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A study of signal transduction events involved in anti CD40 therapy of lymphoma

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES
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A STUDY OF SIGNAL TRANSDUCTION EVENTS INVOLVED IN ANTI CD40 THERAPY OF LYMPHOMA

by Jelena Mann

CD40 is essential in enabling antigen-presenting cells to process and present antigen effectively to T cells. Previous data shows that distribution of antibody raised against CD40 in mice with syngeneic lymphoma resulted in a rapid cytotoxic T-cell response independent of T-helper cells. This response eradicated the established lymphoma and provided protection against secondary tumor re-challenge without further antibody treatment. Although the precise mechanism of in vivo action of anti CD40 antibodies is not clear, indirect evidence suggests that dendritic cells (DCs) play a major role in establishment of the response. It is also possible that tumour cells activated through CD40 signaling may be important. Both of these cell types were assessed for their role in the anti CD40 therapy.

To establish domains in CD40 receptor which may be important in signalling, a range of human CD40 mutants was constructed and stably expressed in mouse B cell lymphoma line A20. Transfected cell lines were tested in *in vivo* therapy using anti human CD40 antibodies. However, this treatment provided no protection to the treated animals over the controls. It was subsequently found that A20 cells were productively infected with retroviruses which could interfere with signalling in the cells, thus making this cell line unsuitable as a model for further studies.

Remaining studies concentrated on the responses evoked in DCs activated through CD40 signalling. Activated DCs not only have the unique ability to sensitise naive T cells, but they also determine the nature of the response. DCs can "instruct" naive T cells to respond in a certain manner through complex interactions of various surface molecules on both DCs and T cells, also through production of appropriate cytokines which can further induce T cell activation and proliferation, such as IL6.

The intracellular signalling events that lead to CD40 ligation-induced activation of IL6 gene transcription in a murine DC line, FSDC, were determined. IL6 RT-PCR and promoter assays established the responsiveness of FSDCs to anti CD40 antibody ligation. Further promoter assays showed that the transcription factors NFκB and AP1 are downstream transcriptional mediators of CD40 induced IL6 gene expression. Anti CD40 treatment of FSDCs stimulated increased expression of specific NFκB (p50:p65) and AP1 (c-Jun, JunB, JunD and c-Fos) DNA:protein complexes. Over-expression of an IκBα dominant negative repressor or a dominant negative JunD resulted in a strong inhibition of CD40 inducible IL6 promoter activity supporting a role for both transcription factors.

Upstream signal transduction events were studied by transfection of wild type and mutant human CD40 expression constructs into FSDCs followed by stimulation with an anti human CD40 antibody. These experiments revealed that anti-CD40 stimulation of NFκB and IL6 gene transcription requires specific amino acid residues in the cytoplasmic region of CD40 involved in the recruitment of TRAF2. Induction of IL6 mRNA by anti CD40 treatment was found to be a transient event and was followed by a diminution of IL6 transcripts to levels below those found in unstimulated cells. This loss of IL6 expression was associated with reduced p50:p65 NFκB binding and elevated binding of CBF1 to a site overlapping the NFκB site. Over-expression of CBF1 resulted in a profound inhibition of basal and anti CD40 induced IL6 promoter activities indicating that prolonged induction of CBF1 may contribute to the transient nature of IL6 response.

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Ovaj rad je posvećen mojim najdražim roditeljima

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Abbreviations

AEBSF	4-(2-Aminoethyl)benzenesulfonyl Fluoride
AP1	Activator protein 1
APC	antigen presenting cell
APS	Ammonium persulphate
ATP	Adenine triphosphate
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyl transferase
CD	cluster of differentiation
CRE	cAMP-response element
DC	dendritic cell
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DNTPs	Deoxynucleoside triphosphates
DTT	Dithiothreitol
EM	electron microscopy
FITC	fluorescein iso-thiocyanate
hCD40	human CD40
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
ICAM	intercellular adhesion molecule
IκBα	inhibitor of kappa binding
IKK	IκB kinase
IL	interleukin
JAK	Janus Kinase
JNK	c-Jun NH_2-Terminal Kinases
L	ligand
LB	Luria Broth
mAb	monoclonal antibody
MAPK	Mitogen activated protein kinases
mCD40	mouse CD40

NFκB	nuclear factor kappa B
NIK	NFκB Inducing kinase
PAGE	Polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RT-PCR	reverse transcript polymerase chain reaction
SDS	Sodium dodecyl sulphate
STAT	Signal transducer and activator of transcription
Taq	Thermus aquatus
TEM	transmission electron microscopy
TEMED	N,N,N',N'-tetramethylethylylenediamine
Th	T helper cell
Th1	T helper type 1
Th2	T helper type 2
TNF	tumour necrosis factor
TRAF	tumour necrosis factor receptor associated factor

CHAPTER 1

General introduction

1.1 General introduction

Cells are the building blocks of an organism. Many cells within the body have the ability to grow and divide. Both of these processes are under a tight control so as to prevent unwanted and possibly detrimental cell proliferation. When cell division becomes unregulated, it leads to formation of a tumour. A tumour, or a neoplasm, is defined as a spontaneous new growth of tissue forming an abnormal mass. In many cases, the tumour remains a benign, non-invasive mass that does not spread. In other instances, tumours can continue to grow and become progressively invasive; these are designated as malignant. The term cancer refers specifically to a malignant tumour. In addition to uncontrolled aggressive growth, malignant tumours exhibit metastasis. Metastasis is a process in which small clumps of cancerous cells bud off from the primary tumour, gain entry into blood or lymphatic vessels, and are then able to gain entry into other organs, where they continue to divide. In this manner the primary tumours can create secondary sites of growth in other organs (Whitehouse M *et al* 1996). Both the primary tumour and the metastasis can eventually cause death by preventing the affected tissue or organ from functioning or by causing severe architectural damage to the surrounding tissue.

Cancer results from mutation in cellular DNA. Mutations can arise during the normal replicative cycle of the cell; they may be inherited, or caused by external stimuli, such as ionising radiation, UV, tobacco and asbestos (WHO report 2000). Although all nucleated cells have the potential to become cancerous, the likelihood of a neoplasm emerging is directly linked to the proliferative capacity of the cell. Therefore, tumours arising from cells which are constantly dividing, such as epithelial cells, are much more common, than those found in cells which are quiescent and terminally differentiated, such as neurons.

Cancer currently kills around 7.5 million people world wide per year. There are an estimated 200 different kinds of cancers, generally grouped according to the embryonic tissue from which the tumour is derived. The majority of cancers are carcinomas, arising from endodermal and ectodermal tissues such as skin or the lining of the internal organs and glands. The leukemias and lymphomas are cancers of hematopoietic cells; leukemias proliferate as single cells, whereas lymphomas grow as

solid tumours. Finally, sarcomas emanate from mesenchymal tissues, such as connective tissues, bone, fat and cartilage and have much lower incidence than the previous groups of cancer (Whitehouse M *et al* 1996).

For a number of years efforts have been made in order to elucidate ways of eradicating cancer. Classical therapeutic modalities for the treatment of cancer are surgery, radiation and chemotherapy. The latter two therapies are based on killing dividing cells and preventing cell division. This therapy is however associated with considerable toxic side effects that result from the loss of normal healthy cells that divide as part of the normal regenerative process of some organs (eg gut mucosa and liver). In addition, these standard treatments fail to cure certain neoplastic diseases, and can lead to severe and debilitating side effects. Furthermore, the high morbidity associated with their use makes them less than ideal treatments.

As many of the existing treatments are not ideal, numerous research groups have turned towards exploring the possibilities of treatment using immunotherapy (Scott and Welt 1997, Stevenson 1993, Stevenson 1992, Grossbard *et al* 1992, Old 1996, Scott and Cebon 1997). Immunotherapy is an approach where the components of the immune system are used to attack cancer cells. The first attempts at a biological therapy of cancer were made approximately 100 years ago and were based on the assumption that tumour cells could be recognised as 'foreign' by the immune system. In an early report dating back to 1898, the use of dog anti-serum to treat cancer was described (Stevenson 1993). Immunotherapeutic approaches have only been realistically considered in the last 40 years since the concept of immune surveillance came to light. This concept proposes that cancerous cells may express products of mutated, amplified or reactivated genes, which can lead to the presence of altered or novel antigens. These altered antigens can then be recognised as foreign by the immune system (Burnett 1970). Once found, the cells carrying novel/altered antigens are targeted by the immune system and destroyed. Evidence to support the idea of immune surveillance has been provided in melanoma models and in the control of virally induced tumours (Boon 1994). Likewise, increased incidence of tumours in immunosupressed individuals, such as seen in AIDS patients, also supports the idea that an intact immune system deals with tumour cells in a very efficient manner. Therefore, finding ways in which the immune system may be aided, supplemented or indeed manipulated may lead to eradication of

tumours which have initially escaped "immune surveillance". This idea gives rise to enthusiasm regarding immunotherapy.

Studies utilising mAbs in the treatment of cancer have been performed in the Tenovus Southampton laboratory for a number of years. We are particularly interested in the treatment of lymphoma, which present a diverse group of neoplasms, accounting for approximately 5-6% of human cancers (WHO report 2000). Most of these cancers originate from T or B cell lineage, and are often restricted to a particular maturation stage of the parental cell (Hiddemann 1996, Harris 2000, Isaacson 2000,see figure 1.1).

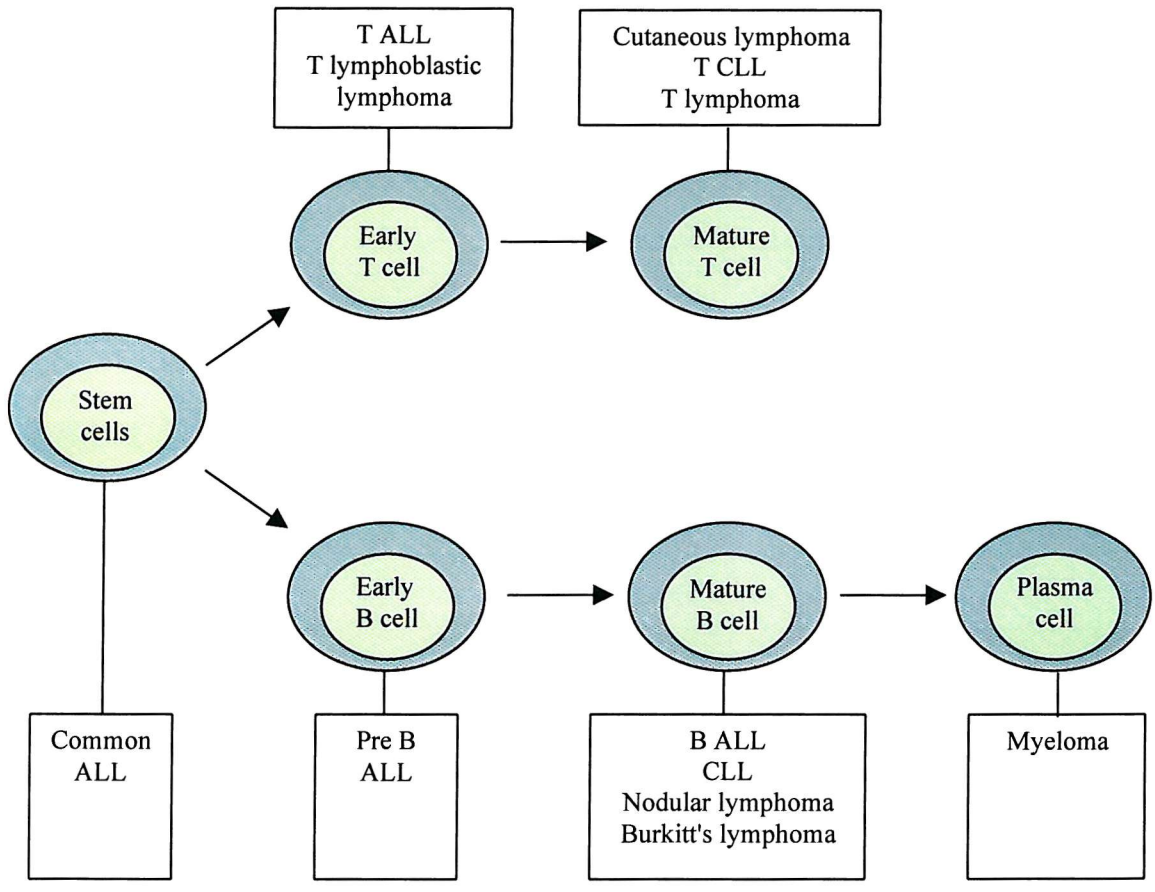


Figure 1.1- Cellular phenotype of human lymphoid malignancies

ALL= acute lymphoblastic leukemia, CLL= chronic lymphocytic leukemia

Lymphomas usually present in the lymphoid organs such as the spleen or lymph nodes, and often have over-spills in blood which makes them easy to isolate and study (Stevenson 1992). In the past 30 years, research at the Tenovus laboratory has been carried to test the efficiency of immunotherapy in treatment of B cell lymphomas. This has been achieved through *in vitro* studies using both human and mouse B cell lymphoma cell lines, and various *in vivo* animal models of the disease. In order to gain full appreciation of the findings so far and the current ideas which have led to work undertaken in this thesis it is necessary to give a basic overview of the immune system. The way in which the various components of the immune system interact with each other, the manner in which they can be re-directed to recognise tumour cells as non-self by the use of immunotherapy and the mechanisms utilised for destruction of tumour cells will also be discussed. The text to follow gives a short overview of the immune system.

1.2 Brief overview of the immune system

From birth, the human body is exposed to a large number of harmful microorganisms and viruses, which can cause diseases and potentially death. In order to protect the body against such insults, an elaborate array of defence measures and mechanisms have been developed, collectively known as the immune system. The immune system is able to generate a large variety of cells and molecules capable of recognising, neutralising and eliminating invading organisms, viruses and altered self-cells (Sher and Ahmed 1995). On the most basic level, the immunity to a disease can be divided into two broad categories: the innate or non-specific immunity and the adaptive or specific immunity (Kuby 1997, see figure 1.2).

1.2.1 Innate immunity

Innate immunity is a set of protection mechanisms that display no specificity for a particular pathogen. Innate responses include anatomic, physiologic, phagocytic and inflammatory barriers, which together help prevent the entry and subsequent establishment of the invading particle. The cellular components of the innate immune

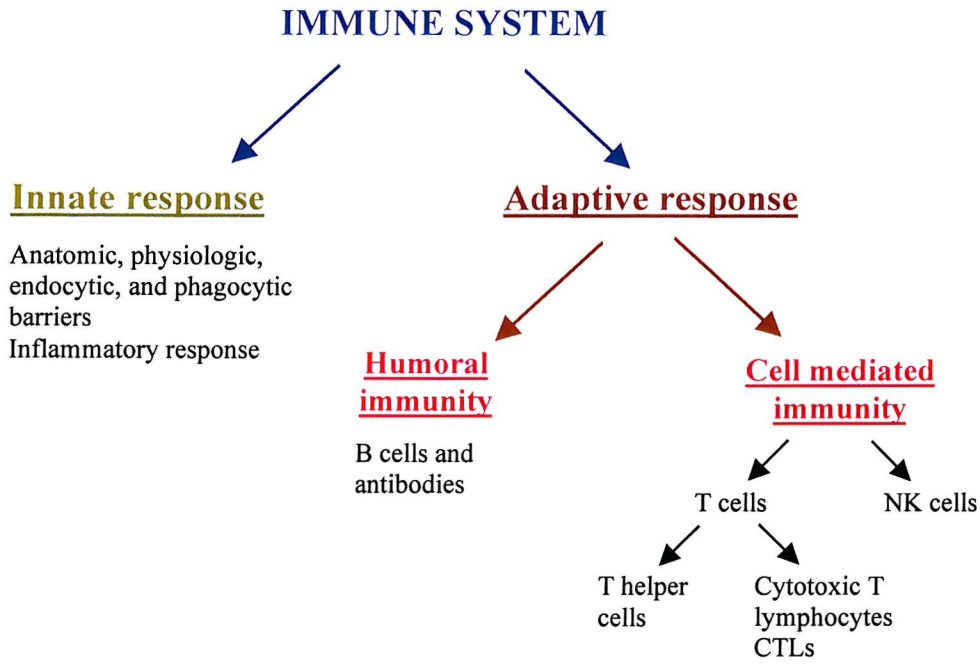


Figure 1.2- The immune system divided into its basic subcomponents

system includes a number of cell types including granulocytes, natural killer (NK) cells and macrophages. The three main types of granulocytes are neutrophils, eosinophils and basophils. These cells can be activated to phagocytose foreign material or to release toxic oxygen radicals which can damage invaders by oxidative damage. Cells of the innate system are the first to be attracted to the site of infection through the action of chemokines released by damaged cells and tissues. Once at the site, macrophages and NK cells secrete cytokines such as interferons (IFNs), interleukin 12 (IL12) and tumour necrosis factor (TNF), which apart from stimulating the action of granulocytes, also recruit lymphocytes into the area (Araik *et al* 1990). The innate response therefore acts as a first line of defence enabling time for the body to mount an acquired response. Due to its relatively non-specific action, the innate immunity has a limited role in tumour immunity.

1.2.2 Adaptive immunity

The acquired or adaptive immune system is highly specific and is achieved by an orchestrated interaction of lymphocytes, which are the central cells of the immune system (Grey *et al* 1989). Lymphocytes interact with each other and other cells via a system of antigen-specific receptors. These interactions bear four hallmarks of adaptive

immune response: specificity, diversity, memory and self/non-self recognition. The system is composed of several components including B cells, T cells, NK cells and antigen presenting cells (APC). Although many cell types in the body have the ability to present antigen, only a limited number of these cells can fully activate lymphocytes and induce an immune response. The cells that have this ability are known as professional APC (pAPC), and this group includes macrophages, dendritic cells and activated B cells. Although all three groups of cells can activate T cells, only dendritic cells have the capacity to activate naive T cells (Steinman *et al* 2000). The importance of T cell activation by the dendritic cell is discussed in further detail in section 1.4.5. Cells other than lymphocytes play an accessory role in the adaptive immune response, serving to augment lymphocyte activity by means of providing an optimal microenvironment.

1.2.3 Cellular and humoral immunity

When the body becomes exposed to a virus or other type of infectious agent for the first time, the innate immunity is the first line of defence. The adaptive response, which is necessary to clear the infection, takes much longer to develop during the primary challenge with a particular agent. Once the acquired response is mounted, the infection is resolved, and in many cases protective immunity against that particular agent is established. This means that the body gains the ability to eradicate a second infection with the same agent before any clinical manifestations occur. This capacity to mount a secondary response can be transferred from an immune individual to a non-immune person. Such procedure has been used to subdivide the acquired response into two types of immunity. Humoral immunity is provided when serum is used to transfer the response (Rudin *et al* 1998). By contrast, cellular mediated immunity requires the transfer of lymphocytes in order to acquire immunity. The cell-mediated branch of the immune system involves effector T cells, which can recognise antigen presented by self APCs in the context of their major histocompatibility (MHC) molecules (Croft *et al* 1994, Griffiths *et al* 1995, Berke 1995). There are two main groups of T cells, namely T helper (Th), which are CD4+ve, and cytotoxic T cells (CTLs),which are CD8+ve. Th cells respond to antigen by producing cytokines, whereas CTLs mediate killing of altered self-cells, such as virally infected cells as well as tumours. Humoral immunity refers to the immunity mediated by immunoglobulins (Ig), or antibodies (Ab). In the

humoral response, B cells interact with the antigen, and then differentiate into Ab-producing plasma cells (Lane 1996, Gallagher and Osmond 1991). The Abs produced by the B cells bind to the antigen thus facilitating its clearance from the body (Brekke *et al* 1995, Kinoshita *et al* 1991, Heyman *et al* 2000, Burton *et al* 2000). The text to follow will examine the details of humoral and cell mediated immunity and their role in tumour clearance.

1.3 Humoral immunity

Humoral immunity refers to the immunity that can be bestowed upon a non-immune individual by transfer of serum Abs from an immune individual. Abs are produced by B cells, which have the ability to produce Abs of high specificity for the given antigen. However, any one B cell can only produce Abs of single specificity. It is thought that the body may be able to generate a total Ab diversity approaching up to 10^{11} (Manser *et al* 1998, Bengten *et al* 2000). The text to follow will attempt to explain what Abs are and how they are produced by B cells during an immune response, as well as how such a vast array of different specificities is achieved.

1.3.1 Immunoglobulins or antibodies

Porter and Edelman deduced the basic structure of immunoglobulin (Ig) during the 1950/60s in a series of independent experiments (Porter 1970, Porter 1971, Cohen *et al* 1964, Edelman *et al* 1968). They hypothesised Ig structure to be an association of two heavy and two light chains, linked by both non-covalent and di-sulphide bonds. These initial findings have been later expounded by electron microscopy and X-ray crystallography studies. Representation of Ig structure is illustrated in Figure 1.

1.3.2 Immunoglobulin structure

Immunoglobulins, or antibodies, are made up of 4 polypeptide chains of two distinct types. These are two light chains, which are smaller, and two heavy chains, which are larger (Virella and Wang 1993). The light chain is common to all classes of Ig whereas the heavy chain is structurally distinct for each class of the antibody. The light chains have been found to exist in two forms called kappa (κ) and lambda (λ). Either of these can

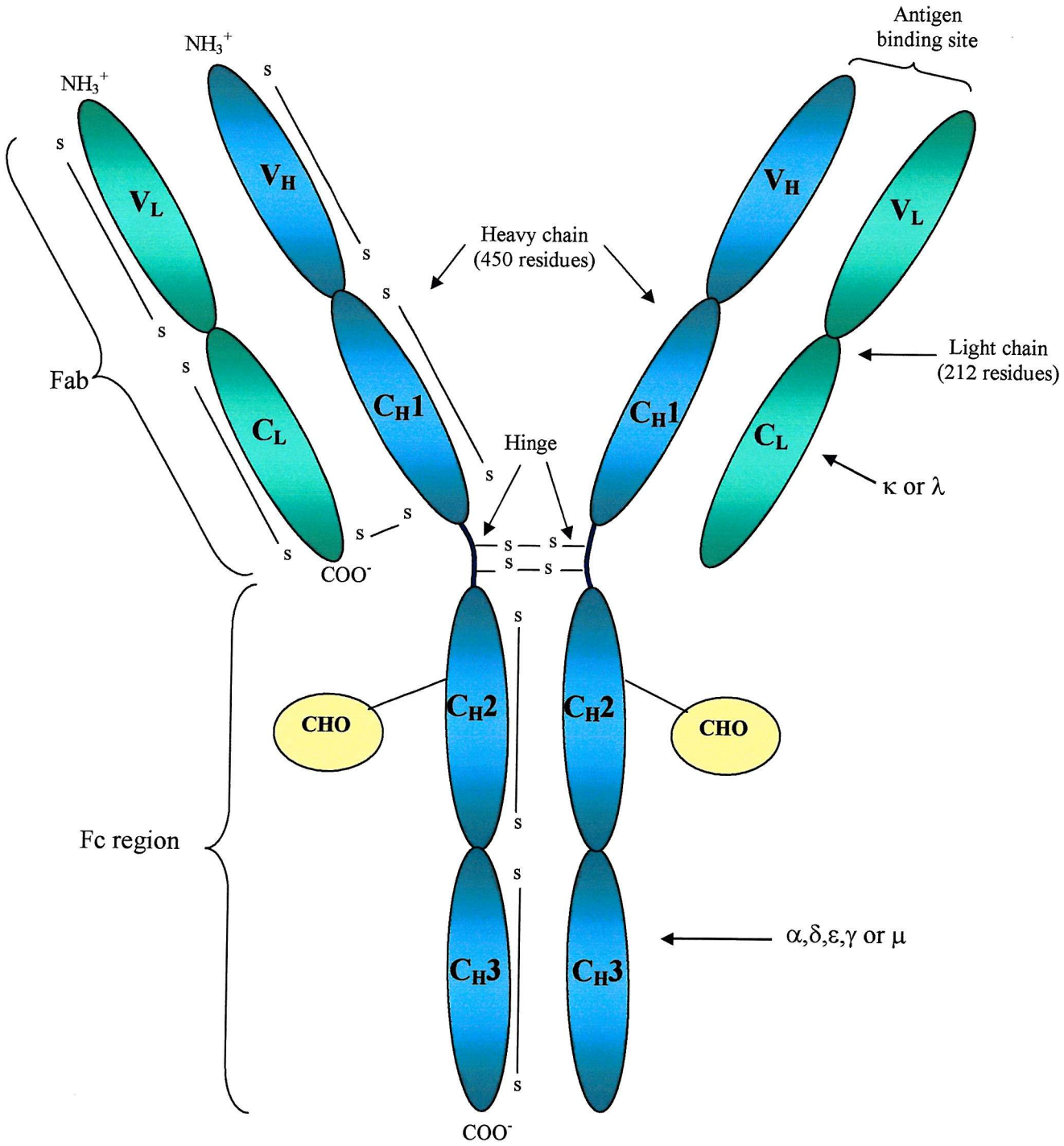


Figure 1.3- Basic diagrammatic representation of an antibody. Shown is an illustration of IgG, detailing heavy and light chain, constant and variable domains, and linking di-sulphide bonds.

combine with any of the heavy chains, but in any one Ab both the heavy and the light chains are of the same isotype (Reth *et al* 1994).

The light chain consists of two regions. The carboxyl terminal portion of the chain is constant except for some limited allotypic and isotypic variation. The amino terminal half shows a high degree of variation between different Abs and is therefore termed variable (V). The heavy chain also consists of two domains. The amino terminal part shows great variation among other heavy chains and is therefore termed the variable heavy domain (V_H). The remaining part of the protein reveals five basic sequence patterns correlating to five different constant regions (C_H). These are termed α, δ, ε, γ and μ. Each C_H region determines the isotype or the class of the Ab, so that μ is the heavy chain associated with IgM, α with IgA, δ with IgD, ε with IgE and γ with IgG. Minor differences in γ and α heavy chains have led to further classification of these into subclasses. In mice there are four IgG subclasses (γ1, γ2a, γ2b and γ3). In humans there are also four IgG subclasses (γ1, γ2, γ3 and γ4) and two subclasses of IgA (α1 and α2) (Jefferis *et al* 1998, Tamm *et al* 1997, Hexham *et al* 1997).

1.3.3 Hypervariable regions

Within the variable regions of both the heavy and light chains of an Ab are short polypeptide segments which show exceptional variability. These are termed hypervariable regions. The hypervariable regions form the antigen-binding site, and are therefore also referred to as complementarity determining regions (CDRs). The sequences intervening the CDRs are designated as framework regions (FRs). In both the heavy and the light chain there are three CDRs (CDR1, CDR2 and CDR3) and four FRs. The V_L and V_H are folded in a way that brings hypervariable, or the CDR, regions together to create the surface that binds the antigen (Sutton *et al* 1999, Kirkham *et al* 1994).

1.3.4 Effector functions

The primary function of an Ab is to bind an antigen in a highly specific manner (Sutton *et al* 1989). In some cases the binding of the Ab to the antigen has a direct effect, for example, by neutralising the toxin, or preventing viral entry into the cells. In most

cases, however, the Ab needs to not only recognise and bind the antigen, but also must also evoke secondary effector responses, which will remove the Ag and kill the pathogen (Bengten *et al* 2000). For this mode of action following Ab/Ag binding, the Fc portion of the Ab becomes of paramount importance. This is because a number of mechanisms in the body have evolved to recognise antigen coated in Abs by recognising the exposed Fc region of that Ab (Unkeless *et al* 1988, Ravetch *et al* 1991). The type of response induced by the Fc domain is dependent on the class of Ab. There are five classes of Ab which differ in their C_H sequences, as well as size, charge, carbohydrate content and consequently their effector function. As effector functions depend on the heavy chain constant domains, not all classes of Abs have the same functional properties (Sogn *et al* 1988).

IgG is the most abundant Ig in the normal human serum constituting ~80% of total Ig. It consists of a single monomer with molecular weight of ~146 kDa. In humans there are four further subclasses of IgG, namely IgG1, 2, 3 and 4. Differences distinguishing subclasses from one another are determined by the size of hinge region and the number of disulphide bonds between the heavy chains. As a result the subclasses have different abilities in activating complement and binding to Fc receptors on phagocytic cells, as summarised in table 1. IgG is the major Ab involved in the secondary immune response and in the only Ab able to cross the placenta, thus conferring early foetal protection.

IgM is composed of five monomeric IgM units held together by a 15 kDa J chain and disulphide bonds. The pentamer has a predominant molecular weight (mw) of 970 kDa and accounts for 5-10% of total serum Ig. It is a predominant Ab secreted during the primary immune response, and is of particular importance in removing invading organisms as well as neutralising viral infectivity. IgM is more efficient than IgG in activating complement at low or threshold Ab concentrations (Soderlind 2000).

IgA constitutes 10-15% of serum Ig, but is the predominant class of Ab in excretions such as saliva, breast milk, tears and mucous secretions of digestive, genitourinary and bronchial tracts. The secreted form of IgA exists mainly as a dimer with a mw of 385 kDa, which, as in IgM, includes a J chain. IgA plays a very important role in defending the body against the invading pathogens by binding to their antigenic sites thus preventing their entry into the body (Hexham *et al* 1997).

IgD accounts for ~0.2% of Ig found in the serum. Together with IgM, it is a major membrane bound Ig expressed on the surface of B cells. The function of IgD remains unclear, although it appears to be an antigen receptor on resting B cells (Boes *et al* 2000).

IgE is present at very low concentrations in the blood. It is found bound to high affinity Fcε receptors on mast cells, basophils and eosinophils. Crosslinking of the Fcε receptor bound IgE results in degranulation of receptor bearing cells, leading to allergic manifestations, asthma and hay fever. IgE is also thought to play a role in providing immunity from parasitic organisms (Dorrington *et al* 1978).

	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	IgD	IgE	IgM
Heavy chain	γ1	γ2	γ3	γ4	α1	α2	δ	ε	μ
Mean serum conc (mg/ml)	9	3	1	0.5	3	0.5	0.03	$5x10^{-5}$	1.5
Mol.wt ($x10^3$)	146	146	170	146	160	160	184	188	970
Half-life (days)	21	20	7	21	6	6	3	2	10
C fixation (classical)	++	+	+++	-	-	-	-	-	+++
Cross placenta	++	+/-	++	++	-	-	-	-	-
Mast cell binding	-	-	-	-	-	-	-	+++	-
Monocyte/MO binding	+++	+	+++	+/-	+	-	-	+	-
ADCC	+++	+	+++	-	-	-	-	-	-

Table 1.1- Biological and physiochemical differences of human Ig classes/subclasses. (adapted from Roitt I.(1997) Essential Immunology, *ninth* edition, Blackwell Science, Oxford, UK, and Stevenson *et al* (1993))

The various functions of the different classes of Ab depend primarily on their interactions with the cytotoxic effector cells and the complement system. A brief summary of these two pathways is given in the table 1.1. Importantly, the ability of Ab to activate these effector mechanisms has been shown to be not only class, but also sub-class dependent.

1.3.5 Monoclonal antibodies

Monoclonal antibodies (mAbs) are Abs of single antigenic specificity which are produced by clones of a single B cell. They were first described in 1975 by Milstein and Kohler, work for which they later shared a Nobel Prize in Medicine and Physiology in 1984 (Kohler and Milstein 1975). In their seminal paper they described a method for making cell lines that secrete a single species of antibody of desired specificity. This is achieved by fusing a normal B cell, which secretes the mAb of interest, with an immortal partner, a myeloma cell, to produce a hybridoma. The process allows for the synthesis of large quantities of mAb in a controlled manner and has permitted the production of a wide range of mAbs targeted against various antigens. Some of the antigens are found on the surface of tumour cells, hence mAbs have found applications both in treatment and diagnosis of cancer (Scott *et al* 1997a, Stevenson 1992, Stevenson 1993, Grossbard *et al* 1992, Old 1996). Their use in both research and medicine is also merited by their fine-specificity of antigen recognition coupled with the relative ease of production *in vitro* as well as *in vivo* (Old 1996, Scott *et al* 1997b). For this reason mAbs have also found applications in the modulation of the immune response, in countering autoimmunity and in transplantation rejection (Mohan *et al* 1995, Larsen *et al* 1997, Kirk *et al* 1999, Liu *et al* 1999).

Since the invention of the technology, hybridoma cells have been cultivated either *in vitro*, or *in vivo* as ascites. mAb production *in vivo* is classified as a procedure that causes discomfort, distress and/or pain to the animals and therefore alternatives have been found where possible (Falkenberg *et al* 1998). Some of the newer "replacement" methods include expression of recombinant Ab fragments in various eukaryotic and insect cell lines, phage display technology, use of translocus mice carrying human heavy and light chains encoded for by yeast artificial chromosome, production of Fv fragments in plants using a potato virus vector and in the milk of transgenic dairy animals (Green 1999, Hudson 1999, Verman *et al* 1998)). Having discussed what Abs and mAbs are, the text to follow briefly considers the development of the cells which produce Abs, the B cells.

1.3.6 B cell development

B cell development occurs in the foetal liver and in the bone marrow of mammals. B cells originate from a lymphoid stem cell which gives rise to the progenitor or pro B cell. The pro B cell then proliferates, stimulated by the various cytokines and interleukins secreted by the stroma of the bone marrow (Rolink *et al* 1996, Rolink *et al* 1999, Rudin *et al* 1998). This leads to differentiation of a pro B cell into a pre B cell. At this stage expression of the Ig μ gene, the first H chain expressed in B cells, is induced. Initially, the D and J genes rearrange at which time an immature, V_H lacking, D-J-Hμ chain may be expressed. This molecule is expressed concurrently with surrogate L chain molecules, λ5 and $V_{pre}B$, which contain C-like and V-like properties respectively. At a later stage of pre B-cell development, a functionally arranged μH is expressed, again with λ5 and $V_{pre}B$. Once a functional H chain is generated, rearrangement of the second H allele is halted, accounting for the phenomenon of allelic exclusion, whereby only single species of H chain are present in the mature Ig. If a functional H gene fails to be generated, the cell is deleted by apoptosis. Although it is not understood how a successful rearrangement is detected, the process by which unsuccessful rearrangement occurs is understood. This latter process involves the V-J joining events; the process of V-J joining is imprecise, and results in an overlap between the V and J regions. Consequently, the sequence in this region will be altered from that in the germ-line. Although this process in part results in hypervariability, it may equally be responsible for shifts out of the coding frame, resulting in a non-functional protein. Additional sequence heterogeneity of Ab may also be introduced at this stage, through the actions of an exonuclease. At the V-D and D-J joins in the H chain, terminal deoxynucletidyl transferase (TdT) can either add or remove a number of random nucleotides, thus increasing the sequence variability.

Once a successful mature H chain has been expressed, rearrangement of the L chain starts. In the L chain rearrangement occurs first between the V_L and the J_L segments. As with the H chain, the successful recombination results in allelic exclusion, whereas unsuccessful recombination ensues in attempted rearrangement of the other L allele. If both processes are non-productive, the cell is deleted by apoptosis. The V_LJ_L region is then joined to the C_L domain, thus accomplishing the production of a mature L chain and the formation of a fully functional B cell receptor (BCR). This causes the cell to

progress to the immature B cell stage of development. The large number of exons, coupled with the imprecise nature of domain joining and the introduction of random bases during development all ensure that each B cell has a unique BCR.

At this stage the immature B cell has its first opportunity to respond to antigen. Firstly, the ligation of randomly generated BCR with a self-antigen in either the periphery or in the bone marrow results in apoptosis. Surviving B cells mature and start expressing IgD on their surface, in addition to IgM. Mature B cells react in a positive manner to the BCR engagement, so that when the BCR encounters an antigen the cells will proliferate, providing there are adequate co-stimulatory signals present. The co-stimulatory signals vary depending on the nature of antigen and according to whether specific T cell help is required for proliferation or not. The details of the co-stimulation events are beyond the scope of this text, and will not be discussed further. In summary, if the correct co-stimulation is not received by the B cells, they may either apoptose or become anergic or unresponsive to that given antigen. If the correct co-stimulation is received following the ligation of the BCR, the mature B cell becomes activated and develops into an IgM-producing plasma cell, or a memory cell (Cushley *et al* 1993). Cells which are to become memory cells undergo class switching and further somatic hypermutation of their V regions (Lane *et al* 1996). This allows the cell to express classes of Ig other than IgM and also promote the development of a higher affinity secondary response. The process of B cell development into memory cells takes place. in germinal centres, through interaction with follicular dendritic cells. Briefly, activated B cells arrive in the GC and unless they receive further signals the cells undergo apoptosis. The two main signals required by the B cell for secretion are BCR ligation and interaction with CD40L. Both of these requirements are met by the follicular dendritic cells, which present antigen in the form of immune complexes and also express CD40L. This type of interaction allows B-cell survival and facilitates maturation into memory cell phenotype (Gallagher *et al* 1991). The memory B cells then provide the immune system with the capacity to respond to a secondary antigen challenge in a fast and powerful manner.

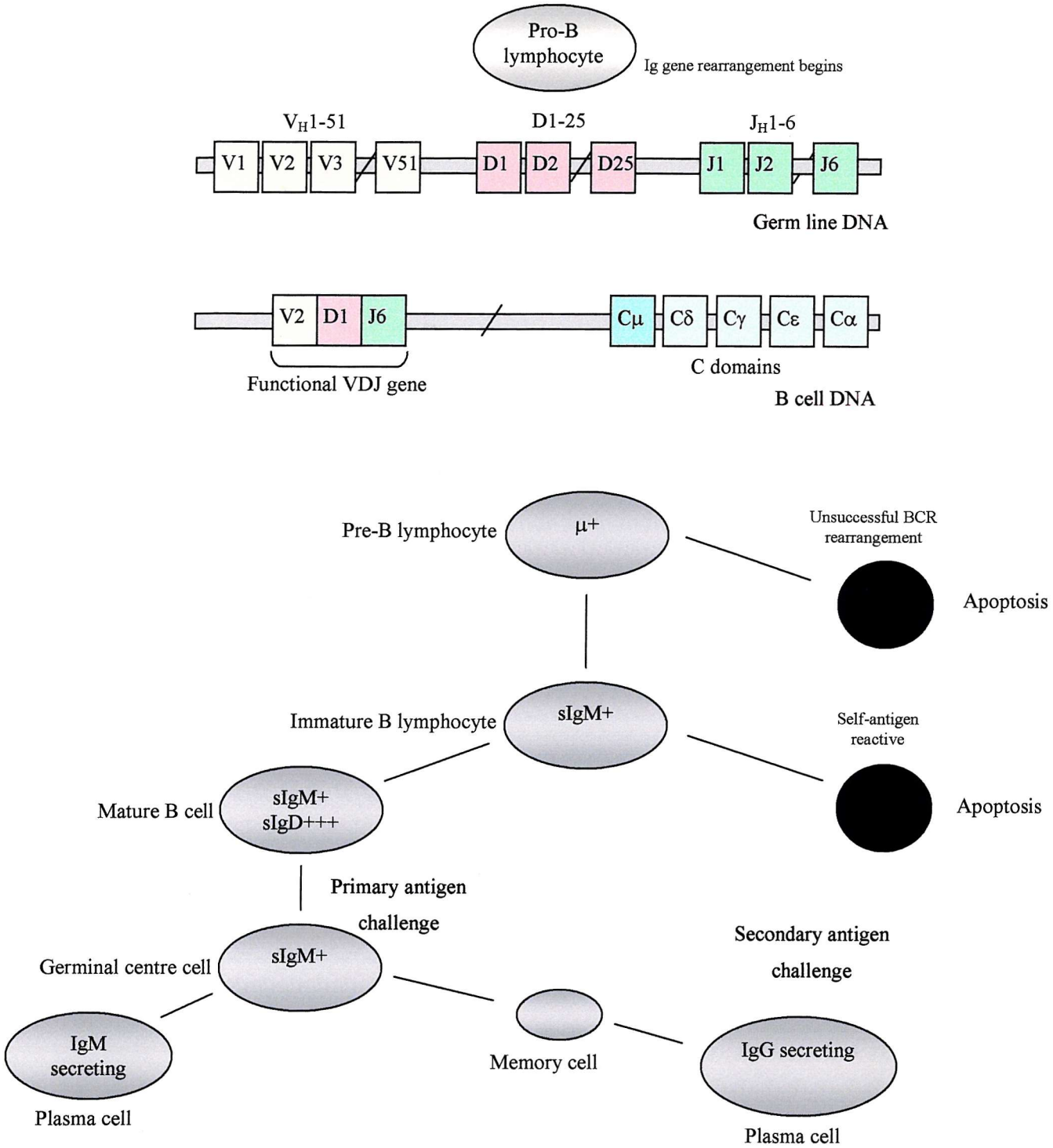


Figure 1.4- Overview of B cell development. Following initial gene rearrangements, primary and secondary exposure to antigen. The recombination events which occur in the V_H regions prior to antigen challenge are illustrated. Analogous process also occurs in V_L. the predominance of IgM isotype throughout B cell development is shown, and the transitory nature of the IgD surface expression.

1.4 Cell-mediated immunity

Cell-mediated immunity is conferred by the action of T and NK cells. T cells are the main cell type involved in cell-mediated immunity, although NK cells also have a large role in deletion of altered and infected cells (Elewant 2000). As T cell immunity is dependent upon activation status of T cells, it is also important to discuss the professional antigen presenting cell which can activate naive T cell, the dendritic cell (DC). How these cell types develop and what their mode of action is will be described in this section.

1.4.1 T cell development

Lymphocytes represent a significant proportion of the cellular immune system. Lymphocytes themselves can be subdivided in B, T and NK cells, progenitors of which are found in the bone marrow (Kuby 1997). During early gestation, chemotactic factors secreted by the thymic epithelium mediate the migration of T lymphocyte progenitors to the maturation sites in the thymus (Anderson *et al* 2000, Akashi *et al* 1998). T cell maturation involves rearrangement of T cell receptor (TCR) genes and the expression of various surface markers. Developing T cells proliferate and differentiate along several alternative pathways that lead to the formation of distinct sub-populations of mature T cells (Kaye *et al* 2000). There are two major processes of T cell selection in the thymus known as positive and negative selection. These selection processes are based on the recognition of an MHC molecule, carrying a peptide derived from a self-protein, by the TCR (Viret *et al* 1999). Positive selection permits survival of those T cells whose TCR recognises self-MHC molecules (Janeway 1999). Negative selection eliminates T cells that react too strongly with a self-MHC or with a self-MHC carrying self-peptides. These combined processes generate a primary T cell repertoire that is self-tolerant (van Oers 1999). During the two processes of selection, T cells also commit to being either CD4 or CD8 surface antigen positive, thus having specificity for MHC class II or MHC class I, respectively. Once differentiated, CD4+ve and CD8+ve cells leave the thymus and enter the circulation (see figure 1.5).

random expression of αβ TCR repertoire		positive selection: exposure to MHC molecules	negative selection: exposure to self antigen	mature T cell pool
precursor	low αβ	low αβ	high αβ	high αβ

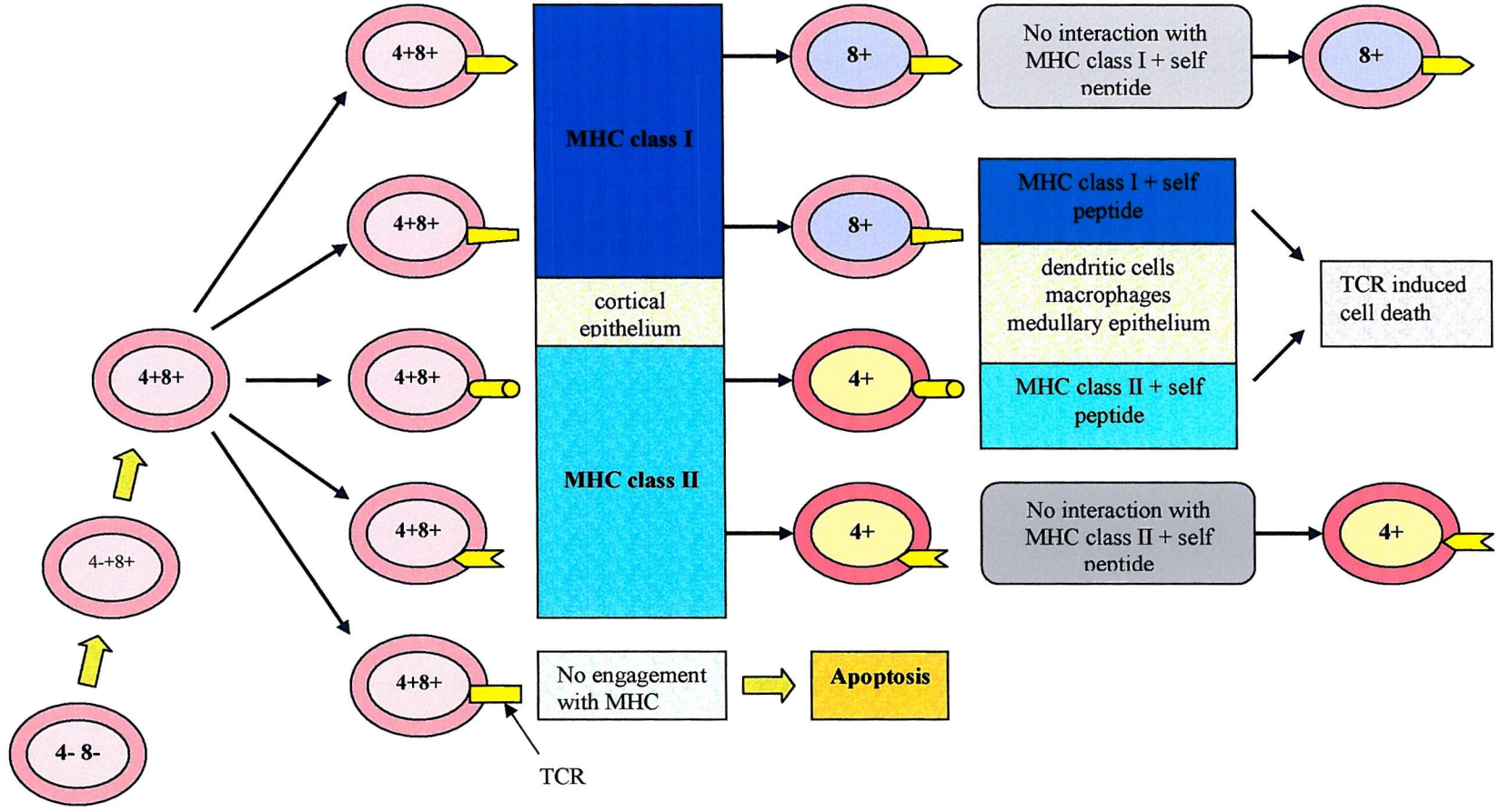


Figure 1.5-Developmental pathway of thymocytes- positive and negative selection (adapted from "Immunology" by Roitt, Brostoff and Male, fifth edition

1.4.2 CD4+ve T cells or T helper cells

CD4+ effector T cells form two sub-populations characterised by the sets of cytokines they secrete (Mossman *et al* 1996). The first subset, also called T helper 1 (Th1) cells, produce predominantly IL12, IL2, IFNγ and TNFβ. These cytokines are responsible for classical cell-mediated functions such as the delayed type hypersensitivity reaction and the activation of CTLs. The Th1 type response is required for deletion of virally infected cells and is thought to have a major role in the eradication of tumour cells. This latter function is achieved either by the direct effect of secreted cytokines (such as IFNγ and TNFα) or by induction of a CTL response through production of the aforementioned cytokines. The other subset of CD4+cells, called the Th2 subset, produces IL4, IL5 and IL10, which facilitate B cell activation. The Th2 response is thought to be induced in the process of controlling and resolving bacterial infections. Some CD4+ cells are found in an uncommitted state, and are therefore termed Th0 cells. However, these cells can be induced to undergo Th1 or Th2 polarisation. Recent publications suggest that the pAPC, mainly dendritic cell, interacting with Th0 cell can determine whether the cell becomes Th1 or Th2 polarised (Khama *et al* 2000, Skok *et al* 1999, Santiago-Schwartz *et al* 2000))(see figure 1.6).

CD4+ve cells, or Th cells, are activated by two sequential signals. Signal one is defined by the Th-TCR interaction with a specific MHC class II-peptide complex found on a pAPC. Following this event, CD40 ligand (CD40L) expression is up-regulated on the surface of Th cells. CD40L binds to CD40 on the pAPC, causing expression of B7.1 and B7.2 glycoproteins on the surface of the pAPC, and secretion of cytokines, such as IL12 and IL6. B7.1/B7.2 ligate with the surface marker molecule CD28 found on the Th cell, thus forming signal two. This occurrence causes Th cells to commence production of cytokines and proliferation. Aside of the Th cell activation, an equally important outcome of this sequence of events is "licensing" of the activated pAPC. This means that once activated, pAPC is enabled to deliver co-stimulatory signal two to other T cells, such as precursor CTLs (Ridge *et al* 1998, Schoenberger *et al* 1998, Bennet *et al* 1998) (see figure 1.7).

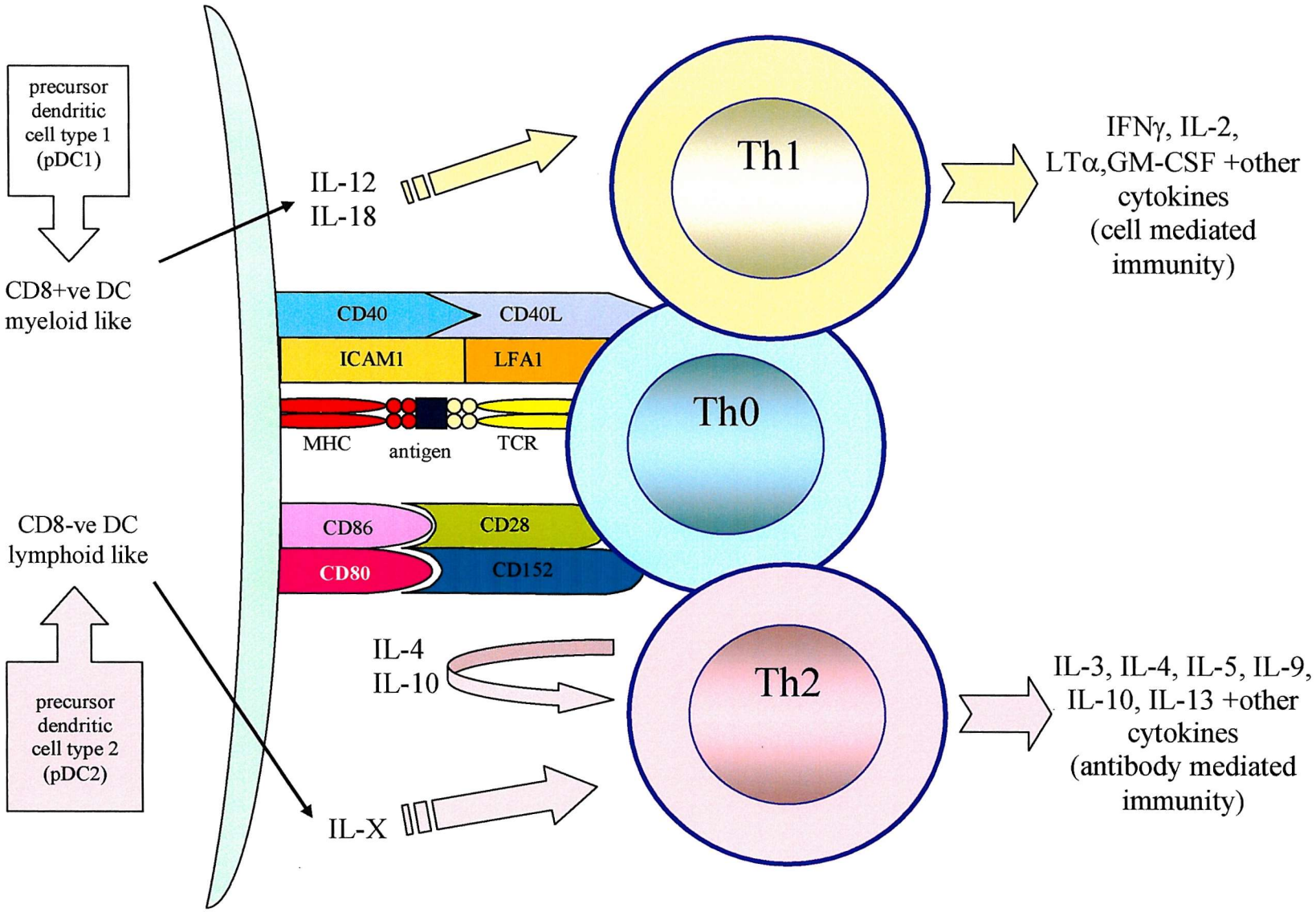


Figure 1.6- DC driven T helper response. Dendritic cells are specialised in presentation of antigen to T helper cells. Their status appears to decide whether Th1 or Th2 type cells are activated. Hence DC1, which predominantly secrete IL12, will differentiate Th0 to Th1, whereas DC2 though production of as yet unspecified cytokine (here called X) favour differentiation to Th2 cells.

1.4.3 CD8+ve T cells or cytotoxic T cells

Naive CD8+ve CTLs are incapable of killing target cells and are therefore called precursor CTLs (pCTL)(Hamann *et al* 1999). These cells acquire cytotoxic activity once they become activated, which is a process requiring three consecutive signals:

- ***Signal one*** represents a set of signalling events that take place in the pCTLs upon TCR recognition of a specific MHC class I-peptide complex on a pAPC,
- ***Signal two*** provides co-stimulation and it consists of CD28-B7 interaction on CTL-P and the pAPC, respectively. The pAPC would itself have to be "licensed" by a Th cell prior to gaining the ability to deliver signal two to CTL-Ps, as already described in 1.4.2
- Ligation of CD28 results in increased IL2 production by the CTL-P. Signal three is induced by IL2 binding to IL2 high affinity receptor, leading to proliferation and differentiation of the antigen-activated CTL-Ps into effector CTLs (see figure 1.7).

Therefore, the CD8+ve CTLs recognise antigens that are presented on target cells by MHC class I. CTLs are responsible for the killing of antigen-bearing target cells such as virally infected or tumour cells. Although CTL effectors can act alone when killing target cells, their differentiation from naive CD8-positive T cells is usually dependent on 'help' from Th cells (Grewal *et al* 1998). Furthermore, for effective CTL priming, this help must be provided in a cognate manner, such that both the Th cell and the CTL recognise antigen on the same APC, although this interaction does not need take place at the same time. One explanation for this requirement is that Th cells are necessary to convert the pAPC into a cell that is fully competent to prime CTLs (Ridge *et al* 1998, Bennet *et al* 1998). The competence of pAPCs is measured in the form of their ability to deliver co-stimulation (signal two) via surface molecules such as B7.1 and B7.2, as well as through production of cytokines (Schoenberger *et al* 1998). In the absence of these signals, the pAPC delivering signal one alone may induce anergy, tolerance and even apoptosis in the precursor CTLs, thus deleting certain populations of CTLs, or indeed Th cells, from the repertoire (Matzinger 1994). This topic is further discussed in section 1.4.5.

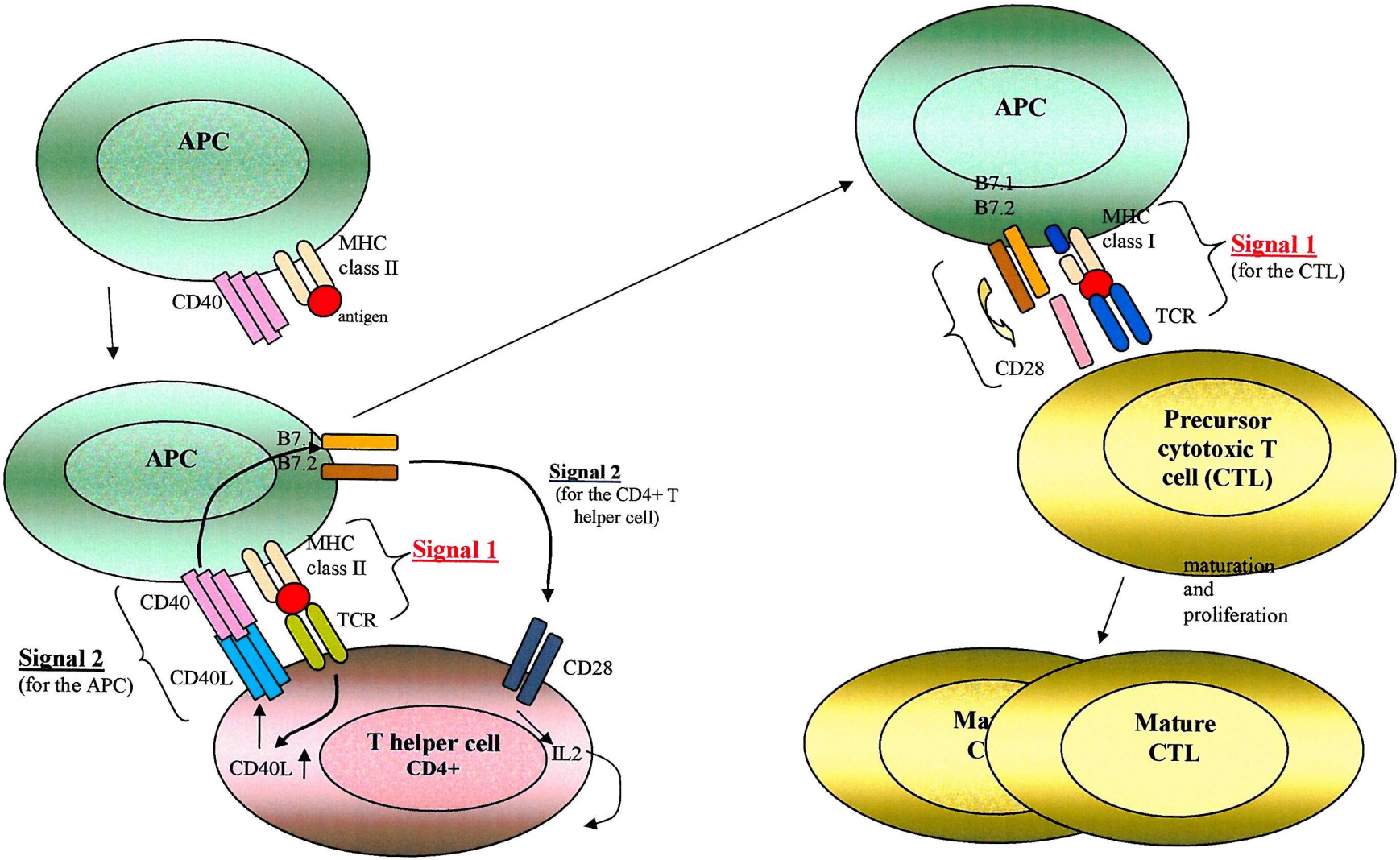


Figure 1.7- Signals one and two involved in pAPC "licencing" and T cell maturation. Please see text in 1.4.2 and 1.4.3 for detailed explanation

In the event of becoming activated, CTLs can utilise a number of mechanisms in order to delete target cells. These mechanisms involve

- granule exocytosis, where granules containing perforin and proteases get released from the CTL and form holes in the target cell membrane thus causing cell death (Kagi *et al* 1996);
- Fas-Fas ligand interaction, where Fas ligand expressed on the surface of activated CTLs binds to Fas on some tumour cells, causing their deletion by apoptosis (Nagata *et al* 1997);
- release of TNFα, which can cause death in cells expressing TNF receptors (Korner and Sedgwick 1996)
- recruitment of Th cells, which when recruited to the site of tumour growth secrete an array of cytokines, such as IL2, thus increasing numbers of CTLs and activating NK cells, also IL4 and IL5 which activate mast cells and eosinophils. Th cells can secrete GM-CSF, which can activate macrophages which can help clear the tumour load, and also TNFα, which can delete tumour cells directly (Geppert and Lipsky 1989).

1.4.4 Natural Killer cells

NK cells stand on the border of innate and adaptive immunity. They demonstrate a cytotoxic activity directed against virally infected and tumour cells (Moretta *et al* 2000). In terms of their activation, they are opposite to CTLs, which require a set of signals in order to lyse target cells. Instead, NK cells can adhere to most myelolymphoid cells and cause their lysis unless an inhibitory signal is received (Ugolini *et al* 2000). The mode by which they cause death of the target cell is granule exocytosis, as described for CTLs. NK cells do not have an antigen specific receptor. Instead they possess an array of activatory and inhibitory receptors on their surface and the functional outcome of interaction with another cell depends on the balance of opposing signals emanating from these receptors. Positive stimulation can be initiated by a number of activating receptors, Fc receptor III (FcRIII) being one of them, but specificity is then provided by a set of inhibitory receptors which recognise MHC class I. Recent evidence suggests that apart from recognising the MHC class I itself, the receptors may be interacting with glycosylated regions on the molecule (Parham *et al* 2000).

There are three distinct families of inhibitory receptors, which recognise MHC class I. They are Ly49, CD94/NKG2 and KIR, which are either Ig like or C-type lectin like molecules (Lanier *et al* 1998). Although these receptors are of diverse types, they have a common inhibitory pathway emanating from immunoreceptor tyrosine based inhibitory motifs (ITIM) which are located in their intracellular domains. Upon ligand binding, the receptors become phosporylated in the ITIM motif, which results in the recruitment of tyrosine phosphatase SHP1 and SHP2, causing inhibition of NK mediated cytotoxicity and cytokine expression (Brumbaugh *et al* 1998).

Many tumours, especially those of myeloid and lymphoid lineage, are good targets for NK cells. In some of these tumours, MHC class I is down-regulated on the surface of tumour cells as a way of evading CTL attack (Rees *et al* 1999). However, some tumours which appear to have a normal level of MHC class I on their surface are also targets for NK cell lysis. It is currently poorly understood which mechanisms allow this to take place.

1.4.5 Dendritic cells

DCs are the pivotal professional antigen presenting cells in the body for a number of reasons. Not only do they possess the unique capacity to sensitize naive T cells to protein antigens, but they can also determine immunogenicity of proteins *per se*. This is to say that DCs can determine if a given protein is going to be immunogenic, or whether it will be immunologically silent. In the context of tumours, the capacity of DCs to present tumour antigens to T cells is probably most important in determining the outcome of immunotherapeutic approach. Although both humoral and cellular responses have been shown to contribute to tumour lysis, the cellular responses involving CTLs have been shown to be responsible for tumour regression in majority of cases (Feltkamp *et al* 1993, Celluzi *et al* 1996, Boon *et al* 1994). The ability of DCs to prime tumour specific CTLs has been demonstrated in a number of animal models (Zitvogel *et al* 1996, Paglia *et al* 1996, Flamand *et al* 1994, Majordomo *et al* 1995). Furthermore, DC based "immunization" against tumours can also lead to immunologic memory which protects the animal against subsequent tumour rechallenges

(Majordomo *et al* 1995). Firstly we will consider DC ontogeny, followed by the manner in which these cells control immune responses.

DCs are derived from CD34+ve hematopoietic stem cells in bone marrow. Although this is the case, there is a lot of evidence indicating that DCs can arise from at least two distinct lineages- myeloid and lymphoid. The relationship between these two lineages remains somewhat controversial, mainly due to the fact that specific surface markers for DC precursors have not been defined as yet. In the light of their antigen presenting capacity, DC precursors also express CD11a (LFA1), CD11c, CD50 (ICAM-2), CD54 (ICAM-1), CD58 (LFA3) and CD102 (ICAM-3) (Hart and Prickett 1993). However, these antigens are not unique to DCs, as they are also expressed on monocytes and macrophages. Human DC precursors can also express CD2, 4, 13, 16, 32 and 33. These are gradually lost during maturation and activation (Takamizawa *et al* 1997).

Myeloid DCs in humans are derived from a granulocyte/monocyte precursor. DC arising from such precursor are found in blood as cells that express high levels of MHC class II and also lack other lineage specific surface markers such as CD19, 20, 24 (B cells), CD56 (NK cells), CD14 (monocytes), CD66b (granulocytes) and CD3 (T cells). Myeloid DCs can also be derived from monocytes and granulocyte precursor when exposed to GM-CSF and TNFα with or without IL4 (Palucka *et al* 1998, Oehler *et al* 1998). This shows that cells, which were previously thought to be terminally committed, can differentiate into DCs.

Lymphoid DC arises from CD8+/CD4+ve lymphoid precursors which can be induced to spontaneously differentiate into DCs *in vitro* (Sus *et al* 1996, Wu *et al* 1996). Once differentiated, these DCs become CD8+ve. Although both myeloid and lymphoid DCs can prime naive T cells *in vivo*, it appears that CD8+ve lymphoid DCs bias Th cell priming towards a Th1 response, whereas CD8-ve myeloid DCs bias towards a Th2 response, as shown in figure 1.6 (Pulendran *et al* 1999, Maldonado-Lopez *et al* 1999).

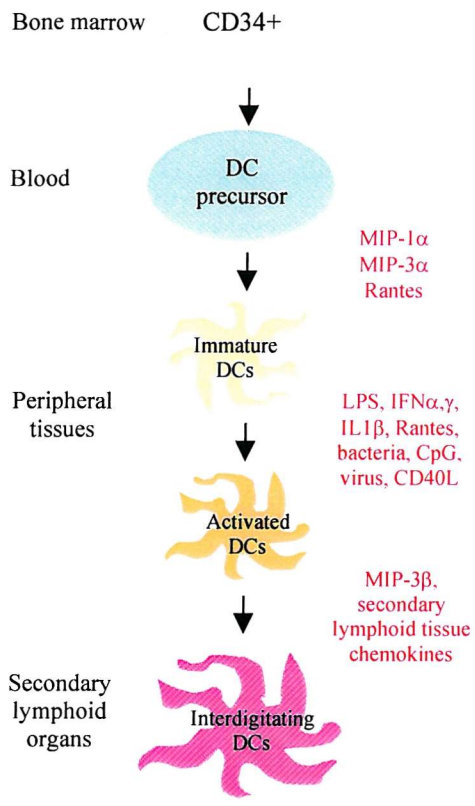


Figure 1.8- DC life cycle. DCs migrate into peripheral tissues through the vascular endothelium, drawn by chemokines. Following stimulation by one or more stimuli, DCs migrate from the tissues to lymph nodes, where interaction with T cells takes place

So how is balance between the DC-driven induction of a vigorous immune response or DC determined tolerance to an antigen controlled? This question is best answered by describing the process of antigen acquisition by DC, through to their maturation, activation and priming of T cells. Immature DCs in all organs continuously sample their environment by taking up proteins, microbes, apoptotic bodies and immune complexes. These antigens are then processed in DCs and presented on the surface in the context of MHC class I or class II. DCs are capable of processing antigens via both of these classical pathways, exogenous antigens via endocytic lysosomes into MHC class II, and endogenous antigens via the proteasome into MHC class I (Hart and McKenzie 1988, Lanzavecchia 1996). However, DCs also have the unique ability to process antigens via an alternative pathway. This allows exogenous antigens to gain entry into the MHC class I pathway. This phenomenon is known as cross-priming (Norbury *et al* 1997). In this manner, antigens arising from immune complexes (Regnault *et al* 1999), bacteria (Svensson *et al* 1997), heat shock proteins (Arnold-Schild *et al* 1999) or apoptotic bodies can be channeled through to the MHC class I pathway (Albert *et al* 1998, Albert *et al* 1998, Ylrid and Wick 2000). The process of cross-priming has been shown to be dependent upon expression of relevant phagocytic receptors on the surface of DCs, such as $\alpha_V\beta_5$ integrin and CD36 (Albert *et al* 1998), and FcγR (Regnault *et al* 1999). However, although immature DCs can take up antigens from their surrounding environment, they are very poor stimulators of immunity due to absence of expression of co-stimulatory molecules on their surface (Schuler and Steinman 1995). In order to stimulate an immune response, DCs must

first undergo a process of activation and maturation. Activation is induced by antigen-independent danger signals such as LPS, IFNα and γ, IL-1β, and some non-specific stimuli such as inflammation, necrosis and infection. Once activated, DCs down-regulate their antigen-uptake, and up-regulate antigen-presenting capacity by increasing the surface expression of MHC class I and II and adhesion molecules. In this state, DCs leave the tissues and move to the draining lymph nodes, drawn by chemokines (Dieu *et al* 1998, Chan *et al* 1999). Within the secondary lymphoid organs, activated DCs attract naive T cells for priming through production of DC-CK1 chemokine (Adema *et al* 1997). Furthermore, they secrete cytokines IL6, IL7 and IL12, which contribute to T cell proliferation and establishment of Th1 type response (Zhou and Tedder 1995, Armitage *et al* 1990, Heufler *et al* 1996, Yawalkar *et al* 1996). The cytokine secretion combined with antigen expression in both MHC class I and II along with the expression of co-stimulatory molecules provides with a generation of strong antigen specific immune response (Steinman 1991). Once attracted to DCs through chemokines, CD4+ve T cells associate with them through TCR/MHC class II and CD40/CD40L interactions. Ligation of CD40 on DCs leads to further activation and maturation as well as providing a survival signal to the DCs (Ridge *et al* 1998, Bennet *et al* 1998, Schoenberger *et al* 1998). More importantly, CD40 ligation "licenses" the DC to prime CTLs in the absence of immediate CD4+ T cell help. The importance of this becomes even more critical when considered in the context of DC ability to cross-prime antigens into MHC class I pathway. It means that DCs can in theory, raise a CTL response to any antigen which can be presented in the context of their MHC class I. This would include antigens arising from virally infected cells and tumours.

It is now clear how the balance between non-responsiveness and a vigorous immune response is achieved. In relation to tumours, it can be hypothesised that tumour antigens gain access to MHC class I on DCs through cross-priming. However, in the absence of DC activation, the end result of tumour antigen presentation is tolerance and anergy of tumour specific T cells induced by DCs. It can be foreseen that stimulation of DC activation and maturation, as is achieved by CD40 signalling, could instead induce a potent anti tumour response.

1.5 Tumour immunity and immunotherapy

Tumours are essentially self cells, and so can remain immunologically silent. It is thought that production of tumour cells is commonplace, however the body eliminates a large number of these altered self cells before they acquire properties which make them tumorigenic. In a way, rather than asking why many tumours are not recognised and eliminated by the body, the more appropriate question might be why aren't there more tumours? The text to follow attempts to explain the requirements which tumours needs to meet in order to be recognised by the body's immune system, as well as the ways in which immunotherapy has been explored as a method of enhancing sub-optimal or inducing non-existent immune responses against tumours.

In order for the idea of immunological surveillance to work, the body has to be able to recognise altered cells with neoplastic potential as non-self. For this to operate, the tumour has to display some new discriminating surface structure which can be recognised by the immune system. There are two groups of such tumour structures. One encompasses antigens which are expressed on tumour cells only, hence called tumour specific antigens. The other group is known as tumour associated antigens, representing antigens which are found not only on tumour cells, but also on healthy tissue. The latter are not likely to stimulate a significant response.

1.5.1 Tumour specific antigens

Tumour antigens can be either intracellular proteins broken down and presented in the context of MHC class I molecule, or extracellular components which are shed from the surface of the tumour and presented in MHC class II molecules. Although the body will be tolerant to self antigens, expanding knowledge of molecular properties of tumours suggests that various proteins may be mutated or otherwise modified during tumour development. Based on this, there are several categories of tumour antigens:

- *point mutations in oncogenes or tumour suppresor genes*- peptides derived from the altered region of the protein can be novel compared to the normal protein, and so recognised by the CTL, as has been found for mutations in p53 gene (Theobald *et al* 1995)

- *chromosomal re-arrangements*- where the breakpoint region of re-arranged gene will encode a novel protein structure, which is able to stimulate T cell responses (Bocchia *et al* 1996)
- *adult expression of foetal genes*- as found in some melanomas and head and neck tumours. These tumours can expresses a family of proteins, MAGE, BAGE and GAGE, normally only found in the foetus or testis. A peptide from MAGE-1 has been found to bind to MHC class I and stimulate T cells (Van der Bruggen *et al* 1991)
- *overexpression of adult genes*- some peptides are normally expressed at levels which are to low to activate T cells. In tumours there can be a marked upregulation of levels of such peptides presented on the surface on MHC class I to a level sufficient for activation of T cells. An example of protein which gives rise to tumour antigen by this mechanism is HER2/neu in breast cancer (Slamon *et al* 1989)
- *changes in carbohydrate structure*- some tumours exhibit altered carbohydrate structures; these changes are potentially recognisable by T cells (Sieling *et al* 1996) and Abs.

The first step in activation of lymphocytes is recognition of antigen, therefore the presence of tumour specific antigens is of fundamental importance for therapeutic strategies to augment the immune response against tumours. Unfortunately, the failure of CTLs, NK cells and Abs to eradicate tumours is often associated with acquired tolerance/anergy to these antigens, just as tolerance can develop to other self antigens. Consequently, most of the therapeutic strategies in cancer therapy are aimed at augmenting T cell activation by these antigens and so reversing the tolerance. These strategies take into account many factors, including

- the existence and nature of tumour specific antigens, the properties of CTLs and Abs which can recognise these antigens,
- the complexity of T cell activation, which require several signals apart from those received through the TCR,
- milieu of cytokines which can amplify the immune response.

Based on all these factors, there have been three main areas of development in biological therapy of cancer. These are gene therapy, active immunotherapy and

passive immunotherapy. Gene therapy is of no concern in this thesis, and will not be discussed further. Active immunotherapy will be mentioned very briefly, and the rest of the text will consider passive immunotherapy.

1.5.2 Active immunotherapy

Active immunotherapy entails various methods of immunisation or vaccination against the tumour. They include approaches such as

- immunisation with protein (Srivastava *et al* 1994, George *et al* 1989),
- DNA vaccines (King *et al* 1998),
- immunisation with lethally irradiated tumour cells (Morton *et al* 1993),
- immunisation with genetically modified tumour cells, which have been engineered to secrete cytokines (Miller *et al* 1994, Colombo and Forni 1994),
- the use of tumour antigen pulsed dendritic cells, which have the ability to activate T cell responses by virtue of high expression of co-stimulatory and adhesion molecules on their surface (Celluzzi *et al* 1996, Hsu *et al* 1996).

1.5.3 Passive immunotherapy

Passive immunotherapy approaches include the use of reagents generated *in vitro*, such as cytokines, monoclonal antibodies and use of effector cells expanded *in vitro* which are then used for adoptive transfer approach. The use of cytokines has been merited by their ability to regulate various aspects of immune response. Some cytokines can induce T, B and NK cell proliferation. Others can heighten the cytotoxic potential of T or NK cells, or regulate Th1/Th2 balance and instigate apoptosis in the target cell. Based on these effects, many cytokines have been evaluated in animal models and clinical trials, including IFNα, IL2 (Rosenberg *et al* 1988), IL4 (Puri and Siegel 1993), TNFα and IL12 (Banks *et al* 1995). Adoptive cellular therapy is mediated by the transfer of lymphocytes sensitised to tumour. There have been various approaches taken, which are described in the review by Ettinghausen and Rosenberg (1995).

The last type of passive immunotherapy concerns the use of mAbs for eradication of cancer. mAbs have been used in cancer immunotherapies either in an unconjugated "naked" form, or conjugated to various other molecules, such as toxins (Goldenberg *et*

al 1995, White *et al* 1996, Knox *et al* 1996). Unconjugated Abs can evoke anti-tumour activity through recruitment of effector cells (Fagerberg *et al* 1995), complement activation and/or directly by inducing growth arrest and /or apoptosis in the target cell (Hess *et al* 1996, Fagerberg *et al* 1996, Pellat-Deceunynck *et al* 1996). Conjugated mAbs are used for delivery of bacterial or plant toxins, radioisotopes or cytotoxic drugs to the targets, and so can be viewed as specific transport vehicles. Although many therapies have been carried out using conjugated mAbs, they are not of concern in this thesis and will not be discussed further.

1.5.4 Classical mechanisms of mAb induced tumour regression

Thus far it was described what Abs and mAbs are and how they are produced. But can these components of humoral immunity contribute or indeed delete tumour cells, and if so, what are the mechanisms utilised by the Abs in order to achieve this? There are ways in which humoral immunity alone can control tumours. The ways in which Abs achieve this can be divided into three categories; complement activation, induction of apoptosis or cell cycle arrest in tumour cells. There is another way in which Abs can directly influence the survival of the target cell, known as antibody dependent cellular cytotoxicity. This mode of action requires effector cells in order to achieve death of the target cell, rather than relying on the effect of Ab alone.

1.5.4.1 Complement activation

The complement system is made up of a large number of distinct plasma proteins that act together to attack extracellular pathogens. It can be activated spontaneously by certain pathogens or by Ab binding to the microbe (Farries and Atkinson 1991). Complement can be activated through three pathways:

- *Classical pathway* – activated by Ab binding to antigen
- *Lectin pathway* – activated by serum lectin binding to mannose containing proteins or to carbohydrates on bacteria on bacteria and viruses,
- *Alternative pathway* – activated when spontaneously activated complement components bind to the surface of pathogens.

Activation of the complement system induces various effector mechanisms, including the formation of lytic membrane attack complexes in the target cell membrane, the

production of anaphylactic substances, and the deposition of other complement components (e.g. C3 fragments) on the target surface. These fragments as well as the C1q itself can be recognised by effector cells such as lymphocytes, endothelial cells, fibroblasts and platelets, leading to the activation or enhancement of a variety of further effector functions (e.g. phagocytosis, cell-cytotoxicity, stimulation of oxidative metabolism, antibody production, etc)(Kinoshita 1991).

1.5.4.2 Cell death and apoptosis

Apoptosis, or programmed cell death, is a physiological, highly conserved process that has been defined as cellular suicide. It is characterised by nuclear condensation, DNA fragmentation, alterations in the distribution of cell membrane phospholipids, membrane blebbing and cellular shrinkage (Hughes and Cidlowski 1996). Apoptotic bodies, resulting from the death of the cell by apoptosis, are readily phagocytosed by neighbouring cells without inducing an inflammatory reaction. Proteases, especially cysteinyl-aspartases or caspases, are the main executioners of apoptosis. Their activity leads to cytoskeleton and membrane damage, and to chromatin cleavage into nucleosomes (Cohen 1997). The machinery for apoptosis exists in all cell types, including tumour cells. Therefore, in terms of tumour eradication, regimes involving induction of apoptosis seem ideal. Ionising radiation, anti-cancer drugs, hormone addition or withdrawal (as is the case in the addition of glucocorticoids to lymphocytic leukemias) and Abs have all been shown to induce apoptosis. As an example of such a process, Abs directed at molecules containing cytoplasmic death domains found on the surface of tumour cells (such as Fas) can induce extensive programmed cell death. Fas is a 45 kDa receptor which belongs to TNF receptor superfamily. It contains a death domain in its intracellular domain, so crosslinking of the receptor can lead to induction of apoptosis in the cell (Trauth *et al* 1989). The major problem associated with the use of anti Fas Abs *in vivo* is that many normal tissues also have Fas on their surface, hence such therapy leads to vast cell death of normal tissues. In mice such a treatment leads to death in 6 hours due to extensive liver damage. Abs directed at the B cell receptor (BCR), such as anti Id Abs, have also been shown to induce apoptosis in lymphoma cells, through the signals emanating from this receptor (Hasbold and Klaus 1990).

1.5.4.3 Cell cycle arrest

The cell cycle defines the path taken by a replicating cell starting from a non-dividing, quiescent state called G0 through G1, a DNA synthesis stage S, G2, and finally ending in mitosis M. An activation signal promotes the cells from G0 to G1. In B cells agents which can promote calcium influx are efficient at producing such a signal. Progression of the cell through the cell cycle is governed by the cyclin proteins and their associated kinases (cdks) (Sherr and Roberts 1995). Once G1 has been passed the cell will normally complete the cycle as long as the appropriate stimuli remain. However, on receiving certain stimuli the cell may be induced to halt cell cycle progression and arrest. This halt occurs at defined check points at the border of G1 and G2/M. At these points, damaged cells are either repaired and allowed to progress through the rest of the cycle, or apoptose (Ashkena and Werb 1996). It has been shown that tumour cells treated with particular anti-cancer drugs, such as chemotherapeutic agents and ionising radiation (Halicka *et al* 1997, Elledge and Lee 1995), can growth arrest and subsequently die.

The best documented method of inducing growth arrest in B cell tumours is that of crosslinking the BCR (Cragg *et al* 1998). The BCR includes surface Ig and associated molecules, as outlined in 1.4.6. The level of arrest signal is proportional to the extent of cross-linking, with hyper-crosslinking of BCR further increasing arrest and apoptosis. Cells can remain in the arrested state for a long time. However, the state of dormancy can reverse, whereby treated cells can re-enter the cell cycle, proliferate and cause a tumour relapse. Some reports indicate that anti CD19 and anti CD40 mAbs can induce similar growth arrest, although the level or growth arrest and/or apoptosis does not increase with the increase in cross-linking of the target receptors (Pezzutto *et al* 1987, Funakoshi *et al* 1994).

1.5.4.4 Antibody Dependent Cellular Cytotoxicity

One of the mechanisms used by the immune system to delete unwanted cells is through the recruitment of effector cells. In relation to the Ab treatment of cancer, this mode of action is called Ab-dependent cellular cytotoxicity, or ADCC. This is the process where Ab binds to its specific target through the Fv region, leaving the Fc domain protruding

out. When there are sufficient numbers of Fc present on the surface of a target cell, they can bind to Fc receptors (FcR) found on an array of potential effector cells. A wide range of cells express FcR, including monocytes, macrophages, dendritic cells, natural killer cells, neutrophils, eosinophils and platelets (Heyman 2000, Ravetch *et al* 1998, Perussia 1998).

There are four classes of Fc gamma(γ) receptor which recognise IgG. The high affinity receptor FcγRI (CD64) is found on macrophages and some monocytes. The low affinity receptor FcγRII (CD32) is found on majority of FcR bearing cells, including basophils, mast cells and platelets. The low affinity FcγRIII (CD16) is found on macrophages, neutrophils and NK cells and finally FcRn is found on endothelial cells (Kuby 2000). All of the aforementioned cell types have varying levels of expression of the different Fcγ receptors and display differing degrees of killing ability (Indik 1995, Fridman *et al* 1992).

Isotype of the Ab is very important in determining whether a given Ab will engage the FcR, as outlined in table 1.1. Furthermore, engagement of the Ab by the FcR may not always result in the Ab coated cell being deleted. The effect of IgG-FcR interaction may be cytostasis, phagocytosis or extracellular lysis, again largely depending on the effector cell type. When cell lysis is the final outcome, the mechanism is thought to be at least in part due to the release of cytotoxic granules. These granules, present in the cytoplasm of resting effector cells, fuse with the plasma membrane in response to target cell recognition, thus releasing their contents at the target cell interface. The constituents of granules are enzymes, such as myeloperoxidases, acid hydrolases and lysozyme, as well as perforin which together cause degradation, toxic oxygen free radical build-up and ultimately death of the target cell. The cells die due to the induction of apoptosis or due to irreparable membrane damage (Henkart 1985).

1.5.5 Problems associated with the antibody treatment

Although there has been much optimism surrounding the use of mAbs in the therapy of cancer (Riethmuller *et al* 1993), immunotherapy has in general failed to deliver significant clinical results, and the initial optimism has been dampened by a series of unsuccessful clinical trials. The possible reasons for this lack of success are shown in

the Table 1.2 and they have been grouped according to the type of obstacle they present (Grossbard *et al* 1992). The following text considers some of these problems.

Obstacles to serotherapy
1. Inability to deliver mAb to the tumour
a) Tumour bulk b) Non-specific antibody binding to normal tissues c) Antigen shedding d) Antigen modulation
2. Inability to kill all tumour cells
a) Lack of endogenous host cytotoxic mechanisms b) Resistance of tumour cells to cytotoxic mechanisms c) Heterogeneity of antigen expression d) Clonogenic tumour cell does not express antigen e) Emergence of antigen negative tumour cells
3. Toxicity of serotherapy
a.) Allergic reactions b.) Antibody/antigen aggregate formation c.) Immediate end organ damage d.) Delayed end organ damage

Table 1.2- Problems associated with mAb therapy. As outlined in Grossbard M.L., (1992), Monoclonal antibody based therapies of leukaemia and lymphoma, *Blood*, Vol.**80**,No4, pp863-878

The first group of problems encountered in immunotherapy relates to poor delivery of mAb to the tumour. There are four main reasons for this, listed a) to d) in section 1 of the table 1.2. One of the major difficulties associated with delivery is linked to the size of mAb molecules. mAbs of IgG isotype have a molecular weight of around 180 kDa and are therefore much larger molecules than chemotherapeutic agents. As such, they encounter complications associated with diffusion of large molecules into the

extravascular sites of solid tumours. Due to this, the mAbs can only "coat" the solid tumours on the outside, with a low efficiency of penetration into the tumour "core".

One of the most significant difficulties is to identify a suitable antigen that is expressed on the surface of the tumour cell, but is absent from the surface of normal tissue. Ensuring this minimal cross-reactivity is a major problem, as tumour cells arise from normal cells, and so often express almost identical surface proteins. When tumour specificity cannot be accomplished, this obstacle can be overcome to some extent by assuring that normal cells which carry the antigen are dispensable to the body and easily replaceable. For example, CD20 is proving a useful target for the treatment of malignant B cells, although it is also found on all normal B cells (Kuehnie *et al* 2000). During the course of therapy, patients receiving anti-CD20 mAb are depleted of their normal B cells, but these cells are rapidly replaced once the treatment is completed.

The third difficulty is presented in the target antigen being shed from the surface of the tumour cell, as is the case with idiotype (Id) in lymphomas. Circulating soluble Id binds most of the anti Id Ab leaving little mAb available to bind to tumour cells (Grossbard *et al* 1992). Also, at times there may be large numbers of circulating tumour cells in the blood, which bind mAbs and so decrease their availability in the circulation. Modulation of the antigen from the tumour cell surface may also present a barrier to therapy, especially as many surface proteins are rapidly internalised after Ab binding. This leads to a decreased cell surface expression of the antigen and consequent loss of certain Ab mediated therapeutic effects. Some malignant cells have also been shown to undergo further mutations, which can result in complete absence of the target antigen from the mutated clone (Grossbard *et al* 1992). In other cases, a subpopulation of tumour cells may lose the expression of target antigen, thus preventing binding of mAbs to the cells.

Inability to kill tumour cells can result from the lack of host cytotoxic mechanisms, or from the resistance of tumour cells to these mechanisms. Some tumours express tumour-associated antigens which can be recognised as non-self. In some circumstances the responses to these antigens can be insufficient due to either

- absence of antigen derived peptides which can be recognised by T cells, or

- due to presence of cytokines, often produced by the tumour itself, which favour Ab, rather than cellular responses.

In some cases the responses against the tumour can be mounted in *in vitro* experiments, whereas such responses are absent from *in vivo* therapies.

Immunotherapy can be associated with varying levels of toxicity, caused in part by Abs themselves being recognised as non-self proteins. Most of the mAbs used for therapies are of rodent origin, hence can induce human anti mouse Ab responses (HAMA), whereby the therapeutic mAb itself is recognised as foreign and cleared out of the circulation. This ceased to be a serious problem since the advent of the process of humanising mAbs, where mouse variable and hypervariable regions of Ig are genetically combined with human framework Ig regions so as to minimise the antigenic determinants (Winter and Harris 1993). In more recent years, ways of producing fully human mAbs have also been developed (Green 1999).

Finally, some mAbs can form large insoluble aggregates by binding to soluble circulating antigen, or circulating tumour cells. When such complexes are formed, they can lead to kidney damage and failure.

1.5.6 Latest developments in immunotherapy -a novel approach

Although early results have been disappointing, the past five years have marked a change in the success of antibody based clinical trials, resulting in a total of eight antibodies now being approved by the FDA for use in clinics (see table 1.3). The reasons for this turnaround are many, and most stem from newly found ways of overcoming the problems associated with the *in vivo* use of murine antibodies in humans as well as the increased knowledge regarding the mechanisms of Ab action *in vivo*. One of the key steps has been the varying degrees of humanisation of murine mAbs, so that today there are

- human-mouse chimeric mAbs (Boulianne *et al* 1984);
- humanised mAbs, where only the CDRs are of murine origin (Jones *et al* 1986),
- fully human mAbs obtained using phage display technology (Rader *et al* 1997), and
- transgenic mice carrying human antibody repertoires (Bruggemann and Taussig 1997).

The process of humanisation of Igs used in immunotherapy has meant that HAMA response can easily be avoided. Furthermore, human or humanised mAbs (i.e. with human constant regions) have an increased half-life, measuring up to 21 days for human IgG, compared to the rodent mAbs half-life of less than 20 hours. This difference is due to the ability of human Igs to be recirculated after they have been internalised, whereas rodent mAbs are destined for intracellular degradation pathway (Ghetie *et al* 1997).

Indication	Target antigen	Antibody name	Product type	Sponsors/ the year FDA approved the Ab
Rheumatoid arthritis	TNFα	InfliximAb	Chimeric IgG1	Centocor 1999
Crohn's disease	TNFα	InfliximAb	Chimeric IgG1	Centocor 1998
Allograft rejection	CD3	Orthoclone/OKT3	Murine IgG2a	Ortho Biotech 1986
	CD25	Zenapax	Humanised IgG1	Protein design lAb/ Hoffman-La Roche 1997
	CD25	Simulect	Chimeric IgG1	Novartis Pharm 1998
Complication of coronary angioplasty	Glycoprotein	ReoPro	Chimeric FAb	Centocor/Lilly 1994
Virus RSV	F protein	Synagis	Humanised IgG1	MedImmune 1998
Breast cancer	HER2/neu	Herceptin	Humanised IgG1	Genentech 1998
Non-Hodgkins lymphoma	CD20	Rituxan	Chimeric IgG1	IDEC Pharm/Genentech 1997

Table 1.3- A list of mAbs approved for clinical use. Adapted from Glennie M.J. and Johnson P.W.M., "Clinical trials of antibody therapy", in *Immunology Today*, Vol.**21**, No.**8**;403-410

In the recent years, a new class of mAbs for treatment of cancer have been developed. These mAbs operate not by exerting a direct effect on the tumour cell, but rather by stimulating the key receptors in the immune system (Leach *et al* 1996, Melero *et al* 1997, French *et al* 1999). Using this approach, the mAbs can potentiate weak,

ineffective anti-tumour responses to a level where effective therapy can be provided. Some of the prime examples of such mAb therapies include the use of anti-CTLA4 mAb (Leach *et al* 1996) where the mAbs acts to block the interaction of CTLA4 on effector T cells with B7.1 proteins on APCs, thus potentiating and prolonging the activated effector phase of T cells.

Another example is anti-CD137 (or 4-1BB) mAb (Melero *et al* 1997) which also aids the activation state of T cells, and finally anti CD40 (French *et al* 1999) which bypasses help normally provided by Th cells and evokes CTL responses against lymphoma. All of these mAbs deliver a stimulatory signal to the target cell which in its nature is very similar qualitatively to the signal provided by the natural ligand. Although so far only tested in mice, the future potential of these mAbs lies in their ability to not only eradicate the existing tumour, but to render the host immunologically protected to a subsequent rechallenge with the same tumour (French *et al* 1999). In this manner, these mAbs act as vaccines.

The therapy accomplished by the use of anti CD40 mAb has been developed in the Southampton Tenovus laboratory over the past three years. The use of anti CD40 mAb to treat B cell lymphoma in animals forms the model on which the work carried out in this thesis was based. The section explains our current knowledge of the events taking place *in vivo* during anti CD40 therapy, as well as a review of knowledge concerning the structure and function of CD40.

1.5.7 Anti CD40 therapy of lymphoma

The biggest problem for most of the mAb-based therapies is the selection of target antigen. Cells of every lineage display specific surface markers, and B cells are no exception. A brief summary of B cell specific markers and the changes in their expression depending on the stage of B cell development are outlined in the table 1.5. Malignant B cells express many of these potential target antigens on their surface and a variety of these antigens have been targeted in the treatment of lymphoma, both in mouse models and in the treatment of humans. The selection of mAb targets has been based on their presence on the surface of lymphoma cells. A panel of potentially therapeutic mAbs, including those raised against CD19, CD22, CD38, CD72, CD86,

MHC class II, CD40 and anti Id, have been evaluated in the Tenovus laboratory over the last few years using a variety of mouse tumour models and *in vitro* cell lines. Based on the results of this work, anti CD40 mAb has emerged as one of the most promising treatments.

Lymphoid cell type								
Markers	Lymphoid stem cell	Pro B-cell	Pre B-cell	immature B-cell	mature B-cell	activated B-cell	memory B-cell	plasma cell
BCR	-	-	cyto μ	sIgM	sIgM/sIgD	sIgM	sIgM/sIgA	cyto Ig
Class II								
CD10								
CD19								
CD20								
CD21								
CD23								
CD25								
CD37								
CD38								
CD40								
CD72								

Table 1.4- Markers expressed on the surface of B cells throughout their development
Adapted from Roitt, Brostoff and Male,Immunology 3rd edition, section 11.11. Filled boxes denote expression.

In the study described by French *et al* (1999) anti CD40 mAb treatment was used to treat mice with syngeneic lymphoma. Three CD40+ve murine B cell lymphoma models, namely A20, A31 and Bcl_1, and two CD40-ve T cell lymphomas, Ten1 and EL4, were tested *in vivo* with this treatment. The therapies were carried out by challenging mice with a large number of tumour cells, followed by a single dose of anti CD40 mAb four days later. After a number of therapies were performed, a relationship between the tumour load and the time of treatment was established. Curiously, it became apparent that the therapeutic activity was best if the mAb injection was given when the tumour load was high in the animals. This relationship between the tumour

load and the mAb protection indicated that the immune system may play a role, by perhaps responding to tumour specific antigens which needed to be present at a sufficient level to evoke a response.

Further evidence confirming the importance of the immune system in the outcome of the therapy was confirmed in two ways. Firstly, the mice that survived for 65 days after the initial tumour inoculum were re-challenged with a fresh tumour load without further mAb treatment. None of the re-challenged mice developed tumour, which indicated that they were rendered immune to the tumour by the initial treatment. Therefore, the mice had memory cells which were able to respond to the secondary tumour challenge. The second piece of evidence concerning the involvement of the immune system came from the treatment of lymphoma in immunocompromised SCID mice where the anti CD40 mAb showed virtually no activity, regardless of when the treatment was distributed. This indicated that the mAb itself has no direct deleterious effect on the tumour itself. The non-responsive state of tumour to anti CD40 mAb therapy could be overcome by reconstituting SCID mice with lymphocytes from normal mice before the treatment, upon which the protective activity of anti CD40 mAb was re-established.

The effector mechanism resulting in the rejection of tumour in these studies was found to involve CD8+ve cytotoxic T cells, or CTLs, as the curative effect of anti CD40 mAb was lost upon depletion of these cells. The therapy was also found to be completely independent of CD4+ve T cells. Therefore, the anti CD40 treatment *in vivo* induced a powerful CTL mediated cytotoxic response against the tumour without the requirement for "help" from CD4+ T cells. Further studies also showed that the positive outcome of the therapy relied upon the presence of cytokines IL12 and IFNγ, as neutralisation of those also abrogated the therapeutic activity of the anti CD40 mAb. Both of these cytokines are potent inducers of Th1 responses, which are known to be necessary for eradication of tumour and virally infected cells.

The studies performed with CD40-ve T cell lymphomas indicate that CD40 expression is not necessary on the tumour cell itself. These findings were further supported by recent data emerging from other research groups (Todryk *et al* 2001). Based on our

findings and those of others, the current working hypothesis of the anti CD40 therapy of B cell lymphoma model is shown in figure 1.9.

It is thought that DCs might be cross-priming tumour antigens into their MHC class I and becoming fully activated and "licenced" through anti CD40 mAb signalling. The tumour antigens can then be presented to precursor CTLs, which are also provided with adequate co-stimulation by the activated mature DC. The activated CTLs then proliferate, thus creating sufficient numbers of tumour antigen specific CTLs, and delete the tumours cells. Some of the CTLs become memory cells, which can act upon a secondary re-challenge with the same tumour to delete the tumour cells without a need for further treatment.

As previously stated, B cell lymphomas, which are CD40+ve, are treated with a higher degree of success than T cell lymphomas which are CD40-ve. It is currently unclear if the presence of CD40 might in some way be contributing to better therapy. CD40 signalling may be in some way exerting a pro-apoptotic signal to the tumour cells, thus creating more apoptotic bodies which can be cross-primed into DCs. Alternativelly, CD40 signaling may be up-regulating co-stimulatory markers on the tumour B cells, thus converting them into self-antigen presenting cells. Alternatively, it is possible that T cell lymphomas are simply less immunogenic due to absence or lower number of tumour specific antigens.

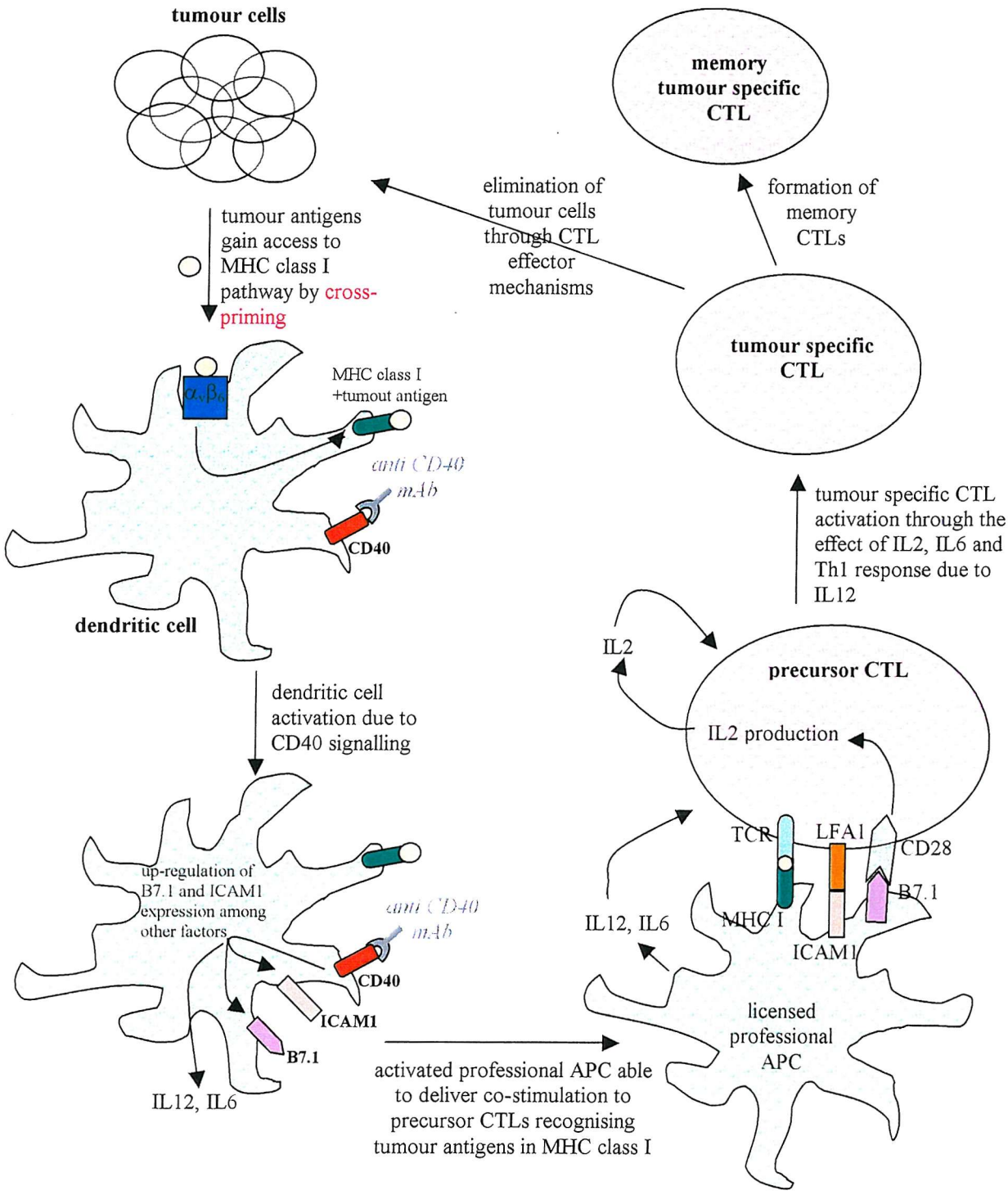


Figure 1.9- Current working hypothesis of the anti CD40 mAb induced tumour clearance *in vivo*.

For full explanation, please refer to text on page 42, and section 1.4.5.

1.6 CD40

1.6.1 Introduction

CD40 was first identified in 1985 by mAbs reacting with carcinomas and B cells (Paulie *et al* 1985). cDNA encoding the antigen was isolated in 1989, and the sequence demonstrated a relationship to the tumour necrosis receptor superfamily (Stamenkovic *et al* 1989). It was then shown that crosslinking of CD40 with addition of IL4 can induce B cells to undergo long term growth, as well as isotype switching (Aversa *et al* 1993). In 1992, use of CD40-Fc fusion protein allowed isolation of CD40L, subsequently designated CD154 (Armitage *et al* 1992). The ligand was found to be expressed on activated T cells, an observation which led to manifestation of the key role of CD40-CD40L interaction in T-cell-dependent B cell activation. In 1993 it was shown that mutation in CD40 or CD40L gene leads to a disorder termed hyper-IgM syndrome, pointing to the critical role of the CD40/CD40L interaction in the T cell-B cell interplay. Hyper IgM syndrome in humans leads to an inability to produce Ab isotypes other than IgM (hence hyper IgM syndrome), an absence of isotype switching and lack of germinal center formation (Allen *et al* 1993, Callard *et al* 1993).

CD40 has initially been considered to be a pan B cell marker. Due to this, the research concerning CD40 has for a long time been focused on humoral immunity. However, in recent years it has become clear that CD40 expression is much broader, as it is found on malignant B cells, some T lymphocytes, monocytes, macrophages, dendritic cells, Reed Stenberg cells (observed in Hodgkin's lymphoma), hematopoitetic progenitors (CD34+ cells), follicular dendritic cells, synoviocytes, basophils, epithelial cells, endothelial cells and fibroblasts where it appears to be involved in regulation and amplification of inflammatory response (van Kooten *et al* 1996). Following these findings, the research into functions of CD40 on different cell types was expanded in various directions, and this later work demonstrated a broader role for CD40 in the immune system contributing to cell-mediated as well as humoral immune responses (van Kooten 2000).

1.6.2 Structure and expression of CD40 and its ligand

CD40 is a 45-50 kDa integral membrane glycoprotein that belongs to the tumour necrosis factor receptor family which includes CD27, CD95 (Fas), OX40, CD30, TNFRI, TNFRII, TNFR Rp, 41-BB, myxVRx and cfECP1 (van Kooten *et al* 1996)(see figure 1.10). Mature human CD40 is composed of 277 amino acids (aa), 193aa forming the extracellular region which contains a 21aa leader sequence. The rest of the molecule is made up of 22aa transmembrane domain and a 62aa intracellular tail, making CD40 a typical type I transmembrane protein. The extracellular region of CD40 displays homology with this region found on other members of TNFR superfamily in respect of having a 22 cysteine residues forming four predicted cysteine repeat rich region each made up of 40aa with 6 cysteines. The intracellular region does not bear any significant sequence homology to any other characterised protein (Paulie 1989). Murine CD40 is composed of 305aa; 193 of those form the extracellular region with a 21aa leader sequence, 22aa transmembrane domain, and 90aa intracellular tail. Human and mouse CD40 have 62% overall gene sequence identity, and 78% identity in the intracellular tail region. The C-terminal 32aa of the human sequence are completely conserved in the mouse, as are all of 22 cysteines, which would suggest that both human and mouse intracellular regions of CD40 fold in the same way and that they can signal interchangeably in human and mouse cells (Hsing *et al* 1997, Hsing *et al* 1999).

As already mentioned, expression cloning using CD40-Fc fusion protein achieved isolation of CD40L from activated T cells (Armitage *et al* 1992). Human CD40L is a protein made up of 261aas including a 215aa extracellular domain with five cysteines, which shares significant amino acid homology with the TNF, particularly in its extracellular domain ("TNF homology region") and is therefore viewed as a member of the TNF ligand superfamily (Noelle *et al* 1992). CD40L forms a symmetric homotrimer similar in organisation to those described for TNFα and LTα (Karpusas *et al* 1995). Although CD40L is mainly expressed on activated CD4+ T cells, it is also found on some activated CD8+ T cells, basophils, eosinophils, mast cells, stromal cells, B cells, B cell lines, macrophages and dendritic cells under certain conditions (Pinchuk *et al* 1996, Grammer *et al* 1995, Mach *et al* 1997, Carbone *et al* 1997). Furthermore, several studies indicate that CD40L can also form soluble trimers, thus suggesting that it may act as a "cytokine"(Graf *et al* 1995, Mazsei *et al* 1995, Ludewig *et al* 1996). It is

currently unclear if there are qualitative differences between the signals initiated from the soluble versus membrane bound CD40L.

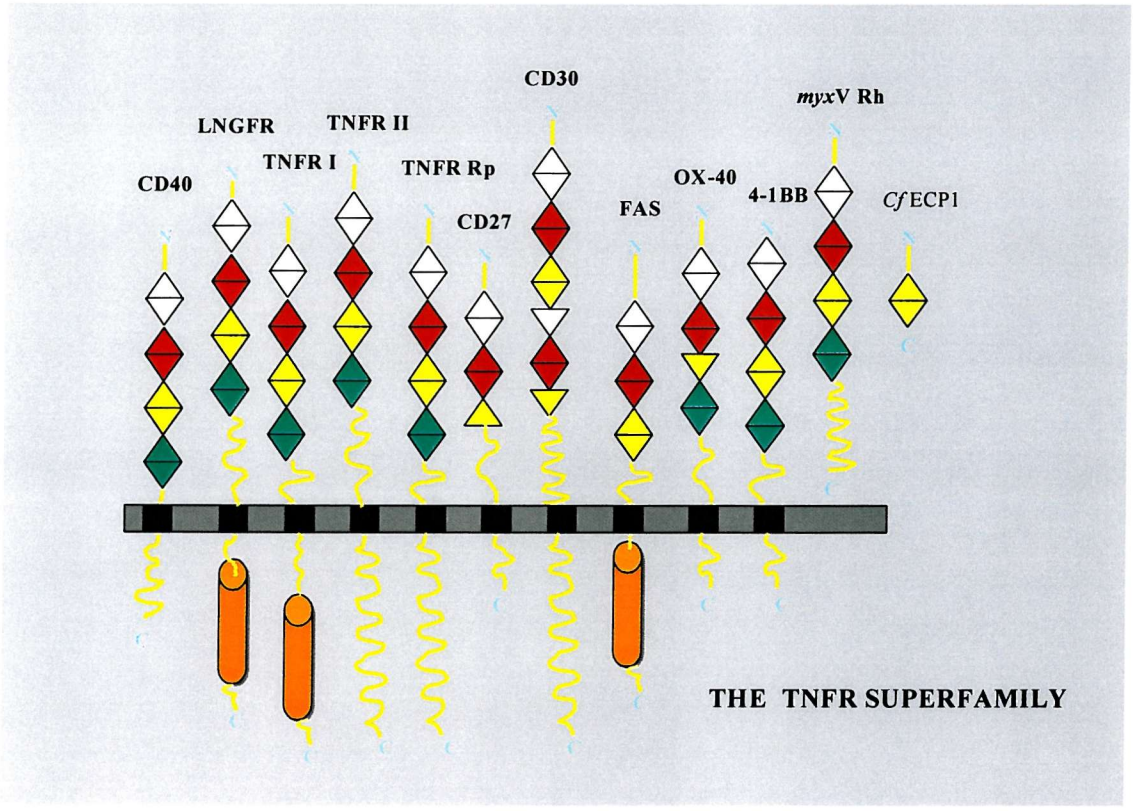


Figure 1.10- The TNFR superfamily members

1.6.3 Signal transduction mediated by CD40

CD40 has been shown to send the signal inside the cell once the extracellular domain has been cross-linked by CD40L or anti CD40 antibody. Its signalling affects numerous functions within the cells of any given cell type bearing the receptor. However, the intracellular domain of CD40 does not appear to contain any enzymatic potential and does not contain a known consensus sequence for binding to kinases. It is therefore likely that its protein kinase activity is mediated via the activity of associated kinases and adaptor molecules, which will be discussed in further detail later.

CD40 ligation has been shown to activate numerous second messenger system and kinases, including protein tyrosine kinases such as PTK, *lyn, syk* and Jak3; also protein kinase A (PKA), phosphoinositide-3 kinase (PI-3 kinase) and phospholipase C (Van

Kooten *et al* 1996). More recently research concerning CD40 signalling has concentrated on involvement of serine/threonine kinases belonging to the MAP kinase family. These kinases include c-jun terminal kinase (JNK) stress activated protein kinase (SAPK), p38 MAPK and extracellular signal-regulated mitogen activated protein kinase (ERK) (Berberich *et al* 1996, Grammer *et al* 1998, Purkerson and Parker 1998, Craxton *et al* 1998). Studies concerning the role of various aforementioned kinases in CD40 signaling have yielded conflicting results in different laboratories. It is therefore hard and somewhat speculative to draw general conclusions based on these results. It is thought that varied experimental results might have arisen because the studies have been performed using different cell lines or transfected cells models. For example, it has been shown that NFκB inducing kinase (NIK) is essential for activation of B cells, yet it appears to be unimportant for activation of DCs (Garceau *et al* 2000). Also, TRAF6 (see text to follow) was found to be an intermediate in the CD40 signalling cascade resulting in NFκB activation in epithelial cells, but not in B cells (Jalukar *et al* 2000). Finally, it was found that different members of NFκB transcription factor family are induced in different cell types due to CD40 signalling (Revy *et al* 1999). The same study showed association of Janus kinase 3 (Jak3) with the CD40 intracellular domain in both B cells and DCs, but only in the latter CD40 ligation results in activation of STAT5a. This cell specific response may explain how CD40 can signal normally in DCs of patients with X-linked hyper IgM syndrome, which affects B cells, as explained previously (Revy *et al* 1998).

In the past several years there has been considerable progress in the identification of intracellular molecules mediating CD40 signalling (Pullen *et al* 1999, Hanissian *et al* 1997, Ishida *et al* 1996, Ishida *et al* 1996). Thus far, association of Jak3 and a group of adapter proteins have been directly shown in various studies (Hanissian *et al* 1997). The adapter proteins in question are collectively known as tumour necrosis factor receptor-associated factors (TRAFs), as they associate with the cytoplasmic domain of other TNFRs, as well as CD40. The text to follow offers a brief overview of the proteins shown to directly interact with the CD40 cytoplasmic domain. These are TRAFs and Jak3. The findings outlined in the text may not apply to all cells expressing CD40, as the receptor appears to be signalling in a cell specific manner. Figure 1.11 offers a map of Jak3/TRAF binding sites, as well as a simplified overview of the signalling events emanating from the CD40 receptor.

1.6.4 Tumour necrosis factor receptor associated factors (TRAFs)

TRAFs are a group of structurally related adapter proteins that link cytosolic domains of some of the TNFR family receptors with downstream signalling pathways (Arch *et al* 1998, Pullen *et al* 1998). There are 6 known TRAF proteins, all of which contain several functional domains, including RING finger, isoleucine zipper and zinc finger domains. In addition, a conserved C-terminal ~150 aa domain known as the TRAF domain plays a role in protein-protein interactions and in formation of homo- or heterodimers (Pullen *et al* 1999). At least 4 TRAFs, namely 2, 3, 5 and 6, have been shown to associate with the cytoplasmic domain of CD40 (CD40cyt)(Rothe *et al* 1994). They initiate signalling after initiation of CD40 receptor multimerization by the ligand or mAb. This is achieved by recruiting members of the TRAF family to the CD40 receptor cytoplasmic domains. The TRAFs are then phosphorylated, thus setting off a cascade of events which leads to activation of kinases and phosphorylation of many other proteins, ultimately leading to the activation of transcription factors NF-AT, AP-1 (activator protein-1) and NF-κB (nuclear factor-kappa B) among others (Hanissian *et al* 1997, Tsukamoto *et al* 1999, Kim *et al* 1999, Padmore *et al* 1997, Goldstein 1997, Hara *et al* 1997). Organisation of this kind provides diversity in the signal created in the different cell types, as activation of signalling pathways will be dependent on the activation and general status of the cell type in question.

Mutagenesis studies on CD40 have determined that TRAF 2, 3 and 5 bind to region spanning residues 246-269 in CD40cyt, whereas TRAF6 binds to a separate domain encoded by residues 230-245 (Ishida *et al* 1996). Further studies showed that a 17-aa sequence ^{250}PVQETJHGCQPVTQEDG^{266} is sufficient for binding of TRAF 2 and 3 as well as for induction of NFkB. Within this sequence a core TRAF binding motif was identified and it was found to be ^{250}PVQET^{254}. Threonine residue in position 254 was shown to be critical for binding of TRAFs as its mutation into alanine abrogated activity of the receptor in most cell types (Galibert *et al* 1998, Pullen *et al* 1998). As the T254A mutation prevented binding of TRAF 2, 3 and 5 to the receptor, it can be assumed that they utilise a similar binding site, as discussed in more detail below (Cheng *et al* 1995).

As already mentioned, interaction of TRAF 6 with CD40 occurs via a binding site located distal to the trans-membrane portion of CD40, made up of residues 230-245. The minimal sequence required for binding of TRAF6 to this region is ^{231}QEPQEINF^{238} (Darnay *et al* 1999, Pullen *et al* 1998)(see figure 1.9).

It has been shown that both the TRAF 2,3 5 and 6 binding sites described are required for optimal NF-kappa B and JNK activation (Cheng *et al* 1996, Rothe *et al* 1995, Lee *et al* 1999). In contrast, p38 mitogen-activated protein kinase activation was primarily dependent upon TRAF6 binding. These studies suggest that the NF-κB and JNK responses to CD40 signalling require integration of signals emanating from binding of TRAF 2, 3, 5 and 6 at both TRAF recognition/ binding sites in the CD40 cytoplasmic domain. However, different studies show varied results, which also appear to heavily depend on the type of cell studied. Having considered the basic facts about these factors, the text to follow will aim to consider each factor individually.

1.6.4.1 TRAF 2 and TRAF 3

The motif necessary for the interaction of TRAF 2 and 3 with CD40 was shown to be ^{250}PVQET^{254}. Various research groups tried to establish the actual binding sites for TRAF 2 and 3, and determine if the two sites contain overlapping sequences (Hostager *et al* 1999). To this aim, a series of mutations were created within PVQET sequence and around it. The effects of amino acid substitutions and deletions in the sequence are presented in table 1.5. Data presented suggests that recognition sites for binding of TRAF 2 and 3 overlap with one another but are distinct. Moreover studies have shown that despite the apparent overlap in recognition sequences, the two proteins do not compete for binding (Pullen *et al* 1999).

To dissect the *in vivo* role of various TRAFs, several members of this group of proteins have been inactivated in mice. These studies have shown that TRAF3 knock out (k/o) mice appear normal at birth, but die 10 days later (Xu *et al* 1996). Signalling in B cells and their proliferation in these animals appeared normal, but T cell activation was impaired.

Mice with deleted TRAF2 were also produced. They were born normal but at lower numbers compared with normal litter (Yeh *et al* 1997). These mice died early in life alike TRAF3 knockouts. Although initial experiments showed TRAF2 to be critical for JNK and NFκB activation, the studies carried out in TRAF2 knockouts mainly suggested suboptimal JNK activation, whereas NFκB responses were almost normal.

Sequence present in CD40cyt or a mutation	Importance for binding of TRAF2	Importance for binding of TRAF3
T254A	No binding	No binding
Pro250	Binding enhanced	Binding moderately enhanced
Val251	Binding enhanced	Binding moderately enhanced
Substitutions in ^{229}PKQEPQE^{235}	No effect	No effect
Alanine substitutions in ^{262}TQEDGK^{267}	No effect	Q263 within this sequence is crucial for binding of TRAF3
Substitution of Gln263	No effect	No binding
G266A	50% reduction in binding	Enhanced binding
CD40cytΔ11	40% reduction in binding	No effect
CD40cytΔ13	40% reduction in binding	Modest reduction in binding
CD40cytΔ15	40% reduction in binding	No binding
^{271}ISVQE^{275}	Motif found in all TRAF2 binding receptors	No effect

Table 1.5- Sequences and individual residues in the hCD40cytoplasmic domain of importance in TRAF2 and TRAF3 binding

In studies using expression constructs of TRAFs, it has been shown that overexpression of TRAF 2, 5 and 6 leads to activation of NF-κB and JNK, whereas overexpression of TRAF3 does not (Leo *et al* 1998). This concurs with data indicating that TRAF 3 mainly functions as a docking factor for TRAF 5.

1.6.4.2 TRAF 5

TRAF 5 binds in the same region as the TRAF2 and 3 as determined by mutational analysis (Ishida *et al* 1995). However, direct binding has not been demonstrated. Studies by Leo *et al* demonstrate that TRAF5 is recruited to CD40cyt by interaction with TRAF3 (Leo *et al* 1993). It therefore appears that TRAF5 has very low affinity for CD40cyt, but can be recruited to the receptor in a TRAF3 dependent manner. Hence, those amino acid residues deemed important for TRAF3 binding apply also to binding of TRAF5. In relation to this, TRAF3 can modulate NF-kB induction by TRAF5, as recruitment of TRAF5 is dependent on TRAF3.

TRAF5 knockout mice had a milder phenotype than either the TRAF2 or TRAF3 knockouts. No other major abnormalities were detected (Nakano *et al* 1999). Although signalling in B cells isolated from these mice showed normal responses in JNK and NF-kB activation, there were defects associated with CD40 mediated proliferation and expression of genes normally regulated by CD40 signalling, such as CD23, CD54, B7.1, B7.2 and Fas. Taken together, these data suggest a critical role for TRAF5 in CD40 function and signalling.

1.6.4.3 TRAF 6

TRAF6 binding takes place at a recognition motif located close to the trans-membrane region of CD40cyt, within the sequence ^{231}QEPQEINF^{238} (Darnay *et al* 1999, Kashiwada *et al* 1998). Effects of mutagenesis of amino acid residues within this region on TRAF6 binding were assessed and the summary of information gathered is shown in table 1.6. Although other residues in the minimal binding site were shown to have an effect on TRAF6 binding to the CD40cyt, residue E235 was found to be critical. TRAF6 has been inactivated in knockout mice. However, due to the fact that TRAF6 and IRAK/MyD88 form critical pathway in the signalling via the Toll/ IL1R family (Cao *et al* 1996), inactivation of this TRAF member *in vivo* formed a phenotype which not only had impact on CD40 signalling, but also on Toll/IL1-R signalling cascades as well. TRAF6 knockout mice had low birth rates, died early and had severe osteoporosis (Lomaga *et al* 1999, Naito *et al* 1999). Dissecting the role of TRAF6 alone in CD40 signalling has been impossible to do, as this factor has a critical role in other signalling cascades.

Sequence present in CD40cyt or a mutation	Importance in TRAF6 binding
CD40Δcyt	No binding
CD40Δ15	No effect
CD40Δ32	No effect
E235A	No binding-critical residue
Q231A	Minor reduction in binding
Q234A	Minor reduction in binding

Table 1.6- Residues and truncations in hCD40 cytoplasmic domain and their effects on TRAF6 binding

1.6.5 Janus kinase 3

Jak3 belongs to the family of non-receptor tyrosine kinases involved in intracellular signalling mediated by cytokines and growth factors (Rane *et al*1994). Association of this kinase with CD40 has been shown in both B cells and monocytes (Revy *et al* 1999). Jak3 constitutively binds to CD40cyt within the domain termed box 1. This is a proline rich sequence in the membrane-proximal region of CD40 with a sequence ^{222}PTNKAPHPK^{230}. Although the binding of Jak3 takes place in box 1, a second region termed box 2 (^{260}PVTQEDGKESR^{270}) is also important for the association of Jak3 with the receptor. This secondary site presumably provides the receptor with a correct conformation or it assists by providing an as yet unidentified interaction (Hanissian *et al* 1997).

Upon CD40 ligation, Jak3 phoshorylates itself and other substrates including STATs, which are transactivators of the promoters of cytokine responsive genes containing IFNγ activation site (Hanissian *et al* 1997, darnell 1997)(see table 1.3). STATs dimerise in the cytoplasm, translocate into the nucleus and affect transcription of CD23, ICAM1 and LTα. All three genes contain both NFκB and STAT sites in their promoter regions (Jabara *et al* 1998).

Name	Function
STAT3	Transcription factor that binds to the interleukin 6 responsive elements identified in the promoters of various acute phase protein genes. Forms a homo- or heterodimers with a related family member. It becomes tyrosine phoshorylated in response to various cytokines; serine phosphorylation is important for the formation of stable DNA-STAT3 homodimers and maximal transcriptional activity. Contains 1 SH2 domain.
STAT5a	Carries out a dual function- signal transduction and activation of transcription. This transcription factor binds to the GAS element and activates PRL-induced transcription. It forms a homo- or heterodimer with a related family member. It translocates into the nucleus upon phosphorylation in response to IL2, IL3, IL7, IL15, GM-CSF, growth hormone, prolactin, erythropoietin and thrombopoietin. Tyrosine phosphorylation is required for DNA binding activity and dimerization. Serine phosphorylation is also required for maximal transcriptional activity. Contains 1 SH2 domain.
STAT6	Carries out a dual function- signal transduction and activation of transcription. It is involved in interleukin 4 signalling. Forms a homo- or heterodimer with a related family member. Tyrosine phosporylated in response to IL4 and IL3. Translocated to the nucleus upon phosphorylation. Contains 1 SH2 domain.

Table 1.7- Brief description of the structure/function of three STAT transcription factors involved in CD40 signalling

There have been conflicting reports with regards to the role of Jak3 in CD40 mediated signalling. Jabara *et al* report a study where the effects of CD40 stimulation on Ig isotype switching, B cell proliferation, up-regulation of CD23, ICAM1, LTα and CD80 have been compared in Jak3 deficient and normal patients. This group reports no changes between the two groups of patients, thus suggesting that Jak3 is not essential for the functions studied (Jabara *et al* 1998). In another study, Jak3 was found to be an absolute requirement for expression of CD23, ICAM1 and LTα in eight different cell lines (Siemasko *et al* 1998). Siemasko *et al* hypothesize Jak3 and STAT6 to be

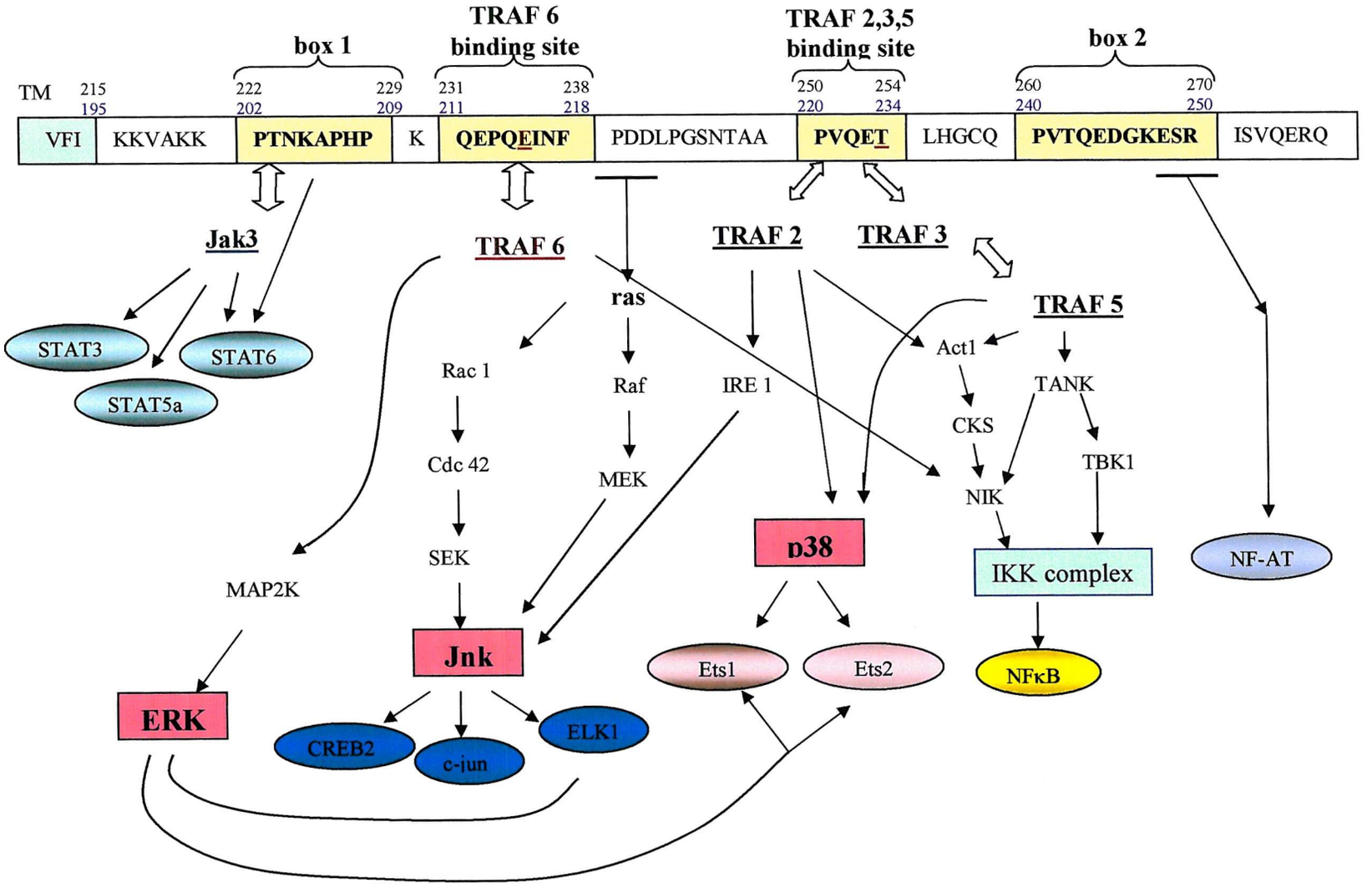


Figure 1.11- Simplified overview of CD40 signalling. CD40 cytoplasmic domain is shown as a boxed sequence. Binding sites for Jak3 and TRAFs are outlined. Residues in CD40 cytoplasmic domain are numbered including (in black) and excluding (in blue) the signal peptide sequence. Various kinases and transcription factors downstream of CD40 are shown in boxes or bubbles.

involved in IgG1 expression as blockade of Jak3 phosporylation leads to inhibition of IgG1 production. Data by Hanissian *et al* demonstrates no Jak3 phosphorylation in murine spleen B cells, whereas it shows induction of STAT6 (Hanissian *et al* 1997). There are reports indicating that inhibition of Jak3 blocks heart allograft rejection, which is in agreement with the reports showing that blockade of CD40 signalling prevents transplant rejection even in strongly mismatched donor-recipient pairs (Wang *et al* 1999, Kirk *et al* 1999). In conclusion, it would seem that Jak3 might be exhibiting cell specific responses in different cell types expressing CD40. Also, STATs may be induced by CD40 in a Jak3 independent manner.

1.6.6 *In vivo* functions of CD40/CD40L interactions

CD40 is expressed on numerous cell types in the body. In each different cell type, CD40 may have a slightly different function, which is dependent on the availability of particular signalling cascades and the function of the cell. In this manner, CD40 signalling and resulting effects can be cell specific. In an *in vivo* situation, CD40/CD40L interactions have an effect on numerous different cells and various aspects of immune response. The receptor/ligand pair has been demonstrated to have a role in inflammation, transplantation, autoimmunity, humoral and cellular responses (Clark 2000, van Kooten 2000, Laman *et al* 1998, Grewal *et al* 1998). The critical importance of CD40 signalling *in vivo* is shown in hyper IgM syndrome, which is normally due to alterations in CD40L function and expression (Kroczek *et al* 1994), but a similar immunodeficiency can also be caused by impairment of CD40 function (Durandy *et al* 1997). Patients with hyper IgM syndrome have an enhanced susceptibility to opportunistic infections, suggesting a major role for CD40/CD40L in cell mediated immune responses. This is further demonstrated in CD40L knockout mice which show a substantial impairment in antigen specific T cell responses (Grewal *et al* 1995). Binding of CD40L on T helper cells to CD40 on B cells results in B cell proliferation in germinal centers and class switching (Kawabe *et al* 1994). Therefore, hyper IgM syndrome is also characterised by absence of germinal centers and immunoglobulin class switching from IgM to other subclasses, hence the name (Garside *et al* 1998). Due to a critical role of CD40/CD40L in humoral responses, various strategies have been employed to manipulate these responses. Thus, blocking anti CD40L antibodies have been used in animal models to inhibit unwanted antibody

production in experimental autoimmune diseases, or alternatively agonistic anti CD40 antibodies have been utilised to promote strong, isotype switched antibody responses against pneumococcal polysaccharides (Dullforce *et al* 1998). Therefore, stimulation of CD40 may be one of the means used in future vaccination strategies against weakly immunogenic, T cell independent antigens. Also, this type of approach might be utilised to boost immune responses in immunocompromised patients.

As previously mentioned, CD40/CD40L signalling has a major role in T cell priming, differentiation and effector functions. This topic has been extensively reviewed elsewhere (Grewal *et al* 1996, Stout *et al* 1996, Mackey *et al* 1998, Heath and Carbone 1999), and already visited in part in section 1.4.5, hence only a brief description will be given here.

It has for a long time been realised that CD4+ T cells are needed for induction of CD8+ cytotoxic T cell responses. In the past, it was thought that a professional antigen presenting cell, a DC, would have to be in contact with both the CD4+ T helper cell, and CD8+ cytotoxic T cells at the same time in order for CD8+ cell to be activated. It was hypothesised that the CD4+ T cell would be activated thought its interaction with a DC (via its TCR and MHC class II on the DC), start producing IL2 which would then cause a by-stander proliferation of CD8+ T cell which is interacting with the same DC through its TCR and MHC class I on the DC (see figures 1.7 and 1.9). However, it was eventually realised that such envisaged events would be very rare, and a new hypothesis was simultaneously presented by a three different research groups (Bennet *et al* 1998, Ridge *et al* 1998, Schoenberger *et al* 1998). In the new concept of CD8+ T cell activation, it is proposed that a "licensed" DC could act as a bridge between the CD4+ T helper cell and CD8+ T cell. The "licensing" is a process of DC activation through its interaction with a CD4+ T helper cell, firstly via TCR/MHC class II interaction, followed by CD40/CD40L binding on DC/helper T cell respectively. As a result of CD40 signalling, the DC start to express co-stimulatory markers and commences production of various cytokines. Such a conditioned DC can migrate away from the helper T cell, and can encounter a CD8+ T cell. This T cell can recognise MHC class I/peptide complex on the DC via its TCR, followed by ligation of CD28, LFA1 and cytokine receptors, thus receiving co-stimulation. These events drive activation of CD8+ T cell, which is further augmented by production of IL2, in turn causing proliferation in an autocrine manner (see figure 1.11). Based on this model,

interaction of the TCR on CD8+/CD4+ T cells with MHC class I/II on a DC in the absence of CD40 signalling leads to tolerance and anergy of the T cells to the antigens presented by the DC. Although some antigens can be efficiently presented to T cells in a CD40 independent manner, most have been shown to depend on this pathway.

CD40/CD40L have also been shown to have a very important role in transplantation and autoimmunity. In many ways, the role of CD40 signalling in both autoimmunity and transplantation is an extension of its role in activation of T cells. In autoimmune disease, inappropriate responses are encountered which are directed against self. Therefore, the tolerance against self is broken in such cases. It has been established that absence of CD40 signalling can lead to tolerance, thus blocking of CD40 triggering is an approach which has been shown to reduce autoimmune responses (Datta *et al* 1997, Desai-Mehta *et al* 1996, Koshy *et al* 1996, Yellin *et al* 1997, Kato *et al* 1999, Gerritse *et al* 1996, Kalled *et al* 1998, Howard *et al* 1999).

Similarly, the problem with transplantation is that the transplant is often rejected due to being recognised as non-self. It was found that tolerance to a transplant can be induced by administration of anti CD40L antibodies which prevent this ligand binding to CD40 thus tolerising T cells to the graft (Larsen and Pearson 1997, Sayegh *et al* 1998, Gudmundsdottir and Turka 1999, Van Kooten 1999, Larsen *et al* 1996, Trambley *et al* 1999, Jones *et al* 2000, Kishimoto *et al* 2000, Sun *et al* 1997, Reul *et al* 1997).

In summary, activation of a number of immune responses is regulated at the level of CD40/CD40L interactions. Multiple disease states can be improved by disruption of interaction between CD40 and the ligand. Currently, this interruption is accomplished by distribution of antibodies which can prevent association of CD40 and CD40L which are being tested in phase II clinical trials. In long term management of CD40 signalling, such an approach may not be optimal. Instead, identification of small synthetic peptides preventing CD40L binding to CD40 would be of interest. Alternatively, reagents which may interfere with the signalling pathways triggered by cross-linked CD40 in cells would also be potentially therapeutic. To the latter aim, in depth knowledge of CD40 signalling in cells of interest would be of paramount importance.

1.7 Aims, objectives and rationale

1.7.1 Rationale

The ways in which the body deals with foreign and altered self-cells have been explained in the previous text. It would seem that the CD40/CD40L interaction regulates a number of such immune responses. In the model upon which this thesis is based, it has been shown that CD40 signalling not only leads to eradication of established tumour, but it also provides the animal with protective immunity against that particular tumour. Although there are a lot of descriptive data regarding this therapy model, there is virtually no mechanistic insight into signalling events taking place in the cell types important in the therapy. Based on indirect evidence, it is thought that host DCs may be responsible for induction of tumour specific CTL responses in treated animals. It is also indicated that CD40 receptor does not have to be on the tumour cell, although anti CD40 therapy in B cells, which have CD40, is more successful than those carried out in T cell tumours, which do not express the receptor. Therefore, CD40 signalling should be studied in DCs and tumour B cells, as the latter may in some way aid the initiation or magnitude of CTL responses.

Which aspect of CD40 signalling should be dissected in DCs? It is known that CD40 is the key regulator of DC activation and maturation. CD40 signalling leads to production of numerous cytokines and up-regulation of many surface markers in DCs. It is impossible to study all of those, so one of the induced genes was chosen for studies in this thesis based on its importance in the immune responses. This gene is IL6. The reasons for looking at CD40 induction of IL6 in DCs are multiple. Interaction of CD40 on DCs with CD40L on naive T cells is critical for DC maturation and the generation of antigen-specific T cell responses (Banchereau et al 2000, Grammer et al 2001). CD40L promotes DC survival, elevates expression of MHC and costimulatory molecules and induces the expression of a variety of cytokines including TNF, IL-1, IL-6, IL-12, IL-15 and IL-18, all of which are involved in T cell activation and proliferation (Banchereau et al 2000, Grammer et al 2001).

IL-6 is a highly pleiotropic cytokine with properties that indicate it is not only a stimulator of the activation, proliferation and survival of T cells (Van Snick 1990,

Akira et al 1993, Gajewski et al 1995, Teague et al 1997, Demoulin et al 1999, La Flamme et al 1999, Teague et al 2000), but is also able to modify DC function and survival (Banchereau et al 2000, Drakesmith et al 1998, Grohmann et al 2001). A direct effect of IL-6 on the proliferation of T cells has been documented by several groups (Van Snick 1990, Akira et al 1993, Gajewski et al 1995, Teague et al 1997, Demoulin et al 1999, La Flamme et al 1999), as has the ability of IL-6 to prevent the death of naïve T cells (Teague et al 2000). Other studies have described the ability of IL-6 to induce IFN-γ secretion in differentiating T cells, indeed lack of this response in IL-6 gene knockout mice renders animals unable to mount T cell responses against *Mycobacterium. tuberculosis* and *Toxoplasma. gonddi* (Suzuki et al 1997, Leal et al 1999). Production of IL-6 by CD40 stimulated DCs (Banchereau et al 2000, Grohmann et al 2001) is therefore likely to be important in promoting the generation and survival of antigen-specific cytotoxic T cells. IL6 can alter the manner in which antigen is processed by DCs enabling the activation of T cells against determinants that were previously cryptic (Drakesmith et al 1998). In addition, Grohmann and colleagues reported that autocrine IL6 mediates most of the anti-tolerogenic effects of CD40 ligation on CD8+ DC (Grohmann et al 2001). Aberrant regulation of autocrine and paracrine stimulation of DCs by IL6 may therefore be important in the generation of anti-self immunity and propagation of autoimmune disease.

The signal transduction events leading to the activation of cytokine gene transcription by stimulation of CD40 have mainly been studied in B cells. By contrast little is understood about these events in other cell types, including DCs (Grammer et al 2001). Thus, studies in DCs could delineate the manner in which CD40 affects activity of IL6 promoter. The study of CD40 signalling in DCs would be done by testing the ability of endogenous CD40 to induce activity of wild type and mutant IL6 promoter/luciferase reporter constructs. Secondly, human CD40 wild type and mutant expression constructs would be tested for their ability to signal to the wild type IL6 promoter/ luciferase reporter. The results of these studies would identify transcription factors important in regulation of IL6 promoter induction, and also domains in CD40 receptor involved in this induction. In this way it may be possible to dissect the signalling pathway important in regulation of IL6 in DCs from both the receptor and promoter end. Further studies would be carried out to identify the transcription factor complexes involved in the signalling events. If IL6 was found to be as important as suggested in this

hypothetical model, then in future it may be possible to control the induction of IL6 by the use of small, specific drugs. This approach would be better than the use of anti CD40 mAbs, which has many undesirable effects associated with major non-specific activation of the immune response.

Work in our laboratory also suggests that the presence of CD40 may be important on the tumour cells. Although protection and a curative outcome are achieved in CD40-ve tumours (French *et al* 1998), it would appear that CD40 signalling in the tumour cells may contribute to the establishment of the immune response. It would therefore also be of interest to study CD40 signalling in the tumour B cells. The approach to this type of study would be in part different to that already described for CD40 in DCs. In these experiments, wild type and mutant hCD40 constructs would be transfected into the tumour cells, and stably transfected cell lines selected. These would be used in *in vivo* studies to establish if mAbs directed against hCD40 are also likely to have an effective therapeutic potential. Results obtained may indicate the relative importance of CD40 signalling in the tumour cells in the attainment of protective immunity.

1.7.2 Aims and objectives

The overall goal of the studies described in this thesis was to gain a better understanding of the mechanisms responsible for the anti-tumour activity of anti-CD40 in mice. The specific aims of the thesis therefore are:

1. To develop an *in vivo* model for studying the signal transduction events which may mediate the therapeutic properties of antibodies directed against human CD40.

2. To determine cellular events resulting from CD40 ligation in B cell lymphoma cells and dendritic cells by:
(i) Determining CD40-induced changes in the expression of mRNA and proteins for effector molecules, mainly IL6.
(ii) Determining the major signal transduction events that mediate CD40-induction of effector molecules utilising transcription activity assays and mutated human CD40 proteins.
To achieve these aims the following objectives were addressed:

Aim 1

1. Produce a series of mammalian expression vectors that encode wild type and mutant human CD40 proteins designed to perturb specific intracellular signal transduction events.
2. Generate stable transfected B cell lymphoma cell lines that constitutively express wild type and human CD40 proteins that are recognised by anti human CD40 antibodies.
3. Determine the ability of B cell lymphoma lines expressing wild type and mutant human CD40 proteins to generate tumours in mice and establish if treatment of these mice with anti-human CD40 mAb is protective.

Aim 2

1. Set up quantitative protein and semi-quantitative mRNA detection assays for a variety of cell surface and secreted effector molecules including IL6, IL12, IL10, IFNγ, IL4 and β actin. These assays would then used to monitor changes in expression induced by engagement of wild type and mutant CD40 molecules.

2. Establish assays for the measurement of transcriptional events occurring at the promoter region of the human IL6 gene. Then utilise mutant human CD40 proteins and mutated promoter constructs to determine membrane proximal and nuclear signalling events mediating CD40-induced changes in B cell and dendritic cell function.

The outline of results chapters contained in the thesis is as follows:

a.) Chapter 3- describes the production of expression vectors for wild type and mutant human CD40 proteins chosen to demonstrate requirement for signalling motifs. It also describes production of stable mouse B cell lymphoma lines expressing wild type and mutant human CD40 proteins.

b.) Chapter 4- describes the use of B cell lymphoma lines expressing human CD40 constructs to develop an *in vivo* therapy model with which to test ability of anti human CD40 mAb to deliver therapy through signalling in tumour B cells alone and to test the requirement for specific signalling motifs on the human CD40 protein.

c.) Chapter 5- describes the *in vitro* analysis of human CD40 expression and signalling in B cell lymphoma lines and outlines the inherent difficulties associated with these studies.

d.) Chapter 6- presents a new hypothesis for anti CD40 therapy based on stimulation of the host dendritic cell. It explains how anti CD40 mAb stimulates signalling events which result in the transcriptional activation of the interleukin 6 gene which can promote anti tumour responses through its effect on activation, proliferation and anti-apoptotic effect on T cells and DCs.

e.) Chapter 7- discusses the overall achievements and problems of the thesis and describes future avenues of research that may lead to an improved understanding of CD40 mediated immunotherapy for cancer.

CHAPTER 2

Materials and methods

2.1. MATERIALS

2.1.1 Antibiotics

Ampicillin	Sigma
Geneticin	Sigma
Penicillin	Glaxo
Streptomycin	Evans
Puromycin	Sigma
Ciproxin	Bayer

2.1.2 Antibodies

NF-κB Transcription Factor Family

Rabbit polyclonal IgG anti human NF-κB p50 (internal region)	Santa Cruz
Rabbit polyclonal IgG anti human NF-κB p65 (amino terminus)	Santa Cruz

CBF1 transcription factor

Goat polyclonal IgG anti mouse CBF1 (internal region)	Santa Cruz

AP1 transcription factor family

Rabbit polyclonal IgG anti mouse c-Jun (amino terminus)	Santa Cruz
Rabbit polyclonal IgG anti mouse Jun D (amino terminus)	Santa Cruz
Rabbit polyclonal IgG anti mouse Jun B (amino terminus)	Santa Cruz
Rabbit polyclonal IgG anti mouse c Fos (amino terminus)	Santa Cruz

Antibody specificity	Clone name	Isotype	Source
Mouse anti human CD40	LOB 7.6	IgG1	Tenovus
Rat anti mouse CD40	3/23	IgG2a	Gerry Klaus

Table 2.1-Antibodies used for *in vitro* stimulation of cells and *in vivo* therapies

Antibody specificity	Clone name	Isotype	Source
Mouse anti human CD40	LOB7.6	IgG1	Tenovus
Mouse anti human CD40	LOB7.1	IgG2a	Tenovus
Rat anti mouse CD40	3/23	IgG2a	Gerry Klaus
Hamster anti mouse CD152 (CTLA-4)	UC10-4F10-11	IgG	American Tissue Culture Collection (ATCC)
Rat anti mouse CD11a (LFA 1)	TIB237	IgG2b	ATCC
Mouse anti mouse MHC class I (D^dK^d)	HB79	IgG2a	ATCC
Rat anti mouse CD54 (ICAM1)	YN1.4.7	IgG2b	ATCC
Hamster anti mouse CD80 (B7.1)	1610A1	IgG	ATCC
Rat anti mouse CD86 (B7.2)	GL-1	IgG2a	ATCC
Hamster anti mouse MHC class II (haplotype d)	N22	IgG	ATCC
Rat anti mouse Bcl_1 idiotype	Mc106A5	IgG2a	Tenovus
Rat anti mouse CD4	GK1.5	IgG2b	ATCC
Rat anti mouse CD4	YTA3.1.2	IgG2a	Steve Cobbold, Oxford
Mouse anti mouse MHC class I(K^k)	HB160	IgG1	ATCC
Mouse anti human CD22	HD6	IgG2a	Dorken
Mouse anti human CD22	4KB128	IgG2a	David Mason, Oxford

Table 2.2- Antibodies used in flow cytometry (FACS)

2.1.3 Chemicals

Acrylamide	Scotlab
Agar	Sigma
Agarose	Gibco
4-(2-Aminoethyl)benzenesulfonyl Fluoride (AEBSF)	Sigma
Ammonium Persulphate (APS)	Sigma
Aprotinin	Sigma
Boric Acid	Sigma

Bovine serum albumin (BSA)	Sigma
Bromophenol Blue	Sigma
Chloroform	Sigma
Deoxynucleoside triphosphates (dNTPs)	Sigma
Dithiothreitol (DTT)	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Ethidium bromide	Sigma
Foetal calf serum (Myoclone Plus)	Gibco
Ficoll 400	Sigma
FITC isomer I	BDH
[γ-^{32}P]dATP	Amersham
L-glutamine	Gibco
Glycerol	Sigma
JM109	Promega
N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid](HEPES)	Sigma
Iso-amyl alcohol	Fisons
Magnesium chloride	Fisons
Methanol	Sigma
Microspin G-25 columns	Amersham
Mineral oil	Sigma
Nonidet P-40 (NP40)	Sigma
Orange G	Sigma
Pfu polymerase	Stratagene
Phenol:chloroform:isoamyl alcohol(25:24:1)	Sigma
Poly dIdC	Sigma
Potassium chloride	Sigma
Reagent A	BioRad
Reagent B	BioRad
Restriction enzymes	Promega
RPMI 1640	Gibco
Sonicated salmon sperm DNA	Stratagene
Sodium chloride	Sigma
Sodium dodecyl sulphate	Sigma

Sodium orthovanadate (NaV)	Sigma
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma
Thermus Aquatus (Taq) polymerase	Promega
T4 DNA ligase	Promega
Tris	Sigma
T4 Polynucleotide kinase	Amersham
Xylene cyanol	Sigma

2.1.4 Disposable equipment

Microfuge tube centrifuge tubes	Elkay
Filter tips	Greiner
Tissue culture plastics	Nunclon
3mm Whatmann paper	Whatmann
X-ray film	GRI

2.1.5 Supplied kits

Effectene transfection kit	Qiagen
QIAprep Plasmid maxi kit	Qiagen
QIAprep miniprep kit	Qiagen
QIAquick gel extraction kit	Qiagen
Quick Prep micro mRNA purification kit	Pharmacia
First strand cDNA synthesis kit	Pharmacia

2.1.6 Buffers and solutions

<u>1x PBS</u>

NaCl	7.01g
Na_2HPO_4	3.44g
KH_2PO_4	0.79g

up to 1 litre with dH_2O

<u>LB medium</u>

NaCl	5g

bactotryptone 10g
bacto yeast exstract 5g
up to 1 litre with dH_2O, aliquot and autoclave immediately at 121°C for 15 minutes.

SOC medium

NaCl 0.4g
KCl 0.19g
$MgCl_2$ 2.03g
$MgSO_4$ 2.46g
glucose 3.6g
bactotryptone 20g
bacto yeast extract 5g
up to 1 litre with dH_2O, autoclave immediately at 121°C for 15 minutes

LB agar

3g of bactoagar into 200 mls of LB medium, autoclave at 121°C for 15 minutes

50x TAE

Tris 242g
glacial acetic acid 57.1mls
NaEDTA 1.9g
up to 1 litre with dH_2O, adjust pH to 8.0

Agarose gel running buffer

20 mls 50x TAE
60µl ethidium bromide
up to 1 litre with dH_2O

10x loading buffer for agarose gels

60µg bromophenol blue
6 mls glycerol
14 mls TE buffer

6x gel loading buffer for EMSA

50mM EDTA
10% Ficoll 400
0.25% bromophenol blue
0.25% xylene cyanol
0.25% orange G

10x forward buffer (for oligonucleotide radiolabeling)

500mM Tris-HCl pH7.5
100mM $MgCl_2$

50mM DTT
1mM spermidine

8% native polyacrylamide gel (for EMSA)

3 mls 5x TBE
10 mls of 40% bis-acrylamide
up to 50 mls with dH_2O
add 0.5 ml 10% APS (1:100) and 50µl TEMED (1:1000)

Dignam A buffer

10mM HEPES(pH7.9)
1.5mM $MgCl_2$
10mM KCl
0.5mM DTT
0.2% NP40

Dignam C buffer

2mM HEPES(pH7.9)
2.5 mls glycerol (in 10mls of Dignam C buffer)
420mM NaCl
1.5mM $MgCl_2$
0.5mM DTT
200nM EDTA

PBS/BSA/azide

1x PBS
1% Bovine Serum Albumin fraction V
20mM NaN3

Tris/EDTA buffer

10 mM Tris-Cl, pH 7.4
1mM EDTA, pH8.0

Uranyl acetate solution for staining grids

Uranyl acetate
50% EtOH

Place uranyl acetate in a polypropylene centrifuge tube. Fill the tube with 50% EtOH and leave to stand in the fridge overnight.

Tri-sodium citrate $Na_3C_6H_5O_7*2H_2O$	1.76g
Fresh distilled water	30mls
Freshly prepared 1M sodium hydroxide NaOH	8ml

Place the nitrate and sodium citrate into the distilled water in a very clean container. Shake for 1 minute and than intermittently for 30 minutes to produce a milky suspension. Add the sodium hydroxide and shake until clear. Make up to 50 mls with fresh distilled water.

Piperazine (PIPES) buffer

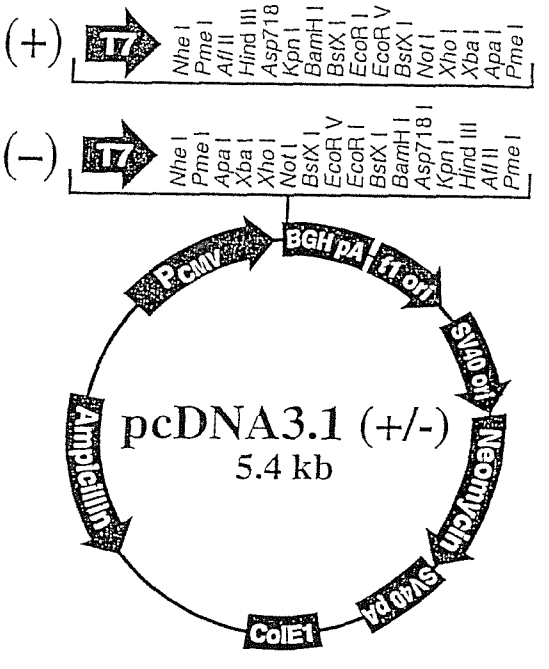
Piperazine-NN'-bis-2-ethanesulphonic acid (PIPES)
10M NaOH

Mix piperazine with 900mls of distilled water. Add 10M NaOH to bring the pH up to 7.2. Make up to 1L with distilled water to produce a 0.2M solution of PIPES buffer.

2.1.7 Vectors, vector maps and sequencing primers

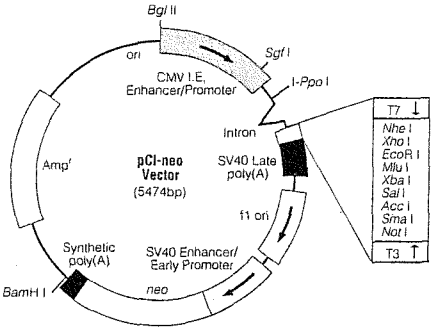
pcDNA3.1, Invitrogen

Sequencing primers T7 and Sp6



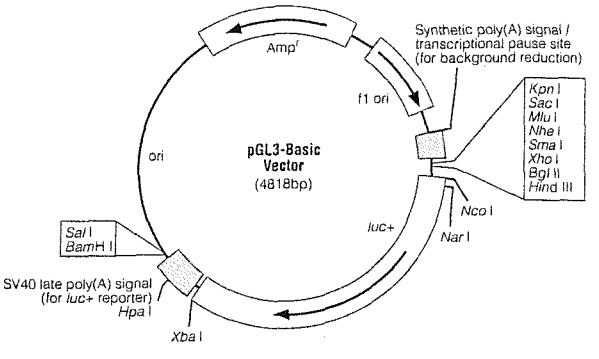
pCIpuro, Promega

sequencing primers T7 and T3



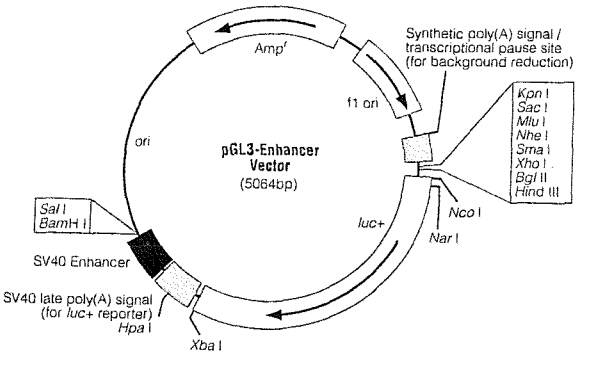
pGL3 Basic, Promega

Sequencing primers RV3 and GL2



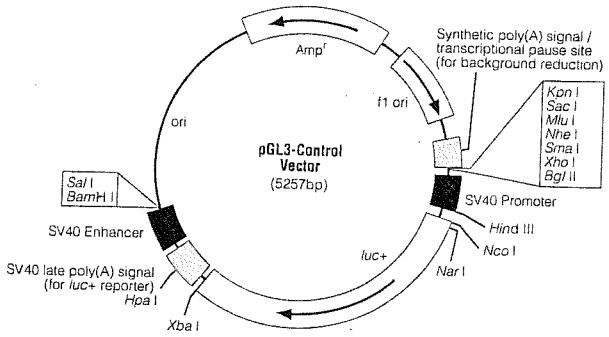
pGL3 Enhancer, Promega

Sequencing primers RV3 and GL2



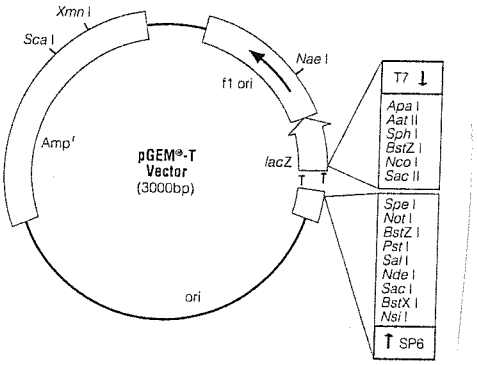
pGL3 Control, Promega

Sequencing primers RV3 and GL2

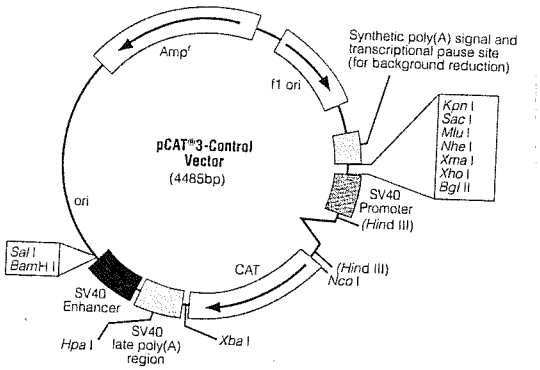


pGEM T vector, Promega

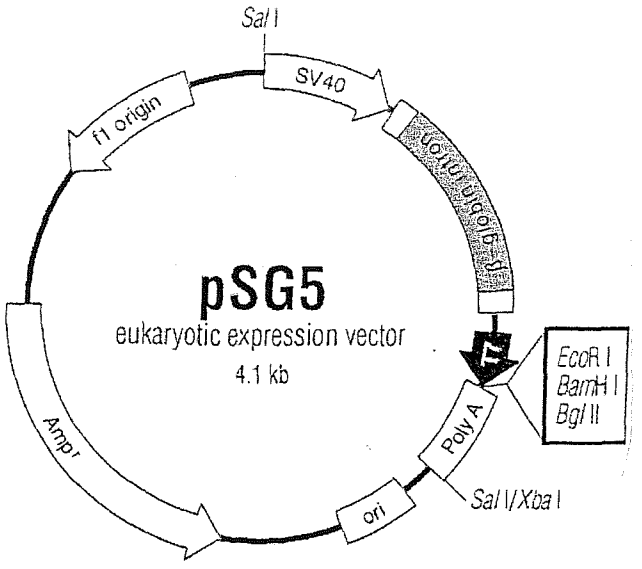
Sequencing primers T7, T3, M13F, M13R



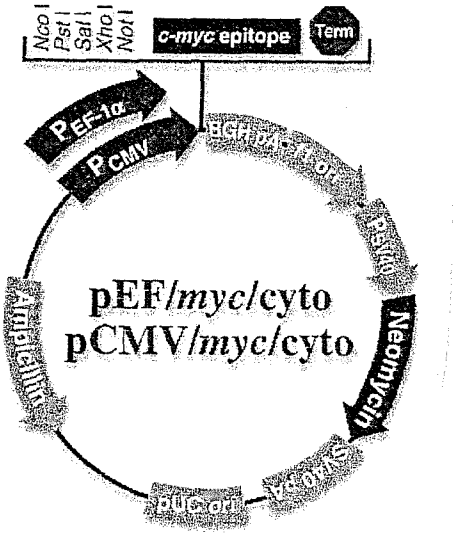
pCAT Control



pSG5



pBos



Sequencing primer name and orientation	Primer sequence
T7 sense	5' taa tac gac tca cta tag gg 3'
T3 antisense	5' att aac cct cac taa agg gga 3'
Sp6 antisense	5' tat tta ggt gac act ata g 3'
M13F sense	5' gtt ttc cca gtc acg ac 3'
M13R antisense	5' cag gaa aca gct atg ac 3'
GL2 antisense	5' ctt tat gtt ttt ggc gtc ttc ca 3'
RV3 sense	5' cta gca aaa tag gct gtc cc 3'

Table 2.3- Sequencing primers

2.1.8 Cell culture materials

All cell lines were cultured in RPMI 1640 medium, supplemented with 100 units (U)/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine and 10% Foetal Calf Serum .

2.1.9 Cell lines

A20, πBcl1 (Illidge *et al*2000.), FSDC (Girolomoni *et al*1995), Daudi, Raji and Ramos (European Collection of Animal Cell Cultures (ECACC), Porton Down) were maintained in culture medium described above at 37^0C in 5% CO_2 humidified incubator. Media was replenished every 2-3 days. Bcl_1 mouse B cell lymphoma line (Slavin *et al*1980, Warnke *et al*1979) was maintained by *in vivo* passage in BALB/c mice. Mice were supplied by Harlan UK (Blackthorn, U.K.). FSDC is an immature myeloid dendritic cell line generated from foetal mouse skin dendritic cells by infecting cell suspensions with a retroviral vector carrying an envAKR-mycMH2 fusion gene (Girolomoni *et al*1995).

2.2 METHODS

2.2.1 mRNA preparation using the "Quickprep micro mRNA Purification kit"

$1x10^7$ cells were harvested by scraping into media and transferred into a Universal tube, pelleted for 3 minutes at 1000rpm in a tabletop centrifuge, and supernatant discarded. Each of the cell samples were washed twice in 3mls of PBS and transferred into a Microfuge tube. 0.4ml of extraction buffer was added to the pellet and mixture vortexed until a homogeneous suspension achieved. The sample was diluted by adding 0.8mls of elution buffer and mixed by vortexing. The tube with the sample was then centrifuged at top speed in the microfuge for 1 minute so to obtain a cleared cellular homogenate. In parallel with this step, a tube containing 1ml of oligo-dT cellulose was centrifuged for one minute. The buffer from oligo-dT cellulose tube was removed by aspiration. The cleared cellular homogenate was transferred to the oligo-dT cellulose pellet, and mixed by pipetting up and down several times. The tube was gently mixed

by inverting for 3 minutes. The sample was then centrifuged at 14000rpm for 1 minute in a microfuge. The supernatant was removed by aspiration, and pellet resuspended in 1ml of high salt buffer. The tube was centrifuged for 10sec, supernatant removed and the 1nl high salt buffer wash step repeated another four times, for a total of five washes. Following this, 1ml of low salt buffer was added, and same procedure as for high salt buffer repeated. The low salt wash was done twice. Following the removal of supernatant, the cellulose resin was resuspended in 0.3 mls of low salt buffer and slurry transferred into a microspin column, which was centrifuged at 14000rpm for 5 seconds. The effluent was discarded, and another 0.5mls of low salt buffer added and sample centrifuged. This step was repeated two more times for a total of three washes. After the final wash, the effluent was removed, and the column transferred into a clean Microfuge tube. 0.2mls of pre-warmed elution buffer (65^0C) was placed on the top of resin bed. The tube was centrifuged for 5 seconds. The eluate contained mRNA.

Prior to proceeding to first strand cDNA synthesis, the obtained mRNA had to be concentrated by precipitation. This was achieved by addition of 10µl of glycogen solution, 1/10 volume of potasium acetate to the mRNA sample. 1ml of 95% ethanol was added, and sample placed at -20^0C for a minimum of 1 hour. The precipitated mRNA was collected by centrifugation in a microfuge at 4^0C for 15 minutes. Supernatant was discarded, and precipitated mRNA re-dissolved in DEPC water so to obtain a concentration of 5µg/20µl.

2.2.2 cDNA preparation utilising "First strand cDNA synthesis kit"

mRNA obtained using the method described in 2.2.1 was used to acquire first strand cDNA. 20µl mRNA solution (containing 5µg mRNA) was placed into a heat block at 65^0C for 10 minutes. The tube was then removed from the heat block and placed on ice. mRNA solution was aspirated into a tip and placed into a clean microfuge tube, to which 11µl bulk first strand cDNA reaction mix was added, as well as 1µl DTT solution and 1µl of $pd(N)_6$ random hexamer primer. The mixture was incubated at 37^0C for 1 hour. The obtained solution contained first strand cDNA and was used as template in subsequent RT-PCR reactions.

2.2.3 PCR primer design

Primers for PCR cloning of the full-length cDNA were constructed to anneal to 5' and 3' ends of the gene of interest. Where appropriate, the end primers were designed to incorporate a restriction site at each end of the resulting PCR product in order to facilitate cloning of the PCR product into an expression vector of choice. Primers that contain restriction enzyme site within their sequence have the enzyme name in their designation, eg H40*HindIII*F.

PCR primers for amplification of gene segments were constructed so to anneal to regions within the given cDNA where that gene shares least homology with other expressed genes. These primers were chosen on the basis of absence of hairpin loops in their sequence, similar GC and AT content and specificity for the chosen region of the gene. The cDNA sequences used for primer design were obtained from the National Institutes of Health data base. Accession numbers of the sequences are included in the tables containing primer sequences.

2.2.4 PCR- Basic protocol

DNA amplification was performed using Pfu or Taq DNA polymerases, where appropriate. Generally, Pfu polymerase was used for cloning where an error free PCR product was required, whereas Taq polymerase was utilised in reactions where inaccuracies in the DNA obtained were of no consequence, such as semi quantitative PCR comparing the levels of particular gene mRNA in the cells. Briefly, the basic PCR reactions were set up in the following manner:

1µl of both sense and antisense primers (at 100ng/ml) were added to 1µl of cDNA/DNA template with 2.5µl of optimised reaction buffer and 1µl of dNTP mix (10mM each dNTP) in a total volume of 24.5µl. This mixture was then pulse spun and 0.5µl Pfu polymerase added (2U/µl), before a further pulse spin and addition of 30µl of mineral oil. The DNA was then denatured with a 5-minute incubation at 94°C. The required number of cycles were performed by denaturing the mixture at 94°C for 45 seconds, followed by annealing step at appropriate temperature for each primer pair for 1 min, followed by elongation step at 72°C for 2 minutes. Total number of cycles

performed ranged from 25-40, with a final elongation reaction of 10 minutes at 72^0C to ensure formation of full-length transcripts. All PCR reactions were loaded onto a 1% agarose gel containing ethidium bromide (0.6µg/ml) and separated for 45min-1h by electrophoresis at 80V, along with known size standards to allow estimation of the fragment size. DNA was visualised under UV light and a photographic record taken.

2.2.5 Reverse transcription PCR (RT-PCR)

mRNA was extracted from $1x10^7$ splenic mononuclear cells or culture cells using "Quickprep micro mRNA Purification kit" . First strand cDNA synthesis was then generated from the mRNA template using random hexamer primer with "First strand cDNA synthesis kit". PCR reactions were performed using Taq or Pfu polymerase in standard conditions (as 2.2.4) and optimised annealing temperatures. The amplified products were separated by electrophoresis in 1% agarose gel containing ethidium bromide (0.6µg/ml) and photographed with UV exposure.

2.2.6 Ligation

5µg DNA to be inserted was mixed with 100ng-1µg of the chosen vector, 3µl manufacturer supplied ligation buffer, 3µl ligase enzyme and dH_2O to make up the final volume of 30µl ligation mix. The mixture was incubated at 4^0C overnight prior to using it for transformation of competent cells.

2.2.7 Transformation

0.1-1µg of plasmid was added to 50µl JM109 cells, and 50µl 0.5M $CaCl_2$. The mix was allowed to incubate on ice for 30 minutes. The water bath was set at 42^0C, and the mix heat shocked for 45 seconds. After the treatment, 0.5ml SOC media was added, and the tube placed in a shaking incubator at 37^0C for 1 ½-2 hours for incubation. The cells were then spun down at 7000 rpm for 1 minute in a microfuge, and most of the media discarded. Cells were resuspended in the remaining media (~150µl), plated out onto an agar plate containing 100µg/ml ampicillin and grown in the incubator at 37^0C overnight.

2.2.8 Small scale preparation of plasmid DNA (mini-prep)

Single bacterial colonies were grown in 5mls LB medium containing 100µg/ml ampicillin overnight. Bacterial cells were harvested by centrifugation at 3000rpm in a table-top centrifuge for 10 minutes at 4^0C. "QIAprep miniprep kit" was used and preparation carried out as per manufacturers instructions. Briefly, the media was discarded, and any excess wiped onto a tissue. The cell pellet was resuspended in 250µl resuspension buffer. 250µl lysis buffer was added to resuspended cells, the tube inverted 4-6 times and cells left to lyse for about 1 minute. Following addition of 350µl neutralisation buffer, the mixture was centrifuged in a microfuge for 10 minutes at top speed. The supernatant obtained after centrifugation was transferred onto a mini column, which was centrifuged for 30 seconds to allow supernatant to flow through. The column was washed twice, first with 500µl buffer PB, then with 750µl buffer PE containing ethanol. The column was centrifuged after each wash for 30 seconds at top speed in a microfuge, and supernatant discarded. After PE buffer wash, the DNA from the column was eluted by placing 50µl TE buffer on top of the column bed, and centrifuging as before. The flow through was collected into a clean microfuge tube, as it contained eluted DNA.

2.2.9 Large scale preparation of plasmid DNA (maxi-prep)

A single bacterial colony was grown in 5mls of LB media containing 100µg/ml ampicillin overnight. 150µl of the starting culture was used to inoculate 100mls LB media supplemented with ampicillin, as before. The culture was grown overnight in a shaking incubator at 37^0C overnight. Bacterial cells were harvested by centrifugation at 3000rpm for 15 minutes at 4^0C in a tabletop centrifuge. "QIAprep plasmid Maxi kit" was used and preparation, elution and ethanol precipitation carried out as per manufacturers instructions. Briefly, the cell pellet was resuspended in 10mls resuspension buffer, followed by addition of 10mls lysis buffer. The mixture was inverted 4-6 times, and the cells left to lyse for 5 minutes. After this time, 10mls of neutralisation buffer were added and solution immediately transferred into Qiafilter cartridge and allowed to incubate for 10 minutes. During the incubation time, Qiagen-Tip 500 was equilibrated by adding 10mls of equilibration buffer, which was left to

empty by gravity. The solution in the Qiafilter was then syringed into the Qiagen-Tip and the contents left to empty by gravity. The tip membrane was washed by applying 60mls of buffer QC. The Qiagen-tip was transferred into a clean 50ml Falcon tube, where the DNA was eluted by applying 15mls of buffer QF on the tip membrane. Such eluted DNA was precipitated by adding 10.5mls of isopropanol. The solutions were mixed, and split into 16 1.5ml microfuge tubes. The tubes were centrifuged at 14000rpm for 30 minutes at 4^0C. Supernatant was carefully decanted off and pellets washed by adding 300µl ethanol to each tube. The tubes were centrifuged again for 10 minutes at 14000rpm in microfuge. All of the supernatant was discarded, and pellets left to air dry for 10 minutes at room temperature. The DNA in the tubes was then resuspended in a total of 600µl TE buffer.

2.2.10 DNA digest

5µg DNA
2µl restriction enzyme buffer of choice
1-5 units restriction enzyme
up to 20µl with dH_2O. Mix was incubated at 37^0C for at least 1 hour. The products were then separated by agarose gel electrophoesis (0.8-3% agarose gel depending on the size of the product expected).

2.2.11 DNA clean up from agarose gel by QIAEX II system

DNA bands were separated on an agarose gel. The band of interest was excised using a clean, sharp scalpel. The gel slice containing the DNA of interest was weighed and three volumes of solubilization buffer added, along with 15µl QIAEX II matrix solution. The tube was placed in a heat block at 50^0C for 10 minutes or until all of the agarose was solubilized. The solution was then centrifuged at 14000rpm for 1 minute. Supernatant was discarded, and pellet resuspended in 500µl of solubilization buffer. The tube was centrifuged as before, and pellet resuspended in 750µl of buffer PE containing ethanol. Following centrifugation, the supernatant was decanted and the pellet left to air dry for 15 minutes. DNA was eluted of by resuspending the pellet in 25µl of TE buffer and incubating this mixture for 5 minutes at room temperature. The

tube was centrifuged for 1 minute and separated DNA containing solution transferred into a clean tube. Typical recovery of DNA using this method was 75-90%.

2.2.12 Cell quantitation

Cell concentrations were determined using a Coulter Industrial D Cell counter (Coulter Electronics, Bedfordshire) as per manufacturers instructions.

2.2.13 Transfection

Transfections were performed using an electroporation method. For electroporation method, the following protocol was followed:

5 x 10^6 cells were washed twice in sterile PBS and resuspended in the final volume of 0.7ml. 10-50µg DNA was placed in the electroporation cuvette, cell suspension added and cuvette chilled on ice for 10 minutes. Electroporation was carried out with a single pulse at 0.25kV and 960µF, with some small modifications for various cell lines. Following the electroporation, cuvette was chilled on ice for further 10 minutes, followed by 10 minutes at room temperature. After that time, for transient transfections, 10% RPMI was added, and cells plated out. Cells were left for 48-72 hours, and surface expression checked using FITC-labelled antibodies on FACS.

For stable transfections, 10%RPMI/ DMEM media was added to the cell mixture to make up 20mls total, which was plated out onto a 96 well plate at 200µl/well. Media was changed after 24 hours and neomycin added at a final concentration of 1mg/ml. Selected stable colonies would appear at around three weeks post transfection. Each of the colonies was cloned out further by serial dilution. Surface expression was checked for by FACS on the selected clones. Positive clones were utilised for further studies.

2.2.14 Determination of the required amount of selection antibiotic for stable transfections

The antibiotics used for selection of stably integrated constructs were neomycin and puromycin. The appropriate amount of selection agent for a particular cell type was determined by setting up a double dilution killing curve. Starting concentration of 50mg/ml for neomycin and 50μg/ml for puromycin was halved sequentially by carrying out eight doubling dilutions bringing the concentration of the antibiotic down to 0.195mg/ml and 0.195μg/ml respectively. The cells were plated into the media containing the selection agent, and left to grow for 3-4 days. After that time, the concentration of antibiotic needed for selection was determined visually by light microscopy, and also by the colour change of the media the cells were grown in. A20 cells required 1mg/ml neomycin and 1μg/ml puromycin in order to select for stable transfectants.

2.2.15 Fluorescein isothyocyanate conjugation of antibodies

Antibody was fluorescein isothyocyanate (FITC) labelled by the method of Holborrow and Johnson (1967). 4.5 ml of normal saline (0.15M NaCl, pH7.4) was added to 50mg of Ab in the presence of 1mg of the FITC isomer I dissolved in 0.5M carbonate buffer pH9.5 and incubated for 45 minutes at 25°C. Unconjugated FITC was then removed by passage of the reaction mixture through a G-25 Fine column, equilibrated with 17.5mM phosphate buffer, pH6.3, followed by a passage through a DEAE-cellulose column. The majority of FITC-conjugated Ab was eluted with 0.5M NaCl in 17.5mM phosphate as determined by measuring conjugate:protein ratios during elution (calculated as absorbance at 495 and 278 nm respectively). Material with a ratio of 0.4-1.0 was pooled for use with final Ab concentration calculated as :

Antibody concentration (mg/ml) = Abs (278 nm) – (0.26 x Abs (495 nm)) / 1.35

2.2.16 Measurement of surface antigens by immunofluorescence

50μl of cells at $1x10^6$/ml concentration were incubated with 15μl of 100μg/ml FITC conjugated Ab of choice at 4°C for 30 minutes. Cells were then washed once in PBS-

BSA-azide and resuspended at approximately 5 x 10^5/ml. Analysis was performed on FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Routinely 10,000 events were collected per sample analysis. Cell debris was excluded by adjustment of the forward scatter threshold parameter. FITC was excited at 488 nm with emission intensity being recorded in the 475-600 nm wavelength region. Samples were analysed using CellQuest software (Becton Dickinson, Mountain View, CA, USA). Based on the forward and side scatter parameter, the population of living cells of interest was gated out. Fluorescence intensities were assessed in comparison to negative control samples and expressed as histograms of fluorescence intensity on a logarithmic scale.

2.2.17 RT-PCR cloning of human CD40

Human CD40 was generated by a PCR method described in 2.2.6. The sequence of human CD40 was obtained from the National Institutes of Health data base, accession number X60592. Based on this sequence, primers for PCR extension were designed using the Oligo primer design program. All of the primers used in the work to be described are contained in the table 2.4.

Human CD40 cDNA was amplified by PCR performed upon cDNA isolated from Daudi cells. Daudi is a human B cell lymphoma cell line, which expresses CD40. mRNA was extracted from 10^7 Daudi cells, following the protocol described in 2.2.1, followed by cDNA synthesis, as outlined in 2.2.2. PCR was performed following the basic protocol , using primers H40*Hind*IIIF (sense) and H40*Xba*R (antisense) at 55^0C for 30 cycles, and Daudi cDNA as a template. The PCR product was ligated into 100ng of T vector overnight as per 2.2.6, followed by a transformation into JM109 competent cells, as per 2.2.7. Six single white colonies were picked and grown in LB/amp overnight for a small scale plasmid preparation. Presence of the PCR product in the vector was established by restriction enzyme digest of the DNA using *Hind*III/*Xba* I enzymes. Three clones found to contain the insert were sequenced using T7 and T3 primers. A clone with the correct sequence was digested with *Hind*III/*Xba*I, and subjected to electrophoresis on 1% agarose gel. The hCD40 insert was excised, cleaned and ligated into pcDNA3 expression vector digested with *Hind*III/*Xba*I restriction enzymes. The ligation mix was transformed into JM109 and grown on a LB

Name of the hCD40 mutant or fusion protein	Primers used	Primer sequence	Annealing temperature	Size of PCR product obtained in the first step reaction	Second step PCR annealing temperature	Size of the final PCR product
hCD40KKV	H40HindIIIF	5' cca agc ttc acc tcg cca tgg ttc gtc tgc 3'	60^0C	678bp	48^0C	862bp
	H40KKVR	5' att ggt tgc ggc cgc tca cac ctt ttt gat aaa 3'				
	H40KKVF	5' aag gtg tga gcg gcc gca acc aat aag gcc ccc 3'	58^0C	197bp		
	H40XbaIR	5' gtg ggt cta gac tca ctg tct ctc ctg cac 3'				
hCD40T234A	H40HindIIIF	5' cca agc ttc acc tcg cca tgg ttc gtc tgc 3'	61^0C	797bp	52^0C	862bp
	H40T234AR	5' atg taa agc ctc ctg cac tgg agc agc 3'				
	H40T234AF	5' cag gag gct tta cat gga tgc caa ccg 3'	57^0C	105bp		
	H40XbaIR	5' gtg ggt cta gac tca ctg tct ctc ctg cac 3'				
hCD40T234S	H40HindIIIF	5' cca agc ttc acc tcg cca tgg ttc gtc tgc 3'	61^0C	797bp	52^0C	862bp
	H40T234SR	5' atg taa gga ctc ctg cac tgg agc agc 3'				
	H40T234SF	5' cag gag tcc tta cat gga tgc caa ccg 3'	57^0C	105bp		
	H40XbaIR	5' gtg ggt cta gac tca ctg tct ctc ctg cac 3'				
hCD40/22	H40HindIIIF	5' cca agc ttc acc tcg cca tgg ttc gtc tgc 3'	63^0C	595bp	63^0C	1029bp
	H40/22R	5' gag tcc cac agc cac tct cag ccg atc ctg 3'				
	H40/22F	5' cag gat cgg ctg aga gtg gct gtg gga ctc 3'	63^0C	434bp		
	H22NotIR	5' tcg cgg ccg cca aac tgg atc agc tct gag taa 3'				

Table 2.4- PCR primers and conditions used in generation of human CD40 mutants

plate containing ampicillin. Colonies were picked for a mini-prep. A clone found to contain the insert was grown in 100 mls LB/amp media for a maxi-prep.

2.2.18 Generation of human CD40 deletion and point mutants

The technique called two step recombinant PCR was utilised to produce mutants and deletion mutants of the human CD40. It was performed by the use of two sets of primers in two separate PCR reactions using human CD40/pcDNA3 as a template. Primers were designed in the following manner:

Primer 2 binds across the area which is to be mutated and it carries the mutated sequence. This primer reads in the 3' to 5' direction (antisense). Primer 3 also binds to the region to be mutated, but it reads 5' to 3' (sense) and also carries the mutation sequence. Primers 2 and 3 are in part complementary to each other, normally within a region no less than 18 base pairs in length. h40*Hind*IIIF and h40*Xba*IR were conveniently used as the end primers. The scheme is represented in figure 2.1.

PCR reactions were set as follows:

Step 1, reaction 1.

1µl DNA template
1µl h40*Hind*IIIF primer
1µl primer 2
0.5µl dNTPs
2.5µl Pfu buffer
0.5µl Pfu polymerase
dH_2O up to 25µl, overlay the reaction with 30µl mineral oil.

Step 1, reaction 2.

1µl DNA template
1µl h4o*Xba*IR primer
1µl primer 3
0.5µl dNTPs
2.5µl Pfu buffer
0.5µl Pfu polymerase
dH_2O up to 25µl, overlay the reaction with 30µl mineral oil.

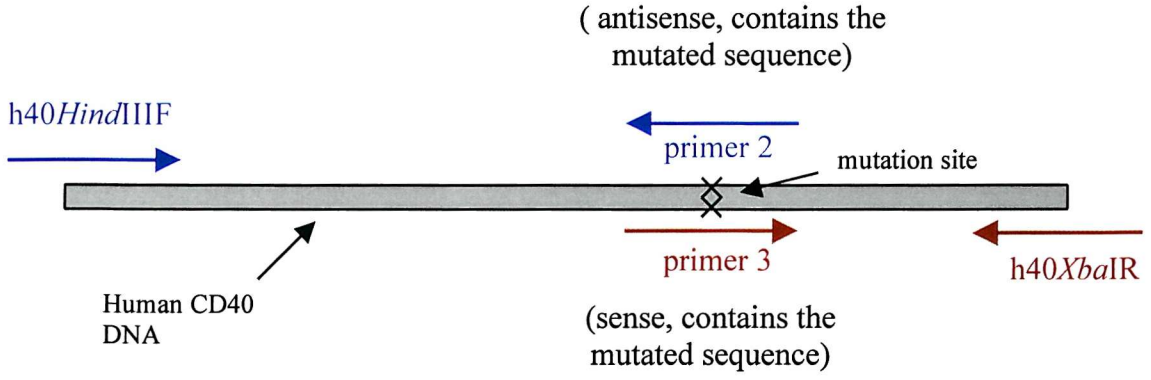


Figure 2.1- Primer design scheme for human CD40 wt and mutants. Primers highlighted in blue or dark red form a pair in the first step of the recombinant PCR. Primers 2 and 3 do overlap, but usually not throughout the full length of the primer.

Primer name	Sequence	Binding site on the hCD40 or hCD22 template
h40*Hind*IIIF sense	5' cca agc ttc acc tcg cca tgg ttc gtc tgc 3'	31-60 bp
h40*Xba*IR antisense	5' gtg ggt cta gac tca ctg tct ctc ctg cac 3'	893-864 bp
h22*Not*IR antisense	5' tcg cgg ccg cca aac tgg atc agc tct gag taa 3'	1999-1965 bp
h40T234AF sense	5' cag gag gct tta cat gga tgc caa ccg 3'	801-828 bp
h40T234AR antisense	5' atg taa agc ctc ctg cac tgg agc agc 3'	815-788 bp
h40T234SF sense	5' cag gag tcc tta cat gga tgc caa ccg 3'	801-828 bp
h40T234SR antisense	5' atg taa gga ctc ctg cac tgg agc agc 3'	815-788 bp
h40KKVF sense	5' aag gtg tga gcg gcc gca acc aat aag gcc ccc 3'	696-728 bp
h40KKVR antisense	5' att ggt tgc ggc cgc tca cac ctt ttt gat aaa 3'	719-687 bp
h40/22F sense	5' cag gat cgg ctg aga gtg gct gtg gga ctc 3'	1565-1579(hCD22) +612-626(hCD40)
h40/22R antisense	5' gag tcc cac agc cac tct cag ccg atc ctg 3'	1579-1565(hCD22) +626-612(hCD40)

Table 2.5-Primers used in cloning of human CD40 and construction of hCD40 mutants

The PCR program was set as described in 2.2.4. Annealing temperature for each set of primers is outlines in the table 2.5. All of the PCR products were separated by electrophoresis on 1% agarose gel, bands were excised, and purified using "QIAEX II Agarose Gel Extraction kit", according to manufacturers instructions. Briefly, the DNA band was excised from the agarose gel with a clean, sharp scalpel. The gel slice was weighed in a microfuge tube, and 3 volumes of Buffer QX1 were added to one volume of gel, ie. 300µl buffer to 100mg gel slice. The mixture was heated in a heating block for 10 minutes at 50^0C, with occasional mixing. The mixture was transferred to a column, and left to incubate for 1 minute. The column was spun for 30 seconds at 13000rpm in a microfuge, followed by a 500µl wash with Buffer QX1 and 500µl wash with Buffer PE which contains ethanol. The column was spun at 13000rpm for 30 seconds after each wash, with an additional spin after the final wash to ensure all traces

of ethanol were removed. DNA was eluted of the column by addition of 20µl Tris-EDTA buffer and a spin at 13000rpm for 30 seconds. The PCR products obtained are used in the second step of the recombinant PCR, where they act as primers and templates for one another. Performing the mutagenesis using this method abolishes the possibility of wild type DNA contamination in the end product. Step 2 PCR was performed using 5-10µl of DNA obtained from each of the reactions 1 and 2 from the step1. The reaction was arranged as follows: 5-10µl reaction 1 PCR product, 5-10µl reaction 2 PCR product, 0.5µl Pfu polymerase, 2.5µl Pfu buffer, 1µl dNTP mix and up to 25µl with dH_2O. Step 2 reaction was amplified over 10 cycles, after which 1µl of h40*Hind*IIIF and h40*Xba*IR were added to the reaction. PCR product obtained from the second step PCR was separated on 1% agarose gel by electrophoresis, excised out, purified using "QIAEXII agarose gel purification kit" and ligated into a T vector overnight. All constructs generated this way were sequenced and clones bearing the correct sequence were subsequently subcloned into pcDNA3 expression vector.

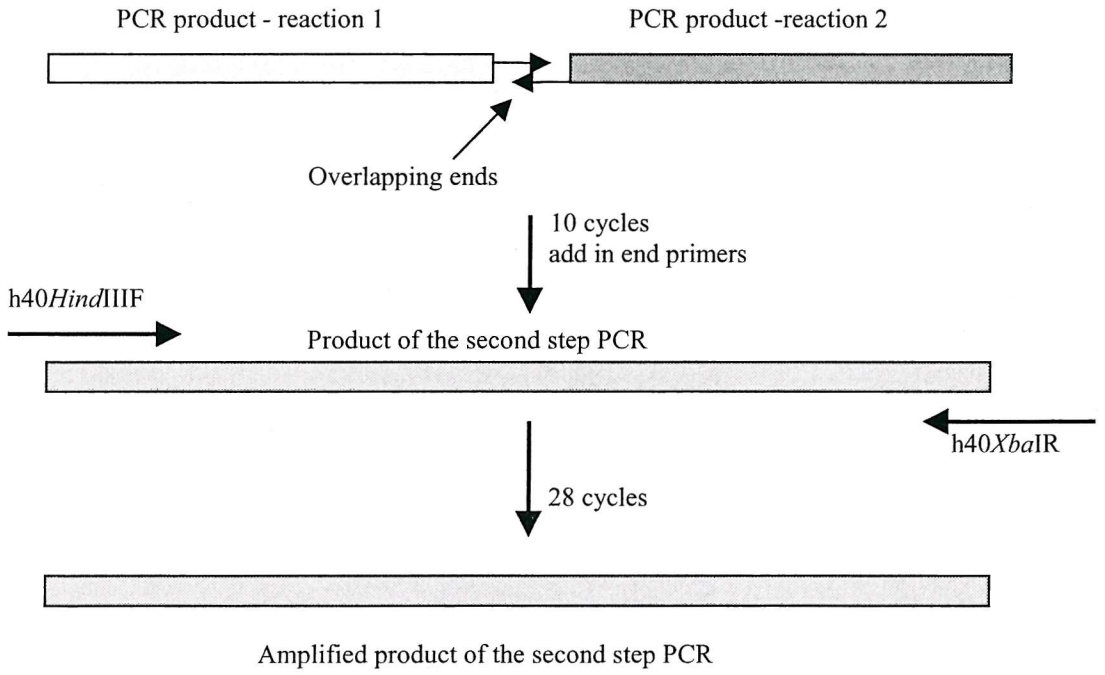


Figure 2.2-Second step of the two step recombinant PCR method.

2.2.19 Generation of hCD40/hCD22 fusion protein

H40/22 fusion protein was produced using already described two step recombinant PCR. Primers H40/22F and H40/22R were designed so to contain 15 bp of CD40 extracellular domain, and 15 bp of CD22 transmembrane domain, as outlined in figure 2.3. PCR was set up as described in 2.2.18.

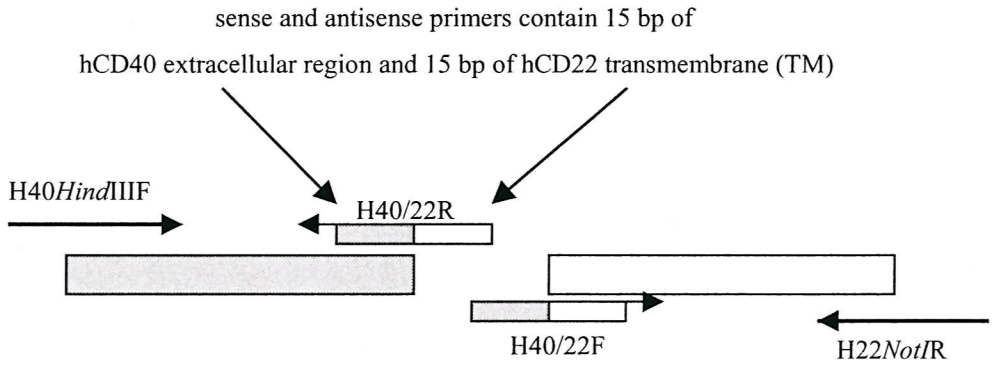


Figure 2.3- Design of sense and antisense primers for h40/22 fusion protein.

Step 1, reaction 1.

1µl hCD40 template
1µl h40*Hind*IIIF primer
1µl H40/22R primer
0.5µl dNTPs
2.5µl Pfu buffer
0.5µl Pfu polymerase
dH_2O up to 25µl, overlay the reaction with 30µl mineral oil.

Step 1, reaction 2.

1µl hCD22 template
1µl h22*Not*IR primer
1µl h40/22F primer
0.5µl dNTPs
2.5µl Pfu buffer
0.5µl Pfu polymerase
dH_2O up to 25µl, overlay the reaction with 30ul mineral oil.

Second step PCR was performed as already described in 2.18.

2.2.20 Modulation of hCD40 and hCD22 from the surface of human B cell lymphoma lines and A20 stably transfected with hCD40 constructs

$1x10^7$ Daudi, Ramos, Raji or A20/hCD40 cells were incubated with 30µg/ml LOB7.1 in a six well plate. At time 0, 0.5, 1.5, 2.5, 4.5, 5.5, 16, 18, 21, 24 and 48 hours a 100µl sample was taken and incubated with 15µl of 100µg/ml FITC labeled LOB7.6 for 25 minutes at 4^0C. The sample was washed after 25 minutes in 3mls of PBS/BSA/azide, and pelleted in a centrifuge at 1500rpm for 5 minutes. The supernatant was poured away, and the cell pellet resuspended in the remaining ~100µl PBS/BSA/azide left in the tube. All of the samples collected on the same day were run together on the FACS using identical instrument settings. Mean fluorescence intensity was determined for each sample. Fluorescence intensity at time 0 was taken as 100% of hCD40 receptors on the surface, and subsequent readings were compared as the percent of receptors remaining on the surface. The data obtained was plotted on a graph and expressed as percent of remaining receptors on the surface of a given cell line against time in hours. The same protocol was followed for determination of hCD22 modulation, except that the cells were initially incubated with HD6 mAb, and the amount of surface expression established by labelling with FITC labelled 4KB128 mAb.

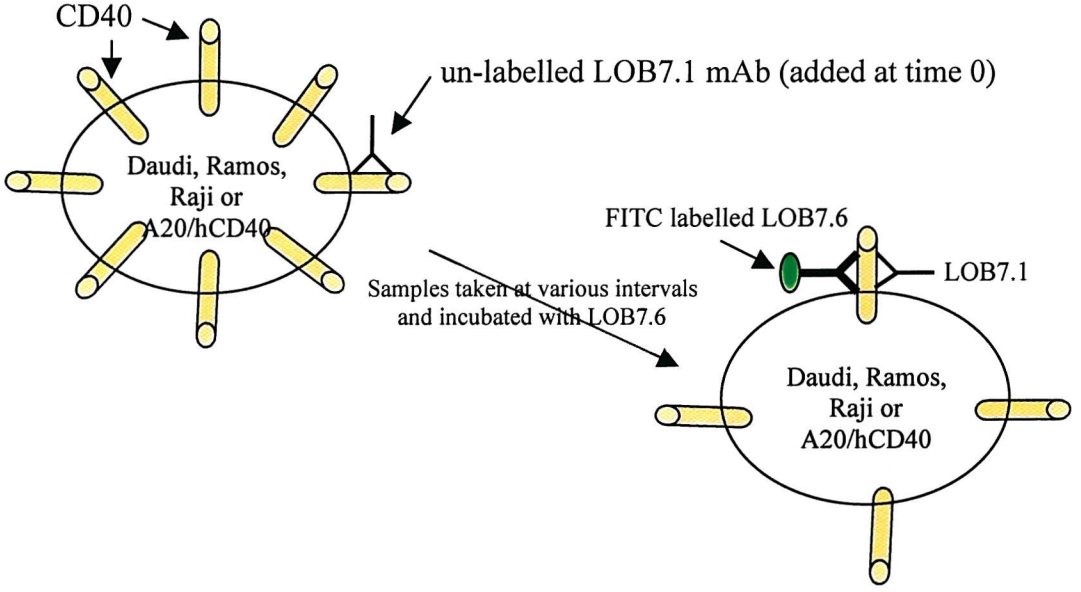


Figure 2.4- Amount of hCD40 internalisation as caused by LOB7.1 and measured by FITC LOB7.6 labelling. LOB7.1 and LOB7.6 mAbs have different binding epitopes on hCD40, therefore the binding of LOB7.1 does not prevent coupling of FITC-labeled LOB7.6 to the same CD40 receptor.

2.2.21 Generation of bone marrow derived dendritic cells (bmdc)

Bone marrow was extracted by syringing PBS through the femur of a Balb/c mouse. The cells obtained in this way were washed twice in sterile PBS and plated out onto tissue culture dishes. Cells were grown in 10% RPMI media supplemented with 5ng/ml GM-CSF (Thomson *et al*1995). Media was changed on alternating days for 10 days. Cells were ready to use on day 10.

2.2.22 PCR analysis of the cytokine levels in bmdc and FSDC cells

Bmdc and FSDC cells were treated with 30μg/ml 3/23 for 24 hours. After this period, mRNA was extracted from cells (as per 2.2.1) and first strand cDNA synthesis accomplished (as in 2.2.2). The concentration of cDNA in all samples was equalised by means of optical density determination at 260nm wavelength, analysis of the samples on a 1% agarose gel and PCR control by amplifying β-actin message in the samples. The levels of IL4, IL6, IL10, IFNγ and IL12 in treated and untreated cells were determined by PCR using primers outlined in the table 2.5. PCR program was as per 2.2.8. 1μl cDNA template was used in each reaction. The amplification was carried out over 30 cycles, except for IL4 and IFNγ where 40 cycles were used. PCR products were separated by electrophoresis on a 1% agarose gel at 80V for 1 hour.

Cytokine	Primer orientation	Primer sequence	Annealing temperature	Expected PCR product size	Accesion number of the sequence
β actin	sense	5' tgg aat cct gtg gca tcc at 3'	55°C	500 bp	M12481
	antisense	5' taa aac gca gct cag taa ca 3'			
IL 4	sense	5' aca gag cta ttg atg ggt ctc aac c 3'	56°C	400 bp	NM021283
	antisense	5' ttt cca gga agt ctt tca gtg atg t 3'			
IL 6	sense	5' act gat gct ggt gac aac 3'	50°C	620 bp	X54542
	antisense	5' tcc aca aac tga tat gct 3'			
IL 10	sense	5' gac cag ctg gac aac ata ctg 3'	54°C	402 bp	NM010548
	antisense	5' tcc tgg agt cca gca gac tca 3'			
IL12 p35	sense	5' ata acc atg ggt ctc cca agg tca 3'	59°C	739 bp	M86672
	antisense	5' gcc tta cta gtt cag gcg gag ctc 3'			
IL12 p40	sense	5' agc acc atg ggt cct cag aag cta 3'	57.5°C	985 bp	M86671
	antisense	5' cgt act agt cta gga tcg gac cct gca 3'			
IFN γ	sense	5' ggc cat ggt ctg aga caa tga acg 3'	56°C	488 bp	K00083
	antisense	5' cct cta gag aat cag cag cga ctc 3'			
IL6 receptor	sense	5' gcc ccg gga agg aag cag 3'	54°C	230bp	X53802
	antisense	5' tcg gac gcc act cac aga 3'			

Table 2.6- Primers used in the RT-PCR determination of cytokine mRNA levels in FSDC cDNA samples

2.2.23 PCR amplification of viral transcripts in the A20 and FSDC cells

Primers specific for the envelope protein (gp70) of an endogenous ecotropic murine leukaemia provirus and primers for murine mammary leukaemia virus were designed. The gp70 primer sequences were as published in Huang *et al.* PCR was carried out as outlined in 2.2.6 using 40 cycles of amplification.

Primer name	Primer sequence	Annealing temperature	Virus
gp70F	5' acc ttg tcc gaa gtg acc g 3'	62^0C	Murine leukemia virus, env gene, ref Huang *et al*
gp70 R	5' gta cca atc ctg tgt ggt cg 3'		
MMLV F	5' gcc gcc gcg aga tga ga 3'	57^0C	Murine mammary leukemia virus, env gene, accesssion number KO1788
MMLV R	5' ggc ccc tga gtt ccc caa agt at 3'		

Table 2.7- Primers used in determination of the presence of viral protein message in A20 and FSDC cells

2.2.24 Transmission electron microscopy (TEM) on A20 and FSDC cells

Cultured A20 or FSDC cells were scraped and transferred into a universal. The cells were pelleted by centrifugation at 1500rpm for 5 minutes in a tabletop centrifuge. The supernatant was discarded and cells resuspended in 3mls of fixative (3% glutaraldehyde, 4% formaldehyde in 0.1M PIPES buffer at pH7.2) where they were left at room temperature for 10 minutes, then transferred into the fridge for 50 minutes. The cells were pelleted after one hour of fixation by centrifugation at 1500rpm for 5 minutes, supernatant discarded and any remaining fixative blotted onto a tissue paper. Two drops of 5% sodium alginate were added onto the cell pellet and gently mixed with a tip of a micropipette. 10µl of the mixture were taken up into a pipette tip, and injected into a glass jar containing a fixation buffer mixed with an equal volume of 0.05 M calcium chloride and left for 15 minutes to set. The buffer was made up by mixing 2mls of 0.2M PIPES buffer, 1ml of 0.2M $CaCl_2$ and 1ml of H_2O making the final concentrations at 0.1M PIPES and 0.05M $CaCl_2$. After 15 minutes the alginate/buffer mixture was discarded and 1 ml of 1% osmium tetraoxide in 0.1M PIPES buffer added

to the cells for a further period of 1 hour. The blocks of cells were then washed twice for ten minutes in 0.1M PIPES, which was followed by washed in increasing concentrations of ethanol. Firstly the blocks of cells were placed in 30%EtOH for ten minutes, followed by the same wash in 50% EtOH, 70% and finally 95% EtOH. After the last wash, the blocks were placed into absolute alcohol for 30 minutes. The alcohol was replaced with more fresh absolute alcohol and blocks left in it for a further 30 minutes. The alcohol was discarded and replaced with 1ml acetonitrile for 10 minutes. After 10 minutes, the acetonitrile was discarded and replaced with a mixture of acetonitrile/TAAB resin at a ratio of 1:1. The cell blocks were left in this mixture overnight. The mixture was discarded the following day, and replaced with neat TAAB resin, which was allowed to infiltrate cell blocks for 6 hours. Following this period, the cell blocks were embedded in fresh resin and polymerised at 60^0C for a further 24 hours.

The blocks of polymerised resin were initially trimmed manually to obtain a flat and square surface which was then cut by ultramicrotome (Leica, Germany). The sections cut in this way were on average 70nm thick. The sections were mounted onto a 200μm copper grid and stained. Firstly the grids were stained for 15 minutes in uranyl acetate in the dark, followed by a wash with double distilled water. The grids were then stained in Reynolds lead stain for exactly five minutes, and washed in water once again. Grids prepared in this way were viewed under TEM under various magnifications and photographic record taken where appropriate (Reynolds 1963, Hayat 1986, Page *et al*1994). Technical help with this part of the project was provided by Dr Anton Page.

2.2.25 Transient transfection of IL6 promoter/luciferase reporter (IL6/pGL3 Enhancer), renilla luciferase (pRLTK), IκB transdominant negative mutant (IκBD/pcDNA3) and human CD40/pcDNA3 expression constructs into FSDC cell line

FSDC cells were collected by scraping into a Universal tube, and pelleted by centrifugation for 5 minutes at 1100rpm. The supernatant was discarded and cells washed in 5 mls of serum and antibiotic free RPMI media, followed by pelleting at 1100rpm for 5 minutes. The supernatant was discarded and cells resuspended at a

concentration of $1x10^7$/0.7ml. IL6 promoter/pGL3 Enhancer, renilla (pRLTK), IκBD/pcDNA3 (IκB transdominant negative mutant), and hCD40/pcDNA3 DNA were prepared by a maxi preparation, and concentrations adjusted to 1mg/ml.

For transfection of FSDC cells, 30μg of IL6 promoter/pGL3 Enhancer vector and 30ng of renilla luciferase expression vector were placed in a sterile 0.4 cm electroporation cuvette. 0.7mls of cell suspension containing $1x10^7$ cells were added to the DNA, and the mixture incubated on ice for 10 minutes. After this time, the cells were subjected to electroporation, using a single pulse at 240V and resistance set at 960μF (as per 2.2.13). The cells were placed on ice for further 10 minutes, followed by 10 minutes at room temperature, after which they were resuspended in 6 mls of 10% fully supplemented RPMI media, and each samples divided onto two wells on a six well plate (3 mls each). The cells were incubated overnight in 37^0C humidified incubator. 24 hours after the transfection, the medium was replenished, and appropriate mAb (LOB7.6 or 3/23) added to one of the two identical wells at a final concentration of 30μg/ml. After further 24 hours, the cells were harvested, lysed and luciferase assay performed as per 2.2.26. All samples were transfected in an identical way, except in the samples where three vectors were co-transfected, ie., IL6 promoter/pGL3 Enhancer renilla pRLTK and IκBD/pcDNA3, where the amount of DNA used per transfection was 30μg, 30ng and 50μg, respectively. The protocol outlined above was also used for transient transfections of FSDC cells with IL6/pGL3 Enhancer and hCD40 expression constructs. The amount of DNA used was 30μg, 30ng and 50μg for IL6/pGL3 Enhancer, renilla pRLTK and hCD40/pcDNA3, respectively. The remaining part of the protocol was the same.

2.2.26 Luciferase assay

FSDC cells were transfected with various constructs, as outlined in 2.2.25, and renilla luciferase, which serves as the internal transfection efficiency control. Cell samples were harvested by scraping into a Universal tube, pelleted for 3 minutes at 1000rpm in a centrifuge, and supernatant discarded. Each of the cell samples was then washed twice, first in 3mls, then in 0.5mls of PBS. All of the PBS was carefully removed and cell pellet resuspended in 50μl of 1x passive lysis buffer (as supplied in Dual luciferase

reporter kit, Promega). Samples were kept on ice and vortexed every two-three minutes for a total of 20 minutes. The luciferase assay was performed in the luminometer, as detailed in the instruction manual, assessing the firefly luciferase first, followed by renilla luciferase activity. Briefly, 100µl of luciferase assay reagent I was added to a luminometer tube, and placed into the luminometer. 20µl of the lysed cell sample was added, and the three readings taken over the next 40 seconds. The numbers obtained reflect the firefly luciferase activity in the sample. Following this, 100µl of Stop&Glo reagent (substrate for renilla luciferase) was added to the existing sample, and a further three readings taken, which reflect the activity of renilla luciferase in the sample. Firefly and renilla luciferases use different substrates and the addition of Stop&Glo substrate for renilla quenches the activity of firefly luciferase by 5000x, therefore both reactions can be carried out in the same tube. The sets of the three readings for each of the two luciferases were averaged, and the result for each sample presented as a ratio of firefly: renilla luciferase activity. The value obtained was expressed as a unit of normalised luciferase activity $x10^{-3}$.

2.2.27 Preparation of nuclear extracts

Nuclear extracts were prepared by the Dignam method (Dignam *et al*, 1983). Briefly, $5x10^6$ to $1x10^7$ cells were harvested by scraping from the plate into the media. The cells were pelleted at 1100rpm in a microfuge for 5 minutes and washed twice in PBS, transferred into a microfuge tube, and pelleted by pulsing for 10 seconds at 7.000rpm. The supernatant was discarded, and the cell pellet resuspended in 50-100µl Dignam A buffer containing a cocktail of protease and phosphatase inhibitors (NaF at 10mM, NaV at 1mM, AEBSF at 300nM, leupeptin at 250µg/ml, pepstatin A at 250µg/ml, aprotinin at 10mM). The mix was then spun at 13000 rpm for 10 sec, and the supernatant discarded. This procedure was repeated twice. Finally, the pellet was resuspended in 20µl Dignam C buffer and placed on ice for 10 minutes with vortexing every 2 minutes. After 10 minutes, the samples were spun for 1 minute at 13000 rpm. The supernatant was taken off and kept, as it contained the nuclear extract, and pellet discarded. The concentration of protein in the nuclear extract was determined by the Bradford protein assay.

2.2.28 γ-^{32}P labelling of the oligonucleotides

End labelling was carried out at 37^{0}C for 45 minutes using the following mixture of reagents:

2.5 pmols PAGE purified sense oligonucleotide

2µl 10x forward buffer

5µl [γ ^{32}P] labelled ATP (50µCi)

1µl T4 Polynucleotide kinase (10 units)

and sterile dH_2O to a total volume of 20µl.

After the 45 minutes, the reaction was stopped by adding 40µl TE buffer. 60µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the stopped reaction by mixing, followed by centrifugation in a microfuge for 5 minute at 13000 rpm. The aqueous top layer was transferred to a clean tube and a second extraction performed by addition and mixing of 60µl of chloroform:isoamyl alcohol (24:1), and 1 minute centrifugation at 13000rpm in a microfuge. The aqueous top layer was transferred onto a G-25 column. The labelled oligonucleotides were collected as the eluate from the column after a 1 minute spin at 4000rpm. The antisense oligonucleotide strand was added to the labelled sense strand oligonucleotide in ratio of 1.5:1 and the strands were annealed by heating the mixture at 90^{0}C for 5 minutes, followed by a slow cooling period of 4 hours in the heated block. The volume of labelled product was made up to 1ng/µl and stored at –20^{0}C until required. This was a stock probe, and was used in EMSAs following a 1/10 dilution.

2.2.29 Electrophoretic mobility shift assay (EMSA)

A probe was generated from a pair of complementary oligonucleotides containing a specific binding site for the transcription factor of interest. Binding of NFκB and AP1 proteins was assessed by the use of annealed, γP^{32} end labelled double stranded oligonucleotides contained in the table 2.6. Sense oligonucleotides were purified by PAGE, and used in γ-P^{32} labelling reaction described in 2.2.28. Nuclear extracts (2-10µg) were made up to 4µl with Dignam C buffer. 1µl polydIdC (1mg/ml) was added

to prevent non-specific protein:DNA binding to the labelled probe and the total volume made up to 20µl with dH_2O. The mixture was incubated on ice for 10 minutes, and then 0.2 to 0.4ng of radiolabeled probe (4µl of 1/10 stock probe dilution) was added to a final volume of 24µl. The mixture was incubated on ice for further 15-30 minutes to allow probe/protein binding to take place. Following the addition of 4µl of 6x loading buffer, the samples were loaded onto a 8% native polyacrylamide gel containing 0.25x TBE buffer. Electrophoresis was carried out at a constant current of 20mA for 2 hours in 0.5x TBE electrophoresis running buffer. The gel was dried on a 3mm Whatmann paper under vacuum for 1 hour at 80^0C. The [γ-^{32}P]ATP labelled DNA was visualised by autoradiography at -70^0C for varying periods of time depending on the activity of the probe.

2.2.30 Competition and supershift EMSA

Competition EMSA was performed by incubating the nuclear extracts with excess concentrations of unlabelled double-stranded oligonucleotides, normally 50-100x, during the first incubation step prior to the addition of the labelled probe. Supershift assays were performed by adding 2µg of supershift antibody after incubation with the radiolabeled probe at 4^0C for 16 hours prior to electrophoresis.

Primer name and orientation	Primer sequence
NFκB sense*	5' caa atg tgg gat ttt ccc atg a 3'
NFκB antisense	5' tca tgg gaa aat ccc aca ttt g 3'
AP1 sense*	5' aaa gtg ctg agt cac taa taa 3'
AP1 antisense	5' tta tta gtg act cag cac ttt 3'
Δp50/p65 sense*	5' agt tga ggc gac ttt ccc agg c 3'
Δp50/p65 antisense	5' gcc tgg gaa agt cgc ctc aac t 3'
ΔCBF1 sense*	5' agt tga ggg gac ttc ccg agg c 3'
ΔCBF1 antisense	5' gcc tcg gga agt ccc ctc aac t 3'
SP1 sense*	5' att cga tcg ggg cgg ggc gag c 3'
SP1 antisense	5' gct cgc ccc gcc ccg atc gaa t 3'

Table 2.8- Oligonucleotides used in EMSA. Oligonucleotides marked with a star were PAGE purified and used for γP^{32} labeling

2.2.31 SDS- polyacrylamide gel electrophoresis (SDS-PAGE)

10-20μg of whole cell or nuclear extracts were made up to 10μl with 1xPBS. An equal volume of SDS-PAGE sample buffer was added, and samples boiled for 10 minutes to denature fully. Samples were loaded on a 9% gel with prestained protein markers alongside. The gels were run for 1 hour at constant voltage of 160V in 1xSDS electrode buffer. Following electrophoresis, the gels were immersed in blotting buffer, and blotted onto Hybond C nitrocellulose membranes for 1hour at 100V using a Transblot semidry protein apparatus. The blotted nitrocellulose was washed in PBS, and incubated in blocking solution made up of 5% Marvell in TBS over night to block non-specific protein binding. Primary Ab was applied at 1μg/ml final concentration in 1xTBS/0.075% Tween and membrane incubated for 1 hour with shaking. The membrane was then washed in three 20 minute washes with 1xTBS/0.075% Tween. Secondary Ab used was anti rabbit IgG-HRP conjugate, which was applied to the nitrocellulose at 1:2000 dilution, again in 1xTBS/0/075% Tween + 3% Marvell, for 1 hour. The membrane was washed three times in 1xTBS/0/075% Tween. The HRP conjugate was then detected by chemiluminescence reaction (ECL system) followed by exposure to a film.

2.2.32 Animal therapies

The animal therapies were constructed in the following manner:
-$1x10^7$ A20 cells, or A20 transfected with pcDNA3, pCIpuro, hCD40wt or hCD40T254S were injected intra-venously into groups of five age and sex matched Balb/c mice on day 0. The groups were then treated with anti mouse CD40 antibody (3/23), or anti human CD40 antibody (LOB7.6), or given PBS intra-venously on day 4 in a total volume of 200μl. Due to the difficulty associated with intravenous injection into a mouse tail vein, these procedures were carried out by experienced staff at the Tenovus animal house.

Transfected cell lines were maintained in neomycin selection media until 10 days before the therapies were set up in order to avoid artefacts arising from the presence of selection antibiotic in the animals. Results of the therapies were presented in a graph

form. Each therapy was planned and set up so to contain a control group of mice, eg A20 +/- the antibody treatment. Each group of five animals represented 100% of the animals on the graph; therefore each animal which succumbed to the tumor was recorded as 20% of the surviving animals. The animals were checked twice daily for signs of pain, distress or discomfort, as well as the development of tumour. The development of tumour in the spleen was monitored by a daily palpating of the abdomenal area. Once the animals have reached the stage where the tumour load was considered to cause them discomfort, they were culled by a schedule 1 method listed in the Home Office rules. Each day of the therapy was recorded as the number of animals left alive at 9am.

2.2.33 Isolation of tumour cells from the spleen

The spleen of a terminally ill tumour-bearing animal was dissected out. The organ was then mashed up through a 70μm diameter grid mesh (Falcon) into some media so to obtain a homogenous cell suspension. 20mls of Lymphoprep (Life Technologies) were placed into a universal tube and the cell suspension carefully overlaid on top. The tube was then centrifuged in a tabletop centrifuge for 15 minutes at 2500rpm. Following this, the tumour cells were found in a uniform layer on top of Lymphoprep solution. The tumour cells were isolated by sucking up into a sterile glass pipette. They were transferred into tissue culture flask supplemented with media, and grown in a humidified incubator at 37^0C.

CHAPTER 3

Production of expression vectors for wild type and mutant human CD40 proteins and selection of stable A20 murine B cell lymphoma lines expressing wild type and mutant human CD40 proteins.

3.1 INTRODUCTION

CD40 is a member of the TNFR superfamily expressed by a range of cells, including B cells, dendritic cells, monocytes, macrophages, epithelial and endothelial cells among others. Mice and humans deficient in CD40 or CD40L genes are immunocompromised in their antibody responses, Ig class switching, germinal center formation and are unable to mount an efficient immune response against certain infectious agents. CD40 is essential in both humoral and cellular arm of the immune response, and its pleiotropic effects are well documented. The importance of CD40 in the treatment of B cell lymphoma in mice has also been established. A20, Bcl_1 and A31 murine B cell lymphoma models described by French (French *et al* 1999) have all shown the paramount importance of CD40 signalling in the alerting and activation of the immune response against tumours which are otherwise immunologically silent.

The next logical step towards improving our understanding of how anti-CD40 therapy can result in the clearance of tumors and promote subsequent protection against further challenge with tumor cells is to investigate the intracellular signalling events that are activated upon ligation of CD40 receptor. One of the key hypothetical issues raised in this thesis is the potential of the B cell lymphoma to function as an APC and to efficiently present tumor-specific antigens to cytotoxic T lymphocytes. This hypothesis includes the idea that one way in which anti-CD40 mAb achieves therapy is to promote the antigen presenting properties of the tumour B cell by stimulating CD40 signal transduction events required for mediating APC function. It was therefore decided to study the consequences of perturbing CD40 signal transduction events in the B cell lymphoma on anti-CD40 therapy. It was also considered important to establish the ability of anti-human CD40: human CD40 (hCD40) mediated signaling events to promote therapy. Therefore the main aim of the first studies presented in this chapter was the production of a series of murine B cell lymphoma lines that constitutively express wild type hCD40 and mutated hCD40 proteins. The mutated hCD40 proteins were selected to carry amino acid deletions or substitutions in regions of the cytoplasmic domain that are required for activation of specific signal transduction events. Previous data by Baccam (Baccam *et al* 1999) and

Jalukar (Jalukar *et al* 2000, also Hsing *et al* 1999 and Hsing *et al* 1997) has shown that mouse and human CD40 signal equally in mouse cells, therefore such an approach would be valid as the route to dissecting the importance of particular domains and residues in the CD40 intracellular region *in vivo* (see figure 3.0)

The first step towards achieving the aim stated above was to generate mammalian expression vectors that provide constitutive and stable expression of hCD40 proteins in B cell lymphoma lines. Two parental B cell lymphoma lines (A20 and πBcl1) were selected for these studies based on their ability to propagate both *in vivo* and *in vitro* (Illidge *et al* 2000). The vector pcDNA3.1 (see section 2.17, page 69) was chosen since it provides high level expression of cloned cDNAs from an upstream CMV promoter/enhancer sequence. In addition, pcDNA3 carries a gene that provides resistance against the antibiotic neomycin enabling selection of stable transfected cell lines on the basis of their antibiotic resistance. Site directed mutagenesis was utilised in order to generate a panel of cDNAs encoding hCD40 mutant proteins and CD40/CD22 fusion proteins. A number of important amino acid residues within hCD40 are known to be required for interaction with downstream effector molecules such as TRAF2/3/5 and 6 as well as Jak3 (Hanissian *et al* 1997, Ni *et al* 2000, Revy *et al* 1999, Lee *et al* 1999, Cheng *et al* 1995, Darnay *et al* 1999, Hostager *et al* 1999, Leo *et al* 1998, Pullen *et al* 1999, Pullen *et al* 1999). TRAFs 2,3 and 5 bind the ^{250}PVQET^{254} motif located in the tail of CD40, while Jak3 binds in the cytoplasmic region termed box 1 (^{222}PTNKAPHPK^{230}) but also requires box 2 (^{260}PVTQEDGKESR^{270}) for its binding and signal transduction function (Jabara *et al* 1998, Hanissian *et al* 1997, Pullen *et al* 1999, Leo *et al* 1998, Cheng *et al* 1995, Hostager *et al* 1999, Pullen *et al* 1999). Overall, the most critical residue for activation of CD40 signaling is known to be Thr254. Based on these observations, it was decided to introduce mutations into the cytoplasmic domain of hCD40 that would disrupt its interaction with TRAFs or Jak3.

The results presented in this chapter describe the production of wild type and mutant hCD40 expression vectors and the subsequent generation of stable transfected cell lines from the parent murine B cell lymphoma line A20.

Human CD40 cytoplasmic domain

KKVAKKPTNKAPHPK**QEPQEINF**PDDLPGSNTAA**PVQET**LHGCQ**PVTQEDGKESR**ISVQER

Mouse CD40 cytoplasmic domain

KKVVKKPKDNEMLPPAARR*QDPQE*MEDYPGHNTAA**PVQET**LHGCQ**PVTQEDGKESR**ISVQERQ VTDSIALRPLV

Figure 3.0- Comparison of human and mouse CD40 amino acid sequence within cytoplasmic domains. Conserved residues are shown in red. Sequences important for binding of TRAFs or Jak3 are underlined. TRAF6 binding site is poorly conserved between the mouse and human CD40. TRAF6 site in human CD40 maps across QEPQEINF residues (underlined in black), whereas in the mouse CD40 that sequence is QDPQE (underlined in italics). Sites for TRAF 2, 3 and 5 binding are fully conserved between the two species.

3.2 RESULTS

3.2.1 Production of expression vectors for wild type and mutant hCD40.

The first requirement was to produce a hCD40 cDNA with which to subsequently generate wild type and mutant expression vectors. This was achieved by amplification of hCD40 cDNA by RT-PCR performed on polyA mRNA extracted from the human Daudi B cell line. Following the generation of first strand cDNA from RNA using a random hexamer primer pd(N)6, PCR was then performed for 30 cycles according to the conditions described in section 2.2.16. Oligonucleotide primers were selected to flank the 5' and 3' ends of hCD40 cDNA and were expected to produce a 862bp product. Following completion of the PCR reaction, the products were then resolved on a 1% agarose gel and were visualised by UV analysis following ethidium bromide staining. As shown in fig 3.1A a major DNA fragment with an approximate size of 862bp was produced. Since this size of DNA fragment was equivalent to the expected size of full length hCD40 cDNA it was excised from the gel, purified according to the protocol described in section 2.2.11 of the methods and was initially ligated into T vector for verification of sequence identity by DNA sequencing. Having verified that the cloned PCR product was full length hCD40 it was then excised from the T vector by restriction endonuclease digestion with *Hind*III and *Xba*I which cut at sequences immediately adjacent to the 5' and 3' ends of the cDNA. A 862bp insert was generated which was then purified by agarose gel electrophoresis (fig 3.1B) and subsequently sub-cloned into the *Hind*III and *Xba*I sites of pcDNA3 to generate hCD40wt/pcDNA3. Fig 3.1C shows a *Hind*III/*Xba*I diagnostic digestion of hCD40wt/pcDNA3 confirming the correct size of the hCD40 cDNA insert.

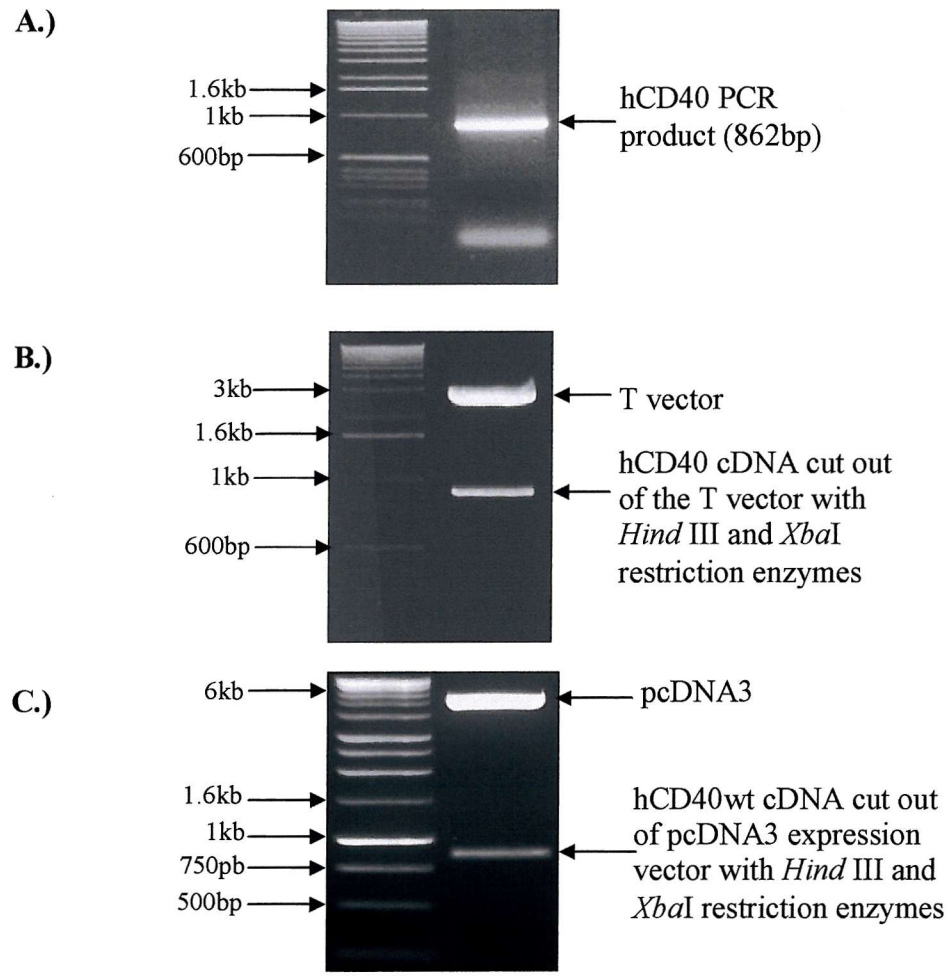


Figure 3.1 -Cloning of human CD40

A.) Human CD40 cDNA was amplified by RT-PCR performed upon cDNA isolated from Daudi cells. PCR was performed using H40*Hind*IIIF sense and H40R antisense primers at 55^0C for 30 cycles. Expected size of the DNA product was ~900bp. Figure 3.1A shows a photograph of the PCR product as separated by electrophoresis on a 1% agarose gel. The product is ~900bp in size, as expected.

B.) Restriction digest of T vector/hCD40wt mini-prep DNA using *Hind*III/*Xba*I restriction enzymes.

C.) Restriction digest of hCD40wt/pcDNA3 mini-prep DNA using *Hind*III/*Xba*I restriction enzymes.

Expression vectors for mutant hCD40 proteins (shown in fig 3.7) were generated by site directed mutagenesis using hCD40wt/pcDNA3 as a template for a two-step recombinant PCR as described in the methods (2.2.17 and 2.2.18). At the end of the two-step PCR the mutant hCD40 cDNAs were digested with *Hind*III and *Xba*I and ligated into pcDNA3. PCR products and final diagnostic *Hind*III / *Xba*I digests for the following mutant hCD40 constructs are shown in figs 3.2 to 3.5. Mutant construct hCD40KKV (fig 3.2) is a deletion mutant in which the final 59 amino acids (A_{219}-R_{276}) of the c-terminal domain of hCD40 have been deleted by insertion of a stop codon after the first 3 amino acids of the intracellular domain leaving amino acid V_{218} as the new terminal residue. Mutant construct hCD40T254A (fig 3.3) in which residue T_{254} has been replaced by an Alanine residue which is not capable of being phosphorylated. Mutant construct hCD40T254S (fig 3.4) carries a single amino acid substitution in which the critical T_{254} residue required for CD40 signaling has been replaced by a Serine residue which would still be expected to be phosphorylated in response to CD40 cross-linking. A further construct, hCD40/hCD22 (fig 3.5), that expresses a chimeric hCD40/hCD22 fusion protein in which the intracellular domain of hCD40 was replaced by the intracellular domain of hCD22 was also generated. Correct sequence identity of the mutated hCD40 cDNAs in these expression vectors was confirmed by DNA sequencing. In addition, three additional mutant hCD40 expression constructs in the pcDNA3 background were donated by Professor Young (Birmingham). These constructs were hCD40T254E in which residue T_{254} was replaced by a Glutamate residue which is unable to be phosphorylated; hCD40Δ262 in which the final 14 amino acids (Q_{263}-R_{276}) at the c-terminal end of hCD40 are deleted; and hCD40T254AΔ262 which also lacks the 14 c-terminal amino acids but in addition carries a threonine to alanine mutation at residue 254. Diagnostic digests for these constructs for these constructs are shown in fig 3.6.

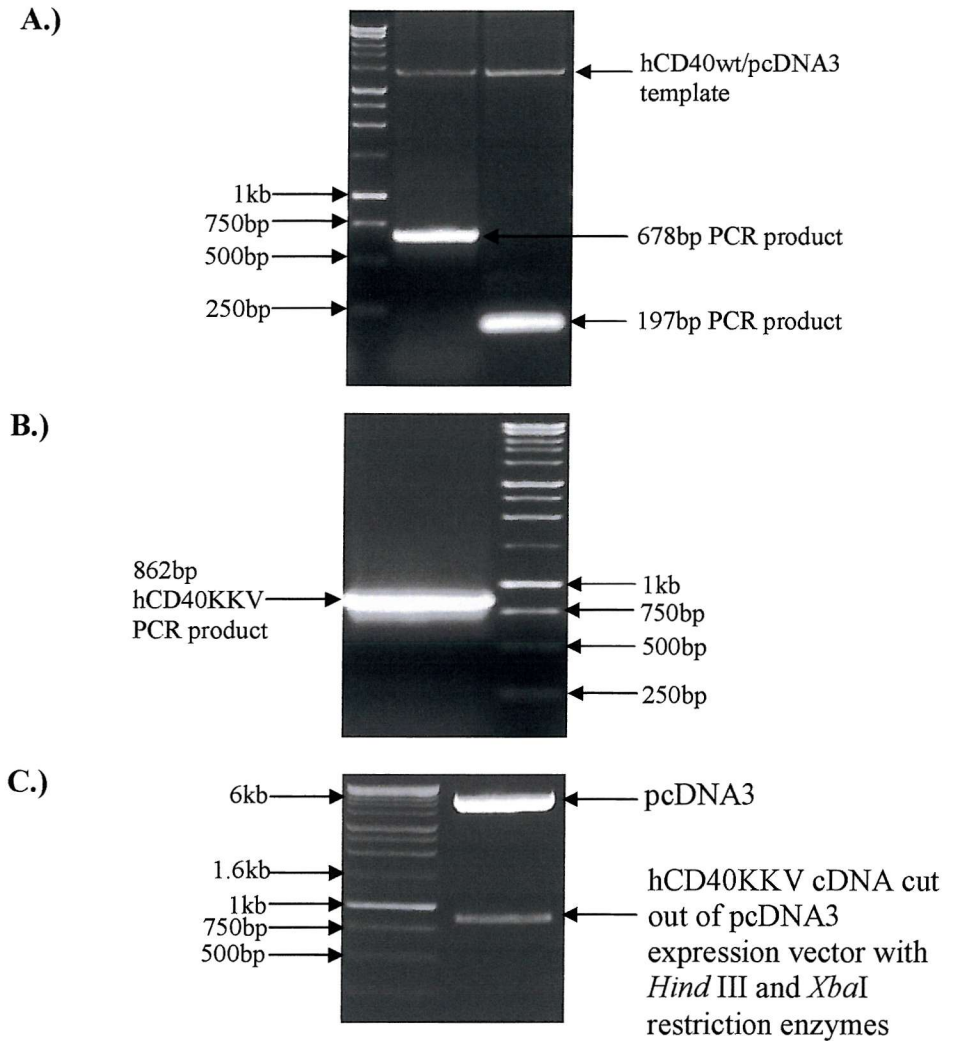


Figure 3.2-Generation of the truncated form of hCD40 containing KKV residues proximal to the transmembrane domain (hCD40KKV)

A.) PCR products obtained by 28 cycles of amplification using H40*Hind*IIIf/ H40KKVr (step 1, reaction 1, expected PCR product size is 678 bp) and H40KKVf/H40*Xba*r (step 1, reaction 2, expected PCR product size is 197 bp) primers, respectively. Expected size of the DNA product coincides with the size of the product obtained.

B.) PCR product resulting from the second step PCR performed at 48^0C for a total of 38 cycles utilizing PCR products obtained as shown in A.) as templates for each other.

C.) Restriction digest of hCD40KKV/pcDNA3 DNA with *Hind*III/*Xba*I restriction enzymes.

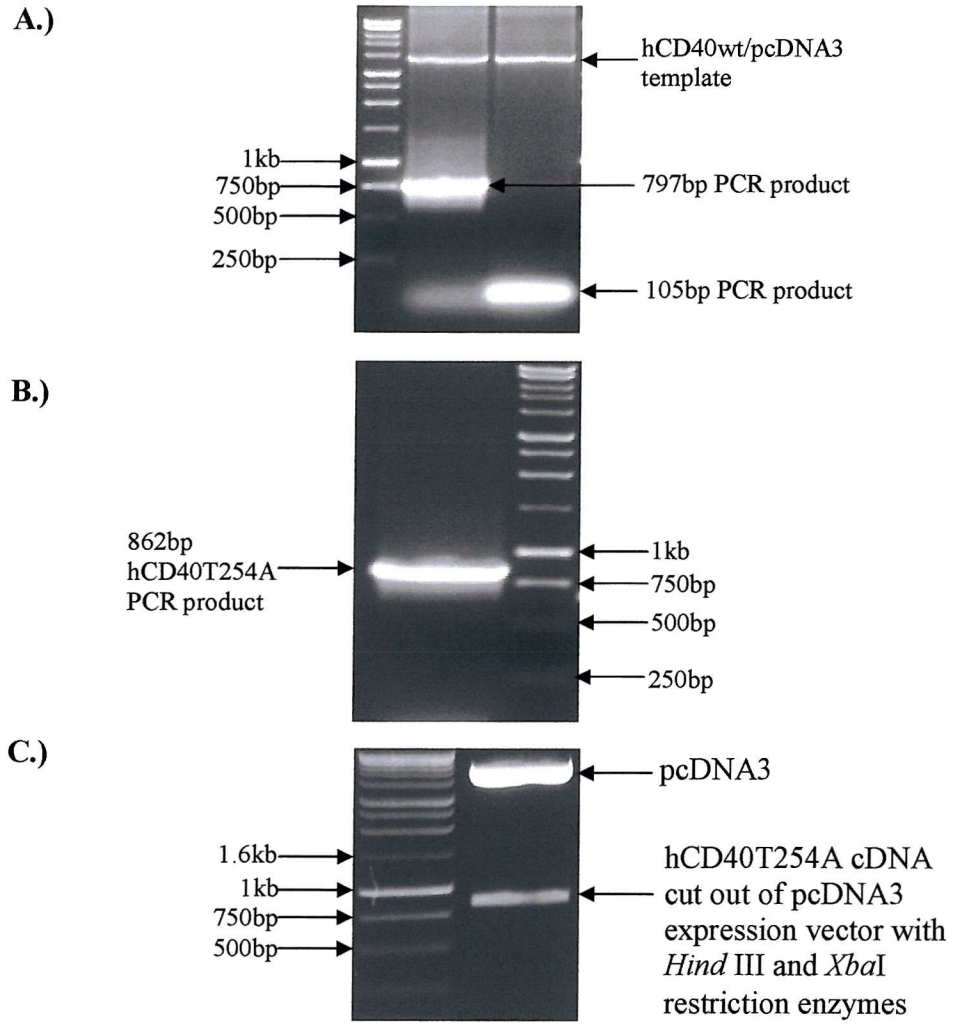


Figure 3.3- Generation of the hCD40T254A construct

A.) PCR products obtained by 28 cycles of amplification using H40*Hind*IIIf/ H40T254Ar (step 1, reaction 1, expected PCR product size 797 bp) and H40T254Af/H40*Xba*r (step 1, reaction 2, Expected PCR product size 105 bp) primers, respectively. Expected size of the DNA product coincides with the size of the product obtained.

B.) PCR product resulting from the second step PCR performed at 52^0C for a total of 38 cycles utilising PCR products obtained as shown in A.) as templates for each other.

C.) Restriction enzyme digest of hCD40T254A/pcDNA3 DNA with *Hind*III/*Xba*I restriction enzymes.

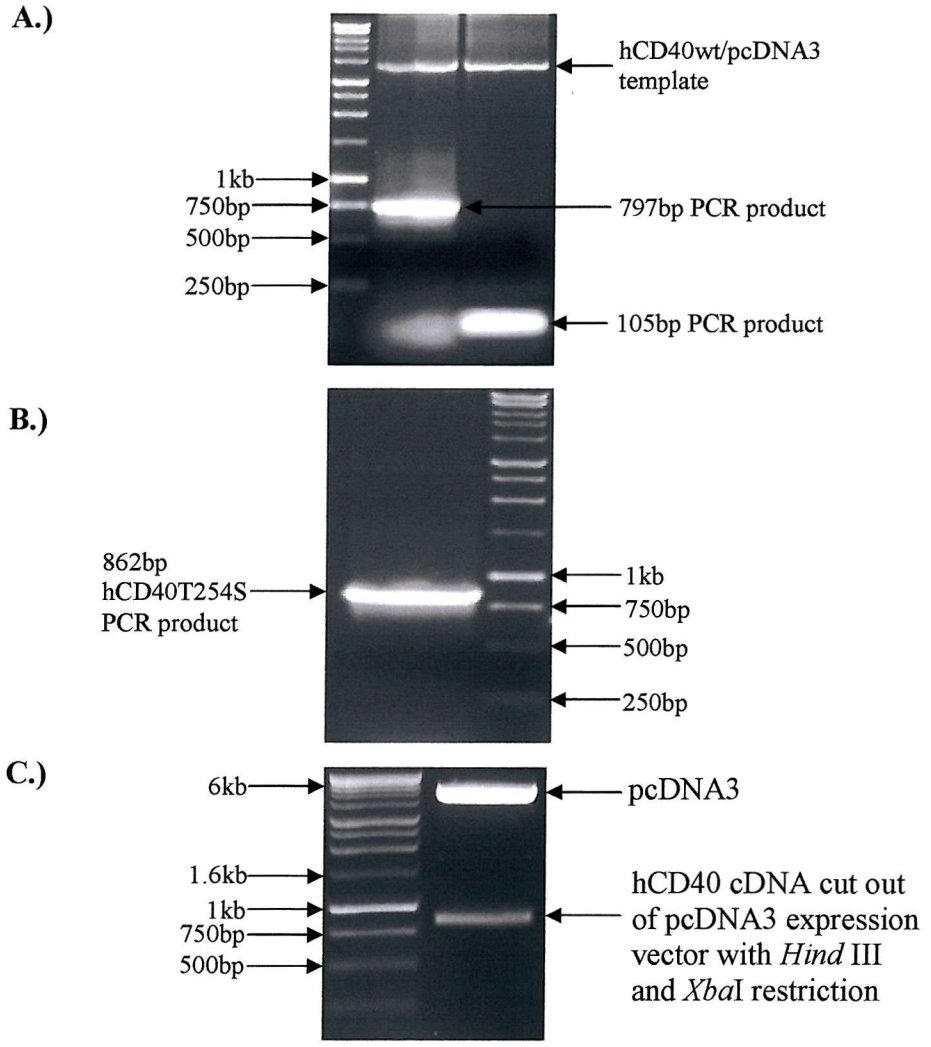


Figure 3.4- Generation of the hCD40T254S construct

A.) PCR products obtained by 28 cycles of amplification using H40*Hind*IIIf/ H40T254Sr (step 1, reaction 1, expected band size 797 bp) and H40T254Sf/H40*Xba*r (step 1, reaction 2, expected band size 105 bp) primers respectively. Expected size of the DNA product coincides with the size of the product obtained.

B.) PCR product resulting from the second step PCR performed at 52^0C for a total of 38 cycles utilising PCR products obtained as shown in A.) as templates for each other.

C.) Restriction enzyme digest of hCD40T254S/pcDNA3 DNA with *Hind*III/*Xba*I restriction enzymes.

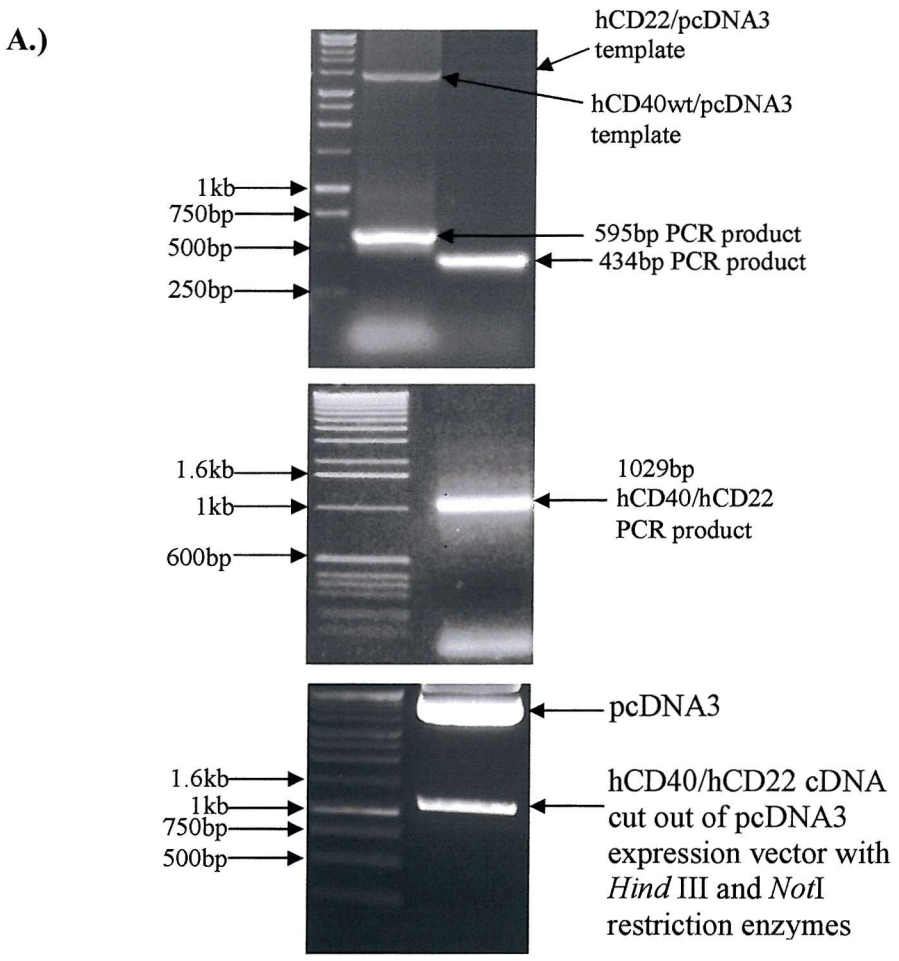


Figure 3.5- Generation of the hCD40/hCD22 fusion protein

A.) PCR products obtained by 28 cycles of amplification using H40*Hind*IIIf/ H40/22r (step 1, reaction 1, expected PCR product size 595 bp) and H40/22f/H22*Not*Ir (step 1, reaction 2, expected PCR product size 434 bp) primers, respectively. Expected size of the DNA product coincides with the size of the product obtained.

B.) PCR product resulting from the second step PCR performed at 63^0C for a total of 38 cycles utilising PCR products obtained as shown in A.) as templates for each other.

C.) Restriction enzyme digest of hCD40/hCD22/pcDNA3 DNA with *Hind*III/*Not*I restriction enzymes.

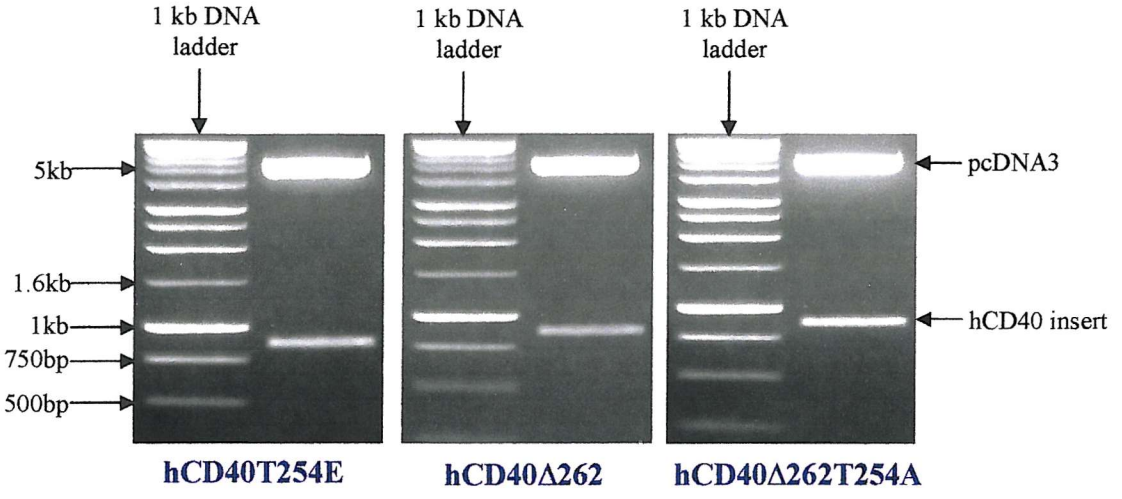


Figure 3.6- Diagnostic digests of hCD40T254E, hCD40Δ262 and hCD40Δ262T254A hCD40 constructs listed above have been kindly donated by Prof. L. Young (Birmingham). The gene constructs have been cloned into pcDNA3 via *Hindl*II and *Not*I restriction enzyme sites. In order to assess for the presence of hCD40 gene in the expression vectors, all of the constructs have been digested with *Hind*III/*Not*I, which releases hCD40 insert from pcDNA3, as shown above.

hCD40wt
KKVAKKPTNKAPHPKQEPQEINFPDDLPGSNTAA**PVQET**LHGCQ**PVTQEDGKESR**ISVQER

hCD40T254S
KKVAKKPTNKAPHPKQEPQEINFPDDLPGSNTAA**PVQES**LHGCQ**PVTQEDGKESR**ISVQER

hCD40T254A
KKVAKKPTNKAPHPKQEPQEINFPDDLPGSNTAA**PVQEA**LHGCQ**PVTQEDGKESR**ISVQER

hCD40T254E
KKVAKKPTNKAPHPKQEPQEINFPDDLPGSNTAA**PVQEE**LHGCQ**PVTQEDGKESR**ISVQER

hCD40Δ262
KKVAKKPTNKAPHPKQEPQEINFPDDLPGSNTAA**PVQET**LHGCQ**PVT**

hCD40T254AΔ262
KKVAKKPTNKAPHPKQEPQEINFPDDLPGSNTAA**PVQEA**LHGCQ**PVT**

hCD40KKV
KKV

hCD40/hCD22 fusion protein
Human CD40 extracellular domain/ hCD22 cytoplasmic domain

Figure 3.7- Protein sequences of cytoplasmic domain in hCD40 wild type and mutant constructs. Mutated residues are highlighted in red; binding site for TRAF 6 is underlined, and residues important for TRAF 2, 3, 5 and Jak3 binding are in bold and underlined. Names of construct kindly donated by Prof. Young are in blue.

3.2.2 Generation of stable transfected A20 cell lines expressing wild type and mutant hCD40 proteins.

Expression constructs hCD40wt/pcDNA3, hCD40KKV/pcDNA3, hCD40T254A/pcDNA3, hCD40/hCD22/pcDNA3 and hCD40T254S/pcDNA3 were transfected into the A20 B cell lymphoma line. The transfection protocol used to achieve stable transfection of this cell line is described in 2.2.13. A20 cells were scraped into media and pelleted by centrifugation in a bench top microfuge for 5 minutes at 1200rpm. The cells were then washed twice in 10 mls of RPMI media supplemented with 2mM L-glutamine, and resuspended so to obtain a density of $5x10^6$ cells/0.7mls of media. The cells were added to the electroporation cuvette containing 30μg DNA encoding hCD40 expression construct of interest, and mixture placed on ice for 10 minutes. The mixture was electroporated with a single pulse at 280V and 960μF. Following this procedure, the cuvette was placed back on ice for 10 minutes, and then placed at room temperature for further 10 minutes. The electroporated cells were transferred from the cuvette into a Petri dish containing 20 mls of fully supplemented media (see 2.1.8). The cell suspension was plated out onto a 96 well plate at 200μl/well. Stably transfected cells were selected by adding neomycin to the cells 24 hours after electroporation at 1mg/ml final concentration. This antibiotic is lethal for eukaryotic cells, but stable transfected cells can live in the medium bearing the antibiotic due to the presence of the neomycin resistance gene in the expression vector carrying the hCD40 construct. The amount of neomycin required for stable selection was established by a double dilution killing curve, as outlined in 2.2.14. Stable clones were obtained approximately 3 weeks after the addition of neomycin to the culture media. Several neomycin-resistant clones were obtained for each of the different hCD40 constructs. Fig 3.8 shows the precise number of clones obtained and the level of surface hCD40 expression detected on these clones by FACS analysis using a FITC labelled anti-hCD40 mAb (LOB7.6). It was now necessary to select representative clones for each construct that provide approximately equivalent levels of surface hCD40 expression so as to ensure that any differential responses observed in the experiments described in chapter 4 would be due to effects of mutagenesis and not differential protein expression. Clones hCD40wt.6, hCD40T254A.4, hCD40T254S.4, hCD40KKV.4 and hCD40/CD22.3 were therefore

selected and expanded on the basis of their similar levels of hCD40 expression (fig 3.8). Representative FACS profiles for each of these 5 clones are shown in figure 3.9. FACS analysis was also performed on the 5 clones and the original parental cell line using an anti-mCD40 mAb (3/23), which showed that the 5 selected clones are still representative of the original line.

The πBcl_1 cell line proved to be impossible to transfect with all available methods, including electroporation, use of lipid reagents for delivery of DNA, DEAE dextran method, calcium phosphate and glycerol shock. Using each of these protocols lack of transient hCD40 expression and inability to generate stable cell lines was observed.

hCD40 construct expressed in A20	Number of hCD40 positive clones obtained	Clone name	Level of hCD40 expression on the surface of A20 expressed as mean fluorescence intensity
hCD40wt	8	hCD40wt.1	9
		hCD40wt.2	30
		hCD40wt.3	40
		hCD40wt.4	50
		hCD40wt.5	55
		hCD40wt.6	60
		hCD40wt.7	70
		hCD40wt.8	140
hCD40T254S	7	hCD40T254S.1	10
		hCD40T254S.2	20
		hCD40T254S.3	50
		hCD40T254S.4	70
		hCD40T254S.5	80
		hCD40T254S.6	110
		hCD40T254S.7	130
hCD40T254A	6	hCD40T254A.1	24
		hCD40T254A.2	40
		hCD40T254A.3	50
		hCD40T254A.4	65
		hCD40T254A.5	70
		hCD40T254A.6	150
hCD40KKV	6	hCD40KKV.1	9
		hCD40KKV.2	40
		hCD40KKV.3	45
		hCD40KKV.4	60
		hCD40KKV.5	80
		hCD40KKV.6	100
hCD40/CD22	5	hCD40/hCD22.1	12
		hCD40/hCD22.2	40
		hCD40/hCD22.3	55
		hCD40/hCD22.4	70
		hCD40/hCD22.5	90

Figure 3.8- Number of stable transfected hCD40 construct clones in A20 cells and the level of their surface expression. Clones highlighted in red were used in subsequent studies.

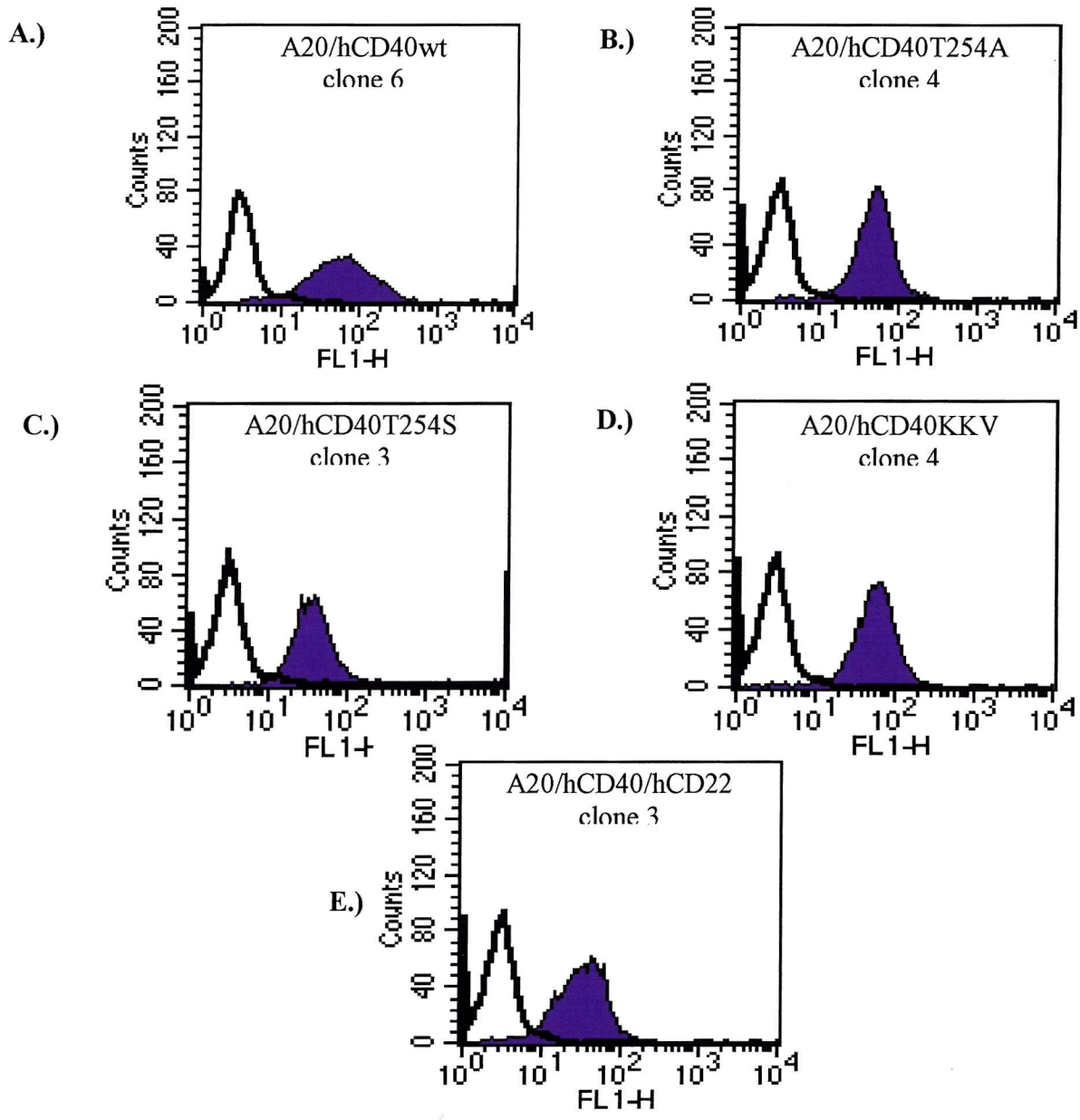


Figure 3.9 -FACS profiles showing levels of human CD40 constructs expression on the surface of A20. A20 was transfected with hCD40 constructs using the method described in 2.2.12. Stable integrations of hCD40 DNA were selected for in the media containing 1mg/ml neomycin. Positive clones were stained with FITC labelled anti human CD40 mAb LOB7.6. The clones with comparable levels of hCD40 expression were chosen. Figures 3.8A to E show a representative clone for each of the transfected hCD40 constructs in A20. Irrelevant mAb used was FITC labelled HB160 (see table 2.2).

3.3 DISCUSSION

This chapter describes the successful production of wild type and mutant hCD40 expression constructs in the background of pcDNA3, a vector that provides high levels of gene expression driven from a strong CMV promoter and implements selection of stable transfected clones by virtue of its neomycin resistance gene. Although some minor problems were present, no significant obstacles were encountered in the production of the constructs or with regard to their transfection into the A20 cell line and subsequent selection of stable transfected clones.

The absolute amounts of mouse and human CD40 expressed in the wild type and transfected cell line were not determined due to absence of antibodies suitable for a western blot. Therefore it was only possible to estimate and compare levels of expressions for both mouse and human CD40 by FACS allowing for the differences in anti mouse and anti human CD40 mAb affinity and intensity/ level of FITC labeling.

It has been possible to select a number of clones for each of the hCD40 constructs, with a wide range of expression levels. Some of the clones had expression levels comparable to those of mouse CD40 on A20 cells (e.g. hCD40wt2). These clones would have been ideal for future use due to the comparable amounts of human and mouse CD40 on the surface, as determined by FACS. However, such clones had a tendency to lose the surface expression of human CD40 over a period of a month after the initial selection. The continuing selection pressure had no bearing on the loss of surface expression in these clones, therefore it proved impossible to resolve this problem and continue working with the low expressing clones. It was also decided to not consider clones expressing very high, unphysiological levels of hCD40 (e.g. hCD40wt8) as signal transduction events in these cells are unlikely to reflect events from lower physiological levels of endogenous mCD40.

A number of clones expressing levels of hCD40 that were roughly 2 to 3 fold higher than that for endogenous mCD40 levels were also obtained, which were suitable for *in vivo* therapy studies. However, a proportion of these clones were found to have significantly

reduced levels of endogenous mCD40. This would have presented a problem in the future *in vivo* therapies, where the anti mouse CD40 mAb is used as a positive control for the treatment of animals. Therefore, only those clones that retained mCD40 expression were selected for the *in vivo* studies. These are shown in figure 3.8 and are highlighted in red.

As it was possible to select clones expressing similar levels of transfected proteins the methodology is therefore suitable for generating reagents that can be used for comparative analysis of the function of wild type and mutated hCD40 in A20 cells. In addition, the expression data indicate that none of the mutations that were introduced into the cytoplasmic domain of CD40 had a significant effect on the stability of the protein. This is clearly an important biochemical parameter to determine when attempting to make an interpretation of the effects of mutagenesis on the function of a transfected protein.

Unfortunately, transfection and selection of stable transfected clones in the πBcl_1 line was unsuccessful despite attempts with a variety of different transfection and selection methods. Two problems were identified. Firstly, transient transfections failed to generate detectable levels of expression as determined by FACS analysis of hCD40 or expression of reporter genes producing highly stable proteins such as chloramphenicol acetyl transferase. Secondly, the cell line appeared to be very sensitive to a variety of antibiotics such that it was not possible to determine the threshold concentrations of antibiotic required for cell death. Based on the double dilution killing curve using neomycin, it appeared that concentrations lower than 2ng/ml of antibiotic were sufficient to cause death of the cells. This concentration is 500 times lower than 1mg/ml, which is the concentration of neomycin used to select for stable transfectants in A20 cells. Precise reasons for lack of transfectability and the high sensitivity of the πBcl_1 line to antibiotics are currently unknown.

CHAPTER 4

The use of B cell lymphoma lines expressing human CD40 for development of an *in vivo* therapy model to test the ability of anti human CD40 mAb to deliver therapy and to investigate the requirement for specific signalling motifs in the human CD40 protein.

4.1 INTRODUCTION

Ligation of CD40 *in vivo* by-passes T cell help provided by Th cells during an immune response (French *et al* 1999, Schoenberger *et al* 1999, Bennet *et al* 1999, Ridge *et al* 1998). It has been confirmed in various studies that CD40 signalling has pleiotropic effects on the immune system, and experiments described in this chapter are an attempt at dissecting these signalling pathways. In chapter 3 the production of tumor cell lines expressing mutant human CD40 proteins was described, make mutations were selected so to disrupt either TRAF 2, 3, 5, 6 or Jak3 binding sites. These molecules have been shown to be associated with CD40 and to be involved in triggering intracellular signal transduction pathways that mediate the induction of the target genes of CD40. The main aim of the experiments in this chapter was to determine if disruption of these signal transduction events in the tumor cell would perturb the ability of anti-CD40 mAb to deliver therapy. This aim is based on the as yet untested hypothesis that anti-CD40 therapy is at least in part mediated by induction of APC properties in the tumor cell (as discussed in sections 1.4.5, 1.6.6 and 3.1).

The experimental model in which anti-CD40 therapy has routinely been tested involves the challenge of inbred strains of mice with tumor. This is considered to be the best model system since the mice provide an identical genetic background, thus allowing any given treatment to be tested many times without the complications incurred as a result of genetic differences between the individuals. The strategy adopted for the studies reported in this chapter was as follows:

1.) Mice were challenged with stable transfected A20 cell lines expressing similar levels of wild type and mutant hCD40 proteins.
2.) Mice were then treated with anti human CD40 mAb, LOB7.6, that specifically recognises an epitope in the extracellular domain of hCD40 which would be expected to be conserved in the mutant hCD40 proteins.
3.) The success or failure of therapy was then monitored over a 80-day period.

It was anticipated that this strategy would elucidate the signaling domains of hCD40 that are critical for mediating therapy in an *in vivo* system.

4.2 RESULTS

4.2.1 Demonstration of the ability of anti-mouse CD40 mAb 3/23 to treat A20 B cell lymphoma in Balb/c mice

Data previously described by French *et al* showed that A20 B cell lymphoma can be successfully treated in Balb/c mice by the use of anti mCD40 mAb 3/23. Prior to testing transfected A20 cell lines, wild type A20 B cell lymphoma cell line was tested in an *in vivo* therapy so to ensure that anti mCD40 mAb treatment can cure mice bearing this tumour. To this aim, two groups of five sex and age matched Balb/c mice were challenged with $1x10^7$ A20 cells contained in 200µl volume of PBS by intravenous tail injection (iv) on day 0. One of the groups was subsequently administered 1mg of anti mCD40 mAb 3/23 on day 4, also iv. The other group of A20 bearing mice was given 200µl of PBS. The tumour cells and mAbs were administered in a 200µl volume of PBS in all cases, for this and all other therapies. Numerous therapies have been performed previously where isotype matched control mAb was used, and those therapies were found to give same results as obtained when administering PBS alone. It was therefore deemed that PBS was a suitable control.

The mice were then checked twice daily for the following 80 days. Each day was recorded on a therapy sheet as the number of animals left alive per group at 9am. The results were expressed in a graph form where the percentage of surviving animals was plotted against time in days. The results of this therapy are shown in figure 4.1. The data shows that mice receiving placebo PBS succumbed to the tumour over a period of 14 days starting at day 24. In contrast, the mice that received anti mCD40 3/23 mAb injection cleared the tumour and lived to day 80 as the last recorded day. These results demonstrate that anti mCD40 mAb treatment is successful in promoting the clearance of A20 B cell lymphoma, thus confirming previously published data and forming the basis for subsequent experiments using A20/hCD40 transfected cell lines.

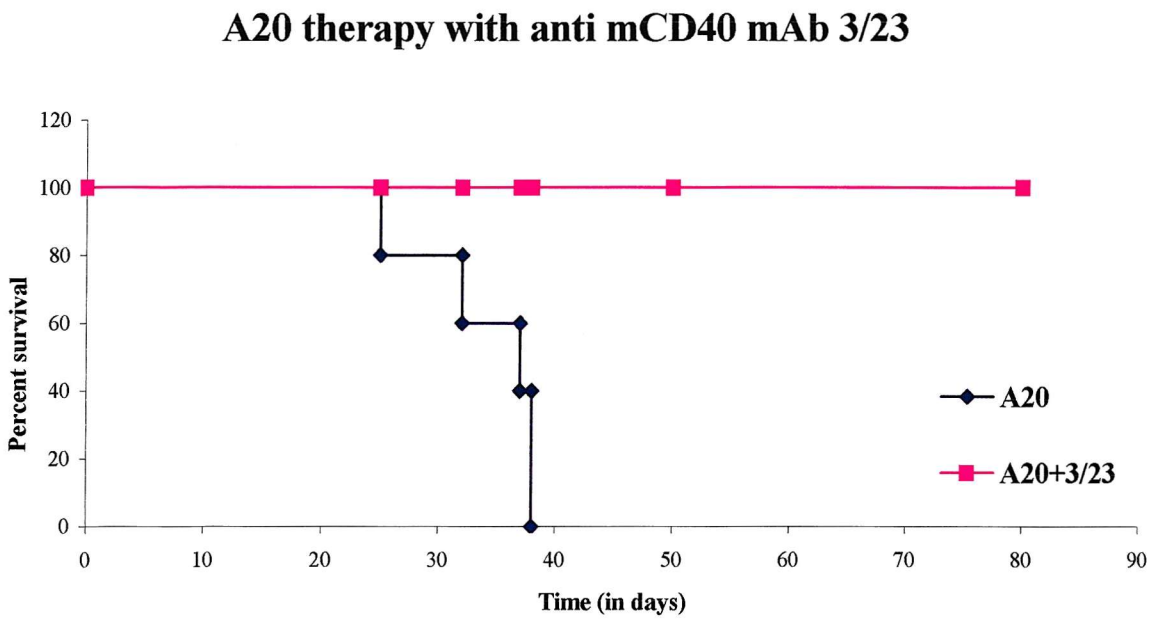


Figure 4.1- A20 therapy with 3/23 mAb. Two groups of five sex and age matched Balb/c mice were injected with $1x10^7$ A20 tumour cells on day 0; one group was treated with 1 mg 3/23 mAb by iv. injection on day 4, and the other group was given PBS iv. of the same volume. The control group succumbed to the tumour over a 14 day period starting day 24. The 3/23 mAb treated group cleared the tumour and lived as healthy animals up to day 80 of the therapy, when the last record was taken.

4.2.2. Testing the ability of anti-human CD40 mAb LOB7.6 to deliver therapy in mice challenged with A20 B cell lymphoma cell lines expressing functional hCD40.

The A20 cell line A20/hCD40wt(6) that expresses wild type hCD40 was selected for the first therapy experiment. Prior to final selection it was important to establish the stability of hCD40 expression in this cell line over an extended period of culture that would reflect the time course for the *in vivo* therapy study. This was considered to be important because if expression of hCD40 was unstable and was attenuated during the course of therapy then ability of anti-human CD40 to clear the tumor would also be attenuated. Expression of both mCD40 and transfected hCD40 protein was therefore monitored by FACS analysis every two weeks over a four-month period of continuous culture. Fig 4.2A shows the level of hCD40 expression in cell line A20/hCD40wt(6) at the end of the four month culture period which was found to be unchanged as compared to the expression profile obtained when the clone was initially established. In addition it was confirmed that the cell line also retained surface expression of mouse CD40 (fig 4.2C).

The therapy study was then set up as follows. To control the experiment two groups of five sex and age matched Balb/c mice were challenged with $1x10^7$ wild type A20 cells iv on day 0. On day 4, one group of these mice were injected iv with 1mg of anti mCD40 mAb 3/23 contained in 200µl volume of PBS, the other group received 200µl PBS alone. For setting up the hCD40 therapy, three groups of five sex and age matched Balb/c mice were challenged with $1x10^7$ cells of clone A20/hCD40wt(6) iv on day 0. On day 4, one group of these mice were iv injected with 1mg of 3/23 mAb; the second group were iv injected with 1mg of anti human CD40 mAb LOB7.6, the third group were iv injected with PBS alone. The results of this study are shown in figure 4.3 and for convenience are depicted on two separate graphs, one for the control A20 line (fig 4.3A) and the other for clone A20/hCD40wt(6) (fig 4.3B). The A20 control group of mice died over a period of 12 days starting at day 20. The A20 group treated with 3/23 mAb, however, cleared the tumour and lived to day 80, as the last recorded date. Mice challenged with clone A20/hCD40wt(6) and injected on day 4 with PBS alone died over a period of 11 days starting at day 21. The group of mice challenged with clone A20/hCD40wt(6) and treated with LOB7.6 mAb also died starting from day 25 to day 41 of the therapy. However, the group of mice challenged

with clone A20/hCD40wt(6) and treated with 3/23 mAb cleared the tumour and lived to day 80. These results show that treatment of clone A20/hCD40wt(6) with anti human mAb LOB7.6 did not achieve therapy *in vivo*. Although LOB7.6 treated group lived an average of 7 days longer than the control PBS injected group, this difference was not statistically significant.

As the hCD40 therapy failed it was possible that this was due to loss of surface hCD40 expression on clone A20/hCD40wt(6) during *in vivo* growth. Therefore tumor cells were isolated from the spleens of mice which succumbed to the tumour in the group treated with LOB7.6 mAb in order to determine the level of surface hCD40 expression by FACS. The isolation procedure is described in the methods (2.2.33). Retrieved cells were labelled with FITC-3/23 and LOB7.6. The FACS results are presented in figure 4.2B and D and for convenience are placed next to the FACS data presented in fig 4.2A and C which allows visual comparison. The levels of surface expression of mCD40 and hCD40 on cells at the end of the therapy were essentially the same as that measured at the start. This suggests that the lack of therapy in mice treated with LOB7.6 mAb did not arise due to absence of hCD40 receptor from the surface of transfected tumour cells.

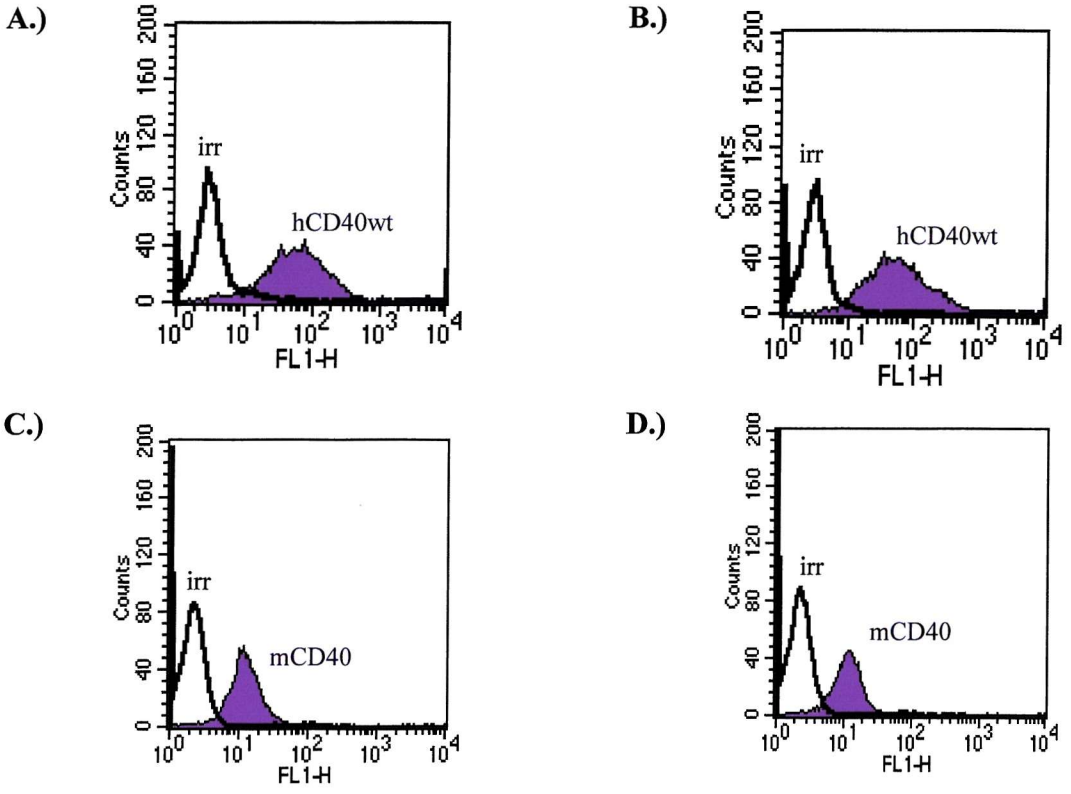


Figure 4.2- FACS profiles showing expression of mCD40 and hCD40wt on the surface of A20/hCD40wt(6) clone prior to and after the therapy shown in figure 4.3

A.) A histogram demonstrating the level of hCD40 expression on the surface of A20/hCD40wt(6) after four months of culturing. Control mAb used in FACS analysis was an isotype matched mAb, HB160, raised against K^k MHC class I molecule (see table 2.2).

B.) The level of hCD40 expression on A20/hCD40wt(6) recovered from a culled mouse in the therapy group treated with LOB7.6 mAb.

C.) A histogram showing the level of mCD40 expression on A20/hCD40wt(6) clone before the therapy described in fig 4.3. The cells were labelled with FITC-3/23 mAb. Control mAb used in FACS analysis was FITC-YTA3.1.2.

D.) The level of mCD40 expression on A20/hCD40wt(6) clone after the therapy. A20/hCD40wt(6) cells were isolated from a culled mouse with a terminal tumour load from the A20/hCD40wt(6) control group.

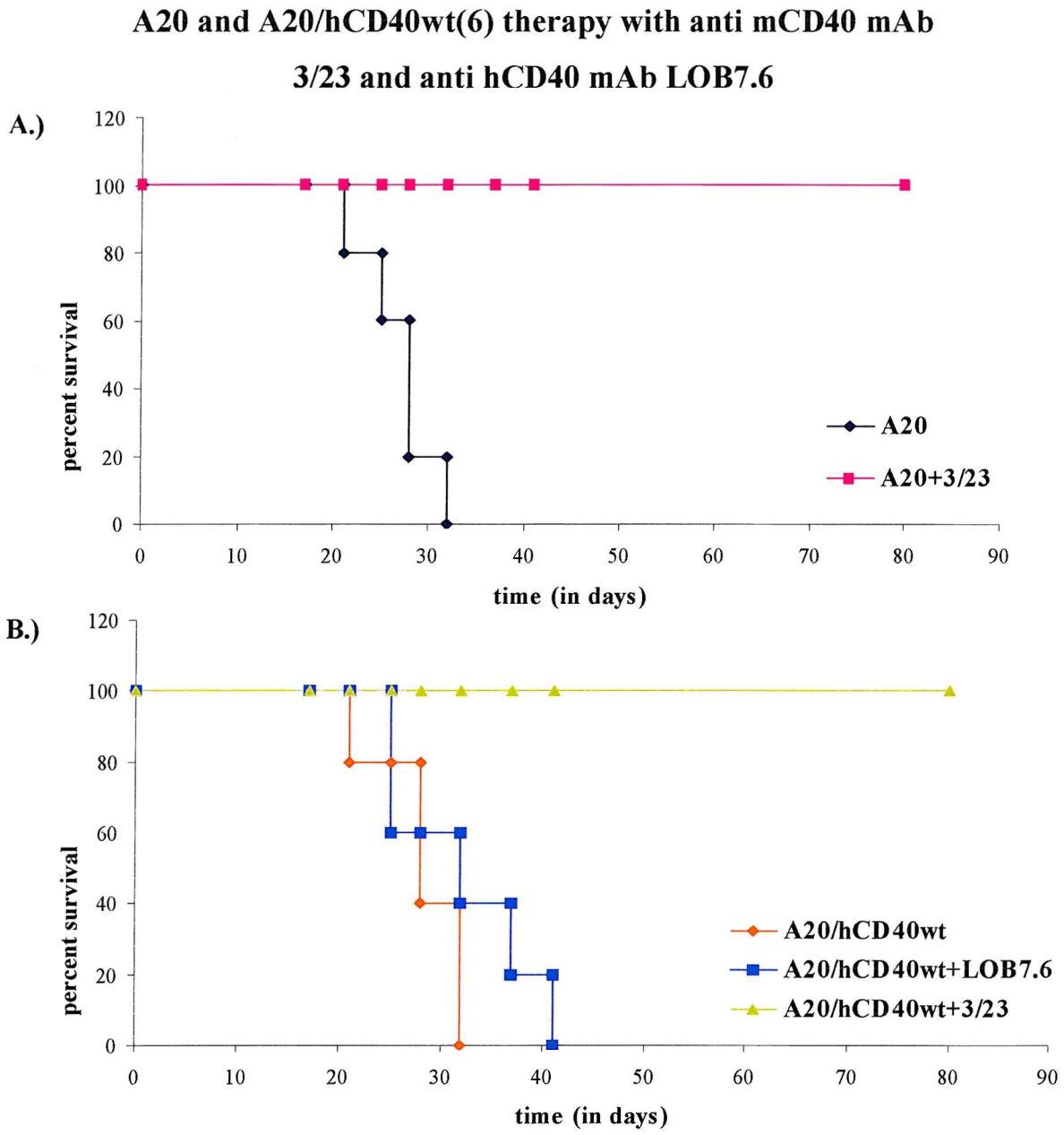


Figure 4.3- A20 and A20/hCD40wt(6) therapy. Two groups of five age and sex matched Balb/c mice were injected with $1x10^7$ A20 tumour cells on day 0 and treated as described in figures 4.1. Three groups of five Balb/c mice were injected with $1x10^7$ A20/hCD40wt transfected tumour. On day 4, one group was treated with 1mg of anti hCD40 mAb LOB7.6 by iv. injection; second group was given 1mg of 3/23 mAb iv., and the third group was given PBS. A20/hCD40wt(6) and wild type A20 both had similar kinetics of growth in animals. A20/hCD40wt(6) tumour was cured by 3/23, but not LOB7.6 mAb treatment.

Following on from the studies described in fig 4.3, the next therapy was carried out with A20/hCD40T254S cell line. In previous studies it has been established that mutation of Thr254 to Ser does not affect signalling through CD40 (Pullen *et al* 1999, Cheng *et al* 1995). Therefore, this mutated molecule would be expected to signal in a similar manner to wild type CD40. The manner in which this therapy was set up was identical to that already described for A20/hCD40wt (fig 4.3). The outcome of this therapy is shown in fig 4.4. The A20 control group of mice died over a period of 9 days starting at day 21. The A20 group treated with 3/23 mAb, however, cleared the tumour and lived to day 80, as the last recorded date. Mice bearing A20/hCD40T254S that were given PBS died over a period of 8 days staring at day 29. The mice treated with LOB7.6 mAb also died starting from day 30 to the day 36 of the therapy. However, the mice given A20/hCD40wt and treated with 3/23 mAb cleared the tumour and lived to day 80. Again, these results suggest that treatment of A20/hCD40T254S cell line with anti hCD40 mAb LOB7.6 did not achieve therapy *in vivo*.

The expression of both human and mouse CD40 on A20/hCD40T254S transfected cells was confirmed prior to the therapy and after the therapy (fig 4.5). As for A20/hCD40wt, the A20/hCD40T254S cells were labelled with FITC conjugated 3/23 and LOB7.6 mAb and tested on FACS. Results showing the expression levels of mouse and human CD40 are contained in the figures 4.5. The results presented show that the levels of surface expression of mCD40 and hCD40 were similar prior to and after the therapy. Therefore the observed lack of therapy in mice treated with LOB7.6 mAb could not be explained by an absence of hCD40T254S receptor on transfected tumour cells.

Following the failure of A20/hCD40wt and A20/hCD40T254S therapies to respond to LOB7.6 mAb, A20 transfected with the remaining hCD40 constructs were not tested *in vivo*, as such course of action was considered to be wasteful. This was due to the very high cost of mAbs used in the therapies (amounting to 15 mg per therapy) and the high numbers of mice (25) required for a statistically significant study. In conclusion to this section of results, mAb LOB7.6 targeted against transfected hCD40 molecule failed to provide therapy in A20/hCD40wt and A20/hCD40T254S.

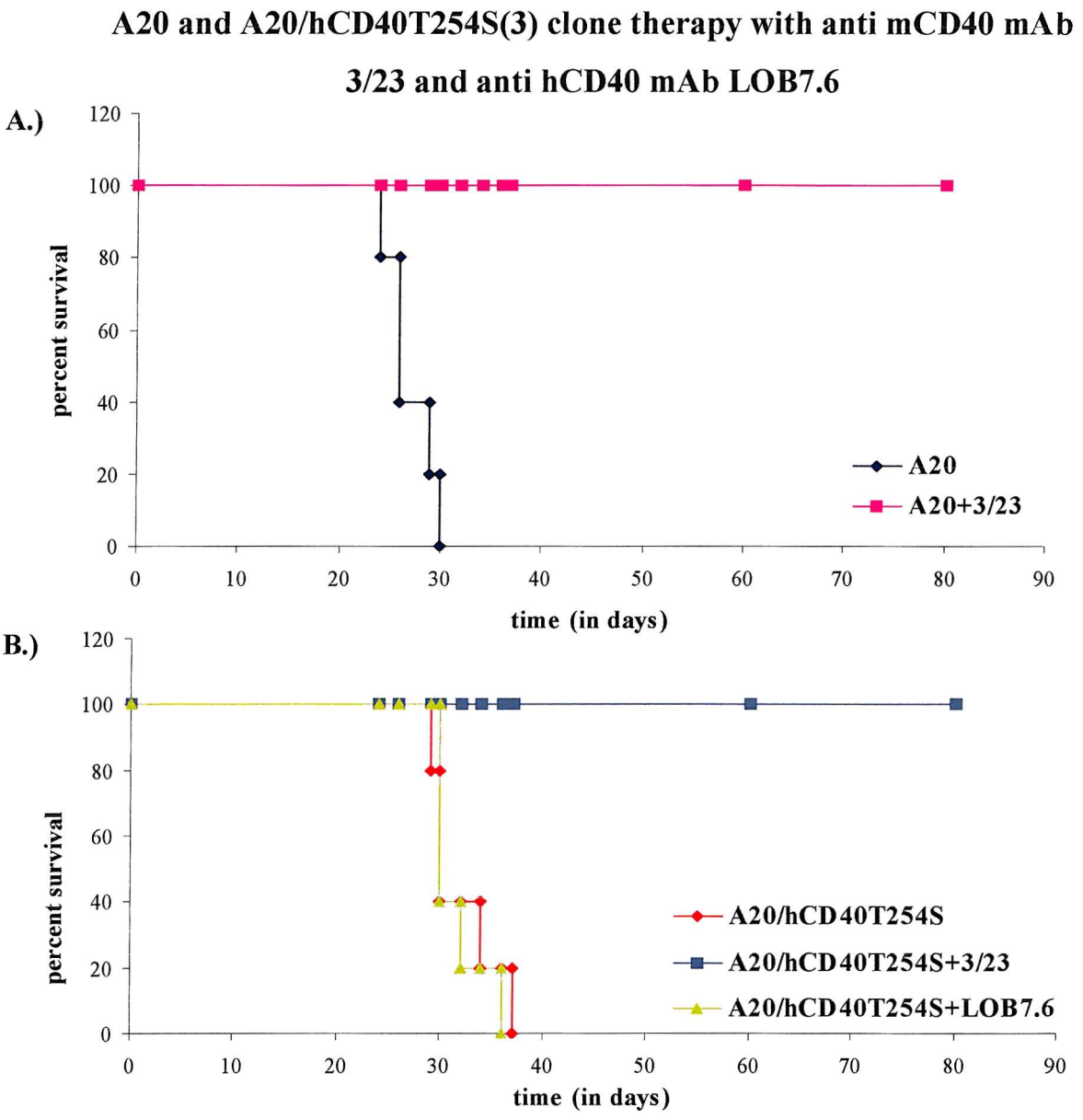


Figure 4.4- A20 and A20/hCD40T254S(3) therapy. Two groups of five age and sex matched Balb/c mice were injected with $1x10^7$ A20 tumour cells on day 0. On day 4, one group was given 1mg 3/23 mAb/mouse iv, and the other PBS in the same volume also iv. Three groups of five Balb/c mice were injected with $1x10^7$ A20/hCD40(T254S) transfected tumour. On day 4 one group was treated with 1mg of anti human CD40 mAb LOB7.6 iv.; second group was given 1mg of 3/23 iv., and the third group was given PBS. As before, in A20/hCD40wt therapy, treatment with anti human CD40 mAb does not seem to give any marked protection over the untreated A20/hCD40T254S. Transfected and wild type A20 both had similar kinetics of growth in animals. A20/hCD40wt transfected tumour was cured by 3/23, but not LOB7.6 mAb treatment.

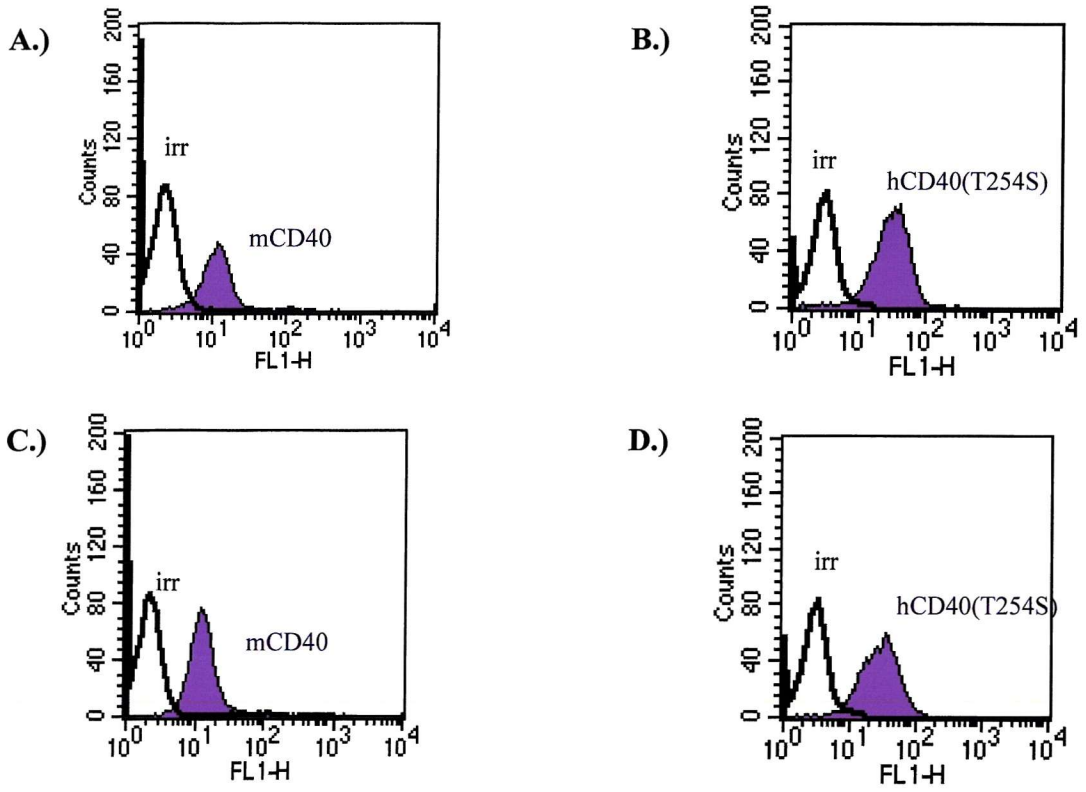


Figure 4.5- FACS profiles showing expression of mCD40 and hCD40T254S on the surface of A20/hCD40T254S(3) clone prior to and after the therapy shown in figure 4.4

A.) A histogram showing the level of mCD40 expression on A20/hCD40T254S(3) prior the therapy described in figure 4.4. Cells were labelled with FITC-3/23 mAb. Control mAb used was FITC-YTA3.1.2 (see table 2.2).

B.) A histogram demonstrating the level of hCD40T254S expression on the surface of A20/hCD40T254S(3) before the therapy in fig 4.4. The cells were labelled with FITC-LOB7.6. Control mAb used in FACS analysis FITC-HB160.

C.) The level of mCD40 expression on A20/hCD40T254S(3) after the therapy. The cells were isolated from a culled mouse with a terminal tumour load from A20/hCD40T254S(3) control group as shown in figure 4.4.

D.) The level of hCD40T254S receptor expression on A20/hCD40T254S(3) recovered from a culled mouse in the LOB7.6 mAb treated group.

4.2.3 Therapies with A20/pcDNA3 as a negative control for the presence of expression vector in the A20/hCD40 transfected cell lines

At the same time as undertaking therapy studies with A20/hCD40wt(6) and A20/hCD40T254S(3) control therapies were also set up in which mice were challenged with a clone (A20/pcDNA31) that had been selected after transfection with pcDNA3 lacking an insert cDNA. This control experiment provided unexpected results that are of potential importance in the design of transfection-based *in vivo* studies.

The therapy was set up as previously described in 4.2.2. Briefly, two groups of five sex and age matched Balb/c mice were challenged with $1x10^7$ wild type A20 cells iv on day 0. On day 4, one group of these mice were injected iv with 1mg of anti mCD40 mAb 3/23 contained in 200µl volume of PBS, the other group received 200µl PBS alone. For A20/pcDNA3(1) therapy, two groups of five sex and age matched Balb/c mice were challenged with $1x10^7$ cells of clone A20/pcDNA3(1) iv on day 0. On day 4, one group of these mice were iv injected with 1mg of 3/23 mAb and the second group were given PBS alone, also iv. The results of this study are shown in figure 4.6 and for convenience are depicted on two separate graphs, one for the control A20 line (fig 4.6A) and the other for A20/pcDNA3(1) (fig 4.6B). The A20 control group of mice died over a period of 12 days starting at day 20. The A20 group treated with 3/23 mAb also died which was unexpected given the ability of 3/23 to clear tumor cell lines A20hCD40wt(6) and A20hCD40T254S(3) (fig 4.3 and 4.4). It was possible that this unexpected result was due to an alteration in the phenotype of the clone relative to its parental A20 cell. In order to test this possibility a polyclonal population of pcDNA3 transfected cells were generated by growing transfected cells in the presence of 1mg/ml neomycin for 3 weeks. These neomycin-resistant cells were then used to challenge mice in the manner described above. As shown in fig 4.7 this polyclonal population of A20/pcDNA3 cells also failed to respond to therapy with 3/23.

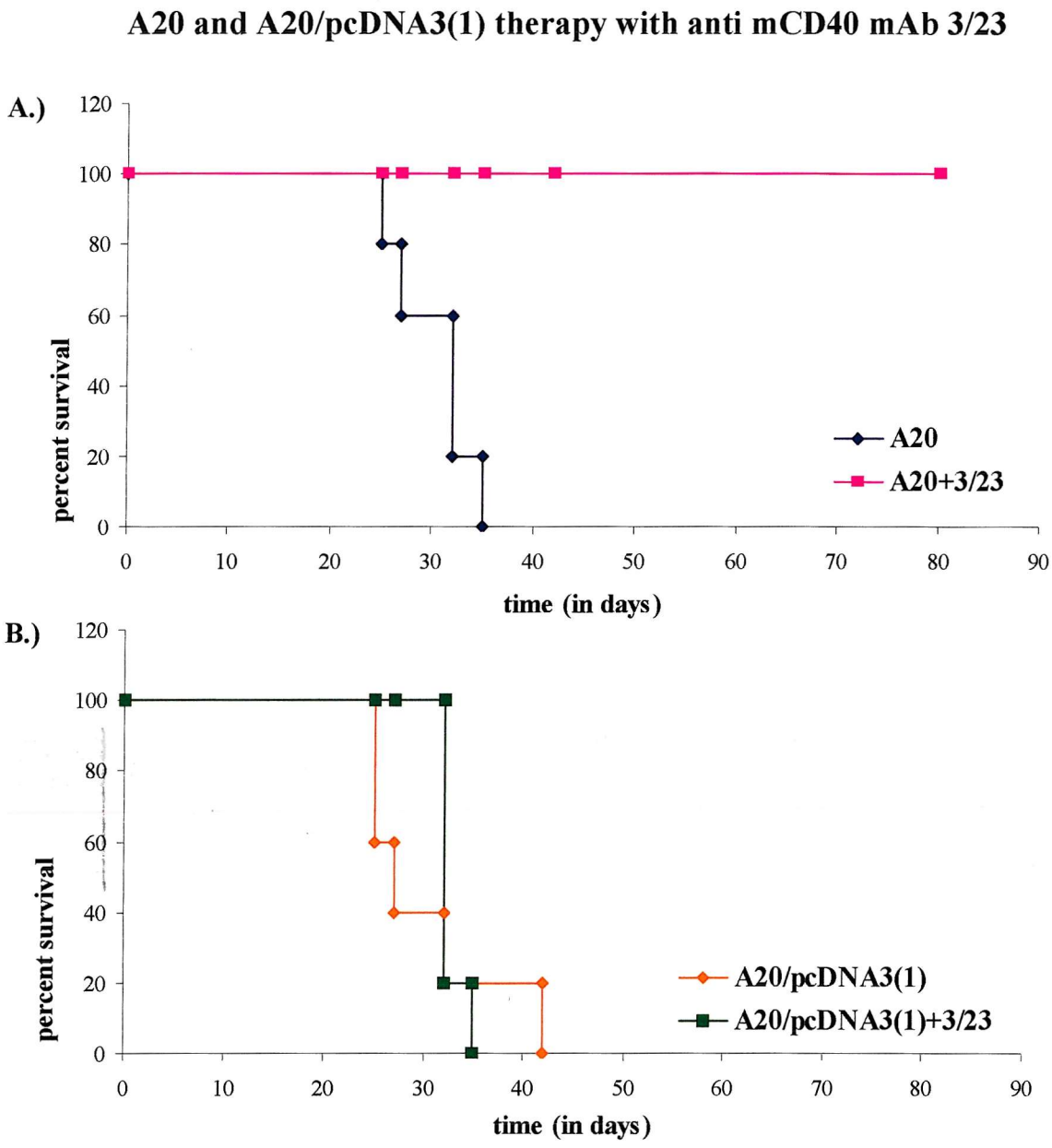


Figure 4.6- A20 and A20/pcDNA3(1) therapy. Single transfected clone of A20/pcDNA3 was selected on the basis of its resistance to neomycin (1mg/ml). Positive colony was cloned out by serial dilution. The therapy was set up as already described in figures 4.1,4.3 and 4.4. Both A20 and A20/pcDNA3 exhibited similar kinetics of tumour growth *in vivo*. A20/pcDNA3 transfected tumour did not respond to the treatment with 3/23 antibody. As a result, the mice in the A20/pcDNA3 treated group succumbed the tumour at similar time as the control groups.

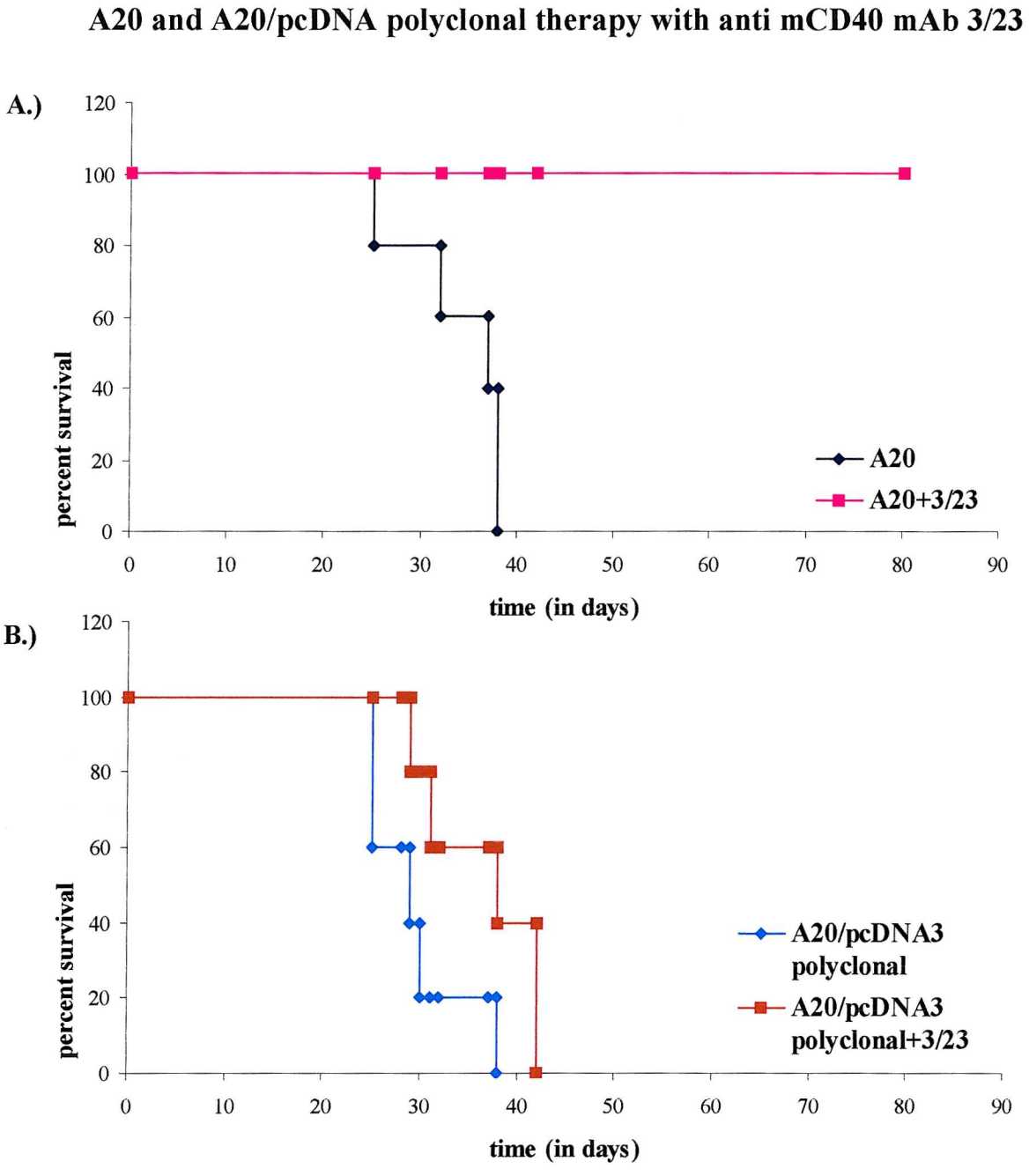
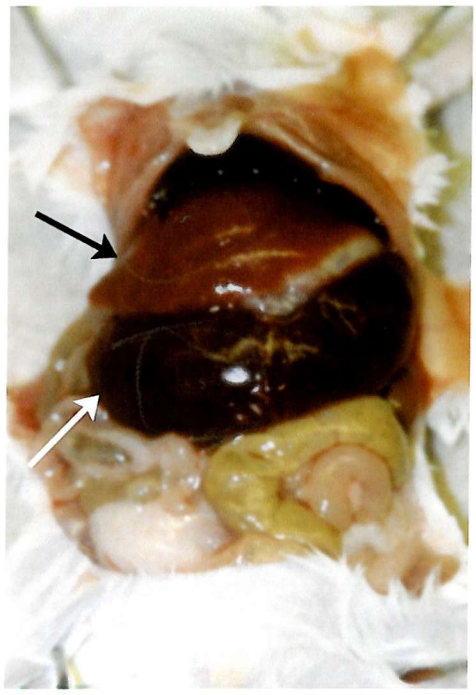


Figure 4.7- A20 and A20/pcDNA3 polyclonal therapy with 3/23 mAb. A20 cells were transfected with pcDNA3, and a polyclonal population selected on the basis of resistance to neomycin (1mg/ml). The therapy was set up with as outlined in 4.2.3. Both A20 and A20/pcDNA3 polyclonal exhibited similar kinetics of tumour growth *in vivo*. Two mice in 3/23mAb treated group died before the untreated controls, but the remaining three mice lived for 17 days longer.

Autopsy of those mice challenged with clone A20/pcDNA3(1) and the polyclonal population of pcDNA3 transfected A20 cells revealed that tumors developed in the liver instead of the usual location, the spleen. Figure 4.8 shows the typical appearance of the spleen and liver of a mouse in the terminal stage of A20-induced tumour. The liver appears normal whereas the spleen is enlarged to between 20 to 30 times its normal size. Figure 4.9 (A and B) shows the liver and spleen of two mice that died from A20/pcDNA3(1)-induced tumors. Although the spleens are enlarged they are significantly smaller than spleens from mice challenged with parental A20 cells. In addition, inspection of the livers of the mice revealed multiple solid tumors across the organ, a pathology that was never observed in mice challenged with parental A20 cells. Figure 4.9 (C and D) shows the liver and spleen from mice challenged with the polyclonal population of pcDNA3 transfected A20 cells. These mice had an almost identical pathology to mice challenged with A20/pcDNA3, ie a slightly enlarged spleen and multiple solid tumors of the liver.

It was also important to rule out the possibility that 3/23 therapy of pcDNA3 transfected cells simply reflected a lack of surface mCD40. Fig 4.10 shows FACS analysis of A20/pcDNA3(1) cells prior to therapy (A) and when recovered from the tumor nodules of the livers of mice at the end of therapy (B). Levels of surface mCD40 were similar. Identity of recovered cells as A20/pcDNA3(1) was confirmed by their ability to proliferate in the presence of neomycin (data not shown) and by their forward and side scatter profiles on FACS (fig C and D). Figure 4.11 (A, B, C and D) shows essentially the same analysis and results for polyclonal pcDNA3 transfected A20 cells, confirming presence of mCD40 at the surface of cells prior to and after therapy.

A.)



B.)

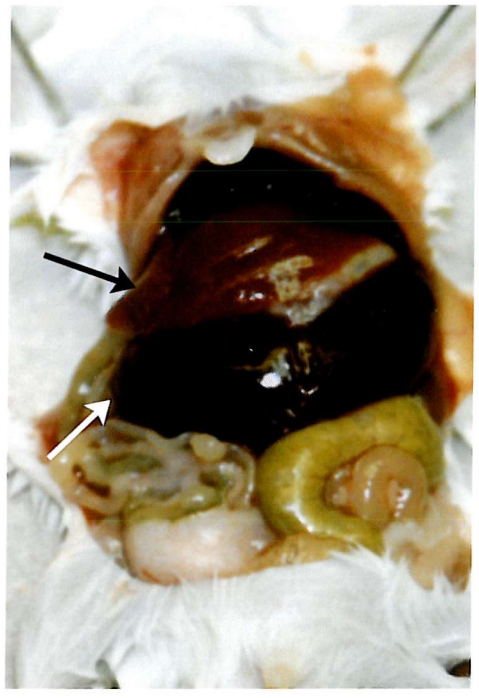


Figure 4.8- The appearance of the internal organs of two dissected mice carrying a terminal load of A20 wild type tumour.

A.) and B.) White arrows are pointing to the enlarged spleens, where most of the tumour mass is located. Due to the presence of a growing tumour load, the spleens of the animals carrying a terminal tumour load are 10-15 times larger than normal. Black arrows show the liver, which in both mice appears healthy and unaffected by the tumour.

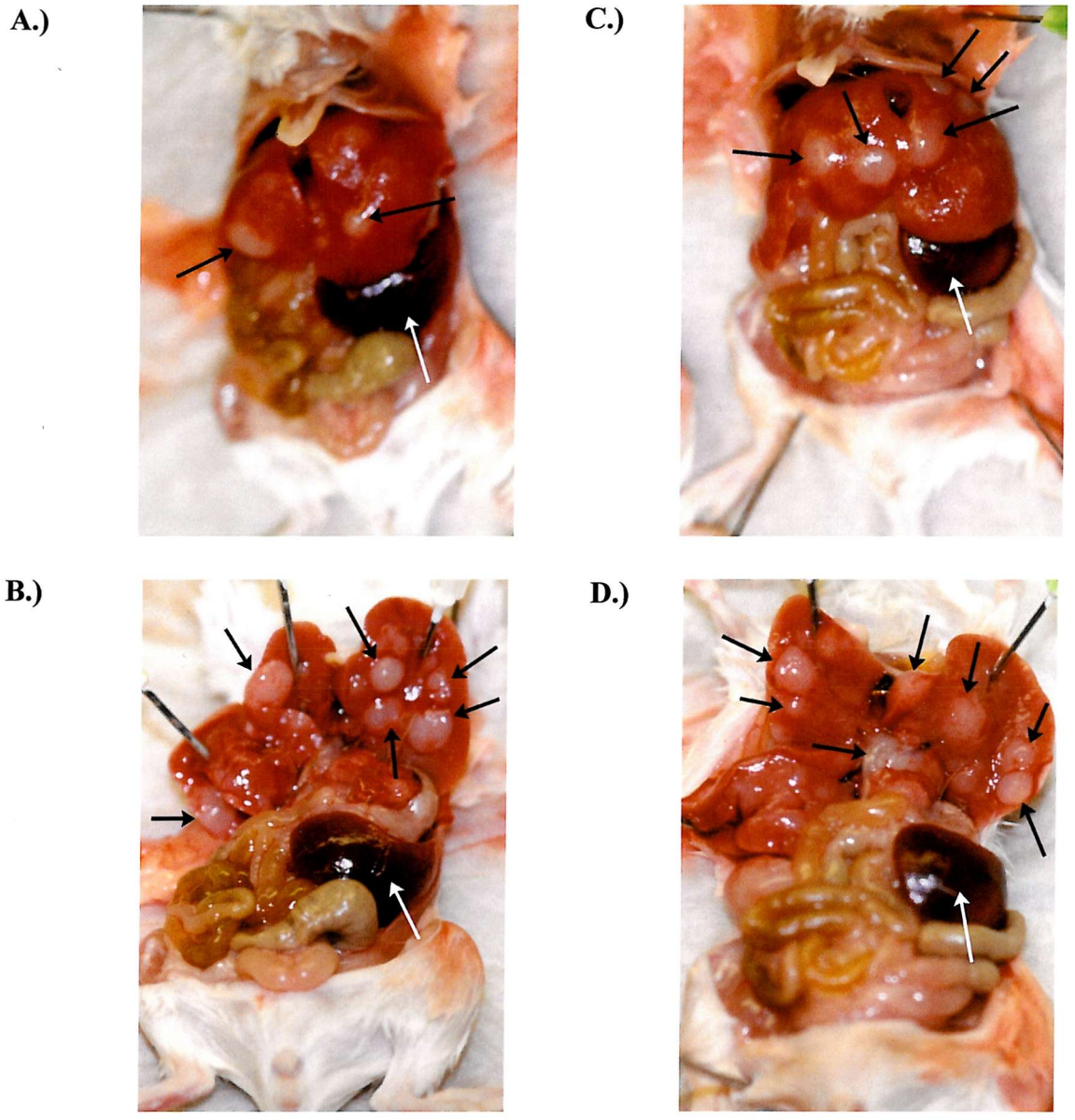


Figure 4.9- The appearance of the internal organs of dissected mice carrying a terminal load of A20/pcDNA3 transfected tumour.

A.) and B.) figures show the livers in two mice given A20/pcDNA3 tumour obtained from a single transfected clone. The animals were treated with 3/23, and were part of the therapy illustrated in figure 4.6. The black arrows point to the solid tumour lumps on the liver. The white arrows point to the spleen which is larger than normal, although not as extensively enlarged as the spleen resulting from the terminal load of A20 wild type tumour (fig 4.8).

C. and D.) show the livers in mice from the group given polyclonal A20/pcDNA3 and treated with 3/23. The therapy is depicted in the figure 4.7. As before, black arrows show tumour lumps on the liver, and white arrow indicate the spleen.

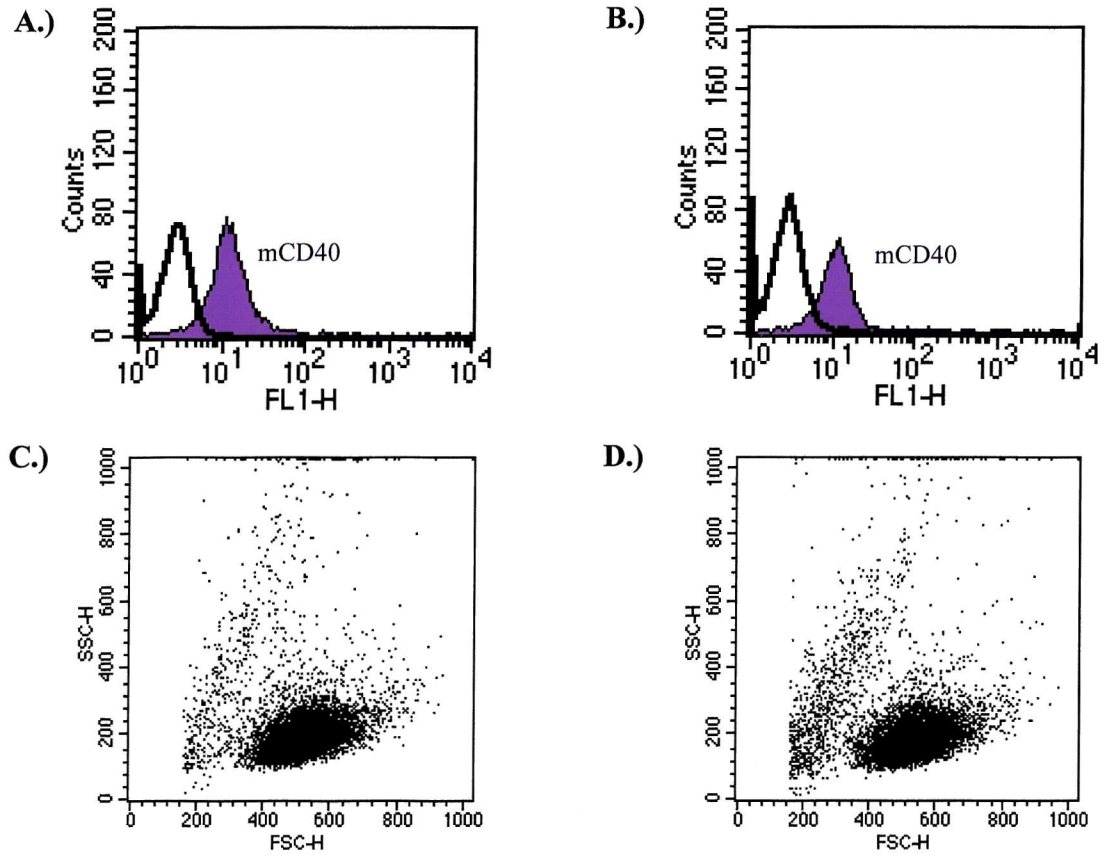


Figure 4.10- FACS profiles showing expression of mCD40 on the surface of A20/pcDNA3(1) clone prior to and after the therapy shown in figure 4.6

A.) The level of mCD40 expression on A20/pcDNA3(1) clone prior to the therapy described in fig 4.6. The cells were labelled with FITC-3/23 mAb. Control mAb used in FACS analysis was FITC-YTA3.1.2.

B.) A histogram demonstrating the level of mCD40 expression on the surface of A20/pcDNA3(1) clone isolated from a culled mouse with a terminal tumour load from the control group as shown in fig 4.6. The cells were labelled in the manner described above.

C.) Forward and side scatter profile of A20/pcDNA3(1) cells prior to the therapy.

D.) Forward and side scatter profile of A20/pcDNA3 cells after the therapy. It shows that the rescued cells upon which the FACS analysis was performed form a uniform population which are of similar size and granularity as the A20/pcDNA3 prior to the therapy.

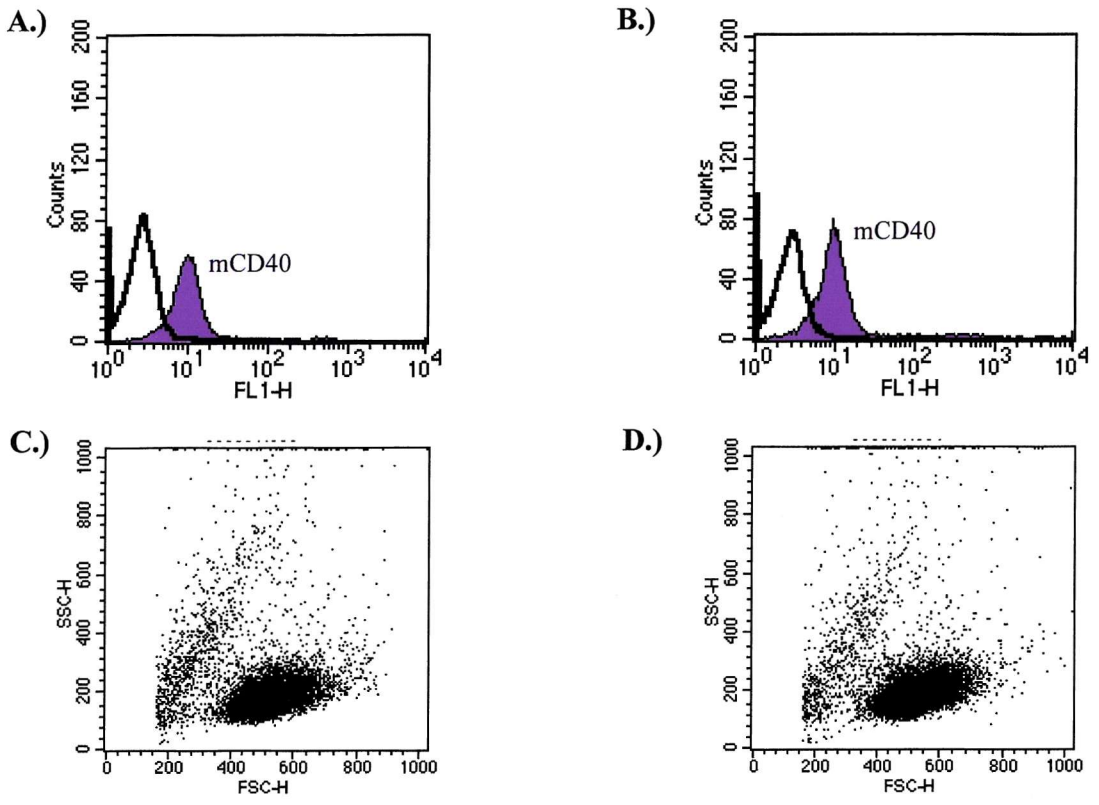


Figure 4.11- FACS profiles showing expression of mCD40 on the surface of A20/pcDNA3 transfected polyclonal population of cells prior to and after the therapy shown in figure 4.7

A.) A histogram showing the level of mCD40 expression on A20/pcDNA3 polyclonal population prior to the therapy described in figure 4.7. The cells were stained as stated in fig 4.10.

B.) A histogram demonstrating the level of mCD40 expression on the surface of A20/pcDNA3 polyclonal population rescued from a mouse with a terminal tumour load from the untreated group as shown in figure 4.7.

C.) Forward and side scatter profile of A20/pcDNA3(1) cells prior to the therapy.

D.) Forward and side scatter profile of A20/pcDNA3 cells after the therapy. It shows that the rescued cells upon which the FACS analysis was performed form a uniform population which are of similar size and granularity as the A20/pcDNA3 prior to the therapy.

4.3 DISCUSSION

The aim of the experiments described in this chapter was to develop an *in vivo* therapy model by using the B cell lymphoma lines expressing human CD40 constructs. In this model the ability of anti hCD40 mAb to deliver therapy would be tested as would the requirement for specific signalling motifs in the hCD40 protein. However, as demonstrated in the "results" section of the text, *in vivo* therapies with transfected cell lines are unlikely to provide a model with which to elucidate the role of CD40 domains in tumour rejection. The major reason for abandoning the model is that the anti hCD40 mAb LOB7.6 failed to prevent growth of A20 tumour cells expressing hCD40. The treatment of mice with the anti mCD40 mAb 3/23 has lead to rejection of A20/hCD40 cells. It can, therefore, be concluded that the lack of response of these cells to LOB7.6 may not have been due to a transfection or selection artefact. Instead, there may be two possible explanations for the lack of therapy using LOB7.6.

Firstly, it could be argued that interaction of LOB7.6 with hCD40 may not lead to activation of signalling pathways in the tumour cell. Although previously published work suggests that the signalling through mCD40 and hCD40 is interchangeable in mouse cells (Hsing *et al* 1997, Hsing *et al* 1999, Jalukar *et al* 2000, Baccam *et al* 1999), it may be that in our hands the hCD40 constructs do not function as expected when transfected into mouse cells. It is also possible that hCD40 constructs do not signal in A20 due to the cells being in some way compromised. This possibility is considered and addressed in the chapter 5. Also, the failure of LOB7. to provide any effect *in vivo* is not due to mAb not binding, as is discussed in chapter 6.

The failure of LOB7.6 to provide therapy in mice injected with A20/hCD40 may suggest that the therapy is not mediated by the tumour cell, but may instead be directed through the activation of a host APCs, such as dendritic cells. Considering the likelihood that a host APCs may be involved, it is important to contemplate the way in which this may be the case. It has been shown that DCs have the ability to internalise apoptotic bodies and cellular debris and channel it into the MHC class I pathway (see 1.4.5). This phenomenon of external antigens gaining access to class I pathway is known as cross-priming. As

outlined in chapter 1, the therapies performed with 3/23 mAb in mouse B cell lymphoma lines only work when there is a high tumour load present at the time of delivering the anti CD40 mAb. This would suggest that there is a threshold level of weak tumour antigens which needs to be reached before an efficient immune response is mounted against the tumour. This threshold effect may be explained in two ways. One is that lymphoma cells can present their own antigen efficiently when the tumour load is high. The cross-linking of CD40 on the tumour cells would enhance their antigen presenting capacity, and due to the large numbers of cells, the immune response may be triggered. The other possibility is that under the circumstances where the tumour load is high, there is an ongoing level of apoptosis/necrosis of the tumour cells. The tumour cell debris may be taken up by the surrounding dendritic cells. They can, through cross-priming, present the tumour antigens in their MHC class I molecules to precursor CTLs. Again, anti CD40 mAb would mediate up-regulation of costimulatory molecules/cytokines on DCs, resulting in generation of CTLs and an onset of a cellular immune response directed against the tumour. It is the second of the two hypothesis that seems more likely to take place *in vivo* due to supporting, but indirect evidence obtained *in vitro* with A20, Bcl_1, πBcl_1 and other B cell lymphomas, and *in vivo* with T cell lymphoma EL4 (French *et al* 1998). In independently carried out *in vitro* experiments, Tutt A. and French R. noted that addition of three different anti mCD40 mAbs, namely IC10, 3/23 and FGK45, did not result in up-regulation of co-stimulatory markers on the surface of these lymphomas (unpublished observations). The manner in which the mAb was added to the cells (i.e. free in solution, cross-linked on a plate or bound by cells expressing Fc receptors) or the final concentration of mAb did not appear to have any impact on the response recorded in the cell lines. Expression of high levels of co-stimulatory proteins, such as B7.1 and B7.2, are necessary for efficient priming of precursor CTLs. It is therefore thought that A20, Bcl_1, πBcl_1 and other B cell lymphomas tested have limited, if any, ability to prime naive T cells. The therapy data obtained with T cell lymphoma EL4 also supports the idea of a host APC presenting the cross-primed tumour antigens to precursor CTLs. This lymphoma cell line does not express CD40 on its surface and being a T cell has no capacity to deliver co-stimulation, and yet more than 50% of anti CD40 mAb treated animals reject EL4 and become immune to a subsequent tumour re-challenge (French *et al* 1998).

In order to establish whether the tumour cell or a host cell is presenting the antigens to the precursor CTL, it is important to find out if hCD40 is signalling in A20 tumour cell line or not. If it is, then it may be that the host APC holds the key to the curative mechanism associated with the use of anti CD40 mAb. Signalling of CD40 in A20 tumour cells is considered in the next chapter. Having considered the nature of the antigen presenting cell in the therapy model, it can be said that the possibilities of T cell priming by tumour B cells or by host professional APCs are not mutually exclusive and it can be envisaged that a multitude of factors and responses can be working together to bring about the effects encountered in the *in vivo* therapies with anti CD40 mAb.

The most perplexing set of observations from this chapter concern the behaviour of A20 cells transfected with pcDNA3 lacking an insert. This "control" did not respond to 3/23 therapy and for reasons unknown developed into several solid tumours in the liver rather than appearing as a dispersed tumour load in the spleen. The initial result obtained with a single clone of A20/pcDNA3 was thought to have arisen as a possible consequence of the vector DNA insertion in the genome in a way that may have altered the expression of adhesion molecules on the surface of the transfected clonal population. A second population of A20/pcDNA3 was obtained after the polyclonal selection of transfected cells. It was thought that a possible artefact of a clonal selection may be eliminated using polyclonal population in the therapy. However, figures 4.6 and 4.7 suggest that there is a minimal difference between the clonal and polyclonal population of A20/pcDNA3 in their *in vivo* responses. It may be possible that the failure of A20/pcDNA3 cells to respond to 3/23 therapy is a direct consequence of the different localisation of the cells, i.e. the liver rather than the spleen. It can be suggested that the nature of immune response to the tumour is different in the liver to that in the spleen, which may lead to the lack of response. However, it has been shown previously by French (personal communication) that there is an almost identical immune response in both the spleen and the liver. This is based on the similar numbers of tumour specific CTLs present in both organs during the therapy. It may therefore be speculated that the reason for the lack of response to the tumour in the liver may be the fact that the tumour was growing as a solid mass. Also, the prospect of a particular tumour antigen being expressed at lower levels in transfected cells cannot be eliminated. Such possibility could occur due to pcDNA3 sequences being incorporated into

the genome next to such antigen, hence affecting its expression. However, since A20/hCD40 transfectants behaved essentially in the same way as untransfected A20 cells and responded to 3/23 mAb therapy, it can be concluded that transfection, selection or expression of pcDNA3 sequences *per se* are not responsible for the altered behaviour of A20/pcDNA3 cells. One might contemplate that the expression of hCD40 along with the expression vector sequences may somehow protect transfected cells from these changes. However, although such a premise is highly unlikely, it can be explained if the following possibility is considered. It may be conceivable that pcDNA3 is expressing very high quantities of the neomycin resistance gene in absence of another insert. This could be tested for by repeating the control therapies with a cell line that is transfected with pcDNA3 carrying an insert such as β galactosidease, which should eliminate the possible detrimental effect of high expression of vector sequences.

CHAPTER 5

The *in vitro* analysis of stable human CD40 expression and signalling in A20 B cell lymphoma line

5.1 INTRODUCTION

The *in vivo* studies using A20/hCD40 stably transfected cell lines in the previous chapter have yielded more questions than answers. The absence of any therapeutic effect delivered by the anti hCD40 mAb LOB7.6 has highlighted various problems and imposed new queries. The biggest unanswered question concerns the ability of transfected hCD40 to signal effectively in A20 transfected cells. A lack of signalling originating from transfected hCD40 may explain the effects observed in the animal therapies presented in the chapter 4. However, the *in vivo* therapy data does not provide any clues with regards to this enigma, as the lack of therapeutic effect using LOB7.6 may simply imply the importance of a host professional APC in the priming of CTLs. The overarching aim of the experiments described in this chapter was therefore to establish the ability of transfected hCD40 expressed at the surface of A20 cells to bind and respond to anti-human CD40 mAb and induce the activation of intracellular signal transduction pathways.

The general experimental approach that was adopted in this chapter was to use anti-hCD40 mAb to cross-link hCD40 at the surface of human B cell lymphoma cell lines and A20/hCD40 cell lines. Antibody-mediated cross-linking of CD40 is a widely used method of inducing CD40 signalling in cells and closely mimics the effects of the physiological CD40: CD40 ligand interaction (Challa *et al* 1999, Dullforce *et al* 1998, van Kooten 1999, Gudmundsdottir *et al* 1999, Larsen *et al* 1996). Two different types of physiological responses to CD40 cross-linking were used to compare the signalling capabilities of hCD40 as an endogenous molecule in human B cells and as a transfected molecule in mouse A20 cell lines:

The first functional assay for transfected hCD40 was to measure the down-modulation of cell-surface hCD40 in response to cross-linking. Following cross-linking by either its ligand or Ab, CD40 is rapidly internalised by receptor-mediated endocytosis resulting in a transient diminution of cell surface levels of the receptor, which can be monitored by FACS analysis. This process requires CD40 to be properly inserted and presented in the plasma membrane and as such provides a suitable assay for assessing the ability of

transfected hCD40 to function as a receptor in A20 cells. Hence, it was reasoned that by comparing the LOB7.6-mediated down-modulation of surface hCD40 in human B cell lines with the same response in A20/hCD40 it would be possible to, in part, determine the function of transfected hCD40 as a receptor for the therapeutic Ab.

A much more specific method of assessing the signaling ability of transfected hCD40 is to monitor the induction of target transcription factors that are activated in response to CD40 cross-linking. Transcription factors represent the end-points of signal transduction pathways in cells and in response to the activity of kinases are stimulated to bind to their DNA recognition sequences in the regulatory regions of responsive genes. In the case of CD40 there are a variety of specific transcription factors that are stimulated in response to its activation of intracellular signal transduction pathways (Honget *et al* 2000, Sutherland *et al* 1999, Jeppson *et al* 1998, Karras *et al* 1997, Huo *et al* 1995, Francis *et al* 1995). Nuclear factor kappa B (NFκB) is the best characterised transcription factor target of CD40 signalling and is activated in response to CD40 cross-linking in a wide variety of cell types (Tsukamoto *et al* 1999, Hsing *et al* 1997, Hsing *et al* 1999, Sha 1998). Active NFκB is responsible for mediating the transcriptional activation of many CD40 responsive genes and is therefore considered to be one of the critical regulators of CD40 responses in cells. There are five known mammalian NFκB proteins: Rel (c-Rel), p65 (RelA), RelB, p50 (NFκB1) and p52 (NFκB2). All members share a 300 amino acid Rel homology domain (RHD) located towards the amino terminus. This region contains sequences essential for DNA binding, homo- and hetero-dimerisation, IκB binding and nuclear localisation (Bose *et al* 1992, Henkel *et al* 1992, Rice *et al* 1992). The NFκB proteins can also be divided on the basis of the presence or absence of a transactivation domain. p65, RelB and c-Rel each have a transactivation domain, whereas p50 and p52 do not and are derived from precursor molecules p105 and p100, respectively (Fan *et al* 1991, Mercurio *et al* 1992). In its non-activated state, NFκB is found in the cytoplasm complexed to Inhibitor kappa B (IκB) which inhibits its transcriptional activity (Arenzana-Seiededos *et al* 1996). There are at least five IκB proteins, named IκBα, IκBβ, IκBε, IκBγ and bcl3, although IκBα and IκBγ are the only members of the family that consistently function as

inhibitors of NFκB. IκBα binds to NFκB in the cytoplasm in a such a way that it masks the nuclear localisation signal sequence of NFκB thus preventing its nuclear translocation (Baldwin *et al* 1996, Ghosh *et al* 1998). However, upon stimulation of cells with a wide variety of stimuli (eg. CD40: CD40 ligand binding,

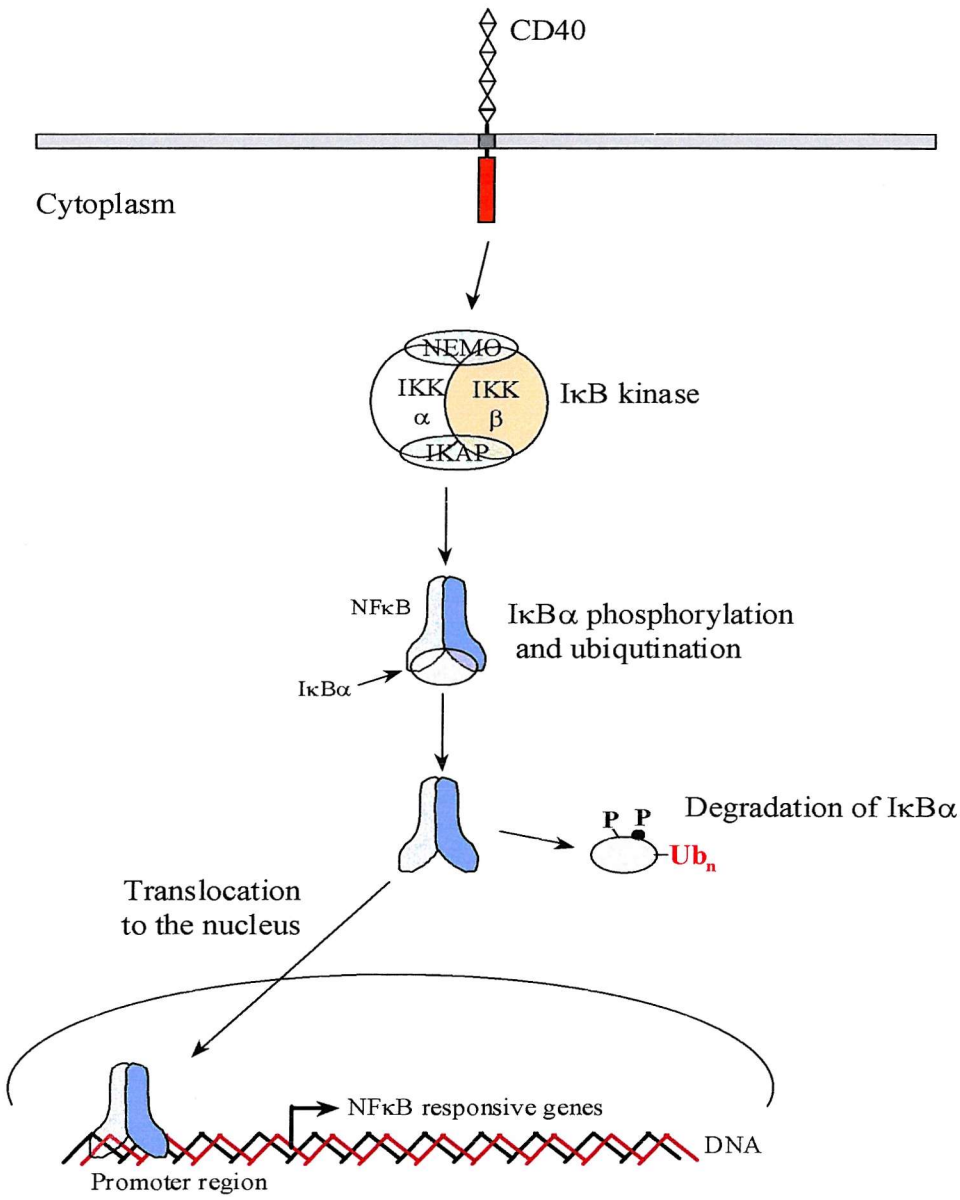


Figure 5.0- Proposed mechanism of NFκB activation See text for details.

cytokines, growth factors, UV, oxidative stress etc) the IKK kinase complex is activated which then catalyses the phosphorylation of IκBα at Ser 32 and Ser 36 residues (Rodriguez *et al* 1996, Roff *et al* 1996). This leads to ubiqitination of IκBα and proteasome-mediated degradation leading to the release of active NFκB. In this activated form, the signal sequence and DNA binding motif on NFκB are revealed enabling it to translocate to the nucleus and affect transcription of genes containing NFκB sites in their promoter regions. One of the promoters regulated by NFκB is IκBα promoter itself (Algarte *et al* 1998, Algarte *et al* 1999). Therefore, when NFκB is activated, it affects transcription of IκBα thus forming a negative feedback loop. Presence of this negative feedback loop prevents continuing activation of NFκB, and limits the extent of responses involving this transcription factor (Singh *et al* 1998).

Based on the information given above and the fact that activation of NFκB is considered to be a pivotal event in CD40 signaling, two experimental protocols were chosen to monitor NFκB activation following mAb-mediated cross-linking of hCD40 in A20/hCD40 cell lines. Firstly, the amount of NFκB in the nucleus can be assayed by electrophoretic mobility shift (EMSA) using a γ P^{32} end labelled oligonucleotide containing a consensus NFκB binding site. Secondly, the increased ability of NFκB to affect the transcription of IκBα associated with increased amount of activated NFκB can be assessed by using IκBα promoter/luciferase reporter. The activity of the promoter is directly linked to the amount of active NFκB present in the nucleus and can be quantified by measuring the activity of the luciferase gene product, which is under the transcriptional control of IκBα promoter in this construct. The activity of the promoter is therefore reflected in the amount of luciferase reporter generated. Hence, if hCD40 is able to induce signal transduction events in transfected A20/hCD40 cell lines, than the EMSA and luciferase assays should detect activation following cross-linking of hCD40 on the surface of A20 by LOB7.6. This strategy would therefore give a clear indication as to the ability of transfected hCD40 to signal in the mouse A20 cell line.

5.2 RESULTS

5.2.1 Modulation of human CD40 receptor from the surface of human B cells and A20/hCD40 transfected cells

Human B cell lymphoma cell lines Daudi, Ramos and Raji were seeded on a six well plate at $5x10^6$ cells/well in the presence of 30µg/ml anti hCD40 mAb LOB7.1. 100µl samples of cells were collected at 0, 0.5, 1.5, 2.5, 4.5, 5.5, 16, 18, 21, 24 and 48 h time points. The cells in each of the samples were labelled with 30µl of anti hCD40 mAb, FITC-LOB7.6 (at a concentration of 100µg/ml). LOB7.1 and LOB7.6 mAbs do not cross-block as they bind different epitopes on hCD40 in such a way that both mAbs can bind to the same receptor at the same time. Mean fluorescence intensity (MFI) was determined for each sample by the use of FACS, and MFI values plotted on a graph versus time in hours. Each of the human B cell lines was assessed separately and at least in triplicate. The results of these experiments are shown in figure 5.1, 5.2 and 5.3. The amount of CD40 at time 0 was taken as 100% surface expression, and subsequent results given as the percentage of receptor remaining on the surface compared to the original MFI. It was possible to compare the data in this manner as the settings on the FACS machine were kept constant, thus allowing direct comparison between various samples.

It can be noted that following addition of LOB7.1 to the media, all of the cell lines internalize CD40 from the surface to a varying degree. The net amount of CD40 remaining on the surface of cells continued to drop over the following 24 hour period, to reach levels of 10%, 8% and 25% in Daudi, Ramos and Raji, respectively. These values were determined by the use of a linear regression curve, which was plotted using data points in the linear range of the graph. The linear regression curve is shown next to each of the data graphs. From 24 hours onwards, the surface expression of CD40 began to recover slowly, this trend was observed in all of the human cell lines. At 48 hours the surface expression of CD40 was found to be 19.45%, 33.5% and 69% in Daudi, Ramos and Raji cell lines, respectively. This result was obtained by determining the amount of CD40 remaining on the surface at 48h time point, rather than by using best fit curve.

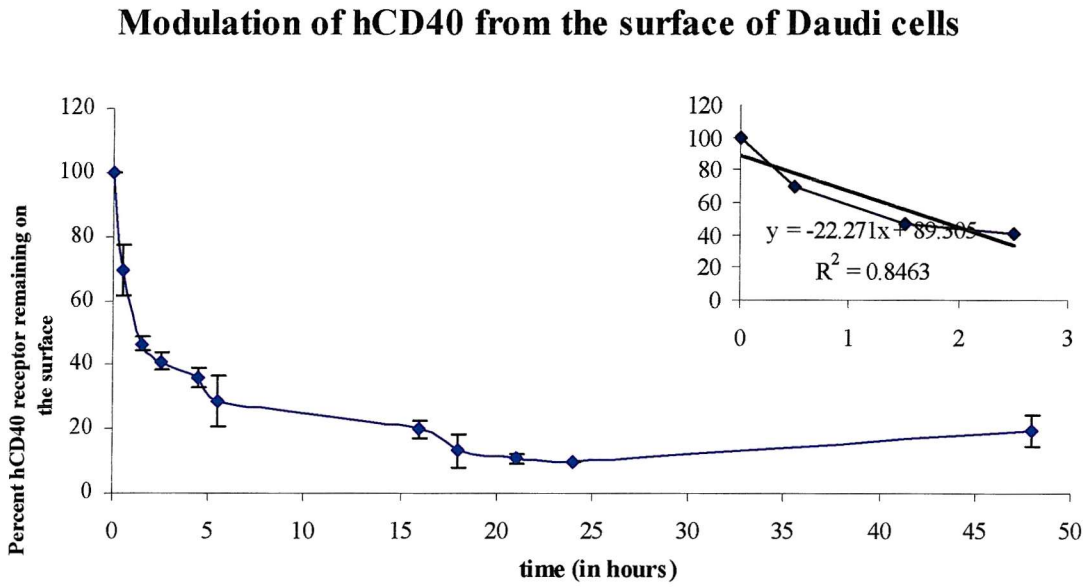


Figure 5.1- Modulation of CD40 receptor from the surface of human B cell lymphoma line Daudi. $5x10^6$ cells were incubated with 30µg/ml LOB7.1 mAb at time 0. 100µl cell samples were taken out at time points as shown on the graph above and incubated with 30µl of 100µg/ml FITC-LOB7.6. The MFI was determined for each sample and plotted on the graph against the time that sample was collected. Within the graph is a linear regression line plotted against the time points to include 50% of the receptor remaining on the surface. Using the equation for the trendline, the time point where 50% CD40 remains on the surface is calculated, and shown in figure 5.8.

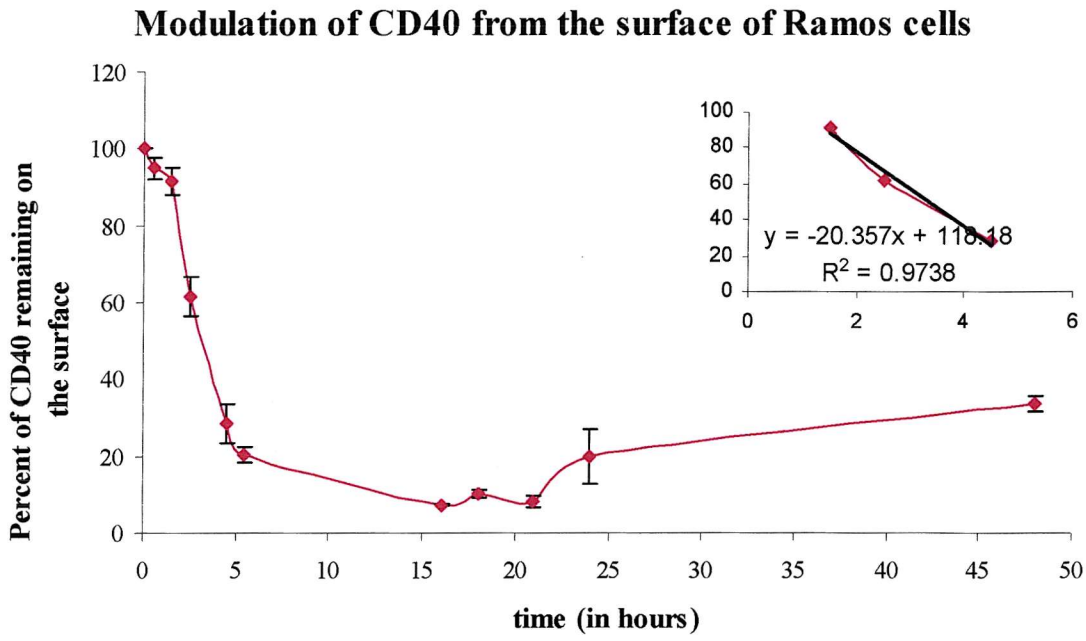


Figure 5.2- Modulation of CD40 receptor from the surface of human B cell lymphoma line Ramos. As for Daudi, $5x10^6$ Ramos cells were incubated with 30µg/ml LOB7.1 mAb at time 0. 100µl cell samples were taken out at time points as shown on the graph above and incubated with 30µl of 100µg/ml FITC-LOB7.6. The samples were analysed on FACS. The MFI was determined for each sample and plotted on the graph against the time that sample was collected. Within the graph is a linear regression line plotted against the time points to include 50% of the receptor remaining on the surface. Using the equation for the trendline, the time point where 50% CD40 remains on the surface is calculated, and shown in figure 5.8.

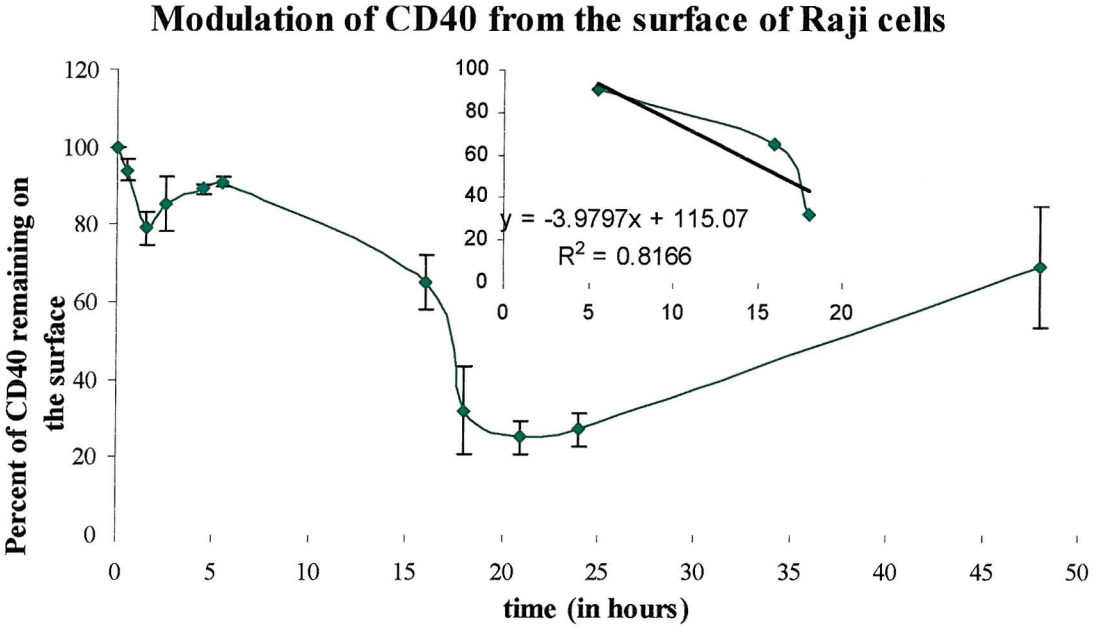


Figure 5.3- Modulation of CD40 receptor from the surface of human B cell lymphoma line Raji. As for Daudi and Ramos, $5x10^6$ Raji cells were incubated with 30μg/ml LOB7.1 mAb at time 0. 100μl cell samples were taken out at time points as shown on the graph above and incubated with 30μl of 100μg/ml FITC-LOB7.6. The samples were analysed on FACS. The MFI was determined for each sample and plotted on the graph against the time that sample was collected. Within the graph is a linear regression line plotted against the time points to include 50% of the receptor remaining on the surface. Using the equation for the trendline, the time point where 50% CD40 remains on the surface is calculated, and shown in figure 5.8.

The experiments described above were repeated using the stable transfected A20/hCD40 cell lines. Briefly, A20/hCD40wt(6), A20/hCD40T254S(3), A20/hCD40KKV(4) and A20/hCD40T254A(4) were seeded on a six well plate at $5x10^6$ cells/well in the presence of 30μg/ml anti hCD40 mAb LOB7.1. 100μl samples of cells were collected at 0, 0.5, 1.5, 2.5, 4.5, 5.5, 16, 18, 21, 24 and 48 h time points. The cells in each of the samples were labelled with 30μl of anti hCD40 mAb, FITC-LOB7.6 (at a concentration of 100μg/ml). Mean fluorescence intensity (MFI) was determined for each sample by the use of FACS, and MFI values plotted on a graph versus time in hours. Each of the transfected cell lines was assessed separately in this manner and at least in triplicate. The results of these experiments are shown in figures 5.4 to 5.7. The amount of CD40 at time 0 was taken as 100% surface expression, and subsequent results given as the percent of receptor remaining on the surface compared to the original MFI.

All of the experiments described using LOB7.1 and LOB7.6 FITC were also carried out using non-labeled LOB7.6 and FITC labelled LOB7.1, but these data did not show differences which were as notable, presumably due to lower affinity of FITC LOB7.1 to hCD40 receptor. This is assumed due to the lower MFI obtained from the studies using LOB7.1 FITC mAb.

The results obtained from these studies show different internalisation and modulation pattern of transfected hCD40 in A20 to the endogenous hCD40 found on human B cells. Following the addition of LOB7.1 to the A20/hCD40 cells there was a fast and relatively large response in all the cell lines tested. The reduction in surface expression of hCD40wt, hCD40KKV, hCD40T254A and hCD40T254S reached 70% of the original levels (found on the surface at time 0) by ~2 hours. Following this initial drop, the amount of hCD40 steadily increased from 2 hours to 5 hours post LOB7.1 addition, which was followed by another decrease in surface expression starting at ~5 hours, and levelling off by 16 hours. The minimum expression level found in A20 expressing hCD40 constructs was found to be ranging from 59% to 69%, as outlined in figure 5.8.

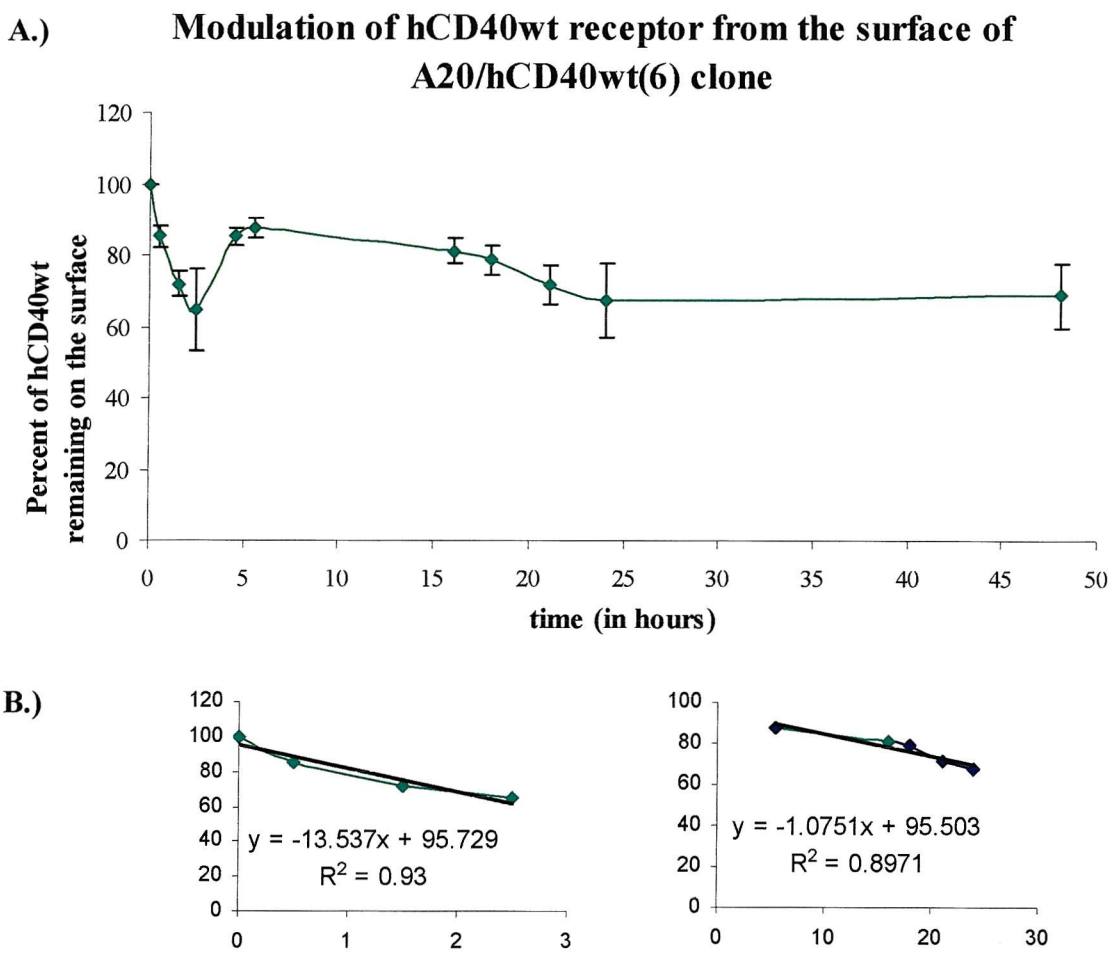


Figure 5.4 A.) and B.) Modulation of hCD40wt receptor from the surface of A20/hCD40wt(6) cell line A.)The A20/hCD40wt(6) cells were incubated with 30μg/ml LOB7.1 mAb at time 0. 100μl cell samples were taken out at time points as shown on the graphs above and incubated with 30μl of 100μg/ml FITC-LOB7.6. The MFI was determined for each sample and plotted on the graph versus the time that sample was collected. Each B.) Below the main graph are linear regression curves showing the initial trend of modulation, and the one established 5 hours following the addition of LOB7.1 mAb. The trendlines include the time where there is 70% of the hCD40wt receptor remaining on the surface. Using the equation for the trendline, the time point when 70% hCD40wt remains on the surface was calculated and shown in fig 5.8.

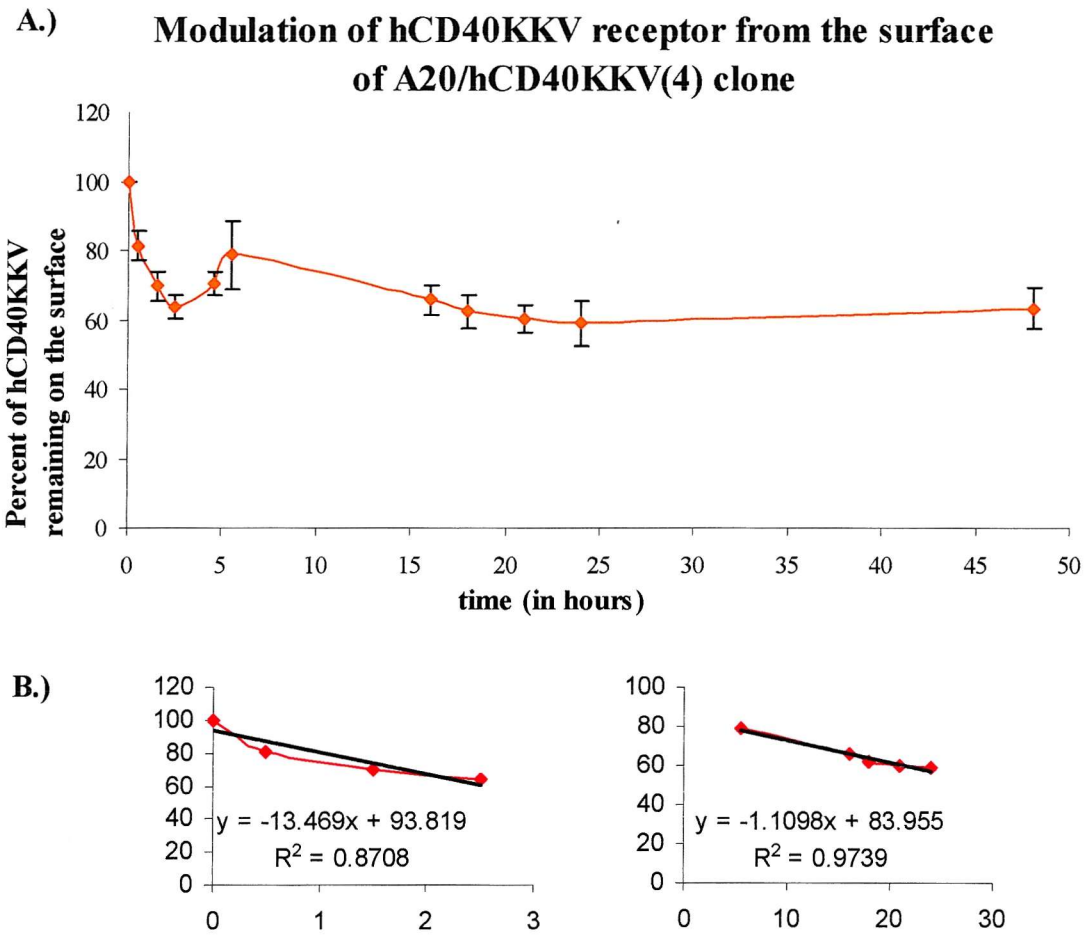


Figure 5.5 A.) and B.) Modulation of hCD40KKV receptor from the surface of A20/hCD40KKV(4) cell line A.) The A20/hCD40KKV(4) cells were incubated with 30μg/ml LOB7.1 mAb at time 0. 100μl cell samples were taken out at time points as shown on the graphs above and incubated with 30μl of 100μg/ml FITC-LOB7.6. The MFI was determined for each sample and plotted on the graph versus the time that sample was collected. B.) Below the main graph are linear regression curves showing the initial trend of modulation, and the one established 5 hours following the addition of LOB7.1 mAb. The trendlines include the time where there is 70% of the hCD40KKV receptor remaining on the surface. Using the equation for the trendline, the time point when 70% hCD40KKV remains on the surface was calculated and shown in fig 5.8.

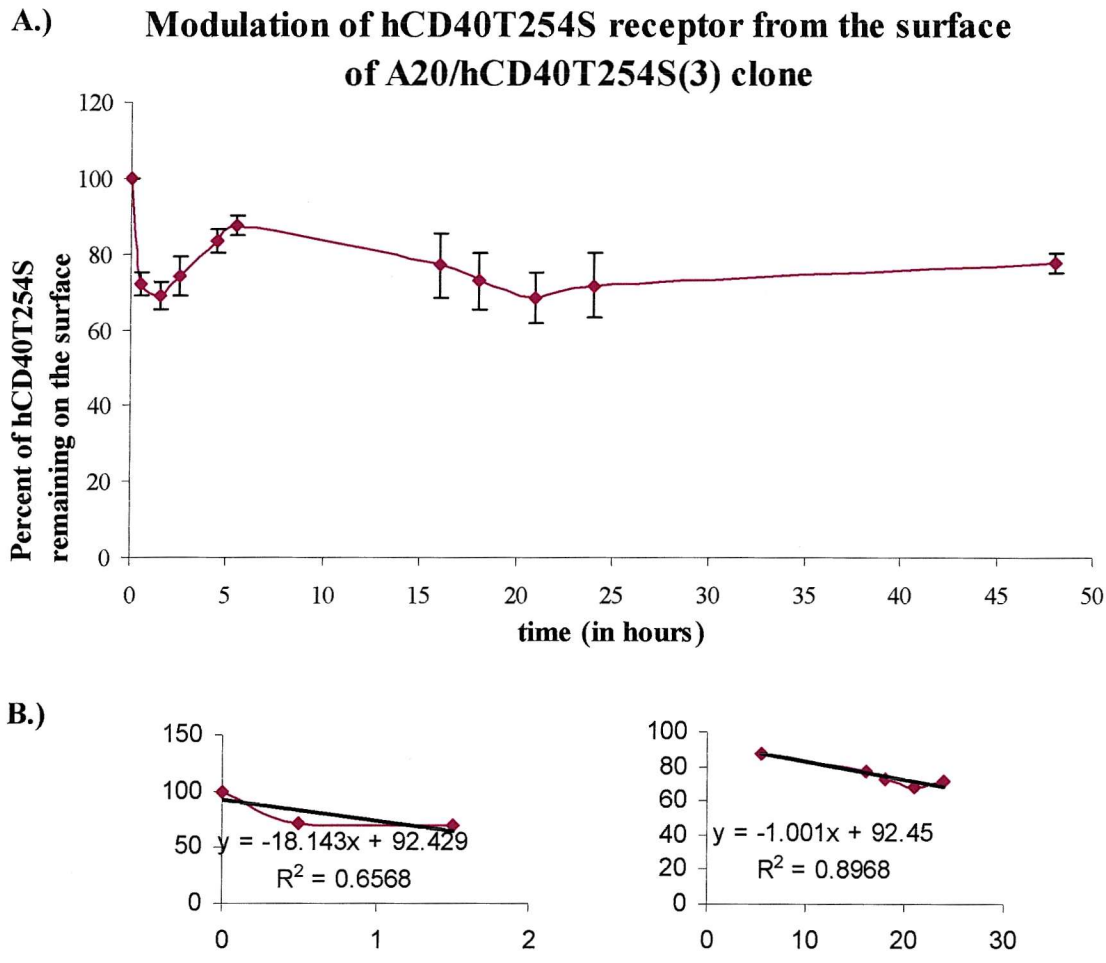
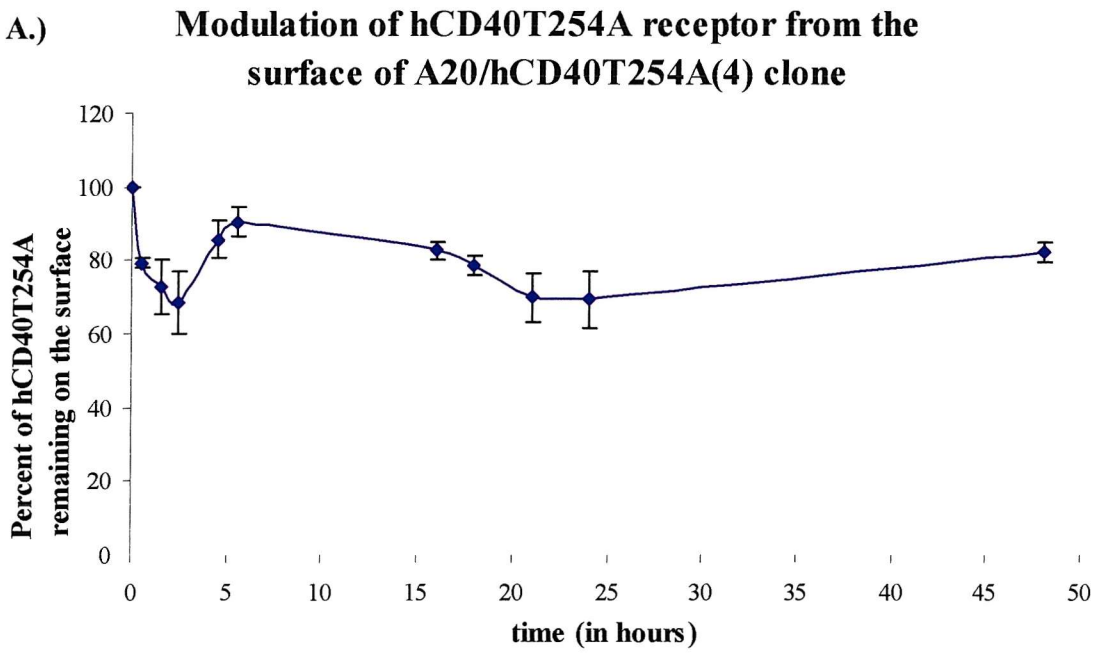


Figure 5.6 A.) and B.) Modulation of hCD40T254S receptor from the surface of A20/hCD40T254S(3) cell line A.) The A20/hCD40T254S(3) cells were incubated with 30μg/ml LOB7.1 mAb at time 0. 100μl cell samples were taken out at time points as shown on the graphs above and incubated with 30μl of 100μg/ml FITC-LOB7.6. The MFI was determined for each sample and plotted on the graph versus the time that sample was collected. B.) Below the main graph are linear regression curves showing the initial trend of modulation, and the one established 5 hours following the addition of LOB7.1 mAb. The trendlines include the time where there is 70% of the hCD40T254S receptor remaining on the surface. Using the equation for the trendline, the time point when 70% hCD40T254S remains on the surface was calculated and shown in fig 5.8.

A.)



B.)

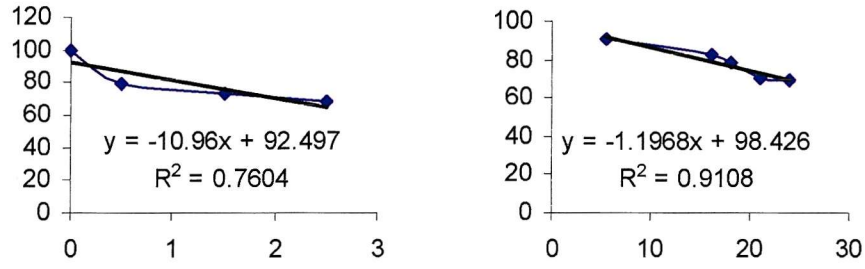


Figure 5.7 A.) and B.) Modulation of hCD40T254A receptor from the surface of A20/hCD40T254A(4) cell line A.) The A20/hCD40T254A(4) cells were incubated with 30μg/ml LOB7.1 mAb at time 0. 100μl cell samples were taken out at time points as shown on the graphs above and incubated with 30μl of 100μg/ml FITC-LOB7.6. The MFI was determined for each sample and plotted on the graph versus the time that sample was collected. B.) Below the main graph are linear regression curves showing the initial trend of modulation, and the one established 5 hours following the addition of LOB7.1 mAb. The trendlines include the time where there is 70% of the hCD40T254A receptor remaining on the surface. Using the equation for the trendline, the time point when 70% hCD40T254A remains on the surface was calculated and shown in fig 5.8.

	Percent of hCD40 remaining on the surface of the cell	Time after LOB7.1 mAb added	Average minimum hCD40 remaining on the surface
Daudi	50%	1 h 20 min	
	70%	28.6 min	10%
Ramos	50%	3 h 20 min	
	70%	2 h 22 min	8%
Raji	50%	16 h 21 min	
	70%	11 h 19 min	25%
A20/hCD40wt(6)	70%	1h 54 min	
	70%	23 h 43 min	64.5%
A20/hCD40KKV(4)	70%	1 h 46 min	
	70%	12 h 34 min	59%
A20/hCD40T254A(4)	70%	2 h 3 min	
	70%	22 h 25 min	69%
A20/hCD40T254S(3)	70%	1 h 14 min	
	70%	23 h 45 min	68.5%

Figure 5.8 Tabulated graph data showing the level of endogenous and transfected hCD40 remaining on the surface of tested cell lines Using the linear regression curves (contained in the graphs in figs 5.1-5.3 and in section B of figs 5.4-5.7) the amount of hCD40 remaining on the surface at various time points was calculated. These numbers are shown in the table above. For human B cell lines the time when 50% and 70% of the receptor remaining on the surface were worked out, and for A20hCD40 lines only the time point where 70% hCD40 receptor remaining was calculated. This is because the receptor expression in these cell lines does not fall below ~70%. Also shown is the minimum percentage of hCD40 remaining on the surface at any time during the period tested. This demonstrates that hCD40 in human B cells can be almost completely modulated from the surface, whereas in A20/hCD40 lines the lowest expression found is 59%.

The results of modulation pattern of endogenous CD40 in human B cells and transfected hCD40 in A20 cells were found to be very different. It was hypothesised that there may be a number of reasons for this:

a.) Human CD40 has a co-stimulatory molecule only present in human cells which is necessary for efficient internalization and modulation, or

b.) The transfection process itself alters the pattern of behavior of the receptor, or

c.) The viral promoter in pcDNA3 expression vector is driving high level expression of the transfected hCD40 receptor, and so although it is being internalized rapidly, the presence of a large pool of cytoplasmic receptors leads to a rapid replenishment of receptors on the surface.

To test these ideas, human CD40/pcDNA3 expression vector was stably transfected into human B cell lymphoma line Daudi, which already has CD40 on the surface. The positive clones were selected on the basis of increased surface expression of hCD40, and acquired resistance to neomycin. If there is requirement for a secondary molecule to interact with CD40 in order for it to be internalised, then such a molecule would be present in Daudi cells. Figure 5.9 shows the results of experiments performed on Daudi cells stably transfected with hCD40. Transfected Daudi cells were found to have altered internalization and modulation pattern of CD40. In figure 5.11 the results of transfected hCD40 in A20 and Daudi modulation experiments were compared. It can be noted that all of the graphs appear very similar, including the data obtained with transfected Daudi.

At this point it was thought that transfected Daudi cells may be in some way compromised by the transfection process. In order to assess this possibility, human CD22 present on Daudi cells was used as an internal control for both transfected and non-transfected cells. Cell surface levels of CD22 are down-modulated by internalisation of the receptor following cross-linking with anti-CD22 (Chan *et al* 1998). To assess the down-modulation of CD22 on Daudi cells receptor internalisation was induced by cross-linking with the anti hCD22 mAb, HD6. For detection of remaining hCD22 on cells, FACS analysis was performed using a second anti CD22 mAb, FITC labeled 4KB128. Figure 5.10 illustrates down-regulation of CD22 from the surface of Daudi cells. At 5h post incubation with HD6

there was ~60% of the receptor remaining on the surface of cells, which was reduced to ~15% at 24h. Similar results are seen in CD22 internalization in Daudi cells transfected with human CD40, which suggests that the transfection process does not alter general mechanisms of receptor modulation and internalization of the cell.

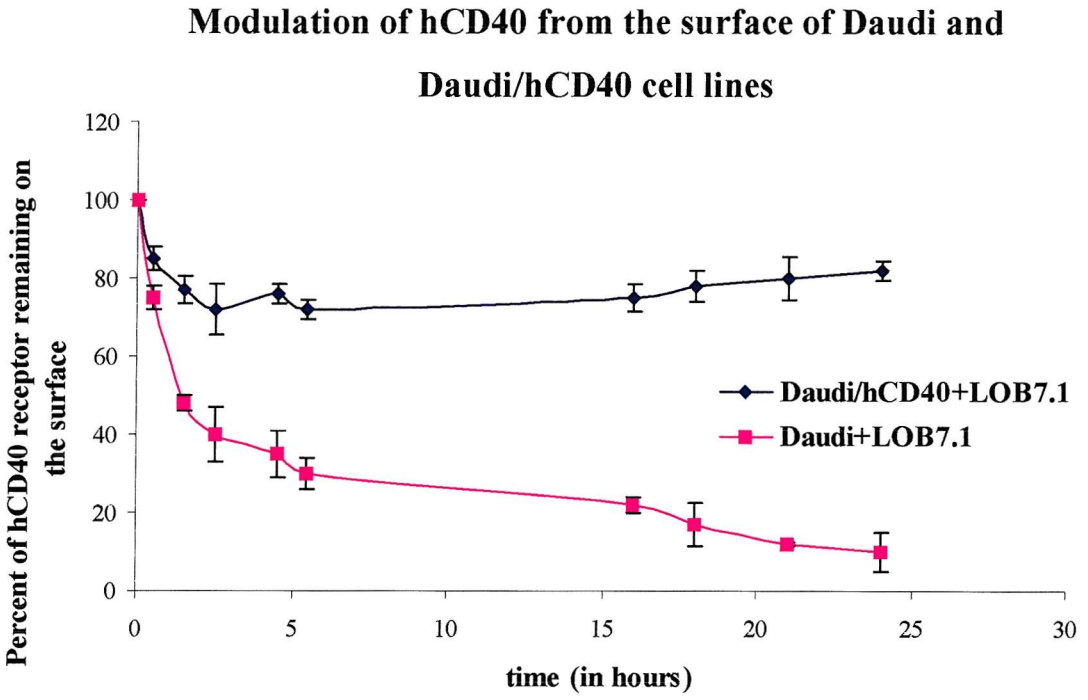


Figure 5.9 Modulation of transfected hCD40 in Daudi cells and Daudi/hCD40 transfected cells. Modulation of hCD40 was compared in transfected and wild type Daudi cells using methods already described in figs 5.1-5.3. Briefly, $5x10^6$ Daudi or Daudi/hCD40 cells were incubated with 30μg/ml LOB7.1 mAb at time 0. 100μl cell samples were taken out at time points as shown on the graph above and incubated with 30μl of 100μg/ml FITC-LOB7.6. The samples were analysed on FACS. The MFI was determined for each sample and plotted on the graph against the time that sample was collected. The transfected hCD40 on Daudi/hCD40 no longer modulates in the manner observed with the endogenous hCD40 on parental cell line.

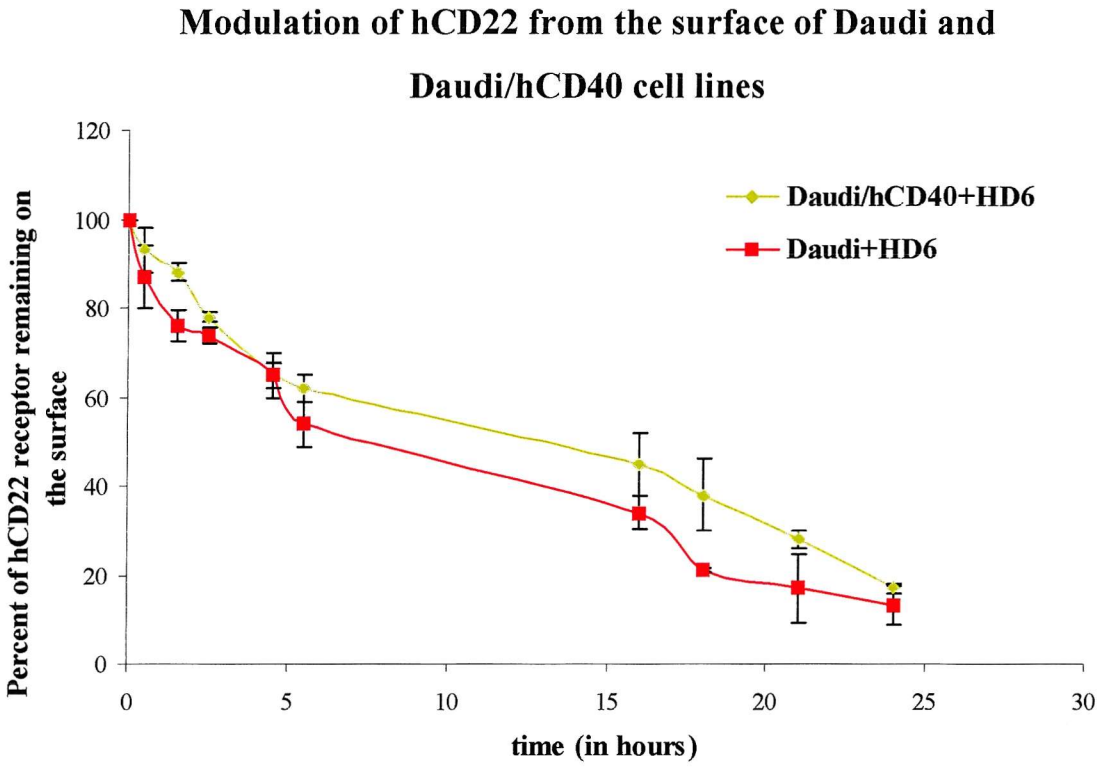


Figure 5.10 Modulation of endogenous hCD22 in Daudi cells and Daudi/hCD40 transfected cells. Modulation of hCD22 was compared in transfected and wild type Daudi cells as an internal control for both cell lines. $5x10^6$ Daudi or Daudi/hCD40 cells were incubated with 30μg/ml anti hCD22 mAb HD6 at time 0. 100μl cell samples were taken out at time points as shown on the graph above and incubated with 30μl of 100μg/ml FITC-4KB128, a non-blocking anti hCD22 mAb. These mAbs recognise different epitopes on hCD22, and can both bind at the same to the same receptor (ie they are non-blocking). The cell samples labelled in this way were analysed on FACS. The MFI was determined for each sample and plotted on the graph against the time that sample was collected. Each experiment was done three times and in triplicate. It can be observed that hCD22 modulates in a similar in both parental Daudi cell line, and the transfected Daudi/hCD40 line.

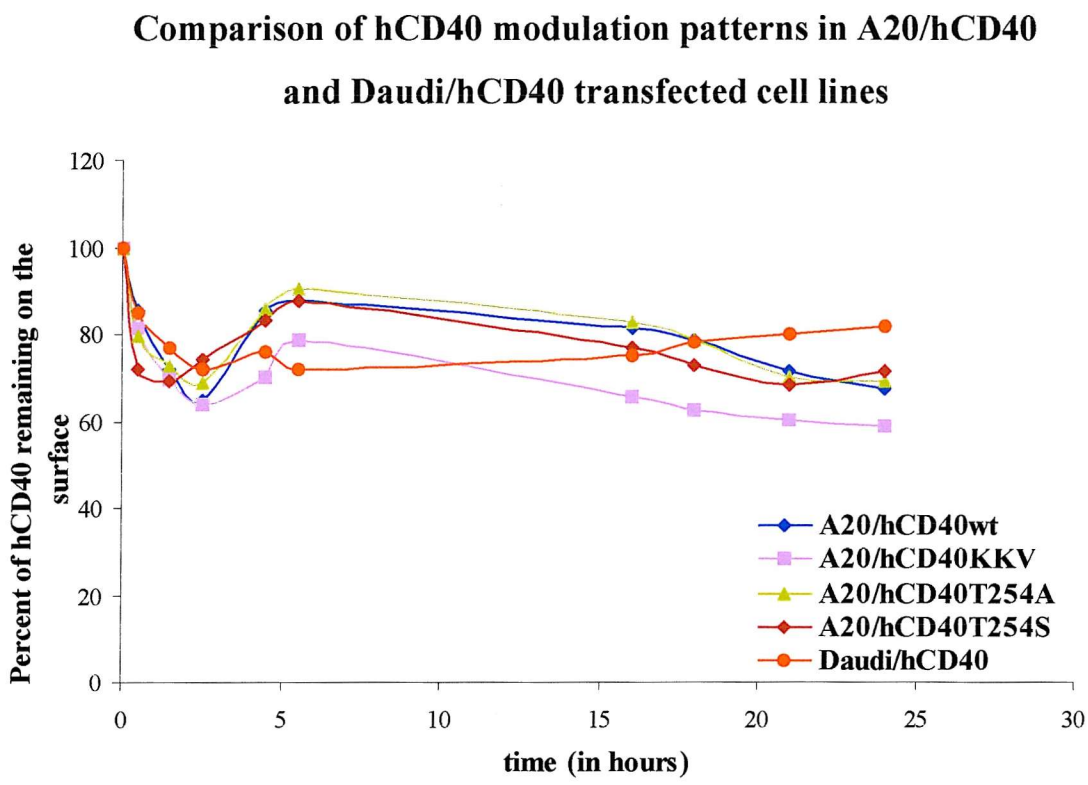


Figure 5.11 Comparison of hCD40 modulation patterns in A20/hCD40 and Daudi/hCD40 transfected cell lines. Graphs previously shown in figures 5.3, 5.4, 5.6, 5.7 and 5.9 are collectively shown on the same graph to allow comparison. It can be seen that all of the transfected hCD40 receptors display similar pattern of modulation in both A20/hCD40 and Daudi/hCD40 cell lines.

5.2.2 The use of electrophoretic mobility shift assay (EMSA) to show effects of hCD40 signalling in A20/hCD40 cells on activation of NFκB

A20 and A20/hCD40wt(6) cells were assayed for the amount of active NFκB in their nuclei in the presence or absence of stimulation mediated by cross-linking of endogenous mouse CD40 on both cell lines and human CD40 on A20/hCD40wt(6) cells. CD40 cross-linking was achieved by incubating A20 and A20/hCD40wt(6) with anti mCD40 mAb 3/23, and the latter also with anti hCD40 mAb LOB7.6. Both mAbs were added to the culture of cells in a soluble form at 30μg/ml final concentration. 24 hours later the nuclei were extracted from cells and total soluble nuclear protein was harvested, as per 2.2.27. 2μg of nuclear extract was incubated with γ-P^{32} end labelled NFκB oligonucleotide, and resultant protein: DNA complexes were separated by electrophoresis through a native polyacrylamide gel. The results of the EMSA analysis are shown in figure 5.12A and B and are highly representative of 3 independent experiments. The gel in fig.5.12A shows NFκB/probe complexes in nuclear extracts from A20, A20+3/23 mAb, A20/hCD40wt(6), A20/hCD40wt(6) + 3/23 mAb and A20/hCD40wt(6) + LOB7.6 mAb. There are two bands in all lanes which are of similar intensity in all samples tested. There is a high constitutive level of activated NFκB in all samples which is non-inducible by either the 3/23 or LOB7.6 mAbs. The specificity of the bands was tested by incubating the nuclear extracts with 100 fold excess of unlabelled NFκB oligonucleotide prior to addition of the labelled NFκB probe (fig.5.12B). This result shows that the complexes found in all cells are specific as they are competed out by the excess unlabelled NFκB oligonucleotide.

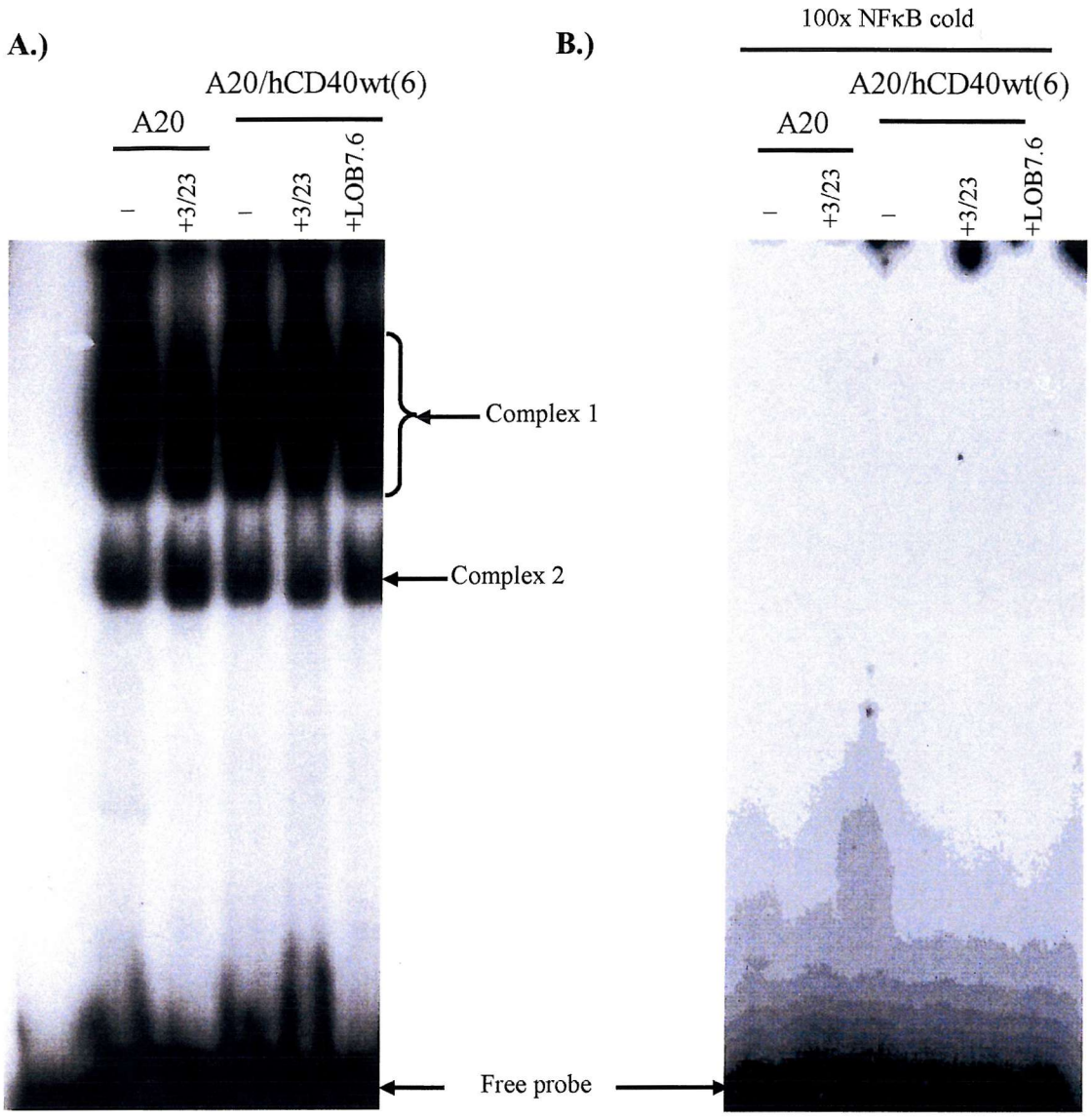


Figure 5.12- Demonstration of constitutively expressed NFκB complexes in A20 and A20/hCD40wt(6) cell line and the effect of 3/23 and LOB7.6 mAb. End labelled NFκB oligonucleotide was incubated with 5μg of nuclear extracts from A20 or A20/hCD40wt(6) cells grown with or without 30μg/ml 3/23 or LOB7.6 mAb for 24 hours, either with no competitor (fig 5.12A), or in the presence of non-labelled competitor oligonucleotide at a 100 molar excess (fig 5.12B). The complexes were separated by electrophoresis on an 8% native polyacrylamide gel. Specific DNA-protein complexes are indicated by arrows. It can be observed that although all of the protein complexes bind specifically to the NFκB oligonucleotide (fig 5.12B), no further induction of NFκB protein/DNA binding activity is achieved through the mAb signalling.

Following the studies described in fig 5.12A and B, the next set of experiments were performed in order to find out which NFκB subunits form the complexes seen in fig 5.12, and also to find out if serum deprivation might reduce the amount of NFκB in the nucleus of the tested cells. To detect the effect of serum deprivation on A20 and A20/hCD40wt(6), the cells were cultured in serum deprived media for 16 hours. Figure 5.13A demonstrates the effect of this treatment on A20 and A20/hCD40wt(6) cells. Nuclear extracts obtained from serum starved cells have two NFκB complexes, in their appearance and intensity similar to those found in cells cultured in 10% foetal calf serum supplemented media (as compared with fig 5.12A). There is also very little difference between the samples incubated with 3/23 or LOB7.6 mAb. All of the samples demonstrate very high levels of active NFκB present in the nucleus, with no effect of mAb treatment or serum deprivation on their quantity, as far as can be determined using the EMSA assay.

The next experiment carried out was to delineate the subunits forming NFκB complexes seen on the gels in figs 5.12A and 5.13A. Nuclear extracts from A20 cells were set up in five identical reactions and incubated with labelled NFκB probe. One of the samples was further incubated with anti p50 NFκB subunit Ab, the second sample had anti p65 subunit Ab added, the third had both anti p50 anti p65 Abs added, the fourth had anti c-jun Ab added as a specificity control, and the final sample had no Ab. The samples were incubated with Abs for 16 hours, and the complexes were then loaded on an 8% native polyacrylamide gel and separated by electrophoresis at 20mA for 2 hours. The results of this experiment are shown in fig 5.13B. This figure indicates that NFκB complexes in A20 cells are made up of both p50 and p65 subunits, which is a classical NFκB heterodimeric complex. Visible supershift was produced by incubation of nuclear extracts with anti p50 Ab, whereas anti p65 Ab reduced the intensity of complex 1 on the gel, rather than producing a retarded band on the gel. Abs raised against both p50 and p65 subunits completely cleared complex 1, but did not reduce complex 2. Anti c-jun Ab had no effect.

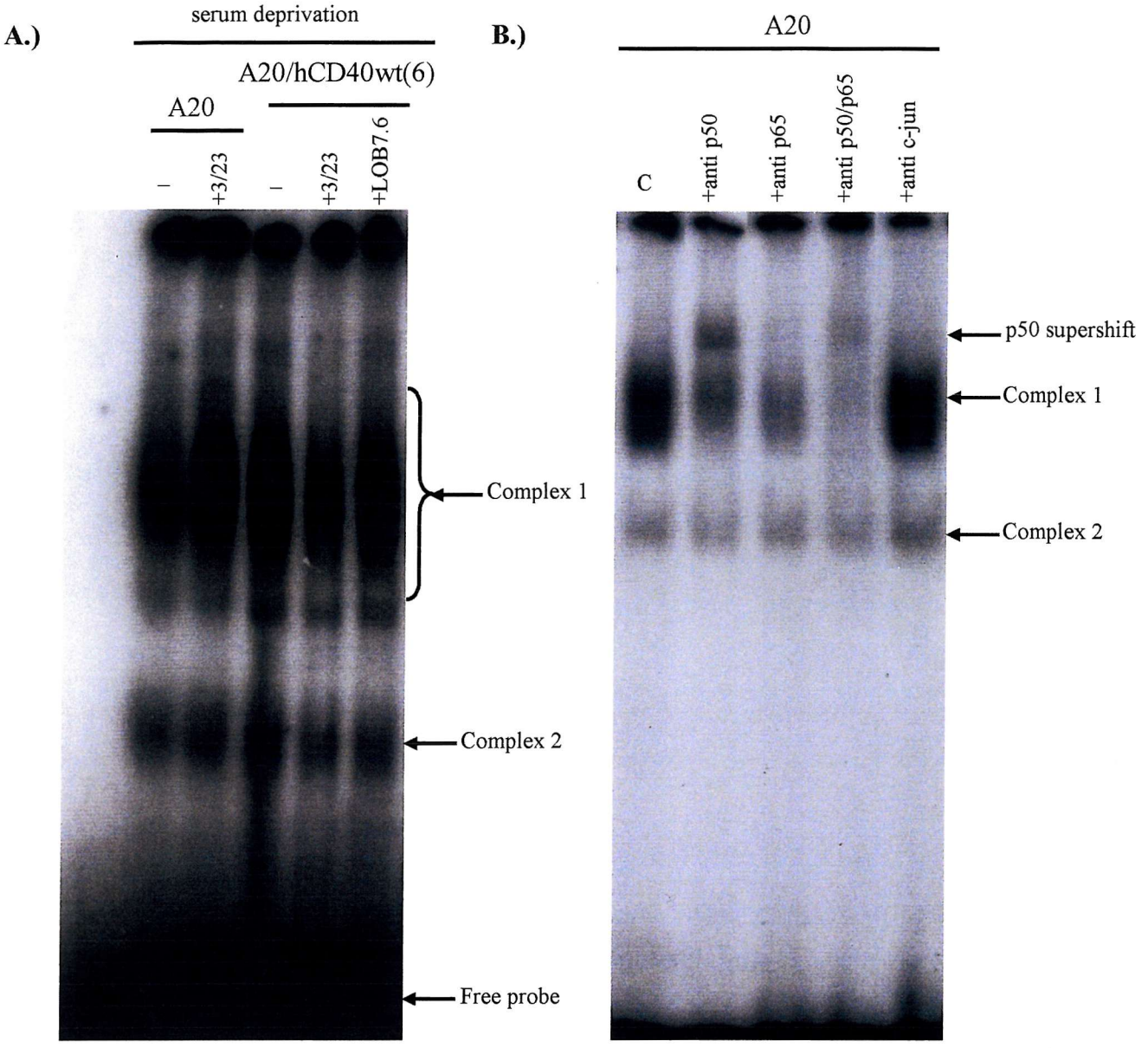


Figure 5.13- Identification of constitutively expressed NFκB complexes in A20 cell line. End labelled NFκB oligonucleotide was incubated with 5μg of A20 nuclear extracts. 2μg od antibody raised against p50 and/or p65 NFκB subunits, or c-jun were added to the complexes as indicated at the top of the figure. The samples were incubated with Ab overnight at 4^0C. The complexes were separated by electrophoresis on an 8% native polyacrylamide gel. Specific DNA-protein complexes are indicated by arrows, as is p50 supershift. Samples incubated with anti p65 Ab have decreased amount of NFκB binding activity in the samples, indicating that p65 is part of the NFκB complex 1 in A20 cells. Incubation of samples with anti c-jun Ab had no effect.

5.2.3 The use of IκBα promoter/luciferase reporter to show effects of hCD40 signalling in A20/hCD40 cells on activation of NFκB

Transcriptional activity of NFκB in cells was determined by quantifying changes in the expression of firefly luciferase that was provided by a transfected plasmid DNA construct in which transcription of the firefly luciferase gene is under the control of the NFκB-responsive human IκBα gene promoter. A pGL3 Basic vector carrying the IκBα promoter was transiently co-transfected with a control renilla luciferase vector, pRLTK, into A20 and A20/hCD40wt(6) cells at time 0, as outlined in 2.2.25. At 24 hours, the cells were incubated with 30μg/ml of 3/23 or LOB7.6 mAb, or were left untreated for further 24 hours, when the cells were harvested. The luciferase assay was performed upon the cell lysates and the data were plotted as the ratio of firefly/renilla luciferase activity.

Figure 5.14 shows the activity of the IκBα promoter in A20 and A20/hCD40wt(6) cells +/- 3/23 or LOB7.6 mAb as determined by the amount of luciferase gene product expressed. The level of IκBα promoter activity found in the cells was high in all samples tested. Treatment of cells with 3/23 or LOB7.6 did not appear to have any significant effect on the level of IκBα promoter activity suggesting that NFκB is in a constitutively active state in the B cell lines. The data also confirm the findings of the EMSA studies and in combination indicate that the constitutively high levels of active NFκB cannot be further elevated in response to cross-linking of either the endogenous mCD40 or transfected hCD40. Since 3/23 failed to induce promoter activity or DNA binding it can be concluded that there may be an inherent problem regarding CD40 signaling in these cells that brings into doubt the ability to further analyse intracellular signal transduction events with A20/hCD40 cell lines.

IκBα promoter/luciferase reporter activity in A20 and A20/hCD40(6) cell lines incubated with anti mCD40 and anti hCD40 mAbs

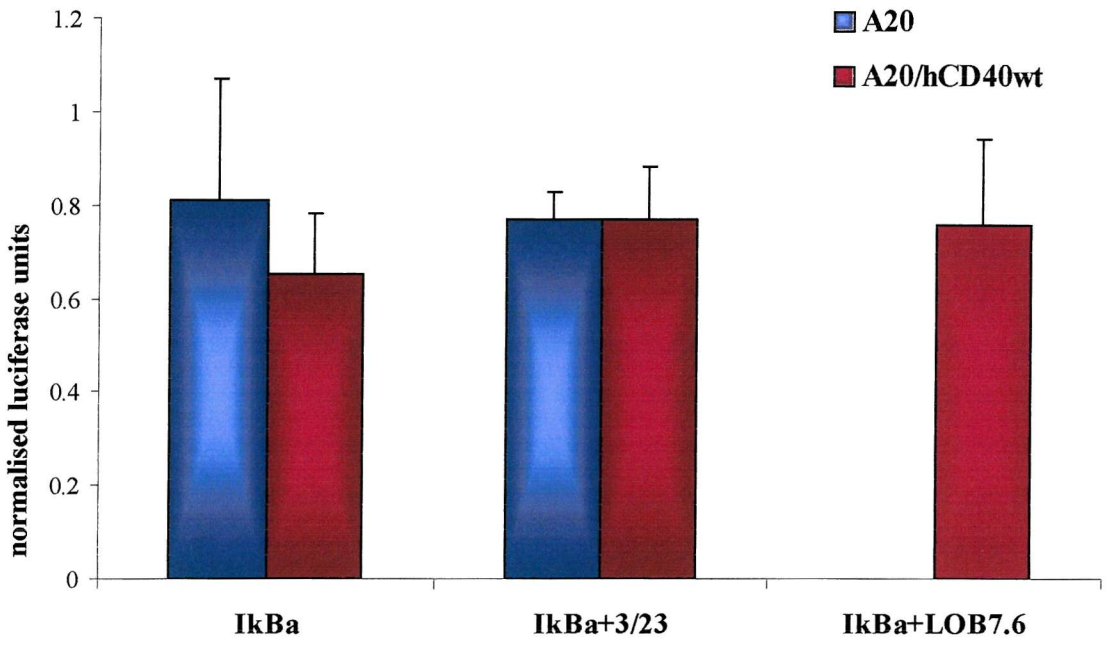


Figure 5.14- IκBα promoter activity in A20 and A20/hCD40wt(6) cells incubated with 3/23 or LOB7.6 mAb. A20 and A20/hCD40wt(6) cells were transiently transfected by electroporation with 30μg IκBα /pGL3 Basic vector encoding a firefly luciferase driven by IκBα promoter. Renilla luciferase driven by a thymidine kinase promoter was co-transfected (30ng per transfection) and used as the transfection efficiency control. 3/23 or LOB7.6 mAb was added 24 hours after transfection at 30μg/ml final concentration. Cells were harvested at 48 hours, and cell lysates assayed for firefly and renilla luciferase activity. The results were expressed as the ratio of firefly/renilla luciferase activity and values above the negative control (promoterless pGL3 Basic) were plotted on the graph. All samples were done three separate times and in triplicate. The activity of IκBα promoter appears to be high irrespective of the presence or absence of anti CD40 mAbs. This indicates constitutively high NFκB activity in the cells tested at the time of these experiments.

5.2.4 Assessment of the presence of expressed viral genes in A20 as a possible cause of problems with signalling in these cells

The data concerning induction of NFκB DNA binding and trans-activation properties suggest that there may be inherent problems associated with the use of A20 cells for NFκB signal transduction studies. Previous studies in other laboratories have shown that mouse B cell lines can be used for the study of NFκB activation in response to CD40 stimulation (Jalukar *et al* 2000, Baccam *et al* 1999, Hsing *et al* 1999, Lin *et al* 1998, Lin *et al* 1996, Schamer *et al* 1996). It is therefore likely that the A20 cell lines used in the present studies are in some way defective or compromised in their ability to generate a NFκB response to CD40 ligation. One possibility that was considered worth exploring was that A20 cells may be productively infected with a virus that would perturb the normal signalling properties of the cell. Two of the viruses known to frequently infect B cells are murine leukaemia virus (MLV) and murine mammary tumour virus (MMTV). To assess for their presence in A20 cells, primers specific for envelope proteins for both of the viruses were designed (see table 2.7). mRNA was isolated from $1x10^7$ A20, FSDC, Bcl_1, πBcl_1, Bcl_1 variant, Jurkat and Daudi cell lines on the dates indicated in the legend of figure 5.15. mRNA isolation was achieved using "QuickPrep Micro mRNA purification kit" following the manufacturers instructions (see section 2.2.1). Bcl_1, πBcl_1 and Bcl_1 variant cell lines were used in order to compare A20 lymphoma to other B cell lymphoma lines available in the laboratory. FSDC cells were to be used in the future studies, so experiments shown in figures 5.15 were included to exclude the possible presence of viruses prior to undertaking further studies with this cell line.

10μg of each of the mRNA samples was used to obtain first strand cDNA. This was carried out by the use of "First strand cDNA synthesis kit" as outlined in section 2.2.2. The concentration of cDNA obtained from murine cell lines (all cell lines except Jurkat and Daudi) was assessed and adjusted to the same concentration in all samples. RT-PCR was performed using cDNAs as template for reactions in which the *env* gene products from MLV and MMTV were amplified over 40 cycles at annealing temperatures of 62^0C and 57^0C, respectively, following the PCR protocol outlined in 2.2.4 and 2.2.5. Briefly, 0.3μg

cDNA, 100ng of each MLV primer F (sense) and R (antisense), 1µl dNTPs (10mM), 2 units of Taq polymerase, 2.5µl Taq buffer (with $MgCl_2$) and dH_2O up to 25µl were placed in a PCR tube. The same procedure was followed for MMTV, except using MMTV primers F and R. The PCR program was set up so to allow for a 5 minutes denaturation step at 95^0C, followed by 40 cycles of 95^0C for 45 seconds, 62^0C (MLV) or 57^0C (MMTV) for 1 minute and 72^0C for 2 minutes. After this, the final elongation step at 72^0C for 10 minutes was carried out, followed by cooling of samples down to 4^0C. The MLV and MMTV viruses only infect murine cell lines, thus using cDNA from human cells provides a control for primer specificity. RT-PCR reactions using cDNA obtained from the human cell lines Jurkat and Daudi are shown in lanes 11 and 12 in 5.15A and B. Control RT-PCR reactions in which primers without template cDNA were used are shown in lane 13 in 5.15A and B. Absence of amplified transcripts in lanes 11-13 indicates specificity of the primers for viral sequences and absence of inter-primer dimerisation. Controls to ensure that all RT-PCR samples using murine cDNA were set up using same amounts of cDNA were carried out by amplifying β actin message in all the samples. The results of the β actin RT-PCR reactions are shown in 5.15C. The amount of amplified PCR product appears to be the same in all lanes, indicating that the amount of cDNA loaded into each PCR reaction was equal. Human cDNA samples were not included in this experiment as their purpose was solely to determine the specificity of primers.

The data presented in 5.15A and B shows the presence of *env* transcripts from both MLV and MMTV in A20, Bcl_1, Bcl_1 variant and πBcl_1 cDNA samples. The transcripts encoding MMTV *env* gene were amplified from A20 cDNA isolated from the cells on 30.4.1999, 15.5.99 and 18.6.99 in lanes 2,3 and 4 respectively as shown in the figure 5.15 legend. The amount of PCR product in all three lanes is similar, suggesting uniform expression of this gene in the cells over the period of 45 days in which the cells were sampled. Transcripts of MLV *env* genes from the same cDNA samples used for analysis of MMTV *env* expression are shown in lanes 2,3 and 4 in figure 5.15B. For two of the cDNA samples (15.5.99 and 18.6.99) the level of *env* gene expression was at a similar high level, by contrast the level of expression in the third sample (30.4.99) was at a much lower, barely detectable level (lane 2).

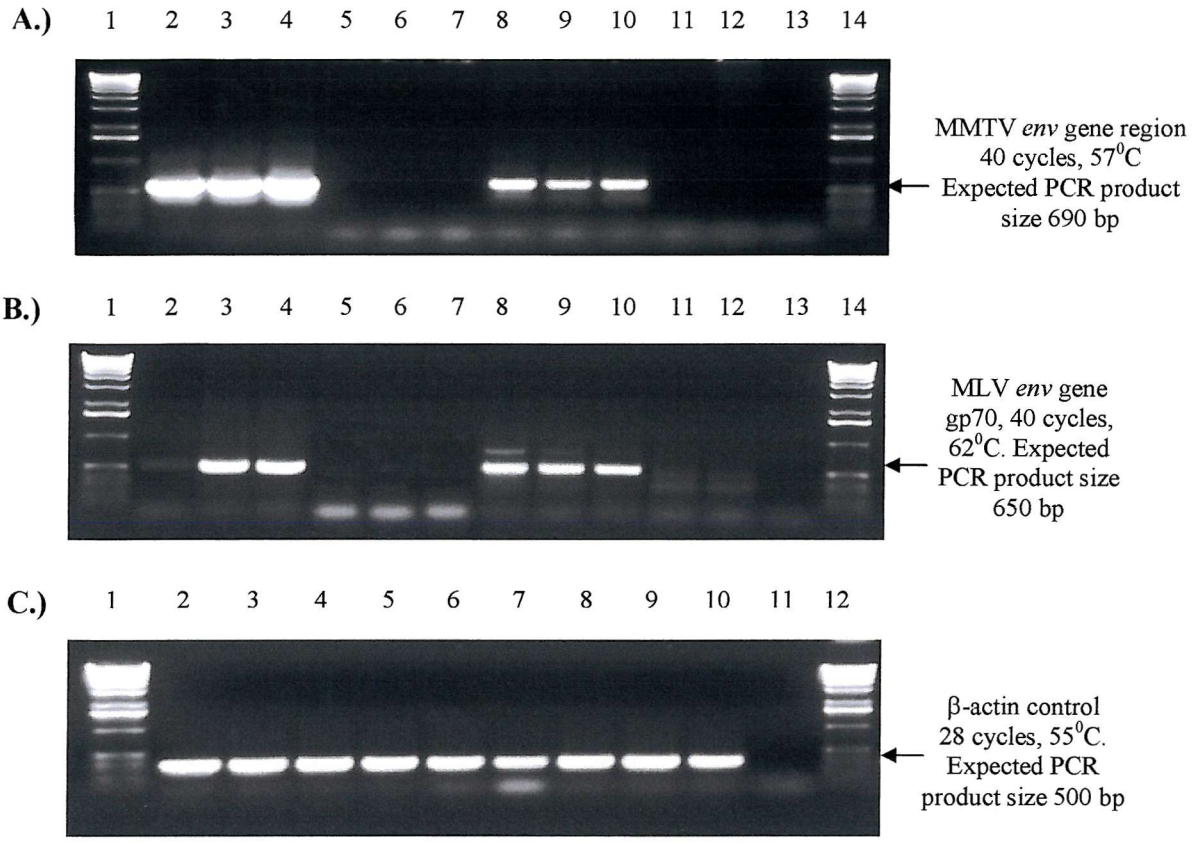


Figure 5.15- RT-PCR amplified cDNA for murine mammary tumour virus (MMTV) and murine leukaemia virus (MLV) envelope genes and β-actin control. The lanes in A.), B.) and C.) are as follows:

1.) 1kb DNA ladder
2.) A20 (cDNA from 30.04.99)
3.) A20 (15.05.99.)
4.) A20 (18.06.99.)
5.) FSDC (22.06.99)
6.) FSDC (12.08.99.)
7.) FSDC (18.02.00)
8.) Bcl_1 (17.06.98.)
9.) Bcl_1 variant (20.05.99)
10.) πBcl_1 (19.03.99.)
11.) Daudi
12.) Jurkat
13.) no DNA
14.) 1kb DNA ladder

The dates next to the cell line denote when mRNA was isolated from the cells. cDNA for β-actin control in C.) was amplified in murine cDNA samples only. Daudi and Jurkat cell lines are of human origin, and were used to establish the specificity of primers for MMTV and gp70 sequences.

5.2.5 Confirmation of productive retroviral infection of A20 cells by transmission electron microscopy (TEM)

The data described in section 5.2.4 indicate that the A20 cells used in therapies and CD40 signaling experiments express the *env* genes of at least two distinct murine retroviruses. However, it is well known that the mouse genome carries a high percentage of DNA that is derived from ancient retroviral infections and that these so-called endogenous retroviral sequences can give rise to viral gene products (Gaubatz *et al* 1991, Hara *et al* 1981). In order to determine if the A20 cell line is productively infected with a retrovirus the presence of viral particles within and around the cells was studied by transmission electron microscopy (TEM). The TEM protocol used for these studies is as described in 2.2.24. Briefly, A20 cells were harvested by scraping into media, and pelleted by centrifugation. Supernatant was discarded, cells resuspended in 3 mls of glutaraldehyde fixative and left for 1 hour. The cells were pelleted after one hour of fixation by pelleting, supernatant discarded and any remaining fixative blotted onto a tissue paper. 2 drops of 5% sodium alginate were added onto the cell pellet and gently mixed with a tip of a micropipette. 10µl of the mixture were taken up into a pipette tip, and ejected into a glass jars containing a fixation buffer containing calcium chloride and left for 15 minutes to set. After 15 minutes the alginate/buffer mixture was discarded and 1 ml of 1% osmium tetraoxide in 0.1M PIPES buffer added to the cells for a further period of 1 hour. The blocks of cells were then washed twice for ten minutes in 0.1M PIPES, which was followed by washed in increasing concentrations of ethanol to finish with absolute alcohol. Alcohol was discarded and replaced with 1ml acetonitrile for 10 minutes, which was then discarded and replaced with a mixture of acetonitrile/TAAB resin at a ratio of 1:1. The cell blocks were left in this mixture overnight. The mixture was discarded the following day, and replaced with neat TAAB resin, which was allowed to infiltrate cell blocks for 6 hours. Following this period, the cell blocks were embedded in fresh resin and polymerised at 60^0C for a further 24 hours.

The blocks of polymerised resin were cut to obtain sections which were on average 70nm thick. The sections were mounted onto a 200µm copper grid and stained, firstly in uranyl

acetate in the dark, then in Reynolds lead stain. Grids prepared in this way were viewed under TEM under various magnifications and photographic records were taken where appropriate.

The data generated in this way is shown in figures 5.16, 5.17, 5.18 and 5.19. Sections of A20 cells were photographed at various magnifications, ranging from 8000 to 120000 times (X). Electron micrographs in figure 5.16 show A20 cells viewed under a lower magnification of 10000X. There are observable viral particles surrounding the cells which can be seen in both 5.16A and B as pointed out with black arrows. Figure 5.17A and B show a different section of the cell sample at 10000X and 40000X magnifications respectively. The viral particles are much more defined, and their hexagonal shape can also be observed. Figure 5.18A and B show more viral particles at 40000X magnification. Again, black arrows point to the viruses surrounding the cells, while white arrows indicate viral particles present within the cell cytoplasm. Black arrows marked with a star in 5.18A indicate viral particles budding off from the surface of the cells and clearly demonstrate that the viral infection in A20 cells is productive and is not due to contamination of the media with viruses. Fig 5.19 confirms this, as more viral particles can be seen budding off from the surface of the cells in this photograph (black arrow marked with a star) taken at a 120000X magnification.

The virions seen in the electron micrographs are enveloped, 90nm in diameter, with a spherical shape and electron dense core. There are no significant visible projections emanating from the envelope. Their shape suggests that these particles are retroviruses. Although retroviruses have a common virion structure, they differ in some aspects of detail. These differences have been used to classify virions into four morphological groups or types, named A, B, C and D. MMTV belongs to the B type virus group, whereas MLV belongs to C type viruses.

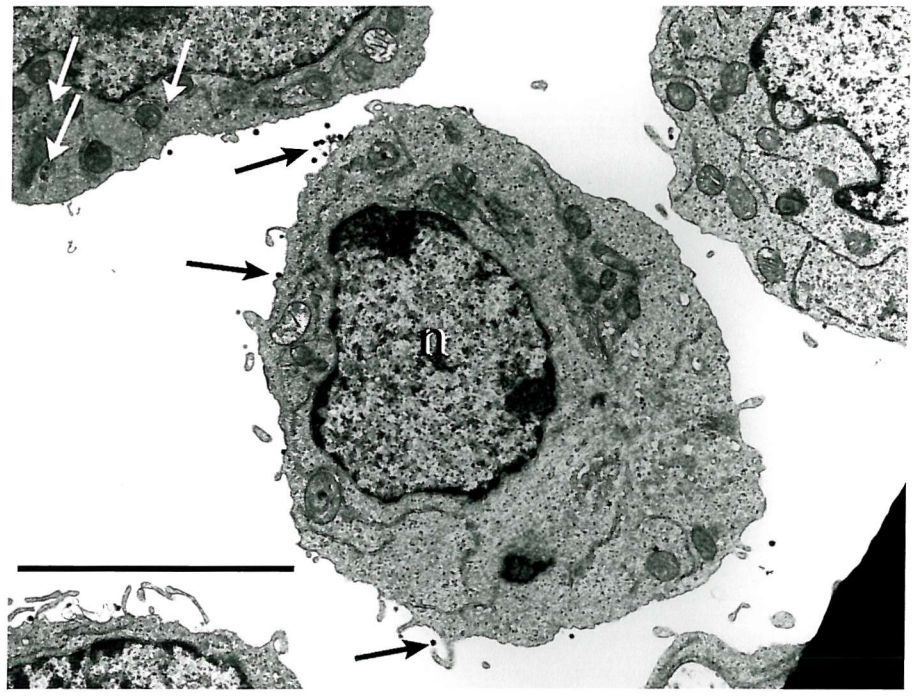
B type particles are enveloped, extracellular form of MMTV. Virions are spherical in shape, measuring 80-100nm in diameter. These particles also have distinct surface projections made up of 8nm long glycoprotein. The projections form spikes which are

uniformly dispersed all over the surface of the virion. MMTV produces only one type of particle, also characterised by tightly condensed, acentric nucleocapsid.

C type particles are of similar size to B type particles, displaying a core with central electron dense structure. These particles have barely visible surface projections. Based on these basic descriptions of the two types of viral particles, and assuming that no other retroviruses were present in A20 cells, it is likely that virions seen in the figs 5.16-5.19 were MLV C type particles.

In summary, A20 cells have been shown to express *env* gene products of at least two separate viruses, MMTV and MLV, furthermore productive retroviral infection of the cells has also been established by TEM. The data presented demonstrates that A20 cells were productively infected with at least one species of retrovirus measuring 90nm in diameter. At this stage it cannot be concluded with certainty which retrovirus is seen in the photographs without further experiments. Based on the results obtained, it can be concluded that A20 cells are compromised due to productive viral infection with possibly more than one retrovirus. For reasons that are expanded on the discussion section of this chapter further *in vitro* signal transduction experiments with the A20 cell line were therefore abandoned.

A.)



B.)

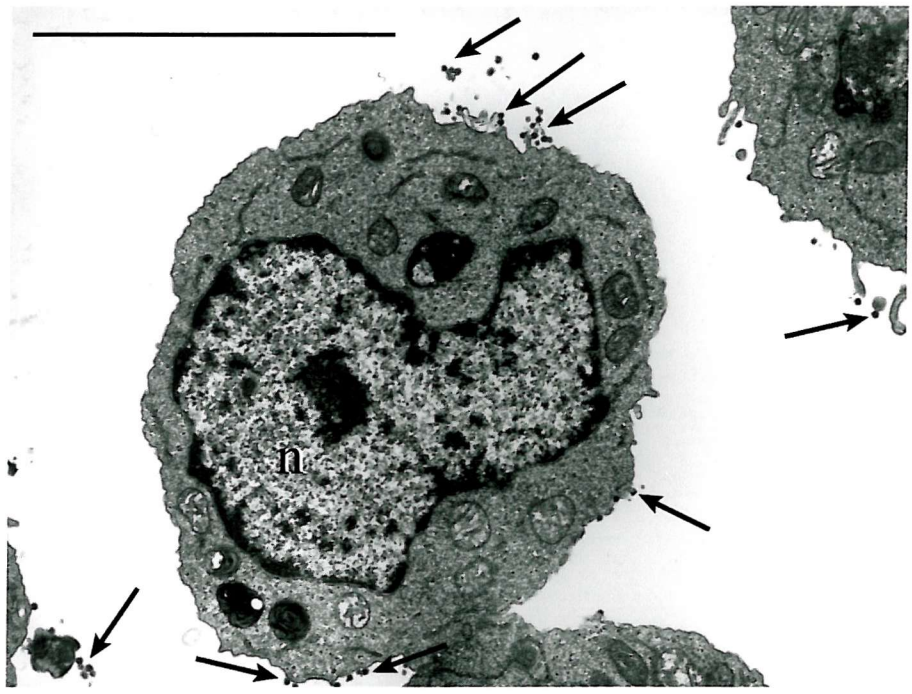
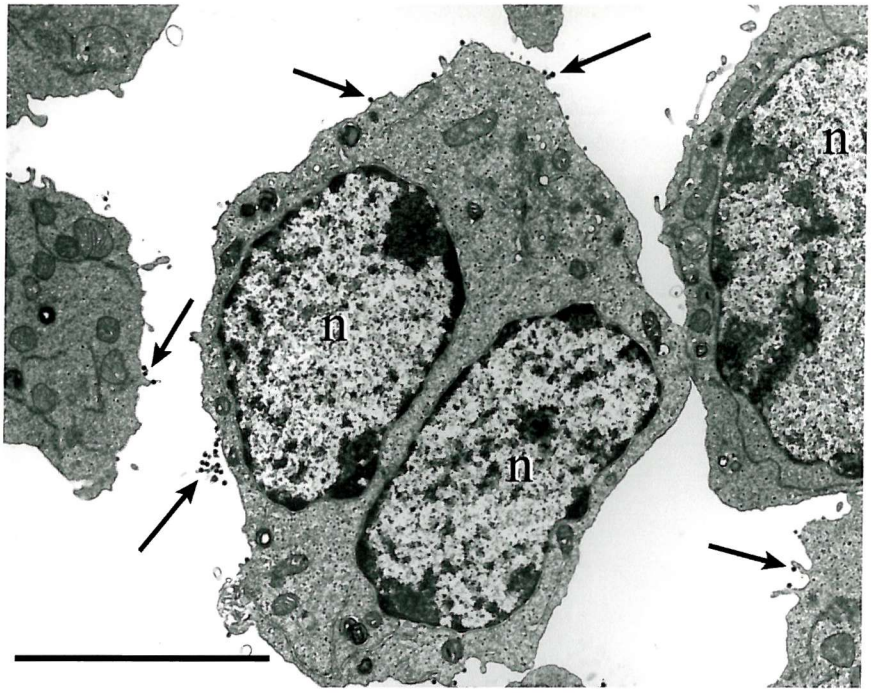


Figure 5.16- Electron microscope pictures of A20 cells viewed at 10000 and 14000X magnifications. The cells were embedded in resin as described in 2.2.24 and sections of <70 microns thickness were cut. Pictures in A.) and B.) show A20 cells and numerous surrounding viral particles. Black arrows point to the clusters of viruses. Sections shown in A.) and B.) were photographed at 10000x and 14000x magnification respectively. The black bar in the corner of each picture is a scale bar representing 5 microns in length.

A.)



B.)

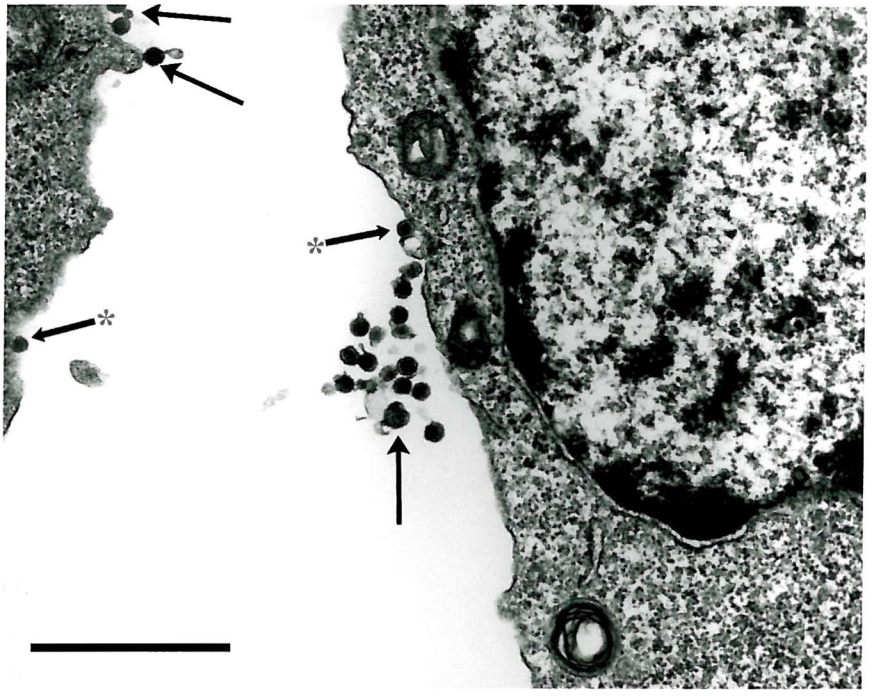
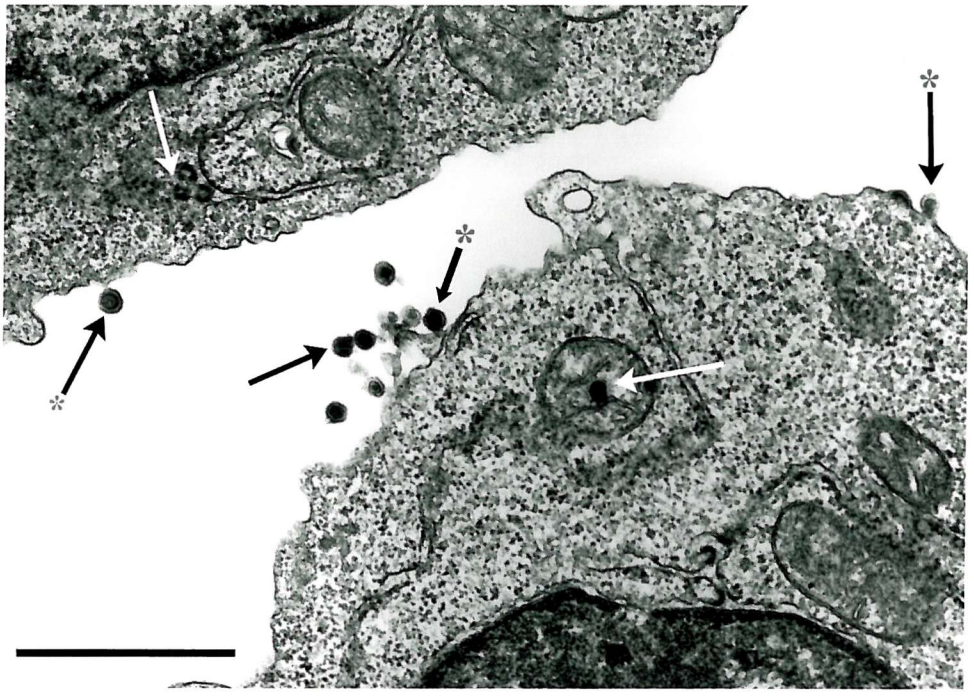


Figure 5.17- Electron micrograph of A20 cells viewed at 10000 and 40000X magnifications. The cells were prepared as described in 5.15. Section shown in a.) was photographed at 10000x magnification; picture in 5.16b.) is an enlargement of the photograph shown in figure 5.16a.). Fig 5.16b.) shows a cluster of viruses next to the cell membrane at 40000x magnification. Black arrows point to the viruses surrounding the cells. White arrows point to the viral particles located inside the cell. The black scale bar in a.) represents 5 microns, whereas the one in b.) is 1 micron in length.

A.)



B.)

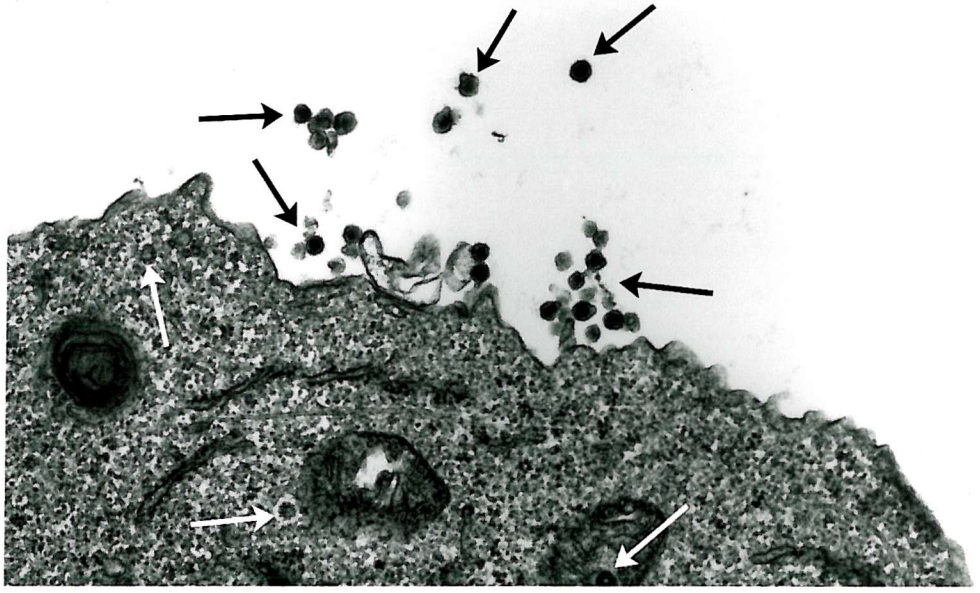


Figure 5.18- Electron micrograph of A20 cells viewed at 40000X magnification. The cells were prepared as described in 5.15. Section shown in a.) and b.) were photographed at 40000x magnification. Black arrows point to the viruses surrounding the cells. White arrows point to the viral particles which are located inside the cell. The black scale bars in a.) and b.) are 1 micron in length.

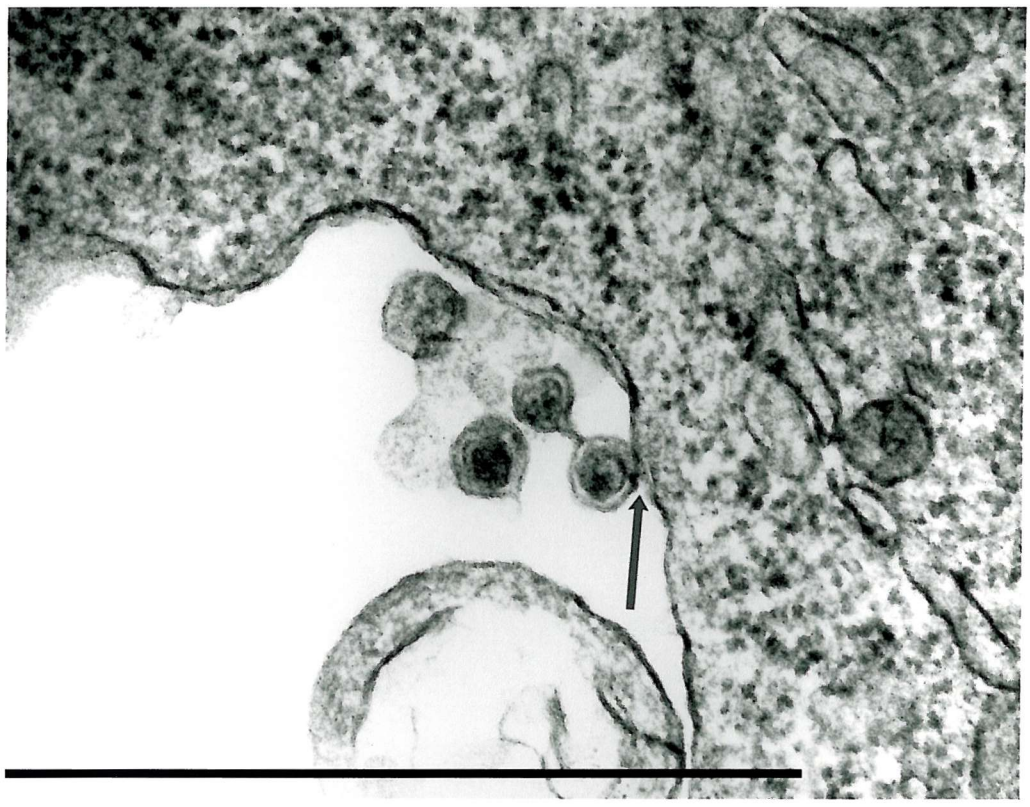


Figure 5.19- Electron micrographs of A20 at 120000x magnification. The cells were prepared as before. The picture shows viruses budding from the surface of A20 cells. Black arrow points to a particular budding virus. A cluster of viruses can be observed next to the budding particle. White arrow points to the virus inside the cell. The scale bar in the corner of the picture is 1 micron long.

5.3 DISCUSSION

5.3.1 Modulation of human CD40 receptor from the surface of human B cells and A20/hCD40 transfected cells

Receptor surface expression is partially regulated by the process of internalisation. In most cases, binding of a ligand or an antibody to a cell surface receptor results in internalisation by receptor-mediated endocytosis (Alberola-Ila *et al* 1993, Brown *et al* 1991). Once internalised, the receptor-ligand complex is delivered to endosomes, where most receptors discharge their ligand due to the acidic environment of the endosome. The receptor is then recycled back to the surface of the cell, whereas the ligand is delivered to lysosomes, where it is degraded. This mechanism represents a major degradation pathway for many signalling molecules (Smythe *et al* 1991, Feger *et al* 1994). Although many receptors are retrieved from endosomes and recycled, some fail to release their ligand and so themselves become targeted for degradation in the lysosomes along with the ligand. In those instances where the concentration of ligand in the microenvironment of the cell is low, the number of receptors degraded in this manner is negligible. However, during a continuous exposure of the cell to high concentration of ligand or mAb, the number of receptors on the surface gradually decreases, as more receptors become degraded, but fail to be replenished. This in turn lowers the sensitivity of the cell to that ligand or mAb. Therefore, by this mechanism, known as receptor down-modulation, the cell is able to adjust its sensitivity to the concentration of a stimulus, which results in desensitisation (Schmid 1992).

Part of the normal life cycle and metabolism of most receptors is made up of endocytosis, recycling, degradation and turnover of the receptor in question. All of these processes have been investigated for their possible involvement in lymphocyte activation and immunoregulation. For example, it has been shown that CD3/TCR complex and BCR are constitutively internalized by the cells whereas other molecules such as CD45 are anchored in the plasma membrane and are not subject to internalization even when an Ab or ligand binds to them (Legendre *et al* 1999, Luton *et al* 1997). This shows diversity of "behaviour"

demonstrated by cell surface proteins. The cellular mechanisms determining which route is taken are currently incompletely understood.

CD40 expressed on human B cells is rapidly modulated from the surface when cross-linked by its mAb or the CD40 ligand. In this chapter, modulation studies were initially used to compare the modulation pattern of transfected hCD40 on transfected A20 cell lines with endogenous hCD40 on human B cell lines. It was expected that both transfected and endogenous molecules would exhibit similar pattern of internalization and modulation, indicating that hCD40 transfected into the mouse cells was "behaving" and therefore at least in part functioning in the similar way as the endogenous CD40 found on human cells. It was found that hCD40 receptor becomes rapidly internalised upon anti hCD40 mAb binding in all three human B cell lines tested. It was found that 30% of the receptor is cleared from the surface in time ranging from 28 minutes (Daudi), 3 h 20 minutes (Ramos) to 16 h 20 minutes (Raji) after mAb addition to the media. The level of hCD40 expression continued to diminish with time in these cells, reaching ~10-15% of the original level at 24 hours. In comparative studies carried out on A20/hCD40 cell lines, the levels of hCD40 down-modulation in the first few hours was found to be very similar. The amount of hCD40 receptor on the A20/hCD40 cell surface diminished by 30% in the first ~2 hours in all of the cell lines tested (figs 5.4 to 5.7), but were then retained at this level for the remaining 46 hours of the experiment. Therefore, the initial response of the endogenous and transfected hCD40 receptors to mAb binding is very similar in all cell lines tested (figs 5.1 to 5.7, also 5.8). However, the data acquired in the later part of the experiments (from ~3 hours onwards) appears to differ between the endogenous hCD40 found on human B cells and transfected hCD40 in mouse B cell line A20. It was hypothesized that there may be at least two reasons for this difference between endogenous and transfected hCD40:

- The transfection process may itself alter the pattern of behavior of the receptor,
- The viral promoter in the hCD40 expression construct may be producing high levels of receptor expression that, in addition to providing high levels of surface expression, also creates a high cytoplasmic pool of protein. This would mean that following ligation-induced internalization of surface hCD40 there would be a more rapid and complete

replenishment of surface CD40 from the cytoplasmic pool without the requirement for *de novo* synthesis of receptor.

To test these ideas, human CD40 was stably transfected into Daudi cells. If the transfection process itself was responsible for altered modulation of receptors in transfected cells, than not only transfected hCD40, but also other receptors in these cells may be affected. As shown in figure 5.9, the Daudi/hCD40 transfectants have an altered pattern of CD40 modulation, similar to that described for A20/hCD40 (fig 5.4-7.7, also 5.11). The likelihood of the transfection process *per se* having an effect on modulation of receptors in transfected cells was addressed by examining the pattern of modulation of hCD22 in wild type and hCD40 transfected Daudi cells. Fig. 5.10 shows modulation of hCD22 in both of the cell lines. The overall pattern of receptor internalization appears very similar in transfected and wild type Daudi cells. It can therefore be concluded that the transfection process does not in itself cause the receptor internalization differences encountered in the studies.

The remaining possibility argues an effect of a CMV promoter driving constitutively high levels of expression of hCD40 in transfected cells. As already mentioned, it can be envisaged that the viral promoter may be providing transfected cells with a cytoplasmic pool of hCD40 receptor, thus abolishing the need for *de novo* synthesis of the protein in order to replenish the number of hCD40 on the surface. This would result in a relatively constant level of transfected hCD40 on the surface. The modulation experiment performed on Daudi/hCD40 (fig 5.9) suggests that this is probably the case. As can be observed, both Daudi and Daudi/hCD40 respond similarly to anti hCD40 mAb cross-linking in the first hour or so. Subsequently, hCD40 in Daudi cells continues to decrease its surface expression, whereas transfected receptor numbers remain relatively constant throughout the experiment. The transfected hCD40 receptor modulation in Daudi/hCD40 is very similar to that found in A20/hCD40 cell lines. Comparison of all transfected lines is shown in fig 5.11, where it can be noted that pattern of modulation in all cell lines is almost identical. It is also very important to note that all of the hCD40 mutants show nearly the same pattern of modulation as the hCD40wt. This would suggest that the introduced mutations do not interfere with the internalisation process.

Modulation studies were originally performed in order to compare responses of hCD40 receptor in human B cells and in transfected mouse B cells. Although initially very similar, the data obtained in the later hours of the experiments (3-48h) shows that hCD40 expressed as a consequence of a viral promoter activity does not behave in the same way as the endogenous hCD40 found on human B cells. It is postulated that cytoplasmic pool of hCD40 causes this discrepancy in the modulation patterns, so that all of the internalised receptors are rapidly replenished, thus retaining relatively constant numbers on the surface. Of course, there is a possibility that the remaining hCD40 receptors are simply not being internalised, which would also result in the same number of receptors on the surface. This question could be resolved by the use of bi-specific mAb (bsAb), where one arm of mAb molecule would be directed against hCD40, and the other against a toxin such as saporin. This bsAb could then be used to deliver saporin to the A20/hCD40 transfected cells. Upon incubation, the bsAb would bind to hCD40 on the surface of A20/hCD40 cells. If the hCD40 receptor was indeed internalised, then the bsAb would be internalised with it, as would saporin, which results in cell death. If transfected hCD40 were not internalised, the cells would live. The final read-out of saporin action in cells would be proliferative capacity of these cells, reflected in the amount of 3H-thymidine incorporation. These types of experiments were not carried out, as further study into modulation of hCD40 receptor would not reveal if this surface protein is actually signalling in A20/hCD40 cells, which was the original question. The end indication from these experiments was that all the receptors were responding to the mAb cross-linking. If a need for further clarification of the ways in which transfected hCD40 receptors modulate, than the types of experiments outlined in figs 5.4-5.7 would be the first step towards answering such query.

5.3.2 High levels of NFκB transcription factor in the nucleus of A20 cells and presence of expressed viral genes in these cells as a possible cause of problems with signalling studies

Results presented in this chapter have highlighted problems encountered with the attempts to dissect signalling events following CD40 cross-linking in A20 cells. One of the end results of CD40 signalling in most cell types expressing the receptor is activation of NFκB.

It has been established by the EMSA and reporter gene studies presented in figures 5.7 to 5.9 that both the DNA binding and transcriptional activities of NFκB in A20/hCD40 cells are in a constitutively active state that cannot be further elevated by stimulation of CD40. All B cells have a basal level of NFκB activation, which is necessary for their survival. However, previous studies have shown that this level of activity in murine B cell lines can be enhanced following ligation of surface CD40 with either antibody or ligand (Jalukar *et al* 2000, Baccam *et al* 1999, Hsing *et al* 1999, Lin *et al* 1998, Lin *et al* 1996, Schamer *et al* 1996). This response was clearly not achievable in A20/hCD40 cells following ligation and stimulation of either the endogenous mCD40 or transfected hCD40 receptor. This profound defect in signaling to NFκB prevented further studies on the function of the wild type and mutant hCD40 constructs generated in chapter 3. It was therefore decided to investigate the possible cause of this defect so as to provide a sound basis for rejecting A20 cells in future studies on CD40 signal transduction.

As detailed in the results section (5.2.4 and 5.2.5) the parental A20 cells used for therapy and for generation of transfected cell lines are productively infected with at least two and possibly more murine retroviruses. RT-PCR analysis detected high levels of the *env* gene product of MMTV and MLV, with the former being found in 3/3 different A20 cell samples and the latter being found at a high level in 2/3 cell samples. MLV is a non-acutely transforming retrovirus. While transforming retroviruses cause tumours by transduction of oncogenes (Hayward *et al* 1981), leukaemia retroviruses lack oncogenes, and therefore employ other mechanisms to the same aim. These include insertional activation of cellular proto-oncogenes by enhancers or promoters located within the retroviral long-terminal repeat (Fan *et al* 1990). However, in many cases the insertion of a viral DNA into the host genome is not site-specific, i.e. not in the vicinity of a cellular oncogene, which may indicate existence of yet more mechanisms leading to unregulated proliferation of host cells. It has been shown that one of the ways in which leukaemia viruses may drive tumorigenesis is persistent activation of NFκB transcription factors in the infected cells (Pak *et al* 1996). NFκB family of transcription factors control expression of a multitude of cellular and viral genes, including Ig κ light chain, IL2, IL6 and IL12 p40 subunit among others (Gilmore *et al* 1996). Various viruses have been shown to activate

NFκB, including HIV1, adenovirus 5, Sendai virus, cytomegalovirus, EBV in humans (Bachelerie *et al* 1991, Riviere *et al* 1991, Roulston *et al* 1992, Cherrington *et al* 1989, Eliopoulos *et al* 1997, Chien *et al* 2000) and most notably for the work presented here, MLV (Pak *et al* 1996). Also, certain viral products have been known to increase NFκB activity in infected cells, such as *tax* protein of human T-cell lymphotropic virus. It can be assumed that increased NFκB activity in infected cells is beneficial to these viruses by enhancing transcription or viral genes and/or cellular genes needed for viral replication and integration (Ross *et al* 1992, Leung *et al* 1988, Nabel *et al* 1987).

Having established the expression of the MLV *env* gene in A20 cells by RT-PCR and based on previous reports of MLV activation of NFκB (Pak *et al* 1996), it is highly likely that this virus is the cause of the constitutive, uninducible NFκB present in A20 cells. However, this cannot be confirmed without undertaking further studies. Experiments which may establish a definitive link between the presence of viral genes and increased NFκB activity would have to be done in presence of strong viral inhibitors, so to show that absence of viral gene transcription and viral replication would affect NFκB activity in the cells. However, the presence of viral inhibitors themselves may cause deviations in normal cellular signalling, so it may be difficult to conclusively demonstrate causes of high NFκB levels in A20 cells.

PCR results presented in figure 5.15 also show presence of MMTV *env* gene transcripts in A20 cells. MMTV is a retrovirus which can exist in two forms; either as exogenous, infectious virus, or as a germline integrated form, or endogenous MMTV. The endogenous form is relatively common in mice (Scherer *et al* 1993). In most cases, the endogenous MMTV loose their infectious character, and the ability to produce infectious viral particles is very rare. As the levels of MMTV *env* transcripts in figure 5.15 are constant, such a result may be indicative of an exogenous, productive infection of A20 with the virus. This is because the template for RT-PCR is mRNA isolated from the cells, suggesting existence of active transcription of the envelope gene, which is normally absent from the endogenous MMTV genome.

The infectious MMTV virus infects cells by entering B cells via as yet un-identified receptor. Once in the cells, it integrates itself into the genome, commencing the transcription of its genes. Among the proteins produced is superantigen (SA), which becomes displayed on the surface of B cells. The presence of SA induces a strong, cognate interaction between the T cells and B cells, resulting in clonal expansion of infected B cells. This process leads to differentiation of B cells into cells of memory phenotype (Held *et al* 1993, Held *et al* 1994, Held *et al* 1994). The long life span and migratory properties of the cells allow the virus to spread to other tissues, such as mammary glands, where it can cause tumours (Jolicoeur *et al* 1998).

In order for MMTV to integrate into the host genome, it is necessary for the host cell to be dividing. As it has been shown that MMTV does not preferentially infect cycling cells, it is thought that the virus has mechanisms which can actively induce B cells to enter the cell cycle and divide (Lopez *et al* 1985, Held *et al* 1993). It is thought that CD40 signalling has a pivotal role in the establishment of this initial infection, as CD40L-/- mice do not allow MMTV to complete its life cycle due to a defect in B cell stimulation (Chervonsky *et al* 1995). Taken together, these observations suggest that MMTV utilises its superantigen to promote a T-B cell interaction through CD40-CD40L, thus activating CD40 signalling and activation of NFκB. Therefore, the productive infection of A20 with MMTV may be another reason for the constitutive NFκB activation observed in figures 5.12, 5.13 and 5.14.

The results obtained so far have shown presence of two different types of viruses in the cells. It is not known how many other different viruses may be found to be transcriptionaly active in A20, or even to be productively infecting the cells at different times. Further experiments would be required to show how many different types of viruses may be present and transcriptionaly active in A20 cells.

Aside from providing a possible cause for unusually high NFκB activity, the presence of MuLV envelope gene product in A20 cells may have added significance for the *in vivo* therapy in terms of providing a tumour associated antigen against which immune response may be mounted. This suggestion can be made based on the data showing that envelope

gene product has been found in many other cell lines, including B16 melanoma, some lymphomas and leukaemias (O'Donnell *et al* 1981, Hartley *et al* 1977, and 3T3 cells whereas normal tissues in Balb/c mice have not been reported to express this protein. A peptide derived from this gene with a sequence SPSYVYHQF mapping to residues 423-431, has been shown to be immunogenic and its sequence confirms to motifs found to bind to L^d MHC class I binding peptides, thus being able to elicit a specific response from CTLs recognising this peptide in the context of L^d MHC class I (Lurquin *et al* 1989). Therefore, the presence of this gene product in A20 may form the basis of its immunogenicity in mice and may provide the cell line with a immunodominant tumour-associated antigen recognised by specific CTLs which are known to be necessary for elimination of tumour cells in the *in vivo* therapy (French *et al* 1998). The presence of MMTV viral proteins in the cells may be excluded from this possibility, as no CTL activity directed against MMTV antigens has ever been found.

Whether the expression of MLV or MMTV envelope gene product is in any way involved in the initiation of transformation or tumorigenesis of A20 cells or other cells expressing the protein is presently unclear. It is possible that tumour cells express these proteins as a result of dysregulation associated with the transformation process.

CHAPTER 6

Investigation of CD40 signaling events in dendritic cells: transcriptional regulation of IL6 gene expression and implications for CD40 therapy.

6.1 INTRODUCTION

The previous chapters describe *in vivo* and *in vitro* studies that were originally intended to dissect the signalling pathways triggered by CD40 stimulation of tumor cells. These studies not only presented problems with respect to the suitability of the tumor cells for signalling work but also cast into doubt the original hypothesis that CD40 therapy is partially due to stimulation of APC properties of the tumor cell. Work carried out by other groups provides evidence which shows that host DCs can cross-prime the tumour antigens into MHC class I, and in this manner prime precursor CTLs to become tumour specific mature CTLs (Heath *et al* 2001, Ferlazzo *et al* 2000, Hoffmann *et al* 2000). Previous chapters support the view that, although theoretically possible, tumour cells probably do not prime precursor CTLs. Furthermore, the studies using the A20 B cell lymphoma cell line found these cells to be unsuitable for signalling studies due to a persistent viral infection with more than one retrovirus.

As outlined in chapter 1, all of the B cell lymphomas, including Bcl_1, A20 and A31, can be cured with anti CD40 mAb treatment, whereas results obtained with T cell lymphomas showed a partial protective effect of the mAb in treatment of CD40-ve lymphomas. At the time of starting these studies, it was not clear if CD40 expression was necessary on the surface of tumour cells in order to obtain a 100% survival, or if T cell lymphomas tested were poorly immunogenic, thus giving only a partial curative outcome with anti CD40 mAb. Since these studies were performed, other work (Todryk *et al* 2001.) has shown that CD40 expression on the tumour cells is not required, and that CD40 therapy is very efficient for treatment of not only lymphomas, but other solid tumours as well. Taken together, evidence so far would indicate that host DCs are at least in part responsible for the effects observed in *in vivo* therapies, and therefore CD40 signalling should further be studied in these cells.

In order to assess how CD40 may affect signal transduction within DCs a new read-out needed to be established. The clues as to the type of assay which may be of interest came from the data previously published by French *et al* in 1998. The paper lists various cytokines which are induced upon CD40 ligation *in vivo,* including IL12 and IFNγ, both of

which have also been shown to be necessary for the curative outcome of the therapy (Tutt A., unpublished data). Another cytokine found to be up regulated upon CD40 cross-linking *in vivo* is IL6.

IL6 is a polyfunctional cytokine which plays a central role in host defence. The biological functions of IL6 include terminal differentiation of B cells (secretion of antibodies), growth promotion of myeloma, plasmacytoma and hybridoma cells, support of multipotent colony differentiation arising form hematopoietic stem cells, induction of acute phase proteins synthesis by hepatocytes, neural differentiation, growth of keratinocytes, synthesis of thrombopoetin by megakariocytes. Of most significance to CD40 therapy of tumor is the ability of IL6 to stimulate the differentiation and/or activation of dendritic cells and T cells. With a view to our lymphoma model, the role of IL6 in DC maturation and activation and likewise its function as a potent inducer of T cell proliferation are of most interest, as both of these events are fundamental in the final curative outcome of therapies. It has been shown by many groups (Hoffmann *et al* 2001, Norbury *et al* 2001, Heath *et al* 2001, Wolfers *et al* 2001, Berard *et al* 2001, Ferlazzo *et al* 2000, Hoffmann *et al* 2000.) that DCs are the only professional APC in the body able to cross-prime antigens into the class I pathway. Also, these cells have 5-fold higher capacity to induce naive T cell responses as compared to macrophages. However, in order for DCs to deliver signal two to T cells, they have to undergo maturation and activation. Several papers have shown that one of the key cytokines required in the maturation and activation phase of DCs is IL6 (Saijo *et al* 1999, Banchereau *et al* 2000, Drakesmith *et al* 1998, Grohmann *et al* 2001). It has also been demonstrated that DCs can produce IL6 in response to CD40 ligation and that DC derived IL6 would then promote maturation in an autocrine manner. It may therefore be postulated that CD40 signalling triggers a series of events leading to maturation of DCs, and that IL6 plays a part in this cascade by controlling one of the stages of the process. If IL6 is absent then the maturation process may not be complete, and so DCs would be unable to initiate T cell responses important in the eradication of tumour. Hence, by studying the signal transduction and gene transcription events that mediate CD40 induction of IL6 gene expression in DCs, we will reveal details of the intracellular events that contribute to DC maturation, APC function and clearance of tumor.

IL6 gene transcription is controlled by a promoter that is located between nucleotides – 750bp to +1 of the human IL6 gene, where +1 represents the start site of transcription. Within the promoter are several DNA sequences involved in transcription factor binding, such as NFκB, NF-IL6, CBF1, Sp1 and AP1 (Beetz *et al* 2000, Ikeda *et al* 2000, Hu *et al* 2000, Matsui *et al* 1999, Dokter *et al* 1996, Galieu *et al* 1996, Ray *et al* 1995, Zhang *et al* 1994, Matsusaka *et al* 1993). It has also been shown that these transcription factor binding sites are perfectly conserved in human and mouse promoters, thus allowing studies in cells of murine origin using a human promoter. Function of the IL6 promoter has been characterised in B cells, macrophages and fibroblasts, however similar studies have not been performed in DCs.

The signalling events leading to induction of IL6 in CD40 stimulated DCs has been studied in this chapter using various methods. They include RT-PCR using cDNA isolated from spleens at various stages of therapy, which provides an indication of the presence of IL6 during an *in vivo* response. Further studies were performed using the FSDC cell line as a model for dendritic cell response to CD40 signalling and IL6 production. The FSDC cell line was chosen for this work, as it has been used previously in numerous studies, and it is well described. This is an immature myeloid dendritic cell precursor line that has the capacity to mature into efficient antigen-presenting cell when provided with an adequate stimulus (Girolomoni *et al* 1995). FSDCs were originally immortalised by transduction with a retroviral vector carrying an envAKR-mycMH2 fusion gene. As a result, these cells have a continuing proliferative capacity, but do not produce retroviral particles (see appendix to chapter 6), which was shown to be a problem with A20 cell line (chapter 5). The FSDC cell line has the phenotype of an immature DC, thus initial experiments were carried out to confirm this by comparing their responses to those found in immature bone marrow derived DCs by RT-PCR amplification of IL12, IL6, IL4, IFNγ and IL10. In these *in vitro* experiments, methods used include RT-PCR to initially establish the effect of CD40 signalling on levels of mRNA transcripts in the cells, followed by FACS analysis of the surface expression of DC maturation markers. Further, promoter studies utilising IL6 wild type and mutated promoter/luciferase reporter vectors and hCD40 constructs were carried out to show effects of mutations in hCD40 on signalling to the IL6 promoter. Having established the transcription factor binding sites within the IL6 promoter which

were important for transcriptional activity of the gene, the presence of these factors was evaluated by EMSA. The DNA: protein complexes found on the EMSAs were then further analysed by supershift assays using antibodies specific to each transcription factor component so to identify the protein subunits involved in the DNA: protein interactions. Expression vectors for the identified transcription factor components or specific inhibitors of these factors were then over-expressed in FSDC in order to confirm a role for the factors as transcriptional mediators of CD40 signalling.

6.2 RESULTS

6.2.1 Assessment of relative levels of IL6 mRNA in the spleen samples during the anti CD40 therapy of lymphoma *in vivo* and levels found in FSDCs and bone marrow derived DCs upon ligation of CD40 *in vitro*

Data by French *et al* (1999) and by Tutt (personal communication) have shown the importance of IL12 and IFNγ cytokines in the anti CD40 mAb therapy *in vivo*. These two cytokines are known to be potent Th1 type response inducers. This study links onto the work performed previously and addresses the possible role of IL6 *in vivo* with regards to DC maturation and induction of T cell proliferation. Effects of CD40 signalling on IL6 induction both *in vivo* and *in vitro* have been assessed in two sets of experiments involving RT-PCR. Levels of IL6 mRNA *in vivo* have been established by RT-PCR performed upon cDNA obtained from whole spleen samples isolated from mice at various stages of tumour development that were either treated with anti CD40 mAb or not. Briefly, two groups of twelve mice have been injected with $1x10^7$ Bcl_1 tumour cells intravenously on day 0. One group was given 1mg of anti CD40 mAb 3/23 on day 4, also iv, while the other group was injected with PBS of the same volume. On days 3, 5, 7, 10 and 12 two mice from each group were culled and spleens isolated. A sample of $1x10^7$ of whole spleen cells was used to extract mRNA. mRNA obtained in this way was used to generate first strand cDNA, which was then used as a template in RT-PCR reactions. The levels of IL6 mRNA were compared in treated and untreated mice by agarose gel electrophoresis of PCR reaction products, as shown in figure 6.1. The results presented show that mice receiving Bcl_1 alone did not produce detectable levels of IL6 mRNA in the spleen (gel A). In contrast, mice given an injection of 3/23 mAb on day 4 displayed a powerful induction of IL6 mRNA that was detectable for three days following the treatment (gel B). This latter result is exemplified by the appearance of discrete IL6 cDNA products obtained from PCR of day 5 and day 7 spleen samples of treated mice. Control amplified β actin cDNA is shown beneath the IL6 PCR gels for samples obtained from both the treated and untreated mice. The levels of β actin in all samples appear to be identical, suggesting that all cDNA

samples from either group have been prepared from same number of cells and are of a similar quality.

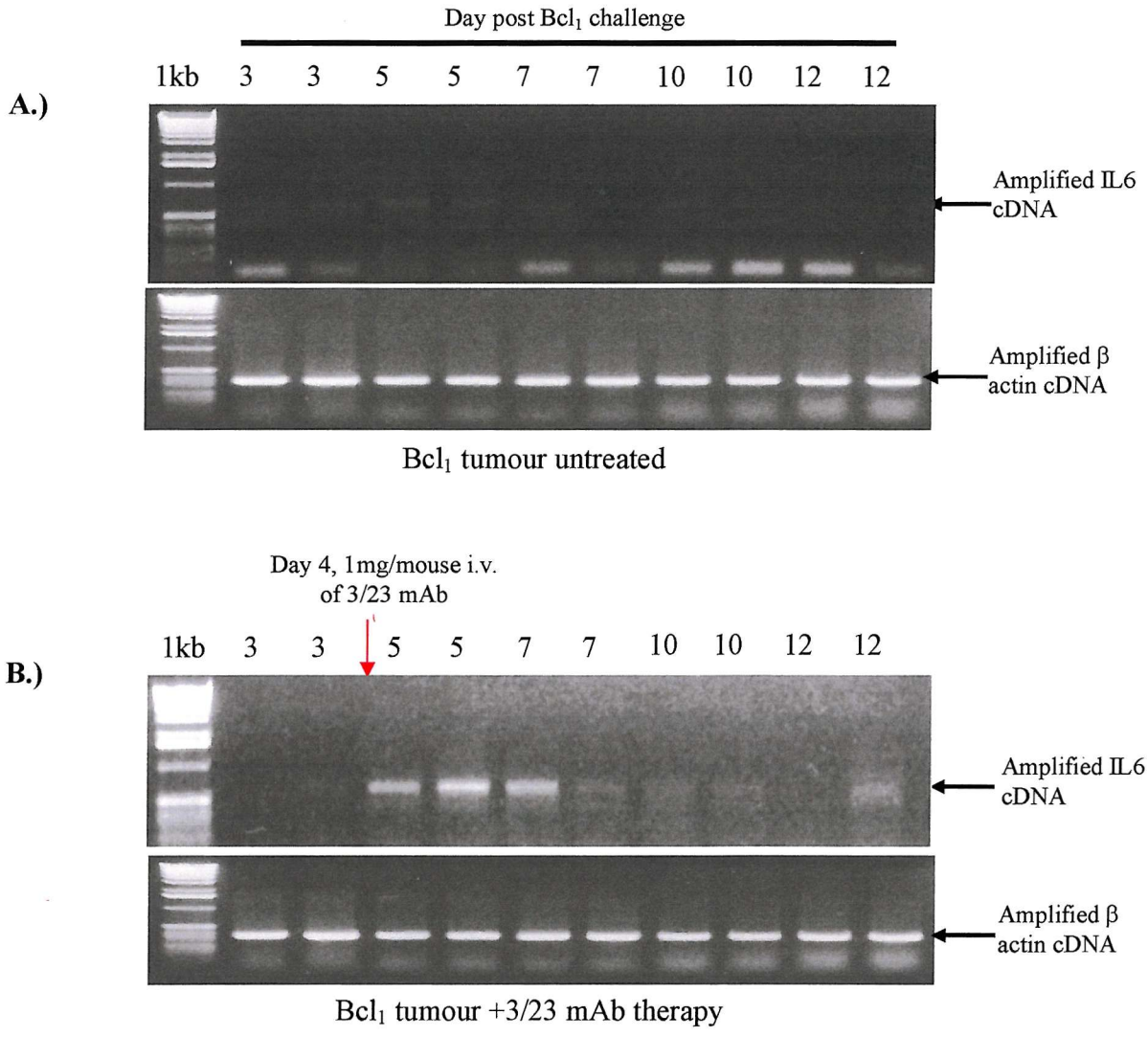


Figure 6.1- Assessment of IL6 levels found *in vivo* during anti CD40 mAb therapy. Two groups of mice were given $1x10^7$ Bcl_1 tumour on day 1. One of the groups was given 1mg of 3/23 mAb iv on day 4. Two animals from each group were culled on days 3, 5, 7, 10 and 12 and spleens taken out. mRNA was extracted from 10^7 total spleen cells (excluding erythrocytes) and first strand cDNA obtained. Semi quantitative PCR was carried out to establish the amount of IL6 found in the spleens of treated and untreated animals at various time points. β actin was used as a control for loading and amount of cDNA used in each reaction. The PCR reactions for IL6 and β actin were run for 28 and 25 cycles respectively. Fig 6.1A demonstrates absence of IL6 transcripts in untreated animals, whereas animals given 3/23 mAb show a transient induction of IL6 lasting ~3 days following the mAb distribution.

A second set of experiments was carried out to establish the effect of CD40 cross-linking on IL6 induction in the FSDC cell line. 6 $x10^7$ FSDC cells were plated out at $1x10^7$ cells per sample. Three of the samples had 30μg/ml anti mCD40 3/23 mAb added at time 0. One sample of cells incubated with 3/23 mAb and one without were harvested at 24, 48 and 72 hours post 3/23 mAb addition to the cell culture. mRNA was isolated from the samples, as before, and cDNA generated, which was used as a template in RT-PCR reaction. The levels of IL6 mRNA in all samples were determined and are shown in figure 6.2. The results show a low and constant level of IL6 transcripts in all of the untreated cell samples, indicating that these cells constitutively produce IL6. Upon endogenous CD40 cross-linking, the level of IL6 transcript increased several fold as shown in the sample of FSDCs incubated with 3/23 mAb for 24 hours. However, 48 hours after the addition of 3/23 mAb, IL6 mRNA was undetectable. By 72 hours the expression of the cytokine mRNA recovered, as demonstrated by a weak PCR product band. This result demonstrates that there is a large response in IL6 production to CD40 signalling, and that this induction is rapid, although transient. It also suggests that 24 hours may be the optimal time point in which to assess the effects of CD40 signalling on IL6 induction in the further experiments to be carried out. Amplifying β actin cDNA in all samples (as shown in 6.2B) controlled for uniformity of samples with regards to the number of cells that mRNA was originally isolated from and the quality of the cDNA used in the PCR reactions. All β actin PCR products appeared identical.

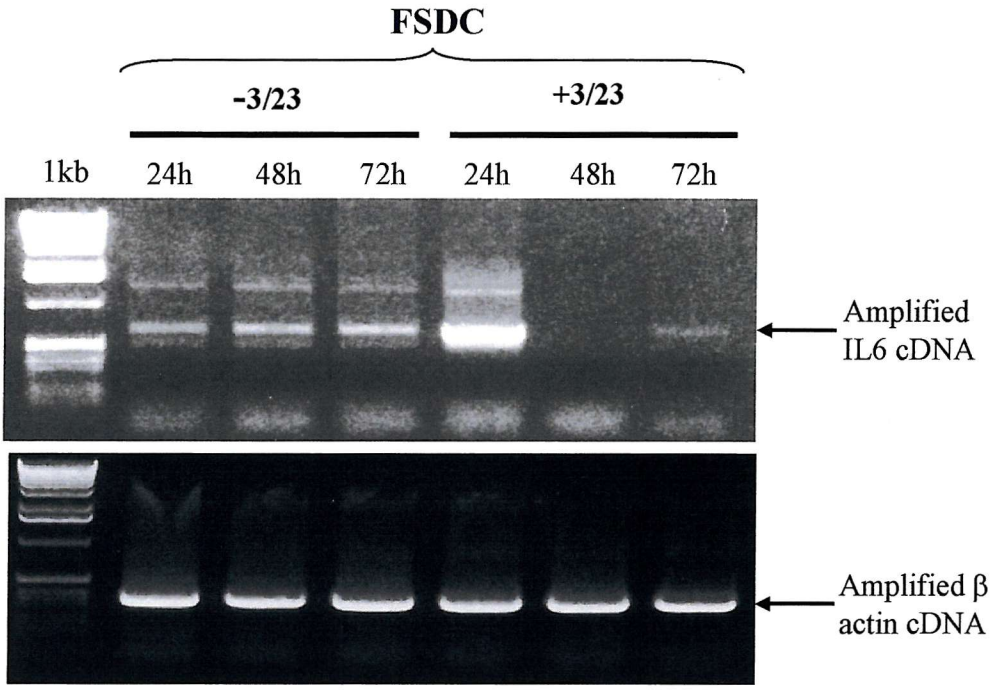


Figure 6.2- Assessment of IL6 transcript induction in FSDC cells incubated with anti mCD40 mAb 3/23. 10^7 FSDC cells were either incubated with 30μg/ml 3/23 mAb, or left untreated for 24, 48 and 72 hours. mRNAs were then extracted form the cells, and first strand cDNA obtained, which was used in RT-PCR reactions. RT-PCR for IL6 and β actin were run over 27 and 25 cycles respectively in a total volume of 25μl reaction. Annealing temperature for IL6 primers was 50^0C, and 55^0C for β actin. 22μl of the RT-PCR product was loaded on 1% agarose gel, and separated by electrophoresis against standard DNA markers. The gels were viewed under UV light and photographic record taken.

6.2.2 Comparison of cytokine production in FSDC and bone marrow derived DCs following CD40 cross-linking by 3/23 mAb

Having demonstrated that IL6 is induced *in vivo* and *in vitro* in FSDCs upon CD40 ligation, the experiments in figures 6.3 and 6.4 show the response at the level of cytokine production in FSDCs and bone marrow derived DCs. These assays were performed in order to assess whether FSDC can be used as a representative model for DCs found *in vivo*. To this aim, stem cells obtained from mouse bone marrow were cultured in GM-CSF and IL4 for 10 days, using a method described previously (Thomson *et al* 1995). Following this period, $1x10^7$ BMDCs were incubated with 30μg/ml 3/23 mAb, and the same number of BMDCs left untreated for 24 hours. FSDCs were subjected to the same protocol. At 24 hours, the cells were harvested, mRNA isolated and first strand cDNA obtained. PCR reactions were set up using these cDNAs as templates for amplification of IL4, IL6, IL10, IL12 p40, IL12 p35 and IFNγ transcripts. These cytokines were chosen due to their Th1 (IL12 and IFNγ) or Th2 type responses (IL4, IL10 and IL6) induction capacity. Amplified β actin cDNA was used as a loading control. Figure 6.3 shows the results of PCR amplification of IL6 and IL12 transcripts. In both BMDCs and FSDCs IL6 appears to be constitutively expressed at a low level, which is markedly induced upon CD40 cross-linking. Careful observation of the PCR products reveals the presence of two distinctive bands in the unstimulated BMDC sample, and only a single band upon stimulation. This may suggest induction of alternative mRNA splicing due to the extracellular stimulus received by the cells. FSDCs exhibit the same result, although the shorter of the two transcripts found in the unstimulated sample was not as distinctive as the one in BMDCs. Further investigations would be needed in order to assess the differences between the two PCR products and the possibility of alternative splicing. IL12 mRNA expression is also shown in this figure. IL12 is comprised of two subunits, p40 and p35. Transcription of both IL12 subunits was strongly induced in stimulated FSDCs and BMDCs. Finally, β actin control shows that same amount of cDNA was used in PCR reactions for stimulated and unstimulated samples.

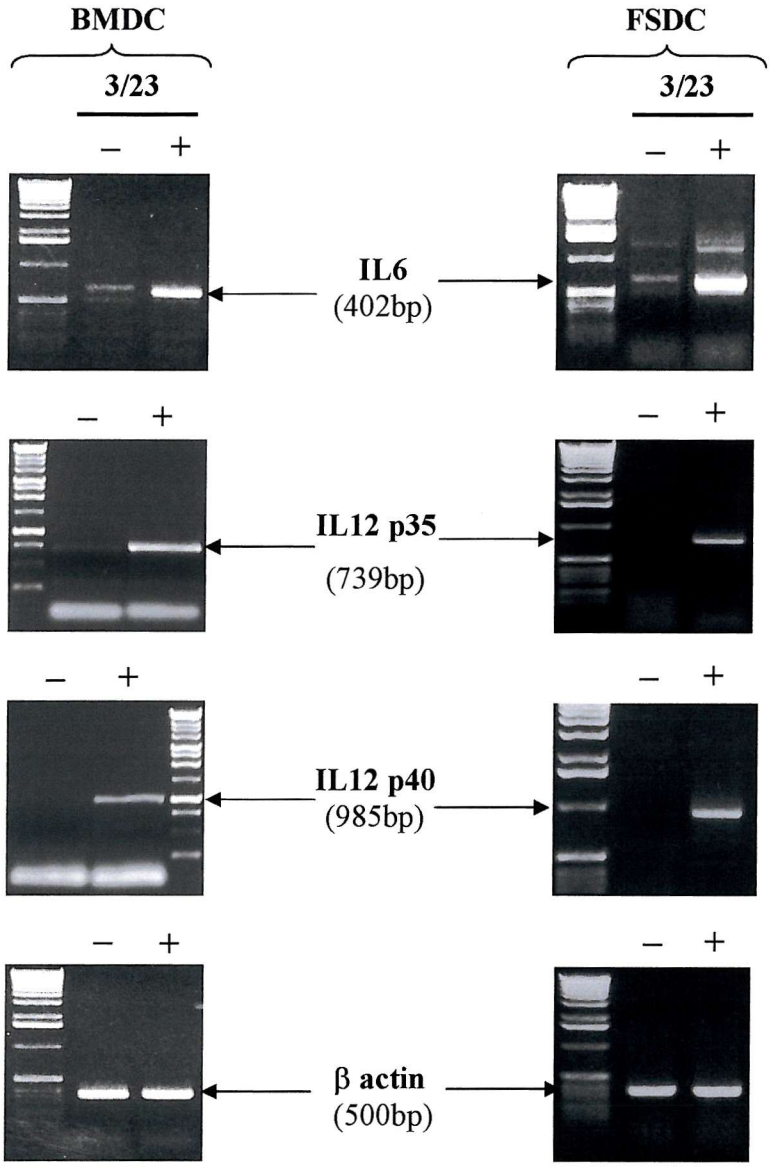


Figure 6.3- Comparison of cytokine induction in FSDCs and BMDCs when stimulated with anti CD40 mAb 3/23. mRNA was isolated form FSDCs and BMDCs treated or untreated with 3/23 mAb for 24 hours (at 30μg/ml final concentration). First strand cDNA was made, and RT-PCR reactions set up as in 2.2.4 and 2.2.5 in a total volume of 25μl. PCR reactions for IL6, IL12p35, IL12p40 and β actin were amplified over 27, 30, 28 and 27 cycles respectively. 22μl of each PCR product was loaded on 1% agarose gel, and separated by electrophoresis. Gels were viewed under UV light and photographic record taken. Both cell types appear to be responding to CD40 cross-linking by production of similar amounts of IL6 and both subunits of IL12.

Figure 6.4 shows more data obtained using RT-PCR. Results for further three cytokines, namely IFNγ, IL4 and IL10 are presented. As can be seen on the photographs, there are no detectable levels of IL4 or IFNγ in FSDC or BMDC cDNA samples. Specificity of the primers for these sequences was performed by RT-PCR using a different template, namely Balb/c cDNA for IL4 and DBA cDNA for IFNγ as a positive control. PCR using the primers for IL10 indicate that BMDCs produce relatively substantial amounts of IL10, which are suppressed upon CD40 cross-linking, while IL10 is undetectable in FSDCs in both samples. Taken together, IL10 suppression and IL12 induction may suggest that both bone marrow derived and FSDCs respond to CD40 ligation by induction of Th1 type cytokines production.

RT-PCR using IL6 receptor primers was also performed in both FSDCs and BMDCs. It was thought that it would be important to show presence of mRNA for the receptor in FSDC and likewise in BMDCs as this chapter deals with effects of IL6 production in DCs and also stipulates the importance of this cytokine on DC maturation. In order for this cytokine ot act on FSDCs or BMDCs, the cells have to have IL6 receptor expressed. Indeed, RT-PCR found a substantial amount of mRNA present encoding for the receptor in both cell types, which does not appear to change following the treatment with 3/23 mAb.

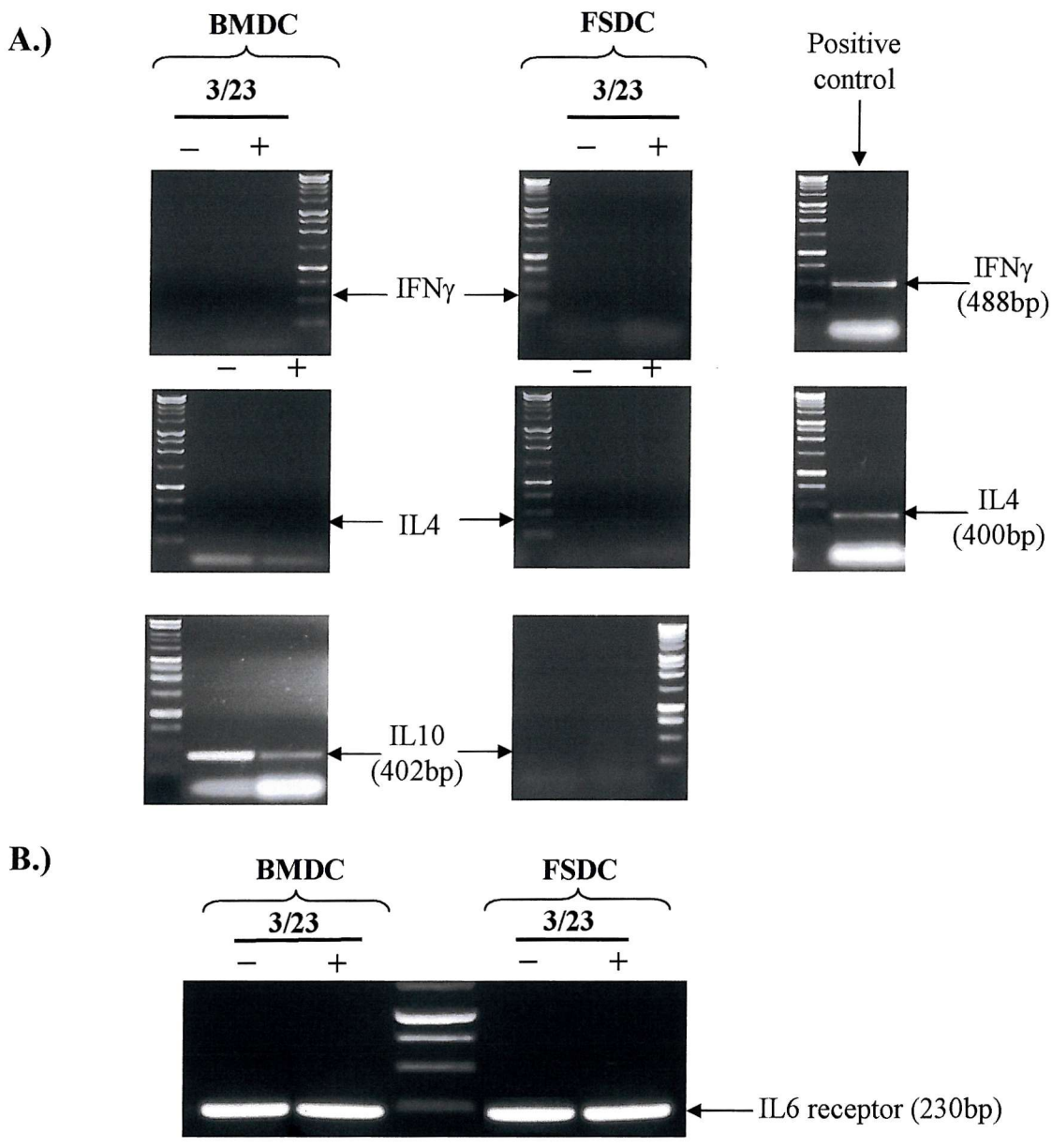


Figure 6.4- Comparison of cytokine induction in FSDCs and BMDCs when stimulated with anti CD40 mAb 3/23 (2). The cells were treated as described for fig 6.3. Neither the FSDCs or BMDCs produce IFNγ or IL4, whereas only the latter produce IL10 in an unstimulated state. This is switched off upon CD40 cross-linking (fig 6.4A). Both cell types have abundance of IL6 receptor transcripts, which remains unchanged with addition of anti CD40 mAb (fig 6.4B).

6.2.3 Evaluation of expression of surface markers associated with DC maturation process by flow cytometry

To confirm the immature phenotype of FSDCs and evaluate the expression of various surface markers including co-stimulatory proteins induced by CD40 cross-linking, cells were stained with FITC labelled anti B7.1 (CD80), B7.2 (CD86), MHC class I (L^d, D^dK^d and D^d), MHC class II, ICAM1 (CD54), mCD40, CD4, Rank and 41BB mAbs.

In order to confirm that anti CD40 mAb treatment of cells induces maturation, the FACS profiles were obtained from untreated cells and cells incubated with 3/23 mAb for 24 hours prior to FACS analysis. The results of these studies are shown in figures 6.5 and 6.6. B7.1, MHC class I and II, CD4 and ICAM1 show marked increase in surface expression after anti CD40 mAb incubation, thus agreeing with previously published work indicating that CD40 signalling leads to maturation of DCs both *in vivo* and *in vitro* (Caux *et al* 1994, Cella *et al* 1996, Banchereau *et al* 1998, Winzler *et al* 1997, Winzler *et al* 1997, Rescigno *et al* 1997) which can be monitored by assessing for increase in these surface proteins. Levels of B7.2 were only marginally affected by CD40 signalling. The binding of irrelevant antibodies to treated and untreated cells remained unchanged, as did surface expression of Rank, 41BB and mCD40, indicating that the increased surface expression of aforementioned surface markers was genuine, and not due to increased non-specific binding of mAbs to activated FSDCs.

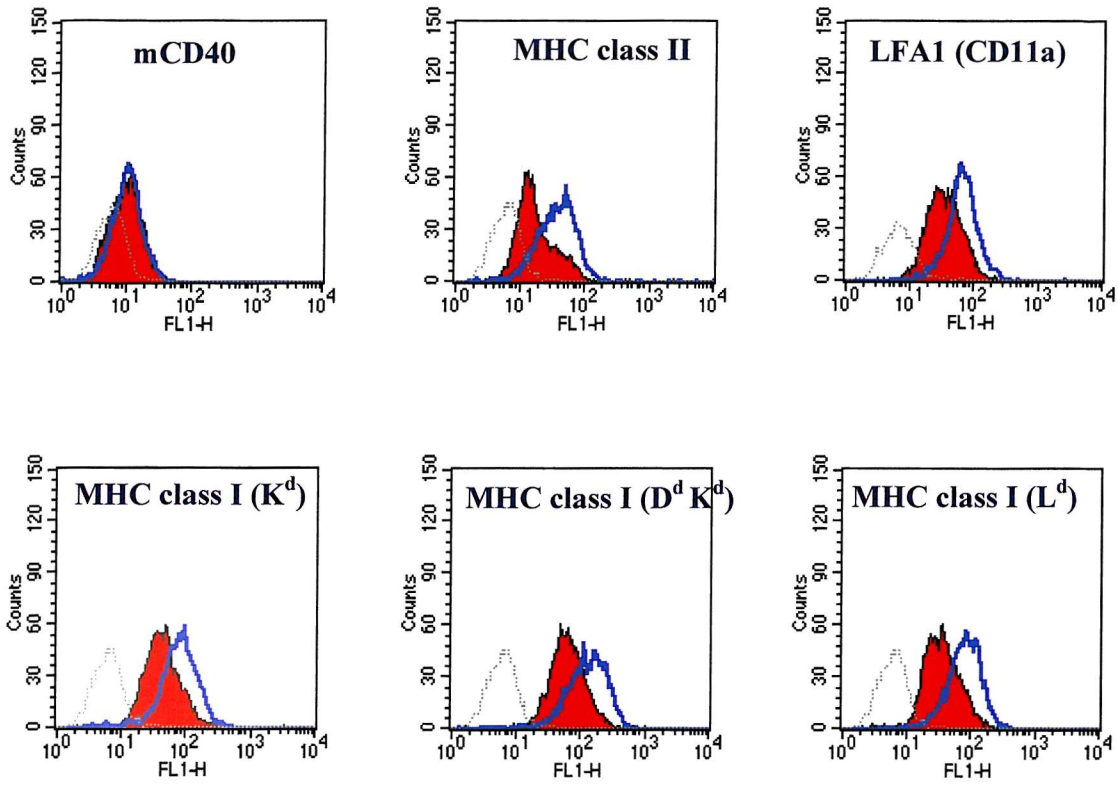


Figure 6.5- Increased expression of selected surface markers following a 24 hour incubation of FSDCs with 30μg/ml 3/23 (anti mCD40) mAb. The cells were seeded so as to achieve around 70% confluency on the day when mAb 3/23 was added to the cultures. The mAb was added in a soluble form at a final concentration of 30μg/ml. Following a 24 hour incubation period, the cells were gently scraped into the media, spun down at 1000rpm for 3 minutes. The pellet was resuspended so to give a cell density of around $1x10^6$/ml. Both treated and untreated cell samples were prepared in this in order to carry out comparison under same conditions. The cell mixtures were divided into a required number of samples, and FITC conjugated mAbs of desired specificity were added for a 30 minute incubation. The cells were then spun down, pellets resuspended, and samples subjected to FACS analysis. The results of the experiments are shown above, where the dotted line represents the level of irrelevant mAb binding, the solid red histogram shows the amount of a particular surface marker present on the cells at time 0, and solid blue line showing the level of the same marker following a 24 hour incubation with 3/23 mAb.

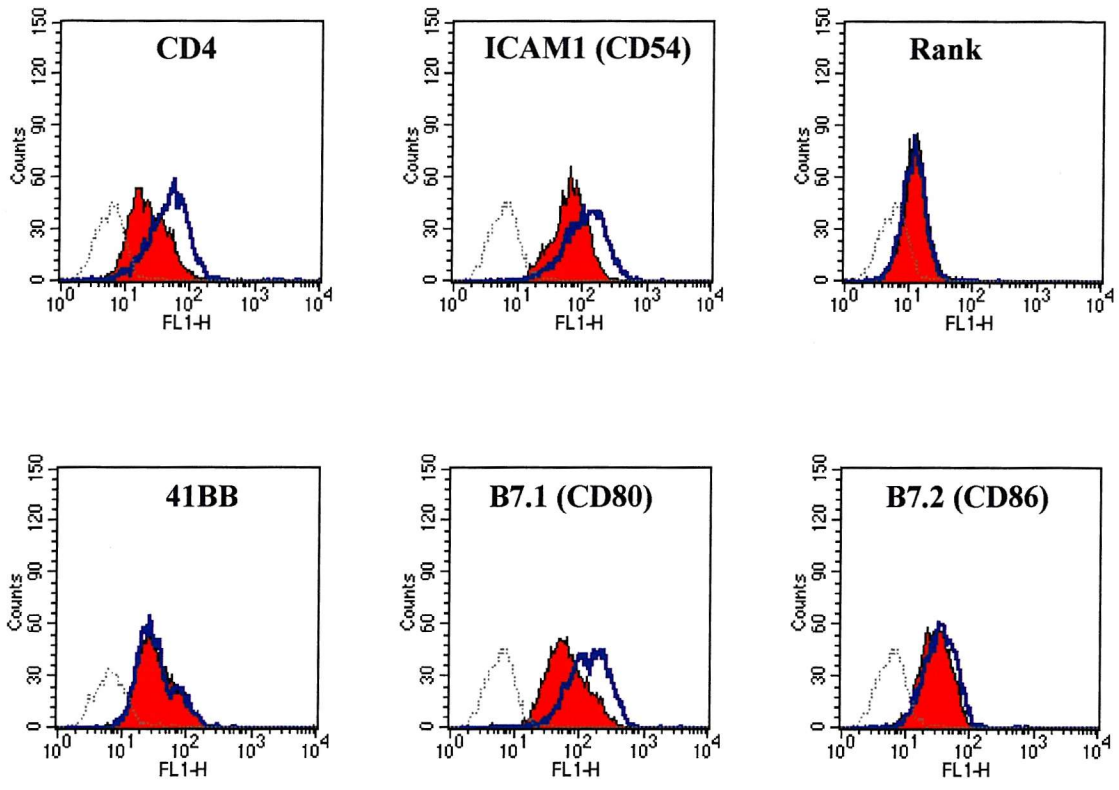


Figure 6.6- Increased expression of selected surface markers following a 24 hour incubation of FSDCs with 30μg/ml 3/23 (anti mCD40) mAb. The cells were treated as described in the previous figure. The results of the experiments are shown above, where as before, the dotted line represents the level of irrelevant mAb binding, the solid red histogram shows the amount of a particular surface marker present on the cells at time 0, and solid blue line showing the level of the same marker following a 24 hour incubation with 3/23 mAb.

6.2.4 Regulation of IL6 gene promoter activity by stimulation of endogenous CD40 in FSDCs.

RT-PCR results and FACS data shown in the previous sections confirmed that the responses of FSDCs to CD40 stimulation mirror the responses found in immature BMDCs. Having demonstrated these FSDCs can serve as a suitable model for studying responses of immature DCs, the following experiments focused on the CD40 signalling events that lead to induction of IL6 gene transcription. The method used to determine the effect of endogenous mouse and transfected human CD40 signalling on IL6 gene transcription was to determine the activity of a transfected firefly luciferase reporter vector in which transcription of the luciferase cDNA is under the control of the human IL6 promoter. Five IL6 promoter/luciferase reporter constructs were used in these experiments, namely IL6 wild type (wt) promoter, and four mutants, IL6ΔNFκB, IL6ΔNF-IL6, IL6ΔNFκB/NF-IL6 and IL6ΔAP1, where the delta sign denotes the absence of a particular transcription factor binding site from the promoter. The human IL6 gene promoter is shown in figure 6.7, whereas the IL6 promoter/luciferase constructs are illustrated in figure 6.8.

In the experiments shown in figure 6.9, 30μg of the IL6 promoter/luciferase constructs and 30ng of pRLTK were transiently transfected into FSDC cells at time 0, as outlined in 2.2.25. At 24 hours, the cells were incubated with 30μg/ml of 3/23 mAb, or were left untreated for further 24 hours, when the cells were harvested. The luciferase assay was performed upon the cell lysates and data plotted as ratio of firefly/renilla luciferase activity. Figure 6.9 compares the activity of IL6wt and mutant promoters. While very little activity of IL6wt promoter was measured in unstimulated FSDCs, the response to CD40 signalling was rapid and was quantified as a 20-fold induction in transcriptional activity. In comparison to the wt construct, activity of the IL6ΔNFκB promoter was not induced by CD40 ligation suggesting that the NFκB site is of paramount importance for CD40-inducible activity of the promoter. The IL6ΔNF-IL6 promoter mutant displayed around 30% of wt promoter activity upon stimulation with CD40 antibody, as did IL6ΔAP1 indicating a requirement for both of these binding sites for high level IL6 gene transcription. The IL6ΔNFκB/NF-IL6 double mutant had similar activity to IL6ΔNFκB.

When the promoter data is considered more carefully, it becomes apparent that there are two types of activity, basal and inducible promoter activities. NFκB seems to be required for the basal activity, as the absence of an NFκB binding site in the promoter results in a loss of transcriptional activity in unstimulated FSDCs. The other promoter constructs displayed a level of basal activity approximately 8 and 7.4 fold higher than found in IL6ΔNFκB and IL6ΔNFκB/NF-IL6, respectively. When the data from figure 6.9 are expressed as the fold-induction of IL6 promoter activity for each promoter construct it becomes clear that stimulation of IL6 gene transcription by CD40 involves AP-1, NF-IL6 in addition to NFκB (fig 6.9). Deletion of the NFκB site resulted in a 68% loss of inducible promoter activity, while loss of the AP-1 site caused a 48% reduction in CD40-induced transcription. Although deletion of the NF-IL6 site alone was without an effect on CD40-induced transcription when combined with deletion of the NFκB site there was a 92% loss of activity, as compared to 68% attributable to NFκB alone. This latter observation indicates that NFκB and NF-IL6 transcription factors may act in synergy to promote high level IL6 promoter activity in CD40 stimulated cells.

ggagtcacacactccacctggagacgccttgaagtaactgcacgaaatttgaggatggccaggcagtctacaacagccgctcaca

gggagagccagaacacagaagaactcagatgactggtagtattaccttcttcataatccaggcttgggggctgcgatggagtca

gaggaaactcagttcagaacatctttggtttttacaaatacaaattaactggaacgctaaattctagcctgttaatctggtcactgaaaa

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AP1

ccaaagtgctgagtcactaataaaagaaaaaaagaaagtaaaggaagagtggttctgcttcttagcgctagcctcaatgacgacct

MRE

aagctgcacttttccccctagttgtgtcttgcgatgctaaaggacgtcacattgcacaatcttaataaggtttccaatcagccccaccc

CRE NF-IL6

gctctggccccaccctcaccctccaacaaagatttatcaaatgtgggatttttccccatgagtctcaatattagagtctcaacccccaat

NFκB CBF1 AP1?

aaatataggactggagatgtctgaggctcattctgccctcgagccaccgggaacgaaagagaagctctatctcccctccaggagc

ccagct***atgaactccttctccacaagtaagtgcaggaaatccttagccctggaactgccagccggtcgagccctgtgtgaggg***

+1

aggggtgtgtggcccagggatgcggggcgccagcagcagaggcaggctcccagctgtgctgtcagtcac

Figure 6.7- Human IL6 promoter sequence. Transcription factor binding sites are highlighted in different colours, underlined and marked with the name of the factor binding the given sequence, except (MRE) which marks multi-response element. The second AP1 site is marked with (?), as it has not yet been conclusively shown that AP1 proteins bind this sequence. +1 site marks the transcription start site; the IL6 gene sequence is written in italics.

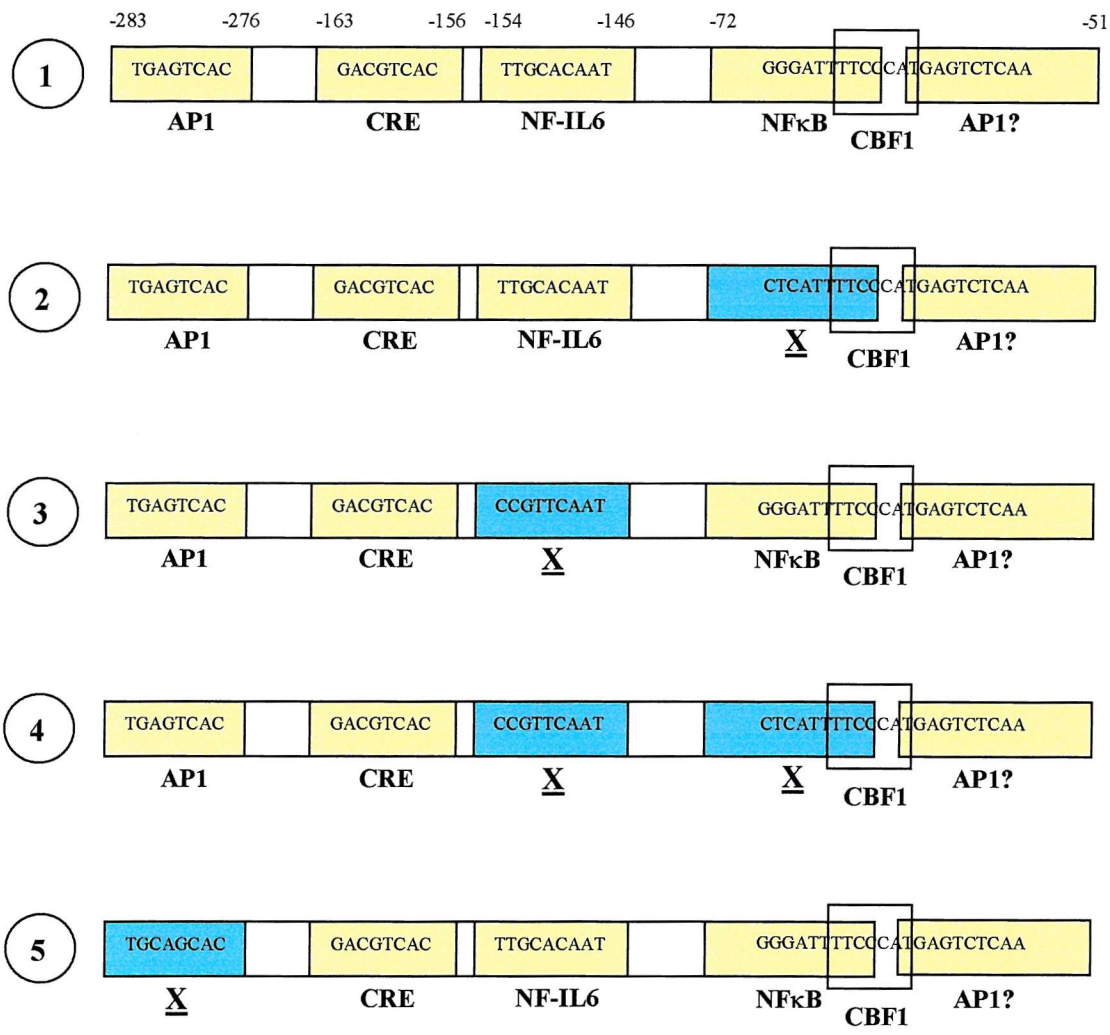


Figure 6.8- A schematic representation of human IL6 promoter constructs. Yellow boxes denote wild type sequences, while the blue boxes contain the mutated promoter sequence, signified by X. The numbers at the top of the page indicate the position of the particular DNA sequence relative to the transcription start site. Numbers next to the constructs mark the alternative name of the construct, for easier labelling on the graphs. The human IL6 promoter constructs are wild type (1), IL6ΔNFκB (2), IL6ΔNF-IL6 (3), IL6ΔNFκB/NF-IL6 (4) and IL6ΔAP1 (5).

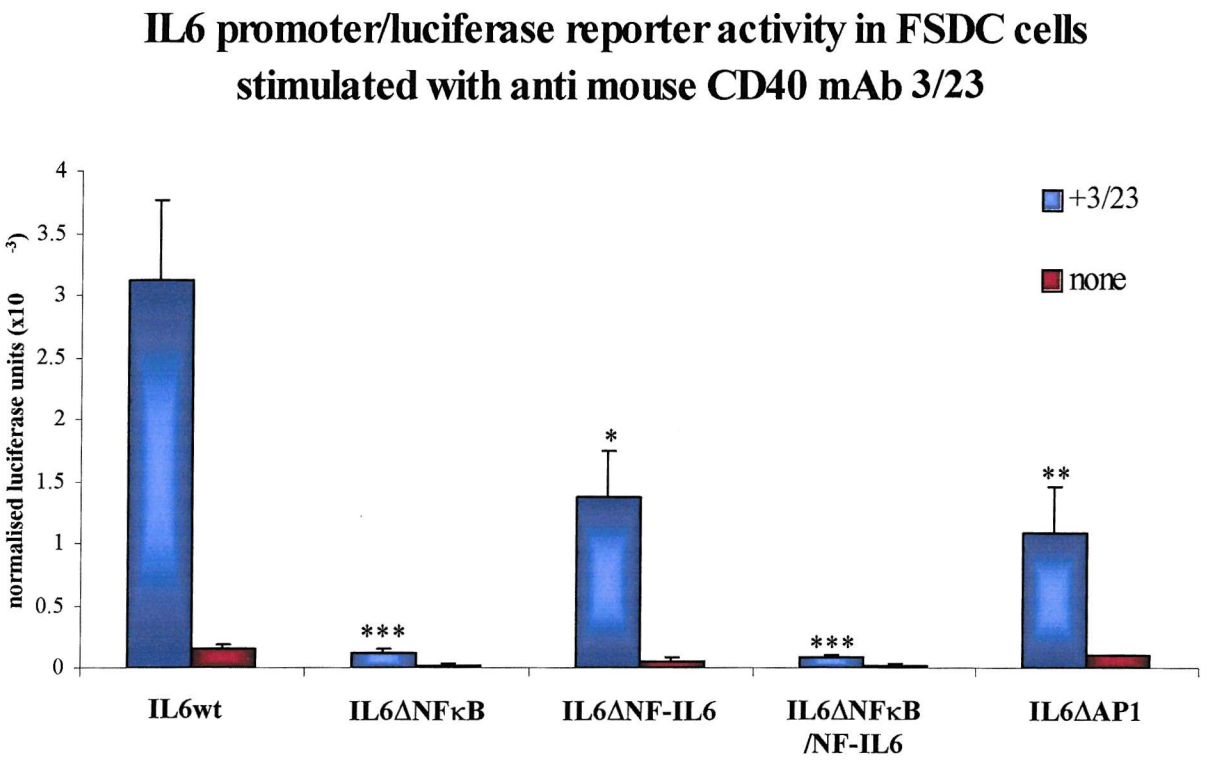


Figure 6.9- Activity of IL6 promoter constructs in FSDC cells stimulated through the endogenous CD40 by incubation with anti mouse CD40 mAb 3/23. FSDC cells were transfected with 30μg IL6/pGL3Enhancer and 30ng of pRLTK by electroporation. Single pulse at 960μF and 280V was used. Each transfected sample was split into two. 3/23 mAb was added to one half of each sample 24 hours after transfection at a final concentration of 30μg/ml. The cells were harvested 24 hours later. Luciferase assay was performed on lysed cell samples and the activity of IL6 promoter expressed as the ratio of firefly/renilla luciferase. n=4 for the samples shown. Statistical analysis was performed by Student's *t* test. *, ** and *** denote $p<0.05$, 0.01 and 0.005 respectively.

6.2.5 Confirmation of the requirement for activation of NFκB for CD40-mediated stimulation of IL6 gene transcription and the identification of NFκB DNA binding proteins in FSDC.

The data in fig 6.9 indicate the importance of the NFκB binding site of the IL6 promoter for induction of transcription in response to CD40 ligation on FSDCs. However, since NFκB binding sites can be the target for transcription factors unrelated to the NFκB (or Rel) transcription factor family, the data do not conclusively prove a role for inducible NFκB as a regulator of CD40-stimulated IL6 gene transcription. In order to build the case for such a role for NFκB, FSDCs were co-transfected with IL6wt promoter and an expression construct for a dominant negative form of IκBα (IκBD). IκBα controls the induction of NFκB activity in cells by forming a direct interaction with the transcription factor that masks domains involved in nuclear transport and DNA binding (Arnold *et al* 1995, Baldwin *et al* 1996). In response to a wide variety of stimulatory events, including ligation of CD40, the IKK complex is activated and brings about the phosphorylation of IκBα at its Ser 32 and Ser 36 residues. These phosphorylation events are pivotal in the induction of active NFκB since they target IκBα for ubiquitination and subsequently for proteasome mediated degradation which releases NFκB allowing it to translocate into the nucleus and affect transcription of genes carrying NFκB binding sites in their promoters (Baldwin *et al* 1996, Ghosh *et al* 1998 and fig 5.0). IκBD is modified such that both serine 32 and 36 are mutated into alanine residues, thus preventing phosphorylation of the protein at these critical sites and blocking the degradation of IκBα. IκBD therefore irreversibly binds NFκB into complexes that cannot be activated via the classical IKK pathway and as a result, cells in which IκBD is over-expressed are unable to mount an NFκB activitation response in the normal manner.

To determine the effects of over-expression of IκBD on induction of IL6 promoter activity by CD40 ligation, FSDC were transiently transfected with a combination of IL6wt promoter/luciferase (30μg), pRLTK (30ng) and either an IκBD (50μg) construct or its control empty vector pcDNA3 (50μg). 3/23 mAb was added to transfected cells 24 hours

later. After a further 24 hours the cells were harvested and luciferase assays performed. The results were expressed as a ratio of firefly/renilla luciferase activity. Figure 6.10 shows that cells transfected with IκBD construct displayed a dramatic reduction in IL6 promoter activity relative to cells transfected with pcDNA3. There appeared to be a low level of residual promoter activity following CD40 cross-linking, which supports the idea that other transcription factors (e.g. AP1) may be able to stimulate a low rate of inducible transcription in the absence of NFκB. However the degree of repression of CD40-inducible IL6 gene transcription by over-expression of IκBD, coupled with the previous data showing the requirement for an intact NFκB DNA binding site is strongly supportive for an absolute requirement for NFκB activation.

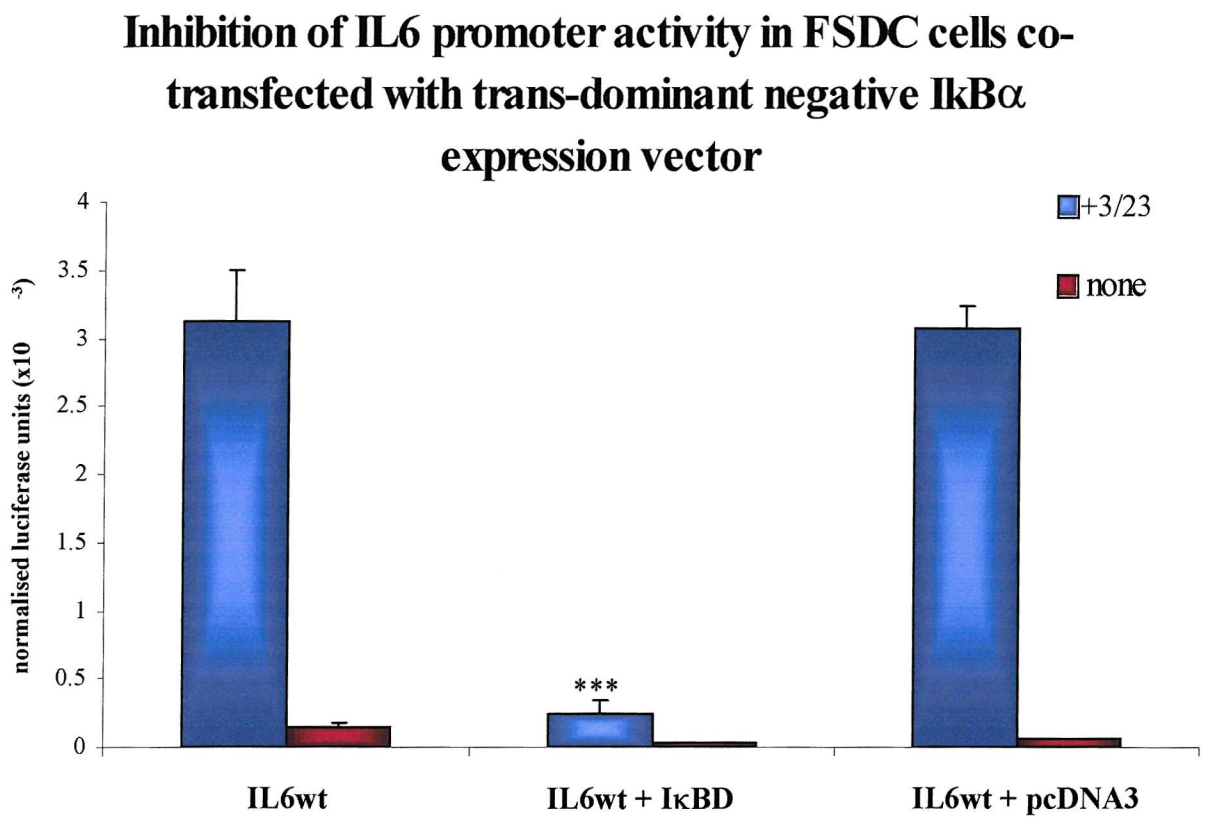


Figure 6.10- Activity of IL6 wild type promoter in FSDC cells co-transfected with trans-dominant negative IκBα expression vector (IκBD) and stimulated with 3/23 mAb. FSDC cells were transfected by electroporation with 30μg IL6/pGL3Enhancer, 30ng pRLTK and 50μg either IκBD/pcDNA3 or pcDNA3 empty vector. Single pulse at 960μF and 280V was used. Samples were split into two, and one half incubated with 30μg/ml 3/23 mAb 24 hours after transfection. Cells were harvested 24 hours after the addition of mAb, and luciferase assay performed on lysed cell samples. The activity of IL6 promoter was expressed as ratio of firefly/renilla luciferase in order to normalise results for transfection efficiency. n=4 for the samples shown. Statistical analysis was performed by Student's *t* test. *** denotes $p < 0.005$.

There have been no studies to date that have investigated the nature of the NFκB protein: DNA interactions that are induced by CD40 ligation in DCs. Indeed the majority of studies concerning activation of NFκB by CD40 have been performed in B cells, which are phenotypically very different from DCs. As active NFκB dimers can be composed of homodimeric and heterodimeric combinations of the different Rel proteins and since the expression of these different NFκB dimers can differ between cell types (Sha *et al* 1998, Karin 1999, Ghosh *et al* 1998, Baldwin *et al* 1996) it was considered important to precisely define the NFκB response in FSDC. To this end, EMSA was employed to determine if assembly of NFκB protein : DNA complexes is stimulated by CD40 ligation in FSDC.

EMSA experiments were carried out using a γ P^{32} end labelled double stranded DNA oligonucleotide corresponding to the NFκB DNA binding sequence of the IL6 promoter. The experiments were conducted according to the methods detailed in 2.2.28, 2.2.29 and 2.2.30 and in the legends describing figures 6.11 and 6.12. The first set of data was obtained using nuclear extracts from untreated and 3/23 mAb stimulated FSDCs. Figure 6.11 shows that FSDCs have active NFκB in the nucleus in their resting, unstimulated state (lane 4). This is apparent from the presence of a single protein: DNA complex (complex 1) on the gel that is seen as a radiolabelled band that is retarded in the gel relative to the free DNA probe that migrates to the bottom of the gel. The absence of this band in the control track (lane 1) that lacks addition of nuclear extract to the NFκB DNA probe shows that it has been assembled from the FSDC nuclear proteins and is not simply an aggregate of the probe. An important criterion when analysing protein: DNA interactions by EMSA is to confirm specificity of the complexes by determining the ability of unlabelled specific and non-specific double stranded oligonucleotides to compete with the radiolablled probe for protein binding. If the NFκB protein: DNA complex observed in FSDC was specific then pre-incubation of the nuclear extract with an excess of unlabelled NFκB oligonucleotides would result in loss of complex formation with the radiolabelled NFκB probe. By contrast, pre-incubation with an unrelated double stranded oligonucleotide would have no effect on protein binding to the NFκB probe, unless the interaction is non-specific in which case protein binding to the probe would be diminished. As shown in figure 6.11 assembly of NFκB complex 1 was competed for by incubation with excess (100-fold molar) unlabelled

NFκB probe (lane 8). However, an unrelated double stranded oligonucleotide corresponding to the binding site for a different transcription factor, Sp1 (lane 6) was unable to compete for complex formation. From these data it can be concluded that unstimulated FSDC express a constitutive specific NFκB DNA binding activity. Upon 24-hour stimulation with 3/23 mAb, the band seen in resting FSDC disappears, and instead two discrete bands (complexes 1 and 2) of lower electrophoretic mobility were observed (fig 6.11, lane 3). Both of these bands were also found to be NFκB specific, as they could not be competed by Sp1 (lane 5), but were competed by unlabelled NFκB oligonucleotide (lane 7).

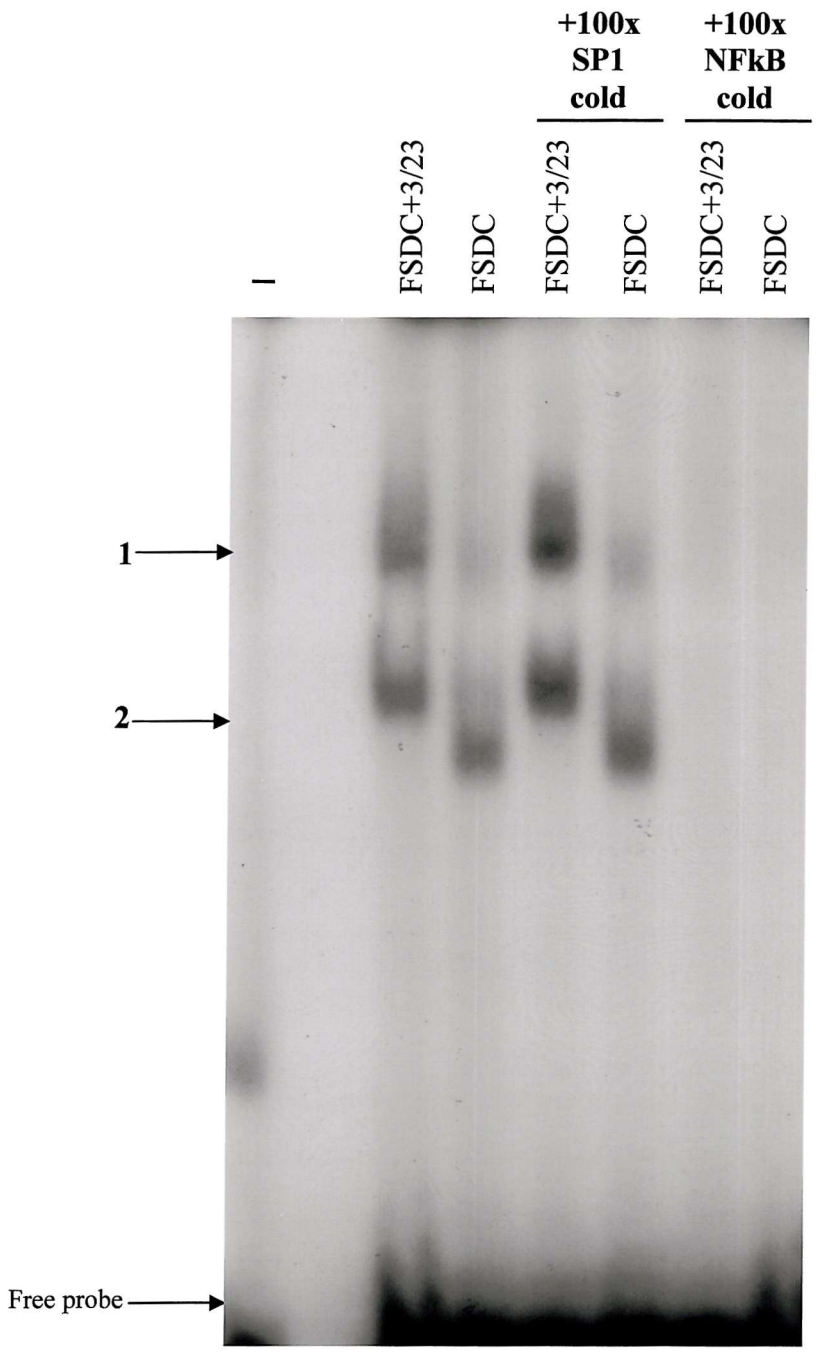


Figure 6.11- Identification of constitutively expressed and anti CD40 mAb inducible NFκB complexes in FSDC cell line. End labelled NFκB oligonucleotide was incubated with FSDC nuclear extracts from cells grown with or without 30μg/ml 3/23 mAb for 24 hours, either with no competitor, or in the presence of non-labelled competitor oligonucleotide at a 100 molar excess. Oligonucleotides used for competition are shown at the top of the figure. The complexes were separated by electrophoresis on an 8% native polyacrylamide gel. Specific DNA-protein complexes (1 and 2) are indicated by arrows.

Further experiments were performed to identify the protein subunits present in the NFκB complexes found in untreated and 3/23 treated cells, as shown in 6.12. This analysis involved the use of the antibody supershift/interference technique in which incubation of pre-formed protein: DNA complexes with Abs with specific immunoreactivity against a transcription factor can be used to confirm presence of the transcription factor in the complex (Kerr 1995). Three possible outcomes are observed with this technique. First the Ab fails to immunoreact with the complex and no change in complex formation is observed. Second, the antibody reacts with a protein component of the complex and forms a larger Ab containing complex. On the EMSA gel this is observed by a diminution in intensity of the original protein: DNA complex and appearance of a lower mobility Ab: protein: DNA complex, this is known as the supershift. The third outcome, referred to as Ab interference, also results from binding of the Ab to the protein component of the complex and involves diminution or loss of the original protein: DNA complex but no supershift is observed. This latter result is explained by either disruption of the protein: DNA complex due to conformational/steric effects of Ab binding or alternatively may arise from the formation of very large Ab: protein: DNA aggregates that are unable to enter the gel.

Nuclear extracts obtained from FSDCs stimulated for 24-hours with 3/23 mAb were used to generate protein: DNA complexes that were then incubated either alone (lane 2) or in the presence of anti-p50 mAb (lane 4), anti-p65 mAb (lane 6), anti-p50 + anti-p65 mAbs (lane 8) or with a mAb recognising the AP-1 transcription factor JunB (lane 10). Figure 6.12 shows that Anti p50 mAb caused a diminution of complex 3 and generated a supershift complex observed as a weak band of slightly lower mobility than complex 3. Anti p65 mAb also caused a reduction in the level of complex 3 but without a visible supershift. However, addition of both of these mAbs resulted in the complete loss of NFκB complex 1. Anti JunB had no effect on complex 1. From these data it can be concluded that the NFκB complex 1 induced by 3/23 ligation of CD40 on FSDCs consists of p50/p65 heterodimers. The second complex associated with 3/23 stimulated FSDCs (complex 2) was neither diminished or supershifted in the presence of any of the Abs. Further investigations into the identity of this complex were carried out in experiments to

follow. In addition, neither anti p50, anti p65, p50 + p65 or anti JunB had detectable immunoreactivity against the NFκB protein: DNA complex present in unstimulated FSDCs.

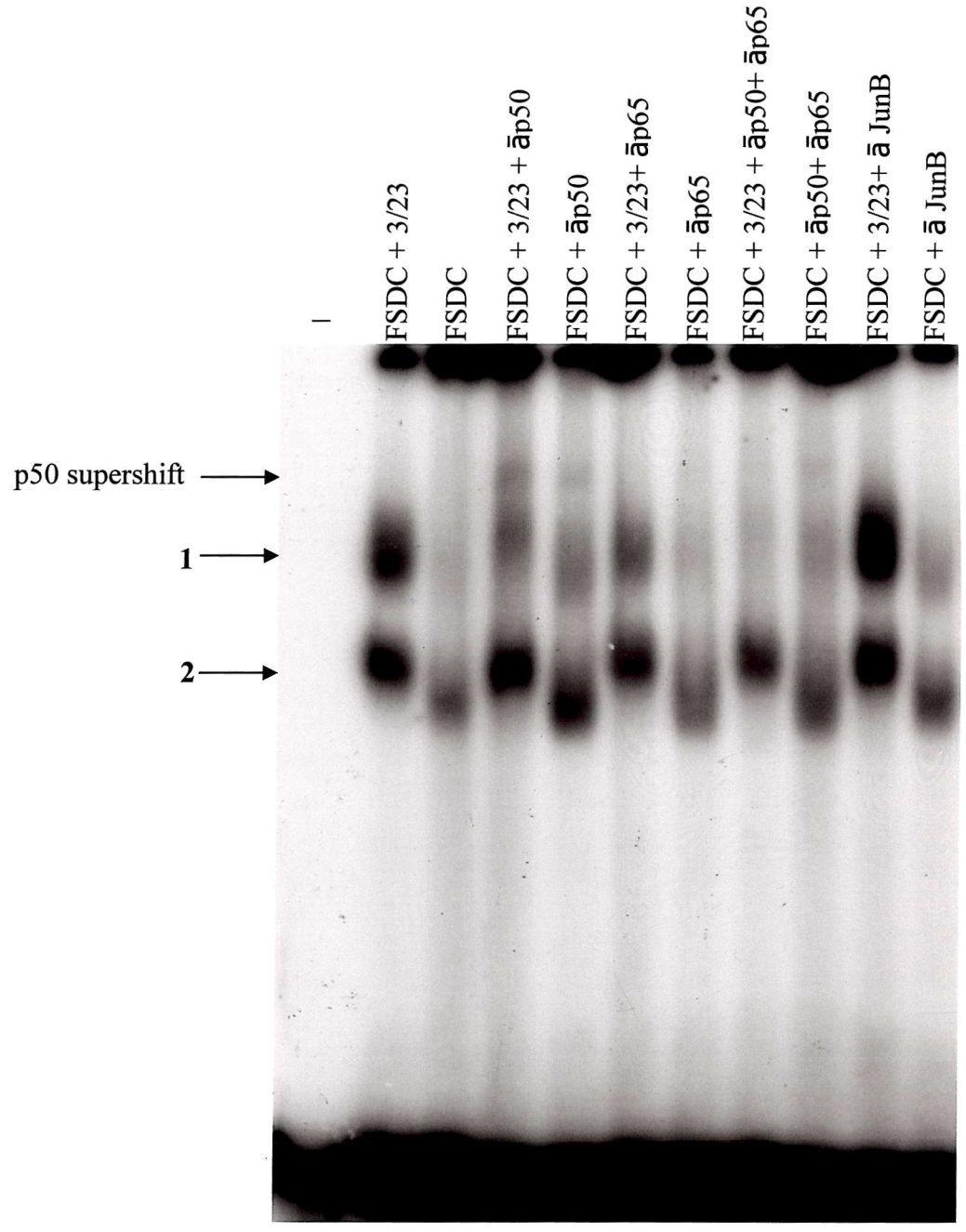


Figure 6.12 -Identification of constitutively expressed and anti CD40 mAb inducible NFκB complexes in FSDC cell line. End labelled NFκB oligonucleotide was incubated with FSDC nuclear extracts from cells grown with or without 30μg/ml 3/23 mAb for 24 hours. 2μg od antibody raised against p50 and/or p65 NFκB subunits were added to the complexes as indicated at the top of the figure. The samples were incubated with Ab for 16 hours at 4^0C. The complexes were separated by electrophoresis on an 8% native polyacrylamide gel. Specific DNA-protein complexes (1 and 2) are indicated by arrows, as is p50 supershift. Samples incubated with anti p65 Ab have decreased amount of NFκB binding activity in the samples, indicating that p65 is part of the complex, but that the anti p65 Ab has prevented the complex from entering the gel, rather than supershifting the subunit.

6.2.6 Discovery of an inhibitory NFκB DNA binding protein (CBF-1) in CD40 stimulated FSDCs.

Previous reports have shown that the NFκB DNA binding sites of the IL6, p52 NFκB subunit and cytochrome p450 gene promoters overlap with the binding site for the CBF-1 transcription factor. Studies in fibroblasts have shown that binding of CBF-1 to this site in the IL6 promoter causes a repression of transcription either by competing with NFκB dimers for DNA binding or/and by recruiting a large multimeric complex that includes histone deacetylases that modify histone structure into a state that is not favorable for transcription (Hsieh *et al* 1999, Kao *et al* 1998, Olave *et al* 1998). The possibility that NFκB complex 2 of 3/23 mAb stimulated cells may contain CBF-1 was therefore investigated since the NFκB oligonucleotide probe used for the EMSAs shown in figures 6.11 and 6.12 includes the overlapping CBF1 site.

To determine if complex 2 the lowest band contains CBF1, two separate experiments were performed. Firstly, a competition EMSA was used in which nuclear extracts from 3/23 stimulated FSDC were preincubated with a 100x molar excess of unlabelled mutated NFκB DNA binding sites prior to incubation with the γP^{32} labelled wt NFκB EMSA probe (fig 6.13). Pre-incubation with an NFκB binding site (NFκBΔp50/p65) lacking a nucleotide (G) critical for interaction of p50:p65 resulted in a failure of this double stranded oligonucleotide to bind p50:p65 confirming the protein identity of this complex as p50:p65 heterodimers. NFκBΔp50/p65 did however bind the protein responsible for assembly of complex 2. Pre-incubation of the same nuclear extract with an NFκB DNA binding site (NFκBΔCBF1) carrying two base point mutations (C and G, as shown in figure 6.13) designed to perturb CBF-1 binding failed to bind complex 2 but retained the ability to bind p50:p65 containing complex 1. This experiment indicates that assembly of complex 2 requires the presence of nucleotide sequences implicated in the specific interaction of CBF-1 with its DNA binding site.

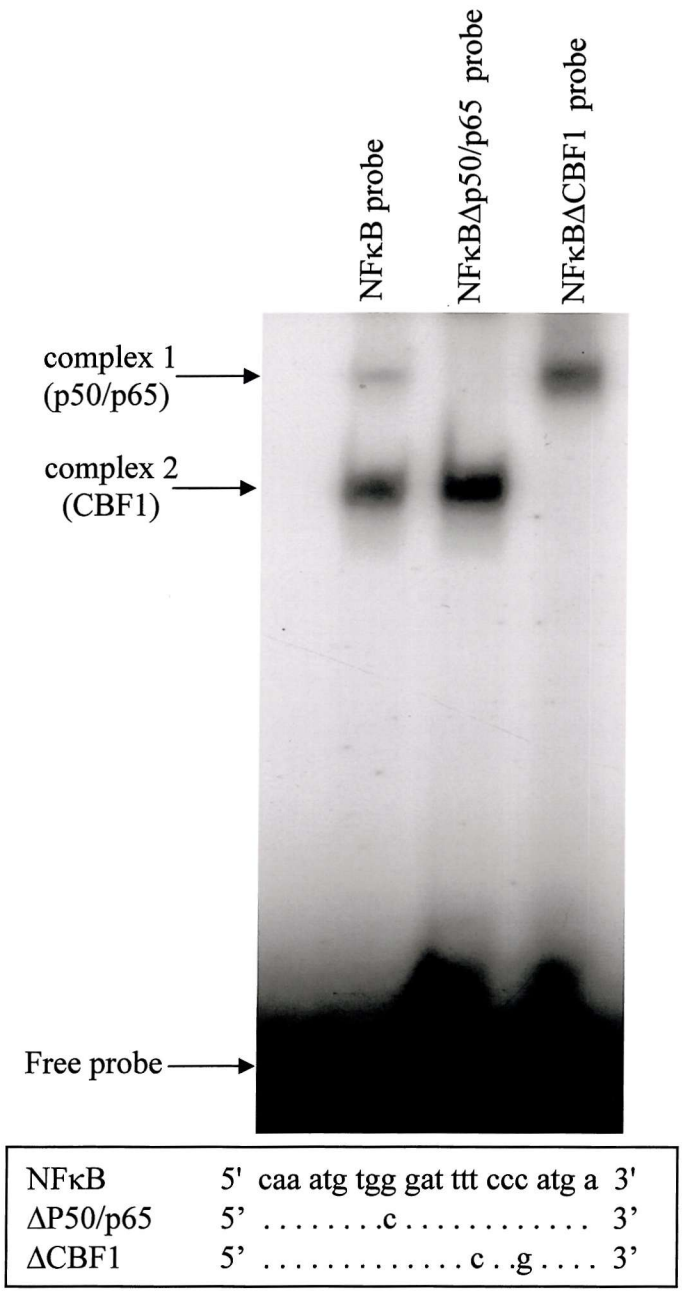


Figure 6.13- Identification of NFκB specific complexes by incubation with probes lacking p50/p65 or CBF1 binding sites. FSDC cells were treated with 30μg/ml of 3/23 mAb for 24 hours, and nuclear extracts isolated. 2μg of nuclear extracts were incubated with γP^{32} end labelled NFκB oligonucleotide (lane 1), or NFκB oligonucleotide lacking p50/p65 binding site (lane 2) or CBF1 binding site (lane 3). The results show absence of a higher complex in lane 2, and absence of lower complex in lane 3. This indicates that higher complex is formed of p50/p65 heterodimers, whereas CBF1 constitutes the lower complex.

Following these results, a second EMSA experiment was carried out in which anti CBF1 Ab was used as a supershift/interference reagent, as shown in figure 6.14. In this experiment, pre-formed NFκB DNA binding complexes were incubated with either anti p50 + anti p65, anti CBF1 or irrelevant (anti JunB) antibodies. The complexes in these reactions were then resolved followed by electrophoretic separation on a gel. The control anti JunB Ab was without effect on either complex 1 or 2 (lane 6) with similar intensities observed to those in the reaction lacking antibody (lane 3). As expected from previous results in this chapter, complex 1 was immunoreactive with anti p50 + anti p65 as demonstrated by a total loss of the complex (interference), whereas complex 2 was not recognised by the Abs (lane 4). However, the intensity of complex 2 was markedly reduced in the presence of anti CBF-1 Ab (lane 5). Lack of effect of anti CBF-1 Ab on the intensity of complex 1 showed that this effect was specific for complex 2. This result in combination with the competition EMSA data shown in figure 6.13 provide strong evidence that the NFκB DNA binding complex 2 of 3/23 mAb stimulated FSDCs is due to recruitment of CBF-1 to the overlapping CBF-1 binding site.

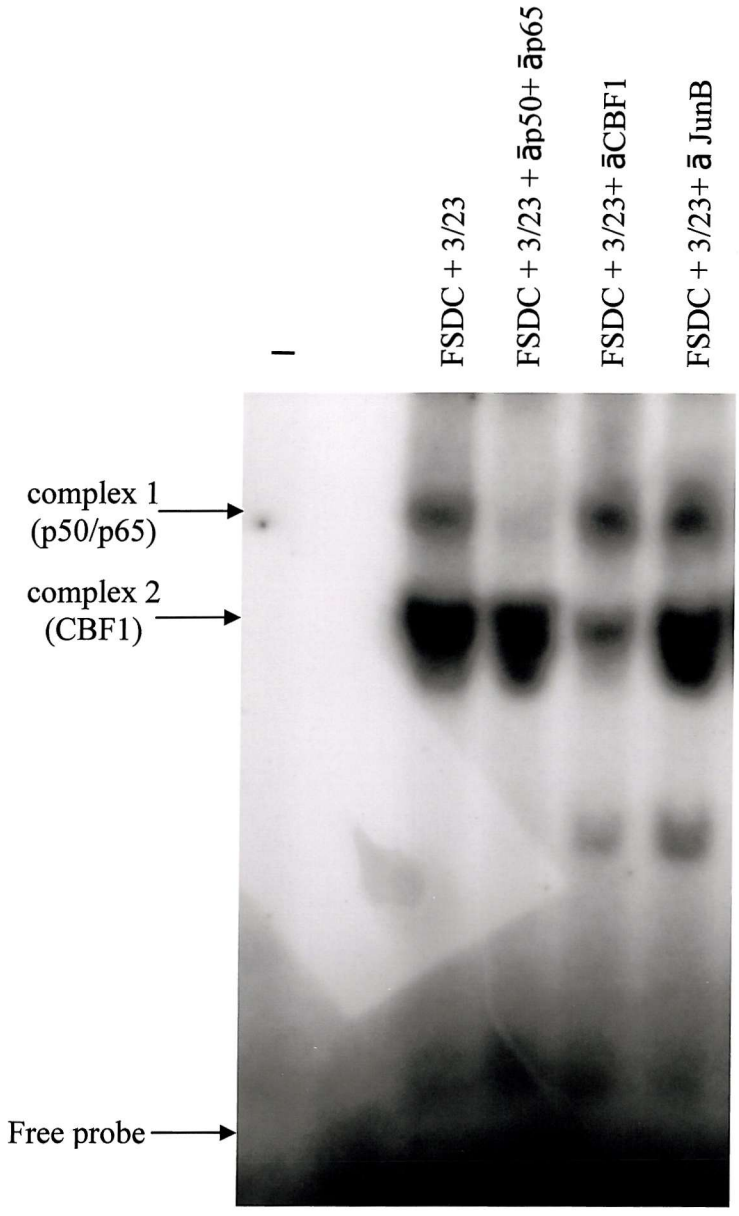


Figure 6.14- Determination of NFκB specific complex 3 identity by supershift. FSDC cells were treated with 30μg/ml of 3/23 mAb for 24 hours, and nuclear extracts isolated. 5μg nuclear extracts were incubated with γP^{32} end labelled NFκB oligonucleotide and 2μg of anti p50/p65, anti CBF1 or anti Jun B antibody. The mixtures were incubated for 16 hours and complexes separated by electrophoresis on a native 8% polyacrylamide gel. As shown in lane 2, cells sample not incubated with antibody has two bands. The sample in lane 3 had anti p50/p65 antibodies added, which caused reduction in the intensity of the top band, demonstrating presence of p50/p65 heterodimers. Lane 4 shows nuclear extracts incubated with anti CBF1 antibody, which caused reduction of the lower band. Anti Jun B antibody had no effect on the complexes. Therefore, the lower band in the sample is CBF1.

The manner in which CBF-1 and NFκB (p50:p65) DNA binding activity develop during the responses induced by 3/23 anti CD40 mAb in FSDCs was assessed. This was achieved by preparing nuclear extracts from cells incubated with 30μg/ml 3/23 mAb at time points 0, 2, 4, 6, 16, 24 and 48 hours after the addition of the mAb. As before, 2 μg of nuclear extracts obtained from the time course were incubated with γP^{32} labelled NFκB probe, followed by an EMSA. The result of this experiment is shown in figure 6.15. It can be observed that the induction of NFκB increased upon the addition of 3/23 mAb, to finally peak at 16h, and slowly reduce over the following 48 hours. However, CBF1 binding activity not only increases upon mAb treatment, but also continues to increase up to 48 hours after mAb, and possibly beyond (nuclear extracts not obtained for a later time point). The effects this may have on the expression of IL6 gene in these cells are discussed later.

Having demonstrated that CBF-1 is indeed present in the nuclear extracts of FSDCs treated with anti CD40 mAb, it was decided to examine what effect this factor may have on the activity of the IL6 promoter in FSDCs. Previous work suggests that CBF-1 acts as a repressor of IL6 promoter activity, however this work was restricted to studies on fibroblasts. If CBF-1 is also inhibitory for IL6 promoter function in FSDCs then forced over-expression of CBF-1 in FSDCs would be predicted to reduce activity of the promoter. To test this possibility, a CBF1 expression construct (pJH282) was co-transfected into FSDCs together with IL6wt promoter/ luciferase reporter. 24-hours post-transfection, cells were then cultured for a further 24-hours in the presence or absence of 3/23 anti CD40 mAb. An equivalent control experiment was carried out using empty expression vector pSG5 in place of pJH282 in order to assess for the effect of the vector on the outcome of the experiment. Data obtained from three separate experiments is shown in figure 6.16. Over-expression of CBF-1 reduced both the basal and inducible activity of IL6 promoter. The basal activity of the promoter was reduced by 3-fold, whereas the inducible activity was reduced by 4.5-fold. This result is in agreement with previous reports (Lee *et al* 2000, Palmieri *et al* 1999, Kannabiran *et al* 1997), which show that CBF1 can bind to the distal part of NFκB binding site within the promoter, thus preventing binding of NFκB, and resulting in repression of activity. In conclusion to these studies the NFκB-dependent

activation of IL6 gene expression by ligation of surface CD40 on FSDCs involves competition between activators (p50:p65) and a repressor (CBF-1) of transcription.

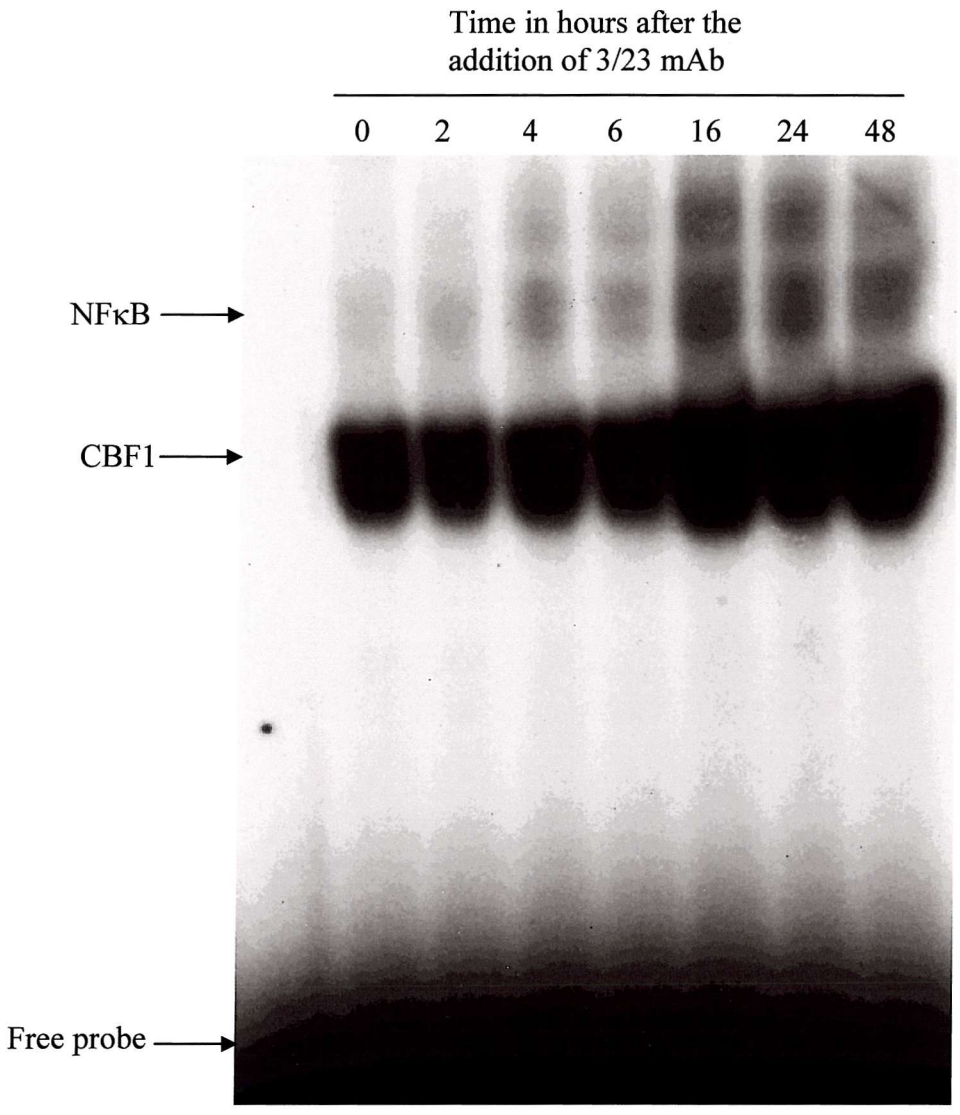


Figure 6.15- Time course of NFκB and CBF1 up-regulation with anti CD40 mAb treatment. FSDC cell samples were treated with 30μg/ml 3/23 mAb at time 0. Nuclear extracts were prepared from treated cells at 2, 4, 6, 16, 24 and 48 hours following the treatment. 5μg of extracts were incubated with γP^{32} end labelled NFκB oligonucleotide and complexes separated by electrophoresis on a native 8% polyacrylamide gel. There are two complexes present in the samples; the top which is NFκB p50/p65 heterodimers, and the lower band which is CBF1, as previously shown in figure 6.13 and 6.14. At time 0 there was a very small amount of NFκB present, which increased upon stimulation with 3/23 mAb to reach the peak of activation at 16 hours. The lower band representing CBF1 was present in a moderate quantity in the untreated cells, and was found to increase with 3/23 mAb stimulation of cells. This increase was also found to be continuous, reaching its maximum in the 48 hour sample. It was not determined if this increase in CBF1 continued after the 48 hour time point.

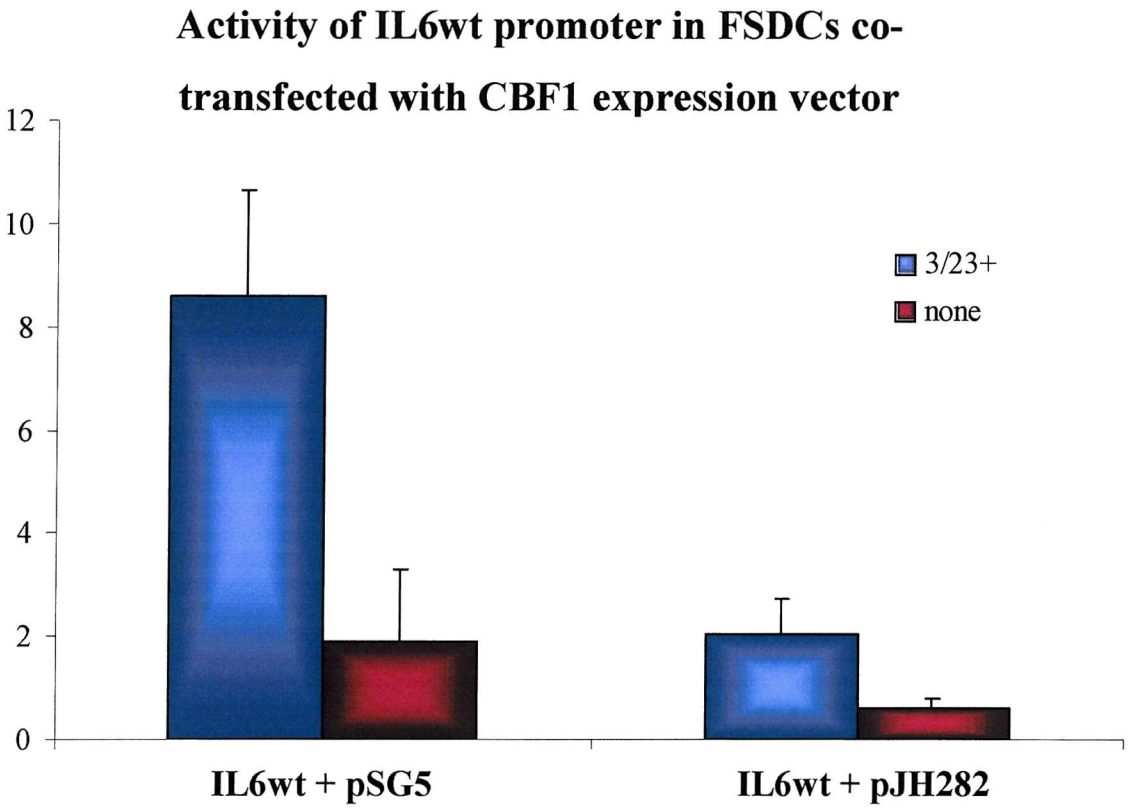


Figure 6.16- Activity of IL6 wt promoter/luciferase reporter in FSDCs co-transfected with CBF1 expression vector pJH282. The cells were transiently transfected by electroporation with 30ng, 30μg and 50μg of pRLTK, IL6wt/pGL3 Enhancer and pJH282, respectively. In the control experiment, empty vector pSG5 was used instead of pJH282. Each sample was split into two, and one half treated with 30μg/ml 3/23 mAb 24 hours after transfection. The samples were harvested 24 hours later, cells lysed and luciferase assay performed. Results obtained were expressed as a ratio of luciferase/renilla (pRLTK) activity, and given in a graph form where value for the untreated cells transfected with empty vector and IL6wt promoter was set as 1, and the remaining values expressed as fold induction or reduction. It can be observed that transient overexpression of CBF1 in this system causes a reduction in both basal and inducible activity of IL6 promoter as compared to the control experiment.

6.2.7 Assessment of the presence of AP1/DNA binding activity and identification of AP1 subunits forming the complexes in FSDC treated with anti CD40 mAb

As mutagenesis of the AP1 site of the IL6 promoter resulted in roughly a 50% drop in anti-CD40 stimulated transcription (fig 6.9) it was of interest to determine if engagement of surface CD40 induces AP1 DNA binding activity in FSDC. EMSA analysis of protein: DNA complex formation was carried out using a double stranded oligonucleotide probe carrying the AP1 binding site from the IL6 promoter. This was achieved by preparing nuclear extracts from FSDC cells incubated with 30μg/ml 3/23 mAb at time points 0, 2, 4, 6, 16, 24 and 48 hours after the addition of the mAb. 10μg of nuclear extracts obtained from the time course were incubated with γP^{32} end labelled AP1 oligonucleotide, followed by an EMSA. Figure 6.17 shows the results of this experiment. There was a small amount of AP1 in untreated FSDCs, which remained at this level during the first 4 hours of anti CD40 stimulation. At 6 hour point, there is a large increase of AP1 binding activity which continues for the following 10 hours. The 16 hour sample shows the peak of AP1 binding activity in FSDCs, which then slowly declines as detected by decreased AP1/DNA band in the 24 and 48 hour samples. Following this initial result, it was decided to assess the specificity of AP1/DNA binding in 0 and 16 hour samples, as they represent the lowest and the highest point of AP1 activity resulting from CD40 signalling. To this end, 10μg of nuclear extracts obtained at 0 and 16 hours were set up in triplicate. 100 fold excess of unlabelled Sp1 oligonucleotide was added to one set of extracts ten minutes before addition of the labelled AP1 probe, and 100 fold excess of unlabelled AP1 oligonucleotide was added to another set. The labelled AP1 probe was added to all three sets of nuclear extracts, which were then separated by electrophoresis on a native polyacrylamide gel. The result of this experiment is shown in figure 6.18. The ability of a 100-fold excess of unlabelled AP1 sites to compete for binding and lack of competition by a 100-fold excess of non-specific (SP1 site) double stranded oligonucleotides confirmed that the AP1 protein: DNA complexes in FSDC were specific and saturable (fig 6.18).

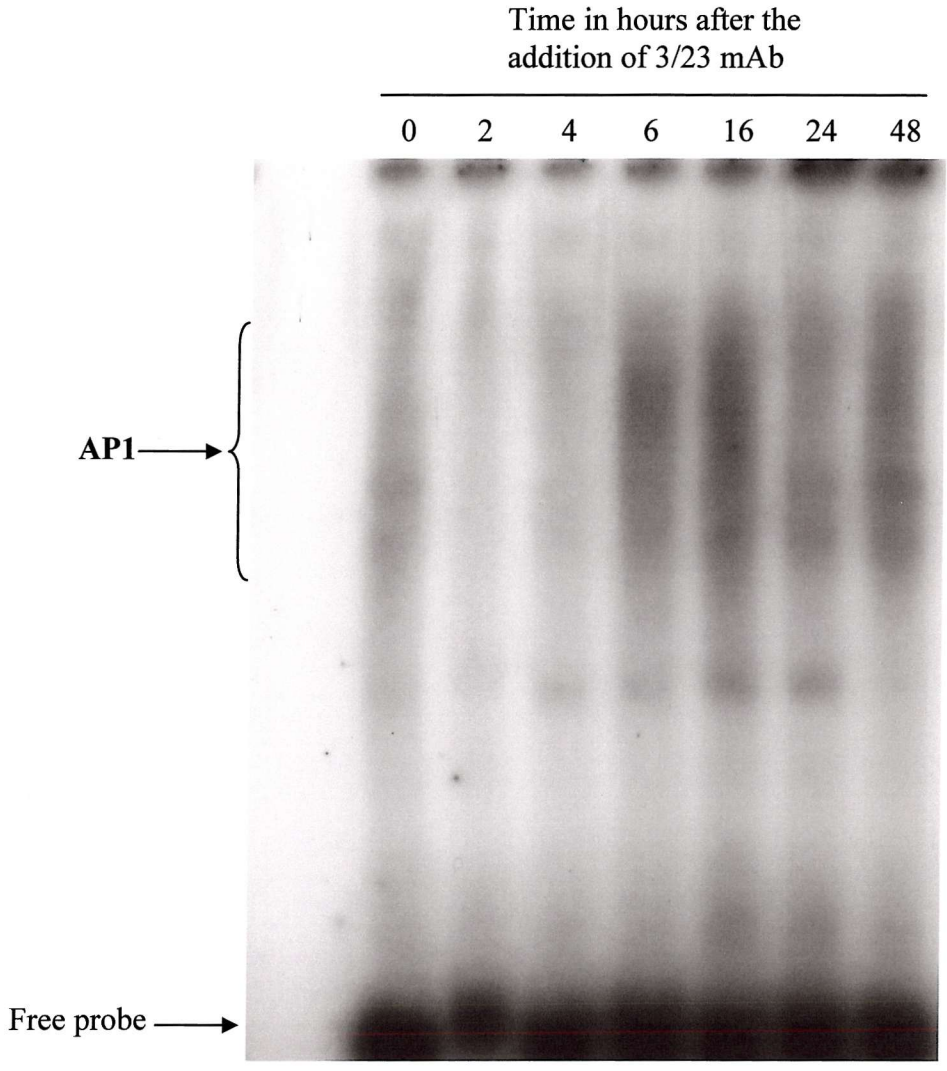


Figure 6.17- Time course of AP1 up-regulation with anti CD40 mAb treatment. FSDC cell samples were treated with 30μg/ml 3/23 mAb at time 0. Nuclear extracts were prepared from treated cells at 2, 4, 6, 16, 24 and 48 hours following the treatment. 5μg of extracts were incubated with γP^{32} end labelled AP1 oligonucleotide and complexes separated by electrophoresis on a native 8% polyacrylamide gel. At time 0 there was a very small amount of AP1 present, which increased upon stimulation with 3/23 mAb to reach the peak of activation at 16 hours.

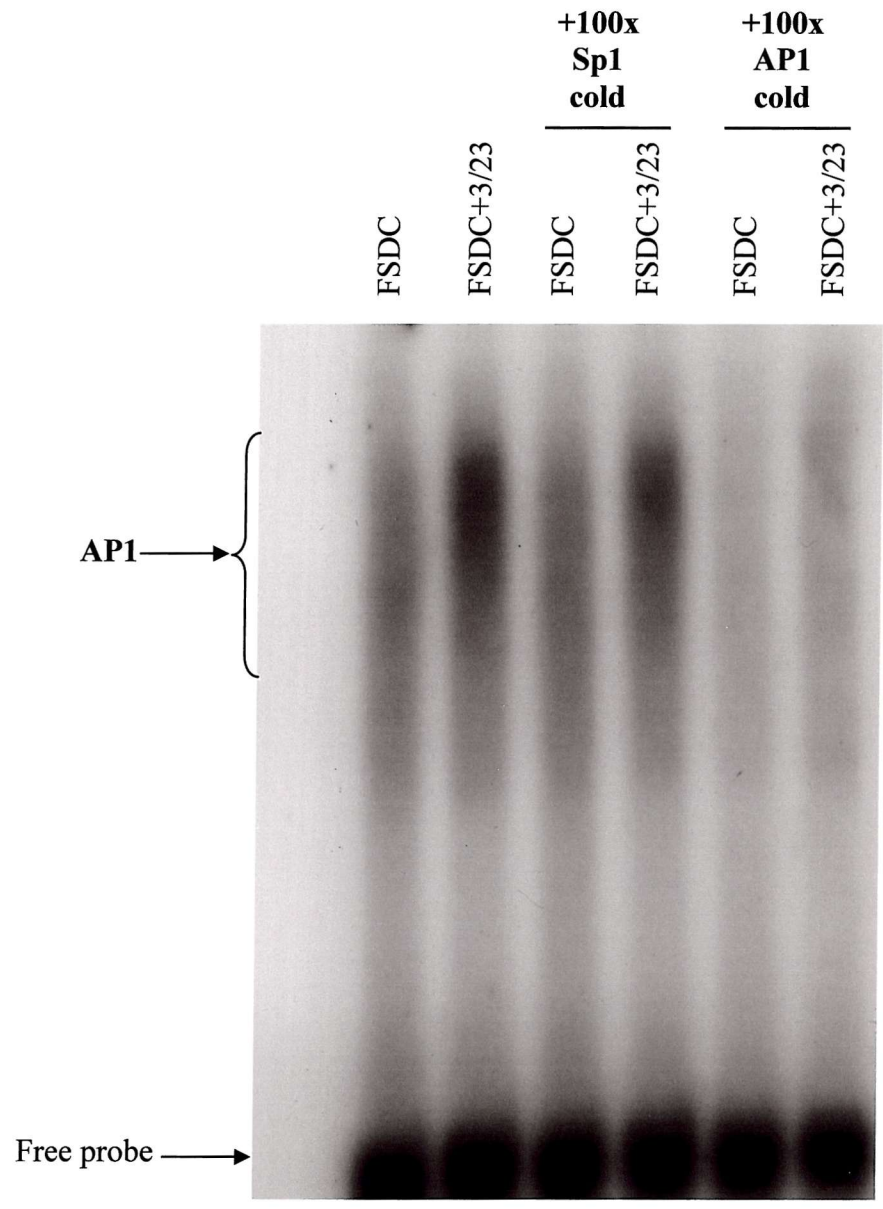


Figure 6.18- Identification of constitutively expressed and anti CD40 mAb inducible AP1 complexes in FSDC cell line. End labelled AP1 oligonucleotide was incubated with FSDC nuclear extracts from cells grown with or without 30μg/ml 3/23 mAb for 24 hours, either with no competitor, or in the presence of non-labelled competitor oligonucleotide at a 100 molar excess. Oligonucleotides used for competition are shown at the top of the figure. The complexes were separated by electrophoresis on an 8% native polyacrylamide gel. Specific DNA-AP1 complexes are indicated by an arrow.

As transcriptionally active AP1 complexes must contain a Jun (c-Jun, JunB or JunD) factor either in homodimeric (Jun:Jun) or heterodimeric (Jun in partnership with a Fos family protein), a supershift/antibody interference was used to identify the Jun components of the CD40-inducible AP1 DNA binding activity of FSDC. Supershift EMSA was set up to identify the subunits involved in the AP1 complexes. 10μg samples of nuclear extract obtained from a FSDCs treated with 3/23 mAb for 16 hours was incubated with AP1 probe. 2μg of Ab raised against c-jun or Jun D or Jun B subunits of AP1 was added to nuclear extracts, and mixture incubated for 16 hours at 4^0C. A control sample was also set up using an irrelevant Ab raised against Sp1. The samples were then separated by electrophoresis on an 8% native polyacrylamide gel, which was dried and exposed to a film. The result of the supershift EMSA is shown in figure 6.19. As shown in figure 6.19, antibodies recognising c-Jun, JunB and JunD reduced the intensity of AP1 DNA binding activity indicating that all three proteins may be present in the complex. However, JunB and JunD antisera not only reduced complex formation to a higher degree than the c-Jun antisera, but they also promoted the assembly of readily detectable supershift complexes. Hence JunB and JunD are likely to be the predominant Jun components of the induced AP1 complexes. Figure 6.19 also shows that addition of an antibody raised against c-Fos interfered with AP1 complex formation, hence CD40 signalling may induce Jun:Jun and Jun:Fos AP1 dimers. Lack of supershift or interference in EMSA reactions incubated with anti-SP1 antisera confirmed specificity of the results obtained using the Jun and Fos antisera.

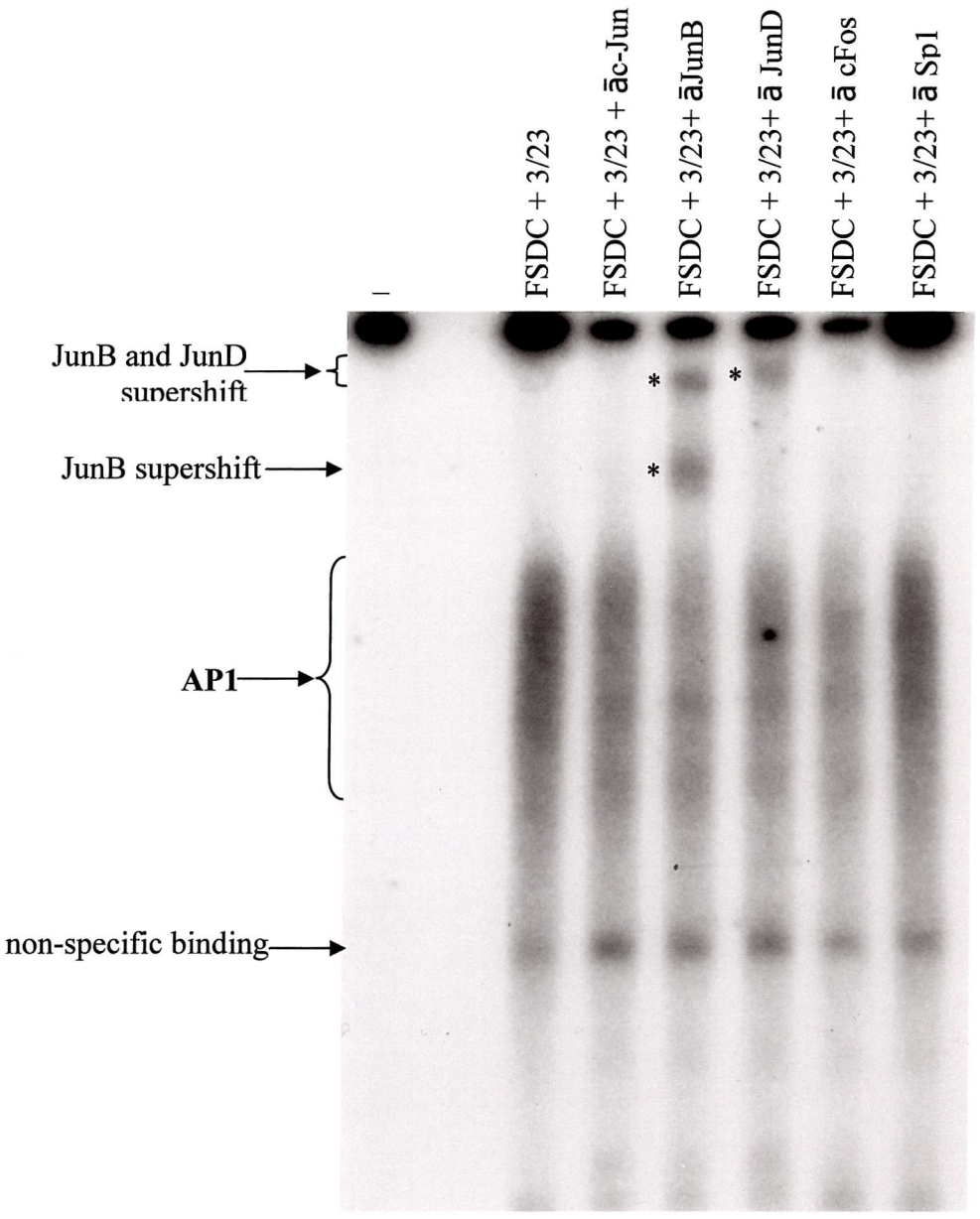


Figure 6.19 -Identification of constitutively expressed and anti CD40 mAb inducible AP1 complexes in FSDC cell line. End labelled AP1 oligonucleotide was incubated with FSDC nuclear extracts from cells grown with or without 30μg/ml 3/23 mAb for 24 hours. 2μg od antibody raised against c-Jun, JunB, JunD or cFos subunits were added to the complexes as indicated at the top of the figure. The samples were incubated with Ab for 16 hours at 4^0C. The complexes were separated by electrophoresis on an 8% native polyacrylamide gel. Specific AP1-protein complexes are indicated by arrow, as are JunD and JunB supershifts. Individual supershifts are marked by a star. Samples incubated with anti c-Jun/cFos Ab have decreased amount of AP1 binding activity in the samples without an observable supershift, indicating that both of these AP1 subunits may form a part of the CD40 induced AP1 complex.

As changes in AP1 activity can be regulated by either transcriptional or post-translational events it was of interest to establish if anti-CD40 treatment of FSDC alters the expression of Jun proteins. Figure 6.20 shows a Western blot analysis of Jun protein expression in the cytoplasmic and nuclear fractions of unstimulated and anti-CD40 stimulated FSDC. Western blots were performed according to section 2.2.31 and as described in 6.20 figure legend. All three Jun factors were expressed in both the cytoplasm and nucleus of FSDC. The cytoplasmic pool of the Jun factors was unchanged upon stimulation with anti-CD40, by contrast nuclear levels of c-Jun were slightly elevated while nuclear JunB expression was strongly induced.

The last experiment involving AP1 was set up in order to asses what consequences the presence of dominant negative Jun D (DN Jun D) might have on the activity of IL6 promoter in FSDCs. The mutant JunD protein expressed from this vector lacks a functional transactivation domain. As the different Jun proteins are able to interact with each other to form functional AP1 binding dimers, over-expression of the dominant negative JunD will sequester c-Jun, JunB and JunD proteins into functionally inactive dimers. To this aim, the cells were transiently transfected with IL6 wt promoter and DN Jun D, stimulated through CD40, and results plotted on a graph shown in figure 6.21. The data indicates that AP1 proteins contribute to the inducible activity of IL6 promoter, but do not regulate its basal activity. The equivalent levels of IL6 wt promoter basal activity in control show this and DN Jun D transfected samples. The inducible activity with anti CD40 mAb measures a 2-fold increase in the presence of DN Jun D, as opposed to a 9.5 fold induction in control samples containing the empty vector. This data, therefore, confirms the findings outlined in the figure 6.9, which shows that the lack of AP1 binding site in the IL6 promoter leads to a reduction in inducible activity of the promoter, without any effect on basal levels of IL6 promoter activity.

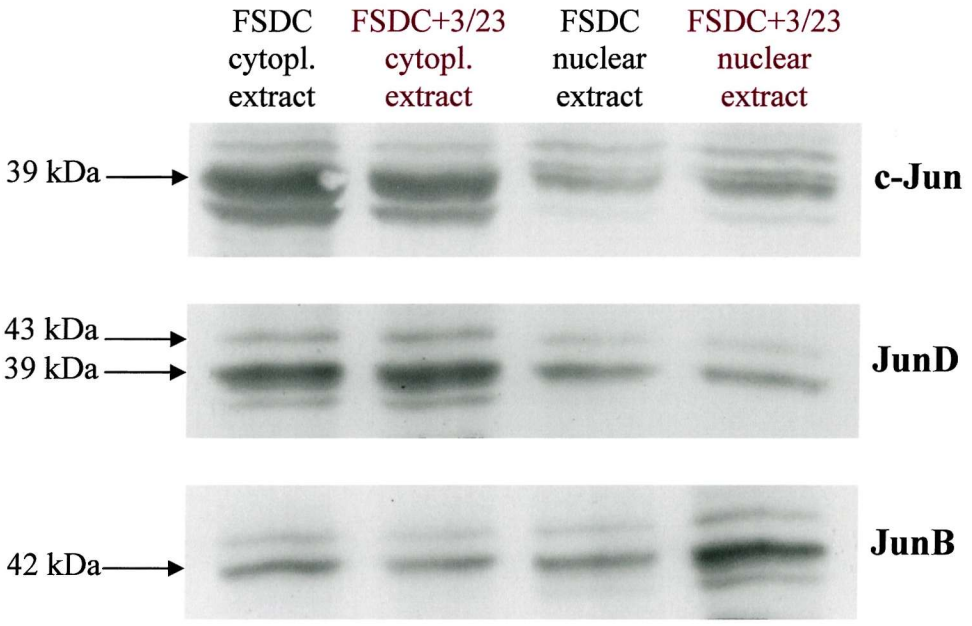


Figure 6.20- Detection of c-Jun, JunD and JunB proteins in the cytoplasm and nucleus of FSDC samples treated with 3/23 mAb or untreated samples. Cytoplasmic or nuclear proteins were isolated from FSDCs treated with 30μg/ml 3/23 mAb for 24 hours, or from untreated cells. 30μg of protein from each sample was denatured in SDS/DTT sample buffer (as outlined in 2.2.31) by boiling, and the samples were loaded onto a 9% SDS polyacrylamide gel alongside prestained protein markers. The gels were run for ~1 hour at a constant 160V. The proteins were transferred onto nitrocellulose membrane (as 2.2.31), which was probed using anti c-Jun, anti JunB and anti JunD Abs at 1μg/ml in blocking buffer. Specific Ab binding was detected by chemiluminescence. Incubation of FSDCs with 3/23 was found to increase the amount of JunB and c-Jun in the nucleus, whereas cytoplasmic pool of Jun proteins appeared to be unaffected by CD40 signalling.

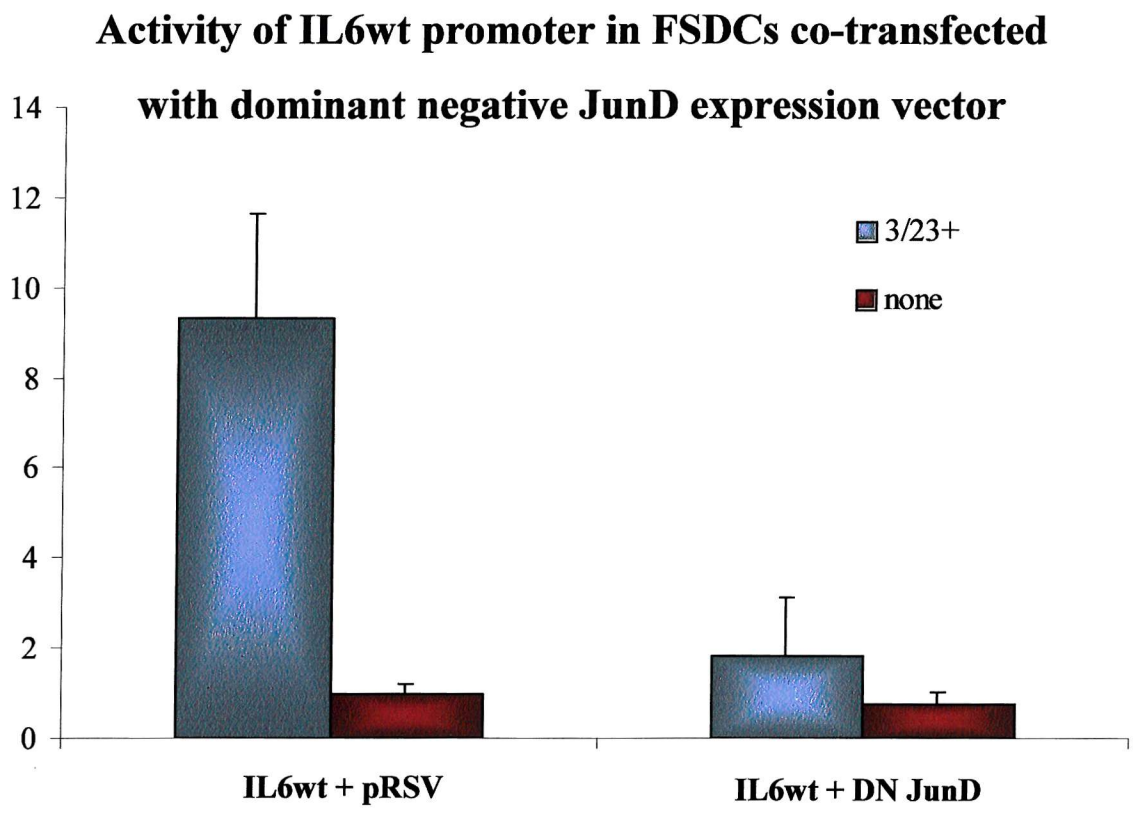


Figure 6.21- Activity of IL6wt promoter/luciferase reporter in FSDCs co-transfected wih dominant negative expression vector (DN JunD). The cells were transiently transfected by electroporation with 30ng, 30μg and 50μg of pRLTK, IL6wt/pGL3 Enhancer and RSV-DN JunD, respectively. In the control experiment, empty vector pRSV was used instead of RSV-DN JunD. Each sample was split into two, and one half treated with 30μg/ml 3/23 mAb 24 hours after transfection. The samples were harvested 24 hours later, cells lysed and luciferase assay performed. Results obtained were expressed as a ratio of luciferase/renilla (pRLTK) activity, and given in a graph form where value for the untreated cells transfected with empty vector and IL6wt promoter was set as 1, and the remaining values expressed as fold induction or reduction. It can be observed that transient overexpression of DN JunD in this system causes a reduction in inducible activity of IL6 promoter as compared to the control experiment. However, no difference is observed in the basal activity, thus confirming the result shown in figure 6.9.

6.2.8 Identification of CD40 residues important in receptor signalling to IL6 promoter

The experiments described so far have established that signalling through endogenous CD40 leads to transcriptional activity of IL6 promoter. The next series of experiments were carried out in order to identify residues within the CD40 molecule that are required for transducing the intracellular signals that impact on the IL6 promoter. The strategy employed was to co-transfect the IL6wt promoter/luciferase reporter with expression vectors for wt and mutant hCD40 proteins (as described in chapter 3) into FSDCs. Following the transient expression of hCD40 proteins on the surface of FSDCs, the receptor was cross-linked using the anti hCD40 mAb, LOB7.6. The relative abilities of the wt and mutant hDC40 proteins to transduce intracellular signal transduction events that stimulate IL6 gene transcription was determined by measurement of luciferase activity.

The residues mutated within the hCD40 receptor were shown in previous studies to affect signalling by preventing binding of TRAFs or Jak3, as discussed in the 1.6.4, 1.6.5 and in chapter 3. The TRAFs 2,3 and 5 bind the ^{250}PVQET^{254} motif in the tail of CD40, while Jak3 binds in the cytoplasmic region termed box 1, ^{222}PTNKAPHPK^{230}, but also requires box 2, with a sequence ^{260}PVTQEDGKESR^{270}, which presumably aids the correct conformation of the receptor (Hanissian *et al* 1997, Pullen *et al* 1998, Pullen *et al* 1999). The most important residue was found to be Thr254. Briefly, hCD40T254A and hCD40Δ262T254A both prevent binding of TRAF 2, 3 and 5 to the receptor, but not TRAF 6 (Ishida *et al* 1997, Lee *et al* 1999, Tsukamoto *et al* 1999, Pullen *et al* 1998). hCD40KKV should abolish binding of all known proteins which interact with CD40 as this deletion mutant only contains three intracellular amino acids, namely lysine-lysine-valine (KKV). This construct was therefore used as a negative control. hCD40T254S mutant retains its ability to be phosphorylated once the receptor is cross-linked, thus allowing TRAF 2, 3 and 5 to bind. Binding of other factors to this mutant should be unaffected. hCD40Δ262 deletion mutant has 17 C-terminal amino acids deleted, some of which form part of box 2, thus may be disrupting the interaction of Jak3 with the receptor (see 1.6.5). This deletion mutant may also affect binding of TRAFs, as Q263 has been shown to be of paramount importance for binding of

TRAF3, therefore hCD40Δ262 cannot bind this adapter protein. Binding of TRAF2 to this mutant is reduced, whereas TRAF6 interaction remains unaffected. The hCD40T254AΔ262 mutant has Q263 removed, thus preventing the binding of TRAF3 and in turn TRAF5. Mutation of T254A within the mutant abolishes the binding of TRAF2 as well, thus any remaining activity of this mutant would be a result of events downstream of TRAF6. Jak 3 binding to this mutant is unaffected in box1, but due to the absence of box 2, the interaction of Jak3 with the mutant receptor is unfavourable. The hCD40T254E mutation should have a similar outcome to that found in hCD40T254A.

Figure 6.22 shows the results of the experiments in which wt and mutant hCD40 proteins were tested for their relative abilities to induce IL6 gene transcription following ligation. Firstly, the results show that FSDC transfected with the IL6 promoter alone and in the absence of stimulation with an anti-CD40 antibody had very little basal activity as previously found in figure 6.9. Experiments performed with IL6 promoter either alone or co-transfected with hCD40KKV showed little basal and no inducible activity upon stimulation with anti human CD40 mAb LOB7.6. These results show that in the absence of transfected hCD40 protein, LOB7.6 mAb is unable to interact with FSDCs in a manner that will induce IL6 promoter activity. Cells transfected with the hCD40KKV expression construct will express a receptor that can specifically bind to LOB7.6 mAb, however lack of a cytoplasmic domain in this protein renders it incapable of triggering intracellular signal transduction cascades required for induction of IL6 gene transcription. In contrast to these negative control experiments, FSDCs transfected with the wt hCD40 expression vector displayed both a higher level of basal (without mAb treatment) IL6 promoter activity and a 5.3-fold induction of IL6 promoter activity upon hCD40 cross-linking with LOB7.6. The increased promoter activity observed in unstimulated cells can be explained as the consequence of spontaneous hCD40 clustering due to a transient over-expression of the receptor. Such clustering can lead to triggering of CD40 signalling in a similar manner to that observed for cells exposed to CD40 mAb or ligand (Kaykas *et al* 2001, Pullen *et al* 1999, McWhirter *et al* 1999, Morris *et al* 1999). The elevation of IL6 promoter activity by LOB7.6 mAb shows that the exogenous hCD40 protein is functional in FSDCs and most importantly provides a model for studying the structural requirements for hCD40 signalling in immature DCs. In

order to further assess the requirement for an intact hCD40 protein FSDC were also transfected with an expression vector for a hCD40/hCD22 fusion protein. This construct consists of the hCD40 extracellular and transmembrane domain fused to the hCD22 intracellular domain, which replaces the cytoplasmic domain of hCD40. Co-transfection with IL6 promoter/luciferase showed that the hCD40/hCD22 fusion protein was unable to stimulate IL6 gene transcription in response to ligation with LOB7.6 mAb. In this respect the fusion protein behaved in a similar manner to the truncated hCD40KKV protein and demonstrates the absolute requirement for structures in the cytoplasmic domain of CD40 that help mediate the activation of IL6 gene expression. Inability of the CD22 cytoplasmic domain to compensate for lack of these structures indicates that they are highly specific to CD40 and critical for signal transduction in FSDCs.

Figure 6.22 also shows the results of cells transfected with the hCD40T254A and hCD40T254E mutants, both of these mutant proteins displayed similar reduced abilities to induce IL6 promoter activity relative to wt hCD40. Over-expression of each mutant receptor gave rise to a low basal level of IL6 promoter activity, which was increased by only 1.43 and 1.5 fold upon hCD40 cross-linking with LOB7.6 in hCD40T254A and hCD40T254E transfected cells respectively. Hence the majority of inducible IL6 gene transcription is lost by mutation of T254 demonstrating the critical nature of this residue for signal transduction in FSDCs. hCD40T254A and hCD40T254E proteins are unable to bind TRAF2, TRAF3 or TRAF5, however they retain the ability to bind TRAF6 and Jak3 (Hanissian *et al* 1997, Tsukamoto *et al* 1999, Pullen *et al* 1999, Ishida *et al* 1996). Therefore, the low level of residual inducible IL6 promoter activity in cells expressing these proteins may be due to TRAF6 and Jak3 signalling to downstream effector molecules. The hCD40T254S mutant represents a more conservative structural attenuation of the cytoplasmic tail, generating a protein that can bind a similar range of adaptor signalling molecules as the wt protein and in studies by other investigators has been shown to support CD40 signalling (Hostager *et al* 1996). However, as shown in figure 6.21, FSDCs co-transfected with the hCD40T254S expression vector and the IL6 promoter/luciferase construct in FSDCs displayed a basal promoter activity that was consistently higher than that measured in cells transfected with the hCD40wt expression vector. Therefore, although

LOB7.6 treated hCD40T254S transfected FSDC expressed 90% of the level of IL6 promoter activity of LOB7.6 treated cells transfected with wt hCD40, the fold induction of non-stimulated versus stimulated hCD40T254S transfected FSDC was only 2.6, as opposed to 5.3 with hCD40wt. The reason for the higher basal activity of the IL6 promoter in cells transfected with hCD40T254S is not known. However, it is tempting to speculate that phosphorylation events and/or adaptor protein binding at the Ser residue may differ from those occurring with the wt Thr residue that lead to an elevated level of basal signalling for the mutant protein.

In order to further define the structural requirements for hCD40 signalling, two additional mutant constructs were tested. hCD40T254AΔ262 is also unable to bind TRAF2, 3 or 5 as it actually lacks the binding site for TRAF3 (Q263) and therefore also TRAF5. The deletion of 17 C-terminal amino acids in this mutant also leads to partial deletion of box 2, which is required for Jak3 binding to the receptor. As hCD40T254AΔ262 induces IL6 promoter activity to the same extent as hCD40T254A, this may imply that Jak3 does not have a role in CD40 signalling to IL6 promoter. Most of the residual activation of these mutant receptors may therefore be attributable to TRAF6 binding, which is unaffected in both hCD40T254A and hCD40T254AΔ262. However, in order to formally rule out a role for Jak3 it would be necessary to attenuate function of this factor while retaining the potential for TRAF2,3 and 5 to bind to CD40. Finally, the hCD40Δ262 mutant was tested in the IL6 transcription assay. The expressed protein supported a fold induction of 2.6 in LOB7.6 treated cultures relative to unstimulated cells. Again, this apparent lower level of induction compared to cells transfected with wt hCD40 can be attributed to a higher level of basal activity of the IL6 promoter in hCD40Δ262 expressing cells rather than a reduced level of CD40-induced transcription. hCD40Δ262 cannot bind TRAF3 or 5, and it also lacks box 2, thus it is also unable to bind Jak3. Binding of TRAF2 to hCD40Δ262 is reduced to about 50% of the normal level, while TRAF6 binding is unaffected (Tsukamoto *et al* 1999, Pullen *et al* 1999). In combination with results obtained with hCD40T254Δ262, the data suggest that TRAF2 is the main adapter protein leading to down-stream effects important in the induction of IL6 promoter activity in FSDC, whereas TRAF3, 5 and 6 can contribute to a lesser extent.

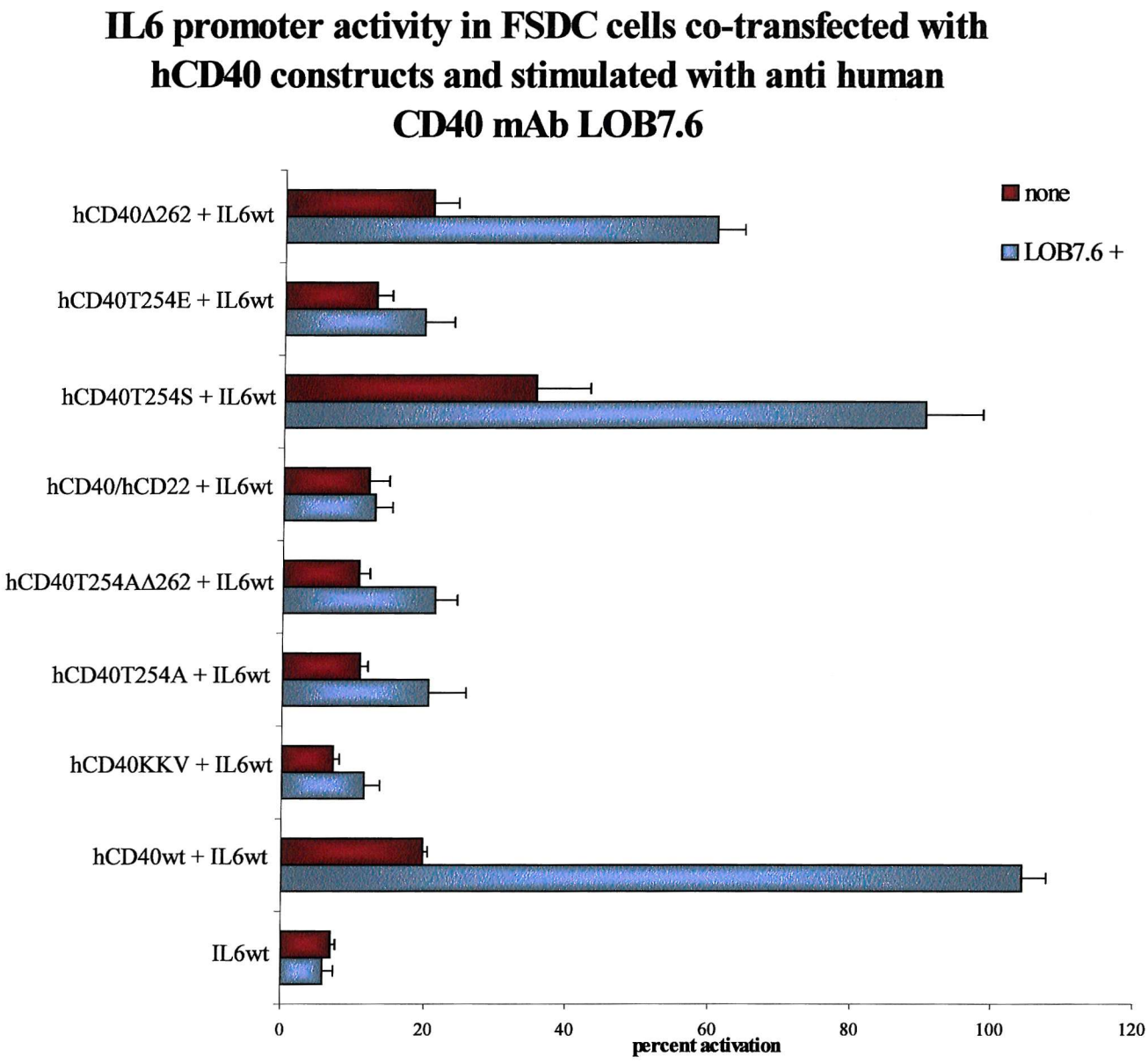


Figure 6.22- IL6 promoter activity in FSDC cells co-transfected with hCD40 constructs and stimulated with anti human CD40 mAb LOB7.6. FSDC cells were transfected with 30μg,30ng and 50μg of IL6/pGL3Enhancer, pRLTK and hCD40 expression construct, respectively, by electroporation. Single pulse at 960μF and 280V was used. The samples were split into two, and LOB7.6 at 30μg/ml was added to one half of each sample 24 hours after transfection, and cells harvested 24 hours later. Luciferase assay was performed on lysed cell samples and the activity of IL6 promoter expressed as the ratio of firefly/renilla luciferase. The number of repeats for the samples varies from 10 for hCD40KKV, hCD40wt and IL6/pGL3 alone, 7 for hCD40T254A and hCD40Δ262, to 5 for the rest of the hCD40 constructs.

6.3 DISCUSSION

The work is this chapter was done to show the effects of CD40 signalling on IL6 promoter activity in FSDCs and to dissect the domains found in CD40 and likewise sites within the IL6 promoter which are necessary for this activity to occur. The data obtained have shown a variety of interactions taking place at a protein/DNA and protein/protein level. However, before discussing these subcellular events and their importance, the cellular interactions and effects of IL6 cytokine *in vivo* situation will be considered, followed by narrowing of attention towards the transcriptional events involved in the regulation of its expression.

At the beginning of this work, it was established that in our *in vivo* therapy system, the tumour antigens are probably cross-primed into DCs, which are in turn activated through the action of anti CD40 mAb (by cross-linking the CD40 receptor on their surface). The activated DCs are then able to present tumour antigens in their MHC class I molecules to precursor CTLs. Due to being activated, DCs are also able to deliver co-stimulation through IL12 and B7.1 and B7.2 glycoproteins, thus inducing maturation and proliferation of CTLs. Fully activated CTLs specific for tumours antigens are then able to clear the remaining tumour by direct action. Therefore, the two key events in the favourable outcome of anti CD40 therapy are activation and maturation of DCs and likewise, activation, maturation and proliferation of CTLs. The question which must be addressed is, what might be the role of IL6? Is there any evidence which would support the study of its induction in DCs in more detail?

There is evidence to suggest that CD40 signalling in DCs leads to their maturation and activation, both of which are thought to be indispensable to the outcome of anti CD40 mAb therapy *in vivo*. CD40 signalling in DCs induces IL6 in these cells. It is therefore of interest to find out how CD40 signalling might be regulating IL6 induction in these cells, thus providing the necessary cytokines for their maturation, which can act on the cells in an autocrine manner. It has been shown in figure 6.3 that DCs have constitutive levels of IL6 receptor, thus making the idea of autocrine action of the cytokine plausible.

IL6 is required for the process of DC maturation (Hoffmann *et al* 2001, Jonuleit *et al* 1997), and it can not only be produced by DCs themselves (Canque *et al* 2000, McRae *et al* 2000, de Saint-Vis *et al* 1998), but is actually a sign of an ongoing maturation process (Salio *et al* 2000, Kitajima *et al* 1996). It has been shown previously that human DCs are one of the major sources of IL6 in draining lymph nodes (Hope *et al* 1995), suggesting that this cytokine may have an important role in the induction of immune responses *in vivo* and that DCs may drive their maturation by secreting the necessary signalling molecules under appropriate conditions. Indeed, IL6 knockout mice have impaired immune and acute phase responses, and are unable to clear certain infections, as well as being increasingly susceptible to others (Nishimura *et al* 1999, Ladel *et al* 1997, Fattori *et al* 1994, Kopf *et al* 1994).

Thus far it has been shown that DCs can and do produce IL6 as a consequence of CD40 cross-linking and signalling. Having established this, the next question is- is there a role for IL6 in the induction of CTLs, which are also the key players in the *in vivo* therapy? A direct effect of IL6 on the proliferation of T cells has been documented by several groups, as has the ability of IL6 to prevent apoptosis of naive T cells. Other studies have described the ability of IL6 to induce IFN-γ secretion in differentiating T cells, indeed lack of this response in IL6 gene knockout mice renders animals unable to mount T cell responses against *Mycobacterium. tuberculosis* and *Toxoplasma. gonddi* (Suzuki *et al* 1997, Leal *et al* 1999). Other work has shown IL6 to be crucial in T cell proliferation (Pankewycz *et al* 1990, Walz *et al* 1991), in the induction of T cell growth (Kuhweide *et al* 1990, Kuhweide *et al* 1990), and as an accessory factor for T cell activation (Vink *et al* 1990). More interestingly, the study by Saijo *et al* (1999) shows that CTLs against human lung cancer can be efficiently generated *in vitro* by IL-1β, IL-4, and IL6 cytokines in combination via possible dendritic cell induction. It can be envisaged that similar a cytokine combination may be present *in vivo* during the induction of CTLs in the anti CD40 mAb therapy system. Production of IL6 by CD40 stimulated DCs is therefore likely to be important in promoting the generation and survival of antigen-specific cytotoxic T cells.

The presence of IL6 *in vivo* has been shown in figure 6.1. The data, shown in this figure, were obtained by RT-PCR performed on spleen cDNA from treated and untreated mice which were given the tumour. Indeed, results of this preliminary study showed that in the absence of anti CD40 mAb treatment there was no IL6 in the spleen populated with increasing tumour load. However, upon anti CD40 treatment, there was a significant although transient induction of this cytokine in the spleen samples. These data were reassuring in suggesting that CD40 signalling treatment did lead to IL6 induction *in vivo*, although by using RT-PCR on whole spleen samples it was not possible to distinguish which particular cell type was producing the cytokine. Many different cell types can produce IL6 (Dechanet *et al* 1997, Hess *et al* 1995, Yellin *et al* 1995, Alderson *et al* 1993). It can therefore be envisaged that B cells, DCs, macrophages, fibroblasts, monocytes and other cell types may be contributing to the level of IL6 seen as the PCR products obtained by amplification of cDNA isolated from mice during the therapy. This initial experiment was performed to show that IL6 can be induced during a response in treated mice. The study which would more definitively show the importance of IL6 in the *in vivo* therapy, could be done by using IL6 blocking antibodies. Performing such a study would show what consequence such treatment might have on the outcome of anti CD40 therapy. It has been shown previously that blocking of IL12 and IFNγ in this manner prevents the therapeutic effect of anti CD40 mAb *in vivo* (Tutt A., unpublished data). Unfortunately, IL6 blocking studies could not be carried out at this time due to a lack of hybridomas producing effective blocking antibodies. It may become possible to carry out this important *in vivo* study in the future.

Prior to engaging in discussion about molecular events, one further idea has to be considered. As already stated, *in vivo* therapy required the presence of Th1 type cytokines. Although IL6 has been implicated in the establishment of Th2 type responses (Rincon *et al* 1997), it would be inadequate to brand any one cytokine as an inducer of only one type of response in an *in vivo* situation. IL6 has in certain models been associated with establishment of Th1 type responses during vaccination (Leal *et al* 1999), and in suppression of Th2 type responses in the lungs (Wang *et al* 2000). It is more likely that many different factors control the nature of immune responses, and cytokines augment such responses

perhaps not only by their presence, but more likely by the ratios of one type of cytokine to another. Therefore, it may be viewed as somewhat of a paradox that IL4 (a typical Th2 type cytokine) can potentate secretion of IL12 in DCs (a typical Th1 type cytokine) (Takenaka *et al* 1997), and that IL12 can induce secretion of IL4 in B cells as a part of a negative feedback loop (Skok *et al* 1999). For these reasons, IL6 and its effects in the context of DC maturation and T cell proliferation should be considered as an effect of a pleiotropic cytokine, rather than a Th2 type cytokine studied in the background of a Th1 response in the tumour rejection model.

Having satisfied the question of whether IL6 is induced *in vivo*, further studies were done to show if such an induction could be observed *in vitro* following CD40 cross-linking in FSDCs. It was shown that engagement of CD40 on FSDCs leads to a powerful, but transient induction of IL6 mRNA expression, which agrees with findings previously published by various groups suggesting that DCs respond to CD40 signalling by induction of IL12 and IL6 (Cumberbatch *et al* 1996, de Saint Vis *et al* 1998, Canque *et al* 2000, McRae *et al* 2000). This induction was maximal at 24 hours after addition of anti-CD40 to cultures and was followed by a rapid diminution of IL6 transcript to levels that were less than those found in unstimulated cultures. This will be further considered in the text to follow. Further experiments were set up to show that FSDC underwent maturation due to CD40 signalling, as detected by surface marker expression. CD40 signalling was found to dramatically increase surface expression of MHC class I and II, CD54, CD80, CD86 and CD4 24 hours after the addition of soluble anti CD40 mAb into the cell cultures.

The RT-PCR and FACS studies mentioned above provided the necessary preliminary data for this work. By investigating the structural requirements at both the CD40 cytoplasmic tail and the IL6 promoter and establishing changes in the activity of specific transcription factors, it was possible to determine the mechanisms underlying these CD40 stimulated responses in DCs. The IL6 promoter was weakly active in FSDC, as expected from the low but detectable level of IL6 transcript found in both FSDC and primary BMDC. Anti-mouse CD40 mAb 3/23 stimulated a powerful induction of IL6 promoter activity in FSDC, as did anti-human CD40 mAb when added to FSDC transfected with a human CD40 expression

construct. These observations contradict an earlier study using the B cell line CH12.LX in which it was shown that while membrane bound CD40L could induce IL6 promoter activity and mRNA expression, anti-CD40 mAb could not (Baccam and Bishop 1999). The precise reasons for the apparent discrepancy between this latter study and the present one are not known, however it is possible that there are inherent differences in either the cell lines or qualities of the antibodies used in the two studies. Whatever these differences may be, this study clearly demonstrates that engagement of endogenous mouse CD40 or transfected human CD40 by specific antibodies can induce IL6 promoter activity in FSDC. This induction was dependent on the presence of intact DNA binding sites for NF-κB and AP-1. Both basal and anti-CD40 inducible activities of the IL6 promoter were regulated by NF-κB. By contrast, AP-1 appeared to be mainly required for inducible IL6 promoter activity, since mutation of the AP-1 site of the IL6 promoter or over-expression of a dominant negative JunD protein only had a significant negative effect on the promoter in cells treated with anti-CD40.

Engagement of endogenous CD40 on FSDC by addition of mAb 3/23 resulted in a transient induction of AP-1 DNA binding activity that was maximal after 16 hours of stimulation. A single, but diffuse AP-1 DNA: protein complex was induced by 3/23 and consisted of all three Jun proteins together with at least one Fos family protein, c-Fos. Examination of Jun protein expression revealed that the major alteration in 3/23 stimulated cells was elevated nuclear expression of JunB. Although no significant changes were detected in the expression of c-Jun or JunD, the presence of both these proteins in the nucleus of FSDC, together with the ability to detect them in the anti-CD40-induced AP-1 complex indicates that post-translational events probably regulate their activity in FSDC. Of relevance to this idea, it is well established that engagement of CD40 results in the activation of the Jun N-terminal kinases (JNKs), which in turn are able to attenuate c-Jun and JunD activities (Schwabe *et al* 2001, Akimura *et al* 2001, Dodgostar *et al* 2000, Sakata *et al* 1999, Leo *et al* 1999, Park *et al* 1999, Sutherland *et al* 1999, Lee *et al* 1998). Previous studies in B cells have shown that engagement of CD40 induces c-Jun, JunB, JunD, c-Fos, FosB and Fra1 at either the mRNA or protein level (Grammer *et al* 1998, Sutherland *et al* 1999, Lee *et al* 1998, Sakata *et al* 1999, Leo *et al* 1999). However, few studies have investigated CD40 mediated induction of

AP-1 activity or Jun and Fos protein family expression in monocytes or DCs. Revy *et al* suggested that CD40 stimulation of human monocytes was associated with induction of NF-κB but not AP-1 (Revy *et al* 1999). Hence it is possible that monocytes and DCs differ in their ability to activate AP-1 in response to engagement of CD40, however confirmation of this idea requires detailed comparative studies. From data presented in this thesis it can be concluded that CD40 signalling in FSDC leads to the activation of AP-1 dimers inclusive of a variety of Jun:Jun and Jun:Fos combinations. It can also be concluded that these AP-1 dimers are transcriptionally active and in combination with NF-κB and NF-IL6 are able to stimulate the elevated levels of IL6 gene transcription observed in anti-CD40 stimulated FSDC.

Experiments shown in figure 6.22 using transfected wild type and mutant hCD40 expression constructs indicate that TRAF2 is the critical mediator of anti-CD40 stimulated NF-κB activity and IL6 gene transcription in FSDC. Having found which transcription factors have the crucial role in the IL6 promoter induction, another interesting set of findings came from the use of hCD40 expression vectors in FSDC co-transfected with IL6 wt promoter/ luciferase reporter. These studies attempted to work out the events regulating the expression of IL6 in FSDCs at the CD40 receptor end. Using anti human CD40 mAb LOB7.6, these studies suggest that TRAF2 regulates the majority of CD40 signalling to IL6 promoter in FSDCs. It was shown that TRAF3 and 5 can contribute about 20-25% of the inducible activity of the promoter, whereas TRAF6 can have a small effect, as can be seen in the graph in figure 6.22. Although TRAFs 2,3,5 and 6 have all been shown to have the capacity to signal to NFκB in other cell types, it would appear that in this system, TRAF2 is required for optimal inducible activity of NFκB transcription factors in the cells. Thus, in FSDCs, TRAF2 binds to hCD40 cross-linked by anti hCD40 mAb, which in turn leads to a signalling cascade resulting in the activation of NFκB and in turn activity of IIL6 promoter. Studies using mutant IL6 promoter have shown that this site is necessary in the promoter for its basal and inducible activity. Therefore, using mutant of both the promoter and receptor, we have been able to narrow down the signalling components in FSDCs which control CD40 induced IL6 gene transcription.

Activation of NF-κB is a widely documented consequence of CD40 signalling and is known to be required for many CD40-induced changes in gene expression (Hatzivassilian *et al* 1998, Hsing *et al* 1997, Schauer *et al* 1996, Hess *et al* 1995, Berberich *et al* 1994). There is also a wide literature concerning the role of TRAFs as mediators of CD40-induced NF-κB activation, however there is controversy regarding the requirement for specific TRAFs in this response (Hostager *et al* 1999, Hsing *et al* 1999, Hsing *et al* 1997, Pomerantz *et al* 1999, Lee *et al* 1999, Cheng *et al* 1996). Studies in 293 cells have shown that NF-κB and JNK can be activated by over-expression of TRAF2, TRAF5 and TRAF6, by contrast TRAF1, TRAF3 and TRAF4 are unable to activate either NF-κB or JNK when over-expressed (Pullen *et al* 1999). Based on the results, the role for TRAF3/5 heterodimers and Jak3 signalling could be ruled out by showing that a hCD40 protein lacking the most c-terminal 15 amino acids that are critical for binding these proteins (Hanissian*et al* 1997, Pullen *et al* 1999) retained the ability to induce IL6 promoter activities. By contrast a hCD40 protein carrying a single amino acid substitution T254A (hCD40T254A) that abolishes binding of TRAF2 in addition to TRAF3/5 and Jak3 displayed a markedly reduced stimulation of IL6 promoter activity in response to anti-CD40 (1.5-fold, compared to a 5-fold response with wild type hCD40). This data is therefore in agreement with the report by Lee *et al*, who showed that binding of TRAF3/5 and Jak3 was dispensable for CD40-induced activation of NF-κB, JNK and ICAM-1 promoter activity. However, other investigators have suggested that TRAF2 is dispensable for NF-κB activation (Hsing *et al* 1997, Hostager *et al* 1999), moreover at least two independent groups have reported that hCD40 carrying the T254A mutation is able to support activation of NF-κB (Pullen *et al* 1999, Hsing *et al* 1997). These latter studies suggested a role for TRAF6 as a potent activator of CD40-induced NF-κB activation since binding of TRAF6 does not require T254. The residual 1.5-fold induction of IL6 promoter activity that was observed in anti-CD40 stimulated hCD40T254A transfected FSDC may be due to TRAF6 signalling. Tsukamoto and colleagues recently showed that TRAF2 and TRAF6 link CD40 to NF-κB via distinct signalling pathways. TRAF2 utilises a pathway that requires activation of the NF-κB-inducing kinase (NIK), by contrast TRAF6 is able to activate NF-κB independently of NIK. Hence it is possible that the observed differences in the apparent requirement for TRAF2 or TRAF6 by different investigators could lie in the differential usage of the NIK-dependent and –independent signalling

pathways. Data shown in fig 6.22 indicates that the TRAF2/NIK pathway may predominate for CD40 activation of NF-κB and IL6 gene transcription in DCs, however further studies using dominant negative inhibitors of this pathway are required in order to confirm this idea.

Analysis of NF-κB DNA binding events induced by anti-CD40 provided a clue as to why induction of IL6 was transient and was followed by a loss of basal expression. The NF-κB site of the IL6 promoter overlaps with a binding site for CBF-1 which can repress NF-κB-dependent transcription driven by p50:p65 heterodimers (Lee *et al* 2000, Palmieri *et al* 1999). CBF1 belongs to a family of highly conserved CSL proteins with homologues in Drosophila (Supressor of Hairless)(Schweisguth *et al* 1992). There is persuasive evidence that both in Drosophila and in mammalian cells CSL proteins play a role in Notch signalling. There are four mammalian Notch proteins forming a family of large transmembrane proteins that control cell fate determination. The extracellular domains of Notch contain tandem EGF-like repeats, while the intracellular domains of Notch contain 6 ankyrin repeats and a carboxy-terminal PEST sequence. Notch proteins interact with their cell-surface bound ligands serrate, jagged 1 and 2 or delta 1-4 (Schroeter *et al* 1998). Interaction of Notch with one of its ligands results in proteolytic release of the Notch intracellular domain (NotchIC), which is transported to the nucleus where it dimerizes with a member of the CSL (CBF-1/ Su(H) / Lag-1) family (Lu *et al* 1996). CBF-1 (also known as Epstein-Barr virus C promoter binding factor 1 and RBP-Jkappa) is the CSL factor expressed in mammalian cells. CBF-1 acts as a repressor of gene transcription in the absence of NotchIC in both transfection and transcriptional assays *in vitro* (Hsieh *et al* 1995). CBF-1 binds to the core DNA sequence GTGGGAA which overlaps with NFkB sites in some genes (Lee *et al* 2000, Palmieri *et al* 1998). The repressor activity of CBF-1 is mediated by its interaction with a corepressor CIR, which recruits histone deacetylase via its interaction with the SAP30 a member of the mSin3A complex (Hsieh *et al* 1999). This implies that CBF-1 causes repression of gene transcription by promoting chromatin modification (Kao *et al* 1998). Dimerization of CBF-1 with NotchIC converts CBF-1 into an activator by an as yet unknown mechanism. Although it is likely that NotchIC displaces CIR, NFkB dimers may also derepress genes by displacing CBF-1 from CBF-1 binding sites that overlap with NFkB binding sites (e.g. IL6 promoter). CBF1 may also prevent activation

of transcription by interacting with two co-activators of RNAPolII complex, TFIID and TFIIA (Olave *et al* 1998).

It has been shown that CBF1 can bind to the NFκB binding site of the IL6 promoter (Palmieri *et al* 1998). This promiscuous binding has been demonstrated in a variety of cell types, where it was shown to act as a repressor of transcription of IL6. CBF1 has a lower affinity for the NFκB binding site than NFκB itself, so it can occupy this site only when NFκB levels are reduced, or when CBF1 levels are markedly increased. When bound to this site, it is thought to repress transcription in various ways- by preventing NFκB binding, which was shown to be necessary for activity of the promoter; possibly by interacting with TFIIA and TFIID thus preventing RNAPolII activity, and also by inducing bending of the promoter, thus influencing transcription by determining a specific conformation of the promoter region. It may also cause histone deposition across this region, thus preventing access of other transcription factors to the promoter.

It was found that anti-CD40 treatment of FSDC generated a prolonged elevation of CBF-1 DNA binding while induction of p50:p65 binding was transient. Since over-expression of CBF-1 was able to repress basal and anti-CD40 induced IL6 promoter activity, it is probable that the prolonged stimulation of CBF-1 activity and the short-lived activation of NF-κB and AP-1 eventually result in a repression of IL6 gene transcription. However, the rapid loss of IL6 transcript between 24 and 48 hours post-stimulation of cells also implies that IL6 mRNA must be rapidly degraded in FSDC. The short half-life of the transcript coupled with repression of IL6 promoter function by CBF-1 is therefore the most likely explanation for the loss of IL6 transcript in FSDC stimulated via CD40 for longer than 24 hours. Transient induction of IL6 in CD40 stimulated DCs may be of great physiological importance. DCs exposed to IL6 acidify their endosomes, which results in altered antigen processing leading to priming of T cell responses against cryptic determinants (Drakesmith *et al* 1998). Prolonged autocrine stimulation of DCs by IL6 could therefore result in the generation of anti-self immunity and propagation of autoimmune disease. It is therefore tempting to speculate that the transient induction of IL6 expression by CD40 stimulated DCs enables activation of naive T cells and maturation of DCs, but also protects against the generation of

anti-self immunity. Further understanding of the regulatory events that control IL6 gene transcription in DCs may lead to the development of experimental strategies for attenuating DC function in pathological conditions and in the production of vaccines.

CHAPTER 7

General discussion

GENERAL DISCUSSION

The work carried out in this thesis was organised in a number of sections, each dealing with a particular aspect of CD40 signalling or its effects in tumour cell lines (chapter 4 and 5) or in FSDC cell line (chapter 6). Each set of findings was discussed in the later section of the given chapter. What remains to be done is to outline the initial ideas and questions which were the driving force behind the work executed, and to also give a summary of work accomplished.

Once again, the work in this thesis is based on the findings published in the "Nature Medicine" paper by French *et al* (1999). This paper showed that anti CD40 mAb can by pass T cell help and induce a powerful CTL mediated response against 3 different established B cell lymphomas on two genetic backgrounds in mice *in vivo*. This work also strongly suggests that DCs may have a role in therapy, but it does not exclude the possibility that tumour cells may provide a sufficient co-stimulatory signal to precursor CTLs to induce their maturation. This is because tumour B cells have CD40 receptor on their surface and may through CD40 signalling, up-regulate co-stimulatory surface markers in this system. What was not addressed in previous work from our laboratory is the mechanism of anti CD40 mAb action. All of the studies published with mAb therapies were of a descriptive nature. It was therefore thought that a mechanistic, sub-cellular approach to dissecting the pathway involved in our system would be appropriate. As it was not clear which cell type was ultimately presenting tumour antigens to precursor CTLs, it was thought that work would best be carried out in both tumour B cells and DCs, in places in a comparative manner.

Therefore, in agreement with "Aims and objectives" on page 52, the tasks of the thesis were:

1. Production of expression vectors for wild type and mutant human CD40 proteins chosen to demonstrate requirement for signalling motifs. Also the production of stable mouse B cell lymphoma lines expressing wild type and mutant human CD40 proteins.

2. To use B cell lymphoma lines expressing human CD40 constructs to develop an *in vivo* therapy model with which to test the ability of anti human CD40 mAb to deliver therapy and to test the requirement for specific signalling motifs on the human CD40 protein
3. To carry out *in vitro* analysis of human CD40 expression and signalling in B cell lymphoma lines
4. To dissect the ways in which anti CD40 mAb stimulates signalling events which result in the transcriptional activation of the interleukin 6 gene which can promote anti tumour responses through its effect on activation and maturation of DCs, as well as proliferation of T cells.

Each one of the set tasks was addressed, and the results obtained were following:

1. Human CD40 constructs were successfully made, as outlined in chapter 3, without problems.
2. Stable expression of these constructs was obtained in the A20 cell line (chapter 3).
3. *In vivo* therapies using transfected A20 cell lines were not responsive to anti human CD40 mAb treatment, although anti mouse CD40 mAb still provided therapy as previously recorded.
4. Problems were also encountered with A20 cell lines transfected with empty vector (pcDNA3). This cell line was intended to provide a transfection control, but instead gave data which could not be fully interpreted (chapter 4). It was thought that the A20 cell line itself may in some way be compromised.
5. It was found that A20 cells were, indeed, compromised by a productive infection with at least one, and possibly more retroviruses. This was confirmed by RT-PCR, transcriptional assays and electron microscopy (chapter 5).
6. It was shown that levels of IL6 increased *in vivo* upon administration of anti CD40 mAb.
7. Levels of IL6 also increased in FSDCs and BMDCs upon CD40 cross-linking using mAb.

8. FSDCs were found to a be suitable model for studying effects of CD40 signaling on DCs, as responses recorded were closely matched by those found in BMDCs.
9. Both cell types were found to express IL6 receptor.
10. Maturation was inferred in FSDCs upon CD40 cross-linking, as observed by the increase of surface expression of MHC class I and II, CD4, CD80 (B7.1), CD86 (B7.2) and CD54 (ICAM1).
11. It was found that IL6 promoter had a low level of activity in non-stimulated FSDCs, which increased 20fold upon CD40 cross-linking with its mAb. The difference was pointed out with regards to basal and inducible activity of the promoter in these cells.
12. NFκB was shown to regulate both the basal and inducible activity of the promoter, while other factors, such as NF-IL6 and AP1 had a role in inducible activity only. Both NFκB binding site within the promoter, and the transcription factor itself were found to be essential for transcriptional regulation of the promoter activity. This was confirmed by the use of mutant promoter constructs and dominant negative IκBα.
13. Sites in the human CD40 important in the induction of IL6 were mapped out. T254 was found to be indispensable for the inducible activity of the promoter, although constructs lacking this residue retained 20% of the activity of the wild type. In agreement with these results, it was inferred that TRAF2 was mainly responsible for induction of NFκB, whileTRAF3, 5 and 6 were contributing in a smaller proportion.
14. NFκB complexes induced in FSDC were identified to be p50/p65 heterodimers. The induction of this band with anti CD40 mAb was found to peak at 16 hours after the addition of mAb, followed by a gradual reduction.
15. The presence of another protein complex, which was specific, but shown not to be NFκB as evident in supershift and EMSA studies. This band was identified as CBF1 (chapter 6).
16. CBF1/DNA binding activity was found to be increased in FSDCs treated with anti CD40 mAb. This increase was constant and continued up to 48-hour time point.
17. Overexpression of CBF1 in FSDCs led to a reduction in both basal and inducible IL6 promoter activities.

18. Anti CD40 mAb was also shown to induce AP1/DNA binding in the cells. AP1 was shown to, in part, control the inducible activity of IL6 promoter. The AP1 complexes were found to consist of JunB, JunD, c-Jun and cFos, as demonstrated in supershift studies by using antibodies directed against these AP1 subunits.
19. Overexpression of dominant negative Jun D, which can bind and inactivate both Jun and Fos proteins, was found to reduce inducible activity of IL6 promoter. Using a mutant promoter construct which lacks AP1 binding site also showed importance of AP1 in the inducible activity of the promoter. This was also found to reduce inducible activity, but, as DN Jun D, had no effect on the basal activity of the promoter.

The questions which remain unanswered are also plentiful. Some of these queries have arisen during the course of work carried out, and others were difficult to address at this time, due to various technical problems. They are subdivided into groups according to the chapter in which they are mentioned.

Chapters 4 and 5

The lack of therapy using anti human CD40 mAb *in vivo* to cure A20/hCD40wt and other transfected cell lines posed a number of questions. Some of those were addressed, and explanation given in terms of the unsuitability of A20 cells for studies due to infection with retroviruses. The remaining questions regarding this work are listed below, along with possible ways in which they may be approached.

1. ***Would signalling through CD40 in tumour B cells alone have an effect on the outcome of the therapy?***

   The work, which would answer this, would have to be carried out on non-infected and non-compromised tumour B cells. In order to make sure that signalling through CD40 in these cells was equivalent to signalling through endogenous CD40, a better approach to the one taken in this thesis would be to produce a human/mouse CD40 chimera. This

fusion protein would have human extracellular domain, and mouse transmembrane and intracellular domains. In this manner, anti human CD40 mAb would recognize this protein only, the signal triggered from this receptor would be the same as the one arising from the endogenous protein. Although the difference in the quantity or quality of signal initiated from a human CD40 in mouse cells has been shown to be to all intents and purposes identical to the endogenous protein, the use of a human/mouse chimera may provide a system where this detail could not be subsequently questioned as a possible source of inadequate signalling in cells. Further work using clean tumour B cells and human/mouse chimera would be carried out as outlined in chapter 4.

2. ***Would signalling through CD40 in host dendritic cells only have any effect on the outcome of the therapy?***

Answering this question would be more important, and also more difficult. If signalling through CD40 in DCs alone can give similar therapy outcome as that described in the paper by French *et al*, than this may mean that the type of tumours which can be cured using this method are essentially limitless. As long as there are some tumour specific antigens which are immunogenic, it can be envisaged that these antigens can be cross-primed into host DCs, and presented to precursor CTLs. Triggering CD40 signalling in DCs by anti CD40 mAb would provide CTLs with a co-stimulatory signal, and lead to eventual eradication of the tumour. In order to find out if CD40 signalling in host DCs alone may be sufficient to give rise to this outcome, the therapy work would need to be done in transgenic mice. These mice would have human/mouse chimera (or even human) CD40 expressed in DCs only. This could be achieved by placing hCD40 under the regulation by a dendritic cell specific promoter, thus ensuring that the protein was only expressed in DCs. Performing therapies in these mice using anti human CD40 mAb would provide with an answer to the question posed above. Although in theory and even technologically simple, this matter may be much more complex than it appears. Firstly, the promoter used to drive human CD40 expression would have to provide these cells with adequate levels of human CD40 expression, and at correct

developmental stages of the cells. Furthermore, most transgenic mice are made on C57BL background, and currently there are not many B cell lymphoma models available for work in these animals. Therefore, even if production of transgenic mice was simple, obtaining cell lines to test in these systems may not be so. Nevertheless, this would be the only convincing method of answering the query regarding the role of host DCs in tumour models.

3. ***What were the retroviruses found in A20 cells in electron micrographs?***

Although RT-PCR data showed presence of two different retroviruses in A20 cells, this did not prove that the viruses seen in the EM pictures belonged to either one of two types. Finding what the infectious particles are would be not only of general interest, but also in terms of commenting on the presence of particular viral antigens with regards to the therapy.

4. ***Might these viruses provide the elusive antigen in these systems, and if so, would it mean that only virally induced tumour might be susceptible to this kind of therapy?***

Repeating the therapies carried out thus far with the same cell lines, which are free of viruses, would provide answers to this. Results of these therapies would answer the question regarding the antigen, and if viral antigens were not necessary for curative outcome, it also would reassure that this therapeutic approach would be valid in attempting to treat tumours other than B cell lymphomas.

Chapter 6

1. ***How do other transcription binding sites within the human IL6 promoter contribute to the activity of the promoter?***

There are at least three more transcription factor binding sites which have not been addressed in chapter 6. Those are Sp1, NF-IL6 and CREB. Although some data were

obtained as to the necessity of NF-IL6 binding to IL6 promoter, due to technical problems, it has been impossible to obtain EMSAs using NF-IL6 labelled probe. DNA binding studies would have provided a more detailed insight into the behaviour of NF-IL6 in FSDCs in presence or absence of anti CD40 mAb.

Sp1 and CREB studies could be approached through a variety of angles. Mutant promoter constructs can be made lacking binding sites for these factors, also overexpression and dominant negative expression constructs can be used in transfection studies. The use of minimal promoter/luciferase constructs driven by several binding sites for Sp1 or CREB can also be used to assess the presence of these in the cells +/- anti CD40 mAb.

Presence of other transcription binding sites within the promoter, as yet unidentified, can be evaluated using techniques such as DNA footprinting. This method would highlight the presence of transcription factor binding sites, which may not have been recognized previously using sequence recognition programs. Although these further studies may be interesting, they are unlikely to find a factor which may be more important to the activity of the promoter than NFκB, which has been shown to control both the basal and to the best part, the inducible activity of this promoter. Nevertheless, for completeness of work, such studies should be carried out, if possible.

2. ***Which kinases are involved in the signalling pathways identified so far?***

TRAF2 on the receptor end, and NFκB on the promoter end were found to be essential for the activity of the promoter. However, the identity of kinases involved in linking the two can only be assumed at this stage. To test for involvement of various kinases in the pathways starting at CD40 and ending at IL6 promoter, further experiments would have to use dominant negative expression constructs of kinases in question, as well specific inhibitors available form pharmaceutical companies. Also, active participation of kinases with the tested pathway can be shown by use of constitutively active forms of

these kinases in transfection studies. Read out for all of these experiments would be activity of IL6 promoter, as already described.

3. ***Does CBF1 have any other role in FSDCs? Is there any Notch signalling in these cells; do they express Notch or its ligands, Jagged or Delta proteins? Is there a role for Notch signalling in these cells with regards to IL6 activity?***

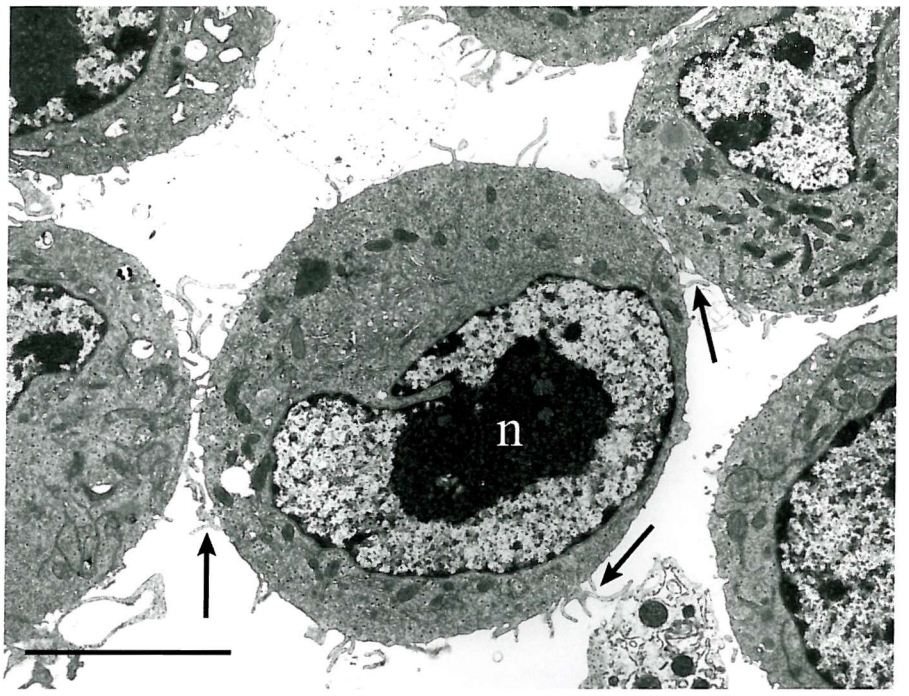
   Results in chapter 6 have convincingly shown that CBF1 interacts with IL6 promoter in these cells. This interaction was demonstrated to occur at around 24-48 hours after the addition of anti CD40 mAb. The result of the interaction of CBF1 with NFκB binding site in IL6 promoter was repression of its activity, as judged by the absence of mRNA transcripts for IL6 gene at 48 hours (figure 6.2). Although figures 6.11 to 6.16 showed effects of CBF1 presence on DNA binding studies and promoter activity, they do not provide explanation to a number of issues, such as

   - how does CD40 signalling regulate CBF1 expression
   - how is CBF1 expression globally regulated, and why is this transcription factor found in large quantities in FSDC, even without CD40 stimulation
   - does CBF1 regulate expression of other genes in FSDCs, and if so, which ones
   - can repressive nature of CBF1 be overcome by Notch signalling
   - is Notch receptor present on the surface of FSDCs
   - are any of the Notch ligands present on the surface, if so, which ones
   - does Notch signalling have any role in control of IL6 gene expression, by forming a negative or positive feedback loop from NFκB signalling as a response to different stimuli and cell environment.

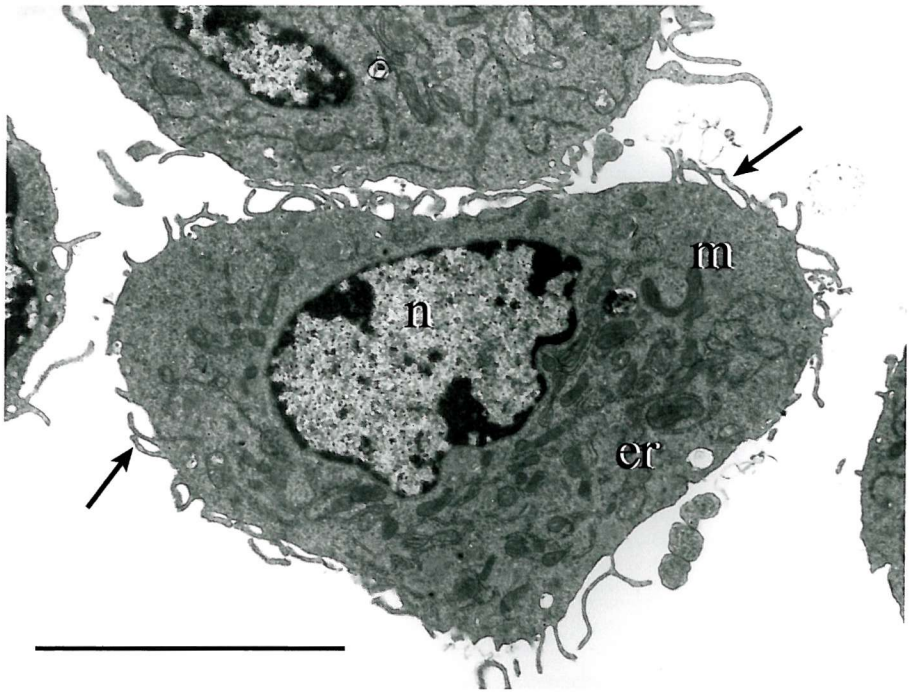
Answering these questions may be possible by the use of constitutively active intracellular domain of Notch, Jagged or Delta transfected feeder cells, soluble Jagged or Delta, using FACS analysis to detect presence of these proteins on cell surface etc. Rather than actually considering the theme further, these questions are asked to suggest that each new finding probably opens more questions than it provides answers.

APPENDIX

A.)



B.)



Appendix figure- Electron micrograph of FSDC cells viewed at 4000X and 8000X magnification. There are no observable viral particles surrounding or inside the cells. Arrrows point to the dendrites which are nicely visible in TEM. The black scale bars in a.) and b.) are 5 microns in length.

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