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| **Title** | CD40 antibodies for the treatment of human malignancy |
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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE HEALTH AND BIOLOGICAL SCIENCES

DIVISION OF CANCER SCIENCES

Doctor of Medicine

# CD40 ANTIBODIES FOR THE TREATMENT OF HUMAN MALIGNANCY

By Melanie Harvey

CD40 is an important antigen involved in immune regulation and anti-CD40 antibody therapies against human CD40 expressing tumours may be particularly advantageous. The antibody could induce tumour response in two ways. Firstly it might have a direct anti-tumour effect and secondly it could 'boost' the immune system to provide a heightened immune response that evades tumour tolerance.

This project has explored the effects of CD40 ligation of a variety of CD40 expressing tumours including transformed human B cell lines (RL and Daudi), human epithelial cell lines (MG79 [ovarian] and Caski [cervical]) and primary human B cell non-Hodgkin's lymphomas obtained, with consent, from patients undergoing excision lymph node biopsy or splenectomy.

The ligation of CD40, on transformed human B cell lines, using chinese hamster ovary cells transfected to express human CD40L and human soluble CD40L has shown significant cellular growth inhibition ($p<0.001$). Ligation of CD40 on human epithelial cell lines using human soluble CD40L has also resulted in significant growth inhibition.

In primary B cell human non-Hodgkin's lymphomas ligation of CD40 with both CD40L expressing CHO cells and human soluble CD40L in the presence of human IL4 has caused significant cellular proliferation ($p<0.001$) and induced upregulation of cell surface and costimulatory molecules including CD80, CD86, CD58, CD54.

Anti-tumour activity has been identified in a xenograft model of a transformed human B cell line (Daudi) treated with both a mouse anti-human CD40 antibody and a chimeric human anti-CD40 antibody.

I have performed important preclinical toxicology studies testing mouse anti-CD40 (3/23) by injecting the antibody intraperitoneally or intravenously in to BALB/c mice. The mice have been culled following treatment and a reversible dose dependent transaminitis associated with microscopic evidence of a reversible lympho-granulomatous hepatitis, that at the highest dose levels is acutely necrotising, has been identified. These anti-CD40 antibody toxicology studies are essential before a protocol for a phase I trial of human anti-CD40 antibody against CD40 expressing human tumours is developed.

I have developed a new chimeric human anti-CD40 antibody containing human constant regions and mouse variable regions. This antibody is currently undergoing large scale production for a proposed trial to treat patients with CD40 expressing tumours (excluding low-grade non-Hodgkin's lymphoma) who have failed conventional therapies.

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I am grateful to Dr Luann McKinney of the Royal Veterinary College, London, for her expert review of the murine pathology specimens.

Finally I must thank my husband Adrian for tolerating me and my family and friends who have supported me in so many ways, including help with IT, child care and simply being there.

# CHAPTER 1

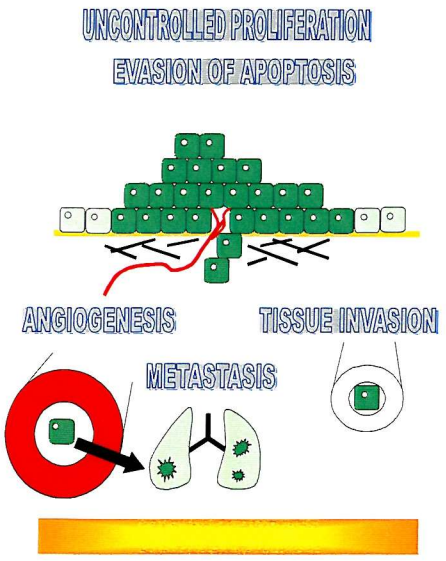
# INTRODUCTION

## CHAPTER 1

### General Introduction

Uncontrolled cellular proliferation generates a cancer. The mechanism by which this occurs is a dynamic, multistep process affecting the genome and resulting in the accumulation of errors in key regulatory intracellular pathways [1]. This succession of genetic change transforms the cell, enabling it to multiply at a rate that exceeds cell death, producing a group of cells with a distinct survival advantage (tumour) [2, 3]. Multiple factors, including cellular differentiation, contacts, extra-cellular matrix, blood supply and immune attack, then influence the behaviour of the tumour. Progression to cancer requires continued uncontrolled proliferation (neoplasia) with invasion of surrounding tissue, and/or spread (metastasis) to distant sites resulting in a relentless condition that threatens life.

**Figure 1**
The acquired capabilities of cancer cells

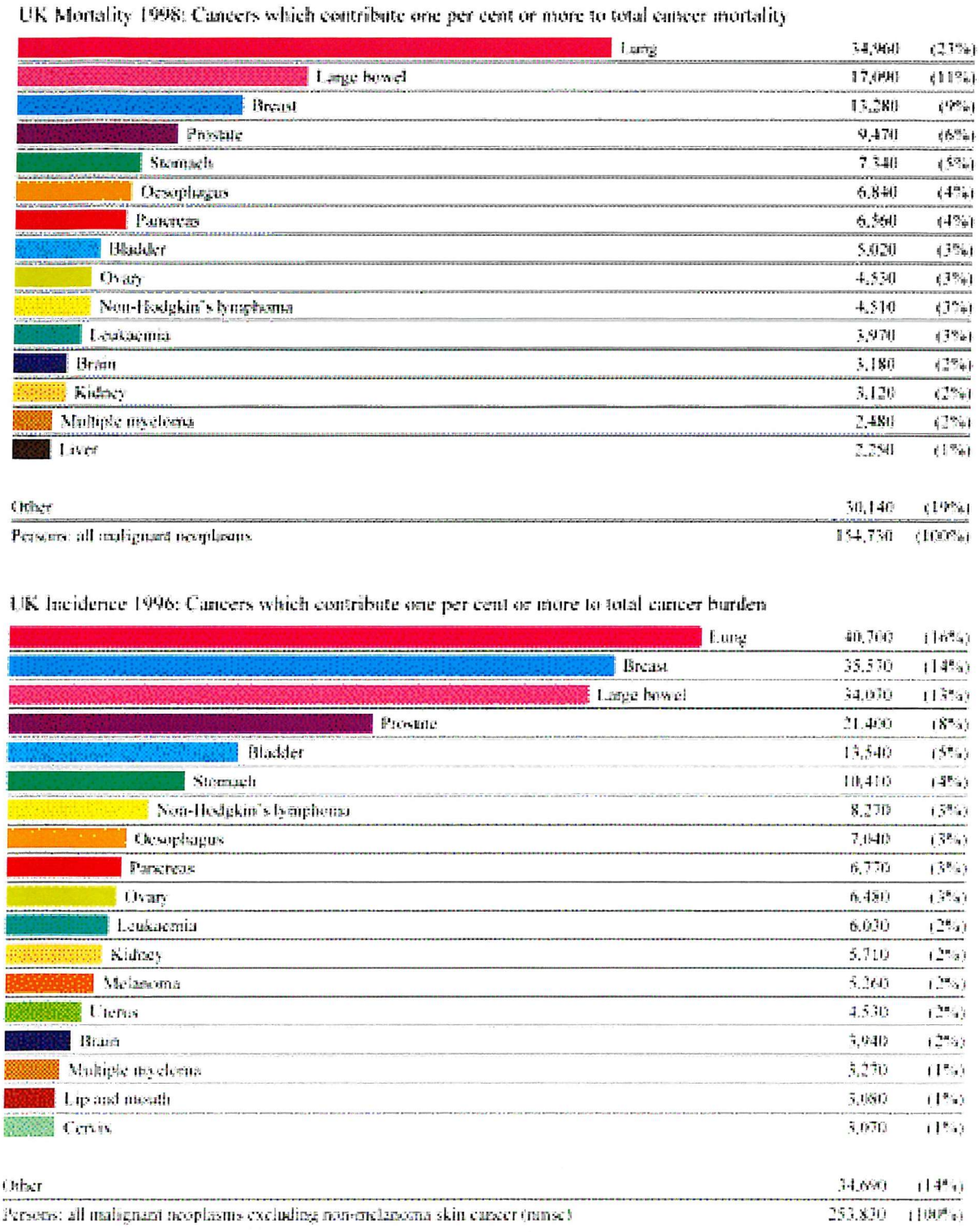


An invasive cancer evolves via a series of premalignant states. These changes have been identified histopathologically in a number of organs[3]. Some cell types are inherently more proliferative and therefore more likely to become cancerous. For example neoplasia with invasion is seen more commonly in cells derived from epithelium, a cell type which rapidly divides.

In modern times longevity and lifestyle have significantly increased the incidence of this disease which causes physical and psychosocial destruction amongst human beings. The result is a frightening condition that terrorises the western world causing considerable morbidity and mortality.

Many advanced stage cancers are incurable. Treatment modalities including surgery, radiotherapy and chemotherapy are employed to palliate symptoms and prolong survival. Surgery has provided the best cure rates for most early stage cancers (e.g. renal cell carcinoma, melanoma, breast carcinoma, bowel carcinoma) with radiotherapy and chemotherapy providing adjuvant therapy, preventing disease recurrence in some malignancies (e.g. breast carcinoma, bowel carcinoma). Good cure rates have been achieved in only a few advanced malignancies such as certain types of lymphoma and testicular germ cell cancers. Clearly better therapeutic options are required for the treatment of many malignancies.

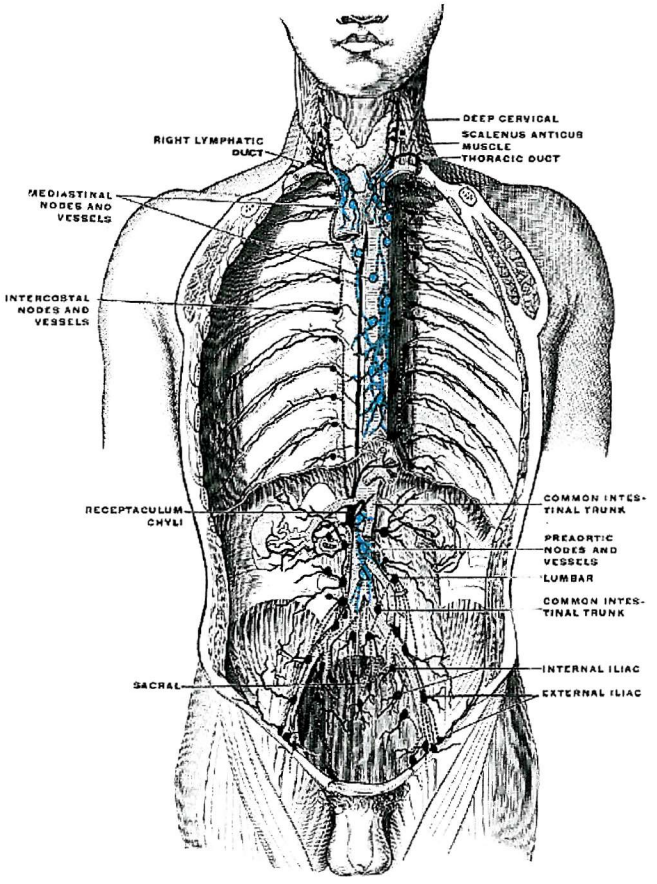
**Figure 2**
UK Cancer incidence and mortality figures




www.crc.org.uk

## The Non-Hodgkin's lymphomas

**Figure 3**
The Thoracic lymphoid system




Grays anatomy

www.bartley.com

The non-Hodgkin's lymphomas (NHL) are a clinically and pathologically heterogeneous group of disorders characterised by the malignant proliferation of lymphoid cells[4]. They include a number of disease entities from indolent NHLs, with a median survival of 8-10 years, which usually do not require immediate

therapy, to very aggressive diseases such as Burkitts lymphoma, which often require emergency therapy.

NHL accounts for around 3% of all cancers in the UK. It is the 7$^{th}$ commonest malignant disease. Approximately 8000 new cases are diagnosed in the UK each year [www.crc.org.uk]. The incidence is increasing but the cause of this is uncertain[5].

A number of conditions predispose to the development of NHL[6]. These include viral infections e.g. Ebstein-Barr (EBV) and the human immunodeficiency virus (HIV), as well as the bacterial infection helicobacter pylori which has been causally linked with primary gastric lymphoma. Congenital and acquired forms of immunosuppression are associated with an increased risk e.g. immunosuppressive therapies following organ transplantation (risk increased up to 33%) and common variable and severe combined immunodeficiency syndromes. It has been speculated that environmental factors such as occupational exposure to chemicals such as those used in agricultural industries, along with prolonged exposure to sunlight and atmospheric pollution may also be influencing the incidence of this disease.

The classification of NHL is complex, and has evolved over recent decades largely because of improved technology and a better understanding of the immune system. The result is the Revised European-American Lymphoma (REAL) classification[7], which is now recognised as the international standard system. This has recently been further revised by the World Health

Organisation[8, 9]. Many distinct entities are recognised in this classification, although in terms of their clinical behaviour, most lymphomas can be categorised as indolent (low grade) or aggressive (including intermediate and high-grade) subtypes.

**Figure 4**

**The Proposed WHO Classification of Lymphoid Neoplasms**

- B-Cell Neoplasms
  - Precursor B-cell lymphoblastic leukemia/lymphoma
  - Mature B-cell neoplasms
    - B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma
    - B-cell prolymphocytic leukemia
    - Lymphoplasmacytic lymphoma
    - Mantle cell lymphoma
    - Follicular lymphoma
    - Cutaneous follicle center lymphoma
    - Marginal zone B-cell lymphoma (MALT type, nodal and splenic type)
    - Hairy cell leukemia
    - Diffuse large B-cell lymphoma
    - Burkitt's lymphoma
    - Plasmacytoma and plasma cell myeloma
- T-Cell Neoplasms
  - Precursor T-cell lymphoblastic leukemia/lymphoma
  - Mature T-cell and NK-cell neoplasms
    - T-cell prolymphocytic leukemia
    - T-cell large granular lymphocytic leukemia
    - Aggressive NK-cell leukemia
    - Mycosis fungoides and Sezary syndrome
    - Angioimmunoblastic T-cell lymphoma
    - Peripheral T-cell lymphomas
    - Adult T-cell leukemia/lymphoma (HTLV1+)
    - Anaplastic large cell lymphoma
    - Primary cutaneous CD30+ T-cell lymphoproliferative disorders
    - Subcutaneous panniculitis-like T-cell lymphoma
    - Enteropathy-type intestinal T-cell lymphoma
    - Hepatosplenic γδ T-cell lymphoma
- Hodgkin's Lymphoma (Hodgkin's Disease)
  - Nodular lymphocyte-predominant Hodgkin's lymphoma
  - Classical Hodgkin's lymphoma
    - Nodular sclerosis
    - Mixed cellularity
    - Lymphocyte depletion

Low-grade (or indolent) lymphomas account for around 40% of NHL and consist of a number of histological subtypes such as small lymphocytic lymphoma/CLL, follicle centre lymphoma (grades 1-3), and mantle cell lymphoma. The majority are follicle centre lymphomas. Mantle cell lymphoma, although considered low grade, usually follows a more aggressive clinical course.

**Figure 5**
A table representing the different types of low-grade lymphomas

| INDOLENT NON-HODGKINS LYMPHOMAS |
|---|
| Follicular |
| Marginal zone B-cell lymphoma (MALT, nodal and splenic type) |
| Mantle-cell lymphoma |
| Small lymphocytic lymphoma |
| Lymphoplasmacytic lymphoma (immunocytoma) |

The condition may affect nodal and extranodal sites (e.g. liver, spleen, lung, bone marrow) but most commonly presents with fluctuating painless lymph node enlargement. The disease process is slowly progressive and many patients present with advanced stage disease. Around 80-85% of patients are initially diagnosed with stage III or IV disease, so bone marrow involvement at diagnosis is common. The clinical course tends to be relapsing and remitting[10].

## Conventional treatments

### Early Stage disease (IA/IIA)

Although only 15-20% of patients with indolent NHL have localised disease at the time of diagnosis, a treatment program including involved field radiotherapy + or –

additional combination chemotherapy is recommended. It can produce long-term disease free survival in 40-50% [11-13].

**Advanced stage disease**

For the majority of patients, who have advanced disease, low grade NHL is incurable. In early studies, single agent therapy with alkylating agents such as chlorambucil or cyclophosphamide was generally used[14]. High response rates were observed, but the disease was characterised by a pattern of continuous relapse. The median survival remained at 6-10 years, with no apparent cures. The addition of doxorubicin based combination chemotherapy did not improve overall survival compared with single alkylating agents[15].

Based on these results, the group at Stanford University, USA, adopted a 'watch and wait' policy[12] for patients with advanced stage low-grade NHL who are asymptomatic at presentation, with no evidence of major organ (including bone marrow) compromise. In the initial study[16] of 83 patients managed in this way, the survival was identical to those treated with alkylating agents immediately after diagnosis.

The watch and wait policy has subsequently been compared with early intensive chemotherapy in a randomised study from the NCI in the USA. Although a high response rate was observed in the patients receiving intensive chemotherapy, there was no survival advantage when compared with the watch and wait policy[17].

For those patients who can initially be managed by a 'watch and wait' policy, the median time to treatment is around 18 months in most series[18]. Single agent therapy is the most common initial treatment, producing response rates of 80-90% with a median response duration of 24-30 months. With successive relapse, response rates fall and response duration also declines. Many patients will receive further treatment with chlorambucil at first relapse, if their initial remission was prolonged. However, in view of the tendency for response duration to decline, most centres use more aggressive therapies including combination chemotherapy such as CHOP (cyclophosphamide, doxorubicin, vincristine, prednisolone), or purine analogues such as fludarabine[19], for second, or subsequent relapses.

**Histological transformation**

Low grade NHL tends to undergo histological transformation from low- to intermediate grade[16]. The exact number of cases that transform is difficult to quantify as many patients do not have repeat lymph node biopsies. Transformation has been reported to occur in up to 30% of cases but this may be an underestimate. In autopsy series, the majority of patients with follicular lymphoma have evidence of histological transformation. Some authorities suggest that transformation occurs at a constant rate throughout the course of the disease whilst others view it as a late event, with most cases occurring more than 6 years from the original diagnosis. Histological transformation is associated with a poor prognosis[20]. Conventionally most patients have been treated with combination chemotherapy, with a response rate of around 60-70%, but relapse is common, and long-term survival occurs in only 10-20%.

## Prognostic factors

Numerous prognostic factors have been identified in various published series. Clinical factors include advanced age, stage IV disease, the presence of B symptoms, involvement of more than 2 extranodal sites, large tumour bulk, bone marrow involvement, evidence of leukaemic overspill, elevated serum alkaline phosphatase, LDH and $\beta_2$ microglobulin.

The international prognostic index was originally developed as a prognostic model for intermediate/high grade NHL. However, it has been applied to low grade NHL and does appear to provide some useful information.

Factors with independent prognostic significance have been identified and include age (<60 years vs > 60yrs), stage (I/II vs III/IV), number of involved extranodal sites (0, 1 vs ≥2), serum LDH (normal vs elevated).

Four risk groups can be identified on the basis of these factors:

**Figure 6**
A table representing the four risk groups

| Risk category | No. of factors | CR % | 5 year DFS % | 5 year OS % |
|---|---|---|---|---|
| **Low** | 0,1 | 87 | 70 | 73 |
| **Low-intermediate** | 2 | 67 | 50 | 51 |
| **High-intermediate** | 3 | 55 | 49 | 43 |
| **High** | 4,5 | 44 | 40 | 26 |

**CR** = Complete response
**DFS** = Disease free survival
**OS** = Overall survival

In summary patients with advanced stage LG NHL and a large proportion with early stage disease are incurable with current treatment regimes. For many, advanced stage, asymptomatic patients the 'watchful waiting' approach is entirely appropriate and treatment with chemotherapy is then reserved for symptomatic/problematic recurrent disease. New therapeutic approaches will be discussed later but have included manipulation of the immune system to enable tumour cell eradication. NHLs have served as useful scientific tools in the understanding of tumour immunobiology and in the development of these treatments. The tumour cells can easily be obtained during diagnostic lymph node excision biopsies or in cases requiring splenectomy and once acquired they may be prepared into cell suspensions suitable for subsequent experimentation. Additionally, many established lymphoma cell lines now exist for in-vitro and in-vivo research.

## Immunology and tumour immunity

### The adaptive and innate immune system

The immune system comprises innate (natural) and adaptive (acquired) components[21]. The innate immune response provides early host defence against pathogens prior to the development of an adaptive response and is mediated by phagocytes, natural killer cells and complement. The adaptive system is defined by specificity for antigenic determinants and immunological memory (the basis of which is determined by clonal proliferation and long cellular life-span). B and T lymphocytes are antigen-specific and function as the mediators of humoral and cell mediated immunity.

**B-lymphocytes**

B-lymphocytes are initially derived in foetal liver and subsequently in bone marrow[22]. They develop into mature cells that carry membrane-bound surface immunoglobulin which act as antigen receptors, the B cell receptor (BCR). The surface immunoglobulin is associated with two transmembrane molecules (CD79a and CD79b) that transduce activation signals to the B cell. Resting B cells migrate throughout the secondary lymphoid tissues, particularly in follicles of lymph nodes and the spleen, to sites of trapped antigen. B cells bearing immunoglobulin specific for antigen enter the T-cell rich paracortical regions of lymphoid tissues, where they capture and process antigen[23]. Activated T cells induce antigen bearing B cells to migrate to B cell follicles. In the follicle B cells interact with follicular dendritic cells to form a germinal centre. In the germinal centres the B cells with high-affinity receptors for antigen are 'selected', and switch from producing IgM to another immunoglobulin class and ultimately become plasma cells, terminally differentiated B cells. Plasma cells have an expanded cytoplasm with characteristic parallel arrays of rough endoplasmic reticulum and are entirely devoted to the production of secreted antibody. This whole process is carefully controlled and relies on interaction with antigen, T cells, accessory cells, and cytokines. The CD40 antigen is important in this regulatory process[24-26]

**Figure 7**
Electron micrograph of a plasma cell

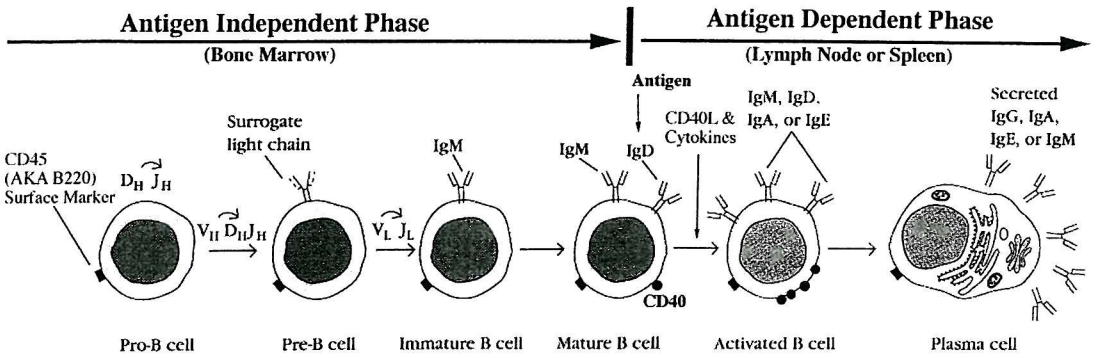
Clinical Medicine

Kumar and Clark

www.doctors.org.uk

There are 2 subsets of B cells, B-1 and B-2. In man the majority of B cells are derived from bone-marrow stem cells, but a minor population, B-1, distinguished by the CD5 marker, appear to form a self-renewing set. B-1 cells develop early, respond to a number of common microbial antigens and sometimes generate auto-antibodies. In adults, the majority of B cells are of the B-2 subset. They generate a greater diversity of antigen receptors and respond well to T cell-dependent antigens.

**Figure 8**
B cell development



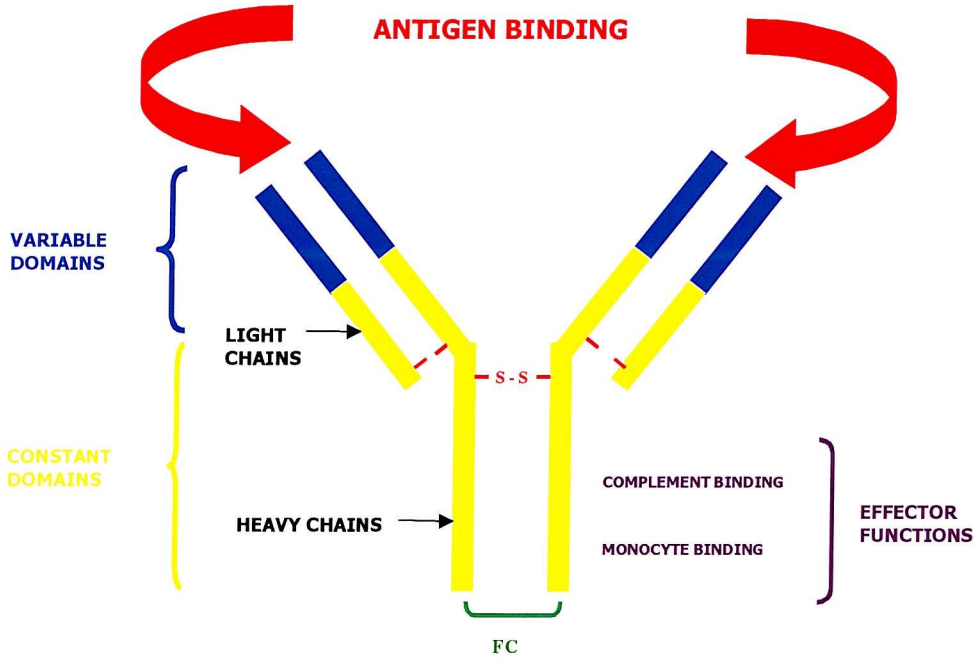

www.wsu.edu/~ms523/ms523s4b.html

Antibodies are serum immunoglobulins that form the humoral element of adaptive immunity[27]. They have a wide range of specificity for different antigens. They can bind to and neutralize bacterial toxins, and also, by binding to the surface of bacteria, viruses, or other parasites, increase their adherence to, and phagocytosis by, myeloid cells. This can be further increased by the ability of many antibodies to activate complement.

Antibody molecules all have a basic structure of four polypeptide chains, consisting of two identical light chains and two identical heavy chains, stabilized and cross-linked by intra-chain and inter-chain disulphide bonds, with glycosylation of the heavy chains[28]. There are five major types of immunoglobulin heavy chains (μ, γ, α, ε, δ), consisting of 450-600 amino-acid residues, and the type determines the class of the antibody. Light chains are of two main types (κ, λ), consisting of about 230 residues, and either type of light

chain may associate with any of the heavy chains. Both heavy and light chains are folded into domains. The domains in antibodies consist of three or four polypeptide loops stabilized by a β-pleated sheet and an intra-chain disulphide bond. Light chains have two domains and heavy chains four or five. This structure is found in many molecules, which are said to belong to the immunoglobulin superfamily. Between the Fc and Fab regions there is a section of heavy chain which contains the inter-heavy-chain disulphide bonds and confers segmental flexibility on the antibody molecule.

**Figure 9**
The structure of IgG




The largest amount of sequence variation, between antibodies, occurs in the N-terminal domains of the light and heavy chains[28]. This is called the variable (V) region. The V regions of a light and a heavy chain form the antigen-binding site. The remaining domains are relatively invariant within a particular class of antibody, and are called the constant (C) regions. The domains of antibody

molecules are named according to whether they are in the variable or constant region of the molecules, and according to whether they are in the light or heavy chain.

Antibodies can be grouped into different classes and subclasses depending on their heavy chains. In man there are five different classes: IgG, IgM, IgA, IgD, IgE. IgG and IgA are further divided into subclasses (IgG1-4 and IgA1,2 and surface IgA) to form antibody isotypes.

IgG is the major serum immunoglobulin. It is the main antibody in the secondary immune response to most antigens and is transferred across the placenta to provide protection in neonatal life. It can activate the classical complement pathway as well as opsonising neutrophils and macrophages by cross-linking immune complexes to Fc receptors. It can sensitise target cells for destruction by K cells.

IgM is a pentamer that is first to beproduced during the development of the immune system and in the primary immune response. It fixes complement and is the main antibody component of the response to T-independent antigens.
IgD is a trace antibody in serum but acts as a cell surface receptor on many B cells.

IgA occurs as monomers, dimers and polymers. It is abundant in secretions, where it protects mucous membranes. It is found in colostrum and is particularly important in protecting neonates.

IgE binds to high affinity receptors on mast cells and basophils, where it sensitises them to release chemical mediators after contact with antigen. It protects against helminth infection and is involved in the type I hypersensitivity reactions, such as asthma and hayfever.

**T-lymphocytes**

Lymphocytic stem cells arising from the bone marrow are sequestred in the thymus during embryonic development. T lymphocytes mature within the epithelium of the thymus. They develop their T cell antigen receptors (TCRs) but remain uncommitted to the CD4 or CD8 lineage until the TCR reacts with HLA molecules[29]. Type I interaction results in CD8 positivity and type II to CD4 positivity. Generally, CD4+ T-lymphocytes are helper T-cells eliciting responses from B cells and macrophages, and CD8+ T lymphocytes become cytotoxic capable of eliminating abnormal cells. T cells recognise antigens originating from within host cells. Cytotoxic T cells are capable of destroying virally infected target cells or foreign cells whilst T helper cells help B cells to divide, differentiate and produce antibody. T helper cells can be further subdivided into 3 groups depending on the blend of cytokines that they produce, TH0, TH1, TH2. TH1 cells interact most effectively with mononuclear phagocytes, while TH2 cells release cytokines required for B cell differentiation. TH1 and TH2 cells are both thought to originate from TH0 cells.

**The Major Histocompatibility Complex**

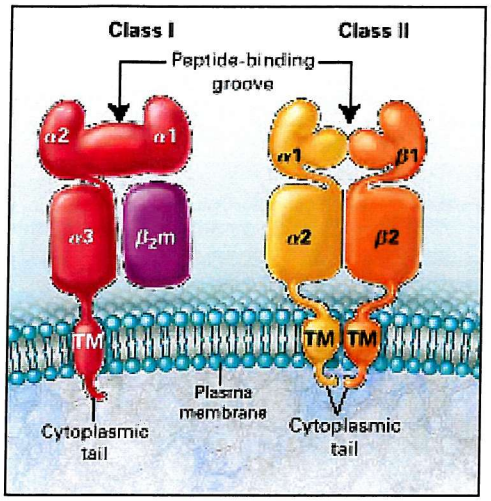
The major histocompatibility (MHC) or human leucocyte antigen (HLA) complex, in man, is a linked cluster of over 200 genes, situated on chromosome 6, the

products of which are essential for intercellular recognition and in discriminating between self (histocompatible) or non-self (non-histocompatible) [27, 30, 31]. Many of the genes encoding leukocyte antigens play a central role in the development of humoral and cell mediated immunity. The HLA genes involved in immunity are divided into 2 groups based on structure and function. Both groups have antigen-presenting functions. Group I is expressed at varying levels on all nucleated cells and presents peptide antigen to cytotoxic T lymphocytes, whilst group II is present only on antigen presenting cells (including B cells, activated T cells, macrophages, dendritic cells, thymic epithelial cells) and some others in the presence of interferon-$\gamma$. They present processed antigenic peptide to T-helper cells.

The class I molecule[32] comprises of an $\alpha$ polypeptide chain, containing extracellular, transmembrane and intracellular domains, and a $\beta$ chain derived from the $\beta$2 microglobulin chain on chromosome 15, consisting of an extracellular domain only. At least 20 class I genes exist but 3 of these are considered to be of utmost importance in the immune system, HLA-A, B, C respectively.

The class II molecule[32] contains $\alpha$ and $\beta$ chains both of which have extracellular, transmembrane and intracellular domains. The gene loci is described by letters: the first (D) indicates the class, the second (M, O, P, Q, or R) the family, and the third (A or B) the chain ($\alpha$ or $\beta$ respectively).

**Figure 10**
HLA class I and II molecules[32].

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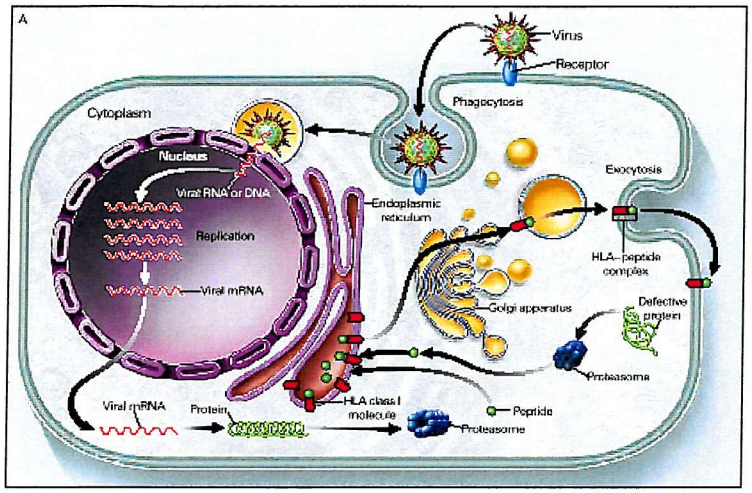
**Antigen processing and presentation**

Worn-out or abnormal intracellular proteins are unfolded into polypeptide chains and fed into proteasomes where they are chopped into short fragments which can be degraded in the cytosol to amino acids or transferred to the endoplasmic reticulum (ER). The intracellular self-peptides are picked up by molecules called the 'transporters associated with antigen processing' (TAPs). These molecules form a channel through which the peptides may cross the external membrane of the ER. Class I molecules wait within the lumen of the ER and, with help, bind to the peptide emerging from the TAP channel. The formed complex then moves to the cell surface where the α-chain of the class I molecule forms the anchor and the peptide, within the binding groove, reveals itself to the immune system

**Figure 11**
The principle pathways of generating peptides for loading onto HLA class I molecules[32].

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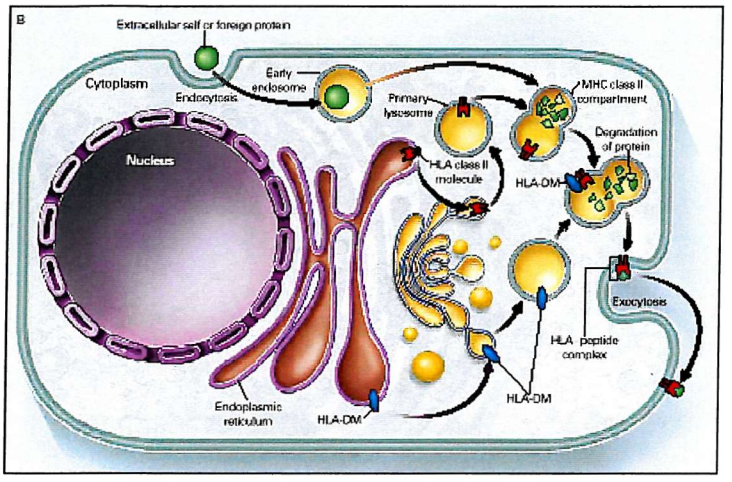
Endstage extracellular proteins enter the cell by invaginating the plasma membrane to form endocytic vesicles that fuse with lysosomes, containing an assortment of proteolytic enzymes, to form endosomes. The proteins can then be digested to peptides and subsequently amino acids for reuse. This system is also useful in distinguishing between self and non-self proteins, an important process if the immune system is to be alerted to foreign invasion, e.g. infection. The endosomes generated via this pathway fuse with vesicles, containing the class II molecules, in the cytosol and form a complex which travels to the cell surface membrane. Both $\alpha$ and $\beta$ chains of the class II molecule anchor the complex and the peptide reveals itself within the binding groove as with class I molecules.

**Figure 12**
The processing of extracellular proteins[32].

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All cells continuously process and present intracellular peptides within class I molecules but the processing and presentation of exogenous proteins on class II molecules is generally, but not absolutely, restricted to B cells, macrophages and dendritic cells. Despite this, class I molecules have been shown to present exogenous peptides (e.g. bacterial) and class II molecules have been found to contain endogenous peptides (e.g.viral). The exact mechanism by which this occurs has not yet been defined.

**Costimulatory Molecules**

Most immune responses are initiated by antigen triggering B or T cells. However, cellular activation also requires other signals. These may be delivered by costimulatory molecules or cytokines:

Two hypotheses exist. The preferred theory is a dynamic process in which the antigen is presented by an antigen presenting cell, expressing a class II molecule,

to a T helper cell. With the help of a second signal, involving costimulatory molecules, the T helper cell becomes activated and upregulates its expression of CD40L. CD40L binds to the CD40 molecule expressed by the antigen presenting cell and thereby activates the cell such that the bound antigen can be recognised and eradicated by cytotoxic T cells. The alternative theory or passive theory suggests that the activated T helper cell can directly influence cytotoxic T cells resulting in clonal proliferation and antigen eradication.

Most of the current literature supports the dynamic theory. Mice lacking T helper cells cannot mount a cytotoxic T cell reponse when injected with antigen presenting cells displaying an antigen recognised by cytotoxic T cells. However, a cytotoxic T cell response can be induced if the same mice are injected with anti-CD40 to provide surrogate help and a helper dependent T-killer response can be blocked in normal mice treated with anti-CD40L. This can be reversed with the addition of anti-CD40.

'Resting' B cells express low levels of costimulatory molecules e.g. CD80 (B7-1) and CD86 (B7-2), these can be significantly increased if the B cell is activated via CD40L expresses on CD4+ T cells. Activated B cells express higher levels of CD80 and CD86 which bind to CD28 and CTLA4 expressed by T cells. CTLA4 is expressed in low levels on resting T-helper cells. It is a second receptor for CD80. Expression is upregulated on activated T-helper cells. Both CD28 and CTLA-4 are important regulators of the immune system, however, their exact functions are controversial. In general it appears that CD28 potently enhances T-cell functions essential for an effective antigen specific immune response, and CTLA-4

counterbalances the CD28 mediated signals preventing overstimulation of the immune system.

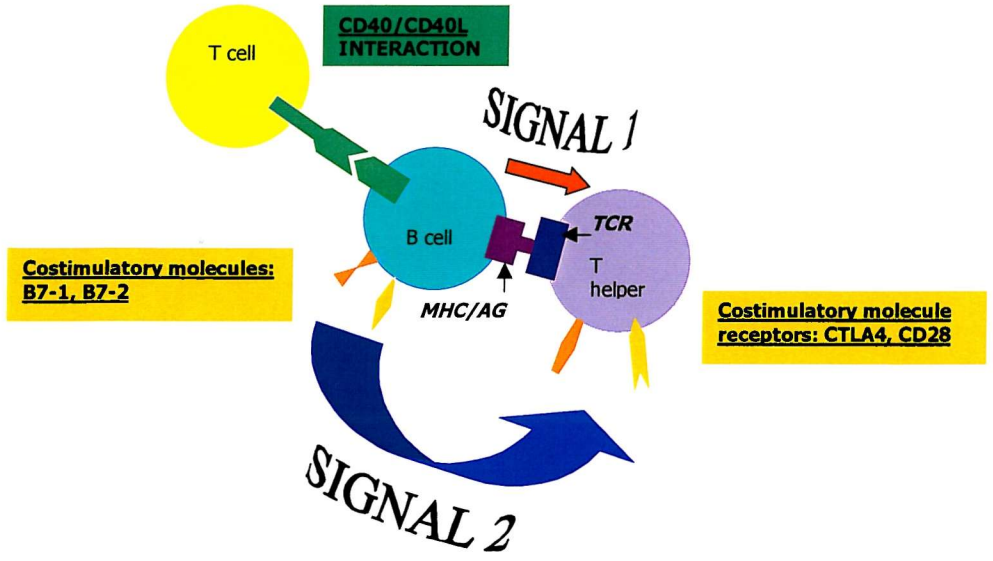
**Antigen presentation by B cells**

In the context of antigen presentation by B cells it is thought that CD80 binds with CD28 and to a lesser extent with CTLA-4. The T-helper cell becomes activated to induce an effective immune response (Th1-IL2 and IFNγ)/cytotoxic T cells). As this immune response proceeds CD28 becomes down-regulated, CTLA-4 upregulated and the signal changes from one of positive activation to negative activation.

It has been hypothesised that CTLA-4 provides an immunomodulatory function enhancing signal transduction by the TCR i.e. an initial signal that positively activates T cells later followed by a negative signal that induces T cell tolerance.

Agonistic anti-CTLA-4 and anti-CD28 have been shown to promote T cell activation synergistically. If CTLA-4 is blocked in mice using anti-CTLA-4 monoclonal antibody T cell activation and clonal expansion is augmented. Purified T cells treated with anti-CD28 monoclonal antibody proliferate. This proliferation can be inhibited by the addition of cross-linked anti-CTLA-4. Mice with a mutation in CTLA-4 show fatal lymphoproliferative disease.

**Figure 13**
Antigen presentation by B cells




**Antigen presentation by dendritic cells**

Dendritic cells are 'professional' antigen presenting cells[36]. Immature dendritic cells are highly phagocytic capturing microbial antigens or fragments from apoptotic cells in the periphery. They then migrate to the T-cell rich zones of local lymph nodes, where, as mature DCs, they express high levels of MHC-antigenic peptide complexes derived from the pathogen. The process is tightly regulated by chemokines produced in the lymph nodes, for which DCs express specific receptors. Once in the lymph node, DCs also begin to express molecules necessary to activate naïve T-cells that continuously flow through the lymph node. Thus, the rare antigen-specific T cells come in contact with antigen-loaded DCs, leading to the initiation of a lymphocyte response.

## CD40

CD40 is an important antigen involved in immune regulation[37]. It is a 48 kDa type I transmembrane protein consisting of extracellular, transmembrane, and cytoplasmic domains (193, 22, 62 aminoacids long respectively). It is a member of the tumour necrosis factor receptor (TNF-R) family and is similar to growth factor receptors, especially nerve growth factor receptor. The cDNA encoding for CD40 was first isolated, using a monoclonal antibody COS cell transient expression and rescue method, by Stamenkovich et al, 1989[38]. It contains an open reading frame of 831 base pairs. The first 30 amino acids at the N-terminus were sequenced by Braesch-Andersen et al, 1989[39].

CD40 is present on a variety of cells including both normal and malignant human B-lymphocytes, dendritic cells, monocytes, reticular endothelial cells, keratinocytes, fibroblasts and some carcinomas[40]. Expression is acquired early in B cell ontogeny, being present on pre-B cell leukaemias but absent on most progenitor B cell leukaemias[36]. It is not expressed on terminally differentiated B cells. It acts as a receptor for anti-CD40 antibodies as well as cellular or soluble CD40 ligand.

Its natural ligand is CD40-ligand (CD40L or CD154), a type II transmembrane protein with a trimeric structure which exists in soluble or membrane bound forms. It can be expressed as a transmembrane protein or as a cell surface heteromultimeric complex. On most cell types the molecular mass of CD40L is 32-

33kDa. However, 2 shorter versions exist (31, and 18kDa respectively) both of which form trimers and can deliver biological signals, indicating that CD40L might function as a cytokine. It is expressed on activated CD4-positive T cells, basophils, eosinophils, activated B cells, and blood dendritic cells. Defective CD40-CD40L interaction results in immunodeficiency[26].

CD40 ligation induces class-switching in B cells, activates dendritic cells to become more effective antigen presenting cells, by upregulating MHC, costimulatory and adhesion molecules, and facilitates CD8+ cytotoxic T-lymphocyte responses[41-43]. In-vitro, CD40 ligation has been shown to promote normal human B lymphocyte proliferation and induce the secretion of Ig, when cultured in the presence of IL4[44, 45].

**CD40 cell signalling**

In-vitro CD40 ligation has been shown to induce both cell survival and cell death. In cell survival pathways ligation of CD40 expressed by B cells induces trimerisation and association or phosphorylation of adaptor molecules including TNFR-associated factors 1, 2, 3, 5, 6 (TRAFS), Lyn, phospholipase Cγ2 and Jak3. These adaptor molecules activate a variety of intracellular kinases including p38 MAPK, c-Jun N-terminal stress activated kinases, phosphatidylinositol 3-kinase and STAT3/6. Transcription factors are then produced, including NFκB which enter the cell nucleus to activate cell survival genes such as BCL-kl, Bfl-1 and A20.

Cell death pathways are less clear but seem to involve activation of caspase-3 and caspase-8 which induce cytotoxic ligands of the TNF family (fas-ligand, TRAIL and TNF). However, apoptosis or rescue probably depends upon the mechanism of ligand binding. Binding to the proximal membrane domain may induce apoptosis whilst binding to the cytoplasmic tail may induce cell survival.

**Tumour Immunogenicity and escape**

Normally the immune system will reject antigens e.g. microbial pathogens and transplanted organs. However, the spontaneous immunological remission of cancer is rare.

The way the immune system responds to new tumours is complicated but seems to depend on where and when tumours develop. In the 1950's the immune surveillance hypothesis suggested that immune surveillance for tumours was a continuous process and tumours were constantly being eradicated but inevitably some escaped recognition and were able to successfully grow unchallenged. This theory was questioned because common cancers such as breast and lung cancer were not seen more frequently in the immunosuppressed. Also nude mice (with no T or B cells) had similar tumour incidences compared with normal mice.

In the 1990's this theory was modified. It was suggested that immunogenic tumours activated tumour specific T cells directly or indirectly by providing danger signals (proinflammatory cytokines and activated antigen presenting cells) to T lymphocytes in local lymph nodes. Non-immunogenic tumours did not provide danger signals resulting in T-cell anergy and therefore tumour tolerance.

The process of cancer rejection can fail for a number of reasons. The cells may not express an immunostimulatory antigen or there may be deficiencies in antigen presentation e.g. the loss of HLA class I expression on some cancer cells. If the malignant cells or their fragments pass to the local lymph nodes they often fail to optimally activate specific B and T cells probably because they lack the capacity to express co-stimulatory molecules. Without adequate co-stimulation CD4+ T cells become tolerant of the tumour. If, however, they do manage to activate specific T and B cells, avoiding T cell tolerance, the effector cells produced must be able to penetrate the tumour. Some cancers have developed their own specific mechanisms for evading an effective immune response, for example the expression of Fas ligand or production of inhibitory cytokines, such as IL10 and tumour necrosis factor β. Others block immunostimulatory cytokines such as IL2 or they associate with tumour-specific macrophages which release toxic oxygen and nitrogen free-radicals.

Given the central role of CD4+ T cells in anti-tumour response, defining the mechanisms responsible for the induction of T-cell tolerance to tumour antigens is necessary for the successful development of immunomodulatory cancer therapies. Host APCs are also required for initiating a productive T-cell immune response, their state of activation and/or differentiation may determine whether T cells are primed or rendered tolerant. In their immature state, APCs such as dendritic cells and B cells have relatively low levels of MHC, co-stimulatory molecules and other adhesion molecules. This may, in par, explain T-cell tolerance to tumour.

**Immunotherapy**

Manipulation of the immune system to 'boost' an anti-tumour response has been extensively investigated. This may be an active or passive process, involving a specific or non-specific approach. Active, specific vaccines are the preferred approach as they induce an immune reaction against a defined antigen with a low probability of adversely affecting normal tissues, but non-specific or passive approaches have also been investigated.

Many tumour antigens have been identified. They may be unique to the affected individual or common to the particular tumour type. Shared rather than unique tumour antigens are better candidates for the development of vaccine strategies. They may be based on whole or lysed tumour cells or polyvalent extracts.

Tumour antigen-peptide vaccination has been clinically studied in a number of trials against metastatic melanoma[46]. Immediate and delayed tumour regression has been seen in some patients but interestingly subsequent disease progression has often been associated with antigen-loss tumour variants.

Various genetic approaches have been investigated to facilitate antigen transfer to APCs. Cytokines, MHC or costimulatory molecules have all been transfected into APCs to enable them to induce potent cellular and humoral anti-tumour immune responses. Recombinant viral and bacterial vaccines have been developed to

present tumour antigens through the MHC pathways and provide danger signals to attract host APC's[46, 47].

CpG oligonucleotides can directly activate APCs, such as B cells, macrophages and dendritic cells, to enhance expression of costimulatory molecules and activate NK cells via IL-12[47]. CpG oligodeoxynucleotides could be used as single agents, in combination with monoclonal antibodies or as an adjuvant to tumour vaccines. Some types of tumours, melanomas, RCC and lymphomas are frequently sensitive to NK-mediated lysis. CpG oligonucleotides will soon be entering phase I trials against these malignancies.

Heat shock proteins (hsp) have important roles in the immune response particularly in MHC class I complex assembly[47]. The hsp, even in the absence of class I expression, as seen in some tumours, may be used to present antigen by cross-priming to involve the MHC class II pathway.

Alternatively, defined tumour antigens can be administered with an adjuvant to provide an additional immuno-stimulatory message to activate APCs and elicit an immune response[47]. Different microbial agents have been used as adjuvants to stimulate the immune system to fight cancer e.g. Bacillus Calmette-Guerin (BCG) and corynebacterium parvum[46]. The use of BCG in the treatment of superficial bladder cancer is well known. Intravesical administration of BCG to these patients results in a recurrence rate of 15-40% compared with 42-100% when surgery alone is used. Chemical adjuvants e.g. Keyhole limpet haemocyanin and oil in water (MF59) enhance antibody response to protein vaccines. Cytokines such as

IL12, Granulocyte-macrophage colony stimulating factor and IL-2 have all improved responses to active specific-peptide vaccines. Cellular adjuvants such as dendritic cells pulsed with acid-eluted peptides have shown potent inhibition of tumour growth in animals.

**Passive immunotherapy**

The use of monoclonal antibodies and engineered antibody derivatives for passive specific or non-specific cancer immunotherapy is interesting.

Immortalisation and cloning of antibody forming cells to produce a single type of antibody, monoclonal antibodies (mAb), each with the same antigen binding site, has been possible since Kohler and Milstein[48] developed the technology in the 1970's. However, as a means of treating malignant disease monoclonal antibodies have only recently begun to realise their potential with promising results emerging using a number of different monoclonal antibodies both alone and in combination with radiotherapy or chemotherapy[49].

A therapeutic anti-cancer effect can be achieved if the mAb selectively binds a specific cell surface receptor, expressed preferentially by tumour cells. Cell death may then result through direct cell signalling or indirectly by stimulation of the host immune system to recruit cell mediated defence mechanisms.

There are several different approaches to mAb therapy. These include unconjugated antibody[50], bispecific antibodies[51], and conjugated forms,

radioactive conjugates[52], immunotoxin conjugates[53] and antibody directed enzyme linked pro-drug therapies (ADEPT) [54]. These treatments may be delivered alone or in combination with conventional therapies.

**Unconjugated monoclonal antibodies**

The first indication that monoclonal antibodies might provide an alternative and effective therapeutic choice in the treatment of human malignancy came with the development of monoclonal antibodies directed against tumour-specific cell surface antigen expressed by B cell malignancies[55]. The surface immunoglobulin expressed by the tumour cells display antigenic determinants within the variable regions of the immunoglobulin (Ig) heavy and light chains. These determinants (idiotypes [Id]) are uniquely encoded by a combination of genes containing several different genetic elements. These malignancies are monoclonal, so all the cells of each tumour produce the same Ig protein, distinguishing neoplastic from normal cells.

An early study of 11 patients treated with individually customised anti-idiotype mAbs, found 1 complete response (CR) resulting in a prolonged clinical remission lasting in excess of 42 months and 5 significant partial responses[56].

Subsequently, another study[57] of 45 patients with low grade B cell lymphoma treated with anti-Id mAbs, alone or in combination with α–Interferon, chlorambucil, or interleukin-2 found an overall response rate (ORR) of 66% (18% CR, 48% PR). Six patients with CRs (13%) experienced prolonged remissions ongoing for

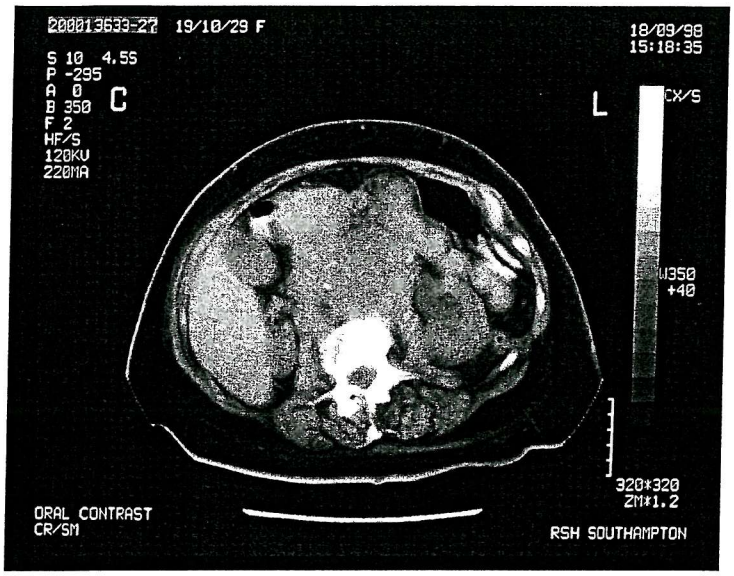
between 4-10 years following their mAb therapy. Low level persistent disease was identified in 5 of the 6 patients (11%) using sensitive investigations including blood and bone marrow flow cytometry, as well as the polymerase chain reaction for clonal gene rearrangements of immunoglobulin sequences or t(14:18) translocations, suggesting the presence of dormant residual tumour.

Although this approach using anti-Id mAb is theoretically attractive each patient requires a, 'custom made', antibody. Clearly, this involves an enormous resource and time burden and presents many technical production difficulties. Additional problems include relapse of the lymphoma with the development of idiotype negative variants following treatment[58]. Other approaches are currently being evaluated including Professor Freda Stevenson's group in Southampton who are attempting DNA vaccination to produce idiotypic protein that initiates an effective anti-tumour immune response.

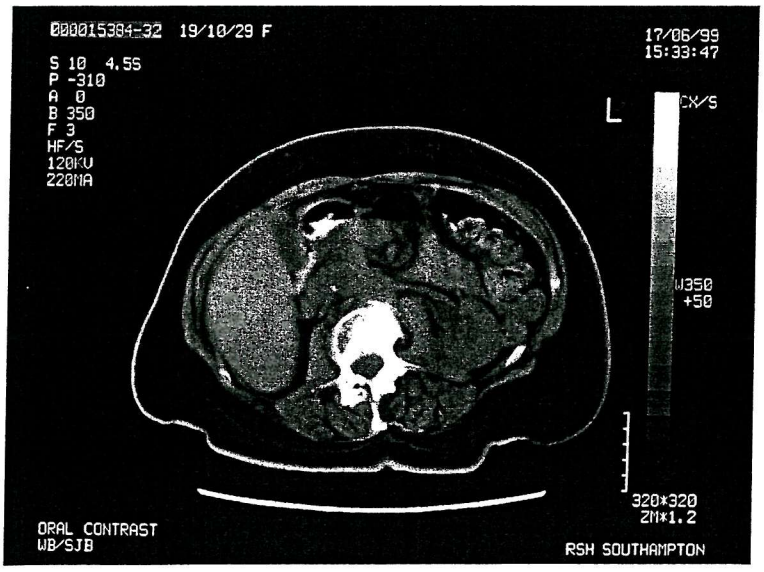
Since these early studies many other murine and engineered monoclonal antibodies, directed against cell surface molecules expressed by tumour cells, have been developed and are at various stages of clinical development. These include anti-CD20, anti-CD19, anti-HLA-DR, anti-CD21, anti-CD22 (NHL); anti-CD52, anti-CD5 (various lymphoproliferative disorders), anti-CD33, anti-CD10, anti-CD25 (acute leukaemia); anti-CD4 (cutaneous T cell lymphoma); anti-VEGF (general anti-cancer); anti-CA125 (ovarian carcinoma); anti-17-1A (colorectal carcinoma); anti-idiotypic GD3 epitope (lung cancer); anti-EGFR (head and neck malignancies); anti-HER2/neu (breast carcinoma)[59].

To date the greatest success has been documented with anti-CD20 mAb in the treatment of NHL, in particular low-grade disease. Response to the mAb occurs in 50% of cases of follicular/indolent NHL even in the presence of bulky disease (masses > 10cm diameter) [60, 61]. When combined with conventional chemotherapy e.g. cyclophosphamide, doxorubicin, vincristine, and prednisolone (CHOP) the ORR can be further improved to around 90% (55% CR), and in some complete responders associated with clearing of Bcl-2/IgH (chromosome 14;18 translocation) positivity from blood and bone marrow assessed with the polymerase chain reaction assay[62] [63].

CT scan of a patient with progressive follicular NHL. It shows a large lymph node mass




The same patient six months after receiving a complete course of rituximab 375mg/m$^2$/week x 4. Interestingly, tumour response continued after completion of the therapy, high-lighting the fact that mAb therapies can continue to provide delayed tumour response even after cessation of therapy.



In newly diagnosed or recurrent mantle cell lymphoma response rates of 30-40% have been achieved with a median duration in some series lasting 1.2 years[64]. Relapsed or refractory diffuse B cell lymphoma, immunocytomas, and small lymphocytic lymphomas have ORR of 37%, 30%, and 15% respectively[65].

In the treatment of post-transplant lymphoproliferative disease data is limited but complete responses have been seen[66].

**Radioimmunotherapy**

The most impressive results in the treatment of NHL have been seen with radiolabelled mAb. This has the theoretical advantage of a 'cross-fire' effect, damaging DNA of adjacent tumour cells regardless of whether they express the target antigen[67]. The result may be a greater cell kill than can be achieved by an unconjugated antibody. The majority of groups have used a non-myeloablative single treatment[52], although fractionated treatments have been implemented. Others have used a myeloablative strategy with autologous, peripheral stem cell transplantation [68, 69]. Despite the clinical success of such therapy the exact mechanisms by which these treatments exert their effects remain largely undefined.

Trials of non-myeloablative radiolabelled anti-CD20 in pretreated recurrent follicular NHL have documented objective response rates of 75-80% (35-45% complete responses) with minimal toxicity, and median response duration of 12

months[68]. In previously untreated patients with advanced low-grade NHL, treated with a single dose of murine $^{131}$I-anti-CD20, 71% had a CR[70].

Myeloablative techniques with autologous stem cell transplantation have demonstrated objective responses in 95% of patients, with complete responses in 85%, progression free survival of 62% and an overall survival of 93% with median follow-up of 2 years[68]. Toxicities are higher than with non-myeloablative techniques but not as severe as conventional bone marrow transplant with total body irradiation.

**Bispecifics Antibodies**

The ability of tumour cells to escape cytotoxic cell mediated attack has been addressed using bispecific antibodies. These are molecularly engineered single molecules that contain two different antibody-based binding specificities[51]. Therefore, the antibody can simultaneously target both the tumour cell surface molecule and a cytotoxic triggering receptor situated on an immune effector cell e.g. T cell receptor. In this manner the effector cell can be redirected to kill the tumour cell that it would otherwise ignore i.e. cellular mechanisms of cytotoxicity are enhanced.

Clinical studies are in various stages of development. Progress has been hampered by toxicity in the initial trials, and cost implications in the production of these antibodies. Bispecific antibodies have been tested in both NHL/CLL and Hodgkins lymphoma.

Following extensive preclinical tests, anti-CD3/CD19 bispecific antibody (BsAb) together with IL2 was administered to 3 patients with B cell NHL in a dose escalating trial[71]. Limited toxicity was identified with WHO grade II fever and chills. These side effects were caused by T cell release of tumour necrosis factor alpha (TNF-α). A subsequent patient with heavily pretreated, chemoresistant CLL received 0.6mg BsAb/$m^2$ as an intravenous infusion preceded by 1MU IL-2/$m^2$. The level of TNF-α was measured to be highest on the first day and side effects included grade II fevers and chills. No response was seen in this patient who had a high tumour burden. Further evaluation in the setting of lower tumour burden was recommended.

In Germany 15 patients with refractory Hodgkins disease were treated with anti-CD16/CD30 BsAb in a phase I/II dose escalation trial[72]. The BsAb was administered 4 times every 3-4 days at a starting dose of 1mg/$m^2$ and the treatment was well tolerated with the maximum tolerated dose not reached at 64mg/$m^2$. Five patients responded to the treatment (1 CR, 1 PR, 3 MRs); the complete and partial responses lasted 6 and 3 months respectively. Six patients suffered mild to moderate side effects with fever, involved node pain, and a maculopapular rash and 8 patients developed a HAMA response while 5 patients showed an allergic reaction after attempted retreatment. There was no dose to side effect or dose-response correlation.

Immunogenicity has been a particular problem encountered with BsAb; HAMA production and non-specific T cell activation occurs commonly. Current work aims

to prevent non-specific T cell activation by modifying BsAb to remove the Fc portion, allowing separate manipulation of T cell targeting and T cell activation.

**Immunotoxins**

Immunotoxins are chimeric or conjugated molecules of 'cancer specific' antibody or growth factor combined with a cytotoxic protein[53]. They were developed to optimise both specific targeting and cell kill by effectively delivering a protein-based toxin directly to the tumour cell. The toxins used are usually derived from bacteria (e.g. diptheria toxin and pseudomonas exotoxin) or plants (ricin and abrin) [73]. They all act at low concentrations inhibiting protein synthesis, by inactivating ribosomes, in both resting and dividing cells. Protein engineering has been used to modify toxin molecules such that they retain their cytotoxic abilities but do not bind to normal human cells. Unlike 'naked' mAb, immunotoxins must be internalised to allow the toxin to reach the cytosol or nucleus to enable disruption of protein synthesis.

Chemically linked immunotoxins and recombinant fusion toxins generated using DNA technologies have been evaluated in phase I/II/III trials[49]. Many problems have been noted including the immunogenicity of the toxin and/or antibody, the poor capacity of the immunotoxin to penetrate large solid tumours, the toxic consequences of therapy, and subsequent disease relapse with the development of antigen-escape variants. Most studies have only included heavily pretreated patients with large tumour burdens. Despite this minor responses have been

identified suggesting activity. However, it is anticipated that immunotoxin therapy will be of most benefit in the setting of minimal residual disease.

Vascular leak syndrome is the major dose limiting toxicity caused by immunotoxins[74]. It is thought to be secondary to toxin mediated endothelial injury and manifests as decreased urinary sodium excretion, hypoalbuminaemia, fatigue, hypotonia, myalgia, and pulmonary oedema. Plasma fibronectin plays a role in maintaining microcirculatory integrity. The most severe cases of vascular leak syndrome occur in those patients with the highest pretreatment levels of fibronectin, the largest post-treatment decrease in fibronectin, increasing post-treatment levels of serum TNF alpha, higher concentrations of circulating immunotoxin and in those with the lowest levels of circulating tumour cells.

Patients with competent humoral immune systems develop human anti-mouse (HAMA) and human anti-toxin antibodies (e.g. human anti-ricin antibodies-HARA) [75]. These can inhibit toxins by increasing their rate of clearance and by blocking binding sites.

The immunotoxins have been administered as continuous infusions or bolus treatments. Generally, there has been no safety or efficacy advantage demonstrated using continuous infusional systems. The most widely clinically tested immunotoxin is B4-blocked ricin (anti-CD19 with a partially blocked ricin toxin). In Boston[76] 75 patients with relapsed B cell NHL patients were treated in a phase II trial with anti-CD19 directed blocked ricin toxin. Three CRs were achieved and 5 PRs. Hepatic enzyme abnormalities and thrombocytopaenia were

dose limiting. A phase II trial was reported in which 49 patients with B cell NHL, in CR following high dose chemotherapy and autologous bone marrow transplantation, received anti-B4 blocked ricin as adjuvant therapy. The immunotoxin was delivered at a dose of 30μg/kg/day as a continuous infusion for seven days. This could be repeated for up to 2 additional courses at 14-day intervals. Thirty-one patients received 2 or more courses. Human anti-mouse antibodies and/or human anti-ricin antibodies developed in 46% of patients. The 4-year disease free survival (DFS) and overall survival was 56 and 72% respectively. Twenty-six patients (53%) remained in CR after a median follow-up of 54.5 months. Toxicities were described as tolerable and reversible, including hepatic enzyyme abnormalities, thrombocytopaenia, myalgias, fatigue, nausea, hypoalbuminaemia, and dyspnoea.

Anti-B4 blocked ricin has been combined with chemotherapy in the treatment of AIDS related NHL[77]. The infusional immunotoxin, 20 μg/kg/day, was administered, to those patients with out progressive disease, for 7 days after the third cycle of low dose methotrexate, bleomycin, doxorubicin, cyclophosphamide, and vincristine (m-BACOD), given every 21-28 days. Twenty-six of 44 received the immunotoxin.The ORR was 57% (13 CRs and 12 PRs), with a median survival for all patients of 8.9 months. Fifty-eight percent of patients developed a significant transaminitis and HAMA or HARA were found in 31%.

Hodgkin and Reed-Sternberg cells consistently express the antigen CD30. Anti-CD30 immunotoxins (Anti-CD30 monoclonal antibody combined saporin, a type-1 ribosome inactivating protein) have been investigated in the treatment of

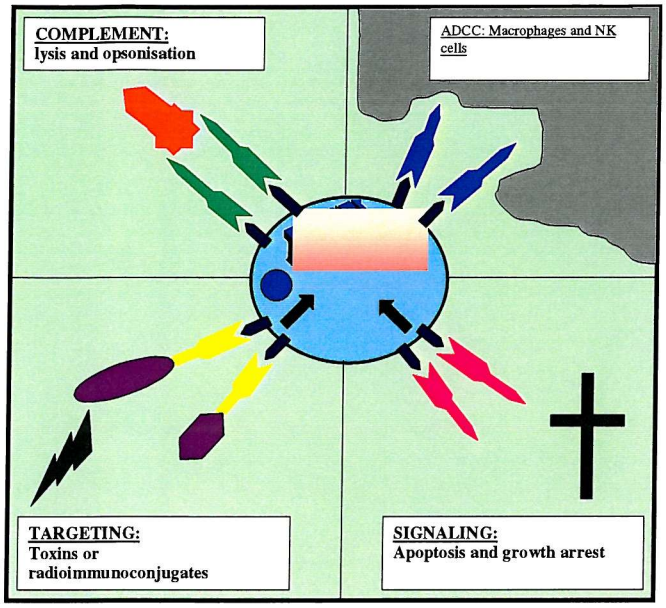
Hodgkin's lymphoma[72]. Four patients were treated with 0.8mg/kg immunotoxin in one or two doses. Three patients had a reduction in tumour size ranging from 50 to greater than 75%. Clinical responses were only transiently maintained with progressive disease occurring 6-10 weeks following treatment. Antibodies to both parts of the immunotoxin developed in all patients.

Immunotoxins have provided some interesting durable partial and even complete responses. However, their immunogenicity and serious side effects have limited their use.

**Mechanisms of action of monoclonal antibodies in the treatment of cancer**

The original concept of anti-cancer mAbs was to specifically target tumour cells causing opsonisation which enabled immune effectors (such as natural killer cells and macrophages) to destroy tumour by a process known as antibody-dependent cellular cytotoxicity (ADCC). The effectiveness of mAbs at inducing ADCC was shown to vary between sub-classes: An early clinical study found that anti-CD52 rat IgG2b (Campath) was superior to IgM or IgG2a variants, of the same antibody, at providing long-lasting depletion of neoplastic cells[78]. More recently the ability of mAb-effector mechanisms to effectively eliminate all tumour, particularly when large numbers of tumour cells are involved, has been questioned and a number of host features have been identified which can protect both normal and malignant cells from antibody attack, including expression of key regulatory proteins e.g. CD55 that provide direct cellular protection or down-regulation of the cytotoxic capabilities of natural killer cells when they engage MHC class I molecules.

**Figure 14**
The mechanisms of action of monoclonal antibodies




It is now recognised that recruitment of effectors is only one of the mechanisms by which monoclonal antibodies act, their cell surface binding can also generate transmembrane signals that directly alter or control tumour growth, in some cases leading to apoptosis[79]. Clinical evidence that transmembrane signalling might play an important role in tumour regression came from studies of the treatment of follicular non-hodgkins lymphoma, using rodent anti-idiotype antibody and demonstrating that tumour response correlated with the ability of the monoclonal antibody to induce intracellular signalling as measured by an increase in tyrosine phosphorylation of intracellular proteins[80].

In-vitro studies also established that proliferation, growth arrest, death or differentiation of a normal or malignant B cell depended, not only on engagement of a suitable mAb with the cell surface receptor, but also on the extent of cross-linking, level of costimulation and duration of the signal. Since the initial

demonstration that anti-CD20 induced growth inhibition was dependent on cross-linking of the mAb[81], subsequent in vitro studies have attempted to improve potency by inducing hyper-cross-linking of the mAb bound to target cells, using multi-valent surface providers such as polyclonal anti-mouse antibody or FcR-bearing cells (such as macrophages) [82].

Animal studies have shown that most anti-cancer mAbs do not function therapeutically when used as Fab' or $F(ab')_2$ fragments. It is unclear whether this lack of activity results from the inability of the antibody fragments to effectively recruit effectors or whether Fc receptor interactions of the whole antibody with normal cells increases the density of antibody cross-linking on target cells. However, normal B cells can be maintained for prolonged periods in culture with either anti-CD40 and IL-4, providing a feeder layer expressing human CD32 (low affinity Fc receptor) is present to cross-link the cells[45, 83], or with trimeric soluble CD40L[84] suggesting that cross-linking rather than cellular interactions is the most important process.

**Target Antigens**

Target selection is crucial to the development of new mAbs. The mAb must be chosen to bind the target antigen, recruit cellular effectors and directly signal the cell to induce growth arrest or cell death. However, the most promising future direction for cancer therapy might be in the production of mAbs that do not depend solely on the direct effects on tumour cells but alternatively, through there cell surface binding, stimulate effector cells of the immune system resulting in the

potentiation of weak, but existent, anti-tumour responses to a level that effectively eradicate tumour. To date some of these new mAbs have only been evaluated in animal models. These include anti-CD40, anti-CTLA4, anti-CD137 and anti-BAT[84].

Theoretically these monoclonal antibodies block or cross-link cytotoxic lymphocytes or APCs in such a way that anti-tumour T cells and inflammatory cells residing in the immune system are stimulated and expand to a level capable or eradicating the metastatic disease. In most cases, the mAb binds to its target on T cells or APCs, and mimics the natural ligand of the target molecule. The resulting expansion of antigen-specific cytotoxic T cells then provides the individual with either complete or partial immune protection in the event of tumour rechallenge.

**Toxicity of monoclonal antibody therapy**

Unconjugated antibodies are generally well tolerated but acute infusion reactions are relatively common, involving up to 50-90% of patients with some antibodies[85]. These reactions are usually mild with the first infusion and with murine mAbs may worsen with subsequent infusions when as a result the patient suffers variable diaphoretic symptoms with fever and occasionally anaphylaxis[49]. Stopping the infusion, administration of diphenhydramine and paracetamol usually abates this reaction within 3 hours and the infusion may then be recommenced at a slower rate.

When rodent mAbs were used therapeutically, humoral host immunity was stimulated resulting in the production of human anti-murine antibodies (HAMA) in 50% of treated patients, these antibodies had the potential to cause serum sickness. Additionally, effector functions of mouse antibodies proved to be less efficient in the human context. Their biological half-life was shorter when compared with human immunoglobulins. These problems could easily be overcome using human antibodies, however, it has been more difficult to produce human antibodies from hybridoma and lymphocyte cell lines. In recent years genetic engineering has allowed the development of techniques to humanise murine mAbs producing chimeric antibodies consisting of human constant and mouse variable regions. These antibodies show the same specificity and affinity of their parental murine antibodies and are capable of efficiently mediating ADCC, antibody-dependent macrophage cytotoxicity and complement fixation in the human context but are themselves much less immunogenic than murine antibodies[86]. In trials of Rituximab (a chimeric human anti-CD20 mAb) HAMAs are detected in only 1% of patients who receive four weekly courses of therapy and acute, severe (WHO grade III and IV) toxicity occurs in just 3-5% [85].

**Chimeric antibody construction and production**

Murine-derived monoclonal antibodies have been used to treat a number of human illnesses with variable success. Two main difficulties have been described[87]. Firstly, although murine mAbs have been found to be specific for their therapeutic targets, they do not have the same ability to trigger human biological effector functions, such as complement and human Fc receptors.

Secondly, treatment of humans has resulted in an immune response with the production of human anti-mouse antibodies (HAMA). These reduce the therapeutic effectiveness of the monoclonal antibody by rapidly clearing the mAb from the blood. In addition re-treatment carries a risk of life-threatening immune reactions (serum sickness). Human monoclonal antibodies would be a more appropriate treatment approach against human disease but these have been technically difficult to produce[28]. Research has therefore been directed into producing engineered monoclonal antibodies, suitable for human therapy, using recombinant DNA technology[88].

One approach has been to clone recombinant DNA containing the promoter, leader and variable-region sequences from a mouse antibody gene and the constant-region exons from a human antibody gene[59, 87]. The antibody encoded by such a recombinant gene is a mouse-human chimera. Its antigenic specificity, which is determined by the variable region, is derived from the mouse DNA. The constant regions are encoded by human genes, resulting in fewer mouse antigenic determinants and less immunogenicity than mouse monoclonal antibodies. Another advantage of this approach is that the chimeric antibody retains the biological effector function of the human antibody and is more likely to trigger human complement activation or Fc receptor binding.

Human anti-human antibodies (HAHA) have arisen, but they have occurred with less frequency, e.g. 1% of patients with B cell NHL treated with chimeric anti-CD20 (rituximab) [85], and in some cases appear clinically irrelevant with no

apparent detrimental clearance of the therapeutic antibody from the serum e.g. mAb against crohns disease – inflixamab (anti-TNF-α)

The variable regions of the therapeutic murine mAb are cloned by PCR and inserted into vectors carrying human constant domains[89]. These vectors can be prepared to include both heavy and light chains for tandem expression. Host cells, usually Chinese Hamster Ovary cells, chosen because of their glycosylation pattern (similar to human cells) and adaptability for high-density, large-scale cultivation in serum and protein-free media, are transfected with the chimeric genetic construct. One difficulty has been the reduced ability of the transfectomas to produce large amounts of antibody compared with hybridomas, and antibody secretion is frequently lost. One approach to this problem has been to use the dihydrofolate reductase (DHFR) selection and amplification system as part of the vector to ensure multi-copy integration and much higher antibody expression. An alternative is to transfect the glutamine synthetase gene in to the expression vector. Glutamine is an essential cellular metabolite but its production can be prevented and cell death results by inhibiting the enzyme glutamine synthetase using the specific inhibitor, methionine sulfoximine. CHO-K1 cells express sufficient glutamine synthetase to survive in a glutamine free medium. A transfected GS gene can act as a dominant selectable marker in CHO cells by conferring resistance to concentrations of MSX that are just sufficient to kill both wild-type cells and natural MSX-resistant variants which result from amplification of the endogenous GS genes. Amplification of vector copy number can subsequently be achieved using elevated levels of MSX. This system has been

preferred to the DHFR system because of the higher levels of expression in initial transfectants.

**CD40 as a suitable therapeutic target**

In-vitro, CD40 ligation has been shown to promote normal human B lymphocyte proliferation and induce the secretion of Ig, when cultured in the presence of IL4[44, 45].

The effect of CD40 ligation on malignant cells is less clear. In-vivo tumour models of B cell malignancies have indicated that CD40 ligation reverses immune tolerance of tumour, inducing an effective immune mediated tumour response (growth arrest with or without apoptosis) [90].

It has been demonstrated that CD40 cross-linking by CD40 mAbs presented on Fcγ RII/CDw32-transfected fibroblasts or CD40L transfectants with or without IL-4 induces proliferation of a variety of resting malignant human B lymphocytes, including follicular lymphoma[84], lymphoplasmacytoid lymphoma[91], multiple myeloma[92], hairy cell leukaemia[93], chronic lymphocytic leukaemia[94], acute lymphoblastic leukaemia[95].

In vitro, CD40L expressing transfectants can stimulate malignant human B cells to express high levels of MHC class I and II, costimulatory (CD80, CD86) and adhesion molecules (CD54 and CD58) [84, 91]. The upregulation of these cell suface costimulatory and adhesion molecules makes the cells potentially more immunogenic.

However, growth inhibition rather than proliferation has been observed with some immortalised human B cell, and human epithelial cell lines, following CD40 ligation in vitro[96-98]. Furthermore, murine xenograft models of human B cell lymphomas, derived from transformed cell lines, have responded to treatment with monoclonal anti-CD40 antibodies, suggesting a direct cytotoxic effect of CD40 cross-linking. In fact, CD40 is the only antibody to consistently eradicate CD40-positive B-cell lymphoma ($BCL_1$) in mice, providing long-term remission[99]. The CD40 ligation seems to reverse immune tolerance of tumour, inducing an effective immune mediated tumour response. The protection appears better when a larger tumour burden is present suggesting that a minimum tumour load is required to provide sufficient antigen to 'prime' the activated immune system to respond. Rechallenge of mice, who previously received monoclonal antibody against CD40 to eradicate $BCL_1$, with tumour, in the absence of further monoclonal antibody, results in no evidence of disease. Administration of these anti-CD40 antibodies to murine lymphoma models can eradicate CD40 positive lymphomas and provide partial protection against some CD40 negative ones. The anti-tumour effect depends on a substantial expansion of CD8-positive effector T cells, and is associated with the sustained production of IL-12 and interferon-$\gamma$ suggesting these cytokines might be influential in the response. Since B cells produce IL-12 after CD40 activation, it was suggested that the stimulatory antibodies induced the lymphoma cells to secrete IL-12, which in turn induced cytotoxic T lymphocytes to secrete interferon-$\gamma$. Whereas mice inoculated with $BCL_1$ without anti-CD40 therapy only transiently make interferon-$\gamma$ suggesting a short-lived ineffectual

cellular response against the tumour and irradiated $BCL_1$ cells (i.e. non-proliferating) fail to induce immunity when given to mice with or without anti-CD40.

Interestingly, CD40 is also expressed at high levels on a variety of human carcinomas, including bladder, breast, cervical and ovarian cancers, as well as malignant melanomas[100, 101]. However, little is known regarding the function of CD40 on these neoplastic cells. Knowing that CD40 ligation induces apoptosis in some tumour cell lines[90, 96, 97, 102-104], we might suspect that stimulation of CD40 expressed on carcinomas could alter neoplastic cell growth. In this situation the use of anti-CD40 monoclonal antibodies or human soluble CD40 ligands would be an attractive approach in the treatment of some solid tumours. In addition the normal CD40 expressing cells might be spared from cell death and in fact could become functionally more efficient. However, to date, the cell surface phenotype induced on carcinoma cells following CD40 activation is not fully capable of inducing T cell proliferation ligation. CD54 and MHC molecules are upregulated but not CD80 or CD86[105]. Despite these findings, further effort must be made to improve carcinoma immunogenicity. CD40 ligation might be effective in those solid tumours that are known to be more immunogenic e.g. Malignant melanoma and renal cell carcinoma.

In summary, it is clear that the CD40 fulfils a central role in cellular immunity. A number of groups have used antibodies to CD40 to specifically boost the immune system to produce an anti-tumour response and induce tumour immunity in mice. This has led to enthusiasm regarding the potential role of anti-CD40 and CD40-ligand therapies for the treatment of human malignancies.

**Potential clinical methods for therapy**

Manipulation of the CD40-CD40L interaction may well be a useful way to overcome tumour tolerance. However, the best approach remains uncertain. Stimulatory anti-CD40 or CD40L based therapies could be administered systemically. The clinical effect would depend upon the balance between tumour growth and enhanced immunogenicity. This approach might be an effective method for reversing tolerance and eradicating disease.

Recently, recombinant human CD40 ligand (rhuCD40L) has been used to treat patients with advanced solid tumours or intermediate/high-grade NHL. The treatment was given in a phase I dose escalating study as a subcutaneous injection for 5 days and repeated until disease progression was identified. The purpose of the study was to determine the maximum-tolerated dose and pharmacokinetics of the ligand. Thirty-two patients were treated at 3 dose levels. The MTD was 0.1mg/kg/day because of a dose-dependent, transient elevation of the serum liver transaminases. These were grade 3 or 4 in 14%, 28%, and 57% of patients treated at 0.05, 0.10, and 0.15mg/kg/day respectively. Two patients had a partial response to treatment (one with laryngeal carcinoma and another with NHL). In the patient with laryngeal carcinoma this was sustained and treatment was discontinued at 12 months. Three months later he had achieved a complete response and remained biopsy-proven disease-free at 24 months. Four patients (12.5%) had stable disease sustained for 4 courses of treatment. [106]

Unfortunately, anti-CD40 antibodies and soluble CD40L therapies could affect normal cells expressing the CD40 marker and if a murine derived anti-CD40

antibody was used it might induce human anti-mouse antibodies. However, this problem could be overcome with a humanised or chimeric antibody.

The non-specific reversal of tolerance might induce a number of diseases including autoimmune phenomena[107], multiple sclerosis[26], atherosclerosis[108], thyroiditis, pulmonary fibrosis, alzeihmers disease[109] and prothrombotic states[110].

The administration of anti-CD40L has been shown to benefit a number of disease models of autoimmunity[107, 111]. In addition patients with SLE have an increased expression of CD40L on circulating lymphocytes, and CD40L expressing cells have been demonstrated in their kidney sections[26]. Anti-CD40L has been shown to interrupt disease progression confirming the role of the CD40-CD40L system in the effector phase of this disease.

This relationship between autoimmune disease and the CD40-CD40L system suggests that administration of anti-CD40 or CD40L therapies might result in the elimination of the tumour but induce autoimmune disease. Similarly activated T-helper cells expressing CD40L and CD40 bearing APCs have been detected in sections of brain taken from patients who suffered multiple sclerosis[26]. However, the complex nature of these disease processes makes it difficult to assess how monoclonal antibodies to CD40 and CD40L based therapies might contribute to the development or exacerbation of these diseases.

Alzheimers disease has a substantial inflammatory component, and activated microglia play a central role in neuronal degeneration. It is thought that amyloid-β is involved in the inflammatory component of Alzeihmers disease. Tan et al[109] investigated whether the CD40-CD40L interaction with amyloid-β activates microglial cells. CD40 expression was increased on cultured microglia cells treated with solubilized amyloid-β and on microglia obtained from a transgenic murine model of alzheimers disease. The amyloid-β activated microglia were treated with CD40L resulting in increased tumour necrosis factor α production and neuronal injury. Microglia from a transgenic mouse model of alzeihmers disease deficient for CD40L had reduced activation suggesting the CD40-CD40L interaction is necessary for amyloid-β induced microglial activation. Finally, abnormal Tau phosphorylation was reduced in the transgenic mouse model of alzheimers disease deficient for CD40L, suggesting that the CD40-CD40L interaction is an early event in alzheimers disease pathogenesis. Suggesting that agonistic CD40 mAbs might induce neurodegenerative syndromes.

CD40 and CD40L are present in-vitro on cells derived from human atheroma and in atheromatous lesions in-vivo. Ligation of CD40 on atheroma associated cells, in vitro, activates functions related to atherogenesis including production of chemokines, cytokine, matrix metalloproteinases, adhesion molecules and tissue factor. All are responsible for lesion progression and plaque destabilization.

Treatment with anti-CD40L antibodies has been shown to reduce the development of atherosclerosis and reduce stromelysin-3 expression in

hyperlipidaemic mice[108]. Mice lacking the LDL receptor were fed a high-cholesterol diet for 12 weeks and then treated with antibody-against mouse CD40L. The antibody reduced the size of the aortic atherosclerotic lesions by 59% and their lipid content by 79%. Furthermore, the atheroma of mice treated with anti-CD40L contained fewer macrophages (64%), T lymphocytes (70%), and exhibited decreased expression of vascular cell adhesion-molecule-1. This data supports the involvement of inflammatory pathways in atherosclerosis and indicate a role for CD40 signalling during atherogenesis in hyperlipidaemic mice. Subsequently, Lutgens et al[112] studied the early and late effects to atheroma following administration of anti-CD40L to mice deficient in low-density lipoprotein receptor (ApoE). They found the initial lesion to be unaffected but the later plaque area was significantly reduced. Advanced plaques in CD40L/Apo-protein E (Apo-E) knock out mice had lower lipid levels and were collagen rich with reduced T-lymphocyte/macrophage component. The data suggested CD40-CD40L signalling is important in late atherosclerotic changes such as lipid core formation and plaque destabilisation.

Treatment of tumours with anti-CD40 antibodies or CD40L might induce or exacerbate atherogenesis with adverse cardiovascular and cerebrovascular consequences.

Anti-CD40L antibodies have been shown to prevent pulmonary inflammation and fibrosis occurring as a consequence of oxygen-induced respiratory distress syndrome, and limit thyroiditis. Stimulation of the CD40-CD40L system could

exacerbate underlying pulmonary inflammatory disorders, induce pulmonary fibrosis, and cause thyroiditis.

Platelets express CD40L within seconds of being activated in vitro and in the process of thrombus formation in vivo. They induce endothelial cells to produce chemokines and adhesion molecules capable of initiating an inflammatory response[110]. Leukocytes are extravasated at the site of injury. Consequently, it could be speculated that anti-CD40 or CD40L therapies might induce a prothrombotic state.

CD40-CD40L interactions play a critical role in T cell priming and have been suggested to prohibit tolerance induction. Therefore, interference with CD40 activation has been extensively investigated to prolong the survival of experimentally transplanted organs. Most of this work has been done in murine models. Treatment with anti-CD40 prolongs surviva[40]l. This can be further improved by simultaneously blocking the CD40 and CD28 pathways resulting in long-term acceptance of skin and cardiac allografts. Local expression of CD40L has been found in human renal and heart allografts and seems to correlate with rejection. In the rhesus monkey model of renal transplantation, beneficial effects have been observed following anti-human CD40L administration. Therapy may provide a novel approach to the prevention of human transplant rejection allowing long-term tolerance.

Therefore, at this stage in the development of anti-CD40 mAb and SCD40L therapies, cancer patients who have previously received organ transplants should

probably not be considered for CD40L or anti-CD40 antibody therapies as organ rejection could result.

Other options for circumventing the problem of systemic administration have been explored. These include cell-based vaccines. Malignant B cells could be activated ex-vivo upregulating costimulatory molecules and improving antigen-presenting functions and these cells, after irradiation to inhibit proliferation, used as a cellular vaccine. A number of phase I trials have commenced using this technique to generate activated B cells for a vaccine preparation, one such trial[113] administered the vaccine intravenously to 9 patients with progressive follicular lymphoma who had failed multiple chemotherapy regimens. The follicular cells were isolated 14 days prior to vaccination, activated in-vitro using CD40 ligand transfectants and then cryopreserved. Analysis of the activated follicular cells revealed upregulation of MHC class I and II, ICAM-1, LFA-3, B7-1 and B7-2. They could stimulate T-cells in vitro after culture and cryopreservation. After vaccination no side effects were reported and there was no apparent disease acceleration. One patient showed a clinical response and continues to receive the vaccine. This group are now assessing the use of vaccination strategies in the setting of minimal residual disease and in less heavily pretreated patients who are likely to be more immunocompetent. The use of soluble CD40L to activate malignant B cells would simplify this technique. Alternatively, these tumour APCs could be used to stimulate autologous T-cells ex-vivo for subsequent adoptive transfer of tumour-specific T-cells[114].

**Summary**

The interaction of CD40 with CD40L is central to immune-regulation. Manipulation of this interaction may be an important method of evading tumour tolerance. However, the best approach to this remains unclear.

This project aims to study different methods of activating normal and malignant human B cells, in vitro, via CD40, making them more immunogenic. These properties could be utilised in the development of immunotherapy (antibody therapy, cellular vaccines and adoptive therapy).

I have investigated, in vitro, a number of CD40 expressing human cell lines. The expressed CD40 has been ligated by various methods including human CD40 ligand expressing transfectants, human soluble CD40 ligand, and human Fc receptor transfectants with the addition of different mouse anti-human CD40 monoclonal antibodies.

Using the same cell lines and antibodies I have studied the therapeutic effects of mouse anti-human CD40 mAbs in mouse xenograft models of high-grade human B cell lymphoma.

I have developed a chimeric human anti-CD40 monoclonal antibody and have performed some of the preclinical murine toxicology necessary before the chimeric human anti-CD40 mAb could be introduced in to a phase I trial treating patients with poor prognosis, CD40 expressing, metastatic tumours such as renal cell carcinoma and malignant melanoma. I propose a protocol for a phase I trial using the chimeric human anti-CD40 mAb described above.

# CHAPTER 2

# MATERIALS AND METHODS

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## Culture materials

All human B-cell and human epithelial cell lines were cultured in RPMI 1640 medium (Gibco), supplemented with 100 IU/ml penicillin and streptomycin (Life Technologies), 50 U/ml amphotericin B (Fungizone; Squibb and Sons), 2 mM L-glutamine (Gibco), 1 mM pyruvate (Gibco) and 10% Myoclone Plus Foetal Calf Serum (FCS; Gibco).

## Cell Lines

### Daudi and RL

Daudi (European Collection of Animal Cell Cultures (ECACC, Porton Down) and RL (ATCC) are transformed high-grade human non-Hodgkins lymphoma B cell lines. RL contains the (14; 18) (q32, q21) translocation. The cells were maintained in culture medium described above at 37°C in a 5% $CO_2$ humidified incubator. Media was replenished every 2-3 days. Experimental cells were maintained in log phase of growth for 24 hours prior to experimentation.

### Caski and MG79

Caski and MG79 (donated by Professor Lawrence Young – Birmingham) are 2 human epithelial cell lines, cervical and ovarian carcinomas respectively. The cells were maintained in culture medium described above at 37°C in a 5% $CO_2$

humidified incubator. The adherent cells were trypsinised, harvested and the media replenished every 2-3 days. Experimental cells were maintained in log phase of growth for 24 hours prior to experimentation.

**CD40L transfectants**

Chinese Hamster Ovary cells transfected with human CD40L (Tenovus) were maintained in supplemented RPMI 1640 culture medium described above, with additional selection media, 1mg/ml Geneticin (G418; Gibco) at 37°C in a 5% $CO_2$ humidified incubator. G418 resistance is conferred by the Neomycin gene encoded in the pcDNA3 plasmid, hence transfected cells will survive in the presence of geneticin. To harvest, adherent cells were scraped from the flask and media replenished every 2-3 days. CD40L expression was confirmed prior to experimentation using direct or indirect flow cytometry described below. These cells were used to provide a feeder layer to enable CD40 cross-linking (see below).

**Human Fc expressing (CD32) transfectants**

NIH3T3 cells transfected with CD32 the low affinity human Fc receptor (donated by Jenny Wood – Dundee University) were maintained in supplemented RPMI 1640 culture medium described above, with additional selection media of 2mls Hypoxanthine 100μM, Aminopterin 400nM, Thymidine 16μM (HAT) per 100mls medium, at 37°C in a 5% $CO_2$ humidified incubator. To harvest, adherent cells were scraped to and media replenished every 2-3 days. CD32 expression was confirmed prior to experimentation using direct flow cytometry described below.

Mouse anti-human CD40 monoclonal antibodies were added to the feeder layer enabling CD40 presentation (see below).

The transfected cells were sorted on the FACSvantage (Becton and Dickenson) to ensure high cell surface expression of the appropriate molecule.

**Cell Counts**

Cell concentrations were determined using a Coulter Industrial D Cell counter (Coulter Electronics, Bedfordshire)

**SCD40L**

Human soluble CD40 Ligand was kindly donated by Immunex, USA. It was provided as a purified trimeric human CD40 ligand/leucine-zipper fusion protein (HuCD40LT). The sterile filtered protein was supplied at a concentration of 13.6 mg/ml in a tris/mannitol/sucrose buffer. The recommended in-vitro concentration was 100-3000 ng/ml.

**Antibodies**

Non-commercial antibodies were produced from hybridoma cell lines, expanded as ascites tumours in pristane-primed BALB/c x CBA (F1) mice.

Purification of the mouse and rat monoclonal antibodies was by fractionation, according to the manufacturer instruction, in a protein A or protein G column respectively (Pharmacia Amersham Biotech UK Ltd). Following elution the IgG

purity was assessed by electrophoresis (EP system, Beckman, Palo Alto CA) and dialysed into PBS.

The panel of mouse anti-human anti-CD40 monoclonal antibodies used (LOB 7/2, 7/4, 7/5, 7/6, 7/7, 7/8) were raised by Lyn O'Brien in the Tenovus Laboratory. Rat anti-mouse CD40 (3/23) was provided by Gerry G.B. Klaus (London), rat anti-mouse CD19 (1D3) by Douglas T Fearon (Cambridge), mouse IgG(2a) anti-rat CD2 (OX35) from Oxford. Polyclonal human IgG and human anti-CD38 was produced in Tenovus.

**Feeder layer plates**

Transfected cells were washed in appropriate media, quantified (see above), diluted appropriately (usually to $2.5x10^5$/ml) and irradiated to prevent proliferation using a cobalt source at 50Gy. The cells were plated accordingly onto a 96 well plate (e.g. $2.5x10^4$/well) and onto 6 well plates (2ml/well). The plates were incubated over night at 37°C in a 5% $CO_2$ humidified incubator to allow the cells to adhere. The following morning the supernatant containing was removed and B cells added for co-culture along with any additional antibodies or cytokines necessary for experimentation

**Ex-Vivo human B cells**

**MACS selection**

Ex-vivo human B cells, obtained from the Cancer Sciences Division tumour bank (collected with informed consent from patients undergoing necessary biopsies), were defrosted rapidly and washed in sterile human B cell culture medium

[Iscove's modified dulbecco's medium (IMDM; Sigma-Aldrich), supplemented with 50μg/ml human transferrin (Sigma-Aldrich), 5μg/ml recombinant human insulin (Sigma-Aldrich), 2mM L-glutamine (Gibco), 1mM pyruvate (Gibco), 0.83ml of 10mg/ml gentamicin (Sigma-Aldrich), and 10% human serum from clotted male whole blood (HS; Sigma-Aldrich)]. The cells were resuspended and then layered on to 5ml of Lymphoprep (Nycomed Pharma AS) and centrifuged at 2,500 r.p.m. for 20 minutes. The cells were removed from the interface, washed and 200μl of sterile FITC-labelled anti-CD3, anti-CD8, and anti-CD16 were placed into a bijou and made up to a volume of 1ml with human B cell medium to a final concentration of 10μg/ml. The cell pellet was then resuspended in this mixture and incubated for 10 minutes before undergoing 2 further washes. The final pellet was resuspended in 100μl of human B cell medium containing 10μl of anti-FITC conjugated MACS microbeads (Miltenyi Biotec). The mixture was incubated at 4°C for 20 minutes and the pellet washed twice before being resuspended in 1ml of human B cell medium. The anti-CD19 enriched (B cell rich) cell population was prepared by depleting T cells and NK cells using primed (2mls PBS/EDTA) MACS $LS^+$ separation columns (Miltenyi Biotec) attached to a midiMACS magnet supported by a MACS multi-stand. The cellular suspension was added to the column and the unselected cells, including CD19 positive cells were collected in a sterile tube held beneath the column. The column was washed with 2mls of human B cell medium to ensure that all the human B cells present in the cellular mixture were collected by this process of negative selection. The positively selected cells (including T cells and natural killer cells) were discarded. The B cells were then centrifuged and the pellet resuspended in human B cell medium. Some of these cells were then assessed using direct flow cytometry to determine

cellular numbers, phenotype and viability prior to use in in-vitro proliferation studies.

**In-Vitro proliferation and growth inhibition studies**

The selected ex-vivo human B cells or cells obtained from established cell lines (Daudi, RL, MG79, Caski) were added to sterile flat-bottomed 96 well culture plates containing the appropriate irradiated feeder layer (see before) +/- antibody or human soluble CD40L as required to meet the defined experimental conditions. Recombinant human interleukin-4 (2ng/ml) (R&D systems) was added to ex-vivo human B cells, according to the method of Banchereau[45], to ensure sustained proliferation. The cells were cultured for 3-5 days depending on cell type and then proliferation assessed using the 3HT-thymidine proliferation assay (described below).

To enable cellular analysis after the culture period, 6 well plates, under the same experimental conditions, were simultaneously prepared and the cells washed and assessed using direct flow cytometry after the proliferative period.

**Fluorescein conjugation of antibodies**

Normal saline, 4.5ml, was added to 50mg of antibody in the presence of 1mg, 0.5ml, of the FITC isomer I (BDH) dissolved in 0.5M carbonate buffer pH 9.5 and incubated for 45 minutes at 25°C. Unconjugated FITC was then removed by passage of the reaction mixture through a G-25 Fine (Pharmacia) column, equilibrated with 0.0175M phosphate buffer, pH 6.3, followed by passage through a DEAE-cellulose column.

The majority of the FITC-conjugated antibody was eluted with 0.25 M NaCl in 0.0175 M phosphate as determined by measuring fluorescein:protein ratios during elution (calculated as absorbance at 495nm and 280nm respectively). Material with a ratio of 0.5-0.9 was pooled for use with final antibody concentration calculated as:

Antibody Concentration (mg/ml) = Abs (280nm) - ((0.26 x Abs (495nm)) / 1.45

**Commercially obtained fluorescein conjugated antibodies**

Some FITC-conjugated antibodies were obtained commercially from Serotec UK. These were all mouse anti-human and included anti-CD80 (B7-1), anti-CD86 (B7-2), anti-CD54 (ICAM-1), anti-CD58 (LFA-3), and anti-CD154 (CD40L).

**Measurement of surface antigens by immunofluorescence**

Measurement of surface antigens by immunofluorescence may be performed directly or indirectly. The first method uses a fluorescent labelled antibody specifically directed against the surface antigen to be studied whilst the indirect method uses unlabelled antibody, against the surface antigen, followed by the addition of a fluorescent labelled antibody against the unlabelled antibody.

Cells at $1x10^6$/ml were incubated at $4^{\circ}C$ for 30 minutes with the FITC-labelled (direct) or unlabelled (indirect) antibody of choice (50μg/ml final concentration). Cells were then washed once (direct) or twice (indirect) in PBS-BSA-Azide (PBS, 1% Bovine Serum Albumin fraction V (BSA; Wilfred Smith Ltd, Middlesex), 20mM

$NaN_3$) and resuspended at approximately $1x10^6$/ml. For indirect immunofluorescence, cells were further incubated for 30 minutes at 4°c with an FITC-conjugated secondary antibody directed to the first antibody e.g. sheep anti-mouse-FITC and washed once in PBS-BSA-Azide before resuspension to $1x10^6$/ml and subsequent analysis

Analysis was performed on a FACScaliber flow cytometer (Becton Dickinson, Mountain View, CA, USA). Routinely, 10,000 events were collected per sample for analysis. Cell debris was excluded by adjustment of the forward scatter (FSC) threshold parameter. FITC was excited at 488nm with emission intensity being recorded in the 515-545nm wavelength region. Samples were analysed using Cellquest software (Becton Dickinson). Fluorescence intensities were assessed in comparison to negative control samples and expressed as histograms of fluorescence intensity versus cell number.

**[$^3$H]Thymidine incorporation assays**

Proliferation status of cells was determined by measuring radiolabelled thymidine incorporation. Only replicating S phase cells incorporate (radiolabelled) thymidine. Therefore, dead cells or those in growth arrest will be identified by a decrease in [$^3$H] thymidine incorporation compared to controls. Cells at differing concentrations depending on cell type and the defined experimental conditions, +/- the irradiated feeder layer, were cultured for 3-5 days with various reagents in a final volume of 200μl in flat-bottomed 96 well plates (Nunc). [$^3$H]thymidine was then added (0.5uCi/well; Amersham, Buckingham) for the final 16-20 hours of

culture and the cells then harvested onto glass fibres (Whatman) using an automated cell harvester.

[$^3$H]thymidine incorporation was subsequently determined via liquid scintillation counting of β-emission. All experiments were set up in triplicate, with the arithmetic means being calculated and compared.

**Enzyme-linked immunosorbant assay (ELISA)**

Enzyme-linked Immunsorbant assays were performed to detect for the presence of various proteins. Primary antibody or coating molecule was diluted in coating buffer (15mM $Na_2C0_3$, 28.5mM $NaHC0_3$, pH 9.6) and added 100μl/well to treated 96 well plates (Maxisorb, Nunc) for 1 hour at 37°C then overnight at 4°C. Unbound antibody was removed by flicking and non-specific binding sites were blocked by the addition of blocking solution (1% (w/v) BSA in PBS) for 1 hour at 37°C. The plate was then washed twice with wash solution (PBS + 0.05% Tween-20) and standards and samples to be assayed added to the plate, in a final volume of 100μl/well, with all dilutions being made in blocking solution. Following incubation for 90 minutes at 37°C, the plate was washed again (x5 in wash solution).

An HRP-conjugated antibody, specific for the samples, was then diluted to a working concentration in blocking buffer and added (100μl/well) for a final 90 minutes. Following washing (x5), HRP substrate (20 mg o-PhenylDiamine free base (o-PD), (Sigma poole UK) 100 ml phospho-buffered citrate pH 5.0 + 20μl (60% w/v) $H_20_2$) was added and incubated in the dark at 37°C. The reaction was

terminated by the addition of 50µl/well 5 M $H_2S0_4$ and the subsequent colour change quantified by measurement of absorbance at 495 nm on an automatic fluorometer (Dynatec 400, Dynatec). Colour change was proportional to protein concentration and unknowns were determined using standard calibration curves of known concentration, on the day of each ELISA.

**Antibodies used for the LOB 7/4 ELISA**

Coating antibody – Rabbit anti-mouse IgG 25µg/ml (Tenovus antibody)

HRP-conjugate - Rabbit anti-mouse $Fab_2$ (Sigma Poole UK)

**Antibodies used for the human chimeric LOB 7/4 ELISA**

Coating antibody – Goat anti-human IgG (γ chain specific). (Sigma Poole UK)

HRP-conjugate – Goat anti-human κ (Sigma Poole UK)

**Dialysis**

Dialysis of samples was performed using Visking tubing (Medicell, London). Samples were dialysed 1:1000 in at least 3 changes of phosphate buffered saline for a time of at least 4 hours per change.

**Measurement of protein concentration by Spectrophotometry**

The concentration of each antibody in a solution was determined by spectrophotometry. The solution to be studied was added to a clean 5mm quartz cuvette. A second cuvette containined PBS alone. They were both placed in to the spectrophotometer (Philips-PU 8620 UV/VIS/NIR). The wavelength was set at 280nm, the difference in absorbance of light at 280nm between the diluent alone

and the diluent plus antibody was measured. The concentration of antibody in the solution was calculated as:

Concentration = Absorbance at 280nm ÷ Absorption of a 1mg/ml solution of rat IgG (1.45) [A=Ecd Beer Lambert London]

**Techniques for concentrating protein**

Antibody solutions were concentrated by 2 techniques depending on the volume and degree of concentration required.

**Amicon**

Is a design technique that concentrates large volumes by ultrafiltration through a 10,000 molecular weight cut off filter under nitrogen gas at 4°C.

**The Vivaspin 15 concentrator**

A volume of 2-15mls of the endotoxin free antibody solution was added to the vivaspin 15 concentrator (R&D - Vivascience) containing a 10,000 molecular weight cut off filter. The concentrator was placed in a vessel to collect the filtrate and the assembly centrifuged at 3000xg until the desired antibody concentration was achieved.

**Endotoxins**

**Endotoxin filtration**

Endotoxins (lipopolysaccharides from the membranes of gram-negative bacteria) were removed from monoclonal antibody solutions by filtration through an ion

exchange membrane system (Q15 Sartobind membrane adsorbers$^{TM}$ – Sartorius) reconstituted with 35mls of pyrogen free 1M sodium hydroxide followed by water, as per manufacturers instructions.

**Endotoxin assay**

The endotoxin content in the antibody solutions was quantified using the Limulus Amebocyte Lysate test (LAL – BioWhittaker). LAL prepared from the circulating amebocytes of the horse shoe crab is standardised to detect the labelled concentration (EU/ml) of the FDA reference standard endotoxin. It was reconstituted prior to use with 1.8ml LAL reagent water. The assay was controlled using a standard endotoxin (from E.Coli strain 055:B5), prepared as per manufacturer instruction. One hundred μl of each serially diluted solution to be tested was added using pyrogen free tips to a pyrogen free tube along with 100μl of LAL. The tubes were placed in a water bath at 37$^{°C}$ for 1 hour and then removed and examined for the presence or absence of gelation. A positive reaction was indicated by a firm gel remaining intact momentarily when the tube was inverted 180$^{o}$. The reaction was recorded as positive or negative. The endotoxin concentration was calculated based on the lysate sensitivity multiplied by the endpoint dilution (EU/ml) and then converted to ng/ml. Less than 1ng/ml of endotoxin was considered acceptable.

**Mouse strains used for therapeutic and toxicological assessment**

All mice were kept in the animal facilities department of Tenovus laboratories under strict regulation by the Home Office department.

**Severe combined immunodeficient mice (SCIDS)**

These mice (Harlan, UK) are homozygous for the autosomal recessive mutation that results in severe combined immunodeficiency. They are deficient in both B and T lymphocytes and generally show lymphopaenia, agammaglobulinaemia, tolerance of foreign tissues and tumours and a high susceptibility to infections of bacteria, viruses, and other opportunistic micro-organisms. Natural killer cells, macrophages and granulocytes are present in normal numbers and appear to function normally. They are bred and maintained in isolators or filter-topped cages and handled under laminar flow hoods.

The mice were injected intraperitoneally (ip) or intravenously (iv) with appropriate amounts of tumour (routinely $5x10^6$/mouse in 200μl PBS of Daudi, RL, Caski and MG79) on day 0 and then received 100μg of the experimental antibody in 200μl of PBS on day 7 via the same route. Untreated and isotype matched control groups were included in each experiment and results documented in terms of survival. Mice were observed twice daily and culled if tumour progression occurred in association with pain or distress.

**BALB/c**

These albino mice (Harlan, UK) are an inbred strain widely used for many scientific disciplines. They have a life-span of around 500-600 days.

**Toxicology studies**

The BALB/c mice were injected IP, on day 0, with varying concentrations of the endotoxin free mouse anti-CD40 antibody (3/23) under investigation. Age-

matched, PBS alone, and isotype matched (1D3-anti-CD19) control groups were also included. According to CRC guidelines 6 male mice were included per group and the mice culled at day 14 (see below)

**Biochemical and Haematological Analysis of blood specimens**

BALB/C mice treated with ACD40 monoclonal antibody (3/23) were culled at day 14 by anaesthetising in halothane followed by rebreathing of carbon dioxide (2L/min). A terminal cardiac bleed was performed to obtain approximately 1ml of blood per mouse. Blood from identically treated animals was pooled to enable adequate samples for analysis in the biochemical and haematological laboratories, Southampton General Hospital. Full blood count, liver function tests, Urea, creatinine and electrolyte results were obtained for each treated group of mice.

**Post-mortem examination**

After culling the mice underwent post-mortem examination largely through a cruciate thoraco-abdominal incision. Any macroscopic abnormalities were documented and the organs (including brain, lungs, heart, liver, spleen, kidneys, bowel, testis, femur, paw) were preserved in formalin and sent for an expert histopathological/toxicological assessment by Dr Luann McKinney at The European Centre for Toxicologic Pathology, The Royal Veterinary College, London.

Following advice trans-tracheal inflation of the lungs was performed with formalin, 1ml, to prevent tissue artefacts that might be detrimental to histological

examination. In addition the skull was split and gently pried from the cortices to allow formalin to penetrate the brain tissue without disturbing the overall anatomy.

Special histochemical stains of 1-micron sections were performed if renal lesions were present that indicated hyper or autoimmune glomerulopathy and likewise for brain sections suggesting demyelination.

**Chimeric human anti-CD40 production**

**DNA fragmentation assessed by Agarose gel electrophoresis**

Following incubation of cells under appropriate conditions, samples containing $1x10^6$ cells were harvested, transferred to 1.5ml sterile, plastic tubes and resuspended in no more than 10μl of supernatant. 20μl of TE-SLS (10mM EDTA, 50 mM Tris Hcl, pH 8.0, 0.5% (v/v) sodium lauryl sarkosinate (SLS, Sigma)) plus 0.5mg/ml Proteinase K (Sigma) was then added, and the samples incubated for 1 hour at 50°C. 10μl of 0.5mg/ml RNase A (Sigma) was added and samples were incubated for a further 1 hour at 50°C. The temperature of the samples was then increased to 70°C for addition of 10μl of loading buffer (10 mM EDTA, 1% (w/v) low melting point agarose, 0.25% (w/v) bromophenol blue, 40% (w/v) sucrose). Samples were loaded into dry wells of 1% (w/v) agarose gel containing 0.5μg/ml ethidium bromide and run either overnight at 30-40 V or for 2-3 hours at 80 V, in TAE (89mM Tris, 89mM boric acid, 2mM EDTA, pH 8.4).

DNA was visualised by UV light.

**Isolation of mRNA**

A single monoclonal antibody secreting hybridoma cell colony was selected and expanded in DMEM (Gibco) supplemented with 100U/ml penicillin (Glaxo), 100ug/ml streptomycin (Evans), 50U/ml amphotericin B (Fungizone; Squibb and Sons), 2mM L-glutamine (Gibco), 1 mM pyruvate (Gibco) and 10% Myoclone Plus Foetal Calf Serum (FCS; Gibco) to obtain $1x10^7$ cells.

The mRNA was isolated using a Quickprep Micro mRNA purification kit (Pharmacia). It is designed for the direct isolation of polyadenylated RNA from eukaryotic cells or tissues, bypassing the need for intermediate purification of total RNA.

$1x10^7$ cells were extracted in a buffered solution containing a high concentration of guanidine thiocyanate (GTC) and N-laurosyl sarcosine, ensuring the rapid inactivation of endogenous Rnases. The extract was then diluted with buffer, 10mM Tris-HCL [pH 7.5], 1mM EDTA, to reduce the GTC concentration and precipitate a number of proteins. The extract was clarified by a short centrifugation, and the supernatant transferred to a microcentrifuge tube containing oligo(dT) cellulose (25mg/ml). After several minutes, during which the poly$(A)^+$ RNA binds to the oligo(dT)-cellulose, the tube was centrifuged at high speed for 10 seconds, and the supernatant pipetted or aspirated off the pelleted oligo(dT)-cellulose. The pelleted material was washed with buffers of various salt concentrations to remove impurities. Purified m RNA is then eluted from the oligo(dT) cellulose with pre-warmed TE buffer (Pharmacia)

**First-strand cDNA synthesis**

The first strand cDNA was synthesised using RNAse-free water (treated with diethyl pyrocarbonate [DEPC]) added to the precipitated mRNA to make a volume of 20μl. The RNA solution was heated to 65°C for 10 minutes and then chilled on ice. The bulk first-strand cDNA reaction mix containing moloney murine leukaemia virus (M-MuLV) reverse transcriptase was gently pipetted to obtain a uniform suspension, and 11μl was added to a sterile 0.5ml microcentrifuge tube along with 1μl of DTT solution (200mM), 1μl of random hexamer primer (100ng/ml) and the heat denatured RNA. The mixture was incubated at 37°C for 1 hour and then used in the polymerase chain reaction to amplify the DNA.

**PCR**

DNA was amplified by polymerase chain reaction (PCR). 100ng of cDNA was mixed with 100ng of primers and 1U of Tag /pfu polymerase in the presence of excess dNTP. PCR reactions were carried out using an AMS "The Protocol" thermal cycler using standard procedure of 30 cycles of 30 second, annealing at 55°C for 1 minute and elongation at 72°C for 2 min, followed by a final 30 minute elongation step to ensure formation of full length transcripts. PCR products were then electrophoresed in 0.7% agarose gel containing ethidium bromide, along with known size standards to allow estimation of fragment size. DNA was visualised under UV light and a photograph record taken.

**Gel Extraction and purification of DNA**

The visualised bands of DNA were excised from the gel and the QIAEX II gel extraction kit (Qiagen) was used to extract and purify the excised DNA. As described in the manufacturers protocol 100ng of purified PCR product (DNA) was used to ligate with 10ng PCR-blunt II-TOPO vector (Invitrogen) for 5minutes at room temperature. The ligation mixture was then mixed with 100μl chemically recombinant E.Coli TOP10 (Invitrogen) and incubated on ice for 30 minutes, followed by a heat shock at 42°C for 30 seconds. The mixture was then immediately put on ice for 2 minutes and 250μl SOC medium was added. It was shaken for 1 hour before being plated on to agar plates containing 50μg/ml kanamycin and left in the 37°C incubator over night. Colonies from the TOPO-transformations were picked and grown in 10ml LB medium containing 50μg/ml kanamycin at 37°C over night. Plasmid DNA was purified from the cultures using QIAprep spin miniprep kit (Qiagen) and subjected to restriction enzyme digests for 2-24 hours. The digested DNA was then analysed in a 0.7% agarose gel containing ethidium bromide.

**Maxiprep**

TOPO-plasmids containing PCR products as inserts were then sequenced using BigDye sequencing reaction kit with T7 and Sp6 primers. Sequencing reactions were run in ABT automated sequencing system and resulting sequences were analysed using DNAstar program.

**Gene-PORTER transfection technique**

One day prior to transfection the CHO cells were harvested by addition of Trypsin/EDTA (10mMTrypsin/0.5mM EDTA) for 5 minutes, at 37°C in the absence of growth media. The cells were then seeded into a 6 well plate at 1 x $10^6$ cells/well in GMEM-S + 10% FCS and incubated until semi-confluent.

On the day of transfection, 2μg of DNA was diluted in 500μl serum free media. The 10μl GenePORTER reagent (Gene Therapy systems) was diluted with 500μl of serum free-medium. The diluted DNA was then added to the diluted GenePORTER reagent and mixed rapidly before incubating at room temperature for 30 minutes. The culture medium was aspirated from the cells and the DNA-GenePORTER reagent mixture was carefully added before incubating for 5 hours at 37°C in a 5-10% $CO_2$ incubator. Subsequently, 1 ml of growth media with 20% FCS was added and the cells incubated overnight. Twenty-four hours post-transfection fresh complete media (10% FCS) was added as required. Seventy-two hours post-transfection the cells were harvested with trypsin/EDTA (as above) and resuspended in 30ml 10% dialysed FCS GMEM-S containing 25-50μM MSX. The cells were plated on to a 96 well plate, the medium changed every 3-4 days as required. Non-transfected cells died within 2 weeks. Antibody production was detected with ELISA 2-3 weeks post-transfection.

# CHAPTER 3

# RESULTS

# GROWTH INHIBITION

## CHAPTER 3

## GROWTH INHIBITION

The ligation of CD40, on transformed human B cell lines, using chinese hamster ovary cells transfected to express human CD40L and human soluble CD40L resulted in significant cellular growth inhibition ($p<0.001$). Ligation of CD40 on human epithelial cell lines (MG79 and Caski) using human soluble CD40L has also produced significant growth inhibition ($p<0.001$).

### Introduction

CD40 is present on a variety of cells including both normal and malignant human B-lymphocytes, dendritic cells, monocytes, reticular endothelial cells, keratinocytes and fibroblasts. The ligand for CD40 (CD40L) is expressed predominantly on activated T cells and the interaction of CD40 with its ligand is critical for normal B lymphocyte differentiation and development[115]. CD40 ligation induces class-switching in B cells, activates dendritic cells to become more effective antigen presenting cells, by upregulating MHC, costimulatory and adhesion molecules, and facilitates CD8+ cytotoxic T-lymphocyte responses[41-43]. In-vitro, CD40 ligation has been shown to promote normal human B lymphocyte proliferation and induce the secretion of Ig, when cultured in the presence of IL4[44, 45].

The effect of CD40 ligation on malignant cells is less clear. In-vivo tumour models of B cell malignancies have indicated that CD40 ligation reverses immune tolerance of tumour, inducing an effective immune mediated tumour response (growth arrest with or without apoptosis) [90].

It has been demonstrated that CD40 cross-linking by CD40 mAbs presented on Fcγ RII/CDw32-transfected fibroblasts or CD40L transfectants with or without IL-4 induces proliferation of a variety of resting malignant human B lymphocytes, including follicular lymphoma[84], lymphoplasmacytoid lymphoma[91], multiple myeloma[92], hairy cell leukaemia[93], chronic lymphocytic leukaemia[94], acute lymphoblastic leukaemia[95].

In vitro, CD40L expressing transfectants can stimulate malignant human B cells to express high levels of MHC class I and II, costimulatory (CD80, CD86) and adhesion molecules (CD54 and CD58) [84, 91]. The upregulation of these cell suface costimulatory and adhesion molecules makes the cells potentially more immunogenic.

However, growth inhibition rather than proliferation has been observed with some immortalised human B cell, and human epithelial cell lines, following CD40 ligation in vitro[90, 96, 97]. Furthermore, murine xenograft models of human B cell lymphomas, derived from transformed cell lines, have responded to treatment with monoclonal anti-CD40 antibodies, suggesting a direct cytotoxic effect of CD40 cross-linking.

Interestingly, CD40 is also expressed at high levels on a variety of human carcinomas, including bladder, breast, cervical and ovarian cancers, as well as malignant melanomas [100]. However, little is known regarding the function of CD40 on these neoplastic cells. Knowing that CD40 ligation induces apoptosis in some tumour cell lines, we might suspect that stimulation of CD40 expressed on carcinomas could alter neoplastic cell growth. In this situation the use of anti-

CD40 monoclonal antibodies or human soluble CD40 ligands would be an attractive approach in the treatment of some solid tumours. In addition the normal CD40 expressing cells might be spared from cell death and in fact could become functionally more efficient. This treatment might be most effective in those solid tumours that are known to be more immunogenic e.g. Malignant melanoma and renal cell carcinoma.

**Materials and Methods**

The proliferation status of all cell lines was determined by measuring the incorporation of radiolabelled thymidine, as described in the materials and methods chapter page 67. The cell lines were cultured for 3 days at a concentration of $2x10^4$/well on a 96 well plate with SCD40L or irradiated feeder layers expressing either human CD40L (TCHO) or human CD32 with additional monoclonal anti-CD40 antibody all in a final volume of 200μl. The [$^3$H] thymidine was added for the final 16-20 hours of culture.

## Results

Daudi and RL are cell lines originally obtained from patients with transformed diffuse large cell lymphomas of B-cell origin. RL contains the t(14;18)(q32;q21) translocation. Both human B cell lines express CD20 and CD19. Examination by direct flow cytometric analysis confirmed the presence of the CD40 molecule.

**Figures 15+16 pg 92**

**CD40 expression of RL and daudi cell lines shown using direct FACS analysis (anti-human CD40-FITC)**

The cell surface phenotype of both cell lines was revealed using direct Facs analysis.

**Figure 17 pg 92**

**A table illustrating the surface phenotype assessed by direct flow cytometric analysis in the B lymphocyte cell lines RL and Daudi**

Incubation of RL and Daudi cells, for 3 days, with an irradiated feeder layer of CHO cells, transfected to express human CD40L (TCHO -1.25x$10^4$/well), significantly inhibited ($p<0.001$) the proliferation of the B lymphocytes with a reduction in thymidine incorporation of 58% (83-25%) and 52% (87-35%) respectively, when compared with the same cells incubated with untransfected CHO cells (CHO -1.25x$10^4$/well).

**Figure 18+19 pg 93**

**Growth inhibition of RL and Daudi cells ($2x10^4$/well) after a 3-day incubation period with varying concentrations of CHO cells expressing the human CD40L. The results are compared with the same cells similarly incubated with CHO cells that did not express the human CD40L.**

Three-day incubation of RL and Daudi cells with varying concentrations of human soluble CD40L provided the most significant growth inhibition in the RL system. The largest reduction in thymidine incorporation was 59% (100-41%) and the degree of growth inhibition was greatest with the highest experimental concentration used (10μg/ml). In the Daudi cells the thymidine incorporation was maximally reduced by 28% (100-72%) at a concentration of 0.3125 μg/ml. Growth inhibition of the RL and Daudi cells was significant ($p<0.001$) at a concentration of 5μg/ml.

**Figures 20+21 pg 94**

**Two figures illustrating the differences in growth inhibition obtained when RL and Daudi cell lines ($2x10^4$/well) were incubated for 3 days with increasing concentrations of human SCD40L.**

An alternative method of studying the effects of signalling via CD40 is to use an irradiated feeder layer of mouse fibroblasts transfected to express the low affinity human Fc receptor on the cell surface (Fcγ RII/CDw32). This feeder layer could then be used to present monoclonal anti-CD40 antibody to the B-lymphocytes.

This sytem could be used to test mouse anti-human CD40 monoclonal antibodies produced from hybridoma cells in the Tenovus laboratory.

**Figure 22** **pg 95**

**The cell surface expression of CDw32 assessed by single fluorescence, direct FACS analysis of mouse fibroblasts transfected to express the human Fc receptor (Fcγ RII/CDw32).**

No significant growth inhibition was identified when anti-CD40 antibodies (1.5μg/ml) were incubated alone with both RL and daudi cells ($2x10^4$/well). However, in both systems there was a trend to reduced proliferation with LOB 7/4. This trend to reduced proliferation was not significant.

**Figure 23+24** **pg 96**

**Anti-CD40 monoclonal antibodies (1.5μg/ml) incubated alone with both RL ($2x10^4$/well) and daudi cells ($2x10^4$/well) do not cause significant growth inhibition. However, in both the RL and daudi systems there was a trend to reduced proliferation with lob 7/4.**

When anti-CD40 antibodies (6μg/ml) were presented on an irradiated (75Gy) feeder layer of fibroblasts expressing the low affinity Fc receptor (CD32) and cultured for 3 days with either RL or daudi cells the results were variable and therefore difficult to interpret. When LOB 7/4 was used in the RL system the results just achieved significance (mean 325692 v 206059; p=0.05).

**Figures 25+26** **pg 97**

**The effect of anti-CD40 antibodies (6μg/ml) on proliferation of RL and daudi cells ($2x10^4$/well) when presented on an irradiated (50Gy) feeder layer of fibroblasts expressing CDw32.**

Caski and MG79 are CD40 expressing cervical and ovarian carcinoma cell lines respectively. Examination of both cell lines using flow cytometric analysis confirmed the presence of surface CD40 expression as well as class I and II MHC molecules.

Both cell lines strongly adhere to plastic culture plates making it difficult to accurately assess the proliferative effects of an adherent CD40L expressing irradiated feeder layer or anti-CD40 antibody presented on an adherent, irradiated feeder layer i.e a cross-linking system. A solution to this problem would be to mix these cells in suspension having first irradiated and washed the TCHO to remove all traces of geneticin, both cell types could then adhere to the well base. However, an alternative is to use human SCD40L. I added the epithelial cells to a 96 well plate at a concentration of $2x10^4$/well and cultured with varying concentrations of human SCD40L. There was significant inhibition of cell growth in both MG79 and Caski cells ($p<0.001$) at 1.25 and 0.625mcg/ml respectively.

**Figure 27+28** **pg 98**

**The effects of increasing concentrations of human soluble CD40L on the proliferation of MG79 and Caski cell lines ($2x10^4$/well).**

The proliferation of MG79 cells was reduced by 57-70% with concentrations of SCD40L ranging from 0.078 and 1.25μg/ml.

**Summary**

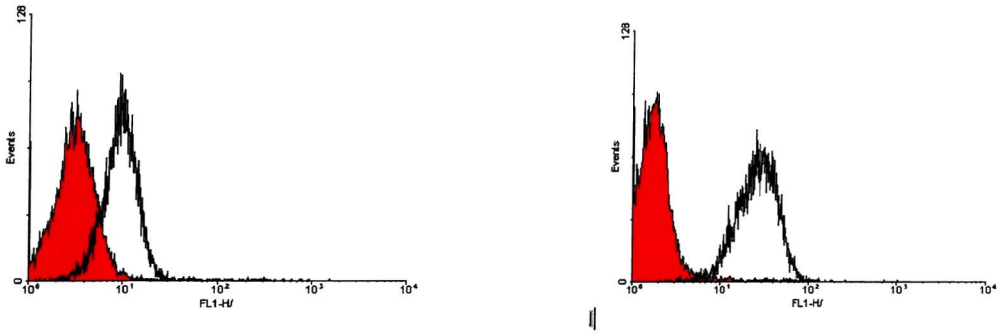
Ligation of CD40, with TCHO or human SCD40L, causes growth inhibition in both RL and Daudi cell lines, however, particularly in Daudi cells, the degree of inhibition was augmented in a system that included a feeder cell layer transfected with the human CD40L rather than the purified trimeric soluble CD40L. It is possible that the feeder cell layer provides a better cross-linking system than the SCD40L. Thymidine incorporation was maximally reduced in the RL cells, using the TCHO system, to 25% compared with 41% in the system using SCD40L. In the Daudi system thymidine incorporation was more impressively reduced after stimulation via TCHO to 35% compared with 72% with SCD40L.

No significant growth inhibition could be identified when these cell lines were incubated with either monoclonal mouse anti-human CD40 (LOB 7/2, 7/6, 7/8) antibodies alone or presented by CDw32 expressed on the cell surface of transfected mouse fibroblasts. When LOB 7/4 was presented by the low affinity human Fc receptor expressed by a feeder layer of transfected NIH3T3 cells significant growth inhibition was just achieved.

Significant growth inhibition was achieved when both MG79 and Caski cell lines were incubated with human soluble CD40L.

**Figure 15+16**
The cell surface expression of human CD40 on 2 transformed human B cell lymphoma cell lines (RL and daudi) shown using direct FACS analysis

**RL** **DAUDI**




FITC-anti-human CD40 (LOB 7/4) IgG1
FITC-anti-saporin IgG1

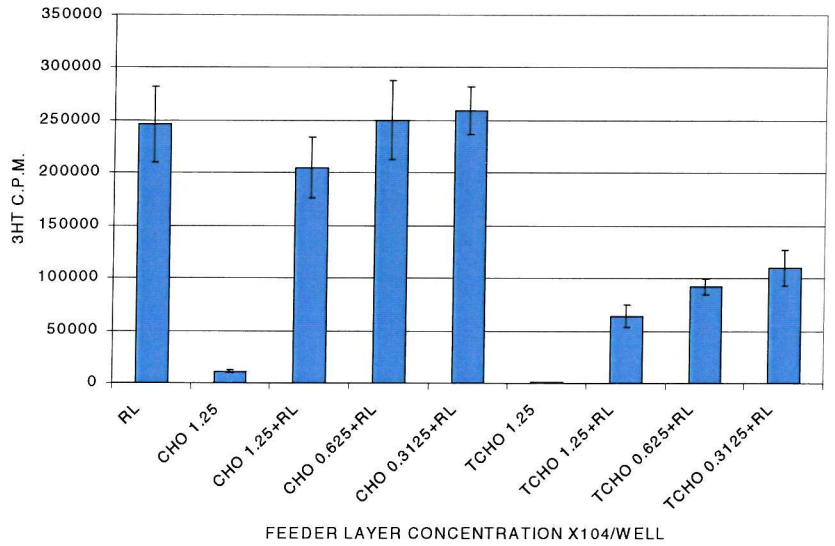
**Figure 17**
A Table to illustrate the surface phenotype of the same RL and Daudi cell lines. This was again shown using the direct FACS analysis method.

| **CELL SURFACE MARKER** | **RL** | **DAUDI** |
|---|---|---|
| **CD20** | ++ | ++ |
| **CD19** | + | + |
| **CD40** | ++ | ++ |
| **CLASS I** | + | - |
| **CLASS II** | +++ | +++ |
| **KAPPA** | + | ++ |
| **LAMBDA** | ++ | - |
| **MU** | ++ | ++ |

**Key - Mean fluorescent intensity**
$10^1$-$10^2$ +
$10^2$-$10^3$ ++
$10^3$-$10^4$ +++

**Figure 18+19**
Growth inhibition of RL and Daudi cells ($2x10^4$/well) after a 3-day incubation period with varying concentrations of CHO cells expressing the human CD40L. The results are compared with the same cells similarly incubated with CHO cells that did not express the human CD40L.

**RL**




**Daudi**

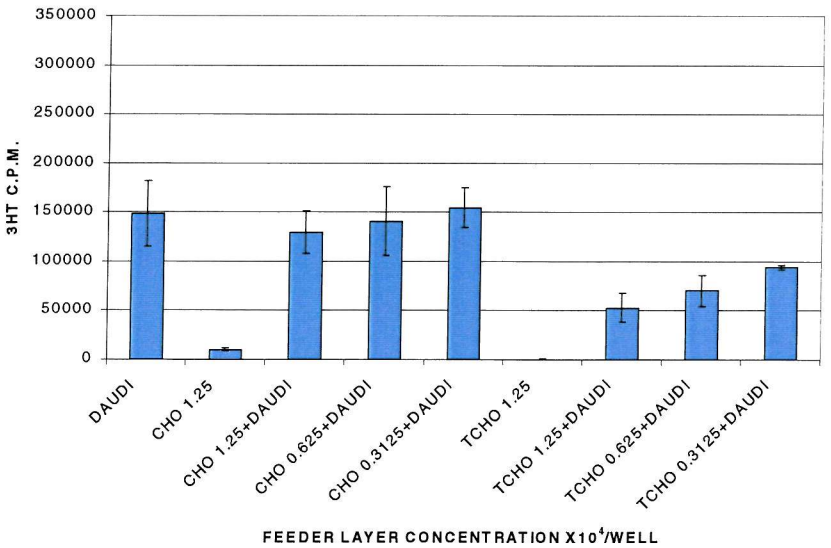
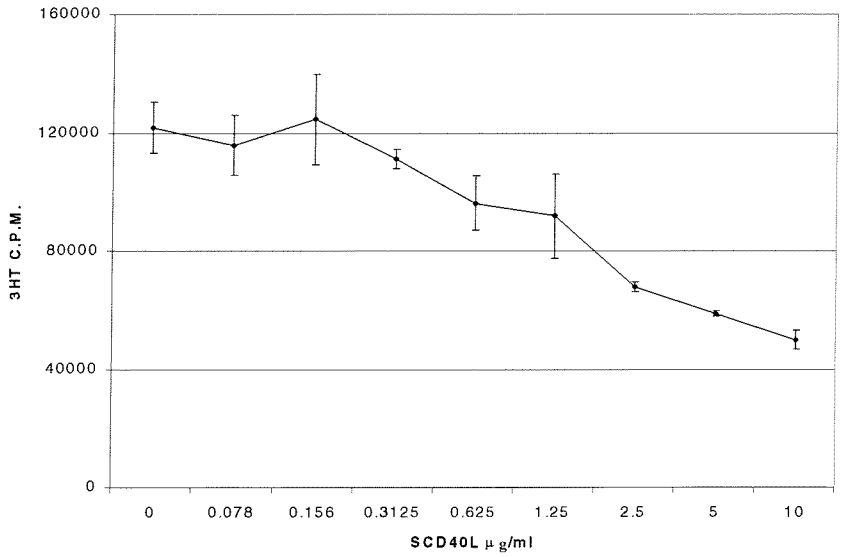


**Figure 20+21**
Two figures illustrating the differences in growth inhibition obtained when RL and Daudi cell lines ($2x10^4$/well) were incubated for 3 days with increasing concentrations of human SCD40L.

**RL**




**Daudi**

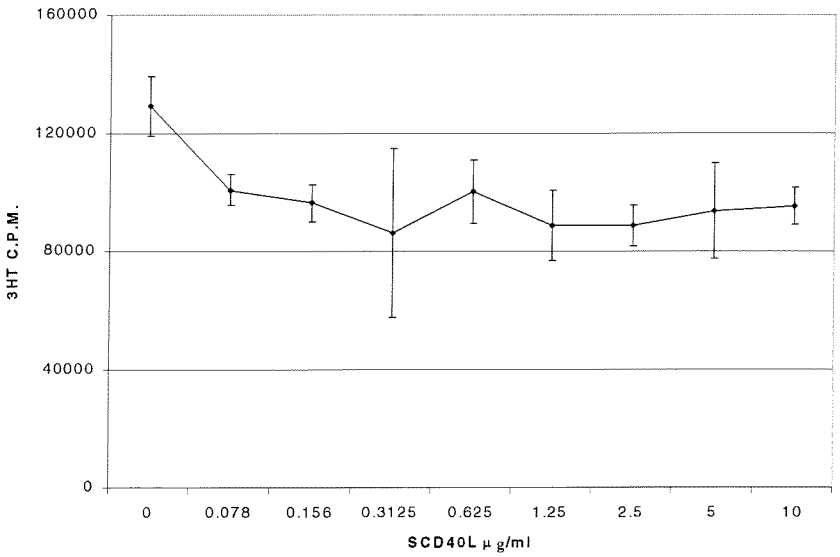
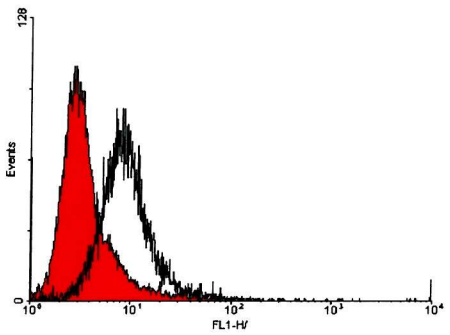


**Figure 22**
Single fluorescence, direct FACS analysis of mouse fibroblasts transfected to express the human Fc receptor (Fcγ RII/CDw32)

This represents the cell surface expression of CDw32.

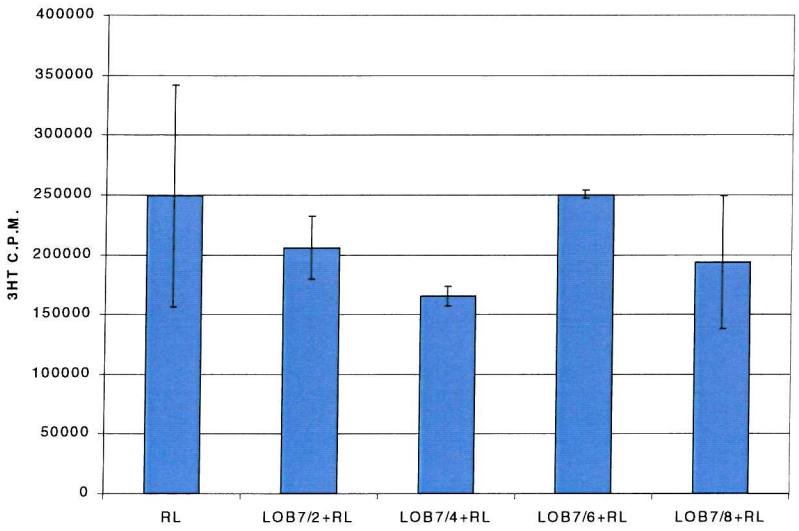



| | |
|---|---|
| FITC-anti-saporin | IgG1 |
| FITC-anti-CD32/$Fab_2$ (AT10) | IgG1 |

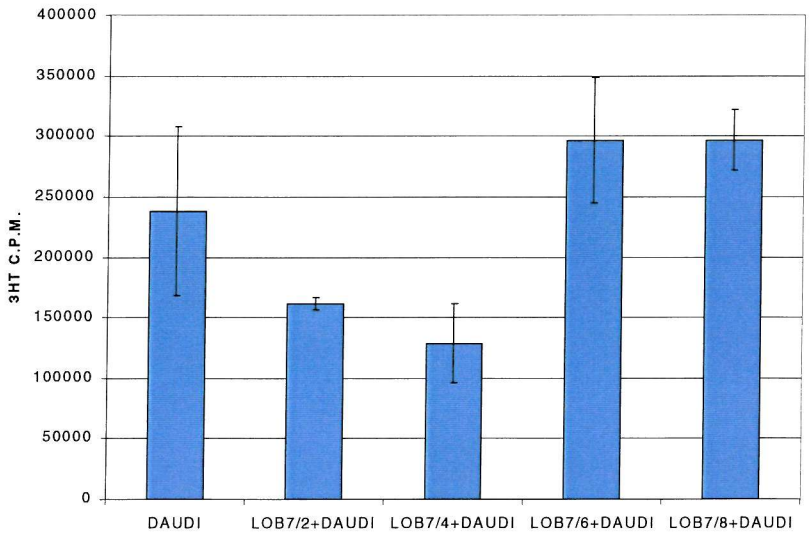
**Figures 23+24**

Two graphs to illustrate that anti-CD40 monoclonal antibodies (1.5 µg/ml) incubated alone with both RL ($2x10^4$/well) and daudi cells ($2x10^4$/well) do not cause significant growth inhibition. However, in both the RL and daudi systems there was a trend to reduced proliferation with lob 7/4.

**RL**



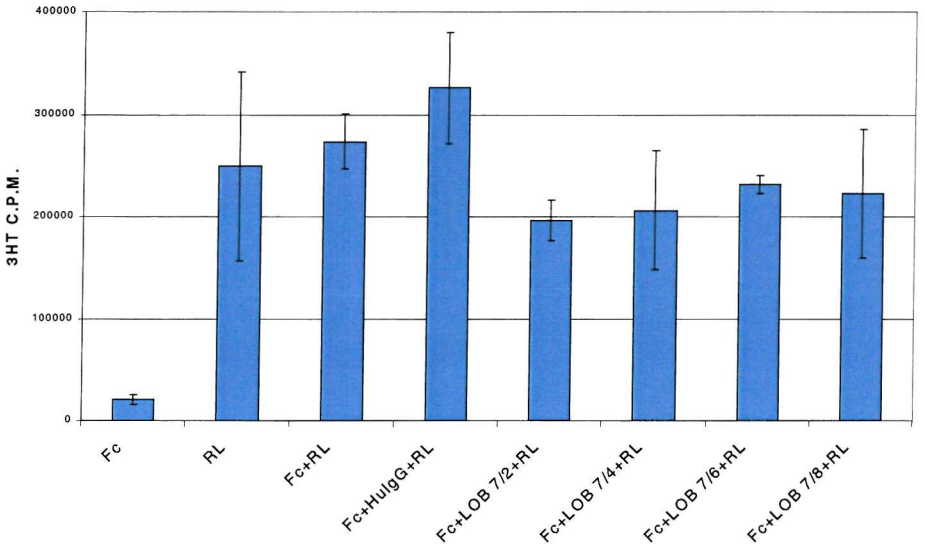

**Daudi**



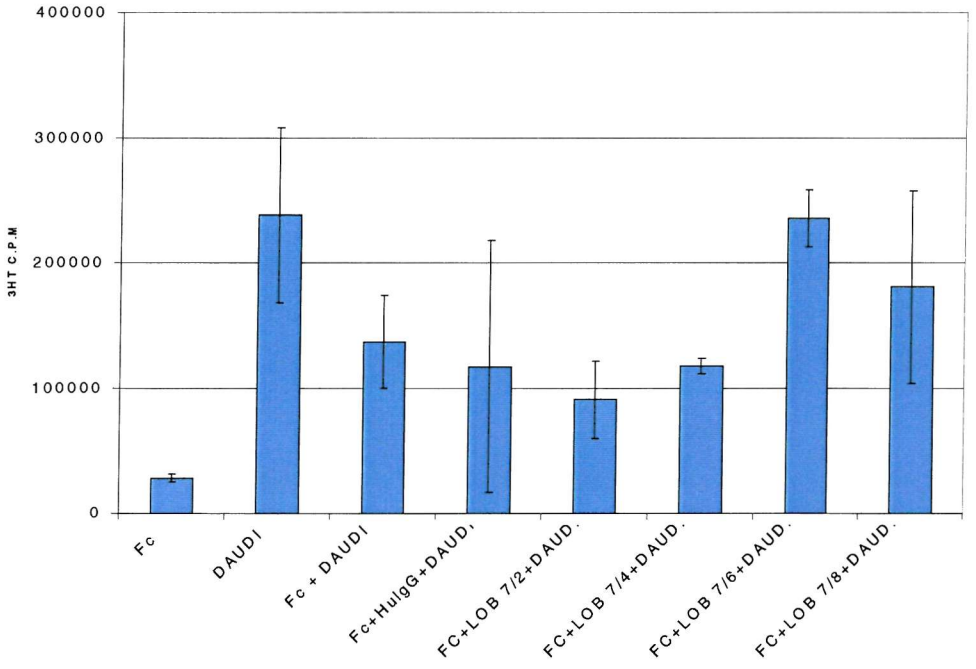
**Figures 25+26**

Figures representing the effects of anti-CD40 antibodies (6µg/ml) on proliferation of RL and daudi cells ($2x10^4$/well) when presented on an irradiated (50Gy) feeder layer of fibroblasts expressing CDw32.

**RL**

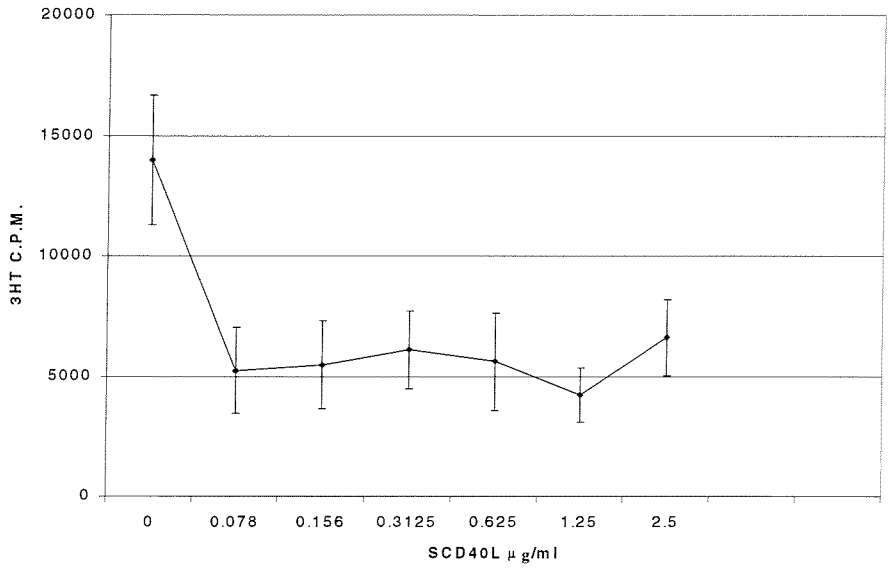



**Daudi**

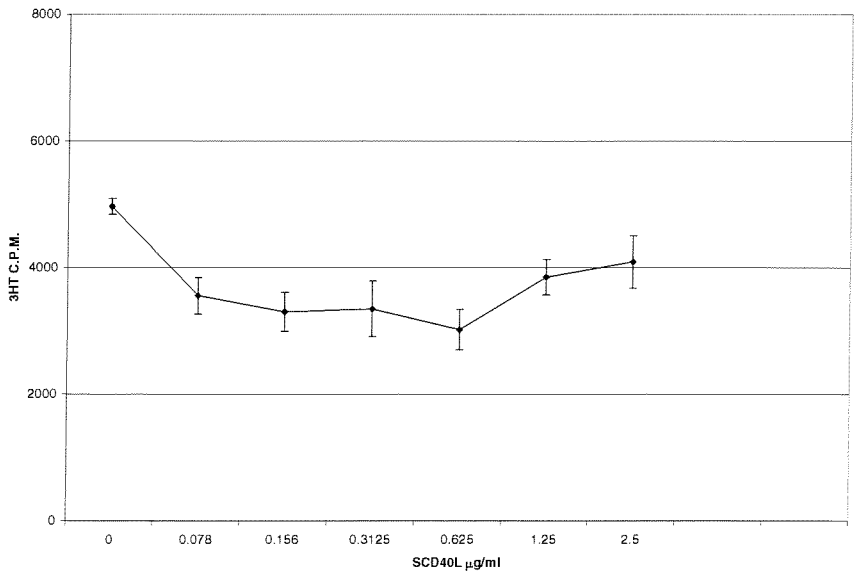


**Figures 27+28**
The effects of increasing concentrations of human soluble CD40L on the proliferation of MG79 and Caski cell lines ($2x10^4$/well).

**MG79**




**CASKI**



# CHAPTER 4

# RESULTS

# EX-VIVO HUMAN B CELL VIABILITY, PROLIFERATION AND CELL SURFACE MOLECULE UPREGULATION

## CHAPTER 4

## EX-VIVO HUMAN B CELL VIABILITY, PROLIFERATION AND CELL SURFACE MOLECULE UPREGULATION

In human primary B cell non-Hodgkin's lymphomas ligation of CD40 with both CD40L expressing CHO cells and human soluble CD40L in the presence of human IL4 has caused significant cellular proliferation ($p<0.001$) and induced upregulation of cell surface and costimulatory molecules including CD80, CD86, CD58, CD54.

### Introduction

Normal human B-lymphocytes are derived in foetal liver and subsequently bone marrow[22]. They mature into cells that carry membrane-bound surface immunoglobulin which act as antigen receptors, the B cell receptor (BCR). The surface immunoglobulin is associated with two transmembrane molecules (CD79a and CD79b) that transduce activation signals to the B cell. Resting B cells migrate throughout the secondary lymphoid tissues to sites of trapped antigen. B cells bearing immunoglobulin specific for antigen enter the T-cell rich paracortical regions below the cortex, of lymphoid tissues, where they capture and process the antigen[23]. Activated T cells induce antigen bearing B cells to migrate in to B cell follicles. In the follicle B cells interact with follicular dendritic cells to form a germinal centre. The B cells with high-affinity antigen receptors are 'selected', and switch from producing IgM to another immunoglobulin class and ultimately become plasma cells, terminally differentiated B cells. Plasma cells are entirely devoted to the production of secreted antibody. This is a carefully controlled

process that relies on interaction with antigen, T cells, accessory cells, and cytokines. The CD40 antigen is important in the regulation of this process[24-26].

In-vitro ligation of CD40 expressed by normal human B cells leads to activation, proliferation, isotype switching, upregulation of cell surface molecules and expression of cytokines[25, 34, 116]. CD40 cross-linking by anti-CD40 antibodies presented by an irradiated feeder layer of Fcγ RII/CDw32 cells induces long-lasting proliferation of normal, ex-vivo, human tonsillar B cells[45, 83]. This proliferation can be enhanced in the presence of IL-4 and IFN-γ. Furthermore, IL-4 induces the CD40-activated B cells to produce IgE, and combinations of IL-4 and IL-2 result in the production of large amounts of IgM and IgA.

Primary malignant human B cells also proliferate when CD40 is ligated by CD40 ligand expressing transfectants[84]. These B cells show increased expression of cell surface costimulatory molecules (CD80, CD86) and adhesion molecules (CD54, CD58) making them efficient antigen-presenting cells.

**Materials and methods**

As described in the materials and methods chapter page 67, ex-vivo human B cells were obtained, with informed consent, at either lymph node excision biopsy or following splenectomy. A cell suspension was formed and the vials of cells were stored in liquid nitrogen. When required the cells were rapidly defrosted, washed and suspended in human B cell medium before undergoing negative MACS selection to ensure a pure population for use in proliferation studies. Viability was confirmed using propidium iodide (PI). The cells were cultured under

defined experimental conditions appropriate for each particular experiment. B cell viability was again assessed after culture.

The proliferation status of all human B cells was determined by measuring the incorporation of radiolabelled thymidine. The human B cells with additional human IL4 (final concentration 2ng/ml) were cultured for 5 days at a concentration of $1x10^5$/well on a 96 well plate with human SCD40L or irradiated feeder layers expressing the human CD40L (TCHO) or human CD32 with additional monoclonal anti-CD40 antibody all in a final volume of 200μl. The [$^3$H] thymidine was added for the final 16-20 hours of culture.

B cells were also incubated for 5 days under similar experimental conditions on a 6 well plate with TCHO. These cells were then studied for evidence of cell surface co-stimulatory and adhesion molecule up-regulation using flow cytometric analysis.

**Results**

Cell viability was confirmed in 50-80% of the MACS selected B cell populations using propidium iodide (10μcg/ml). Ten μl of the PI solution was added to 100μl of the MACS selected primary human B cells. The presence of viable B cells was confirmed using direct FACS analysis.

**Figure 29 pg 109**

**Three examples of B cell viability assessed using propidium iodide (10μcg/ml). Ten μi of the PI solution was added to 100μl of MACS selected primary human B cells, including normal human B cells, B cells from a patient with splenic marginal zone NHL and B cells from a patient with follicle centre cell NHL.**

The surface phenotype of all MACS selected ex-vivo human B cells was confirmed using direct FITC-labelled anti-CD20, anti-CD19, anti-CD40, anti-κ, anti-λ, anti-class I, anti-class II in flow cytometric analysis. The successful removal of T-cells was confirmed using anti-CD3-FITC.

**Figure 30**

**A table to show the cell surface phenotype of a variety of MACS selected primary human B cells**

| Cell surface marker | Normal B cells (splenic) | SMZ NHL | FCC NHL |
|---|---|---|---|
| CD3 | - | - | - |
| CD20 | ++ | + | ++ |
| CD19 | + | + | + |
| CD40 | + | + | + |
| IgG κ | - | + | + |
| IgG λ | - | - | - |
| HLA class I | ++ | ++ | ++ |
| HLA class II | ++ | ++ | ++ |

**Key - Mean fluorescent intensity**

$10^1$-$10^2$ +

$10^2$-$10^3$ ++

$10^3$-$10^4$ +++

Normal and malignant B cells ($1x10^5$/well), in the presence of human IL4 (2ng/ml), were shown to proliferate when incubated on a 96 well plate for 5 days with irradiated (75Gy) CHO cells ($2x10^4$/well) expressing the human CD40L (TCHO). The degree of proliferation, for both normal and malignant cells, was significant when compared with the same cells incubated with CHO cells that did not express human CD40L (CHO).

P value Normal means 125421v1448; $p \leq 0.01$

Maligant means 14065v1072; $p < 0.001$

The cell numbers used in these experiments (B cells [$1x10^5$/well] and CHO/TCHO [$2x10^4$/well]) were chosen to be in keeping with similar experimental work performed by other groups[45].

**Figure 31+32 pg 110**

**Macs selected ex-vivo normal and malignant human B cells ($1x10^5$/well), in the presence of human IL4 2ng/ml, proliferate significantly when cultured on a 96 well plate for 5 days with an irradiated (75gy) feeder layer of CHO cells transfected to express the human CD40L (TCHO).**

Subsequent titration of the irradiated TCHO feeder layer, on a 96 well plate, established that malignant human B cells (FCC NHL - $1x10^5$/well) proliferated significantly (mean 14065v1072; $p<0.001$) after a 5 day incubation with a concentration of TCHO cells of $1.25x10^4$ - $6x10^3$/well. This data was reproducible.

**Figure 33 pg 112**

**A graph to show the effect of different TCHO feeder layer concentrations on the proliferation of ex-vivo FCC NHL B cells.**

The different proliferative abilities of B cells obtained from patients with a range of normal and malignant B cells were compared by calculating their individual proliferation indices when incubated with irradiated TCHO cells and IL4 using the same experimental design as described above. Each system was appropriately controlled by culturing the same B cells alone and with a control feeder layer that did not express human CD40L (CHO). The concentration of feeder layer used was $2x10^4$/well in keeping with work by other groups who have found this

concentration of feeder layer to be optimal for the proliferation of $1x10^5$ B cells per well on a 96 well plate. These results were compared with the same human B cells cultured with human SCD40L 1.25μcg/ml. The results have been expressed as proliferation indices.

**Figure 34 pg 113**

**The proliferation indices of 4 different types of malignant human B cells and 2 normal types ($1x10^5$/well) after 5-day culture on a 96 well plate with either CHO cells transfected to express the human CD40L ($1.25x10^4$/well) or human soluble CD40L (1.25μcg/ml). All in the presence of human IL4 2ng/ml**

Normal (splenic) and malignant human B cells (splenic marginal zone NHL) were incubated for 5 days with increasing concentrations of human soluble CD40L (immunex). The cells proliferated and the degree of proliferation was highly significant ($p<0.001$) from concentrations $\geq$0.156mcg/ml.

**Figure 35 + 36 pg 114**

**Two graphs illustrating the proliferation of normal and malignant human B cells ($1x10^5$/well) cultured for 5 days with increasing concentrations of human SCD40L and IL4 2ng/ml.**

There was no evidence of significant proliferation when malignant human B cells (FCC NHL) were incubated with a panel of anti-CD40 antibodies produced in the Tenovus laboratory.

When malignant human B cells (FCC NHL - $1x10^5$ /well) were incubated with IL4 (2ng/ml) and the panel of anti-CD40 antibodies, presented on an irradiated (75Gy) feeder layer of NIH3T3 cells transfected to express CD32 ($2x10^4$/well), uptake of tritiated thymidine was higher than that achieved when the B cells were incubated alone. However, the B cells proliferated equally as well when simply incubated with the irradiated, transfected NIH3T3 cells in the absence of any anti-CD40 antibody. This effect was not blocked by the addition of polyclonal human IgG. The results were no different when other human B cells were cultured in the same system.

**Figure 37 pg 114**

**$1x10^5$ human FCC NHL cells/well were incubated with IL4 2ng/ml and the panel of anti-CD40 antibodies, presented on an irradiated (75Gy) feeder layer of NIH3T3 cells transfected to express CD32 ($2x10^4$/well).**

After the 5-day incubation period with TCHO the viability of the ex-vivo human B cells could be reassessed using propidium iodide and flow cytometric analysis as discussed previously. From this data it was discovered that 60-70% of the total cell population of cultured cells remained viable.

**Figure 38 pg 115**

**Three examples of human B cell viability after a 5-day incubation period with TCHO and IL4 (2ng/ml)**

The cell surface phenotype was analysed after the incubation period to confirm that the population of proliferated cells were malignant B cells i.e. CD20, CD19 positive and kappa or lambda light chain restricted. FITC-anti-CD3 was used to ensure that no T-cells had proliferated to contaminate the sample.

**Figure 39**

**A table to show the cell surface phenotype after the 5 day incubation period with TCHO and IL4 (2ng/ml)**

| Cell surface markers | Normal B cells (splenic) | SMZ NHL | FCC NHL |
|---|---|---|---|
| CD3 | - | - | - |
| CD20 | + | +++ | +++ |
| CD19 | + | + | + |
| CD40 | + | + | ++ |
| IgG κ | - | + | _ |
| IgG λ | - | - | + |
| HLA class I | ++ | ++ | +++ |
| HLA class II | + | ++ | +++ |

Evidence of cell surface co-stimulatory and adhesion molecule up-regulation could be found in the cultured malignant B cells. This data was generated from the cells obtained from cases of follicular NHL and splenic marginal zone NHL incubated for 5 days with TCHO.

**Figure 40** **pg 116**

**Flow cytometric data illustrating the up-regulation of some cell surface co-stimulatory and adhesion markers, including CD58, CD54, CD80, CD86, on B cells obtained from a patient with SMZ NHL and FCC NHL and cultured for 5 days with TCHO and IL4 (2ng/ml).**

**Summary**

Normal and malignant B cells were obtained from ex-vivo human B cells stored as cell suspensions in liquid nitrogen. These cells were rapidly defrosted, resuspended and negatively selected using a MACS separation technique to obtain a purified population of human B cells. Their cell surface CD40 was ligated, in the presence of human IL4, with either CHO cells transfected to express cell surface human CD40L or human soluble CD40L, and resulted in B cell proliferation.

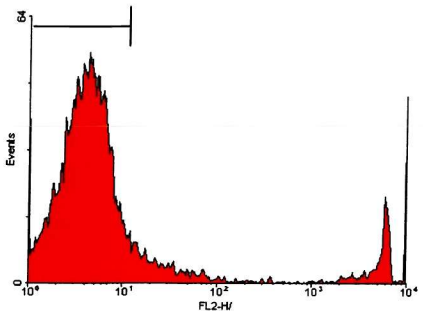
Human B cell proliferation was not shown when irradiated NIH3T3 cells transfected to express the low affinity human Fc receptor, in the presence of human IL4, were used to present anti-CD40 antibodies. The technique appeared to be an unsatisfactory method for testing the effects of our anti-CD40 monoclonal antibodies on, ex-vivo, human B cell proliferation.

After a 5-day culture period with TCHO the human B cells were shown to be viable and their cell surface phenotype had changed. CD58, CD54, CD80 and CD86 were all upregulated in both cases of NHL (splenic marginal zone, and follicle centre cell).

**Figure 29**
Examples of cell viability post MACS selection but pre-culture

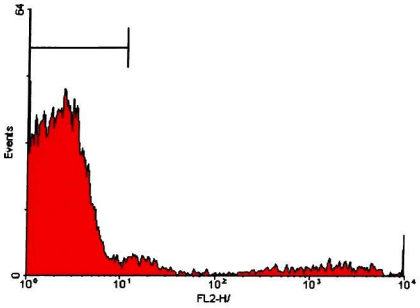
**Splenic marginal zone NHL**

**80% viable**




**Follicle centre cell NHL**

**90% viable**




**Normal splenic B cells**

**65% viable**

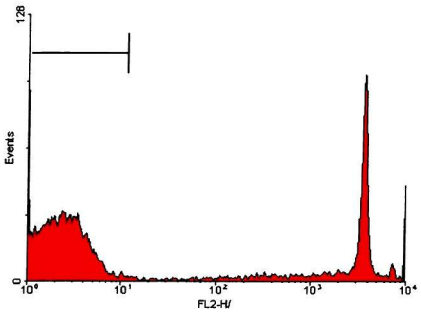
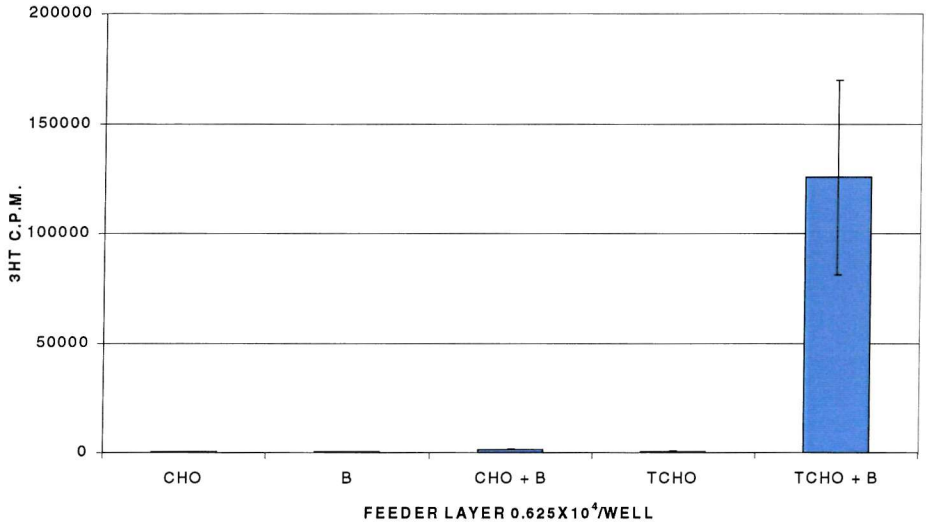


**Figure 31+32**
Macs selected ex-vivo normal and malignant human B cells ($1x10^5$/well), in the presence of human IL4 2ng/ml, proliferate significantly when cultured on a 96 well plate for 5 days with an irradiated (75gy) feeder layer of CHO cells transfected to express the human CD40L (TCHO).

**Normal**




**Malignant**

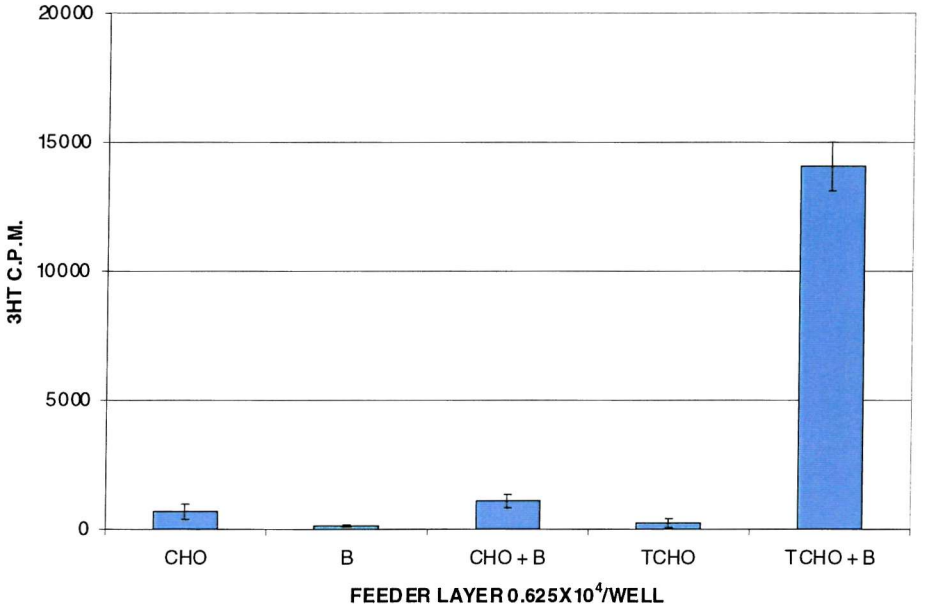


**Figure 33**
A graph to show the effect of different TCHO feeder layer concentrations on the proliferation of ex-vivo FCC NHL B cells.

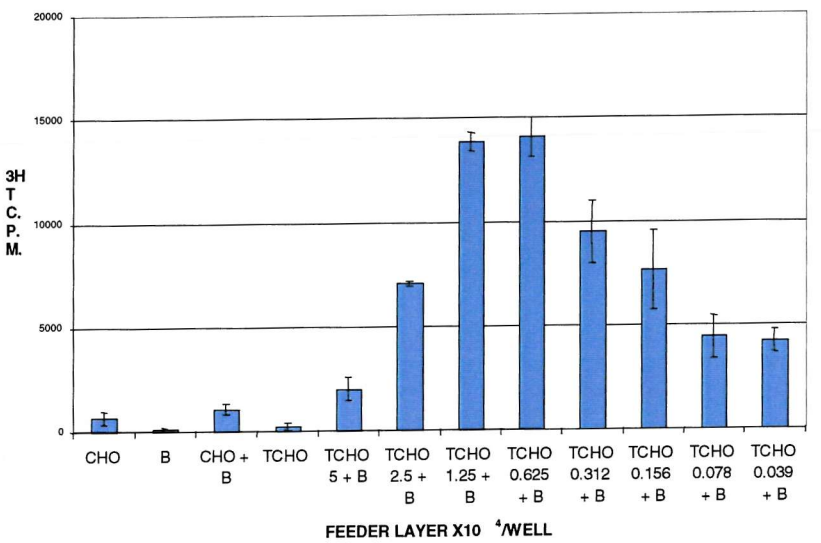



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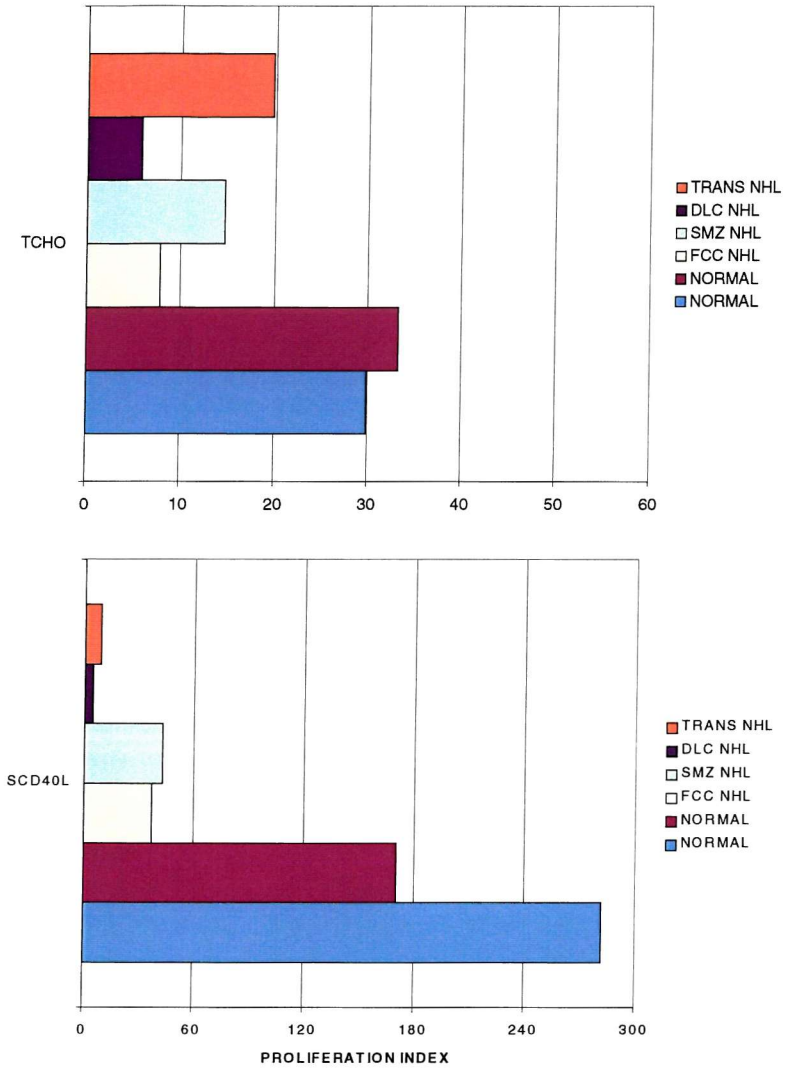
**Figure 34**
The proliferation indices of 4 different types of malignant human B cells and 2 normal types ($1x10^5$/well) after 5-day culture on a 96 well plate with either CHO cells transfected to express the human CD40L ($1.25x10^4$/well) or human soluble CD40L (1.25µcg/ml). All in the presence of human IL4 2ng/ml

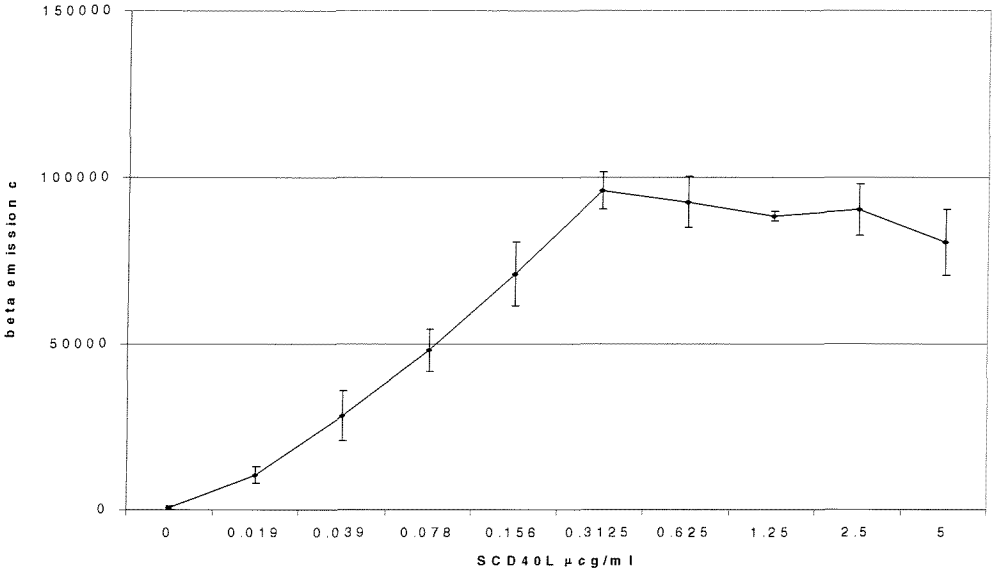



Each bar represents the mean proliferation index of 3 experiments

| | | |
|---|---|---|
| **NORMAL** | Normal splenic B cells | |
| **FCC NHL** | Follicle centre cell | non-Hodgkins lymphoma |
| **SMZ NHL** | Splenic Marginal Zone | |
| **TRANS NHL** | Transformed low-grade | |
| **DLC NHL** | Diffuse large cell | |

**Figures 35+36**
Two graphs illustrating the proliferation of normal and malignant human B cells ($1x10^5$/well) cultured for 5 days with increasing concentrations of human SCD40L and IL4 2ng/ml.

**Normal**




**Malignant**

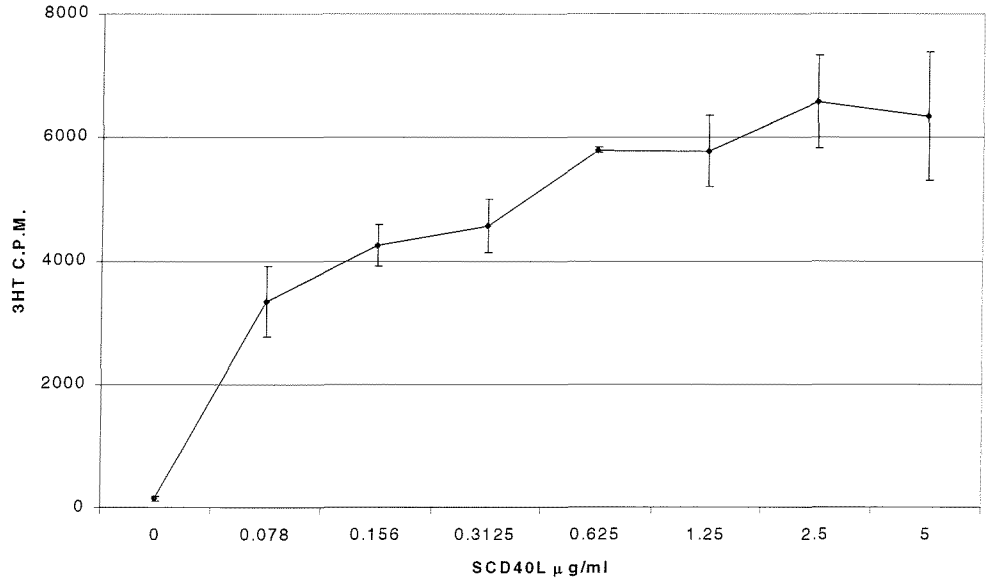


**Figure 37**

1x$10^5$ human FCC NHL cells/well were incubated with IL4 2ng/ml and the panel of anti-CD40 antibodies, presented on an irradiated (75Gy) feeder layer of NIH3T3 cells transfected to express CD32 (2x$10^4$/well).

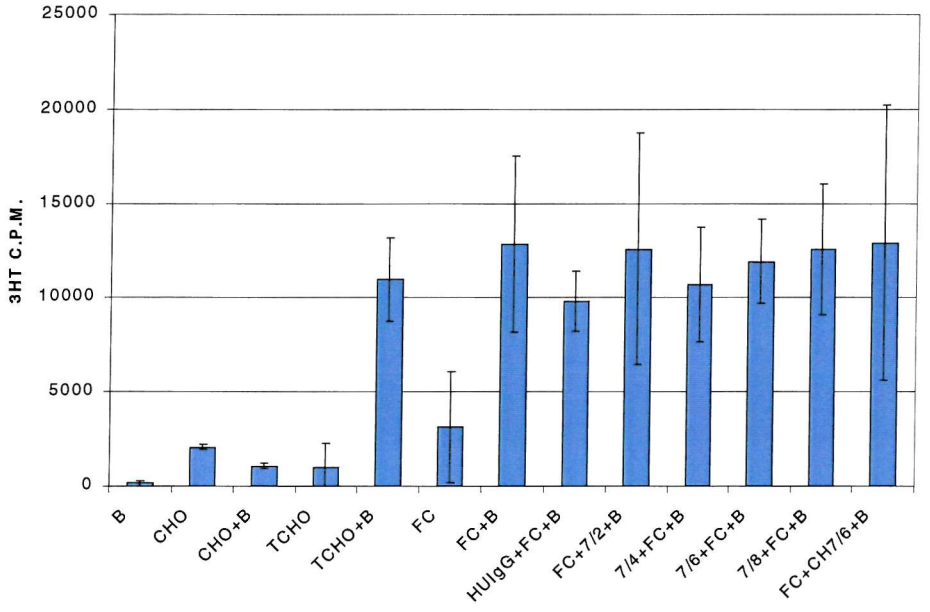
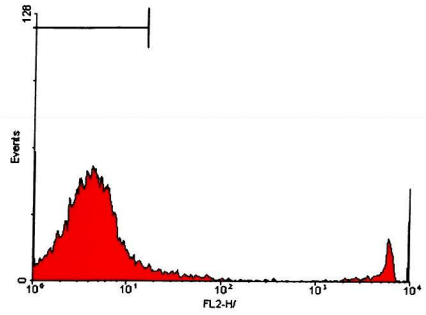


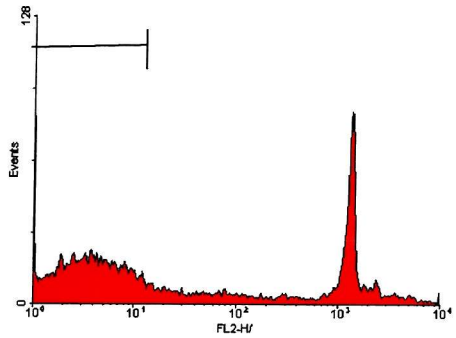
**Figure 38**
Cell viability after a 5-day incubation period with TCHO and IL4 (2ng/ml)

**Splenic marginal zone NHL**



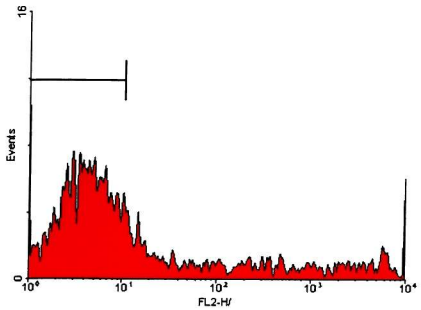

**70% viable**

**Follicle centre cell NHL**




**60% viable**

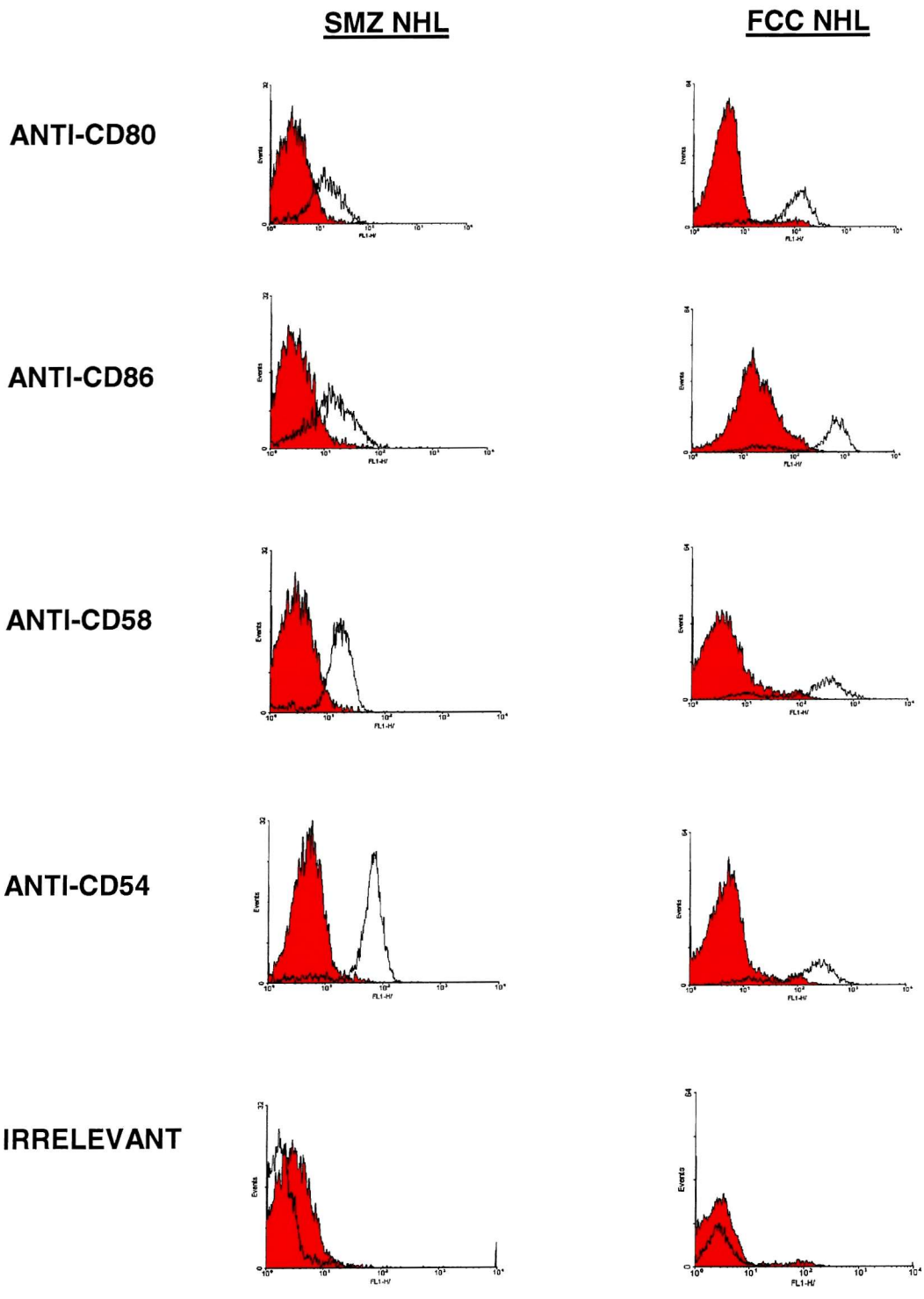
**Normal splenic B cells**




**60% viable**

**Figure 39**
Flow cytometric data illustrating the up-regulation of some cell surface co-stimulatory and adhesion markers on B cells obtained from a patient with SMZ NHL and FCC NHL.

CD58, CD54, CD80, CD86 all upregulated.



# CHAPTER 5

# RESULTS

## PRODUCTION OF A CHIMERIC HUMAN MONOCLONAL ANTIBODY

## CHAPTER 5

## PRODUCTION OF A CHIMERIC HUMAN MONOCLONAL ANTIBODY

This chapter details the development of a new chimeric human anti-CD40 antibody containing human constant regions and mouse variable regions. This antibody is currently undergoing large scale production for a proposed trial to treat patients with CD40 expressing tumours (excluding low-grade non-Hodgkin's lymphoma) who have failed conventional therapies.

### Introduction

Murine-derived monoclonal antibodies have been used to treat a number of human illnesses with variable success. Two main difficulties have been described[87]. Firstly, although murine mAbs have been found to be specific for their therapeutic targets, they do not have the same ability to trigger human biological effector functions, such as complement and human Fc receptors. Secondly, treatment of humans has resulted in an immune response with the production of human anti-mouse antibodies (HAMA). These reduce the therapeutic effectiveness of the monoclonal antibody by rapidly clearing the mAb from the blood. In addition re-treatment carries a risk of life-threatening immune reactions (serum sickness). Human monoclonal antibodies would be a more appropriate treatment approach against human disease but these have been technically difficult to produce[28]. Research has therefore been directed into producing engineered monoclonal antibodies, suitable for human therapy, using recombinant DNA technology. [88]

One approach has been to clone recombinant DNA containing the promoter, leader and variable-region sequences from a mouse antibody gene and the constant-region exons from a human antibody gene[59, 87]. The antibody encoded by such a recombinant gene is a mouse-human chimera. Its antigenic specificity, which is determined by the variable region, is derived from the mouse DNA. The constant regions are encoded by human genes, resulting in fewer mouse antigenic determinants and less immunogenicity than mouse monoclonal antibodies. Another advantage of this approach is that the chimeric antibody retains the biological effector function of the human antibody and is more likely to trigger human complement activation or Fc receptor binding.

Human anti-human antibodies (HAHA) have arisen, but they have occurred with less frequency, e.g. 1% of patients with B cell NHL treated with chimeric anti-CD20 (rituximab) [85], and in some cases appear clinically irrelevant with no apparent detrimental clearance of the therapeutic antibody from the serum e.g. The mAb used to treat Crohns disease – inflixamab (anti-TNF-$\alpha$)

In our laboratory a chimeric anti-CD40 antibody (chimeric LOB 7/6) was successfully produced by Dr Claude Chan. He chose to chimerise LOB 7/6 from a panel of anti-CD40 antibodies because the hybridoma cell line secreting LOB 7/6 was the best antibody producer when compared with the other hybridomas secreting anti-CD40 antibodies. The antibody was successfully chimerised and the expression cassette transfected into CHO-K1 cells. Initial chimeric human LOB 7/6 production appeared promising but unfortunately this was not sustained and production failed.

Since this time some of the growth inhibition work using the high grade human B cell line (RL) has suggested that LOB 7/4 might be might be more potent in terms of signalling via CD40 than LOB 7/6.

The panel of LOB anti-CD40 antibodies were also sent to Jan Fisher and Chris Tretter at the Dartmouth-Hitchcock medical center, New Hampshire, USA. This group kindly tested our anti-CD40 antibodies in a system using elutriated monocytes obtained from a normal donor and cultured at a density of $1.5x10^6$/ml in T-165 flasks containing serum free defined media. GMCSF 10ng/ml and IL4 20ng/ml were both added to the flasks. Fresh cytokine was subsequently added on days 3 and 6. On day 6 TNF$\alpha$ 50ng/ml was added to all but one flask (GMCSF/IL4 control flask) and anti-CD40 antibodies were added to all but the control flask at concentrations of 1 and 10ng/ml. The panel of anti-CD40 antibodies were compared with a known agonistic mouse anti-human CD40 antibody (s2c6) obtained from The Randy Noelle Laboratory. After 9 days of culture the dendritic cell phenotype was assessed by flow cytometric analysis.

The results were tabulated and expressed as a percentage of dendritic cells positive for a selected antibody.

**A table to show the dendritic cell phenotype after 9 day culture with a variety of antibodies (Jan Fisher and Chris Tretter at the Dartmouth-Hitchcock medical center, New Hampshire, USA. Personal Communication)**

| Staining Ab-FITC | Flask 1 % | Flask 2 % | Flask 3 % |
|---|---|---|---|
| **MIgG1** | 1.90 | 1.10 | 1.30 |
| **Anti-CD14** | 19.3 | 1.10 | 1.50 |
| **Anti-CD54** | 98.4 | 99.7 | 99.6 |
| **Anti-CD1a** | 6.30 | 4.80 | 5.80 |
| **Anti-HLA-DR** | 84.3 | 98.0 | 98.8 |
| **HLA-ABC** | 99.9 | 99.8 | 99.6 |
| **Anti-CD40** | 98.5 | 95.6 | 74.8 |
| **Anti-CD80** | 53.4 | 91.9 | 91.0 |
| **Anti-CD86** | 59.7 | 99.1 | 98.4 |
| **Anti-CD83** | 8.20 | 87.1 | 89.8 |

**Flask 1** – 10ng/ml GMCSF + 20ng/ml IL4
**Flask 2** – GMCSF/IL4 + day 6 50ng/ml TNFα and anti-CD40 (s2c6) 1μg/ml
**Flask 3** – GMCSF/IL4 + day 6 50ng/ml TNFα and anti-CD40 LOB 7/4 1μg/ml

The majority of dendritic cells (>90%) cultured with LOB 7/4 were up-regulated to express the costimulatory molecules CD80 and CD86. These results were similar to those achieved with the control anti-CD40 antibody s2c6. Increasing the concentration of anti-CD40 in the culture system to 10μg/ml did not significantly influence the results. On the basis of this data LOB 7/4 was chosen for attempted chimerisation. Successful production of this chimeric human anti-CD40 antibody would enable the necessary preclinical in-vitro and toxicology work to commence prior to development of an appropriate protocol for a phase I trial of this antibody in the treatment of CD40 expressing human solid tumours.

## The production of chimeric human anti-CD40 monoclonal antibody

### mRNA preparation

A hybridoma cell colony secreting mouse anti-human CD40 (LOB 7/4) was selected and expanded to obtain $1x10^7$ cells as described in the materials and methods chapter. The mRNA was then purified using the Quickprep micro-mRNA kit (Pharmacia; St Albans, Herts). This system utilises an oligo(dT) matrix which selectively binds the poly(A) tail of mRNA.

### cDNA preparation

cDNA was prepared from mRNA obtained from the above method, using the First strand cDNA synthesis system (Pharmacia; St Albans, Herts), under the manufacturers guidelines.

### DNA amplification

It was necessary to first identify the leader and frame-work 4 sequences of both the heavy and light chains of LOB 7/4 so that subsequent cloning could be achieved. The variable regions of both heavy (Vγ) and light chains (Vκ) of the mouse anti-human CD40 (LOB 7/4) were amplified using a family of Vγ and Vκ primers in a polymerase chain reaction (PCR) using Taq polymerase (Promega) and the cDNA was prepared as a template. Twelve heavy and 11 light chain 5' primers were used all of which included the restriction enzyme site Sal1 (GTCGA) and the initiation codon (ATG). The 5' primers contained sequences of the whole family of heavy and light chains. Of the 12 heavy chain 5' primers MHV-7

identified the leader sequence of the heavy chain. The reverse (3') primer used for the heavy chain was MCγ1, it binds to the hinge region of the heavy chain and enables identification of the frame-work 4 sequence. The leader sequences of the light chains were identified with 2 of the 11 5' primers MKV-2 and MKV-4. The reverse (3') primer used for both was MκCR, it binds to the amino acid terminal end of the constant region of the kappa chain.

Preliminary amplification using the above primer pairs yielded amplified PCR bands of 420-450 b.p. when the primer MHV-7 was used for the heavy chain and primers MKV-2 and MKV-4 were used for the light chain.

**MHV-7:**

5' ACTAGTCGACATGG (A/G) ATGGAGC (T/G) GGA (A/T) CTTT (A/C) TCTT 3'

**MKV-2:**

5' ACTAGTCGACATGGA (T/A) CAGACACTCCTG (T/C) TATGGGT 3'

**MKV-4:**

5' ACTAGTCGACATGAGG (A/G)CCCCTGCTCAG (A/T) TT (C/T) TTGG (A/C) (A/T) TCTTG 3'

**Figure 41 pg 129**

**Two electrophoretic gels to show the variable light and heavy chain DNA of LOB 7/4**

The heavy and light chain DNA was then further amplified using PCR with MHV-7 and MKV-4 primers and Pfu polymerase. MKV-2 was initially utilised but resulted in the detection of an aberrant Vκ chain revealed at the later stage of sequencing. The PCR products were analysed by agarose gel electrophoresis and visualised under UV light. The bands in the gel representing the PCR fragments of 400 and 420bp indicated specific amplification of the Vγ and Vκ chains. The bands were extracted from the gel and purified using the QIAEX II agarose gel extraction protocol (Qiagen). The extracted PCR product (100ng) was ligated with the TOPO blunt II vector (Invitrogen) and transformed into chemically competent Escherichia Coli (E.Coli), TOP-10 cells (Invitrogen) and cultured on plates of agar containing kanamycin. The TOPO-transformants contain a resistance gene to kanamycin, therefore only the appropriately transformed cells grow and establish themselves as a purified colony of the plasmid. The plasmid DNA was then purified from the cultures using QIAprep spin miniprep kit (Qiagen). The purified plasmid DNA was verified by digesting with suitable restriction enzymes (5' enzyme -Sal I and 3' enzyme –Xho I) and the presence of the inserted DNA was confirmed using agarose gel electrophoresis.

The inserted DNA was then sequenced using T7 and Sp6 primers and the leader sequences aligned so that suitable primers could be designed for subsequent use in the specific amplification of the heavy and light chains of the LOB 7/4. The following primers were then designed. The 5' primers contained HIND III (AAGCTT) restriction sites, the Kozak sequence (CACCA), and the initiation codon (ATG). The 3' primers contained restriction enzyme sites SpeI (ACTAGT) and BsiWI (CGTACG) for the heavy and light chains respectively. Amplification of

the mouse variable regions was then performed using the new primer pairs and cDNA as a template. The DNA obtained was ligated with TOPO blunt II vector as described above and the inserted DNA sequenced.

The designed primers used:

**Heavy chain:**

5': TGAAGCTTCAGGACCTCACCATGGGATGGAGCTGG

3': TGACTAGTGTTCCTTGACCCCAGTAGTCCA

**Light chain:**

5': TGAAGCTTCAGGACCTCACCATGAGGGCCCCTGCT

3': CCCGTACGTTTTATTTCCAGCTTGGT

The DNA obtained was similarly ligated with TOPO blunt II vector, as described above. Restriction enzyme digests were then performed to confirm the presence of the PCR products within the plasmid (HINDIII, SpeI [Vγ], BsiwI [Vκ]).

**Figure 42 pg 130**

**An electrophoretic gel representing the digestion of the ligated TOPO and LOB 7/4 $V_H$ with the restriction enzymes HIND III/SpeI and the ligated TOPO and LOB 7/4 $V_K$ with the restriction enzymes HIND III/BsiWI**

The DNA was then sequenced using T7 and T6 primers as described above.

**Figure 47 pg 135**

**The final consensus sequences of LOB 7/4 $V_H$ and LOB 7/4 $V_K$**

**Chimerisation**

The confirmed $V_H$ and $V\kappa$ chains in TOPO blunt II vector were digested with HINDIII/SpeI and HINDIII/BsiWI restriction enzymes respectively. The digested variable regions were then ligated with pre-digested pUC plasmids containing either the human heavy chain constant region (pUC$\gamma$) or the human kappa chain constant region (pUC$\kappa$) to form the chimeric heavy and light chain products. The ligation mixture was then used to transform competent E.Coli JM109 cells. The transformants contain the ampicillin resistance gene and were, therefore, selected to grow when cultured on ampicillin containing agar plates. The presence of the chimeric DNA construct was confirmed by performing a restriction enzyme digest and gel analysis (HINDIII and EcoRI).

For stable expression of the chimeric antibody the chimerised heavy and light chain constructs needed to be subcloned into mammalian expression vectors. The vectors (pEE6.1, pEE12.1, pEE14.1) contain the promoter, poly-A signal and other sequences necessary for expression in mammalian cell lines. The chimerised constructs were digested with HINDIII/EcoRI and subcloned into pEE6.1 (heavy) and pEE12.1/pEE14.1 (light) using the same restriction enzyme sites.

**Figure 43 pg 131**

**Chimeric LOB 7/4 H in pEE6.1, chimeric LOB 7/4 K in pEE12.1 or pEE14.1 all digested with HINDIII and EcoR1 restriction enzymes**

Stable expression transfectants can be obtained by co-transfecting CHO-K1 cells with the chimeric heavy and light chains into separate vectors. However, it is more convenient to have the 2 chimerised chain within 1 plasmid vector. This was achieved by digesting the chimerised heavy chain expression cassette in pEE6.1 with NotI/BamHI enzymes and ligating into PEE12.1/pEE14.1 plasmids containing the chimerised light chain via the same restriction sites. The resulting plasmid would contain both chimerised heavy and light chains in one expression cassette.

**Figure 44 pg 132**

**Chimeric LOB 7/4H in pEE6.1 and chimeric LOB 7/4κ in pEE12.1 or 14.1 all digested with NOT1 and BamH1**

The DNA was extracted, ligated and transformed as above to produce the chimeric heavy and light chains within one plasmid. The plasmid was then transfected into CHO-K1 cells using the Gene-Porter technique.

Transient expression and subsequently stable expression of the human chimeric anti-CD40 antibody (chLOB 7/4) was identified using ELISA and indirect FACS analysis.

The chimeric LOB 7/4 was purified on a 1.5ml protein-A sepharose column equilibrated with 40mM tris/HCL, 2mM EDTA and 200mM sodium chloride buffer at a pH of 9.0. Initially 1.6 litres of supernatant was passed through the column with 50ml of the tris buffer. The peak was eluted with glycine 200mM/EDTA buffer 2mM at a pH of 3.0. The antibody was dialysed against PBS. The optical density was recorded as 0.9, equivalent to a concentration of 0.67mg/ml. The presence of the antibody was confirmed by gel electrophoresis. The supernatant was then reloaded onto the column and re-eluted with the glycine buffer. The eluted solution was dialysed against PBS and pooled with the previous eluent. The optical density was recorded as 0.662, equivalent to 0.49mg/ml of antibody. The total quantity of antibody obtained from 1.6L of supernatant was 9.8mg contained in 20mls of PBS.

## Summary

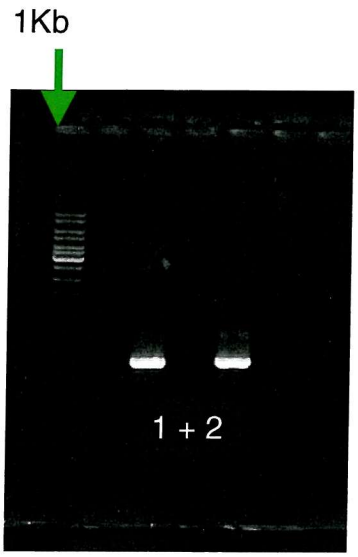
The chimeric human anti-CD40 antibody (ch LOB 7/4) was successfully produced. It has now been sent to Professor J Hales in Oxford for larger scale production.

## Summary figures

**Figure 41**

**Gel 1A**

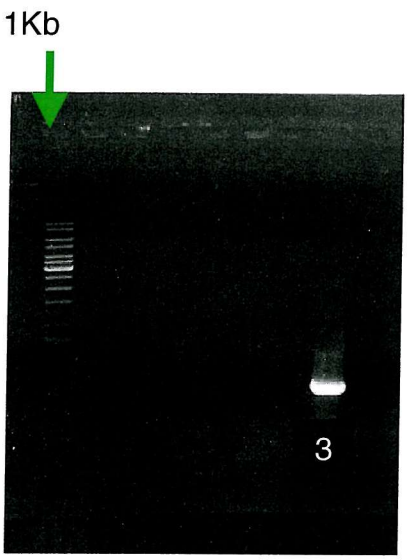
The variable light chain DNA




(1 + 2) $V_K$ - 400 b.p.

**Gel 1B**

The variable heavy chain DNA

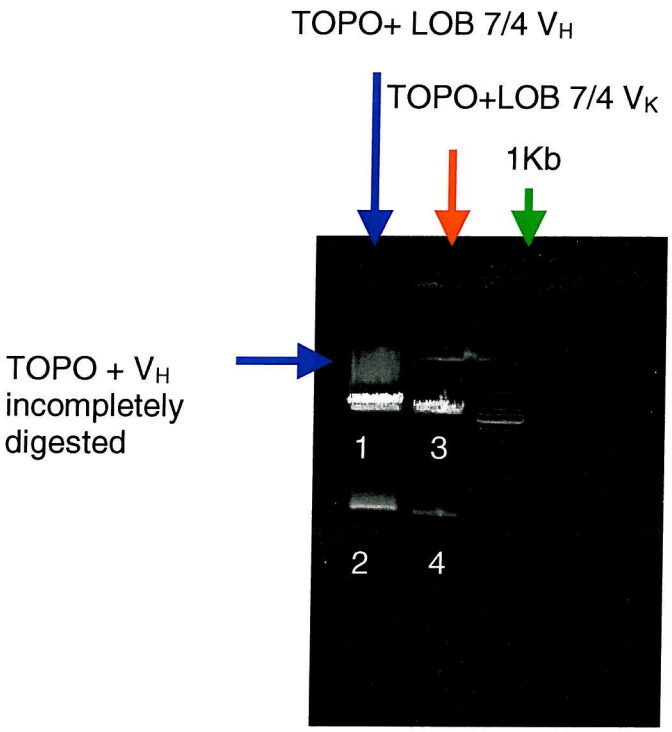



(3) $V_H$ - 420 b.p.

**Figure 42**

**Gel 2**

TOPO and LOB 7/4 $V_H$ digested with HIND III/SpeI and TOPO and LOB 7/4 $V_K$ digested with HIND III/BsiWI

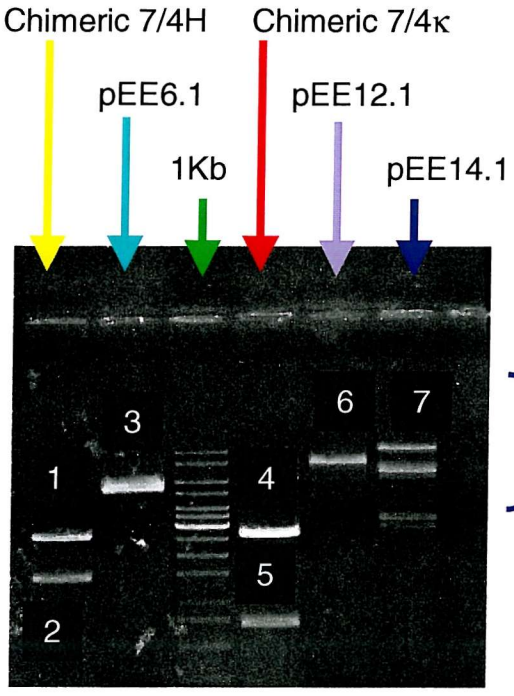



| | | |
|---|---|---|
| (1) TOPO | 3500 | b.p. |
| (2) LOB 7/4 $V_H$ | 420 | b.p. |
| (3) TOPO | 3500 | b.p. |
| (4) LOB 7/4 $V_K$ | 400 | b.p. |

**Figure 43**

**Gel 3**

Chimeric LOB 7/4 H in pEE6.1, chimeric LOB 7/4 in pEE12.1 or pEE14.1 all digested with HINDIII and EcoR1




pEE14.1
Incompletely cut
The top (linearized) band was extracted

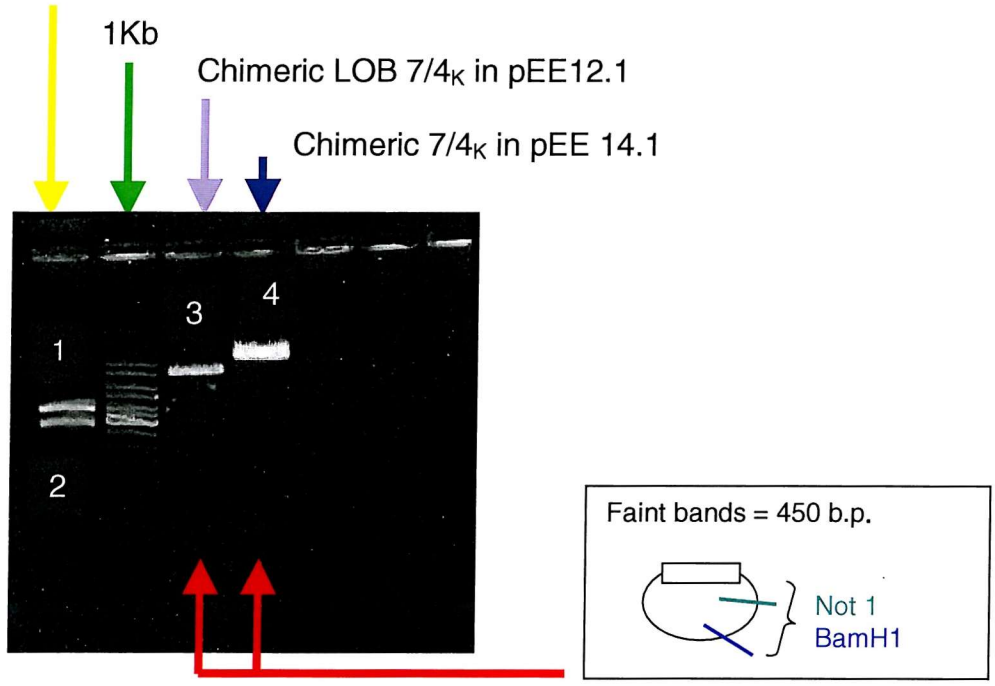
| | |
|---|---|
| (1) pucγ | 3000 b.p. |
| (2) Chimeric LOB 7/4H | 1500 b.p. |
| (3) pEE6.1 | 5079 b.p. |
| (4) pucκ | 3000 b.p. |
| (5) Chimeric LOB 7/4κ | 720 b.p. |
| (6) pEE12.1 | 7583 b.p. |
| (7) pEE14.1 | 10157 b.p. |

**Figure 44**

**Gel 4**

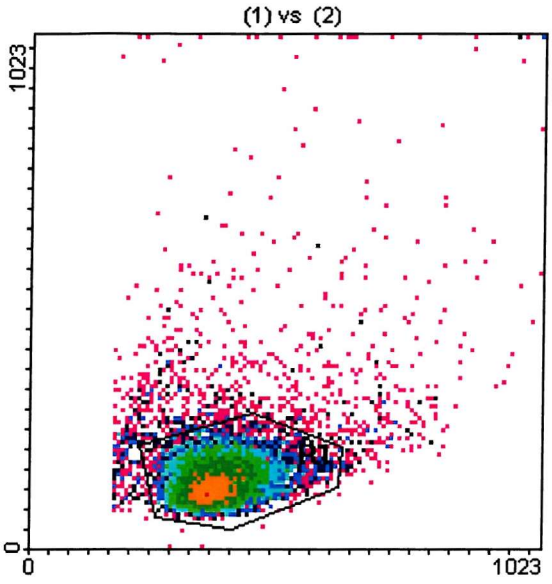
Chimeric LOB 7/4H in pEE6.1 and chimeric LOB 7/4κ in pEE12.1 or 14.1 all digested with NOT1 and BamH1

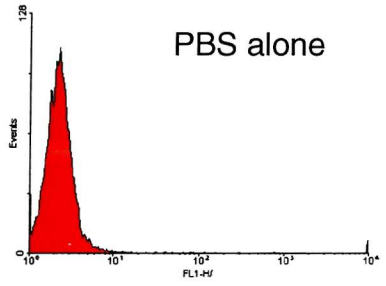
Chimeric LOB $7/4_H$

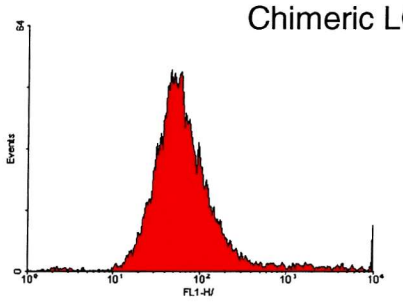


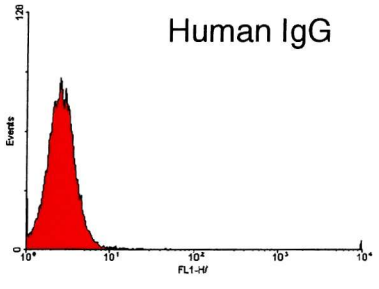

(1) Expression cassette for chimeric LOB 7/4H 3790 b.p.
(2) pEE 6.1
(3) Chimeric LOB 7/4κ/pEE12.1
(4) Chimeric LOB 7/4κ/pEE14.1

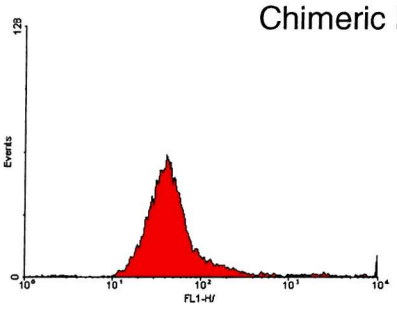
**Figure 45**
Indirect flow cytometric analysis of murine B cells transfected to express human CD40 and used to confirm the presence of the chimeric human anti-CD40 monoclonal antibody in the supernatant obtained from the transfected CHOK1 cells



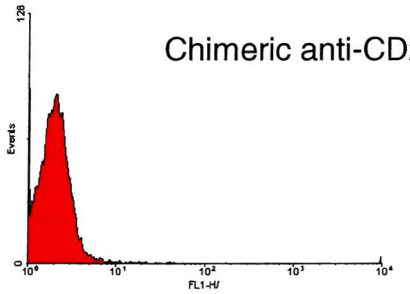
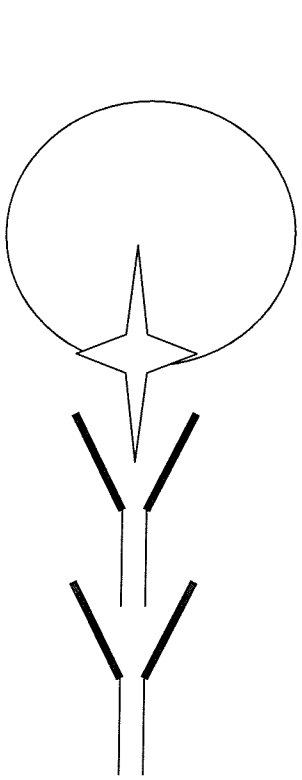



**Figure 46**
A schematic to represent the method of indirect flow cytometric analysis used to confirm the presence of chimeric human anti-CD40 antibody (ch LOB 7/4)



Murine B cell transfected to express human CD40

If anti-CD40 antibody is present in the supernatant it binds to the expressed CD40 molecule

Sheep anti-human IgG-FITC

## Figure 47

The final consensus sequences

TOPO + Lob7.4 V heavy with HindIII and SpeI site

AAGCTTCAGGACCTCACC ATG GGA TGG AGC TGG ATC TTT CTC TTT CTC
CTG TCA GGA ACT GCA GGT GTC CTC TCT GAG GTT CAG CTA CAA CAG
TCT GGA CCT GAC CTG GTG AAG CCT GGG GCT TCA GTG AAG ATA TCC
TGC AAG ACT TCT GGA TAC ACA TTC ACT GAA TAC ATC ATG CAC TGG
GTG AAG CAG AGC CAT GGA AAG AGC CTT GAG TGG ATT GGA GGT ATT
ATT CCT AAC AAT GGT GGT ACT AGC TAC AAC CAG AAG TTC AAG GAC
AAG GCC ACG ATG ACT GTA GAC AAG TCC TCC AGC ACA GGT TAC ATG
GAA CTC CGC AGC CTG ACA TCT GAG GAT TCT GCA GTC TAT TAC TGT
ACA AGG CGA GAG GTG TAC GGG AGG AAT TAC TAT GCT TTG GAC TAC
TGG GGT CAA GGA ACA CTA GT

TOPO + Lob7.4 $V_{kappa}$

AAGCTTCAGGACCTCACCATG AGG GCC CCT GCT CAG TTC CTT GGT CTC
CTG TTG CTC TGT TTT CAA GGT ACC AGA TGT GAT ATC CAG ATG ACA
CAG ACT ACA TCC TCC CTG TCT GCC TCT CTG GGA GAC AGA GTC ACC
ATC ACT TGC AGT GCA AGT CAG GGC ATT AAC AAT TAT TTA AAC TGG
TAT CAG CAG AAA CCA GAT GGA ACT GTT AAA CTC CTG ATC TAT TAC
ACA TCA AGT TTA CAC TCA GGA GTC CCA TCA AGG TTC AGT GGC AGT
GGG TCT GGG ACA GAT TAT TCT CTC ACC ATC AGC AAC CTG GAA CCT
GAA GAT ATT GCC ACT TAC TAT TGT CAG CAG TAT AGT AAC CTT CCG
TAC ACG TTC GGA GGG GGG ACC AAG CTG GAA ATA AAA CGT ACG G

The variable regions, sequenced above, were digested and subcloned into vectors containing the human constant region sequence.

**Figure 48**

Initial stages in the production of human chimeric anti-CD40 (ch LOB 7/4)

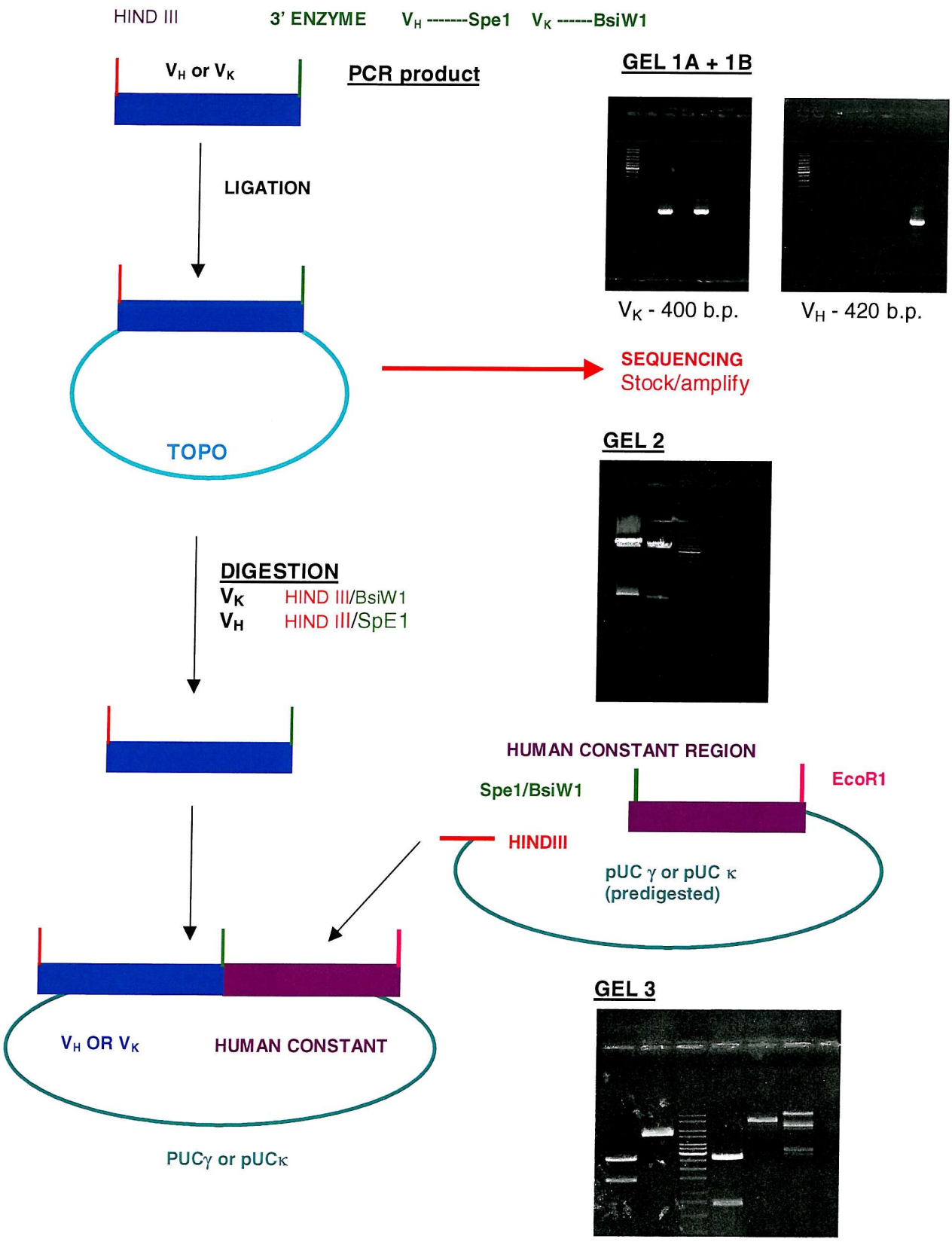
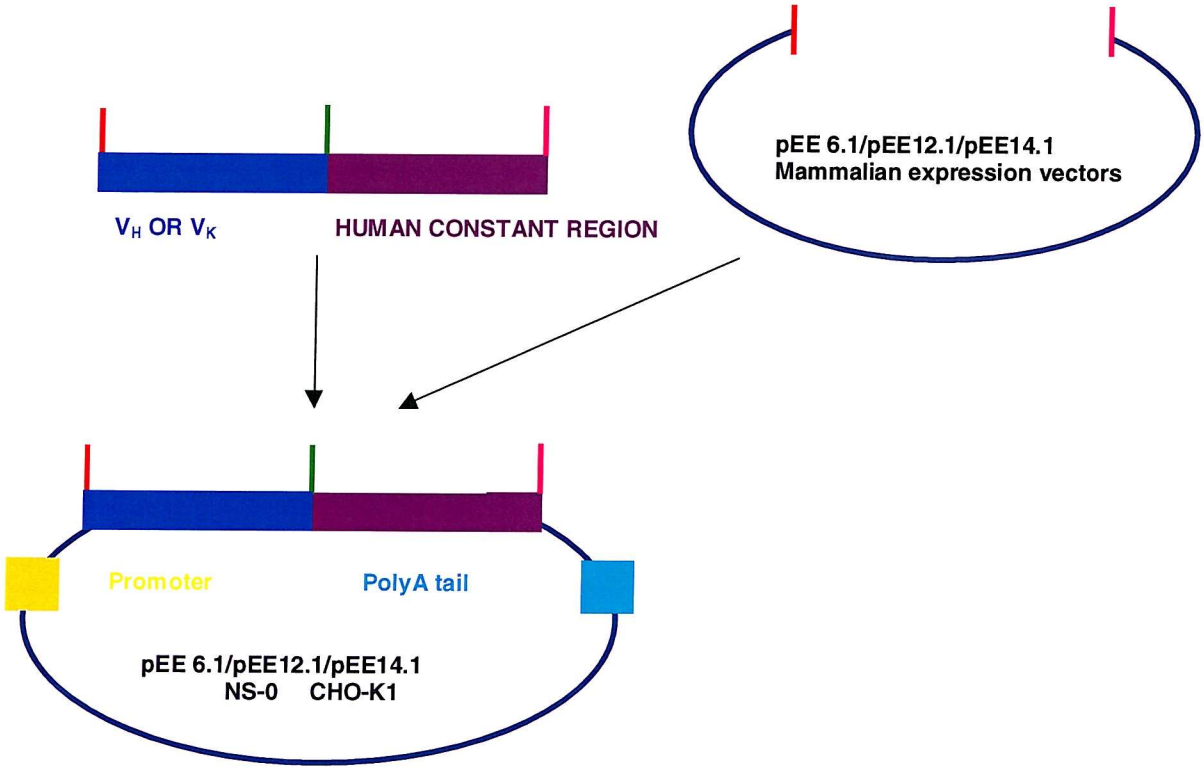


**Figure 49**

Insertion of chimeric LOB 7/4 heavy and light chains into the appropriate mammalian expression vectors




**GEL 4**

Chimeric LOB 7/4 H with pEE6.1 and chimeric LOB 7/4 K with pEE12.1 and pEE14.1

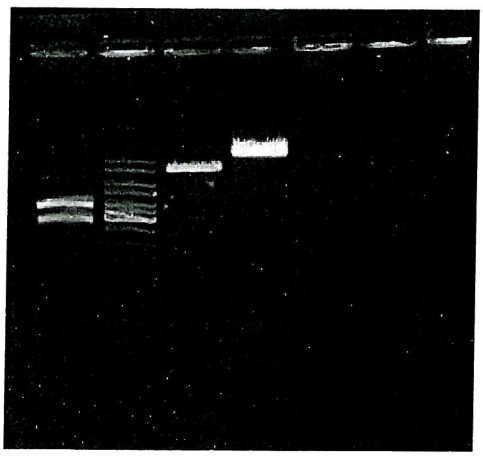
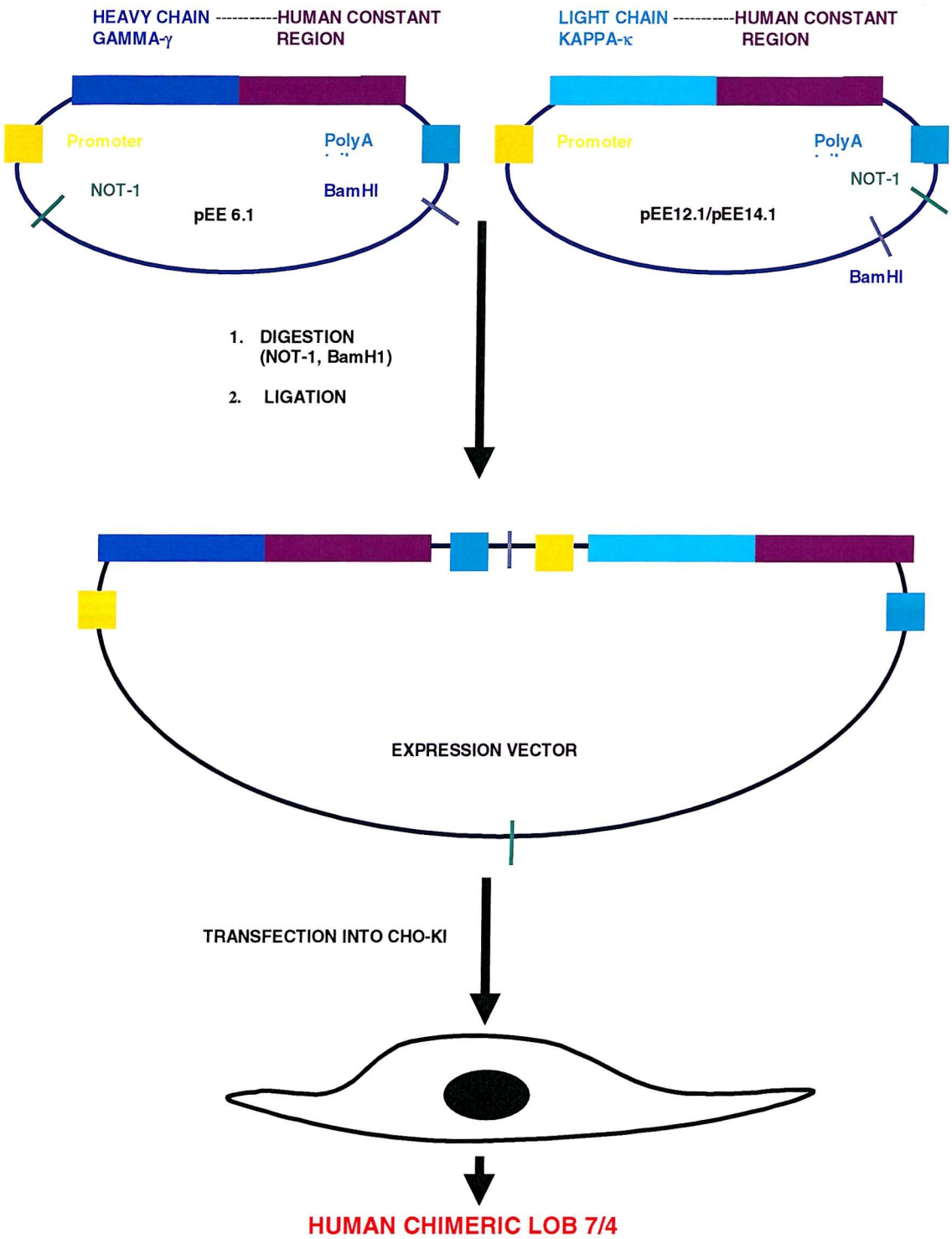


**Figure 50**

Final stages in the development of the chimeric human anti-CD40 antibody (Ch LOB 7/4)



# CHAPTER 6

# RESULTS

# IN-VIVO TOXICOLOGY AND SCID THERAPIES

## CHAPTER 6

## IN-VIVO TOXICOLOGY AND SCID THERAPIES

The results of important preclinical toxicology studies testing mouse anti-CD40 (3/23) in BALB/c mice are described in this chapter. The mice have been culled following treatment and a reversible dose dependent transaminitis associated with microscopic evidence of a reversible necrotising lympho-granulomatous hepatitis, has been identified. These anti-CD40 antibody toxicology studies are essential before a protocol for a phase I trial of human anti-CD40 antibody against CD40 expressing human tumours is developed.

Anti-tumour activity has been identified in a xenograft model of a transformed human B cell line (Daudi) treated with both a mouse anti-human CD40 antibody and a chimeric human anti-CD40 antibody.

### Introduction

The efficacy, safety and quality of monoclonal antibodies, directed against human tumour-associated antigens, must be established before a potentially effective treatment can be offered to patients.

The widespread expression of CD40 and CD40L could cause difficulties if CD40 agonists were used against human CD40 expressing cancers. The non-specific reversal of tolerance might induce a number of diseases including autoimmune

syndromes[107, 111], multiple sclerosis, atherosclerosis[112], thyroiditis, pulmonary fibrosis, alzheimers disease and prothrombotic states[110].

The phase I/II clinical trials committee of the Cancer Research Campaign was established in 1980. It aimed to expedite the early clinical evaluation of novel cancer therapies. In 1986 they published the results of a working party regarding the clinical use of antibody treatments[117]. The issued guidelines advised on the control of production of antibodies and drug-antibody conjugates, necessary preclinical toxicology studies and the principles on which phase I trials of these new agents should be conducted. Subsequently, further control recommendations were published for products derived from recombinant DNA technology prepared for administration to patients with cancer in phase I trials[118].

Animal models of human cancers treated with knew therapeutic agents do not reliably predict the response of similar cancers in man. In addition, the routine use of non-rodent species in preclinical toxicology studies has been shown to be unnecessary[119]. Unhelpful animal toxicology studies must be avoided but some evidence of anti-tumour activity is valuable and localisation of the antibody or antibody conjugate in xenografts of human tumours is useful. The aim of animal research models should be to define the maximum safe drug levels with which to commence human toxicity and anti-cancer trials. However, clear evidence of therapeutic activity in the clinical setting can only be obtained when the treatment is carefully assessed in human beings.

The guidelines regarding minimum preclinical toxicology studies for unconjugated antibodies have been summarised as follows[117]:

1 The purity and specificity of the unconjugated antibody should be confirmed

2 The antibody should be administered ip or iv

3 One sex of experimental animal should be studied (usually males)

4 Six-ten animals should be treated per dose

5 At least 10x the proposed dose in man (mg/kg) should be administered to the animals

6 A second dose could be administered in some circumstances

7 The aim should be to identify any untoward or unexpected effects of the antibody

8 The animals should be sacrificed 14 days after treatment

9 Histopathology should be examined on any macroscopically abnormal tissues

10 Blood should be obtained for haematological analysis

In summary, the minimum toxicology studies should satisfy and assist the clinician contemplating a phase I trial. Experimental systems should reveal clear evidence of special or general anti-tumour activity. Evidence of in-vivo tumour localisation of the antibody is required and/or the antibody or drug-antibody conjugate should have a selective effect on a normal system which could be active against a human tumour recognised as a neoplastic transformation.

## Materials and Methods

### Balb/c Toxicology

As described in the materials and methods chapter page 74 Balb/c mice were treated on day 0 with the appropriate dose of murine anti-CD40 antibody (3/23), they were observed daily, and then, according to CRC guidelines, culled at day 14. Blood was obtained and sent for haematological and biochemical analysis. Post-mortem examination was undertaken on all animals and organs including brain, heart, lungs, liver, spleen, kidney, bowel, testis, femur, and paw were removed, preserved in formalin and sent for expert histopathological analysis at the London Veterinary College.

## SCID Therapies

As described on page 74, the severe combined immunodeficient mice (SCIDS) were injected on day 0 with 200μl of PBS containing $5x10^6$ the appropriate tumour cells under investigation. Then 200μl of PBS containing 100μg of the experimental antibody was injected via the same route on day 7. The experiments were carefully controlled with isotype matched and untreated groups and results documented in terms of survival.

## Results

## Balb/c toxicology

### Observations

All mice treated with murine anti-CD40 (3/23) were observed by trained animal laboratory staff to be non-specifically unwell (lethargy, reduced feeding) on day 3 following treatment. The severity was noted to be dose dependent and all features had fully resolved by day 5 or 6.

### Pathology

Splenomegaly, described as moderate splenic enlargement was observed in mice treated with murine anti-CD40 antibody (3/23). The degree of enlargement was dose dependent. Splenomegaly was not observed in the control animals receiving 1D3.

Microscopically the balb/c mice showed no evidence of background disease and the tissues were appropriately fixed with minimal artefact present.

Microscopic abnormalities were noted in the liver, kidney and spleen of the mice treated with 3/23. The majority of these changes were dose dependent. Similar abnormalities were not seen in the control groups, including untreated mice and those who received a control antibody, 1D3.

Liver changes were first observed in the group treated with 200μcg of 3/23. They consisted of Lymphocyte aggregates (including 5-10 lymphocytes) located around degenerate/necrotic hepatocytes or small fragments of cellular/nuclear debris. These aggregates were scattered throughout the liver parenchyma in 50% of the treated group. Lymphocyte infiltrates were observed in the portal tracts of 66% of the treated animals and in 33% of the same group small aggregates of neutrophils and macrophages centred round hepatocellular debris were seen.

The randomly scattered parenchymal lymphocyte aggregates increased with increasing dose from 1/10x microscopic field (low power) at 500μcg to >10/10x field from dose levels ≥2mg.

The infiltration of portal tracts with lymphocytes increased with increasing dosage. At doses ≥1mg/mouse the infiltrates abridged the portal plate surrounding and infiltrating individual periportal hepatocytes (piecemeal necrosis) in 83-100% of treated mice. At dose levels ≥4mg 25-30% of the liver parenchyma was replaced with lymphocyte aggregates and diffuse portal infiltrates.

Microgranulomas consisting of a cluster of macrophages with abundant cytoplasm and large vesicular nuclei mixed with a few lymphocytes were identified at a level of 4/10x field in 33% of mice treated with 500μcg of antibody. These became more prominent in mice treated with doses ≥1mg. They consisted of round to oval, sometimes confluent clusters of large macrophages with abundant pale cytoplasm and a variable number of lymphocytes. They engulfed 30% of the surface area of the hepatic parenchyma and extended into the portal tracts at doses ≥2mg in 83-100% of treated mice. At doses ≥4mg confluent microgranulomas replaced much of the portal lymphoid infiltrates in ≥83% of treated mice.

Overall the microscopic hepatic changes revealed a dose dependent lympho-granulomatous hepatitis. The inflammatory change randomly focused on individual hepatocytes. At low dose levels the lesions included small numbers of neutrophils or macrophages clustered on necrotic hepatocytes or cellular debris. This developed into large numbers of confluent or coalescent microgranulomas that randomly effaced upto 30% of the cross-sectional area of the liver sections. As the number of microgranulomas increased the population of lymphoid cells in the portal tracts increased. The lymphoid population abridged the portal plate, and surrounded individual, often degenerate or necrotic hepatocytes. These changes were compatible with piecemeal necrosis. Coalescent microgranulomas replaced the portal tract lymphoid infiltrates at the highest dose levels.

Interestingly, in the group of mice treated with 1mg of 3/23 on days 0 and 10 who were culled at 3 months following their initial therapy, no evidence of liver abnormality was found. This suggested a complete resolution of the acute hepatitis.

Minor microscopic abnormalities were found in the kidneys of 16-50% of mice treated with 3/23. These changes were first noted in the mice receiving 500mcg of 3/23. They included a minimal to mild tubulo-interstitial lymphocytic nephritis that was not apparently dose dependent. The glomerular tufts were noted to be prominent and contained round nuclei, which could represent mesangial lymphocyte infiltration. Peri-tubular lymphocytes were seen. The glomerular change was said to be notable but not quantifiable.

Again, in the group of mice treated with 1mg of 3/23 on days 0 and 10 who were culled at 3 months following their initial therapy, no evidence of of renal abnormality could be detected suggesting a complete resolution of the minimal-moderate tubulo-interstitial lymphocytic nephritis.

Microscopic splenic changes were first noted at dose levels of 2mg. These changes included prominence of the marginal zone, with displacement of the splenic red pulp in 66% of treated animals. At doses $\geq$4mg the marginal zones were uniformly hypertrophied (upto 30% wider) and contained large macrophages with abundant pale cytoplasm.

A few lymphocytes were also noted beneath the lining of the cerebral ventricles in 16-33% of mice treated at doses ≥4mg. The significance of this is uncertain.

No other remarkable abnormalities were seen in any other organs removed.

**Figure 51** **pg 152**

**Sections of murine liver (Balb/c mice) taken 14 days after treatment with anti-mouse CD40 antibody (3/23). A dose dependent lympho-granulomatous hepatitis developed following treatment. Microscopic evidence of this hepatitis include:**

**A** **Scattered parenchymal and periportal lymphocyte aggregates**

**B** **A portal tract with lymphocyte aggregates and parenchymal lymphocyte infiltration**

**C** **Two microgranulomas within the liver parenchyma**

**D** **Hepatocyte piecemeal necrosis**

**Blood analysis**

The circulating blood volume in a balb/c mouse is small. A cardiac stab provided 0.5-1ml of blood from each mouse. Therefore it was necessary to pool the blood from each group of 6 identically treated mice. The pooled blood was then divided between tubes for haematological and biochemical analysis. Preliminary investigations found the blood from balb/c mice to be hypercoagulable. Heparinisation of the samples did not improve this and many of the blood samples clotted prior to analysis. The results obtained were unreliable and uninterpretable

with no significant difference in platelet count noted between the 2 treatment groups.

Aminotransferases are liver enzymes that reside in hepatocytes. They leak into blood with hepatocellular damage. There are 2 measurable types of enzymes, aspartate aminotransferase (AST) present in the mitochondria and alanine aminotransferase (ALT) a cytosolic enzyme. ALT is more specific to liver than AST which is also present in heart, muscle, kidney and brain. Alkaline phosphatase (ALP) is present in the canalicular and sinusoidal membranes of the liver, but is also present in many other tissues e.g. bone, intestine, and placenta. It is elevated in cholestasis of any cause (intra or extrahepatic). Levels are highest in conditions of liver infiltration e.g. Liver metastases and in conditions which disrupt liver architecture e.g. cirrhosis.

Murine liver ALT levels were measured on the pooled blood of 6 identically treated Balb/c mice. The levels of ALT appeared to be elevated 2 weeks after treatment with endotoxin-free 3/23 at doses greater than or equal to 1mg/mouse.

**Figure 52 pg 153**

**Two graphs indicating the level of murine liver transaminase, ALT, 2 weeks after intraperitoneal treatment with murine anti-CD40 antibody (3/23). The results are compared with the administration of murine anti-CD19 (1D3). The first graph expresses the results as an absolute value of ALT whilst the second as percentage of a mean value obtained from a group of untreated balb/c mice**

Murine liver ALP levels appeared unaffected following treatment with endotoxin free 3/23 when compared with the control antibody 1D3. The use of pooled blood makes it impossible to confirm this statistically. These experiments would need to be repeated to confirm this.

**Figure 53 pg 154**

**Murine alkaline phosphatase levels (ALP) 2 weeks after intraperitoneal treatment with endotoxin free 3/23 compared with endotoxin-free murine anti-CD19.**

**SCID therapies**

SCID mice aged ≥10 weeks, were inoculated intravenously on day 0 with high grade transformed non-Hodgkins lymphoma cell lines (either RL or Daudi). They were treated on day 7 with 100μg of intravenous mouse anti-human CD40 (LOB 7/6) or chimeric LOB 7/6. Significant and reproducible increases in mouse survival ($p<0.001$) were noted in those mice who received the Daudi cell line followed by either LOB 7/6 or chimeric LOB 7/6. The experimental groups were compared with 3 age-matched control groups who received PBS alone, PBS containing an isotype matched antibody for LOB 7/6 (OX35-IgG2a) or PBS containing an isotype matched antibody for the human chimeric LOB 7/6 (Human IgG-IgG1).

**See figure 54 pg 155**

**This figure is a survival curve to represent the anti-tumour effects of 100mcg of either mouse anti-human CD40 (LOB 7/6) or human chimeric anti-CD40 (chimeric LOB 7/6) in SCID mice bearing a transformed, high grade human B-cell lymphoma (Daudi-5x10$^6$cells/mouse)**

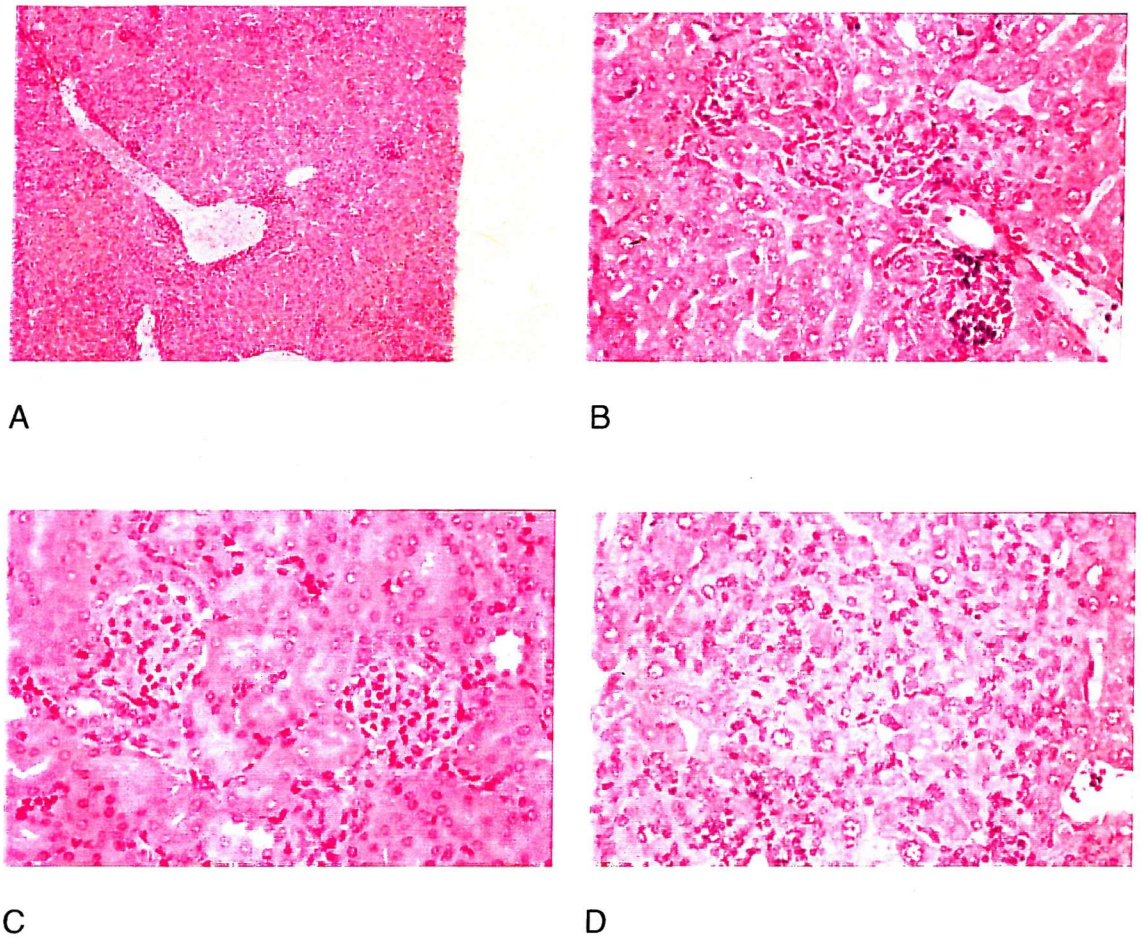
Unfortunately the RL cell line did not engraft and was therefore unsuitable for survival analysis.

**Summary**

Mouse anti-CD40 (3/23) causes a reversible dose dependent transaminitis associated with macroscopic evidence of a reversible lympho-granulomatous hepatitis that at the highest dose levels is acutely necrotising.

In the xenograft model of a transformed human B cell NHL (daudi) evidence of anti-tumour activity was shown with both human anti-CD40 (LOB 7/6) and chimeric human anti-CD40 (Ch LOB 7/6) antibody therapies.

**Figure 51**
Liver sections showing the dose dependent lympho-granulomatous hepatitis developed after treatment with anti-mouse CD40 antibody (3/23)



A

B

C

D

A A low power slide showing scattered parenchymal and peri-portal lymphocyte aggregates

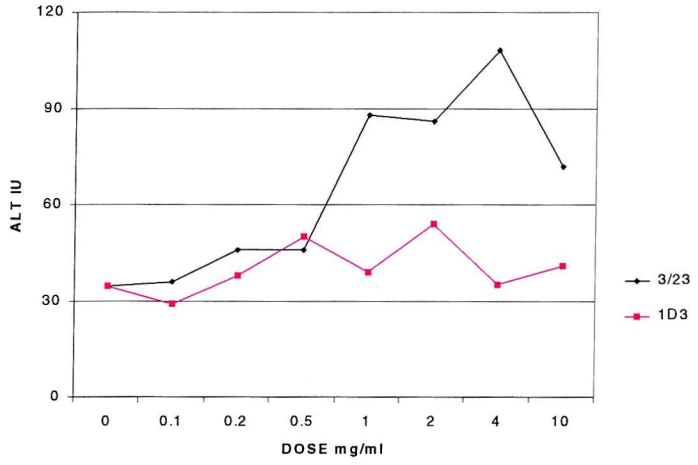
B A high power slide of a portal tract with lymphocyte aggregates and parenchymal lymphocyte infiltration

C A high power slide showing two microgranulomas situated within the liver parenchyma

D An example of hepatocyte piecemeal necrosis

## Figure 52

Two graphs to show the levels of murine liver transaminase, ALT, 2 weeks after intraperitoneal treatment with murine anti-CD40 antibody (3/23). The results are compared with the administration of murine anti-CD19 (1D3). The first graph expresses the results as an absolute value of ALT whilst the second as percentage of a mean value obtained from a group of untreated balb/c mice



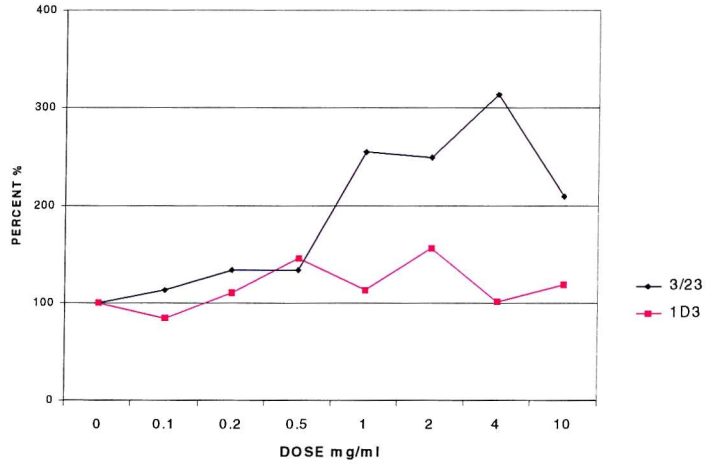
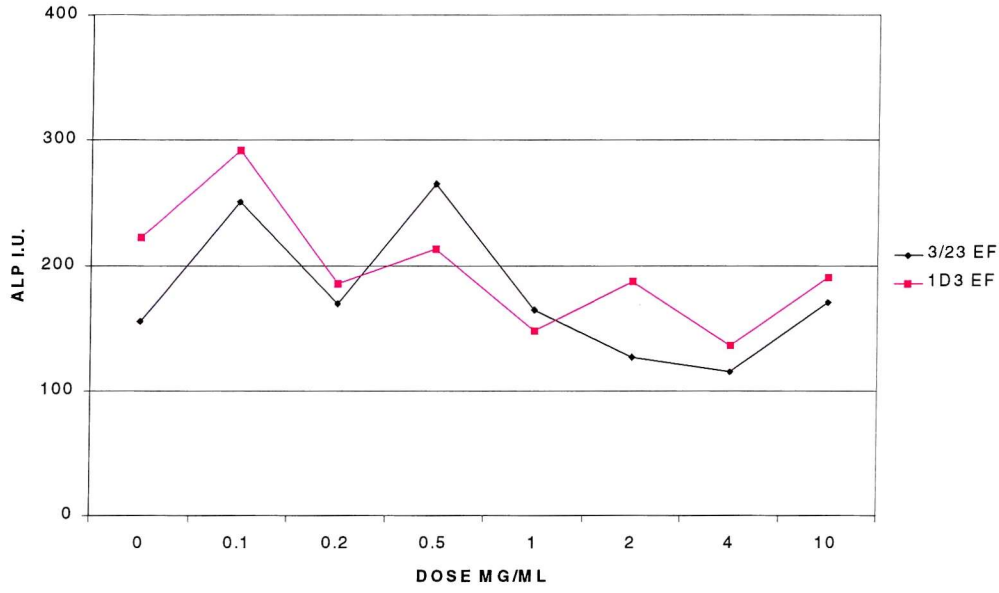



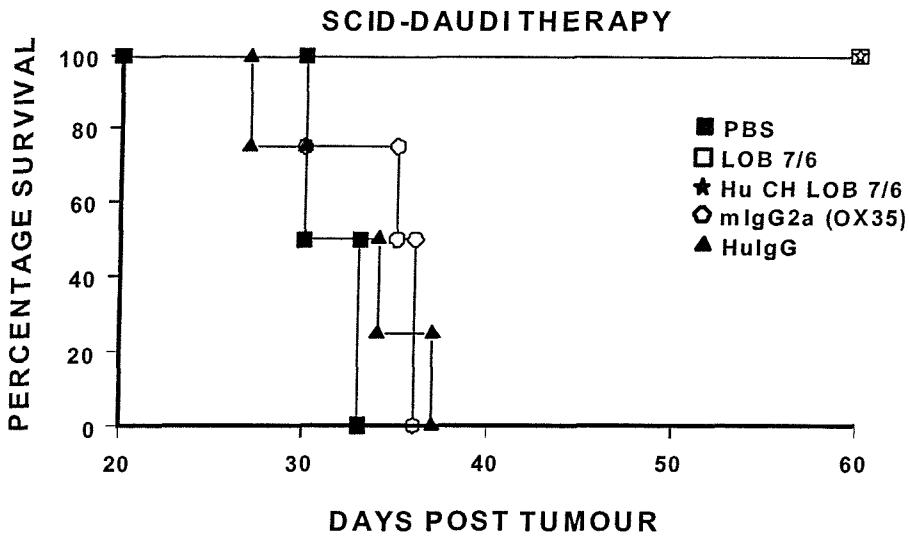
**Figure 53**

Murine alkaline phosphatase levels (ALP) 2 weeks after intraperitoneal treatment with endotoxin free 3/23 compared with endotoxin-free murine anti-CD19.



## Figure 54

A survival curve to represent the anti-tumour effects of 100mcg of either mouse anti-human CD40 (LOB 7/6) or human chimeric anti-CD40 (chimeric LOB 7/6) in SCID mice bearing a transformed, high grade human B-cell lymphoma (Daudi-$5x10^6$cells/mouse)



# CHAPTER 7

# DISCUSSION

# CHAPTER 7

## DISCUSSION

Cancer, in all its forms, causes significant morbidity and mortality. Many advanced stage cancers are incurable. Notable exceptions exist, as discussed previously, but in cases of metastatic disease surgery, radiotherapy and chemotherapy treatments usually serve only to palliate symptoms and prolong survival. Alternative and improved therapeutic options are needed to treat many disseminated malignancies. These treatments must aim to improve cure rates or at least provide prolonged disease free survival. The toxic consequences of treatment must be minimised to enable the quality of life to be maintained.

One approach has been to use monoclonal antibodies to specifically target cell surface receptors expressed by cancer cells to achieve an anti-tumour response. To date anti-CD20 antibody (rituximab) has provided the most impressive results in the treatment of non-Hodgkin's lymphoma[60-63].

A better method might be to use an antibody that targets both the tumour cells and the cells that induce an immune response. The tumour may then be directly attacked and indirectly challenged by the induction of an effective immune response that evades tumour tolerance. One such antibody, CD40 antibody, has the potential to fulfil this role [59].

I have studied the effects of anti-CD40 antibodies, in-vitro using both transformed human B cell lines and ex-vivo human B cells. I have assessed the possible toxic consequences of an anti-CD40 antibody therapy in-vivo using a mouse model and shown evidence of anti-tumour activity in a xenograft model of a transformed human B cell line (daudi) treated with human anti-CD40 antibody and chimeric human anti-CD40 antibody. In addition I have developed a human chimeric anti-CD40 antibody.

In vivo CD40 ligation on normal B cells causes B cell proliferation, isotype switching, immunoglobulin production and rescue from apoptosis [41-43].

In vitro CD40 stimulation can induce proliferation and apoptotic rescue in normal B cells, some malignant B cell lines, follicular lymphoma, mantle cell lymphoma and CLL cultures [44,45, 83, 84]. Whilst in other non-Hodgkin's lymphoma cell lines, carcinomas and mouse models of lymphoma CD40 activation can induce growth arrest, apoptosis and tumour eradication [90, 96, 97]. The molecular mechanisms of cell survival versus cell death induced by CD40 remain unclear. They may be explained by differential expression and/or regulation of apoptosis controlling proteins, in particular the Bcl-2 protein family. These include anti-apoptotic proteins (Bcl-2, Bcl-xl, Bfl-1, A1, Mcl-1) and pro-apoptotic proteins (Bax, Bak, Bik).

Rescue from apoptosis with increased Bcl-2 expression has been shown in germinal centre B cells following CD40 ligation whilst in a range of other B cells

increased Bcl-xl has been documented following CD40 ligation induced apoptotic rescue.

To date increased levels of pro-apoptotic proteins have not been shown in B cells following CD40 ligation. However, CD40 ligation on CD40 positive breast carcinoma cells has induced upregulation of Bax [102].

**Growth inhibition**

In-vitro CD40 ligation directly inhibits the proliferation of transformed human B cell lines (RL and Daudi). This appears to be further enhanced in a system that includes cross-linking (e.g. TCHO). The chimeric anti-CD40 antibody (chLOB 7/6), like the standard mouse anti-human CD40 monoclonal antibody, is able to reproducibly cure a xenograft model of transformed high grade human B cell non-Hodgkins lymphoma (Daudi). In future work this xenograft model could be tested using the newly developed chimeric anti-CD40 antibody (chLOB 7/4).

When LOB 7/4 was presented by the low affinity human Fc receptor, expressed by a feeder layer of transfected mouse fibroblasts (Fcγ RII/CDw32), significant growth inhibition was just achieved in the RL system. This was not identified when LOB 7/4 was substituted with some of our other mouse anti-human CD40 antibodies (LOB 7/2, 7/6, 7/8). There are several reasons why LOB 7/4 might be a better inducer of growth inhibition. Possible explanations include steric orientation, agonist activity, affinity, avidity, and the different epitopes of the antibodies.

CD40 ligation with human SCD40L, in vitro, caused significant growth inhibition with both ovarian and cervical cell lines (MG79 and Caski respectively). Future expansion of this work could include further in-vitro experiments using other methods of CD40 ligation and the development of a mouse model of human CD40 expressing solid malignancy treated with anti-CD40 monoclonal antibodies (standard and chimerised variants).

It is known that cross-linking, in vitro, markedly enhances the inhibitory signals of anti-CD40 antibodies[90]. Experiments have been performed with Burkitt's lymphoma cell lines showing that soluble anti-CD40 does not significantly inhibit cell growth but immobilized anti-CD40 does. In addition, anti-CD40 significantly prolongs the life of mice bearing this tumour. This explains the different growth inhibitory effects seen when RL and daudi cells were cultured with TCHO, SCD40L and anti-CD40 antibody alone. However, it is more difficult to explain the different observations seen when these same cells were cultured with Fcγ RII/CDw32 cells and anti-CD40 monoclonal antibody. This system should provide appropriate cross-linking of the antibody, yet in my experiments growth inhibition was not clearly apparent and only just achieved significance with LOB 7/4. The relative insensitivity of these particular Fcγ RII/CDw32 cells to the effects of radiation, delivered to prevent cellular proliferation, was problematic but even with successful irradiation the proliferation of the B cells was not influenced with the addition of anti-CD40 antibody. The results were variable and difficult to assess in this more complicated system. Further work should include pre-incubation of the B cells with polyclonal human IgG to block non-specific antibody binding sites. A new line of Fcγ RII/CDw32 cells could be produced and compared with the original

Fc expressing cells. Alternatively the Fcγ RII/CDw32 cells could be substituted with Fc expressing beads. This would simplify the technique, eliminating the cell irradiation step and still provide a cross-linking system for the anti-CD40.

**Ex-vivo human B cell proliferation, upregulation and viability**

In keeping with work by other groups[84], in-vitro ligation of CD40, using CD40L transfectants or human SCD40L, on normal and primary malignant human B cells resulted in B cell proliferation.

The optimum concentration of CD40L transfectant feeder layer ($1.25x10^4$/well) was found to be slightly less than others have recommended ($2.5x10^4$/well) but these groups have generally used mouse fibroblast cells rather than chinese hamster ovary cell transfectants[45, 83]. It may be that, at high concentrations, TCHO produce cytokines that directly reduce B cell proliferation or they simply overcrowd the wells and with their low level of background proliferation consume nutrients effectively 'starving' these cells and inhibiting their growth.

I could not produce a reliable model, using irradiated Fcγ RII/CDw32 cells, to test our panel of human anti-CD40 antibodies. A large variation in results occurred and, as with the growth inhibition work, the transfectants required high levels of irradiation (75Gy) to halt their proliferation. The high level of irradiation did not affect the cell surface expression of the human Fc receptor as shown using flow cytometry pre- and post- irradiation. The most confounding factor occurred because ex-vivo human B cells incubated with transfected NIH3T3 cells

proliferated equally regardless of the presence or absence of anti-CD40 making it impossible to assess the effect of the antibody. As before, further work should include pre-incubation of the B cells with polyclonal human IgG to block non-specific antibody binding sites. Another source of Fcγ RII/CDw32 cells could be obtained or the cells could be substituted with Fc expressing beads to simplify the technique, as previously discussed.

In the presence of human IL4, increased expression of MHC I /II, CD80, CD86, CD54 and CD58 was identified on the cell surface of human B cells obtained from patients with splenic marginal zone NHL and follicle centre cell NHL, following culture with CD40L chinese hamster ovary cell transfectants. These findings are in keeping with other groups who have shown that CD40 cross-linking, by CD40 mAbs presented on CD32-transfected fibroblasts or CD40L transfectants, induces proliferation and activation of a variety of freshly isolated malignant B cells. The malignant B cells studied include follicular lymphoma[84], lymphoplasmacytoid lymphoma[91], multiple myeloma[92], hairy cell leukaemia[93], chronic lymphocytic leukaemia[94], and acute lymphoblastic leukaemia[95].

The increased cell surface expression of MHC I and II, along with adhesion and co-stimulatory molecules, make these cells potentially capable of efficient antigen-presentation. A chimeric anti-CD40 antibody, injected systemically, might, therefore, prevent immune tolerance to tumour, allowing the immune system to recognise and eliminate the tumour. Alternatively, in view of the widespread expression of CD40L, primary malignant human B cells could be CD40 ligated in-vitro, irradiated and then inoculated as efficient antigen presenting cells capable of

'boosting' the immune response to tumour. Activated tumour cells could also be used to stimulate the ex-vivo production of autologous human T-cells, these could be adoptively transferred. The potential effects of widespread CD40 ligation would then be avoided.

Future, in-vitro, work, as discussed previously, should include the development of a better system for studying the effect of anti-CD40 antibodies on primary malignant human B cells. The newly developed chimeric anti-CD40 antibody could then be tested to see if it causes malignant human B cells to proliferate and upregulate their expression of cell surface molecules making them efficient antigen-presenting cells.

**Anti-tumour activity**

Anti-tumour activity was shown with both human anti-CD40 (LOB 7/6) and chimeric human anti-CD40 (Ch LOB 7/6) antibody therapies when they were used in the xenograft model of a transformed human B cell NHL (daudi). This may be a direct anti-tumour effect provoked by the antibody. Alternatively or in addition the antibody might be able to bind Fc receptors expressed by NK cells inducing an NK mediated response in the SCID mice. Future therapies of this nature should include a method for blocking NK cells or the Fc receptor sites.

## Toxicology

When balb/c mice were treated with mouse anti-CD40 (3/23) a clear pattern of dose dependent hepatitis was identified in the livers of mice culled 14 days after the initial injection, this was associated with elevated serum transaminase levels. The inflammation was characterised by an acute, non-suppurative, necrotizing, lympho-granulomatous hepatitis. However, in the livers of mice treated on day 1 and day 10 with 1mg of 3/23, and culled 3 months from the first injection, no evidence of chronic active hepatitis could be found, suggesting that the hepatitis was short-lived and resolved completely.

Mouse hepatitis can be caused by a variety of toxins and infections[120]. Viruses causing liver inflammation include mouse hepatitis virus and mousepox, whilst bacterial causes include bacillus piliformis and helicobacter hepaticus. Clearly it is important to note that at the time of these mouse experiments no known infections were present in the Tenovus Laboratory mice that might be responsible for the documented pathological changes. In addition the livers obtained from the control mice, including the groups receiving no treatment, PBS alone, and 1D3 showed no evidence of hepatitis. The transaminase levels in these mice were all within normal limits. In future work serology and blood culture tests could be performed to exclude viral and bacterial infection respectively. For helicobacter hepaticus infection the definitive diagnosis depends upon cultural isolation of the organisms from the liver or lower intestinal tract. If only fixed tissue is available, silver staining to identify spiral bacteria in the liver or identification of murine

helicobacter species by polymerase chain reaction (PCR) and restriction enzyme analysis would be an appropriate alternative.

A recent phase I dose escalation study[106] of recombinant human CD40L, given subcutaneously to 32 patients with a range of malignancies, found the major dose-limiting toxic event to be a transient but dose-dependent transaminitis. Grade 3 or 4 elevations in liver transaminases were identified in 14%, 28%, and 57% of patients treated for 5 days at 0.05, 0.01, and 0.15 mg/kg/day, respectively. In this study 1 patient with laryngeal carcinoma achieved a complete response following 12 month of therapy.

The effects of anti-CD40 therapy in mice have been documented[121]. Anti-CD40 causes splenomegaly. The initial changes can be seen within 24 hours and the spleen enlarges to four times its original size in 4 days. The white-pulp enlarges first as $IgD^+$ B cells are retained in the follicles. The red pulp initially reduces, possibly because of compression of the blood sinusoids by the expanding white pulp. After 3 days the red pulp expands with the growth of several cell types and by day 6 the red pulp venous sinuses are noticeably wider. T and B-lymphocytes initially (<24hours) proliferate within the follicles. The B cells then migrate to the red pulp (>3days). $CD11b^+$ macrophages increase dramatically in the red pulp by days 3-6 following the administration of anti-CD40. Marginal zone metallophils, which characteristically form a single layer beneath the marginal sinuses, increase after anti-CD40 treatment thickening the marginal zone in association with marginal sinus endothelial growth (by day 6). The $CD11c^{high}$ dendritic cell subset

also resides in the red pulp. These dendritic cells progressively increase their numbers after anti-CD40 treatment and occupy much of the red pulp by day 6.

In my results with mice treated by increasing doses of 3/23 which were culled 14 days after therapy, a marked increase in the marginal zone was noted. This actively displaced the red pulp although the follicles were also larger than in control samples.

The renal histopathological findings were inconclusive consisting of minimally hypercellular glomerular tufts apparently infiltrated by lymphocytes and thin lymphocytic infiltrates centred on either basophillic or degenerate tubules. Clearly there is a possibility that CD40 ligation might cause tubulo-interstitial nephritis. However, in my data the findings were subtle and did not appear to correlate with increasing dose of 3/23. In addition their was no evidence of tubulo-interstitial nephritis in the mice treated with 1mg of 3/23 on days 0 an 10 and culled 3 months later.

If the developed human chimeric anti-CD40 (LOB 7/4) were to be used in a phase I trial against CD40 expressing human solid tumours, such as malignant melanoma and renal cell carcinoma, I anticipate that the monoclonal antibody would induce a dose-dependent transaminitis that would limit the maximum-tolerated dose. However, I would anticipate that the transaminitis would be short-lived and would not result in persistent chronic active hepatitis.

CD40 ligation has a profound effect on the cellular nature and distribution within the spleen[121]. The long-term consequences of this are unknown but the induced hypersplenism could result in excessive red blood cell destruction and platelet sequestration within the spleen causing anaemia and thrombocytopaenia respectively.

The widespread expression of CD40 is a concern. It is possible that CD40 ligation might result in many other sequelae, in particular auto-immune phenomena and depletion of normal B cells, monocytes, endothelial cells, dendritic cells and other normal cell types[96]. However, in this mouse model, and in the phase I study using SCD40L no other immediate treatment consequences were found. Further pre-clinical toxicology must include similar pathological investigations using the developed chimeric anti-CD40 given to balb/c mice and another species[119], such as guinea pigs or hamsters. However, a human monoclonal antibody given to another species is unlikely to reveal significant toxic side effects. The only reliable data will come when the antibody is tested in humans.

The developed chimeric anti-CD40 antibody has now been sent to Professor J Hales laboratory in Oxford for larger scale production and quality control. If current levels of production are maintained we will propose an appropriate phase I protocol to the new drugs committee with the intention of treating selected patients in the near future.

# Proposed Phase I Protocol

**Aim**

To determine the toxicity, maximum tolerated dose (MTD), and pharmacokinetics of the human chimeric anti-CD40 monoclonal antibody (chLOB 7/4).

**Patients**

Appropriate patients include those with advanced, CD40 expressing solid tumours or non-Hodgkin's lymphoma (excluding low-grade NHL) who have developed disease progression despite any number of conventional cancer therapies.

**Method**

Patients will receive intra-venous injection of chLOB 7/4 on day 1 (T1/2 likely to be >7 days) in a dose escalation study

Subsequent courses will be given every 4 weeks until disease progression

The starting dose will be 10mg/kg D1 q4weekly**
The dose will be increased by 5mg/kg for subsequent courses until MTD is reached

Treatment will stop if patient shows evidence of disease progression, they have unacceptable side effects or they reach 12 months from their initial start date

**This dose was calculated to be equivalent to 1/10 of the dose below the level that caused the first sign of toxicity in mice

**Monitoring**

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| Clinical assessment | Clinical evaluation will be performed on a regular basis, as outlined below |
| Weekly | Research led nurse evaluation (including measurements of measurable lesions)<br>To be discussed with the assigned, responsible doctor if clinical evaluation or investigations highlight a problem |
| Monthly | Full clinical evaluation by the responsible Doctor |

**Blood**

| | |
|---|---|
| Weekly | Full blood count |
| | Urea and electrolytes |
| | Liver function tests |
| | Urinalysis |
| Monthly | Auto-immune profile |
| | Serum protein electrophoresis |
| | Reticulocyte count |
| | Blood film |
| | C-reactive protein |
| | Erythrocyte sedimentation rate |
| | T-cell and dendritic cell activation studies |

**Radiology**

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| 3 monthly | CT scans chest/abdomen/pelvis |
| | CT head if considered appropriate |

| | |
|---|---|
| **Toxicity** | Toxic side effects will be graded according to the World Health Organisation classification.<br><br>They will include: |
| Haematological | Haemoglobin and Platelet assessment |
| Renal | Serum creatinine assessment and Urinary casts, protein etc |
| Liver | Liver function tests |
| Autoimmune | Autoimmune profile |

## Summary

I have shown evidence to support the role of CD40 ligation as an immunotherapeutic strategy against human cancers. This approach could have direct anti-tumour effects and the ability to 'boost' the immune system to overcome its inherent tolerance of malignant cells. This 'double hit' approach should eliminate the need for conjugation of the antibody or soluble ligand with radionuclide or immunotoxin, and bispecific types would offer no additional advantage. The toxic sequelae of immunotoxins and radionuclides would be avoided, and the additional financial and other resource burden of conjugating antibodies or developing bispecific types would be eliminated.

I have developed a human chimeric anti-CD40 monoclonal antibody that could be tested in the proposed phase I trial against human CD40 expressing malignancies, providing further preclinical toxicology reveals no unexpected complications that might be to the detriment of the patients we intend to treat.

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