and CD8 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 3 experiments.

[A] *Day 0*



[B] *Day 9 syngeneic*



[C] *Day 9 allogeneic*

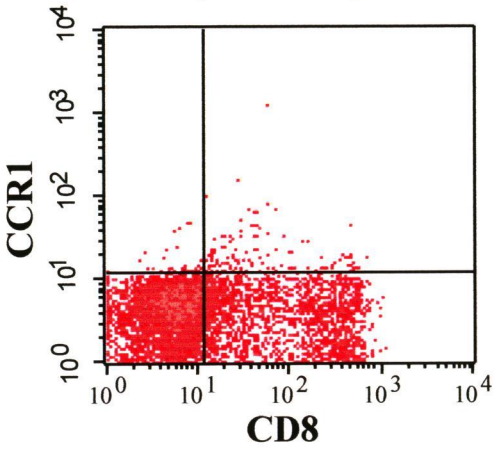
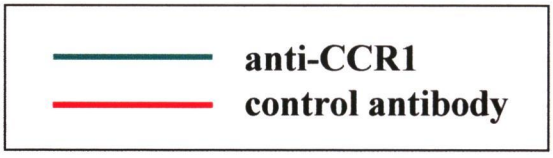
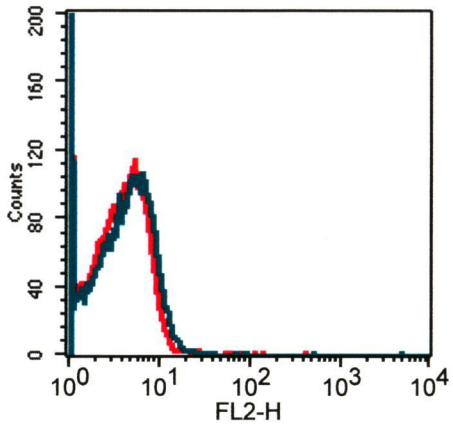


Figure 3.6. *Comparison of CCR1 staining on lymphocytes and monocytes.* PMBC were purified, labelled with anti-CCR1 or an isotype-matched negative control antibody and analysed by flow cytometry. Lymphocytes and monocytes were respectively gated using forward and side scatter characteristics and the level of staining with the control antibody (red) or anti-CCR1 (green) assessed for each population. Data shown are representative of 3 experiments.



[A] *lymphocytes*



[B] *monocytes*

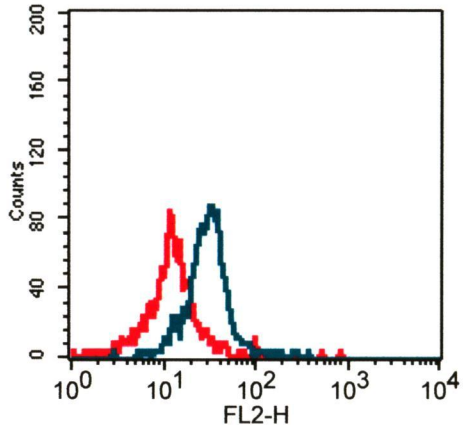


Figure 3.7. *CCR2 expression on $CD4^+$ T cells in allogeneic and syngeneic MLR cultures: time-course.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. At the initiation of culture, or after the indicated period of time, cells were labelled with antibodies to CCR2 and CD4 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the percentage of cells positive for CCR2 determined in comparison to staining with an isotype-matched control antibody. The percentage of $CD4^+$ T cells that expressed detectable levels of CCR2 was then calculated as a proportion of total $CD4^+$ T cells. Data are presented as mean $\pm$ SEM (n = 3-4 at each time-point).

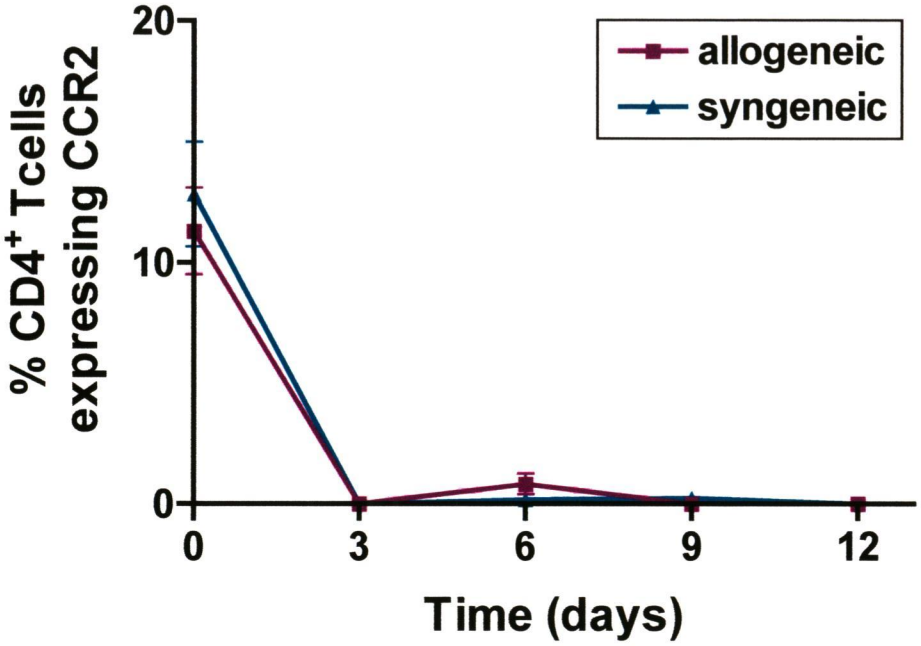
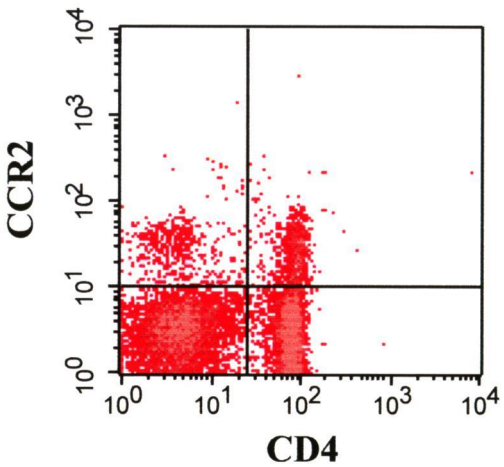
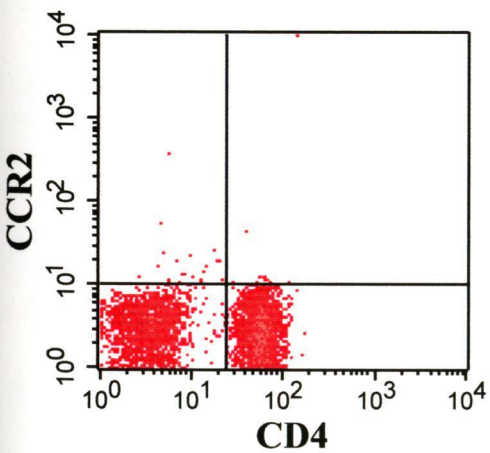

% CD4+ Tcells expressing CCR2
20
10
0
0
3
6
9
12
Time (days)
allogeneic
syngeneic

Figure 3.8. *Expression of CCR2 on $CD4^+$ T cells in allogeneic and syngeneic MLR cultures: representative density plots.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 days of culture under syngeneic [B] or allogeneic [C] conditions, cells were labelled with antibodies to CCR2 and CD4 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 3 experiments.

[A] *Day 0*



[B] *Day 9 syngeneic*



[C] *Day 9 allogeneic*

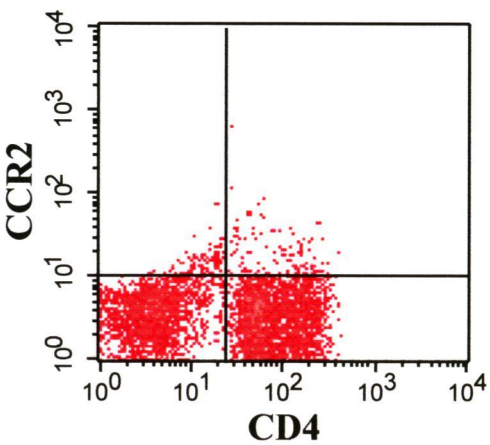


Figure 3.9. *CCR2 expression on $CD8^+$ T cells in allogeneic and syngeneic MLR cultures: time-course.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. At the initiation of culture, or after the indicated period of time, cells were labelled with antibodies to CCR2 and CD8 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the percentage of cells positive for CCR2 determined in comparison to staining with an isotype-matched control antibody. The percentage of $CD8^+$ T cells that expressed detectable levels of CCR2 was then calculated as a proportion of total $CD8^+$ T cells. Data are presented as mean $\pm$ SEM (n = 3-4 at each time-point).

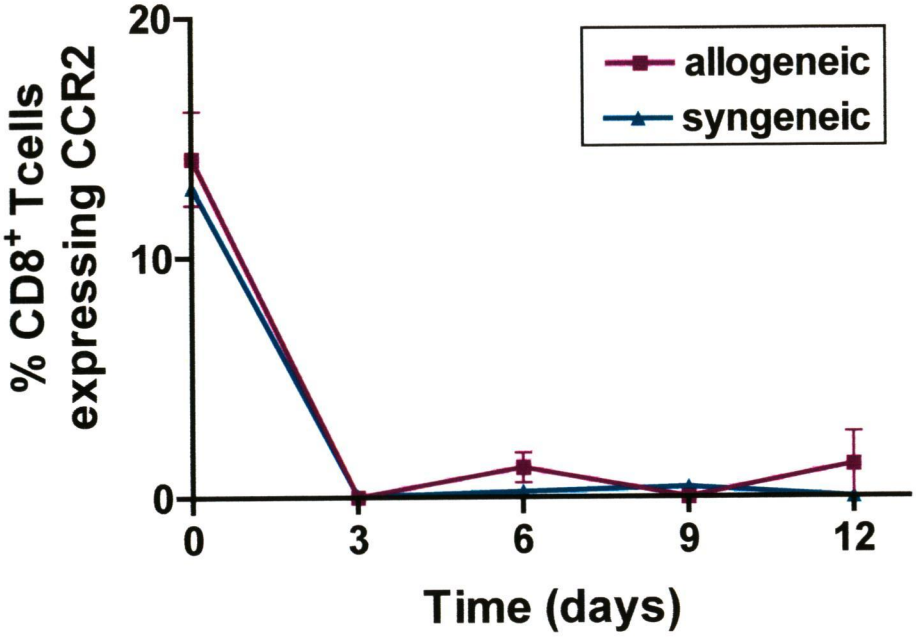
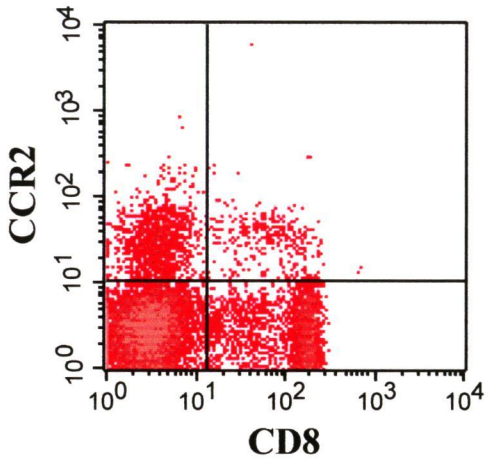
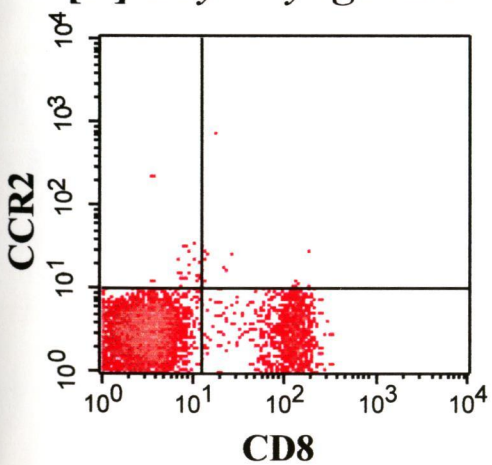

% CD8+ Tcells expressing CCR2
20
10
0
0
3
6
9
12
Time (days)
allogeneic
syngeneic

Figure 3.10. *Expression of CCR2 on $CD8^+$ T cells in allogeneic and syngeneic MLR cultures: representative density plots.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 days of culture under syngeneic [B] or allogeneic [C] conditions, cells were labelled with antibodies to CCR2 and CD8 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 3 experiments.

[A] *Day 0*



[B] *Day 9 syngeneic*



[C] *Day 9 allogeneic*

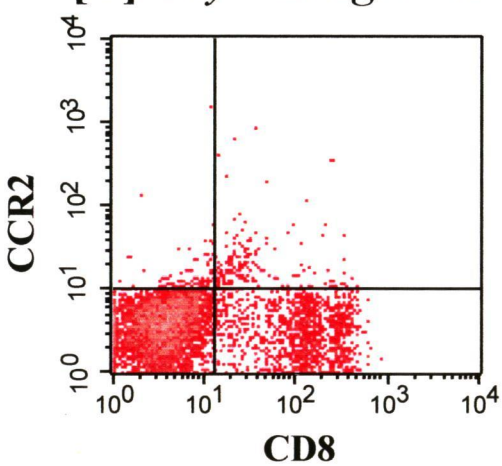
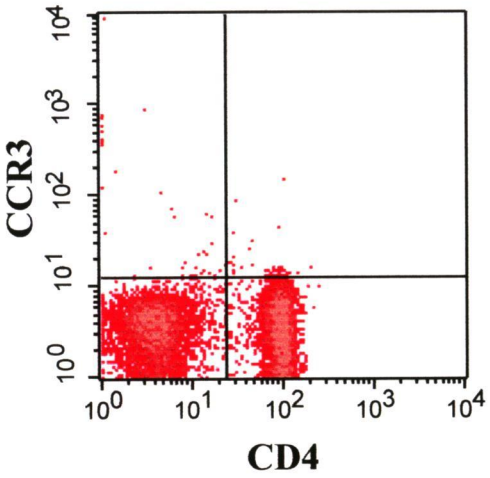
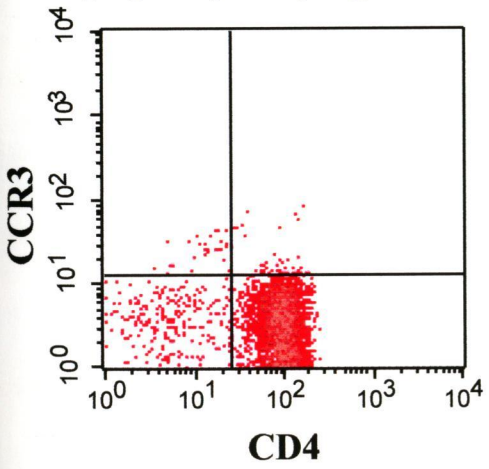


Figure 3.11. *Expression of CCR3 on $CD4^+$ T cells in allogeneic and syngeneic MLR cultures.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 days of culture under syngeneic [B] or allogeneic [C] conditions, cells were labelled with antibodies to CCR3 and CD4 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 3 experiments.

[A] *Day 0*



[B] *Day 9 syngeneic*



[C] *Day 9 allogeneic*

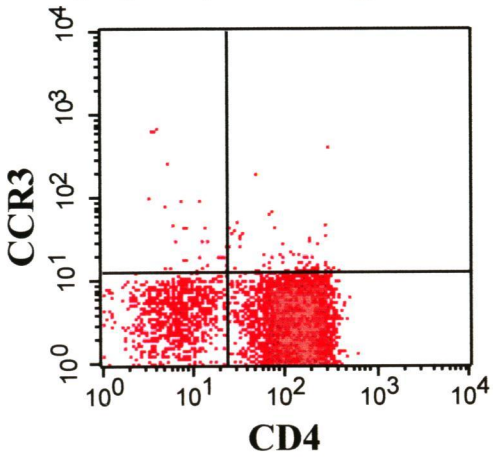
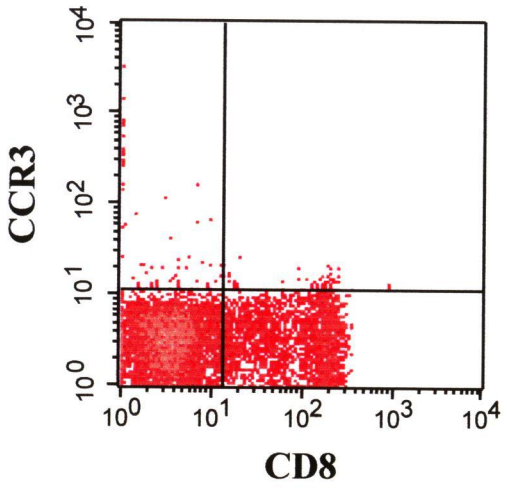
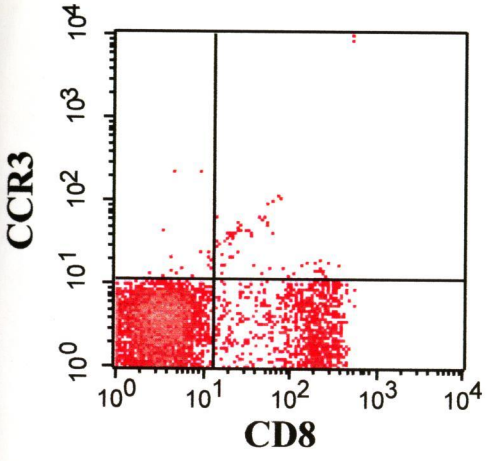


Figure 3.12. *Expression of CCR3 on $CD8^+$ T cells in allogeneic and syngeneic MLR cultures.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 days of culture under syngeneic [B] or allogeneic [C] conditions, cells were labelled with antibodies to CCR3 and CD8 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 3 experiments.

[A] *Day 0*



[B] *Day 9 syngeneic*



[C] *Day 9 allogeneic*

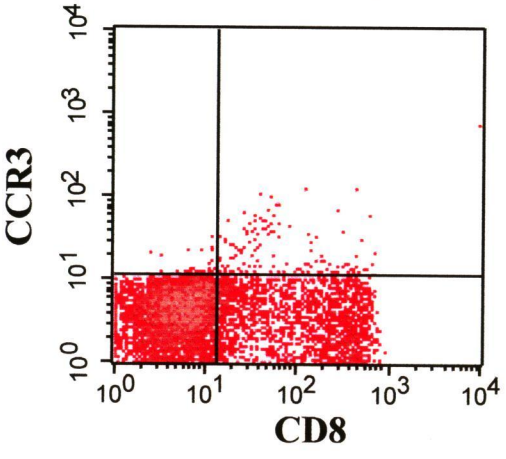


Figure 3.13. *One-site ELISA to determine the success of the anti-CCR3 biotinylation reaction.* The anti-CCR3 monoclonal antibody was labelled with aminohexanoyl-biotin-N-hydroxysuccinimide ester, as described in Chapter 2. After extensive dialysis, both the biotinylated and unlabelled antibodies were coated onto triplicate wells of a high-binding 96 well assay plate. Following incubation with streptavidin-HRP followed by the addition of OPD substrate, the extent of biotinylation was quantified by measuring absorbance at 485nm.

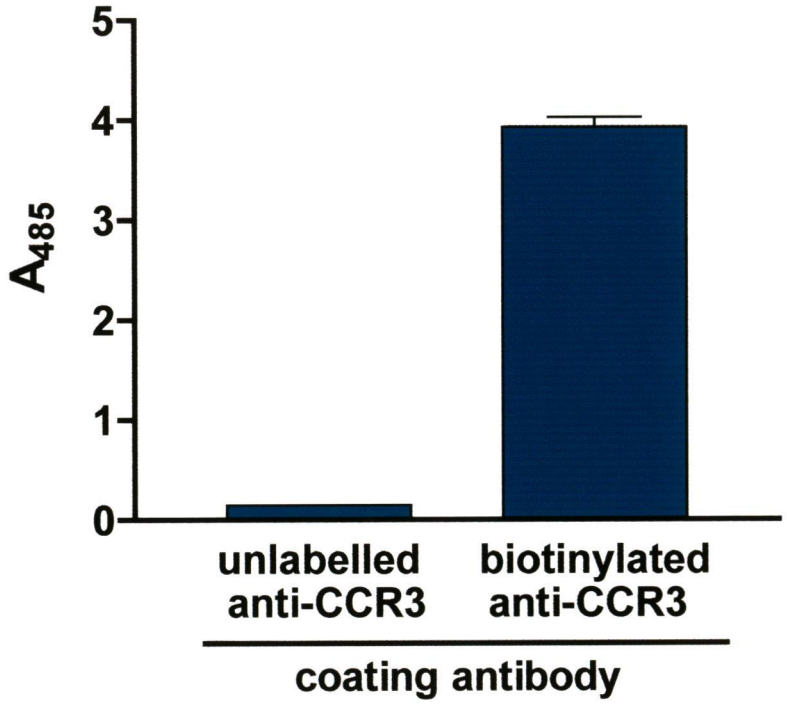
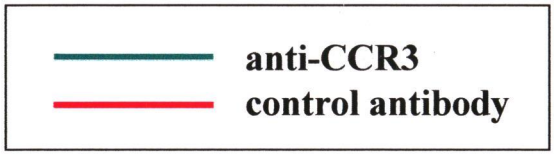
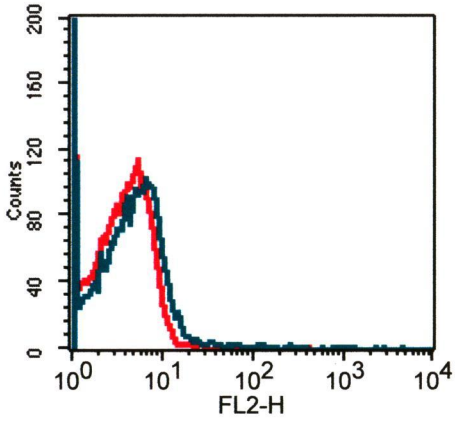

5
4
3
2
1
0
A_{485}
unlabelled anti-CCR3
biotinylated anti-CCR3
coating antibody

Figure 3.14. *Comparison of CCR3 staining on lymphocytes and monocytes.* PMBC were purified, labelled with anti-CCR3 or an isotype-matched negative control antibody and analysed by flow cytometry. Lymphocytes and monocytes were respectively gated using forward and side scatter characteristics and the level of staining with the control antibody (red) or anti-CCR3 (green) assessed for each population. Data shown are representative of 3 experiments.



[A] *lymphocytes*



[B] *monocytes*

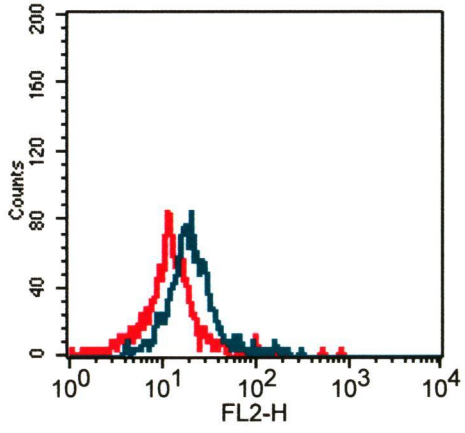
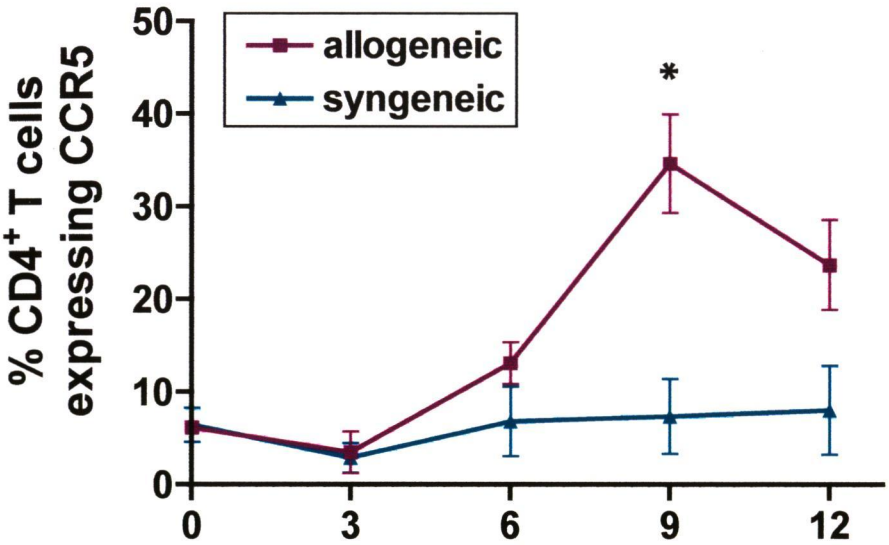


Figure 3.15. *CCR5 expression on $CD4^+$ T cells in allogeneic and syngeneic MLR cultures: time-course.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. At the initiation of culture, or after the indicated period of time, cells were labelled with antibodies to CCR5 and CD4 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the percentage of cells positive for CCR5 determined in comparison to staining with an isotype-matched control antibody. In [A], the percentage of $CD4^+$ T cells that expressed detectable levels of CCR5 were calculated as a proportion of total $CD4^+$ T cells. In [B], the geometric mean of CCR5 fluorescence intensity was determined for the $CD4^+$ $CCR5^+$ population. A statistically significant difference between allogeneic and syngeneic conditions is indicated by an asterisk. Data are presented as mean $\pm$ SEM ($n \geq 4$ at each time-point).

[A]


allogeneic
syngeneic
% CD4+ T cells expressing CCR5
50
40
30
20
10
0
0
3
6
9
12
*

[B]

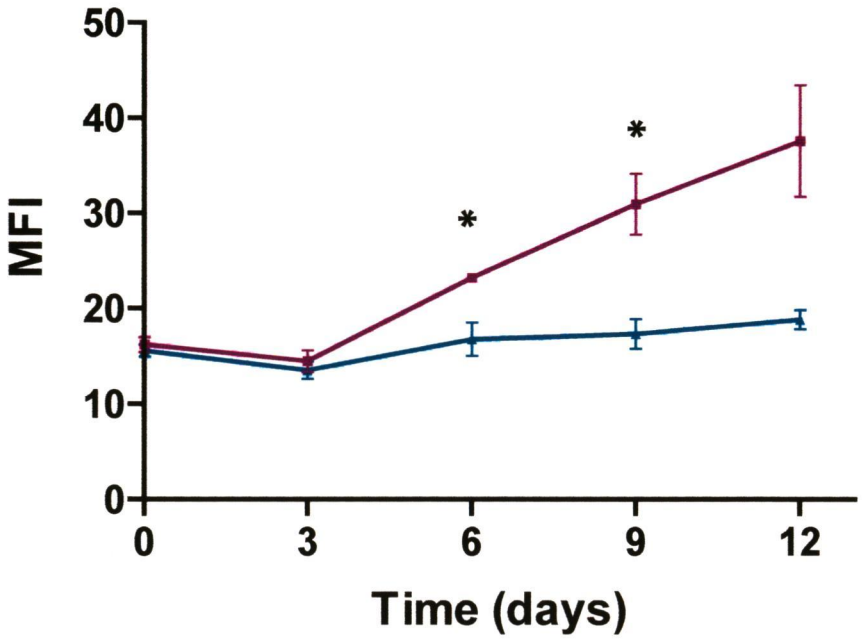
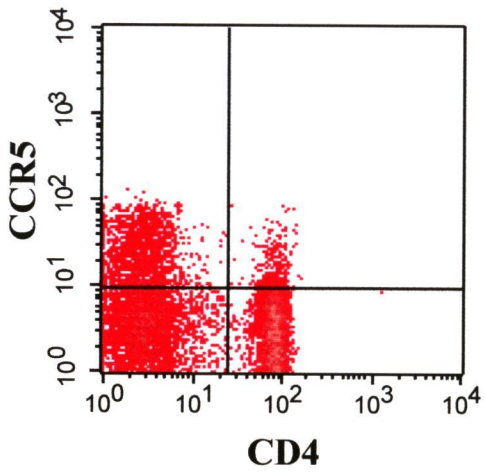
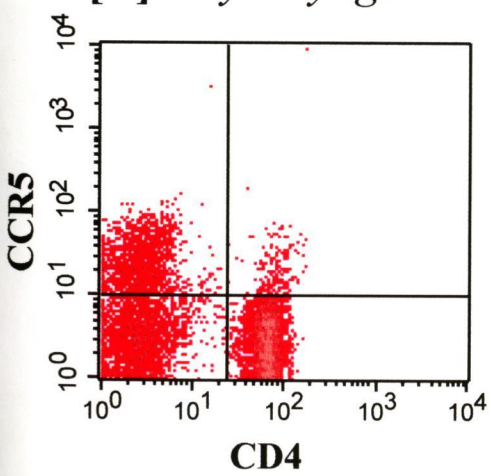

MFI
50
40
30
20
10
0
0
3
6
9
12
Time (days)
*
*

Figure 3.16. *Expression of CCR5 on $CD4^+$ T cells in allogeneic and syngeneic MLR cultures: representative density plots.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 days of culture under syngeneic [B] or allogeneic [C] conditions, cells were labelled with antibodies to CCR5 and CD4 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 4 experiments.

[A] *Day 0*



[B] *Day 9 syngeneic*



[C] *Day 9 allogeneic*

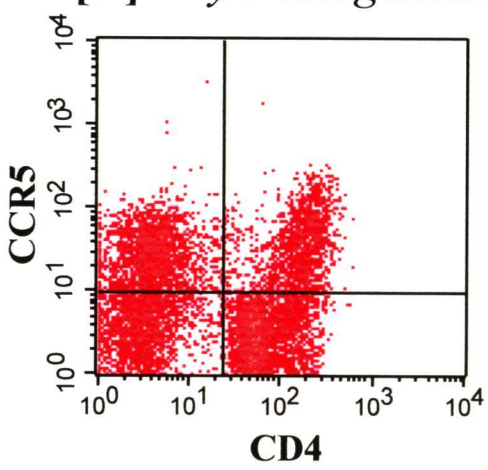
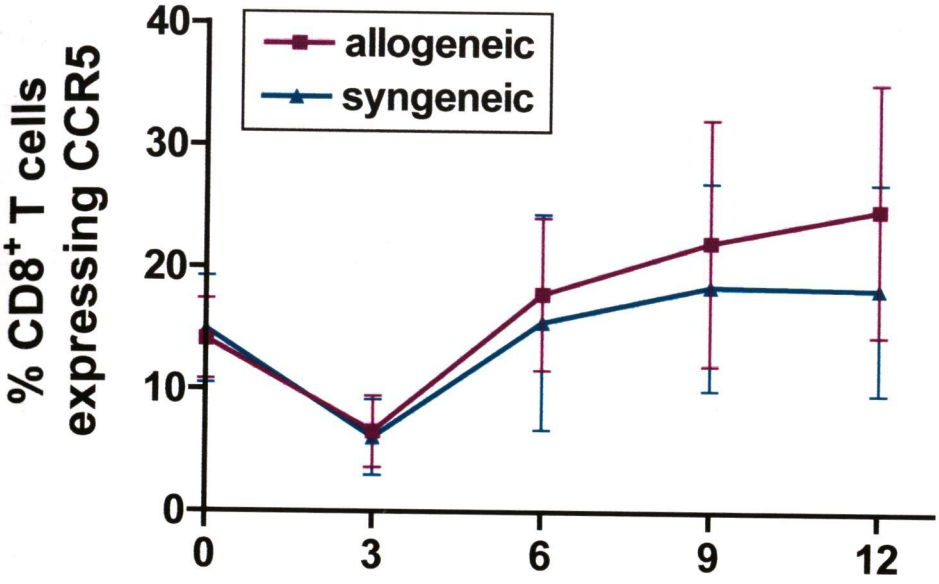


Figure 3.17. *CCR5 expression on $CD8^+$ T cells in allogeneic and syngeneic MLR cultures: time-course.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. At the initiation of culture, or after the indicated period of time, cells were labelled with antibodies to CCR5 and CD8 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the percentage of cells positive for CCR5 determined in comparison to staining with an isotype-matched control antibody. In [A], the percentage of $CD8^+$ T cells that expressed detectable levels of CCR5 were calculated as a proportion of total $CD8^+$ T cells. In [B], the geometric mean of CCR5 fluorescence intensity was determined for the $CD8^+$ $CCR5^+$ population. Data are presented as mean $\pm$ SEM (n = 4 at each time-point).

[A]


% CD8+ T cells expressing CCR5
allogeneic
syngeneic
40
30
20
10
0
0
3
6
9
12

[B]

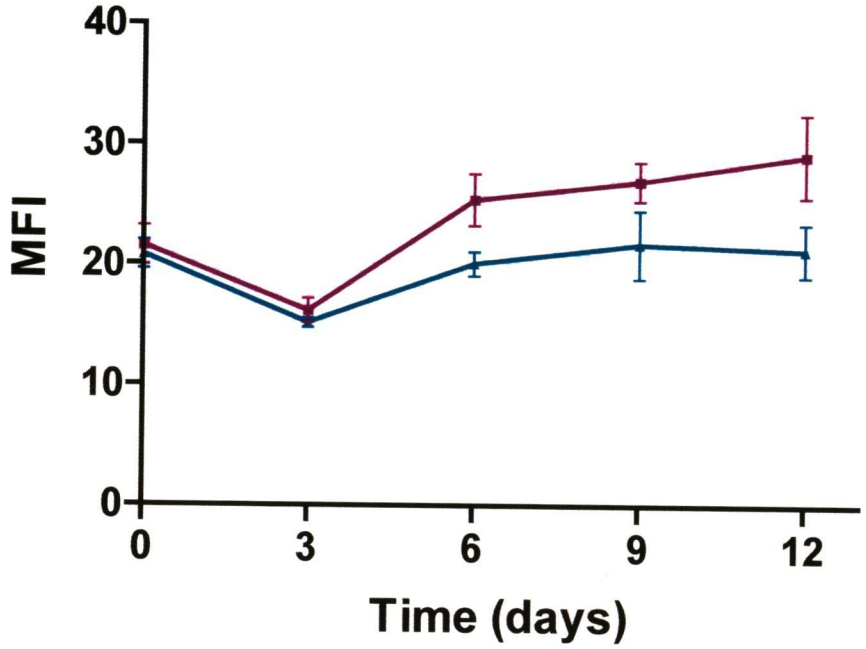

MFI
40
30
20
10
0
0
3
6
9
12
Time (days)

Figure 3.18. *Expression of CCR5 on $CD8^+$ T cells in allogeneic and syngeneic MLR cultures: representative density plots.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 days of culture under syngeneic [B] or allogeneic [C] conditions, cells were labelled with antibodies to CCR5 and CD8 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 4 experiments.

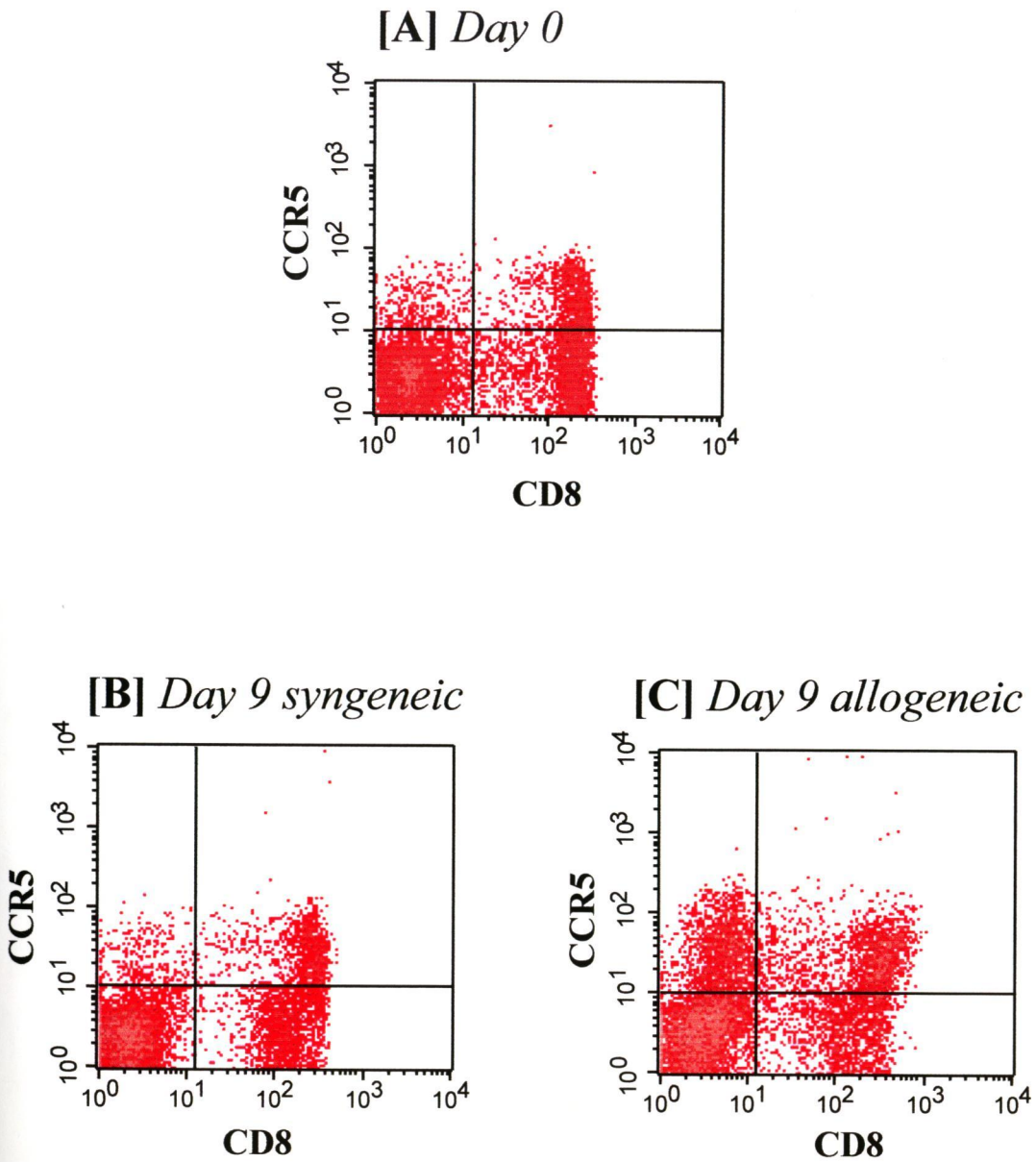
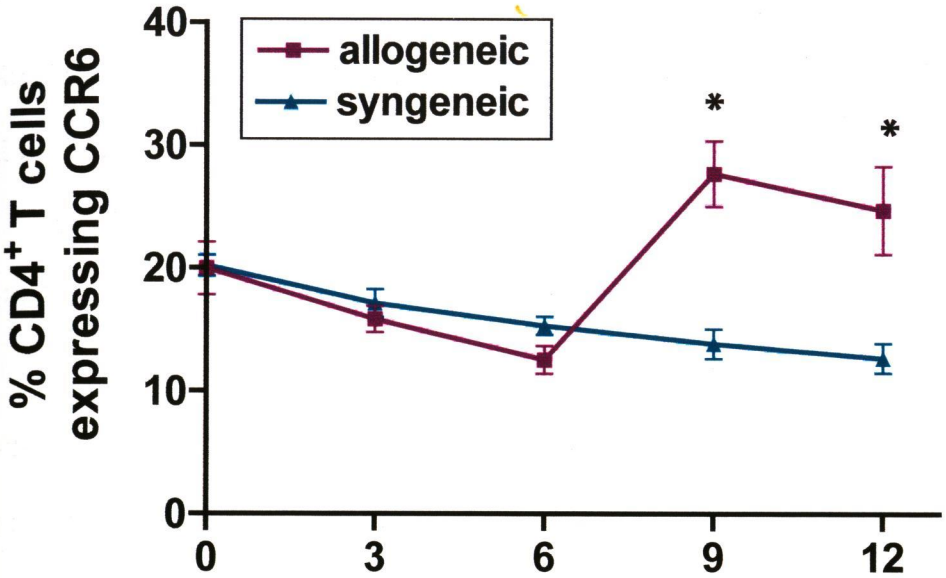

[A] Day 0
CCR5
CD8
10^0
10^1
10^2
10^3
10^4
[B] Day 9 syngeneic
CCR5
CD8
[C] Day 9 allogeneic
CCR5
CD8

Figure 3.19. *CCR6 expression on $CD4^+$ T cells in allogeneic and syngeneic MLR cultures: time-course.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. At the initiation of culture, or after the indicated period of time, cells were labelled with antibodies to CCR6 and CD4 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the percentage of cells positive for CCR6 determined in comparison to staining with an isotype-matched control antibody. In [A], the percentage of $CD4^+$ T cells that expressed detectable levels of CCR6 were calculated as a proportion of total $CD4^+$ T cells. In [B], the geometric mean of CCR6 fluorescence intensity was determined for the $CD4^+$ $CCR6^+$ population. A statistically significant difference between allogeneic and syngeneic conditions is indicated by an asterisk. Data are presented as mean $\pm$ SEM ($n \geq 4$ at each time-point).

[A]


allogeneic
syngeneic
% CD4+ T cells expressing CCR6
40
30
20
10
0
0
3
6
9
12
*
*

[B]

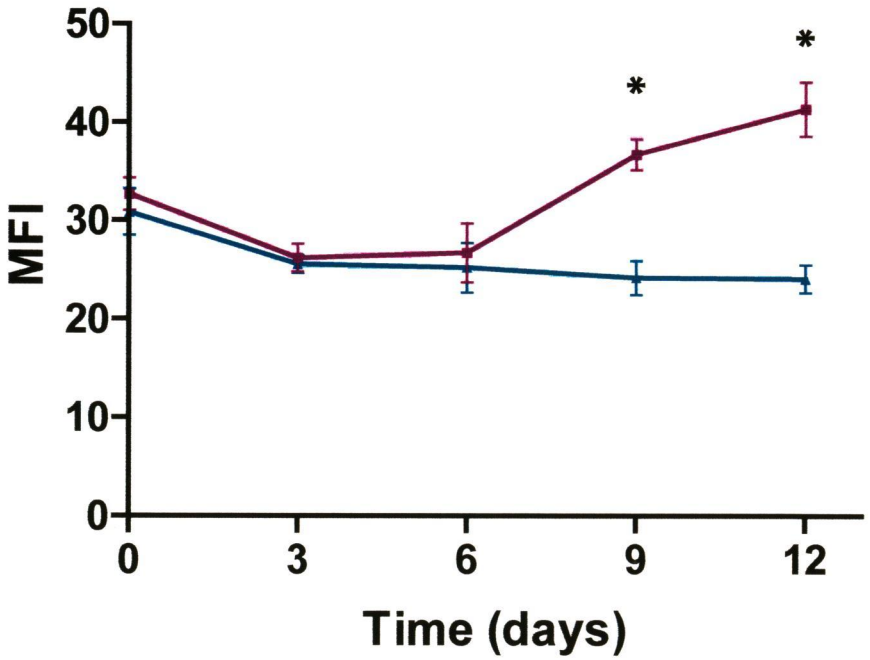
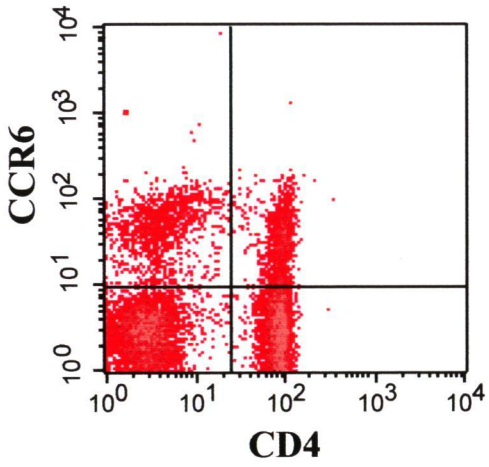
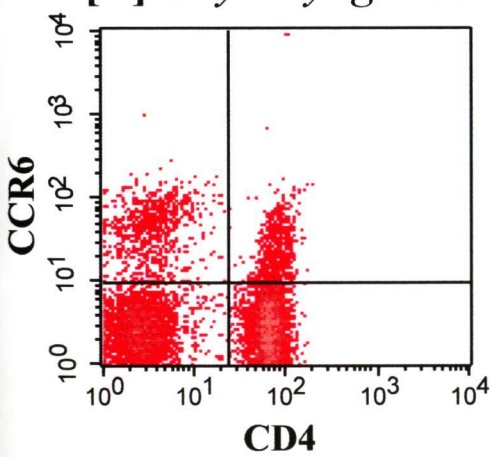

MFI
50
40
30
20
10
0
0
3
6
9
12
Time (days)
*
*

Figure 3.20. *Expression of CCR6 on $CD4^+$ T cells in allogeneic and syngeneic MLR cultures: representative density plots.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 days of culture under syngeneic [B] or allogeneic [C] conditions, cells were labelled with antibodies to CCR6 and CD4 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 4 experiments.

[A] *Day 0*



[B] *Day 9 syngeneic*



[C] *Day 9 allogeneic*

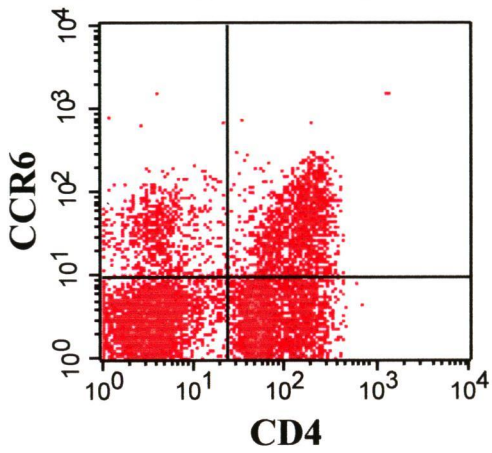
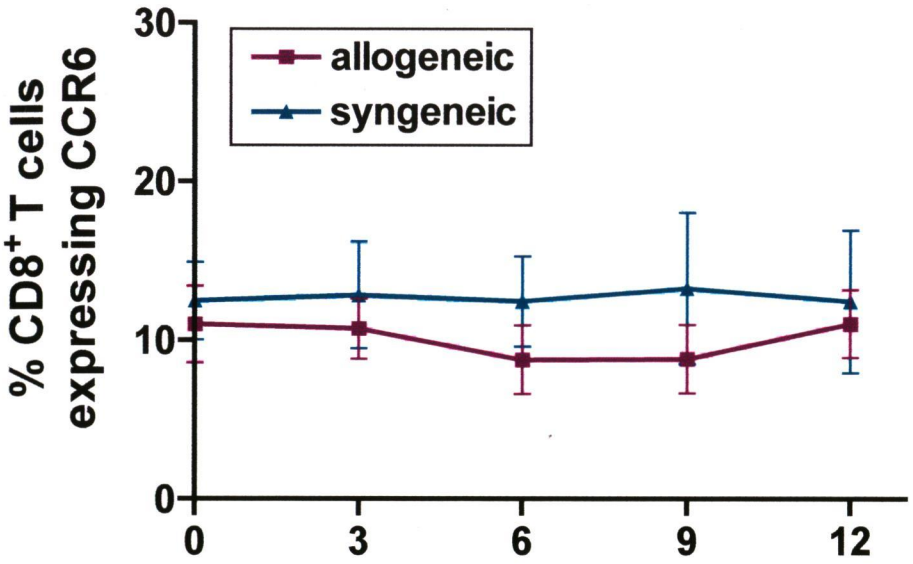


Figure 3.21. *CCR6 expression on $CD8^+$ T cells in allogeneic and syngeneic MLR cultures: time-course.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. At the initiation of culture, or after the indicated period of time, cells were labelled with antibodies to CCR6 and CD8 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the percentage of cells positive for CCR6 determined in comparison to staining with an isotype-matched control antibody. In [A], the percentage of $CD8^+$ T cells that expressed detectable levels of CCR6 were calculated as a proportion of total $CD8^+$ T cells. In [B], the geometric mean of CCR6 fluorescence intensity was determined for the $CD8^+$ $CCR6^+$ population. A statistically significant difference between allogeneic and syngeneic conditions is indicated by an asterisk. Data are presented as mean $\pm$ SEM (n = 4 at each time-point).

[A]


30
20
10
0
% CD8+ T cells expressing CCR6
allogeneic
syngeneic
0
3
6
9
12

[B]

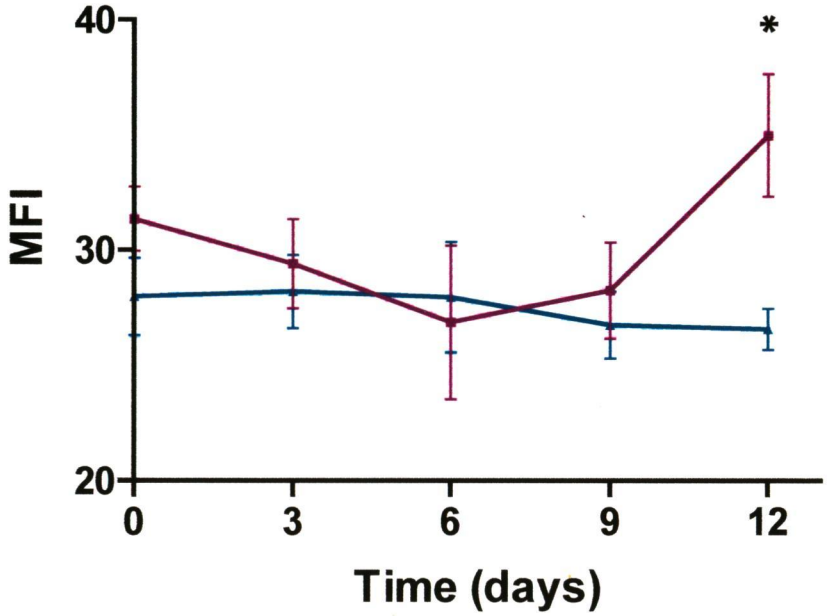
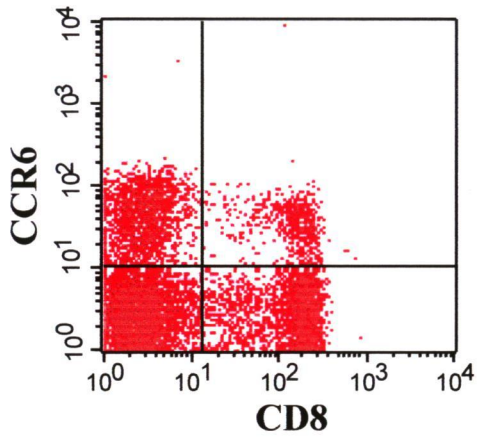
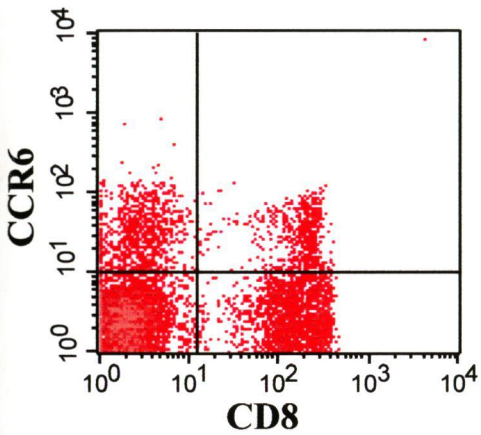

40
30
20
MFI
*
0
3
6
9
12
Time (days)

Figure 3.22. *Expression of CCR6 on $CD8^+$ T cells in allogeneic and syngeneic MLR cultures: representative density plots.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 [B-C] or 12 [D-E] days of culture under either syngeneic [B and D] or allogeneic [C and E] conditions, cells were labelled with antibodies to CCR6 and CD8 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 4 experiments.

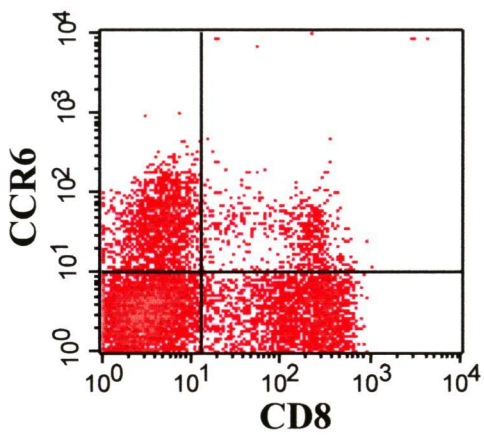
[A] *Day 0*



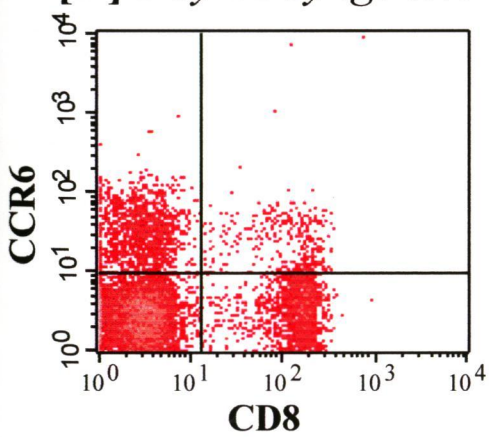
[B] *Day 9 syngeneic*



[C] *Day 9 allogeneic*



[D] *Day 12 syngeneic*



[E] *Day 12 allogeneic*

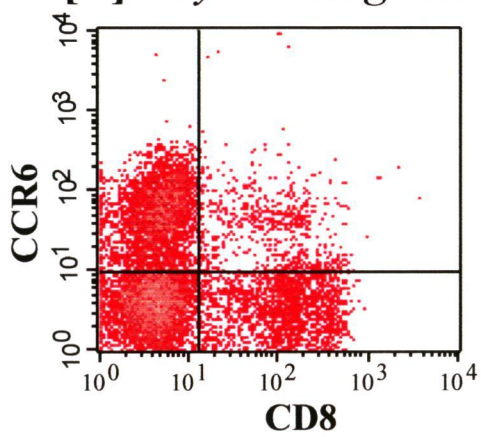
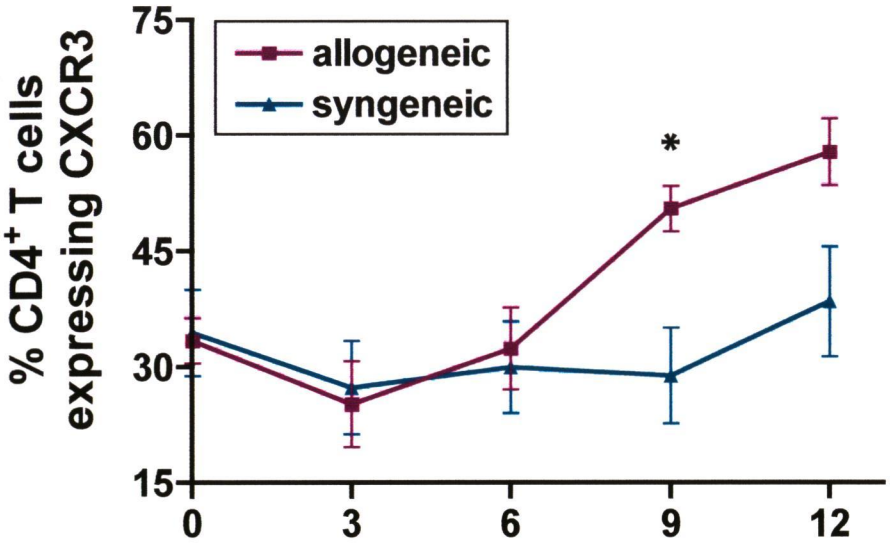


Figure 3.23 *CXCR3 expression on $CD4^+$ T cells in allogeneic and syngeneic MLR cultures: time-course.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. At the initiation of culture, or after the indicated period of time, cells were labelled with antibodies to CXCR3 and CD4 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the percentage of cells positive for CXCR3 determined in comparison to staining with an isotype-matched control antibody. In [A], the percentage of $CD4^+$ T cells that expressed detectable levels of CXCR3 were calculated as a proportion of total $CD4^+$ T cells. In [B], the geometric mean of CXCR3 fluorescence intensity was determined for the $CD4^+$ $CXCR3^+$ population. A statistically significant difference between allogeneic and syngeneic conditions is indicated by an asterisk. Data are presented as mean $\pm$ SEM (n = 4-5 at each time-point).

[A]


allogeneic
syngeneic
% CD4+ T cells expressing CXCR3
75
60
45
30
15
0
3
6
9
12
*

[B]

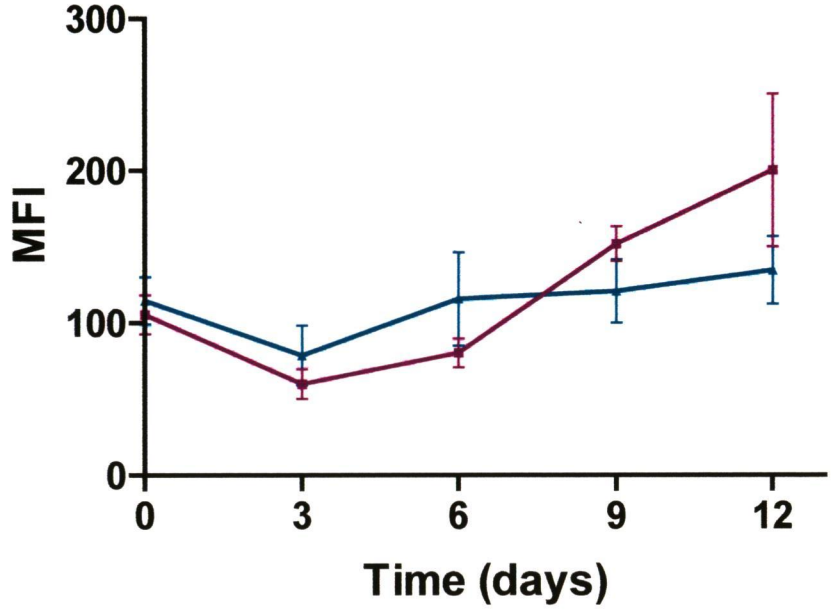
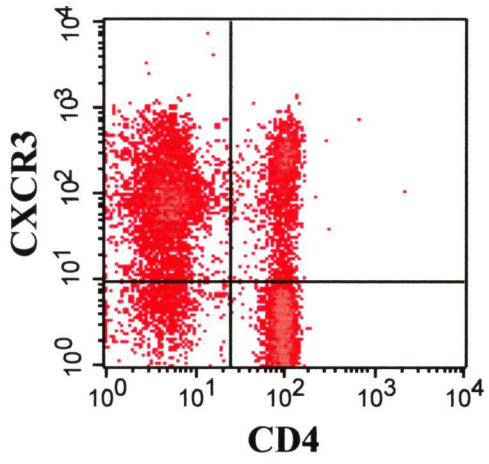
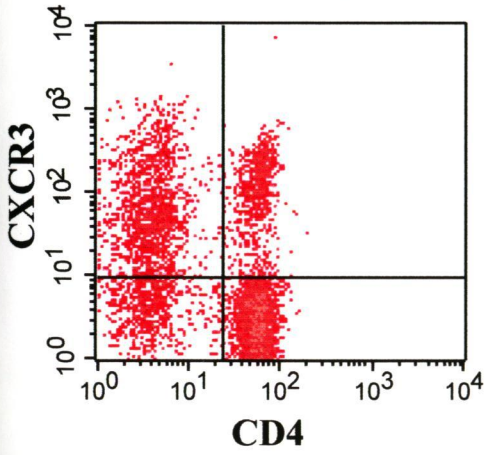

MFI
300
200
100
0
0
3
6
9
12
Time (days)

Figure 3.24. *Expression of CXCR3 on $CD4^+$ T cells in allogeneic and syngeneic MLR cultures: representative density plots.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 days of culture under syngeneic [B] or allogeneic [C] conditions, cells were labelled with antibodies to CXCR3 and CD4 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 4 experiments.

[A] *Day 0*



[B] *Day 9 syngeneic*



[C] *Day 9 allogeneic*

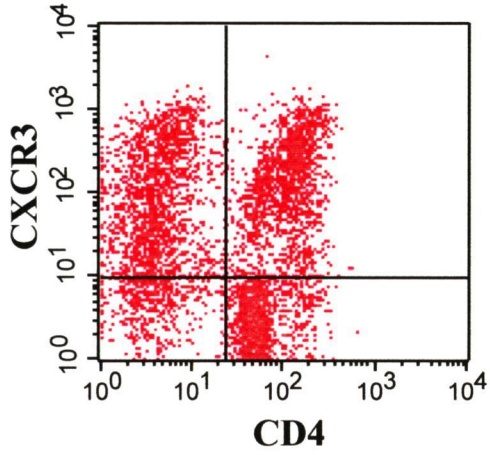
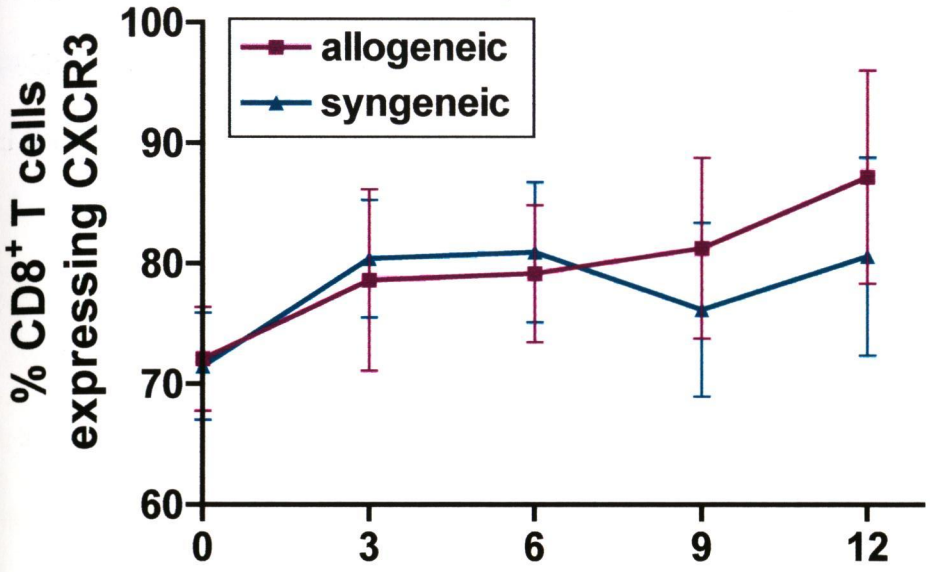


Figure 3.25. *CXCR3 expression on $CD8^+$ T cells in allogeneic and syngeneic MLR cultures: time-course.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. At the initiation of culture, or after the indicated period of time, cells were labelled with antibodies to CXCR3 and CD8 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the percentage of cells positive for CXCR3 determined in comparison to staining with an isotype-matched control antibody. In [A], the percentage of $CD8^+$ T cells that expressed detectable levels of CXCR3 were calculated as a proportion of total $CD8^+$ T cells. In [B], the geometric mean of CXCR3 fluorescence intensity was determined for the $CD8^+$ $CXCR3^+$ population. Data are presented as mean $\pm$ SEM (n = 4-5 at each time-point).

[A]


% CD8+ T cells expressing CXCR3
100
90
80
70
60
allogeneic
syngeneic
0
3
6
9
12

[B]

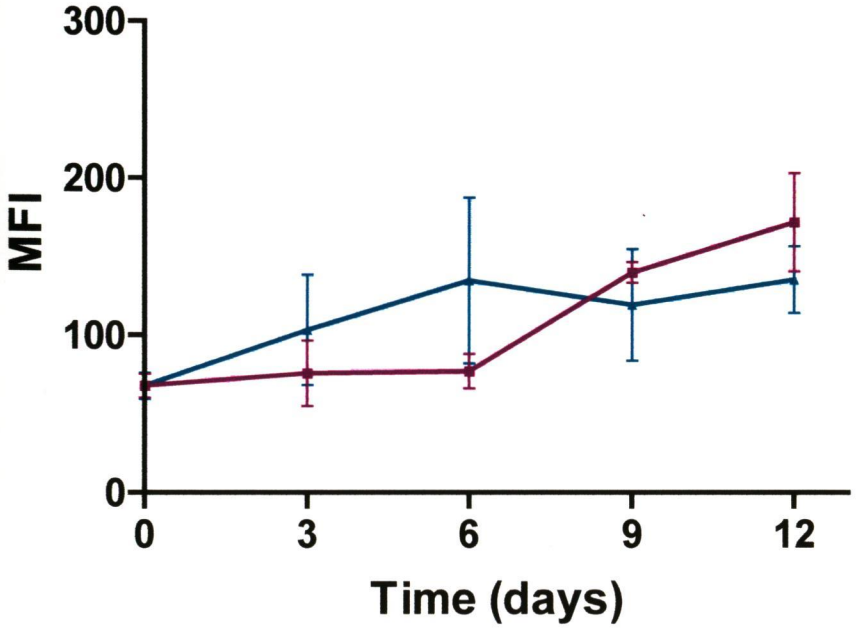
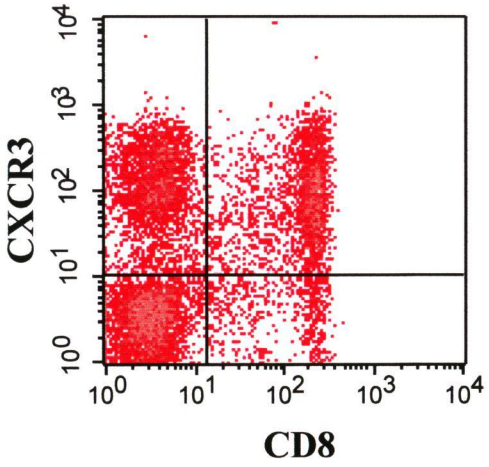
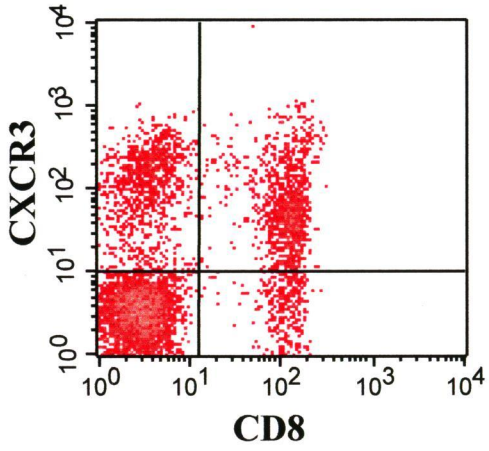

MFI
300
200
100
0
0
3
6
9
12
Time (days)

Figure 3.26. *Expression of CXCR3 on $CD8^+$ T cells in allogeneic and syngeneic MLR cultures: representative density plots.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 days of culture under syngeneic [B] or allogeneic [C] conditions, cells were labelled with antibodies to CXCR3 and CD8 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 5 experiments.

[A] *Day 0*



[B] *Day 9 syngeneic*



[C] *Day 9 allogeneic*

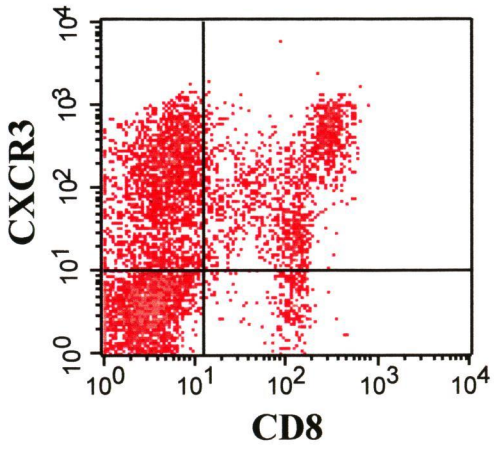
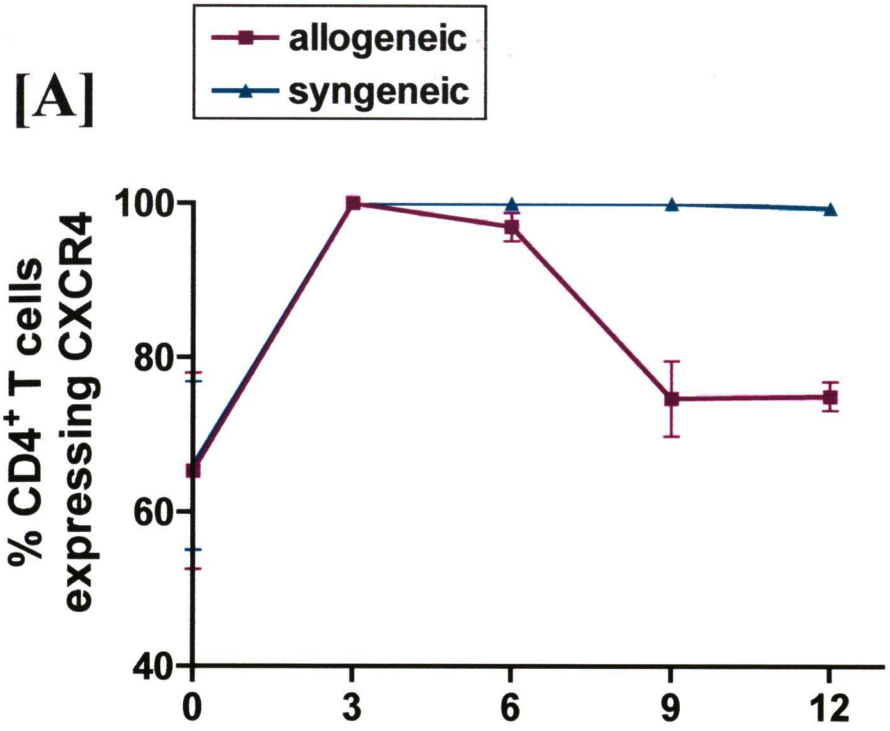


Figure 3.27 *CXCR4 expression on $CD4^+$ T cells in allogeneic and syngeneic MLR cultures: time-course.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. At the initiation of culture, or after the indicated period of time, cells were labelled with antibodies to CXCR4 and CD4 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the percentage of cells positive for CXCR4 determined in comparison to staining with an isotype-matched control antibody. In [A], the percentage of $CD4^+$ T cells that expressed detectable levels of CXCR4 were calculated as a proportion of total $CD4^+$ T cells. In [B], the geometric mean of CXCR4 fluorescence intensity was determined for the $CD4^+$ $CXCR4^+$ population. Data are presented as mean $\pm$ SEM (n = 4-5 at each time-point). It was not possible to perform T-test calculations on this data, as the uniformity of the syngeneic values excluded the calculation of error values.

[A]


allogeneic
syngeneic
% CD4+ T cells expressing CXCR4
100
80
60
40
0
3
6
9
12

[B]

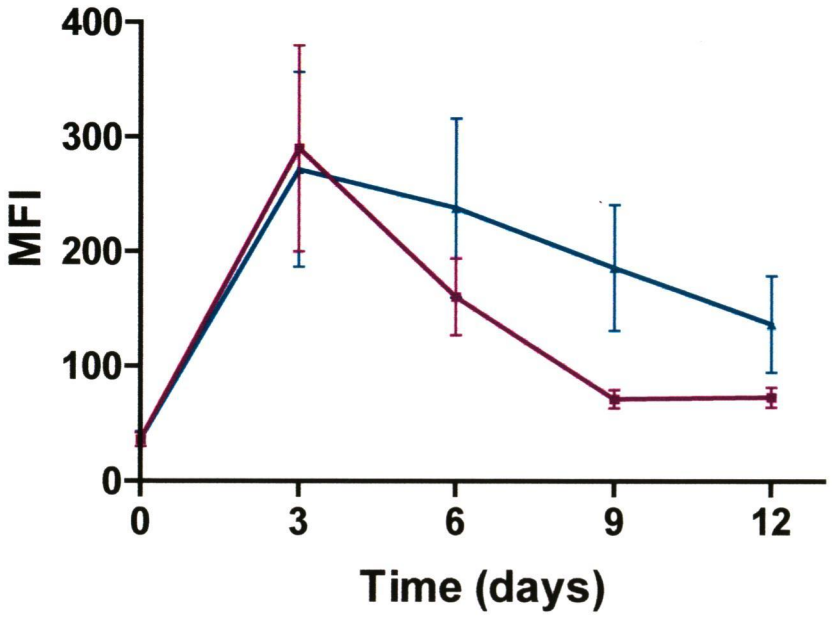
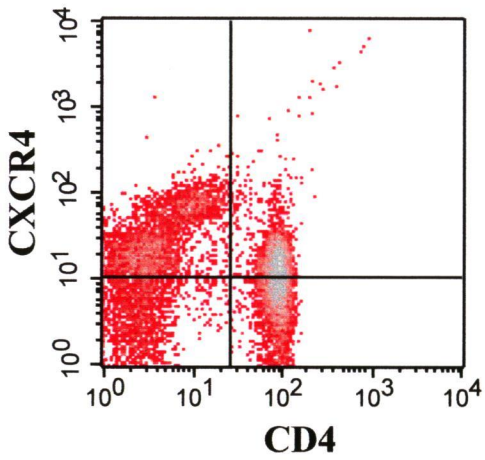
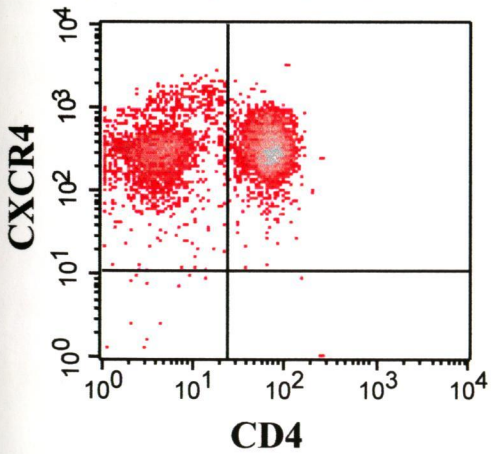

MFI
400
300
200
100
0
0
3
6
9
12
Time (days)

Figure 3.28. *Expression of CXCR4 on $CD4^+$ T cells in allogeneic and syngeneic MLR cultures: representative density plots.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 days of culture under syngeneic [B] or allogeneic [C] conditions, cells were labelled with antibodies to CXCR4 and CD4 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 4 experiments.

[A] *Day 0*



[B] *Day 9 syngeneic*



[C] *Day 9 allogeneic*

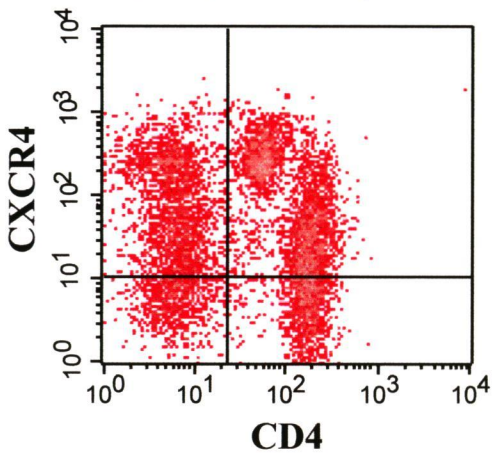


Figure 3.29. *CXCR4 expression on $CD8^+$ T cells in allogeneic and syngeneic MLR cultures: time-course.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. At the initiation of culture, or after the indicated period of time, cells were labelled with antibodies to CXCR4 and CD8 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the percentage of cells positive for CXCR4 determined in comparison to staining with an isotype-matched control antibody. In [A], the percentage of $CD8^+$ T cells that expressed detectable levels of CXCR4 were calculated as a proportion of total $CD8^+$ T cells. In [B], the geometric mean of CXCR4 fluorescence intensity was determined for the $CD8^+$ $CXCR4^+$ population. Data are presented as mean $\pm$ SEM (n = 4-5 at each time-point). It was not possible to perform T-test calculations on this data, as the uniformity of the syngeneic values excluded the calculation of error values.

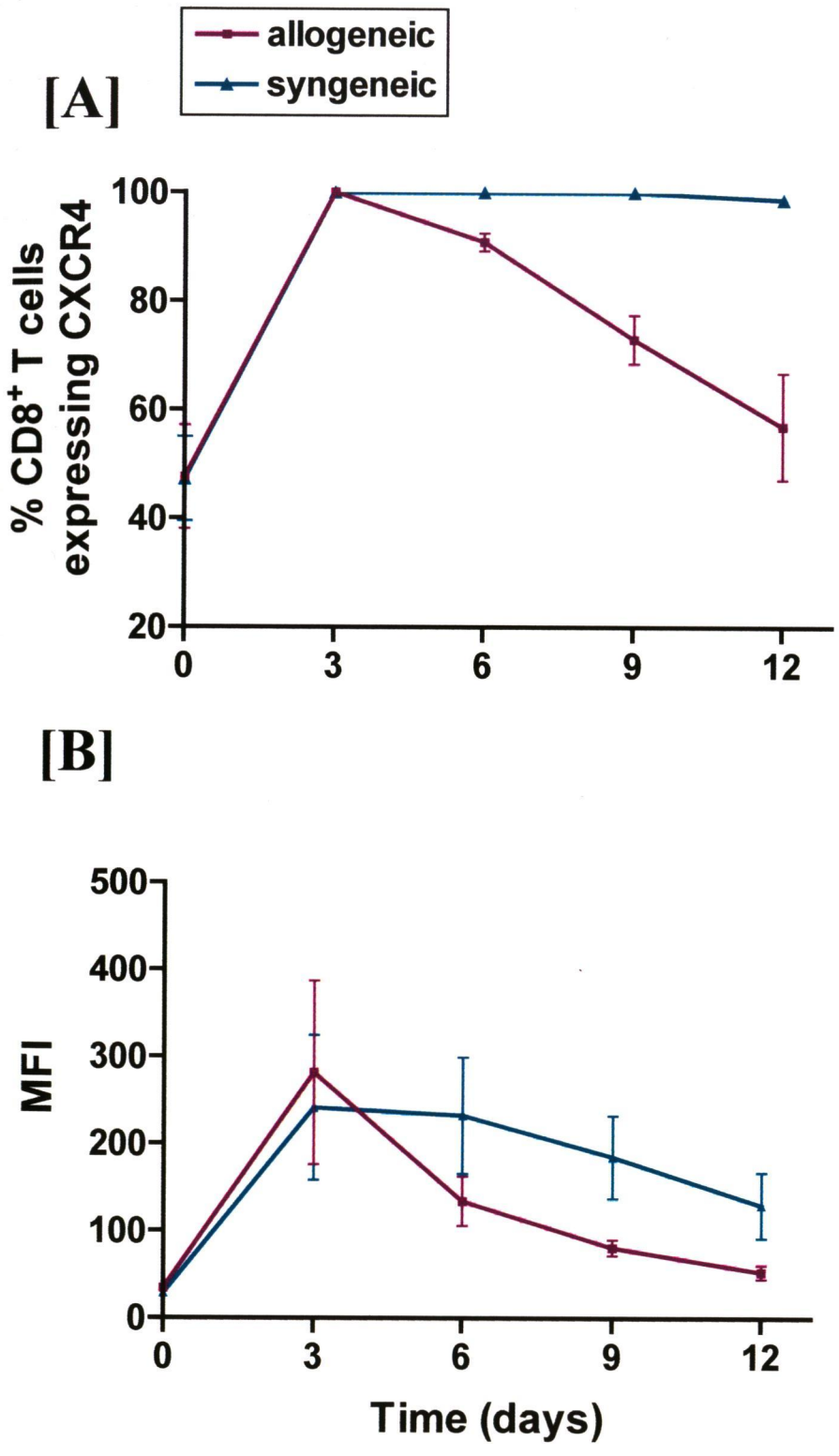
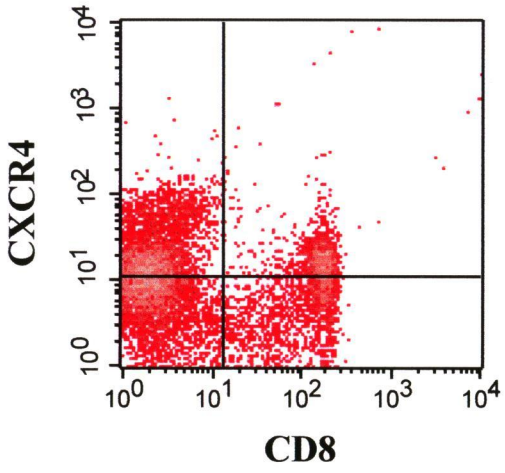
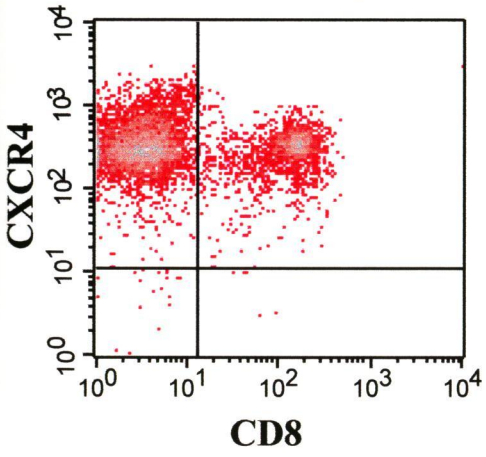

[A]
allogeneic
syngeneic
% CD8+ T cells expressing CXCR4
100
80
60
40
20
0
3
6
9
12
[B]
MFI
500
400
300
200
100
0
0
3
6
9
12
Time (days)

Figure 3.30. *Expression of CXCR4 on $CD8^+$ T cells in allogeneic and syngeneic MLR cultures: representative density plots.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 days of culture under syngeneic [B] or allogeneic [C] conditions, cells were labelled with antibodies to CXCR4 and CD8 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 5 experiments.

[A] *Day 0*



[B] *Day 9 syngeneic*



[C] *Day 9 allogeneic*

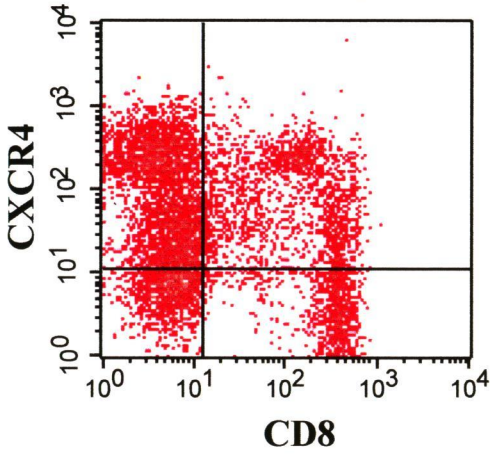
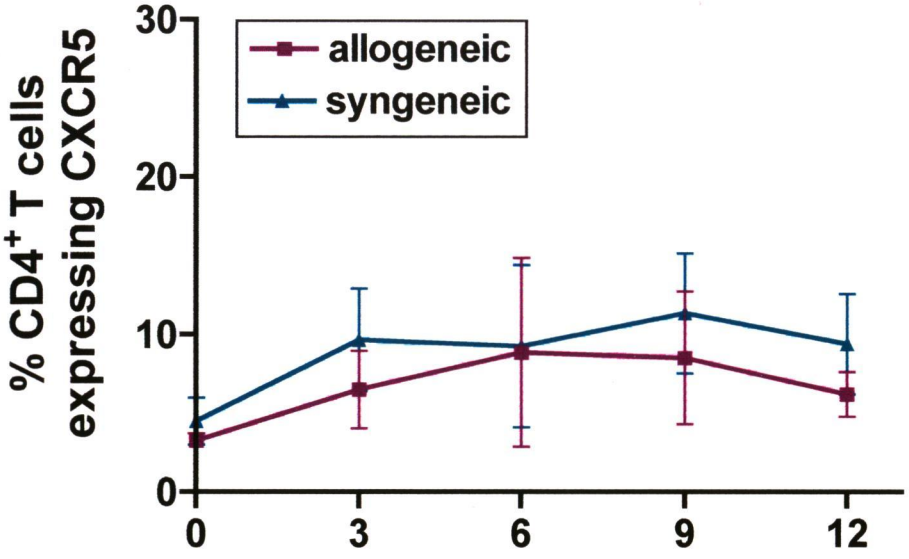


Figure 3.31 *CXCR5 expression on $CD4^+$ T cells in allogeneic and syngeneic MLR cultures: time-course.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. At the initiation of culture, or after the indicated period of time, cells were labelled with antibodies to CXCR5 and CD4 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the percentage of cells positive for CXCR5 determined in comparison to staining with an isotype-matched control antibody. In [A], the percentage of $CD4^+$ T cells that expressed detectable levels of CXCR5 were calculated as a proportion of total $CD4^+$ T cells. In [B], the geometric mean of CXCR5 fluorescence intensity was determined for the $CD4^+$ $CXCR5^+$ population. Data are presented as mean $\pm$ SEM (n = 3-4 at each time-point).

[A]


% CD4+ T cells expressing CXCR5
allogeneic
syngeneic
30
20
10
0
0
3
6
9
12

[B]

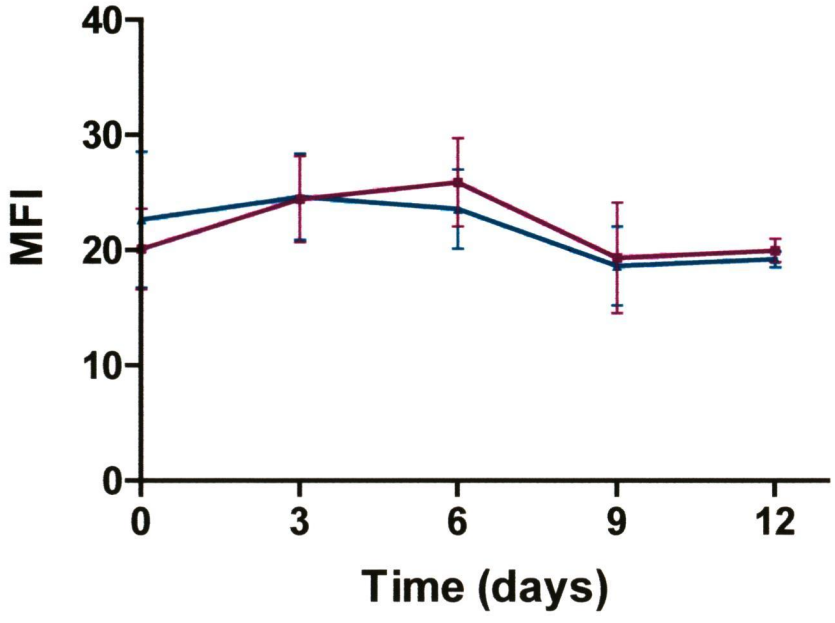
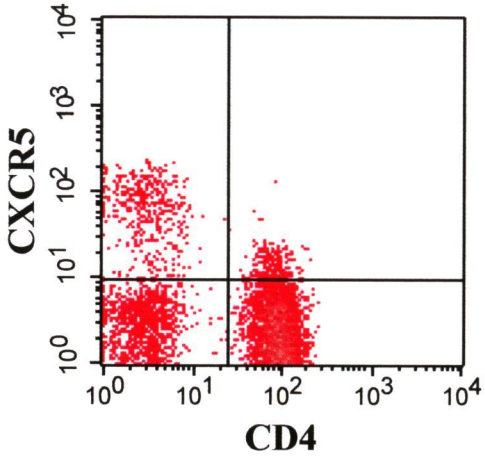
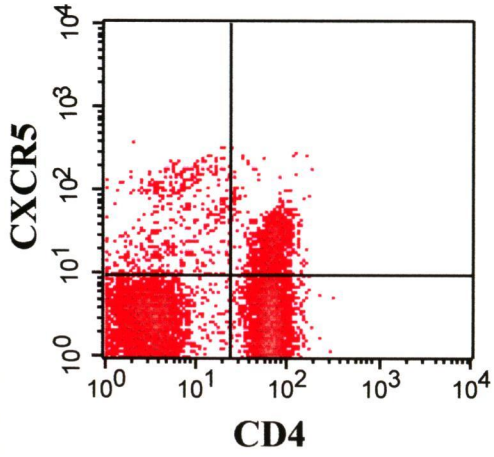

MFI
40
30
20
10
0
0
3
6
9
12
Time (days)

Figure 3.32. *Expression of CXCR5 on $CD4^+$ T cells in allogeneic and syngeneic MLR cultures: representative density plots.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 days of culture under syngeneic [B] or allogeneic [C] conditions, cells were labelled with antibodies to CXCR5 and CD4 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 4 experiments.

[A] *Day 0*



[B] *Day 9 syngeneic*



[C] *Day 9 allogeneic*

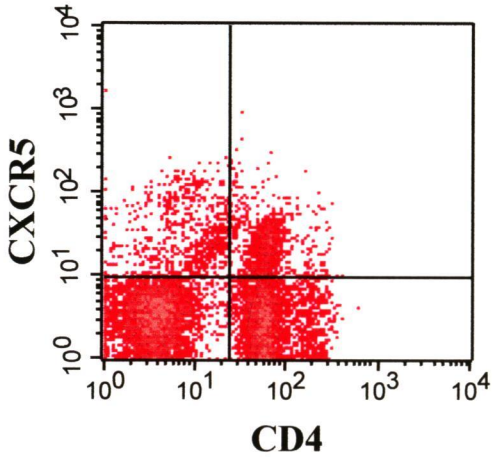
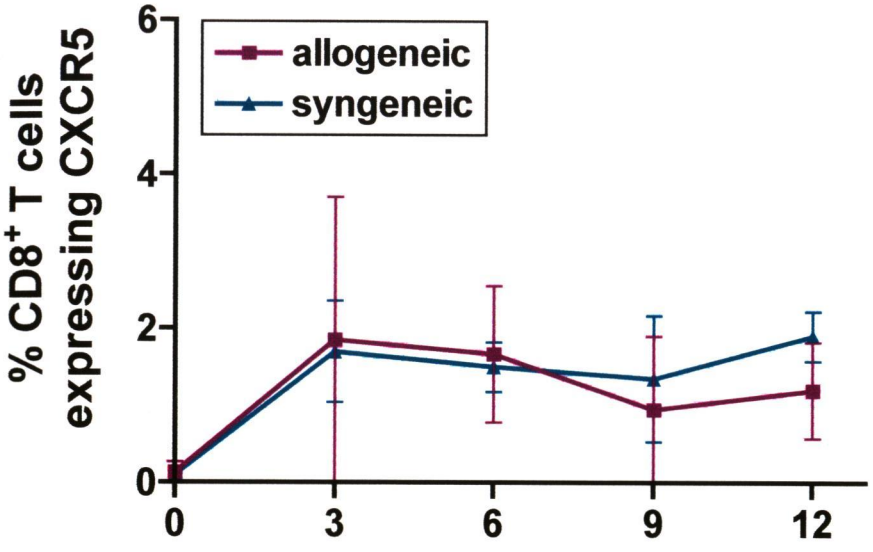


Figure 3.33. *CXCR5 expression on $CD8^+$ T cells in allogeneic and syngeneic MLR cultures: time-course.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. At the initiation of culture, or after the indicated period of time, cells were labelled with antibodies to CXCR5 and CD8 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the percentage of cells positive for CXCR5 determined in comparison to staining with an isotype-matched control antibody. In [A], the percentage of $CD8^+$ T cells that expressed detectable levels of CXCR5 were calculated as a proportion of total $CD8^+$ T cells. In [B], the geometric mean of CXCR5 fluorescence intensity was determined for the $CD8^+$ $CXCR5^+$ population. A statistically significant difference between allogeneic and syngeneic conditions is indicated by an asterisk.. Data are presented as mean $\pm$ SEM (n = 3-4 at each time-point).

[A]


% CD8+ T cells expressing CXCR5
allogeneic
syngeneic
6
4
2
0
0
3
6
9
12

[B]

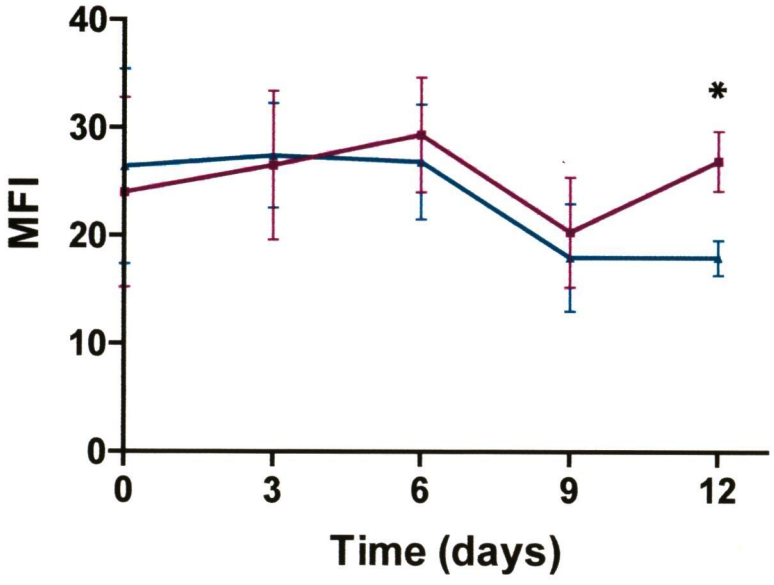
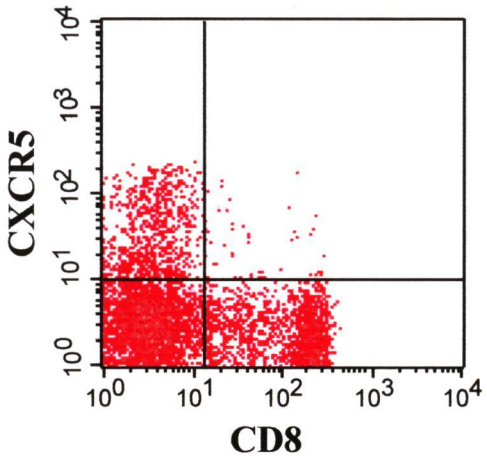
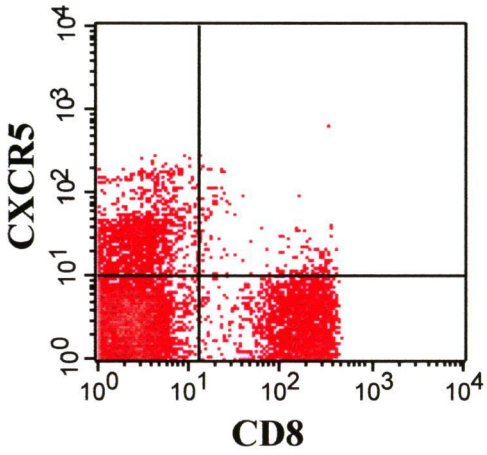

MFI
40
30
20
10
0
*
0
3
6
9
12
Time (days)

Figure 3.34. *Expression of CXCR5 on $CD8^+$ T cells in allogeneic and syngeneic MLR cultures: representative density plots.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures [A], or after 9 days of culture under syngeneic [B] or allogeneic [C] conditions, cells were labelled with antibodies to CXCR5 and CD8 and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with an isotype-matched negative control antibody. Data shown are representative of 4 experiments.

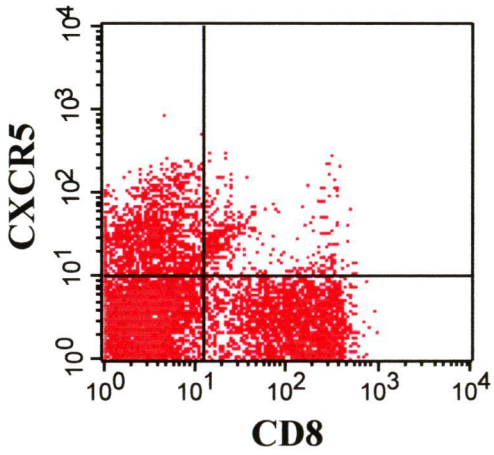
[A] *Day 0*



[B] *Day 9 syngeneic*



[C] *Day 9 allogeneic*



CHAPTER 4

DETAILED CHARACTERISATION OF CCR5, CCR6, CXCR3 AND CXCR4 EXPRESSION AND FUNCTION ON ACTIVATED $CD4^+$ T CELLS

Summary – Chapter 4

- Elevated expression of CD4 was shown to be a reliable marker of $CD4^+$ T cell activation in the MLR.
- The modulation of chemokine receptor expression on allo-activated $CD4^+$ T cells was closely correlated with a range of markers of T cell memory and activation, including the $CD4^{hi}$ phenotype.
- The modulation of CXCR3 and CXCR4 expression occurred in close parallel with the cell division process, while the up-regulation of CCR5 and CCR6 expression was more dependent on time in culture.
- The pattern of chemokine receptor expression acquired within the first 9 days was retained for extended periods of time.
- The observed changes in cell surface chemokine receptor expression were paralleled by altered levels of transcripts for CCR5, CCR6 and CXCR3, but not CXCR4.
- Stores of CCR6 and CXCR3 receptor protein could be detected intracellularly, while CCR5 and CXCR4 were localised exclusively to the cell surface.
- Allogeneic activation of T cells resulted in altered chemotactic responsiveness toward the ligands for CCR5, CXCR3 and CXCR4, but not the ligand for CCR6.
- In accord with the results using human lymphocytes, the up-regulation of CCR5 and CXCR3 expression also occurred on murine $CD4^+$ T cells. However, no significant modulation of CCR6 or CXCR4 could be detected in the murine system.

4.1 Introduction

The aim of the experiments presented in Chapter 3 was to identify chemokine receptors that demonstrated altered patterns of expression on T cells following interaction with APCs in the MLR. The results of these experiments identified four chemokine receptors (CCR5, CCR6, CXCR3 and CXCR4) that demonstrated significant alterations in expression in allogeneic compared to syngeneic cultures. Accordingly, all further experimentation will be focussed upon these four receptors. Further, with the exception of CXCR4 modulation, the acquisition of novel patterns of chemokine receptor expression was a phenomenon restricted to the $CD4^+$ T cell population. Accordingly, all subsequent studies will specifically examine the regulation of chemokine receptor expression on the $CD4^+$ T cell population.

The aim of this present series of experiments was to examine in detail the regulation of CCR5, CCR6, CXCR3 and CXCR4 expression on $CD4^+$ T cells during allogeneic activation. A major goal of these studies was to determine the extent to which modulation of chemokine receptor expression was coordinated with the acquisition of an activated/memory T cell phenotype, as well as the process of cell division. Furthermore, studies were performed to determine whether the observed changes in chemokine receptor expression were controlled at the level of messenger RNA, protein trafficking, or both. Also, the biological outcome of altered patterns of chemokine receptor expression was explored, by comparing the chemotactic responsiveness of cells from allogeneic and syngeneic cultures to the relevant chemokine ligands. Finally, studies were conducted in the murine MLR system, to determine whether the observed patterns of receptor regulation would be conserved across the human-murine species barrier.

4.2 Results

4.2.1 Regulation of chemokine receptor expression is limited to CD4^{hi} T cells

4.2.1.1 Time-course of CD4 expression in the MLR

Experiments presented in the previous chapter demonstrated that the expression of CD4 and CD8 in allogeneic cultures was somewhat heterogeneous. Moreover, the divergent expression patterns noted on the CD4^{+} T cell population apparently correlated well with the level of expression of CCR5, CCR6, CXCR3 and CXCR4. Thus, it appears that CD4 is up-regulated on a sub-population of T cells in the MLR, and that this up-regulation is linked to the acquisition of novel patterns of chemokine receptor expression. As CD4 is not generally considered to be an activation marker, a series of experiments were designed to explore the phenomenon of CD4 up-regulation in more detail. First, a time-course analysis (Fig 4.1) demonstrated that the CD4^{hi} population developed with similar kinetics to the observed changes in chemokine receptor expression, being first detectable at day 6 and increasing dramatically by day 9. Of note, the CD4^{hi} population did not develop in syngeneic cultures (Fig 4.1 [G]), suggesting that the up-regulation of CD4 requires cellular activation. Furthermore, although distinct sub-populations of CD4^{normal} and CD4^{hi} T cells were not clearly discernible at very late time-points (day 24; Fig 4.1 [F]), the level of CD4 expression was still clearly higher than the level observed at day 0.

4.2.1.2 Confirmation that CD4^{hi} cells are T lymphocytes

As DCs and monocytes also express CD4, it was important to confirm that the CD4^{hi} cells were indeed T cells. Accordingly, cells from day 9 allogeneic MLR cultures were

stained with antibodies to CD3, a T cell specific marker, and CD4 (Fig 4.2). These experiments demonstrate that both the $CD4^{hi}$ and the $CD4^{normal}$ populations were 99.5% positive for CD3, thus confirming that $CD4^{+}$ cells, whether they expressed normal or elevated levels of CD4, were indeed T cells. Of interest, the histograms presented in Figure 4.2 suggest that the level of expression of CD3 was also slightly increased in the $CD4^{hi}$ population compared to the $CD4^{normal}$ population. However, it is likely that the slight apparent increase in CD3 staining intensity is mostly or entirely due to the increased level of autofluorescence that is inherent in populations of activated T cells, as will be discussed in more detail below.

4.2.1.3 Phenotypic analysis of $CD4^{hi}$ T cells

Further analysis of $CD4^{hi}$ T cells revealed that they have many characteristics of activated T cells. As summarised in Table 4.1, this population was characterised by a larger size (indicative of blast formation) and a memory phenotype, with the majority of $CD4^{hi}$ T cells staining positive for the memory T cell marker CD45RO yet negative for the naïve T cell marker CD45RA. Moreover, the $CD4^{hi}$ T cell population was greatly enriched in cells expressing the activation markers CD25 and CD69, compared to the $CD4^{normal}$ T cell population. Finally, the CFSE dye dilution technique demonstrated that the vast majority of $CD4^{hi}$ T cells had undergone division in the MLR. Thus, the up-regulation of CD4 is coupled to cell division, blast formation and the acquisition of an activated/memory phenotype.

4.2.1.4 The increase in CD4 staining intensity is not simply due to the increased cellular autofluorescence of activated T cells

The observation that $CD4^{hi}$ T cells were larger than $CD4^{normal}$ T cells is not surprising, given the activated phenotype of these cells. It does, however, raise the possibility that the apparent increase in CD4 expression is due simply to blast formation, which increases cell size and results in alterations to cellular cytoplasmic contents, both of which are likely to increase endogenous cellular autofluorescence. In order to assess the possibility that the apparent increase in CD4 expression was simply due to the increased autofluorescence resulting from blast formation, the autofluorescence of small and large lymphocytes from day 9 allogeneic MLR cultures was compared (Fig 4.3). The results demonstrate that there was indeed a small increase in autofluorescence within the large lymphocyte population compared to the small lymphocyte population, resulting in a slight rightward shift in the fluorescence intensity of unstained cells (Fig 4.3 [B]). However, when the cells were labelled with anti-CD4, the shift in fluorescence was greatly magnified (Fig 4.3 [C]). This difference is expressed numerically in the graph in Figure 4.3 [D], which shows the fluorescence intensity ratio of large lymphocytes over small lymphocytes. This analysis demonstrates that there was a statistically significant ($p = 0.0005$) increase in the fluorescence intensity ratio of cells stained with the anti-CD4 antibody, compared to unstained cells. Thus, while activated T cells are characterised by slightly higher levels of endogenous autofluorescence compared to resting T cells, this difference alone is not sufficient to account for the observed up-regulation of CD4 expression.

4.2.1.5 *Chemokine receptor expression on $CD4^{hi}$ versus $CD4^{normal}$ T cells*

A comparison of chemokine receptor expression on $CD4^{hi}$ T cells and $CD4^{normal}$ T cells in allogeneic cultures revealed that the up-regulation of CCR5, CCR6 and CXCR3, and the down-regulation of CXCR4, were events limited exclusively to the $CD4^{hi}$ T cell population (Fig 4.4). When the $CD4^{normal}$ and $CD4^{hi}$ populations from allogeneic cultures were compared, a statistically significant difference in the percentage of cells staining positive was observed for each of the four chemokine receptors (CCR5, $p < 0.01$; CCR6, $p < 0.001$; CXCR3, $p < 0.0001$; CXCR4, $p < 0.005$). Of note, the $CD4^{normal}$ T cells expressed an almost identical pattern of chemokine receptor expression to that observed on $CD4^+$ T cells from syngeneic cultures ($p > 0.05$ for all). Thus, the $CD4^{hi}$ phenotype marks a population of activated T cells that displays a distinct repertoire of chemokine receptor expression compared to that observed on non-activated ($CD4^{normal}$) bystander cells in the same cultures, or resting T cells from syngeneic cultures.

4.2.2 *Coordinated regulation of chemokine receptors and T cell memory/activation markers*

4.2.2.1 *Expression of memory/activation markers in allogeneic versus syngeneic cultures*

The studies discussed above provide clear evidence that the expression of certain chemokine receptors is co-ordinately regulated with the expression of CD4. Thus, it was of particular interest to determine the extent to which the expression of chemokine receptors was coordinated with the expression of other markers of T cell memory and activation. Initially, the percentage of $CD4^+$ T cells expressing various memory and activation markers was compared between day 0, day 9 allogeneic and day 9 syngeneic cultures, to confirm that allogeneic activation resulted in the development of a

population of $CD4^+$ T cells with the characteristics of activated lymphocytes. For these studies, CD45RA was used as a marker of naïve status, while reciprocal expression of CD45RO was used as an indicator of memory status (190). Two markers of cellular activation were used. The first, CD25, is the IL-2R α-chain, expressed during activation (191) but also present on a sub-population of memory T cells in peripheral blood (192, 193). The second, CD69, is almost undetectable on resting T cells, but rapidly up-regulated in response to activation (194).

Figure 4.5 demonstrates that the percentage of $CD4^+$ T cells expressing the naïve T cell marker CD45RA decreased significantly in allogeneic cultures over the 9-day incubation period ($p < 0.0005$), while the percentage expressing the memory T cell marker CD45RO was significantly increased ($p < 0.05$). These changes indicate the transition of a sub-population of $CD4^+$ T cells from naïve to memory status during allogeneic culture. It is unlikely that the increased proportion of memory cells is due to the selective outgrowth of existing memory cells, as both naïve and memory T cell subsets are known to respond in the MLR (163, 195, 196). No significant change to the expression of these markers was observed in syngeneic cultures. The two activation markers investigated, CD25 and CD69, were both up-regulated on a significant proportion of $CD4^+$ T cells in allogeneic cultures ($p < 0.001$ and $p < 0.0005$, respectively). There were, however, obvious differences between the expression patterns of these two markers, with CD25 being gained by a greater proportion of activated cells than CD69, and also being expressed on a larger fraction of the starting population. Neither activation marker was significantly altered on $CD4^+$ T cells in syngeneic cultures.

4.2.2.2 *Analysis of chemokine receptor and memory/activation marker co-expression*

Next, the extent to which these markers of memory and activation were co-expressed with chemokine receptors on $CD4^+$ T cells was assessed (Table 4.2 and Figs 4.6 – 4.7). As can be seen in Figure 4.6, the $CD45RA^-$ $CD45RO^+$ phenotype that is typical of memory cells correlated remarkably well with the observed patterns of chemokine receptor modulation. Thus, $CD4^+$ T cells that were positive for CCR5, CCR6 or CXCR3, or negative for CXCR4, generally expressed low to undetectable levels of the naïve marker CD45RA, but readily detectable levels of the memory marker CD45RO.

The expression of the activation markers CD25 and CD69 also correlated well with the expression of these chemokine receptors (Fig 4.7). The majority of $CD4^+$ T cells that were positive for CCR5, CCR6 or CXCR3, or negative for CXCR4, expressed CD25. The relationship between the expression of CD69 and chemokine receptors was not as clear, as a marked proportion of $CD4^+$ T cells that expressed CCR5, CCR6 or CXCR3, or had lost CXCR4, remained negative for this activation marker. However, in general, around half of the $CCR5^+$, $CCR6^+$, $CXCR3^+$ and $CXCR4^-$ cells expressed CD69 by day 9, indicating that this molecule was up-regulated to some extent by the cells that had modulated chemokine receptor expression patterns.

4.2.3 *Temporal aspects of chemokine receptor modulation in relation to the cell division process*

4.2.3.1 *Determining the number of rounds of cell division undertaken in culture using the CFSE dye dilution technique*

It was noted in Chapter 3 that the observed changes in chemokine receptor expression peaked at around day 9, in contrast to cellular proliferation (as determined by

incorporation of [^{3}H]-thymidine), which peaked at day 6. This contrast raised the possibility that these two processes may be temporally distinct. Thus, in order to determine the nature of the relationship between cell division and the observed changes in chemokine receptor expression, the CFSE dye dilution technique was used to compare chemokine receptor expression on sub-populations of $CD4^+$ T cells that had undergone a defined number of cell divisions. As this dye is diluted two-fold with every successive cell division (197), the number of divisions that an individual cell has undergone in culture can be determined.

Figure 4.8 [A] demonstrates that, by plotting CFSE fluorescence intensity against a measure of cell size (forward scatter), 8 populations of $CD4^+$ T cells could be defined after 6 days of culture in an allogeneic MLR. The population R1 represents undivided cells, which still contain maximal levels of CFSE and are relatively small, indicative of a resting state. Populations R2 to R7 represent cells that have undergone between 1 and 6 divisions, respectively. The cells within these populations are larger and contain progressively lower levels of the CFSE dye. The population contained in R8 represents cells that have undergone at least 7 divisions. However, due to the very low levels of dye contained by cells within this population it is not possible to further sub-divide this population according to the number of divisions. Figure 4.8 [B] demonstrates that the intensity of CFSE fluorescence decreases in a precise linear fashion with each successive round of cell division, as expected.

4.2.3.2 Comparison of chemokine receptor expression on $CD4^+$ T cells that have undergone a defined number of divisions

Allogeneic cultures of CFSE-labelled cells were analysed for chemokine receptor expression at the day 6 time-point, a time at which the cells were still actively

dividing, resulting in a flow cytometric profile akin to that presented in Figure 4.8. Accordingly, the percentage of $CD4^+$ T cells expressing a particular chemokine receptor could be individually determined for each of the populations R1 – R8. As a comparison, CFSE-labelled cells were also assessed at the day 9 time-point, to detect late changes in chemokine receptor expression. By this time, most cells had undergone 7 or more rounds of division, whereas only up to 6 divisions can be accurately enumerated using this technique. Accordingly, cells are simply designated as divided or undivided.

As shown in Figure 4.9 [A], the percentage of cells positive for CCR5 at day 6 increased progressively over the first two rounds of cell division (the difference between populations which had divided 0 and 2 times was statistically significant; $p < 0.005$), but then remained relatively stable over subsequent division cycles. Even after 7 or more rounds of cell division, there were no further increases in CCR5 expression at the day 6 time-point. In contrast, after another three days in culture (day 9), a striking increase in the percentage of CCR5-expressing cells was observed in the divided population ($p < 0.001$ compared to the undivided population). These observations suggest that there are two distinct phases to CCR5 up-regulation; one that occurs immediately following the initiation of cell division and another, more marked increase, that occurs many days after the T cell activation process is initiated.

In contrast to the patterns observed for CCR5, no up-regulation of CCR6 was detectable on $CD4^+$ T cells at the day 6 time-point, even after at least 7 rounds of cell division (the difference between, for example, divisions 0 and 2 was not statistically significant; $p > 0.05$; Fig 4.9 [B]). By the day 9 time-point, however, the percentage of divided cells staining positive for CCR6 had increased significantly ($p < 0.005$

compared to the undivided population), indicating that CCR6 up-regulation is highly dependent on the time that has elapsed since cellular activation, and occurs as a late activation event, temporally distinct from the cell division process.

In apparent contrast to the results obtained for CCR5 and CCR6, the regulation of CXCR3 and CXCR4 was intimately dependent on the cell division process. As seen in Figure 4.9 [C], there was a progressive increase in the percentage of $CD4^+$ T cells expressing CXCR3 over the first two rounds of cell division, with no further significant increase detectable over subsequent divisions, nor after a further 3 days in culture (day 9). The most dramatic decrease in CXCR4 expression also occurred over the first two rounds of cell division, such that only ~25% of $CD4^+$ T cells expressed detectable levels of this receptor following the first two rounds of cell division (Fig 4.9 [D]). There was also a less dramatic loss of CXCR4 over subsequent divisions, such that after 7 or more rounds of cell division, the percentage of $CD4^+$ T cells that expressed detectable levels of CXCR4 was less than 5%. However, no further significant changes were detected following another 3 days of culture (day 9).

4.2.3.3 Hierarchy in chemokine receptor expression on divided $CD4^+$ T cells

During the analysis of chemokine receptor expression on divided cells at day 9, it became apparent that there was a hierarchy in the expression of the four receptors. This trend is illustrated in Figure 4.10. The most uniform change observed on activated $CD4^+$ T cells was the loss of CXCR4, with 90.1 $\pm$ 0.8% of divided cells becoming negative for this receptor. This was closely followed by the up-regulation of CXCR3, such that 81.1 $\pm$ 4.0% of divided cells expressed this receptor by the day 9 time-point. The acquisition of CCR5 was less complete, with 64.7 $\pm$ 4.5% up-regulating this

receptor, while the acquisition of CCR6 by activated cells was even more variable, such that only 30.4 ± 2.1% of divided cells expressed detectable levels at day 9.

4.2.4 *Patterns of chemokine receptor and memory/activation marker expression following extended culture*

In order to determine whether the observed changes in chemokine receptor expression persisted for an extended period of time, allogeneic MLR cultures were maintained for 24 days with regular changes of culture medium. As illustrated in Figure 4.11, there were no significant changes in the percentage of $CD4^+$ T cells expressing any of the four receptors between the day 9 and day 24 time-points. Although there appeared to be a slight up-regulation of CCR5, CCR6 and, to a lesser extent CXCR3, between days 9 and 24, these differences were not statistically significant ($p > 0.05$ for all). There was, however, a significant decrease in the percentage of $CD4^+$ T cells expressing CD45RA, and a significant increase in the percentage of $CD4^+$ T cells expressing CD45RO, between days 9 and 24 (Fig 4.12 [A], $p < 0.0005$ for both), such that the vast majority of $CD4^+$ T cells in extended culture were characterised by a memory phenotype. At day 24, the percentage of $CD4^+$ T cells expressing CD25 was also modestly increased compared to day 9 ($p < 0.01$).

4.2.5 *Regulation of chemokine receptor messenger RNA levels*

To determine if the modulation of chemokine receptor expression was due to altered levels of mRNA for the receptors, RNA was purified from cell pellets collected from allogeneic and syngeneic cultures after a 9-day incubation period, and the relative levels of receptor mRNA determined using RT-PCR. Initially, preliminary studies

were conducted for each set of primers at a range of cycle numbers, to ensure that the PCR reaction was in the linear range (not shown). Subsequently, the level of mRNA for the four receptors (normalised to that of the housekeeping gene GAPDH) was compared between allogeneic and syngeneic cultures. A representative photomicrograph illustrating the relative density of PCR products following agarose gel electrophoresis is presented in Figure 4.13 [A], while a graph summarising the densitometry results of three separate experiments is presented in Figure 4.13 [B].

In keeping with the acquisition of CCR5, CCR6 and CXCR3 by allo-activated $CD4^+$ T cells, there was a significant increase in the level of messenger RNA for these three receptors in allogeneic compared to syngeneic samples ($p < 0.05$ for all). The down-regulation of CXCR4 surface expression following allogeneic activation was not, however, mirrored by a corresponding decrease in CXCR4 mRNA. In fact, the levels of CXCR4 message were consistently and significantly higher in allogeneic compared to syngeneic samples ($p < 0.05$). Similar results were obtained with an additional set of CXCR4 primers, directed at a different region of the target sequence (not shown).

4.2.6 Chemokine receptor expression in intracellular versus extracellular compartments

The amount of chemokine receptor protein on the cell surface is likely to reflect not only mRNA levels, but also the amount and nature of receptor trafficking between the cell surface and intracellular compartments. Experiments were therefore conducted to test whether these receptors were present intracellularly in $CD4^+$ T cells, and if so, whether the levels of intracellular protein differed between cells cultured under allogeneic and syngeneic conditions. Cells cultured for 9 days were processed for flow

cytometric analysis of chemokine receptor expression, with or without prior permeabilisation to allow detection of intracellular as well as extracellular receptors, as described previously (198) (Fig 4.14). Additionally, the localisation of receptor protein was assessed using immunofluorescence microscopy, following the staining of permeabilised cells with the relevant anti-chemokine receptor antibodies (Fig 4.15).

When comparing permeabilised to non-permeabilised $CD4^+$ T cells, the percentage of cells of staining positive for CCR5 or CXCR4 was identical, for both syngeneic (Fig 4.14 [A]) and allogeneic (Fig 4.14 [B]) cultures, indicating that these two receptors were not found intracellularly under either culture condition. The lack of intracellular CCR5 and CXCR4 was confirmed by immunofluorescence microscopy, which demonstrated that the staining for these receptors was primarily restricted to the cell surface in both syngeneic and allogeneic cultures (Fig 4.15 [A - D]), despite the inclusion of a permeabilisation step prior to labelling. As lymphocytes have only a small amount of cytoplasm, it was possible that low level intracellular staining may be mistaken for surface staining. However, the patterns of CCR5 and CXCR4 staining were comparable to those observed when non-permeabilised cells were labelled with anti-CD4 (Fig 4.15 [E]), suggesting that these receptors are in fact localised exclusively on the cell surface.

In contrast to the results for CCR5 and CXCR4, permeabilisation of $CD4^+$ T cells from syngeneic or allogeneic cultures resulted in an increased number of cells staining positive for CCR6 compared to non-permeabilised cells ($p < 0.05$ for both; Fig 4.14), suggesting that this receptor is stored intracellularly or is undergoing continual recycling between the cytoplasmic and cell surface compartments. The intracellular localisation of CCR6 protein was confirmed by immunofluorescence microscopy, as

illustrated in Figure 4.15 [F - G]. In both syngeneic and allogeneic cultures, a punctate distribution of protein was evident throughout the cell. Surprisingly, surface staining around the circumference of the cells was not obvious. However, many of the pockets of staining appeared to be localised close to the cell surface, suggesting that extracellular CCR6 protein may be arranged in distinct foci on the cell membrane.

With regard to the expression of CXCR3, in both syngeneic and allogeneic cultures the percentage of $CD4^+$ T cells stained was significantly higher in permeabilised compared to non-permeabilised samples ($p < 0.0001$ and $p < 0.005$, respectively; Fig 4.14), indicating that CXCR3 protein was present intracellularly under both culture conditions. The presence of intracellular stores of CXCR3 was also confirmed by immunofluorescence microscopy of permeabilised cells; for both allogeneic and syngeneic samples, CXCR3 was shown to have a punctate distribution throughout the cell, in addition to surface staining (Fig 4.15 [H - I]).

Thus, both CCR6 and CXCR3 were detected intracellularly, in contrast to CCR5 and CXCR4, which were localised exclusively to the cell surface. Notably, when cells were permeabilised, the percentage positive for CCR6 or CXCR3 was virtually identical between allogeneic and syngeneic cultures. This is in clear contrast to the results obtained using non-permeabilised cells, which demonstrate (as observed in Chapter 3) that a higher proportion of $CD4^+$ T cells expressed CCR6 or CXCR3 when cultured under allogeneic conditions compared to syngeneic controls. These observations therefore suggest that, unlike the case with surface protein, the expression of intracellular receptor protein is not subject to activation-dependent regulation.

4.2.7 *Functional consequences of altered patterns of chemokine receptor expression*

4.2.7.1 *Allogeneic activation results in altered migration toward chemokine ligands for CCR5, CXCR3 and CXCR4 but not CCR6*

The most obvious reason for T cells to begin expressing alternative patterns of chemokine receptors following cellular activation is to allow them to migrate in response to a distinct range of chemokine ligands. Accordingly, allo-activated T cells would be expected to demonstrate enhanced chemotactic responsiveness toward CCR5, CCR6 and CXCR3 ligands, and a reduced ability to migrate toward CXCR4 ligands. To test this hypothesis, cells from day 9 allogeneic and syngeneic MLR cultures were compared in Transwell® chemotaxis assays for their ability to migrate toward MIP-1β/CCL4 (a CCR5 ligand), MIP-3α/CCL20 (the only identified CCR6 chemokine ligand), I-TAC/CXCL11 (a CXCR3 ligand) and SDF-1/CXCL12 (the only identified CXCR4 ligand). In contrast to some chemokines, such as MIP-1α/CCL3, RANTES/CCL5 and IL-8/CXCL8, all of the chemokines to be tested in these experiments are agonists only for the receptors stated. Hence, any observed differences in responsiveness to these chemokines can be ascribed to defined receptors. In order to establish the optimal dose of each chemokine to be used, pilot dose-response chemotaxis experiments were conducted using a range of chemokine concentrations (not shown). Subsequently, the ability of cells to migrate toward an optimal concentration of each chemokine was compared between allogeneic and syngeneic cultures. In order to correct for any differences in spontaneous migration between the two culture conditions, the data are expressed as migration index (MI; see Chapter 2).

As shown in Figure 4.16, migration toward the CCR5 ligand MIP-1β/CCL4 was virtually undetectable in syngeneic cultures but increased significantly following allogeneic activation ($p < 0.005$), in keeping with the observed up-regulation of CCR5 expression in allogeneic cultures. Similarly, as may be expected given the up-regulation of CXCR3 expression following allo-activation, the ability of cells from allogeneic cultures to migrate toward I-TAC/CXCL11 was significantly increased compared to syngeneic controls ($p < 0.05$). Furthermore, the migration toward SDF-1/CXCL12 was significantly decreased in allogeneic compared to syngeneic cultures ($p < 0.001$), in keeping with the observed down-regulation of CXCR4 upon allogeneic activation. However, despite the significant increase in CCR6 expression in allogeneic cultures, these cells did not display an increased chemotactic response toward MIP-3α/CCL20 compared to syngeneic controls.

In order to confirm the negative result with respect to MIP-3α/CCL20, more detailed chemotaxis studies were undertaken. A highly sensitive fluorescent-based assay was used to test migration at a range of MIP-3α/CCL20 concentrations, and the cells labelled with anti-CD4 before and after migration, such that the specific response of $CD4^+$ T cells could be assessed in the absence of other potentially contaminating lymphocyte populations (Fig 4.17). In agreement with the data presented in Figure 4.16, these studies confirmed the lack of enhanced migration toward MIP-3α/CCL20 in allogeneic compared to syngeneic cultures. At the lowest concentration of MIP-3/αCCL20 tested, there appeared to be slightly enhanced chemotaxis in the allogeneic population compared to the syngeneic controls, but this difference was not statistically significant ($p > 0.05$).

4.2.7.2 *A CCR6 receptor antagonist does not alter the extent of apoptosis occurring at day 12 in the allogeneic MLR*

Despite the fact that significantly more $CD4^+$ T cells expressed CCR6 following allogeneic activation, this difference did not allow these cells to migrate more efficiently toward the only known chemokine ligand for CCR6 (MIP-3α/CCL20). This observation suggests an alternative function for this receptor on allo-activated T cells. Given that the up-regulation of CCR6 was a late event, occurring many days after the cell division process had peaked, it seemed possible that CCR6 may be involved in down-regulation of the T cell immune response. Accordingly, the ability of a CCR6 receptor antagonist (MIP-3α 4-70) to inhibit or enhance apoptosis at the end of the culture period was assessed. This receptor antagonist is a truncated synthetic peptide, corresponding to the amino acid sequence of the human chemokine minus the first 3 amino acids. This peptide has been shown in previous studies in our laboratory to have antagonist activity for human MIP-3α/CCL20 (Caon & McColl, unpublished data). Accordingly, any alteration to the levels of apoptosis occurring in allogeneic MLR cultures in the presence of this peptide would implicate CCR6 in the regulation of T cell apoptosis.

Cells were cultured under allogeneic conditions in the presence of varying concentrations of either MIP-3α 4-70 or a control peptide (MCP-Ala), which has neither agonist nor antagonist activity (199). After 12 days in culture, the percentage of cells undergoing apoptosis was determined using a combination of Annexin-V and ethidium monoazide bromide (EMA) staining. Cells that stain with Annexin-V only are considered apoptotic, while cells that also stain with EMA have lost membrane integrity and are therefore considered dead (200, 201). As illustrated in Figure 4.18,

the MIP-3α 4-70 peptide had no effect on the percentage of cells undergoing apoptosis at any concentration tested, suggesting that CCR6 is not involved in regulating apoptosis in this system.

4.2.8 Similar to the human MLR, CCR5 and CXCR3 are also up-regulated in the murine MLR

In order to test the species-specificity of the observed patterns of chemokine receptor regulation, murine lymphocytes were cultured in an MLR and any changes in chemokine receptor expression assessed using flow cytometry (CCR5, CXCR3 and CXCR4) or RT-PCR (for CCR6, as no suitable antibody for muCCR6 was available at the time this study was conducted). As pilot studies (not shown) indicated that the murine MLR proceeded more rapidly than the human MLR, proliferation was assessed at day 5 while expression of chemokine receptors was quantified at day 7. For most of these studies, an immortalised DC line (tsDC) was used as a source of stimulator cells (202). This cell line was derived from a transgenic mouse harbouring a thermolabile form of the simian virus 40 (SV40) large tumour antigen (203). Expression of this gene immortalises cells, thereby enabling them to be grown in continuous cell culture at the permissive temperature of 33°C. However, upon transfer to 39°C, expression of the transgene is lost and the cells undergo a program of terminal differentiation. These cells have been shown previously to be potent stimulators of the MLR, and accordingly, were used here (after differentiation at 39°C for 24 hours) to stimulate splenocytes from an MHC-mismatched mouse strain (BALB/c). Supporting data was also obtained using a more conventional MLR culture system, in which splenocytes obtained from [BALB/c x CBA] F1 mice were used to stimulate splenocytes from BALB/c mice.

Initial studies utilising incorporation of [^{3}H]-thymidine as an indicator of cell division confirmed that a significant proliferative response was mounted when splenocytes were cultured with tsDCs (allogeneic) compared to splenocytes cultured alone (syngeneic) (Fig 4.19 [A]). Subsequently, flow cytometric analysis demonstrated that CCR5 and CXCR3 were both expressed on a greater proportion of $CD4^+$ T cells in allogeneic compared to syngeneic cultures ($p < 0.05$ and $p < 0.005$, respectively). However, in contrast to the results obtained in the human system, no significant alteration to the expression of CXCR4 was observed. Subsequent studies using splenocytes from [BALB/c x CBA] F1 mice as stimulator cells produced essentially identical results (Fig 4.19 [C-D]), thereby adding further weight to these observations. The representative density plots in Figure 4.20 illustrate the up-regulation of CCR5 and CXCR3 observed in allogeneic cultures, and demonstrate the lack of alteration to the expression of CXCR4. Furthermore, it is evident from these plots that – similar to the human MLR – changes in chemokine receptor expression were restricted to T cells expressing elevated levels of CD4.

The expression of CCR6 in murine MLR cultures could not be assessed using flow cytometry, as a suitable antibody was not available. Although polyclonal antibodies were raised against all four of these murine chemokine receptors early in this study, none were found to be reactive with the native receptor in flow cytometry, although in ELISA assays, all recognised the cognate antigenic peptide used for immunisation (not shown). Thus, in the absence of a suitable flow cytometric assay, RT-PCR was used to compare the levels of CCR6 mRNA in allogeneic and syngeneic cultures. These experiments demonstrate that, in contrast to the human MLR, CCR6 mRNA levels were not increased in allogeneic compared to syngeneic cultures (Fig 4.21).

4.3 Discussion

4.3.1 Increased expression of CD4 as a marker of T cell activation

Although the principle aim of this study was to examine the regulation of chemokine receptor expression on T cells, an interesting observation was made regarding the regulation of CD4 expression that warranted further investigation. Initially, in the studies presented in Chapter 3, a bimodal pattern of CD4 expression was noted in allogeneic, but not syngeneic cultures. Further analysis revealed that the $CD4^{hi}$ population developed predominantly over the days 6 – 9 time-points, and that these cells had many characteristics of activated/memory T cells. The down-regulation of CD4 on human T cells in response to phorbol ester treatment is well documented (204, 205), thereby providing evidence that the levels of CD4 on peripheral blood T cells are not necessarily constant. However, extensive literature searches suggest that this study is the first to document the increased expression of CD4 on human T cells in response to cellular activation. On the other hand, the up-regulation of CD4 on murine T cells has been observed following activation *in vitro* and *in vivo* (206), suggesting that this observation is not simply a peculiarity of the MLR, and that it is a phenomenon conserved between the human-murine species barrier.

The biological significance of CD4 up-regulation is not clear at present. One possibility is that it may represent a mechanism to maintain a constant density of CD4 molecules on the cell surface as cell size increases with activation. This may be important in maintaining a sufficient threshold of TCR signalling over the extended period of time required for the activation of naïve T cells (207). Alternatively, it is possible that the increased levels of CD4 are required for interactions distinct from

those mediated by class II MHC ligation. In this regard, IL-16 is a functional ligand for CD4, and upon binding is able to induce a variety of effector functions in $CD4^+$ T cells, including chemotaxis and induction of IL-2R expression (208). Additionally, the ligation of CD4 in the absence of TCR triggering can result in the transmission of a negative signal, preventing subsequent activation through the TCR. Thus, the up-regulation of CD4 may allow the efficient binding of IL-16 in the absence of antigen signalling, thereby regulating the degree of T cell stimulation. This concept is supported by the development of the $CD4^{hi}$ T cell population relatively late in the MLR culture, a time at which the proliferative response declines. The increase in the expression of CD4 may also be a disadvantage during HIV infection. CD4 is the primary receptor for cellular entry of HIV-1 (209). Thus, up-regulation of CD4 may enhance the efficiency with which HIV can infect activated T cells, although the contribution of simultaneous alterations in the expression of HIV co-receptors, such as CCR5 and CXCR4, must also be considered (210).

4.3.2 Modulation of chemokine receptor expression is coordinated with cell division and the acquisition of an activated/memory phenotype

The acquisition of CCR5, CCR6 and CXCR3, and the loss of CXCR4, by $CD4^+$ T cells in allogeneic but not syngeneic cultures suggested that the regulation of chemokine receptor expression was dependent on T cell activation and division, which occur only under allogeneic conditions. Hence, a number of experiments were conducted in order to investigate the relationship between modulation of chemokine receptor expression and the T cell activation and division processes in detail.

4.3.2.1 *The memory/activation status of $CD4^+$ T cells with the $CCR5^+$ $CCR6^+$ $CXCR3^+$ $CXCR4^-$ phenotype*

It was shown that $CD4^+$ T cells from allogeneic cultures that expressed CCR5, CCR6 and CXCR3, and that had lost CXCR4, were predominantly characterised by a memory ($CD45RA^-$ $CD45RO^+$) phenotype. This observation suggests that only memory – and not naïve – $CD4^+$ T cells expressed this panel of chemokine receptors. This is in keeping with the documented expression patterns of these receptors on peripheral blood T cells, which demonstrate that CCR5, CCR6 and CXCR3 are predominantly expressed by memory $CD4^+$ T cells, with limited or undetectable levels of expression on the naïve population (72, 74, 107, 118, 130, 134, 171). In contrast, CXCR4 appears to be expressed preferentially on naïve $CD4^+$ T cells, with reduced levels of expression on memory cells (74, 107, 171). The expression of CCR5, CCR6 and CXCR3, and the reduced levels of CXCR4, observed on memory T cells in these previous studies suggested, but did not prove, that T cells acquired this panel of chemokine receptors during the activation process. The present study has confirmed that this is indeed the case, as a population of memory $CD4^+$ T cells was shown to develop during the course of the allogeneic MLR, and to express the $CCR5^+$ $CCR6^+$ $CXCR3^+$ $CXCR4^-$ phenotype. It is likely, however, that not all memory $CD4^+$ T cells present at day 9 with the $CCR5^+$ $CCR6^+$ $CXCR3^+$ $CXCR4^-$ phenotype developed *de novo* during allogeneic culture, as a reasonable proportion of the starting population had a memory phenotype. Thus, the pool of memory cells detected at day 9 with the $CCR5^+$ $CCR6^+$ $CXCR3^+$ $CXCR4^-$ phenotype likely consists partially of existing memory cells that were not activated in the MLR but already expressed this panel of chemokine receptors, and partially of recently-activated cells that have acquired the memory phenotype in culture and altered their expression of chemokine receptors accordingly.

Similar to that observed with respect to the memory $CD4^+$ T cells, a readily detectable population of $CD4^+$ T cells expressing CD25, albeit at low levels, was detectable at the initiation of culture. This observation is in keeping with previous reports of CD25 expression on a sub-population of memory $CD4^+$ T cells in peripheral blood (192, 193). At the day 9 time-point, this marker showed an excellent correlation with altered patterns of chemokine receptor expression, such that the vast majority of $CCR5^+$, $CCR6^+$, $CXCR3^+$ and $CXCR4^-$ cells exhibited a $CD25^+$ phenotype. This observation suggests that $CD4^+$ T cells with the $CCR5^+$ $CCR6^+$ $CXCR3^+$ $CXCR4^-$ phenotype had undergone cellular activation. Again, however, it is unclear what proportion of the pool of CD25-expressing cells with the $CCR5^+$ $CCR6^+$ $CXCR3^+$ $CXCR4^-$ phenotype present at day 9 existed as a result of activation in the MLR, and what proportion already had this phenotype at the beginning of the culture period.

In contrast to CD25, CD69 is a more strict marker of acute activation, and accordingly, was almost undetectable at the day 0 time-point. The expression of this marker was also more restricted at the day 9 time-point compared to CD25. Thus, although the $CD69^+$ phenotype did correlate with the acquisition of the $CCR5^+$ $CCR6^+$ $CXCR3^+$ $CXCR4^-$ phenotype to some extent, a readily detectable proportion of $CD4^+$ T cells expressed altered patterns of chemokine receptors but did not express CD69. There are a number of possible explanations for this observation. First, CD69 is known as an early activation marker (194), and it is therefore conceivable that the level of CD69 expression may have been reduced beyond the limits of detection by the day 9 time-point. However, this is unlikely, as in previous studies of CD69 expression in the MLR, expression of this marker had only just begun to decline at day 8 (194). Alternatively, it is possible that distinct pathways of T cell activation are characterised by the acquisition of either CD25 or CD69, and that the pathway associated with CD25

up-regulation is more closely coupled to the observed changes in chemokine receptor expression than the pathway associated with CD69 up-regulation. In this regard, there is some evidence that the up-regulation of CD25 and CD69 involve distinct activation pathways in the MLR, as co-depletion of both $CD25^+$ and $CD69^+$ cells reduces the amount of IL-2 produced in MLR cultures to a significantly greater extent than depletion of $CD25^+$ or $CD69^+$ cells alone (211).

Thus, the up-regulation of CCR5, CCR6 and CXCR3, and the down-regulation of CXCR4, correlated well with expression of the $CD45RA^-$ $CD45RO^+$ $CD25^+$ $CD69^+$ phenotype, which is typical of activated/memory T cells. However, as described above, the expression of elevated levels of CD4 also appears to be a reliable marker of T cell activation. Accordingly, all changes in the expression of CCR5, CCR6, CXCR3 and CXCR4 were shown to be restricted to the $CD4^{hi}$ population, while the $CD4^{normal}$ T cells expressed almost identical patterns of chemokine receptor expression at day 9 compared to $CD4^+$ T cells from syngeneic cultures. The $CD4^{hi}$ phenotype may be a particularly good marker of acute T cell activation, as (similar to CD69 expression) it was not detectable at the initiation of culture, suggesting that all $CD4^{hi}$ T cells that were detectable at day 9 had been activated in the MLR. However, in contrast to CD69, the $CD4^{hi}$ phenotype demonstrated a remarkably efficient correlation with the up-regulation of CCR5, CCR6 and CXCR3 and the down-regulation of CXCR4, suggesting that this phenotype is far less restricted than the $CD69^+$ phenotype, and is comparable to that of CD25 or CD45RO.

4.3.2.2 *The relative proportions of divided $CD4^+$ T cells that have acquired CCR5, CCR6 and CXCR3, and lost CXCR4*

Virtually all $CD4^+$ T cells that expressed CCR5, CCR6 and CXCR3, and that had lost CXCR4, had a memory ($CD45RA^-$ $CD45RO^+$) phenotype, had up-regulated CD4 and expressed CD25, and a large proportion also expressed CD69. It was also of interest, however, to consider the relationship between chemokine receptor expression and T cell activation/division from the reciprocal perspective, and ask what proportion of $CD4^+$ T cells that were activated in the MLR adopted the $CCR5^+$ $CCR6^+$ $CXCR3^+$ $CXCR4^-$ phenotype. To address this question, cell division, as assessed by CFSE dye dilution, was taken as the most definitive measure of activation in culture. The results indicated that CXCR3 and CXCR4 were modulated on the majority of divided cells (~81% and ~91% respectively), CCR5 on ~65% of the divided population and CCR6 on ~30% of divided cells. Thus, there appears to be a hierarchy in the extent of receptor regulation, such that while some receptors were modulated on the vast majority of divided cells, others were modulated on only a minority of the divided population. CXCR4 and CCR6 represented these two extremes.

As discussed in Chapter 3, the acquisition of CXCR3 and the loss of CXCR4 are events that appear to occur whenever T cells are triggered through the TCR (72, 106, 118, 136), suggesting that these changes are directly linked to the T cell activation process. This is in keeping with the observation that modulation of these two receptors occurred on the majority of $CD4^+$ T cells that had divided in the MLR. There were, however, small populations of T cells that underwent division but did not begin expressing detectable levels of CXCR3, and did not lose expression of CXCR4, indicating some degree of heterogeneity in the regulation of these receptors on activated T cells. This issue will be explored further in Chapter 6.

In comparison to the modulation of CXCR3 and CXCR4 expression, the acquisition of CCR5, and especially CCR6, was less complete, as a marked proportion of $CD4^+$ T cells did not adopt a $CCR5^+$ or $CCR6^+$ profile, despite undergoing a program of cellular activation and division. This is in keeping with the notion that the acquisition of these receptors requires signals in addition to stimulation through the TCR, such as those provided by appropriate co-stimulatory molecules or cytokines (see Chapter 3), and is therefore more likely to be subject to fine-tuning mechanisms, rather than being an automatic consequence of TCR triggering. These results are also supported by the observation that all peripheral blood $CCR5^+$ T cells express CXCR3, but not all $CXCR3^+$ T cells express CCR5 (118), suggesting that the expression of CCR5 by circulating memory T cells is more restricted than the expression of CXCR3.

4.3.2.3 Regulation of chemokine receptor expression is coordinated with the cell division process

The initial time-course studies using incorporation of [^{3}H]-thymidine as a measure of cell division indicated that proliferation peaked around the day 6 time-point, and had markedly declined by day 9. In contrast, the up-regulation of CCR5, CCR6 and CXCR3, and the loss of CXCR4, were most apparent over the days 9-12 time-period (Chapter 3). These observations indicated that a program of T cell activation/division must commence prior to novel patterns of chemokine receptor expression being adopted. However, it was not clear from these studies whether changes in chemokine receptor expression become apparent immediately before, co-incident with, or sometime after, a T cell has begun dividing. As this distinction could have important implications for the reasons for, and the molecular mechanisms of, chemokine receptor

modulation, the relationship between these two events was examined in detail using the CFSE dye dilution technique.

These studies revealed that the relationship between cell division and modulation of chemokine receptor expression was slightly different for each of the four receptors examined. Thus, at one end of the spectrum, the loss of CXCR4 expression was intimately dependent on cell division, such that populations of T cells that had undergone more rounds of cell division contained more $CXCR4^-$ cells. The acquisition of CXCR3 expression was also closely related to the cell division process, as a greater proportion of T cells that had divided once expressed this receptor compared to undivided cells, and a greater proportion of cells that had divided twice expressed this receptor compared to cells that had divided only once. With subsequent cell divisions, however, there was no further significant increase in the proportion of $CD4^+$ T cells expressing CXCR3.

Of note, neither CXCR3 nor CXCR4 was subject to further modulation of expression between days 6 and 9, suggesting that the regulation of these receptors was independent of the time in culture. This conclusion may, at first inspection, seem to contradict the time-course studies presented in Chapter 3, which demonstrated that the greatest difference in expression of all four receptors in allogeneic compared to syngeneic cultures occurred at day 9, not at day 6. However, it is likely that by day 6, the proportion of activated T cells was quite small, and therefore although the expression of CXCR3 and CXCR4 had been modulated on these cells, they were not readily detectable when analysing the total $CD4^+$ T cell population. In contrast, by day 9, sufficient $CXCR3^+$ and $CXCR4^-$ cells would have accumulated to be readily detectable within the bulk population. The observed association between cell division

and regulation of CXCR3 and CXCR4 expression is remarkably similar to previous investigations into the relationship between T cell division and cytokine production (169). This previous study demonstrated that the ability of *in vitro*- stimulated human T cells to produce a wide range of cytokines was intimately dependent on the cell division number, and independent of the time the cells had been in culture. Thus, it is possible that the mechanisms controlling the expression of these two chemokine receptors share common signalling pathways with the mechanisms that regulate cytokine producing phenotype and cell division. This may provide an explanation for the observed correlation between CXCR3 expression and the Th1 cytokine-producing phenotype (73, 74).

In contrast to CXCR3 and CXCR4, the regulation of expression of CCR5 and CCR6 appeared to be more dependent on time in culture than the number of divisions undertaken. Thus, there was either limited (CCR5) or undetectable (CCR6) up-regulation of these receptors at the day 6 time-point, even after at least 7 rounds of cell division. However, by day 9, both receptors were expressed on a significantly greater proportion of the divided $CD4^+$ T cell population compared to $CD4^+$ T cells at the initiation of culture. These observations suggest that the up-regulation of CCR6, and to a lesser extent CCR5, is only initiated after a defined period of time has elapsed since cellular activation and the initiation of proliferation. Despite the temporal distinction, however, cellular activation is clearly a pre-requisite for up-regulated expression of CCR5 and CCR6, as the undivided population did not alter expression of these receptors, irrespective of the length of time in culture. It is unclear at this stage what mechanism controls the timing of up-regulation of these receptors. However, it is possible that the delay between cell division and the acquisition of CCR5 and CCR6 is

due to the time required for cytokines that drive the expression of these receptors to accumulate in the culture.

4.3.3 *Extended culture of allo-activated cells does not result in further alteration to chemokine receptor expression*

Previous analyses of chemokine receptor expression on peripheral blood lymphocytes have shown that resting, memory $CD4^+$ T cells are more likely to express CCR5, CCR6 and CXCR3 compared to naïve $CD4^+$ T cells (72, 74, 107, 118, 130, 134, 171). These observations suggest that the CCR5, CCR6 and CXCR3 receptors shown here to be acquired by activated $CD4^+$ T cells during the MLR would be retained over long periods of time, even after the cells have returned to a resting, memory phenotype. On the other hand, it is likely that the complete loss of CXCR4 expression during T cell activation observed here would be reversed to some extent as these cells returned to a resting state, as peripheral blood memory T cells have been shown to express this receptor, albeit at lower levels and in a less uniform manner compared to the naïve population. However, these predictions have never been directly tested. Therefore, experiments were conducted to examine chemokine receptor expression on $CD4^+$ T cells that had been cultured for an additional 18 days after the peak of proliferation at day 6.

The studies presented here demonstrate that no significant changes in the expression of any of the four receptors occurred between days 9 and 24. Thus, expression of CCR5, CCR6 and CXCR3 was maintained (but not notably increased), and CXCR4 failed to be re-expressed. This suggests that the $CCR5^+$ $CCR6^+$ $CXCR3^+$ $CXCR4^-$ phenotype is retained by activated T cells for extended periods of time. However, it is difficult to

ascertain the state of activation of $CD4^+$ T cells at day 24. The proliferation time-course presented in Chapter 3 indicates that cell division had ceased by day 12, suggesting that the cells remaining at day 24 were no longer in a state of acute activation analogous to that observed during the peak of the proliferative response. Furthermore, although the percentage of $CD4^+$ T cells expressing CD25 did not decrease between days 9 and 24, this cannot be considered as conclusive evidence that the cells had not begun to return to a resting state, as a relatively large proportion of circulating memory $CD4^+$ T cells, which presumably are in a resting state, maintain expression of CD25 (192, 193). Thus, there is no evidence that day 24 cells are in an activated state, but on the other hand, the ability of cultured primary T cells to ever return to a true, quiescent state, analogous to the long-lived memory T cell population found *in vivo*, is not clear at this stage. Further studies are required to examine this issue in a more physiological setting.

It is also interesting to note that there was a marked increase in the percentage of $CD4^+$ T cells expressing CD45RO, as well as a comparable decrease in the percentage of $CD4^+$ T cells expressing CD45RA by day 24, such that the proportion of $CD4^+$ T cells with a memory phenotype had increased to ~90%. It is likely that the increase in the proportion of memory T cells in extended culture is attributable to the preferential survival of memory cells, rather than continued *de novo* activation, as proliferation was undetectable past the day 12 time-point. Regardless of the mechanism, however, it is surprising that the expression of CCR5, CCR6 and CXCR3 did not further increase, and the expression of CXCR4 did not further decrease, in co-ordination with the increase in the proportion of memory T cells over the day 9 – 24 time-period. This difference suggests that some of the memory T cells at day 24 did not express the

$CCR5^+$ $CCR6^+$ $CXCR3^+$ $CXCR4^-$ phenotype, although further studies would be required to confirm this.

4.3.4 Mechanisms of chemokine receptor regulation

4.3.4.1 Molecules involved in the regulation of chemokine receptor expression

Thus far, the pattern of chemokine receptor regulation that occurs during T cell activation, and the way in which this pattern is coordinated with other aspects of T cell biology, have been examined in detail. However, the biological mechanisms that account for the observed pattern of receptor regulation have not been discussed. Although an investigation into the molecules responsible for altering chemokine receptor expression on activated T cells would have been highly informative, screening the large number of cytokines and co-stimulatory and signalling molecules that may potentially be involved in this process was beyond the scope of this study. However, given the Th1-associated nature of CCR5 and CXCR3 expression (73, 74, 111), it was considered likely that the principal Th1-inducing cytokine IL-12 would play a role in the regulation of these two receptors. Accordingly, the effect of an anti-IL-12 monoclonal antibody on the up-regulation of CCR5 and CXCR3 expression in the allogeneic MLR was investigated. The presence of this antibody during culture consistently had no effect on the proliferative response or the up-regulation of CXCR3 expression (not shown). This is in keeping with the hypothesis that the acquisition of this receptor is a default pathway triggered whenever T cells are activated through the TCR, as discussed in Chapter 3. In contrast, in some experiments, anti-IL-12 appeared to inhibit the up-regulation of CCR5 in the allogeneic MLR, although this effect was not consistently reproducible in subsequent experiments (not shown). These results are

in keeping with a recent analysis of chemokine receptor expression on murine T cells (188). This study demonstrated that the addition of rIL-12 to *in vitro*-stimulated T cells had no effect on the up-regulation of CXCR3, yet enhanced the up-regulation of CCR5. Enhanced up-regulation of CCR5 in response to IL-12 has also been observed in the human system (122).

4.3.4.2 Regulation of chemokine receptor mRNA levels

Chemokine receptor expression is frequently regulated at the mRNA level. For example, differential expression of CCR5, CCR7, CXCR3, CXCR4 and CXCR5 at the cell surface has been shown to correlate with differential expression at the mRNA level (119, 122, 134, 135). However, this is not always the case, as in some systems chemokine receptors are subject to complex patterns of protein trafficking between the extracellular and intracellular compartments, as will be discussed shortly. Therefore, to determine if the chemokine receptors under investigation in the present study were regulated at the level of transcription, mRNA levels were compared between allogeneic and syngeneic cultures for each receptor using RT-PCR. Although this is not a quantitative technique, a number of steps were taken (see Chapter 2) to ensure that the results allow an accurate comparison of the approximate levels of chemokine receptor gene expression.

In keeping with the up-regulation of cell surface expression of CCR5, CCR6 and CXCR3, the level of mRNA corresponding to each of these receptors was significantly increased in allogeneic compared to syngeneic cultures. These observations suggest that the enhanced cell surface expression of these receptors is controlled at the level of increased transcription, although it is also possible that the increased level of mRNA

additionally or alternatively results from enhanced transcript stability. In contrast, the down-regulation of cell surface expression of CXCR4 in allogeneic cultures was not mirrored by a corresponding decrease in level of CXCR4 mRNA. In fact, the level of CXCR4 transcripts was significantly increased following allogeneic activation. In this regard, the detection of abundant CXCR4 transcripts in the face of reduced CXCR4 surface protein has been observed previously (139), and a decrease in cell surface expression of CCR1 and CCR5 has been demonstrated following TCR triggering despite an increase in the level of the corresponding transcripts (212). Thus, it is clear that chemokine receptors may be subject to multiple levels of regulation, and that an increase in mRNA level does not always correspond to increased expression of the receptor on the cell surface.

4.3.4.3 Intracellular versus extracellular localisation of chemokine receptor protein

The previous observation of a constant level of CXCR4 mRNA despite a decreased level of cell surface protein was proposed to be due to internalisation of surface CXCR4 protein through a protein kinase C (PKC)-responsive mechanism (139). Indeed, a number of previous studies have detected a rapid internalisation of CXCR4 upon activation of T cells with various stimuli, including phorbol esters and mitogens (106, 213, 214). Moreover, the internalisation of chemokine receptors in response to ligand binding (desensitisation) has been extensively documented (60, 213, 215-217), suggesting that the loss of CXCR4 from the cell surface may be due to the release of SDF-1/CXCL12 during culture, and subsequent ligand-mediated internalisation of CXCR4. In the MLR culture system, however, intracellular stores of CXCR4 protein could not be detected at the day 9 time-point in either allogeneic or syngeneic cultures. The lack of intracellular CXCR4 protein during the time at which CXCR4 down-

regulation peaked suggests that CXCR4 internalisation is not a major mechanism of receptor down-regulation in this system. The mechanism accounting for the observed decrease in CXCR4 surface protein therefore remains unclear. It is possible that an increase in CXCR4 transcription is coupled with a decrease in translation of the message, or an increased rate of protein degradation, thereby resulting in lower levels of surface protein. Another possibility is that CXCR4 is shed from the cell surface. In this regard, microvesicles containing CCR5 protein have been shown to bud from the surface of PBMCs and fuse with cells that normally lack CCR5 expression, thereby rendering them $CCR5^+$ (218) Hence, it is possible that CXCR4 may similarly bud from the cell surface of allo-activated $CD4^+$ T cells, thereby accounting for the loss of cell surface expression despite the high level of CXCR4 transcripts.

Similar to the observations made for CXCR4, CCR5 was also not detected intracellularly in $CD4^+$ T cells from either allogeneic or syngeneic cultures, suggesting that the up-regulation of this receptor is simply due to the observed increase in the level of CCR5 mRNA, leading to increased levels of protein that are translocated directly to the cell surface. In contrast, intracellular stores of both CCR6 and CXCR3 were detected in $CD4^+$ T cells from allogeneic as well as syngeneic cultures. This observation suggests that the traffic of these receptors between the cell surface and intracellular compartments is highly regulated, such that in a proportion of $CD4^+$ T cells, receptor protein is prevented from reaching the cell surface, or is internalised almost immediately after reaching the cell surface.

Given the existence in intracellular stores of CCR6 and CXCR3, it is not clear whether the increase in surface expression of these receptors is due to translocation of pre-formed receptor protein from the cytoplasm, or to enhanced *de novo* protein production

resulting from increased transcription. In this regard, however, it is interesting to note that following permeabilisation (ie, taking total receptor protein into account), the percentage of cells staining positive for each receptor did not differ between allogeneic and syngeneic cultures. This is in distinct contrast to the results obtained using non-permeabilised cells, which demonstrate, as observed in Chapter 3, that CCR6 and CXCR3 were expressed on more $CD4^+$ T cells in allogeneic compared to syngeneic cultures. These results suggest that a proportion of resting $CD4^+$ T cells constitutively express these receptors intracellularly in the absence of detectable surface expression. Following allogeneic activation, some – but not all – of these cells may begin expressing the receptors on the cell surface, resulting in a significant difference in the expression of surface protein when comparing allogeneic to syngeneic cultures. The precise mechanisms that allow a cell to switch from expressing the receptor intracellularly to expressing it extracellularly are not clear at this stage. However, a possible scenario is that the increased transcription of the CCR6 and CXCR3 genes upon allogeneic activation saturates the mechanisms that maintain the receptor protein inside the cell, thereby enabling expression on the cell surface.

Of note, only ~50% of $CD4^+$ T cells expressed CCR6 following permeabilisation, while ~80% of $CD4^+$ T cells were positive for CXCR3 following permeabilisation, in both allogeneic and syngeneic cultures. This observation is particularly interesting in the context of the observation that far fewer $CD4^+$ T cells that underwent cell division in the MLR acquired the expression of CCR6 compared to the number that acquired CXCR3. Thus, it is possible that only a defined proportion of peripheral blood $CD4^+$ T cells have the capacity to express CCR6. Those that do not express CCR6 intracellularly in a constitutive manner may not be able to express it at all, thereby

accounting for the significant proportion of $CD4^+$ T cells that underwent cell division in the MLR but did not up-regulate CCR6.

4.3.5 Functional consequences of altered patterns of chemokine receptor expression

Although chemokines have been proposed to play a number of roles in T cell biology, chemotaxis is still generally considered to be the primary function of the chemokine gene superfamily. Therefore, it was considered likely that allogeneic activation would result in enhanced migration toward CCR5, CCR6 and CXCR3 ligands, and reduced migration toward the CXCR4 ligand, SDF-1/CXCL12. Chemotaxis assays confirmed these predictions, with the exception of increased migration toward the CCR6 ligand, MIP-3α/CCL20. Surprisingly, despite the up-regulation of CCR6 on allo-activated $CD4^+$ T cells, chemotaxis toward this ligand was not enhanced. However, it is possible that an increased migratory response of the $CD4^+$ T cell population may have been masked by the lack of migration of the $CD8^+$ subset, as CCR6 was only expressed on a very small proportion of $CD8^+$ T cells. To address this concern, cells were labelled with anti-CD4 before and after migration in response to MIP-3α/CCL20, such that the specific migration of $CD4^+$ T cells could be assessed. In addition, a wide range of chemokine doses were used, to test for the possibility that a difference in chemotaxis between allogeneic and syngeneic culture conditions would only be seen at one or two particular concentrations of MIP-3α/CCL20. However, again no significant difference in migration was observed between the allogeneic and syngeneic populations.

The lack of increased migration toward MIP-3α/CCL20 following allogeneic activation suggested that the up-regulation of CCR6 plays an alternative function in the

biology of activated T cells. Although a number of possibilities exist, as will be discussed in Chapter 6, regulation of apoptosis was initially explored, as a number of chemokines have been shown previously to play a role in the regulation of apoptosis of T cells and other leukocytes. For example, SDF-1/CXCL12 induces the apoptosis of the Jurkat T cell line (219), while the presence of lymphotactin/XCL1 during anti-CD3 mediated T cell stimulation similarly enhances apoptosis (86). Conversely, other chemokines appear to inhibit apoptosis. For example, fractalkine/CX3CL1 has been shown to act as a survival factor for brain microglia (220), while IL-8/CXCL8 has the ability to reduce apoptosis in neutrophils (221). However, in the present study, a CCR6 receptor antagonist failed to either increase or decrease the level of apoptosis occurring at day 12 of the allogeneic MLR, suggesting that CCR6 is not involved in the regulation of apoptosis in this system.

4.3.6 *Conservation of CCR5 and CXCR3 up-regulation across the human-murine species barrier*

In keeping with the up-regulation of CCR5 and CXCR3 expression observed to occur in the human MLR, these receptors were also shown to be up-regulated by allo-activated murine $CD4^+$ T cells. Furthermore, similar to observations in the human MLR system, the up-regulation of CCR5 and CXCR3 on allo-activated murine T cells was restricted to a sub-population of T cells expressing elevated levels of CD4. Conversely, up-regulation of CCR6 expression, and down-regulation of CXCR4 expression, could not be detected in the murine MLR as they were in the human MLR. However, it must be noted that it was not possible to examine the surface expression of CCR6, due to the lack of an antibody suitable for flow cytometry. Therefore, the possibility remains that the expression of CCR6 on the cell surface is increased upon

activation of murine T cells, despite the lack of increased levels of mRNA. This possibility is worth investigating, considering that intracellular stores of CCR6 were detected in human T cells that could potentially be translocated to the cell surface in the absence of altered gene expression. Furthermore, it is possible that subtle changes in gene expression would not be detected using the RT-PCR technique. Therefore, these observations must be confirmed when an antibody becomes available.

To date, far less is known about the regulation of chemokine receptor expression on murine T cells compared to human T cells. In support of the present findings, however, the up-regulation of CCR5 expression on murine $CD4^+$ T cells has recently been observed in response to *in vitro* stimulation with a combination of anti-CD3, anti-CD28 and rIL-12 (188). Furthermore, murine TCR-transgenic T cells activated *in vitro* under Th1 polarising conditions acquired the ability to migrate toward the CXCR3 ligand IP-10/CXCL10 *in vivo*, suggesting that expression of CXCR3 had been up-regulated on these cells, as was observed in the present study. In contrast, investigations into the regulation of CXCR4 expression have produced distinctly different results to those presented here. Thus, PHA activation of murine $CD4^+$ T cells was shown to result in an early up-regulation of CXCR4 expression, followed by a significant decline, and then re-expression a few days later (222). It is possible that the distinctly different T cell activation regimes used in that previous study compared to the present one are responsible for the observed differences.

4.3.7 Conclusions

The aim of this series of experiments was to characterise the expression and function of four chemokine receptors that were identified in the previous chapter as being

modulated on $CD4^+$ T cells during allogeneic activation. Also, the possibility of using elevated expression of CD4 as a marker of T cell activation was assessed, on the basis of preliminary findings in Chapter 3. It was shown that the up-regulation of CCR5, CCR6 and CXCR3 expression, and the down-regulation of CXCR4 expression, was closely correlated with the acquisition of a memory phenotype. Furthermore, the modulation of these receptors occurred in coordination with the expression of various markers of cellular activation, although the $CD4^{hi}$ phenotype appeared to show the closest association with chemokine receptor expression. The modulation of expression of CXCR3 and CXCR4 occurred in close parallel with the cell division process, while in contrast, the up-regulation of CCR5 and CCR6 expression appeared to be more dependent on time in culture, as little or no evidence of increased expression was seen at early time-points, even after extensive cell division. In extended culture, it was shown that the pattern of chemokine receptor expression acquired within the first 9 days was retained for extended periods of time, suggesting that the up-regulation of CCR5, CCR6 and CXCR3 expression, and the loss of CXCR4 expression, were events not limited to a state of acute activation.

The increased expression of CCR5, CCR6 and CXCR3 on the cell surface was associated with an increase in mRNA levels, while in contrast, the down-regulation of CXCR4 protein was not mirrored by a corresponding decrease in CXCR4 transcripts. The possibility that CXCR4 down-regulation was effected via internalisation of surface protein was accordingly explored, and while intracellular stores of CXCR4 were not found, both CCR6 and CXCR3 could be detected intracellularly. The up-regulated expression of CCR5 and CXCR3 in allogeneic cultures was associated with an increased chemotactic response to the cognate ligands, while the down-regulation of CXCR4 expression accordingly reduced chemotactic responsiveness toward SDF-

1/CXCL12. However, the up-regulation of CCR6 did not result in enhanced migration toward MIP-3α/CCL20, and studies using a CCR6 antagonist failed to reveal a role for this receptor in the modulation of activation-induced apoptosis. Analysis of two models of the murine MLR revealed that the up-regulation of CCR5 and CXCR3 expression was conserved across the human-murine species barrier, while in contrast, the down-regulation of CXCR4 expression was not detected in either model. It was difficult to analyse the expression of CCR6 on murine T cells, due to the lack of a suitable antibody; however, RT-PCR analysis did not detect any up-regulation of this receptor on allo-activated murine T cells. Further studies are required to determine whether or not this receptor is up-regulated on the cell surface.

Table 4.1: Comparison of T cells expressing normal and elevated levels of CD4, following 9 days of culture in the allogeneic MLR[1]

Phenotype	CD4 expression	
	High	**Low**
Size (forward light scatter; FSC)	Large (high FSC)	Small (low FSC)
% of cells divided	93.8 ± 3.7 %	24.8 ± 13.7%
% expressing CD45RA (naïve)	4.5 ± 1.7%	56.1 ± 3.1%
% expressing CD45RO (memory)	86.8 ± 4.6%	37.9 ± 3.7%
% expressing CD25 (activated)	95.4 ± 0.7%	28.2 ± 2.5%
% expressing CD69 (activated)	50.6 ± 9.1%	6.0 ± 0.7%

[1] PMBC were purified from two unrelated donors, mixed to generate an allogeneic MLR and either labelled with CFSE or left untreated. On day 9 of culture, cells were labelled with anti-CD4 and (where appropriate) anti-CD45RA/anti-CD45RO/anti-CD25/anti-CD69 and analysed by flow cytometry. Sub-populations of T cells expressing high or low levels of CD4 were electronically gated and the forward light scatter characteristics, percentage of cells expressing activation markers and the percentage of cells showing a reduction in CFSE fluorescence were determined. Values represent mean ± SEM (n = 3).

Table 4.2: Co-expression of chemokine receptors and markers of memory and activation[1].

	Percentage co-expressing:			
	CD45RA	CD45RO	CD25	CD69
CCR5	14.6 ± 2.8	91.3 ± 5.2	90.3 ± 1.1	49.8 ± 2.0
CCR6	19.1 ± 4.8	93.7 ± 3.2	80.1 ± 1.6	33.1 ± 1.7
CXCR3	17.9 ± 1.0	85.7 ± 3.4	82.3 ± 5.0	42.9 ± 4.5
CXCR4	6.0 ± 0.7	86.3 ± 3.9	98.6 ± 0.4	56.5 ± 11.5

[1] PMBC were purified from two unrelated donors and mixed to generate an allogeneic MLR. On day 9 of culture, cells were labelled with anti-CD4 and the other antibodies as indicated and analysed by flow cytometry. $CD4^+$ T cells were electronically gated, and the percentage of cells double positive for the chemokine receptor and the memory/activation marker determined as a proportion of total chemokine receptor-positive cells. Values represent mean ± SEM (n = 4).

Figure 4.1. *$CD4^{hi}$ T cells: time-course of development.* PBMC were purified from two unrelated donors and cultured under syngeneic or allogeneic conditions for up to 24 days. Immediately after preparation of cultures (day 0), or at the time-points indicated, cells were labelled with anti-CD4 antibodies and analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. Shown are fluorescence histograms demonstrating the development of the $CD4^{hi}$ population in allogeneic cultures at day 0 [A], day3 [B], day 6 [C], day 9 [D], day 12 [E] and day 24 [F]. Also shown, for comparison, is the staining for CD4 observed in syngeneic cultures at day 12 [G]. Data are representative of >6 experiments.

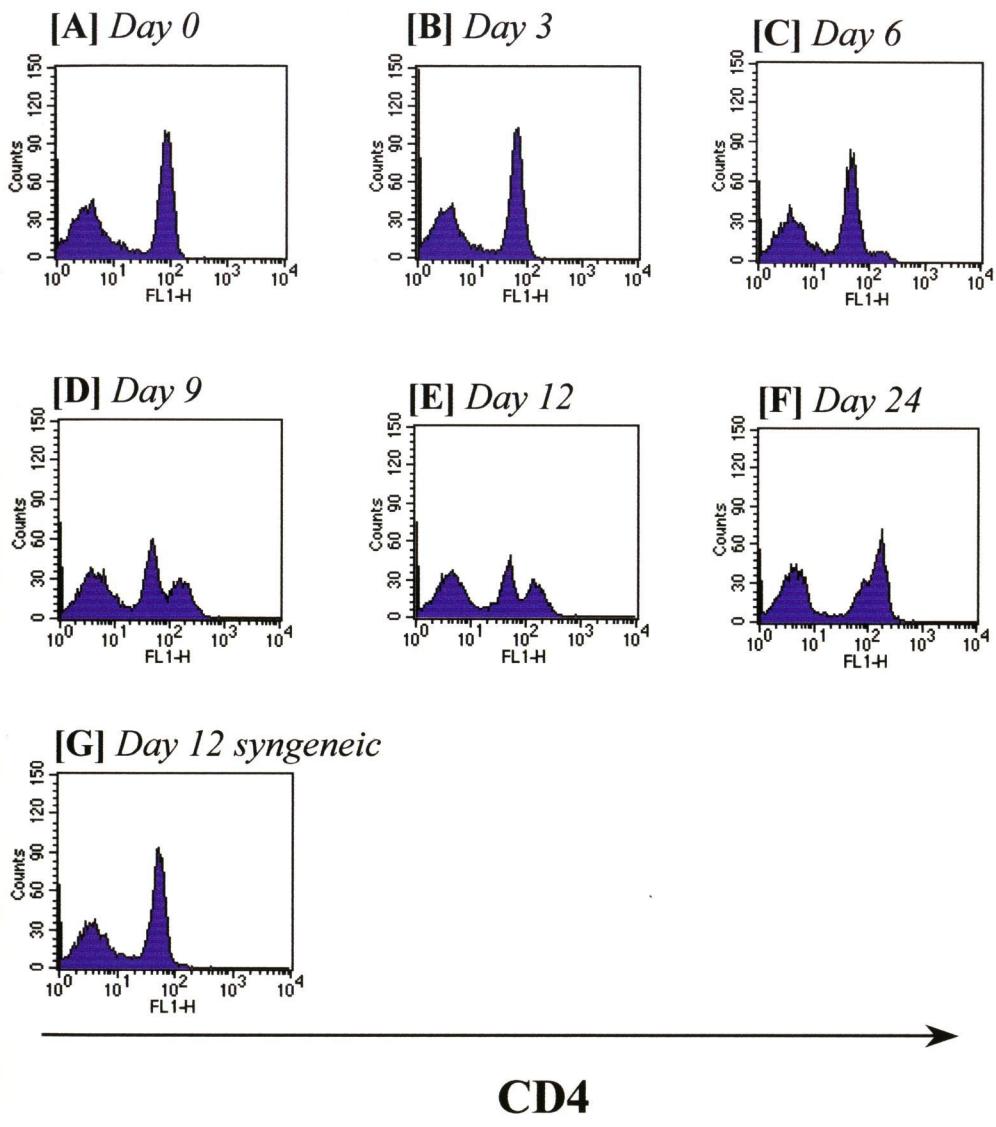
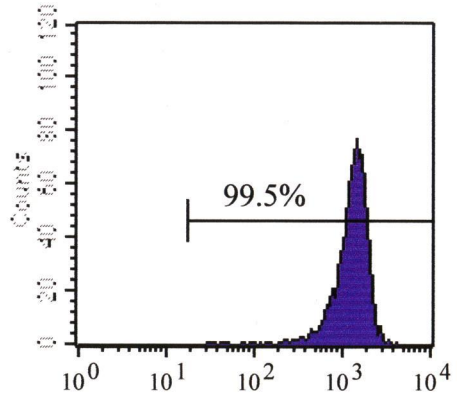

[A] Day 0
[B] Day 3
[C] Day 6
[D] Day 9
[E] Day 12
[F] Day 24
[G] Day 12 syngeneic
Counts
FL1-H
CD4

Figure 4.2. *$CD4^{hi}$ and $CD4^{normal}$ T cells co-express CD3*. PBMC were purified from two unrelated donors and mixed to generate an allogeneic MLR. After 9 days of culture, cells were labelled with antibodies to CD3 and CD4 and analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. Lymphocytes with normal [A] or elevated [B] levels of CD4 expression were electronically gated, and the expression of CD3 on each population determined. Data are representative of 3 experiments.

[A] *gated on CD4 normal*



[B] *gated on CD4 hi*

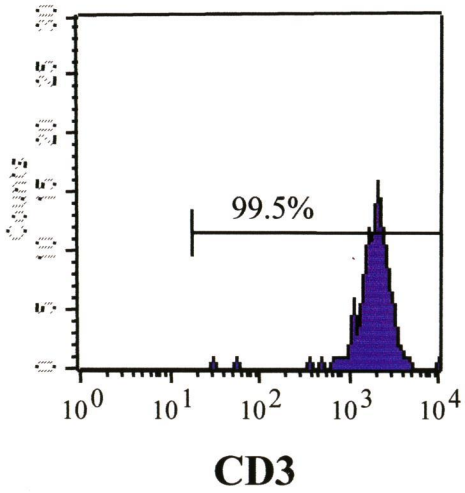


Figure 4.3. *Fluorescence intensity of small and large lymphocytes: comparison of autofluorescence and staining with anti-CD4.* PBMC were purified from two unrelated donors and mixed to generate an allogeneic MLR. After 9 days in culture, cells were either left unstained or labelled with anti-CD4, and subsequently analysed by flow cytometry. Small and large lymphocytes were electronically gated on the basis of forward and side scatter characteristics [A], and the green ('FL1') fluorescence determined for each population. Histograms represent the fluorescence intensity of small (red) and large (green) lymphocytes within populations of unstained [B] and anti-CD4 labelled [C] cells. In [D], the MFI of large lymphocytes has been divided by the MFI of small lymphocytes to give a ratio value for unstained and anti-CD4-labelled samples of cells. Values represent mean $\pm$ SEM (n = 6).

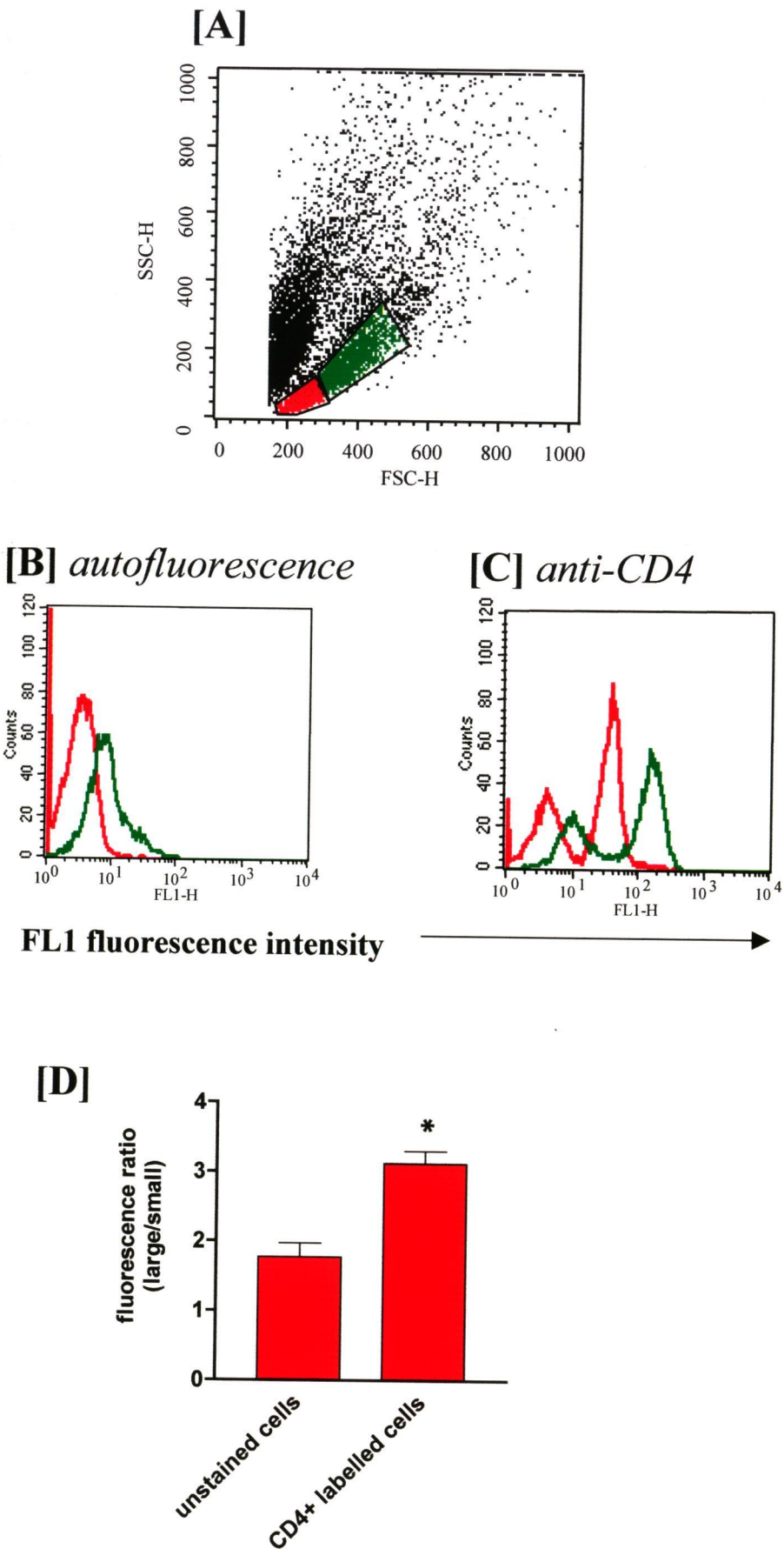

[A]
SSC-H
FSC-H
0
200
400
600
800
1000
[B] autofluorescence
[C] anti-CD4
Counts
FL1-H
FL1 fluorescence intensity
[D]
fluorescence ratio (large/small)
*
unstained cells
CD4+ labelled cells

Figure 4.4. *Differential expression of chemokine receptors on $CD4^{normal}$ and $CD4^{hi}$ T cell sub-populations.* PBMC were purified from two unrelated donors and cultured under syngeneic or allogeneic conditions. After 9 days, cells were labelled with anti-CD4 and antibodies to chemokine receptors and analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. Sub-populations of $CD4^{normal}$ and $CD4^{high}$ T cells were gated, and the percentage of cells expressing the relevant chemokine receptor was determined for each population. Values represent mean $\pm$ SEM (n = 7).

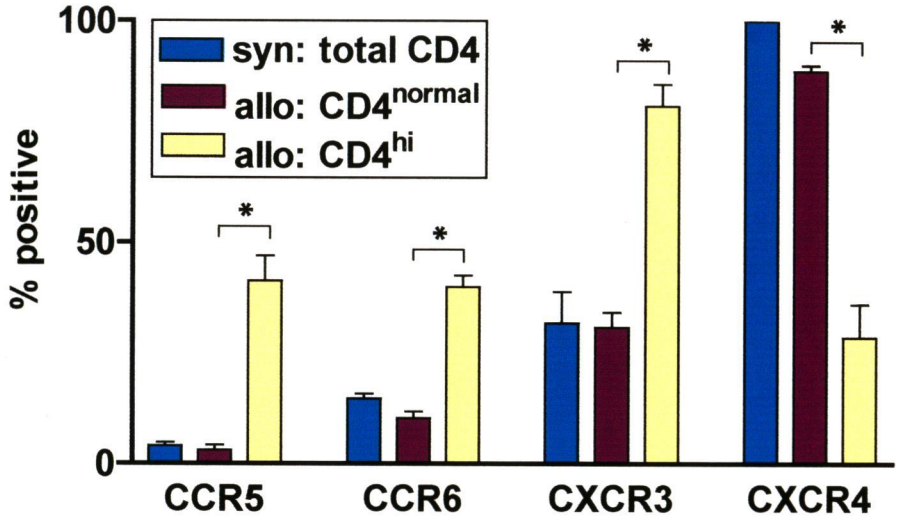

100
50
0
% positive
syn: total CD4
allo: CD4normal
allo: CD4hi
*
*
*
*
CCR5
CCR6
CXCR3
CXCR4

Figure 4.5. *Comparison of memory/activation marker expression in day 0, day 9 syngeneic and day 9 allogeneic MLR cultures.* PMBC were purified from two unrelated donors and mixed to generate syngeneic or allogeneic MLR cultures. Immediately after the preparation of allogeneic cultures, or after 9 days of culture under syngeneic or allogeneic conditions, cells were labelled with anit-CD4 in combination with antibodies against CD45RA, CD45RO, CD25 or CD69, and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the percentage of $CD4^+$ T cells expressing each memory/activation marker determined as a proportion of total $CD4^+$ T cells. A statistically significant difference between day 0 and day 9 values is indicated by an asterisk. Values represent mean $\pm$ SEM (n = 3-6 at each time-point).

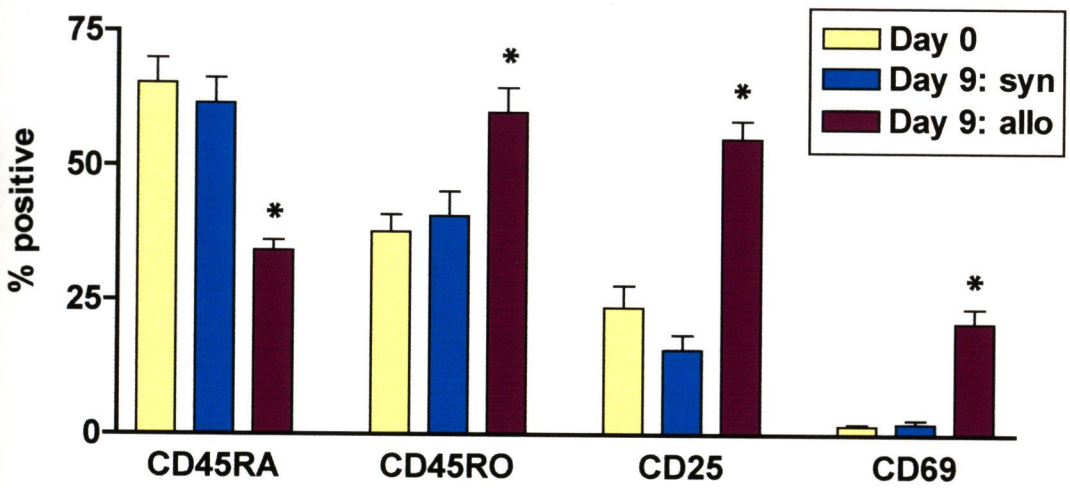

75
50
25
0
% positive
Day 0
Day 9: syn
Day 9: allo
*
*
*
*
CD45RA
CD45RO
CD25
CD69

Figure 4.6. *Co-expression of chemokine receptors and isoforms of CD45R on allo-activated $CD4^+$ T cells.* PMBC were purified from two unrelated donors and mixed to generate an allogeneic MLR. On day 9 of culture, cells were labelled with anti-CD4 and either anti-CCR5 [A & B], anti-CCR6 [C & D], anti-CXCR3 [E & F] or anti-CXCR4 [G & H] and either anti-CD45RA [A, C, E and G] or anti-CD45RO [B, D, F and H], followed by flow cytometric analysis, gating on lymphocytes using forward and side scatter characteristics. $CD4^+$ T cells were gated, and staining for the remaining markers was displayed on bivariate density plots. Data shown are representative of 4 experiments.

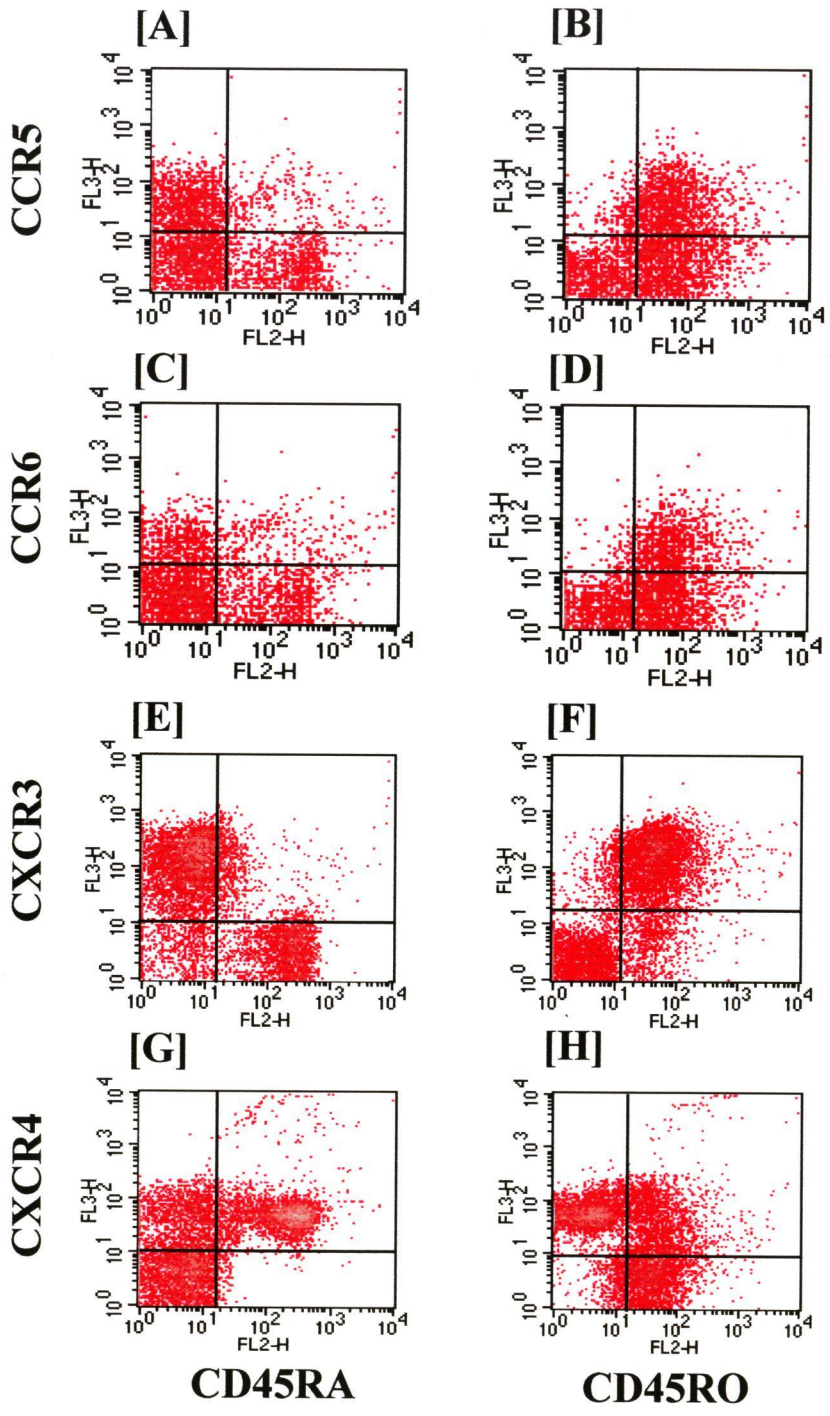

[A]
[B]
[C]
[D]
[E]
[F]
[G]
[H]
CCR5
CCR6
CXCR3
CXCR4
FL3-H
FL2-H
CD45RA
CD45RO

Figure 4.7. *Co-expression of chemokine receptors and activation markers on allo-activated $CD4^+$ T cells.* PMBC were purified from two unrelated donors and mixed to generate an allogeneic MLR. On day 9 of culture, cells were labelled with anti-CD4 and either anti-CCR5 [A & B], anti-CCR6 [C & D], anti-CXCR3 [E & F] or anti-CXCR4 [G & H] and either anti-CD25 [A, C, E and G] or anti-CD69 [B, D, F and H], followed by flow cytometric analysis, gating on lymphocytes using forward and side scatter characteristics. $CD4^+$ T cells were gated, and staining for the remaining markers was displayed on bivariate density plots. Data shown are representative of 4 experiments.

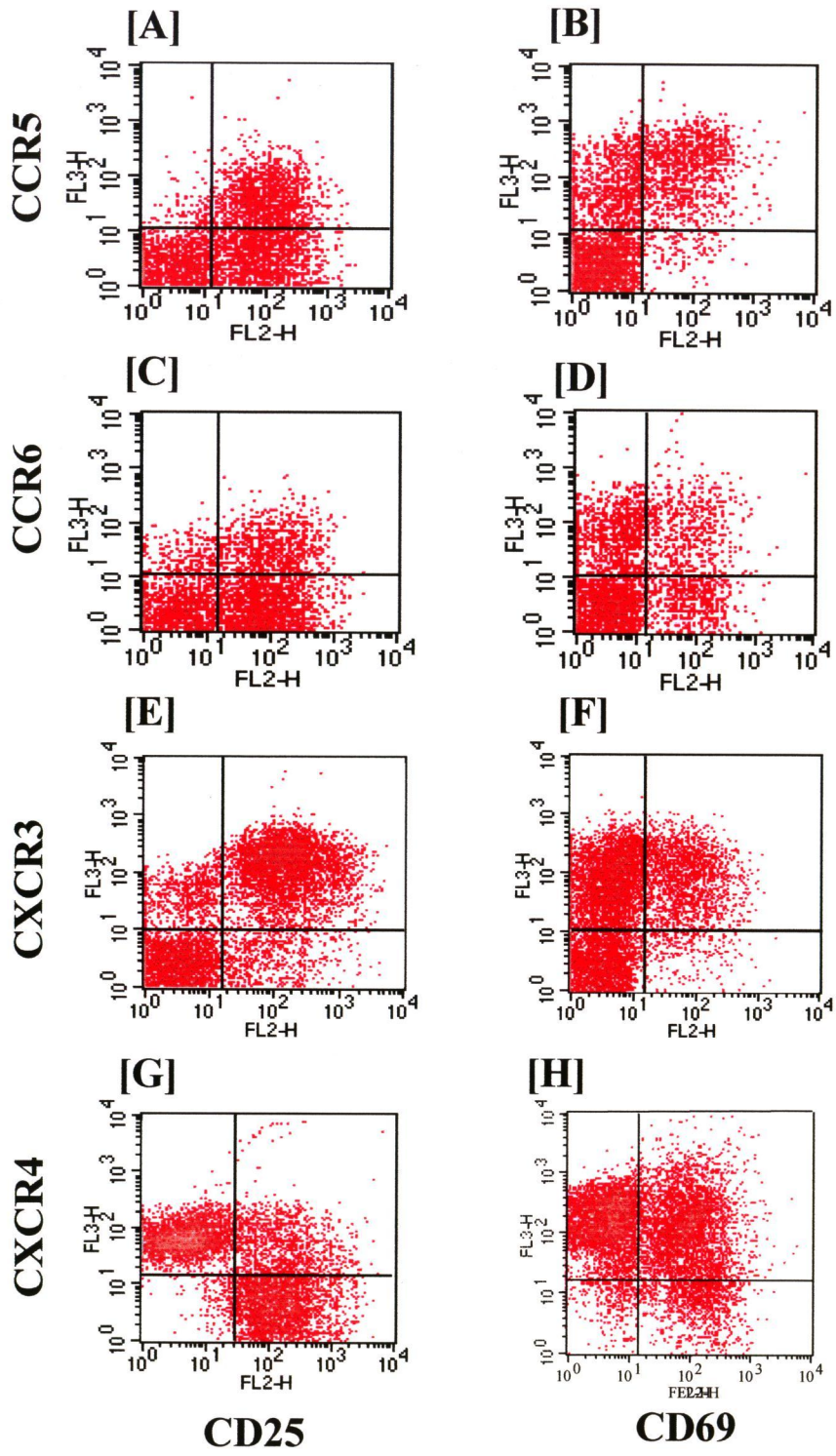
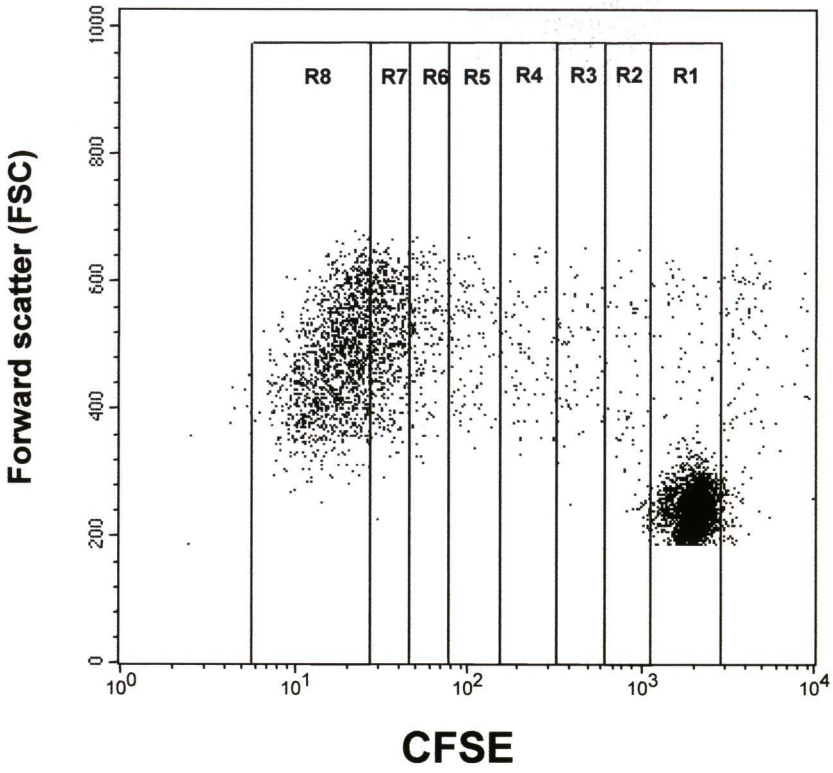

[A]
[B]
[C]
[D]
[E]
[F]
[G]
[H]
CCR5
CCR6
CXCR3
CXCR4
FL3-H
FL2-H
CD25
CD69

Figure 4.8. *Determination of $CD4^+$ T cell division using the CFSE technique.* PMBC were purified from two unrelated donors and mixed to generate an allogeneic MLR. Cells were labelled with CFSE, cultured for 6 days, then labelled with anti-CD4 and analysed by flow cytometry. $CD4^+$ T cells were electronically gated, and dot plots of CFSE fluorescence intensity versus forward scatter generated [A]. By visual inspection, regions R1 – R8 were generated and used to define cells that had undergone a set number of rounds of cell division. Subsequently, the mean of CFSE fluorescence intensity was plotted against division number and a line of best fit generated using non-linear regression. Data are representative of 6 experiments.

[A]


R8
R7
R6
R5
R4
R3
R2
R1
Forward scatter (FSC)
0
200
400
600
800
1000
10^0
10^1
10^2
10^3
10^4
CFSE

[B]

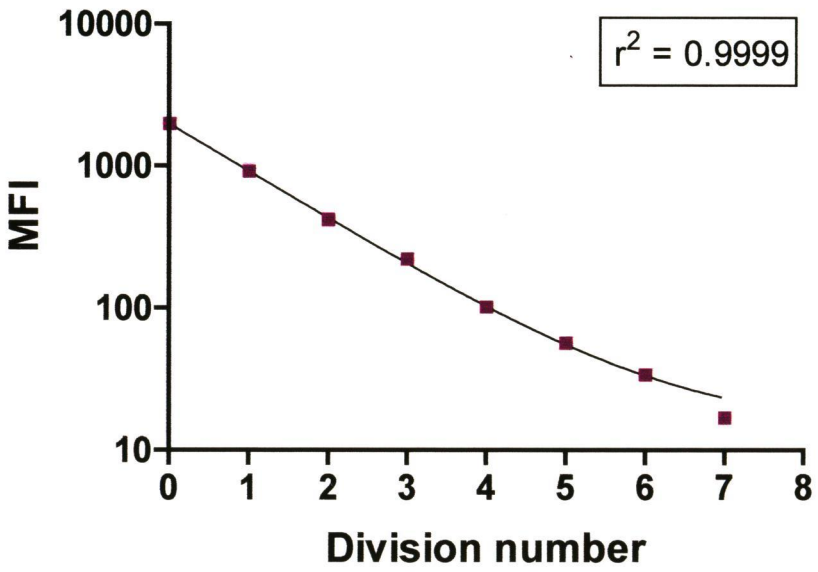
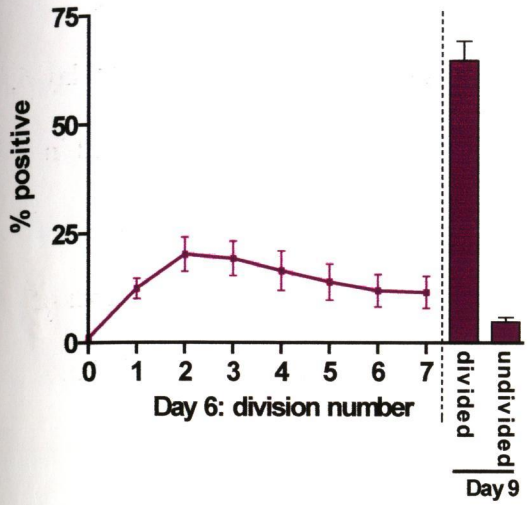
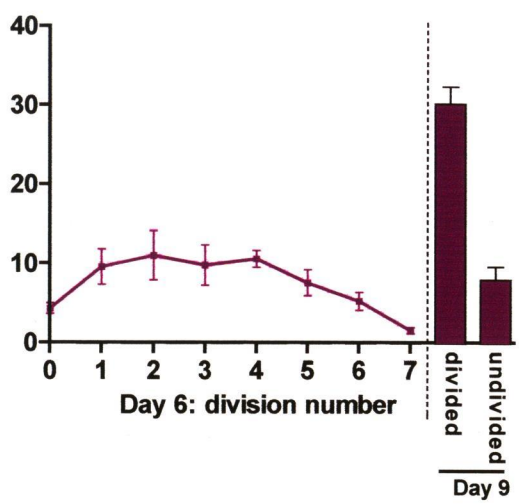

r^2 = 0.9999
MFI
10000
1000
100
10
0
1
2
3
4
5
6
7
8
Division number

Figure 4.9. *The expression of chemokine receptors on CD4$^+$ T cells that have undergone a defined number of cell divisions.* PMBC were purified from two unrelated donors and mixed to generate an allogeneic MLR. Cells were labelled with CFSE, cultured for 6 or 9 days, then labelled with anti-CD4 and anti-chemokine receptor antibodies and analysed by flow cytometry, gating on CD4$^+$ T cells. At day 6, cells that had undergone 0 – 7+ rounds of cell division were defined as illustrated in Figure 4.8, and the percentage of CD4$^+$ T cells expressing chemokine receptors determined for each population. At day 9, it was not possible to define the number of rounds of cell division undertaken, and therefore, cells are designated as divided or undivided. Values represent mean $\pm$ SEM (n = 4-6 at each time-point).

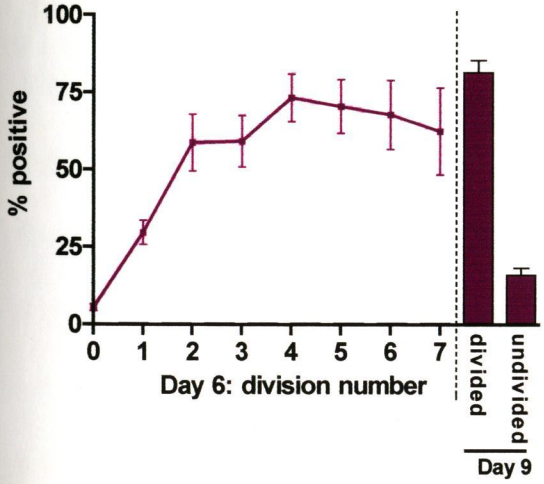
[A] *CCR5*



[B] *CCR6*



[C] *CXCR3*



[D] *CXCR4*

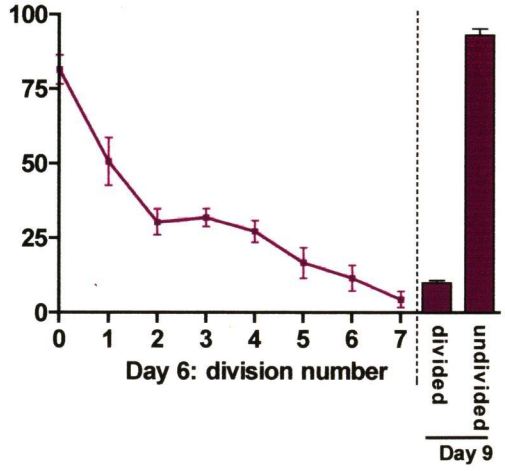


Figure 4.10. *The percentage of divided CD4$^+$ T cells expressing each chemokine receptor.* PMBC were purified from two unrelated donors and mixed to generate an allogeneic MLR. Cells were labelled with CFSE, cultured for 9 days, then labelled with anti-CD4 and anti-chemokine receptor antibodies and analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. The percentage of CD4$^+$ T cells that had divided in culture and expressed the indicated chemokine receptor phenotype were determined as a proportion of total divided CD4$^+$ T cells. Statistical significance is indicated by an asterisk. Values represent mean $\pm$ SEM (n = 4).

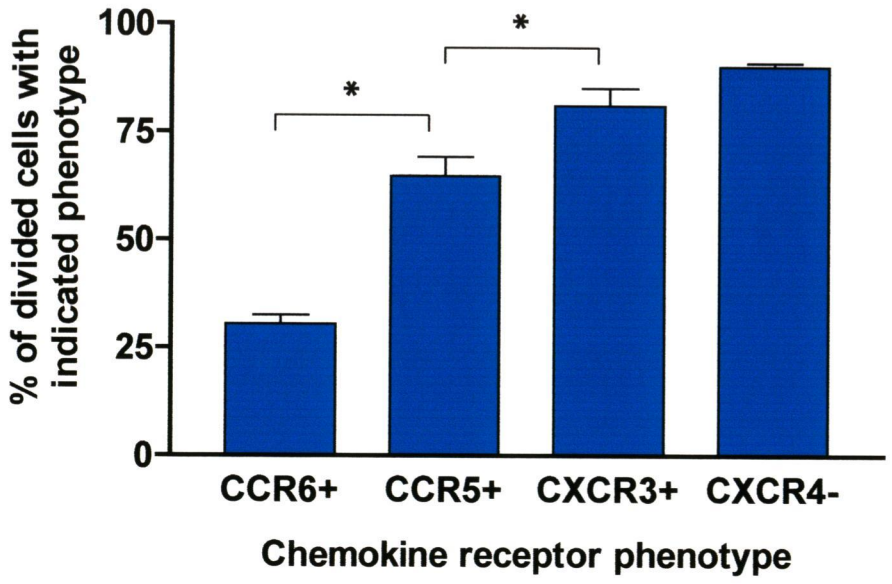

% of divided cells with indicated phenotype
100
75
50
25
0
*
*
CCR6+
CCR5+
CXCR3+
CXCR4-
Chemokine receptor phenotype

Figure 4.11. *Expression of chemokine receptors on $CD4^+$ T cells in extended MLR culture*. PMBC were purified from two unrelated donors and mixed to generate an allogeneic MLR. After either 9 or 24 days in culture, cells were labelled with anti-CD4 and anti-chemokine receptor antibodies and analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. The percentage of $CD4^+$ T cells expressing each chemokine receptor was determined as a proportion of total $CD4^+$ T cells at each time-point. Values represent mean $\pm$ SEM ($n \geq 4$ at each time-point).

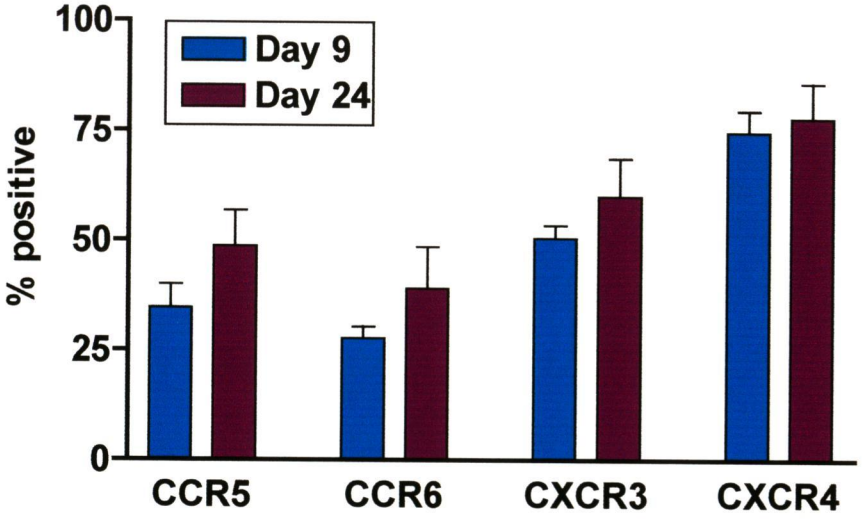

100
75
50
25
0
% positive
Day 9
Day 24
CCR5
CCR6
CXCR3
CXCR4

Figure 4.12. *Expression of memory/activation markers on $CD4^+$ T cells in extended MLR culture*. PMBC were purified from two unrelated donors and mixed to generate an allogeneic MLR. After either 9 or 24 days in culture, cells were labelled with anti-CD4 and either anti-CD45RA, anti-CD45RO or anti-CD25, and then analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. The percentage of $CD4^+$ T cells expressing each memory/activation marker was determined as a proportion of total $CD4^+$ T cells at each time-point. A statistically significant difference between day 9 and day 24 values is indicated by an asterisk. Values represent mean $\pm$ SEM (n = 4-6 at each time-point).

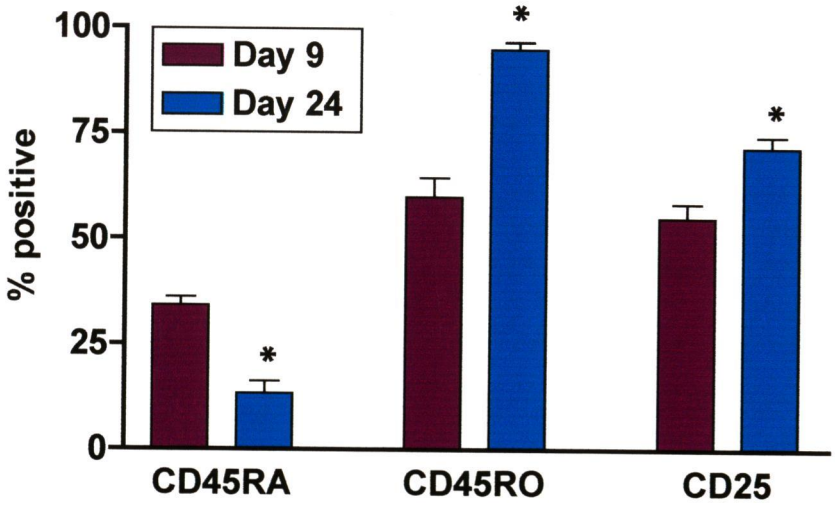
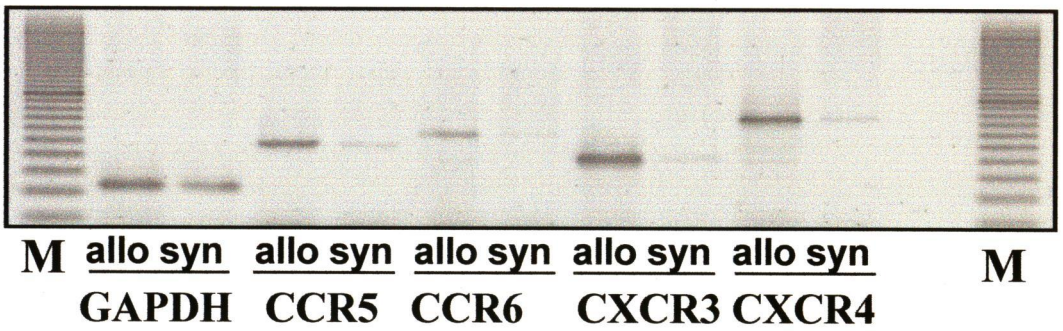

100
75
50
25
0
% positive
Day 9
Day 24
*
*
*
CD45RA
CD45RO
CD25

Figure 4.13. *RT-PCR analysis of chemokine receptor mRNA levels in day 9 allogeneic and syngeneic MLR cultures.* PMBC were purified from two unrelated donors and cultured under syngeneic or allogeneic conditions for 9 days. RNA was extracted from cell pellets, reverse-transcribed, and used in PCR with primers specific for CCR5, CCR6, CXCR3, CXCR4 or GAPDH. A representative SYBR-gold stained agarose gel is shown in [A], while a densitometric analysis is presented in [B]. The data shown in [B] represent the chemokine receptor band density as a ratio of GAPDH band density from the same sample. A statistically significant difference between syngeneic and allogeneic conditions is indicated by an asterisk. Values represent mean $\pm$ SEM (n = 3)

[A]


M
allo syn
GAPDH
allo syn
CCR5
allo syn
CCR6
allo syn
CXCR3
allo syn
CXCR4
M

[B]

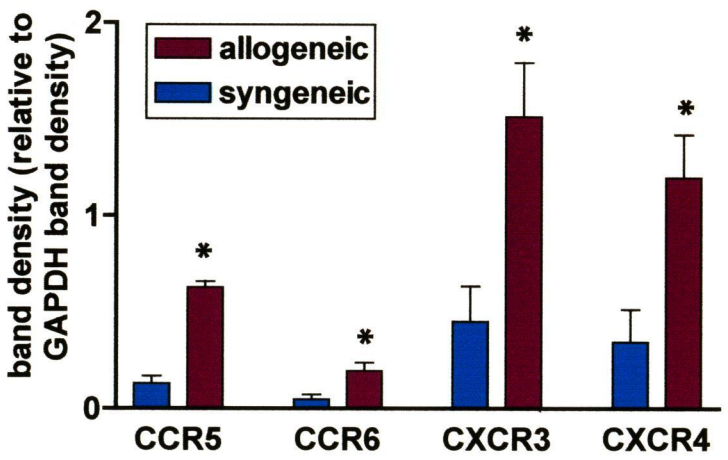
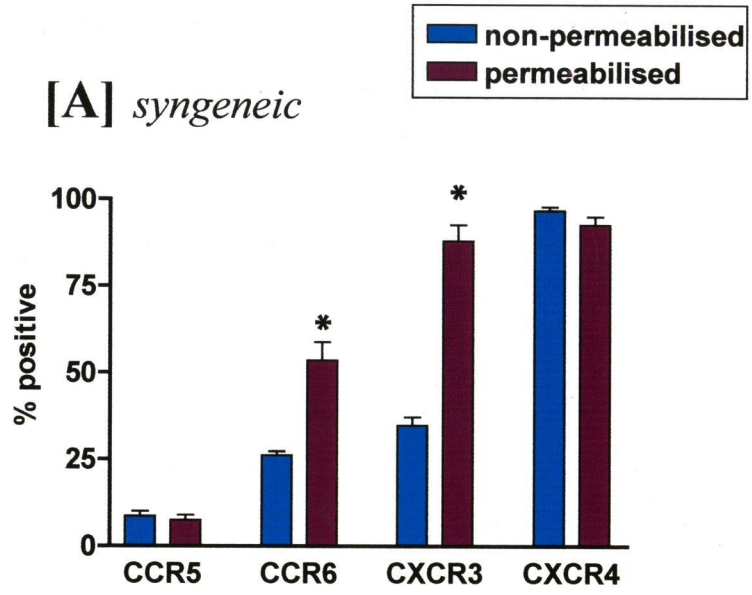

band density (relative to GAPDH band density)
2
1
0
allogeneic
syngeneic
*
*
*
*
CCR5
CCR6
CXCR3
CXCR4

Figure 4.14. *Comparison of chemokine receptor protein in intracellular versus extracellular compartments.* PMBC were purified from two unrelated donors and cultured under syngeneic [A] or allogeneic [B] conditions. After 9 days in culture, cells were collected, fixed in PFA, then either permeabilised with saponin or left untreated. Permeabilised and non-permeabilised cells were labelled with anti-CD4 and anti-chemokine receptor antibodies and subsequently analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. The percentage of $CD4^+$ T cells expressing each chemokine receptor was calculated as a proportion of total $CD4^+$ cells for each condition. A statistically significant difference between permeabilised and non-permeabilised conditions is indicated by an asterisk. Data are presented as mean $\pm$ SEM (n = 4-8).


non-permeabilised
permeabilised
[A] syngeneic
100
75
50
25
0
% positive
*
*
CCR5
CCR6
CXCR3
CXCR4

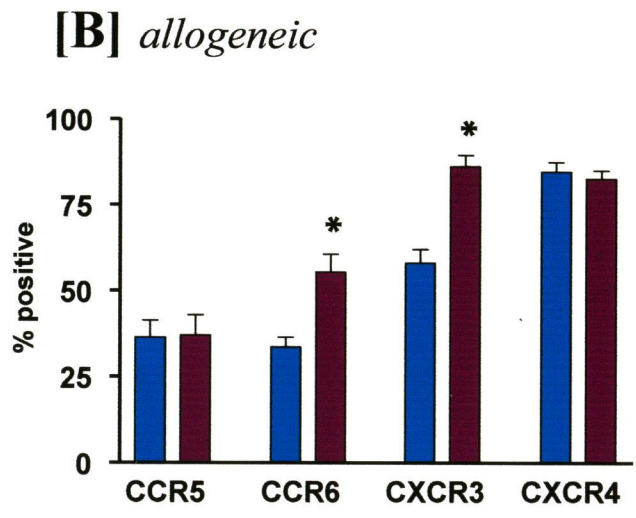

[B] allogeneic
100
75
50
25
0
% positive
*
*
CCR5
CCR6
CXCR3
CXCR4

Figure 4.15. *Localisation of chemokine receptor protein by immunofluorescence microscopy.* PMBC were purified from two unrelated donors and cultured under syngeneic [A, C, F and H] or allogeneic [B, D, E, G and I] conditions. After 9 days in culture, cells were spun onto microscope slides, fixed, permeabilised and stained with anti-CCR5 [A-B], anti-CXCR4 [C-D], anti-CCR6 [F-G] or anti-CXCR3 [H-I] and viewed by fluorescence microscopy. Images shown are representative of 4 independent experiments. In [E], cells were fixed but not permeabilised, and then labelled with anti-CD4 and analysed as described above.

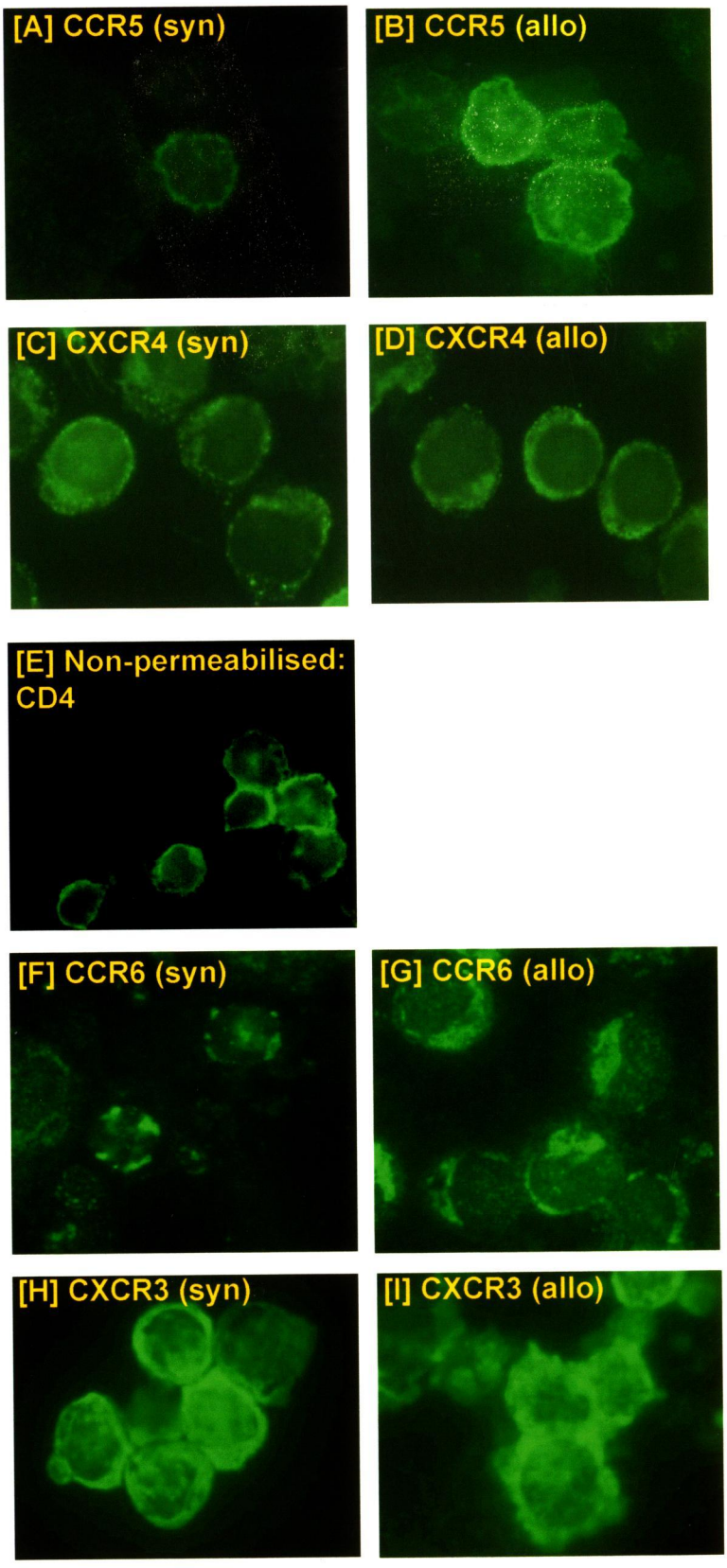

[A] CCR5 (syn)
[B] CCR5 (allo)
[C] CXCR4 (syn)
[D] CXCR4 (allo)
[E] Non-permeabilised: CD4
[F] CCR6 (syn)
[G] CCR6 (allo)
[H] CXCR3 (syn)
[I] CXCR3 (allo)

Figure 4.16. *Transwell chemotaxis assays to compare chemotactic responsiveness of cells from syngeneic and allogeneic day 9 cultures.* PMBC were purified from two unrelated donors and cultured under syngeneic or allogeneic conditions. On day 9 of culture, cells were collected and subject to Transwell chemotaxis assays using MIP-1β/CCL4, MIP-3α/CCL20, I-TAC/CXCL11, SDF-1/CXCL12 or diluent alone in the lower chamber. The absolute number of migrated cells was determined by duplicate haemocytometer counts, and the migration index calculated, as described in Chapter 2. A statistically significant difference between syngeneic and allogeneic conditions is indicated by an asterisk. Values represent mean ± SEM (n = 6).

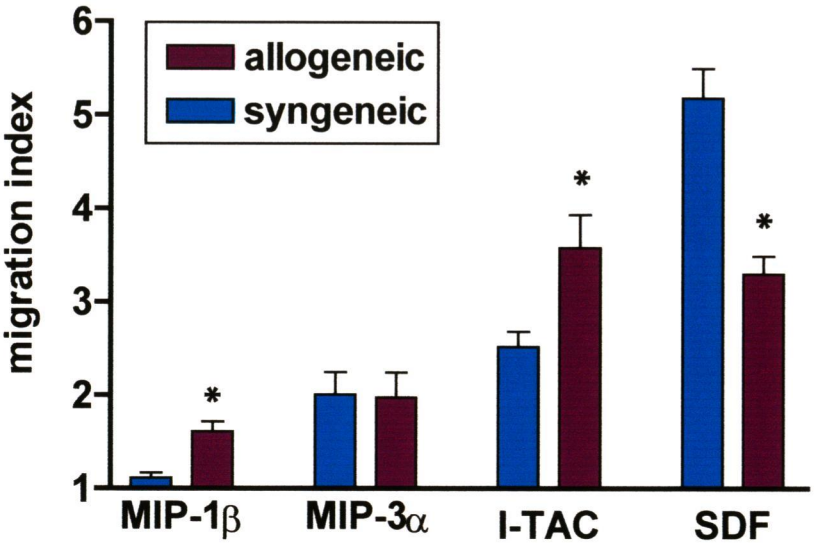

migration index
6
5
4
3
2
1
allogeneic
syngeneic
*
*
*
MIP-1β
MIP-3α
I-TAC
SDF

Figure 4.17. *Dose-response of $CD4^+$ T cell migration toward MIP-3α/CCL20 following culture under syngeneic or allogeneic conditions.* PMBC were purified from two unrelated donors and cultured under syngeneic or allogeneic conditions. On day 9 of culture, cells were collected, labelled with Calcein and subject to Transwell chemotaxis assays. Migration through the filter was determined fluorometrically, as described in Chapter 2. Following the chemotaxis assay, the migrated cells (as well as a sample of the starting population) were labelled with anti-CD4 and analysed by flow cytometry, thereby allowing the percentage of $CD4^+$ T cells migrated to be calculated. Values represent mean $\pm$ SEM (n = 4).

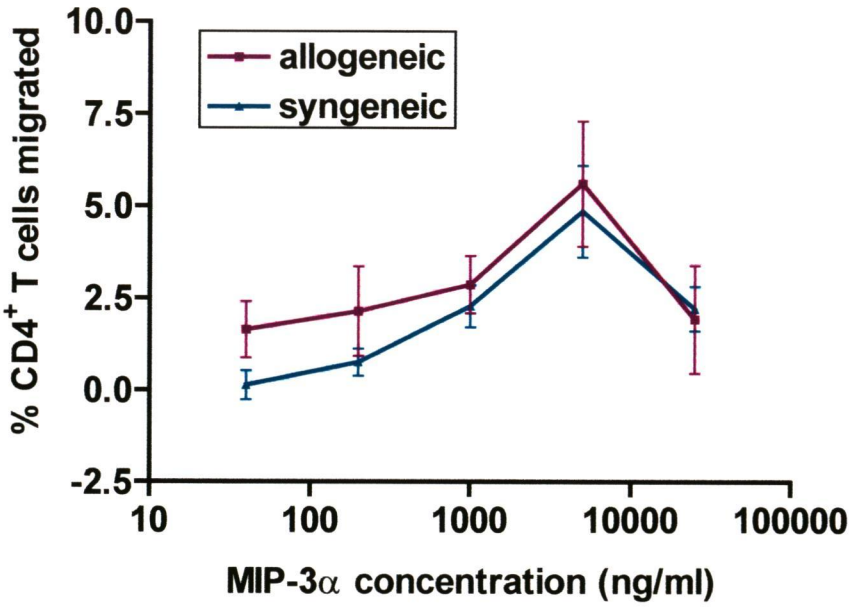
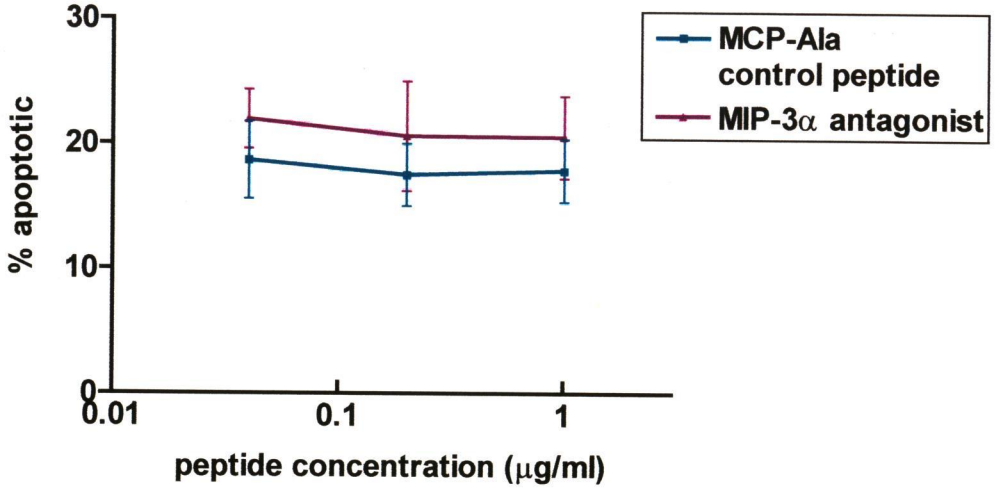

10.0
7.5
5.0
2.5
0.0
-2.5
% CD4+ T cells migrated
allogeneic
syngeneic
10
100
1000
10000
100000
MIP-3α concentration (ng/ml)

Figure 4.18. *The effect of a MIP-3α antagonist on apoptosis in day 12 allogeneic MLR cultures.* PMBC were purified from two unrelated donors and mixed to generate an allogeneic MLR. Peptide (MIP-3α 4-70 or MCP-1 Ala) was added at the initiation of culture to the indicated final concentration. After 12 days in culture, cells were labelled with annexin-V and EMA and analysed by flow cytometry. The percentage of apoptotic cells in each sample was defined as the percentage stained positive for annexin-V but negative for EMA. A dose-response is presented in [A] (n = 4), while representative density plots of cells treated with 1μg/ml of MCP-1 Ala or MIP-3α 4-70 are presented in [B] and [C], respectively.

[A]



[B] *control peptide*

[C] *MIP-3α antagonist*

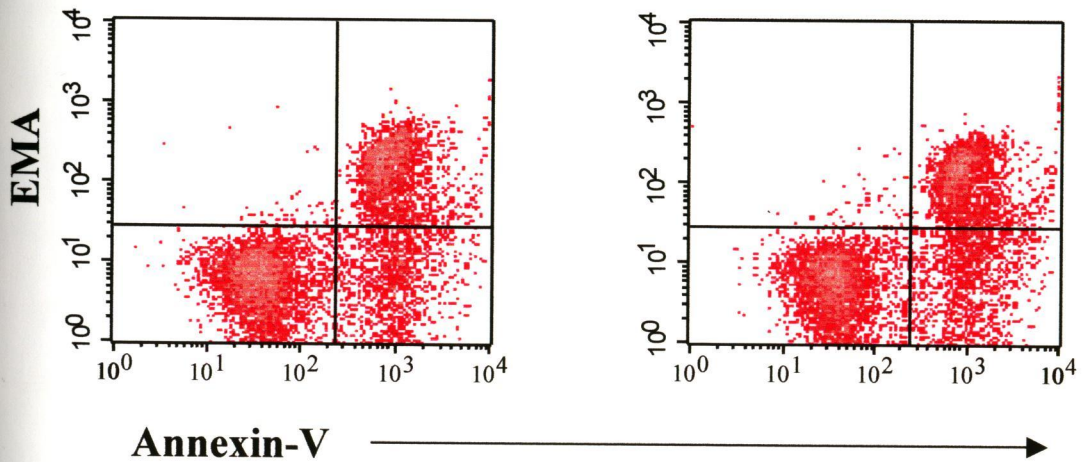
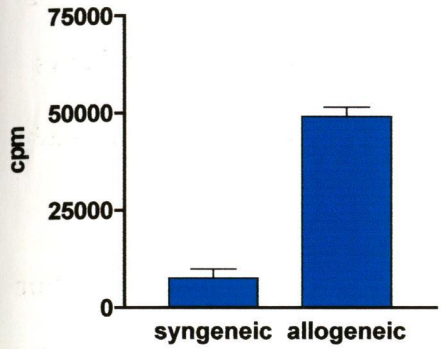
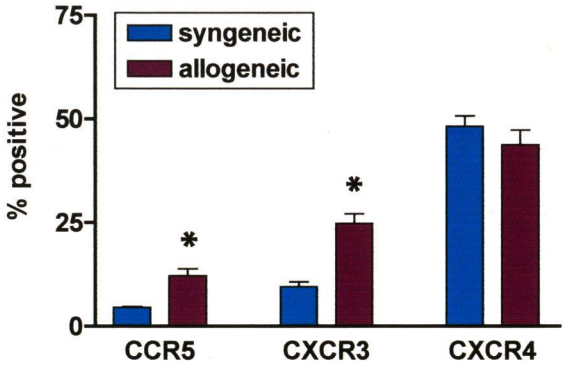


Figure 4.19. *Proliferation and chemokine receptor expression in murine MLR cultures*. Splenocytes were prepared from BALB/c mice and cultured alone (syngeneic) or mixed with either tsDCs [A-B] or splenocytes from BALB/c x CBA F1 mice [C-D] (allogeneic). To quantify cellular proliferation [A and C], cells were cultured for 5 days, with 1μCi of [^{3}H]-thymidine being added to each well for the last 18 hours. Incorporation of [^{3}H]-thymidine was determined by scintillation counting. To assess chemokine receptor expression [B and D], cells were cultured for 7 days and then stained with anti-CD4 and anti-chemokine receptor antibodies, followed by flow cytometric analysis, gating on lymphocytes using forward and side scatter characteristics. The percentage of $CD4^+$ T cells expressing each chemokine receptor was determined as a proportion of total $CD4^+$ T cells. Values represent mean $\pm$ SEM (n = 4 for [A-B] and n = 2 for [C – D]). On [B], a statistically significant difference between syngeneic and allogeneic conditions is indicated by an asterisk. Statistical tests were not conducted for [D], as only two experiments were performed.

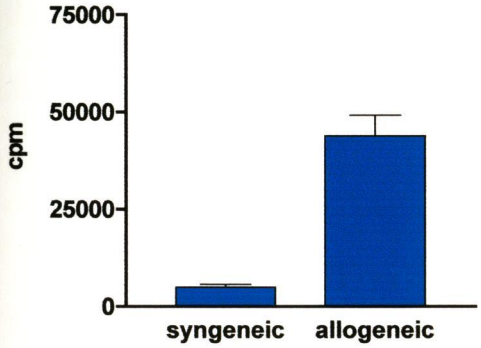
[A]


75000
50000
25000
0
cpm
syngeneic
allogeneic

[B]


75
50
25
0
% positive
syngeneic
allogeneic
*
*
CCR5
CXCR3
CXCR4

[C]


75000
50000
25000
0
cpm
syngeneic
allogeneic

[D]

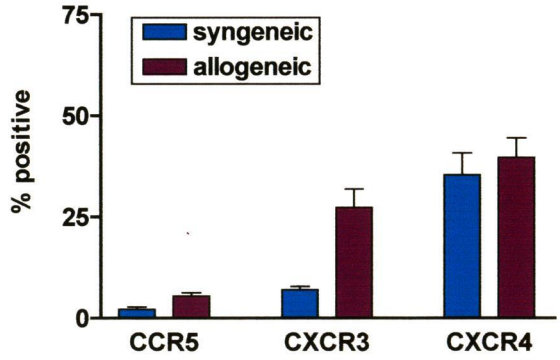

75
50
25
0
% positive
syngeneic
allogeneic
CCR5
CXCR3
CXCR4

Figure 4.20. *Chemokine receptor expression on $CD4^+$ T cells in the murine MLR: representative density plots.* Splenocytes were prepared from BALB/c mice and either cultured alone [A-C] or mixed with tsDC stimulator cells to generate allogeneic cultures [D-F]. Cells were cultured for 7 days and then stained with anti-CD4 and either anti-CCR5 [A and D], anti-CXCR3 [B and E] or anti-CXCR4 [C and F] and analysed by flow cytometry. Lymphocytes were gated using forward and side scatter characteristics and the quadrant markers set on the basis of staining with control goat IgG.

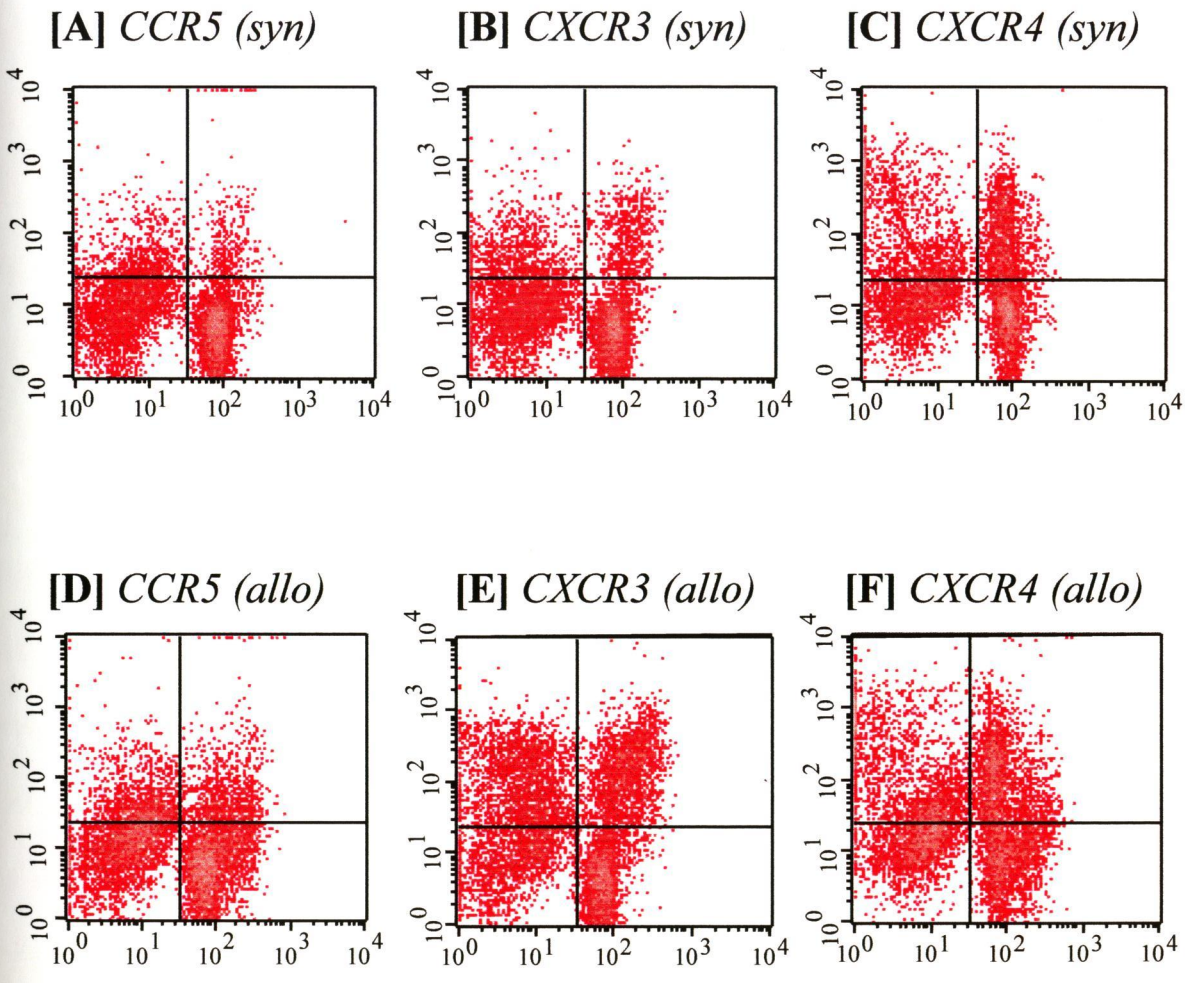
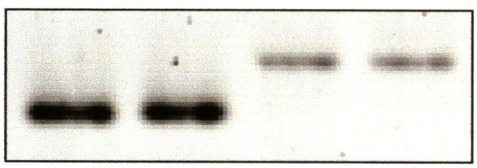

[A] CCR5 (syn)
[B] CXCR3 (syn)
[C] CXCR4 (syn)
[D] CCR5 (allo)
[E] CXCR3 (allo)
[F] CXCR4 (allo)
10^0 10^1 10^2 10^3 10^4

Figure 4.21. *RT-PCR analysis of CCR6 mRNA levels in the murine MLR.* Splenocytes were prepared from BALB/c mice and either cultured alone (syngeneic) or with tsDC stimulator cells (allogeneic) for 7 days. RNA was extracted from cell pellets, reverse-transcribed, and used in PCR with primers specific for CCR6 or GAPDH. A representative SYBR-gold stained agarose gel is shown in [A], while a densitometric analysis is presented in [B]. The data shown in [B] represent the CCR6 band density as a ratio of GAPDH band density from the same sample. Values represent mean $\pm$ SEM (n = 4).

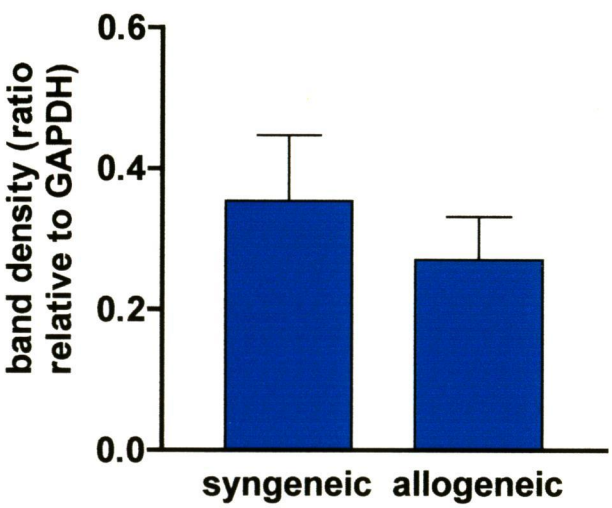
[A]



allo syn allo syn

GAPDH CCR6

[B]



Summary – Chapter 5

- The sub-cutaneous injection of allogeneic DCs into the footpads of naïve mice resulted in a dramatic increase in the cellularity of the draining LN at days 5 and 8 post-immunisation.
- Part of this increase was due to the local proliferation of $CD4^+$ and $CD8^+$ T cells, while the remainder was due to the influx of cells from the circulation.
- This influx resulted in a large increase in the absolute numbers of $CCR5^+$, $CXCR3^+$ and $CXCR4^+$ $CD4^+$ T cells in the LN.
- However, analysis of the relative proportions of these cell populations suggested that the entry of cells expressing CCR5 or CXCR3 was more efficient than the entry of CXCR4-expressing cells.
- Within the population of dividing $CD4^+$ T cells in the LN, the proportion expressing CCR5 and CXCR3 was strikingly increased compared to the non-dividing cells. CXCR4 was only modestly up-regulated on the dividing cells.
- The injected DCs were retained at the site of immunisation for at least 8 days, and this was associated with a modest accumulation of $CD4^+$ T cells within the footpad tissue.
- In preliminary studies, a number of these infiltrating $CD4^+$ T cells were shown to express CCR5 and CXCR3, supporting the notion that the acquisition of these receptors upon activation may contribute to the ability of T cells to migrate to peripheral sites of immunisation.

CHAPTER 5

REGULATION OF CHEMOKINE RECEPTOR EXPRESSION ON MURINE $CD4^+$ T CELLS ACTIVATED BY ALLO-ANTIGEN *IN VIVO*

5.1 Introduction

Thus far, starting with a panel of nine chemokine receptors, four have been identified that demonstrate altered patterns of expression on $CD4^+$ T cells upon allogeneic activation. The regulation of these four receptors has been investigated in detail in the human MLR, and, in the case of CCR5 and CXCR3, an analogous pattern of up-regulation on the surface of T cells was shown to occur in the murine MLR, although the expression of CXCR4 was unchanged. It is clearly important, however, to confirm and extend upon these observations in an *in vivo* model. Accordingly, the aim of the present series of experiments was to analyse the expression of CCR5, CXCR3 and CXCR4 on $CD4^+$ T cells in secondary lymphoid tissue during the generation of a primary immune response. CCR6 could not be readily studied in this system, due to the current lack of an anti-murine CCR6 antibody.

In keeping with the *in vitro* studies conducted thus far, an *in vivo* version of the MLR was used to assess the regulation of chemokine receptor expression in a whole-animal system. Thus, the tsDC cell line (see Section 4.2.8) was utilised as a source of APCs to stimulate an allogeneic immune response upon subcutaneous (s.c.) injection into an MHC-mismatched strain of recipient mice (BALB/c). The s.c. injection of allogeneic splenocytes is a commonly used model of allogeneic activation. The availability of the tsDC cell line, however, provided the opportunity for a similar model of immune activation to be developed using a homogeneous, and readily available, source of allogeneic stimulator cells. The tsDC cell line has been shown previously (202), as well as in Chapter 4, to be a potent stimulator of the MLR *in vitro*. Moreover, the ability of these cells present exogenous antigen, and induce a protective immune response, *in vivo* has also been demonstrated (223).

5.2 Results

5.2.1 Establishment of optimal immunisation conditions

Although the tsDC cell line is known to stimulate an MLR *in vitro*, the conditions required for the induction of an allogeneic immune response *in vivo* are not known. Accordingly, a dose-response study was conducted in order to confirm that the injection of tsDCs resulted in T cell activation, and to optimise the immunisation conditions to be used in subsequent analyses. The tsDCs were differentiated at the non-permissive temperature of 39°C for 24 hours, and then injected s.c. in PBS into the hind footpads of BALB/c mice at a range of cell concentrations. Control mice received an equal volume of PBS.

It is acknowledged that the injection of diluent (PBS) alone does not control for the various non-specific effects that may be induced by the injection of foreign cells into sub-cutaneous tissue, such as the secretion of pro-inflammatory cytokines. Initially, it was hoped that the injection of tsDCs into mice of the same genetic background from which the cell line was derived (CBA) would provide a more rigorous control. However, a significant immune response was mounted in these mice also (not shown), presumably due to antigenic changes that may have occurred during the long-term culture of tsDCs, and possibly proteins from the FCS in the culture medium that may have non-specifically bound to the surface of the cells. Therefore, in the absence of a more appropriate alternative, the injection of PBS alone was used as a control in all experiments, with the understanding that any observed differences between mice injected with PBS only and those injected with tsDCs would be due to a combination of factors, and not purely to the recognition of allo-antigen by T cells. However, as any

immune response is unlikely to involve antigen presentation in the absence of non-specific inflammation, this is still a realistic and biologically relevant model.

The extent of the immune response occurring in the draining (popliteal) LN was initially evaluated by enumerating the number of viable cells recovered per LN. As a second, more specific, measure of immune activation, an *in vitro* cytotoxicity assay was employed, utilising a monolayer of tsDCs as targets. At all doses tested, the injection of tsDCs resulted in a significant increase in the number of viable cells recovered from the draining popliteal LN at day 5, compared to injection with PBS alone (Fig 5.1; $p < 0.01$ for all). Although the number of cells recovered appeared to increase slightly with higher doses of tsDCs, these differences were not statistically significant. The cytotoxicity assay confirmed that an immune response had been mounted in LNs draining sites of tsDC injection. Cells recovered from LNs following the injection of PBS alone induced only minimal target cell destruction. In contrast, cells recovered from LNs sensitised with all doses of tsDCs mounted a significant cytotoxic response against tsDC target cells, with destruction of up to 86% of the target cell monolayer being observed in some samples (Fig 5.2; $p < 0.05$ for all).

On the basis of the results presented in Figures 5.1 and 5.2, the highest dose of tsDCs (5×10^5) was chosen for all further studies, as although the lower doses also induced T cell activation, the yield of LN cells recovered following injection of the highest dose was slightly higher and more consistent. After establishing the optimal dose of tsDCs to be injected, an experiment was conducted to determine if the co-injection of lipopolysaccharide (LPS) would enhance the immune response to tsDCs further, as LPS is known to have an adjuvant effect (5, 224, 225). However, when tsDCs were co-

injected with 1μg of LPS (as used previously; see ref (225)), no enhancement of cytotoxicity or LN cellularity was observed in comparison to the injection of tsDCs alone (not shown). Therefore, LPS was not used in subsequent experiments.

5.2.2 Characterisation of the immune response

5.2.2.1 Time-course of viable cell yield

In order to assess the time-frame over which the immune response to tsDCs developed, the number of viable cells recovered per LN was determined at days 2, 5 and 8, subsequent to injection of either PBS or 5 x 10^5 tsDCs (Fig 5.3). Following injection of tsDCs, there was no significant change in the number of cells recovered from LNs collected at day 2 post-immunisation, in comparison to the number recovered at the same time-point following PBS injection. However, by day 5, the mean number of cells recovered from sensitised LNs was 3.0-fold greater than the mean number recovered following PBS injection ($p < 0.0001$). At day 8 post-immunisation, the number of viable cells recovered was 2.9-fold above the control value for the same time-point ($p < 0.0001$).

5.2.2.2 The percentage of $CD4^+$ and $CD8^+$ T cells undergoing division in the popliteal LN

The time-course analysis of cell yields discussed above revealed an ~3-fold increase in the total number of lymphocytes recovered from sensitised LNs compared to control LNs at days 5 and 8. In order to assess what proportion of this increase was due to local proliferation in response to antigen, and what proportion was due to the non-specific influx of lymphocytes in response to inflammatory stimuli, proliferating cells

were detected by means of 5-bromo-2-deoxyuridine (BrdU) incorporation. BrdU is a halogenated DNA precursor analogue which, when introduced into the circulation of immunised animals, is incorporated into cells that are actively synthesising DNA. These proliferating cells can subsequently be detected by means of an anti-BrdU antibody, followed by – in this case – flow cytometric analysis (226, 227).

Mice were injected s.c. with tsDCs or PBS, and then on the day prior to LN collection, BrdU was administered by means of two intraperitoneal (i.p.) injections, 8 hours apart. The following day (day 5 or day 8), LNs were harvested and labelled with anti-BrdU as well as anti-CD4 or anti-CD8, followed by flow cytometric analysis. As this BrdU injection regime will only detect cells that have divided in the 24-hour period prior to LN collection, T cells that have been activated by allo-antigen but are not actively proliferating in this 24-hour period will not be detected. However, it is relevant to note here that the i.p. injection regime of BrdU administration resulted in levels of BrdU incorporation virtually identical to those obtained when BrdU was administered continuously, from the time of immunisation to the time of LN collection, in the animals' drinking water (not shown). This suggests that the i.p. injection regime did permit the detection of all antigen-activated T cells in the LN at the time of collection. It is likely that, once antigen-activated T cells have stopped proliferating, they migrate out of the LN, thereby explaining why $BrdU^+$ T cells did not accumulate to much higher numbers following long-term continuous administration of BrdU.

In the absence of immunisation with tsDCs, there was a low-level turnover of T cells, such that ~1.7% of $CD4^+$ T cells, and ~2% of $CD8^+$ T cells, were found to have divided in the 24-hour period between the initial injection of BrdU and the collection

of LNs. As expected, these background proliferation values did not significantly vary between days 5 and 8 (Fig 5.4 [A] and [B]). Five days after immunisation with tsDCs, there was a 2.5-fold increase in the percentage of $CD4^+$ T cells that had divided, and a 1.8-fold increase in the percentage of $CD8^+$ T cells that had divided, compared to non-sensitised LNs (Fig 5.4 [A]; $p < 0.005$ for both). By day 8, the relative proportion of $CD4^+$ T cells that had divided was similar to day 5, suggesting the maintenance of similar levels of $CD4^+$ T cell activation between days 5 to 8 (Fig 5.4 [B]; $p < 0.005$ compared to the control value). In contrast, the percentage of divided $CD8^+$ T cells present at day 8 was 4.4-fold greater than the value obtained for control LNs (Fig 5.4 [B]; $p < 0.0005$), suggesting that the proliferation of $CD8^+$ T cells had markedly increased between days 5 and 8. The representative density plots presented in Figure 5.4 [C-D] illustrate the presence of readily detectable populations of $CD4^+$ and $CD8^+$ T cells that had incorporated BrdU at day 8.

5.2.2.3 Absolute numbers of divided and non-divided T cells in the popliteal LN

The data presented in Figure 5.4 demonstrate that the relative proportions of $CD4^+$ and $CD8^+$ T cells undergoing proliferation in the popliteal LN was significantly increased following immunisation with tsDCs. However, in the context of a dynamic system such as the LN, percentage values can sometimes be misleading, as changes in an unrelated population can profoundly affect the relative proportions of the population under investigation. To address this concern, in Figure 5.5, the BrdU data have been combined with the total cell count values, such that the absolute numbers of divided and non-divided T cells in the LN can be estimated. This analysis demonstrates that the extent of the T cell proliferative response was greater in terms of absolute numbers than as suggested by the percentage values. Based on these calculations, at day 5 there

were 6.8 times as many proliferating $CD4^+$ T cells, and 5.5 times as many proliferating $CD8^+$ T cells, in sensitised LNs compared to controls ($p = 0.0005$ and $p < 0.0001$, respectively; Fig 5.5 [A]). At the day 8 time-point, there was a 7.5-fold and 16.3-fold increase in the numbers of proliferating $CD4^+$ and $CD8^+$ T cells, respectively ($p = 0.0001$ and $p < 0.0001$, respectively; Fig 5.5 [B]).

Thus, upon immunisation with tsDCs, there was a large increase in proliferation of both $CD4^+$ and $CD8^+$ T cells. However, when compared to the number of T cells present in the sensitised LN that were not proliferating (Fig 5.5 [C-D]), it is clear that the majority of the increase in LN cellularity observed following immunisation was not due to local T cell proliferation. At day 5, there was a 2.6-fold increase in the number of non-dividing $CD4^+$ T cells, and a 3.0-fold increase in the number of non-dividing $CD8^+$ T cells, in the sensitised LN compared to controls ($p < 0.0001$ and $p < 0.0005$, respectively; Fig 5.5 [C]). The results at the day 8 time-point were similar, such that there was a 3.1-fold and 3.4-fold increase in the numbers of non-dividing $CD4^+$ and $CD8^+$ T cells, respectively, upon immunisation ($p < 0.0001$ for both). Although the magnitude of these relative increases appear less than those observed within the divided $CD4^+$ and $CD8^+$ T cell populations, they occurred on a much larger scale. For example, upon immunisation, the number of dividing $CD4^+$ T cells at day 8 increased, on average, from 0.1×10^5 cells to 0.9×10^5 cells, whereas the number of non-divided $CD4^+$ T cells at the same time-point increased, on average, from 7.1×10^5 cells to 21.7×10^5 cells. Therefore, although a large proliferative response was mounted in the popliteal LN following immunisation with tsDCs, the majority of the increased cellularity observed in sensitised nodes was due to the recruitment of lymphocytes from the circulation.

5.2.3 Lack of detectable migration of tsDCs to the draining LN

Given that the injection of tsDCs into the footpad resulted in the development of an immune response in the draining LN, it seemed likely that tsDCs were migrating to the popliteal LN and presenting allo-antigen to T cells within the secondary lymphoid tissue. To test this hypothesis, tsDCs were labelled with CFSE prior to injection and the presence of green fluorescent cells in the popliteal LN assessed either 1 day (Fig 5.6 [A-C]) or 5 days (Fig 5.6 [D-F]) later. CFSE-labelled tsDCs were cultured *in vitro* for the same length of time (1 or 5 days), and the flow cytometric profile of parallel-cultured cells used to define a gate ('R1') in which tsDCs present in the LN would be expected to localise. Surprisingly, at neither time-point could CFSE-labelled tsDCs be detected in the draining LN.

At high concentrations, however, CFSE can affect cellular function (not shown) and therefore, although a relatively low concentration of CFSE was used in these experiments, it was nevertheless important to confirm that CFSE-labelled tsDCs were still functional. It was not possible to test the *in vitro* migration capacity of the labelled cells, as previous studies in our laboratory have shown that tsDCs are too adherent to migrate in chemotaxis assays. Therefore, the ability of labelled tsDCs to stimulate an MLR was assessed in comparison to unlabelled cells, as a surrogate measure of functional capacity (Fig 5.7). This experiment confirmed that CFSE-labelled tsDCs were just as efficient at presenting allo-antigen as were unlabelled tsDCs, thereby providing evidence that CFSE-labelled tsDCs were not functionally compromised.

5.2.4 *Analysis of chemokine receptor expression on CD4$^+$ T cells following immunisation with tsDCs*

5.2.4.1 Time-course of chemokine receptor expression in the popliteal LN

The expression of CCR5, CXCR3 and CXCR4 on CD4$^+$ T cells within the popliteal LN was assessed at days 2, 5 and 8 using flow cytometry. As seen in Figure 5.8, the percentage of CD4$^+$ T cells expressing CCR5 at day 2 was virtually identical for control and tsDC-sensitised LNs. By day 5, the percentage positive for this receptor appeared to be greater in tsDC-sensitised LNs than control LNs, although this difference was not statistically significant. At day 8, however, the extent of CCR5 up-regulation was considerably more pronounced compared to that observed at day 5. Thus, when comparing sensitised and control LNs at the day 8 time-point, immunisation was shown to result in a significant increase in the proportion of CD4$^+$ T cells expressing CCR5 ($p < 0.0001$). Furthermore, there were significantly more CCR5$^+$ CD4$^+$ T cells in tsDC-sensitised LNs at day 8 compared to day 5 ($p < 0.01$).

The up-regulation of CCR5 at day 8 is further illustrated in Figure 5.8 [B-C], in which typical flow cytometric profiles are compared for control and sensitised LNs at day 8. These plots also demonstrate that the expression of CCR5 on the CD4$^-$ population (which is likely to consist of CD8$^+$ T cells and B cells) appeared to be higher in tsDC-sensitised LNs compared to control LNs. However, this issue was not specifically investigated, as the primary intention of these studies was to study the CD4$^+$ T cell population. A final interesting piece of information obtained from these density plots is the observation that, as observed in the MLR, the up-regulation of CCR5 expression appeared to be limited to a sub-population of T cells expressing elevated levels of CD4. Similar observations were made with regard to CXCR3 expression (see Fig 5.9).

The pattern of CXCR3 expression observed following immunisation with tsDCs was similar to the pattern observed for CCR5. The percentage of $CD4^+$ T cells expressing CXCR3 was not altered from control values at day 2, but a significant increase in the percentage of $CD4^+$ $CXCR3^+$ T cells was observed at both day 5 and day 8 ($p < 0.05$ and $p = 0.0001$, respectively; Fig 5.9 [A]). Furthermore, similar to CCR5, the up-regulation of CXCR3 was significantly greater at day 8 than at day 5 ($p < 0.05$). It is interesting to note that – at all time-points – the percentage of $CD4^+$ T cells expressing CCR5 was quite similar to the percentage expressing CXCR3 (~8-10% at day 2, ~13-16% at day 8). In contrast, however, the density plots illustrated in Figure 5.9 [B-C] demonstrate that the expression of CXCR3 on $CD4^-$ lymphocytes was far more extensive than the expression of CCR5. The large numbers of $CXCR3^+$ lymphocytes, which were apparent in both control and sensitised LNs, could represent $CD8^+$ T cells or B cells.

In contrast to the up-regulation of CCR5 and CXCR3 expression that was observed in response to immunisation with tsDCs, the expression of CXCR4 on $CD4^+$ T cells did not vary significantly between control and sensitised LNs at any time-point (Fig 5.10 [A]). At all time-points tested, ~50-60% of $CD4^+$ T cells expressed CXCR4, regardless of whether the LNs had been sensitised with tsDCs or not. As seen in the density plot analysis of representative day 8 data (Fig 5.10 [B-C]), the distribution of staining for CXCR4 was virtually identical between sensitised and control LNs.

In Figure 5.11, the percentage of $CD4^+$ T cells expressing each chemokine receptor has been combined with total cell count data, similar to the analysis of the BrdU data

presented in Figure 5.5. This has allowed the absolute number of cells expressing each chemokine receptor to be calculated. The number of $CCR5^+$ $CD4^+$ T cells present in LNs that had been immunised with tsDCs was significantly greater than the number present in control LNs at all time-points ($p < 0.01$ for day 2; $p < 0.0001$ for days 5 and 8; Fig 5.11 [A]). Similarly, the number of $CXCR3^+$ $CD4^+$ T cells was also significantly higher in sensitised LNs compared to control LNs, at all three time-points ($p < 0.005$ for day 2; $p < 0.0001$ for days 5 and 8; Fig 5.11 [B]). These trends are in accordance with the observed increase in the relative proportions of CCR5-expressing and CXCR3-expressing $CD4^+$ T cells. However, the changes observed here are greatly magnified in comparison to the percentage data, as the present analysis takes into account the large increase in LN cellularity that occurred following immunisation with tsDCs.

With regard to CXCR4, at all time-points tested there was a significant increase in the absolute number of $CXCR4^+$ $CD4^+$ T cells present in tsDC-sensitised LNs compared to control samples ($p < 0.0005$ for day 2; $p < 0.0001$ for days 5 and 8; Fig 5.11 [B]). This is in distinct contrast to the results presented in Figure 5.10, which demonstrated that there was no change to the relative proportion of $CXCR4^+$ $CD4^+$ T cells present in the popliteal LN in response to immunisation with tsDCs. Thus, consistent with the fact that a large proportion of $CD4^+$ T cells express CXCR4, the increase in LN cellularity that occurred upon immunisation resulted in an increase in the number of CXCR4-expressing $CD4^+$ T cells, but the relative proportion of $CXCR4^+$ $CD4^+$ T cells present was not altered in comparison to resting LNs.

5.2.4.2 *Chemokine receptor expression on $CD4^+$ T cells undergoing cell division in sensitised LNs*

Using the BrdU technique, it was demonstrated in Figure 5.5 that the large increase in cellularity observed within sensitised LNs was due mostly to the influx of lymphocytes into the node in response to inflammation, with only a small component of the increase attributable to local proliferation in response to antigen. Thus, in order to directly study the regulation of chemokine receptor expression during T cell activation *in vivo*, it was necessary to specifically analyse receptor expression on $CD4^+$ T cells that were undergoing cell division. Accordingly, LN cells from mice that had been sensitised with tsDCs and given BrdU i.p. were collected at day 8 post-immunisation, labelled with antibodies to CD4, BrdU and the chemokine receptors, and subsequently analysed by flow cytometry.

Figure 5.12 demonstrates that the population of $CD4^+$ T cells that had undergone cell division in the preceding 24 hours were significantly more likely to express CCR5 and CXCR3 compared to cells that had not divided. Thus, within the population of $CD4^+$ T cells that had incorporated BrdU, CCR5 and CXCR3 were expressed on a significantly greater percentage of cells compared to the population of $CD4^+$ T cells that did not incorporate BrdU ($p < 0.0001$ for both). The expression of CXCR4 also differed between the dividing and non-dividing $CD4^+$ T cell populations, such that the percentage of divided $CD4^+$ T cells expressing this receptor was significantly greater than the percentage of non-divided $CD4^+$ T cells expressing this receptor ($p < 0.0005$). However, the magnitude of CXCR4 up-regulation on the divided population was far less striking than the extent of up-regulation of either CCR5 or CXCR3. Thus, the percentage of divided $CD4^+$ T cells expressing CCR5 was 6.8 times greater than the percentage of non-divided $CD4^+$ T cells expressing this receptor, and the percentage of

divided $CD4^+$ T cells expressing CXCR3 was 6.9 times greater than the percentage of non-divided $CD4^+$ T cells expressing this receptor. In contrast, the percentage of divided $CD4^+$ cells expressing CXCR4 was only 1.4-fold above the percentage of non-dividing $CD4^+$ cells expressing this receptor.

5.2.4.3 The percentage of $CD4^+$ T cells expressing CCR5, CXCR3 or CXCR4 that have divided

Figure 5.11 demonstrated that, upon immunisation with tsDCs, there was a large increase in the absolute numbers of $CD4^+$ T cells in the popliteal LN expressing CCR5, CXCR3 and CXCR4. Subsequently, in Figure 5.12, it was shown that $CD4^+$ T cells that had undergone cell division had up-regulated the expression of CCR5, CXCR3 and – to a lesser extent – CXCR4. The observation that proliferating T cells had up-regulated chemokine receptor expression suggests that the acquisition of these receptors by dividing T cells contributes to the observed increase in the number of $CD4^+$ T cells expressing CCR5, CXCR3 and CXCR4 in the tsDC-sensitised LN. It was therefore important to assess the contribution of receptor up-regulation on locally-proliferating $CD4^+$ T cells, as opposed to the influx of cells already bearing these receptors, to the observed numerical increase in $CD4^+$ T cells expressing these receptors. Accordingly, for each of the receptor-positive $CD4^+$ T cell populations, the percentage that had undergone division (ie, had incorporated BrdU) was determined.

Figure 5.13 demonstrates that an average of 20.4% of $CD4^+$ T cells that expressed CCR5 had incorporated BrdU, while an average of 18.3% of $CD4^+$ T cells that expressed CXCR3 were $BrdU^+$. Thus, a relatively large proportion of $CD4^+$ T cells that expressed these two receptors had undergone division, suggesting that the local up-

regulation of these receptors on activated $CD4^+$ T cells makes a considerable contribution to the numbers of $CD4^+$ $CCR5^+$ and $CD4^+$ $CXCR3^+$ T cells present in sensitised LNs. In contrast, only an average of 5.1% of CXCR4-expressing $CD4^+$ T cells had incorporated BrdU. This observation suggests that the increased number of $CXCR4^+$ $CD4^+$ T cells in tsDC-sensitised LNs is due mainly to the influx of cells already expressing CXCR4, rather than the novel acquisition of this receptor by locally proliferating $CD4^+$ T cells.

5.2.5 *The BrdU technique allows the detection of T cell proliferation with high efficiency*

It was observed that a considerable proportion of $CD4^+$ T cells in the sensitised LN that expressed CCR5, CXCR3 and – most notably – CXCR4, had not incorporated BrdU. However, it was important to confirm that the BrdU technique was enabling the detection of proliferating T cells with high efficiency, and therefore had not underestimated the proportion of $CD4^+$ T cells in the LN undergoing division. Accordingly, the incorporation of BrdU by thymocytes was assessed in parallel with each BrdU-labelling experiment, as thymocytes undergo rapid, continual turnover and therefore incorporate BrdU with high efficiency (228).

As demonstrated in Figure 5.14, a large proportion of thymocytes incorporated BrdU in the 24-hour period between the first injection of BrdU and the collection of thymus samples. In the experiment shown, 38.0% of thymocytes were $BrdU^+$, while the average from three experiments was 34.9%. Thus, although only a small proportion of cells from LNs were found to incorporate BrdU, in the same animals a much larger fraction of thymocytes were shown to be $BrdU^+$. The proportion of thymocytes

observed to stain positive for BrdU is comparable to the proportion of $BrdU^+$ thymocytes detected in previous studies after similar BrdU administration regimes (229, 230). Thus, in the present study, the BrdU technique was indeed capable of detecting T cell proliferation with high efficiency.

5.2.6 Preliminary analysis of the site of antigen deposition (footpad)

5.2.6.1 Evidence for retention of tsDCs in footpad tissue at day 8

It was observed that CCR5 and CXCR3 were acquired by $CD4^+$ T cells undergoing cell division in the LN in response to stimulation with tsDCs. It therefore seemed likely that these novel patterns of chemokine receptor expression would allow activated $CD4^+$ T cells to relocate to the original site of antigen deposition in the periphery, where inflammatory chemokines may be expected to be produced. In the present study, the outcome of this would be the migration of CCR5- and CXCR3-expressing $CD4^+$ T cells to the footpad, where the tsDCs were originally injected. However, the extent to which this occurs will be dependent on the level of retention of tsDCs within the footpad tissue.

In order to determine if tsDCs were still present in the footpad at the end of the time-course, samples of footpad tissue were collected from mice that had been injected with CFSE-labelled tsDCs or PBS 8 days earlier. Subsequently, cryostat sections of tissue samples were examined by fluorescence microscopy for the presence of CFSE-labelled cells. Three representative photomicrograph images of each condition are shown in Figure 5.15. Compared to the PBS-injected footpads (Fig 5.15 [A]), readily detectable numbers of fluorescent cells were observed in all tsDC-injected footpads examined

(Fig 5.15 [B]). Interestingly, in one of the images presented, the cells were clustered in one location, while in the other two, the cells were scattered throughout the tissue. It is possible that clusters of tsDCs also existed in these samples, but by chance, were not observed in the cryostat sections taken.

5.2.6.2 Detection of $CD4^+$ T cells expressing CCR5 or CXCR3 in footpad tissue

The analysis of CFSE-labelled tsDCs indicated that these cells were retained in the footpad in detectable numbers 8 days after the initial injection. Thus, it was of interest to determine if tsDC-injected footpad tissue was infiltrated with $CD4^+$ T cells, and whether or not these $CD4^+$ T cells expressed CCR5 and CXCR3. To this end, cryostat sections of footpad tissue from immunised and control mice were labelled with antibodies to CD4 and the chemokine receptors, and subsequently analysed by fluorescence microscopy.

Initially, a single colour analysis was performed to determine whether $CD4^+$ T cells were present in higher numbers in tsDC-injected footpad tissue compared to PBS-injected footpad tissue (Fig 5.16). In these images, the presence of $CD4^+$ T cells is indicated by small, bright blue dots within the tissue. For illustrative purposes, one of these dots is distinguished in each panel of [B] by a green-coloured arrow. The larger areas of diffuse blue colouration can likely be attributed to the high levels of autofluorescence emitted by regions of fatty tissue when viewed through this filter. Similar regions of diffuse blue colouration were also evident within sections of tissue stained with control antibodies (see Fig 5.17 [A-B]) and were therefore not considered to indicate the presence of $CD4^+$ T cells in the present analysis.

$CD4^+$ T cells were present in very low numbers in samples of PBS-injected footpad tissue (Fig 5.16 [A]). Thus, only ~3 - 6 positive cells could be detected in each of the photomicrograph images presented. When samples of tsDC-injected footpad tissue were examined (Fig 5.16 [B]), the number of $CD4^+$ T cells detected was generally higher. In the upper and middle panels, ~15 - 20 cells stained positive, although the number of positive cells in the lower panel was slightly less (~8). Therefore, although this analysis was not quantitative, there was a clear trend toward increased numbers of $CD4^+$ T cells in tsDC-injected footpad tissue compared to control tissue.

In Figure 5.17, a 2-colour analysis is presented, in which the staining patterns obtained for two antibodies, each detected with a different fluorochrome, are overlayed. The staining obtained with anti-CD4, or the relevant control monoclonal antibody, is presented in blue, while the staining obtained with anti-chemokine receptor antibodies, or the relevant control goat IgG, is presented in red. Where the two colours overlap, a pink/purple colour is obtained. In [D] and [F], an example of each staining pattern has been highlighted with an arrow. Cells that have stained with anti-CD4 only appear blue, and are indicated with a green-coloured arrow. Cells that have only stained with anti-CCR5 or anti-CXCR3 appear red, and are indicated by a white-coloured arrow. Cells that have stained with both antibodies appear pink/purple, and are indicated by a yellow-coloured arrow.

Presented in [A] and [B] are photomicrographs of 3 samples of PBS-injected footpad tissue, and 3 samples of tsDC-injected footpad tissue, stained with the two negative control antibodies. In general, there was very little background staining with either control antibody, although in some panels, a small number of fluorescent cells were

evident, which must be taken into account when analysing the samples stained with specific antibodies (presented in [C - F]). Also, in most samples, regions of fatty tissue appeared moderately bright in the blue channel, probably as a result of high levels of autofluorescence, as discussed above. Finally, in both channels, the outer edges of the tissue have stained relatively brightly, most likely due to the non-specific trapping of antibody along the tissue edges. This bright fluorescence along the outer edges of the tissue, as well as the diffuse blue autofluorescence, were therefore not considered to indicate specific staining in subsequent images.

When PBS-injected footpad tissue was stained with anti-CD4 and anti-CCR5 (Fig 5.17 [C]), very few $CCR5^+$ cells were detected in the upper and lower panels. Of particular note, the few cells that stained positive for CCR5 did not, in general, co-stain with anti-CD4, suggesting that the small number of $CCR5^+$ cells detected in PBS-injected tissue were not $CD4^+$ T cells. The tissue sample presented in the centre panel clearly stained with the anti-CCR5 antibody to a greater extent than the other two panels, and a small number of $CCR5^+$ $CD4^+$ T cells were detected. However, it is important to note that this particular sample also gave a higher level of staining with the control antibodies (Fig 5.17 [A], centre panel), suggesting that at least some of the staining detected is non-specific.

Compared to PBS-injected tissue, the staining for CCR5 in samples of tsDC-injected footpad tissue was generally more extensive, such that $CCR5^+$ cells could be detected in all 3 of the samples presented (Fig 5.17 [D]). Furthermore, in all 3 samples, some of the $CCR5^+$ cells also stained with the anti-CD4 antibody (yellow arrows), indicating the presence of $CCR5^+$ $CD4^+$ T cells. As was observed for the PBS-injected samples,

however, there was also considerable variation in the tsDC-injected samples, as the centre panel in Figure 5.18 [B] was more extensively stained with the anti-CCR5 antibody than the other 2 samples. Thus, in the centre panel, almost all $CD4^+$ T cells were $CCR5^+$, and there were also a large number of $CCR5^+$ cells that were not $CD4^+$.

With regard to CXCR3 staining, readily detectable numbers of $CXCR3^+$ cells could be detected in all 3 samples of PBS-injected footpad tissue (Fig 5.17 [E]), although the numbers were higher in the centre and lower panels compared to the upper panel. Of note, however, most of this staining did not co-localise with staining for CD4, suggesting that the majority of $CXCR3^+$ cells were not $CD4^+$ T cells. Extensive staining for CXCR3 was also evident in the tsDC-injected footpad tissue samples (Fig 5.17 [F]), especially in the centre panel (this sample also stained particularly well with the anti-CCR5 antibody; see Fig 5.17 [D], centre panel). In contrast to the PBS-injected samples, however, a number of the $CXCR3^+$ cells did co-stain with the anti-CD4 antibody (yellow arrows), indicating the presence of $CXCR3^+$ $CD4^+$ T cells in immunised footpad tissue.

5.3 Discussion

5.3.1 The nature of the immune response to allogeneic DC

In the present study, the sub-cutaneous injection of DCs was used to stimulate an allogeneic T cell-mediated immune response. Although the ability of tsDCs to stimulate an allogeneic immune response *in vivo* had not been previously demonstrated, these cells have been shown to be competent at inducing an immune response to bacterial antigens. Thus, tsDCs loaded with *Mycobaterium tuberculosis* cells *in vitro* induced a protective immune response, characterised by high levels of IL-2 and IFN-γ production and the generation of specific antibodies, when injected i.p. into syngeneic mice (223). Furthermore, tsDCs are effective stimulators of the MLR *in vitro* ((202), and this study). It therefore seemed likely that the s.c. injection of tsDCs into the footpad would result in the activation of allo-reactive T cells in the draining (popliteal) LN and indeed, a large increase in LN cellularity was observed following immunisation with tsDCs, in addition to the generation of a significant cytotoxic response.

The immune response observed in the popliteal LNs following the injection of tsDCs involved the activation of both $CD4^+$ and $CD8^+$ T cell subsets. Thus, using the BrdU technique, both subsets were shown to undergo a significant proliferative response, at both day 5 and day 8. Furthermore, the development of an effective cytotoxic response also suggests the activation of both $CD4^+$ and $CD8^+$ T cells, as although the effector function measured in the cytotoxicity assay can almost certainly be ascribed to $CD8^+$ T cells, efficient cytotoxicity would not be expected to develop in the absence of an

adequate $CD4^+$ helper T cell response. Finally, while B cell activation was not specifically studied here, it is likely that there was also a significant humoral component to the immune response generated, as a number of lymphocytes that incorporated BrdU did not express either CD4 or CD8 markers. Moreover, when sections of LN tissue were examined for BrdU incorporation, clusters of $BrdU^+$ cells could be detected in regions morphologically consistent with B cell follicles (not shown). It may be interesting in future studies to assess the expression of chemokine receptors on populations of B cells and $CD8^+$ T cells undergoing activation in this system.

The concurrent activation of $CD4^+$ T cells, $CD8^+$ T cells and B cells suggests that the immune response mounted against tsDCs mimics quite closely the events that occur during graft rejection, as this process is generally characterised by a combination of $CD4^+$ T cell-mediated DTH function, $CD8^+$ T cell-mediated cytotoxicity and an allo-antibody humoral response (231). The characteristics of the immune response observed in the present study are also comparable to previous studies of immune activation induced by the injection of allogeneic splenocytes. For example, the injection of C57BL/6 splenocytes into the footpads of BALB/c mice resulted in an increase in LN cellularity of a similar magnitude to that observed here (232), while the injection of B6 x D2 splenocytes into the footpads of C x D2 mice resulted in significant T cell proliferation in the popliteal LN, although the relative contributions of $CD4^+$ and $CD8^+$ T cells to the proliferative response were not assessed (233).

5.3.2 *Despite inducing an effective immune response, migration of tsDCs to the popliteal LN was not detected*

The lack of detectable tsDC migration to the popliteal LN following s.c. injection into the footpad was surprising, given that a specific immune response was mounted against tsDCs in the LN, and previous studies have shown that DCs grown *in vitro* from precursors in blood (234) or bone marrow (235) and injected s.c. are capable of migrating to the draining LN and localising in the T cell areas. Using lymphoid DCs purified from spleen, however, no evidence of DC migration to the LN was found following s.c. injection, and depots of DCs could still be detected at the site of injection for at least 7 days post-immunisation (236). It is therefore likely that the ability of DCs to migrate to the draining LN is highly dependent on their lineage and state of maturation. Migration, however, does not appear to be critical for the generation of an immune response, as in the present study, as well as previous studies (159, 236), T cell activation has been shown to occur in the absence of DC localisation in the LN. In these instances, T cell activation is likely to be mediated following the transfer of antigen from the injected DCs to host DCs (159, 237), which subsequently carry the antigen to the LN and present it to T cells. Alternatively, the possibility remains that, in the present study, T cell activation was mediated directly by allogeneic tsDCs that migrated to the LN, but that the number of cells arriving in the LN was below the limit of detection of the assay.

One factor that may be particularly important for the ability of DCs to migrate to the LN is the expression of CCR7. This chemokine receptor is up-regulated by DCs in response to inflammation (238) and appears to be critical for the migration of DCs to the T cell areas of LNs (97, 239). Thus, it is conceivable that tsDCs do not express

CCR7, and therefore are not equipped to localise within the LN. In support of this hypothesis, RT-PCR analysis failed to detect CCR7 expression in a sample of tsDC cDNA, although every other chemokine receptor tested produced a readily detectable PCR product (not shown). In this regard, experiments are currently being conducted in our laboratory to determine whether the transfection of tsDCs with an expression plasmid encoding murine CCR7 will allow them to migrate to the popliteal LN following s.c. injection into the footpad.

5.3.3 The expression of chemokine receptors on $CD4^+$ T cells within the popliteal LN

5.3.3.1 Preferential influx of $CCR5^+$ and $CXCR3^+$ T cells into the tsDC-sensitised LN

Flow cytometric analysis of LN cell preparations revealed a significant increase in the absolute number, as well as the relative proportion, of $CD4^+$ T cells expressing CCR5 or CXCR3 following immunisation with tsDCs. With regard to CXCR4 expression, an increase in the absolute number of $CXCR4^+$ $CD4^+$ T cells was observed, but the relative proportions did not alter significantly. In order to appreciate the biological significance of these observations, it is important to consider them in the context of the large increase in LN cellularity that was observed to result from immunisation.

BrdU studies indicated that only a small proportion of the observed increase in LN cellularity resulted from local T cell proliferation. This observation was not unexpected, as antigen-stimulated LNs have been known for many years to be subject to altered patterns of lymphocyte trafficking that result in significant increases in LN cellularity (240-243). Changes that occur within the LN, as well as changes to the lymphocytes themselves under conditions of inflammation, both contribute to the

influx of lymphocytes into sensitised LNs. Thus, during inflammation, blood flow to the LN increases (244) and the expression of ligands for L-selectin on HEVs is enhanced (245). Furthermore, fever-range temperatures associated with the inflammatory response result in changes to L-selectin avidity on lymphocytes, leading to a dramatic increase in the efficiency with which lymphocytes roll along HEVs during immune activation (246). The net result of these changes is an increased number of lymphocytes entering the node via the HEVs, resulting in a large increase in LN cellularity, as observed in the present study. Furthermore, early during an immune response, the number of lymphocytes exiting the LN via the efferent lymphatics decreases, which may also contribute to the increased cellularity of the node following immunisation (242, 243). However, as this phase of 'lymphocyte shutdown' generally only occurs for the first few days following immunisation, it is unlikely to contribute to the increase in LN cellularity observed in the present study at days 5 – 8.

Considering the large influx of lymphocytes into tsDC-stimulated LNs, it is not surprising that the number of $CCR5^+$, $CXCR3^+$ and $CXCR4^+$ $CD4^+$ T cells was increased in comparison to resting LNs. If an increase in any of these populations was not observed, this would suggest that cells bearing that receptor were specifically excluded from the immunised LN, presumably because of a failure to cross the HEVs. This was clearly not the case. In fact, to the contrary, the increased proportion of $CD4^+$ T cells expressing CCR5 and CXCR3 in immunised LNs suggests that these cells were preferentially selected for entry into the node. This was not, however, the case for CXCR4-expressing cells, as the proportion of $CXCR4^+$ $CD4^+$ T cells was not altered in LNs following tsDC injection, compared to injection of PBS. It is important to acknowledge here that at least part of the increase in CCR5- and CXCR3-expressing

$CD4^+$ T cells in the sensitised LN was due to the up-regulation of these receptors on activated cells, as will be discussed below. However, given that only ~20% of $CD4^+$ T cells expressing CCR5 or CXCR3 were dividing, it is highly unlikely that the local up-regulation of these receptors was the sole factor responsible for the increased number of $CCR5^+$ and $CXCR3^+$ $CD4^+$ T cells detected in the immunised LN.

5.3.3.2 *Regulation of chemokine receptor expression on antigen-activated $CD4^+$ T cells undergoing cell division*

In addition to the influx of circulating lymphocytes into the LN upon immunisation with tsDCs, it is clear that a significant proliferative response was also mounted, and that cell division was associated with the up-regulation of CCR5, CXCR3 and – to a lesser extent – CXCR4. Many more $CD4^+$ T cells that had undergone cell division in response to allo-antigen expressed CCR5 and CXCR3 compared to non-dividing cells, thereby providing direct evidence for the up-regulation of these receptors upon T cell activation *in vivo*. With regard to the expression of CXCR4, a relatively large proportion of $CD4^+$ T cells expressed this receptor in the resting LN, and this fraction was increased only modestly upon activation. It is unclear at this stage whether or not this observation has any biological significance.

The observed up-regulation of CCR5 and CXCR3 on antigen-activated $CD4^+$ T cells *in vivo* is in keeping with previous studies demonstrating that the expression of these chemokine receptors is often associated with T cell-mediated inflammatory pathologies. For example, the rheumatoid synovium has been shown to be greatly enriched in T cells expressing CXCR3 and CCR5 (111, 118), and large numbers of CXCR3-expressing skin-homing $CD4^+$ T cells can be isolated from psoriatic epidermis

(37). Further, in a murine model of bacterial-induced hepatic failure, liver-infiltrating $CD4^+$ T cells are predominantly $CCR5^+$ $CXCR3^+$ (103). Importantly, however, while these studies suggest that activated T cells expressing CCR5 and CXCR3 are associated with sites of immune activation, they do not provide evidence that these receptors are acquired during T cell activation. It is quite possible that T cells already expressing these receptors would be selected for localisation at inflammatory sites, in response to production of the cognate chemokine ligands.

Although little information has existed until now regarding the regulation of CCR5 and CXCR3 expression on $CD4^+$ T cells *in vivo*, the regulation of other chemokine receptors on $CD4^+$ and $CD8^+$ T cells has been investigated to some extent. Using an adoptive transfer/TCR-transgenic system, $CD8^+$ T cells have been shown to down-regulate the expression of CCR7 following lymphocytic choriomeningitis virus (LCMV) infection, which was associated with a dramatically reduced ability to enter the splenic white pulp (110). Of interest, however, no change in the expression of CXCR3 was noted. In another study, ovalbumin-specific TCR-transgenic T cells were shown to up-regulate expression of CXCR5 and responsiveness to the cognate ligand, BLC/CXCL13, following immunisation with ovalbumin in complete Freund's adjuvant (CFA), but not in the absence of adjuvant (247). Furthermore, activation of T cells in this system was also associated with reduced chemotactic responsiveness to ELC/CCL19, SLC/CCL21 and SDF-1/CXCL12. While expression of the cognate receptors was not assessed, this observation nevertheless raises the possibility that CCR7 and CXCR4 were down-regulated upon T cell activation.

The suggestion of CXCR4 down-regulation is in contrast to the present findings, which in fact demonstrated a subtle up-regulation of CXCR4 on the divided $CD4^+$ T cell population. The results from the present study are, however, supported by the observation that immunisation of mice with the T-dependent antigen TNP-KLH did not result in any significant change to the percentage of $CD4^+$ T cells in the draining LN expressing CXCR4 (222). This observation is identical to that made in the present study when the bulk $CD4^+$ T cell population was analysed (see Figure 5.11). Thus, in at least two different experimental systems, down-regulation of CXCR4 upon *in vivo* T cell activation has not been demonstrated, in contrast to the observation of reduced chemotaxis toward the CXCR4 ligand in the study by Ansel *et al* (247). This difference may potentially be explained by uncoupling of the CXCR4 receptor from intracellular signalling cascades, such that levels of receptor expression remain constant but cells are no longer chemotactically responsive (72). Alternatively, the regulation of CXCR4 expression may differ depending on the nature of the immune response.

5.3.4 The acquisition of CCR5 and CXCR3 may contribute to activated $CD4^+$ T cell relocation to the footpad

5.3.4.1 Retention of tsDCs at the site of injection

Following the injection of CFSE-labelled tsDCs into the footpad, fluorescence microscopy revealed that detectable numbers of cells persisted at the site of injection for at least 8 days. The retention of tsDCs within the footpad tissue may explain why the proliferative response in the draining LN was still increasing at day 8, whereas the response may be expected to be decreasing by this stage if all antigen had been cleared (248). The persistence of s.c.-injected DCs in the footpad for at least 7 days has also

been observed previously (236), suggesting that this phenomenon is not unique to the tsDCs. Clustering of the tsDCs within the footpad tissue was observed in one of the tissue samples examined, and it is conceivable that similar clusters existed in the other samples, but by chance, were not observed in the cryostat sections taken. This apparent tendency of the tsDCs to cluster may contribute to their persistence in the tissue, and also to the failure of the cells to migrate to the draining LN.

5.3.4.2 *Preliminary evidence that $CD4^+$ T cells expressing CCR5 and CXCR3 localise to the footpad*

Thus, tsDCs were still present at the site of injection at day 8, a time at which T cell proliferation had been occurring for a number of days, and the expression of chemokine receptors had been significantly increased on allo-activated $CD4^+$ T cells. Accordingly, it seemed possible that activated T cells bearing inflammatory chemokine receptors (CCR5 and CXCR3) may be attracted to the site of antigen deposition, where inflammatory chemokines may be expected to be produced in response to the presence of foreign material. CXCR4 was not investigated in these preliminary studies, as the up-regulation of this receptor upon immunisation was minimal. Furthermore, a role for this receptor in the specific relocation of activated T cells to sites of inflammation seemed unlikely, given the widespread, constitutive expression of its ligand, SDF-1/CXCL12 (46, 47).

The immunofluorescence analysis of footpad tissue suggested that the area was infiltrated with small numbers of $CD4^+$ T cells following the injection of tsDCs. In this context, it is interesting to note that our study of a bacterial-induced DTH response in the footpad revealed a far more extensive infiltration of $CD4^+$ T cells into the tissue

than that observed in the present study (Ebert *et al*, manuscript submitted). It is likely that mammalian cells from the same species as the recipient induce a local inflammatory response of a much lower magnitude compared to that induced by bacteria. Thus, although an effective T cell response is clearly mounted in response to the injection of foreign DCs, fewer activated cells may migrate to the site of antigen deposition due to the relatively mild inflammation occurring there.

In general, $CD4^+$ T cells detected in tsDC-injected footpad tissue expressed CCR5 and CXCR3, while the few $CD4^+$ T cells detected in PBS-injected footpad tissue generally did not express these chemokine receptors. This observation provides preliminary evidence that the recruitment of $CD4^+$ T cells to a site of antigen deposition in the peripheral tissues is associated with the expression of CCR5 and CXCR3. At this stage, this association may be taken as indirect evidence that the acquisition of these chemokine receptors by activated $CD4^+$ T cells in the LN is required for the relocation of these cells to peripheral sites such as antigen-challenged footpad tissue. However, it is clear that further studies are required to confirm this hypothesis.

First, more quantitative methods are required to enumerate the $CD4^+$ T cell influx. The enumeration of cells within a solid tissue such as the footpad is inherently difficult, especially given that the footpad tissue is not homogeneous. Thus, the accurate characterisation of the cellular influx in response to tsDC immunisation will probably require the injection of tsDCs into a site more amenable to the collection of single cells, such as the peritoneal cavity or a subcutaneous air pouch. Second, it will be important to distinguish between an influx of allo-activated $CD4^+$ T cells and the recruitment of circulating memory T cells that are not reactive with the antigen but are

able to localise at an inflammatory site by virtue of expression of the appropriate range of chemokine receptors and adhesion molecules. The administration of BrdU during priming, followed by the detection of $BrdU^+$ $CD4^+$ T cells at the site of immunisation, would address this issue.

Finally, blocking studies are required to determine whether or not CCR5 and/or CXCR3 are critical for the migration of activated T cells to the site of antigen deposition. To this end, a number of attempts were made to develop a model of T cell transfer of allo-activation, such that the role of CCR5 and CXCR3 in T cell localisation could be definitively addressed. $CD4^+$ T cells activated *in vitro* (MLR) or *in vivo* (intravenous injection of tsDCs, followed by collection of peripheral LNs) were radiolabelled with ^{125}I-UdR and transferred to recipient mice. Subsequently, the recipient mice were injected in one footpad with tsDCs and in the other with PBS, in the anticipation that sensitised, radiolabelled T cells would specifically localise to the challenged footpad, as has been observed previously (152). Blocking the function of CCR5 or CXCR3, followed by quantification of the amount of swelling and radioactivity in the challenged footpad, would therefore allow the relative contributions of the chemokine receptors to the T cell localisation process to be assessed. However, this regime did not lead to the development of a DTH swelling response, and radioactive counts in the challenged footpad tissue were not significantly different to counts in control tissue (not shown). Thus, the role of CCR5 and CXCR3 in activated $CD4^+$ T cell migration remains to be definitively addressed in the model of allogeneic activation used here. Of relevance, however, our studies of bacterial-induced DTH demonstrated that a RANTES receptor antagonist, which blocks CCR5 function, can

reduce the migration of activated T cells to a site of secondary challenge in the footpad (Ebert *et al*, manuscript submitted).

5.3.5 Conclusions

In this series of experiments, a murine model of allogeneic activation in response to foreign DCs was developed, characterised, and used to study the regulation of chemokine receptor expression on $CD4^+$ T cells *in vivo*. Initial analyses revealed that the total number of lymphocytes within the draining LN was increased dramatically at days 5 and 8 following immunisation. A small part of the increase in LN cellularity was due to the local proliferation of $CD4^+$ and $CD8^+$ T cells, while the remainder was likely to be due to the influx of cells from the circulation. This influx resulted in a large increase in the absolute numbers of $CCR5^+$, $CXCR3^+$ and $CXCR4^+$ $CD4^+$ T cells. However, analysis of the relative proportions of these cell populations suggested that the entry of $CD4^+$ T cells expressing CCR5 or CXCR3 was more efficient than the entry of CXCR4-expressing cells.

Within the population of locally-dividing $CD4^+$ T cells, approximately half acquired the ability to express CCR5 and CXCR3, which represented a dramatic up-regulation of these receptors in comparison to the non-dividing population. There was also a less striking, but statistically significant, increase in the percentage of $CD4^+$ T cells expressing CXCR4 in the divided compared to the non-divided population. Analysis of the site of immunisation revealed that the tsDCs were retained within the tissue for at least 8 days, and that this was associated with a modest accumulation of $CD4^+$ T cells. In preliminary studies, a number of these infiltrating $CD4^+$ T cells were shown to express CCR5 and CXCR3, supporting the notion that the acquisition of these

receptors upon activation may contribute to the ability of T cells to migrate to peripheral sites of immunisation.

Figure 5.1. *Total cells recovered from popliteal LNs following injection of PBS or tsDCs.* BALB/c mice were injected into the hind footpad with endotoxin-free PBS or the indicated number of tsDCs. Five days later, popliteal LNs were removed and single cell suspensions prepared. The total number of viable cells recovered from each LN was then determined by counting on a haemocytometer after staining with trypan blue. A statistically significant difference between control and tsDC-stimulated LNs is indicated by an asterisk. Data are presented as mean $\pm$ SEM (n = 4).

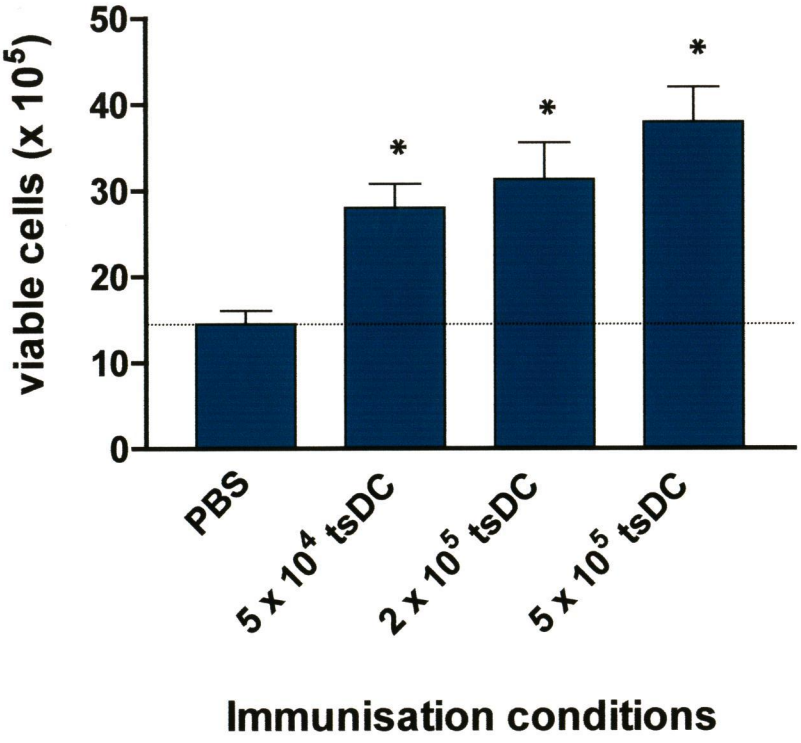

viable cells (x 10^5)
50
40
30
20
10
0
*
*
*
PBS
5 x 10^4 tsDC
2 x 10^5 tsDC
5 x 10^5 tsDC
Immunisation conditions

Figure 5.2. *Cytotoxic effector function of cells recovered from popliteal LNs following injection of PBS or tsDCs.* BALB/c mice were injected into the hind footpad with endotoxin-free PBS or the indicated number of tsDCs. Five days later, popliteal LNs were removed and single cell suspensions prepared. LN cells were added to monolayers of Calcein-labelled tsDCs, and the cells co-cultured for 48 hours. Subsequently, following extensive washing in PBS, the amount of tsDC monolayer remaining was quantified by measuring fluorescence emission at 520nm and percentage cytotoxicity values calculated, as described in Chapter 2. A statistically significant difference between control and tsDC-stimulated LNs is indicated by an asterisk. Data are presented as mean $\pm$ SEM (n = 4).

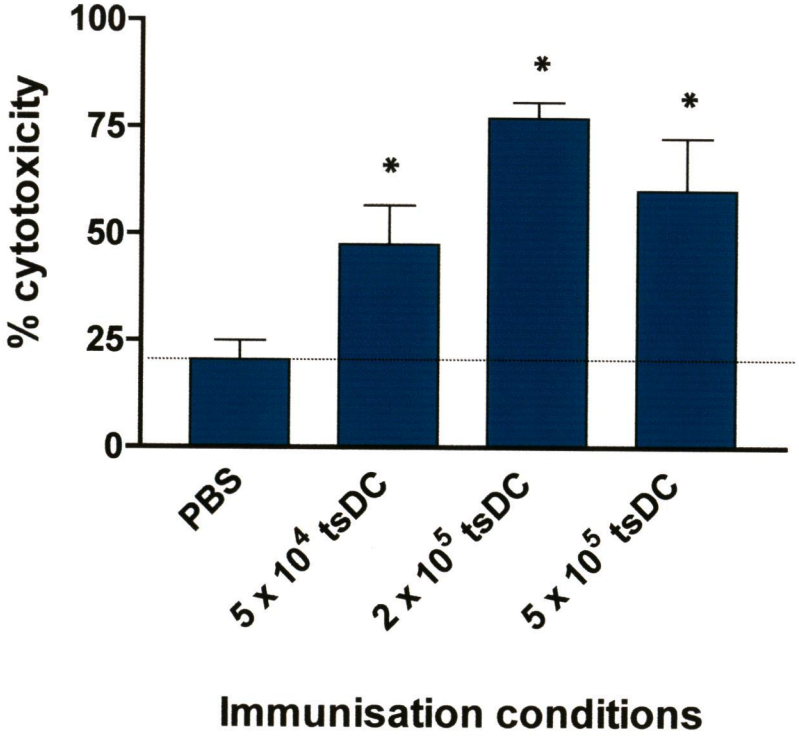

100
75
50
25
0
% cytotoxicity
*
*
*
PBS
5 x 10^4 tsDC
2 x 10^5 tsDC
5 x 10^5 tsDC
Immunisation conditions

Figure 5.3. *Time-course of total cell yields from popliteal LNs following injection of PBS or tsDCs.* BALB/c mice were injected into the hind footpad with endotoxin-free PBS or 5 x 10^5 tsDCs. After the indicated period of time, popliteal LNs were removed and single cell suspensions prepared. The total number of viable cells recovered from each LN was then determined by counting on a haemocytometer after staining with trypan blue. A statistically significant difference between control and tsDC-stimulated LNs is indicated by an asterisk. Data are presented as mean $\pm$ SEM (n = 11-15, from 3 independent experiments).

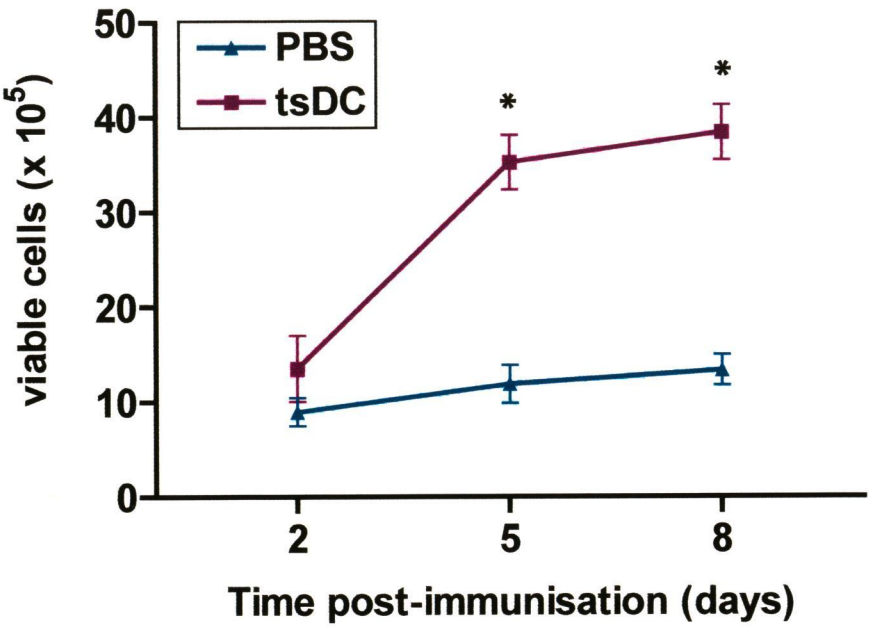
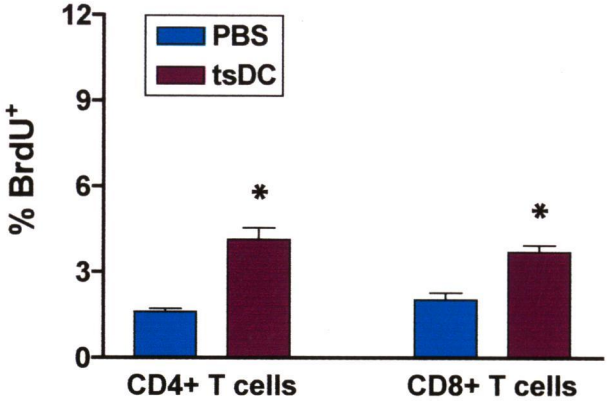
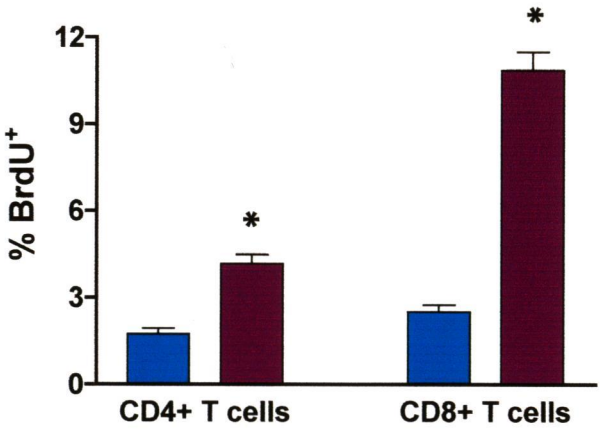

PBS
tsDC
viable cells (x 10^5)
50
40
30
20
10
0
*
*
2
5
8
Time post-immunisation (days)

Figure 5.4. *The percentage of $CD4^+$ and $CD8^+$ T cells undergoing cell division in control and tsDC-stimulated LNs.* BALB/c mice were injected into the hind footpad with endotoxin-free PBS or 5 x 10^5 tsDCs, and BrdU administered as described in Chapter 2. Five days [A] or 8 days [B] later, popliteal LNs were removed and single cell suspensions prepared. Cells were labelled with anti-BrdU and either anti-CD4 or anti-CD8 and analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. The percentage of $CD4^+$ or $CD8^+$ T cells that had incorporated BrdU at day 5 [A] and day 8 [B] was determined as a proportion of the total $CD4^+$ or $CD8^+$ populations, accordingly. A statistically significant difference between control and tsDC-stimulated LNs is indicated by an asterisk. Data are presented as mean $\pm$ SEM (n = 3-4). Representative density plots of day 8 tsDC-stimulated LN cells stained with anti-BrdU and either anti-CD4 or anti-CD8 are presented in [C] and [D], respectively.

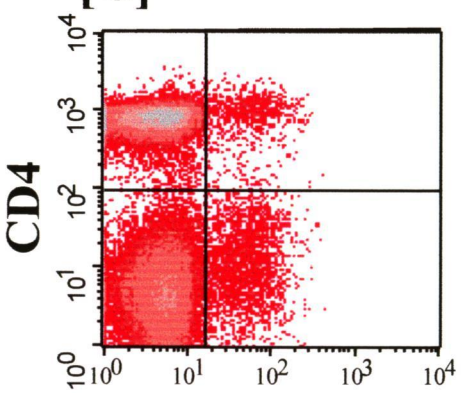
[A] *Day 5*



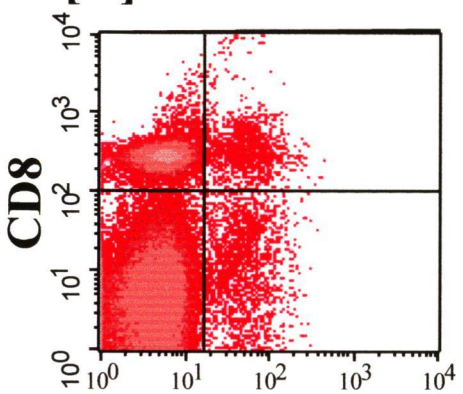
[B] *Day 8*



[C]



[D]



BrdU ⟶

Figure 5.5. *The absolute numbers of divided and non-divided $CD4^+$ and $CD8^+$ T cells in control and tsDC-stimulated LNs.* BALB/c mice were injected into the hind footpad with endotoxin-free PBS or 5 x 10^5 tsDCs, and BrdU administered as described in Chapter 2. Five days [A and C] or 8 days [B and D] later, popliteal LNs were removed and single cell suspensions prepared. Cells were labelled with anti-BrdU and either anti-CD4 or anti-CD8 and analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. The percentage of double positive ($CD4^+$ or $CD8^+$ and $BrdU^+$, [A-B]), and the percentage of single positive ($CD4^+$ or $CD8^+$ and $BrdU^-$, [C-D]) cells was multiplied by the total cell count value for the same time-point, such that the absolute number of cells of each phenotype could be determined. A statistically significant difference between control and tsDC-stimulated LNs is indicated by an asterisk. Data are presented as mean $\pm$ SEM (n = 3-4).

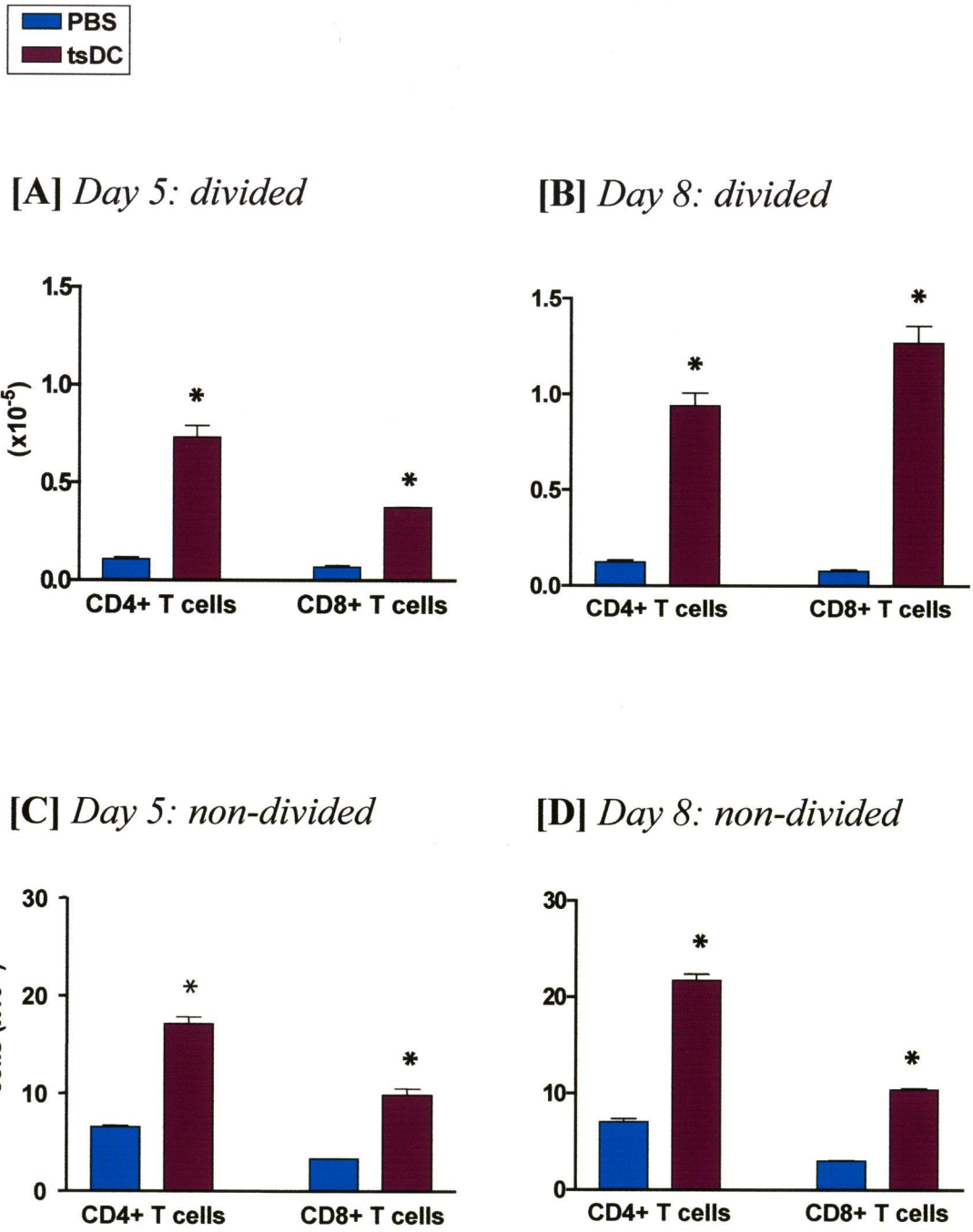
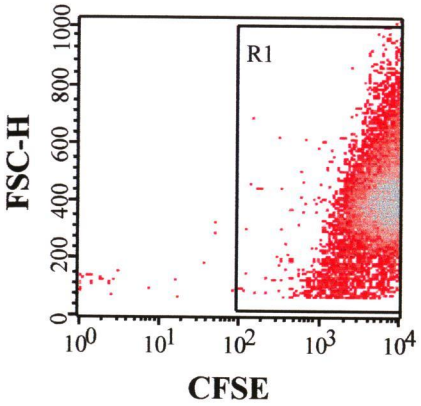
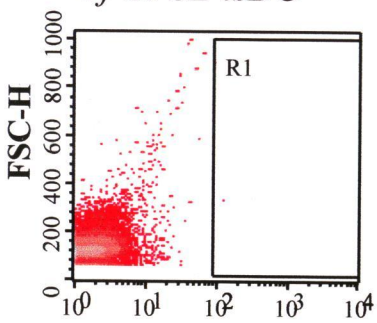

PBS
tsDC
[A] Day 5: divided
number of BrdU+ cells (x10-5)
1.5
1.0
0.5
0.0
*
*
CD4+ T cells
CD8+ T cells
[B] Day 8: divided
1.5
1.0
0.5
0.0
*
*
CD4+ T cells
CD8+ T cells
[C] Day 5: non-divided
number of BrdU- cells (x10-5)
30
20
10
0
*
*
CD4+ T cells
CD8+ T cells
[D] Day 8: non-divided
30
20
10
0
*
*
CD4+ T cells
CD8+ T cells

Figure 5.6. *Lack of migration of CFSE-labelled tsDCs from the footpad to the popliteal LN.* BALB/c mice were injected into the hind footpad with endotoxin-free PBS or 5 x 10^5 CFSE-labelled tsDCs. A sample of tsDCs was also maintained in culture after labelling with CFSE. Twenty-four hours [A-C] or 5 days [D-F] later, cells were prepared from popliteal LNs, and parallel-cultured CFSE-labelled tsDCs were detached from the culture flask. Both were subject to flow cytometric analysis, and forward scatter plotted against CFSE fluorescence intensity to enable detection of CFSE-labelled tsDCs. Parallel-cultured CFSE-labelled tsDCs [A and D] were used to define a region (R1) in which CFSE-labelled tsDCs that had migrated to the LN should be detectable [B and E]. For comparison, the flow cytometric profile of a control LN is also presented for each time-point [C and F]. Data are representative of $\geq$ 3 mice at each time-point.

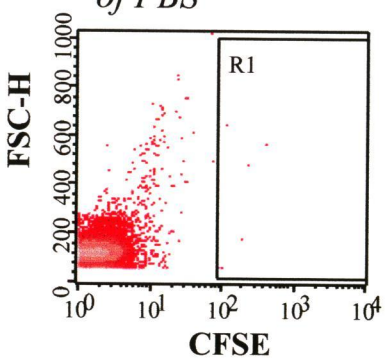
[A] *tsDCs cultured for 24 hrs*



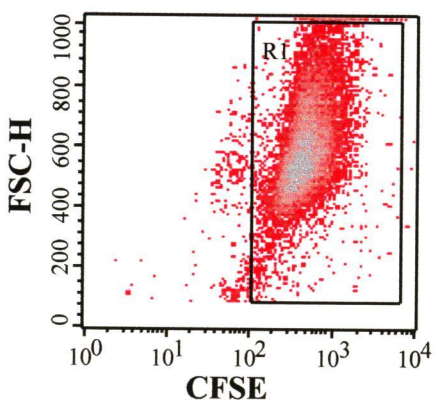
[B] *LN cells 24hrs post-injection of CFSE-tsDC*



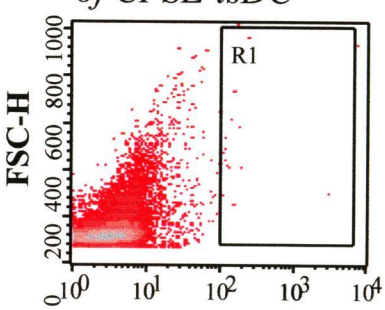
[C] *LN cells 24hrs post-injection of PBS*



[D] *tsDCs cultured for 5 days*



[E] *LN cells 5 days post-injection of CFSE-tsDC*



[F] *LN cells 5 days post-injection of PBS*

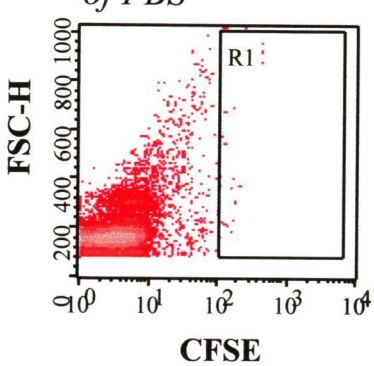


Figure 5.7. *Labelling tsDCs with CFSE does not affect their ability to stimulate an MLR.* Splenocytes were prepared from a BALB/c mouse and cultured alone or mixed with tsDCs that had either been labelled with CFSE or left untreated. The ability of untreated or CFSE-labelled tsDCs to induce the proliferation of BALB/c lymphocytes was quantified by culturing cells for 5 days, with 1μCi of [^{3}H]-thymidine being added to each well for the last 18 hours. Incorporation of [^{3}H]-thymidine was subsequently determined by scintillation counting. Data are presented as mean ± SEM (n = 3).

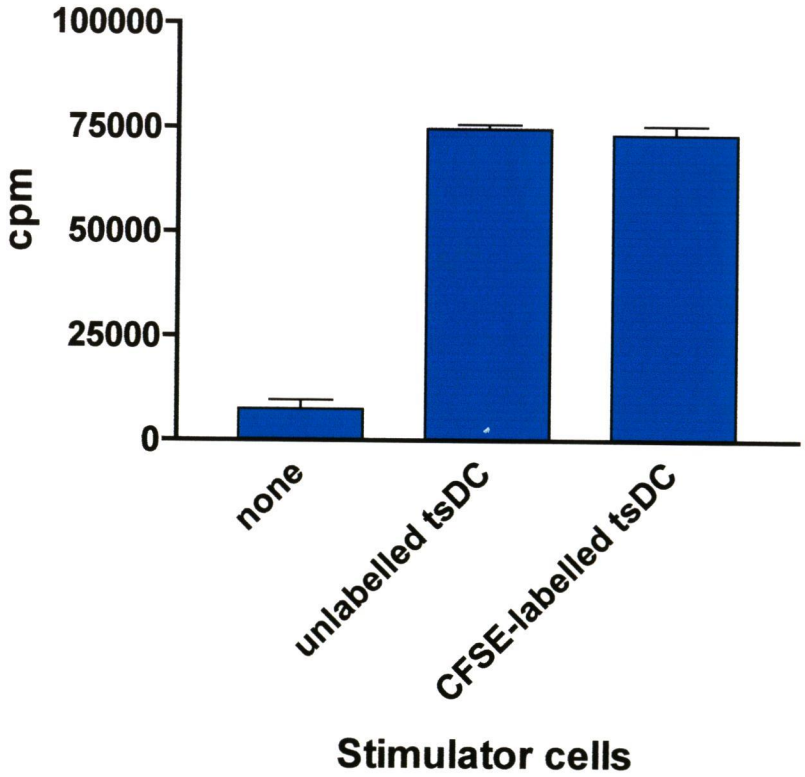
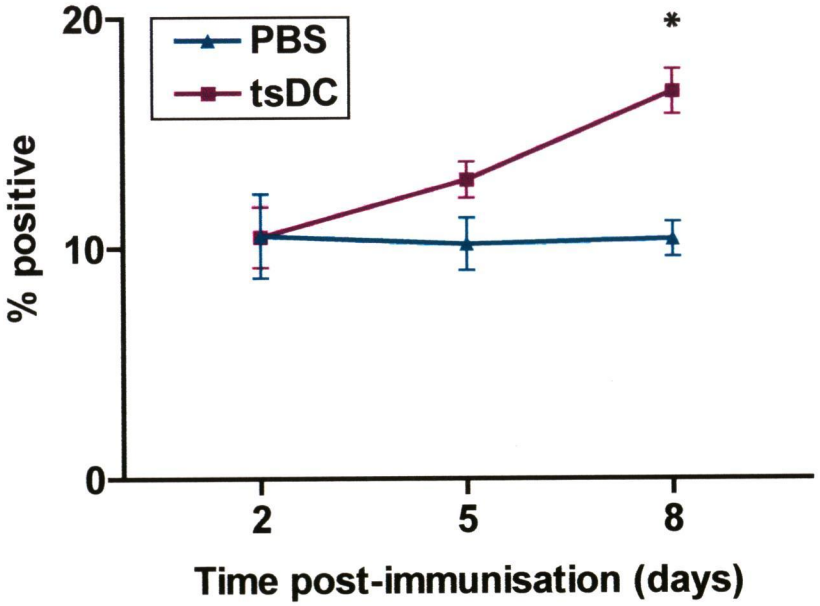

100000
75000
50000
25000
0
cpm
none
unlabelled tsDC
CFSE-labelled tsDC
Stimulator cells

Figure 5.8. *Time-course of CCR5 expression on $CD4^+$ T cells in control and tsDC-sensitised LNs.* BALB/c mice were injected into the hind footpad with endotoxin-free PBS or 5 x 10^5 tsDCs. After the indicated period of time, popliteal LNs were removed and single cell suspensions prepared. Cells were labelled with antibodies to CD4 and CCR5 and analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. In [A], the percentage of cells positive for CCR5 was determined in comparison to staining with control goat IgG. The percentage of $CD4^+$ T cells that expressed detectable levels of CCR5 was then calculated as a proportion of total $CD4^+$ T cells. A statistically significant difference between control and tsDC-stimulated LNs is indicated by an asterisk. Data are presented as mean $\pm$ SEM (n = 11, from 3 independent experiments). Representative density plots of day 8 LN cells from control [B] and tsDC-sensitised [C] are also shown.

[A]



[B] *PBS (Day 8)*

[C] *tsDC (Day 8)*

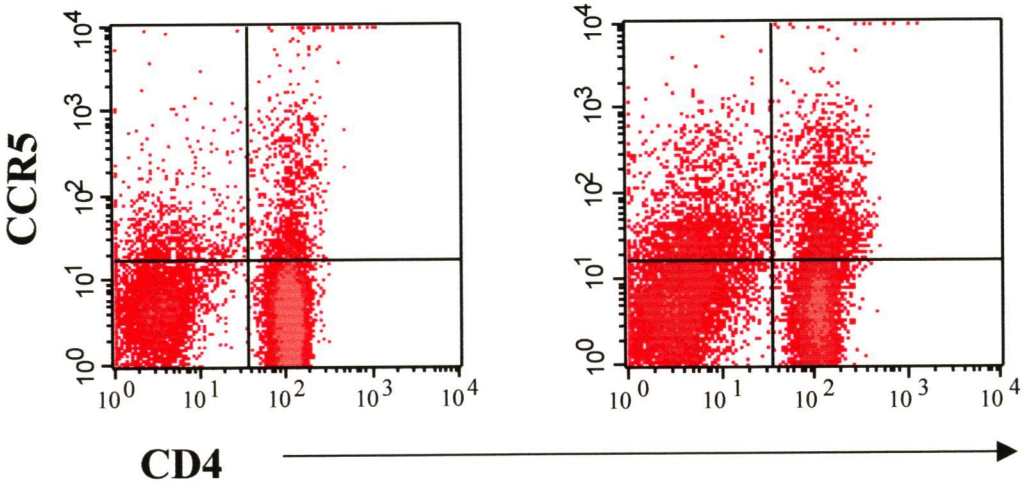
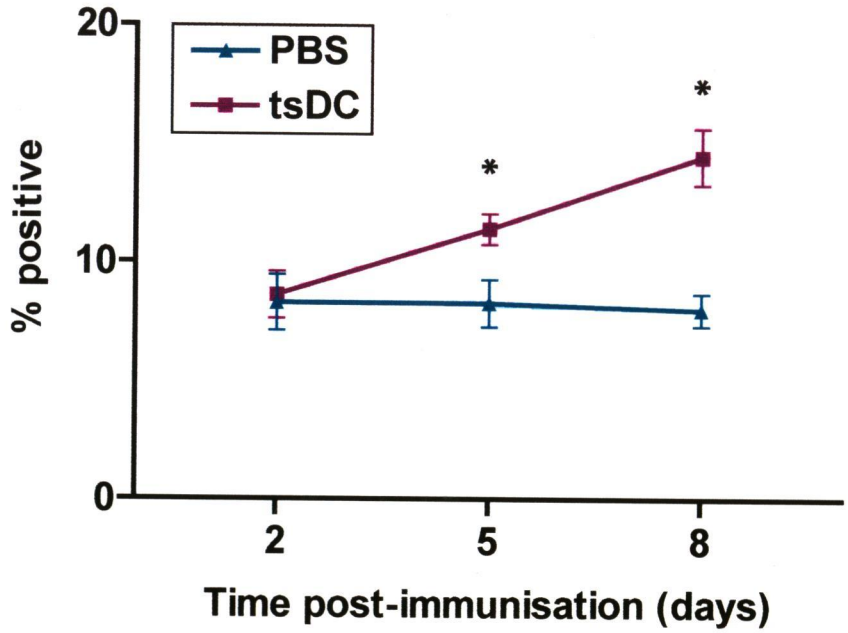


Figure 5.9. *Time-course of CXCR3 expression on $CD4^+$ T cells in control and tsDC-sensitised LNs.* BALB/c mice were injected into the hind footpad with endotoxin-free PBS or 5 x 10^5 tsDCs. After the indicated period of time, popliteal LNs were removed and single cell suspensions prepared. Cells were labelled with antibodies to CD4 and CXCR3 and analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. In [A], the percentage of cells positive for CXCR3 was determined in comparison to staining with control goat IgG. The percentage of $CD4^+$ T cells that expressed detectable levels of CXCR3 was then calculated as a proportion of total $CD4^+$ T cells. A statistically significant difference between control and tsDC-stimulated LNs is indicated by an asterisk. Data are presented as mean $\pm$ SEM (n = 11, from 3 independent experiments). Representative density plots of day 8 LN cells from control [B] and tsDC-sensitised [C] are also shown.

[A]



[B] *PBS (Day 8)*

[C] *tsDC (Day 8)*

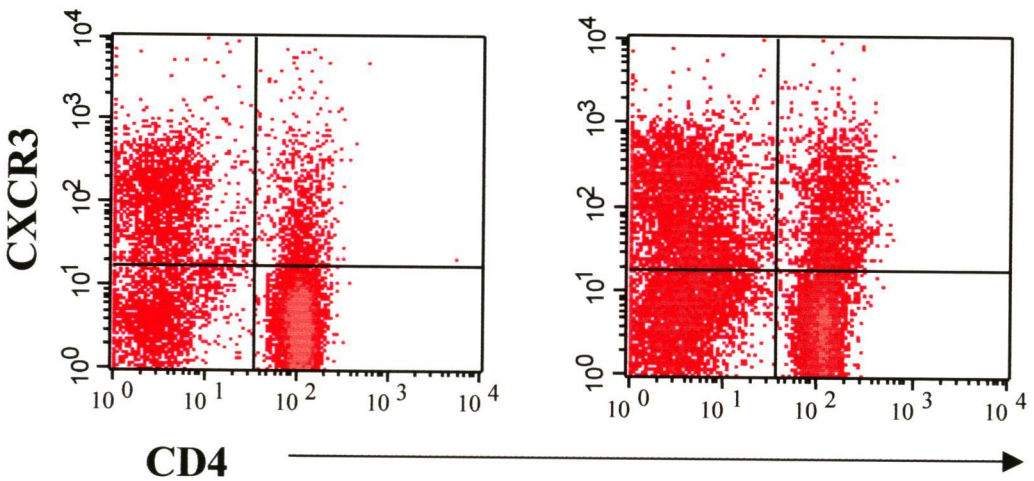
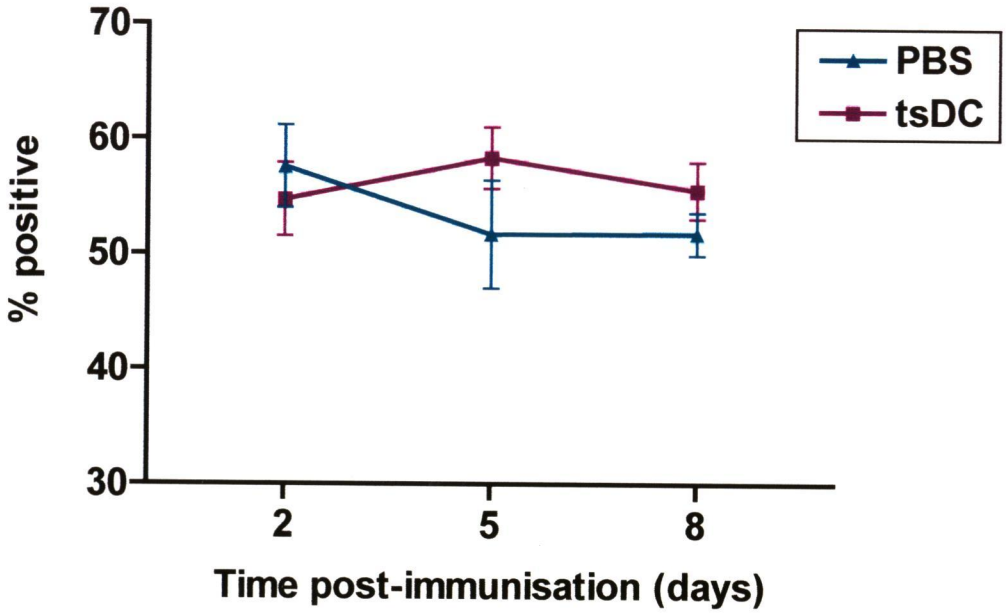


Figure 5.10. *Time-course of CXCR4 expression on $CD4^+$ T cells in control and tsDC-sensitised LNs.* BALB/c mice were injected into the hind footpad with endotoxin-free PBS or 5 x 10^5 tsDCs. After the indicated period of time, popliteal LNs were removed and single cell suspensions prepared. Cells were labelled with antibodies to CD4 and CXCR4 and analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. In [A], the percentage of cells positive for CXCR4 was determined in comparison to staining with control goat IgG. The percentage of $CD4^+$ T cells that expressed detectable levels of CXCR4 was then calculated as a proportion of total $CD4^+$ T cells. A statistically significant difference between control and tsDC-stimulated LNs is indicated by an asterisk. Data are presented as mean $\pm$ SEM (n = 11, from 3 independent experiments). Representative density plots of day 8 LN cells from control [B] and tsDC-sensitised [C] are also shown.

[A]



[B] *PBS (Day 8)*

[C] *tsDC (Day 8)*

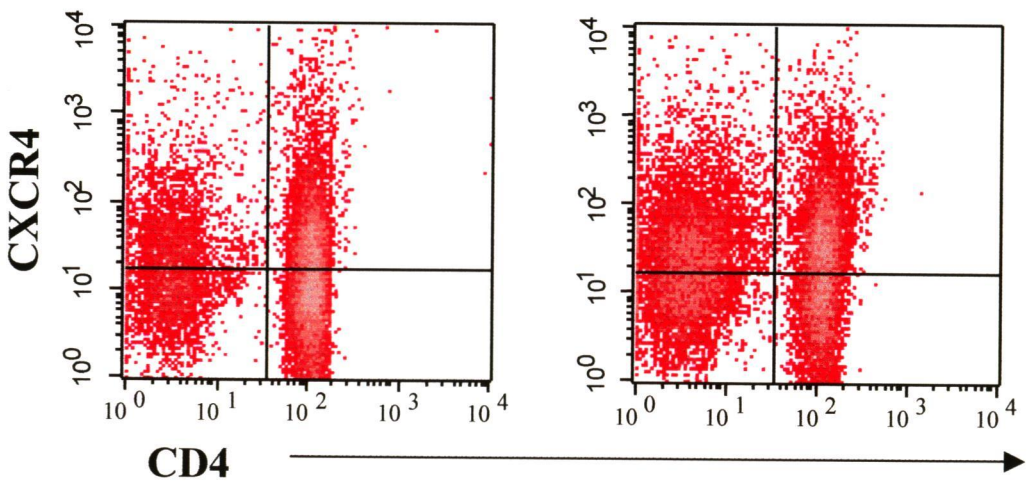
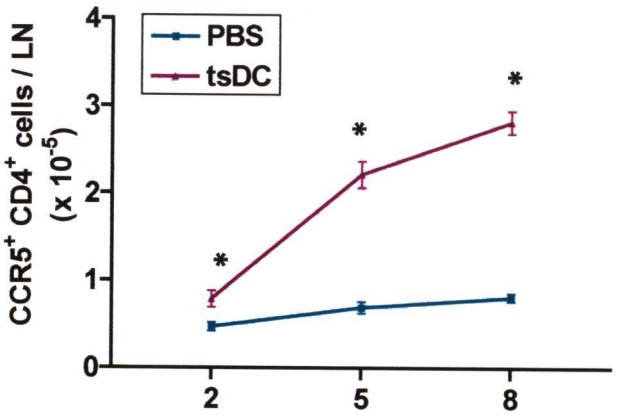
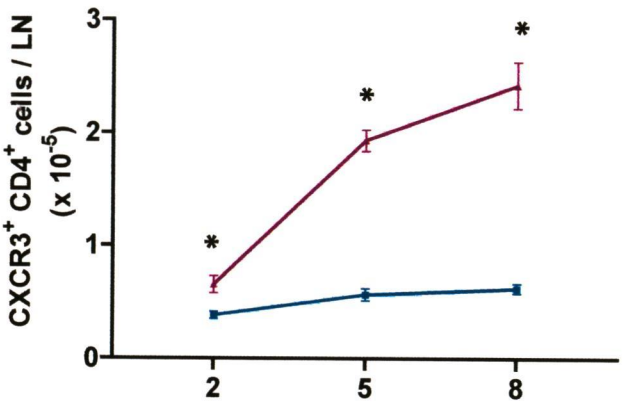


Figure 5.11. *Absolute number of $CD4^+$ T cells expressing CCR5, CXCR3 and CXCR4 in control and tsDC-sensitised LNs.* BALB/c mice were injected into the hind footpad with endotoxin-free PBS or 5 x 10^5 tsDCs. After the indicated period of time, popliteal LNs were removed and single cell suspensions prepared. Cells were labelled with antibodies to CD4 and either CCR5 [A], CXCR3 [B] or CXCR4 [C], and analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. The percentage of $CD4^+$ T cells expressing the relevant chemokine receptor was determined as a proportion of total lymphocytes, and this value multiplied by the total cell count value for the same time-point, such that the absolute number of cells of each phenotype could be determined. A statistically significant difference between control and tsDC-stimulated LNs is indicated by an asterisk. Data are presented as mean $\pm$ SEM (n = 11, from 3 independent experiments).

[A] CCR5



[B] CXCR3



[C] CXCR4

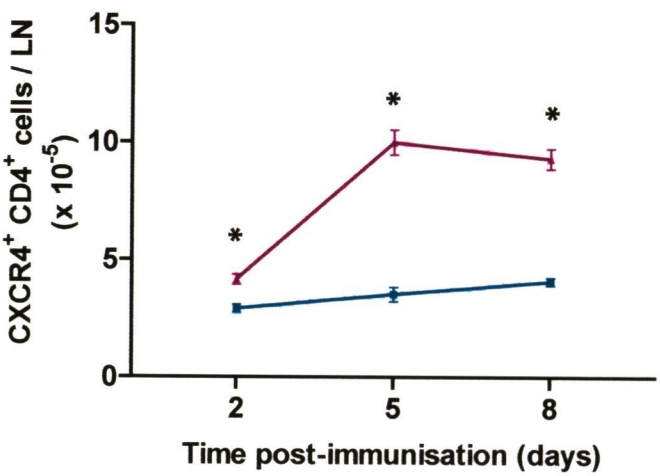


Figure 5.12. *Comparison of chemokine receptor expression on proliferating and non-proliferating CD4$^+$ T cells in tsDC-sensitised LNs.* BALB/c mice were injected into the hind footpad with 5 x 10^5 tsDCs, and BrdU administered as described in Chapter 2. Eight days later, popliteal LNs were removed and single cell suspensions prepared. The cells were labelled with anti-BrdU, anti-CD4 and anti-chemokine receptor antibodies, and then analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. Within the CD4$^+$ T cell population, the percentage of BrdU$^+$ (divided) and BrdU$^-$ (non-divided) cells expressing each chemokine receptor was determined as a proportion of the total BrdU$^+$ or BrdU$^-$ population, accordingly. A statistically significant difference between the divided and non-divided populations is indicated by an asterisk. Data are presented as mean $\pm$ SEM (n = 9, from 3 independent experiments).

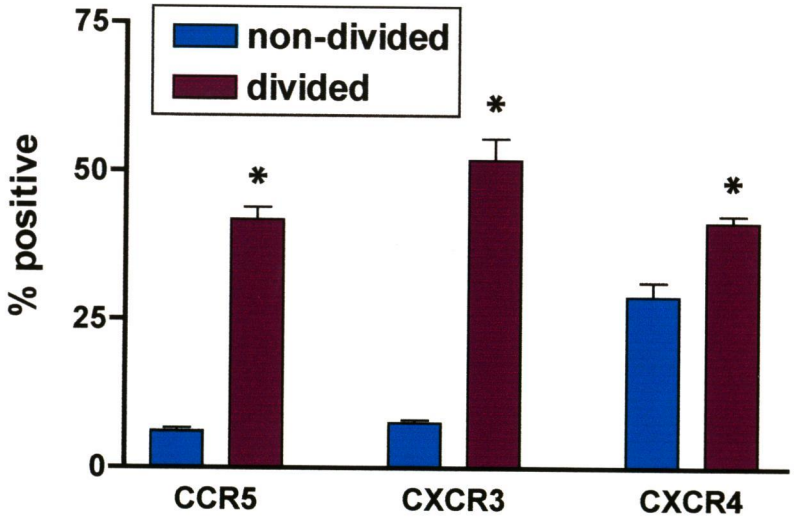

75
50
25
0
% positive
non-divided
divided
*
*
*
CCR5
CXCR3
CXCR4

Figure 5.13. *The percentage of chemokine receptor-expressing $CD4^+$ T cells that are proliferating.* BALB/c mice were injected into the hind footpad with 5×10^5 tsDCs, and BrdU administered as described in Chapter 2. Eight days later, popliteal LNs were removed and single cell suspensions prepared. The cells were labelled with anti-BrdU, anti-CD4 and anti-chemokine receptor antibodies, and then analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. Within the $CD4^+$ T cell population, the percentage of $CCR5^+$, $CXCR3^+$ and $CXCR4^+$ cells that had incorporated BrdU was determined as a proportion of the total $CCR5^+$, $CXCR3^+$ and $CXCR4^+$ populations, respectively. Data are presented as mean $\pm$ SEM ($n = 9$, from 3 independent experiments).

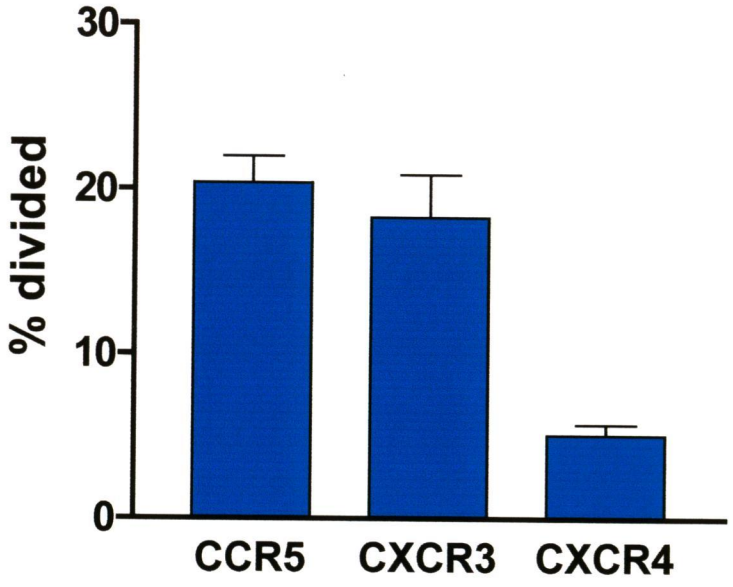

30
20
10
0
% divided
CCR5
CXCR3
CXCR4

Figure 5.14. *Incorporation of BrdU by thymocytes.* BrdU was administered to BALB/c mice as described in Chapter 2, and 24 hours after the first injection, the thymus was removed and single cell suspensions prepared. The cells were labelled with anti-BrdU and then analysed by flow cytometry, gating on lymphocytes using forward and side scatter characteristics. The percentage of cells that had incorporated BrdU was determined in comparison to staining with an isotype-matched negative control antibody. Data are representative of 3 samples.

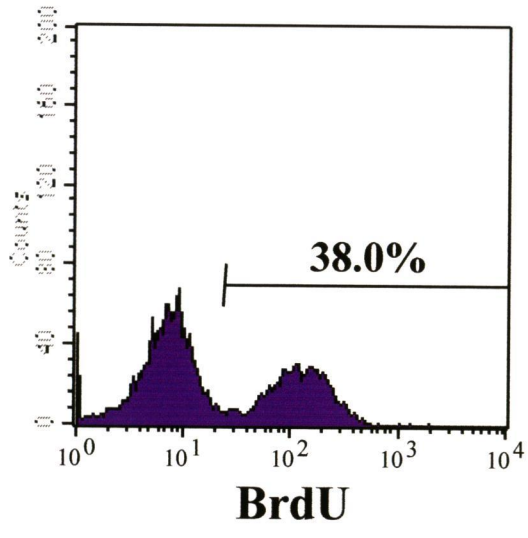

38.0%
10^0
10^1
10^2
10^3
10^4
BrdU

Figure 5.15. *Retention of tsDCs within footpad tissue.* BALB/c mice were injected into the hind footpad with endotoxin-free PBS [A] or 5 x 10^5 CFSE-labelled tsDCs [B]. Eight days later, the footpad tissue was removed, and 6μm cryostat sections were prepared and analysed by fluorescence microscopy. Shown are 3 representative images, captured using the 10X (top panel) or 20X (centre and bottom panels) objective.

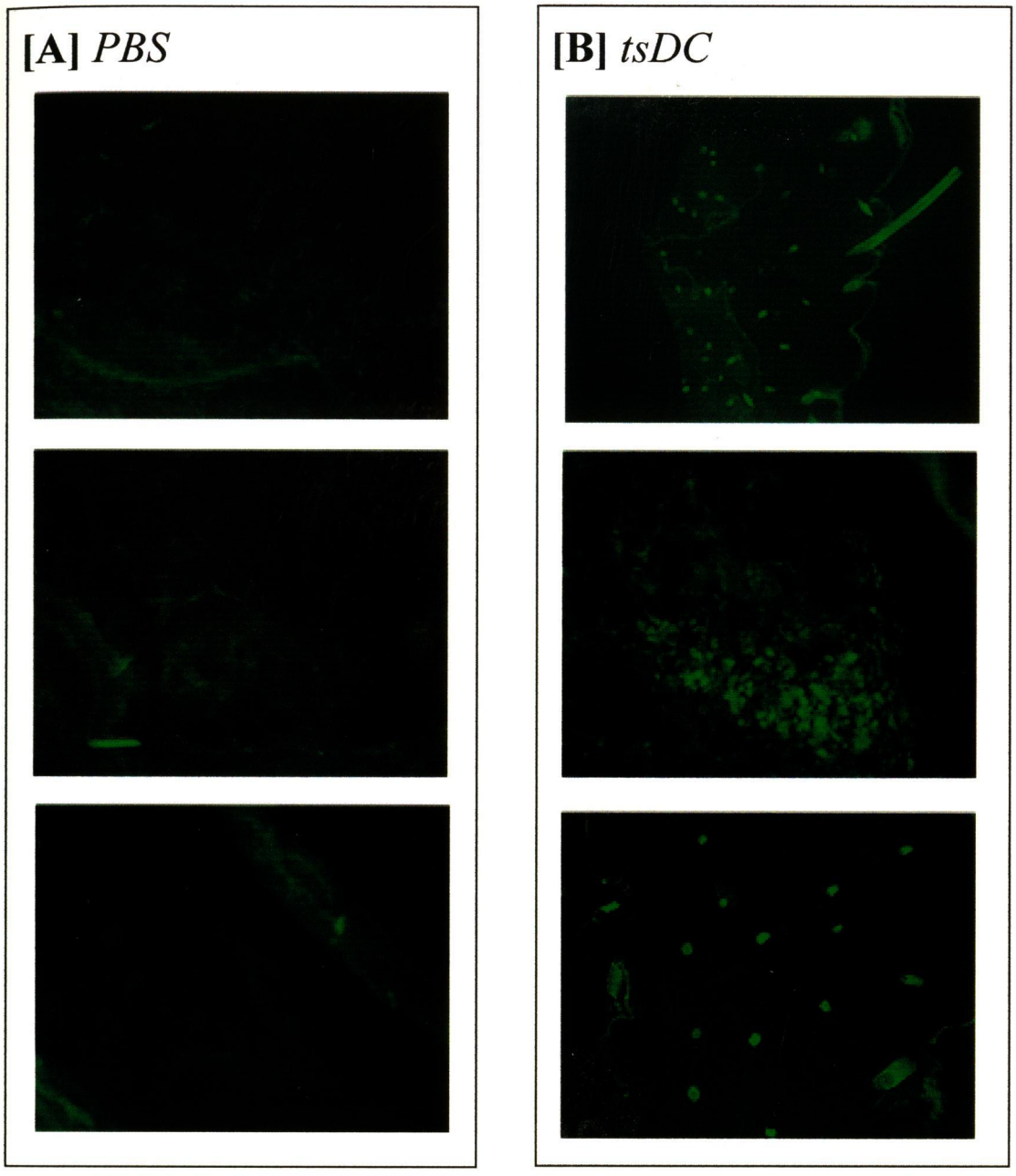

[A] PBS
[B] tsDC

Figure 5.16. *Comparison of $CD4^+$ T cell numbers in footpad tissue injected with PBS or tsDCs.* BALB/c mice were injected into the hind footpad with endotoxin-free PBS [A] or 5 x 10^5 tsDCs [B]. Eight days later, the footpad tissue was removed and 6μm cryostat sections prepared. The sections were fixed, stained with anti-CD4 or negative control monoclonal antibody and analysed by fluorescence microscopy. Shown are 3 representative images, captured using the 10X objective. In [B], arrows indicate $CD4^+$ T cells.

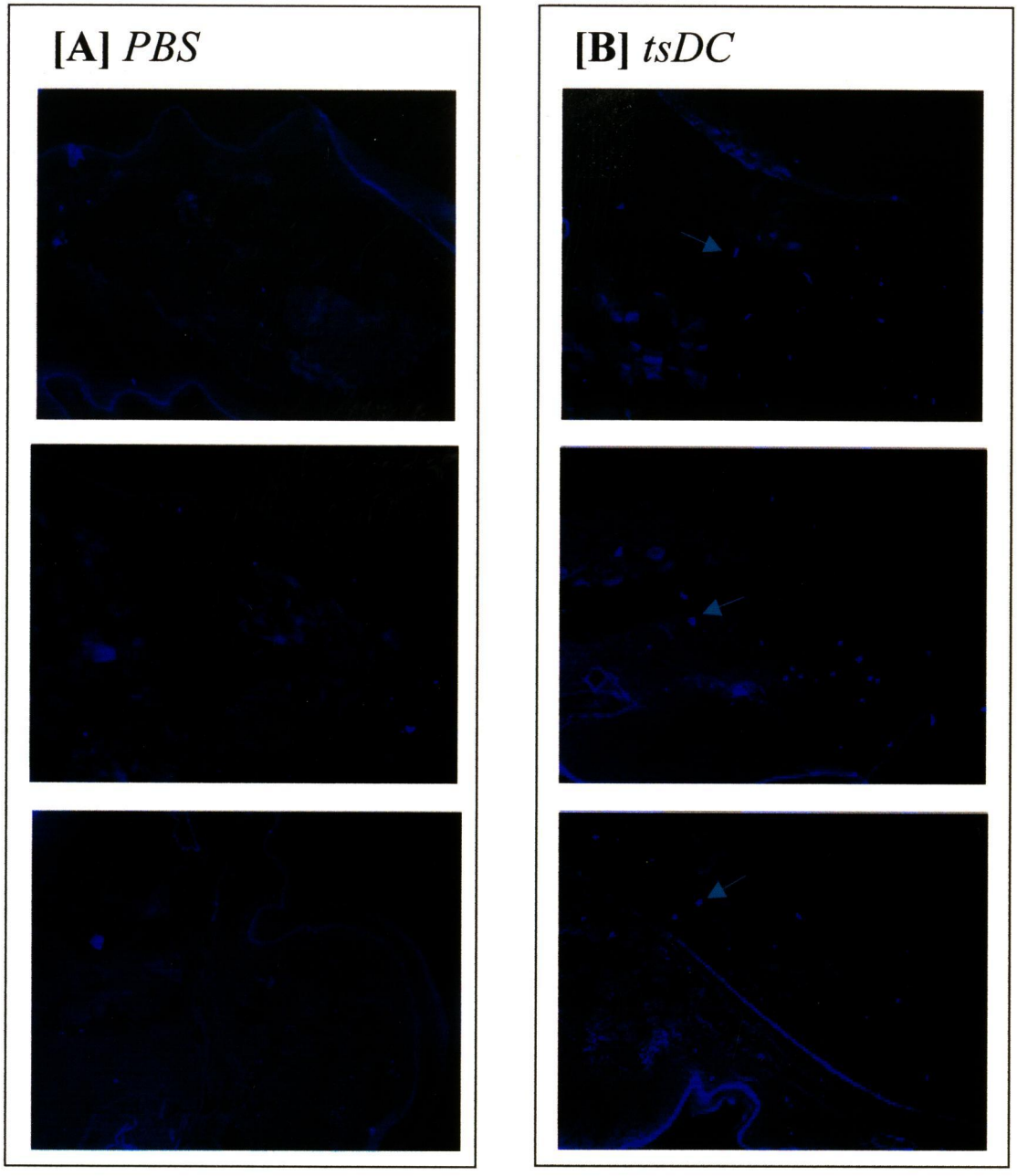
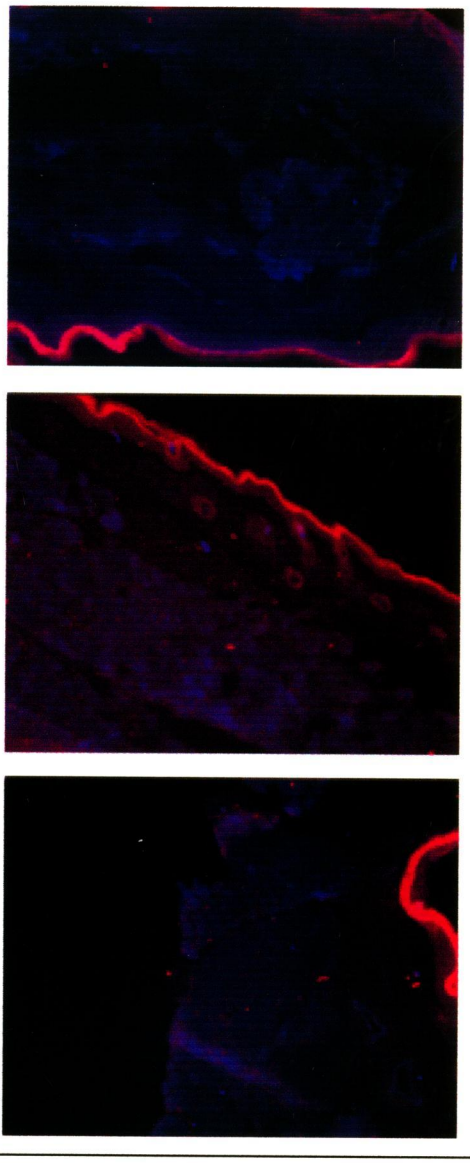

[A] PBS
[B] tsDC

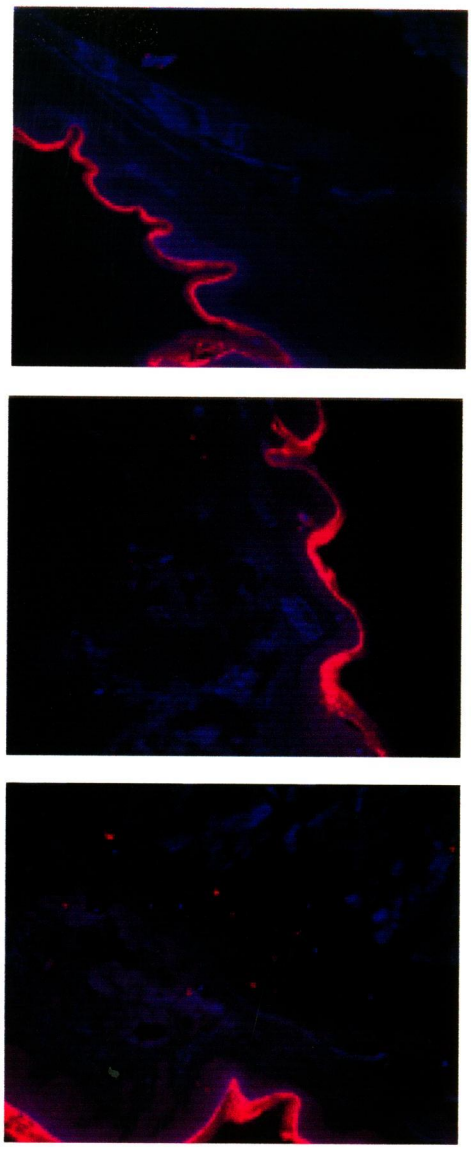
Figure 5.17. *Expression of CCR5 and CXCR3 by $CD4^+$ T cells within footpad tissue.* BALB/c mice were injected into the hind footpad with endotoxin-free PBS [A, C and E] or 5 x 10^5 tsDCs [B, D and F]. Eight days later, the footpad tissue was removed and 6μm cryostat sections prepared. The sections were fixed and then stained with one of the following combinations of antibodies: negative control monoclonal antibody and control goat IgG [A-B], anti-CD4 and anti-CCR5 [C-D] or anti-CD4 and anti-CXCR3 [E-F]. The sections were analysed by fluorescence microscopy, two images were collected from each sample using different filters, and the images overlayed. Cells stained with anti-CD4 only appear blue (green arrows), and cells stained with anti-CCR5 or anti-CXCR3 only appear red (white arrows). Where staining with the two antibodies coincides, cells appear pink (yellow arrows). Shown are 3 representative images of each condition, captured using the 10X objective.

In some instances, the colour resolution of the printing was not sufficient to allow a clear discrimination between red and pink colours. To exclude any ambiguity in this respect, the original monochrome images (ie, before overlaying) are presented in Appendix I, allowing a side-by-side comparison of staining for CD4 and either CCR5 or CXCR3.

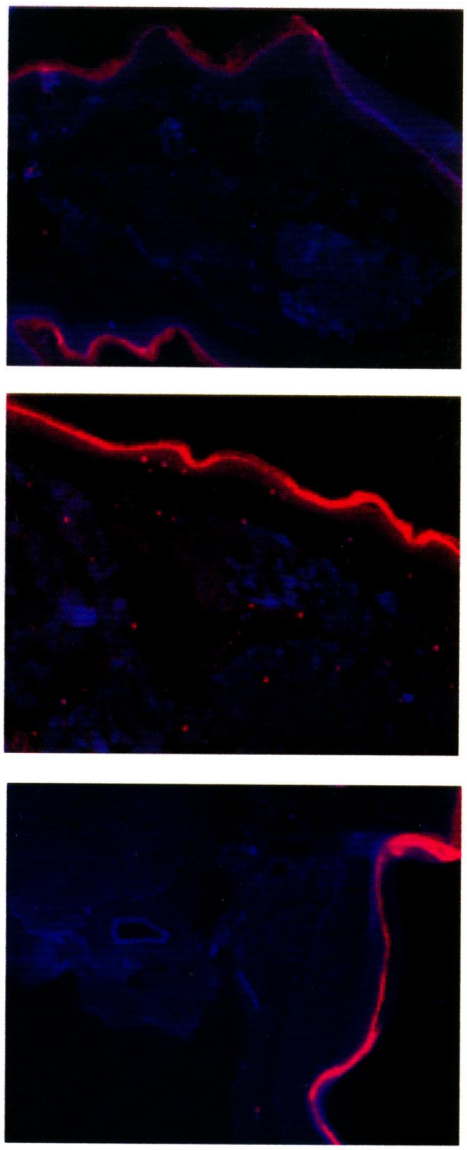
[A] *PBS: staining control*



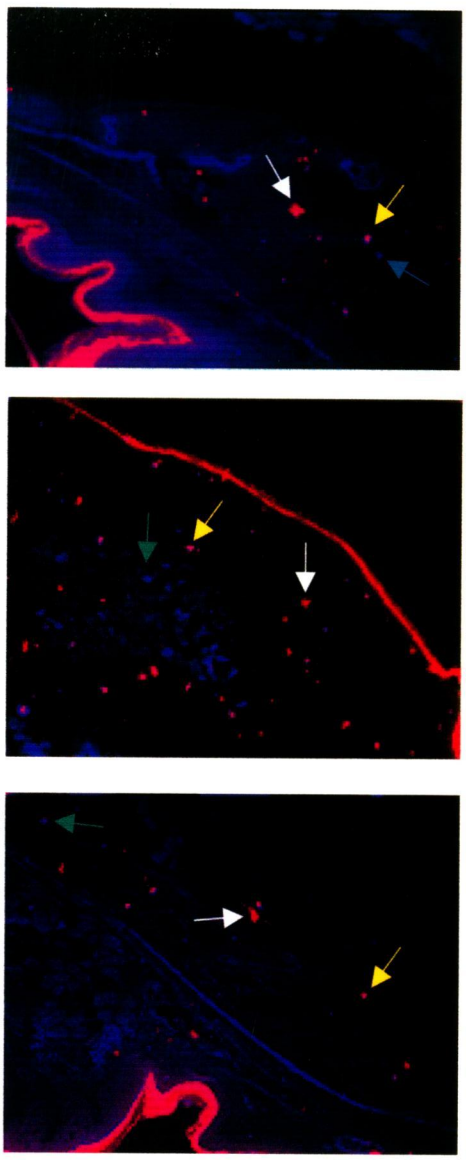
[B] *tsDC: staining control*



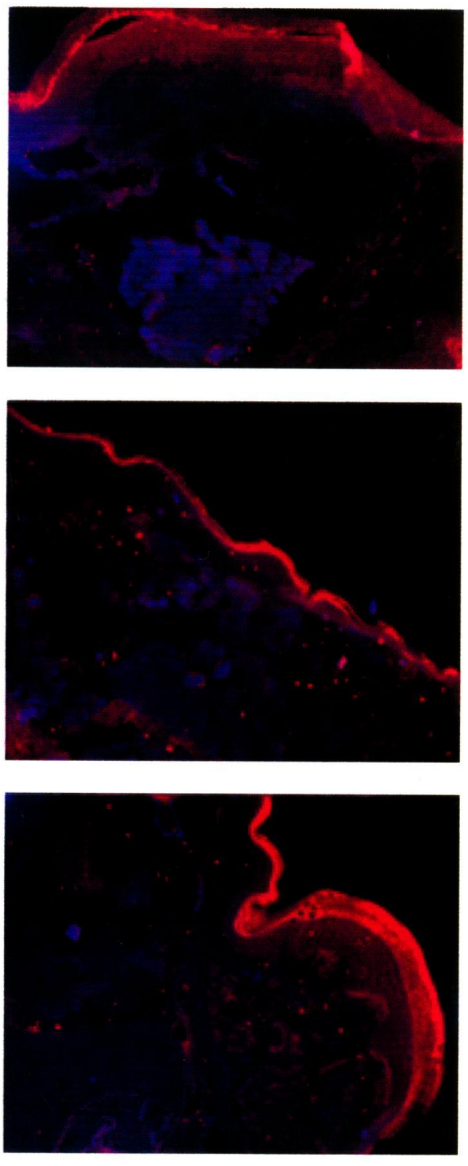
[C] *PBS: CCR5 + CD4*



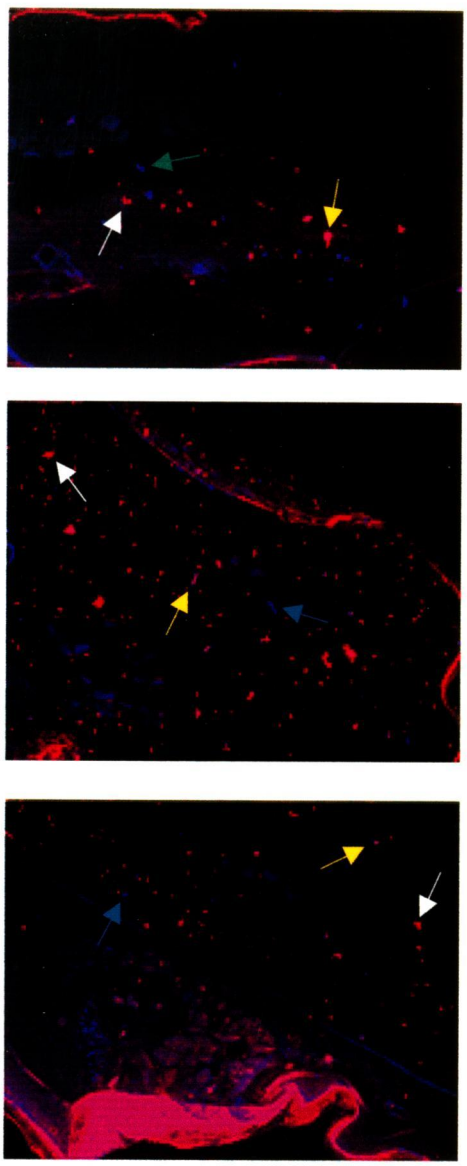
[D] *tsDC: CCR5 + CD4*



[E] *PBS: CXCR3 + CD4*



[F] *tsDC: CXCR3 + CD4*



CHAPTER 6

GENERAL DISCUSSION

6.1 Preamble

The chemokine gene superfamily is rapidly becoming recognised as one of the most important and diverse gene superfamilies within the immune system. However, with over 45 ligands and 19 receptors already cloned, the function, regulation and interplay of the chemokine network *in vivo* is likely to be extremely complex, and is thus far poorly understood. One of the major ways in which the chemokine system is likely to be regulated is at the level of receptor expression, for a potential target cell can clearly only respond to a chemokine if it expresses the cognate receptor. Thus, while the cell surface expression of a particular chemokine receptor does not always guarantee responsiveness to the cognate ligand (72), it is a critical prerequisite, and accordingly, is regulated to a high degree.

Although the ultimate goal of these investigations is to understand how the chemokine system functions within the whole animal, the majority of the studies have been conducted in an *in vitro* culture system. This was done for two reasons. First, a simplified system such as this is more amenable to detailed analysis in the absence of the extraordinary degree of complexity inherent to an *in vivo* model system. Second, for the first two years of this study, antibodies with which to study rodent chemokine receptors, and hence conduct *in vivo* experiments, were not available commercially, and although an intensive effort was made in the first 6 months of this project to generate such reagents, the resulting antibodies – while having high affinity for the immunising peptides – did not recognise the native receptors in flow cytometry. When antibodies to murine chemokine receptors did become available commercially, the

experiments within the human culture system had provided a sufficient depth of information to allow directed experimentation in a whole animal system.

The MLR was chosen for the *in vitro* studies as it involves cell-cell interactions between DCs and resting peripheral blood T cells. The mechanism of T cell activation is therefore biologically relevant, yet polyclonal in nature and consequently easily measurable. For the *in vivo* studies, a model of allogeneic activation was developed based on the s.c. injection of allogeneic DCs, such that the theme of allogeneic activation could be followed through to a whole animal system. Therefore, these results are likely to be highly relevant in the context of allogeneic transplant rejection, and provide evidence that CCR5 and CXCR3 may be useful targets in the control of such reactions, a concept supported by previous studies (49, 50, 155). However, the findings of the present study cannot automatically be applied to other situations of T cell activation, such as those mounted in response to viral, bacterial, tumour and multicellular parasite challenge, as it is likely that the nature of chemokine receptor regulation will be fine-tuned according to the characteristics of the antigen detected.

6.2 Variable patterns of chemokine receptor regulation on activated $CD4^+$ T cells

One interesting observation that was made consistently throughout the study of chemokine receptor expression on human $CD4^+$ T cells was the enormous degree of variation in the patterns of regulation of the various receptors. Thus, at the simplest level, in response to cellular activation some receptors (CCR1, CCR3, CXCR5) were

not modulated on $CD4^+$ T cells at all, while others were modulated on a small (CCR6), moderate (CCR5) or large (CXCR3, CXCR4) proportion of the activated $CD4^+$ T cell population. Two of the receptors, CXCR3 and CXCR4, were modulated in close parallel with cell division, while in the case of CCR5 and CCR6, division and receptor regulation were temporally distinct. The up-regulation of CCR5, CCR6 and CXCR3 was associated with an increase in mRNA levels, while in contrast, the down-regulation of CXCR4 protein was not mirrored by a corresponding decrease in CXCR4 transcripts, suggesting more complex regulation of this receptor. CXCR3 and CCR6, but not the other two receptors, were found to be stored intracellularly, although even here there were differences between the two; the percentage of $CD4^+$ T cells expressing intracellular CXCR3 was nearly 100%, while only around half that expressed CCR6 intracellularly. This variability suggests that the expression of each receptor may be subject to distinct control mechanisms, and strongly justifies the choice of flow cytometry as an indication of protein expression, rather than assessment of mRNA as an index of expression.

The observation that not all $CD4^+$ T cells that had divided in the MLR had altered expression of CCR5, CCR6 and – to a lesser extent – CXCR3, suggests that distinct populations of receptor-positive and receptor-negative activated T cells are generated during the immune response. This is unlikely to be an artefact of the *in vitro* culture system, as the modulation of chemokine receptors on $CD4^+$ T cells activated *in vivo* was similarly heterogeneous. Thus, only 42% of $CD4^+$ T cells that had undergone cell division in response to tsDC injection had acquired the ability to express either CCR5 or CXCR4, and only 52% had begun expressing CXCR3.

Further studies are required to elucidate the mechanisms by which a pool of T cells activated by the same stimulus (for example, allogeneic cells) can express distinct repertoires of chemokine receptors. A number of factors may contribute to this heterogeneity. First, subtle differences in the local cytokine environment, or in the nature and level of expression of co-stimulatory molecules expressed by individual APCs, could affect the range of chemokine receptors expressed by activated T cells. For example, a T cell that is exposed to a concentration of IL-12 above a certain level may express a distinct range of chemokine receptors compared to a T cell that was not exposed to a sufficient level of IL-12. Alternatively, it is conceivable that quantitative differences in affinity for antigen could lead to qualitative differences in chemokine receptor expression. Thus, the expression of CCR6, for example, may be limited to T cells with a high affinity for antigen, while T cells that recognise antigen with only moderate affinity may be unable to initiate expression of this receptor.

It is also not clear at this stage what advantage is provided by the generation of sub-populations of activated T cells expressing distinct repertoires of chemokine receptors. One possibility is that sub-populations of activated T cells expressing different patterns of chemokine receptors play distinct roles in the immune response. This concept is supported by recent studies demonstrating that the presence or absence of CCR7 appears to define sub-populations of effector/memory T cells with distinct homing and effector functions (109). Thus, $CCR7^{+}$ memory T cells express LN homing receptors and are therefore likely to be capable of entering secondary lymphoid tissue, but have limited immediate effector function. Accordingly, these cells are thought to be important in the initiation of an immune response to recall antigens that have localised in the secondary lymphoid tissues. In contrast, those cells that lack CCR7 express a

range of inflammatory chemokine receptors, suggesting that they circulate through peripheral tissues, and possess immediate effector function, implicating this population in the generation of rapid protective responses at the site of antigen deposition. The expression of CXCR5 is also thought to define sub-populations of activated T cells with distinct effector functions, as only $CXCR5^+$ T cells appear to be efficient at providing B cell help (119-121). Similarly, it is possible that the development of sub-populations of allo-activated $CD4^+$ T cells that differ in expression of CCR5, CCR6, CXCR3 and CXCR4 also possess distinct effector functions. In this regard, it would be informative to separate allo-activated $CD4^+$ T cells into receptor-positive and receptor-negative subsets and compare the effector functions of these sub-populations, particularly in terms of cytokine producing phenotype.

Thus, it is possible that sub-populations of activated T cells expressing distinct patterns of chemokine receptors are generated during immune response induction to provide unique effector functions *in vivo*. However, the generation of activated T cells expressing a range of chemokine receptors, and possibly combinations of chemokine receptors, is also consistent with the concept that the acquisition of distinct homing phenotypes by sub-populations of effector/memory T cells occurs by a process of selection, rather than instruction (249). If this model is applied to chemokine receptor expression, it could be hypothesised that, following activation, a pool of T cells expressing a range of different chemokine receptors would be generated, as observed in the present study. Subsequently, the most appropriate cells would be selected for relocation to the site of antigenic challenge, by means of production of the relevant chemokine ligand/s, and subsequent ligation of the appropriate combination of chemokine receptors on a limited number of activated T cells. Meanwhile, the

remaining activated T cells that did not express the appropriate combination of chemokine receptors would not have access to antigen in the peripheral tissues and would therefore be expected to undergo apoptosis due to lack of antigen stimulation (250). It is important to note here that this model is not inconsistent with an important role for cytokines or other factors in the regulation of chemokine receptor expression. To the contrary, it seems likely that a degree of instruction-based regulation of chemokine receptor expression would work in co-ordination with the selection-based model, such that the repertoire of chemokine receptors expressed by activated T cells would be tailored to some extent according to information available in the secondary lymphoid tissue (for example, the patterns of cytokines produced by APCs).

As an example of the selection-based model, in an immune response in the skin, E-selectin-dependent adhesion to vascular endothelium would be expected to be important for T cell extravasation. As such, the expression of MIP-3α/CCL20 at the site of challenge would select for T cells that had acquired expression of CCR6, which is particularly good at inducing adhesion to vascular endothelium under these conditions (82). Another chemokine receptor such as CXCR3 may be the most appropriate for the efficient localisation of activated T cells within the skin, and accordingly, the expression of one or more CXCR3 ligands at defined locations in the dermis or epidermis (39) would select for those T cells that had up-regulated this receptor. In this way, only the T cells which co-expressed CCR6 and CXCR3 would be recruited to the site of immune challenge.

In this respect, a comprehensive analysis of the various combinations of chemokine receptors expressed on activated T cells would have been informative, but was beyond

the scope of the present study. However, the patterns of CCR5 and CCR6 co-expression were studied (not shown), and this analysis revealed that activated $CD4^+$ T cells could be divided into four sub-populations on the basis of CCR5 and CCR6 expression: small numbers of double-negative cells and CCR6 single-positive cells and larger populations of CCR5 single-positive and $CCR5^+$ $CCR6^+$ double-positive cells. Furthermore, previous studies have demonstrated that the co-expression patterns of other chemokine receptors can similarly define distinct T cell sub-populations (109, 118, 123). It is likely that further analysis of the co-expression patterns of many different chemokine receptors would reveal an extraordinary number of sub-populations expressing various combinations of receptors, in keeping with the large number of effector phenotypes that would need to be generated for a selection-based model of regulation to be feasible.

6.3 Location and timing of chemokine receptor regulation

As an isolated *in vitro* culture system, the human MLR provided information about the nature of chemokine receptor regulation, but could not be used to definitively establish the location in the body where the observed changes in chemokine receptor expression would take place. However, in the case of CXCR3 and CXCR4, the close parallels observed between the regulation of their expression and the cell division process suggests that these receptors are modulated in the secondary lymphoid tissue, as this is likely to be the location at which T cell proliferation occurs, at least during a primary immune response. In contrast, the most notable changes in the expression of CCR5 – and especially CCR6 – occurred many days after the initiation of cell division. This

temporal distinction suggests that the up-regulation of CCR5 and CCR6 occurs after activated T cells have left the secondary lymphoid tissue environment, as it seems unlikely that activated T cells, which would be expected to be fully-armed by this stage, would be permitted to stay for this length of time in secondary lymphoid tissue (251-253).

Studies in mice confirmed that the up-regulation of CXCR3 on activated $CD4^+$ T cells occurred in the LN, therefore supporting the results from the human MLR. However, these studies also revealed a dramatic up-regulation of CCR5 expression on proliferating $CD4^+$ T cells within the popliteal LN. This finding therefore argues against the possibility that CCR5 (and perhaps CCR6) are acquired by activated $CD4^+$ T cells only after they have left the LN. A more likely explanation for the observed temporal distinction between cell division and regulation of CCR5 and CCR6 expression in the MLR is that this delay is exaggerated in the *in vitro* culture system. Thus, in an *in vivo* setting, CCR5 and CCR6 may indeed be acquired only after the cells had undergone extensive proliferation, but after a sufficient number of cell divisions had occurred, these receptors would be immediately expressed, rather than being subject to the extensive delay observed *in vitro*. As discussed previously (see, for example, Section 3.3.3), a number of observations suggest that cytokines play an important role in the regulation of CCR5 and CCR6 expression. Accordingly, it is conceivable that the up-regulation of these receptors occurs only after certain cytokines have accumulated to effective concentrations, and this may occur within the LN microenvironment much faster than within a culture well. Delaying chemokine receptor up-regulation until after T cells have completed (or are well progressed in) the cell division process may be a mechanism to prevent activated T cells becoming

prematurely responsive to a novel set of chemokines that are expressed in the periphery, and thereby leaving the secondary lymphoid tissue before having undergone sufficient proliferation and differentiation in an appropriate microenvironment.

6.4 Comparison of chemokine receptor regulation in humans and mice

The conservation of a biological mechanism across a species barrier may be considered to indicate the relative importance of this mechanism to the functioning of the organism, as generally only the most successful evolutionary adaptations are retained during the process of natural selection. Thus, if the patterns of chemokine receptor regulation observed using human cells are also observed using mouse cells, it may be considered that these mechanisms are biologically successful, as they have been retained through evolution. Conversely, a pattern that occurs in humans but not in mice is likely to have developed relatively recently, and its suitability to optimal functioning of the immune system is yet to be determined by evolutionary forces.

The patterns of CCR5 and CXCR3 expression observed on $CD4^+$ T cells were well conserved across the human-murine species barrier, and furthermore, were similar *in vitro* and *in vivo*. Thus, it appears that, at least for these two receptors, the activation of human T cells in the MLR models quite well the events occurring during immune response induction in mice, and probably also humans. It is not clear from these studies whether the regulation of CCR6 is similar in mice and humans. The RT-PCR analysis of CCR6 mRNA levels in the murine MLR did not detect any up-regulation of this

receptor in allogeneic cultures compared to syngeneic controls. This observation is in contrast to other studies within our laboratory using the murine model of EAE, which have demonstrated that *in vitro* re-stimulation of LN cells from mice immunised with an immunogenic peptide of proteolipid protein (PLP) results in increased levels of CCR6 mRNA, compared to non-stimulated cells from a naïve mouse (Liston and McColl, unpublished observations). Therefore, in contrast to the results of the present study, the induction of a secondary immune response to a peptide antigen resulted in the up-regulation of CCR6, at least at the mRNA level. This distinction suggests that the up-regulation of CCR6 is dependent on the nature of the antigen, or perhaps only occurs during secondary stimulation. Alternatively, the possibility remains, as discussed already, that cell surface levels of CCR6 expression are modulated on allo-activated cells, even though a change in expression of the transcript was not detected. In future studies of the MLR, until an anti-murine CCR6 antibody becomes available, a potential way to address the regulation of CCR6 expression indirectly would be to test the ability of cells from allogeneic and syngeneic cultures to migrate toward the CCR6 ligand, MIP-3α/CCL20.

The regulation of CXCR4 expression differed significantly between the human and murine MLR, as the striking loss of CXCR4 expression from activated human T cells observed in this study and others (106, 136) was not observed in the murine MLR. This difference therefore highlights a notable species-specific distinction between the regulation of CXCR4 in mice and humans. Furthermore, the *in vivo* studies revealed a subtle up-regulation of CXCR4 expression on activated $CD4^+$ T cells that was not revealed in either the human or murine MLR.

Although difficult to test directly, it would appear likely that the down-regulation of CXCR4 expression observed in the human MLR does occur during immune response induction in humans, as memory T cells express lower levels of this receptor compared to the naïve population (74, 107), suggesting (as discussed in Chapter 1) that CXCR4 is down-regulated during T cell activation *in vivo*. However, on the basis of the present study, this is clearly not the case in mice, either *in vitro* or *in vivo*. It is therefore possible that the down-regulation of CXCR4 expression upon T cell activation is a relatively recent evolutionary adaptation. It is not clear at this stage whether the distinct patterns of CXCR4 regulation in mice and humans produce a different outcome in terms of functioning of the immune system, or whether this difference represents the development of distinct mechanisms to produce the same net result.

6.5 Why is the expression of chemokine receptors altered following T cell activation?

6.5.1 Up-regulation of receptors for inflammatory chemokines

As discussed in Chapter 1, naïve T cells are characterised by a distinctly different route of recirculation compared to effector and memory T cells (6-8). Thus, naïve T cells recirculate primarily through the lymphatics, passing directly from the blood to the secondary lymphoid tissues and back to the blood again. In contrast, effector and memory T cells are also able to enter peripheral tissues, a migratory route which is required for the clearance of antigen and effective surveillance to detect secondary infections. Given the apparent importance of chemokines and their receptors in

controlling leukocyte migration, it seemed likely that, upon activation, T cells would be subject to changes in the patterns of chemokine receptors expressed, and that these changes would contribute to the altered patterns of migration seen for naïve versus effector/memory cells.

Clear evidence has now been obtained that CCR5, CCR6 and CXCR3 are up-regulated on antigen-activated $CD4^+$ T cells. The ligands for all of these receptors are inflammatory chemokines, and as such, are produced in defined peripheral tissues under conditions of inflammation. For example, the induction of EAE in mice is associated with the increased production of ligands for CCR5 and CXCR3 in the central nervous system, including MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5 and IP-10/CXCL10 (49), and a similar range of chemokines are found in the central nervous system of patients with the related disease, multiple sclerosis (49, 254). Ligands for CXCR3 are also readily detectable at sites of various skin pathologies in humans (39). The only known chemokine ligand for CCR6, MIP-3α/CCL20, is expressed at high levels within the rheumatoid synovium (255), and is up-regulated in the intestine in response to bacterial infection (256). Also, of particular relevance to the present study of allogeneic activation, MIP-1β/CCL4, RANTES/CCL5, as well as all three ligands for CXCR3 have been shown to be expressed at elevated levels following allogeneic transplantation (49).

Thus, considering that CCR5, CCR6 and CXCR3 were up-regulated on antigen-activated $CD4^+$ T cells, and that ligands for these receptors are known to be produced at sites of T cell-dependent inflammatory pathologies, it seems likely that the observed changes in chemokine receptor expression may contribute to the ability of activated T

cells to migrate to peripheral sites of inflammation. In support of this hypothesis, the up-regulation of CCR5 and CXCR3 on human allo-activated $CD4^+$ T cells resulted in a significant increase in the migration of these cells toward the relevant chemokine ligands, MIP-1β and I-TAC. Furthermore, analysis of footpad tissue sections in mice demonstrated that the injection of tsDCs was associated with a low-level infiltration of $CD4^+$ T cells, and that most of these cells expressed CCR5 and CXCR3. Thus, the recruitment of $CD4^+$ T cells to a site of antigen deposition in the peripheral tissues was associated with the expression of CCR5 and CXCR3, although a causal role for these receptors in $CD4^+$ T cell migration was not able to be demonstrated within the time constraints of this study. Potential directions for future studies that would extend and confirm these preliminary observations have been extensively discussed in Chapter 5.

A number of studies have suggested that CCR5 and CXCR3 play a role in the development of T cell-mediated immune responses. For example, a naturally occurring defect in the expression of CCR5 within the human population is associated with delayed onset of multiple sclerosis (257) and reduced severity of rheumatoid arthritis (258). Furthermore, of particular relevance to the present study, $CCR5^{-/-}$ and $CXCR3^{-/-}$ mice show enhanced acceptance of allografts in comparison to wildtype animals (50, 155). However, it is not clear whether the reduction in T cell effector function observed in these studies is associated with an inability of the cells to localise to the site of antigen challenge, or to other defects. In fact, T cells from $CXCR3^{-/-}$ mice demonstrate reduced proliferation in the MLR (155), implying that the enhanced acceptance of allografts in these animals is at least partly due to reduced T cell activation, rather than an inability of activated cells to localise to the graft. The

contribution of CCR5 and CXCR3 function to the ability of activated T cells to migrate to peripheral sites of inflammation therefore remains to be definitively determined.

The biological significance of CCR6 up-regulation on allo-activated human $CD4^+$ T cells is unclear, as this alteration was not associated with an increased chemotactic response toward MIP-3α/CCL20. Furthermore, studies with a MIP-3α receptor antagonist revealed that CCR6 did not play an apparent role in the regulation of apoptosis in the MLR. A number of other possible functions for CCR6 on activated T cells exist, and although these could not be specifically tested in the present study due to time constraints, future studies addressing these possibilities will be important in gaining a better understanding of this receptor. One of the most likely functions of CCR6 on activated T cells is the activation of integrin molecules on the T cell surface. MIP-3α/CCL20 has been shown to induce the rapid adhesion of memory T cells to intercellular adhesion molecule-1 (I-CAM) (78), and a recent study by Fitzhugh *et al* suggests that the interaction between CCR6 and MIP-3α/CCL20 is essential for the firm adhesion of memory T cells to dermal microvascular endothelium (82). This observation implies that the acquisition of CCR6 by activated T cells, as demonstrated here, would be a pre-requisite for migration through the endothelial wall in the skin, and possibly at other sites as well. Alternatively, the possibility remains that the purpose of CCR6 up-regulation is to mediate chemotaxis toward MIP-3α/CCL20, but that a further signal, such as that provided by a cytokine, is required to couple the newly-acquired receptor to the appropriate intracellular signalling cascades. In this context, it is interesting to note that while CCR6 is expressed at readily detectable levels by freshly isolated B cells, no chemotactic or calcium flux response to MIP-3α is observed (130).

Consistent with a role for CCR6 in either chemotaxis or vascular adhesion, studies using $CCR6^{-/-}$ mice have revealed an important function for this receptor in the localisation of activated T cells to sites of inflammation. Thus, the DTH response to allogeneic splenocytes injected into the footpad was significantly reduced in $CCR6^{-/-}$ mice compared to wildtype animals (152). Furthermore, when $CD4^+$ T cells were transferred from sensitised $CCR6^{-/-}$ mice to wildtype recipients, the recipients were unable to develop a DTH response to allogeneic splenocytes injected into the footpad, while mice that received sensitised $CD4^+$ T cells from wildtype animals mounted a strong DTH response. Of note, the inability of $CD4^+$ T cells from $CCR6^{-/-}$ mice to transfer DTH reactivity was unlikely to be due to sub-optimal T cell activation, as *ex vivo* stimulation of the cells in an MLR was as efficient as wildtype cells, and did not increase the ability of the $CCR6^{-/-}$ cells to induce a DTH response upon transfer to wildtype recipients. These observations suggest that the reduction in DTH responsiveness in $CCR6^{-/-}$ mice was due to an inability of the activated T cells to reach the site of antigen challenge, or possibly to perform an appropriate effector function when they arrived there. Further studies in these mice, as well as other chemokine receptor knock-out mice, using radioactive or fluorescent-based methods to track effector T cell migration, would be particularly informative.

Previous analyses of peripheral blood lymphocytes suggest that activated T cells retain the expression of CCR6 and CXCR3 as they return to a resting, memory phenotype, as these receptors are expressed by a large proportion of peripheral blood memory T cells (72, 74, 118, 130, 134). Expression of CCR5 also appears to be retained to some extent by memory T cells, although a number of studies have shown that the expression of this receptor by memory cells is more restricted than that of CCR6 or CXCR3 (72, 74,

118), suggesting that CCR5 is lost by some activated T cells as they return to a resting state. In the present study, analysis of extended MLR cultures revealed that the expression of CCR5, CCR6 and CXCR3 was retained for at least 24 days, long after T cells had stopped proliferating, thereby suggesting that the expression of these receptors is not restricted to a state of acute activation. This observation therefore provides preliminary support for the hypothesis that activated T cells retain expression of CCR6 and CXCR3 as they return to a resting state. However, the mechanisms leading to the putative loss of CCR5 expression by some memory T cells are unclear at this stage, as no evidence of this effect was observed in the extended MLR cultures.

It is relevant to note here that, at least in the case of CCR5 and CCR6 expression, allogeneic activation resulted in an increase in the level of receptor expression, as well as the number of receptor-expressing cells, compared to syngeneic controls (Chapter 3). This observation therefore provides evidence for a quantitative difference in the expression of inflammatory chemokine receptors on activated versus resting memory T cells. Thus, while memory T cells do appear to retain the expression of some inflammatory chemokines receptors, the level of expression is reduced in comparison to acutely activated T cells.

The expression of inflammatory chemokine receptors by resting memory T cells suggests a role for inflammatory chemokines and their receptors in maintaining a basal level of memory T cell traffic through peripheral tissues in the absence of inflammation. In support of this concept, low-level constitutive expression of CXCR3 ligands (Mig/CXCL9 and IP-10/CXCL10) and CCR6 ligand (MIP-3α/CCL20) has been detected within normal human colon epithelium, and is associated with the

presence of $CXCR3^+$ mononuclear cells, and $CCR6^+$ T cells, within the epithelial tissue or lamina propria, respectively (36, 256). Thus, it is conceivable that the expression of inflammatory chemokine receptors acquired during the activation process, as demonstrated here, allows memory T cells to circulate though peripheral tissues under conditions of homeostasis. However, it is important to note that the ability of memory T cells to enter peripheral tissues will also be controlled at the level of adhesion molecule expression, thereby providing the potential for multiple levels of control (6, 16, 17). It will therefore be important to perform blocking studies to determine the relative contributions of chemokine receptors and adhesion molecules to the trafficking of T cells through peripheral tissues in normal animals. Of relevance here is the observation, in two separate studies, that $CCR6^{-/-}$ mice have increased numbers of intra-epithelial lymphocytes (151, 152). It is possible that the increased number of gut-resident T cells results from a corresponding decrease in the localisation of T cells to other specific sites, such as the skin. However, it is important to acknowledge that any observed differences in lymphocyte homeostasis in knock-out mice may be caused by developmental defects, due to the gene being absent throughout embryogenesis.

A potential way to minimise this complication and address the role of chemokine receptors in the control of basal T cell trafficking would be to adoptively transfer resting, memory T cells from chemokine receptor null mice to wildtype recipients and assess any differences in the migratory patterns of these cells compared to wildtype cells, under normal, non-inflammatory conditions. Labelling the transferred cells with ^{125}I-UdR would provide a sensitive means to track the cells throughout all tissues of the body. In preliminary experiments using this technique, readily detectable radioactive counts were observed in a wide variety of tissues from normal mice,

including the small and large intestines, stomach, lungs, kidneys, liver and skin. It would be of significant interest to determine the mechanisms by which T cells reach these varied tissues in the absence of inflammation.

All of the roles for inflammatory chemokine receptors discussed thus far have focussed on the movement of activated T cells to sites in the periphery. However, it is also possible that these receptors are important for the ability of memory T cells to enter secondary lymphoid tissues during immune response induction. In Chapter 5, it was observed that immunisation of mice with tsDCs resulted in a large influx of lymphocytes into the popliteal LN. This was associated with an increase in the percentage of $CD4^+$ T cells in the LN expressing CCR5 and CXCR3, thereby suggesting that $CCR5^+$ and $CXCR3^+$ cells entered the LN with greater efficiency compared to cells that did not express these receptors. This observation is of particular interest, given that the entry of memory T cells into the LN is known to increase during an immune response (243, 259), presumably to allow the memory population access to antigen that has drained to the LN. It is therefore conceivable that the expression of CCR5 and CXCR3 plays a role in the entry of memory T cells into the inflamed LN. In this context, it is interesting to note that CCR6 and CXCR3 (although not CCR5) are expressed on the $CCR7^+$ subset of memory T cells that is proposed to recirculate through LNs (109).

The possibility that $CD4^+$ T cells expressing CCR5 and CXCR3 were preferentially recruited into the tsDC-immunised LN suggests that chemokine ligands for these receptors were produced in the LN following immunisation. Although not specifically examined here, this concept is supported by the finding that the CXCR3 ligands

Mig/CXCL9, IP-10/CXCL10 and I-TAC/CXCL11 are expressed in the LNs of rats during the development of EAE (McColl et al, manuscript submitted), while ligands for CCR5 have been identified in the draining LN during elicitation of contact hypersensitivity in mice (260). It is also possible that the expression of inflammatory chemokine ligands in the LN has a role in microenvironmental positioning of lymphocytes, as has been proposed for homeostatic chemokines such as BLC/CXCL13 (34), or in the control of T cell activation and/or polarisation (84, 86-88). Finally, a role for chemokines and their receptors in regulating immunological synapse stability has been proposed. Normally, upon initial contact with antigen/MHC complexes on an APC, a T cell immediately stops all migration, such that a stable interaction between the two cells can occur, leading to the formation of the immunological synapse. However, recent studies show that signalling through CXCR3, but not CCR5, is capable of over-riding this 'stop' signal, thereby allowing an activated T cell to be released from its intimate association with the APC (261). This observation is particularly interesting, given that CXCR3 up-regulation was shown in the present study to occur immediately upon the initiation of cell division, whereas the majority of CCR5 up-regulation occurred only after extensive proliferation. Thus, it is conceivable that, following 1-2 rounds of cell division, the activated T cell is ready to move away from the APC, possibly for relocation to another region of the secondary lymphoid tissue. Accordingly, CXCR3 expression is initiated, thereby allowing the activated T cell to receive signals from CXCR3 ligands (which, as discussed above, appear to be produced in the LN during an immune response) and resume its migratory abilities.

6.5.2 Down-regulation of CXCR4 (homeostatic chemokine receptor)

Given the constitutive expression of homeostatic chemokines in the LN, it seems likely that the down-regulation of the corresponding receptors, such as CXCR4, upon activation would reduce the propensity of effector/memory T cells to recirculate through the lymphatics. Thus, inflammatory chemokines produced at peripheral sites would be able to recruit these T cells efficiently, without having to compete with opposing signals that encourage entry into the lymphatics from the peripheral blood (via HEVs). As discussed already, a similar scenario has recently been proposed for CCR7, based on the suggestion that $CCR7^-$ effector/memory T cells are excluded from secondary lymphoid tissue (108, 109).

The present study has shown that CXCR4 was indeed lost by virtually all activated (dividing) $CD4^+$ T cells in the human MLR, thereby providing evidence that this homeostatic chemokine receptor is down-regulated by activated T cells. Furthermore, the observation that this occurred, with remarkable efficiency, on both $CD4^+$ and $CD8^+$ T cell subsets, suggests that it is a ubiquitous, and therefore perhaps critical, mechanism. Finally, the finding that allo-activated T cells migrated with reduced efficiency toward the ligand, SDF-1/CXCL12 supports the concept that the reason this receptor is lost from activated T cells is to prevent migration toward sites of homeostatic chemokine production.

However, the biological significance of CXCR4 down-regulation *in vivo* will be difficult to address, given that, at least in the present study, CXCR4 down-regulation did not occur during immune response induction in mice. Further studies in other animal models, such as rats or primates, will be required to determine whether

alternative *in vivo* model systems will be more relevant. Also, the interpretation of CXCR4 down-regulation is complicated by the striking, non-specific up-regulation of this receptor that occurs upon placing peripheral blood T cells into culture ((106, 136, 137, 189), and this study). Thus, although the loss of CXCR4 expression on activated T cells in the present study was clearly a specific effect, in that it did not occur on non-dividing T cells in the same cultures, it occurred against a background of non-specific up-regulation that appears to be an artefact of *in vitro* culture.

While the expression of other homeostatic chemokines is primarily restricted to specific sites within secondary lymphoid tissue (43, 69, 70, 184), the expression of SDF-1/CXCL12 is far more extensive, with constitutive expression detected in a wide range of normal tissues (46, 47). Thus, while the loss of CXCR4 expression may indeed discourage effector/memory T cells from recirculating through the lymphatics, this change may additionally play a more general role in preventing the migration of activated T cells into multiple tissues. It is conceivable that activated T cells would be required only at the specific site of antigen deposition, while memory T cells would require access to a wider range of peripheral tissues in order to maintain effective surveillance for evidence of re-infection. Accordingly, the loss of CXCR4 expression by activated T cells will prevent these cells from responding chemotactically to the SDF-1/CXCL12 produced in non-target sites, and therefore specifically home to the site of antigen deposition, presumably in response to inflammatory chemokines and the expression of appropriate adhesion molecules. In contrast, although memory T cells from peripheral blood do appear to express CXCR4 at lower levels, and in a more heterogeneous manner, compared to naïve cells (74, 107, 109), this receptor is clearly not completely absent, in contrast to the effector T cells generated in the MLR.

Therefore, by responding to SDF-1/CXCL12, memory T cells are afforded a much less restricted surveillance of peripheral tissues. This hypothesis is supported by the observation that preventing the cell surface expression of CXCR4 blocks the multi-organ dissemination of a T cell hybridoma (262).

6.6 Concluding remarks

Using an *in vitro* model of antigen presentation, three inflammatory chemokine receptors have been shown to be up-regulated on human $CD4^+$ T cells in response to activation, while one homeostatic chemokine receptor has been shown to be down-regulated on activated $CD4^+$ T cells. Detailed characterisation of these receptors revealed that modulation of their expression generally occurs in co-ordination with the expression of memory and activation markers, although dependence on the progression of cell division was variable. Three out of four of the receptors were shown to be regulated at the transcriptional level, although additional mechanisms of regulation are suggested for CCR6 and CXCR3 by the observation of intracellular stores of receptor protein. In an *in vivo* model of immune activation, the expression of CCR5 and CXCR3 was shown to be increased in LNs draining the site of immunisation. This was likely to be due to both the preferential entry of $CD4^+$ T cells expressing these receptors into the LN during an immune response, as well as the local up-regulation of expression by dividing T cells. A modest up-regulation of CXCR4 expression on divided $CD4^+$ T cells was also noted, in contrast to the findings with human $CD4^+$ T cells.

In vitro chemotaxis assays demonstrated that the up-regulation of CCR5 and CXCR3 expression resulted in enhanced chemotaxis toward the relevant ligands, while the loss of CXCR4 expression reduced chemotaxis toward SDF-1/CXCL12. In contrast, despite the up-regulation of CCR6 expression following allogeneic activation, activated cells showed an identical chemotactic response toward MIP-3α/CCL20 compared to resting cells. The up-regulation of CCR6 also did not appear to be involved in the regulation of apoptosis. *In vivo*, the injection of allo-antigen into the footpad was associated with a modest infiltration of $CD4^+$ T cells, most of which expressed CCR5 and CXCR3. When interpreted in the context of findings from the chemotaxis assays, this result suggests a role for these chemokine receptors in the relocation of activated T cells to sites of peripheral inflammation.

It will be important to extend these findings to other models of immunity, in order to compare the patterns of chemokine receptor regulation observed *in vitro* and *in vivo* in the present study to the patterns of regulation that occur following stimulation with different antigens. Considering that the expression of CCR5 and CXCR3 has been suggested to be associated with a polarised Th1 phenotype *in vitro* (73, 74, 111), it would be particularly interesting to determine if the up-regulation of these receptors *in vivo*, as observed in the present study in response to allogeneic activation, would also occur during an immune response toward a characteristic Th2 antigen, such as ovalbumin. It will also be important to study the regulation of other chemokine receptors, such as CCR4, CCR7 and CCR8, which could not be assessed in the present study. Given the degree of variation observed in the patterns and mechanisms of regulation of the four receptors studied here, it is reasonable to expect that each

chemokine receptor will be uniquely controlled, and must therefore be specifically studied.

Finally, it will be critical in future studies to definitively determine the biological reasons for the observed patterns of chemokine receptor regulation. In the present study, *in vitro* chemotaxis assays and preliminary *in vivo* studies suggested that the acquisition of inflammatory chemokine receptors by activated T cells allowed them to respond chemotactically to a novel set of chemokines, and therefore migrate to a site of antigen deposition in the periphery. However, more detailed *in vivo* studies are required to confirm and extend upon these observations. Blocking studies, in which the homing patterns of activated T cells are compared in the presence or absence of an agent that prevents chemokine receptor function, will be particularly important. Such agents may include receptor antagonists (199, 263), neutralising antibodies, and intrakine approaches (in which the chemokine receptor of interest is prevented from reaching the cell surface by binding to a modified chemokine with an endoplasmic reticulum retention signal) (264). Studies of chemokine receptor knockout animals, particularly using adoptive transfer techniques (152) to reduce the complicating effects of potential developmental defects, will also be highly informative.

Given the complexity and diversity of the chemokine system, it is likely that these molecules will play critical roles in controlling the migration of T cells, as well as regulating many other biological functions, both within and outside of the immune system. Accordingly, a thorough understanding of how this family of molecules is regulated, as well as the precise roles that each member plays in the control of immunity, should be an important research focus. In addition to contributing to our

understanding of how the immune system functions, such research efforts may also lead to the development of novel therapeutics for the control of autoimmunity, infectious disease and transplantation (49).

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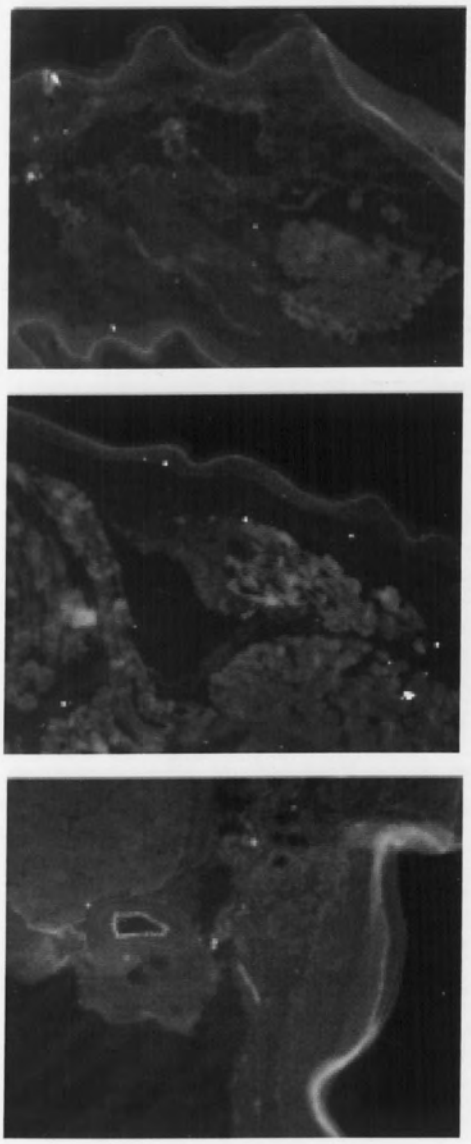
Appendix I: supplementary material for Figure 5.17.

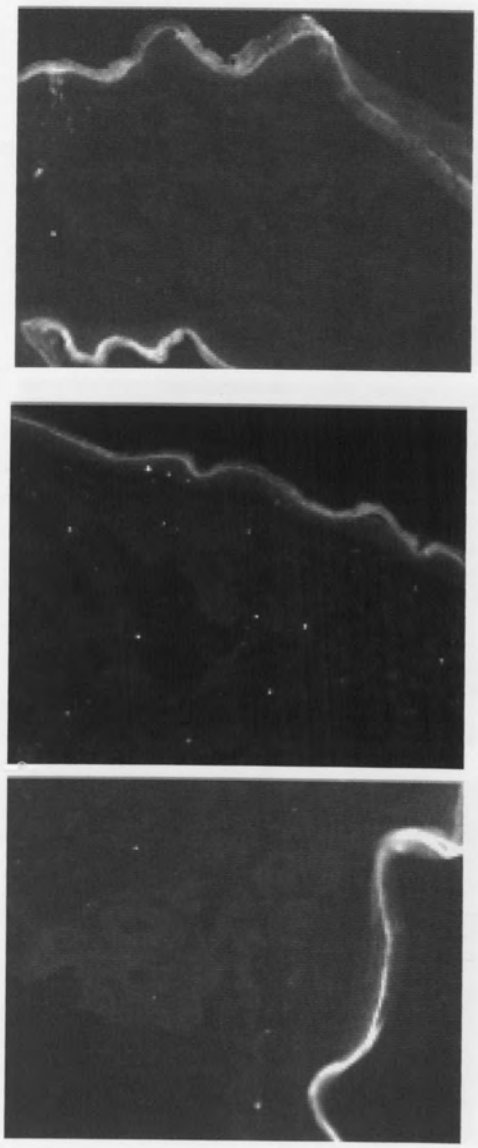
BALB/c mice were injected into the hind footpad with endotoxin-free PBS [*1* and *3*] or 5 x 10^5 tsDCs [*2* and *4*]. Eight days later, the footpad tissue was removed and 6μm cryostat sections prepared. The sections were fixed and then stained with one of the following combinations of antibodies: anti-CD4 and anti-CCR5 [*1* - *2*] or anti-CD4 and anti-CXCR3 [*3* - *4*]. The sections were analysed by fluorescence microscopy and two images were collected from each sample using different filters. For each sample, the image collected using the blue filter is presented in [A], while the image collected using the red filter is presented in [B]. The position and orientation of the tissue in [A] and [B] are identical, allowing a direct comparison of staining patterns. Thus, a stained cell that is in the same position in both [A] and [B] can be considered to be double positive.

1. PBS: CD4 + CCR5

[A] *PBS: CD4 staining (blue channel)*

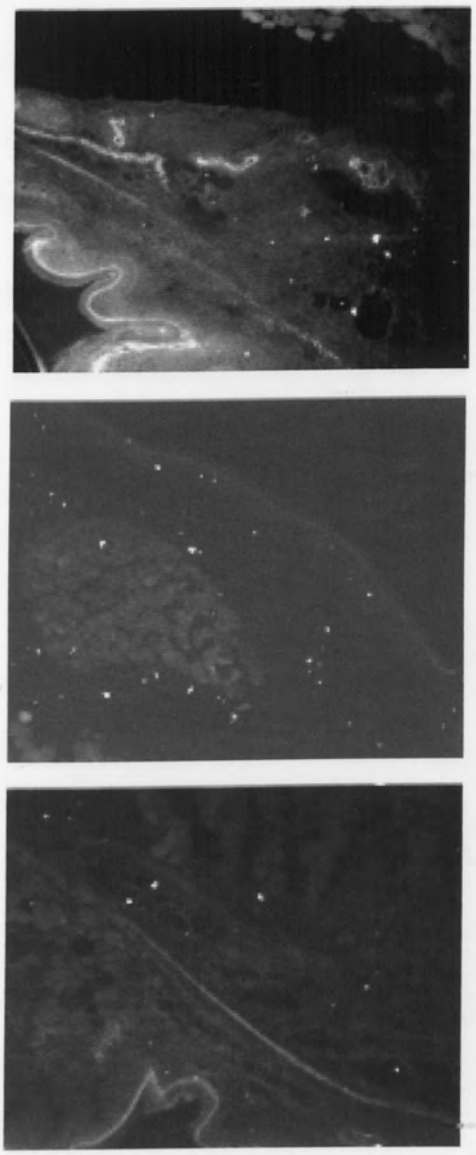


[B] *PBS: CCR5 staining (red channel)*

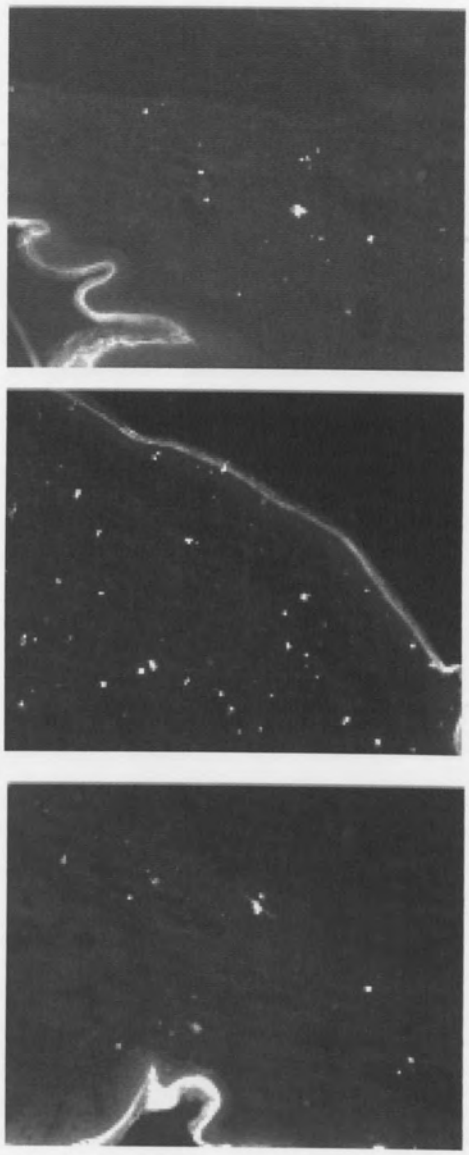


2. *tsDC: CD4 + CCR5*

[A] *tsDC: CD4 staining (blue channel)*

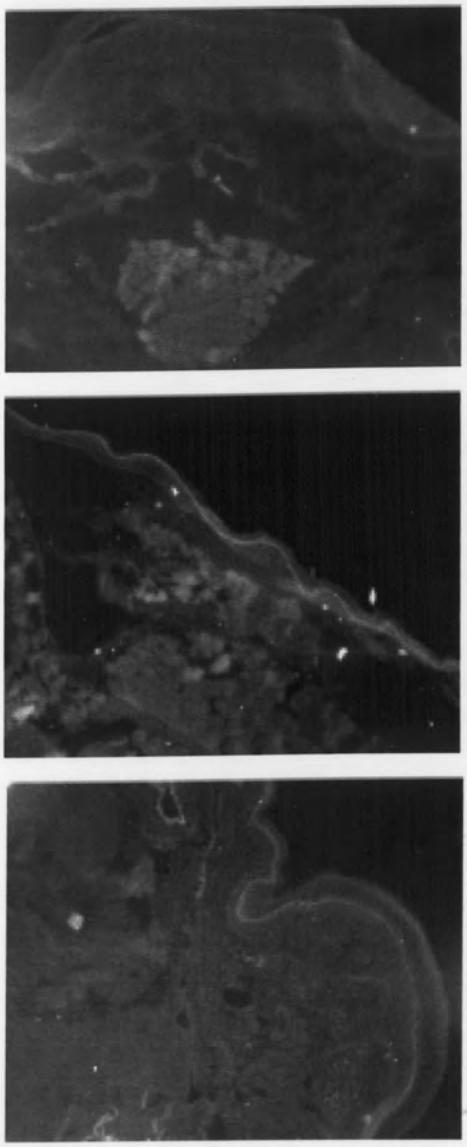


[B] *tsDC: CCR5 staining (red channel)*

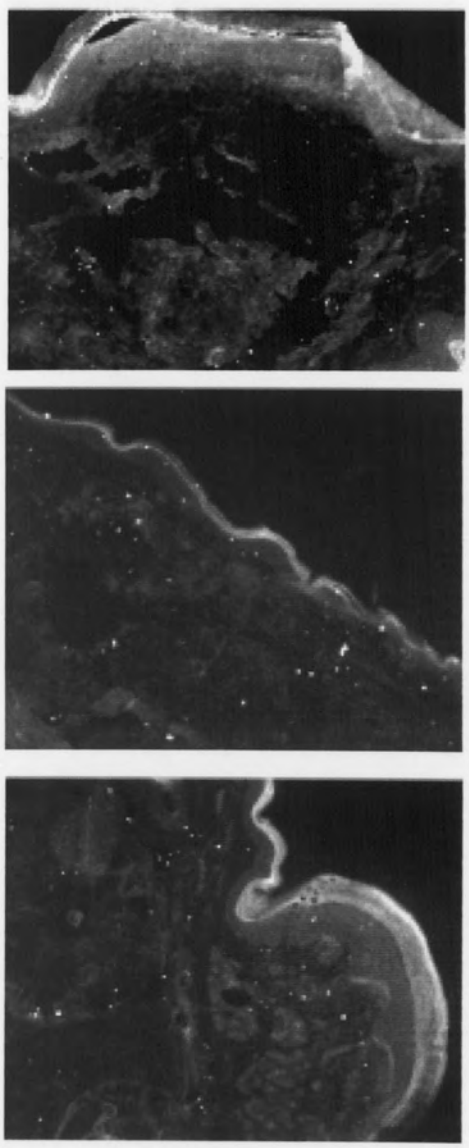


3. PBS: CD4 + CXCR3

[A] *PBS: CD4 staining (blue channel)*

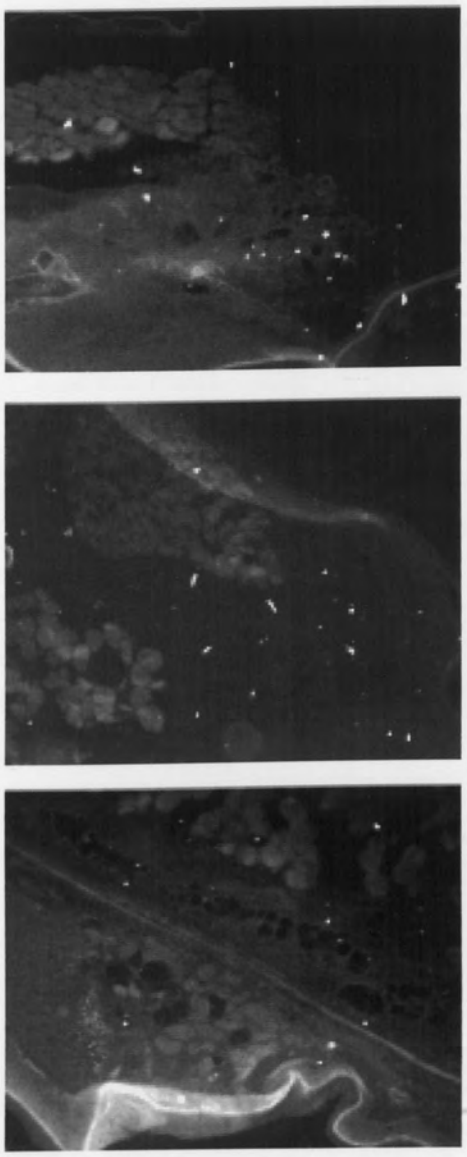


[B] *PBS: CXCR3 staining (red channel)*

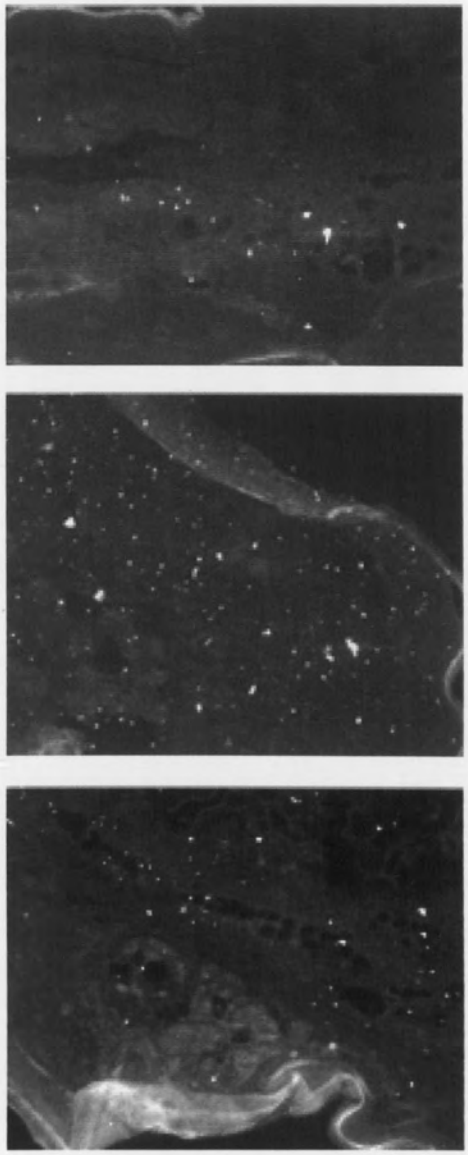


4. tsDC: CD4 + CXCR3

[A] *tsDC: CD4 staining (blue channel)*



[B] *tsDC: CXCR3 staining (red channel)*



Appendix II: publication reprints

Gale, L. M., & McColl S. R. (1999). Chemokines: extracellular messengers for all occasions? *BioEssays 21*(1), 17-28.

Ebert, L. M. & McColl S. R. (2001). Coregulation of CXC chemokine receptor and CD4 expression on T lymphocytes during allogeneic activation. *Journal of Immunology, 166*(8), 4870-4878.

Ebert, L. M. & McColl S. R. (2002). Up-regulation of CCR5 and CCR6 on distinct sub-populations of antigen-activated CD4+ T lymphocytes. *Journal of Immunology 168*(1), 65-72.