Investigation of the Therapeutic Potential of Transgenic CD40 Ligand Expression

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THESIS ABSTRACT

The CD40 ligand (CD40L) molecule is central to innate and adaptive immunity. CD40L expression is very tightly regulated whereas its CD40 receptor is constitutively expressed by many different cell types. CD40L is expressed transiently on helper T cells (Th) only after activation by specific immune recognition molecules carried by professional antigen presenting cells, in particular, dendritic cells (DC). CD40L subsequently binds to CD40 on DC to enable full Th activation. CD40 ligated DC produce interleukin-12 (IL-12) and contribute both to the development of IFNγ-secreting natural killer cells, a vital component of innate immunity, and of IFNγ-secreting type 1 Th (Th_1) cells. CD40 ligated DC also contribute to the development of IL-4- and IL-10-secreting Th_2 cells. CD40L on Th cells also binds CD40 on macrophages to enhance their cytotoxic functions. CD40L-expressing Th cells provide the 'help' pivotally required to activate other components of adaptive immunity responsible both for clearing invading pathogens and generating the memory cells required to prevent re-infection. Th-supplied CD40L binds (i) B cell CD40 to switch production of antibodies to more potent effector molecules that have higher avidity for antigen, and (ii) DC CD40 to prime then expand antigen-specific cytotoxic T lymphocytes (CTL). Activated NK cells and CTL are required both to eradicate malignant cells and cells infected with viruses or other intracellular pathogens.

Genetic CD40L deficiency causes the very rare HyperIgM Syndrome Type 1 (HIGM1), which is realistically modelled by genetically engineered CD40L-deficient mice. Neither CD40L-deficient patients nor mice make effective antibodies or mount cellular immune responses that would defend them against intracellular pathogens such as parasites. Consequently, the only potentially curative therapy is allogeneic stem cell transplantation or CD40L gene replacement. Here, we used a retroviral vector, which constitutively expressed CD40L, to genetically modify CD40L-deficient bone marrow cells, which were used to reconstitute partially the immunity of CD40L-deficient mice. The crucial importance of tight regulation of CD40L expression was revealed when these mice later developed lethal thymic T cell malignancy.

Growing tumours escape immune vigilance by genetic alterations that reduce their sensitivity to IFNγ. Using murine tumour models, we incorporated transgenic CD40L expression in therapeutic tumour vaccines to show that CD40L gene transfer augmented the immunogenicity of the host's tumour thus reducing its tumorigenicity. We translated this finding clinically to safety and immunogenicity testing of a transgenic CD40L- and IL-2-expressing leukaemia vaccine.

Finally, the common viral respiratory pathogen, respiratory syncytial virus (RSV) mainly infects young infants and the elderly to cause potentially lethal pneumonia. Both groups have reduced cellular and humoral immunity, which predisposes them to re-infection with RSV. Using a murine model, we showed first that simultaneous adenoviral expression of CD40L augmented primary RSV-specific Th1 responses that were associated with accelerated pulmonary viral clearance. Second, we showed that expression of CD40L in RSV-F and RSV-G subunit DNA vaccines elevated antibody and cellular immune responses to RSV challenge four and eight months after the initial immunisation.

These results demonstrate the potent ability of CD40L gene transfer to solve the absolute immune deficiency caused by genetic lesions of CD40L. However, physiological regulation of the transgene is required to prevent serious adverse consequences. In contrast, no adverse effects were observed after transgenic CD40L expression was used to overcome relative immune deficiencies imposed by malignancy and RSV infection.

The crucial significance of the ligand for CD40

The ligand for CD40 is a molecule critical in human biology and medicine (Chapters 1 and 2). CD40 ligand (CD40L) acts at the point of specific immune recognition of an antigen to activate the immune system and so produce immunity. If immune activation results then a chain of events is set in motion that result in elimination of antigen from the body. An integral feature of immunity is immunological memory, which is the ability to mount a similar or augmented immune response in the event that the antigen is again encountered. CD40L plays a vital role in coordinating, amplifying and recalling the immune response, which necessarily includes CD40L-mediated cooperation between the innate and adaptive arms of the immune system. If CD40L does not act and immune activation does not occur then the immune system sleeps in a state of immunological tolerance for the antigen. Although there are other causes of immunological tolerance, tolerance is the functional outcome as tumours grow in the body.

After antigen recognition, the decision within the immune system to activate, or not, may be considered a binary 'on' or off' response that may produce outcomes deleterious to the organism's survival if the decision is made out of its proper context. Hence, an evolutionary imperative will have dictated that appropriate mechanisms be built into the organism's immune system to control appropriately 'on' or 'off' decisions and so maximise the organism's ability to survive and reproduce. The term antigen is nominally an experimental construct. In the real world, antigens are part of infectious pathogens, the altered body components of cancer, and normal body components subject to autoimmune attack. Consequently, failure of the organism's immune system to make correct 'on' or 'off' decisions in response to antigen recognition may impair the organism's chances of survival.

How is this key 'on' or 'off' decision made and controlled?

The antigen recognition event initiates the decision making. A peptide derived from the antigen is bound by the major histocompatibility complex (MHC) molecule on the surface of an antigen presenting cell (APC), which is required to be a dendritic cell (DC) in order to prime rather than recall the T cell responses of immunity or tolerance. If the 'key fits the lock' then the peptide-MHC (pMHC) complex interacts with the T cell receptor (TCR) on the surface of a CD4-expressing T helper lymphocyte. The duration and strength of this cognate interaction determines the probability that T cell activation will occur. The first essential signal after T cell activation is the induction of CD40L expression. By ligating its CD40 receptor on the DC surface, CD40L then conditions the DC to engage further in crosstalk with the T cell so that a more durable immunological synapse between the two cells is assembled. Subsequently, the DC is licensed to prime the killer CD8-expressing T lymphocyte, which kills virally infected cells and tumour cells. Thus, CD40L expression promotes the development of type 1 immune responses, which are cytolytic. The CD4- and CD40L-expressing T lymphocyte can also provide 'help' to B lymphocytes in the form of ligation of its CD40 receptor so that it can produce more effective antibodies able to neutralise pathogens. Finally, antigen-experienced and CD40L-expressing T lymphocytes traffic to peripheral tissues at the site of antigen challenge where, *via* the ligation of CD40 on accessory immune cells, inflammation is promoted. In the permissive inflammatory microenvironment, CD40L is also expressed by other accessory immune cells and, *via* CD40 ligation, inflammation is reinforced and the ongoing development of immunity is enhanced.

To control this cascade of possibly injurious events, it will be noted that CD40L expression is for the most part only inducible in the most temporally and spatially restricted circumstances following lymphocyte activation whereas its CD40 receptor is constitutively expressed by both immune and non-immune cells such as endothelial cells, fibroblasts and epithelial cells

throughout the body. This compartmentation of the CD40L activating signal ensures that the responding cells receive it only in the context of immune recognition.

What are the implications if the CD40L-dependent activation signal is not made and it should be?

Absolute loss of the CD40L-dependent activation signal results in profound cellular and humoral immunodeficiency, which may be complicated by lethal opportunistic infections and, less comprehensibly, by malignancy of the hepatobiliary tree. The most straightforward although rare cause of absolute signal loss is inherited mutations in the genes for CD40L or its receptor. *Thus, a clear rationale exists for the replacement of the CD40L gene in the case of its genetic deficiency (Chapters 2 and 3).*

Relative loss of the CD40L-dependent activation signal occurs in the neonatal period and early infancy when CD40L expression is less responsive to T cell activation. A teleological explanation for the relative deficiency of CD40L expression would be that the immature immune system is less responsive because it has the important tasks of positively selecting lymphocytes that recognise foreign antigens and negatively selecting autoreactive lymphocytes. 'Hair-trigger' induction of CD40L expression may compromise the elimination of autoreactive lymphocytes and predispose to autoimmune disease. To continue the teleological argument, while thymic T cell education is underway, transfer of mature maternal antibodies *via* the placenta and breast milk compensates for the relative lack of CD40L-dependent antibody maturation and so reduces the infant's susceptibility to infection. However, the most common respiratory pathogen of early infancy is respiratory syncytial virus (RSV), which does not evoke effective immunity and predisposes to re-infection even in adults, which includes the mothers of susceptible infants. RSV infection tends toward the induction of type 2 rather than type 1 immune responses and so deviates the host immune response toward a less effective anti-viral response. Given that peak exposure to RSV occurs in early infancy, relative CD40L deficiency may also contribute to the development of ineffective RSV-specific immune responses. *In the presence of ineffective RSV-specific immunity and immune deviation, it is reasonable to test the hypothesis that CD40L gene augmentation will correct the immune deviation and provoke effective RSV-specific immunity (Chapter 4).*

A plethora of defects in immune function accompany malignancy. One of the most outstanding is the state of immunological tolerance or even frank immune suppression that progresses as the malignancy itself progresses. Here, it is important to note that abundant evidence now exists to indicate that immune recognition of tumour associated antigens does occur. Therefore, the host's immunological tolerance of the malignancy belies the threat that it poses to life.

A distinction will be made between the malignancies that express CD40 and those that do not. CD40-expressing malignancies more commonly originate from the lympho-haemopoietic system although a significant proportion arise from the epithelium, which is the most important source of malignancy overall. In many instances, CD40 ligation of CD40-expressing malignancies induces at least one of two effects. First, CD40 ligation induces upregulation of MHC, costimulatory and cell adhesion molecules, which together directly enhance the antigen processing and presentation functions of the tumour cell itself. Second, CD40 ligation induces tumour cell death, which indirectly promotes antigen processing and presentation by bystander APC. In this second event, it is both the greater efficiency in APC uptake of tumour antigens and CD40L-mediated enhancement of bystander APC function that promote anti-tumour immunity. On the other hand, in CD40-negative malignancies, CD40 ligation of bystander APC is most important, and anti-tumour immune effects may be reinforced if another modality of anti-tumour treatment such as cytotoxic chemotherapy induces tumour cell death. Of course, in any one malignancy, tumours may or may not express CD40 and any of the abovementioned mechanisms may operate to increase anti-tumour immunity. *Hence, it is reasonable to test the hypothesis that genetic augmentation*

with the gene for CD40L, which is a critical positive regulator of immunity, will induce effective anti-tumour immune responses (Chapter 5).

What are the implications if the CD40L-dependent activation signal is made and it should* not *be?

Several studies show that transgenic unregulated overexpression of CD40L induces autoimmunity. However, although retroviral gene transfer partially corrected CD40L deficiency in the mouse model, unregulated transgenic expression of CD40L by bone-marrow derived cells, which had repopulated the CD40L-deficient mice, produced thymic lymphoproliferations and lymphoblastic lymphoma after a latent period (Brown MP *et al*., 1998). In contrast, a later study of regulated transgenic CD40L expression also corrected the immunodeficiency but did not cause malignancy and, therefore, vindicated the physiological relevance of regulated CD40L expression *(Chapter 6)*.

Summary of the laboratory research program

The complementary DNA (cDNA) for murine CD40L (mCD40L) was cloned (Dilloo D *et al*., 1997) and the cDNA for human CD40L (hCD40L) was obtained. The mCD40L cDNA was subcloned into the retroviral shuttle plasmid, pG1a, which was subsequently used to make and clonally select a high-titre retroviral producer cell line (Grossmann ME *et al*., 1997). The supernatants obtained from this mCD40L retroviral producer clone were frozen in aliquots and stored for later use. The pG1a.mCD40L was provided to make a fibroblast cell line, which stably expressed mCD40L (Dilloo D *et al*., 1997). The mCD40L retroviral producer supernatants were provided to make a neuro2a cell line, which stably expressed mCD40L (Grossmann ME *et al*., 1997). The mCD40L retroviral producer supernatants were used to transduce CD40L-deficient murine bone marrow cells in readiness for transplantation (Brown MP *et al*., 1998). Bone marrow transplantation, measurement of gene transfer efficiencies, harvesting and phenotypic analysis of thymic tumours, and analysis of dinitrophenol-specific IgE responses were done as described (Brown MP *et al*., 1998). The mCD40L cDNA was subcloned into an adenoviral transfer plasmid, which was used to make a mCD40L-expressing adenoviral vector (Ad-mCD40L) after triple-plaque purification. An empty vector control (Ad-VC) was made in a similar way. Scaled-up stocks of these vectors were provided for co-infection experiments with RSV in BALB/c mice (Tripp RA *et al*., 2000) and a scaled-up stock of Ad-mCD40L was also supplied to perform transductions of MB49 murine bladder cancer cells (Loskog A *et al*., 2001). The hCD40L cDNA was subcloned into an adenoviral transfer plasmid and used to make a hCD40L-expressing adenoviral vector (Ad-hCD40L), which after triple-plaque purification was used subsequently to perform adenoviral transductions of human myeloma cell lines *in vitro* (Dotti G *et al*., 2001). The same vector was used to create the clinical leukaemia vaccines reported by Rousseau RF *et al*. (2006).

Significance of the major research findings

A recent survey conducted under the auspices of the U.S. National Cancer Institute ranked CD40L and anti-CD40 monoclonal antibodies fourth out of 20 molecules with high potential for use in treating cancer (http://web.ncifcrf.gov/research/brb/site/home.asp.). The publications presented herein were the first to show that:

(i) Genetic correction of CD40L deficiency was possible by gene replacement (Brown MP *et al*., 1998)

(ii) Deleterious effects resulted from constitutive CD40L transgene expression (Brown MP *et al*., 1998)

(iii) Transgenic CD40L gene expression conferred anti-tumour properties using *in vivo* models of CD40-positive and CD-negative malignancies (Dilloo D *et al*., 1997; Grossmann ME *et al*., 1997)

(iv) CD40 was not detected on primary human prostate cancer cells (Moghaddami M *et al*., 2001)

(v) Transgenic CD40L gene expression enhanced anti-RSV immunity in a model of RSV infection (Tripp RA *et al*., 2000; Harcourt JL *et al*., 2003).

Consequently, the nine publications included in this thesis have been cited 251 times in total.

STATEMENT OF ORIGINALITY

This work 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 and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis being made available in the University Library.

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Signed: Date: 23 May 2007

DEDICATION

This work is dedicated to the memories of my late father, Reginald Frederick Brown, my late aunt, Heather Gabrielle Brown, and my late father-in-law, Peter William Gage, who all at various stages of my life provided the inspiration or impetus for this work.

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STATEMENTS OF THE CONTRIBUTIONS OF JOINTLY AUTHORED PAPERS

Each publication included in this thesis was jointly authored. As permitted by Professor Richard Russell, Dean of Graduate Studies, University of Adelaide, appended to this thesis (Appendix A) is a statement for each publication, which gives written and signed permission by each author for the paper to be included in the thesis and which provides a detailed description of the contribution made by the PhD candidate as an author on each paper.

CHAPTER 1

INTRODUCTION

1.1 The emergence of molecular medicine

In 1953, Watson and Crick solved the crystal structure of deoxyribonucleic acid (DNA) revealing the genetic code that serves as the blueprint for life and thus ushering in the era of molecular medicine. Later, it was more completely understood that translation of the information contained in DNA to the information contained in proteins, which constitute the fundamental machinery of cellular action, required intermediary transcription of DNA to messenger ribonucleic acid (mRNA). Messenger RNA can be reverse-transcribed *in vitro* to create complementary DNA (cDNA), which is a much more stable and tractable entity than its parental RNA and which can be copied or cloned.

The process by which protein is generated from the code specified by the DNA sequence is termed 'expression'. A transgene is defined here as a segment of cloned cDNA that has been introduced into an organism or a vector construct in which it was previously unknown. Revolutionary advances in recombinant DNA technology, which is popularly known as genetic engineering or gene splicing, in the final quarter of the twentieth century are realising the full potential of the original discovery of the genetic code to treat and cure human diseases.

1.2 A primer on recombinant DNA technology – the concept of the cloning vector

Recombinant DNA technology enables DNA fragments from higher eukaryotes to be excised from their host genome with bacterial restriction enzymes and then to be recombined using DNA ligase enzymes with the genome of bacterial viral vectors called recombinant bacteriophages. When bacteria are infected with phage at a low ratio (<1) of phage particles to bacteria, which is described as the multiplicity of infection (MOI), each bacterium tends to harbour a unique recombinant bacteriophage, which results in the cloning of the cDNA upon replication of the phage containing the cDNA. Phages individually containing different cDNA fragments derived from a particular tissue or organism constitute a 'library' of cDNA clones in total. The phage library is used to infect bacteria in culture, which are subsequently diluted in liquid agar and overlaid on a bed of solid nutrient agar. As the liquid agar cools and solidifies, this method restricts the mobility of the infected bacteria among the 'lawn' of uninfected bacteria. The phage inside infected bacteria replicate, lyse their host bacterial cells and propagate a limited lytic infection among the surrounding bacteria to produce a clear plaque. These plaques are picked and used to re-infect bacteria in cycles of amplification, isolation and purification until a pure isolate of the phage (or clone) containing the eukaryotic cDNA is obtained. Two main methods are used to identify the phage containing the eukaryotic cDNA of interest. Plaques are first transferred onto nitrocellulose membranes. Then, (i) to identify the cDNA of interest directly, the membrane is hybridised with a specific DNA probe or (ii) to identify the expressed protein product of the cDNA of interest, the membrane is probed with specific antibody, which has been previously been generated against the biochemically purified protein.

1.3 Translating recombinant DNA technology to therapeutic recombinant proteins

Similarly, recombinant DNA techniques have enabled heterologous DNA to be expressed *via* bacterial, yeast and other eukaryotic expression vectors in various cell 'factories', which has facilitated the industrial scale production of recombinant proteins derived from many species including humans. The leading example of the therapeutic benefit deriving from recombinant

DNA technology is recombinant human insulin. Banting, Best and Collip discovered in the early 1920s that the insulin protein was the anti-diabetic principle inherent in pancreatic extracts. Insulin, which was biochemically purified from porcine or bovine pancreatic islets, kept diabetic patients alive but humoral immune responses against the foreign insulin limited the effectiveness of the treatment in many patients. This complication of heterologous immunisation was largely prevented by recombinant human insulin (Humulin), which was cloned in 1978 and marketed in 1982 as the first recombinant DNA drug by the world's first biotechnology company, Genentech, Inc (www.gene.com).

1.4 Translating recombinant DNA technology to therapeutic gene transfer technology

1.4.1 The promise and limitations of current gene therapy

Distinct from the use of recombinant DNA technology for the *ex vivo* production of therapeutically active recombinant proteins has been the use of recombinant DNA technology to clone and express cDNA in viral and non-viral vectors for therapeutic purposes. These purposes are presented, described and discussed herein and include the therapeutic use of gene transfer vectors as gene replacement in a genetic immunodeficiency model of human disease (Brown MP *et al.*, 1998) and as genetic augmentation in the 'acquired' immunodeficiency states of cancer (Grossmann ME *et al.*, 1997; Dilloo D *et al.*, 1997; Loskög A *et al.*, 2001; Dotti G *et al.*, 2001; Rousseau RF *et al.*, 2006) and respiratory syncytial virus infection (Tripp RA *et al.*, 2000; Harcourt JL *et al.*, 2003). In the broadest sense, these efforts are considered to be gene therapy, which unlike the use of recombinant therapeutic proteins, remains at the lagging edge of the vanguard of molecular medicine.

Gene therapy may be defined as the modification of the biological behaviour of cells by the transfer of new genetic material to achieve therapeutic objectives. Nonetheless, in spite of the arduous birthing process of gene therapy as a genuine therapeutic modality, considerable advantages will attend the development of a successful means of delivering therapeutic genes to cells *in vivo* so that they are expressed at the correct level when and where they are needed. Ideally, the target cells will need to be transduced specifically, rapidly and efficiently with the gene delivery vector. Sometimes, it may even be necessary to ensure that the site of integration of the gene and its associated control elements permit the gene to be expressed in the correct amounts at appropriate times. No available vector can meet these requirements, and current gene therapy protocols represent compromises determined by the limitations of current gene transfer technologies.

1.4.2 Gene delivery systems

1.4.2.1 Non-viral gene delivery systems

DNA may be transferred to cells directly as either naked DNA or DNA bound to lipids and/or targeting proteins, which are designed to increase the efficiency of transfer. *In vitro* gene transfer efficiencies may be high using these methods but transgene expression is short-lived because of the episomal disposition of the plasmid DNA. In contrast, *in vivo* gene transfer rates in clinical studies are often disappointingly low, for example, in cystic fibrosis [1]. However, the ability to deliver DNA by non-viral means is limited less by the type of cell or tissue than by the transfection efficiency and repeated administration should be possible without immunogenicity developing.

The 'gene gun' is a ballistic device that delivers plasmid DNA coated onto fine gold particles to the skin or other tissues by bombardment (reviewed in ref. 2). Its particular advantage lies in the efficient priming of immune responses, including anti-tumour responses [2], by skin-derived dendritic cells (DC) that have taken up only small quantities of DNA [3 4 5]. Priming efficiency is enhanced by unmethylated CpG motifs in plasmid DNA that have potent immune adjuvant activity [6 7] by binding to the innate immune activating receptor on DC called Toll-like receptor-9 (TLR9) [8].

Mixing cationic lipids with negatively charged plasmid DNA has produced clinically useful improvements in transfection efficiency. For example, direct intra-tumoral injection of cationic lipid complexed with plasmid DNA that encoded an allogeneic major histocompatibility complex (MHC) molecule (HLA-B7 and β2-microglobulin) was safe and elicited partial tumour responses in approximately 50% of patients with treatment-resistant head and neck cancer and metastatic melanoma [9] [10] [11] and is now proceeding to a phase III clinical trial in advanced melanoma. A preclinical study showed that liposomes containing the wild type p53 gene induced regression of endobronchial tumours in nude mice harbouring p53-null human lung tumour xenografts and suggested that DNA/liposome complexes may have wider clinical application, particularly when locoregional control of tumours is desirable [12]. Recent improvements in the design of DNA/liposome complexes have increased gene transfer efficiency, particularly *in vivo*. A bilamellar liposome that contains condensed DNA between the two lipid bilayers enhanced reporter gene expression after intravenous injection into mice. This novel structure offers the possibility of targeted gene delivery *in vivo* through the placement of ligands on the exterior surface [13].

In vivo electroporation or electrogene therapy (EGT) is a technique that delivers DNA to cells *via* transient pores in the cell membrane created by micro- or millisecond electrical pulses [14]. In some applications, gene transfer efficiency was superior to DNA delivery by gene gun, *in vivo* lipofection or direct injection [15] [16]. In one EGT study of potential clinical relevance, plasmid DNA was injected into the internal carotid arteries of rats with implanted cerebral tumours that had been electroporated between two electrodes. Intra-tumoral reporter gene expression was observed only if the electroporation step was included [17]. A related *in vitro* gene transfer technique uses laser beams to produce tiny transient holes in the cell membrane and has been termed optoporation [18].

1.4.2.2 Viral gene delivery systems

Murine retrovectors, adenoviral vectors, adeno-associated viral vectors, pox virus and herpes virus vectors have all been approved for clinical use. Murine retroviral and adenoviral vector systems have complementary properties: retroviral vectors (retrovectors) infect (transduce) dividing target cells with low efficiency, but integrate in the host cell genome, and so enable long-term expression of transgenes in the transduced cell and its progeny. Adenoviral vectors (adenovectors) transduce susceptible target cells at high efficiency, regardless of their proliferative status, but do not integrate and produce only transient expression of transgenes. For many applications in cancer therapy and also in genetic immunisation, long-term gene expression is not necessary and reliable expression of short duration is acceptable.

1.4.2.3 Murine retrovectors

Nearly all retroviral vectors used in clinical trials are based on the Moloney Murine Leukemia Virus (MoMuLV). The *gag*, *pol* and *env* genes that are responsible for retroviral replication are lacking in the retrovector so that the gene of interest can be cloned into the space they formerly occupied thus providing up to 10 kilobases (kb) of cloning capacity. The retrovector is then packaged in a cell line that contains the complementing genes necessary for replication and infectivity. Infectious virus from the packaging cell line or 'producer cell' now contains the gene of interest and can be used to transduce target cells (reviewed in ref. 20). It is important to note that the vector is the only genetic material transferred to the target cell and no virus-derived coding sequences are transferred thus avoiding cytotoxic T lymphocyte (CTL) reactions directed at viral antigens. Limited or no homology with the packaging elements in the producer cells ensure that the retrovector has little or no chance of recombining to make replication competent retrovirus (RCR). The strong retroviral promoter in the long terminal repeat (LTR) or other viral, housekeeping or tissue/tumour specific promoters may be used to control the gene of interest. Moreover, a retrovirally integrated transgene may express its protein product for at least 9 years *in vivo* although often only at moderate levels (Rill DR *et al.*, Proceedings of American Society of Hematology, 2000).

Until recently, retrovectors had an impressive clinical safety record without any adverse vector-related events being reported in over a thousand patients. Insertional mutagenesis had not been reported and contamination of clinical retrovector preparations with replication competent retrovirus (RCR) was believed to be the major concern [19]. Stringent quality control checks had prevented RCR from being released for clinical use. Recombination either within the host or with the parent virus was considered highly unlikely because there was minimal homology with endogenous retroviral elements. Now, however, new concerns have been raised about the safety of retrovectors after two cases of insertional mutagenesis at or near a proto-oncogene were reported in the most successful trial of gene therapy to date [20 21]. Nine boys with X-linked severe combined immunodeficiency (XSCID) due to common gamma (γ_c) chain deficiency had their immunodeficiency corrected by MoMuLV-mediated transfer of the γ_c gene to $CD34^+$ bone marrow cells [22]. Subsequently, two children developed pauci-clonal acute T cell leukaemias, which were associated with vector insertions at or near the *LMO2* proto-oncogene [21], aberrant expression of which had earlier found in childhood leukemia [23]. The youngest two of the children developed leukaemia and it is believed that rare insertional events occurred in retroviral enhancer elements in open chromatin of the *LMO2* promoter and conferred a proliferative advantage on upon γ_c-expressing cells early during T cell ontogeny in these γ_c-deficient children [20] (see Section 6.1.1).

1.4.2.4 Adenovectors

Adenovectors generally have a much higher titre than retrovectors, in the range of 10^{11} or 10^{13} viral particles (VP) per mL. The higher titre and the biology of adenovirus itself make transduction more efficient than for retrovectors (reviewed in ref. 26). Adenovectors infect both replicating and non-replicating cells because the double-stranded DNA genome is transported into the nucleus. However, the virus remains as a non-replicating episome so that it is lost from the cell after cell division and, consequently, expression from the transgene is transient in dividing cell populations. The major drawback of adenovectors is the acute inflammatory response that is evoked by viral proteins and the subsequent immune response that limits the duration of expression and prevents repeated administration. Clinical studies of adenovectors have employed the non-oncogenic adenoviral serotype 5 (Ad5) in which genes in the E1 and E3 regions of the vector have been deleted to reduce the chances of replication competent adenovirus contaminating the clinical product and to enable MHC class I molecules to be expressed in transduced cells. The E3/19K gene product, in particular, interferes with trafficking of MHC class I molecules in adenovirally infected cells [24].

The safety record of adenovectors has been blemished by a death on a clinical study. An 18 year-old patient with ornithine transcarbamylase (OTC) deficiency was given a high dose of an adenovector (10^{11} VP/kg), which encoded the wild type *OTC* gene, *via* the hepatic artery. Despite its regional administration, a significant amount of adenovector was detected in extrahepatic tissues. The patient developed hepatocellular injury and coagulopathy and subsequently died from multi-organ failure [25]. Endothelial injury, hepatocellular damage and coagulopathy were also observed in nonhuman primates that received large intravenous doses (10^{11} plaque forming units) of adenovector [26]. Together, these observations dramatically illustrate the narrow therapeutic index of adenovectors. Nonetheless, adenovectors have characteristics that favour their application as gene transfer vectors for the treatment of cancer, particularly high transduction efficiency and reliable and transient expression of therapeutic genes. It may be possible to retain these beneficial characteristics while minimising toxicities by more specific targeting of vectors that would allow the delivery of vector at a lower systemic dose [27].

1.4.3 Vector modifications to improve vector targeting

The non-viral and viral (retro- and adeno-) vectors reported herein were ‘standard issue’ gene transfer vectors. However, depending on the particular application, vector modifications may be made to alter (i) the tropism of the vector and hence the scope and efficiency of vector targeting and/or (ii) the transgene control elements to guide temporal and/or spatial regulation

of transgene expression. For example, cancer is generally a disseminated disease and it may be necessary for a gene therapeutic to be administered systemically. To obtain optimal specificity, therefore, the vector will need to be targeted to tumour cells and the transgene will need to affect only the malignant process. Hence, these requirements may be met by the use of genetic engineering either to create tissue-specific promoters or modify or replace viral envelope proteins with ligands for cell-specific receptors.

1.4.3.1 Pseudotyped murine retrovectors and other retroviral backbone modifications

Pseudotyping a retrovector, in which the envelope protein in the retroviral coat is genetically modified to alter the vector's tropism, may target gene delivery. For example, pseudotyping of MoMuLV-based vectors with truncated HIV-1 or HIV-2 envelope proteins can narrow the host range of the vector to cells that express CD4/CXCR4, such as T cells, [28] [29] [30]. Conversely, pseudotyping with the vesicular stomatitis virus G protein (VSV-G) can broaden the tropism of MoMuLV-based vectors. The VSV-G envelope has the additional advantage of being mechanically stable so that virus can be concentrated to high titre by ultracentrifugation [31]. In an experimental demonstration of the utility of this approach, rat gliomas were injected under stereotactic guidance with only 9μL of a high titre preparation (>10^{10} infectious colony forming units per mL) of a VSV-G pseudotyped Moloney-based retrovector that encoded HSV-TK. After administration of the pro-drug GCV, two-thirds of the rats had prolonged tumour-free survival [32]. However, attempts to improve specificity by targeting retrovectors to cell surface receptors have met with limited success because either the affinity of interaction was too low or the interaction was sterically unfavourable for the processes of viral binding and fusion (reviewed in ref. 36).

The safety, duration and tissue-specificity of retrovector expression may be improved by vector backbone modifications, particularly in the LTR region, which contains the strong retroviral promoter that is transcriptionally active in many cell types. Self-inactivating (SIN) retrovectors have 3'LTR deletions that result in inactivation of the 5'LTR promoter once the virus integrates in the genome of the target cell [33]. Thus, the strong LTR is no longer capable of generating transcriptional interference, which can suppress the activity of tissue specific promoters contained in the vector. For example, specific expression in mature muscle cells was achieved *in vitro* and *in vivo* with a SIN vector containing a minimal creatine kinase promoter and enhancer [34]. The use of a myeloproliferative sarcoma virus (MPSV) LTR together with removal of negative regulatory elements produced enhanced and sustained expression in murine haemopoietic cells [35]. Nonetheless, viral regulatory elements may inhibit tissue specific promoters and the position of viral integration may also affect transciptional control. A not uncommon observation concerning transgene expression from integrated retrovectors is hypermethylation of the retroviral promoter, which produces silencing of the transgene [36].

1.4.3.2 Adenovector backbone modifications

Adenoviral vectors normally enter a cell in a two-step process: (1) attachment of the viral surface fibre protein to the Coxsackie-B adenovirus receptor (CAR) and (2) internalisation *via* an RGD motif on the viral penton base protein, which binds cell surface integrins (usually $\alpha_V\beta_3$ and $\alpha_V\beta_5$) [37] [38]. The tissue tropism of Ad5 adenovectors is restricted by CAR expression and these vectors do not efficiently transduce endothelium, smooth muscle cells, haemopoietic cells or brain tissue. Tumour tissue expression of these receptors is variable. For example, of surgical specimens of bladder cancer, all (27/27) were positive for CAR with the strongest staining on superficial tumours. Most bladder tumours (14/24) expressed the $\alpha_V\beta_5$ integrin but few tumours (3/20) expressed the $\alpha_V\beta_3$ integrin [39]. However, genetic modification of the fibre or penton base proteins can redirect binding of adenoviral particles to other cell surface receptors that have either a broader or narrower range of expression than the natural receptor [40] [41] [42] [43]. It is possible to generate chimeric adenovectors by incorporating the fibre head from one subtype into the backbone of another. For example, genetic substitution of the Ad35 fibre for the Ad5 fibre (Ad5/F35) altered viral tropism so that non-cycling human

$CD34^+$ sub-populations with potential stem cell capacity were efficiently transduced by a CAR- and α_V integrin-independent pathway [44]. Moreover, Ad5/F35 vectors efficiently transduced the Hoechst-negative 'side population' of bone marrow cells, which are believed to the very earliest multipotential haemopoietic progenitor cells, with low toxicity [45].

Modifying the fibre protein to incorporate a polylysine motif increased binding to heparan sulphate receptors, which are expressed by cells not usually efficiently transduced by adenovectors such as macrophages, endothelial, smooth muscle or T cells. Modifying the fibre protein to incorporate an RGD motif increased binding to α_V integrins that can be aberrantly expressed in malignant glioma and metastatic melanoma [41]. T cells are usually resistant to adenoviral transduction because they lack the receptors for attachment and internalisation. However, T cell transduction efficiency can be markedly enhanced by combining an adenovector that contains the FLAG peptide epitope tag in the penton base, with an adaptor molecule, which is a bispecific antibody that recognises both CD3 and FLAG [43]. Peptide ligands that can redirect the binding of viral and non-viral gene transfer vectors to cell surface receptors can be generated by phage display library technology. For example, random peptide-presenting phage libraries were used to select peptide ligands capable of binding and entering specific cell types without knowing which cell surface receptors were involved [46]. Phage display libraries were also used to target peptide ligands to cell surface integrins that were expressed preferentially in tumour vasculature or in the vascular beds of certain organs (reviewed in ref. 50).

Novel bilamellar DOTAP:cholesterol liposomes, which are potentially useful for non-viral gene transfer, may also be used to overcome limitations imposed by adenovectors, which include lack of attachment receptors such as CAR and pre-existing or induced anti-vector humoral immune responses. Unlike other liposomal systems, adenovectors, which were enscapulated by these liposomes, permitted CAR-independent transduction of otherwise resistant cells and expressed transgenes *in vitro* and *in vivo* despite the presence of neutralising anti-adenoviral antibodies [47].

1.4.4 Other clinically applicable viral vectors

Although the greatest clinical experience has been acquired with the use of retroviruses and adenoviruses as gene transfer vectors, other viral gene transfer vectors are clinically applicable and some have demonstrated features that will improve the clinical utility of gene transfer vectors.

1.4.4.1 Lentiviral vectors

MoMuLV based retrovectors integrate only in dividing cells, which in experimental models allows selective *in vivo* transduction of proliferating brain tumour cells situated among normal post-mitotic neurons. This gene therapy involved the injection into the tumour of a retroviral packaging fibroblastic cell line, which produced replication-defective retroviral vectors. These retrovectors expressed the herpes simplex virus-1 thymidine kinase (*HSV-tk*) gene product, which converts the pro-drug, ganciclovir (GCV), to cytotoxic metabolites [48]. However, a phase III trial of this technology used adjunctively to the standard treatment of surgery and radiation in glioblastoma multiforme patients showed that adding the adjuvant treatment was no better than standard treatment alone probably because transduction efficiencies were very low [49]. Furthermore, the growth fraction of many solid tumours is less than 10% and indicates that other kinds of retroviral vectors may be useful for oncology applications.

Lentiviral vectors, which are based mainly on the human immunodeficiency virus (HIV) backbone, transduce a broad range of non-dividing cells because nuclear entry of the viral integrase does not require mitosis. Hence, lentiviral vectors may be particularly useful in cancer gene therapy. Significant concerns about the safe clinical use of HIV-based lentiviral vectors exist because replication competent and pathogenic viruses can be created by recombination in the producer cell or the target cell. However, recent progress in lentiviral vector development has now increased the likelihood of clinical safety. In particular, third-

generation vectors based on extensive deletion of the native HIV genome and the splitting of helper functions among a number of packaging cell lines has produced only short regions (<50 base pair) of sequence overlap that could facilitate recombination *in vitro* or *in vivo*.

The safety and utility of lentiviral vectors has been further enhanced by self-inactivating modifications of the LTR, which reduce the risks both of the emergence of RCR and transcriptional interference. These lentiviral vectors have been to shown to produce efficient transduction of human haemopoietic progenitor cells and primary malignant cells of the haemopoietic lineage [50]. A VSV-G-pseudotyped HIV-based lentiviral vector was created (VRX496), which used the HIV LTR to drive expression of an anti-sense version of the HIV *env* gene that is highly transactivated by the HIV tat protein upon HIV infection of the T cell. Consequently, $CD4^+$ T cells were rendered resistant to destruction by HIV infection [51]. Encouraging results from the first clinical trial of lentiviral gene therapy were reported recently when five HIV-infected patients were given a single infusion of autologous $CD4^+$ T lymphocytes genetically modified with VRX496. Viral loads were stabilised and in one patient a dramatic fall in viral load was recorded. $CD4^+$ T cell counts were at least maintained or increased [52].

Transduction of human DC has proven difficult using most currently available gene transfer methods. A replication-defective VSV-G pseudotyped HIV-1-based vector transduced 70-90% of immature monocyte-derived dendritic cells leading to stable integration of the HIV genome in contrast to transduction rates of 2-8% for VSV-G pseudotyped MoMuLV vectors [53]. Transfer of GM-CSF and IL-4 genes by lentiviral vector to human monocytes produced stable and functional DC-type antigen presenting cells [54].

1.4.4.2 Herpes virus vectors

Herpes simplex virus vectors have recently been shown to transduce human tumour cells and bone marrow progenitor cells efficiently [55]. HSV vectors integrate in the host genome at low frequency, so their primary value may be in the efficient delivery of transient gene expression to dividing and non-dividing cells. HSV vectors are based on DNA viruses and have large cloning capacity (>100kb), which enables the insertion of multiple transgenes or long segments of genomic DNA. Although HSV has broad tissue tropism, it is a neurotropic virus that exists in a stable episomal state in neurons and produces life-long latency in neurons of sensory ganglia. This vector property can be exploited to generate prolonged expression of transgenes for the therapy of primary brain tumours [56]. Currently, the correction of genetic deficiencies relies upon expression of a cDNA transgene driven by a heterologous promoter. Ideally, gene replacement strategies should provide all of the native promoter and regulatory elements in genomic DNA to recreate physiological expression of the transgene. Recent evidence indicates that HSV-1 amplicon vectors have sufficient cloning capacity to deliver genomic inserts >100kb in size to cells *in vitro* to rescue phenotypes in cellular models of genetic disease [57].

1.4.4.3 Adeno-associated virus (AAV) vectors

Although the AAV vector remains a clinically immature gene transfer technology, the solution of certain technical problems has made it clinically applicable (reviewed in ref. 62). The major advantage of the AAV vector is that it mediates stable integration of heterologous genes into the genome of quiescent somatic cells such as muscle, brain and liver with little or no inflammatory response. However, because transduction efficiencies are very low and expression of the transgene is delayed, 'niche-specific' applications may be preferred for cancer gene therapy, for example, in treatment of gliomas [58] [59] [60] and the production of secreted proteins such as anti-angiogenic proteins [61].

Hybrid AAV vectors have been developed, which combine favourable properties from different vectors. For example, homologous inverted repeat sequences contained in two different adenovectors mediated precise genomic rearrangements that removed all viral genes including those that encode cytotoxic viral proteins from adenovector genomes to create 'gutless' adenovectors that were then packaged into infectious adenoviral capsids. The

inverted repeats had been inserted into the E1 region of E1-deleted vectors and the genomic rearrangements depended upon viral DNA replication [62]. This method of generating gutless adenovectors was adapted to create hybrid adenoviral-AAV vectors that contained AAV inverted terminal repeat sequences that flanked a reporter gene. Because gutless vectors lack cytotoxic adenoviral proteins, sensitive target cells may be transduced at very high multiplicities of infection. The hybrid vectors integrated into the genomic DNA of cultured cells with a frequency comparable to that of recombinant AAV vectors [63]. The successful *in vitro* transduction of human haemopoietic stem cells by Ad5/F35 chimeric adenovectors [45] facilitated the development of gutless Ad.AAV hybrid vectors that expressed the human γ-globin gene at low levels after stable integration in a haemopoietic cell line [64]. Such hybrid vectors may potentially be useful for the stable integration of chemoprotective genes in human haemopoietic stem cells. Clinical studies of AAV-based gene therapy have been conducted in neurological conditions such as Parkinson's disease, genetic disorders such as haemophilia and heart failure rather than cancer-related applications [65] [66].

1.4.4.4 Poxvirus vectors

Poxviruses as gene transfer vectors have mainly been applied to cancer immunotherapy. They are large DNA viruses, which have a large cloning capacity and so can be engineered to express genes for an antigen together with several immunomodulatory genes in the cytoplasm of mammalian cells. Consequently, attention has turned to avipox virus vectors such as the ALVAC strain of canarypox and fowlpox, which undergo abortive replication in mammalian cells [67]. Recombinant fowlpox virus (FPV) vectors have been shown to transfer genes efficiently to human DC [68] and to induce CTL efficiently [69] [70]. Autologous DC were genetically modified *ex vivo* with FPV vectors, which expressed carcinoembryonic antigen (CEA) together with CD80, intercellular adhesion molecule-1 (ICAM-1) and leukocyte function-associated antigen-3 (LFA-3) (TRICOM™). The gene-modified DC were used to immunise colorectal cancer patients who subsequently demonstrated CEA-specific immune responses among both $CD4^+$ and $CD8^+$ T cells [71]. A phase III clinical trial is currently underway of poxvirus vaccines expressing CEA, mucin-1 and TRICOM™ for pancreatic cancer patients. Patients are being primed with the vaccinia form of this vector and then being serially boosted with the FPV version of the vector in conjunction with subcutaneous injections with GM-CSF [72].

1.5 The CD40 receptor and its ligand

CD40 ligand and its CD40 receptor are members of the tumour necrosis receptor (TNF) and TNF receptor (TNFR) superfamilies, respectively. Since a homologue of CD40 has been cloned from the jawed vertebrate, Japanese flounder [73], the phylogeny of CD40 appears to have accompanied the evolution of adaptive and anticipatory immunity, which is first recognisable as a lymphocyte-based recombinatorial rearrangement of unique antigen receptors in jawed vertebrates [74]. TNF and TNFR superfamily members are involved in a range of critical biological processes that include lymphoid and bone organogenesis, inflammation, host defence, and other aspects of homeostasis. Abnormalities of TNF/TNFR superfamily member function contribute to a wide range of pathological processes that include acute and chronic inflammatory diseases, autoimmune diseases, and cancer [75]. TNF superfamily protein monomers form homotrimers that interact non-covalently around a three-fold axis of symmetry [76]. After ligation by the homo- or hetero-trimeric ligands, TNFR trimerise and signal *via* the recruitment of intracellular adaptor proteins [77]. TNF and TNFR superfamily members also commonly shed soluble forms of membrane proteins by extracellular cleavage [78].

CD40L is unlike the TNF superfamily members, TNF, TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL; CD95L), which are death receptor ligands. Their corresponding receptors recruit death domain (DD) containing adaptors such as TNFR associated DD (TRADD) and Fas (CD95)-associated death domain (FADD) to transduce apoptotic signals [77].

The critical costimulatory role that CD40L/CD40 interactions play in the development of immune responses will be described in greater detail below. However, several other members of the TNFR family, OX40 (CD134), 4-1BB (CD137), and CD27, are equally important to the effective generation of many types of T cell response. These molecules are induced some time after T cell activation and control cell division to prevent early termination of the T cell response [79]. Some TNF superfamily members have a more specialised role in immune homeostasis. For example, OX40L participates in homing of follicular helper T cells [80] whereas B cell activating factor (BAFF) is a critical survival and maturation factor for peripheral B cells [81]. Other TNF superfamily members play a redundant role in host defence. For example, TRANCE (TNF-related activation-induced cytokine) also known as RANKL (receptor activator of NFκB ligand) or OPGL (osteoprotegerin ligand) is most closely related to CD40L [82]. TRANCE is also expressed on activated T cells and binds RANK on DC to induce similar effects of DC survival and differentiation, and Th1 development. TRANCE-RANK signalling was able to substitute for CD40L/CD40 interactions in the development of T cell responses to infection with lymphocytic choriomeningitis virus (LCMV) [83].

1.5.1 The key concepts of immunological specificity and memory

The key concepts that define the operation of the vertebrate immune system are specificity and immunological memory. Louis Pasteur's germ theory advanced that specific diseases had specific causes, and Paul Erlich's concept of the 'magic bullet' indicated that specific protection against infectious diseases could be conferred by a specific chemical reaction between antibody and its target antigen. Similarly, the later discovery of MHC restriction by Peter Doherty and Rolf Zinkernagel established the basis for understanding how T lymphocytes interact specifically with antigen. Experiments performed by Max Delbruck's phage group showed that genetic mutations were not induced specifically but were generated randomly, which emphasised the role of selection in driving phenotypic change. Immunologically, the most developed expression of this thought was Talmage's and Macfarlane Burnet's Clonal Selection Theories, which explained that the specificity of the antibody was the same as that of the antigen receptor of its originating cellular clone. After selection from a huge repertoire of 10^{10}-10^{11} clones, the clone bearing the correct antigen receptor underwent clonal expansion.

Primary adaptive immune responses require at least seven days [84] and may not contribute to the clearance of the pathogen. Therefore, the value added by CD40L/CD40 interactions during adaptive immune responses may derive from the heightened efficiency of the secondary anamnestic response occurring upon re-challenge of the organism with the pathogen. At that time, components of the adaptive immune system will complement innate immune elements in the line of first defence. Long-lived antibody-producing plasma cells in bone marrow together with circulating and mucosal antibodies provide the effector functions required for prompt and efficient pathogen clearance. Moreover, a pool of memory CTL and class-switched memory B cells ensure that new CTL, plasma cells and antibodies are generated more rapidly than in the primary immune response. In particular, CD40L/CD40 interactions are required for strong anti-viral humoral immune responses, and for the establishment and/or maintenance of $CD8^+$ memory [85].

1.5.2 Characteristics of the CD40 receptor and its ligand

The CD40/CD154 receptor-ligand pair has been described as a dyad [86], which suggests a mutually exclusive interaction. However, it is now clear that CD40 has several other ligands whereas CD40L is thus far known only to bind the CD40 receptor. However, CD40 also binds C4b binding protein (C4BP) and the 70 kilo-Dalton (kDa) microbial heat shock protein (HSP70). Microbial HSP70 binds CD40 on human DC, induces DC maturation and elicits DC production of chemokines, interleukin (IL)-12, TNF, and nitric oxide (NO). As a substitute for CD40L, HSP70/CD40 interactions may convert tolerance to immunity, and help to protect against *Mycobacterium tuberculosis* [87] or bacterial sepsis [88]. Human C4BP activates CD40 on human B cells at a different site from CD40L to induce proliferation, differentiation, and IL-

4-dependent IgE isotype switching [89]. Since CD40 has a much broader tissue distribution than CD40L, it may be less surprising that other ligands for the CD40 receptor exist (Table I). Human CD40L exhibits 78% amino acid identity with murine CD40L [90] whereas human and murine CD40 share 63% overall homology [86].

Characteristic	CD40	CD40L (CD154)	References
Gene symbol	*tnfrsf5*	*tnfsf5*	91
Chromosomal location	20q12-Xq13.2	Xq26.3-q27.1	86
Genomic organisation	9 exons	5 exons	86
Protein superfamily	TNFR	TNF	92
Transmembrane glycoprotein type	I	II	92
Mature protein size (kDa)	50	39	92
Expression	constitutive/inducible	inducible	86
Cell types expressing	haemopoietic progenitor cells, T and B cells, monocyte/macrophages, dendritic cells, endothelial cells, fibroblasts, neurones, epithelial and many of their counterpart carcinoma cells	$CD4^+$ T cells, some $CD8^+$ T cells, NK cells, NKT cells, dendritic cells, B cells, platelets, basophils, mast cells, and eosinophils	92, 86

Table I: Characteristics of CD40 ligand and CD40

The essential characteristics of the human CD40 receptor and its ligand are tabulated. tnfsf, tumour necrosis factor superfamily; tnfrsf, tumour necrosis factor receptor superfamily; kilo-Dalton, kDa; NK, natural killer.

1.5.3 Regulation of the CD40L/CD40 interaction

1.5.3.1 Overview of CD40 signalling

CD40 activation has pleiotropic effects because of the many different cell types that express CD40 together the engagement of different cell type-dependent intracellular signal transduction pathways, which regulate cell growth, differentiation, survival and proliferation.

Common to other TNFR superfamily members, the CD40 receptor exists in trimeric form and ligand-dependent trimer formation is governed by the extracellular domain of CD40. Ligation of CD40 induces clustering of CD40 at the plasma membrane in lipid-rich rafts called 'signalosomes' [91]. Since the intracellular domain of CD40 does not have intrinsic kinase activity, oligomerisation of the cytoplasmic tail of CD40 results in recruitment of various tumour necrosis factor receptor-associated factor (TRAF) adaptor molecules, which bind to particular motifs located on the intracytoplasmic tail of CD40. The TRAF molecules trimerise and thereby activate a range of signalling pathways such as those involving the c-Jun N-terminal kinase (JNK), the extracellular regulated kinase (ERK) and p38 mitogen-activated protein kinases (MAPK), phosphatidylinositol 3-kinase (PI3K), and the transcription factors,

signal transducer and activator of transcription (STAT) and NFκB. These pathways regulate transcription and translation and, consequently, CD40 signalling mediates a variety of cellular activities, which include survival, proliferation and differentiation [92]. TRAF 2, 3, 5 and 6 molecules associate with CD40. TRAF2 is required for CD40-mediated activation of NFκB. On the other hand, recruitment of TRAF6 was needed for NFκB and JNK activation in an epithelial cell line but not in B cell lines. Although TRAF molecules are not required for early CD40-activated B cell phenomena such as proliferation and immunoglobulin production, TRAF6 is required for the later events of germinal centre formation, generation of long-lived plasma cells and affinity maturation of antibodies [93].

1.5.3.2 CD40L expression is induced by activation of $CD4^+$ T cells

CD40L expression by activated $CD4^+$ T cells is tightly controlled. Deregulated CD40L expression has been observed in systemic autoimmune diseases such as systemic lupus erythematosus (SLE) [94 95], and may contribute to the immunopathogenesis of this disorder because sustained expression of CD40L *in vivo* triggered autoantibody production [96]. Interestingly, quantitative effects of CD40L expression have been observed. In transgenic mice in which the murine CD40L gene was expressed under the control of the IL-2-promoter, the fraction of activated $CD40L^+$ T cells increased by 1.1- to 2-fold and corresponded to an increase in the abundance of CD40L mRNA. Accordingly, the transgenic mice produced a 4- to 5-fold increase in antibody titres in response to a T-dependent antigen and a similar increase in B cell proliferation and monocyte matrix metalloproteinase production. Hence, CD40L is a rate-limiting factor in the induction of high-affinity and class-switched antibody responses [97].

Despite the evidence that CD40L expression is inducible and that its CD40 receptor is expressed constitutively, both ligand and receptor appear to be expressed on a bewildering number of different cell types [86]. Hence, at first, it seems difficult to understand how expression of a tightly regulated molecule such as CD40L can be controlled sufficiently to prevent adverse consequences in most tissues. However, factors such as cytokines may operate to 'yoke' expression of CD40 with that of CD40L so that 'CD40 priming' enhances the subsequent effects of CD40 ligation [98]. As illustrated for the case of T cell activation (Figure 6), if the CD40 receptor is ubiquitously and constitutively expressed then the control of CD40 activation will be more stringent if the expression of biologically potent CD40L is contingent upon the appearance of a first signal (signal 1). Signal 1, which is the cognate interaction between the antigen receptor and antigen, induces CD40L expression and thus ensures that CD40L expression is coupled to signal 1. Then as CD40 ligation induces CD40 expression, expression of the ligand becomes yoked to expression of the receptor.

Physiologically, CD40L expression is induced by T cell activation, which is initiated by the interaction between the peptide-MHC (pMHC) molecule on the antigen presenting cell (APC) and the T cell receptor (TCR). CD40L subsequently engages the CD40 receptor on the APC surface and induces up-regulated expression of the B7 family members, CD80 or CD86, which in turn engage CD28 on the T cell surface. CD28 expression together with the induced expression of other costimulatory and cell adhesion molecules contributes to the formation of the immunological synapse. To simulate these conditions *in vitro*, human T cells have been activated with anti-CD3 and anti-CD28 monoclonal antibodies (mAb) or pharmacologically with phorbol 12-myristate 13-acetate (PMA), which activates PKC, and the calcium ionophore, ionomycin. Consequently, CD40L mRNA is induced rapidly without requiring *de novo* protein synthesis [99]. These *in vitro* studies indicated that, unlike induction of cytokine secretion by $CD4^+$ T cells, optimal CD40L expression required both TCR engagement and combined treatment with PMA and ionomycin [100].

While there has been evidence for reverse signalling by CD40L, the mechanism is unknown and its physiological significance is uncertain [101]. Although the 22 amino acid-long cytoplasmic tail of CD40L lacks enzymatic activity, it activates JNK, p38 MAPK, neutral sphingomyelinase, and protein kinase C (PKC) in human and murine T cells [101 102]. Cross-

linking of CD40L results in the co-endocytosis of CD40L with a splice variant of CD28 (CD28i), which retains an intact transmembrane region and cytoplasmic tail, but which lacks the B7 interaction domain. After CD40L cross-linking, CD28i becomes phosphorylated on tyrosine. In addition to binding CD40L, CD28i also associates with wild type CD28 and cross-linking of CD28 transactivates CD28i. CD40L activates NFκB, which is enhanced by CD28i. Co-clustering of CD28i with CD28 and CD40L enhances activation of the p21-activated kinase 2 (PAK2)-JNK pathway. Hence, reverse signalling *via* CD40L may enhance the formation of a focal adhesion-like structure within the T cell at the site of the immunological synapse with APC, which is essential for T cell costimulation. Together with CD28i, CD40L may help to regulate the T cell activation threshold [102]. However, studies of T cells from female type 1 hyperIgM syndrome (HIGM1) carriers support the hypothesis that the function of CD40L in T cell priming is to induce other costimulatory molecules rather than act as an intrinsic T cell costimulator. Because of random X-inactivation, T cells from HIGM1 carriers express CD40L or not after T cell activation. After T cell priming, little difference was observed among the CD40L-deficient and CD40L-expressing T cells of HIGM1 carriers in their expression of CD45RO or production of IFNγ and TNF. Therefore, these results indicate that reverse signalling *via* CD40L has a subsidiary rather than critical physiological function [103].

1.5.3.3 Molecular regulation of CD40L expression by activated CD4$^+$ T cells

Peak levels of CD40L mRNA were expressed eight hours after stimulation in a T cell-specific fashion [104]. After T cell activation with anti-CD3 mAb *in vitro*, CD4$^+$ T cell surface expression of CD40L was described as transient, lasting less than 48 hours. Later studies suggested that CD40L expression *in vivo* may extend to 72 hours or more (see Section 1.5.3.7). The stability of CD40L mRNA was considerably enhanced by greater T cell activation using PMA and ionomycin [105], which is the same regimen that induced extracellular proteolytic processing of membranous CD40L to sCD40L [78] (see Section 1.5.3.5).

CD40L expression is transcriptionally regulated by the binding of nuclear factor of activated T cells (NFAT) sites in the CD40L promoter [106]. In addition to transcriptional regulation by NFAT proteins, early growth response (Egr) transcription factors bind sites in the CD40L promoter adjacent to an NFAT site. *In vitro* experiments using primary human CD4$^+$ T cells and *in vivo* experiments using Egr-1-deficient mice demonstrate that Egr-1 is required for CD40L transcription in CD4$^+$ T cells [94]. NFκB also binds the CD40L promoter in activated T cells, and pharmacological inhibition of NFκB activation down-regulates CD40L mRNA and protein levels in concert with cyclosporin A, which is an inhibitor of NFAT activation [99].

However, it has become apparent that the differential regulation of CD40L expression depends less on transcriptional regulation than on post-transcriptional regulation [100]. Post-transcriptional regulation is manifest as alterations in mRNA decay rates during the period of T cell activation, and has been described as 'regulated instability' of CD40L mRNA [107]. Polypyrimidine tract-binding protein (PTB) [107 100] and nucleolin are integral to the stability of CD40L mRNA and are components of the CD40L ribonucleoprotein particle associated with actively translating ribosomes [108]. CD28 costimulation was shown to accelerate and then stabilise CD40L expression on T cells *in vitro* probably *via* stabilisation of CD40L mRNA. However, it has also been suggested that interactions between the B7 family members, CD80/CD86, and CD28 augment CD40L expression in the two to eight hours after T cell activation by facilitating more avid T cell engagement rather than by directly inducing CD40L expression [109]. Although CD28-mediated signalling is not required for the induction of CD40L expression, sustained interaction with CD80/CD86-expressing APC appears necessary for prolonged T cell expression of CD40L, which is otherwise rapidly down-regulated by CD40 on B cells, for example. Feed-forward amplification serves to maximise the effects of the CD40L/CD40-mediated interaction between APC and T cells because CD40 activation increases APC expression of CD80/CD86 [110].

1.5.3.4 Pre-formed CD40L contributes to the regulation of T cell activation

Like other TNF superfamily members, CD40L may be stored pre-formed in the cytoplasm of cells for prompt release, which is appropriately scaled to the activating signal. CD40L protein has been observed to be sequestered in normal thymocytes and in thymocytes that express a transgene directing T cell expression of CD40L under the control of the Lck promoter. However, unlike normal thymocytes, *in vitro* activation of transgenic thymocytes was not required to induce surface expression of CD40L. As transgene copy number increased, the proportion of transgenic thymocytes that spontaneously expressed cell surface CD40L increased, and suggested that the mechanism for intracellular sequestration of CD40L was saturable [111]. Activation-induced elaboration of pre-formed CD40L was previously observed in human tonsillar T cells *in vitro* [112]. Within five minutes of TCR stimulation, pre-formed CD40L stored in intracellular microsomes is exported to the surface of antigen-experienced $CD45RO^+$ $CD4^+$ T cells derived from human tonsil and remains there for one to two hours. After two hours of TCR stimulation, expression of $CD4^+$ T cell surface CD40L derives from synthesis of CD40L protein, which peaks at six hours and was observed to persist for up to 20 hours [112].

Rapid delivery of CD40L to the plasma membrane would facilitate the early phases of T cell interaction and would initiate immunological responses in the absence of new gene transcription. Moreover, this sequestration mechanism restricts the appearance of this potent regulatory signal and therefore protects against protracted or indiscriminate signalling with $CD40^+$ cells [111] because the surface expression of pre-formed CD40L is limited by its internalisation after T cell engagement with CD40-expressing APC [109]. For example, CD40 on the B cell surface down-modulates T cell surface expression of CD40L by endocytosis [113].

1.5.3.5 Soluble CD40L may act as a cytokine-like agonist locally

Soluble versions of CD40L (sCD40L) may provide cytokine-like amplification of CD40 signalling locally [114]. Both platelets and T cells give rise to sCD40L. Soluble CD40L has been shown to have agonist rather than antagonist properties and promotes B cell proliferation, differentiation, antibody production and DC survival. Soluble forms of both CD40L and CD40 are shed from membrane-bound forms by extracellular protein cleavage. Shedding may regulate CD40L activity by (i) down-modulating surface CD40L, (ii) generating cytokine-like sCD40L, and (iii) quenching CD40L with a soluble CD40 'decoy' [78].

Elevated serum levels of sCD40L are found in atheroma and SLE and cytokine-like effects of sCD40L may contribute to the pathogenesis of these disorders by acting on $CD40^+$ bystander cells. The membrane and soluble isoforms of CD40L are differentially regulated depending upon activation stimulus. A candidate protease, ADAM (a disintegrin and metalloproteinase)-10, was found to cleave membrane-bound CD40L, and is related to ADAM-17 or TNF converting enzyme (TACE) that cleaves the CD40 receptor. The activity of these enzymes is PKC-dependent and the generation of sCD40L from T cells depends on PKC activation and does not occur if T cells are activated only in response to intracellular calcium release. However, no differences in expression of membrane or soluble forms of CD40L were observed after T cell stimulation with anti-CD3 and anti-CD28 mAb *in vitro* and the physiological relevance of these findings is uncertain [78].

1.5.3.6 C4b-Binding Protein is an alternate CD40 ligand that modulates CD40 signalling

C4b-Binding Protein (C4BP), which is produced as an acute phase reactant by the liver, does not bind CD40 but forms stable high molecular weight complexes with sCD40L. These C4BP/sCD40L complexes bind CD40 and prevent activation of AP-1 or STAT 3, sustained activation of which are required to overcome transient NFκB activation in order to induce the Fas-mediated apoptosis of cholangiocytes that is otherwise initiated by sCD40L alone. In contrast, CD40 activation of hepatic endothelial cells produces sustained up-regulation of NFκB and an absence of AP-1 activation and results in proliferation. C4BP is expressed strongly in inflammatory cells and proliferating biliary ductules in primary sclerosing cholangitis, and in the reactive stroma near CD40-expressing liver tumour cells. Hence, C4BP

could suppress epithelial cell apoptosis, which although it may facilitate normal protective immune responses, risks facilitating malignant transformation. C4BP may also modulate CD40 signalling in other cells such as endothelial cells, infiltrating leukocytes and stromal cells and so dampen inflammation [115] (see Chapter 2).

1.5.3.7 The duration of CD40L expression determines biological outcomes

Although IL-12, which is produced by APC, up-regulates CD40L expression by human peripheral blood T cells, which have been activated *in vitro* with anti-CD3 mAb, optimal induction of CD40L requires that IL-12 work in conjunction with IL-2 and B7/CD28 costimulatory interactions [116]. More than 24 hours after activation, CD40L expression by T cells is reciprocally regulated by IL-12 and IL-4. IL-12 sustains CD40L expression whereas IL-4 has the opposite effect. In particular, in the first phase (<24 hours), expression of CD40L is down-modulated by CD40 on the APC surface then further CD40L expression extending up to 72 hours post-activation is regulated by IL-12 and prevented by IL-4. Consequently, sustained expression of CD40L prevents terminal differentiation of B cells into antibody producing plasma cells. In this scenario, CD40 ligation would still enhance antibody production because Th1 cells would continue to activate B cells and drive proliferation and isotype switching in germinal centres (GC), which are CD40-dependent structures. Subsequently, activated B cells would migrate to lymph node areas devoid of CD40L to facilitate antibody secretion. Similarly, the strong Th1-mediated inflammation and cell-mediated immunity required to eliminate many micro-organisms, derives from IL-12-directed polarisation of Th1 cells and would be reinforced by a positive feedback loop. Sustained CD40L expression induced by IL-12 would increase APC production of IL-12 *via* ligation of CD40 on APC. Sustained CD40 signalling on macrophages may also synergise with IFNγ, which augments expression of CD40 [117], to further increase macrophage activation and cytotoxic functions [109].

1.5.3.8 CD40 expression is modulated by alternate splicing and cytokines

The expression of multiple isoforms of CD40 mRNA, which are generated by alternative splicing, is differentially regulated in activated macrophages and DC. Soon after CD40 activation, signalling competent CD40 mRNA is produced. But at 24 hours, a non-signalling isoform lacking the membrane-associated endodomain negatively feeds back on the amount of signalling competent CD40 present on the cell surface, which may provide a mechanism to regulate the activation threshold of CD40 signalling. A model was proposed to take account of these data. Quiescent cells constitutively express low levels of functional CD40 mRNA, which after stimulation with lipopolysaccharide (LPS), for example, increases in amount before the non-functional CD40 mRNA increases to interfere with formation of a signalling cell surface receptor [118].

The cytokine milieu of CD40-expressing cells modulates the level of CD40 expression. CD40 ligation itself often up-regulates expression of CD40. CD40 expression on human monocytes is up-regulated *in vitro* by IFNγ, GM-CSF, and IL-3 [119] whereas IL-10, which some tumours produce, down-regulates DC expression of CD40 and thus contributes to the immunosuppression of the tumour microenvironment [98]. The significance of CD40 expression levels on monocytes was illustrated by differences among the polar tuberculoid and lepromatous variants of human leprosy. Tuberculoid leprosy is often self-healing, pauci-bacillary and associated with cell-mediated immune responses. Conversely, lepromatous leprosy is persistent, multi-bacillary and is not associated with cell-mediated immunity. IL-10 predominates in lepromatous lesions and blocks IFNγ up-regulation of CD40 on monocytes whereas both IFNγ and GM-CSF, which up-regulate monocyte expression of CD40, are preferentially expressed in tuberculoid lesions. The low-level expression of CD40 on monocytes from lepromatous lesions did not result from a cell-autonomous defect because CD40 expression on monocytes from both tuberculoid and lepromatous lesions was inducible with IFNγ. These data suggested that the levels of CD40 expression on monocytes were

determined by the relative balance of stimulatory type 1 cytokines such as IFNγ and GM-CSF, and the inhibitory type 2 cytokine, IL-10 [120].

Recent studies of NFκB-inhibited human monocyte-derived DC and murine models of tolerance induction indicate that the level of CD40 expression is a critical determinant of immune activation. During T-DC interactions, NFκB activation controls feed-forward amplification of CD40 expression, which contributes to the induction of productive immunity [121] (see Section 1.5.5.3)

1.5.3.9 Control of CD40L/CD40 interactions is compartmented and process-dependent

While CD40 is expressed constitutively by a more diverse range of cell types than CD40L, CD40L expression is inducible and largely restricted to lymphoid organs and the inflammatory microenvironment. CD40L expression is usually contact-dependent and, particularly in lymphoid organs, depends on antigen-specific interactions to initiate cell-cell contact thus directing the specificity of signalling. However, in the inflammatory microenvironment located in the immunological periphery, although CD40L expression may be independent of antigen, it acts synergistically with innate immune stimuli such as IFNα and IL-1β on DC to produce IL-12 [122] [123]. The DC is the cell type in the immunological periphery that is tasked with integrating various immune stimuli to couple innate and adaptive immune responses.

Tissue injury will usually produce a breach in the integument, which provides a portal of entry for microbes and which leads to thrombin formation, platelet activation and induction of CD40L expression on platelets. Platelet CD40L is pre-formed and its rapid induction on the platelet surface induces an inflammatory reaction of $CD40^+$ endothelial cells. Simultaneously, CD40L binds constitutively expressed CD40 on platelets to cleave membrane-bound surface CD40L within minutes to hours, and release sCD40L that can no longer induce an inflammatory reaction of endothelial cells [124]. Consistent with CD40L expression on eosinophils, basophils and mast cells [125], the control of which is presumed to be inducible and tissue-dependent (like in atheroma), CD40L/CD40 interactions have a role to play in the induction of both Th1 [126] and Th2 [127] [128] immune responses. Moreover, CD40 stimulation on DC has been found to up-regulate surface expression of functional CD40L on DC [129]. Nevertheless, it is likely that CD40L control mechanisms, perhaps similar to those operating for platelets, apply to other CD40L-expressing cells in the inflammatory microenvironment.

The local induction of CD40L expression in the immunological periphery may give the adaptive immune system a 'head start'. For example, optimal secretion of IL-12 by DC *in vitro* after CD40 activation occurs after the addition of T cell-derived IFNγ and IL-4. However, CD40L is also expressed by non-T cells during inflammation at the primary infection site in conjunction with innate immune cell-derived cytokines such as IL-1β. IL-1β is constitutively produced by monocytes and monocyte-derived DC (moDC), and increases in response to intact *E. coli* or CD40L. In the absence of T cell-derived cytokines, IL-1β synergises with CD40 ligation of DC to secrete more IL-12, which results in higher T cell secretion of IFNγ. Hence, these *in vitro* results indicate that T cells are not required for DC activation during the earliest stages of inflammation [130]. Furthermore, type I interferons such as $IFN\alpha_{2a}$, which are also present at sites of infection and inflammation, were also shown *in vitro* to act in concert with IL-1β and/or IL-4, and CD40L to activate DC and enhance secretion of IL-12, which results in higher T cell production of IFNγ. Therefore, at sites of infection and inflammation, IFNα, IL-1β, and CD40L are rapidly induced and collaborate to mature cytokine-secreting DC that can migrate to the draining lymph nodes and exercise potent antigen presenting functions [123]. Further *in vitro* studies showed that the strength and persistence of CD40 signalling differentially determines migratory capacity and cytokine secretion of DC. For example, moDC migrated in response to a weak and transient CD40 activation signal but required a strong and persistent CD40 signal to block migration and induce cytokine secretion [122].

The mechanisms engaged to regenerate and repair tissue injury, which is associated with pathogen entry and which may include CD40 signalling, may be subverted in the case of internal injury to endothelium, which may occur at places of shear stress to cause and/or exacerbate atheromatous lesions. In atheromatous lesions, the inducible expression of CD40L together with the constitutive expression of CD40 by different cell types appears to promote inflammation once initiated by other mechanisms [131]. Although human endothelial cells (EC), smooth muscle cells (SMC) and macrophages constitutively express CD40, none expressed CD40L in the normal arterial wall. However, during *in vitro* culture, EC, SMC and macrophages co-expressed CD40L and CD40, and stimulation with the pro-inflammatory cytokines, IL-1β, TNF and IFNγ, increased surface expression by these cells of biologically active CD40L. Furthermore, CD40 ligation of SMC, which constitutively express CD40, induced expression of pro-inflammatory cytokines. Finally, as an indication of the *in vivo* significance of these findings, all of these cell types were often found to express both CD40 and CD40L in atheromatous plaques [131].

However, while another study confirmed CD40 expression on EC, SMC and macrophages in atheromatous lesions, CD40L was found not to be expressed by these cell types. Instead, CD40L expression was discovered only on activated $CD4^+$ T cells in intimal tissue in association with macrophage infiltrates and neovascularisation, and on platelets in the intima and in plaque ruptures. *In vitro* flow chamber experiments suggested a model in which inflammation is initiated by $CD40L^+$ platelets, which bind to damaged endothelium and stimulate EC *via* CD40 to recruit neutrophils and T cells from blood, but in which inflammation is sustained in the atheromatous lesion by CD40L-expressing $CD4^+$ T cells because platelet CD40L has a short half-life [132].

Once CD40L expression is induced in the inflammatory microenvironment by stimuli such as pro-inflammatory cytokines, autocrine and paracrine CD40L/CD40 interactions may create a feed-forward loop to maintain and amplify inflammation. While sCD40L may contribute to this amplification, the signalling effects of sCD40L will depend on its local concentration and will be less strong than the signalling effects exerted by membrane-bound CD40L. The strength and duration of CD40 signalling is also likely to have a critical effect on cell behaviour with stronger and longer signalling producing inhibitory effects [133]. Thus, potentially explosive effects of the CD40L/CD40 interaction are mitigated by physiological factors that include signalling strength, temporal regulation of membrane-bound surface CD40L [124], together with the kinetic delay in CD40 signalling that is mediated *via* the alternative NFκB pathway [134]. Together, these factors may provide a means both of controlling the effects of the interaction *in situ* and limiting its geographic extent.

Finally, production of T-dependent antibodies critically depends on intact CD40L/CD40 interactions, and for the most part, is limited to the specialised microenvironment of the B cell follicle of secondary lymphoid organs such as spleen and lymph node. Experienced human $CD4^+$ $CD45RO^+$ T cells, which mainly display intracellular expression of CD40L, are found predominantly in the outer zone of the GC, and in the T cell-rich zone adjacent to the follicular mantle that surrounds the GC. The T cell-rich zone contains interdigitating or follicular dendritic cells (FDC). Antigen is held on FDC and internalised by the cognate B cell *via* its specific B cell receptor (BCR), processed, and presented on B cell surface MHC class II molecules to cognate $CD4^+$ T cells. Even in established GC, blockade of CD40L/CD40 interactions prevents B cell memory formation [135]. In rodents, naïve and antigen-experienced B cells migrate to the T cell-rich zone where cognate interactions with T cells occur, and where antigen-specific B cells can activate memory T cells. Short-lived plasma cells and B cell blasts that are generated at this site then migrate to the GC where they will undergo apoptosis within hours unless they are rescued by CD40 stimulation [135].

Hence, the supply of membrane-bound CD40L in the vicinity of the GC of the lymph node seems to be strictly limited. Membrane-bound CD40L may ensure that strong and durable CD40 signalling is only supplied to antigen-reactive B cells that interact with antigen-laden FDC in the GC thus avoiding bystander effects on nearby non-antigen specific clones.

Although the physiological function of inducible CD40L expression on activated B cells is not well established [136], a cytokine-like function for sCD40L emanating from B cells is not excluded. Any effects of diffusible sCD40L may depend on a concentration gradient that delivers a basal level of pro-survival signals [133] to ambient $CD40^+$ cells in the follicle.

1.5.4 CD40L/CD40 interactions and innate immunity

Janeway developed a hypothesis stating that: "the immune system has evolved specifically to recognise and respond to infectious microorganisms, and that this involves recognition not only of specific antigenic determinants, but also of certain characteristics or patterns common on infectious agents but absent from the host" [137]. Medzhitov from Janeway's own laboratory later vindicated this hypothesis by the discovery on host APC of pattern recognition receptors that bind invariant microbial structures to activate APC and generate signal 2 [138] [139] (see Section 1.5.5.1). Janeway later refined his hypothesis to indicate that: "the immune system evolved to discriminate infectious non-self from noninfectious self" [140]. However, this hypothesis did not take account of how immune responses are made to transplants, tumours or the host's own tissues in the case of the autoimmune diseases. Hence, in counterpoint to Janeway's 'Stranger hypothesis' [141], Matzinger developed her 'Danger hypothesis' to explain how the immune system evolved to discriminate non-dangerous from dangerous antigens, which may be 'self' as well as 'non-self' (or 'stranger' or 'foreign') antigens. Here, cellular injury contributes endogenous signals that alert the immune system to a threat to immune homeostasis by activating APC to become effective T cell stimulators. Therefore, although cellular injury may be caused by infectious pathogens or other sterile disease processes such as ischaemic necrosis, immune responses do not depend on a distinction being made between foreign and self molecules [142]. Now, sufficient experimental evidence supports the danger hypothesis. Endogenous danger signals released during tissue injury include heat shock proteins, monosodium urate, and alarmins such as high mobility group box protein 1 (HMGB1) [141] [143]. Nevertheless, the Stranger and Danger hypotheses need not be mutually exclusive, and current evidence would indicate that both together have sufficient explanatory power.

Pattern recognition receptors (PRR) on innate immune cells interact with the microbe-associated molecular patterns (MAMP) that are the invariant features of microbes such as cell wall structures, non-methylated DNA and double-stranded RNA. Among PRR, TLR are the primary sensors of innate immunity [144] (see Section 1.5.4.2). PRR do not discriminate between pathogens, opportunistic and symbiont microbes, and indeed pathogens are defined by their ability, at least initially, to overcome innate immune defences and cause disease. Instead pathogenicity is mediated by virulence factors encoded in the bacterial genome at sites separate from MAMP. Rather, the concept is emerging that the development of an innate immune response in a given organ is specifically regulated in that organ so that the threshold for activation of the organ's innate immune response is governed by its bacterial load under steady state conditions. For example, in the non-sterile environment of the gut, peaceful co-existence with symbionts is required for the good health of the organism and a 'homeostatic circuit' feeds back on inappropriate TLR-induced inflammation. For example, endotoxin tolerance, or TLR cross-tolerance, describes a phenomenon in which repeated TLR stimulation results in dampened TLR-induced inflammatory responses in the non-sterile gut but not in the sterile spleen. In the gut, other regulatory mechanisms may operate to blunt responses to microbes and include (i) the lack of expression by lamina propria macrophages of CD14, which is a co-receptor with TLR4 for LPS, (ii) expression of TLR by mucosal epithelial cells as well as innate leukocytes, (iii) differential expression profiles of intracellular negative regulators of TLR signalling, and (iv) gut tissue expression of anti-inflammatory cytokines such as transforming growth factor β (TGFβ) and IL-10 [145] [146].

In defending the organism against tissue injury and invading pathogens, immune cells co-operate with the frontline cells of the epithelium, which maintains the integrity of the internal milieu. Tissue injury is frequently caused by infectious pathogens, and independently, may facilitate infection with opportunistic pathogens. Given their proximity to the epithelium, cells

of the innate immune system respond first to invading pathogens and any associated breaches of the epithelium. New evidence now indicates that normal tissues harbour molecules, which when released into the internal milieu after tissue injury, send activating signals to the host immune system. Cytosolic messengers such as monosodium urate may be released irrespective of whether tissue injury produces primary necrosis, or apoptosis followed by secondary necrosis. Circumstances in which cells had been stressed prior to apoptotic cell death may result in elevation of monosodium urate levels as purines in RNA and DNA degrade [147] [141]. Necrosis-inducing factors include physical insults such as heat and mechanical trauma, acute energy depletion resulting from ischaemia, and severe membrane damage resulting from reactive oxygen species generated in acute inflammation. Here, the disruption of tissue organisation may result in the release of immunostimulatory factors such as fibrinogen, heparin-sulphate proteoglycans, and hyaluronan oligosaccharides, all of which seem to signal macrophages and/or DC *via* TLR4. In addition, tissue trauma will also result in platelet activation and platelet expression of CD40L and thus also supply a danger signal [148] (see Section 1.5.3.9).

Alarmins are a structurally diverse set of host molecules, which are released rapidly in response to infection and tissue injury. Alarmins both recruit and activate APC, particularly DC, and augment antigen-specific immune responses *in vivo*. Hence, similarly to the alerting effects of MAMP, alarmins mobilise the innate and adaptive immune system [143]. Immune effector cells may secrete alarmins in response to activation by MAMP or other alarmins, and alarmins may interact with some of the same TLR as MAMP. Accordingly, MAMP have been united together with alarmins and other endogenous danger signals to form a group termed damage-associated molecular patterns (DAMP) [149] [150]. However, some immunologists consider the evidence that cites binding of endogenous danger signals such as heat shock proteins to TLR to be controversial and these concerns may apply to experimental studies of other endogenous danger signals. For example, some heat shock proteins such as HSP60 and HSP70 signal through the LPS receptor complex comprising TLR4/MD2/CD14. Some studies now indicate that LPS-free preparations of HSP are not immunostimulatory and that immunostimulation is restored by small amounts of LPS. Experiments to test if neutralisation of HSP *in vivo* prevents adjuvant activity of HSP have not been performed [141].

Alarmins include human neutrophil protein, the β-defensins, and high mobility group box protein 1 (HMGB1) (see Section 1.9.7.3). Defensins such as the β-defensins are produced, for example, by epithelial cells in response to TLR signalling [151], and act by disrupting the cytoplasmic membrane of micro-organisms [152]. In addition, human β-defensins bind CCR6, which is expressed preferentially by immature DC and memory T cells and which is also bound by the MIP-3α chemokine. Subsequently, β-defensins may recruit immune cells to the site of microbial invasion and thus promote adaptive immunity [153]. Murine β-defensin-2 is also a TLR4 agonist and may attract immature DC *via* CCR6 and then activate the DC *via* TLR4 [152].

Innate immune cells such as neutrophils, macrophages and DC acquire pathogen-derived and necrotic material using pinocytosis and phagocytosis. The pathogens and debris are cleared and the material is transported to the secondary lymphoid organs, which are specialised structures housing the site of the adaptive immune response. The increased complexity of the adaptive immune response causes it to develop more slowly than the innate immune response but, once fully developed, the multiplicity and exquisite specificity of its effector components completes a wall of defence that incorporates more fail-safe features. Ultimately, the changing cytokine profile of a fully developed adaptive immune response guides innate immune cells that contribute to the resolution of inflammation by healing, repair and regeneration of damaged tissue.

During the innate immune response in the immunological periphery, professional scavenger cells such as macrophages and DC operate in ‘antigen capture mode’. Necessarily, this mode of operation precedes the ‘antigen presentation mode’ adopted by macrophages and DC in secondary lymphoid tissues during the initiation of the adaptive immune response.

Consequently, the temporal and spatial coordination of innate and adaptive immune responses is achieved by separate signal transduction mechanisms that mediate the transition between the modes of antigen capture and antigen presentation [154] (see Section 1.5.4.4).

1.5.4.1 The dendritic cell is a unique cell type

The dendritic cell (DC) is a relatively rare cell type, which is distributed throughout most body tissues and which functions as a professional APC. Uniquely as an APC, the DC initiates the immune response by priming T cells. No one property of the DC defines its role in T cell priming. Rather, a constellation of biological properties, which include cytokine secretion together with the cell surface expression of stable pMHC complexes and costimulatory and cell adhesion molecules, optimise the efficiency of antigen processing and presentation [155]. DC have a vital sentinel function in the non-lymphoid tissues of the 'immunological periphery'. Here, DC sample the external environment, which includes the lumen of the gut, by capturing and processing antigen, and then migrating with its antigen load to secondary lymphoid organs such as lymph nodes, spleen and the mucosal associated lymphoid tissues (MALT). In secondary lymphoid organs, increased DC production of T cell stimulatory cytokines and chemokines attracts other immune accessory cells and T cells within a microenvironment that already contains a high density of lymphocytes. Lymphocytes constantly migrate throughout the body and interact with antigen-loaded DC until immune recognition occurs and the processed antigen is presented to a specific lymphocyte. In particular, vigorous 'random-walk' motility of the lymphocytes allows exploration of large areas within lymphoid tissues and so efficiently surveys for the presence of antigen-bearing DC [156].

Hence, when it is located in the immunological periphery, the DC integrates multiple inputs and upon its relocation to secondary lymphoid organs, the DC generates multiple outputs that modulate the outcome of the immune response. These sophisticated properties of the DC enable it to provide a crucial link between the innate and adaptive arms of the immune system. Consequently, DC have been described as 'nature's adjuvant' [157].

1.5.4.2 Dendritic cells operate pathogen sensors

According to Janeway's hypothesis, innate immune recognition is required for the initiation of adaptive immune responses and provides a mechanism to allow discrimination of infectious non-self from non-infectious self [137]. The DC 'senses' the presence of microbes and subsequently initiates and coordinates the innate and adaptive immune responses by using PRR to bind MAMP that are the invariant features of micro-organisms not found in the host. TLR are the best characterised PRR, and 13 TLR have been identified in mammals [158]. TLR are expressed in varying patterns by DC depending on DC subtype and anatomical location and each TLR recognises a chemically-distinct set of MAMP [159]. For example, TLR3 binds double-stranded (ds)RNA, single-stranded (ss)RNA is bound by TLR7 and TLR8, and TLR9 specifically binds dsDNA. All four TLR play a crucial role in recognition of viral DNA and RNA. On the other hand, other TLR interact with such diverse ligands as LPS and peptidoglycans [160].

Exposure of DC to microbes initiates a maturation program, which is regulated by signalling both *via* TLR and CD40. The maturation program alters chemokine secretion patterns and the cell surface expression profile of chemokine receptors, MHC molecules, costimulatory molecules such as CD80, CD86 and CD40, and cell adhesion molecules such as lymphocyte function antigen (LFA)-1 [161]. Consequently, more DC are recruited to the site of infection, and DC switch in the periphery from antigen capture mode using macropinocytosis and phagocytosis to antigen-presentation mode, and migrate to the T cell zone of the draining lymph node [161] where T cell priming occurs [139].

MyD88 is an intracellular adaptor protein that links TLR binding to the downstream signalling events that produce DC activation. T cells in MyD88-deficient mice fail to proliferate or produce IFNγ in response to subcutaneous immunisation of ovalbumin protein in Complete Freund's Adjuvant (CFA). Moreover, MyD88-deficient DC do not increase

expression of MHC, CD80, CD86 or IL-12 after stimulation with heat-killed H37RA *M. tuberculosis*, which is the major microbial component of CFA that activates TLR2 and TLR4. Hence, a distinct TLR signalling pathway is responsible for activation of type 1 cytokine responses [162]. Although the TLR expression profile of $CD11b^+$ dermal DC, which would respond to subcutaneous immunisation, is not known [139], it has been demonstrated that murine monocytes residing in subcutaneous tissue can become lymph-borne DC that localise in draining lymph nodes [163].

1.5.4.3 Dendritic cells integrate innate immune signals and drive adaptive immunity

Like other cells in the haemopoietic system, DC are produced in various lineages to meet steady state requirements of tissue homeostasis, and then in emergency demand situations such as acute inflammation. Immediate precursors of tissue DC, or precursor DC (pre-DC), are generated from bone marrow, and differentiate further in the host tissue. DC are specialised for different functions, and functional specialisation may originate as DC develop in separate lineages. Evidence for this concept of DC development has been established most completely in murine experimental systems so that different DC subsets with different functional properties have been defined, and will be described below [164]. Similar DC subsets have been defined in humans but a firm one-to-one correspondence with murine DC subsets has not yet been established [165]. So conventional DC (cDC), which include migratory DC such as Langerhans and dermal DC residing in peripheral tissues, respond to pathogen challenge by trafficking pathogen-derived antigen to the draining lymph nodes [166].

In contrast, lymphoid DC reside in lymph nodes and present foreign and self antigens [167]. In mice, lymphoid DC may be further characterised based on CD8α expression: $CD8\alpha^+$ DC are present in the T cell areas of secondary lymphoid tissue, and are closely associated with cross-presentation of antigens particularly from viruses, tumours and DNA vaccines [168]. Lymphoid DC play an important role in the induction of adaptive anti-viral immune responses, and it has been proposed that $CD8\alpha^+$ DC prime CTL responses to both cytolytic and non-cytolytic viruses [169]. The plasmacytoid DC (PDC) arises from a distinct cellular lineage and is the most important cell type in innate anti-viral immunity [170]. In the steady state, PDC exist as a precursor population that selectively express TLR7 and TLR9. After exposure to virus, PDC secrete large amounts of type I interferons, which led to inhibition of viral replication within infected cells [171] and which activate a number of indirect anti-viral responses including natural killer (NK) cell cytotoxicity, proliferation, IFNγ production [172], and IFNα/β receptor-mediated stimulation both of antigen-specific $CD8^+$ CTL [173] and B cells [174].

Emerging evidence suggests that integration by DC of innate immune inputs may occur across more than one cell type, and thus may require apportionment of tasks by a particular DC subset ('division of labour' model) as well as extensive cooperation between different DC subsets [175]. Although it remains uncertain as to whether PDC *per se* can directly prime naïve T cell responses [176] [177], PDC may induce anti-viral and anti-bacterial immunity *via* cross-talk with other DC subsets [178]. The complexity of DC involvement in natural viral infection is illustrated by herpes simplex virus (HSV) infection of mice. After infection of murine vaginal mucosa with HSV2, PDC acted not as APC but as anti-viral effector cells because adaptive Th1 immunity developed in the absence of PDC [179]. Priming of virus-specific CTL in response to HSV1 infection of murine epidermis did not depend on direct presentation by Langerhans cells, which reside in infected skin, but rather on cross-presentation to lymph node-resident $CD8\alpha^+$ DC [180] [181]. However, blocking egress of migratory DC from HSV-infected skin did show that CTL priming depended on transport of HSV antigens from infected skin to skin-draining lymph nodes. It was hypothesized that antigen presentation can be amplified if relatively small numbers of skin-emigrant DC transfer antigen to a higher density of lymphoid-resident DC, which subsequently elicit robust CTL activation [181].

1.5.4.4 CD40-dependent cross-talk between innate immune cells completes adaptive immunity

Innate natural killer T (NKT) lymphocytes may be activated *in vivo* using the glycolipid, α-galactosylceramide (αGalCer), which binds CD1d on DC and in turn binds the invariant NKT

cell receptor, Vα14. Activated NKT cells then mature DC in a fashion analogous to TLR signalling. Subsequently, DC produce IL-12, TNF and IFNγ, increase MHC class I and II-restricted antigen presentation of ovalbumin (OVA) protein epitopes, and up-regulate expression of CD40, CD80 and CD86 costimulatory molecules to drive productive $CD4^+$ and $CD8^+$ T cell immunity in response to a small amount of OVA protein antigen presented in association with dying cells. This immunity is potent enough to afford durable protection against challenge with live OVA-expressing tumour cells [182].

However, after αGalCer treatment, induction of OVA-specific T cell immunity requires CD40L/CD40 interactions. Studies of splenic DC in αGalCer-treated CD40L- and CD40-deficient mice show that although NKT cell activation was intact in CD40-deficient mice, OVA-specific T cell immunity did not occur. Despite presentation of specific pMHC complexes (signal 1), and up-regulation of membrane and cytokine costimulators (signal 2), which were induced by cytokines rather than CD40 ligation, OVA-specific T cells were not primed. Moreover, CD40 expression was required on DC for OVA-specific $CD4^+$ and $CD8^+$ T cell responses, which indicates that CD40L was essential for full DC maturation. After αGalCer treatment, CD40L expression by NKT cells, which is presumably induced by interaction with glycolipid-presenting DC and which reciprocally induces DC maturation, is sufficient for OVA-specific $CD8^+$ T cell priming [182].

Expression of CD80 and CD86 is also required for the induction of OVA-specific αβT cell immunity in this system, and indicates that at least basal levels of these costimulatory molecules are needed for T cell priming [182]. Perhaps, CD80/CD86 expression on DC is required for both CD28 binding and sustained induction of CD40L expression by either NKT cells or αβT cells. For this reason, other potential sources of CD40L, which include activated platelets, mast cells, or HSP70, seem not to be physiologically significant. Consistent with the view that CD28 stimulation and consequently prolonged CD40L expression is required for $CD4^+$ T cell priming, is the argument that co-recognition of antigen on the same DC by both $CD4^+$ and $CD8^+$ T cells is essential because the period taken by the $CD4^+$ T cell to activate DC is sufficiently long to require that only one DC is activated during the period that $CD8^+$ T cells are primed [183]. In further in support of this notion, experiments were conducted *in vivo* using transgenic T cells that lacked CD28 or CD40L. While CD28-deficient T cells failed to expand after immunisation, CD40L-deficient T cells initiated a response that could not be sustained, which indicated that CD80/CD86-CD28 interactions were critical for initiating T cell responses but that CD40L/CD40 interactions were required to sustain Th1 responses, in particular [184].

That CD40 stimulation of DC bypasses the need for CD4 T cell help in CTL priming suggests that CD40 signalling induces immunity by increased formation of pMHC complexes or increased expression of costimulators (membrane-bound CD80 and CD86 and/or soluble IL-12 or IFNα) as well as increased DC survival and migration [185] [186] [187]. Unexpectedly, this report showed that both $CD4^+$ and $CD8^+$ T cell immunity depended on CD40 expression by DC in the presence of CD40-independent antigen presentation and costimulation. Hence, the two-signal model of T cell activation seems inadequate to explain these findings. This model describes an escalating induction of immunity, which depends first on antigen presentation in the steady state (signal 1), then inflammatory cytokine-dependent increases in costimulators (signal 2), and finally an as yet unidentified and distinct CD40/CD40L signal involving DC is required to generate fully functioning $CD4^+$ Th1 cells and $CD8^+$ CTL [182].

1.5.4.5 NFκB signalling pathways critically underpin inflammation and immunity

The NFκB transcription factors are central regulators of inflammatory and immune responses, and thus coordinate innate with adaptive immune responses. In the immunological periphery, surface receptors on innate immune cells sense immune challenges, and use the IκBα kinase (IKK) complex containing the IKKβ catalytic subunit in association with subunits of IKKα and IKKγ (*alias* NFκB essential modulator, NEMO) to signal to the nucleus *via* the classical NFκB pathway and so activate the innate immune cells. Classical NFκB pathway activation

causes innate immune cells such as DC to migrate from the immunological periphery to organised lymphoid tissue where DC activate $CD4^+$ T cells to express CD40L, which in turn completes DC activation *via* the alternative NFκB pathway using the IKK comprising an IKKα homodimer (Figure 1).

The primary function of the innate immune system is to provide a rapid response to exogenous and endogenous stimuli that perturb immune homeostasis within the organism. Exogenous stimuli include viruses, other pathogens, and their products that alert the immune system by binding TLR or antigen receptors, pro-inflammatory cytokines, and endogenous stimuli such as double-stranded DNA breaks. Receptor recognition and the consequent intermediary signalling converge on the IKKβ-containing kinase complex, which facilitates a rapid response by initiating activation of the dimeric p50/RelA NFκB transcription factor within minutes [134] [188].

Inhibitory IκB proteins sequester NFκB family members in an inactive state in the cytoplasm until a signal is transduced by an IKK complex. The activated kinase complex phosphorylates serine residues on IκBα thus targeting the protein for ubiquitin-mediated proteosomal degradation. Destruction of IκBα reveals the nuclear localisation sequence (NLS) on the NFκB protein, releasing dimeric NFκB transcription factors to translocate to the nucleus, bind to promoters of target genes and thus implement pro-inflammatory gene expression programs. NFκB proteins function only as dimers because one member of the pair (RelA or RelB) contains the transcription activation domain whereas the other member (p50 or p52) contain the Rel homology domain (RHD) that harbours the dimerisation motif, the DNA recognition motif, the NLS, and the IκBα binding site [134].

Inflammation is amplified by feed-forward effects of secreted IL-1β and TNF on the activation of the classical NFκB pathway. The inflammatory proteins expressed include cytokines (IL-6, IL-1β, TNF, granulocyte-macrophage colony stimulating factor [GM-CSF]), chemokines (IL-8, regulated on activation normal T Cell expressed and secreted [RANTES], macrophage inflammatory protein-1α [MIP-1α], monocyte chemotactic protein [MCP-1]), enzymes (inducible nitric oxide synthase [iNOS], cyclooxygenase 2 [COX-2], phospholipase A2 [PLA2]) and adhesion molecules (vascular cell adhesion molecule 1 [VCAM-1], 1 ICAM-1 and E-selectin). Although many NFκB target genes have been identified in haemopoietic cell types, many of the innate immune functions mediated by IKKβ and RelA mainly occur within non-haemopoietic cells (probably epithelial) of the gut, liver and lung where many of the NFκB target genes remain to be identified. Moreover, an additional important property of the classical NFκB pathway is to enhance the survival of lymphocytes and accessory immune cells during infection and acute inflammation by suppressing apoptosis in response to cytokines such as TNF [189].

On the other hand, the alternative NFκB pathway plays a central role in the expression of genes such as lymphotoxin (LT)-α and β, and TNF, which are concerned with the development and maintenance of the secondary lymphoid organs. Lymphotoxins and TNF are organogenic chemokines, which are responsible for the normal positioning of stromal cells in secondary lymphoid tissue, and so guide the subsequent normal organisation of lymphocytes. Lesions affecting the alternative pathway disrupt formation of secondary lymphoid organs and formation of GC after immunisation. In particular, lesions in the alternative pathway prevent the formation in GC of the network of long-lived antigen-trapping FDC, which are believed to contribute to immunological memory [189]. In contrast, genetic disruption of the other major alternative pathway activator, CD40L, does not result in lymphoid structural abnormalities other than absence of GC [190] [191]. Hence, in the mature organism, *via* its activation of the alternative NFκB pathway, CD40L has the vital operational role of coordinating adaptive immune responses within the confines of the structural framework predetermined by developmental activation of the same alternative pathway by organogenic chemokines.

In the regulation of the alternative pathway, IKKα, and its upstream regulator, NFκB-inducing kinase (NIK), cooperate in a phosphorylation-dependent kinase cascade to process the precursor p100 protein to p52 that dimerises with RelB. The kinetics of p100 processing takes several hours in contrast to degradation of IκBα, which takes minutes and releases p50/RelA. The processing of p100 is negatively regulated by TRAF2 and TRAF3. TRAF3 sequesters NIK resulting in its ubiquitination and degradation. Then CD40 activation, and the consequent CD40-mediated sequestration of TRAF3 increases steady-state levels of NIK, allowing it to bind and phosphorylate IKKα, which enhances p100 processing and the subsequent activation of the alternative NFκB pathway *via* the nuclear translocation of the p52/RelB heterodimer [134]. Even if antigen-charged DC arriving in secondary lymphoid tissue have been activated by CD40L in the immunological periphery, the slower kinetics of alternative NFκB pathway activation ensure that DC are becoming fully competent as APC upon entry to the lymph node.

In evolutionary terms, the function of IKKα in lymphoid organogenesis and adaptive immunity is a more recent adaptation because it is dispensable for innate immune responses and suppression of apoptosis. Under the control of the classical NFκB pathway, the innate immune system marshals the recruitment of neutrophils, macrophages, DC and lymphocytes to sites of infection and inflammation in response to pro-inflammatory chemokine secretion by the infected tissue. As the innate immune task of destroying and clearing the infectious pathogen is undertaken, an adaptive immune response, which involves antigen processing and presentation, develops to consolidate defences against the pathogen and to provide protective immunity upon later re-challenge.

TNF family members such as LTα and LTβ [129] [189] and CD40L, which are under IKKα-dependent control, are regulated by the classical NFκB pathway. The IKKα substrate p100 is also under the control of the classical NFκB pathway and IKKγ, which is part of the classical IKKβ-containing trimeric complex, negatively regulates IKKα-dependent processing of p100 [189]. IKKβ-dependent control of the alternative NFκB pathway is reciprocated by IKKα homodimers, which determine the duration of NFκB activation through phosphorylation and subsequent ubiquitin-mediated degradation of its RelA and c-Rel subunits. Consequently, the inability of IKKα complexes to terminate NFκB activation results in exaggerated inflammatory responses to challenge with microbial pathogens [154].

CD40L expression during the inflammation attending infection and/or tissue injury in the immunological periphery would help to amplify and refine the evolving inflammatory response. For example, cell types such as platelets that respond almost immediately to tissue injury express CD40L that can interact with CD40 on endothelial cells and also on epithelial cells. CD40 stimulation of these cell types induces secretion of cytokines and chemokines, which promote angiogenesis and the recruitment of additional innate immune cells. Consequently, CD40 signalling in the immunological periphery would enlarge the dynamic range of the inflammatory response and thus hasten the clearance of invading pathogens as well as contribute to wound healing and the resolution of inflammation *via* processes of tissue repair and regeneration (Figure 2). Other CD40L-expressing innate immune cell types such as eosinophils and mast cells may be become involved in the event of parasitic infestation or allergic inflammation. Ideally, the innate immune response leads to an adaptive immune response and eventual resolution of the acute insult. However, failure or subversion of signalling shut-off mechanisms may enable CD40L-amplified responses to contribute to ongoing pathology such as atheroma formation (see Section 1.5.3.9).

Moreover, the slower kinetics of activation of the alternative NFκB pathway, and its subjugation to the control of the NFκB classical pathway delays induction of the organogenic chemokines and the consequent migration of antigen-laden APC to secondary lymphoid organs [189]. Without this understanding [122] [123] [130], the expression of CD40L in the immunological periphery, even for therapeutic purposes, may seem counterintuitive (Figure 2).

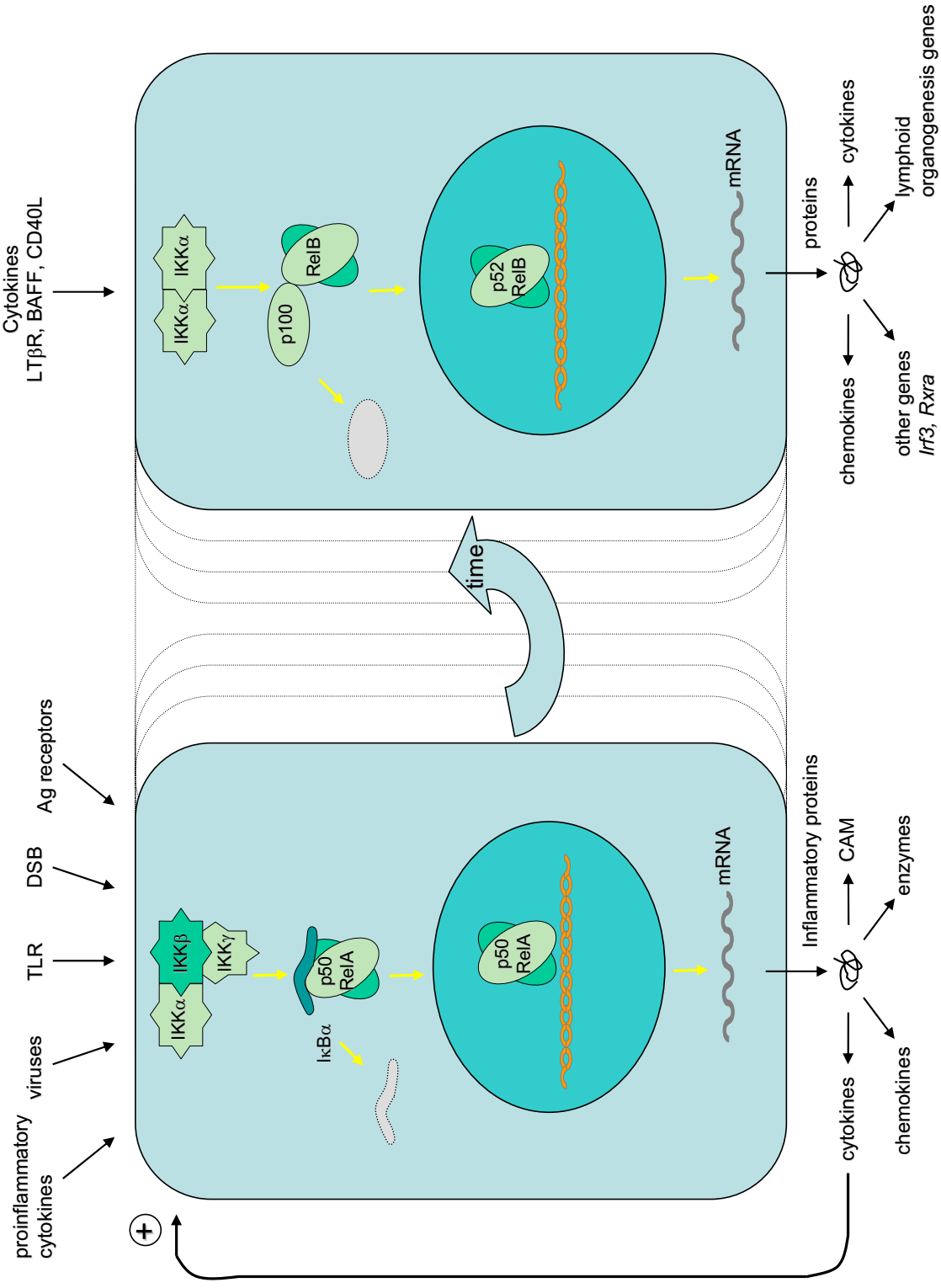


Figure 1: NFκB signal transduction machinery

Schematic diagram illustrates that the NFκB signal transduction machinery recruited in response to activators of the innate immune system, the classical NFκB pathway (**A**), is separate from the alternative NFκB pathway (**B**), which is utilised in response to CD40 stimulation or stimulation by lymphoid organogenic cytokines. In particular, signalling flux through the alternative pathway is considerably slower (hours) than that through classical pathway (minutes). TLR, Toll-like receptor; Ag, antigen; DSB, double-stranded DNA break; LTβR, lymphotoxin β receptor; BAFF, B cell activating factor; IKK, IκB kinase; CAM, cell adhesion molecules (adapted from refs. 140 and 193).

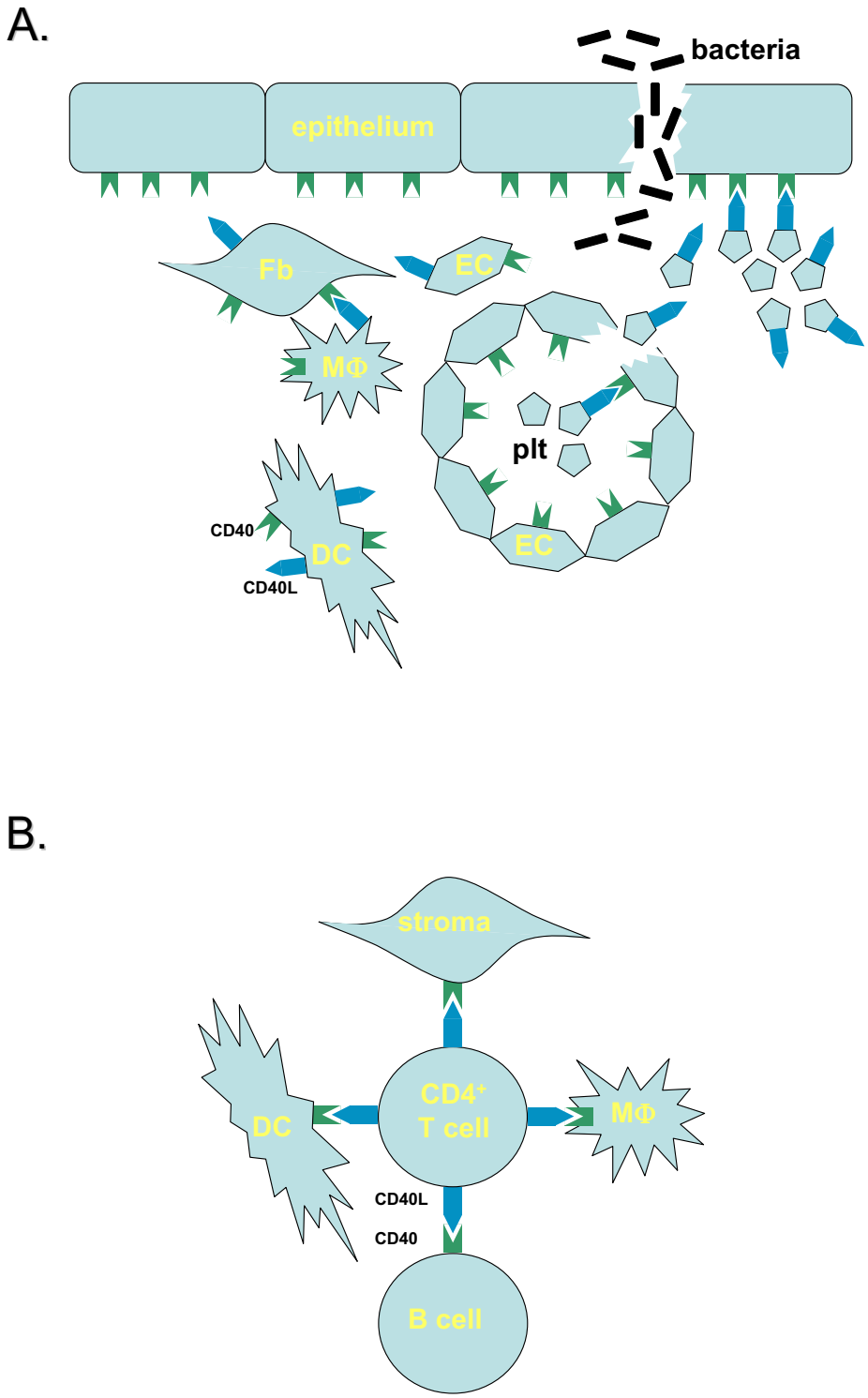


Figure 2: CD40 ligand plays critical roles in both innate and adaptive immunity

(**A**) In the immunological periphery, CD40L is induced on different cell types in an inflammatory microenvironment and contributes to clearance of invading pathogens, and to the resolution of inflammation and wound healing. The different cell types include dendritic cells (DC), macrophages (MΦ), fibroblasts (Fb), platelets (plt), and endothelial cells (EC). (**B**) In organised lymphoid tissue, CD40L is induced after activation of $CD4^+$ T cells and contributes to diversification and amplification of the immune response by the 'licensing' of dendritic cells (DC), the activation of macrophages (MΦ) and B cells, and the activation of stromal elements such as thymic epithelial cells.

1.5.4.6 CD40-dependent and CD40-independent effects on NK cells

The cytolytic activity of NK cells is regulated by a balance of signals delivered by inhibitory and activating receptors. MHC class I molecules bind the inhibitory receptors and down-regulate NK cell cytotoxicity and cytokine production so that absent or low MHC expression by target cells activates NK cells, which subsequently lyse the target cells. Activating receptors such as natural killer group 2D (NKG2D) recognise families of immunoglobulin-like or lectin-like molecules such as H60 or RAE1 in mice or MICA/B in humans [192]. NK cell activation depends on interactions between DC and NK cells, which may or may not involve CD40 signalling.

Early data indicated that CD40 expression by target cells resulted in cytotoxicity by NK cells in which prior exposure to IL-2 had induced NK cell expression of CD40L. For example, human IL-2-activated and CD40L-expressing NK cell lines and NK cell clones killed CD40-transfected but not CD40-negative P815 cells. Similarly, P815 target cells were killed by normal peripheral blood NK cells, which had been cultured for 18 hours in the presence of IL-2, but not by CD40L-negative fresh NK cells. Moreover, cross-linking of CD40L on IL-2-activated NK cells induced redirected cytolysis toward CD40-negative and otherwise resistant P815 cells. NK cell-mediated cytolysis of transporter associated with antigen processing (TAP)-deficient T2 cells could be blocked by anti-CD40 antibodies as well as by reconstitution of MHC class I expression, indicating that the CD40-dependent pathway for NK activation can be downregulated, at least in part, by MHC class I molecules on the target cells [193].

An initially puzzling finding of protection against challenge with pulmonary metastases was made in mice that had been immunised with DC *not* loaded with tumour antigens. Although this protection was mediated by NK cells, it was durable and depended also on the presence of $CD4^+$ T cells. However, tumour protection did not depend on CD1d-expressing DC that induce NKT cells but rather on CD1d-negative DC that expressed CD40, CD80 and CD86, which suggested a role for CD40L/CD40 interactions in the tumour protection [194].

However, another study showed that DC-NK cross-talk primed anti-tumour $CD8^+$ CTL and thus bypassed the need for $CD4^+$ T cells and CD40L/CD40 interactions. Bone marrow-derived DC, which were not loaded with tumour-derived antigens, resulted in the rejection of A20 leukaemia cells and induced long-term tumour-specific memory. Neither $CD4^+$ T cells nor CD40 expression were required for primary tumour rejection or long-term CTL memory. Primary tumour rejection required both $CD8^+$ CTL and NK cells, and NK cell-mediated rejection depended on A20 expression of NKG2D ligands. It was hypothesised that anti-tumour memory CTL, which were directed against A20-expressed antigens, were induced because DC activated NK cell, which in turn produced IFNγ and primed DC to secrete IL-12 and thus activate CTL [192].

1.5.5 CD40L/CD40 interactions and adaptive immunity

1.5.5.1 The role of the CD40L/CD40 interaction in tolerance and immunity

The signals responsible for immune recognition and subsequent immune activation were decoded by Doherty and Zinkernagel [195 196 197], Bretscher and Cohn [198], and Lafferty and Cunningham [199]. Doherty and Zinkernagel discovered that cellular immune recognition of virally infected cells was restricted by major histocompatibility complex molecules, which represented signal 1. Bretscher and Cohn postulated that a second signal was needed to help activate B cells. Lafferty and Cunningham postulated that two signals were supplied by APC to activate T cells, and the second signal was antigen-independent and species-restricted. Moreover, Schwartz and co-workers discovered that delivery of signal 1 in the absence of signal 2 resulted in sustained hyporesponsiveness or tolerance of T cells [200 201].

In a simplified schema of immunological responsiveness (Figure 3), tolerance is an active state of acquired immunological non-responsiveness (Figure 3A) whereas immunity describes the state in which activation of the immune system generates a productive immune response

(Figure 3B). Costimulatory molecules have the central role in determining lymphocyte activation or tolerance. Immune tolerance may result from the deletion of T cells, T cell anergy or the generation of regulatory T cells. Illustrated is T cell anergy (Figure 3A) in which tolerance is produced because the immune recognition event (signal 1) occurs in the absence of immune activation (addition of signal 2). On the contrary, productive immunity results when immune recognition is coupled with activation (Figure 3B) leading to clonal expansion and the recruitment of other immune effector elements such as killer T cells and B cell production of antibodies (Figure 3C).

1.5.5.2 Dendritic cells, cross-presentation, cross-priming and cross-tolerance

Cross-presentation is the immunological phenomenon whereby exogenous antigens gain access to the MHC class I antigen-processing machinery that is otherwise reserved for processing of endogenous viral or transformed cellular antigens for presentation to $CD8^+$ CTL. In mice, the $CD8^+$ DC is the critical cell type responsible for cross-presentation. Cross-presentation may serve the physiological purposes of (i) cross-priming CTL to infections or malignant transformations of cell types other than DC [202] [203], and (ii) cross-tolerising lymphocytes to normal tissue antigens [204]. Recent studies have begun to elucidate the mechanisms underlying the phenomenon of cross-presentation. Cross-presented antigens may either be particulate or cell-associated and taken up by DC into phagosomes using phagocytosis or soluble and taken up by DC into endosomes using pinocytosis. Particulate [205] and cell-associated antigens are presented very efficiently [206] whereas soluble antigens are presented much less efficiently [205]. Ultimately, in each case, the antigen is delivered to organelles that contain endoplasmic reticulum (ER), which supplies the complete antigen processing machinery. While the peripheral ER-like phagosomes are already competent for presentation, soluble antigens utilise a different antigen presentation pathway [205]. Soluble protein antigens are internalised by macropinocytosis and enter the lumen of the ER *via* early pinosomes. Dedicated machinery in the ER retrotranslocates the endocytosed antigen to the cytosol where it is degraded by proteosomes to peptides, which are then translocated to the ER *via* the TAP complex for loading onto MHC class I molecules and eventual cell surface presentation to $CD8^+$ T cells [207] (Figure 4). This constitutive retrograde transport process may facilitate DC-mediated peripheral tolerance [205].

Nevertheless, the immune system rarely develops pathogenic autoimmune responses to self antigens that are released by dead cells. High-avidity autoreactive T cells are generally deleted in the thymus to produce central tolerance. Some low-avidity autoreactive T cells may escape central tolerance and circulate in the lymphoid tissues. However, autoimmune disease is prevented because either the self antigen is 'ignored' [208] [209], or a peripheral tolerance mechanism such as cross-tolerance operates [203]. Cross-tolerance results from re-presentation or cross-presentation of antigen by tolerogenic APC. It is proposed that in the steady state, APC in peripheral tissues constantly capture self antigens, which may be generated by a low turnover rate of apoptotic cell death in the tissue. These cells then traffic constitutively to the draining lymph nodes where they exert cross-tolerance *via* either the deletion or anergy of autoreactive T cells or the induction of regulatory T cells [157]. Consequently, noxious autoimmune responses to self antigen, which are released during tissue damage caused by infection, are prevented by the previously established state of antigen-specific tolerance [204]. A cross-tolerance mechanism is probably exploited by growing tumours to evade an immune response [210].

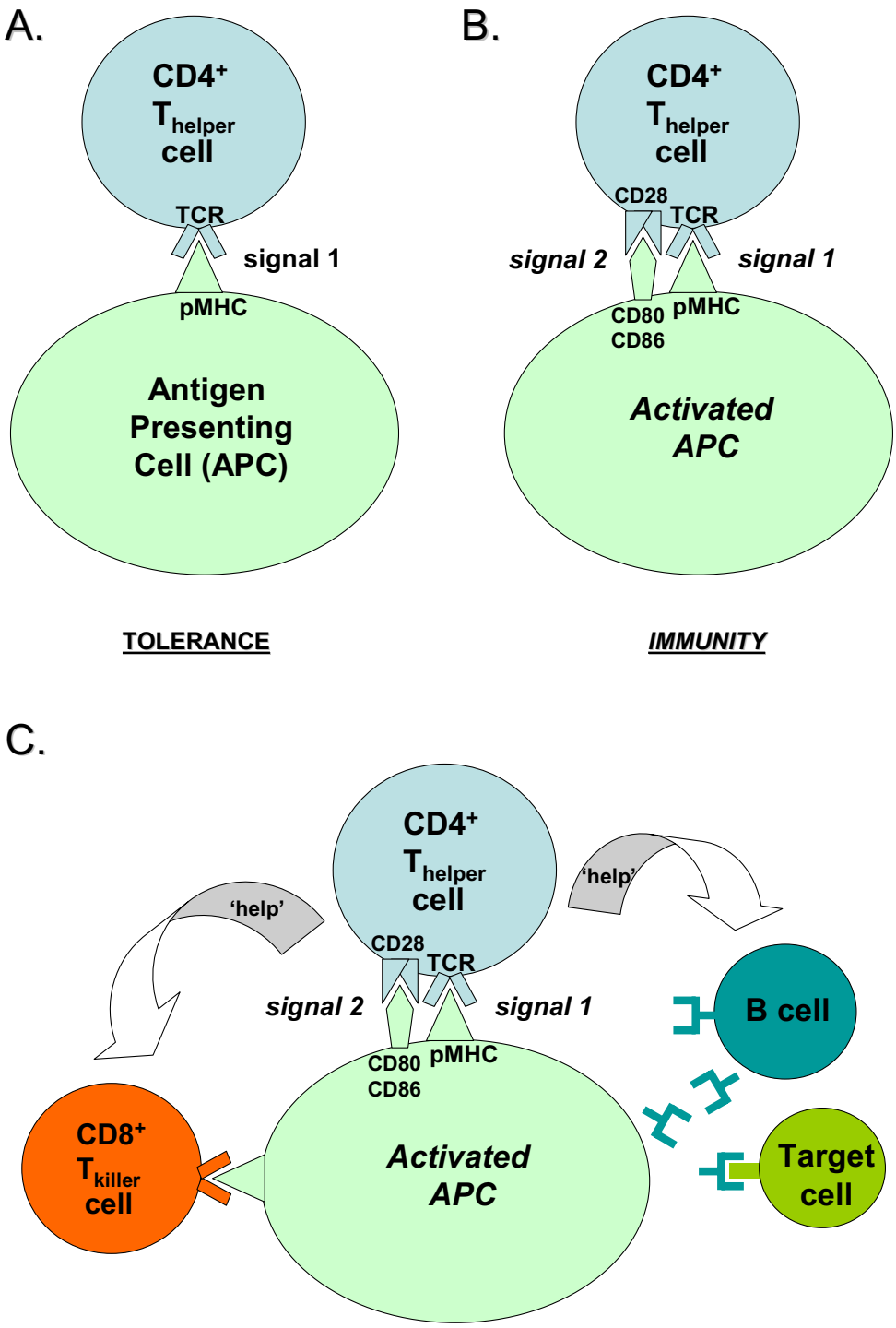


Figure 3: Costimulation results in productive immunity and prevents tolerance induction

Once costimulation has resulted in sustained $CD4^+$ T cell expression of CD40L, the T cells clonally expand and CD40L/CD40 and additional costimulatory interactions produce the full panoply of adaptive immune responses. More DC become licensed by CD40L-expressing $CD4^+$ T helper cells to engage $CD8^+$ T cells in a cognate interaction, and so produce clonal expansion of cytolytic effector killer cells with eventual formation of memory $CD8^+$ T cells. In response to thymus-dependent antigens, CD40L-expressing $CD4^+$ follicular T helper cells interact with DC and B cells to initiate the germinal centre reaction with subsequent production of mature isotype-switched and high-affinity antibodies and memory B cells. Depending on the cytokine balance in the milieu of the draining lymph node, CD40L/CD40 interactions tend to polarise the developing immune response toward a type 1 cell-mediated immune response, which engages and activates innate immune cells such as macrophages and NK cells (not shown).

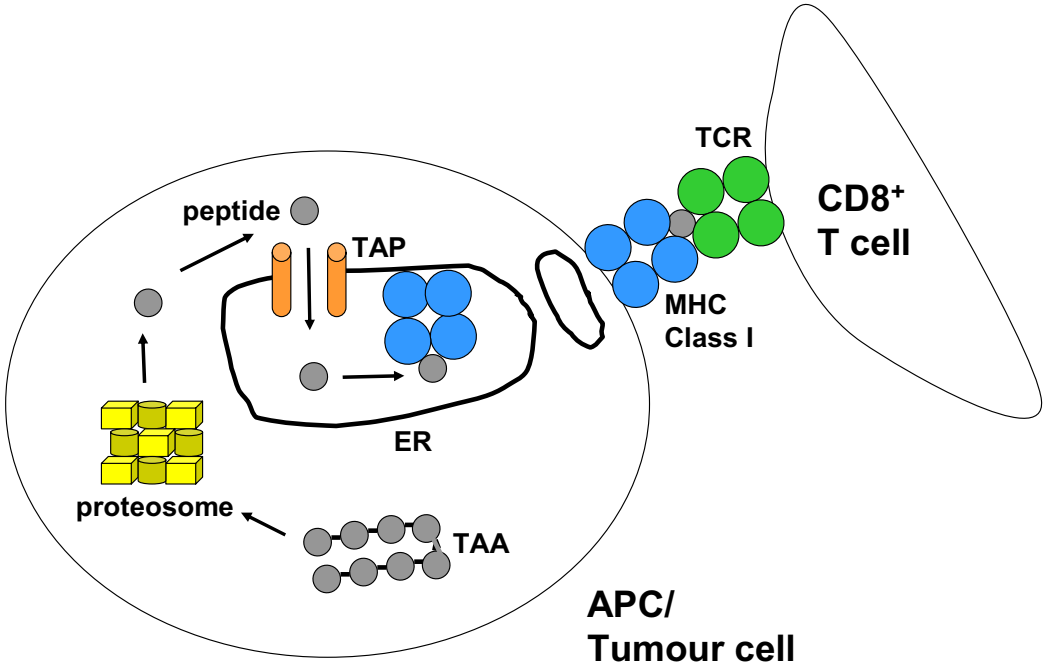


Figure 4: Antigen processing and presentation *via* the IFNγ-responsive MHC class I pathway

Intracellular antigens include tumour associated antigens (TAA). TAA may be processed endogenously in a tumour cell and presented directly to a T cell or, after phagocytosis, TAA may be processed by an antigen presenting cell (APC) and cross-presented to a T cell. TAA may first be processed by a proteosome, which becomes an immunoproteosome in response to IFNγ by changing its composition and generating a different range of peptides [211]. Finally, peptides are translocated by the IFNγ-responsive transporter associated protein (TAP) from the cytosol to the endoplasmic reticulum (ER) where they are loaded onto MHC class I molecules for passage to the surface of the APC or tumour cell, and presentation to the TCR of a MHC class I-restricted $CD8^+$ T cell.

1.5.5.3 Dendritic cells, CD40L/CD40 interactions and peripheral tolerance

Murine DC obtained from long-term *in vitro* cultures down-regulate expression of CD40 and spontaneously produce IL-10, but not IL-12, and induce hyporesponsiveness of alloreactive T cells both *in vitro* and *in vivo* [212]. Cells freshly isolated from normal murine prostate after treatment of the mice with the DC expansionary cytokine, Flt-3 ligand, are unresponsive in an allogeneic mixed lymphocyte reaction unless stimulated with soluble CD40L [213], which suggests that the 'default state' of DC including those residing in tissues is immaturity, and thus this state contributes to the maintenance of peripheral tolerance [214]. CD40 stimulation of human monocyte-derived DC produces sustained translocation to the nucleus of the RelB/p50 heterodimer of the alternative NFκB activation pathway and is essential for T cell activation. In contrast, TNF produces briefer NFκB activation that, however, becomes sustained with blockade of autocrine IL-10 [215]. CD40 expression is up-regulated when microbial signals stimulate DC to migrate to draining lymph nodes. A feed-forward loop then links CD40-stimulated NFκB activation with DC expression of CD40 [216].

CD40 may also play an important role in the generation of regulatory $CD4^+$ T cells that are thought to maintain peripheral self-tolerance *in vivo*. RelB activation is required for myeloid DC differentiation. On the other hand, when RelB function is inhibited in antigen-exposed DC, cell surface CD40 expression is lost and the DC prevent priming of immunity and suppress previously primed immune responses [217], which is the same phenotype exhibited by CD40-deficient DC [218]. Regulatory $CD4^+$ T cells, which are induced by the RelB-inhibited DC, transfer antigen-specific and IL-10-dependent 'infectious' tolerance to primed recipients. This report indicated that the state of CD40 signalling, which converges on the alternative

NFκB pathway, regulates the decision between tolerance and immunity that is inherent in antigen-loaded DC. These data support a model of peripheral tolerance, which is maintained by the constitutive traffic of NFκB-silenced DC from immunological periphery to secondary lymphoid tissue [219] [214]. Interestingly, in response to microbial stimuli and pro-inflammatory cytokines *in vitro*, monocyte-derived DC from CD40-deficient patients mature to show expression of CD83 and DC-LAMP. But in comparison with normal DC, CD40-deficient DC have reduced surface expression of MHC class II, secrete lower levels of IL-12 and higher levels of IL-10, and demonstrate poor costimulation of naïve allogeneic T cells. In addition, CD40-deficient PDC produce little IFNα in response to HSV-1 infection [218]. Pharmacological inhibition of RelB transcriptional activity in a Burkitt lymphoma cell line produced very similar effects in that expression of CD40 and MHC class I molecules was suppressed. Hence, the RelB transcriptional activity of the alternative NFκB pathway seems to mediate a positive feedback loop that facilitates productive APC/T cell interactions [220].

NFκB-inhibited CD40-negative $CD86^{lo}$ MHC class II^{+} DC resemble the CD40-negative $CD80/86^{lo}$ DC, which constitutively populate resting tissues and lymphoid organs *in vivo* and which may retain their phenotype by constitutive ingestion of apoptotic bodies. Low-level expression of CD80/CD86 by DC is NFκB-independent in contrast to DC expression of CD40, which is NFκB-dependent. However, up-regulation of the expression of CD80/86 or CD40 by DC in response to TLR signalling depends on NFκB activation. Deriving from *in vitro* data, a model has been proposed in which the function of NFκB-inhibited CD40-negative $CD86^{lo}$ MHC class II^{+} DC is to prime, ready or sensitise T cells, which is evident as TCRζ phosphorylation, calcium mobilisation, weak CD40L expression, growth arrest and lack of IFNγ production. Then additional signals can be received *in vivo* that will result in the induction either of regulatory T cells or effector T cells. In this model, it is proposed that rather than CD40-negative DC initiating a default pathway for regulatory T cell development, transient weak expression of CD40L by T cells in response to CD40-negative $CD86^{lo}$ DC may enable the T cells to engage B cells or PDC and confer upon these APC the capacity to induce tolerance perhaps by induction of antigen-specific regulatory T cells. Conversely, activation of NFκB within DC by TLR signalling, for example, up-regulates DC expression of CD40, which provides a crucial subsequent stimulus for T cell proliferation and IFNγ production [121].

Moreover, if the same considerations apply to tumour tissue where additional mechanisms may down-regulate CD40 expression on intra-tumoral DC then failure to activate NFκB may reinforce peripheral tolerance to tumour associated antigens (TAA). However, a therapeutic course of action suggests itself if NFκB-silenced intra-tumoral DC, which may be additionally loaded with apoptotic tumour cells engendered by cytotoxic chemotherapy, can be converted to fully competent APC by CD40 stimulation [221].

Consequently, an emerging paradigm in cancer immunology suggests that while common immunological processes underlie immunity against infectious pathogens, autoimmunity and cancer immunity, the control settings that govern the degree of immunological responsiveness in each set of disease processes is different. In this sense, whereas tolerance and immunity may be considered flip sides of the same coin, so may cancer immunity and autoimmunity also be considered. It may be inferred that the same controls and checkpoints that protect against autoimmunity provide the most parsimonious explanation for the lack of immunity toward tumours (Figure 5).

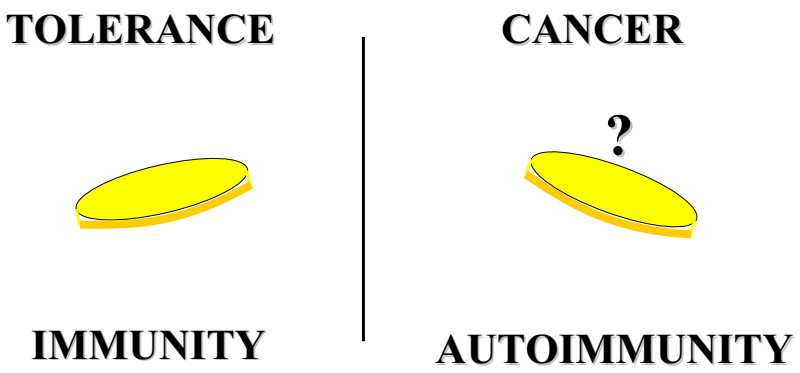


Figure 5: Can cancer be considered the immunological 'flipside' of autoimmunity?

Immunological tolerance is an active process that depends on antigen recognition but is marked by consequent failure of immune activation. It is an appropriate response to self antigens. In contrast, immunity is the product of immune activation, and results in an initial primary immune response and a characteristic anamnestic secondary immune response. Autoimmunity is inappropriate immune activation that results in immunity directed at self antigens. On the other hand, cancer is characterised by altered self antigens but immune responses against these antigens results in tolerance.

1.5.6 The role of the CD40L/CD40 interaction in T cell physiology

The central function of CD40L is to bind the surface CD40 receptor of professional APC such as DC, macrophages and B cells to enhance their antigen presenting, costimulatory and effector functions. CD40 ligation of DC is crucial for the initiation of T cell-dependent immune responses to many pathogens and tumours, which produce both cytotoxic (killer) T cells and class-switched high-affinity antibodies.

1.5.6.1 CD40L/CD40 interactions are required for T cell priming

The specificity of the cognate interaction between the TCR on the $CD4^+$ helper T cell and the pMHC complex on the surface of the DC determines the strength of signal 1, which initiates the process of T cell activation. The strength of TCR signalling determines the magnitude of the calcium flux that activates IL-2 secretion and the T cell surface expression of CD40L. Using the technique of extended four-dimensional confocal imaging of a lymph node *in vivo*, naïve $CD4^+$ T cells were observed to have a long-lived attachment (of at least 15 hours duration) to a single antigen-pulsed DC. This union was associated with immunological synapse formation and evidence of immune activation, and is consistent with a model of lymphocyte activation requiring reciprocal communication between the two cell types [222]. Sustained T cell surface expression of the non-cognate molecule, CD40L, is contingent on the specificity of signal 1 and is reinforced by the positive feedback of up-regulation of CD40, and CD80/CD86 that engage CD28. All of these interactions occur within the 'safe harbour' of the immunological synapse (Figure 6).

For example, the crucial role played by CD40L/CD40 interactions in T cell priming was demonstrated for cellular immune responses to adenoviral challenge. Anti-adenoviral immune responses depend first on $CD4^+$ T cell activation before adenovirus-specific $CD8^+$ effector CTL and B cells can be generated. An adenoviral vector expressing the *lacZ* reporter gene was given by intra-tracheal administration to CD40L-deficient mice and, unlike wild type mice that demonstrated transient transgene expression, resulted in pulmonary and hepatic transgene expression lasting at least 28 days. Anti-adenoviral neutralising antibodies were not

detected in the CD40L-deficient mice. Second administration of an adenoviral vector expressing a different reporter gene was possible at 28 days and resulted again in stable transgene expression. *In vitro* T cell assays indicated that failure of $CD4^+$ T cell priming was responsible for the lack of B cell and CTL responses [223].

1.5.6.2 CD40L/CD40 interactions are required for T helper 1-type immune responses

After encounter with antigen-laden DC in lymph nodes, naïve T helper (Th) cells differentiate into one of several functional subsets that differ in their cytokine secretion patterns and effector functions. Th1 cells secrete IL-2 and IFNγ and promote cellular immunity by activating CTL, NK cells, and macrophages. In contrast, Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 to induce immunoglobulin class switching to IgE and to promote eosinophil-mediated inflammation. Th3 or T regulatory (Tr1) cells secrete IL-10 and TGFβ and control Th1 and Th2 responses to limit pathogen-induced immunopathology [224]. The balance between these Th subsets determines the nature, strength, and duration of immune responses [225 226].

Treatment with anti-CD40L mAb in murine models of arthritis and graft *versus* host disease, which are considered typical of Th1-mediated immunopathology, impaired Th1 function whereas typical Th2 responses were unaffected. These data suggest that CD40L/CD40 interactions are more important for Th1 than Th2 T cell development [227]. In addition, CD40L/CD40 interactions were found to be necessary for *in vivo* priming of IFNγ-secreting Th1 cells in a murine model of experimentally induced and Th1-mediated colitis. Anti-CD40L mAb treatment during the induction phase of the disease, but not once the disease was established, prevented colitis by inhibition of IL-12 secretion by APC in the inflammatory tissue of the bowel wall [228].

In vitro studies have established that IL-12 produced by DC is the dominant cytokine involved in the development of the IFNγ-producing T cells that contribute to Th1-directed cell-mediated immune responses [229]. After activation by cell surface CD40L, *in vitro* generated human DC develop features consistent with the mature interdigitating DC of the lymph node. CD40 ligation improves DC survival, confers dendritic morphology, increases surface expression of MHC class II and CD58 (LFA3) molecules, and CD80 and CD86 costimulatory molecules, high-affinity IL-2Rα (CD25), and increases secretion of TNF, IL-8, and MIP-1α [230], which is a chemokine known to induce the migration of activated and memory $CD8^+$ T cells [231]. Moreover, CD40 ligation of human DC *in vitro* greatly increases production of bioactive IL-12, and stimulates proliferation and IFNγ production by T cells [232]. Together, these CD40L-induced changes simulate the physiological maturation of DC. Further *in vitro* experiments suggest that control of IL-12 production by C40L/CD40 interactions occurs at the level of the DC. In particular, IL-10 reduces CD40-induced secretion by DC of IL-12p40, which is required for production of the bioactive heterodimer, IL-12p70. Hence, together with IL-4, which also inhibits IL-12 secretion, IL-10 may favour Th2 development [227].

While neither microbial stimuli nor CD40 ligation alone is sufficient to elicit IL-12 production by DC *in vivo*, IL-12 is produced efficiently if microbial stimuli are administered first. After DC activation by microbial stimuli, DC expression of CD40 increases together with DC production of IL-12p40. Subsequent CD40 ligation significantly increases DC production of IL-12p35 together with IL-12p70 [233]. In particular, CD40L/CD40 interactions are necessary for production of IL-12 by $CD8\alpha^+$ DC and subsequent induction of Th1 cell proliferation in mice [126]. The dependence of DC activation on the sequence of innate immune stimulation followed by cognate interaction in association with CD40 ligation is consistent with the progression of DC activation that is mediated by the classical then the alternative pathways of NFκB activation [134 188]. Hence, infectious pathogens such as bacteria, protozoa, or viruses may activate DC to drive the Th1 phenotype *via* DC-T cell interactions that then incorporate T cell-derived CD40L [230 232], which is a vital component of the Th1 response during infection [234].

CD40L/CD40 interactions are also critical for the T cell-dependent activation of monocytes and macrophages. The stimulation of CD40 on human peripheral blood monocytes with soluble CD40L *in vitro* increased cell survival, cell surface expression of MHC class II, CD54 (ICAM-1), CD86, and CD40 molecules, and secretion of TNF, IL-1β, IL-6, and IL-8. Together with the observation that IFNγ increases monocyte expression of CD40, positive feedback of type 1 cytokines on CD40 activation would be expected to reinforce and prolong inflammatory responses [235]. CD40-dependent stimulation by previously activated T cells of antigen-bearing monocytes or macrophages induces the secretion of IL-12, which would favour the persistence of Th1 immune responses [236]. IL-12 production by murine macrophages requires both CD40L/CD40 interactions and the cytokines, GM-CSF and IFNγ, and IL-12-secreting macrophages in turn were required for Th1 cell proliferation [237].

The CD40L/CD40 interaction represents the 'help' given by the helper $CD4^+$ T cell *in vivo* to activate the killer $CD8^+$ CTL and depends on the intermediary step of ligation of CD40 expressed by DC (Figure 3). Although a subset of $CD8^+$ CTL express CD40, *in vivo* priming of CTL still depends on ligation of CD40 expressed by DC [238]. To deliver CTL activation, cell-cell contacts both between the $CD4^+$ T cell and the DC, and the $CD8^+$ CTL and the DC, are required. These shared, although not necessarily simultaneous, interactions are made in the lymph node with an antigen-laden DC that has recently arrived from the immunological periphery. An MHC class II-restricted cognate interaction of the $CD4^+$ T cell with the antigen-loaded DC initiates T cell activation resulting in expression of CD40L on the helper $CD4^+$ T cell surface. Helper T cell-derived CD40L then signals to produce a fully competent DC that is conditioned to supply the activating signals to the $CD8^+$ CTL when it engages in an MHC class I-restricted interaction with the same DC. These events, which have been termed 'DC licensing' of the killer cell, provide the lymph node-based mechanism by which a rare antigen-specific $CD4^+$ T cell signals to an equally rare $CD8^+$ CTL. The signal that unifies this trinity of helper T cell, killer T cell, and DC, is CD40 ligation [185 186 187].

For $CD8^+$ T cells, signals 1 and 2 are sufficient for proliferation and IFNγ production but activated CTL but $CD8^+$ T cell tolerance may result if clonal expansion is not sustained. Instead, a third signal, IL-12 or IFNα, is required for strong clonal expansion (i.e. survival), cytotoxic effector function, and memory by naïve CTL. CD40-dependent up-regulation by DC of signal 3 cytokines permits full activation of CTL, which although they do not produce their own IL-2, will continue to expand if antigen remains present and IL-2 is provided by $CD4^+$ T cells. Then, once antigen is cleared, these CTL will finally convert to responsive memory cells. However, antigen persistence in the absence of IL-2 prevents the development of a responsive memory population [239]. This result is redolent of observations made about proliferating but non-cytolytic tumour antigen-specific $CD8^+$ T cells in tumour bearing mice [240 241]. In addition, using the read-out of IFNγ production by $CD4^+$ T cells as a measure of Th1 effector function, Mescher *et al.* previously suggested that IL-1β could act as a third signal for $CD4^+$ T cells [242]. The mandatory contribution of signals other than antigen to T cell activation supports the current model in which interaction of the innate and adaptive immune systems determines the outcome of antigen exposure not only at sites of tissue inflammation and destruction, but also at the time of antigen presentation [243] (Figure 6).

1.5.6.3 CD40L/CD40 interactions are required for tumour-specific T cell priming

The essential function of CD40 ligation in the initiation of cell-mediated and humoral immunity was confirmed by studies of mice, which were immunised with a tumour vaccine and which subsequently failed to mount protective systemic immunity against tumour challenge if the CD40L/CD40 interaction was impaired. CD40L blockade using anti-CD40L mAb fails to protect mice against subcutaneous growth of MCA 105 cells after prior immunisation with irradiated MCA 105 cells. *Corynebacterium parvum* (*C. parvum*) is a tumour vaccine adjuvant that is at least as effective as cytokine-transduced tumour cells in the generation of protective immunity. However, CD40L/CD40 interactions were required for a *C. parvum*-enhanced MCA 105 tumour cell vaccine to generate protective tumour immunity.

GM-CSF gene-modified B16 melanoma cells were also used as a tumour vaccine. GM-CSF contributes to the recruitment and enhancement of the antigen presenting function of DC at the immunisation site. Again, CD40L blockade prevented the development of protective tumour immunity. Similar results were obtained in CD40-deficient mice after immunisation with a *C. parvum*-enhanced TS/A tumour cell vaccine failed to protect mice against parental tumour challenge. These data suggest that CD40L/CD40 interactions are necessary for the development of $CD4^+$ and $CD8^+$ T cell responses that are normally protective after immunisation with the MCA 105 and B16 tumour vaccines [244].

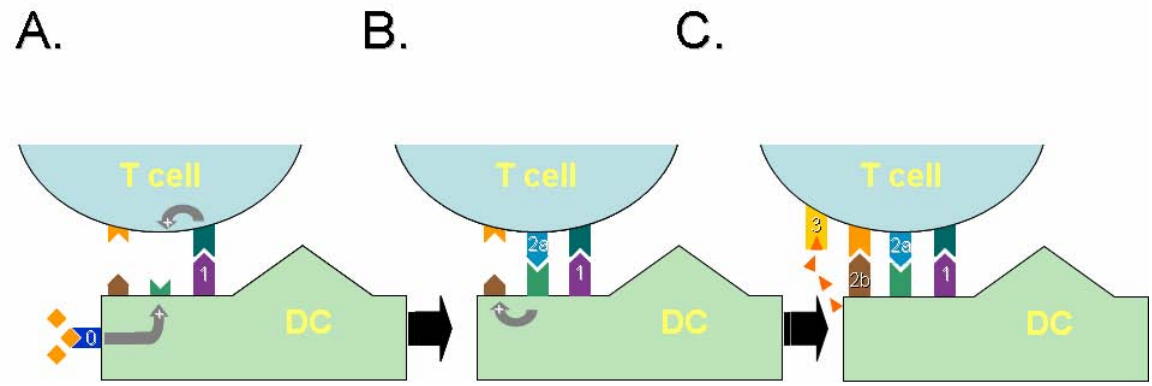


Figure 6: Conditional and reciprocal interactions prime T cells

(**A**) The dendritic cell (DC) is the first point of contact between pathogens (◆) and the immune system. Microbe-associated molecular patterns bind to pattern recognition receptors such as Toll-like receptors (TLR) (0) on the DC surface and activate the classical NFκB signalling pathway to up-regulate expression of CD40 on the DC surface. The subsequent cognate interaction between the peptide-MHC complex on the DC and the T cell receptor on the T cell provides signal 1, which initiates T cell activation and the induction of T cell surface expression of CD40L (1). (**B**) CD40L (2a) binds CD40 on the DC to up-regulate expression of costimulatory molecules such as CD80 and CD86 (2b). (**C**) These costimulatory molecules interact with CD28 on the T cell surface to provide signal 2 that completes T cell activation. In addition, after CD40 ligation, the DC secretes IL-1β, or IL-12 and/or IFNα to supply signal 3 that enables full effector function and memory formation of $CD4^+$ T cells and $CD8^+$ T cells, respectively (3).

Since both $CD4^+$ and $CD8^+$ T cells were required for protection against live MCA 105 sarcoma challenge [244], further studies suggested that CD40L blockade prevented development of protective tumour immunity to the *C. parvum*-enhanced MCA 105 tumour cell vaccine because Th1 priming failed. After CD40L blockade, little IFNγ was produced in tumour-restimulated cultures of cells derived from the draining lymph nodes of vaccine-primed mice. It was hypothesised that T cell priming may depend on stimulation of CD40 on DC. After prior immunisation with a vaccine comprising irradiated TS/A tumour cells, approximately 50% of wild type mice were protected against live tumour challenge whereas virtually no CD40-deficient mice were protected. In the next experiment, wild type mice were immunised again with this vaccine, which had been mixed on this occasion with CD11c-enriched DC that expressed CD40 or not. Although almost 90% of tumour-challenged mice were protected if the vaccine mixture contained $CD40^+$ DC, only about 10% of mice were protected if CD40-deficient DC were mixed with the vaccine instead. These data indicate both the superior potency of vaccine preparations that contain DC and the requirement for DC to express CD40 if the vaccine is to confer protective tumour immunity [245].

Immunisation with live MB49 murine bladder cancer cells is sufficient to protect against live tumour challenge in virtually all mice if immunisation is performed in the foot pad and then the tumour-inoculated leg is amputated 10 days after immunisation. Conversely, CD40L blockade prevented tumour protection in most of the mice immunised in this way. Tumour protection was partially restored if the immunising MB49 tumour cells expressed IL-12

because then little more than half of the immunised mice were protected. Hence, the ability of IL-12 to bypass the requirement for CD40L/CD40 interactions indicated that a major immunostimulatory function of CD40 ligation on APC is the production of IL-12, which subsequently directs tumour protective Th1 responses. However, these experiments did not exclude a role for IL-12 in the activation of NK cells [245].

Subsequent studies further defined the vital T helper function as the supply of IL-12 by DC. These data also indicated that CD40 ligation was required to generate fully competent antigen presenting DC because although transgenic expression of GM-CSF up-regulated expression of CD80 and CD86 costimulatory molecules, blockade of CD40L/CD40 interactions *in vivo* prevented optimal T cell priming after immunisation with a GM-CSF gene modified B16 tumour vaccine [244 245]. However, protective anti-tumour immunity was restored in CD40-deficient mice given CD40-expressing DC with the tumour vaccine, which showed that CD40 signalling in DC was required for successful tumour vaccination. IL-12 expression by the tumour vaccine induced systemic tumour immunity in CD40L-deficient mice, which suggested that CD40L/CD40 interactions activate DC to provide IL-12 required for Th1-mediated anti-tumour immunity [245]. Together, these reports demonstrated that CD40L/CD40 interactions were essential for protective tumour immunity in murine subcutaneous transplantable tumour models and supplied a sound rationale for testing the transgenic expression of CD40L in tumour vaccines without the requirement for CD40 expression by the tumour cells.

1.5.7 The generation of antibody diversity

The study of the primary immunodeficiency diseases clearly indicates the need for high-affinity antibodies to protect against lethal bacterial infections. The development of the antigen recognition repertoire of the BCR and its secreted protein product, the antibody, occurs as a two-step process. In the first T cell- and antigen-independent step of stochastic recombination, the primary antibody repertoire is generated in the primary lymphoid organs of the foetal liver and bone marrow. In each immature B cell, an active V gene locus is created from the random rearrangement of hundreds of germ line-encoded variable (V) and dozens of diversity (D) and joining (J) elements within the heavy and light chain immunoglobulin genes. Thus, the diversity of the primary repertoire depends on the number of rearranged heavy and light chain genes. The rearranged V gene locus of each heavy and light chain immunoglobulin gene encodes a β-pleated sheet secondary protein structure, which together create a β-sandwich tertiary protein structure. The β-sandwich of the variable domain of each heavy and light chain constitutes a stable framework for the variable loops arising from the end of each sheet. These loops comprise the three complementarity determining regions (CDR) of each variable domain, which interact with the antibody binding site on the antigen. The CDR from the heavy and light chain of each antibody dimer may be considered as three fingers from each hand holding an apple, which is the antigen.

The second T cell- and antigen-dependent step of antibody maturation produces the secondary repertoire of effective antibodies. Antibody maturation is accomplished by the processes of class switch recombination (CSR) and somatic hypermutation (SHM), which occur independently of each other in the GC of the secondary lymphoid organs of the spleen, lymph nodes and mucosal associated lymphoid tissues. CSR generates the different classes of antibodies each of which is distinguished by a separate set of effector functions. SHM provides the substrate for the selection of high-affinity antibodies [246]. CSR and SHM both occur in the GC and have several processes in common, which include activation after the engagement of CD40 and BCR, and transcription and cleavage of target DNA in the switch (S) and variable (V) regions of the genomic DNA of the immunoglobulin loci, respectively, although different DNA repair processes operate in each case [247]. Immature B cells, which express cell surface IgM, mature when they acquire cell surface IgD and migrate from primary to secondary lymphoid tissues. There, after its IgM BCR specifically binds antigen, the B cell proliferates in conjunction with the formation of the unique structure known as the GC. In the GC, proliferating B cells remain in close contact with cognate T cells from which

they obtain the signals previously known as T cell help in order to undergo CSR and SHM. It is now known that this T cell help is equivalent in molecular terms to the CD40/CD40L interaction [246].

1.5.7.1 The processes of class switch recombination and somatic hypermutation

CSR is the somatic rearrangement of immunoglobulin heavy chain (IgH) constant (C) region DNA that occurs during B cell activation. CSR results when the S region upstream of the μ constant region recombines with an S region upstream of the γ, α or ε C regions and the intervening DNA is deleted. Thus, the same variable gene, which encodes the antibody recognition domain that confers specificity and affinity, is 'disconnected' from the C region of IgM and 'hooked up' to the C regions of IgG, IgA or IgE. The constant domains of the different classes of immunoglobulin then confer different effector functions on the antibody such as complement fixing ability, and Fc receptor binding that determines recruitment of phagocytes and other leukocytes. CSR occurs in three main steps:

(i) The target DNA is transcribed. Activated T cells secrete cytokines that signal to open the chromatin at S regions so that each cytokine specifically activates an 'I' promoter upstream of each S region and germ-line transcripts are run off the targeted DNA. In humans, for example, IL-4 and IL-13 are specific for Sε or Sγ4; IL10 for Sγ1, Sγ3, and Sα; and TGFβ for Sα.

(ii) The targeted DNA is cleaved in each S region. Cytokine-specific germ-line transcripts form RNA/DNA hybrids on the template DNA leaving the non-template single-stranded DNA to form an R loop that is targeted by activation-induced cytidine deaminase (AID). AID deaminates deoxycytosine (dC) residues of the R loop to deoxyuracil (dU) residues, which are deglycosylated and subsequently removed by uracil-N-glycosylase (UNG). The loss of the uracil residues facilitates access of an endonuclease that creates single-stranded DNA breaks or nicks in the R loop, which are processed subsequently into a blunt-ended double-stranded DNA breaks (DSB) and which are then repaired *via* an unexplained mechanism.

(iii) Specialised DNA repair machinery is engaged to join the DSB at Sμ and the targeted S region together [246 247].

SHM introduces mainly missense mutations and occasionally small insertion or deletion mutations into the rearranged V(D)J gene segments at a frequency of approximately 10^{-3} mutations per base per round of cell division [248]. The mutations are generated mainly within the DNA encoding the CDR of the variable domains of the light and heavy chains to produce amino acid substitutions that alter the affinity of the antibody for its antigenic determinant. Consequently, these BCR variants will result in positive or negative selection of B cells if the affinity for antigen is high or low, respectively. Selection of B cells within the GC occurs in close association with FDC. Again, SHM is a three-step process:

(i) The targeted DNA is transcribed and the mutation rate correlates with transcription efficiency.

(ii) Cleavage of the targeted DNA probably occurs as DSB.

(iii) The repair of the broken DNA ends probably involves mismatch repair enzymes and certain error-prone polymerases. It is during the DNA repair phase that mutations are introduced [246 247].

AID is an enzyme that is critical for both CSR and SHM. AID has been identified as a DNA cytidine deaminase, which preferentially deaminates deoxycytidine to deoxyuridine on the non-transcribed DNA strand during CSR and SHM. Although AID is homologous to an enzyme known to have an RNA editing function, most recent experimental evidence supports a DNA editing rather than a RNA editing function for AID. How AID induces DSB in S regions remains unclear although the two DNA-repair pathways of base-excision repair and mismatch-repair appear to be involved. A model has been proposed to explain how CSR and

SHM behave as physiological examples of DNA damage and repair. The DSB-sensing complex of Mre11, Rad50 and Nbs1 (MRN complex) is recruited to S regions and results in activation of the master regulator of the DNA damage response, ataxia-telangectasia (ATM), and phosphorylation of the histone 2A family member X, H2AX. ATM activation stabilises the MRN complexes at DSB foci and facilitates cell-cycle arrest and DNA repair, and phosphorylated H2AX (γH2AX) may mediate the synapsis of distant S regions. Ligation of the S region DSB results in class switching from Cμ to the downstream Cα, Cγ or Cε, and DNA repair occurs by non-homologous end joining (NHEJ) with consequent excision of the intervening DNA as an episomal DNA circle. Although DSB are a necessary intermediate for CSR, the requirement of DSB for SHM is less clear. Currently, it is suggested that AID may induce DSB with staggered ends, which results in repair by homologous recombination so that somatic mutations are introduced by error-prone DNA polymerases [248].

1.5.8 The role of CD40L/CD40 interactions in B cell physiology

CD40L/CD40 interactions are required for the formation of highly functional antibodies, which have acquired higher avidity *via* SHM and versatile protein domains for complement-mediated cytotoxicity (CMC) and antibody-dependent cell-mediated cytotoxicity (ADCC) *via* CSR (Figure 7). Similarly, CD40L/CD40 interactions are required for the formation of GC mainly in lymph nodes, which are the principal although not exclusive sites of SHM and CSR (Figure 8).

However, as clinical observations indicated (Section 1.6.1.1), CD40L/CD40 interactions are not absolutely required for CSR. For example, interactions between TLR9 and microbial DNA containing hypomethylated CpG motifs may initiate or amplify T cell-independent IgG responses against pathogens. TLR9 is expressed by IFNα-secreting PDC and B cells, and TLR9 signalling on B cells results in B cell activation, proliferation, and IgM production. In conjunction with IL-10, TLR9 signalling results in activation of NFκB and AID, and generation of class-switched Cγ1, Cγ2, and Cγ3 germ-line transcripts. Subsequent IgG production requires additional BCR- and BAFF-derived signals, which are produced by IFNα-exposed DC [249].

CD40 was first identified as a human bladder cancer antigen using a mAb, which cross reacted with the same 50kDa band in B cell neoplasms [250] [251]. Anti-CD40 mAb induced human B cell proliferation. That activated T cells induced B cell proliferation and differentiation in a contact-dependent manner [252] resulted in the natural ligand of CD40 being identified in the plasma membrane fractions of activated T cells as a 39kDa molecule [253], which was soon cloned [254]. When the cloned molecule was expressed by a non-immune cell type and cultured in IL-4 with murine splenic B cells, IgE production [254] indicated that CD40L was the molecule responsible for T cell help to B cells. Identical results were obtained when human CD40L was cloned [90].

After GC B cells undergo somatic mutation and antigen selection, they become either memory B cells or plasma cells. CD40 expression is lost from the fully differentiated plasma cell, which is not an APC but an antibody factory [255]. CD40 stimulation of B cells promotes their survival particularly in GC [255] and prevents the terminal differentiation of B cells to plasma cells preventing immunoglobulin secretion [256]. When purified human GC cells were cultured with IL-2, IL-10, and CD40L-expressing cells, memory-like B cells were generated, and CD40L withdrawal produced terminal differentiation of GC B cells into plasma cells (PC). These results indicate that CD40L directs the differentiation of GC B cells toward memory B cells rather than toward PC [257].

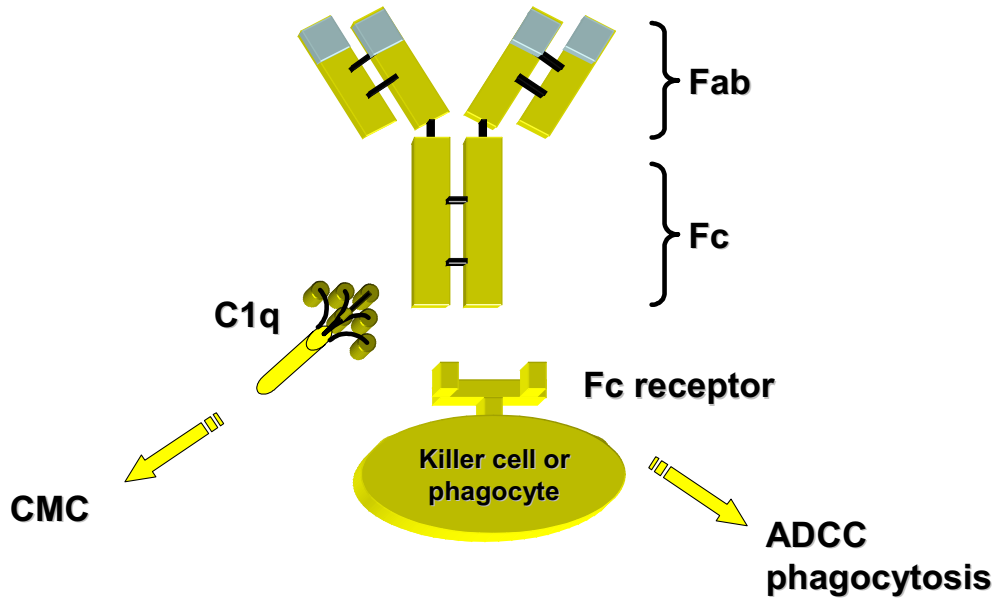


Figure 7: CD40L/CD40 interactions produce mature highly functional antibodies

Antibodies bind to antigenic determinants or epitopes *via* Fragment-antigen binding (Fab) domains and exert effector functions *via* the Fragment-crystalline (Fc) moiety. The Fab domain includes both the immunoglobulin heavy and light chains that each contribute a framework region on which are fixed the loops or complementarity determining regions (CDR), which interact with the epitope. Antibody affinity may be increased during the process of somatic hypermutation as mutations are introduced into the CDR. During the process of class switch recombination, the Fab domains may be assorted with different Fc moieties each having different functional capabilities. For example, in humans, the Fc moieties of IgM, IgG_1 and IgG_3 are the most efficient inducers of complement-mediated cytotoxicity (CMC). Activation of the classical complement pathway is initiated by binding of the first component, C1q, to the Fc region. After opsonising bacteria, for example, *via* the Fab domain, the Fc moiety of IgG can also bind Fc receptors on phagoyctes such as neutrophils and macrophages to initiate phagocytosis. Similarly, antibody-bound cells or micro-organisms are bound by Fc receptors on NK cells to elicit antibody-dependent cell-mediated cytotoxicity (ADCC).

Signalling strength determines the fate of B cells and the modulating signals occur mainly *via* the BCR, CD40 or the receptor for B cell activation factor of the TNF family (BAFF). The strength of BCR signalling depends on the quantity and avidity of antigen. Antigens that are part of immune complexes, or are fixed rather than soluble, or that have repetitive structures will engage BCR with greater intensity. These antigens activate B cells in a T cell-independent manner and so generate low avidity antibodies with limited isotype switching. Conversely, humoral responses to soluble protein antigens require T cell input. Thus, for thymus-dependent antigens, the CD40 receptor may act as a rheostat to determine the intensity, specificity and duration of the B cell response. For example, the reduced strength of CD40 signalling resulting from blockade of CD40/CD40L interactions may terminate the GC response and antibody formation and, similarly, heightened CD40 signalling may abort the GC response [255].

1.5.8.1 T-B interactions and the CD40-dependent germinal centre reaction

Affinity-matured and isotype-switched antibody-forming B cells tend to be generated in the GC and then seed to the long-lived and CD40-negative PC compartment to provide enduring protection against pathogens. Despite their terminally differentiated state, PC may survive in bone marrow and elaborate protective antibody for many years. The transformed equivalent of this cell type is the myeloma cell, which has also down-regulated surface expression of BCR, MHC class II molecules, CD45 and CD19 [255].

CD40 is constitutively expressed on professional APC that are involved in the GC reaction, in particular, DC and B cells. Following immunisation or pathogen challenge in the immunological periphery, thymus-dependent humoral immune responses take place in draining lymph nodes. DC arriving from the periphery present antigen to naïve $CD4^+$ T cells patrolling in the parafollicular T zones and activate them. Subsequently, the activated T cells remain *in situ* or migrate to the border of the B cell follicles where they engage and activate naïve antigen-specific B cells. Some of the activated B cells together with a subset of the activated $CD4^+$ T cells, so-called follicular T helper (T_{FH}) cells, enter the B cell follicles to initiate the GC reaction [80], which results in expansion of the antigen-specific B cell population by both increased proliferation and enhanced survival to generate a differentiated output of memory B cells and long-lived antibody-producing PC [80].

Radiation bone marrow chimera experiments show that the entire multi-step process depends on CD40L/CD40 interactions. Recruitment to B follicles of T_{FH} cells, which are needed for the development of high-avidity antibodies and memory B cells, depends only on the presence of CD40-expressing DC. In order to generate GC, and the formation of isotype-switched and high-avidity antibodies, CD40 expression is required only on B cells. Activated T cells rather than CD40L-expressing B cells comprise the only required source of CD40L [136].

To enable the GC response, and the formation of highly functional antibodies and memory B cells, the following model incorporating an exchange of signals between DC and T cells has been proposed to explain the migration of T_{FH} cells to B follicles. First, DC prime T cells that subsequently up-regulate expression of CD40L. Ligation of CD40 on DC induces up-regulation of the TNF superfamily member, OX40 ligand (OX40L), which in turn stimulates T cell expression of the T cell homing molecule, CXCR5; and confers T cell survival by up-regulated expression of Bcl-2 and Bcl-X_L. TLR ligands, such as LPS and unmethylated CpG DNA motifs that are carried on pathogens such as bacteria in the immunological periphery, may provide alternative DC-activating stimuli to prime T cells. Although T cell priming by TLR-activated DC is evident in T cell proliferation and IgM production, CD40 signalling by DC is required for the acquisition of T helper functions, which are manifest as the formation of GC and IgG. TLR signalling did not result in DC up-regulation of OX40L expression and did not substitute for CD40 signalling in the follicular homing of T cells [80].

However, this model does not yet provide a complete mechanistic explanation of CD40-dependent signalling events in B cell physiology. OX40-deficient mice have a T cell follicular homing defect because T cell expression of CXCR5 is not induced but GC and T-independent antibody responses are normal. Hence, CD40-dependent induction of another APC costimulatory molecule is required to mediate the GC reaction although it remains possible that B cell-derived chemokines may result in the later recruitment of T_{FH} cells to GC [80]. Perhaps, a contribution to the GC reaction may be made by the inducible co-stimulator (ICOS) molecule, which is highly expressed by activated $CXCR5^+$ T cells in GC and which binds constitutively expressed ICOS ligand on B cells. ICOS expression marks a subset of T_{FH} cells associated with follicular helper function [258]. Lack of ICOS produces a profound deficiency in the GC reaction, isotype switching and memory B cell formation in humans. In addition, data generated from ICOS-deficient mice indicate that ICOS has an essential role in the differentiation of $CXCR5^+$ $CD4^+$ T cells. $CXCR5^+$ $CD4^+$ T cells are a marker of GC formation and are absent in the secondary lymphoid tissue and blood of ICOS-deficient mice and humans, and in the blood of CD40L-deficient humans [259].

1.6 The primary immunodeficiency diseases

The story of how some genetic lesions produce discrete forms of immunodeficiency, contribute to the immunopathogenesis of the resulting primary immunodeficiency disease (PID), and drive the search for curative cell and/or gene therapy exemplifies molecular medicine in action [260].

Using the classification scheme developed by an expert committee of the International Union of Immunological Societies, over 100 PID have now been described in most of which the

underlying genetic abnormalities have been identified[261] [262] [260]. The study of PID has provided unique insights into the workings of the immune system and, in particular, its division into cell-mediated and humoral arms [261] [260]. The clinical manifestations of PID are highly variable, and range from susceptibility to microbial infections, allergy, autoimmune diseases and lymphoproliferative disorders [262]. Most PID are rare diseases of childhood with the most severely affected individuals, who combine defects of both cellular and humoral immunity, having the shortest survival. These combined immune defects expose patients to serious and life threatening infections with the full gamut of viral, bacterial, protozoal and parasitic infectious pathogens. Nonetheless, the most prevalent PID are selective IgA deficiency (often asymptomatic) and common variable immunodeficiency (CVID) [263] [264]. The more selective nature of the immune defects associated with these conditions ensure that most affected individuals survive well into adulthood. CVID, in particular, has complex and ill-defined immunogenetics so that an inherited susceptibility together with an acquired component is required for the disorder to manifest fully [263]. The observed mode of inheritance, the extent and severity of the clinical phenotype, and the laboratory findings together form the basis for classifying the PID [261]. Although most of the described PID are transmitted as X-linked or autosomal recessive Mendelian traits, at least ten kinds of PID with autosomal dominant inheritance have been reported [265].

The laboratory abnormalities of deficient T lymphocytes, B lymphocytes, phagocytes or complement have traditionally been used to define PID. However, the growing molecular understanding of PID enables re-classification on the basis of common pathophysiological mechanisms. For example, primary immunodeficiency states may arise from failures of lymphocyte development, cell migration, antigen presentation or immunoglobulin class switch recombination. In addition, defects of DNA repair may shorten survival of lymphocytes and other cell types, and defects in the cytolytic pathway, the oxidative burst and intracellular signalling pathways may impair the killing of both intra- and extra-cellular pathogens. Ultimately, the pathophysiological basis of PID is being understood using both careful clinical studies and experimental studies of gene-disrupted mice [260].

1.6.1 HyperIgM syndromes

The Hyper-IgM (HIGM) syndromes are a rare and heterogeneous group of primary immunodeficiency diseases, which occur with an estimated frequency of 1/500,000 births [247]. In HIGM patients in whom a molecular lesion can be characterised, all genetic lesions affect the CD40L/CD40 signalling axis (Figure 8). CD40 signalling may be defective because CD40L does not bind CD40, or because the CD40 molecule itself or its dependent intracellular signalling apparatus is defective [266]. HIGM was originally described in 1961 when there were two independent reports of boys who had recurrent bacterial infections and 'dysgammaglobulinemia'. Dysgammaglobulinemia was used to describe a deficiency of 7S gamma-globulin (IgG) together with an elevated 19S gamma-globulin (IgM) [267] [268]. Over 200 patients with HIGM have now been reported world wide [269].

There are few clinical differences between CD40L- and CD40-deficient patients, and CD40L and CD40 gene-disrupted mice have virtually identical phenotypes. Together, these observations suggest that CD40L is the only physiologically significant ligand for the CD40 receptor. Nonetheless, mycobacterial heat shock protein-70 and the C4 binding protein are two other ligands for the CD40 receptor that have been identified (Section 1.5.2), which suggests that microbial challenge experiments using CD40L and CD40 gene-disrupted mice may yet result in the discovery of more subtle phenotypic differences to indicate the involvement of other ligands, or other signalling systems.

Because CD40 is expressed by a wide range of cell types from APC to non-haemopoietic cells such as endothelial and epithelial cells, the most severe immunopathology results from genetic lesions of CD40L and CD40, which are proximally located in the signal transduction pathway and which produce defects in both cellular and humoral immunity. Conversely, lesions of B cell-specific signalling molecules such as AID and UNG, which are located downstream of CD40, affect only the processes of CSR and/or SHM and, therefore, produced

isolated defects in humoral immunity. An exception is the genetic lesion of NFκB essential modulator (NEMO) or IKKγ, which is an integral component of the IκBα kinase (IKK) complex of the classical NFκB signal transduction pathway (Figure 8). Because NEMO is expressed more widely than in the immune system, lesions affecting NEMO produce not only a combined T and B cell immune defects but also abnormalities of skin including loss of sweat glands, and abnormal dentition. Nonetheless, all HIGM syndromes have a core element of disordered class switch recombination. The absence of GC is a hallmark of CD40L deficiency [269] whereas GC are hyperplastic in AID deficiency. Together, these data indicate that although AID gene function is not required for GC formation, another CD40-dependent signalling pathway is required [269] (Table II).

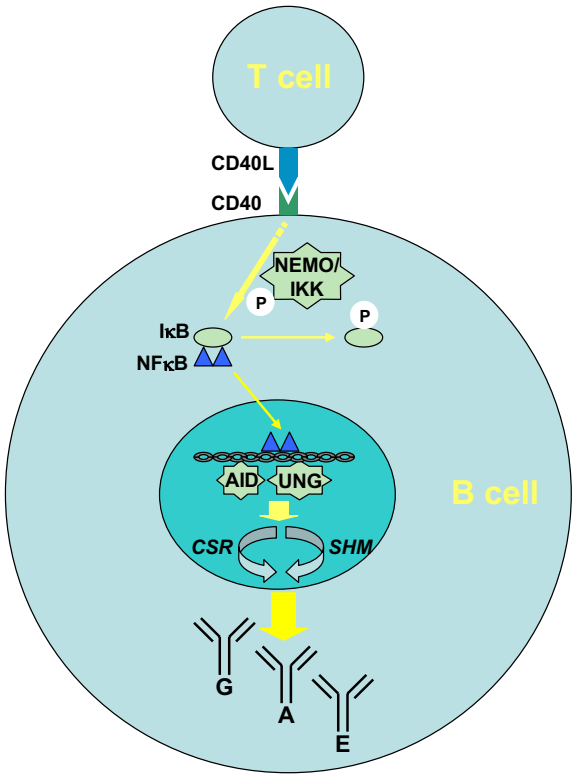


Figure 8: Schematic diagram of CD40-dependent signalling in B cells

CD40 stimulation of B cells produces isotype-switched IgG, IgA and IgE classes of immunoglobulin of high avidity because NFκB activation is associated with activity of intra-nuclear DNA editing enzymes that facilitate isotype switching and somatic hypermutation. The inhibitory factor, IκB, is phosphorylated (P) by the NEMO/IKK complex thus releasing the NFκB transcription factor to translocate to the nucleus. Subsequently, AID and UNG participate in CSR and SHM. AID, activation-induced cytidine deaminase; CSR, class switch recombination; IKK, IκB kinase; NEMO, NFκB essential modulator; SHM, somatic hypermutation; UNG, uracil-N-glycosylase (adapted from refs. 275 and 252).

1.6.1.1 HyperIgM syndromes 1 and 3

The hyperIgM syndromes were numbered in order of discovery. HIGM1 or X-linked HIGM is caused by defective expression or, less commonly, defective function of the CD40 ligand, which prevents CD40L binding to CD40. Peripheral blood B cell numbers and the numbers of T cells in total and in subsets are normal in HIGM1. HIGM1 T cells respond normally to mitogens [266] but not to recall antigens [93]. Identification of the disease locus accelerated identification of the genetic cause of HIGM1 because CD40L was a candidate gene. Five independent research groups identified CD40L gene mutations and defective CD40L expression as the cause of HIGM1 in 1993 [270] [271] [272] [273] [274]. HIGM1 patients comprise approximately 30% of the total number of HIGM cases [269] [266]. Autosomal recessive cases of HIGM comprise the majority of the remaining patients with HIGM of which AID deficiency

or HIGM2 is the most well described example. Nonetheless, HIGM3, which is an autosomal recessive form of HIGM caused by defective CD40 signalling, only comprises <1% of patients with HIGM. Four HIGM3 patients, who came from three families and who have normal CD40L expression and mutations in CD40, have been described [275] [276] [247].

The clinical presentation of patients with HIGM1 and HIGM3 is very similar and the severity and diverse clinical manifestations of the disorders reflect the critical role that CD40/CD40L interactions play in the maintenance of a normal immune system. The phenotype observed in CD40L [190] [191] and CD40 [277] [278] gene-disrupted mice corresponds to the clinical observations made in CD40L- and CD40-deficient patients. Together, the clinical and experimental data indicate that CD40/CD40L interactions are essential for efficient antibody production, B cell memory and APC-derived stimulatory signals that are required for T cell priming and differentiation.

HIGM syndrome	Mode of inheritance	Immune defect	Gene(s) affected
1	XL	Combined (T^- B^-)	CD40L
2	AR/AD	Humoral (B^-)	AID
3	AR	Combined (T^- B^-)	CD40
4	AR	Humoral (B^-)	unknown
	AR	Humoral (B^-)	UNG
HIGM-ED	AR	Combined (T^- B^-)	NEMO

Table II: The HyperIgM syndromes

Hyper-IgM (HIGM) syndromes are numbered in order of discovery. The severity of the clinical phenotype varies according to how proximally located the genetic lesion is in the CD40L/CD40 signalling axis. ED, ectodermal dysplasia; XL, X-linked; AR, autosomal recessive; AD, autosomal dominant; AID, activation-induced cytidine deaminase; UNG, uracil-N-glycosylase; NEMO, NFκB essential modulator.

Affected patients most commonly present in infancy with manifestations of humoral and cell-mediated immunodeficiency. The most common presentation is recurrent bacterial sinopulmonary infections in the first two years of life [269]. Chronic diarrhoea, which is often caused by opportunistic infection with *Cryptosporidium parvum*, is the next most common manifestation [266]. Alain Fischer observed that HIGM1 patients have a unique pattern of susceptibility to infections with particular intracellular organisms such as *Pneumocystis carinii*, *C. parvum* and *Toxoplasma gondii*. Particularly striking is the cholangitis caused by chronic cryptosporidiosis, which predisposes to sclerosing cholangitis, and subsequently in the second and third decades of life to hepatic cirrhosis and failure, and hepatobiliary carcinomas [260]. Consequently, few affected individuals survive beyond the early part of their third decade [266]. How CD40L mediates defence against these micro-organisms is not yet

known but may involve intracellular killing in macrophages *via* CD40 activation, IL-12 production and the engagement of T and NK cells [260]. A hypothesis will be developed to link the critical function of the CD40L/CD40 dyad in the defence against intracellular pathogens to a possible function in the control of inflammation-related neoplasia (Chapter 2). Non-immune manifestations of HIGM1 include chronic neutropenia and associated stomatitis. As the result of the combined cellular and humoral immune deficiencies, the prognosis is clearly worse than that conferred by the selective humoral deficiency of X-linked agammaglobulinaemia, which is more readily treated with intravenous immunoglobulin (IVIG).

The genetic lesions that involve CD40L are heterogeneous and range from point mutations and truncations to in-frame and out-of-frame deletions [279]. Most of the genetic lesions affect the exons encoding the CD40L extracellular domain and result in a defective protein that is either not expressed or expressed in a form that is unable to bind CD40. There is no apparent correlation of the location of the mutations in the CD40L gene and the phenotype [266]. The heterogeneity of CD40L mutations would suggest that many cases may arise *de novo*. Also, the fact that many female carriers of the trait have normal immune function, suggest that carrier detection and prenatal diagnosis may not be useful.

Both HIGM1 and 3 patients have low or absent levels of IgG, IgA and IgE and normal to high levels of IgM [266]. It is believed that the syndrome-defining elevation of serum IgM results from chronic persistent infections because IgM levels tend to fall with IVIG treatment [269]. While signalling that stems from the activation of BCR and CD40 initiates CSR and SHM, CD40 ligation is not absolutely required for these processes. Infrequent observations have been made in CD40L-deficient patients of (i) IgA and IgE in the serum, which indicates that other mechanisms have produced CSR, and (ii) unswitched mutated IgM-bearing B cells, which indicates that SHM has also occurred. T cell independent CSR is known to occur both in response to stimulation with bacterial polysaccharides or viral glycoproteins or to BAFF expressed by cytokine-stimulated DC. Since T cell-independent CSR occurs in the splenic marginal zone and the intestinal lamina propria, the burden of uncleared pathogens in CD40L-deficient patients may contribute to T cell independent CSR. Unswitched (IgM^+) and mutated (memory) B cells are generated in the marginal zone of the spleen in a T cell-independent manner in defence against encapsulated bacteria, which are not engulfed efficiently by phagocytes [247].

1.6.1.2 HyperIgM syndrome 2, UNG deficiency, and hyperIgM syndrome 4

HIGM1 patients lack lymph node GC, which are not only present but hyperplastic in HIGM2 patients [280] [281]. Although the exact mechanism of GC formation is not known, these data indicate that CD40-mediated signalling events independent of AID function are responsible for GC formation. Unlike CD40L deficiency in which CD40 stimulation of B cells induces isotype switching, the isotype switching defect in HIGM2 is intrinsic to B cells and is not associated with opportunistic infections.

AID deficiency is the most common autosomal recessive hyperIgM syndrome and usually causes defective CSR and SHM. The *AIDCA* gene, which encodes the enzyme, is expressed selectively in GC B cells. Mutations are found throughout the *AIDCA* gene and are responsible for susceptibility to recurrent bacterial infections, which reflects selective defects in CSR and/or SHM. Hence, the humoral immunodeficiency is almost fully corrected by IVIG. However, unlike the combined immunodeficiency of HIGM1 and HIGM3, there is gross GC enlargement in HIGM2, which results from an excess of proliferating GC founder cells that express IgM, IgD and CD38. The *AIDCA* gene product seems to suppress this B-cell proliferation, which may be driven by antigens derived from gut flora, for example, and which may be reversible with IVIG treatment. The CSR lesion caused by *AIDCA* deficiency occurs before the DNA cleavage step and may also impair DSB formation in SHM. Interestingly, mutations in the N-terminal domain of the AID protein impair SHM but leave CSR intact, which suggests that a cofactor interacts with the N-terminal domain of the AID protein to mediate SHM. Conversely, mutations in the C-terminal domain of the AID protein

impair CSR and indicate that a cofactor may interact with the C-terminal domain of the AID protein to target the S regions and thus mediate CSR. One model suggests that the short inverted repeats, which are found in the S regions, provide the secondary structure needed for the AID protein to interact with the single-stranded non-template DNA. In turn, the AID protein requires domain-specific cofactors to target single strand DNA in the S and V regions appropriately and enable CSR and SHM, respectively [247].

More recently, another autosomal recessive form of HIGM has been discovered, which results from UNG deficiency and which provides further evidence for the DNA editing function of AID. UNG deglycosylates uracil residues on DNA and forms part of a multimeric complex that functions in DNA base excision repair. UNG deficiency results in an absolute CSR defect associated with defective DNA cleavage in Sμ regions. Although the frequency of SHM is normal, the mutations are biased toward transitions in which G is converted to A and C to T. The biased pattern of SHM may result from the failure of uracil residue removal that permits these residues to be replicated and/or repaired in a faulty manner [247] (see Section 1.5.7).

HIGM4 is an unidentified form of HIGM in which patients have a B cell intrinsic defect with lymphoid tissue hyperplasia, absent GC and a selective block in CSR but normal SHM. HIGM4 is believed to result from a selective defect either in a CSR-specific factor of the DNA repair machinery or in survival signals delivered to switched B cells [282].

1.6.1.3 HyperIgM syndrome associated with hypohydrotic ectodermal dysplasia

HIGM with hypohydrotic ectodermal dysplasia (HIGM-ED) is a severe X-linked deficiency of IKKγ/NEMO [283] [284] [285]. While nullizygous NEMO deficiency causes the prenatal lethality in males known as familial incontinentia pigmenti, a hypomorphic mutation of the tenth exon of NEMO is responsible for the viable phenotype of HIGM-ED. Normally, CD40L binding to CD40 activates the CD40 receptor and results in the ubiquitin-mediated degradation of IκBα, which releases NFκB so that it migrates to the nucleus to activate the transcription of its many target genes. If NEMO/IKKγ is defective then NFκB release in response to CD40 signalling prevents the activation of NFκB-dependent genetic programs such as CSR and synthesis of IL-12 and TNF by APC. Consequently, affected patients are prone to atypical mycobacterial infections and have recurrent bacterial infections [269].

1.6.1.4 Therapy of hyperIgM syndrome 1

Supportive measures for HIGM1 patients include passive immunotherapy using IVIG, which reduces the frequency and severity of recurrent bacterial infections but not the incidence of sclerosing cholangitis or hepatobiliary carcinomas. Regular subcutaneous injections with granulocyte colony stimulating factor (G-CSF) can reduce the incidence of infectious complications of chronic neutropenia. Administration of cotrimoxazole-trimethoprim provides effective *P. carinii* pneumonia prophylaxis. The most applicable curative option for all clinical manifestations of HIGM1 is early allogeneic stem cell transplantation, which has been successfully employed using haploidentical bone marrow, matched unrelated bone marrow and cord blood as sources of stem cells [269] [286] [287].

1.7 Respiratory syncytial virus evades host immunity

1.7.1 Clinical features of respiratory syncytial virus infection

RSV is the commonest and most important cause of serious respiratory tract infection in infants and children in the world. It is a highly infectious and ubiquitous pathogen, and infections occur in all human populations as annual and seasonal epidemics. RSV is the most common cause of hospitalisation among infants in developed countries where it accounts for approximately 80% and 50% of infant cases of bronchiolitis and pneumonia, respectively. Similarly, RSV is a leading cause of morbidity in the developing world. Almost all individuals will be infected with RSV by two years of age with the peak incidence occurring in the third and fourth months of life. Few infants are infected before the age of two months,

although premature infants are particularly susceptible [288]. In most immunocompetent children, RSV infection is limited to the upper respiratory tract infection [288].

RSV is an important cause of morbidity and mortality in immunocompromised patients of all ages [289] [290] mainly because the infection is more likely to spread to the lower respiratory tract and cause bronchiolitis and pneumonia. Children and adults who have human immunodeficiency virus (HIV) infection, primary immunodeficiency diseases, and secondary immunodeficiency following chemotherapy or stem cell transplants are particularly susceptible as are those whose defences are impaired by chronic lung diseases such as cystic fibrosis or by congenital heart disease [288] [290]. RSV has been identified as a cause of recurrent pneumonia in children who have primary immunodeficiency disease including HIGM [291]. Immunocompromised and RSV-infected children shed more virus and for longer periods [289]. However, in a recent registry survey of 79 HIGM1 patients, 64 patients experienced pneumonia, which was caused by *Pneumocystis carinii* pneumonia in 38 patients (often before the HIGM1 diagnosis was made) but only by RSV in one case [292].

The potency and specificity of immunological memory to respiratory viruses is best illustrated by the story of Faroe Islanders who had been infected with measles, which is a paramyxovirus like RSV. In 1846, after an interval of 65 years, none of individuals previously infected with measles was re-infected during a new outbreak [293]. Unusually, therefore, RSV generates limited rather than robust immunity and re-infection is common. Although re-infection is common in immunocompetent individuals, the infection is usually limited to the upper respiratory tract and has a milder clinical course. Re-infection rates decline with age and re-infection less commonly involves the lower respiratory tract [288]. Nevertheless, RSV causes repeated infections in old age when immunity wanes and so remains an important cause of morbidity and mortality in the elderly [294]. RSV not only causes acute morbidity and risk of long-term pulmonary damage but may predispose to later development of allergic asthma in susceptible atopic individuals [295].

1.7.2 Treatment of respiratory syncytial virus infection

Little effective treatment exists for the treatment of acute and lower respiratory tract RSV infections. The most effective agent for the treatment and prevention of severe RSV disease, although not necessarily viral infection, is a mAb called palivizumab (Synagis®), which has high affinity for the highly conserved F protein [296] [297]. In further support of the critical importance of humoral immunity, passive immunotherapy using palivizumab significantly reduces both the rate and impact of RSV-related hospitalisation in premature infants and in those with bronchopulmonary dysplasia [298]. The highly conserved nature of the palivizumab epitope was confirmed in a large study of clinical RSV isolates, which even included infants who had received palivizumab prophylaxis [299]. In contrast, RSV-G sequences readily mutate under selection pressure [300].

1.7.3 Respiratory syncytial virus vaccines

The first RSV vaccine trial proved to be a disastrous experience, which while it provided a spur to basic RSV research, inhibited the clinical development of other vaccine candidates for many years. The vaccine contained a formaldehyde-inactivated form of RSV (FI-RSV), which not only failed to prevent RSV infection , when that infection did occur, eventuated in enhanced and sometimes fatal RSV infection. Although the immunology of this phenomenon remains obscure, studies in murine models of RSV indicate that the FI-RSV vaccine induce an even greater shift of the anti-RSV response to a type 2 cytokine response than does natural infection. Since further clinical development of killed virus vaccines has been precluded, live attenuated vaccines have been studied in humans. Although live attenuated vaccines are immunogenic, upper respiratory tract symptoms have prevented their evaluation in young infants [301]. Hence, there has been a move away from the development of whole virus vaccines toward subunit vaccines, which are more readily combined with immunomodulatory molecules particularly in virally vectored and DNA vaccines. Nevertheless, although subunit

vaccines have been found to be effective in seropositive children, they also induce upper respiratory symptoms [301]. Hence, no safe and effective vaccine is yet available.

1.8 Cancer kills, in part because it subverts immune controls

Never before in human history have so many aging and relatively affluent people been alive when the possibility for the effective treatment of human disease is greatest. The aging populations of developed nations will ensure that malignancy is a growing cause of morbidity and mortality. While recent advances in cancer treatment have produced incremental improvements in the survival of patients with metastatic carcinomas over the last 50 years, most patients will die from their disease within several years of diagnosis [302].

First, in this section, the nature, complexity and scale of the problems confronting the development of systemic approaches to the treatment of cancer will be discussed. In the next section, recent accounts, which describe in molecular detail how cancer may be controlled by the immune system, will be reviewed. This new understanding coupled with technological advances provides cause for optimism that future developments in the systemic treatment of cancer will improve outcomes for cancer patients. Finally, systemic treatment approaches to cancer, in particular, immunogene therapy, will be discussed. It is now apparent that conventional approaches using anti-mitotic agents will be combined with novel approaches including gene therapy, vaccines and biologic agents because of previously unrealised synergies that result from their combined use.

1.8.1 The nature of malignancy

The cardinal features of cancer are initially autonomous growth and later invasion and metastasis, which are due to loss of homeostatic control of cell number, differentiation state and proper anatomic context. Recently, Hanahan and Weinberg defined six characteristics of the cancer phenotype: (1) limitless replicative potential, (2) self-sufficiency in growth signals, (3) sensitivity to anti-growth signals, (4) evading apoptosis, (5) sustained angiogenesis, and (6) tissue invasion and metastasis [303]. To this list may be added other hallmarks of cancer, which include aneuploidy, altered energy metabolism (A Ulrich, personal communication), and immune defence evasion [304].

The cancer phenotype typically becomes manifest after a series of lesions in multiple classes of genes that include proto-oncogenes, anti-apoptotic genes, tumor suppressor genes, and genes for cell adhesion molecules. For example, the genetic lesions of single point mutation, deletion, amplification, rearrangement and translocation may subvert the crucial role that oncogenes play in signal transduction and transcription. Only one copy of a proto-oncogene need be damaged to produce ‘gain of function’ effects that contribute to the malignant phenotype. On the other hand, the genetic lesions of methylation, mutation, deletion or a combination thereof, must damage both copies of a tumor suppressor gene to cause ‘loss of function’ effects and malignant change [305] [306] [307]. Tumor suppressor genes may positively regulate apoptosis and DNA repair and negatively regulate cell cycle progression. One critically important tumor suppressor gene, p53, is known as ‘guardian of the genome’ because its disruption promotes genetic instability. Consequently, self-perpetuating genetic changes entrench the cancer phenotype and are associated with tumor aggressiveness and resistance to radiation and conventional cytotoxic chemotherapy [308]. In addition, it is becoming clear that epigenetic changes such as hypermethylation have an important influence on the malignant phenotype and may even be heritable [309].

1.8.2 The nature of metastases

Most cancer deaths result from the uncontrolled growth of metastatic disease. As cancer cells grow in an uncontrolled way to invade normal tissues, they are afforded the opportunity to metastasise successfully to distant sites where growth of the metastases beyond 1-2 mm in diameter depends on their acquisition of a blood supply *via* the process of angiogenesis. With few exceptions, solid tumours are not usually curable once they have metastasised throughout the body.

Although solitary malignant cells may give rise to later overt metastases, extensive preclinical studies and some clinical studies also support the notion that overt metastatic disease may arise from dormant pre-angiogenic micro-metastases as the result of an 'angiogenic switch' [310 311]. Preclinical studies of xenograft micro-metastases in particular, and supported by some clinical data, indicate that pre-angiogenic lesions maintain a stable size of 0.5-1.0mm because the rates of proliferation and apoptosis within the lesion balance each other [310].

1.8.3 Infection, inflammation and malignancy

While some viruses such as human papilloma virus (HPV), Epstein-Barr virus (EBV) and human T-lymphotropic virus-I (HTLV-I) are simply oncogenic, inflammation resulting from chronic infection with other agents may also play a role in the initiation and/or promotion of various malignancies. For example, there are associations between hepatitis B virus (HBV) and/or hepatitis C virus (HCV) and hepatocellular carcinoma, schistosomiasis and bladder cancer, EBV and nasopharyngeal carcinoma, *H. pylori* and gastric adenocarcinoma or mucosal-associated lymphoid tissue (MALT) lymphoma, and chronic inflammatory bowel disease and colorectal cancer [312].

Various mechanisms have been postulated to relate infection and inflammation to malignancy and include (i) chronic antigen-driven immune activation resulting in overproduction of pro-inflammatory cytokines, and (ii) induction of cyclo-oxygenase-2 (COX-2) expression, which has been observed in lung, breast, colorectal, gastric and pancreatic tumours. COX-2 suppresses cell-mediated immune responses, and promotes pro-angiogenic and anti-apoptotic effects, cell proliferation and motility and, ultimately, disease progression [313].

Clinical epidemiological studies provide further support for this hypothesis. Large retrospective and prospective population-based studies show that the use of both non-selective non-steroidal anti-inflammatory drugs (NSAID) and selective COX-2 inhibitors was associated with a decrease in both the incidence and mortality rate of colorectal cancer. Randomised controlled double-blind trials show that NSAID and COX-2 inhibitors reduce the number and size of colonic polyps in familial adenomatosis polypi (FAP) patients [314]. Non-randomised studies show that long-term exposure to aspirin and other NSAID reduces the incidence of oesophageal, gastric, bladder and lung cancers.

Macrophages are both an important source of inducible COX-2 activity and a significant constituent element of many tumours. While an earlier view held that tumour-associated macrophages (TAM) had tumoricidal activities, a more recent evidence-based consensus [315] indicates that inflammatory cells such as macrophages, which comprise the stromal component of many cancers, play a crucial role in cancer progression and metastasis. Indeed, the trophic and tissue remodelling functions of macrophages are subverted to support tumour growth so that cancer becomes 'the wound that never heals' [316].

For example, transgenic mice, which develop breast cancer and which are deficient in the production of the macrophage survival factor, colony simulating factor-1 (CSF-1), have a reduced incidence of spontaneous pulmonary metastases [317]. Clinical studies indicate that both increased tumour microvessel and macrophage content, and also overexpression of CSF-1 and its receptor, portend a worse prognosis in breast cancer patients [318]. Together with other supporting experimental data, tumour infiltrating macrophages can now be viewed as critical pro-angiogenic actors in a tumour [315]. Hence, even in malignancies in which there is not an evident inflammatory antecedent to the cancer [154], the biological behaviour of most inflammatory cells may be manipulated to slow the progression of malignancy [319].

1.8.4 The limitations of current anti-cancer treatments

The best chance that a patient has for cure of a solid tumour remains the use of local treatments such as surgery and/or radiotherapy when the solid tumour is localised to its originating lining and has not spread either to the lymph nodes that drain the tumour or *via* the bloodstream. Nonetheless, even at this early stage, and particularly if the tumour has spread to the draining lymph nodes, micrometastases may already be established and, in most cases, led to the death of the patient. In this sense, cancer is a systemic disease that requires systemically

administered treatments. So that, of the patients who receive surgery and/or radiotherapy as definitive local treatment for their primary tumour and who have micrometastases, a minor proportion may be cured or at least achieve a durable remission from cancer by the addition of systemic treatments such as cytotoxic chemotherapy or hormonal drugs. Clearly, the poor survival prospects of patients with metastatic disease indicate that new approaches to the systemic treatment of cancer are needed.

The mainstay of systemic treatment of cancer remains cytotoxic chemotherapy despite its ultimate ineffectiveness in the treatment of most cases of metastatic cancer [320]. Cytotoxic chemotherapy may reduce a patient's tumour burden by many orders of magnitude and induce a complete remission which, despite the lack of evident disease by conventional medical imaging criteria, is illusory because residual drug-resistant cells survive to grow another day. Chemo-resistance may occur effectively in part because of (i) tumour repopulation, whereby chemo-sensitive tumour cells repopulate between cycles of chemotherapy, and (ii) defective tumour perfusion, which impairs drug delivery. Thus, as cytotoxic drugs induce apoptosis and kill a constant fraction of tumour cells with each cycle of chemotherapy, the efficacy of treatment levels off [320].

A model was developed to account for this phenomenon, which proposed that a constant fraction of tumour cells is killed with each cycle of chemotherapy but after an initial delay, tumour cells repopulate at a rate that accelerates with successive cycles of chemotherapy. Consequently, the tumour size reduces to the extent that a complete or partial radiographic remission results but then accelerated tumour cell repopulation eventually results in tumour regrowth despite ongoing chemotherapy [321]. The proportion of tumour cells killed may be increased by dose intensification of cytotoxic drugs, particularly if drugs with non-cross reactive mechanisms of action are used. More fundamentally, pre-existing or acquired mechanisms of intrinsic cellular resistance, such as expression of pro-survival factors, drug efflux pumps or drug detoxifier enzymes, are responsible for cytotoxic chemotherapy failures [308 322]. Moreover, it seems that features marking the residual drug-resistant repopulating tumour cell have more in common with the newly revived concept of the cancer stem cell.

1.8.5 How current anti-cancer treatments are being improved

Although somewhat 'blunderbuss' in approach because they interfere with the cell cycle of both normal and malignant cells, cytotoxic drugs are relatively selective for malignant cells because normal cells tend to recover more rapidly from cytotoxic drug damage between the applications of treatment. In contrast, biotherapeutic agents often have greater efficacy than conventional agents in the sub-population of patients whose cancers bear the target [302]. Therefore, targeted therapy of cancer aims to improve the therapeutic ratio of cancer treatment by enhancing its specificity for and/or precision of delivery to malignant tissues while minimising adverse consequences to normal non-malignant tissues. The two major currently approved classes of targeted therapy are (i) the small molecule inhibitors such as the tyrosine kinase inhibitor subset of signal transduction inhibitors including imatinib mesylate, gefitinib and erlotinib, and (ii) the mAb including rituximab and trastuzumab (see Section 1.9.7.1). Other classes of biotherapeutic agents include growth factor modulators, cancer vaccines and cell and gene therapies.

1.8.5.1 Combined modality treatment: exploiting synergies to improve cancer treatment

Cancer treatment is often more effective when its modalities are combined to exploit potentially synergistic interactions. Because treatment efficacy is improved, combined modality treatment may have the additional benefit of elevating the therapeutic ratio for the combined treatment beyond that for each of the individual treatments. For example, combined modality treatment using radiation and radiosensitising chemotherapeutic drugs such as 5-fluorouracil and cisplatin (chemoradiotherapy) has improved survival in a number of solid tumours such as those of head and neck, lung, oesophagus, stomach, pancreas and rectum because of both improved local tumour control and reduced rates of distant failure [323]. Chemoradiotherapy may overcome mutually reinforcing resistance mechanisms, which may

only manifest *in vivo*. For example, the potent new generation radiosensitisers, gemcitabine and docetaxel, induce tumour cytotoxicity and so re-oxygenate and radiosensitise the tumour [324] [325] [326]. However, the benefit that radiosensitisation confers *via* increased tumour response may occur at the cost of increased toxicity to adjacent normal tissues, which may be mitigated by improved radiotherapy techniques [323].

Increasingly, local and systemic biotherapeutic approaches to cancer treatment are being integrated with conventional treatments and becoming standard of care for some cancers. For example, the combination of cytotoxic chemotherapy and the anti-B cell mAb rituximab increases cure rates for patients with aggressive diffuse large B cell lymphoma, and the combination of cytotoxic chemotherapy and the anti-C-ERBB2 mAb trastuzumab prolongs survival in metastatic breast cancer patients whose cancers have *c-erbB2* gene amplification. On the whole, the more favourable toxicity profile of biotherapeutic agents means that extension of life is accompanied by improvement in its quality because of an increased probability of therapeutic benefit resulting from synergistic interactions between its conventional and biotherapeutic components. While some biotherapeutic agents may help to cure patients of cancer, other such agents have the worthy objective of controlling rather than curing cancer in much the same way as HIV infection has been converted to a chronic disease with combination anti-retroviral therapy [302].

1.8.6 The cancer stem cell is the main obstacle to curative treatment for metastatic cancer

Virtually all cancers are believed to have a clonal origin. While all the cells in a tumour mass originate from a single neoplastic clone, only relatively few of their number has the capacity to initiate tumours and are known as cancer stem cells. Like normal stem cells, cancer stem cells (CSC) are defined by two major characteristics, (i) the ability to renew themselves (self-renewal capacity), and (ii) the ability to expand to generate many progenitors, which give rise to larger numbers of cells in varying states of differentiation (multi-potency).

1.8.6.1 The hierarchy model of the leukaemic stem cell

The most sophisticated understanding of tumour initiating cells has been achieved using non-obese diabetic severe combined immunodeficiency disease (NOD-SCID) models of human acute myeloid leukaemia (AML). In this xenotransplantation model system, immunodeficient NOD-SCID mice were infused with different fractions of human AML cell populations. Leukaemia stem cells (LSC) gave rise to full blown leukaemia and would do so repeatedly upon serial transplantation into immunodeficient mice. Hence, the LSC sits at the apex of the neoplastic hierarchy, which recapitulates in a disordered way the hierarchy of normal haemopoietic cell differentiation [327] [328].

The division of stem cells is a stochastic event. Asymmetric division of a stem cell yields a stem cell daughter (self renewal) and a transit amplifying cell, which subsequently divides to yield proliferating and differentiated progeny. On the other hand, symmetric division of a stem cell can yield two stem cells or two transit amplifying cells. Studies using retroviral insertion sites to mark the LSC clonally have shown that the LSC sub-population is functionally heterogeneous. Earlier studies had shown that gene marked neuroblastoma cells, which had contaminated the bone marrow inocula of neuroblastoma patients receiving allogeneic bone marrow transplantation with curative intent, were the source of relapse and contributed a multiplicity of malignant cells to relapse [329]. Self-renewal capacity and thus the ability to expand among LSC vary to the extent that, with time, the number of progeny of some LSC will become depleted, while other LSC will maintain the number of progeny, and yet others will continue to expand. Hence, among the LSC compartment is a rarer type of leukaemic stem cell, which has the unusual clonal properties of a high level of self-renewal capacity but a low level of commitment to the progenitor pathway and/or a slow rate entry into the cell cycle. Because they are so quiescent, these rare LSC may escape the induction chemotherapy used to eradicate the large number of blasts in presenting AML patients and so contribute to late (greater than one year) relapse in these patients [330].

These particular properties of LSC also complicate treatment of chronic myeloid leukaemia (CML) in which the pathogenic event is a chromosomal translocation that produces an oncogenic fusion product called BCR-ABL. BCR-ABL is a tyrosine kinase that is targeted with high affinity by the small molecule tyrosine kinase inhibitor, imatinib mesylate (Glivec®). Despite the high rates of complete molecular remission, which may be achieved with the use of imatinib, LSC containing high levels of BCR-ABL persist, which indicates that these LSC do not require BCR-ABL at least for survival or proliferation [331]. Nonetheless, other strategies may provide promising avenues for intervention. For example, LSC require specialised niches to survive. Using the NOD-SCID model, a mAb that targeted CD44 on the surface of LSC prevented the interaction of the LSC with its supportive microenvironment and thus prevented the LSC surviving to repopulate another mouse in a xenotransplantation assay [332].

1.8.6.2 The hierarchy model likely applies to cancer stem cells

Although the bulk of evidence suggests that the LSC arises from the normal stem cell compartment, LSC may also arise from progenitors that have acquired additional mutations. However, while the number of progeny generated from the normal stem cell is maintained, the capacity for self-renewal among LSC is deregulated so that the neoplastic clone becomes expanded. Like leukaemia, many metastatic carcinomas probably harbour relatively quiescent but multi-potent tumour initiating cells. Studies also in NOD-SCID mice support the hierarchy model for cancer stem cells in breast cancer and glioma. Therefore, many of the considerations relating to treatment resistance probably apply also in solid tumours [333].

Recently, a more developed model of CSC behaviour in solid tumours was presented [334]. This model posits the existence of migrating CSC that exit the leading invasive front of a primary tumour and spread *via* the blood stream or lymphatics to establish distant metastases. The histological appearances of these secondary tumours are often very similar to those of the primary tumour, which the authors hypothesise results because the migrating CSC recapitulates the organisation of the primary tumour. Thus, while the progeny of the CSC may be diminished by cytotoxic chemotherapy to the point of a complete clinical and/or radiological remission, CSC themselves are very likely to survive to repopulate the patient with overt metastatic disease.

1.8.7 Why does adjuvant systemic treatment cure patients with micrometastases?

Given the previous discussion about the ineradicable cancer stem cell in overtly metastatic carcinoma, why can this cell type be eradicated in early-stage disease, which is marked by the presence of systemic micrometastases? Large randomised controlled trials have shown a survival benefit for those patients with early-stage cancer who receive systemic cytotoxic chemotherapy adjunctive to the use of potentially curative surgery. The clear inference to be drawn from these many studies is that approximately 10% of patients, who were otherwise destined to die from metastatic disease, were rescued by the use of cytotoxic drugs. This striking observation does not have an evident explanation. While it is possible that the cancer stem cells and their differentiated progeny in these patients were killed outright by the cytotoxic drugs or induced into a senescent state, a role for the immune system in the elimination of micrometastases is not precluded particularly in light of the resurgent view that the immune system has cancer surveillance properties. In addition, the likelihood that tumour cell death would have occurred in those patients who were cured may suggest a mechanism by which anti-tumour immune activation may occur (see Section 1.9.3).

1.8.7.1 Micrometastatic disease is curable in early-stage breast and colorectal cancers

The development of metastatic disease is usually what prevents the cure of women who present with early-stage breast cancer. The women who do relapse after definitive treatment for early-stage breast cancer are presumed to have had micro-metastases at the time that the primary tumour was removed at surgery. Many clinical trials have now been done in women with early-stage breast cancer who were randomised to receive adjuvant cytotoxic chemotherapy and/or hormonal treatments or not. The results convincingly demonstrate that a

minor proportion of women who would have relapsed with metastatic disease are converted to a cure presumably because the micro-metastases were eradicated by the adjuvant therapy. The 30-year follow-up data, in particular, clearly indicate that the benefit of adjuvant cytotoxic chemotherapy persists [335]. Although, hormone receptor-positive breast cancer may relapse very late, the data for the use of chemotherapy strongly support the view that women at significant risk of relapse were cured of their systemic disease. However, what is less clear is whether the adjuvant chemotherapy itself contributed to the eradication of the presumed micrometastases in the small proportion of patients who did not relapse. Could the immune system be contributing to the survival benefit of patients receiving adjuvant treatment? An additional consideration is that chemotherapy-induced tumour cell death may be sufficient to activate an anti-tumour immune response in some instances, which may potentially be reinforced by other immunostimulatory treatment such as anti-CD40 mAb.

Adjuvant chemotherapy has had a long history of success in the treatment of resected node-positive colon cancer where again up to 10% of patients at risk of relapse obtain an absolute survival benefit from the addition of 5-fluorouracil (5FU)-based chemotherapy to definitive surgery [336]. Similar results are now also being obtained with adjuvant cisplatin-based chemotherapy in patients with resected stage II and stage IIIA non-small lung cancer [337]. Adjuvant passive immunotherapy of resected colon cancer using a murine mAb called edrecolomab, which is directed against epithelial cell adhesion molecule (EpCAM), was tested in a randomised controlled trial. In comparison with the observational arm, edrecolomab improved overall survival in stage III patients [338], which suggests that the immune system contributes to the eradication of micrometastases, perhaps *via* the recruitment of ADCC. Although stage II patients did not benefit [339], the greatest impact of this therapy in stage III patients was on prevention of metastatic relapse rather than locoregional recurrence [338]. In patients with metastatic colorectal cancer treated with edrecolomab, pre-treatment NK cell function correlated significantly with survival of edrecolomab responders [340]. Responding metastatic disease patients were also observed to develop an idiotype network of antibody responses and associated idiotype-reactive T cell responses [341] [342]. Nevertheless, the combination of edrecolomab with standard adjuvant 5FU/folinic acid chemotherapy did not improve overall survival in comparison with standard chemotherapy alone, which may suggest redundancy in the mechanism of action of each modality of treatment [343]. Finally, despite its efficacy as adjuvant treatment, edrecolomab monotherapy compared unfavourably with standard adjuvant chemotherapy [343].

In conclusion, these clinical studies show that up to 10% long-term survival benefit is conferred upon patients who have had primary resection of cancers of breast, colon or lung and adjuvant systemic therapy. Although the mechanism is not evident, these patients were cured presumably because the adjuvant chemotherapy contributed to the eradication of micrometastases, which would otherwise have produced relapsed disease.

1.9 The rationale for cancer immunotherapy

While the history of cancer immunotherapy has been filled with false dawns followed by disappointment and disparagement, a number of recent advances have led to resurgent interest in this approach. The discovery that human tumours contain tumour specific or tumour associated molecules (such as mutant oncogenes, fusion proteins and viruses) coupled with the realisation that these molecules may be processed and presented in association with MHC molecules, has provided a rationale for generating tumour specific immune responses [344] [345]. Multiple lines of laboratory and clinical evidence support the contention that the immune system has an important role to play in the prevention and control of tumours. For example, it is now evident that cancer patients make spontaneous albeit ineffective immune responses against their own tumours *in vivo*, which thus broadens the scope for therapeutic intervention [346] [347] [240]. Advances in our understanding of the molecular basis of immune system recognition, activation and amplification have provided us with the tools to manipulate components of the immune system to enhance its responsiveness.

Cancer immunotherapy is intrinsically appealing because of the frequently observed and powerful ability of the immune system to generate, amplify and maintain a specific and damaging attack upon any foreign and/or self antigens in the infectious and autoimmune diseases. The development of immunological memory with the first episode ensures that the ferocity of the second episode of immunologically mediated disease at least equals that of the first episode. Repeated episodes may be associated with the phenomenon of 'epitope-spreading' with consequent widening of the extent of immunologically mediated tissue damage [348]. Moreover, the demonstration that transplanted lymphocytes exert clear-cut anti-neoplastic activity in patients with leukemia and lymphoma has encouraged the exploitation of these mechanisms in other malignant disorders. Nonetheless, in the face of inadequate spontaneous anti-tumour responses and an immunosuppressive tumour microenvironment, the major challenge confronting the field of cancer immunotherapy is to develop and maintain immunological memory in order to prevent tumour recurrence and/or metastases.

Immunogene therapy remains under active investigation as an approach to the immunotherapy of cancer. Current gene transfer vectors are poorly targeted and inefficient. Hence, gene transfer may be used to recruit the host's immune system, the characteristics of which effectively compensate for the limitations of available vectors. Whereas previously the development of cancer immunotherapeutics had been empirical, the rationale now exists for the design of a new generation of cancer immunotherapeutics based on the adoption of new monoclonal antibody and gene transfer technologies.

1.9.1 Cancer immunosurveillance and the updated concept of cancer immunoediting

Because malignant cells are identifiably different from normal cells, it has been theorised that the immune system would detect the foreignness of malignant cells and eliminate them. MacFarlane Burnet developed an immunosurveillance hypothesis postulating that a major function of the immune system was to eliminate transformed cells. Earlier, Lewis Thomas had envisaged that the process of tumour elimination would be similar to homograft rejection [349]. Conversely, it has been argued that the immune system evolved to combat infectious pathogens rather than reject tumours. Now, however, new insights provided by molecular carcinogenesis studies into the role of chronic inflammation in the induction and promotion of malignancy [188] have begun to blur the distinctions between immune and other defences that have evolved to deal with infectious pathogens directly and those that evolved to deal with indirect consequences such as virally infected and/or transformed cells and cells otherwise altered as the result of an host inflammatory response (see Chapter 2).

Studies in gene-targeted mice demonstrate that the immune system has a significant tumour suppressor function that depends on the presence of lymphocytes [350], natural killer T cells [351], the effector molecule perforin [352] and intact type I and II interferon (IFN) signalling pathways [353] [350]. Studies of mice with disruption of the genes for *Rag2* and *Stat1* demonstrated that lymphocytes and IFNγ signalling, respectively, were required to protect against spontaneous carcinomas and to control fibrosarcomas, which had been induced by the chemical carcinogen methylcholanthrene (MCA) [350]. The *Rag2* gene controls genomic rearrangement of TCR genes and its disruption prevents lymphocyte development whereas the *Stat1* gene is an essential activator and transducer of IFNγ signalling [354]. Lack of interferon responsiveness allows tumours to evade the immune system by reducing their immunogenicity. In IFN-*responsive* tumour cells, IFNγ promotes increased expression of TAP1, which in turn facilitates peptide loading of MHC class I molecules, and enables MHC class I-restricted cytolytic anti-tumour immune responses (Figure 4). Conversely, in IFN-*unresponsive* tumour cells, IFNγ is unable to elicit TAP1-mediated processing of MHC class I-restricted peptides and, hence, anti-tumour immune responses are abrogated [350]. The clinical significance of this finding in genetically manipulated mice was demonstrated using gene expression profiling studies of prostate adenocarcinoma samples to show that IFN-inducible genes were down-regulated in approximately 30% of samples [355].

Similarly, mice deficient in NK and NKT cells were unable to control MCA-induced fibrosarcomas [351], and epithelial T lymphocytes, which express the γδ T cell receptor, were required for the control of chemical carcinogen-induced skin tumours [356]. In addition, mice deficient for the molecule, perforin, which is required for effector function of cytolytic lymphocytes, were not protected against lymphomagenesis [352]. However, these studies do not make clear the relative contributions of the innate and adaptive arms of immunity.

While this evidence supports Burnet's earlier theory of tumour immunosurveillance, a new concept termed 'cancer immunoediting' has emerged and explains the apparent paradox of tumours evolving in an immunocompetent host [350]. By selecting for tumour cells that are less immunogenic [350], insensitive to IFNγ and more tumorigenic [353], an intact immune system favours the growth of tumour cells that evade immune destruction. Hence, while some tumours may be completely eliminated, a state of immunologic tolerance or anergy would develop toward other tumours [350]. Thus, the cancer immunoediting concept accounts for the duality of anti-tumour immunity that both eliminates early-stage tumours and sculpts the growth of tumours that escape immune control. Consequently, three phases of immune action with, parenthetically, corresponding effects on tumour growth may be described: elimination (protection), equilibrium (persistence) and escape (progression) [304].

In further support of immunosurveillance by the adaptive immune system, a recent clinical study of genetic and immunohistochemical analyses of colorectal tumours, which had been surgically removed with curative intent from of 490 patients, was reported. Tumour gene expression profiles indicative of Th1 immunity were associated with beneficial effects on clinical outcome. In agreement, patients who had the highest densities of CD3-, CD8-, granzyme B-, and CD45RO-staining lymphocytes in the central and infiltrating regions of their tumours tended not to develop tumour recurrences. Indeed, a strong *in situ* immune reaction correlated with a favourable prognosis irrespective of the T and N stages of the tumours. Conversely, a weak *in situ* immune reaction correlated with a poor prognosis even in patients with stage I tumours. In multivariate analysis, high tumour density of $CD3^+$ lymphocytes was the only independent parameter associated with overall survival. Hence, TIL density outperformed conventional histological staging criteria and these data were validated in two additional patient populations [357].

1.9.2 Spontaneous anti-tumour immune responses and tumour cell death

1.9.2.1 The implications of apoptotic versus necrotic cell death

Generally, cell death presents as either apoptosis or necrosis, each of which has characteristic morphological appearances. Tumours, in particular, often have high rates of apoptosis and areas of necrosis. In the adult human, virtually all of the 10^5 normal cells that die an apoptotic death each second are removed efficiently with scant evidence of their passing on microscopic examination of tissues [358]. Apoptosis is a physiological form of cell death that prevents adjacent cells being exposed to potentially toxic intracellular substances. Therefore, the intracellular contents of the apoptotic cell are re-ordered and packaged into membrane-bound parcels known as apoptotic bodies (Figure 9), which are engulfed aphlogistically by nearby semi-professional and professional scavenger cells. During apoptosis, the cell membrane of the dying cell becomes everted to reveal phosphatidylserine (PS), which provides an 'eat me' signal for phagocytic cells [359]. Usually, the phagocytic clearance of apoptotic cells is very efficient [360]. However, if phagocytosis is insufficient to remove apoptotic cells then secondary necrosis may ensue with accompanying loss of membrane integrity, spillage of injurious intracellular material and the risk of autoimmune disease [361]. Loss of membrane integrity may result in NFκB activation in neighbouring viable cells, and subsequently, pro-inflammatory genes such as those for neutrophil chemokines may be induced [362].

Multiply redundant receptor systems mediate the phagocytosis of apoptotic cells and inhibition of any one of them will not abrogate phagocytosis. Several different kinds of receptor mediate the clearance of apoptotic cells by macrophages, which include the PS receptor, vitronectin receptor ($\alpha_v\beta_3$ integrin), class A scavenger receptor, CD36 (class B

scavenger receptor), and CD14. In addition, various serum proteins, which include collectins such as the first component of the complement cascade, C1q, opsonise apoptotic cells and contribute to phagocytic clearance by bridging the apoptotic cells to the corresponding receptor such as the collectin receptor, CD91 [363]. After phagocytosis of apoptotic cells, macrophages, which are the professional scavenger cells, produce TGFβ1 that suppresses pro-inflammatory cytokine and chemokine expression, thus facilitating the immunologically silent clearance of apoptotic cells [364] [365]. Epithelial tumours such as human prostate cancer also frequently produce TGFβ1, which may contribute to down-regulation of tumour antigen presentation by DC and the generation of ineffective anti-tumour T cell responses. Uptake of apoptotic cells by DC leads to IL-10 production and may also down-modulate DC immunostimulatory functions [366].

Unlike macrophages, immature DC utilise CD36 and the $\alpha_v\beta_5$ integrin to ingest apoptotic cells *in vitro*, which then enables cross-priming of T cells [367]. Similarly, DC can engulf apoptotic prostate cancer cells *in vitro* and elicit primary T cell responses to PSA. Ingestion by murine macrophages of apoptotic cells *in vitro* led to the active suppression of pro-inflammatory cytokines such as TNF and chemokines such as the C-X-C neutrophil chemoattractants (KC, MIP_2) and the C-C chemokine, MIP-1α [368]. A major role for TFGβ1 in mediating the suppression of these pro-inflammatory mediators was suggested by the use of neutralising TFGβ1 antibodies [368]. PS exposure on apoptotic cells was demonstrated to be crucial for the *in vivo* production of TFGβ1 by inflammatory murine peritoneal macrophages [369]. *In vitro* studies using cultured human peripheral blood mononuclear cells (PBMC) indicate that macrophages, which have ingested apoptotic cells, inhibit DC presentation of antigen [370]. Moreover, human *in vitro* cell cultures indicate that apoptotic primary cells or tumour cells do not mature DC, only necrotic tumour cells [371]. Similarly, CD36-mediated uptake of apoptotic cells by murine DC *in vitro* prevented T cell activation [366]. Recently, the potency of apoptotic cells in the generation of tissue-specific tolerance mediated by regulatory T cells was demonstrated when diabetes was prevented in diabetes-prone mice after streptozocin-induced apoptosis of β-islet cells [372].

There are other reports that conflict in whether apoptotic cells are immunogenic or not. Although the reasons are not clear, there are several possible explanations. Apoptotic cells may have undergone secondary necrosis during the assay and released endogenous adjuvants. Accordingly, studies suggest that late apoptotic cells rather than early apoptotic cells activate DC. Cells stressed before apoptosis are more immunogenic, and apoptotic cell death engendered by cytotoxic drugs is an example in which immunogenicity is enhanced [141]

1.9.2.2 Spontaneous anti-tumour immune responses

Studies using adoptive transfer of transgenic tumour antigen-specific T cells to tumour-bearing mice showed that tumours were actively recognised rather than ignored, although only a feeble effector response developed that waned rapidly. Moreover, a proliferative T cell response to tumour inocula that were not subsequently palpable was detected six months after tumour inoculation and indicated that tumour antigen presentation to draining lymph nodes was constitutive [240].

One of the earliest indications of spontaneous recognition of tumours by the human immune system came from studies of sera from cancer patients. In the laboratory, these sera were discovered to bind not only autologous tumour tissue but also tumour tissue derived from patients with the same type or other types of tumour [373]. Hence, it was realised that some tumour antigens were shared and could, therefore, represent therapeutic targets. These tumour antigens were subsequently identified using tumor mRNA and autologous patient serum in a powerful cloning technology called serological analysis of recombinant cDNA expression libraries (SEREX) [374].

Spontaneous responses of tumour-specific T cells have been observed in cancer patients *in vivo* although these responses have been ineffective [347] [375]. For example, circulating $CD8^+$ T cells, which were specific for normal melanocyte differentiation antigens, were identified by

MHC tetramer technology in melanoma patients but were anergic [346]. However, adoptive transfer of *ex vivo* expanded melanoma antigen-reactive $CD8^+$ T cell clones produced destruction of patient melanoma cells and normal melanocytes *in vivo* [376]. Moreover, the tumour-specific immune responses detected in cancer patients may spontaneously cause autoimmune pathology. For example, circulating $CD8^+$ cytolytic T cells specific for the onconeural antigen, cdr2, were identified by MHC tetramer technology in paraneoplastic cerebellar degeneration patients and may be responsible for the Purkinje cell degeneration [347]. Then, in patients with active/progressive paraneoplastic cerebellar degeneration, activated cdr2-specific T cells were found in CSF and were subsequently reduced in number in CSF and blood after tacrolimus treatment [377].

Similarly, autoantibodies have been detected in cancer patients with the sera of many cancer patients carrying autoantibodies specific for nuclear antigens [378 379]. Other cancer patient sera contain antibodies specific for malignancy-associated antigens such as p53 and cyclin B1 [380 381]. A study of prostate cancer patients discovered prostate tissue-reactive IgG antibodies, which were not present in patients with benign prostatic diseases and some of which recognised cancer-specific carbohydrates [382]. IgG antibodies specific for the prostate tissue differentiation antigen, prostatic acid phosphatase (PAP) were found in approximately 5% of prostate cancer patients and male controls [383]. PAP-specific T cell proliferative responses were detected in 11% of prostate cancer patients [384], and MHC class II-restricted T helper epitopes from PAP were identified [385].

The presence of similar mainly nuclear antigen-specific autoantibodies has long been known in patients with such systemic autoimmune diseases as SLE and Sjögren's syndrome. These diseases are marked by the presence of high-titre and high-affinity serum autoantibodies, which are often not pathogenic [386]. A large body of evidence suggests that these nuclear antigens may be revealed to the immune system by apoptosis [387]. A hypothesis that unifies preclinical and clinical data is the waste disposal hypothesis, which states that the disposal of apoptotic cells is defective in systemic autoimmune diseases [388]. It may be that a relative excess of apoptotic tumour cells that are unable to be cleared efficiently are the source of autoantigen for these humoral immune responses [361].

Strikingly, a wealth of tumour-derived material is found circulating in the blood of cancer patients. Not surprisingly, apoptotic cells have been found in the blood filtering organ of the spleen of metastatic cancer patients. Monoclonal antibody staining for the M30 neo-epitope, which is revealed only during the caspase-mediated cleavage of keratin-18, was found in splenic red pulp [389]. As tumours grow and outstrip their blood supply, dead cells commonly feature in many malignancies.

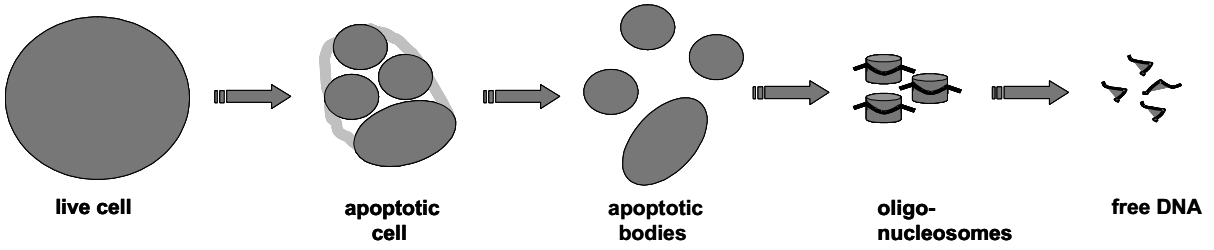


Figure 9: Schematic depiction of the progression of apoptosis through various stages *in vitro*

After an apoptotic stimulus, apoptotic cells shrink and fragment into membrane bound parcels known as apoptotic bodies that become increasingly leaky or secondarily necrotic with time. Eventually the apoptotic bodies disintegrate to oligonucleosomes and then free DNA.

The sequence of apoptotic changes illustrated in Figure 9 is recapitulated in the blood of cancer patients. Live circulating tumour cells (CTC) were detected in the blood of small cell lung cancer patients and declined with ongoing chemotherapy [390]. The majority of CTC found in prostate cancer patients were $M30^+$ apoptotic cells [391]. The highest levels of circulating

mono- or oligonucleosomes were detected by ELISA in serum of untreated lung cancer patients [392] and serum nucleosome concentrations peaked 24-72 hours after the initiation of chemotherapy [393]. The highest levels of circulating double-stranded DNA fragments were found in cancer patients,, and the proportion of tumour-specific DNA varied from >90% to <10% of total plasma DNA. Non-tumour DNA may originate from degenerating stromal cells in the tumour [394]. Gel electrophoresis studies of plasma DNA from metastatic breast cancer patients indicated that the plasma DNA originated from necrotic and/or apoptotic cells with the latter demonstrating DNA laddering patterns characteristic of an association with mono- or oligonucleosomes [394].

Could the circulating tumour material originate from the same source as the tumour antigens driving spontaneous anti-tumour immune responses? DC that constitutively transport apoptotic intestinal epithelial cell remnants to T cell areas of mesenteric lymph nodes have been identified *in vivo* [395]. In tumour xenograft studies, dormant tumours were found to contain a significant proportion of apoptotic tumour cells in a dynamic balance with proliferating tumour cells [310 311]. Tumour antigen-specific T cell proliferative responses were detected in mice six months after subcutaneous tumour inoculations were performed and because the inoculated tumour cells were not palpable, tumour antigens were likely to have originated from dormant tumours [240]. Together, these data suggest that spontaneous but non-pathogenic autoimmune responses to tumour-derived antigens may be a variant of the same constitutive process of DC sampling of normal body tissues that appears to create peripheral tolerance [214].

1.9.3 Induced anti-tumour immune responses and tumour cell death

Clinical examples of induced immune responses provoking anti-tumour immunity include the use of high dose intravenous interleukin-2 (IL-2) for the treatment of metastatic melanoma and renal cell carcinoma by Rosenberg and his group at U.S. National Cancer Institute in the late 20th century. Durable complete remission rates of approximately 5% were obtained but with an incidence of lethal toxicities that approximated 1% [396]. The beneficial effects of IL-2 may have resulted from recruitment of lymphokine-activated killer (LAK) cells and may help to explain why no further survival benefit was obtained by the addition of LAK cells [397]. The major toxicity was a capillary-leak syndrome but now a vasogenic peptide has been identified within IL-2 that can be deleted and not affect the biological activity of IL-2 [398 399]. High dose IL-2 treatment in patients with metastatic melanoma produced objective anti-tumor responses that were significantly associated with the autoimmune phenomenon of vitiligo [400]. Autologous tumour infiltrating lymphocytes (TIL) expanded *ex vivo* in IL-2 induced regression of metastatic melanoma lesions [401]. Rosenberg and his group also used the TIL that produced melanoma regression to clone TAA and the most commonly identified TAA were normal melanocyte differentiation antigens [400]. In an important demonstration that T cell anergy can be reversed, $CD8^+$ T cell clones specific for a normal melanocyte differentiation antigen were expanded *ex vivo* and infused into a melanoma patient and later identified in association with melanocyte destruction in both tumour deposits and areas of vitiligo [376].

In their studies, Rosenberg and co-workers demonstrated in human melanoma and renal cell carcinoma that both normal tissue differentiation antigens and mutant proteins generated MHC class I-restricted T cell epitopes [401 402]. The earlier work of Thierry Boon and colleagues in murine models indicated that mutated TAA existed as the target of MHC class I-restricted cytolytic T cell immune responses [403]. Unique tumour antigens are the product of somatic mutations in cancer cells because cancer results from the accumulation of mutations in genes that directly control cell birth or cell death. The origin of cancer mutations is unclear but two non-mutually exclusive possibilities include the 'mutator phenotype' model in which the characteristic genomic instability of cancer allows mutation to beget mutation, and a 'Darwinian' model in which selection operates on normal rates of mutation followed by waves of clonal expansion of mutant sub-populations [404]. A tumour may accumulate at least 10^{12} mutations although many will not be deleterious and will be present in only a small proportion of tumour cells [405]. Particular evidence for this conclusion was obtained from a

genetic survey of human breast and colorectal cancers in which an average of 11 frequently mutated genes were found per tumour that contributed to the neoplastic process [406]. Thus, human cancers contain a mutational (non-self) repertoire of immunogenic potential [405].

In addition to genetic alterations, epigenetic changes also contribute significantly to carcinogenesis. For example, patients may be predisposed to malignancy because of an inherited mutation in one allele of a tumour suppressor gene, and loss of the other allele produces malignant transformation according to the Knudson two-hit hypothesis. While a genetic lesion in the other allele may underlie the malignancy, genetic silencing of the other allele by epigenetic changes such as promoter hypermethylation is also a common cause [407]. Conversely, epigenetic changes such as loss of gene methylation or histone acetylation may derepress the expression of genes that may contribute to carcinogenesis. Important classes of epigenetically modified genes include the cancer-testis (CT) antigens, which are normally expressed only in germ cells but which may be re-expressed in malignancy in a non-lineage restricted manner. CT antigens were originally identified by T-cell epitope cloning but SEREX and differential mRNA expression analysis have thus far identified approximately 20 CT antigens or antigen families. Spontaneous cellular and humoral immunity against several CT antigens such as NY-ESO-1 and MAGE-A has been observed. Importantly, the cancer-selectivity of these antigens has resulted in their investigation as therapeutic targets particularly for active immunotherapy [408].

In the mid-twentieth century, George Klein and colleagues demonstrated that tumours were immunogenic in the primary autochthonous tumour host. Tumours were induced using the chemical carcinogen MCA. Then the tumour was resected and cultured *in vitro* before it was used to re-inoculate the original host mouse or to inoculate a naïve syngeneic mouse. The mice previously treated with surgery survived the autochthonous tumour challenge and were cured whereas the naïve syngeneic mice died after the same tumour challenge [409]. Pramod Srivastava and his colleagues went onto discover the therapeutic principle inherent in the autochthonous tumour challenge, which likely resulted in the generation of injured and/or necrotic cells during implantation [141]. Fractionation of Klein's tumour cell preparations identified heat shock protein (HSP)-peptide complexes as the biochemical entity that conferred protective tumour immunity. HSP behave as 'chaperokines', which at least *in vitro* bind cell surface receptors such as the common heat shock protein receptor (CD91) on DC to deliver the chaperoned and unique tumour-specific peptides, and to induce NFκB activation [410], cytokine-like maturation of DC, and DC migration to draining lymph nodes. The authors argued that HSP behave as messengers of necrotic cell death and CD91 acted as a sensor of damage to the intracellular milieu [411]. It was hypothesised that these autochthonous MCA-induced murine tumours were rejected immunologically because of unique tumour antigens that were chaperoned by heat shock proteins [412]. While the physiological relevance of these findings remains in contention [141], 'pharmacological' preparations of tumour-derived HSP are being tested in phase III clinical trials [412].

Nevertheless, in the 1970's, Hewitt set out to determine if tumours evoked an immune response, particularly since many previous researcher workers may have transplanted tumours that were immunogenic because the tumours had been induced by viral or chemical carcinogens. He obtained 27 different types of spontaneously arising murine tumours and did not find a single instance of spontaneous tumour regression among 20,000 isotransplants of these tumours in mice [413]. These results are consistent with Schreiber's seminal findings that spontaneous tumours may not be immunogenic [350]. So, what allows a tumour to be rejected?

A relatively trivial explanation may reconcile Klein's results with those of Hewitt if note is taken of Klein's acts of tumour excision and *in vitro* culture of the excised tumour, which may have increased the immunogenicity of the autochthonous tumour challenge inoculum by exposing endogenous immunological 'danger' signals (Figure 10).

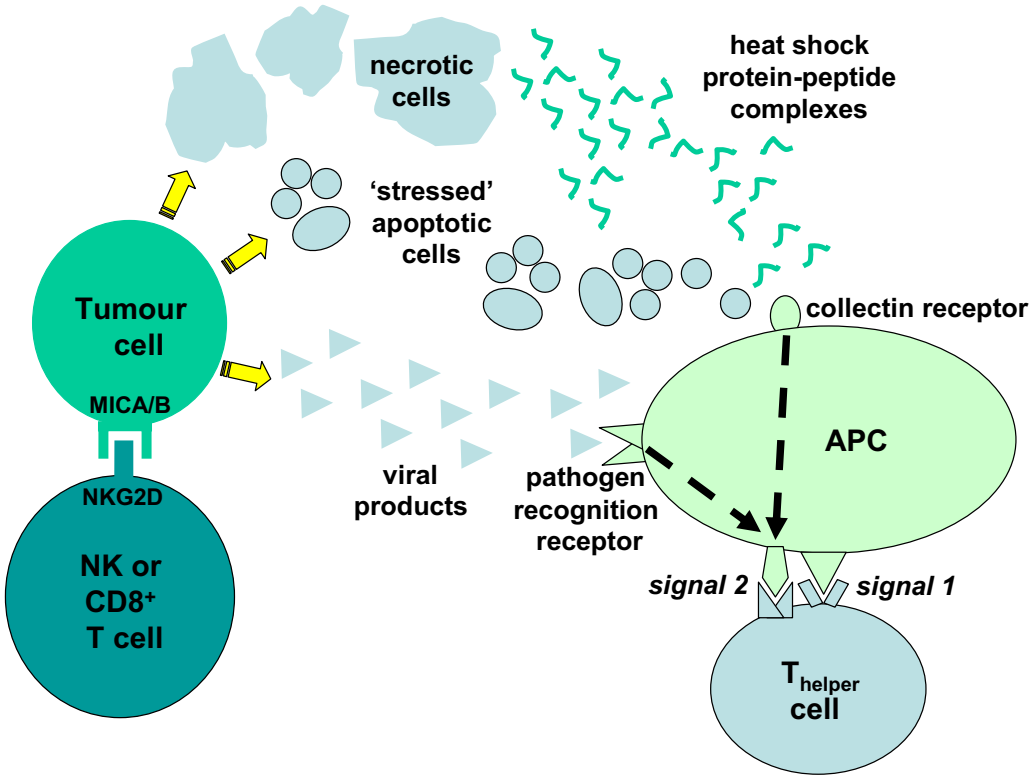


Figure 10: Endogenous 'danger' may supply additional dendritic cell maturation signals

In addition to exogenous danger signals such as viral products that bind pattern recognition receptors, endogenous danger signals may be expressed or become available in tumours. For example, necrosis may release heat shock protein-peptide complexes, which bind endogenous pattern recognition receptor family of collectin receptors such as common heat shock protein receptor (CD91). Similarly, tumour cells that have been stressed because of hypoxic and acidotic conditions may express on their surface heat shock protein-70, or MHC-like molecules such as MICA and MICB that engage activating natural killer group 2D (NKG2D) receptors on NK or $CD8^+$ T cells to elicit anti-tumour immune responses In conjunction with these antigen-presenting cell (APC)-activating signals, the tumour-derived material may be ingested by dendritic cells and cross-presented to T cells.

Earlier, in the 1890s, William Coley pioneered cancer immunotherapy by using what came to be known as "Coley's toxins", which were undefined and non-standardised bacterial lysates to treat patients with advanced malignancy. Coley obtained remission rates of approximately 40% in approximately 900 patients with some durable remissions although the results were not readily reproducible and the treatment was toxic [414]. Consequently, James Ewing's radiotherapy triumphed. Nevertheless, Coley's toxins presumably contained therapeutically promising immunological danger signals, which inspired the original commercialisation of TLR9 agonists by Coley Pharmaceuticals. Hypomethylated CpG motifs found in bacterial DNA are TLR9 ligands, which stimulate APC to induce potent cell-mediated immune responses [6]. Human non-small lung cancer cells express TLR and human tumour xenograft studies showed that treatment of mice with TLR9 ligands had anti-tumour effects, which may have occurred *via* direct action on tumour-expressed TLR9 and which were augmented by cytotoxic chemotherapy [415]. A ligand for TLR9 is currently being tested phase III clinical trials in conjunction with standard chemotherapy for advanced non-small lung cancer.

The oldest continuous cancer immunotherapy has been the use of Bacille Calmette-Guerin (BCG), which is an attenuated form of *Mycobacterium tuberculi*, in the treatment of early-stage bladder cancer. A randomised controlled clinical trial of intra-vesical doxorubicin *versus* intra-vesical BCG showed that BCG was superior in preventing recurrences of superficial transitional cell carcinoma (TCC) of the bladder. Of patients with Ta and T1 lesions of TCC bladder, 17% of those who received intra-vesical doxorubicin remained disease free at 5 years whereas 37% of patients who received intra-vesical BCG remained

disease free [416]. However, BCG induces a non-specific inflammatory reaction in the bladder wall, which involves $CD4^+$ and $CD8^+$ T cells but which does not confer protective immunity in a murine model [417]. On the other hand, adenovirally mediated CD40L gene transfer to bladder cancer cells does induce protective immunity (Loskög A *et al*., 2001) and may provide superior treatment to BCG [418].

1.9.4 Transplanted allogeneic lymphocytes treat leukemia and lymphoma

A distinct graft *versus* leukemia (GvL) effect was observed in allogeneic bone marrow transplant patients that was T cell-dependent and directly related to the degree of MHC-mismatch between donor and recipient [419]. Relapsed leukaemia patients were salvaged by infusions of donor lymphocytes [420], and these results have encouraged the exploitation of these mechanisms in other malignant disorders [421]. Although T cell depletion of the allograft prevents severe graft *versus* host disease (GvHD), it increases the incidence of leukaemic relapse, graft rejection and viral infection. Conversely, delayed infusions of donor lymphocytes post-transplant abrogate leukaemic relapse, particularly in CML, and infectious complications but severe GvHD may still result [419]. Notwithstanding the correlation of GvH and GvL effects with the extent of histo-incompatibility between donor and recipient, recent evidence suggests that these effects may be separable. Consequently, T cells that are specific either for overexpressed leukemia antigens such as PR3 and WT1 or haemopoietic lineage-restricted minor histocompatibility antigens, which are present on recipient and not donor bone marrow cells, have been expanded *ex vivo* for adoptive transfer to leukaemia patients [422].

The toxicity of stem cell transplants may be reduced by non-myeloablative conditioning of the recipient ('mini-allotransplant') followed by graded infusions of donor lymphocytes to maintain graft *versus* tumour effects mediated by donor lymphocytes. The mini-allotransplant approach has been extended to the treatment of leukaemia and lymphoma and, more recently, to the treatment of solid tumours such as metastatic renal cell carcinoma and metastatic melanoma. Although a 10% complete response rate was achieved using mini-allotransplants to treat metastatic renal cell carcinoma, the transplant-related mortality was 10-20%. This approach was not considered suitable for metastatic melanoma, which usually progressed too rapidly for any potential graft *versus* tumour effect to occur [421]. The latter clinical observation is consistent with the rationale for adoptive T cell immunotherapy for solid tumours developed from studies of tumour-bearing mice after adoptive transfer of tumour-specific exogenous lymphocytes. These studies indicate that CTL wane in number and function within the tumour microenvironment, perhaps because of tumour secretion of immunosuppressive cytokines such as TGFβ, and that, based on murine studies, high numbers of activated CTL with a high-affinity TCR are required to exert tumour control because a 'kinetic race' exists between CTL and tumour cells [423].

1.9.5 Immunosurveillance of virus-induced malignancies

Chronically immunosuppressed renal transplant patients had a significantly higher incidence of malignancy particularly those that are virally induced [424]. A wealth of evidence indicates that failure to adequately protect against infectious pathogens because of immunocompromise predisposes to malignancy. Renal transplant registry data show that chronic immunosuppression produces an excess of cancers of skin and anogenital region as well as lymphomas, hepatocellular carcinomas and Kaposi's sarcoma. HPV probably predisposes to squamous cell carcinomas of the cervix and anogenital region, and chronic infection with hepatitis B and/or C viruses predispose to hepatocellular carcinoma, which is the commonest malignancy in China. Infection with human herpes virus-8 (HHV-8) is strongly associated with Kaposi's sarcoma. HIV-infected patients suffer opportunistic viral infections and virus-induced malignancies. Most AIDS-related malignancies result from opportunistic infections with EBV, HHV-8 or HPV. Chronic infection with *Helicobacter pylori* predisposes to gastric adenocarcinoma and MALT lymphoma [312]. EBV is associated with Burkitt lymphoma, Hodgkin disease, and nasopharyngeal carcinoma. Post-transplant lymphoproliferative disease (PTLD) is caused by uncontrolled EBV infection, which can be prevented and treated by the

adoptive transfer of donor-derived EBV-specific CTL [425].

1.9.6 Cancer immunotherapy

The development of immune-based treatments for cancer has been empirical. The earliest and most successful have been passive immunotherapy using mAb or allogeneic lymphocytes with graft *versus* tumour activity. Active immunotherapy will increasingly play an important role in cancer treatment and includes both prophylactic and therapeutic anti-cancer vaccines. Clearly, the most effective anti-cancer vaccines have been those that prevent cancers caused by infectious agents such as HBV and HPV, which are the most common causes of hepatocellular carcinoma and cervical carcinoma, respectively.

Successful active immunotherapy of cancer will require a combination of strategies that (i) deliver powerful costimulatory signals that prevent T cell anergy following T cell recognition of tumour antigens, (ii) overcome or bypass mechanisms used by the tumour to evade the immune system, and (iii) develop immune effector functions that are tumour-specific.

Among the range of active and passive immunisation strategies currently being tested preclinically and clinically, which will produce optimal anti-tumour immunity is not yet known. Autologous whole tumour cells are preferred for the creation of gene-modified tumour vaccines because the 'universe' of known and unknown TAA in the patient's tumour is available to be presented in the context of both MHC class II and I molecules on the same APC in order to optimise the generation of $CD4^+$ and $CD8^+$ T cells, respectively, for potent anti-tumour cytotoxicity. While genetic immunisation with individual tumour-specific antigens may arguably be less effective, this approach facilitates quantification of both the amount of antigen delivered and the associated immune response, which may require further careful clinical characterisation in order to understand and manipulate it for therapeutic purposes [426 427]. On the other hand, the adoptive transfer to patients of CTL generated against tumour cells *ex vivo* may obviate the need for generating active immune responses.

The ultimate expectation for any approach to cancer treatment, which includes gene therapy, is that it be curative and preventative. It might be expected also that cancer treatment strategies be versatile enough to match the diverse genetic lesions, biologic behaviours and clinical presentations inherent in the malignant diseases. These strategies will be most limited if their successful application depends on every cancer cell being targeted directly. Unless all malignant cells are targeted, non-transduced cells may obtain a selective growth advantage and out-grow the gene modified cells. Hence, cancer treatment strategies will more likely be successful if they induce a 'bystander effect' to ensnare non-targeted cells in the trap.

Bystander effects apply to a number of cancer gene therapy approaches. Replication-conditional viral vectors or oncolytic viruses often have deletions favouring viral replication in tumour cells because these viruses harbour a metabolic abnormality that genetically complements the virus. For example, ONYX-015 is a mutant adenovirus that replicates preferentially in tumour cells, which lack p53 or p14/ARF thus selectively causing lysis only of these cells [428 429]. Viral fusogenic membrane glycoproteins, which are found in varicella-zoster virus, measles virus, HIV, VSV-G envelope and a mutated version of the Gibbon Ape Leukemia Virus (GALV) envelope, induce formation of lethal multi-nucleated syncytia by fusing target cells to neighbouring cells [430]. Gene-directed enzyme pro-drug therapy (GDEPT) may convert a previously harmless drug to an injurious metabolite that is emitted from the targeted tumour cells to affect neighbouring non-targeted cells even if gene transfer efficiencies are limiting. For example, HSV-TK phosphorylates the nucleoside analogue, ganciclovir, to produce metabolites that interfere with cell division. Local bystander effects result mainly from intercellular gap junction transfer of these metabolites between cells (reviewed in 433). Subsequently, apoptotic and necrotic cell death results in the expression of cytokines, costimulatory and adhesion molecules and heat shock proteins thus producing 'danger' signals that promote distant bystander effects [431 432 433] mediated by $CD4^+$ and $CD8^+$ T lymphocytes [431 434 435].

1.9.6.1 Passive cancer immunotherapy

Monoclonal antibodies exert their therapeutic effects by both direct and indirect means, which tend to be mediated by their fragment-antigen binding (Fab) and fragment-crystalline (Fc) moieties, respectively. Fab binding to the target antigen can induce signalling changes in the target tumour cell, which induce apoptosis, inhibit proliferation, and sensitise the tumour cells to cytotoxic effects of some chemotherapy drugs. In addition, the tumour cell bound by the Fab end of the mAb may be destroyed by immune effector mechanisms that are engaged by the Fc or 'business' end of the mAb. These mechanisms include CMC and ADCC [436], and opsonisation of tumour cells for phagocytosis with consequent processing and presentation of ingested tumour antigens for induction of anti-tumour T cell responses (Figure 7).

Two major examples of therapeutic anti-cancer mAb include rituximab and trastuzumab. Rituximab targets CD20, which is expressed by normal mature B lymphocytes and more than 95% of malignant B lymphocytes of non-Hodgkin lymphoma (NHL). Treatment with rituximab produces profound B lymphopenia for six to nine months but not hypogammaglobulinaemia. Treatment with rituximab produces clinical responses in low grade NHL, which may be delayed by months and the efficacy of which may be related to Fc receptor polymorphisms [437]. For low grade NHL patients, while rituximab monotherapy is active in chemorefractory or relapsed disease with overall response rates of approximately 40% [438], it is more effective in combination with chemotherapy [439]. Addition of rituximab to CHOP (cyclophosphamide, doxorubicin, vincristine, prednisolone) chemotherapy significantly prolongs overall survival of diffuse large B cell lymphoma patients both below [440] and above [441] 60 years of age.

Trastuzumab targets c-ERBB2 or HER2, which is a cell surface molecule overexpressed in approximately 25% of breast cancers. Patients with c-ERBB2^{+} metastatic breast cancer have a significantly worse median survival of approximately three years compared with approximately six years for patients whose disease lacks c-ERBB2 overexpression. Overexpression of c-ERBB2 results from amplification of the *c-erbB2* gene, which produces a constitutively active proto-oncogene receptor. Consequently, trastuzumab monotherapy is effective only in c-ERBB2^{+} metastatic breast cancer patients. Trastuzumab has *in vitro* synergy with cytotoxic drugs including docetaxel and platinum-based agents, and trastuzumab and chemotherapy combinations prolong the overall survival of c-ERBB2^{+} metastatic breast cancer patients by approximately 25% [442]. These results have also translated to dramatic 40-50% reductions in risk of relapse in patients who received the combination of trastuzumab and chemotherapy as adjuvant treatment after definitive breast cancer surgery. The use of trastuzumab essentially obviated tumour c-ERBB2 expression as a risk factor for relapse [443].

1.9.6.2 Active cancer immunotherapy

Working from the principle that "prevention is better than cure", vaccination to prevent cancer is probably the most effective public health measure that could be taken to reduce the morbidity and mortality of cancer. A universal HBV immunisation program in Taiwan produced a significant decline in the incidence of childhood hepatocellular carcinoma (HCC) and serves as an indicator of an expected decline in adult HCC, which peaks in the sixth decade. This study provides evidence that HBV infection is causal in HCC and that immunisation against a viral infection prevents a specific cancer [444]. The oncogenic strain of HPV, HPV-16, infects 20% of adults and is present in 50% of cervical cancers and high grade cervical intra-epithelial neoplasia (CIN). A double blind study in 2400 women aged 16 to 23 years of placebo *versus* a synthetic HPV-16 virus-like-particle (VLP) vaccine demonstrated 100% efficacy at a median follow up of 17.4 months. All 41 cases of new HPV-16 infection occurred among placebo recipients and included nine cases of HPV-16-related CIN. The HPV-16 VLP vaccine comprised empty viral capsids, which displayed L1 protein conformational determinants that elicited neutralising antibodies [445]. Although this study provided evidence that HPV infection was preventable, further follow-up indicated that, moreover, the vaccine provided high-level protection against persistent HPV16 infection and

HPV16-related CIN2-3 for at least 3.5 years after immunisation and thus is likely to reduce risk of cervical cancer [446].

Evidence from recent genetic studies strengthens the rationale for active cancer immunotherapy as an approach to tumour eradication. IFNγ signalling defects were observed in tumour cells and IFNγ made tumours immunogenic by up-regulating MHC class I and TAP expression [350]. Restoring TAP function in IFNγ-insensitive tumours resulted in tumour rejection that was mediated by $CD4^+$ and $CD8^+$ T cells. Significantly, mice that rejected these tumours were also protected against challenge with IFNγ-insensitive parental tumours, which indicated that a higher level of antigen expression was required for induction of the immune response than for recognition by the immune effector cells [350]. Tumour immunogenicity may be improved by increasing costimulation by tumour cells and/or by correcting defects in antigen presentation and processing. Accordingly, cancer vaccines may comprise any of the following components:

(i) autologous or allogeneic tumour cells

(ii) heat shock protein preparations

(iii) defined tumour-associated antigens

(iv) non-viral DNA or viral vectors

(v) DC or DC/tumour hybrids

(vi) immunostimulatory molecules or genes e.g. cytokines, chemokines, costimulators [447].

It is likely that cancer vaccines will be most effective in the adjuvant clinical setting after definitive surgery and/or radiotherapy when micrometastatic disease is probable and thus, the tumour burden and its associated production of an immunosuppressive microenvironment is least. Consequently, the proof of cancer vaccine efficacy as adjuvant therapy would be a long and expensive exercise. Of particular interest are the DC-based anti-cancer vaccines because the DC is the professional APC that uniquely initiates immune responses, which may be tolerogenic or immunogenic. Objective tumour response rates of 25-30% were achieved using autologous melanoma-loaded DC vaccines and may be associated with melanoma-specific immune responses [448] [449] [450].

1.9.6.3 A primer on adjuvants and vaccines

From Jenner's original observation in 1796 of the ability of immunisation with material from cowpox viral pustules (vaccination) to protect against natural infection with smallpox virus, the therapeutic potential of vaccines has been evident. Indeed, in the age of recombinant DNA technology, viruses continue to be employed in vaccines but now more often as vectors for the expression of genes for heterologous antigen and immunomodulatory molecules. Virally vectored vaccines have the advantage of intrinsic adjuvanticity because they are microbial products [67]. In addition, cells *per se*, and in particular tumour cells, are used in vaccines often without a requirement for adjuvant. The immunogenicity of tumour cell vaccines seems to depend on the combination of 'endogenous adjuvant' activity acting on DC that engage in potent cross-presentation of tumour cell-derived antigens [141].

Early experimental studies dating from the 1920s showed that the magnitude of immune responses to antigens could be boosted by the addition of adjuvants, which included lipids and detergents, and microbial products such as LPS, the killed *Mycobacterium tuberculi* preparation, BCG, and *Corynebacterium parvum*. Adjuvant derives from 'adjuvare', which is Latin for 'to help' [451]. Until recently, the mechanisms of adjuvant action have been unknown, and for that reason, adjuvants have been described as the Immunologist's "dirty little secret" [137]. It had been hypothesised that some adjuvants such as those involving emulsions may act as a depot to allow sustained release of antigen, and to recruit leukocytes to the site of deposition. While support remains for this hypothesis, it did not explain durable immunological effects of some adjuvants. Recent advances in our molecular understanding of immune system function now also provide insights into mechanisms of adjuvant action. Some

adjuvants, particularly those of microbial origin, are best considered as exogenous adjuvants that act by binding APC pattern recognition receptors such as TLR. Other adjuvants originate from dying cells *in vivo* and hence may be considered as endogenous adjuvants, which act on overlapping pattern recognition receptors as well as other APC receptors (see Sections 1.5.4 and 1.5.4.2).

'Suicide gene therapy' studies of tumour cells that were genetically modified to express pro-drug converting enzymes such as thymidine kinase or cytosine deaminase demonstrated that the tumour cell immunogenicity was promoted after tumour cell death was induced *in situ* by treating the tumour-bearing mice with a pro-drug, which was converted in the tumour to a cytotoxic metabolite. In some cases, the immunostimulation was sufficient to induce protective tumour immunity [452] [453] [454] (see Section 1.9.6).

The adjuvanticity of syngeneic cells was first demonstrated when soluble ovalbumin protein, which is known to be poorly immunogenic alone [455], was mixed with either irradiated or mitomycin C-treated apoptotic cells or freeze-thawed necrotic syngeneic cells and induced $CD8^+$ CTL and $CD4^+$ T cell responses [456]. This effect was not simply the result of aggregation of the ovalbumin protein with cellular material to form particulate antigen, which is known to be more immunogenic than soluble ovalbumin because the same effect was achieved if ovalbumin-conjugated particles were mixed with freeze-thawed cells. Moreover, antigen-specific $CD4^+$ and $CD8^+$ T cell responses were further augmented if particulate antigen was injected at a separate site from the cellular material, which indicated that a systemic immune process had been induced by the endogenous adjuvant. Moreover, this classical adjuvant activity was augmented significantly further if the cells were injured several hours before injection [457]. Eventually, monosodium urate was identified as the active low molecular weight cytosolic constituent that was responsible at least in part for these immune enhancing effects. It is likely that, during the tissue injury associated with infection, endogenous activators such as monosodium urate amplify the response to infection [147].

HMGB1 is another cellular constituent that has endogenous adjuvant activity. HMGB1 is DNA binding protein, which binds to the receptor for advanced glycation end-products (RAGE) on DC and macrophages and which may also bind TLR2 and TLR4. HMGB1 is released by necrotic cells and induces the migration of human immature moDC *via* its interaction with RAGE. HMGB1 also activates immature moDC by up-regulating the expression of IL-6, IL-8, IL-12p70, TNF as well as cell surface MHC class I, CD80, CD86, and CD83 molecules. Consequently, DC switch from responsiveness to inflammatory chemokines such as RANTES to a lymph node-homing phenotype [458]. HMGB1 demonstrated molecular adjuvant activity by enhancing antibody responses in mice to ovalbumin, and by protecting mice against tumour challenge after HMGB1 was mixed with apoptotic tumor cells. Activated DC have been reported to secrete HMGB1 after LPS stimulation, and blocking studies suggest that DC may require HMGB1 and RAGE to become fully stimulatory APC [152].

In a subcutaneously inoculated transgenic CD40L-expressing tumour cell vaccine, alarmins and other endogenous danger signals provide an immediate chemotactic stimulus to attract immune cells including APC to the immunisation site for subsequent activation [152]. CD40L itself activates DC and macrophages to produce chemokines such as IL-8 and MIP-1α [230] and so may amplify the chemotactic and immunostimulatory activity of endogenous adjuvants.

In preceding sections, immune deficits were described in relation to the congenital lesion of CD40L deficiency (Section 1.6.1), the poor induction of protective immunity to RSV infection, which makes infants and the elderly particularly susceptible to re-infection with RSV (Section 1.7), and to the immunosuppression characteristic of advanced cancer in particular (Section 1.9.1). Vaccine technologies, which include genetically modified vaccines, enable the decision that the immune system makes between tolerance and immunity after antigen exposure to be manipulated for therapeutic purposes. Hence, therapeutic intervention in model systems and in leukaemia patients using genetically modified cellular vaccines, or

genetically modified virally vectored vaccines, will be described and discussed as an approach to treat the immune deficits of congenital CD40L deficiency (Chapter 3), RSV infection (Chapter 4), and cancer (Chapter 5).

1.9.6.4 An introduction to immunogene therapy

For many malignancies, antigens have not been defined or may be heterogeneous between individual tumours. Consequently, whole tumour cells have frequently been used as a source of antigen. The genetic modification of tumour cells with immunomodulatory genes is designed to increase the specificity and potency of the anti-tumour immune response by overcoming avoidance mechanisms and by providing costimulatory signals. Although autologous tumour vaccines should ideally provide the relevant antigens for an anti-tumour immune response, many cancers cannot be grown, expanded, or transduced easily in culture to allow for the development of autologous genetically modified tumour vaccines. Mixing irradiated autologous tumour cells with gene-modified autologous or allogeneic fibroblasts, or the use of allogeneic tumour cell lines or partially matched allogeneic tumour cell lines (transduced with additional HLA genes), may help to circumvent this problem. Fibroblasts are easier to obtain and have been shown to satisfactorily express cytokine genes, which can achieve high local concentrations, which can induce an immune response against co-injected tumour cells. Several classes of immunologically related genes have been used in clinical tumour vaccine studies and include cytokines such as IL-2, which activates T and NK cells, or GM-CSF, which activates APC; costimulatory surface molecules such as CD80; chemokines such as lymphotactin, which attracts lymphocytes [459 460], and allogeneic MHC molecules such as HLA-B7 [11].

The clinical efficacy of cancer vaccines that include a non-specific immune stimulus such as BCG suggests that optimal anti-tumour effects may be produced by the transfer of a combination of immunomodulatory genes with minimally overlapping functions. For example, gene transfer studies in murine tumour vaccine models support this concept established that the combination of CD80 and IL-4 genes produced superior anti-tumour effects compared with the use of either gene alone or the non-specific immune adjuvant, *Corynebacterium parvum* or BCG [461]. Furthermore, combinations of IL-2 with , or of IL-2 with CD40 ligand, had significantly greater anti-tumour activity than either agent used singly in murine models of ALL, AML and neuroblastoma [460] (Dilloo D *et al*., 1997; Grossmann ME *et al*., 1997). Clinical trials of these combinations of cytokine and chemokine, or cytokine and costimulator, have now been conducted in childhood neuroblastoma [460] and ALL (Rousseau RF *et al*., 2006), respectively.

Phase I clinical studies of IL-2 gene-modified neuroblastoma vaccines in patients with relapsed stage IV disease demonstrate the safety of the vaccines and also illustrate the relative advantages of allogeneic and autologous tumour vaccines [462 463]. Although allogeneic vaccines are easier to prepare, standardise and package, autologous vaccines are more likely to generate tumour-specific rather than allogeneic immune responses, and to allow the appropriate tumour antigens to be presented in the context of the correct MHC. The allogeneic vaccine was an IL-2-expressing neuroblastoma cell line made by retroviral transduction. Autologous vaccines were prepared by adenoviral transduction of *ex vivo*-expanded neuroblasts. Undesirable effects of under- or over production of IL-2 were avoided by controlling the IL-2 production rate at 500-2000pg/10^6 cells/24 hours for both vaccines [464]. Of twelve evaluable patients given the allogeneic vaccine, one had a partial response and five had partial responses after oral etoposide was given. Four patients were alive with disease 77-726 days after vaccination [462]. Of ten evaluable patients given autologous vaccines, one had a complete response, one had a partial response and one had stable disease. Additionally one patient had a complete response, and two had partial responses after oral etoposide was given. Eight patients were alive 110-510 days after vaccination, six with disease [463]. $CD4^+$ T cell infiltrates were observed at the vaccination sites and neuroblast-specific CTL and humoral responses were detected [462 463].

Although the allogeneic vaccine was active, the autologous vaccine generated greater local and systemic immune responses, which were both specific and non-specific. The observed immune responses may have produced anti-tumour responses and, indeed, complete remissions were seen only with the autologous vaccine. These precursor studies supplied the rationale for combination immunogene therapy using an autologous leukaemia vaccine (Rousseau RF *et al*., 2006).

1.9.7 Genetic enhancement and correction strategies for cancer gene therapy

The genetic lesions of cancer may be categorised as gain of function lesions such as oncogene mutations, or as loss of function lesions such as mutation or deletion of tumour suppressor genes. Small molecule inhibitors such as the BCR-ABL tyrosine kinase inhibitor, imatinib mesylate, may be the most effective way of treating gain of function lesions. Conversely, gene replacement approaches may be the most effective means of remedying loss of function lesions such as the p53 tumour suppressor gene, which is mutated or functionally impaired in approximately 50% of common human cancers [465]. Replacement of the p53 gene may be a particularly potent approach to cancer therapy [466] [467]. Nevertheless, powerful new gene transfer technologies such as single chain variable fragments of antibodies (scFv) or intrabodies [468] [469], ribozymes (reviewed in ref. 473, 474), DNAzymes [470], or small interfering RNA [471] may be used to inhibit oncogenes or drug resistance genes.

CHAPTER 2

CD40L, CRYPTOSPORIDIOSIS, CHOLANGITIS, AND CANCER

Janeway proposed that successful adaptive immunity depended on innate immunity [137]. Here, not only will this proposition be discussed, but using the peculiar association of chronic cryptosporidiosis and hepatobiliary malignancy in CD40L-deficient patients, it will be argued that a CD40L-dependent interplay between innate and adaptive immunity defends against malignant transformation.

The mechanisms initiating immune activation together with the cellular and molecular patterns associated with chronic inflammation resemble those described in acute inflammatory host responses to infectious pathogens, which although self-amplifying, are self limiting, and resolve within days [154]. During acute inflammation, the classical NFκB pathway-dependent expression of inflammatory chemokines directs the migration of DC to sites of inflammation, where they take up antigens. There, pro-inflammatory cytokines such as TNF and pattern recognition receptor ligands drive the maturation and migration of DC to draining lymph nodes. CD40L, which signals more slowly *via* the alternative NFκB pathway, may be induced in inflammatory sites and prepare the delivery to draining lymph nodes of antigen-laden and fully competent DC. Similarly, CD40L that is expressed also in draining lymph nodes will lead to the induction of a fully fledged, productive immune response [188]. Consequently, signalling *via* the alternative NFκB pathway may switch an acute, short-lived inflammatory response to a delayed and sustained lymphocyte-driven response that is classically associated with chronic inflammation. In these circumstances, the critical inflammatory mediator, TNF, links chronic inflammation and cancer [154].

2.1 CD40L deficiency, cryptosporidiosis, hepatobiliary inflammation and malignancy

Despite chronic hypogammaglobulinaemia, boys with HIGM1 commonly develop chronic hepatitis and cholecystitis and die in their teenage years with malignancies of the hepatobiliary system and pancreas. This observation would appear to result directly from the cellular immune deficiency caused by lack of CD40L, because chronic hypogammaglobulinaemia is more often associated with development of lymphoma and gastric carcinoma. Cholangitis progresses with fibrosis to sclerosing cholangitis then to biliary cirrhosis and ultimately liver failure. The progression of hepatobiliary disease in boys with HIGM1 was age-related so that of the original 53 reported patients, 16 reached the age of 20 and only four did not have liver disease. Although this was not a prospective study, the observed frequency of hepatobiliary disease clearly exceeded that described in X-linked hypogammaglobulinaemia and common variable immunodeficiency. While *Cryptosporidium parvum* (CP) infection was not sought systematically, it was identified in 14 patients, all of whom had cholangitis or cirrhosis. Infection with cytomegalovirus (CMV) and hepatitis viruses was also identified in several of these patients [472].

Nine of the 53 HIGM1 patients had pre-existing cirrhosis or sclerosing cholangitis and developed 10 incurable gastrointestinal malignancies, which were diagnosed at a median age of 17.5 years. The tumours included three cases of cholangiocarcinoma together with two cases of hepatic metastases of poorly differentiated adenocarcinoma that were probably of biliary origin, two cases of primary hepatocellular carcinoma, and three cases of tumours of neuroendocrine origin. Since bile ducts, and the endocrine and exocrine pancreas all have a common embryological origin in the foregut, malignancies of these cell types may differentiate as neuroendocrine tumours or adenocarcinomas. CD40 expression was not detected on normal bile duct epithelium using immunohistochemical methods but it was detected in regenerating biliary epithelium, sclerosing cholangitis and in intrahepatic bile

ducts of HIGM1 patients with liver metastases. The authors argued that, because two cases of bile duct tumours had normal appearing bile ducts near the tumours, chronic inflammation was unlikely to be sufficient explanation for the induction of malignancy [472]. In a later registry report of 69 HIGM1 patients enrolled between 1997 and 2002 in the USA, five patients had sclerosing cholangitis, four of whom had associated cryptosporidiosis. Two patients died from hepatocellular carcinoma and one from cryptosporidium-associated sclerosing cholangitis [292].

2.2 Gastrointestinal infection with the cryptosporidial parasite

Although CP is the one of the most commonly reported enteric pathogens worldwide [151], the pathogenesis of CP infection remains poorly understood. CP is a ubiquitous and highly infectious protozoal parasite, which causes transient and trivial diarrhoea in healthy subjects. Infection occurs with CP oocysts that subsequently discharge sporozoites. Sporozoites attach to enterocytes, and then invade to form parasitophorous vacuoles. Subsequently, sporozoites replicate within enterocytes and disrupt enterocyte handling of electrolytes to cause diarrhoea. Cellular immunodeficiency reveals CP to be an opportunistic pathogen that causes chronic persistent infections and life-threatening illness for which no effective treatment exists [473]. Chronic enteric infection in immunodeficient individuals causes disabling diarrhoea. Infection often extends beyond the gut probably *via* the portal circulation to the biliary tree where CP is the most commonly identified pathogen in AIDS cholangiopathy [151].

In tissue-sections of human liver, biliary epithelial cells (cholangiocytes) express all known members of the TLR family of pattern recognition receptors. Using an SV40-transformed human cholangiocyte cell line *in vitro*, CP was shown to induce recruitment of and subsequent signalling by TLR2 and TLR4. Although these TLR were not required for attachment and invasion, downstream signalling effects included translocation of the p50/RelA NFκB heterodimer to the nucleus with consequent secretion of antimicrobial β-defensin molecules, cytokines such as TNF, chemokines such as IL-8, and pro-inflammatory mediators such as products of prostaglandin (PG)-E_2. In addition, NFκB activation has anti-apoptotic effects, which presumably favour parasite propagation [151]. CP parasites do not infect monocytes or macrophages and are unlikely to stimulate IL-12 production [474], which is likely instead to depend on additional stimuli such as CD40 ligation of myeloid cells.

2.3 Cell mediated immunity defends against cryptosporidiosis

Although normal individuals secrete anti-CP antibodies, which seem to reduce parasite numbers in the intestine, antibodies do not protect AIDS patients from heavy parasite burdens. Animal models have confirmed the vital role that cell mediated immunity plays in recovery from CP infection [473]. BALB/c severe combined immunodeficiency (SCID) mice, which lack B and T cells because of failed genomic antigen receptor rearrangements but which retain NK cells, have been a very useful model for the study of CP infection. Whereas CP organisms are cleared within four to six weeks of infection in normal mice, CP persists in the gut of SCID mice and extends to the biliary tree within six weeks of gut infection [475].

2.3.1 The role of NK cells in cryptosporidial infection

Despite chronic persistent CP infection of SCID mice, clinical illness was not evident, which indicates that CP was not itself directly toxic to murine tissues. Moreover, CP-infected SCID mice did not develop biliary tract pathology other than mild triaditis [476]. IFNγ-producing mononuclear cells were observed among the inflammatory infiltrate in the submucosa of intestine and gallbladder of CP-infected SCID mice. The authors reasoned that these $IFN\gamma^+$ mononuclear cells were $IFN\gamma^+$ NK cells because, at that time, only T and NK cells were believed to produce IFNγ [477]. In the confirmed absence of T cells in CP-infected SCID mice, NK cells would appear to be the only other source of IFNγ to protect against clinical illness and biliary tract pathology [477].

Subsequently, however, it has been revealed that other cell types produce IFNγ with appropriate stimulation. IFNγ may be produced by T cells, NK cells, NKT cells and, B cells if

stimulated with IL-12 [478]. Macrophages may produce IFNγ if stimulated with IL-12 and IL-18 [479]. DC may produce IFNγ if stimulated with IL-12 [480] and IL-18, or with IL-12 and IL-4, or *via* CD40 and with IL-12 [481]. Moreover, the recently discovered unique cell type of the interferon-producing killer DC (IKDC) identifies a significant source of IFNγ [482] [483] (see Section 2.6.3). Although mice deficient in NK cells (neonatal SCID and beige mice) were observed to be susceptible to CP infection, NK cell function was not studied in these mice [484]. Depletion of splenic NK cells in CP-infected SCID mice using the anti-asialo-GM1 antibody did not worsen the clinical course of infection in these animals. In the study, it was suggested that gut NK cells made the most important contribution to protection against disease because (i) gut NK cells may have been isolated from the effects of anti-asialo-GM1 antibody treatment, and (ii) IFNγ$^+$ mononuclear cells were found in the intestinal submucosa of CP-infected SCID mice [485] [477]. Therefore, these studies do not exclude a role for other IFNγ-producing cell types such as IKDC in defending against CP infection.

2.3.1.1 IFNγ may be required for the initial response to cryptosporidial infection

Adoptive transfer of CD4$^+$ T cells cleared CP infection in SCID mice [486] [487] [488] but recovery from infection was impaired by neutralising antibodies against IFNγ [486] [489] [490], IL-4 [491] or IL-12 [492]. Depletion studies in adult CP-infected BALB/c mice using anti-CD4 and/or anti-IFNγ antibodies suggested that IFNγ production by a non-T cell contributed to host immunity [490]. It was concluded that IFNγ-producing intestinal NK cells may be responsible for the lack of illness in CP-infected SCID mice [477]. In IFNγ-deficient mice, the response to CP infection was variable: approximately half of the mice cleared the infection, but mice with persistent infection had severe enteritis [472]. Moreover, IFNγ-deficient mice did not display immunity upon re-challenge with CP. Thus, it seems that IFNγ may be required for the initial response to infection because recovery could later proceed in its absence [477].

Other possible mechanisms of IFNγ-mediated resistance to infection include reducing susceptibility to infection of epithelial cells, inducible nitric oxide synthase (iNOS) production by macrophages [472], or promotion of apoptosis in conjunction with TNF [476]. However, iNOS-deficient mice recovered normally from CP infection, which indicates that iNOS is not required for CP immunity [477]. Nevertheless, the combination of IFNγ, which is induced by CD40L/CD40 interactions, and TNF result in apoptosis of CP-infected epithelial cells [477],

2.3.2 The role of T cells and T cell interactions in cryptosporidial infection

2.3.2.1 Non-cognate CD4$^+$ T cell expression of CD40L suffices for cryptosporidial clearance

RAG-deficient mice also lack B and T cells because genomic antigen receptor rearrangements cannot be made and, like SCID mice, RAG-deficient mice cannot clear CP infection. To examine the effect of competent CD4$^+$ T cells of irrelevant specificity on CP infection, RAG-deficient mice were bred to have CD4$^+$ T cells bearing a rearranged transgenic T cell receptor (DO11.10), which is specific for the chicken ovalbumin (OVA) antigen and which does not cross-react with CP antigens. In contrast to RAG$^{-/-}$ mice, these DO11.10 RAG$^{-/-}$ mice can clear CP infection. Similar results were obtained in AND RAG$^{-/-}$ mice, which is another RAG-deficient transgenic CD4$^+$ T cell model in which the AND T cell receptor is specific for pigeon cytochrome C [493].

CP-infected SCID mice were reconstituted with splenocytes from DO11.10 RAG$^{-/-}$ mice, which contain CD4$^+$ T cells bearing only the OVA-specific TCR. Surprisingly, these reconstituted SCID mice cleared the CP infection unless the OVA-specific transgenic T cells were tolerised by administration of soluble OVA protein to the infected mice. This result suggests that only the transgenic CD4$^+$ T cells among the splenocyte preparation contributed to clearance of the CP infection. Moreover, proliferating and activated OVA-specific transgenic CD4$^+$ T cells were found in the intestinal submucosa within 48 hours of adoptive transfer only in CP-infected and not in uninfected SCID mice [475].

Furthermore, CP infection in reconstituted SCID mice was not cleared if the OVA-specific transgenic T cells were CD40L-deficient [475], which is similar to the result previously obtained when the adoptive transfer of CD40L-deficient splenocytes to CP-infected SCID mice failed to eliminate CP infection [473]. These data indicate that the CP-infected environment of SCID mice suffices to activate T cells of irrelevant specificity, which subsequently supply CD40L to enable clearance of the CP infection [475]. CP-infected SCID mice, which are reconstituted with transgenic $CD8^+$ T cells, which bear a TCR specific for a Listeria antigen and which do not express CD40L after activation, also do not clear CP infection. In all, these data indicate that an antigen-specific immune response is not required for recovery from CP infection, although the mechanism by which transgenic T cells are activated to express CD40L was not identified. Candidate mechanisms include low affinity TCR-self MHC interactions occurring in a favourable costimulatory environment or antigen-independent T cell activation. Although the mechanism for CP elimination from CP-infected SCID mice remains unknown, these data indicate that $CD4^+$ T cells are the source of CD40L required for elimination of CP infection [475].

2.3.2.2 MHC class II is required for T cell activation and cryptosporidial clearance

MHC class II-deficient mice, which lack the restriction element for thymic selection of $CD4^+$ T cells and so lack $CD4^+$ T cells, cannot clear CP infection [494]. As cryptosporidial sporozoites invaginate into intestinal epithelial cells and remain separated from the cytoplasm by an intact cytoplasmic membrane [477], it is unlikely that the MHC class I pathway would be utilised in immunity against CP. So it was found that neither MHC class I molecules [494] nor MHC class I-restricted CD8 T cells [487 488 490] were required for cryptosporidial clearance.

It is known that CP-infected MHC class II-deficient mice remain infected after adoptive transfer of wild type splenocytes. The adoptively transferred $CD4^+$ T cells among the wild type splenocytes were not activated, and presumably were thus unable to eradicate the CP infection. Because T cells of irrelevant specificity were able to clear CP infection [493], experiments were designed to test the dependence of CP clearance on CP-derived MHC class II bound peptides. To achieve this aim, the authors used $A\beta E\alpha^{-/-}Ii^{-/-}$ genetically manipulated mice, which lack the invariant chain essential for peptide loading of MHC class II molecules and which are also transgenic for the I-A (MHC class II) molecule to which a captive I-E peptide is bound. Hence, $A\beta E\alpha^{-/-}Ii^{-/-}$ mice express a single irrelevant peptide-MHC class II complex that blocks specific antigen presentation, for example, from CP. This transgenic MHC class II molecule is sufficient to select $CD4^+$ T cells in the thymus, which then amount to approximately 50% of the normal number of $CD4^+$ T cells. In both AND $RAG^{-/-}$ and $A\beta E\alpha^{-/-}Ii^{-/-}$ transgenic TCR models, clearance of CP was preceded by transgenic $CD4^+$ T cell activation, which included expression of CD40L, in the draining mesenteric lymph nodes (MLN). Hence, it appears that a TCR-mediated recognition event occurs in the MLN of CP-infected mice that results in activation and is followed by clearance of CP. Since high-avidity and self-reactive T cells are negatively selected in the thymus and low avidity self T cells are positively selected and exported from the thymus, it was hypothesised that irrelevant T cells would cross-react with self antigens and become activated. Hence, the experiments using $A\beta E\alpha^{-/-}Ii^{-/-}$ mice demonstrated that MHC class II was required for thymic selection and subsequent T cell activation in the MLN irrespective of whether the MHC class II molecules were loaded with self or irrelevant peptides [493].

2.3.2.3 CD40 is required only on bone marrow-derived cells for cryptosporidial immunity

While the eradication of CP infection depends on CD40L expression by $CD4^+$ T cells, immunity to CP does not depend on CD40 expression by epithelial cells but rather by bone marrow-derived cells [495], which presumably express MHC class II molecules.

2.4 CD40L-deficient mice model cryptosporidiosis and hepatobiliary inflammation

CP-infected HIGM1 patients develop sclerosing cholangitis and one third of these patients died from hepatobiliary malignancy [476]. Similarly, one third of CD40L-deficient mice

developed cholangitis with dense inflammatory infiltrates, which produced bile duct injury, sclerosing cholangitis and jaundice, and contrasts with the lack of biliary tract inflammation in SCID mice [473].

2.4.1 Is CD40 required on biliary epithelial cells for cryptosporidial clearance?

Since the chronic hepatobiliary inflammation observed in CD40L-deficient boys was associated with up-regulated CD40 expression on biliary epithelium, the ligation of CD40 expressed on epithelial tissue may serve a defensive function. Hence, studies of the effects of CD40 ligation on CP-infected $CD40^+$ HepG2 cells, which resemble cholangiocytes, were undertaken *in vitro*. The number of HepG2 cells recovered from *in vitro* culture was reduced after CP infection. Incubation of CP-infected HepG2 cells with trimeric CD40L, a CD40L-CD8 fusion protein or fixed and activated (CD40L-expressing) T cells normalised the recovery of cells from the culture and significantly reduced the number of CP-infected cells. The specificity of the CD40L-dependent anti-CP effect of activated T cells was confirmed using anti-CD40L antibody blockade. In comparison with the proportion of apoptotic HepG2 cells found in CP-infected cultures, addition of CD40L to the infected cultures significantly increased the proportion of apoptotic cells. On the other hand, CD40 ligation of uninfected HepG2 cells did not increase HepG2 apoptosis unless protein synthesis was blocked simultaneously with cycloheximide, which suggested that anti-apoptotic protein synthesis resisted pro-apoptotic effects of CD40 ligation.

Together, these data suggest that CP infection interferes with anti-apoptotic protein synthesis in HepG2 cells, which renders CP-infected HepG2 cells susceptible to ligation by exogenous CD40L [472]. HepG2 cells infected with CP and treated with CD40L fusion protein demonstrated apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) whereas neither CP-infected HepG2 cells nor CD40-ligated HepG2 cells developed apoptosis [473]. These *in vitro* data suggest that CD40 ligation may play a role in the elimination of CP-infected cholangiocytes. Although genetic evidence would suggest that CD40 expression by epithelial cells is not required for CP clearance (see Section 2.4.3), in the absence of CD40 ligation, CP-infected cholangiocytes may persist to receive other signalling that assists malignant transformation of cholangiocytes.

2.4.2 CD40L deficiency may prevent apoptosis of cholangiocytes

During chronic hepatobiliary inflammation, whether it originates from chronic infection, autoimmune pathology or transplant rejection, some data indicate that mechanisms exist to control potentially harmful effects of sustained inflammation, which include free oxygen radical generation and DNA damage. First, by inducing apoptosis, CD40L may protect against the inappropriate survival of injured epithelial cells. Second, the acute phase reactant, C4b binding protein (C4BP) may limit the pro-apoptotic activity of CD40L during chronic hepatobiliary inflammation.

The results of earlier *in vitro* studies had suggested a mechanism by which CD40 activation may amplify Fas-mediated apoptosis. CD40 ligation of hepatocytes [496] or cholangiocytes [497] induced up-regulation of epithelial FasL expression and, subsequently, Fas-mediated apoptosis of these cells in an autocrine and/or paracrine fashion. CD40-mediated apoptosis induction applies to both hepatocytes [498] and primary human cholangiocytes [499], and depends on sustained signalling *via* STAT3 and cFos/cJun (AP-1) to overcome initial transient activation of the RelA NFκB transcription factor, which delivers a survival signal [498]. CD40 stimulation of hepatocytes converges on AP-1 activity *via* phosphorylation of the mitogen-activated protein kinases: ERK1/2 and JNK. Accordingly, inhibition of ERK and JNK reduces apoptosis of both hepatocytes [498] and cholangiocytes [499]. Similarly, inhibition of JAK2, which lies upstream of STAT3, prevents CD40-mediated cholangiocyte apoptosis [499]. Accordingly, *in vivo* sources of CD40L have been identified in chronic hepatobiliary inflammation. During hepatocyte apoptosis occurring in chronic allograft rejection, CD40L seems to be expressed mainly by $CD68^+$ macrophages [496]. During cholangiocyte loss in primary biliary cirrhosis, the source of CD40L may be T cells and macrophages observed in the portal mononuclear

infiltrate [497]. Together, these data indicate that sustained CD40-dependent activation of these pathways impairs survival of primary human hepatic epithelial cells. On the other hand, CD40 activation of intrahepatic endothelial cells (IHEC) induces proliferation and chemokine and cell adhesion molecule expression by IHEC in association with sustained NFκB activation (>24 hours) rather than pro-apoptotic AP-1 activity. To demonstrate the dependence of signalling alterations in IHEC on CD40 activation, blockade of CD40 ligation prevented NFκB activation, and produced apoptosis rather than proliferation of IHEC [498].

C4BP is an acute phase reactant produced mainly by the liver and activated monocytes. It has been proposed as an alternate ligand of CD40 [89]. C4BP is expressed together with CD40 by hepatocytes and infiltrating inflammatory cells in inflammatory conditions such as primary sclerosing cholangitis and in the reactive stroma of liver tumours. While C4BP was shown to bind CD40 on B cells to promote class switching [89], it did not bind CD40 on cholangiocytes unless it was complexed with sCD40L. Whereas treatment of CD40-expressing cholangiocytes with sCD40L resulted in their apoptosis *via* a Fas-mediated mechanism, addition of C4BP at physiologically relevant concentrations prevented apoptosis and did not induce proliferation. Hence, this C4BP control mechanism may ensure that epithelial integrity is maintained in the face of persistent inflammation [115].

Despite the *in vitro* data that CD40 ligation promotes apoptosis of CP-infected human cholangiocyte cell lines [472 473] or primary murine gall bladder epithelium [476], radiation bone marrow chimera experiments indicate that only CD40 expression by bone marrow-derived cells, and not by epithelial cells, is required for CP clearance [495]. For similar reasons, up-regulation by IFNγ of CD40 on infected cells would not seem to be important for CP immunity. An earlier *in vitro* study had suggested that the pro-apoptotic effects of CD40 ligation on human biliary epithelial cells depended on Fas/FasL interactions [496]. However, Fas- or FasL-deficient and CP-infected mice recovered in less than six weeks from CP infection and, therefore, Fas-mediated apoptosis was not apparently required to clear CP infection [477]. Nonetheless, a circuit that comprises the IFNγ-induced up-regulation of epithelial CD40 together with CD40-induced up-regulation of epithelial FasL may, in the presence of CD40L, protect against the inappropriate survival of injured or transformed epithelial cells.

2.4.3 CD40L/CD40 interactions are required for cryptosporidial immunity

Neither CD40- nor CD40L-deficient mice cleared CP infection, which persisted in the gut and liver of CD40L-deficient mice. In these mice, CP infection extended from the crypts and villi of the jejunum and ileum, where there was no inflammatory infiltrate in the bowel wall, to the gallbladder epithelium and large and small intra-hepatic bile ducts, where infection was associated with a predominantly mononuclear cellular infiltrate in the submucosa. The dependence of CP immunity on CD40L expression by T cells was demonstrated by the adoptive transfer of CD40-deficient T cells, which cleared CP-infected SCID mice of infection whereas SCID recipients of CD40L-deficient T cells failed to recover from CP infection. Similarly, mice lacking the genes for CD40 or CD40L were unable to eliminate CP [473].

Radiation bone marrow chimera experiments indicate that CD40 expression was required only on bone marrow-derived cells, and not on CP-infected epithelial cells, for clearance of the infection [495]. These results show that direct antigen presentation by CP-infected epithelial cells to T cells is not sufficient for immunity to CP and, importantly, refute the *in vitro* data suggesting that CD40L-expressing T cells act directly on epithelium to clear CP infection by inducing apoptosis of CP-infected $CD40^+$ epithelial cells [472 473 476]. In addition, the fact that CD40-deficient recipients of bone marrow from IgM (μ) chain-deficient mice, which are B cell-deficient, were able to clear CP infections indicate that antibody is not required for CP clearance, and thus that the $CD40^+$ bone marrow-derived population required for CP clearance may be a macrophage or DC. However, the data do indicate that an interaction between a

CD40L-expressing T cell and a $CD40^+$ bone marrow-derived cell is required for immunity to CP [495].

To test the requirement of CD40 engagement on APC for T cell activation in the draining MLN of CD40-deficient and CP-infected mice, MLN $CD4^+$ T cells were examined for their surface expression of the very early activation marker, CD69, as well as CD40L. Irrespective of whether the mice were wild type or CD40-deficient, the MLN of CP-infected mice contained significantly more activated $CD69^+$ $CD40L^+$ $CD4^+$ T cells than uninfected mice. Next, wild type spleen and lymph node cells were adoptively transferred to CP-infected and wild type or CD40-deficient mice. These cells had been labelled with the fluorescent tracking dye, carboxyfluorescein succinimidyl ester (CFSE), which permits evaluation of cell division *in vivo*. Corresponding to the T cell activation observed in the MLN of CP-infected mice, cellular proliferation of $CD4^+$ T cells was observed only in the MLN of CP-infected mice regardless of whether these mice were CD40-deficient or CD40-sufficient. Moreover, CFSE-labelled cells were found only in the intestinal lamina propria of CP-infected mice, again regardless of their CD40 status. Despite these data, other features of APC or T cell activation, such as IL-12 production by APC or generation of IFNγ by the primed T cells, were not further characterised in this report [495].

First, these results [495] discount the possibility that afferent or reverse signalling through CD40L on T cells [101] is required for an immune response directed toward CP. Second, the results show that CD40 is not required for T cell activation in this experimental system [495]. As CD40-deficient T cells were primed only in CD40-deficient mice that had been infected with CP, the fact of T cell priming suggested that at least signal 2 had been supplied as CD80/86-CD28 interactions. Therefore, CD40-independent APC activation had occurred perhaps because of CP-related TLR signalling and/or the production of pro-inflammatory mediators that are generated in CP-infected gastrointestinal tissues. However, data obtained in other experimental systems suggest that T cell expression of CD40L and cell division are not necessarily indicative of productive immunity [498 500]. Low-level expression of CD80 and CD86 by DC is NFκB-independent, and DC up-regulation of CD80, CD86 or CD40 in response to TLR signalling is NFκB-dependent. Therefore, MHC class II^+ human monocyte-derived DC, in which NFκB had been inhibited pharmacologically, expressed low levels of CD80 and CD86 but did not express CD40. *In vitro* contact with NFκB-inhibited and CD40-negative DC in the absence of exogenous antigen was sufficient to prime or sensitise T cells, which was manifest as the induction of CD40L expression and survival by T cells [498].

In conclusion, these results indicate that (i) antigen presentation by intestinal epithelial cells is insufficient for induction of immunity, and (ii) CD40L/CD40 interactions are not required in the afferent limb of the immune response to CP. Earlier, it was discussed that T cell activation was required to clear CP infection [493] (Section 2.3.2.2), and that the dependence of CP clearance on CD40L/CD40 interactions [495] suggested that the primary function of $CD4^+$ T cell activation may be to supply CD40L. Therefore, CD40L/CD40 interactions are most likely required for the efferent limb or effector phase of the immune response to CP. IL-12 neutralisation experiments suggest that CD40-mediated production of IL-12 by $CD40^+$ APC is an important contributor to CP immunity. IL-12 mediates IFNγ production by T cells, NK cells and IKDC [483]. Similar to the data indicating that IFNγ was required mainly for the initial response to CP, since recovery could proceed in its absence [491], treatment of SCID mice with IL-12 prior to CP infection prevented or greatly reduced infection but did not effectively treat established infections [492]. Consequently, failure of Th1 immunity in CD40L-deficient mice would be expected to reduce production of IL-12 and IFNγ, and thus prevent initial clearance of CP infection. Hence, there is an essential CD40L-dependent cellular mediator of CP clearance.

2.5 T cells contribute to Cryptosporidium-related hepatobiliary inflammation

2.5.1 IFNγ protects against cryptosporidium-related biliary tract pathology

T cells are required not only for clearance of CP but also for the development of biliary tract pathology. Lack of IFNγ promotes clinical illness resulting in death and marked biliary tract pathology [476]. In contrast to the clearing effect that adoptive transfer of intact T cells had upon CP infection in SCID mice, adoptive transfer of IFNγ-deficient T cells to CP-infected SCID mice produced severe pathology of gut or liver depending on the genetic background of the SCID mice. Adoptive transfer of IFNγ$^{-/-}$ splenocytes of a C57BL/6 background to CP-infected SCID mice produced rapid and lethal enteritis without hepatic pathology, whereas adoptive transfer of IFNγ$^{-/-}$ splenocytes of a BALB/c background to CP-infected SCID mice produced biliary tract pathology. The latter mice sickened with triaditis, sclerosing cholangitis, lobular hepatitis and dysplastic (premalignant) biliary epithelial changes. These data clearly showed that the IFNγ gene was required for the survival phenotype of SCID mice [476].

Adding an equal number of wild type splenocytes to the IFNγ$^{-/-}$ splenocytes resulted in clearance of CP infection and no liver abnormalities. To demonstrate the dependence of the effect on CP infection, no abnormalities resulted if IFNγ$^{-/-}$ splenocytes were transferred to uninfected BALB/c SCID mice. IFNγ-deficient mice that were re-challenged with CP did not clear the infection and exhibited dysplastic changes in the biliary tract epithelium together with changes of squamous and mucinous-gastric metaplasia. It was presumed that chronic immune activation stimulated cytokine production, which promoted biliary epithelial cell proliferation and metaplasia [477]. These results indicate that the inflammatory and sclerosing pathology that follows CP infection is induced by T cell cytokines other than IFNγ. Rather, IFNγ dampens inflammation by accelerating CP clearance and/or down-regulating pro-inflammatory cytokine production [476].

2.5.2 CD40L/CD40 interactions protect against biliary tract pathology

In liver of CP-infected CD40L-deficient mice, triaditis, sclerosis and obliteration of the bile ducts were observed. As in HIGM1 patients, the bile duct epithelium of CD40L-deficient mice expressed CD40 [473]. No apoptotic cells were identified in the biliary epithelium of CD40L- or CD40-deficient mice. *In vitro*, only gallbladder epithelium of CP-infected CD40L-deficient mice became apoptotic in response to CD40 ligation [476].

2.5.2.1 Biliary tract pathology in CD40L-deficient mice is mediated by TNF signalling

Evidence for TNF production by T cells in the biliary tract was found. Mice bred with targeted disruptions in the genes for either or both of the TNF receptors (TNFR) 1 and 2 cleared CP infections. In CD40- and CD40L-deficient mice, and in CD40L-deficient mice also deficient in TNFR1 *or* TNFR2, biliary ductular proliferation, sclerosis and fibrosis were found. However, although CD40L-deficient mice that were also deficient in TNFR1 *and* TNFR2 did not clear CP infection, these mice lacked bile duct abnormalities. In all cases, CP forms were associated with the biliary epithelial border. These results clearly indicate a requirement for TNF signalling in the biliary tract pathology that is observed in CD40L-deficient mice [501].

2.6 Other innate immune resistance mechanisms to cryptosporidial infection

2.6.1 The role of IL-12, IL-15, IL-18 and IL-23 cytokines in cryptosporidial infection

IL-12p40-deficient mice lack both IL-12 and IL-23, which are key cytokines in the production of IFN-γ, the cytokine that is critical for clearance of CP in mice. IL-12p40-deficient mice on a C57BL/6 genetic background are highly susceptible to CP infection, and unlike wild type C57BL/6 mice, which are mostly refractory to infection, recover within three weeks of infection. CP infection is established in these mice within four days presumably because of the reduced IFNγ expression, which peaked seven days post-infection. Hence, IL-12p40-deficient mice provide a model of delayed recovery from CP infection that allows evaluation of the role of other cytokines. Quantitative RT-PCR was used to show early peak expression

of IL-15 and TNF mRNA three days post-infection [502]. IL-15 is suspected of playing an early part in the clearance of primary CP intestinal infection in humans (*vide infra*).

Further study of IL-12p40-deficient mice indicated that IL-12-independent mechanisms may exist for both resolution of and resistance to CP infection because, despite the lack of IL-12, IFNγ expression was up-regulated in the intestine and MLN of CP-infected mice, although to a lesser extent than in wild type mice. For example, both IL-18 and IL-23 have been implicated in host resistance to intracellular parasites and both increase IFNγ production. IL-18 may be generated by epithelial cells in response to TLR signalling, and IL-18 may induce IFNγ production by NK cells, or by inducing proliferation of intra-epithelial lymphocytes. In this study, IL-18 was identified as the dominant cytokine that may elicit alternative activation mechanisms for IFNγ production. IL-18 mRNA was expressed in the intestinal tissue of mice lacking IL-12, and mice lacking the IL-18 gene are susceptible to CP infection. Treatment of CP-infected IL-12p40-deficient mice with recombinant IL-18 (rIL-18) produced significant early clearance of the CP parasite burden, which was similar to the treatment effect achieved with rIL-12 [503]. A recent study showed that human intestinal epithelial cells (IEC) constitutively expressed IL-18, and also IL-18R subunits. CP infection of IEC *in vitro* increased IL-18 secretion and rIL-18 treatment of CP-infected IEC retarded the intracellular development of CP parasites perhaps *via* IL-18-mediated elaboration of defensin molecules [504]. Although IL-23 also contributes to IFNγ production in CP-infected IL-12p40-deficient mice, treatment of these mice with rIL-23 produced lower CP clearance rates than treatment with either rIL-12 or rIL-18 [503].

In jejunal mucosal biopsies obtained from naïve human volunteers who were given an oral CP challenge, IL-15 was detected at both mRNA and protein level within the epithelial cell layer. Although IL-15 was expressed by IEC, IL-15 expression in these biopsy samples appeared to be associated with intra-epithelial DC since IEC are not known to express IL-15. Gut expression of IL-15 was associated with control of parasite burden in symptomatic disease, and hence IL-15 may play a role in limiting parasite burden during a primary infection of humans. IL-15 preferentially stimulates chemotaxis, proliferation, survival, and activation of NK cells, NKT cells, CTL, and γδT cells and thus may be expected to draw in cytotoxic effector cells to the site of infection to eliminate parasitised epithelial cells. In contrast, IL-15 was not detected in AIDS patients with chronic cryptosporidiosis. Interestingly, increased numbers of intestinal NK cells were observed in symptomatic CP-challenged volunteers. In contrast, little IFNγ was expressed in human intestinal tissue during primary CP infection [474].

In vitro, IL-15 activates human NK cells to up-regulate expression of the NK activating receptor, NKG2D, and on CP-infected primary IEC, IL-15 up-regulates expression of the NKG2D ligands, and stress-induced MHC class I-related molecules, MICA and MICB. Moreover, IL-15 enhances NK-mediated cytolysis of CP-infected targets in a physiologically relevant range of IL-15 concentrations, and significantly reduces the number of CP-infected cells in culture *in vitro*. Thus, IL-15 may act to target infected cells for killing by NK cells or γδT cells [484]. Finally, IL-15 costimulates lymphoid cells with other cytokines such as IL-12 or IL-18 to produce cytokines including IFNγ [483 505].

2.6.2 CD40 activation and IL-15 may collaborate to mediate NK cell effector function

For some hours after microbial stimulation, and enhanced by CD40 ligation, murine DC were shown to produce IL-2, which contributes to T cell priming [506], and which is also required for DC-mediated NK cell activation [507]. Unlike murine DC, the regulation of IL-2 secretion by human DC is more tightly regulated, and human moDC do not produce IL-2 in response to microbial stimulation alone. Nevertheless, the production of IL-2 by both murine and human DC is regulated by IL-15 [155]. Only human moDC, which were derived in the presence of GM-CSF and IL-15 and stimulated with CD40L, were able to generate IL-2 *in vitro* even in the absence of inflammatory cytokines. Interestingly, human moDC treated in this way did not generate IL-12 [155].

Human NK cell activation and expansion *in vitro* is enabled by IL-12 and IL-15. While DC secretion of IL-12 mainly induces NK cell production of IFNγ *in vitro*, NK cell proliferation depends on IL-15 being bound *via* the IL-15Rα to the DC membrane. Maturation of human moDC or murine splenic DC with pro-inflammatory cytokines such as TNF, IL-1β and IL-6, and microbial stimuli such as LPS or BCG induce DC membrane-bound IL-15, which was not detectable on immature DC or monocyte/macrophages. However, the highest levels of DC membrane-bound IL-15 and the greatest NK cell proliferation occurred after CD40 ligation. At least two days of *in vitro* maturation of DC were required to produce high-level IL-15 expression and NK cell proliferation. Because mature DC are the most efficient stimulators of NK cell activation and expansion, and mature DC home to T cell areas of secondary lymphoid tissue after activation in the periphery, it was hypothesised that the secondary lymphoid tissue is the main site for DC-mediated NK cell activation. Consistent with this hypothesis is the observation that DC are co-localised with cells of the $CD56^{bright}CD16^{-}$ NK cell subset in the T cell zones of both inflamed and non-inflamed human secondary lymphoid tissue, and it is presumed that some of these DC have migrated to secondary lymphoid tissue upon maturation [508]. Similarly, in mice, NK cell cytotoxicity is stimulated by DC production of type I interferons, and NK cell production of IFNγ is stimulated by IL-2 and/or IL-15 together with IL-12. Moreover, a lymphoid microenvironment enriched in IFNγ would support Th1 priming and polarise immune responses toward a Th1 phenotype [508]. Therefore, in CP-infected tissues, which are replete with IL-2, 1L-12, IL-15, and CD40 activating signals, DC-NK cross-talk may facilitate NK cell targeting of CP-infected cells.

2.6.3 Interferon-producing killer dendritic cells and a potential role in cryptosporidiosis

Activated T cells have been recognised as an important source of IFNγ, which stimulates antigen processing and presentation by tumour cells thus enhancing their recognition and destruction by T cells [350] [353]. However, mice with lesions in the genes for *Rag2* and *Stat1*, which render them T cell-deficient and unresponsive to IFNγ, respectively, exhibit a higher incidence of primary tumours than mice deficient in *Rag2* function alone. This result indicates that activated T cells are not the only source of IFNγ [350], and suggests NK cells as the source of IFNγ [482] [483]. Depending on cytokine stimulation and tumour cell expression of NKG2D ligands, NK cells lyse tumour cells using Fas- or perforin-mediated mechanisms, or TRAIL, the expression of which is controlled by IFNγ. For example, IL-2 or IL-12 stimulate NK cells to exert anti-tumour effects *via* NKG2D ligand recognition and perforin-mediated cytotoxicity [509]. In addition, it is known that NK and NKT cells, and DC can activate each other [510]. Furthermore, while tumour cell death induced by TRAIL has been thought to be a major mechanism of tumour immunosurveillance conducted by NK cells, the recent identification of a new species of cell with NK properties, which has been termed the interferon-producing killer DC (IKDC), suggests that NK cells may not be the only significant source of either IFNγ or TRAIL [482].

IKDC have unique morphology and share some immunophenotypic features of DC and NK cells. The developmental origin of IKDC differs from (i) conventional and plasmacytoid DC because IKDC are absent in mice lacking the gene for *IL-2Rβ*, and from (ii) conventional NK cells because IKDC are present in mice, which lack the genes for *Rag2* and *IL-2Rγ* and, therefore, which lack NK cells. In addition, approximately half of lymph node IKDC express high levels of MHC class II and CD40, CD80 and CD86 costimulatory molecules normally lacking on murine NK cells. After stimulation with influenza virus, IKDC produce IFNα but at lower levels than PDC. Also *in vitro*, IKDC produce IFNα in response to stimulation with anti-CD40 mAb or a TLR9 ligand, but only produce IL-12 in response to stimulation with a TLR9 ligand, and not with anti-CD40 mAb. Similar to NK cells, IKDC produce IFNγ in response to stimulation with IL-15 and IL-12 although *in vivo* stimulation with these cytokines would occur in the presence of IL-2 [483]. However, it is important to note that despite previous reports of IFNγ production by murine DC, fully developed and steady-state DC cDC and PDC that interact with naive T cells produce little IFNγ [511].

IKDC can recognise tumour cells that are not well recognised by NK cells. Upon contact with transformed cells, IKDC produce IFNγ, which controls expression of TRAIL. IKDC expression of TRAIL then induces lytic death of tumour cells. Since IKDC are major producers of IFNγ, and NK cell expression of TRAIL is induced by IFNγ, IKDC may collaborate with NK cells in anti-tumour responses. Hence, IKDC have a role both as sensors and effectors of the innate anti-tumour immune response [482].

In vitro, a temporal association was found between decreasing cytolytic capacity and increasing MHC class II expression by IKDC. Correspondingly, in mice infected with *Listeria monocytogenes*, IKDC that migrate to lymph nodes down-regulate NKG2D and up-regulate expression of MHC class II and costimulatory molecules. In addition, lymph node IKDC displayed antigen presenting function *in vivo*, which indicates loss of NK function and acquisition of DC-like APC function as the IKDC mature [483]. IKDC present antigen after signalling *via* TLR4 or TLR9 ligands, and by acting as 'tumour scavengers' may cross-present antigen to tumour cells after TRAIL-mediated tumour cell death [482]. However, the relatively weak T cell responses in the reported assays [483] suggested that IKDC, like PDC, may have limited antigen presenting function of uncertain significance [505].

The characteristics described for IKDC may match the profile of the $CD40^+$ effector cell required for CP clearance [495]. Similarly, the MHC class II^+ APC required to activate CD40L expression required by $CD4^+$ T cells to clear CP [493] may represent a collaborative network of MHC class II^+ DC and IKDC. For example, PDC or IKDC may produce IFNα after CD40 stimulation, which together mature cDC, which then supply IKDC with IL-12 in conjunction with other cytokines such as IL-15 to activate potent IKDC production of IFNγ [483 512].

2.7 IL-12 and IFNγ protect against malignancy

As IFNγ is central to host resistance to infection, so it is a critical element of cancer immunosurveillance. In primary tumorigenesis models, genetic ablation of IFNγ signalling in mice accelerated the formation of sarcomas induced by the MCA carcinogen and, depending on genetic background, predisposed mice to spontaneous lymphomas or carcinomas. Similar results were obtained in RAG2-deficient mice lacking lymphocytes, which indicated that cancer immunosurveillance functions of IFNγ and lymphocytes overlapped [228]. Epithelial γδ T cells appear to be a particularly important source of the IFNγ that protects against the formation of MCA-induced sarcomas [513]. IL-12 is a critical regulator of IFNγ production and IL-12 has anti-tumour effects probably *via* its induction of IFNγ [228]. Moreover, IL-12 and TNF cooperate to induce greater expression of IFNγ by NK cells [514]. For example, in the MC-38 tumour model, IL-12-dependent rejection of tumours was abrogated by treatment with neutralising anti-IFNγ antibody [515]. MCA-exposed mice had both increased latency and frequency of tumours if they were also treated with exogenous IL-12, which was associated with high production of IFNγ by $CD8^+$ T cells [516]. Furthermore, IL-12p40-deficient mice heterodimer developed significantly more MCA-induced tumours than wild type mice [517].

2.7.1 Cytokine imbalance is associated with unopposed inflammation

CP infection of mice results in the production of TNF in the submucosa of bile ducts [501]. Similarly, CP infection of human intestine that was xenografted in SCID mice resulted in the production of TNF and IL-8 [518]. However, the lack of biliary tract pathology in CP-infected SCID mice indicated that CP-induced pro-inflammatory cytokine production alone was not sufficient to induce chronic changes such as sclerosing cholangitis. Therefore, the major contribution made by TNF to chronic biliary inflammation derives from T cells. Hence, if CD40L/CD40 interactions fail to occur then less IL-12 will be produced. Consequently, insufficient IL-12 will be available to counterbalance the pro-inflammatory and pro-tumorigenic effects of IL-23, and will also result in insufficient production of IFNγ [514]. Although TNF will still be produced, a relative deficiency of IFNγ would make cells less sensitive to TNF-induced and caspase-mediated apoptosis, which may contribute inappropriately to the survival of some epithelial cells [519].

IL-12 and IL-23 cytokines share the p40 subunit, and their receptors share the IL-12βR1 subunit. IL-12 and IL-23 are produced mainly by activated DC and phagocytes and the receptors for both cytokines are expressed on T cells, NK cells, and NKT cells. Whereas IL-12 drives development of IFNγ-producing Th1 cells, IL-23 drives the generation of pro-angiogenic and pro-inflammatory IL-17-producing $CD4^+$ T cells. IL-23 promotes tumorigenesis *via* the generation of pro-inflammatory factors and the recruitment of tumorigenic cell types. IL-12 and IL-23 antagonise the function of each other and, in particular, IL-12 promotes intra-tumoral influx of $CD8^+$ T cells whereas IL-23 reduces the number and function of cytotoxic cells in transformed tissue [520]. Conversely, IL-12 has an important role to play in the elimination of transformed cells mainly *via* its promotion of adaptive Th1 immunity and IFNγ production [521].

2.7.2 TNF-induced NFκB activation in chronic inflammation is associated with cancer

How direct the link is between infection, inflammation and malignancy will depend on whether a microbe encodes its own oncogene, toxins or growth factors. Otherwise, microbes act through major host-encoded pattern recognition receptors of the TLR family. Signalling pathways activated by TLR and pro-inflammatory cytokines such as IL-1 and TNF converge on the NFκB family of transcription factors and activate anti-apoptotic gene expression, which is the most important factor underlying tumour promotion mediated by NFκB activation resulting from chronic TNF signalling [522].

A murine carcinogenesis model of hepatocellular carcinoma (HCC) is most relevant to the present discussion of chronic TNF signalling and predisposition to hepatobiliary malignancy in CD40L-deficient patients. The model is based on multi-drug resistance 2 (*mdr2*)-deficient mice in which absence of the MDR2 transporter results in the accumulation of pro-inflammatory bile acids and phospholipids, cholangitis and the development of HCC with a latency period of 8-10 months. It is important to note that the origin of the inflammation in this model is the biliary system. In order to study the effects of NFκB on the natural history of chronic inflammation and malignancy, *mdr2*-deficient mice were bred with transgenic mice in which classical pathway NFκB function was inactivated exclusively in hepatocytes by the hepatocyte-specific expression of a non-degradable form of IκB called IκB-super-repressor (IκB-SR), which was inducible using a tetracycline-regulated element.

In *mdr2*-deficient mice, an orderly progression of pathology similar to human HCC ensues: cholangio-hepatitis, dysplasia, carcinoma localised then metastatic. T cell-rich peri-portal mixed inflammatory infiltrates are prominent in the hepatitis. NFκB activation, which was identified by RelA immunostaining in hepatic parenchymal cells surrounding the peri-portal inflammation, was induced by paracrine expression of TNF by portal inflammatory and endothelial cells. *Mdr2*-deficient hepatocytes had a higher proliferative rate, accelerated hyperploidy and dysplasia. When the *mdr2*-deficient mice were bred to allow hepatocyte-specific inhibition of NFκB function, the non-suppurative cholangitis of the *mdr2*-deficient mice was unaffected. Similarly, ablation of NFκB activity did not affect the extent of hepatocyte proliferation, hyperploidy and dysplasia, and indicated that NFκB was not required for early neoplastic events. At seven months of age, the livers of *mdr2*-deficient mice displayed similar dysplastic changes and a similar incidence of early tumours irrespective of whether hepatocyte NFκB activity had been repressed. Therefore, NFκB activation was not required for tumour initiation. However, if tetracycline treatment was used at seven months of age to switch off the IκB-SR transgene thus allowing NFκB activity in *mdr2*-deficient mice, the rate of tumour progression increased significantly.

NFκB activation exerts anti-apoptotic effects to protect both immune and non-immune cells from immune-mediated destruction or genotoxic stress, and stimulates production of pro-inflammatory cytokines, which activate NFκB creating a feed forward loop for the production of pro-inflammatory cytokines [134 188]. The pro-inflammatory cytokines such as TNF then

signal damaged or transformed cells to promote their survival and proliferation, and their production of pro-inflammatory cytokines [523].

The rate of apoptosis was higher in the dysplastic livers of seven month-old *mdr2*-deficient mice than in the livers of wild type mice, presumably because of the toxic effects of inflammation. Moreover, in the dysplastic livers of *mdr2*-deficient mice, ablation of NFκB activity tripled the rate of apoptosis, which was similar to the effect observed with anti-TNF mAb treatment. These data suggest that, although NFκB is activated early during hepatobiliary inflammation in *mdr2*-deficient mice, it is not critically required until premalignant cells acquire oncogenic mutations that render them susceptible to pro-apoptotic factors such as p53 and c-Jun, which may then be neutralised by activated NFκB and thus contribute to tumorigenicity. As described for TNF superfamily members in general [524], continuous TNF signalling stimulates pro-apoptotic and anti-apoptotic pathways, the balance of which decides the fate of the cell. Hence, anti-TNF treatment or NFκB inhibition allows pro-apoptotic signalling by p53 and c-Jun to dominate and thus tips the balance toward cell death [525].

In conclusion, TNF-dependent NFκB activation drives tumour promotion in this cholestatic liver cancer model by inhibiting hepatocyte apoptosis. This model resembles the TNF-driven cholangitis and biliary epithelial dysplasia of CD40L-deficient mice, which may apply as precursor lesion to hepatobiliary malignancy in CD40L-deficient patients.

2.8 A model to link cryptosporidial-related inflammation with malignancy

Although there may be species-specific differences in the mechanisms of CP clearance that, for example, depend more on IFNγ in mice than in humans [484], a blended model is proposed to illustrate the interdependence between innate and adaptive immunity for both eradication of CP infection and protection against hepatobiliary malignancy. The proposed model is illustrated schematically (Figure 11) and pictorially (Figure 12).

The study of chronic CP infection in SCID and $RAG^{-/-}$ murine models indicates that, despite recruitment of some inflammatory cells to the gut and biliary tree *via* CP-induction of TLR signalling, local expression of CD40L by innate immune cells such as DC, macrophages and NK cells is either not triggered or is not sufficient to result in CP clearance. Hence, for the full utility of the CD40L signal to be realised requires that it be amplified by a T cell-derived source. Moreover, NK cells alone cannot clear CP infection unless additional activating signals are supplied indirectly by CD40L-expressing T cells. For example, stimulation of DC by T cell-derived CD40L would augment secretion of IL-15 and IL-2 to activate NK cells so that they produce anti-infective and anti-neoplastic IFNγ, which may act with T cell-derived TNF to induce apoptosis of CP-infected epithelial cells.

In the absence of CD40L/CD40 interactions, CP infection persists; chronic inflammation ensues, and results in molecular changes in epithelial cells that favour malignant transformation. If a lack of CD40L/CD40 interactions result in a relative IFNγ deficiency during chronic persistent hepatobiliary CP infection, then IFNγ-responsive immunogenic tumours may arise in the chronic inflammatory microenvironment. In particular, failure of CD40 signalling that depletes IFNγ in the inflammatory microenvironment may undermine mechanisms that protect against malignant transformation. First, IFNγ up-regulates CD40 on injured or transformed epithelial cells to increase their receptivity to CD40 signalling, and thus to Fas-mediated apoptosis [497], and second, IFNγ sensitises cells to TNF-induced and caspase-mediated apoptosis [519].

Certain CD40-dependent signals, which would be required for resistance to CP infection and for elimination of transformed biliary epithelial cells, may be absent in CD40L-deficient mice or humans. First, IL-12 is not likely to be produced in significant amounts as a result of CP infection unless CD40 stimulation of macrophages or DC supplies it. An adequate supply of IL-12 is sufficient to clear CP infection, and IL-12 also contributes to the elimination of transformed cells mainly *via* its induction of IFNγ. Second, IL-15 production by DC in human

and murine systems is optimised by CD40 stimulation, and its relative lack will impair the maturation and function of cytotoxic cells including IKDC, NK cells, NKT cells, γδT cells and CTL. Ultimately, the effects of a relative deficiency of IL-12 and IL-15 converge to produce an inadequate supply of IFNγ. In addition to reducing the immunogenicity of infected and transformed epithelial cells, diminished IFNγ production limits both direct cytotoxic effects of IFNγ on infected and transformed epithelial cells and the activities of TRAIL-expressing effector cells such as IKDC and NK cells. Notwithstanding the relative absence of IL-12 and IFNγ, IL-23 and TNF would continue to be produced in chronic persistent CP infection and thus would drive chronic inflammation to promote tumorigenesis.

Therefore, the same mechanisms that promote CP clearance may prevent development of neoplasia. Both tasks require that the activities of multiple cell types, which include epithelial cells, cDC and PDC subsets, IKDC, T cells, NKT cells and NK cells, are coordinated in a cytokine network. The resulting cellular collaboration is required to clear CP and eliminate transformed epithelial cells. For example, CD40 activated DC secrete IL-2, IL-12 and IL-15 to activate IKDC to express IFNγ and TRAIL, and to activate and induce proliferation of NK cells.

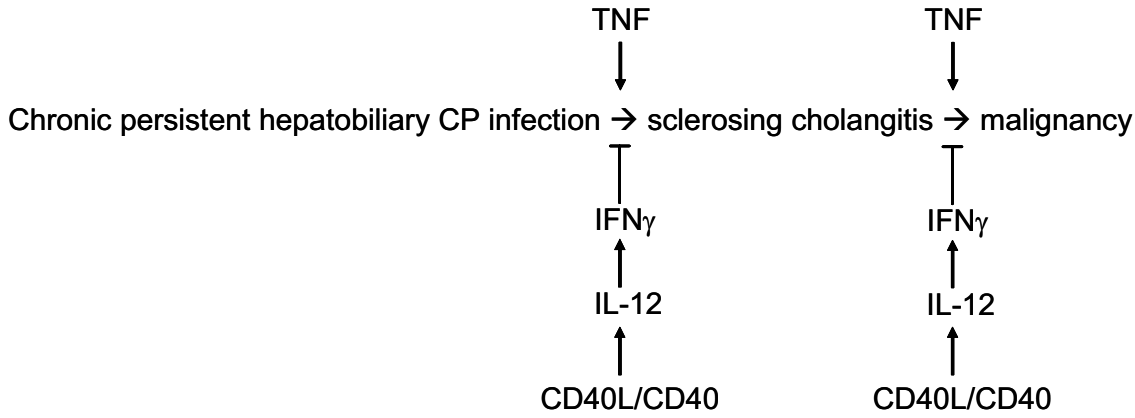


Figure 11: CD40L/CD40 interactions supply protective IL-12 and IFNγ cytokines

Chronic persistent infections of *Cryptosporidium parvum* (CP) in the gastrointestinal tract are prevented by IFNγ, which is generated in response to IL-12, which in turn results from CD40L/CD40 interactions. If CD40L/CD40 interactions are lacking then IFNγ is not generated, and TNF production by T cells predominates to drive chronic inflammatory pathology such as sclerosing cholangitis. Similarly, inadequate IFNγ generation results in the failure of tumour immunosurveillance and the unbalanced production of TNF, which then enables the anti-apoptotic effects of constitutive NFκB activation to drive tumorigenesis.

Other cytokines present in the CP-induced inflammatory microenvironment such as IL-18 would cooperate with IL-12 or IL-15 derived from CD40 stimulation of innate immune cells to induce IFNγ production by NK, NKT, T cells, and perhaps IKDC. IFNγ would have direct effects on infected or transformed cells, and up-regulate CD40 expression on innate immune cells. Cytokines such as IL-2, IL-12 and IL-15 would also cooperate to activate NK cells to exert cytotoxic activity against infected or transformed targets *via* a perforin-mediated mechanism or TRAIL. Similarly, IFNγ would induce TRAIL expression by IKDC with subsequent killing of transformed target cells [482]. Conversely, if CD40 activation is lacking then ineffectual clearance of CP-infected epithelial cells by NK cells, NKT cells or IKDC in conjunction with reduced availability of counter-regulatory IFNγ would permit sustained TNF signalling and NFκB activation.

Genetic approaches have exposed TNF as the major actor in the chronic hepatobiliary inflammation of CP-infected and CD40L-deficient mice [495] [473]. CP infection of an enterocyte or cholangiocyte results in activation of anti-apoptotic NFκB and subsequent induction of chemokines, which initiate the inflammatory response. Consequently, the TNF that is produced both by the CP-infected intestinal cells and the recruited T cells sustains NFκB activation and cellular survival unless IFNγ is available to induce apoptosis. Moreover, the key role of TNF in chronic NFκB activation, hepatobiliary inflammation and predisposition to HCC was later demonstrated in an experimental murine model [525]. Together with the protective function that IFNγ exercises against both the onset of spontaneous malignancy [350] and the development of dysplastic changes in biliary epithelium [477], these data support the notion that a cytokine imbalance adds momentum to tumour initiation and promotion.

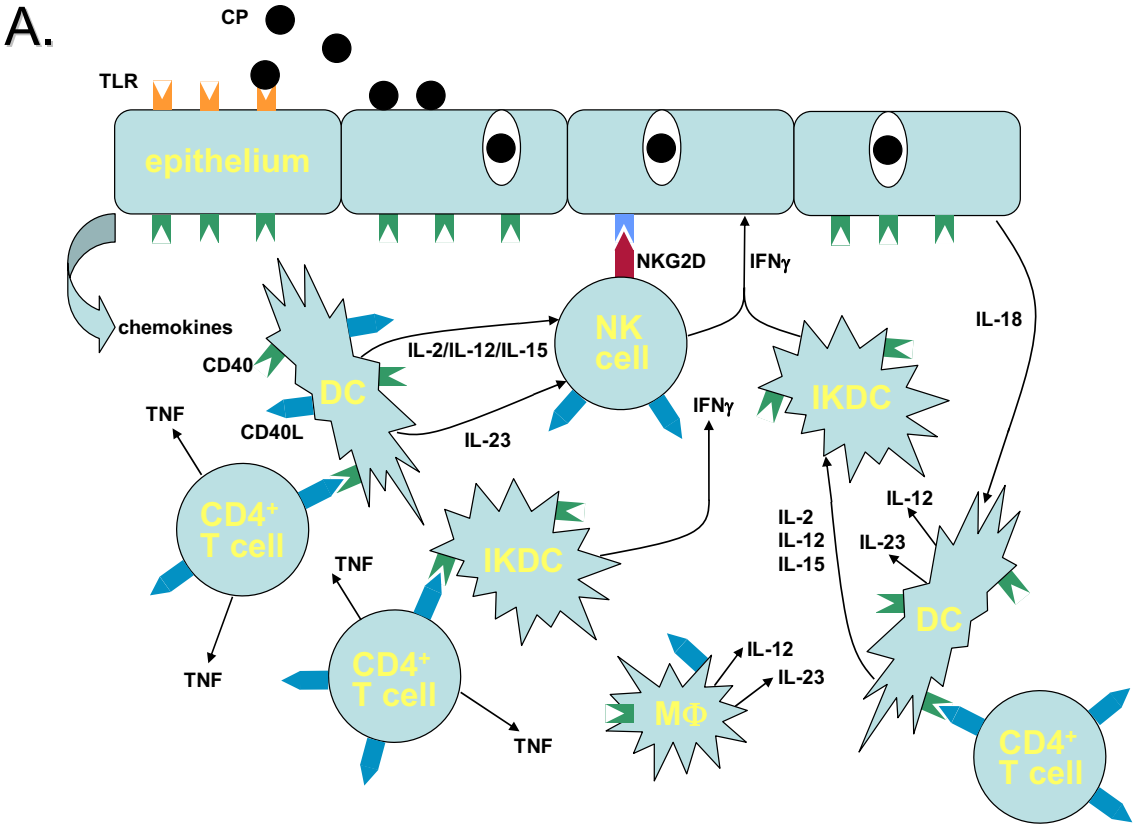
2.8.1 Conclusions and future directions

In conclusion, it was shown that a $CD40^+$ effector cell was required for CP clearance because a $CD40^+$ bone marrow-derived cell was required for CP clearance, and CD40-negative T cells were unable to eradicate CP infection in a CD40-deficient background [495]. It is not clear if the characteristics of this $CD40^+$ effector cell would be embodied in a single cell type such as the CD40-expressing IKDC, or would represent the output of a collaborative effort involving $CD40^+$ DC communicating via cytokines with IFNγ-producing effector cells such as the IKDC, NK or NKT cells. Nevertheless, in the most parsimonious hypothesis, it is argued that the same effector cell type is responsible both for CP eradication and protection against the progression of hepatobiliary malignancy, although different effector mechanisms may be implicated. For example, together with NK or NKT cells, the IKDC may supply IFNγ to limit CP infection, and TRAIL and/or IFNγ to eliminate transformed cells.

Dysplastic changes were the severest manifestation of neoplastic change in the hepatobiliary epithelium, which was observed only in IFNγ-deficient mice [476]. Severe TNF-induced chronic inflammatory changes, but no evident premalignant changes, were evident in CD40- or CD40L-deficient mice. Hence, to investigate whether cell types such as IKDC, NK, or NKT cells play a role in protection against malignancy, it will be necessary to use a 'sensitised' cancer model. For example, in mice harbouring a p53 mutant allele, chronic intermittent toxin exposure provoked bile duct injury and necrosis, ductular proliferation, and a fibrotic reaction, which was followed by progressive hepatic cirrhosis and then intra-hepatic cholangiocarcinoma. [526]. Perhaps, p53 mutant mice bred with CD40L-deficient mice may be used to develop a suitable cancer model in which earlier onset malignancy is induced by chronic CP infection and its associated inflammation. Then, to test which of the various effector cell types may be responsible for CP eradication, lethally irradiated CD40L-deficient mice would be reconstituted with $CD40^+$ bone marrow from (i) IL-2Rβ-deficient mice, which are specifically deficient in IKDC, and not cDC or PDC [483], or (ii) Rag2- and IL-2Rγ-deficient mice, which lack both T and NK cells, but which retain IKDC [482], or (iii) Jα18-deficient C57BL/6 mice, which lack $V\alpha14^+$ NKT cells [527].

Figure 12 (over page): CD40 signalling defends against infection, inflammation and cancer

(**A**) *Cryptosporidium parvum* (CP) parasitise gastrointestinal epithelium and signal *via* Toll-like receptors (TLR) to produce chemokines and cytokines such as interleukin (IL)-18. Consequently, immune cells such as dendritic cells (DC), natural killer (NK) cells, interferon-producing dendritic cells (IKDC), and $CD4^+$ T cells are recruited to the gastrointestinal mucosa and submucosa. These cell types produce cytokines such as IL-23 and, in addition, CD40L/CD40 interactions between these cell types augment the production of cytokines such as IL-2, IL-12, and IL-15. Together, these cytokines contribute to the activation of effector cells such as NK cells, IKDC, and $CD4^+$ T cells and also result in the production of IFNγ by these cell types, and tumour necrosis factor (TNF) production by $CD4^+$ T cells. (**B**) In the absence of CD40L/CD40 interactions, there is a relative insufficiency of IL-12 and IFNγ production so that TNF and IL-23 dominate in the inflammatory microenvironment. Consequently, under the influence of these cytokines, CP infection persists and inflammation proceeds unchecked. (**C**) CP-infected epithelial cells may become apoptotic in response to combined stimulation with TNF and IFNγ, or after perforin-mediated cytolysis by NK cells that have used the activating NKG2D receptor to bind conserved MHC class I-related molecules such as MICA/B in humans or Rae in mice. If an epithelial cell were to become transformed then in an IFNγ-rich inflammatory microenvironment, the transformed cell would be recognised by TNF-related apoptosis inducing ligand (TRAIL)-expressing NK cells or IKDC and eliminated. (**D**) However, in the absence of CD40L/CD40 interactions, CP infection will persist and the related chronic inflammation will endure. Relatively low quantities of cytokines such as IL-12 and IFNγ will prevent tumour-eliminating mechanisms such as perforin- and TRAIL-mediated cytotoxicity from operating effectively. Instead, the relative surfeit of TNF and IL-23 will prevail and, consequently, transformed epithelial cells will survive inappropriately bathed in a cocktail of pro-tumorigenic cytokines.


A.
CP
TLR
epithelium
NKG2D
IFNγ
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chemokines
CD40
DC
IL-2/IL-12/IL-15
NK cell
IKDC
TNF
CD40L
IL-23
IFNγ
CD4+ T cell
IL-12
IL-2
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MΦ
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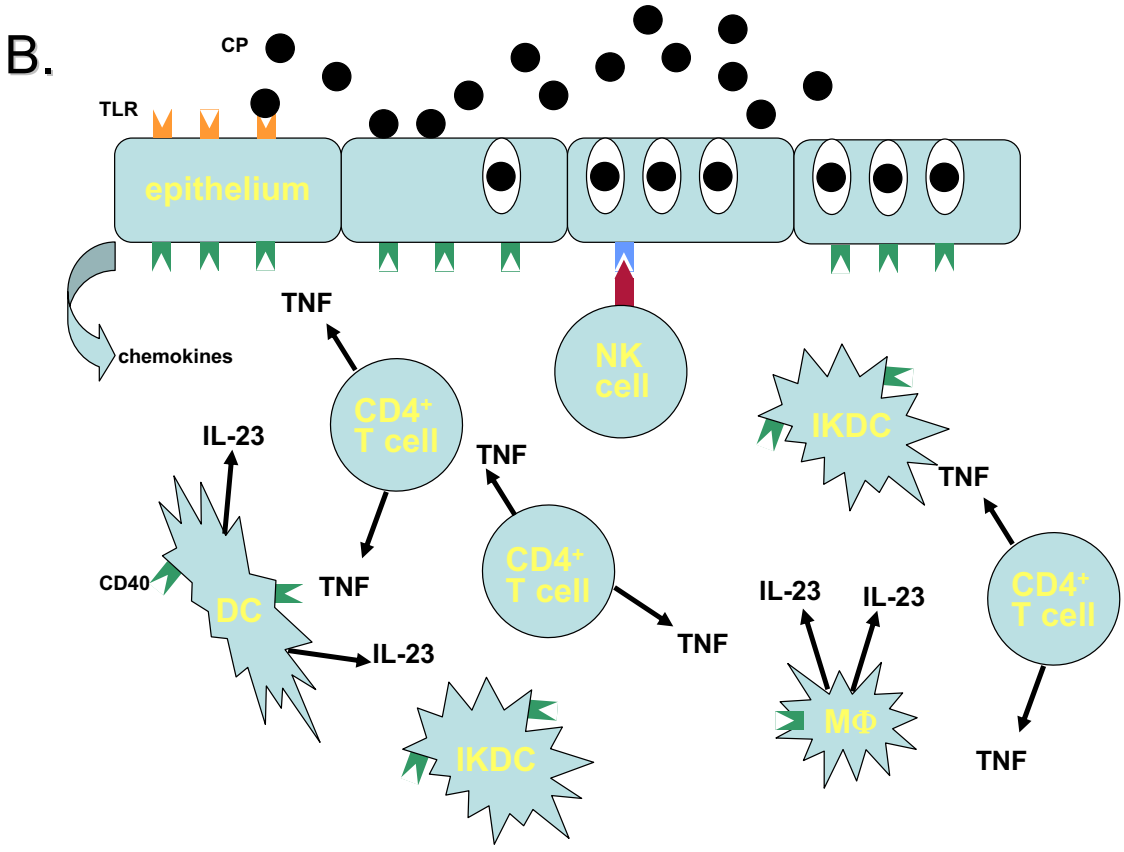
CD4+ T cell


B.
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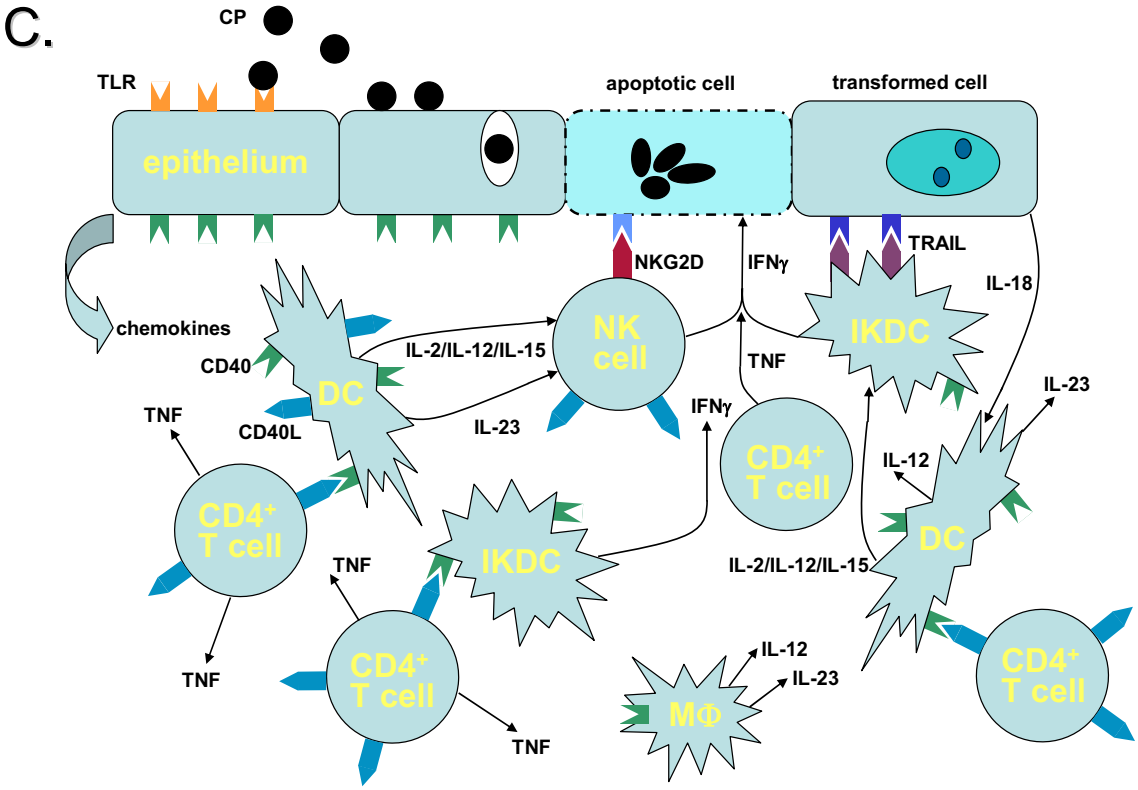
C.
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NK cell
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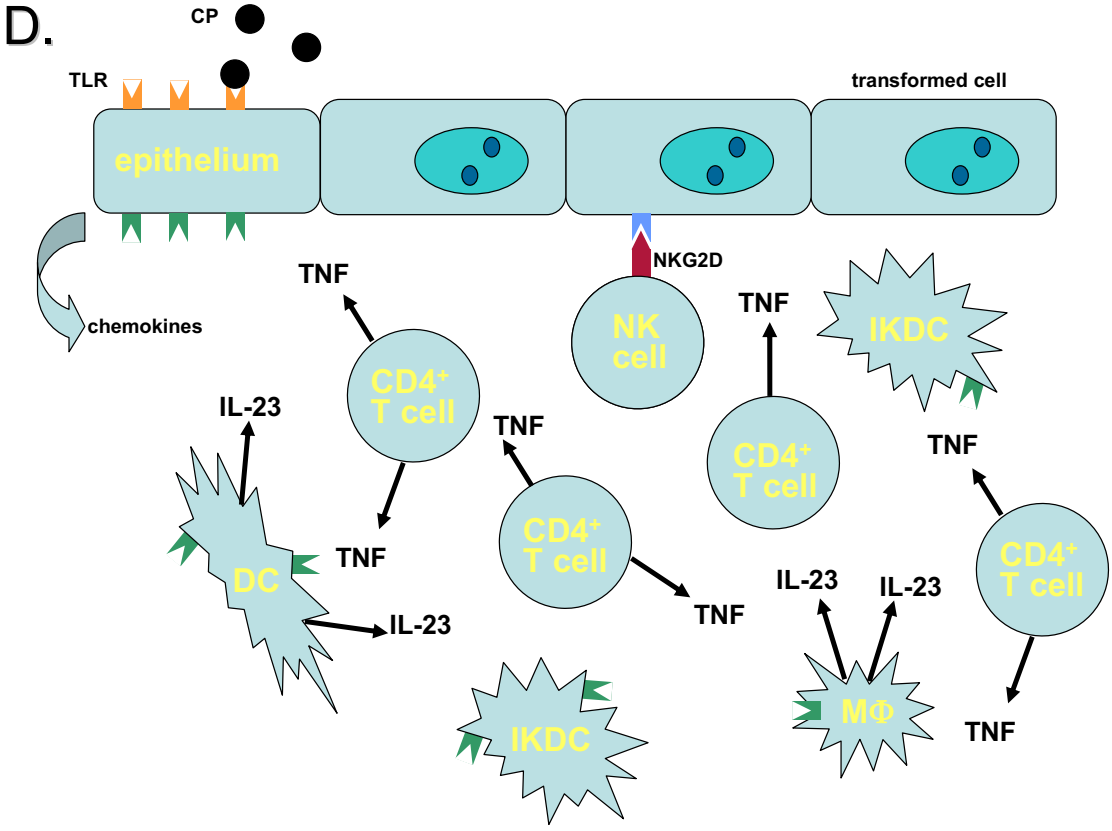
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TNF

CHAPTER 3

GENETIC CORRECTION OF CD40L DEFICIENCY INDUCES THYMIC MALIGNANCY

3.1 Background and Rationale

X-linked or Type 1 HyperIgM Syndrome is a rare human primary immunodeficiency, which is caused by genetic lesions in the CD40L gene. Hence, CD40L gene-disrupted mice are a realistic model in which to test the safety and feasibility of retrovirally-mediated CD40L gene replacement. In particular, immunoglobulin isotype switching fails in CD40L-deficient mice, and the development of isotype-switched immunoglobulin in response to immunisation becomes a sensitive and readily measurable indicator of successful CD40L gene correction. Since both CD40L-deficient mice and humans are susceptible to viral infections, the ability of CD40L gene-corrected mice to develop humoral and cell-mediated immunity to challenge with influenza virus becomes an important test of restoration of immune function.

Retroviral gene transduction of bone marrow is a clinically applicable technology that offered the best prospect of genetic correction of CD40L-deficiency. The clinically approved retroviral vector backbone was derived from the Moloney murine leukaemia virus (MoMuLV) and used a strong constitutively active retroviral promoter originating in the long terminal repeat (LTR) of MoMuLV to drive CD40L transgene expression. Transgene expression depended on integration of the retroviral vector in dividing cells. Hence, during retroviral transduction, donor bone marrow cells were induced to proliferate in culture with growth factors. Consequently, multi-potential bone marrow progenitor cells were transduced with the retroviral vector to give rise to progeny that included murine CD40L (mCD40L)-expressing T cells, which are the physiological source of CD40L required for normal immune function.

3.2 Aims

The primary aim of this study was to restore normal immune function in a murine genetic model of CD40L deficiency.

Although this aim was partially achieved, longer follow-up of the CD40L gene-corrected mice demonstrated that retroviral gene transduction of CD40L-deficient bone marrow cells or thymic cells had been complicated by the development of thymic lymphoproliferative disease and overt thymic lymphoblastic lymphoma. The secondary aims of the study then became to characterise the nature of the thymic malignant transformation and to explore possible causes.

3.3 Experimental Approach

The cDNA for mCD40L was cloned from an activated T cell library and subcloned into a retroviral transfer vector under the control of the LTR promoter of MoMuLV. A retroviral producer cell line was created and cloned to provide supernatants containing the mCD40L-expressing retrovector for transduction of CD40L-deficient bone marrow and thymic cells. As a negative control, a retrovector expressing the neomycin resistance gene (*neo*) was also used for the transductions. The transduced cells were transplanted into CD40L-deficient recipients. Transfer of the mCD40L gene to bone marrow and thymic cells was confirmed by fluorocytometric analysis of surface mCD40L expression. Levels of transduction among bone marrow and thymic cells some months after low or high efficiency gene transfer conditions were evaluated by mCD40L-specific genomic and reverse transcriptase-polymerase chain reaction (RT)-PCR analyses.

The immunological integrity of the transplanted recipients was tested by the presence of influenza-specific humoral, proliferative and cytolytic responses after viral challenge. In particular, isotype switching of influenza-specific serum IgG antibodies was assayed. In some of the recipients, immunoglobulin class switching was also evaluated by the detection of serum IgE in response to immunisation with the neoantigen, dinitrophenol (DNP).

Once thymic tumours were discovered, the nature of the malignancy was evaluated using histopathology, immunophenotypic analysis, and secondary transplantation studies. The presence of mCD40L expression in lymphoproliferations and tumours was confirmed by flow cytometry and immunohistochemistry.

3.4 Major Findings

Brown MP, Topham DJ, Sangster MY, Zhao JF, Flynn KJ, Surman SL, Woodland DL, Doherty PC, Farr AG, Pattengale PK, Brenner MK. "Thymic lymphoproliferative disease after successful correction of CD40 ligand deficiency by gene transfer in a murine model." Nature Medicine 4, 1253-1260, 1998.

3.4.1 Retroviral transduction partially corrects CD40L-dependent immune defects in vivo

Unlike CD40L-deficient mice that received bone marrow transduced with the *neo* control retrovector, CD40L-deficient recipients of CD40L-deficient bone marrow transduced with the mCD40L-expressing retrovector had partial restoration of CD40L-dependent immune functions, in particular, the generation of influenza-specific isotype-switched antibodies of the different IgG subclasses. The bone marrow, thymus and spleen of these mice contained at least 5% transgene-positive cells and produced mCD40L mRNA. Despite another cohort of seven CD40L-deficient mice receiving corrected bone marrow transduced at 100-fold lower efficiency, bone marrow, thymus and spleen of these mice all had detectable mCD40L mRNA by RT-PCR. A further cohort of six CD40L-deficient mice received thymic cells transduced with the CD40L-expressing retrovector so that 1.8% of these cells initially displayed surface expression of CD40L. Only one of five evaluable mice from this cohort had detectable CD40L mRNA in lymphoid tissue. In the latter two cohorts of CD40L-deficient mice receiving corrected cells transduced at lower efficiency, three of seven transplanted with corrected bone marrow, and four of six given corrected thymic cells, displayed isotype-switched DNP-specific antibodies, after immunisation with DNP-keyhole limpet haemocyanin (KLH) hapten-carrier complex.

Hence, only very low-level constitutive expression of CD40L identified in lymphoid tissues was required to support isotype switching in a CD40L-deficient background. The type of CD40L-expressing cell responsible for isotype switching was not identified but it was presumably present in the lymph node(s) draining the site of influenza virus challenge (lung) or DNP-KLH immunisation (peritoneum).

3.4.2 Retroviral transduction unexpectedly induces thymic malignancy

After a latency period of six to nine months, mice that had received CD40L gene-modified bone marrow or thymic cells sickened. These mice were necropsied as were all recipients of gene-modified cells. Only mice that had been treated with the CD40L-expressing retrovector had histopathological evidence of thymic lymphoproliferative disease, which ranged in severity from pre-lymphoma to overt lymphoblastic lymphoma. Of 19 CD40L-deficient mice receiving CD40L gene-modified cells, 12 developed thymic T-lymphoproliferative disease: 4/6 mice given CD40L gene-modified bone marrow at moderate gene transfer efficiency, 3/7 mice given CD40L gene-modified bone marrow at low gene transfer efficiency, and 4/6 mice given CD40L gene-modified thymic cells. In contrast, none of seven mice that received marrow transduced with the *neo* control retrovector developed thymic lymphoproliferative disease. All of the tumours were genotypically and phenotypically T cell lymphomas that appeared to reflect different stages of thymocyte development but predominantly involved a $CD4^+$ $CD8^+$ population, which is also typically the manifestation of other oncogenic transformations. Larger, metastatic lesions were marked by oligo- or mono-clonality of the Vβ chain of the T cell receptor. The malignant nature of the lesions was confirmed by

secondary transplantation studies in CD40L-deficient mice, which demonstrated lethal proliferations of the same lymphomatous lesions including their propensity for growth in the thymus.

3.4.3 Non-transgene-related causes of thymic malignancy are unlikely

Thus, these findings were made irrespective of the efficiency of the original gene transfer method and indicate that, just as the CD40L-dependent outcome of isotype switching occurred at low levels of gene transfer efficiency, so the delayed malignant outcomes of deregulated CD40L transgene expression occurred with equally great efficiency. In order to make this statement as true as possible, it was necessary to exclude as many other causes of thymic malignancy as were identifiable. These included radiation, replication competent virus (RCR) and insertional mutagenesis. Radiation was considered unlikely as a cause because thymic lymphomas did occur in unirradiated mice that received CD40L gene-modified thymic cells but did not occur in irradiated control mice. Three assays using independent methodologies, PCR, XC plaque assay and electron microscopy, were done to show that no RCR was present either in the retrovector preparations or the tumours themselves. Insertional mutagenesis was also considered unlikely because (i) the proportion of proviral DNA detected in tumours did not exceed that in the input bone marrow, and (ii) a minor proportion (≤15%) of tumour cells expressed the CD40L transgene.

3.4.4 CD40L transgene likely has a role in the immunopathogenesis of thymic malignancy

The lymphomas contained a phenotypically heterogeneous mix of cells, which may indicate the course of tumour evolution. Particularly striking was the observation that the CD40L transgene product, and its CD40 receptor, was expressed by $CD4^+ \pm CD8^+$ and $Thy1^+$ $B220^+$ $TCR\alpha\beta^+$ $CD4^{lo}$ tumour sub-populations, respectively. $Thy1^+$ $B220^+$ $TCR\alpha\beta^+$ tumour cells were also discovered in the thymus of a secondary transplant recipient. Hence, these data suggest that paracrine stimulation of CD40 on $Thy1^+$ $B220^+$ $TCR\alpha\beta^+$ tumorigenic cells by the CD40L transgene product contributed to the initiation and/or promotion of the malignancy. Moreover, T-lymphoma expression of the CD40L transgene indicates that proviral integration occurred in T cell precursors and, subsequently, in the mature T cells that would have mediated immunoglobulin class switching, and cellular and humoral anti-influenza immune responses.

Brown MP et al., 1998

Brown MP, Topham DJ, Sangster MY, Zhao JF, Flynn KJ, Surman SL, Woodland DL, Doherty PC, Farr AG, Pattengale PK, Brenner MK. “Thymic lymphoproliferative disease after successful correction of CD40 ligand deficiency by gene transfer in a murine model.” Nature Medicine 4, 1253-1260, 1998.

Brown, M.P., Topham, D.J., Sangster, M.Y., Zhao, J.F., Flynn, K.J., Surman, S.L., Woodland, D.L., Doherty, P.C., Farr, A.G., Pattengale, P.K. & Brenner, M.K. (1998) Thymic lymphoproliferative disease after successful correction of CD40 ligand deficiency by gene transfer in a murine model.
Nature Medicine, vol. 4(11), pp. 1253-1260.

CHAPTER 4

CD40L GENE AUGMENTATION OF REPIRATORY SYNCYTIAL VIRUS IMMUNITY

4.1 Background and Rationale

By the age of two, most humans have been infected with respiratory syncytial virus (RSV). However, immunity against RSV is often incomplete. While the immune response to RSV is insufficient to prevent re-infection, the infection tends to be confined to the upper respiratory tract in young, healthy individuals. On the other hand, young infants, who have an immature immune system and who may not be fully protected by maternal IgG, and the elderly, who have waning immunity, are particularly susceptible to RSV-induced bronchiolitis and pneumonia. No effective RSV vaccine yet exists and there is little effective treatment for acute RSV infection. Adequate levels of neutralising antibodies particularly directed against the surface fusion (F) and attachment (G) proteins of RSV may be sufficient to protect a human host against re-infection with RSV. In addition, cytolytic T cells help to destroy virally infected respiratory epithelial cells and thus contribute to viral clearance and resolution of the infection. Recent approaches to the development of RSV vaccines have included attenuated live virus vaccines as well as subunit vaccines. Killed whole virus vaccines are typically used in viral vaccine development but are excluded because of the disastrous experience in the 1960's when a formaldehyde-inactivated RSV vaccine produced enhanced pulmonary disease and death in immunised children upon re-exposure to RSV.

Studies of patients with XHIGM1 and 3, and of gene-targeted mice with corresponding lesions in CD40L and CD40, indicate that CD40L/CD40 interactions are required for antibody maturation and the generation of type 1 cellular immune responses. Since the lack of CD40 signalling in professional APC causes failure of effective humoral and cell-mediated immunity against infectious pathogens including some viruses, we hypothesised that the transgenic expression of CD40L concomitantly with expression of RSV antigens would augment antibody responses and cell-mediated immunity against RSV. We hypothesised that transgenic expression of CD40L would strengthen immunological priming against RSV and its F and G antigens and augment the primary immune response to RSV infection. We present evidence to show that transgenic CD40L expression offset the inherent tendency of RSV infection toward a type 2 response by biasing the immune response to concurrent or subsequent primary RSV infection toward a type 1 response.

4.2 Aims

4.2.1 To evaluate the effects of adenovirally expressed CD40L on the primary immune response to RSV infection of BALB/c mice (Tripp RA *et al*., 2000).

4.2.2 To examine the effect of transgenic CD40L expression in DNA subunit vaccines on the potency of specific cellular and humoral immune responses to RSV challenge of BALB/c mice four and eight months post-immunisation (Harcourt JL *et al*., 2003).

4.3 Experimental Approach

4.3.1 To evaluate the effects of adenovirally expressed CD40L on the primary immune response to RSV infection of BALB/c mice.

The effects of adenovirally mediated expression of CD40L on the primary immune response to RSV were tested in BALB/c mice. Mice in experimental groups of 4-6 animals each were challenged intranasally with wild type RSV and, concurrently, given intraperitoneal (IP) injections of a recombinant adenovirus expressing murine CD40L (Ad-CD40L) or empty vector control adenovirus (Ad-VC) or phosphate-buffered saline (PBS) diluent control. The IP route of delivery was chosen for adenoviral vectors because the pulmonary inflammation and clinical status of the animals worsened if RSV and adenovectors were both given intranasally. To elicit humoral responses, mice were inoculated intradermally with 20μg plasmid DNA at each of two sites on the abdominal skin using a ballistic device. Mice were then boosted every week with the same total of 40μg DNA for a total of three weeks.

Mice were killed at 3, 6, 7, 10, or 14 days post-infection and immunological analyses were performed on the harvested tissues. Splenocytes and the cellular content of broncho-alveolar lavage (BAL) fluid were analysed directly *ex vivo* for surface expression of T cell subset markers and intracellular expression of cytokines by T cells. Cytokine concentrations in cell-free BAL fluid were measured using a cytokine bead array system and calibrated against standard curves of recombinant cytokines. The BAL fluid content of nitrite, which is a product of nitric oxide (NO) catabolism, was measured using a modified Greiss reaction. RSV-specific antibodies were measured using ELISA. ELISA plates were coated with lysates of Vero cells alone or lysates of Vero cells infected with RSV. The reported absorbance reading was obtained after subtraction of background absorbance values generated by binding to Vero cell lysates. Viral lung titres were determined by methylcellulose plaque assay on Vero cell monolayers and the plaques enumerated using Crystal Violet staining. CTL precursor (CTLp) frequencies in BAL were measured using limiting dilution analysis. At different time points, BAL cells were restimulated *in vitro* for seven days in IL-2 with RSV-infected autologous splenocytes before cytolytic activity was determined using a ^{51}Cr-release assay (Tripp RA *et al*., 2000).

4.3.2 To examine the effect of transgenic CD40L expression in DNA subunit vaccines on the potency of specific cellular and humoral immune responses to RSV challenge of BALB/c mice four and eight months post-immunisation.

In experimental groups of 4-5 mice, BALB/c mice 4-5 weeks old were inoculated intramuscularly with 20μg plasmid DNA (pDNA) in each thigh weekly for three weeks to a total of 120μg pDNA per mouse. The pDNA expressed either the viral capsid glycoproteins, RSV-F or -G, as pDNA-F or pDNA-G, respectively, or murine CD40L (pDNA-CD40L). RSV-F is the highly conserved fusion protein that elicits neutralising antibodies and protective immunity whereas RSV-G is the mutation-susceptible attachment protein, which is the next most effective viral protein in inducing neutralising antibodies but which affords only limited protection. The DNA vaccines were given singly or in combination. The combinations are represented as follows: pDNA-F + pDNA-G = pDNA-F/G, pDNA-F + pDNA-CD40L = pDNA-F/CD40L, pDNA-G + pDNA-CD40L = pDNA-G/CD40L, and pDNA-F + pDNA-G + pDNA-CD40L = pDNA-F/G/CD40L. The DNA vaccine control was the empty vector encoding the neomycin resistance gene, pDNA*neo*. Four and eight months after immunisation, the mice were left unmanipulated or challenged intranasally with live RSV. At 3, 5, 7, and 14 days after RSV challenge, mice were killed and pulmonary viral clearance, pulmonary leukocyte content and serum RSV-specific antibody levels were measured. Immunophenotyping and intracellular cytokine staining of BAL and spleen cells was done by flow cytometry. RSV-specific IgG was analysed by ELISA and splenic RSV-specific IgG antibody forming cells (AFC) were measured using a modified ELISPOT assay. Viral lung titres were determined by plaque assay on Vero cell monolayers and the plaques enumerated using anti-RSV-F and anti-RSV-G immunostaining.

4.4 Major Findings

Tripp RA, Jones L, Anderson LJ, Brown MP. "CD40 Ligand (CD154) Enhances the Th1 and Antibody Response to Respiratory Syncytial Virus in the BALB/c Mouse." Journal of Immunology 164, 5913-5921, 2000.

4.4.1 CD40L gene transfer augments RSV-specific type 1 responses and viral clearance

Mice were challenged with RSV and simultaneously immunised with Ad-CD40L, or with PBS diluent or empty adenovector controls. In all experimental groups, intracellular cytokine levels in BAL and spleen peaked 7-14 days post-infection and a type 1 cytokine profile (IL-2, IL-12, IFNγ, TNF) generally dominated over a type 2 cytokine profile (IL-4, IL-5, IL-6). On the other hand, in Ad-CD40L-treated mice, type 1 intracellular cytokine production in BAL was further exaggerated, which was particularly significant as the countervailing type 2 intracellular cytokine production did not increase above levels found in control mice. The proportions of BAL $CD3^+$ T cells expressing IL-2 or IL-12 at 10 and 14 days post-infection, respectively, were significantly higher in Ad-CD40L-treated mice than in control mice. However, similar effects were not observed among $CD3^+$ splenic T cells. Significantly higher levels of BAL IFNγ, which peaked 10 days post-infection, were found only in Ad-CD40L-treated mice. Ad-CD40L-treated mice also consistently demonstrated higher BAL nitrite levels than control mice. BAL nitrite levels peaked at 5 days post-infection but remained almost as high until 10 days post-infection in mice infected with either adenovirus but not in saline-treated mice. Moreover, Ad-CD40L-treated mice had begun to clear RSV from lung more rapidly than control mice by day 6 post-infection. The next day, Ad-CD40L-treated mice had completely cleared the RSV infection whereas it took another week for PBS control mice to clear the infection when Ad-VC control mice still retained infectious pulmonary RSV. The kinetics of enhanced viral clearance in Ad-CD40L-treated mice matched the kinetics of the enhanced pulmonary content of type 1 cytokine-producing T cells and the elaboration of NO and type 1 cytokines. The same Ad-CD40L vector was used in studies of murine MB49 bladder cancer and its expression of CD40L was sustained for at least 6 days *in vitro* (Loskög A *et al*., 2001), which suggests that transgenic CD40L was available to exert biological effects *in vivo* in RSV-infected mice.

In addition, Ad-CD40L-treated mice had a two-fold higher frequency of RSV-specific and MHC class I-restricted precursor CTL than control mice at 21 days post-infection, which may have resulted from a positive effect of virally mediated CD40L expression on the generation of memory CTL. In summary, simultaneous adenoviral gene transfer of CD40L augmented RSV-specific type 1 cellular immune responses and accelerated RSV clearance.

4.4.2 CD40L gene transfer augments RSV-specific humoral immunity

Although simultaneous adenoviral gene transfer of CD40L resulted in a two-fold higher titre of RSV-specific serum antibodies in immunised mice than in control mice, there was no evident skewing of the isotype profile of these antibodies, which has been reported for dominant type 1 cytokine responses. Overall, these results indicate that intraperitoneal delivery of virally mediated CD40L expression at a site distant from the site of pulmonary infectious challenge with RSV enhanced both RSV-specific immunity and innate immunity in the lung with consequently accelerated pulmonary clearance of RSV. Although, Ad-CD40L treatment produced a heightened type 1 cytokine response among RSV-specific pulmonary T cells, an increase in type 1 T cells in the spleen was not observed. Nonetheless, despite the increased BAL concentration of IFNγ in Ad-CD40L-treated mice, the proportion of IFNγ-expressing pulmonary $CD3^+$ T cells did not increase with Ad-CD40L treatment, which suggests that cells other than T cells were responsible for the increased pulmonary output of IFNγ.

Harcourt JL, Brown MP, Anderson LJ, Tripp RA. "CD40 Ligand (CD154) improves the durability of respiratory syncytial virus DNA vaccination in BALB/c mice." Vaccine 21, 2964 – 2979, 2003.

4.4.3 RSV subunit DNA vaccines alone do not augment antibody responses to RSV infection

Immunisation of mice with plasmid DNA encoding RSV-F and/or G proteins four or eight months previously produced frequencies of splenic IgG AFC that approached 1:20,000 to 1:30,000 before RSV challenge. In contrast, mice immunised with pDNA*neo* displayed IgG AFC frequencies in spleen of 1:360,000 to 1:833,000 before RSV challenge. Nonetheless, after RSV challenge, all DNA-immunised mice demonstrated similar frequencies (approximately 1:20,000) of splenic IgG AFC, which indicated that DNA priming *per se* did not augment the humoral immune response to RSV antigens. Four or eight months after DNA-immunisation and before RSV challenge, serum RSV-specific IgG was barely measurable. Following RSV infection, serum RSV-specific IgG was manifest although antibody titres were two-fold to four-fold higher at four months than eight months post-immunisation. Serum RSV-specific IgG titres four months post-immunisation were not significantly different irrespective of the immunising plasmid DNA including pDNA*neo*. Hence, genetic immunisation with RSV subunit antigens alone was not sufficient to augment humoral immune responses to RSV infection.

4.4.4 Genetic immunisation accelerated pulmonary viral clearance

As previously reported [528], pulmonary viral clearance among mice immunised with plasmid DNA including pDNA*neo* was significantly greater than age-matched unimmunised mice. Only in mice that had received pDNA-F four months previously was clearance of virus significantly greater than in mice receiving pDNA*neo* 14 days post-infection. Among mice receiving pDNA-G, viral clearance was significantly more delayed than in control mice receiving pDNA*neo* whereas viral clearance was not significantly different between mice immunised four months previously with pDNA-F/G or pDNA*neo*. In contrast, DNA-immunisation with any RSV subunit antigen eight months previously resulted in significantly greater viral clearance than if mice had been immunised with pDNA*neo*. Therefore, while immunisation with DNA itself accelerated pulmonary viral clearance, genetic immunisation using RSV subunit antigens further enhanced pulmonary clearance of RSV.

4.4.5 RSV subunit DNA vaccines induce cellular responses to clear RSV

Altogether, these results suggested that while prior DNA immunisation itself may have contributed to enhanced viral clearance after RSV challenge, prior immunisation with the RSV-F sequences further contributed to viral clearance. However, the lack of an effect of DNA priming on RSV-specific antibody titres implied that humoral responses alone were not responsible for the positive effects of DNA immunisation on pulmonary viral clearance 14 days after infection. In RSV-infected mice that had been immunised four months previously with RSV subunit DNA vaccines, and particularly in mice immunised with pDNA-G, pulmonary $CD4^+$ T cell infiltrates were significantly higher than in mice immunised with pDNA*neo*. Pulmonary NK cell numbers were higher in pDNA-F-immunised mice at four and eight months post-immunisation than in mice immunised with pDNA*neo*. Otherwise, in comparison with pDNA*neo* immunisation, no distinct patterns of pulmonary trafficking of $CD8^+$ T-lymphocytes, B-lymphocytes, monocyte-macrophages, and neutrophils were apparent in lungs of RSV-infected mice four and eight months after DNA-immunisation with RSV subunit vaccines. Although skewing toward a Th1 or Th2 response was not apparent, the proportions of pulmonary $CD3^+$ T-lymphocytes expressing type 1 or type 2 cytokines were variously 1.5 to 3-fold higher after RSV challenge in mice previously immunised with DNA encoding RSV-F and/or RSV-G proteins than in mice previously immunised with control pDNA*neo*. Hence, an augmented effector T cell response is more likely to account for the enhanced viral clearance found after RSV-specific DNA-immunisation. In summary, RSV-specific cellular immune responses may explain the accelerated pulmonary viral clearance that occurred after genetic immunisation with RSV subunit antigens.

4.4.6 CD40L-expressing DNA vaccines enhance RSV immunity and pulmonary viral clearance

Less vigorous RSV-specific antibody responses were encountered after RSV challenge in mice genetically immunised with RSV subunit vaccines eight months earlier than in mice immunised four months earlier. Nevertheless, at eight months post-immunisation, antibody titres were significantly boosted in mice immunised with pDNA-F/CD40L or pDNA-G/CD40L than in mice immunised with pDNA-F or pDNA-G. However, only in mice immunised eight months previously with pDNA-F/G/CD40L rather than pDNA-F/G were significantly higher frequencies of splenic IgG AFC observed 14 days post-infection. Pulmonary viral titres were significantly reduced in all mice previously co-immunised with RSV-specific and CD40L-expressing plasmid DNA than in mice immunised with RSV-specific plasmid DNA alone. At four months post-immunisation, the most significant enhancement of viral clearance occurred in mice that had been immunised with pDNA-F/CD40L. However, in 2/4 and 3/4 mice immunised with pDNA-F/G/CD40L four and eight months earlier, respectively, virus was completely cleared 14 days post-infection. At the observed peak in viral clearance 14 days post-infection, significantly more pulmonary $CD4^+$ and $CD8^+$ T cells were found in mice receiving pDNA-F/G/CD40L rather than pDNA-F/G eight months earlier, which suggests that an augmented adaptive immune response contributed to the observed acceleration in viral clearance. Pulmonary $CD3^+$ T cells expressing type 1 cytokines (IFNγ foremost) and less so, type 2 cytokines, predominated in association with the enhanced pulmonary viral clearance induced by prior immunisation with pDNA-F/CD40L and pDNA-F/G/CD40L. Interestingly, in RSV-infected mice four months after immunisation with pDNA-G, co-immunisation with CD40L-expressing plasmid DNA abolished the pulmonary ingress of $CD4^+$ T cells and was associated with significantly enhanced pulmonary viral clearance and peaks both of $CD8^+$ T cells and IFNγ-expressing $CD3^+$ T cells in lung 14 days post-infection.

Significantly more $CD11b^+$ cells, presumably monocyte-macrophages and DC, and significantly more $CD4^+$ T cells together with IL-12-expressing cells, were detected five days post-infection in the lungs of mice immunised four months previously with pDNA-F/CD40L than in mice immunised with pDNA-F alone. For these immunisation conditions, viral clearance, splenic IgG AFC and RSV-specific IgG titres 14 days post-infection were all significantly enhanced by adjunctive CD40L. Prior adjunctive use of CD40L may expand the pool of RSV-specific memory $CD4^+$ T cells, which interact soon after infection with $CD11b^+$ APC to generate IL-12 and subsequently induce T cell production of IFNγ. IFNγ can be expected to contribute to the further maturation of antigen presenting and effector functions of $CD11b^+$ cells. Similarly, a pool of RSV-specific memory B cells may be expanded to produce IgG AFC and elevated serum titres of RSV-specific IgG.

Adjunctive genetic immunisation using CD40L opposed effects that were induced in response to sole immunisation with plasmid DNA encoding the G protein, which facilitates virus infection and induces leukocyte chemotaxis *via* its fractalkine-like activity. After co-immunisation with CD40L-expressing plasmid DNA, viral clearance was increased in association with pulmonary ingress of $CD8^+$ T cells and $IFN\gamma^+$ $CD3^+$ T cells. Hence, given the effects of adjunctive CD40L genetic immunisation on immune responses to both RSV-F and RSV-G proteins, it would not be surprising to find that the most effective vaccination regimen was pDNA-F/G/CD40L. Immunisation with pDNA-F/G/CD40L was associated with accelerated pulmonary viral clearance four and eight months after immunisation. Immunological correlates included significantly more splenic IgG AFC, increased pulmonary $CD8^+$ T cell numbers, and modestly increased pulmonary Th1 (IL-2, IL-12 and IFNγ) cytokine production, all of which may have aided in early viral clearance. In conclusion, adjunctive genetic immunisation with CD40L enhances RSV-specific humoral and type 1 cellular immune responses and pulmonary viral clearance.

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CHAPTER 5

CD40L GENE AUGMENTATION INDUCES ANTI-TUMOUR IMMUNITY

5.1 Background and Rationale

In the therapeutic vaccination of malignancy, we tested the value of forced expression of CD40L in order to direct ligation of its CD40 receptor *in vitro* or *in vivo*. While CD40 is expressed constitutively on all the cells on which it is expressed, CD40L expression is mainly induced within the strict temporal and spatial confines of helper T cell activation. CD40 is expressed on all professional APC, epithelial cells and other common cell types such as endothelial cells and fibroblasts. Consequently, CD40 is expressed by many of the primary cell types that may become malignant. Of human malignancies, the commonest are the carcinomas, which are a heterogeneous group of malignant transformations of epithelial tissues. Less prevalent are the leukaemias, which are malignant transformations of circulating myeloid or lymphoid cells, and the lymphomas, which are usually malignant transformations of lymph node B cells.

While the physiology of CD40 expression by the professional APC of DC, monocyte/macrophages and B cells is reasonably well understood, it is less well understood for CD40 expression by epithelial cells. The linked observations that (i) CD40L-deficient HIGM1 patients commonly develop chronic hepatobiliary cryptosporidiosis, sclerosing cholangitis and hepatitis, and (ii) CD40- and CD40L-deficient mice also fail to eradicate cryptosporidial infection and subsequently develop chronic hepatobiliary inflammation, indicate that CD40L/CD40 interactions involving bone marrow-derived cells are needed to clear a parasitic infection, the persistence of which induces chronic and destructive inflammation. Furthermore, HIGM1 patients with hepatobiliary disease are particularly susceptible to hepatobiliary malignancy. This clinical example supported by the results of realistic preclinical model studies suggests that different protective functions conferred by CD40 expression on bone marrow-derived cells or epithelial cells may be subserved by different signal transduction pathways (Chapter 2).

In the absence of CD40 ligation, CD40-expressing epithelial cells ensconced in a chronic inflammatory environment may continue to survive inappropriately and undergo malignant transformation. Once malignant transformation has occurred, different consequences of CD40 ligation may ensue depending on the CD40 receptor status of the malignant cells. Among CD40-expressing carcinoma cell lines, CD40 ligation *in vitro* may induce apoptotic cell death or promote survival and proliferation, which suggests that in some carcinoma cells, the signal transduction pathways downstream of CD40 activation may be ‘uncoupled’ from the cell’s death machinery. In addition, the loss of CD40 expression by some carcinomas has been suggested to be a mechanism by which carcinoma cells evade host tumour defence mechanisms such as activated tumour-specific CD40L-expressing T cells. To begin addressing some of these questions, we analysed primary human prostate cancer specimens for expression of CD40 (Moghaddami M *et al*., 2001).

The immunobiological effects of constitutive transgenic CD40L expression were investigated in preclinical models of myeloma, neuroblastoma, bladder cancer and leukaemia (Dotti G *et al*., 2001; Grossmann ME *et al*., 1997; Loskög A *et al*., 2001; Dilloo D *et al*., 1997), and finally in an early phase clinical trial of a CD40L gene-modified vaccine in post-transplant leukaemia patients (Rousseau RF *et al*., 2006).

The 'tumour protection' models of murine neuroblastoma (Grossmann ME *et al.*, 1997) and murine bladder cancer (Loskög A *et al.*, 2001) demonstrate immunobiological principles rather than represent clinical reality. Here, mice were immunised with live CD40L gene-modified tumour cells, which were not tumorigenic *per se* but which did act as a therapeutic vaccine to protect mice against subsequent challenge with parental tumour. Furthermore, in these models, co-inoculation of mice with parental or control tumour cells together with CD40L gene-modified tumour cells inhibits the growth of the parental or control tumour cells. In the first example, the presence of as few as 1.4% CD40L-expressing tumour cells significantly retarded the growth of the dominant admixed population of *neo* control neuroblastoma cells (Grossmann ME *et al.*, 1997). In the second example, the simultaneous inoculation of mice with CD40L gene-modified tumour cells and parental tumour cells at two different sites results in retarded growth of the parental tumour (Loskög A *et al.*, 2001). Thus, these tumour protection data may usefully be applied to the clinical investigation of primary or secondary tumour masses, which may be accessible to injection with CD40L gene transfer vectors and so subsequently induce systemic anti-tumour immunity toward distant metastatic deposits.

The murine leukaemia models (Dilloo D *et al.*, 1997) most closely resemble the clinical reality of pre-existing malignancy (Rousseau RF *et al.*, 2006) and are, therefore, called 'tumour treatment' models. In the murine models, pre-existing leukaemia was present as an impalpable tumour inoculum at the time of therapeutic vaccination (Dilloo D *et al.*, 1997) whereas in high-risk leukaemia patients, minimal residual disease was presumed to exist after remission induction (Rousseau RF *et al.*, 2006). Importantly, as a realistic model of human leukaemia, A20 cells express MHC class I and II molecules but not the costimulatory molecule CD80. Hence, although leukaemia cells such as A20 may express unique TAA such as oncogenic fusion proteins and mutant proteins for presentation on MHC molecules to the host immune system after immunisation, anergy rather than immunity is more likely to be induced in the absence of concomitant costimulation. Upon cognate interaction between the TCR and the peptide/MHC complex borne by the APC, CD40L is known to initiate the costimulatory cascade leading to productive immunity. Therefore, artificial CD40L expression may induce anti-leukaemic immunity directly by ligation of CD40 on A20 leukaemia cells and/or indirectly by ligation of CD40 on host APC.

The preclinical findings of Dilloo *et al*. (1997) were clinically translated in a phase I trial of an autologous leukaemia vaccine in high-risk acute leukaemia patients in cytologic remission following allogeneic stem cell transplantation or chemotherapy (Rousseau RF *et al*., 2006).

We anticipate that the therapeutic consequences of a surfeit of CD40 ligation would differ according to whether tumours express CD40 or not. For CD40-negative and positive tumours, CD40 ligation would initiate and/or augment the recruitment of immune effector mechanisms *via* the activation of professional APC. In the case of a $CD40^+$ tumour, further enhancement of immune activation and immunological memory induction would be expected *via* increased levels of CD40-mediated tumour cell death with a subsequent increase in antigen uptake, processing and presentation by APC to cognate T cells.

Therefore, in order to study the effects of CD40L gene-modified tumour vaccines in controlled circumstances, we used preclinical systems to model different clinical circumstances in which CD40L-expressing vaccines may be used:

1. CD40-expressing malignancies
    (i) RPMI 8226 human myeloma cell line *in vitro* (Dotti G *et al*., 2001)
    (ii) A20 murine B-leukaemia cell line *in vivo* (Dilloo D *et al*., 1997)
    (iii) MB49 murine bladder carcinoma cell line *in vivo* (Loskög A *et al*., 2001)
2. Malignancies that do not express CD40
    (i) U266B1 human myeloma cell line *in vitro* (Dotti G *et al*., 2001)
    (ii) WEHI-31 murine myeloid leukaemia cell line *in vivo* (Dilloo D *et al*., 1997)

(iii) Neuro2a murine neuroblastoma cell line *in vivo* (Grossmann ME *et al*., 1997)

5.2 Aims

5.2.1 To ascertain if primary adenocarcinoma of the human prostate gland expressed CD40 (Moghaddami M *et al*., 2001).

5.2.2 To investigate differential effects of transgenic CD40L expression by human myeloma cell lines on the immunogenicity and biological responses of both CD40-expressing and non-expressing human myeloma cells *in vitro* (Dotti G *et al*., 2001).

5.2.3 To study the effects of varying the proportion of CD40L transgene-expressing neuroblastoma cells among vector control cells of a CD40-negative murine neuroblastoma cell line on tumorigenicity and host immune responses *in vivo* (Grossmann ME *et al*., 1997).

5.2.4 To compare the tumorigenicity of and the host immune responses to a CD40-expressing murine bladder cancer cell line genetically modified to express CD40L, CD80 or IL-12 (Losközg A *et al*., 2001).

5.2.5 To investigate the effects of *in vivo* bystander transgenic CD40L and/or IL-2 expression on the tumorigenicity, host immune responses and host survival of murine CD40-expressing murine B leukaemia and CD40-negative myeloid leukaemia cell lines (Dilloo D *et al*., 1997).

5.2.6 To evaluate the safety and immunogenicity of a leukaemia vaccine comprising autologous leukaemia cells and autologous fibroblasts genetically modified to express CD40L and IL-2 in high-risk leukaemia patients in remission following allogeneic stem cell transplantation or chemotherapy (Rousseau RF *et al*., 2006).

5.3 Experimental Approach

5.3.1 To ascertain if primary human prostate cancer expressed CD40.

Immunochemical and immunofluorescence techniques were used to examine the expression of basal cell-specific cytokeratins and CD40 in sections of normal, benign and malignant prostatic tissue and in short-term *in vitro* cultures of epithelial cells derived from benign or malignant prostatic tissues (Moghaddami M *et al*., 2001).

5.3.2 To investigate effects of human myeloma cell expression of transgenic CD40L on myeloma cell behaviour in vitro.

Two human multiple myeloma cell lines (HMCL) were chosen that differed in their expression of CD40: RPMI 8226 was $CD40^+$ and U266B1 was negative or weakly positive for CD40 expression. HCML were transduced at a multiplicity of infection (MOI) of 500 viral particles (VP)/cell with a serotype 5 adenovector expressing human CD40L (AdhCD40L), or with a control adenovector expressing the green fluorescent protein (AdGFP). Transgene expression was analysed by flow cytometry. Cell viability and proliferation were determined by trypan blue staining and tritiated thymidine incorporation, respectively. Apoptosis was measured by binding of annexin V and/or by uptake of nucleic acid binding dyes. Immunoreactivity to gene-modified HCML was evaluated by autologous or allogeneic mixed lymphocyte reactions. Immunophenotyping of HCML, CTL and DC populations was analysed by flow cytometry. Monocyte-derived DC were co-cultured with gene-modified HCML and apoptotic cell uptake was determined by fluorescence staining methods. Co-cultivated DC and gene-modified HCML acted as autologous stimulators for the generation of effector cells, the cytokine output of which was measured by ELISA and cytometry bead array (Dotti G *et al*., 2001).

5.3.3 To study effects of murine neuroblastoma expression of transgenic CD40L on tumorigenicity and host immune responses.

Neuro2a is a CD40-negative neuroblastoma cell line syngeneic with A/J mice. To analyse the effects of tumour expression of CD40L on tumorigenicity and anti-tumour immune responses, stable neuro2a cell expression of CD40L (neuro2a/CD40L) was achieved by transduction

with a CD40L-expressing retrovector then enrichment by fluorocytometric sorting. The proportion of neuro2a/CD40L cells in the tumour inoculum was varied by mixing them with control neuro2a/neo cells, which had been transduced with the control retrovector expressing the neomycin resistance gene (*neo*) and maintained under G418 selection. Tumour challenge with unmodified neuro2a cells was done in naïve mice and compared with challenge in mice, which had been inoculated 30 and 60 days previously with 70% $CD40L^+$ neuro2a cells. The nature of systemic anti-tumour immunity induced by inoculation with neuro2a/CD40L cells was studied by *in vivo* depletion of $CD4^+$ or $CD8^+$ T cells at the time of tumour inoculation. To determine if inoculation of mice with neuro2a/CD40L cells altered indicators of systemic immune activation, immunohistochemical and fluorocytometric analyses of spleen and splenocytes, respectively, were performed nine days after tumour inoculation (Grossmann ME *et al*., 1997).

5.3.4 To compare the tumorigenicity of and the host immune responses to a CD40-expressing murine bladder cancer cell line genetically modified to express CD40L, CD80 or IL-12.

The MB49 cell line, which was derived from a transitional cell carcinoma of bladder of C57BL/6 mice, was transduced with recombinant adenoviral vectors of serotype 5, which expressed CD80, CD40L or IL-12 (Ad-CD80, Ad-CD40L, or Ad- IL-12). The MOI of the IL-12-expressing adenovector was varied to create low and high dose levels of IL-12 production by transduced MB49 tumour cells. Surface expression of CD80 and CD40L was measured by flow cytometry. IL-12 production was measured by ELISA and its bioactivity confirmed by a T cell proliferation assay The tumorigenicity of the transduced tumours was evaluated by daily palpation and a tumour was confirmed when its size exceeded $10mm^3$. Tumour challenge experiments were performed two weeks after a tumour immunisation program, which included up to four sequential injections 17 days apart using tumour cells transduced with Ad-CD40L alone or in combination with Ad-IL-12, or three sequential injections with lysed parental tumour cells. To demonstrate specificity of the anti-tumour immune response, mice immunised four times with Ad-CD40L-transduced tumour cells were challenged simultaneously in each flank with parental tumour or an unrelated syngeneic tumour. Immunohistochemical analyses were performed on Ad-CD40L-transduced and parental tumours at various stages of their formation (Loskög A *et al*., 2001).

5.3.5 To investigate the effects of in vivo bystander transgenic CD40L and/or IL-2 expression on the tumorigenicity, immune responses and survival in murine leukaemia.

To ascertain if CD40 expression by tumour cells *per se* increased the therapeutic efficacy of a CD40L-expressing tumour cell vaccine, comparison was made of vaccines prepared using cell lines derived from the CD40-positive A20 B lymphoblastic leukaemia or the CD40-negative WEHI-3 myeloid leukaemia, which are syngeneic to BALB/c mice. The vaccines were studied *in vivo* in a treatment model in which unmodified leukaemia cells were transplanted as subcutaneous tumours. Live cells of the A20 or WEHI-3 lines were inoculated subcutaneously in the flanks of mice, which were subsequently immunised four and 14 days later with the corresponding A20 or WEHI-3 therapeutic tumour vaccine. This vaccine comprised a lethally irradiated (10Gy) mixture of A20 or WEHI-3 leukaemia cells and retrovirally transduced sublines of a syngeneic fibroblast cell line, which expressed as a negative control the *neo* gene, or individually the murine genes for IL-2 or CD40L. The chosen A20 tumour cell dose was double that found to produce tumours in 90% of control mice.

Tumours were measured bi-dimensionally and the tumour endpoint of the study was tumour growth beyond 30% of animal weight, ulceration or animal distress. *In vivo* depletion studies were performed using mAb specific for CD4, CD8 or asialo-GM1 (NK cells) and commenced the day before tumour inoculation and continued for four weeks. Fluorocytometric analysis of spleen and blood confirmed that the efficacy of the depletion method was >98%. A20 cells were co-cultured with *neo* or $CD40L^+$ fibroblasts for three days and A20 expression of CD80,

Fas, and MHC molecules measured by flow cytometry. After the period of co-culture, Fas-mediated killing of A20 cells *in vitro* was tested by incubation with anti-Fas antibody. Immunophenotyping of single cell suspensions prepared from A20 tumour explants was performed using flow cytometry (Dilloo D *et al*., 1997).

5.3.6 To evaluate the safety and immunogenicity of a leukaemia vaccine expressing CD40L and IL-2 in high-risk leukaemia patients.

The vaccine comprised a mixture of adenovirally transduced autologous skin fibroblasts, which had been propagated *in vitro* from a punch biopsy of skin, together with autologous leukaemic blasts, which had been prepared from peripheral blood or bone marrow. The leukaemic blast preparation was considered adequately enriched if >65% of cells carried the immunophenotypic profile characteristic of the leukaemic clone. Leukaemic blast preparations were irradiated (40Gy) and stored in liquid nitrogen until further use. The fibroblasts were transduced with E1a/b and E3-deleted serotype 5 adenoviral vectors expressing human IL-2 (AdhIL-2) or human CD40L (AdhCD40L) at MOI values of 500 or 1000 VP per cell, respectively. As an index of the number of competent vectors, the VP to infectious unit ratio of both vectors was <30:1. Transduction was considered adequate if, at 24 hours (24h) post-transduction, the IL-2 production rate was >150pg/10^6 cells/24h or the CD40L expression level was >20%. Then transduced fibroblasts were irradiated (30Gy) and stored in liquid nitrogen before use.

Prior to immunisation a fixed dose of leukaemic blasts (2 x 10^7/injection) was mixed with a fixed dose of hIL-2-gene-modified fibroblasts (2 x 10^7/injection) and a variable dose of hCD40L-gene-modified fibroblasts, which escalated in log increments from 2 x 10^5/injection at dose level 1 to 2 x 10^7/injection at dose level 3. A conventional '3 + 3' phase I trial design was adopted so that 3 patients were dosed at each of levels 1, 2 and 3. Up to six subcutaneous injections of the leukaemia vaccine were administered and the schedule of vaccine administration was weekly for the first three injections followed by a two-week 'rest' interval then fortnightly for the remaining three injections (Rousseau RF *et al*., 2006).

Skin punch biopsies of the immunisation sites were performed in all patients one week after the first and the second vaccine injections, fixed in formalin, processed and analysed by immunohistochemistry, and compared with controls obtained from normal healthy volunteers. Immunophenotyping of PBMC was done by flow cytometry and the profiles were compared with historical controls at the same institution. Pre- and post-immunisation granzyme B (GrB), IFNγ and IL-5 responses to the immunising leukaemic blasts were measured among the thawed patient PBMC using enzyme-linked immunospot (ELISPOT) assays. MHC restriction was tested by CD4 and CD8 blockade. Target cells included unmodified pre-chemotherapy leukaemic blasts, and as controls, the NK-specific K562 myeloid cell line, recipient-derived skin fibroblasts, and phytohaemagglutinin (PHA) blasts obtained from each patient's pre-immunisation PBMC. Testing for the presence of leukaemic blast-binding IgG antibodies in post-immunisation plasma samples was performed by flow cytometry (Rousseau RF *et al*., 2006)

5.4 Major Findings

Moghaddami M, Cohen P, Stapleton AMF, Brown MP. "CD40 is not detected on prostate cancer cells by immunohistologic techniques." Urology 57, 573-578, 2001.

5.4.1 Human prostate cancer does not express CD40

We found that CD40 was expressed on normal and benign hyperplastic prostatic tissue and most strongly on the basal layer of the epithelium where it was co-expressed with the basal cell-specific keratins, keratin-14 (K14), and high molecular weight keratins identified by the mAb clone, 34βE12. In contrast, neither CD40 nor these basal cell-specific keratins were expressed on any prostate adenocarcinoma irrespective of the grade of differentiation. Interestingly, an early pre-neoplastic version of prostate adenocarcinoma known as prostatic intra-epithelial neoplasia (PIN) had basal discontinuities in the expression of CD40 and

34βE12 staining, which suggested that CD40 expression contributes to the basal phenotype and that loss of the basal phenotype may be an early manifestation of malignant transformation.

Dotti G, Savoldo B, Takahashi S, Goltsova T, Brown M, Rill D, Rooney C, Brenner M. "Adenovector induced expression of human CD40-ligand (hCD40L) by multiple myeloma cells: A model for immunotherapy." Experimental Hematology 29, 952-961, 2001.

5.4.2 Transgenic CD40L expression induces death among CD40-expressing myeloma cells

Expression of GFP or hCD40L by HCML was stable for 10 days and, 72 hours after transduction, 38% and 51% of RPMI 8226 and U266B1 cells, respectively, were GFP^+, and 96% and 78% of RPMI 8226 and U266B1 cells, respectively, were $hCD40L^+$. In comparison to transduction with the control AdGFP adenovector or no transduction, AdhCD40L transduction of CD40-expressing RPMI 8226 cells induced significant levels of apoptosis and significantly reduced rates of RPMI 8226 cell viability and proliferation. At 72-96 hours post-transduction, 37% of AdhCD40L-transduced RPMI 8226 cells bound annexin V (AV) and propidium iodide (PI) whereas only 20% of non-transduced cells were AV^+ PI^+. The proportion of RPMI 8226 cells expressing CD40 was significantly reduced among AdhCD40L-transduced cells 72-96 hours after transduction compared with AdGFP-transduced or non-transduced cells. This result was interpreted to mean that death of CD40-expressing cells had occurred. An alternative not mutually exclusive interpretation of these data is that CD40 expression was down-modulated by transgenic expression of CD40L.

In contrast, AdhCD40L transduction had a less dramatic impact on the *in vitro* behaviour of CD40-negative U266B1 cells. The proportions of apoptotic U266B1 cells were not significantly different among U266B1 cells transduced 72-96 hours previously with the AdhCD40L adenovector, or non-transduced cells. Nevertheless, AdhCD40L transduction significantly reduced both the proliferation and viability of CD40-negative U266B1 cells 96 hours post-transduction, which suggested that paracrine CD40L expression by U266B1 cells may have ligated CD40 molecules expressed at low level on U266B1 cells to exert smaller or delayed apoptotic or anti-proliferative effects. The results of these experiments also indicate that at an MOI of 500 VP/cell, toxicity from adenoviral proteins was unlikely to have contributed to cell death or growth inhibition because cell viability, apoptosis and proliferation rates were not significantly different between AdGFP-transduced and non-transduced cells. However, adenoviral transduction *per se* did have an observable biological effect on HCML because transduction of RPMI 8226 cells with AdhCD40L or AdGFP up-regulated expression of CD86 on these cells to a similar degree.

5.4.3 CD40L-expressing myeloma cells induce alloreactivity via APC activation

The RPMI 8226 line was poorly immunogenic because AdGFP-transduced or non-transduced RPMI 8226 cells failed to induce significant proliferation of PBMC in the allogeneic mixed lymphocyte reaction (alloMLR) despite varying the ratio of RPMI 8226 cells to PBMC, or using multiple different normal donors as a source of PBMC. Instead, AdhCD40L transduction of RPMI 8226 cells was required to stimulate significant proliferation of PBMC in the alloMLR. The increased proportion of apoptotic RPMI 8226 cells among cultures of AdhCD40L-transduced RPMI 8226 cells was insufficient *per se* to induce proliferation in the alloMLR because RPMI 8226 cells rendered apoptotic by ultraviolet light exposure or serum deprivation failed to induce proliferation in the alloMLR. In spite of the profound effects of AdhCD40L transduction on the survival and proliferation of HCML, AdhCD40L transduction had no other detectable effect on the surface immunophenotype of RPMI 8226 or U266B1 cells, which included the expression of MHC class I and II molecules, CD80 and the cell adhesion molecules, CD11a, CD18, CD50, CD54 and CD102. Furthermore, since purified $CD3^+$ lymphocytes were unable to proliferate in the alloMLR in response to AdhCD40L-transduced RPMI 8226 cells, it is unlikely that AdhCD40L-transduced RPMI 8226 cells were themselves able to act as professional APC but rather that proliferation in the alloMLR was

induced *via* CD40 ligation of professional APC. On the other hand, U266B1 cells were immunogenic and AdhCD40L transduction did not significantly enhance immunoreactivity to U266B1 cells in the alloMLR. In conclusion, adenovirally mediated CD40L expression by human myeloma cells induced alloreactivity *in vitro* probably *via* activation of APC.

5.4.4 Transgenic CD40L expression stimulates autologous cell cultures

Human moDC were used to study the effects of transgenic CD40L expression on professional APC. In contrast to AdGFP-transduced or non-transduced cells, moDC co-cultured for 48 hours with AdhCD40L-transduced RPMI 8226 cells up-regulated expression of the MHC class II molecule, HLA-DR, CD40, CD80 and CD83, which indicated that DC matured in response to CD40 ligation. Similar results were obtained with AdhCD40L-transduced U266B1 cells. Moreover, it was evident from co-culture of fluorescently stained moDC and RPMI 8226 cells, which had been transduced 48-72 hours previously with AdhCD40L, this HMCL population contained a sub-population of apoptotic bodies that were almost entirely taken up by the moDC within a 24-hour period. Similar results were obtained using AdhCD40L-transduced U266B1 cells that were subsequently rendered apoptotic by ultraviolet light exposure or serum deprivation.

To demonstrate the functional consequences of CD40L-mediated up-regulation of MHC and costimulatory molecule expression, moDC that had been co-cultured with AdhCD40L-transduced HMCL (RPMI 8226 or U266B1 cells) for 48 hours induced significantly greater proliferation of autologous PBMC than AdGFP-transduced or non-transduced HMCL averaged over five different MLR. Unlike the results of the alloMLR, enrichment of DC in these mixed lymphocyte reactions produced a clear demonstration of the immunostimulatory potency of CD40 ligation mediated by CD40L gene-modified U266B1 cells. These data also indicate that after AdhCD40L transduction of U266B1 cells, moDC activated by CD40 ligation presented U266B1-related antigens to autologous PMBC. Here, it must be emphasised that although AdhCD40L transduction of U266B1 cells did not itself induce significant levels of U266B1 cell death, all HCML preparations were γ-irradiated (80Gy) before addition to MLR cultures, which will ensure that DC in the culture are supplied with dead HMCL. In summary, CD40L-expressing human myeloma cells stimulated autologous cell cultures *via* CD40 activation of DC that had ingested dead myeloma cells.

5.4.5 Transgenic CD40L expression induces a type 1 cytokine response in vitro

Long-term co-culture of moDC with adenovirally transduced or non-transduced HMCL for four to five weeks followed by short term 48-hour re-stimulation with AdhCD40L-transduced, AdGFP-transduced or non-transduced HMCL produced a significant and MHC class I-restricted increase in the secretion of type 1 cytokines (IFNγ, IL-2 and TNF) and a significant decrease in the secretion of the type 2 cytokine, IL-5, but only for the cultures that had been re-stimulated with AdhCD40L-transduced HMCL. The only immunophenotypic difference detected in co-cultures of DC with AdhCD40L-transduced rather than AdGFP-transduced or non-transduced HMCL was a $CD3^+CD56^+$ sub-population, although NK killing was not detected. The lytic capacity of the restimulated PBMC cultures was also analysed. However, the target HMCL cells were allogeneic to the effectors contained in the restimulated PBMC cultures so that no effect of AdhCD40L-transduction was discernable above the high background levels of killing even though the killing was shown to be MHC class I-restricted. Finally, it was shown that CD40L-expressing human myeloma cells induced MHC class I-restricted and type 1 cytokine production in restimulated autologous cell cultures.

Grossmann ME, Brown MP, Brenner MK. “Anti-tumour responses induced by transgenic expression of CD40 ligand.” Human Gene Therapy 8, 1935-1940, 1997.

5.4.6 Minimal transgenic CD40L expression is required to abrogate tumorigenicity

Five and 14 weeks after *in vitro* selection, the proportions of transduced neuro2a cells that remained $CD40L^+$ were 70% and 30%, respectively. In contrast with tumour growth in mice

inoculated subcutaneously with neuro2a/neo cells, tumour growth in mice inoculated with 35% $CD40L^+$ neuro2a (neuro2a/CD40L) cells was significantly retarded. The mean time to tumour detection in neuro2a/CD40L tumour-bearing mice was double (~6 weeks) that of neuro2a/neo tumour-bearing mice (~3 weeks). As was shown for $CD40L^+$ neuro2a cells *in vitro*, outgrowth of neuro2a/CD40L tumours may have occurred because of an ongoing decline in CD40L expression, which suggests that a threshold level of CD40L-expressing tumour cells was required to suppress overall tumour growth presumably *via* the recruitment of immune effector mechanisms.

This question was explored more formally by mixing neuro2a/CD40L cells in varying proportion with control neuro2a/neo cells. A level of 1.4% neuro2a/CD40L tumour cells in the inoculum was sufficient to suppress tumour growth so that 4/8 mice were tumour-free three weeks after inoculation compared with 0/8 mice and 1/8 mice remaining tumour-free after receiving 0.3% and 0% CD40L-expressing neuro2a cells, respectively.

5.4.7 Transgenic CD40L expression induces systemic anti-tumour immunity

Prior challenge with CD40L-expressing tumour cells protected against parental tumour challenge. Naïve mice or mice previously inoculated with neuro2a/CD40L cells were challenged with unmodified neuro2a tumour cells. All naïve mice grew sizeable tumours by 3.5 weeks after challenge whereas none of the mice previously inoculated with neuro2a/CD40L cells grew tumours at that time although 2/4 mice had palpable tumours five weeks after challenge.

5.4.8 $CD8^+$ T cells mediate the reduced tumorigenicity of CD40L-expressing tumours

In vivo depletion of $CD8^+$ and not $CD4^+$ T cells prevented the suppression of tumour growth normally seen after inoculation of 70% $CD40L^+$ neuro2a cells. In contrast to naïve mice and mice challenged with neuro2a/neo cells, mice challenged with neuro2a/CD40L cells demonstrated increased proportions of both $CD4^+$ and $CD8^+$ T cells among their splenocytes. The largest increase was in $CD8^+$ T cells most of which expressed the T cell activation marker, CD25. In addition, mice challenged with neuro2a/CD40L cells displayed a three-fold increase in splenic $CD45R/B220^+$ cells expressing the activation marker, CD86. Although the majority of these $CD45R/B220^+$ $CD86^+$ splenocytes probably represented activated B cells, it is worth noting that activated T and NK cells can also express CD45R/B220 and that expression of CD86 can be induced on activated T cells [529], so that a proportion of the $CD45R/B220^+$ $CD86^+$ splenocytes may actually represent activated T lymphocytes, particularly in view of the larger proportion of activated $CD8^+$ T cells in the spleens of neuro2a/CD40L-challenged mice. Nevertheless, these systemic effects confirm the biological activity of the CD40L transgene. Hence, it may at least be concluded that CD40L expression by neuro2a cells within the first week after tumour inoculation produced marked and systemically evident immune activation.

To draw the experimental observations made by Grossmann *et al*. together:

(i) Cross-reactive systemic immunity was generated against neuro2a/CD40L tumours because prior inoculation with neuro2a/CD40L cells protected against challenge with parental neuro2a tumour.

(ii) Generation of systemic immunity against neuro2a/CD40L tumours depended on the integrity of the $CD8^+$ T cell population.

(iii) Inoculation with neuro2a/CD40L cells increased the proportion of activated splenic $CD8^+$ T cells before detectable tumour growth.

(iv) While some of the $CD45R/B220^+$ $CD86^+$ splenocytes over-represented in neuro2a/CD40L-challenged mice may be activated T lymphocytes, other $CD45R/B220^+$ $CD86^+$ splenocytes may represent activated APC including B cells and plasmacytoid DC [530].

Losköog A, Björkland A, Brown MP, Malmstöm P-U, Korsgren O, Tötterman T. "Immunotherapy of murine urinary bladder cancer using adenovirus vector transduced cell vaccines." Journal of Urology 166, 1093-1097, 2001.

5.4.9 Transgenic expression of CD40L and IL-12 abrogates tumorigenicity

The *in vitro* expression of CD80 or CD40L peaked between 3-4 days post transduction and lasted up to at least 6 days. By increasing the MOI, the production rate of IL-12 by transduced MB49 cells nearly doubled from 6 to 11ng/mL/10^6 cells in a 48 hour period. No IL-12 production was observed after transduction of MB49 cells with the other adenoviral vectors. CD80 was expressed weakly on non-transduced MB49 cells and on MB49 cells transduced with the control adenovector. CD80 expression increased substantially after Ad-CD80 transduction and CD80 expression was also increased by Ad-CD40L transduction or irradiation.

The tumorigenicity of transduced MB49 tumour cells varied markedly according to the kind of cytokine or costimulatory molecule expressed. Parental MB49 tumour cells and MB49 cells transduced with the control adenovector grew in all inoculated mice. Ad-CD40L transduction alone prevented tumour growth in 94% of mice. Transduction with Ad-IL-12 vector at low MOI rendered 10% of mice tumour-free whereas at high MOI, 40% of mice remained tumour-free. Combining transduction of Ad-CD40L with Ad-IL-12 abrogated tumour growth completely. In contrast, Ad-CD80 transduction did not alter the pattern of tumour growth at all. Hence, while transgenic IL-12 expression reduced tumorigenicity in a dose-dependent manner, the combined transgenic expression of CD40L and IL-12 completely prevented MB49 tumour formation.

5.4.10 Transgenic CD40L and IL-12 expression paradoxically prevents tumour protection

Prior inoculation with Ad-CD40L-transduced MB49 tumour cells protected against parental tumour challenge completely if four inoculations were given. This protection was specifically related to MB49 tumour antigens because little protection was afforded against challenge with an unrelated syngeneic tumour. Approximately 60% of challenged mice remained tumour free after two or three consecutive doses of Ad-CD40L-transduced MB49 cells. Although significant parental tumour growth delay occurred after challenge in mice receiving a single prior inoculation with Ad-CD40L-transduced MB49 cells, none of the mice was protected. In a control experiment, no protection was obtained after prior inoculation with three consecutive doses of lysed parental MB49 cells. Concomitant tumour immunity was also induced because significant growth delay of parental MB49 tumour was only observed if Ad-CD40L-transduced MB49 cells, and not control adenovector-transduced or lysed unmodified MB49 cells, were inoculated simultaneously at a distant site. However, unexpectedly, no mice were protected after prior inoculation with four consecutive doses of MB49 cells that had been transduced with both Ad-CD40L and Ad-IL-12.

5.4.11 Transgenic CD40L produces early-onset and heavy lymphocytic infiltrates

MB49 tumours that were able to grow because they contained 15% Ad-CD40L-transduced MB49 cells contained a heavy infiltrate of both $CD4^+$ and $CD8^+$ T cells early in their growth (<5mm^3 in size) in addition to large numbers of $F4/80^+$ macrophages also observed in parental tumours. In contrast, only after the parental tumour size exceeded 5mm^3 was infiltration with $CD4^+$ T cells and a few $CD8^+$ T cells observed.

Dilloo D, Brown MP, Roskrow M, Zhong WY, Holden W, Holladay M, Brenner MK. "CD40 ligand induces an antileukemia immune response in vivo." Blood 90, 1927-1933, 1997.

5.4.12 Transgenic CD40L and IL-2 expression cured mice of pre-existing leukaemia

In comparison with mice receiving *neo* control fibroblasts in the tumour vaccine, mice receiving tumour vaccines expressing IL-2 or CD40L had significantly delayed A20 tumour growth. Furthermore, when these mice were followed longer to 42 days post-tumour inoculation, it became apparent that the IL-2- and CD40L-expressing vaccines each prevented

tumour outgrowth in one of seven mice per group whereas the combination synergised to prevent tumour outgrowth in four of seven mice. Finally, in comparison with the *neo* control vaccine, the combined IL-2- and CD40L-expressing vaccine produced long-term tumour-free survival in approximately 30% of a larger group of 28 mice.

5.4.13 Induced anti-leukaemia immunity was mediated by T and NK cells

In vivo depletion studies indicated that the anti-leukaemic activity of the combination vaccine depended on the integrity of the major components of a cellular immune response, which included $CD4^+$ and $CD8^+$ T cells and NK cells.

5.4.14 The IL-2 and CD40L vaccine produced intra-tumoral activation of APC and T cells

Co-culture of A20 cells with CD40L-expressing but not *neo* fibroblasts *in vitro* induced the expression of CD80 and Fas, and also increased the expression of MHC class I and II molecules. CD40L-mediated up-regulation of Fas on A20 cells subsequently sensitised those cells to Fas-induced death *in vitro*. However, $B220^+$ A20 cells derived from the tumour inoculum *in vivo* did not differ in their expression profile of CD80, Fas, and MHC class I and II molecules irrespective of whether the mice were previously administered the IL-2- and CD40L-containing tumour vaccine, or the *neo* vaccine. Perhaps, Fas-related cell death occurred too rapidly to be detectable *in vivo*. On the other hand, evidence for activation of APC and T cells was found *in vivo*. In comparison with mice that had received the *neo* tumour vaccine, mice that had received the IL-2- and CD40L-containing vaccine demonstrated up-regulated expression of CD86 and of MHC class II molecules on intra-tumoral $CD11a^+$ APC and $CD3^+$ T cells, respectively.

The same treatment model was applied using the CD40-negative WEHI-3 myeloid leukaemia cell line. In contrast to the A20 leukaemia model, immunisation with the combination IL-2- and CD40L-expressing vaccine did not protect any of the mice bearing WEHI-3 tumours, which suggested that activation of intra-tumoral DC alone by transgenic CD40L expression was insufficient to offer the mice protective immunity. This result also suggested that CD40 expression by the leukaemia cells themselves contributed to the induction of anti-leukaemia immunity, perhaps by facilitating leukaemia cell death and subsequent leukaemia antigen processing and presentation. Nevertheless, the vaccine mixture of C40L-transduced fibroblasts was mixed and irradiated (10Gy) before injection. This preparation period may have been sufficient to have initiated CD40 and/or Fas-mediated cell death, which would have been consolidated by γ-irradiation. In conclusion, mice receiving the leukaemia vaccine expressing IL-2 and CD40L demonstrated intra-tumoral activation of APC and T cells, which was associated with probable leukaemia cell death *in vivo*.

Rousseau RF, Biagi E, Dutour A, Yvon ES, Brown MP, Lin T, Mei Z, Grilley B, Popek E, Heslop HE, Gee AP, Krance RA, Popat U, Carrum G, Margolin JF, Brenner MK. “Treatment of High-Risk Acute Leukemia with an Autologous Vaccine Expressing Transgenic Human CD40L and IL-2.” Blood 107, 1332-41, 2006.

5.4.15 The clinical leukaemia vaccine relied on bystander transgenic expression

The approach of using skin fibroblasts as a “transfection partner” in the creation of the leukaemia vaccine was necessitated by the poor transducibility of primary human leukaemic blasts *per se*. After freeze-storage of the vaccine, thawing did not impair transgene expression by the transduced fibroblasts so that transgene expression was still detectable 10 days after thawing and peaked six days post-thawing with either an IL-2 production rate of $50ng/10^6$ cells/24h or a CD40L expression level of 40%.

5.4.16 The clinical leukaemia vaccine was relatively safe

Only one patient (no. 10) suffered clinically significant toxicity after a sterile abscess was drained surgically at the site of the fifth immunisation. GvHD was an exclusion criterion and no patients developed GvHD on study. No laboratory indices changed to indicate the development of autoimmunity or self immunoreactivity. In particular, neither anti-nuclear

antibodies nor anti-red cell antibodies were detected, and nor were there significant differences in IFNγ ELISPOT responses between pre- and post-immunisation PBMC toward autologous PHA-stimulated blasts or skin fibroblasts.

5.4.17 Most leukaemia patients remained in remission after immunisation

Of the ten patients entered into the study, eight patients were evaluable for the immunological endpoints because one patient (no. 1) had rapidly progressed and died on study, and the other patient (no. 5) had too few blasts after vaccine preparation for later immunological analysis. Of the remaining nine patients, four (no. 2, 8, 9 and 10) had acute myeloid leukaemia (AML) and five (no. 3, 4, 5, 6 and 7) had acute lymphoblastic leukaemia (ALL). All of these nine patients had achieved complete cytologic remissions after definitive therapy, which included chemotherapy in one patient and allogeneic stem cell transplantation using matched-related donors in five patients, matched-unrelated donors in three patients and a mismatched-unrelated donor in one patient. All of the eight evaluable patients had ceased immunosuppressive therapy at least 14 days (median of 45 days) prior to receiving the first immunisation. Patient no. 3, who had had ALL and had received dose level 1, relapsed solely in the scalp 152 days after entering the study and was reinduced into complete remission with chemotherapy. At the time of reporting, all nine patients remained alive with no evident disease with a median follow-up of 41 months to give an actuarial 5-year overall survival rate of 90%. It cannot be concluded, however, that the lack of leukaemia progression resulted from anti-leukaemia immune responses because the study was an uncontrolled safety study.

5.4.18 The leukaemia vaccine induces local immune responses to immunisation

Immunisation sites were punch-biopsied one week after each of the first and second weekly immunisations. No leukaemic cells were identified but dermal perivascular and subcutaneous infiltrates of lymphocytes were observed, which were graded as mild (<10 cells per high power field [hpf]), moderate (10-50/hpf) or severe (>50/hpf). The infiltrates mainly consisted of $CD8^+$ T cells in the skin punch biopsies of patient no. 2, 9 and 10. In the skin of three patients, there was also evident infiltration of phenotypically immature $CD1a^+$ DC, which had strong perinuclear but relatively weak membranous HLA-DR staining, and which lacked CD83 staining.

5.4.19 The leukaemia vaccine induces leukaemia-specific immune responses

Of the evaluable eight patients, all were lymphopenic before immunisation with a mean absolute circulating lymphocyte count (± SEM) of 533 ± 99/μL and all remained lymphopenic post-immunisation with a mean lymphocyte count of 722 ± 139/μL. However, post-immunisation, there were significant 1.5-fold and 1.6-fold increases in the number of circulating $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocyte sub-populations, respectively, and, in particular, a significant 1.2-fold increase in the number of circulating activated $CD3^+$HLA-DR^+ lymphocytes. Immunisation did not alter the frequencies either of regulatory T cells ($CD4^+$ $CD25^{dim}$ LAG-3^+) or NK and B cells. Release of granzyme B (GrB) by post-immunisation PBMC in response to stimulation with autologous leukaemic blasts indicated the presence of cytotoxic effector cells. Preincubation of these PBMC with blocking anti-CD4 or anti-CD8 mAb confirmed that these cytotoxic effector responses were restricted by MHC class I and class II molecules, respectively. It is clear from these experiments that blockade of CD4 or CD8 did not account for most of the observed GrB^+ ELISPOT responses, which suggested that other cytotoxic effector cell types such as NK cells may have been responsible for the GrB secretion among PBMC from the five responding patients (Table III). While it was apparent in two of three responding patients that IL-5 secretion derived from $CD4^+$ cells, among four responding patients, IFNγ secretion may have originated from NK cells or $CD4^+$ cells or, less commonly, from $CD8^+$ cells (Table III). The anti-leukaemic immune responses appeared to be leukaemia-specific because no changes in GrB or cytokine responses were observed after stimulation with third-party allogeneic blasts. Moreover, the development of anti-leukaemic immune responses did not appear only to be a function of immunological recovery after allogeneic transplantation because among PBMC collected from recipients of

CMV-seropositive donor stem cells, no significant differences were found in IFNγ ELISPOT responses between pre- and post-immunisation samples, which had been stimulated with the potent pp65 CMV antigen.

Patient No.	Type of leukaemia	Lymphocytic infiltrate at immunisation site	Leukaemia-specific IgG response	Leukaemia-specific cellular immune response (ELISPOT)		
				GrB^+	$IFN\gamma^+$	$IL\text{-}5^+$
2	AML	+++§*	-	+	+	+
3	ALL	+++§	-	+	-	-
4	ALL	+++§	-	+	+	+
6	ALL	++	+	-	-	-
7	ALL	+++§	+	-	+	+
8	AML	+	-	-	-	-
9	AML	+*	-	+	+	-
10	AML	+*	NE	+	-	-

Table III: Local and systemic anti-leukaemia immune responses

The major local and systemic immune responses to immunisation with the leukaemia vaccine are listed for immunologically evaluable patients. NE, not evaluable; Post-immunisation lymphocytic skin infiltrate: +, <10 cells per high power field (hpf); ++, 10-50/hfp; +++, >50hpf.
*the infiltrate was predominantly $CD8^+$
§post-immunisation skin infiltrate increased in one week from ++ to +++

5.4.20 Local and systemic anti-leukaemia immune responses correlate

The data presented in Table III suggest an association of more marked local cellular responses in four patients with systemic leukaemia-specific cellular immune responses. Of particular interest, a predominant $CD8^+$ infiltrate at the immunisation site was associated with anti-leukaemic GrB and IFNγ responses. Only two patients developed mild erythema at the subsequently biopsied immunisation sites, which suggests that lymphocytic skin infiltrates at the immunisation sites were not part of a classic delayed type hypersensitivity reaction. In addition, leukaemia cells administered in the vaccine were no longer evident at the immunisation site, which indicated that they had been removed. It is possible that subcutaneous immunisation with adenovirally transduced fibroblasts or the fibroblasts themselves explain the non-specific local and systemic immune effects, which were observed post-immunisation. Indeed, data presented by Dotti *et al*. (2001) indicate that adenoviral transduction itself had immunomodulatory effects on human monocyte-derived DC *in vitro* because DC up-regulation of CD86 was observed. However, non-specific local cellular infiltrates are unlikely to have generated leukaemia-specific immune responses. Local and systemic anti-leukaemia immune responses appeared to correlate (Table III), and were induced after clearance of the dead leukaemic cells in the vaccine, which suggests the following hypothesis in the absence of a significant inflammatory response at the immunisation site. Minor local cellular infiltrates indicate initial recruitment of APC, which participated in clearance of dead leukaemic cells to the draining lymph nodes, and then recirculation and arrest of leukaemia-specific effector cells at the immunisation site where APC remain to present leukaemia-specific antigens.

Moghaddami M et al., 2001

Moghaddami M, Cohen P, Stapleton AMF, Brown MP. “CD40 is not detected on prostate cancer cells by immunohistologic techniques.” Urology 57, 573-578, 2001.

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CHAPTER 6

CONCLUSIONS

6.1 CD40L gene replacement corrects CD40L deficiency and induces thymic malignancy

6.1.1 Prerequisites for successful gene therapy of primary immunodeficiency diseases

Gene therapy of primary immunodeficiency diseases is most likely to be successful if transgenic expression is correctly regulated and/or it provides a selective growth advantage to the corrected cells. Gene therapy was first applied successfully to severe combined immunodeficiency (SCID), which is the most severe primary immunodeficiency disease. SCID is fatal in infancy unless the child is isolated in an extremely expensive 'germ-free' environment, which in the case of a child with X-linked SCID gave rise to the media description of 'bubble-boy' disease. Nonetheless, the cell therapy of allogeneic bone marrow transplantation (alloBMT) also transfers perfectly regulated genes to SCID patients. SCID patients were among the first to be cured by alloBMT [531] and, until the advent of gene therapy, alloBMT provided the only curative treatment for SCID.

Bone marrow cells from major histocompatibility complex (MHC)-matched sibling donors have been used to transplant SCID patients for over 35 years. For about 25 years, it has been possible also to treat SCID patients using T cell-depleted bone marrow cells from haploidentical parents without the requirement for pre-transplant myeloablative conditioning or graft *versus* host disease (GvHD) prophylaxis. In recipients of T cell-depleted haploidentical marrow, donor-derived T cells were apparent in the circulation three to four months post-transplant and 94% of recipients achieved normal T cell function. However, donor-derived B cells were less commonly seen, and 63% of patients continued to require intravenous immunoglobulin (IVIG) replacement therapy [532].

More detailed analysis of thymus-dependent T cell reconstitution in SCID recipients of alloBMT was performed. The detection in peripheral T cells of extra-chromosomal DNA excision circles or "thymic excision circles", which are formed during V(D)J recombination of TCR genes, serves as evidence of T cell export from the thymus. Thymic excision circles were detected in 88% of SCID recipients within three to six weeks post-transplant, and peaked at one to two years before declining to low levels within 14 years, which contrasts with the gradual decline observed in normal subjects from birth to 80 years of age [533]. Thus, while normal T cell immune function may be restored, its longevity may be less than that of the normal lifespan, and at least a 20-year observation period is required to properly assess outcomes of therapy [534]. Questions remain about whether the hypoplastic thymic tissue of SCID patients is adequate to the task of T cell reconstitution, and whether the SCID patients have actually engrafted with multipotential stem cells rather than more mature-stage common lymphoid progenitors or pre-T cells. Moreover, these studies of the long-term outcomes of SCID patients treated with stem-cell transplants provide the earliest indication of what may be required for the successful gene therapy of SCID [534].

SCID is a favoured candidate disease for gene therapy because both the use of alloBMT and cases of genetic reversion indicate that T cells efficiently repopulate a SCID host. Spontaneous reversion of the genetic mutations causing common-gamma chain (γc) and adenosine deaminase (ADA) deficiencies produces sustained although partial correction of these immunodeficiencies indicating that a selective growth advantage for T lymphocyte precursors can produce long-lived progeny [535] [536]. This rationale drove the pioneering gene therapy of French Anderson and his colleagues. ADA deficiency, which accounts for 15–20% of patients with SCID, was the first immunodeficiency for which a genetic aetiology was

identified [534]. In 1990, retrovirally mediated transfer of the ADA gene to autologous lymphocytes of ADA-deficient patients was the first successful demonstration of gene therapy [537]. Although Alain Fischer and colleagues were more successful in their retrovirally mediated correction of the common-gamma chain (γc) deficiency of XSCID, retroviral insertion into an oncogene spectacularly induced leukaemia in the youngest recipients of this treatment [534] (see Section 1.4.2.3).

The biological rationale for the genetic correction of γc-related signalling defects has been provided by detailed studies of gene-targeted mice. Jak3 is required downstream of γc to transduce signalling from the γc-dependent cytokines of IL-2, IL-4, IL-7, IL-9 and IL-15. The development of T cells, but not B or NK cells, is abrogated in mice in which the gene for the α chain of the IL-7 receptor (IL-7Rα) is disrupted [538]. Similarly, disrupting the genes for γc or Jak3 further downstream in this signalling pathway causes T cell development to fail. Hence, restoration of IL-7 signalling by γc or Jak3 gene replacement enables dramatic expansion of the thymus and the export of mature T cells, which function in the periphery because other γc-dependent cytokines such as IL-2, IL-4 and IL-15 can still signal.

In experimental murine models, a selective growth advantage has been demonstrated for gene-corrected immune cells in the case of SCID due to deficiencies of the genes for γc [539] or Jak3 [540], and for X-linked agammaglobulinaemia (XLA) using the equivalent murine *xid* model [541]. For example, the genetic correction of Jak3 deficiency in mice was first attempted using a retroviral vector using the Moloney murine leukaemia virus (MoMuLV) long terminal repeat (LTR) promoter to drive Jak3 expression. Jak3-deficient bone marrow cells were transduced with the Jak3-expressing retrovector and used to transplant irradiated Jak3-deficient mice. No evidence of immune reconstitution was found in these transplanted mice (Brown MP and Nosaka T, unpublished data). It had earlier been observed that retrovectors with the MoMuLV backbone did not transduce haemopoietic progenitor cells efficiently whereas retrovectors with the myeloproliferative sarcoma virus (MPSV) backbone had a broader host expression range and a LTR that was highly active in murine thymus [542]. Subsequently, irradiated Jak3-deficient mice were reconstituted with bone marrow cells, which had been retrovirally transduced with a Jak3-expressing MPSV retrovector. Here, it was evident that this method conferred a marked selective growth advantage upon genetically corrected T cells restoring parameters of immune function [540] so that gene-corrected mice resisted influenza virus infection to the same extent as Jak3-deficient mice transplanted with wild type bone marrow [543]. Similarly, a selective growth advantage for gene-corrected precursor cells with correction of the immunodeficiency was obtained in Jak3-deficient mice after transplantation with retrovirally transduced liver haemopoietic stem cells without prior myeloablation [544].

6.1.2 Requirements for successful gene therapy of inherited CD40 ligand deficiency

In contrast, there is no failure of T cell development in CD40L deficiency, therefore, no selective growth advantage would be expected from genetic correction. CD40L deficiency does not impair T cell survival or function, which is confirmed by the clinical observation that random inactivation of the X chromosome, on which the CD40L gene is encoded, still occurs in female carriers of the trait [545] [546]. Nevertheless, lack of a selective growth advantage need not hinder successful gene therapy for CD40 ligand deficiency. Random X inactivation to the extent that 30% of a carrier's T cells express wild type CD40L was compatible with normal immune function. Moreover, it was not until random X inactivation was skewed to the extent that only 5% of T cells expressed wild type CD40L that a carrier exhibited mild hyperIgM (HIGM) syndrome [547]. Therefore, these observations establish a threshold level of at least 5% CD40L gene-modified T cells before full immune reconstitution can be expected in CD40L-deficient patients.

Another potential obstacle to successful gene therapy for CD40 ligand deficiency is dominant negative or interfering effects of mutant CD40L proteins. Like other members of the TNF family, such as TNF and lymphotoxin-α, CD40L exerts its ligand function as a trimer [548].

However, many CD40L gene mutations produce both full-length and truncated mutant proteins that can associate with wild type CD40L on the cell surface of co-transfected COS cells *in vitro*. Given the abnormal clinical phenotype of the patients from whom the mutant CD40L cDNA was derived, these data suggest that the association between wild type and mutant CD40L proteins may produce inefficient cross-linking of CD40 [252]. In particular, point mutations in CD40L may result in reduced surface oligomerisation of CD40L, which is then insufficient to trigger productive signalling *via* CD40 [244]. Consequently, the therapeutic efficacy of wild type CD40L gene transfer into a cell making mutant CD40L protein may be reduced if wild type CD40L protein oligomerises with mutant CD40L protein into poorly or non-functioning trimers.

It also follows that dominant negative effects may represent a more important obstacle to successful gene therapy in the absence of a selective growth advantage for genetically corrected cells. If a selective growth advantage does apply to genetically corrected cells then it may compensate for the expected low levels of retrovirally mediated gene transfer. However, if genetic correction does not confer a selective growth advantage then therapeutic benefit will depend on low-level gene transfer efficiency approximating the 5% threshold level for CD40L expression, which is associated with relative immunocompetence.

Other than treatment of opportunistic infections, the only available and potentially curative approach to treatment of HIGM1 is alloBMT, which must be performed before significant liver damage occurs unless a liver transplant is also performed [287]. The severity of the combined humoral and cellular immunodeficiency of HIGM1, which approaches that of SCID, is sufficient to warrant consideration of alloBMT and to develop an optimal gene therapeutic approach [549].

6.1.3 The cause of thymic malignancy in CD40L gene therapy remains unknown

Although the cause of the thymic T-lymphoproliferative disease in CD40L gene-corrected mice remains unknown (Brown MP *et al*., 1998), a study showed that transgenic expression of CD40L in thymocytes produced disruption of normal thymic architecture [550] and at least hints at conditions that may have been formative for thymic malignancy. Similar to the thymic pathology of these transgenic mice, pre-lymphomatous thymic lesions showed densely stained collections of $CD3^+$ cells that co-localised especially at the corticomedullary junction with $CD40^+$ and $CD86^+$ thymic DC and macrophages (Brown MP *et al*., 1998).

CD40L has been detected in normal thymus mainly on the $CD4^+$ $CD8^-$ subset of thymocytes, which are believed to accumulate within the CD40-rich medullary compartment during their maturation into $CD4^+$ T cells [111]. Thymic CD40L/CD40 interactions may enable TCR-mediated negative selection and so shape the T cell repertoire *via* thymocyte deletion [551]. Consistent with this hypothesis, transgenic overexpression of CD40L dramatically depleted thymocytes [111]. Conversely, defective negative thymic selection in CD40L-deficient mice [551] may allow the survival of thymocyte clones, which could subsequently act as a substrate for inappropriate stimulation by transgenically expressed CD40L protein.

Brown *et al*. (1998) found that administration of CD40L gene-modified thymic cells to CD40L-deficient mice also resulted in thymic lymphoproliferative disease. Although transplants of thymic cells transduced with the control retrovector were not performed in this study (Brown MP *et al*., 1998), transplantation of three out of 30 SCID patients with cultured allogeneic thymic epithelial cells resulted in fatal B cell lymphoma, in which, however, Epstein-Barr virus infection could not be excluded [552]. However, in a separate study, of five athymic patients with DiGeorge syndrome who were transplanted with cultured normal thymic cells, two survived with normal T cell function. The other three died from infections or other abnormalities but not from transplant-related events or tumours [553].

Although secondary transplantation of thymic lymphomas was performed in CD40L-deficient hosts, secondary transplantation in wild type hosts may have given additional information about the immunopathogenesis of the malignancy (Brown MP *et al*., 1998). Since the tumours

were clearly not rejected in CD40L-deficient hosts then rejection in wild type hosts would have indicated that the tumours remained immunogenic and, therefore, IFNγ-sensitive [228].

Finally, although deregulated transgenic expression of CD40L may have drastic consequences (Brown MP *et al*., 1998) (see Section 6.1.4), only one other report indicates that constitutive CD40L expression *in vivo* results in malignancy [554]. Even though the MoMuLV LTR promoter expresses poorly in the thymus [542] (Brown MP and Nosaka T, unpublished data), it was evident that the CD40L transgene was expressed in malignant thymocytes of CD40L-deficient mice (Brown MP *et al*., 1998). This finding would suggest that CD40L expression was more than an epiphenomenon, particularly given the susceptibility of the thymus to oncogenic insults. In particular, thymus has a highly organised microenvironment for generating a non-self-reactive T cell repertoire that maximises the host's chances of survival in a hostile world of potential pathogens [555], and this microenvironment is characterised by very high rates of both cell proliferation and cell death [556].

For example, thymic lymphoma is reliably induced in C57Bl/Ka mice using radiation. That radiation perturbed the thymic microenvironment to de-repress control of 'pre-leukaemic' thymocytes was suggested because either allogeneic bone marrow transplantation or treatment with IFNγ or TNF prevented radiation-induced lymphoma formation [557]. Although irradiated recipients of bone marrow transduced with the control retrovector did not develop thymic lymphoproliferative disease (Brown MP *et al*., 1998), it is possible that radiation may have acted as a lymphomagenic co-factor given that deregulated CD40L expression is known to seriously perturb thymic organisation [550] [111]. Hence, it would not perhaps be surprising to learn that persistent, low-level and deregulated expression of CD40L in the sensitive and abnormal microenvironment of the CD40L-deficient thymus would predispose to malignant transformation (Brown MP *et al*., 1998).

6.1.4 Deleterious consequences of deregulated transgenic expression of CD40 ligand

Transgenic expression of CD40L *in vivo* produces a variety of mainly autoimmune pathologies. The administration of a CD40L-expressing adenovector to the liver represents a model of human fulminant hepatic failure in mice. In this model, hepatocellular apoptosis depends on CD40L/CD40 interactions, and the hepatic damage is T cell-mediated and depends on CD40L/CD40 interactions [558].

Transgenic mice in which CD40L was expressed on β-cells of the pancreatic islets under control of the rat insulin promoter (RIP) develop insulitis and diabetes, which depends on the presence of T cells and B cells. Since activated DC were found only in association with transgenic islets containing CD40L-expressing β-cells and in pancreatic draining lymph nodes, it was suggested that transgenic CD40L induced lymphocyte-mediated destruction of the islets after *in situ* activation of islet DC [559]. Transgenic mice with basal keratinocyte-directed expression of CD40L spontaneously developed autoimmune dermatitis, which was ascribed to CD40L-mediated activation of Langerhans cells resulting in keratinocyte-specific $CD8^+$ T cell responses. Transgenic mice had massive lymphadenopathy containing increased numbers of B cells and DC, which probably migrated from the dermis after CD40 ligation. Markers of systemic autoimmunity were present and included anti-nuclear antibodies and anti-double stranded (ds)DNA antibodies, renal immunoglobulin deposits, proteinuria, and pulmonary fibrosis [560].

Evidence of systemic autoimmunity was also obtained after repeated immunisation of mice with a CD40L-expressing cellular vaccine. Fibroblast cell lines that constitutively expressed CD40L or IL-2 were mixed with monocyte-derived DC, which had been loaded with peptides acid-eluted from A20 B-leukaemia cells, were given by three consecutive subcutaneous injections to syngeneic BALB/c mice. The immunised mice were then challenged subcutaneously with live A20 cells, and all mice eradicated the leukaemia. However, 60 days after the A20 challenge, all immunised mice developed a $CD8^+$ CTL-dependent illness, which was characterized by cachexia, fur loss, gross hepatosplenomegaly, ascites, proteinuria, lymphopenia, anaemia and thrombocytopenia. The mice exhibited peripheral blood

cytotoxicity against the fibroblast cell line, and together these findings described a systemic autoimmune disease that resembled GvHD [561].

The proximal Lck promoter was used to direct transgenic CD40L expression in murine thymocytes and peripheral T cells. The Lck promoter functions in a dose-dependent manner, and the observed steep decline in thymic cellularity depended on CD40L transgene copy number. Similarly, the loss of thymic cortical epithelium and the retention of a CD40-rich medullary epithelium directly depended on transgene copy number. Radiation bone marrow chimera experiments showed that CD40L overexpression significantly reduced T cell development in a normal thymic environment. Prominent inflammatory changes with mononuclear cell infiltrates developed in lung, liver, pancreas, and gastrointestinal tract. Mice with the highest transgene copy numbers developed a lethal wasting disease with morphological features of chronic inflammatory bowel disease. In the bowel, an excess of $CD40L^+$ cells, $CD40^+$ B cells, macrophages and DC were found. Other abnormalities included a membranous glomerulopathy with IgG deposition, and lymphadenopathy marked by loss of normal follicular architecture, B cell hyperplasia, and clusters of activated T cells and B cells [111].

Transgenic mice were created that expressed CD40L on B cells. Some transgenic mice older than 12 months spontaneously produced anti-nuclear antibodies reactive with dsDNA and histones, and approximately half of the mice developed glomerulonephritis with immune-complex deposition. Thus, this lupus-like disease depended on unidentified environmental factors, and suggested that CD40L expression observed on the B cells of SLE patients may be contribute to the pathogenesis of SLE [562]. In some cases, in particular, in which transgenic CD40L expression caused autoimmunity and/or inflammation, it was proven formally [558 563] that the effects of transgenic CD40L expression resulted from CD40L/CD40 interactions directly activating the immune system, which is how the anti-tumour effects of transgenic CD40L expression have been mediated (Chapter 5).

In a further study of deregulated transgenic CD40L expression, both ubiquitous and lineage-restricted expression of CD40L was created in transgenic mice. In the first transgenic construct, the phosphoglycerokinase promoter directed ubiquitous expression of CD40L but post-natal lethality of all transgenic lines ensued. In the second transgenic construct, the CD40L gene was placed under the control of the LTR of human lymphotropic virus-1 (HTLV1) to provide constitutive expression mainly in mature $CD4^+$ T cells. Expression of the transgene was detected in lymph nodes, spleen, and thymus. One-third (35/105) of these mice developed clinically palpable inguinal masses from six to 12 months of age. Other lymphadenopathy was also present and histological examination revealed atypical lymphoid hyperplasia of mature class-switched and $CD40^+$ B cells. Three mice developed a Burkitt-like lymphoma with clonal rearrangement of the immunoglobulin heavy chain gene. The atypical lymphoid cells had a centroblastic appearance, which would be consistent with the hypothesis that deregulated CD40L transgene expression inappropriately activated and drove proliferation of germinal centre B cells. Eventually, this expanded polyclonal population of B cells would suffer additional somatic mutations and result in the occurrence of tumorigenic B cell clones. In support of this hypothesis, expression of the CD40L transgene was not detected among the lymphoma cells. Other secondary lymphoid tissues were histologically normal and there was no clinical evidence of autoimmunity [554]. Nonetheless, these findings would suggest that the immunopathogenesis of malignancy in this transgenic model differs significantly from that described in the thymic malignancy affecting CD40L-deficient mice given CD40L gene therapy (Brown MP *et al*., 1998).

In the HTLV1 LTR-CD40L transgenic model [554], high-level constitutive expression of CD40L appears to have driven uncontrolled proliferation of lymph node B cells. An interesting parallel is suggested by the observation that oncogenic latent membrane protein-1 (LMP1) of EBV mimics constitutively active CD40 in its ligand-independent proliferative and transforming effects on EBV-infected B cells [564 565]. Moreover, EBV infection of normal B cells and T cells induces their expression of CD40L and CD40, respectively. These EBV-

induced changes in CD40L and CD40 expression were shown to contribute to the transforming potential of EBV. Unlike normal B cells, however, EBV does not transform HIGM1 B cells because EBV fails to induce expression of the genetically absent CD40L even though these B cells are infected with EBV [566].

Common to these transgenic studies is high-level constitutive CD40L expression that, however, produced different effects. The cell type in which the transgene is predominantly expressed or the kind of promoter used to drive transgene expression may explain some of the differences. For instance, both Lck and HTLV1 promoters result in T cell expression of the CD40L transgene. Whereas Lck-driven CD40L expression produced thymocyte depletion and chronic inflammatory disease [111], HTLV1-driven CD40L expression produced B cell hyperplasia and neoplasia [554]. Perhaps, stage-specific or subset-specific differences in T cell expression result in different trafficking behaviours. In HTLV1-CD40L transgenic mice, perhaps only lymph node B cells were affected because the trafficking of peripheral T cells, which were efficiently targeted by the HTLV1 promoter, was confined to lymph nodes. In contrast, the mCD40L-expessing MoMuLV retrovector may have only efficiently targeted the lymphoid progenitors that were required to migrate *via* the thymus (Brown MP *et al*., 1998).

6.1.5 Normally regulated transgenic CD40 ligand expression avoids thymic malignancy

A lentiviral vector was used to deliver an mRNA *trans*-splicer, which enables two different pre-mRNA species to be joined using the cell's splicing apparatus. The method allows *in situ* correction of a mutant mRNA, which is still regulated by the cell's endogenous control elements thus maintaining natural regulation of the transgene. Normal murine CD40L pre-mRNA contains exons 1-5 whereas CD40L pre-mRNA from genetically engineered CD40L-deficient mice lacks exons 3 and 4. A vector-encoded hybridisation domain, which is anti-sense to a short segment of intron 1 of the mutant CD40L pre-mRNA, allows the vector-encoded *trans*-splicer sequences to hybridise to the target pre-mRNA and so connect exon 1 of target pre-mRNA with exons 2-5 of the *trans*-splicer transcript to yield a corrected CD40L mRNA within the targeted cell [567].

Vector-transduced bone marrow cells from CD40L-deficient mice were transplanted into lethally irradiated CD40L-deficient hosts. Marrow transduction efficiency using the lentiviral vector was estimated using a comparable vector that expressed enhanced green fluorescent protein (EGFP) and revealed that 20% of peripheral T cells were $EGFP^+$ five weeks post-transplant. However, quantitative RT-PCR showed that an average 1.2% of total CD40L mRNA was in the *trans*-spliced form 10 weeks post-transplant. Sequencing of the RT-PCR products of *trans*-spliced CD40L showed that *trans*-splicing had occurred correctly *in vivo*. Sixteen hours after *ex vivo* activation, 67% and 5% of splenic $CD4^+$ T cells obtained from wild type and CD40L gene-corrected mice, respectively, were $CD40L^+$; whereas <1% of negative control $CD4^+$ T cells were $CD40L^+$. Together, these data indicated that both transduction efficiency and *trans*-splicing efficiency were relatively low. Transduction of primitive haemopoietic cells was confirmed by successful secondary transplantation of CD40L-deficient mice. In secondary transplant recipients, the average number of *trans*-splicer gene copies per splenic T cell was 0.27, which demonstrated that long-term repopulating cells had expanded the number of gene-corrected cells. Normally regulated expression of *trans*-spliced CD40L was demonstrated in activated $CD4^+$ T cells of primary as well as secondary transplant recipients. The kinetics of CD40L expression among gene-corrected splenic $CD4^+$ T cells was similar to that of wild type cells although it was not as prolonged (i.e. <40 hours). Other haemopoietic lineages did not express the CD40L transgene [567].

Partial post-immunisation isotype switching to IgG was confirmed in gene-corrected CD40L-deficient mice. These mice also resisted infection with *Pneumocystis carinii* showing attenuated pulmonary inflammation commensurate with a reduced parasite burden. None of 26 gene-corrected mice developed gross or microscopic evidence of lymphoproliferative disease 12 months after transplantation. Despite the low efficiency of *trans*-splicing CD40L therapy, partial rescue of the phenotype was obtained thus again indicating the potency of the molecular action of CD40L (Brown MP *et al*., 1998). This gene therapy technique ensures

that the amount of corrected mRNA produced is normally regulated because it is necessarily proportionate to the total amount of mutant mRNA produced under the same conditions [567]. This technique also has the advantage of overcoming dominant interfering effects of some CD40L mutants by correcting the pre-mRNA before it is translated to a mutant protein product.

The continued growth of large government and privately funded cord blood banks that would allow matched unrelated donor stem cell transplants [568] together with the development of improved methods for both pre-transplant sub-myeloablative conditioning [569] [570] and post-transplant GvHD prophylaxis may provide an acceptable and feasible means for the immune reconstitution of HIGM patients [286]. First, this method would circumvent the need for a gene therapy approach in which perfect regulation of a CD40L transgene is required. Second, the demand for limited and expensive resources would tend to be balanced by the low frequency of HIGM and its associated propensity to arise *de novo*.

However, an HIV-based lentiviral vector was shown recently to be safe and effective in HIV-infected patients [52]. Hence, lentiviral transfer of a CD40L *trans*-splicer transgene offers the realistic prospect of effective and safe treatment for HIGM1 patients particularly since many of the known CD40L gene mutations are located in exons 2-5 and are clustered in the regions encoding either the CD40 binding domain or oligomerisation interfaces [571]. Furthermore, this study indicates that cell therapy is now not the only approach to the relatively safe supply of tightly regulated CD40L *in vivo*.

6.1.6 Other approaches to therapy of CD40L deficiency

A phase I clinical study of recombinant human CD40L trimer (rhuCD40L) given subcutaneously was conducted in 32 patients with advanced malignancies. One partial response and one complete remission were achieved [572]. However, systemic CD40L administration was limited by serious hepatic toxicity because the maximum tolerated dose of rhuCD40L was reached at 0.1mg/kg/day every four weeks based on transient grade 3-4 elevations of hepatic transaminases. The dose-dependent ALT elevations peaked six days after administration and then normalised within two weeks. Although a direct cytotoxic effect of CD40L on $CD40^+$ hepatocytes was proposed as the cause of the 'transaminitis' [572], the murine model of fulminant hepatic failure induced by adenoviral expression of CD40L suggests rather that the hepatic damage was immune-mediated [558]. Hence, CD40L replacement therapy using repeated administration of exogenous doses of rhuCD40L may be neither safe nor feasible for treatment of CD40L deficiency.

6.2 CD40 ligand gene augmentation promotes RSV immunity

6.2.1 Immunopathogenesis of RSV infection

The immunology of RSV infection is highly unusual and its immunopathogenesis is still not well understood [573]. Given that RSV infection is virtually universal in infancy, what are the relative contributions of the host immune response and of RSV itself to the poor immunity that develops after natural RSV infection? Does the immature immune system of the neonate and infant contribute significantly to limited anti-RSV immune responses?

6.2.1.1 Viral proteins modulate host immune responses to infection.

RSV contains a single-stranded negative RNA genome and is a member of the *Pneumovirinae* subfamily of the *Paramyxoviridae* family that includes the parainfluenza viruses together with the measles and mumps viruses. The viral genome encodes 11 proteins, nine of which are structural proteins and surface glycoproteins and two of which encode proteins responsible for the replicative machinery of the virus [288]. The viral capsid glycoproteins, the attachment G protein and the fusion F protein, are the major RSV protein antigens that induce neutralising antibodies. Whereas the F protein has an invariant structure, the structure of the G protein varies more widely among the strains and subtypes of RSV, thus limiting an effective immune response and potentially facilitating frequent re-infections. The F protein inserts into the membrane of infected cells where it facilitates intercellular fusion and syncytial formation,

which promotes covert spread of the replicating virus [288]. Virally encoded proteins and the innate immune response together are the most important determinants of the outcome of acute RSV infection and its long-term sequelae of recurrent wheezing [574] and perhaps, asthma.

6.2.1.2 The host immune response to RSV infection produces pulmonary disease

RSV predominantly infects ciliated respiratory epithelium. Infection is initiated with attachment and fusion of the virus to the cell membrane followed by its entry into the cell. Then the virus uncoats itself to enable replication of the viral genome, assembly of new virions, and lysis of the infected cell with release of the virions into the extracellular environment. After epithelial cell infection, expression of RSV early genes promotes viral replication by counter-acting the potent anti-viral activity of the type I IFNα/β interferons and the type III IFNλ interferons, which are promptly secreted after RSV infection of epithelial cells and macrophages [575]. IFNβ also up-regulates epithelial cell expression of MHC class I molecules [574].

In naïve individuals, the infection begins in the upper respiratory tract with coryzal symptoms then progresses to infection of the lower respiratory tract where it may cause bronchiolitis and pneumonia. Infection produces lysis of infected cells and consequently impaired function of the mucociliary elevator. Sloughed epithelial cells, accumulating mucous secretions and the inflammatory cell infiltrate, which is recruited by release of chemokines and inflammatory mediators from the RSV-infected respiratory epithelium, together contribute to the 'catarrhal congestion', which was the first clinical term to describe RSV infection [288]. RSV is particularly associated with pulmonary neutrophilia and eosinophilia and the single-stranded RNA of RSV may have a role in reducing granulocyte apoptosis by binding Toll-like receptor (TLR) 8 [238].

6.2.1.3 Innate immune responses to RSV infection guide the adaptive immune response

Innate immune defences against RSV are present in respiratory secretions and include lactoferrin, and collectins such as surfactant protein-A, which binds the heavily glycosylated F protein to opsonise the virus for disposal by phagocytes [84]. Pattern recognition receptors such as TLR are also particularly important. RSV-F protein binds to the TLR4 and CD14 co-receptor complex and initiates innate immunity to the virus [576]. RSV-infected TLR4-deficient mice displayed defects in NK cell function, impaired IL-12 expression, and impaired virus clearance compared to mice expressing TLR4 [577 576]. However, a subsequent study of the relative effects of IL-12 hyporesponsiveness in the genetic background of the TLR4-deficient strains raised questions about the precise contribution of TLR4-mediated signalling to RSV clearance [578].

Nasal wash samples from children hospitalised with acute infection showed that RSV mobilised both conventional DC (cDC) and plasmacytoid DC (PDC) to the nasal mucosa. The DC contained RSV-F protein and DC numbers significantly correlated with RSV load. Even higher numbers of both DC subsets were found in respiratory secretions weeks after the acute infection had subsided [579]. Similarly, RSV infection increased the number of cDC and PDC in lung and pulmonary draining lymph nodes and the increased PDC numbers lasted until 30 days after infection. Depletion of PDC resulted in decreased RSV clearance and exacerbated immune-mediated pathology caused by RSV infection, which suggested that PDC played a protective role during RSV infection by modulating local immune responses [580].

PDC sense pathogens *via* a complement of TLR molecules and produce IFNα, which has potent anti-viral effects. RSV-F protein enables the virus to enter the cytosol of DC and induce potent secretion of IFNα by PDC but not cDC. IFNα secretion by PDC occurred independently of known TLR including the ssRNA-specific TLR8 [581]. PDC are poorly immunogenic but migrate to the draining lymph nodes where they contribute to the induction of antigen-specific immunity. For example, as a 'third signal', IFNα stimulates $CD8^+$ CTL directly during cross-priming [173], and IFNα also exerts paracrine effects to mature cDC [512].

RSV suppresses T cell proliferation to both itself and other antigens. RSV was shown to suppress the proliferation of $CD4^+$ T cells *in vitro* to *Staphylococcus* enterotoxin B after RSV infection of moDC *via* DC production both of type I IFNα/β and type III IFNλ. This paradoxical *in vitro* observation of RSV-induced and interferon-dependent immunosuppression may be explained by interferon signalling that preferentially activates anti-proliferative effects rather than the expected anti-viral effects because of differences in context and timing. Hence, it was suggested that RSV modulates host gene expression to strike a fine balance between impairing the anti-viral properties of interferons and allowing their suppressive activity to persist. Consequently, this immunosuppressive activity may contribute to the phenomena of re-infection with RSV and may be mechanistically linked to Th2 immune deviation induced during primary RSV infection [582] [583].

Initial chemokine expression by RSV-infected epithelial cells results in the recruitment of NK cells within three days [574]. Then, as viral replication peaks at five days, T cells are recruited maximally by seven days post-infection, and antibody responses develop by seven days post-infection [84]. Other innate immune cells drawn in by the chemokines include neutrophils, eosinophils, basophils, and macrophages. Established RSV infection is controlled primarily by the T cell response. Because of the difficulty of performing studies in young infants, studies in BALB/c mice indicate that both $CD4^+$ and $CD8^+$ T cells control infection with $CD8^+$ effector T cells playing the dominant role by inducing death of virally infected cells. In RSV pneumonia, in particular, while aiding viral clearance, the recruited immune cells also contribute significantly to the pathogenesis of the infection *via* inflammation and tissue damage [574]. Primary RSV infection in BALB/c mice induces a mixed Th1/Th2 cytokine response and pulmonary disease that is controlled by IFNγ secretion. In the absence of IFNγ during primary or secondary RSV infections, a predominant Th2 cytokine response results and is associated with aggravated pulmonary disease [84]. Moreover, consistent with this view, infants hospitalised with RSV pneumonia had reduced nasopharyngeal aspirate levels of IFNγ [84]. Moreover, infants who were infected with RSV and developed RSV-specific CTL and heightened production of IFNγ were less likely to suffer RSV-induced lower respiratory tract disease in their second year [584].

After the acute infection has resolved, a small pool of memory T cells is maintained together with B cells and antibodies that help to resist re-infection. Resistance to RSV infection in the upper airways is mediated by transitory mucosal secretion of IgA but neutralising IgG antibodies are most important [84]. The presence of neutralising antibody correlates with protection against RSV infection, and most neutralising antibodies are directed against the invariant RSV-F protein [288]. Full term infants carry maternal antibody, which reduces the incidence of RSV infection in the neonatal period [288]. After RSV infection of infants, serum and mucosal antibody titres are generally lower than in adults and wane in the several months post-infection and so may increase the susceptibility of infants to re-infection [84]. However, passive immunotherapy using high titre RSV immunoglobulin intravenous (RSV-IGIV), which is prepared from immune sera, reduces the incidence and severity of RSV infection in high-risk infants [288].

6.2.1.4 The RSV-G protein has a significant immunomodulatory role

Unlike many other respiratory virus infections, RSV infection produces severe lower respiratory tract infection early and late in life, and re-infections with the same or different strains of the virus are common. Thus, it is apparent that RSV itself modulates the immune response to promote viral replication. In particular, upon re-exposure to the virus, the RSV-G protein is known to sensitise mice to enhanced pulmonary disease, which is marked by severe weight loss, pulmonary eosinophilia and a type 2 cytokine response. Adoptive transfer experiments suggest that it is T cells specific for the G protein that cause the enhanced pulmonary disease because RSV-F-specific T cells cause minimal disease. Both murine and clinical studies have associated the type 2 cytokine, IL-4, with enhanced pulmonary disease.

On the other hand, in keeping with the Th1/Th2 paradigm of immune responses to infection, the type 1 cytokines, IL-12 and IFNγ, oppose the actions of IL-4 [84].

RSV infection induces respiratory epithelial cell expression of the chemokine, CX3CL1 (fractalkine), and also expression of its receptor, CX3CR1, on macrophages, NK and Th1 cells, which exert anti-viral immunity. Fractalkine exists as both secreted and membrane-bound forms and is unique in having both chemokine and cell adhesion activities. The secreted form creates a gradient to attract $CX3CR1^+$ cells to the site of infection where the membrane-bound form promotes adhesion of $CX3CR1^+$ cells to infected cells. The RSV-G protein, which also exists as both secreted and membrane-bound forms, contains a CX3C chemokine motif and mimics the activity of fractalkine. RSV-infected cells also secrete G protein, which disrupts the fractalkine gradient and, in its membrane-bound form, G protein behaves as a fractalkine decoy to attenuate binding of highly expressing $CX3CR1^+$ cells to infected epithelium. In contrast, Th2 cells have low levels of CX3CR1 and poor anti-viral activity. Therefore, it was hypothesised that G protein may act *via* CX3CR1 to alter the activation and/or trafficking of NK and Th1 cells and so favour a less potent anti-viral Th2 response [84]. Specifically, during RSV infection *in vivo*, RSV-G protein reduced the pulmonary frequency of RSV-specific MHC class I-restricted $IFN\gamma^+$ cells [585]. However, immunisation against RSV-G protein may interfere with its function and may, in part, restore normal immune responses to RSV (Harcourt JL *et al.*, 2003). RSV-G-specific antibodies, which are induced by natural infection or immunisation, are associated with inhibition both of RSV-G protein CX3C-CX3CR1 interactions and the chemotaxis of leukocytes toward RSV-G protein [586].

While a minor proportion of RSV-infected children may have wheezing and documented bronchial hyperreactivity that extends five years beyond the initial infection, no definite link has been made between RSV infection and the later development of atopic asthma. Prospective, randomised studies, which include an analysis of the cytokine profile of bronchoalveolar lavage (BAL) fluid, would be required to answer this question [288]. Nonetheless, despite the lack of conclusive epidemiological evidence, studies of both murine models of RSV infection, and the cytokine and cellular content of BAL lavage fluid of RSV-infected infants suggest that bias toward type 2 immune responses in the immature immune system of the infant may predispose to allergic disease [288 575].

6.2.2 Immunological immaturity of the neonate

6.2.2.1 Neonatal mice later develop enhanced pulmonary disease after re-infection with RSV

BALB/c mice that were infected with RSV as one and seven day-old neonates developed severe illness during re-challenge unlike mice that were first infected at four or eight weeks of age. Although RSV-specific CTL were primed in neonatal mice, IFNγ mRNA and IFNγ-producing $CD4^+$ T cells were significantly reduced in lung during the primary RSV infection. In spite of this finding, pulmonary viral load was not increased during primary infection of neonates. These data indicated that the predisposition of neonates to enhanced pulmonary disease was immune-mediated because the primary response was not marked by a vigorous immune response or delayed viral clearance [583].

During re-infection, and in contrast to mice primed as adults, neonatally primed mice developed a greater pulmonary inflammatory infiltrate, which included neutrophilia, a marked eosinophilia, an increased proportion of $CD8^+$ T cells, and a reduced proportion of $CD4^+$ T cells. For mice first infected as neonates, the proportion of pulmonary $IFN\gamma^+$ $CD4^+$ T cells was significantly reduced, and the proportion of pulmonary $IL\text{-}4^+$ $CD4^+$ T cells was significantly increased after re-challenge. In contrast, mice first infected as adults displayed a reverse pattern of intracellular cytokine expression by $CD4^+$ T cells [583].

These data support the notion that natural RSV infection in the post-natal period *per se* is sufficient to enhance RSV disease and may result in subsequent recurrent wheeze. These data suggest that an RSV-induced bias toward Th2 memory responses may direct subsequent pulmonary responses to other antigens toward a more ‘allergic’ phenotype [583]. The source of

the Th2 bias in neonates is not clear but neonatal DC are deficient in IL-12 production. Neonatal moDC that were generated from cord blood expressed lower levels of MHC class II, CD80, and CD40. CD40 ligation resulted in a selective defect in IL-12 synthesis, which was corrected, however, by the addition of IFNγ and LPS. Consequently, neonatal moDC demonstrated impaired induction of IFNγ secretion by T cells unless IL-12 was supplied [587]. Additional T cell-related factors include DNA methylation changes in neonatal $CD4^+$ T cells that may explain reduced IFNγ secretion [588], and impaired induction of CD40L expression by neonatal $CD4^+$ T cells after activation (see Section 6.2.2.2).

6.2.2.2 Hyporesponsiveness of CD40L expression may predispose to RSV infection

CD40L expression by activated T cells is age-related [107]. CD40L expression was generally not inducible in cord blood T cells but could be triggered by activation of T cells from infants more than three weeks of age [108]. Attainment of the ability to express CD40L progressively increased during the first months of life, reached a plateau in the second decade, and was significantly correlated with the expression of the CD45RO antigen found on experienced T cells [589]. In addition, switching of cord-blood B cells to IgG or IgA in the presence of cytokines was reduced after stimulation with anti-CD40 mAb or soluble CD40L [590].

A factor that may contribute to the observed Th2 bias of the immature immune system is the reduced CD40L expression that follows activation of human neonatal $CD4^+$ T cells. The induction of CD40L expression by purified, naive ($CD45RA^{hi}$ $CD45RO^{lo}$) $CD4^+$ T cells, which were obtained from adult or cord blood, was compared after the T cells were activated with the calcium ionophore, ionomycin, or by triggering of the TCR with anti-CD3 mAb. In comparison with adult $CD4^+$ T cells, reduced cell-surface CD40L expression by neonatal $CD4^+$ T cells resulted from a reduction in both the total number of CD40L-expressing cells as well as the level of CD40L expression per cell, which suggested that the threshold for the induction of CD40L expression had been elevated in neonatal $CD4^+$ T cells. Similar results were found for expression of the early activation marker, CD69, which indicated that the impairment of neonatal $CD4^+$ T cell activation was generalised. Moreover, immobilised polyclonal anti-TCR Vβ antibodies together with an anti-CD28 mAb, which were used to simulate physiological APC-T cell interactions, also did not rescue CD40L expression by purified, naive neonatal $CD4^+$ T cells.

Reduced CD40L expression by neonatal $CD4^+$ T cells was likely to have functional significance. Neonatal $CD4^+$ T cells, which were activated with anti-CD3 and anti-CD28 mAb, failed to induce CD40-dependent B-cell line expression of CD54. Reduced CD40L protein was associated with reduced levels rather than reduced stability of CD40L mRNA. Interestingly, Northern blot analysis of the time course of CD40L expression after anti-CD3 mAb stimulation revealed that the second mRNA peak at six hours post-activation rather than the first mRNA peak at two hours post-activation was most deficient in neonatal T cells in comparison with adult T cells. In addition to reduced CD40L expression, a smaller than expected rise of intracellular free calcium was found after TCR-triggering of neonatal $CD4^+$ T cells. Increased intracellular calcium is required for calcineurin-dependent NFAT-mediated transcription of CD40L. Nonetheless, bypassing this block in calcium mobilisation by the use of ionomycin did not correct the defect in CD40L expression, which indicated that as yet unidentified factors, which act distally to calcium mobilisation, impaired CD40L gene transcription in neonatal $CD4^+$ T cells [591].

6.2.3 Aging is associated with more extensive RSV infection and type 2 cytokine responses

Intranasal RSV infection was compared in young (2-4 months old) and old (20-22 months old) BALB/c mice. Four to six days post-infection, in comparison with young mice, old mice had significantly higher virus titres in lung than in nose washes. Also, lung titres were higher in old mice than in young mice (P=0.07). Using an *in vitro* recall assay, old mice also had significantly lower RSV-specific and MHC class I-restricted cytolytic activity in spleen than young mice. In supernatants of unfractionated lymphocyte cultures in the same *in vitro* recall

assay, old mice had significantly lower levels of IFNγ (P=0.03), and higher levels of IL-4 than young mice (P=0.08) [592].

There is a hypothesis that ageing is associated with deviation toward a type 2 cytokine response, which may be responsible for the increased morbidity and mortality of infectious diseases in the elderly. However, little evidence was found to support this hypothesis. Peripheral blood mononuclear cells (PBMC) from 21-30 year-old individuals (n=26), 81 year-old individuals (n=15), and 100 year-old individuals (n=25) were analysed for cytokine production after polyclonal T cell activation with the protein kinase C (PKC) activator, PMA, and ionomycin. [593]. After *ex vivo* stimulation, the elderly displayed an increased proportion of both IFNγ- and IL-4-producing lymphocytes, which correlated with the increased proportion of antigen-experienced $FasL^+$ $CD45RO^+$ lymphocytes. Per cell production of cytokines was not affected by age. Some evidence for a shift in the balance between type 1 and type 2 cytokine-producing cells was found among the elderly. Centenarians had a significant reduction of $IFN\gamma^+$ $CD8^+$ T cells and IL-4-producing $CD8^+$ whereas 81 year-old individuals had significantly reduced supernatant levels of IFNγ compared with IL-4 after 48 hours stimulation. The clinical relevance of these findings is uncertain but the cytokine balance at the time of lymphocyte activation may determine subsequent lymphocyte differentiation. In conclusion, these data suggest that non-immune factors such as cardio-respiratory comorbidities may be important than immune factors in predisposing elderly individuals to extensive and serious RSV infection.

6.2.4 Respiratory syncytial virus vaccine options

Although a safe and effective respiratory syncytial virus (RSV) vaccine active in the first six months of life may reduce the severe disease burden of lower respiratory tract infection and hospitalisation, the presence of maternal antibody may reduce the ability of the infant to respond to a vaccine [594]. An alternative approach to prevent RSV disease in young infants is maternal immunisation [296]. Immunisation of pregnant women with viral vaccines for polio, influenza, and rubella is safe for both mother and foetus [595]. IgG antibodies cross the placenta well during the third trimester and are also available to the infant in breast milk [596]. Protection against RSV has been demonstrated in babies born to mothers with high levels of neutralising RSV antibody and RSV-F-specific IgG correlates well with immunity [596]. A study of post-partum women immunised with purified RSV-F protein demonstrated that the vaccine was safe and immunogenic and resulted in the detection of RSV-specific antibody in breast milk [595]. However, a conundrum faces the RSV vaccine field. While maternal immunisation may protect susceptible young infants, passive maternal transfer of RSV-specific antibody may inhibit an infant's response to natural infection or active immunisation, particularly when young infants are least responsive to vaccines [596].

6.2.5 Transgenic CD40L expression may reverse reduced type 1 immune responses to RSV

RSV immunity depends on neutralising antibodies and cytolytic effector cells. CD40L orchestrates and amplifies the inflammatory microenvironment, which facilitates the induction and expansion of antigen-specific immune responses including effector activities. CD40 ligation of DC induces secretion of IL-12, which polarises T cell differentiation toward IFNγ-producing Th1 cells, which clear virus. CD40 ligation of DC also induces secretion of chemokines, which direct recruitment (i) of cells to secondary lymphoid organs to participate in the developing immune response, and (ii) of effector cells to sites of inflammation [597]. CD40 ligation of macrophages activates functions such as production of matrix metalloproteinases (MMP), reactive oxygen species (ROS) and pro-inflammatory cytokines such as TNF [269]. CD40 ligation of B cells is critical for the survival of B cells and thus their eventual differentiation into plasma cells and memory B cells [257].

Since CD40 ligation is a vital stimulus to the production of the type 1 polarising cytokines, IL-12 and IFNγ, would the incorporation of CD40L into RSV subunit vaccines polarise the anti-RSV immune response to a type 1 phenotype? Since CD40L is critical for the induction and maintenance of both humoral and cellular immunity, would the incorporation of CD40L

into RSV subunit vaccines augment both humoral and cellular immune responses to subsequent RSV challenge, and induce more effective and durable immunity?

Repeat infections with RSV occur despite a limited number of subtypes and a genome that is not prone to mutation unlike influenza. A confluence of factors appears to determine the limited immunity generated in response to RSV infection, which predisposes to re-infection throughout life.

(i) Viral factors include the ubiquitous and highly infectious nature of the pathogen, which leads to infection early in life, and the RSF-G protein, which induces a decreased RSV-specific type 1 pulmonary cytokine response.

(ii) Host factors include (a) the early age at first infection, which is marked by immunological immaturity and includes hyporesponsive DC and T cells with reduced CD40L expression after T cell activation, and (b) the reduced type 1 response to RSV associated with ageing.

These studies (Tripp RA *et al*., 2000; Harcourt JL *et al*., 2003) supported earlier data showing a mixed Th1/Th2 response without eosinophilia during primary RSV infection of BALB/c mice. Incorporation of transgenic CD40L expression either concurrently with primary RSV infection, or preceding the primary infection by four to eight months, had the practical consequence of accelerating pulmonary viral clearance, which was associated with evidence of a strong RSV-specific type 1 cytokine response. In particular, concurrent transgenic CD40L expression markedly augmented the pulmonary content of IFNγ, which has potent anti-viral effects. IFNγ is one of the most potent inducers of inducible nitric oxide (NO) synthase (iNOS), which is produced by many cell types including NK cells, DC, macrophages, and neutrophils [598]. If memory B cells are absent in HIGM patients, can transgenic expression of CD40L enhance B cell memory? We find some evidence to suggest that transgenic CD40L expression expanded the pool of antigen-specific B cells from which recall humoral immune responses are generated. Genetic immunisation did not itself increase the frequency of splenic antibody forming cells (AFC). However, incorporation of transgenic CD40L expression in the DNA vaccine, which contained both RSV-F and -G subunits, produced significantly higher frequencies of RSV-specific splenic IgG AFC 14 days after genetically immunised mice were challenged with live RSV four or eight months post-immunisation (Harcourt JL *et al*., 2003).

6.2.6 The prospects and hazards of transgenic CD40L expression in RSV vaccines

Human neonates dampen T cell expression of CD40L after T cell activation [591], and neonatal mice infected with RSV develop enhanced pulmonary disease and type 2 pulmonary cytokine responses upon re-challenge with RSV [583]. Together, these observations suggest that hyporesponsiveness of CD40L expression may contribute to the neonatal immunological immaturity that predisposes to severe RSV infection. Hence, assuming that neonatal murine T cells display similar hyporesponsiveness of CD40L expression after activation, experiments could be done to test hypotheses that transgenic CD40L expression at the time of the initial RSV infection (i) deviates the pattern of the subsequent pulmonary cytokine response to RSV re-infection toward a type 1 profile, and (ii) ameliorates RSV-related pulmonary disease. Given, the known effects of transgenic CD40L expression on immune development and function [550] [111] (Brown MP *et al*., 1998), careful and prolonged observation of experimental mice for the development of autoimmune and malignant phenomena would be required.

Data showing adverse outcomes for mice that were re-infected with RSV after a first RSV infection as neonates suggests that measures to delay RSV infection until later in infancy may allow the infant's immune system to prime for type 1 rather than type 2 cytokine responses after RSV infection [583]. Moreover, it seems that marginal improvements in the host immune response that would clear infections in the upper respiratory tract and prevent their passage to lower respiratory would also be beneficial. These considerations may favour the development of RSV immunisation strategies for pregnant and nursing mothers with the intention of boosting transfer of RSV-specific IgG *via* placenta and/or breast milk. However, the use of

CD40L as a molecular adjuvant remains potentially hazardous for mother, foetus or infant. Certainly, deregulated CD40L expression has been shown to induce systemic autoimmunity in experimental models. The danger may be particularly acute in the neonate and infant when the delicate and necessary process of thymic education to select lymphocytes reactive with foreign antigens, and to eliminate lymphocytes reactive with self antigens, may be perturbed and produce the devastating consequences of autoimmune disease.

6.3 CD40 ligand gene augmentation induces anti-tumour immunity

Since CD40 was first identified in 1984 as an antigen on human bladder cancer by the S2C6 murine mAb [250 253], the wheel has now turned full circle on its use as a target for the treatment of malignancy. After CD40 and CD40L were cloned in 1985 and 1992, respectively [93], the therapeutic potential of the CD40L/CD40 interaction has become more evident. The conditional nature of CD40 ligation, together with improved knowledge of CD40-related signalling events in diverse cell types including carcinoma, have indicated ways in which the CD40L/CD40 signalling axis may be manipulated as a therapeutic target [599 600].

The physiological expression of CD40L is distinctly limited in time and space. Within the confines of the lymphoid organs, which are organised to maximise interactions between rare cell types [601], the non-choreographed appearance of a potent activating signal such as CD40L may have devastating consequences (Brown MP *et al*., 1998). Conversely, in the disorganised tumour microenvironment, DC-mediated peripheral tolerance mechanisms that operate in normal tissues are subverted to create immunosuppression that is reinforced by ongoing low-level apoptosis in association with the elaboration of additional factors that facilitate tumour escape. Here, transgenic expression of CD40L would appear as a 'bolt from heaven'; its deregulated expression may be less hazardous in the setting of massive tumour-related immunosuppression so that there may be equipoise for the risk of autoimmunity, which may even be a desirable therapeutic objective [574].

6.3.1 The significance of CD40 expression by tumour cells

CD40 expression is confined to the basal layer of normal epithelium, which suggests that CD40 complements its well-characterised role in immune regulation with a role in host defence. Although CD40 is commonly expressed by malignant lymphoid cells, CD40 is expressed consistently on some carcinomas such as those of breast, ovary, nasopharynx, liver and bladder [602] but not on others (Moghaddami M *et al*., 2001) [603] for reasons that remain unclear.

CD40 stimulation has differential cell type-dependent effects, which include proliferation and differentiation, growth inhibition, apoptosis, and sensitisation to apoptotic stimuli such as cytotoxic drugs and death receptor ligands [524 603]. For example, CD40 ligation induces proliferation of normal B cells but growth arrest of malignant B cells. CD40 activation of primary epithelial cells *in vitro* inhibits growth, promotes differentiation, and induces the secretion of pro-inflammatory cytokines such as IL-6, IL-8, and TNF [604]. Growth retardation of breast and ovarian cancer cells occurs in response to treatment with sCD40L, which also promotes the endogenous production of other TNF superfamily cytotoxic ligands, TNF, FasL and TRAIL, with subsequent *in vitro* and *in vivo* induction of low-level apoptosis. Recently, CD40 has been discovered to have a novel role in the regulation of protein synthesis [524], which plays a necessary role in cancer cell survival and growth [605]. The initial laboratory observation was made that CD40 ligation of fibroblasts induced apoptosis if protein synthesis was also inhibited by cycloheximide [606]. This observation suggests that CD40 signalling triggers a regulatory circuit, which balances survival and death signals *via* control of the protein synthesis machinery and which is a feature of other TNFR superfamily members. That is, CD40 activation induces *de novo* synthesis of survival proteins that counteract the pro-apoptotic effects of CD40 activation [524]. Moreover, this study revealed a cancer vulnerability that may be exploited as a therapeutic target if the mechanisms of CD40-dependent survival of malignant cells could be understood and disrupted.

As indicated, recent *in vitro* studies of cancer cell lines suggest that CD40 ligation simultaneously activates pro-apoptotic and counteracting anti-apoptotic signalling pathways. CD40 activation of HeLa/CD40 cells activates the well-characterised PI3K/Akt survival pathway as well as the ERK pathway to converge on the regulation of the protein synthesis machinery and so enhance initiation and elongation of protein translation. Among the proteins synthesized in a PI3K- and ERK-dependent manner in response to CD40 ligation was the truncated or short-form of the cellular FADD-like IL1β-converting enzyme inhibitory protein (cFLIP), which is an anti-apoptotic protein that demonstrates a pro-survival function in CD40-activated carcinoma cells. The short form of FLIP also confers resistance to apoptosis mediated by other TNF superfamily members, TNF, as well as FasL [524]. Interestingly, CD40-mediated induction of Fas expression by murine A20 B leukaemia cells and the subsequent induction of Fas-mediated apoptosis of these cells were augmented by cycloheximide treatment, which suggested that *de novo* synthesis of an anti-apoptotic protein had occurred (Dilloo D *et al.*, 1997). This observation is clinically relevant because CD40 stimulation of primary human B-ALL blasts induces the short isoform of c-FLIP and inhibits the onset of Fas-induced apoptosis despite high cell surface levels of Fas expression [607].

6.3.1.1 Malignant transformation alters CD40-dependent signalling circuits

CD40 signalling commences with CD40L-induced oligomerisation of plasma membrane CD40 monomers and results in recruitment of intracellular TRAF adaptor proteins to specific binding sites on the cytoplasmic tail of CD40. The cytoplasmic tail of CD40 does not have intrinsic catalytic activity, and CD40 signalling depends on TRAF proteins to 'bridge' the ligated receptor with downstream signal transduction pathways. In normal murine B cells, CD40 signalling results in TRAF2-mediated degradation of cytoplasmic IκBα, which releases the p50/p65 NFκB heterodimer to exercise its transcriptional control function in the nucleus. However, in B lymphoma cells, CD40 signalling is 'rewired' and TRAF2 is not required for NFκB activation. Using mutation analysis of CD40 receptors, gene 'knock-down' and 'knock-out' techniques, CD40 signalling was studied in carcinoma cell lines and primary fibroblasts as examples of non-haemopoietic cells. These experiments showed that TRAF2 played the major physiological role in CD40 signal transduction in non-haemopoietic cells. TRAF2 was required for CD40-mediated activation of NFκB, p38 MAPK, PI3K/Akt, and to a lesser extent, JNK signalling pathways, all of which control transcription and translation and, therefore, which will regulate such CD40-mediated functions as cell growth, survival, and cytokine secretion. A novel finding in these experiments was the critical role played by TRAF6 in the activation of these signalling pathways. While TRAF6 binding to the cytoplasmic tail of CD40 played a minor role in CD40 signalling, TRAF6 interacts with TRAF2 downstream of the CD40/TRAF2 signalling complex and modulates its function to activate the kinases responsible for the engagement of the NFκB, p38, Akt and JNK pathways [608].

6.3.1.2 Aberrant CD40 signalling prevents antigen presentation of CD40$^+$ tumours

The improved antigen presenting function exhibited after CD40 stimulation of B-lymphoma and chronic lymphocytic leukaemia cells of B cell type (B-CLL) seems to be the exception rather than the rule for other malignancies, even for those that commonly express CD40 such as multiple myeloma (MM). As in acute leukaemia, MM may be treated with high dose chemotherapy and autologous stem cell transplantation to induce a complete remission but most patients relapse indicating the presence of minimal residual disease. Hence, active immunotherapy may be most effective in the post-treatment setting of lymphopenia and low tumour burden.

In vitro experiments have shown that CD40 activation of different MM cell lines produces dichotomous outcomes of proliferation, or growth arrest and apoptosis [609]. The explanation may lie in the p53-dependent regulation of the myeloma cell cycle that follows CD40 activation of myeloma cells [609]. *In vitro* studies of the CD40$^+$ human myeloma cell lines (HMCL), RPMI 8226 and HS Sultan, demonstrated that these HMCL have wild type p53

function at the permissive temperature of 30°C and defective p53 function at the restrictive temperature of 37°C, where p53 missense mutations disrupt the conformation of wild type p53. At the permissive temperature, trimeric CD40L, which was expressed on the surface of the NIH3T3 fibroblast cell line, was used to stimulate CD40 and induce G_1/S cell cycle progression and proliferation of HMCL. At the restrictive temperature, CD40 activation induced growth arrest, which was associated with transcription of p53 and the p21 cell cycle checkpoint protein, reduced cell viability, and sub-G_1 phase accumulation of cells suggestive of apoptosis. The involvement of p53 in these effects was confirmed using a p21 reporter assay. HMCL were transfected with a p21 reporter plasmid, which expresses luciferase in response to p53 binding of p21-related transactivation elements upstream of the luciferase gene. Consequently, CD40 activation induced luciferase activity only at the permissive temperature in the presence of wild type p53. Together, these data indicate that CD40 activation may induce growth arrest and apoptosis of MM cells with defective p53 but proliferation of MM cells with non-functioning p53. In late stage myeloma, p53 mutations are more common and associated with evasion of apoptosis and drug resistance. Hence, there is a risk that CD40-activating therapy may contribute to the pathogenesis of late-stage myeloma, and the authors considered that determination of the p53 status of myeloma cells may be relevant to the consideration of this therapy [609].

Abnormalities in CD40 signal transduction pathways of MM cells extend to CD40-mediated antigen presenting functions. For example, CD40 activation by transgenic expression of CD40L failed to up-regulate expression of CD80 or CD86 by either $CD40^+$ human MM cell lines (Dotti G *et al.,* 2001) [610] or primary MM cells [611]. However, transduction of the RPMI 8226 HMCL with the combination of CD40- and CD40L-expressing adenovectors increased expression of CD70, CD54 and CD86 and increased proliferation in an alloMLR in contrast to transduction with either vector alone, which indicated that intracellular effector pathways downstream of CD40 remained intact. In addition, CD40L-mediated activation of bystander APC was also found in this *in vitro* system, which would be expected to apply *in vivo* [612].

6.3.1.3 CD40 ligation increases the immunogenicity of $CD40^+$ tumour cells

Carcinoma of the uterine cervix is almost invariably caused by HPV infection, and in particular, by oncogenic viral types expressing E6 protein that interferes with normal p53 function. Using immunohistochemistry, the expression of CD40 was discovered on normal cervical epithelium, cervical intraepithelial neoplasia (of varying grades) and invasive cervical carcinoma. As in other normal human epithelia, CD40 was expressed on basal cells of normal cervical epithelium in six out of ten cases. In HPV-infected, dysplastic, and frankly malignant epithelial cells, CD40 was overexpressed on individual cells, and expressed on most epithelial cells rather than just basal epithelial cells. Similar to other non-haemopoietic cells lines [524], CD40 ligation of cervical cancer cell lines activated the NFκB, and JNK and ERK mitogen-activated protein kinase pathways.

Treatment of cervical cancer cell lines *in vitro* with soluble CD40L trimer produced slight increases in the pre-existing expression of surface MHC class I molecules and CD54, and in the intracellular expression of the TAP1 protein. In contrast, there was little change in the pre-existing expression of CD40, and neither expression of CD80 nor MHC class II molecules was induced by CD40 ligation. CD40 ligation of cervical cancer cell lines dramatically increased CD40-dependent cytolysis of cervical cancer cells by a CTL clone, which recognised a TAP-dependent and HLA-A2-restricted epitope of the E6 protein introduced into the cells using a vaccinia viral vector. At an effector:target ratio of 12:1, CD40 ligation produced approximately 50% specific lysis compared with 5% lysis for untreated control cells. In contrast, CD40 ligation did not affect cytolysis if the $CD8^+$ T cell epitopes were TAP-independent but proteosome-dependent [602].

In these studies of cervical cancer, it is important to note first that CD40 stimulation did not cause apoptosis of cervical cancer cells [602]. Second, CD40 signalling probably mediates TAP-dependent antigen processing *via* NFκB activation because TAP1 promoter activity depends

critically on NFκB binding. Although not excluded formally, the inability of CD40 ligation to enhance CTL responses to TAP-independent epitopes suggested that CD40 stimulation did not simply sensitise the carcinoma cells to Fas-mediated killing by CTL [602]. Hence, of the two major cytolytic pathways, it is more likely that CTL killing in this *in vitro* model system used the perforin-dependent pathway, which has the major role in tumour immunosurveillance in comparison with Fas-mediated cytolysis [613]. Conversely, the Fas-dependent pathway has a minor role in immunosurveillance probably because many tumour cells overexpress the anti-apoptotic cFLIP protein, which confers resistance to Fas-mediated killing. CD40 ligation induces cFLIP in cervical cancer cells thus promoting resistance to Fas-mediated apoptosis although these cells retain sensitivity to perforin-mediated lysis [602]. Perforin-dependent cytolysis applies to killing both by MHC class I-restricted CTL and NK cells, which target MHC class I-negative tumours [613].

These findings are consistent with previous observations that CD40 stimulation up-regulated TAP-dependent antigen processing and, consequently, specific CTL killing of lymphoma cells. For example, sCD40L up-regulated expression of TAP1 and MHC class I molecules on Burkitt lymphoma (BL) cells, and improved the processing efficiency of endogenously synthesized EBV antigens to the extent that brisk MHC class I-restricted cytolysis by EBV-specific CTL resulted [614]. In murine models in which mice carried a high burden of syngeneic lymphoma cells, treatment with anti-CD40 mAb expanded CTL to eradicate the lymphoma burden, and to provide immunity that was cross-protective to challenge with another lymphoma cell line. *In vivo* blocking studies indicated that the treatment effect resulted from a Th1 response involving costimulation and IFNγ production [615]. Similarly, CD40 ligation of $CD40^+$ human melanoma cells enhanced their immunogenicity. CD40 expression in these melanoma cell lines was augmented by IFNγ and TNF, but not by CD40 stimulation itself. CD40 stimulation triggered NFκB activation, up-regulated melanoma surface expression of MHC class I and II molecules and CD54, and induced melanoma cell secretion of GM-CSF, IL-6, IL-8, and TNF. In response, MHC class I-restricted and melanoma antigen-specific CTL clones lysed CD40-activated melanoma cells [616].

6.3.1.4 Transgenic CD40L expression enhances tumour apoptosis and antigen presentation

Although B-CLL cells express unique immunoglobulin molecules as well as MHC class I and II molecules, they do not express costimulatory molecules and are unable to stimulate an alloMLR. The poor immunogenicity of B-CLL cells may result, in part, from an excess of circulating CD40-expressing B-CLL cells, which down-modulate surface expression of CD40L by activated T cells to cause an acquired CD40L deficiency [617].

Nevertheless, CD40 signalling in many B-CLL and B-lymphoma cells remains as intact as it does in normal B cells [618]. CD40 activation of follicular lymphoma cells up-regulates expression of MHC class II, cell adhesion, and CD80 and CD86 costimulatory molecules to the extent that allogeneic T cells are primed *in vitro* and can subsequently recognise unmanipulated lymphoma cells [619]. Similarly, B-CLL cells that were transduced with murine CD40L (mCD40L)-expressing adenovector up-regulated expression of Fas, CD80, CD86, CD70 (CD27 ligand), and the cell adhesion molecules, CD54 and CD58, which blocking experiments determined resulted from activation in *trans*. Moreover, this activation in *trans* extended to the activation of bystander non-transduced B-CLL cells *in vitro*. In comparison with control cells, mCD40L-expressing B-CLL cells stimulated IFNγ production and proliferation in an alloMLR, which was inhibited if CD80/86-CD28 or CD54-CD11a/18 interactions were blocked by specific antibodies. Only T cells co-cultured with autologous mCD40L-expressing B-CLL cells for five days, and not control B-CLL cells, developed specific and MHC class I-restricted CTL activity for non-modified B-CLL cells that was not crossreactive with allogeneic B-CLL cells [620]. Similar autologous T cell recognition of acute lymphoblastic leukaemic or follicular lymphoma cells was obtained *in vitro* if these cells were first stimulated with hCD40L-expressing fibroblasts [621 622].

However, B-CLL cells, which were transduced with an adenovector expressing hCD40L, did not express CD40L even after transduction at a high multiplicity of infection (MOI) (10^4) [620]. In order to overcome poor transfer efficiency of the human CD40L (hCD40L) gene to B-CLL cells, hCD40L expression by the leukaemic cells was achieved *in vitro* by intercellular transfer of hCD40L protein from the MRC5 fibroblast cell line, which had been transduced with an hCD40L-expressing adenovector [623]. Moreover, although hCD40L-expressing B-CLL cells induced activation of autologous T cells *in vitro*, these T cells did not recognise parental leukaemic cells. This situation was rectified by co-transduction of the leukaemia cells to express both hCD40L and hIL-2 [624].

6.3.1.5 Transgenic CD40L expression modulates B-CLL in vivo inducing clinical responses

The approach of using CD40L gene-modified B-CLL cells was tested clinically in a phase I dose-escalation study of a single intravenous infusion of autologous mCD40L gene-modified B-CLL cells. Clinical responses were observed in most patients and correlated with leukaemia-specific immune responses. Acute reductions (within 48 hours) of ≥80% in circulating leukaemic cells were considered to result from apoptosis rather than primary T cell responses. However, short-term *in vitro* recall assays [620] indicated that primed leukaemia-specific T cells probably existed in most patients and thus may have contributed to early clearance of peripheral leukaemic cells. Sustained reductions in the peripheral B lymphocytosis of >50% were achieved for at least three months and were associated with similar sustained partial responses in the malignant lymphadenopathy. Toxicity was minimal and no haematological autoimmune phenomena were observed [625].

It was estimated that the body burden of B-CLL cells exceeded the number of mCD40L-transduced B-leukaemic cells by 10^4:1. Notwithstanding the small proportion of mCD40L-transduced cells, most circulating bystander B-leukaemic cells had up-regulated expression of costimulatory and cell adhesion molecules probably as a result of interactions with mCD40L-transduced cells in lymphoid tissue because an *in vitro* experiment excluded a role for serum mediators [625].

Approximately 50% of B-CLL cells expressed the mCD40L transgene *in vitro* before infusion, and peripheral blood samples analysed after the infusion showed that both transduced and non-transduced B cells demonstrated *de novo* expression of CD54, CD80, CD86 and Fas. While B cell expression of CD54 and CD80 lasted less than one week, Fas expression persisted for at least two weeks. A threshold dose of CD40L gene-modified B-CLL cells was required to observe these phenotypic changes on bystander B-CLL cells, the level of which was not subsequently increased by increasing the dose of infused cells. Therefore, it was reasoned that repeated infusions may be needed to elicit durable anti-leukaemic responses. Plasma levels of IL-12 and IFNγ peaked <48 hours after the infusion. An earlier peak was observed for IL-12, which may have resulted from CD40-activation of macrophages and DC in secondary lymphoid tissues since IL-12 did not appear to have originated from the transduced leukaemic cells *per se*. Several days after peak levels of Th1-type cytokines were attained; the absolute numbers of both $CD4^+$ and $CD8^+$ T cells rose and remained elevated for several weeks. Peak cytokine levels correlated with the absolute number of $CD4^+$ T cells at the time of treatment suggesting that the patient's immune status may be an important determinant of response to the gene therapy, which may, therefore, be more effective in early-stage disease patients who have more intact immune systems [625].

Leukaemia-specific immune responses were observed. A direct IFNγ ELISPOT assay of CD4- and CD8-immunoselected T cells demonstrated a treatment-related increase in IFNγ spot-forming cells in response to CD40-activated autologous B-CLL cells four weeks post-infusion. Similarly, a post-treatment increase in proliferation and IFNγ production by T cells in response to AdmCD40L-transduced B-CLL cells was observed in an autologous MLR six months post-infusion. In both sets of analyses, involvement of $CD8^+$ CTL was suggested by the observation that treatment-related effects were partially blocked with a monoclonal antibody specific for MHC class I.

A later report from the same laboratory suggested that the therapeutic effect of AdmCD40L-transduced B-CLL cells resulted from CD40L-mediated up-regulation of Fas on transduced and bystander B-CLL cells [626]. Fas-mediated apoptosis of CD40-activated autologous and allogeneic B-CLL cells occurred promptly (≤4 hours) in an MHC class I-unrestricted manner if it was induced *in vitro* by B-CLL primed $CD4^+$ T cells, which at least express FasL. In contrast, Fas-induced death of B-CLL cells was delayed (>72 hours) if the FasL was expressed by Chinese hamster ovary (CHO) cells. These data suggested that $CD4^+$ T cells may express molecules other than FasL that contribute to B-CLL cell death [626]. Latent apoptotic B cell responses to Fas ligation is a physiological response of CD40-activated normal B cells, which is adopted by B-CLL cells and which depends on CD40-mediated expression of the anti-apoptotic cFLIP protein. If this finding is physiologically relevant then FasL, which is expressed by T cells and NK cells *in vivo* [626], may be available for a sufficiently long time in secondary lymphoid tissues to cause Fas-mediated apoptosis of CD40-activated B-CLL cells.

In contrast, in another study using the same hCD40L-expressing adenovector described herein, successful expression of hCD40L was obtained in primary B-CLL cells [623] [624]. Autologous B-CLL cells were transduced to express both hCD40L and hIL-2 and repeatedly administered (three to eight times) as a subcutaneous vaccine to nine patients in a phase I trial. Post-immunisation leukemia-specific T cell responses, which comprised markedly enhanced frequencies of IFNγ, granzyme-B, and IL-5 spot-forming cells by ELISPOT, were detected in seven patients. Three patients demonstrated partial responses of malignant lymphadenopathy to immunisation. However, anti-leukaemia immune responses, which waned after immunisation ceased, may have been suppressed by $CD4^+$ $CD25^+$ LAG-3^+ FoxP-3^+ regulatory T cells, which were consistently observed to be circulating, because removal of these cells *in vitro* increased anti-leukaemic T cell reactivity [627].

Interestingly, one patient, who had leukaemia-specific T cell reactivity and a transient response in lymphadenopathy, developed a complete response approximately 12 months post-immunisation, which was associated with the development of B lymphopenia. Levels of circulating T regulatory cells had remained unchanged during this period (MK Brenner, personal communication). This result is somewhat reminiscent of the autoimmunity that was observed in leukaemia-bearing mice, which had been immunised with a mixture of CD40L- and IL-2-expressing fibroblasts and leukaemia-derived peptide-loaded DC [561].

6.3.2 CD40L/CD40 interactions may be pro-tumorigenic

Many CD40-expressing cancers including those of breast [318] and uterine cervix [604] are marked by macrophage infiltration and *in situ* expression of macrophage chemotactic protein-1, MCP-1. In breast cancer, increased tumour content of TAM is directly correlated with vascular grade and is significantly associated as an independent prognostic factor with worsened overall survival [318]. In primary invasive breast cancer extracts, MCP-1 concentration was measured by ELISA and correlated significantly with TAM accumulation and the level of vascular endothelial growth factor (VEGF), thymidine phosphorylase (TP), TNF, and IL-8, which are potent angiogenic factors. MCP-1 expression was observed in both infiltrating macrophages and tumour cells. Both high expression of MCP-1 and VEGF provided significant indicators of early breast cancer relapse [628].

Similarly, cervical cancer lesions displayed extensive epithelial expression of CD40 and MCP-1, and contained heavy infiltrates of macrophages. CD40L was detected on a population of tumour infiltrating lymphocytes. Synergistic up-regulation of carcinoma cell secretion of MCP-1 and another T cell chemoattractant, interferon-γ inducible protein (IP-10), was observed in response to stimulation of CD40 and concurrent treatment with IFNγ. Hence, it was suggested that CD40L/CD40 interactions may contribute to progression of cervical cancer [604]. A clinical epidemiological study demonstrated that expression of CD40 by most tumour cells in soft tissue sarcomas dramatically worsened prognosis [629] and, recently, several other pro-tumorigenic effects of CD40 signalling have been discovered.

6.3.2.1 CD40 activation is pro-angiogenic and pro-tumorigenic

CD40 is expressed by endothelial cells (EC), and a murine transgenic tumour model showed that CD40-mediated angiogenesis was essential for early tumour formation [630]. CD40, which was expressed weakly on murine EC *in vivo*, was up-regulated by TNF and IFNγ on a murine EC cell line *in vitro*. When this cell line was activated using sCD40L, expression of VCAM-1 and VEGF was up-regulated similarly to the effect of CD40 stimulation of human EC *in vitro*.

The significance of EC expression of CD40 was discovered when, using the matrigel plug assay, angiogenesis was found to be attenuated in CD40-deficient mice. Next, BALB/NeuT mice, which express the HER2/*neu* oncogene under the control of the mammary murine tumour virus (MMTV) promoter and which spontaneously develop breast cancer, were crossed onto a CD40-deficient background. Although breast development was normal, the resulting tumours were smaller, slower in onset, reduced in number, and contained fewer larger blood vessels compared with BALB/NeuT mice. Radiation bone marrow chimera experiments indicated that host CD40 expression rather than CD40 of bone marrow origin was required for tumorigenicity. Because of the delayed onset and late progression of some tumours in CD40-deficient BALB/NeuT mice, these data suggested that CD40 triggering of EC occurred early rather than late in tumour formation [631].

Few T cells, which may express CD40L, were observed in the tumours of BALB/NeuT mice. Consequently, it was hypothesised that platelets may provide CD40L in this experimental system. Human platelets release sCD40L upon activation, and thrombin-activated murine platelets, but not resting platelets, express surface CD40L. Platelets often contain pro-angiogenic molecules such as VEGF, and after platelet activation and thrombosis, platelet CD40L induces EC to express tissue factor, cell adhesion molecules, and chemokines, all of which favour recruitment and extravasation of leucocytes at the site of injury [631].

Platelet expression of CD40L is blocked by the adenosine diphosphate receptor (ADP) antagonist, clopidogrel. Hence, BALB/NeuT mice were continuously treated with clopidogrel before tumour formation. Intra-tumoral thrombi were reduced, and tumour size and multiplicity were reduced to levels seen with CD40-deficient BALB/NeuT mice. No additional effect of clopidogrel treatment was observed in CD40-deficient BALB/NeuT mice, which indicated that clopidogrel probably acted *via* inhibition of platelet CD40L [631].

In the BALB/NeuT model, disruption of CD40L/CD40 interactions prior to tumour formation demonstrated that the dominant effect of CD40 activation was pro-angiogenic and thus pro-tumorigenic. Nonetheless, tumours arising in BALB/NeuT mice grew more aggressively when transplanted into CD40-deficient than in wild type hosts, which suggested that CD40 has a role in tumour immunosurveillance [631]. Moreover, these data are consistent with the hypothesised role of T cells in the control of larger tumours that have escaped intrinsic tumour suppressor mechanisms [304]. Although the results of the BALB/NeuT model would suggest that caution be exercised in the application of anti-CD40 therapy, anti-tumour effects of intentional CD40-mediated APC activation are not precluded.

However, the weight of evidence, which is derived from 15 studies of transgenic CD40L therapy in murine tumour models, argues against the dominance of pro-angiogenic and pro-tumorigenic effects arising from CD40 activation of endothelial cells. Rather, an average tumour-free efficacy rate of 72% was obtained in these 15 studies [599].

6.3.2.2 CD40L/CD40 interactions may operate autocrine and/or paracrine growth loops

Given that expression of CD40 and CD40L has been observed in the same tissue, autocrine and/or paracrine growth loops have been proposed that involve CD40L/CD40 interactions in the initiation, promotion or progression of malignancy. Low-level expression of CD40L in malignant B cells such as B-CLL, NHL, and BL leads to constitutive CD40 activation and evidence of oncogenicity [632 91 633].

The outcome of CD40 activation does seem to depend on the strength and duration of CD40 engagement. For example, a BL cell line that was stimulated with high concentrations of CD40L underwent growth arrest. Conversely, below the population threshold at which cell

death occurred, low picomolar quantities of exogenous CD40L sustained the proliferation of the clone. Endogenous production of CD40L was detected once cell numbers in culture became limiting and neutralisation of endogenous CD40L activity accelerated the decline of the cell population at lower cell densities, which suggested that an auto-inducible survival mechanism may operate within an appropriate environmental niche. This mechanism was likened to "quorum sensing" among gram-negative bacteria in which diffusible molecules communicate to coordinate gene expression with population density [634]. These observations leads to speculation that a physiological role for the cytokine-like function of sCD40L may be survival without proliferation of responsive cell types in microenvironments such as the secondary B cell follicle, which is somewhat analogous to the homeostatic function that colony stimulating factor-1 is suspected to have in maintenance of tissue macrophages [133].

Although normal B cells may express CD40L, its physiological function remains unknown. However, B cell lymphomas may also express CD40L, but in contrast to normal B cells, NFκB is constitutively active [91]. Gene expression profiling studies have shown that constitutively active NFκB may account for the worsened prognosis of patients with the activated B cell-like (ABC) genotype of diffuse large B cell lymphoma (DLBCL) [635]. Constitutive expression of nuclear NFκB appears to be maintained by a CD40 signalosome, which is a scaffold-like signalling platform extending from the cell membrane within a lipid raft microdomain, the assembly of which is initiated by autocrine stimulation by CD40L. Anti-CD40 or anti-CD40L mAb disrupt CD40 signalosomes, inhibit NFκB activity, and induce cell death and inhibit lymphoma cell growth [91]. In addition, DLBCL cells contain constitutively active NFATc1 and cRel NFκB transcription factors that interact to bind the CD40L promoter and synergistically activate CD40L gene transcription. Inhibition of CD40L gene transcription using NFATc1 or cRel small interfering RNA (siRNA) also inhibited lymphoma cell growth [632]. Similarly, some cases of B-CLL express both CD40 and CD40L. In these B-CLL cells, NFκB is also constitutively active and CD40 ligation further augments NFκB activation and extends CLL cell survival *in vitro*. Again, anti-CD40L mAb treatment of the B-CLL cells inhibited NFκB activation and accelerated CLL cell death [633].

Although normal breast epithelium does not express CD40L, in 160 cases of invasive breast cancer, 85% of specimens expressed CD40 and 86% of specimens expressed CD40L. CD40 and CD40L were expressed in 76% of the same cases, and only 9% of biopsies stained positively for CD40 and not for CD40L. CD40L staining was both membranous and cytoplasmic, which indicated the presence of cytoplasmic stores of CD40L.

To explore potential oncogenic effects of CD40 activation further, the CD40-expressing SCC12F human carcinoma cell line was studied. SCC12F cells are immortalised but not tumorigenic and were stably transfected with a plasmid DNA vector to create inducible expression of CD40L using a tetracycline-regulated expression system. Enforced sustained, but not transient, expression of CD40L by the SCC12F cell line *in vitro* induced NFκB activation, increased its proliferation rate, and enhanced its transwell migration, which was blocked by anti-CD40L antibody. However, sustained CD40 stimulation was not sufficient to transform the SCC12F cell line fully because it was still unable to grow in an anchorage-independent fashion in soft agar.

Since, it is known that fewer oncogenic stimuli are required to transform fully rodent cells than human cells, the Rat-1 immortalised rodent fibroblast cell was fully transformed with a chimeric CD40 receptor, which contained the transmembrane domains of LMP1 of EBV fused to the intracytoplasmic domain of CD40 to create LMP:CD40. LMP1 is constitutively active independently of ligand binding because its transmembrane regions promote the oligomerisation of the TRAF-interacting cytoplasmic domain of LMP1 so that it binds TRAF molecules directly to activate the NFκB and MAPK pathways [636]. LMP1 fully transforms Rat-1 fibroblasts *via* NFκB activation [637], and partially mimics CD40 function by spontaneously producing B cell differentiation and antibody formation in CD40-deficient mice although germinal centre formation was prevented [637]. Activation of the LMP:CD40 receptor in Rat-1

fibroblasts was constitutive and ligand-independent and resulted in activation of the NFκB, JNK and MAPK pathways. Moreover, unlike control Rat-1 cells, LMP:CD40 Rat-1 cells displayed anchorage-independent growth in soft agar and were tumorigenic in nude mice. Further experiments demonstrated that transformation was largely mediated by aberrant activation of the NFκB pathway. Together, these observations suggested that although constitutive engagement of CD40 may contribute to carcinogenesis, additional oncogenic stimuli may be required for the expression of full malignant potential [636].

6.3.3 The potential consequences of epithelial loss of CD40 expression

Our findings of the expression of CD40 in normal prostate epithelium, its complete absence in prostate cancer, and its patchy expression in two cases of prostatic carcinoma *in situ* (Moghaddami M *et al*., 2001) were confirmed in an immunohistochemical study of 57 archived specimens of invasive prostate adenocarcinoma [603]. Given that CD40 expression is associated with the basal phenotype in normal prostatic epithelium and in premalignant prostatic intra-epithelial neoplasia (PIN) (Moghaddami M *et al*., 2001), CD40 signalling may be associated with the commitment of epithelial stem cells to differentiation. There are three potentially significant consequences of loss of CD40 expression. First, loss of CD40 may prevent CD40-mediated growth inhibitory or differentiation signals, and thus contribute to invasive cancer.

Second, CD40 expression may be an indicator of tumour immunogenicity, which would be consistent with the susceptibility of the highly CD40-expressing cancers of bladder and melanoma to immunotherapy [603]. Normal urothelium does not express CD40. However, in 131 cases of transitional cell carcinoma of the bladder, 89% of early-stage Ta and T1 tumours were $CD40^+$ whereas only 62% of T2-T4 tumours were $CD40^+$. Although CD40 expression was not an independent prognostic marker, its association with early-stage tumours may signify clinically less aggressive tumours, which are more immunogenic and which may help to explain the responsiveness of early-stage tumours to BCG immunotherapy [638]. CD40L-expressing activated T-cells that patrol the epithelium may trigger CD40-mediated apoptosis of epithelial cells and thus prevent the outgrowth of premalignant cells. Thus, loss of CD40L/CD40 interactions may contribute to immune evasion by malignant cells (Chapter 2).

Third, CD40 loss may confer resistance to treatment with cytotoxic drugs, which sensitise carcinoma cells to CD40 ligation by disruption of protein synthesis. For example, while neither CD40 ligation nor treatment with 5-fluorouracil (5-FU) or etoposide alone caused significant apoptosis of cervical cancer cell lines in *vitro*, the combination of the treatments synergised to increase apoptosis. Etoposide or 5-FU acted similarly to cycloheximide and PI3K inhibitors in reducing CD40-mediated initiation of protein synthesis [602]. Hence, restoration of CD40 signalling, for example, by CD40 gene transfer may enhance sensitivity to a number of apoptotic stimuli including cytotoxic drugs [639] (see Section 6.3.8).

6.3.3.1 Anti-myeloma effects of transgenic CD40L expression irrespective of CD40 status

The CD40-negative X24 and the CD40-expressing MPC-11 and S107 murine plasmacytoma cell lines were studied using a vaccine formulation similar to that described by Dilloo *et al*. (1997). Moreover, CD40-dependent signalling in S107 cells was defective and CD40 ligation of S107 cells did not result in activation of the alternative NFκB pathway unlike in primary murine B cells [640]. The CL7.1 fibroblast cell line was retrovirally transduced to stably express mCD40L or *neo* as a negative control and then lethally irradiated (10Gy). The transduced fibroblasts were mixed with sub-lethally irradiated (3.5Gy) myeloma cells before subcutaneous inoculation. Sub-lethal irradiation of $CD40^+$ myeloma cells was necessary because of the aggressive nature of these tumours. Transgenic CD40L expression retarded myeloma growth *in vivo* but not *in vitro*, and both $CD4^+$ and $CD8^+$ T cells were required for this tumour inhibitory effect. Moreover, tumour-specific protective immunity against live tumour cell challenge was conferred by prior immunisation with two doses of either CD40L-expressing MPC-11 or S107 myeloma vaccines. The partial protection against challenge with live S107 cells afforded to MPC-11-immune mice confirmed the role of the adaptive immune

system in mediating tumour-specific immunity, and also indicated that TAA were shared between the two tumours.

Tumour cells were required in the vaccine because no anti-tumour effects were seen after injection of CD40L-expressing CL7.1 fibroblasts alone. Moreover, *in vitro* co-culture experiments indicated that these CD40L-mediated effects appear not to have resulted from up-regulated myeloma cell expression of MHC class II and costimulatory molecules. Similar levels of CD40L-mediated reduction in tumorigenicity and improvement in protection against live tumour cell challenge were observed for the incorporation of live CD40-negative X24 murine plasmacytoma cells in the vaccine, which supported the notion that ligation of myeloma cell CD40 was not required for anti-tumour effects of transgenic CD40L expression.

The effects on tumorigenicity were similar irrespective of whether each of the three plasmacytoma cell lines had been first irradiated *ex vivo* with UV light or γ-rays before each line was mixed with CD40L-expressing or *neo* fibroblasts. The outgrowth of UV-irradiated tumour cells in all mice that had received the mixture containing *neo* fibroblasts indicated that viable tumour cells had been present in the tumour inoculum, which did not survive if they were pre-mixed with CD40L-expressing fibroblasts. However, given the lack of an inhibitory effect of transgenic CD40L expression on their growth *in vitro*, it seems unlikely that CD40 ligation of $CD40^+$ myeloma cells resulted in the death of these cells in the tumour inoculum. Presumably, viable tumour cells were destroyed by immune effector mechanisms elicited by transgenic expression of CD40L and involved $CD4^+$ and/or $CD8^+$ T cells [641].

6.3.3.2 Rationale for investigating transgenic CD40L expression in CD40-negative tumours

Subsequently to the original studies by Grossmann *et al*. (1997) of transgenic CD40L expression by CD40-negative tumour cells, other investigators reproduced the results using CD40L gene-modified tumour cells and further explored the mechanisms underlying the anti-tumour immune responses. The CD40-negative murine tumour cell lines, MCA 207 or TS/A, were retrovirally transduced to express CD40L. CD40L-expressing MCA 207 and TS/A cells stimulated splenic cell proliferation *in vitro*, which demonstrated the biological activity of the transgene. CD40L gene-modified tumour cells reduced tumorigenicity or increased tumour protection when used in therapy or vaccination models, respectively [642].

A significant finding made by Grossmann *et al*. (1997) was that incorporation of only 1.4% CD40L gene-modified tumour cells in the tumour inoculum reduced tumorigenicity, which provided support for subsequent testing of intra-tumoral injections of CD40L gene transfer vectors as a therapeutic approach. These studies showed that *in vivo* injection of CD40-negative tumours with a CD40L-expressing adenovector produced tumour regression and concomitant tumour immunity and that these effects were mainly mediated by DC (see Section 6.3.3.3). In addition, the results of these studies suggested that this approach may be applied to the treatment of metastatic cancers in which injection of a CD40L gene transfer vector at one tumour site may induce concomitant tumour immunity at remote tumour sites. The involvement of DC in CD40L-mediated anti-tumour effects may be particularly relevant given that many tumours lack DC. For example, in cancers of breast and prostate, tumour masses contain a paucity of DC, which are not activated [643] [644]. Together with other recent evidence, tumour-induced DC dysfunction seems to be a generalized phenomenon that has been demonstrated clinically and in preclinical models [98]. *In vitro* incubation of human DC with prostate cancer cell lines, normal prostate cells or peripheral blood white cells showed that prostate cancer cells accelerated DC apoptosis, which was resisted if DC had been previously stimulated with CD40L for 24 hours. The increased resistance of DC to prostate cancer-induced apoptosis was associated with increased DC expression of the anti-apoptotic protein Bcl-x_L [643], which was similar to the reported effects of CD40 ligation on WEHI-231 murine B cells, which had rapidly up-regulated Bcl-x_L expression in response [645].

6.3.3.3 Transgenic CD40L-mediated effects on CD40-negative tumours via APC activation

Mice with established subcutaneous tumours of syngeneic B16 melanoma, CT26 colon cancer or Lewis lung carcinoma (LLC) received intra-tumoral injections of an adenovector

expressing murine CD40L, AdmCD40L. These tumours do not express CD40 [646] [647], and only B16 weakly expresses CD40 in response to IFNγ treatment [647]. Regression and tumour remission were achieved in >60% of mice bearing B16 or CT26 tumours. On the other hand, although remissions of LLC were not obtained, LLC tumour growth was significantly suppressed. In addition, concomitant tumour immunity was induced by CD40L gene modification of each tumour type. Subcutaneous inoculation of mice with tumour cells, which had been transduced *ex vivo* with AdmCD40L and irradiated, produced significant growth delay of parental tumours, which had previously been inoculated in the contralateral flank of the same mice. Direct intra-tumoral injection with AdmCD40L produced a tumour infiltrate of cells expressing CD86, CD25, and CD8 and suggested that AdmCD40L treatment had invoked infiltration of activated APC and $CD8^+$ T cells, and had elicited tumour-specific CTL. Adoptive transfer of splenocytes from mice that had received intra-tumoral injections of AdmCD40L, but not the control adenovector, protected 80% of naïve mice against parental tumour challenge [648].

The anti-tumour effects of *in vivo* tumour transduction using AdmCD40L that were observed using the CT-26 colon cancer model were confirmed in another study [649]. The incidence of regression of subcutaneous CT-26 colon cancers after intra-tumoral injection of AdmCD40L was shown to depend directly on vector dose. *In vivo* depletion methods were used to demonstrate that the regression of treated primary CT-26 tumours depended on $CD8^+$ CTL rather than $CD4^+$ T cells. In contrast, $CD4^+$ and $CD8^+$ T cells were required to mediate protection against tumour re-challenge, which suggested that endogenous CD40L is important for $CD8^+$ memory cell generation. Moreover, rejection of the primary tumour elicited by intra-tumoral injection with AdmCD40L correlated with the intra-tumoral expression of IL-12, IFNγ and chemokines such as MIP-1α, MIP-1β, MIP-2, RANTES and eotaxin. MIP-1α, in particular, attracts activated $CD8^+$ CTL as well as immature DC, macrophages, NK cells, and neutrophils [649]. In these studies, given that the tumour cells themselves did not express CD40 receptor, transgenic CD40L substituted for activated helper $CD4^+$ T cell expression of CD40L, and *via* CD40 stimulation, activated APC to present TAA to naive $CD8^+$ CTL [648]. The dependence of transgenic CD40L-mediated anti-tumour effects on the induction of systemic T cell-mediated immunity toward CD40-negative tumours reproduces the original findings of Grossmann *et al*. (1997), in which potent anti-tumour activity was also obtained after low-level CD40L gene transfer.

The *in vivo* effects of transgenic CD40L expression by tumour cells on intra-tumoral DC was analysed more specifically [650]. CD40-negative MC38 colon carcinoma cells were retrovirally transduced with the CD40L gene or with the *neo* control gene and syngeneic mice were inoculated subcutaneously with the transduced cells. MC38 expression of CD40L significantly retarded tumour growth. Compared with unmodified MC38 tumours, immunohistochemistry demonstrated an increase in $CD11c^+$ DC and $CD8^+$ T cells in CD40L-expressing MC38 tumours. This *in vivo* result correlated with the *in vitro* finding of decreased apoptosis *in vitro* of DC, which were co-cultured with $CD40L^+$ MC38 cells, and suggested that intra-tumoral accumulation of DC may have resulted from a CD40L-mediated reduction in tumour-induced apoptosis of DC [650].

6.3.3.4 CD40 activation is potentiated by the addition of dendritic cells

In our studies of transgenic CD40L expression (Dilloo D *et al*., 1997; Grossmann ME *et al*., 1997; Loskog A *et al*., 2001), we had not conducted detailed analyses of the role of DC, which are critical mediators of immunomodulation by CD40L. The potent ability of transgenic CD40L to interact with CD40 on DC *in vivo* to prime tumour-eradicating $CD8^+$ CTL was graphically demonstrated when the intra-tumoral injection of AdmCD40L synergised with the intra-tumoral injection of DC [651]. B16 or CT-26 tumours that were injected either with a 100-fold lower dose of AdmCD40L than in the previous study [648], or bone marrow-derived DC (BMDC), failed to regress. However, mice bearing B16 or CT-26 tumours, which were injected with this dose of AdmCD40L then with BMDC two days later, demonstrated complete tumour regression although survival rates of the mice were not

reported [652]. The dependence of these anti-tumour effects on the CD40L/CD40 interaction was confirmed by anti-CD40L mAb blockade. Moreover, it was demonstrated that transgenic CD40L expression bypassed the need for $CD4^+$ T cell-derived CD40L in this system [651].

This approach was extended by CD40L gene modification of DC *per se*, which when injected into B16 or CT-26 tumours also effectively induced sustained tumour regression at a dose that was 10-fold lower than that of non-genetically modified DC [652 651]. Only CD40L gene-modified DC, and not control DC, up-regulated expression of CD54, CD80 and CD86, and secreted IL-12 and MIP-1α, presumably *via* a paracrine effect of transgenic CD40L expression. It should be noted that the cure rates of B16 or CT-26 tumour-bearing mice, which received an intra-tumoral injection of *ex vivo* AdmCD40L-transduced DC, were lower than those of tumour-bearing mice that had received an intra-tumoral injection of an approximately 100-fold higher dose of AdmCD40L alone [652 651]. Moreover, there was no obvious dose effect of CD40L gene-modified DC on the survival of tumour-bearing mice, which suggests that the recruitment of anti-tumour effector mechanisms is limited in some way using this approach. Nevertheless, the anti-tumour effects of intra-tumoral injection of CD40L gene-modified cells were shown to depend on the transfer of DC because CD40L gene-modified and syngeneic fibroblasts did not cause regression of primary CT-26 tumours. Evidence of migration of the CD40L gene-modified DC to spleen was found three days after injection and was associated with $CD25^+$ cells in the spleen. Again, anti-tumour effects were mediated by tumour-specific $CD8^+$ CTL, which transferred passive immunity to naïve tumour-bearing mice. Concomitant tumour immunity was also demonstrated using this approach [652].

6.3.4 Transgenic CD40L expression counteracts tumour immunosuppression

In addition to the recruitment/retention and activation of intra-tumoral DC mediated by transgenic CD40L expression, transgenic expression of CD40L within a tumour may counteract its immunosuppressive microenvironment.

Genetic studies have shown that IFNγ has a vital role in tumour elimination [350]. IFNγ is produced by T and NK cells, and interferon-producing killer dendritic cells (IKDC) [482 483], and its secretion is mainly induced by IL-12 in Th1-driven immune responses [653]. Immunosuppressive factors produced by tumours themselves may counteract Th1 immune responses. For example, the cytokine profile of tumours may be skewed toward production of the cytokines, IL-10 and TGFβ, which are associated with regulatory T cells and which produce the T regulatory-1 (Tr1) or T helper-3 (Th3) response. Tr1/Th3 responses negatively regulate Th1 responses by inhibiting DC maturation [654]. IL-10 is now considered to be more a Th3 or T regulatory-1(Tr1) cytokine than a Th2 cytokine, although its secretion favours Th2-supported activation of B cells [599]. Earlier studies had shown that mature human epidermal Langerhans cells (LC), which were used to simulate lymph node interdigitating DC, died spontaneously from apoptosis in culture and were rescued from cell death by CD40 ligation. In contrast, IL-10 accelerated the apoptosis of LC *in vitro* and counteracted CD40-mediated up-regulation of MHC, costimulatory and cell adhesion molecule expression by LC [655]. If human bladder cancer cells, which secrete IL-10 or TGFβ, were co-cultured *in vitro* with CD40L gene-modified human DC that secreted IL-12, the down-regulation of DC secretion of IL-12 was resisted perhaps *via* reduced DC expression of TGFβ and IL-10 receptors [654]. Furthermore, *in vivo* studies reinforced the important negative regulatory effects of IL-10 on DC maturation and also showed the significant countervailing influence of transgenic CD40L expression.

In the MC38 tumour model, the tumour cells produced IL-10, and exogenous IL-10 down-regulated DC expression of CD40. CD40 expression on splenic DC isolated from MC38 tumour-bearing mice was significantly reduced in comparison with non-tumour-bearing mice. Treatment of tumour-bearing mice with neutralising anti-IL-10 antibody de-repressed CD40-dependent IL-12 production by DC. Furthermore, treatment of MC38 tumour-bearing mice

with sCD40L reversed deficient expression *in vivo* by DC of CD40 and IL-12p40, which together with IL-12p35 comprises bioactive IL-12p70 [98].

In the MB49 murine bladder cancer model, the tumour cells inhibited the spontaneous maturation of BMDC *in vitro*, which was measured by DC surface expression of CD83 and MHC class II molecules. This maturation defect was reversed by first transducing MB49 cells with AdmCD40L, which significantly increased supernatant levels of levels of IL-12 but did not alter IL-10 levels. Similar to the effect that the adenovector control had on CD86 expression of human moDC (Dotti G *et al*., 2001), MB49 cells, which were transduced with the control adenovector, also reverted expression of CD83 and MHC class II molecules by BMDC, which confirmed that adenoviral proteins had adjuvant activity. *In vivo*, MB49 tumour cells escape immunosurveillance by inducing IL-10 at the tumour site, which is functionally significant because MB49 tumour rejection is increased in IL-10 gene-disrupted mice [653].

It was hypothesised that introduction of the DC-activating molecule CD40L into the MB49 tumour milieu would overcome the inhibitory effects that IL-10 has on DC maturation. In contrast to intra-tumoral injection with the control adenovector, small subcutaneous MB49 tumour nodules (2.5-5mm) regressed when injected four times with AdmCD40L, which was the same vector reported herein (Loskog A *et al*., 2001). Tumour cytokine mRNA levels were analysed by quantitative PCR (qPCR), and whereas the levels of the immunosuppressive cytokines, IL-10 and TGFβ, were decreased by injections of AdmCD40L and empty control adenovectors, only AdmCD40L significantly increased levels of IL-12. Moreover, only intra-tumoral injection with AdmCD40L reduced serum IL-10 to levels that were indistinguishable from those of naïve mice. CD8-immunoselected splenocytes, which were obtained from tumour-bearing mice that had received four intra-tumoral injections with AdmCD40L or the empty vector control, were restimulated *in vitro* with MB49 tumour cells. Supernatants from these co-cultures demonstrated significantly higher levels of IL-12 and IFNγ, and the co-cultured cells demonstrated tumour-specific and non-NK-mediated cytolysis, which was inhibited by the addition of IL-10 [653].

In a further demonstration of the clinical potential of this approach, MB49 cells were implanted in the murine bladder and subsequently mice were treated intravesically with AdmCD40L or control adenovector 1, 7 and 14 days after tumour installation. The orthotopic tumours were cured with AdCD40L gene therapy in 60% of mice and in only 10% of mice receiving the control adenovector. AdmCD40L transduction induced bladder expression of IL-12 and conferred protective systemic tumour-specific immune responses. These effects were associated with a reduction in the tumour-draining lymph node content of regulatory $CD4^+$ $CD25^+$ T cells together with reduced suppressor activity in the draining lymph nodes [418].

Hence, the positive indirect effects of CD40 activation resulted from (i) enhancement of DC survival and antigen presenting functions in an immunosuppressive tumour microenvironment, and (ii) the recruitment and amplification of host immune responses *via* intra-tumoral secretion of chemokines and cytokines that favoured the development of Th1 responses.

6.3.5 Salient features of transgenic CD40L-expressing tumour vaccines

Active immunisation with a CD40L-expressing autologous whole tumour cell vaccine may be functionally equivalent to "*in vivo* immunisation" in which anti-CD40 mAb treatment is coupled with a cytotoxic chemotherapy-induced supply of dead autologous tumour cells [656]. Although these two approaches may differ in the scale and extent of sites of immune interaction, it may be assumed that the relatively small proportion of CD40 ligation events occurring on each occasion will necessitate either repeated active immunisation, or multiple cycles of combination treatment with anti-CD40 mAb and cytotoxic chemotherapy. Hence, the greatest utility of anti-CD40 treatment may be in its cross-priming action, which includes uptake of tumour cells dying as a result of chemotherapy and/or CD40 stimulation. Moreover,

while CD40 ligation may increase the antigen processing and presenting capacity of the tumour cells themselves, most studies described herein indicate that generation of anti-tumour immunity is dominated by CD40 ligation of host professional APC.

Consequently, a potential limitation of therapeutic CD40 ligation would be overcome if not only CD40-expressing malignancies such as acute B leukaemia, multiple myeloma and some carcinomas could be treated (Dilloo D *et al*., 1997; Rousseau RF *et al*., 2006; Dotti G *et al*., 2001; Loskög A *et al*., 2001) but also tumours that have lost CD40 expression during carcinogenesis (Moghaddami M *et al.*, 2001). In these circumstances, for an anti-tumour response to be generated, cross-priming would be required and DC uptake of apoptotic tumour cells provides the most effective means of cross-priming if combined with the enabling signal supplied by CD40 ligation. Similarly, cross-priming may surmount the limitation imposed by tumour heterogeneity in which tumour cell CD40 expression may be variable. In conclusion, since the interaction with tumour cell CD40 is neither universal nor required for the immune enhancement mediated by transgenic CD40L, CD40 ligation may be useful therapeutically for both CD40-positive and CD40-negative forms of malignancy.

Our publications that describe the therapeutic vaccination of malignancy *in vivo* have a number of features in common, which may be used to develop a model of the therapeutic action of transgenic CD40L expression in subcutaneously administered tumour vaccines:

6.3.5.1 Syngeneic or autochthonous tumour cells were used to immunise against malignancy

Immunogens in the tumour vaccines are contained within syngeneic or autochthonous tumour cells, which were irradiated before immunisation of leukaemia-bearing mice (Dilloo D *et al.*, 1997) or leukaemia patients (Rousseau RF *et al.*, 2006), or inoculated as live tumour cells in the neuroblastoma (Grossmann ME et *al.*, 1997) and bladder cancer models (Loskög A *et al.*, 2001). Hence, the TAA to be presented to the host immune system may include unique mutated TAA as well as non-mutated but overexpressed tissue differentiation antigens. In addition, the radiation dose may induce necrotic cell death among tumour cells in the inoculum and so may provide DC maturational stimuli *in situ via* binding of HSP/peptide complexes to the DC common heat shock protein receptor (CD91) [411 410 657], or by provision of chromatin-associated HMBG1 protein [458] or cytosol-associated monosodium urate [147 658].

6.3.5.2 Tumour vaccines were deposited subcutaneously

The tumour vaccines were deposited into what may be considered a 'low-activity' anatomic compartment. Nevertheless, the presence of lymphatic vessels will ensure that the deposited material eventually drains to regional lymph nodes where the primary immune recognition events will occur [659 660]. In addition, sentinel DC and tissue macrophages exist in this compartment and will ensure that the deposited material is digested before its passage to the draining lymph nodes [661 163]. If there are an insufficient number of phagocytes to cope with the phagocytic burden then mechanisms exist to ensure that more cells are recruited to clear the deposited material [662]. In any subcutaneous inoculum of tumour cells, a sizeable proportion of the inoculum is expected to die [663], and *via* the caspase-3 mediated release of chemoattractant molecules such as lysophosphatidylcholine (LPC), macrophages will be drawn in to engulf and remove the dead tumour cells [664].

6.3.5.3 Efficacy of transgenic CD40L expression and tumour cell death in situ

After subcutaneous immunisation with live or irradiated cells, it is likely that a major proportion of the tumour cells in the inoculum are destined to die *in situ*. Even though murine neuro2a neuroblastoma and MB49 bladder cancer cells were alive at the time of injection, other subcutaneous tumour implantation models suggest that many if not most tumour cells die soon after injection [663]. Although MB49 cells express CD40, no data are available to indicate if these cells die from apoptosis following CD40 stimulation. *In vitro* evidence was presented to indicate that A20 B-leukaemia death occurred in response to CD40 ligation and hence was likely to occur *in vivo* even though direct evidence was lacking (Dilloo D *et al.*, 1997). Similar observations may apply to the leukaemia vaccines for patients although the

CD40 expression status of the leukaemic blasts in the vaccines was not stated (Rousseau RF *et al.*, 2006). In the absence of CD40 expression by neuro2a and WEHI-3 cells, tumour cell death is not likely to be augmented by transgenic CD40L expression.

All of the leukaemic vaccines were irradiated before subcutaneous immunisation. In the case of the A20 and WEHI-3 leukaemia vaccines, the radiation dose was 10Gy whereas the dose was 80Gy for the patient vaccines [658]. In the case of *in vitro* studies that were performed with human myeloma cell lines such as RPMI 8226, a γ-radiation dose of 80Gy was used (Dotti G *et al*., 2001). After 10Gy or 20Gy γ-irradiation *in vitro*, trypan blue exclusion analysis showed that ~50% of RPMI 8226 cells remained viable at 72 hours [665]. In another study, ~30% RPMI 8226 cells were apoptotic at 72 hours after 6Gy γ-irradiation [666]. Although no direct evidence is available that ionising radiation has DC maturation and immunostimulatory effects in its own right, ionising radiation may be immunostimulatory because of secondary factors, which include the induction of (i) pro-inflammatory mediators such as TNF, IL-1β and PGE_2, and of (ii) apoptosis or necrosis of tumour cells with consequent release of endogenous adjuvants such as monosodium urate and/or HMGB1 (see Section 1.9.7.3). Together, these effects may facilitate presentation of TAA and the subsequent cross-priming of TAA-specific T cells [658] (see Section 1.5.5.2).

In addition, similar to effects that DNA damaging drugs such as cisplatin have on tumour cell induction of death receptors such as Fas [667], ionising radiation can up-regulate tumour-cell expression of Fas thus sensitising tumour cells to Fas-mediated killing by CTL [658]. Hence, although the addition of ionising radiation was not always controlled experimentally in these studies (Dilloo D *et al*., 1997; Rousseau RF *et al*., 2006; Dotti G *et al*., 2001), it is reasoned that the critical molecular adjuvant that induced anti-tumour immune responses in these studies was transgenic CD40L expression. This may be particularly evident for the murine transgenic CD40L-expressing leukaemia vaccine studies in which the A20 vaccine was compared with the WEHI-3 vaccine. Although both vaccines were irradiated before administration to the mice, only the A20 vaccine induced therapeutically effective immunity perhaps because transgenic expression of CD40L exerted its effects *via* A20 tumour cell expression of CD40.

6.3.5.4 Cellular recruitment at the immunisation site may influence effector mechanisms

It may be inferred that induction of protective $CD8^+$ T cell immunity in response to immunisation with CD40L gene-modified tumour cells resulted from the transgenic expression of CD40L, which then licensed the DC that presented TAA on MHC class I molecules. What are the CD40L-directed steps that lead to induction of T cell immunity? As professional scavenger cells, macrophages will probably arrive first 'on the scene' at the immunisation site and will be recruited in force until the dead material is cleared [668]. The longevity of these macrophages may decline after the task of phagocytosis is completed [669] and then they and their partially digested cargo may be available for cross-presentation by DC in the draining lymph nodes (DLN). Given that APC at the immunisation site will ingest dead or dying tumour cells resulting in the recruitment of more APC, cytokines emitted by irradiated dying tumour cells together with signalling *via* endogenous PRR that activates NFκB may be sufficient to induce some DC maturation [410] [670], which will be reinforced by proximate transgenic expression of CD40L. CD40 ligation will be the most potent stimulus that converts the DC from antigen capture to antigen presentation mode. Subsequently, in the DLN, the CD40-activated DC will be conditioned to help B cell and killer T cell responses.

However, recent studies in murine models of cutaneous viral infection suggest that some antigens captured in the immunological periphery are cross-presented in the DLN because DC that capture the antigens are not the same as the DC that present the antigens to T cells in the DLN [180] [181]. Hence, it is possible that transgenic CD40L expression at the antigen deposition site in the immunological periphery may aid antigen presentation efficiency in at least a two-step process. First, CD40L protein of transgenic origin may increase the local supply of dead tumour cells by apoptosis induction, and accelerate the migration to DLN of fully competent

APC. Second, in the DLN, CD40L protein of transgenic origin may supplement physiologically available CD40L to amplify antigen presentation and, therefore, activation and clonal expansion of antigen-specific T cells.

6.3.5.5 Tumour vaccine immunogenicity was augmented by transgenic CD40L expression

The complete tumour vaccine included CD40L gene-modified tumour cells in the neuroblastoma (Grossmann ME et *al.*, 1997) and bladder cancer models (Loskög A *et al.*, 2001). Both IL-2- and CD40L gene-modified bystander syngeneic or autologous fibroblasts mixed with leukaemia cells comprised the complete leukaemia vaccine for use in A20 and WEHI-3 leukaemia-bearing mice (Dilloo D *et al.*, 1997) and in leukaemia patients (Rousseau RF *et al.*, 2006). In the A20 leukaemia model, IL-2 secreting fibroblasts synergised with CD40L-expressing fibroblasts to prevent outgrowth of pre-existing A20 leukaemia cells (Dilloo D *et al.*, 1997). IL-2 is transiently produced by both human and murine myeloid DC in response to microbial stimulation, and its secretion depends on T cell-derived IL-15. In addition, IL-2 secreted by murine DC plays a key role in NK cell activation [155 671]. Therefore, apart from the well-described effects of local IL-2 production on the clonal expansion and survival of T cells [672 463], transgenic IL-2 may also help to activate NK cells [464], an observation which was inferred in the report by Dilloo *et al.*, (1997) and which may apply to the clinical study reported by Rousseau *et al.*, (2006). Transgenic IL-2 likely explains how the efficacy of the combination IL-2- and CD40L-expressing vaccine depended on NK cells together $CD4^+$ and $CD8^+$ T cells (Dilloo D *et al.*, 1997). In this sense, the local transgenic production of IL-2 may simulate a microbial challenge to provide an additional immunostimulatory boost. In contrast, the effects of transgenic CD40L expression by neuro2a cells on murine neuroblastoma tumorigenicity depended only on the integrity of $CD8^+$ T cells (Grossmann ME et *al.*, 1997).

The results derived from the MB49 murine bladder cancer model provide insight into the conditions required for a CD40L-expressing tumour vaccine to induce immunological memory (Loskög A *et al.*, 2001). These data suggest that antigen presentation first occurs *in situ* and involves professional APC. Attempting to improve the antigen presenting function of MB49 tumour cells *per se* by transgenic CD80 expression was not sufficient to recruit anti-tumour immune effector mechanisms because transgenic CD80 expression had no effect on tumorigenicity. Hence, professional APC in the vicinity of the vaccine deposit may be critical for the generation of systemic immunity because memory generation may depend on which cells arrive at the 'scene of the accident' first.

Using the MB49 model, transgenic expression of IL-12 exhibited threshold effects because the tumorigenicity of IL-12-expressing MB49 tumour cells was reduced by increasing the per cell production of IL-12. The additive effect that transgenic CD40L expression had on reducing tumorigenicity may be explained by an increase in the local production of IL-12 that is mediated by CD40 ligation. Control experiments using either MB49 tumour cell lysates or tumour cells transduced with the empty adenovector showed lack of an effect on tumorigenicity and indicated that IL-12 and/or CD40L gene transfer engaged adaptive tumour-specific immunity rather innate or non-specific mechanisms such as TAM. Then, the striking observation was made that repeated immunisation of mice with both CD40L and IL-12 gene-modified live MB49 cells did not protect mice against parental tumour challenge whereas repeated immunisation of mice with CD40L gene-modified live MB49 cells did protect the mice (Loskög A *et al.*, 2001). The immediate inference is that tumour cells were destroyed locally before cross-priming could elicit adaptive immunity engendered by transgenic expression of CD40L. A working hypothesis is that IL-12 expression by live tumour cells rapidly induces NK cell recruitment and activation to clear tumour cells before professional APC transport the associated tumour antigens from the tumour site, which may be more likely if CD40 stimulated APC are delayed in their migration because of slower IKKα-dependent signalling [134].

That productive and systemic anti-tumour immunity was invoked by CD40L gene-modified MB49 tumour cells indicates that transgenic expression of CD40L did not induce maturation of DC before DC had captured TAA since CD40 ligation is one of the signals that converts DC from antigen capture to antigen presentation mode. Among the cell types likely to be constitutively present in the vicinity of a subcutaneously implanted and transgenic CD40L-expressing tumour vaccine are macrophages, sentinel DC such as dermal or interstitial DC, which are the APC able to activate naïve T cells [204]. In addition, monocytes recruited from the circulation may differentiate *in situ* to form phagocytically competent $CD11c^+$ DC [163]. One of the most efficient means for antigen loading of DC is phagocytosis of apoptotic cells [673 674 675 676 677]. Then, CD40L expressed by tumour cells or bystander fibroblasts in the vaccine will activate these infiltrating monocytes/macrophages and DC to augment antigen presenting functions and IL-12 production. Hence, a dynamic process is envisaged in which the early initiation of CD40-mediated tumour cell apoptosis, which may be enforced by paracrine CD40L expression, facilitates antigen capture as resident DC phagocytose the dying tumour cells. As more DC arrive at the scene to capture TAA, residual viable CD40L-expressing tumour cells provide the CD40-mediated maturation stimulus to accelerate passage to the draining lymph nodes of antigen-loaded DC now in efficient antigen presentation mode with up-regulated expression of MHC class I and II molecules, costimulatory molecules, cell adhesion molecules and immunostimulatory cytokines. As described in Section 1.5.4.5, the slow kinetics of CD40-induced IKKα-dependent signalling presumably ensures a smooth transition between antigen capture and antigen presentation modes of DC function (Figure 3).

Importantly, evidence that DC are present at the subcutaneous immunisation site was obtained in the clinical leukaemia vaccine study. One week after immunisation with the vaccine, three patients had evidence of a dermal infiltrate of phenotypically immature $CD1a^+$ DC, which had strong perinuclear but relatively weak membranous HLA-DR staining, and which lacked CD83 staining (Rousseau RF *et al.*, 2006). These findings are most consistent with immature DC operating in the immunological periphery in antigen capture mode. MHC class II-peptide complexes do not form unless immature DC are exposed to inflammatory stimuli such as TNF, LPS or CD40L. Then peptide is loaded in the MHC class II compartments of late endosomes and lysosomes and transported to the DC surface as TCR ligands [678].

6.3.5.6 CD40L gene-modified tumour vaccines induced systemic tumour-specific immunity

Immunity engendered by the IL-2- and CD40L-expressing leukaemia vaccine in mice included adaptive cellular immunity mediated by $CD4^+$ and $CD8^+$ T cells and innate cellular immunity mediated by NK cells (Dilloo D *et al.*, 1997). A comparable vaccine, which was used in leukaemia patients, elicited leukaemia-specific cellular immunity, which included leukaemia-specific $CD8^+$ T cells in peripheral blood and which was associated with a $CD8^+$ T cell infiltrate at the immunisation site (Rousseau RF *et al.*, 2006). $CD8^+$ T cells induced by CD40L-expressing tumour cells were responsible for tumour control in the murine neuroblastoma model (Grossmann ME et *al.*, 1997). Protective and systemic tumour-specific immunity was elicited by repeated immunisation with CD40L gene-modified tumour cells in the murine MB49 bladder cancer model, which may have been mediated by $CD4^+$ and/or $CD8^+$ T cells discovered early in tumour growth (Loskög A *et al.*, 2001).

6.3.5.7 IFNγ and tumorigenicity and immunogenicity

Studies of MCA-induced fibrosarcomas show that rejection of a tumour depends on its sensitivity to IFNγ. Conversely, tumours that are IFNγ-insensitive are poorly immunogenic and highly tumorigenic. Rejection of MCA-induced fibrosarcomas by LPS depends on IFNγ and not TNF. The mechanism of rejection depends in part on the IFNγ-mediated up-regulation of the MHC class I pathway of antigen processing and presentation in tumour cells because forced expression of components of this pathway can render IFNγ-insensitive tumours immunogenic. Other studies show that IFNγ treatment of tumours reduces tumour cell expression of H60, which is the murine ligand for the activating NKG2D receptor of NK cells, simultaneously with increased tumour cell expression of MHC class I molecules, which

are recognised by $CD8^+$ CTL. These results suggest that small tumours may be recognised and eliminated by NK cells whereas elimination of growing tumours may depend on the activity of the adaptive immune system. Other anti-tumour effects mediated by IFNγ include inhibition of cellular proliferation, induction of apoptosis *via* expression of Fas, caspases or TRAIL, and inhibition of angiogenesis *via* elaboration of the chemokine, IP-10. In addition, IFNγ further polarises the immune system toward type 1 cell-mediated immune responses [228]. Hence, CD40L transgene expressing vaccines may be successful because they induce professional APC to induce expression of IFNγ, which subsequently reduces tumorigenicity and increases immunogenicity of IFNγ-sensitive tumours.

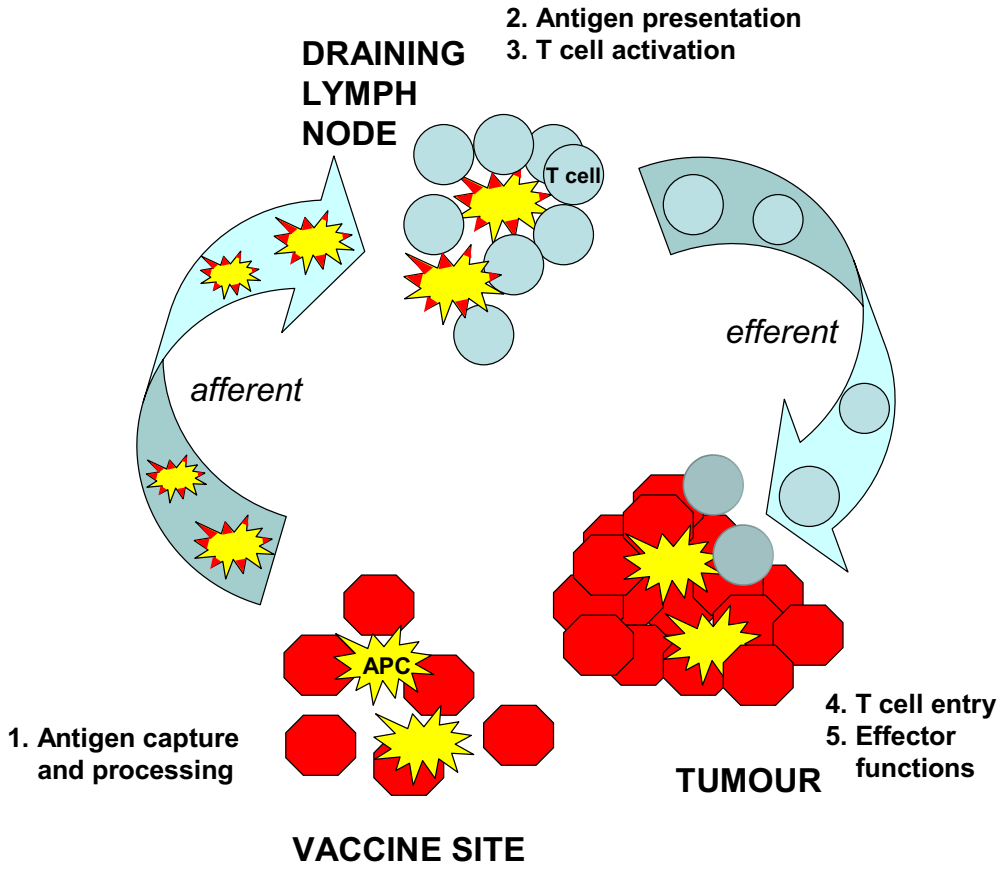

Figure 13: Anti-tumour cellular immune responses require effective antigen presentation

Spontaneous adaptive anti-tumour immune responses are often detected but are rarely associated with anti-tumour activity. Illustrated schematically are the steps required for the induction of tumour-directed effector T cell responses. Defects in each of these critical steps have been described in tumours and may be remedied by the use of therapeutic manoeuvres such as active and passive immunotherapies. Furthermore, CD40L gene-modified tumour vaccines may augment each step of immune activation to produce effective tumour-specific immunity (adapted from ref. 660).

In conclusion, subcutaneous deposition of antigens derived from dying tumour cells results in antigen capture by DC, and perhaps partial maturation of DC *in situ*. Transgenic CD40L expression *in situ* is hypothesised to augment antigen capture and presentation first by facilitating the attraction of DC, and second by accelerating the maturation and migration of DC to DLN. Consequently, T cell activation and clonal expansion is enhanced by CD40-activated and antigen-laden DC, and effector T cells enter the tumour to exert anti-tumour effects aided by CD40 activation. In the experiments in which anti-CD40 mAb was administered to tumour bearing mice following gemcitabine chemotherapy, an increase in tumour-infiltrating lymphocytes was observed [221]. This influx may have resulted from a chemotherapy-related inflammatory microenvironment, which may have included antigen-laden APC capable of restimulating and expanding intra-tumoral effector CTL. Furthermore,

using the same tumour model, anti-CD40 mAb treatment alone was shown to cause dissemination to the tumour of $CD8^+$ CTL, which had been activated in the TDLN. The ability of these CTL to promote tumour regression depended on continual CD40 stimulation [597] (Figure 13).

6.3.6 Lymphopenia and immunisation efficiency

Cytotoxic chemotherapy would seem to be incompatible with active immunotherapy because of its known and presumed effects on immune responses. For example, chemotherapy reduces rates of seroconversion in response to influenza immunisation particularly if patients are immunised at the time chemotherapy is given rather than between cycles of chemotherapy [679]. However, cytotoxic chemotherapy can enhance cancer immunotherapy *via* several potential mechanisms [680 656] to:

(i) induce cell death to increase the supply of tumour antigens, which allows the tumour to act as its own vaccine

(ii) overwhelm dead cell clearance mechanisms resulting in secondary necrotic cells, which induce partial DC maturation [681]

(iii) debulk the tumour chemically to reduce tumour-related immunosuppression, which may promote intra-tumoral T cell trafficking

(iv) deplete negative regulatory populations such as T regulatory cells using low-dose cyclophosphamide or murine splenic myeloid suppressor cells by using gemcitabine [682]

(v) induce lymphopenia with resultant homeostatic proliferation of potentially tumour-reactive lymphocytes thus providing a 'window of opportunity' for post-chemotherapy vaccination.

6.3.6.1 Anti-tumour effects of homeostatic proliferation in sub-lethally irradiated mice

Homeostatic proliferation of lymphocytes reliably occurs in mice in which lymphopenia is induced using sub-lethal irradiation (6Gy). It is believed that the homeostatic proliferation of lymphocytes is driven by the same process of self-MHC/peptide ligand recognition that underlies positive selection of lymphocytes in the thymus and so involves polyclonal proliferation of self-reactive lymphocytes of low avidity. Sub-lethally irradiated mice were infused with autologous or syngeneic naïve lymphocytes 24 hours after irradiation and then challenged with live syngeneic melanoma cells. Tumour growth was significantly retarded in a manner directly related to the dose of lymphocytes transferred. These anti-tumour effects were associated with induction of vitiligo in some cases. The anti-melanoma lymphocytes were $CD8^+$ and IFNγ-producing, and resulted in memory T cells because adoptive transfer also conferred protection against later re-challenge with melanoma. Moreover, the anti-tumour effects were shown to depend on the homeostatically proliferating T cells encountering tumour antigens in the tumour draining lymph nodes (TDLN). Sub-lethally irradiated mice bearing established melanomas also demonstrated significant tumour growth retardation after adoptive transfer of autologous or syngeneic naïve lymphocytes.

The authors suggested that immunisation against cancer antigens may be most effective in the immediate aftermath of lymphopenia-inducing anti-cancer treatment such as cytotoxic chemotherapy and/or irradiation when homeostatic proliferation is maximal [683]. While sub-lethal irradiation of mice may simulate lymphopenia-inducing cytotoxic chemotherapy and may seem similar to the lymphopenic state attending allogeneic bone marrow transplantation (alloBMT), the biology of immune reconstitution in these two different experimental settings may be sufficiently different for one not to assume that lymphocyte expansion in each setting has the same cause or set of effects.

6.3.6.2 Pre-transplant myeloablative conditioning; post-transplant anti-tumour immunisation

AlloBMT is effective primary or salvage treatment for haematological malignancies and, more recently although less satisfactorily, as treatment for metastatic renal cell carcinoma and melanoma [421]. Graft *versus* leukaemia/tumour effects have been exploited by the use of 'mini-

alloBMT'. In this regimen, T cell depletion of the donor inoculum is performed in conjunction with sub-myeloablative (or reduced-intensity) pre-transplant conditioning and post-transplant donor lymphocyte infusions. While T cell depletion reduces the incidence of GvHD and improves post-transplant immune reconstitution by relieving the immunosuppressive effects of GvHD, the risk of leukaemic relapse is heightened unless donor lymphocyte infusions are also given. Mini-alloBMT regimens establish a state of mixed chimerism in which the reconstituted immune system originates from both donor and recipient and causes donor lymphocytes to become tolerant to recipient antigens.

The post-transplant application of cancer vaccines would then seem to be favoured by two major conditions: (i) before transplantation, a state of minimal residual disease is created by induction chemotherapy and the transplant conditioning regimen, and (ii) expansion of donor lymphocytes that reconstitute the host during the lymphopenic post-transplant period. Just as clinical BMT studies indicate the need for post-BMT immunisation against pathogens to compensate for the inevitable reduction in donor-derived immunity; a case can be made for immunisation against minimal residual disease. In the study reported by Rousseau *et al*. (2006), fully myeloablative pre-transplant conditioning including total body irradiation (TBI) was administered. The leukaemia vaccine was administered more than 100 days after alloBMT when all patients were lymphopenic (lymphocyte range: 158-1,071/μL) and when all patients had been free of immunosuppressive GvHD prophylactic therapy for a range of 14-195 days. Although we presented evidence to support the immunogenicity of the transgenic CD40L-expressing leukaemia vaccine, an effect of this vaccine on clinical outcomes of the immunised patients could not be deduced because of the non-randomised nature of the study (Rousseau RF *et al*., 2006).

To analyse the effects of myeloablative conditioning upon the response to post-transplant immunisation, mice were lethally irradiated (11Gy) and reconstituted with syngeneic bone marrow. Full reconstitution with $CD4^+$ T cells and partial reconstitution with $CD8^+$ T cells occurred six weeks post-transplant. These transplanted mice were then immunised with irradiated GM-CSF gene-modified B16 melanoma cells four or six weeks post-transplant and then challenged with live B16 melanoma cells after a further one to four weeks. Of the mice immunised four weeks post-transplant, 39% were protected from tumour challenge but 77% of mice were protected if they had been immunised at the time of full $CD4^+$ T cell reconstitution six weeks post-transplant. In contrast, if lethally irradiated mice were transplanted with allogeneic bone marrow, immunisation with the GM-CSF melanoma vaccine six weeks post-transplant did not protect any mice. It was inferred that the high incidence of GvHD after alloBMT prevented tumour protection because GvHD markedly delays immune recovery. However, if the lethally irradiated mice received T cell-depleted allogeneic bone marrow (TCD-alloBMT) then GvHD did not occur and 71% of mice, which had been immunised with the GM-CSF melanoma vaccine six weeks post-transplant, were protected against live tumour challenge. At the time of immunisation, lymphocyte counts were normal apart from reduced $CD8^+$ T cell counts [684].

In TCD-alloBMT recipients that also received the GM-CSF melanoma vaccine, anti-melanoma T cell proliferative and cytokine responses were induced together with immunological memory because the mice resisted re-challenge with melanoma five months post-transplant. During the post-transplant period, mixed chimerism developed in these mice and tolerance to recipient antigens was confirmed. Therefore, to show that vaccine efficacy did not simply result from a lack of tolerance induction, the transplanted mice were exposed to melanoma antigens in the form of irradiated B16 cells during the period of post-transplant immune reconstitution, and tumour protection still occurred. Moreover, despite acquisition of the tolerance to host antigens in the recipient thymus that prevents post-immunisation GvHD, the immunisation with the tumour vaccine stimulated donor T cells to generate anti-tumour immunity. Although anti-melanoma immunity may have been directed against minor histocompatibility differences, a role for NK cell-mediated immunity in the protection of mice against live tumour challenge was not formally excluded. It was hypothesised that the anti-

tumour effects observed in this model system required a functional thymus for the *de novo* generation of T cells from donor stem cells because expansion of donor T cells was not considered likely after T cell depletion [684].

In adult humans, impairment of T cell reconstitution from age-related reductions in thymic regenerative capacity may be overcome by manipulations such as transient medical castration, which augments lymphopoiesis and the responses to immunisation [685]. The impact on immunisation efficiency of immune reconstitution that results from expansion of mature lymphocyte populations (homeostatic proliferation) in sub-lethally irradiated mice appears not to have been tested. However, the quality of immune reconstitution resulting from homeostatic proliferation may differ significantly from that resulting from the *de novo* generation of lymphocytes when successful immunisation post-TCD-alloBMT only occurred after full reconstitution with $CD4^+$ T cells [684]. These data suggest that further study is required to understand how best to harness both effects in the service of effective immunisation against malignancy.

6.3.7 Chemotherapy and CD40 activation synergise to produce anti-tumour immunity

Phagocytosis of apoptotic cells is an efficient and aphlogistic process, which prevents inadvertent autoimmune responses [686] [687], and which may be exploited for therapeutic purposes. Macrophages, which ingest apoptotic cells *in vitro*, subsequently present peptides derived from antigens in the engulfed apoptotic cells on surface MHC class I molecules in a TAP-dependent manner [688]. Similarly, DC engulf apoptotic cells and process them for presentation to both MHC class I- and class II-restricted T cells. When DC are incubated with a great excess of apoptotic cells, which simulates failure of *in vivo* clearance, the DC secrete IL-1β and TNF and undergo partial maturation [662] [681]. These data suggest that apoptotic cells, which have not been sequestered *in vivo,* may undergo secondary necrosis and thus become immunogenic.

The *in vivo* consequences of excess tumour cell death were studied in a murine model. In order to study tumour antigen cross-presentation *in vivo*, murine mesothelioma cells were genetically 'marked' with the nominal antigen, influenza haemagglutinin (HA), which allowed proliferation of an HA-specific $CD8^+$ T cell clone in the TDLN to be measured as an index of cross-presentation. Radiation bone marrow chimera experiments confirmed that the tumour-expressed HA was cross-presented by host APC rather than presented directly. Moreover, cross-presentation of the HA tumour antigen in the TDLN was constitutive because it was observed throughout the period of tumour growth, and produced little in the way of an anti-tumour immune response [240]. Importantly, cross-presentation ceased within several days if mice were cured by gemcitabine chemotherapy. Gemcitabine is a false nucleoside, which is phosphorylated inside the cell and which becomes incorporated into DNA thus terminating chain synthesis and disrupting cell division. Gemcitabine induces apoptotic cell death of mesothelioma cells [689], and in a murine syngeneic tumour model of a subcutaneously implanted mesothelioma, gemcitabine promptly and markedly reduced tumour volume.

Gemcitabine treatment of gemcitabine-sensitive tumours substantially increased T cell proliferation in TDLN, which indicated that more tumour antigen had become available for cross-presentation. In contrast, gemcitabine treatment of resistant mesotheliomas did not reduce tumour growth and did not result in cross-presentation of tumour-derived HA in the TDLN. In transgenic models in which HA is a self antigen, deletional tolerance of adoptively transferred clonogenic T cells occurs. Conversely, in the murine mesothelioma model, HA is a foreign antigen and, after gemcitabine treatment, the adoptively transferred HA-reactive T cell clone transiently expanded. Moreover, T cells in TDLN, but not spleen, displayed *in vivo* cytolytic activity in response to a specific MHC class I-restricted HA peptide, which indicated that tolerance had not been induced [241].

However, the induction of cytolytic activity in the TDLN did not produce anti-mesothelioma effects until CD40 was ligated. The combination of gemcitabine chemotherapy and the

activating FGK45 anti-CD40 antibody (chemoimmunotherapy) synergised to cure 40-80% of mice with established subcutaneous tumours of transplanted mesothelioma whereas neither treatment alone cured mice, although tumour growth was retarded. In addition, in comparison with gemcitabine treatment alone, the combination treatment increased tumour infiltration with $CD4^+$ and $CD8^+$ T cells. Cured mice resisted re-challenge with parental tumour indicating that immunological memory had developed. The success of the chemoimmunotherapy was schedule-dependent: anti-CD40 mAb treatment was only effective if it was administered after chemotherapy, and chemical debulking of the tumour was essential for this effect. Neither the combination of anti-CD40 mAb treatment with gemcitabine treatment of resistant mesothelioma nor surgical debulking of mesothelioma produced cures [221].

In vivo depletion of $CD4^+$ T cells indicated that anti-CD40 mAb treatment bypassed the requirement for CD4 T cell help. Moreover, *in vivo* depletion of $CD8^+$ T cells showed that successful chemoimmunotherapy depended on an intact $CD8^+$ T cell population. The dramatically positive effect of CD40 ligation on the survival of mesothelioma-bearing mice treated with gemcitabine suggested that tumour cell death had primed host APC for full activation of $CD8^+$ CTL [221]. These data would be most consistent with the hypothesis that gemcitabine treatment created an excess of dead tumour cells, which would be engulfed by phagocytes including DC locally and/or in the TLDN, and that the associated tumour antigens would be processed and presented to cognate T cells. Subsequently, ligation of CD40 on these antigen-laden DC primed the tumour-reactive CTL.

As discussed in Section 6.3.3.3, intra-tumoral injection of adenovirally expressed mCD40L (AdmCD40L) inhibited growth of the poorly immunogenic Lewis Lung Carcinoma (LLC) but did not induce remissions in LLC-bearing mice [648]. However, 40% of LLC-bearing mice, which were given a single intraperitoneal injection of cisplatin concomitantly with a single intra-tumoral injection of AdmCD40L, were cured whereas no mice were cured with cisplatin alone, and only 10% were cured with AdmCD40L alone. Cisplatin treatment of LLC cells *in vitro* induced expression of Fas, and Fas-expressing LLC cells were more susceptible to Fas-mediated apoptosis. Moreover, the cure of tumour-bearing mice treated with the combination of cisplatin and AdmCD40L was abrogated in a FasL-deficient genetic background. The lytic capacity of splenocytes, which were obtained from LLC-bearing mice after an intra-tumoral injection of AdmCD40L, was tested against cisplatin-treated or untreated of LLC target cells in an *in vitro* recall assay. Using appropriate controls, significant lysis was observed only against cisplatin-treated LLC targets in the presence of splenocytes from AdmCD40L-exposed wild type mice and not from FasL-deficient mice [667]. The up-regulation of Fas in response to DNA-damaging cisplatin treatment was found to be p53 dependent [690]. Together, these data suggested that cure of LLC after combination treatment with cisplatin and AdmCD40L resulted from the induction of LLC-specific effector CTL that exerted Fas-mediated destruction of LLC cells [667].

6.3.8 Future systemic CD40-directed chemoimmunotherapy

Our published models (Grossmann ME *et al.*, 1997; Dilloo D *et al.*, 1997; Loskög A *et al.*, 2001; Dotti G *et al.*, 2001; Rousseau RF *et al.*, 2006) illustrate that transgenic CD40L expression may directly or indirectly produce therapeutic effects *via* a number of possible mechanisms, which include:

1. for non-CD40-expressing malignancies
    (i) Recruitment and activation of bystander APC
    (ii) Paracrine effects derived from CD40L-expressing bystander fibroblasts
2. for CD40-expressing malignancies
    (i) Activation of the antigen presenting functions of malignant cells
    (ii) Induction of CD40-mediated apoptotic cell death, and enhanced APC uptake of apoptotic tumour cells to increase cross-priming efficiency

(iii) Recruitment and activation of bystander APC

Hence, CD40L/CD40 interactions may promote tumour immunogenicity by at least activating normal host APC functions. In addition, CD40 stimulation may enhance the antigen processing and presenting functions of tumour cells *per se* and may, after CD40 stimulation induces tumour cell apoptosis, include cross-presentation by CD40-activated APC of apoptotic tumour cells. In this respect, cross-presentation by DC of TAA derived from dead tumour cells is more efficient than cross-presentation of soluble protein antigens by several orders of magnitude [206]. Consequently, the positive impact that CD40L/CD40 interactions have on tumour eradication and tumour protection suggests that CD40L/CD40 interactions play a significant role in tumour immunosurveillance (Chapter 2).

Preclinical and clinical data suggest that CD40-expressing haematological tumours, which often have a surface complement of immunostimulatory molecules and which may readily undergo apoptosis in response to CD40 stimulation, may be more effective inducers of anti-tumour immunity upon CD40 ligation than non-haematological tumours. Nevertheless, favourable results of intra-vesical AdmCD40L immunogene therapy in the murine MB49 orthotopic bladder cancer model [418] have led to the institution of a phase I/II trial in bladder cancer patients of AdCD40L and the transduction enhancer, Clorpactin, which is already used clinically to treat interstitial cystitis (T Tötterman, personal communication). In contrast, conventional BCG treatment for *in situ* bladder cancer does not induce CTL and requires repeated intra-vesical treatments to reduce bladder cancer progression [417].

Clinically promising preclinical murine data demonstrate that schedule-dependent use of chemotherapy and anti-CD40 mAb treatment produces anti-tumour synergy, which is based on a cellular immune mechanism [221]. Moreover, *in vitro* data show that combining CD40 stimulation with inhibitors of protein synthesis such as the cytotoxic drugs, etoposide and 5-FU (but not doxorubicin) [602], or the mammalian target of rapamycin (mTOR) inhibitor, rapamycin [524], synergise to cause apoptosis. Apoptosis is hypothesised to result because CD40-induced synthesis of a pro-survival factor such as cFLIP [602 524] fails because protein synthesis overall is blocked by the cytotoxic drug or mTOR inhibitor. Here, the mechanism of synergy depends on a 'synthetic lethality' effect. The concept of synthetic lethality derives from yeast genetics when it was discovered that, whereas mutations in two genes individually did not cause cell death, mutations in the two genes together did. When applied to cancer treatment, synthetic lethality describes the circumstances in which mutations in certain tumour suppressor or metabolic genes cause tumour cells to rely for their viability on one other druggable mechanism whereas normal cells depend on more than one mechanism to remain viable and, therefore, normal cells are affected minimally by the drug of interest. Hence, the concept of synthetic lethality applies particularly to tumours in which, although the founding mutation is not itself druggable, the tumour is 'set up for a fall' because it depends for its survival on a druggable mechanism [691].

The mTOR pathway is of particular interest because it plays a critical role in the control of cell growth and proliferation [692] by integrating mitogenic signals and intracellular nutrient levels in a PI3K/Akt-dependent manner to control protein translation and cell cycle progression [693]. Inhibitors of mTOR such as everolimus and temsirolimus are already in clinical trials of lung and breast cancer patients, and potentially could be applied in combination with CD40 activating treatments such as anti-CD40 monoclonal antibodies, which have been tested clinically [694].

A single dose of a CD40 agonist mAb was evaluated in a phase I dose-escalation study of 29 patients with advanced solid tumors. The maximum tolerated dose (MTD) was determined to be 0.2mg/kg because dose-limiting toxicity of venous thrombo-embolism and grade 3 headache was observed in two of seven patients at the 0.3mg/kg dose level. Transient liver function abnormalities were observed 24 to 48 hours after infusion. Peripheral blood B cells were depleted by 93% for less than one week at the MTD, and the remaining B cells demonstrated a dose-related up-regulation of costimulatory molecules after treatment. Four patients with melanoma had objective partial responses when they were restaged at day 43 [694].

Two major global pharmaceutical companies each have an anti-CD40 mAb and these products are currently being tested in phase II clinical trials in a variety of carcinoma indications. On the other hand, clinical development of recombinant human CD40L trimer was not further pursued after asymptomatic grade 3 transaminitis was demonstrated at a dose of 0.1mg/kg/day [572] (see Section 6.1.6).

Genetically engineered whole tumour cell vaccines may have the advantage of being individualised but preparation of these vaccines is expensive, resource-intensive, un-standardised, and, therefore, these vaccines are not likely to be widely marketable as commercial products. Therefore, future clinical use of CD40-directed immunotherapy may involve mass-marketed pharmaceutical products such as CD40 agonist monoclonal antibodies. Hence, CD40-directed immunotherapy may not only have direct effects on $CD40^+$ tumour cells and indirect effects *via* $CD40^+$ APC but may also operate in conjunction with cytotoxic chemotherapy and/or pro-apoptotic drugs. Ultimately, further potential for synergy may exist if CD40 signalling contributes to tumour cell apoptosis, which then fuels a developing anti-tumour immune response.

Nonetheless, the clinical development of anti-CD40 mAb in cancer indications may show that other treatments are needed in the anti-cancer regimen to maximise its therapeutic impact such as cytokines, TLR agonists, or neutralising antibody or small molecule inhibitor approaches to overcome tumour immunosuppression mounted by regulatory T cells, TGFβ, IL-10, suppressor of cytokine signalling-1 (SOCS1) or programmed death ligand-1 (PDL1) [152].

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APPENDIX A

STATEMENTS OF THE CONTRIBUTIONS OF JOINTLY AUTHORED PAPERS

Each publication included in this thesis was jointly authored. As permitted by Professor Richard Russell, Dean of Graduate Studies, University of Adelaide, appended to this thesis is a statement for each publication, which gives written and signed permission by each author for the paper to be included in the thesis and which provides a detailed description of the contribution made by the PhD candidate as an author on each paper.