THE ROLE OF CD40 AND CD137 IN IMMUNE RESPONSES TO CANCER: A STUDY OF MONOCLONAL ANTIBODY MEDIATED IMMUNOMODULATION IN TWO MURINE TUMOUR MODELS.

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Doctor of Medicine

February 2003

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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SCHOOL OF MEDICINE

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THE ROLE OF CD40 AND CD137 IN IMMUNE RESPONSES TO CANCER: A STUDY OF MONOCLONAL ANTIBODY MEDIATED IMMUNOMODULATION IN TWO MURINE TUMOUR MODELS.

By Michael Green, MB ChB, FRCS

Immunomodulation is the alteration of an immune response to a desired level. In the context of cancer immunotherapy this implies the amplification of a potential but unfulfilled response. CD40 and CD137 are cell surface receptors thought to be critical in the control of cellular immune responses. Specific monoclonal antibodies (mAb) against CD40 and CD137 were used to deliver a signal through the ligation and crosslinking of the surface receptor. The character of these responses and potential for immunotherapy were investigated *in vitro* and *in vivo* using two murine cancer models, CT26 and B16.

FACS analysis of CT26 and B16 demonstrated that neither expresses CD40 or CD137. Furthermore the mAb used, FGK45, 3/23 (CD40) and Lob 12 (CD137), had no effect on tumour growth *in vitro*.

CT26-bearing BALB/c and B16-bearing C57Bl6 mice were treated with anti-CD40 mAb. Growth of established subcutaneous (sc) and intradermal (id) CT26 was significantly reduced. In contrast there was no protection from pulmonary CT26. Pulmonary B16 deposits were significantly reduced with anti-CD40 mAb.

The pulmonary $CD8^+$ lymphocyte population increased in response to anti-CD40 mAb. In BALB/c mice this was partly tumour dependent, but tumour independent in C57Bl6 mice. The $CD19^+$ population also expanded but the $CD4^+$ cell number was unaffected by anti-CD40 mAb.

The response to anti-CD40 mAb was found to involve IFN-γ, but this cytokine alone had no *in vivo* effect on the growth of CT26, suggesting that anti-CD40 mAb was not effective merely through increased levels of IFN-γ.

Selective depletion of $CD4^+$ and $CD8^+$ cells demonstrated that tumour abrogation with anti-CD40 mAb was dependent on $CD8^+$ cells, but did not require $CD4^+$ cells. The specific effector cells could not be isolated *in vitro*.

Transfection of CD40 ligand into CT26 cells gave greatly enhanced protection against tumour growth compared with anti-CD40 mAb.

Anti-CD137 mAb abolished growth of id CT26 and offered long-term protection against re-challenge with CT26. It abrogated sc tumour growth but failed to protect against pulmonary tumour. Selective depletion of $CD8^+$ and $CD4^+$ lymphocytes confirmed that both were required for therapy.

Immunomudulation through CD40 and CD137 can result in a powerful and enduring response to tumour antigen that may be useful in the development of novel strategies in cancer immunotherapy.

Contents

Acknowledgements

I would like to thank Martin Glennie, Alison Tutt and Ruth French for all their time and help with my research and the preparation of this thesis, and James Smallwood for getting things started. I would also like to thank Claude Chan for his practical help with the PCR and tumour cell transfection.

I am indebted to the Wessex Cancer Trust for their funding of this research.

Abbreviations used:

APC	Antigen presenting cell
ADCC	Antibody dependent cell-mediated cytotoxicity
AFP	Alpha-fetoprotein
AICD	Activation-induced cell death
AIDS	Aquired Immune Deficiency Syndrome
ANOVA	Analysis of variance
BCG	Bacillus Calmette-Guerin
BCR	B cell receptor
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CT26	Colon tumour 26
CTL	Cytotoxic T lymphocyte
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
EGP	Epithelial glycoprotein
ELISA	Enzyme-linked immunsorbant assay
ER	Endoplasmic reticulum
FASC	Fluorescence activated cell scanning
FCS	Foetal calf serum
FITC	Fluorescein iso-thiocyanate
FSC	Forward scatter cells
GM-CSF	Granulocyte monocyte colony stimulating factor
GTC	Guanidinium thiocyanate
HAMA	Human anti-mouse antibodies
HGPRT	Hypoxanthine:guanidine phosphoribosyl transferase
HIGM	Hyper-IgM syndrome
HLA	Human leucocyte antigen
HSP	Heat shock protein
ICAM	Intercellular adhesion molecules

id	Intradermal
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iv	Intravenous
LAK	Lymphokine activated killer
LFA	Lymphocyte function-associated antigen
mAb	Monoclonal antibody
MHC	Major histocompatability complex
MMP	Matrix metalloproteinase
NK	Natural killer
NO	Nitrous oxide
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PE	Phyco-erythrin
PEG	Polyethylene glycol
PTK	Protein tyrosine kinases
sc	Subcutaneous
SCID	Severe combined immunodeficiency disorder
sIg	Surface immunoglobulin
SLE	Systemic lupus erythematosis
SSC	Side scatter cells
TAA	Tumour associated antigen
TAP	Transporters associated with Antigen Presentation
TCC	Transitional cell carcinoma
TCR	T cell receptor
TGF	Transforming growth factor
TIDC	Tumour infiltrating dendritic cells
TNF	Tumour necrosis factor
TRAF	TNF-R associated factors
TSA	Tumour specific antigen

Chapter 1

The priniciples of tumour immunology and immunotherapy

1.1 Introduction

Our understanding of tumour immunology has advanced considerably since the time of Hericourt and Richet, who in 1895 reported their attempts to vaccinate against tumour using the sera from a patient with sarcoma [1]. This met with little success, but the idea that tumours are 'foreign' in the sense of infectious diseases has persisted. Although we now know that malignant disease is not infectious, many tumours express antigens that may be recognised and therefore targeted by the immune system. This characteristic remains central to attempts to treat cancer by immunotherapy.

In this chapter modalities of immunotherapy will be discussed in more detail, including tumour antigens of clinical significance, the basic immunology of these antigens and the immune responses to them. From this the development and use of successful immunotherapeutic strategies will be outlined, many of which are entering mainstream clinical practice, including that of monoclonal antibodies.

Early investigations (reviewed by Robins[2]) revealed strong circumstantial evidence, such as the infiltration of tumours by immune cells, that the immune system responds to cancer but gave little insight into why this was occurring or why it failed to eliminate the disease. Despite over a century of research, immunotherapy of cancer remains inadequate when compared with surgery, radiotherapy and chemotherapy. This is perhaps surprising when compared with the advances made in vaccination against infectious disease, the treatment of immune diseases such as rheumatoid arthritis and in organ transplantation.

1.2 Principles of tumour immunotherapy.

Tumour immunotherapy can be specific or non-specific. Specific immunotherapy relies on the expression of unique antigens by the tumour; therapy is directed at these specific antigens. This may be passive, as in the early experiments of Hericourt and Richet and more recently with monoclonal antibody (mAb) such as 17-1A in colorectal cancer[3], or active, by vaccination with specific antigen to induce an immune response against the tumour, such as melanoma fragments[4].

Non-specific immunotherapy relies on the assumption that cancer cells constitutively express antigens with the potential for an immune response that is not realised without intervention. Various methods have been employed to activate or up-regulate this underlying potential. Unlike specific immunotherapy it is not necessary for the relevant antigen to be identified and characterised in the design of the therapeutic strategy.

The underlying pathways and signalling sequences involved in determining the character of any immune response, whether it be humoral or cellular, stimulatory or inhibitory, are open to manipulation (immunomodulation). Recent focus has been on utilising a combination of both specific and non-specific approaches to immunotherapy[5]. It is anticipated that the introduction of a non-immunogenic antigen combined with appropriate stimulation will activate immune cells against this antigen. This amounts to the induction of controlled auto-immunity [6].

1.3 Tumour antigens

Tumours produce a few abnormal proteins that may serve as antigenic epitopes under the right circumstances. The expression of these antigens may be critical to the malignant potential of the cell, or may be independent of the growth and spread of the cancer. The aim of immunotherapy is to eliminate tumour cells expressing these antigens and leave only cells that are not a threat, avoiding collateral or bystander tissue damage.

Tumour antigens may be tumour specific (TSA) or tumour associated (TAA). They may be expressed as intracellular (cytosolic or nuclear) molecules, or on the cell surface as cell membrane components. Transformations due to chemical and physical insults result in unique TSA specific to the clonal tumour cell line. If such a tumour elicits an immune response then the general rule is that this is not transferable except in tumour clones in syngeneic animal models. Clearly this has implications for the use of these models in the study of tumour immunology and the development of immunotherapeutic strategies against such cancers.

Virally induced tumours also express TSA but because these antigens are derived from viral components they are common to all tumours induced by that virus. The nature of these antigens has been studied in animal models using SV40 and polyoma viruses [7]. The role of virus dependent malignant transformation in humans is less

clear, but Epstein-Barr and human papilloma virus are strongly implicated in Burkitt's lymphoma [8] and cervical cancer respectively [9].

TSA are less commonly found in association with spontaneous tumours, and this may be a result of the early elimination of these malignant cells by immunosurveillance. Despite the limitations of using TSA as targets for immunotherapy, some human tumours do have common specific antigens[10], described in table 1.2, which might be suitable. However these have had limited clinical impact although MUC-1 [11, 12] and melanoma antigens [13] have been found to elicit a reproducible immune response, confirmed by the recognition of the tumour antigen by autologous T lymphocytes from patients with tumours expressing these antigens.

B cell surface Ig (sIg) is unique to a B cell, as it corresponds to a specific antigenic epitope. Similarly the sIg from a B cell lymphoma is unique to that patient's tumour cells and is called the idiotype. The accessibility of this antigen has made it the target for 'custom made' tumour vaccines [14].

The majority of the antigens described in table 1.1 are internal proteins and only expressed on the tumour cell surface in the context of MHC I, implying that any immune response will cellular rather than humoral. This limits the use of mAb against many tumour antigens, as it will be ineffective against epitopes that are internal or MHC-bound.

Embryonic antigens are peptides normally only expressed during foetal development, at a time of immunological immaturity. Expression is switched off in mature tissue before the immune system develops any memory of the antigen. At some point in the malignant transformation of a cell the gene responsible becomes re-activated and the embryonic gene product is expressed once again.

Abnormalities in nuclear regulatory proteins such as Ras and p53 may be considered as tumour antigens. However non-malignant cells also express the abnormal protein, defining them as TAA rather than TSA. They are usually specific to an individual but may be inherited, betraying a strong family history of cancer. Their clinical role is in identification of such a risk and genetic counselling, but they may be useful in immunotherapy or gene therapy.

The fusion protein BCR-ABL is associated with chronic myeloid leakaemia and is the result of the formation of abnormal gene product by the chromosome translocation t(9:22). This is also a TAA in that normal cells from these individuals express it.

Type of antigen	Antigen	Nature of antigen	Associated tumour
Embryonic (oncofetal)	MAGE BAGE CAGE	Germ cell line	Melanoma, breast, Glioma
Abnormal post-transformational modification	MUC-1	Underglycosylated mucin	Breast, pancreas
Differentiation	Tyrosinase	Melanin synthesis	Melanoma
Differentiation	Surface Ig	Specific antibody in B cell clone	Lymphoma
Mutated oncogene	Ras	GTP-binding protein (signal transduction)	Many tumours
Mutated oncogene	p53	Cell cycle regulator	Lung, breast, gastrointestinal, brain, haematological
Fusion protein	BCR-ABL	Tyrosine kinase activity; Philadelphia chromosome	Chronic myeloid leukaemia
Oncoviral protein	HPV type 16 E6 and E7 proteins	Viral transforming gene products	Cervical carcinoma

Table 1.1. Human tumour specific antigens. (adapted from Immunobiology, Janeway&Travers[15] and Boone et al[10]).

Most cancers do not express common TSA, but individual tumours from an individual may express unique TSA. This limits the use of tumour specific immunotherapeutic strategies to tailor made, antigen-specific immunotherapy. At current levels of technology and resources this would be impractical. By the time an antibody or DNA vaccine (see below) had been constructed it might be too late for any therapeutic benefit to be realised.

There is increasing evidence that the majority of tumours express TAA (reviewed in by Nawrocki et al[16]). These antigens are expressed at low levels by normal cells but are over-expressed by malignant cells. The fact that they are known to the mature immune system implies that they are recognised as self rather than foreign. However their overexpression may render tumour cells vulnerable to attack by the immune system (reviewed by Ionnades et al[17]).

TAA (table 1.2) may be classified according to the nature of the protein from which they are derived, in a similar way to TSA. Several embryonic antigens have been identified that are expressed at low level by normal tissues, but at greatly increased levels by several tumour types. Carcinoembryionic antigen (CEA) and alpha-fetoprotein (AFP) are soluble and membrane-bound proteins that have a significant

clinical role in monitoring the remission of colonic and testicular carcinoma following treatment[18, 19]. Because levels are variable between healthy individuals they are not helpful in initial diagnosis.

Type of antigen	Antigen	Nature of antigen	Associated tumour
Cell surface protein	CD20	B cell trans-membrane molecule	B cell lymphoma
Cell surface protein	17.1A	Epithelial glycoprotein	Colon
Cell surface receptor	P97	Epithelial growth factor receptor	Melanoma
Cell surface receptor	HER2/Neu	Epithelial growth factor receptor	Breast
Embryonic	CEA	Membrane glycoprotein	Breast, Colon, Pancreas, Lung
Embryonic	AFP	Membrane glycoprotein	Hepatocellular carcinoma

Table 1.2. Human TAA (adapted from Immunobiology, Janeway&Travers[15] and Boone et al[10]).

Table 1.2 is not an exhaustive list of TAA. It is representative of the better known antigens of clinical significance. The immune system will often ignore TAA as they are recognised as self. It is interesting to note that many of these antigens are expressed on the cell surface, and there are reasons why these rather than intracellular antigens have been characterised. Their presence on the cell surface has facilitated their identification through the generation of antibodies by immunisation. An abnormal increase in the expression of intracellular (cytosolic or nuclear) proteins is more difficult to identify as only MHC I restricted fragments are expressed on the cell surface. This makes identification of the whole protein very difficult. However, with the increasing importance of cellular immune responses to immunotherapy and new techniques for the identification of MHC-restricted TAA[10], the list of potential antigens is rapidly increasing.

1.4 Immune responses to tumour antigens

The immune response to antigen is classified according to the type of helper T cells involved. Cellular responses are primarily effected by cytotoxic T cells (CTL) and are dependent on antigen recognition by the T_H1 subset of helper T cells. Humoral responses are effected through antibody production by B cells. Each response has a

characteristic cytokine profile. The cellular response to tumour antigens is discussed in detail in chapter 3.

The immune system is not inert, awaiting activation through a specific signal. It is dynamic, constantly responding to endogenous and exogenous antigenic stimuli. However the use of artificial stimuli or inhibitions modulates the response of the immune system, favouring a specific outcome.

Humoral immune responses to a variety of tumour antigens were the first to be observed, and may be induced through vaccination. Tumour antigen binds to the B cell receptor (BCR) of naïve B cells (figure 1.1). The BCR/antigen complex is then internalised, processed and MHC II restricted antigen epitopes presented to helper T cells. If the T cell recognises the antigen then it is able to help the B cell to mature through specific cellular signals and cytokine release. The activated B cell proliferates, matures and secretes antigen specific antibody. Because more than one epitope on an antigen may be recognised by a number B cells and T cells, the overall profile of the Ig response is polyclonal, i.e. more than one epitope on an antigen may be recognised.

Antibody dependent tumour cell killing by the immune system requires that antibody binds tumour cell surface antigen and is able to recruit either natural effectors, such as natural killer (NK) cells (figure 1.2), macrophages and neutrophils, or complement. The effector cell is then able to bind to the antibody Fcγ (see section 3.2). The mechanism of tumour cell killing depends on the character and armoury of the effector cell. Macrophages and particularly neutrophils release a battery of toxins including H^+, NO, O_2^-, H_2O_2 and specific competitors to essential co-enzymes and substrates. This results in tumour (and effector and bystander) necrosis. Natural killer (NK) cells are more selective and release granules onto the surface of individual tumour cells. These granules contain perforin, which creates holes in the cell membrane. NK cells signal through Fas and TNF-α, which cause tumour cell apoptosis. There is also evidence that NK cells have specific receptors for changes in the tumour cell membrane, such as down regulation of MHC I, that allow for similar programmed tumour cell death [20].

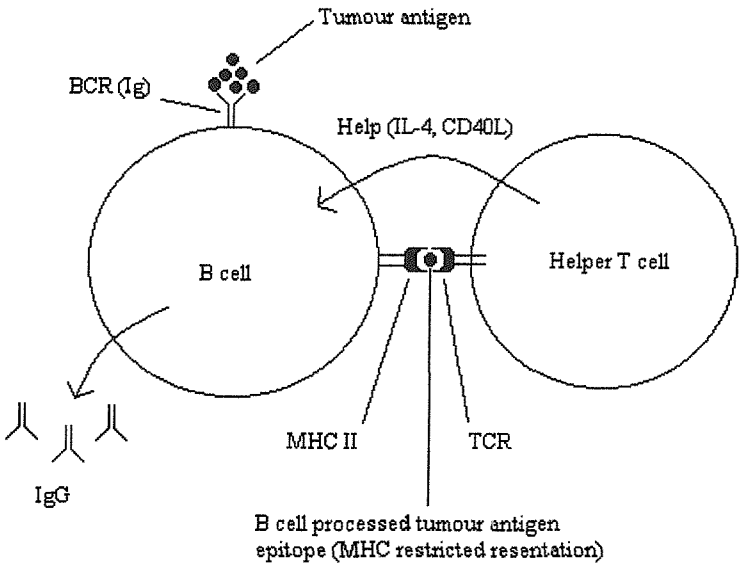


Figure 1.1. MHC II restricted presentation of tumour antigen to T_H2 cells results in a humoral immune response.

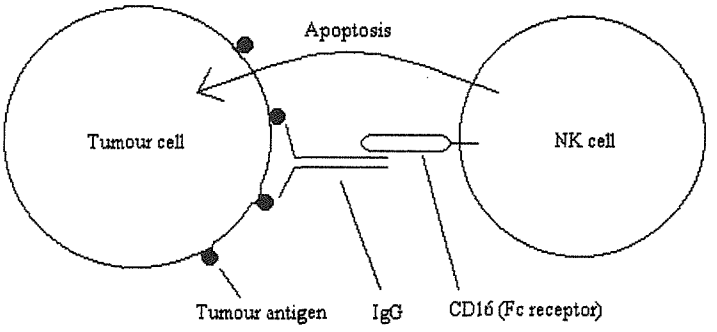


Figure 1.2. Tumour antigen recognition by NK cells is IgG dependent.

The classical complement pathway is activated by antigen-bound antibody and results in the complement cascade releasing opsonins and chemokines capable of augmenting the response of natural effectors.

An important limitation of these systems for the recognition of tumour antigens, and subsequent elimination of tumour cells expressing these antigens is that they are only effective against cell-surface antigens. Furthermore a complex system of control proteins prevents natural effectors engaging self-antigen bound antibody.

An important concept in immunology is that of immunosurveillance, postulated by Paul Erlich in the early 1900s and redefined in the 1970s[21]. This process relies on

the constant 'inspection' of MHC I restricted antigen, which is presented on the cell surface, by the immune system. All normal eukaryotic cells express MHC I. The immune response to MHC I restricted antigen is cellular (T_H1). Furthermore it is generally recognised that T_H1 cytotoxic responses to tumour antigen are the most effective in inhibiting tumour growth and tumour cell killing[10]. The role of humoral immunity and natural effectors in immunosurveillance is less certain but has been invoked in immunotherapeutic strategies.

Potential antigens are processed by APCs and presented to cytotoxic T cells through a mechanism called 'cross-priming' (section 3.4, and figure 3.3). If an antigen is recognised as foreign then armed CTL are generated. These CTL recognise antigen epitopes in the context of MHC I restriction and will induce apoptosis in cells expressing that antigen. IFN-γ released by CTL increases MHC I expression by cells, enhancing antigen presentation. The advantage of antigen presentation through MHC I is that both intracellular and membrane bound antigenic epitopes will be represented. Furthermore tumour cell killing is specific and localised, minimising bystander damage.

1.5 The development of monoclonal antibodies and their role in cancer treatment.

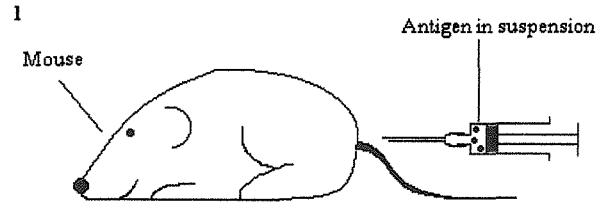
1.5.1 Introduction

Although cellular immune responses are of primary importance to this project, it is important that they are seen in the context of immunotherapy as a whole and the importance of antibodies to immunotherapy. Furthermore the methods used in this project are dependent on monoclonal antibodies. In 1975 Kohler and Milstein[22] announced the first mAb. For the first time specific mAb could be produced in limitless quantities. Antigens on a cell surface could be recognised and targeted, paving the way for unique strategies in the battle against cancer.

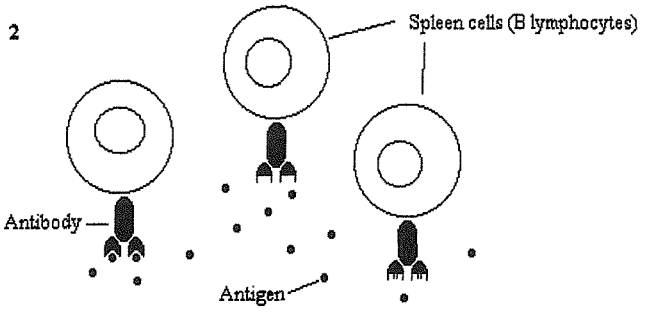
1.5.2 The production of monoclonal antibodies

Monoclonal antibody can be produced through immunisation and cell fusion or by genetic engineering. Kohler and Milstein described a process where splenic cells from an immunised mouse are fused with an immortal cell line, resulting in a hybridoma. The cell secreting the specific antibody can be identified and cloned. When grown in bulk the clones will secrete a unique monoclonal antibody (figure 1.3).

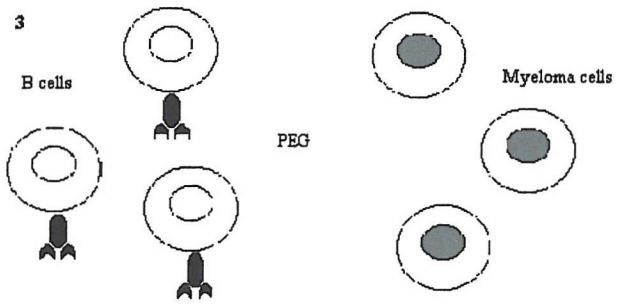
Figure 1.3. The production of monoclonal antibodies:



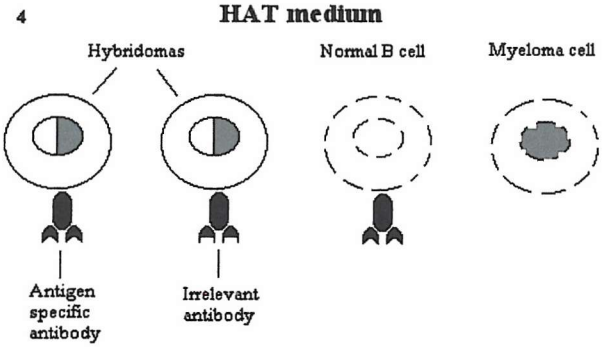
1. Mice are immunised with the antigen against which an antibody is required, usually with a booster injection three days before they are required.



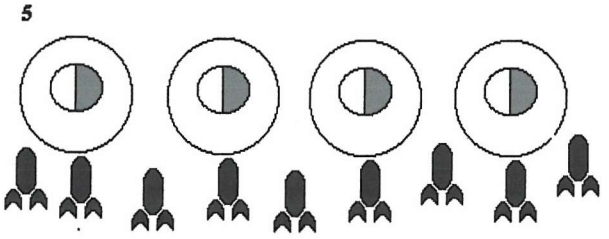
2. The immunised mice are killed and spleen cells are harvested including a large number of B cells, some of which produce antibody.



3. The spleen cells are mixed with immortal myeloma cells in medium containing polyethylene glycol (PEG). Myeloma cells for this procedure are selected on the basis that they do not secrete antibody themselves and that they lack the enzyme hypoxanthine:guanidine phosphoribosyl transferase (HGPRT). This causes a proportion of the cells to fuse.



4. The cells are transferred into a medium containing hypoxanthine-aminopterin-thymidine, which kills un-fused myeloma cells. Un-fused B cells are not immortal and die after a few days.



5. The surviving hybridomas are cloned individually, then screened for the specificity of secreted antibody. The monoclonal population is massively expanded to yield adequate quantities of mAb.

Early uses of mAb involved the identification of common tumour antigens. Libraries have been established to catalogue mAb that are able to bind to specific tumours antigens in addition to a vast range of other tissue surface markers (e.g. Serotec antibody location service, www.serotec.co.uk/anti). The therapeutic benefits of anti-tumour mAb have been investigated and a diverse range of experimental and clinical models has been developed. Although early results were promising in some diseases, particularly lymophoma [23] and colon cancer [3] they have not necessarily equalled or exceeded the therapeutic benefits of conventional modalities of treatment. The limitations of mAb are well documented and are determined by the characteristics of mAb, the immune system and target tumours.

Murine immunoglobulin has a short half-life, particularly IgG, which is the most commonly used mAb isotype, and repeated infusions of large quantities are required. Larger mAb constructs have prolonged half-life but reduced tissue penetration.

For an anti-tumour mAb to be an effective therapy it must have a large volume of distribution. Such systemic therapy allows effective targeting of metastatic disease but it also requires the administration of large amounts of potentially toxic mAb,

Although there is considerable structural homogeneity between the Fc regions of antibody from other species there is sufficient difference to prevent effective cellular activation or complement cascade. Furthermore, human anti-rodent antibodies (e.g. human anti-mouse antibodies, HAMA) are produced which may recognise epitopes on xeno-mAb which is then more rapidly cleared from the circulation and may even result in an anti-serum type reaction, although this is rare. This has limited the use of xeno-mAb raised against human antigen.

There is also concern that the mAb molecule may be unable to diffuse through capillary walls and penetrate larger tumours at an adequate concentration because of its molecular size and the high interstitial pressure found in many tumours[24] [25].

Methods to overcome these problems and to potentiate the effect of mAb have been proposed. These are directed at increasing tumour penetration, enhancing antigen presentation and introducing enhanced effector cells. Induction of a local inflammatory response, for example with cytokines, allows extravasation of mAb through leaky blood vessels and an influx of effector cells. Interferon-γ may increase expression of antigen, enhancing mAb binding.

Adoptive transfer of enhanced effector cells such as lymphokine activated killer (LAK) cells also increases mAb efficacy. Under normal circumstances only a small number of natural killer cells, with the ability to recognise Fc and thus kill tumour cells, are in circulation. This limits the therapeutic potential of simple monoclonal antibodies. NK cells can be cultured *in vitro* in cytokine-enriched medium and the expanded population of cells is then returned to the circulation of the tumour-bearing individual.

Tumour defence from the immune system including internalisation of the antigen/mAb complex and antigen modulation (see below) further limits the use of mAb against tumour antigen.

1.5.3 Unconjugated mAb

There are three mechanisms by which simple unconjugated mAb directly facilitate tumour cell killing (figure 1.4). Following ligation of the variable region to antigen, effector cells that express Fc receptor such as natural killer (NK) cells, granulocytes and macrophages can be activated to kill those cells. This process, known as antibody dependent cell-mediated cytotoxicity (ADCC), is most effective in conjunction with IgG mAb (the isotype varies between species). There are several trials involving such monoclonal antibodies, and Rituximab (anti-CD20 chimerised mAb) has recently been approved for use in America for lymphoma therapy [26]. Antibodies to the melanoma antigen gp95, gp97 and several cell-surface gangliosides have not shown clear therapeutic benefit in melanoma (reviewed by Atkins[4]), but the colorectal tumour antigen, 17.1A, has been targeted and therapeutic benefits similar to the use of adjuvant chemotherapy has been demonstrated [3].

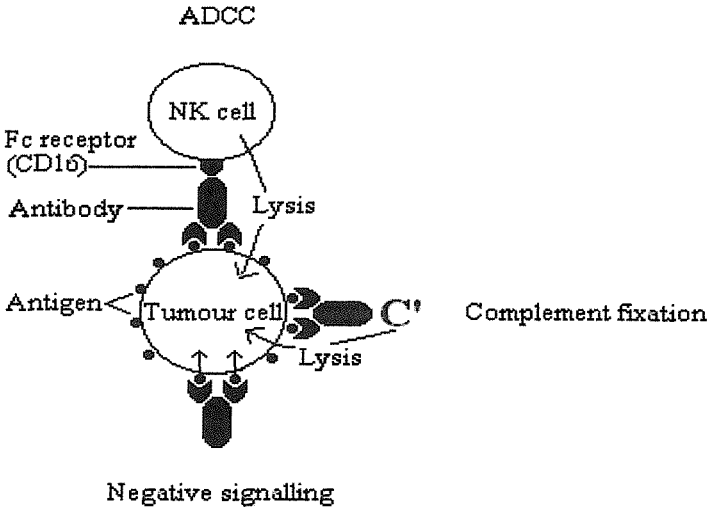


Figure 1.4. Unconjugated antibody is effective in tumour killing through three mechanisms. The variable region of the antibody binds to the antigen on the cell surface of the tumour. In ADCC the Fc of the invariant region bind to a natural effector cell such as an NK cell through the CD16 receptor. The NK cell is then able to send a death signal to the tumour cell. Alternatively the antibody may fix complement through Fc binding. The complement cascade causes disruption of the cell membrane and recruits natural effector cells. If the antigen is a cell surface receptor then the antibody may ligate the receptor and mimic the normal ligand. This effect is enhanced by the ability of antibody to cross-link through Fc:FcR interaction. Targeting specific receptors is thought to inhibit cell growth and even induce apoptosis.

Fc may also mediate a complement (C') cascade resulting in C'-dependent tumour cytolysis.

Antigen on a cell surface may function as a receptor, and ligation with mAb may induce transmembrane signalling into the cell. Antigens have been identified, such as Fas, that will induce cell cycle arrest or even apoptosis when bound to mAb[27]. The ubiquitous expression of many such antigens means that systemic administration of such mAb is lethal[28]. Others, such as the B cell marker CD20, induce cell cycle arrest when crosslinked by anti-isotype mAb. These are expressed only by B cells and have proved effective in the treatment of some B cell lymphoma [23]. This is distinct from anti-idiotype mAb [14].

Similarly many carcinomas depend on autocrine or paracrine promotion by growth factors such as epidermal growth factor, EGF, or transforming growth factor-α (TGF-α). Blockade of these receptors by mAb prevents tumour survival, and anti-EGF mAb

may be of therapeutic benefit in certain breast (Herceptin), vulval, cervical and squamous carcinomas expressing EGF receptors (EGF^+) (reviewed Farah et al[29]).
An increasingly important use of mAb in immunology is the identification of specific receptors on immune cells. Ligation of these receptors by mAb may modulate immune responses by mimicking normal cell interactions. This is not as a result of receptor blockade but of direct activation of intra-cellular signals. This is of relevance to the action of anti-CD40 and anti-CD137 mAb, and is discussed in more detail in chapter 4.

1.5.4 Recombinant mAb

Fusion proteins are synthetic molecules with structural and functional properties of immunoglobulin but designed to reduce or eliminate the effects of their own immunogenicity. By isolating the relevant mRNA from hybridomas producing specific mAb, cDNA encoding specific regions of the immunoglobulin molecule can be cloned into immortalised cells that become factories for that region. For example the grafting of the V-regions region of a mouse anti-human antibody onto the human constant region. The resultant chimeric antibody has less epitopes for the induction of an unwanted immune response. Even less immunogenic are humanised mAb. These use the same principles as chimeric mAb in their construction, but the hypervariable sequences of the specific mAb are grafted into the framework of the whole immunoglobulin molecule. Low yields and errors in protein folding limit recombinant technology.
The cDNA can also be incorporated into the genetic code of bacteriophage which, when infected into bacteria and allowed to proliferate, will display the variable region on their surface; the phage display library. Phage can be selected for strong antigen binding affinity and the relevant DNA for that variable region isolated. These genes can then be incorporated into plasmids including genes for other molecules such as the invariant region of immunoglobulin. When transfected into immortalised B cells, these will combine, creating a complete mAb. This is one means of developing chimeric antibodies in which the variable region from species of animal is combined with the constant region of the species in which it is to be used. Alternatively the variable region alone can be synthesised in these cells and incorporated into novel

structures through which payloads attached to the variable region deliver toxic agents or specific signals to target cells (see below).

In another approach to this problem, mice have been genetically engineered to produce human antibody.

An additional advantage of recombinant molecules is that they can be made smaller, allowing far greater tissue penetration, particularly into tumours. However with the reduction in size there may be reduction in their circulating half-life.

More than one antigen may be recognised by a mAb construct; and bispecific and trispecific antibodies have been developed [30]. These may be constructed by the chemical cross-linking of two different monoclonal antibodies creating a tetravalent molecule with two binding sites for each antigen. Alternatively two hybridomas can be fused resulting in a mixture of immunoglobulin with either binding sites on the heavy or light chains of the immunoglobulin. Finally novel multivalent molecules can be synthesised from components of different immunoglobulin cDNA using similar technology to that for other recombinant immunoglobulin[29].

When these multi-specific molecules are used for tumour immunotherapy one region recognises a specific tumour antigen. The other region may recruit an effector cell to activate a cytotoxic response against the tumour cell, for example through ligating CD3 on cytotoxic T cells. Because the molecules may be multivalent a variety of signals can be induced through the use of more than one specific Fv.

The binding of mAb to an effector cell receptor will not necessarily induce a response from that cell. Other factors such as the cytokine environment, receptor cross-linking and the underlying level of activity of the effector cell may determine the outcome. As our understanding of the processes involved in these cellular responses becomes more comprehensive, so specific signals may be incorporated into recombinant anitbody fusion proteins to increase therapeutic efficacy.

1.6 Cellular immunotherapy.

Because the cellular immune system recognises MHC I restricted antigenic epitopes, intracellular antigens can be targeted for immunotherapy. Again this may be specific, through the identification of a tumour antigen and subsequent vaccination, or non-specific to augment an underlying but anergic immune response. The advantage of specific cellular immunotherapy is that the response is confined to the target antigen.

This is limited by the inherent difficulties involved in identifying and synthesising the most effective MHC restricted epitopes. Non-specific activation of the cellular immune system leads to unwanted T cell mediated damage of healthy tissue. This has stimulated research into the sequence of events and signals to arm T cells against potential tumour antigens whilst excluding unwanted actvation against normal tissue antigens.

1.6.1 Non-specific cellular immunotherapy

Early non-specific cellular immunotherapeutic strategies focussed on the observation that some bacterial products increased the resistance of subjects to certain tumours[31], acting as immunostimulants and causing the activation of cytotoxic lymphocytes and macrophages through what is now known to be a cellular mechanism[32]. An early example of this is melanoma and bacillus Calmette-Guerin mycobacterium (BCG). Local application of BCG to scarified tissue around a tumour skin deposit causes tumour regression in a significant proportion of patients. Immunostimulant therapy that has entered common clinical practice is that of superficial transitional cell carcinoma (TCC) of the bladder with intra-vesical BCG [33]. A modest effect has also been observed in lung tumours with *Mycobacterium Smegmatis*[34]. A common observation is that the therapeutic benefit is only seen following local administration of the immunostimulant.

It was thought that the activity of these immunostimulants was cytokine dependent, and that greater therapeutic benefit might be seen using the responsible cytokines, including interferons (IFN), interleukins (IL) and tumour necrosis factors (TNF). These chemical messages provide a means of transient, short-range communication between cells. TNF-α, IL-1, IL-2, IL-4 IL-6, I-12, GM-CSF, IFN-α, IFN-β and IFN-γ have all been investigated in animal models and clinical settings (reviewed by Ben-Efraim[35]). However there are no generally accepted clinical uses for systemic cytokine-based tumour immunotherapy, one of the limitations being the side effects.

It is likely that the inflammatory response induced by cytokines leads to an effective immune response by providing a "danger signal"[36] in association with the tumour antigen. It is the danger signal rather than the cytokine that activates the cellular immune system (chapter 4). The influx and activation of granulocytes and macrophages in response to the local cytokine environment may result in the

debulking of the tumour through non-specific mechanisms. Clinically an inflammatory response to a tumour does not necessarily correlate with an improved prognosis.

The TNF family of proteins and their receptors are central to many signalling sequences in the immune system. Their biology is discussed in more detail in chapter 4. *In vitro* studies have shown that TNF-α has direct anti-tumour effects in models of malignant melanoma, gastrointestinal adenocarcinoma, lung carcinoma, sarcoma and lymphoma, but no clinical benefits have been demonstrated except in lymphoma[35]. Combinations of TNF-α with other cytokines in phase I studies have not improved on this. More recent work has focussed on the role of TNF-α in the activation of cytotoxic monocytes, along with IFN-α, IFN-γ and IL-1.

IL-2 is a critical cytokine in the maturation of cytotoxic immunocytes, including T lymphocytes and natural killer cells. Effective anti-tumour responses with LAK cells can be induced *in vitro* with IL-2, but despite promising results in animal models this strategy has failed to realise its potential in clinical applications[37].

Because IFN is involved in the induction and execution of cellular anti-tumour responses it has been hoped that therapy with interferon would enhance any underlying immune response to tumour antigens. In phase I studies of advanced neoplastic disease IFN has been shown to slow disease progression in a number of patients with renal cell carcinoma and malignant melanoma[38]. In a similar study of advanced lung cancer the immune response was enhanced, but this did not correlate with any clinical benefit[39]. Other studies have come to similar conclusions.

Neutrophils and macrophages do not recognise specific tumour antigens. However they can be induced to be effective against tumours through antibody bound (T_H2) antigen recognition (see section 2.4). The activity of these cells is dependent on the cytokine environment in which they find themselves. Although there is evidence that granulocyte monocyte colony stimulating factor (GM-CSF) enhances monocyte killing of human colonic adenocarcinoma cells, and increases the number of intraperitoneal neutrophils clinical studies have been disappointing[40] [41].

The correlation between tumour infiltration with dendritic cells and improved survival has lead to interest in the ability of these cells to kill tumour cells and to direct an immune response. This is discussed in detail in chapter 3.

Advances in molecular biology have allowed the transfection of DNA into murine tumour cells such that they can be induced to secrete cytokines, including GM-CSF and IL-2. These cells have been used as live tumour vaccines, protecting against wild-type tumours (reviewed by Dalgleish et al[42]). Again it can be argued that the mechanism involved is indirect and that without the danger signal engendered by the cytokine there will be no immune response. These techniques have been applied to human carcinoma cell lines but live tumour vaccination in humans has not been attempted for reasons of ethics and human leucocyte antigen (HLA) incompatibility.

As understanding of the sequence of signals involved in the immune response to tumour becomes more sophisticated (though with this increased complexity it is not necessarily becoming complete) key events in the cascade can be identified and manipulated. The aim is to direct the immune system toward effective tumour regression in response to tumour antigen without inducing unwanted autoimmune effects. It is likely that the effects of the cytokines described are too broad to be of use other than in providing a non-specific danger signal to alert and activate immune cells. However in the past decade the intimate signalling mechanisms between immune cells have been described and can be invoked through receptor ligation with monoclonal antibodies, fusion proteins and recombinant peptides (section 2.4). It is now possible to provoke specific responses from discrete populations of immune cells. Effective anti-tumour immune responses can be generated in tumour bearing animals by activation of tumour-recognising but anergic CTL through signals such as CD40/CD40L and 4-1BB/CD137L ligation (chapter 4).

Despite the limitations described above there is much hope that adjuvants such as bacterial proteins, cytokines and cell-surface ligands may yet find a role in clinical practice in directing the immune response. This may be in combination with vaccination against specific tumour antigens, some of which are described below. As the sequence in signals leading to effective anti-tumour immune responses is unravelled it will be possible to be more sophisticated and specific in the manipulation and directing of events to achieve clinical benefits.

1.6.2 Specific cellular immunotherapy.

The use of tumour protein as a vaccine has a long history dating back to the treatment of sarcoma with a suspension of tumour cells. In general the immune response to injected antigen is humoral, but recently attempts have been made to generate cell-mediated immunity. It is now realised that the key to an effective cellular immune response is in the antigen presentation to the relevant T cells. The pathways involved are described in chapter 4.

Identification of tumour antigens has lead to their use in attempts at vaccination. Tumour homogenate, comprising a mixture of tumour antigens has met with little success but an allogenic cell lysate was used in metastatic melanoma and claimed regression in up to 20% of patients, associated with an increase in tumour-specific cytotoxic T cells[43]. This effect was augmented when the lysate was prepared by infection of the cell line with vaccinia virus[44].

More recently individual peptides have been synthesised for use as tumour vaccines. These have only recently entered clinical study. Preparation of adequate amounts of antigen is possible with improvements in recombinant technology. Early studies using melanoma-associated gangliosides demonstrated the presence of anti-tumour antibody but only limited therapeutic benefit. The MAGE-3 TAA has been shown to induce regression in melanoma[45] and to generate tumour-specific CTL. This effect is enhanced when adjuvant is co-administered. However under other circumstances such vaccines lead to tolerance and tumour progression. To improve vaccines it will be necessary to thoroughly understand the presentation of tumour antigen and to elucidate the circumstances leading to an effective anti-tumour immune response without risking clinical deterioration.

To this end recent research has focused on how antigen presentation can be improved to yield effective anti-tumour immune cells. Antigen presenting cells can be cultured from bone marrow cells or blood monocytes for *in vitro* study. Antigen is then presented as fragments and in the context of adjuvants such as heat shock proteins (HSP) or cytokines. The APC is then returned to a tumour-bearing animal where it induces effective CTL and tumour regression [46]. The optimal modality of antigen presentation can then be determined.

One method of vaccination is to pulse cultured APC with ovalbumin. The APC are then returned to syngeneic mice which become immune to tumour cells transfected to

express ovalbumin. It has been confirmed that immunity is CTL-dependent. However it is more difficult to generate an effective immune response against tumours through pulsing dendritic cells with antigen alone, and additional cytokines are required, such as IL-3[47]. A more sophisticated approach has been to identify the specific epitope recognised by CTL and combine this with heat shock protein (HSP) (Chapter 4) to pulse the APC before they are returned to the experimental animals.

The use of polymerase chain reaction (PCR)[48] and vectors for genetic transfection has lead to the development of DNA vaccines[49, 50]. A bacterial plasmid incorporating DNA encoding tumour antigen is injected into normal tissue, such as leg muscle. The muscle cells take up the DNA and synthesise the tumour antigen. Presentation of the antigen alone will not necessarily induce an immune response; these antigens are not normally immunogenic. However, if a danger signal such as BCG is incorporated into the plasmid the immune system will respond. Because the muscle cell presents the antigen as an intracellular peptide a cellular immune response will be effected. Alternatively a cell-surface signal, such as a cytokine, can be incorporated. Again the immune system will be recruited and directed toward an appropriate response.

1.7 Evasion of the immune system by tumour cells

Malignant transformation of normal cells may be avoided through self-regulation, for example through p53 mediated apoptosis [51]. These mechanisms are unaffected by immunosuppression, and their effectiveness is demonstrated by the minimal increase in incidence of cancer in immunosuppressed patients. However there is good evidence for the existence of immune surveillance for malignant cells that escape this initial safety net and that subsequent immune responses are effective in tumour cell killing [52]. This is despite the majority of potential tumour antigens being either self-antigens or differing from the original protein by a minimal number of amino acids.

The initiation of an immune response to a tumour is through presentation of antigen by APC. The characteristics of the immune response are determined by the interaction between APC, lymphocytes and other immune cells (section 3.4). A new, non self-antigen will be immunogenic and induce a positive response. This does not normally occur for self-antigens; immune cells recognise the antigen but remain anergic or are

deleted from the population through inhibitory mechanisms that protect against autoimmune damage.

The lack of an effective immune response to tumours is because the immune system fails to recognise the threat of a malignant cell and because of strategies used by tumour cells to avoid the immune system. This is categorised in table 1.3.

Failure of the immune system to respond to malignant cells	1. Local tissue environment 2. Characteristics of tumour antigens
Strategies employed by malignant cells to evade the immune system	1. Down-regulation and variation in tumour antigen expression (antigenic modulation). 2. Direct evasion of the immune system. 3. Indirect evasion of the immune system.

Table 1.3. Why the immune system is unable to control malignant growth.

Normal tissue around the tumour may mask the presence of tumour cells. Tissue stroma may physically conceal tumour cells and without local inflammation or other danger signal there is no stimulus for the immune system to become involved with otherwise normal tissue.

Tumour antigen may become available to immune cells following local tissue damage by an invasive tumour with an inflammatory response. However, the presence of immune cells, including infiltrating dendritic cells and lymphocytes, does not correlate with an effective immune response [53]. Analysis of the surface of these cells has shown that they fail to express the necessary co-stimulatory molecules (section 2.6). It is likely that this is because the majority of antigens are treated as self by APC, which determine the initial presentation of antigen, and the reasons for this are discussed in chapter 3.

Even if effective immune cells are introduced into the tumour environment they may remain ineffective in killing tumour cells, which employ a variety of strategies to avoid detection and death. These may be passive, through changes in antigen expression, or active, through expression of inhibitory costimulatory molecules and release of cytokines[27, 54].

A fundamental principle of immunology that antigen may be immunogenic, treated as foreign and rejected by one individual but treated as self and tolerated by another. This applies to many tumour antigens.

The expression of antigen by tumour cells is variable, within any one tumour and between tumours of any origin. For example breast cancers vary in levels of oestrogen receptor, but individual tumours with high levels will often down-regulate expression. Furthermore the antigenic epitope may vary through genomic instability (reviewed by Breivik et al)[55]. Even though a humoral response to tumour antigen can be demonstrated, antibody fails to bind to the cells at sufficient levels to engage natural effectors. Changes in intracellular transport proteins such as TAP, (see below and chapter 4) alter MHC-restricted antigen presentation to the immune system such that it is ineffective or inappropriate (section 3.2). Down regulation of the surface expression of MHC I prevents effector cells from targeting and killing tumour cells[56].

Evasion of the immune system by tumours may be direct or indirect. It has been shown that tumour antigen-specific T cells from the immediate tumour environment are often deficient in T cell receptor (TCR)ζ expression (section 3.3), with corresponding reduced levels of signal transduction molecules including NF-κB and $p56^{lck}$ [57]. The mechanism and signals involved in this process have not been fully elucidated. When these lymphocytes are removed from the tumour environment their surface phenotype returns to that of a fully armed T cell, suggesting a direct, local role for the tumour cells in this process, and also that the process can be blocked and reversed. Tumours have been identified that secrete cytokines, particularly IL-10[58]. This may result in the down regulation of MHC I and TAP by the tumour, and diverts the immune response from T_H1 to T_H2 (see section 2.2). Transforming growth factor β (TGFβ) is another powerful immunosuppressor that protects tumour cells from immune effector cells. Secretion of TGF-β by certain tumours has a similar effect on down-regulating T cell function (reviewed in Lord[54]), particularly cytotoxic T cells. This effect can be reversed with IL-2.

A more complex issue is the expression of Fas ligand (FasL) by some tumour cells. T cells express Fas, which enables them to be deleted from the lymphocyte population through apoptosis should they be self-recognising. When armed lymphocytes encounter tumour cells that bear FasL they apoptose. However this relationship is extremely delicate and introduction of FasL into FasL negative tumour has been shown to induce such a strong inflammatory response that the tumour regresses

through granulocyte tumour killing, with extensive collateral damage to normal tissue (reviewed by Lamhamedi-Cherradi et al[59]).

Indirect evasion of the immune system occurs through changes induced in the stroma around the tumour. Immune cells leave the circulation and infiltrate normal and tumour tissue by means of tissue adhesion molecules. Tumour cell mediated down-regulation of endothelial cell ligands including L-selectin and integrins prevents lymphocytes from marginalising and then migrating through capillary walls.

The immune system also displays undesirable characteristics that propagate tumour growth, and that certain tumours exploit these characteristics. Oxidative products such as nitric oxide, produced by macrophages, act as mutagens and facilitate tumour growth and metastasis[60].

Vascular growth into the tumour is promoted by angiogenic factors secreted by macrophages, which also convert plasminogen into angiostatin[61]. Other growth factors capable of driving tumour growth result from a non-specific inflammatory response.

The reasons why the immune system fails to engage with tumour cells are complex and extend far beyond the simple concept that tumour antigens are too similar to self-antigens to be effective targets. The strategies required by tumours for evasion demonstrate that the immune system poses a real threat, which reinforces the belief that immunotherapy has considerable potential. The underlying pathways and signalling sequences involved in determining the character of the immune response to tumours, whether it be humoral or cellular, stimulatory or inhibitory, are open to manipulation (immunomodulation). It is anticipated that the introduction of an antigen with appropriate stimulation will activate immune cells against this antigen. This amounts to the induction of controlled auto-immunity [6] and is discussed in more detail below and, in the context of this project, in chapter 3.

Although the immune system is not often an effective barrier to the growth and spread of a mature tumour, the humoral and cellular immune systems still play both positive and negative roles in tumour biology. As the key steps in the sequence of events leading to or preventing an effective immune response are identified clinically valuable tumour immunotharapy will become a reality.

1.8 The role of animal tumour models

Our understanding of tumour immunology and the development of immunotherapy is dependent on studies of animal models. These models are powerful tools but have their limitations, which must be understood if experimental results are to be interpreted[62].

Tumour models used in animals for the study of cancer are classified into one of three groups. Syngeneic tumours may be transplanted from one syngeneic mouse to another of the same strain. Spontaneous tumours may be induced through the use of mitogens, or in strains of animals with a propensity for a particular tumour. Xenograft tumours can be studied *in vivo* by transplantation into immunodeficient mice.

1.8.1 Syngeneic models.

The majority of syngeneic tumours are studied in mice. Many were first described in the 1970s and were grown from chemically induced tumours that were then passaged into syngeneic mice or cultured *in vitro*. The advantages of studying these tumours *in vitro* and *in vivo* are that tumour growth is predictable and reproducible. The timing of investigations and treatments can be planned according to the known growth patterns of the tumour, and experimental work repeated for statistical validation of results. Because the immune system of these mice is intact we can be sure that observations of the cellular processes involved in the growth of the tumour and its relationship with the host immune system are complete.

The disadvantages lie in the fact that tumours found in a clinical setting are unique, with variable biological properties and patients have diverse immune responses to their malignancy. In contrast each syngeneic model of cancer will be the same in all the animals in which it is studied. This lack of individuality raises serious questions as to the validity of observations derived from single models in their application to other malignancies.

The time-scale of the growth of spontaneous tumours in clinical settings cannot be determined. Tumours may, often through chance, present very early in their development when still small and pre-invasive, or at a much later stage with extensive local and metastatic spread of disease. With regard to this the timing of treatment cannot be predicted in the way that it can in syngeneic models, although in these models it can be varied to take this into account.

More specific concerns for the use of syngeneic models for the study tumour immunology lie in the nature of the antigens recognised by the immune system of the experimental animals. It has been observed that many of these tumours, and the mice in which they are studied, are infected with retrovirus[63]. These may lie dormant in the tumour cell line until activated by a non-specific signal, such as a change in the cytokine environment, after which they provide an antigenic stimulus for the immune system. These retroviral antigens themselves may yet be of interest to tumour immunologists. It is not clear to what extent retro-viral infection has been responsible for the vast areas of seemingly redundant genetic code in the human genome. A full description of these antigens will be provided by the Human Genome Project in due time, and they may offer new possibilities for tumour immunotherapy[64].

Another concern is that of antigenic drift. Although the major MHC antigens (section 2.4) remain constant in strains of syngeneic mice and tumours arising from them, over time the minor MHC antigens change. Alone these may be inadequate to induce tumour rejection through MHC antigen mismatch, but with a generalised, non-specific upregulation of the immune system they may be adequate.

Many important clinical and experimental concepts have been derived from the study of the immune system in syngeneic tumour bearing mice. Concomitant immunity was first demonstrated in 1984[65]. Specific anti-tumour T cells were raised against MethA sarcoma and P815 plasmacytoma. In both cases CTL were effective in adoptive immunotherapy but did not inhibit tumour growth in the donors themselves because of the presence of suppressor T cells that recognised the same antigen.

The concept of cross-priming of antigen (section 3.4) has been developed through the study of CTL responses to exogenous antigens in mice over the past ten years. The characteristics of the antigens processed in this way are also being studied (section 3.2).

Techniques in PCR and proteomics have demonstrated that homology exists between murine and human molecules involved in antigen recognition, signal transduction and effector mechanisms (chapter 4). More recent advances in molecular biology have demonstrated that function is conserved across species, although for humans this is mostly limited to *in vitro* studies.

Human antigens have been transfected into immortal murine tumour cell lines and specific immunotherapy, such as mAb or fusion protein, can be studied in syngeneic animals.

Most immunotherapies developed in animals have been disappointing when transferred from animal studies to clinical trials. However, techniques developed to target specific tumour antigens in mice may allow for the improvement of the promising results seen for mAb therapy against specific human antigens such as CD20 and 17.1A.

1.8.2 Spontaneous tumour models

The argument that spontaneous tumour animal models mimic human neoplasia more closely than syngeniec model is flawed for several reasons. The artificial circumstances, such as the use of powerful mitogens that induce these tumours are not necessarily relevant to the mechanisms of carcinogenesis that apply to spontaneous tumours in humans. The biological properties of artificially induced tumours may be different from those of human spontaneous tumours. The growth of the tumours is less predictable, making it difficult to account for variations in tumour size and spread when comparing experimental treatment of therapeutic and control groups. It is not clear whether the induction of tumours in itself leads to a degree of immunomodulation, or affects the experimental animals in any other way. In most cases a single strain of (syngeneic) mice will be used, so that the problem remains of genetic identity between study animals.

1.8.3 Human tumour xenograft models

Immunodeficient mice used in the study of xenografted tumours are usually nude or SCID (severe combined immunodeficiency disorder). They lack T and/or B cells but their innate immune system, including natural effectors, is intact. These mice provide a useful halfway house for the study of tumour growth. The lack of MHC-recognising immune cells means that human tumours grafted into these animals will be treated as self. This has been of particular use in the study of mouse anti-human TAA or TSA mAb in the killing of grafted human tumours by murine natural effector cells. Interpretation of results must not be considered in isolation as a substantial proportion of the immune system is completely absent in these animals.

It is not just tumour cells that can be grafted into SCID mice, and various forms of adoptive immunotherapy have been investigated, including the use of human macrophages and human tumour specific CTL, as effector cells. Furthermore the

importance of specific cytokines such as IL 2, 4, 6 and 12, TNF-α and TNF-β in the functions of these cells can be studied.

The efficacy and toxicity of other specific immunotherapies has been studied, in particular the use of immune fusion proteins such as immunotoxins (section 2.4). These treatments are not dependent on intact components of the immune system, and efficacy can be studied *in vitro.* However they are potentially toxic to the recipient and a correlation between toxicity and therapeutic efficacy can only be achieved *in vivo.* This can be studied in transfected immortal tumour cell lines, as described above, but a more simple and equally valid approach is to graft and treat the human tumours with the immunotoxin or similar fusion protein in SCID mice.

1.8 Conclusions

This chapter has presented immunology and immunotherapy in their broadest context. The role of cellular signals such as CD40 and CD137 can be seen to be small in the scale of the whole subject. However, with the realisation that some fields of immunotherapy are no longer likely to yield the therapeautic benefits they promised it is essential that the mechanism of action of these signals be fully described and that the possibilities for incorporation into other immuntherapeutic strategies are thoroughly explored.

Chapter 2

An overview of antibody, the immunoglobulin superfamily and other polypeptides involved in antigen recognition and processing.

2.1 Introduction

Members of the immunoglobulin superfamily are diverse proteins involved in the recognition and processing of antigen. They include antibody, the TCR, components of MHC molecules, and co-receptors and co-stimulatory molecules involved in signalling between cells of the immune system. They are constructed from constant (c) and variable (v) domains (figure 2.1) with considerable structural homology. These are linked by amino acid chains, allowing for flexibility within the molecule, and by disulphide bonds. Specific regions of some molecules, including antibody, have considerable structural variability within the same class of polypeptide. This enables the molecules to interact with a diverse range of antigen, the epitope being the specific region of the antigen that is recognised.

Although this project has focused on cellular immune responses it is important to appreciate that the molecular mechanisms of antigen recognition, processing and induction of such an immune response are inextricably linked to those of humoral immune responses. The molecules involved are structurally similar and often involved in both. It is the balance between cellular and humoral responses that determines the final outcome. This chapter describes the structure and basic molecular biology of the molecules involved in antigen recognition, including antibody, the TCR, the MHC, and important adhesion and co-stimulatory molecules involved in cellular immune responses. The function of these molecules in the generation of a cellular immune response is described in more detail in Chapter 3, whilst Chapter 4 will focus on the structure and function of the CD40 molecule, the CD137 molecule and their ligands.

Antibody was the first member of the immunoglobulin superfamily to be fully described, and so it will be used it as a prototypic example for more detailed discussion. Its relationship with the TCR, MHC and relevant costimulatory molecules will be discussed.

3.2 Antibody

Interest in antibody began in the nineteenth century following the development of vaccination by Jenner, Koch and Pasteur. In 1890 Emil von Behring and Shibasaburo Kitasato identified a substance in the serum of vaccinated individuals that would bind specifically to the relevant pathogen. They named this substance "antibody"[66].

Although it was soon realised that a specific response could be raised against heterogeneous substances it was not until 1939 that Tiselius and Kabat[67] identified the fraction of serum proteins responsible. They used ovalbumin to precipitate antibody from the serum of ovalbumin-vaccinated rabbits, and compared the elecrtophoresis band of this serum with that of whole serum. They found that the second band, gamma (γ) globulin, was significantly reduced in the first aliquot. Because considerable protein remained in this band even after precipitation they distinguished the responsible proteins by naming them immunoglobulins.

The Nobel Prize winners Edelman and Porter [68] investigated the functional components of the immunoglobulins. Ultracentrifugation separated γ-globulin into a high and low (150,000) molecular weight fraction which they called immunoglobulin G (IgG). Using the proteolytic enzyme papain they cleaved the low molecular weight protein into three fractions. The first two identical weight fragments (45,000MW) retained the ability to bind antigen, and were named Fab (antigen binding fragment). The third, slightly larger fragment (50,000MW) did not have an easily identifiable function other than that it crystallised in the cold, and was therefore named Fc. Digestion with pepsin resulted in a large (100,000MW) fragment also capable of precipitating the relevant antigen but this time there was no Fc fragment, but rather multiple small fragments due to pepsin digestion. Reduction of IgG disulphide bonds with mercaptoethanol yielded four protein chains; two light (25,000MW each) and two heavy (50,000MW each). The relationship of the Fab and Fc fragment to the heavy and light chains was determined by vaccinating goats with Fab or Fc derived from rabbit IgG. Antibody to Fab would precipitate both heavy and light chains but antibody to Fc would only precipitate heavy chains.

The final piece of the puzzle was the relationship between the fragments yielded by enzyme digestion and the chains yielded by reduction of the disulphide bonds. Porter immunised goats with rabbit Fab or Fc and found that the resulting anti-Fab antibody would bind to both chains, but the anti-Fc antibody would only bind to the heavy

chain. It was found that both the light and heavy chains consisted of a variable (V) region and a constant (C) region.

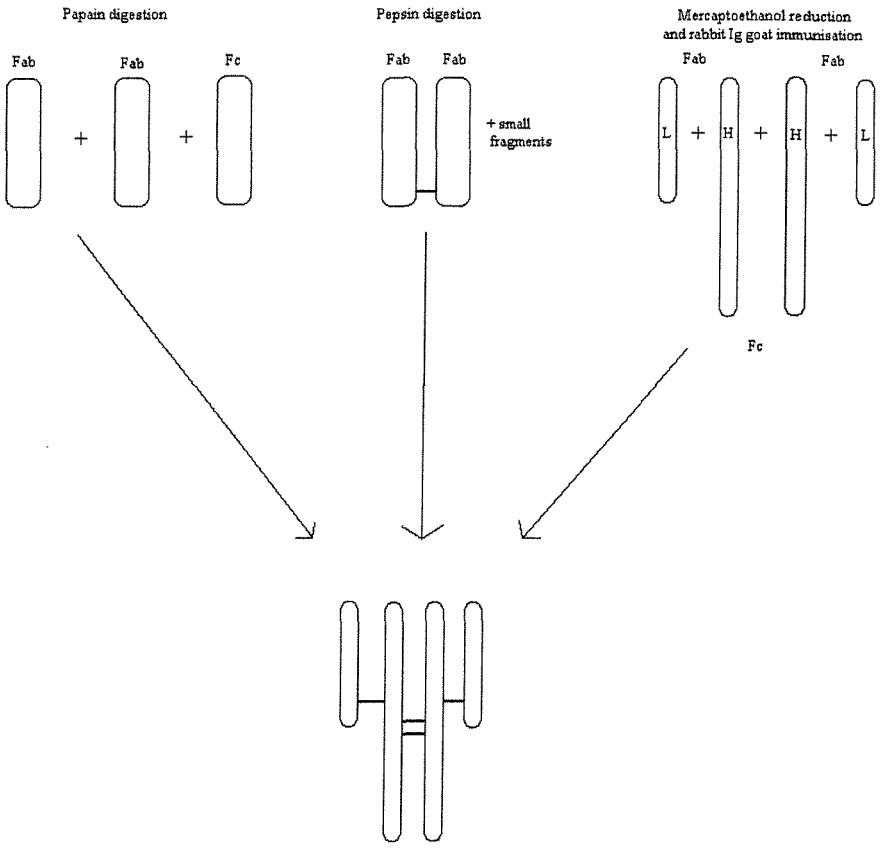


Figure 2.1. Edelman and Porter established that antibody is a protein constructed of four domains consisting two identical light chains and two identical heavy chains capable of binding the same antigen (see text for detail).

Gene rearrangement and somatic mutation causes variations in the amino acid sequence of the V region, giving rise to structural heterogeneity that in turn allows a diverse range of antigens to bind antibody. Vaccination with a single antigen results in the recognition of a number of epitopes by different antibodies, but each plasma cell that produces each antibody is unique. The realisation that multiple myeloma is caused by a malignant plasma cell line producing a monoclonal antibody meant that antibody could be produced in sufficient quantity to sequence the amino acids (see chapter 1).

Each light chain has one variable region, V_L, and one constant region, C_L, each consisting of one domain, and the heavy chain one variable region, V_H and three to four constant regions, C_H1-4. The class of immunoglobulin to which an antibody

belongs is determined by the combinations of heavy and light chains: IgM, IgG, IgA, IgD or IgE.

X-ray crystalography and electron microscopy have determined the shape of the immunoglobulin molecule. Each region, whether from variable or constant, is folded three or four times into a beta-pleated sheet. The two sheets lie parallel to each other so that the final structure is a tube called a β-barrel (Figure 2.2).

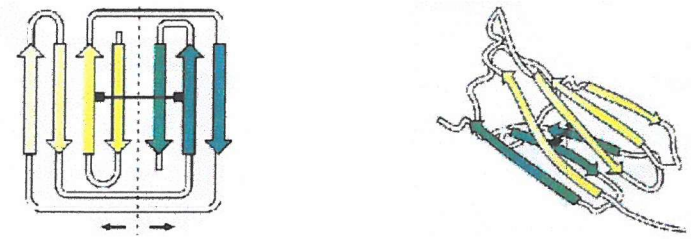


Figure 2.2. Structure of an immunoglobulin domain illustrating how the amino acid chain is folded into two β-pleated sheets linked by a disulphide bond (the black dumbbell in each diagram) to form a β-barrel (from Janeway and Travers, Immunobiology[15]; Chapter 3).

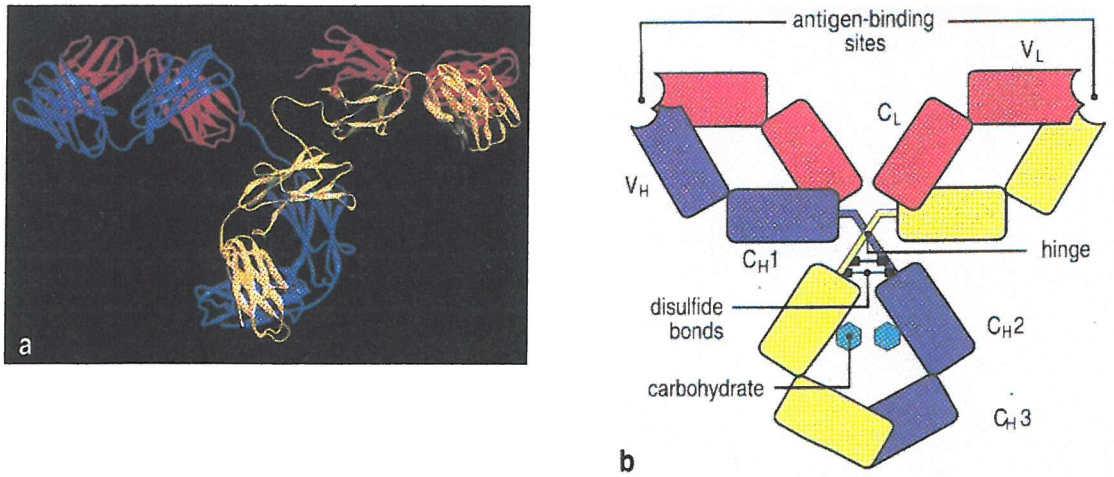


Figure 2.3. The 3-dimensional structure of the immunoglobulin molecule (a) is determined by the relationship between each domain of the heavy and light chains (b) (from Janeway and Travers[15], Immunobiology; Chapter 3).

Diversity in antigen recognition arises from rearrangement of the variable region genes, and from somatic mutation.

The structure of the regions allows non-covalent interaction between neighbouring β-barrels on each chain, which contribute to the three-dimensional relationship between the heavy and light chains (Figure 2.3).

2.3 The T cell receptor

The T cell receptor (TCR) is a member of the immunoglobulin superfamily. It is expressed by helper and cytotoxic T cells and is associated with a number of polypeptides (CD3) to form the T cell receptor complex. That part of the receptor involved in antigen recognition shares structural similarities with the Fab region of immunoglobulin. It comprises two chains, α and β, each with two regions (figure 2.4)[69] resembling the constant and variable regions of immunoglobulin domain. The two variable regions lie adjacent to one another and this comprises the antigen-biding site. The transmembrane and cytosolic components resemble that of the B cell receptor. They are responsible for signal transduction following successful antigen binding. Furthermore the genes for the T cell receptor resemble those of antibody. Extensive variable-region gene rearrangement takes place to increase antigen-binding variation, but somatic mutation does not occur. This may reduce the incidence of self-recognising T cells, the majority of which have to be deleted from the T cell population. Much of the variability in antigen binding arises through an increase in the number of joining (J) regions of the T cell receptor. This concentrates the variability on the central, CDR3, region of the molecule. The outer, CDR1 and CDR2 regions interact with the MHC molecule, and it is variability in these regions that accounts for transplantation rejection.

CD3 is a complex of polypeptides γ, δ, ε and ζ, (figure 2.4) with structural similarities with other immunoglobulin. The cytosolic tails of CD3 molecules have regions called immunoreceptor tyrosine-based activation motifs (ITAM). Following TCR activation these regions associate with protein tyrosine kinases, facilitating transduction of the signal from the TCR to the T cell. The CD3 complex is also essential for transportation of the TCR to the cell surface.

The TCR recognises both MHC I and II, but the effector function of the T cell (helper or cytotoxic) is determined by the expression of CD4 or CD8. This enables the T cell to discriminate between MHC I and MHC II. CD4 is a single chain molecule expressed by helper T cells. It comprises four immunoglobulin-like domains, arranged as two rods linked by a flexible hinge region.

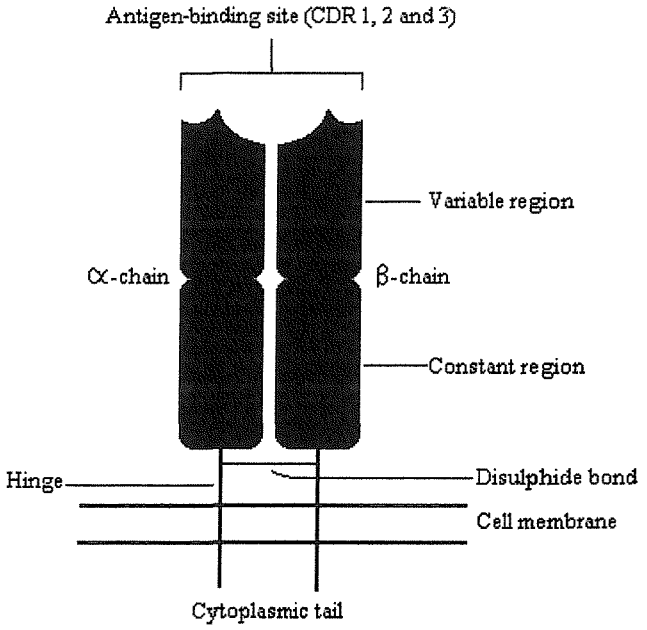


Figure 2.4. The T cell receptor is constructed of two chains, α and β, each with a constant and a variable region. The inner part of each of the two variable regions, CDR3 is responsible for antigen recognition. The outer areas, CDR1 and CDR2, are responsible for recognition of, and restriction to MHC I and MHC II. Each chain is linked by a disulphide bond between the extracellular areas of their cytoplasmic tails.

The CD8 molecule is expressed by cytotoxic T cells. It comprises two immunoglobulin-like regions connected to the cell membrane by a polypeptide chain with a cytosolic tail.

CD4 and CD8 will bind weakly to MHC but this is ineffective without TCR/antigen.

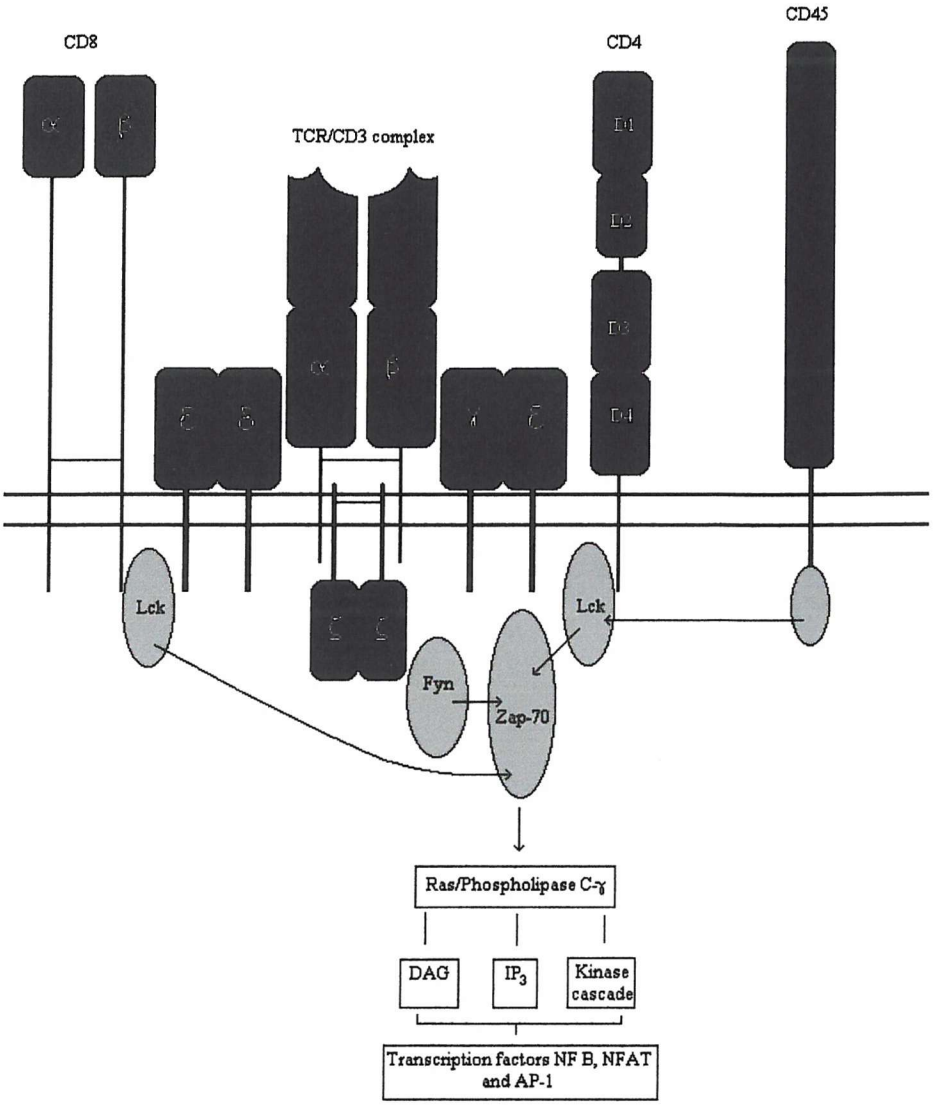


Figure 2.5. The T cell receptor complex (T_H1 and T_H2). When MHC presents an antigen that is recognised by the TCR the associated CD3 co-receptor complex augments tyrosine kinase activity. This is only effective following MHC II/CD4 or MHC/CD8 binding, which increases kinase activity by 100-fold. The greater the number of MHC molecules presenting that antigen, the more TCR will bind leading to receptor aggregation, which also enhances kinase activity in a synergistic fashion. CD45 is transmembrane molecule that contributes to signal generation by removing inhibitory phosphate groups.

Fyn and Lck phosphorylate residues on CD3 ε and ζ molecules, which allows a cytosolic tyrosine kinase, Zap-70, to bind to them. Zap-70 and Fyn activate Ras, which cleaves phosphatidylinositol to diacylglycerol (DAG) and inositol triphosphate (IP_3). These then act through a cascade of protein kinases and phosphatases that ultimately up-regulate nuclear transcription factors, particularly NF κB, NFAT and AP-1. These initiate gene transcription causing further differentiation of the T cell, proliferation and upregulation of the cellular machinery required for effector function.

2.4 The Major Histocompatability Complex

The MHC molecule facilitates the presentation of potential antigens to the immune system. The MHC is a cluster of genes identified through their role in the immune response to allograft antigens[70]. There are two distinct classes of MHC molecule, MHC I and MHC II. They have similar antigen binding properties, but different tissue distribution and functions within the immune system.

MHC I is normally expressed by all nucleated cells. MHC I molecules collect and presented antigenic epitopes derived from any polypeptide being synthesised by the cell at that time.

MHC II is expressed by antigen presenting cells including macrophages, dendritic cells and B cells. MHC II molecules present exogenous antigen that has been ingested by the cell and processed in intracellular vesicles.

The MHC molecules are cell surface glycoproteins each consisting of four subunits similar those of immunoglobulin (figure 2.5). MHC I ligates CD8, MHC II CD4, MHC I has only one transmembrane domain, and the antigen binding sites of MHC II are more open. This conventional wisdom has recently been challenged and mechanisms demonstrated by which exogenous antigen is presented on MHC I through a process called cross-priming (see Chapter 3).

Gene rearrangement of the MHC complex does not occur. However there is considerable variability in the amino-acid sequence and shape of the antigen-binding groove. This is a result of the extreme polymorphism of the genes encoding for MHC molecules. It is difficult to find a teleological explanation for this variability, as recognition of a specific antigen is dependent on the TCR and immunoglobulin. It is most likely that it has arisen from the need to present any one antigen to T cells in a variety of MHC-dependent contexts. It has been demonstrated that certain pathogens have altered critical antigens such that they will no longer bind to a particular MHC molecule. However other individuals, with different MHC alleles will be able to present the antigen and mount a response to the threat.

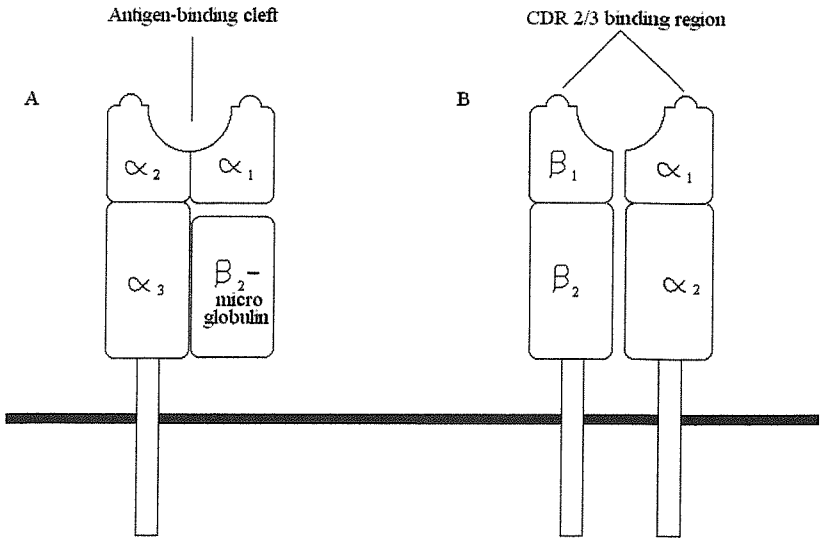


Figure 2.6. MHC I (A) and MHC II (B) molecules. The α_1 and α_2 domains of the MHC I molecule are contiguous and form the peptide binding cleft. They also form the binding site for CDR2 and CDR3 of the TCR. Although the α_1 and β_1 of the MHC II molecule are not contiguous they still form a cleft for antigen binding, in addition to binding CDR2 and CDR3.

2.5 Adhesion and co-stimulatory molecules

Activation or inhibition of T cells is not exclusively dependent on MHC molecules and the TCR. There exists a diverse range of cell-surface molecules that augment or inhibit signals arising from MHC-restricted antigen recognition. They play no direct role in the recognition of the antigen and are classed as co-stimulatory and adhesion molecules.

Adhesion molecules are divided into groups of polypeptides with similar structure and function. In the context of the immune system they enable immunocytes to navigate through different tissues and to identify and initiate communication with other cells. They may provide stimulatory or inhibitory signals between cells and are essential to most immunological processes.

The selectins are a group of small proteins expressed on the cell-surface of most immunocytes. Their ligands are the addressins, primarily expressed by endothelial cells. The selectins and addressins guide lymphocytes to their destinations.

Initial interaction between T cells and APC is through two further groups of adhesion molecules. The integrins are cell-surface proteins expressed by most cells. They

mediate adhesion between cells, and between cells and the extracellular matrix during inflammation. The integrin ligands are either members of the immunoglobulin superfamily or other polypeptides with similar structural features. They include CD2, which is expressed by all T cells. Its ligand is CD53, or LFA-3. Their weak binding is the first step in interactions between T cells and APC.

Intercellular adhesion molecules (ICAMs) are members of the immunoglobulin superfamily that bind LFA-1 on either the T cell or the APC. They are synergistic with CD2/LFA-3 in maintaining a connection between the two cells. The TCR is then able to come into contact with the MHC molecule of the APC. If antigen presentation is unsuccessful the T cell will break away from the APC and is able to encounter other APC.

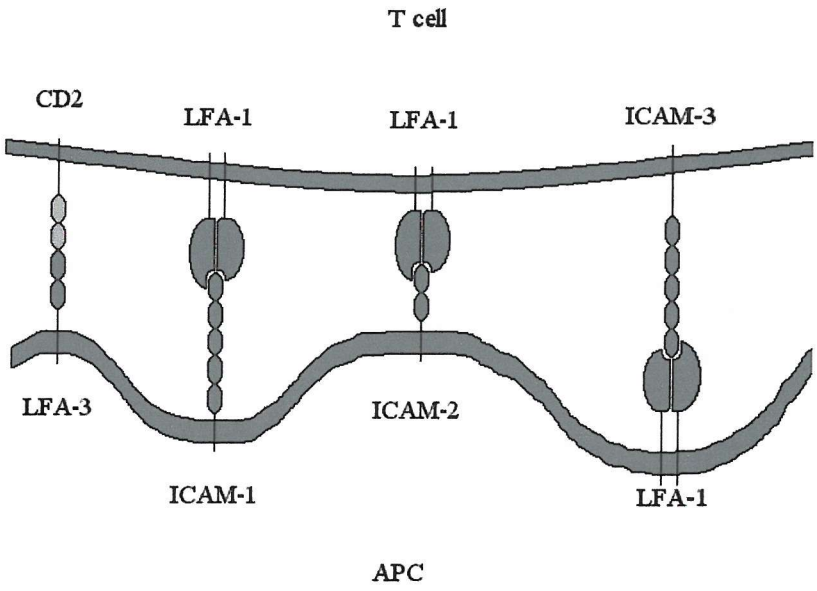


Figure 2.7. A number of adhesion molecules are expressed by mature APC. They facilitate early interaction between APC and T cells. Similar interactions also occur between mature, armed T cells and their targets.

Costimulatory molecules provide a second signal that helps determine the outcome of successful antigen recognition by the T cell. In the context of CTL the best-described co-stimulatory molecules are B7.1 (CD80) and B7.2 (CD86). These are homodimeric members of the immunoglobulin superfamily. Their ligands are CD28 and CTLA-4. CD28 is another member of the immunoglobulin superfamily and it delivers an essential signal to the T cell allowing full maturation. CTLA-4 binds to B7 molecules with 20 times the avidity of CD28, and indirectly delivers an inhibitory signal to the T

cell by blocking synthesis of the cytokine IL-2 (see Chapter 3, figure 3.3). Although this does not affect the function of the individual T cell it limits T cell proliferation following activation by the APC.

Many other co-stimulatory molecules have recently been described, including CD40 and CD137, which are described in more detail in Chapter 4.

Chapter 3

The induction of cytotoxic T lymphocyte responses to tumour antigens

3.1 Introduction

Although humoral and natural effector immune responses to cancer have been demonstrated there is increasing evidence that powerful cytotoxic T lymphocyte (CTL) responses will offer the best hope for the development of clinically useful immunotherapy [10].

The sequence of events and molecular signals involved in the uptake and recognition of antigen by the cells of the immune system and the subsequent priming of CTL have been described (figure 3.1). As our understanding of these processes increases it can be turned to therapeutic advantage.

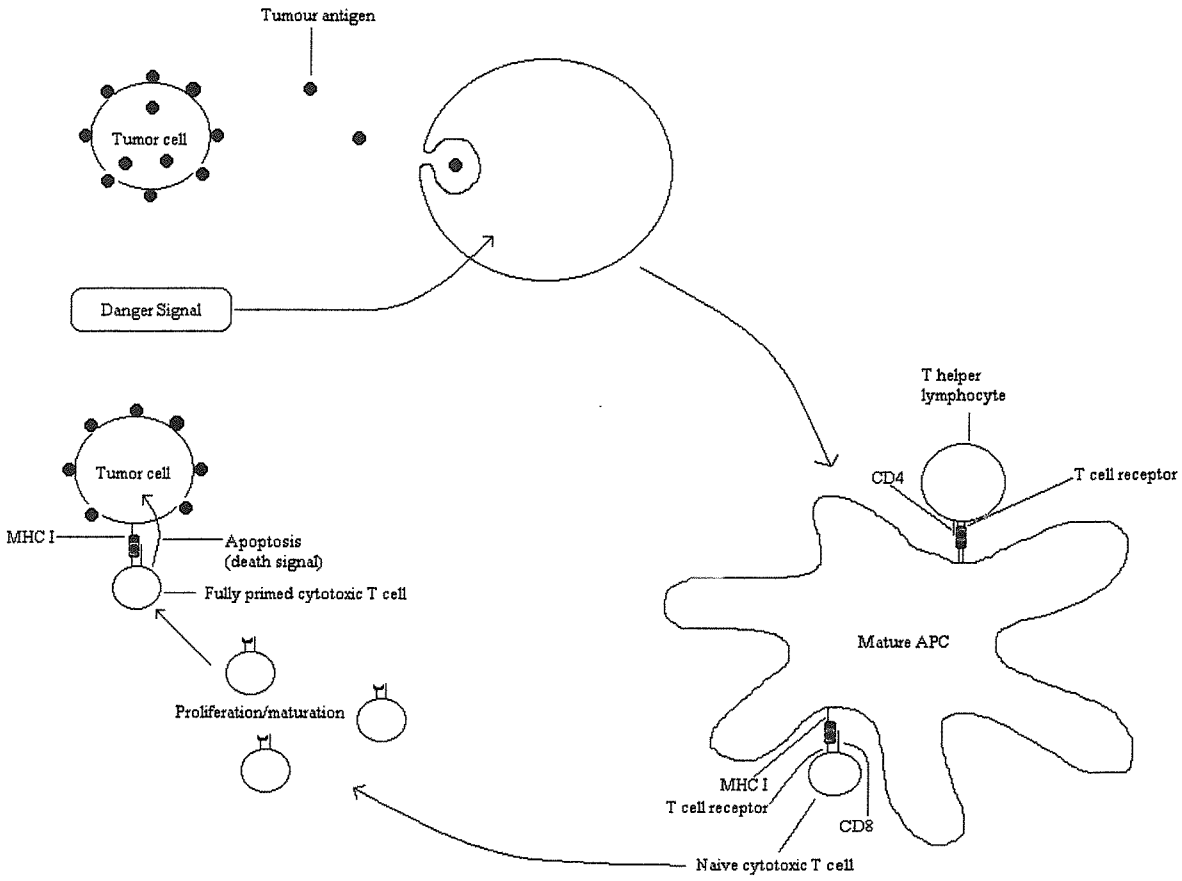


Figure 3.1. Tumour antigen is ingested by a naïve APC. If the APC receives the appropriate danger signals it migrates to the regional lymphoid tissue, where it matures. Antigen is processed and presented to lymphocytes. Antigen specific cytotoxic T cells may be primed and armed by the APC, after which they are able to proliferate, mature and migrate back to the site of the tumour, where they are effective in killing tumour cells.

3.2 Ingestion and presentation of antigen by APC to the immune system

Naïve professional APC such as dendritic cells are distributed throughout the body. They constantly sample antigens from their environment, including secreted proteins and components of dead cells but are ineffective in their antigen presentation. The change to effective antigen presentation requires a 'danger signal'[36]. These are not specific and are most often components of the innate immune system that are produced as a result of tissue damage. They include inflammatory mediators such as cytokines, complement, heat shock protein (HSP) and antigen/antibody complexes[71]. Some antigens, such as bacterial cell wall components, can serve as both antigenic epitope and danger signal.

Tumours also have characteristics that provide the danger signals to activate APC. They often display high levels of cell turnover, with correspondingly high cell death rates. Under normal circumstance scavengers such as macrophages clear these cells, but this system may be overwhelmed. This results in considerable cellular debris, mimicking non-specific tissue damage and causing inflammation[72]. The process is exacerbated as the tumour outgrows its blood supply, resulting in further tissue destruction through ischaemia, which is particularly potent in the induction of inflammation through tissue necrosis and the release of free radicals. Some tumours try to overcome this with vasoproliferative cytokines to encourage vascular ingress, but these often have inflammatory activity in themselves. The growth of the tumour may cause local tissue death through its physical size (pressure necrosis), and through the release of enzymes that facilitate invasion and metastatic growth.

Once an APC has been activated by a danger signal it migrates to the local lymph nodes and undergoes dramatic changes in phenotype. The surface area of the cell is increased by the dendritic extensions of the cell membrane. The cell surface bristles with MHC I and II, and the co-stimulatory molecules required for effective antigen presentation. A single APC is able to interact with many thousands of lymphocytes[73]. However, other factors than the antigen are important in determining whether an immune response is positive and humoral or cellular.

Cytotoxic T cells only recognise MHC I restricted antigenic epitopes and any tumour antigen may be represented in this context. Tumour antigen is abundant for ingestion by an APC following tumour cell damage.

All metazoic cells, including tumour cells, are liable to apoptosis, which is the physiological mechanism of programmed cell death. It causes the release of cellular antigens. Apoptosis is induced through intrinsic mechanisms by which cells monitor the translation and repair of DNA, such as the regulatory protein p53. It also occurs following exposure to cytotoxins (see Section 3.5) or extrinsic signals through specific receptor/ligand (such as Fas/FasL) interactions with immune cells. Not only are tumour cells vulnerable to apoptosis but the majority demonstrates greatly increased cell turnover, which further increases available antigen.

Under normal circumstances apoptotic cells and cellular antigens are carefully removed from healthy tissue by professional scavenger cells such as macrophages. Although macrophages are capable of antigen presentation they express low levels of the co-stimulatory molecules necessary to induce a competent immune response even in the presence of inflammatory cytokines. This limits the potential for unwanted immune responses to self-antigens. Large amounts of cell debris may overwhelm the macrophages, exposing antigens not normally encountered in the context of a danger signal by dendritic cells. These are encouraged to infiltrate the tumour (tumour infiltrating dendritic cells, TIDC) by inflammatory cytokines. Such a mechanism has been shown to exist in SLE, a prototypic autoimmune disease. In SLE a state of increased tissue inflammation exists resulting in increased tissue damage, apoptosis and reduced scavenger cell efficacy. This results in the generation of autoreactive CTL which mediate further tissue destruction through the recognition of neo-antigen and thus the perpetuation of a vicious circle (see also section 4.7).

Apoptotic bodies are collections of cellular material bodies, including intact antigens normally hidden from the immune system, contained by cell membrane that breaks away from damaged tumour cells. Further cell disruption through the action of apoptotic enzymes such as caspases releases intracellular proteins and forms new peptides. These neo-antigens provide epitopes not previously encountered by immune cells and are treated as foreign antigen even though they are derived from self-antigen.

With uncontrolled cell destruction (necrosis) smaller cellular components are released including potentially harmful enzymes that exacerbate the process and which may also generate neo-antigens.

Continued growth of the tumour implies that these APC are not able to induce an fully effective immune response[73].

Because apoptosis and necrosis results in a large and diverse range of antigens, APC must have mechanisms for selective presentation of antigens from this melange. A teleological explanation might be that antigens are selected through the context in which they are ingested. For example tumour antigens are most often self-antigens and so it would be unlikely for such a mechanism to rely on antibody bound antigen since self-reactive B cells will have undergone early negative selection.

The ideal tumour antigen for presentation to helper T cells and CTL is the same antigen that is presented by the tumour cell MHC I, as this is the antigen against which the CTL will be effective. Even if a tumour manufactures a wide range of potential tumour antigens, if those antigens are not represented on the tumour cell's surface in the context of a MHC I restricted epitope then CTL will be unable to kill the tumour cell.

Moore et al[74] demonstrated that although fully effective CTL against target cells are most likely to occur in response to antigens actively synthesised by the target cells the antigen alone is insufficient to induce effective CTL. If mice are inoculated with ovalbumin then no specific CTL are generated but if immortalised tumour cells are transfected with a plasmid encoding DNA for ovalbumin, and those cells inoculated into syngeneic mice then a specific anti-ovalbumin CTL response is generated. This implies that the ovalbumin, or an epitope thereof is only recognised in conjunction with another component of the tumour cell. The limiting step in the generation of the observed CTL response is most likely to be at ingestion by the APC, as beyond this the antigen is fragmented and constituent parts only are presented to lymphocytes in the context of MHC I or II restriction. This may also explain why simple tumour vaccination yields an evident but ineffectual humoral immune response.

Cellular polypeptides and glycoproteins have been identified that function as such an additional component. At any stage in the synthesis of a polypeptide, molecules exist to chaperone it, helping with folding of proteins and guarding against unwanted enzyme activity. One group of such chaperone molecules was first identified in heat damaged tissue, hence the appellation "heat shock protein"[75, 76]. It has been shown that some of the neo-antigens released by dying tumour cells are only immunogenic in association with HSP[77].

A subset of APC exists with receptors (CD91) that allow it to identify and ingest antigens chaperoned by HSP[77]. Most MHC I restricted antigen on a tumour cell's surface is processed in the endoplasmic reticulum (ER). These antigens are

transported to the ER by 'Transporters associated with Antigen Presentation' (TAP) molecules. Specific HSP exist for the chaperoning of antigens within the ER and during apoptosis the HSP remains bound to the antigen after dissociation from TAP. Antigen that is bound to HSP and ingested by the APC will be processed for MHC I restricted presentation to CTL and MHC II restricted presentation to helper T cells. This allows for the selective cross-priming of CTL against endogenous tumour antigen presented by tumour cells in the context of MHC I. Exogenous antigens are ignored these APC resulting in a more efficient system for the processing and surveillance of cellular antigens.

Activation of CTL is not limited to ER derived antigen. In a TAP-knockout murine model tumour cells were still capable of cross-priming CTL, even though the lack of MHC I restricted antigen on those tumour cells protected them from CTL dependent lysis[78]. Other mechanisms must exist to guide tumour antigens into APC MHC I restricted presentation and a CTL response.

The adaptive immune response may be positive leading to the generation of primed effector cells, or negative, causing immune cell anergy or deletion of potentially reactive effector cells. This is determined by the response of the two subsets of helper T cells, T_H1 or T_H2. APC are able to direct the presentation of antigen to specific cell types and are responsible for the activation of effector cells[73]. Early evidence of the importance of professional APC in controlling the immune response is in the observation that those found near mucosal surface express high levels of Fc receptor[79, 80]. These cells are best suited for ingestion of antibody bound antigen such as that from pathogens to which the immune system has prior exposure and antigen presentation can be efficiently directed to appropriate memory T and B cells and a humoral immune response.

Surman et al [81] found that viral transfection of a murine tumour cell line would induce functioning T_H1 and T_H2 cells but that only the T_H1 cells were capable of inducing a therapeutic immune response. Furthermore the response was found to be CTL dependent and required the expression of both MHC I and II by the APC. This implies that any humoral response was inadequate or lacked specificity, either because of reduced tumour antigen expression by the tumour or because of the failure of natural effector cells to abrogate tumour growth despite antigen/antibody binding.

3.3 Lymphocyte selection

Although the processes to determine whether a tumour antigen will result in a T_H1 or T_H2 immune response have not been fully elucidated, they involve dialogue between APC and T cells, rather than being dependent on the APC alone[81]. Before this can occur T cells undergo clonal selection or deletion at an early stage in their development (figure 3.2). There is also evidence for inhibitory helper T cells that recognise self-antigen to prevent particular responses. It is neither clear at what point these T cells differentiate nor the factors involved in determining this differentiation [82]. The human bone marrow produces 5×10^7 T cells each day[15] and the majority are deleted or rendered anergic through negative selection. This prevents unwanted immune responses to self-antigens. The process of T cell selection is intimately associated with MHC restriction of antigen.

Naïve T cells initially express neither CD4 nor CD8 (double negative). They migrate to thymic tissue where they encounter self-MHC I and II in the context of thymic epithelial cells. At this point the T cells upregulate surface expression of the TCR and express both CD4 and CD8 (double positive). Only those T cells that recognise self-MHC undergo positive selection. The remaining cells, approximately 95% of the total, undergo apoptosis. A number of cells remain able to undergo further TCR α chain rearrangement, allowing them to be salvaged if this leads to self-MHC recognition. The β chain remains constant. There are also subtle differences between MHC I and MHC II so that even at this stage the T cell is acquiring the necessary characteristics for helper and cytotoxic T cell function even though it expresses both CD4 and CD8. In a murine model without MHC II the $CD8^+$ population is normal but the $CD4^+$ population is severely depleted, implying that the naïve T cell receives a positive signal when it interacts with self MHC but will apoptose in the absence of this signal.

Helper T cells are treated more ruthlessly than cytotoxic lymphocytes and a greater proportion is deleted from the lymphocyte pool. This means that a greater number of potentially self reactive CTL will reach the peripheral immune system. However the absence of the relevant helper T cell results in impotent, anergic CTL, and the question as to whether these anergic cells can be reactivated continues to be discussed.

Subsequent development of the T cell is determined by MHC antigen restriction; T cells will only recognise antigen with the MHC haplotype to which they were restricted at this stage. This phenomenon also leads to the rejection of MHC mismatched transplantation antigens. Acute allograft rejection occurs because as many as 10% of armed T cells will recognise and be effective against foreign MHC molecules despite MHC restriction and positive self-selection. These T cells are able to engage the foreign MHC even though they are not restricted to that haplotype. The TCR then binds the foreign MHC as though it was self-MHC presenting foreign antigen and the T cell induces apoptosis in the allogenic transplanted cells.

In terms of antigen recognition the surviving T cell population remains pluripotential at this stage. There are two further stages in the selection of T cells; central and peripheral negative selection. This selection of antigen specific helper and cytotoxic T cells is critical in the development of immune responses to tumour antigens. If all self-recognising T cells are deleted from the T cell pool at an early stage then effective cytotoxic T cell responses to tumours antigens would be entirely dependent on tumour specific antigens, tumour associated antigens being included as self-antigens. A mechanism must exist by which potentially self-reactive cytotoxic T cells are able to mature to a certain point, but are only effective against self-antigens under exceptional circumstances. They must continue to be produced by the bone marrow and be available to the peripheral immune system if cytotoxic T cell dependent immune surveillance is to be effective.

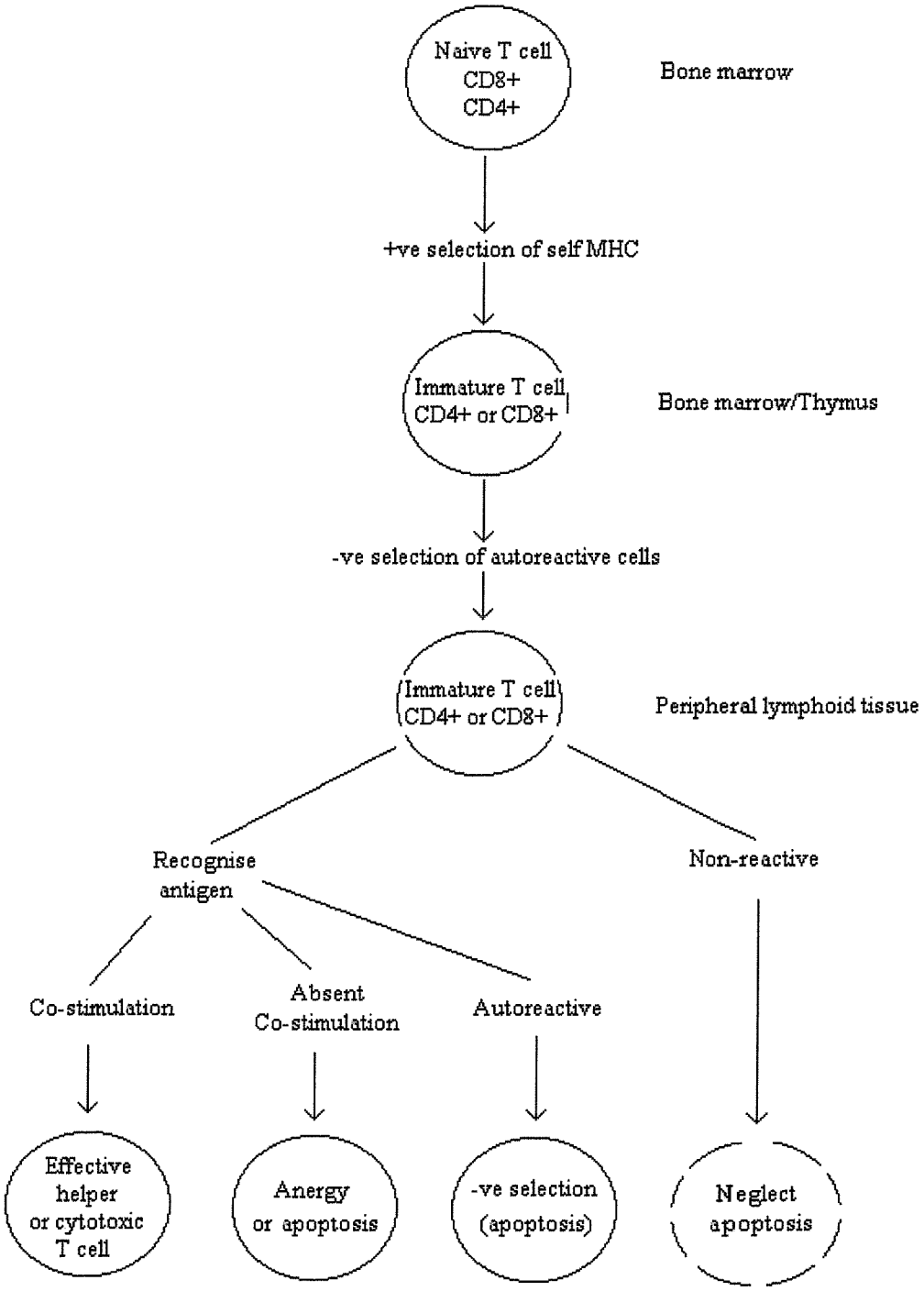


Figure 3.2. Flow chart summarising the sequence of events involved in the maturation of T lymphocytes. Initial selection is by positive selection of those cells that recognise self-MHC I. This is followed by negative selection of strongly autoreactive cells. The final outcome for the T cell is determined by a combination of positive and negative selection.

The first and most powerful step in the process of negative selection of self-recognising T cells occurs in the bone marrow, where the double positive naïve T cell encounters professional APC. These present a library of self-antigens restricted to

both MHC I and II. Most T cells that recognise self-antigen undergo apoptosis. It is not clear whether the apoptotic signal is dependent on physical properties of antigen itself, or on the strength and frequency of binding of the TCR to the MHC/antigen and a proportion of self reactive T cells escape. Furthermore there are a number of self-antigens, minor histocompatability antigens, which are not presented in the central immune system. T lymphocytes that recognise these antigens undergo negative selection in the periphery.

It is likely that central negative selection is incomplete. The process is also dependent on the frequency of antigen expression by the APC, and self-reactive T lymphocytes to less common antigens escape to the periphery without encountering their MHC-restricted antigen. For example T cells will survive that recognise tumour associated antigens occurring at a low frequency in normal tissue but over expressed by tumour cells. Similarly, lymphocytes that only weakly bind to MHC-restricted antigen will be rescued from apoptosis, and reach the periphery.

There is strong evidence that a number of self-reactive T lymphocytes do reach the peripheral immune system and are able to enter the circulation[83]. They do not cause autoimmune disease for a number of reasons. As discussed above the antigens they recognise are uncommon, and they only weakly bind MHC-restricted antigen. If peripheral lymphocytes do not encounter MHC-restricted antigen they soon undergo apoptosis through neglect. If they do encounter APC expressing MHC-restricted antigen, without an appropriate danger-signal that APC will not express the necessary co-stimulatory signals to effect a competent immune response on the part of helper and cytotoxic T cells. These cells remain anergic, but their life expectancy is greatly increased.

The subsequent function of effective, mature helper T cells is determined by the cytokines and cell surface receptors that enable them to interact with other immune cells including B cells, APC and CTL (figure 3.3). The general character of an immune response is determined by the profile of cytokines synthesised by a helper T cell following antigen recognition. A T_H1 cytokine profile is characterised by high levels of IFN-γ, IL-12 and TNF-β, and a T_H2 profile is characterised by high levels of IL-4, IL-5, IL-10and IL-13. Specific signals are communicated to individual cells through the expression of membrane proteins (figure 3.3), in particular the tumour necrosis factor (TNF) family, which is described in chapter 4.

Anergic lymphocytes may in turn contribute to inhibition of effective cytotoxic T cell responses. They may do this through physically blocking other lymphocytes at the MHC and preventing them from interacting with both APC and target cell. It is uncertain whether these lymphocytes can be rescued from anergy, although this may be important if we believe that CTL are to be important immunotherapeutic tools.

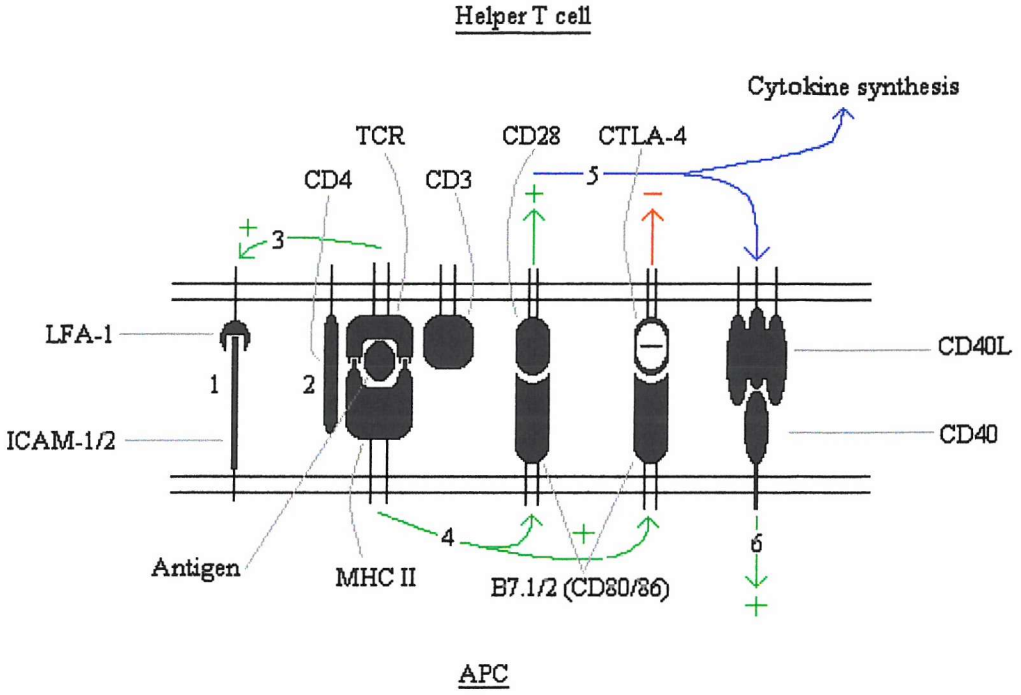


Figure 3.3. Antigen presentation to helper T cells.

1. T cells bind weakly to mature APC through cell adhesion molecules such as LFA-1/ICAM-(1-2), CD2/LFA-3 and ICAM-3/LFA-1.
2. This brings the TCR/CD3 complex on the helper T cell into contact with the antigen/MHC II complex on the APC.
3. If the antigen is recognised by the T cell conformational changes in the adhesion molecules induce greater affinity between the adhesion molecules.
4. Positive signalling through the MHC II results in the upregulation of surface co-stimulatory molecule expression on both the APC and the T cell. Immediately following antigen recognition B7 ligates CD28 on the helper T cell, but subsequent to this CTLA-4 expression is upregulated.
5. Positive signalling through CD28 allows the helper T cell to become fully functioning. There is further upregulation of co-stimulatory molecules on the T cell, particularly the TNF family, which includes CD40L, and the synthesis of cytokines relevant to the required immune response.
6. CD40 ligation on the APC induces further maturation of the APC and synthesis of cytokines. In the case of the dendritic cells this will 'license' the APC to fully prime, or arm, cytotoxic T cells.

3.4 Cross-priming of CTL by APC

Until recently convention would have that only antigen endogenous to the APC, such as that resulting from viral infection of the APC, could be presented in the context of MHC I. Furthermore APC would only present exogenous antigen, such as ingested tumour antigen, to helper T cells in the context of MHC II restriction and only these T cells would be responsible for the priming of CTL. Tumour antigen-specific helper and cytotoxic lymphocytes are extremely rare, with a frequency of 1/100,000 or less[73]. Generation of a T_H1 response would then require this rare tumour antigen-specific helper T cell to interact simultaneously with equally rare specific cytotoxic T cells, a statistically improbable event despite the high surface area available for antigen presentation by the APC. It was proposed that a mechanism exists by which the APC presents antigen to both helper and cytotoxic T cells at different times[84-86]. The helper T cell is able activate the APC in such a way that it is able to activate CTL independently of the helper T cell. However, as CTL only recognise MHC I restricted antigen, such a mechanism requires that antigen is presented in the context of both MHC I and II and therefore exogenous antigen must be able to enter the endogenous processing pathways of the APC for MHC I restricted presentation.

The term 'cross-priming' was coined 25 years ago to describe the induction of host CTL responses to MHC mismatched graft antigens through indirect antigen presentation[87]. It is now used to describe the process by which an APC is able to ingest exogenous antigen and to present it to and prime CTL through MHC I restriction[88]. This is discussed in more detail in chapter 4.

MHC I restricted antigen presentation by APC to naïve CTL is not helper T cell dependent, but without appropriate helper T cell signalling there is a lack of the co-stimulatory molecules on the surface of the APC. This results in anergic CTL that are unable to kill tumour cells and which may contribute to tolerance of tumour antigens by blocking tumour MHC I. They may represent a considerable proportion of the lymphocyte population seen in and around many tumours.

In a process called 'cross-tolerance', antigen specific CTL are deleted when inhibitory helper T cells recognise MHC II restricted self-antigen presented by an APC [89].

A final mechanism by which a T_H1 immune response may be promoted is through CD137/CD137L ligation. Signalling through CD137 following MHC I restricted

antigen recognition by CTL results in increased activity by the helper T cell including CD40 expression and IL 2 synthesis. This is discussed in more detail in chapter 4.

3.5 CTL-dependent cytotoxicity

Once CTL have been fully primed and armed by the APC they migrate back to the site of the tumour. CTL are highly efficient, selective serial killers of target cells. The mechanisms by which they are able to target and destroy their targets depend on effector molecules that are either cytokines or cytotoxins. The cytokines effect the potential targets of the CTL, the CTL themselves and other immune cells. IFN-γ is the predominant CTL cytokine. In particular it up-regulates the surface expression of MHC I by target cells which increases the library of antigen epitopes presented to the CTL. Macrophages are activated and become able to destroy phagocytosed structures. They increase nitric oxide (NO) synthesis and are more effective in antigen presentation. B cells undergo differentiation and immunoglobulin isotype switching to IgG2a under the influence of IFN-γ, which also activates NK cells. NK cells are particularly effective against target cells that do not express MHC I. The combination of increased IgG2a with an increase in NK cells may be a way in which the immune system is able to overcome the problem of evasion by tumour cells through down-regulation of MHC I expression.

Cytotoxins include perforins, the granzymes and cell membrane polypeptides. The perforins are released by CTL only in the immediate vicinity of the target cell. They polymerise into cylinders which are hydrophobic at each end but hydrophilic through the centre; they bridge the cell membrane to create a breach 16 nm across that allows the free passage of water, salt and small peptides across the cell membrane. At high *in vitro* concentrations of perforin the osmotic change in the cell is adequate to kill the cell, but *in vivo* the concentration is much lower. Instead the perforins allow granzymes to enter the cell. These are proteins with structural and functional similarities to the digestive enzymes trypsin and chymotrypsin. They do not directly digest the cell but cleave enzymes to initiate a cascade that leads to apoptosis. As the target cell undergoes apoptosis it is ingested by macrophages activated by CTL. Direct signals may be passed from CTL to target cell by cell surface molecules including TNF family members and their ligands.

The efficiency and effectiveness of CTL in killing their targets poses a dilemma. Given that many tumour antigens are self-antigens, it would seem inevitable that inducing a CTL-dependent immune response would result in considerable collateral damage to normal cells also expressing that antigen. However recent research has shown that the CTL response to some antigen is dependent on the frequency of MHC-restricted antigen presentation by the target cell [90]. Normal cells, with a low, background level of antigen synthesis will therefore escape CTL killing. Tumour cells, which over-express the antigen, will be killed, the level of antigen expression being reflected in their MHC I.

3.6 Conclusions

Successful CTL-based immunotherapeutic strategies depend on the identification and manipulation of key junctures in the sequence of events leading to the generation of armed CTL. They require suitable tumour antigen for presentation but this must also be encountered in the correct context in terms of both danger signals and additional (non-antigenic) molecules. Even if antigen presentation is effective, further dialogue is necessary for the production of armed CTL. This is dependent on the APC, the helper T cell and the CTL. Finally the tumour and its environment must be suitable for the CTL; antigen expression by normal tissue may lead to unacceptable collateral damage, and the tumour itself must be receptive to the CTL, through MHC I and antigen expression.

Chapter 4

An over-view of the roles of CD40, CD137 and their respective ligands in the immune system.

CD40 and CD137 are members of the tumour necrosis factor receptor (TNF-R) family. This group of receptors includes Fas, CD30, CD27 and TNF-R1&2 [91]. Their ligands, CD40L and CD137L are members of the TNF family.

4.1 CD40

CD40 was identified by expression cloning from a library of the Burkitt lymphoma Raji [92-94]. The murine cDNA was cloned from stimulated murine B cells using a probe derived from the human cDNA and shares 62% amino acid identity. More importantly extracellular cysteines are conserved suggesting that protein folding is similar. The CD40 gene is on human chromosome 20 and mouse chromosome 2, which is syntenic.

At first it was thought that CD40 distribution was limited to B cells and function restricted to B cell proliferation and isotype determination. It is now known that it is expressed by APC including macrophages and dendritic cells, endothelial cells and some epithelial cells. It has also been found on tumour cells including melanoma and carcinoma.

The protein is 48kDa with some characteristics of the TNF-R family. The extracellular region, which binds to CD40L, is folded into four domains, each consisting of two modules, A and B. However the intracellular region has few structural similarities with other members of the TNF-R family.

4.2 CD40L

The CD40 ligand (CD40L) was identified in 1992 [95]. CD40L is also referred to as CD154, gp39, TRAP or T-BAM. Murine CD40L cDNA was isolated from thymoma cells expressing high levels of CD40L. From this a DNA probe was developed to identify the gene for CD40L on the human X chromosome in stimulated blood T cells. Human and murine CD40L differ primarily in that human CD40L has five cysteines in the extra cellular domain, the murine only four[96].

The predicted molecular mass is 29 kDa[97] but the mass of CD40L isolated from most cells is 32-33 kDa suggesting post-translational modification[98]. Human CD40L has been found to have 78% amino acid identity with murine CD40L. X-ray crystallography has shown structural homology of CD40L with tumour necrosis factor, TNF-α and TNF-β[99]. It forms a threefold symmetric homotrimer such that it ligates three CD40 molecules. Smaller, soluble forms of CD40L have also been identified. These molecules retain the ability to trimerise and to crosslink CD40 on the cell membrane, suggesting a cytokine function for CD40[100].

CD40L is expressed by mature activated helper T cells (T_H1 and T_H2). Expression can be induced in basophils, eosinophils, platelets and some $CD8^+$ cells[101]. The distribution of cells expressing CD40L is tightly restricted, presumably to minimise the chance of unwanted bystander immune cell activation. Immunohistochemistry has demonstrated $CD40^+$ $CD4^+$ cells in the germinal centre light zones and interfollicular T cell-rich area of peripheral lymphoid tissue[102]. Strong CD40L expression by lymphocytes has also been found in certain inflammatory conditions (SLE) and allograft transplant rejection[103].

Stimulation of T cells via CD3/TCR leads to a rapid but transient expression of CD40L[104]. This would indicate that early expression is dependent on pre-formed protein. Subsequent expression is preceded by an increase in mRNA coding CD40L, 1-2 hours after the initial signal. Stimulation via CD28 enhances and stabilises CD40L expression.

Regulation of the CD40L signal is thorough. On ligation with CD40 there is down regulation of CD40L mRNA and receptor mediated endocytosis of the complex, followed by lysosomal and proteolytic degradation[104]. A soluble form of CD40, sCD40, is released which binds to and blocks CD40L[105]. Although immunosupressants such as cyclosporin are effective *in vitro* at down regulating CD40L there is still strong CD40L immunostaining in allograft tissue from transplant patients undergoing rejection[106].

CD40/CD40L ligation results in cross-linking of CD40 and subsequent signalling cascade in B cells and other APC (see below), but little is known about the pathways activated in T cells following CD40L cross-linking. Undoubtedly there are changes to the T cells as *in vitro* cross-linking results in enhanced cytokine production[107]. There is evidence that CD40L itself is involved in the promotion of memory helper T cells[108].

4.3 Signal transduction following CD40/CD40L ligation

The signalling cascade is very much downstream from $CD40L^+$ cells to $CD40^+$ cells. Following crosslinking of CD40 on the target cells a signal cascade is induced (reviewed by van Kooten[109]), the end-point of which is an increases in the expression of the transcription factor nuclear factor κ B (NFκB)[110]. There is no protein kinase domain in the intracellular region of CD40, but there are several areas at which various secondary messengers may interact (figure 4.1)

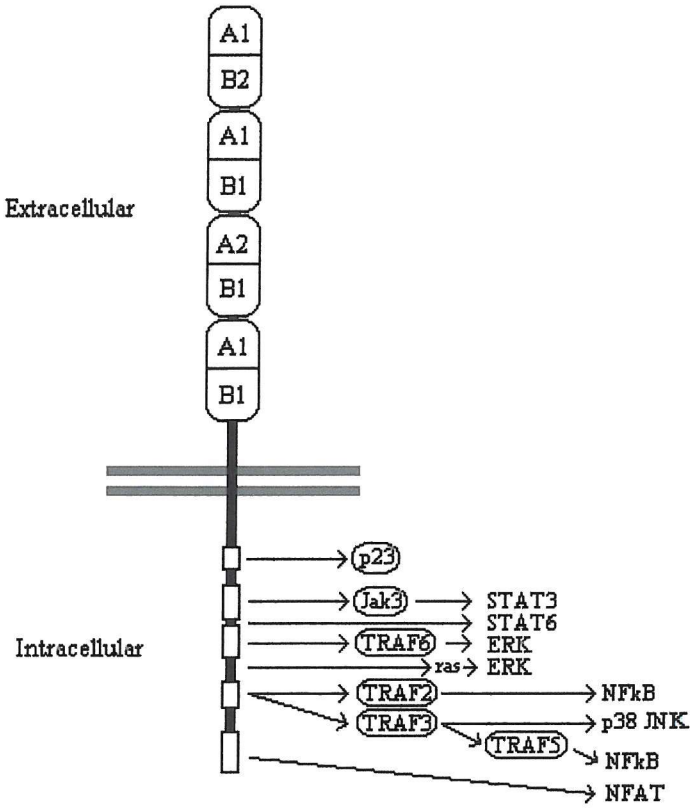


Figure 4.1. Diagram representing the structure of the CD40 molecule. See text for details.

The first detectable events following CD40 crosslinking involve protein kinases, including the activation of protein tyrosine kinases (PTK, e.g. Jak3). A family of proteins, TNF-R associated factors (TRAF), has recently been identified[111]. Unlike the extracellular regions of TNF-R, which show little cross-reactivity despite structural homogeneity, TRAF will associate with other TNF-R to form a complex of

homo- and heterodimers. These proteins have two types of functional domains. One is able to interact with CD40 and other TRAF, and the other is involved in signal transduction. The final links in the chain of signals are nuclear transcription factors, particularly NFκB and NFAT. The pathways involved are highly complex and have not been fully elucidated, but it is clear that there is multiple redundancy within the system. The cellular response varies according to the type of cell and its level of activation. For example T cell activation is inhibited in TRAF3 knockout mice but B cells are normal, whilst TRAF5 deletion results in impaired B cell proliferation and an inability to up-regulate certain cell-surface receptors.

4.4 Biological effects of CD40/CD40L ligation

Cell type	Change in function/cytokine production following recepto ligation
Pre B cell	CD23 expression, proliferation
Naïve mature B cell	Proliferation, differentiation and isotype switching
Germinal centre B cells	Proliferation, differentiation Fas expression and selection
Plasma cells	IL-6
Monocytes/macrophages	Cytokine secretion, NO/ MMP production and procoagulation.
Synovial monocytes	TNF
Alveolar macrophages	High level CD40 expression in sarcoidosis
Dendritic cells/Langerhans cells	Growth and survival, cytokine and costimulatory molecules
CD34+ precursors	Proliferation. Develop into dendritic cells
T cells	Proliferation, CD25 expression, cytokines
Eosinophils	Survival, GM-CSF
Endothelial cells	Tissue factor, thrombomodulin, IL-1 IL-6 IL-8, GM-CSF
Epithelial cells: Thymus	GM-CSF
Renal	IL-6 IL-8 GM-CSF
Skin	CD54 IL-8
Carcinoma	Growth inhibition, apoptosis
Fibroblasts	IL-6 IL-8 GM-CSF Cox-2
Follicular dendritic cells	Growth CD54
Vascular smooth muscle	MMP
Hepatocytes	Fas-L

Table 4.1. Expression of CD40 by various cell types (adapted from van Kooten [109]).

4.4.1 B cells

The clinical significance of CD40 is demonstrated by individuals with a defective gene for CD40L, who develop X-linked hyper-IgM syndrome (HIGM)[112]. HIGM is characterised by high levels of serum IgM, with some IgD but only trace levels of

other isotypes. The B and T cell populations develop normally but no germinal centres are formed in the lymph nodes. Patients are susceptible to infection with extracellular bacteria and opportunistic pathogens. These two clinical observations suggest that the predominant results of CD40/CD40L cross-linking are B cell maturation and isotype switching, and activation of macrophages. Similar patterns of disease are found in experimental mice in which CD40L has been deleted, suggesting that a murine model for investigation of the functions of CD40 and CD40L in humans would be valid[113].

In vitro studies of CD40 have elaborated on the function of CD40 in B cells, and have demonstrated the critical role that CD40/CD40L plays in antigen presentation to helper T cells and the subsequent activation, proliferation, differentiation and maturation of B cells (figure 4.2) [109].

CD40 cross-linking alone results in B cell proliferation and maturation with up-regulation of surface costimulatory and adhesion molecules (CD23, B7.1, B7.2 and ICAM), MHCI/II and cytokine production [114]. Fas is also up-regulated and will bind Fas ligand (FasL) expressed by T cells[115], causing B cell apoptosis unless the BCR is cross-linked by antigen. This prevents activation of bystander B cells, but prolonged cross-linking of the BCR in the absence of CD40 dependent T cell stimulation also results in B cell apoptosis.

Increased cytokine production by T cells and APC critical for isotype switching of B cells following antigen presentation[116] is enhanced by CD40 cross-linking.

4.4.2 Endothelial cells, epithelial cells and fibroblasts.

Expression of CD40 by non-haematopoietic cells is generally very low but it can be induced *in vitro* in some epithelial cell lines by adding IFN-γ or IL-1 to the culture medium. Its role is not clear but is probably related to local production of cytokines and inflammatory mediators[117]. Expression of CD40 by endothelial cells and of CD40L by activated thrombocytes suggests a role in mediating coagulation.

Melanocytes express low levels of both CD40 and CD40L. High levels of expression of both molecules by melanoma tumour cells is associated with improved patient survival, but it is not clear whether this is as a result of a direct effect of CD40L/CD40 or whether it is as a result of an immune meditated cytotoxic mechanism (see below). There are neither gross organ abnormalities nor specific organ dysfunction in patients with HIGM suggesting a degree of redundancy in cellular mechanisms involving CD40/CD40L in these tissues.

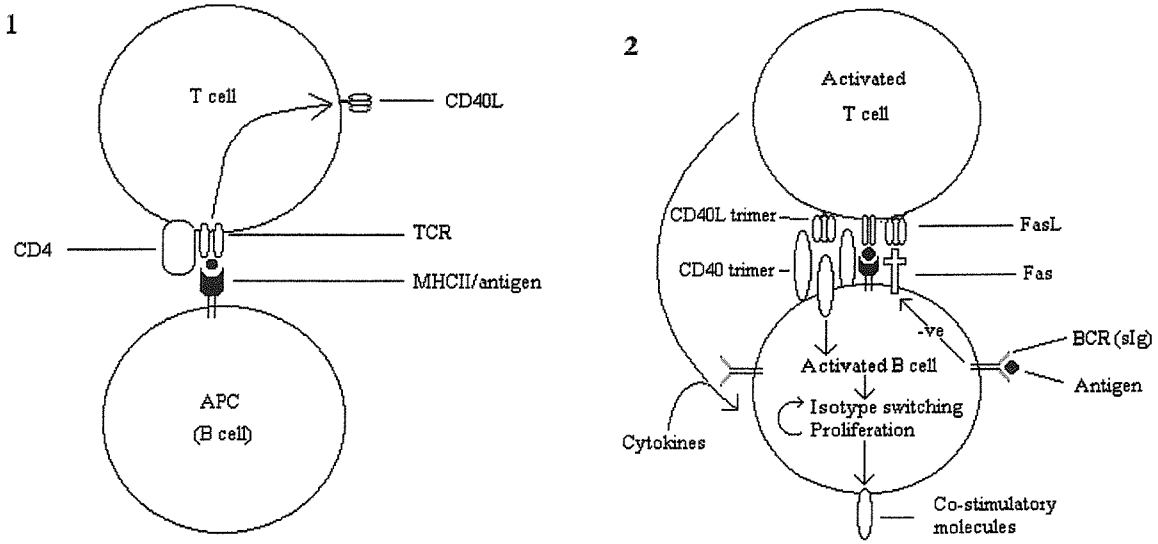


Figure 4.2.

1: When a helper T cell recognises an epitope presented by an APC (e.g. a B cell) in conjunction with MHCII/TCR complex, the T cell may up-regulate expression of CD40L.

2: CD40L then cross-links CD40 on the B cell. If the B cell receptor (surface Ig, sIg) is cross-linked by antigen relevant to that recognised by the MHC II/TCR complex, Fas is inhibited, preventing apoptosis.

4.4.3 Professional APC

CD40 cross-linking on APC including monocytes and dendritic cells results in profound changes in their phenotype and function. Immature cells in peripheral tissue are phagocytic, with few surface co-stimulatory molecules or appendages of the cell wall. Following the uptake and processing of antigen they migrate, usually to regional lymphoid tissue. Here their function changes from antigen uptake to antigen. The cell surface becomes 'hairy' in appearance (hence the appellation dendritic) which greatly increases the cell surface area for antigen presentation on MHC. The MHC II interacts with the TCR of helper T cells, and if antigen is recognised by the T cell there is a similar chain of events to that seen in B cell/T cell interactions (figure 4.2). This has several effects on the APC[118]. Cell survival is enhanced and cytokine production is increased. Monocytes may also initiate production of enzymes such as matrix metalloproteinase (MMP) and nitrous oxide (NO). Dendritic cells become fully activated to become the most powerful APC in the induction of CTL responses to antigen, including tumour antigens. The cellular mechanisms by which these changes are effected have not been fully elucidated.

Despite the powerful response that CD40 ligation induces in B cells the general response of CD40 ligation on APC is characterised by a T_H1 cytokine profile, that is to say IFN-γ and IL-12 predominate over IL-4[119].

Patients with HIGM are also susceptible to infections with intracellular organisms such as *Pneumocystis carinii.* Effective immune responses to these organisms are cytotoxic rather than humoral, confirming a role for CD40L/CD40 in the generation of armed CTL. The realisation that CD40 was expressed by APC and its central role in the licensing of the CTL advanced our understanding of the mechanisms involved in antigen presentation (figure 4.3).

Peptide derived from tumour antigen is presented by a naïve APC to a specific helper T cell. If the MHC II restricted antigen is recognised by T cell, it will up-regulate CD40L expression and cross-link CD40 on the APC. The APC then becomes fully licensed (cross-primed) to arm any cytotoxic T cells that recognise MHC I restricted antigen through its TCR. Surface co-stimulatory molecules including B7.1, B7.2 and LFA1 are up-regulated. Cytokine secretion by APC, helper T cells and cytotoxic T cells promotes proliferation of the cytotoxic T cells which then migrate to the periphery to kill tumour cells bearing that MHC I restricted antigen.

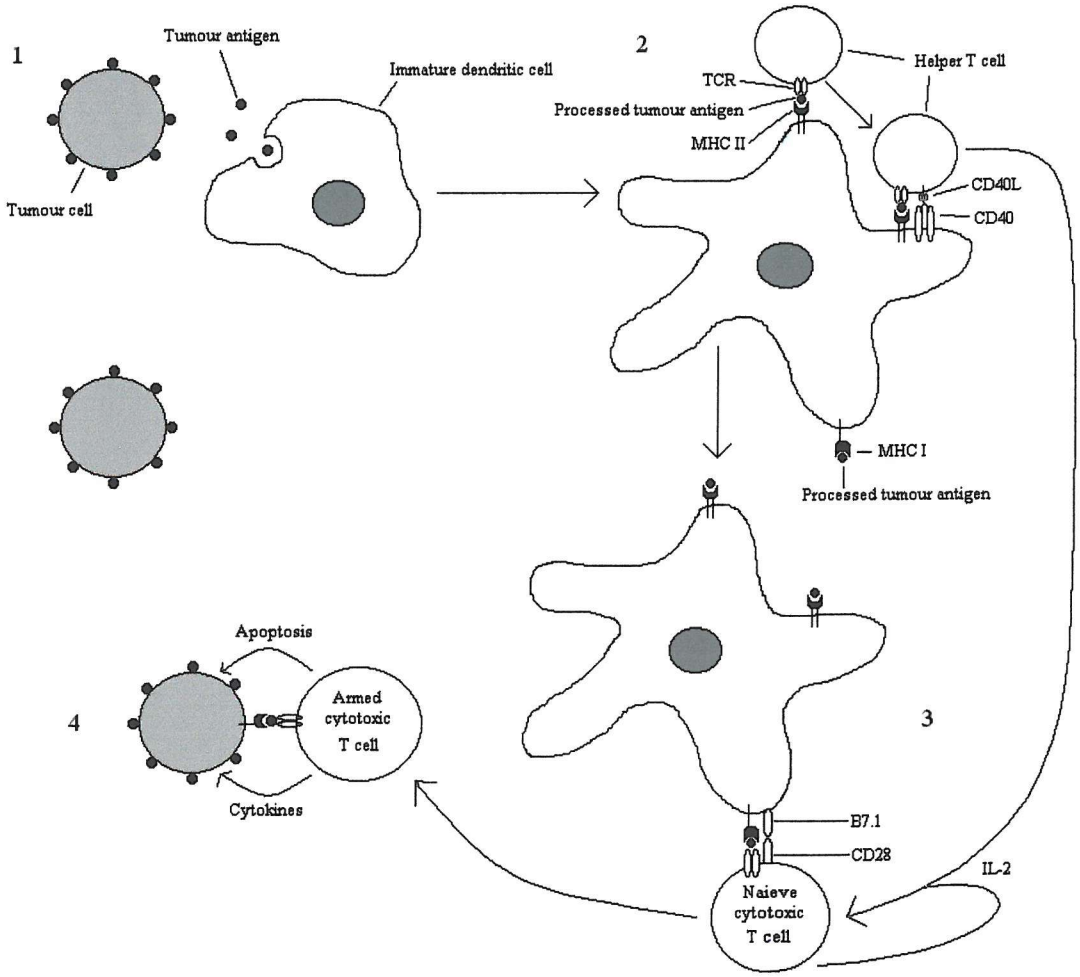


Figure 4.3. Antigen, in this case shed by a tumour cell, is processed by dendritic cells resulting in anti-tumour cytotoxic T cells, a process called "cross-priming".
1: Polypeptides and proteins (tumour antigens) shed by tumour cells are ingested by dendritic cells, which then migrate to the regional lymphoid tissue.
2: The dendritic cell processes the antigen and presents a library of epitopes on MHC II to helper T cells. The morphology of the dendritic cell has already changed to increase the cell surface area over which this may occur. CD40L expression by the T cell is increased and dendritic cell CD40 is cross-linked.
3: The dendritic cell is now 'licensed' to 'arm' naïve cytotoxic T cells. Antigen is presented to cytotoxic T cells in the context of MHC I and co-stimulatory molecules e.g. B7.1/CD28. The armed T cells are further stimulated to proliferate by autocrine and helper T cell secreted cytokines, e.g. IL-2 and IL-12.
4: The armed T cells leave the lymphoid tissue to migrate to site of tumour growth where the engage the tumour cells. The cytotoxic T cells recognise antigen restricted to MHC I and a signal is sent to the tumour cell resulting in apoptosis of that cell. Secretion of cytokines, e.g. IFN-γ and TGF-β, enhance expression of MHC I and recognition of death signals by tumour cells.

It has been proposed that the arming of self-recognising cytotoxic T cells is CD40-dependent[120]. If a cytotoxic T cell recognises that antigen on a naïve APC without CD40 ligation then it is rendered anergic. It is still able to recognise and bind MHC I restricted antigen on a tumour cell's surface but will not have any effect on that cell.

4.5 Immunotherapy

There is increasing evidence that CD40 may be of considerable use in the development of novel immunotherapeutic strategies[121,122]. It is hoped that immunomodulation of the immune system through CD40 ligation will yield cytotoxic T cells capable of killing tumour cells. Very strong evidence that this is possible has been demonstrated in the treatment of murine models of lymphoma. French et al[123] showed that treatment with anti-CD40 mAb lead to the *in vivo* eradication of established B cell lymphoma (figure 4.4). Three syngeneic cell lines were used. Their results confirmed that although the B cells were $CD40^+$ themselves the mechanism of action was dependent on $CD8^+$ cells, but not $CD4^+$ cells. The antigen recognised by the $CD8^+$ cells was not CD40 and animals cured of lymphoma were resistant to re-challenge with the same lymphoma, implying immune memory.

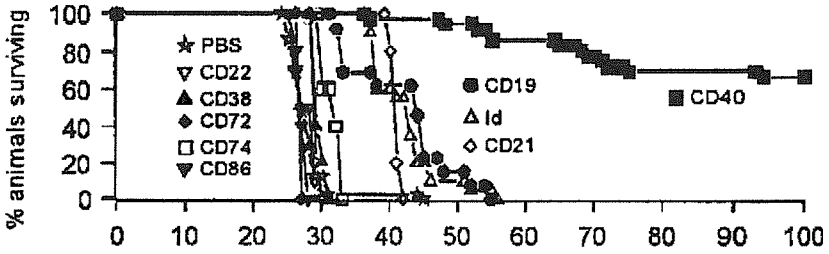


Figure 4.4. When mice with established B cell lymphoma (in this case BCL1) were treated with mAb against different B cell antigens, anti-CD40 was by far the most effective (French et al[123]).

Although this has profound implications for our understanding of the activation of cellular immune responses, lymphoma is an atypical model of malignancy. Being derived from a B cell line it presents antigen in the context of MHC I and MHC II. It may be that the lymphoma cells are presenting their own tumour antigen to $CD8^+$ and thus inducing an effective cellular response though CD40 cross-linking. For this reason it is imperative to study CD40 cross-linking in tumours that do not function as their own APC.

What is less certain is the generation of immune memory. When murine models of B cell lymphoma are treated with anti-CD40 mAb there is rapid tumour clearance, particularly following large tumour antigen loads. When mice that have been cured of lymphoma are re-challenged with the same lymphoma they are resistant to that lymphoma for many weeks, and the resistance is abrogated by depletion of cytotoxic T cells. Clearly the duration of the immune response extends considerably beyond that of the anti-CD40 mAb, the half-life of which can be measured in days. As CD40-directed therapy is developed for clinical trials the duration of auto-reactive T and B cells must be carefully monitored for the toxicity to be determined.

An unusual situation has recently been observed in Aquired Immune Deficiency Syndrome (AIDS). In AIDS there are reduced levels CD40 and CD40L and an increased incidence of both lymphoma and Kaposi's sarcoma[124-126]. These tumours usually express CD40, and therapy with CD40L has been investigated, however CD40 cross-linking on Kaposi's sarcoma is associated with disease progression [127].

4.6 Autoimmunity and inflammation: the clinical implications of immunomodulation through CD40 cross-linking.

There are real concerns that therapies based on the activation of immune mechanisms through CD40L/CD40 will result in autoimmune disease. This possibility has been investigated but there is little evidence that disease is can be induced through artificial CD40 cross-linking[128]. Given that immunomodulation through cross-linking of CD40 is apparently indiscriminate, and that CD40/CD40L participate in a range of responses involving both the cytotoxic (T_H1) and humoral (T_H2) responses this is perhaps surprising.

However there is growing evidence of the role of CD40/CD40L in autoimmune disease in the experimental treatment of animal models of autoimmunity. Several models have been studied using either anti-CD40L mAb to prevent CD40 cross-linking, or CD40 and CD40L knockout-mice. Murine models of collagen arthritis[129], systemic lupus erythematosus (SLE)[130], nephritis [131] and autoimmune diabetes[132] have all shown therapeutic benefit from anti-CD40L mAb. Similarly pulmonary inflammation is abrogated in CD40 knockout mice with asthma.

In all of these models the immune responses are heterogeneous, making it difficult to demonstrate at what point anti-CD40L may be acting.

The diseases that demonstrate the most promising response to CD40/CD40L blockade are those chronic illnesses characterised by prolonged periods of indolent inflammation, rather than those that are acute and aggressive with more florid inflammation[128]. With regard to the use of anti-CD40 mAb or similar therapies based on the direct activation of the immune system, it may be that a short period of immune activation, whilst effective in mediating tumour cell killing, is not an adequate stimulus to induce chronic inflammation.

Histological studies of human lupus nephritis (SLE)[133] and multiple sclerosis[134] have demonstrated increased levels of CD40L on helper T cells, and of $CD40^+$ APC. These disease processes are characterised by their chronicity, with periods of acute exacerbation and anti-CD40L mAb is entering clinical trials.

A similar pattern is seen in other chronic inflammatory processes such as atherosclerosis[135] and lung fibrosis[136]. The histology of these diseases is characterised by tissue infiltration with both T and B cells, suggesting a role for the adaptive immune system in the disease process. Again, although in animal models treatment with anti-CD40L mAb has reduced both inflammation and fibrosis, the prolonged period of inflammation is critical in the pathogenesis of the disease. This suggests that should such a disease process be induced by stimulation through CD40, that stimulation would have to be over a considerable period of time.

4.7 CD137

The receptor CD137, which is also referred to as 4-1BB (used to describe the cDNA isolated from an inducible T cell transcript) was identified more recently than CD40, firstly in the mouse. Less is known about this molecule and its ligand, CD137L. CD137 is a type 1 membrane protein that is found as either a 30-kDa glycoprotein monomer or a 55-kDa dimer [137]. Like CD40 it is a member of the TNF receptor superfamily. Human CD137 was identified shortly thereafter and its amino acid sequence is 60% homologous to murine CD137. However extracellular and intracellular regions of the molecules are identical in the position of cysteine residues confirming close structural similarities [138].

CD137 is expressed by mature T cells, identified by expression of CD45RO (chapter 2, figure 2.5). It has not been found on naïve T cells. Expression is first detected by increasing levels of mRNA following TCR modulated stimulation by MHC, with a peak at 60 hours[139]. The functional response (see below) takes 4 to 5 days. Although up-regulation is TCR dependent there is no functional response in the absence of CD28 ligation. Expression is amplified through IFN-γ, for which there is a positive feedback loop. However IFN-γ alone will not induce CD137 expression [140].

An interesting note is that the human gene resides in 1p36, and mutations within this cluster are associated with several malignancies[141].

4.8 CD137L

CD137L is a type 2 membrane protein expressed primarily by APC, including dendritic cells, B cells and macrophages[20]. Expression follows CD40 ligation, usually on fully mature, licensed APC. Inducible expression has also been demonstrated in T cells but the functional significance of this has not been described[138].

4.9 Signal transduction and biological effects of CD137/CD137L ligation.

Signal transduction from CD317 is similar to that for CD40. Ligation of the receptor cross-links the molecules on the cell surface. Receptor cross-linking by anti-CD137 mAb has a similar effect, inducing a positive signal rather than blocking activity. CD137 does not have a signalling region but relies on TRAF 1 TRAF 3 and particularly TRAF 2 to activate PTK[142]. Signal transduction can be observed in the nucleus through increased NF-κB.

Cross-linking CD137 on T cells *in vitro* increases T cell populations: the CD4+ and $CD8^+$ populations quadruple. There is a CD28-dependent T_H1 cytokine response from CTL, with significantly increased (×30) levels of IFN-γ[143]. *In vivo* in mice there is an increase in levels of IL-2 and IL-4[144]. T_H2 responses are inhibited, but this effect may be secondary to a CD137-dependent increase in IFN-γ [145]. It is significance that expression of CD30, a T_H2 cell marker, is mutually exclusive of CD137, reinforcing the role of CD137 in T_H1 responses. Furthermore, T cell apoptosis in these cells may be induced by CD137 ligation, further inhibiting inappropriate T_H2

responses [145]. However its role has been shown to be far more sophisticated than the co-stimulatory support of T_H1 immune responses (figure 4.5).

Interestingly the signal may be bi-directional. Resting B cells expressing CD137L proliferate following CD137L binding. The mechanism by which this occurs has not been demonstrated[145]. A similar mechanism almost certainly occurs in APC but has not been conclusively demonstrated. It probably reinforces positive signals from the APC to $CD8^+$ cells, enhancing T_H1 responses to a given antigen.

Cross-linking of CD137L on macrophages leads to a rise in IL-6, IL-8 and TNF-α. Whilst these cytokines are not directly related to T_H1 responses, they are strongly implicated in inflammation and in the generation of danger signals to the APC[138].

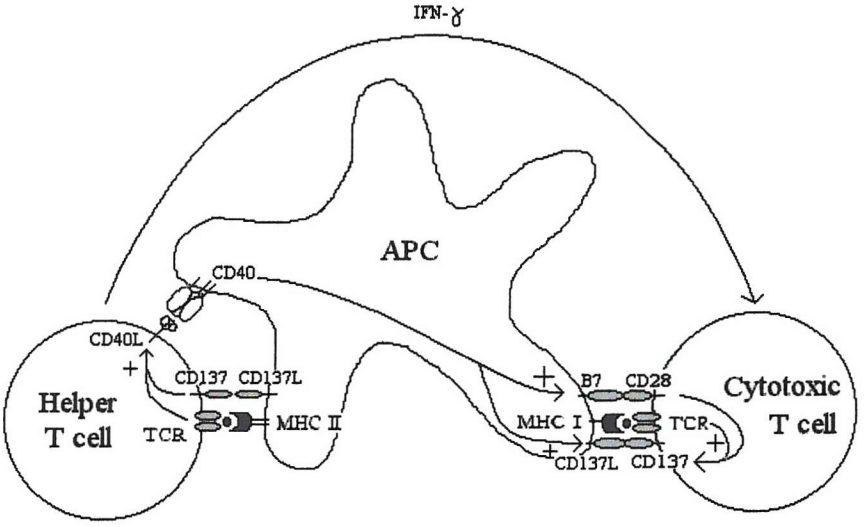


Figure 4.5. CD137 ligation is important in antigen recognition by both helper and cytotoxic T cells. TCR/MHC II restricted antigen recognition results in increased expression of CD40L. If this in conjunction with CD137-crosslinking the response is augmented. Subsequent CD40 cross-linking 'licenses' the APC (see above and chapter 4). CD137 cross-linking on cytotoxic T cells restricted to the same antigen through TCR/MHC I, in conjunction with CD28 co-stimulation, results in an armed T cell that is resistant to AICD.

Repeated co-stimulation through CD28, whilst necessary for an effective T cell response, also leads to activation-induced cell death (AICD). Fas rather than IFN-γ mediate this, in conjunction with down-regulation of CD28 [146]. Such a mechanism would serve to dampen T cell response to antigens and minimise the potential for T cell mediated autoimmune responses. Co-stimulation of T cells through CD137 protects them from CD28 dependent AICD, leading to an increase in the T cell population. The synergistic effect of IFN-γ and CD137 suggests that these cells are also resistant to inhibition of T cell proliferation by IFN-γ. Blocking IL-2 does not

prevent CD137 dependent protection from AICD, suggesting that the protected cells are mature and long-lived[147].

The immune system of CD137 knockout mice is able to function at an adequate level. The qualitative response by T cells to viral challenge is normal, but T cell numbers are reduced. Immune memory is preserved[148].

Anti-CD137 mAb has recently been demonstrated to induce tumour regression in murine models of tumour. Melero et al[143] demonstrated that established Ag104A sarcoma and P815 mastocytoma could be cured, and that the cured animals were resistant to re-challenge. The resistance is tumour specific. Studies in $CD4^+$ and $CD8^+$ mAb-depleted mice have shown that both T cell sub-sets are required for anti-CD137 mAb to be effective. There was no T cell response, other than proliferation, in animals that had not been exposed to tumour antigen. Whilst tumour antigen primes T cells the response is weak. CD137 ligation promotes and amplifies an effective T_H1 response to tumour antigen. Moreover it provides a survival signal to $CD8^+$ (but not $CD4^+$) T cells which blocks AICD and induces long term immune memory[147].

Although CD137 has not been strongly implicated in autoimmune disease processes a soluble form, sCD137 has been identified in increased levels in some patients with rheumatoid arthritis. The expression of sCD137 lags behind that of membrane bound CD137 by 24 hours and provides a negative control by increasing AICD[149].

4.10 Summary

Endogenous antigens, such as tumour antigens, may enter APC processing pathways and be presented to $CD8^+$ T cells in the context of MHC I through cross-priming. This is an extremely powerful means of presenting antigen, including self-antigens, and the undesirable consequences of successful presentation and subsequent T_H1 immune response include devastating autoimmune disease such as systemic lupus erythematosis. Therefore MHC-restricted antigen presentation to T cells via the TCR complex leads to T cell anergy unless there is a second signal.

The second, or co-stimulatory, signal is provided by CD28 on the APC. This is only expressed by mature APC. Other co-stimulatory signals are also involved in determining the outcome, and CD28 co-stimulation alone will lead to an impotent immune response.

The immune system exists as a dynamic entity. Antigen is constantly processed and presented to helper and effector cells, and a balance exists between positive and negative (anergic or apoptotic) responses, and between cellular and humoral responses. This balance can be tipped, and with our increasingly sophisticated understanding of the processes involved it can be manipulated to achieve an effective response to antigen. CD40 and CD137 are two receptors of fundamental importance in the generation of CTL to endogenous antigens presented by cross-priming. Cross-linking of these receptors by mAb has been shown to prevent $CD8^+$ T cell anergy, yielding fully armed cytotoxic T cells with the capacity for immune memory. For the most part this has been demonstrated only in models wherein the antigen has been artificially introduced though cell transfection. Much work remains to demonstrate whether a similar effect can be elicited in *in vivo* tumour models of low immunogenicity.

Chapter 5

Materials and methods

5.1 Culture medium

All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco) supplemented with 2mM L-glutamine (Gibco), 1mM pyruvate (Gibco) and 10% foetal calf serum (FCS)(Myoclone plus, Gibco), referred to in the text below as DMEM/10%FCS. Antimicrobials were added to all medium, unless otherwise stated, at the following concentrations: 100units/ml penicillin (Glaxo), 50 units/ml streptomycin (Evans) and 50 units/ml amphotericin B (Fungizone, Squibb and sons). Lymphocytes were cultured *in vitro* in DMEM with 5% FCS and 50 μM mercaptoethanol, referred to as DMEM/5%FCS/mercaptoethanol.

5.2 Cell lines

B16F10 (supplied by International Cancer Research Fund) and B16.c215 melanoma cell lines are syngeneic with C57Bl6 mice, and Colon tumour 26 (CT26) (supplied by International Cancer Research Fund) are syngeneic with BALB/c mice.

Cells were maintained in DMEM/10%FCS at 37^0 in a 5% CO_2 humidified incubator. The medium was changed every 3-5 days, when the cells were seen to be growing as a monolayer. To maintain both tumour cell lines in a log growth phase the cell cultures were split on a regular basis. CT26 cells were washed off the bottom of the plastic culture flask with medium from a movette and the total cell number reduced by 80%. The B16 cells require a cell-scraper to lift them off the plastic. Again the cell number was then reduced by 80%.

Cells to be used for *in vitro* or *in vivo* experiments were prepared in such a way to give high numbers of viable cells in log phase of growth. Three to four days before any cells were required for an experiment the cells were washed off the bottom of the culture flask by gently agitating them in sterile phosphate buffer solution (PBS) with 0.2mM ethylenediamine tetra-acetic acid (EDTA) for 10 minutes. Approximately 90% of the resulting suspension was discarded. The remaining cells were washed once in DMEM and re-suspended in DMEM/10% FCS. They were incubated for a further two to three days. 24 hours before the cells were required the medium was

changed. The cells were finally harvested by washing them off the culture flask with PBS/0.2mM EDTA, as above then washing and re-suspending in DMEM. Enzymatic separation of cells with trypsin was avoided as it has been shown to alter cell-surface antigens, particularly in B16 cell lines [150].

5.3 Cell quantitation and viability

Cell viability was measured by adding 50 µl of trypan blue to 50µl of a suspension of 10^6 cells/ml and counting the ratio of live (clear) cells to non-viable (blue) cells on a haemocytometer. The cells were counted with a Coulter Industrial D Cell Counter (Coulter Electronics, Bedfordshire) and an appropriate volume of medium added to dilute the cell suspension to the required concentration.

5.4 *In vivo* tumour growth

The *in vivo* growth characteristics of the tumour models were determined by the inoculation of age and sex matched syngeneic mice with the relevant tumour cell line and is described in Chapter 6.

5.4.1 Pulmonary tumour model

Tumour cells were grown *in vitro* as described above (section 5.2). They were harvested as they reached confluence by washing the cell layer with 0.2 mM EDTA two to three times and then gently agitating the flask with 0.2 mM EDTA for ten minutes. After this the cells were centrifuged once and re-suspended in 1 ml DMEM without additives. The viability and concentration of the cell suspension was checked (section 5.3).

Once its concentration had been corrected 200 µl of the tumour cell suspension was inoculated into the dorsal tail vein (intravenous, iv) of the mouse (day 0). BALB/c mice inoculated with CT26 tumour were observed on a twice-daily basis and culled when determined to be unwell due to the tumour load. The date of death was the recorded endpoint for these studies. On the basis of previous experience with iv injection of B16.c215 the C57Bl6 mice were culled on day 20 after tumour inoculation and the tumour deposits in their lungs counted (J. Alberts, personal

communication). A proportion of mice was dissected to exclude wide spread dissemination of tumour.

5.4.2 Subcutaneous (sc) and intradermal (id) models

CT26 tumour in log growth phase was prepared as described above (6.2) and 200µl of a suspension of 10^6 cells/ml injected into the subcutaneous tissue of the shaven left flanks of two BALB/c mice. Once the tumours had reached adequate size (approximately 15mm in any one dimension) the mice were culled and tumour dissected from the subcutaneous tissue. It was then cut into $1mm^3$ cubes, avoiding the tumour capsule and areas of necrosis. One cube was immediately passaged into the subcutaneous tissue of the shaved left flank of anaesthetised mice using a trocar. The small wound was closed with a Michelle clip, which was removed after two days. $1mm^3$ represents a large tumour load, but it was not possible to cut smaller pieces with any consistency.

When tumour is injected into the intradermal tissue it does not disperse and the initial tumour growth is contained. Only 50µl of tumour suspension may be inoculated into the intradermal tissue of each mouse, and so much higher concentrations of tumour were required.

Measuring the maximum dimensions of the tumour in two axes followed the growth of passaged subcutaneous and intradermal tumours. Digital callipers were used and measurements made to 0.1 mm.

One mouse from each group was dissected to establish whether tumour growth extended beyond the lungs for those mice receiving intravenous tumour and away from the site of injection for those mice receiving local injection of tumour. Mice were dissected to exclude wide spread dissemination of tumour.

5.5 Fluorescence activated cell scanning (FACS) analysis of cell phenotype

5.5.1 Single channel

Cells that had been growing *in vitro* were harvested, washed in DMEM and re-suspended in PBS at a concentration of 5×10^6 cells/ml. 300µl of this suspension was incubated at 4^0 C for 15 minutes with the relevant mAb at a final concentration of 50µg/ml. In most cases the antibodies used were conjugated with fluorescein iso-thiocyanate (FITC) or phyco-erythrin (PE). If the primary antibody was not fluorescence-conjugated then a specific secondary fluorescent mAb was used to label the primary mAb. The method for the secondary labelling was the same as for the primary. After washing the cells in PBS they were re-suspended in 300µl PBS/azide (20 mM NaN_3) to stabilise antibody binding.

Analysis was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Negative (irrelevant mAb) control samples were included to calibrate forward and side scatter of the cells (FSC and SSC), and to set the gain for the fluorescence channel. Cell debris was excluded by adjusting the FSC threshold parameter. FITC is excited at a wavelength of 488 nm and emission intensity was recorded at 515-545 nm (FL1). 10,000 events were collected per analysed sample and the results expressed as a histogram of fluorescence intensity (FL1 or log FL1 on the X-axis) versus cell number (events on the Y-axis).

5.5.2 Dual Channel

Cells were prepared as described in sections 6.3 or 6.10. The resulting washed cell pellet was resuspended in PBS at a concentration of 5×10^6 cells/ml. They were labelled with the relevant FITC conjugated mAb as described in section 6.5.1. After the first wash the cells were re-suspended in 100µl PBS and 10µl of the appropriate PE conjugated mAb at a concentration of 100µg/ml was added. Again cells were incubated at 4^0 C for 15 minutes before a final wash and re-suspension in 300µl PBS azide. Negative controls for both channels were included to calibrate both FCS and SSC, and also to set the gain and the compensation for each fluorescence channel.

10,000 events were recorded per sample and analysed with CellQuest (BD Immunocytometry Systems) software. Dot-plots for log FL1 (x-axis) and log FL2 (y-

axis) were used to calculate the proportion of cells of each phenotype studied by gating discrete populations, and thus total cell numbers.

5.6 PCR

Reverse transcription PCR amplification and analysis of tumour cell mRNA was performed using standard commercial kits. A 'QuickPrep' mRNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) was used to extract and purify the mRNA. Sterile (RNAase-free) conditions were maintained. Tumour cells were grown *in vitro* (6.2) and 10^6 pelleted in a 30 ml universal tube by centrifugation. 1.5 ml extraction buffer (guanidinium thiocyanate, GTC) added at room temperature. The cells were homogenised and the homogenate diluted with 3 ml elution buffer. The extract was then centrifuged (12,000×g) for 5 minutes.

The Oligo(dT)-cellulose column was centrifuged (350×g) for 2 minutes in a 15 ml centrifuge tube, then emptied, to prepare a matrix of oligo(dT)-cellulose resin for mRNA isolation. 4 ml of extract supernatant was added to the spun column. The column was gently agitated to resuspend the resin and mixed for ten minutes. The column was then centrifuged (350×g) in a 15 ml tube for 2 minutes. The supernatant was discarded without disturbing the resin and 3 ml high salt buffer added to resuspend and wash the matrix. The tube was centrifuged (350×g) for 2 minute. This wash was repeated twice.

A further two washes were performed, this time using the low salt buffer.

At this stage the bottom closure of the column was removed and a 1.5 ml eppendorf tube placed in the 15 ml centrifuge tube, beneath the column. The bound poly(A)$^+$ RNA was eluted from the matrix by adding 0.25 ml elution buffer to the spun column and centrifuging (350×g) for 2 minutes. This was repeated twice more, giving 0.75 ml elution buffer/mRNA in the eppendorf. The collected sample was placed on ice (~4^0 C).

The mRNA elution was confirmed by spectrophotometry. 0.25 µl, or a dilution thereof, of the elutant was analysed by measuring the absorbency at 260 nm (A_{260}) and calculating the concentration of the mRNA using the formula [mRNA]=A_{260}×D×40 µg/ml, where D is the final dilution.

The mRNA was precipitated by the addition of 100 µl sample buffer, 10 µl Glycogen solution and 2.5 ml ice-cold ethanol. After 2 hours incubation at -20^0C the sample was centrifuged at 4^0C, 12000×g for 10 minutes, and the pellet retrieved.

A cDNA library was prepared from the mRNA using a 'First-Strand cDNA Synthesis Kit' (Amersham Pharmacia Biotech, Buchinghamshire, UK). The mRNA was resuspended in 20 µl sterile distilled water at 65^0 C for 10 minutes, then chilled on ice. One µl 'DTT Solution' was added to 11µl of 'Bulk First-Strand cDNA Reaction Mix' and 1 µl primer in a 1.5 ml eppendorf tube. The heat–denatured mRNA was mixed in and the tube incubated at 37^0 for one hour.

The PCR reaction was performed with TAQ polymerase. The amplified products were separated by electrophoresis in 1.5% agarose gel containing ethidium bromide at 0.6 µg/ml. The separation of the bands was demonstrated by UV photography.

5.7 Transfection of CT26

CT26 cells were transfected according to the methods of Andreason and Evans [151, 152].

30µg of DNA encoding for a plasmid vector and geneticin resistance gene was incubated with DNA encoding the target antigen in the presence of Puv I digestion enzyme.

CT26 cells that had been growing *in vitro* were harvested and washed as above (section 6.2). Two batches of 2×10^6 cells were re-suspended with the DNA in 0.8 ml DMEM each without antibiotics or GP in a 4 mm electroporation cuvette. After the mixture had stood for 5 minutes at 4^0 C the cells were electroporated with a single pulse of 960 microfarads at 300V. The cells were then stood at 4^0 centigrade for a further 10 minutes and then at room temperature for 4 minutes, before the suspension was added to 30ml standard medium; 200µl of this suspension was dispensed into each well of a flat-bottomed 96 well plate (Nalge Nunc International). The plate was incubated in a humidified incubator at 37^0 C and 5% CO_2.

Selection medium was prepared as followed: 100mg of Geneticin (neomycin, Life Technologies, Inc.,Canada) was added to 100 ml of standard medium (1mg/ml). This was then filter-sterilised (0.2 µm filter). The medium was changed by removing 100-

150µl from each well and replacing with 150 µl selection medium. This was repeated whenever the medium was seen to have changed from pink to yellow.

Once a colony were seen to be established in the bottom of a well, the cells were carefully washed off and the colony expanded by transferred it to a well in a 24 well plate in 2 ml selection medium. Once the cells approached confluence they were washed of the plate and checked for expression of the desired antigen by FACS analysis with an appropriate mAb (section 5.6.1).

To confirm that CT26 does not express constitutive resistance to the selection medium, wild type cells were incubated in selection medium at a range of concentrations of geneticin from 0.001 mg to 1 mg/ml. 1 ml of a suspension of 10^6 cells/ml in standard medium was added to each well of a 24 well plate and a further 1 ml of selection medium added to make up the final concentration of geneticin for that well. The medium was supplemented as required and the cells observed by microscopy on alternate days for four weeks.

5.8 Selection of transfected cells

Cells that were found to express the target antigen at low levels underwent further selection by FACS. 5×10^6 cells were labelled under sterile conditions with the relevant filter-sterilised FITC conjugated mAb (0.2 µm filter) (section 5.6.1). The cells were then sorted by FACS (FACScaliber, Becton Dickinson, Mountain View, CA, USA). An irrelevant FITC-conjugated mAb was used to calibrate FL-1 gain and cell debris excluded through the FSC threshold. Cells in the top 10% of the FL-1 positive population were collected. 10,000 cells (events) were collected into a flat bottomed 96 well plate and the resulting colonies grown up in selection medium to adequate cell numbers for re-analysis by FACS.

5.9 Tritiated thymidine assay of growth inhibition

The proliferation of cells over a period of time is proportional to the incorporation of thymidine by those cells. Only replicating, S-phase cells will incorporate thymidine. This may be determined by measuring β emissions from cells that have been growing in radiolabelled [^{3}H] thymidine-enriched medium. Those cells that have growth

arrested or died as a result of experimental intervention will yield less [^{3}H] thymidine than controls.

Tumour cells (CT26, B16F10) growing *in vitro* as above (section 6.2) were re-suspended in 10 ml of medium at 2×10^5 cells/ml. 100µl of this suspension was added to each well of a flat-bottomed 96 well plate (Nalge Nunc International). They were incubated for 24 hours with reagent (eg control or study mAb) at a range of final concentrations in triplicate, at a final volume of 200µl/well.

After 24 hours 50µl of [^{3}H] thymidine (Amersham Pharmacia, Buckingham) containing medium (100µl [^{3}H] thymidine in 5ml DMEM/10% FCS) was added to each well (0.5 µCi/well). The plate was then incubated for a further 16 hours. After this time the plates were washed through a cell harvester (10 wash/dry cycles) so that the cells were absorbed on to individual disks on a sheet of glass fibre filter paper (Whatman, Springfield Mill, Kent); each disk on the filter paper represented a well from this sheet. The disks were cut from the paper and placed in individual β-counter tubes with 200µl scintilin. β-emissions were counted for one minute per tube, with the control being the average of three counts from wells without the test reagent. An average count was taken for each concentration of reagent and the growth expressed as a percentage of the control.

5.10 Preparation of lymphocytes for *in vitro* assays

5.10.1 Preparation of splenic lymphocytes

Mice were culled in CO_2 and a midline incision used to dissect the spleen intact. Single cell suspensions were prepared from each spleen by passing them through a 70 µm cell filter in 30 ml DMEM. The resulting suspension was centrifuged at 1500 rpm for 5 minutes. The cell pellet was re-suspended in 7 ml light Percol which was layered onto 7 ml of heavy Percol in a 30 ml universal container. A top layer of 7 ml DMEM was added (figure 5.1).

The Percol gradient was then centrifuged at 1900 rpm for 30 minutes. The cell layer between the heavy and light Percol was removed with a pipette and suspended in DMEM. The suspension was spun at 1500rpm for 5 minutes and the cell pellet re-suspended in 10 ml PBS.

200 µl of this suspension was removed and the cells counted on a Coulter Industrial D Cell Counter (Coulter Electronics, Bedfordshire) to give the total lymphocyte count.

5.10.2 Preparation of lung lymphocytes.

The mediastinum from each mouse was removed with the heart and great vessels intact. The right ventricle was canulated before significant coagulation had occurred and the lungs irrigated with 2 ml DMEM to remove circulating blood cells. The lungs were then dissected from the heart and thymus and macerated with a razor. The macerated lung was placed in a 30 ml universal container with 5 ml stock enzyme (0.1% collagenase (bovine type IV, Sigma) and 0.01% hyalurinidase (bovine type IV, Sigma)). This was incubated for one hour at 37^0 C.

25 ml DMEM was added to the digested lung and a single cell suspension prepared by passing it through a 70 µm cell filter. The cell suspension was centrifuged at 1500 rpm for 5 minutes and the resulting cell pellet re-suspended in 7 ml light Percol. This was then layered onto 7 ml heavy Percol in a 30 ml universal container. A top layer of 7 ml DMEM was added and the universal container centrifuged at 1900 rpm for 30 minutes.

The cell layer between the heavy and light Percol was carefully removed with a pipette and washed once in DMEM. The final cell pellet was re-suspended in 1 ml PBS. The total lymphocyte count per mouse lung was then measured on a Coulter Industrial D Cell Counter.

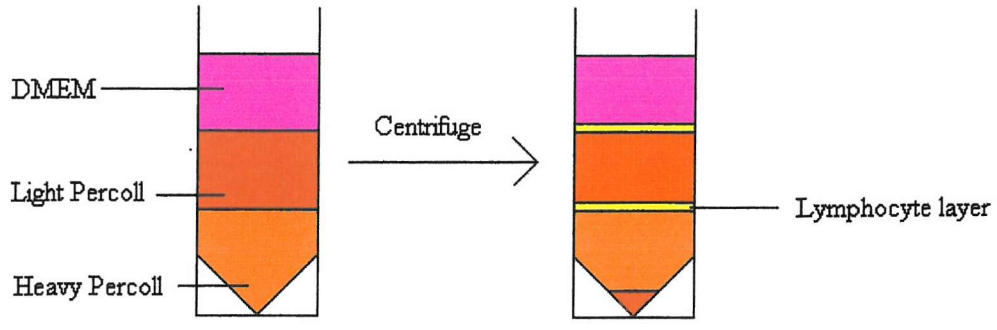


Figure 5.1 Percol preparation of lymphocytes.

5.11 ^{51}Cr release assay

Effector cells were prepared from experimental animals according to the methods described in sections 6.10.1 and 6.10.2.

Cells were labelled at 2×10^6 cells/ml in 2 ml standard medium with 200 µl $Na_2{}^{51}CrO_4$ (Amersham, UK) for 30 minutes at 37^0C. Excess ^{51}Cr was removed by washing the cells four times in standard medium, with appropriate waste disposal. The cells were resuspended at 10^5 cells/ml.

Assays were planned for three effector (lymphocytes)/target (CT26) ratios: 100:1, 50:1 and 25:1. The high ratios were thought to be required as the total number of cells from the effector preparation was taken, rather than discriminating between potential effector cells including NK cells, B lymphocytes and CTL.

The concentration of targets was constant, at 10^5/ml, and 100µl of this suspension was added to each of 39 wells of a flat-bottomed 96 well plate. The effector cells were suspended at a concentration of 10^7 cells/ml, and 100, 50 or 25 µl added to each well to give the required effector/target ratio, each in triplicate. Medium was added to each well to give a total volume of 200µl/well. The remaining 12 wells were controls to measure background release of ^{51}Cr, and maximum of release ^{51}Cr.

The plates were incubated for five hours under standard conditions, then 50 µl of 1% Nonidet P40 (NP40) (Amersham Pharmacia Biotech) was added to the (maximum) control wells. The plates were sedimented at 520g for five minutes. 100 µl of supernatant was taken from each well and placed in a standard vial, which was then placed in a counting tube for the automated gamma scintillation counter. Each sample was counted for three minutes. An average for each effector/target ratio was calculated and the percent specific chromium release calculated by the formula:

$$\%\ \text{specific chromium release} = \frac{\text{cpm(sample)-cpm(background)}}{\text{cpm(maximum)-cpm(background)}}$$

5.12 *In vitro* assay of tumour cell killing by mouse lymphocytes following stimulation with anti-CD40 mAb.

5.12.1 Preparation of BALB/c lymphocytes for *in vitro* activation with anti-CD40 mAb for use in ^{51}Cr release cytotoxicity assays.

One mm^3 pieces of CT26 tumour was passaged sc into the flanks of two age and sex matched BALB/c mice (section 6.4.2). The mice were culled after 14 days, by which time the tumours had each reached a volume of approximately 2 cm^3. Lymphocytes were harvested from the spleens of these mice as described in section 6.10. The lymphocytes were re-suspended in 5% DMEM/5%FSC/mercaptoethanol at a concentration of 6×10^6 cells/ml. 1 ml of this suspension was added to each well of a 24 well plate for incubation with growth arrested tumour cells.

10 µl of anti-CD40 mAb at 2mg/ml (20µg), control mAb (20µg MC106A5) or PBS was added to eight each of the 24 wells.

Growth-arrested tumour cells were prepared by incubating 10^7 cells in 1 ml DMEM/mitomycin-c at 50µg/ml for 30 minutes. These cells were then thoroughly washed in DMEM to remove all of the mitomycin-c (4 cycles at 1500 rpm for 5 minutes each). The tumour cells were re-suspended in DMEM/FCS5%/mercaptoethanol at a concentration of 2×10^5 and 1 ml added to each of the 24 wells.

After 5 days the cells were harvested and washed once in DMEM5%/mercaptoethanol. The total number of effector cells from each of the three groups was counted on a Coulter Industrial D Cell Counter.

5.12.2 Preparation of mouse lymphocytes following *in vivo* and *in vitro* activation with anti-CD40 mAb for use in ^{51}Cr release cytotoxicity assays.

Eight age and sex matched (BALB/c or C57Bl6) mice were divided into four groups of two mice each (A,B,C&D). Two groups were inoculated with 2×10^6 tumour cells iv. (CT26 or B16F10) on day 0 as described in section 5.4.1, and each of the four groups received 0.5 mg iv. anti-CD40 mAb or control mAb on days 2,4 and 6 (table 5.1).

Group:	Inoculation
A	Tumour cells + anti-CD40 mAb.
B	Tumour cells + control mAb
C	Anti-CD40 mAb only
D	Control mAb only

Table 5.1. Groups of mice studied in section 5.12.

The mice were culled on day 14 and lymphocytes (effector cells) prepared from the lungs and spleens as described in section 6.10. These were incubated with growth arrested tumour cells for five days, with either anti-CD40 mAb, control mAb or PBS as described in section 5.12.1. After this period of incubation the suspension of effector cells was prepared and incubated with ^{51}Cr labelled tumour cells (target cells, see below, 5.12.3) in a 96 well plate for five hours as described in section 6.11. The lymphocytes from the spleens were incubated at effector/target ratios of 100/1, 50/1 and 25/1, in triplicate, as above. Because the lungs yielded less effector cells (a minimum of 4.2×10^6 lymphocytes from one control group) a single effector/target ratio of 25/1 was used. It was hoped that the number of tumour-specific lymphocytes would be sufficient to compensate for the overall reduction in effector cell numbers.
The incubated plates containing lymphocytes prepared from the lungs were examined under a microscope: in none of the wells were live tumour cells seen growing, confirming that the Percol gradient was effective in discriminating between live tumour cells and lymphocytes.

5.12.3 Preparation of target cells

Live CT26 or B16F10 tumour cells were incubated in DMEM/10%FCS with 20μg/ml IFN-γ, for 24 hours before they were required for use as targets. They were harvested and cell viability checked as described in section 6.2 and 6.3. The inclusion of IFN-γ was following the observation that tumour cells express low levels of MHC I, vital for successful tumour killing by cytotoxic T cells but not necessary for other potential effectors such as NK cells. Target cells were labelled with ^{51}Cr as described in section 5.11.

5.13 Enzyme-linked immunsorbant assay (ELISA) for cytokine analysis.

One hundred ml of 5 μg/ml primary mAb in coating buffer (15 mM Na_2CO_3, 28.5 mM $NaHCO_3$, pH 9.6) was dispensed into each well of a (flat) 96 well ELISA plate (Maxisorb, Nunc) which was then incubated at 37^0 C for one hour, then 16 hours at 4^0 C, coating the bottom of the wells. After this time the coating solution was thrown off and 100μl BSA/PBS added to each well for 1 hour at 37^0 C to block non-specific binding sites. The plate was then washed ×5 with PBS/Tween and the contents shaken off just before the plate was required.

Samples to construct a standard curve were made by double diluting stock cytokine over 7 dilutions from 4 ng/ml to 62.5 pg/ml, and 100 μl of each sample dispensed in duplicate into the first two columns of the 96 well plate. 100 μl BSA/PBS was dispensed into the last two wells of these columns. 100μl of each experimental sample was dispensed into individual remaining wells. The plate was then incubated at 37^0 C for 90 minutes.

Following incubation the plate was washed ×5 with PBS/Tween and 100 μl biotinylated anti-cytokine mAb (1/400 dilution) with avidin-HRP (1/1000 dilution). The plate was incubated at 37^0 for a further 90 minutes. Following washing (×5, PBS/Tween), HRP substrate (20 mg o-Phenyldiamine free base (o-PD), 100 ml phospho-buffered citrate pH 5.0 + 20 μl (60% w/v) H_2O_2) was added and incubated at room temperature in the dark for 90 minutes. The reaction was terminated by the addition of 50 μl/well 5 M H_2SO_4. The subsequent colour change was quantified by reading the absorbance of each well at 495 nm on an automated fluorometer (Dynatec 400, Dynatec). Colour change was proportional to protein concentrations and unknowns were determined using the standard calibration curves from each plate.

5.14 Selective depletion of helper and cytotoxic T lymphocytes

Age and sex matched BALB/c or C57Bl6 mice were depleted of helper T cells, cytotoxic T cells or both by injection with a specific mAb. YTA312 binds to CD4, depleting mice of helper T cells, and YTS169 binds to CD8, depleting cytotoxic T cells. Depletion of these cells was initiated two days before the mice were inoculated with tumour (day –2). The mice received intraperitoneal injection of 1mg anti-CD4 or 0.5mg anti-CD8, or both 1mg anti-CD4 and 0.5mg anti-CD8. This was repeated every

five days until the conclusion of the experiment. Mice were inoculated with tumour on day 0 (section 5.4).

5.15 Monoclonal antibodies

Non-commercial mAb are produced at Tenovus from immortalised hybridoma cell lines.

5.16 Statistical analysis

All data was recorded in a GraphPad Prism (GraphPad Software, Inc., San Diego CA) database, which was subsequently used for all calculations and to generate the appropriate figures.

The most important assumption in the statistical analysis of the growth of tumours, whether it is the measurement of cutaneous tumour or counting the number of tumour deposits, is that the distribution is Gaussian. Clearly samples sizes of only six mice will not demonstrate this, but common sense and an understanding of biological patterns would suggest that this is the case. This allows us to use parametric tests (t tests and ANOVA) to discriminate between populations without resorting to tests of the distribution. The only significant problem in the interpretation of these results with parametric tests is that the standard deviations of the two populations are not going to be the same for the treatment and control groups. If a treatment is successful then the standard deviation will be numerically less as the tumour size or number of tumour deposits tends toward zero.

Student's unpaired t test was used to compare study groups when just two variables were included, e.g. control versus anti-CD40 mAb. When more than one variable was compared an ANOVA table of variance was calculated and from this the t value for the difference between each group calculated using Bonferonni's post test. This allows comparison of multiple groups. Again the standard deviations of the two populations are not going to be the same for the treatment and control groups, but ANOVA and Bonferonni' post test are robust from this point of view. Bonferroni's post test is more conservative than Student's t test leading to high P values, but this may be an advantage in interpreting the significance or otherwise of results.

Chapter 6

The *in vitro* and *in vivo* characteristics of Colon Tumour 26 and melanoma B16 mouse tumour cell lines.

6.1 Introduction

Colon Tumour 26

Colon tumour 26 is a murine colonic adenocarcinoma that was first described in 1976 [153]. It was induced in BALB/c syngeneic mice as a model of chemical carcinogenesis by the oral administration of N-methyl-N-nitrosourethane. Because of the relative ease with which the cells grow *in vitro* and *in vivo* it has become ubiquitous as a model for the study of adenocarcinoma in mice. However there is little evidence in the literature of successful treatment of wild-type CT26 with immunomodulation: for this reason it remains a challenge to immunotherapy and a powerful research tool.

The first reported investigation of immunotherapy for CT26 was by Colomerauer et al in 1980[154], who found that BCG toxin did not induce a therapeutic immune response. However they made the unusual observation that if the primary explant was removed those mice that had received BCG succumbed to metastatic tumour before either control mice or those that had received BCG but were left with the primary tumour intact. Similarly ambiguous results were described by Fukushima et al[155] who administered combinations of CT26 derived antigen, CT26 mRNA and splenocytes from tumour bearing mice and found that only a strict sequence of specific doses of each would induce a modest (20%) therapeutic response. However other combinations often lead to diminished survival compared with control groups.

More convincing evidence of an effective immune response was reported by Schackert et al[156]. They transfected CT26 with a murine influenza virus, which would not grow *in vivo*. However the transfected cell line vaccinated against wild-type CT26 cells, indicating that the influenza virus antigen was functioning as an immunological adjuvant, inducing an immune response to a native CT26 antigen.

In 1990 Rodolfo *et al*[157] demonstrated that recombinant IL-2 reduced the number of pulmonary tumour deposits but did not prolong survival. However IL-2 combined with adoptive transfer of lymphocytes from BALB/c mice with subcutaneous tumours cured 80% of BALB/c mice with 3-day pulmonary tumour. Furthermore this response was IFN-γ dependent. Neither adoptive transfer alone nor LAK cells offered

protection, implying either a cellular component to the immune response or that IFN-γ might in some way directly affect CT26 tumour cells.

In contrast, Fujiwara et al[158] reported enhanced survival of mice with hepatic CT26 deposits following prolonged IL-2 treatment alone, with or without LAK cells. ^{51}Cr release assays implicated a cellular rather than a humoral response. Contrasting results were obtained by Rabau et al[159] who demonstrated that IL-6 inhibited the *in vivo* growth of CT26 tumour cells, implying a humoral response. They observed increased infiltration of non-specific lymphocytes around the tumour deposits but found no evidence of a direct effect of IL-6 on CT26. They did report increased surface expression of CEA (a candidate tumour associated antigen, see Chapter 3) by HT 29 human colon carcinoma cells in response to IL-6. A similar response was observed by Itsuki et al[160] who engineered CT26 tumour cells to express IL-4, although no mice were cured by this treatment. Immunohistochemistry demonstrated that the infiltrating cells were initially eosinophils but that after eight days the majority of infiltrating cells were lymphocytes. A strong CTL response against wild type CT26 tumour cells was demonstrated in tumour bearing mice. The hypothesis was that the IL-4 induced a humoral response, with B cell proliferation and IgE isotype switching, and that a tumour antigen was targeted, rather than a transfected antigen. This would not explain the subsequent CTL response, which would be inhibited by IL-4 but it may be that the initial humoral immune response lead to an increase in available tumour antigen and providing a danger signal to induce a cellular response.

A candidate tumour antigen for CT26 has been described by Okamoto et al[161]. A heat shock protein, HSP 70, isolated from CT26, immunised mice against CT26. This protection was enhanced when additional HSP 70 was added to a preparation of heat-treated CT26. No mice were cured but tumour growth was abrogated by vaccination. It is likely that certain forms of cellular injury, such as heat treatment or immunological attack, result in the release of tumour antigens that are better for cross priming of CTL by APC.

<u>B16 melanoma</u>

B16 melanoma is a poorly immunogenic and aggressive murine tumour cell line that is syngeneic with C57Bl6 strain of mice. The cell line has been investigated as a model of melanoma over many years, and this has resulted in diverse strains with varying *in vitro* and *in vivo* characteristics. Early strains were amelanocytic and grew

poorly. Subsequent lines grow consistently and produce pigment. The most commonly used strains of B16 are derived from the cell line B16F1, including B16.c215 and B16.F10, used in these experiments and described below.

Early *in vivo* investigation of the immune response to B16 melanoma suggests that it is predominantly T_H2 in character though this may be a result of the suppression of T_H1 responses by the tumour itself (see below). A number of antigens have been identified[162] and early studies focused on humoral responses using specific mAb (ADCC) with or without LAK cells. Indirect but effective immune responses have also been achieved through IL-6, which is thought to act by promoting a T_H2 response to such antigens [163].

Some of these antigens are now known to be of retroviral origin[164]. These include the ubiquitous murine leukaemia viral antigens and other, melanoma-specific retroviral antigens[162]. They are expressed as surface proteins and in the cytosol and have played an important role in the investigation of immune responses to tumours in general.

Poor immunogenicity is probably due to a range of strategies that allow B16 cells to maintain a low immunological profile. In particular they expresses low levels of MHC I, critical for cytotoxic T cell antigen recognition[165]. Most studies have found native CTL dependent immune responses to be ineffective, but that this is overcome in some strains of B16 melanoma through IFN-γ. IL-2 leads to the strongest CTL response, being most effective when B16 cells are engineered to secrete the cytokine[166]. Sytemic administration of IL-12 also induces an effective CTL response, and a tyrosinase-related protein, TRP-2, has been identified as the tumour antigen[167, 168]. Unlike other B16 candidate tumour antigens this is a normal tissue differentiation antigen (see table 1.1) similar to human melanoma differentiation antigen, and not a retroviral antigen. These observations lend credibility to B16 models of indirect immunotherapy that offer strategies that are more widely applicable.

B16.c215 is B16F10 cell line that has been transfected to express a human epithelial glycoprotein, 17.1A, which could be used as a known antigen for the investigation of T cell responses.

CTLA-4 (figure 3.3) blockade by specific mAb, in combination with GM-CSF, will inhibit *in vivo* tumour growth[169]. This is further evidence that CTL anti-tumour responses to B16 can be indirectly induced. As with anti-CD40 mAb (chapter 4), the

mechanism involved bypasses the need for helper T cells, reinforcing the hypothesis that a population of tumour-antigen specific CTL exists that is anergic but can be rescued by an appropriate signal.

Vaccines have been created by pulsing naïve bone marrow dendritic cells with B16 cell lysates and then incubating the dendritic cells in GM-CSF. Injection of these cells into mice resulted in CTL that were effective *in vitro* but did not abrogate tumour growth *in vivo*. The antigens recognised by the CTL have not been identified. Heat shock proteins as a source of B16 antigen for dendritic cells have not been investigated, although it has been shown that heat shock treatment of B16 cells alone yields a partially effective vaccine [170].

In summary CT26 and B16 cell lines provide an excellent tool for the *in vitro* and *in vivo* study of immunotherapeutic mechanisms. Previous workers have demonstrated consistent and reproducible tumour growth characteristics and that a range of immune responses may be generated, suggesting a diversity of tumour antigen. They have found it possible to transfect tumour cells with a range of genes and that expression of the gene product remains stable *in vitro* and *in vivo*. Despite this the tumours have proven difficult to cure by immunotherapy and vaccination has met with only limited success, suggesting that only the most robust of strategies will be effective.

6.2 Aims

The first aim was to establish the optimal conditions for *in vitro* and *in vivo* study of CT26 and B16, and measure expression of CD40 and MHC I by these cells.

The second was to investigate the possibility that anti-CD40 mAb might act directly on tumour cells. Should the tumour itself express CD40 then the use of anti-CD40 mAb will make that tumour a potential target for natural effector cells (section 2.5) or even provide a direct signal to the tumour cells (section 1.4)[171]. We established levels of CD40 expression by FACS analysis and PCR, and investigated direct effects of anti-CD40 mAb by *in vitro* measurement of tumour growth inhibition.

Next MHC I expression by the tumour cells was measured with and without the addition of IFN-γ to the culture medium.. Low levels of MHC I on the cell surface would explain why explanted tumour would grow in syngeneic mice even though they might be engineered to express foreign antigen such as the human epithelial glycoprotein EGP 2 (B16.c215).

MHC I expression is upregulated by IFN-γ and the kinetics of any response to IFN-γ by tumour cells must be known to plan the *in vitro* assessment of cytotoxicity by T cells. If CTL thought to be effective against a tumour antigen are cultured *in vitro* with tumour cells that do not express MHC I then tumour killing will only occur if those tumour cells respond to the cytokine environment and increase MHC I expression.

Finally reliable and reproducible *in vivo* growth of both tumour models was established by inoculating mice with tumour cells intravenously (iv), intradermally (id) and subcutaneously (sc) at a range of tumour cell concentrations.

6.3 Methods

FACS analysis was used to measure CD40 expression by CT26 and B16 melanoma cells. Cells that had been growing *in vitro* were labelled with FITC conjugated anti-CD40 or control mAb according to the methods described in sections 5.2 and 5.6.1.

Reverse transcription PCR of first strand cDNA library taken from CT26 cells was performed to validate initial results using the methods described in section 5.7. A 'Quick Prep' kit (Amersham) was used to extract mRNA from proliferating CT26 cells, and cDNA synthesized from this. PCR of the cDNA was performed using Taq polymerase and CD40 primers under standard conditions. The BALB/c B cell lymphoma cell lines A30 and A31 were used as positive controls.

Growth inhibition of tumour cells by anti-CD40 mAb was determined by measuring the uptake of [^{3}H] thymidine, described in section 5.9. The anti-CD40 mAb, 3/23 or control mAb, MC106A5, were used at 6 concentrations between 0 and 10 μg/ml

Interferon-γ was purchased from Serotec. Tumour cells were prepared for *in vitro* use following the methods described in section 5.2. Specific mAb were used for FACS: HB11 binds to the D^bK^b MHC I haplotype expressed on nucleated cells from C57Bl6 mice and HB79 binds to the D^dK^d haplotype of BALB/c mice.

To measure the *in vitro* response of cells to IFN-γ eight small flasks of tumour cells (either CT26 or B16) in were prepared for *in vitro* use as described in section 5.2. The tumour cells were incubated in standard conditions for 24 hours at a range of concentrations of IFN-γ from 0 to 100 ng/ml. After this time the cells were harvested from the flasks. The cells were labelled with the appropriate mAb and analysed by FACS (section 5.6.1).

To measure the kinetics of the response to IFN-γ three small culture flasks of tumour cells were prepared for *in vitro* use as described in section 5.2. They were incubated under standard conditions for 6, 24 and 48 hours in DMEM/10% supplemented with 20 ng/ml IFN-γ. At each time point tumour cells were harvested from a flask and labelled with FITC mAb. FACS analysis of these cells was compared with un-stimulated cells and irrelevant mAb with changes in MHC I expression being represented by the change in fluorescence staining.

Tumour cells were grown in vitro and prepared for in vivo use as described in section 6.2. The cells were centrifuged once and re-suspended in DMEM without additives to make up the required cell concentration and the cell viability checked (section 5.3).

It was already known that *in vivo* growth of B16 could be measured by killing the mice after 21 days, dissecting the lungs and then counting the number of tumour deposits visible on each lobe, the total number of tumour deposits for each mouse being the measured outcome. Previous experimental work had suggested that tumour (lymphoma) should be given four days before anti-CD40 mAb therapy is begun[123], and that tumour cell eradication is not seen until at least five days after the first dose of mAb. It was planned to give three doses of mAb, as for the lymphoma model, with two days between doses. Thus the fifth day after the final dose of mAb would be eleven days after the initial dose of tumour. Therefore the mice must be able to survive at least eleven days after tumour inoculation in the first instance.

To determine the tumour dose that would give even and reproducible growth, C57Bl6 mice were inoculated iv with B16F10 (wild-type) and B16.c215 cells on day 0. Tumour doses were 10^5, 10^6 and 10^7 cells per mouse in 200μl. The mice were culled on day 20 and the tumour deposits in their lungs were counted.

The same principle was observed for the CT26 except that in this case BALB/c mice were inoculated with tumour and then observed twice a day, as tumour deposits are difficult to discriminate from normal lung tissue. If their health deteriorated (section 5.4.1) they were culled, with the day after initial inoculation being the measured outcome. As there was no previous experience with this tumour a wider dose range was given, from 10^3 to 10^7 cells per mouse but on one occasion a mouse died within minutes of inoculation of 10^7 cells, probably from clumps of cells causing fatal tumour embolism.

Because anti-CD40 mAb failed to confer any survival advantage in the pulmonary CT26 tumour model (see below) sc and id models of tumour growth were investigated.

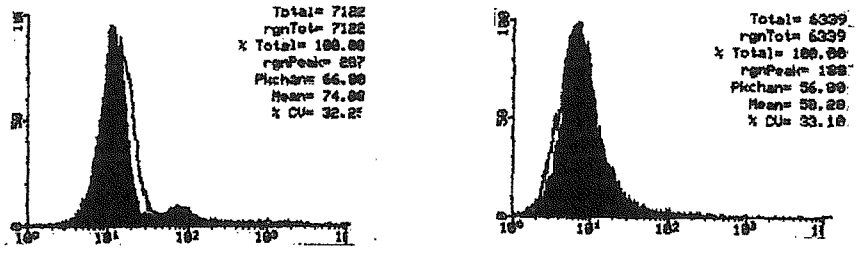
Subcutaneous injection of a tumour suspension was found to give uneven growth of tumour, probably as a result of dispersal of the tumour cell suspension through the tissue planes. Solid tumour was passaged into the subcutaneous tissue of the shaved left flank of each of two anaesthetised mice (section 5.4.2). Tumour doses of 10^4, 10^5 or 10^6 cells were injected id into the shaved left flanks of two age and sex matched BALB/c mice per tumour load. Tumour growth was measured on alternate days.

One mouse from each group was dissected to establish whether tumour growth extended beyond the lungs for those mice receiving iv tumour and away from the site of injection for those mice with sc or id tumour.

6.4 Results

In vitro characteristics

The FACS analysis of both cell lines (figure 6.1) demonstrates that neither expresses measurable levels of CD40. The PCR of the mRNA from CT26 (figure 6.2) confirms that the small amount of fluorescence at 10^2 is artefact, and that CD40 mRNA is not being synthesised. The B cell lymphoma A30 and A31 used as positive controls for the PCR express CD40 on FACS analysis (results not shown).



CT26 B16F10

Figure 6.1. Surface expression of CD40 by CT26 and B16F10 tumour cells assessed by direct FACS using FITC labelled anti-CD40 mAb (3/23). The X-axis is fluorescence intensity, the Y-axis cell numbers. The solid histogram represents CD40 expression, the open histogram the control antibody (ID3).

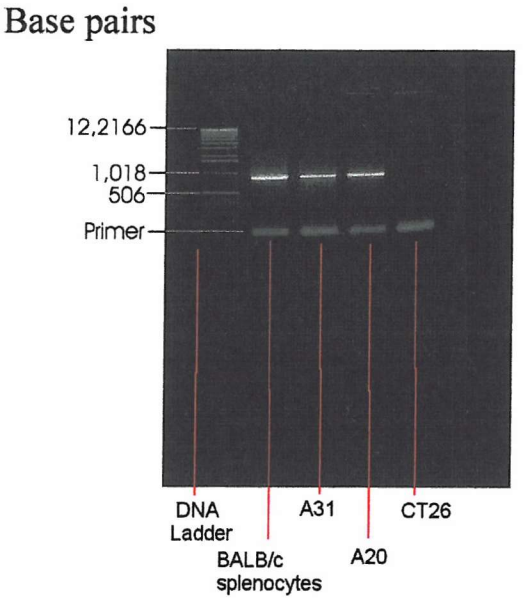


Figure 6.2. Photograph of the electrophoresis gel from the rtPCR of BALB/c mice tumour cells. A31 and A20 are B cell lymphoma (positive controls) and CT26 tumour cells.

Figures 6.3 and 6.4 confirm that even at extremely high concentrations of mAb, neither anti-CD40 mAb nor control mAb had any effect on the growth of either tumour cell line. Observations of the *in vivo* growth of these tumour cell lines in mice that have been treated with anti-CD40 mAb is most unlikely to be due to a direct cytotoxic effect of anti-CD40 mAb. The less reliable [^{3}H] thymidine uptake (and therefore release) seen with B16 is probably a reflection of its slightly slower rate of growth *in vitro* compared with that of CT26.

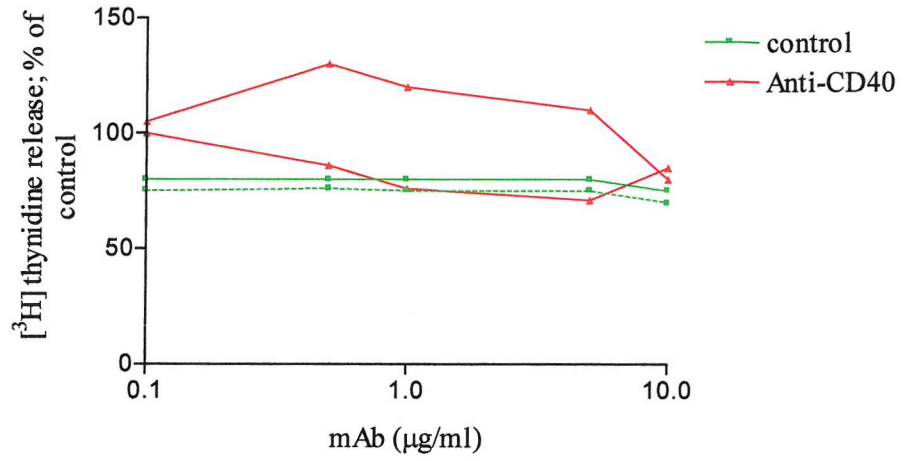


Figure 6.3. Growth of CT26 tumour cells incubated with varying concentrations of control (MC106A5) or anti-CD40 mAb. The graph shows the results of two experiments, with thymidine release expressed as a percentage that with no antibody ([^{3}H] thymidine release=100%).

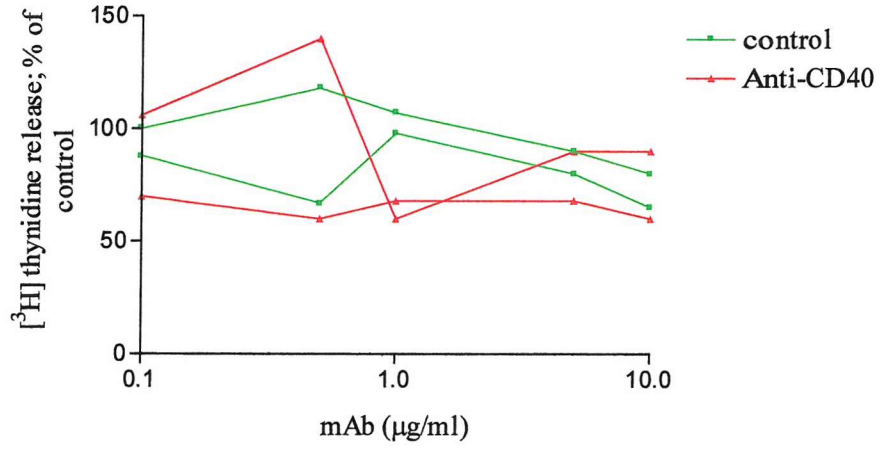


Figure 6.4. Growth of B16.c215 tumour cells incubated with varying concentrations of control (MC106A5) or anti-CD40 mAb. The graph shows the results of two experiments.

CT26 and B16 cultured *in vitro* express very low (CT26) or no (B16) MHC I on the cell surface (figures 6.5 and 6.6). However they remain sensitive to concentrations of IFN-γ as low as 20 pg/ml, with a maximum level of expression being achieved at a concentration of 20 ng/ml and above. We can predict that these tumour cells will not evade cytotoxic T cells through failure to express MHC I.

Although the cells are sensitive to IFN-γ, figure 6.7 shows that the increase in MHC I expression takes greater than 6 hours. *In vitro* assays of tumour cell killing that rely

on cytotoxic T cells as the effectors will not be effective if the targets do not express MHC I, but activated cytotoxic T cells will secrete IFN-γ. Therefore cytotoxicity assays involving CT26 or B16 cells should be at least 24 hours in duration to allow for up-regulation of MHC I by target cells, or target cells should be cultured in medium enriched with IFN-γ at a concentration of 20 ng/ml for 24 hours before use.

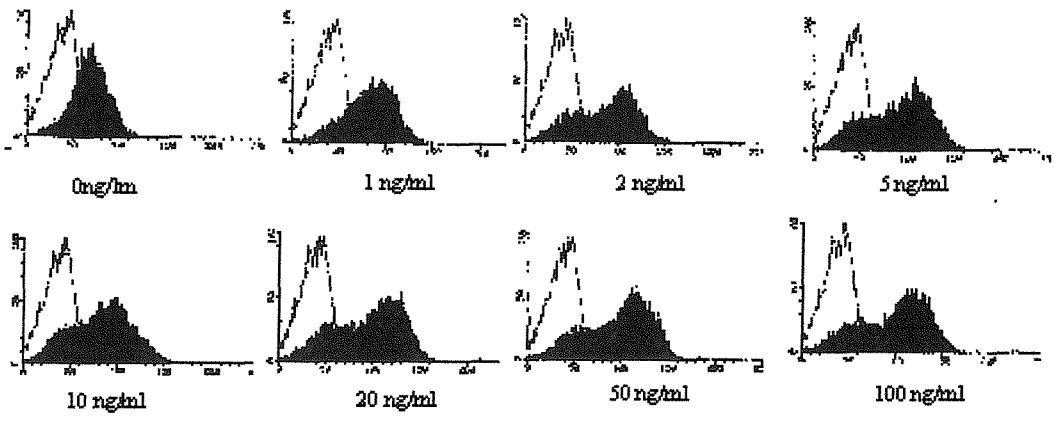


Figure 6.5. FACS analysis of the expression of MHC I by CT26 cells measured with FITC/HB11 mAb after 24 hours incubation with increasing concentrations of IFN-γ (x-axis). The X-axis is fluorescence intensity, the Y-axis cell numbers. MHC I expression is represented by the solid histogram, the open histogram being control mAb.

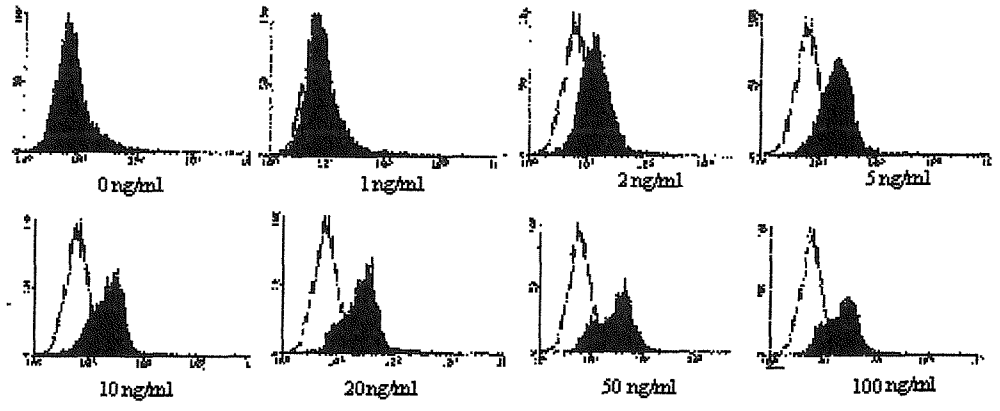


Figure 6.6. FACS analysis of the expression of MHC I by B16F10 melanoma cells measured with FITC/HB79 mAb after 24 hours incubation with increasing concentrations of IFN-γ (x-axis). The X-axis is fluorescence intensity, the Y-axis cell numbers. MHC I expression is represented by the solid histogram, the open histogram being control mAb.

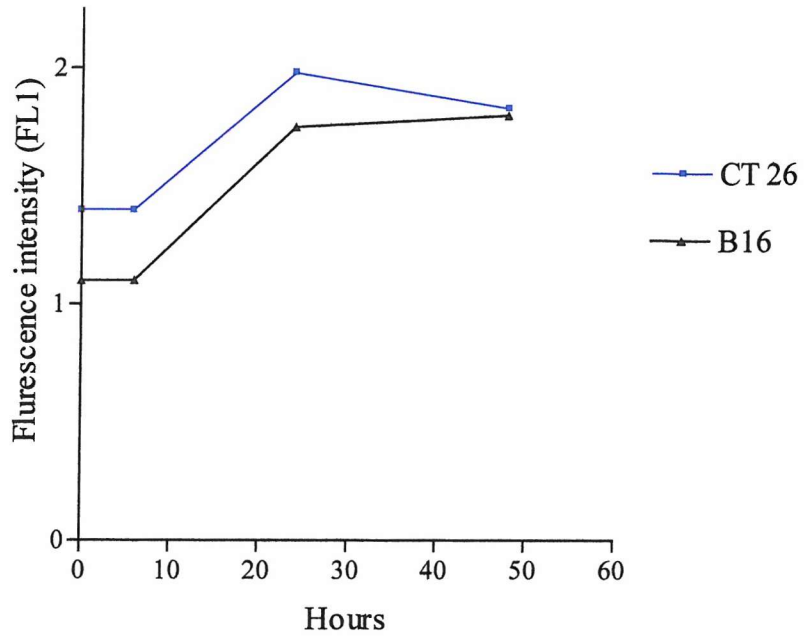
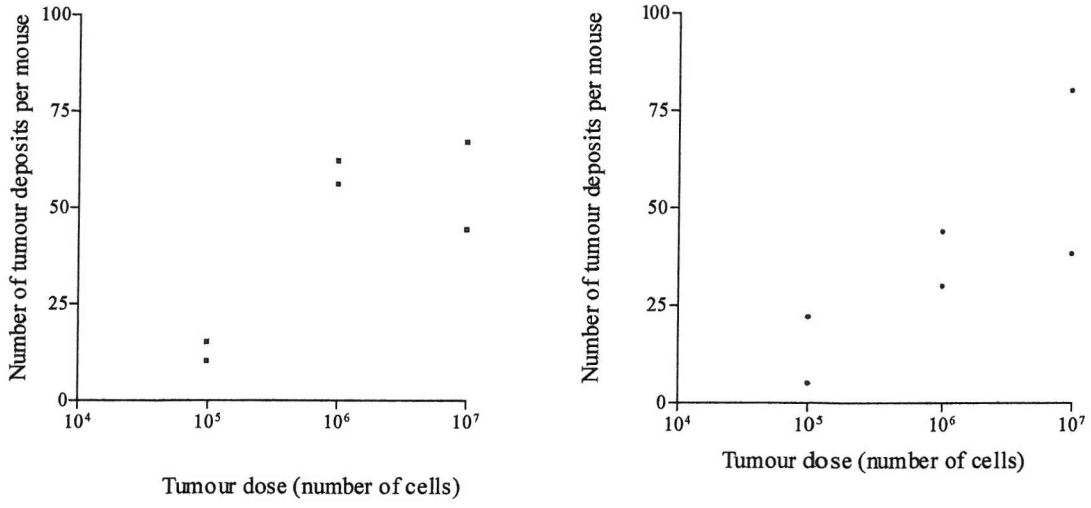


Figure 6.7. Measurement of MHC I expression by FACS analysis as a function of time by CT26 and B16F10 tumour cells following incubation with IFN-γ at 20 ng/ml over two days.

In vivo characteristics

With regard to the numbers of B16 melanoma tumour deposits (figure 6.8) for any initial tumour dose there is no significant difference between the wild-type and the transacted cell lines. However the former was much more aggressive in its ability to spread throughout the pleural cavity and mediastinum following a tumour dose of greater than 10^6 cells per mouse. Whilst a tumour dose of 10^5 B16F10 cells only gave a small number of tumour deposits, there was no advantage in inoculating with a tumour load greater than 10^6 cells per mouse as the extensive extra-pulmonary spread of tumour made it difficult to count the deposits with any accuracy. One mouse that had received 10^7 B16F10 had intra-peritoneal tumour deposits.



B16f10 B16.c215

Figure 6.8. Number of tumour deposits in the lungs of C57Bl6 mice after iv injection with a range of B16 tumour doses.

Intravenous injection of 10^5 or more CT26 cells killed all mice within three weeks (figure 6.9). Even the most dilute suspension of tumour cells was potentially lethal, but large numbers of mice would be required to demonstrate a difference in survival between treated and untreated tumour-bearing animals. For subsequent investigations a 100% lethal tumour dose (10^5 cells) was used.

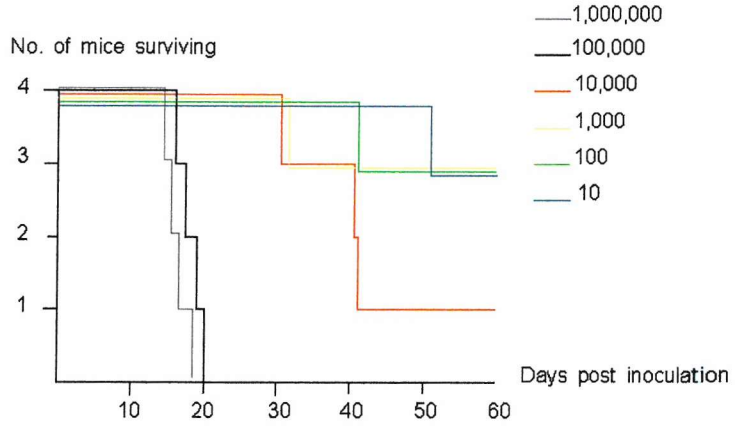


Figure 6.9. Survival of BALB/c mice following iv injection with increasing CT26 tumour doses. Mice receiving less than 10^4 CT26 tumour cells were capable of clearing the tumour load and surviving greater than 60 days.

Despite the considerable tumour load posed by subcutaneous passage of solid CT26 the resulting tumours were well tolerated by the mice (figure 6.10). Tumour growth was consistent and progress easily followed. The intradermal tumour dose was much

smaller but demonstrated consistent growth after the injection of 5×10^5 cells (figure 6.11).

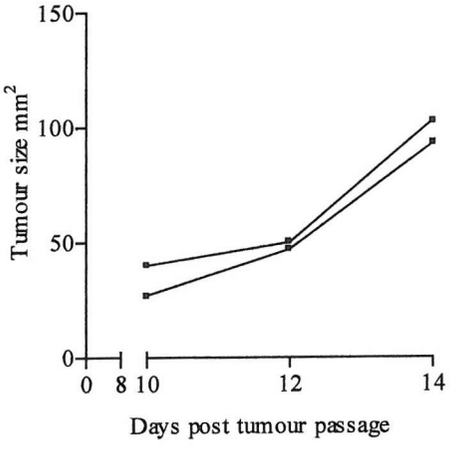


Figure 6.10. Growth of sc passaged CT26 tumours in BALB/c mice.

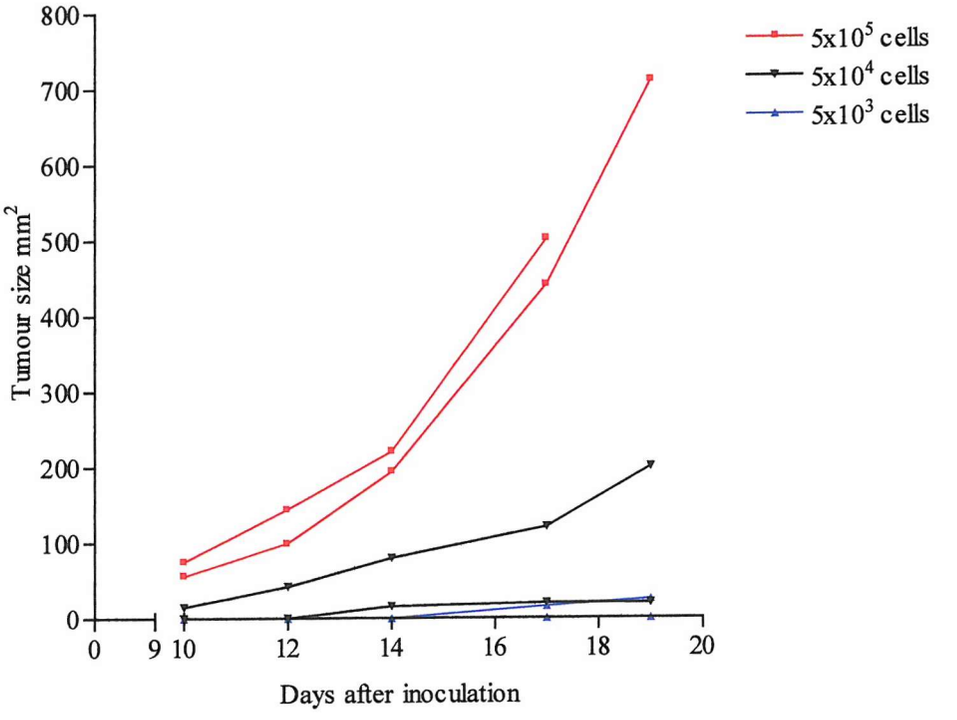


Figure 6.11. Growth of id CT26 tumours in 6 BALB/c mice. One mouse that had received 5×10^6 tumour cells developed an ulcerating tumour by day 18 and was culled.

Chapter 7

Can an effective anti-tumour therapy be induced through CD40 signalling?

7.1 Introduction

Anti-CD40 mAb has been proven effective treatment of lymphoma in murine models [123], but not in other solid malignancies. It was essential to determine whether anti-CD40 mAb therapy would have any effect *in vivo* against solid tumours. CT26 adenocarcinoma and B16 melanoma were used as the murine tumour models.

Previous workers have induced an effective CTL response to CT26 tumour cells *in vivo* through genetic engineering [172] [173, 174]. The resulting CTL are effective against both engineered and wild-type CT26 tumour cells and CT26 cells expressing or secreting receptors or cytokines including IL 2, IFN–γ, TNF-α, and B7 have been effective tumour vaccines. Immunohistochemistry of many tumours has demonstrated that APC infiltrate tumours[175] but remain ineffective in arming CTL, resulting in anergic T cells. The APC process antigen from the tumour environment, but without an adequate danger signal or T cell interaction, they do not cross-prime CTL in association with the necessary co-stimulatory receptors for arming CTL. If tumour cells are modified to express CD40L at an adequate level to cross-link CD40 on the APC cell surface then those APC might become licensed to arm CTL against antigen from their immediate surroundings (i.e. the unidentified CT26 tumour antigen) and vaccinate against CT26. The localised mechanism reduces the likelihood of arming of CTL against a broader range of self-antigens and induction of autoimmune disease. Provided there are adequate numbers of naïve CTL in the tumour environment then this mechanism may obviate the need for the APC to migrate to the regional lymph nodes. Furthermore the arming of CTL would not be dependent on the cytokine environment provided by helper T cells in those lymph nodes, but on the APC at the site of the tumour.

The obvious disadvantage with this strategy is that most of the CTL infiltrating the tumour are believed to be anergic, having survived negative selection (discussed in section 4.6). It is not clear whether these CTL can still be rescued from this state of anergy through CD40 dependent signalling by an APC. However CD40 dependent

signalling may provide the APC with the stimulus or danger signal (chapter 4) to migrate to the regional lymph nodes where it will encounter and arm naïve CTL.

7.2 Aims

The immediate aim was to demonstrate inhibition of tumour growth by anti-CD40 mAb in the B16 and CT26 tumour models. If this was successful it was intended to introduce the same signal by incorporating CD40L into the tumour cells as a prototype tumour vaccine.

7.3 Methods

7.3.1 *In vivo* therapy of CT26 and B16 tumours with anti-CD40 mAb

CT26 tumour cells were prepared for *in vivo* use as described in section 5.2. Pulmonary, sc and id tumours were studied.

For the pulmonary tumour model, 3 groups of 6 BALB/c mice were inoculated iv with 2×10^5 or 2×10^6 CT26 tumour cells per mouse on day 0, prepared as described in section 5.4.1 (200 µl from a suspension of 10^7 or 10^6 cells/ml.). Each group received 0.5mg anti-CD40 mAb in 200µl PBS, 0.5mg control IgG2a (MC106A5) in 200µl PBS or 200µl PBS per mouse on days 2,4 and 6. The mice were examined twice per day and culled according to protocol, with the day of death being the measured outcome.

For the sc model eighteen age and sex matched BALB/c mice were inoculated with solid tumour on day 0, as described in section 5.4.2. They each received iv injections of 200 µl comprising 0.5 mg anti-CD40 mAb, 0.5 mg control mAb or PBS into the dorsal tail vein on days 2, 4 and 6. Tumour growth was recorded on alternate days. This was repeated under identical conditions and the tumour measurements from each animal analysed with Prism Graph Pad statistical software. The statistical significance of any difference between each group on each day was calculated with Bonferonni's post-ANOVA test (un-paired).

For the id model CT26 tumour cells were prepared for *in vivo* use as described in section 5.2 and re-suspended in DMEM at a concentration of 10^7cells/ml. 50 µl of this suspension was injected into the intradermal tissue of the shaved left flanks of twelve age and sex matched BALB/c mice on day 0 and the mice divided into two groups of six. Each mouse received intravenous injections of 200 µl comprising 0.5 mg anti-

CD40 mAb, 0.5 mg control mAb or PBS into the dorsal tail vein on days 2, 4 and 6. Tumour growth was recorded on alternate days. This was repeated under identical conditions and the tumour measurements from each animal analysed with Prism Graph Pad statistical software. The significance of any difference between either group on each day was calculated with an un-paired Students T test.

For the pulmonary melanoma model, B16f10 (wild type) and B16.c215 (human epithelial glycoprotein II transfected) tumour cells were used (section 5.2 and 5.3). 2×10^5 (B16F10) or 2×10^6 (B16.c215) cells were inoculated iv in 200µl DMEM into 12 age and sex matched C57Bl6 mice on day 0. The mice were divided into 2 groups of 6 mice. The first group received 0.5mg iv of control mAb on days 3,5 and 7, the second group received anti-CD40 mAb. The mice were culled on day 20 and the lungs removed and divided into individual lobes. The number of tumour deposits on the surface of each lobe of the lungs was counted and the total for each mouse recorded. The significance of any difference between either group on each day was calculated with an un-paired Students T test.

7.3.2 Protype tumour vaccine: CD40L expressing tumour cells

CT26 tumour cells were transfected with a bacterial plasmid encoding murine CD40L following the methods described in section 5.7 and 5.8 resulting in the engineered cell line CT26.CD40L. Once stable expression of CD40L was achieved (figure 7.13) the cells were cultured *in vitro* for *in vivo* use, as described in section 5.2 and 5.3. Wild type CT26 and CT26.Hu40 (see appendix 1) were used as controls.

Eighteen age and sex matched BALB/c mice received intradermal injections of 2×10^5 tumour cells in 50 µl DMEM. Six received wild-type cells, six received CT26.Hu40 and six received CT26.CD40L.

Tumour growth was measured on alternate days from day 10, as described in section 5.4.2.

7.4 Results

Anti-CD40 mAb failed to confer any survival advantage to mice with pulmonary CT26 tumours (figure 7.1). Even at a tumour dose of 2×10^5 cells per mouse (figure 7.2) it was not possible to demonstrate any survival advantage with anti-CD40 mAb therapy. Although the mice do survive beyond the minimum time predicted for the

immune response to be initiated tumour growth in this model is far too aggressive. This experience is similar to that of other investigators who have either assessed numbers and size of pulmonary tumour deposits or measured subcutaneous tumour growth [163, 166, 176].

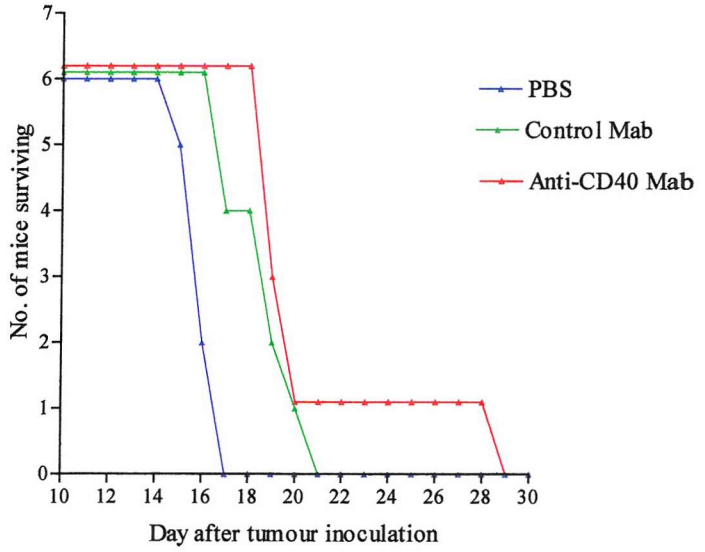


Figure 7.1 Survival of BALB/c mice following iv inoculation with 2×10^6 CT26 tumour cells. Mice that have been treated with anti-CD40 mAb do not survive longer than controls.

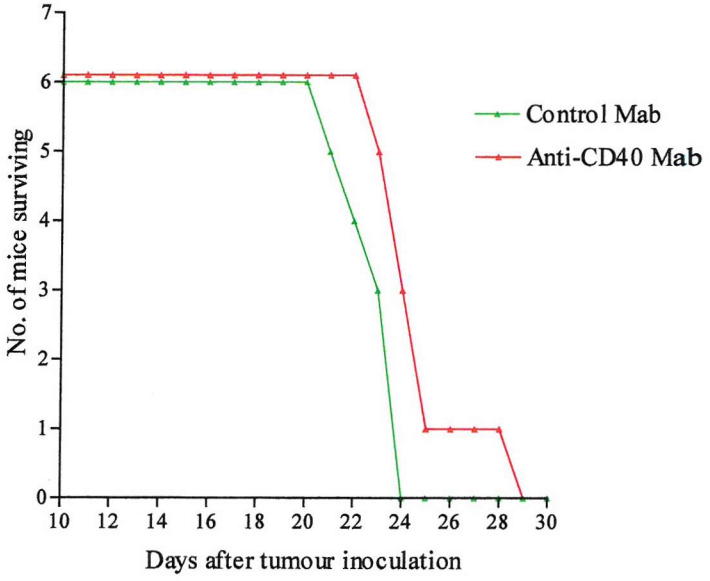
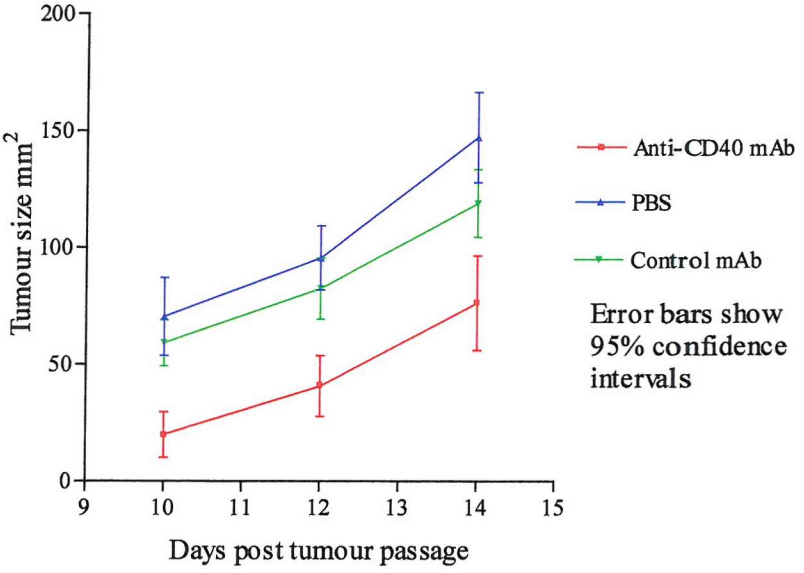


Figure 7.2. Survival of BALB/c mice following iv inoculation with 2×10^5 CT26 tumour cells. Even after a smaller tumour dose, mice that have been treated with anti-CD40 mAb do not survive longer than controls.

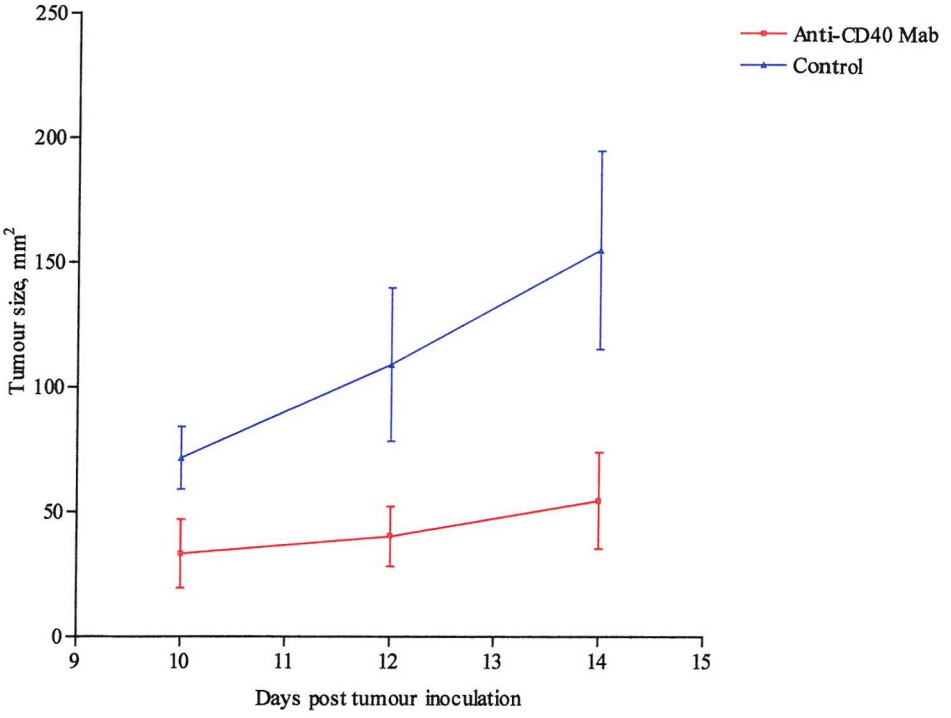
Although no survival advantage could be demonstrated for anti-CD40 mAb therapy in mice with pulmonary CT26 tumours anti-CD40 mAb is highly effective in suppressing the growth of sc and id CT26 tumours (figures 7.3 and 7.4). However the response generated is not adequate to induce tumour regression. These results help to explain the lack of therapeutic benefit seen when anti-CD40 mAb is used to treat established pulmonary CT26. Although tumour size in the treated group is at best 5 days behind that of the control groups the growth curves appear to be similar. The pulmonary model was not sensitive enough to show this difference.



Bonferroni post ANOVA test:

10	Anti-CD40 MAb vs Control Mab	P < 0.001
	Anti-CD40 MAb vs PBS	P < 0.001
	Control Mab vs PBS	P > 0.05
12	Anti-CD40 Mab vs Control Mab	P < 0.001
	Anti-CD40 Mab vs PBS	P < 0.001
	Control Mab vs PBS	P > 0.05
14	AntiCD40 MAb vs Control Mab	P < 0.001
	AntiCD40 MAb vs PBS	P < 0.01
	Control Mab vs PBS	P > 0.05

Figure 7.3. Anti-CD40 mAb inhibits the growth of sc CT26 tumour explants. The modified T test confirms that there is no difference between control mAb and PBS but a highly significant difference between controls and anti-CD40 mAb. (Combined results from 2 experiments)



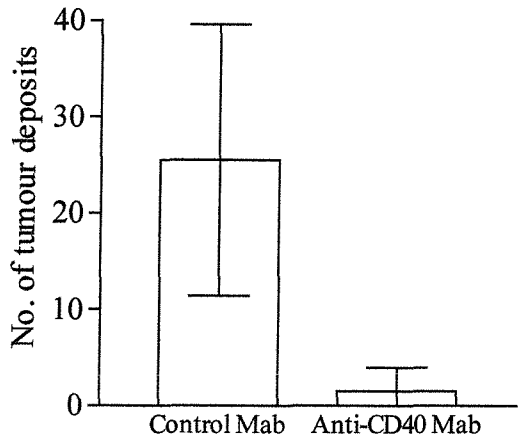
10	Unpaired t test P value	0.0513
12	Unpaired t test P value	0.0485
14	Unpaired t test P value	0.0334

Figure 7.4. Anti-CD40 mAb inhibits id tumour growth. Error bars show the standard error of the mean. At days 12 and 14 there was a significant difference ($P<0.05$) in the growth of tumours in mice from each group.

Two mice from one intradermal/treatment group did not develop tumour and were kept for re-challenge with tumour. Two months after the initial inoculation with tumour they both received intradermal injections of 2×10^5 CT26 tumour cells, but both mice succumbed to tumour within 21 days of re-challenge. It may have been that these mice had escaped tumour growth in the first instance for technical reasons, such as accidental subcutaneous rather than intradermal injection of tumour cells. Alternatively anti-CD40 mAb may have offered the mice protection from the tumour but this effect was short-lived and failed to induce immunological memory. This contrasts with the result in the lymphoma models of anti-CD40 mAb therapy[123], and those following anti-CD137 therapy (Chapter 10).

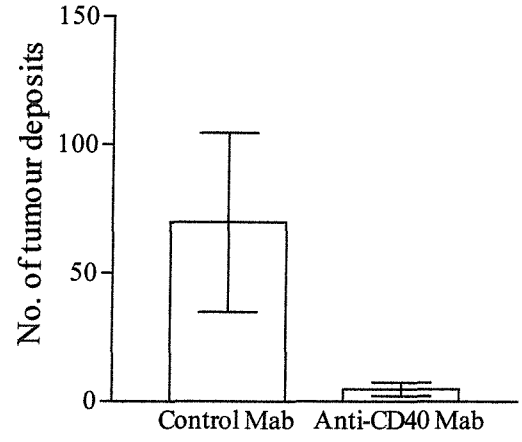
Anti-CD40 mAb is also effective in reducing the numbers of B16 tumour pulmonary deposits even when the tumour has had two days in which to become established

(figures 7.5 and 7.6). Furthermore half the mice given B16F10 were cured of tumour by anti-CD40 mAb.



Unpaired t test: P value=0.0015

Figure 7.5. Anti-CD40 mAb significantly reduces the number of B16.F10 tumours in the lungs of C57Bl6 mice when compared with controls. Error bars show 95% confidence intrervals.



Unpaired t test: P value=0.0008

Figure 7.6. Anti-CD40 mAb significantly reduces the number of B16.c215 tumours in the lungs of C57Bl6 mice when compared with controls. Error bars show 95% confidence intervals.

Expression of a foreign antigen, EGP II, by B16.c215 might be expected to enhance any immune response directed against the tumour, particularly as the aggressive behaviour by B16F10 *in vivo* is accounted to its poor immunogenicity. However, even taking into account that ten times the tumour load of B16.c215 was used the response generated by anti-CD40 mAb is no greater in terms of reducing the number of either B16F10 or B16.c215 tumour deposits, suggesting that other tumour antigens are being recognised. None of the mice receiving B16.c215 were cured of tumour.

CD40 ligand was successfully transfected into CT26 cells, and stable expression achieved (figure 7.12)

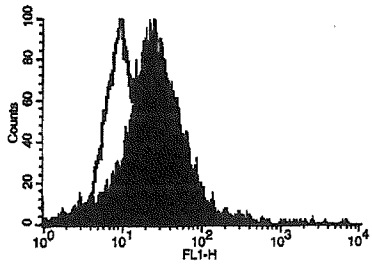
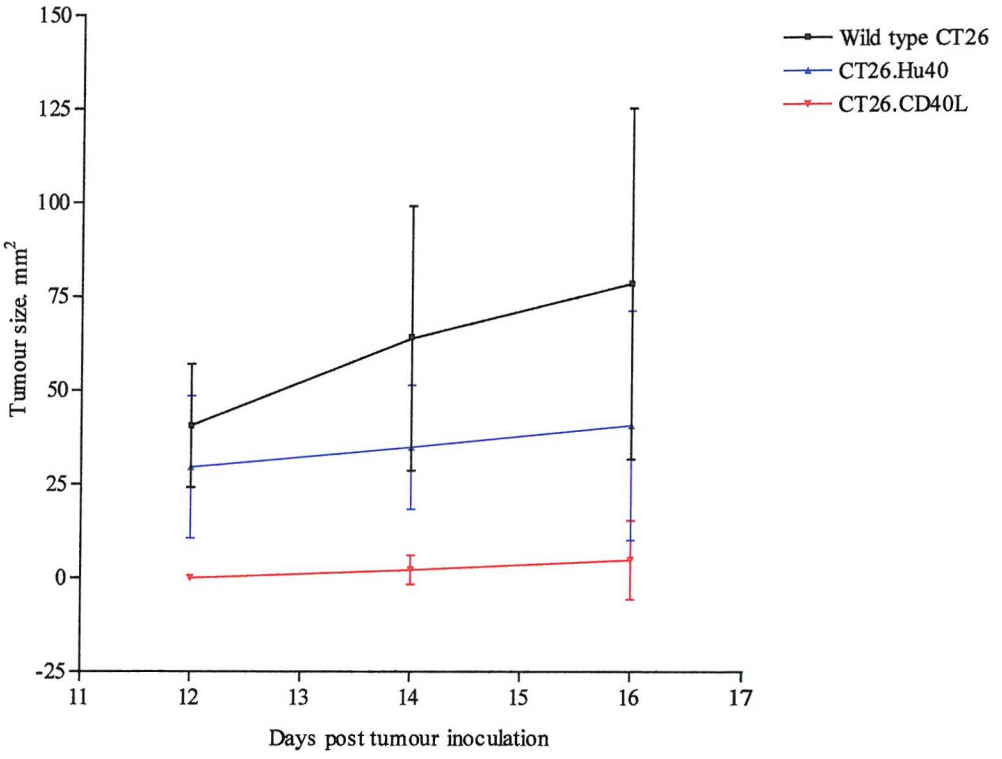


Figure 7.12. FACS measurement of the expression of CD40L by CT26.CD40L. The Solid histogram represents CT26.CD40L, the hollow histogram wild-type. The X-axis is fluorescence intensity, the Y-axis cell numbers.

The expression of CD40L on the cell surface of the tumour cells inhibits tumour growth more effectively than anti-CD40 mAb (figure 7.13), but the response, although highly promising, is less than perfect. All the animals eventually succumbed to tumour despite the initial survival advantage. For this reason it was not possible to demonstrate an effective response against re-challenge with wild type CT26 tumour cells. Given that the control engineered cells, CT26.HuCD40 tumour cells grow *in vivo* at the same rate as wild-type cells it is probable that the engineered tumour cells invoke same mechanism of action as anti-CD40 mAb and that the concentrated, localised action of the transfected CD40L is more effective in inhibiting tumour growth.



Bonferroni's post ANOVA t test:

Wild type CT26 vs CT26.Hu40	$P > 0.05$
Wild type CT26 vs CT26.CD40L	$P < 0.001$
CT26.Hu40 vs CT26.CD40L	$P < 0.01$

Figure 7.13. Growth of intradermal CT26.CD40L (C) tumours compared with that of two control groups (A and B). Four of the six mice inoculated with CT26.CD40L had no visible tumour after three weeks, but eventually developed tumour after six weeks and were all culled after eight weeks. This contrasts with the long-term protection following anti-CD137 mAb (Chapter 10) and in the lymphoma models (described in section 5.)

Chapter 8

The role of lymphocyte sub-populations in anti-CD40 mAb therapy

8.1 Introduction.

Anti-CD40 mAb affects the *in vivo* growth of both solid tumour models, through a mechanism that is indirect, i.e. not through ADCC, but the immune mechanism involved needs further investigation.

The lymphocyte response to anti-CD40 mAb is well documented[123, 177]. In mice treated with anti-CD40 mAb there is B cell proliferation and a modest increase in the number of $CD8^+$ lymphocytes that is tumour-dependent, but no change in the number of $CD4^+$ T cells (figure 8.1). Furthermore, successful immunotherapy is $CD8^+$ dependent and $CD4^+$ independent.

If cytotoxic T cells are responsible for therapeutic responses then it might be expected that these cell populations would expand following anti-CD40 mAb, and that this expansion would be most marked in tumour bearing animals, in particular in tumour-bearing organs from these animals.

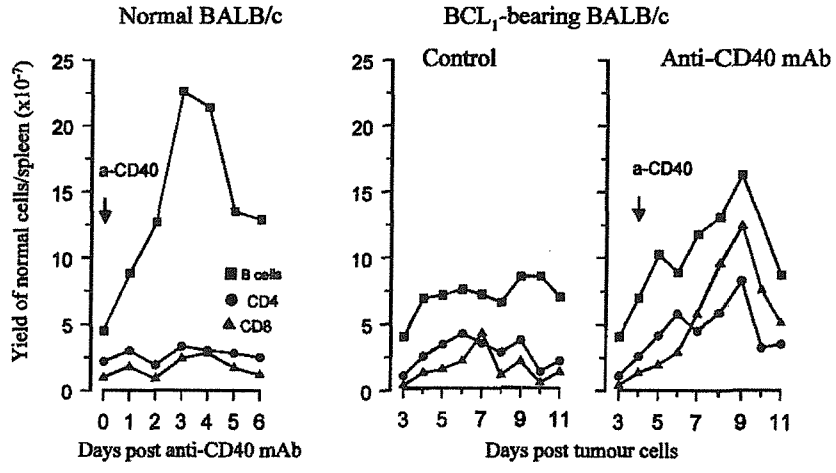


Figure 8.1. Anti-CD40 mAb in BALB/c mice causes a rapid increase in the number of B cells in the spleen. The number of T cells remains constant except in the presence of tumour, which, in combination with anti-CD40 mAb, causes a rise in the $CD8^+$ population [123].

8.2 Aims

The first aim was to establish any changes in the pulmonary lymphocyte populations following inoculation with anti-CD40 mAb, and to compare the results with those seen in lymphoma. An understanding of the kinetics of any expansion in T cell populations would also allow us to optimise the timing of harvesting of T cells for in-vitro cytotoxicity assays (see appendix 2).

Secondly, by repeating successful therapeutic strategies in mice that have had specific lymphocyte populations depleted ($CD4^+$, $CD8^+$ or both) it should be possible to demonstrate the importance of either cell type in abrogating tumour growth.

Finally it was of interest to demonstrate a corresponding increase in IFN-γ. This was on the assumption that anti-CD40 mAb promotes a T_H1 response, essential for effective cytotoxic T cell responses. This also raises the question of whether the therapeutic effect is merely a result of IFN-γ production by cells following engagement of CD40.

8.3 Methods

8.3.1 Lymphocyte population kinetics

Eight BALB/c mice were inoculated iv with 2×10^6 CT26 tumour cells, as described in section 5.4.1 and divided into two groups, A and B. This was repeated with B16f10 melanoma tumour cells in C57Bl6 mice. A further two groups of four mice each, from the same breeding group, were included as healthy mice controls, C and D.

Day 0	A and B inoculated with tumour.
Day 3	A and C treated with 0.5 mg/day anti-CD40 mAb. (3/23 mAb in sterile PBS) B and D treated with 0.5 mg/day MC106A5 mAb (rat anti-mouse anti-idiotype IgG2a mAb)
Day 8,10,12,14	One mouse from each group was culled and the lungs and spleen harvested.

Lymphocytes from the lungs of these mice were harvested as described in section 5.10 and counted on a Coulter Industrial D Cell Counter. The lymphocytes were then resuspended at 5×10^6 cells/ml. 900 µl of this lymphocyte suspension was divided

equally between three FACS tubes and labelled with anti-CD4, anti-CD8 or anti-CD19 PE conjugated mAb as described in section 5.5.2. They were all counter-labelled with anti-CD3 FITC conjugated mAb.

The cells were analysed by FACS. Negative controls were included for each day for FACS calibration and to allow for the calculation of non-specific binding. Amplification, detection and compensation were set from a standard calibration. The dot-plot FL1/FL2 was gated to calculate the percentage and then the total number of CD4, CD8, and CD19 cells in each group.

8.3.2 Selective lymphocyte depletion

Lymphocytes were depleted from BALB/c or C57Bl6 mice as described in section 5.14. Twenty-five mice were divided into five groups of five age and sex matched mice, groups A-E:

A	Tumour only
B	Tumour and anti-CD40 mAb therapy
C	Tumour and anti-CD40 mAb therapy: $CD4^+$ depletion
D	Tumour and anti-CD40 mAb therapy: $CD8^+$ depletion
E	Tumour and anti-CD40 mAb therapy: $CD4^+$ and $CD8^+$ depletion

Table 8.1. Five groups were studied to demonstrate the lymphocyte sub-population responsible for the therapeutic effect of anti-CD40 mAb.

Lymphocyte depletion of groups C, D and E was initiated two days before tumour inoculation (day –2). BALB/c mice were inoculated id with CT26 tumour cells as described in sections 5.4.2 on day 0. C57Bl6 mice were inoculated with B16F10 melanoma cells as described in sections 5.4.1 on day 0. Mice in groups B, C, D and E each received 0.5 mg anti-CD40 mAb in 200μl PBS on days 2, 4 and 6. The id tumours on the BALB/c mice were measured on alternate days from day eight. The C57Bl6 mice were humanely culled on day 21, the lungs dissected and the tumour deposits from each mouse were counted. All measurements were recorded and analysed with Prism Graph Pad statistical software.

8.3.3 The role of IFN-γ in CD40-dependent immune responses

BALB/c mice were inoculated iv with CT26 (section 5.4.1) and given either anti-CD40 mAb or control mAb (section 7.1.1). The same number of naïve mice also received anti-CD40 mAb or control mAb. After 10 days they were culled and a suspension of lymphocytes was prepared from their lungs (section 5.10.2). 5×10^6 lymphocytes were then cultured *in vitro* for 48 hours in a small culture flask with CT26 after which the culture supernatant was removed and centrifuged. The supernatant IFN-γ was measured by ELISA (section 5.13).

This was repeated, but instead of ELISA the supernatant was added at 3 ratios, 1:1, 1:10 and 1:100, to growth medium for culture of fresh CT26, with or without anti-IFN-γ mAb to block the effect of IFN-γ (table 8.2). The tumour cells were then harvested (sections 5.3 and 5.4) and MHC I expression measured by FACS (section 5.5.1).

1. Mice inoculated with CT26 and given anti-CD40 mAb.
2. Mice inoculated with CT26 and given anti-CD40 mAb. Anti-IFN-γ is added to the CT26 culture medium.
3. Mice given anti-CD40 mAb.
4. Mice given anti-CD40 mAb. Anti-IFN-γ is added to the CT26 culture medium.
5. Mice inoculated with CT26.
6. Mice inoculated with CT26. Anti-IFN-γ is added to the CT26 culture medium.
7. Naïve mice.
8. Naïve mice. Anti-IFN-γ is added to the CT26 culture medium.

Table 8.2 Groups used to study the response of CT26 cells to supernatant from different lymphocyte cultures.

To investigate a therapeutic role for IFN-γ, 12 age and sex matched BALB/c mice were inoculated id with CT26 (section 5.4.2). On days 2,4 and 6 they received subcutaneous injections, at the site of the tumour, of IFN-γ or PBS. Tumour growth was recorded as above.

8.4 Results

Both BALB/c and C57Bl6 mice show a similar response to stimulation by anti-CD40 mAb. Interpretation of the lymphocyte kinetics from the lungs is not straightforward. Anti-CD40 mAb results in an expansion of the $CD8^+$ population in these mice that is not dependent on the presence of tumour (figures 8.2 and 8.3), but for the C57Bl6 the rise in $CD8^+$ cells may be early in the presence of tumour. The relative increase in numbers of $CD8^+$ lymphocytes is greater than the increase in the total number of lymphocytes and of B cells. The timing of this rise in $CD8^+$ cells is delayed by up to two days compared with that seen in the spleen in lymphoma (figure 1). The optimum time for harvesting $CD8^+$ lymphocytes is 8 to 10 days after stimulation with anti-CD40 mAb.

The expansion of B cells numbers is less well defined although the timing of lymphocyte harvesting may have missed this. Anti-CD40 mAb has no effect on the $CD4^+$ population. The presence of tumour alone has no additional effect to the anti-CD40 mAb, nor does it significantly increase the cell numbers in the Percol fraction on its own.

The response is close to that predicted by results from murine lymphoma models (figure 1) [123]; in the spleens of BALB/c mice there is considerable proliferation of B cells after anti-CD40 mAb is given, and a lesser increase in $CD8^+$ T cells from this time. There is no change seen in $CD4^+$ T cell population. Cell numbers begin to decline after 5 days.

The delay in the increase in the $CD8^+$ population may be explained by anatomical considerations. In the lymphoma model the tumour, APC and lymphocytes are immediately related to one another. The lungs do not have a large pool of APC and naïve lymphocytes but rely on regional lymph nodes for the generation of new immune responses.

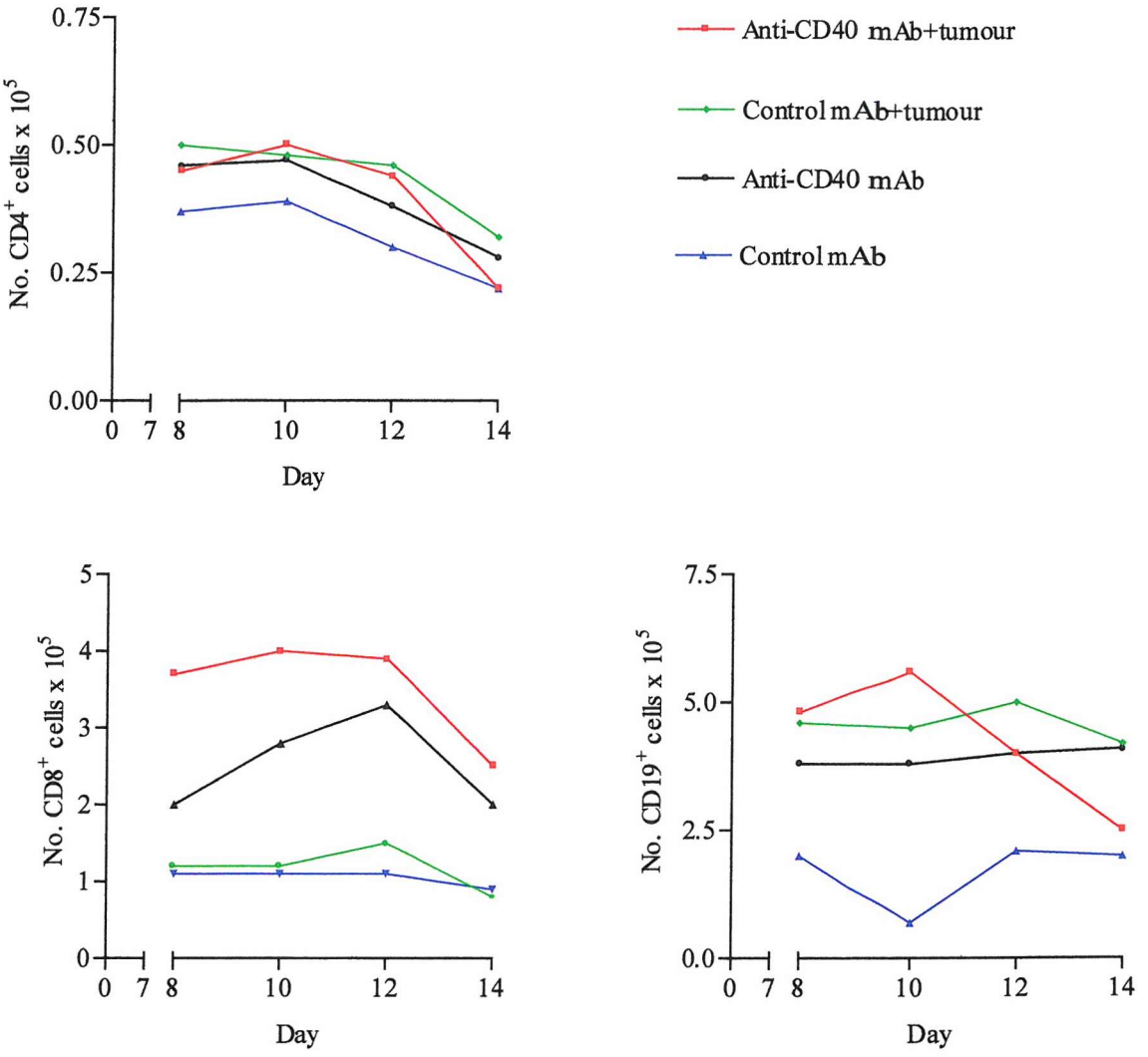


Figure 8.2. Kinetics of the $CD4^+$, $CD8^+$ and $CD19^+$ cell populations from the lungs of C57Bl6 mice. The relevant mice received tumour on day 0 and mAb on day 3. Neither anti-CD40 mAb nor tumour have an effect on $CD4^+$ cell numbers. The initial rise in $CD8^+$ cells is dependent on tumour but this is followed by a rise that is independent of tumour. Tumour alone has no effect on the $CD8^+$ population There is a modest increase in the number of $CD19^+$ cells, but this is not as marked as that seen in the spleen (figure 8.1).

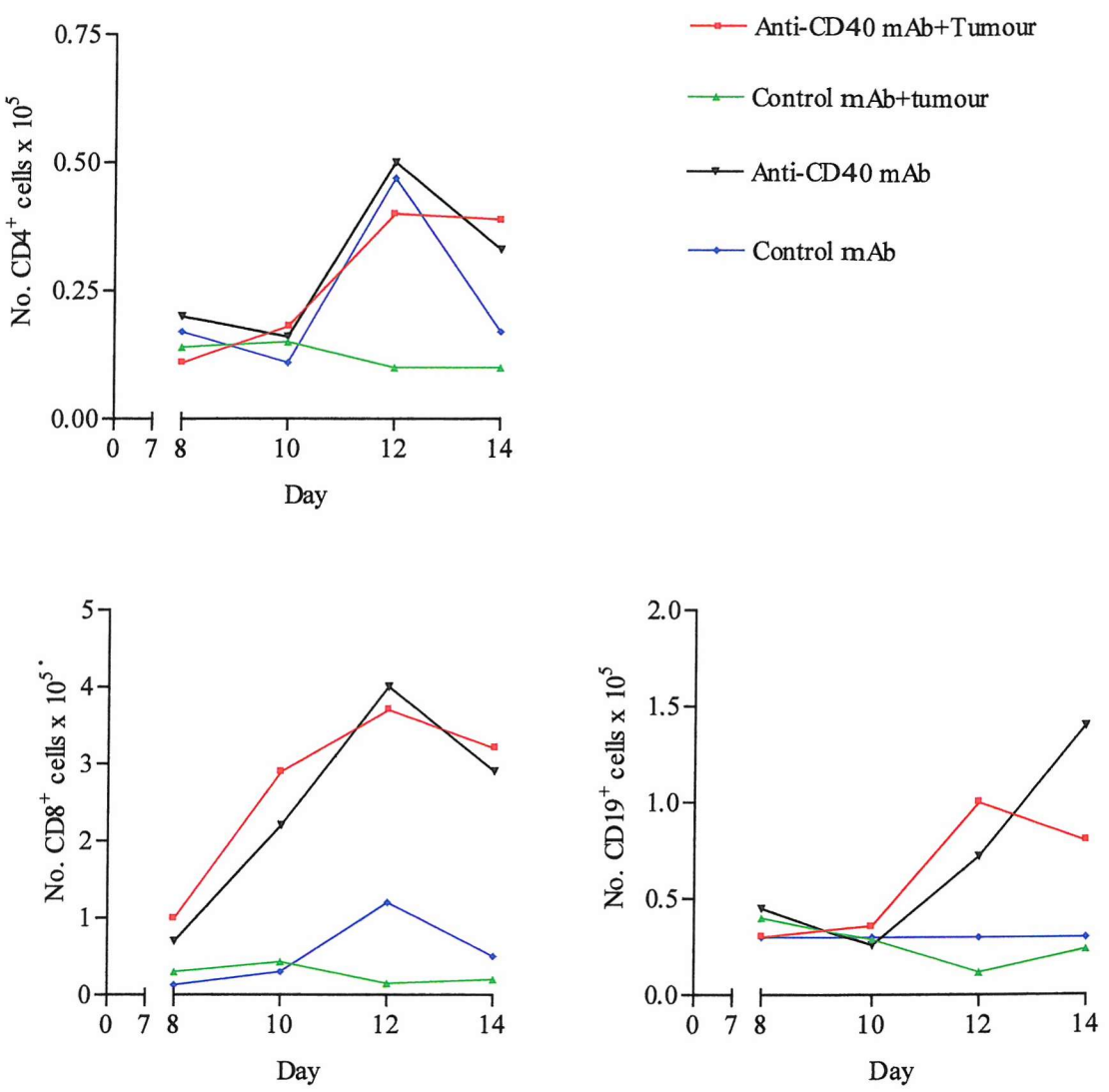
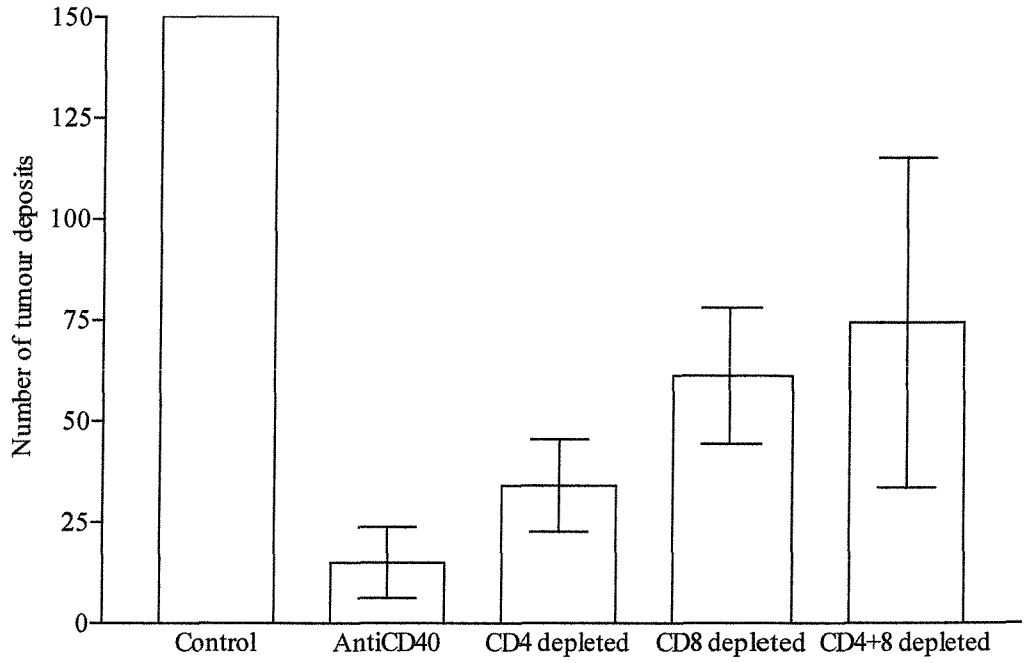


Figure 8.3. Kinetics of the $CD4^+$, $CD8^+$ and $CD19^+$ cell populations from the lungs of BALB/c mice. The relevant mice received tumour on day 0 and mAb on day 3. Neither anti-CD40 mAb nor tumour have an effect on $CD4^+$ cell numbers. There is a rise in $CD8^+$ cell numbers with anti-CD40 mAb that is independent of tumour, but tumour alone has no effect on the $CD8^+$ population. There is a modest increase in the number of $CD19^+$ cells, but this is not as marked as that seen in the spleen (figure 8.1).

The increase in $CD8^+$ cell numbers suggest that the therapeutic response to anti-CD40 mAb involves these cells. However this is independent of tumour and there is no direct evidence that they are responsible for any therapeutic effect. Figure 8.4 shows that depletion of $CD8^+$ cells abrogates the therapeutic effect of anti-CD40 mAb in B16 tumour models. Although statistically there was no clear difference between

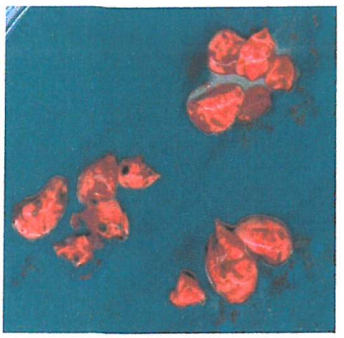
$CD4^+$ and $CD8^+$ depleted mice the results also indicate that anti-CD40 mAb remains effective despite depletion of $CD4^+$ cells, illustrated by photographs of the lungs of mice from each group (figure 8.5).



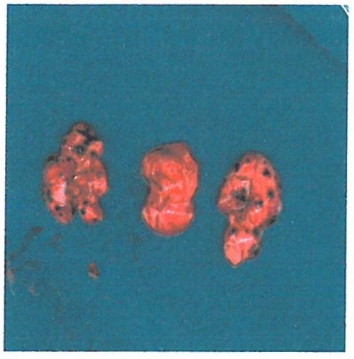
Bonferroni's Multiple Comparison Test	P value
Control vs CD40	P < 0.001
Control vs CD4 depleted	P < 0.001
Control vs CD8 depleted	P < 0.01
Control vs CD4+8 depleted	P > 0.05
CD40 vs CD4 depleted	P > 0.05
CD40 vs CD8 depleted	P > 0.05
CD40 vs CD4+8 depleted	P > 0.05
CD4 depleted vs CD8 depleted	P > 0.05
CD4 depleted vs CD4+8 depleted	P > 0.05

Figure 8.4. B16F10 melanoma tumour deposits in the lungs of C57Bl6 mice after treatment with anti-CD40 mAb and following selective depletion of $CD4^+$ and $CD8^+$ lymphocytes. The impression that these results give is that $CD4^+$ depletion fails to abrogate the therapeutic effect of anti-CD40 mAb, but that much of the therapeutic effect is lost when $CD8^+$ lymphocytes are depleted. However there is no significant difference between the number of lung metastases observed in each of these groups. Indeed statistical differences arise between the control group and all four of the experimental groups. The reasons for this are almost certainly due to the small numbers in each group and the single observation made for each animal. Three mice were culled early and not for tumour during the course of the experiment.

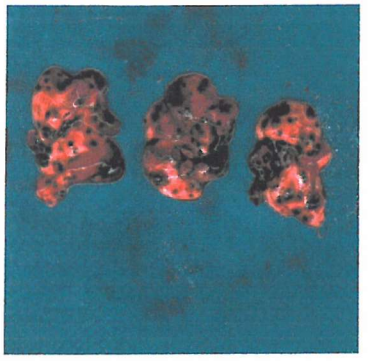
Lungs from the control group, A.



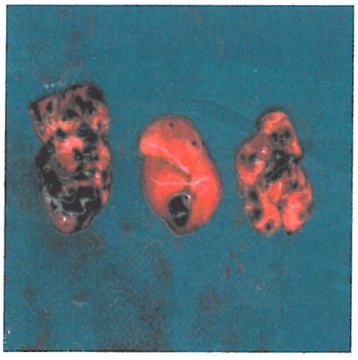
Lungs from the anti-CD40 mAb treated group, B.



Lungs from the anti-CD40 mAb treated/$CD4^+$ lymphocyte depleted group, C.



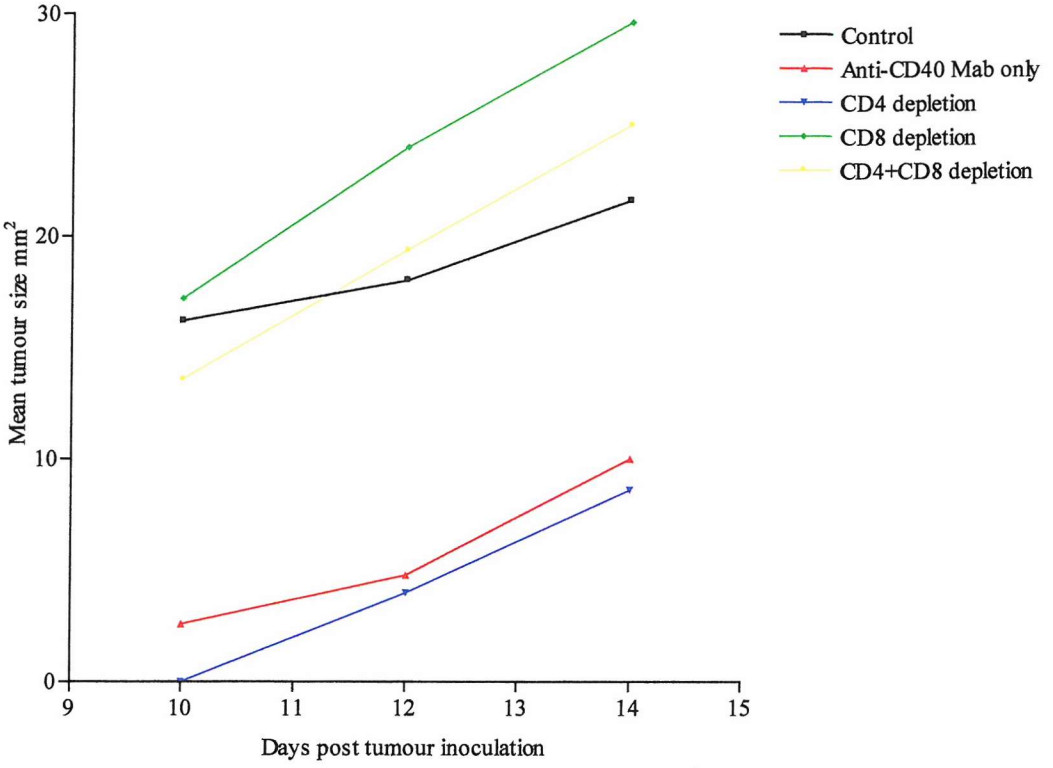
Lungs from the anti-CD40 mAb treated/$CD8^+$ lymphocyte depleted group, D.



Lungs from the anti-CD40 mAb treated/$CD4^+$ & $CD8^+$ lymphocyte depleted group, E.

Figure 8.5. Photographs of the lungs from B16F10 tumour-bearing C57Bl6 mice. The lungs from the control group (A) are solid with tumour, whilst there is very little tumour in the lungs from mice that have been treated with anti-CD40 mAb (B). Depletion of $CD4^+$ cells has little effect on anti-CD40 mAb treatment (C), but depletion of $CD8^+$ cells negates most of the therapeutic benefit (D and E).

The results for the treatment of CT26 are stastistically more convincing (figure 8.6). The therapeutic benefit of anti-CD40 mAb is lost without $CD8^+$ cells. Depletion of $CD4^+$ cells has no effect on therapy.



Bonferroni's Multiple Comparison Test	P value
Control vs Anti-CD40 Mab only	P > 0.05
Control vs CD4 depletion	P < 0.05
Control vs CD8 depletion	P > 0.05
Control vs CD4+CD8 depletion	P > 0.05
Anti-CD40 Mab only vs CD4 depletion	P > 0.05
Anti-CD40 Mab only vs CD8 depletion	P < 0.01
Anti-CD40 Mab only vs CD4+CD8 depletion	P > 0.05
CD4 depletion vs CD8 depletion	P < 0.01
CD4 depletion vs CD4+CD8 depletion	P < 0.05
CD8 depletion vs CD4+CD8 depletion	P > 0.05

Figure 8.6. Inhibition of CT26 growth by anti-CD40 mAb following selective depletion of $CD4^+$ and $CD8^+$ lymphocytes. The table summarises the results of the statistical analysis of each group incorporating all three measurements from each mouse using one–way ANOVA with Bonferroni's post-test for significance. This confirms that the mechanism of action of anti-CD40 mAb in this model is dependent on $CD8^+$ cells, but is able to by-pass $CD4^+$ help. It might have been expected, given that the tumour antigens recognised by the effector cells may be self-antigens, that the depletion of inhibitory $CD4^+$ T cells, whose normal function is to prevent autoreactive CTL activation, would either enhance the effect of anti-CD40 mAb or result in catastrophic autoimmune disease. Although there was the suggestion that initial CT26 growth was delayed in $CD4^+$ lymphocyte depleted mice, there was no statistical evidence that this was the case.

These results confirm that anti-CD40 mAb therapy of these two tumour models is dependent on $CD8^+$ lymphocytes, and that the mechanism of action bypasses the need for $CD4^+$ cells.

Because this implies a T_H1 immune response it was anticipated that there would be high levels of IFN-γ produced by immunocytes from mice that had received anti-CD40 mAb. However ELISA failed to demonstrate this (results not shown).

When CT26 cells were grown *in vitro* with the addition to the growth medium of supernatant derived from a lymphocyte culture from those mice that had received anti-CD40 mAb the MHC I expression on the tumour cells increased (figure 8.7). This increase was blocked by anti-IFN-γ mAb, confirming that IFN-γ is produced by these lymphocytes. The most effective lymphocyte culture in eliciting an increase in MHC I was that from mice with both tumour and anti-CD40 mAb. Lymphocytes derived from naïve mice that had not received anti-CD40 mAb did not raise MHC I on CT26. It was of interest that the culture supernatant from those mice that had received tumour but not anti-CD40 mAb also upregulated MHC I expression by tumour cells, although to a lesser extent. This suggests a degree of immunogenicity on the part of CT26 alone.

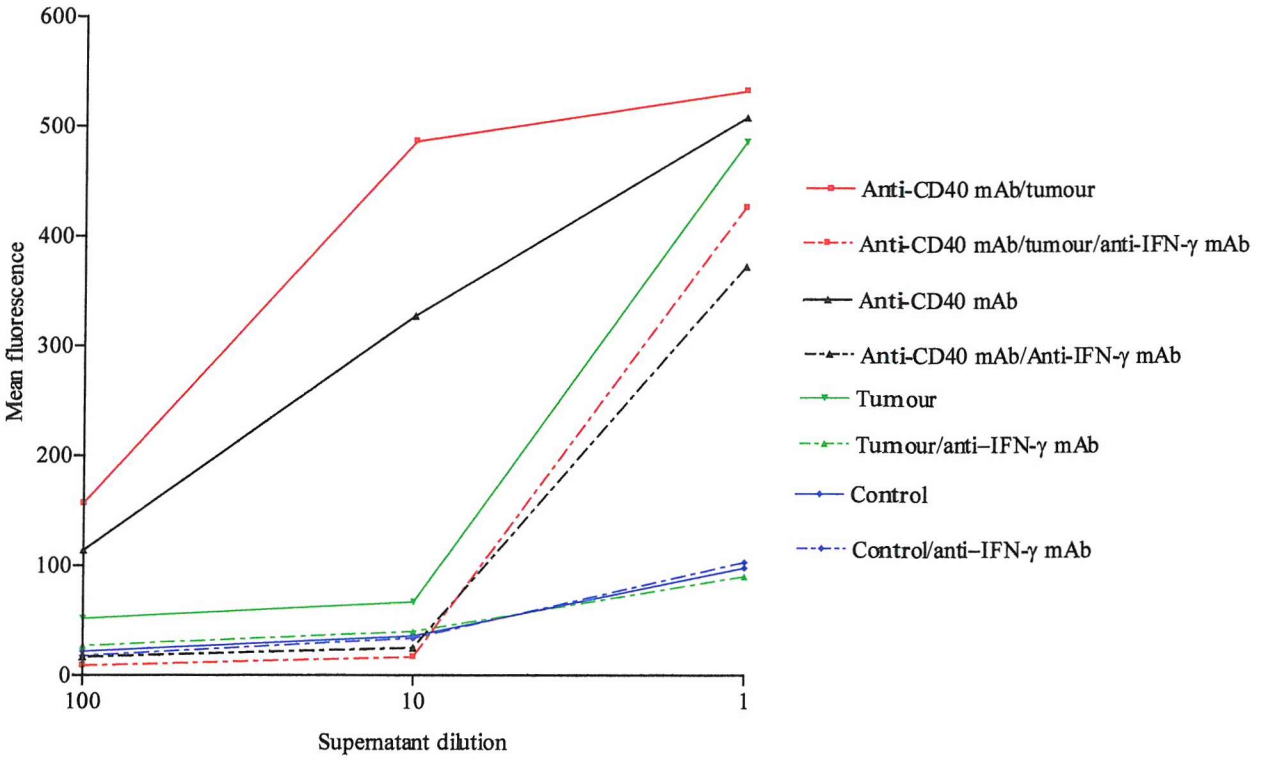


Figure 8.7. MHC I expression by CT26 cells following incubation with lymphocyte culture supernatant. The X-axis shows the dilution of supernatant (1:1, 1:10 and 1:100) and the Y-axis the mean fluorescence of FL1 channel. When tumour cells are cultured *in vitro* in the groups described above their MHC I expression is greatest with the supernatant from lymphocyte culture derived from mice that have received anti-CD40 mAb. This effect is blocked by adding anti-IFN-γ mAb to the tumour cell culture medium, confirming that anti-CD40 mAb *in vivo* leads to an increase in IFN-γ. The greatest response is seen when mice have received both tumour and anti-CD40 mAb. There is a modest increase in MHC I expression with tumour alone, which is also blocked by anti-IFN-γ mAb, suggesting that CT26 is immunogenic.

IFN-γ is involved in the therapeutic response to anti-CD40 mAb, but is not responsible (figure 8.8). Inoculation of IFN-γ around id CT26 tumours has no effect on tumour growth when compared with PBS.

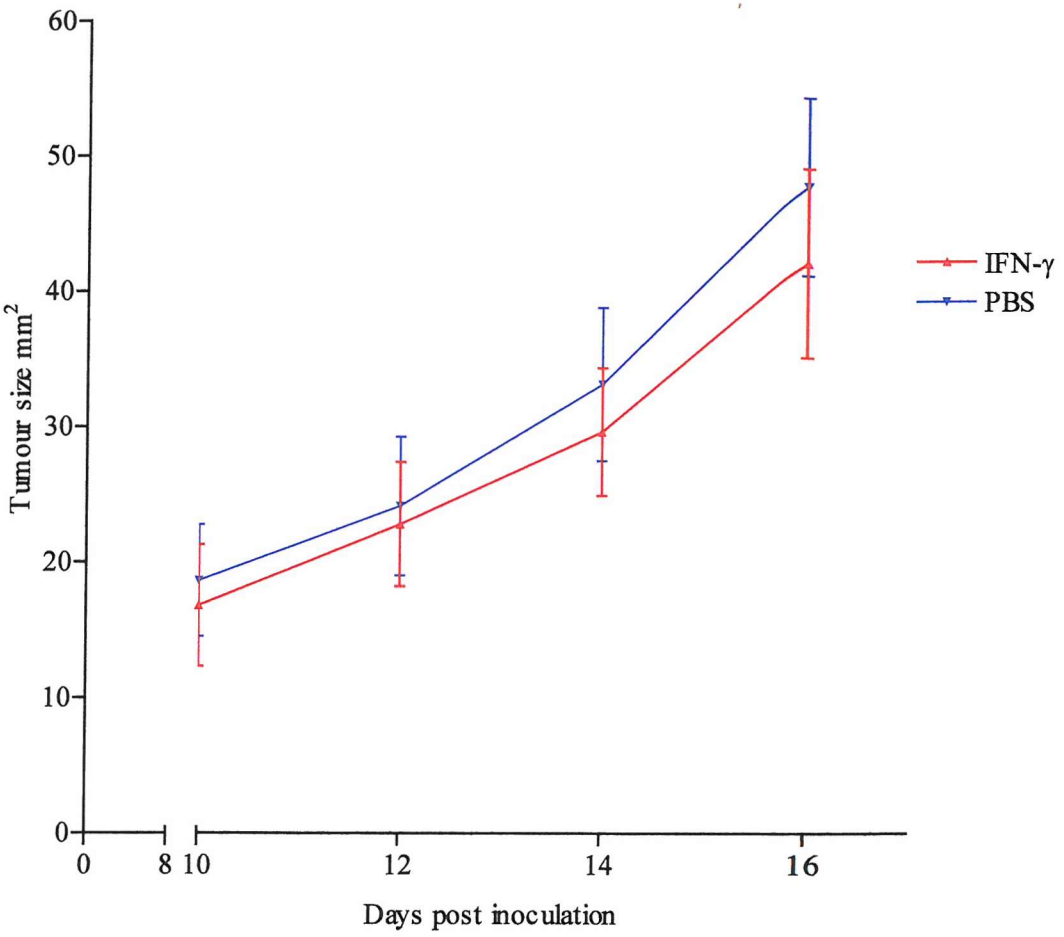


Figure 8.8. CT26 id growth (Y-axis) over 16 days (X-axis) is unaffected by high concentrations of IFN-γ (see text above for methods). This suggests that the mechanism of anti-CD40 mAb is not just increasing the local concentration IFN-γ.

The immune response to anti-CD40 mAb involves an increase in lymphocytes, predominantly $CD8^+$ but also $CD19^+$ cells. Therapy with anti-CD40 mAb is dependent on $CD8^+$ cytotoxic T lymphocytes, but bypasses the need for $CD4^+$ helper T lymphocytes. The increase in IFN-γ reinforces the hypothesis that anti-CD40 mAb is augmenting a T_H1 immune response to an unidentified tumour antigen. However, anti-CD40 mAb is not effective merely through increased levels of IFN-γ.

Chapter 9

Can an effective therapy be induced through CD137 signalling?

9.1 Introduction

CD 137, or 4-1BB, is expressed primarily by mature T cells. Its ligand, CD137L, is expressed by APC. Its function is detailed in Chapter 4. In the context of inducing an immune response to tumour antigen the activation of helper T cells through CD137 ligation greatly increases the efficacy of those T cells in promoting a T_H1 response. This is mediated through their interaction with the APC. Furthermore ligation of CD137 on cytotoxic T cells promotes further maturation of these cells.

9.2 Aims

The first aim was to investigate the therapeutic potential and mechanism of action of anti-CD137 mAb alone and in combination with anti-CD40 mAb, the growth of subcutaneous, intradermal and pulmonary CT26 tumours was measured. Because CD40 and CD137 and their ligands are critical in the generation of armed CTL, it was hoped that they might have a synergistic effect. Surviving mice exposed to intradermal CT26 were re-challenged with tumour.

Anti-CD137 mAb therapy was then repeated in mice following the selective depletion of helper and cytotoxic T cells, to determine which, if any, cells were required for therapy.

Finally the kinetics of the T cell response was studied in the pulmonary model of CT26 and included measurement of CD62L, a marker of mature, armed cytotoxic T cells (see appendix 3).

9.3 Methods

Pulmonary model of CT26.

Eighteen age and sex matched BALB/c mice received intravenous inoculation with 2×10^6 CT26 tumour cells as described in section 5.4.1, and divided into three groups of six mice. On days two and seven they received intraperitoneal injections of PBS,

0.5 mg anti-CD137 mAb (Lob 12) or 0.5mg anti-CD40 mAb and 0.5 anti-CD137 mAb. They were closely observed and culled when they became unwell, with the day of death being the recorded outcome.

Subcutaneous and intradermal models of CT26 growth

For each model eighteen age and sex matched BALB/c mice were inoculated with tumour as described in section 5.4.2. The sc tumour mice received intraperitoneal injections of 0.5ml PBS, 0.5 mg anti-CD137 mAb (Lob 12) or 0.5mg anti-CD40 mAb and 0.5 anti-CD137 mAb on days two and seven. The intradermal tumour mice received intraperitoneal injections of PBS, 0.5 mg anti-CD137 mAb (Lob 12) or 0.5mg anti-CD40 mAb on days two and seven. Tumour growth was measured for each mouse on alternate days.

Selective depletion of $CD4^+$ and $CD8^+$ lymphocytes

Twenty-five age and sex-matched BALB/c mice were divided into five groups of five mice (A, B, C, D, and E) and selectively depleted of either, or both, $CD4^+$ and $CD8^+$ lymphocytes, as described in section 5.13 and 8.2, beginning two days before tumour inoculation. All of the mice were inoculated id with 2×10^5 CT26 tumour cells in 50 µl PBS as described in section 5.4.2. Groups B, C, D, and E received intraperitoneal injections of anti-CD137 mAb on days 2 and 7. Tumour growth was measured for each mouse on alternate days.

9.4 Results

Non-specific immunotherapy with anti-CD137 mAb alone or in combination with anti-CD40 mAb failed to provide any survival advantage to mice bearing pulmonary CT26 tumour (figure 9.1). Anti-CD137 mAb in combination with anti-CD40 mAb significantly inhibits, but does not prevent tumour growth in the sc model of tumour growth, and appears to be better than anti-CD137 alone in respect of this (figure 9.2). However the results do not reach statistical significance. By far the most interesting results are those from the id model of tumour growth (figure 9.3). The initial growth of the tumour in mice treated with anti-CD137 mAb was similar to that in mice treated with anti-CD40 mAb. In the majority of mice tumour was visible by day 10.

After this point the intradermal tumours in the anti-CD137 mAb treatment group regressed and by day 14 were no longer visible except in the small number of mice in which the tumour failed to respond to treatment.

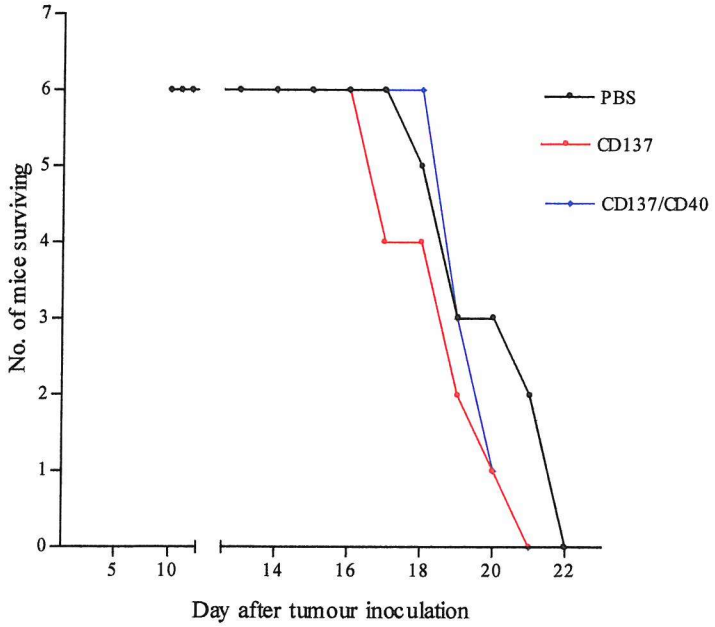


Figure 9.1. Survival of BALB/c mice after intravenous inoculation with CT26. The mice were treated with intraperitoneal PBS, anti-CD137 mAb or anti-CD137 mAb in combination with anti-CD40 mAb. Anti-CD137 mAb does not confer any survival advantage.

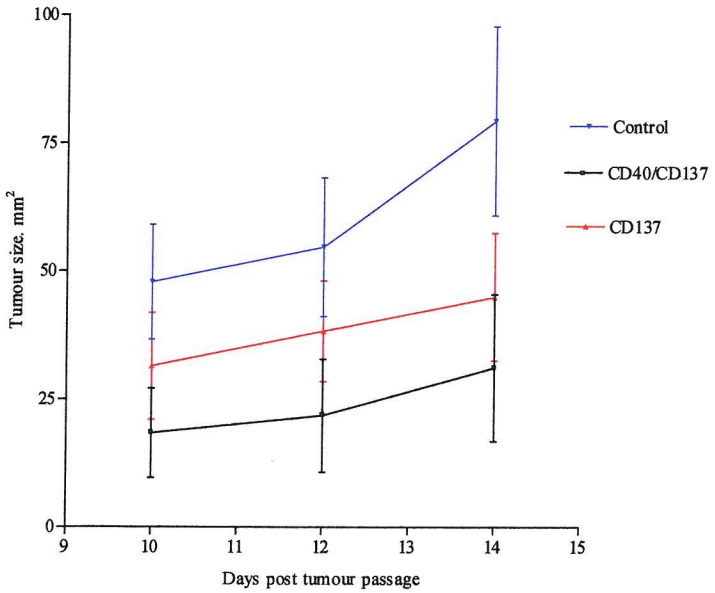
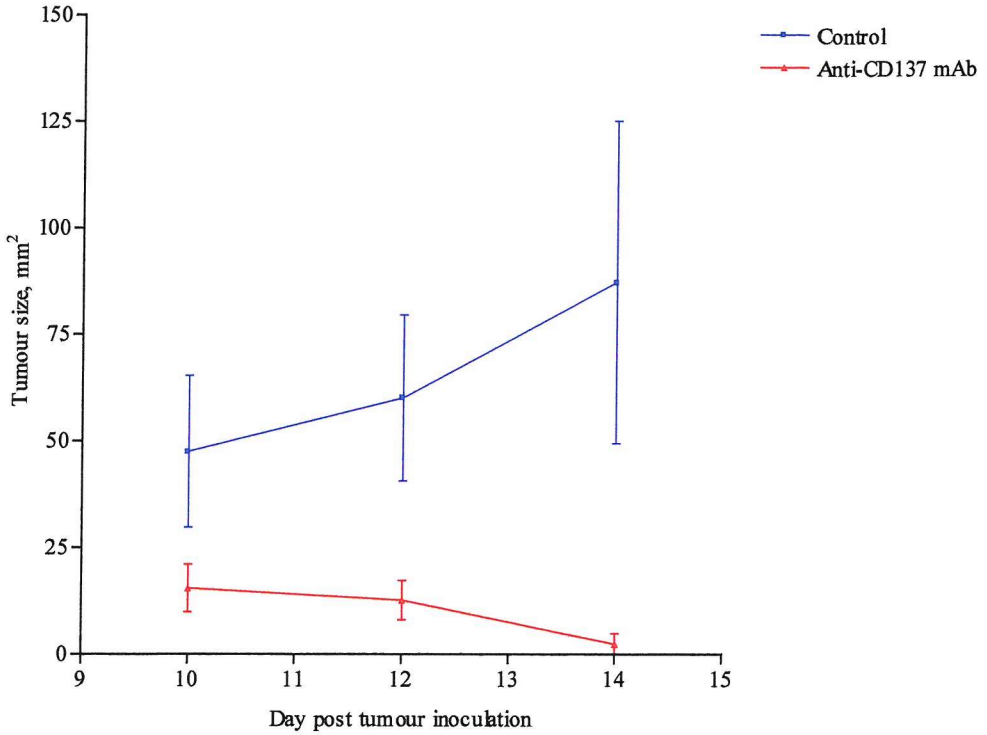


Figure 9.2. Growth of sc passaged CT26 tumour cells in BALB/c mice after treatment with intraperitoneal injections of PBS, anti-CD137 mAb or anti-CD137 mAb in combination with anti-CD40 mAb. Error bars show the standard error of the mean. Anti-CD137 abrogates tumour growth. This is enhanced by anti-CD40 mAb, although the effect does not reach statistical significance



10	Unpaired t test P value	0.0037
12	Unpaired t test P value	0.0001
14	Unpaired t test P value	P<0.0001

Figure 9.3. Growth of id CT26 tumours in BALB/c mice after treatment with intraperitoneal injections of PBS, anti-CD137 mAb. By day 14 there was no evidence of tumour on mice treated with anti-CD137 mAb.

The specific depletion of $CD4^+$ and $CD8^+$ lymphocyte sub-populations confirmed that, as with the anti-CD40 mAb therapy, the response is dependent on cytotoxic T cells (figure 9.4). The hypothetical mechanism of action of anti-CD137 mAb therapy also requires $CD4^+$ cells (see above and section 5). However there was no significant difference in tumour growth between the $CD4^+$ lymphocyte depleted anti-CD137 mAb treated mice and intact anti-CD137 mAb treated mice. The lack of a significant difference in tumour growth between and $CD4^+$ lymphocyte depleted anti-CD137 mAb treated mice is explained by tumour regression in two mice from that group. The other three mice developed tumours that grew at a similar rate to those in the control group. This implies that in addition to the helper T cell signalling through CD137

there may be other mechanisms that allow helper T cells to be bypassed, as with anti-CD40 mAb therapy. Alternatively the depleting mAb had not been entirely effective in those two mice (see appendix 2).

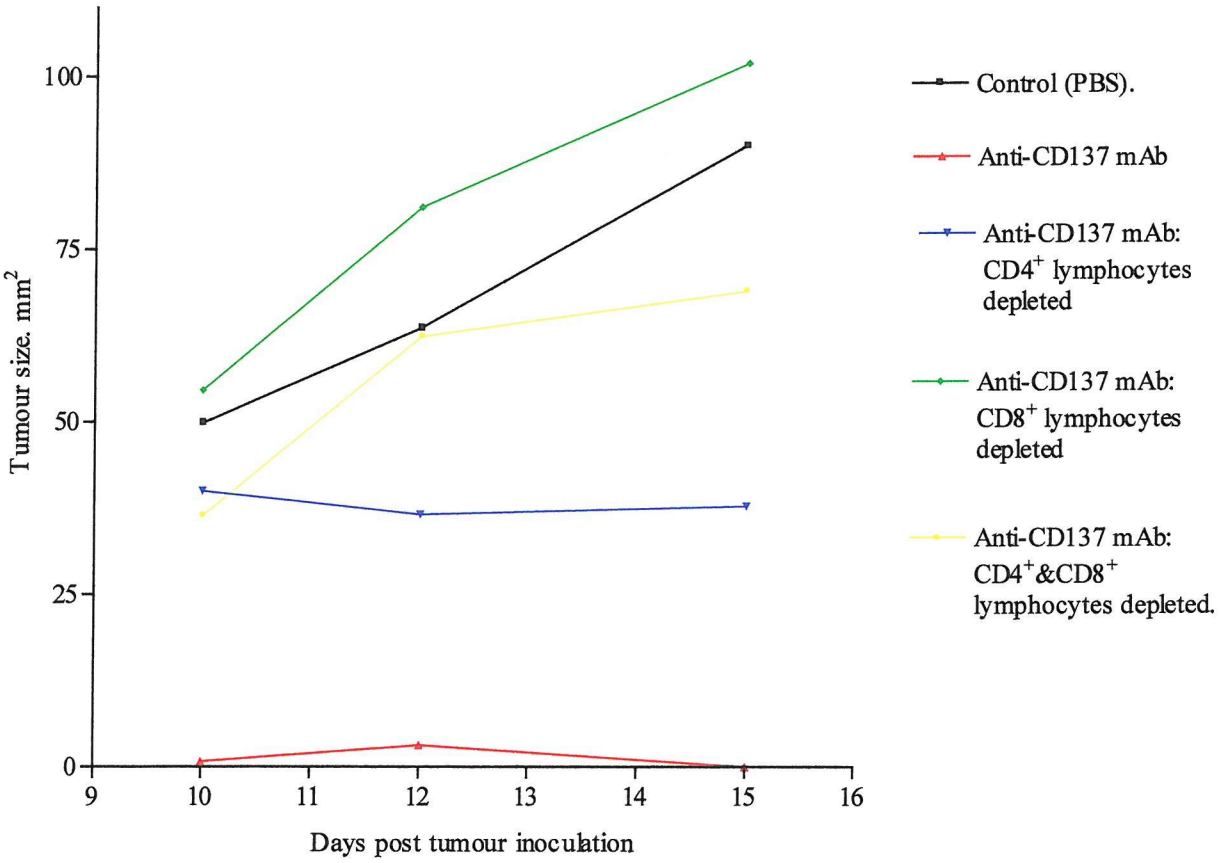


Figure 9.4. Anti-CD137 mAb therapy of intradermal CT26 tumours following selective depletion of $CD4^+$ and/or $CD8^+$ lymphocytes with specific mAb. Although statistical significance is not achieved, the results suggest that $CD4^+$ lymphocytes are required in addition to $CD8^+$ T lymphocytes for the therapeutic effect of anti-CD137 mAb. (Two mice from the $CD8^+$ and $CD4^+/8^+$ depleted groups died from causes other than tumour growth before completion of the study).

9.4 Re-challenge of cured mice with tumour

Thirteen of eighteen (72%) mice with intradermal CT26 tumours that received intraperitoneal anti-CD137 mAb were re-challenged after 1-3 months with 5×10^5 CT26 tumour cells in 50 µl DMEM, as described in section 6.3. Three mice from control groups that had also survived received the same tumour re-challenge. All of the mice from the anti-CD137 mAb treatment groups were protected from CT26

tumour, and were all still alive after a further three months. None of the mice from the control groups survived.

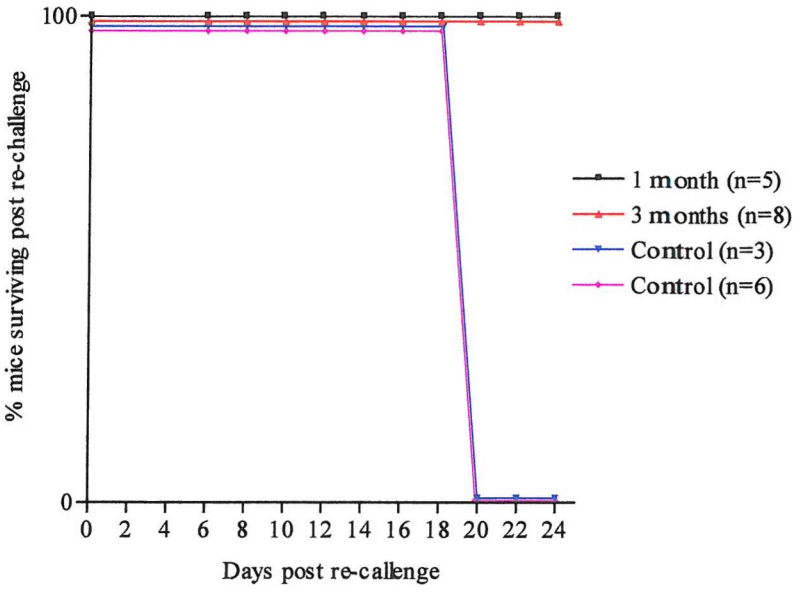


Figure 9.9. Mice that had received both CT26 tumour cells and anti-CD137 mAb, and survived the initial tumour challenge by 1 or 3 months, were re-challenged with intradermal CT26. Three mice from control groups from these experiments were also re-challenged with the same tumour load, but all developed tumours after re-challenge. Six naïve mice were included as a control.

Immunologically intact mice cured of tumour by anti-CD137 mAb remained healthy for at least one month, after which they were re-challenged. All of these mice were protected from CT26 tumour for up to 3 months, and remained healthy for at least three months after this time. This is in contrast to the small number of mice that did not develop tumours following treatment with anti-CD40 mAb. None of these animals had any protection from re-challenge. The best CD40-dependent response was seen following CT26.CD40L tumour inoculation (section 7.4). In this case the growth of the genetically engineered tumour was severely abrogated but not entirely abolished, and so it was not possible to demonstrate immunological memory to other tumour antigens.

It has been hypothesised that in the case of the lymphoma models of anti-CD40 mAb cytotoxic T cell dependent immunological memory protects against re-challenge with tumour. The reduced levels of protection afforded by anti-CD40 mAb against CT26 meant that, with few long-term survivors, there was no opportunity to investigate long term protection. Anti-CD137 mAb provides clear evidence of lasting protection

against CT26 tumour, and it is likely, from what we know about the function of CD137 and the effects of $CD4^+$ depletion on anti-CD137 mAb therapy, that this effect is, at least in part, helper T cell dependent. Ideally this should be investigated further by curing mice of CT26 tumour with anti-CD137 mAb, and then re-challenge mice with tumour following selective $CD4^+$ lymphocyte depletion (it is already clear that $CD8^+$ lymphocytes are required for anti-CD137 mAb dependent protection against this tumour).

Chapter 10
Discussion

Solid tumours can usually be resected or safely included in a radiotherapy field but advanced disease may involve structures that prevent safe excision and metastatic tumour deposits may be too small to be identified and thus treated. Chemotherapy is a means of systemic treatment of such advanced disease, but although it may control spread for a period of time it is to be expected that tumours will eventually become chemo-resistant and escape control. There is a pressing need for novel therapies that combine the specificity of surgery and radiotherapy with the systemic availability of chemotherapy. Thus the argument for immunotherapy, which offers the potential of systemic specific anti-tumour therapy, is persuasive.

Immunotherapy through immunomodulation has been the subject of many previous studies, but few have led to significant clinical success. Inducing an effective anti-tumour CTL response is a particular challenge, but signalling through CD40 or CD137 promises to deliver such a response through their mechanisms of action (discussed in chapter 4). Antibodies to CD40 cure mice of lymphoma and it appears that cytotoxic T cells generated after exposure to T_H1 cytokines, are the main effectors in this immunotherapy. However we must take into account the mechanism of action of the costimulatory molecules and cytokines to avoid the re-invention of a square wheel. It may be that all that has been achieved is an excessively complicated method of increasing cytokine levels, with no added benefit when compared with simple immunomodulation using those cytokines alone.

In vivo therapy with anti-CD40 and anti-CD137 mAb

There was no significant survival advantage for anti-CD40 or anti-CD137 mAb therapy in the CT26 pulmonary tumour model, but id and sc tumour growth was inhibited. This effect was most dramatic in the anti-CD137 mAb therapy of id CT26 where initial tumour growth was followed by tumour regression and long term survival.

CT26 tumour growth *in vivo* was almost completely abrogated by genetic modification to express CD40L. This suggests that APC can interact directly with tumour and that engagement of tumour CD40L by APC leads to an immune response against CT26. The characteristics of that response, such as the cells involved, could

not be described and it was unexpected that tumour was found to grow so long after control mice had died. Ideally microscopic eradication of tumour and resistance to rechallenge would be demonstrated.

Others have found similar results with CT26 transfected to express CD40L [172]. In a similar model, using a murine lung carcinoma (3LLSA) transfected to express CD40L, $CD4^+$, $CD8^+$ and NK cells infiltrated the tumour. The tumour was rejected and mice were protected against re-challenge with wild-type 3LLSA. Tumour rejection and subsequent immunity was CTL dependent.

Anti-CD40 mAb therapy of both of the B16 tumour models resulted in considerable reduction in tumour deposits and a proportion of mice inoculated with B16F10 tumour cells were macroscopically tumour-free following treatment.

Therapeutic mechanism of action of anti-CD40 and anti-CD137 mAb

Murine lymphoma can be cured through anti-CD40 mAb mediated immunomodulation. The mechanism of action is thought to be activation of APC and subsequent arming of cytotoxic T cells. The lymphoma studied were B cell and T cell in origin, and at least in the case of the B cells, may have been able to function as their own professional APC. In this situation, crosslinking of CD40 appears to prime APC so that they can to arm CTL which are then able to kill the tumour [123]. Furthermore lymphoma cells express unique tumour antigen, their idiotype, but it is yet to be confirmed that the epitope recognised by the T cells is derived from the cell surface receptor.

FACS analysis of surface expression of B16 melanoma cell lines confirmed that they do not express CD40 or CD137. Although initial FACS of CT26 suggested it might express low levels of CD40, there was no evidence of this with PCR of their cDNA. Thus any therapeutic benefit is a result of indirect activation of the immune system mediated by crosslinking of CD40 or CD137 by the respective mAb (Chapter 4). However, others have found $CD40^+$ B16[178], and expression is variable in human melanoma

Although there was no evidence that anti-CD40 mAb has a direct effect on B16 or CT26 cell lines, carcinoma cells expressing CD40 have been identified[179]. T cells co-cultured with $CD40^+$ carcinoma showed impaired function indicated by reduced IFN-γ secreting cells, reduced IL-2 secretion, impaired proliferation, and a lack of CD40L re-expression on restimulation.

The possibility that the anti-CD40 mAb may also recruit FcR-bearing effectors for ADCC is also a concern. To investigate this CT26 cells were transfected with human CD40 (Appendix 1). There was no evidence of ADCC with anti-human CD40 mAb, although the levels of human CD40 expression by CT26 were low. There is no evidence in the literature of ADCC with anti-CD40 mAb, and Dotti et al [180] found that the immune response is not dependent on tumour expression of CD40 (discussed below).

The reduced efficacy of anti-CD40 mAb in the treatment of B16 and CT26 tumours, compared with that for lymphoma, may in part be because these tumours do not express CD40, and are unable to function as APC, unlike lymphoma.

Increased IFN-γ in response to anti-CD40 mAb provided strong circumstantial evidence of a T_H1 immune response. However it was unlikely that IFN-γ was the sole arbiter of therapy as it had no demonstrable effect *in vitro* or *in vivo* on the growth CT26 or *in vitro* on B16F10. Others have found similar results[166]. Indeed it has been found that the malignant potential of CT26 is enhanced by IFN-γ[181].

With regard to anti-CD40 mAb, it is evident that $CD8^+$ cells are essential. Depletion of $CD4^+$ cells had little effect on its therapeutic efficacy. Depletion of $CD8^+$ cells appeared to permit tumour growth when compared to that of the controls, but this was not statistically significant. Further study of this effect might support theories of immunosuppressor T cells. Although there was a slight decrease in the initial growth of tumour in $CD4^+$ depleted mice, there was no evidence for inhibitory $CD4^+$ cells. The role of NK cells was not investigated, although these cells have been implicated as effectors after CD40 signalling[182].

Depletion of $CD4^+$ and $CD8^+$ cells suggested that anti-CD137 mAb mediated abrogation of tumour growth was dependent on both T cell sub-sets. These results are explained in part by what is known of the function of CD137 and the molecular signalling that occurs following ligation with CD137L (section 4.9). CD137 is effective through $CD4^+$, $CD8^+$ and APC; signalling is bi-directional. In particular the cross-linking of CD137 on $CD4^+$ cells leads to up-regulation of CD40 (chapter 4). However there is not the increase in B cells seen following CD40 cross-linking. May et al [183] demonstrated that anti-CD137 mAb can be effective in the absence of $CD4^+$ cells; tumour-bearing/$CD4^+$ depleted mice were treated with tumour-antigen

specific CTL. Their efficacy was greatly enhanced by anti-CD137 mAb through reduced AICD.

Others have found that although immunomodulation through CD137 leads to a T_H1 helper cell response, the effectors were NK cells, not $CD8^+$ cells[184, 185]. Melero et al[185] demonstrated that NK cells are involved in the anti-tumour CTL response seen after ligation of CD137 with anti-CD137 mAb. They studied P815 plasmacytoma in a syngeneic murine model and found that depleting mice of NK cells abrogated tumour killing to the same extent as $CD8^+$ depletion. The mechanism of action of the NK cells was not confirmed. The NK cells did not effect tumour killing, and although NK cells were found to augment the levels of T_H1 cytokines this was not significant to the CTL response. $CD8^+$ and $CD4^+$ cell numbers and function were otherwise unaffected by NK depletion.

In contrast, Chen et al [186] found that CD137 signalling switched the immune response to in a murine colorectal cancer model from NK cell dependent to CTL-dependent.

A combined NK cell/CTL-dependent anti-tumour mechanism is exciting, as it would prevent the escape of tumour cells from CTL. The role of NK cells following CD137 signalling requires further study.

Lymphocyte kinetics in response to anti-CD40 and anti-CD137 mAb

Anti-CD40 mAb induces similar changes in the lung lymphocyte populations to those seen in the spleens of the murine lymphoma models[123]. The absolute numbers of lymphocytes harvested from the lungs is far less than from the spleens, giving a reduced yield of immune cells, particularly B cells. However the relative increase in B and $CD8^+$ T cells following anti-CD40 mAb is similar in both organs.

An important difference between the pulmonary tumour models and the lymphoma models is an increase in the number of $CD8^+$ cells in tumour-free mice that recieved anti-CD40 mAb. This may be explained by the presence of inhaled antigen, which may be adequate for antigen presentation and arming of CTL following stimulation with anti-CD40 mAb.

It cannot be determined whether the cells are from the lung parenchyma or hilar lymph nodes. Idiosyncratic results, particularly low cell yields, may be explained by a lack of lymphoid tissue after dissection.

Crude cell numbers alone cannot be used to characterise the immune response to anti-CD40 mAb. However the proportional increase in pulmonary $CD8^+$ cells is greater than that of B cells, which would further support the argument that the response is T_H1.

The kinetics of the immune response to anti-CD137 mAb was unclear, as there was little change in cell numbers (appendix 3). Further study should investigate changes in activation markers on immune cells. There is little evidence that CD137 crosslinking results in significant cellular proliferation and May et al [183] suggest that the effect on CTL is prolonged survival rather than proliferation. However, Kwon et al[187] demonstrated enhanced T cell proliferation in CD137 deficient mice, although immune responses by these cells were diminished.

Failure to demonstrate CTL (appendix 2)

It is disappointing not to have confirmed that CTL are responsible for the anti-tumour immune response in these tumour models despite the presence of the transfected tumour antigen EGF II in the B16.c215 cell line, that other MHC-restricted B16 antigens have been identified and that CT26 expresses viral antigens. However it must be remembered that the aim of this project was to demonstrate that immunomodulation through CD40 and CD137 could be effective in inhibiting or even eliminating established tumours, and this has been achieved. By definition non-specific immunotherapy is not dependent on specific characteristics of either the tumour or the immune response: never-the-less optimisation of immune response is best approached once the response has been characterised.

It is likely that the reasons for this failure are multi-factorial. Although there is strong circumstantial evidence that the cytokine response to anti-CD40 mAb is T_H1 in profile, T_H2 cytokines were not excluded in these two tumour models and this may have been critical. However French et al [123] demonstrated that in murine models of lymphoma the profile was T_H1 and not T_H2 despite the proliferation of B cells.

The number of CTL harvested was small, and only a proportion will have been tumour specific. Although previous experience suggested that the ratio of target to effector cells was adequate, this may not be relevant given the incomplete therapeutic effect observed in the CT26 and B16 tumour models.

Other cells may be involved in the generation of a CTL response and lack of effective CTL function may in part be due to loss of NK cells or NK cell function in the experimental cell population (see below). Introducing an environment favourable to NK cells would undoubtedly bias results, as they require T_H1 cytokines for successful culture. Selective depletion of NK cells would be feasible.

Although these results, and those of others discussed in Chapter 4, indicate that engagement of CD40 results in a T_H1 cytokine and costimulatory response, it has been difficult to demonstrate which cells are responsible. CTL have been shown to be effective in killing tumour cell [188], but other cells have been implicated. For example NK cells are also activated by T_H1 cytokines. Although they lack specificity against tumour antigen and do not express the TCR, they have other characteristics that allow them to recognise and kill tumour cells.

These results indicate that the arming of anti-tumour CTL is more sophisticated than the mechanisms described in Chapters 2 and 4. The role of NK cells in the activation of CTL is not well described, and in the context of CD40 and CD137 further investigation is essential. Naïve and mature NK cells express CD40L but not CD40 itself[189]. The anti-tumour cytotoxicity of NK cells following anti-CD40 mAb is almost certainly in response to increased levels of T_H1 cytokines [190] rather than a result of direct activation of NK cells through CD40 ligation. The possibility that NK cells have a role in the generation of T cell cytotoxic responses by CD40 ligation has not been thoroughly investigated.

The future of CD40 and CD137 in immunotherapy

It is apparent that conventional approaches cancer immunotherapy through immunomodulation such as signalling through CD40 or CD137 are unlikely to be successful. Attempts to upregulate or induce an immune response with cytokines are well documented (reviewed by Hellstrand [193] and discussed in sections 1.5 and 1.6) but have rarely demonstrated consistent therapeutic benefits.

The study of immunosuppression provides further evidence that this is the case. Although immunosuppressed patients and experimental animals are prone to malignancy, the disease seen is for the most part esoteric. If reducing cellular immune responses does not predispose to common malignancies then why should non-specific augmentation of cellular immune responses be effective in treating malignancy? An

example is Kaposis' sarcoma in AIDS[191]. It was the epidemiological study of this previously rare malignancy that raised suspicions of an infectious aetiology.

The largest cohort of immunosuppressed patients is that of allograft transplant recipients. These patients are of particular relevance as commonly used immunosuppresants including cyclosporin and FK506, suppress the cellular immune system by blocking cytokine responses. The activity of cytotoxic cells is obtunded and they suffer from opportunistic intracellular bacterial and viral infections. They are also prone to esoteric malignant disease but the incidence of common cancers such as colon, breast or lung is not greatly increased[192]. This would suggest that malignancy in those with an otherwise intact immune system is not a result of evasion of immune cells by tumour cells, and that there is no easily corrected deficiency.

Further attempts at immunotherapy through immunomodulation must be considered in the light of these observations, when reviewing experiment results and planning further studies.

Might immunomodulation have a role in combination with other modalities of immunotherapy? In Chapters 2 and 4 the mechanisms were discussed by which effective CTL are generated. Simple vaccination with tumour antigen alone is unlikely to result in an effective cytotoxic response, but when combined with an immunomodulating agent this may generate effective CTL; a dual-function vaccine. There are several stages at which such an agent may act. Initial activation of the immune system requires a non-specific danger signal, some of which are better at promoting T_H1 responses than others. Secondly the antigen must be recognised in the correct context, such as being chaperoned by a heat shock protein, for crosspriming to take place. From this point a number of signals including interleukins, interferons and costimulatory molecules may be invoked to promote the desired response.

The scene is set for a role in tumour vaccination for CD40 and CD137. Signalling through CD40 and CD137 induces an anti-tumour immune response with memory. Tumour antigens must normally be recognised by both $CD4^+$ and $CD8^+$ cells for anti-tumour CTL to be fully armed, and engagement of CD40 or CD137 allows bypassing of many of the early signalling steps involved in this process. However immunomodulation through IFN-γ alone has no therapeutic effect on CT26 or B16 *in vivo* and there is little evidence in the literature that other cytokines, whether biased to T_H1 or T_H2, have any significant effect on either tumour. Ribas [194] et al provide an

elegant example of this in their work with murine melanoma antigen transfected dendritic cells. These cells provide protection against tumour that is lost when mice are depleted of $CD4^+$ or $CD8^+$ lymphocytes. Crosslinking CD40 on the dendritic cells restored their efficacy in $CD4^+$ depleted mice, but transduction of the dendritic cells with T_H1 cytokines including IL-2, IL-7 and IL-12 did not.

Signalling through CD40 and CD137 is certainly effective but remains inadequate. It is likely that a degree of redundancy incorporated in the immune system prevents a single step from inducing such a radical change in the status quo that self-antigens, even in the context of a tumour, become immunogenic. However, another reason for this might be the context in which the antigen and subsequent immune signals are processed. With CD40 and CD137 being croslinked by injected antibody the immune response is systemic. However the tumour, and therefore the tumour antigen, is localised. It might be preferable for the signal to be delivered exclusively at the point where the immune system encounters the antigen. A vaccine incorporating both antigen and immunomudulating signal is a tantalising possibility that has been investigated in the past. This possibility is only being realised as we understand more of the immune responses to tumour antigens and develop new techniques to deliver such a vaccine.

CD40L transfected CT26 (chapter 7) functions as a crude tumour vaccine of this sort. The mechanism of action is probably through local activation of dendritic cells and CTL through CD40 signalling. Recently considerable interest has developed in the literature in the use of CD40L in the development of new vaccines. Gurunathan et al [195] described a DNA plasmid encoding both CD40L and antigen that induced both cellular and humoral immune responses to that antigen, but found that the CD40L plasmid alone had some therapeutic effect. At the very least it demonstrated that DNA vaccines incorporating CD40L would upregulate immune responses.

Kikuchi et al [172] developed an adenoviral vectored CD40L which, when injected into established B16 and CT26 subcutaneous tumours, lead to 60% tumour-free mice. Adoptive transfer of splenocytes to naïve mice conferred immunity from the relevant tumour. This was maintained whether CD40L expression was by the tumour cells or by dendritic cells [196]. Sun et al observed a similar effect in a CT26 tumour model with an adenoviral vectored CD40L. The immune response was characterised by T_H1 cytokines and required both $CD4^+$ and $CD8^+$ lymphocytes. Dotti et al[180] observed a systemic anti-tumour immune response with CD40-dependent signalling in a CD40+/-

plasmacytoma model. CD40 signalling was through a fibroblast expressing CD40L, but the response was not dependent on expression of CD40 by the tumour. They hypothesised that the response was due to upregulation of costimulatory molecules on professional APC.

The transition of murine studies to clinical trials involving CD40L in tumour vaccination is progressing. Toxicity studies are underway, but what are of more interest are the proposed tumour antigens. The diverse characteristics of tumour antigens have been discussed in chapter 1, and whilst they provide a variety of options to immunologists, careful selection of candidate antigens for immunisation is essential. However, tumour antigens that might attract T_H1 responses are difficult to identify.

Known tumour antigens to murine tumour cell lines can be incorporated into DNA vaccines. These have the advantage that they are specific to that tumour, and can be used in normal sysngeneic mice. The disadvantage is that the antigen will not be of use against human disease. Human tumour antigens can be studied in transgenic mice, but the murine immune response to human antigen may still be atypical.

A dual function DNA vaccine has been developed by Xiang et al [197] that incorporates CEA and CD40L trimer. This confers immunity from CEA-expressing colon tumours in CEA transgenic mice. They demonstrated that T cell tolerance to CEA was broken by this vaccine through *in vivo* tumour rejection, up-regulation of B7.1, B7.2 and ICAM-1 expression by dendritic cells and an increase in CEA-restricted CTL with up-regulation of their activity markers including LFA-1, CD25, CD28 and CD69. Although the majority of colorectal tumours produces CEA, it is mostly found as a serum protein. Such an antigen might be expected to engender a T_H2 immune response of little value against tumour cells expressing low levels of surface CEA.

P53 protein is a strong contender as it accumulates in tumour but not normal cells. Immunisation would be effective against a diverse range of tumours. Dendritic cells transduced with an adenoviral construct incorporating p53 protected 60% of mice against MethA sarcoma [198]. This response was enhanced by CD40 crosslinking, suggesting that incorporating CD40L would improve the efficacy of the vaccine.

A concern with CD40 is that its effects are broad reaching, given its involvement in both T_H1 and T_H2 immune responses. None of the mice receiving anti-CD40 mAb or CT26.CD40L displayed significant side effects but rigorous toxicity studies will be

essential for clinical trials. This is particularly true for vaccines that incorporate self-antigens such as CEA or p53. Although this is a tumour antigen it is expressed at low levels by a wide range of normal tissue, particularly in the gut.

The results comparing anti-CD40 mAb with anti-CD137 mAb in the treatment of CT26 suggest that immunotherapy based on CD137 will be more effective as it provided long term protection against tumour in contrast to merely limiting tumour growth. An advantage of CD137 over CD40 signalling is its narrow sphere of influence compared with CD40, particularly in terms of B cell activity. Immunomodulation through CD137 is better restricted to cellular responses. Bertram et al[199] compared CD28 knock-out (-/-) mice with CD137L(-/-) mice. The CD137(-/-) mice had normal primary CTL and antibody responses to influenza virus, but were deficient in their ability to maintain an effective cellular immune response. Tan et al demonstrated similar results with a lymphocytic choriomeningitis vaccine[200]. Although T cell expansion is limited following CD137 stimulation, armed CTL are protected from AICD giving a relative expansion in the $CD8^+$ population[149]. These CTL are long lived, suggesting that CD137L will be useful in the development of dual-signal vaccines for tumours similar to those developed around CD40L. Another important but little understood role of CD137 is in NK cell responses to tumours. If tumour cells escape CTL following CD137-dependent immunomodulation, they may be dealt with by NK cells[185, 186]. The combination of armed CTL with NK cells would provide comprehensive cellular immunity from cancer.

The first step in the development of such a vaccine might be the transfection of tumour cells to express CD137L in the manner of CT26.CD40L. Although the DNA sequence and primers for CD137L have been described there is little published literature describing such a vaccine. Electroporation is a crude but affective means of achieving CD137L expression but viral transfection or transduction the more popular as it can be used *in vivo* as well as *in vitro*. CD137L transfected tumour cells provide a diverse source of antigen, avoiding the need for a specific tumour antigen as part of the initial vaccine.

The disciplines of genomics and proteomics are identifiying an increasing number of differences between malignant and normal cell, any one of which might provide an epitope against which CTL are effective. Ultimately a vaccine incorporating CD40L

or CD137L will require clinical trial, but this will only be possible when toxicity following immunomodulation with either has been fully described.

Immunomudulation through CD40 and CD137 can result in a powerful and enduring response to tumour antigen that is T_H1 in character and predominantly CTL dependent. However it is unlikely to be clinically useful as a sole agent against carcinoma, as it shows little significant improvement over previous cytokine-based immunomodulation. The full potential of immunomodulation through CD40 and CD137 signalling may lie through their incorporation into vaccines, although their function is yet to be fully described at a molecular level and their toxicity is uncertain.

Appendix 1

The activity of anti-CD40 mAb against tumour cell-bound CD40

Ligation of CD40 expressed by epithelial and tumour cells has been shown to induce cell death directly [201] (discussed in Chapter 4). Furthermore anti-CD40 mAb may recruit natural effector cells such as NK cells or macrophages that then kill cells expressing CD40 by ADCC. Whilst this may be desirable with regard to CD40-expressing tumours such as lymphoma, it may result in collateral damage to normal CD40-expressing tissue. In order to study this possibility a model was devised to confirm that the mechanism of action was not through binding of anti-CD40 mAb to the tumour cells, using murine cells engineered to express human CD40.

To investigate the possibility that anti-CD40 mAb might act on other cells expressing CD40, CT26 cells were transfected with a DNA plasmid encoding human CD40, and those cells with stable expression of the highest levels of human CD40 were selected. Green fluorescence protein, GFP, is an algal protein that fluoresces under ultraviolet light facilitating easy identification of positive colonies. It is excited between 515 and 545 nm and cells expressing GFP can therefore be detected on channel 1 (FL 1) of the FACS. CT26 cells expressing GFP, CT26.GFP were used to study the *in vitro* and *in vivo* growth of transfected cells.

Methods

CT26 tumour cells were grown *in vitro* under optimal conditions and harvested as described in sections 5.2 and 5.3. A plasmid vector encoding complete cDNA for human CD40, or for GFP, and resistance to a selection agent (genetecin) was transfected into these cells (section 5.7) to engineer CT26 cells with surface expression of human CD40, CT26.Hu40, or expression of GFP, CT26.GFP. Colonies were selected by their resistance to geneticin in the selection medium.

Non-transfected cells were incubated with selection medium to confirm that the wild-type cells did not display constitutive resistance to selection medium.

Expression of human CD40 was confirmed by single channel FACS using FITC conjugated anti-human CD40 (Lob 7.6) as described in section 5.5.1. Cells with high levels of surface human CD40 or GFP expression were selected by FACS as described in section 5.8. These cells were grown in selection medium, then standard medium for two months, with regular examination by FACS to monitor levels of

human CD40 or GFP expression. Those colonies that demonstrated stable expression were selected for *in vitro* and *in vivo* use.

Two age and sex-matched BALB/c mice were inoculated with 2×10^6 CT26.GFP or CT26.Hu40 tumour cells into the subcutaneous tissue of their shaved right flanks. When the tumours reached 15 mm in any dimension the mice were culled and the tumour dissected. Cells were extracted by homogenising the tissue through a 70 μm filter into selection medium. Two more mice were inoculated intravenously with 2×10^6 CT26.GFP or CT26.Hu40 tumour cells, and the lungs harvested after 14 days. The lungs were digested with collagenase/hyaluronidase as described in section 5.10.2. The cell suspensions were incubated for 16 hours in selection medium, after which time the tumour cells would be adherent to the bottom of the culture flask. They were then washed from the flasks and re-suspended in PBS. GFP expression was measured by FACS on channel FL1.

The intradermal growth of CT26.HuCD40 was measured in mice that then received either anti-human CD40 mAb or control mAb. 2×10^5 cells were injected into the shaved right flanks of age and sex matched BALB/c mice on day 0, as described in sections 5.2, 5.3 and 5.4.2. They were injected intravenously with mAb on days 2, 4 and 6. The tumour growth was followed by measurement of two dimensions on alternate days.

Although CT26.Hu40 express human CD40, the molecule might not be functional in the murine cells. CD40 signal transduction is dependent on TRAF, which is not necessarily expressed by these cells and not included in the transfection vector. It was essential to establish whether the anti-human CD40 mAb would have a direct effect on CT26.Hu40 tumour cells. To confirm this tritiated thymidine uptake was measured in CT26.Hu40 cells following incubation with anti-human CD40 (LOB 7.6) or control mAb, using the methods described in sections 5.2, 5.3 and 5.9, at a mAb concentration of up to 20 μg/ml.

Results

Stable transfection of CT26 by electroporation with the plasmid to incorporate human CD40 was achieved (figure A1.1). However FACS analysis of CT26.HuCD40 cells re-cultured *in vitro* after *in vivo* growth did not demonstrate continuing surface expression of human CD40. For this reason cells were transfected GFP (figure A1.2).

Although GFP is highly immunogenic FACS can be used to detect it at very low intracellular levels. There was minimal evidence of fluorescent cells after intravenous inoculation of tumour but low levels of fluorescence could be detected in cells harvested from solid subcutaneous tumour, confirming that the method of transfection at least was stable.

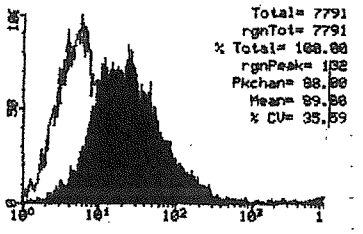
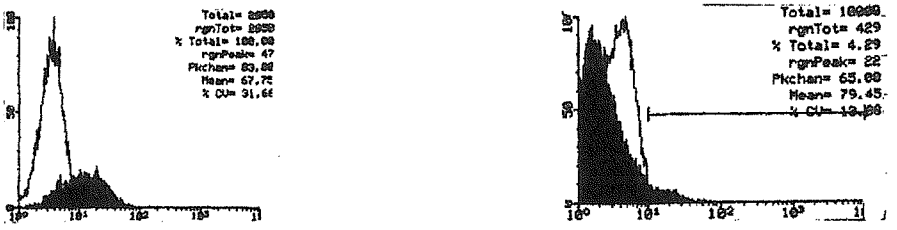


Figure A1.1. FACS histogram (FL1) of CT26.Hu40 (solid histogram) compared with the wild-type CT26 (hollow histogram) after labelling with FITC anti-human CD40 mAb. The X-axis is fluorescence intensity, the Y-axis cell numbers.



A B

Figure A1.2. FACS histograms (FL1) of CT26. The X-axis is fluorescence intensity, the Y-axis cell numbers.
A: CT26.GFP cells (solid histogram) re-cultured in selection medium after *in vivo* tumour growth.
B: FACS histograms (FL1) of homogenised lung cells (solid histogram) from mice receiving intravenous CT26.GFP cells.

Anti-CD40 mAb has no direct effect *in vitro* on the growth of CT26 cells expressing CD40 (figure A1.3). Anti-human CD40 mAb did not inhibit tumour growth in either the iv (pulmonary) or the id models (figures A1.4 and A1.5). This may be due to the survival of only those cells expressing low levels of human CD40, which are then able to grow normally. However the growth of the id tumours and the survival of the mice with pulmonary tumour was no different to that seen with wild-type tumour (chapter

8). Furthermore the lack of therapeutic response seen in the intradermal model suggests that LOB 7.6 is not able to induce ADCC through the human CD40 molecule expressed by CT26.HuCD40.

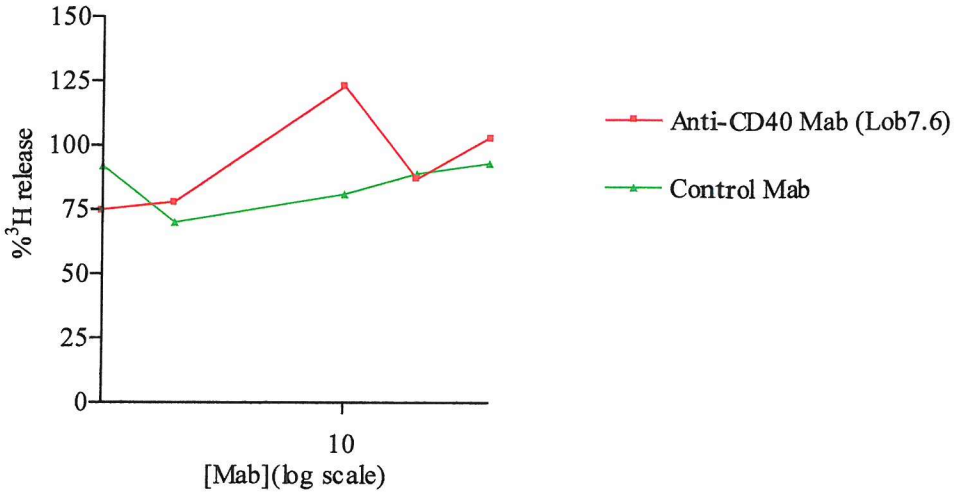


Figure A1.3. Anti-human CD40 mAb (LOB 7.6) does not inhibit the growth of murine tumour cells transfected to express human CD40.The log concentration of mAb is shown on the X-axis. [^{3}H] thymidine release (Y-axis) is proportional to cell growth and is expressed as a percentage of that released by tumour cells cultured without mAb.

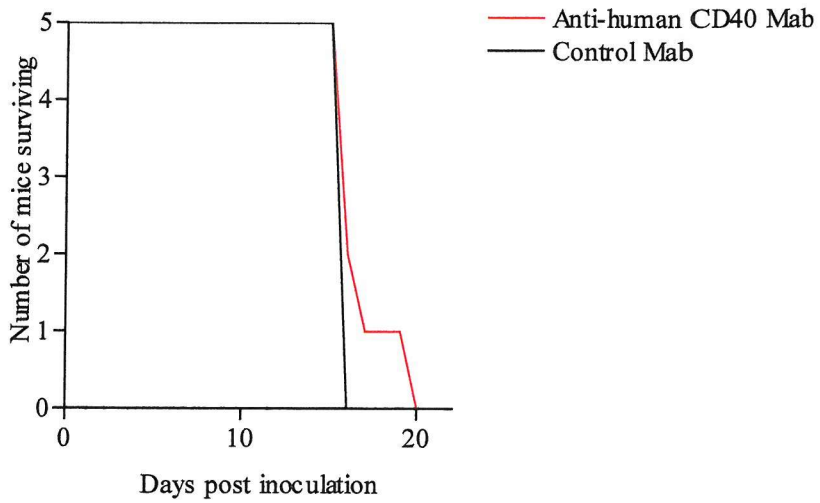


Figure A1.4. Anti-human CD40 mAb therapy of pulmonary CT26.HuCD40 does not offer any survival advantage to CT26.Hu40 bearing mice. The number of mice surviving (Y-axis) is the same for any day after tumour inoculation (X-axis), whether they receive anti-human CD40 mAb or control mAb.

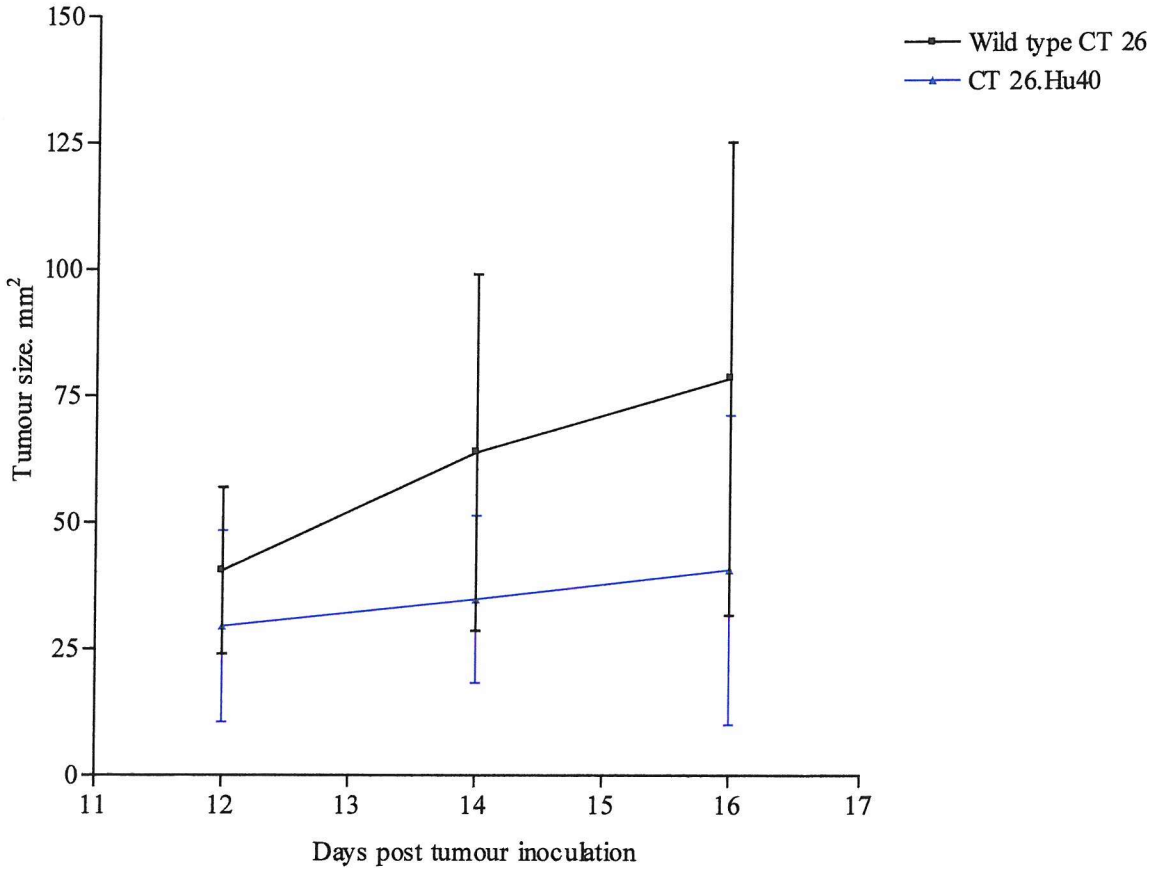


Figure A1.5. Although the growth of id CT26.Hu40 appears to be slightly less than that of the wild-type CT26 after treatment with anti-human CD40 mAb, there is no statistical difference between the two tumours.

These results would suggest that therapy with anti-CD40 mAb will not cause unwanted bystander damage to cells expressing low levels of CD40, such as endothelial cells, through undesirable ADCC.

Appendix 2

Can an effective anti-tumour lymphocyte population be identified *in vitro?*

Anti-CD40 mAb abrogates the growth of CT26 and B16 melanoma tumours. This is dependent on $CD8^+$ cells, but the mechanism of action of these cells and the tumour antigen they target remains unclear. An *in vitro* model was developed from the methods of Donepudi *et al* [202] to further study both the effector cells involved and their target antigens following immunomodulation with anti-CD40 mAb.

Methods

In the first experiments mouse lymphocytes were harvested from the spleen and cultured *in vitro* with antigen in the form of killed tumour cells, and/or anti-CD40 mAb. After five days *in vitro* culture the lymphocytes were incubated with live ^{51}Cr labelled tumour cells to measure their cytotoxicity by ^{51}Cr release.

There were several concerns with this methodology. The immune response to anti-CD40 mAb may require more than five days, even if the immunocytes are taken from animals previously exposed to tumour antigen. Secondly, and perhaps more importantly, the method involved harvesting a large number of naïve APC and effector cells, and the proportion of cells previously exposed to tumour antigen would be small. These lymphocytes would no longer be active, as they had been deprived of much of the normal cytokine environment that they require. Therefore in a second experiment mice were inoculated iv with both tumour cells and anti-CD40 mAb. It was hoped that a cytotoxic response would be demonstrated better if the effectors were fresh from an environment of both tumour antigen and licensed antigen presenting cells. Lymphocytes from the spleen and lungs were harvested and their *in vitro* cytotoxicity measured by ^{51}Cr release following incubation with live ^{51}Cr-labelled tumour cells.

In vitro activation of effector cells with anti-CD40 mAb and cytotoxicity assay

Lymphocytes were prepared from a homogenate of the spleens from tumour-bearing mice as described in section 5.12.2. The homogenate, which included T lymphocytes, B lymphocytes and other potential APC, was incubated with anti-CD40 mAb, control mAb or PBS, with or without growth arrested tumour for five days, giving six groups.

After this time the resulting cell suspension was incubated with ^{51}Cr labelled tumour at three effector(lymphocytes)/target(CT26) ratios: 100:1, 50:1 and 25:1 and the specific chromium release calculated. High ratios were thought to be required, as there was no discrimination between the number of tumour specific effector cells and the total cell population. Tumour killing resulted in ^{51}Cr release by the tumour cells, measured by an automated γ counter.

9.2.2 *In vivo* activation of effector cells with anti-CD40 and cytotoxicity assay

Lymphocytes were prepared from the lungs and spleens of tumour bearing mice as described in section 5.12. Previous results confirmed that the cell numbers from the lungs would be adequate and the kinetics of the T cell response in these models had been established (section 8.1).

Target tumour cells were labelled with ^{51}Cr as described in section 5.12.3. Lymphocytes harvested from the spleen were incubated with ^{51}Cr labelled tumour cells at three effector(lymphocytes)/target(CT26) ratios: 100:1, 50:1 and 25:1, as described above. 5×10^6 pulmonary lymphocytes were incubated for five hours with ^{51}Cr labelled tumour cells at an effector/target ratio of 25/1 in 200µl medium in triplicate and the specific chromium release calculated.

Results

In vitro activation of effector cells with anti-CD40 mAb and cytotoxicity assay

The maximum ^{51}Cr release by labelled CT26 was 2943 (n=6, SEM=147), and background 252 (n=6, SEM=6), confirming reliable uptake and release of the isotope (second of two experiments) by the target cells.

Although this experiment was repeated, each time with viable effectors and consistent chromium uptake and release, there was no evidence that effective cytotoxic cells could be generated by the anti-CD40 mAb (table A2.1). Because of the lack of positive results with this approach and the difficulty in establishing reliable subcutaneous growth of B16F10 without metastatic tumour growth, this method was not repeated for the melanoma tumour cell line.

maximum ^{51}Cr release=2943 (n=6, SEM=147)

background ^{51}Cr release=252 (n=6, SEM=6)

	% chromium release		
	100/1	50/1	25/1
Anti-CD40 mAb+tumour	0.0	0.0	0.0
Control mAb+tumour	0.0	0.0	0.0
PBS+tumour	0.16	0.0	0.0
Anti-CD40 mAb	0.0	0.6	0.0
Control mAb	0.0	0.0	0.0
PBS	0.0	0.0	0.0

Table A2.1 Six groups were studied, and the lymphocytes from each group incubated with ^{51}Cr-labelled target cells at three ratios. However there was no evidence of cytotoxicity by the effector cells, as there was no incresase in the γ radiation count after *in vitro* anti-CD40 mAb compared with controls.

In vivo activation with anti-CD40 mAb

Even though the effector cells were used immediately after preparation from the lungs, there was no evidence of cytotoxicity for any of the four groups studied (tables A2.2-A2.5). The target cells consistently incorporated adequate ^{51}Cr, confirmed by the high maximum γ count compared with the background γ count.

Maximum ^{51}Cr release=3554 (n=6, SEM=243)

Background ^{51}Cr release=399 (n=6, SEM=14)

	% chromium release		
	100/1	50/1	25/1
Anti-CD40 mAb+tumour	0.0	0.0	0.0
Control mAb+tumour	0.0	0.3	0.0
Anti-CD40 mAb	0.0	0.6	0.0
Control mAb	0.0	0.0	0.0

Table A2.2. Splenic lymphocytes from BALB/c mice with pulmonary CT26. There was no increase in the γ radiation count suggesting that these lymphocytes were ineffective against target tumour cells despite *in vivo* anti-CD40 mAb.

Maximum ^{51}Cr release=2100 (n=6, SEM=318)

Background ^{51}Cr release=510 (n=6, SEM=28)

	% chromium release		
	100/1	50/1	25/1
Anti-CD40 mAb+tumour	0.0	0.0	0.25
Control mAb+tumour	0.0	0.0	0.0
Anti-CD40 mAb	0.0	0.0	0.0
Control mAb	0.0	0.0	1.2

Table A2.3. Splenic lymphocytes from C57Bl6 mice with pulmonary B16F10. There was no increase in the γ radiation count suggesting that these lymphocytes were ineffective against target tumour cells despite *in vivo* anti-CD40 mAb.

Maximum ^{51}Cr release=3554 (n=6, SEM=243)

Background ^{51}Cr release=399 (n=6, SEM=14)

	% chromium release
	25/1
Anti-CD40 mAb+tumour	0.0
Control mAb+tumour	0.0
Anti-CD40 mAb	0.0
Control mAb	0.0

Table A2.4. Lung lymphocytes from BALB/c mice with pulmonary CT26. There was no increase in the γ radiation count suggesting that these lymphocytes were ineffective against target tumour cells despite *in vivo* anti-CD40 mAb.

Maximum ^{51}Cr release=1770 (n=6, SEM=128)

Background ^{51}Cr release=450 (n=6, SEM=18)

	% chromium release
	25/1
Anti-CD40 mAb+tumour	0.0
Control mAb+tumour	0.5
Anti-CD40 mAb	2.1
Control mAb	0.0

Table A2.5. Lung lymphocytes from C57Bl6 mice with pulmonary B16F10. There was no increase in the γ radiation count suggesting that these lymphocytes were ineffective against target tumour cells despite *in vivo* anti-CD40 mAb.

There is no satisfactory evidence of a cell mediated cytotoxic mechanism. It was felt that the methodology used was robust as the chromium uptake by the targets was consistent and adequate and the background release was low. The reason for the negative results is likely to be multifactorial. A combination of low effector cell numbers and a weak effector response with lack of sensitivity in the method would mean that no significant release of chromium could be detected. The findings concur with those of Zoller et al [165], who observed heterogeneity in the *in vitro* response to different B16 strains following incubation of the tumour cell with IFN-γ. They observed that although CTL were effective against B16F1 cells, IFN-γ failed to confer a similar effect in B16F10 cells.

Although the anti-CD40 mAb treated group had no visible melanoma in their lungs, the predominant effect could be tumour-inhibitory rather than cytotoxic. Subsequent tumour clearance would be by other cells, for example macrophages. Furthermore the response may require B cells and FACS of the Percol fraction used has shown relatively small numbers of B cells. Most likely is that essential co-factors for the promotion and maintenance of a cytotoxic response are lost during lymphocyte preparation, and these may not be generated during *in-vitro* lymphocyte culture. This has been explored by Todryk et al [188] who demonstrated cytotoxicity in a similar model with the addition of IL-2 to the culture medium (figure A2.1). It has already been discussed that IL-2 alone may induce an immune response to CT26 antigens (section 6.1). Similarly, Maeda et al[203] found that IL-2 was required to elicit CTL-dependent lysis of B16F10 tumour cells *in vitro*.

The use of cytokine enriched culture medium is an added variable and creates a artificial environment, although in the proposed model for CD40 dependent arming of cytotoxic T cells cytokine production is not necessarily dependent on CD40 ligation. Donepudi et al were successful in generating an *in vitro* cytotoxic T cell response that is independent of additional cytokines or other stimuli, but we were unsuccessful in duplicating this in different models.

Other cells critical to the induction of an effective cytotoxic response to tumour cells might be found in a different layer of the Percol gradient. This possibility was

investigated by FACS analysis of the lymphocytes extracted from the percol gradient (figure A2.2) and found to be unlikely as both APC and NK cells were present. Finally, it may be that an entirely different population of effector cells is responsible for the initial CD40-dependent therapeutic response to tumour.

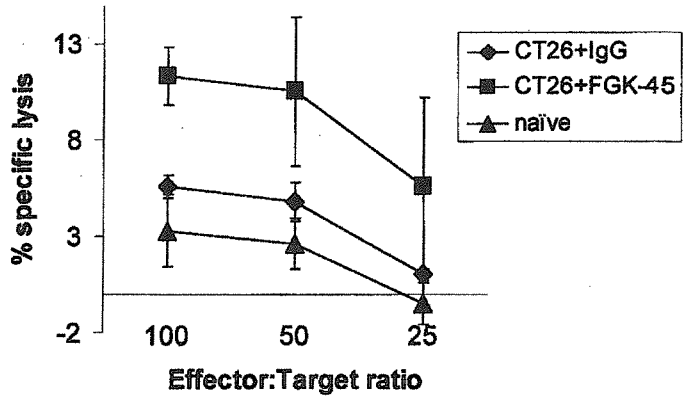


Figure A2.1 (From Todryk et al[188]). Specific chromium release by CT26 tumour cells following incubation with lymphocytes from tumour bearing mice. Cells from mice treated with anti-CD40 mAb (FGK-45) showed a two-fold higher cytotoxicity than mice treated with control mAb. Error bars show one standard deviation.

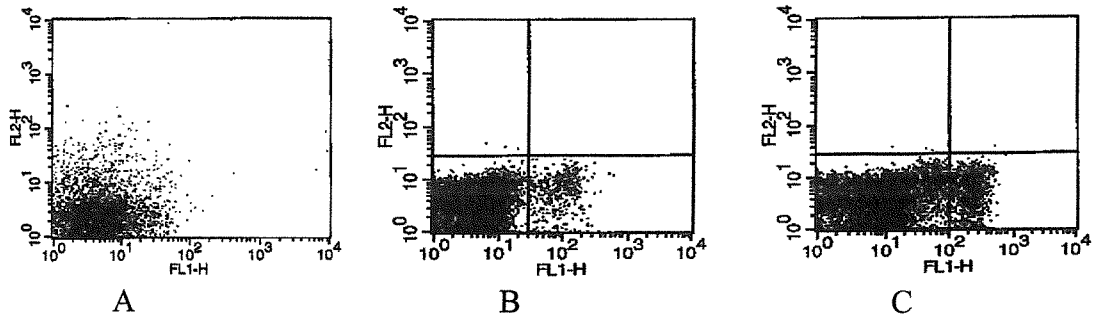


Figure 9.2. A: Control FACS profile of lung cells labelled with irrelevant FITC and PE mAb.
B: FACS of lung cells labelled with the FITC conjugated mAb DX-5, which labels NK cells.
C: FACS of lung cells labelled with FITC conjugated F4-80 mAb, which labels antigen presenting cells including dendritic cells and macrophages.

Appendix 3

Kinetics of the lymphocyte response to anti-CD137 mAb in BALB/c mice

Ten BALB/c mice were injected intravenously with 2×10^6 CT26 cells, as described in section 5.4.1 and divided into two groups A and B. A further two groups of five mice each from the same breeding group were included as healthy mice controls, C and D.

Day 0:	A and B inoculated with tumour.
Day 3	A and C treated with 0.5 mg/day anti-CD40 mAb. (3/23 mAb in sterile PBS)
Day 3	B and D treated with 0.5 mg/day MC106A5 mAb (rat anti-mouse anti-idiotype IgG2a mAb)
Day 6,8,10,12,14:	One mouse from each group was culled and the lungs harvested.

Lymphocytes from the lungs of these mice were prepared as described in section 5.10. The total cell numbers from each organ from each mouse were counted on a Coulter Industrial D cell counter (section 5.3). The lymphocytes from each organ were then resuspended at 5×10^6 cells/ml. 900 µl of lymphocyte suspension was divided between three FACS tubes and labelled with fluorescent mAb as described in section 5.5.2. Each sample of lymphocytes from the lungs and spleen of each mouse was labelled with anti-CD4, anti-CD8 or anti-CD62 PE mAb, and then counter-labelled with anti-CD3 FITC mAb. Negative controls were included for each day for FACS calibration and as a control to allow for the calculation of non-specific binding.

The cells were analysed by FACS. Amplification, detection and compensation were set from a standard calibration. The dot-plot FL1/FL2 was gated (figure 10.2) to calculate the percentage and thus the total number of $CD4^+$, $CD8^+$ cells in each group, and the number of activated cytotoxic T cells (CD62).

Mice with intradermal CT26 tumours that received intraperitoneal anti-CD137 mAb and survived long-term were re-challenged after 1 or 3 months with 5×10^5 CT26 tumour cells in 50 µl DMEM, as described in section 6.3.

Interpretation of the lymphocyte kinetics in the lungs of tumour bearing BALB/c mice is difficult because of inconsistencies in the total cell numbers from the Percol

separation. The total number of lymphocytes increases, and this is mirrored by the increase in number of $CD8^+$ lymphocytes. However the proportion remained unchanged. There was a small, proportional increase in the number of activated $CD8^+$ cells. This modest increase in the $CD8^+$ population may be explained by a reduction in AICD (section 4.9) rather than clonal expansion. The total number of helper T cells remained constant, although the proportion fell. The increase in cells appears to be polyclonal, as for anti-CD40 mAb, with no clear increase in activated, mature cytotoxic T cells.

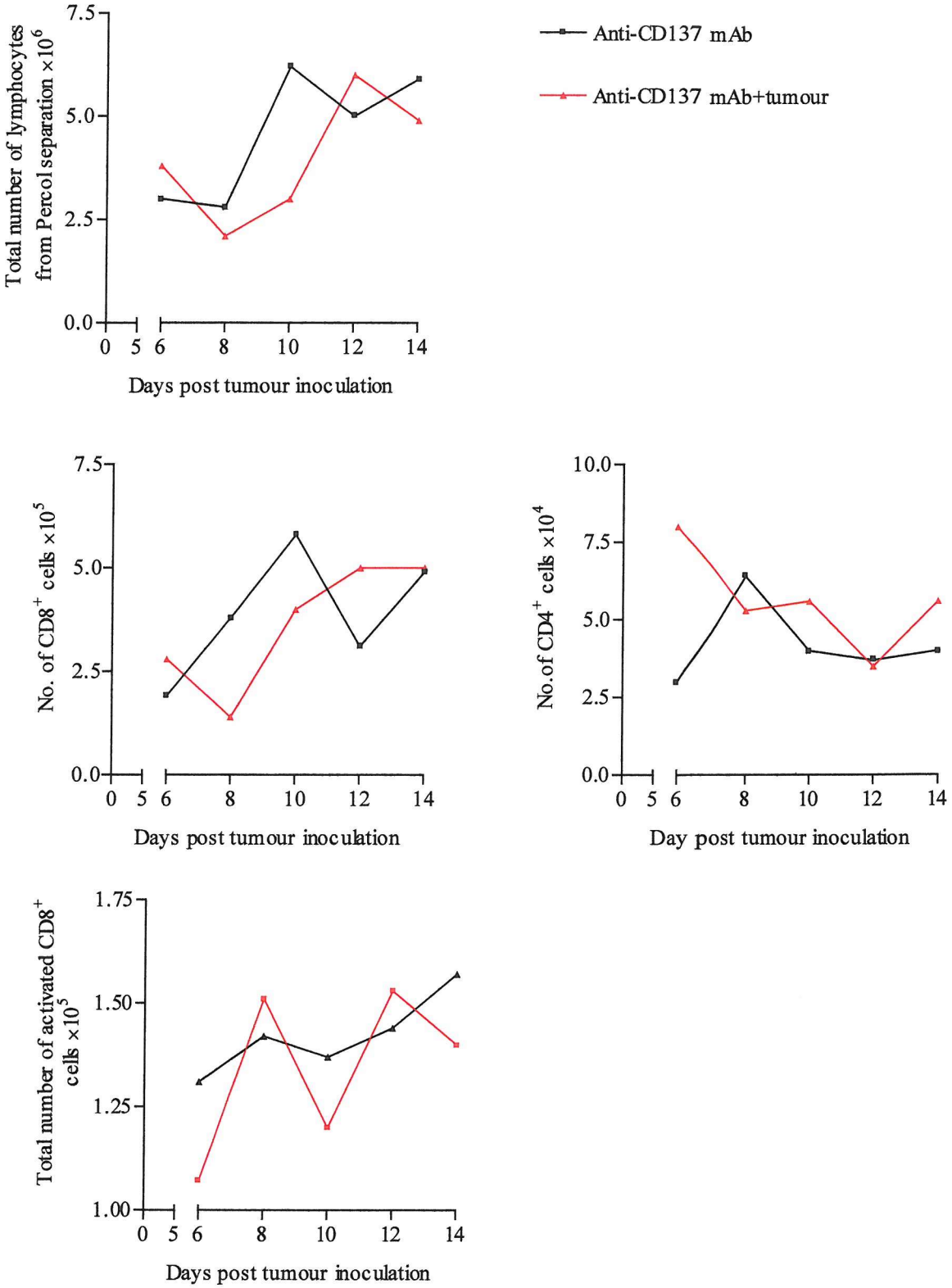


Figure A3.1. The total number of lymphocytes from the lungs of BALB/c mice rises after 8-10 days, and remains elevated beyond the 14th day following ip injection of anti-CD137 mAb. This is unaffected by the presence of CT26. There is a modest rise in the number of CD8$^+$ cells and of activated CD8$^+$ cells. The CD4$^+$ population is unaffected by anti-CD137 mAb.

References

1. Hericourt, J. and C. Richet, *De la serotherapie dasn le traitement du cancer.* C R Hebd Acad Sci, 1895. **120**: p. 567-569.
2. Robins, R.A., *T-cell responses at the host: tumour interface.* Biochim Biophys Acta, 1986. **865**(3): p. 289-305.
3. Riethmuller, G., et al., *Monoclonal antibody therapy for resected Dukes' C colorectal cancer: seven-year outcome of a multicenter randomized trial.* J Clin Oncol, 1998. **16**(5): p. 1788-94.
4. Atkins, M.B., *Immunotherapy and experimental approaches for metastatic melanoma.* Hematol Oncol Clin North Am, 1998. **12**(4): p. 877-902, viii.
5. Sogn, J.A., *Tumor immunology: the glass is half full.* Immunity, 1998. **9**(6): p. 757-63.
6. Montero, E., et al., *Tumor-specific immunotherapy based on dominant models of natural tolerance.* Med Hypotheses, 2000. **54**(4): p. 531-6.
7. Dilworth, S.M., *Cell alterations induced by the large T-antigens of SV40 and polyoma virus.* Semin Cancer Biol, 1990. **1**(6): p. 407-14.
8. Epstein, M.A., Y.M. Barr, and B.G. Achong, *Studies with Burkitt's lymphoma.* Wistar Inst Symp Monogr, 1965. **4**: p. 69-82.
9. Josey, W.E., A.J. Nahmias, and Z.M. Naib, *Viruses and cancer of the lower genital tract.* Cancer, 1976. **38**(1 SUPPL): p. 526-33.
10. Boon T., v.d.B.P., *Human tumour antigens recognised by T lymphocytes.* J. Exp. Med., 1996. **183**: p. 725-729.
11. Kontani, K., et al., *Modulation of MUC1 mucin as an escape mechanism of breast cancer cells from autologous cytotoxic T-lymphocytes.* Br J Cancer, 2001. **84**(9): p. 1258-64.
12. Agrawal, B., M.A. Reddish, and B.M. Longenecker, *In vitro induction of MUC-1 peptide-specific type 1 T lymphocyte and cytotoxic T lymphocyte responses from healthy multiparous donors.* J Immunol, 1996. **157**(5): p. 2089-95.
13. Gillespie, A.M. and R.E. Coleman, *The potential of melanoma antigen expression in cancer therapy.* Cancer Treat Rev, 1999. **25**(4): p. 219-27.
14. Hsu, F.J., et al., *Tumor-specific idiotype vaccines in the treatment of patients with B-cell lymphoma-long termresults of a clinical trial.* Blood, 1997. **89**: p. 3129-3135.
15. Janeway, C. and P. Travers, *Immunobiology : the immune system in health and disease.* 1997, London ; San Francisco: New York : Current Biology Limited ; Garland Pub. Inc. 1 v (various pagings) , ports.
16. Nawrocki, S. and A. Mackiewicz, *Genetically modified tumour vaccines--where we are today.* Cancer Treat Rev, 1999. **25**(1): p. 29-46.
17. Ioannides, C.G. and T.L. Whiteside, *T cell recognition of human tumors: implications for molecular immunotherapy of cancer.* Clin Immunol Immunopathol, 1993. **66**(2): p. 91-106.
18. Holubec, L., Jr., et al., *The significance of CEA, CA19-9 and CA72-4 in the detection of colorectal carcinoma recurrence.* Anticancer Res, 2000. **20**(6D): p. 5237-44.
19. Fossa, S.D., et al., *Prognostic factors in patients progressing after cisplatin-based chemotherapy for malignant non-seminomatous germ cell tumours.* Br J Cancer, 1999. **80**(9): p. 1392-9.

20. Gruss, H.J. and S.K. Dower, *Tumor necrosis factor ligand superfamily: involvement in the pathology of malignant lymphomas.* Blood, 1995. **85**(12): p. 3378-404.
21. Burnett, F.M., *The concept of immunological surveillance.* Prog. Exp. Tumor Res., 1970. **13**: p. 1-27.
22. Kohler, G. and C. Milstein, *Continuous cultures of fused cells secreting antibody of predefined specificity.* Nature, 1975. **256**: p. 495.
23. Grillo-Lopez, A.J., *Rituximab: an insider's historical perspective.* Semin Oncol, 2000. **27**(6 Suppl 12): p. 9-16.
24. Netti, P.A., et al., *Enhancement of fluid filtration across tumor vessels: implication for delivery of macromolecules.* Proc Natl Acad Sci U S A, 1999. **96**(6): p. 3137-42.
25. Baxter, L.T. and R.K. Jain, *Transport of fluid and macromolecules in tumors. IV. A microscopic model of the perivascular distribution.* Microvasc Res, 1991. **41**(2): p. 252-72.
26. Anderson, D.R., et al., *Targeted anticancer therapy using rituximab, a chimeric anti-CD20 antibody (IDEC-C2B8) in the treatment of non-Hodgkin's B-cell lymphoma.* Biochem Soc Trans, 1997. **25**: p. 705.
27. Hahne, M., et al., *Melanoma cell expression of Fas (Apo-1/CD95) ligand: implications for tumour immune escape.* Science, 1996. **274**: p. 1363-1366.
28. Yonehara, S., A. Ishii, and M. Yonehara, *A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor.* J Exp Med, 1989. **169**(5): p. 1747-56.
29. Farah, R.A., et al., *The development of monoclonal antibodies for the therapy of cancer.* Crit Rev Eukaryot Gene Expr, 1998. **8**(3-4): p. 321-56.
30. Glennie, M.J., et al., *Preparation and performance of bispecific F(ab' gamma)2 antibody containing thioether-linked Fab' gamma fragments.* J Immunol, 1987. **139**(7): p. 2367-75.
31. Coley, W., *The treatment of malignant tumours by repeated inoculations of erysipelas: with a report of ten original cases.* Am J Med Sci, 1893. **105**: p. 487-511.
32. Bast, R.C., Jr., et al., *BCG and cancer.* N Engl J Med, 1974. **290**(26): p. 1458-69.
33. Ratliff, T.L., *Role of the immune response in BCG for bladder cancer.* Eur Urol, 1992. **21**(suppl 2): p. 17-21.
34. Decroix, G., et al., *Adjuvant immunotherapy with nonviable Mycobacterium smegmatis in resected primary lung carcinoma. A randomized clinical trial of 219 patients.* Cancer, 1984. **53**(4): p. 906-12.
35. Ben-Efraim, S., *One hundred years of cancer immunotherapy: a critical appraisal.* Tumour Biol, 1999. **20**(1): p. 1-24.
36. Gallucci, S., M. Lolkema, and P. Matzinger, *Natural adjuvants: endogenous activators of dendritic cells.* Nature Medicine, 1999. **5**(11): p. 1249-1255.
37. Schultze, J.L., et al., *Autologous tumor infiltrating T cells cytotoxic for follicular lymphoma cells can be expanded in vitro.* Blood, 1997. **89**(10): p. 3806-16.
38. Tsavaris, N., et al., *The prognostic significance of immune changes in patients with renal cancer and melanoma treated with interferon-alpha 2b.* Tumour Biol, 1995. **16**(6): p. 365-73.

39. Byhardt, R.W., et al., *Recombinant human interferon-beta (rHuIFN-beta) and radiation therapy for inoperable non-small cell lung cancer.* J Interferon Cytokine Res, 1996. **16**(11): p. 891-902.
40. Andreesen, R., et al., *Adoptive transfer of tumor cytotoxic macrophages generated in vitro from circulating blood monocytes: a new approach to cancer immunotherapy.* Cancer Res, 1990. **50**(23): p. 7450-6.
41. Hennemann, B., et al., *Intrahepatic adoptive immunotherapy with autologous tumorcytotoxic macrophages in patients with cancer.* J Immunother Emphasis Tumor Immunol, 1995. **18**(1): p. 19-27.
42. Dalgleish, A.G. and M.J. Browning, *Tumor immunology : immunotherapy and cancer vaccines*. Cancer, clinical science in practice. 1996, Cambridge ; New York, NY, USA: Cambridge University Press. xiv, 355.
43. Mitchell, M.S., et al., *Active specific immunotherapy for melanoma: phase I trial of allogeneic lysates and a novel adjuvant.* Cancer Res, 1988. **48**(20): p. 5883-93.
44. Wallack, M.K., S.D. Scoggin, and M. Sivanandham, *Active specific immunotherapy with vaccinia melanoma oncolysate.* Mt Sinai J Med, 1992. **59**(3): p. 227-33.
45. Marchand, M., et al., *Tumor regression responses in melanoma patients treated with a peptide encoded by gene MAGE-3.* Int J Cancer, 1995. **63**: p. 883.
46. Celluzi, C.M., et al., *Peptide pulsed dendritic cells induce antigen-specific CTL mediated protective tumor immunity.* J Exp Med, 1996. **183**: p. 283.
47. Yeh, K.-Y., et al., *B7.1 enhances natural killer cell-mediated cytotoxicity and inhibits tumour growth of a poorly immunogenic murine carcinoma.* Cell Immunol, 1995. **165**: p. 217.
48. Mullis, K., et al., *Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction.* Cold Spring Harb Symp Quant Biol, 1986. **51 Pt 1**: p. 263-73.
49. Hellstrom, K.E., et al., *On the role of costimulation in tumor immunity.* Ann N Y Acad Sci, 1993. **690**: p. 225-30.
50. Magarian-Blander, J., N. Domenech, and O.J. Finn, *Specific and effective T-cell recognition of cells transfected with a truncated human mucin cDNA.* Ann N Y Acad Sci, 1993. **690**: p. 231-43.
51. DeLeo, A.B., *p53-based immunotherapy of cancer.* Crit Rev Immunol, 1998. **18**(1-2): p. 29-35.
52. Svane, I.M., M. Boesen, and A.M. Engel, *The role of cytotoxic T-lymphocytes in the prevention and immune surveillance of tumors--lessons from normal and immunodeficient mice.* Med Oncol, 1999. **16**(4): p. 223-38.
53. Chaux, P., et al., *Tumour infiltrating dendritic cells are defective in their antigen presenting function and inducible B7 expression in rats.* Int J Cancer, 1997. **72**(4): p. 619-624.
54. Lord, E.M. and J.G. Frelinger, *Tumour immunotherapy: cytokines and antigen presentation.* Cancer Immunol Immunother, 1998. **46**: p. 75-81.
55. Breivik, J. and G. Gaudernack, *Genomic instability, DNA methylation, and natural selection in colorectal carcinogenesis.* Semin Cancer Biol, 1999. **9**(4): p. 245-54.
56. Rivoltini, L., et al., *Quantitative correlation between HLA class I allele expression and recognition of melanoma cells by antigen specific cytotoxic T lymphocytes.* Cancer Res, 1995. **55**(14): p. 3149-3157.

57. Mizoguchi, H., et al., *Alteration of signal transduction molecules in T lymphocytes from tumour bearing mice.* Science, 1992. **258**: p. 1795-1798.
58. Petersson, M., et al., *Constitutive IL-10 production accounts for the high NK sensitivity, low MHC class I expression, and poor transporter associated with antigen processing (TAP)-1/2 function in the prototype NK target YAC-1.* J. Immunol, 1998. **161**: p. 2099-2105.
59. Lamhamedi-Cherradi, S.E. and Y. Chen, *Fas (CD95, Apo-1) ligand gene transfer.* J Clin Immunol, 2001. **21**(1): p. 24-9.
60. Lala, P.K., *Significance of nitric oxide in carcinogenesis, tumor progression and cancer therapy.* Cancer Metastsis Rev., 1998. **17**: p. 1-6.
61. Dong, Z., et al., *Macrophage derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma.* Cell, 1997. **88**: p. 801-810.
62. Newell, D.R., et al., *Evaluation of rodent-only toxicology for early clinical trials with novel cancer therapeutics.* British Journal of Cancer, 1999. **81**(5): p. 760-768.
63. Resor, L., T.J. Bowen, and A. Wynshaw-Boris, *Unraveling human cancer in the mouse: recent refinements to modeling and analysis.* Hum Mol Genet, 2001. **10**(7): p. 669-75.
64. Becker, Y., *Endogenous retroviruses in the human genome--a point of view.* Virus Genes, 1995. **9**(3): p. 211-8.
65. North, R.J., *Gamma-irradiation facilitates the expression of adoptive immunity against established tumors by eliminating suppressor T cells.* Cancer Immunol Immunother, 1984. **16**(3): p. 175-81.
66. Behring, E.K., S., *Untersuchungen ueber das Zustandekommen Diptherie-Immunitat und der Tetanus-Immunitat bei Thieren.* Med Wochenschr, 1890. **16**: p. 1145-1148.
67. Tiselius, A.K., E., *An electrophoretic study of immune sera and purified antibody preparations.* J Exp Med, 1939. **69**: p. 119-131.
68. *The 1972 Nobel Prize in Physiology or Medicine.* 1972, Karolinska Institutet: www.nobel.se/medicine/laureates/1972/press.html.
69. Bentley, G.A. and R.A. Mariuzza, *The structure of the T cell antigen receptor.* Annu Rev Immunol, 1996. **14**: p. 563-90.
70. Accolla, R.S., et al., *The molecular biology of MHC genes.* Immunol Today, 1991. **12**(4): p. 97-9.
71. Colombo, M.P. and G. Forni, *Immunotherapy I: cytokine gene transfer strategies.* Cancer Metastasis Rev, 1997. **16**: p. 412-432.
72. Ren, Y. and J. Savill, *Apoptosis: the importance of being eaten.* Cell Death Diff., 1998. **5**: p. 563-568.
73. Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of immunity.* Nature, 1998. **392**(6673): p. 245-52.
74. Moore, M.W., F.R. Carbone, and M.J. Bevan, *Introduction of soluble protein into the class I pathway of antigen processing and presentation.* Cell, 1988. **54**: p. 777-785.
75. Ellis, R.J., *The molecular chaperone concept.* Semin Cell Biol, 1990. **1**(1): p. 1-9.
76. Schedl, P., et al., *Two hybrid plasmids with D. melanogaster DNA sequences complementary to mRNA coding for the major heat shock protein.* Cell, 1978. **14**(4): p. 921-9.

77. Blachere, N.E., et al., *Heat shock protein-peptide complexes, reconstituted in-vivo, elicit peptide-specific cytotoxic T lymphocyte response and tumour immunity.* J Exp Med, 1997. **186**: p. 1315.
78. Schoenberger, S.P., et al., *Cross-priming of CTL responses does not require antigenic peptides in the endoplasmic reticulum of immunising cells.* Journal of Immunology, 1998. **161**: p. 3808-3812.
79. Stingl, G., et al., *Epidermal Langerhans cells bear Fc and C3 receptors.* Nature, 1977. **268**(5617): p. 245-6.
80. Rowden, G., M.G. Lewis, and A.K. Sullivan, *Ia antigen expression on human epidermal Langerhans cells.* Nature, 1977. **268**(5617): p. 247-8.
81. Surman, D.R., et al., *CD4+ T cell control of CD8+ T cell reactivity to a model tumour antigen.* Journal of Immunology, 2000. **164**: p. 562-565.
82. Rissoan, M.C., et al., *Reciprocal control of T helper cell and dendritic cell differentiation [see comments].* Science, 1999. **283**(5405): p. 1183-6.
83. Shimizu, J., S. Yamazaki, and S. Sakaguchi, *Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity.* J Immunol, 1999. **163**(10): p. 5211-8.
84. Lanzavecchia, A., *Licence to kill.* Nature, 1998. **393**(6684): p. 413-4.
85. Bennett, S.R., et al., *Help for cytotoxic-T-cell responses is mediated by CD40 signalling.* Nature, 1998. **393**(6684): p. 478-80.
86. Ridge, J.P., F. Di Rosa, and P. Matzinger, *A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell.* Nature, 1998. **393**(6684): p. 474-8.
87. Bevan, M.J., *Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay.* J Exp Med, 1976. **143**(5): p. 1283-8.
88. Ronchetti, A., et al., *Role of antigen-presenting cells in cross-priming of cytotoxic T lymphocytes by apoptotic cells.* J Leukoc Biol, 1999. **66**(2): p. 247-51.
89. Heath, W.R., et al., *Cross-tolerance: a pathway for inducing tolerance to peripheral tissue antigens.* J. Exp. Med., 1998. **187**: p. 1549.
90. Lethe, B., et al., *MAGE-1 expression threshold for the lysis of melanoma cell lines by a specific cytotoxic T lymphocyte.* Melanoma Res, 1997. **7**: p. 83-88.
91. Smith, C.A., T. Farrah, and R.G. Goodwin, *The TNF receptor superfamily of cellular and viral proteins: activation, costimulation and death.* Cell, 1994. **76**: p. 959-962.
92. Beiske, K., et al., *Triggering of neoplastic B cells via surface IgM and the cell surface antigens CD20 and CDw40. Responses differ from normal blood B cells and are restricted to certain morphologic subsets.* Int J Cancer, 1988. **42**(4): p. 521-8.
93. Stamenkovic, I., E.A. Clark, and B. Seed, *A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas.* Embo J, 1989. **8**(5): p. 1403-10.
94. Paulie, S., et al., *The human B lymphocyte and carcinoma antigen, CDw40, is a phosphoprotein involved in growth signal transduction.* J Immunol, 1989. **142**(2): p. 590-5.
95. Armitage, R.J., et al., *Identification of a source of biologically active CD40 ligand.* Eur J Immunol, 1992. **22**(8): p. 2071-6.

96. Hollenbaugh, D., et al., *The human T cell antigen gp39, a member of the TNF gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity.* Embo J, 1992. **11**(12): p. 4313-21.
97. Armitage, R.J., et al., *Molecular and biological characterization of a murine ligand for CD40.* Nature, 1992. **357**(6373): p. 80-2.
98. Fanslow, W.C., et al., *Structural characteristics of CD40 ligand that determine biological function.* Semin Immunol, 1994. **6**(5): p. 267-78.
99. Bajorath, J., et al., *Analysis of gp39/CD40 interactions using molecular models and site-directed mutagenesis.* Biochemistry, 1995. **34**(31): p. 9884-92.
100. Pietravalle, F., et al., *Human native soluble CD40L is a biologically active trimer, processed inside microsomes.* J Biol Chem, 1996. **271**(11): p. 5965-7.
101. Pinchuk, L.M., et al., *Functional CD40 ligand expressed by human blood dendritic cells is up-regulated by CD40 ligation.* J Immunol, 1996. **157**(10): p. 4363-70.
102. Carbone, A., et al., *The expression of CD26 and CD40 ligand is mutually exclusive in human T-cell non-Hodgkin's lymphomas/leukemias.* Blood, 1995. **86**(12): p. 4617-26.
103. Hancock, W.W., et al., *Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection.* Proc Natl Acad Sci U S A, 1996. **93**(24): p. 13967-72.
104. Casamayor-Palleja, M., M. Khan, and I.C. MacLennan, *A subset of CD4+ memory T cells contains preformed CD40 ligand that is rapidly but transiently expressed on their surface after activation through the T cell receptor complex.* J Exp Med, 1995. **181**(4): p. 1293-301.
105. Bjorck, P., S. Braesch-Andersen, and S. Paulie, *Antibodies to distinct epitopes on the CD40 molecule co-operate in stimulation and can be used for the detection of soluble CD40.* Immunology, 1994. **83**(3): p. 430-7.
106. Biancone, L., et al., *Expression of inducible lymphocyte costimulatory molecules in human renal allograft.* Nephrol Dial Transplant, 1998. **13**(3): p. 716-22.
107. Brenner, B., et al., *Evidence for a novel function of the CD40 ligand as a signalling molecule in T-lymphocytes.* FEBS Lett, 1997. **417**(3): p. 301-6.
108. Borrow, P., et al., *CD40L-deficient mice show deficits in antiviral immunity and have an impaired memory CD8+ CTL response.* J Exp Med, 1996. **183**(5): p. 2129-42.
109. van Kooten, C. and J. Banchereau, *CD40-CD40 ligand.* J Leukoc Biol, 2000. **67**(1): p. 2-17.
110. Berberich, I., G.L. Shu, and E.A. Clark, *Cross-linking CD40 on B cells rapidly activates nuclear factor-kappa B.* J Immunol, 1994. **153**(10): p. 4357-66.
111. Baker, S.J. and E.P. Reddy, *Modulation of life and death by the TNF receptor superfamily.* Oncogene, 1998. **17**(25): p. 3261-70.
112. Fuleihan, R., et al., *Localization of the genetic defect in X-linked immunoglobulin deficiency with normal or elevated IgM (HIGMX-1) to the CD40 ligand gene.* Trans Assoc Am Physicians, 1993. **106**: p. 91-5.
113. Aruffo, A., et al., *The molecular basis of X-linked agammaglobulinemia, hyper-IgM syndrome, and severe combined immunodeficiency in humans.* Curr Opin Hematol, 1994. **1**(1): p. 12-8.

114. Maliszewski, C.R., et al., *Recombinant CD40 ligand stimulation of murine B cell growth and differentiation: cooperative effects of cytokines.* Eur J Immunol, 1993. **23**(5): p. 1044-9.
115. Garrone, P., et al., *Fas ligation induces apoptosis of CD40-activated human B lymphocytes.* J Exp Med, 1995. **182**(5): p. 1265-73.
116. Grewal, I.S. and R.A. Flavell, *The role of CD40 ligand in costimulation and T-cell activation.* Immunol Rev, 1996. **153**: p. 85-106.
117. Pammer, J., et al., *CD40 antigen is expressed by endothelial cells and tumour cells in Kaposi's sarcoma.* Am. J. Pathol, 1996. **148**: p. 1387-1396.
118. Caux, C., et al., *Activation of human dendritic cells through CD40 cross-linking.* J Exp Med, 1994. **180**(4): p. 1263-72.
119. Kelsall, B.L., et al., *Interleukin-12 production by dendritic cells. The role of CD40-CD40L interactions in Th1 T-cell responses.* Ann N Y Acad Sci, 1996. **795**: p. 116-26.
120. Van Gool, S.W., et al., *CD80, CD86 and CD40 provide accessory signals in a multiple-step T-cell activation model.* Immunol Rev, 1996. **153**: p. 47-83.
121. Sinkovics, J.G. and J.C. Horvath, *Vaccination against human cancers (review).* Int J Oncol, 2000. **16**(1): p. 81-96.
122. Costello, R.T., J.A. Gastaut, and D. Olive, *What is the real role of CD40 in cancer immunotherapy?* Immunol Today, 1999. **20**(11): p. 488-93.
123. French, R.R., et al., *CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help.* Nat Med, 1999. **5**(5): p. 548-53.
124. Ihata, A., et al., *Immunomodulatory effect of a plasmid expressing CD40 ligand on DNA vaccination against human immunodeficiency virus type-1.* Immunology, 1999. **98**(3): p. 436-42.
125. Vanham, G., et al., *Decreased CD40 ligand induction in CD4 T cells and dysregulated IL-12 production during HIV infection.* Clin Exp Immunol, 1999. **117**(2): p. 335-42.
126. Wolthers, K.C., et al., *Functional B cell abnormalities in HIV type 1 infection: role of CD40L and CD70.* AIDS Res Hum Retroviruses, 1997. **13**(12): p. 1023-9.
127. Biancone, L., et al., *Activation of CD40 favors the growth and vascularization of Kaposi's sarcoma.* J Immunol, 1999. **163**(11): p. 6201-8.
128. Noelle, R.J., et al., *CD40 and its ligand in autoimmunity.* Ann N Y Acad Sci, 1997. **815**: p. 384-91.
129. Durie, F.H., et al., *Prevention of collagen-induced arthritis with an antibody to gp39, the ligand for CD40.* Science, 1993. **261**(5126): p. 1328-30.
130. Datta, S.K., *Production of pathogenic antibodies: cognate interactions between autoimmune T and B cells.* Lupus, 1998. **7**(9): p. 591-6.
131. Banu, N., Y. Zhang, and C.M. Meyers, *Immune reactivity following CD40L blockade: role in autoimmune glomerulonephritis in susceptible recipients.* Autoimmunity, 1999. **30**(1): p. 21-33.
132. Balasa, B., et al., *CD40 ligand-CD40 interactions are necessary for the initiation of insulitis and diabetes in nonobese diabetic mice.* J Immunol, 1997. **159**(9): p. 4620-7.
133. Koshy, M., D. Berger, and M.K. Crow, *Increased expression of CD40 ligand on systemic lupus erythematosus lymphocytes.* J Clin Invest, 1996. **98**(3): p. 826-37.

134. Balashov, K.E., et al., *Increased interleukin 12 production in progressive multiple sclerosis: induction by activated CD4+ T cells via CD40 ligand.* Proc Natl Acad Sci U S A, 1997. **94**(2): p. 599-603.
135. Mach, F., U. Schonbeck, and P. Libby, *CD40 signaling in vascular cells: a key role in atherosclerosis?* Atherosclerosis, 1998. **137 Suppl**: p. S89-95.
136. Agostini, C., et al., *Expression of tumor necrosis factor-receptor superfamily members by lung T lymphocytes in interstitial lung disease.* Am J Respir Crit Care Med, 1996. **153**(4 Pt 1): p. 1359-67.
137. Goodwin, R.G., et al., *Molecular cloning of a ligand for the inducible T cell gene 4-1BB: a member of an emerging family of cytokines with homology to tumor necrosis factor.* Eur J Immunol, 1993. **23**(10): p. 2631-41.
138. Vinay, D.S. and B.S. Kwon, *Role of 4-1BB in immune responses.* Semin Immunol, 1998. **10**(6): p. 481-9.
139. Pollok, K.E., et al., *Inducible T cell antigen 4-1BB. Analysis of expression and function.* J Immunol, 1993. **150**(3): p. 771-81.
140. Pollok, K.E., S.H. Kim, and B.S. Kwon, *Regulation of 4-1BB expression by cell-cell interactions and the cytokines, interleukin-2 and interleukin-4.* Eur J Immunol, 1995. **25**(2): p. 488-94.
141. Schwarz, H., K. Arden, and M. Lotz, *CD137, a member of the tumor necrosis factor receptor family, is located on chromosome 1p36, in a cluster of related genes, and colocalizes with several malignancies.* Biochem Biophys Res Commun, 1997. **235**(3): p. 699-703.
142. Saoulli, K., et al., *CD28-independent, TRAF2-dependent costimulation of resting T cells by 4-1BB ligand.* J Exp Med, 1998. **187**(11): p. 1849-62.
143. Melero, I., et al., *Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors.* Nat Med, 1997. **3**(6): p. 682-5.
144. Vinay, D.S. and B.S. Kwon, *Differential expression and costimulatory effect of 4-1BB (CD137) and CD28 molecules on cytokine-induced murine CD8(+) Tc1 and Tc2 cells.* Cell Immunol, 1999. **192**(1): p. 63-71.
145. Shuford, W.W., et al., *4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses.* J Exp Med, 1997. **186**(1): p. 47-55.
146. Collette, Y., et al., *Distinct regulation of T-cell death by CD28 depending on both its aggregation and T-cell receptor triggering: a role for Fas-FasL.* Blood, 1998. **92**(4): p. 1350-63.
147. Takahashi, C., R.S. Mittler, and A.T. Vella, *Cutting edge: 4-1BB is a bona fide CD8 T cell survival signal.* J Immunol, 1999. **162**(9): p. 5037-40.
148. Tan, J.T., et al., *4-1BB ligand, a member of the TNF family, is important for the generation of antiviral CD8 T cell responses.* J Immunol, 1999. **163**(9): p. 4859-68.
149. Michel, J. and H. Schwarz, *Expression of soluble CD137 correlates with activation-induced cell death of lymphocytes.* Cytokine, 2000. **12**(6): p. 742-6.
150. Tzeng, J.J. and R.F. Barth, *Sensitivity of B16 melanoma sublines to lymphokine-activated killer cells as determined by 51Cr-release and clonogenic assays.* J Immunol Methods, 1990. **128**(2): p. 257-66.
151. Andreason, G.L. and G.A. Evans, *Introduction and expression of DNA molecules in eukaryotic cells by electroporation.* Biotechniques, 1988. **6**(7): p. 650-60.
152. Andreason, G.L. and G.A. Evans, *Optimization of electroporation for transfection of mammalian cell lines.* Anal Biochem, 1989. **180**(2): p. 269-75.

153. Sato, N., M.C. Michaelides, and M.K. Wallack, *Characterization of tumorigenicity, mortality, metastasis, and splenomegaly of two cultured murine colon lines.* Cancer Res, 1981. **41**(6): p. 2267-72.
154. Colmerauer, M.E., J.A. Koziol, and Y.H. Pilch, *Enhancement of metastasis development by BCG immunotherapy.* J Surg Oncol, 1980. **15**(3): p. 235-41.
155. Fukushima, M., et al., *Immunotherapy of a murine colon cancer with syngeneic spleen cells, immune RNA and tumor antigen.* Int J Cancer, 1982. **29**(1): p. 107-12.
156. Schackert, H.K., et al., *Systemic immunity against a murine colon tumor (CT-26) produced by immunization with syngeneic cells expressing a transfected viral gene product.* Int J Cancer, 1989. **43**(5): p. 823-7.
157. Rodolfo, M., et al., *Adoptive immunotherapy of a mouse colon carcinoma with recombinant interleukin-2 alone or combined with lymphokine-activated killer cells or tumor-immune lymphocytes. Survival benefit of adjuvant post-surgical treatments and comparison with experimental metastases model.* Cancer Immunol Immunother, 1990. **31**(1): p. 28-36.
158. Fujiwara, T., et al., *Application of an interleukin 2 slow delivery system to the immunotherapy of established murine colon 26 adenocarcinoma liver metastases.* Cancer Res, 1990. **50**(21): p. 7003-7.
159. Rabau, M., et al., *Inhibition of CT-26 murine adenocarcinoma growth in the rectum of mice treated with recombinant human interleukin-6.* J Immunother Emphasis Tumor Immunol, 1994. **15**(4): p. 257-64.
160. Itsuki, Y. and S. Suzuki, *Expression of interleukin-4 in colon 26 cells induces both eosinophil mediated local tumor killing and T-cell mediated systemic immunity in vivo.* Nippon Ika Daigaku Zasshi, 1996. **63**(4): p. 275-85.
161. Okamoto, M., et al., *The combined effect against colon-26 cells of heat treatment and immunization with heat treated colon-26 tumour cell extract.* Int J Hyperthermia, 2000. **16**(3): p. 263-73.
162. Rappaport, I., et al., *Syngeneic monoclonal antibodies to B16 melanoma viral antigens.* Cancer Res, 1987. **47**(20): p. 5391-6.
163. Katz, A., et al., *Abrogation of B16 melanoma metastases by long-term low-dose interleukin-6 therapy.* J Immunother, 1993. **13**(2): p. 98-109.
164. Stackpole, C.W. and A. Demsey, *Multiple antigens related to the major envelope glycoprotein of murine leukemia virus expressed on B16 melanoma cells as targets of host immune response.* Invasion Metastasis, 1984. **4**(1): p. 28-46.
165. Zoller, M., *IFN-treatment of B16-F1 versus B16-F10: relative impact on non-adaptive and T-cell-mediated immune defense in metastatic spread.* Clin Exp Metastasis, 1988. **6**(5): p. 411-29.
166. Arca, M.J., et al., *Diverse manifestations of tumorigenicity and immunogenicity displayed by the poorly immunogenic B16-BL6 melanoma transduced with cytokine genes.* Cancer Immunol Immunother, 1996. **42**(4): p. 237-45.
167. Harada, M., et al., *Characterization of B16 melanoma-specific cytotoxic T lymphocytes.* Cancer Immunol Immunother, 1998. **47**(4): p. 198-204.
168. Bloom, M.B., et al., *Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma.* J Exp Med, 1997. **185**(3): p. 453-9.
169. van Elsas, A., A.A. Hurwitz, and J.P. Allison, *Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-*

producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. J Exp Med, 1999. **190**(3): p. 355-66.

170. Santini, R., et al., *Increasing antigenicity of B16 melanoma cell fraction with microwave hyperthermia.* J Microw Power Electromagn Energy, 1986. **21**(1): p. 41-4.
171. Hirano, A., et al., *Inhibition of human breast carcinoma growth by a soluble recombinant human CD40 ligand.* Blood, 1999. **93**(9): p. 2999-3007.
172. Kikuchi, T. and R.G. Crystal, *Anti-tumor immunity induced by in vivo adenovirus vector-mediated expression of CD40 ligand in tumor cells.* Hum Gene Ther, 1999. **10**(8): p. 1375-87.
173. Weber, S.M., et al., *Interleukin-12 gene transfer results in CD8-dependent regression of murine CT26 liver tumors.* Ann Surg Oncol, 1999. **6**(2): p. 186-94.
174. Bash, J.A., *Recombinant vaccinia interleukin-2-infected tumor cell vaccines in immunotherapy of murine colon adenocarcinoma.* J Immunother, 1993. **14**(4): p. 269-72.
175. Schwaab T, W.J., Schned AR, Barth RJ Jr., *Dendritic cell infiltration in colon cancer.* J Immunother, 2001. **24**(2): p. 130-7.
176. Harada, M., et al., *Role of the endogenous production of interleukin 12 in immunotherapy.* Cancer Res, 1998. **58**(14): p. 3073-7.
177. Banchereau, J., et al., *Functional CD40 antigen on B cells, dendritic cells and fibroblasts.* Adv Exp Med Biol, 1995. **378**: p. 79-83.
178. Bohm, W., et al., *T cell-mediated, IFN-gamma-facilitated rejection of murine B16 melanomas.* J Immunol, 1998. **161**(2): p. 897-908.
179. Batrla R, L.M., Rudy W, Stumm S, Wallwiener D, Guckel B., *CD40-expressing carcinoma cells induce down-regulation of CD40 ligand (CD154) and impair T-cell functions.* Cancer Res, 2002. **62**(7): p. 2052-7.
180. Dotti G, S.B., Yotnda P, Rill D, Brenner MK., *Transgenic expression of CD40 ligand produces an in vivo antitumor immune response against both CD40(+) and CD40(-) plasmacytoma cells.* Blood, 2002. **100**(1): p. 200-7.
181. Kelly SA, G.S., East N, Balkwill FR., *Enhancement of metastatic potential by gamma-interferon.* Cancer Res, 1991. **51**(15): p. 4020-7.
182. Martin-Fontecha, A., et al., *Triggering of murine NK cells by CD40 and CD86 (B7-2).* J Immunol, 1999. **162**(10): p. 5910-6.
183. May KF Jr, C.L., Zheng P, Liu Y., *Anti-4-1BB monoclonal antibody enhances rejection of large tumor burden by promoting survival but not clonal expansion of tumor-specific CD8+ T cells.* Cancer Res, 2002. **62**(12): p. 3459-65.
184. Ye Z, H.I., Hayden-Ledbetter M, Dahlin A, Ledbetter JA, Hellstrom KE., *Gene therapy for cancer using single-chain Fv fragments specific for 4-1BB.* Nat Med, 2002. **8**(4): p. 333-4.
185. Melero, I., et al., *NK1.1 cells express 4-1BB (CDw137) costimulatory molecule and are required for tumor immunity elicited by anti-4-1BB monoclonal antibodies.* Cell Immunol, 1998. **190**(2): p. 167-72.
186. Chen, S.H., et al., *Rejection of disseminated metastases of colon carcinoma by synergism of IL-12 gene therapy and 4-1BB costimulation.* Mol Ther, 2000. **2**(1): p. 39-46.

187. Kwon BS, H.J., Lee ZH, Kwack KB, Seo SK, Choi BK, Koller BH, Wolisi G, and V.D. Broxmeyer HE, *Immune responses in 4-1BB (CD137)-deficient mice.* J Immunol, 2002. **186**(11): p. 5483-90.
188. Todryk, S.M., et al., *CD40 ligation for immunotherapy of solid tumours.* J Immunol Methods, 2001. **248**(1-2): p. 139-47.
189. Carbone, E., et al., *A new mechanism of NK cell cytotoxicity activation: the CD40-CD40 ligand interaction.* J Exp Med, 1997. **185**(12): p. 2053-60.
190. Turner, J.G., et al., *Anti-CD40 antibody induces antitumor and antimetastatic effects: the role of NK cells.* J Immunol, 2001. **166**(1): p. 89-94.
191. Gyorkey F, S.J., Melnick JL, Gyorkey P., *Retroviruses in Kaposi-sarcoma cells in AIDS.* N Engl J Med, 1984. **311**(18): p. 1183-4.
192. Saigal S, N.S., Muiesan P, Rela M, Heaton N, O'Grady J., *Evidence of differential risk for posttransplantation malignancy based on pretransplantation cause in patients undergoing liver transplantation.* Liver Transpl, 2002. **8**(5): p. 482-7.
193. Hellstrand K, H.S., Naredi P, Mellqvist UH, Brune M., *Histamine and cytokine therapy.* Acta Oncol, 1998. **37**(4): p. 347-53.
194. Ribas A, B.L., Amarnani SN, Dissette VB, Kim D, Meng WS, Miranda GA, and M.W. Wang HJ, Glaspy JA, Economou JS., *CD40 cross-linking bypasses the absolute requirement for CD4 T cells during immunization with melanoma antigen gene-modified dendritic cells.* Cancer Res, 2001. **61**(24): p. 8787-93.
195. Gurunathan, S., et al., *CD40 ligand/trimer DNA enhances both humoral and cellular immune responses and induces protective immunity to infectious and tumor challenge.* J Immunol, 1998. **161**(9): p. 4563-71.
196. Kikuchi T, M.N., Moore MA, Crystal RG., *Tumor regression induced by intratumor administration of adenovirus vector expressing CD40 ligand and naive dendritic cells.* Cancer Res, 2000. **60**(22): p. 6391-5.
197. Xiang R, P.F., Ruehlmann JM, Niethammer AG, Silletti S, Lode HN, Dolman CS, and R.R. Gillies SD, *A dual-function DNA vaccine encoding carcinoembryonic antigen and CD40 ligand trimer induces T cell-mediated protective immunity against colon cancer in carcinoembryonic antigen-transgenic mice.* J Immunol, 2001. **167**(8): p. 4560-5.
198. Nikitina EY, C.S., Muro-Cacho C, Fang B, Zhang R, Roth JA, Gabrilovich DI., *An effective immunization and cancer treatment with activated dendritic cells transduced with full-length wild-type p53.* Gene Ther, 2002. **9**(5): p. 345-52.
199. Bertram EM, L.P., Watts TH., *Temporal segregation of 4-1BB versus CD28-mediated costimulation: 4-1BB ligand influences T cell numbers late in the primary response and regulates the size of the T cell memory response following influenza infection.* J Immunol, 2002. **168**(8): p. 3777-85.
200. Tan, J.T., et al., *4-1BB costimulation is required for protective anti-viral immunity after peptide vaccination.* J Immunol, 2000. **164**(5): p. 2320-5.
201. Hess, S. and H. Engelmann, *A novel function of CD40: induction of cell death in transformed cells.* J Exp Med, 1996. **183**(1): p. 159-67.
202. Donepudi, M., D.D. Quach, and M.B. Mokyr, *Signaling through CD40 enhances cytotoxic T lymphocyte generation by CD8+ T cells from mice bearing large tumors.* Cancer Immunol Immunother, 1999. **48**(2-3): p. 153-64.
203. Maeda, K., R. Lafreniere, and L.M. Jerry, *Production and characterization of tumor infiltrating lymphocyte clones derived from B16-F10 murine melanoma.* J Invest Dermatol, 1991. **97**(2): p. 183-9.