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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

CANCER SCIENCES DIVISION

Doctor of Medicine

EFFICACY AND TOXICITY OF ANTI-CD40 MONOCLONAL ANTIBODIES IN THE TREATMENT OF MALIGNANCY

Thomas Richard Geldart

In recent years, monoclonal antibodies (mAbs) have established themselves as the most rapidly expanding class of therapeutic agents for a wide range of human diseases including cancer. This project has examined the efficacy and toxicity of mAbs directed against the CD40 antigen. CD40 is a type I transmembrane protein belonging to the tumour necrosis factor receptor (TNFR) super-family. It is expressed primarily on antigen presenting cells but is also expressed on a broad range of malignancies. CD154 (CD40L) is the natural ligand for CD40 and is expressed predominantly on activated CD4+ T helper lymphocytes. Normal, bi-directional CD40-CD154 interactions are central to the generation of both T cell dependent, humoral immune responses and cytotoxic T cell responses. Tumour expression of CD40 and the important functional role of CD40-CD154 in-vivo make CD40 an attractive target for antibody based anti-cancer immunotherapy.

I have demonstrated in-vitro that a mouse / human chimeric anti-CD40 mAb developed within the Cancer Sciences Division (Chi Lob 7/4) is able to cause growth inhibition and effectively recruit immunological effector mechanisms such as complement-dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC) against a number of malignant cell lines. I have evaluated the toxicity of anti-CD40 mAb administration in a comparative mouse model and found a reversible hepatitis and splenomegaly to be the principal toxicities of treatment. I have developed and validated an immunohistochemical method for the detection of CD40 expression on normal and malignant human tissue and developed an enzyme linked immunosorbant assay for the quantitative detection of serum Chi Lob 7/4.

Substantial funding has been acquired from the New Agents Committee of Cancer Research UK for the development of a phase I clinical trial of Chi Lob 7/4 in the treatment of CD40 positive malignancies refractory to conventional therapy. A preliminary trial protocol has been developed and clinical grade antibody production is underway. It is hoped that the trial will open to recruitment in 2004.

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LIST OF ABBREVIATIONS

Ab	Antibody
ADCC	Antibody dependent cellular cytotoxicity
ADEPT	Antibody dependent pro-drug therapy
AE	Adverse event
AEC	3-amino-9-ethylcarbazole
Ag	Antigen
ALK PHOS	Alkaline phosphatase
ALT	Alanine aminotransferase
AML	Acute myeloid leukaemia
APC	Antigen presenting cell
AST	Aspartate aminotransferase
ATCC	American type culture collection
AUC	Area under the curve
BCG	Bacille Calmette Guerin
BCR	B cell receptor
BSA	Bovine serum albumin
C	Celsius
C1-9	Components of complement pathway
CA	Cancer antigen
CD	Cluster designation
CD40L	CD154 / CD40 ligand
CDR	Complementarity determining region
CDC	Complement dependent cytotoxicity
CEA	Carcinoembyronic antigen
Chi	Chimeric
CLL	Chronic lymphocytic leukaemia
CR	Complete Response
CR1	Complement receptor one

CRC	Cancer Research Campaign
CRP	C Reactive protein
CRUK	Cancer Research UK
CT	Computerised tomography
CTC	Common Toxicity Criteria
CTLA	Cytotoxic T lymphocyte antigen
DAF	Decay accelerating factor
DDO	Drug Development Office
dL	Decilitre
DLT	Dose Limiting Toxicity
DMSO	Dimethyl sufoxide
DNA	Deoxyribonucleic acid
ECACC	European collection of animal cell cultures
ECG	Electro-cardiogram
EDTA	Ethylene diamine tetra-acetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbant assay
EMEM	Eagles minimum essential medium
ERK	Extracellular signal-regulated mitogen-activated protein kinase
ESR	Erythrocyte sedimentation rate
Fab	Fragment antigen binding
FACS	Fluorescence activated cell sorter
Fc	Fragment crystallisable
FcγRI-III	Fragment crystallisable gamma receptor I-III
FCS	Foetal calf serum
FITC	Fluorescein isothyocyanate
FNA	Fine needle aspiration
g	Gram
GGT	Gamma glutamyl transferase
Gm-CSF	Granulocyte macrophage colony stimulating factor

GMA	Glycol methacrylate
GvHD	Graft versus Host disease
HACA	Human anti-chimeric antibody
HAMA	Human anti-mouse antibody
HARA	Human anti-rat antibody
Hb	Haemoglobin
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HPLC	High pressure liquid chromatography
HRF	Homologous restriction factor
HRP	Horseradish peroxidase
HSP	Heat shock protein
Hu	Human / Humanised
H&E	Haematoxylin and Eosin
ICAM	Intracellular cellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Intraperitoneal
Irr	Irrelevant
iU	International units
IV	Intravenous
JNK	c-jun amino terminal kinase
L	Litre
LAL	Limulus amoebocyte lysate
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LREC	Local Research Ethics Committee
MAC	Membrane attack complex
MASP	Mannan binding lectin associated serine protease

MBL	Mannose binding lectin
MALT	Mucosal associated lymphoid tissue
MARA	Mouse anti-Rat antibody
MCP	Membrane co-factor protein
M	Molar
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
mg	Milligram
MHC	Major histocompatibility complex
min	Minute
MIP	Macrophage inflammatory protein
ml	Millilitre
mM	Millimolar
MREC	Multi- Research Ethics Committee
MRI	magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MTD	Maximum Tolerated Dose
MTT	Tetrazolium bromide
NEAA	Non-essential amino acids
ng	Nanogram
NK	Natural Killer
nM	Nanomolar
nm	Nanometres
NCI	National Cancer Institute
NHL	Non Hodgkin's Lymphoma
PALS	Periarteriolar lymphoid sheath
PAMM	Pharmacology and Molecular Mechanisms Group
PAS	Periodic Acid Schiff
PBS	Phosphate buffered saline
PD	Progressive Disease

PECAM	Platelet / endothelial specific cell adhesion molecule
Plts	Platelets
PK	Pharmacokinetics
PR	Partial Response
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SAE	Serious Adverse Event
SAR	Serious Adverse Reaction
SD	Stable Disease
SMA	Smooth muscle actin
SOP	Standard Operating Procedure
TAA	Tumour associated antigens
TBS	Tris buffered saline
TCR	T cell receptor
TD	Thymus dependent
TI	Thymus independent
TNF	Tumour Necrosis Factor
TNF-R	Tumour Necrosis Factor Receptor
TRAF	Tumour Necrosis Factor-Receptor associated factor
TSA	Tumour Specific Antigens
UK	United Kingdom
ULN	Upper limit of normal
VCAM	Vascular cellular adhesion molecule
VEGF	Vascular endothelial growth factor
WBC	White Blood Cells
WHO	World Health Organisation
µg	Microgram
µCi	Microcurie
µl	Microlitre

CHAPTER ONE

1 INTRODUCTION

1.1 Cancer

Cancer may be defined as an uncontrolled proliferation of cells with the capacity to invade locally and spread to distant sites (metastasise). The development of cancer is a multi-step process with each step conferring a growth advantage to the cell driving its transformation from a normal human cell into its malignant counterpart [1, 2].

Hanahan and Weinberg suggest that six essential alterations in cell physiology are acquired by all malignant cells during their development [2]: Self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis (programmed cell death), limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. It is suggested that these changes in physiology may be obtained via a number of cellular strategies but that they are shared in common by potentially all human cancers.

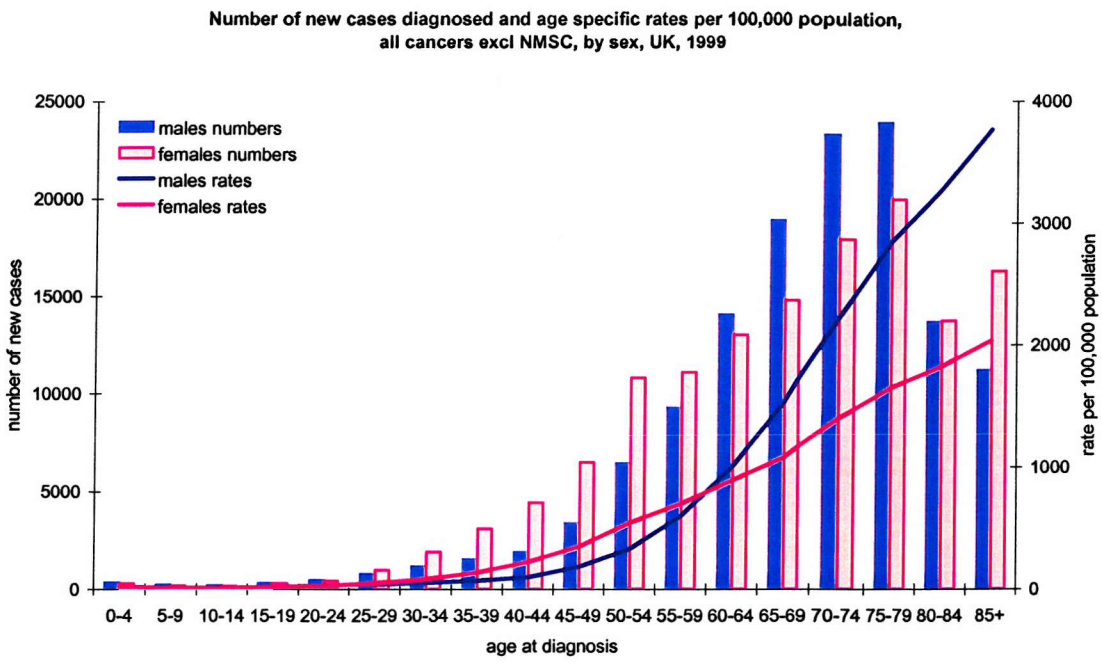
Several hundred distinct types of cancer have been described. Cancers may arise from virtually any cell within the body but may be categorised into three main groups. Carcinomas, the most common types of cancer, arise from epithelial cells (ectodermal or endodermal cells); sarcomas arise from bone or connective tissues (mesodermal cells); leukaemias and lymphomas arise from cells of the bone marrow and immune system.

1.1.1 Cancer Statistics

Cancer is a major cause of morbidity and mortality worldwide. In the United Kingdom, an individual's lifetime risk of developing cancer is about I in 3[3]. Each year, around 267,000 new cases of cancer are diagnosed in the United Kingdom (1999 figures) with the majority of cases (65%) being diagnosed in individuals aged 65 years or over (figure 1.1).

Figure 1.1

UK cancer incidence 1999 (excluding non-melanotic skin cancer) [3].



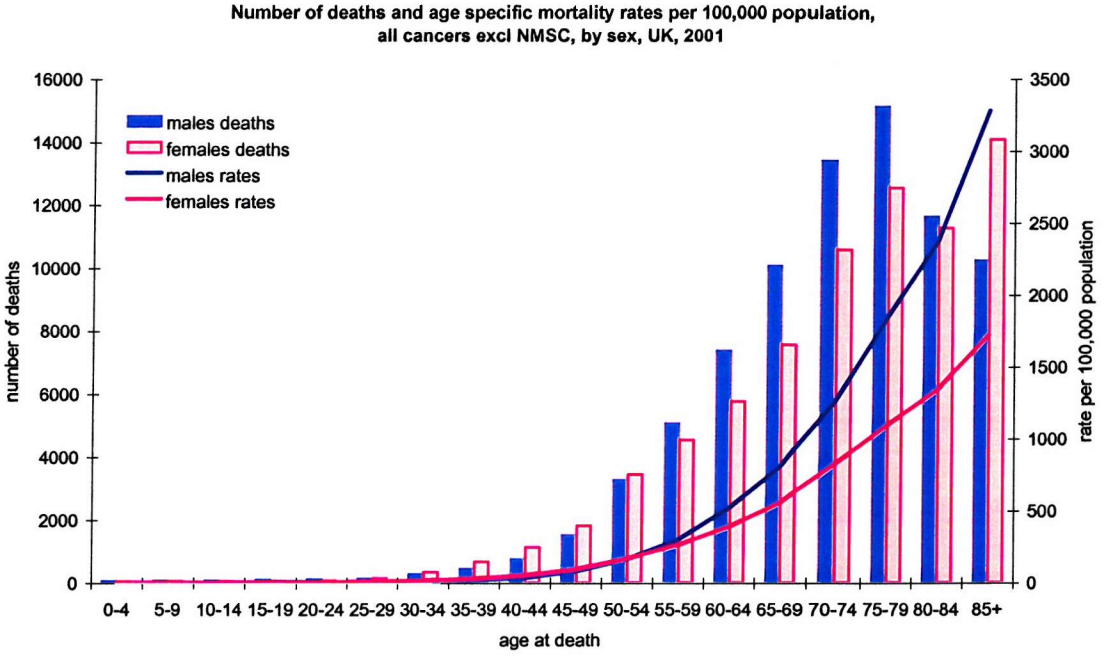
Four cancers; breast cancer, lung cancer, colorectal cancer and prostate cancer account for over 50% of all newly diagnosed cancers. In men, prostate cancer accounts for the largest proportion (19%) closely followed by lung cancer (18%) and colorectal cancer (14%). In women, breast cancer accounts for 30% of all newly diagnosed cancers followed by colorectal cancer (13%) and lung cancer (12%) (1999 figures).

Cancer is responsible for a quarter of all deaths in the UK [3]. In 2001, 154,000 people were registered as dying from cancer (figure 1.2). In both men and women, lung cancer is the leading cause of mortality accounting for 22% of all cancer deaths. For men, lung cancer mortality is followed by prostate cancer (12%) and colorectal cancer mortality (11%). For women, breast cancer mortality (17%) is close to that of lung cancer.

Colorectal is the third most common cause of cancer mortality in women accounting for 10% deaths.

Figure 1.2

UK cancer mortality 2001 (excluding non-melanotic skin cancer) [3].



1.1.2 Cancer treatment

The treatment of cancer requires a multidisciplinary approach with surgery, chemotherapy and radiotherapy representing the main treatment modalities. Surgery alone is often curative in early stage cancer with adjuvant chemotherapy and / or radiotherapy improving cure rates still further. However, except for a few notable exceptions such as testicular germ cell cancer and lymphoma, a large number of malignancies present at an advanced stage, are often incurable and are ultimately fatal.

The immunotherapy of cancer represents a fourth treatment modality that has been used alongside surgery, chemotherapy and radiotherapy for many years. An improved

understanding of the immune system and its relationship to cancer coupled with important developments in laboratory techniques has led to a resurgence of interest in immunotherapeutic strategies. This interest in immunotherapy has not been confined to the treatment of cancer; in particular, monoclonal antibodies have established themselves as the most rapidly expanding class of therapeutic agents for a wide range of human diseases [4].

1.2 The Immune System and Cancer

A relationship between the immune system and malignancy has long been the subject of research. In the late 19th century, physician William Coley (1862-1936) noted regressions of tumours in patients who had co-incidentally developed infections such as erysipelas. He subsequently administered early bacterial vaccines (live streptococcus erysipelas; Coley's toxins) to patients with cancer and noted occasional tumour responses.

In the early 1900s Paul Ehrlich proposed the concept of immune surveillance, hypothesising that we would all die from cancers if the immune system did not recognise and remove "aberrant germs" (nascent tumours). Subsequently, Burnett and Thomas re-stated Ehrlich's theory of immune surveillance suggesting that malignant cells frequently arose within the body but were generally recognised as "foreign" and eradicated by the immune system. They hypothesised that most malignant cells possessed distinct antigenic qualities from the cell type from which they derived and that these antigenic differences could be recognised by the immune system and so provoke an immune response.

A number of observations seemed to support the theory of immune surveillance. These included the occurrence of cancers at times of relative immunodeficiency (i.e. the very young and old), the observation of high rates of malignancy in patients who have received significant, long term immunosuppression and the phenomenon of spontaneous tumour regressions[5, 6]. Although immune surveillance is an attractive theory, alternative explanations are likely to account for some of the above observations. The

differential age incidence is likely to be related to the increased incidence of aberrant genetic change occurring during times of rapid cell division (early life) or following prolonged exposure to external carcinogens (older age). Tumours associated with immunosuppression are not the commonly seen epithelial tumours but those cancers thought to be associated with a viral aetiology such as Post Transplant Lymphoproliferative Disease, (Epstein Barr virus). This observation suggests that the immune surveillance operates principally against oncogenic viruses rather than cancer per se [6].

Spontaneous tumour regressions, although rare, do occur and support the observation that the immune system can recognise and respond to some tumour antigens. Resected tumour specimens commonly show some evidence of an immune response in the form of tumour infiltrating lymphocytes. However, the majority of tumours left untreated will grow and kill the host despite an intact immune system. Most immune responses to tumour antigens are likely to be weak, occurring late on in the disease process and as such are unable to effectively prevent tumour growth. Nevertheless, that an immune response to tumour antigens can occur, raises the prospect of immunotherapy; the manipulation of the immune system as a therapeutic approach to treating cancer.

1.3 The Immune System

The immune system acts as the body's defence against invasion by pathogens and cancer and can be subdivided into innate (non-specific) and adaptive (specific) components.

1.3.1 Innate Immunity

An innate immune system exists in some form in most organisms. Innate immunity comprises a set of general, non-specific resistance mechanisms that provide a first line of immunological defence.

1.3.1.1 Anatomical Barriers

Anatomical barriers such as the skin and mucous membranes and the secretion of sweat, sebaceous secretions and mucus provide a defence against most pathogens. Anatomical barriers play an extremely important role in defence against pathogens but are not considered important in cancer immunity; as such they will not be discussed further.

1.3.1.2 Activation of the innate immune system

Cells of the innate immune system possess a limited number of receptors that may recognise a variety of highly conserved structural motifs expressed by microbial pathogens, called pathogen-associated microbial patterns (PAMPs). These receptors or pattern recognition molecules (PRMs) include the toll like receptors (TLRs) [7, 8]. Ten human TLRs have been identified to date responsible for the recognition of a range of microbial molecules expressed by viruses, bacteria and protozoa. Lipopolysaccharide (LPS, endotoxin), a constituent of gram negative bacterial cell walls is recognised by TLR4, the first TLR to be identified. LPS exposure has a clear relationship to septic shock, a life threatening situation that may arise following severe infection by gram negative bacteria. It is characterised by fever, hypotension, metabolic acidosis and organ failure. LPS binding by TLR4 is thought to promote the development of septic shock through prompting the release of IL1, IL6 and TNF [9].

In addition to LPS, a variety of other molecules may be recognised by TLRs. These include bacterial peptidoglycan, lipoproteins, flagellin and DNA with unmethylated CpG motifs, viral double stranded RNA, yeast zymosan, and the antiviral immune response modifier, imiquimod. Each TLR may recognise one or more ligand and different TLRs may form heterodimeric complexes. TLRs are endowed with Toll/IL1 receptor/resistance motif domains (TIRs). Five such cytoplasmic adaptor proteins (MyD88, MAL, TRIF, MyD88-4 and MyD88-5) have been identified. TIR signalling, predominantly via the MyD88 pathway, leads to cellular activation with the production of cytotoxic reactive oxygen species, pro-inflammatory chemokines and cytokines, and the up-regulation of co-stimulatory molecules that effectively link early innate responses to the activation of a subsequent, more specific, adaptive immune response.

1.3.1.3 Phagocytosis

Phagocytosis (engulfment and digestion of invading micro-organisms) is an important innate immune defence mechanism. Polymorphonuclear neutrophils and cells of the mononuclear phagocyte system (monocytes / macrophages) are the cell types chiefly responsible for phagocytosis. Neutrophils are produced by haematopoiesis in the bone marrow and released into the bloodstream where they are the dominant white blood cell. Neutrophils circulate for about 7 – 10 hours before migrating into tissues where they have a lifespan of a few days. Mononuclear phagocytes also develop in the bone marrow and are initially released into the bloodstream as pro-monocytes. Pro-monocytes further differentiate into mature monocytes in the bloodstream where they circulate for approximately 8 hours before migrating into the tissues where they differentiate into tissue macrophages. Both neutrophils and macrophages are highly effective at phagocytosing exogenous antigens such as whole micro-organisms in addition to endogenous cellular matter such as dying or dead cells.

Although macrophages / monocytes and neutrophils lack immunological specificity and memory they express membrane receptors that recognise the Fc fragment of antibody. Antibody binding therefore can effectively direct these and other (e.g. Natural Killer Cells) non-antigen specific effector cells to a target dictated by antibody specificity. Once localised to the target cell, these cells are able to secrete a variety of lytic enzymes, tumour necrosis factor and other cytotoxic substances that ultimately lead to target cell killing, a process known as antibody dependent cellular cytotoxicity (ADCC). This process is thought to be of considerable importance in the immunotherapy of malignancy and is discussed in more detail later in this chapter.

1.3.1.4 Soluble factors

A variety of soluble factors contribute to innate immunity. These include the interferons and complement.

1.3.1.4.1 Interferons

Interferons (IFNs) are a family of proteins with a number of immunoregulatory properties. Three major types of IFN have been identified; IFNα, β and γ.

Viral infection of a cell (with resultant exposure of the cell to viral double stranded RNA; dsRNA) leads to production of IFNα / β and the activation of antiviral mechanisms enabling infected and neighbouring cells to resist infection. These mechanisms include the activation of intracellular enzymes such as 2'-5'-oligo-adenylate synthetase (which in turn activates a ribonuclease able to degrade viral RNA) and the induction of a specific dsRNA-dependent protein kinase able to inhibit viral protein synthesis.

IFNs have a number of immunoregulatory effects considered to be important in the immunotherapy of malignancy. In particular, IFNs have stimulatory effects on macrophages, NK cells, T lymphocytes and neutrophils and are able to facilitate antigen presentation through up-regulation of class I and class II major histocompatibility complex expression (see 1.3.2.2.3). Interferons may also inhibit angiogenesis, induce cellular differentiation and inhibit the proliferation of tumour cells. Interferon-α has been used for the treatment of a number of haematological and epithelial malignancies for many years [10].

1.3.1.4.2 Complement

Complement exists as a number of circulating serum glycoproteins which, when activated via a tightly regulated enzymatic cascade, promote the development of an inflammatory response, target cell opsonisation and cellular lysis. Complement activation is an important innate defence mechanism against pathogens and is a potentially important effector mechanism for antibody directed immunotherapy.

The main components of complement are designated by numerals (complement 1 – 9; C1-9) and circulate as functionally inactive forms. Many components of complement

are pro-enzymes, activated by proteolytic cleavage. Peptide fragments generated through cleavage are designated by small letters. With the exception of C2, the smaller component is designated by the letter a, the larger component by the letter b, e.g. C3a and C3b.

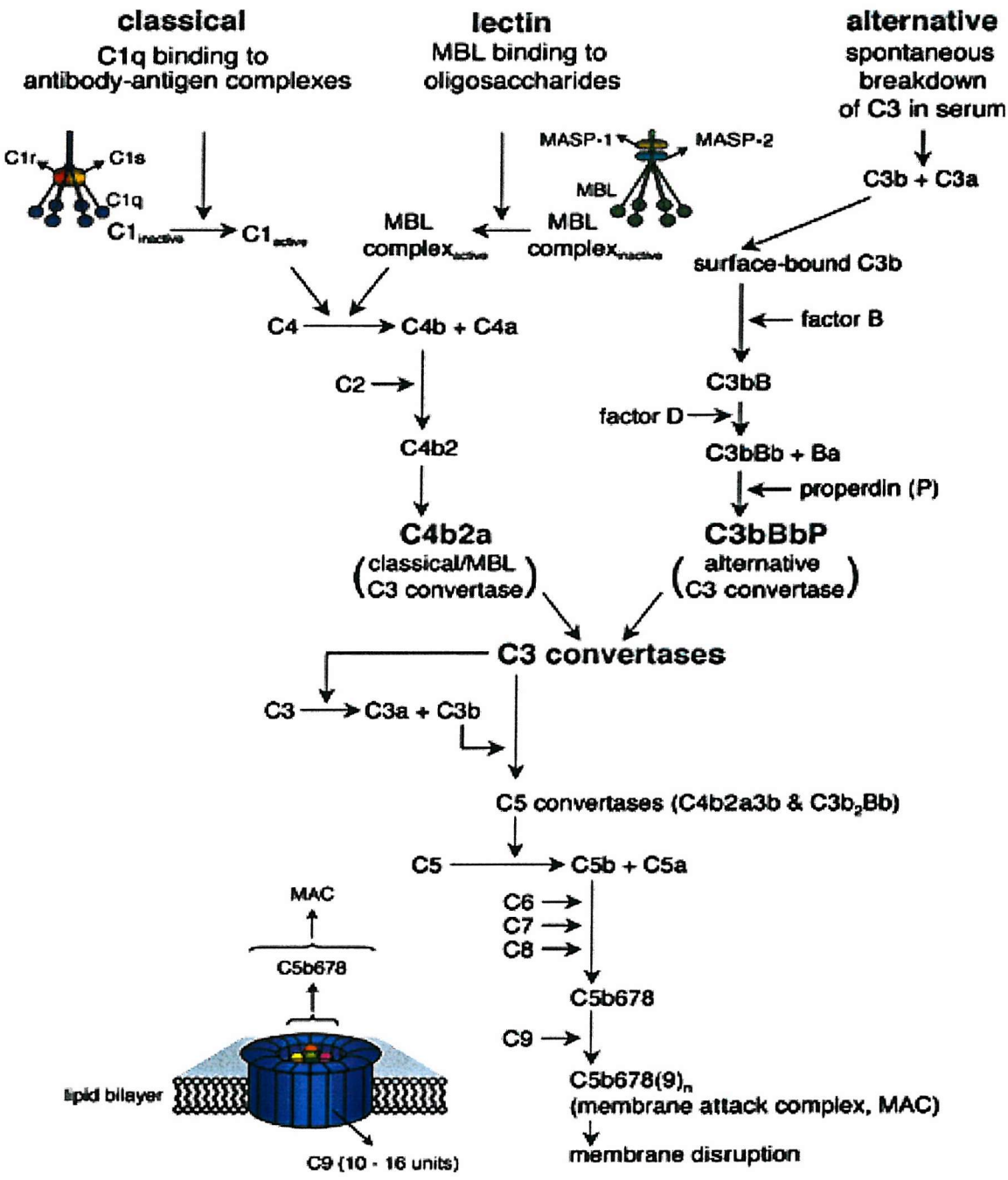
1.3.1.4.2.1 Complement activation

Complement can be activated through three different pathways; the classical pathway, the alternative pathway and the lectin pathway (figure 1.3). The alternative and lectin activation pathways may be thought of as components of the innate immune system whilst the classical pathway is dependent upon activation via antibody. The final common pathway of complement activation is the same regardless of the initial activating pathway and results in the production of the membrane attack complex (MAC).

The MAC is a macromolecular complex able to perforate target cell membranes and cause cell death by profoundly disrupting cellular osmotic stability. In addition to MAC formation, the complement cascade also leads to the generation of anaphylotoxins (e.g. C3a, C4a and C5a) that play an important role in the development of an effective inflammatory response. Complement fragments such as C3b can also act as opsonins, coating antigen and antigen-antibody complexes.

Figure 1.3

Overview of complement activation pathways. Adapted from [11]



1.3.1.4.2.1.1 The Classical Pathway

The "classical" pathway of complement activation is so named because it was discovered first, although on an evolutionary scale it was probably the last pathway to develop. The pathway is dependent upon the adaptive immune system and in particular, the binding of antibody to antigen on a suitable target such as the surface of a micro-organism or a malignant cell. The classical pathway of complement activation may be activated by therapeutically administered antibodies targeting tumour antigens.

Once a sufficient quantity of antibody has bound target antigen, C1 is able bind to the Fc portions of the aggregated antibody molecules. C1 is a macromolecular complex made up from C1q in association with 2 molecules of C1r and C1s ($C1qr_2s_2$). C1q is composed of 18 polypeptide chains that form 6 triple helical arms able to bind antibody. In order to from a stable C1-antibody interaction and activate the classical pathway of complement, each C1 molecule must bind at least 2 Fc antibody sites. Binding of C1q leads to a conformational change in C1 that converts it to C1r, an active serine protease enzyme which may then cleave C1s into its active form. Activated C1s cleaves C4 to C4a and C4b. C4a diffuses away but the larger C4b fragment is able to bind the target surface close to C1. The cleavage of C4 exposes a binding site on C4b to which C2 may bind. C2 may then be cleaved by neighbouring C1s, allowing C2a to diffuse away leaving the active C4b2a complex (also known as C3 convertase). A single C4b2a or C3 convertase molecule is able to cleave several hundred C3 molecules into C3a and C3b and this is a major amplification step in the complement activation pathway. C3b may bind C4b2a to form C4b2a3b (also known as C5 convertase) allowing cleavage of C5 into C5a and C5b. C5b may then bind C6 initiating formation of the membrane attack complex.

1.3.1.4.2.1.2 The Lectin Pathway

The lectin pathway is activated following the binding of the collectin protein, Mannan Binding Lectin (MBL) to mannose carbohydrate residues present on the surface of micro-organisms. After binding, the collagen like domain of MBL is able to interact with serine proteases known as MASP and MASP2 (mannan binding lectin associated serine protease). The active complexes formed by this association lead to activation of C4 which in turn leads to antibody independent activation of the classical pathway described above.

1.3.1.4.2.1.3 The Alternative Pathway

The alternative pathway of complement activation is reliant upon the stabilisation of activated serum C3b by foreign micro-organisms. Normally, C3 is subject to spontaneous hydrolysis to C3a and C3b. C3b is rapidly inactivated by sialic acid present on the membranes of most mammalian cells thereby preventing spontaneous complement activation. Foreign micro-organisms however express only low levels of sialic acid and consequently, C3b bound to foreign cell surfaces may stay active long enough to bind factor B. C3b binding exposes an active site on factor B that acts as a substrate for Factor D. Factor D in turn cleaves C3b-Factor B generating C3bBb and releasing a small fragment, Ba. C3bBb has C3 convertase activity and acts in a manor analogous to C4b2a in the classical pathway described above.

1.3.1.4.2.1.4 Formation of the Membrane Attack Complex

Activation of the complement cascade via the classical, alternative and lectin pathways leads to the generation of C5 convertase and the cleavage of C5 to C5a and C5b. C5b is able to bind the target cell where it acts as a docking site for components of the MAC. C6 binds to C5b stabilising its activity and forming C5b6. The subsequent binding of C7 leads to a structural change in the resulting complex allowing C5b67 to insert into the phospholipid bi-layer of the plasma membrane. Binding of C8 to this complex leads to further structural change and the development of a small pore (10Å diameter) in the plasma membrane. Binding and polymerisation of C9 to C5b678 results in further

structural change and completion of the MAC, a transmembrane pore of 70-100 Å that ultimately leads to target cell lysis through the disruption of cellular osmotic stability.

1.3.1.4.2.2 Complement inhibitors

Complement is a component of the innate immune system and is therefore non-specific. In order to avoid widespread inflammation and cellular death, a variety of regulatory mechanisms exist. In general, many components of complement are extremely labile and become spontaneously inactivated as they diffuse a short distance away from their site of activation. In addition to this general regulatory mechanism, a number of specific inhibitors of complement have been identified.

An important family of complement inhibitory proteins regulate the activity of C3 convertase, a major amplification step in the complement cascade. These C3 convertase regulatory proteins or regulators of complement activation (RCA) all contain short consensus repeats of amino acid sequences and act to prevent assembly of C3 convertase. C4b binding protein (C4bBP), complement receptor type 1 (CR1) and membrane co-factor protein (MCP), act by binding C4b and preventing its association with C2a to form C3 convertase in the classical and lectin pathways. In the alternative pathway, CR1, MCP and factor H act to prevent the binding of C3b to factor B. CR1, C4bBP, Factor H and decay accelerating factor (DAF) also act on assembled C3 convertase leading to its dissociation and inactivation.

Other regulatory proteins act at the level of the MAC; protein S is able to bind C5b67 and prevent its insertion into the cell membrane. Two other proteins, membrane inhibitor of reactive lysis (CD59) and homologous restriction factor (HRF) protect cells from complement mediated lysis by binding C8 thereby inhibiting the polymerisation of C9 and MAC formation.

1.3.2 Adaptive Immunity

In contrast to the innate immune system, the adaptive immune system is restricted to vertebrates. It is defined by antigenic specificity, diversity and immunological memory.

The cellular basis for adaptive immunity is provided by lymphocytes (B and T cells) and antigen presenting cells.

1.3.2.1 Humoral Immunity

B lymphocytes derive from lymphoid progenitor cells that in turn have differentiated from haematopoietic stem cells. B cells are produced throughout an individual's lifespan, initially in the foetal liver and subsequently in the bone marrow. Immature B cells exit the bone marrow via the sinusoids and migrate to the secondary lymphoid organs to complete their development.

B cells express a unique antigen binding site on their membrane, the B cell receptor, a membrane bound antibody (surface immunoglobulin). B cell receptors confer unique antigenic specificity to B cells. Approximately 80,000 B cell receptors, each with an identical antigen binding site are expressed on the surface of a single B cell. Interaction between specific antigen and surface immunoglobulin on mature naïve B cells (in the presence of T helper cells and appropriate cytokines) will induce B cell division, maturation and differentiation into a clonal population of memory B cells and plasma cells (see 1.3.2.1.6).

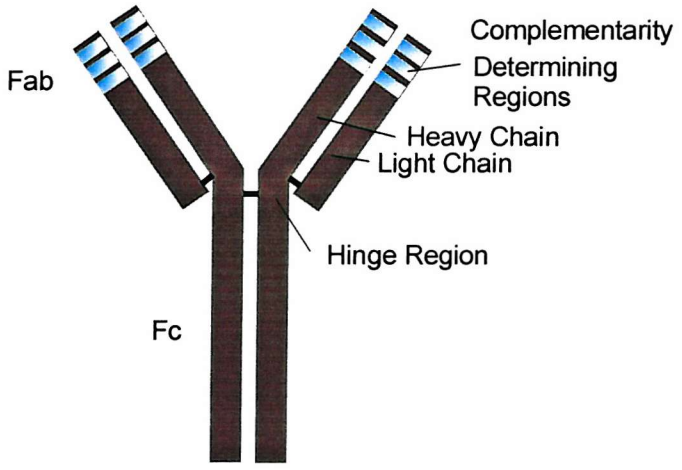
Plasma cells lack membrane bound immunoglobulin, but are able to synthesise and secrete high levels of soluble monoclonal antibody (mAb) with identical antigen specificity to that of the parent B cell. Circulating soluble antibodies are the mediators of humoral immunity, binding foreign antigen and neutralising it or targeting it for elimination. Because of the unique way in which antibody diversity is generated, the immune system is capable of producing antibodies specific to virtually any antigen encountered, a property that makes antibodies immensely powerful tools for experimental, diagnostic and therapeutic use.

1.3.2.1.1 Antibody Structure

Antibodies are large "Y" shaped glycoproteins composed of four polypeptide chains made up from two identical heavy chains combined with two identical light chains (figure 1.4).

Figure 1.4

Schematic representation of an antibody.



The chains are folded into a number of distinct domains linked together by a series of disulphide bonds. Heavy chains consist of one variable domain and three or four constant domains representing regions of relatively variable or constant amino acid sequence respectively.

Five types of heavy chain (grouped according to heavy chain constant region sequences) have been identified in humans; alpha (α), delta (δ), epsilon (ε), gamma (γ) and mu (μ). On the basis of these heavy chain constant region sequences, antibodies are classified into one of five main classes or isotypes; immunoglobulin A (IgA), IgD, IgE,

IgG and IgM. Minor differences in the heavy chain amino acid sequences of IgA and IgG allow further sub-classification of these antibody isotypes into IgA_1 and IgA_2 and IgG_1, IgG_2, IgG_3 and IgG_4.

Light chains are smaller and consist of one variable and one constant domain. Two types of light chain are described in humans; kappa (κ) and lambda (λ). Any two identical light chains may be combined with any two identical heavy chains to form a complete antibody monomer; e.g. IgG kappa or IgG lambda, IgE kappa or IgE lambda etc.

IgD, IgE and IgG are produced and secreted as monomers. IgM is expressed on the cell surface of B cells as a monomer but secreted by plasma cells as a pentamer. IgA exists primarily as a monomer but may form dimeric, trimeric or tetrameric forms. The differing biological properties of various antibody isotypes are described in section 1.3.2.1.2.

The two arms that make up the top of the Y shaped immunoglobulin structure are identical and are known as Fab fragments (Fragment antigen binding). Each Fab fragment is composed of one variable region and an adjacent constant region from one light chain and one heavy chain. Within each variable domain, three short polypeptide sequences show immense variability. These hypervariable regions make up the specific antigen binding site of the antibody and are known as the complementarity determining regions (CDRs). Intervening polypeptide sequences called framework regions act as a scaffold for the CDRs. The two Fab fragments of IgA, IgD and IgG are joined at an area of structural flexibility known as the hinge region (replaced by an additional constant domain in IgM and IgE). Beyond the hinge region is the remainder of the antibody, the Fc fragment (fragment, crystallisable), made up from the remaining heavy chain constant regions. The Fc fragment is responsible for mediating the immune effector functions of antibody such as opsonisation, complement mediated cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC).

1.3.2.1.2 Biological properties of antibody isotypes

The biological properties of the different antibody isotypes are described below and illustrated in table 1.1.

IgM accounts for only 5-10% of the total immunoglobulin fraction but is the antibody that is predominant in the early immune response. Monomeric IgM is expressed as membrane bound protein on B cells. IgM is secreted as a pentamer composed of five H_2L_2 antibody monomer units held together by disulfide bonds linking their $C_\mu 3$ / 4 domains. The five Fc regions sit in the centre of the pentamer and ten antigen binding sites on the outside of the molecule. IgM is particularly good at activating the classical complement pathway.

IgG is the major serum immunoglobulin and accounts for approximately 80% of total serum immunoglobulin. Four IgG subtypes exist, characterised by differences in their γ chain and numbered according to decreasing serum concentration. The biological activity of IgG varies according to subclass. IgG_1 and IgG_3 bind with high affinity to Fcγ receptors and are therefore good at mediating opsonisation and antibody dependent cellular cytotoxicity (ADCC). IgG_4 has intermediate affinity, IgG_2 low affinity. IgG_3 is the most effective complement activator followed by IgG_1 and IgG_2. IgG_4 is unable to activate complement.

IgA constitutes only 10 -15% of the serum immunoglobulin fraction but predominates in secretions where it performs an important effector role at mucous membrane surfaces, the main entry site for pathogenic organisms. IgA exists primarily as a monomer but may form dimeric, trimeric or tetrameric forms.

IgD constitutes less than 1% of serum immunoglobulin. Together with IgM it is expressed on the B cell surface but is not thought to function in the activation of B cells. No biological effector function for IgD has been identified.

Table 1.1

Selected properties of human antibody isotypes. (Adapted from [12])

Isotype / property	IgG_1	IgG_2	IgG_3	IgG_4	IgM	IgA_1	IgA_2	IgD	IgE
Heavy chain	γ1	γ2	γ3	γ4	μ	α1	α2	δ	ε
Present on membrane of mature B cells	-	-	-	-	+	-	-	+	-
Serum level (mg/ml)	9	3	1	0.5	1.5	3	0.5	0.03	0.00005
Serum half life (days)	21	20	7	21	10	6	6	3	2
Activates complement via classical pathway	++	+	+++	-	+++	-	-	-	-
Binds FcγRIIIa (CD16) Mediates ADCC	+	-	+	-	-	-	-	-	-

IgE is a trace serum immunoglobulin. IgE binds with high affinity to Fc receptors on mast cells and basophils. Antigenic crosslinking of receptor bound IgE induces degranulation of these cells, the release of pharmacologically active substances such as histamine and is responsible for immediate hypersensitivity reactions (e.g. anaphylaxis, perennial allergic rhinitis, asthma.). IgE is thought to play a protective role in defence against some parasitic infections.

1.3.2.1.3 Generation of antibody diversity

The heavy and light immunoglobulin polypeptide chains that together make up a complete antibody molecule are encoded by the recombination of different versions of gene segments rather than a single contiguous DNA gene sequence. This and other mechanisms discussed below allow the production of antibodies with specificity for an almost infinite number of antigens.

The gene segments encoding heavy and light immunoglobulin polypeptide chains are categorised and grouped into leader (L), variable (V), diversity (D), joining (J) and constant (C) sets with each set containing a number of different versions of the gene segment. During B cell ontogeny, individual versions of these gene segments are randomly selected and brought together to form a contiguous functional gene, a process known as somatic recombination. This process allows the generation of immense antibody diversity even in the absence of exposure to specific antigen.

For light chains, a complete gene results from the recombination of single V_L and J_L gene segments (together encoding for the light chain variable region) with a C_L gene (encoding for the constant region). For kappa light chains, approximately 40 versions of the V_κ gene segment have been identified and these may be combined with any of 5 J_κ gene segments and one C_κ gene to form a complete kappa light chain gene. For lambda light chains, approximately 30 V_λ gene segments, 4 J_λ gene segments and 4 C_λ gene segments have been described.

Human heavy chain genes are similar to, but more complex than light chains. They possess an additional gene segment (D_H), encoding for part of the variable region along with the V_H and J_H gene segments. About 50 versions of the heavy chain V_H gene segment have been identified, along with 27 D_H gene segments and 6 J_H gene segments. Any VDJ recombination may combine with any of the C_H genes that encode for the 5 different immunoglobulin isotypes. Leader (L) gene segments encode for a short leader peptide responsible for guiding the heavy or light chain through the endoplasmic reticulum; this peptide is cleaved prior to immunoglobulin assembly.

1.3.2.1.4 Somatic Recombination and B cell ontogeny

Progenitor (pro-) B cells are the earliest committed B cells and are characterised by the expression of CD45 (leucocyte common antigen), a transmembrane tyrosine phosphatase and CD19, part of the B cell co-receptor. Recombination of D_H and J_H gene segments occurs at this stage (figure 1.5)

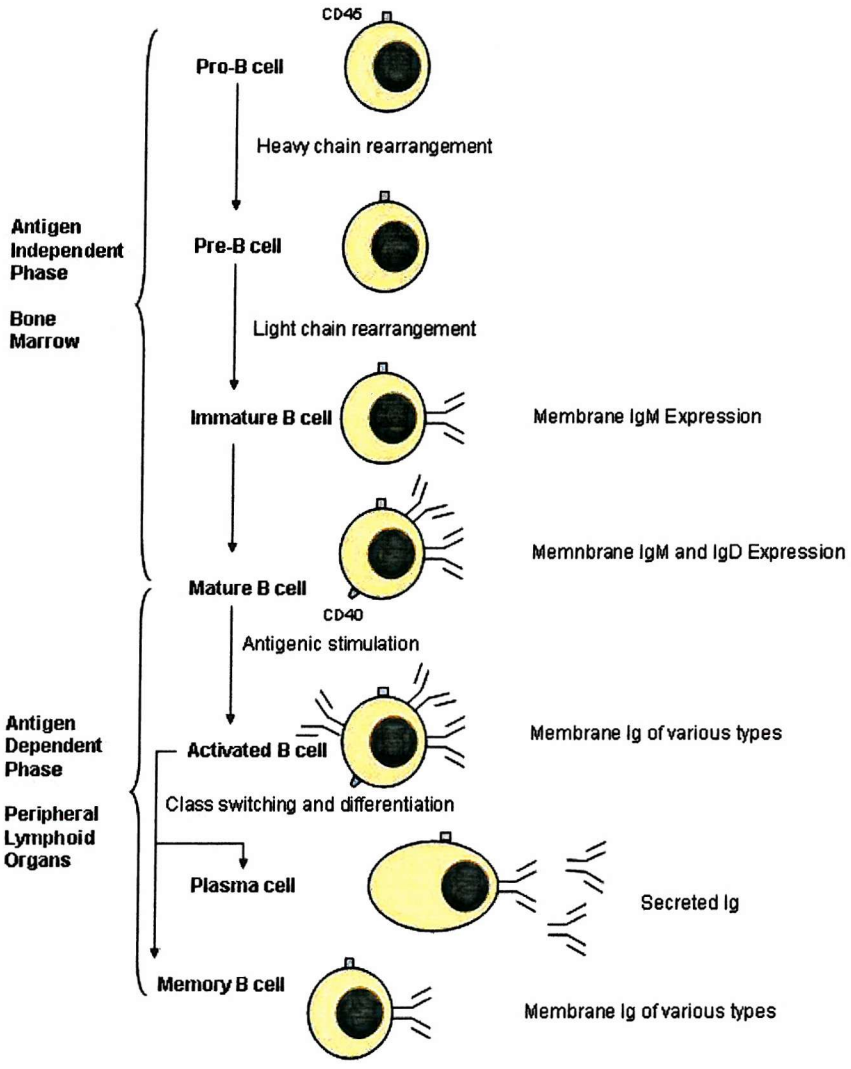
Pro-B cells differentiate into precursor (pre-) B cells in the presence of bone marrow stromal cells and appropriate cytokines. The generation of a contiguous V_H region gene (from which a μ heavy chain is eventually produced) occurs at this stage following V_H to D_HJ_H recombination.

Development of a pre-B cell into an immature B cell requires light chain V_L and J_L rearrangement and the appearance on the cell surface of paired light and heavy chain polypeptides constituting the B cell receptor (surface IgM).

Immature B cells develop into mature B cells as they migrate from the bone marrow to the periphery. During this development, surface IgM expression is increased and IgD begins to appear on the cell surface as a result of alternative splicing of heavy chain transcripts. As immature B cells begin to encounter self-antigens, those with reactivity to bone marrow self-antigens are deleted through apoptosis in a process termed negative selection; This process induces tolerance so that B cells which recognise self-antigens do not continue development and do not become activated.

Figure 1.5

An overview of B cell ontogeny. Adapted from [13]



1.3.2.1.5 B cell activation

Activation of antigen specific, naïve B cells (B cells that have not encountered foreign antigen) generally takes place in secondary lymphoid organs such as the lymph nodes, spleen and mucosa associated lymphoid tissues (MALT). Secondary lymphoid organs provide an environment where antigen may be concentrated and displayed to B and T lymphocytes. Depending on the nature of the antigen, B cell activation may occur dependently or independently of T helper cells.

Antigens that can activate B cells in the absence of specific T cell help are known as thymus independent antigens. TI antigens are able to activate B cells regardless of their specificity (polyclonal activation) and are generally made up of highly repetitious molecules. TI antigens include polymeric proteins and cell wall polysaccharides that are able to extensively cross-link IgM receptors. The response to TI antigens is early and specific but generally weak, predominantly IgM in nature and memory cells are not formed.

B cell activation and response to "thymus dependent" (TD) antigens (generally polypeptides or proteins) is dependent on T cell help. Recognition and binding of a TD antigen by the B cell receptor on naive B cells leads to receptor mediated antigen endocytosis and internal antigen processing into short peptide sequences. Processed antigenic peptide may then be coupled to the class II major histocompatibility complex (MHC) and transported to the cell surface where it is expressed within the cleft of MHC II and may be recognised by specific responding T helper cells. The affinity of this interaction is enhanced by the CD4 co-receptor molecule binding MHC II and facilitating the formation of a B – T cell conjugate.

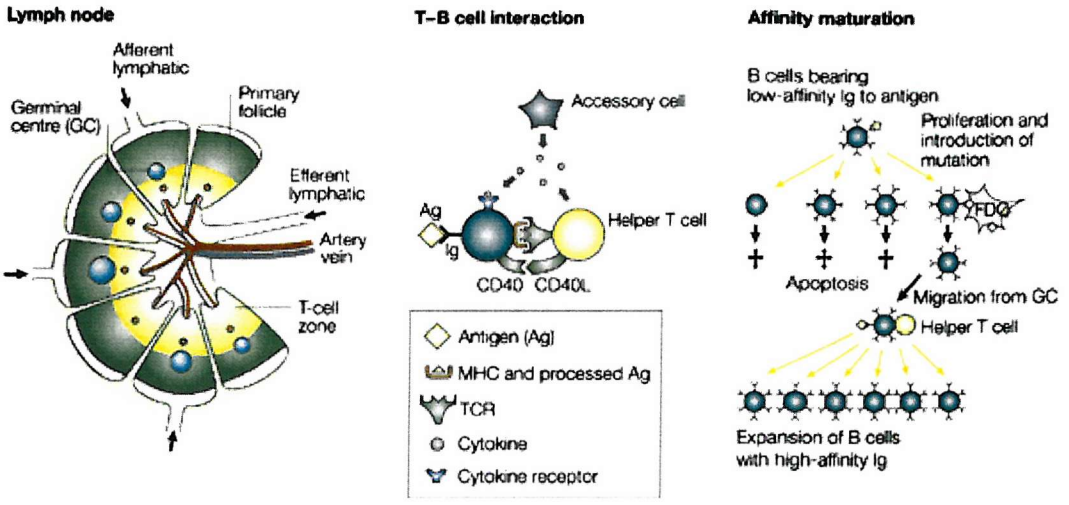
B cell receptor antigen binding also leads to up-regulation of co-stimulatory B7 molecules. Recognition of specific peptide-MHC II in concert with B7 by responding T helper cells leads to activation of the T helper cell. Activation and clonal expansion of CD4+ T helper cells is a central event in the generation of thymus dependent, B cell

immune responses. T helper cell activation induces expression of CD154 (CD40 ligand) on the T cell. Interaction between CD154 and its natural receptor CD40 expressed on naïve B cells provides an essential second signal to the B cell, initiating activation, driving it into G_1 of the cell cycle (see 1.6.1). T helper cells also secrete a variety of cytokines such as interleukin-2 (IL-2) IL-4 and IL-6, able to bind receptors expressed by the activated B cell and induce B cell activation and proliferation.

Initial activation of B cells takes place in the T cell rich paracortex of lymph nodes (or periarteriolar lymphoid sheaths (PALS) of the spleen), (figure 1.6). Following B cell activation, small foci of activated B cells proliferate at the edge of the T cell rich zone and may differentiate into IgM and IgG secreting plasma cells. Following the formation of these foci, a number of activated B and T helper cells migrate to primary follicles which in turn develop into secondary follicles. Activated B cells may migrate to the centre of the follicle to form a germinal centre. Germinal centre formation is crucially dependent on previous interaction of B cells with T helper cells via CD40-CD154 interaction (figure 1.6). Within germinal centres, B cells undergo rapid proliferation and somatic hypermutation, a mechanism that allows the introduction of point mutations into the antibody V region, resulting in antibodies with varying affinity to antigen. B cells producing antibody with high affinity to specific antigen may then undergo antibody isotype switching (e.g. IgM to IgG) and be selected for maturation into memory B cells or antibody forming (plasma) cells. Memory B cells do not secrete antibody but may be quickly reactivated following repeat exposure to antigen.

Figure 1.6

B cell activation and development [14].



Plasma cells are terminally differentiated and eventually home to the bone marrow or intestinal lamina propria where they produce large quantities of soluble antibody for several weeks.

1.3.2.1.6 Antibody function

Antibodies are the mediators of humoral immunity. Following the recognition of specific antigen, antibodies may trigger a number of immunological effector mechanisms that include antigen opsonisation, complement mediated cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC).

1.3.2.1.6.1 Opsonisation

Opsonisation describes the process whereby target cells expressing antigen are coated by specific antibody which in turn attracts phagocytic cells such as neutrophils and

macrophages. Phagocytosis of antibody coated cells is an important defence against common bacterial pathogens.

1.3.2.1.6.2 Complement mediated cytotoxicity (CDC)

Activation of complement by antibody-antigen complexes via the classical pathway is a major effector component of humoral immunity promoting the development of an inflammatory response, target cell opsonisation and cell lysis. CDC may be an important mechanism through which therapeutic antibodies used for the treatment of malignancy may achieve their anti cancer effect.

1.3.2.1.6.3 Antibody dependent cellular cytotoxicity (ADCC)

Natural killer cells, macrophages / monocytes, neutrophils and eosinophils lack immunological specificity and memory but express membrane receptors that recognise the Fc fragment of antibody. Fc receptor binding can effectively direct these non-antigen specific effector cells to a target dictated by antibody specificity. Once localised to the target cell, the secretion of lytic enzymes, tumour necrosis factor and other cytotoxic substances by effector cells mediates target cell killing, a process known as antibody dependent cellular cytotoxicity (ADCC). ADCC is thought to be an important mechanism of action for therapeutic antibodies used in the treatment of malignancy.

1.3.2.2 Cellular immunity

In contrast to humoral immunity where antibody is able to recognise extracellular or cell membrane expressed antigens, cell mediated immunity is able to recognise and directly eliminate those cells expressing atypical antigens such as virally infected cells, allogeneic cells (transplants) and tumour cells. Humoral and cell mediated responses are not mutually exclusive; antibody acts as an essential component of some cell mediated immune responses (e.g. ADCC) and T cell responses are critically involved in the initiation of specific antibody responses. Both antigen specific (T cells) and non-specific (e.g. natural killer cells, macrophages) cells are involved in cellular immunity.

1.3.2.2.1 T Cells

T cells arise from stem cells within the bone marrow. Unlike B cells, progenitor T cells migrate to the thymus to differentiate and mature. Only a small proportion (~2%) of progenitor T cells or thymocytes exit the thymus as self-tolerant and self-restricted mature T cells.

In common with B cells, mature T cells possess an antigen specific, clonally restricted antigen receptor – the T cell receptor (TCR). The TCR however differs from B cell surface immunoglobulin in two important ways. Firstly, the TCR is exclusively membrane bound and is not secreted in a soluble form. Secondly, the TCR recognises antigen only when presented in combination with molecules of the major histocompatibility complex (MHC), (see 1.3.2.2.3). The TCR is a heterodimer composed of either α and β chains or γ and δ chains associated on the cell surface with CD3, a signal transducing complex. Two well defined sub-populations of T cell have been identified – T helper cells and cytotoxic T cells, distinguished from each other by function and the expression of either CD4 or CD8 glycoproteins on their cell surface.

1.3.2.2.1.1 T cell development

Bone marrow derived thymocytes migrating to the thymus do not express classical T cell markers (CD3, CD4, and CD8). In a process analogous to the production of the B cell receptor, thymocyte TCR gene rearrangements begin to occur and cells become committed to αβ or γδ T cell lineage. Approximately 95% of circulating T cells are αβ T cells. γδ T cells make up only 5% of circulating T cells but are common in the skin and mucosal surfaces.

Following commitment to αβ or γδ lineage, developing T cells begin to express both CD4 and CD8. T cells that express a TCR able to recognise self-MHC are positively selected at this point but negatively selected (i.e. deleted) if they recognise self-antigens in combination with self-MHC. Final lineage commitment and differentiation to a

mature (naïve) CD4+, CD8– or CD4–, CD8+ T cell depends on whether the TCR interacts with class I or class II MHC and the quality / intensity of any such interaction.

1.3.2.2.1.2 T cell activation

Mature, naïve T cells have not yet encountered specific antigen and generally do not do so until they leave the thymus, circulate in the bloodstream and migrate to lymphoid organs such as the spleen, MALT and lymph nodes. Activation of antigen naïve T cells requires two signals. The primary signal is delivered following the interaction of the TCR-CD3 complex and CD8 or CD4 with a specific antigenic peptide presented in combination with class I or class II MHC. A secondary co-stimulatory signal is delivered between membrane bound molecules on the T cell and the antigen presenting cell. This second, co-stimulatory signal is not antigen specific and is provided primarily by interaction between CD28 on the T cell and B7-1 (CD80) and B7-2 (CD86) molecules on the antigen presenting cell. The B7 molecules are members of the immunoglobulin family with similar extracellular domains but differing intracellular domains. Signalling by B7 molecules through CD28 delivers a positive co-stimulatory signal to the T cell. Engagement of the T cell receptor with MHC – peptide leads to the up-regulation of another molecule CTLA-4 (CD152) also able to bind B7. CTLA-4 is an inhibitory receptor acting antagonistically to CD28 downregulating T cell activation. Once activated, T cells undergo clonal expansion and differentiation into short lived effector cells and longer lived memory T cells.

1.3.2.2.1.3 T helper cells

Activation and clonal expansion of CD4+ T helper cells is a central event in the generation of both humoral and cell mediated immune responses. T helper cell activation is initiated following recognition by the TCR of a specific peptide bound to MHCII on the surface of a professional antigen presenting cell (APC) such as a dendritic cell, B cell or macrophage. In the presence of an appropriate co-stimulatory signal provided through CD28 on the T cell and B7 on the APC, T helper cells may become fully activated. The absence of an appropriate co-stimulatory signal will lead to T cell anergy. In addition to the important role of CD40 – CD154 interactions in B cell

development discussed earlier, activation of CD40 on APCs is important in the regulation of cell mediated immunity. CD40 activation on APCs leads to upregulation of MHCII expression, and other key accessory and costimulatory molecules such as B7-1 and B7-2 on the APC and the subsequent production of immunoregulatory cytokines such as IL-12 (see 1.6.2).

T cell activation triggers entry of the T helper cell into G_1 of the cell cycle and the production of various cytokines. CD4+ cells may differentiate into one of two helper T cell subsets, T_H1 or T_H2 depending on the cytokine environment in which they develop. These subsets may be distinguished on the basis of the cytokine profile the T helper cell secretes. T_H1 cells secrete interleukin-2 (IL-2), interferon γ (IFNγ) and tumour necrosis factor β (TNFβ) and are responsible for orchestrating cell mediated immunity such as the activation of cytotoxic T cells. T_H2 cells on the other hand secrete IL-4, 5, 6 and 10 and are important for the generation of an effective, B cell mediated, humoral response.

1.3.2.2.1.4 Cytotoxic T cells

The activation and generation of cytotoxic T cells (CTLs) from CTL precursors requires recognition of antigenic peptide – class I MHC complex by the T cell receptor-CD3 complex. In common with T helper cell activation, a second co-stimulatory signal (CD28 – B7) is also required. Full activation of CTLs generally requires the participation of CD4+ T helper cells in a process called cross-priming. CD154-CD40 interaction between T helper cells and APCs is of critical importance here, effectively empowering the APC to present processed antigen to responding CTLs[15] (see 1.6.2). Following antigenic activation, the IL-2 receptor (and to a lesser extent IL-2 production) is up-regulated on CTL precursors. IL-2 is the principal cytokine required for CTL proliferation, differentiation and full CTL activation.

Cytotoxic T cells achieve killing through a number of mechanisms that include the secretion of perforin, granzyme and the expression of Fas ligand. Perforin is able to form a pore within target cells (like the MAC of complement) leading to cell death through osmotic instability. Granzyme (able to gain access to target cells through the

perforin pore) contains proteolytic enzymes able to digest host cell proteins and activate the pro-apoptotic caspase cascade. Fas ligand is a potent inducer of apoptosis and when expressed on activated T cells is able to cause cellular killing through the stimulation of Fas (and subsequent activation of caspases via their cytoplasmic death domains) on target cells.

1.3.2.2.2 NK cells

Natural Killer cells (NK) make up 5-10% of the circulating lymphocyte population. They are large granular lymphocytes and are involved in cellular immune responses to virally infected cells and malignant cells.

NK cells do not possess antigen specific receptors and are thus part of the innate immune system. However, NK cells do express receptors for the Fc region of IgG, FcγRIII (CD16) and as such are able to recognise IgG bound to target antigens on cells thus triggering antibody directed NK cell mediated killing (antibody directed cellular cytotoxicity, ADCC). NK cells also possess inhibitory receptors. Two major groups of inhibitory receptors have been recognised, C-type lectin –inhibitory receptors (CLIR) and Ig superfamily inhibitory receptors, together referred to as the inhibitory receptor superfamily (IRS). IRS receptors recognise molecules of the class I major histocompatibility complex. Recognition of MHC I on the cell surface effectively blocks NK mediated killing as NK cells preferentially recognise and kill cells with absent MHC. This is particularly important in virally infected cells or malignant cells which often downregulate MHC in order to escape T cell mediated immune attack. Cytotoxic mechanisms used by NK cell are identical to those used by T cells.

1.3.2.2.3 Major histocompatibility complex

The major histocompatibility complex (MHC) plays a key role in the development of both humoral and cell mediated immune responses. The MHC is known as the human leucocyte antigen (HLA) in humans where it is located on chromosome 6. There are three major classes of MHC in humans; class I, II and III.

Class I and II MHC molecules have structural and functional similarities. Both types are membrane glycoproteins with roles as specialised antigen presenting molecules. Classical class I MHC molecules are encoded by the A, B and C loci in humans and are expressed on the surface of nearly all nucleated cells. Their major function is the presentation of antigenic peptide fragments to CD8 expressing cytotoxic T cells. Class I molecules are non-covalently associated heterodimers formed from an α chain encoded by the MHC and a β chain (β_2-microglobulin) encoded by a gene on chromosome 15. The α chain comprises intracellular, transmembrane and extracellular domains; the β chain an extracellular domain only. Processed peptides of about 8 – 11 amino acid residues are presented bound within a groove within the α chain.

Classical class II MHC molecules are encoded by the DP, DQ and DR gene loci and are expressed primarily on professional antigen presenting cells (dendritic cells, monocytes and B cells). Their major function is the presentation of antigenic peptide fragments to CD4 expressing T helper cells. Class II molecules are non-covalently associated heterodimers formed from an α and a β chain encoded by the MHC. Both chains have intracellular, transmembrane and extracellular domains. Processed peptides of 13 amino acid residues or longer may be presented bound within a groove created by both α chain and β chains.

Class III genes encode a variety of molecules important to the immune response but have little in common with class I or II MHC. Class III MHC molecules include a variety of secreted proteins such as cytokines (tumour necrosis factor α and β), heat shock proteins, complement components (C2, C4 and BF) and steroid 21-hydroxylases.

There is extensive polymorphism in the MHC. MHC gene loci are also closely linked and for this reason individuals tend to inherit MHC alleles as two sets (haplotypes), one from each parent. In contrast to immunoglobulin and the TCR, MHC alleles are co-dominantly expressed.

Exogenous antigens taken up by antigen presenting cells (B cells, monocytes, dendritic cells) by phagocytosis / endocytosis are degraded to peptides within the endocytic processing pathway. Within this pathway, they may bind to the peptide cleft of class II MHC and peptide – class MHC II complexes may then be exported to the cell surface for presentation. T (helper) cells displaying CD4 are able to recognise and respond to peptide – class II MHC.

Endogenous protein antigens (such as viral antigens or cancer antigens) are produced within the host cell itself. These antigens may be degraded within the endoplasmic reticulum and complexed to class I MHC prior to transportation to the cell surface. Class I MHC is expressed on the cell surface of all nucleated cells. CD8 positive (cytotoxic) T cells are able to recognise and respond to MHC class I complexed to peptide.

1.4 CD40

CD40 is a type I transmembrane protein belonging to the tumour necrosis factor receptor (TNFR) super-family. Its expression was first described on human urinary bladder carcinomas and normal and malignant B cells in 1985[16]. Since its discovery, CD40 is now known to be expressed primarily on antigen presenting cells (APCs) such as dendritic cells, B lymphocytes and monocytes[17, 18]. CD40 has also been found on a variety of other cells that include endothelial and epithelial cells, fibroblasts, eosinophils, most B cell malignancies (e.g. non-Hodgkin's lymphomas) and many epithelial malignancies (e.g. breast, lung, ovary, bladder and melanoma)[16, 19-29]

cDNA encoding human CD40 was first isolated by expression cloning from a Burkitt's non-Hodgkin lymphoma cell line Raji[30]. The CD40 gene has been mapped to chromosome 20 using human-rodent somatic cell hybrids and to 20q11-20q13.2 by in-situ hybridisation. The human CD40 gene is expressed as a single 1.5kb mRNA species.

CD40 is a 48kDa phosphorylated glycoprotein composed of 277 amino acids (AA) with a 193 AA extracellular domain that includes a 21 AA leader sequence, a 22 AA

transmembrane domain and a 62 AA intracellular cytoplasmic domain. The extracellular segment of CD40 shares close homology to other members of the TNF-R superfamily.

1.5 CD154

CD154 (CD40L) is the natural ligand for CD40 and a member of the tumour necrosis factor (TNF) family. It exists in both a soluble and type II transmembrane bound form where it appears to form a trimeric protein structure[31]. CD154 was first identified on a mutant T cell line and subsequently found to be expressed on activated, normal human CD4+ T cells[32-34]. CD154 is expressed predominantly on activated CD4+ T helper lymphocytes, but has also been described on other cells such as basophils, eosinophils, platelets, activated B cells, monocytes and Natural Killer cells[17, 18, 33, 35-37]. Significant CD154 expression has not been found on CD8+ T cells or non-lymphoid tissues[38].

cDNA encoding human CD154 has been isolated through the screening of stimulated human T cell libraries[39, 40]. The gene for human CD154 is located on the X chromosome at position Xq26.3-Xq27.1. It spans 5kb of DNA and consists of 5 exons [41]. Two mRNA species of 2.1 and 1.4kb have been identified through analysis of northern blotting[40].

CD154 is a 32-33kDa protein. Its structure, resolved by x-ray crystallography reveals a trimeric structure similar to that described for TNFα [31]. Apart from its 33kDa form, two biologically active, soluble versions of CD40L (31kDa and 18kDa) have been identified[42, 43].

1.6 CD40 – CD154 signalling

Normal, bi-directional CD40-CD154 interactions are central to the generation of both T cell dependent, humoral immune responses and cytotoxic T cell responses.

1.6.1 CD154-CD40 signalling and humoral immunity

CD40 is expressed on B cells throughout their development from CD34+ B cell precursors until terminal differentiation into plasma cells[44]. CD154 (expressed on CD4+ helper T cells following the recognition of specific processed antigen in combination with class II MHC on the B cell surface) ligation of CD40 provides an essential effector signal to naïve B cells that promotes B cell growth, differentiation and maturation. More specifically, CD154-CD40 signalling leads to up-regulation of key B cell co-stimulatory molecules such as CD80 (B7.1) and CD86 (B7.2) and surface activation molecules that include CD23 (FcεRII), CD54 (ICAM-1) and CD95 (Fas) [45] [46] [47]. CD40 signalling leads to rescue of B cells from apoptosis[48-50] and the induction of immunoglobulin class switching with production of high affinity antibody to T-cell dependent antigens[51, 52]. The critical role of this interaction is illustrated by the X-linked hyper IgM syndrome, a serious, congenital immunodeficiency disease arising from a mutation in the gene encoding CD40L [53, 54]. It is characterised by severe impairment of T cell dependent, B cell antibody responses, a lack of B cell memory, absence of lymph node germinal centres and no circulating IgA, IgE or IgG. Patients with this syndrome suffer from an increased incidence of recurrent pyogenic infections.

1.6.2 CD154-CD40 signalling and cellular immunity

CD154-CD40 signalling is of critical importance to the regulation of cellular as well as humoral immune responses[18]. Recognition of specific peptide complexed to MHC II on the surface of resting APCs by CD8+ T helper cells in the context of appropriate co-stimulation leads to T helper cell activation and expression of CD154. CD154 – CD40 signalling upregulates the antigen presenting function of APCs, inducing high levels of MHC II, upregulating key co-stimulatory molecules such as B7-1 and B7-2, accessory molecules such as LFA-3 (CD58) and increasing the production of activating cytokines such as, IL-8, IL-12, TNF-α and macrophage inflammatory protein 1 α (MIP1-α)[55]. IL-12 in turn upregulates CD154 expression and also functions as a major cytokine governing the differentiation of T helper cells to a T_H1 phenotype[56].

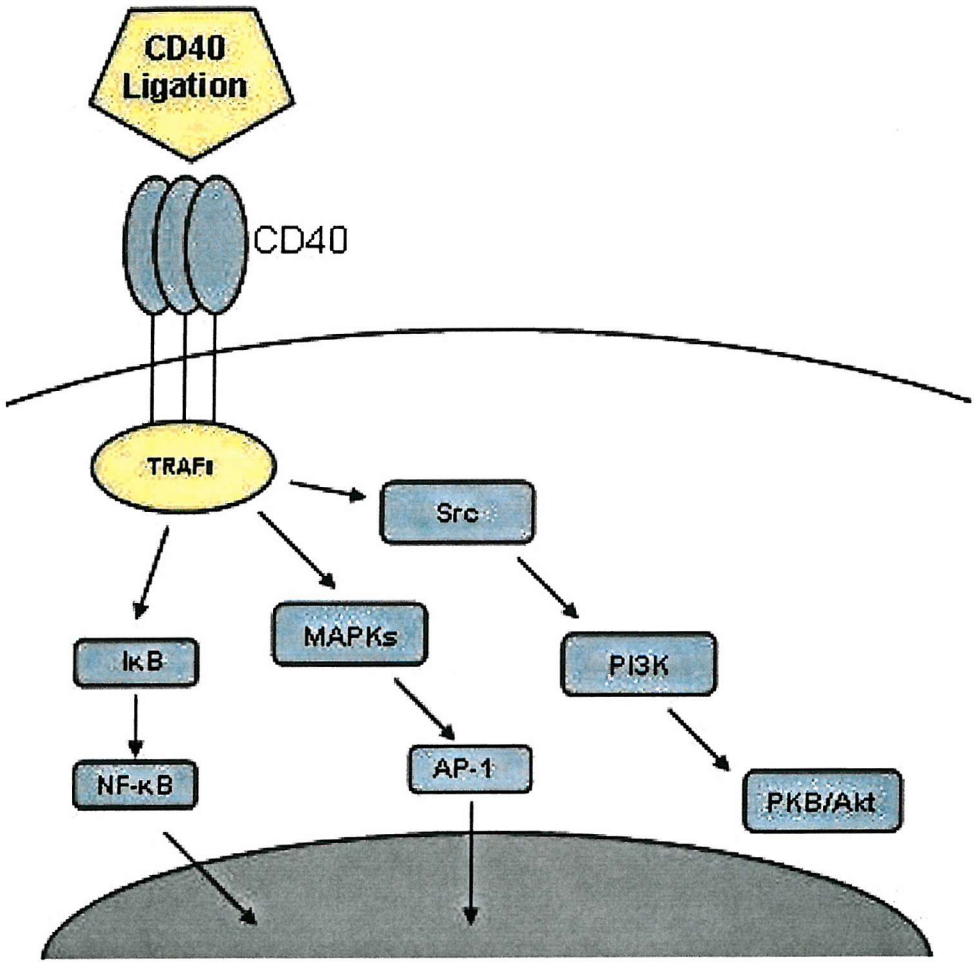
CD154-CD40 signalling is essential for the cross-priming and activation of CD8+ cytotoxic T cells. Activated CD4+ helper T cells signal via CD154-CD40 to the APC and effectively empower or license the APC (through the mechanisms described above) to effectively present antigen to and activate antigen specific responding CTL precursors[57-59].

1.6.3 CD40 signal transduction pathways

In common with other members of the TNF-R superfamily, the cytoplasmic C terminus of the CD40 molecule lacks intrinsic enzymatic activity. Signalling via CD40 is mediated through its interaction with a family of proteins, known as Tumour Necrosis Factor-Receptor associated factors (TRAFs)[60], (figure 1.7). TRAFs themselves have no intrinsic enzymatic activity, but are thought to act as adaptor proteins promoting intracellular transduction through their ability to bind to receptors and potentiate the recruitment of proteins to a signalling complex. The TRAF family consists of 6 members (TRAF1-6), of which 1, 2, 3, and 6 can bind directly to the cytoplasmic tail of CD40 through their C-terminal TRAF domain[61]. TRAF1 and 5 may also interact with CD40 indirectly through the formation of hetero-oligomers, with TRAF2 and TRAF3. With the exception of TRAF1, the N-terminal portion of TRAF proteins contains a RING finger and several zinc finger motifs that are involved in mediating downstream signalling events[60].

Figure 1.7

Tumour necrosis factor receptor associated factor (TRAF) mediated CD40 signalling.



TRAFs are thought to be capable of activating a variety of kinases including IκB kinase (IKK); mitogen activated protein kinases (MAPKs) which include serine/threonine kinases such as, c-jun amino terminal kinase (JNK), p38 mitogen activated protein kinase and extracellular signal-regulated mitogen-activated protein kinase (ERK) and protein tyrosine kinases, including the Src family kinases [62, 63]. These differing activation pathways may result in the activation of a number of transcription factors that include NF-κB and AP1 and the antiapoptotic kinase PKB/Akt [64, 65].

1.7 CD154 - CD40 expression and function outside the immune system

The expression of CD40 has been reported on a variety of non-haematopoietic cells that include fibroblasts, endothelial cells and epithelial cells. Expression of CD40 in these tissues is generally low although it may be upregulated in a variety of disease states. The functional relevance of CD40 in these tissues remains relatively obscure; in patients suffering with hyper IgM syndrome or in CD40 / CD154 knockout mice, no gross abnormalities are observed in organs that have been demonstrated to express CD40[66].

CD40 expression on non-haematopoietic tissues may be important for the induction of inflammatory responses. In-vitro experiments on endothelial cells and fibroblasts have demonstrated the ability of CD154 to up-regulate the expression of a variety of cellular adhesion molecules such as intercellular adhesion molecule I (ICAM-1; CD54) and vascular cell adhesion molecule I (VCAM-1; CD106)[18, 67]. CD154 may also augment fibroblast cytokine production (e.g. IL-6), and trigger collagen / collagenase production. Epithelial CD40 expression has been reported in a diverse variety of tissues that include lung, kidney, thymus and cornea[68-71]. CD40 has also been identified on the basal layer of stratified squamous epithelium where it is associated with expression of Fas[21]. Expression is likely to relate to the cycling nature of these cells and receptor activation is likely to relate to cellular growth and differentiation.

CD40 has also been found on a variety malignant cells that include most B cell malignancies (e.g. leukaemia, lymphoma, myeloma) and the majority of epithelial malignancies (e.g. breast, nasopharynx, lung, ovary, bladder, melanoma and sarcoma)[16, 19-29, 72]. In contrast to the potent survival signal delivered to B cells through the ligation of CD40, considerable published data attests to the observation that ligation of CD40 on malignant cells by CD154 or agonistic anti-CD40 mAbs leads to growth inhibition or apoptosis[19, 21, 23, 25, 29, 73-82].

1.8 Evasion of the Immune system by cancer

The majority of tumours fail to elicit an adequate immune response with cancer arising and developing in spite of an intact immune system. A variety of mechanisms exist

which facilitate the escape of tumours from host immunity. Effective immunity requires the recognition and presentation of immunostimulatory antigens in an appropriate co-stimulatory environment. Tumours many not express immunogenic antigens or express them only weakly; malignant transformation is often associated with loss of MHC class I and therefore candidate antigens are poorly presented to CD8+ cytotoxic T cells. Additionally, critical co-stimulatory signals required for the activation of an effective cellular response may not be expressed; presentation of tumour antigens in the absence of effective co-stimulation will lead to a state of immunological tolerance or anergy.

1.9 Immunotherapy of malignancy

The immunotherapy of malignancy seeks to break immunological tolerance and augment, stimulate or mimic host immunity in the hope of treating or preventing the development of cancer. Cancer immunotherapy may be sub-divided into a number of broad categories that include the use of non-specific biological response modifiers (e.g. interferons and interleukins), specific and non-specific vaccination strategies and the use of monoclonal antibodies.

1.9.1 Biological Response Modifiers

1.9.1.1 Interferons

The interferons have wide ranging and important effects on the immune system (see 1.3.1.3.1). They have stimulatory effects on macrophages, NK cells, T lymphocytes and neutrophils. Interferons upregulate MHC class I and II expression facilitating antigen presentation. Interferons may also inhibit angiogenesis, induce cellular differentiation and inhibit the proliferation of tumour cells. Interferon-α is used in the treatment of a number of haematological malignancies (e.g. hairy cell leukaemia, chronic myeloid leukaemia, multiple myeloma) and some solid tumours (e.g. AIDS related Kaposi's sarcoma, malignant melanoma and renal cell carcinoma)[83-87]. Interferon-α treatment is commonly associated with systemic side effects. These side effects may be severe and dose limiting and include flu-like symptoms such as headache, fever, shivering and fatigue.

1.9.1.2 Interleukins

A number of interleukins have been used in cancer immunotherapy with Interleukin 2 (IL-2) being the most widely studied. IL-2 is a critical cytokine in the activation of cellular and humoral immune responses inducing proliferation of antigen primed CD4 and CD8 positive T cells and enhancing the cytotoxic activity of NK cells. The largest clinical experience with IL-2 has been in the treatment of malignant melanoma and renal cell carcinoma where it is associated with modest response rates [87-91]. Toxicity such as hypotension, fever and oedema is however dose limiting and often profound. In an attempt to limit systemic toxicities of IL-2, attempts have been made to isolate host lymphocytes and culture them ex-vivo in the presence of IL-2 prior to re-infusion. This approach has been shown to be feasible but remains experimental.

1.9.1.3 Tumour Necrosis Factor Family Members

In addition to the key role of CD40-CD154 in the regulation of immune responses, other members of the tumour necrosis factor (TNF) family of ligands / receptors are critically important in the regulation of cellular homeostasis through their ability to induce apoptosis or enhance cellular growth and proliferation. At least 26 receptors and 18 ligands belonging to the TNF family have been identified to date, with a number of ligands binding more than one receptor and some receptors being shared between one ligand.

The biologically active forms of TNF ligands and receptors consist of protein trimers which may exist as transmembrane or soluble forms. TNF family receptors are characterised by the presence of 40 amino acid, cysteine rich repeats in their extracellular domains. In contrast, intracellular domains do not share sequence homology but interact with two major groups of intracellular proteins, TNF receptor associated factors (TRAFS) (see section 1.6.3) and death domain (DD) containing proteins, through which TNF family members may mediate their diverse biological actions.

A number of TNF family members show direct anticancer effects[92]. Tumour necrosis factor α, a cytokine produced by monocytes / macrophages and TNFβ exhibits direct cytotoxic effects in-vitro when cultured with malignant cell lines[93]. TNFα has also been shown to inhibit tumour angiogenesis through damage to vascular endothelial cells. The therapeutic use of TNFα remains experimental; systemic administration has been found to be severely limited by significant toxicity, particularly hepatotoxicity, capillary leak syndrome and profound hypotension.

Therapeutic targeting of other TNF family members may be associated with less toxicity and thereby prove more useful in the treatment of malignancy. Such targets include CD30-CD30 ligand, receptor activator of nuclear factor-κB (RANK)-RANK ligand and TNF related apoptosis inducing ligand (TRAIL)-TRAIL receptor[92].

CD30 expression is restricted to activated B and T lymphocytes. CD30L is expressed on a range of haematopoeic cells. Both CD30 and CD30L are expressed on a number of lymphoproliferative and epithelial malignancies. The exact biological function of CD30 – CD30L interactions remains uncertain although recent studies suggest a role in T and B cell co-stimulation and cytokine secretion. The restriction of CD30 to a small number of normal cells and its expression on a range of malignancies make it a good target for mAb therapy and early phase clinical trials in human malignancies are currently in progress[94, 95].

RANK expression has been demonstrated on dendritic cells, CD8 and CD4+ cells and osteoclast precursor cells. RANK ligand is expressed predominantly on activated T cells and osteoblasts. RANK ligand (also known as osteoclast differentiation factor) binds to both RANK and osteo-protegrin (OPG) and signalling between RANK and its two receptors is critical for bone and calcium homeostasis. RANK knockout mice demonstrate defects in lymph node formation, B cell development and in bone resorption. Blockade of RANK signalling may well be of use in the treatment (or prevention) of lytic bone metastases or malignant hypercalcemia[96, 97].

TRAIL receptor activation leads to cell death through the activation of death receptors or direct activation of caspases. TRAIL is primarily expressed by activated T cells and NK cells. Many tumours express TRAIL receptors in contrast to normal human tissue where expression is unusual. TRAIL interactions are thought to have an important physiological role in tumour immune surveillance. Soluble TRAIL and mAbs directed against TRAIL receptors show in-vitro and in-vivo efficacy against a number of malignancies[98-100].

1.9.2 Vaccination Strategies

Vaccination strategies rely on the identification of suitable tumour antigens. Tumour antigens may be sub-divided into tumour specific antigens and tumour associated antigens.

Tumour specific antigens (TSAs) are unique to tumour cells and are not expressed on normal cells. In contrast, tumour associated antigens (TAAs) are not unique to tumour cells. TAAs include oncofoetal antigens (antigens expressed during foetal development but not normally expressed in the adult) and proteins normally expressed at very low levels in normal cells but at significantly higher levels on malignant cells. Tumours may express both tumour specific and tumour associated antigens. Following the identification of an appropriate tumour antigen, a variety of approaches may be taken in an attempt to stimulate host immunity through vaccination and a variety of TAAs are the subject of clinical vaccination trials (table 1.2).

Table 1.2

Examples of Tumour-Associated Antigens in Vaccine Trials. Adapted from [101]

Class of antigen	Example
Cancer testis antigens (melanoma, breast, lung and other cancers)	• MAGE-1 • MAGE-3 • NY-ESO-1 • LAGE-1/CAMEL
Melanocyte-differentiation antigens	• MART-1/MelanA • gp100/pmel-17 • Tyrosinase • Tyrosinase-related protein-1 (gp75) • Tyrosinase-related protein-2
Normal self-proteins	• Carcinoembryonic antigen • Prostate-specific antigen • Prostate-specific membrane antigen • Human chorionic gonadotropin-beta • Melanocyte-stimulating hormone receptor • HER-2/neu • CA125 • GA733 • Gp72

Much of the clinical experience in tumour vaccination has been in patients with malignant melanoma using autologous or allogeneic tumour cells as a source of antigen for immunisation[102]. Preparation of individualised, autologous vaccines is significantly more labour intensive but offers the chance of immunising with patient / tumour specific antigens; allogeneic vaccines offer a more uniform source of antigen but allow targeting of only shared antigens. Immunological adjuvants such as bacille Calmette-Guerin (BCG), Corynebacterium parvum or alum are generally added to the vaccine in an attempt to promote adequate antigen presentation and enhance immune responsiveness[103, 104].

A variety of specific peptide antigens known to be presented in combination with MHC have been identified from tumour antigens and have been generated for use in vaccination strategies. In an attempt to further enhance the immunogenicity of vaccines, efforts have been made to directly transfer such antigens into antigen presenting cells such as dendritic cells (DCs). DCs are key mediators of vaccine function, activating T cells through antigenic capture, processing and presentation in concert with appropriate co-stimulatory signals. A variety of experimental techniques have been utilised and theses include the expression of tumour cell RNA in host APCs, pulsing of APCs with peptides eluted from the surface of tumour cells and the creation of DC-tumour cell fusions[105, 106].

The use of heat shock proteins (HSPs) derived from tumour cell lines represents another treatment approach. HSPs have been shown to carry antigenic peptide determinants of cellular proteins (including tumour antigens) to the MHC molecules of the endoplasmic reticulum. When administered as a vaccine HSPs may gain access to both class I and II MHC antigen processing pathways of APCs thereby inducing specific T cell responses[107].

Vaccination with naked DNA is an alternative approach. Intramuscular injection with naked DNA encoding a tumour antigen can effectively induce cellular and humoral immune response due to efficient transcription and translation into peptide and

presentation of antigen by APCs. Immune responses may be augmented through the attachment of DNA encoding an immune alert signal such as that encoding fragment C of tetanus toxoid [108, 109].

1.9.3 Antibodies

Over the past 25 years, monoclonal antibodies (mAbs) have revolutionised not only experimental and diagnostic laboratory techniques but have established themselves as the most rapidly expanding class of therapeutic agents for a wide range of human diseases [4]. Although only a handful of mAbs have been licensed for routine therapeutic use, several hundred are currently in clinical development, many of them aimed at treating cancer.

See http://archive.bmn.com/supp/ddt/glennie.pdf

1.9.3.1 Polyclonal antibodies

Naturally occurring, soluble antibodies are generated in-vivo following exposure of foreign antigen to the immune system. In the laboratory, antibodies may be raised artificially by injecting antigen into an animal and collecting the resultant antibody rich serum several weeks later. This serum (also known as antiserum) will contain a heterogeneous, polyclonal mixture of antibodies. Polyclonal antibodies, by definition, are produced by the clonal populations of a number of different B lymphocytes whose surface immunoglobulin recognise any of the epitopes (specific antibody binding sites) present on the given antigen.

1.9.3.2 Monoclonal antibodies

Antibodies produced from identical copies or clones of a single B lymphocyte will have identical antigen epitope specificity and are known as monoclonal antibodies (mAbs). In 1975, an experimental technique was devised that enabled stable and permanent production of mAb [110]. This technique opened the doorway to the widespread introduction of mAbs into experimental, diagnostic and therapeutic work and earned its developers, Köhler and Milstein, the Nobel prize. Köhler and Milstein's method immunises animals, generally rodents, with an antigen of interest in order to elicit an

antibody response. A cell suspension containing antibody producing B lymphocytes is then prepared from these animal's spleens and mixed in-vitro with a myeloma (immortal B lymphocyte tumour) cell line. The addition of polyethylene glycol to the cell suspension will result in cell-cell fusion and the generation of hybridomas - the fusion products of normal, antibody producing B cells and myeloma cells. Through the use of myeloma cell lines that lack the ability to synthesise endogenous immunoglobulin, hybridomas may be created that secrete antibody specific to the antigen used for the original immunisation and possess the immortal growth properties of the myeloma cell line. Once antibody secreting hybridomas have been selected, they can be screened to identify and isolate those hybridomas producing antibodies with the desired antigenic specificity. This is generally achieved by diluting out hybridoma cell suspensions to single cell dilutions which are subsequently grown up as clonal cell populations. The presence of specific antibody within the cell supernatant can be assayed by a variety of techniques such as enzyme linked immunosorbant assay (ELISA) or radioimmunoassay. By repeat limiting dilution, truly monoclonal cell populations may be obtained thereby enabling stable and permanent production of large quantities of mAb through modern continuous tissue culture techniques.

1.9.3.3 Antibody humanisation

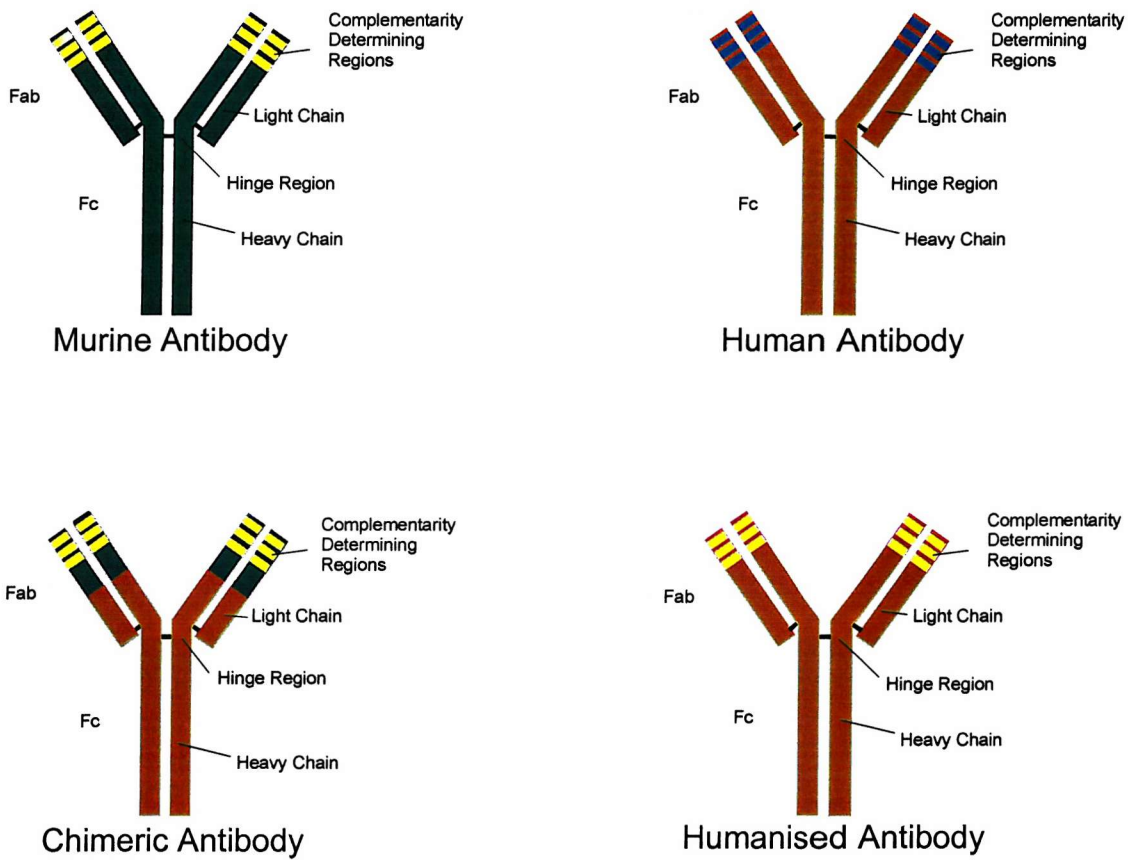
The techniques of Kohler and Milstein rely on the immunisation of animals (generally mice), to derive a population of antibody producing B lymphocytes. It follows that the mAb produced by this system will be of mouse origin (murine). Although murine mAbs are of immense importance in experimental and diagnostic techniques, their use in human therapy is problematic. Murine antibodies may be highly immunogenic to humans and repeated administration commonly leads to the generation of human anti-mouse antibody (HAMA) responses. HAMA responses not only adversely affect the clinical efficacy and half life of antibody but are also implicated in the clinical symptoms of allergy and anaphylaxis. In addition, because the Fc fragment of the mAb is of murine rather than human origin, its therapeutic administration may fail to activate appropriate immune effector functions such as CDC and ADCC, and as such prove ineffective.

1.9.3.4 Chimeric monoclonal antibodies

In an attempt to overcome the HAMA response and improve the clinical efficacy of murine derived mAbs for human therapy, genetic engineering techniques have been developed to "humanise" murine mAbs (figure 1.8).

Figure 1.8

Schematic representation of murine, human, chimeric and humanised antibodies.



Genes that encode for mouse antibody variable regions specific to an antigen of interest may now be identified, cloned and inserted into a vector along with the genes encoding for human immunoglobulin constant domains. Transfection of this vector into an appropriate cell line such as Chinese hamster ovary cells will result in expression of these transfected genes and synthesis of a chimeric mAb containing mouse variable and human constant regions. These cells may be screened, cloned and expanded like conventional hybridomas to provide a stable source of chimeric mAb.

1.9.3.5 Humanised monoclonal antibodies

The humanisation of murine derived mAbs has been further developed to produce "complementarity determining region (CDR) grafted" or humanised mAbs. This approach requires the synthesis of a novel variable region utilising gene sequence information for the three epitope specific murine CDRs combined with compatible sequences from human variable framework regions. The newly created humanised variable regions can then be linked to human constant region genes that when expressed in an appropriate cell line will produce a humanised mAb. In practice, grafting of murine CDRs alone usually results in some loss of antigen binding affinity and a number of the original framework region amino acid residues may also need to be retained alongside the CDRs to maintain mAb affinity.

1.9.3.6 Fully human monoclonal antibodies

The production of fully human mAbs is now possible through the use of phage display libraries or transgenic mice carrying human immunoglobulin gene loci. Phage display technology requires the isolation of gene segments encoding for human variable mAb domains and their fusion to genes encoding for bacteriophage coat proteins. By infecting bacteria with these transfected bacteriophages, phage particles containing immunoglobulin variable domain proteins will be synthesised and expressed. Phage display libraries may be built up consisting of a large collection of phages ($> 10^{10}$), each expressing a variable domain specific for an individual antigen. By challenging these libraries with antigens of interest and isolating phages that express appropriate antigen binding domains, the gene encoding the variable region of interest may be recovered

from the isolated phage, joined to the remaining parts of a given human immunoglobulin gene and transfected into an appropriate host cell capable of secreting the resultant fully human mAb. In addition to the fully human nature of mAbs produced through phage display techniques, other advantages of this technique include the number and diversity of mAbs that can be generated in a relatively short time frame and the ability to use selection and screening strategies to isolate mAbs with desirable characteristics (e.g. high affinity) [111, 112].

An alternative approach in development is the production of fully human mAbs utilising transgenic animals [113, 114]. This technology requires deletion of an animals own immunoglobulin genes and the subsequent introduction of human immunoglobulin gene segments. As a result of this genetic manipulation, antigen immunisation results in the de novo generation of a fully human immunoglobulin which may be modified using standard somatic fusion technology to generate mAbs of any class desired.

1.9.4 Immunotherapy with antibodies

Antibodies are the mediators of humoral immunity. Following the recognition of specific antigen, antibodies may trigger a number of immunological effector mechanisms that include antigen opsonisation, complement mediated cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC). CDC and ADCC are likely to play an important role in the clinical efficacy of therapeutic antibodies. In addition to these classical functions, therapeutic mAbs provide scope for additional modes of action. These include blockade or augmentation of cell signalling by tumour cells or by cells of the immune system and the delivery of a variety of cytotoxic immunoconjugates (figure 1.9)

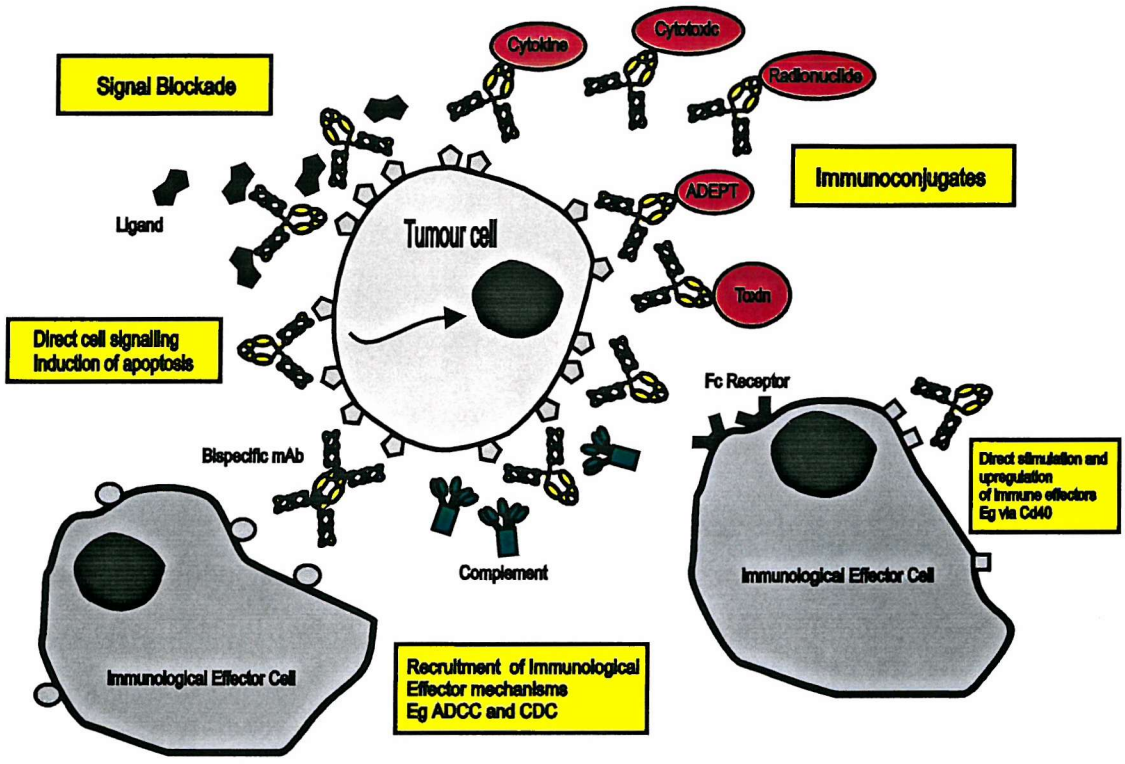
1.9.4.1 Complement mediated cytotoxicity (CDC)

As discussed in section 1.3.1.3.2, activation of complement by antibody-antigen complexes via the classical pathway is a major effector component of humoral immunity promoting the development of an inflammatory response, target cell opsonisation and cell lysis. CDC may be an important mechanism through which

therapeutic antibodies used for the treatment of malignancy may achieve their anti cancer effect[115]. However, the expression of complement inhibitors (see 1.3.1.3.2.2) on tumour cells may be problematic, limiting the ability of therapeutic anti-cancer monoclonal antibodies to effectively activate complement dependent cytotoxicity against target malignant cells[116].

Figure 1.9

Potential mechanisms of action of therapeutic antibodies.



1.9.4.2 Antibody dependent cellular cytotoxicity (ADCC)

ADCC is thought to be an important mechanism of action for therapeutic antibodies used in the treatment of malignancy. ADCC refers to the ability of antigen bound antibody to recruit Fc receptor expressing NK cells and macrophages / monocytes to a target dictated by antibody specificity. Once localised to the target cell, the secretion of lytic enzymes, tumour necrosis factor and other cytotoxic substances by effector cells mediate target cell killing (see 1.3.2.1.6.3). Supporting evidence for the role of ADCC in immunotherapy comes from the observation that antibody isotypes that effectively recruit ADCC in-vitro generally exhibit good in-vivo activity[117, 118].

1.9.4.3 Cell signalling and blockade

Therapeutic antibodies may be raised against a variety of cell surface receptors expressed on the surface of malignant cells. Upon receptor binding, mAbs can stimulate or block receptor signalling and in doing so provide growth inhibitory or apoptotic signals or block / prevent growth signals. The development of Trastuzumab (Herceptin), a humanized IgG_1 mAb directed against the extracellular domain of the HER-2/neu (c-erbB-2) epidermal growth factor receptor (EGFR) and licensed for the treatment of metastatic breast cancer is an example of this approach (see 1.9.7.5).

MAbs can also be raised against key cytokines or ligands and prevent cell-cell signalling through their elimination or by preventing their binding to target receptor. Of considerable interest are mAbs that have been developed to mimic or block ligand / receptor interactions that are capable of promoting strong cellular immune responses against tumours, irrespective of tumour antigen expression. These molecules, amongst others, include the immune cell co-receptors CD152 (CTLA4) and CD40 (see 1.10). Normal signalling through CTLA4 (cytotoxic T lymphocyte antigen 4), expressed on the surface of T cells, down regulates T cell activation. It appears possible through the development of mAbs that can bind and block CTLA-4 signalling, to promote T cell activation and proliferation and significantly enhance tumour cytotoxicity [119].

1.9.5 Immunoconjugates – Arming mAbs

Laboratory techniques exist that allow the conjugation of a wide variety of molecules to specific antibodies and this approach is discussed below. Given careful selection of a target tumour antigen, this technology permits the selective delivery of an anticancer agent directly to antigen expressing tumour cells maximising tumour delivery and minimising systemic toxicity.

1.9.5.1 Immunotoxins

A variety of plant and bacterial toxins have been conjugated to mAbs and tested in pre-clinical and early phase clinical trials as anticancer therapies. These have included the ribosome inactivating proteins, ricin and saporin, and bacterial toxins such as Pseudomonas exotoxin [120, 121]. These molecules are highly toxic and without conjugation and selective delivery by mAb, their systemic use would not be feasible. Plant or bacterial toxins are potentially immunogenic and antibody responses to toxin immunoconjugates have been noted. Despite selective toxin delivery, clinical trials have encountered problematic toxicity, predominately with vascular leak syndrome [122]. This syndrome, manifested by widespread oedema that may be dose limiting and severe, is thought to be secondary to toxin mediated endothelial injury. Encouragingly, recent animal data suggest that for ricin, it may be possible to modify a short amino acid motif in the ricin A chain and abolish vascular leak syndrome without loss of tumour cytotoxicity [123].

1.9.5.2 Cytotoxic Agents

A variety of conventional chemotherapy agents have been conjugated to mAbs and some of these cytotoxic immunoconjugates have been evaluated in clinical trials. Doxorubicin, a well established anthracycline cytotoxic in routine systemic use, has been conjugated to a mAb that recognises the oncofoetal antigen Lewis Y, expressed on many common solid tumours. Preclinical testing revealed promising activity but early phase clinical trials in the treatment of breast and gastric malignancies have been disappointing [124-126]. The absence of significant clinical activity may be related to the low proportion of antibody (and therefore cytotoxic drug concentration) that can

effectively localise to target. In contrast to conventionally administered doxorubicin, the dose limiting toxicities of the immunoconjugate were found to be gastrointestinal rather than haematological, a finding thought to be related to binding of normal tissue by mAb. Gemtuzumab ozogamicin (Mylotarg), a humanised IgG_4 mAb directed against the CD33 antigen and conjugated to the tumour antibiotic calicheamicin, (an agent several hundred fold more potent than doxorubicin) has met with more clinical success and is discussed later (see 1.9.7.3).

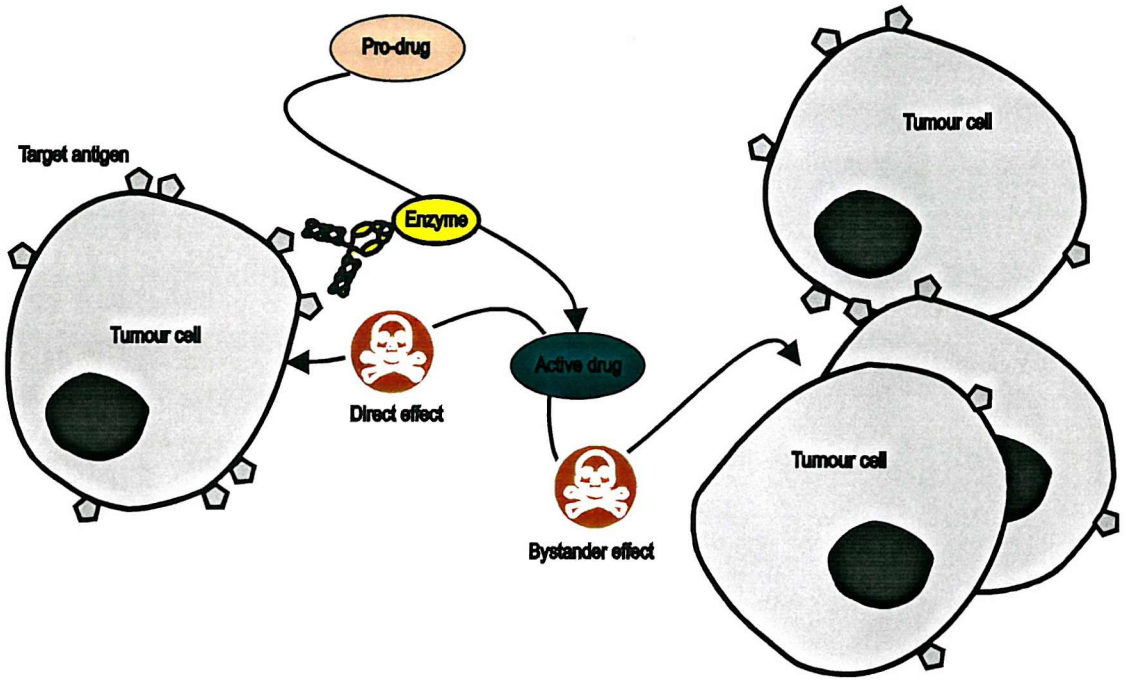
1.9.5.3 Antibody directed enzyme-prodrug therapy (ADEPT)

An alternative approach with the aim of selectively delivering cytotoxic drug to the site of tumour is known as antibody directed enzyme-prodrug therapy (ADEPT). This approach involves the activation of a cytotoxic pro-drug at the tumour site by an enzyme that has been targeted to a tumour through its conjugation to a specific mAb (figure 1.10)

This approach has a number of theoretical advantages. Exceptionally high concentrations of active drug can be selectively delivered to any number of tumour sites whilst minimising active drug exposure to normal tissues (and hence toxicity). In contrast to the delivery of mAb-toxin/chemotherapy conjugates, a much larger number of active cytotoxic molecules can, in theory, be generated at the tumour site by a single mAb-enzyme conjugate. The generation of high concentrations of active drug within the interstitial space of a tumour site allows for effective drug diffusion and exposure to adjacent tumour cells, without the need for antibody binding (bystander effect). This can help to overcome the limited diffusion capabilities of antibodies within tumour sites and the considerable variability in intra-tumoral antigen expression. However, in common with other immunoconjugates immunogenicity has been a problem. This may be overcome through the use of human(ised) enzymes and mAbs, an approach already being undertaken [127]. A number of early phase I clinical trials have been undertaken and clinical development is ongoing [128, 129]. Some tumour responses have been seen but myelosuppression appears to be dose limiting, a feature likely to be related to diffusion of activated drug from the site of tumour into the systemic circulation.

Figure 1.10

A schematic representation of ADEPT therapy. Inactive, pro-drug is converted to active drug at the tumour site by an enzyme conjugated to a mAb. Active drug may have a direct effect on tumour cells bound by mAb and is also able to diffuse to and act on adjacent tumour cells not bound by mAb (bystander effect).



1.9.5.4 Cytokines

A number of mAb immunoconjugates have been developed to target cytokines (including interleukin-2 (IL-2), interleukin 12 (IL-12) and granulocyte / macrophage colony-stimulating factor (GM-CSF)) to the tumour micro-environment. This approach has the aim of enhancing the recipient's immune response to tumour whilst minimising the substantial side effects that are associated with systemic cytokine delivery [130]. Promising preclinical results have been achieved using this approach to deliver IL-2, a cytokine normally produced by responding T helper cells that is able to stimulate T cells

to proliferate and become cytotoxic. Early clinical trials with IL-2 immunoconjugates are ongoing.

1.9.5.5 Radioimmunotherapy

By arming antibodies with radionuclides, effective tumour cell killing may be augmented through the delivery of radiotherapy to target and bystander cells (tumour cells in close proximity, but not bound to mAb) in addition to any endogenous, anti-tumour activity of the naked carrier antibody [131]. Radioimmunotherapy, in common with ADEPT may overcome some of the potential problems of variable tumour antigen expression and poor mAb penetration in poorly vascularised or bulky tumours. Clinical studies in non-Hodgkin's lymphoma of ^{90}Yttrium labelled Ibritumomab, a radiolabelled anti CD20 mAb, have demonstrated its superior efficacy over naked mAb [132] (see 1.9.7.2). Radioimmunotherapy is also under investigation in the treatment of solid tumours. Pemtumomab (Theragyn) is a murine IgG_1 mAb that has been conjugated to the radioisotope Yttrium-90. It binds specifically to a glycoform of MUC1 (CD227), a cell surface glycoprotein overexpressed on the surface of epithelial tumour cells, including ovarian, gastric, breast and lung. The results of a recently completed large phase III study (SMART trial) evaluating its efficacy in addition to standard adjuvant therapy for epithelial ovarian carcinoma are awaited. An alternative radioimmunotherapy approach that has been examined in early clinical trials involves the use of tumour specific mAbs conjugated to streptavidin [133]. Tumour selective delivery of radionuclide can then be accomplished through its conjugation to biotin, a molecule with extremely high affinity for streptavidin.

1.9.6 Bispecific antibodies

MAbs have been generated that can bind to and activate the T cell receptor, independent of MHC or antigen. Bispecific antibodies, immunoglobulin constructs that can effectively bind two epitopes, have been created that combine this T cell activating property whilst also recognising a tumour antigen. In doing so, bispecific antibodies can activate and retarget cellular immune responses towards a pre-selected tumour antigen. T cell activation in this situation requires effective cross-linking by antibody and by

designing bispecific antibodies with only a single T cell arm, widespread T cell activation can be limited; only those bispecific antibodies that have bound to tumour cells and are presented in a multimeric array will effectively activate T cells. Bispecific antibodies that can target natural killer cells (via CD16 / FcγRIII) and macrophages (via FcαRI / CD89) have also been designed. This approach is not limited to the recruitment of cellular immunity; in theory, bispecific antibodies can be designed to recruit and deliver an array of cytotoxic agents such as radionuclides, toxins, cytokines and cytotoxic drugs [134, 135]. Although this approach has shown promising results in vitro, in vivo animal and human studies have met with more limited success [136].

1.9.7 Successful antibody targeting: MAbs licensed for clinical use

A number of mAbs have now been developed for the treatment of malignancy in humans and their use is discussed below. A much larger number of mAbs are currently being evaluated in a range of Phase I, II and III trials.

1.9.7.1 Rituximab

Four mAbs currently in routine clinical use target cluster designation (CD) molecules expressed on a variety of haematological malignancies. Rituximab (Rituxan) was the first mAb to be approved for the treatment of a malignant condition. It was rapidly incorporated into clinical use and quickly became the most successful selling new anticancer drug ever. Licensed for the treatment of B-cell lymphoma, it is a chimeric IgG_1 anti-CD20 mAb. CD20 is a transmembrane protein expressed on normal and malignant B cells but is absent from stem cells, plasma cells and non-lymphoid tissue. Rituximab binds CD20 with high affinity and is thought to achieve its clinical effects through the activation of CDC, ADCC and the induction of apoptosis [137-139]. Both normal and malignant B cells are targeted but the regeneration of normal B cells from pluripotent stem cells effectively leads to the selective depletion of the malignant clone. Rituximab has demonstrated efficacy in the treatment of both low grade and intermediate grade non-Hodgkin's lymphoma (NHL), as a single agent or in combination with standard chemotherapy regimens. In the pivotal phase II study of Rituximab therapy for follicular NHL, response rates of 48% were achieved in relapsed

disease, with minimal toxicity [140]. Significantly higher response rates have subsequently been demonstrated in trials of first line therapy [141]. Retreatment with Rituximab at relapse seems well tolerated and effective with a very low rate of human anti-chimeric antibody (HACA) development [142]. For intermediate grade NHL, the pivotal phase III study evaluating the treatment of diffuse large B cell NHL in elderly patients, demonstrated that the addition of Rituximab to standard CHOP chemotherapy significantly improved response rates, event free survival and overall survival without an increase in clinically relevant toxicity [143].

1.9.7.2 ^{131}Iodine-Tositumomab and ^{90}Yttrium-Ibritumomab

^{131}Iodine-Tositumomab (Bexxar) and ^{90}Yttrium-Ibritumomab (Zevalin) also target the CD20 molecule but in contrast to Rituximab have been conjugated to radionuclides. Both ^{90}Yttrium labelled Ibritumomab, a murine IgG_{2a} anti-CD20 mAb (the parent mAb of Rituximab) and ^{131}Iodine labelled Tositumomab, a murine IgG_1 anti-CD20 mAb are in use for the treatment of relapsed or refractory low-grade, follicular, or transformed B-cell non-Hodgkin's lymphoma. A randomised phase III trial of ^{90}Y-Ibritumomab against Rituximab in patients with refractory or relapsed low grade, follicular or transformed NHL, demonstrated that the addition of a radionuclide to naked mAb significantly improved overall response rates and complete response rates [132]. Treatment with ^{90}Y-Ibritumomab was well tolerated but associated with an increased incidence of reversible myelosuppression.

1.9.7.3 Gemtuzumab ozogamicin

Gemtuzumab ozogamicin (Mylotarg) is a humanised IgG_4 mAb conjugated to novel enediyene tumour antibiotic, calicheamicin. It is directed against another CD molecule, CD33, a sialic-acid-binding Ig-like lectin found on the surface of 80-90% of acute myeloid leukaemia (AML) cells and myeloid progenitor cells. It is licensed for the treatment of first relapse, CD33 expressing AML in elderly patients. In common with CD20 it is not expressed on pluripotent stem cells or normal non-haematopoietic tissues [144]. Calicheamicin becomes inactivated when conjugated to a mAb but is selectively reactivated within the tumour following receptor binding and antibody internalisation.

Calicheamicin is an extremely potent inducer of double-stranded DNA breaks [145]. Remission rates of 30% have been demonstrated using Gemtuzumab ozogamicin in patients with relapsed AML although significant myelosuppression and hepatotoxicity has been encountered [146].

1.9.7.4 Alemtuzumab

Alemtuzumab (Campath-1H) is a humanised IgG_1 mAb directed against the CD52 antigen, a glycopeptide highly expressed on the majority of normal and malignant B and T lymphocytes but not normal stem cells. It is thought to achieve its therapeutic effects through a combination of CDC and ADCC [118, 147, 148]. It is licensed for the treatment of patients with B-cell chronic lymphocytic leukaemia who have been treated with alkylating agents and have failed fludarabine therapy. In a large phase II study, objective response rates of 33% were seen [149]. In contrast to Rituximab, the treatment of B-CLL by Alemtuzumab is associated with significant levels of immunosuppression and an increased risk of infective complications. A proportion of these infections are opportunistic in nature, a finding, at least in part, related to the T cell depletion that results from Alemtuzumab treatment. This property of Alemtuzumab has been exploited in studies evaluating the effects of T cell depletion in allogeneic bone marrow transplant grafts [150].

1.9.7.5 Trastuzumab

Trastuzumab (Herceptin) is approved for the treatment of metastatic breast cancer and is a humanized IgG_1 mAb directed against the extracellular domain of the HER-2/neu (c-erbB-2) epidermal growth factor receptor (EGFR). HER-2/neu is overexpressed on approximately 25% of breast cancers and its overexpression correlates well with a number of adverse histological prognostic factors that include tumour grade, size, ploidy, and lack of steroid receptor expression [151]. The antibody shows moderate efficacy with a response rate of around 15% when used as a single agent in the treatment of metastatic breast cancer [152]. When combined with conventional cytotoxic chemotherapy, Trastuzumab and has been shown to provide significant additional benefit. In the pivotal phase III trial, combination therapy resulted in

significantly higher response rates, longer median duration of response, better one year survival rates and improved overall survival [153]. The effects of Trastuzumab are likely to be mediated through a number of mechanisms that include signal blockade and immune effector recruitment [154, 155]. Of note, Trastuzumab, particularly when used in combination with anthracycline chemotherapy, has been associated with an increased incidence of cardiac dysfunction. The basis for the observed cardiotoxicity is, as yet, not fully explained. HER-2/neu is also overexpressed on a number of other epithelial malignancies that include lung, prostate, ovary and gastrointestinal tract and clinical trials using Trastuzumab in these diseases are underway.

1.9.8 MAbs in development and target antigens

In addition to those mAbs in routine clinical use, a large number of therapeutic anticancer mAbs are in pre-clinical and clinical development and many are being evaluated in clinical trials. Interest has been focused on a variety of tumour antigens that include cell surface receptors, oncofoetal antigens, cellular adhesion molecules and antigens associated with tumour neo-vasculature.

1.9.8.1 Growth factor receptors

In common with HER-2/neu, HER-1 is another member of the EGFR family and the target of Cetuximab (Erbitux) a chimeric IgG_1 mAb. HER-1 is implicated in tumour cell invasion, proliferation, metastasis and angiogenesis and its blockade by Cetuximab is associated with clinically useful activity in a number of solid tumours [156]. Cetuximab has shown therapeutic efficacy as a single agent or in combination with radiotherapy or chemotherapy and its evaluation continues in Phase III trials of the treatment of colorectal cancer and head and neck cancer.

1.9.8.2 Oncofoetal antigens

Carcinoembryonic antigen (CEA) is one of the best known and most extensively studied oncofoetal antigens. Oncofoetal antigens are proteins normally expressed transiently during embryonic development of normal tissues that are also expressed on the surface of many malignant cells. CEA is expressed on the cell surface of many

adenocarcinomas, particularly those of the gastrointestinal tract and its measurement in serum is a useful marker in the clinical management of colorectal cancer.

CeaVac, a murine IgG anti-idiotype mAb, is currently being evaluated in phase III clinical trials. In contrast to other therapeutic mAbs, the approach of CeaVac is one of vaccination rather than direct mAb mediated therapy. CeaVac is an anti-idiotype antibody that acts as an image of the true CEA antigen. It was developed by immunising Balb/C mice with a mAb (8019) against human CEA to produce a mAb recognising the idiotype of the 8019 mAb [157]. Following administration, it can act as a surrogate tumour antigen and lead to the development of "anti-anti"-idiotype antibodies that in turn recognise and bind the original tumour antigen CEA. Clinical studies have shown that administration of CeaVac is associated with the development of a potent humoral and cellular response against CEA in patients with resected colorectal cancer [158]. Disappointingly, a recent phase III clinical trial has failed to show any significant survival benefit following the addition of CeaVac to 5FU based chemotherapy for metastatic colorectal cancer, despite the development of anti-CeaVac antibodies in more than 75% of patients treated [159]. Antibodies to a number of other oncofoetal antigens are currently being evaluated. These include the Lewis X and Y antigens and tumour-associated glycoprotein-72, expressed on a variety of common solid tumours types.

1.9.8.3 Cellular Adhesion Molecules

A variety of antigens associated with cellular adhesion molecules have been identified as potential targets for therapeutic mAbs. Edrecolomab (Panorex) is a murine IgG_{2a} mAb that recognises Ep-Cam (17-1A antigen), an epithelial cellular adhesion molecule expressed on normal epithelial cells and various malignancies. Following the publication of a Phase III clinical trial demonstrating its efficacy as adjuvant therapy in the treatment of surgically resected Duke's C colorectal cancer, Edrecolomab was licensed for this indication in Germany [160]. However, initial clinical optimism has been met with disappointment following the results of a subsequent, much larger European study that has shown it to be inferior to standard chemotherapy and that its addition to chemotherapy does not improve overall or disease free survival [161]. The

targeting of endothelial cell adhesion molecules in an attempt to disrupt tumour angiogenesis is also under investigation. Target molecules include the integrins (αVβ3/ αVβ5), E-selectin, platelet/endothelial-specific cell adhesion molecule (PECAM) and vascular endothelial cadherin. Preclinical and clinical trials are in varying stages of development.

1.9.8.4 Tumour vasculature

Targeting tumour vasculature is an attractive alternative approach to cancer therapy with potential application to a broad spectrum of tumours. Vascular endothelial growth factor (VEGF) a potent pro-angiogenic factor produced by many types of tumour cells has been targeted by Bevacizumab, a humanised IgG_1 mAb currently in phase III clinical trials of the treatment of a variety of solid tumours. A recently reported phase III trial of Bevacizumab in the first line treatment of colorectal cancer has shown that the addition of this mAb to chemotherapy results in significantly improved survival, progression free survival, response rate and duration of response, compared to chemotherapy alone [162]. Bevacizumab has also shown promising activity in renal cell carcinoma[163]. Therapeutic mAbs against basic fibroblast growth factor (bFGF), another potent endothelial cell mitogen, are also in development.

1.9.9 Potential problems with antibody therapy

Although mAbs have shown great promise in the treatment of cancer, a number of problems have been encountered that have limited their success.

1.9.9.1 Antibody targeting and delivery

Tumours masses generally sit within a dense packing of stromal tissue, are poorly vascularised and lack a lymphatic circulation. These factors make effective delivery of mAb to target antigen difficult and represent one way in which tumours can avoid effective therapy. Tumour antigens may be internalised or shed by cells with a resultant decrease in tumour cell expression and circulating free antigen. Shed antigen is a more accessible target for circulating mAb, effectively reducing the quantity of mAb that can reach the malignant cell and potentially increasing mAb toxicity. If therapeutic mAb is

able to penetrate tumour, the expression of a target antigen may be highly variable amongst cells within a tumour or within histologically related tumours in different individuals. Poor targeting of cells with low or absent antigen expression will limit response to treatment and may lead to treatment resistance. The direct arming of mAbs through the development of immunoconjugates and utilisation of the "bystander effect" is one approach taken to try and overcome the problems of poor antibody penetration and low tumour antigenicity.

1.9.9.2 Antibody immunogenicity

Antibody immunogenicity has been an additional problem encountered during the development of mAb therapy. Human anti mouse antibody (HAMA) responses are commonly encountered following multiple infusions of mouse mAbs and will adversely affect the clinical efficacy and half life of antibody. HAMA responses are also implicated in the clinical symptoms of allergy and anaphylaxis. Chimerisation, humanisation and the production of fully human antibodies have dramatically reduced the incidence of this neutralising antibody response although the addition of cytotoxic conjugates in an attempt to improve therapeutic efficacy may once again increase immunogenicity.

1.10 CD40 as a target for monoclonal antibody cancer immunotherapy

The expression pattern of CD40 on a broad range of malignancies and the important functional role of CD40-CD154 in-vivo make CD40 an attractive target for antibody based immunotherapy. The potential mechanisms of action of anti-CD40 antibody immunotherapy are summarised below and represented schematically (figure 1.11).

1.10.1 Recruitment of immune effectors

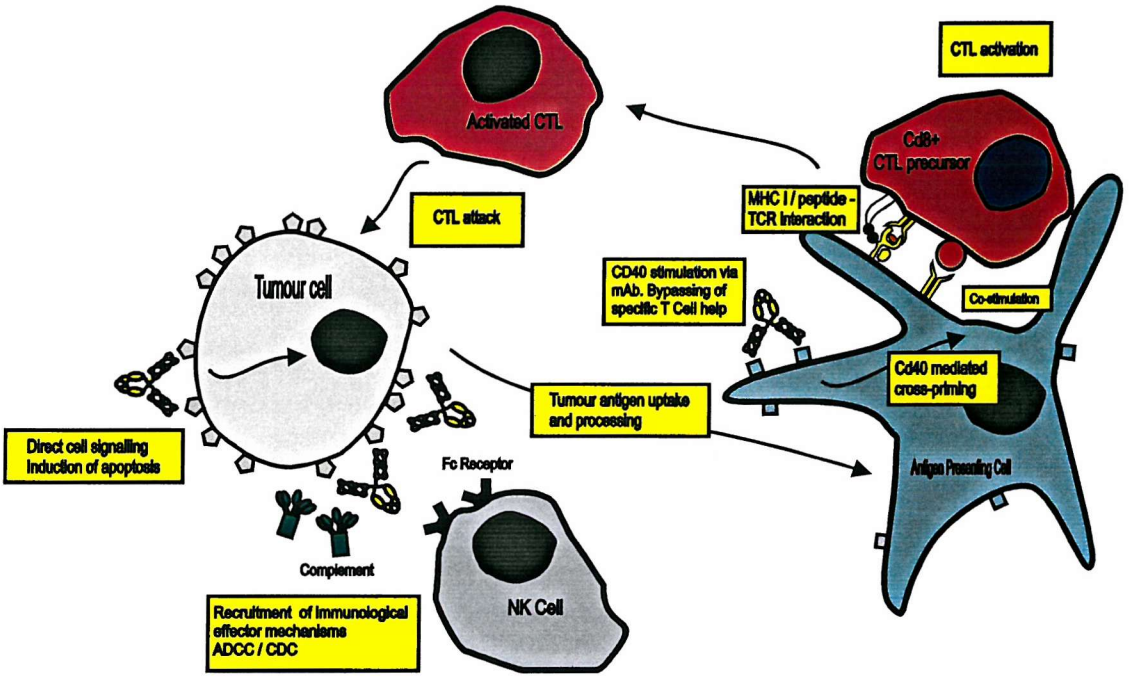
Anti-CD40 antibodies will target and bind tumour cells that express surface CD40. In common with other therapeutic mAbs, anti-CD40 mAbs binding their target antigen may effectively recruit immune effectors mechanisms such as complement-dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC).

1.10.2 Cell signalling

CD40 signalling plays an important role in the regulation of cellular growth. Considerable published data has demonstrated, that a variety of malignant epithelial and lymphoma cell lines undergo apoptosis or growth arrest in-vitro when treated with anti-CD40 therapy [19, 23, 25, 29, 73, 75-82].

Figure 1.11

Schematic representation of the potential mechanisms of action of anti-CD40 monoclonal antibody therapy.



1.10.3 Direct conditioning of APCs

CD154 – CD40 signalling provides an essential effector signal to naïve B cells that promotes B cell growth, differentiation and maturation. CD154-CD40 signalling is also of critical importance to the regulation of cellular as well as humoral immune responses upregulating the antigen presenting function of APCs, inducing high levels of MHCII,

key co-stimulatory molecules such as B7-1 and B7-2, accessory molecules such as LFA-3 (CD58) and increasing the production of activating cytokines such as, IL-8, IL-12, TNF-α and macrophage inflammatory protein 1 α (MIP1-α)[55]. CD154-CD40 signalling is essential for the cross-priming and activation of CD8+ cytotoxic T cells. Activated CD4+ helper T cells signal via CD154-CD40 to the APC and effectively empower or license the APC to effectively present antigen and activate antigen specific responding CTL precursors [57-59].

Stimulation of CD40 by therapeutic agonistic mAb can effectively bypass the need for specific T cell help, directly priming helper dependent CD8+ cytotoxic T cell responses [58, 59]. In animal models of malignancy, this effect translates into an impressive anti-cancer immunotherapeutic approach.

Published preclinical in-vivo experimental work from the Tenovus research laboratory in syngeneic mouse models of malignancy suggest that the most powerful therapeutic effect of anti-CD40 antibodies may relate to their ability to effectively license or condition APCs, completely bypassing the need for specific CD4+ T cell help to activate CD8+ cytotoxic T cell precursors [164] [165]. These comparative mouse model experiments were performed using the rat anti-mouse CD40 monoclonal antibodies 3/23 or FGK45. When these anti-CD40 antibodies were used to treat mice with syngeneic lymphoma, a rapid CD8+ cytotoxic T cell response occurred independent of CD4+ T cell help [164]. CD8+ cells recovered from responding mice showed powerful cytotoxic activity in-vitro against the target B-cell lymphoma. In-vivo, this response effectively eradicated lymphoma and provided protection against further tumour re-challenge without further antibody treatment. The model was reproducible in three different CD40+ murine lymphomas (A20, A31 and BCL1). Partial protection was seen in one of two CD40- tumours tested (EL4 and Ten1). Of considerable interest was the improvement seen in therapeutic activity when animals with a higher tumour burden were treated (i.e. later in the course of their lymphoma or when a larger primary tumour inoculum is given). In mice inoculated with a range of syngeneic CD40 negative epithelial tumour cell lines, FGK-45 provides significant therapeutic effects as well as

protection against tumour re-challenge [165]. The effectiveness of treatment was seen to correlate with the intrinsic immunogenicity and aggressiveness of the inoculated tumour and was critically reliant on the presence of CD8+ T cells.

Clearly, anti-CD40 mAb therapy has a number of advantages over conventional tumour antigen targeting by mAb. Although many tumours express CD40, the upregulation of antigen presentation and direct cross-priming of cytotoxic T cell responses by anti-CD40 mAb therapy means that virtually all tumours may be targeted regardless of tumour CD40 expression, with the potential for the development of persistent, long term, anti-tumour immunological memory.

1.10.4 Potential toxicities of anti-CD40 therapy

Widespread activation of CD40 through the use of CD154 or agonistic anti-CD40 mAbs may have deleterious effects. CD40-CD154 interactions are critically important for the generation of humoral and cellular immune responses and it could be hypothesised that widespread CD40 ligation and activation might give rise to immune mediated disease. Studies with CD154 blocking agents such as anti-CD154 mAbs suggest that aberrant CD40 activation may well be important to the pathogenesis of diseases as diverse as systemic lupus erythematosus [166], rheumatoid arthritis [167], type I diabetes mellitus [168], atherosclerosis [169-172], neurodegenerative disorders [173-175], graft versus host disease and allograft rejection [176, 177].

Much of the evidence relating to CD40 and autoimmunity relates to the ability of CD154 blockade to prevent the development of animal models of auto-immune disease. It remains to be seen whether short term treatment of patients with malignancy with CD154 or agonistic anti-CD40 mAbs would be capable of inducing clinically relevant autoimmune disease.

1.11 Clinical trials of anti-CD40 therapy

No clinical trials of anti-CD40 monoclonal antibody therapy in the treatment of human malignancy have been published.

One phase I clinical trial of systemic CD154 (CD40 ligand) therapy in humans has been published [178]. Vonderheide et al used recombinant human CD154 in patients with advanced intermediate or high grade non-Hodgkin's lymphomas or solid tumours. Treatment was administered sub-cutaneously, daily for 5 days and repeated again six weeks later. Subsequent treatments were given to responding patients at four weekly intervals. Treatment was generally very well tolerated. The maximum tolerated dose was established as 100 μg/kg/day; the dose limiting toxicity was a transient, reversible elevation of serum liver transaminases. Thirty-two patients were treated. One patient with heavily pre-treated squamous carcinoma of the larynx achieved a complete response; one patient with large cell immunoblastic T cell non-Hodgkin's lymphoma achieved a partial response. Twelve patients achieved stable disease. Induction of autoimmune disease was not observed.

An alternative treatment approach utilising CD154 gene transfection rather than systemic CD154 administration has also been undertaken [179, 180]. Wierda et al developed a replication defective adenoviral vector (Ad-CD154) able to induce recombinant CD154 expression in chronic lymphocytic leukaemia (CLL) cells. Having demonstrated that CLL cells transduced to express CD154 and bystander CLL cells could become highly effective antigen-presenting cells able to induce CLL-specific autologous cytotoxic T lymphocytes responses in-vitro, they investigated the immunologic and clinical responses in-vivo to the infusion of autologous Ad-CD154-CLL cells in patients with CLL. After a single bolus infusion of autologous Ad-CD154-transduced cells, increased or de novo expression of immune accessory molecules on bystander, non-transfected CLL cells was demonstrated. Treated patients developed high plasma levels of interleukin-12 and interferon-gamma. On average, patients experienced a greater than 240% increase in absolute blood T-cell counts within 1 to 4

weeks of treatment. Treatment increased the numbers of leukemia-specific T cells, demonstrated by autologous ELISPOT assay and mixed lymphocyte reactions. Immunological effects were associated with reductions in leukemia cell counts and lymph node size. Treatment did not induce autoimmune disease and no dose-limiting toxicity was observed.

Takahashi et al also transferred the human CD154 gene in association with the IL-2 gene to non-Hodgkin's lymphoma (NHL) cells through the use of adenoviral vectors [180]. Expression of transgenic human CD154 on these CD40-positive cells was associated with up-regulation of co-stimulatory molecules including B7-1 and B7-2. CD154 and IL-2 gene transfection enhanced T-cell activation and generated autologous T cells capable of specifically recognizing and killing parental (unmodified) cells via MHC restricted cytotoxic T lymphocytes. These findings suggest that the combination of CD154 and IL2 gene-modified B-NHL cells could induce a cytotoxic immune response in vivo directed against unmodified tumor cells.

1.12 Development of a therapeutic anti-CD40 monoclonal antibody

1.12.1 Lob 7/4

A panel of murine derived anti-human CD40 IgG_1 monoclonal antibodies were initially developed within the Tenovus Research Laboratory, Southampton by Dr L. O'Brien. Antibodies were produced in accordance with the method of Kohler and Milstein [110] (see 1.9.3.2) Mice were immunised with human CD40 - human Fc fusion protein (HuCD40-Fc). Animals were subsequently sacrificed and isolated spleen fused with cells from the mouse myeloma cell line NS1. Antibody secreting hybridomas were selected by limiting dilution and screened against the HuCD40-Fc fusion protein by enzyme linked immunosorbent assay (ELISA) and against a range of human cell lines. Positive clones were re-cloned at least twice.

The panel of Lob anti-CD40 antibodies were assessed against a known agonistic mAb s2c6 (courtesy of Professor Randy Noelle, Dartmouth-Hitchcock Medical Centre, New Hampshire, USA) for their ability to upregulate the key co-stimulatory molecules B7-1 and B7-2 in a dendritic cell culture system (developed by Jan Fisher and Chris Tretter, Dartmouth-Hitchcock Medical Centre, USA). Lob 7/4, an IgG_1 mAb was found to up-regulate B7-1 and B7-2 in >90% of cultured dendritic cells, results similar to those obtained with s2c6. On the basis of this data, in-vitro potency data and hybridoma secretion, the Lob 7/4 mAb clone was selected for chimerisation.

1.12.2 Production of Chimeric Lob 7/4

Chimerisation of Lob 7/4 was performed by Melanie Harvey and Claude Chan within the Tenovus Research Laboratory. A hybridoma cell colony secreting the mouse anti-human CD40 monoclonal antibody Lob7/4 was selected and mRNA was purified using the Quickprep micro-mRNA kit (Amersham Bioscience, Little Chalfont, UK) under the manufacturer's guidelines. cDNA was synthesized from the purified mRNA using the First-Strand cDNA synthesis system (Amersham Bioscience, Little Chalfont, UK). The variable regions of the immunoglobulin heavy and light chains were then amplified in a polymerase chain reaction (PCR) using primer sets that bind to the leader and constant regions of the two chains. These were cloned into pCR-BluntII TOPO vector (Invitrogen, UK) and sequenced. Specific primers with restriction sites HindIII/SpeI and HindIII/BsiWI (for Vλ and Vκ respectively), were designed and used to amplify the variable chains. The amplified variable chain sequences were verified by sequencing and then subcloned via the respective restriction sites into expression vectors containing the human heavy and light chain constant regions (Huλ-pEE6.1 and Huκ-Pee14.1 respectively). The chimeric heavy chain expression cassette was then subcloned into the vector containing the chimeric light chain via NotI/BamHI sites. The resulting plasmid contained both chimeric heavy and light chains in 2 expression cassettes and was used to transfect into Chinese Hamster Ovary (CHO) cells using GenePorter (Cambridge Bioscience, UK). Transfectants were selected on GMEM-S medium containing 25μM methionine sulfoximine. Expression of chimeric Lob7/4 antibody was confirmed using ELISA and indirect flow cytometry.

1.13 Conclusions and Research Aims

Cancer remains a major cause of morbidity and mortality worldwide. Although surgery, chemotherapy and radiotherapy can provide a cure for early stage disease and palliation for more advanced disease, significant developments in treatment and outcome are only likely to be achieved with novel therapeutic approaches.

A relationship between the immune system and malignancy has long been the subject of research. Until recently however, immunotherapeutic approaches to the treatment of cancer have been generally disappointing. Normal, bi-directional CD40-CD154 interactions are central to the generation of both T cell dependent, humoral immune responses and cytotoxic T cell responses and probably essential for any effective anti-tumour immune response. Treatment with agonistic anti-CD40 monoclonal antibodies can effectively by pass the need for specific T cell help and in animal models can successfully stimulate the immune system to eradicate established malignancy and provide protection against further tumour re-challenge without further antibody treatment.

A chimeric human/mouse anti-human CD40 monoclonal antibody, Chi Lob 7/4 has been developed within the Tenovus Research Institute. This thesis has been primarily concerned with the translational development of Chi Lob 7/4 into a human, phase I, clinical dose escalation trial in the treatment of patients with advanced malignancies. My research aims were to evaluate the in vitro properties of Chi Lob 7/4 against a variety of human malignant cell lines in a number of in vitro systems. Additionally, I aimed to investigate the toxicities of anti-CD40 mAb therapy in a comparative mouse model using the rat anti mouse mAb 3/23. I set out to develop an immunohistochemical technique to assess the tissue expression of CD40 in normal human tissue and human tumour samples and a specific ELISA to assess serum concentrations of Chi Lob 7/4 thereby enabling pharmacokinetic analysis within any subsequent clinical trial.

During the course of my research, I prepared and submitted a proposal to the New Agents Committee of Cancer Research UK for large scale production of Chi Lob 7/4 and the development of a phase I clinical trial to examine its safety, tolerability and biological effects in patients with CD40 expressing malignancies refractory to conventional therapy. This proposal was accepted and production of clinical grade antibody is now underway at the Therapeutic Antibody Centre, Oxford under the direction of Professor Geoff Hale. I remain involved with the development of the phase I clinical trial and it is envisaged that this will be open to recruitment in 2004.

CHAPTER TWO

2 MATERIALS AND METHODS

2.1 Tissue Culture

2.1.1 Cell Lines

The following cell lines were used for in-vitro experimentation.

2.1.1.1 Human non-Hodgkin's lymphoma cell lines

Daudi is a high grade Burkitt's non-Hodgkin's lymphoma cell line (European Collection of Animal Cell Cultures; ECACC). RL is a transformed follicular non-Hodgkin's lymphoma cell line containing the t(14;18) (q32;q21) translocation (American Type Culture Collection; ATCC).

2.1.1.2 Human epithelial cancer cell lines

EJ138 is a human bladder carcinoma cell line (ECACC). Caski is a human cervical carcinoma cell line and MG79, a human ovarian cancer cell line (kindly donated by Professor Lawrence Young, Birmingham University).

2.1.2 Culture materials

Daudi, RL, Caski and MG79 cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, (Gibco), supplemented with 2 mM L-glutamine (Gibco), 1 mM pyruvate (Gibco), 10% myoclone plus foetal calf serum (Gibco) 100 IU/ml penicillin and streptomycin (Life Technologies) and 50 u/ml amphotericin B (Fungizone; Squibb and Sons). EJ138 was cultured in Eagles Minimum Essential Medium (EMEM), (Bio-Whittaker) supplemented with 2 mM L-glutamine (Gibco), 1% non-essential amino acids (NEAA), (Sigma) and 10% myoclone plus foetal calf serum (Gibco).

2.1.3 Culture techniques

Cell lines were maintained in culture medium described above at 37°C in a 5% CO_2, humidified incubator. Cell cultures were split and media replenished every two to three days. Adherent cell lines (EJ138, MG79 and Caski) were detached from the surface of plastic cell culture flasks using trypsin-EDTA (Gibco) prior to handling. Cells were maintained in log phase growth for 24 hours prior to experimentation.

2.1.3.1 Storage, freezing and thawing of cell lines

Cell lines were stored frozen in a liquid nitrogen storage facility at -180°C. Cells were prepared for cold storage in 1ml aliquots at a concentration of 1 x 10^7/ml in appropriate culture medium supplemented with 10% foetal calf serum (Gibco) and 10% dimethyl sulfoxide (DMSO), (Gibco). Cells were initially frozen at -80°C and then transferred to the main storage facility several days later. When required, cells were defrosted into warmed, supplemented culture medium, washed and then resuspended in appropriate medium for culture.

2.1.3.2 Cell counting and viability

Cells were counted using an automated Coulter industrial D cell counter (Coulter electronics, UK) or through the use of a haemocytometer. Cell viability was assessed by trypan blue exclusion under light microscopy.

2.2 Antibodies

Antibodies from a variety of sources were used for flow cytometry, in-vitro experimentation and in-vivo toxicology studies.

2.2.1 Non-commercial antibodies

Non-commercial antibodies were produced in-house from hybridoma cell lines, expanded as ascites tumours in pristane-primed BALB/c x CBA (F1) mice. Antibodies were purified by protein A or protein G columns (Pharmacia Amersham, UK). Immunoglobulin purity was assessed by electrophoresis (EP system, Beckman, USA) following elution. Antibodies were dialysed into phosphate buffered saline prior to use.

2.2.2 Mouse anti-human CD40 antibodies

Lob 7/4 and Lob 7/6 are mouse anti-human CD40 monoclonal antibodies (mAbs) raised within the Tenovus Research Laboratory by Dr Lyn O'Brien. Lob 7/4 is an IgG_1 mAb. Lob 7/6 is an IgG_{2a} mAb.

2.2.3 Mouse / Human chimeric anti-human CD40 antibody

Chi Lob 7/4 is a chimeric mouse / human IgG_1 kappa anti-human CD40 mAb. It was produced within the Tenovus Research Laboratory through the recombination of variable region gene sequences from Lob 7/4 with human constant regions from a human immunoglobulin gene and transfection of the resultant gene product into a Chinese hamster ovary cell line [181]. Antibody chimerisation was performed by Dr Melanie Harvey and Dr Claude Chan.

2.2.4 Additional antibodies

3/23 is an IgG_{2a}, rat anti-mouse CD40 mAb (kindly provided by Gerry Klaus, London). Mc39-16 (Tenovus) is a rat anti-mouse IgG_{2a} mAb raised against the idiotype of the mouse lymphoma A31. Rituximab is a human / mouse chimeric IgG_1 anti-human CD20 mAb (Roche). DB7-18 (Tenovus) is a mouse anti-saporin IgG_{2a} mAb and was used as an irrelevant, negative control antibody. M359 F10 (Tenovus) is a mouse anti-rat immunoglobulin mAb. Additional mAbs used for flow cytometric analysis of cell surface antigens by immunofluorescence (anti-CD19, CD22, CD37, MHCII, Mu) were produced in-house. All fluorescein isothyocyanate (FITC) conjugated mAbs used were FITC labelled in-house by Mrs Maureen Power.

2.2.5 Measurement of antibody concentrations by spectrophotometry

Quantification of antibody concentration in buffer solutions was determined by spectrophotometry. Samples were added to a 1 cm quartz cuvette. A second identical cuvette was filled with buffer alone (generally PBS). Both cuvettes were placed in a spectrophotometer (Phillips-PU 8620 UV/VIS/NIR) with the wavelength set at 280 nm. Antibody concentration was calculated from the difference in absorbance between buffer alone and buffer plus antibody using an adaptation of the following Beer-

Lambert equation (A=εbc) where A represents absorbance, ε, molar absorbtivity, b, the path length of the sample in cm and c, the concentration of the antibody in solution.

For human antibodies the following formula was used:

$$\text{Antibody concentration (mg/ml)} = \frac{\text{Absorbance at 280nm}}{1.35}$$

(Where 1.35 = absorbtion of 1 mg/ml solution of human IgG)

For rodent antibodies the following formula was used:

$$\text{Antibody concentration (mg/ml)} = \frac{\text{Absorbance at 280nm}}{1.45}$$

(Where 1.45 = absorbtion of 1 mg/ml solution of rat IgG)

2.2.6 Dialysis of antibodies

Dialysis of antibodies was performed using either Visking tubing (Medicell) or slide-a-lyzer dialysis cassettes (Pierce). Samples were loaded into tubing or cassettes and dialysed 1:1000 in at least 3 changes of dialysis solution for at least four hours per change of solution.

2.2.7 Concentration of antibodies

Antibodies in solution were concentrated by one of two techniques. Large volumes were concentrated using an Amicon ultrafiltration device (Biomax). This device concentrates proteins by ultrafiltration through a 10,000 molecular weight cut off filter (Millipore) under nitrogen gas at 4°C. Smaller concentrations were concentrated through a vivaspin 20 concentrator (Vivascience Sartorius) using a 10,000 molecular weight cut off filter. This device was centrifuged 3000 rpm until the desired concentration was reached.

2.2.8 Removal of endotoxin

Bacterial endotoxin (lipopolysaccharide from the cell membrane of gram negative bacteria) was removed from all antibodies prior to their use in-vivo through the use of an albumin – polymixin column. Prior to use, the column was sterilised with 1M NaOH and rinsed with endotoxin low PBS. 1M ammonium thiocyanate was then passed through the column to elute off any bound protein followed by a further rinse in PBS. Antibody samples were then passed through the column and additionally filtered through two 0.2 micron filters. The emergence of antibody from the column was detected ex-column using high pressure liquid chromatography (HPLC) and antibody was collected in a sterile vessel. Antibody concentration was then determined spectrophotometrically and the presence or absence of endotoxin assessed using the Limulus Amoebocyte Lysate (LAL; Bio Whittaker) endotoxin test.

2.2.9 Limulus Amoebocyte Lysate assay for the presence of endotoxin

The presence or absence of endotoxin in antibody solutions was characterised through the use of a Limulus Amoebocyte Lysate endotoxin test kit (Bio Whittaker, USA). This assay is a qualitative test for Gram negative bacterial endotoxin and its use is based on the observation that Gram negative infection of the horseshoe crab *Limulus polyphemus* causes fatal intravascular coagulation. Clotting occurs as a result of the interaction between endotoxin and a clotable protein in the circulating amoebocytes of Limulus blood. The LAL kit uses a lyophilized lysate prepared from the circulating amoebocytes of *Limulus polyphemus* that has been standardised to detect a known concentration of reference endotoxin (USA Food and Drug Administration; FDA).

Limulus Amoebocyte Lysate (LAL) was reconstituted with 1.8 ml of LAL reagent water as per manufacturer's instructions. Standardised endotoxin samples (from E. coli strain 055:B5) were supplied with the kit and used as a positive control and reference range. Sample solutions in a volume of 100 µl were prepared in duplicate in pyrogen free test tubes and diluted in endotoxin free dH_2O using endotoxin free pipette tips. 100 µl of prepared LAL was then added to the samples and the tubes were placed in a 37°C water bath for one hour. The presence of endotoxin was assessed by the

development of a firm gel within the tube that remained momentarily intact when the sample tube was inverted by 180 degrees. The concentration of endotoxin was calculated by reference to LAL sensitivity against reference range dilutions, and expressed in ng/ml. A concentration of endotoxin of less than 1 ng/ml was considered acceptable.

2.3 Flow cytometric analysis of cell surface antigens by immunofluorescence

The expression of cell surface antigens was measured by direct flow cytometric analysis. Cells of interest were incubated in phosphate buffered saline (PBS) at a concentration of 1 x 10^6/ml at 4°C for 30 minutes with a FITC labelled antibody of choice at a final antibody concentration of 50 μg/ml. Cells were then washed in PBS – bovine serum albumin 1% w/v (BSA; Wilfred Smith, Middlesex) – azide (20 mM NaN_3); (PBS-BSA-Azide). Flow cytometric analysis was performed on a FACScaliber flow cytometer (Becton Dickinson, USA). 5,000 to 10,000 events were collected for each sample. Collected data was analysed using the Cellquest software program (Becton Dickinson). Fluorescence intensity of labelled cells was assessed in comparison to negative and positive control samples and expressed as histograms of fluorescence intensity against cell number.

2.4 In-vitro growth inhibition studies

Selected cell lines were added to sterile, flat bottomed 96 well tissue culture plates pre-loaded with antibody or cisplatin. All antibodies were presented on superparamagnetic, polystyrene, polyurethane coated microbeads (Dynabeads M-450, Dynal AS) to optimise antibody cross linking. 100 μg of each antibody was linked to 2 x 10^8 beads and between 5 x 10^2 and 5 x 10^5 beads per well used in individual assays. Cisplatin was used at a final concentration ranging from 0.01 to 10 μg/ml. Cells were cultured for five days at 37°C in a 5% CO_2, humidified incubator. Growth inhibition / proliferation was measured using [3^H methyl] thymidine incorporation or tetrazolium bromide conversion assays.

2.4.1 [3^H methyl] thymidine incorporation assay

Growth inhibition of non-Hodgkin's lymphoma cell lines was determined through the measurement of [3^H methyl] thymidine incorporation into cells. Replicating cells in S phase of the cell cycle will incorporate thymidine into DNA in contrast to apoptotic cells or cells in growth arrest. The measurement of [3^H methyl] thymidine incorporation into cells incubated with a variety of test antibodies allows calculation of relative growth inhibition when compared to cells incubated with negative or positive control antibodies. Selected cell lines were cultured for five days at a starting concentration of 5 x 10^3 / ml in flat bottomed 96 well plates (Nunc) in combination with a variety of antibodies in a final volume of 200 µl. All antibodies were presented on superparamagnetic, polystyrene, polyurethane coated microbeads (Dynabeads M-450, Dynal AS) to facilitate optimal antibody cross-linking. Lob 7/4 and Chi Lob 7/4 were compared to isotype matched, negative control mouse (DB7-18) or human (Irr Hu IgG) mAbs or the mouse-human chimeric mAb rituximab. Plates were maintained at 37°C in a 5% CO_2, humidified incubator. 50 µl of [3^H methyl] thymidine (Amersham, UK) was added at a concentration of 0.5 µCi per well for the final sixteen to twenty four hours of culture. Cells were harvested onto glass fibre mats using an automated cell harvester (Packard, USA). [3^H methyl] thymidine incorporation was determined by liquid scintillation counting of β emission. Each assay was performed in triplicate and each experiment repeated on two or three occasions.

2.4.2 Tetrazolium bromide conversion assay

The tetrazolium bromide conversion assay was used as an alternative to the [3^H methyl] thymidine incorporation assay for adherent epithelial cell lines. Tetrazolium bromide (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), (MTT; Sigma) is a water soluble tetrazolium salt yielding a yellow solution when dissolved in RPMI 1640 medium lacking phenol red (Sigma). Dissolved MTT is converted to water insoluble purple formazan crystals by active mitochondrial dehydrogenases in living cells. Formazan crystals may then be solublised in dimethyl sulfoxide (DMSO) and the absorbance of the resultant purple solution measured by spectrophotometry using a wavelength of 570nm. Optical density correlates with the concentration of converted

dye in turn correlating with cell number. Selected cell lines were cultured at a concentration of 5 x 10^3 / ml in flat bottomed 96 well plates (Nunc) in combination with Lob 7/4, Chi Lob 7/4, isotype matched, negative control human (Irr Hu IgG) mAb or cisplatin, in a final volume of 200 µl for five days at 37°C in a 5% CO_2, humidified incubator. All antibodies were presented on superparamagnetic, polystyrene, polyurethane coated microbeads (Dynabeads M-450, Dynal AS) to facilitate optimal antibody cross-linking. 20 µl of MTT was added to each well for the final three hours of culture at 37°C. The cell supernatant was removed and the remaining formed crystals dissolved with DMSO. The plates were analysed at 570 nm on an automated plate reader (Dynatec 400, Dynatec). Growth inhibition was assessed by observing the difference in optical densities between treatment and control wells. Each assay was performed in triplicate and each experiment repeated on two or three occasions.

2.4.3 Linkage of antibody to microbeads

Selected cell lines were added to sterile flat bottomed 96 well tissue culture plates pre-loaded with antibody presented on superparamagnetic, polystyrene, polyurethane coated microbeads (Dynabeads M-450, Dynal AS) to facilitate optimal antibody cross-linking.

Dynabeads M-450 are coated with a polyurethane surface activated by p-toluenesulphonyl chloride to provide reactive groups for the covalent binding of proteins such as antibodies. 100 µg of antibody was linked to 2 x 10^8 beads for in-vitro assays, with an expectation of 40-80% antibody binding. Between 5 x 10^2 and 5 x 10^5 beads were added to each well of a 96 well plate.

Microbeads were supplied at a concentration of 4 x 10^8/ml. Prior to use, beads were resuspended by vortexing for one - two minutes. 500µl of beads was then pipetted off into a labelled, sterile eppendorf tube and placed into a dedicated magnet (Dynal AS) for one minute effectively separating beads from supernatant. All supernatant was removed by careful pipetting and the beads resupended and washed in 1 ml sterile filtered (0.2 micron filter) buffer "A" (2.62 g NaH_2PO_4 and 14.42 g Na_2HPO_4 dissolved in 1 Litre DH_20) for two minutes. The tube was then placed in the magnet for a further

one minute, buffer A removed, after which beads were resuspended in a further 400 µl of buffer A.

Experimental antibodies were dialysed into sterile filtered buffer "A" and used at a concentration of 1 mg/ml. 100 µg (100µl) of sterile, filtered antibody added to an individual eppendorf containing beads prepared as above. The tube was then vortexed for a further one - two minutes and then incubated for ten minutes at 37°C. 50 µl of sterile filtered 2% BSA (in buffer A) was added and the mixture incubated at 37°C for sixteen to twenty four hours. Following this incubation, the tube was placed in the magnet for two to three minutes and the supernatant removed. Antibody coated beads were then washed twice in sterile filtered buffer "D" (0.88 g NaCl plus 100 mg BSA added to 10 mls buffer A diluted with 90 mls dH_20) for five minutes at 4°C. A further wash with sterile filtered buffer "E" (2.42 g TRIS added to 80 mls dH20, adjusted to pH 8.5 using 1M HCl, 100 mg BSA, final solution made up to 100 ml with dH20) was carried out for four hrs at 37°C. A final wash in buffer D was performed for five minutes at 4^0C. Antibodies conjugated to beads were stored short term at 4°C in buffer D and prior to use resuspended in sterile 10% RPMI 1640 medium (Gibco).

2.4.4 Cisplatin

The cytotoxic drug cisplatin (Faulding DBL, UK) was used as a positive control in growth inhibition studies at a final concentration of 0.01 to 10 µg/ml.

2.5 Complement dependent cytotoxicity assay

Lysis of target cells through the activation of complement by antigen bound antibody was measured using a Chromium^{51} (^{51}Cr) release assay. Human serum was used as a source of complement and obtained from whole blood donations from healthy volunteers. Blood (generally 50 – 100 ml) was allowed to clot in glass for one hour at room temperature and then two hours at 4°C. The sample was then centrifuged at 2000 rpm for 10 min and the resultant serum aspirated off for use in the assay. Prior to use, serum was diluted 1 in 3 in un-supplemented Eagles minimum essential medium (EMEM, Bio-Whittaker).

Target cells (malignant cell lines) were harvested and re-suspended at 1 – 5 x 10^7 /ml EMEM and incubated with 100-200 µl of 1 mCi/ml radioactive Chromium^{51} (^{51}Cr) (Amersham, UK) for thirty minutes at 37°C. Cells were then washed four times in EMEM. Radiolabelled cells were counted by haemocytometer and re-suspended at a final concentration of 1 x 10^6 / ml.

Antibodies of interest (Lob 7/4, Chi Lob7/4, Irr Hu IgG and Rituximab) were prepared in phosphate buffered saline to give a final concentration range from 10 µg/ml to 0.016 µg/ml. 100 µl of antibody was dispensed into individual flat bottomed tubes, in duplicate. 100 µl of target cells was added and cells and antibodies incubated for fifteen minutes at 4°C. Diluted human serum was added to each tube in a volume of 300 µl. This was followed by a thirty minute incubation in a 37°C water-bath. Tubes were then removed to 4°C and centrifuged for five minutes at 1500 rpm. 200 µl of supernatant was carefully aspirated from each tube into a fresh labelled tube.

Supernatant radioactivity, expressed as counts per minute (cpm) was measured using a gamma counter. As a positive control, 400 µl of NP40 detergent was added to duplicate tubes containing 100 µl of target cells just prior to the final centrifuge spin. Tubes containing 100µl of target cells, 100 µl of medium and 300 µl of diluted human serum acted as a negative, background control. Percentage specific ^{51}Cr release as a measure of cell lysis was calculated from the arithmetic mean of duplicate samples using the following formula:

$$\% \text{ Specific } {}^{51}\text{Cr release} = \frac{\text{Experimental sample c.p.m. - background sample c.p.m.}}{\text{NP40 sample c.p.m. - background sample c.p.m.}}$$

Each assay was performed in duplicate and each experiment repeated on two or three occasions.

2.6 Antibody dependent cellular cytotoxicity assay

Lysis of target cells through the activation of antibody dependent cellular cytotoxicity by antigen bound antibody was measured using a Chromium^{51} (^{51}Cr) release assay. Peripheral blood mononuclear cells isolated from whole blood donations from healthy volunteers were used as a source of immune effector cells. Whole blood (generally 50 – 100 ml) was heparinised with 5000 iu heparin sodium (CP pharmaceuticals, UK) and diluted 1:2 with Dulbecco's Modified Medium (DMEM; Gibco) supplemented with 2 mM L-glutamine (Gibco) and 1mM pyruvate (Gibco). The sample was then layered onto an equal volume of lymphoprep (Nycomed Pharma AS) and centrifuged at 2000 rpm for twenty minutes. Following centrifugation, mononuclear cells were removed using a pasteur pipette to aspirate cells from the sample – lymphoprep interface, washed in supplemented DMEM and re-suspended at a concentration of 5 x 10^6/ml.

Target cells (malignant cell lines) were harvested, re-suspended at 1 – 5 x 10^7/ml in DMEM and incubated with 100-200 µl of 1 mCi/ml radioactive Chromium^{51} for thirty minutes at 37°C. Cells were then washed 4 times in supplemented DMEM, counted by haemocytometer and re-suspended at a final concentration of 2 x 10^5/ml to give a final effector cell : target cell ratio of 50:1. In some experiments, cells were resuspended at alternative concentrations to give a range of effector : target ratios from 100:1 to 5:1.

Antibodies of interest (Lob 7/4, Chi Lob 7/4, Irr Hu IgG and Rituximab) were prepared in supplemented DMEM to give a final concentration range from 10 µg/ml to 0.001 µg/ml. For each assay, antibodies were dispensed into a U bottom 96 well plate (Nunc) in triplicate in a volume of 50 µl. 50 µl of target cells was then added and cells and antibodies incubated for fifteen minutes at 4°C. Effector cells were then added to each tube in a volume of 100µl. This was followed by a three and a half hour incubation at 37°C in a 5% CO_2, humidified incubator. Plates were centrifuged for five minutes at 1500 rpm and 100 µl of supernatant carefully aspirated from each well into a fresh labelled, flat bottomed tube.

Supernatant radioactivity, (cpm) was measured using a gamma counter. As a positive control, 150 µl of NP40 detergent was added to triplicate wells containing 50 µl of target cells just prior to the final centrifuge spin. Triplicate wells containing 50 µl of target cells, 50 µl of medium and 100 µl of effector cells acted as a negative, background control. Percentage specific ^{51}Cr release as a measure of cell lysis was calculated from the arithmetic mean of triplicate samples using the following formula:

$$\% \text{ Specific } {}^{51}Cr \text{ release} = \frac{\text{Experimental sample c.p.m. - background sample c.p.m.}}{\text{NP40 sample c.p.m. - background sample c.p.m.}}$$

Each assay was performed in triplicate and each experiment repeated on two or three occasions.

2.7 Toxicology studies

2.7.1 Experimental animals

Toxicology studies were carried out in two strains of mice, C57/BLK$_6$ and BALBc, and one other rodent species, Syrian Hamsters (Harlan, UK and Charles River, UK). Animals were kept in local facilities under Home Office regulations.

2.7.2 Toxicological study methods

General toxicological study methods are described below. Specific methods are described in the methods section of chapter 4.

2.7.3 Administration of antibody

Animals were injected intraperitoneally or intravenously with varying concentrations of endotoxin free antibody in PBS at a standard volume of 200 µl. Intravenous injections were administered via animal tail veins. Animals were culled by halothane anaesthesia and carbon dioxide rebreathing.

2.7.4 Biochemical and haematological analysis of blood specimens

Terminal cardiac bleeds were performed on some animals and samples collected for biochemical and haematological testing. In order to establish a local reference range, ten untreated animals of each strain / species were bled for haematological and biochemical testing.

Biochemical testing was performed on serum samples from clotted blood. Samples were processed using an automated analyser by the Department of Chemical Pathology, Southampton University Hospitals. Parameters measured included urea, creatinine, electrolytes (sodium and potassium) and liver function tests (alanine transaminase, aspartate transaminase).

Haematological testing was performed on whole blood anti-coagulated with EDTA. Samples were processed using an automated analyser (Coulter, UK) by the Department of Haematology, Southampton University Hospitals. Parameters measured included haemoglobin, total white blood cell count, differential white blood cell count and platelet count.

2.7.5 Post mortem examination of animals

Post mortem examination was performed on all animals through a midline, anterior thoraco-abdominal incision. Macroscopic abnormalities were noted and selected organs (liver, spleen and kidneys) retained for subsequent histological analysis.

2.8 Histochemistry

Histochemical analysis was performed on organs retained from animal experiments. Specimens were processed either into paraffin or glycol methacrylate blocks. Prior to processing into paraffin blocks, samples were initially fixed in 10% formalin for a minimum of 24 hours. Prior to processing into glycol methacrylate samples were fixed overnight at -20°C in acetone containing 2mM phenylmethylsulphonyl fluoride and 20mM iodoacetamide (Merck; UK).

Histochemistry was also performed on a variety of paraffin embedded, anonymised normal and malignant human tissues obtained from the Department of Histopathology, Southampton Hospital following local ethics committee approval.

2.8.1 Tissue processing

2.8.1.1 Glycol methacrylate (GMA) resin

Two millimetre diameter tissue specimens were obtained fresh and fixed overnight at -20°C in acetone containing 2 mM phenylmethylsulphonyl fluoride and 20 mM iodoacetamide. Fixative was then replaced with room temperature acetone for 15 minutes followed by methyl benzoate for 15 minutes. Specimens were then infiltrated for 2 hours on 3 occasions with processing solution (5% methyl benzoate in glycol methacrylate plus 250 µl GMA solution B; Polysciences, UK) at 4°C. Specimens were then embedded in freshly prepared embedding solution (10 mls processing solution, 70 mg benzoyl peroxide;) in flat bottomed, capped tubes. Samples were allowed to polymerise at 4°C for 48 hours and stored at -20°C until use.

2.8.1.2 Paraffin Wax

Following fixation, samples were embedded into liquid paraffin which was then allowed to set into solid paraffin blocks. This processing was kindly performed by the staff within the University Histochemistry Research Unit.

2.8.2 Cutting of paraffin and GMA sections

All sections were cut by staff within the University Histochemistry Research Unit. For paraffin sections, 4-5 µm sections were cut from blocks using a microtome, floated onto a waterbath and picked up onto labelled aminopropyltriethoxysilane (APES; Sigma) coated slides. Sections were dried for at least 48 hours in a 37°C dry incubator. For GMA sections, blocks were removed from embedding capsules, excess resin was trimmed away and 2 µm sections cut using a microtome and floated out onto an ammonia water bath (1 ml ammonia in 500 ml dH_2O). Sections were picked up onto labelled, poly-L-lysine (Sigma) coated slides, dried for 1 hour at room temperature and stored wrapped in aluminium foil at -20°C for up to one week prior to use.

2.8.3 Tinctorial Staining

Paraffin embedded specimens from animals used in toxicology specimens were stained using a variety of tinctorial stains.

2.8.3.1 Haematoxylin and Eosin Stain

Haematoxylin and Eosin (H&E) stains were performed using a semi-automated method in the University Histochemistry Research Unit. Paraffin embedded sections were de-waxed by immersion in xylene for twenty minutes and rehydrated through graded alcohols to water. Sections were then stained in haematoxylin for five to ten minutes, washed in water for ten minutes then counterstained in 1% eosin for ten minutes. Sections were then washed in water for one to five minutes, dehydrated through graded alcohols, cleared in xylene and mounted in DPX (Merck, UK). H&E staining results in blue/black staining of cell nuclei with pink cytoplasmic staining.

2.8.3.2 Periodic Acid Schiff (PAS) - Diastase Stain

Paraffin embedded sections were de-waxed by immersion in xylene for 20 minutes and rehydrated through graded alcohols to water. Sections were treated with 1% diastase in dH_2O for three hours followed by a dH_2O wash. Sections were then treated with periodic acid for five minutes followed by several washes with dH_2O. Sections were then treated with Schiff's reagent (Feulgen stain), washed in tap water and then lightly counterstained with Mayer's haematoxylin. Following a final wash in tap water, sections were dehydrated through graded alcohols, cleared in xylene and mounted in DPX (Merck, UK). PAS-Diastase staining will stain neutral mucins and other reactive carbohydrates (but not glycogen) magenta.

2.8.3.3 Reticulin Stain

Paraffin embedded sections were de-waxed by immersion in xylene for twenty minutes and rehydrated through graded alcohols to water. Sections were then treated with acidified potassium permanganate solution (47.5 ml 0.25% aqueous potassium permanganate and 2.5 ml 3% aqueous sulphuric acid) for five minutes. Sections were washed with dH_2O and then treated with 5% aqueous oxalic acid solution for one minute, washed with dH_2O and then treated with 2% aqueous ferric ammonium sulphate for five minutes. Following three further washes in dH_2O, sections were treated with ammoniacal silver solution (5ml 10% aqueous silver nitrate, 5 ml 3.1% sodium hydroxide plus concentrated ammonia (added until formed precipitate re-dissolves) made up to 50 ml with dH_2O) for five seconds and then washed with three changes of dH_2O. Sections were then treated with 10% formalin for thirty seconds to one minute, washed in dH_2O and fixed with 5% sodium thiosulphate. Following a final wash in tap water, sections were dehydrated through graded alcohols, cleared in xylene and mounted in DPX (Merck, UK). Reticulin staining stains reticulin black and collagen yellow brown.

2.8.4 Immunohistochemical Techniques

2.8.4.1 Antigen Retrieval

Fixation in formalin is known to cause changes in protein shape and protein cross-linking. These changes can potentially mask target antigens from immunohistochemical techniques and therefore, a number of techniques are employed to "retrieve" antigens of interest, breaking down protein cross-linking and restoring antigen epitope conformation. A number of approaches were employed to determine the best technique for retrieving antigens of interest enabling subsequent detection by immunohistochemistry. Prior to the employment of antigen retrieval techniques, sections were de-waxed by immersion in xylene for twenty minutes and rehydrated through graded alcohols to water. Endogenous peroxidase was inhibited with 100 µl

0.5% hydrogen peroxide in 5.9 ml methanol for ten minutes followed by rinsing with TRIS buffered saline.

Fixation of sections in acetone (containing 2 mM phenylmethylsulphonyl fluoride and 20 mM iodoacetamide) and subsequent processing into glycol methacrylate avoids inducing changes in protein shape and protein cross-linking and therefore, such sections do not require antigen retrieval techniques

2.8.4.1.1 Pronase antigen retrieval

A stock solution of 1% pronase was prepared by dissolving 100 mg of pronase (Dako) in 10 ml of TBS. 100 µl aliquots were then stored at -20 °C until required. A working solution of pronase was prepared by thawing 100 µl of stock pronase and diluting it in 1.9 ml of TBS. All sections were covered with working pronase for ten minutes before washing with TBS and continuing with the immunohistochemical staining procedure.

2.8.4.1.2 Heat induced antigen retrieval

2.8.4.1.2.1 Microwave

Slides were placed in plastic staining racks and immersed in a standard volume (330 ml per 24 slide rack) of 0.01 M pH 6 citric acid buffer (citric acid crystals 2.1 g in 1 L dH_2O adjusted to pH 6 with 1 M NaOH). To maintain a constant load for the microwave, a total of 72 slides were placed in 3 vessels containing 330 ml buffer on each occasion. Blank slides were used to make up numbers if necessary. Each vessel was placed onto the microwave baseplate in a standardised position. The microwave (Tecnolec Superwave 800) was set at medium power and run for twenty five minutes. Slides were then removed from the microwave, washed in cold running tap water for several minutes and then rinsed in TBS for ten minutes before continuing with the immunohistochemical staining procedure.

2.8.4.1.2.2 Pressure Cooker

Citric acid buffer (0.01 M, pH 6) was brought to the boil in a domestic pressure cooker with the lid fitted but not locked. Slides positioned back to back in pairs in stainless steel staining racks were placed into the boiling citric acid buffer and the lid fitted and locked. Pressure was allowed to build to 13 lb psi and slides were heated at this pressure for two minutes. The pressure cooker was then removed from the heat and cooled rapidly under tap water until atmospheric pressure was reached. The cooker lid was then removed and slides washed and cooled in running tap water. Slides were rinsed in TBS for ten minutes before continuing with the immunohistochemical staining procedure.

2.8.5 Immunohistochemical staining

2.8.5.1 Paraffin embedded tissue

Paraffin embedded specimens were stained using a variety of immunohistochemical stains. All staining was carried out in humidified, covered staining trays. Sections were de-waxed by immersion in xylene for twenty minutes and rehydrated through graded alcohols to water. Endogenous peroxidase activity was inhibited with 100µl 0.5% hydrogen peroxidase in 5.9 ml methanol for ten minutes followed by rinsing with TRIS buffered saline (TBS; NaCl 80 g, TRIS 6.05 g, 1M HCl 38 ml (Merck, UK) in dH_2O 10 L, pH 7.6). Depending on the antigen selected, antigen retrieval techniques were employed as detailed above.

Following antigen retrieval, sections were washed in TBS. Non-specific binding was blocked with avidin solution (Vector, USA) for twenty minutes, biotin solution (Vector, USA) for twenty minutes and culture medium (DMEM 80 ml; Sigma, foetal calf serum 20 ml; PAA and BSA 1 g; Sigma) for twenty minutes. Each of these steps was followed by three washes in TBS.

Primary antibodies to detect an antigen of interest were then applied at appropriate dilutions and sections incubated overnight at 4°C. Primary antibody was omitted on

negative control specimens. Following three, five minute washes with TBS, a biotinylated second stage antibody was applied at an appropriate concentration for thirty minutes. This stage was followed by three further five minute washes with TBS and subsequently streptavidin biotin – peroxidase complex was applied for thirty minutes. After another three, five minute TBS wash, 3-amino-9-ethylcarbazole (AEC; Biogenex, USA) chromogen was applied for ten minutes. Sections were then rinsed once in TBS, washed in running tap water and counterstained for two minutes in Mayer's Haematoxylin. Sections were then blued in running tap water, covered in crystal mount permanent aqueous mounting medium (Biomeda, USA) and baked for twenty minutes at 80°C. Sections were then allowed to cool and mounted in DPX (Merck, UK).

2.8.5.2 GMA embedded tissue

GMA embedded specimens were stained using a variety of immuno-histochemical stains. Endogenous peroxidase activity was inhibited using a solution of 0.1 % sodium azide and 0.3 % hydrogen peroxide in dH_2O for 30 minutes followed by a TBS wash. Antigen retrieval techniques were not employed with GMA embedded specimens. Non-specific binding blocked with avidin solution (Vector, USA) for thirty minutes, biotin solution (Vector, USA) for thirty minutes and culture medium (DMEM 80 ml; Sigma, foetal calf serum 20 ml; PAA and BSA 1 g; Sigma) for thirty minutes. Each of these steps was followed by three washes in TBS. Primary antibodies to detect an antigen of interest were then applied at appropriate dilutions and sections incubated overnight at 4°C. Following three, five minute washes with TBS, a biotinylated second stage antibody was applied at an appropriate concentration for 2 hours. This stage was followed by three further five minute washes with TBS and subsequently streptavidin biotin – peroxidase complex was applied for two hours. After another three, five minute TBS washes, 3-amino-9-ethylcarbazole (AEC; Biogenex, USA) chromogen was applied for twenty minutes. Sections were rinsed once in TBS, washed in running tap water and counterstained for two minutes in Mayer's Haematoxylin. Sections were then blued in running tap water, covered in crystal mount permanent aqueous mounting medium (Biomeda, USA) and baked for twenty minutes at 80°C. Sections were then allowed to cool and mounted in DPX (Merck, UK).

2.8.6 Antibodies used for immunohistochemistry

2.8.6.1 Primary antibodies

A variety of commercial and in-house antibodies were used as primary antibodies for the detection of antigens by immunohistochemistry.

2.8.6.1.1 Mouse sections

CD3 (Murine T cell marker). KT3 (ImmunoKontact), a rat anti-mouse CD3 IgG_{2a} monoclonal antibody was used at a variety of concentrations from 1:25 to 1:1000 dilutions of a 500 μg/ml solution.

CD4 (Murine T cell marker). RM4-5 (ImmunoKontact), a rat anti-mouse CD4 IgG_{2a} monoclonal antibody was used at a variety of concentrations from 1:25 to 1:1000 dilutions of a 500 μg/ml solution.

CD8a (Murine T cell marker). KT15 (ImmunoKontact), a rat anti-mouse CD8α chain IgG_{2a} monoclonal antibody was used at a variety of concentrations from 1:25 to 1:1000 dilutions of a 500 μg/ml solution.

CD19 (Murine B cell marker). 1D3 (BD Biosciences) a rat anti-mouse CD19 IgG_{2a} monoclonal antibody was used at a variety of concentrations from 1:25 to 1:2000 dilutions of a 500 μg mg/ml solution.

CD40 Four, rat anti-mouse CD40 IgG_{2a} monoclonal antibodies 1C10, 4F11, FGK45 and 3/23 (gift from Gerry Klaus) were used at a variety of concentrations from 1:25 to 1:2000 dilutions of a 1 mg/ml solution

2.8.6.1.2 Human sections

Two mouse anti-human CD40 monoclonal antibodies, Lob 7/4 an IgG_1 mAb and Lob 7/6 an IgG_{2a} mAb were used as primary antibodies for CD40 detection. Optimal staining concentrations were determined from a variety of dilutions from 1:25 to 1:2000 of a 1mg/ml solution. Once antibodies had been applied at appropriate dilutions, sections were incubated overnight at 4°C. Lob 7/6 yielded consistently good results at a working dilution of 1:800.

2.8.6.2 Biotin labelled secondary antibodies

2.8.6.2.1 Mouse sections

Biotin labelled $F(ab')_2$ fragment donkey anti-rat IgG (Jackson Immunoresearch, USA) was supplied at a concentration of 1.5 mg/ml and used at a dilution of 1:1500 for GMA embedded sections and 1:3000 for paraffin embedded sections.

2.8.6.2.2 Human sections

Biotin labelled $F(ab')_2$ fragment rabbit anti-mouse IgG (Dako) was supplied at a concentration of 0.8 mg/ml and used at a dilution of 1:200 for paraffin embedded sections following application of primary mouse antibody and following three, five minute washes with TBS.

2.8.7 Reporting of histochemical staining

All sections were expertly reviewed and reported by Dr Norman Carr, Consultant Histopathologist, and Professor William Roche, Southampton University Hospitals.

2.9 Enzyme linked immunosorbant assays (ELISAs)

Enzyme linked immunosorbant assays were used to quantitatively detect the presence of a variety of proteins / antibodies. Specific primary antibodies / capture proteins were diluted into coating buffer (28.5 nM $NaHCO_3$, 15 nM Na_2CO_3 in dH_2O, pH 9.6) and added to 96 well, flat bottomed ELISA plates (Maxisorb, Nunc) in a volume of 100 µl per well. Plates were incubated at 37°C for one hour then overnight at 4°C. Unbound antibody was thrown off and non-specific binding sites blocked by the addition of blocking solution (1% w/v bovine serum albumin; BSA in phosphate buffered saline; PBS) for one hour at 37°C. Plates were then washed three times in PBS-Tween (PBS plus 0.05% Tween 20). Meanwhile serial dilutions of sample specimens and standard specimens (specimens containing protein of known concentration) were prepared in blocking solution and added to the plate in duplicate, in a final volume of 100 µl per well. Plates were then incubated for ninety minutes at 37°C followed by a further five washes in PBS-Tween. A horseradish peroxidase (HRP) conjugated antibody, specific to the protein of interest was added to the wells at an appropriate concentration in a volume of 100 µl and incubated for ninety minutes at 37°C followed by five further washes in PBS-Tween. HRP substrate (20 mg o-phenylenediamine free base (o-PD)), (Sigma, Poole) in 24.7 ml of ELISA citrate (19.2 g/L citric acid in dH_2O), 25.3 ml ELISA phosphate (24.8 g/L Na_2HPO_4 in dH_2O), 50 ml dH_2O and 40 µl 30% H_2O) was then added to the wells in a volume of 100 µl and incubated for thirty minutes in the dark at room temperature. The reaction was terminated by the addition of 50 µl per well 2.5 M H_2SO_4. Colour change was quantified in an automated plate reader (Dynatec 400, Dynatec). A standard curve of absorbance against known protein concentration of standards was constructed for each ELISA. Concentrations of unknown proteins could then be determined by reference to the standard curve.

2.9.1 Detection of Chi Lob 7/4 through the development of a specific ELISA

An enzyme linked immunosorbant assay (ELISA) was designed to quantitatively detect the concentration of Chi Lob 7/4 in serum and enable pharmacokinetic studies for the

Phase I trial. For this assay, human immunoglobulin Fc fragment – CD40 fusion protein[1] (manufactured in-house) was used as the primary capture protein diluted into coating buffer (28.5nM $NaHCO_3$, 15nM Na_2CO_3 in dH_2O, pH 9.6) at a concentration of 1 μg/ml and added to 96 well, flat bottomed ELISA plates (Maxisorb, Nunc) in a volume of 100 μl per well. Plates were incubated at 37°C for one hour then overnight at 4°C. Unbound HuFc – CD40 was thrown off and non-specific binding sites blocked by the addition of blocking solution (1% w/v bovine serum albumin; BSA in phosphate buffered saline; PBS) for one hour at 37°C. Plates were then washed three times in PBS-Tween (PBS plus 0.05% Tween 20).

Serial dilutions of Chi Lob 7/4 from 1:10 to 1:32,000 (2 μg/ml to 0.6 ng/ml) were prepared in blocking solution and added to the plate in duplicate, in a final volume of 100μl per well. Plates were then incubated for ninety minutes at 37°C followed by a further five washes in PBS-Tween.

As an initial titration and checking procedure, titrations of peroxidase conjugated rabbit $F(ab')_2$ fragment anti-human $F(ab')_2$ fragment (Jackson ImmunoResearch, USA) were carried out in an ELISA designed to detect the $F(ab')_2$ fragment of the anti-CD20 IgG_1 mAb Rituximab (Roche; digestion prepared in house). Rituximab $F(ab')_2$ fragment was diluted into coating buffer (28.5 nM $NaHCO_3$, 15 nM Na_2CO_3 in dH_2O, pH 9.6) and 100 μl used to coat each well of a 96 well, flat bottomed ELISA plate (Maxisorb, Nunc) at a concentration of 1 μg/ml to 15.6 μg/ml. Plates were then incubated at 37°C for one hour then overnight at 4°C. Unbound Rituximab $F(ab')_2$ fragment was thrown off and non-specific binding sites blocked by the addition of blocking solution (1% w/v bovine serum albumin; BSA in phosphate buffered saline; PBS) for one hour at 37°C. Plates were then washed three times in PBS-Tween (PBS plus 0.05% Tween 20).

[1] Human immunoglobulin Fc fragment – CD40 fusion protein consisting of human Fc ($C_H2 - C_H3$) and the extracellular region of human CD40

Peroxidase conjugated rabbit F(ab')$_2$ fragment anti-human F(ab')$_2$ fragment (Jackson ImmunoResearch, USA) was added to wells at a dilution of 1:5,000 to 1:80,000 in a volume of 100 µl and incubated for ninety minutes at 37°C followed by five further washes in PBS-Tween. HRP substrate (20 mg 0-phenyldiamine free base (o-PD), (Sigma, Poole) in 24.7 mls of ELISA citrate (19.2 g/L citric acid in dH_2O), 25.3 ml ELISA phosphate (24.8 g/L Na_2HPO_4 in dH_2O), 50 ml dH_2O and 40 µl 30% H_2O) was then added to the wells in a volume of 100 µl and incubated for thirty minutes in the dark at room temperature.

The reaction was terminated by the addition of 50 µl per well 2.5M H_2SO_4. Colour change was quantified in an automated plate reader (Dynatec 400, Dynatec). A standard curve of absorbance against known Rituximab F(ab')$_2$ fragment concentration was constructed and the optimal dilution of peroxidase conjugated rabbit F(ab')$_2$ fragment anti-human F(ab')$_2$ fragment (1:5,000) selected for use in subsequent assays including assays of Chi Lob 7/4.

2.9.2 Detection of Chi Lob 7/4 in human serum by ELISA

The ELISA method detailed above was followed and a standard curve generated from samples of Chi Lob 7/4 at a concentration of 20 µg/ml, diluted 1:10 to 1:16,000 (2 µg/ml to 1.25 ng/ml) in PBS / BSA and added to the plate in duplicate, in a final volume of 100µl per well. Three human serum samples were obtained from healthy volunteers. Blood (30 - 50 ml) was allowed to clot in glass for one hour at room temperature and then two hours at 4°C. The sample was then centrifuged at 2000 rpm for 10 minutes and the resultant serum aspirated off for use in the assay. Serum samples alone were diluted 1:10 to 1:16,000 and added to the plate in duplicate, in a final volume of 100 µl per well. A further series of serum samples were spiked with Chi Lob 7/4 20 µg/ml and diluted 1:10 to 1:16,000 in an identical fashion to the Chi Lob 7/4 PBS / BSA standard curve.

At the completion of the assay, colour change was quantified in an automated plate reader (Dynatec 400, Dynatec). A standard curve of absorbance against known Chi Lob

7/4 concentration was constructed and compared to the curve of absorbance against Chi Lob 7/4 concentration / dilution of serum for spiked and unspiked serum samples.

2.9.3 Detection of mouse anti 3/23 and Mc39-16 antibodies by ELISA

General ELISA methods described above were followed. 3/23 or Mc39-16 were diluted into coating buffer at a concentration of 2 μg/ml and used as primary capture antibodies. Serial dilutions of serum sample specimens were prepared and added to the plate in duplicate, in a final volume of 100 μl per well. Sample specimens were diluted 1:1000 and then further diluted by doubling dilution out to 1:128,000. M359 F10, a monoclonal mouse anti-rat immunoglobulin was used as a positive control / standard and was diluted out in doubling dilutions from 100 ng/ml to 12.5 ng/ml. Horseradish peroxidase (HRP) conjugated rat anti-mouse immunoglobulin was added in a volume of 100 μl per well.

CHAPTER THREE

3 IN-VITRO PROPERTIES OF CHI LOB 7/4

3.1 Introduction

Chi Lob 7/4 is an IgG_1 mouse / human chimeric anti-CD40 monoclonal antibody (mAb) developed for the immunotherapy of CD40 expressing human malignancies [181]. The therapeutic administration of Chi Lob 7/4 could theoretically achieve an antineoplastic effect through a variety of mechanisms.

3.1.1 Growth inhibition

Therapeutic antibodies that target cell surface receptors can effectively induce receptor mediated cell signalling and in doing so provide growth inhibitory or apoptotic signals to the cell. The development of the therapeutic mAb Trastuzumab, (a humanized IgG_1 mAb directed against the extracellular domain of the HER-2/neu (c-erbB-2) epidermal growth factor receptor and licensed for the treatment of metastatic breast cancer) is an example of the effectiveness of such an approach [155].

The majority of human B cell malignancies and most epithelial malignancies express surface CD40 [16, 19-29, 72]. Considerable published data has demonstrated that ligation of surface CD40 on malignant (particularly epithelial) cell lines (generally by soluble CD154) leads to growth inhibition or apoptosis [19, 21, 23, 25, 29, 73-82]. The ability of Chi Lob 7/4 to cause direct growth inhibition in-vitro has been investigated in a number of human, non-Hodgkin's lymphoma and malignant epithelial cell lines through the use of [3^H methyl] thymidine incorporation and tetrazolium bromide conversion assays.

3.1.2 Complement dependent cytotoxicity

Antibody mediated activation of complement via the classical pathway is a major effector component of humoral immunity promoting the development of an inflammatory response, target cell opsonisation and cell lysis. Although the expression

of complement inhibitors by tumour cells may limit the ability of some therapeutic anti-cancer mAbs to effectively activate complement, a number of therapeutic mAbs such as the anti-CD20 mAb rituximab, are thought to achieve at least some of their therapeutic effect through the activation of complement dependent cytotoxicity (CDC) [116, 139].

The binding of Chi Lob 7/4 to CD40 on tumour cells might effectively activate complement and mediate killing through CDC. The ability of Chi Lob 7/4 to activate CDC in-vitro has been investigated in several human, non-Hodgkin's lymphoma and malignant epithelial cell lines through the use of a complement dependent cytotoxicity chromium ^{51} release assay.

3.1.3 Antibody dependent cellular cytotoxicity

Antibody dependent cellular cytotoxicity (ADCC), the recruitment of Fc receptor expressing natural killer cells and macrophages / monocytes to a target dictated by antibody specificity is thought to be an important mechanism of action for therapeutic antibodies used in the treatment of malignancy. Significant ADCC has been demonstrated in-vitro for both Alemtuzumab, (anti-CD52) and Trastuzumab (anti-Her2-neu), mAbs licensed for the use of chronic lymphocytic leukaemia and breast cancer respectively[118, 154]. Antibody isotypes that effectively recruit ADCC in-vitro have also been shown to exhibit good in-vivo activity[117, 118].

Chi Lob 7/4 binding to surface CD40 on tumour cells might effectively recruit immune effector activity through ADCC. The ability of Chi Lob 7/4 to activate ADCC has been investigated in several human, non-Hodgkin's lymphoma and malignant epithelial cell lines through the use of an antibody dependent cellular cytotoxicity chromium ^{51} release assay.

3.1.4 Stimulation of CD40 on antigen presenting cells (APCs)

The therapeutic use of mAbs that mimic or block important ligand / receptor interactions between cells of the immune system could promote the development of strong cellular immune responses in-vivo that might be effective against tumours, irrespective of tumour antigen expression. These receptors, amongst others, include the immune cell co-receptors CD154 and CD40.

CD40 mediated signalling is of critical importance to the regulation of cellular as well as humoral immune responses up-regulating the antigen presenting function of APCs, inducing high levels of MHC II, key co-stimulatory molecules such as B7-1 and B7-2, accessory molecules such as LFA-3 (CD58) and increasing the production of activating cytokines such as, IL-8, IL-12, TNF-α and macrophage inflammatory protein 1 α (MIP1-α)[55]. CD40 signalling is essential for the cross-priming and activation of CD8+ cytotoxic T cells; activated CD4+ helper T cells signal via CD154-CD40 to the APC and empower or license the APC to present antigen to and activate antigen specific responding CTL precursors[57-59]. Stimulation of CD40 by therapeutic agonistic mAb can effectively bypass the need for specific T cell help, directly priming helper dependent CD8+ cytotoxic T cell responses[58, 59]. In animal models of malignancy, this effect translates into an impressive anti-cancer immuno-therapeutic approach [164, 165].

Lob 7/4, the parent antibody of Chi Lob 7/4 has previously been assessed against a known agonistic anti-CD40 mAb s2c6 for its ability to upregulate the key co-stimulatory molecules B7-1 and B7-2 in a dendritic cell culture system developed by Jan Fisher and Chris Tretter, Dartmouth-Hitchcock Medical Centre, New Hampshire, USA. Lob 7/4, was found to up-regulate B7-1 and B7-2 in >90% of cultured dendritic cells, results similar to those obtained with s2c6 [181].

3.2 Materials and methods

Detailed materials and methods are described in chapter two.

3.3 Results

3.3.1 Surface phenotype

The surface phenotype of all cell lines was assessed using direct flow cytometry. All cell lines evaluated were shown to express surface CD40.

Figure 3.1a-e

3.3.2 Growth Inhibition

3.3.2.1 Bead - antibody binding to cells

Light microscopy of beads linked to Lob 7/4 or Chi Lob 7/4 and incubated with CD40 expressing cells (Daudi) showed obvious antibody mediated bead - cell binding. Beads linked to negative control mAbs (DB7-18 or Irr Hu IgG) did not bind to CD40 expressing cells.

Figure 3.2

3.3.2.2 Human non-Hodgkin's lymphoma cell lines

Growth inhibition of human non-Hodgkin's lymphoma cell lines was assessed using [3^{H} methyl] thymidine incorporation assays. Incubation of RL and Daudi cell lines for 5 days with Lob 7/4 and Chi Lob 7/4 led to a significant inhibition of cellular proliferation when compared to incubation with irrelevant, isotype matched murine (DB7-18) or human (Irr Hu IgG) mAb or to cells incubated without antibody. All antibodies were presented linked to microbeads; (100 μg linked to 2 x 10^{8} beads). Maximal growth inhibition occurred at a bead concentration of 50,000 beads per well.

Figures 3.3 and 3.4

3.3.2.3 Human epithelial cancer cell lines

Growth inhibition of human epithelial cancer cell lines was assessed using the tetrazolium bromide conversion assay. Incubation of EJ138 and Caski cell lines for 5 days with Lob 7/4 and Chi Lob 7/4 led to a significant inhibition of cellular proliferation when compared to incubation with irrelevant, isotype matched murine (DB7-18) or human (Irr Hu IgG) mAb or to cells incubated without antibody. All antibodies were presented linked to microbeads; (100μg linked to 2 x 10^8 beads). Maximal growth inhibition occurred at a bead concentration of 500,000 beads per well.

Figures 3.5 and 3.6

3.3.3 Complement mediated cytotoxicity

3.3.3.1 Human non-Hodgkin's lymphoma cell lines

The ability of Lob 7/4 and Chi Lob 7/4 to mediate complement mediated cytotoxicity (CDC) was assessed using the CDC chromium^{51} release assay. Chi Lob 7/4 was able to induce significant CDC (as measured by specific chromium^{51} release) in both RL and Daudi cells. In RL cells, Chi Lob 7/4 specific chromium^{51} release was maximal (22%) at a final antibody concentration of 0.4 μg/ml. In Daudi cells, Chi Lob 7/4 specific chromium^{51} release was maximal (65%) at a final antibody concentration of 2 μg/ml. Lob 7/4 did not mediate effective CDC.

Figures 3.7 and 3.8

3.3.3.2 Human epithelial cancer cell lines

Effective Chi Lob 7/4 mediated CDC could not be demonstrated in EJ138 or MG79 cells lines (data not shown).

3.3.4 Antibody directed cellular cytotoxicity

3.3.4.1 Human non-Hodgkin's lymphoma cell lines

The ability of Lob 7/4 and Chi Lob 7/4 to mediate antibody dependent cellular cytotoxicity (ADCC) was assessed using an ADCC chromium^{51} release assay. Chi Lob 7/4 was able to induce significant ADCC (as measured by specific chromium^{51} release)

in both RL and Daudi cells. In RL cells, maximal Chi Lob 7/4 mediated specific chromium^{51} release (65%) was seen at a final antibody concentration of 10μg/ml and an effector : target ratio of 50:1. Chi Lob 7/4 was significantly better at mediating ADCC than rituximab at equivalent antibody concentrations and effector : target ratios. In Daudi cells, maximal Chi Lob 7/4 mediated specific chromium^{51} release (71%) was seen at a final antibody concentration of 10 μg/ml at an effector target ratio of 50:1. Lob 7/4 did not mediate effective ADCC in either cell line.

Figures 3.9 and 3.10

3.3.4.2 Human epithelial cancer cell lines

Effective Chi Lob 7/4 mediated CDC could not be demonstrated in EJ138 or MG79 cells lines (data not shown).

3.4 Summary of results

Flow cytometry confirms the expression of CD40 on the surface of the human non-Hodgkin's lymphoma (NHL) cell lines RL and Daudi and the epithelial cancer cell lines EJ138 and Caski. Ligation of surface CD40 by Lob 7/4 or Chi Lob 7/4 presented on M-450 Dynabeads caused significant growth inhibition in the human NHL cell lines RL and Daudi and in the malignant epithelial cell lines EJ138 and Caski. Binding of Chi Lob 7/4 to surface CD40 on RL and Daudi cells was able to effectively activate complement and mediate CDC. This effect could not be reproduced in human epithelial cell lines. Due to its murine Fc domain, Lob 7/4 was unable to mediate CDC with human complement. Binding of Chi Lob 7/4 to surface CD40 on RL and Daudi cells was able to effectively mediate ADCC. This effect could not be reproduced in human epithelial cell lines. Due to its murine Fc domain, Lob 7/4 did not mediate significant ADCC with human effectors cells.

From these studies we can be confident that Chi Lob 7/4 is able to bind in a similar manner to the parent mAb Lob 7/4 and is fully functional. Furthermore, the human constant regions of the chimeric antibody allow it to effectively interact with human effector mechanisms.

Figure 3.1a

RL cell line. Cell surface phenotype assessed by flow cytometry using FITC labelled irrelevant, anti-CD40, CD19, CD22, CD37, CD20, Class II MHC and IgM antibodies. Top image, forward scatter (X axis), side scatter (Y axis). Subsequent images mean fluorescent intensity (x axis), cell count (Y axis).

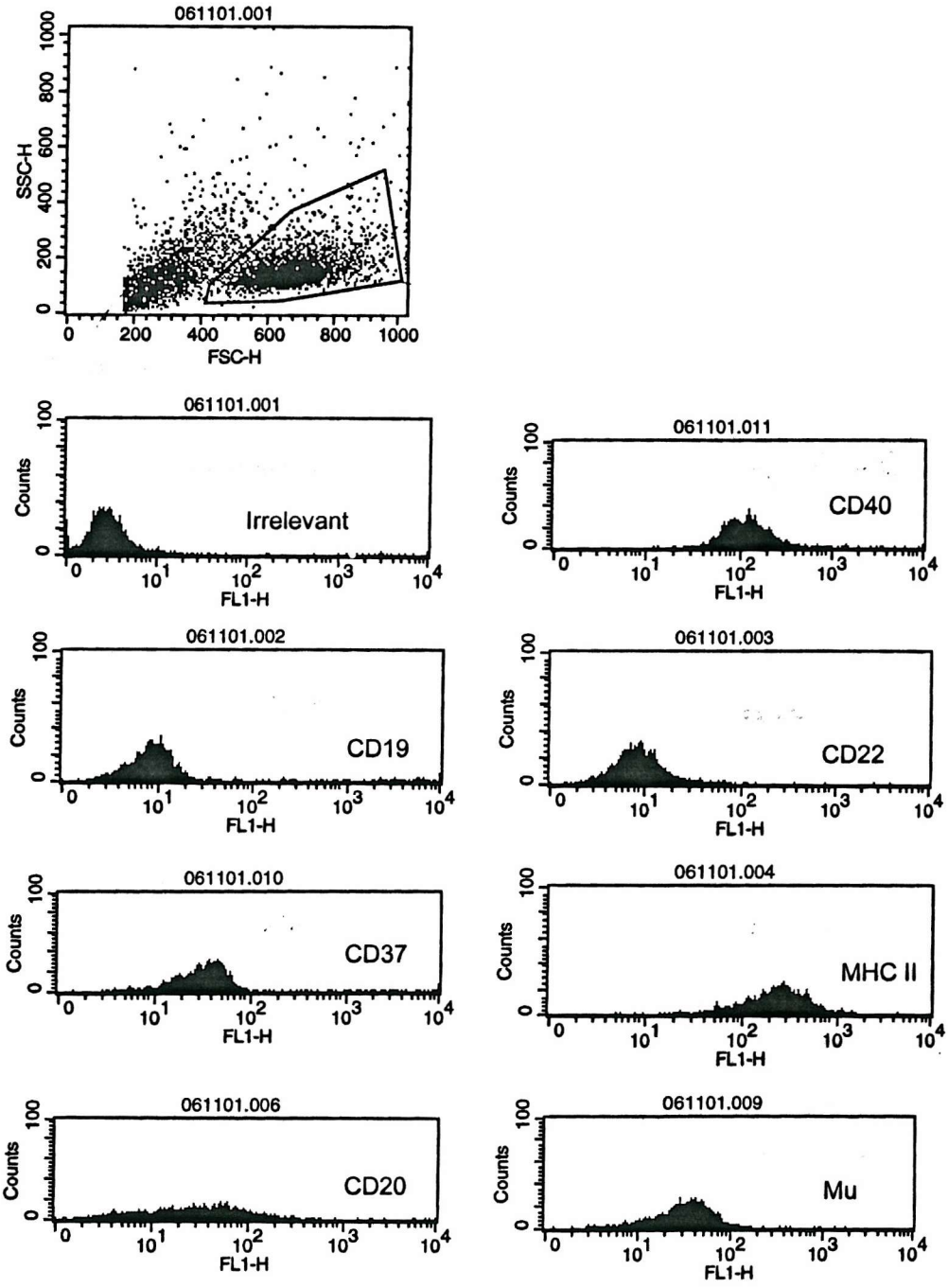


Figure 3.1b

Daudi cell line. Cell surface phenotype assessed by flow cytometry using FITC labelled irrelevant, anti-CD40, CD19, CD22, CD37, CD20, Class II MHC and IgM antibodies. Top image, forward scatter (X axis), side scatter (Y axis). Subsequent images mean fluorescent intensity (x axis), cell count (Y axis).

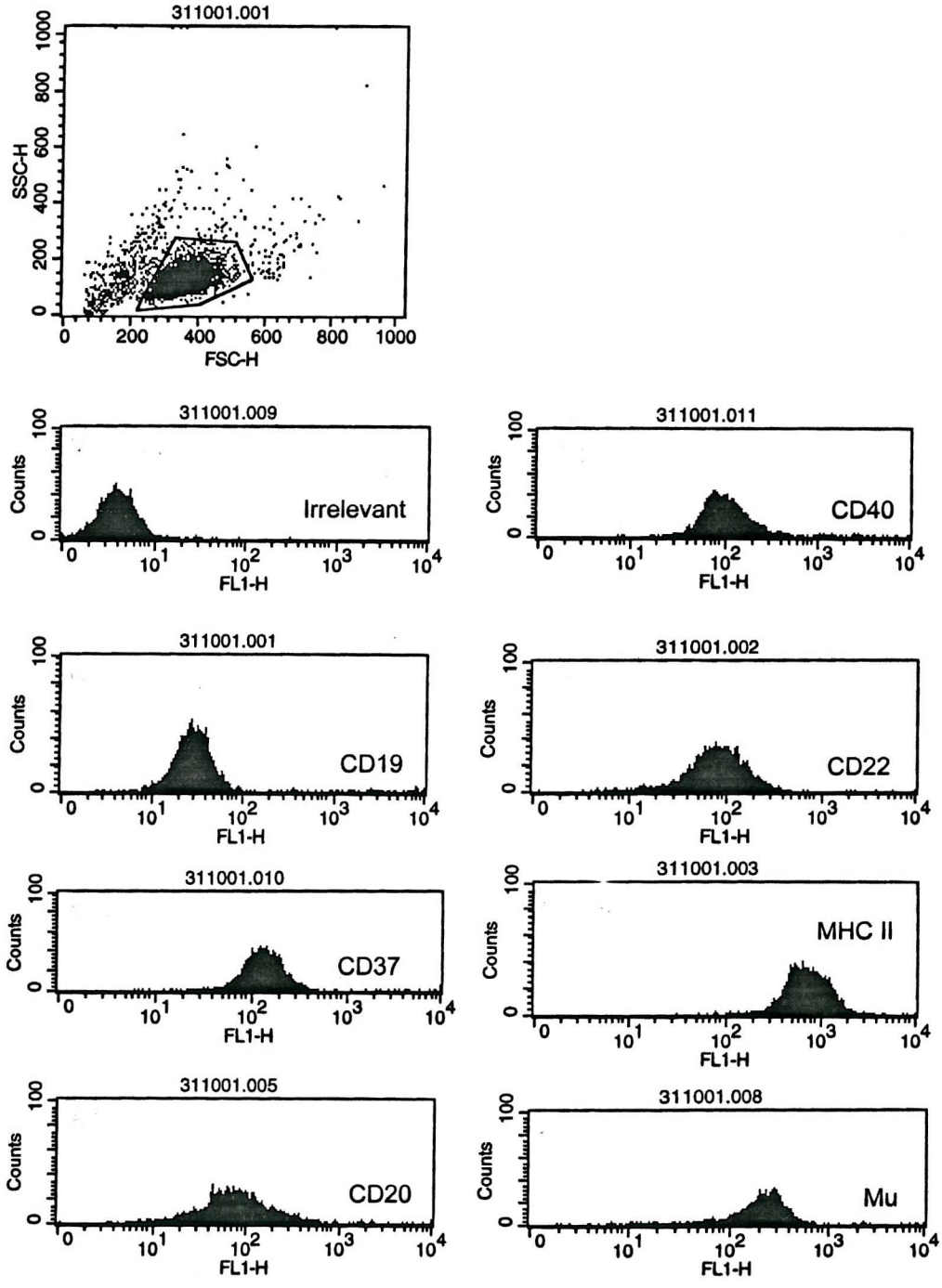


Figure 3.1c

Caski cell line. Cell surface phenotype assessed by flow cytometry using FITC labelled irrelevant and anti-CD40 antibodies. Top image, forward scatter (X axis), side scatter (Y axis). Subsequent images mean fluorescent intensity (x axis), cell count (Y axis).

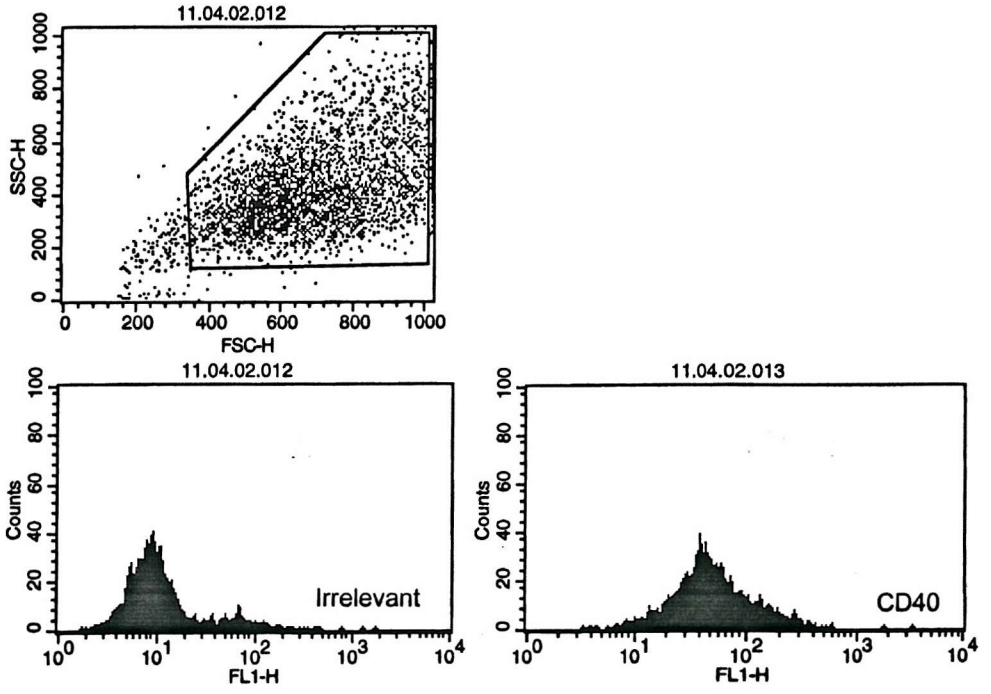


Figure 3.1d

MG79 cell line. Cell surface phenotype assessed by flow cytometry using FITC labelled irrelevant and anti-CD40 antibodies. Top image, forward scatter (X axis), side scatter (Y axis). Subsequent images mean fluorescent intensity (x axis), cell count (Y axis).

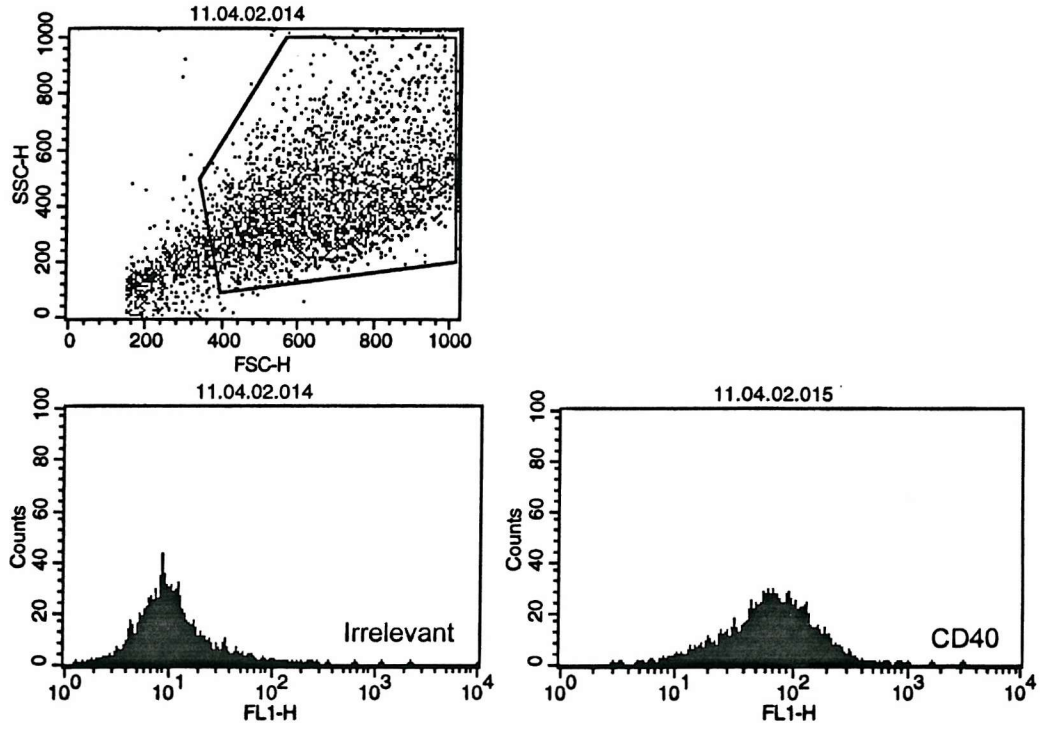


Figure 3.1e

EJ138 cell line. Cell surface phenotype assessed by flow cytometry using FITC labelled irrelevant and anti-CD40 antibodies. Top image, forward scatter (X axis), side scatter (Y axis). Subsequent images mean fluorescent intensity (x axis), cell count (Y axis).

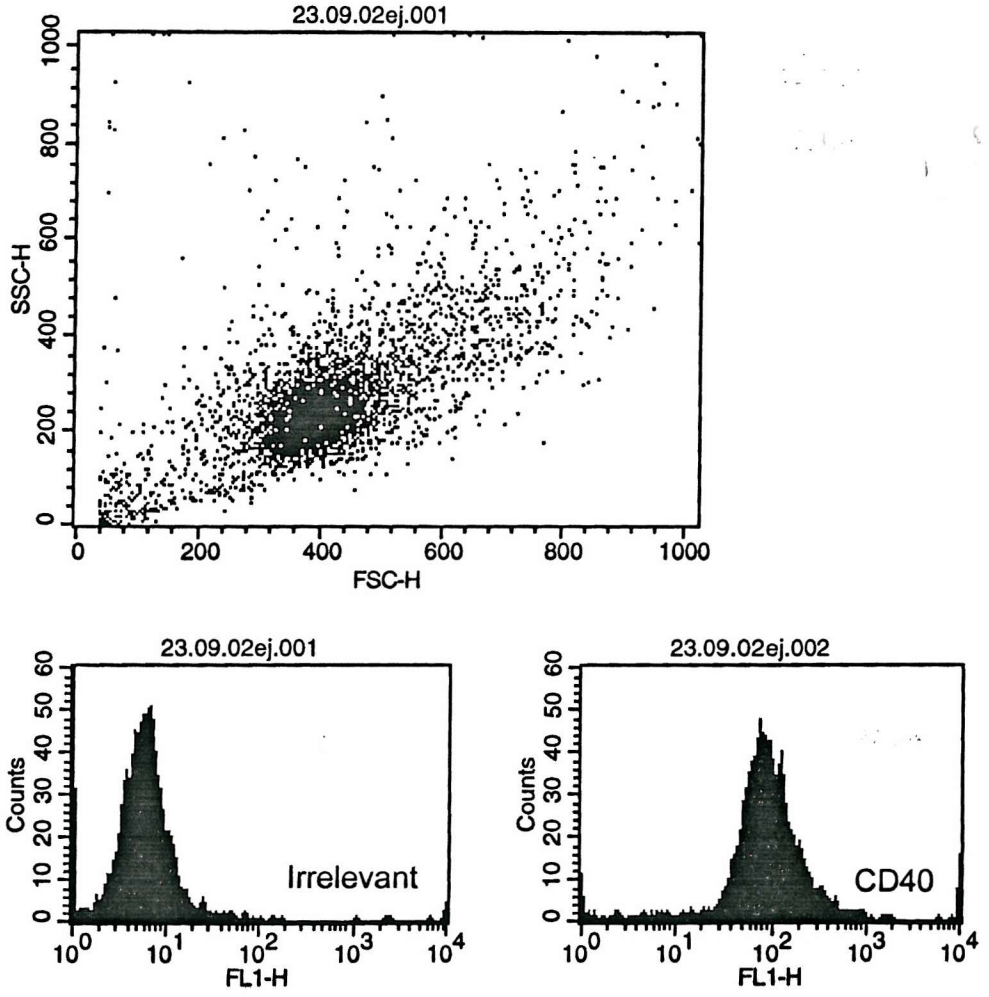
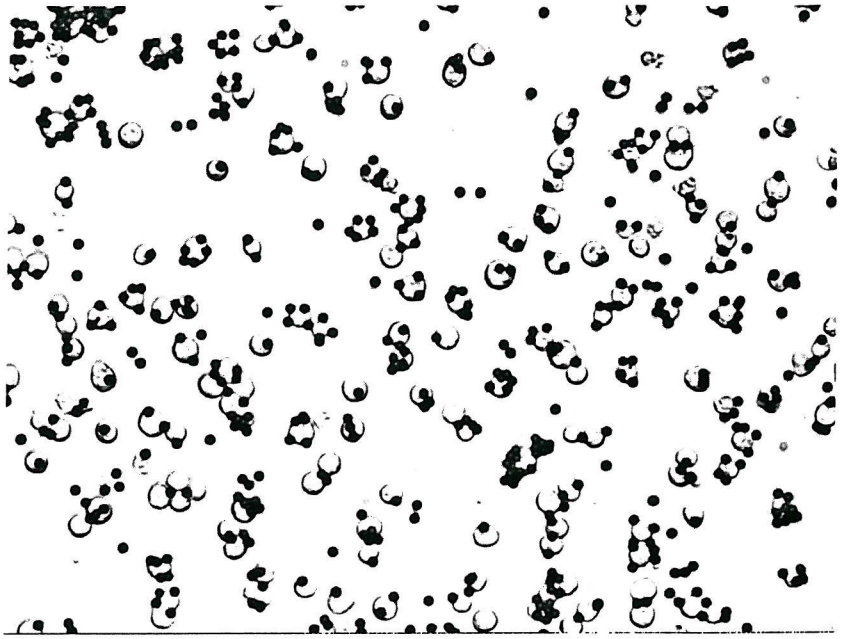


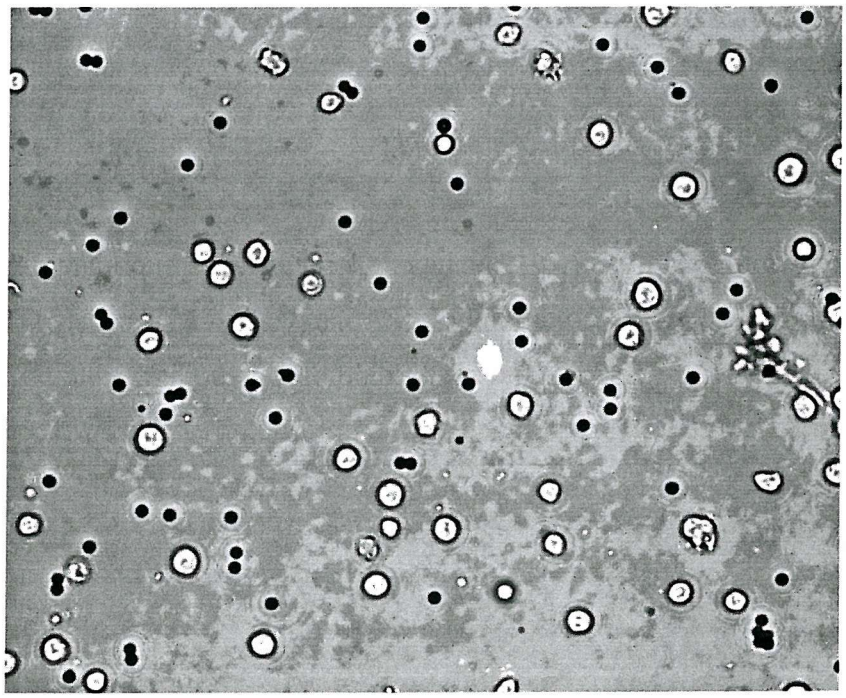
Figure 3.2

Antibody presentation on superparamagnetic, polystyrene, polyurethane-coated microbeads (Dynabeads M-450, Dynal AS). Light microscopy (x10 magnifcation).

a) Chi Lob 7/4: Antibody / bead binding to Daudi cells.



b) DB7-18 (negative control): No antibody / bead binding to Daudi cells.



Figures 3.3 and 3.4

Growth inhibition of RL and Daudi cells by Chi Lob 7/4 compared to Lob 7/4 and isotype matched negative control antibodies (Irr Hu IgG and DB7-18) assessed by relative uptake of [3^{H} methyl] thymidine. Mean values plus standard deviation.

Figure 3.3 RL cells

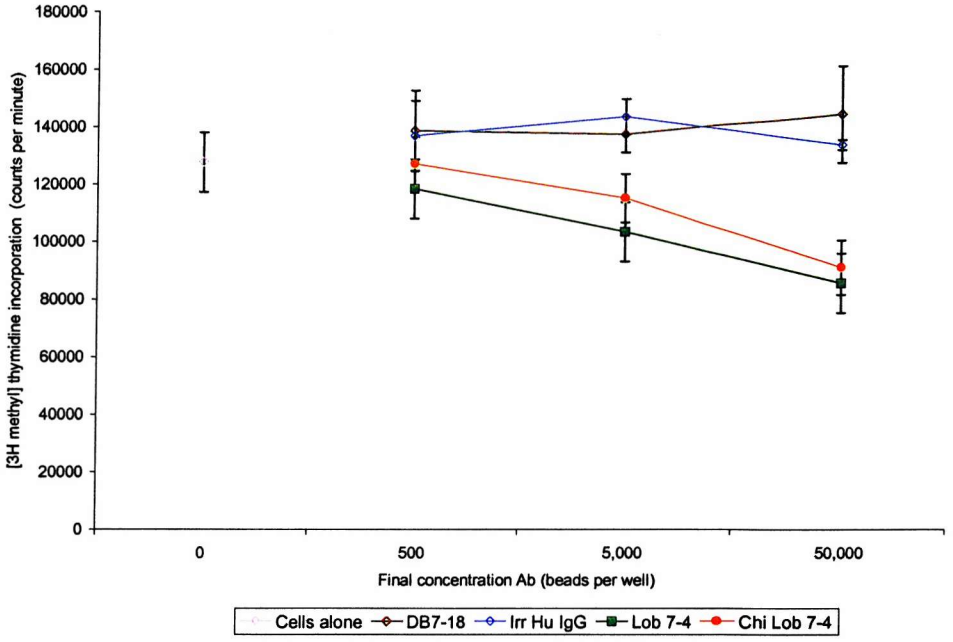


Figure 3.4 Daudi cells

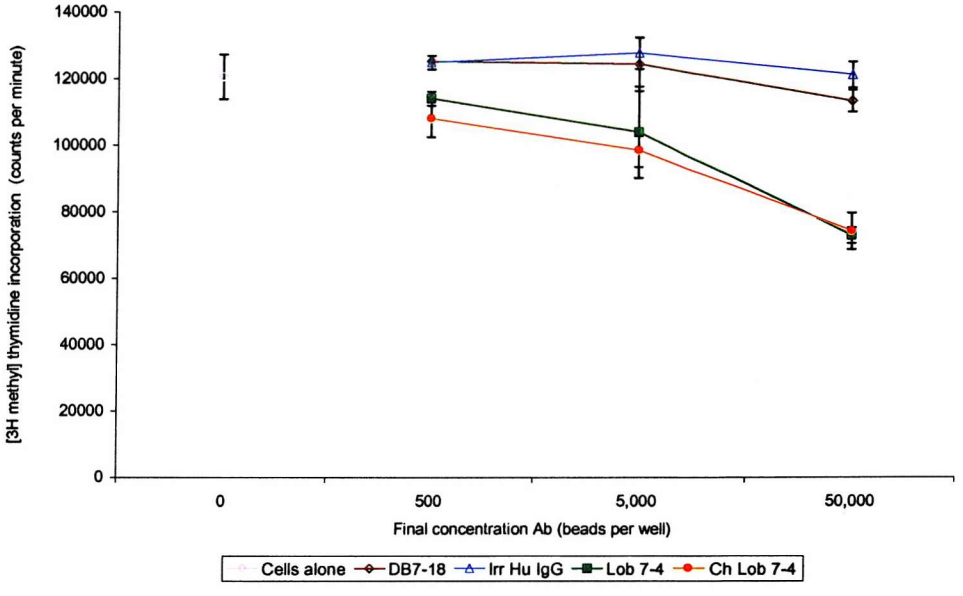


Figure 3.5 and 3.6

Growth inhibition of EJ138 and Caski cells by Chi Lob 7/4 compared to Lob 7/4, an isotype matched negative control antibody (Irr IgG) and a positive control (Cisplatin) assessed by tetrazolium bromide conversion assay. Mean values plus standard deviation

Figure 3.5 EJ138 cells

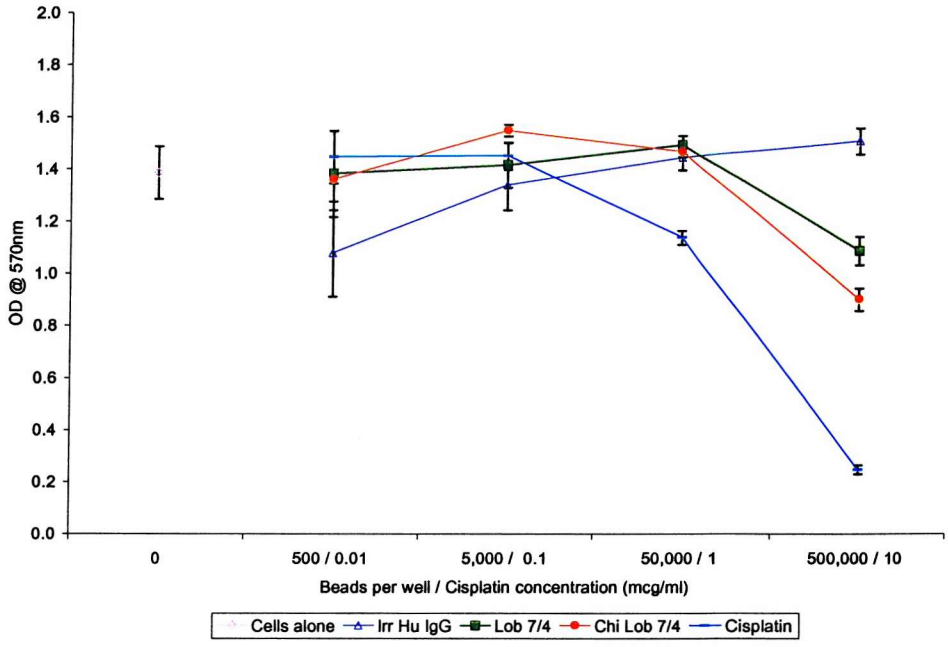
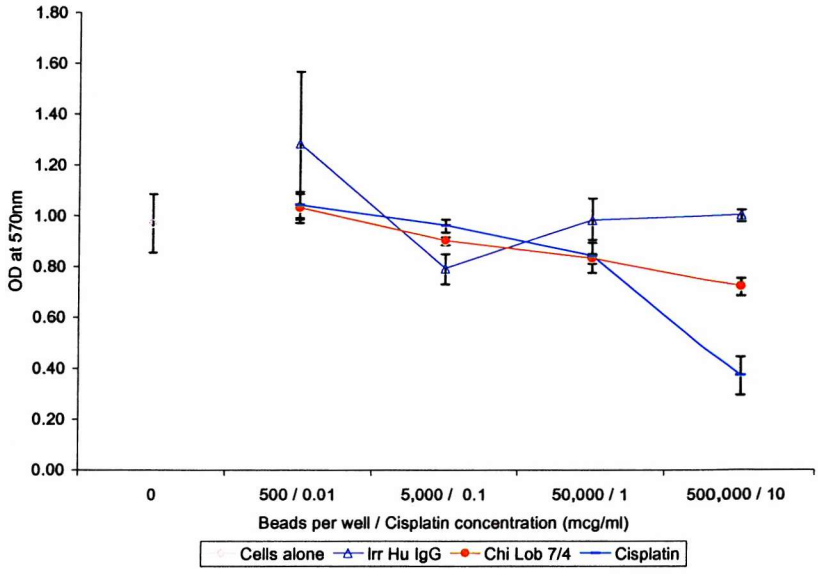


Figure 3.6 Caski cells



Figures 3.7 and 3.8

Complement mediated cytotoxicity of RL and Daudi cell lines by Chi Lob 7/4 compared to Lob 7/4 and isotype matched positive (rituximab) and negative (irrelevant IgG) control mAbs by chromium^{51} release assay.

Figure 3.7 RL cells

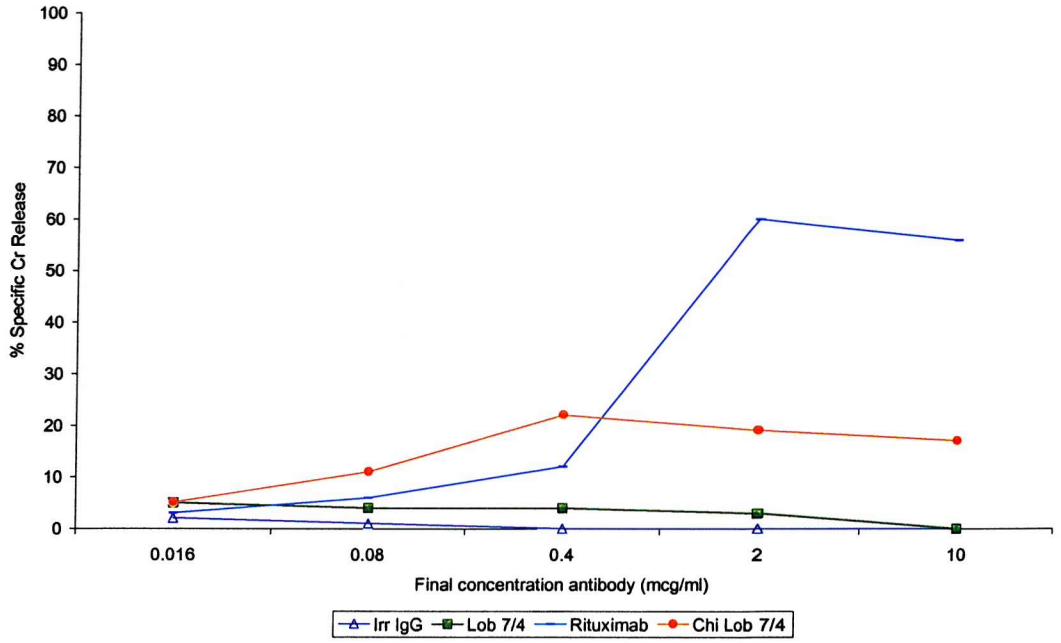
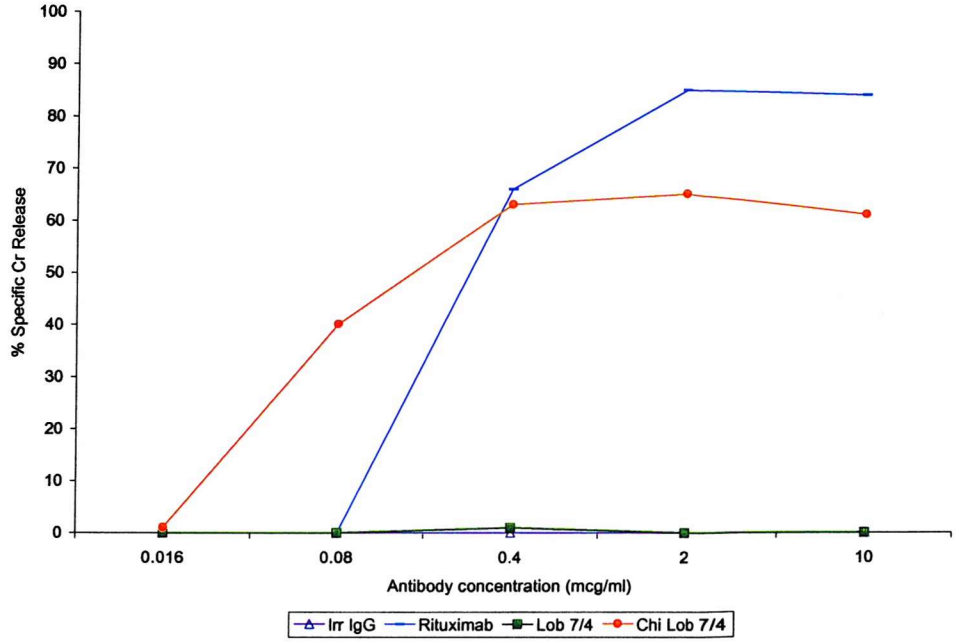


Figure 3.8 Daudi cells



Figures 3.9 and 3.10

Antibody dependent cellular cytotoxicity of RL and Daudi cell lines by Chi Lob 7/4 compared to Lob 7/4 and isotype matched positive (rituximab) and negative (irrelevant IgG) control antibodies assessed by chromium^{51} release assay. Mean values plus standard deviation.

Figure 3.9 Daudi cells. Effector : target ratio 50:1. Variable mAb concentration.

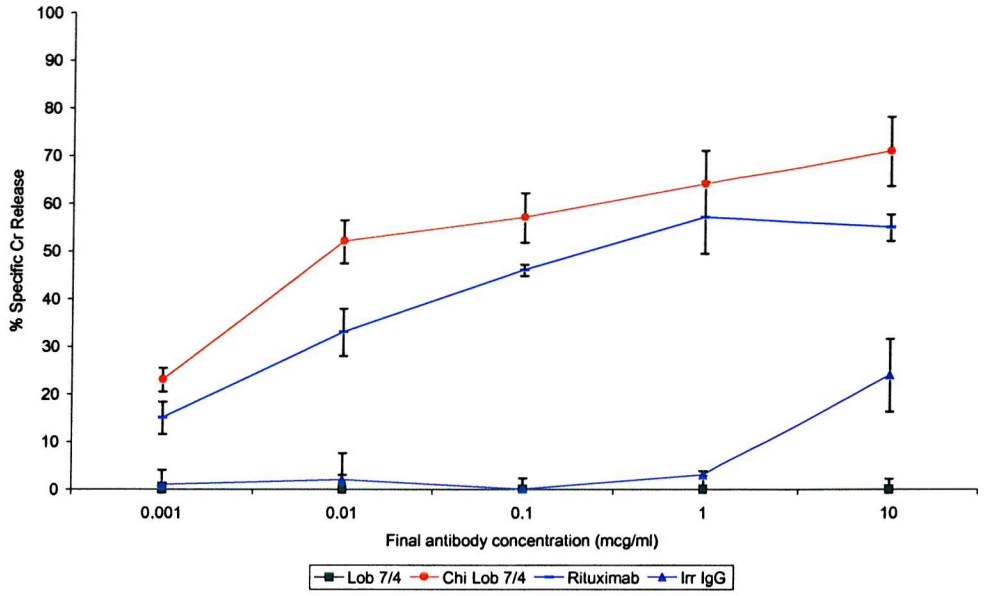


Figure 3.10a RL cells. Effector : target ratio 50:1. Variable mAb concentration.

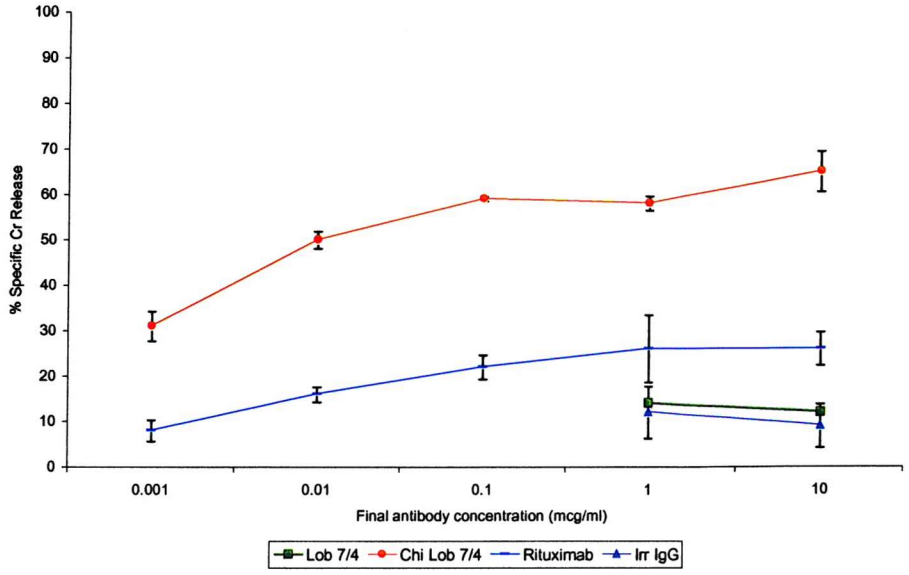
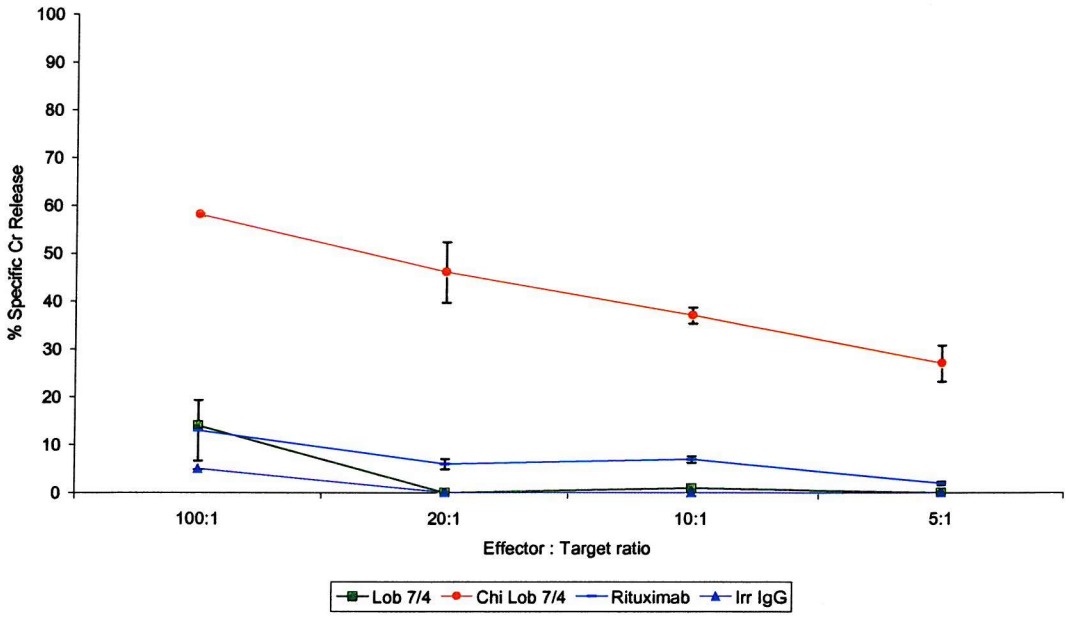


Figure 3.10b RL cells. Variable effector : target ratios.
Antibody concentration 1 μg/ml.



CHAPTER FOUR

4 TOXICITY OF ANTI-CD40 MONOCLONAL ANTIBODY THERAPY

4.1 Introduction

As part of the development of any new therapeutic agent, an assessment of the potential toxicity of therapy must be made before human phase I trials can be undertaken. Given the nature and mechanisms of action of Chi Lob 7/4, conventional preclinical toxicological testing in animals is unlikely to provide much useful toxicological data. However, the development of a mouse model using the rat anti-mouse CD40 monoclonal antibody 3/23 has allowed substantial comparative preclinical toxicology testing to be performed.

In general, monoclonal antibody therapy is well tolerated. Significant side-effects are rare. Common toxicities include infusion related hypersensitivity reactions which may be abrogated through slowing of the infusion and the co-administration of antihistamines and corticosteroids. However, repeated infusion of murine mAbs may be problematic. Murine antibodies may be highly immunogenic to humans with repeated administration commonly leading to the generation of human anti-mouse antibody (HAMA) responses. HAMA responses may not only adversely affect the clinical efficacy and half life of therapeutic antibodies, but are also implicated in the clinical symptoms of allergy and anaphylaxis. For the most part, the chimerisation / humanisation of murine monoclonal antibodies has successfully overcome these problems.

Widespread activation of CD40 through the systemic use of CD154 or agonistic anti-CD40 mAbs may result in toxicity as a result of CD40 signalling. CD40-CD154 interactions are critically important for the generation of humoral and cellular immune responses and it might be hypothesised that widespread CD40 ligation and activation could give rise to immune mediated disease. Studies with CD154 blocking agents such as anti-CD154 mAbs suggest that aberrant CD40 activation may well be important to the pathogenesis of auto-immune diseases; CD154 blockade can help to prevent the development of animal models of auto-immune

disease as diverse as systemic lupus erythematosus [166], rheumatoid arthritis [167], type I diabetes mellitus [168], atherosclerosis [169-172], neurodegenerative disorders [173], graft versus host disease and allograft rejection [176, 177]. It remains to be seen whether short term treatment of patients with malignancy with agonistic anti-CD40 mAbs could be capable of inducing clinically relevant autoimmune disease. In the single published phase I clinical trial of systemic administration of CD154, autoimmune sequelae were not observed[178].

Previous exploratory work carried out in The Tenovus Research Institute by Dr Melanie Harvey, (a previous Cancer Research Campaign Clinical Research Fellow) had begun to examine the acute toxicities of agonistic anti-CD40 mAb therapy using rat anti-mouse CD40 mAb 3/23 in a mouse model [181]. In these studies, 3/23 was administered to a number of mice by intraperitoneal (IP) injection at a range of doses (100 μg to 10 mg). Animals were sacrificed 14 days after injection of 3/23 and subjected to haematological, biochemical and histopathological analysis.

No toxicity was seen in animals treated with 100 μg of 3/23. Animals treated with 1 mg or more of 3/23 however demonstrated a dose dependent, acute hepatitis with an associated elevation in serum liver transaminases (figure 4.1). Peak elevations in serum liver transaminases were seen at the 4 mg dose. Other toxicity included splenomegaly (noted in animals receiving 1 mg or more of 3/23) and a mild nephritis (in animals receiving 200 μg or more of 3/23). No other toxicity was observed and there were no toxic deaths.

This chapter presents a detailed assessment of the toxicity (and its reversibility) of anti CD40 mAb therapy (3/23) in a comparative mouse model. The results of formal toxicological testing of the chimeric anti human CD40 mAb, Chi Lob 7/4 in two rodent species are also presented.

4.2 Materials and methods

General methods are described in chapter two. Specific methods are described below. Toxicity studies were performed using eight week old, pathogen free, male, C57BLK/6 or BALB/c mice or Syrian Hamsters (Harlan, UK and Charles River, UK). Prior to use, bacterial endotoxin was removed from stocks of Chi Lob 7/4 and 3/23 by affinity chromatography. Antibodies were confirmed to be free of endotoxin prior to administration through the use of a Limulus Amoebocyte Lysate (LAL) endotoxin test kit (Bio Whittaker, USA).

4.2.1 Comparative mouse model

4.2.1.1 Intraperitoneal mAb therapy

BALB/c and C57BLK/6 mice in cohorts of three, were injected via the intraperitoneal (IP) route with a single dose of either 1 mg or 4 mg of endotoxin free 3/23 in a volume of 200 µl phosphate buffered saline (PBS). Mice were sacrificed in groups of three at time intervals of one, two, three, four, six, eight and twelve weeks after injection. Animals were culled by halothane anaesthesia followed by carbon dioxide re-breathing. Terminal cardiac bleeds were performed and samples subjected to haematological and biochemical analysis within the relevant laboratories of Southampton General Hospital. Post-mortem examination was performed on all animals and selected pathological specimens retained for expert review by Dr Norman Carr, Consultant Histopathologist, Southampton General Hospital.

The feasibility of repeated IP dosing with 3/23 was also assessed. 3/23 was administered IP weekly at a dose of 25 or 100 µg (the highest dose at which no toxicity was seen in single dose studies) on four occasions, to cohorts of five C57BLK/6 in a volume of 200 µl PBS. All animals were sacrificed one week after the final injection.

4.2.1.2 Intravenous mAb therapy

The repeated administration of 3/23 via the intravenous route (tail vein) was evaluated in C57BLK/6 mice. Mice were treated in cohorts, on four occasions, at a variety of doses and by a variable administration schedule as outlined below.

4.2.1.2.1 Weekly schedule

3/23 was administered IV weekly at doses of 25, 50, 75 and 100 μg (the highest dose at which no toxicity was seen in single dose studies) on four occasions, to cohorts of five C57BLK/6 mice in a volume of 200 μl PBS. An isotype matched, negative control mAb, Mc39-16 (a mAb recognising the A31 mouse lymphoma idiotype) and Chi Lob 7/4 were given to further cohorts of animals using an equivalent route of administration, dose and schedule. Additionally, assessment was made of the toxicity of administering 100 μg 3/23 IV for two injections followed by 100 μg Mc39-16 for two injections over a four week period. All animals were sacrificed one week after the final injection.

4.2.1.2.2 Variable schedule

A comparison was made between various schedules of IV administration of 3/23. 100 μg of 3/23 in a volume of 200 μl PBS was administered IV to cohorts of five C57BLK/6 mice at fortnightly, three weekly, four weekly and six weekly intervals. Animals received up to four injections in total. Animals were sacrificed one week after the final injection.

4.2.2 Assessment of mouse anti rat antibody (MARA) responses

The development of mouse anti-rat antibody responses in animals treated with the rat anti-mouse mAb 3/23 or control mAb Mc39-16 was assessed by an enzyme linked immunosorbant assay (ELISA) as described in chapter two. Serum samples were obtained from tail bleeds from cohorts of four C57BLK/6 mice one week after three separate 100 μg IV mAb injections (given weekly over three weeks). As an additional control, serum from two untreated experimental control animals was evaluated.

4.2.3 Chi Lob 7/4 toxicity studies

In accordance with Cancer Research UK guidelines, limited toxicological testing with Chi Lob 7/4 was performed in two separate rodent species. 10 mg of endotoxin free Chi Lob 7/4 was administered to six C57BLK/6 mice and six Syrian hamsters. Animals were culled at 14 days by halothane anaesthesia followed by carbon dioxide re-breathing. Terminal cardiac bleeds were performed on all animals and haematological and biochemical analysis performed within the relevant laboratories of Southampton General Hospital. Post-mortem examination was performed on all animals and specimens retained for expert review by Dr Norman Carr, Consultant Histopathologist, Southampton General Hospital.

4.3 Results

4.3.1 Comparative mouse model: Single dose intraperitoneal 3/23 treatment

There were no animal deaths at doses at either the 1 mg or 4 mg dose level. All animals injected with 3/23 were observed to be non-specifically unwell (lethargy / reduced feeding) after injection. The severity was dose dependent and resolved by 2 to 3 days post injection.

4.3.1.1 Biochemistry - Renal function

No significant elevation in biochemical markers of impaired renal function such as serum urea or creatinine were observed in C57BLK/6 or BALB/c mice at the either the 1 mg or 4 mg dose levels.

Figures 4.2 and 4.3

4.3.1.2 Biochemistry - Liver function

All animals treated with 3/23 developed reversible elevations of serum liver transaminases (alanine transaminase, ALT; aspartate transaminase, AST) compatible with a degree of reversible liver damage. Serum ALT (the more liver specific of the two transaminases measured) levels peaked at one to three weeks. Animals receiving 4 mg of 3/23 demonstrated the highest levels of ALT rise (up to 5 fold rise from baseline). ALT levels reached a higher peak level in C57BLK/6 mice than in

BALB/c mice. ALT levels returned to normal by four weeks. A similar pattern of change was seen in AST levels.

Figures 4.4 and 4.5

4.3.1.3 Haematology - Full blood count

At the 4 mg dose level, moderate falls (~30% reduction from baseline) in haemoglobin levels were observed over time. Mean levels fell to a nadir two to three weeks post 3/23 recovering to normal by week twelve. No consistent change was seen in total white blood cell count, white blood cell differential count or platelet count (not shown).

Figure 4.6

4.3.1.4 Histopathology

Up to six weeks after treatment, macroscopic changes were apparent in the spleens and livers of most animals at post mortem. Microscopic changes were visible in spleens, livers and kidneys retained for examination. All macroscopic and microscopic changes had resolved by eight weeks after treatment. No significant differences in the nature or severity of histopathological changes were seen between BALB/c and C57BLK/6 mice.

4.3.1.4.1 Spleen

Macroscopic

Mean splenic size / weight was seen to increase by up to six fold at the 4 mg dose level, peaking one to three weeks post injection and returning to normal by week eight. Changes in splenic weight were more pronounced in BALB/c mice compared to C57BLK/6 mice.

Figure 4.7

Microscopic

Microscopic splenic changes consisted of marginal zone hypertrophy / hypertrophy of the periarteriolar lymphoid sheaths and occasional microgranulomata. Changes were maximal at week two to three, returning to normal by weeks four to six.

Figure 4.8

4.3.1.4.2 Liver

Macroscopic

The majority of livers removed from animals at post mortem within the first four to six weeks post 3/23 demonstrated an abnormal pale, mottled appearance. Livers removed five weeks or more after 3/23 appeared normal.

Microscopic

Within the liver, a dose dependent, acute lympho-granulomatous hepatitis was apparent from one week after treatment. Small aggregates of inflammatory cells and degenerate hepatocytes were apparent. In more severely affected livers, microgranulomata became confluent and features compatible with piecemeal necrosis were seen.

Figure 4.9

Diastase periodic acid Schiff staining revealed an increase in seroid laden macrophages and Kupffer cells consistent with uptake of necrotic hepatocytes by these cells. Reticulin staining confirmed piecemeal hepatic necrosis to be lying predominantly within zone one of the liver. Three months after treatment with 3/23, a slight increase in fibrous tissue was seen in the portal triads but there was no bridging / linkage fibrosis.

Figure 4.10 and 4.11

Immunohistochemical staining revealed large numbers of CD3 positive T cells infiltrating the liver parenchyma and portal tracts. The majority of these cells were CD8 positive cytotoxic T cells.

Figure 4.12

Immunohistochemical staining for α smooth muscle actin, a marker of activated hepatic stellate cells, showed widespread stellate cell activation within the liver.
Figure 4.13

By three weeks after injection of 3/23, many of the above features had begun to resolve. By week five, all livers appeared histologically normal demonstrating complete resolution of all previously noted abnormalities.
Figure 4.14

4.3.1.4.3 Kidney

Macroscopic

All kidneys removed from 3/23 treated animals appeared macroscopically normal.

Microscopic

Mild renal changes were apparent one week after treatment. Changes were limited to a minimal / mild tubulo-interstitial lymphocytic nephritis. The lymphocytic infiltrate was predominantly made up of CD3 positive, CD8 positive cytotoxic T cells.
Figure 4.15

By week five, all kidneys appeared histologically normal demonstrating complete resolution of all previously noted abnormalities (not shown).

4.3.2 Multiple dose studies

4.3.2.1 Intraperitoneal 3/23 administration: Weekly schedule

Animals administered 3/23 at a dose of 25 or 100 μg by IP injection weekly on four occasions remained well, without signs of systemic toxicity. All animals continued to feed normally and gain weight as expected, (data not shown).

4.3.2.2 Intravenous 3/23 administration: Weekly schedule

Animals receiving 3/23 by IV injection appeared outwardly well until the third injection of 3/23. Following the third injection, animals became acutely unwell within minutes of treatment. Animals appeared crouched, still and relatively unresponsive. Their breathing became shallow and laboured. One animal from the

25 μg group and four animals from the 100 μg group failed to recover and died within one hour of injection. Within two to three hours, all remaining animals recovered fully. Following the fourth injection, all animals became similarly unwell. The four remaining animals from the 25 μg group recovered fully within two to three hours. A fifth animal from the 100 μg group failed to recover and died within one hour of injection.

Table 4.1

4.3.2.3 Intravenous 3/23 administration: Variable schedule

In addition to the weekly schedule of four injections described above, cohorts of C57BLK/6 mice were injected IV with 100 μg of 3/23 on four occasions at fortnightly, three weekly, monthly and six weekly intervals. Animals appeared well following the first injection but unwell following all other injections. Animals appeared crouched, still and relatively unresponsive and their breathing became shallow and laboured. There were three animal deaths: Two animals in the four weekly schedule cohort died, one following injection two, the other following injection three; one animal in the three weekly schedule cohort died following the third injection. All other animals made a complete recovery within two to three hours of each injection.

Table 4.1

4.3.2.4 Intraperitoneal / intravenous Mc39-16 administration

All animals receiving the irrelevant, negative isotype control mAb, Mc39-16 by IV or IP injection remained well throughout the course of their treatment regardless of dose and there were no animal deaths in any of these treatment groups, (data not shown).

4.3.2.5 Intravenous 3/23 followed by intravenous Mc39-16 administration

For the cohort of animals treated weekly with 100 μg of 3/23 IV for two injections followed by 100 μg Mc39-16 for two injections, animals appeared acutely unwell as described above following their third and fourth injection (first and second Mc39-16 injections). One animal died within one hour of the fourth and final injection. All remaining animals recovered fully within two to three hours.

Table 4.1

Survival of C57BLK/6 mice treated with four doses of intravenous rat anti-mouse mAb 3/23 at a variety of doses and schedules.

Schedule	Dose (μg)	Dose 1		Dose 2		Dose 3		Dose 4	
		Animals receiving mAb	Animals surviving	Animals receiving mAb	Animals surviving	Animals receiving mAb	Animals surviving	Animals receiving mAb	Animals surviving
Weekly	25	5	5	5	5	5	*4*	*4*	*4*
	50	5	5	5	5	5	5	5	5
	75	5	5	5	5	5	5	5	5
	100	10	10	10	10	10	*6*	*6*	*5*
Two weekly	100	5	5	5	5	5	5	5	5
Three weekly	100	5	5	5	5	5	*4*	*4*	*4*
Four weekly	100	5	5	5	*4*	*4*	*3*	*3*	*3*
Six weekly	100	5	5	5	5	5	5	5	5

4.3.3 Mouse anti rat antibody (MARA) responses: 3/23

Antibodies against rat immunoglobulin (3/23) were detectable by ELISA in animals treated with 3/23 (100 μg 3/23 given IV weekly for three weeks). Antibodies were detectable at the highest serum dilution (1:128,000). A control / standard mouse anti rat monoclonal antibody, M359 F10 was detectable by ELISA (using the same 3/23 coated ELISA plate) at a concentration of 50 – 100 ng/ml. Mouse anti-rat antibodies were not detected in an untreated control animal.

Figure 4.16a

4.3.4 Mouse anti rat antibody (MARA) responses: Mc39-16

Significant antibodies against rat immunoglobulin (Mc39-16) were not detectable by ELISA in the majority (three of the four animals) treated with Mc39-16 (three 100μg Mc39-16 doses given IV weekly over three weeks). A low level MARA response was seen in mouse two at the strongest serum dilutions (1:1,000 – 1:2000). The control / standard mouse anti rat monoclonal antibody, M359 F10 was detectable by ELISA (using the same Mc39-16 coated ELISA plate) at a concentration of 50 - 100ng/ml. Mouse anti-rat antibody responses were not detected in an untreated control animal.

Figure 4.16b

4.3.5 Chi Lob 7/4

4.3.5.1 Multiple dose studies

Animals receiving a total of four, weekly treatments of up to 100 μg Chi Lob 7/4 by IV or IP injection remained well throughout the course of their treatment regardless of dose and there were no animal deaths in any treatment groups, (data not shown).

4.3.5.2 High dose single dose studies

Six pathogen free C57BLK/6 mice and six Syrian hamsters were administered a single 10 mg IP dose of endotoxin free Chi Lob7/4. Animals did not appear unwell following antibody injection and continued to feed normally and gain weight. Terminal bleeds were performed for biochemical and haematological analysis and

results compared to untreated control animals. No significant biochemical or haematological abnormalities were seen. No macroscopic abnormalities were seen at post-mortem. Given the toxicities observed in 3/23 treated animals, specimens of liver, kidney and spleen were taken from each animal, preserved in formalin and processed into paraffin blocks. Slides cut from each of these blocks were stained with haematoxylin and eosin; no significant histopathological abnormalities were observed in any tissue examined.

Figure 4.17 and 4.18

4.4 Summary of results

4.4.1 Comparative mouse model: 3/23 single dose studies

Single dose toxicity studies in mice reveal the major toxicity of intraperitoneal treatment with the rat anti mouse CD40 mAb 3/23 to be a reversible, dose dependent, acute hepatitis. Serum biochemistry and pathological examination of animal livers revealed that peak hepatic toxicity occurred within one to three weeks of 3/23 treatment. Histopathology confirmed a lymphogranulomatous hepatitis with infiltration of predominantly CD8 positive T cells. Widespread stellate cell activation was seen. Piecemeal necrosis was seen in the livers of more severely affected animals. Regardless of severity, all changes were completely reversible with most hepatic changes resolving by five weeks after treatment. There was no evidence of significant liver fibrosis.

Treated animals developed significant splenomegaly, maximal one to three weeks post treatment and normalising by week eight. Microscopic evaluation revealed marginal zone hypertrophy / hypertrophy of the periarteriolar lymphoid sheaths.

A mild lymphocytic nephritis (without associated change in serum biochemistry) was seen within one to three weeks of 3/23 treatment. The lymphocytic infiltrate was made up predominantly of CD8 positive T cells.

All biochemical, haematological and histopathological changes were completely reversible, resolving completely over the three month study period. There were no animal deaths in this group of animals receiving a single IP dose of 3/23.

4.4.2 Comparative mouse model: Multiple 3/23 and Mc39-16 dose studies

4.4.2.1 Intraperitoneal dosing

Animals receiving up to 100 μg of 3/23 weekly for four injections remained well without signs of systemic toxicity. There were no animal deaths in this group.

4.4.2.2 Intravenous dosing

Animals treated with multiple doses of intravenous 3/23 became significantly unwell following repeat doses of 3/23 given two or more weeks after the initial dose. For animals receiving 3/23 weekly, this occurred on their third dose; for animals receiving 3/23 on fortnightly, three weekly, monthly or six weekly schedules, this occurred on their second dose. Animals became unwell within minutes of their injection appearing crouched, still and relatively unresponsive. The majority of animals recovered spontaneously and fully within a few hours of their injection but there were a number of animal deaths in the weekly, three weekly and monthly treatment schedules.

No animal receiving the negative control antibody Mc39-16 alone, given by either IP or IV injection weekly for four injections appeared unwell or died. Animals treated with 3/23 IV for two doses followed by Mc39-16 IV for two doses appeared unwell after their third (first Mc39-16) injection. One animal in this group died.

Animals receiving multiple doses of 3/23 IV developed a marked antibody response against mouse anti-rat immunoglobulin (3/23). In contrast, only one out of four animals receiving multiple doses of Mc39-16 IV developed a very low level mouse anti rat antibody response with no detectable antibody response developing in the remaining animals examined.

4.4.3 Chi Lob 7/4

Six C57BLK/6 mice and six Syrian hamsters received 10mg IP of Chi Lob 7/4 without haematological, biochemical of histopathological evidence of toxicity.

Figure 4.1

Mean serum alanine transaminase levels of BALB/c mice treated with a single IP dose of 3/23 (rat anti-mouse CD40 mAb) or a negative control mAb (1D3). Data courtesy of Dr M Harvey[181].

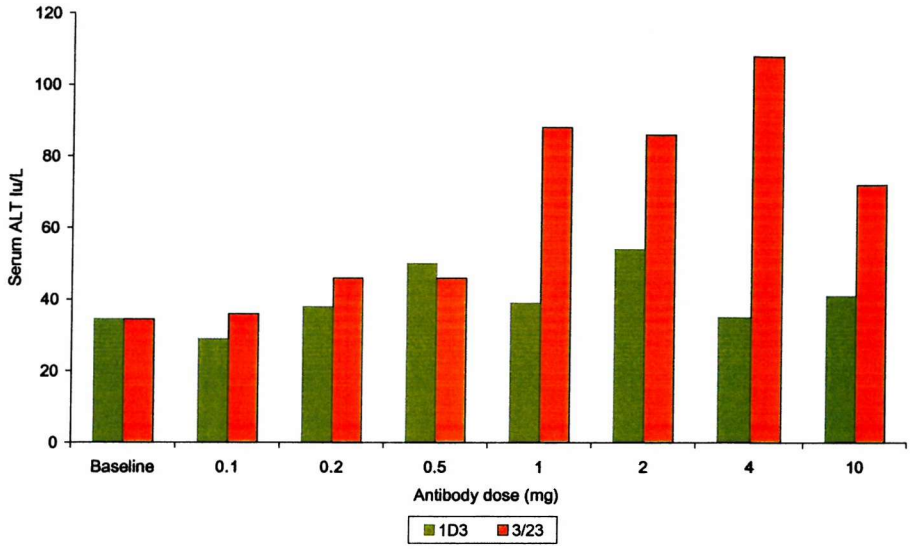


Figure 4.2

Mean serum urea levels of C57BLK6 and BALB/c mice treated with a single IP dose of the rat anti-mouse CD40 mAb 3/23.

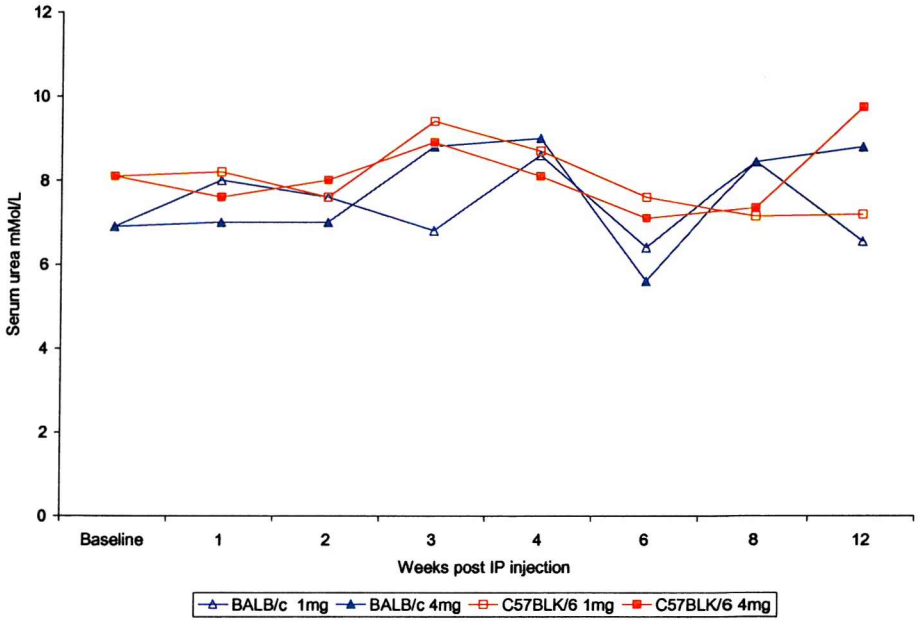


Figure 4.3

Mean serum creatinine levels of C57BLK6 and BALB/c mice treated with a single IP dose of the rat anti-mouse CD40 mAb 3/23.

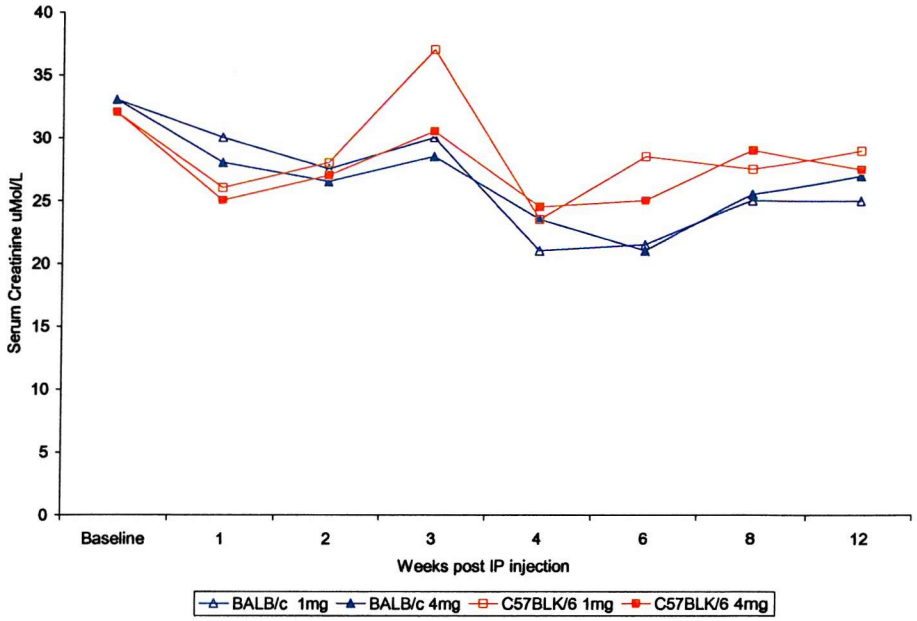


Figure 4.4

Mean serum alanine transaminase levels of C57BLK6 and BALB/c mice treated with a single IP dose of the rat anti-mouse CD40 mAb 3/23.

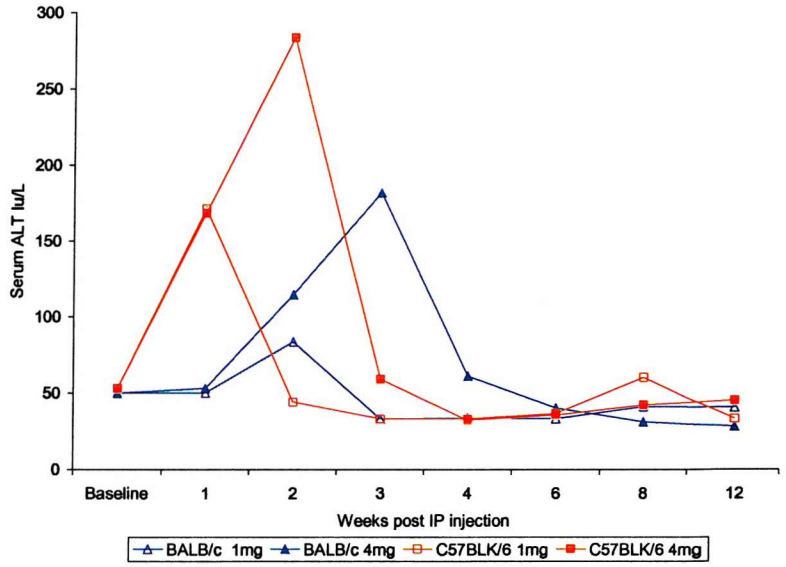


Figure 4.5

Mean serum aspartate transaminase levels of C57BLK6 and BALB/c mice treated with a single IP dose of the rat anti-mouse CD40 mAb 3/23.

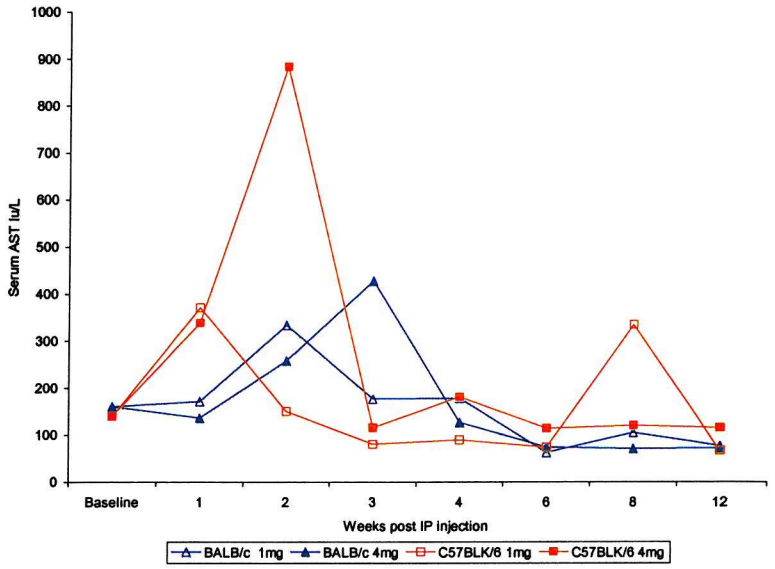


Figure 4.6

Mean haemoglobin levels of C57BLK6 and BALB/c mice treated with a single IP dose of the rat anti-mouse CD40 mAb 3/23.

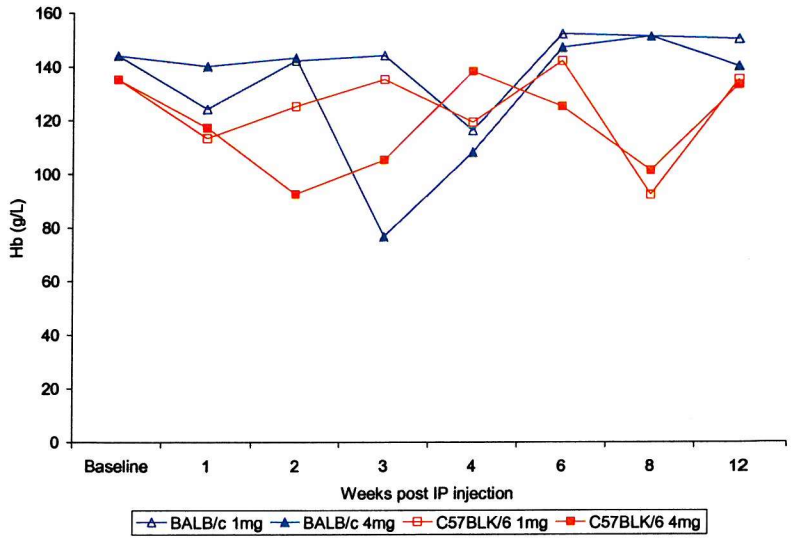


Figure 4.7

Mean spleen weights of C57BLK6 and BALB/c mice treated with a single IP dose of the rat anti-mouse CD40 mAb3/23.

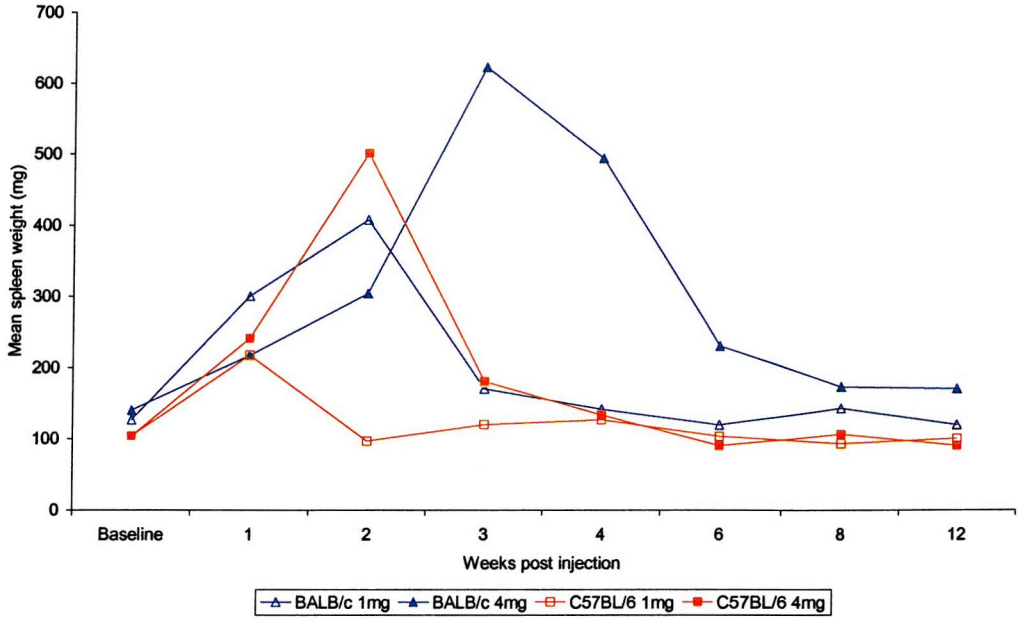
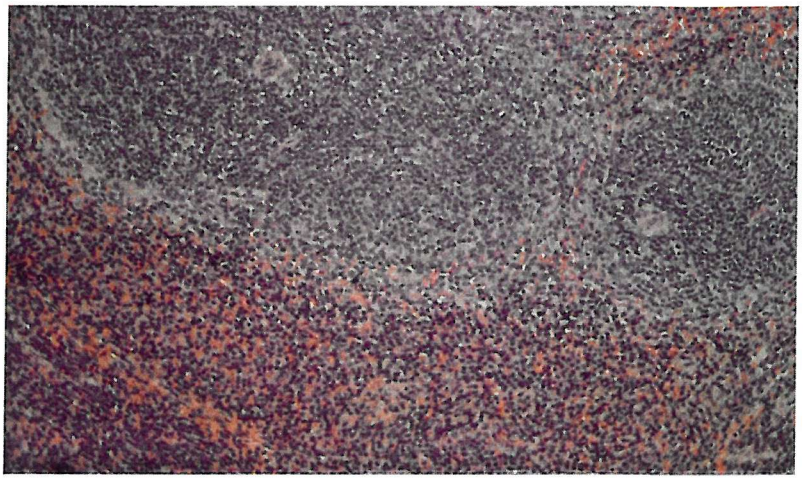
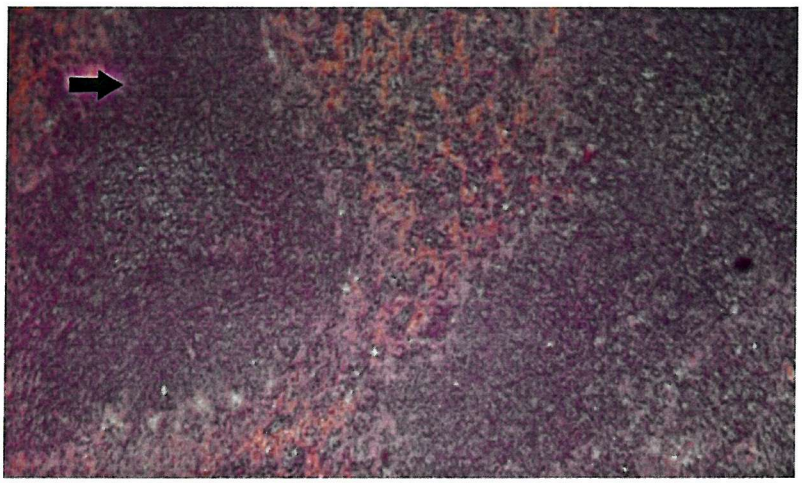


Figure 4.8

Paraffin sections of C57BLK/6 mice spleens. Haematoxylin and eosin stain. Medium power magnification.



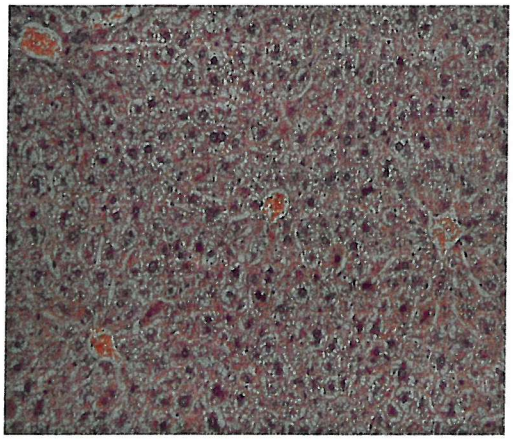
A) Normal untreated mouse.



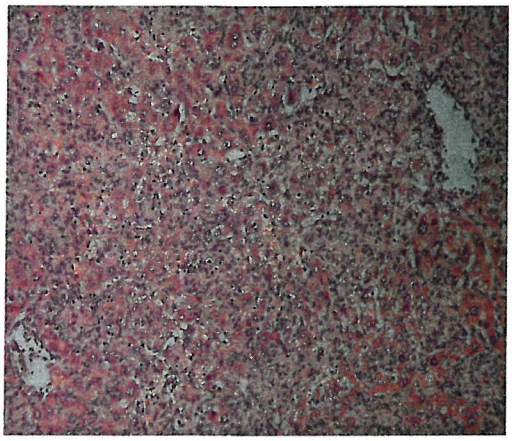
B) Two weeks after a single 4 mg IP dose of the rat anti-mouse CD40 mAb3/23. Note hypertrophy of the periarteriolar lymphoid sheaths / marginal zone (arrow).

Figure 4.9

Haematoxylin and eosin stained paraffin sections of C57BLK/6 mouse livers.
Section A) Normal untreated mouse liver
Section B) Two weeks after a single 4mg IP dose of the rat anti-mouse CD40 mAb 3/23. Medium power view reveals an intense lymphocytic infiltrate with multiple micro-granulomata (top left and bottom right), becoming confluent (centre).



A) Normal liver.



B) Following 4 mg 3/23 treatment.

Figure 4.10

Paraffin section of C57BLK/6 mouse liver, two weeks after a single 4 mg IP dose of the rat anti-mouse CD40 mAb3/23. High power view. Diastase periodic acid schiff stain revealing seroid laden macrophage (centre).

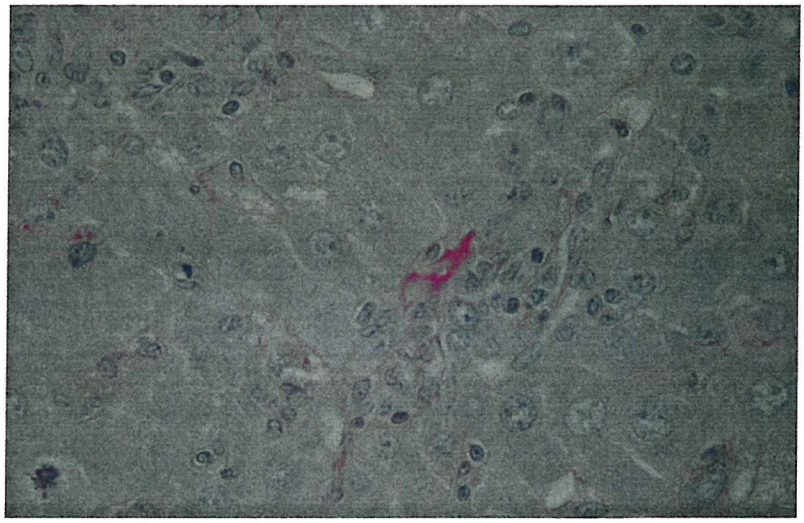
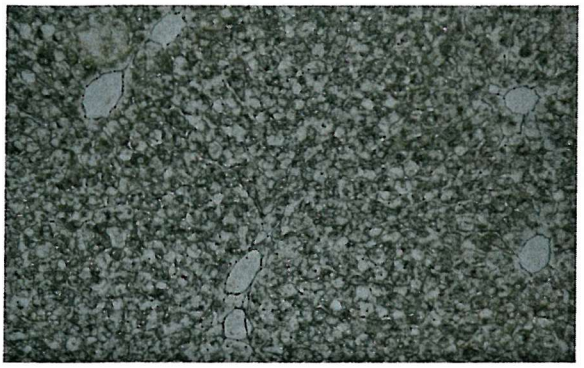
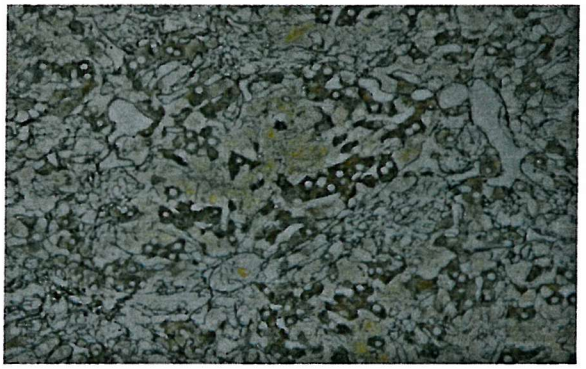


Figure 4.11

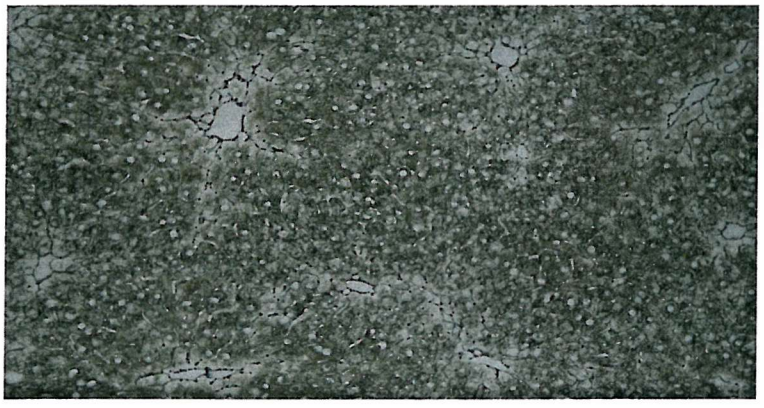
Paraffin sections of C57BLK/6 mouse liver. Reticulin stain.



A) Normal untreated mouse liver. Medium power view.



B) Liver, two weeks after a single 4 mg IP dose of the rat anti-mouse CD40 mAb3/23.

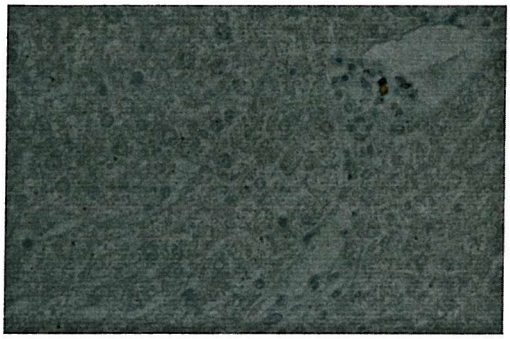


C) Liver, three months after single 4mg IP dose of the rat anti-mouse CD40 mAb3/23. Section shows slight increase in fibrous tissue in portal triads but no bridging / linkage fibrosis.

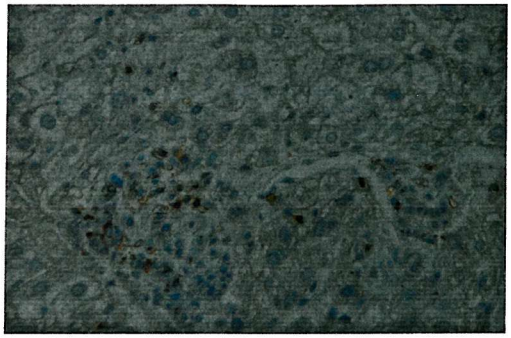
Figure 4.12

Glycol methacrylate sections of C57BLK/6 mouse liver stained for the T cell markers CD3, CD4 and CD8.

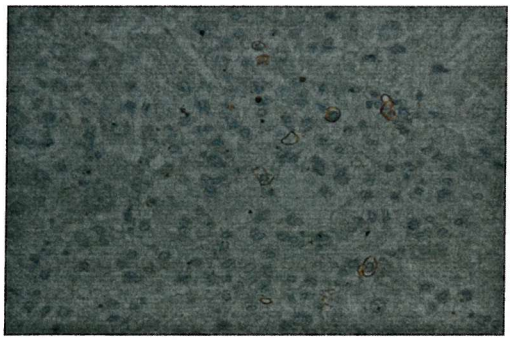
Section A	Normal mouse liver.
Sections B – D	Liver two weeks after a single 4 mg IP dose of the rat anti-mouse CD40 mAb 3/23. Note increase in number of CD3, CD4 and CD8 positive cells.



A) CD3 stain.



B) CD3 stain.



C) CD4 stain.

D) CD8 stain.

Figure 4.13

Paraffin sections of C57BLK/6 mouse liver stained for α smooth muscle actin.

A) Normal, untreated mouse liver.

Positive staining is seen in vascular smooth muscle only (internal positive control).

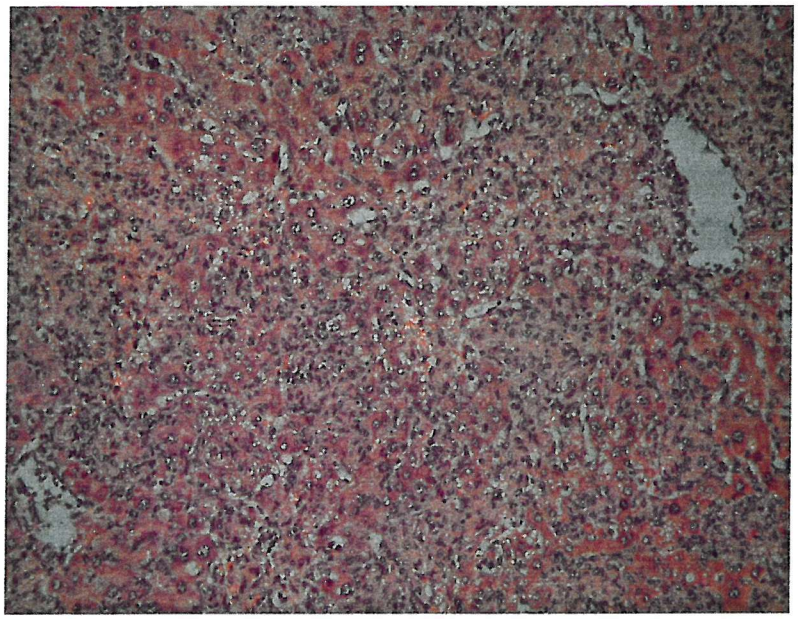
B) Liver two weeks after a single 4 mg IP dose of the rat anti-mouse CD40 mAb 3/23.

There is widespread positive staining of activated stellate cells.

C) Liver twelve weeks after a single 4 mg IP dose of the rat anti-mouse CD40 mAb 3/23. Stellate cell activation is no longer seen. The liver appears normal.

Figure 4.14

A) Paraffin section of C57BLK/6 mouse liver, two weeks after a single 4 mg IP dose of the rat anti-mouse CD40 mAb3/23. Haematoxylin and eosin stain. Section shows an intense lymphocytic infiltrate and multiple, coalescing microgranulomata.



B) Paraffin sections of C57BLK/6 mouse liver, five weeks after a single 4 mg IP dose of the rat anti-mouse CD40 mAb3/23. Haematoxylin and eosin stain. There is complete resolution of the previously noted abnormalities.

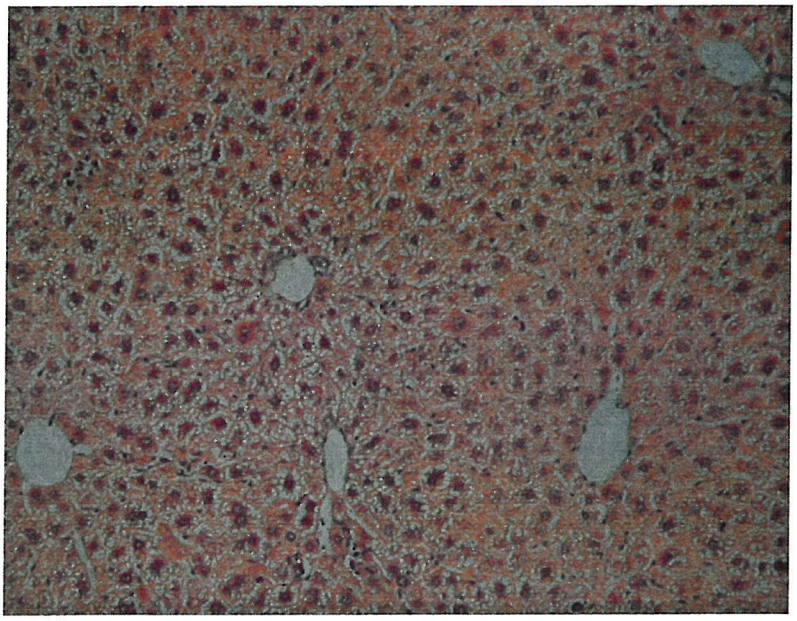


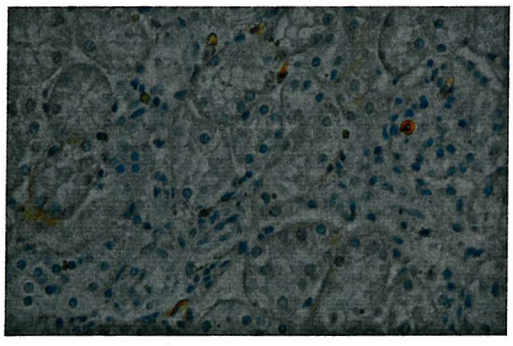
Figure 4.15

Glycol methacrylate sections of C57BLK/6 mouse kidney stained for the T cell markers CD4 and CD8.

Sections A and B	Normal untreated mouse kidney.
Sections C and D	Mouse kidney two weeks after a single 4 mg IP dose of the rat anti-mouse CD40 mAb3/23. Note increase in numbers of both CD4 and CD8 positive cells.

A) CD4 stain.

B) CD8 stain.

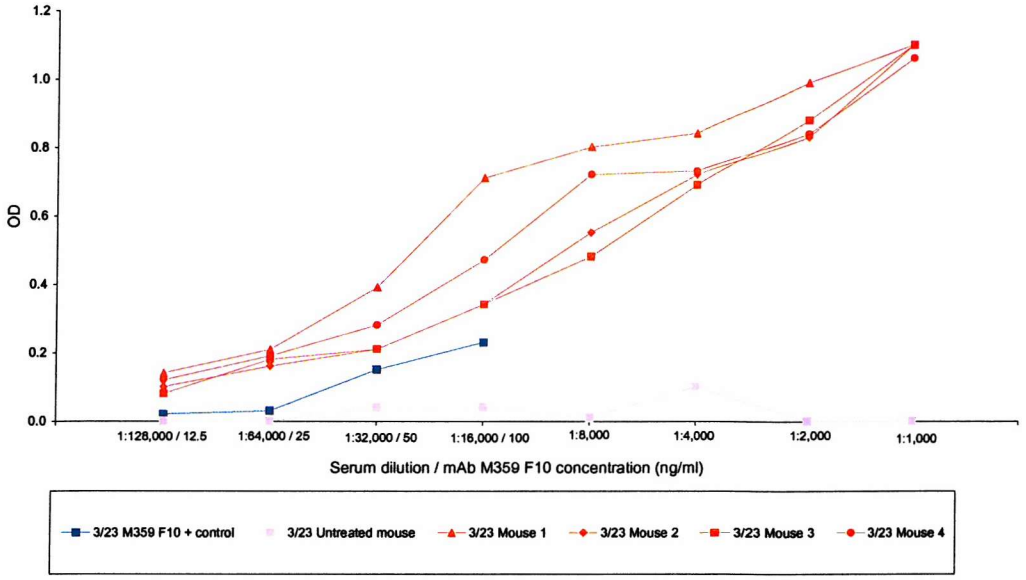


C) CD4 stain.

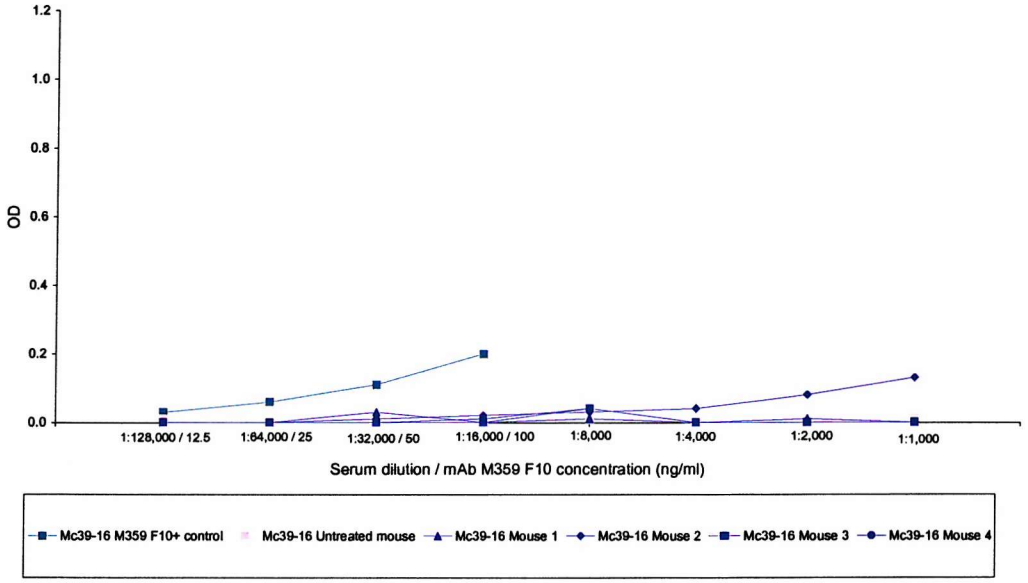
D) CD8 stain.

Figure 4.16

Mouse anti rat antibody (MARA) responses by ELISA



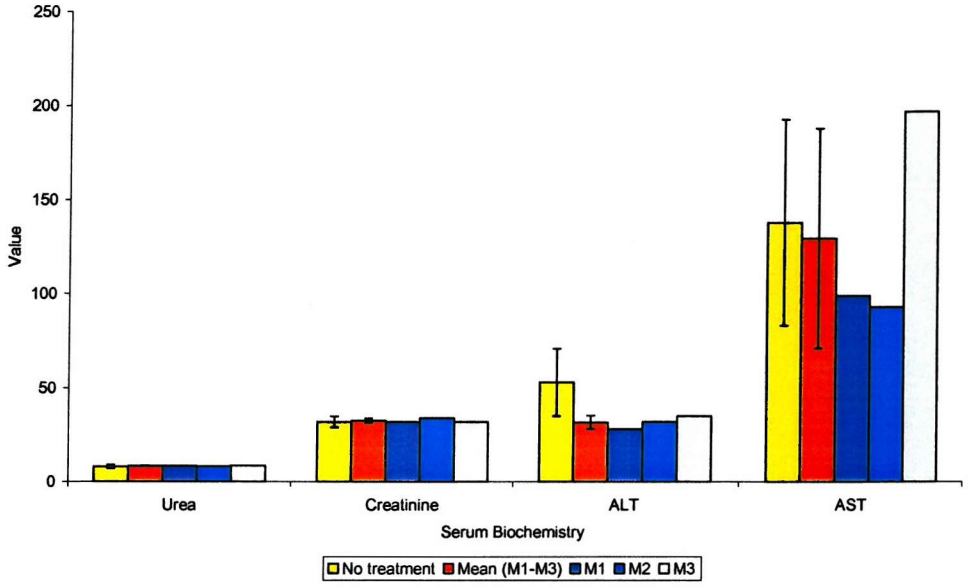
A) Significant serum MARA (3/23) responses were seen in all C57BLK/6 mice treated with 100 µg IV 3/23 given weekly for three doses.



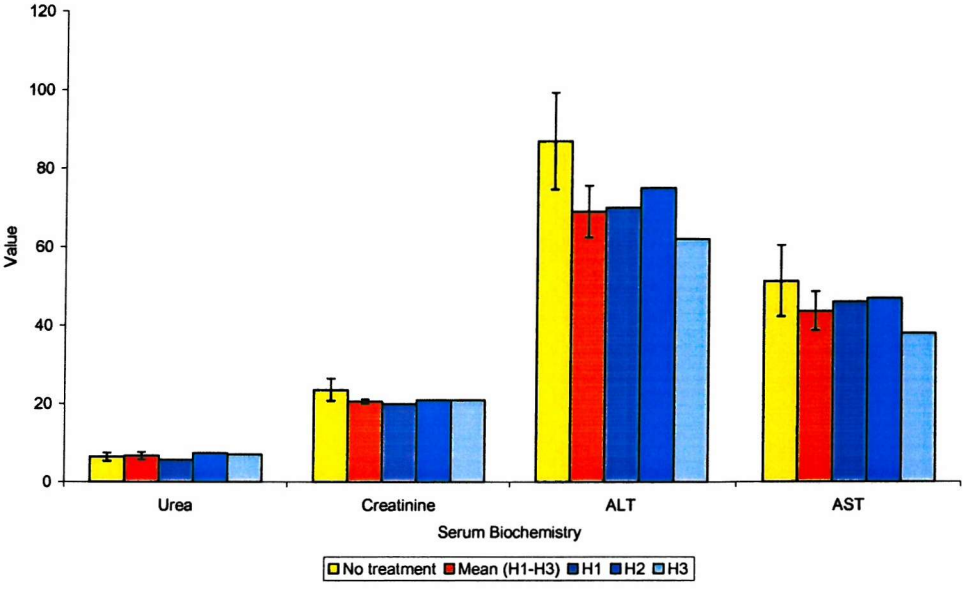
B) Serum MARA (Mc39-16) responses were not seen in three of four C57BLK/6 mice treated with 100 µg IV Mc39-16 given weekly for three doses. A low level MARA response is seen in mouse 2 at the lowest serum dilutions (1:1,000 – 1:2000).

Figure 4.17

Renal and hepatic function (assessed by serum urea, creatinine, alanine transaminase; ALT and aspartate transaminases levels; AST) of C57BLK6 mice / Syrian hamsters, treated with a single 10 mg IP dose of the chimeric anti-human CD40 mAb Chi Lob 7/4 compared to normal untreated animals.



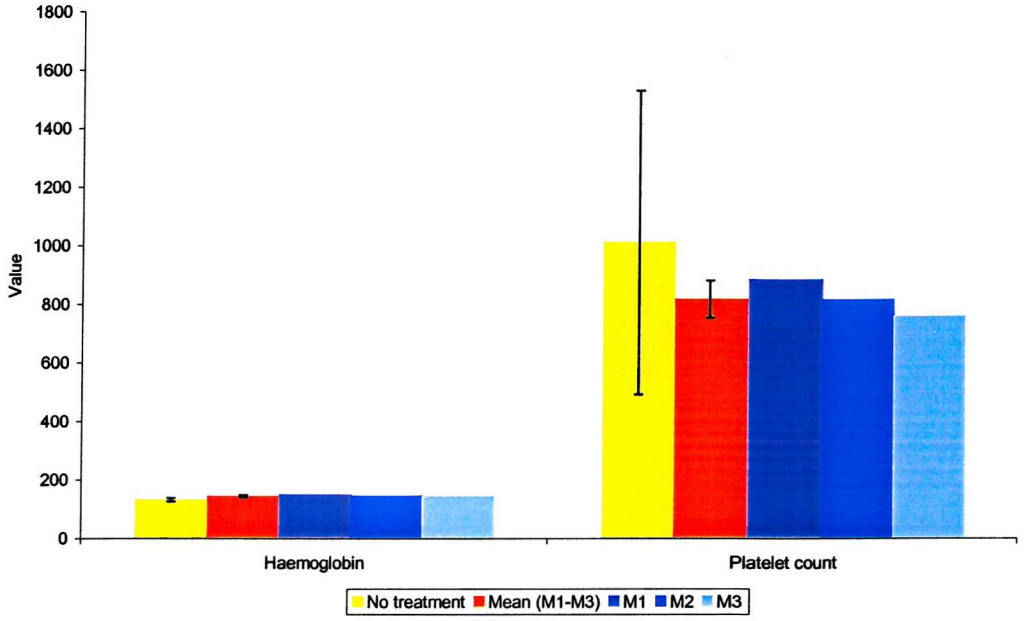
A) C57BLK/6 mice



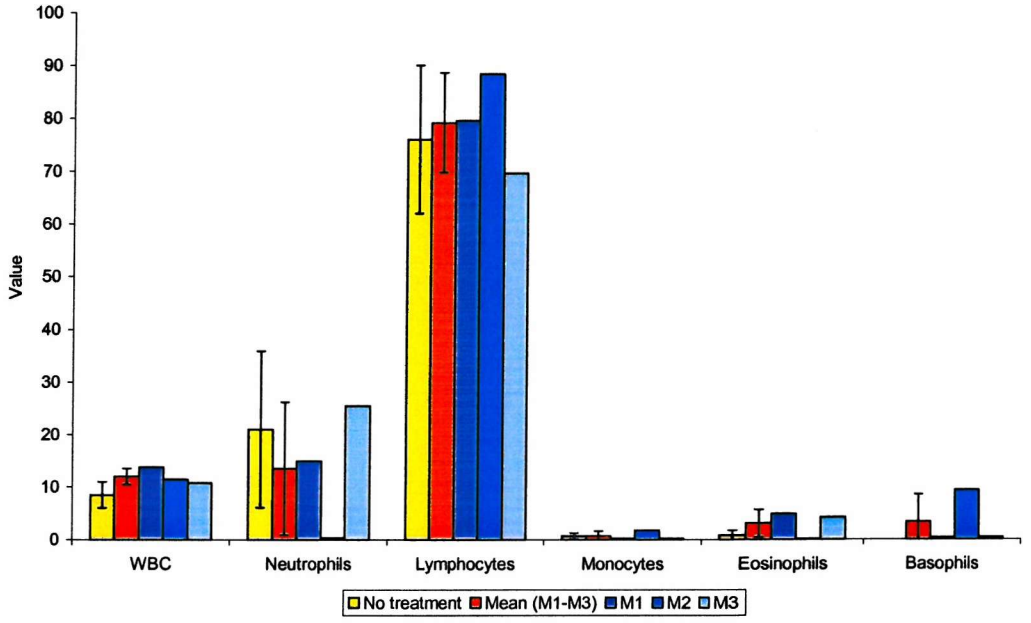
B) Syrian hamsters

Figure 4.18

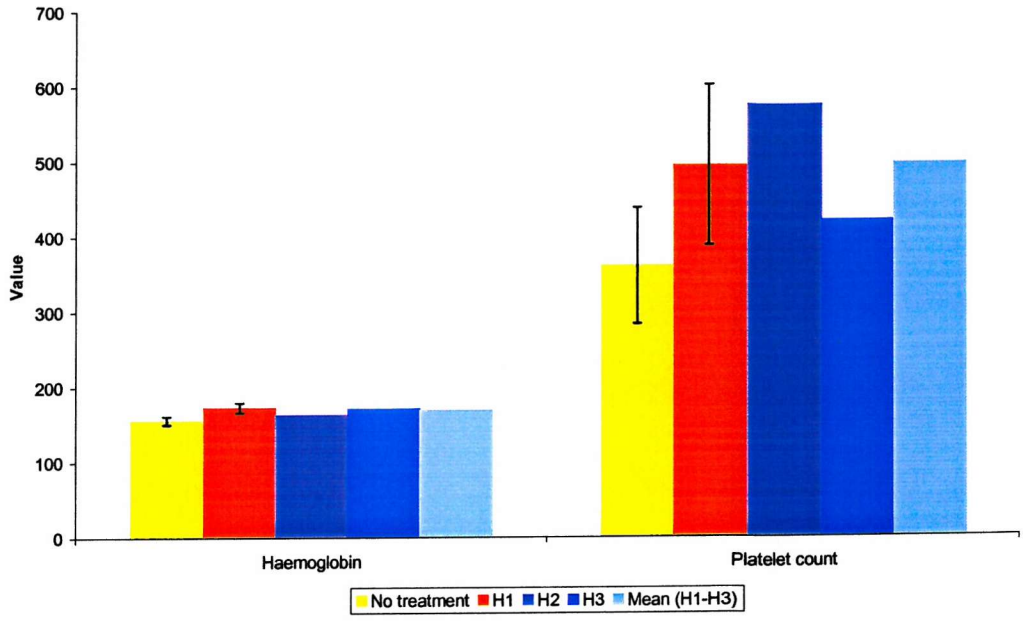
Full blood count of C57BLK6 mice / Syrian hamsters, treated with a single 10 mg IP dose of the chimeric anti-human CD40 mAb Chi Lob 7/4 compared to normal untreated animals.



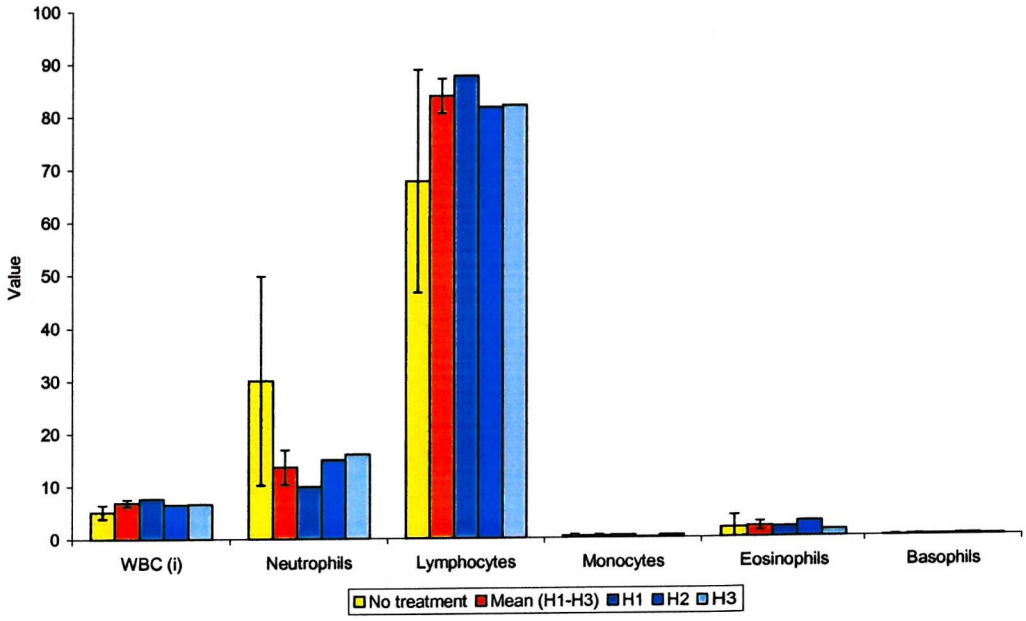
A) C57BLK/6 mice: Haemoglobin level (g/L) and platelet count (x 10^9/L).



B) C57BLK/6 mice: Total (x10^6/L) and differential (%) white blood cell count.



C) Syrian hamsters: Haemoglobin level (g/L) and platelet count (x 10^9/L).



D) Syrian hamsters: Total (x10^6/L) and differential (%) white blood cell count.

CHAPTER FIVE

5 CD40 TISSUE EXPRESSION AND SERUM CHI LOB 7/4 DETECTION

5.1 Introduction

The expression of CD40 was first described on human urinary bladder carcinomas and normal and malignant B cells in 1985[16]. Since then, normal CD40 expression has been demonstrated primarily on antigen presenting cells (APCs) such as dendritic cells, B lymphocytes and monocytes[17, 18]. CD40 has also been found on a variety of other cells that include endothelial and epithelial cells, fibroblasts, eosinophils, most B cell malignancies (e.g. non-Hodgkin's lymphomas) and some epithelial malignancies (e.g. breast, lung, ovary, bladder and melanoma)[16, 19-29]

In preparation for a phase I clinical trial of Chi Lob 7/4 therapy in CD40 expressing malignancies, immunohistochemical methods were developed for the detection of CD40 expression in paraffin embedded, human tissue samples. Following the development of a reliable and reproducible method, CD40 expression was investigated in normal human tissues and in a range of human tumour types selected from anonymised, historical specimens.

Phase I clinical trials are principally aimed at evaluating the safety and toxicity of novel agents. Pharmacokinetic analysis forms an integral part of this evaluation. In preparation for a phase I clinical trial of Chi Lob 7/4 therapy, it was necessary to develop a detection method to accurately measure serum levels of Chi Lob 7/4 following therapeutic administration. An enzyme linked immunosorbant assay (ELISA) was developed for this purpose. The ability of this assay to detect Chi Lob 7/4 in normal human serum was evaluated and validated.

5.2 Methods

5.2.1 Human tissue samples: CD40 expression

Following appropriate local ethical committee approval (LREC No 159/02), a range of anonymised, normal and malignant, paraffin embedded human tissue blocks were

obtained from the Department of Histochemistry, Southampton General Hospital. Representative tissue blocks were selected by Dr Norman Carr and Professor William Roche and sections cut by the University Histochemistry unit. Lob 7/6 was used as the primary detection antibody for immunohistochemistry at a variety of dilutions using the methods detailed in chapter two.

5.3 Results

5.3.1 CD40 Tissue Expression

5.3.1.1 Normal human tissue samples

A wide range of normal human tissues were selected for evaluation of normal CD40 expression. Lob 7/6 yielded consistently good results at a working dilution of between 1:600 and 1:1000. Details of positive staining are summarised below (table 5.1) and illustrated in figures 5.1 to 5.9. Antigen presenting cells (B cells, macrophages) were generally CD40 positive regardless of tissue site. Weak focal CD40 positivity was also seen on urothelial transitional epithelium.

Table 5.1

Normal human CD40 tissue expression by immunohistochemistry.

Tissue type	Positive Staining	Figure
Tonsil	B cells / Macrophages	5.1
Lymph node	B cells / Macrophages	-
Spleen	B cells / Macrophages	5.2
Liver	Inflammatory cell infiltrate	5.3
Kidney	-	-
Urothelial tract	Transitional cell urothelium (weak, focal)	5.4
Skin	Inflammatory cells	5.5
Colon	B cells	5.6
Uterus	-	-
Stomach	B cells	5.7
Lung	Alveolar macrophages	5.8
Oesophagus	-	-
Small intestine	Macrophages	-
Muscle	-	-
Parotid	Inflammatory cells	5.9
Ovary	-	-
Thyroid	-	-
Pancreas	-	-
Salivary gland	-	-
Brain	-	-

5.3.2 Malignant human tissue samples

A selection of malignant human tissues were selected for evaluation of tumour CD40 expression. Lob 7/6 yielded consistently good results at a working dilution of

between 1:600 and 1:1000. CD40 positivity was demonstrated in a range of paraffin embedded B cell non-Hodgkin's lymphoma and solid tumour specimens as described below.

5.3.2.1 Diffuse large B cell lymphoma

Tissue sections from three different patients were evaluated. Positive CD40 expression was demonstrated in all sections examined.

Figure 5.10

5.3.2.2 Melanoma

Tissue sections from three different patients were evaluated. Positive CD40 expression was demonstrated in two out of three sections examined.

Figure 5.11

5.3.2.3 Non-small cell lung carcinoma

Tissue sections from eleven different patients were evaluated. CD40 expression was positive in five patients and negative in six patients.

Figure 5.12

5.3.2.4 Invasive ductal breast carcinoma

Tissue sections from a single patient was evaluated. Positive CD40 expression was demonstrated in the section examined.

Figure 5.13

5.3.2.5 Renal cell carcinoma

Tissue sections from four different patients were evaluated. Positive CD40 expression was not demonstrated in any of the sections examined.

Figure 5.14

5.3.2.6 Colorectal adenocarcinoma

Tissue sections from three different patients were evaluated. Positive CD40 expression was not demonstrated in any of the sections examined.

Figure 5.15

5.3.2.7 Transitional cell carcinoma bladder

Tissue sections from three different patients were evaluated. Positive CD40 expression was not demonstrated in any of the sections examined.

Figure 5.16

5.3.3 Detection of Serum Chi Lob 7/4

5.3.3.1 Enzyme linked immunosorbant assay

5.3.3.1.1 Titration of peroxidase conjugated rabbit F(ab')2 anti-human F(ab')$_2$

Using the method described in chapter two, standard curves were generated for a range of dilutions of peroxidase conjugated rabbit F(ab')$_2$ anti-human F(ab')$_2$. A 1:5,000 dilution was found to be optimal and this was used for subsequent assays.

Figure 5.17

5.3.3.1.2 Detection of Chi Lob 7/4 and generation of a standard curve

Having established an optimal concentration for peroxidase conjugated rabbit F(ab')$_2$ anti-human F(ab')$_2$, Hu Fc – CD40 fusion protein was used as a capture protein in an ELISA for Chi Lob 7/4. A standard curve was generated for Chi Lob 7/4 (diluted in PBS/BSA) at a starting concentration of 20 μg/ml, diluted 1:10 to 1:32,000 (2 μg/ml to 0.6 ng/ml) in PBS / BSA. Chi Lob 7/4 was detectable at a concentration as low as 0.6 ng/ml. The range of detection was optimal from 0.6 to 19.5 ng/ml.

Figure 5.18

5.3.3.1.3 Detection of Chi Lob 7/4 in human serum

To ensure Chi Lob 7/4 could be detected reliably in human serum, three human serum samples were spiked with Chi Lob 7/4 20 μg/ml, diluted 1:10 to 1:16,000 and evaluated by ELISA. Spiked serum was compared to unspiked serum and a Chi Lob 7/4 standard curve (in PBS / BSA alone) at a identical dilutions. The addition of serum did not influence the detection of Chi Lob 7/4. Chi Lob 7/4 was reliably detected at the lowest concentration evaluated (1.25 ng/ml).

Figure 5.19

5.4 Summary of results

A method for evaluating CD40 tissue expression by immunohistochemistry was developed and used to assess CD40 expression in a range of normal and malignant human tissue samples.

In normal tissues, strong CD40 expression was noted in antigen presenting cells / inflammatory cells such as B cells and macrophages. CD40 expression was not seen elsewhere apart from weak focal weak positivity apparent in urothelial transitional cell epithelium.

CD40 expression was demonstrated in a number of malignant tissue types. Expression varied both within tumour types and between tumour types. In the sample selected, CD40 expression was seen in diffuse large B cell non-Hodgkin's lymphoma, malignant melanoma, non-small cell lung carcinoma and breast adenocarcinoma. Expression was not seen in colorectal adenocarcinoma, renal cell carcinoma or bladder transitional cell carcinoma.

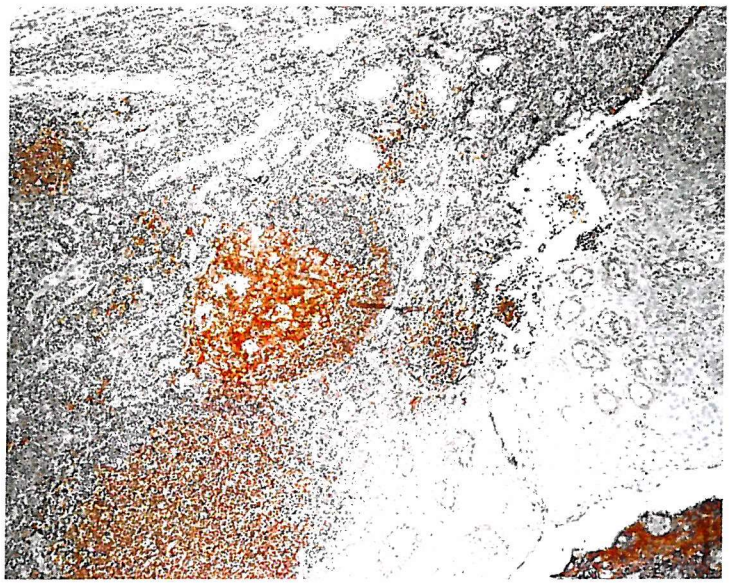
An effective ELISA was developed for the detection of Chi Lob 7/4. Using this ELISA, Chi Lob 7/4 could reliably be detected down to low ng/ml levels. Addition of normal human serum did not adversely effect assay results.

Figure 5.1

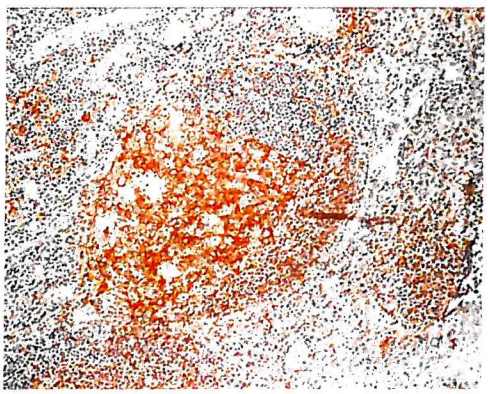
Normal Human Tonsil.

CD40 expression assessed by immunohistochemistry.

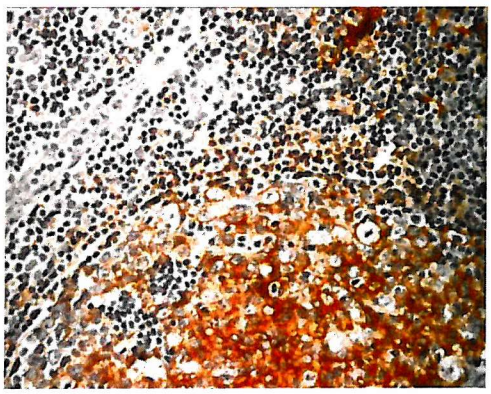
Paraffin embedded section stained with Lob 7/6, 1:600.



A) Low power view (x10). Note intense staining of CD40 on B cells within lymphoid follicles.



B) Medium power view (x20)



C) High power view (x40)

Figure 5.2

Normal Human Spleen.

CD40 expression assessed by immunohistochemistry.

Paraffin embedded section stained with Lob 7/6, 1:800.

Medium power view (x20) demonstrating CD40 positive staining of B cells within B cell area of white pulp.

Figure 5.3

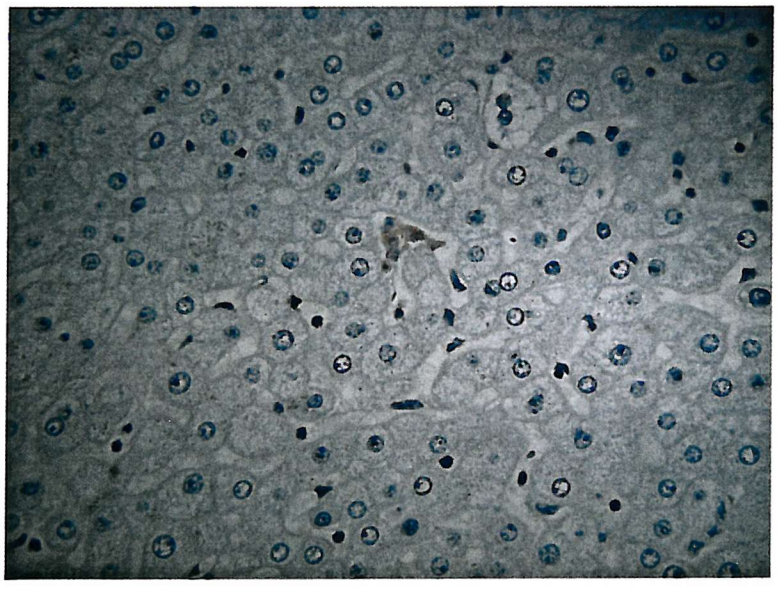
Normal Human Liver.

CD40 expression assessed by immunohistochemistry.

Paraffin embedded section stained with Lob 7/6, 1:800.

a) Low powered view (x10) centred on portal tract.

No CD40 positive staining is seen.



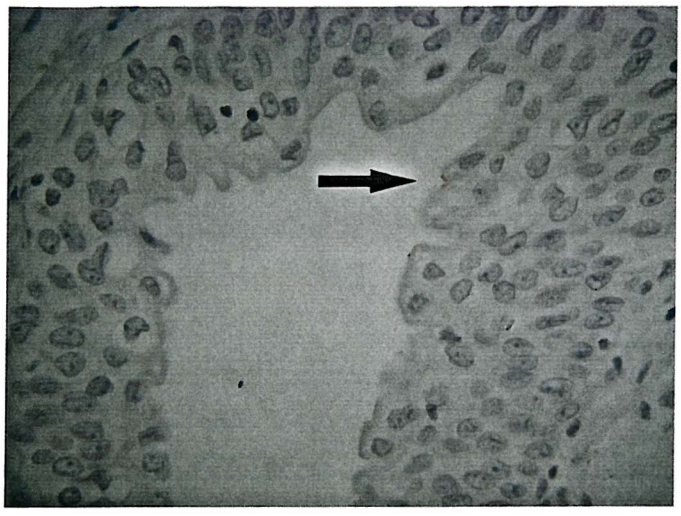
b) High power view (x40) centred on small CD40 positive inflammatory focus within liver. Surrounding hepatocytes do not stain for CD40.

Figures 5.4 and 5.5

Normal Human Ureter.

CD40 expression assessed by immunohistochemistry.

Paraffin embedded section stained with Lob 7/6, 1:800.

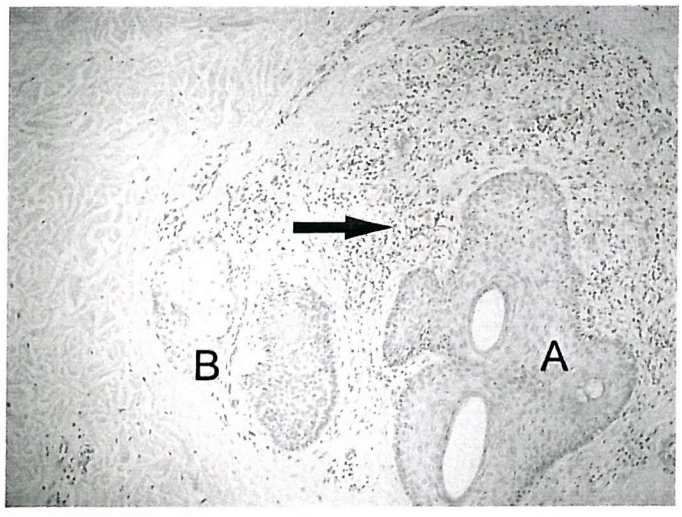


High power view (x40) demonstrating patchy weak focal CD40 positive staining of transitional cell epithelium (arrow).

Normal Human Skin.

CD40 expression assessed by immunohistochemistry.

Paraffin embedded section stained with Lob 7/6, 1:800.



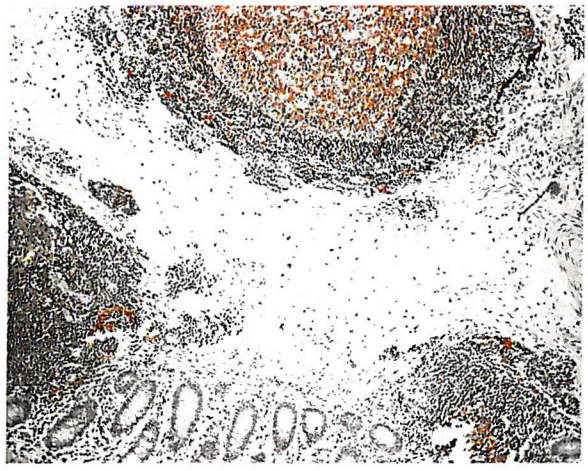
Low power view (x20) demonstrating CD40 positive staining of inflammatory cell infiltrate (arrow). Surrounding structures including hair follicle (A) and sebaceous gland (B) do not stain for CD40.

Figures 5.6 and 5.7

Normal Human Colon.

CD40 expression assessed by immunohistochemistry.

Paraffin embedded section stained with Lob 7/6, 1:800.

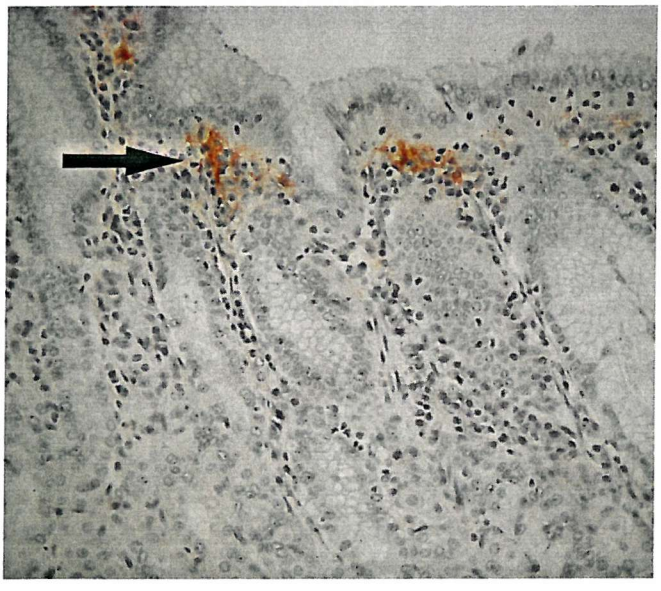


Low power view (x10) demonstrating CD40 positive staining of B cells within lymphoid follicle (top of picture). Surrounding colonic glandular tissue (bottom of picture) does not stain for CD40.

Normal Human Stomach.

CD40 expression assessed by immunohistochemistry.

Paraffin embedded section stained with Lob 7/6, 1:800.



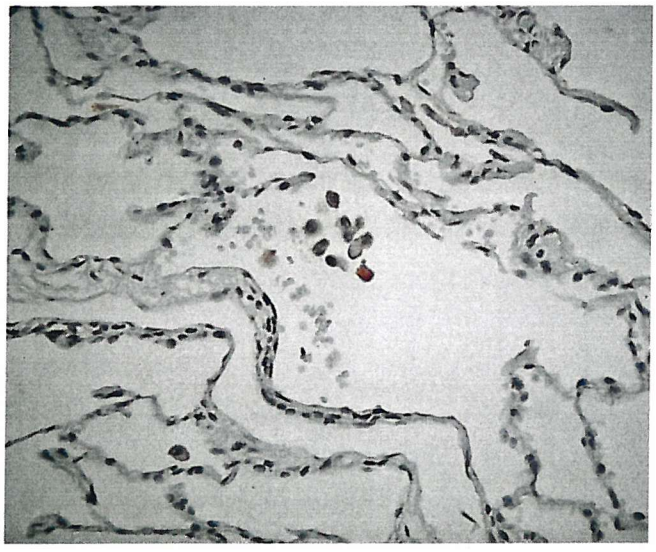
Medium power view (x20) demonstrating CD40 positive staining of B cell infiltrate within stomach wall (arrow). Surrounding tissues do not stain for CD40.

Figures 5.8 and 5.9

Normal Human Lung.

CD40 expression assessed by immunohistochemistry.

Paraffin embedded section stained with Lob 7/6, 1:800.

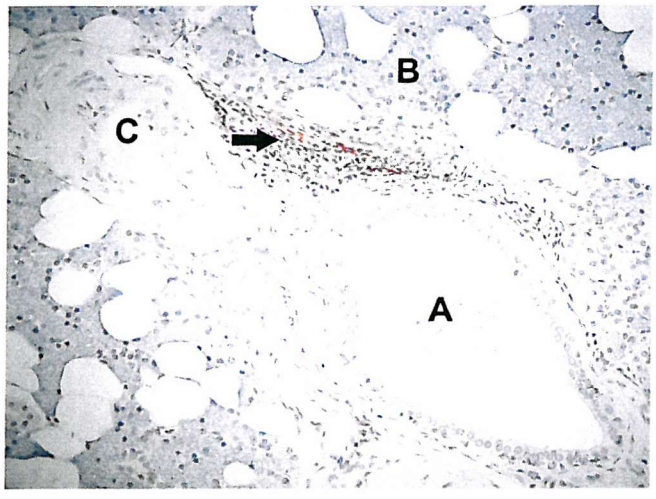


Medium power view (x20) demonstrating CD40 positive staining of alveolar macrophages (centre). Surrounding pneumocytes do not stain for CD40.

Normal Human Parotid.

CD40 expression assessed by immunohistochemistry.

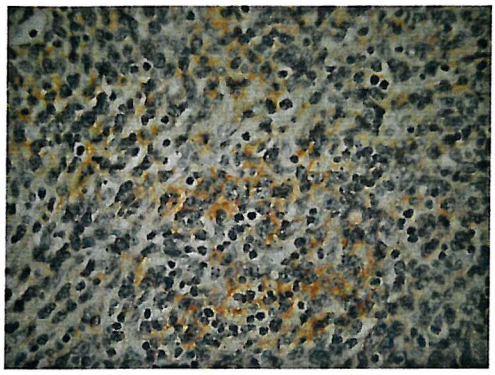
Paraffin embedded section stained with Lob 7/6, 1:800.



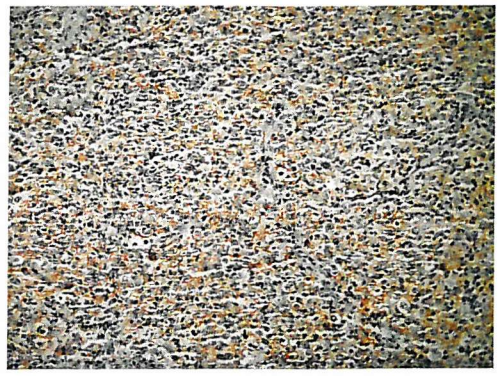
Medium power view (x20) demonstrating CD40 positive staining of inflammatory infiltrate (arrow). Surrounding structures including parotid acinar cells (A), parotid duct (B), and blood vessel (C) are CD40 negative.

Figure 5.10

Diffuse large B cell lymphoma. CD40 expression by immunohistochemistry.
Paraffin embedded sections from two patients stained with Lob 7/6, 1:600.



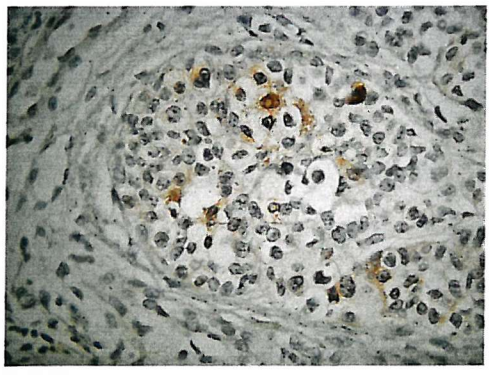
A) High power view.
Positive staining of malignant cells.



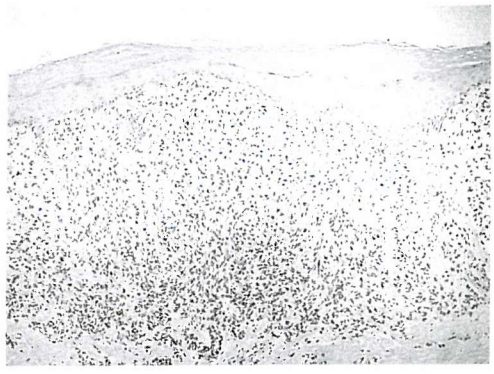
B) Low power view.
Diffusely positive staining.

Figure 5.11

Malignant melanoma. CD40 expression by immunohistochemistry.
Paraffin embedded sections from two patients stained with Lob 7/6, 1:600.



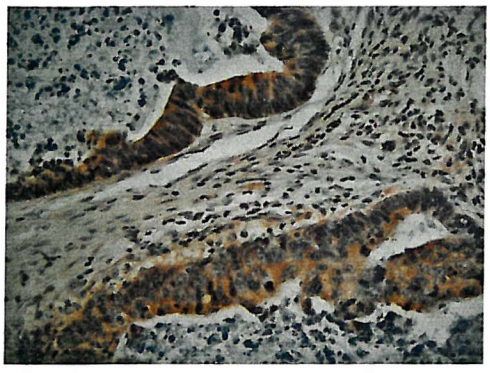
A) High power view.
Malignant cells show focally positive staining.



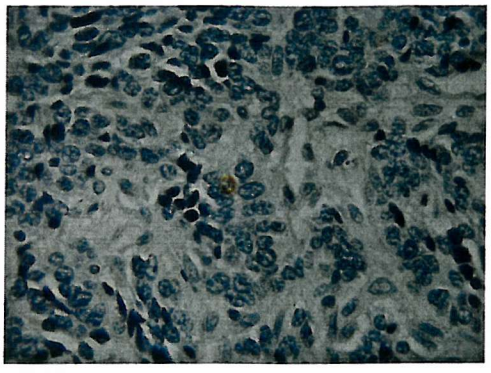
B) Low power view.
Negative staining.

Figure 5.12

Non-small cell lung carcinoma. CD40 expression by immunohistochemistry. Paraffin embedded sections from two patients stained with Lob 7/6, 1:600.



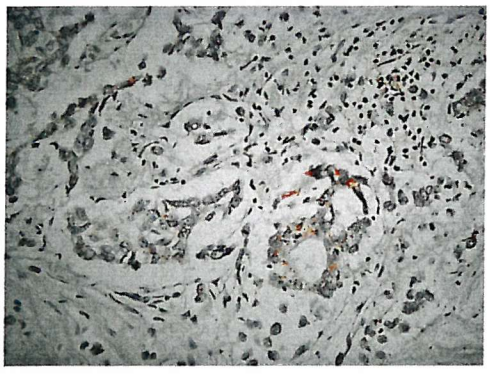
A) Low power view.
Positive staining of malignant epithelial cells. Note fibrotic reaction (centre) and areas of necrosis (top left, bottom right).



B) High power view.
Negative staining of malignant cells. Single positively staining lymphocyte at centre acts as an internal positive control.

Figure 5.13

Invasive ductal breast carcinoma. CD40 expression by immunohistochemistry. Paraffin embedded section stained with Lob 7/6, 1:600.

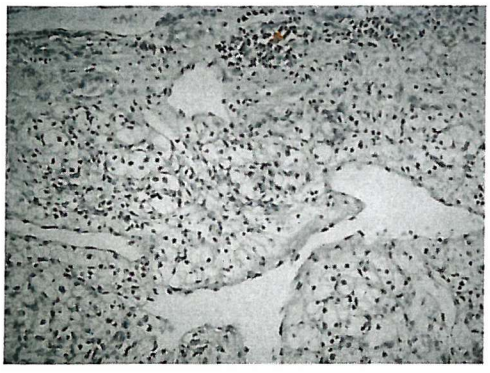


Low power view.
Malignant cells show positive staining.

Figure 5.14 – 5.16

CD40 expression by immunohistochemistry.

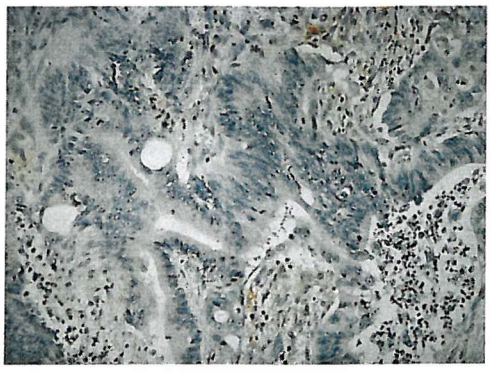
Paraffin embedded sections from patients stained with Lob 7/6, 1:600.



5.14) Renal cell carcinoma

Negative tumour staining.

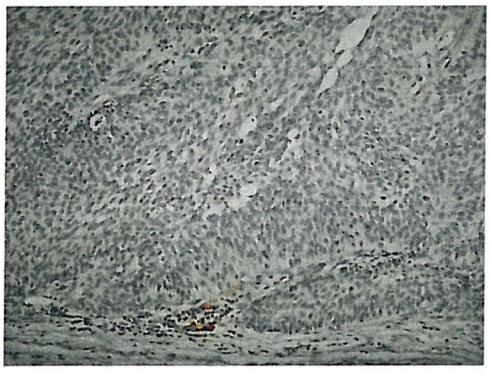
Lymphocytes are positive (top centre) and act as an internal positive control.



5.15) Colonic adenocarcinoma

Negative tumour staining.

Lymphocytic infiltrate is positive and acts as an internal positive control.



5.16) Transitional cell carcinoma bladder

Negative tumour staining.

Lymphocytes are positive (bottom centre) and act as an internal positive control.

Figure 5.17

Detection of rituximab $F(ab')_2$ fragment using varying peroxidase conjugated rabbit $F(ab')_2$ anti-human $F(ab')_2$ dilutions. Optimal dilution 1:5,000.

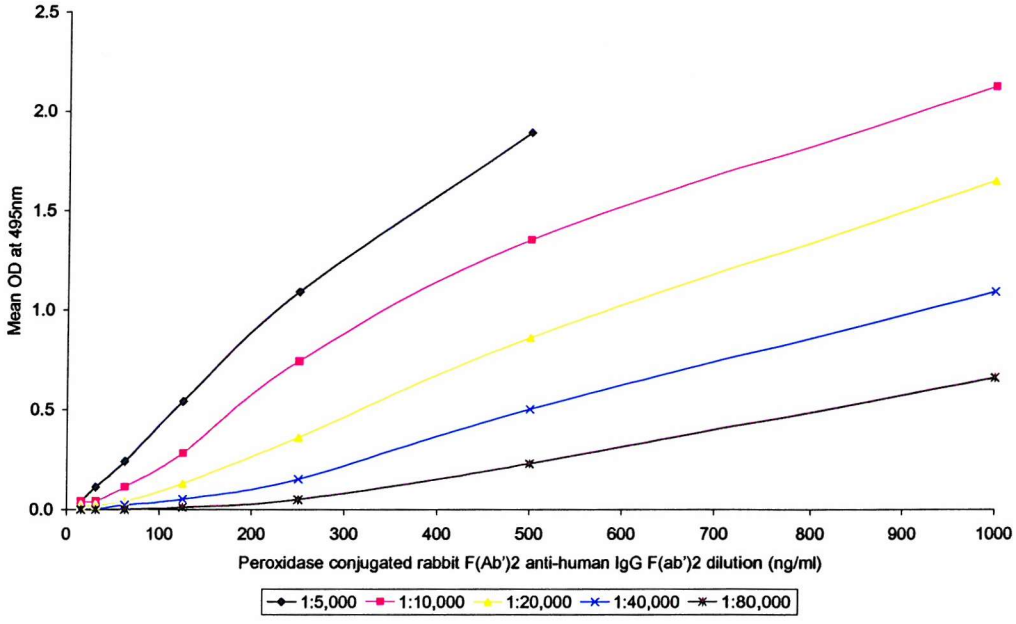


Figure 5.18

A standard curve was generated for Chi Lob 7/4 (diluted in PBS/BSA) at a concentration of 20 µg/ml, diluted 1:10 to 1:32,000 (2µg/ml to 0.6ng/ml) in PBS / BSA. Range of detection optimal at 0.6 to 19.5ng/ml.

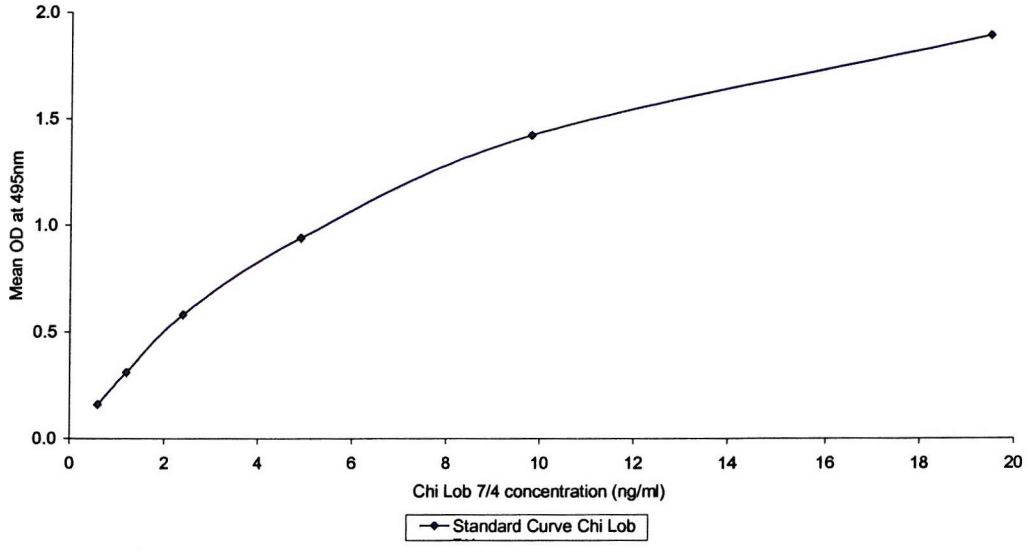
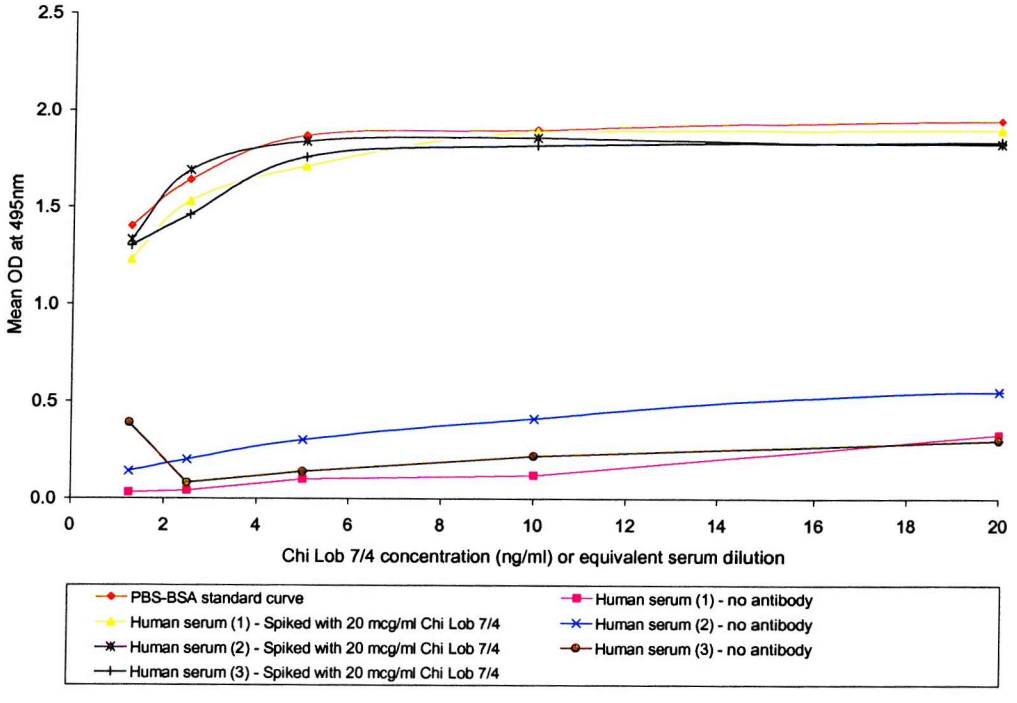


Figure 5.19

Chi Lob 7/4, 20 µg/ml, diluted 1:10 to 1:16,000 and evaluated by ELISA. Spiked serum compared to unspiked serum and a Chi Lob 7/4 standard curve (in PBS / BSA alone) at a identical dilutions. The addition of serum did not influence the detection of Chi Lob 7/4. Chi Lob 7/4 could be reliably detected at the lowest concentration evaluated (1.25 ng/ml).



CHAPTER SIX

6 DISCUSSION

6.1 Introduction

Cancer remains a major cause of morbidity and mortality. In the UK, an individual's lifetime risk of developing cancer is about 1 in 3. Cancer is responsible for a quarter of all deaths [3]. A modern multidisciplinary approach to the treatment of cancer with appropriate use of surgery, chemotherapy and radiotherapy has improved the outlook for many patients however many cancers present at an advanced stage and are ultimately fatal.

The development of laboratory techniques allowing stable and permanent production of monoclonal antibodies (mAbs) in the 1970s, revolutionised investigative and diagnostic laboratory techniques [110]. The subsequent development of genetic engineering techniques that allowed the chimerisation and humanisation of mAbs, has led to the production of large numbers of therapeutic mAbs. MAbs have established themselves as the most rapidly expanding class of therapeutic agents for a wide range of human diseases[4]. Several hundred therapeutic mAbs are currently in human clinical trials with a significant proportion of these antibodies being evaluated in the treatment of cancer.

Therapeutic anti-cancer mAbs may achieve their effects through a variety of mechanisms that include complement dependent cytotoxicity (CDC), antibody dependent cellular cytotoxicity (ADCC) and through the augmentation or blockade of cell signalling. One further mechanism is the development of mAbs able to mimic or block key receptor – ligand interactions of the immune system such as CD40 – CD154 and in doing so promote strong, cellular anti-tumour immune responses.

The expression pattern of CD40 on a broad range of malignancies and the important functional role of CD40-CD154 in-vivo make CD40 an attractive target for antibody based immunotherapy. The ability of anti-CD40 mAbs to recruit CDC and ADCC, provide a direct growth inhibitory cell signal and upregulate antigen presentation

and cytotoxic T cell responses means that a wide variety of tumours may be targeted with the potential for the development of persistent, long term, anti-tumour immunological memory.

I have evaluated the in-vitro effects of a chimeric mouse-human anti-CD40 mAb, Chi Lob 7/4 against a variety of human malignant non-Hodgkin's lymphoma and epithelial cell lines. I have studied the potential toxicities of anti-CD40 mAb therapy in a comparative mouse model and demonstrated that the toxicity seen is fully reversible. I have developed an immunohistochemical method for the detection of CD40 expression in human tissue and validated the method in a range of normal and malignant human tissues. I have developed an ELISA for the detection in serum of therapeutically administered Chi Lob 7/4 enabling pharmacokinetic analysis in a proposed phase I clinical trial of Chi Lob 7/4 in the treatment of patients with CD40 expressing tumours. On the basis of these and other results, a full application for funding of a phase I clinical trial has been approved by the New Agents committee of Cancer Research UK. Production of clinical grade Chi Lob 7/4 is now underway and it is hoped that the trial will open to recruitment in 2004.

6.2 In-vitro activity of Chi Lob 7/4

Chi Lob 7/4 effectively inhibited the in-vitro growth of two B cell, non-Hodgkin's lymphoma cell lines (RL and Daudi) and two malignant epithelial cell lines (EJ138 and Caski) when presented cross-linked on polyurethane coated microbeads (Dynabeads M-450). No significant difference was noted between Chi Lob 7/4 and the parent murine antibody Lob 7/4. These findings are in line with considerable published data demonstrating, that a variety of malignant epithelial and lymphoma cell lines undergo growth arrest in-vitro when treated with anti-CD40 therapy [19, 23, 25, 29, 73, 75-82]. The use of microbeads to present and crosslink antibody was novel and chosen because of its ability to provide reproducible results. Alternative techniques using an irradiated fibroblast layer transfected with the CD32 (FcγRII) receptor proved unreliable and problematic. Similarly, results obtained using presentation of antibody pre-bound to plastic 96 well plates did not prove reliable.

In assays of complement dependent cytotoxicity (CDC), Chi Lob 7/4 was effective at activating complement when bound to RL and Daudi cells. In contrast, Lob 7/4 was unable to activate complement. This finding demonstrates the importance of antibody chimerisation and the requirement (in this system) of human IgG_1 Fc antibody regions in binding and activating complement. Cellular lysis via CDC was maximal in Daudi cells (65% specific Cr^{51} release), but effective cell lysis was also seen in RL cells (22% specific Cr^{51} release). CDC could not be demonstrated in the malignant epithelial cell lines EJ138 or MG79. This finding may relate to expression of complement defence molecules or inhibitory proteins, acting predominately to regulate C3 convertase and prevent binding of the membrane attack complex. Complement defence molecules are vital to normal cells and act to contain complement activation and prevent widespread inflammation and cell death. Their expression by malignant cells however, may act as an important mechanism through which malignant cells can escape CDC. Further studies could evaluate the contribution complement defence molecules make to evasion of CDC mediated by Chi Lob 7/4.

Chi Lob 7/4 was able to effectively recruit cytotoxic cells such as NK cells via Fc receptors in assays of ADCC. Effective ADCC (as measured by specific Cr^{51} release) was demonstrated for both RL cells and Daudi cells and a clear relationship was apparent between ADCC, antibody concentration and effector cell to target cell ratio. Lob 7/4 was unable to activate ADCC once again demonstrating the importance of antibody chimerisation and the requirement of human IgG_1 Fc antibody regions to effectively recruit Fc receptor bearing cytotoxic cells. Effective ADCC could not be demonstrated in either MG79 or EJ138 cell lines. Assays using epithelial cell lines were technically difficult and significant problems were encountered with cell adherence and clumping.

CD40 mediated signalling is of critical importance to the regulation of cellular as well as humoral immune responses, up-regulating the antigen presenting function of APCs, inducing high levels of MHC II, key co-stimulatory molecules, accessory molecules and increasing the production of activating cytokines[55]. CD40 signalling is essential for the cross-priming and activation of CD8+ cytotoxic T

cells. Stimulation of CD40 by therapeutic agonistic mAb can effectively bypass the need for specific T cell help, directly priming helper dependent CD8+ cytotoxic T cell responses; in animal models of malignancy, this effect translates into an impressive anti-cancer immuno-therapeutic approach [58, 59, 164, 165].

Lob 7/4 has previously been assessed against a known agonistic anti-CD40 mAb s2c6 for its ability to upregulate the key co-stimulatory molecules B7-1 and B7-2 in a dendritic cell culture system. Lob 7/4, was found to up-regulate B7-1 and B7-2 in >90% of cultured dendritic cells. Attempts were made to investigate the effects of Chi Lob 7/4 on cultured normal B lymphocytes obtained from healthy volunteers. These experiments were repeated on numerous occasions under a variety of conditions but were unsuccessful. A similar approach looking at follicular / low grade non-Hodgkin's lymphoma cells obtained following consent from routine patient biopsies was also unsuccessful.

6.3 Toxicity of monoclonal antibody therapy

Clinical experience with therapeutic monoclonal antibody therapy demonstrates that such treatment is generally very well tolerated. Although infusion related hypersensitivity reactions are common, these are generally mild and significant side-effects are rare. The chimerisation and humanisation of therapeutic mAbs has overcome the problem of human anti-mouse antibody (HAMA) responses associated with repeated administration of murine antibodies.

6.3.1 Anti CD40 mAb therapy: Single dose studies

Previous exploratory work carried out in The Tenovus Research Institute by Dr Melanie Harvey, (a previous Cancer Research Campaign Clinical Research Fellow) had begun to examine the acute toxicities of agonistic anti-CD40 mAb therapy in a comparative mouse model using a single dose of intraperitoneal rat anti-mouse CD40 mAb 3/23[181].

No toxicity was seen in animals treated with a 100 μg dose of 3/23 however, animals treated with dose of 1 mg or more of 3/23 however demonstrated dose dependent, acute hepatitis and splenomegaly. A mild nephritis was also noted in animals

receiving 200 μg of 3/23 or more. No other toxicity was observed and there were no toxic deaths.

A more detailed assessment of the toxicity of anti CD40 mAb therapy (3/23) in a comparative mouse model has confirmed the principal toxicity of 3/23 treatment to be a dose dependent acute hepatitis, peaking in severity at one to three weeks following a single intraperitoneal injection but recovering fully (biochemically and histopathologically) by around week five. Detailed histochemical analysis revealed a widespread acute lymphogranulomatous hepatitis progressing to piecemeal necrosis in the most severely affected livers. The lymphocytic infiltrate contained large numbers of CD3 positive, CD8 positive cytotoxic T cells. Widespread stellate cell activation was also noted. Apart from a very slight increase in fibrous tissue within the portal triads there was no evidence of subsequent chronic fibrotic change.

The underlying mechanism of 3/23 mediated hepatic damage remains uncertain. Studies performed to investigate the tissue distribution of CD40 in mice by immunohistochemistry were plagued by technical difficulties and it was not possible to be certain of the CD40 expression status of mouse hepatocytes. Interestingly, the dose limiting toxicity of the published phase I clinical trial of CD154 (CD40L) in humans was a hepatic transaminitis akin to the results seen in the comparative 3/23 mouse model[178]. Liver biopsies were not performed in this study and as such underlying histopathological change was not determined. However, my finding that CD40 was not expressed on normal human hepatocytes however suggests that hepatic toxicity from anti-CD40 therapy is unlikely to be related to a "direct hit" by anti CD40 treatment.

One possible mechanism that could explain CD40 mediated hepatic damage involves the interaction of Fas and Fas ligand. This death receptor-ligand pair are members of the tumour necrosis factor family. FasL is expressed on activated cytotoxic T cells and is important in mediating cellular cytotoxicity thorough cross-linking of the Fas receptor on target cells. The liver has been shown to be a site for clearance of lymphocytes and as such is an organ at potential risk of damage by infiltrating "inappropriately activated" cytotoxic T lymphocytes[182, 183]. Large

numbers of CD8 positive lymphocytes were apparent the livers of mice treated with 3/23. Both human and murine hepatocytes do express Fas and are particularly susceptible to Fas mediated death[183-185]; indeed, experimental injection of anti-Fas mAb in mice leads to massive liver injury without significant damage to other organs[186]. In graft versus host disease (GvHD), the liver is a conspicuous target of allografted cytotoxic T cells and hepatocyte injury is thought to be dependent on Fas – FasL interaction[187]. Also of interest is the upregulation and expression of CD40 on human hepatocytes in patients with chronic GvHD (in contrast to absence of expression on normal hepatocytes). Ligation of CD40 in these cells may well amplify hepatic damage in this situation through Fas dependent mechanisms[184].

Staining for α smooth muscle actin (α SMA) revealed widespread but reversible hepatic stellate cell (HSC) activation. Hepatic stellate cells reside in the perisinusoidal space of Disse and are thought to have four major functions in the normal liver: Production and homeostasis of extracellular matrix protein, control of sinsusoidal microvascular tone, storage of retinoids and a role in liver regeneration[188]. Several groups have demonstrated that in response to injury there is HSC expansion, neo-expression of the cytoskeletal antigen (α SMA) and activation of HSCs with production of matrix proteins[189-191]. HSC activation is considered to be a key process in hepatic fibrogenesis [188]. In chronic models of hepatic injury, HSC numbers proliferate and plateau, however, if injury is not sustained, HSC numbers and activation returns to normal [192]. Although α SMA activation was marked in animals treated with higher doses of 3/23, it was reversible and did not lead to significant hepatic fibrosis.

Splenomegaly was noted in all animals receiving 1mg or more of 3/23. Spleen size increased significantly (up to six fold increase in weight) within one to two weeks of 3/23 treatment but returned to normal by week eight. Histopathological examination of enlarged spleens revealed hypertrophy of the periarteriolar lymphoid sheaths and marginal zones. These findings are consistent with B and T cell activation and proliferation secondary to CD40 cross-linking.

A mild lymphocytic infiltrate was seen in the kidneys of animals treated with 200μg or more of 3/23. This did not appear to be dose dependent. The lymphocytic infiltrate was made up mainly of CD8 positive T cells but did not appear to result in any functional abnormality (biochemical measures of renal function were not affected). In animals examined greater than five weeks after injection the lymphocytic infiltrate was no longer apparent

6.3.2 Chi Lob 7/4

Six C57BLK/6 mice and six Syrian hamsters received a single 10mg IP dose of Chi Lob 7/4 without haematological, biochemical of histopathological evidence of toxicity. These findings are not surprising in an antibody with human Fc fragment and a variable region that does not target a murine antigen. Such studies were required by Cancer Research UK and justified as "lethality studies" in preparation for the proposed phase I clinical trial.

6.3.3 Anti CD40 mAb therapy: Multiple dose studies

Studies were performed to evaluate the feasibility of delivering anti-CD40 mAb therapy on multiple dose schedule. Using the intraperitoneal route, 25 or 100μg 3/23 or Chi Lob 7/4 could be delivered on a weekly schedule for four weeks without clinically obvious toxicity. However, delivery of 3/23 via the intravenous route was problematic. Animals became significantly unwell following repeat doses of 3/23 given two or more weeks after an initial dose. For animals receiving 3/23 weekly, this occurred on their third dose; for animals receiving 3/23 on fortnightly, three weekly, monthly or six weekly schedules, this occurred on the second dose. Animals became unwell within minutes of these injections; although the majority of animals recovered spontaneously and fully within a few hours of their injection. There were however, a number of animal deaths in the weekly, three weekly and monthly treatment schedules.

Similar responses to multiple dose intravenous (but not local / sub-cutaneous) anti-CD40 mAb treatment have been described by other groups and attributed to cytokine release from stimulated CD40 positive cells [193]. An alternative explanation supported by the time course and clinical appearance of animals reacting

to intravenous antibody therapy is that of an acute anaphylactic response to antibody (augmented by the immunostimulatory properties of anti CD40 therapy), rather an acute response to anti-CD40 crosslinking. This explanation is supported by a number of observations. Firstly, the absence of such a response in animals receiving 3/23 therapy via the IP route where antibody would enter the systemic circulation over a significantly longer time period. Secondly, animals receiving multiple doses of 3/23 IV developed a marked antibody response against mouse anti-rat immunoglobulin (3/23). In contrast, only one out of four animals receiving multiple doses of negative control Mc39-16 mAb IV developed a low level mouse anti-rat antibody response with no detectable antibody response developing in the remaining animals. Finally, animals treated with 3/23 IV for two doses followed by Mc39-16 IV for two doses appeared unwell after their third (first Mc39-16) injection and one animal in this group died. This final observation clearly suggests an acute immunological response to elements of rat immunoglobulin common to the two IgG_{2a} antibodies rather than an acute response to CD40 cross-linking per se.

6.3.4 Implications for clinical administration of Chi Lob 7/4

Overall, the findings of these toxicity studies have particular relevance to the proposed administration of Chi Lob 7/4 in a human phase I clinical trial. In keeping with the published phase I clinical trial of SCD40L, the dose limiting toxicity of Chi Lob 7/4 are likely to be hepatic in nature[178]. Close attention will need to be paid to serum markers of hepatic function such as alanine and aspartate transaminases, γ glutamyl transferase and alkaline phosphatase. Additionally, serum markers of renal function such as urea and creatinine will need to be closely monitored.

The administration of intravenous mAb therapy is known to be associated with allergic responses and this may be particularly true for the administration of Chi Lob 7/4. Infusions should be started at a slow rate and the administration of prophylactic paracetamol and antihistamine may well be required.

6.4 CD40 Immunohistochemistry

The development of a reproducible method for assessing CD40 expression in paraffin embedded histological specimens was crucial for the proposed clinical trial

in which treatment will be limited to those patients whose tumours express CD40. An appreciation of the normal tissue distribution pattern of CD40 in humans is also clearly of value. The expression pattern of CD40 seen in normal and malignant tissues is broadly in line with published data (reviewed in [194]) and provides a validated method for examination of CD40 expression in tumour tissue specimens from patients who are potential candidates for treatment within the phase I trial. A more expansive and detailed investigation may well confirm CD40 expression in those tumour types where CD40 was not seen (renal, bladder and colorectal caner) in the relatively small number of samples examined.

6.5 Chi Lob 7/4 ELISA

Pharmacokinetic evaluation forms part of any clinical phase I trial evaluating novel therapeutic agents and in preparation for a phase I clinical trial of Chi Lob 7/4, an ELISA was developed for this purpose. My results demonstrate that Chi Lob 7/4 can be detected in human serum at least down to low nanogram/ml levels.

6.6 Future Directions

A clinical trial protocol for the phase I evaluation of Chi Lob 7/4 in the treatment of patients with CD40 positive malignancy refractory to conventional therapy was submitted to the New Agents Committee of Cancer Research UK in early 2003. The proposal was accepted by committee and production of clinical grade Chi Lob 7/4 has been funded and is now underway at The Therapeutic Antibody Centre, University of Oxford under the direction of Professor Geoff Hale. A provisional clinical trial proposal is detailed in appendix one. It is hoped that the clinical trial will open for recruitment in 2004.

The primary objective of the trial will be to establish the safety and tolerability of Chi Lob 7/4 administered as a slow intravenous infusion weekly for four doses. The starting dose will be low (5 mg per dose; cumulative dose 20 mg) and represents the equivalent human dose (on a mg/kg or 2000 times multiplier) to one tenth of the highest dose of 3/23 (100 μg) that was given to mice without toxicity. Subsequent individual dose levels will increase to 10 mg, 20 mg, 40 mg, 80 mg and 160 mg providing dose limiting toxicity is not observed. Secondary objectives will be to

evaluate tumour responses and a number of immunological endpoints pertinent to the proposed mechanisms of action of anti-CD40 monoclonal antibody therapy. These immunological endpoints are detailed in the clinical trial protocol and will include flow cytometric analysis of peripheral blood mononuclear cells and APCs, serum cytokine levels, intratumoral effects (presence of tumour infiltrating lymphocytes / mononuclear cells, cytokine levels, apoptotic cells and expression of FasL) development of human anti Chi Lob 7/4 mAbs and Chi Lob 7/4 pharmacokinetics.

6.7 Summary

The expression pattern of CD40 on a broad range of malignancies and the important functional role of CD40-CD154 in-vivo make CD40 an attractive target for antibody based immunotherapy. Chi Lob 7/4 a chimeric anti-human mAb was developed within the Cancer Sciences Division of the University of Southampton as a potential therapeutic anti CD40 mAb for the treatment of cancer. I have shown that Chi Lob 7/4 can effectively cause growth inhibition in-vitro in variety of malignant cell lines. I have also shown that Chi Lob 7/4 can target and mediate tumour cell lysis through the recruitment of complement and targeting of antibody directed cellular cytotoxicity.

I have performed toxicity studies in a comparative mouse model using the rat anti mouse mAb 3/23 and confirmed the major toxicities of treatment to be a dose dependent lymphogranulomatous hepatitis, splenomegaly and a mild lymphocytic nephritis. I have demonstrated that these toxicities are fully reversible.

I have successfully developed and validated an immunohistochemical technique for the evaluation of CD40 tissue expression in paraffin embedded normal and malignant human tissue sections. I have developed an ELISA for the evaluation of pharmacokinetic evaluation of serum Chi Lob 7/4.

On the basis of these and other studies, I have submitted a successful application to the New Agents Committee of Cancer Research UK and obtained funding for production of clinical grade Chi Lob 7/4 and a phase I clinical trial of Chi Lob 7/4 therapy in patients with CD40 expressing malignancies refractory to conventional therapy.

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8 OUTLINE CLINICAL TRIAL PROPOSAL

TITLE

A phase I research study evaluating the safety, tolerability and biological effects of the chimeric anti-CD40 monoclonal antibody Chi Lob 7/4 given weekly, for four weeks in the treatment of patients with advanced, CD40 positive malignancies, refractory to conventional anti-cancer treatment.

DESIGN

Dose escalation, phase I study.

PRIMARY OBJECTIVES

To determine the dose limiting toxicity (DLT) and maximum tolerated dose (MTD) of Chi Lob 7/4.

SECONDARY OBJECTIVES

To examine biological effects, pharmacokinetics and response rates of Chi Lob 7/4.

Immunological / biological effects

i) Analysis of effects on peripheral blood mononuclear cells: FACS analysis using labelled anti-CD3, CD4, CD8, CD14, CD16, CD19, CD25, CD56 and CD69 monoclonal antibodies.

ii) Analysis of effects on cytokine levels: Interleukin 2, (IL-2), IL-4, IL-6, IL-10, IL-12, interferon-γ (IFN-γ), macrophage inflammatory proteins MIP1α and MIP1β, RANTES and tumour necrosis factor TNFα.

iii) Analysis of effects on antigen presenting cells (APCs): FACS analysis of markers of APC activation using labelled anti CD11c, MHCII and B7.1/2 monoclonal antibodies if adequate numbers of circulating APCs can be isolated to enable analysis.

iv) Assessment of the development of human anti chimeric antibody (HACA) response against Chi Lob 7/4: Analysis by ELISA.

v) Assessment of intratumoral effects (subject to additional patient consent). Serial biopsies / fine needle aspiration (FNA) of easily accessible tumour sites to assess the presence of tumour infiltrating lymphocytes / mononuclear cells, cytokine profiles, apoptotic bodies and expression of FasL by immunohistochemistry.

Pharmacokinetics

Serum samples for pharmacokinetic studies will be obtained at baseline, immediately following the infusion and at 30 minutes, 1 hour, 3 hours, 6 hours, 24 hours, 48 hours and 72 hours after completion of the infusion. Concentration of serum Chi Lob 7/4 will be measured by ELISA. Parameters will include half-life, maximum concentration and area under the curve.

Response Rate

Evaluation of response rate by RECIST criteria.

STUDY TIMELINES AND ACCRUAL

It is expected that the study will commence in 2004 and last for a duration of 18-24 months. It is expected that accrual rate will be 1-3 patients per month.

TREATMENT REGIMEN

In line with other established antineoplastic, chimeric monoclonal antibody therapies such as Rituximab, Chi Lob 7/4 will be given by slow intravenous infusion once every week for a total of four weeks. This treatment regimen will facilitate early, rapid and dose dense administration of antibody to a patient group with advanced malignancy refractory to conventional treatment.

The starting dose for each infusion of Chi Lob 7/4 will be 5 mg (giving a total dose per patient of 20 mg divided over 4 weeks). The starting dose represents the equivalent human dose (on a mg/kg or x 2000 multiplier basis) to one tenth of the highest 3/23 dose (100 μg) given to mice without toxicity. Subsequent individual

dose levels will be 10 mg, 20 mg, 40 mg, 80 mg and 160 mg resulting in total patient doses of 40 mg, 80 mg, 160 mg, 320 mg and 640 mg respectively.

A minimum of 3 patients will receive treatment at each dose level. Escalation from one treatment dose level to another will be based on tolerance of lower doses and will only be permitted when at least 3 patients have completed four full weeks of treatment and four weeks of follow-up without dose limiting toxicity.

DLT will be defined as any grade 3 or 4 non-haematological toxicity (common toxicity criteria) occurring during the treatment period and up to 28 days of follow-up post treatment. Dose limiting toxicity will require discontinuation of treatment for that individual. Should one patient experience DLT at a given dose level then 3 further patients should be enrolled at that dose level. If 2 or more patients experience DLT at a given dose level then the dose level preceding the one at which DLT was observed will be considered the MTD.

INCLUSION, EXCLUSION AND WITHDRAWAL CRITERIA

Inclusion Criteria

i) Histological evidence of unresectable, metastatic or locally advanced solid tumour or intermediate grade non-Hodgkin's lymphoma for whom conventional treatment options have been exhausted.
ii) Positive tumour CD40 expression determined by immunohistochemistry.
iii) Adequate bone marrow function with platelets > 75 x 10^9/l, Neutrophils > 1 x 10^9/l at the time of study entry.
iv) Measurable or evaluable disease.
v) Satisfactory renal function; calculated creatinine clearance > 40 mls/min.
vi) Normal liver function in the absence of liver metastases. For those patients with hepatic involvement by malignancy ALP and ALT should be < 3 x upper limit of normal. All patients should have a normal bilirubin.
vii) ECOG performance status 0, 1 or 2.

viii) Women of child bearing potential must not be pregnant and agree to adequate contraception for the duration of the trial.

ix) Patients must give written, informed consent.

Exclusion criteria

i) Patients with CD40 negative tumours. Patients with low grade or transformed B cell, non-Hodgkin's lymphoma.

ii) Patients on long term immunosuppressant therapy (including steroids).

iii) Acute toxicities from prior chemotherapy must have resolved prior to randomisation.

iv) Prior radiotherapy must be completed 4 weeks prior to randomisation.

v) Prior anti-tumour biological therapies must be completed 4 weeks prior to randomisation.

vi) Significant medical or psychiatric conditions that compromise the patient's ability to give informed consent or complete the study.

vii) Prior treatment with murine or murine derived monoclonal antibodies.

viii) A history of autoimmune disease.

ix) Known HIV infection.

Withdrawal criteria

Patients should be withdrawn from the study if any of the following occur:

i) Interruption of therapy for whatever reason resulting in a delay of scheduled therapy greater than 3 weeks.

ii) Intolerable adverse effects that are judged by the investigator to be either physically or psychologically detrimental to the patient.

iii) Patient decision to discontinue treatment.

iv) Pregnancy.

v) Patient non-compliance.

vi) Unresolved or recurrent grade III or IV toxicity.

vii) Treatment with other chemotherapeutic or investigational antineoplastic agents.

viii) Progressive disease.

ix) Serious systemic allergic response to Chi Lob 7/4.

TREATMENT EVALUATION

Baseline pre-treatment

i) Full history and examination, height, weight, performance status.

ii) Full blood count to include full WBC differential.

iii) Clotting screen, ESR, CRP.

iv) Serum biochemistry to include GGT, ALT, ALP, Bilirubin, Albumin, LDH, Calcium, Sodium, Potassium, Urea and Creatinine.

v) Whole blood and serum samples for peripheral blood lymphocyte, APC and cytokine analyses.

vi) Serum immunoglobulins and electrophoretic strip.

vii) ECG and chest x-ray.

viii) Bi-dimensional evaluation of any measurable disease (CT) performed not more than 4/52 prior to entry to the study.

ix) Biopsy specimens should be obtained for analysis of CD40 expression by immunohistochemistry (historical biopsy material acceptable). Where possible, biopsy / fine needle aspiration of an accessible tumour site should be performed pre-treatment to enable subsequent comparative analysis of intratumoral treatment effects.

On treatment

Prior to each infusion of Chi Lob 7/4, patients should undergo the following:

i) Evaluation of toxicity using common toxicity criteria.

ii) Full blood count to include full WBC differential.

iii) ESR and CRP.

iv) Serum biochemistry to include GGT, ALT, ALP, Bilirubin, Albumin, LDH, Calcium, Sodium, Potassium, Urea and Creatinine.

v) Serum immunoglobulins and electrophoretic strip.

Following week two and week four infusions of Chi Lob 7/4

Where possible, biopsy / fine needle aspiration of an accessible tumour site should be performed pre-treatment to enable analysis of intratumoral treatment effects.

One month after completion of four infusions of Chi Lob 7/4

i) Bi-dimensional evaluation of measurable disease (CT).

ii) Evaluation of toxicity using common toxicity criteria.

iii) Full blood count to include full WBC differential.

iv) ESR and CRP.

v) Serum biochemical testing to include GGT, ALT, ALP, Bilirubin, Albumin, LDH, Calcium, Sodium, Potassium, Urea and Creatinine.

vi) Serum immunoglobulins and electrophoretic strip.

TREATMENT MODIFICATION

Treatment related toxicity should be measured using National Cancer Institute Common Toxicity Criteria (NCI CTC version 2.0) and in the event of toxicity treatment may be modified as follows:

Grade I or II toxicity (Haematological or Non-haematological)
No dose modification necessary.

Grade III or IV toxicity (Non-haematological)
Dose limiting toxicity will have been reached and treatment with Chi Lob 7/4 should be discontinued.

Grade III or IV toxicity (Haematological)
Treatment with Chi Lob 7/4 should be deferred until recovery of full blood count to grade I toxicity or better. Treatment may be delayed for a maximum of 2 weeks. Patients experiencing grade III or IV haematological toxicity lasting greater than 2 weeks should not receive further treatment with Chi Lob 7/4.

ETHICAL CONSIDERATIONS

Before the study is undertaken ethical approval will be sought from the appropriate ethics committee(s). Written informed consent will be obtained from all patients entering this study.