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The Role of CD40 in the Immune Response

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THE ROLE OF CD40 IN THE IMMUNE RESPONSE

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Abstract

A successful humoral immune response requires reciprocal signaling between T helper cells and B cells. The first signal is antigen specific and is mediated by the interaction between the T cell receptor and antigen in association with major histocompatibility complex on B cells. The subsequent signals are provided by the costimulatory molecules CD40 and CD28 and their respective ligands. CD40L which is expressed on activated T helper cells interacts with CD40 on B cells providing the essential signal for the induction of B cell activation and immunoglobulin production. The importance of the CD40-CD40L interaction has been shown in patients suffering from X-linked immunodeficiency with hyper-IgM (HIGM1). The disease is characterized by the inability of B cells to undergo immunoglobulin isotype switching. Affected males experience recurrent infections and most infections are of bacterial origin, but HIGM1 patients are also unusually susceptible to infections with opportunistic pathogens and often suffer from *Pneumocystis carinii* pneumonia and *Cryptosporidium* intestinal infection. Mutations in CD40L molecules are responsible for hyper IgM syndrome in humans.

To study the role of the CD40-CD40L interaction *in vivo* and to derive an animal model for HIGM1, I generated CD40 deficient mice using homologous recombination in embryonic stem (ES) cells. A targeting vector was constructed using an 8 kb CD40 genomic fragment. The G418 resistance gene (NEO) was inserted into the 3rd exon to disrupt the CD40 gene and to allow selection. The targeting vector was transfected into ES cells and G418 resistant clones were isolated and screened for homologous recombination by Southern blot analysis. Chimaeric mice were generated by injection of targeted ES cell clones into blastocysts. Germline transmission was obtained and heterozygous mutant mice bred to generate CD40 deficient mice.

Flow cytometric analysis of lymphocytes in CD40 deficient mice revealed normal development of B and T lymphocytes. CD40 deficient mice were immunized with KLH

and assessed for germinal centre formation. CD40 deficient mice did not generate germinal centres. Analysis of serum immunoglobulin levels showed that CD40 deficient mice displayed reduced levels of isotype switched immunoglobulins compared to wild-type mice. These results confirm the crucial role of the CD40-CD40L interaction in humoral immunity.

CD40 is also expressed on other antigen presenting cells and the involvement of the CD40-CD40L interaction in cell-mediated immunity was determined in CD40 deficient mice infected with *Mycobacterium Bovis* (BCG). Although CD40 deficient mice survived mycobacterial infection, the increased numbers of bacilli in spleen and lungs and the reduced production of IFN-γ in response to mycobacterial infection indicates that CD40 deficient mice are more susceptible to infection with BCG than control mice.

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Practicalities

All the work in this thesis was my own with the following exceptions:

Staining of liver-sections by the Ziehl-Neelsen method for acid fast bacilli and with hematoxylin and eosin (H&E) was performed by Simon C. Biddolph. Department of Cellular Science, John Radcliffe Hospital.

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Chapter 1: Introduction

1.1 Acquired immune responses

The immune system can be divided into innate and acquired immunity. The effectors of innate immunity consists of non-specific activities of complement, natural killer cells and different phagocytic cells. In contrast, acquired, or specific, immunity is capable of recognizing and selectively eliminating foreign microorganisms and molecules. It displays specificity, diversity, memory and self/nonself recognition. Specific immunity can be divided into humoral and cell-mediated immunity. The humoral branch of the immune system involves B lymphocytes which recognize whole antigen via cell-surface immunoglobulin receptors. Antigen uptake and processing by B lymphocytes via surface immunoglobulin, is followed by presentation on MHC class II and interaction with T helper cells. This subsequently leads to proliferation and differentiation of B lymphocytes into antibody-secreting plasma cells and memory cells. Cell-mediated immunity involves both T-cytotoxic (Tc) and T-helper (Th) lymphocytes, and, in contrast to B lymphocytes, T lymphocytes only recognize antigen in association with MHC class I and II via surface T cell receptors. Activation of T helper cells via their T cell receptors requires presentation of antigen in association with MHC class II on antigen presenting cells (APCs), such as B cells, dendritic cells and macrophages. These cognate interactions lead to secretion of cytokines which aid in the activation of antigen specific cytotoxic T cells.

The interaction between the T cell receptor (TCR) and antigen in association with MHC class II on B cells is the first step in the mutual activation of antigen specific T and B cells. In order to complete this mutual activation several costimulatory molecules are needed. The most important receptor-ligand molecules on T and B cells are CD40-CD40 Ligand, and CD28/CTLA-4-CD80/CD86.

1.2 Identification of cells required for induction of humoral immunity

Experiments performed in the 1960s and 1970s demonstrated that different types of cells must cooperate to generate a humoral immune response. Before B and T cells were defined, evidence that distinct subpopulations of cells were required to generate an antibody response came from adoptive-transfer experiments done by Henry Claman and co-workers. In the these experiments, irradiated mice were reconstituted with either syngeneic thymus cells alone, or bone marrow cells alone, or spleen cells alone, or with a mixture of thymus and bone marrow cells. The mice were then immunized with SRBCs (Sheep Red Blood Cells) and the ability of these reconstituted mice to produce antibodies against SRBCs was tested in a hemolytic plaque assay. Mice reconstituted with thymus cells alone had only a slight restoration in their ability to produce anti-SRBC antibodies, and mice reconstituted with bone marrow cells alone failed to produce anti-SRBC antibodies. However, mice reconstituted with both thymus and bone marrow cells gave a response as good as mice reconstituted with spleen cells (Claman *et al.,* 1966; Claman *et al.,* 1967). These experiments demonstrated that both thymus and bone marrow cells were necessary for the induction of the humoral response.

Claman's experiments showed that both thymus and bone marrow cells were necessary to generate a humoral antibody response, but they did not identify which population of cells produced antibodies. The importance of an intact thymus, for the antibody response to SRBC, was shown by performing neonatal thymectomy in mice. Neonatal thymectomy in mice reduced the ability to make antibodies against SRBC to about 5-10 percent of the normal response. This defect could be corrected in the adult mice by injection of either thymus cells or thoracic-duct cells, but not by bone marrow cells (Miller *et al.,* 1968). Mitchell and J. F. A. P. Miller (1968) subsequently demonstrated that bone marrow cells were the source of antibody producing cells. Irradiated and thymectomized mice were reconstituted with various populations of cells and immunized with SRBC, after which the spleen cells were assayed for plaque formation in response

to SRBC. Instead of thymus cells, thoracic-duct lymphocytes were used as an enriched source of T cells. These experiments showed that the thoracic-duct cells and bone marrow cells in combination could generate the antibody response, confirming Claman's findings. They then went one step further to identify the antibody-producing population by repeating the experiment with thoracic-duct lymphocytes and bone marrow cells of different MHC haplotypes. CBA ($H-2^k$) mice were thymectomized and irradiated and reconstituted with CBA ($H-2^k$) bone marrow cells. Two weeks later, these mice were injected with (CBA x C57BL/6)F1 thoracic-duct cells ($H-2^{k/b}$) and SRBCs. Five days later the spleens of the reconstituted mice were removed and tested for the presence of cells producing antibodies against SRBC. By treating the spleen cells with antibody to the $H-2^k$ or $H-2^b$ MHC, it was possible to deplete the two populations selectively and determine the effect of each depletion on the antibody response to SRBC. Depletion of $H-2^k$ cells removed both populations and abolished the antibody response to SRBC. However, removal of $H-2^b$ thoracic-duct cells did not affect the response. These results demonstrated that the bone marrow cells provided the antibody-forming cells (now known as B cells) and that the thoracic-duct cells provided T helper cells for generating the humoral response.

Further proof of the existence of two interacting subsets of lymphocytes involved in antibody production was provided by experiments using hapten-carrier conjugates which enabled immunologists to determine that the generation of a humoral antibody response required the associative recognition by T helper cells and B cells, each recognizing different epitopes on the same antigen (Rajewsky *et al.* 1969, Mitchison *et al.* 1971, Katz *et al.* 1970). One of the earliest findings with hapten-carrier conjugates was that a hapten (small organic compound) had to be chemically coupled to a carrier (protein) molecule to induce an antibody response to the hapten. A second important observation was that in order to generate a secondary antibody response to a hapten, the animal had to be immunized with the same hapten-carrier conjugate used for the primary immunization. If the secondary immunization was with the same hapten but conjugated to a different

unrelated carrier, no secondary anti-hapten antibody response occurred. Thus, even though the animal was seeing the hapten for the second time, it was only able to recognize the hapten when coupled to the same carrier. This phenomenon is called the carrier effect. The carrier effect could be circumvented by immunizing the animal separately with the unrelated carrier. For example, the animal was immunized with DNP-OVA (day 0) and then immunized with BGG (unrelated carrier, day 7) and finally immunized with DNP-BGG (DNP-unrelated carrier, day 28). The secondary anti-DNP antibody response was now as high as if the animal had been immunized with DNP-OVA on day 0 and day 28 with an intermediate immunization of OVA (day 7) (Rajewsky *et al.,* 1969, Katz *et al.,* 1970). The carrier effect was also demonstrated in an adoptive-transfer model (Mitchison 1971a, Paul *et al.,* 1970). Mice were immunized with NIP-CGG and spleen cells from these mice (donors) were injected into irradiated syngeneic recipients. These recipients were then immunized with NIP-CGG (homologous conjugate) or NIP-BSA (heterologous conjugate) and the anti-NIP antibody response was measured. An anti-NIP antibody response was only elicited if the recipients were immunized with the homologous conjugate (NIP-CGG). Mitchison (1971b) also showed that the injection of spleen cells obtained from mice immunized with BSA (carrier-primed) together with spleen cells from mice immunized with NIP-OVA (hapten-primed) into irradiated syngeneic recipients immunized with NIP-BSA elicited an anti-hapten antibody response, thereby bypassing the carrier specificity of the primary NIP-OVA immunization. These results demonstrated that carrier-primed cells could be raised independently of hapten-primed cells (i.e. carrier-primed and hapten-primed cells are distinct populations) and that carrier-primed cells could interact with hapten-primed cells (in the recipient) raised independently of the carrier-primed cells. By repeating the adoptive-transfer experiment described above, using an allotype marker on carrier-primed cells, Mitchison demonstrated that carrier-primed cells (BSA-primed) did not produce an anti-hapten antibody (Mitchison, 1971b). Thus, the carrier-primed cells appeared to serve only as helpers and not as precursors of the anti-hapten antibody forming cells. Adoptive transfer experiments investigating the effect of anti-T-cell

antiserum and complement treatment of carrier-primed cells or hapten-primed cells, revealed that the secondary anti-hapten antibody response was inhibited if carrier-primed cells were treated with anti-T-cell antiserum and complement, whereas treatment of hapten-primed cells had no effect on the anti-hapten antibody response. This provides further evidence that carrier-primed cells (helper cells) are thymus-derived cells while the anti-hapten producing cells are thymus independent (marrow-derived) (Raff, 1970, Mitchison, 1970c).

These experiments concerning thymus-bone marrow interactions and hapten-carrier conjugates led to the concept that two types of lymphocytes cooperate in the induction of an antibody response. As induction of an antibody response required the hapten to be chemically coupled to the carrier molecule it was assumed that antigen (hapten-carrier conjugate) was recognized by two specific receptors, one on the B cell and one on the T helper cell. This cellular cooperation hypothesis envisioned a B cell binding to the hapten and a T helper cell binding to carrier epitopes, with the hapten-carrier conjugate bridging the two cells, which would then lead to activation of the B cell (Mitchison, 1971b; Rajewsky, 1971). The discovery of MHC restriction between T helper cells and B cells (described below) meant that the cellular cooperation hypothesis was not sufficient and that any model of T-B cell interaction had to include a role for MHC class II molecules. The current model of T-B cell cooperation in the generation of antibody responses to protein antigens and hapten-protein conjugates involves presentation of peptides in association with MHC class II molecules. Antigen specific B cells bind the hapten-protein conjugate (via the hapten determinant) on membrane Ig molecules. After binding, the hapten-protein conjugate is internalized, processed in an endosomal pathway and peptides are presented to T helper cells in association with MHC class II molecules on the surface of the B cell. A T helper cell then recognize the processed peptide in association with MHC class II molecules via the T cell receptor and B cell activation results from interaction between molecules on the surface of the T and B cell, and by cytokines released by the T cells.

The use of hapten-carrier systems enabled Katz and co-workers (Katz *et al.*, 1973) to show that carrier-primed T cells could only cooperate with hapten-primed B cells, if the T and B cells had identical MHC haplotypes. Adoptive transfer of BGG-primed T cells from strain A (H-2^a) into an (A x B)F1 (H-2$^{a/b}$) recipient, followed by the transfer of DNP-KLH-primed B cells derived from strain A (H-2^a) or from strain B (H-2^b). The recipient mice were then immunized with DNP-BGG, and the secondary response to DNP was measured. One complication which initially made the experiment unworkable was the allogeneic effect. The transferred strain A T cells recognized the strain B allogeneic MHC antigens of the F1 recipient as foreign and could potentially activate the host B cells nonspecifically. This problem was solved by irradiating the (A x B)F1 recipient after transfer of the BGG-primed strain A T cells. The irradiation eliminated unprimed alloreactive T cells whereas primed T cells, which were less susceptible to irradiation, survived. Strain A (H-2^a) or strain B (H-2^b) B cells from DNP-KLH-primed mice were now transferred into the F1 recipient, and the secondary response to DNP was measured. The results revealed that carrier-primed T helper cells with haplotype H-2^a could only assist hapten-primed B cells in generating an anti-DNP antibody response if the B cells shared the same MHC haplotype (H-2^a). Subsequent experiments using congenic H-2 mouse strains revealed that the portion of the H-2 complex which was crucial for the interaction between carrier and hapten–primed cells involved the I region, now known to code for MHC class II genes (Katz *et al.*, 1974).

The discovery of monoclonal antibodies produced by hybridomas (Kohler *et al.*, 1975) allowed the identification and analysis of lymphocyte populations expressing different membrane proteins. These surface proteins were initially recognized as markers for lymphocytes subpopulations, but further analysis revealed that they also played important roles in the effector functions of these cells (The Leucocyte Antigen Facts Book, Barclay *et al.*, 1997).

1.3 Reciprocal signaling between T helper cells and B cells

The critical role of CD4$^+$ T helper cell activity for B cell activation and function became apparent when it was shown that direct physical interaction between T cells and B cells was necessary, since B cells proliferated when co-cultured with mitogen activated T cells, but not when cultured with the supernatant from these activated T cells (Clement *et al.*, 1984). The use of membranes from activated T cells could replace whole activated T cells, and induce B cell proliferation (Brian, 1988). Suggesting that membrane molecules on activated T cells interacted directly with membrane molecules expressed on B cells, in order to induce B cell proliferation. The critical role of CD40 was later confirmed when activated T cells were rendered incapable of inducing B cell proliferation, due to the inhibitory effect of a soluble CD40-Ig molecule. The ligand on T helper cells was found, by use of the soluble CD40-Ig, to be expressed only on activated T helper cells (Noelle *et al.*, 1992).

In addition to the CD40-CD40L interaction, another T and B cell receptor/ligand interaction has been found to be important for the mutual regulation of T and B cells. This involves CD28/CTLA-4 which is expressed on T cells and CD80/CD86 which is expressed on B cells (Lenschow *et al.*, 1996).

Interactions between T and B cells must be regulated in order to prevent activation of self-reactive or bystander cells and experiments have shown that, to initiate activation of antigen specific T and B cells, distinct signals are required. The first signal is antigen specific and is mediated by the interaction between the T cell receptor/CD3 complex and antigen bound on MHC class II expressed by B cells. This induces the expression of CD40L on T cells. CD40L then interacts with CD40 which then upregulates the expression of CD80 and CD86 on the B cell. The T cell receives a signal through CD28 mediated by the upregulated expression of CD80 and CD86 on the B cell. CD80/CD86 interacting with CTLA-4, on the T cell, is thought to be a negative regulator of T cell activation. The activated T cell starts to secret cytokines, which further induces the

upregulation of CD80/CD86 on the B cell. The final result is fully activated antigen specific T and B cells (Clark & Ledbetter, 1994; Durie *et al.*, 1994; Lenschow *et al.*, 1996). Another possible sequence of events after the initial interaction between the T cell receptor/CD3 complex and antigen bound on MHC class II, is that CD80/CD86 binds to CD28/CTLA-4. This induces the upregulation of CD40L on the T cell and CD40L would then signal through CD40, inducing CD80/CD86 upregulation of expression, which would then result in amplification of the T cell response (Hollenbaugh *et al.*, 1994)

Experimental evidence that supports the two models described above is presented here. The presence of CD40 and MHC class II on resting B cells, or the T cell receptor/CD3 complex and CD28 on T cells is insufficient to initiate mutual activation (Fuchs & Matzinger, 1992). Antibodies that cross-link CD3, resembling activation through TCR, induce expression of CD40L (Roy *et al.*, 1993) which then interact with CD40 on the B cell. CD40 cross-linking induces the expression of CD80 (Ranheim & Kipps, 1993) and CD86 (Azuma *et al.*, 1993) on the B cell. *In vitro* studies on the regulation of CD40L were carried out using pigeon cytochrome C (PCC) TCR transgenic T cells. (Roy *et al.*, 1995). In the presence of antigen presenting cells (APC), the addition of PCC induced CD40L expression on the T cells, an effect which could be inhibited by the addition of antibodies to MHC class II, CD4 or LFA-1. Antibodies that blocked CD80, CD86 and CTLA-4-Ig did not interfere with the PCC-induced upregulation of CD40L. As a result of PCC-induced upregulation of CD40L, B cells were induced to upregulate both CD80 and CD86. The upregulation of CD80 and CD86 could be blocked by an anti-CD40L mAB. These results suggest that interaction between the T cell receptor/CD3 complex and antigen bound on MHC class II induces the expression of CD40L and that costimulation by the CD80-CD28 interaction is not required for the expression of CD40L. This is in contrast to the findings that human T cells activated by mAb to the TCR/CD3 complex require a CD80-CD28 signal for the upregulation of CD40L (de *et al.*, 1993).

The models for mutual activation of T and B cells described above are obviously very simplified models. CTLA-4 has a higher affinity for CD80/86 than CD28 and the addition of anti-CTLA-4 mAb to *in vitro* model systems of T cell activation generally leads to increased T cell proliferation, indicating that CTLA-4 is a negative regulator of T cell activation. This is confirmed by experiments showing that CTLA-4 deficient mice die at 2-4 weeks of age due to uncontrolled lymphocyte proliferation (Lenschow *et al.*, 1996; Linsley & Golstein, 1996; Waterhouse *et al.*, 1995). Adhesion molecules, such as CD11a/18-CD54 (LFA-1-ICAM-1), are also required for the interaction between T and B cells to occur (Barrett *et al.*, 1991). This reciprocal signaling may occur in the peripheral lymphoid T cell area, where CD40L$^+$ T cells have been identified in both mice and humans, or in the light zones of germinal centres, where CD40L$^+$ T cells have also been found (Lederman *et al.*, 1992a; Van *et al.*, 1993).

The central role of CD40L in initiating the clonal expansion and differentiation of B cells into antibody forming cells and memory cells is important, but it is equally important that B cells mature into "competent" antigen presenting cells by expressing high levels of costimulatory molecules (CD80/CD86), thereby inducing maximal T cell activation and cytokine production.

1.4 Structure of CD40 and cellular distribution

CD40 is a member of the tumor necrosis factor receptor (TNFR) family of receptors that include TNFR1, TNFR2, nerve growth factor receptor (NGFR), CD30, CD27, Fas and others. Members of the TNF receptor family are type-I membrane proteins and have a characteristic repeating extracellular cysteine-rich domain (Beutler & van, 1994). CD40 is a 50 kDA protein that was first identified in 1986 by monoclonal antibodies which were found to induce proliferation in B cells (Clark & Ledbetter, 1986; Ledbetter *et al.*, 1987). A human cDNA clone encoding CD40 was isolated and it was found to encode a type-I membrane protein of 277 amino acids. Type-I membrane proteins are characterized by having the C-terminal part of the protein on the intracellular side of the

membrane and the N-terminal part of the protein on the extracellular side of the membrane. The amino terminal region contains a 19 amino acid hydrophobic signal peptide, followed by a 193 amino acid extracellular domain including 22 cysteine residues and two potential N-linked glycosylation sites. The transmembrane part of CD40 consists of 22 hydrophobic residues and the cytoplasmic domain a 62 amino acids (Stamenkovic *et al.*, 1989).

A cDNA encoding murine CD40 has also been isolated. A comparison of the amino acid sequence of human and murine CD40 showed some differences. The murine CD40 protein contains a 21 amino acid amino terminal signal peptide, a 172 amino acid extracellular domain, a 22 amino acid hydrophobic transmembrane domain and a 90 amino acid cytoplasmic domain. The extracellular domain of murine CD40 contains 23 cysteine residues, 22 of which are conserved with respect to the human sequence and one N-linked glycosylation site which is shared with the human sequence. Overall, it was found that the extracellular human and murine domains are 62 % identical while the cytoplasmic domains are 78 % identical (Torres & Clark, 1992). Chromosomal mapping of the human and murine CD40 has shown that the human gene is located on chromosome 20 (Ramesh *et al.*, 1993) whilst the murine gene is located on chromosome 2 (Grimaldi *et al.*, 1992). Analysis of the murine CD40 genomic structure revealed the presence of 9 exons spanning a region of 16 kb (Grimaldi *et al.*, 1992).

CD40 is constitutively expressed on many cells including B cells, macrophages, follicular dendritic cells, thymic epithelium, endothelial cells and Langerhans cells (Alderson *et al.*, 1993; Caux *et al.*, 1994; Clark, 1990; Galy & Spits, 1992; Hollenbaugh *et al.*, 1995; Karmann *et al.*, 1995). This molecule plays a central role in both T and B cell activation. Studies show that activating human dendritic cells *in vitro* with TNF-α or soluble CD40L antibody increase their surface expression of CD40, CD80, CD54 and MHC class II two- to three-fold, suggesting that maturation of dendritic cells to become "effective" antigen presenting cells requires increased surface expression of CD40. Not only does the surface expression of CD40 increase, but it seems that further signals

through CD40 are required for DC's to become fully effective (Sallusto & Lanzavecchia, 1994). CD40 has also been reported to be expressed by human B cells in a soluble form, indicating that signaling to B cells through CD40 might be regulated by secretion of a soluble form of CD40 (van *et al.*, 1994b). It has been suggested that CD40L binds to CD40 which is expressed at very low levels on T cells. CD4$^+$ T cells appear to respond to this CD40L cross-linking by proliferation and secretion of IFN-γ, TNF-α and IL-2 (Armitage *et al.*, 1993).
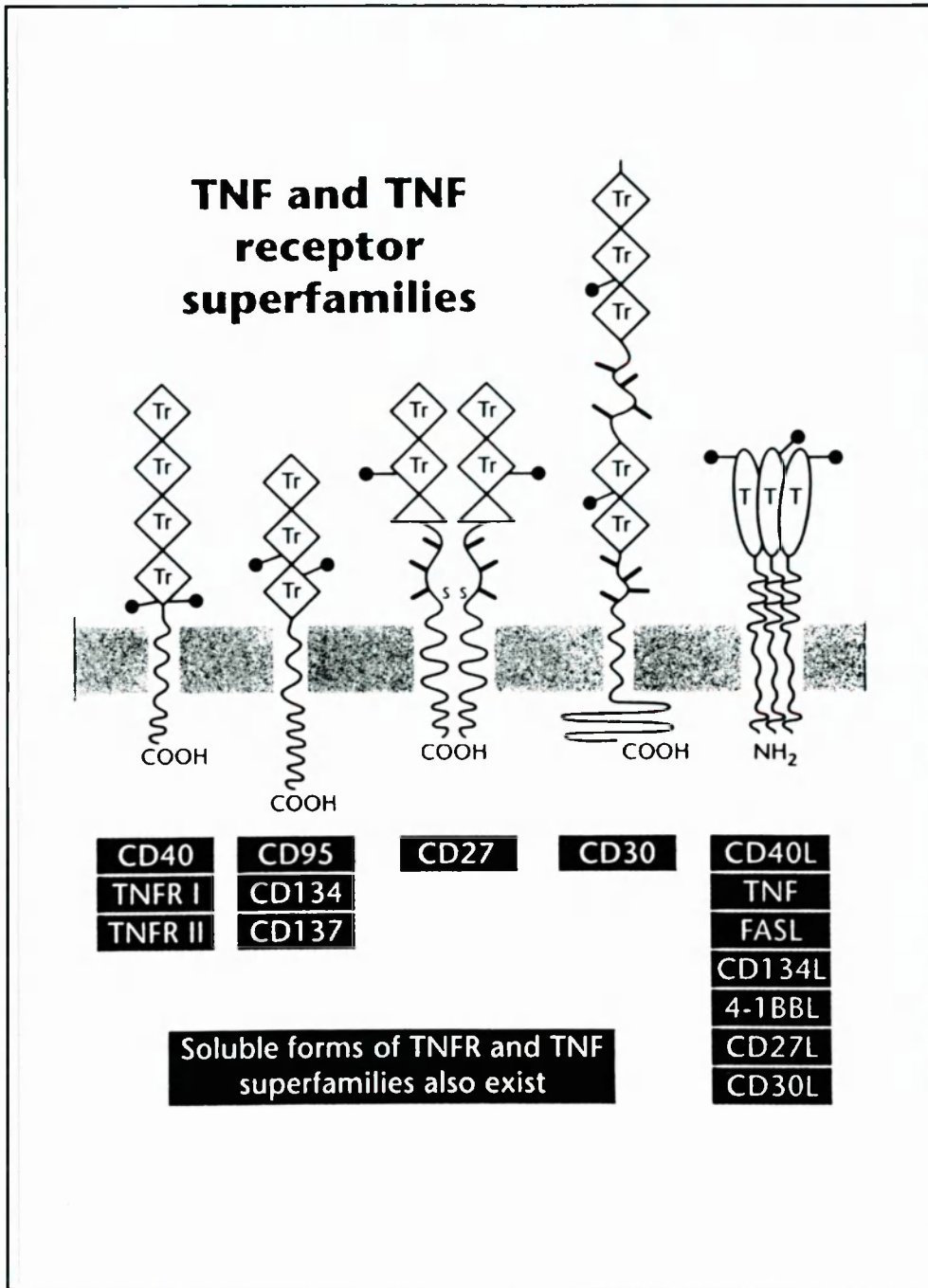
Figure 1. TNF and TNF receptor superfamilies. The receptors CD40, TNFR I and II, CD95, CD134, CD137, CD27 and CD30 are all type I membrane proteins containing cysteine-rich repeats (Tr). The ligands, shown here as trimers, are type II membrane proteins. Trimer domain (T). Potential N-linked glycosylation sites are shown as ball on stick. (Figure taken from Immunology Today, October 1997).

1.5 Structure and expression of CD40 ligand

The ligand for CD40, CD40 ligand, is a member of the tumor necrosis factor (TNF) gene family, and was identified using monoclonal antibodies. Monoclonal antibodies were selected on the basis of their ability to block T cell induced B cell proliferation. This led to the identification of two antibodies, an anti-human antibody (5c8) (Lederman *et al.*, 1992b) and an anti-murine antibody (MR1) (Noelle *et al.*, 1992) both of which blocked the ability of activated T cells to induce B cell proliferation. Both antibodies recognized a protein of 30-39 kDa. The cDNA encoding the murine CD40 ligand was identified by screening a cDNA expression library using a CD40-Ig fusion protein (Armitage *et al.*, 1992a). The human cDNA encoding CD40 ligand was later isolated by subtractive hybridization and comparison with the murine sequence for CD40 ligand (Graf *et al.*, 1992; Hollenbaugh *et al.*, 1992; Spriggs *et al.*, 1992). A cDNA encoding the human CD40 ligand was isolated using the 5c8 mAB (Lederman *et al.*, 1993).

Analysis of the cDNA clone containing the murine CD40 ligand showed that it encoded a 260 amino acid polypeptide with typical characteristics of a type II membrane protein. Murine CD40 ligand is composed of a 22 amino acid cytoplasmic domain followed by a 24 amino acid hydrophobic transmembrane domain and a 214 amino acid extracellular domain which contains a single potential N-linked glycosylation site (Armitage *et al.*, 1992a). Analysis of the cDNA clones of human CD40 ligand showed that it encodes a polypeptide of 261 amino acids. This polypeptide consists of a 22 amino acid cytoplasmic domain, a 24 amino acid transmembrane domain, and a 215 amino acid extracellular domain containing a single potential N-linked glycosylation site, in common with the murine CD40 ligand. Analysis of the genomic structure of human and murine CD40L revealed 5 exons with 4 intervening introns spanning a region of 13-14 kb (Tsitsikov *et al.*, 1994; Villa *et al.*, 1994). Chromosomal mapping of the murine CD40 ligand showed that the gene was linked to *HPRT* on the X chromosome (Padayachee *et al.*, 1993), while the human CD40 ligand mapped to the q26 band of the long arm of the

X chromosome (Aruffo *et al.*, 1993; Graf *et al.*, 1992). Comparison of the murine and human CD40 ligand amino acid sequences showed 73% identity at the amino acid level. Comparison of the amino acid sequence of the extracellular domain of CD40 ligand with that of other known protein sequences showed that CD40 ligand is related to TNF-α and TNF-β (Beutler & van, 1994; Hollenbaugh *et al.*, 1992).

CD40 ligand functions as a membrane-bound protein, however it has been reported that the supernatant from a murine T cell line, which expresses the membrane-bound form of CD40 ligand, also contains a soluble form of CD40L which induces B cell activation. This effect could be blocked using a CD40-Ig fusion protein. The biological function of the soluble form of CD40L is unknown (Armitage *et al.*, 1992b; Graf *et al.*, 1995). CD40L is not constitutively expressed on the cell surface, but can be detected on CD4^{+} T cells after *in vitro* activation with anti-CD3 mAb or PMA/ionomycin. Detectable levels of CD40L were found 2-4 hours following activation and maximal levels were detected 6-8 hours post-activation. Subsequent down regulation occurred 24-48 hours post-activation. Anti-CD3 activation of human CD8^{+} T cells did not induce expression of CD40L, although CD8^{+} T cells activated with PMA/ionomycin did express CD40L (Castle *et al.*, 1993; Lane *et al.*, 1992; Roy *et al.*, 1993; Sad *et al.*, 1997).

Murine CD4^{+} Th1 and Th2 clones were shown to express high levels of CD40L following *in vitro* activation with anti-CD3 mAb and the expression of CD40L on these T helper subsets could also be regulated by cytokines. TGF-β downregulated the expression of CD40L on both subsets, whereas IFN-γ only downregulated CD40L expression in Th2 cells. In this study IL-1, IL-2 and IL-4 had no effect on CD40L levels (Roy *et al.*, 1993). Immunohistology of the spleens of mice immunized with Keyhole Limpet Hemocyanin (KLH) revealed that CD40L expressing T cells were predominantly found in the outer periarteriolar lymphocyte sheaths (outer-PALS) and around the terminal arterioles. The greatest number of CD40L expressing T cells were observed 3-4 days after immunization with KLH, and these cells produced IL-2, IL-4 and IFN-γ with similar kinetics to those seen for CD40L expression. It was shown that B cells producing

antibodies to KLH were located in close proximity to the CD40L expressing T cells (van *et al.*, 1994a).

CD40L is expressed on activated basophils (Gauchat *et al.*, 1993) and eosinophils (Gauchat *et al.*, 1995). In both cases, these activated cells could induce the production of IgE by B cells in the presence of IL-4. Functional CD40L has also been found on human blood dendritic cells. These dendritic cells could induce B cell IgG and IgA production, suggesting that CD40L on dendritic cells may regulate B cell activation and differentiation (Pinchuk *et al.*, 1996). CD40L is also expressed on human B cell lines, which are able to induce resting B cells to produce immunoglobulin in a CD40-dependent manner. These results indicate that human B cells express a ligand for CD40 identical to that expressed on activated CD4$^+$ T cells (Grammer *et al.*, 1995).

1.6 The CD40-CD40L interaction

The crystal structures of CD40 and a complex of CD40-CD40L are still unknown. However, X-ray structures of TNF-α, TNF-β (Eck & Sprang, 1989; Eck *et al.*, 1992; Jones *et al.*, 1989), and the TNF-β/TNFR complex have been elucidated (Banner *et al.*, 1993) and these structures have served as templates for three-dimensional models of the extracellular domains of CD40, CD40L, and CD40-CD40L complex (Bajorath *et al.*, 1995b). Using structure-based sequence alignments and computer modeling, surface residues in CD40 and CD40L were selected to be targeted for site-directed mutagenesis. Binding studies with the mutant proteins resulted in the identification of five CD40 residues (E74, Y82, D84, N86, E117) and five CD40L residues (K143, Y145, Y146, R203, Q220) whose replacement with Alanine substantially affected the interaction of the two molecules (Bajorath *et al.*, 1995a; Bajorath *et al.*, 1995b). In all the binding experiments carried out, the structural integrity of the mutant proteins were determined using anti-CD40 and anti-CD40L mAbs, recognizing different conformational epitopes. These models predict that CD40L forms a trimer and that CD40 binds to the CD40L trimer at the interface between each of the monomers so that each CD40L trimer binds

three CD40 molecules (Bajorath *et al.*, 1995b). This is analogous to the TNF-β/TNFR complex (Peitsch & Jongeneel, 1993). In the TNF-β/TNFR complex, ligand binding has been proposed to result in receptor clustering and signal transduction (Banner *et al.*, 1993), a similar mechanism might be responsible for CD40 signaling (Bajorath *et al.*, 1995b).

A crystal structure of an extracellular fragment of human CD40 ligand has now been determined. CD40L forms a trimeric molecule like other members of the TNF family, such as TNF-α and TNF-β, and exhibits a similar overall folding pattern. Two residues (K143, Y145) appear to be directly involved in CD40 binding and the area immediately surrounding these two residues are also important for CD40 binding, whereas CD40L residues (S131, N180, F201, E202, N240) were not directly involved in CD40 binding. The CD40L structure suggests that most of the point-mutations occurring in patients suffering from hyper IgM syndrome (see "X-linked immunodeficiency with hyper-IgM") affect the folding and stability of the whole protein rather than directly affecting the binding site (Karpusas *et al.*, 1995).

1.7 CD40 intracellular signaling

The mechanism of signal transduction by members of the tumor necrosis factor (TNF) receptor family has only just begun to be elucidated. The cytoplasmic domains of the receptors in this family do not contain any obvious enzymatic activities, such as a tyrosine kinase domain (Tewari & Dixit, 1996). The mechanism by which they couple to downstream cellular signaling events is still an enigma.

The role of the CD40 cytoplasmic domain in signal transduction was examined by Inui and co-workers (Inui *et al.*, 1990). A cDNA encoding human CD40 was transfected into a murine B-cell lymphoma and a murine thymoma. Cross-linking of CD40 using anti-CD40 mAb resulted in inhibition of growth of these transfectants. By mutagenesis of the cytoplasmic domain of CD40 it was shown that a CD40 deletion mutant lacking Thr[234], or a point mutation in which Thr[234] was substituted with Ala, were unable to transmit

downregulatory signals in this system. This suggests that Thr^{234} or a region around this residue is critical for CD40 signal transduction. The two major effects of CD40 activation on B cells is the activation of NF-κB and the activation of protein tyrosine kinases (PTKs). Cross-linking CD40 on resting human tonsillar B cells and on B-cell lines results in the activation of transcription activator proteins p50, p65 (RelA), c-*rel* and NF-κB (Berberich *et al.*, 1994). Furthermore, coculture of human tonsillar B cells with various concentrations of PTK inhibitors led to inhibition of anti-CD40 mAb mediated activation of NF-κB, demonstrating that PTK activity is required for CD40-dependent NF-κB activation (Berberich *et al.*, 1994). The increase in PTK activity following cross-linking and activation of CD40 may play an important role in mediating the biological effects of CD40, as PTK inhibitors attenuate B-cell aggregation and immunoglobulin isotype switching in response to CD40 cross-linking (Kansas & Tedder, 1991; Ren *et al.*, 1994a). Phosphorylation of the *src* related kinase *lyn* has been shown to occur within one minute of CD40 cross-linking, whereas phosphorylation of other *src* related kinases such as *fyn*, *fgr* and *lck* remains unchanged. CD40 cross-linking also results in phosphorylation of phospholipase Cγ2 and phosphatidylinositol (PI)-3-kinase. Phosphorylation of PI-3-kinase was shown to be associated with an increase in its enzymatic activity. These findings suggest that *lyn*, phospholipase Cγ2 and PI-3-kinase play an important role in CD40 signal transduction (Ren *et al.*, 1994b). As *lyn* does not appear to associate directly with CD40, it seems likely that other molecules are required for the initial stage of signaling by CD40 (Ren *et al.*, 1994b).

Two molecules have been identified which bind the cytoplasmic domain of CD40. The first, TRAF2 (TNFR-associated factor 2), binds to the cytoplasmic domain of CD40 and to the cytoplasmic domain of TNFR2. The second, TRAF3 (TNFR-associated factor 3) is identified as a 64kDa protein that binds to the cytoplasmic domain of CD40 (Cheng *et al.*, 1995; Hu *et al.*, 1994; Mosialos *et al.*, 1995). This factor also binds to the LMP1 protein of the Epstein-Barr virus, which is known to be a potent inducer of B cell activation and proliferation. TRAF2 and TRAF3 share a region of homology near their

carboxyl termini, which binds the cytoplasmic domain of CD40, termed the TRAF domain (Rothe *et al.*, 1994). In addition to the TRAF domain, TRAF2 and TRAF3 contain an amino-terminal ring finger motif which may mediate protein-protein interactions. A possible role for TRAF2 is NF-κB activation. Overexpression of TRAF2 activates NF-κB, whereas expression of the ring-finger truncated version of TRAF2 attenuates CD40-mediated NF-κB activation (Rothe *et al.*, 1995). A truncated version of TRAF3 lacking the ring-finger motif can act as a dominant negative inhibitor, blocking the induction of CD23, a low affinity receptor for IgE, which is activated by CD40 ligation (Cheng *et al.*, 1995), suggesting that TRAF3 is involved in CD40-mediated signaling. In contrast, overexpression of full-length TRAF3 blocks CD40-mediated activation of NF-κB (Rothe *et al.*, 1995). The reason for the discrepancy in TRAF3 signaling is not clear. In addition to TRAF3 a new protein, TRAF5, has been found that binds the cytoplasmic tail of CD40. *In vitro* binding assays reveal that TRAF5 binds the cytoplasmic tail of CD40, but not the cytoplasmic tail of TNFR2. Overexpression of TRAF5 activates NF-κB, in contrast to TRAF3. The differences in signaling between TRAF3 and TRAF5. i.e. suppression versus activation of NF-κB and the fact that they both bind CD40, suggests that they may be involved in both common and distinct signaling pathways (Ishida *et al.*, 1996b). Yet another molecule, TRAF6, has been identified by use of a yeast two-hybrid system. TRAF6 seems to bind a region on the cytoplasmic tail of CD40 that is different from the region that binds TRAF2, TRAF3 and TRAF5. Despite the difference in binding, TRAF6 is also involved in NF-κB activation (Ishida *et al.*, 1996a). What still remains to be determined is, which molecular interactions link TRAF2, TRAF3, TRAF5 and TRAF6 to NF-κB activation.

1.8 Functional consequences of CD40 engagement

1.8.1 CD40 induced immunoglobulin production

Antibodies against CD40 in the presence of IL-4 were found to induce proliferation in human B cells (Clark & Ledbetter, 1986; Ledbetter *et al.*, 1987). Other studies showed

that stimulation via CD40 in the presence of IL-4 induced immunoglobulin class switching from IgM to IgE and the combination of IL-2 and IL-4 induced the production of large quantities of IgM and IgA (Rousset *et al.*, 1991; Zhang *et al.*, 1991). The ability of CD40 to induce B cell proliferation and isotype switching *in vitro*, suggested that the CD40-CD40L interaction *in vivo* might play a role in antibody production. This was tested by use of a CD40L antibody (MR1), which blocks the CD40-CD40L interaction. Following immunization with either T cell dependent or T cell independent antigens, mice were treated with the MR1 antibody. It was shown that the antibody blocked both primary and secondary antibody responses to T cell dependent antigens, but had no effect on the antibody response to T cell independent antigens (Foy *et al.*, 1993).

1.8.2 CD40 and Fas-induced apoptosis

Activation of B cells through CD40 was found to result in the induction of Fas expression, rendering B cells susceptible to Fas-induced apoptosis (Garrone *et al.*, 1995). Another study showed the importance of signals received through the B cell receptor, Fas and CD40 for the survival or death of B cells interacting with T cells. When the B cell receptor is triggered simultaneously with CD40 and Fas, Fas induced apoptosis is inhibited. It is proposed that B cell receptor triggering leads to upregulation of CD86, which interacts with CD28 on the T cell. This increased activation induces upregulation of CD40L and Fas and secretion of cytokines, leading to further differentiation of the B cells into plasma cells or memory cells. However, lack of triggering of the B cell receptor and continued CD40 and Fas ligation, leads to apoptosis. Prolonged triggering of the B cell receptor, CD40 and Fas, in the absence of cytokines also leads to apoptosis (Rathmell *et al.*, 1996).

1.8.3 CD40 induced Th1 development

Dendritic cells (DC) are professional antigen presenting cells (APC) specialized in antigen capture, migration to secondary lymphoid organs and T cell priming (Steinman, 1991). CD40 cross-linking on human dendritic cells by CD40L was shown to upregulate

the expression of CD80, CD86 and CD54, leading to increased capacity of DCs to stimulate proliferation of autologous or allogeneic T cells and IFN-γ production by T cells. In addition, DCs were induced to produce high levels of IL-12 upon CD40 cross-linking (Cella *et al.*, 1996; Peguet *et al.*, 1995). A similar study with murine dendritic cells showed that CD40 cross-linking induced IL-12 production by dendritic cells, and that IL-12 production could be down regulated by IL-4 and IL-10 (Koch *et al.*, 1996). IL-12 production by human monocytes could be triggered by activated T cells and this could be inhibited by a soluble CD40 (CD40-Ig) antibody blocking the CD40-CD40L interaction (Shu *et al.*, 1995). It has been established that IL-12 is responsible for the development of Th1 responses (Trinchieri, 1995) and these results suggest that the CD40-CD40L interaction is important for the priming of Th1 T cells via the stimulation of IL-12 secretion by antigen presenting cells. This hypothesis was tested in an *in vivo* model for a Th1-mediated disease, the hapten reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis. This experimental animal model of colitis has been shown to be Th1-mediated as the majority of the infiltrating CD4$^+$ T cells predominantly secrete IFN-γ. Treatment with anti-CD40L antibodies during the induction of the Th1-mediated colitis was able to prevent the disease and a decrease in IL-12 production was observed. When mice treated with TNBS and anti-CD40L were also administered recombinant IL-12, the effect of anti-CD40L was reversed and severe disease resulted. This study confirmed the role of CD40-CD40L in the priming of Th1 effector cells (Stuber *et al.*, 1996).

1.9 Germinal Centres

Germinal centres are the anatomical sites in which B cells undergo somatic hypermutation and immunoglobulin isotype switching, followed by differentiation into either plasma cells or memory cells (Kelsoe, 1996). When an organism is challenged with a pathogen or antigen via the skin or mucosal surfaces, the antigen is captured by immature dendritic cells (Steinman, 1991). The dendritic cells then mature during migration into the T cell rich areas of secondary lymphoid organs, such as the spleen, lymph nodes and Peyer's patches. Once they have reached the T-cell rich area (Figure 2.

A.), the mature dendritic cells, now called interdigitating dendritic cells (IDC), present antigen derived peptides bound to MHC class II, to naive antigen specific CD4$^+$ T cells (MacLennan *et al.*, 1997). These antigen activated CD4$^+$ T-cells either leave the T cell rich area to become recirculating effector- and memory-cells, or migrate to the outer zone of the T cell rich area. In the outer zone the antigen activated CD4$^+$ T cells associate with naive antigen specific B cells that have been activated through their B cell receptor (BCR) by free antigen (MacLennan *et al.*, 1997). After this cognate interaction between activated CD4$^+$ T cells and activated B cells, the activated CD4$^+$ T cells migrate into the light zone of the germinal centre or become recirculating effector- or memory-cells. The activated B cells migrate to extrafollicular sites where they either differentiate into plasma cells that are mainly short-lived (Smith *et al.*, 1996), or into centroblasts to form the dark zone of the germinal centre (Liu *et al.*, 1991; MacLennan *et al.*, 1990). B cell growth in extrafollicular sites is not associated with somatic mutation in Ig variable-region genes (Jacob *et al.*, 1991a), but memory B cells with mutated Ig variable-region genes can be induced in secondary responses to proliferate in extrafollicular sites and differentiate into plasma cells (Toellner *et al.*, 1996).

In the dark zone the centroblasts activate a somatic hypermutation mechanism that acts on their Ig variable region genes, which changes the affinity and specificity of the B cell receptor (Berek *et al.*, 1991; Jacob *et al.*, 1991b). The centroblasts continue to proliferate with a cell cycle time of 6-7 hours. There is no increase in numbers of centroblasts, however, as they continually give rise to non-dividing centrocytes, which then migrate into the follicular dendritic cell (FDC) network (Liu *et al.*, 1991; Zhang *et al.*, 1988). Having undergone affinity maturation of the Ig variable region in the dark zone, the centrocytes are now selected in the light zone of the germinal centre on the basis of their ability to bind and process antigen held on FDC and make cognate interaction with antigen activated germinal centre T cells (MacLennan, 1994). There is a high death rate among B cells in germinal centres. This occurs among centroblasts as well as centrocytes but the greatest concentration of dying cells is found among the centrocytes in the light

zone (Hardie *et al.*, 1993). Apoptosis in these germinal centre B cells seems to correlate with high expression of apoptosis-inducing genes, such as c-*myc*, p53, Bax and Fas in the absence of Bcl-2. Apoptosis of cultured centrocytes can be delayed for some hours by crosslinking their BCR with anti-Ig coated onto erythrocytes (Martinez *et al.*, 1996). The fact that CD40L is expressed by activated T cells (Armitage *et al.*, 1992a) pointed to the possibility that germinal centre T cells might be involved in the selection of centrocytes. This hypothesis was supported by results showing that a more efficient inhibition of apoptosis in centrocytes could be achieved by CD40 cross-linking with CD40 monoclonal antibody (Liu *et al.*, 1989) or recombinant CD40L (Holder *et al.*, 1993). The survival signal delivered through CD40 does not act by upregulating Bcl-2 expression, but apoptosis is inhibited for several hours before Bcl-2 starts to be expressed in centrocytes (Holder *et al.*, 1993).

The role of CD40-CD40L in the development of germinal centres and the generation of memory B cell development was examined *in vivo*. Mice were immunized with sheep red blood cells (a thymus-dependent antigen), treated with anti-CD40L antibody (MR-1), and the development of germinal centres was evaluated using immunohistochemical staining for the presence of germinal centres 9-11 days after immunization. The results showed that the formation of germinal centres was completely inhibited as a result of treatment with anti-CD40L antibody. Furthermore, adoptive transfer experiments demonstrated that the generation of antigen-specific memory B cells was inhibited by blocking the CD40-CD40L interaction, with no IgG1 production by memory B cells (Foy *et al.*, 1994). Another study demonstrated that *in vivo* treatment with anti-CD40L antibody inhibited both primary and secondary immunoglobulin responses to thymus-dependent antigens, whereas the immunoglobulin response to thymus-independent antigens was unaltered (Foy *et al.*, 1993). The CD40-CD40L interaction was found to be necessary for memory B cell development to thymus-dependent antigens but not germinal centre formation, in experiments using a soluble CD40 fusion protein (CD40-Ig) that blocked the CD40-CD40L interaction (Gray *et al.*, 1994). The reason for the

discrepancy in the requirement for the CD40-CD40L interaction in germinal centre formation is unknown, but it is argued that the soluble CD40 fusion protein may be incapable of efficient blocking of the CD40-CD40L interaction involved in germinal centre formation (Foy *et al.*, 1994).

Germinal centre T cells not only express CD40L (Casamayor *et al.*, 1995), but may also express Fas-ligand. *In vitro* experiments have shown that CD40L is not able to protect germinal centre B cells from Fas-mediated apoptosis and even primed resting B cells are sensitive to Fas-mediated apoptosis (Garrone *et al.*, 1995), however anti-Ig could inhibit this Fas-mediated apoptosis. Therefore, germinal centre B cells will die by apoptosis if they do not interact with T cells expressing CD40L within a short period of time or if they interact with T cells expressing CD40L and Fas-ligand in the absence of B cell receptor triggering (Rathmell *et al.*, 1996). These results support a model for selection in the germinal centres. Centrocytes that have undergone somatic hypermutation but only have low-affinity antigen receptors die by apoptosis due to their failure to bind antigens on follicular dendritic cells. Centrocytes with high-affinity antigen receptors will bind antigen on FDCs, take up the antigen and present it to germinal centre T cells. These T cells will then deliver a rescue signal via CD40L and the centrocytes start to differentiate into plasma cells or memory B cells (MacLennan, 1994).

In vitro experiments have shown that in addition to the involvement of CD40L in preventing Fas-mediated apoptosis and it's role in subsequent plasma cell and memory cell development, CD40L also produces a negative signal that prevents human germinal centre B cells from differentiating into plasma cells. Germinal centre B cells undergo strong proliferation in the presence of IL-2 and IL-10, as long as CD40L is provided. Upon withdrawal of the CD40L, germinal centre B cells rapidly differentiate into plasma cells (Arpin *et al.*, 1995). A similar study examined the role of CD40 cross-linking on human centrocytes co-cultured with either naive T cells or memory T cells. Both T cell populations were induced to express CD40L, before co-culture with centrocytes. It was found that centrocytes formed conjugates with the memory T cells and the cells in these

conjugates differentiated into memory B cells. The effect was lost if the CD40-CD40L interaction was blocked by an anti-CD40L antibody. Centrocytes co-cultured with naive T cells did not differentiate into memory B cells. Taken together these experiments suggest that CD40 cross-linking induces centrocytes to differentiate into memory B cells and differentiation of centrocytes require memory T cells (Casamayor *et al.*, 1996).
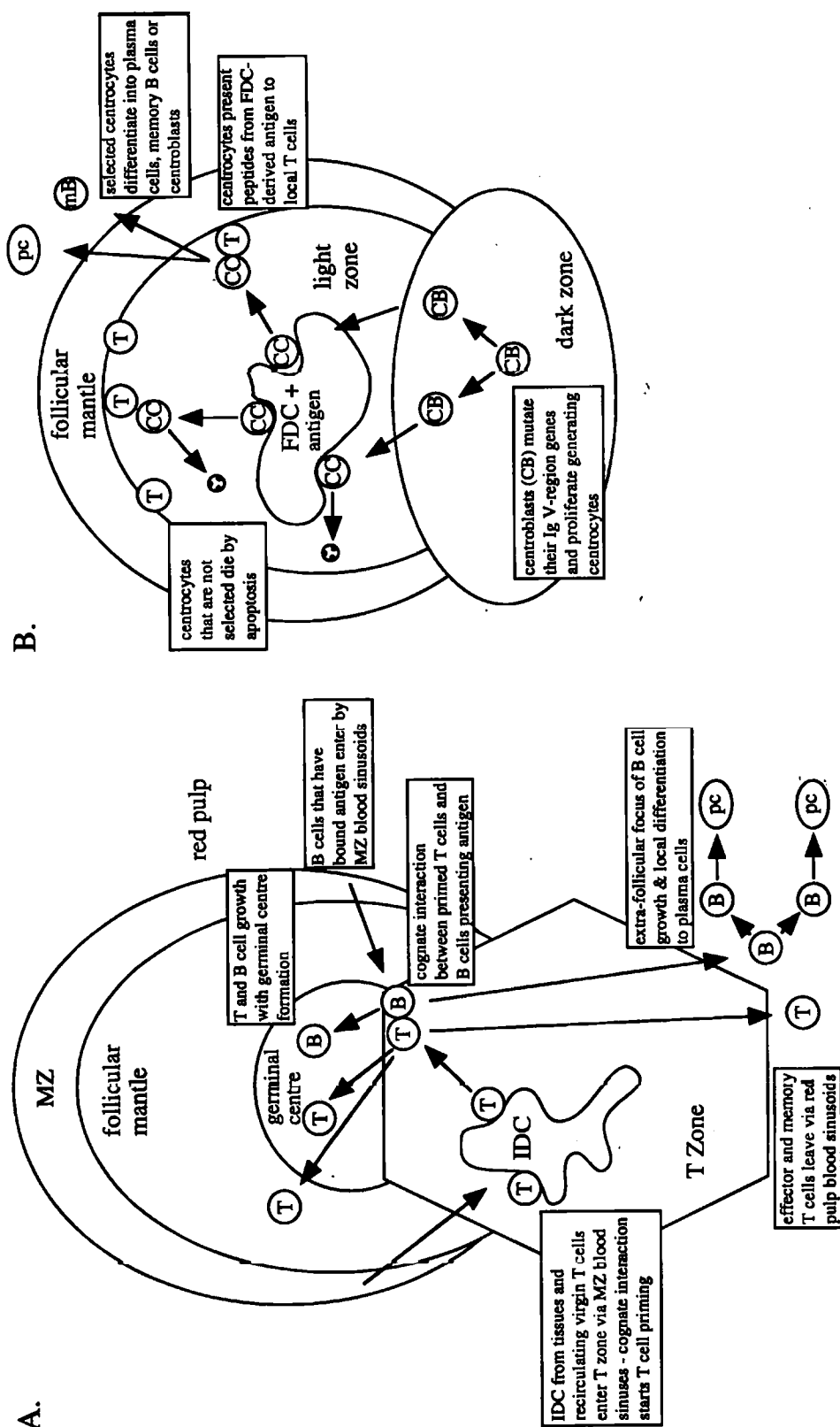
Figure 2. Schematic drawing of cell-interactions in the T zone of the spleen (A.) and germinal centre (B.).
A. Cells involved in cognate interactions in the T zone of the spleen. IDC from tissues and recirculating naive T cells enter the T zone via MZ blood sinuses and this interaction starts T cell priming. Effector and memory T cells leave via the red pulp blood sinusoids or interact with antigen specific B cells in the T zone. Antigen activated T and B cells initiate the germinal centre formation. MZ: marginal zone; T: T cell; B: B cell; pc: plasma cell; IDC: interdigitating dendritic cell.
B. Hypermutation and selection of germinal centre B cells. Centroblasts mutate their Ig V-region genes and proliferate into centrocytes. Centrocytes are then selected in the light zone of the germinal centre on the basis of their ability to bind and process antigen held on FDCs and interact with antigen activated germinal centre T cells. Centrocytes which fail to bind antigen on FDCs or present antigen to germinal centre T cells die by apoptosis. Successful interaction between centrocytes and T cells results in antigen specific plasma and memory B cell production. CB: centroblasts; cc: centrocytes; T: CD4+ T cell; pc: plasma cell; mB: memory B cell; FDC: follicular dendritic cell.

1.10 Immunoglobulin class switching

Immunoglobulin class switching occurs when IgM⁺IgD⁺ B cells switch expression to a different heavy-chain constant region (C_H), which results in a change in both the class of antibody and in the effector function. The expressed variable region (V(D)J-region) of both heavy- and light-chains do not change, therefore the specificity of antigen binding is unaltered (Stavnezer, 1996a). Immunoglobulin class switching in IgM⁺IgD⁺ B cells starts approximately 6 days after activation by thymus dependent antigens *in vivo* (Stavnezer, 1996a). The mechanism resulting in class switching is a switch recombination event, in which the V(D)J region recombines with a downstream heavy-chain constant region ($C_{\gamma,\varepsilon,\alpha}$) and the intervening DNA is deleted, resulting in the following isotypes; IgG, IgE and IgA. The switch recombination event occurs between tandemly repeated sequences, called switch regions, which are located upstream of each heavy-chain constant region. Sterile "germline" transcripts, are produced prior to the switch recombination event and have been shown to be essential for class switching to occur (Coffman *et al.*, 1993; Sideras *et al.*, 1989). Germline transcripts lack the V(D)J-encoded sequence and therefore cannot direct synthesis of immunoglobulin. Transcription of these germline transcripts starts 5' to the switch region, proceeds through the heavy-chain constant region ($C_{\gamma,\varepsilon,\alpha}$) and terminates at the normal poly(A) sites for secreted or membrane bound Ig heavy-chain mRNAs. After switch recombination, DNA 5' to the switch region is deleted from the chromosome and thus germline transcripts cannot be produced after class switching, instead a functional V(D)J-C_H mRNA is produced (Liu *et al.*, 1996; Lutzker & Alt, 1988; Stavnezer, 1996b). Class switching to IgG, IgA and IgE occurs by DNA recombination, in contrast to an alternative mechanism, RNA processing, that occurs for production of IgD, which is co-expressed with IgM on mature B cells (Stavnezer, 1996a).

Cultured mouse and human IgM⁺ B cells can be induced to switch to all classes of antibody isotypes by B-cell mitogens such as lipopolysaccharide (TI-1) for mouse B

cells and by Staphylococcus aureus Cowan I for human B cells, in the presence of the appropriate cytokine (Stavnezer, 1996b). Immunoglobulin class switching in B cells is influenced by both CD40 cross-linking and the presence of different cytokines. *In vitro* studies have shown that purified human B cells could be induced to produce IgE by cross-linking CD40 (CD40 mAb) in the presence of recombinant IL-4 (Jabara *et al.*, 1990). IL-4 also augments class switching to IgA in murine B cells activated in vitro with LPS or CD40L in the presence of IL-5 and transforming growth factor-β (TGFβ) (McIntyre *et al.*, 1995). IL-10 induces isotype switching to IgG1 and IgG3 in human CD40 activated B cells (Malisan *et al.*, 1996) and to IgG3 in murine LPS activated B cells (Shparago *et al.*, 1996). CD40 activation of murine splenic B cells has been shown to induce germline γ_1 and ϵ transcripts independently of cytokines (Warren & Berton, 1995). Cross-linking surface-Ig on B cells with dextran (TI-2) induces germline γ_1, γ_3 and γ_{2a} transcripts, but not γ_1 and ϵ (Zelazowski *et al.*, 1995). Thus, it appears that both CD40 and surface-Ig signaling contribute to the isotype specificity of class switching.

1.11 X-linked immunodeficiency with hyper-IgM (HIGM1)

The X chromosome-linked form of the hyper IgM syndrome (HIGM1) is a rare disorder characterized by the inability of B cells to undergo immunoglobulin isotype switching. Affected males experience recurrent infections, usually within the 1st year of life, when levels of maternally-derived antibodies decline. Most infections are of bacterial origin, but HIGM1 patients are also unusually susceptible to infections with opportunistic pathogens and often suffer from *Pneumocystis carinii* pneumonia and *Cryptosporidium* intestinal infection. These are diseases that are often observed with T-cell immunodeficiencies but not with other forms of hypogammaglobulinemia (Notarangelo *et al.*, 1992). Haematological disturbances, such as anaemia, thrombocytopenia, and neutropenia are common in HIGM1 patients. Autoimmunity and increased susceptibility to neoplasms are also well documented in HIGM1. HIGM1 patients have normal numbers of circulating B cells. The overall early-mortality rate in HIGM1 patients is not known, but the figure appears to be around 10% from a survey of 67 patients. HIGM1

patients often suffer from life-threatening infections in spite of intravenous immunoglobulin and antibiotic prophylactics (Notarangelo *et al.*, 1992).

Affected individuals have normal or elevated serum concentrations of IgM and decreased serum levels of IgG, IgE, and IgA (Geha *et al.*, 1979; Levitt *et al.*, 1983). The primary antibody response, that of IgM, develops normally upon *in vivo* antigenic stimulation. However, boosting results in poor secondary responses, with no expression of IgG specific antibodies (Nonoyama *et al.*, 1993). It has been shown that peripheral blood mononuclear cells from HIGM1 patients, when stimulated with pokeweed mitogen *in vitro*, only secrete IgM, thus resembling the *in vivo* situation (Levitt *et al.*, 1983; Mayer *et al.*, 1986). Patients suffering from HIGM1 show no development of germinal centres in the lymph nodes (Notarangelo *et al.*, 1992). The lack of immunoglobulin class switching seems to be a defect in B cell function, but the primary role of defective T helper cell activity was also established, when it was shown that B cells from HIGM1 patients differentiated into IgG-secreting cells if cocultured with activated T cells (Mayer *et al.*, 1986). With the exception of helper T cell activity, the *in vitro* and *in vivo* activation of T cells as measured by mitogen induced proliferation, response to alloantigen and delayed-type hypersensitivity is usually normal in HIGM1 patients (Benkerrou *et al.*, 1990).

Molecular genetic analysis of HIGM1 patients revealed that the gene responsible for HIGM1 mapped to Xq24-27 (Mensink *et al.*, 1987), and was shown to be distinct from the XLA gene (Malcolm *et al.*, 1987). The study of a larger number of HIGM1 patients allowed for further refinement of the HIGM1 gene location to Xq26-27 (Padayachee *et al.*, 1993). Mapping of the CD40L gene to the q26.3-27.1 region of the X-chromosome by fluorescence *in situ* hybridization (Aruffo *et al.*, 1993; Graf *et al.*, 1992; Kroczek *et al.*, 1994), provided the initial evidence that CD40L was involved in HIGM1. The characteristics of the disease, low levels of isotype switched immunoglobulins, IgG, IgA, and IgE, indicated a failure in the ability to switch from IgM to other immunoglobulins. This was compatible with the finding that stimulation through CD40

in the presence of cytokines was required to induce immunoglobulin class switching and secretion of immunoglobulins (Rousset *et al.*, 1991; Zhang *et al.*, 1991).

To exclude a defect in the B cells of HIGM1 patients, the capacity of B cells to secrete immunoglobulin was compared in HIGM1 patients and age-matched controls. Peripheral blood lymphocytes (PBL) were cultured in the presence of pokeweed mitogen, a compound inducing immunoglobulin synthesis by activating B and T cells and allowing them to cooperate. PBL of HIGM1 patients were unable to secret IgG, IgA or IgE in contrast to the controls, who were able to switch normally from IgM secretion to other Ig isotypes. However, when B cells from HIGM1 patients were cross-linked directly through CD40 in the presence of *Staphylococcus aureus* Cowan (SAC) and IL 4 or IL 10, the B cells produced IgG, IgA and IgE normally (Durandy *et al.*, 1993; Korthauer *et al.*, 1993). These findings confirm the unimpaired intrinsic capacity of B cells in HIGM1 patients to undergo immunoglobulin class-switching *in vitro* and to produce immunoglobulin of all isotypes (Korthauer *et al.*, 1993). Earlier analysis of immunoglobulin heavy-chain genes in B cells from HIGM1 patients by use of genetic probes of constant and switch regions showed normal gene patterns (Mayer *et al.*, 1986). These results show that the inability to switch in these patients is due to ineffective helper T cell activity for B cell differentiation.

The cloning of the human CD40L molecule (Graf *et al.*, 1992; Hollenbaugh *et al.*, 1992; Spriggs *et al.*, 1992) and the development of a soluble CD40-Ig fusion protein (Fanslow *et al.*, 1992; Lane *et al.*, 1992; Noelle *et al.*, 1992) has enabled the analysis of the defect in HIGM1 patients at the molecular level. Cell surface analysis of activated T cells from three males, all from unrelated families, demonstrated that CD40L expression was defective, since all three HIGM1 patients failed to bind soluble CD40-Ig (Korthauer *et al.*, 1993; Kroczek *et al.*, 1994). However, the use of CD40L-specific rabbit antiserum against a recombinant polypeptide derived from the first 137 amino acids of the extracellular portion of the CD40L protein showed that non-functional CD40L was expressed on activated T cells in some, but not all patients (Korthauer *et al.*, 1993). In

order to identify the basis for the failure of activated T cells from HIGM1 patients to bind CD40-Ig, the CD40L mRNA was analysed. All three patients had point mutations, leading either to protein truncation, complete abrogation of cell surface expression or drastic amino acid changes (Korthauer *et al.*, 1993). Other groups have also identified mutations in the CD40L protein from patients with HIGM1 (Aruffo *et al.*, 1993; Di *et al.*, 1993). Apart from a Met_{36}-Arg substitution that affects the transmembrane portion of the protein, all other mutations appear to be located to the extracellular part of the protein, especially within the TNF homology domain, corresponding to amino acids 123-261 (Kroczek *et al.*, 1994). Three patients have been identified with a point mutation in the same codon (Trp_{140}) leading either to stop codons or a non-conservative amino acid substitution (Kroczek *et al.*, 1994). This point mutation leads to complete abrogation of CD40-Ig binding. These findings are in agreement with the finding that amino acids 143 and 145 in the CD40L protein are important for the binding of CD40 (Bajorath *et al.*, 1995a; Bajorath *et al.*, 1995b).

In contrast to the above mentioned studies, hyper-IgM syndrome patients with functional CD40L have also been studied (Callard *et al.*, 1994; Conley *et al.*, 1994; Durandy *et al.*, 1997). T cells from these patients were all able to bind to the CD40-Ig fusion protein and sequencing data showed that the CD40L cDNA appeared normal. However, serum IgG, IgA and IgE was undetectable and histology showed lack of germinal centres in the spleen. B cells from these patients were unable to synthesize IgG, IgA and IgE *in vitro* when stimulated with anti-CD40 mAb in the presence of IL-4 or IL-10. Sequence analysis of CD40 and CD40 binding proteins, TRAF2 and TRAF3, revealed no abnormalities in the protein coding regions in B cells from CD40L positive patients. Further studies traced the defect to phosphatidyl-inositol 3 (PI3) kinase activation and subsequent induction of NF-κB (Durandy *et al.*, 1997). Altogether, these results show that patients suffering from HIGM1 have a defect either in CD40L or in the CD40-triggered activation cascade, both of which account for the lack of germinal centre formation and immunoglobulin isotype switching.

Patients with HIGM1 have normal or elevated levels of serum IgM, indicating that IgM synthesis and secretion can proceed in the absence of a functional CD40-CD40L interaction. In HIGM1 patients with elevated IgM, levels of IgM often fluctuate, and increases in IgM levels often coincide with infection. This probably reflects the response to antigenic stimulation (Kroczek *et al.*, 1994).

1.12 Generation of a gene-deleted mouse by homologous recombination in embryonic stem cells

The most definitive way of establishing the function of a gene *in vivo* is the generation of a gene-deleted mouse ("knockout mouse") by targeted mutation of the gene. The whole process of making a gene-deleted mouse is dependent upon the occurrence of a homologous recombination event that occurs between the targeting vector and the endogenous gene in mouse embryonic stem (ES) cells.

The targeting vector contains sequences homologous to the endogenous gene, and once introduced into ES cells by electroporation, the sequences that are homologous can recombine with and replace the endogenous gene. Positive and negative selection markers are used to select for cells in which a homologous recombination event have taken place (Figure 3.). The positive-selection marker, a neomycin resistance gene (NEO) is inserted into an upstream exon and the negative selection-marker, a viral thymidine kinase gene (TK) is inserted outside the homologous sequences. If a homologous recombination event occurs, the ES cells will be resistant to G418 (protein synthesis inhibitor) and insensitive to FIAU/gancyclovir (nucleotide analogue incorporated by HSV-tk). The presence of the inserted DNA (NEO) in the upstream part of the endogenous gene ensures that transcription is disrupted, leading to a non-functional gene. Targeting vectors can be designed such that one or more exons will be deleted if homologous recombination takes place and the presence of the desired recombination can be verified by Southern blot analysis or PCR.

ES cells in which the endogenous gene has been disrupted are injected into mouse blastocysts, and these blastocysts are then implanted into a pseudopregnant foster mother (Figure 4.). The foster mother will give birth to chimaeric mice. The tissues in these chimaeric mice are a mixture of the gene disrupted ES cells derived from one strain of mice (129Sv, agouti coat colour) and cells from the wild-type blastocysts (C57BL/6, black coat colour). Chimaeric mice where most of the tissue is derived from the gene disrupted ES cells (mainly agouti coat colour) will have a sex bias towards males, since the ES cell line is male-derived (XY ES cells). In combination with a female blastocyst, male ES cells will often produce a fertile, phenotypic male chimaeric mouse. These sex-converted chimaeric mice are advantageous for breeding since they will only transmit the ES cell genotype. If the ES cells colonize a male blastocyst the resultant chimaeric mouse will transmit both the wild-type genotype and the ES cell genotype. Chimaeric males are then mated with wild-type females. The resultant offspring will be either black (wild-type) or agouti ("germline transmission"). Only 50% of the agouti offspring will be heterozygous for the gene disruption. Mice heterozygous for the gene disruption can then be mated to produce offspring homozygous for the gene disruption. Such "knockout mice" are deficient in the expression of the targeted gene.
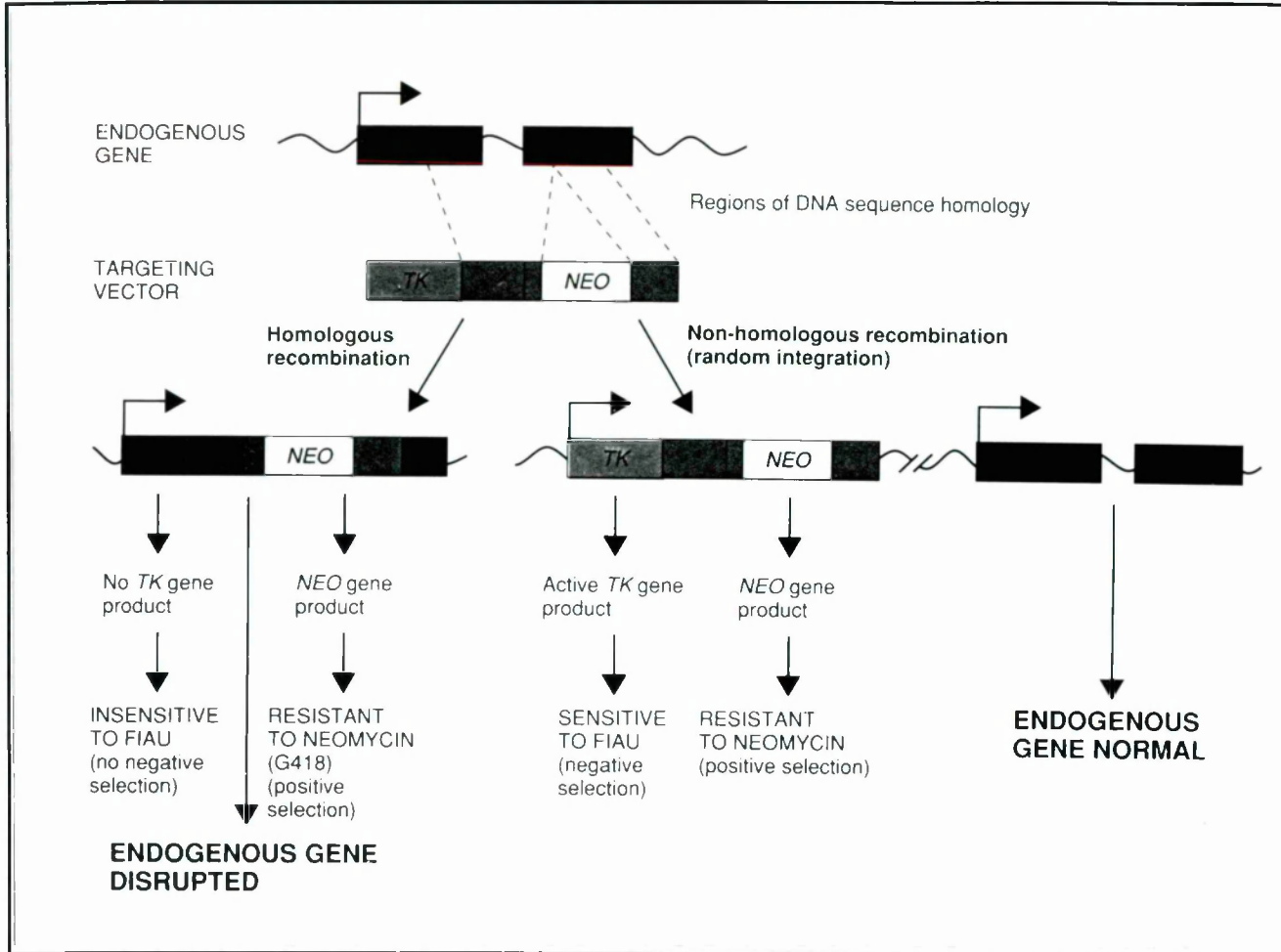
Figure 3. Disruption of the endogenous locus with a sequence replacement vector. The targeting vector can integrate into the genome either by homologous recombination or random integration. The positive selection marker (NEO) selects for cells which have integrated the targeting vector and the negative selection marker (TK) ensures that only cells which have integrated the vector at the correct locus are resistant to FIAU. Taken from Cellular and Molecular Immunology by Abul Abbas, Jordan S. Pober, Andrew H. Lichtman. 1994.
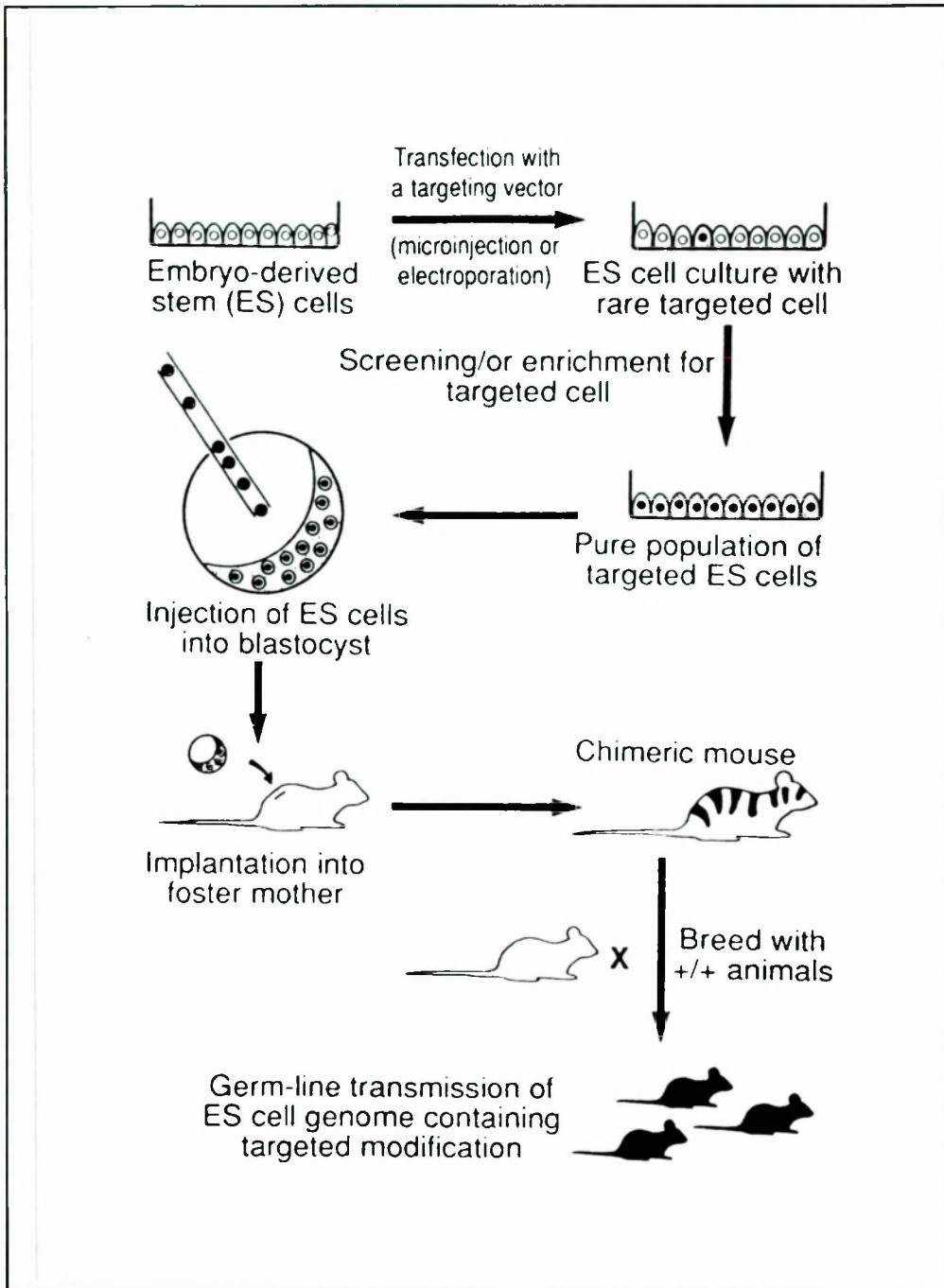
Figure 4. Generation of germ-line chimaeras from embryonic stem (ES) cells. ES cells are transfected with the targeting vector. This is followed by enrichment for targeted ES cell clones (positive and negative selection markers, Figure 3) which have integrated the targeting vector at the correct locus. Targeted ES cells are then injected into blastocysts and the blastocysts are transferred into a foster mother. The resulting chimaeric mouse is bred with wild-type mice to check for germline transmission. Taken from (Capecchi, 1989).

1.13 CD40 deficient mice

To study the role of CD40 *in vivo* and to produce an animal model for hyper IgM syndrome, I generated CD40 deficient mice using homologous recombination in embryonic stem cells.

The analysis of CD40 deficient mice will provide a definite proof of whether the CD40-CD40L interaction is important for germinal centre formation and immunoglobulin class switching. Patients suffering from hyper-IgM syndrome fail to show germinal centres, and this is likely to be caused by a defect in the expression of CD40L (Korthauer *et al.*, 1993). Furthermore, blocking the CD40-CD40L interaction *in vivo* with soluble anti-CD40L antibody abrogated germinal centre formation in mice (Foy *et al.*, 1994). Patients with hyper-IgM syndrome have elevated or normal levels of IgM and low levels of isotype switched immunoglobulins, IgG, IgA, and IgE, indicating a failure in the ability to switch from IgM to other immunoglobulins (Geha *et al.*, 1979; Levitt *et al.*, 1983). The treatment of mice with anti-CD40L antibody inhibited the expression of all immunoglobulins in secondary responses to KLH (Foy *et al.*, 1993), confirming the role of CD40-CD40L in isotype switching. Immunization of CD40 deficient mice with KLH and immunohistochemical analysis of spleens in CD40 deficient mice will determine whether the CD40-CD40L interaction is important in germinal centre formation. Analysis of serum from CD40 deficient mice will provide the answer to whether immunoglobulin class switching is regulated by the CD40-CD40L interaction.

Patients with hyper-IgM syndrome are unusually susceptible to infections with opportunistic pathogens and often suffer from *Pneumocystis carinii* pneumonia and *Cryptosporidium* intestinal infection. These are diseases that are often observed with T-cell immunodeficiencies but not with other forms of hypogammaglobulinemia (Notarangelo *et al.*, 1992). It will be interesting to determine whether the absence of the CD40-CD40L interaction leads to functional T cell abnormalities directly or indirectly through defective activation of antigen-presenting cells. Cross-linking of CD40 on

dendritic cells and monocytes induces secretion of IL-12 (Cella *et al.*, 1996; Peguet *et al.*, 1995; Shu *et al.*, 1995), which is important for the development of Th1 responses (Trinchieri, 1995). This suggests that the CD40-CD40L interaction might be involved in cell-mediated immunity (Th1 development) as well as humoral immunity.

To determine whether the CD40-CD40L interaction is important for cell-mediated immunity to *Bacillus Calmette-Guerin* (BCG). CD40 deficient mice were infected with BCG. Survival and bacterial counts in spleen, lung, and liver of CD40 deficient mice were analysed. The analysis of tuberculin Purified Protein Derivative (PPD) induced IFN-γ, IL-12, TNF-α and nitric oxide production by splenocytes of BCG infected CD40 deficient mice will reveal any defect in Th1 development.

Chapter 2: Materials and Methods

2.1 Molecular Biology

2.1.1 Agarose gel electrophoresis

1 % (w/v) agarose gels were made by dissolving agarose and ethidium bromide (60 μg, 10 μg/μl) in boiling 1x Tris-acetate buffer and pouring the solution into a gelform with an appropriate slot former. The gels were run with DNA samples in 1x Tris-acetate buffer at 50-200 mA. After electrophoresis, gels were photographed under UV-illumination (Stratagene: Eagle Eye; Mitsubishi: video copy processor).

2.1.2 Isolation of DNA fragments from an agarose gel

After electrophoretic separation in an agarose gel and visualisation by ethidium bromide staining and UV-illumination, the relevant fragment(s) were isolated using a strip of GFC-filter and dialysis tubing. The agarose gel was cut below the fragment band and the GFC-filter on top of the dialysis tubing was placed in the crack below the fragment band. Electrophoresis was continued until all the DNA had transferred to the GFC-filter as judged by UV-illumination. The GFC-filter (Whatmann, glass microfibre filters, cat. no. 1822025) was transferred to a 0.5 ml Eppendorf tube without a tip and then placed on top of a 1.5 ml Eppendorf tube. The Eppendorf tube was centrifuged at 14000 rpm (Hereaus Biofuge) for 5'. The solution of approximately 30 μl containing the fragment was extracted with phenol/chloroform and the aqueous phase was passed through a Sephadex G50 spin column in preparation for ligation.

2.1.3 Isolation of plasmid DNA (miniprep)

Harvesting and lysis of bacteria was based on the principle of the alkali-SDS method in which SDS binds and denatures proteins and lipids and NaOH denatures the DNA, resulting in lysis of the cells. The cellular debris including chromosomal DNA is precipitated by centrifugation and removed and plasmid DNA is recovered from the

supernatant by ethanol precipitation and dissolved in TE. A single colony was inoculated into 2 ml LB medium containing 40 µg/ml Ampicillin in 3 ml wells of a microtiter plate. These were grown for 7-14 h at 37 °C. with constant shaking (200 rpm). The cultures were centrifuged 2600 rpm for 3' and the supernatant discarded (Sorvall Centrifuge). The pellet was resuspended in 100 µl Sol I and lysed with 200 µl Sol II, then 150 µl Sol III was added and the plate was shaken flat on a bench. Cell fragments and genomic DNA will be in a well defined aggregate. The plate was centrifuged at 2600 rpm for 4' and the supernatant was transferred to a clean 3 ml well. 1 ml 96% ethanol was added to precipitate the plasmid and centrifuged for 4' at 2600 rpm. The ethanol was removed and the DNA was resuspended in 80 µl TE and transferred to a small microtiter plate. 80 µl 5M LiCl was added to precipitate proteins for 5' at -20 °C. The samples were centrifuged 2600 rpm 3' and transferred to an Eppendorf tube. The DNA was ethanol precipitated once again and dissolved in 50 µl TE, yielding 25-50 µg plasmid DNA.

2.1.4 Isolation of plasmid DNA (maxiprep)

A single colony was inoculated into 10 ml of LB containing 40 µg/ml ampicillin and grown o/n at 37 °C with vigorous shaking. The overnight culture was seeded into 400 ml LB/amp and grown 37 °C o/n. The culture was transferred to 500 ml Beckmann bottles and centrifuged at 4000 rpm (Beckmann J6 MC centrifuge) for 20' at 4 °C. The supernatant was discarded and the pellet resuspended in 4 ml solution I and transferred to 50 ml tubes (Beckmann centrifuge).A further 0.5 ml of solution I was added containing 4 mg/ml lysozyme, vortexed and incubated RT for 10'. 8 ml of solution II was added to the mix with vortexing and then incubated on ice for 5'. Then 4.5 ml of solution III was added with vortexing and the sample incubated on ice for 15'. The supernatant was recovered after centrifugation at 4000 rpm for 20' at 4 °C. 11 ml isopropanol was added to the supernatant and the DNA was pelleted by centrifugation at 14000 rpm for 20' at 4 °C. The supernatant was discarded and the DNA pellet dried. The pellet was resuspended in 3 ml ddH$_2$O plus (44 µl of 1 M Tris pH 8.0 + 27 µl of 0.5 M EDTA pH8.0), allowing approximately 1 hr for resuspension. 4.4 g CsCl + 300 µl EtBr (10mg/ml) was added

before loading the contents into a 13x48 mm Optiseal tube (Beckmann 362185). This was centrifuged for 3.5 hr at 70K rpm in a VTi80 rotor at 20 °C. Extraction of CsCl band was performed according to the procedure described in Maniatis (Molecular Cloning: A laboratory Manual, Sambrook, Fritsch, Maniatis). The plasmid DNA was then butanol extracted to remove contaminating EtBr, followed by ethanol precipitation, air drying and resuspension in 500 µl ddH$_2$O. Protein and RNA were removed by digestion with 20 µg/ml RNAse for 60' at 37 °C followed by 60' digestion with 100 µg/ml proteinase K. Phenol/chloroform extraction followed by ethanol precipitation was used to remove the enzymes. Lastly the DNA pellet was washed in 70 % ethanol, air dried and resuspended in 500 µl TE and the DNA concentration determined.

2.1.5 Plating and screening genomic library

Bacteria (LE392) were grown in 10 ml LB-medium containing 0.2 % maltose and 10 mM MgCl$_2$, o/n at 37 °C. The bacteria were infected with lambda phage containing the genomic library (Lambda Dash 9H-129) by mixing 100 µl MgSo$_4$/CaCl$_2$ buffer (10 mM final), 300 µl bacteria, 100 µl SM buffer, and phage (5.0 x 10^4 pfu/plate) and incubated 10' at 37 °C. 10 ml Top agar (45-48 °C) was added to the infected bacteria, mixed gently and poured onto LB-agar plates (15 150mm LB-agar plates had been pre-prepared for this purpose). These were incubated at 37 °C for 12-16 hr. The plates were then incubated at 4 °C for 1 hr. Duplicate filters (HybondN+) were made for each plate by placing the filters on the plates making sure of the orientation of the filters by making holes through filter and agar using a 25 gauge needle. The corresponding holes in the filters were marked on the bottom of the plate. The filters were gently peeled off and dried. Phage DNA bound to the filters was denatured by soaking in 0.5 M NaOH, 1.5 M NaCl and neutralised in 1.0 M Tris-HCl pH 7.5, 1.5 M NaCl. Any agarose was removed using gauze. Filters were rinsed in 2x SSC and dried on 3MM paper and baked at 80 °C for 2 h, ready for hybridisation. Any positive plaques that were identified were extracted and a subscreening performed if necessary. For a detailed description of this

procedure please see Maniatis (Molecular Cloning: A Laboratory Manual, Sambrook, Fritsch, Maniatis).

2.1.6 Phage maxiprep

Bacteria (LE392) were grown o/n at 37 °C in LB-medium containing 10 mM $MgCl_2$ and 0.2 % maltose. Five different dilution's of phage were made and mixed with 1 ml of bacteria plus 10 μl $MgSo_4/CaCl_2$ buffer (1 M) and incubated 10' at 37 °C. 100 ml of LB-medium containing 10 mM $MgSo_4/CaCl_2$ was added to the infected bacteria and incubated at 37 °C for 5-8 hr until cell lysis occurred. The phage dilution giving complete cell lysis was identified and another 4 x 100 ml bacterial cultures containing the appropriate phage dilution were set up and incubated at 37 °C for 5-8 h until lysis. The 5 lysates (500 ml) were pooled, 2 ml of chloroform was added and the mix shaken for 15'. 2 mg of DNAse and RNAse were added and incubated for 30' at 37 °C. 30 g of NaCl was added and incubated for 15' on ice and the cellular debris was pelleted by centrifugation at 4000 rpm for 30', 4 °C (Beckmann Centrifuge). 50 g PEG (8000) was added to the supernatant and incubated 60' on ice. The intact phage were pelleted by centrifugation at 4000 rpm for 30',4 °C and resuspended in 12 ml SM buffer. Chloroform extraction followed by centrifugation at 3000 rpm for 10', 4 °C was performed once. The aqueous phase was centrifuged 25000 rpm for 30' (SW40 rotor, Beckmann ultracentrifuge) and the phage pellet resuspended in 1 ml SM buffer. Phage were purified on a CsCl density gradient and phage DNA extracted according to Maniatis (Molecular Cloning: A Laboratory Manual, Sambrook, Fritsch, Maniatis).

2.1.7 DNA extraction with phenol/chloroform

The standard way of removing proteins from DNA solutions is to extract once with phenol/chloroform/isoamylalcohol (25:24:1,v/v/v). The use of more than one organic solvent ensures a more efficient deproteinization. The organic solvents and aqueous phase (DNA containing solution) were mixed and then centrifuged for 1-2' in an eppendorf centrifuge (Heareus Biofuge) at 14000 rpm. The aqueous phase was re-

extracted with one volume of chloroform/isoamylalcohol (24:1, v/v) to remove traces of phenol, centrifuged and then ethanol precipitated.

2.1.8 Ethanol extraction of DNA

Ethanol precipitation is an efficient way to concentrate and desalt a DNA sample. The DNA sample (e.g. after phenol/chloroform extraction) was adjusted to 300 mM NaCl and 2.5 volumes of 96% ethanol was added. The DNA was precipitated for 2 h at -20 °C or for 15' at -70 °C. The sample was centrifuged for 30' (Heraeus Biofuge), the supernatant discarded and the pellet washed twice with 70% ethanol to remove as much salt as possible. Finally, the residual ethanol was evaporated by air drying.

2.1.9 DNA extraction with n-butanol

It is possible to concentrate DNA by extraction with water-free n-butanol as DNA and salt is insoluble in the organic phase but water is not. The DNA was extracted with two rounds of 3-10 volumes of n-butanol, reducing the volume of the DNA-containing aqueous phase considerably. After centrifugation the n-butanol was removed and the aqueous phase ethanol precipitated to remove the residual salt.

2.1.10 5'-end labeling with T4 polynucleotide kinase

T4 polynucleotide kinase catalyses the transfer of γ-phosphates from ATP to 5'-hydroxyl ends of DNA. The enzyme works equally well on single-stranded and double-stranded DNA. However, in the latter case the transfer is more efficient on protruding 5'-ends than on blunt ends and does not work on recessed 5'-ends. 1-40 pmol of oligonucleotides were incubated with 1x kinase buffer, 50 μCi (γ-^{32}P)ATP, 2 units of T4 polynucleotide kinase in a total volume of 20 μl. The reaction mixture was placed at 37 °C for 30' and stopped by addition of EDTA to 10mM. The volume was adjusted to 100 μl with TE buffer and the enzyme was removed by ethanol extraction. The 5'-end labeled oligonucleotides were separated from unincorporated nucleotides on a sephadex G50 spin-column and ethanol precipitated. DNA was recovered in TE buffer.

2.1.11 Dephosphorylation of DNA

To be able to label DNA fragments (except synthetic oligonucleotides) with T4 polynucleotide kinase or to prevent religation of compatible DNA ends it is necessary to remove a 5'-phosphate group. This can be done with Calf Intestinal alkaline phosphatase, CIP. Normally 0.01 units of CIP is added to dephosphorylate 1 pmole of 5'-ends. If the DNA is to be run on an agarose gel for purification, no further treatment is necessary prior to addition of glycerol loading buffer. Otherwise CIP must be inactivated and salts from CIP preparation removed.

1.0 unit of CIP was added to a total volume of 50 µl 1x CIP buffer containing 1.0-3.0 µg DNA (immediately after the restriction enzyme digestion) and incubated for 20' at 37 °C. The CIP was then inactivated by heating the reaction to 68 °C in the presence of 1% SDS, extracting with phenol/chloroform and passing the aqueous phase through a sephadex G50 spin-column.

2.1.12 Restriction enzyme digestion

1-3 units of restriction enzyme is normally used to digest 1 µg of DNA. Due to the inhibitory effect of glycerol, the restriction enzyme must always contribute less than 1/10 volume of the final reaction mixture. Restriction enzyme digestion was carried out in a total volume of 50 µl 1x TA buffer (100 mM Tris-HCl, pH 7.9, 200 mM K-acetate, 100 mM Mg-acetate), 1-3 units of the appropriate enzyme and 1ug DNA and incubated for 1-2 h at 37 °C. Restriction digestion was stopped by the addition of glycerol gel-loading buffer containing a final concentration of 10 mM EDTA.

2.1.13 Ligation of DNA fragments (vector and insert)

DNA ligation is catalyzed by T4 DNA ligase. A phosphodiester bond is made between juxtaposed 5'-phosphoryl and 3'-hydroxyl DNA ends. High backgrounds of religated vector can thus be reduced either by dephosphorylation with CIP or by cutting the vector with restriction enzymes that create noncompatible ends.

The ligation reaction was performed in 1x ligase buffer, 10 mM ATP and 3 units of T4 ligase, in a total volume of 15 µl and incubated for 6 h at 15 °C. As controls, reaction mixtures containing either vector or insert alone were included. The ratio of vector/insert was varied in order to achieve a high insertion efficiency.

2.1.14 Transformation of competent E.coli cells

Preparation of competent E.coli cells (JM109, Promega).

A single colony was inoculated into 25 ml LB/amp medium and incubated 37 °C o/n with vigorous shaking. 5ml of the overnight culture was then seeded into 500 ml LB medium and shaken at 37 °C until an OD A_{600} of 0.45-0.55 was reached. The cells were chilled on ice for 2 h. and collected by centrifugation at 2500x g for 15'-20',4 °C. The cells were resuspended in 10-20 ml ice cold trituration buffer and diluted to 500 ml with the same solution. The cells were centrifuged at 1800x g for 10' and gently resuspended in 50 ml ice cold trituration buffer. 80 % glycerol was added dropwise with gentle swirling to a final concentration of 15 % (v/v). 0.2-1 ml aliquots of the cells were made and frozen on dry ice prior to long term storage at -70 °C.

Transformation of competent cells.

The ligation mixture was added to 200 µl competent E.coli cells and placed on ice for 10' and then heat shocked at 37 °C for 5'. 500 µl LB medium (37 °C) was then added and the cells incubated 37 °C for a further 45'. The mixture was centrifuged 5-10'' (Heraeus biofuge) at 14000 rpm and 500 µl supernatant was removed. The cells were resuspended and spread on agar plates supplemented with 40 µg/ml ampicillin. Plates were incubated inverted at 37 °C for 12 h. Colonies of transformed cells were then subjected to "Mini Prep" analysis, to isolate positive clones.

2.1.15 Random primer labeling of hybridization probes

(Stratagene, Prime It RmT Random Primer)

25-50 ng of DNA (probe) was added to a reaction tube containing, unlabelled dNTPs, random prime buffer and random sequence nonamers. ddH$_2$O was added to a final volume of 42 μl. The mixture was boiled 5', centrifuged briefly to collect the condensation and then 5 μl of (α-^{32}P)dCTP (50 μCi) and 3 μl of magenta DNA polymerase (4U/μl) was added. The sample was mixed and incubated at 37 °C for 5'-30'. After incubation, 150 μl of ddH$_2$O was added to the reaction mixture. To remove unincorporated radioactive nucleotides, the 200 μl reaction mixture was passed through a sephadex G50 spin column, 1700 rpm for 2' (Sorvall centrifuge). The labeled DNA was denatured by heating to 95-100 °C for 5', then chilled on ice before hybridisation. Probes with a specific activity of 1.0x10^9 dpm/μg can be produced with most DNA substrates.

2.1.16 Isolation of high molecular weight DNA from mouse tails

1 cm of tail was cut and placed in a 1.5 ml eppendorf tube. The tail was minced using a small pair of scissors. 700 μl of mouse tail buffer and 35 μl of 10 mg/ml solution of Proteinase K was added and the tail was incubated at 55-60 °C overnight. After Proteinase K digestion, 20 μl of RNAse A was added and incubated at 37 °C for 1-2 h before organic extraction twice in phenol/chloroform (1:1) (200 μl) and once in chloroform (200 μl).The sample was centrifuged 2' at 13000 rpm (Heraeus Biofuge) and the aqueous phase transferred to a clean eppendorf tube. DNA was precipitated by the addition of an equal volume of isopropanol at room temperature (invert several times to ensure complete mixing) and centrifugation at 13000 rpm 10' (Heraeus Biofuge) The supernatant was discarded and the DNA pellet air dried. The pellet was resuspended in 150 μl ddH$_2$O at 4 °C o/n. A 1:100 dilution of the DNA was made and the OD A$_{260/280 \text{ nm}}$ determined. The yield is usually between 50-100 μg DNA.

2.1.17 Restriction enzyme digest of mouse genomic DNA

10 μg of DNA was digested in a total volume of 40 μl containing 1x BamHI buffer, 1 mM spermidine, 100 μg/ml BSA, 3 U/μg of BamHI restriction enzyme and ddH$_2$O. The

sample was incubated at 37 °C o/n, centrifuged briefly to collect the condensation before adding 4 μl gel-loading buffer and heating to 56 °C for 2' prior to loading onto a 0.7 % agarose gel.

2.1.18 Agarose gel electrophoresis of mouse genomic DNA

After restriction enzyme digest, the DNA was loaded onto a 0.7 % agarose gel (1x TAE) and electrophoresed at 80 V (1.85 V/cm) for 4-5 hours. This gives good separation of DNA fragments of 5 -23 kb. The DNA was visualised by staining with ethidium bromide and then photographed under UV-illumination. The gel was then submerged in denaturing buffer for 30', followed by a quick rinse in H_2O and then submerged in neutralizing buffer for a further 30' and rinsed in H_2O. This denatured the DNA in preparation for Southern blotting.

2.1.19 Southern blotting

A glass dish was filled with blotting buffer (20x SSC) and a glass plate placed across the dish, covered with two sheets of Whatmann 3MM filter paper, saturated with blotting buffer. The gel was placed on the 3MM filter paper and trapped bubbles were removed. The gel was surrounded with Saran wrap to prevent the blotting buffer being absorbed directly into the paper towels above. A sheet of Hybond-N+ filter (positively charged nylon membrane, Amersham) was cut to the exact size of the gel and placed on top of the gel. Again, trapped bubbles were removed. Two sheets of 3MM paper wetted with 5x SSC were placed on top of the filter, and a stack of dry paper towels was placed over the filter paper. A glass plate was placed on top of the paper towels with a 0.5-1.0 kg weight on top. Transfer of DNA was allowed to proceed for 2-16 h. The filter was then placed with the DNA side up on 3MM paper soaked in 0.4 M NaOH. Efficient DNA fixation was achieved after 15'-30'. Lastly the filter was rinsed gently in 5x SSC for not more than 1', prior to hybridisation.

2.1.20 Hybridization protocol

The Hybond N⁺ filter was placed in a hybridization oven (Techne: Hybridiser HB-1) in 40 ml of hybridization buffer (church) at 65 °C for 1 h. The 40 ml of hybridization buffer was then poured away and replaced with 20 ml of hybridization buffer and the denatured labeled probe and hybridized o/n at 65 °C. The filter was washed in 2x SSC, 0.1 % SDS for 15' at RT, followed by another wash in 0.1x SSC, 0.1 % SDS for 30' at 65 °C. The filter was removed from the hybridization cylinder, wrapped in Saran warp and place under film overnight. The autoradiograph was developed to visualise probe hybridisation. If an oligonucleotide probe was used, the hybridization temperature was 48 °C and the filter was washed 2 times in 2x SSC, 0.1 % SDS at 48 °C.

2.1.21 Filter stripping protocol

For successful removal of probes, filters must never be allowed to dry out during or after hybridization and washing. Filters were washed at 45 °C for 30' in 0.4 M NaOH, then transferred to a solution containing 0.1x SSC, 0.1 % SDS, 0.2 M Tris-HCl and incubated for a further 15'. Autoradiography for a normal exposure time was used to check that all probe had been removed. The filter was then available for further hybridization.

2.1.22 Polymerase chain reaction (PCR)

The polymerase chain reaction is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. 50-100 ng of genomic DNA or 1-2 ng of cloned DNA was added to a reaction tube containing, 200 µM dNTPs, 1x *Taq* buffer, 1 µM of each primer, 1 µl *Taq* 5U/µl (*Thermus aquaticus*, heat stable DNA polymerase) and ddH₂O, to a final volume of 50 µl. Mineral oil was layered on top to avoid evaporation during the heating and cooling cycles. The denaturation step was normally performed at 94 °C and the annealing temperature depended on the oligonucleotide primers used and was normally in the range

of 60-68 °C. Extension was carried out at 72 °C (PCR Machine: Hybaid Omnigene). 25 to 35 cycles were sufficient to produce 100 ng-1 µg of DNA.

2.1.23 Isolation of genomic DNA from cultured cells

The purification procedure is based on the selectivity of the QIAGEN resin which allows isolation of pure genomic DNA from cultured cells. Genomic DNA purified with QIAGEN resin columns has an average length of 80-100 kb, and is free of contaminants. 2 ml of ice-cold buffer C1 was added to every 2 ml of cell suspension (1.0 x 10^7 cells/ml) and mixed by inverting several times. The cells were incubated for 10' on ice and then centrifuged at 4 °C for 15' at 1300xg and the supernatant discarded. 1 ml of ice-cold buffer C1 and 3 ml of ice-cold ddH$_2$O were added to the pelleted nuclei which were then resuspended by vortexing. The nuclei were centrifuged again at 4 °C for 15' 1300g and the supernatant discarded. 5 ml of buffer G2 was added and the nuclei resuspended by vortexing for 10''-30''. 95 µl of QIAGEN protease (20 mg/ml) was added and the nuclei incubated at 50 °C for 30'-60'. The sample was applied to a QIAGEN resin column, entering the resin by gravity flow. The column was washed twice with 7.5 ml of buffer QC and the genomic DNA was eluted with 5 ml of buffer QF. The DNA was precipitated by the addition of 3.5 ml of isopropanol ensuring thorough mixing and then centrifuged at 5000g 4 °C for at least 15'. The supernatant was discarded and the DNA pellet washed with 2 ml of cold 70 % ethanol, air dried and resuspended in 150 µl TE pH 8.0 o/n at 4 °C. DNA concentration was measured at A$_{260nm}$. For pure DNA the A$_{260nm}$/A$_{280nm}$ ratio should be 1.7-1.9. (Spectrophotometer Cecil, CE 2040).

2.1.24 Linearizing DNA construct for electroporation

Restriction enzyme digest of the targeting DNA construct was carried out at 37 °C for 2 h. A small aliquot was run on an agarose gel, to check for complete restriction digestion. The DNA construct was precipitated by adding 1/10 volume of sodium acetate plus 2 1/2 volume of 100 % ethanol at -20 °C for 10'. The DNA was centrifuged at 13000 rpm (Heraeus Biofuge) for 10'.The DNA was kept sterile so supernatant was discarded and

the pellet dried in a sterile tissue culture hood. The DNA was resuspended in 100 µl sterile PBS in preparation for electroporation.

2.1.25 Screening for homologous recombination using PCR

The strategy is to amplify a novel DNA fragment created by the correct homologous recombination of endogenous genomic DNA with a targeting vector containing the G418 resistance gene (NEO). One primer binds to the *neo* cassette and the second primer just past the short arm of the targeting vector, within the endogenous locus. After eight days of G418 selection, ES colonies were visualized by holding the plate up to the light and individual colonies marked on the bottom of the plate. Half of each colony was transferred with a 10 µl tip. 20 colonies were pooled. The master plate was replaced in the incubator and the pooled ES cells centrifuged at 13000 rpm (Heraeus biofuge) for 15'' and the supernatant removed. Cells were resuspended in 30µl ddH$_2$O and boiled for 10' followed by centrifugation at 13000 rpm 10''. 1 µl of Proteinase K (10 mg/ml) was added and the cells incubated at 50 °C for 2 h. The cells were boiled for 10' and centrifuged 13000 rpm 10', ready for PCR amplification. The PCR programme used was: Denaturing temp.; 94 °C for 1'. Annealing temp.; 62 °C for 1'. Elongation temp.; 72 °C for 2' for 40 cycles. The reaction mix contained 200 µM specific primers, 1x Taq reaction buffer, 1 µM of each dNTP, 1 µl *Taq* 5U/µl. 10 µl of the PCR reaction was analysed on an agarose gel. The DNA was blotted onto Hybond-N+ membrane and hybridized with a probe spanning the expected PCR fragment. Membrane was washed and placed under film for o/n exposure. If positive pools were identified, the PCR and Southern blot was then repeated for each individual colony in the positive pool. Positive ES colonies were transferred to plates containing fibroblasts, expanded and then frozen down. The homologous recombination event was confirmed by Southern blot analysis.

2.1.26 Southern blot analysis using DNA prepared directly in a 96 well plate

The ES cells on the gelatin-coated plates were grown until the medium was very yellow, and the medium was changed every day for 4-5 days. The wells were rinsed twice with PBS and 50 µl of lysis buffer was added per well. The plates were incubated o/n at 60 °C in a humid atmosphere. 100 µl of NaCl/ethanol solution (75 mM NaCl in ethanol) was added and the plate was incubated for 30' at RT without mixing. The nucleic acids were precipitated as a filamentous network. The plate was inverted carefully to discard the solution, leaving the nucleic acids attached to the plate. Excess liquid was blotted on paper towels and the DNA rinsed 3 times with 150 µl of 70 % ethanol per well. The ethanol was discarded by inversion of the plate each time. After the final wash, the plate was inverted and allowed to dry on the bench. The DNA was cut with restriction enzymes. Each restriction digestion mix contained 1x BamHI buffer, 1 mM spermidine, 100 µg/ml BSA, RNAse (100 µg/ml), 3 U/µg of BamHI restriction enzyme, and ddH$_2$O. 30 µl of the restriction digest mix was added to each well, mixed and then incubated at 37 °C o/n in a humid atmosphere. Gel electrophoresis loading buffer was added to each well and electrophoresed as described in section "2.1.18 Agarose gel electrophoresis of mouse genomic DNA".

2.2 Tissue culture

2.2.1 Preparation of primary embryonic fibroblasts

Twenty 15-17 day old fetuses were dissected in PBS. Heads, liver and attached internal organs were removed and as much blood as possible was removed by washing twice in PBS. Carcasses were minced with scissors in a small volume of PBS. 50 ml Trypsin-EDTA was added and the mixture transferred to 500 ml conical flasks containing sterile glass beads (Borosilicate solid-glass beads, Aldrich cat.no. Z14392-8). The flasks were incubated at 37 °C, with stirring, for 30'. This step was repeated twice with the addition of more Trypsin-EDTA. The solution was decanted from the beads and centrifuged 1200 rpm 5' (Sorvall) and washed twice in PBS. The cells were resuspended in complete

medium and viable cells counted. Cells were plated at 5×10^6 cells/150 mm plate and medium changed after 24 h. When confluent (2-3 days), each plate was plated onto 5 further plates. When these plates were confluent they were frozen in liquid nitrogen (1 plate/1 vial, freezing medium: complete medium, 30 % FCS, 10 % DMSO).

2.2.2 Growth of primary embryonic fibroblasts

1 Nunc tube of frozen fibroblasts was thawed rapidly in a waterbath (37 °C) and transferred to 10 ml of medium. Cells were centrifuged at 1200 rpm for 5' (Sorvall RT 6000 D). Supernatant was removed and the pellet resuspended in 10 ml of medium. The cells were transferred to a 150 mm tissue-culture and a further 15 ml of medium added. The fibroblasts were grown at 37 °C, 5 % CO_2 for 48 h. or until confluent. Spent medium was removed and replaced with 10 ml of medium containing 0.01 mg/ml Mitomycin C (to inhibit further differentiation of the fibroblasts) and incubated for a further 2-3 h at 37 °C, 5 % CO_2. Medium was removed and the cells washed twice in 15 ml PBS. Cells were treated with Trypsin-EDTA for 2' at 37 °C, 5 % CO_2 to make a single cell suspension. Cells were aliquoted into 8-10 60 mm tissue-culture dishes and incubated 37 °C, 5 % CO_2 for 3 h. These fibroblasts were then used as feeders for ES cells.

2.2.3 Culture of ES cells on fibroblasts

Newly thawed ES cells usually grow slowly. The growth depends on the cell density. As a general rule, the more dense the cells, the faster the growth. It is important to keep the time in culture to a minimum and to dissociate clumps of cells at each passage. Check the cells frequently, ideally twice a day and change the medium every day. The important thing is that the ES cells remain totipotent and can thus contribute both to somatic tissues and most importantly, to the germline of mice. Totipotent cell colonies are oval and look shiny under phase contrast microscope. Individual cells can not be seen within undifferentiated cell colonies. In contrast, differentiated ES cell colonies are flat, granular and appear grayish under the microscope.

One vial of ES cells was thawed as described below. ES cells were transferred to a 30 mm tissue-culture dish containing fibroblasts and grown at 37 °C, 5 % CO_2. When the ES cells were confluent they were treated with Trypsin-EDTA for 2' at 37 °C, 5 % CO_2 to make a single cell suspension and transferred to a 60 mm tissue culture dish containing fibroblasts. Further expansion of ES cells was carried out using 60 mm tissue culture dishes containing fibroblasts. Each 60 mm dish was split into 2 or 3 60 mm dishes.

2.2.4 Freezing ES cells

ES cells (on feeders) were washed twice in PBS and treated with Trypsin-EDTA for 2' at 37 °C, 5 % CO_2. 10 ml of medium was added to make a single cell suspension which was centrifuged 1200 rpm (Sorvall) for 5'. The supernatant was discarded and the cells resuspended in 2 ml freezing medium (4 °C) at a density of approximately 2-3x 10^6 cells/ml (10 % DMSO, 30 % FCS, 70% complete medium). 1 ml of ES cells were aliquoted into each Nunc freezing vial. These were wrapped in bubble-plastic and frozen slowly o/n at -80 °C before transfer to liquid nitrogen for long term storage.

2.2.5 Thawing ES cells

1 Nunc tube of frozen ES cells were thawed quickly in a waterbath (37 °C) and transferred into 10 ml of medium. Cells were centrifuged at 1200 rpm for 5' (Sorvall RT 6000 D). The supernatant was removed and the cells resuspended in 1 ml of medium. ES cells were transferred to a 30 mm tissue-culture dish containing fibroblasts and grown at 37 °C, 5 % CO_2.

2.2.6 Electroporation of ES cells and G418 selection

DNA can be transfected into ES cells by application of a high voltage electrical pulse to a suspension of cells and DNA. The electrical pulse punches holes through the cell membrane through which the DNA passes. This procedure results in the death of about 50 % of the ES cells.

ES cells were passaged a day or two before the electroporation. The medium on ES cells growing on fibroblasts was replaced approximately 3 h before the electroporation (5x 10^6 cells/60 mm tissue culture dish), these were washed twice with PBS treated with Trypsin-EDTA for 2' at 37 °C, 5 % CO_2. and 5 ml of complete medium was added to make a single cell suspension. Cells were centrifuged at 1200 rpm (Sorvall RT 6000 D) for 5'. The supernatant was removed and the cell pellet resuspended in 5 ml of complete medium and then counted on a haemocytometer. The volume of the resuspended ES cells was adjusted to give 5x10^6 cells/0.7 ml suspension. 0.7 ml of suspension was removed as a control for G418 resistance. The appropriate amount of linearized DNA construct (32 μg in 100 μl PBS/5x 10^6 cells) was added to the cell suspension and mixed well. The 0.8 ml cell/DNA mix was transferred to an electroporation cuvette (BioRad, Cat. no. 165-2088). The settings used were: Capacitance 250 μF, Voltage 340, Time constant 3ms (BioRad, gene pulser). Electroporated cells were allowed to recover at RT for 15' and then 0.4 ml of electroporated cells was plated onto a 100 mm gelatinized tissue culture dish containing complete medium plus LIF (Leukaemia Inhibitory Factor). Cells were incubated at 37 °C, 5 % CO_2 for 48 hr at which point G418 selection was commenced. Complete medium containing LIF and 300 μg/ml G418 was added and the medium changed every day for 7-8 days. G418 resistant colonies began to appear at this time and at day 9 or 10 the colonies were ready for screening by PCR or by Southern blot analysis.

2.2.7 Picking and expanding ES cell colonies

After electroporation, the ES cell colonies were allowed to grow for 8-12 days to become visible. Single colonies were seeded into each well to avoid a further cloning step. Plates containing the colonies were washed with PBS and then PBS was added to cover the plate. Colonies were visualized by holding the plate up to the light and marked on the bottom of the plate. A 96 well U-bottomed plate was prepared by adding 25 μl of Trypsin-EDTA solution to each well. The original 10cm plate was placed on a microscope in a Laminar Flow Hood and individual colonies were picked with a micro-

pipetter and disposable sterile tips (1-10 µl) in a maximum volume of 10 µl. Each colony was transferred to the trypsin solution in the 96 well plate prepared earlier. After 96 colonies were picked, the 96 well plate was placed at 37 °C, 5 % CO_2 for 10'. During the incubation , a previously prepared 96 well feeder plate (flat bottomed wells), containing 200µl of medium and fibroblasts was aspirated to remove the medium and 150 µl of fresh complete medium was added per well. The trypsinized colonies were retrieved from the incubator and add 25 µl of complete medium was added per well. Clumps of cells were broken up by moving the cell suspension up and down with the multi-channel pipetter 5-10 times. The entire contents of each well were transferred to a well in the pre-prepared feeder plate. The plate was transferred to the incubator and the ES cells grown to confluence (approximately 2-3 days). When the ES cells approached confluence, they were washed twice with PBS and trypsinized using 50 µl of trypsin solution per well for 10'. 50 µl of complete medium was added to each well and clumps were broken up by vigorous pipetting. 50 µl of the cell suspension was replated onto a gelatinized 96 well (flat bottomed) plate without feeder cells. The remaining cells in the original 96 well plate were frozen by adding 50 µl of 2x freezing medium as described in "Freezing and thawing ES cells". The gelatinized plate was grown to confluence for DNA preparation and analysis by Southern blot analysis. Once the targeted clones were identified, the appropriate wells (colonies) were retrieved from the freezer and expanded for blastocyst injection.

2.2.8 Freezing and thawing ES cells in 96 well plates

The medium was changed 4 hours before freezing. The medium was removed by aspiration and cells were rinsed twice with PBS. 50 µl trypsin was added to each well and the plate incubated for 10' at 37 °C, 5 % CO_2. 50 µl of 2x freezing medium was then added per well and the colonies were resuspended evenly 100 µl of sterile mineral oil was placed over each well to prevent degassing and evaporation during storage at -70 °C. The 96 well plate was sealed with parafilm and stored at -80 °C. The optimum storage time was less than 2-3 weeks before retrieval of targeted clones.

To thaw, the 96 well plate was removed from the freezer and placed at 37 °C for 10'-15'. Targeted clones were transferred to a 1 cm well (24 well plate) with feeder cells containing 2 ml of complete medium. The medium was changed next day to remove the DMSO and the oil. Cells were grown to confluence and cultured as described in section "2.2.3 Culture of ES cells on fibroblasts".

2.2.9 Karyotyping

ES cells that were split the previous day onto gelatinized 60 mm tissue culture dishes were used for karyotyping. 0.1 ml of colcemid (Gibco, Karyomax colcemid, 10 µg/ml) was added to the cells and incubated for 2 h at 37 °C, 5 % CO_2 to arrest the cells in late prophase, making it easier to count individual chromosomes. Cells were treated with Trypsin-EDTA for 2' at 37 °C and a single cell suspension made. Cells were centrifuged at 1200 rpm (Sorvall) for 5', the supernatant removed and the cells resuspended in PBS. Cells were centrifuged 1200 rpm for 5', the supernatant discarded and the pellet resuspended gently in 5 ml 0.075 m KCl at RT for 10'. Cells were centrifuged at 1200 rpm for 5' and the supernatant removed and the pellet resuspended by gentle flicking. 1 ml fixative (3 parts of methanol to 1 part of acetic acid) was added and the cells incubated on ice for 10'. The cells were centrifuged 1200 rpm for 5' and the supernatant removed and resuspended in 200 µl fixative. Cells were added drop-wise onto an ethanol-washed slide from a height of 1-3 feet using a pasteur pipette. Slides were stained in 1 % lacto-acetic orcein for 15', rinsed in 45 % acetic acid/water and allowed to dry. The number of chromosomes in each individual cell were counted using light microscopy (magnification x 25).

2.3 Introduction of mutations into the mouse germline

2.3.1 Production of mouse blastocysts

C57BL/6 (B6) mice were used to obtain the recipient blastocysts. Mating pairs were set up 4 days before the injection, and vaginal plugs checked the next morning. 2 females

were mated per male (60 females). Plugged females were identified and separated from the males and kept in a separate cage. These females were sacrificed 3 days later to obtain the 3.5 day blastocysts.

2.3.2 Preparation of foster mothers for blastocyst injection

F_1 hybrid females (B6 x CBA) were used as foster mothers for the chimeras as they have a very high pseudopregnancy rate and provided good care for their litters. Foster mothers were used at 2.5 days of pseudopregnancy and therefore Therefore were set up for mating with sterile males on the same day that the plugs were checked in the B6 females (see previous section). 2 F_1 hybrid females were mated per vasectomised male (30 females). The next morning plugged females were collected into a separate cage.

2.3.3 Obtaining the blastocysts

The B6 females were sacrificed by cervical dislocation. The abdomen was opened and the genital system, including the uterus, oviducts and ovaries were pulled out. The fat pad and mesenteric blood vessels were cut away from the uterus. The 2 horns of the uterus were separated by cutting between the ovary and the oviduct at the proximal end and at the bifurcation of the uterine horns at the distal end. The blastocysts were flushed out of the uterus into a 6 cm tissue culture plate using a 2 ml syringe and a 25 gauge needle inserted into the oviduct-end of the uterus. Secum medium was used for culturing and flushing the blastocysts When all the uteri were flushed, the blastocysts from the plates were collected with a finely drawn Pasteur pipette and put together in a drop of Secum medium covered with mineral oil. These were placed in the incubator (37 °C, 5 % CO_2) until fully expanded for injection.

2.3.4 Injection needles and holding pipettes

The injection needle will carry the ES cells into the blastocoele. To work properly, it should have a diameter of 20 μm at the sharp end. A good needle is the key to a successful injection day, if the needle is not working properly (the flow of the cells

cannot be controlled properly or the point is not sharp enough to penetrate the blastocysts) it is best to prepare a new one. The holding pipette will keep the blastocyst steady while the needle penetrates it and injects the ES cells. A holding pipette should be prepared with an external diameter of 100 μm, and an internal diameter of 20 μm. The very end of the pipette should be polished to permit good holding and to avoid damaging the blastocysts. For a detailed description of injection needle and holding pipette preparation please see "Guide to Techniques in Mouse Development" Edited by Paul M. Wasserman, and Melvin L. DePamphilis. 1993.

2.3.5 Blastocyst injection

The ES cells were fed with complete medium 3 hours before the injection and then washed and trypsinized for 2' at 37 °C. The trypsin was washed off the cells which were then centrifuged and the supernatant discarded. Complete medium was added to the cell pellet and a single cell suspension made by vigorous pipetting. One drop of ES cells was transferred to a 3 cm tissue culture dish (the remainder were stored at 4 °C) just above where a drop of Secum medium containing 5-10 blastocysts had been placed. The 3 cm tissue culture plate was covered with mineral oil and placed under the microscope (Leitz, Labovert, microinstruments Ltd.) prior to injection. 16 ES cells were collected with the injection needle and moved to the drop containing the blastocysts. A blastocyst was picked up with a gentle vacuum on the holding pipette and lowered until it touched the bottom of the plate. The microscope was focused on an intercellular junction at the equatorial plane of the blastocyst, and the height of the needle was adjusted to bring it into focus in the same plane. The injection needle was inserted through the intercellular space and the ES cells were injected slowly. When finished, the needle was withdrawn and the injected blastocyst separated from the uninjected group. This procedure was completed with each blastocyst in turn. For a detailed description of Blastocyst injection please see "Guide to Techniques in Mouse Development" Edited by Paul M. Wasserman, and Melvin L. DePamphilis. 1993.

2.3.6 Blastocysts transfer into foster mothers

When the injections were completed, the blastocyst were left in the incubator for 1-2 h to allow reexpansion to occur. A foster B6xCBA female was anaesthetized by intraperitoneal injection with Hypnorm/Hypnovel. The back of the mouse was rinsed with ethanol and a small incision was made in the back, 1/3 of the length of the mouse from the base of the tail. The skin at the incision was moved ventrally with a needle until the ovary was seen through the body wall as a pink structure surrounded by fat. A small incision in the body wall was performed and the ovary and oviduct pulled out, handling the reproductive tract by the attached fat. The injected blastocysts were transferred to the uterus by making a hole with a 25G needle, close to the oviduct end. Using a finely drawn Pasteur pipette, 8 injected blastocysts were transfered into the uterus. If possible only one uterine horn was used in each female. The uterus was returned to the abdominal cavity and the skin closed with a metal clip. The female was placed on a warm surface to allow her to recover and then transferred to a cage. The gestation time is approximately 17 days. For a more detailed description please see "Guide to Techniques in Mouse Development" Edited by Paul M. Wasserman, and Melvin L. DePamphilis. 1993.

2.4 Functional assays

2.4.1 Antibody staining of mouse peripheral blood lymphocytes

Blood was harvested into a heparinised capillary tube and transferred to a 1.5 ml eppendorf tube containing 0.2 ml 0.9 % saline and heparin (20 IU/ml, 20000 IU/L). The blood was transferred to a polystyrene tube (round bottom tube, 12x75 mm, Falcon 2052) and 1.25 μg of CD40 mAb was added, mixed and incubated 5' on ice. 0.5 μg of B220 mAb was then added, mixed and incubated for a further 30' on ice. 4 ml of Geys lysis buffer was then added for 5' at RT to lyse red blood cells. The tube was inverted 3-4 times and centrifuged at 1200 rpm (Sorvall RT 6000 D) at 4 °C for 7'. The supernatant was discarded and the blood quickly resuspended on a whirlimixer. The cells were washed once in FACs buffer (PBS, 1 % BSA, 0.1 % azide), centrifuged 1200 rpm at 4

°C for 7' and the supernatant discarded before final resuspension in 0.5 ml FACs-buffer. Samples were stored in the dark at 4 °C prior to flow cytometric analysis within 18 h of the staining procedure (FACScan, Becton Dickinson, CellQuest).

2.4.2 Flow cytometric analysis of lymphatic organs and peripheral blood

Single cell suspensions were prepared as described (see section "*In vitro* B cell proliferation"). 4 ml of Geys lysis buffer was then added for 5' at RT to lyse red blood cells. The tube was inverted 3-4 times and centrifuged at 1200 rpm (Sorvall RT 6000 D) at 4 °C for 7'. The supernatant was discarded and the cells quickly resuspended on a whirlimixer. The cells were washed once in FACs buffer (PBS, 1 % BSA, 0.1 % azide), centrifuged 1200 rpm at 4 °C for 7' and the supernatant discarded before final resuspension in 0.5 ml FACs-buffer. The cells were then counted and 1.0×10^5 cells were aliquoted into to a polystyrene tube (round bottom tube, 12x75 mm, Falcon 2052). The cells were labeled with antibodies to the following cell surface markers: CD4, CD8, CD40, B220, IgM, and lastly αβ–TCR. The same labeling method was used as described for red blood cells (see section "Antibody staining of mouse peripheral blood lymphocytes") the only difference being that 1μg of each antibody was used for staining splenocytes and cells from the lymph nodes and thymus. Samples were stored in the dark at 4 °C prior to flow cytometric analysis within 18 hours of the staining procedure (FACScan, Becton Dickinson, CellQuest).

2.4.3 In vitro B cell proliferation

B lymphocytes were purified from the spleens of wt and CD40 deficient mice. A single cell suspension was made and washed in buffer (PBS, 2 mM EDTA, 0.5 % BSA) and B lymphocytes were purified using MACS (Magnetic Cell Sorting of Mouse Leukocytes) CD45R microbeads and positive selection (RS+) columns. A single cell suspension was made from spleens by breaking up the spleens and passing the cells through a 70 μm nylon mesh using a 2 ml syringe plunger in 5 ml buffer. The cells were centrifuged at 1200 rpm 5' (Sorvall RT 6000 D) and the supernatant discarded. The pellet was

resuspended to give 10^7 total cells per 90 µl of buffer. 10 µl of MACS CD45R (B220) microbeads were added per 10^7 total cells, mixed well and incubated for 15' at 6-12 °C. Cells were washed by adding 1 ml of buffer, centrifuging at 1200 rpm for 5', removing the supernatant completely and resuspending the resulting cell pellet in 500 µl of buffer per 10^8 total cells. The magnetically labeled cell suspension was pipetted onto an RS+ column, inserted into the magnetic field of a VarioMACS magnet. The cell suspension was allowed to run through the column which was then washed with 500 µl of buffer. The effluent was collected as the negative fraction. The column was washed twice more with 500 µl buffer and this effluent was pooled with the previous negative fraction. The column was removed from the VarioMACS magnet and placed over a new collection tube. 1 ml of buffer was applied to the reservoir of the RS+ column and the cells flushed out using a 2 ml syringe plunger. The resultant cell preparations were more than 95 % pure B cells as determined by flow cytometric analysis (FACScan, CellQuest) for surface CD45R, CD40, CD4 and CD8.

B cells were cultured in RPMI 1640 containing 10 % FCS, L-Glutamine, penicillin/streptomycin, β-mercaptoethanol. For proliferation assays, B cells (3.5 x 10^5 cells/well; 200 µl) were cultured for 72 hours in 96 well round-bottom tissue culture plates in the presence of 2 µg/ml LPS, 50 U/ml IL-4, 25 µg/ml CD40 mAB. Cultures were pulsed with 1 µCi/well of (^{3}H)thymidine (Amersham) for the last 18-24 hours of a 72 hour culture period and then harvested onto glass fiber filters. (^{3}H)thymidine uptake was measured by a liquid scintillation counter (1205 Betaplate, Wallac).

2.4.4 Immunohistochemistry

Mice were immunized with 100 µg KLH in complete Freund's adjuvant. Ten days later the spleens were removed and placed in embedding compound and snap-frozen in liquid nitrogen and then stored at -80 °C. Up to 48 tissue sections of 5-10 µm were cut using a cryostat (Kryostat 1720, LEITZ) and left until the next day at RT. The sections were fixed in acetone (AnalaR acetone, BDH) for 10' and air dried for 30'. Slides were

wrapped in foil and stored at -20 °C. Slides were thawed for 10' and a two-step indirect staining method was used. Primary antibody was diluted to the correct concentration and applied to the tissue section for 30'-45' at RT. The section was washed in TBS and an enzyme-conjugated secondary antibody was then added and incubated for 30'-45' at RT. The sections were washed and substrate (Diaminobenzidine or Fast red) was added and incubated until the desired color intensity had developed. Counterstaining was carried out using haematoxylin and the section was then placed under a coverslip.

Primary antibodies: 1.) Biotinylated Peanut agglutinin, 2.) FITC conjugated rat anti-mouse CD40, 3.) Rabbit anti-CD3, 4.) Rabbit anti-CD79a.

Secondary antibodies: 1.) Avidin horseradish peroxidase conjugate, 2.) Rabbit anti-FITC horseradish peroxidase conjugate, 3.) Goat anti rabbit horseradish peroxidase conjugate. 4.) Avidin alkaline phoshatase conjugate.

Substrate: DAB (Diaminobenzidene) and Fast red. (Sigma).

2.4.5 Preparation of serum from blood

The mouse was sacrificed and bled out using a 1 ml syringe. The blood was clotted in a 15 ml Falcon tube for 4 h at RT and placed o/n at 4 °C to allow clot retraction. The clot was gently loosened from the sides of the tube with a wooden applicator stick without breaking up the clot which was then discarded and the serum was transferred to a 15 ml Falcon tube. Remaining blood cells and debris were pelleted by centrifugation for 10' at 2700g 4°C (Sorvall RT 6000 D). The serum was then ready for analysis of immunoglobulin levels.

2.4.6 ELISA

Detection of serum immunoglobulin isotypes was performed using a sandwich-ELISA. NUNC polysorp plates were coated with anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3 and IgE diluted in PBS/0.05% NaN_3 to a final concentration of 20 µg/ml and incubated

overnight at 4 °C. Plates were then blocked with PBS containing 1% BSA, 0.3% Tween, 0.05% NaN_3 and incubated for 2 hrs at 37 °C. Plates were then washed 3x in ELISA wash buffer (see buffers and solutions). Mouse immunoglobulin standards for each of the isotypes and serum from wild-type and CD40 deficient mice were diluted in PBS, 0.1% BSA, 0.3% Tween, 0.05% NaN_3 and added to the plates and incubated for 2 hrs at 37 °C. The starting concentration of the standards was 500 ng/ml and these were then diluted 2 fold with a total of 11 dilutions. The starting dilution of serum for the detection of the different immunoglobulin isotypes was: IgM: 1:500; IgG1: 1:1000; IgG2a: 1:1000; IgG2b: 1:1000; IgG3: 1:500; IgE: undiluted. These were then diluted 2 fold with a total of 11 dilutions. Plates were washed 5x with ELISA wash buffer and alkaline phosphatase-conjugated isotype specific antibodies (anti-mouse Ig-AP) were then added at a final concentration of 2 µg/ml (diluted in PBS) and incubated 1 hr at 37 °C. Plates were then washed 10x in ELISA wash buffer and PNPP (p-nitrophenyl phosphate, 1mg/ml) substrate was added and color intensity determined at 405 nm in an ELISA microplate reader (Dynex MRX TC2). Serum immunoglobulin isotype concentrations were calculated using the software program Revelation 3.04.

2.4.7 Mouse ELIspot (IFN-γ)

MAHA plates were coated with 50 µl of 10 µg/ml R4 monoclonal antibody in sterile PBS. Incubated overnight at 4 °C. Wells were washed 5 times with 200 µl of PBS, followed by blocking of nonspecific binding by complete RPMI-1640 medium containing 10 % FCS for 2 h at room temperature. 1.0×10^6 cells in 50 µl were added to the per well and the antigen were added on top, either 250 ng/ml or 1 µg/ml Tuberculin PPD (Tuberculin purified protein derivative BP) in a total of 200 µl of medium. Cells plus antigen were incubated undisturbed for 18-24 h at 37 °C. Next day, cells were flicked out and the wells washed 3 times with PBS, then once with water and then twice more with PBS. 50 µl of biotin conjugated rat anti-mouse IFN-γ antibody in PBS (1 µg/ml) were added to the wells and incubated 2 h at room temperature or overnight at 4 °C. Wells were washed 5 times in PBS and 50 µl of streptavidin poly alkaline

phosphatase (1 μg/ml) were added and the plate were incubated 2 h at room temperature. Wells were washed 5 times with PBS and 50 μl of developing reagent (BIORAD: AP-conjugate substrate kit) were added. Colour developed at room temperature for 1-2 h, wells were then washed 5 times with tap water and allowed to air dry, before counting spots.

2.4.8 Mycobacterial counts (colony-forming units)

Homogenates of spleen, liver and lung were prepared in 1 ml of PBS/Tween-80 (0.05%). 200 μl of homogenized tissue was plated at 2.5, 5- or 10-fold serial dilution (diluted in PBS) onto Middlebrook 7H10 agar plates. Colony-forming units (bacterial colonies) were counted 2-3 weeks later.

2.4.9 Histology (BCG infected mice)

Tissue sections were fixed in 10% formalin and embedded in paraffin blocks. Sections (5 μm) were stained with hematoxylin and eosin. Hematoxylin stains the nucleus blue, whereas eosin stains the cytoplasm red. Sections were also stained by the Ziehl-Neelsen method for acid fast bacilli using the following method. Take sections to water. Rinse in distilled water. Cover sections with filter paper dampened with distilled water. Apply staining solution to filter paper on sections. Rinse in distilled water. Decolourise sections in 1% acid alcohol. Rinse in distilled water. Counterstain in 1% methylene blue in 1% acetic acid. Dehydrate, clear and mount. Result: acid-fast bacilli stain red and nuclei stain blue.

2.4.10 Statistical analysis:

The results from the various analyses carried out in this thesis were compared using Student's t-test. This was used to evaluate whether the differences between the means of the parameters studied were significantly different. It was assumed that the results obtained from the two populations (wild-type and mutant mice) were normally distributed and that the standard deviations of the two populations were equal. A

significance level of 5% was chosen, and P values below this indicated that the differences in the two populations being compared were significant, (Essentials of Medical Statistics, Betty R. kirkwood).

2.5 Buffers and Solutions

2.5.1 Molecular biology

Solution I: 50 mM Glucose
                               25 mM Tris-Cl pH 8.0
                               10 mM EDTA

Solution II: 0.2 M NaOH
                                 1 % SDS

solution III: 3 M KOAc
                                 2 M HOAc

10x kinase buffer: 500 mM Tris-Cl pH 7.6
                               100 mM $MgCl_2$
                                50 mM DTT
                                 1 mM Spermidine
                                 1 mM EDTA pH 8.0

10x TA buffer: 1   M Tris-acetate
                                 2   M K-acetate
                                 1   M Mg-acetate

10x Ligation buffer: 500 mM Tris-Cl pH 7.4
                               100 mM $MgCl_2$
                               100 mM DTT
                                10 mM Spermidine
                                10 mM ATP
                                 1 mg/ml BSA

10x CIP buffer: 500 mM Tris-Cl pH 9.0
                                10 mM $MgCl_2$
                                 1 mM $ZnCl_2$

	10 mM	Spermidine

1xTE buffer:

10 mM	Tris-Cl pH 7.6
1 mM	EDTA pH 8.0

1xTBE buffer:

89 mM	Tris-Cl pH 7.8
89 mM	Borate
2 mM	EDTA pH 8.0

10x Loading buffer, agarose gel:

15	%	Ficol
0.25	%	Bromphenol blue
0.25	%	Xylen cyanol
100	mM	EDTA pH 8.0

Hybridisation buffer (church):

0.5	M	NaPo$_4$ pH 7.2
7	%	SDS
5 mM		EDTA
(1	%	BSA)

Mousetail buffer:

50 mM	Tris-HCl pH 8.0
100 mM	EDTA
100 mM	NaCl
1	% SDS

Lysis buffer (ES cells):

10 mM	Tris pH 7.5
10 mM	EDTA
10 mM	NaCl
0.5	% sarcosyl

Add Proteinase K to a final concentration of 1 mg/ml just prior to use.

Denaturing solution:

1.5	M	NaCl
0.5	M	NaOH

Neutralising solution:

1.5	M	NaCl
0.5	M	Tris-HCl pH 7.2
1 mM		EDTA

20x SSC: 3   M   NaCl

                           0.3   M Na_3citrate

Buffer C1:   Dissolve 109.54 g sucrose, 1.02 g $MgCl_2.6H_2O$, 1.211 g Tris base in 800 ml ddH_2O. Add 100 ml 10 % Triton X-100 solution. Adjust pH to 7.5 with HCl. Adjust the volume to 1 liter with ddH_2O.

Buffer G2:   Dissolve 76.42 g GuHCl, 11.17 g Na_2-EDTA-2H_2O, 3.633 g Tris base in 600 ml ddH_2O. Add 250 ml 20 % Tween-20, 50 ml 10 % Triton X-100. Adjust the pH to 8.0 with HCl. Adjust the volume to 1 liter with ddH_2O.

Buffer QC: Dissolve 58.44 g NaCl, 10.46 g MOPS in 800 ml ddH_2O. Adjust the pH to 7.0 with NaOH. Add 150 ml pure ethanol. Adjust the volume to 1 liter with ddH_2O.

Buffer QF: Dissolve 73.05 g NaCl, 6.055 g Tris base in 800 ml ddH_2O. After the addition of 150 ml pure ethanol adjust the pH to 8.5. Adjust the volume to 1 liter with ddH_2O.

50xTAE: 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0. Adjust the volume to 1 liter with ddH_2O.

SM Buffer   5.8 g NaCl

                        2.0 g $MgSo_4.7H_2O$

                        50 ml 1 M Tris.HCl (pH 7.5)

                        5   ml 2 % gelatin solution

                        H_2O to 1 liter

Plasmids: pGEM-7Zf(+) (Promega)

               pGEM-11Zf(+) (Promega)

               pMC1 Neo (contains the *neo* structural gene from Tn5)

2.5.2 Tissue culture

ES cell lines: D3: derived from mouse strain 129/Sv.

R1: derived from mouse strain (129/Sv x 129/SvJ) F_1.

Medium for ES cells (complete medium):

DMEM - 500 ml (Gibco-BRL; 1x; 41965-039).
Fetal Calf Serum - 80 ml (Sigma; F-4884; lot. 51H-0546).
100 % FCS batch tested, heat inactivated at 56 °C for 30'.
L-Glutamine - 5 ml (Gibco-BRL; 100x; 25033-010).
Pen/Strep - 5ml (Gibco-BRL; 5000IU; 15070-022).
Sodium Pyruvate - 5 ml (Gibco-BRL; 100mM; 11360-039).
β–mercaptoethanol - 0.5 ml (0.1 M) (Sigma; 14.3 M; M7522).

G418 selection medium:

Complete medium.
G418 added to complete medium at a final concentration of
300 µg/ml. Geneticin (G418 sulfate; Gibco-BRL; 11811-031).
LIF (Leukaemia Inhibitory Factor) 500 U/ml

Gelatin: 1 % (Sigma; Type A from porcine skin; G-2500)

Mitomycin C: (Sigma; M0503; 2mg).(10 µg/ml complete medium).

PBS: Phosphate buffered Saline. PBS tablets (OXOID PBS Dulbecco
                  A tablets; BR14a; Ca^+ and Mg^+ free.

Trypsin-EDTA: (Gibco-BRL; 1x; 45300-019)

2.5.3 Functional assays

Geys lysis buffer: Solution A: (1 liter)

                     35   g NH_4Cl
                     1.85 g KCl
                     0.56 g Na_2HPO_4
                     0.12 g KH_2PO_4
                     5.0   g Glucose

Sterilise by filteration: 0.22 μm filter.

Solution B:(500 ml)

$$
\begin{array}{ll}
2.1 & \text{g } MgCl_2\text{-}6H_2O \\
0.7 & \text{g } MgSO_4\text{-}7H_2O \\
2.25 & \text{g } CaCl_2\text{-}2H_2O
\end{array}
$$

Autoclave.

Solution C: (500 ml)

$$
11.25 \quad \text{g } NaHCO_3
$$

Autoclave.

Mix solutions:

A:	100	ml
B:	25	ml
C:	25	ml
ddH$_2$O:	350	ml
total	500	ml

FACS buffer: PBS, 1 % BSA, 0.1 % azide.

IL-4: Interleukin-4 (50 units/ml) (Pepro Tech inc.; cat.no. 214-14)

LPS: Lipopolysaccharide 1 mg (Sigma; cat.no. L 6529)

PPD: Tuberculin purified protein derivative BP.(EVANS medical)

BCG: Percutaneus BCG vaccine. BCG: *bacillus Calmette-Guerin* (Copenhagen sub-strain 1077). 1.0×10^7 viable units per I.V. injection. (EVANS medical).

MACS CD45R microbeads:

MACS colloidal super-paramagnetic microbeads conjugated to monoclonal rat anti-mouse CD45R (B220) antibodies. Isotype: rat IgG$_{2a}$. Clone: RA3-6B2. (Miltenyi Biotec; cat no. 495-01).

MACS separation columns:

RS+ positive selection separation columns. (Miltenyi Biotec; cat. no. 413-01).

RPMI 1640 medium:

        Medium for B cell proliferation.
        RPMI 1640- 500 ml (Sigma; R 8758).
        Fetal Calf Serum - 50 ml (Sigma; F-4884; lot. 51H-0546).
        100 % FCS batch tested, heat inactivated at 56 °C for 30'.
        L-Glutamine - 5 ml (Gibco-BRL; 100x; 25033-010).
        Pen/Strep - 5ml (Gibco-BRL; 5000IU; 15070-022).
        β–mercaptoethanol - 0.5 ml (0.1 M) (Sigma; 14.3 M; M7522).

ELISA wash buffer: 0.9% NaCl, 0.005M K_2HPO_4, 0.005M KH_2PO_4, 0.025% Tween. (For 10L: 90g NaCl, 100 ml 0.5M K_2HPO_4, 100 ml KH_2PO_4, 25 ml 1:10 diluted Tween 20. Adjust to pH 7.0).

ELISA substrate: PNPP (Sigma) diluted at 1 mg/ml in diethanolamine buffer.

Diethanolamine buffer:

        48.5 ml diethanolamine, 400 mg $MgCl_2$ x $6H_2O$, 100 mg NaN_3, add 450 ml H_2O. Stir for 1 hr in the DARK, pH 9.8 (with con. HCL) add ~ 500 ml to 1 litre. Store at 4 °C.

2.5.4 Introduction of mutations into the mouse germline

Mineral oil: Embryo tested (Sigma; cat.no. M8410)

Glass tubing: Holding pipette, injection needle (World Precision Instruments; 1.0/0.75, RTW 100-4)

Glass capillary tubes: Transfer pipettes (BDH; cat.no. 314/1100/12)

Silicone oil: Dow Corning 200/200cS (BDH; cat. no. 63008-4C)

Hypnorm (Fentanyl)/Hypnovel (midazolam), 0.2 ml/20 g mouse (veterinary surgeon)

Injection microscope: Leitz, Labovert FS, Microinstruments Ltd.

Pipette puller: Vertical pipette puller, model 720C, David Kopf Instruments, Tujunga,California, U.S.A.

Microforge: Alcatel-Annecy , TY 4160, Microinstruments Ltd.

Beveller: 1300 M Beveller, World Precision Instruments.

Secum Medium:

	g/1000 ml
EDTA	0.037
NaCl	5.546

KCl	0.356
KH_2PO_4	0.162
$MgSO_4.7H_2O$ 0.294	
$NaHCO_3$	2.106
Glucose	1.000
Na-lactate (D,L)	3.05 ml
Ca-lactate.$5H_2O$	0.527
Na-pyruvate	0.028
G-penicillin	0.075
Streptomycin.SO_4	0.050
BSA	3.000
Phenol Red	1 ml/1% solution

Weigh out all ingredients, except Phenol red, Na-lactate, BSA, in one weighing boat. Add 980 ml of ddH_2O in a sterile tissue culture flask. Add Phenol red and Na-lactate, stir until all chemicals are dissolved. Sprinkle BSA on top of medium. DO NOT STIR. Leave o/n at 4 $^{\circ}$C. Filter the medium slowly, in order to avoid bubbles. Store at 4 $^{\circ}$C for not more than one month.

Chapter 3: Generation of CD40 deficient mice

3.1 Generation of a targeting vector for CD40

When a fragment of genomic DNA is introduced into a mammalian cell it can locate and recombine with the endogenous homologous sequences and this is called gene targeting. Gene targeting has been widely used in mouse embryonic stem (ES) cells to make mutations at different loci (genes) so that the phenotypic consequences of a specific genetic modification can be assessed *in vivo* (Figures 3. and 4.). In this chapter the generation of a vector to specifically target CD40 and the subsequent production of CD40 targeted embryonic stem cell clones is described. The production of chimaeric mice, the establishment of germline transmission and lastly the interbreeding of mice heterozygous for the mutant allele to generate CD40 deficient mice is also described.

The first experimental evidence for the occurrence of gene targeting in mammalian cells was made in a fibroblast cell line where a selectable artificial locus introduced by Lin and coworkers, corrected a deficiency in the thymidine kinase gene of that cell line (Lin *et al.*, 1985). Another study demonstrated that homologous recombination could also occur between a plasmid containing sequences from the β-globin gene and the endogenous β-globin gene in erythroleukaemia cells (Smithies *et al.*, 1985). In general the frequencies of gene targeting events in mammalian cells are low. This is probably related to the fact that transfected DNA can also integrate into random chromosomal sites. The relative frequency of targeted to random integration events will determine the ease with which targeted clones may be identified in a gene targeting experiment. A targeting vector is designed to recombine with and mutate a specific chromosomal locus. The minimal requirements of a targeting vector are that it should have homologous sequences to the desired chromosomal integration site and positive and negative selection markers providing strong selection for cells with a targeted integration. The positive selection marker in a targeting vector may serve two functions. Its primary function is as a selection marker to isolate the rare transfected cells that have integrated DNA. Secondly

the positive selection marker can serve as a mutagen, for instance, if it is cloned into a coding exon of the gene or replaces coding exons. Many targeting vectors also use a negative selection marker which is positioned at the end of the homologous sequences. The negative selection marker renders cells, which have integrated the targeting vector into a random chromosomal site, sensitive to FIAU/gancyclovir. The *neo* gene, encoding a bacterial aminoglycoside phosphotransferase, renders a given cell resistant to the antibiotic G418 (an inhibitor of protein synthesis), so that it can be positively selected (Colbere *et al.*, 1981). The thymidine kinase gene (*tk*) is of viral origin (herpes simplex virus, HSV) and facilitates the incorporation of antiviral drugs such as gancyclovir and FIAU (1-(2'-deoxy-2'-fluoro-b-D-arabinofuranosyl)-5-iodouracil) into DNA during replication. These base analogs block the incorporation of additional bases and thereby induce a termination of replication that will lead to cell death. Thus, cells which have randomly integrated the targeting vector and the *tk* gene can be negatively selected (Borrelli *et al.*, 1988; St *et al.*, 1987).

Two distinct designs are commonly used for gene targeting in mammalian cells, replacement and insertion vectors. These vector-types are constructed differently so that following homologous recombination they yield different integration products. The basic elements of a replacement vector are, homologous sequences to the target locus, a positive selection marker, bacterial plasmid sequences and a linearization site outside of the homologous sequences of the vector (Figure 6 and 8). The final recombinant mutant allele can be described as a consequence of double reciprocal recombination between the vector and the chromosomal sequences. This is equivalent to a replacement of the chromosomal homology with all components of the vector which are flanked on both sides by homologous sequences. Any heterologous sequences at the ends of the vector homology are excised from the vector and are not recovered as stable genomic sequences following targeting. The recombinant allele generated in a gene targeting experiment using a replacement vector typically has a positive selection marker inserted into a coding exon or a coding exon is deleted and replaced by the positive selection marker.

Disruption of the coding sequence by the positive selection marker will in most instances ablate a gene's function. However, in some situations a truncated protein may be generated which retains some biological activity. Therefore, null alleles are more likely to occur if the positive selection marker is inserted into an upstream exon rather than a downstream exon. This ensures that only the very N-terminal portion of the polypeptide should be made. Another important consideration is exon skipping. An artificially large exon with an inserted positive selection marker may not be recognized by the RNA splicing mechanism and could be skipped (Robberson et al., 1990). Thus, transcripts initiated from the endogenous promoter may delete the mutated exon from the mRNA. If this involves a coding exon, containing the positive selection marker, without a unit number of codons, then the net result will be both a deletion and a frame-shift mutation of the gene, which should generate a null allele. However, if the disrupted coding exon has a unit number of codons which is spliced out, this would result in a protein with a small in-frame deletion which may retain partial or complete function.

The position of the positive selection marker with respect to the homologous sequences of the vector will determine the type of screen that must be used to detect the ES cell clones targeted with a gene replacement event. One common screening method for targeted ES cell clones is based on PCR amplification. This is accomplished by using one primer which hybridizes to the positive selection marker and a second primer which hybridizes to the target chromosomal sequences just beyond the homology used in the vector (Figure 7.). Thus, replacement vectors designed for screening by PCR amplification require that the positive selection marker is inserted at an asymmetric location near one end of the homologous sequences, while still leaving sufficient homology for the formation of a crossover. This gives a vector with one long arm and one short arm of homologous sequences. Another common screening method for ES cell clones targeted with a gene replacement vector uses Southern blot analysis (Figure 9.). For this it is important to design the vector and identify a unique probe(s) and restriction sites so that such analysis is unambiguous and can discriminate between the wild-type

allele and the predicted targeted allele. This analysis is ideally performed with a probe that is not contained in the homologous sequences of the targeting vector (external probe, probes A and B in Figure 7. and 9.) and a restriction enzyme digest that does not cut in the homologous sequences of the vector or where at least one restriction enzyme site is located outside the homologous sequences of the vector (Figure 7. and 9.).

The basic elements of an insertion vector are, a region of homologous sequence to the target locus which has a unique linearization site, a positive selection marker and a bacterial plasmid backbone. The major difference between the two vector types (replacement and insertion vectors) is that the linearization site of an insertion vector is made in the homologous sequences of the vector. An insertion vector undergoes single reciprocal recombination (vector insertion) with its homologous chromosomal target which is stimulated by a double stranded break (linearization site) in the vector. Since the entire insertion vector is integrated into the target site, including the homologous sequences of the vector, the recombinant allele generated by such a vector becomes a duplication of the target homology separated by the heterologous sequences in the vector backbone. The duplication of exon sequences is usually sufficient to generate a null allele. However, to ensure that exon duplication will create a mutation at the protein level it may be necessary to introduce stop codons into a single exon in the targeting vector. Screening for the recombinant allele generated by an insertion vector can be done by PCR amplification or Southern blot analysis. The best restriction enzyme digest for Southern blot analysis will be with an enzyme that does not cut within either the homology of the target locus or the vector backbone. As I have described previously, the use of an external probe in Southern blot analysis is necessary. An ES cell clone which has inserted one unit of the vector into the target locus will show an increase in the size of the restriction fragment which matches exactly the size of the targeting vector.

To obtain sequences from the murine CD40 gene, a murine genomic library containing genomic sequences from the mouse strain 129/Sv (Lambda Dash 9H-129/Sv) was plated and screened using a 930 bp cDNA probe (Plating and screening procedure is described

in section 2.1.5). The CD40 cDNA probe was produced by PCR amplification, as described in section 2.1.22, using primers hybridizing to exon 1 (Primer 1) and just downstream of exon 9 (Primer 9) on cDNA isolated from mouse splenocytes. The mammalian genome contains approximately 3×10^9 bp and one phage, on average, contains a genomic insert of approximately 12 kb (12×10^3 bp). To ensure that the entire genome was represented in this screening, a minimum 2.5×10^5 clones (phages) would need to be plated out and screened for the gene of interest. 7.5×10^5 clones were plated and screened for the presence of CD40 genomic sequences. This yielded 3 positive phage clones (Figure 5.). The 3 positive phage clones were isolated and amplified in the phage maxiprep method described in section 2.1.6. Restriction enzyme digests, using enzymes *Bam*HI, *Sal*I, *Sac*I, *Kpn*I, *Bgl*II, *Eco*RI, *Xho*I, *Xba*I, *Hind*III, and agarose gel electrophoresis on phage DNA (described in section 2.1.1 and 2.1.12) and subsequent Southern blot analysis (described in section 2.1.19-2.1.20) using oligo-nucleotide primers (primers 1-8) to probe for the different exons, revealed that phage clone 1 contained exon 1, whereas phage clone 2 contained exons 2-6 and phage clone 3 contained exons 2-9 (Figure 5.). Both clones 2 and 3 were ideal for the generation of a replacement targeting vector as they contained sufficient sequence to allow homologous recombination to occur and the presence of an *Eco*RI restriction site in exon 3 allowed for the insertion of the positive selection marker and stop-codons.

A. CD40 Gene
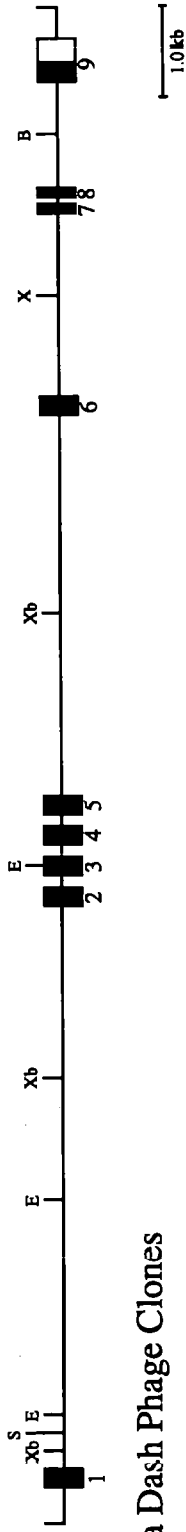
B. Lambda Dash Phage Clones
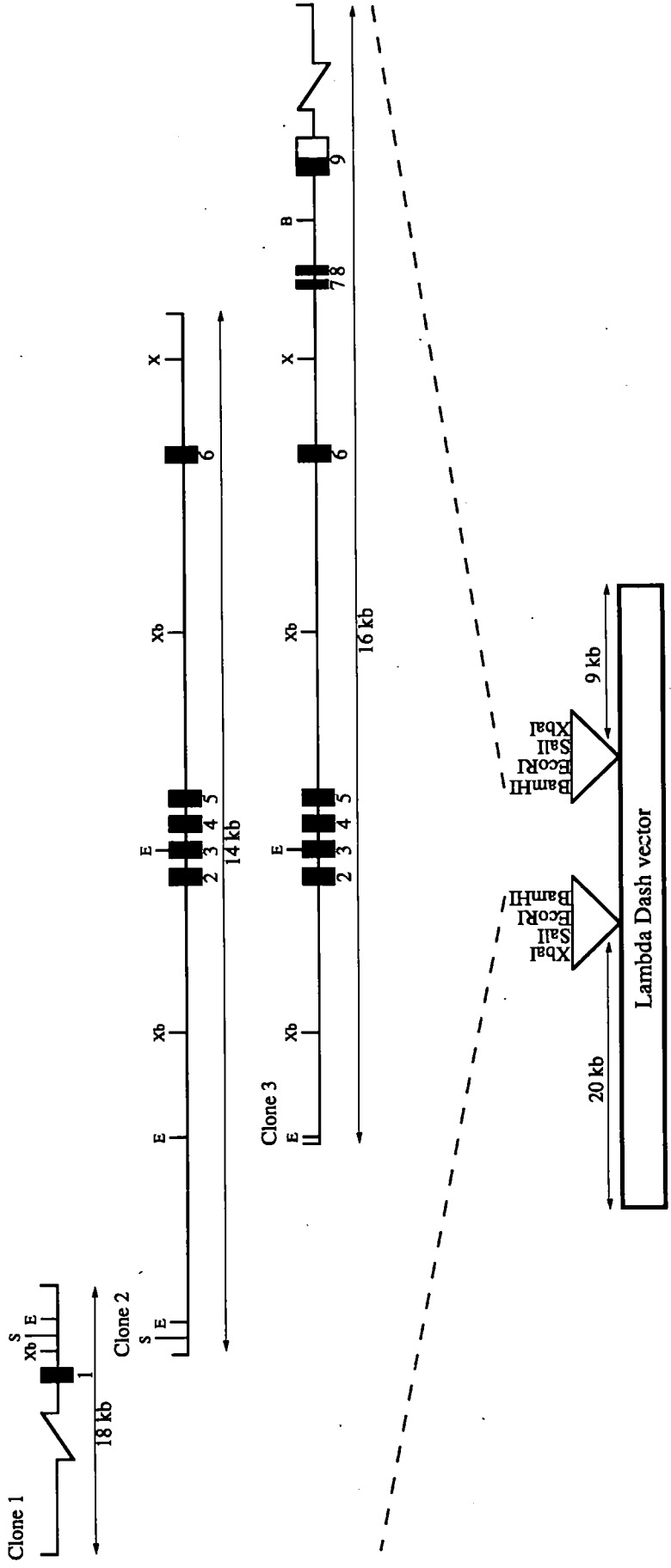
Figure 5. Phage clones obtained by screening Lambda Dash library. A.) Partial restriction map of the CD40 gene. B.) Phage clones were cloned into the *Bam*HI restriction sites of the Lambda Dash vector (shown by dashed lines). The size of each genomic fragment is given below each clone. Black boxes represent exons 1-9. Restriction enzyme sites: Xb. *Xba*I: S. *Sac*I: E. *Eco*RI: B. *Bam*HI: X. *Xho*I.

Two different targeting vectors were made, construct 1 (Figure 6.) and construct 2 (Figure 8.). Construct 1 was made by ligating the *Xba*I restriction fragment from the CD40 gene, containing exons 2-5, into a pGEM-11Zf(-) vector (Figure 6.B.1.) as described in section 2.1.12-2.1.13. The cloning step was followed by PCR amplification of a 3 kb region spanning exons 2-4. The primers used were the T7 polylinker primer (primer 12) and a primer hybridizing downstream of exon 4 (primer 4). The resulting PCR fragment was then cloned into a pGEM-7Zf(+) vector (Figure 6.B.2.). The last step in generating construct 1 was to clone the G418 resistance gene (NEO) into the *Eco*RI site of exon 3. The G418 resistance gene consisted of the *neo* structural gene (aminoglycoside phosphotransferase, derived from the bacterial transposon Tn5) and the promoter driving the expression of this G418 resistance gene (derived from the herpes simplex virus thymidine kinase gene (HSV-*tk*)). To increase the efficiency of the *tk* promoter a 65 bp fragment derived from the polyoma virus enhancer was inserted upstream of the *tk* promoter (Thomas & Capecchi, 1987). The G418 resistance gene confers resistance to Geneticin (G418) which is an inhibitor of protein synthesis.

The pGEM-7Zf(+) vector containing the cloned CD40 PCR fragment (Figure 6.B.2.) was cut with the enzyme *Eco*RI and an oligonucleotide linker with a *Sal* I restriction site was ligated into the *Eco*RI restriction site. The linker, consisting of primers 13 and 14, contains a *Sal*I restriction site and stop-codons in all three reading frames. Ligation of the linker into the *Eco*RI restriction site abolishes this restriction site due to nucleotide changes in the linker sequence. The vector containing the cloned PCR fragment (Figure 6.B.2.) and the linker fragment was subjected to restriction digestion with *Eco*RI, and *E. coli* was transformed with the restriction digest. This ensures that only vectors containing the cloned PCR fragment and the linker fragment (*Sal*I restriction site) will support bacterial growth, whereas vectors without the linker, still containing the EcoRI site, will be linearized and as a result, cannot support bacterial growth. The correct vector was then amplified in a miniprep (described in section 2.1.3) and then cut with *Sal*I restriction enzyme to remove concatamers of the linker and to prepare the vector for ligation of the

G418 resistance gene into the *Sal*I restriction site. The G418 resistance gene (NEO) was cut out of the plasmid pMC1Neo by a *Sal*I-*Xho*I restriction digest and ligated into the *Sal*I site of the vector containing exons 2-4 of the CD40 gene. The *Sal*I and *Xho*I restriction sites are compatible for ligation purposes. The final outcome of the cloning procedure is a targeting vector containing a 3 kb fragment of the CD40 gene with a G418 resistance gene (1.2 kb) inserted into the engineered *Sal*I site (previously *Eco*RI site) of exon 3 (Figure 6.B.3.). The linker served two purposes, it introduced a *Sal*I site enabling the cloning of the G418 resistance gene (*Sal*I–*Xho*I restriction digest) into exon 3, and the linker also contained stop-codons in all three reading frames, terminating transcription of the CD40 gene in the event of homologous recombination. The G418 resistance gene was inserted upstream of the putative binding site (residues E74, Y82, D84, N86, E117) for CD40L, thereby abolishing any ligand binding of a truncated CD40 protein (Bajorath *et al.*, 1995b).

A. CD40 Gene

```
      S
   Xb | E         E      Xb              E                    Xb                      X           B
   |  |  |         |      |              |                    |                       |           |
  [■]            [_]    [_]          [■][■][■][■]           [_]    ... [■] ...       [■][■]      [■][□]
   1                                  2 3 4 5                        6              7 8          9
```

B. Cloning of Targeting construct no.1 |———|
                                                                          1.0 kb

1.)

```
  Xb                    E                      Xb
  |                     |                      |
  [        [■][■][■][■]          ]
           2  3  4  5
```

T7↓ SfiI SacI EcoRI SalI XhoI BamHI ApaI XbaI NotI NsiI HindIII SP6↑

pGEM-11Zf(-)
vector
3.2 kb

PCR product
cloned into
pGEM-7Zf(+)

2.)

```
  B Xb                 E       X
  |  |                 |       |
  [      [■][■][■]  ]
          2  3  4'
```

NsiI BstXI SacI BamHI HindIII ClaI Csp45 I SmaI KpnI EcoRI XhoI XbaI SphI AatII ApaI

pGEM-7Zf(+)
vector
3.0 kb

G418 resistance
gene cloned into
SalI restriction
site
→

3.)

```
                    [NEO]
                     Sa
  B Xb                 ▽       X
  |  |                 |       |
  [      [■][■][■]  ]
          2  3  4'
```

NsiI BstXI SacI BamHI HindIII ClaI Csp45 I SmaI KpnI EcoRI XhoI XbaI SphI AatII ApaI

pGEM-7Zf(+)
vector
3.0 kb

Figure 6. Cloning of targeting construct 1. A.) Partial restriction map of the CD40 gene. B.)
Cloning procedure for construct 1. XbaI fragment of the CD40 gene was cloned into pGEM-
11Zf(-) vector (B. 1.), followed by PCR amplification of a 3 kb region spanning exons 2-4. The
PCR product was cloned into the BamHI-XhoI restriction site of a pGEM-7Zf(+) vector (B. 2.).
Finally, cloning of the G418 resistance gene into the SalI restriction site of exon 3 (B. 3.). NEO:
G418 resistance gene, The antisense primer (primer 4, exon 4) and the T7 primer (primer 12)
used in the PCR amplification are shown by small arrows (B. 1.). Black boxes represent exons
1-9. Restriction enzyme sites: Xb, XbaI; S, SacI; E, EcoRI; B, BamHI; X, XhoI; Sa, SalI.
Dashed lines show cloning sites in the polylinker.

Construct 1 was designed so that the initial screening for the homologous recombination could be carried out using PCR amplification (Figure 7.C.). Homologous recombination was detected using PCR amplification on genomic DNA extracted from electroporated ES cells using a primer (primer 11) inside the G418 resistance gene (NEO) and a primer to exon 5 (primer 5) outside the region of homology, giving a 1 kb product (Figure 7.C.). Screening for homologous recombination by PCR is described in detail in section 2.1.25. Subsequent confirmation of the homologous recombination event would be carried out by *Eco*RV restriction enzyme digest on genomic DNA, followed by Southern blot analysis using a 820 bp fragment spanning exon 6 as an external probe (probe B). A targeted mutation would yield a 6.2 kb fragment, whereas the non-targeted wild-type allele would give a 5 kb fragment (Figure 7.C.).

Construct 2 was made by cloning an *Xba*I-*Xho*I restriction fragment of the CD40 gene, containing exon 6, (Figure 8.B.1.) into a pGEM-11Zf(+) vector. Then an *Xba*I restriction fragment of the CD40 gene containing exons 2-5 was cloned into the *Xba*I site of the vector, resulting in an 8 kb genomic fragment spanning exons 2-6 of the CD40 gene (Figure 8.B.2.). Cloning of the G418 resistance gene into the *Eco*RI restriction site of exon 3 requires that only one *Eco*RI restriction site is present in the vector and the CD40 fragment (exons 2-6). Due to the presence of an *Eco*RI restriction site in the polylinker of the pGEM-11Zf(+) vector, the 8 kb genomic fragment (*Hind*III-*Xho*I, exons 2-6) was excised and then cloned into a pGEM7Zf(+) vector, having no *Eco*RI restriction site in the polylinker (Figure 8.B.3.). This facilitated the cloning of the G418 resistance gene into the exon 3 *Eco*RI restriction site (Figure 8.B.4.), as described for construct 1.

Screening for homologous recombination occurring with construct 2 was only possible using *Bam*HI restriction enzyme digestion followed by Southern blot analysis (Figure 9.). A targeted mutation would give a 10 kb fragment, with a *Bam*HI restriction site introduced by the G418 resistance gene targeted into the CD40 gene, whereas the non-

targeted wild-type allele would give a 23 kb fragment when probed with the external probe, probe A, which spans exons 7-8 (Figure 9.C.). The *Bam*HI restriction site in the wild-type allele that generates this 23 kb fragment lies 5' (upstream) to the CD40 gene. This latter *Bam*HI restriction site is not shown in Figure 9.

Screening of ES cell clones for a homologous recombination event by Southern blot analysis should be unambiguous, therefore identification of a probe suitable for verification of a homologous recombination event is crucial. The probe used in the Southern blot analysis must hybridize outside the homologous sequences of the targeting vector. The criteria for which restriction enzyme to use is that it should generate a fragment unique to the homologous recombination event. This unique fragment should be labeled by the probe (Figure 7 and 9). In this study I found that external probes (probes A and B) derived from exon sequences of the CD40 gene by PCR amplification specifically labeled both the wild-type allele and the targeted allele, whereas probes derived from intron sequences would bind several restriction enzyme fragments or give a "smear" in a Southern blot analysis (data not shown). The non-specific binding of probes derived from intron sequences was probably caused by repetitive sequences. I also found that the size of the probe was important for detection of restriction enzyme fragments in a Southern blot analysis. Probes ranging from 80-280 bp, even when derived from exon sequences, failed to detect any restriction fragments in a Southern blot analysis (data not shown). Thus, probes used in Southern blot analysis should be derived from exon sequences and the length of the probe should be in the range of 0.5-1.2 kb. Ideally the Southern blot strategy and probe(s) should be defined and checked for suitability in the Southern blot analysis before the targeting vector is made.

Large quantities of construct 1 and 2 were made using the maxiprep method and purified on a CsCl gradient as described in section 2.1.4. This yielded very clean plasmid DNA which was necessary for efficient transfection of ES cells. Both targeting vectors were linearized, using an enzyme restriction site outside the homologous sequences, prior to transfection. Constructs 1 and 2 were linearized with *Xba*I and *Hind*III, respectively,

and prepared for electroporation (described in section 2.1.24). Previous studies have shown that linearizing the targeting vector increases the targeting frequency, however there is no advantage in isolating the homologous sequences from the bacterial plasmid DNA constituting the rest of the vector (Hasty *et al.*, 1991b). The advantage of construct 1 over construct 2, is that the screening procedure using PCR amplification is quicker than restriction enzyme digestion and southern blotting. More G418 resistant clones can be screened in a shorter time. The constructs are now ready for transfection into embryonic stem cells.

A. CD40 gene
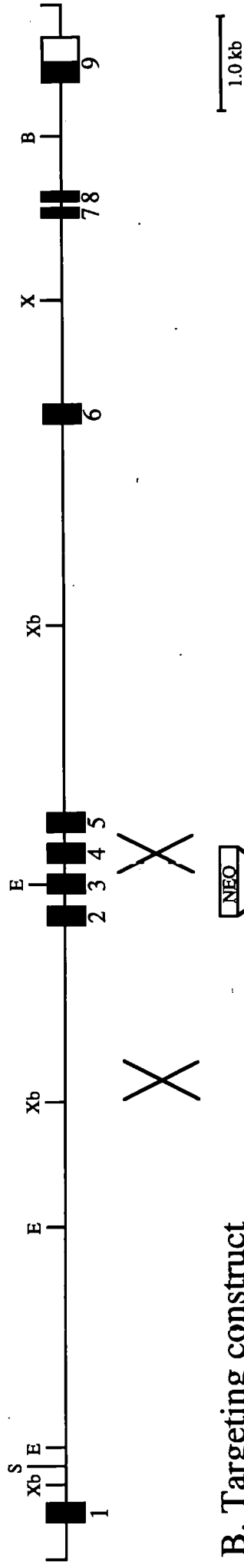
B. Targeting construct
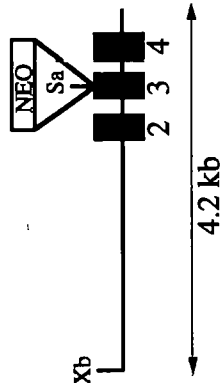
C. Targeted mutation of CD40 gene
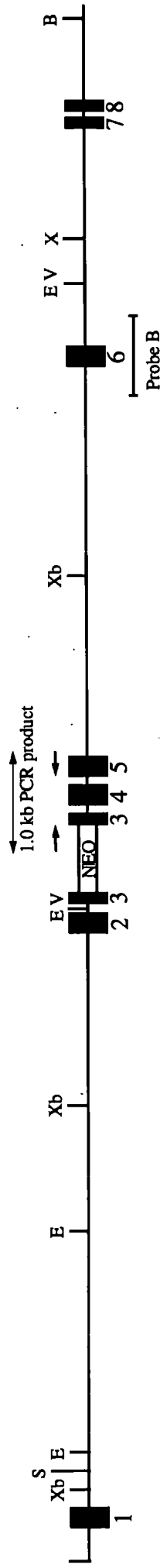
Figure 7. Targeting of the CD40 gene by homologous recombination using construct 1. A.) Partial restriction map of the CD40 gene, B.) the CD40 targeting vector, C.) the CD40 locus after homologous recombination. NEO: G418 resistance gene. Black boxes represent exons 1-9. Restriction enzyme sites: Xb, *XbaI*; S, *SacI*; Sa, *SalI*; E, *EcoRI*; B, *BamHI*; X, *XhoI*; E V, *EcoRV*. PCR primers are shown as small arrows above NEO (primer 11) and above exon 5 (primer 5), yielding a 1 kb PCR product. An 820 bp fragment at the 3' end of the genomic sequence was used as external probe in Southern blot analysis (probe B).

A. CD40 Gene
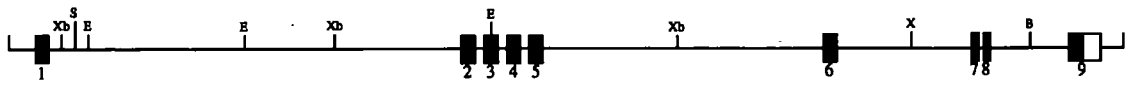
B. Cloning of Targeting Construct no.2
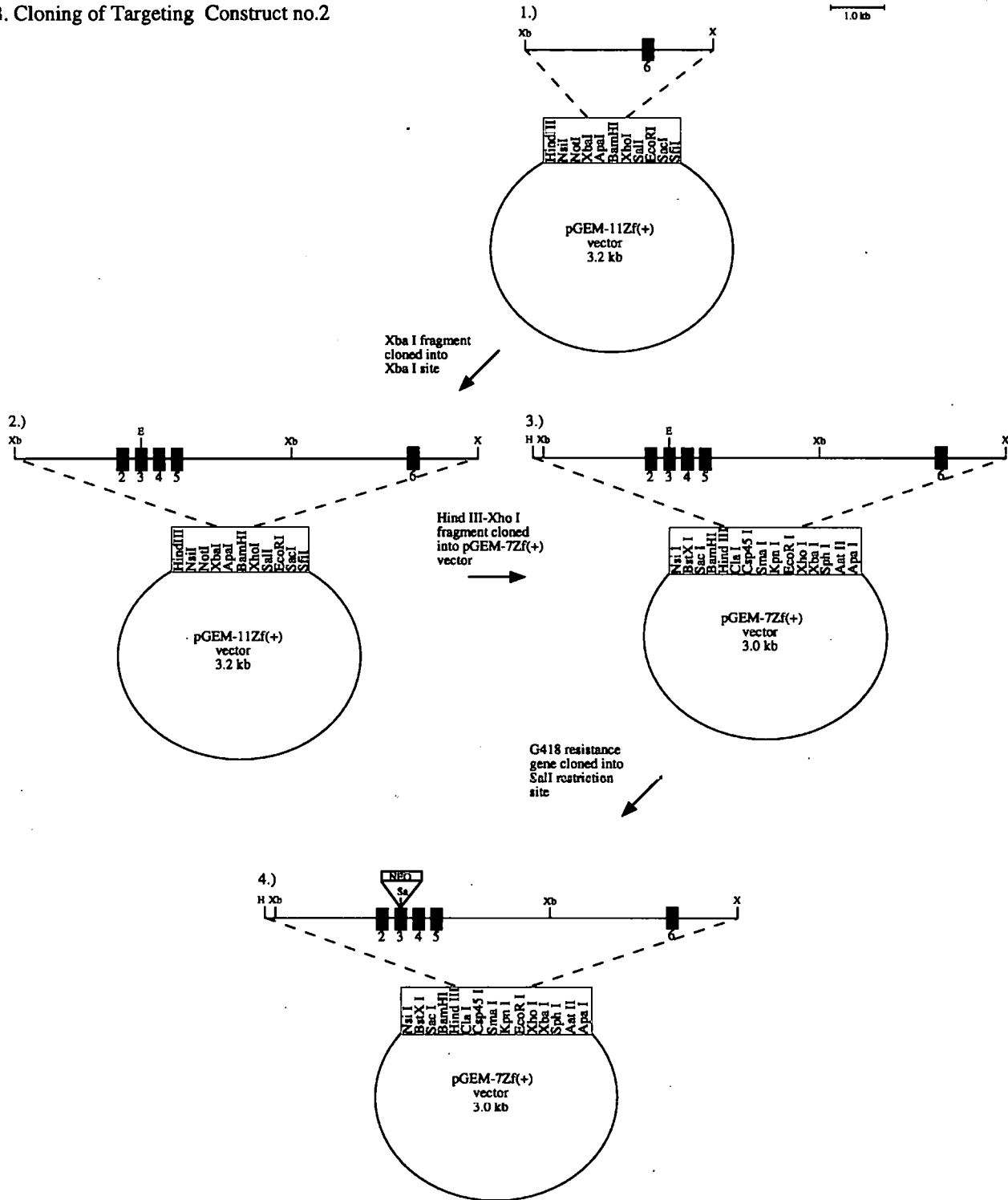
Figure 8. Cloning of targeting construct 2. A.) Partial restriction map of the CD40 gene. B.) Cloning procedure for construct 2. *XbaI-XhoI* restriction fragment of the CD40 gene cloned into pGEM-11Zf(+) vector (B. 1.), followed by cloning of *XbaI* restriction fragment of the CD40 gene into the *XbaI* site of the pGEM-11Zf(+) vector (B. 2.). The 8 kb CD40 genomic fragment (*Hind*III-*XhoI*) was then cloned into a pGEM-7Zf(+) vector (B. 3.) and the G418 resistance gene cloned into the *SalI* restriction site (B. 4.). NEO: G418 resistance gene. Black boxes represent exons 1-9. Restriction enzyme sites: Xb, *XbaI*; S, *SacI*; Sa, *SalI*; E, *EcoRI*; B, *BamHI*; X, *XhoI*. Dashed lines show cloning sites in the polylinker.

A. CD40 gene

B. Targeting construct

C. Targeted mutation of CD40 gene

Figure 9. Targeting of the CD40 gene by homologous recombination using construct 2. A.) Partial restriction map of the CD40 gene, B.) the CD40 targeting vector, C.) the CD40 locus after homologous recombination. Black boxes represent exons 1-9. Restriction enzyme sites: Xb, XbaI; S, SacI; Sa, SaII; E, EcoRI; B, BamHI; X, XhoI. A 540 bp fragment at the 3' end of the genomic sequence was used as an external probe in Southern blot analysis (probe A). A 10 kb BamHI restriction fragment is obtained on Southern blot analysis when homologous recombination has occured, whereas a 23 kb fragment occurs if the wild-type allele is present.

3.2 Production of targeted embryonic stem cell clones

Pluripotent mouse embryonic stem (ES) cells derived from inner cell mass (ICM) cells of mouse blastocysts were isolated and conditions for maintaining them in culture were initially developed by two laboratories (Evans & Kaufman, 1981; Martin, 1981). The *in vitro* culture of ES cells is dependent on the cytokine leukaemia inhibitory factor (LIF) (Smith *et al.*, 1988; Williams *et al.*, 1988). This protein is essential for maintaining the growth of ES cell *in vitro* since, in its absence, ES cells will differentiate and eventually cease to proliferate. Leukaemia inhibitory factor can be applied to the ES cells in two different ways. Currently the best approach, and the most effective one for long-term culture, is to culture the ES cells on a feeder layer of mouse primary embryonic fibroblasts. The feeder layers synthesizes and secretes LIF into the culture medium, which facilitates the continuous proliferation of undifferentiated ES cells. The alternative is to culture ES cells in medium which is supplemented with recombinant LIF. Apart from the requirement for LIF, ES cells are very sensitive to the type and quality of fetal calf serum (FCS) in which they are grown. Because the quality of fetal calf serum can vary from batch to batch, it is essential to test each batch of serum to ensure that it is optimal for ES cell growth. The use of stringent tissue culture conditions ensures that these cells maintain their ability to contribute to all tissues in chimaeras, even after genetic manipulation. The important thing is that the ES cells maintain their pluripotency (remain undifferentiated) and can thus contribute both to somatic tissues and most importantly, to the germline of mice. Newly thawed ES cells usually grow slowly and the growth depends on the cell density. As a general rule, the more dense the cells, the faster the growth. It is important to keep the time in culture to a minimum and to dissociate clumps of cells at each passage. Check the cells frequently, ideally twice a day and change the medium every day. Undifferentiated ES cell colonies are oval and look shiny under the phase contrast microscope. Individual cells can not be seen within undifferentiated cell colonies. In contrast, differentiated ES cell colonies are flat, granular and appear grayish under the microscope. However, even if an ES cell culture appears undifferentiated, this

does not guarantee germline transmission. Some ES cells have been found to be able to form somatic, but not germline cells in chimeras (this was found to be the case in this study). The reason for this is unknown. Such cells cannot be distinguished from germline-competent ES cells by morphology, karyotype or any other *in vitro* test. Therefore, prior to use in gene targeting experiments, the germline competence of an ES cell line should be confirmed by the generation and test breeding of chimaeras.

The ES cell lines used in the experiments described here were D3 (Doetschman *et al.*, 1985) and R1 (Nagy *et al.*, 1993). D3 was derived from mouse strain 129/Sv and R1 was derived from mouse strain (129/Sv x 129/SvJ)F$_1$. D3 ES cells were electroporated with construct 1 (Figure 7.) and G418 resistant colonies appeared after 8-10 days. Electroporation of ES cells and G418 selection is described in section 2.2.6. In total, 1600 G418 resistant clones were screened by PCR using the primer (primer 11.) hybridizing inside the NEO gene and primer 5 (exon 5) hybridizing outside the region of homology (Figure 7.C.). In the event of homologous recombination occurring, a 1 kb PCR amplification product would have been seen. Screening for homologous recombination using PCR is described in section 2.1.25. Unfortunately a 1 kb PCR product was never detected in the initial screening. PCR products of 1 kb would occasionally appear after gel electrophoresis, but Southern blot analysis using a specific oligo-nucleotide primer 4 (primer 4) failed to confirm the presence of any specific 1 kb PCR products. I therefore decided to use construct 2 for the generation of targeted ES cell clones.

D3 and R1 ES cells were electroporated with construct 2 (Figure 9.) as described in section 2.2.6 and G418 resistant clones were screened for homologous recombination events by *Bam* HI restriction enzyme digestion and Southern blot analysis on genomic DNA. The procedure used for screening by restriction enzyme digestion and Southern blot analysis follows. The G418 resistant clones were picked into individual wells of a 96-well plate and left to grow until confluent. The ES cell clones were then split into a "masterplate" and a "replica plate". The "masterplate" was frozen down whereas the

"replica plate" was used for the Southern blot analysis on the individual ES cell clones. A detailed description of picking and expanding ES cell clones in 96-well plates is shown in section 2.2.7. Extraction of genomic DNA from ES cell clones grown in 96-well plates is described in section 2.1.26.

Southern blot analysis was performed on each individual ES cell clone using the external probe A (Figure 9.C.) after digestion with BamHI restriction enzyme. Those cells that had undergone a homologous recombination event would yield a 23 kb fragment corresponding to the single intact wild-type CD40 allele and also a 10 kb fragment corresponding to the mutant targeted allele (Figure 10). To confirm that the G418-resistance gene (NEO) was part of the 10 kb mutant restriction fragment, a probe consisting of NEO coding sequence was used as a radioactive probe in the Southern blot analysis. This would hybridize to the mutant allele giving a single 10 kb band. Randomly integrated constructs yielded restriction fragments of different sizes (Data not shown). The correctly targeted clones were retrieved from the "masterplate" (described in section 2.2.8), expanded and frozen in liquid nitrogen. Targeted ES cell clones were now ready for karyotyping and subsequent blastocyst injection.

Table 1. shows 4 electroporations and the number of targeted clones obtained in each experiment. In total 12 ES cell clones were found to have a targeted disruption of the CD40 gene. As seen in Table 1., the frequency of homologous recombination in G418-resistant clones varied from 1/82 to 1/580 and the overall targeting frequency was 1/172.

Table 1. Electroporation of ES cells with construct 2. The individual targeted clones were given numbers, shown in brackets.

Electroporation:	ES cell line:	G418-resistant clones:	Number of targeted clones:	Targeting frequency of G418-resist. clones:
1.	R1	580	6 (34, 40, 55, 61, 65, 77)	1/96
2.	D3	580	1 (21)	1/580
3.	D3	580	1 (55)	1/580
4.	D3	329	4 (3, 5, 85, 89)	1/82

Electroporation of ES cells with construct 1 (Figure 7.) yielded no targeted clones. The lack of homologous recombination events using construct 1 could be explained by the presence of short homologous sequences which only allowed a 3 kb overlap. Another possibility was that the detection of homologous recombination by PCR amplification was not sensitive enough. The conditions for the PCR could have been optimized by making a control targeting construct containing exon 5 (this exon is absent in construct 1). ES cells could then be transfected with the control targeting construct and PCR amplification conditions optimized. However, this may have introduced other problems such as a risk of contamination, giving rise to many false positive PCR products. Several studies have described the relationship between the length of homology and the targeting frequency (Hasty *et al.*, 1991a; Thomas & Capecchi, 1987). As a general rule it has been shown that, the greater the length of homology, the higher the targeting frequency. The ideal length of the homologous sequences in a targeting vector is approximately 8-10 kb. This will also allow for unique restriction enzyme sites which can be used to linearize the vector prior to electroporation and also to allow unambiguous detection of targeted clones using Southern blot analysis of G418 resistant clones. The targeting frequency is probably dependent on both the length of the homologous sequences and access to the endogenous locus. Another important factor may be the use of isogenic DNA.

Polymorphic variations between the targeting vector and the chromosome have been shown to affect targeting frequencies adversely (te *et al.*, 1992). This study showed that the frequency of homologous recombination events in 129/Sv derived ES cells was twenty times higher with a targeting construct containing isogenic DNA (129/Sv derived construct) compared with a targeting vector containing polymorphic DNA (BALB/c derived construct). There seems to be a great deal of variability between different loci even with isogenic constructs of the same homology. In this study I used isogenic DNA for my targeting vector, avoiding the possible reduction in targeting frequency through the influence of polymorphic sequences.

Positive selection (NEO) was used to select targeted clones arising from transfection with both constructs 1 and 2 (Figure 7. and Figure 9.) A negative selection marker (TK), although not increasing the targeting frequency, could have been used to decrease the number of clones in the screening procedure. I decided not to use a negative selection marker based on information showing that FIAU/gancyclovir (nucleotide analogue incorporated by HSV-*tk*) might reduce the potential of the targeted ES cells to go into germline (Rahemtulla, personal communication).
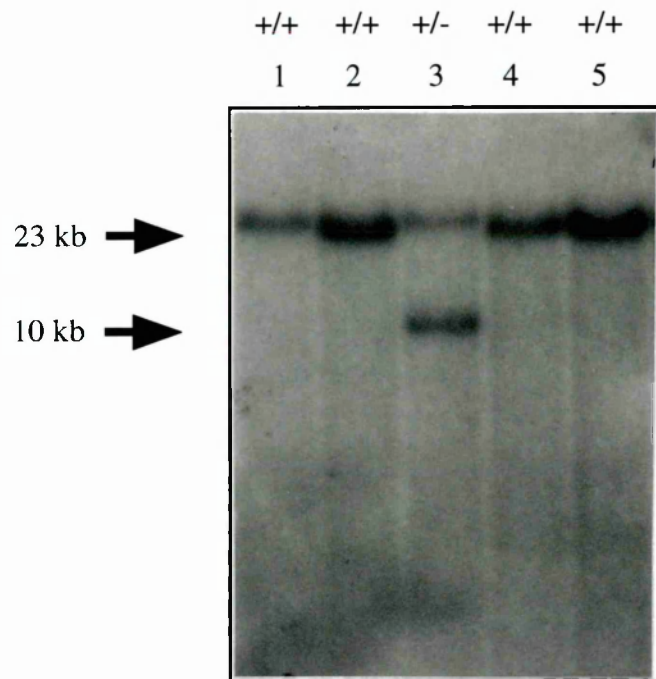
Figure 10. Southern blot analysis of G418-resistant ES cell clones. Lanes 1-5: *Bam*HI restriction enzyme digest of genomic DNA from G418-resistant clones, blotted onto Hybond-N+ and hybridized with a radioactively labeled 3'-flanking probe (probe A.). Lanes 1-2 and 4-5 all display a 23 kb restriction fragment corresponding to the wild-type allele. Lane 3 displays both a 23 kb and a 10 kb restriction fragment indicating the presence of a normal allele and a targeted mutant allele.

3.3 Generation of germline chimeras from targeted ES cells

3.3.1 Karyotyping of ES cells

The continuous culture of ES cells is a very delicate process and care must be taken to keep them in an undifferentiated state, to secure germline transmission. The only way of distinguishing undifferentiated from differentiated ES cells is to look at their morphology. As described previously, pluripotent ES cell colonies are oval and look shiny under phase contrast microscopy and individual cells cannot be seen within undifferentiated cell colonies. In contrast, differentiated ES cell colonies are flat, granular and appear grayish under the microscope. One problem that can occur is that although ES cells are undifferentiated (as defined by their morphology) they can lose or acquire chromosomes during extended culture. To ensure the correct number of chromosomes, once targeted ES cell clones have been identified and expanded, they must be karyotyped. Karyotyping is achieved by blocking actively growing ES cells in late prophase, using the method described in section 2.2.10., and then counting the number of chromosomes in each individual cell. Normal mouse cells contain 40 chromosomes. R1 and D3 ES cell lines were karyotyped (Figure 11.). R1 targeted ES cell clones (34, 65, 77) and D3 targeted ES cell clones (3, 5, 21, 55) were selected on the basis of their morphology and karyotyped (Figures 12. and 13.). Both R1 and D3 have a normal distribution of cells with 40 chromosomes, but D3 seems to have a higher percentage of cells (70%) with a normal number of chromosomes (shown in Figure 11.). Of the R1 targeted ES cell clones shown in Figure 12., 34 was found to have 80% of it's cells with a normal karyotype compared to only 55% in the R1 ES cell line (Figure 11). This was probably due to the fact that the homologous recombination event happened by chance to take place in an ES cell with a normal karyotype. Independently of this event, the ES cell was then able to maintain a stable karyotype throughout culture. Of the D3 targeted ES cell clones shown in Figure 13., only clones 5 and 55 showed a normal distribution,

around 40 chromosomes, with a lower percentage of cells having a normal karyotype, compared to the original D3 ES cell line (Figure 11).

Figure 11. Karyotyping of R1 and D3 ES cell lines. The number of chromosomes in each individual cell was counted and a total of 50 cells were counted for each cell line. The bars represent the percentage of those 50 cells having the number of chromosomes indicated.
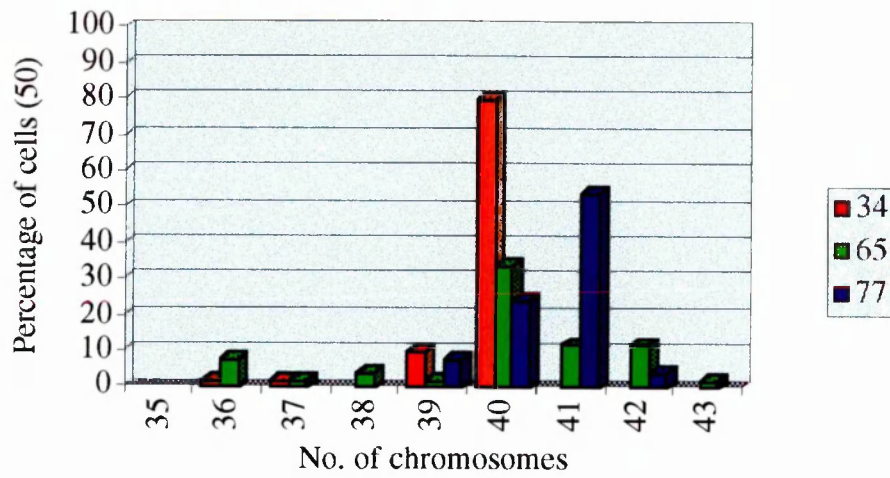
Figure 12. Karyotyping of targeted R1 ES cell clones. The number of chromosomes in each individual cell was counted and a total of 50 cells were counted for each cell line. The bars represent the percentage of those 50 cells having the number of chromosomes indicated.
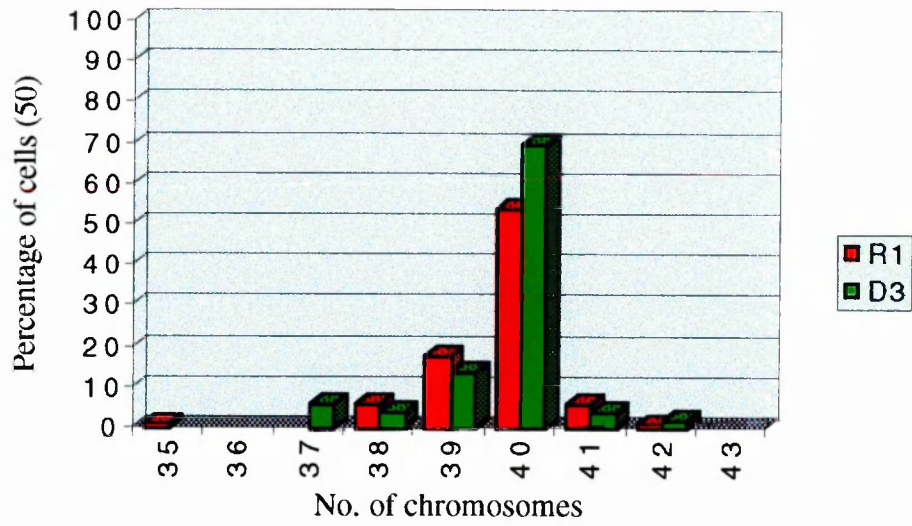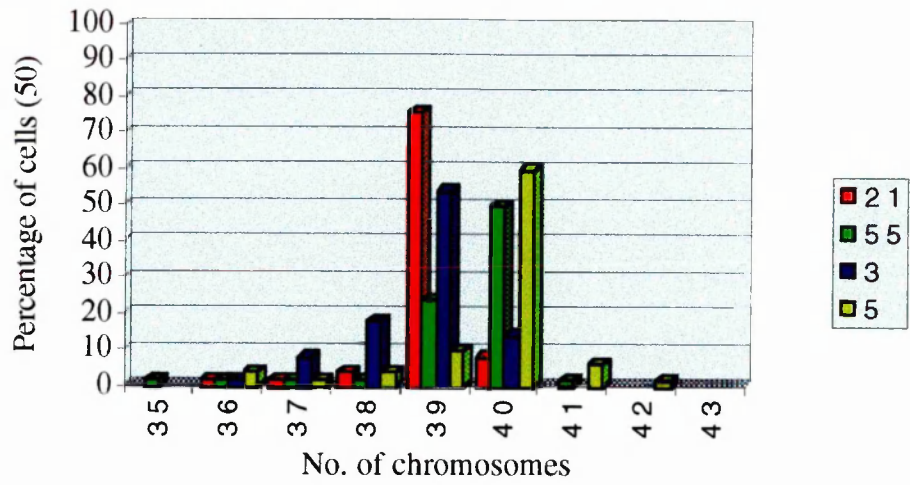
Figure 13. Karyotyping of targeted D3 ES cell clones. The number of chromosomes in each individual cell was counted and a total of 50 cells were counted for each cell line. The bars represent the percentage of those 50 cells having the number of chromosomes indicated.

3.3.2 Injection of targeted ES cell clones into C57BL/6 blastocysts

Most ES cell lines that are currently in use have a male genotype (XY). This has two advantages. The first is that the male ES cell lines, when injected into female blastocysts (XX) , will tend to bias the development of the resulting chimaera toward a male phenotype. In phenotypically male chimaeras, XY germ cells (i.e. those derived from the ES cells) will form functional gametes, whereas XX germ cells (i.e. those derived from the host blastocyst) will not form functional gametes. This sex conversion presumably occurs when XY cells colonize sufficient portions of the various tissues which determine sex in the developing embryo. This sex conversion is not always complete in chimaeras and occasionally results in an infertile hermaphrodite. Secondly, a male chimaera can produce more offspring over its reproductive life span than a female, so that even chimaeras with a relatively low percentage contribution of the ES cells to the germline can be detected. Transmission of the male derived ES cell genotype has been reported to occur from female chimaeras. Since XY cells do not normally undergo oogenesis in chimaeras, this phenomena may be due to the loss of part or all of the Y chromosome, resulting in XO cells which are capable of forming ovaries (Kuehn *et al.*, 1987).

Another important consideration is the strain of mouse from which the ES cells are derived and the strain of embryo (blastocyst) into which they will be introduced. This is particularly relevant for producing germline chimaeras since certain strains have, in combination with others, a competitive advantage in their development. Thus, many chimaeras will be predominantly composed of cells derived from the dominant strain, increasing the likelihood that the ES cells will contribute to the germline (Schwartzberg *et al.*, 1989). ES cell lines derived from the 129 strain of mice, when injected into blastocysts of the C57BL/6 strain, tend to predominate in the chimeras. The 129 ES cells have an agouti coat color genotype, whereas the C57BL/6 embryos is black. Therefore, the chimaeras produced from this combination will be a colour mix of agouti and black (Figure 14.). If the ES cell line is particularly "good" at making chimaeras, extreme

individuals composed almost entirely of the ES genotype can be produced (95%-99% agouti coat colour). ES cell lines have also been derived from the C57BL/6 strain of mice (Ledermann & Burki, 1991) and the BALB/c strain of mice (Raul M. Torres, Laboratory Protocols .., Appendix C). The choice of which ES cell line to use may be dictated by the genetic background necessary for the experiments or the source of DNA used in the targeting construct. The appropriate genetic background can always be obtained by backcrossing, although at a substantial price in terms of time. If the targeted mutation has been generated in 129 ES cells and germline transmission has been obtained, then mice homozygous for the targeted mutation must be backcrossed (e.g. onto C57BL/6) 10 times to gain 99.91% homozygosity on all alleles. Ultimately, the goal is to deliver the targeted mutation to the germline.

The targeted ES cell clones were selected for injection into C57BL/6 blastocysts on the basis of the result of the karyotyping and their morphology. The clones selected were 34 R1, 65 R1 and 55 D3. The targeted ES cell clones (stored in liquid nitrogen) were thawed and grown until confluent (section 2.2.5). On the day of injection, the blastocysts were recovered as described in section 2.3.1 and the chosen targeted ES cell clone was prepared by trypsinization and washing in complete medium. 16 ES cells were injected into each blastocyst (section 2.3.5) and 12-16 injected blastocysts were transferred into a pseudo-pregnant foster mother (section 2.3.6). 17 days later the foster mother gave birth to both wild-type and chimaeric mice. Although all blastocysts had been injected, only some of these became colonised by the targeted ES cells, giving rise to chimaeric mice. Table 2. shows the number of blastocysts injected with each ES cell clone and the number of chimaeras born. 24% of the progeny were chimaeric for ES cell clone 34 R1, however 68% were chimaeric for ES cell clone 55 D3. It was important that the targeted ES cells contributed to most of the differentiated tissue in the chimaeric mouse and more importantly, that they contributed to the germline cells of that mouse. The targeted ES cell clones were derived from a male ES cell line (R1 and D3) and male ES cells in combination with a female blastocyst often produce a fertile phenotypic male

chimaera. Thus those male chimaeric mice with a mainly agouti coat colour were most likely to pass on the mutated allele and comparison of the male to female ratio among the chimaeric mice gave a good indication of the likelihood of germline transmission. 70% of the chimaeric mice generated from injection with the 55 D3 ES cell clone were males compared to 66% in those chimaeras generated by injection with the 34 R1 ES cell clone.

Table 2. Production of chimaeras. The number of chimaeras is shown as a percentage of the total number of offspring (in brackets). The number of male chimaeras is shown as a percentage of total number of chimaeras (in brackets).

ES cell clone	Total no. of injected blastocysts	Total no. of offspring born	Number of chimaeras born	No. of male and female chimaeras	Germline-transmission
34 R1	279	91	24 (26%)	16 males (66%) 8 females	
65 R1	165	28	4 (14%)	1 male (25%) 3 females	
55 D3	107	69	47 (68%)	33 males (70%) 14 females	1 male (95% agouti)
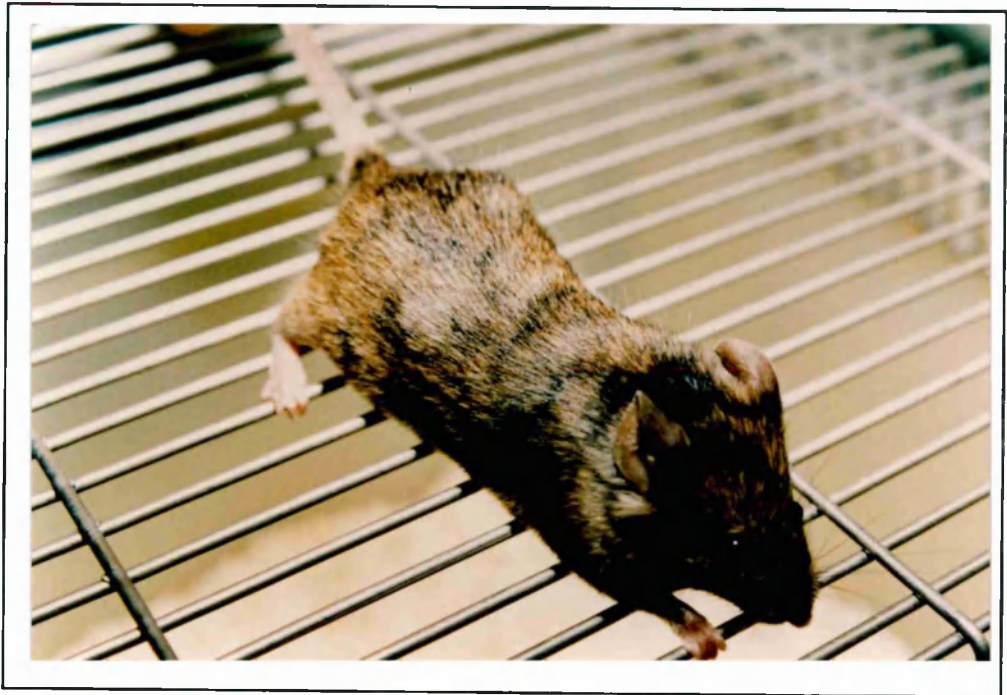
Figure 14. shows a male chimaera, notice the appearance of both black (blastocyst derived) and agouti (ES cell derived) coat colour.

3.3.3 Breeding chimaeras to check for germline transmission

Males with 70-95% agouti coat colour were set up for breeding with (C57BL/6xDBA2)F$_1$ females in preference to C57BL/6 females, to check for germline transmission. The reason that F$_1$ females were used rather than C57BL/6 female mice was because they have larger litters and are less likely to cannibalize their offspring. 1 male chimaera (95% agouti) generated from the 55 D3 ES cell clone finally resulted in germline transmission. Once germline transmission was achieved the male chimaera was bred with C57BL/6 females. This allowed backcrossing of the targeted deletion onto a C57BL/6 genetic background. The offspring were either black (wild-type) or agouti (germline transmission) and only 50 % of the agouti offspring were heterozygous for the mutant allele. The agouti offspring were screened for the presence of the mutant allele by PCR amplification (section 2.1.22) using primer 2 (hybridizing to exon 2) and primer 11 (hybridizing to the NEO gene) resulting in a product of 500 bp (data not shown). PCR amplification was carried out on genomic DNA extracted from the tails of the mice (section 2.1.16). Mice heterozygous for the mutant allele were interbred and DNA was extracted from the tails of the offspring. This was used to screen for the presence of homozygous mutant alleles using *Bam*HI restriction enzyme digestion and Southern blot analysis. Figure 15 shows a representative result from this screening process. A wild type C57BL/6 DNA control and a targeted ES cell clone DNA control was included (lanes 1 and 10 respectively). Lanes 2-9 contain DNA from offspring derived from the heterozygote interbreeding. Lanes 6 and 7 are the only two showing the presence of mutant alleles at both loci. Mice homozygous for the targeted disruption of CD40 were set up for mating to produce a colony of CD40 deficient mice.
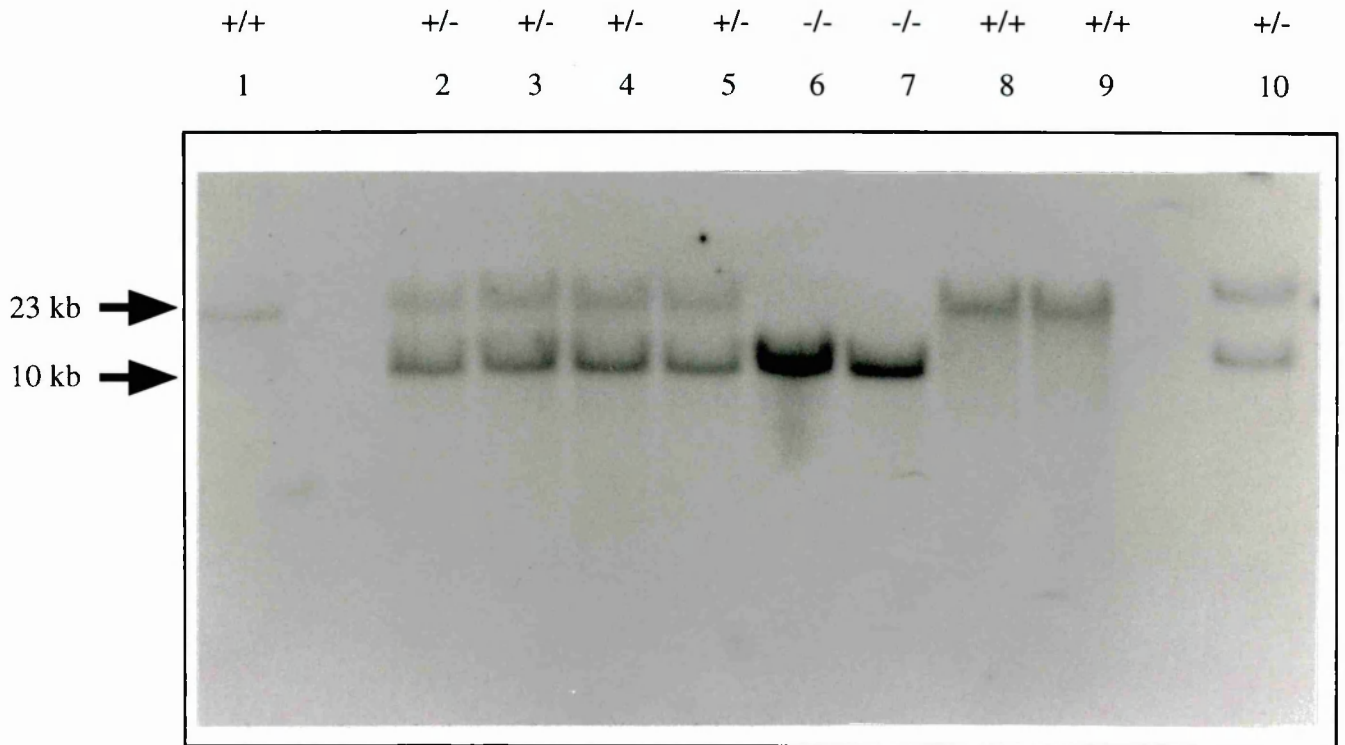
Figure 15. Southern blot analysis of the offspring resulting from interbreeding of mice heterozygous for the mutant CD40 allele. *Bam*HI restriction enzme digests of genomic DNA extracted from mouse tails, blotted onto Hybond-N+ and hybridized with a radioactively labeled probe (probe A). Lane 1: wild-type control +/+. Lanes 2-5: heterozygotes +/-. Lanes 6-7: homozygotes -/-. Lanes 8-9: wild-type +/+. Lane 10: ES cell clone 55 D3 +/-.

Chapter 4: Analysis of CD40 deficient mice

4.1 Phenotypic analysis of lymphocytes in CD40 deficient mice

CD40 is expressed throughout most stages of B cell development, including progenitor B cells expressing CD34 (Saeland *et al.*, 1992) and ligation of CD40 induces tyrosine phosphorylation, proliferation and expression of CD23 in precursor B cells (Saeland *et al.*, 1993; Uckun *et al.*, 1991). Thymic epithelial cells also express CD40 and cross-linking of these cells with an anti-CD40 antibody increases Granulocyte/macrophage-colony stimulating factor (GM-CSF) secretion (Galy & Spits, 1992). These observations suggested that CD40 may play an important role in both B and T lymphocyte development. Therefore it was important to examine whether the absence of CD40 would affect the surface phenotypes of T and B lymphocytes in peripheral blood and lymphoid organs and also whether the proportions of these cells in the periphery and organs would change.

To examine the surface expression of CD40 on lymphocytes in the mutant mice, the capacity of peripheral blood lymphocytes to bind a CD40 mAb was analysed (Figure 16.A.). Peripheral blood lymphocytes were isolated as described in section 2.4.1 from wild-type and mutant mice. The peripheral blood lymphocytes from both sets of mice were then stained with monoclonal antibodies to the following surface molecules, CD40, B220, CD4 and CD8 (as described in section 2.4.1, Appendix B). The same total number of events were collected for each individually stained sample from both mutant and wild-type mice, allowing me to determine the proportion of cells in the peripheral blood that were B220 positive (B lymphocytes) or that were CD4 or CD8 positive (T lymphocytes). The intensity of the staining observed also allowed me to assess whether the levels of surface marker expression were different in the CD40 deficient mice as compared to the wild-type mice. Flow cytometric analysis of the subsets of cells within peripheral blood lymphocytes of wild-type and mutant mice was carried out on the

FACScan (Becton Dickinson) using CellQuest software. The analysis revealed that B lymphocytes from wild-type mice expressed both B220 and CD40 at the cell surface, resulting in a double-positive population. However, the mutant mice only expressed B220 on the surface of their B cells, resulting in a single-positive population (Figure 16. A.). This confirms that the targeted gene disruption has abrogated the expression of functional CD40 in the mutant mice (the lack of CD40 expression in mutant mice is also shown in Figure 18. B. and E.). The availability of a specific antibody for CD40 made the search for surface expression relatively straight forward. If no monoclonal antibody had been available, expression of the CD40 gene could have been tested by a Northern blot analysis using CD40 cDNA as a probe or reverse transcriptase (RT) PCR on splenocytes.

Flow cytometric analysis using the B220 monoclonal antibody to look for B cells in the peripheral blood, showed that the proportion of B cells expressing B220 in the wild-type mice was equal to the proportion of B cells expressing B220 in the mutant mice and that the relative levels of B220 at the surface was similar in both groups of mice. This was quantitated by staining a fixed volume of blood and ten thousand cells were then counted on the flow cytometer. Also flow cytometric analysis of T lymphocyte subsets in the peripheral blood expressing CD4 and CD8 in wild-type and mutant mice was found to be the same (Figure 16. A.). To confirm that the proportions of T and B lymphocytes in the peripheral circulation of CD40 deficient mice remained the same, despite the lack of functional CD40 expression, a single cell suspension of mesenteric and popliteal lymph nodes was stained with CD4 mAb, CD8 mAb, B220 mAb and IgM mAb. Flow cytometric analysis (Figure 16. C.) revealed two single positive populations for CD4 expression and CD8 expression and no difference in the proportions of each of the CD4 positive and CD8 positive lymphocytes were found in wild-type compared with mutant mice. The proportion of B lymphocytes, as determined by B220 and IgM staining of the lymph nodes in both wild-type and mutant mice, were the same. Splenocytes from wild-type and mutant mice were also stained with monoclonal antibodies to the following

surface markers CD4, CD8, B220 and IgM. The proportions of B and T lymphocytes in spleens of wild-type and mutant mice were indistinguishable (data not shown).

The total number of T and B cells in the peripheral lymphoid organs of both groups of mice was indistinguishable. This was assessed by counting the total number of cells in each organ (hemocytometer) and 1.0×10^5 cells were then stained with the appropriate antibody (described in section 2.4.2). 1.0×10^4 events were then counted on the flow cytometer. This gave me the proportion of T cells (or B cells) in this sample. This proportion could then be used to calculate the total number of T or B cells in the organs (data not shown). This suggested that the lack of functional CD40 had no effect on the proportions and numbers of T or B lymphocytes in the peripheral organs of the mutant mice.

The involvement of CD40 in thymocyte development was assessed by staining the thymus with specific antibodies. A single cell suspension of cells from the thymus was made as described in section 2.4.2 and the cells stained with monoclonal antibodies specific for CD4, CD8 and $\alpha\beta$-TCR. Double staining for CD4 and CD8 expression (Figure 16. B.) revealed a double-positive CD4+CD8+ population of thymocytes and two single positive populations, CD4+CD8- and CD8+CD4- thymocytes. Also double staining for CD4 and $\alpha\beta$-TCR showed a gradual increase in expression of TCR as thymocytes matured (determined by an increase in the intensity of TCR staining). This showed the development of double-positive immature thymocytes (CD4+CD8+TCR^{low}) into (CD4+CD8-TCR^{high}) or (CD8+CD4-TCR^{high}) mature single-positive thymocytes, which then become circulating T lymphocytes (Anderson *et al.*, 1996). Staining of thymocytes with antibodies to the cell surface antigens CD4, CD8 and $\alpha\beta$-TCR showed that there were no abnormalities in thymocyte development in the CD40 deficient mice compared to the wild-type mice.

Figure 16.   A. Peripheral blood
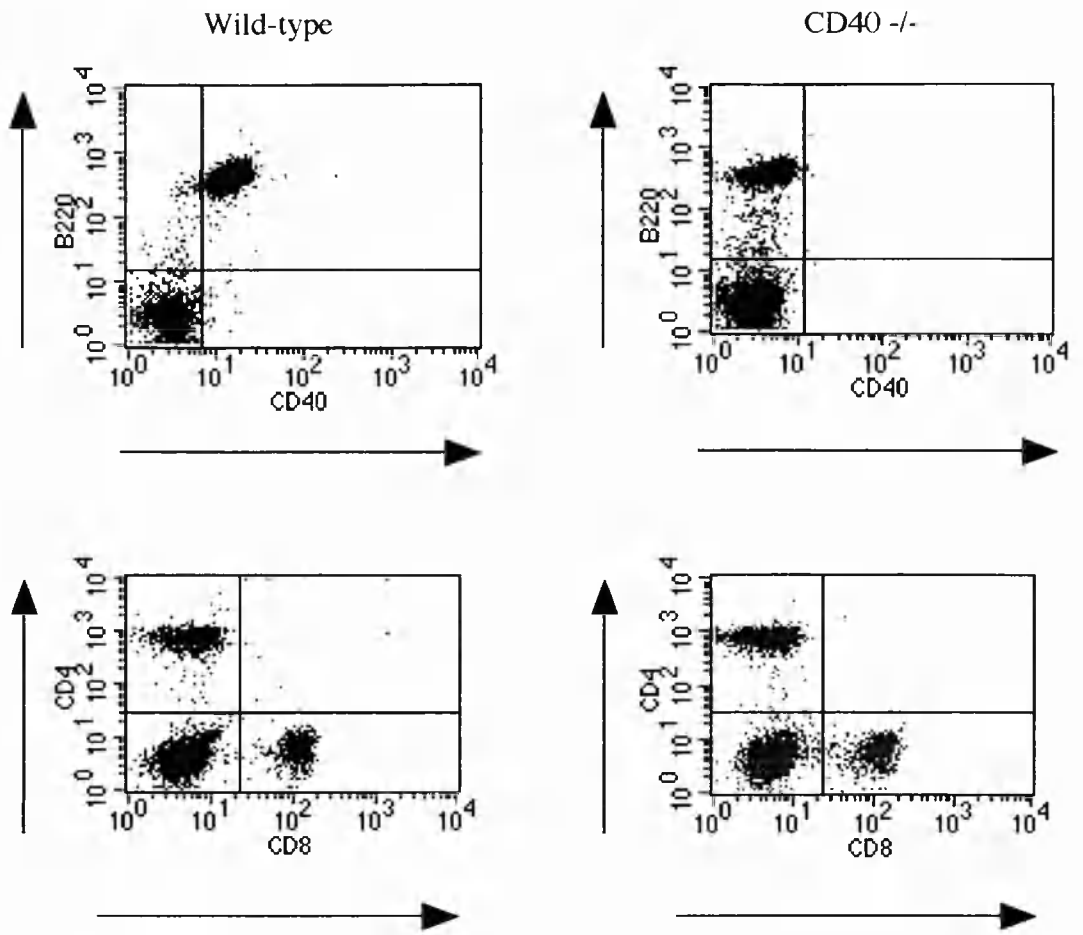
B. Thymus
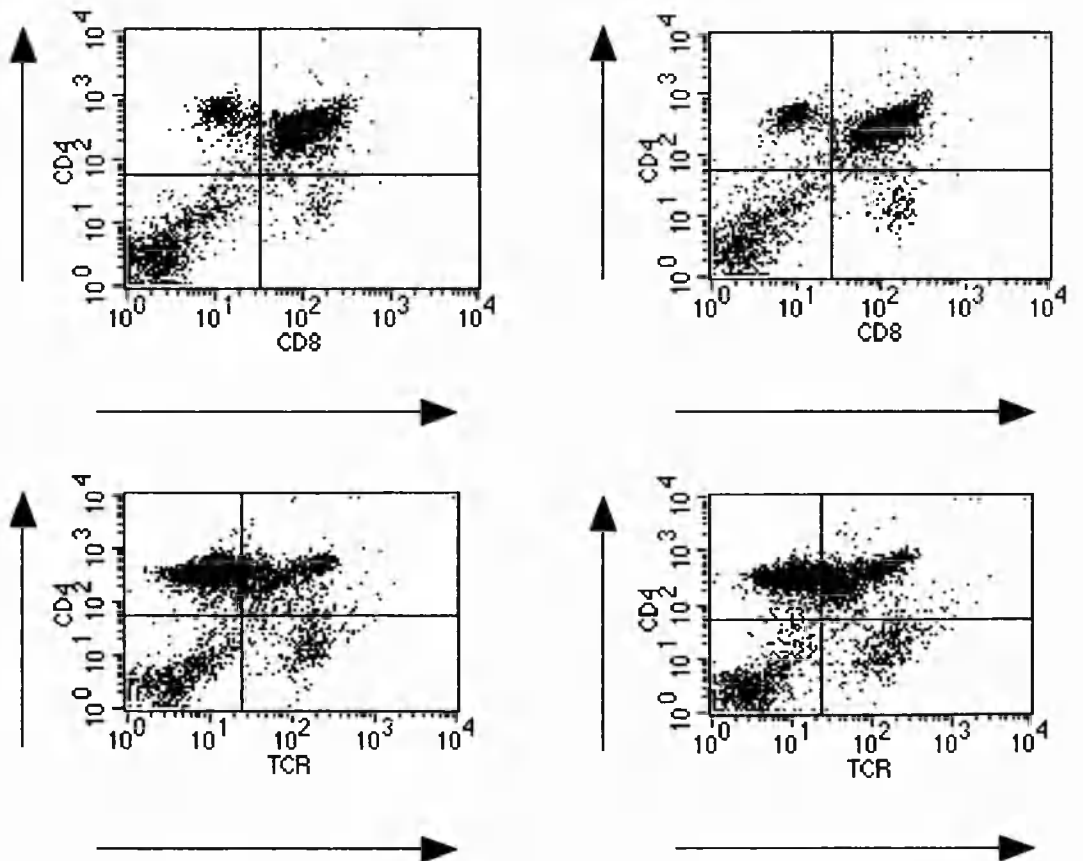
C. Lymph nodes
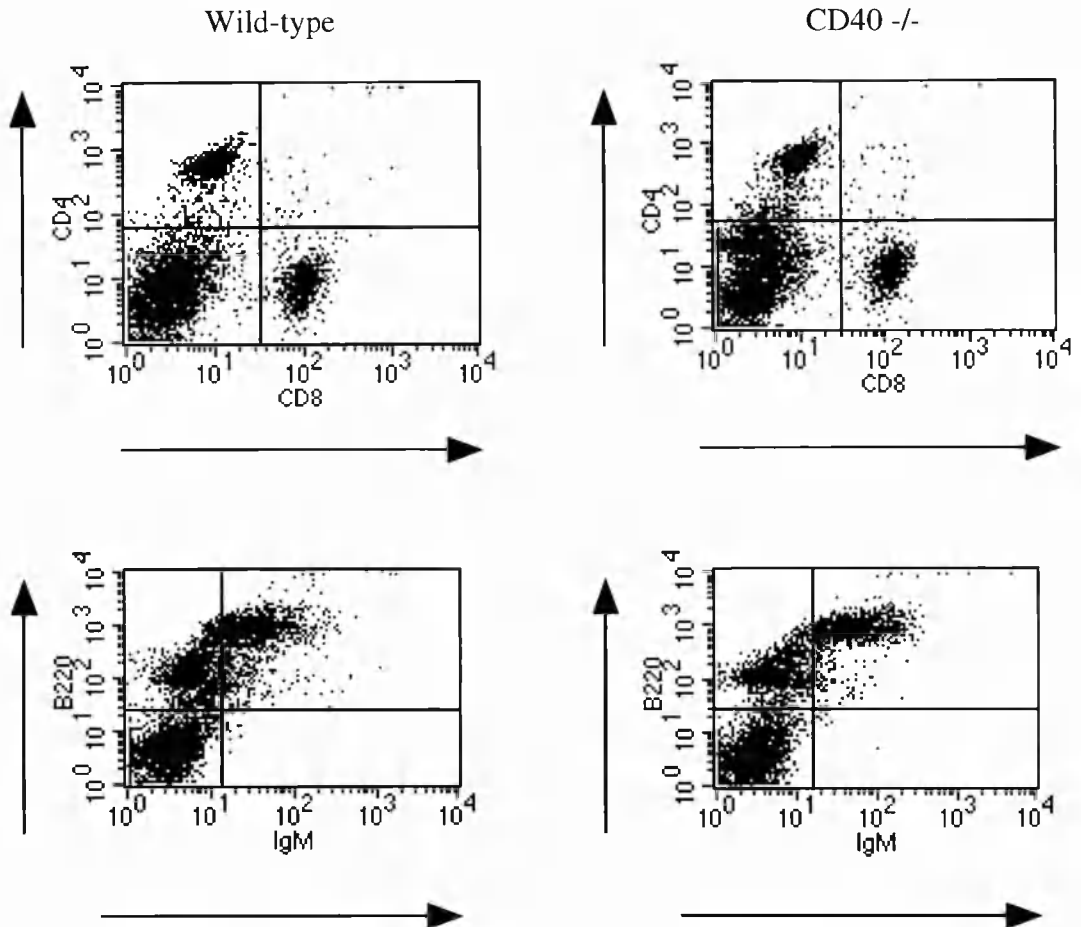
Wild-type

CD40 -/-

Figure 16. Flow cytometric analysis of lymphocytes from wild-type and CD40-/- mice, A.) peripheral blood, B.) Thymus, C.) Lymph nodes. Peripheral blood, thymus and lymph nodes were isolated from 8-week old mice and stained with following antibodies: A.) PE-anti-B220, FITC-antiCD40, PE-anti-CD4, FITC-anti-CD8; B.) PE-anti-CD4, FITC-anti-CD8, FITC-anti-TCR; C.) PE-anti-CD4, FITC-anti-CD8, PE-anti-B220, FITC-anti-IgM. Cells were analyzed by FACS (Becton Dickinson, CellQuest). Results are presented as two-dimensional dot-plots in which each dot represents an individual cell. Similar results were obtained in three experiments.

4.2 *In vitro* B cell response to CD40 engagement

Antigens which stimulate proliferation and antibody production by B cells can be divided into two categories. Thymus-dependent or thymus-independent antigens. Thymus-dependent antigens are protein derived peptides, which can only induce the production of antigen specific antibody, if the peptide is recognized in association with MHC class II by antigen specific T cells, this leads to mutual activation of both B and T cells, and the production of antigen specific antibody. Therefore, antibody production in response to protein antigens requires T cell help. The activation and proliferation of B cells in response to protein antigens require that B cells recognize the antigen via the B cell receptor and also require a second signal through CD40 (Rathmell *et al.*, 1996). Antigens that stimulate proliferation and antibody production by B cells in the absence of MHC class II restricted T cell help are classified as thymus-independent antigens (Mond *et al.*, 1995). Included within the category of thymus-independent antigens are lipopolysaccharide (LPS) derived from gram-negative bacterial cell walls, dextran, pneumococcal capsular polysaccharide, and Ficoll. In vitro studies have shown that B cells proliferate in response to CD40 engagement by anti-CD40 monoclonal antibody (Ledbetter *et al.*, 1987) and that B cells proliferate and undergo cytokine-dependent immunoglobulin isotype switching in response to ligation by a soluble CD40 ligand (Armitage *et al.*, 1992a). To confirm the absence of CD40 in the mutant mice, I examined the capacity of B cells from these mice to proliferate in response to various stimuli.

B lymphocytes were purified from the spleens of wild-type and CD40 mutant mice using MACS (Magnetic Cell Sorting of Mouse Leukocytes) B220 (CD45R) microbeads and positive selection (RS+) columns as described in section 2.4.3. The resulting cell preparations were found to consist of greater than 95% pure B cells as determined by flow cytometric analysis (Becton Dickinson, CellQuest) of cell surface expression of B220, CD40, CD4 and CD8. Purified B lymphocytes from both wild-type and mutant

mice (3.5×10^5 B cells/well; 200 μl RPMI 1640) were then cultured for 72 hours with different stimuli (Figure 17.). Medium alone was used as a negative control (1.), LPS alone (2.), IL-4 alone (3.), LPS and IL-4 together (4.), CD40 monoclonal antibody alone (5.) and lastly CD40 monoclonal antibody with IL-4 (6.). Proliferation was measured by thymidine incorporation over a fixed time period (18-24 hours). 1 μCi (^{3}H)thymidine was added to each culture well for the last 18-24 hours of a 72 hour culture period and at the end of the 72 hour culture period the cells were harvested and counted on a Betaplate counter as described in section 2.4.3. B cells were purified from three wild-type and three mutant mice and stimulated as described above (Figure 17). The results shown are from three different mice in each group with each mouse tested in triplicate wells. The results represent the mean proliferation of the three mice and the error bars represent the standard error of this mean. The Student t-test was used to calculate whether the results for B cell proliferation were significantly different in the two groups of mice. A P value of 0.05 or below was taken to be significant. The results of this assay show that B lymphocytes from both sets of mice proliferated strongly when stimulated with LPS. A slight increase in B cell proliferation was observed, if LPS and IL-4 were added together, however no synergistic effect on B cell proliferation seemed to occur. The B cell proliferation induced by LPS is thought to occur via cross-linking of surface IgM, leading to activation. This is a T-independent antigen requiring no T cell help for activation of the B cell and as a result of this the proliferation is independent of a functional CD40 protein. B cells from both mutant and wild type mice were able to proliferate in response to LPS and LPS together with IL-4. Although LPS is a T independent antigen, there does seem to be a slight impairment of the ability of mutant B cells to proliferate in response to LPS (2. P=0.025, 4. P=0.036, 5 % significance level). This reduced *in vitro* proliferative response of mutant B cells to LPS as compared to wild-type B cells, was not observed by Kawabe *et al.* (1994). However, in support of the data shown here, Castigli *et al.* (1994) showed that in 2 out of 4 of their experiments, spleen cells from CD40 deficient mice exhibited a small reduction in proliferation when stimulated with LPS or LPS and IL-4 as compared with wild type mice. In addition,

Hasbold *et al.* (1994) demonstrated that stimulation of wild type mouse B cells with LPS led to substantial increases in the levels of surface expression of CD40, however it is still not known if CD40 is involved in B cell activation in response to LPS.

The CD40 monoclonal antibody used in this proliferation assay has been shown previously to induce proliferation in B lymphocytes (Klaus *et al.*, 1994) even when used as a soluble molecule. Other experiments with different antibodies found that optimal activation of B cells via CD40 required antibody cross-linking, or presentation of the antibodies on a matrix (Banchereau *et al.*, 1991). In the proliferation assays performed here, the CD40 monoclonal antibody was added directly to the cultures. Wild-type B cells were able to proliferate in response to addition of CD40 monoclonal antibody, however B cells from mutant mice showed very low levels of proliferation that were not much above background (5. $P<0.001$, 6. $P<0.01$). This confirmed that B lymphocytes from mutant mice were unable to proliferate in response to CD40 cross-linking as the CD40 molecule was absent.
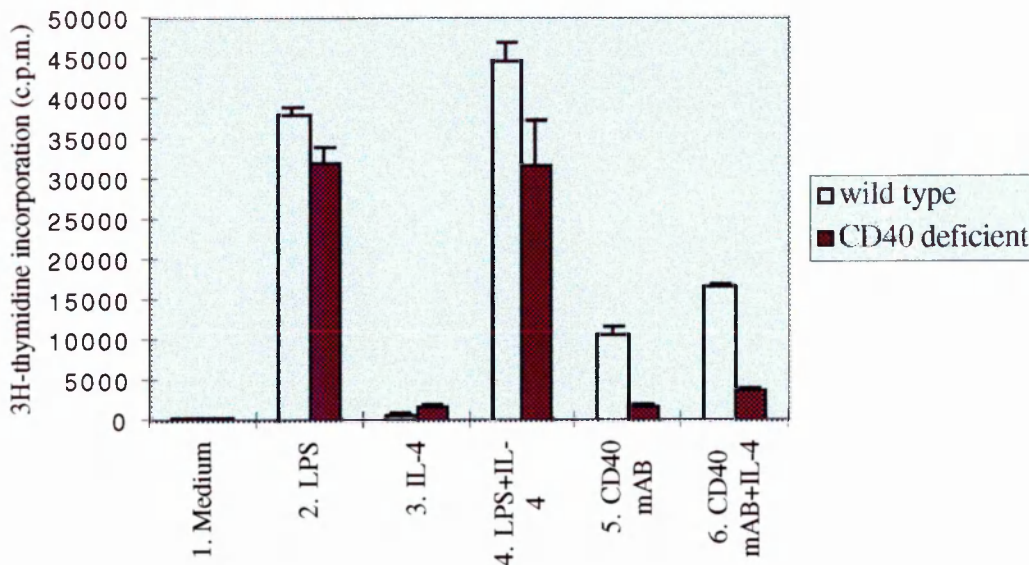
Figure 17. *In vitro* proliferative responses of B cells from wild-type mice and CD40 deficient mice measured by incorporation of (^{3}H)thymidine over a 18-24 hour period. LPS: Lipopolysaccharide, IL-4: Interleukin-4, CD40 mAB: CD40 monoclonal antibody, c.p.m.: counts per minute. B cells (3.5×10^5 cells/well; 200 µl) were cultured for 72 hours in 96 well round-bottom tissue culture plates in the presence of 2 µg/ml LPS, 50 U/ml IL-4, 25 µg/ml CD40 mAB. Cultures were pulsed with 1 µCi/well of (^{3}H)thymidine (Amersham) for the last 18-24 hours of a 72 hour culture period and then harvested onto glass fiber filters. (^{3}H)thymidine uptake was measured by a liquid scintillation counter (1205 Betaplate, Wallac). The values shown are means ± s.e.m. of 3 mice in each group.The P values for each comparison were determined using the Student's t-test. 1. P=0.96; 2. P=0.025; 3. P<0.01; 4. P=0.036; 5. P<0.001; 6. P<0.01. Results are representative of three experiments.

4.3 Germinal Centre formation in CD40 deficient mice

As described in more detail in the introduction, germinal centres are the anatomical sites in secondary lymphoid tissues where B cells undergo somatic hypermutation and immunoglobulin isotype switching and differentiate into plasma cells or memory cells (Kelsoe, 1996). The formation of germinal centres is initiated by interaction between interdigitating dendritic cells (IDC) presenting antigen to antigen specific CD4$^+$ T cells (Figure 2. A.). These activated CD4$^+$ T cells then associate with naive antigen specific B cells (MacLennan *et al.*, 1997). After this interaction between T and B cells, the activated T cells migrate into the light zone of the germinal centre or become recirculating effector- or memory-cells. Activated B cells differentiate into centroblasts forming the dark zone of the germinal centre. Centroblasts mature into centrocytes which are selected in the light zone of the germinal centre on the basis of their ability to bind and process antigen held on follicular dendritic cells (FDC) and also interact with activated germinal centre T cells. These T cells deliver a rescue signal via CD40L that allows centrocyte differentiation into plasma cells or memory B cells (MacLennan, 1994).

Patients suffering from hyper-IgM syndrome exhibit low levels of isotype switched immunoglobulins, IgG, IgA, and IgE, indicating a failure in the ability to switch from IgM to other immunoglobulins (Geha *et al.*, 1979; Levitt *et al.*, 1983). Affected males experience recurrent infections, usually within the 1st year of life, when levels of maternally-derived antibodies decline. More importantly it was found that these patients failed to form germinal centres. This was discovered to be caused by a defect in expression of CD40L or point mutations in CD40L (Korthauer *et al.*, 1993). Further evidence that blocking the CD40-CD40L interaction *in vivo* with soluble anti-CD40L antibody was able to abrogate germinal centre formation was described more recently in mice (Foy *et al.*, 1994). There is some controversy over the role of CD40-CD40L interaction in the formation of germnal centres as other experiments show that mice treated with a soluble CD40-Ig fusion protein, which should block the CD40-CD40L

interaction, developed germinal centres after immunization with a thymus-dependent antigen (Gray *et al.*, 1994). CD40 deficient mice will provide definitive evidence to support hypotheses showing that the CD40-CD40L interaction is important for the formation of germinal centres.

In the spleen, the splenic white pulp surrounds the arteries, forming a periarteriolar lymphoid sheath (PALS) consisting mainly of T lymphocytes. Closely associated with the PALS is a B cell rich area, called the marginal zone, containing primary lymphoid follicles. During antigenic stimulation, these primary follicles develop into secondary follices containing germinal centres (GC). Cellular interactions occuring in the germinal centre induce the B cell antigen receptor to mutate and switch isotype. Positive selection of B cells expressing high affinity receptors occurs, producing memory B cells and plasma B cells.

Currently the only way of determining if germinal centres are forming in primary follicles of secondary lymphoid tissues is by staining with peanut agglutinin (PNA). PNA is a plant lectin with specificity for terminal galactosyl residues which has been shown to bind germinal centre B cells (Kraal *et al.*, 1982; Rose *et al.*, 1980). In the experiments shown here, the formation of germinal centres was tested by immunizing wild-type and mutant mice with KLH in complete Freund's adjuvant, a T-dependent antigen. Ten days after immunization, spleens were removed and placed in embedding compound and snap-frozen in liquid nitrogen (section 2.4.4). Tissue sections were cut using a cryostat and then fixed in acetone, stained with primary antibodies or biotinylated PNA. The sections were then washed in TBS and enzyme-conjugated secondary antibodies added. Sections were washed once more and substrate added (Diaminobenzidine or Fast red) and incubated until the desired colour intensity had developed. Counterstaining was carried out using hematoxylin, which stains nuclei blue. The staining procedure is described in detail in section 2.4.4.

PNA staining of splenic tissue from wild-type and mutant mice is shown in Figure 18.A. top: wild-type; bottom: CD40-/-). In the wild-type mice primary follicles developed extensive germinal centres becoming secondary follicles. These germinal centres are composed of large clusters of actively proliferating B cells expressing high levels of PNA binding sites. Figure 18.A. (top: wild-type) shows a secondary follicle containing germinal centre B cells which stained brown, confirming that germinal centres (GC) do develop after antigenic challenge (KLH). Germinal centre B cells (GC), stained with PNA appear brown and lie in the middle of the lymphoid follicle. Biotinylated PNA binds germinal centre B cells, Avidin horseradish peroxidase conjugate then binds the biotin molecule and catalyses the oxidation of diaminobenzidene (substrate), resulting in a brown colour (Figure 18.A.). At the follicle periphery is a narrow zone of small lymphocytes called the mantle zone (Wheater's Functional Histology, A text and Colour Atlas) which also showed PNA staining. Staining of the mantle zone with PNA occured in both wild-type and mutant mice (Figure 18.A. top: wild-type; bottom: CD40-/-). Experiments performed by Foy on wild-type mice treated with blocking anti-CD40L antibody (Foy *et al.*, 1994) also exhibited PNA staining of the mantle zone. Furthermore, in CD40 deficient mice generated by Castigli, mantle zone staining was also seen (Castigli *et al.* 1994).

Staining of germinal centres in wild-type and CD40 deficient mice clearly showed (Figure 18.A. top: wild-type; bottom: CD40-/-) the absence of germinal centres in the CD40 deficient mice compared to wild-type mice. This was confirmed by double staining of splenic tissue with both PNA and with a monoclonal antibody specific for CD40 (Figure 18.B. top: wild-type; bottom: CD40-/-). Biotinylated PNA binds germinal centre B cells, Avidin alkaline phoshatase conjugate then binds the biotin molecule and catalyses the conversion of fast red, resulting in a red colour. The FITC-conjugated anti-CD40 mAb stains CD40 positive cells, anti-FITC horseradish peroxidase conjugate then binds the FITC molecule and catalyses the oxidation of diaminobenzidene (substrate), resulting in a black staining (the diaminobenzidene substrate can be enhanced from

brown to black by nickel chloride as shown in Figure 18. B. top: wild-type). Figure 18 B. (top: wild-type) shows a secondary follicle with germinal centre B cells, although less obvious than in Figure 18.A. (top: wild-type) The germinal centre B cells stained red with PNA (GC) and black (CD40 positive). In CD40 deficient mice, no clusters of germinal centre B cells were detected (Figure 18.B. bottom: CD40-/-). As shown in Figure 18.A. mantle zone lymphoid cells binds PNA in both wild-type and CD40 deficient mice (Figure 18. B. top: wild-type; bottom: CD40-/-).

The splenic architecture, as assessed by the distribution of T and B cells (apart from germinal centre B cells) were similar in both wild-type and CD40 deficient mice (Figure 18.C and D. top: wild-type; bottom: CD40-/-). In these experiments spleen sections were stained with a monoclonal antibody specific for CD79a to detect B cells and a CD3 mAb to detect T cells. The CD79a mAb binds the α–chain of the co-receptor of surface IgM. Rabbit anti-CD3 and rabbit anti-CD79a binds T and B cells respectively (Jones *et al.*, 1993). Goat anti rabbit horseradish peroxidase conjugate then binds the rabbit antibodies and catalyses the oxidation of diaminobenzidene (substrate) resulting in a brown colour (Figure 18. C. and D.). In these experiments I did not distinguish between resting and actively proliferating follicular B cells using anti-IgM, anti-IgD antibodies (as the B cells proliferate they lose surface expression of IgD).

In summary, wild-type mice, immunized with KLH displayed lymphoid follicles with germinal centres (secondary follicles). This was shown by intense staining of germinal centre B cells with PNA. In CD40 deficient mice, immunized with KLH, the lymphoid follicles resembled primary follicles with no recognizable germinal centres (Figure 18.B. bottom: CD40-/-). In occasional follicles, I observed faint staining of some cells with PNA (Figure 18.A. bottom: CD40-/-). This faint staining of cells with PNA in follicles was also observed by Castigli *et al.* (1994) in CD40 deficient mice.

If it is the case that the faint staining with PNA in CD40 deficient mice resembles germinal centre B cells, then one would have to assume that germinal centre B cells

become activated independently of the CD40-CD40L interaction with alternative costimulatory molecules signalling during B-T or B-B cell interactions, leading to proliferation and differentiation of germinal centre B cells. Such a candidate molecule could be the signaling lymphocytic activation molecule (SLAM), a 70 kD glycoprotein constitutively expressed on CD45RO+ memory T cells, immature thymocytes, and a proportion of B cells. SLAM is a member of the immunoglobulin gene superfamily and belongs to the CD2 family of cell surface molecules. Activated CD4+T cells express both a membrane-form of SLAM (mSLAM) and a soluble secreted form of SLAM (sSLAM). Engagement of SLAM by specific mAB or soluble SLAM (SLAM is a high-affinity self-ligand) induced proliferation of human activated CD4+ T cells in a CD28-independent fashion (Cocks *et al.,* 1995). In addition, the expression and function of SLAM on B cells was also investigated. The expression of mSLAM was significantly upregulated on both peripheral blood and splenic B lymphocytes when stimulated with anti-IgM mAb or anti-CD40 mAb. The most rapid effect was observed in response to anti-IgM mAb, which induced high levels of mSLAM expression on B cells after a 6 hour culture period. These data indicate that mSLAM is strongly upregulated on B cells after activation, and suggests that mSLAM is rapidly induced after recognition of antigen by surface Ig (Punnonen *et al.,* 1997). Like T cells, B cells are capable of producing a soluble form of SLAM (sSLAM). The addition of recombinant sSLAM to cultures of B cells resulted in B cell proliferation in a dose-dependent manner. sSLAM also strongly enhanced proliferation of B cells cultured in the presence of anti-IgM mAb or anti-CD40 mAb plus IL-4. Furthermore, sSLAM significantly enhanced IgM, IgG and IgA produced by B cells, but the effects of sSLAM were generally more potent when anti-CD40 mAb were added. These results indicate that SLAM-SLAM interactions enhance not only B cell proliferation, but also Ig synthesis by B cells (Punnonen *et al.,* 1997). I must point out that these experiments were performed on human T and B cells and that a murine form of SLAM has not yet been published. Nevertheless, one can speculate that, in CD40 deficient mice, T cells and B cells may become activated in a CD40-CD40L independent way in the T zone (PALS) (Figure 2. A.), via homophilic interaction

between SLAM-SLAM during B-T cell interactions. The fact that SLAM is rapidly induced after activation (anti-IgM mAb) and that high expression levels are sustained for several days suggest that SLAM-SLAM interactions may play a role in mediating homophilic B cell contacts in germinal centres resulting in expansion of antigen specific B cells. B cells activated through SLAM-SLAM interactions could then start to express PNA binding sites, explaining the faint staining in occasional follicles in the absence of CD40.

The observation that B cell activation after SLAM-SLAM interaction was enhanced by anti-CD40 mAb supports the view that fully developed germinal centres would only appear if a functional CD40-CD40L interaction was present. The results described in Figure 18. confirm the pivotal role of the CD40-CD40L interaction in the formation of germinal centres and also showed that no other splenic architecture was affected.
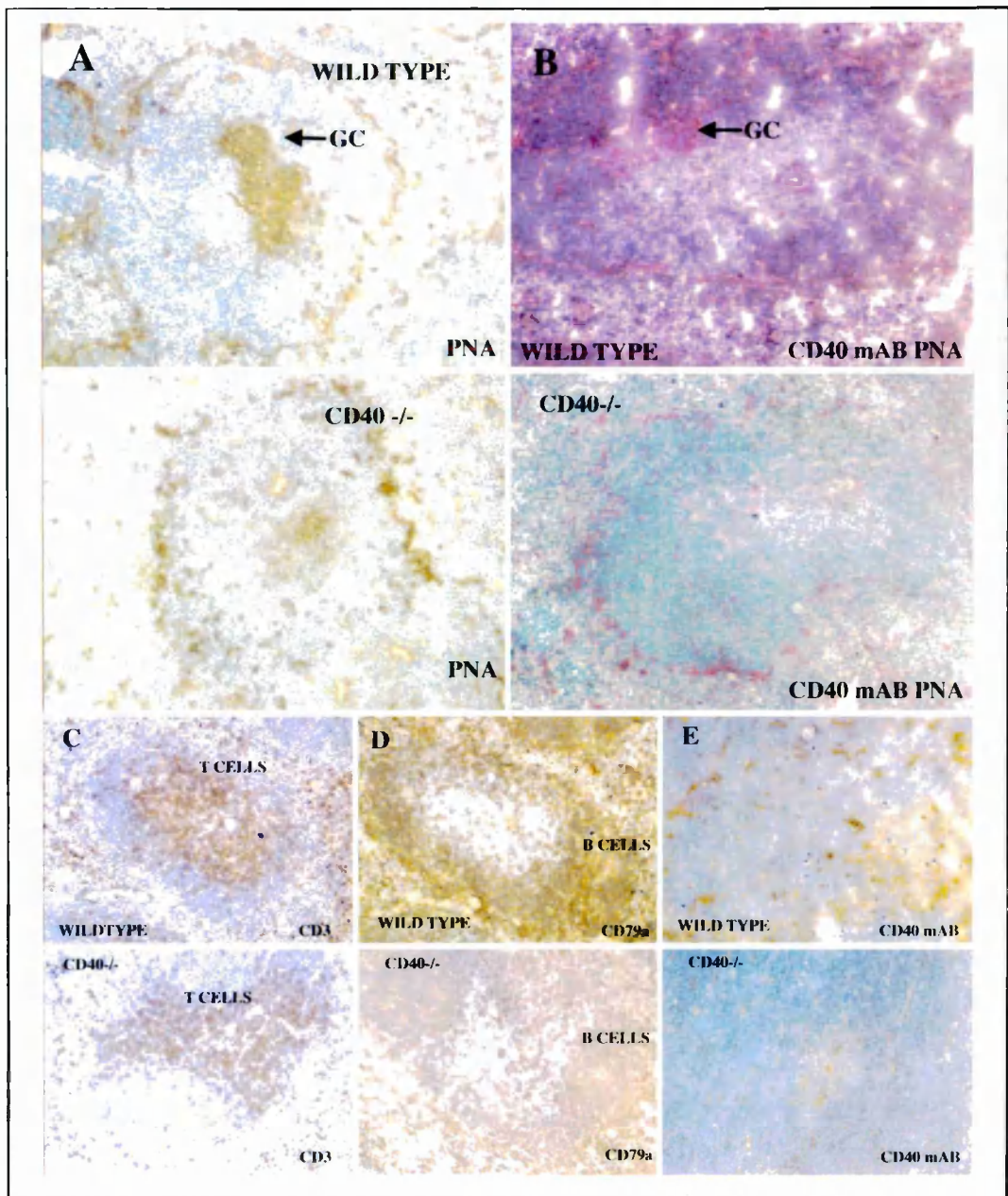
Figure 18. No development of germinal centres in CD40 deficient mice. Immunohistochemical staining of spleen tissue sections. Cryostat sections of spleens from KLH immunized wild-type and CD40 deficient mice. Sections were fixed in acetone, stained with primary antibodies or biotinylated PNA. The sections were then washed in TBS and enzyme-conjugated secondary antibodies added. Sections were washed once more and substrate added (Diaminobenzidine or Fast red) and incubated until desired colour intensity had developed. Counterstaining was carried out using hematoxylin, which stains nuclei blue. A.) Top: wild-type; bottom: CD40-/-. Staining of germinal centre (GC) with biotinylated PNA and Avidin horseradish peroxidase conjugate, germinal centres stain brown. Magnification 25x. B.) Top: wild-type; bottom: CD40-/-. Staining of germinal centres with biotinylated PNA and CD40mAB. Germinal centre B cells stain red and black. B cells positive for CD40 stain black. Magnification 25x. C. and D.) Top: wild-type; bottom: CD40-/-. Staining of T and B cell areas with a monoclonal antibody to CD3 and to CD79a, respectively. T and B cell areas are shown by the brown colour staining.

Magnification 10x. E.) Top: wild-type; bottom: CD40-/-. Cryostat sections of thymus tissue. Staining of CD40-positive cells in the thymus with anti-CD40 mAB. Magnification 10x.

4.4 Serum immunoglobulin levels in CD40 deficient mice

Class, or isotype, switching is the process whereby a B cell changes the heavy chain class of the antibody it synthesizes by changing the heavy chain constant (C_H) region expressed, but not the light chain or heavy chain variable region (V_H) regions. The change in antibody class, except to IgD, is effected by a DNA recombination event called switch recombination, which occurs between tandemly repeated sequences called switch regions. Switch recombination causes the recombined V(D)J gene segment, which is initially expressed with the C_μ gene, to be subsequently expressed with one of six (mouse) or seven (human) downstream C_H genes (Stavnezer, 1996a). Because antibody specificity is determined by the variable regions of antibodies, class switching does not change the antigen binding specificity. However, class switching does change the effector function of the antibody because these are determined by the C_H regions. Soon after emerging from the bone marrow, immature B cells which express IgM on their surface also start to express surface IgD, with the same V_H region as the IgM. This event occurs in immature B cells in the absence of antigen stimulation (Stavnezer, 1996a). T-dependent antigens induce class switching during cognate interactions between T and B cells in the T cell zone of the spleen (Toellner *et al.*, 1996) and class switching can also be triggered in centrocytes when they interact with T cells in the light zone of germinal centres (Liu *et al.*, 1996). T-independent antigens induce class switching, probably by signals from surface Ig cross-linked by multivalent antigens. T-independent antigens do not stimulate germinal centre formation within follicles of lymphoid tissue. Thus B cells in which class switching is induced by T-independent antigens may be located in the splenic marginal zone (Mond *et al.*, 1995). In addition to the absence of germinal centres in individuals with hyper-IgM syndrome it was also found that the patients had elevated or normal levels of IgM and low levels of isotype switched immunoglobulins, IgG, IgA, and IgE. This indicated a failure in the ability to switch from IgM to other immunoglobulins (Geha *et al.*, 1979; Levitt *et al.*, 1983).

To determine the influence of a non-functional CD40-CD40L interaction on the production of isotype switched immunoglobulins in CD40 deficient mice, serum was taken from 12 week old wild-type and also from CD40 deficient mice and the levels of the different immunoglobulin isotypes in the two groups were compared. Immunoglobulin levels were determined by isotype-specific ELISA as described in section 2.4.6. These results are shown in Figure 19 and represent the mean immunoglobulin levels found in five wild-type and five CD40 deficient mice. The error bars represent the standard error of this mean. The Student's t-test was used to calculate whether the results for each immunoglobulin isotype were significantly different in the two groups of mice. A P value below 0.05 was taken to be significant.

Elevated levels of IgM and IgG3 were found to occur in the mutant mice compared to the wild-type mice (1. P<0.02; 5. P=0.037). In contrast to this, levels of IgG1 and IgG2b were significantly reduced in CD40 deficient mice compared with wild-type mice (2. P<0.001, 4. P<0.001). The levels of IgE expression in wild-type mice were extremely low and were only just detectable by ELISA. In the mutant mice, IgE levels were undetectable. Unexpectedly, the levels of IgG2a were not significantly different in the two groups of mice (3. P=0.36), and this expression was very low in both wild-type and mutant mice.

CD40 deficient mice exhibited elevated levels of IgM, confirming the findings in patients suffering from hyper-IgM syndrome who may also have elevated levels of IgM (Notarangelo *et al.,* 1992). The presence of elevated levels of IgM and IgG3 in CD40 deficient mice could be due to stimulation with T cell independent antigens, as it was found that IgM and IgG3 were the major isotypes of antibodies in T cell independent responses (Mongini *et al.,* 1981). Serum IgE was absent and significantly reduced levels of IgG1 and IgG2b were found in CD40 deficient mice. The presence of elevated levels of IgG3 and normal levels of IgG2a indicates that isotype switching may also occur independently of the CD40-CD40L interaction. See "Discussion" for immune responses to T cell independent antigens in CD40 deficient mice.

The results presented here (Figure 19.), investigating the serum immunoglobulin levels in unimmunized CD40 deficient mice, were compared to the results obtained in similar studies of CD40 deficient mice by Kawabe *et al.,* 1994 and also by Castigli *et al.,* 1994. Kawabe *et al.* observed normal IgM levels and higher IgG3 levels in CD40 deficient mice compared to control mice. Serum levels of IgG1, IgG2a, IgG2b and IgA were significantly reduced (Kawabe *et al.,* 1994). Castigli *et al.* also found that the IgM levels in CD40 deficient mice were similar to wild-type mice. Serum levels of IgG1 and IgG2a were significantly reduced and IgE was absent. Normal levels of IgG3 and slightly reduced levels of IgG2b and IgA were observed (Castigli *et al.,* 1994). There were slight differences in the levels of the immunoglobulins measured in these studies however, the general trends in immunoglobulin production in CD40 deficient mice (reduced isotype switched immunoglobulins) were the same. The slight differences in the levels of serum immunoglobulins seen in these studies compared with the results presented in this thesis could be due to the different conditions in which these CD40 deficient mice were kept and the different pathogens that they may be exposed to.

The normal or elevated levels of serum IgM that were seen in patients with hyper IgM syndrome and also in CD40 deficient mice indicates that IgM antibody responses in both systems were independent of the CD40-CD40L interaction. The reduced levels of isotype switched immunoglobulins in the serum of CD40 deficient mice suggests that the CD40-CD40L interaction is important for the isotype switching from IgM to other immunoglobulins.

4: Analysis of CD40 deficient mice
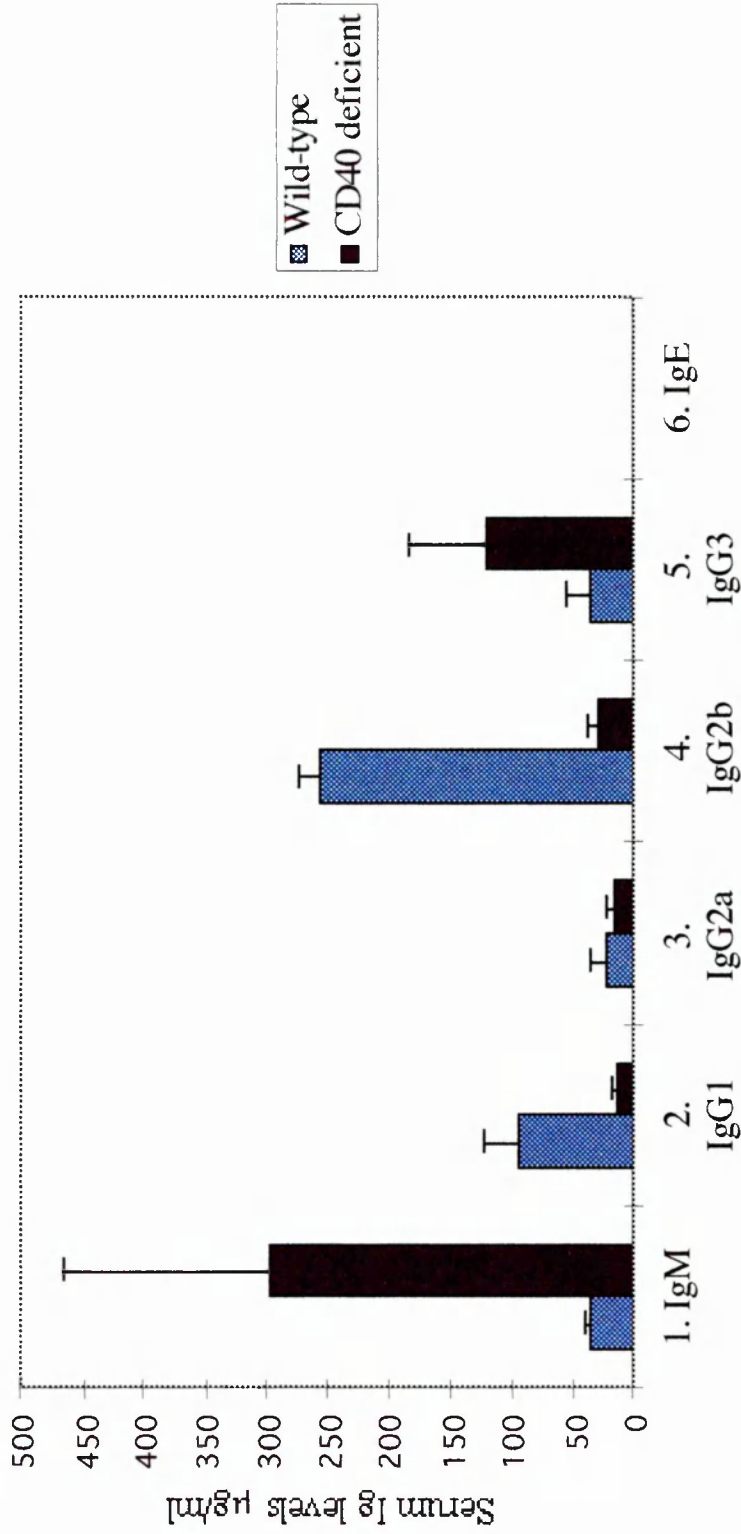
Figure 19. Serum levels of immunoglobulin isotypes. Mice were bled at 12 weeks of age and serum isolated. Serum immunoglobulin isotype levels were determined by isotype-specific ELISA. Concentration of serum immunoglobulin is given in µg/ml. The values shown are means ± s.e.m. of 5 mice in each group. The P values for each comparison were determined using the Student's t-test. 1. P<0.02; 2. P<0.001; 3. P=0.36; 4. P<0.001; 5. P=0.037. The mean of the serum IgE level in wild-type mice was 0.246 µg/ml. Serum IgE was undetectable in CD40 deficient mice.

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Chapter 5: Cell-mediated immunity in CD40 deficient mice infected with *bacillus Calmette-Guerin*

5.1 Introduction

5.1.2 CD40-CD40L interaction in Th1 development

Patients having hyper IgM syndrome experience recurrent infections, usually within the 1st year of life, after levels of maternally-derived antibodies have declined. Most infections are of bacterial origin, but HIGM1 Patients are unusually susceptible to infections with opportunistic pathogens and often suffer from *Pneumocystis carinii* pneumonia and *Cryptosporidium* intestinal infection. These diseases are often observed with T-cell immunodeficiencies but not with forms of hypogammaglobulinemia other than hyper IgM syndrome (Notarangelo *et al.*, 1992). This observation led researchers to ask whether the interaction between CD40-CD40L was involved in T cell-mediated immunity? It has been established that IL-12 is a key cytokine for the development of a Th1 response as it induces the production of IFN-γ in NK cells and T cells (Trinchieri, 1995). Results suggest that the CD40-CD40L interaction is important for the priming of Th1 T cells via the stimulation of IL-12 secretion by antigen presenting cells. CD40 cross-linking on human dendritic cells by CD40L upregulated the expression of CD80, CD86 and CD54, leading to an increased capacity of DCs to stimulate proliferation of autologous or allogeneic T cells and also of IFN-γ production by these T cells. In addition, secretion of high levels of IL-12 by human DCs was induced upon CD40 cross-linking (Cella *et al.*, 1996; Peguet *et al.*, 1995). A similar study, this time with murine dendritic cells showed, that CD40 cross-linking induced IL-12 production by the dendritic cells, and that IL-12 production could be downregulated by IL-4 and IL-10 (Koch *et al.*, 1996). IL-12 production by human monocytes could be triggered by activated T cells and this could be inhibited by a soluble CD40 (CD40-Ig) antibody blocking the CD40-CD40L interaction (Shu *et al.*, 1995). An *in vivo* model of colitis (induced by the hapten reagent (2,4,6-trinitrobenzene sulfonic acid (TNBS)) is a Th1

mediated disease. This was shown by the fact that the majority of infiltrating CD4^{+} T cells in this condition secreted IFN-γ. Treatment with anti-CD40L antibody during the induction of the disease was able to prevent colitis occurring and a reduction in the levels of IL-12 was also observed (Stuber *et al.*, 1996). In addition, CD40 cross-linking on dendritic cells induced the production of TNF-α, IL-8 and macrophage inflammatory protein (MIP-1α). The production of cytokines such as IL-8 and MIP-1α by dendritic cells may be important for the recruitment of cells to a site of inflammation (Stout & Suttles, 1996). A direct role for CD40-CD40L interactions in the induction of nitric oxide (NO) production by macrophages is indicated by the ability of anti-CD40L antibody to inhibit the induction of nitric oxide by fixed activated lymphocytes (Tian *et al.*, 1995). This was supported by the observation that T cells from CD40L deficient mice were impaired in their ability to induce macrophages to produce TNF-α and nitric oxide (Stout *et al.*, 1996).

5.1.3 Impaired T cell priming in CD40 and CD40L deficient mice

Two reports have described the impairment of antigen-specific T cell priming in CD40 and CD40L deficient mice. To determine whether CD40L influences T cell responsiveness, CD40L deficient mice and wild-type mice were immunized with KLH and T cell *in vitro* recall proliferative responses were tested. The *in vitro* proliferative responses of T cells from CD40L deficient mice were considerably reduced compared to wild-type mice. Furthermore, a dramatic reduction in IL-4 and IFN-γ production was seen in proliferative responses with T cells from CD40L deficient mice. The reduced response in CD40L deficient mice could either be caused by insufficient activation of the antigen presenting cells (APCs), no expression of costimulatory molecules (CD80, CD86), or a defect in the T cells. To test whether the APCs were causing the defect in T cell activation, APCs from wild-type mice were used to activate T cells *in vitro* from CD40L deficient mice immunized with KLH. Neither wild-type APCs nor lipopolysaccharide (LPS) activated B cells, which express costimulatory activity and thus might bypass a CD40L requirement, were able to restore the defect in proliferation.

Whether APCs are intrinsically defective in CD40L deficient mice, was tested by adoptive transfer of wild-type T cells (CD4^{+}) to CD40L deficient mice. Following immunization of the CD40L deficient mice with KLH, these transferred wild-type T cells responded vigorously to KLH. Furthermore, the proliferative response of CD40L deficient T cells and wild-type T cells is similar when stimulated with anti-CD3 or concanavalin A, confirming that CD40L deficient T cells do not have an intrinsic defect. These observations suggest that a signal through CD40L to the T cell is necessary for *in vivo* priming of T cells and that the reduced proliferation in CD40L deficient mice is not due to a defect APC activation or intrinsic T cell defect in CD40L deficient mice (Grewal *et al.*, 1995). Another study showed that simultaneous cross-linking of CD3 and CD40L on human CD4^{+} T cells upregulated IL-4 synthesis. This indicated that CD40L might serve as a receptor which transduces a costimulatory signal to the T cell (Blotta *et al.*, 1996).

To test the ability of T cells primed in the absence of CD40 to provide T cell help for B cells, CD40 deficient mice and wild-type mice were immunized with KLH. Purified T cells from these mice were then injected into lightly irradiated CD40 deficient mice (hosts) with B cells expressing CD40 (wild-type B cells) from non-immune IgH[a] congenic donors. These allotype-distinct "indicator" B cells enable the donor/recipient source of serum antibodies to be determined. Adoptive hosts were immunized with dinitrophenylated KLH (DNP-KLH). Ten days after cell transfer, the mice that had received T cells primed in a wild-type (CD40+/+) mouse produced anti-DNP IgM, IgG1, IgG2a and IgG2b antibodies and formed germinal centres in their spleens. Mice that received T cells primed in CD40 deficient mice produced only IgM antibodies and exhibited no splenic germinal centres. This indicates that T cells primed in the absence of CD40 are unable to provide the help required for normal B cells to class switch or to form germinal centres (van *et al.*, 1995).

5.1.4 Leishmaniasis in CD40 and CD40L deficient mice

Mice deficient for CD40 and CD40L have been used to establish that the CD40-CD40L interaction is required for protective immunity to the intracellular protozoan, *Leishmania Major*. This parasite infects macrophages and the severity of disease in different mouse strains infected with L. major is genetically determined. In the resistant strains such as C57BL/6 and CBA, Th1 effector T cells are generated upon infection, whereas Th2 responses are generated in the susceptible strain BALB/c (Heinzel *et al.*, 1993). It has been shown that Th1-derived IFN-γ and Th2-derived IL-4 play a role in protection from and exacerbation of experimental leishmaniasis, respectively (Sadick *et al.*, 1990). These results were confirmed by the fact that experimental leishmaniasis could not be controlled in IFN-γ deficient mice, which succumbed upon infection with *L. major* (Wang *et al.*, 1994). Another important cytokine in the control of *L.major* infection is IL-12. *In vivo* IL-12 treatment can cure BALB/c mice of *L.major* infection by changing a Th2 response to a Th1 response (Heinzel *et al.*, 1993). The infection of CD40 deficient and CD40L deficient mice with *Leishmania major* provided a means of determining the mechanisms involved in the production of IL-12 and generation of Th1 responses to the parasite and also which molecules other than IFN-γ were required for activation of macrophages. CD40L deficient mice bred onto a resistant background (C57BL/6), control C57BL/6 (resistant) and BALB/c (susceptible) mice were infected with *Leishmania major*. CD40L deficient mice were susceptible to infection with *L. major*, and like the susceptible strain BALB/c, developed ulcerating lesions within 7 weeks of parasite challenge. The levels of IFN-γ, IL-4 and IL-12 were measured. Lymph nodes cells taken from CD40L deficient mice secreted low levels of IFN-γ when stimulated with a soluble leishmanial antigen *in vitro*, compared to control mice. The levels of IL-4 in CD40L deficient mice were comparable to susceptible BALB/c mice. Furthermore, T cells from CD40L deficient mice failed to induce IL-12 production by macrophages when stimulated with soluble leishmanial antigen *in vitro*. Complete protection from *Leishmania major* in CD40L deficient mice could be obtained by intraperitoneal injection of recombinant IL-12

(Campbell *et al.*, 1996). A similar study, where CD40 deficient mice were infected with *Leishmania major* showed that CD40 deficient mice developed systemic leishmaniasis. Lymph node cells from infected CD40 deficient and BALB/c mice produced significantly higher levels of IL-4 in response to *L. major* antigens than C57BL/6 (controls) mice. Cells from resistant C57BL/6 mice produced high levels of IFN-γ and in contrast, only low levels of IFN-γ were produced by CD40 deficient mice in response to *L. major* antigens. As shown for CD40L deficient mice infected with *L. major*, lymph node cells from CD40 deficient mice infected with *L. major* showed levels of IL-12 production that were reduced considerably compared to C57BL/6 mice and the IL-12 secretion was even lower than in BALB/c mice.

Nitric oxide produced by activated macrophages has been implicated as one of the effector molecules involved in killing Leishmania parasites (Liew *et al.*, 1990; Wei *et al.*, 1995). The activation of macrophages to exert antiparasitic responses such as nitric oxide production was investigated in CD40L deficient mice infected with *Leishmania amazonensis*. Unlike *L. major*, where selected strains of mice are resistant to infection, most inbred strains of mice are susceptible to *L. amazonensis*. Infection of CD40L deficient mice with *L. amazonensis* resulted in a tissue parasite burden 50-fold higher than in the wild-type (C57BL/6) mice, and CD40L deficient mice failed to generate parasite specific immune responses. Lymph node cells from CD40L deficient mice, stimulated with parasite lysates for 72 h in vitro, failed to produce IFN-γ, TNF-α and lymphotoxin in response to parasite antigen. Moreover, macrophages isolated from CD40L deficient mice did not produce any significant levels of nitric oxide after stimulation with parasite antigen, whereas macrophages from the control mice produced high levels of nitric oxide. Unlike the studies cited above, infection with *L. amazonensis* showed that the production of IL-12 in CD40L deficient mice was comparable to wild-type mice. One of the major contributing factors to the diminished resistance to *L. amazonensis* in CD40L deficient mice is probably the reduced capacity of macrophages to exert antiparasitic responses such as nitric oxide production (Soong *et al.*, 1996).

The CD40-CD40L interaction appears to be important for the induction of IL-12 and the production of nitric oxide (NO) by activated macrophages in response to infection with Leishmania species. A possible sequence of events could be that during infection of resistant strains of mice, APCs present leishmanial antigens to T cells, resulting in activation-induced expression of CD40L. CD40L positive T cells, possibly in combination with IFN-γ, interact with CD40 on APCs and induce IL-12 production (Koch *et al.*, 1996; Shu *et al.*, 1995). IL-12 then stimulates NK cells and CD4$^+$ T cells to secrete IFN-γ, which drives CD4$^+$ T cells towards a Th1 phenotype (Trinchieri, 1995). Simultaneously, CD40L positive T cells would interact with macrophages via CD40 and induce the expression of nitric oxide, thereby increasing the microbicidal capacity of the macrophages (Stout *et al.*, 1996; Tian *et al.*, 1995).

5.1.5 Immune response to Mycobacterial infection

Mycobacterium tuberculosis and *M. bovis* are intracellular bacteria capable of persisting and replicating within resting macrophages. The precise mechanisms by which the cell-mediated immune response operates to effectively contain the bacillus are not fully understood. The requirement of both CD4$^+$ and CD8$^+$ T cells in the protective immune response to *M. tuberculosis* has been established. The *in vivo* depletion of either CD4$^+$ or CD8$^+$ T cells in mice (C57BL/6) reduced resistance to *M. tuberculosis* infection, whereas intact control mice were able to clear the infection (Muller *et al.*, 1987; Orme *et al.*, 1992). However, the contributions of the different components of the T cell response are unclear. Patients with human immunodeficiency virus (HIV) have reduced CD4$^+$ T cell counts and CD4$^+$ T cells are thought to play a major role in controlling infections with *M.tuberculosis,* as HIV patients are more susceptible to *M. tuberculosis* infections than the non-HIV infected population (Hopewell, 1992). β2-microglobulin deficient mice which lack an efficient CD8$^+$ T cell response, also show increased susceptibility to *M. tuberculosis* infection (Flynn *et al.*, 1992). Several groups have shown, using gene-deleted mice, that nitric oxide, IFN-γ, TNF-α and IL-12 are all essential for development of resistance to *M. tuberculosis*. Mice deficient for nitric oxide synthase (NOS2), the

gene producing nitric oxide, succumbed to *M. tuberculosis* infection within 50 days, whereas control (C57BL/6) mice survived the infection. The lack of NOS2 did not have any effect on granuloma formation and levels of IFN-γ and TNF-α, as they appeared similar in control and NOS2 deficient mice (MacMicking *et al.*, 1997). IFN-γ deficient mice succumbed to infection with *M. bovis* (BCG) and the defect in IFN-γ production led to an impaired production of nitric oxide by macrophages in response to *M. bovis* (Dalton *et al.*, 1993). Upon infection with *M. tuberculosis*, IFN-γ deficient mice developed granulomas but they failed to produce nitric oxide and eventually succumbed, due to the unrestricted growth of bacilli. Recombinant IFN-γ was injected intramuscularly in an attempt to rescue IFN-γ deficient mice from unrestricted growth of bacilli, but the treatment only delayed and did not prevent the fatal outcome of *M. tuberculosis* infection (Cooper *et al.*, 1993; Flynn *et al.*, 1993). The role of TNF-α was investigated by infecting BALB/c mice with BCG (*bacillus Calmette-Guerin*). Upon infection with BCG the mice developed granulomas and the production of TNF-α was shown to coincide with these granulomas. *In vivo* treatment with anti-TNF-α antibody prevented the development of granulomas and led to accumulation of TNF mRNA in BCG infected mice, leading to massive replication of BCG. This study indicates that TNF-α is essential for granuloma formation, which is thought to be required for localization and control of mycobacterial replication in tuberculosis (Kindler *et al.*, 1989). In addition to the role of TNF-α in granuloma formation, TNF-α with IFN-γ, induces the production of microbicidal reactive nitrogen intermediates (RNI) by macrophages, one of which is nitric oxide (Flesch & Kaufmann, 1990). The only demonstrated mechanism *in vitro* by which macrophages kill *M. tuberculosis* bacilli is activation by IFN-γ and LPS or TNF-α to produce RNI (Flesch & Kaufmann, 1991). The use of mice lacking the TNF receptor confirms the role of TNF-α in protection against *M. tuberculosis*. TNF receptor deficient mice succumb to *M. tuberculosis* infection within 30 days, whereas the control mice survive the duration of the experiment. After 2 weeks of infection the TNF receptor deficient mice contained 10- to 50-fold more bacilli in the organs, compared with the control mice. Granuloma formation

did occur in the TNF receptor deficient mice and equivalent numbers of granulomas were found in TNF receptor deficient mice and control mice, 12 days postinfection. However, the onset of granuloma formation in TNF receptor deficient mice was slightly delayed. Likewise, the production of RNI was delayed in TNF receptor deficient mice until 14 days postinfection although once again levels of RNI production were similar to those observed in wild-type mice. The same results were obtained in mice, where TNF-α was neutralized *in vivo* by an anti-TNF-α antibody (Flynn *et al.*, 1995a). This conflicts with the previous report (Kindler *et al.*, 1989) where neutralization of TNF *in vivo* by antibody to TNF-α abolished granuloma formation following infection with BCG. The reason for the discrepancy is unknown. Clearly, TNF-α is required early in infection to limit replication of bacilli within the organs and the way that this replication is limited is through the production of RNI. Complete absence of TNF-α or TNF signalling (TNF receptor) within the two first weeks of a mycobacterial infection was found to be fatal (Flynn *et al.*, 1995a).

Finally, the role of IL-12 in resistance to *M. tuberculosis* infection was investigated. C57BL/6 mice normally survive more than 140 days when infected with *M. tuberculosis*, where as BALB/c mice are susceptible to the infection and only survive around 58 days (Flynn *et al.*, 1995b). When BALB/c mice were given recombinant IL-12 at the initiation of infection with *M. tuberculosis*, their mean survival time doubled from 58 to 112 days. IL-12 treated mice had diminished bacterial burdens, whereas treatment with recombinant IFN-γ had no effect on survival or bacterial burden. Treatment of IFN-γ deficient mice with recombinant IL-12, did not increase survival, indicating that IL-12 does not induce protection against tuberculosis in mice in the absence of IFN-γ. This supports the hypothesis that IL-12 increases resistance to tuberculosis by inducing IFN-γ production from NK and/or T cells (Cooper *et al.*, 1995; Flynn *et al.*, 1995b).

Tuberculosis is the world's leading cause of death in humans from a single infectious agent. *Mycobacterium tuberculosis* currently infects 2 billion people worldwide and

causes 8 million new cases of tuberculosis and 2.9 million deaths annually (Kochi, 1991). The disease is a major health problem in developing countries and has reemerged in recent years in many industrialized countries. This has been linked to the spread of the human immunodeficiency virus (HIV) and to a combination of social and economic factors (Fenton & Vermeulen, 1996). The only currently available vaccine, *bacillus Calmette-Guerin* (BCG) does not provide protection against *M. tuberculosis* infection in all individuals (Fine, 1989) and the lack of an effective vaccine is at least in part responsible for the reemergence of tuberculosis in industrialized countries. However, the development of an effective vaccine against *Mycobacterium tuberculosis* has been hampered by a poor understanding of the immunlogical mechanisms of protection and the pathogenesis of this disease. It is therefore important to investigate the precise mechanisms by which the cell-mediated immune response operates to contain and eliminate a mycobacterial infection. As resistance to both tuberculosis and leishmaniasis was associated with production of similar CD40-CD40L-associated mediators of immunity, including IFN-γ, IL-12, TNF-α and nitric oxide (NO), this study was carried out to assess the role of the CD40-CD40L interaction in the induction of cell-mediated immunity and control of *Mycobacterium tuberculosis.*

Performing experimental work with *Mycobacterium tuberculosis* requires category 3 facilities which were not available in our department, therefore I chose to use *Mycobacterium bovis* (BCG) as a model system for *Mycobacterium tuberculosis.*

5.2 Results

5.2.1 Survival of CD40 deficient mice infected with BCG

To investigate the role of CD40 in resistance to BCG (*bacillus Calmette-Guerin*) I compared the course of BCG infection in CD40 deficient mice (CD40-/-) and in sex- and age-matched heterozygous (CD40+/-) littermates. Heterozygous littermates (CD40+/-) were used as controls because the CD40 deficient mice were backcrossed onto the C57BL/6 background for 2 generations (129/Sv x C57BL/6)F_2. This only gave

homozygosity on 75% of all alleles, therefore the degree of homozygosity had to be the same in the control mice (CD40+/-) as in the CD40 deficient mice. Five 10 week old male mice were used in the CD40-/- group and in the CD40+/- control group. Mice were infected intravenously (tail vein) with 1×10^7 colony-forming units (CFU) of *bacillus Calmette-Guerin* and three parameters of resistance were analyzed, survival, weight gain or loss and bacterial load in the spleen, liver and lungs. The health of the mice in each group was assessed by monitoring the weight loss or gain regularly throughout the infection, shown in Figure 20. The weight of the mice on each day of measurement is presented as the percentage weight gain as compared with the day zero levels (day zero is the first day of infection). There was no net weight loss during the course of infection in either group of mice, with both groups putting on weight during the 256 day observation period and surviving for the entire observation period.

An initial period of 11 days with equal percentage increase, followed by a decrease in percentage weight gain for the CD40 deficient mice compared to the control mice (day 11-20). This is followed by a period of equal increase in percentage weight gain. Unfortunately, the mice were not weighed individually and therefore the values for the standard error of the mean cannot be given for each percentage increase.

There is a difference in weight, with the CD40 deficient mice being slightly heavier. This weight difference may be due to differences in the genetic backgrounds of individual mice. The CD40-/- mutation was generated in ES cells derived from a 129 genetic background and injected into a blastocyst with a C57BL/6 genetic backround. This results in mutant mice with a mixture of 129 and C57BL/6 genetic backgrounds. These mutant mice (129/C57BL/6) will be backcrossed onto a C57BL/6 genetic background, but backcrossing has to (selecting for the mutation) have taken place for 10 generations in order to achieve 99.91 % homozygosity of alleles. Therefore, the lower the number of backcrosses the more genes are expected to segregate and any variations seen in the weight may be due to differences in the genetic backgrounds of individual mice.

The bacterial load in the spleen, liver and lungs was determined in both groups of mice, 4 weeks after infection. Spleen, liver and lungs were removed into sterile PBS and homogenized as described in section 2.4.8, followed by plating out onto Middlebrook agar plates. These plates were incubated for 2-3 weeks at 37 °C. Middlebrook agar selects for growth of mycobacteria. The number of mycobacteria are presented as colony forming units (CFU, defined as bacterial colonies) and these were counted after 2-3 weeks of culture. The results represent the mean of CFU from six mice in each group and the error bars represent the standard error of this mean. The Student t-test was used to calculate whether the results for CFU counts were significantly different in the two groups of mice. A P value of 0.05 or below was taken to be significant. The results of the CFU counts are shown in Figure 21.

The number of BCG CFU recovered from 1. spleens, 2. lungs and 3. livers of heterozygous control mice (CD40+/-) were compared with those recovered from CD40 deficient mice (CD40-/-). The results are presented in Figure 21. CD40 deficient mice had a significantly higher parasite burden in the spleens $(6.78 \times 10^3 \pm 0.90 \times 10^3)$ compared to control mice $(2.96 \times 10^3 \pm 1.09 \times 10^3)$ (P<0.001). The CFU counts in the lungs of CD40 deficient mice $(6.60 \times 10^3 \pm 2.08 \times 10^3)$ compared to the control mice $(2.80 \times 10^3 \pm 0.96 \times 10^3)$ were also significantly different (P<0.001). The CFU counts in the livers of CD40 deficient mice $(10.77 \times 10^3 \pm 6.15 \times 10^3)$ compared to control mice $(7.23 \times 10^3 \pm 3.16 \times 10^3)$ appear different in Figure 21., but a Student's t-test revealed that this difference was not significant (P=0.27). These results show that CD40 deficient mice have raised parasite burdens in the spleen and lungs, suggesting that CD40 deficient mice, despite long-term survival (256 days), are more susceptible to infection with BCG than control mice.
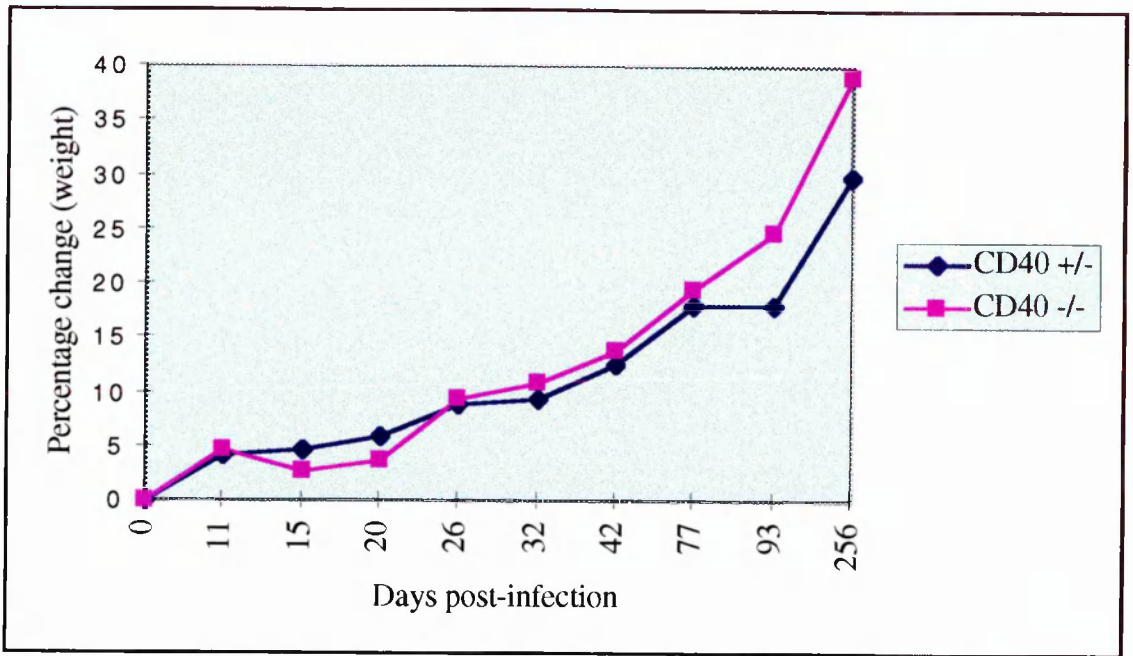
Figure 20. Percentage weight gain of heterozygote littermate controls (CD40+/-) and CD40 deficient mice infected with *bacillus Calmette-Guerin* (BCG). The weight gain is calculated as a percentage of the weight at the start of the experiment. CD40-/- (deficient) and CD40+/- (control) mice were infected i.v. with 1×10^7 CFU of *bacillus Calmette-Guerin* and the course of infection, survival and weight, were monitored for 256 days. There were five mice in each group and the weight is the cumulative weight of 5 mice.
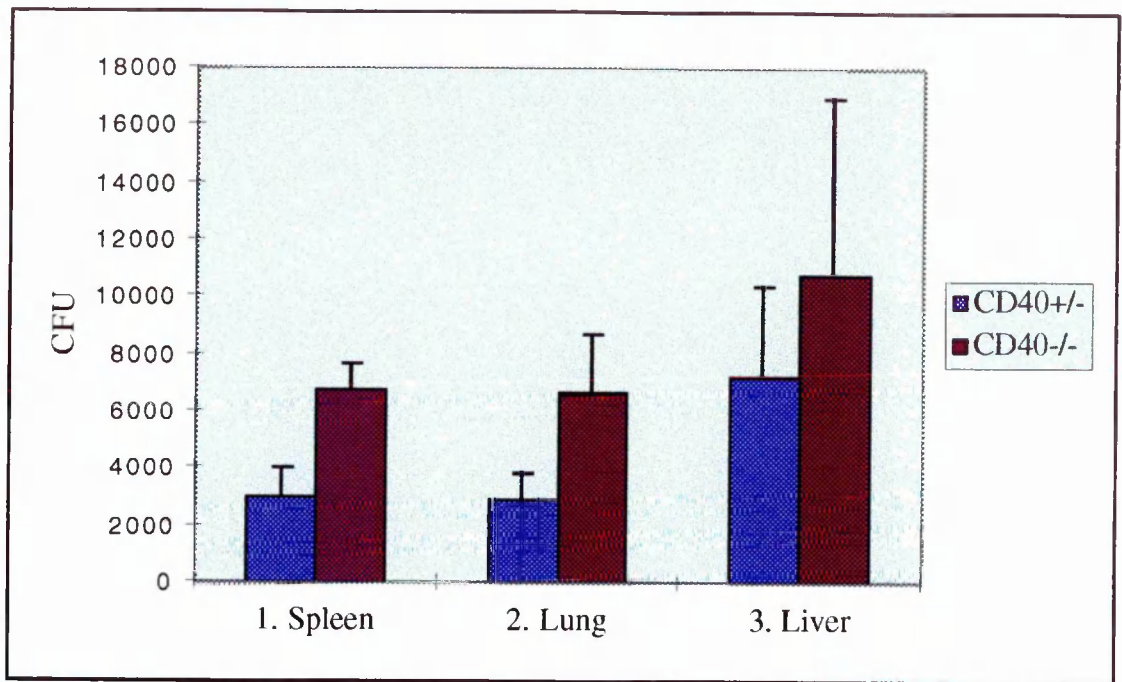
Figure 21. Bacterial burdens in *bacillus Calmette-Guerin* infected CD40+/- control mice and CD40-/- mice. CFU (colony-forming units) in 1. Spleen, 2. Lung and 3. Liver were determined 4 weeks after infection. The values shown are means of CFU counts ± s.e.m. of 6 mice in each group. The P values for each comparison were determined using the Student's t-test. 1. Spleen: $P < 0.001$; 2. Lung: $P < 0.001$; 3. Liver: $P = 0.27$.

5.2.2 IFN-γ production in CD40 deficient mice infected with BCG

The CD40-CD40L interaction has been directly implicated in pathways for the production of IL-12, nitric oxide and macrophage activation (Shu *et al.*, 1995; Stout *et al.*, 1996; Tian *et al.*, 1995). In addition, the enhanced susceptibility of CD40L and CD40 deficient mice to Leishmaniasis was associated with an impaired generation of a Th1 response, reflected by the production of low levels of IFN-γ, TNF, IL-12, and nitric oxide (Campbell *et al.*, 1996; Kamanaka *et al.*, 1996; Soong *et al.*, 1996). In view of these observations I infected CD40 deficient mice and control mice with BCG and then measured the induction of IFN-γ production in response to PPD (Tuberculin Purified Protein Derivative) *in vitro*. IFN-γ was detected using an ELIspot assay. The ELIspot assay relies on an anti-IFN-γ antibody which is bound to the bottom of a 96-well tissue culture plate. Cells and antigen (PPD) are then added and incubated overnight. Cells recognizing the antigen will secrete IFN-γ, which will be bound by the anti-IFN-γ antibody. The cells are then removed and bound IFN-γ can now be recognized with a secondary antibody coupled to alkaline phosphatase. A substrate is added and the alkaline phosphatase then converts this substrate to produce a coloured compound. A series of dilution's of cell numbers allows very sensitive detection such that each spot represents a single cell secreting IFN-γ. 1 x 10^6 splenocytes were isolated from mice 4 weeks post-infection and cultured overnight with PPD (described in section 2.4.7). Triplicate wells per mouse were counted and the values shown are means ± s.e.m. of 5 mice in each group. The P values for each comparison were determined using the Student's t-test. A P value of 0.05 or below was taken to be significant. The results shown in Figure 22. represent the mean number of IFN-γ producing cells in response to PPD in control mice (CD40+/-) and CD40 deficient mice (CD40-/-). The control mice exhibit approximately 225 IFN-γ producing cells per 1.0 x 10^6 splenocytes, whereas CD40 deficient mice exhibit less IFN-γ producing cells, only 100 per 1.0 x 10^6 splenocytes. These results show a significant reduction in the IFN-γ response to PPD (BCG infection) in CD40 deficient mice (P<0.001). The reduced levels of IFN-γ in the

CD40 deficient mice in response to a mycobacterial infection may explain the raised parasite burdens found in this study (Figure 21.). The results are discussed further in relation to other studies (see Discussion).

Histology was performed on control and CD40 deficient mice four weeks after infection with BCG. Spleen, lungs and liver were taken out and fixed in formalin, embedded in paraffin and tissue sections stained with hematoxylin and eosin (H&E). In addition, tissue sections were stained for the presence of acid-fast bacilli (mycobacteria) as described in section 2.4.9. Figure 23. (A.1.: CD40+/- and B.1.: CD40-/-) shows the presence of localized mycobacteria in macrophages of liver-sections from control (A.1.) and CD40 deficient mice (B.1). The bacilli appear as bright pink dots which is due to the high concentration of lipids in the mycobacterial walls which are responsible for the acid resistant staining with fuchsin red (hence acid-fast bacilli) and can easily be localized. A comparative quantitative analysis of the number of bacilli (BCG) can be performed by choosing at random ten optical fields per histological slide and counting the number of bacilli. Such an analysis should have 5 mice in each group and the result should be expressed as a mean value ± s.e.m (Kindler *et al.*, 1989). In this instance, the slides should be coded so that they can be read blind. The code should only be broken once all the slides have been analyzed. If the number of bacilli present in the tissue is so high that a quantitative histological determination is impossible, then a colony forming unit (CFU) assay should be used (Figure 21.).

A BCG infection results in formation of granulomas consisting of large numbers of activated macrophages, surrounded by lymphocytes in the organs of infected mice. The formation of granulomas contains the mycobacterial infection and prevents the continued growth and dissemination of bacilli (Nibbering *et al.*, 1989; Pelletier *et al.*, 1982).

In order to asses the formation of granulomas in CD40 deficient and control mice H&E staining of liver-sections (Figure 23. A.2.: CD40+/- and B.2.: CD40-/-) was performed. Hematoxylin preferentially stains acidic structures blue/purple, therefore activated cells

with high levels of DNA and RNA stain heavily and are blue. Eosin stains basic structures pink, so that proteins and the cytoplasm of cells appears pink. The accumulation of many activated macrophages and lymphocytes with high concentrations of RNA, DNA and ribosomes can be readily seen as heavily blue stained cells in tissue sections, denoting the presence of a granuloma.

The H&E staining revealed the formation of granulomas in both control mice and CD40 deficient mice. There appeared to be fewer granulomas in control mice and these were smaller and less densely packed with activated macrophages and lymphocytes than the granulomas in CD40 deficient mice. A comparative quantitative analysis of the number of granulomas in control and mutant mice can be performed by counting at random ten optical fields per histological slide. In the same way as is described for determination of bacilli numbers, the slides should be blinded. Such an analysis should have 5 mice in each group and the result should be expressed as a mean value ± s.e.m (Kindler *et al.*, 1989). In addition, the size of the granulomas can be estimated if the area of the optical field is known (Nibbering *et al.*, 1989).

Figure 23. (A.3.: CD40+/- and B.3.: CD40-/-) shows granulomas from control and mutant mice at a higher magnification (400x). This allows us to see that the individual granuloma in the mutant mouse (Figure 23. B.3.) is larger and more densely packed with blue-stained cells, indicating the presence of more activated macrophages and lymphoctes than the individual granuloma shown in the control (Figure 23. A.3.). Spleen- and lung-sections showed the same pattern with raised numbers of granulomas in the CD40 deficient mice (data not shown).

Infection of control and CD40 deficient mice with BCG results in significantly increased numbers of bacilli (CFU) in the spleen and lungs of CD40 deficient mice (Figure 21.). The ability of the CD40 deficient mice and control mice to generate a Th1 response towards mycobacterial antigens was assessed *in vitro*. This was carried out by looking at the ability of splenocytes from BCG infected mice from each group to make IFN-γ in

response to culture with PPD. This revealed that the splenocytes of CD40 deficient mice secreted significantly less IFN-γ in response to PPD (Figure 22.). Granuloma formation occurred in response to BCG infection in both control and CD40 deficient mice, however the number and size of granulomas appeared increased in CD40 deficient mice (Figure 23). CD40 deficient mice survived infection with BCG (Figure 20.), nevertheless the increased numbers of bacilli in spleen and lungs, and the reduced production of IFN-γ in response to mycobacterial infection indicates that CD40 deficient mice are more susceptible to infection with BCG than control mice.
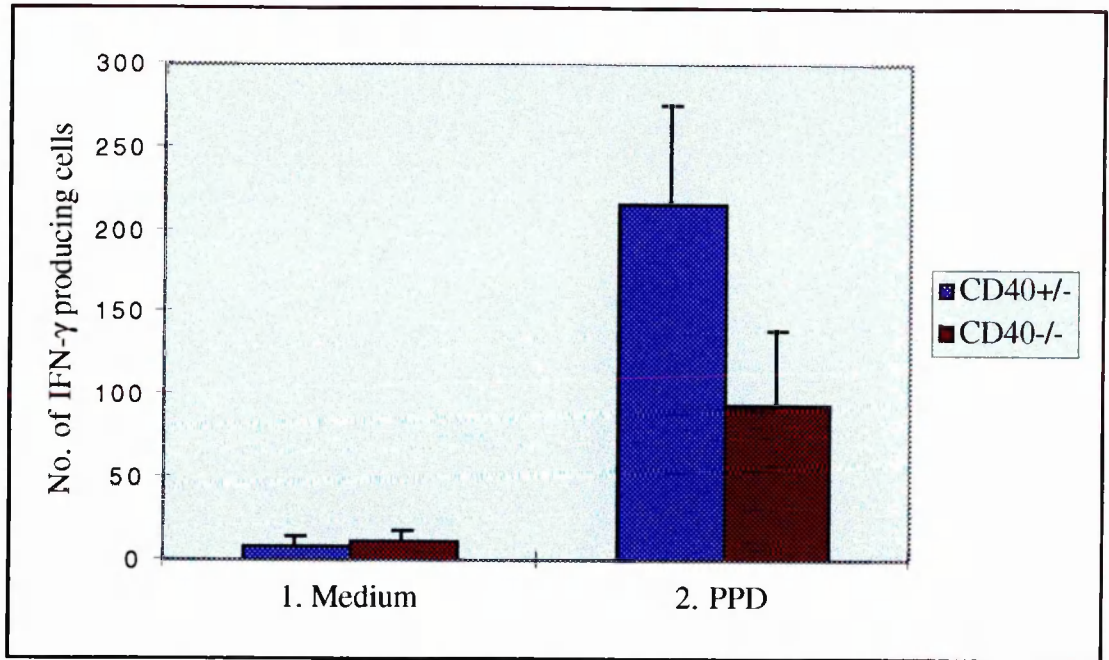
Figure 22. IFN-γ production by spleen cells of BCG infected control (CD40+/-) and CD40 deficient (CD40-/-) mice. Four weeks after infection with BCG, spleens were removed and 1.0 x 10^6 splenocytes were stimulated with PPD (1 µg/ml). The number of cells producing IFN-γ in response to PPD was detected in an ELIspot assay. Results are given as the number of IFN-γ producing cells per 1.0 x 10^6 splenocytes (2. PPD). The control (1. Medium) is splenocytes cultured without PPD. Triplicate wells per mouse were counted and the values shown are means ± s.e.m. of 5 mice in each group. The P values for each comparison were determined using the Student's t-test. 1. Medium: P=0.36; 2. PPD: P<0.001.
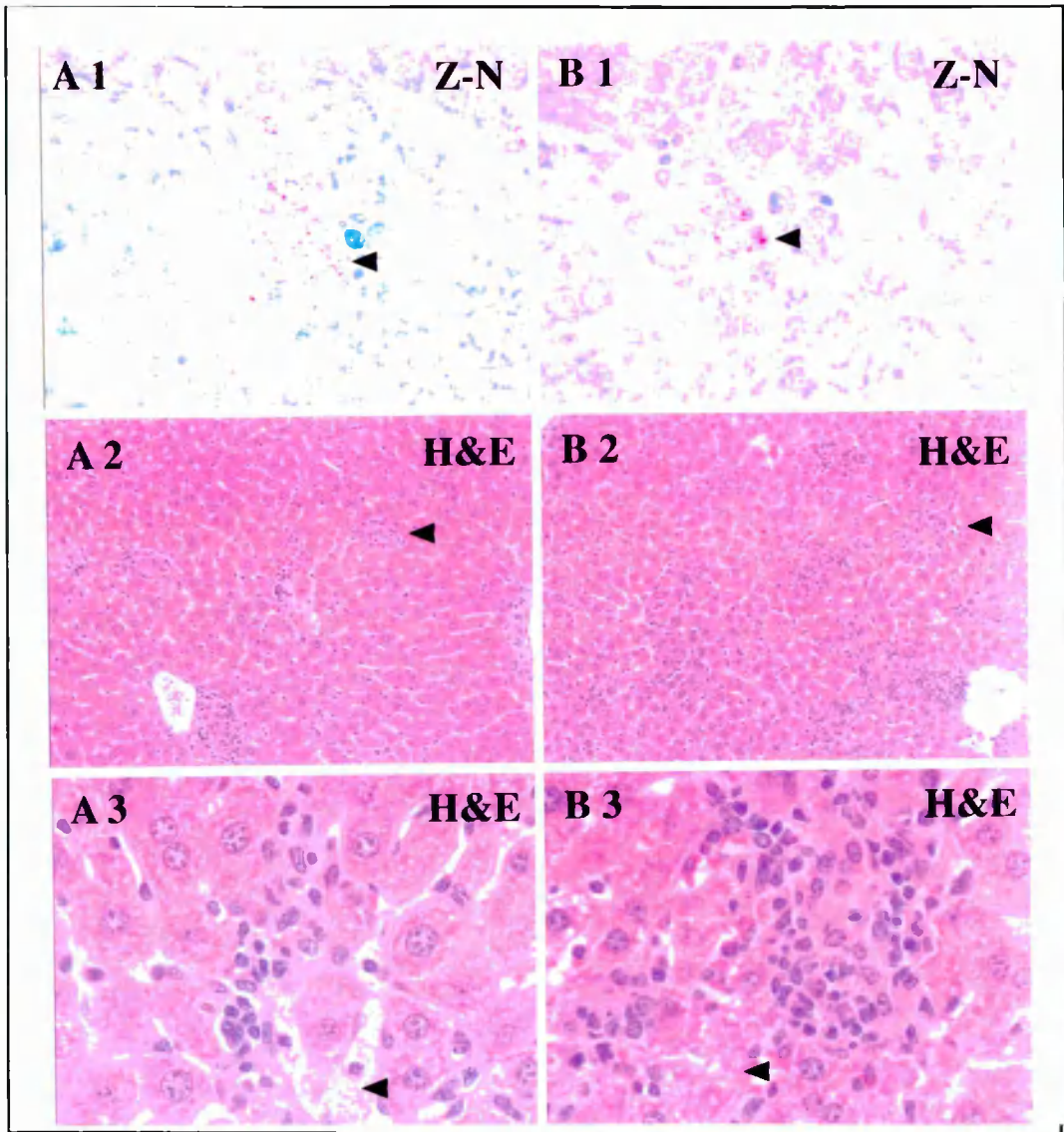
Figure. 23. Histology of liver-sections from *bacillus Calmette-Guerin* infected CD40+/- control mice and CD40-/- mice. Mice were infected i.v. with 1 x 10^7 CFU of *bacillus Calmette-Guerin* and killed four weeks later. Liver-sections were stained by the Ziehl-Neelsen method for acid fast bacilli (mycobacteria), A.1.: CD40+/-; B.1.: CD40-/-. Arrows indicate bacilli localized in macrophages. Magnification 100x. Liver-sections were also stained with hematoxylin and eosin (H&E), A.2: CD40+/-; B.2. CD40-/-. Arrows indicate granuloma. Magnification 40x. A.3.: CD40+/-; B.3.: CD40-/-. Arrows indicate disintegrated liver tissue. Magnification 400x.

Chapter 6: Discussion

6.1 Generation of CD40 deficient mice

There are many factors that must be considered when making a gene-deleted mouse. Firstly, the time that is required for the process is lengthy. It is probably best therefore to plan to delete a gene that has been cloned in your own or a closely collaborating laboratory. This ensures extra time so that changes to the different steps involved in the long process can be made. Secondly, gene-deleted mice may well be more susceptible to infections than intact mice. This means that the conditions under which the animals are housed must be very well regulated. SPF (specific pathogen free) facilities must be used if the specific deletion affects immune functions. However even specific pathogen free facilities may have to be modulated as deficient mice may display a different panel of infections that are not normally tested for under normal SPF requirements. Lastly, clean, pathogen-free mice which are used to provide blastocysts for microinjection will give better yields of blastocysts than infected mice and this will aid in the process of making chimaeric animals.

The two most difficult processes involved in making a gene-deleted mouse are the generation of targeted ES cells and the subsequent germline transmission of this targeted mutation. Generation of targeted ES cells is dependent on the targeting frequency of the targeting vector which is directly correlated to the length of homologous sequences present in the targeting vector (Hasty *et al.*, 1991a). The results of the homologous recombination events that I found with the two different constructs that I made would tend to support this view since the length of homology in construct 2 (Figure 8.) was greater than that in construct 1 and it was only with this construct that I was able to generate any targeted ES cell clones. The length of the construct is governed by other considerations such as the need for unique restriction enzyme sites and sequences for an external probe that allows unambiguous Southern blot analysis. Another factor that can be considered when making a targeting vector is the use of a negative selection marker

(TK) in the targeting vector outside the region of homology. This would reduce the number of G418 resistant clones considerably. The advantages of this negative selection would be the decreased the number of clones that needed to be screened to obtain targeted ES cell clones and this may shorten the process by several weeks.

The culture conditions that need to be used to keep ES cells growing in an undifferentiated state are crucial. Even with stringent culture conditions abnormal variant cell clones will appear in the ES cell population with increased time in culture. Some of these variants will have an obvious abnormal karyotype and are unlikely to be able to contribute to the germline. For this reason, karyotyping was performed on both ES cell lines and targeted ES cell clones (Figure 11-13.) to ensure a normal karyotype. As shown in Table 2. and Figure 12., a seemingly normal karyotype in the 34 R1 ES cell clone did not guarantee germline transmission. It should be noted that both the R1 and D3 ES cell line (unmanipulated) was tested for germline transmission prior to generation of targeted ES cell clones. In contrast to the D3 ES cell line, the R1 ES cell line seemed to grow slower, otherwise it displayed normal morphology.

The results shown in Table 2. indicates how rare it was to achieve germline transmission despite the fact that all targeted ES cell clones had been karyotyped. The best indicator of the chance of germline transmission is the male to female ratio of chimaeras. As mentioned earlier, the conversion of a female blastocyst to a male chimaera indicates that the male ES cells are capable of colonizing the germline cells and possibly passing on the mutated allele. Therefore in this case more than 50% of the chimaeras will be male which I found to be the case for chimaeras generated from the 55 D3 ES cell clone. Of a total of 33 male chimaeras, derived from the 55 D3 ES cell clone, only one male chimaera managed to pass on the mutated allele.

The ratio of male to female chimaeras is a better indicator of the chance of germline transmission than coat colour. All 16 male chimaeras derived from the 34 R1 ES cell clone were 80-95% agouti (Figure 14.). This indicates that most of the tissues in these

mice were ES cell derived, however when bred to check for germline transmission, some were sterile or only produced wild-type offspring. The ES cells were obviously capable of differentiating into somatic cells but could not differentiate into germline cells and the reason for this is unknown. Ideally the majority of targeted ES cell clones should contribute to the germline. The resulting homozygous mutant mice, derived from two independent ES cell clones, can then be analysed and found to have the same phenotype. This confirms that the phenotype of the homozygous mutant mice really is a result of the targeted mutation. One last important factor to consider is that all cells grown in culture can become infected with mycoplasma. Mycoplasma does not always have an obvious effect on cell growth or morphology but can cause chromosome damage and severely reduce the efficiency of obtaining ES cell chimaeras. All the ES cell lines and clones used in this study were rigorously screened for mycoplasma and the results were always found to be negative.

The production of chimaeras by blastocyst injection, as described in Chapter 3, is the prevalent method used by many laboratories. While this method is effective for the production of chimaeras, including germline chimaeras, it does have some practical limitations. The main limitations are the cost of the equipment used and the man-hours required to acquire the skills needed to make good holding and injection needles. This is time-consuming but critical to the success of the injection process. In addition, successful injection of 25-30 blastocysts per day requires several months of practise. This technique alone required 1-2 months practise before I became proficient.

These considerations have lead to the investigation of alternative methods of chimaera production. A simpler technique has been developed that requires no expensive equipment and much less practice (Wood *et al.*, 1993). This method is based on the observation that ES cells aggregate with morulae (the zona pellucida has been removed) and therefore all that is required for chimaera production is to bring the two cell populations into contact. Although the ES cells initially attach to the outside of the morulae they are efficiently internalized and by the blastocyst stage chimaeric embryos

contain ES cell-derived cells in the inner cell mass. The chimaeras (morulae and ES cells) are then cultured overnight to the blastocyst stage before transfer into a pseudopregnant foster mother. Comparison of germline chimaeras produced by blastocyst injection and morulae aggregation revealed that an equal number of germline transmitters occurred with both methods. R1 ES cells were used in these experiments. The production of chimaeras by the aggregation method is well worth considering if no one in the laboratory has experience in blastocyst injection.

6.2 Analysis of CD40 deficient mice

6.2.1 Lymphocyte development in CD40 deficient mice

When I was screening ES cells for homologous recombination events, two papers were published showing that CD40 deficient mice had impaired immunoglobulin class switching and germinal centre formation (Castigli *et al.*, 1994; Kawabe *et al.*, 1994). Another paper on CD40 ligand deficient mice (Xu *et al.*, 1994) was also published with essentially the same findings. The rational behind continuing to make the gene-deleted mouse was that it was becoming clear that CD40 expression was much wider than first thought and that many different cell types within the immune system could potentially be regulated by the CD40-CD40L interaction, providing opportunities for the investigation of a complex issue. I will go on to discuss my results and compare them with those found in these other papers.

In this study, analysis of lymphocytes in the periphery (peripheral blood and lymph nodes) and thymus (Figure 16.) of CD40 deficient mice revealed no change in the proportions and phenotype of B and T lymphocytes. B cells made up similar proportions of the lymphocyte population of peripheral blood and lymph nodes in both wild-type and CD40 deficient mice. The B cells in the CD40 deficient mice expressed normal levels of B220$^+$ and IgM indicating that B cells lacking CD40 were still able to undergo normal maturation. Likewise no abnormal development of T lymphocytes in the thymus was observed. This was confirmed by staining thymocytes with T cell surface markers (CD4,

CD8 and $\alpha\beta$-TCR). CD40 was also found to be expressed on thymic epithelial cells (Figure 18. E.) and dendritic cells (Galy & Spits, 1992), suggesting that the CD40-CD40L interaction might also play a role in thymic selection, the process by which self-reactive T cells are deleted from the T cell repertoire. Thymic selection involves both positive selection and negative selection. Positive selection is the process in which thymocytes whose TCRs bind self MHC molecules associated with self or foreign peptides are permitted to survive and all those that have no affinity for self MHC molecules die. Negative selection involves the deletion of thymocytes whose TCRs bind with high affinity to self peptide antigens in association with self MHC molecules (Anderson *et al.*, 1996). It is clear that both positive and negative selection in the thymus requires recognition of peptide associated with MHC complexes by the TCR, but the requirement for additional costimulatory molecules is unclear. This is in contrast to the activation of naive peripheral T cells, where it has been demonstrated that recognition of peptide associated with MHC by TCR is insufficient for optimal T cell activation and that costimulatory molecules provided by APCs are necessary for complete T cell activation (Durie *et al.*, 1994). To determine if CD40-CD40L interactions influenced thymic selection, the deletion of T cells expressing TCRs reactive against endogenous retroviral Mls (minor lymphocyte stimulating) antigens was examined in mice treated with anti-CD40L antibody. Due to the expression of Mls antigens encoded by endogenous mouse mammary tumor viruses, young adult BALB/c mice normally delete TCR expressing $V\beta3$, $V\beta11$, and $V\beta12$, but not TCR using $V\beta8$. Mice treated from birth with anti-CD40L antibody were analysed for T cells expressing $V\beta3$, $V\beta11$, $V\beta12$, and $V\beta8$, by flow cytometric analysis. Thymocytes expressing $V\beta3$, $V\beta11$, and $V\beta12$ were absent in the $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive thymocyte populations obtained from normal untreated BALB/c mice. However, analysis of $V\beta3$, $V\beta11$, and $V\beta12$ expression on single positive thymocytes from BALB/c mice treated with anti-CD40L antibody showed the presence of single positive thymocytes expressing $V\beta3$, $V\beta11$, and $V\beta12$. The expression of these TCRs was similar to that seen in a nondeleting strain, C57BL/6. These results demonstrated that Mls-mediated deletion of self-reactive T cells was

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prevented as a result of anti-CD40L treatment. To further confirm the role of CD40L in Mls mediated deletion of thymocytes, analysis of TCR Vβ usage in CD40L deficient mice was performed. Normal CBA/J mice (Mls^{a,c,f}) and CD40L heterozygous CBA/J F$_1$ mice delete a range of Vβ-expressing T cells, including Vβ3, Vβ5, Vβ6, Vβ7, Vβ9, Vβ11, and Vβ12. In contrast, there was an increase in CD4$^+$ and/or CD8$^+$ single positive thymocytes using Vβ5, Vβ6, Vβ7, Vβ11, and Vβ12 in CBA/J F$_1$ mice deficient in CD40L (Foy *et al.*, 1995). To investigate the role of the CD40-CD40L interaction in the selection of T cells to other self antigens, an *in vivo* system of negative selection involving TCR transgenic mice (AND TCR Tg) expressing an α/β TCR specific for pigeon cytochrome c (PCC) in association with H-2E^K class II MHC molecules and transgenic mice endgenously expressing PCC in the thymus were used. This system allows examination of the selection of transgenic thymocytes to an endogenously encoded "self antigen". Mating the AND TCR transgenic mice with transgenic mice expressing PCC results in the recognition of PCC self antigen by thymocytes bearing the TCR specific for PCC and results in their deletion. To determine whether the CD40-CD40L interaction was required for the deletion of mature transgenic thymocytes in this model of negative selection, (AND TCR Tg X PCC Tg)F$_1$ mice were treated from birth with anti-CD40L antibody, and the thymocyte populations were examined four weeks later. The results, demonstrated a dramatic decrease in the percentage of CD4$^+$ thymocytes in (AND TCR Tg X PCC Tg)F$_1$ mice compared with AND TCR transgenic mice. The administration of anti-CD40L antibody completely restored the CD4$^+$ thymocyte compartment in the (AND TCR Tg X PCC Tg)F$_1$ mice. These results demonstrate that blockade of the CD40-CD40L interaction interferes with antigen-mediated deletion of thymocytes in this transgenic model of negative selection. Exogenous administration of PCCF (PCC peptide fragment) to AND TCR transgenic mice results in the deletion of DP (double positive) thymocytes. To determine whether administration of anti-CD40L antibody interfered with high-dose PCCF-mediated deletion of thymocytes, AND TCR transgenic mice were injected with PCCF, with or without coadministration of anti-CD40L antibody. The results showed that although

administration of PCCF resulted in loss of DP thymocytes, coadministration of anti-CD40L antibody did not prevent this deletion. The results demonstrate that deletion of DP TCR transgenic thymocytes by high concentrations of antigen does not appear to be dependent upon the CD40-CD40L interaction. The involvement of the CD40-CD40L interaction in negative selection mediated by exogenous superantigens was also investigated. Administration of SEB (Staphylococcus Enterotoxin B) to neonatal BALB/c mice causes the deletion of Vβ8-bearing T cells. Neonatal BALB/c mice were administered SEB alone or in combination with anti-CD40L antibody. SEB caused a dramatic loss in the Vβ8-bearing T thymocytes in the CD4 and CD8 single-positive compartment. Coadministration of SEB and anti-CD40L antibody did not alter selection of Vβ8-bearing thymocytes, suggesting that SEB-mediated deletion occurs independently of the CD40-CD40L interaction. These results showed that negative selection mediated by exogenously administered antigen/superantigen appeared to be independent of CD40-CD40L interactions, whereas negative selection mediated by endogenously expressed antigens was dependent on the CD40-CD40L interaction. Foy *et al.* (Foy *et al.*, 1996) hypothesized that the clonal deletion that occurs following administration of supraphysiological concentrations of antigens/superantigens overrides any requirement for CD40L-mediated signaling. Furthermore, CD40L-dependent selection may also be related to the affinity/avidity of the interactions between TCR and MHC/Ag. In the model of viral-superAg-mediated deletion, mature antigen-specific T cells proliferated poorly in response to Mls, suggesting that thymocytes bearing these TCRs also interacted with Ags/SAgs in a low affinity/avidity fashion. Therefore CD40-CD40L interactions might provide additional costimulatory signals necessary to facilitate the deletion of thymocytes bearing TCRs of low affinity/avidity. In contrast, CD40-CD40L interactions may not be required for deletion mediated by high-affinity TCR ligands such as high dose PCC peptide and SEB, which elicit strong proliferative responses from mature T cells.

Expression of CD86 in the thymus of CD40L deficient mice and in mice treated with anti-CD40L was also investigated (Foy *et al.*, 1995). The results of quantitative immunohistochemical analysis revealed that the expression of CD86 in the medulla region was substantially reduced. This suggested that CD40L expression was critical for CD86 expression and that the reduced expression may have contributed to the defects in negative selection observed in the absence of CD40-CD40L interactions. Two models have been proposed which would explain the involvement of the CD40-CD40L interaction in negative selection of thymocytes. A negative signal is delivered by APCs expressing CD40 to the T cell via CD40L, inducing apoptosis in the T cell. Negative selection will not take place if CD40L is missing. The second model predicts that TCR recognition induces CD40L expression, which in turn engages CD40 and induces the upregulation of costimulatory molecules (CD86), which then induces a signal causing thymocyte death (Foy *et al.*, 1996).

The analysis of CD40 deficient mice in this thesis and by Kawabe *et al.* (1994) and of CD40L deficient mice (Xu *et al.*, 1994) revealed that proportions and numbers of B and T lymphocytes in peripheral lymphoid organs was normal and the pattern of thymocytes was indistinguishable in these mice compared with wild-type mice. Further experiments are obviously needed to clarify the role of the CD40-CD40L interaction in thymic selection.

The results in this thesis and by Kawabe *et al.* (1994) also showed that B cells retained their capacity to proliferate in response to LPS and LPS together with IL-4 (Figure 17.), however there was a slight reduction in the ability of CD40 deficient B cells to respond to LPS and LPS plus IL-4 (see results for further details). Cross-linking of mutant B cells with anti-CD40 antibody, did not induce proliferation. T cells from CD40L deficient mice and control mice responded equally well to mitogenic stimulation with PMA and/or ionomycin, or anti-CD3 antibody, indicating that CD40L deficient T cells are not defective in their ability to respond to mitogens (Xu *et al.*, 1994).

6.2.2 Germinal centre formation in CD40 deficient mice

Patients suffering from hyper-IgM syndrome do not have germinal centres (Korthauer *et al.*, 1993) and blocking the CD40-CD40L interaction *in vivo* with soluble anti-CD40L antibody has been shown to abrogate germinal centre formation in mice (Foy *et al.*, 1994). In this study the formation of germinal centres was tested by immunizing wild-type and mutant mice with KLH (Figure 18. A and B.). The absence of peanut agglutinin-binding B cells in CD40 deficient mice revealed that no germinal centres developed in CD40 deficient mice. Histological analysis with specific staining for T and B cell areas of the spleens of CD40 deficient mice revealed normal splenic architecture, indicating that a general disruption of splenic architecture did not account for the inability of CD40 deficient mice to form germinal centres. The lack of peanut-agglutinin binding in CD40 deficient mice, after immunization with KLH, was also demonstrated by Kawabe *et al.* (1994). In addition, CD40L deficient mice, immunized with SRBC (Sheep Red Blood Cells), failed to develop germinal centres. An interesting finding concerning the role of the CD40-CD40L interaction in germinal centre maintenance is that the administration of anti-CD40L antibody in mice causes the disappearance of preexisting germinal centres (Han *et al.*, 1995). It has been established that germinal centre B cells apoptose upon isolation and that CD40 cross-linking can "rescue" them from death (Liu *et al.*, 1992). Therefore, the immediate assumption is that anti-CD40L antibody treatment causes the loss of germinal centres due to increased apoptosis or it is possible that loss of the CD40-CD40L interaction in germinal centres causes B cells to migrate to the bone marrow and terminally differentiate into plasma cells. This hypothesis would be consistent with a recent *in vitro* study showing that human germinal centre B cells, when cultured with CD40L expressing T cells, differentiate into memory cells but the removal of CD40L expressing T cells resulted in terminal differentiation of germinal centre B cells into plasma cells (Arpin *et al.*, 1995).

Since it is clear that CD40L plays an important role in germinal centre formation and maintenance, it is important to determine which cells within the germinal centres are

capable of expressing CD40L. Early studies in mice immunised with KLH showed no expression of CD40L in the germinal centres and marginal zones of their spleens. Instead, the expression of CD40L was restricted to T cells in the outer periarteriolar lymphocyte sheaths (outer-PALS), and around the terminal arterioles (TA). Antibody-producing B cells specific to KLH have been found in close proximity to CD40L expressing T cells (Van *et al.*, 1993). Furthermore, studies in human tonsils have shown a subset of T cells which contain preformed CD40L that can be mobilized upon activation. The T cells containing preformed CD40L were found predominantly in the outer zone of the germinal centres and could be important for the maintenance of germinal centre integrity (Casamayor *et al.*, 1995). The different results in murine and human systems may be due to species differences or lack of sensitivity of the murine anti-CD40L antibody. Activated T cells expressing a membrane-bound form of CD40L can also secrete a soluble form (Graf *et al.*, 1995). The soluble form of CD40L may be the active factor in maintaining germinal centre B cell proliferation. In addition, human B cells can express CD40L (Grammer *et al.*, 1995) and mouse B cells express CD40L in their cytoplasm which is released into the supernatant when B cells are activated with anti-Ig and anti-CD40 antibodies (Wykes *et al.*, 1998). Whether soluble CD40L secreted by either T cells or B cells is important for germinal centre formation and maintenance is currently unknown.

The interpretation of these studies suggests that signals via CD40 are responsible for germinal centre formation and maintenance. Experiments with CD40 deficient mice have revealed new information about the CD40-CD40L interaction. To investigate CD40L-transduced signals (signals to the T cell via CD40L), CD40 deficient mice were immunized with DNP-KLH and injected with a soluble CD40-Ig fusion protein to cross-link the CD40L. Ten days after immunization, mice treated with soluble CD40-Ig had formed germinal centres, whereas mice injected with human IgG1 had not. Interestingly, CD40 deficient mice treated with CD40-Ig were still unable to make class switched antibodies (van *et al.*, 1995). This indicated that CD40 signaling to B cells was required

for isotype switching but was not necessary for the initiation of germinal centre formation. These data may explain the discrepancy between experiments in CD40 and CD40L deficient mice, which do not form germinal centres or memory B cells, and those in normal mice treated with soluble CD40-Ig (to block the CD40-CD40L interaction) which do form germinal centres but still have no memory B cells (Gray *et al.*, 1994). The observation that CD40L can also be expressed on B cells (Grammer *et al.*, 1995) means that one cannot distinguish whether the effect of the soluble CD40-Ig is due to CD40L transduced signals to T cells or B cells. However as a defect in T helper cell function was also seen in CD40 deficient mice (see Chapter 5), this signal is most likely to be directed to T cells. The germinal centres that formed after soluble CD40-Ig treatment of CD40 deficient mice were underdeveloped, there were fewer and smaller than in wild-type mice. It is possible that the CD40 signal to B cells is important for their progression within the germinal centres as indicated in the experiments described earlier (Arpin *et al.*, 1995; Liu *et al.*, 1992). These experiments suggest that transduction of signals by CD40L to T cells can initiate germinal centre formation but for full maturation of germinal centres and the production of class switched immunoglobulins, signals to B cells via CD40 are required.

6.2.3 Serum immunoglobulin levels in CD40 deficient mice

A characteristic feature of hyper IgM syndrome in humans is the difference in serum immunoglobulins levels as compared with healthy individuals. Serum from hyper IgM syndrome patients contains normal or elevated levels of IgM, but low to undetectable levels of other immunoglobulin isotypes. To determine whether mice deficient in CD40 exhibited similar alterations in serum immunoglobulin levels, wild-type and CD40 deficient mice were bled and the levels of serum IgM, IgG1, IgG2a, IgG2b, IgG3, and IgE were determined (Figure 19). The results demonstrate that, like HIGM1 patients, CD40 deficient mice express elevated levels of serum IgM. This was also shown to be the case in the CD40 deficient mice studied by Kawabe *et al.* (1994). In contrast, CD40L deficient mice displayed completely normal serum IgM levels (Xu *et al.*, 1994).

In this thesis, the serum immunoglobulin levels in unimmunized CD40 deficient mice, were compared to the results obtained in two other studies of CD40 deficient mice (Castigli *et al.*, 1994. Kawabe *et al*, 1994). Both studies showed normal levels of IgM in CD40 deficient mice compared to control mice. Serum levels of IgG1, IgG2a, IgG2b and IgA were significantly reduced, however IgG3 was significantly higher (Kawabe *et al.*, 1994). Castigli *et al.* (1994) also found that levels of IgG1 and IgG2a were significantly reduced and they found that IgE was absent in the CD40 deficient mice . Normal levels of IgG3 and slightly reduced levels of IgG2b and IgA were observed (Castigli *et al.*, 1994). There were slight differences in the levels of the immunoglobulins measured in these two studies and also in the results presented in this thesis, which could be attributed to the different conditions that the mice for the different studies were kept in, however the general trends in immunoglobulin production in CD40 deficient mice (reduced isotype switched immunoglobulins) were the same.

The normal or elevated levels of serum IgM that were seen in patients with hyper IgM syndrome and also in CD40 deficient mice indicates that IgM antibody responses in both systems were independent of the CD40-CD40L interaction. The reduced levels of isotype switched immunoglobulins in the serum of CD40 deficient mice suggests that the CD40-CD40L interaction is important for the isotype switching from IgM to other immunoglobulins.

Antigens which stimulate antibody production by B cells can be divided into two categories, thymus-dependent or thymus-independent antigens. Thymus-dependent antigens are protein derived peptides, which can only induce the production of antigen specific antibody if the peptide is recognized in association with MHC class II by antigen specific T cells. This leads to mutual activation of both B and T cells and the subsequent production of antigen specific antibody. Therefore antibody production in response to protein antigens requires T cell help. Antigens that stimulate antibody production in the absence of MHC class II restricted T cell help are classified as thymus-independent antigens (Mond *et al.*, 1995). Included within this category are lipopolysaccharide (LPS)

derived from gram-negative bacterial cell walls, dextran, pneumococcal capsular polysaccharide, and Ficoll.

To investigate the role of the CD40-CD40L interaction in humoral immune responses to both thymus-dependent and thymus-independent antigens. CD40 deficient mice were immunized with a thymus-dependent antigen, ovalbumin, on day zero and day 22 of the experiment. Antigen specific antibody responses were measured by isotype-specific ELISA on day 7, 14 and 28. CD40 deficient mice completely failed to mount primary (day 7) and secondary (day 28) anti-ovalbumin antibody responses of all immunoglobulin isotypes except for the IgM class. Anti-ovalbumin IgM responses were normal or slightly higher than IgM responses in wild-type mice. These results indicate that immunoglobulin class switching did not take place in response to thymus-dependent antigens in CD40 deficient mice. However, the ability to produce antibodies in response to thymus-independent antigens, such as LPS or Ficoll, did not appear to be affected in these mice. When immunized with LPS or Ficoll, CD40 deficient mice developed anti-LPS or anti-Ficoll antibody responses of IgM as well as IgG immunoglobulin isotypes (IgG1, IgG2a, IgG2b, IgG3) (Kawabe *et al.*, 1994).

This suggests that the signal through CD40 is not required for *in vivo* thymus-dependent IgM responses and thymus-independent responses but is required for immunoglobulin class switching in primary and secondary immune responses to thymus-dependent antigens.

Similar experiments were performed in CD40L deficient mice (Xu *et al.*, 1994). CD40L deficient mice were immunized with a thymus-dependent antigen, sheep red blood cells (SRBC), and the primary anti-SRBC antibody response was analysed 5 days later. CD40L deficient mice failed to produce any anti-SRBC IgM antibody responses. This is consistent with earlier studies in which *in vivo* CD40-CD40L interactions were blocked by an anti-CD40L antibody (Foy *et al.*, 1993). To confirm the defect in primary humoral responses, CD40L deficient mice were immunized with the thymus-dependent antigen,

KLH. On day 7 and 19 the antibody responses to KLH were analysed. The primary (day 7) anti-KLH IgM response was reduced compared to the wild-type response. Analysis of the secondary (day 19) anti-KLH IgG1 response demonstrated that CD40L deficient mice were incapable of mounting a secondary IgG1 response to this antigen. There is obviously a difference in the way that CD40L deficient mice respond to different thymus-dependent antigens. KLH may have some mitogenic activity independent of the CD40-CD40L interaction. In addition, CD40L deficient mice were immunized with the thymus-independent antigen Ficoll, and anti-Ficoll IgM and IgG3 responses were determined on day 6 after immunization. CD40L deficient mice developed similar anti-Ficoll IgM and IgG3 responses as control animals, confirming that antibody responses to thymus-independent antigens are independent of the CD40-CD40L interaction.

There are significant similarities between the results observed with CD40 and CD40L deficient mice and patients suffering from hyper IgM syndrome. In all cases, low levels of IgG isotypes, IgA, and IgE have been observed. CD40 and CD40L deficient mice have normal or slightly elevated levels of IgM. In HIGM1 patients the IgM levels are significantly elevated, and this may reflect the persistent response of these patients to pathogens such as bacteria. In mice, IgM and IgG3 are known to be the major antibody isotypes stimulated in thymus-independent responses (Mongini *et al.*, 1981). The normal capability of B cells to produce IgM and IgG antibodies to thymus-independent antigens and IgM to thymus-dependent antigens may be the reason why normal or slightly elevated serum levels of IgM and IgG3 are observed in CD40 and CD40L deficient mice.

Patients with hyper IgM syndrome make a poor antibody response to thymus-dependent antigens but mount strong humoral response to thymus-independent antigens (Notarangelo *et al.*, 1992). Both CD40 and CD40L deficient mice are capable of eliciting normal antibody response to thymus-independent antigens, and show normal switching of antibody responses from IgM to IgG isotypes, IgA, and IgE. In contrast, immunoglobulin class switching from IgM to IgG isotypes, IgA, and IgE in both primary and secondary immune responses to thymus dependent antigens is impaired in

CD40 and CD40L deficient mice. The capability of CD40 and CD40L deficient mice to mount a normal IgM response to thymus-dependent antigens in both primary and secondary immune responses indicates that IgM secretion is independent of the CD40-CD40L interaction.

These results suggests that in order for immunoglobulin class switching to occur in response to thymus-dependent antigens germinal centres are required. Germinal centres are only formed in response to thymus-dependent antigens and constitutes the site in which antigen stimulated B cells undergo somatic mutation and immunoglobulin class switching (MacLennan *et al.*, 1997). The germinal centre reaction generates two populations of B cells, plasma B cells which are short-lived and memory B cells which are long-lived. The plasma cells secrete high-affinity isotype switched antibodies. Upon a secondary antigenic stimulation, high-affinity memory B cells differentiate into plasma cells, resulting in clearance of the antigen. Thymus-independent antigens do not stimulate germinal centre formation, but immunoglobulin isotype switching does take place and the capacity to generate memory B cells to thymus-independent antigens is associated with the appearance of antigen specific B cells in the marginal zones of the spleen (Mond *et al.*, 1995).

It has been established that the generation of memory B cells is dependent on the formation of germinal centres and the current dogma is that memory B cells express isotype switched immunoglobulins (IgG isotypes, IgA and IgE) and that B cells expressing IgM or IgD contribute little to the memory pool of B cells (Gray, 1993; Gray *et al.*, 1996). Adoptive transfer experiments by Foy *et al.* (1994) and Gray *et al.* (1994) confirmed the role of the CD40-CD40L interaction in the generation of memory B cells. CB17 (Igh[b]) mice were immunized with TNP-BSA and treated with Hamster Ig or anti-CD40L antibody. After four weeks, splenic B cells from these mice were adoptively transferred into KLH-primed BALB/c (Igh[a]) recipients. The recipients were subsequently challenged with TNP-KLH and the level of donor-specific IgG1[b] anti-TNP antibodies determined. The results showed that B cells from untreated or Hamster Ig-treated mice

immunized with TNP-BSA produced high levels of donor-specific IgG1[b] antibody, whereas mice which received B cells from mice treated with anti-CD40L antibody displayed an 80% decrease in the level of donor-specific IgG1[b] antibody produced. These results demonstrated that blocking the CD40-CD40L interaction inhibited the generation of functional memory B cells. As described earlier both CD40 and CD40L deficient mice failed to mount a secondary antibody response to thymus-dependent antigens. This confirms that memory B cells do not develop if the CD40-CD40L interaction is non-functional.

The role of the CD40-CD40L interaction in the generation of immune responses to viral infection was investigated by infecting CD40L deficient mice with lymphocytic choriomeningitis virus (LCMV). The primary anti-LCMV specific antibody responses were severely impaired in CD40L deficient mice, with reduced levels of IgG1, IgG2a, IgG2b and IgG3. Unlike wild-type mice infected with LCMV, CD40L deficient mice were unable to sustain virus-specific antibody responses and showed a gradual decline in serum antibody levels over time. The CD40L deficient mice were also deficient in the generation of memory B cells as measured by a limiting dilution assay 60 days postinfection. In contrast to the severely impaired humoral responses, CD40L deficient mice were still able to generate a virus-specific CD8^{+} cytotoxic T cell response after LCMV infection and were able to clear the infection (Whitmire *et al.*, 1996). A similar study examined the immune responses to LCMV, Pichinde virus and vesicular stomatitis virus (VSV) in CD40L deficient mice. The antibody responses to these viruses were severely compromised in CD40L deficient mice, however CD40L deficient mice did produce some virus-specific IgM and IgG2a. The antiviral antibody production in virus infected CD40L deficient mice took place in the absence of germinal centres and titres of virus-specific antibody decreased over time. Whether CD40L deficient mice failed to develop memory B cells after virus infection was investigated in adoptive transfer experiments. The results demonstrated that no virus-specific memory B cells were present in CD40L deficient mice 4 months postinfection in contrast to wild-type control

mice. CD40L deficient mice mounted strong primary cytotoxic T lymphocyte (CTL) responses after infection with all of these viruses and cleared the infection with similar kinetics to wild-type control animals. LCMV-specific memory CTL activity was assessed by restimulating splenocytes from CD40L deficient and wild-type mice infected 2 months previously with LCMV for 6 days *in vitro* and then testing their ability to mediate virus-specific MHC-restricted CTL lysis in a ^{51}Cr-release assay. Splenocytes from CD40L deficient mice did mediate MHC restricted CTL lysis of LCMV-infected target cells, but the level of CTL activity was reduced considerably compared to wild-type mice. These results indicate that the CD40-CD40L interaction plays a role in the establishment and/or maintenance of CD8$^+$ cytotoxic T lymphocyte (CTL) memory (Borrow *et al.*, 1996). Taken together, these results confirm the crucial role of the CD40-CD40L interaction in B cell activation, antibody response to thymus-dependent antigens, germinal centre formation, immunoglobulin isotype switching and generation of memory B cells. Furthermore, the CD40-CD40L interaction appears to be important in the establishment of memory CTL.

6.3 Cell-mediated immunity in CD40 deficient mice infected with *bacillus Calmette-Guerin*

6.3.1 Susceptibiity of CD40 deficient mice infected with BCG

The results presented in Chapter 5 indicate an increased susceptibility of mice with a non-functional CD40-CD40L interaction. This statement is based on the following observations. Infection of control and CD40 deficient mice with BCG revealed that CD40 deficient mice have a significantly increased number of mycobacteria in the spleen and lungs compared to control mice (Figure 21.). The development of a Th1 response towards mycobacterial antigens following infection with BCG was assessed by an *in vitro* assay system (Eli-spot assay). Splenocytes from CD40 deficient mice infected with BCG secrete significantly less IFN-γ in response to PPD (Figure 22.). Lastly, histopathological staining of liver-sections revealed that CD40 deficient mice had more

granulomas that were bigger and more densely packed with cells than those seen in control mice (Figure 23.).

The increased susceptibility of CD40 deficient mice to BCG is likely to be due to the reduced ability of these mice to secrete IFN-γ in response to a mycobacterial infection. The importance of IFN-γ in the control of mycobacterial infections has been shown clearly in IFN-γ deficient mice who are unable to survive infections with BCG or *M.tuberculosis* (Cooper *et al.*, 1993; Dalton *et al.*, 1993). The death of IFN-γ deficient mice was shown to be due to insufficient activation of macrophages resulting in a reduced production of nitric oxide. This resulted in these mice having a very high parasite burden. A plausible explanation for the reduced production of IFN-γ in CD40 deficient mice upon infection with BCG, is a reduced production of IL-12 by activated macrophages and dendritic cells. The CD40-CD40L interaction was shown to be important for IL-12 secretion following activation of dendritic cells and macrophages (Cella *et al.*, 1996; Peguet *et al.*, 1995; Shu *et al.*, 1995). IL-12 would then induce the production of IFN-γ in T cells and NK cells, resulting in the development of a Th1 response towards a mycobacterial infection. IFN-γ and TNF-α in conjunction with activated T cells would result in macrophage activation and clearence of the infection. The enhanced susceptibility of CD40L and CD40 deficient mice to Leishmaniasis was associated with an impaired generation of a Th1 response, reflected by the production of low levels of IFN-γ, TNF, IL-12, and nitric oxide. From these studies it was concluded that the major contributing factor to increased susceptibility to Leishmania sp. was a diminished macrophage activation and nitric oxide production (Campbell *et al.*, 1996; Kamanaka *et al.*, 1996; Soong *et al.*, 1996). Thus, in order to assess the level of macrophage activation, levels of TNF-α, IL-12, and nitric oxide must be measured in CD40 deficient mice infected with BCG. Furthermore the cells involved in the IFN-γ production must be identified and characterised. The involvement of CD4^{+} or CD8^{+} T cells in IFN-γ production can be examined by blocking each subset of T cells with anti-CD4 or anti-CD8 antibodies and determining their effect.

As shown by the experiments described above the development of protective immunity against a leishmanial infection is dependent on the development of a Th1 response, which would lead to macrophage activation and eradication of the intracellular parasite. This is also the case for mycobacterial infections where the development of a Th1 response is crucial for protective immunity. One of the key cytokines for protective immunity against mycobacteria is IFN-γ as discussed at the beginning of this section (Cooper *et al.*, 1993; Dalton *et al.*, 1993), and this is correlated with a decrease in macrophage reactive nitrogen intermediates (RNI) production in these mice (Flynn *et al.*, 1993). Another key cytokine in protective immunity aganist mycobacteria is IL-12. BALB/c mice, a strain highly susceptible to infection with *M. tuberculosis*, were given recombinant IL-12 at the initiation of infection with *M. tuberculosis*. This treatment increased the mean survival time from 58 days to 112 days, indicating a role for IL-12 in protection against a mycobacterial infection. In contrast to the findings in the BALB/c model, IL-12 treatment of IFN-γ deficient mice infected with *M. tuberculosis* did not increase survival time. This indicates that IL-12 does not induce protection against *M. tuberculosis* in the absence of IFN-γ (Bloom *et al.*, 1995).

These experiments confirm the importance of IFN-γ in protective immunity aganist a mycobacterial infection. CD40 deficient mice infected with BCG (this Thesis) displayed a reduced production of IFN-γ in response to mycobacterial antigens (PPD) indicating that CD40 deficient mice are impaired in their ability to generate a Th1 response. Granulomas consisting of activated macrophages and lymphocytes serve as an effective means of containing the mycobacteria and preventing their continued growth and dissemination (Fenton & Vermeulen, 1996). In CD40 deficient mice following BCG infection, I found that the numbers of granulomas appeared increased compared to control mice (Figure 23.). As granulomas are produced to control the infection why was an increase in the number of CFU recovered from CD40 deficient mice compared with wild-type mice? (Figure 21.). A hypothesis that could explain this apparent inconsistency is that macrophage activation is a key component of the protective immune response to

M. tuberculosis infection. The only demonstrated mechanism by which macrophages kill mycobacteria is through activation by IFN-γ and LPS or TNF-α to produce reactive nitrogen intermediates (RNI) (Flesch & Kaufmann, 1991). It is possible that the reduced levels of IFN-γ in CD40 deficient mice would lead to reduced activation of macrophages. Without optimal macrophage activation upon infection with mycobacteria, the bacilli would continue to multiply, leading to increased numbers of bacilli in the organs. In CD40 deficient mice, the sub-optimal levels of IFN-γ although not clearing the parasitaemia, would still lead to granuloma formation.

A recent study was published while the BCG experiments described in this thesis were being carried out. This study investigated the immune response of CD40L deficient mice to *Mycobacterium tuberculosis* (Campos *et al.*, 1998). CD40L deficient mice infected with *M. tuberculosis* showed 100% survival during a 30 week period and mycobacterial counts after 3 or 6 weeks of infection in spleen, lung, and liver were indistinguishable in control and CD40L deficient mice. However later in infection (week ten), the CD40L deficient mice presented slightly elevated CFU in all three organs. Histopathologic examination of tissue sections taken at 6 weeks of infection revealed differences between the granulomas developing in CD40L deficient mice compared with those in control mice. The number of granulomas forming in all three organs of CD40L deficient mice was the same. This was also the case for all three organs in normal mice. However, the number of granulomas observed in the liver of CD40L deficient mice was reduced compared to control mice and the granulomas appeared disintegrated, lacked epithelioid cells and were reduced in size. Since the survival and mycobacterial counts were similar in control and CD40L deficient mice, these results indicated that organized granulomas are not essential for protection against *M. tuberculosis*. *In vitro* T cell proliferation and IFN-γ production by splenocytes in response to PPD was identical in control and CD40L deficient mice and the production of IFN-γ was abrogated by anti-CD4 mAb, but not by anti-CD8 mAb. This indicates that CD4$^+$ T cells proliferate and produce IFN-γ in response to *M. tuberculosis* antigens, independently of the CD40-CD40L interaction.

Campos *et al.* (1998) could not detect IL-4 in the supernatants of any of the splenocyte cultures stimulated with PPD. The production of IL-12 and TNF-α was measured *in vitro* after stimulation of splenocytes with *M. tuberculosis* antigens. Similar levels of IL-12 were present in the supernatants of cultures of both control and CD40L deficient mice, in contrast to which TNF levels were slightly elevated in CD40L deficient mice. Finally, CD40L deficient mice did produce nitric oxide, albeit to a slightly lesser extent than control mice in response to *M. tuberculosis* antigens. The title of the paper in which this data appears states that the CD40-CD40L interaction does not affect the development of protective immunity to *M. tuberculosis* and that CD40L deficient mice are resistant to infection with *M. tuberculosis*. Perhaps to confirm this, granuloma formation and cytokine production will have to be measured at week 10 when the authors showed that *M. tuberculosis* CFU counts were different in CD40L deficient and normal mice.

In CD40L deficient mice infected with *M. tuberculosis*, the reduced number of granulomas which appeared small, disintegrated and lacking in epithelioid cells, was not caused by a decrease in TNF-α levels as these mice displayed slightly elevated levels of TNF-α. This is surprising given that TNF-α was shown to be crucial for granuloma formation in a study where mice were infected with BCG and then treated with anti-TNF-α antibody. Anti-TNF-α treatment of mice resulted in a 10-fold decrease in the number of granulomas and those that formed were small and lacked epithelioid cells, compared to untreated mice. It was proposed that TNF-α released from macrophages into the microenvironment of developing granulomas could be involved in a process of autoamplification. TNF-α enhances its own synthesis and release, thereby inducing further macrophage accumulation and activation. This would then lead to fully developed granulomas and mycobacterial elimination (Kindler *et al.*, 1989). TNF-receptor deficient mice infected with *M. tuberculosis* also showed granulomas which lacked epithelioid cells and seemed more loosely organized (Flynn *et al.*, 1995a). Since the levels of TNF-α in CD40L deficient mice infected with *M. tuberculosis* were slightly higher compared to the control mice, TNF-α does not appear to be the only effector needed for fully

developed granulomas. Other cytokines such as GM-CSF (Granulocyte/Macrophage Colony Stimulating Factor), IL-1 and IL-8, have been found in granulomas (Bergeron *et al.*, 1997) and are probably involved in their development. Whether the production of these cytokines are influenced by the CD40-CD40L interaction is currently unknown. It will be interesting to see if CD40 deficient mice infected with BCG show reduced levels of TNF-α. I would not expect to see this, if further experiments confirm that more granulomas develop in CD40 deficient mice compared to control mice. What also remains to be determined is whether the granulomas in the CD40 deficient mice infected with BCG contain epithelioid cells, determined by silver staining for reticulum fibres, indicating fully developed granulomas.

CD40L deficient mice are susceptible to infection with *Leishmania* but are able to control infection by *M. tuberculosis*. The difference between these two infections is that there is no apparent T cell response following infection of CD40L and CD40 deficient mice with *Leishmania* (Campbell *et al.*, 1996; Kamanaka *et al.*, 1996; Soong *et al.*, 1996), whereas there is priming for T cell proliferation and IFN-γ production following *M. tuberculosis* infection in CD40L deficient mice (Campos *et al.*, 1998). If the CD40-CD40L interaction is not crucial for the induction of a Th1 response to a mycobacterial infection, then there must be some compensatory mechanism, which would lead to a Th1 response and macrophage activation.

The interaction of *M. tuberculosis* with human dendritic cells was studied *in vitro* (Henderson *et al.*, 1997). Cultured human dendritic cells were capable of phagocytosing live *M. tuberculosis* and this resulted in increased surface expression of costimulatory molecules, including CD80, CD40, and CD54 as well as MHC class I molecules. In addition, infected dendritic cells secreted elevated levels of cytokines, including TNF-α, IL-1, and IL-12. These results suggest that in addition to activation of dendritic cells to become competent antigen presenting cells in response to *M. tuberculosis*, they also secrete cytokines which could potentially induce macrophage activation and Th1 development. IL-12 would induce antigen specific T cells to secrete IFN-γ, which

together with TNF-α induces activation of macrophages. Another study has shown that IL-12 is not the first cytokine to be produced in a mycobacterial infection (Flesch *et al.*, 1995). Murine macrophages were infected with BCG *in vitro* to assess whether IL-12 production depended on other cytokines. Murine macrophages infected with BCG *in vitro* were only capable of producing IL-12 if they had been primed with rIFN-γ. Stimulation with rIFN-γ alone or infection with BCG alone did not induce expression of IL-12. To investigate the role of endogenously produced TNF-α in the induction of IL-12, an anti-TNF-α antibody was used. Addition of anti-TNF-α to infected murine macrophages in the presence of rIFN-γ significantly reduced IL-12 production, indicating that IL-12 production by macrophages is dependent on both IFN-γ and TNF-α. To investigate the influence of IFN-γ and TNF-α on IL-12 induction *in vivo*, IFN-γ-Receptor deficient- and TNFR1 deficient-mice were infected with BCG and spleen cells were analysed for IL-12 mRNA and IL-12 protein 3 h after infection. Splenocytes from both mutant strains failed to express IL-12 (determined by ELISA) after infection with BCG as analysed by RT-PCR. *In vitro* stimulation of spleen cells with rIFN-γ or BCG did not induce IL-12 production, whereas splenocytes from control mice did produce IL-12. The authors argue that IL-12 production by macrophages in response to mycobacterial infection depends on IFN-γ and TNF-α, therefore macrophage derived IL-12 is not the first cytokine to induce a protective antimycobacterial immunity mediated by Th1 cells. In view of the two studies discussed above, one can speculate that dendritic cells which have phagocytosed *Mycobacterium sp.* would secrete sufficient levels of IL-12, which would activate antigen specific NK and T cells to secrete IFN-γ. IFN-γ and TNF-α would then activate IL-12 production in macrophages.

It has recently been reported that *Toxoplasma gondii* another intracellular pathogen, induces *in vivo* IL-12 production by dendritic cells independently of the CD40-CD40L interaction (Sousa *et al.*, 1997). Macrophages infected *in vitro* with *T. gondii* did not produce any detectable levels of IL-12 unless primed with exogenous IFN-γ, in contrast, whole splenocytes produced significant levels of IL-12 after infection with *T. gondii*.

When spleen cells were separated into adherent and nonadherent cells, *T. gondii* induced IL-12 production was found in the adherent fraction composed mainly of macrophages and dendritic cells. To determine if IL-12 production by spleen cells in response to *T. gondii* also occurred *in vivo*, mice were infected with *T. gondii* and splenocytes were analysed for IL-12 production. Maximum IL-12 production could be detected as early as three hours after infection. The IL-12 produced *in vivo* appeared to be active because spleen cells from *T. gondii* infected mice produced increased levels of IFN-γ when restimulated *in vitro* with *T. gondii*. This enhancement of IFN-γ production was specifically dependent on IL-12 induction *in vivo* as it was not seen in IL-12 deficient mice. These results demonstrate that spleen cells can produce IL-12 in response to *T. gondii*, and that IL-12 production *in vivo* can prime an IFN-γ response. Immunohistochemical staining of spleen sections from *T. gondii* infected mice revealed that dendritic cells were responsible for the production of IL-12 but not by splenic macrophages since production of IL-12, by macrophages, could not be detected *in vitro* three hours after infection. The major mechanisms involved in IL-12 induction appear to be signaling through dendritic cell surface CD40, after cross-linking by T cell expressed CD40L. Spleen cells from CD40L deficient mice infected with *T. gondii* secreted substantial levels of IL-12, suggesting that *T. gondii* induced IL-12 production by dendritic cells did not require cross-linking of CD40 on dendritic cells by CD40L on T cells. To exclude the possibility that other cognate T cell-dendritic cell interactions may be responsible for IL-12 production, SCID mice were infected with *T. gondii*. SCID spleen sections showed IL-12 staining comparable to wild-type controls and spleen cells stimulated in vitro with *T. gondii* also produced high levels of IL-12. Based on these results, a model is proposed for the role of macrophage versus dendritic cell derived IL-12 in immunity to microbial infections. Dendritic cells produce IL-12 early in infection due to direct stimulation by microbial stimulation, and present antigen to antigen-specific T cells. During the DC-T cell interaction, engagement of CD40 on the DC by CD40L on the T cell induces the continued production of IL-12 by DC, further driving Th1 development. DC derived IL-12 might also activate NK cells in lymphoid tissue, which

then produce IFN-γ, further stimulating T helper cells to differentiate towards Th1 effectors. On the other hand, IL-12 produced by macrophages in response to microbial infection could be important at the site of infection. Macrophage derived IL-12 could then further stimulate NK and T cells to produce IFN-γ, which would then increase the microbicidal activity of the macrophages.

The results presented in this thesis indicate that the CD40-CD40L interaction is important for the development of protective immunity against infection with *Mycobacterium Bovis* (BCG). This is in contradiction to experiments where CD40L deficient mice were infected with *Mycobacterium tuberculosis*. These experiments revealed that protective Th1 immunity against *M. tuberculosis* developed in CD40L deficient mice (Campos *et al.*, 1998). The discrepancy in these two studies could be due to the different genetic backgrounds of the two groups of mice. The CD40 deficient mice used for experiments in this thesis were on a (129SvxC57BL/6)F2 background, whereas the CD40L deficient mice were on a C57BL/6 background (backcrossed for seven generations). C57BL/6 and BALB/c mice both have the same allele of the *bcg* gene. In a study by Pelletier and co-workers, resistance or susceptiblity was determined by the number of CFU (BCG) recovered from the spleens of infected mice within the first 3-4 weeks after infection (Pelletier et al. 1982). However *bcg* is only one of the many genes that may govern the susceptibility or resistance of a certain strain of mouse to an infectious disease. If these two strains of mice are infected with *M. tuberculosis*, C57BL/6 mice survive more than 140 days, whereas BALB/c mice succumb after 42 days. Therefore, despite the same susceptibility gene at the *bcg* locus , C57BL/6 mice are highly resistant to infection with *M. tuberculosis*. The increased susceptibility to infection with BCG in CD40 deficient mice could be attributed to a 129Sv susceptible genetic background. This explanation is called into question by some unpublished observations quoted by Flynn *et al.* (1996) who found that 129Sv and (129SvxC57BL/6)F1 mice have similar infection profiles as C57BL/6 mice, when infected with *M. tuberculosis*.

Chapter 7: Conclusion

It is well established that cell-cell interactions play a pivotal role in the regulation of the immune response. Attempts to identify interacting molecules on B and T cells led to the discovery of the receptor-ligand pair CD40-CD40L, which in recent years has been shown to be important in both humoral and cell-mediated immunity. Antibodies cross-linking CD40 on B cells were found to induce proliferation and cross-linking CD40 in the presence of cytokines was shown to induce immunoglobulin class switching and secretion of immunoglobulins. The phenotype of hyper IgM syndrome, low or absent IgG, IgA and IgE, indicated a failure in the ability to switch from IgM to the other immunoglobulin isotypes. The development of a soluble CD40 (sCD40) molecule, by fusing the extracellular domain of human CD40 to the constant region of human IgG1, was an important step in the identification of CD40L function. The use of this chimaeric protein in the analysis of T cells from patients suffering from hyper IgM syndrome, revealed that T cells from these patients were unable to bind sCD40. The lack of sCD40 binding was due to mutations in the CD40L protein. In addition to low or absent isotype switched immunoglobulins, patients with hyper IgM syndrome displayed no germinal centres.

The discovery of homologous recombination in embryonic stem (ES) cells and the subsequent generation of "knock-out" mice made it possible for me to produce an *in vivo* model for hyper IgM syndrome by generating mice deficient for CD40 (Chapter 3). Analysis of CD40 deficient mice (Chapter 4) revealed an impaired ability to produce isotype switched immunoglobulins (IgG isotypes, IgA and IgE) and an absence of germinal centres. Immunoglobulin class switching in response to thymus-dependent antigens, in both a primary and secondary response, did not occur in CD40 deficient mice. IgM secretion to thymus-dependent antigens were seen in both primary and secondary immune responses. Immunoglobulin class switching in response to thymus-independent antigens was unaffected by a disrupted CD40-CD40L interaction. As a

result of no germinal centre formation and impaired immunoglobulin class switching, memory B cells did not occur in CD40 deficient mice. Similar results were obtained with CD40L deficient mice. The results presented in this thesis confirm the crucial role of the CD40-CD40L interaction in the initiation of humoral immune responses to thymus-dependent antigens.

When the CD40-CD40L receptor-ligand was first discovered, it was thought that a receptor-ligand pair with a single function (the regulation of humoral immunity) and no redundancy had been found. Since then many studies with CD40L and CD40 deficient mice have revealed that the CD40-CD40L interaction is involved in cell-mediated immunity as well as humoral immunity. Disruption of the CD40-CD40L interaction resulted in impaired T cell activation, either by direct signaling via CD40L or by a reduced production of IL-12, which would impair the development of a Th1 response. It was established that the CD40-CD40L interaction was required for protective immunity to *Leishmania major* and *Leishmania amazonensis*. The lack of protective immunity to Leishmania in CD40L and CD40 deficient mice was due to a reduced production of IL-12 and IFN-γ, resulting in a reduced macrophage activation. The susceptibility to infections with *Pneumocystis carinii* and *Cryptosporidium* in patients suffering from hyper IgM syndrome and the results from the Leishmania studies mentioned above, prompted my investigation of whether CD40 deficient mice were capable of developing a protective Th1 response to infection with mycobacteria (BCG). The studies presented in Chapter 5 indicate that although CD40 deficient mice do develop a Th1 response to BCG, this response is less efficient and these mice produce lower levels of IFN-γ when compared with normal control mice. The CD40 deficient mice are more susceptible to BCG infection and this is indicated by the increased numbers of bacilli found in the spleen and lungs, compared to control mice.

Ever since the discovery of the CD40-CD40L interaction, the different effector functions in which the CD40-CD40L interaction are involved, has increased dramatically. Although the initial studies on CD40 focused on its role in humoral immunity, several

observations suggested that CD40-CD40L interactions played a broader role in the immune system. Studies have shown that APCs are activated following CD40 cross-linking, leading to upregulated expression of costimulatory molecules and cytokines. Likewise, macrophage effector functions are induced following CD40 cross-linking. The CD40-CD40L interaction is also involved in cell-mediated inflammatory responses. Cross-linking CD40 on endothelial cells induces the expression of CD62E (E-selectin), CD106 (vascular cell adhesion molecule; VCAM) and CD54 (intercellular adhesion molecule; ICAM). This demonstrates that endothelial cells respond to stimulation through CD40 by expressing selectins and adhesion molecules that promote homing and extravasation of peripheral blood leukocytes (Stout & Stuttles, 1996).

Three recent studies have shown that T cell help for cytotoxic T lymphocytes (CTL) is mediated by the CD40-CD40L interaction (Bennet *et al.*, 1998; Ridge *et al.*, 1998; Schoenberger *et al.*, 1998). It was shown that mice lacking T helper cells (MHC class II deficient mice) could not mount a CTL response when injected with professional APCs that displayed an antigen recognized by antigen specific CTLs. However, a CTL response could be induced by injecting the mice with an antibody against CD40. Cross-linking CD40 leads to activation of the APCs, a function normally carried out by CD40L expressed on the surface of T helper cells. The anti-CD40 antibody in these experiments acted as a substitute for T helper cells. Mice lacking either CD40 or CD40L could not mount T helper-dependent CTL responses, confirming the findings described above. The traditional model of CTL activation requires that T helper cells and CTLs recognize antigen on the same APC. The T helper cell is activated and secretes IL-2 which contributes to activation of the CTL, a three cell interaction. A new model has been proposed, the sequential two-cell interaction where the APC becomes activated via CD40 ligation by the T helper cell and is "conditioned" or activated in some way such that it is then able to activate CTL's. There is therefore no need for both the T helper cell and the CTL to recognize antigen on the same APC. The ever widening effector functions of the

CD40-CD40L interaction in the regulation of the immune system means that CD40 deficient mice will prove a very valuable tool for future experiments.

One of the first things that I would like to do is to follow on from the studies described in this thesis on CD40 deficient mice infected with BCG. I would like to look at the immune response to *Mycobacterium tuberculosis* in my CD40 deficient mice and I would like to perform a thorough investigation of the levels of different cytokines produced by these mice in response to this infection.

Another interesting study would be the infection of my CD40 deficient mice with the malaria parasite *Plasmodium chabaudi chabaudi* . The course of infection in C57BL/6 mice is characterized by two phases of parasite control, the first is a rapid phase (7-14 days) which decreases the parasitaemia from the peak of infected erythrocytes of 30% to 1-2%, and the second is a long chronic phase (18-60 days) with a small recrudescence in which the parasites are maintained at very low levels before being eliminated. In the first phase, the predominant parasite-specific CD4$^+$ T cell response is that of a Th1 type, producing IFN-γ and IL-2. As the infection progresses the frequency of Th1 cells decreases and the frequency of Th2 cells increases and the second phase of the immune response is predominantly CD4$^+$ T cells producing increased levels of IL-4 which increase production of specific anti-malaria antibodies (Langhorne *et al.*, 1989). The infection of CD40 deficient mice would provide information about the importance of either a Th1 or Th2 response in the development of protective immunity to the erythrocytic stages of a *P. chabaudi chabaudi* infection.

Gene targeting in ES cells is now commonly used to inactivate genes and the resulting "knock-out" mice have become standard tools to understand gene function. The field of gene targeting has advanced enormously with the discovery of a site-specific recombinase (Cre-recombinase) for gene modification in ES cells and mice (Kuhn & Schwenk, 1997). This recombinase allows a much greater flexibility in gene modification. Cre-mediated recombination excises a DNA segment flanked by

recognition sites (*loxP* sites, 34 bp each) as a circular molecule, leaving a single 34 bp *loxP* site in the genome. The modification of the ES cell genome using the CRE/*loxP* recombination system involves two steps: a genetic modification with *loxP* sites (targeting construct with *loxP* sites) is first introduced into the ES cell genome by homologous recombination, subsequently the desired, final structure of the targeted locus is generated by site-specific recombination between two *loxP* sites by transient expression of Cre recombinase. An important application of Cre-mediated recombination is the removal of the selection marker gene to avoid its possible interference with the expression of the targeted gene. Cre-mediated recombination provides the opportunity to study the effects of targeted point-mutations, deletions or insertions on gene function. The ES cells resulting from Cre-mediated recombination would then be injected into blastocysts giving rise to chimaeras, which would be bred for germline transmission. The Cre/*loxP* recombination system can also be used for conditional gene targeting. Conditional gene targeting can be defined as a gene modification which is restricted to certain cell types or developmental stages of the mouse. Conditional gene targeting requires the generation of a mouse strain containing a *loxP*-flanked segment of a target gene (both alleles must be *loxP*-flanked) and a second strain (transgenic) expressing Cre recombinase. The recombinase activity can be expressed constitutively or upon induction in specific cell types or tissues or at different developmental stages by using a cell, tissue or stage specific promoter controlling Cre. A conditional mutant is then generated by crossing these two strains so that the modification occurs in those tissues in a controlled fashion through the activity of the Cre recombinase. The specificity of conditional gene targeting makes this an extremely powerful technology that is now replacing conventional gene targeting methods.

methods.

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Appendix A

Primer sequences

(Genosys Biotechnologies Ltd.)

Primer 1.
CD40 sense, exon 1:
> 5'-cagtcaagcttcccgaagaccccgccctcttcc-3'

Primer 2
CD40 sense, exon 2:
> 5'-agggcagtgtgttacgtgcagtga-3'

Primer 3
CD40 antisense, exon 3:
> 5'-gggttcacagtgtctgtgctg-3'

Primer 4
CD40 antisense, downstream of exon 4:
> 5'-cagtctgagctgactcccaagggagcc-3'

Primer 5
CD40 antisense, exon 5:
> 5'-gatgactgattggagaagaagccg-3'

Primer 6
CD40 sense, exon 6:
> 5'-cagtggatccggggaagtcttcccagaagac-3'

Primer 7
CD40 sense, exon 7:
> 5'-gcgtttaaagttcccctagtgacc-3'

Primer 8
CD40 antisense, exon 8:
> 5'-ggcgggatccaatacaagctctct-3'

Primer 9
CD40 antisense, exon 9:
> 5'-tgcagtgtctcctgcactggagca-3'

Primer 10
CD40 antisense, downstream of exon 9:
> 5'-aagcagttccagggttcagaccag-3'

Primer 11

Neo F3 sense:

5'-cccgattcgcagcgcatcgccttc-3'

Primer 12

T7 primer sense (Promega):

5'-gggcgaattcgagctcggtacccg-3'

Primer 13

Linker oligo (*Sal*I restriction site):

5'-aattttagctagcgtcgactaga-3'

Primer 14

Linker oligo (*Sal*I restriction site):

5'-aatttcagtcgacgctagctaa-3'

Appendix B

Antibodies

FITC-conjugated Rat anti-mouse CD40 monoclonal antibody
Isotype: Rat IgG$_{2a}$, κ. Clone: 3/23. (Pharmingen).
PE-conjugated Rat anti-mouse CD45R/B220 monoclonal
antibody. Isotype: Rat IgG$_{2a}$, κ. Clone: RA3-6B2.
(Pharmingen).
PE-conjugated Rat anti-mouse CD4 (L3T4) monoclonal
antibody. Isotype: Rat (Lou/WS1) IgG$_{2a}$, κ. Clone: H129.19.
(Pharmingen).
FITC-conjugated Rat anti-mouse Ly-2 (CD8a) monoclonal
antibody. Isotype: Rat IgG$_{2a}$. Clone: 53-6.7. (Boehringer
Mannheim).
FITC-conjugated Rat anti-mouse IgM monoclonal antibody.
Isotype: Rat IgG$_{2a}$. Clone: II/41. (Pharmingen).
FITC-conjugated Hamster anti-mouse αβ TCR monoclonal
antibody. Isotype: Armenian hamster IgG. Clone: H57-597.
(Pharmingen).

R4 hybridoma: secreting anti-mouse IFN-γ antibody.
Biotin-conjugated Rat anti-mouse IFN-γ antibody. Cat. no.
18112 D. (Pharmingen).
Streptavidin poly alkaline phosphatase. Cat. no. S-5795.
(Sigma).
Biorad: AP-conjugate substrate kit. Cat.no. 170-6432.

Antibodies used in Immunohistochemistry

Primary antibodies:

> 1.) Biotinylated Peanut agglutinin (Vector laboratories).
>
> 2.) FITC conjugated rat anti-mouse CD40, Clone: 3/23.
>
> (Pharmingen).
>
> 3.) Rabbit anti-CD3 (Jones *et al.*, 1993).
>
> 4.) Rabbit anti-CD79a (Jones et al., 1993).

Secondary antibodies:

> 1.) Avidin horseradish peroxidase conjugate (DAKOPATTS P
>
> 364).
>
> 2.) Rabbit anti-FITC horseradish peroxidase conjugate
>
> (DAKOPATTS P 404).
>
> 3.) Goat anti rabbit horseradish peroxidase conjugate
>
> (DAKOPATTS P 162).
>
> 4.) Avidin alkaline phoshatase conjugate (DAKOPATTS P 365).

Substrate:

> DAB (Diaminobenzidene) (Sigma D 5637)
> Fast red. (Sigma F 1500).

Antibodies used for ELISA

Coating antibodies: Goat anti-mouse IgM (Fc)/7s, goat anti-mouse IgG1 (Fc)/7s, goat anti-mouse IgG2a (Fc)/7s, goat anti-mouse IgG2b (Fc)/7s, goat anti-mouse IgG3 (Fc)/7s, goat anti-mouse IgE (Fc)/7s, (Nordic Immunology).

Standards: Mouse IgM (Sigma, cat. no. M-7394), mouse IgG1 (Sigma, cat. no. M-1398), mouse IgG2a (Sigma, cat.no. M-9144), mouse IgG2b (Sigma cat.no. M-7644), mouse IgG3 (Sigma, cat no. M-1645), mouse IgE (Pharmingen cat.no. 05121D).

Anti mouse-Ig conjugates. (Alkaline phosphatase conjugated):

Goat anti-mouse IgM-AP (cat. no. 1020-01), goat anti-mouse IgG1-AP (cat. no. 1070-04), goat anti-mouse IgG2a-AP (cat. no. 1080-04), goat anti-mouse IgG2b-AP (cat. no. 1090-04), goat anti-mouse IgG3-AP (cat. no. 1100-04), rat anti-mouse IgE-AP (cat. no. 1130-04). Southern Biotechnology Associates.

Substrate: PNPP (p-Nitrophenyl Phosphate). Sigma cat. no. N-9389.

Appendix C

Books

Essentials of Medical Statistics. Betty R. Kirkwood. Blackwell Science. 1988.

Flow Cytometry. A Practical Approach. M. G. Ormerod. Oxford University Press. 1994.

Gene Targeting. A Practical Approach. A. L. Joyner. Oxford University Press. 1993.

Guide to Techniques in Mouse Development. Edited by Paul M. Wasserman, and Melvin L. DePamphilis. 1993. Academic Press.INC.

Laboratory Protocols for Conditional Gene Targeting. Raul M. Torres and Ralf Kuhn. Oxford University Press. 1997.

The Leucocyte Antigen Facts Book. A. Neil Barclay. Academic Press. Second Edition 1997.

Appendix D

Abbreviations

bp	base pair
BGG	Bovine Gamma Globulin
BSA	Bovine Serum Albumin
ddH_2O	double distilled water
DMEM	Dulbecco's Modified Eagle's Media
DMSO	DiMethyl SulfOxide
DNP	2,4-DiNitrophenyl
cDNA	complementary DNA
CD	Cluster of Differentiation

CGG	Chicken gamma globulin
EDTA	EthyleneDiamine Tetra-Acetic acid
ES cells	Embryonic Stem cells
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FITC	Fluorescein IsoThioCyanate
G418	geneticin[tm], Gibco
IU	International Units
kb	kilobase
KLH	Keyhole Limpet Hemocyanin
LIF	Leukaemia Inhibitory Factor
LMP1	Latent infection Membrane Protein 1
mAb	monoclonal Antibody
MHC	Major Histocompatibility Complex
NEO	Neomycin (G418 resistance gene)
NIP	4-hydroxy-3-iodo-5-nitrophenyl
OVA	Ovalbumin
PBS	Phosphate-Buffered Saline
PE	PhycoErythrin
PCR	Polymerase Chain Reaction
PNPP	P-Nitrophenyl PhosPhate.
RNase	Ribonuclease
RT	Room Temperature
RT-PCR	Reverse Transcriptase PCR
s.e.m.	standard error of the mean
SDS	Sodium Dodecyl Sulphate
TBS	Tris Buffered Saline
TK	Thymidine Kinase
TM	Melting Temperature

TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TRAF	TNFR-Associated Factor
UV	Ultraviolet