

T cell function: effects of MHC level, APC and apoptosis

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Submitted for the examination for the degree of PhD

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All the work in this thesis was carried out in the

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This work was supported by the Medical Research Council

Abstract:

A number of models have been used to study the effects of different developmental factors and regulatory mechanisms on T cell function.

During selection in the thymus, there is thought to be a window of 'avidity', into which the TCR/MHC interaction must fall, a combination of the number of TCR/MHC interactions and the affinity of the interaction, to allow positive and negative selection. The influence of the level of MHC class II on selection was examined by generating mice expressing class II at low levels, altering the avidity selection thresholds. Examining the repertoire of TCRs selected showed that in such mice, certain V α /V β regions are used preferentially. CDR3 β regions were sequenced, to see whether there were other alterations in repertoire. The data suggest that decreased MHC class II expression influences the structure of the TCRs selected, adding further evidence for the avidity theory. Alloreactivity was also examined in these mice, by determining their ability to generate MLR and to mount graft rejection responses in the presence of reduced MHC class II levels.

Dexamethasone is commonly used as an immunosuppressive steroid, with a wide range of effects. Dexamethasone was used to induce apoptosis in male thymocytes, and it was shown that apoptotic cells when administered *in vivo* are less immunogenic than untreated thymocytes and can induce non-responsiveness in female mice. In work with the HY- skin-graft system, the mechanism of tolerance induction was investigated. The role of dexamethasone on dendritic cell (DC) development was also examined. The addition of dexamethasone early in BMDC cultures appears to inhibit development, preventing them from stimulating CD4⁺ but not CD8⁺ T cell clones. When DEXDCs are re-cultured in the absence of DEX, they regain full stimulatory capacity. Suppression of the *in vitro* response is less effective as female mice injected with male DEXDCs show rapid graft rejection similar to that seen when untreated male DCs are given.

Acknowledgements:

"I do not feel obliged to believe that the same God who has endowed us with sense, reason, and intellect has intended us to forgo their use." -Galileo

I would like to thank my supervisors, Dr. Julian Dyson and Prof. Liz Simpson for all their help not only through this entire project, but also for reading this thesis for me on a few occasions. Thank you Julian for your direction, encouragement, supervision and enthusiasm even when things weren't quite going as we'd 'hoped' (and I don't want to do any more mini-preps for quite a while!). I would also like to thank Liz for your help, being always ready to check out new data as well as being a great devil's advocate, thinking of all possibilities and to both Julian and Liz for answering the many questions of a non-immunologist trying to learn immunology.

Thanks to everybody else in, or who used to be in T.B: especially Diana (for all your help at the beginning... and then through the rest of it), Edd (for help with grafting and immunisations etc and as a companion novice skier) and Hamlata for all your help with grafting; Jean for all the typing; to Halima, Selina and Soumaya for the fun and amusement, together with Nate; to Di for the chats in TC and many thanks to Caroline, Maggie, Istvan, Chai, Cristina, Stefan, Faisal, Danny, James, Viv, Phil, sPam and Reshma for all your help, advice and for making it a great place to work. Special thanks must go to Mum, Dad, Steve and Grandparents, as well as other friends and relatives who have been supportive and encouraging throughout this work, including my best friend- "who sticks closer than a brother". Also this is for Aunt Rose and Uncle Jack, my 'adopted grandparents', who want to see me graduate this time around.

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Chapter 1.

Introduction

The immune system is a complex, multi-organ system, comprised of a number of cell-types that interact to prevent, and if that fails, to clear infections from the body. The first line of defence is the skin and the layers of epithelial cells, forming a physical barrier inhibiting pathogen entry. These are found both at the external surface of the skin and at internal epithelia on mucous membranes such as in the lung. In addition to a barrier function, cilia on the surface of epithelial cells enable the movement of secreted mucus, inhibiting both pathogen adhesion and subsequent penetration. This is commonly observed in infections such as influenza, where the production of mucus by epithelia in the respiratory tract facilitates removal of both dead cells as well as virus from the tract.

Cells in the epithelium secrete a variety of compounds that exhibit anti-bacterial properties. These include fatty acids that alter the pH of the surface such that it is inhospitable to many pathogens; sweat and tears containing lysozyme to cleave bacterial cell walls; and cryptidins, anti-bacterial peptides produced by Paneth cells in the small intestine. A third form of protection associated with the epithelium is other bacteria that make up the normal flora of the epidermis. The presence of normal flora aids in preventing pathogen colonisation and entry by competition for nutrients and attachment to the host epithelial cells, as well as in some cases also producing anti-microbial substances such as colicins made by some *E. coli*.

If the outer layer of defence is breached, other arms of the immune system can come into play. There are two main branches of the immune system, innate and adaptive that for many years have been treated as separate entities, yet more recently it has been seen that they are probably linked far more closely than was previously thought.

1.1: The innate and adaptive immune systems- a brief overview

The innate immune system is characterised by being constitutively expressed, independent of whether an infection is occurring or not, allowing a rapid response when required. Many factors make up the innate response, including the complement protein complex, which can be activated in two ways.

The classical pathway of complement activation is dependent on production of antibodies by the adaptive immune system (B cells) and subsequent, consecutive binding of the various subunits to the surface of pathogens. The alternative pathway is able to respond in a quicker manner to infection, such that the C3 subunit is spontaneously cleaved to initiate complex formation and attack of the pathogen. Host cells are protected from attack and subsequent lysis or opsonization by various constitutively expressed complement inhibitory proteins.

Other, important players in the innate response include production of interferons that induce an anti-viral state in non-infected cells inhibiting viral replication, and phagocytes such as macrophages and neutrophils. Macrophages patrol tissues, able to opsonize pathogens (pathogens coated in complement complex or antibodies) as well as both apoptotic and necrotic host tissues that they subsequently ingest by either phagocytosis or endocytosis. The ingested material is degraded in lysosomes within the phagocyte, either to prevent subsequent infection, or to alert the immune system by presentation of antigen to the adaptive immune system. On ingestion, phagocytes are stimulated to secrete a number of immunomodulatory cytokines in order to recruit other phagocytes to the site of infection, as well as triggering an inflammatory response within the infected tissue. Binding of antibodies to infected cells triggers binding and subsequent killing by specialised cells including natural killer (NK cells). Fc receptors on the surface of NK cells are cross-linked by surface-bound antibody, triggering release of perforin and granzymes from within the NK cell and death of the infected cell.

The second arm of the immune response is the adaptive system that is highly specific, yet diverse, and able to respond to a vast range of potential antigens. It includes lymphocytes such as T and B cells and is characterised by rapid, enhanced response on second exposure to a non-self antigen, a phenomenon known as immunological memory. On initial encounter with a foreign antigen, the initial immune response is the innate, while the adaptive system takes longer to mount a primary response, ~7 days after infection has begun. On second challenge with the same antigen, the adaptive response has developed 'memory' of the first encounter through a number of mechanisms, enabling the system to respond more quickly and with a higher magnitude of response. An important feature of the adaptive response is the ability to discriminate between self and non-self antigens and respond appropriately. Loss of this control and regulation can have drastic consequences, through the development of inappropriate responses to self, known as autoimmunity.

Both B and T cells develop from multi-potent stem cells in the bone marrow and express specific receptors on their cell surface. B cells express specific receptors at the cell surface known as antibodies or immunoglobulins. These are cell-surface bound, but can also be secreted by the B cell into the surrounding environment. On initial encounter with an appropriate antigen, the naïve B cell is stimulated to divide and produce B cell blasts (or plasma cells) that secrete vast quantities of the specific antibody made by the B cell; and memory B cells that remain for long periods of time in case of subsequent encounter with the same antigen. B cells undergo a process of affinity maturation, such that mutations in the receptor genes allow formation and selection of higher affinity variants of the antibody, improving the effectiveness of the B cell with increasing time of exposure. In order to function optimally, B cells require co-ordinating signals from T cells that regulate their function.

T cells develop from the bone marrow, but undergo processes of selection and development in the thymus and express a specific receptor on the cell surface known as the T cell receptor (TCR). TCRs are able to interact with peptide antigens bound to molecules called major histocompatibility complex (MHC) class I or II on the surface of antigen-presenting cells (APCs) such as dendritic cells (DCs), macrophages (involved in phagocytosis of dead cells, debris and pathogens) and B cells (involved in production of antibodies). MHC class I is almost ubiquitously expressed, both on APCs and on other cell-types, while MHC class II is induced in other tissues during inflammatory responses. One role of the MHC class I/II molecule is to ensure self/non-self discrimination, such that TCRs should be able to bind to antigens presented in the context of self-MHC. On encountering appropriate antigen/MHC complexes, signals are transduced through TCR-associated transmembrane signalling molecules to activate the T cell. If this is accompanied by appropriate activating co-stimulatory signals such as through the CD28 co-receptor, the T cell will proliferate, stimulating B cells or killing infected cells depending on the function of the T cell. When an infection has been successfully cleared, most cells of the specific clones that have expanded to deal with the pathogen will die by apoptosis, leaving a small population of memory T cells on guard against subsequent encounter with the same antigen.

1.2: MHC class I and II- structure and gene regulation

The human MHC locus is a complex, highly polymorphic region located on the short arm of chromosome 6, spanning about 4Mb of DNA. Known as HLA (Human Leukocyte Antigens), there are around 80 known genes, many of the products of which are involved in antigen processing and presentation. The HLA locus is divided into three main sections. The first, located towards the telomere contains around 20 MHC class I loci, while towards the centromere is a second region containing at least 15 MHC class II loci. In between the two segments is a third, gene-dense group known as MHC class III, which encodes mainly secreted proteins such as complement components (C2/4) and tumour necrosis factors A and B. The human MHC class I loci are divided into three clusters, the classical class I loci known as HLA-A, -B and -C, while class II contains around 13 different loci, encoding both α and β -chains, subdivided into four separate clusters of genes, known as HLA-DR, -DQ, -DP and -DZ (Klein 1986). In addition to classical HLA- class I, the human locus also contains other, less polymorphic class-I-like genes such as HLA-E and -F. Other non-class I/II genes include the two transmembrane transporter proteins TAP1 and 2 (transporter associated with antigen processing) that are critical for the transfer of peptides into the lumen of the ER for loading onto MHC class I molecules, as well as LMP2 and 7 that are subunits of the proteasome, a large proteolytically active complex, involved in degradation of proteins into peptides for antigen presentation (Trowsdale 1993).

The murine MHC locus, known as H2 is located on chromosome 17 spanning approximately 2Mb. Chromosome 2 (*B2m*- encoding β 2-microglobulin) and 18 (*Ii*- encoding the γ -chain of class II molecules) encode genes essential for the cell surface expression of MHC class I and II molecules. The murine MHC class I locus is divided into two sections, split by regions encoding MHC class II and III loci. The classical murine H2- class I genes are H2-K, -D and -L, and the class II are known as H2-A and H2-E (Klein 1986). As in the human HLA-locus, the mouse H2 class I locus also contains non-classical class I-like molecules, located at the telomeric end of the H2 region, known as Q, T and M. In humans and to a similar extent in mouse, some of the non-classical molecules are pseudogenes, that are thought to be decaying with time, with increasing numbers of mutations and thus are not expressed as functional protein products.

MHC class I molecules are transmembrane heterodimers expressed on the surface of many cell-types. The heavy α -chain is ~45kDa and has three extracellular

domains $\alpha 1$ -3, which associate non-covalently with $\beta 2$ -microglobulin, a ~12kDa single immunoglobulin domain with limited polymorphism to form the functional receptor. Other domains of the α -chain span the plasma membrane and lie within the cytoplasm, whilst $\alpha 1$ and $\alpha 2$ domains form a peptide-binding groove composed of eight β -strands and two α -helices. The $\alpha 1$ and $\alpha 2$ domains each contribute four β -strands and one α -helix to the peptide-binding groove (Bjorkman, Saper et al. 1987). Peptides either of viral or self-origin, most commonly 8-11 amino acids in length, are loaded into the peptide-binding groove of MHC class I molecules before transportation to the cell surface (Maffei and Harris 1998). The MHC class I binding groove is closed at both ends, and peptides are anchored in various binding pockets in the groove, but longer peptides can bulge at the centre (Guo, Jardetzky et al. 1992).

Peptide motifs are restricted by specific pockets in the binding groove, such that there are a number of binding pockets, defined by highly polymorphic residues at the N-terminal region of the heavy chain such that only peptides with certain amino acid anchor residues can be bound. The bound peptide is therefore influenced by the sequence of the MHC class I allele, such that amino acids with certain side chains are excluded by size, hydrophobicity or charge from particular binding pockets (Maffei and Harris 1998).

Presentation of non-self peptides at the cell surface in the context of MHC class I act as a warning signal to the immune system that something is amiss. Recognition of the MHC class I/peptide complex by the TCR on specific $CD8^+$ cytotoxic T cells (T_{cyt}) in combination with costimulatory molecule interactions such as CD80/86 (B7-1 and B7-2) with CD28 stimulates the cytotoxic T cell to proliferate, differentiate and produce perforin, a multi-subunit attack complex that will insert into the membrane and kill the infected cell, before the virus has had a chance to replicate if directed against early proteins.

The MHC class II molecule is a transmembrane heterodimer of α - (~35kDa) and (~30 kDa) β -chains, expressed on the surface of APCs such as DCs, macrophages and B cells, as well as on thymic epithelia. MHC class II expression on APCs, particularly DCs is upregulated in response to various activating/maturation stimuli such as bacterial lipopolysaccharide (LPS), double-stranded DNA and CD40 ligation. Each of the chains has two extracellular domains with the N-terminal domains of each chain forming the binding groove. This is made up from an anti-parallel β -sheet floor surrounded by two

α -helices, with the binding groove open at either end allowing longer peptides of 10-24 amino acids to be presented (Maffei and Harris 1998).

Unlike the precise cleavages in MHC class I-associated peptides, those associated with class II are often sets of related peptides differing at both amino and carboxy termini. Similar to MHC class I, polymorphism in the peptide-binding groove influences the amino acid anchor pockets and thus the spectrum of peptides that can be bound by particular MHC isotypes and haplotypes.

1.3: Antigen processing and presentation- endogenous processing

In order for antigens to be presented in the context of MHC molecules at the cell surface, proteins must first be digested into suitably sized peptides, loaded into the peptide-binding groove and transported to the plasma membrane for presentation at the cell surface.

Peptides destined for presentation in the context of MHC class I molecules generally enter the endogenous pathway (Pamer and Cresswell 1998). When a cell is virally infected, proteins from the virus found within the cytoplasm are degraded into MHC class I binding peptides. A number of different features mark cytosolic proteins for degradation. Proteins containing PEST sequences (enriched for proline, glutamine, serine and threonine residues) are quickly degraded in the cytosol, while other proteins have regions known as destruction boxes. The most well characterised method of directing proteins for destruction is ubiquitination (Pamer and Cresswell 1998). Ubiquitination involves modification of proteins at targeted lysine residues in the protein by addition of ubiquitin, catalysed by three enzymes E1-3. Once ubiquitin has been attached, the protein unfolds and enters the degradative pathway. However not all MHC class I-presented peptides derive from proteins that are ubiquitinated. After being marked for destruction, proteins are degraded by the proteasome, a multi-subunit proteolytic complex, comprised of a number of catalytic and regulatory subunits including LMP2 and LMP7, both encoded within the MHC locus. It is thought that LMP subunits may be able to direct the specificity of cleavage in a protein to different consensus sequences, so that cleavage occurs after hydrophobic or basic residues (Pamer and Cresswell 1998). LMP subunits are inducible by IFN- γ and replace some of the constitutively expressed products.

Once proteins are degraded into constituent peptides, they are transported to the site of MHC class I loading. Transporter associated with antigen processing (TAP) is a heterodimer of TAP1 and TAP2, encoded by genes within the MHC locus and is a transmembrane transport protein of the ABC transporter family, able to translocate peptides from the cytosol to the lumen of the endoplasmic reticulum (ER) in an ATP-dependent manner. It is suggested that longer peptides are transported into the lumen of the ER by TAP, where further degradation catalysed by endopeptidases occurs. A model for the assembly of MHC class I/peptide has been proposed involving interactions between newly synthesized MHC class I heavy chain, $\beta 2m$ and a number of chaperone proteins associated with complex formation including calnexin and calreticulin. It is thought that newly synthesized heavy chain associates with calnexin. On interaction with $\beta 2m$, calnexin and calreticulin are exchanged and subsequently interact with the TAP peptide transporter for peptide loading. In the absence of heavy chain it is thought that $\beta 2m$ associates with another chaperone, tapasin, which interacts with TAP. On formation of heavy chain/ $\beta 2m$ and exchange of calnexin for calreticulin, tapasin is thought form a multimer (possibly four tapasin molecules) forming a multi-heavy chain/ $\beta 2m$ complex, locating the complex close to TAP for peptide loading (Pamer and Cresswell 1998). Peptides are loaded into the binding groove before dissociation from the chaperones and vesicular transport from the ER to the surface of the cell for presentation. MHC class I molecules contain peptides derived both from pathogens if infected and of self-origin, while the processes of positive and negative selection remove thymocytes expressing TCRs capable of interacting with self-MHC/peptide complexes.

An alternative pathway of antigen processing, cross-priming, also operates (Bevan 1976), in which antigen derived from exogenous proteins is presented. This process is thought to occur in macrophages and DCs (Lane and Brocker 1999) either in a TAP/proteosome dependent or independent manner. It is thought that antigens are internalised by phagocytosis or micropinocytosis and escape into the cytosol for presentation by MHC class I.

1.4: Exogenous antigen processing

Exogenous antigen processing is the process by which proteins that have been phagocytosed or internalised by APCs are degraded and loaded principally onto MHC class II molecules for presentation. Professional APCs including DCs are capable of

antigen uptake by a number of mechanisms including specific receptor-mediated endocytosis through a clathrin-pit dependent mechanism, phagocytosis and macropinocytosis. Phagocytosis and macropinocytosis are involved in the uptake of large particles or large volumes of liquid respectively into vesicles within the APC (Watts 1997). Vesicles known as early endosomes migrate from the surface of the cell and as they mature, the pH in the endosome decreases (known as late endosomes). A number of proteases are associated with degradation in acidic endosomes including cysteine and aspartate proteases, both exopeptidases such as cathepsins H and B; and endopeptidases such as cathepsins D, E and L. However the dominant roles are thought to be played by cathepsins D and E so that the epitopes are not destructively degraded, but are processed to peptides of 13-18 amino acids in length (Geuze 1998). Recent work has found an IFN- γ inducible, lysosomal thiol reductase (GILT) enzyme in both human and murine endocytic vesicles involved in breaking disulphide bonds in proteins. GILT-deficient mice were shown to have defective presentation of HEL-derived peptides to T cell hybridomas (Maric, Arunachalam et al. 2001). MHC class II molecules are synthesised and assemble in the ER as heterodimers, associated with the type-II transmembrane protein, invariant chain (Ii). This is thought to act as a chaperone, aiding in folding of MHC class II, preventing peptides in the ER from binding through association of CLIP, a region of Ii (class II associated Ii peptide) with the peptide-binding groove (Roche, Teletski et al. 1993), as well as directing the newly synthesised dimer to the endocytic compartments. Three Ii molecules form a trimer, binding three MHC class II molecules, forming a nonamer (Roche, Marks et al. 1991).

After assembly, the complex moves into the Golgi and then into the endocytic pathway. It is currently unclear which vesicles are associated with peptide loading, as evidence for MHC class II enriched compartments (MIIC) or class II associated vesicles (CIIV) have both been described (Geuze 1998). Both MIIC and CIIV vesicles have been isolated and found to have intracellular MHC class II and little Ii (although CIIV may have less Ii than MIIC). As the endosome matures and proteolytic activity increases, Ii is degraded leaving the CLIP bound in the peptide-binding groove (Watts 1997). Both compartments are thought to have peptide-bound MHC class II as it has been shown that MHC class II, in the absence of peptide, is relatively unstable (Germain and Hendrix 1991).

Endocytic vesicles are thought to fuse with the transport endosomes, delivering the peptides to the MHC class II loading compartments and, in the acidic environment of the vesicles, CLIP and antigenic peptides are exchanged in a process mediated by the

class II-like molecule DM (H2-M). Loaded MHC class II dimers are transported to the cell surface by vesicles, fusing with the plasma membrane, delivering class II to the surface by exocytosis. It is also thought that in this manner, vesicles known as exosomes can be secreted. In addition to using newly synthesised MHC class II for peptide loading, surface MHC class II molecules may be internalised by endocytosis and thus recycled and fed back into the peptide-loading pathway. Inhibitors of protein synthesis do not prevent peptides from being loaded indicating that *de novo* class II synthesis is not always essential for peptide presentation.

1.5: Direct and indirect antigen recognition

In transplantation of tissues between unrelated individuals of the same species, it is clear that there are two pathways of antigen recognition, direct and indirect. When both MHC haplotypes are shared between donor and recipient, rejection responses are weaker than when there are MHC differences. Direct recognition is the process by which recipient T cells recognise MHC/peptide complexes on the surface of donor cells as being non-self and are stimulated to proliferate and respond. Evidence for this pathway comes from the strong proliferation observed in a primary allogeneic H2 mixed lymphocyte reaction, in which T cells are mixed with APCs of a different MHC type. High frequencies of T cells are capable of responding to allogeneic MHC molecules (Liu, Sun et al. 1993). In addition, if a graft is depleted of MHC class I or II positive cells prior to transplantation (or *in vitro* with MLR), the response is reduced such that graft survival is longer and the MLR response decreased (Gould and Auchincloss 1999).

In the absence of MHC class I and II from donor tissue, grafts can still be rejected (Lee, Grusby et al. 1997). In this situation, the indirect pathway is the process by which recipient T cells are stimulated by recipient APCs presenting peptides derived from donor sources, commonly, although not exclusively donor MHC molecules (Gould and Auchincloss 1999). It is thought that donor APCs or T cells traffic to draining lymph nodes from the graft, where they are phagocytosed by host APCs and presented in the context of recipient MHC molecules. In order to tolerate transplanted grafts, interference with one or other of the pathways of presentation would be necessary (Womer, Sayegh et al. 2001).

Recent work in the male-specific HY system has demonstrated using HY-specific, H2^d-restricted, TCR transgenic mice that indirect recognition is due to antigens

from the surface of male cells being presented by recipient female APCs (Braun, Grandjean et al. 2001). Transgenic T cells did not respond, either when cultured with H2^k expressing male cells or when injected into H2^k male mice, however female TCR transgenic mice were capable of rejecting male H2^k skin, suggesting that the HY antigen is being processed and presented by host APCs. When cultured, primed transgenic T cells from H2^k male graft rejecting, H2^b mice were capable of responding to H2^k male splenocytes but if the H2^b APCs were depleted, they did not respond. This suggests that in this model, the indirect pathway plays an essential role in the graft rejection process.

1.6: Vesicular transport and transfer of MHC class II molecules

During the processes of exogenous antigen processing and presentation, there are a number of vesicle-mediated transport steps between the site of formation of MHC class II complexes in the endoplasmic reticulum and expression of MHC class II/peptide complexes at the cell surface. MHC class II α and β -chains are dimerized in the endoplasmic reticulum, after which a nonamer is formed using a trimeric invariant chain (Ii) to bind into the peptide-binding groove, stabilising the molecules (Pierre and Mellman 1998). After formation of the nonamer, it is transported in vesicles to the trans-golgi network. From the Golgi, the MHC/Ii enters the endocytic pathway involving a number of intermediate lipid vesicles (endosomes) for transport to the cell surface.

Between the Golgi and the cell surface, the MHC molecules are loaded with class II peptides in vesicles known as MIIC (major histocompatibility complex class II-enriched compartment). This occurs as the endosome matures, on transit to the cell surface. Proteins are phagocytosed and degraded into appropriate length peptides by endopeptidases within lysosomes, and subsequent fusion of the lysosomes with MHC/Ii-containing vesicles allows binding of antigenic peptides for presentation at the cell surface (Watts 1997).

This is followed by transport to the cell surface and presentation on the APC MHC class II molecules, regulated in DCs by stimuli causing maturation such as TNF- α , CD40-ligand and LPS. Activation and maturation causes upregulation of MHC class II expression at the cell surface with subsequent increases in antigen presentation capability (Inaba, Turley et al. 1998). The vesicle-mediated process is useful, allowing sequestration of MHC class II molecules in the non-lysosomal vesicles known as CIIVs,

together with co-stimulatory co-receptors including B7-family and MHC class I. When CIIVs arrive at the cell surface the MHC class II and costimulatory molecules remain concentrated in lipid rafts, facilitating interactions with T cells (Turley, Inaba et al. 2000).

The exogenous pathway of antigen processing is of particular interest in the indirect pathway of antigen presentation in graft rejection. As described previously, it is known that there are two forms of presentation, the direct pathway where non-self MHC molecules on the surface of a graft are recognised as foreign by alloreactive T cells, and the indirect pathway where antigens from the graft are internalised by host APCs, re-processed and presented in the context of self MHC molecules (Gould and Auchincloss 1999). Due to the highly polymorphic nature of MHC molecules, a proportion of the peptides that are reprocessed and recognised are derived from graft MHC (Liu, Sun et al. 1993). It is therefore clear that phagocytosis of graft material by host APCs, particularly DCs and macrophages (Mitchell, Nair et al. 1998) are important in indirect presentation, contributing both MHC class I and II epitopes (Auchincloss, Lee et al. 1993; Gould and Auchincloss 1999; Boisgerault, Liu et al. 2001; Braun, Grandjean et al. 2001).

Similarly, phagocytosis of material by APCs *in vivo* in the absence of inflammation is thought to be involved in maintaining peripheral tolerance both to self-antigens and to those from commensal organisms such as in the intestine (Alpan, Rudomen et al. 2001; Bu, Keshavarzian et al. 2001). It is thought that phagocytosis of antigen in the 'steady-state', such as from apoptotic cells can create a tolerogenic population of immature DCs that are capable of inducing antigen-specific tolerance (Hawiger, Inaba et al. 2001) and that while immature DCs are highly phagocytic, the maturation/activation state of the DC post-phagocytosis plays a regulatory role as to whether T cell responses are initiated or tolerance occurs (Banchereau and Steinman 1998; Sauter, Albert et al. 2000; Steinman, Turley et al. 2000; den Boer, Diehl et al. 2001; Heath and Carbone 2001).

In 1997, Arnold *et al* (Arnold, Davidian et al. 1997), showed that using irradiated rat APCs, it was possible to transfer antigen from the surface of professional APCs to T cells. T cells specific for myelin-basic protein were derived from Lewis rats and cultured with irradiated, peptide-pulsed, syngeneic splenocytes. The peptides used were the cognate peptide MBP and an unrelated conalbumin antigen. When both peptides were presented together on the APCs and cultured with MBP-specific T cells, it was possible to detect transfer of both antigens to the T cells, although the transfer of

the non-cognate antigen was dependent on the presence of the cognate MBP. It is thought that the acquisition of antigen by T cells may be involved in presentation to other T cells (T-T presentation) leading to: cytotoxic function, apoptosis and generation of anergy, for the maintenance of peripheral tolerance.

During T cell activation through ligation of the TCR by MHC molecules, the TCR and costimulatory molecules on the T cell and peptide/MHC molecules on the APC cluster together in lipid-rafts, recently described as the 'immunological synapse' (reviewed in (Bromley, Burack et al. 2001)). MHC class I-green fluorescent protein (GFP) fusion proteins expressed in a dendritic cell-line, *Drosophila* or RMA-S cells were used to present antigen to transgenic 2C T cells. Within five minutes of contact between cells, clusters of class I formed at the site of interaction and in 30 minutes, clusters of MHC class I could be observed in the transgenic T cells, while the size of the surface clusters decreased (Huang, Yang et al. 1999). Transfer of MHC class I is linked to the T cells becoming sensitive to peptide-specific CTL by neighbouring T cells or 'fratricide'. The process of MHC class II molecules moving from APCs to responding T cells has also been shown, using rat T cells specific for MBP (Patel, Arnold et al. 1999). Use of both transformed T cell clones (constitutively expressing MHC class II) and macrophages as APCs, showed that increases in MHC class II expression on responder T cells was regulated in a cycloheximide (and thus protein synthesis)-independent manner. This suggested that the MHC class II was being transferred from the surface of the APCs onto the responder T cells. It was also possible to track this process using a lipophilic dye to show transfer of dye between cells. This was mediated *in vitro* by vesicles containing both MHC class II and peptides, and the vesicles themselves were capable of stimulating in an antigen-specific manner (Arnold and Mannie 1999). T cells acquiring MHC class II/peptide were then capable of presenting peptides to other T cells.

Use of CD28^{-/-} mice has recently shown that transfer of cell surface molecules from APCs to T cells occurs in a CD28-dependent process, such that CD28^{-/-} T cells (either CD4 or CD8) were unable to acquire CD80. It is thought that interaction of CD28 with CD80 on APCs may enable the T cell to co-absorb any co-expressed molecules. Absorption is dependent on the interaction between MHC class II/peptide and TCR (Hwang, Huang et al. 2000). Not surprisingly, these methods of intercellular transfer are dependent on direct cell-cell contact.

A number of antigen presenting cells are capable of producing and secreting vesicles. Work by Raposo *et al* (Raposo, Nijman et al. 1996) showed that transformed B

cell lines secreted MIIC-like vesicles *in vitro*, named exosomes. MIIC vesicles within the B cells could fuse with the plasma membrane of the cells and were purified by a multiple-centrifugation process. Purified exosome preparations were enriched for MHC class II, and when pulsed with a peptide from *Mycobacterium leprae* could directly stimulate specific T cell clones.

Dendritic cells are also known to produce vesicles. Human DCs stimulated by CD8⁺ T cells can be stimulated to secrete lysosomes. In a Ca²⁺ dependent process, the lysosomes are produced, secreting both IL-1 β and cathepsin D, a lysosomal enzyme (Gardella, Andrei et al. 2001). Exosomes (50-90nm in diameter) capable of antigen presentation, are produced by the murine splenic dendritic cell line D1 (Thery, Boussac et al. 2001). Exosomes were purified from the D1 cell line by multiple step centrifugation or filtration protocols and proteins contained within the exosomes analysed by trypsin digestion and mass spectrometry. Apart from MHC class II and costimulatory molecules, a number of interesting proteins were found including: cytoskeletal proteins such as cofilin and tubulin, thought to be involved in exosome formation; membrane transport and signalling molecules such as various annexins and GTPases (e.g. rab7); heat shock proteins known to be immunostimulatory and molecules involved in apoptosis such as thioredoxin peroxidase II (Thery, Boussac et al. 2001). Interestingly if DCs are pulsed with exosomes produced by mouse tumour cells, they are able to stimulate anti-tumour CTL, suggesting that tumour antigens contained within the exosomes are capable of transferring antigens for subsequent presentation by APCs (Wolfers, Lozier et al. 2001). In this model, introduction of the tumour exosome-pulsed BMDCs into mice with existing tumours led to decreased tumour growth and increased survival.

Supernatants from DC cultures stimulated primary allogeneic mixed leukocyte reactions (MLR) that could be blocked with anti-MHC class II antibodies, while DCs incubated with allogeneic supernatant were shown to acquire MHC class II molecules at the cell surface. These data suggest that DCs can act both as donors and acceptors of MHC class II molecules, which may play a role in the indirect presentation pathway in graft rejection (Bedford, Garner et al. 1999).

Recent work (Harshyne, Watkins et al. 2001) used co-culture assays with monkey DCs to examine transfer of membrane-bound material between DCs. Different populations of DCs were labelled with a cytoplasmic (CMFDA) or a lipophilic dye (DiD) and co-cultured under different conditions. Immature DCs were capable of acquiring both membrane-bound and cytoplasmic material from immature or mature

DCs, macrophages, activated T cells or B cells in a cell contact-dependent manner, while macrophages were inefficient at accepting material. The cells from which material had been extracted survived, and the process of transfer could be visualised. Melanoma antigens expressed on DCs were also transferred to other DCs, subsequently stimulating melanoma-specific CTL.

Analysis of a number of different DC-types has suggested that while both splenic and lymph node DCs are capable of donating and receiving MHC molecules, bone-marrow derived DCs are capable only of donating exosomes (Knight and Bedford 2001). These processes have been demonstrated *in vitro*, but until recently, little has been known about the activity of exosomes *in vivo*. Follicular DCs (FDCs), a specific population of DCs involved in presentation of antigen to B and T cells in germinal centres are thought to be unable to synthesize MHC class II molecules themselves. Isolation of FDCs from human tonsil showed that they were closely associated with membrane vesicles expressing MHC class II. Characterisation of the vesicles found a number of other exosome or MIIC-associated proteins. It is suggested that as FDCs are incapable of MHC class II synthesis, they may be a target for MHC class II-bearing exosomes *in vivo* for antigen presentation in germinal centres (Denzer, van Eijk et al. 2000).

Together, these data show that MHC class I and II molecules, as well as costimulatory molecules and antigen, can be transferred between different cell-types as well as within types. This can occur in several ways, either through direct cell-cell contact as in APC-T cell transfer, mediated by TCR and CD28 interactions; by immature DCs 'stripping' material from the membrane and cytoplasm of neighbouring cells as in Harshyne *et al*; or through secreted vesicles known as exosomes. Together, these phenomena (if shown to be relevant *in vivo*) add further complexity to antigen presentation and may play an important role in the indirect pathway of antigen recognition in the graft rejection process.

1.7: Thymocyte development

T cells develop in the thymus from a pluripotential stem cell that originates in the bone marrow. These cells seed the thymus by migrating through blood vessel walls and into the stroma of the thymus. Murine bone marrow cells have recently been tracked (Mori, Shortman et al. 2001) using the marker Ly-5 and transferring bone marrow from Ly-5.2 mice into irradiated Ly-5.1 mice, then performing a secondary transfer of the developing thymocytes into Ly-5.1 mice. They found that T cell precursors were scarce initially after transfer, but after a longer period T cell precursors appeared. Secondary thymocyte transfer showed mainly lymphoid precursors with little evidence for myeloid or multipotent stem cells suggesting that commitment to the T cell lineage occurs preferentially in the thymus.

1.8: Thymocyte differentiation and thymus anatomy

Thymocytes are characterised by expression of three cell surface receptors, CD4, CD8 and the TCR. Upon entering the thymus, the precursor cells are negative for these surface markers. There has been conflicting data as to the precise anatomical point of entry of thymocyte precursors from the blood. The thymus is a highly vascularised dual-lobed organ, positioned above the heart and is made up of distinct anatomical regions. At the centre is the medulla, surrounded by the cortex, made up from specialised thymic medullary or cortical epithelia and protected by a capsular membrane. Studies using bromodeoxyuridine (BrdU) to label the DNA of murine thymocytes (Penit 1988), together with labelling of surface markers suggested that the precursors enter into the subcapsular region of the thymus. However recent work (Lind, Prockop et al. 2001) used BrdU labelled, Ly-5.1 bone marrow injected into Rag-deficient Ly-5.2 mice and examination of the thymus by immunohistochemistry for the presence of labelled, proliferating cells and examined their surface phenotype. This approach suggested that thymocyte precursors enter the thymus at the perimedullary cortex and during subsequent differentiation and maturation migrate through four distinct layers in the cortex, which may correlate with the state of differentiation of the developing thymocyte, each layer representing a point in the maturation pathway of the thymocyte.

At early stages of differentiation, thymocytes are also characterised by CD44 (Pgp-1) and IL-2 receptor- α (IL-2R α - CD25) expression. There are four intermediate stages in the CD4/CD8 double-negative (DN) state of development. DN1 thymocytes express CD44, but low CD25, then express both markers (DN2), lose expression of CD44 (DN3) and finally become negative for both CD44 and CD25 (DN4). During this DN state, induction of TCR β -chain gene rearrangement begins, between DN2 and DN3 (Sebzda, Mariathasan et al. 1999).

1.9: TCR α and β -chain gene rearrangement

The T cell receptor is made up from two chains, either α/β or γ/δ , that pair to form a heterodimer. α - and β -chains of the TCR are generated by rearrangement of a number of gene segments contained in the α - and β -chain loci that are recombined to form the functional chain. The α -chain locus comprises 42 Variable (AV), 61 Joining (J) and 1 Constant (C) segments, while the β -chain locus is made from 47 BV, 2 Diversity (D), 13 J and 1C segments in humans. In mice there are around 87V, 50J and 1C in the α -locus while in the β -locus there are approximately 23V, then two D-J-C clusters, each containing 1D, 6J and 1C regions (Bell, Owen et al. 1995). Further diversity in the potential TCR pool is brought about by the random deletion and addition of P and N nucleotides in junctional regions between gene segments by exonuclease activity and terminal deoxynucleotide transferase (TdT) respectively. Further diversity is achieved by the random pairing of α - and β -chains to form the heterodimer. Davis *et al* (Davis and Bjorkman 1988) have calculated the potential diversity for the α/β TCR as being up to 10^{15} in humans, while recent work (Arstila, Casrouge et al. 1999) has compared TCR usage in both the naïve and memory pools of human TCRs by amplifying and sequencing TCRs containing a particular V β , V β 18. In human blood there were calculated to be around 10^6 different β -chains, each of which was able to pair with at least 25 different α -chains, giving a final diversity of at least 25×10^6 . However although the memory pool (CD45RO+) was around 37% of the total T cells, the diversity only contributed around 1% of the total diversity in the sample tested. In mice, it was recently calculated that in a naïve mouse spleen (DBA/2 or C57BL/6 were used in this analysis) there were approximately 2×10^6 different clones each with around 10 cells/clone. This analysis used Immunoscope, followed by

isolation of the various fluorescent bands and sequencing of the CDR3 regions (Casrouge, Beaudoin et al. 2000).

TCR gene segment rearrangements require RAG1 and 2, protein products of the recombination activating genes 1 and 2 (*Rag1* and 2) (Schatz, Oettinger et al. 1989; Oettinger, Schatz et al. 1990) together with other proteins, and were first discovered by their ability to induce V(D)J recombination in fibroblasts. These *Rag* genes and their products are highly conserved in vertebrates and are crucial for the correct functioning of the adaptive immune response. Indeed, the critical role for RAG1/2 in V(D)J recombination and thymocyte development has been shown both in *Rag1*^{-/-} (Mombaerts, Iacomini et al. 1992) and *Rag2*^{-/-} mice (Shinkai, Rathbun et al. 1992). Both strains have a block in thymocyte development at the CD3/CD4/CD8 negative stage, have reduced thymic cellularity and do not develop peripheral T cells. RAG1/2 are also involved in the rearrangement of immunoglobulin genes in B cells and consequently, defects in RAG genes also prevent the maturation and development of mature B cells from pro-B cell precursors.

In humans, mutations in *RAG1* or 2, particularly in the catalytic region of the enzyme (Schwarz, Hansen-Hagge et al. 1991; Schwarz, Gauss et al. 1996) also prevent maturation of T and B cells, leading to a number of conditions including SCID (severe combined immunodeficiency) and Omenn's Syndrome (OS) (Notarangelo, Villa et al. 1999), both of which are characterised by a lack of circulating B cells and deficiencies and/or malfunction in peripheral T cells and a consequent susceptibility to infection. These conditions are fatal unless treated with allogeneic bone marrow transplantation.

Recombination of the gene segments encoding the TCR chains comprises two stages. The first is a double-strand break (DSB), a cut in the DNA between a defined signal sequence known as a recombination signal sequence (RSS) comprising a heptamer and a nonamer motif, separated by either 12 or 23 b.p., and the coding segment being used. The 12/23 rule, in which recombination will only occur between a gene segment which has an RSS with a 12 b.p. spacer region and one that has a 23 b.p. spacer, ensures that recombination does not occur between same-type segments. The second step involves recombination of the signal segment with the coding region of the joined gene segment. This involves formation of a hair-loop structure that is cleaved out by the RAG proteins (Ramsden, van Gent et al. 1997).

It is currently unclear as to the signal involved in triggering the first wave of RAG expression, function and subsequent β -chain gene rearrangement, although

interleukin- 7 (IL-7) has been implicated in stimulating TCR rearrangement and RAG expression (Muegge, Vila et al. 1993).

Successful rearrangement of β -chain gene segments to form a functional β -chain expressed at the cell surface initiates the next of the stages of development. The β -chain is transported to the surface of the developing thymocyte in complex with a pre-T α -chain, a 33kDa transmembrane protein that pairs with the β -chain allowing temporary cell-surface expression, along with associated molecules of the pre-TCR complex including CD3 family proteins (Borst, Jacobs et al. 1996). The pre-T α chain intracellular domain contains signalling motifs including an SH3 domain and two possible target sequences for protein kinase-C (PKC) phosphorylation. Recent work using retroviral insertion of either constitutively active PKC or a dominant negative form of PKC into RAG-deficient foetal thymic organ cultures (FTOC) have shown that formation of the pre-TCR complex is important in activation of PKC in thymocytes and that PKC then acts as an important control of subsequent stages in development (Michie, Soh et al. 2001), including differentiation to the CD4/CD8 double positive stage and allelic exclusion.

It is thought that tyrosine kinase p56^{lck} plays a role in intracellular signalling at this stage in development; possibly post TCR- β chain rearrangement. Work with either p56^{lck} deficient mice (Molina, Kishihara et al. 1992), or expressing a dominant-negative p56^{lck} (Levin, Anderson et al. 1993), showed that both inhibit thymocyte development beyond the DN stage; while over expression of p56^{lck} (Anderson, Abraham et al. 1992) appears to mimic signalling of the TCR β -rearrangement, as it initiates allelic exclusion of the β -chain locus, allowing TCR α -rearrangement and development of thymocytes to the DP stage. By altering various domains of LCK, it has been possible to show that it functions at several stages in thymocyte development. Chimeric constructs containing alternative Fyn domains have shown that the non-catalytic domains of lck are crucial to differentiation from DN to DP, while the catalytic domains appear to be influential in development from DP to SP (Lin, Longo et al. 2000).

Transition from the DN to DP stage is regulated by expression of integrins, receptors important for adhesion to extracellular matrix proteins such as fibronectin. Expression of a trans-dominant inhibitor of integrins, blocking multiple integrin heterodimers, causes a block of thymocytes being able to adhere to fibronectins and subsequently a partial block in differentiation between DN and DP after expression of CD25. This suggests that integrins may link with LCK-mediated differentiation. In

addition, these mice showed decreased CD4SP thymocytes compared to wild type, suggesting a role for integrin-mediated adhesion in lineage commitment (Schmeissner, Xie et al. 2001).

T cell receptor transgenic mice regularly show decreased thymic cellularity and TCR⁺, CD4/8-negative thymocytes. Recent work has revealed that this may be due to premature expression of the TCR α/β complex, causing inhibition of the DN-DP transition. It was suggested that this occurs in two stages. Formation of the TCR α/β complex initially inhibits the pre-TCR complex formation, while an interaction between a TCR α/β on DN cells with MHC molecules causes a block in the proliferation/differentiation step (Lacorazza, Tucek-Szabo et al. 2001).

1.10: Allelic exclusion

After successful β -chain rearrangement, the non-expressed β -chain locus undergoes a process known as allelic exclusion. When a successful recombination event has occurred, the β -chain loci are blocked from undergoing further recombination events, preventing production of a second β -chain. To investigate the basis for allelic exclusion, a rearranged human TCR β -chain transgene under control of the mouse TCR β enhancer was introduced into mice. There was no expression of mouse TCR β in the periphery of these mice, although hybrid mouse/human-TCRs were observed, showing that expression of the transgenic rearranged β -chain was able to inhibit endogenous β -rearrangements (Rothe, Ryser et al. 1993). This process is also known to occur in rearrangement of the α -locus, although in this case allelic exclusion is incomplete. Incomplete exclusion allows some cells to express two functional α -chains that pair with the β -chain, forming dual V α -T cells, with dual specificity (Elliott 1998).

Recent work (Huang and Kanagawa 2001) has suggested that incomplete exclusion may be an important way of forming the T cell repertoire, by showing that the process is highly co-ordinated, first by rearrangement of a 3'- V α segment followed by a 5'-J α on both chromosomes, then using upstream V α and downstream J α segments until positive selection is achieved, thus optimising the process of selection.

Further work has examined control of allelic exclusion using a transgenic V β 13 segment together with 5' and 3' putative control regions inserted into the mouse locus only 6.8 k.b. upstream of the D β 1 gene segment. The authors (Sieh and Chen 2001)

showed that in the segment inserted, there were control regions that regulated the frequency of recombination. The frequency of recombination of the V β 13 transgene was the same as that for the normal V β 13 locus. However differences were observed in the control of allelic exclusion, as in the presence of a rearranged, functional V β transgene, the inserted V β 13 gene still recombined at the same frequency, implying the existence of distinct allelic exclusion control sequences outside of the region inserted. These findings suggest that recombination control and allelic exclusion are regulated by distinct sequences.

A process known as 'functional allelic exclusion' has been recently suggested by Sant'Angelo *et al* (Sant'Angelo, Cresswell *et al.* 2001). Double TCR transgenic mice (AND/MBP TCRs) were examined by flow cytometry. The AND TCR recognises an E^k-restricted, pigeon cytochrome C peptide and expresses V α 11/V β 3, while the MBP TCR is V α 4/V β 8.2 and specific for a peptide from myelin basic protein. Although both transgenes were present in the T cells of the double transgenic mice, only one TCR was expressed at the cell surface as a functional α/β -heterodimer, either MBP or AND. The protein products of the rearranged TCRs were expressed in the cell and a possible reason for the lack of surface expression may be disruption of formation of one heterodimer in the cell.

Termination of β -chain allelic exclusion is thought to trigger a burst of proliferation (possibly involving p56^{lck} signalling), after which initiation of α -locus rearrangement in the CD4/8 DN thymocyte population occurs (Dudley, Petrie *et al.* 1994; von Boehmer and Fehling 1997), together with upregulation of CD4 and CD8 co-receptors, while TCR expression remains low. In mice, the burst of proliferation that occurs causes an expansion of between 20 and 50-fold in the number of thymocytes over a period of around 2 days between β and α -rearrangement (Penit, Lucas *et al.* 1995; Rodewald and Fehling 1998).

When thymocytes have reached the CD4/CD8 DP stage they undergo two processes of selection known as positive and negative selection (discussed in section 1.12) in order to ensure that the TCRs expressed on the cell surface are effective at binding to self-MHC class I/II molecules. After the processes of selection have occurred, thymocytes that have interacted with MHC class I/peptide complexes down-regulate expression of the CD4 co-receptor while maintaining expression of CD8, while those that interact with MHC class II/peptide down-regulate CD8 and maintain CD4 expression. In this manner thymocytes have become either CD8 or CD4 single positive

cells and are then able to migrate from the thymus into the periphery where they exist as naïve T cells until encounter with appropriate antigen/MHC complexes.

1.11: Mechanisms of CD4/CD8 lineage commitment

1.11.1: The instructive model

The process by which DP cells are selected to become either CD4 or CD8SP is influenced by many different factors. Studies using different TCR transgenic mice have shown a strong link between whether a TCR interacts with MHC class I or II and the co-receptor it expresses on the cell surface. Berg *et al* (Berg, Pullen et al. 1989) showed that a transgenic mouse expressing H2-E^k, as well as a TCR that recognises a Pigeon Cytochrome C (PCC) epitope in the context of H2-E^k, selected a population of CD4 positive T cells that were specific for the PCC epitope, but if the transgenic MHC class II was not present, as in H2-A^b mice, then there was a block in thymocyte development at the CD4/8 DP stage and 10-fold fewer transgene expressing T cells were selected. If H2-E^k expression was limited to the surface of epithelial cells in the thymic cortex, selection of the transgenic cells was restored. Similarly, Kaye *et al* (Kaye, Hsu et al. 1989) used the PCC-specific TCR and H2-E^k double transgenic mouse to show a larger-than-normal CD4SP population in the thymus, together with a population of CD4⁺ T cells in the periphery specific for the PCC epitope.

There are two main models currently considered to account for lineage commitment. The instructive model proposes that engagement of any DP thymocyte with MHC class I/peptide during selection will instruct the DP thymocyte to become a CD8SP thymocyte by downregulation of CD4 gene expression. Conversely those that interact with MHC class II/peptide will become CD4SP by downregulating CD8 expression. Evidence for this model includes work by Itano *et al* (Itano, Salmon et al. 1996) who used a fusion protein known as CD884, a combination of the extracellular domain of CD8 with the intracellular domain of CD4 into transgenic mice. Thymocytes in these mice expressed MHC class I restricted CD4⁺ T cells, similar to those of Seong *et al* (Seong, Chamberlain et al. 1992). This suggested that a signal transduced by ligation of the hybrid co-receptor induces differentiation independent of the TCR-specificity.

1.11.2: The stochastic and asymmetric models

The stochastic model suggests that the process of co-receptor downregulation is random and independent of TCR specificity. DP thymocytes that have downregulated the appropriate co-receptor will survive (Bluestone 1998).

A third way has been suggested, the semi-instructive or asymmetric model, which suggests that commitment to the CD4 lineage is selective, while CD8 is instructive, implying a default pathway for CD4 selection, while in order to become CD8⁺, interaction with MHC class I is necessary. Work by Chan *et al* (Chan, Correia-Neves et al. 1998) used a β -galactosidase reporter gene (LacZ) inserted in place of CD4 to examine the role of CD4 in lineage commitment. By crossing the β -gal/CD4 mice with MHC class II negative or β 2m-deficient, mice expressing MHC class I-restricted transgenic TCRs specific for HY and OVA, or CD4 deficient mice and examining thymic development, various observations were made. In MHC class II deficient mice there was a population of CD4-committed cells, while a smaller population of CD8-committed cells were detectable in β 2m-deficient animals. By restoring CD8 expression to a population of CD4-committed cells in the CD8 transgenic models used (normally these cells would not survive selection), it was possible to rescue the CD4⁺ cells. It was argued that these data showed that there was no evidence for any default CD4-selective pathway.

Work from Sha *et al* (Sha, Nelson et al. 1988) examined selection of CD8⁺ TCR transgenic thymocytes (2C), which showed selective expression on CD8⁺ T cells in the periphery when selected on the appropriate MHC class I molecule. This could be accounted for either by the instructive or stochastic models of selection.

Other reports using MHC-deficient and TCR-transgenic mice have shown that from DP thymocytes it is possible to select TCRs, normally MHC class I-reactive, to the CD4⁺ lineage, suggesting that the mechanism may be of the selective nature. These included mice that constitutively express either transgenic CD4 or CD8. Using MHC class I and/or class II deficient mice, Chan *et al* (Chan, Cosgrove et al. 1993) showed that a population of CD4⁺ thymocytes intermediate between DP and SP occurs in MHC class II-deficient mice, and a similar population of CD8⁺ thymocytes in the absence of MHC class I. Other work has shown that in the absence of CD4 (Matechak, Killeen et al. 1996) transgenic T cells normally restricted by MHC class II can develop along the CD8 pathway, suggesting that differentiation to the CD4 lineage is the default pathway for this particular TCR, but in the absence of CD4 it will direct CD8⁺ differentiation.

Work has also been performed using a transgenic mutant MHC class II-A^b, unable to interact with CD4 because of mutations in the β 2-domain. When crossed with the transgenic TCR specific for a PCC epitope (AND), normally CD4⁺, a population of CD8⁺ thymocytes develop expressing the AND TCR (Riberdy, Mostaghel et al. 1998).

1.11.3: Signalling molecules and lineage commitment

A second factor in the process of lineage commitment is the signals received by the cell. It is currently unclear as to whether the signals received that cause thymocytes to differentiate are quantitatively (alterations in signal intensity) or qualitatively different (distinct signalling molecules for each pathway). As previously described, the tyrosine kinase p56^{lck} is thought to be involved in the development of thymocytes from the DN to DP stage. It has been shown that p56^{lck} is the main signalling molecule activated by engagement of either CD4 or CD8 co-receptor, and it has been suggested that p56^{lck} may play a crucial role in lineage commitment by modulating the amount of signal available to the engaged TCR. It is known that CD4 has a higher affinity for p56^{lck} than CD8 and that around 40% of CD8 molecules are unable to interact with p56^{lck} in the thymocyte due to the absence of a cytoplasmic tail. In line with these observations, interactions between CD4/TCR and MHC class II induces more p56^{lck} activation than CD8/TCR with MHC class I (Basson and Zamoyska 2000). Work with two different transgenic mice strains, the AND (PCC) and the OT-1 (OVA-specific) (Sharp and Hedrick 1999) using foetal thymic organ cultures (FTOC), together with inhibitors of various intracellular signalling molecules including Erk, MAP kinases as well as p56^{lck} showed it was possible to divert MHC class I restricted transgenic T cells to a CD4⁺ phenotype. Introduction of an overactive form of p56^{lck} caused thymocytes from OT-1 mice to develop into CD4⁺ rather than CD8⁺ (Sharp and Hedrick 1999). With expression of an inactive p56^{lck} in MHC class II-restricted TCR transgenic mice (AND), thymocytes no longer developed into CD4⁺ but differentiated to CD8⁺ (Hernandez-Hoyos, Sohn et al. 2000). p56^{lck}-deficient mice showed reduced numbers of DP thymocytes and few SP cells, especially CD4⁺. Introduction of a doxycycline-inducible transgenic p56^{lck} with a human CD2 (T cell lineage) promoter followed by induction with doxycycline causes restoration of CD4SP thymocytes and subsequently peripheral CD4⁺ T cells. Less effect was seen on CD8 selection (Legname, Seddon et al.

2000). Taken together, these data highlight the importance of differential p56^{lck} signalling in the process of lineage commitment.

Other signalling molecules and pathways have been implicated in the process of lineage commitment. It has been shown that if the MAP (mitogen activated protein)-kinase pathway is inhibited through inhibitors of MEK-1 (MAP/Erk kinase) including PD98059 in FTOC, it was possible to inhibit differentiation to CD4SP in AND TCR transgenic mice, while enhancing differentiation to CD8SP (Sharp and Hedrick 1999). It is thought that this process occurs through Erk1 and Erk2 and that the MAP kinase signalling cascade plays a crucial role in differentiation into CD4 but less of a role in CD8 differentiation (Basson and Zamoyska 2000) except at later stages.

It has been suggested that the Notch family of receptors may play a role in lineage commitment, (Robey 1999). Expressing a constitutively active form of Notch (Robey, Chang et al. 1996) led to an increase in CD8SP and a decrease in CD4SP thymocytes suggesting that Notch may enhance selection of CD8⁺ cells, while inhibiting selection of CD4⁺. While an inducible model of Notch1 deficiency has been shown to have dramatic effects on T cell development, work on other cell-types in the thymus, such as thymic DCs, appear not to be affected (Radtke, Ferrero et al. 2000). This suggests that Notch1 may play a specific role in T cell differentiation, whereas other thymic populations are unaffected by Notch deficiency despite suggested common precursors.

It has been suggested that the process of signalling through Notch is modulated by signals through MHC class I/II interacting with TCR and either CD4 or CD8. Two models have been proposed for this process. The lateral signalling model suggests that both Notch and an appropriate ligand are expressed on thymocytes. Interaction of MHC class I with TCR/CD8 complex provides a positive signal that is then amplified by interaction of Notch on the thymocyte undergoing selection with a ligand on other thymocytes causing differentiation to CD8. If the signal is through TCR/CD4 it is a negative signal, again amplified by Notch/ligand interactions causing a CD4 fate. The second model suggests that the Notch ligand is expressed on thymic epithelial cells, available to all cells undergoing selection. Notch signalling would be regulated by MHC recognition such that interaction between MHC class I with TCR/CD8 would enhance Notch signalling, leading to CD8 differentiation, while MHC class II recognition would cause inhibition of Notch signalling and subsequent differentiation to CD4 (Robey 1999).

However more recent data suggested that Notch is not critical for early development. Wolfer *et al* (Wolfer, Bakker et al. 2001) used tissue-specific targeting utilising the *Cre/loxP* system to inactivate Notch in T cell precursors and showed that Notch was not required for T cell development beyond the DN stage. This suggests that some of the initial work maybe affected by the constitutive expression or repression of Notch.

Together these data suggest that it is a combination both of multiple external receptor-ligand interactions together with internal signalling cascades, interact in a complex network to induce commitment to either CD4 or CD8 lineages.

1.12: Positive and negative selection

In order to ensure that thymocytes expressing a TCR are functionally competent to become naïve peripheral T cells, thymocytes undergo processes known as positive and negative selection. These act to eliminate two populations of thymocytes. In order to ensure self-restriction, thymocytes expressing TCRs that do not interact with self-MHC/peptide complexes in the thymus die through a process of neglect, through lack of survival signals through the TCR (programmed cell death). TCRs that interact strongly, with high affinity to self-MHC, are potentially autoreactive and an active process of cell death by apoptosis removes most autoreactive cells. This process is known as central tolerance (Sprent and Kishimoto 2001). The two processes of selection are so stringent that it is thought that only 3-5% of thymocytes survive selection to become mature peripheral T cells (Huesmann, Scott et al. 1991; Shortman, Vremec et al. 1991) and are known as positive and negative selection.

Positive selection of thymocytes is the process through which developing thymocytes expressing TCR interact with MHC/peptide complexes and are signalled to survive, through the TCR. In contrast, negative selection is the process of deletion through active-TCR mediated apoptosis through upregulation of pro-apoptotic and downregulation of a number of anti-apoptotic genes (Williams and Brady 2001).

Many experiments have been done to assess the importance and effects of different parameters in positive and negative selection, including: the cell types involved with respect to thymic anatomy, role of MHC/peptide complexes both of the peptides bound and the level of expression, role of TCR and signalling molecules, timing of positive and negative selection as well as general theories of how the processes occur; yet many of the answers remain unclear.

One of the major questions to be answered is whether the location of positive and negative selection in the thymus and the cells involved are the same or different. Laufer *et al* (Laufer, Glimcher et al. 1999) have recently suggested that the two processes occur in distinct locations and on different cell types with positive selection occurring before negative selection, with positive selection solely on MHC molecules expressed on thymic cortical epithelial cells. Three main types of cells are implicated in selection: cortical epithelium, medullary epithelium and cells derived from bone marrow (B cells, macrophages and DCs). Cosgrove *et al* (Cosgrove, Chan et al. 1992) used mice with altered MHC class II expression: MHC^{-/-}, only expressing H2-E, mice with H2-E only in thymic medulla not cortex and the opposite, mice with H2-E only in the cortex and not medulla, each crossed to a strain with a transgenic TCR selected on H2-E. When the thymic profiles were examined, mice with H2-E in the cortex had normal CD4SP (and CD4⁺ in the periphery), while mice with H2-E only on medulla had almost no CD4SP thymocytes or peripheral T cells. Similar experiments with a number of double TCR transgenic mice in which MHC class II expression was restricted to particular cell types (Laufer, DeKoning et al. 1996), suggested that only cortical epithelial cells regulate positive selection and not medullary epithelia, or bone marrow-derived DCs. This work suggested that the absence of MHC class II from other cell types in the thymus allows escape of autoreactive cells from negative selection and entry into the periphery. Recently MHC class I molecules were expressed only on cortical epithelia and were unable to induce central tolerance, allowing autoreactive CD8⁺ cells to develop, able to lyse syngeneic target cells both *in vitro* and *in vivo* (Capone, Romagnoli et al. 2001). Regulatory CD4⁺CD25⁺ T cells may also be selected on cortical epithelium. Using restricted expression MHC class II molecules, the development of this regulatory population was dependent on MHC class II selection on cortical epithelium. Although CD4⁺CD25⁺ cells are present in MHC class II-deficient mice they are unable to inhibit proliferation of CD4⁺CD25⁻ *in vitro* (Bensinger, Bandeira et al. 2001).

1.13: Cell types and timing of selection

Under certain circumstances, including transplantation of allogeneic bone marrow or bone marrow stem cells, other cell types are thought to play a role in positive selection. In these experiments allogeneic bone marrow was transplanted into mice and the selection of allo-restricted T cells measured, either by graft rejection or *in vitro*

proliferation assays. Allogeneic bone marrow derived cells are able to migrate to the thymus and there participate in positive selection (Li, Hisha et al. 2000; Shizuru, Weissman et al. 2000). Another approach has shown that MHC class I expressing fibroblasts injected into the thymus of MHC class I-deficient mice can induce positive selection (Anderson, Moore et al. 1996). In contrast, MHC class I expression on cells of haematopoietic origin poorly positively selected a small population of CD8⁺ T cells compared with thymic epithelia (Bix and Raulet 1992), while Markowitz *et al* (Markowitz, Auchincloss et al. 1993) used MHC class II deficient mice expressing MHC class II only on bone marrow-derived or thymic stromal cells and showed that in the absence of class II from thymic epithelia, i.e. class II was only expressed on bone marrow derived cells, positive selection of CD4⁺ T cells did not occur. These data suggested that positive selection signals for CD4⁺ and CD8⁺ T cells differ slightly in tissue restriction. The consensus is that while other cells may be capable of inducing positive selection artificially, or even be involved as accessories, the most efficient cell type for inducing positive selection are thymic epithelia.

Negative selection appears mainly to be regulated by bone marrow derived APCs, particularly DCs located in the thymic medulla and corticomedullary junction (Laufer, Glimcher et al. 1999) or in the cortex (Sprent and Kishimoto 2001), while the role of thymic epithelia is more controversial. Earlier work from Lo & Sprent (Lo and Sprent 1986) showed that if macrophages/DCs were selectively destroyed in the thymus, tolerance could no longer be generated, even though epithelial cells still survived, while more recently they have suggested that there is a particular population of medullary epithelium that may be involved (Lo, Reilly et al. 1997). Viret *et al* (Viret, Sant'Angelo et al. 2001) have used TCR transgenic mice crossed with transgenic H2-E α mice expressing a negatively-selecting peptide conjugated to MHC class II with differing cellular localisation in the thymus. All of the double transgenic mice had decreased thymic cellularity and the absence of peripheral CD4⁺ T cells, suggesting that the T cells were being negatively selected in the cortex. Comparison of expression in different cell types showed that both thymic cortical epithelia and thymic DCs were capable of inducing negative selection *in vivo*. In normal B6 mice, due to the absence of self H2-E α the cells develop normally. While APCs may play the dominant role in negative selection, other cell types may also play a role, dependent on the model used and whether *in vitro* or *in vivo*.

Another important factor of selection is the timing of positive and negative selection with respect to each other and also during thymocyte maturation. A number of

groups have used transgenic thymocytes and varied expression of MHC in the thymus to show that positive selection appears to occur late in thymocyte differentiation. Earlier work from Benoist and Mathis (Benoist and Mathis 1989) suggested that positive selection occurs after the transition from DP to SP using restricted H2-E expressing mice. Other authors used human CD4⁺ transgenic mice, with interactions between human CD4 and mouse MHC in MHC class I⁻ mice, to show that positive selection occurs after lineage commitment, i.e. late in thymocyte development (Paterson, Burkly et al. 1994). Male and female HY-specific transgenic TCR mice were examined at different stages in foetal development. At birth, male mice had around 50% of the number of thymocytes compared to females and most were DN, suggesting that negative selection had occurred prior to lineage commitment (Teh, Kishi et al. 1990), although late in development. Zal *et al* (Zal, Volkman et al. 1994) used TCR transgenic mice specific for a complement component C5 and showed that in the absence of C5, CD4SP cells develop that are functional in the periphery, while in the presence of C5 they do not, suggesting that negative selection has occurred after the DP-SP transition, late in development.

In contrast, more recent work using tetramers to examine thymocyte development detected tetramer positive cells early in TCR rearrangement, and that in the presence of the specific peptide cells that bind most strongly to the tetramer are deleted early and that negative selection appears to occur throughout development (Baldwin, Trenchak et al. 1999). Pircher *et al* (Pircher, Burki et al. 1989) showed that transgenic mice expressing a TCR with dual specificity for both the lymphocytic choriomeningitis virus (LCMV) in the context of H2-D^b and the mixed lymphocyte stimulatory antigen (Mls^a) could be tolerised by two different antigens at different stages in differentiation. Interestingly, work with HD (Helper T cell deficient) mice (incapable of generating peripheral CD4⁺ cells, although without defects in antigen presentation or CD4 function, possibly through a mutated signalling pathway molecule) showed that if HD^{-/-} and HD^{+/+} bone marrow were transferred into RAG^{-/-} mice, HD^{-/-} gave rise to only CD8⁺ cells, while HD^{+/+} gave both CD4 and CD8 (Keefe, Dave et al. 1999). Using MHC class II-restricted transgenic T cells it was shown that when on a HD-deficient background, the thymocytes were redirected to CD8 independently of MHC class I. This suggests that the signals that define lineage commitment are separate from those inducing positive selection. Douek *et al* (Douek, Corley et al. 1996) used TUNEL staining to visualise thymocyte apoptosis in transgenic mice, showing that negative selection could occur at different points within the thymus, in the cortex,

medulla or at the cortico-medullary junction. These data suggested that the localisation of selection was dependent on the avidity of the interaction and/or the timing and localisation of the MHC/peptide expression, which would account for the conflicting data previously discussed.

1.14: The role of peptides in selection

The role of the peptide bound to the MHC molecules in thymocyte selection has been analysed in detail. Amino acid side-chains that protrude from the binding groove interact with, and determine, the strength of interaction between the MHC/peptide and the TCR, thus influencing the outcome of selection. A number of groups have examined mutations in the peptide binding groove of MHC class I and shown that this influences the repertoire of CD8⁺ T cells, presumably by altering the self-peptides that can be bound in the groove and that can therefore be involved in positive selection (Goldrath and Bevan 1999). Alterations in either peptide processing or MHC expression in thymic organ cultures are also known to affect selection such that few cells can be selected. However selection was restored if a mixture of peptides were added to thymic organ cultures from TAP-deficient mice (Goldrath and Bevan 1999).

Interestingly a number of genes linked to autoimmune disease are transcribed in the thymus. These include insulin, myelin-basic protein (MBP) and thyroglobulin. Thyroglobulin and MBP mRNA are found in MHC class II⁺ thymic epithelia and DCs (Heath, Moore et al. 1998) and are thought to be involved in tolerance induction to autoantigens. Sebzda *et al* (Sebzda, Wallace et al. 1994) used TCR transgenic thymic cultures with either low or high doses of the cognate peptide and suggested that low doses of peptide allowed positive selection while higher doses induced deletion and therefore tolerance. This implies that the diversity of peptides is an important factor. A number of groups have examined the repertoire of peptides expressed in the thymus and attempted to equate this to function in selection. Bender *et al* (Bender, Mitchell et al. 1999) transferred CFSE-labelled T cells into sub-lethally irradiated hosts and showed that division of CD4⁺ T cells did not occur in the periphery in the presence of MHC class II with only the selecting peptide, derived from E α , covalently bound to A^b. This work suggested that division of CD4⁺ T cells in the periphery was dependent on MHC class II expression and that the peptides stimulating division were different from those involved in selection.

A number of papers from the group of Ignatowicz (Chmielowski, Muranski et al. 2000; Gaszewska-Mastalarz, Muranski et al. 2000; Kraj, Pacholczyk et al. 2001; Pacholczyk, Kraj et al. 2001) have examined the density and diversity of expression of peptides involved in selection. Mice with low levels of transgenic MHC expression and 50% of MHC class II molecules occupied by a single peptide were compared with mice that lack invariant chain. The repertoire of T cells were examined by V β flow cytometry and sequence analysis and showed that the repertoires were similar (Chmielowski, Muranski et al. 2000). It was concluded that diverse, low abundance peptides in the thymus play a more significant role than high abundance peptides that predominate. Other work used a transgenic MHC class II molecule with a covalently bound peptide and bone marrow chimaeras, suggested that in the presence of a high abundance peptide, cells survived due to impaired negative, rather than enhanced positive selection due to the decreased abundance in other self-peptides. Cells in the periphery had a memory phenotype and exhibited some autoreactive phenomena because of the low abundance of self-peptides in the thymus due to the high expression of the selecting ligand (Gaszewska-Mastalarz, Muranski et al. 2000). More recently (Pacholczyk, Kraj et al. 2001) examined mice with a transgenic TCR β -chain and endogenous α -chains with two alternative peptides on MHC class II in the thymus, showing that the two peptides select different repertoires of T cells independently, and if both were expressed, a third repertoire was generated. This suggested that as the complexity of peptide repertoire expressed increases, the repertoire of T cells selected is increased in an additive fashion. Using a TCR transgenic mouse specific for a peptide from Pigeon Cytochrome C (PCC), they examined the role of peptide analogues in selection. In the absence of H2-M and Ii, as well as the cognate peptide, thymocytes remain at DP stage. Injection of the peptide restored positive selection and migration of transgenic CD4⁺ cells into the periphery. Strongly agonistic homologues induced negative selection while co-injection of antagonist peptides inhibited positive selection.

The importance of peptide recognition is clear, that the presence of a peptide filling the binding groove does not automatically trigger selection and that interaction with TCR is important in determining the strength of the interaction. Mice were made expressing transgenic HEL, in which a HEL peptide residue, crucial for fitting in the MHC class II binding pocket, was altered. When immunised with altered HEL, they made no response, while wildtype HEL induced proliferation of T cells *in vitro*, showing that subtle alterations in peptide presentation affects the selected TCR repertoire (Peterson, DiPaolo et al. 2001). Use of soluble TCR with MHC/peptide

bound to Biacore has enabled classification of interaction 'strength' into three groups: high-affinity deleting ligands, low affinity positively selecting ligands and those where the interaction was too low to measure (Bevan 1997). Many groups used restricted peptides and transgenic TCRs to examine thresholds regulating selection, yielding information concerning how the repertoire is selected. Other work comparing mice with a 'normal-diverse' repertoire of peptides with MHC class II containing mainly the CLIP peptide showed that the repertoire selected in the MHC/CLIP mice was much more limited in terms of V α /J α usage (Bevan 1997). Recently Viret *et al* (Viret, He et al. 2001) used the TCR transgenic mouse, specific for E α 52-68 peptide in the context of A^b, and showed that expression of the MHC class II A^b with only the covalently attached peptide induced positive, but surprisingly, not negative selection of transgenic T cells.

1.15: Receptors and signalling molecules involved in selection

While the role of the peptide and the MHC molecule in selection are not disputed, other factors are important. These include the expression of costimulatory and accessory molecules, which together with the TCR determine whether a signal through the TCR induces survival, or death through activation or repression of genes. These include cyclins (e.g. Cdk2) and Bcl2 family members (e.g. Bcl2 and Bcl-x_L) (Williams and Brady 2001). The repertoire of CD8⁺ T cells was compared recently in a number of different MHC mice, wild type, H2^k-deficient, H2^d-deficient and double knockouts (leaving only non-classical class I molecules). Interestingly, when the peripheral repertoire was examined by Immunoscope, the various V α and V β rearrangements were used in similar proportions in all the mice, suggesting that even when classical MHC class I molecules are removed, it is possible to select a 'normal' or at least non-biased repertoire on non-classical MHC molecules (Laouini, Casrouge et al. 2000). Similarly, Maurice *et al* (Maurice, Gould et al. 2001) used 2C TCR transgenic mice and showed that in FTOCs from double knockout K^b^{-/-}D^b^{-/-} mice CD8⁺ cells were selected and were also found in the periphery, implying that non-classical MHC were capable of mediating positive selection of this TCR. Both classical and non-classical MHC molecules seem able to play a role in selection.

CD69 was also shown to be a marker both of positive and negative selection (Merkenschlager, Graf et al. 1997). Using thymus reaggregate cultures between MHC^{-/-} thymocytes (DP) and MHC⁺ thymic stromal cells, approximately 20% of the DP

thymocytes upregulated expression of CD69 and also possibly CD5 on interaction with MHC⁺ stroma and thus entry into selection. However whether CD69 is a participant or simply a result of entry into selection is unclear.

Other receptors thought to be involved in selection include costimulatory molecules such as CD5, CD80 and CD86 which when blocked, prevent negative selection of a transgenic MHC class II-restricted TCR CD4⁺ population in a dose-dependent manner (Page 1999). CD4⁺ T cells rescued from negative selection *in vitro* in this manner were shown to respond when stimulated with the negatively selecting ligand by proliferating *in vitro*. More recently, negative selection in CD5^{-/-}, CD28^{-/-} and CD40L^{-/-} mice was examined (Li and Page 2001). CD5^{-/-}, CD28^{-/-} and double knockout mice, as well as CD40L^{-/-} showed increased CD4SP and CD8SP thymocyte populations, indicating that negative selection was being altered. In the presence of superantigens however, CD40L was most effective in rescue of V β -expressing thymocytes from superantigen-mediated deletion. This suggests that by blocking the interactions with co-receptors, particularly CD40-CD40L, but also CD5 and CD28 to an extent, it is possible to alter thresholds involved in survival determination for thymocytes.

Not surprisingly, the specificity of the TCR, together with the degree of TCR stimulation, is thought to play a role in determining selection. In the cell, positive and negative selecting ligands have been shown to differentially trigger signals such that positive selection is thought to induce ERK activation, while negative selection induces stronger but less sustained ERK activation (Mariathasan, Zakarian et al. 2001) as well as phospholipase-C and a large network of other intracellular signalling molecules (Sebzda, Mariathasan et al. 1999). The number of immunoreceptor tyrosine-based activation motifs (ITAMs) signalling motifs in the ζ -chain of the TCR signalling complex, can also influence selection (Love, Lee et al. 2000). By altering the signalling potential of the cell (presumably as a result of the affinity of interaction), it is possible to modulate the number of ITAM motifs that are phosphorylated, thus altering the 'signal-strength' in the cell and thereby the outcome of the selection process.

1.16: Theories of selection- altered ligand hypothesis and differential avidity model

1.16.1: Qualitative/peptide model and altered ligand hypothesis

There are two principal theories of the mechanisms regulating positive and negative selection (Sebzda, Mariathasan et al. 1999). The qualitative/peptide model suggests that positive selection and negative selection are induced by interaction with different, distinct peptides, with lower affinity, antagonistic peptides that initiate unique signals in thymocytes leading to survival and positive selection while agonistic peptides induce clonal deletion and negative selection. Evidence for this model includes work with agonist/antagonist peptides previously described, as well as antibodies used to mimic agonist and antagonist interactions. This model would suggest that different peptides are capable of inducing distinct intracellular signals depending on the interaction, leading to either positive or negative selection. However peptides can only fall into defined categories for a particular TCR, and the outcome depends on the system being tested. This would suggest that for one TCR a peptide might exert agonistic properties, while for another it may function as an antagonist (Sebzda, Mariathasan et al. 1999). Closely linked with this idea is the altered-ligand hypothesis (Marrack and Kappler 1988), suggesting that positive and negative selection occur on distinct cell populations in the thymus (which could express different peptides). The cortical epithelium is thought to be specialised for inducing positive selection on MHC/self-peptides, while bone marrow-derived cells (APCs) in the medulla induce negative selection. This theory suggests that self-MHC in the thymic epithelia contain self-peptides that differ from those on thymic APCs.

1.16.2: The avidity theory of selection

The second major theory of selection is the quantitative or avidity theory (Ashton-Rickardt, Bandeira et al. 1994; Ashton-Rickardt and Tonegawa 1994) (Figure 1.1 a). Avidity is a combination between the affinity of interaction between TCR and MHC and the number of interactions occurring. It is suggested that there is an avidity window into which the interaction must fall in order for a thymocyte to survive

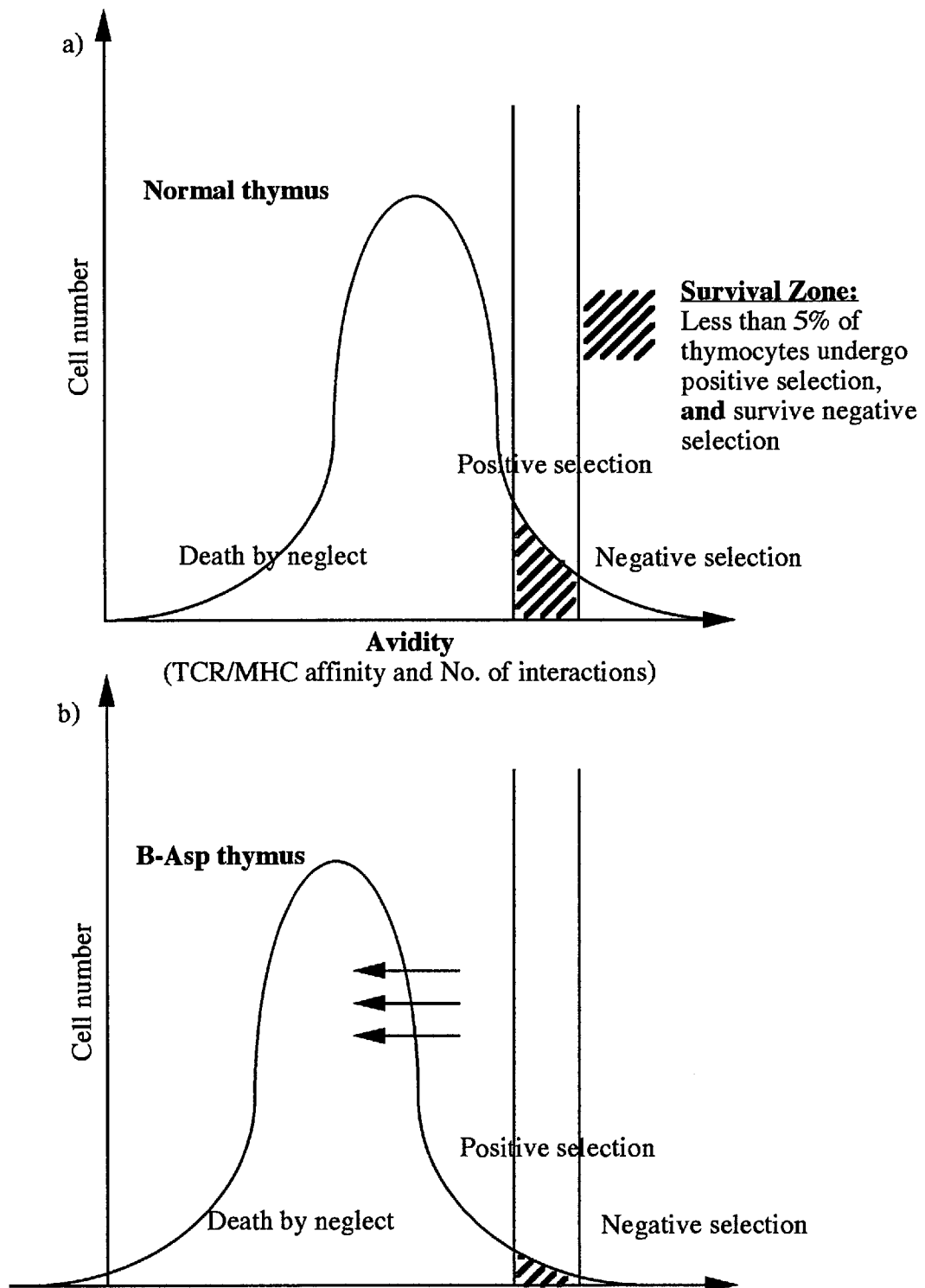


Figure 1.1. The Avidity model of T cell selection. a) A combination of affinity and the number of interactions determines whether T cells bearing TCRs survive positive and negative selection in the thymus. b) By decreasing the number of TCR/MHC interactions in the B-Asp mixed haplotype MHC class II model, the overall avidity range is shifted and the number of CD4⁺ thmocytes surviving selection decreases.

selection, and that the parameters of the window can be influenced by glucocorticoids (Vacchio and Ashwell 1997; Tolosa, King et al. 1998; Vacchio, Lee et al. 1999; Ashwell, Lu et al. 2000). Cells that do not interact with sufficient avidity are not positively selected, while negative selection is triggered by high avidity interactions. In order to fall into the moderate avidity window for survival there is a spectrum of interaction 'strengths' that can allow moderate avidity. At one extreme, moderate avidity can be attained by a large number of low affinity interactions, while at the other extreme a small number of high affinity interactions will also allow survival. The window of avidity however, is unlikely to include the extremes. The 'strength' of the interaction will therefore be the sum of two main factors: TCR/MHC affinity and level of surface expression of both receptors.

Evidence for this theory comes from experiments with addition of different doses of cognate peptide to FTOC from TCR transgenic (TAP-deficient) mice and showing that low and high doses of peptide induce positive and negative selection respectively, while addition of peptide even at low levels in the presence of TAP induces negative selection (Ashton-Rickardt, Bandeira et al. 1994). More recently a number of synthetic thymic self-peptides were used with HY-tetramers to examine the outcome of selection. The affinity of the peptide for the TCR was examined in transgenic mice. While the cognate HY peptide bound the TCR with high affinity, other self-peptide complexes bound with lower affinity and did not trigger negative selection (Ober, Hu et al. 2000). By altering the level of TCR expression at the cell surface, Watanabe *et al* (Watanabe, Arase et al. 2000) found that the level influences TCR signal strength, thus affecting selection. Crossing DO11.10 TCR transgenic with CD3 ζ -deficient mice, they generated mice that expressed reduced levels of TCR of known specificity. Subsequent injection of the cognate high affinity peptide restored positive selection leading to an increased CD4SP population in a dose-dependent manner. In a similar manner Dave *et al* (Dave, Allman et al. 1999) used dual TCR transgenic mice, assuming that expression of both TCRs would cause decreased expression of each TCR compared to single transgenic mice. Positive selection of both TCRs was reduced while negative selection of the HY transgene was partially blocked in male double-transgenic mice.

From the ligand side of the TCR/MHC interaction, it is known that differences in the level of MHC class I on the surface of thymic epithelia and DCs can influence selection (Delaney, Sykulev et al. 1998). FTOC with the 2C transgenic TCR cultured with thymic epithelia depleted of DCs showed that positive selection occurred but with

thymic DCs, deletion occurred. When examined by flow cytometry epithelial cells had a 10-fold decreased expression of MHC class I compared with thymic DCs. However, there is evidence that very low levels of MHC class II expression on thymic DCs are able to induce negative selection and remove autoreactive T cells from the repertoire (de Bont, Reilly et al. 1999). Liu *et al* (Liu, Fairchild et al. 1995) examined the role of low affinity peptides and their effects on selection and showed that a peptide commonly linked to the development of autoimmune encephalitis binds to the H2^u molecule with low affinity and that T cells recognising this peptide in the context of H2^u escape deletion. Treatment of TCR transgenic mice with peptide did not affect the thymocytes, while higher affinity analogues induced deletion. Similarly, recent work with OT-1 transgenic TCR mice showed that transgenic expression of an antagonist peptide could induce positive selection *in vivo* (Stefanski, Mayerova et al. 2001).

Together these data suggest that the outcome of selection is within a gradient influenced by many different factors, particularly avidity of MHC/peptide for TCR, but also additive effects of other co-receptors and the peptide affinity for the MHC. In combination these factors induce differential TCR signalling and altered gene expression in the cell.

1.16.3: The role of avidity in the periphery

After undergoing positive and negative selection, lineage commitment and full differentiation into SP thymocytes, thymocytes are able to migrate into the periphery where they exist as naïve T cells circulating throughout the secondary lymphoid network of spleen and lymph nodes until encounter with an appropriate antigen in the context of the restricting self-MHC molecule.

Interestingly, avidity has also been suggested to be involved in T cell responses in the periphery. Immunisation with wild-type (low affinity for MHC) MBP peptides induced autoimmune encephalomyelitis by clonally expanding high affinity TCR-bearing cells, while administration of high affinity analogues induced apoptosis of high affinity TCR-cells, preventing disease development (Anderton, Radu et al. 2001). Low avidity interactions in the periphery may induce anergy (non-responsiveness) of memory T cells (Mirshahidi, Huang et al. 2001). Increased avidity for MHC/peptide was detected on activated T cells compared to naïve T cells by the binding of fluorescently labelled, peptide-MHC dimers. This was thought to be a mechanism

allowing T cells to be able to respond to minimal amounts of cognate antigen in the periphery.

1.17: CD4⁺ T cell function (Th1/2)

MHC class II/peptide complexes are recognised by specific TCR on the surface of CD4⁺ T cells or T helper (T_h) cells. These perform several functions involving regulation and recruitment of CD8⁺ cells and components of the innate immune response as well as regulating B cell functions such as antibody production. Ligation of the TCR by MHC class II/peptide with appropriate co-stimulatory molecules induces transcription of various cytokine genes depending on the type of CD4⁺ cell.

Classically, Th cells have been classified into two types, Th1 and Th2 depending on the profile of cytokines secreted (Constant and Bottomly 1997). Th1 cells are characterized by production of IL-2, IFN- γ and TNF- β while Th2 secrete IL-4, IL-5, IL-6, IL-10 and IL-13. While there appears to be no overlap between Th1 and 2 cells, it is suggested that a third population Th0 exists that may have overlapping functions. Th1 cells are commonly associated with inflammation and responses to intracellular infections while Th2 are linked with regulation of B cells and involvement in the humoral response to extracellular pathogens through antibody production. It is thought that the two populations of cells are derived from a common precursor population and that skewing of a CD4⁺ cell is influenced by a number of factors. These include the local cytokine milieu such that localised production of IFN- γ and IL-2 are thought to influence differentiation to Th1, while IL-4 with a possible contribution from IL-10, are thought to cause Th2 cells to differentiate (Constant and Bottomly 1997).

Altered peptide ligands are thought to skew CD4 function. A number of groups have examined the role of alterations in the residues contacting the TCR, resulting in altered stimulation through the TCR and signalling in the cell. Pfeiffer *et al* (Pfeiffer, Stein et al. 1995) showed that strong interactions between TCR/peptide/MHC skew towards Th1 while weaker interactions skew to Th2. Mutant versions of the cognate peptide were administered to TCR transgenic mice, inducing a switch from Th1 to Th2 phenotype. Altered peptide ligands may be a mechanism used by viruses to manipulate the immune system by antagonizing T cell responses. The affinity of an agonist peptide might also play a role in skewing.

Further influences are the dose of antigen and the activation threshold of the TCR/peptide/MHC interaction. It is unclear whether high or low doses of antigen induce Th1 or Th2 responses, indeed both have been suggested. Work using naïve transgenic T cells found that different doses of a cognate peptide caused secretion of different cytokine profiles by cultured T cells. Low doses of peptide induced high concentrations of IL-4 (Th2), while higher doses of the same peptide induced IFN- γ (Th1) (Constant, Pfeiffer et al. 1995). This is thought to be linked with the degree of signalling induced via the TCR.

Short TCR stimulation in the presence of IL-12 can induce Th1 skewing (also if present after TCR stimulation), while Th2 skewing is thought to be induced by more prolonged stimulation as well as the presence of IL-4 only during TCR signalling (Iezzi, Scotet et al. 1999). Consensus of opinion suggests a relationship between the strength of interaction between TCR/peptide/MHC, the length of time of interaction (and thus the degree of TCR signalling) as well as the cytokine environment prevailing at the time. One current theory is that a threshold of activation exists such that abundant TCR/peptide/MHC interactions or high affinity peptides induce Th1 skewing, while less abundant interactions or lower affinity peptides will not reach a signalling threshold in the cell, inducing a Th2 response (Constant and Bottomly 1997).

Linked to MHC/TCR interactions and cytokine environment, the nature of the APC involved in triggering the T cell is also thought to be involved with Th1/2 outcome. DCs can be divided into two populations (known as DC1 and DC2) that skew T cells to Th1/2 phenotypes. The 'type' of DC population involved in stimulation, the activation stimulus and subsequently the cytokines secreted by the DC as well as costimulatory molecule expression are proposed to influence skewing (Kalinski, Hilkens et al. 1999) for example CD8a⁻ (myeloid DCs) are thought to induce Th1 responses while CD8a⁺ (lymphoid DCs) are thought to induce Th2. Both populations have been described in mouse spleen (Reid, Penna et al. 2000). Populations of monocyte-derived DCs were described in human cultures characterised by cell-surface expression of CD1a. CD1a⁻ DCs (DC2) secrete IL-10 but no IL-12 while CD1a⁺ (DC1) secrete IL-12 and little IL-10. Both were potent APCs in MLR, yet after activation when cultured with CD4⁺ cells mDC1 induced Th1 differentiation while mDC2 skewed either towards a Th2 or Th0 profile (Chang, Wright et al. 2000).

1.18: T cell receptor structure and function

In recent years understanding of the function of the T cell receptor and its interaction with MHC has been advanced by determination of the crystal structure of the TCR, both alone, and in combination with MHC class I and II. The rearrangement of the TCR gene segments, and their homology with immunoglobulin genes, suggests that the TCR forms a similar domain structure to that of immunoglobulin (Fields and Mariuzza 1996). Different groups have attempted to crystallize fragments of the TCR providing some information, but it is difficult to crystallize transmembrane proteins due to the presence of the lipid-associated transmembrane region. A number of crystal structures have been elucidated including: the murine 2C TCR in complex with H2-K^b plus peptide (Garcia, Degano et al. 1996), as well as the alternate ligand for the 2C TCR, H2-L^d with peptide (Speir, Garcia et al. 1998); the human A6 TCR in complex with HLA-A2 with the Tax peptide from the HTLV virus (Garboczi, Ghosh et al. 1996); and more recently the first TCR in complex with MHC class II- the D10 TCR bound to the mouse H2-A^k molecule with a peptide derived from conalbumin (Reinherz, Tan et al. 1999). Other groups have examined the way in which exogenous superantigens interact with the β -chain of the TCR and determined residues that interact with sites on the superantigen protein (Fields, Malchiodi et al. 1996).

The TCR is a transmembrane glycoprotein heterodimer of an α - and β -chain, both members of the Ig superfamily. The N-terminal region of each chain is located outside of the plasma membrane; there is a short (~20 amino acid) transmembrane domain and a cytoplasmic tail. The cytoplasmic tail does not have inherent signalling capacity, but interacts with chains of the CD3 complex that are involved in transducing the signal from the TCR into the cell. In part, association of TCR with CD3 is mediated by one or two positively charged amino acids in the TCR β - and α -transmembrane domains (Allison and Lanier 1987).

The α - and β -chains of the TCR have close homology with immunoglobulin structures (Chothia, Boswell et al. 1988). The structure is in many ways very similar to that of immunoglobulin, with conserved structures and similar residues in the variable domains. Four hypervariable regions are in the TCR $\alpha\beta$ heterodimer and three of these are analogous to complementarity determining regions (CDR) in immunoglobulins (Patten, Yokota et al. 1984). CDR1 and 2 are encoded by the variable gene segments, while CDR3, the most variable, is encoded by the joining regions between V-J in the α -chain and V-D-J in the β -chain. From structural studies the CDR3 hypervariable loop

lies close to, and interacts with, the peptide side chains protruding from the peptide-binding groove, while CDR1 and 2 regions interact mainly with the MHC molecule itself. Two of the MHC class I/TCR structures have revealed that the TCR binds to MHC class I in a diagonal manner with respect to the MHC binding groove and peptide. The V α CDR1 and 2 interact with the MHC α -helices of the binding groove, and the amino-terminal end of the peptide. V β CDR1/2 interacts with the carboxy-terminal end of the peptide, while the CDR3 regions straddle the central portion of the peptide (Garcia, Degano et al. 1996). This arrangement enables the TCR to interact both with the MHC class I molecule and to discriminate between different peptides (Wilson 1999).

Each TCR has two main domains, Variable and Constant, resembling two linked Ig folds. The surface of the V β domain that interfaces with the V α domain is mainly hydrophobic residues, while the C β domain face that interacts with the C α domain is comprised mainly of polar, positively charged residues, able to form inter-chain salt-bridges (Fields and Mariuzza 1996). The TCR V α domain overlays the MHC class I α 1 helix, while V β overlays the α 2 helix. It was thought that this model would be applicable to all TCR/MHC interactions (Garboczi, Ghosh et al. 1996), yet when the first TCR/class II structure was solved, it showed that the TCR was oriented in an orthogonal manner with respect to the peptide-binding groove on the MHC class II molecule (Reinherz, Tan et al. 1999).

Analysis of the crystal structures of TCR/MHC/peptide complexes, together with alanine scanning mutagenesis of different TCR residues, have identified the importance of particular residues in interacting with MHC, to shed further light on the processes of thymic selection, peripheral T cell function and allorecognition (Speir, Garcia et al. 1998; Manning and Kranz 1999; Manning, Parke et al. 1999; Reiser, Darnault et al. 2000).

1.19: Mixed haplotype MHC class II molecules

MHC class II molecules are expressed as cell surface heterodimers of α - and β -chains. Mice express two different MHC class II isotypes, H2-A and H2-E. In addition, further complexity is added by having different alleles, in an isotype such that theoretically, 16 $\alpha\beta$ pairs could form in an MHC heterozygote. While it had been assumed that functional pairing between α - and β -chains occurred only between matched haplotype and isotype molecules, mixed haplotype molecules are quite commonly expressed (Lechler 1988).

(B10 x B10.D2) F_1 mice form hybrid molecules *in vivo* (Lafuse, McCormick et al. 1980), while in a different model, mixed isotype (E α^d A β^d) molecules were detectable at much lower levels than either parent molecule on both murine B cells and macrophages (Spencer, Freed et al. 1993) and were able to present sperm whale myoglobin (SwM) peptides to T cell hybridomas derived from clones specific for the SwM peptides presented by mixed isotype class II (Ruberti, Sellins et al. 1992). This was also shown *in vitro* using L cells transfected with different murine isotype and haplotype MHC chain genes, and although expressed at lower levels than the matched pairs, mixed isotype molecules were still able to form at significant levels on the cell surface (Germain and Quill 1986; Sant and Germain 1989). Human mixed isotype molecules were first shown using B cell lines (Lotteau, Teyton et al. 1987), while hybrid molecules between human and mouse MHC have been shown using transfected cell lines (Lechler, Sant et al. 1990).

A number of factors are thought to cause the decreased surface expression of mismatched MHC molecules. Inefficient pairing and expression of mixed molecules can be caused partly by incompatible amino-terminal regions of each chain (Sant, Braunstein et al. 1987; Lechler, Sant et al. 1990). Sant *et al* (Sant, Hendrix et al. 1991) used metabolic labelling and immunoprecipitation to show that both isotype and haplotype-mismatched MHC do not undergo post-translational modifications such as N-linked glycosylation, typical of mature MHC class II molecules. As a consequence mismatched dimers were retained intracellularly, possibly in the endoplasmic reticulum and are not efficiently transported to the cell surface.

The peptide binding capacity of mismatched molecules was examined using a number of synthetic peptides (Moore, Zauderer et al. 1997). H2 ^{d} mice were injected with four different peptides. The peptides were one from SwM and a peptide derived from the cytoplasmic domain of the invariant chain, previously shown to be capable of

binding to the isotype mismatched molecule $E\alpha^dA\beta^d$, and two synthetic peptides YL2 and FL2. The two synthetic peptides were able to bind to the mismatched molecule but not to either parental molecule. SwM was able to bind both to the parental and the mixed isotype molecule, but the invariant chain peptide bound to MHC with high affinity only at low pH. Such interactions were considered rare *in vivo*, and conclusions were drawn that there may be a population of T cells with high-affinity TCRs, capable of responding to peptide bound to MHC class II expressed at a low level.

This conclusion is interesting because if only high affinity TCR-expressing T cells are able to respond to the low levels of MHC class II/peptide, selection on low self-MHC may result in a repertoire of higher affinity, autoreactive TCRs, while the pairing of mixed isotype or haplotype α - and β -chains would form novel binding pockets in the antigen binding groove, influencing the peptides presented. In (NZB x NZW) F_1 mice there is a high incidence of autoimmune systemic lupus erythematosus (SLE) (Nygard, McCarthy et al. 1993) and the mixed haplotype MHC class II expressed in these mice ($A\alpha^dA\beta^z$) is thought to be in part responsible for the disease. Most of these F_1 mice develop anti-nuclear antibodies, infiltration and eventually glomerulonephritis and die within one year, a condition that is not observed in either parental strain NZB or NZW. Antigen specific T cell clones isolated from F_1 mice were restricted by mixed haplotype MHC molecules (Gotoh, Takashima et al. 1993), and of these, some were autoreactive (Kimoto 1996), responding to only the mixed haplotype molecule and neither parental strain MHC. When transferred into young F_1 mice, the autoreactive clones induced anti-DNA antibody production (Tokushima, Koarada et al. 1994)

The peptide-binding motifs of these mixed haplotype class II were also examined (Mine, Koarada et al. 1998) and found to exclude charged residues from positions 3, 4 and 6, while large residues were also excluded from positions 3 and 4.

While the presence of mixed haplotype molecules and peripheral T cells that can recognise them has been extensively documented, it has not been established whether such MHC class II molecules can select T cells. Low expression MHC class II molecules may select high affinity TCRs and this has implications on allo- and autoreactivity, structural parameters of the TCRs, as well as the function of the T cell. $CD4^+$ T cells are divided into two main functional groups, Th1 and Th2, dependent on the cytokines secreted. It has also been suggested that strong TCR/peptide/MHC interactions skew the phenotype towards Th1 while weaker interactions skew to Th2-type responses (Pfeiffer, Stein et al. 1995). It is possible that if T cells are selected on

low levels of mixed MHC class II molecules they are towards the lower end of the avidity spectrum and may skew responses preferentially towards Th2-type responses, while higher avidity interactions may skew towards the Th1-response.

1.20: Effects of low-level MHC class II expression on T cell selection (B-Asp mice)

Mice expressing only a mixed haplotype MHC class II molecule ($\alpha^b\beta^{g7(asp)}$) in the absence of matched pairs have been analysed in this laboratory (Schoendorf 2000). Due to poor pairing of the α and β -chains, the MHC class II heterodimer is expressed at low levels on the cell surface, as shown using monoclonal antibodies (Y3P- anti H2- $A\alpha^b$ and OX-6- anti H2- $A\beta^{g7/g7(asp)}$) specific for α and β -chains respectively. Expression of the mixed haplotype molecule, are about 10% of the expression levels of either parental strain or normal littermate control mice. Decreased expression was found both in the thymus on CDR1⁺ thymic cortical epithelia and also in peripheral B cells (Schoendorf 2000).

Due to the dramatic decrease in MHC class II expression, as suggested by the avidity theory of T cell selection (Ashton-Rickardt and Tonegawa 1994) thymic development is altered dramatically in these mice (Figure 1.1 b). In normal mice CD4SP thymocytes account for 5-10% of thymocytes. However, when selected on the mixed haplotype MHC class II molecule, the number of CD4SP thymocytes is decreased to less than 1% of total thymocytes, similar to that seen in MHC class II knockout mice (Grusby, Auchincloss et al. 1993; Schoendorf 2000).

Lymph nodes and spleen of these mice were found to contain a population of mature peripheral CD4⁺ T cells that had migrated from the thymus. CD4⁺ T cells selected in B-Asp spleen were around 7%. MHC^{-/-} mice have a similar proportion of CD4SP in the thymus, yet fewer CD4⁺ in the periphery (around 2-3%). Primary, initial analysis of CD4⁺ cells selected by B-Asp did not appear to have skewed V β gene segment usage (Schoendorf 2000).

In order to further characterise B-Asp peripheral CD4⁺ T cells, they were examined for various phenotypic markers. While the cells were negative for both CD69 and CD62L expression (markers of having encountered antigen), they expressed CD44 at a higher level than that seen in the littermate controls. CD44 is known to be an activation marker and/or a memory marker on T cells (Dutton, Bradley et al. 1998) and was also shown to be upregulated on CD4⁺ T cells in MHC class II-deficient mice,

possibly due to homeostatic expansion, while increased expression was not seen on the CD8⁺ T cells in B-Asp (Schoendorf 2000).

Although some splenomegaly was observed in some of the B-Asp mice, there did not appear to be any gross defects in spleen architecture or proportion of spleen cell populations, except for an increase in the numbers of megakaryocytes. Apart from splenomegaly, there was no other evidence of autoimmunity, such as anti-nuclear antibodies, tissue infiltration or diabetes. This was surprising, as the avidity theory predicts that under conditions of low MHC class II expression, TCRs selected may be of higher affinity in order to pass through positive and negative selection, and thus be potentially more autoreactive. In addition, it was suggested that with decreasing stability of MHC molecules, increased autoimmunity may be observed (Ridgway, Fasso et al. 1999), possibly due to defective negative selection (Ridgway and Fathman 1999) as seen in NOD mice. However by expressing a low level of transgenic MHC class II on the cell surface of thymic dendritic cells, then backcrossing onto an autoreactive mouse strain, other work has revealed that even with limited expression of MHC class II, deletion of autoreactive cells from the thymus is extremely efficient (de Bont, Reilly et al. 1999), which may explain the absence of autoimmunity in B-Asp mice. These mice were maintained in a SPF environment, and it is also possible that autoimmunity might be observed if maintained in a normal 'dirty' environment, exposed to environmental pathogens.

In order to determine whether the CD4⁺ T cells were functional, several analyses were performed. Proliferation assays to test alloreactivity were conducted using irradiated allogeneic splenocytes as APCs, showing that T cells were capable of proliferating in response to an alloreactive stimulus. Analysis of antibody production in response to injection with keyhole-limpet haemocyanin (KLH) revealed an ability to produce IgM, as well as IgG1, but not IgG2a. Littermate controls were able to produce IgG2a, suggesting that the defect may be due to defective T cell help by the low number of CD4⁺ cells or a defect in Th1 CD4⁺ cells. Cytokine ELISAs in response to KLH also suggested that there was a skewing of T cell function towards a Th2 phenotype, producing IL-10 and IL-4, but not IFN- γ , while littermate control mice exhibited a more 'normal', mixed Th1/2 response (Schoendorf 2000).

1.21: Methods of TCR repertoire analysis

1.21.1: CDR3 background

Various methods have been applied to examine the repertoire of T cells involved in immune responses. The pre-selected TCR comprises heterodimers of randomly associated pairs of α and β -chains. The α -chain is made from gene rearrangements from V, J and C gene segments with further diversity through junctional diversity, while the β -chain is made from V, D, J and C segments also with nucleotide additions. The CDR1 (6 amino acids in length) and CDR2 loops (8-12 amino acids in length) in both α - and β -chains are produced from germ-line V gene segments. However the CDR3 region of the β -chain (6-13 amino acids) is encoded by the V(D)J junction regions together with N-nucleotide additions and is consequently the most diverse region of the TCR (Davis and Bjorkman 1988). Structural studies have revealed that the hypervariable region, the CDR3 of the β -chain interacts closely with the peptide in the binding groove of the MHC molecule (Fields, Malchiodi et al. 1996; Garcia, Degano et al. 1996).

Wang *et al* (Wang, Ono et al. 1998), using a transgenic TCR α -chain with a peptide from vesicular stomatis virus (VSV) presented by H2-K^b, showed that position 98 in the CDR3 loop of the TCR β -chain is crucial in determining specificity of the interaction between the TCR and peptide/MHC complex. TCR α transgenic mice were injected with wild type and mutated VSV peptides, CTL clones isolated and the CDR3 β region sequenced. By altering the peptide at position 6 (but not position 1) in the binding groove, it was possible to induce changes both in the CDR3 β -region sequence at position 98, as well as altering the CDR3 length necessary to allow interaction with the MHC/peptide. However it is likely that this interaction may be specific both for the particular TCR-peptide/MHC complex, as well as for CD8 TCR/MHC interactions and not necessarily be applicable as a general rule for contact sites between TCR and MHC/peptide complexes.

1.21.2: Flow cytometry-based methods

Where peptide/MHC complexes are defined, it is possible to examine an immune response by using tetramers, a multimeric, fluorescently labelled MHC/peptide complex. Applied to viral immune responses, such as HIV specific CD8⁺ T cells in human blood (Altman, Moss et al. 1996) and recently murine MHC class I tetramers to examine anti-HY responses (Millrain, Chandler et al. 2001).

An alternative method of repertoire analysis that has been commonly used (and used in this thesis) is flow cytometry with monoclonal antibodies specific for V α or V β gene segments on the surface of CD4⁺ or CD8⁺ peripheral lymphocytes. Genevée *et al* (Genevée, Farace et al. 1994) used monoclonal antibodies to examine the dependence of V α and V β selection in normal human blood samples on various polymorphic and non-polymorphic HLA genes, particularly in HLA-identical and non-identical siblings. They suggested that HLA-haplotype differences between siblings had effects on the overall T cell repertoire. However this method has some limitations in that while it is able to examine the gross repertoire and skewing of the repertoire to particular V α or V β , it can be influenced by the presence of superantigens and subsequent deletion of particular V β families (described later), as well as not providing detailed information about other regions in the TCR such as CDR3 length or J β gene usage.

1.21.3: RNA-based methods

Okada and Weissman (Okada and Weissman 1989) used RNase protection assays to examine the repertoire of different V β in thymus, spleen and different lymph nodes from a number of mouse strains. This showed a 20-fold range in transcript levels in the thymus. The differences in the periphery were even larger in most mice tested. They also used this method to compare selection of different V β in the presence of H2-E and minor lymphocyte-stimulating antigens (MIs), now known to be encoded by mouse mammary tumour pro-viruses (*Mtv*) integrated into the genome.

Singer *et al* (Singer, Balderas et al. 1990) used a multi-probe RNase protection assay to quantitate the expression of a number of V β genes in unselected thymocytes, and in selected CD4⁺ and CD8⁺ subsets. This had an advantage over the previous method in that it allowed analysis of repertoire skewing to different compartments in the periphery. It showed that the V β repertoire differed in a number of H2^k mouse

strains, implying that the repertoire is influenced not only by MHC, but also by polymorphic regions other than the MHC.

1.21.4: PCR methods, sequencing and Immunoscope

Genevée *et al* (Genevee, Farace et al. 1994) also used a second method to examine the TCR repertoire. Peripheral blood lymphocyte cDNA was made and PCR reactions run using primers specific for a number of different V α and V β regions in combination with specific C region primers. The samples were then analysed by Southern Blot using C α or C β oligonucleotide probes and the relative contribution of each V α or V β as a percentage of the total repertoire was analysed. This method however also suffers from a lack of detailed information beyond the V α /V β usage. It would also detect out of frame gene-rearrangements.

Semi-quantitative anchored PCR reactions have been useful in analysing the V β repertoire in non-obese diabetic (NOD) mice. Monoclonal anti-V β antibodies were used to stimulate splenocytes and lymph node cells isolated from NOD and the NOD-E transgenic strain (expressing H2-E class II molecules and protected from diabetes). This method showed that there were few detectable differences on stimulation between the strains (Parish, Acha-Orbea et al. 1993). The repertoire of NOD mice was further analysed (Sarukhan, Gombert et al. 1994) using anchored PCR, comparing the repertoire in thymus, spleen and pancreatic islets that are infiltrated by autoreactive T cells as part of the disease progression. This showed that certain V β were selected preferentially from the thymus into the spleen, particularly V β 2 showing higher expression in the spleen in these mice compared with C57BL/6. When the islets were examined, high expression of V β 12 was observed. V β 8 is expressed at high levels in the spleen both of NOD mice and C57BL/6 mice and showed excellent correlation when compared with flow cytometry data. In the presence of *Mtv-3*, V β 3 containing TCRs are deleted in NOD mice (McDuffie, Schweiger et al. 1992). If V β 17a was introduced into the NOD background (NOD mice do not have H2-E molecules), V β 17a⁺ T cells are deleted. It had previously been thought that H2-E was necessary to mediate deletion of V β 17a.

Other methods that are available involve PCR reactions with V β - and J β -specific primers using either ³²P-labelled or fluorescently labelled oligonucleotides. This can be used in 'run-off reactions' followed by electrophoresis and sequence

analysis (Pannetier, Delassus et al. 1993), (Cochet, Pannetier et al. 1992) or first with unlabelled primers, then a run-off reaction with fluorescently labelled V β and J β primers in a method known as Immunoscope, both for human TCRs (Pannetier, Even et al. 1995; Manfras, Rudert et al. 1997) and also mice (Pannetier, Cochet et al. 1993). This is a useful method enabling more detail of the TCR to be analysed in that it provides information about the CDR3 length as well as the V and J β gene usage in a sample. It has the advantage of using known primers, initially unlabelled V β and C β primers to amplify all CDR3 regions in the particular V β pool. A subsequent reaction is run using an additional fluorescently labelled either C β (for lower resolution analysis) or J β -specific primer (for more detailed analysis). The amplified products are subjected to automated sequencing and it is possible to analyse the fluorescence intensity in each sample and display the fluorescence intensity for different CDR3 lengths contained in either the V β -C β reaction or the V β -J β reaction. Under 'normal' circumstances the values measured will display a Gaussian or normal distribution with the maximum peak at around 10 amino acids in length. The advantage of this technique means that it is possible to compare a normal repertoire with a clinical sample or in mice, repertoires that have developed due to immunisation with either specific peptides or infectious agents. If particular T-cell clonal expansions have occurred due to exposure to the antigen, these may be detected by an increase in fluorescence intensity of one clone compared to others in a pool and a shift in the distribution in a pool (Pannetier, Even et al. 1995).

In parallel with Immunoscope, the authors recommended that other techniques be used, including quantitative PCR (Pannetier, Delassus et al. 1993). An interesting development of the Immunoscope process called TcLand (or T cell Landscape) has been published (Sebille, Gagne et al. 2001). This process used Immunoscope to measure the changes in TCR clonality in mixed lymphocyte reactions (MLR) using as responders both human PBLs and rat splenocytes. Analysis of the cDNA using human or rat V β and C β primers followed by comparison with a standard curve for each V β in unprimed samples has enabled a three-dimensional 'landscape' of changes in CDR3 length frequencies to be plotted across all the different V β . This allows comparison of unstimulated cells with different stimuli such as MLR, Con A that stimulate across V β families, superantigen (TSST) that stimulates expansion in one V β family only. The expression was plotted relative to HPRT.

All of the sequences of the different V β genes are known, both in humans (Arden, Clark et al. 1995) and mice (Arden, Clark et al. 1995) and have been compared for sequence homology (Clark, Arden et al. 1995). It is possible to design V β -specific primers both in mouse and man, either for Immunoscope as described above or for using PCR to amplify and then directly sequence CDR3 regions contained between particular V β and C β regions (Candeias, Waltzinger et al. 1991). Although this analysis is more labour intensive than other methods and cannot be used to examine the repertoire at low resolution, it does provide more detailed, high-resolution information about the CDR3. Work by Candéias *et al* (Candeias, Waltzinger et al. 1991), has examined the thymic, pre- and post-selection V β 17⁺ repertoire, as well as in bulk peripheral and purified CD4⁺ and CD8⁺ T cells. This was compared in mice that expressed H2-E and those that did not. This will be discussed in greater detail in Chapter 3.

1.22: NOD and NOD-Asp mice

Autoimmune diseases are commonly linked to MHC where candidate-MHC class I and II play a role in pathogenesis and disease progression (Vyse and Todd 1996). One of the most studied models of human type-I diabetes is the non-obese diabetic (NOD) mouse strain. NOD mice spontaneously develop autoantibodies to antigens in pancreatic beta cells, including insulin, followed by infiltration of autoreactive T cells, insulinitis and destruction of the beta cells. Around 80% of female mice develop diabetes by six months and die through an inability to modulate glucose levels. The first diabetes-linked locus was called *Idd1* (Wicker, Todd et al. 1995) and was linked to the MHC. A number of further loci predisposing to diabetes have subsequently been mapped. NOD mice express the MHC molecule H2-A^{E7}, the α -chain of which is identical to A^d, while the β -chain (β ^{E7}) is expressed in NOD, Biozzi strain (Liu, Baker et al. 1993) and non-obese normal (NON) mice. There is a structural link between A^{E7} and the human MHC allele DQ*0302, both linked to diabetes, sharing a non-Asp residue at position 57 of the β -chain (Todd, Bell et al. 1987).

Much work has been performed using the NOD mouse as a model for human type I diabetes. The presence of the NOD MHC is necessary, but not sufficient for disease progression, and expression of H2-E molecules abrogates disease (Hattori, Buse et al. 1986). Expression of H2-E on the surface of B cells alone protects mice from

diabetes. However, mice with mixed H2-E⁺ and H2-E⁻ populations of B cells were susceptible to disease. Further analysis using variable H2-E expression on macrophages and dendritic cells showed that if H2-E expression was restricted to only 50% of cells, disease was prevented (Johnson, Silveira et al. 2001). The MHC region was also linked to disease progression, using crosses between NOD and the related (NON) mice (Prochazka, Serreze et al. 1989) as well as between NOD and B10 mice (Wicker, Appel et al. 1992).

Two groups have recently examined the structure of the H2-A^{g7} MHC. It is an unstable molecule, expressed on the surface of both peripheral B cells and also when transfected into immortalized B cells, with a short half-life in comparison to other MHC molecules (Carrasco-Marin, Shimizu et al. 1996). Peptides bind only transiently to the H2-A^{g7} molecules. When the structure was examined, the binding groove was shown to have a P1 pocket that was partially solvent-exposed, and the P9 pocket was wider than in other MHC class II molecules, due to the absence of a salt bridge between α - and β -chains (Latek, Suri et al. 2000). This may be the cause of poor peptide binding and presentation. The structure was also resolved to 2.6Å in complex with the glutamic acid decarboxylase (GAD) 65 peptide, a common peptide in autoreactive T cells from NOD mice. The peptide-binding groove was confirmed as being wide, particularly near position 57 of the beta-chain (Corper, Stratmann et al. 2000), suggesting a link to the poor peptide binding capacity of the H2-A^{g7} molecule. One hypothesis is that the instability of the MHC class II molecule plays a significant role in disease by altering thymic selection, allowing autoreactive cells to survive negative selection and enter the periphery leading to disease (Ridgway, Fasso et al. 1999). In line with this, it was shown that in NOD mice with a transgenic H2-A^k molecule, A^{g7} (Ridgway, Ito et al. 1998) restricted all autoreactive cells.

In the H2-A^{g7} molecule, β 56 and β 57 are histidine and serine residues respectively. By introducing transgenic H2-A^{g7} β -chains expressing charged residues such as proline at position 56 (Lund, O'Reilly et al. 1990) or aspartic acid at position 57 (Quartey-Papafio, Lund et al. 1995) it is possible to protect from diabetes. Mice expressing transgenic A β ^{g7(asp)} at position 57 are described in the remainder of this thesis as NOD-Asp. It has been suggested that mutation to an acidic residue allows formation of an intra-chain salt-bridge to the arginine at position 76 of the α -chain, increasing both stability and half-life of the molecule, as well as altering the peptide-binding characteristics (Reizis, Eisenstein et al. 1998). Similarly, mutagenesis to residues found in diabetogenic MHC preventing salt-bridge formation in the related H2-

A^d affected stability such that the MHC was not expressed at the cell surface (Nalefski, Shaw et al. 1995).

Absence of the salt-bridge alters the peptide-binding groove. Expression of empty and peptide-filled H2-A^{g7} molecules in *Drosophila* cells, using phage-display technology, suggested that due to a large P9 pocket in the binding groove, the binding of peptides is 'promiscuous' when compared with H2-A^d (Stratmann, Apostolopoulos et al. 2000). Others have suggested that loss of the salt-bridge allows formation of a number of distinct conformations to be achieved, dependent on the peptide in the groove, thus altering potential contact-sites with TCR (Arneson, Peterson et al. 2000).

Human HLA class II molecules have been analysed to compare their stability and while alleles associated with susceptibility to insulin-dependent diabetes (IDDM) (including DR3/4) are unstable in SDS, other alleles including HLA-DQA1*0102/DQB1*0602 are much more stable (Ettinger, Liu et al. 1998). This suggests that type-I diabetes susceptibility in humans is also linked to the stability of the MHC class II molecule. The structure of the human DQ8 class II molecule in complex with an insulin-derived peptide has also recently been elucidated (Lee, Wucherpfennig et al. 2001), linking both the P9 pocket as well as position 57 of the β -chain in the human molecule to that seen in mouse. This allows close correlation to be drawn between the structural features of the molecules that predispose to diabetes, the shape of the peptide-binding pockets and the peptides that bind in mouse and man.

A number of T cell clones were isolated from NOD mice to examine the peptide binding and TCR interaction capacity of H2-A^{g7}. Kanagawa *et al* (Kanagawa, Shimizu et al. 1997) showed that positions 56 and 57 were important both in binding of peptides as well as TCR recognition of the MHC/peptide complex. NOD mice are able to recognize polymorphism at position 57, by rejecting skin grafts from NOD-Asp mice, while grafts from mice expressing a mutant β -chain at position 56 (histidine to proline) are not recognized or rejected. Analysis of the selected repertoire by flow cytometry suggested that the presence of Asp at position 57 affects T cell selection, both in terms of V α /V β usage and in the CD4/CD8 ratio of T cells (Antoniou, Elliott et al. 1998). Two NOD-Asp restricted T cell clones, 1F8 and 2E4, characterised by Antoniou *et al* are used in chapter 5 of this thesis. NOD mice are capable of mounting anti-HY responses; about 60% of naïve female mice were able to reject male skin grafts. Female NOD mice immunised with male splenocytes also make anti-HY specific CD8⁺ responses as measured with ⁵¹Cr-release assays *in vitro* (Chandler, Fairchild et al. 1988).

1.23: Superantigens

Superantigens (SAGs) are divided into two main classes. The first group are exogenous soluble proteins such as bacterial toxins including staphylococcal enterotoxins A-I and toxic shock syndrome protein (TSST-1), globular proteins of between 22-29 kDa, resistant to both protease digestion and heat denaturation (Papageorgiou and Acharya 2000).

The second group are encoded by endogenous retroviruses integrated in the host genome. When first discovered in 1974, they were named minor lymphocyte stimulating antigens (Mls), and were later shown to be products of integrated proviruses, *Mtv*, of the mouse mammary tumour virus (MMTV) an infectious agent expressed in breast tissue and then transmitted to the offspring through milk, by infecting gut lymphocytes that subsequently traffic to the mammary gland (Fraser, Arcus et al. 2000). Mice are protected from infection by MMTV if they express *Mtv* pro-virus in thymic epithelial and dendritic cells (Moore, Anderson et al. 1994). This causes deletion of *Mtv*-reactive cells during negative selection, preventing the presence of MMTV activated T cells in the gut, without which, an MMTV infection cannot be transmitted to the pups. Almost all strains of mice have integrated *Mtv* pro-viral genomes. The open reading frame in the 3' long terminal repeat of *Mtv* encodes the viral superantigen (vSAG). Although the various *Mtv* integrations are highly homologous at a sequence level, they differ in C-terminal residues, a polymorphic region conferring V β specificity. As they are integrated into the genome, they are inherited like host genes, and cause thymic and peripheral deletion of specific V β -containing TCRs. The crystal structure of several TCR β -chain/MHC/superantigen complexes have now been analysed, including mouse TCR V β 8.2 in complex with staphylococcal enterotoxin B (SEB) and MHC (Li, Llera et al. 1998) and TCR β -chain in complex with two different staphylococcal enterotoxins C2 and C3 (Fields, Malchiodi et al. 1996). This information, together with other methods of analysis has enabled the mechanism of T cell deletion by SAGs to be explored.

Massive T cell V β -specific activation by SAGs can cause a number of conditions including toxic shock syndrome and scarlet fever, by expansion and activation of T cells expressing TCRs with particular V β , followed by production of various inflammatory cytokines such as TNF- α and - β and IL-2 (Marrack and Kappler 1990).

SAGs bind to MHC class II in several ways. Crystal structures have shown that they bind to MHC class II molecules on the surface of antigen presenting cells, and form a bridge to the T cell, binding specifically to the V β region of the TCR, bypassing the need for specific TCR/peptide/MHC interactions. SEB has been shown to have two globular domains, one of which exhibits MHC class II binding capacity through a hydrophobic groove in the α 1 domain of the human MHC class II molecule HLA-DR. Between the two globular domains is a groove that connects to the TCR CDR2 and another position on the TCR. In this way, SEB is able to bridge between the TCR and MHC, altering binding position and orientation. A second mechanism is used by TSST which binds to the MHC class II molecule in a similar manner to that seen in the SEB, yet also appears to lie on top of the MHC class II, thus preventing the intimate interaction between the two molecules (Fraser, Arcus et al. 2000).

1.24: HY minor transplantation antigens

While the major histocompatibility molecules have been shown to be involved in the immune responses, autoimmunity and transplantation, reviewed by (McDevitt 2000), other antigens, known as minor histocompatibility antigens are involved in allograft responses. While mismatched major antigens in a transplantation setting cause a rapid immunological response and graft rejection process, minor antigens can cause rejection, even when the major antigens have been matched. The minor histocompatibility antigen HY has been extensively studied. This minor antigen is encoded on the Y chromosome of humans and mice. In mice it can provoke the rejection of male tissues by syngeneic females (Simpson, Scott et al. 1997). As well as graft rejection (host versus graft, HvG), HY can play an important role in graft versus host disease (GvHD) and graft versus leukaemia (GvL), important clinical aspects of bone marrow transplantation therapy for human leukaemia (Simpson, Roopenian et al. 1998).

First demonstrated in the 1950s, HY antigens provide a useful model for investigating transplantation responses using inbred mouse strains, as the only differences between male and female mice are proteins encoded by genes on the Y-chromosome. It was shown that female C57BL/6 mice reject syngeneic male tail skin grafts (Bailey 1971; Bailey and Hoste 1971).

The Y-chromosome contains a relatively small number of genes, some involved in testis formation and spermatogenesis. At present, three genes in mice *Dby*, *Uty* and *Smcy*, all located on the short arm of the Y-chromosome, in a deletion interval known as *Sxr^b*, have been defined as encoding MHC class I and II-associated HY peptides (Simpson, Scott et al. 1997).

Uty is a constitutively expressed protein and one MHC class I epitope of HY, presented in the context of H2-D^b, has been defined (Greenfield, Scott et al. 1996). The peptide sequence WMHHNMDLI has been elucidated and various T cell clones, including CTL-10, respond by proliferating maximally with 10-100nM peptide presented on irradiated female splenocytes. These clones do not respond to the related peptide from the female *Utx* gene (Personal Communication- Dr D. Scott). The CTL-10 clone and others of this specificity are capable of killing peptide-pulsed cells in a ⁵¹Cr cytotoxicity (CTL- cytotoxic T lymphocyte) assay.

More recently, dendritic cells were used in experiments to identify two HY class II peptides. COS cells were transfected with the *Dby* gene and then allowed to express the protein. After 48 hours female day 6 bone marrow DCs were added (either H2^b or H2^k) and allowed to process and present relevant HY peptides, before being used to stimulate an HYA^b-specific hybridoma to produce IL-2, and a CD4⁺ HY E^k-specific T cell clone to produce IFN-γ. The cognate peptide epitopes were found to be NAGFNSNRANSSRSS (H2-A^b-*Dby*) and REEALHQFRSGRKPI (H2-E^k-*Dby*) (Scott, Addey et al. 2000) both of which were shown to stimulate appropriate CD4⁺ T cell clones at nanomolar concentrations, while female epitope homologues do not stimulate.

The CD4⁺ B9 T cell clone, responding to the A^b-*Dby* peptide and CD8⁺ CTL-10 clone responding to the D^b-*Uty* peptide have been used in this thesis. Together with rejection of male grafts by syngeneic female mice, assays with these clones provide useful tools for examination of modulating influences on CD4 and CD8⁺ T cell responses, both *in vitro* and *in vivo*.

1.25: Effects of glucocorticoids and immunosuppressive treatments on immune responses

A number of different compounds are known to have immunomodulatory effects, leading to suppression of immune responses. Cyclosporin (CsA) is a fungal product, commonly used as a long-term immunosuppressive to prevent graft rejection responses in humans, inhibiting T cell function by interfering with intracellular signalling. However, such agents tend to cause blanket immunosuppression, causing problems such as susceptibility to infection during the therapeutic regime.

Glucocorticoids (GC) are steroid hormones known to have a wide number of roles in the immune system. Glucocorticoids are able to diffuse through cell membranes, binding to specific glucocorticoid receptors (GR) found in the cytoplasm. On binding a glucocorticoid, the receptor is translocated to the nucleus, forming a homodimer, which binds to glucocorticoid response elements (GREs) associated with glucocorticoid-regulated genes. GR also interact with other transcription factors such as AP-1, normally acting as antagonists, although GR have also been shown to enhance AP-1 activity (Ashwell, Lu et al. 2000).

Glucocorticoid receptors are part of a large family of steroid hormone receptors including thyroid hormone, vitamin D₃ and retinoic acid, many of which have also been examined for immunoregulatory properties. Synthetic glucocorticoids such as dexamethasone and cortisone are commonly used immunosuppressive compounds, for treatment of a number of autoimmune and inflammatory conditions including rheumatoid arthritis. However, they too suffer from inducing non-specific immunosuppression (Ashwell, Lu et al. 2000). Glucocorticoids have also been shown to prolong skin graft survival (Belldegrun, Cohen et al. 1981). As many different cell-types express GR and many different genes contain GREs in their regulatory elements it paints a complicated picture. Glucocorticoids can inhibit T cell proliferation *in vitro*, as well as influencing the secretion of a large number of cytokines including IL-2. Dexamethasone was recently used to block IL-2 induced binding of Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, inhibiting IL-2 receptor expression and thus blocking a number of other cytokines using IL-2 receptor components (Bianchi, Meng et al. 2000). Other glucocorticoid targets include a mouse gene known as *SRG3*, linked to the sensitivity of a thymoma cell line to apoptosis by dexamethasone (Han, Choi et al. 2001). Overexpressing the *SRG3* gene in peripheral T cells, made them sensitive to GC-induced apoptosis and stress-induced cell death. The

SRG3 protein associates with GR in the thymus, and if blocked with an 89 amino acid fragment of SRG3, thymocytes are no longer sensitive to GC-induced apoptosis. Linking closely with this work, it was shown that use of activated Notch1 induces down regulation of SRG3 and thus sensitivity to apoptosis, while expression of transgenic SRG3 in thymocytes expressing transgenic Notch1 was able to restore GC-sensitivity (Choi, Jeon et al. 2001). This suggests that SRG3 is a downstream target of Notch1 in modulating GC-sensitivity of thymocytes or T cells to apoptosis. GCs have also been shown to inhibit TCR-stimulated tyrosine phosphorylation events, inhibiting kinases including ZAP70 kinase, affecting localisation of TCR-associated signalling molecules at the plasma membrane in the lipid rafts (Van Laethem, Baus et al. 2001).

Results of work using KLH-immunised rats, has questioned whether GCs are always inhibitory. After removal of the adrenal gland (source of most GC hormones), rats were given replacement corticosterone in their drinking water. In the absence of GC replacement, rats had reduced anti-KLH IgG and IgM responses compared to untreated controls, while those that received replacement had less reduced levels of IgM (Fleshner, Deak et al. 2001). GCs have also been implicated in influencing Th lineage commitment, through favouring generation of Th2 cells (Ashwell, Lu et al. 2000). Other effects have been shown on both thymocytes and antigen presenting cells such as DCs, the subject of part of this thesis.

1.26: Effects of GCs on thymocyte apoptosis

Many groups have examined the role of GC-induced apoptosis of thymocytes due to its link to T cell selection in the thymus. The use of dexamethasone to induce apoptosis in thymocytes is a classical model for apoptotic death and is often used for examining inhibitors of apoptosis, and other parameters of apoptosis.

A number of groups have examined treatment of thymocytes *in vitro* and *in vivo*, showing that populations in the thymus have differential sensitivity. *In vivo* treatment of C57BL/6 mice with dexamethasone caused a decrease in thymic cell yield, with a decrease in the DP population and proportional increase in SP and DN thymocytes (Screpanti, Morrone et al. 1989). If the treatment regime was continued over a longer time (5-10 days as opposed to 7-48 hours) effects were seen in the DN and CD4SP populations, with less cells detectable. If the time-course was continued beyond 10 days, the thymic yield was very low.

Several groups have analysed the time taken for cultured thymocytes to become apoptotic and shown that apoptosis can be observed within 4-6 hours *in vitro*. Thymocytes cultured for 6 hours in the presence of 0.1 μ M dexamethasone were analysed for propidium iodide (PI) staining, a non-specific DNA-binding agent that will only bind if the cell membrane has become leaky as happens in the latter stages of apoptotic death. After 6 hours, around 16% of thymocytes had spontaneously undergone apoptosis, while with dexamethasone 52% were PI positive (Jan, Wing et al. 1999). Others have performed a more detailed time-course of thymocyte apoptosis using both dexamethasone and another hormone involved in thymocyte death, diethylstilbesterol. After 4 hours in culture, apoptosis was obvious for both treatments using criteria including: 7-AAD staining (staining DNA) and annexin V binding (measuring phosphatidyl serine exposure at the cell surface); both are indicative of the early stages of apoptosis. There was no evidence at the early time point of PI staining (Donner, Becker et al. 1999). Maximal apoptosis was observed after 12 hours exposure to dexamethasone.

The activity of different caspase enzymes can be monitored during apoptosis of thymocytes using confocal microscopy. Synthetic rhodamine-based caspase substrates were introduced into thymocytes undergoing dexamethasone-induced apoptosis. On activation of the caspase, the substrate rhodamine ring is cleaved, becoming fluorescent indicating activation of the specific caspase. Using a number of different substrates, the order of caspase activation within the cascade was determined (Komoriya, Packard et al. 2000).

Thymocytes are thought to be sensitive to GC-induced apoptosis due to the role of GCs in the processes of thymic selection, particularly in the DN-DP transition when secreted by thymic epithelial cells (Ashwell, Lu et al. 2000). It is thought that while high concentrations of GCs are able to induce apoptosis, low doses may antagonise TCR-mediated apoptosis in thymocytes (Iwata, Hanaoka et al. 1991). A number of groups have examined the role of GCs in selection. Vacchio *et al* (Vacchio and Ashwell 1997) used foetal thymic organ cultures (FTOC) with TCR transgenic mice, specific for D^b in the presence or absence of corticosterone, inhibiting endogenous GC secretion using myterapone, an inhibitor of enzymes used to convert inactive GC-precursors into active GCs. Presence of the inhibitor caused a decrease in the DP population, that could be reversed by addition of physiological concentrations of GCs. By inhibiting GC production in the transgenic mice, it was shown that the loss was both antigen and MHC-specific, from cells that would otherwise undergo positive selection, suggesting

that a balance exists in selection between the TCR signal and GC signalling, allowing TCRs interacting with low-moderate avidity to survive. More recently, the same group have suggested that GCs within the thymus affect the thresholds of avidity in selection, altering the effects of TCR signalling. They proposed that while thymocytes with high avidity TCR/MHC interactions are deleted and those with moderate avidity survive, a large population with low-moderate self-reactivity are selected yet do not become SP because of GC-mediated repression of the effects of TCR occupancy, preventing further differentiation. By lowering the local concentration of GC, it is suggested that the effects of TCR occupancy are increased, thus shifting the window of avidity in selection (Vacchio, Lee et al. 1999). Further evidence for the role of GC in altering selection thresholds was shown using a spontaneously autoimmune mouse model (MRL-*lpr/lpr*) (Tolosa, King et al. 1998). Expression of an antisense transgenic GR in thymocytes causes loss of cells that are normally positively selected, thus inhibiting many of the symptoms of autoimmunity in these mice.

Work has shown that thymocytes can undergo spontaneous apoptosis *in vitro*, possibly mimicking death-by-neglect within selection. Although caspases are known to be involved in this death mechanism, it has been shown that alternative pathways are involved, such that caspases-1 and -3 are dispensable, with death occurring through mitochondrial release of *cytochrome c* and activation of other caspases (Zhang, Mikecz et al. 2000).

Some of these ideas have generated controversy, considering that other work has shown that in GR^{-/-} mice, thymocyte differentiation, selection and T cell development appear to occur normally (Purton, Boyd et al. 2000). Doubts were raised over the validity of using FTOC or newborn thymus cultures, as well as using antisense GR transgenes to examine GC function and comparing this to the role of GC within the adult (Godfrey, Purton et al. 2001). It appears that the issue of the precise role of GCs in thymic selection is still not clear.

It cannot be doubted however, that both *in vitro* and *in vivo* treatment of thymocytes with GCs causes significant apoptosis particularly in DP thymocytes, decreased thymic output and *in vivo*, thymic involution. These processes occur in a complex pathway, downregulating survival genes and upregulating pro-apoptotic genes. This involves a number of different intermediates including downregulation of NF- κ B binding to DNA (Wang, Wykrzykowska et al. 1999) as well as other transcription factors; activation of the caspase cascade, activation of cyclins such as Cdk2 and alterations in activity of Bcl2 family members such as Bcl2, Bcl-x_L and Bax, all of

which have been shown to affect thymocytes, either preventing or assisting apoptosis (Williams and Brady 2001).

1.27: Effects of immunosuppressive treatments on dendritic cell maturation and function

A number of treatments have been examined *in vivo* and *in vitro* and shown to have immunosuppressive effects on murine, human and rat DCs.

Aspirin is a commonly used analgesic and anti-inflammatory drug. Murine bone marrow derived DCs (GM-CSF and IL-4) were treated from day 2 of culture with 0.5-2.5mM aspirin. At day 7, their maturation was inhibited when compared to untreated controls by examining cell surface markers, showing decreased expression of CD40, CD80, CD86, MHC class II on CD11c positive cells in a dose-dependent manner. *In vitro*, aspirin-treated DCs showed decreased stimulatory capacity as well as displaying morphological differences. Such DCs were less able to stimulate immune responses *in vivo* (Hackstein, Morelli et al. 2001).

IL-10 is an immunosuppressive cytokine thought to have a number of roles including modulation of DC function. Groups have examined the effects of IL-10 on human PBL-derived DCs. Steinbrink *et al* (Steinbrink, Jonuleit et al. 1999) showed that if DCs were cultured for 7 days with IL-10 for the final 2 days, the DCs were less stimulatory in an allogeneic MLR and inhibited proliferation of CD3-antibody-stimulated T cells. It was also shown that CD8⁺ T cell lines when treated with IL-10 DCs became anergic and unable to respond to secondary challenges with the same antigen in an alloantigen-specific manner that could be overcome by addition of IL-2 to the culture. However mature DCs were not susceptible to the effects of IL-10. Melanoma-specific CD8⁺ T cells that had been made anergic in this manner were no longer able to kill melanoma cells. Previous work from the same group had shown that upregulation of a number of costimulatory markers including CD86 and CD58 was inhibited in IL-10 treated DCs (Steinbrink, Wolfl et al. 1997), suggesting that IL-10 blocks complete maturation of DCs. More recently, work on monocyte-derived DCs has suggested that an autocrine IL-10 loop may exist, limiting activation of DCs by a number of different stimuli, leading to increased IL-10 production upon activation (Corinti, Albanesi et al. 2001).

1 α , 25 dihydroxyvitamin D₃ (D₃) is also thought to inhibit DC maturation. Treatment of monocyte DCs with 10nM D₃ during differentiation *in vitro* showed limited effect on a number of surface markers. However after induction of maturation with LPS, D₃DCs were less able to upregulate class I, II, CD80, CD86 and CD40 than untreated controls. D₃DCs were better at endocytosis of a model antigen, suggesting immaturity and were less able to stimulate an MLR. Responder T cells were shown to be anergic in a non-specific manner, as they were unable to respond in a subsequent MLR with untreated DCs (Piemonti, Monti et al. 2000). Immature D₃-treated murine DCs have been shown to be resistant to maturation stimuli in a similar manner to that in humans, while injection of male D₃ DCs into female mice and subsequent challenge with a male graft showed prolongation of survival in a number of the mice, while untreated DCs primed a rejection response (Griffin, Lutz et al. 2001). Treatment of mice with a combination of D₃ and Mycophenolate Mofetil *in vivo* induced tolerance to mismatched islet grafts, while mice that maintained islet grafts subsequently accepted mismatched heart grafts. Both DCs and macrophages from within the graft area were found to express decreased levels of a number of costimulatory molecules. Tolerance was thought to be mediated by CD4⁺CD25⁺ regulatory cells and was transferable to a proportion of naïve recipients (Gregori, Casorati et al. 2001).

Glucocorticoids such as dexamethasone (DEX) are known to cause immunosuppression in a number of ways including affecting DC maturation. Epithelial DCs from airways are known to be potent at uptake and processing antigens. Epithelial DCs isolated from rat respiratory tract and treated with dexamethasone showed an inhibited ability to uptake and process exogenous antigens (OVA) when stimulated by GM-CSF. However in an MLR, pre-processed self-antigen could still be presented to alloreactive T cells and no decrease in MHC class II or CTLA-4 expression was seen. These data suggest that the effects of dexamethasone (at least in this case) may play a role in processing of antigen but not in presentation (Holt and Thomas 1997). This conflicts somewhat with other work showing decreased functional ability of DEXDCs, although this may be a result of the model used or specific properties of epithelial DCs when compared to other populations of DCs. Work using a murine epidermal DC-line showed that DEX inhibited a number of different characteristics associated with maturation including upregulation of CD86 and secretion of IL-1 β and TNF- α . This maturation process is known to be dependent on interaction with T cells (Kitajima, Ariizumi et al. 1996).

Thymic DCs from rats have been shown to express GC receptors, but are insensitive to GC-induced apoptosis unlike thymocytes. When treated *in vitro* with DEX, they show decreased expression of a number of costimulatory molecules and when used as APCs in an MLR, show reduced IL-1 β and TNF- α but not IL-6 or IL-10 production (Sacedon, Vicente et al. 1999). This suggests that within the thymus, GC may not only play a role in thymocyte apoptosis during selection, but also in modulating thymic DC function.

Work on murine splenic DCs showed that treatment with DEX *in vitro* led to decreased viability, decreased surface expression of CD80 and CD86 that could be blocked with steroid antagonists, but limited effects on CD11c or class II. When used as APCs, DEXDCs were unable to stimulate naïve T cells, but were able to stimulate IL-2 production by a T cell hybridoma. *In vivo* treatment with DEX caused a decrease in splenic DC numbers and decreased responses of splenocytes to stimulation with anti-CD3 or superantigen (Moser, De Smedt et al. 1995). This work agrees with work from Matyszak *et al* (Matyszak, Citterio et al. 2000), which showed that immature DCs when treated with DEX were unable to undergo maturation when assessed by cell surface markers and showed both a decreased ability to stimulate T cells and altered cytokine secretion patterns by the T cells. This work suggested that repeated stimulation with DEXDCs gave rise to a regulatory population of T cells *in vitro*, secreting IL-10. This compares with others who have shown that repeated stimulation with allogeneic immature human DCs was able to induce T cells secreting large amounts of IL-10 in the absence of IFN- γ , IL-2 and IL-4. Initial T cell proliferation when stimulating with immature DCs was reduced compared to that with fully matured DCs (Jonuleit, Schmitt et al. 2000).

A number of groups have examined the effects of glucocorticoids on different human cell-types. Human neutrophils are known to be less sensitive to GC effects than both T cells and DCs due to increased expression of GR β compared to GR α . There are two isoforms of GC receptor in humans GR α and GR β . GR β is unable to bind GC, but acts as an inhibitor of GC binding to GR α , possibly forming an antagonistic heterodimer and is expressed at high levels in neutrophils, increasing on stimulation with IL-8. This renders neutrophils less sensitive to apoptosis than other cell-types and on transfection into mouse neutrophils (that do not express GR β) protects from DEX-induced death (Strickland, Kisich et al. 2001).

Human monocyte-derived DCs were cultured for 7 days in the presence or absence of DEX. When antigen uptake capacity of the DCs was examined, it was found

that DEXDCs showed enhanced antigen capture, both through receptor-mediated endocytosis and fluid-phase macropinocytosis. This was measured using two fluorescently labelled uptake substrates, FITC-dextran for receptor-mediated uptake and Lucifer yellow for macropinocytosis. A number of different receptors involved in receptor-mediated uptake were upregulated on DEX treatment. After maturation with TNF- α or CD40-L expression of these markers remained higher than controls suggesting an initial immature phenotype, with reduced ability to mature as was shown in murine DCs. DEXDCs were examined for the ability to present soluble tetanus toxin to T cells and were shown to have decreased stimulatory capacity compared to untreated controls (Piemonti, Monti et al. 1999). Subsequently, the same group showed that DCs cultured for 7 days in the presence of DEX expressed decreased levels of CD1a, CD40, CD80 and CD86 (Piemonti, Monti et al. 1999), the latter three molecules being involved in costimulation. Molecules involved in antigen uptake were upregulated, correlating with the enhanced uptake ability shown in the previous work. It was therefore suggested that DEX functions by blocking DCs in an early stage in differentiation, preventing maturation into fully functional APCs and inhibiting activation.

This work links well to data from Woltman *et al* (Woltman, de Fijter et al. 2000) showing that the presence of DEX or prednisolone (another corticosteroid) in monocyte cultures even for the first 48 hours alone were able to block DC maturation. As before, DEXDCs showed decreased expression of costimulatory molecules and MHC as well as an inability to respond to activation by LPS or CD40L and decreased ability to stimulate T cells. This was compared with other treatments commonly used in conjunction with steroids such as CsA or FK506. While the DEXDCs showed significant changes in surface markers, functional capacity and some changes in cytokine production, CsA and FK506 treatments led to decreased production of some cytokines and no other effects. This suggests that CsA/FK506 and DEX act in different manners to induce immunosuppression.

Rea *et al* (Rea, van Kooten et al. 2000) have used a CD40L/CD8 fusion protein as a reagent to activate immature human DCs in the presence or absence of DEX. Their work showed that activation through CD40L in the presence of DEX showed decreased costimulatory molecules expression, but also altered the cytokines secreted by DCs so that they produced significant amounts of IL-10. T cells stimulated by DEXDCs showed reduced proliferation in MLR compared to CD40L-fusion activated DCs in the absence of DEX and that the T cells were unable to respond when challenged again in

an antigen-specific manner. This approach extends previous observations and shows that CD40L/CD40 stimulated activation of DCs can be inhibited.

Together, these various strands of evidence show that *in vitro* and *in vivo* DC function is modulated by multiple pathways. It has been shown that DEX is able to inhibit DC maturation *in vitro* from precursors, while other work has shown that immunosuppressive compounds such as DEX, IL-10 or D₃ can modulate activation of DCs. It appears that if DCs are not completely activated or are prevented from differentiating completely, they may induce a tolerogenic phenotype *in vivo* partly by inducing non-responsiveness (anergy) in an antigen specific or non-specific manner and preventing further activation of such T cells; or alternatively by inducing regulatory populations of T cells (such as CD4⁺CD25⁺) that are able to inhibit responses to DCs. A third potential mechanism from this data, not necessarily mutually exclusive from the others is that immature DCs may secrete IL-10 (or induce regulatory T cells to secrete IL-10) which can act both as an immunosuppressive cytokine on surrounding T cells, but also feedback in an autocrine loop preventing differentiation of DCs.

1.28: Tolerance induction and the APC

Dendritic cells, in their role as professional APCs have, in recent years become recognised as playing a key role in the immune response and have been much studied as tools for the induction of peripheral tolerance. Different subtypes of DC and approaches to immunomodulation have been explored and used to induce tolerance either through anergy or inducing populations of regulatory T cells (Jonuleit, Schmitt et al. 2001). The effects of the immunosuppressive cytokine IL-10 on DC development have been discussed, showing that treatment of immature DC cultures with IL-10 inhibits secretion of a number of inflammatory cytokines such as IL-1 β , IL-6 and TNF- α . However if DCs are already mature, they are more resistant to the effects of IL-10 (Jonuleit, Schmitt et al. 2001). IL-10 treatment of DCs has been implicated in tolerance through the induction of anergy, a feature shared with immature DCs that are thought to induce anergy (Steinbrink, Wolfl et al. 1997). In a number of systems, immature DCs have been implicated in tolerance mechanisms, possibly through generation of CD4⁺CD25⁺ T cells, while CD4⁺CD25⁺ T_{reg} induced through repeated TCR stimulation are also thought to function by secreting IL-10 (Jonuleit, Schmitt et al. 2001).

Fully activated DCs are capable of inducing a potent immune response, and are considered to be the only APCs capable of priming a naïve immune response (Banchereau and Steinman 1998). Maturation can be induced by a number of stimuli including microbial and inflammatory products such as bacterial LPS and DNA, cytokines such as IL-1, GM-CSF and TNF- α ; as well as artificial activation using receptor ligation such as CD40. DC maturation downregulates antigen uptake capacity and at the same time enhances antigen presentation ability, upregulating expression of cell surface receptors involved in presentation of antigen such as MHC class I/II, CD80/86 and signalling lymphocytic activation molecule (SLAM) (Bleharski, Niazi et al. 2001). Maturation of dendritic cells *in vivo* is associated with migration from the site of antigen challenge and uptake of antigen to lymphoid tissue such as spleen and lymph nodes for presentation of antigen to T and B cells (Banchereau and Steinman 1998).

Recently cultured mouse bone marrow DCs have been examined by ELISA and FACS, at different stages in maturation for expression of cytokine both at mRNA and protein levels *in vitro*. This work sorted CD86⁻ immature DCs from CD86⁺, and used either LPS or CD40 ligation to induce terminal maturation and showed that immature DCs expressed high levels of IL-1 α and -1 β , TNF- α , transforming growth factor β 1 (TGF β 1) and macrophage inhibitory factor (MIF). In contrast, mature CD86⁺ DCs upregulated IL-6, -15, -12 and -18. The pattern of cytokine secretion also differed depending on whether LPS or CD40 was used to terminally mature the DCs. Both treatments induced production of IL-6, IL-15 and TNF- α , while treatment with LPS induced IL-1 α , β and MIF (Morelli, Zahorchak et al. 2001). Interestingly, it was recently shown that while mature DCs normally stimulate immune responses, if the epitope presented is an altered peptide ligand, it is able to induce anergy in a human autoreactive T cell clone, implying that DC maturation is not necessarily always indicative of a potent immune response (Quaratino, Duddy et al. 2000).

Several groups have analysed immune responses to immature DCs and showed that they are capable of inducing T_{reg} or anergy. Hawiger *et al* (Hawiger, Inaba et al. 2001) used a fusion protein between a monoclonal antibody to DEC-205, one of the endocytic receptors characterised as being restricted to DCs, and a hen-egg lysozyme peptide (HEL) to target antigen delivery to DCs. This method was shown to be more efficient at antigen delivery than peptide in Complete Freund's Adjuvant (CFA). Use of anti-DEC-205 to target peptide to DCs in this manner allows delivery in the absence of either inflammation (inducible by CFA), or other activation stimuli. When T cell anti-HEL responses were examined after 2 days, mice that had received HEL in CFA

produced strong proliferative responses, as did the DEC/HEL fusion. However 7 days after treatment, the DEC/HEL no longer exhibited responses even to HEL/CFA, while HEL/CFA still maintained good proliferation. This suggests that the stimulation by DEC/HEL was transient, and even with repeated injections of DEC/HEL, prolonged responses could not be detected. The conclusions drawn from this work suggested that antigen presentation by DCs in steady-state (or in the absence of inflammation) was able to induce only transient T cell activation, after which deletion or anergy occurred *in vivo*.

Lutz *et al* (Lutz, Kukutsch et al. 2000), using BMDCs *in vitro* showed another method of producing immature DCs apart from IL-10 and dexamethasone/D₃. They cultured murine BMDCs from day 0 with GM-CSF as normal, but with 1µg/ml LPS in the culture. The DCs that developed appeared to be blocked in development as they expressed low levels of MHC class II and other markers of maturation when compared with DCs cultured in the absence of LPS from day 0-6 then LPS added to induce maturation for the last day. Using FITC-dextran as a model antigen, the immature DCs showed high phagocytic capacity and when used in an MLR showed decreased stimulation. They were able to induce alloantigen specific anergy of CD4⁺ T cells *in vitro* such that while the T cells were no longer able to respond to the alloantigen they were able to proliferate in response to a third-party APC or high doses of IL-2. The same group also showed that low doses of GM-CSF in the absence of IL-4 could produce immature DCs capable of inducing cardiac allograft survival *in vivo* (Lutz, Suri et al. 2000).

Immature DCs have also been shown to induce antigen-specific non-responsiveness in humans *in vivo* (Dhodapkar, Steinman et al. 2001). Injection of immature DCs pulsed with a peptide derived from influenza (MP) or KLH into healthy humans induced reduced MP or KLH-specific responses directly from CD8⁺ T cells. It also induced MP-specific T cells that secreted IL-10 by ELISPOT. When the cells were cultured, clones were expanded that bound specific tetramers, yet were unable to lyse target cells.

These data suggest that DCs constitutively phagocytose antigen in the steady state (in the absence of inflammation or in the absence of 'danger') and in the absence of activating stimuli remain in an immature state with low levels of MHC and costimulatory molecules. Interaction with T cells in an immature state may be able to induce tolerance via several mechanisms including anergy or induction of regulatory T cells. However it is also possible that populations of DCs may exist that are specialised

in inducing T cells to become regulatory T cells. A number of groups have characterised what they classify as DC2 cells that are able to cause T_{reg} cells to produce IL-10 or IFN- α , possibly during immune responses to viruses to regulate the immune responses preventing excessive responses (Roncarolo, Levings et al. 2001). Hara *et al* (Hara, Kingsley et al. 2001) showed that IL-10 was crucial for T_{reg} function when tolerance was induced with depleting anti-CD4 antibody treatments. This used both cardiac and skin allografts and showed that when treated with anti-IL-10 or anti IL-4 in tolerance transfer, anti-IL-4 had no effect while anti-IL-10 prevented tolerance on adoptive transfer of T_{reg}.

Finally it is also thought that T_{reg} are involved in a number of different tolerance protocols. Use of either low antigen dose i.v. or oral tolerance, where the antigen is introduced in drinking water in TCR transgenic mice induced populations of CD4⁺CD25⁺ T cells that played a role in antigen-specific tolerance. In contrast if the same antigen was introduced in the presence of LPS as an adjuvant, no T_{reg} were generated (Thorstenson and Khoruts 2001). T_{reg} were also demonstrated to be involved in an oral tolerance model in rats. Rats fed allogeneic splenocytes prior to kidney transplantation, showed prolonged graft survival. When infiltrating T cells were isolated from the graft, CD8⁺ cells on transfer into naïve animals were able to prolong graft survival (Zhou, Carr et al. 2001).

Although there are a number of strategies involved in the role of APCs in peripheral tolerance, it cannot be denied that DCs play a crucial role in regulating immune responses, both in infection and also in tolerance. Whether they are involved in induction of anergy, clonal deletion, masking of epitopes or regulatory T cells, it is clear that DCs are an important part within a complex network of interacting mechanisms, although there is still much to be investigated. The phenotype of an 'immature' DC is normally described as low expression of MHC and costimulatory molecules, however, it is likely that there are a large number of molecules yet to be characterised that may be involved in maturation, regulation and effector function.

1.29: Apoptosis and necrosis, cellular death mechanisms and antigen presentation

1.29.1: Apoptosis

Two processes of cell death have been described, apoptosis and necrosis. Programmed cell death or apoptosis is an orderly process, through which a cell becomes separated from neighbouring cells and undergoes nuclear condensation and shrinkage. The chromatin undergoes condensation, through two parallel pathways of activation (Susin, Daugas et al. 2000) and is eventually cleaved into fragments of about 180bp in length, known as DNA laddering (Rathmell and Thompson 1999). The plasma membrane begins to bleb, allowing division of the cell into apoptotic bodies. Induction of apoptosis in a cell also induces changes in the symmetry of the plasma membrane, such that phosphatidyl serine molecules are flipped from the cytoplasmic face to the extracellular face of the membrane, by the action of enzymes known as scramblases. This asymmetry can be detected using Annexin V that binds specifically to phosphatidyl serine in the membrane (used within this thesis as an indicator of apoptosis in thymocytes). Apoptotic bodies containing DNA and cellular organelles, displaying phosphatidyl serine at the surface are then marked for phagocytosis by cells including macrophages and neutrophils and subsequent digestion of the contents by lysosomes. One of the important factors of apoptosis is that it is a relatively 'clean' process, such that cellular contents are not spilled into the surrounding environment inducing immunological responses.

Apoptosis is thought to occur through a number of pathways, either through activation of a number of 'death' receptors expressed on the cell surface such as Fas or through cellular 'stress' mechanisms such as DNA damage by UV irradiation, and also through programmed cell death during development. After an immune response, excess T cells formed through clonal expansion are removed, leaving a small population of memory cells (Lenardo, Chan et al. 1999). Defects in this clearance mechanism has been linked to a number of autoimmune conditions including autoimmune lymphoproliferative disease in which antigen-induced apoptosis is defective in T cells; and systemic lupus erythematosus.

When triggered by appropriate signals, such as ligation by Fas-ligand, exposure to TNF- α or even cytokine withdrawal, a number of intracellular cascades are activated. Caspases are proteases constitutively expressed as inactive pro-caspases within cells

(Thornberry and Lazebnik 1998), peculiar for a particular cleavage-site motif involving a tetrapeptide motif to the N-terminal side of an aspartic acid residue. Positive and negative feedback loops exist such that activation and cleavage of the first pro-caspase enzyme into the active caspase initiates cleavage of other caspases within a cascade. The caspase cascade is regulated by a number of proteins including inhibitor of apoptosis proteins (IAPs) (Green 2000). Caspases induce apoptosis by inactivating inhibitors (such as Bcl-2) of other proteins that are functional in apoptosis, by destroying structural proteins such as the nuclear lamina proteins and also by deregulating other proteins by cleaving regulatory and catalytic domains (Thornberry and Lazebnik 1998).

A second method of activating apoptosis is through triggering release of cytochrome C from mitochondria by treatments with anti-cancer drugs or irradiation. Cytochrome C released into the cytosol is thought to bind to apoptotic protease-activating factor (Apaf-1), occurring within 1 hour of apoptosis induction (Renz, Berdel et al. 2001). Apaf-1 undergoes conformational change exposing a caspase recruitment domain, which is able to activate pro-caspase 9. The mitochondrial/Apaf-1 pathway is thought to link into the death-ligand pathway further down the caspase cascade such that lower events in apoptosis are common to both mitochondrial/Apaf-1 and 'death'-receptor mediated apoptosis (Green 1998). More recently, other processes of non-apoptotic, yet programmed cell death have been suggested (Sperandio, de Belle et al. 2000)

1.29.2: Necrosis

The second principal type of cell death is known as necrosis. This is a more 'messy' and unregulated form of death, and can induce inflammation through the uncontrolled release of cellular contents. Necrotic cells take up water through the membrane and swell in stark contrast to apoptotic shrinkage, leading to the cell bursting and release of cytoplasmic contents. Although the chromatin does not undergo condensation, the DNA is still degraded although in a less structured manner to apoptosis (Rathmell and Thompson 1999). Necrosis can be triggered by a number of events including absence of energy in the form of ATP to maintain the membrane potential, as well as uncontrolled viral replication and subsequent cell-lysis and release of virions as part of the viral replication cycle.

1.29.3: The effects of cell death on immune responses

Apoptosis and necrosis are thought to have different consequences in immune responses. Several groups have shown that apoptosis of T cells is essential for development of peripheral T cell tolerance. Mice with defective apoptotic pathways when treated with either co-stimulation blockade such as CD28-B7 and CD40-CD40L, or rapamycin (normally both treatments can be used to induce peripheral tolerance) were no longer effective, suggesting that induction of T cell apoptosis was crucial for development of tolerance through these protocols (Li, Li et al. 1999; Wells, Li et al. 1999). The controlled nature of apoptosis is thought to limit inflammation, although it can still occur through IL-1 and IL-18 mediated pathways. Apoptosis inhibits pathogen release from infected cells, while necrosis is associated with release of pathogens and intracellular proteases, subsequent inflammation and tissue damage as well as the presence of immune activating stimuli such as LPS and inflammatory cytokines.

It is currently unclear as to the effect of apoptotic death on antigen presentation and immune responses. Interesting work from Albert *et al* (Albert, Sauter et al. 1998) showed that DCs could acquire antigen *in vitro* from apoptotic monocytes infected with influenza and present in the context of MHC class I molecules, inducing CTL. Antigen presentation was dependent on apoptosis as CTL were inhibited in a dose-dependent manner by ZVAD, an inhibitor of apoptosis, while monocytes and macrophages could not induce CTL in this manner. Previous work had shown that feeding apoptotic cells to macrophages *in vitro* could stimulate CTL (Bellone, Iezzi et al. 1997). More recently, apoptotic T cells were found to overexpress vinculin, and subsequent phagocytosis induced maturation of DCs, allowing cross priming of vinculin-specific CTL in a CD40L-dependent process (Propato, Cutrona et al. 2001). This was also examined in the peripheral blood of HIV patients and a direct correlation found between the number of CD40L⁺ cells and the magnitude of the CTL-response to vinculin. Together with other work involving various models of infection and apoptosis, this suggested that CTL-epitopes could be generated by an exogenous cross-presentation pathway in APCs particularly DCs and macrophages, allowing presentation of epitopes from phagocytosed apoptotic cells on MHC class I molecules (Larsson, Fonteneau et al. 2001).

In addition to MHC class I epitopes, Inaba *et al* (Inaba, Turley et al. 1998) showed that epitopes from phagocytosed cells could also be presented in the context of

MHC class II. Murine BMDCs were fed B cell blasts or human HLA-DR α ⁺ cells and presentation of a peptide derived from H2-E, by H2-A, recognised by the Y-Ae antibody was shown to be dependent on antigen processing and phagocytosis. This was shown to be a highly efficient process, more efficient than pulsing with peptide.

This work has led a number of groups to propose the use of (Nestle, Banchereau et al. 2001) and begin clinical trials with human DCs presenting tumour derived peptides (Berard, Blanco et al. 2000; Banchereau, Palucka et al. 2001) and DCs 'fed' apoptotic tumour cells (Palucka 2001), as well as hybrids between renal carcinoma cells and human DCs (Kugler, Stuhler et al. 2000) as clinical vaccines to aid in the clearance of tumours.

A number of groups have examined the interaction between different mechanisms of cell-death and APC function. Heat shock proteins (HSP) have often been implicated in the generation of immunity through stimulation of DC maturation (Bhardwaj 2001; Somersan, Larsson et al. 2001), released during necrotic cell death. However it has been suggested that apoptotic leukaemia cells, when heat stressed can also induce expression of HSP72 and HSP60 at the cell surface (Feng, Zeng et al. 2001). When apoptotic and viable tumour cells were injected into mice, leukaemia developed. However if the apoptotic cells were also heat-stressed prior to injection, allowing expression of HSP, the leukaemia cells were removed. DCs fed the heat-shocked apoptotic cells were also capable of inducing anti-tumour CTL. However the presence of HSP begs the question as to whether true apoptosis was induced in this model.

It was shown that if murine CTLL-2 cells were grown in the absence of IL-2, apoptosis could be induced and measured by Annexin V staining. When apoptotic CTLL-2 cells were cultured with a PMA-treated macrophage cell-line (THP-1), they were able to phagocytose the CTLL-2 cells, leading to secretion of IL-8 and IL-1 β , both pro-inflammatory cytokines but no anti-inflammatory cytokines (Kurosaka, Watanabe et al. 1998). Phagocytosis and production of cytokines could be inhibited by using phospo-L-serine and RGDS, both of which are inhibitors of known phagocytosis receptors.

Double stranded, but not single stranded DNA has recently been shown to activate APCs inducing upregulation of MHC class I/II and costimulatory molecules, while dsDNA-treated DCs were able to present OVA peptides to T cells *in vivo* and induce anti-OVA antibodies (Ishii, Suzuki et al. 2001). This suggests a difference between apoptosis and necrosis, as dsDNA is not released into the environment in

apoptosis, unlike necrotic death. Others have compared the effects of lysed (necrotic) and apoptotic cells on APC function and shown that macrophages could be induced to secrete macrophage inflammatory protein-2 (MIP-2) and TNF- α by adding lysed neutrophils or proteases derived from cytoplasm of neutrophils, but not apoptotic cells (Fadok, Bratton et al. 2001). Gallucci *et al* (Gallucci, Lolkema et al. 1999) examined murine BMDCs cultured for 24 hours with healthy, apoptotic or necrotic fibroblasts and showed that while healthy and apoptotic fibroblasts do not induce upregulation of class II and CD80/2, necrotic cells do, to a similar extent to LPS. These data, together with other work suggests that different signals are encountered by APCs in the local environment of apoptotic and necrotic cells, where release of factors (possibly HSPs) from necrotic cells induced activation, while apoptotic cells do not. This conflicts somewhat with the use of apoptotic cells to induce antigen-specific CTL and kill tumour cells, although if tumour cells are killed in a non-apoptotic manner, one might envisage anti-tumour CTL.

These data compare well with that from a number of groups who have suggested that phagocytosis of apoptotic cells induces a non-immunogenic and even immunosuppressive phenotype (Voll, Herrmann et al. 1997). This work compared co-cultures between apoptotic, fresh or fixed peripheral blood lymphocytes (PBL) with either peripheral blood mononuclear cells (PBMC) or monocytes. The apoptotic cultures caused the PBMC/monocytes to secrete more IL-10 (anti-inflammatory) and less TNF- α , IL-1 β and IL-12 (pro-inflammatory) than fresh or fixed cells. Secretion of IL-10 in culture could be mimicked using monoclonal anti-CD36 antibodies to bind to the phagocytic thrombospondin receptor. Sauter *et al* (Sauter, Albert et al. 2000) showed that if immature DCs phagocytose apoptotic cells they do not undergo maturation, while necrotic cells are able to induce upregulation of CD83, DC-LAMP, CD40 and CD86. DCs exposed to necrotic cells are able to cross-present antigens from the cells to induce CTL.

More recently, it has been shown that if mice are injected intravenously with irradiated apoptotic splenocytes, after receiving sub-lethal total body irradiation and prior to receiving an allogeneic bone marrow transplantation, that engraftment occurred to a greater extent, in the absence of graft-versus host disease (GvHD) (Bittencourt, Perruche et al. 2001). Surprisingly, this appeared to be independent of the source of apoptotic cells, in that donor, recipient, third-party and even human PBMCs could induce enhanced engraftment in the recipient mice.

These data have suggested that apoptosis and necrosis are processes that induce different phenotypes in the cells involved in phagocytosis. It is thought that while necrotic cells are able to induce maturation and cross-presentation of antigens, possibly via release of HSPs or intracellular proteases and subsequent activation of the APCs, apoptosis does not release these factors, such that phagocytosis occurs in a 'non-threatening' milieu. It is thought that as apoptosis is a 'normal' regulated process, the uptake of apoptotic cells is involved both in immune suppression and tolerance to antigens (Steinman, Turley et al. 2000), through absence of maturation stimuli and subsequent presentation of antigen to T cells in the absence of sufficient co-stimulation. It has also been suggested that apoptosis is involved in oral tolerance mechanisms, such that in the absence of inflammatory signals, T cells in the lamina propria do not respond to antigenic stimulation and undergo apoptosis normally. In patients with inflammatory bowel disease, including Crohn's disease or colitis, decreased apoptosis was found. This suggests that apoptosis may play a crucial role in tolerance in the gut to environmental antigens, and that immune responses are induced by the presence of pathogenic bacteria, causing local inflammation and necrosis (Bu, Keshavarzian et al. 2001).

The 'Danger model' suggests that APCs are activated in the presence of injury or inappropriate cell death (Matzinger 2001). Release of 'alarm' or 'danger' signals by cells undergoing necrosis or infection with pathogens and subsequent phagocytosis by APCs induces maturation and activation of the APC, stimulating upregulation of costimulatory molecules. This allows complete activation of specific T cells in the presence of costimulatory molecules and the initiation of the adaptive immune response. Uptake of antigen in the absence of 'danger' (such as apoptosis) would mean the absence of suitable activating signals, and subsequent presentation of antigens without upregulation of costimulatory molecules and maturation of the APCs. Ligation of the TCR/MHC in the absence of appropriate costimulation would induce incomplete activation of the T cells and either complete absence of a response or active tolerance.

Phagocytosis of apoptotic cells is mediated by a number of specific cell surface receptors and intracellular mediators. At the plasma membrane of the apoptotic cell, phosphatidyl serine is flipped to the extracellular surface and it is thought that this acts as a ligand for scavenger receptors on the surface of macrophages, although it is unclear how this occurs. CD14 is thought to be a potential receptor allowing attachment of apoptotic B cells to macrophages, although CD14 is also a receptor for bacterial LPS. Scavenger receptors are divided into six categories, each with different domain structures and ligand specificities. One of the best characterised is CD36, binding a

number of ligands, but also involved in malaria allowing the parasite to enter the cell (Platt, da Silva et al. 1998). Macrophages are highly phagocytic cells and express a number of receptors including SR-A one of the scavenger receptors that was shown to be important for the phagocytosis of fluorescently labelled apoptotic thymocytes *in vitro* (Platt, Suzuki et al. 1996); and Mer, a receptor tyrosine kinase also shown to regulate thymocyte uptake *in vitro* (Scott, McMahon et al. 2001). The Wiskott-Aldrich syndrome protein (WASP) is expressed in haemopoietic cells and is involved in signal transduction from the cell membrane to the cytoskeleton involved in receptor mediated endocytosis. Macrophages from WASP^{-/-} mice have reduced phagocytic capability both *in vitro* and *in vivo* (Leverrier, Lorenzi et al. 2001).

1.30: Mechanisms of tolerance induction: apoptosis, anergy and regulation

1.30.1: Tolerance background

A number of different mechanisms have a role in the induction and maintenance peripheral tolerance to antigens in infection, autoimmunity and transplantation and have been exploited to suggest approaches to tolerance induction (Waldmann 1999). These include the application of antibodies to disrupt receptor-ligand interactions such as CD4, MHC class II or CD40-CD40L (Honey, Cobbold et al. 1999; Graca, Honey et al. 2000; Ito, Kurtz et al. 2001). The first point of tolerance induction is central tolerance, the processes of positive selection in the thymus, selecting only cells that express TCRs interacting with moderate avidity with self-MHC peptides and deleting those that interact with high affinity as previously discussed. However this process is incomplete, such that a number of autoreactive cells escape selection and enter the periphery (Sprent and Kishimoto 2001), while not all of the peripheral antigens are expressed in the thymus. Autoimmune disease is rare in the population, despite the presence of autoreactive cells and there are thought to be three main mechanisms maintaining peripheral tolerance, none of which are mutually exclusive, but when defective, can allow disease to occur.

1.30.2: Apoptosis and tolerance

The first mechanism is apoptosis. As previously described, this is an ordered process linked to peripheral tolerance. Apoptosis of T cell clones is a normal part of T cell homeostasis, as when an infection has been cleared large numbers of specific T cells exist which must be removed. Clonal deletion happens in several ways including activation-induced cell death (AICD) and cytokine withdrawal. Blockade of costimulation and deprivation of growth factors such as IL-2, as well as decreased expression of anti-apoptotic proteins such as Bcl-xL induces passive death of T cells. Repeated stimulation of the TCR induces apoptosis through Fas and TNF-mediated pathways. As previously described, apoptosis of T cells has been shown to be necessary for induction of peripheral tolerance in a number of transplantation models (Li, Li et al. 1999; Wells, Li et al. 1999; Li, Wells et al. 2000). Clonal deletion is thought to influence tolerance by impairing the pool of potential effector cells, possibly through costimulation blockade, reducing the frequency of effectors and reducing the survival capacity of existing cells (Wells, Li et al. 2001).

1.30.3: Anergy and tolerance

A second mechanism is anergy, T or B cell unresponsiveness to antigen either by proliferation or effector function. It has been suggested that T cell tolerance can be mediated through “a state of long-lasting, partial or total unresponsiveness induced by partial activation” (Lechler, Chai et al. 2001). A number of mechanisms have been shown to induce anergy *in vitro* including recognition in the absence of costimulatory molecules. This has been examined using anti-CD3 stimulation of both naïve and memory murine T cells in the absence of costimulation *in vitro* and showed that some cells underwent apoptosis and the surviving CD4⁺ cells were no longer able to produce or respond to IL-2 by proliferating (Chai and Lechler 1997). *In vitro*, anergic cells were shown to inhibit proliferation of normal T cells independent of cytokine secretion. Transfer of the anergic CD4⁺ T cells into mice followed by skin grafting showed prolonged survival of allogeneic skin grafts (Chai, Bartok et al. 1999). *In vivo* induction of tolerance by continuous antigenic stimulation is thought to be dependent on IL-10 production by anergic T cells (Jooss, Gjata et al. 2001).

Other mechanisms for induction of anergy include using antagonist peptide analogues, which bind to MHC molecules but prevent T cell activation and in some cases induce anergy. Such peptide ligands are called altered-peptide ligands (APL) and are thought to incompletely activate T cells, inducing anergy. There is interest in the use of APL as therapeutics in both transplantation and autoimmunity, although a limitation of this approach is that all of the potential peptides would have to be known for each response (Lechler, Chai et al. 2001).

A third mechanism involves the stimulation of T cells either without IL-2 or in the presence of IL-10. IL-2 is important in causing T cell division and it has been shown that if T cell clones are stimulated in the absence of IL-2 signalling, either through IL-2 receptor blockade or with anti-IL-2 antibodies, they do not proliferate and may become anergic. Similarly, IL-10 is known as an immunosuppressive cytokine and if the TCR is stimulated in the presence of IL-10, CD4⁺ T cells become unresponsive (Lechler, Chai et al. 2001).

Finally presentation of antigen by T cells to T cells has been shown to induce anergy in T cell clones. The mechanism underlying this phenomenon is currently unclear (Lechler, Chai et al. 2001).

1.30.4: Tolerance and regulatory T cells

In vivo tolerance is also thought to be mediated by regulatory T cells (T_{reg}) that influence other populations of T cells and APCs, either through direct interaction or through cytokine secretion. Around 10 years ago, the idea of suppressor T cells lost favour in the immunological community, yet in the past 2-3 years, they have reappeared in another guise as regulatory T cells. A number of populations of CD4⁺ T_{reg} have been described to be involved in tolerance (Sakaguchi 2000). One of the populations of T_{reg} is thought to be contained in the CD4⁺CD25⁺ T cell cohort. CD25 is the IL-2 receptor α -chain, transiently expressed on CD4⁺ T cells after activation. However a population of about 5-10% of CD4⁺ T cells, constitutively expressing CD25 are thought to be regulatory. Adoptive transfer experiments have shown that nude mice develop autoimmunity if CD4⁺CD25⁻ cells are transferred. However if either CD4⁺CD25⁺ cells or a mixed (CD25⁺ and CD25⁻) population are transferred, disease does not occur, suggesting that the CD25⁺ cells regulate the CD25⁻ population (Shevach 2001). Regulatory T cells have been characterised both *in vitro* and *in vivo* to cause

suppression of immune responses in mice, both in transplantation models (Hara, Kingsley et al. 2001) and in autoimmunity (Shevach 2001).

The mechanisms by which regulatory T cells are thought to develop is unclear, and depends upon the model used, whether *in vitro* or *in vivo* and the method used to induce tolerance.

1.31: Aims of the project.

The aims of this work were to use a number of model systems to examine the roles of several important factors involved in T cell function: MHC class II expression in the thymus and periphery, and the mode of antigen presentation for the induction of immunity and tolerance.

The first system examined the role of the level of MHC class II surface expression on T cell development and function, both in the thymus on T cell selection and in the periphery on antigen presenting cells. B-Asp mice express the mixed haplotype MHC class II molecule $\alpha^b\beta^{g7(asp)}$ at approximately 10% of either of the parental molecules in the absence of other MHC class II. The reduced expression MHC molecules were used to examine the role of the level of MHC class II expression on the T cells selected, particularly in terms of the T cell receptors that survive thymic selection. V α and V β gene usage in B-Asp mice were examined and compared to other mouse strains, with detailed sequence analysis of the β -chain CDR3 region to determine whether the MHC class II has had any influence on the structure of the TCR. In addition, the function of the T cells selected was examined by assessing the degree of alloreactivity, the cytokines they produced and whether altering the level of MHC class II influences the ability of the T cells to induce an effective immune response for skin-graft rejection.

NOD-Asp transgenic mice were used to examine a possible MHC class II transfer mechanism, as well as the roles of both the direct and the indirect antigen-presentation pathways in the graft rejection process. In normal circumstances, both the direct and indirect pathways are thought to play important roles, with the intra-graft APCs stimulating an anti-graft response in the host (direct presentation), while uptake, processing and presentation of graft antigens by host APCs (indirect presentation) has also been implicated in graft rejection. When NOD female mice are given a NOD-Asp male graft, both the direct pathway and indirect pathways can be brought into play. By manipulating the antigens in the graft, an attempt has been made to examine the role of the direct pathway in isolation from the indirect pathway.

Thirdly, a transplantation system was used to examine APCs as an important regulator of T cell function. The HY system was used to examine the role of the immunosuppressive glucocorticoid (dexamethasone) on APC and subsequent T cell function. By treating cultured bone-marrow dendritic cells *in vitro*, dexamethasone was

used to modulate the responses of various HY-specific T cell clones to treated APCs. This effect was also examined *in vivo*.

The HY system was also used to examine the role of apoptosis, antigen uptake and processing. This model was used to examine the role of apoptosis in inducing peripheral tolerance to minor allo-antigens. Using dexamethasone to induce apoptosis, the uptake of apoptotic thymocytes by different APCs both *in vitro* and *in vivo* was studied, and possible mechanisms by which tolerance may be induced in this manner were suggested.

Chapter 2.

Materials and methods

2.1: Mice

B-Asp mice were bred by a backcross between NOD-Asp transgenic mice (Quartey-Papafio, Lund et al. 1995) and $MHC^{-/-}$ ($A\beta^{-/-}$ and $\beta 2m^{-/-}$) mice (a kind gift from Dr. M. Merckenschlager, MRC, CSC). F_1 progeny were then backcrossed with $MHC^{-/-}$. Backcross mice were typed using tail DNA in PCR reactions by Dr. J. Picard, (MRC CSC) for three markers: NOD MHC class II α -chain, $A\beta^{g7(asp)}$ transgenic β -chain and $\beta 2m$. Mice that were NOD negative and Asp positive were called B-Asp, while mice that were NOD positive and Asp negative were called BN. Both mice occur in the presence or absence of $\beta 2m$ (MHC class I) (Figure 3.1), and are used as stated in the text.

C57BL/6, CBA/Ca and BALB/c mice were purchased from Harlan Olac, UK. NOD, NOD-Asp, SJL, B10.BR, B10.Ea, B10.GD and FVB mice were bred in the BSU at Hammersmith Hospital, where animals were maintained.

2.2: Growth media and culture conditions

RPMI 1640 medium (Gibco BRL) was used for all cell-culture and was with 10% Foetal Calf Serum (FCS) (Biogen), Hepes buffer (10mM), Penicillin (100 I.U./ml), 100mg/ml Streptomycin (Gibco BRL), $10^{-5}M$ 2-Mercaptoethanol (2-ME) and 2mM L-glutamine (Gibco BRL). Unless stated otherwise all cultures were performed at 37°C with 5% CO₂.

2.3: Dynabead purification: B cells, T cells and thymocytes

Dynabeads were purchased from Dynal and used according to the manufacturer's instructions. Briefly, single cell suspensions were made from spleen, LN or thymus of mice, washed in RPMI (unless specified in the text, this involved resuspending in at least 10ml RPMI or BSS with 5-10% FCS, then centrifugation for 5 minutes at 1500 rpm) and resuspended in 3ml RPMI.

For depletion of B cells, 0.2ml of sheep anti-mouse IgG beads were washed twice in Balanced Salts Solution (BSS) and resuspended in 2ml cold RPMI. In order to deplete populations of T cells, single cell suspensions were treated similarly, with either Pan-T (Thy1/2) beads to remove all T cells, anti-mouse CD4 or anti-mouse CD8 to remove a single population. For depletion of MHC class II positive cells from C57BL/6 thymocytes, the single cell suspension was counted and resuspended in BSS. Cells were resuspended in 4ml of BSS + 2% FCS together with 1 μ g per 10⁶ target cells of purified Y3P (anti-class II- A^b), or 2ml per 10⁶ target cells of hybridoma supernatants containing Y3P, HB26 (mouse-anti-H2^b) and/or TIB120 (rat-anti mouse-class II). These were filter-sterilised before use and allowed to incubate at room temperature for 20 minutes while shaking gently. Thymocytes were washed twice in BSS + FCS (10 minutes, 1000 rpm) and then beads (pan-mouse IgG, sheep-anti-mouse Ig or sheep-anti-rat IgG beads) equivalent to 10 times the number of target cells were used.

Cells were mixed with appropriate dynabeads and incubated at 4°C shaking for 30 minutes (or 20 minutes at room temperature for thymocytes) after which the cell suspension/beads were placed in the magnetic separator for 5 minutes. Supernatant containing unbound cells was removed, transferred to a fresh tube and placed in the separator for a further 5 minutes. Supernatant was again removed and cells centrifuged before use. In order to ensure that appropriate cells had been removed, samples were either examined under the microscope to ensure that dynabeads were present in 10-fold excess as recommended or examined by flow cytometry.

2.4: RNA purification and BV2/8S2/8S3 PCR optimisation

2.4.1: RNA purification and cDNA

Thymocytes and spleen were removed from B-Asp and BN MHC class I⁺ mice. Any CD8⁺ T cells were depleted from the spleen as described using CD8 dynabeads. Total mRNA was isolated from the cell populations using the RNeasy RLTTM kit (Qiagen), according to the manufacturers instructions. Briefly, cells were washed in Phosphate Buffered Saline (PBS) and resuspended at 10⁷ cells per 2ml RNeasy RLT. The RNA was solubilised by pipetting and 0.2ml chloroform was added to 2ml of RNA solution. Samples were mixed and stored on ice for 5 minutes, then centrifuged at 4°C at 12000g for 15 minutes. After centrifugation, the clear aqueous phase containing the

RNA was removed to a fresh tube, an equal volume of isopropanol added, and placed on ice for 15 minutes. After centrifuging at 4°C at 12000g for 15 minutes, the RNA formed a pellet at the base of the tube. The supernatant was removed, the pellet washed in 75% ethanol by vortexing, then centrifuging for a further 8 minutes at 4°C. The ethanol was removed, the pellet dried and the RNA resuspended in RNA-free dH₂O (distilled water) with RNAGuard (Pharmacia).

After quantifying the RNA, cDNA was made by RT-PCR. Briefly 1-2µg total RNA, 200ng random hexamers (Gibco BRL) and dH₂O (to a final volume of 40 µl) were incubated at 70°C for 10 minutes. After cooling to room temperature, remaining ingredients were added as follows: 2µl 100mM DTT (dithiothreitol), 5x 1st strand buffer, 1µl Superscript (200 U/µl) (Gibco BRL), 2µl dNTPs (10mM stock solution), 1µl RNAsin (40 U/µl) (Pharmacia), incubated for 1 hour at 37°C, then 5 minutes at 95°C. dH₂O was added to a final volume of 100µl. Quality of cDNA was confirmed, using HPRT control primers:

R 5' GTC AAG GGC ATA TCC AAC AAC AAA C
F 5' CCT GCT GGA TTA CAT TAA AGC ACT G

These were used under the following conditions: 1x 95°C- 5 minutes, 30x (95°C- 50 seconds, 60°C- 50 seconds, 72°C- 1 minute), 1x 72°C- 7 minutes. When run on an agarose gel this PCR gave a product of 352 b.p. in size (Figure 3.7 b).

2.4.2: BV-specific PCR reactions and cloning

Sequences for Vβ2, 8S2 and Cβ specific primers were taken from (Pannetier, Cochet et al. 1993) and purchased from Genosys. Sequences are:

Vβ2: 5' TCA CTG ATA CGG AGC TGA GGC
Vβ8S2: 5' CAT TAT TCA TAT GGT GCT GGC
Cβ: 5' CTT GGG TGG AGT CAC ATT TCT C

Vβ-specific PCR reactions were optimised using cDNA isolated from the spleen of a C57BL/6 mouse and performed using the Expand High Fidelity PCR system (Boehringer Mannheim). According to the manufacturer's protocol, two separate

mixtures are made: Mixture 1: 200 μ M dNTPs, 0.1-0.75 μ g cDNA, 300nM of each primer (V β 2 and C β , V β 8S2 and C β) and dH₂O to a final volume of 25 μ l; and Mixture 2: 2.6 U High-Fidelity enzyme mix, 10x Expand buffer with 15mM MgCl₂ and dH₂O to 25 μ l. Just before use, the two were mixed and run at the following PCR conditions: 1x 94°C- 2 minutes, 10x (94°C- 15 seconds, 58°C- 30 seconds, 72°C- 45 seconds), 20x (94°C- 15 seconds, 58°C- 30 seconds, 72°C- 45 seconds + 20 seconds increment per cycle), 1x 72°C- 7 minutes. The PCR products were run on a 0.8% low-melting-point agarose gel (Amresco) and appropriate sized bands (BV2 ~140 b.p. and BV8S2 ~210 b.p.) excised under low power U.V. light. PCR products were purified from the agarose using the QIAquick™ gel extraction kit (QIAGEN) according to the manufacturer's protocols, first heating to dissolve the agarose, then passing through a spin-column to isolate and then elute the DNA. Purified DNA was run on a 2% agarose gel to confirm the band purified was of the correct size (Figure 3.7 a).

Purified PCR product was cloned into the pCR®2.1 vector supplied with the TA cloning kit (Invitrogen) according to the manufacturer's protocols. Briefly, 1.8ng fresh PCR product was set up in a ligation reaction with ligation buffer, 50ng vector, 4.0 Weiss units of T4 DNA ligase and dH₂O to 10 μ l final volume. The ligation reaction was incubated for 4-12 hours at 14°C, then used to transform INV α F' cells. Cells were thawed on ice and 2 μ l of 0.5M 2-ME was added. 2 μ l of the ligation reaction was added and incubated on ice for 30 minutes. The cells were heat-shocked in a 42°C water bath for 30 seconds, then returned to ice for a further 2 minutes. 250 μ l of SOC medium was added to each reaction and shaken at 37°C for 1 hour. 50 and 100 μ l of the reaction was plated onto LB-agar (50 μ g/ml ampicillin) plates that had been pre-treated with 40 μ l of 40 mg/ml X-Gal. Once the liquid was absorbed, the plates were inverted and incubated overnight at 37°C.

2.4.3: Plasmid DNA isolation and sequencing

Individual white or pale blue colonies were picked from the LB agar plates and inoculated into 3-4ml LB-broth (50µg/ml ampicillin) and grown overnight shaking at 37°C. Plasmid DNA was isolated from the cultures using mini-prep kits (QIAGEN, Bio/Spin – Biogene or NucleoSpin- Macherey-Nagel). Briefly, overnight cultures were spun down, the pellet resuspended in buffer and the cells alkaline-lysed to isolate plasmid DNA. The reaction was neutralised, adjusted to high-salt conditions and spun onto a silica-gel membrane. The plasmid DNA remains bound to the membrane while other impurities are eluted. DNA was washed with ethanol and eluted with dH₂O. Presence of an insert was confirmed by digesting a sample of each isolate with EcoRI for 3-4 hours or overnight, then running on a 2% agarose gel (Figure 3.8). The sequence for the insert for positive samples was analysed using the Thermo Sequenase II dye terminator cycle sequencing kit (Amersham). Sequencing mix including dye-labelled dNTPs and DNA polymerase were mixed with 0.2-2µg plasmid DNA and 5-20µM M13 reverse primer: 5' CAG GAA ACA GCT ATG AC.

Samples were overlaid with mineral oil and run as follows: 96°C- 1 minute, 20-30x (96°C- 30 seconds, 45°C- 15 seconds, 60°C- 4 minutes). DNA was precipitated using ice-cold ethanol and centrifuged, then analysed by the DNA core laboratory CSC, MRC. This process was repeated for each Vβ gene until 60-130 sequences had been collected from each tissue sample. The sequences were translated (GCG- HGMP resources) and in-frame sequences analysed for CDR3 length, amino acid usage and Jβ gene usage. When the sequence data for Vβ8S2 were analysed, it was discovered that the primers had cross-reacted with the closely related family member Vβ8S3, such that approximately 60% of sequences analysed from the Vβ8S2 reaction were Vβ8S3. Both Vβ8S2 and Vβ8S3 were therefore analysed separately.

2.5: Flow cytometry

2.5.1: General information

In most cases, 10^6 cells per sample were washed in 2-3ml sterile PBS wash (Phosphate Buffered Saline with 5% FCS and 0.02% Sodium Azide (Sigma)) and centrifuging for 1000 rpm for 4 minutes. The cells were incubated with appropriate antibodies for 30 minutes in the dark on ice unless stated otherwise and washed in PBS wash. Unless stated, all antibodies used for flow cytometry were purchased from Beckton Dickinson, Pharmingen. If biotinylated antibodies were used in the first step, cells were incubated for a further 30 minutes in the dark with either streptavidin-QR (Sigma) or streptavidin-APC. Samples were then washed twice and analysed by flow cytometry using either a FACScan or a FACScalibur (Beckton Dickinson). Data were analysed using CellQuest software. Controls included unstained cells, cells with streptavidin-QR/APC alone and individually stained samples.

2.5.2: V α /V β analysis

Mesenteric lymph nodes were removed from mice and immediately fixed in 1ml fix solution (PBS with 1% BSA and 0.15% paraformaldehyde) and incubated at room temperature for 15 minutes. Cells were then washed twice in PBS wash and stained with CD4- PerCP and V α /V β -specific antibodies as follows: V α 2-PE, 3.2-FITC and 8-FITC, V β 2-PE, 4-PE, 5.1/5.2-PE, 6-PE, 7-PE, 8.3-PE, 10-PE, 11-PE, 12-FITC, 13-PE and 14-FITC for 20 minutes at room temperature. Cells were again washed twice and analysed for CD4 and V α /V β expression.

2.5.3: *In vitro* *in vivo* Carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling and tracking

Thymocytes (treated or untreated) were harvested, washed in PBS, centrifuged at 1500rpm for 5 minutes and resuspended in PBS at 10^7 cells/ml. CFSE (Molecular Probes) was added at 5 μ M for *in vivo* and 100nM for *in vitro* and incubated at 37°C for

15 minutes in the dark. Cells were washed three times in PBS and resuspended in an appropriate volume for the experiment.

In vivo thymocyte tracking

C57BL/6 male thymocytes were harvested and put in culture overnight with 100nM dexamethasone (Water soluble, stocks at 1mM in RPMI, Sigma) in RPMI. After treatment the cells, along with fresh thymocytes were harvested, washed twice and labelled with CFSE as described above. Cells were resuspended in PBS and injected into B6 female mice at 1×10^7 cells/mouse in the tail vein (i.v.). The three groups were untreated male thymocytes, untreated thymocytes with an injection of 0.1mg dexamethasone intraperitoneally (i.p.) and apoptotic thymocytes. 1, 3, 6 and 24 hours and 1 week after injection, two mice were sacrificed from each group, the spleen, mesenteric lymph nodes (LN) and thymus harvested, washed in PBS wash, aliquoted into FACS tubes and stained with CD11c-PE, B220-CyChrome and CD11b-biotin with streptavidin- APC as described previously. Untreated controls were analysed and used to set a background level. Other samples were stained with CD4-PerCP to ensure that CFSE-labelled thymocytes had been internalised. 100,000 cells were analysed in each sample, and the percentage of CD11c, CD11b or B220 positive cells also CFSE positive were plotted.

In vitro thymocyte uptake

Thymocytes were treated +/- dexamethasone for 5 hours *in vitro*, then labelled with CFSE. 5×10^6 thymocytes were added to each well with equal numbers of BMDCs, splenic DCs, peritoneal macrophages or T cell depleted (Dynabead) splenocytes. Some wells had 100nM dexamethasone added to the untreated thymocytes overnight. Cells were co-cultured overnight and harvested the following day. Depending on the APC, samples were labelled with the antibodies described above.

2.5.4: BMDC analysis

C57BL/6 BMDCs were harvested on day 7-8 of culture and washed twice in RPMI at 1500 rpm for 5 minutes. Cells were then stained on ice with the following antibodies: CD11c-PE, B7.1 (CD80)- FITC, CD40- FITC, MHC class II A^b-biotin + SA-APC or PerCP, B7.2 (CD86)-biotin + SA-APC/PerCP, MHC class I K^b- FITC or CD11b (Mac1)- FITC.

2.5.5: Tetramer analysis of spleen cells

Spleen cells were depleted of B cells using sheep anti-mouse IgG Dynabeads and washed in PBS wash as described. Resuspended cell pellets were incubated with HYD^b (^{Uty}) tetramer-PE conjugated (made by Maggie Millrain, Transplantation Biology CSC, MRC) for 20 minutes at room temperature in the dark. Samples were washed twice in PBS wash and resuspended then CD8-QR/PerCP was added and incubated for 30 minutes at 4°C in the dark. Samples were then washed twice and analysed.

2.5.6: Annexin V staining

Treated/untreated cells were washed in 1% Annexin V binding buffer (Pharmingen) with 1% FCS. To 100µl of cells, 3µl of Annexin V-FITC and 1µl of CD8-QR was added. Cells were incubated in the dark at room temperature for 15 minutes. After 14 minutes, 1µl of propidium iodide was added (in some experiments) and the cells were washed twice in binding buffer before analysis.

2.5.7: MHC class II upregulation

B-Asp or C57BL/6 splenocytes were cultured in 24 well plates (3ml/well) with various doses of rIL-4, IFN-γ or IL-2. After 24 hours in culture, the splenocytes were stained with CD11b-PE, CD11c-FITC and MHC class II A^b (Y3P)-biotin and SA-QR. Cells were gated as described and analysed for mean fluorescence intensity (MFI) of MHC class II staining in various populations.

2.6: Hybridoma formation and analysis

T cell hybridomas were made from B-Asp MHC class I⁺ splenocytes; B-Asp and BN purified CD4⁺ T cells. Between 5×10^6 and 3×10^7 splenocytes or purified CD4⁺ T cells were mixed together with BW7CD4 fusion partners (Letourneur and Malissen 1989) at a ratio of 1:1. Cells were washed three times (1200 rpm for 5 minutes) in BSS without FCS. On the last wash all the supernatant was removed and the pellet resuspended gently. 0.7ml polyethylene glycol (PEG) at 37°C was added over 90 seconds, shaking gently. 5ml of BSS at 37°C was added drop-wise over 2 minutes, then 15ml over 3 minutes shaking gently. The cells were then centrifuged for 5 minutes at 200g. The cell pellet was gently resuspended in RPMI (60ml/ 2×10^7 cells in the fusion). 1:3 serial dilutions were made and 100µl plated into each well of a 96 well flat-bottomed plate and incubated overnight. 100µl of RPMI with 2x HAT supplement (Gibco BRL) was added to each well and incubated for 8-14 days. Fused hybridomas were harvested into 24 well plates containing 0.8ml RPMI with 1x HAT. Once growing well, the hybridomas were transferred into 12 well plated with RPMI with 1x HT (Gibco BRL). Again the hybridomas were allowed to grow, then maintained in RPMI.

Hybridomas were tested by flow cytometry for expression of CD4 and TCR. As described above, they were washed and stained with CD4-FITC and CD3ε-PE (Figure 3.14). Double positive hybridomas were stored in liquid nitrogen until needed and used in alloreactivity assays.

Alloreactivity assay by IL-2 production were performed in two ways using APCs or IL-4 treated splenocytes as APCs. L cells expressing MHC class II molecules- A^d, A^b, A^k and A^s (a gift from Dr. M. Merckenschlager, CSC, MRC.) were maintained in flasks in RPMI. 2.5×10^5 cells/ml (100µl/well) were plated into 96 well flat-bottom plates and cultured overnight. The following day hybridomas were harvested and added to the L cells at $1-2 \times 10^5$ cells/ml (100µl/well) in triplicate. After 24 hours in culture at 37°C, 100µl supernatant was harvested from each well into a new 96 well plate, frozen at -70°C for 2 hours then thawed. The B9 CD4⁺ IL-2 responsive HY-specific clone (Simpson and Tomonari 1989) was used as a readout and 100µl added to each well at 2×10^5 /ml. Controls included RPMI with rIL-2 at 1 I.U./ml. Cells were cultured at 37°C for 24 hours, then 0.5µCi of ³H-Thymidine was added to each well and cultured for a further 18 hours before harvesting on a Tomtec cell harvester onto glass-fibre mats and counting in a beta-counter.

The alternative method used splenocytes from a variety of different mouse strains. Splenocytes were harvested and resuspended at 5×10^6 cells/ml in 12 well plates with rIL-4 at 1.25ng/ml to upregulate MHC class II and cultured overnight. Cells were harvested, washed twice, irradiated (all irradiation of APCs was done with 30Gy) and plated out at 2×10^6 cells/ml (100 μ l/well). Hybridomas were then added at 2×10^5 cells/ml as before and the same protocol as described above was followed.

2.7: Growth and maintenance of cell-lines and clones

2.7.1: KLH-specific cell lines

B-Asp and BN mice that had been previously immunised with keyhole-limpet haemocyanin (KLH) (Schoendorf 2000) were taken and the mesenteric (LN) removed. LN cells were washed and cultured at 5×10^6 cells/ml in RPMI in 24 well plates. Irradiated syngeneic splenocytes were used as APCs and resuspended at 5×10^6 cells/ml, then added to the responder cells. KLH was added to each well at 100 μ g/ml final concentration. Cells were incubated at 37°C for 10-14 days, harvested, washed and resuspended in the same manner with irradiated APCs. After 3-4 stimulations 20 I.U./ml rIL-2 was added and the responder concentration was decreased to 2.5×10^5 cells/ml. 72 hours after stimulation supernatant was harvested and used in IFN- γ , IL-10 and IL-4 ELISAs.

2.7.2: HY and NOD-Asp specific T cell clones

CD4⁺ (B9: A^b-*Dby*, (Simpson and Tomonari 1989)) and CD8⁺ (CTL10: D^b-*Uty*) HY-specific T cell clones were maintained by Dr. D Scott. 1F8 and 2E4 NOD-Asp specific clones (Antoniou, Elliott et al. 1998) were maintained with the assistance of Dr. J-G Chai. Briefly, the clones were harvested and resuspended at $1-2 \times 10^5$ /ml in RPMI and plated at 1ml/well in 24 well plates. Irradiated APCs (H2^b-male splenocytes for B9/CTL-10, NOD-Asp splenocytes for 1F8/2E4) were resuspended at 5×10^6 /ml (1ml/well), with 20 I.U./ml rIL-2 (B9, 1F8 and 2E4). Clones were restimulated every 2-3 weeks and were used as responders in proliferations assays 1-2 weeks after restimulation.

2.8: Skin grafting

Mice were skin grafted according to the procedure of Billingham and Medawar (Billingham and Medawar 1951). Briefly, tail skin was removed from donor mice and cut into 1cm² pieces for grafting. Recipient mice were anaesthetised with a mix of 1:3:1 of Hypnorm (Jensen Pharmaceutical Ltd), dH₂O and Midazolam (Roche Products Ltd) injected i.p. Mice were shaved and a piece of skin from the right flank was cut, the size of a graft. The skin graft was put in place, covered with a square of paraffin-impregnated gauze, and the mouse was then wrapped in a plaster of Paris bandage. After 8-12 days, the plaster was removed and the graft survival scored according to the following criteria:

‘+’ - represents a healthy graft with hair, pigmentation and without shrinkage. ‘+/-’ -

represents a graft that had lost hair, pigment and was shrunken, but still present. ‘-’ -

represents a graft that has been completely rejected, with no remaining graft tissue.

Statistical analyses of graft rejection times were performed using the Prism programme, using the Log-Rank method. Median survival time of the grafts is the time at which 50% of the grafts had been rejected.

2.9: Cytokine ELISA

As previously described, 72 hours after restimulation, supernatants were harvested from B-Asp and BN KLH-specific T cell lines and tested using Quantikine ELISA kits (R&D Systems) according to the manufacturer’s protocols. Briefly, dilutions of supernatant were incubated on antibody-coated plates and allowed to incubate for 2 hours at room temperature. The plate was then washed five times, after which a second layer anti-mouse cytokine-conjugate was added for a further 1 ½ - 2 hours. Wells were again washed five times. Conjugated to the second layer antibody was an enzyme that induces a colour change in the substrate mixture solution that was added after washing. A ‘stop’ solution was finally added when the colour change had developed sufficiently in the control-cytokine curve samples and the plates were read on a microtiter plate reader at 450nm.

2.10: Isolating antigen presenting cell populations for assays

2.10.1: Bone Marrow Dendritic Cells (BMDCs)

Bone marrow was isolated from the femur and tibia of donor mice. Bones were removed and cleaned of the majority of the muscle and cut above and below the kneecap. Bone marrow was flushed from inside the bone with 5ml RPMI using a syringe with a 25G needle. Cells were harvested, filtered and washed in RPMI (1700 rpm for 5 minutes), then resuspended to 5×10^5 cells/ml in RPMI with 5% GM-CSF supernatant and 6ml put into each well of 6 well plates. Cells were then cultured for 6-8 days before use, allowing expansion of dendritic cells.

2.10.2: Peritoneal macrophages

Donor mice were sacrificed, then 5ml of RPMI was injected i.p. The needle on an empty syringe was then inserted into the peritoneal cavity and the media carefully withdrawn. The RPMI then contained a high proportion of peritoneal macrophages.

2.10.3. Making splenocyte suspensions

The spleen was removed into a petri dish with 5-10 ml RPMI and was cut into two pieces. With the edge of two bent needles the spleen was gently 'teased' to release the splenocytes into suspension. A single cell suspension was made by aspirating the cells in a pipette several times, before passing them through a cell strainer. Cells were washed by centrifuging as previously described.

2.11: GM-CSF supernatant

The mouse GM-CSF gene was cloned into the expression vector (BCMGSNeo) (Karasuyama, Kudo et al. 1990) and transfected into the plasmacytoma line X63-Ag8. The GM-CSF hybridoma was grown in selection in RPMI with 1mg/ml G-418 geneticin sulphate (Sigma). In order to produce GM-CSF supernatant the hybridoma was washed

in RPMI twice and then grown in RPMI in the absence of G-418 in large flasks or roller bottles (5×10^7 cells/bottle). When the supernatants had turned yellow (hybridomas were grown to exhaustion) they were harvested (generally 7 days) and centrifuged to remove cells. Supernatant was stored at -20°C until needed, then thawed and stored at 4°C . The GM-CSF was quantified by Sofia de Noronha (I.C.H., London) using ELISA, and used at final concentration of 100ng/ml (approximately 5%).

2.12: B-Asp/NOD co-culture experiments

B-Asp and NOD splenocytes were harvested and cultured either in 12 well plates (3 ml/well) or 24 well plates (2ml/well) for 24 or 48 hours prior to use in an assay. Cells were co-cultured at 1:1 (1×10^6 of each cell-type/well) or 3:1 (3×10^6 B-Asp and 1×10^6 NOD). In later experiments when other strains of mice were used, the cells were cultured at 1:1 for 48 hours in 24 well plates.

B-Asp and NOD splenocytes were also cultured with the same ratios in the presence or absence of a transwell (Costar). 1ml of B-Asp splenocytes were cultured in the main well and 0.5ml NOD splenocytes in the transwell. After co-culture, the cells from separate chambers were harvested, washed and irradiated, then resuspended at 1.5×10^6 cells/ml (100 μl /well) in 96 well flat-bottomed plates. If fewer splenocytes were available, round bottom plates were used and splenocytes used at 5×10^5 /ml. The two NOD-Asp specific clones were harvested, washed and plated at 2×10^5 /ml (100 μl /well). The cells were cultured for 48 hours, then 0.5 μCi of ^3H -Thymidine added to each well and harvested after a further 18 hours in culture.

2.13: MACS purification and immunisation of naïve B cells

In order to have purified naïve B cells from the splenocytes of male NOD-Asp mice, MACS cell sorting using CD43 (Ly-48) microbeads (Miltenyi Biotec) was applied with depletion-type (CS) columns according to the manufacturer's protocols. Splenocytes were put into single-cell suspension and washed in PBS with 2mM EDTA (MACS buffer). After centrifugation, the cells were resuspended in 90 μl /10 ^{7} cells MACS buffer, 10 μl CD43 MACS beads added and incubated at 4°C for 15-20 minutes, after which the cells were washed in 10-20x labelling volume of MACS buffer and centrifuged at 300g for 10 minutes. The CS column was set up in the VarioMACS

magnet with a 22G needle attached and loaded with MACS buffer. Cells were resuspended in 2ml of buffer and applied to the column. The CD43 negative fraction was collected by washing the column with 30ml buffer, the column removed from the magnet and the CD43 positive fraction eluted. 10^6 cells of each type, as well as unseparated splenocytes were analysed by FACS using B220-CyChrome, CD3 ϵ -FITC, CD11c-PE and CD11b-biotin and SA-APC to ensure purity before resuspending the CD43 negative fraction at 5×10^7 cells/ml in PBS and injecting 100 μ l i.v. into female NOD mice.

2.14: Direct and indirect antigen presentation

Female NOD mice were injected i.v. with either 1×10^7 unpurified NOD-Asp male splenocytes on day (-3) or 5×10^6 CD43 negative naïve NOD-Asp male B cells on days (-15) and (-1) in PBS. The mice were grafted on day 0 as described above with NOD-Asp male, NOD-Asp female, NOD male or NOD-Asp female and NOD male grafts in the same graft-bed.

2.15: Treatment of HY- BMDCs and use *in vitro* and *in vivo*

2.15.1: Treatment of BMDCs *in vitro*

As previously described, BMDCs were made from various donor mice. For analysis of the effects of dexamethasone and lipopolysaccharide (LPS) (*E. coli* 0111:B4, Difco) in the HY system, DCs were made from C57BL/6 female or male mice. In some experiments BMDCs were treated from day 7-8 with various doses of LPS in PBS. In dexamethasone time course experiments, BMDCs were treated from days 0-7, 6-7 or 7-8 with doses of dexamethasone ranging from 10^{-9} M to 10^{-6} M or rIL-10. In some experiments, the BMDCs were treated from day 0-6 with 10^{-6} or 10^{-7} M dexamethasone, harvested and washed three times (1500 rpm for 7 minutes) to remove dexamethasone. Cells were then plated out in the same volume of RPMI with 5% GM-CSF supernatant and 10 μ g/ml LPS overnight and examined by flow cytometry as described in section 2.5.4 and for stimulating proliferation of HY-specific T cell clones. Other BMDCs were cultured from day 0-7 with 10^{-6} / 10^{-7} M dexamethasone, washed three

times, plated out in the same volume of RPMI with 5% GM-CSF and cultured for a further 7 days. They were then analysed by flow cytometry and for stimulatory capacity.

2.15.2: *In vitro* proliferation

BMDCs that had been treated with dexamethasone (DEXDCs), LPS or untreated were harvested and washed three times. Female BMDCs were resuspended in 1-2ml RPMI and incubated with 20nM HY D^{b(Uty)} (WMHHNMDLI) and 50nM HY A^{b(Dby)} peptide (NAGFNSNRANSSRSS) for 3-4 hours. Peptide-pulsed BMDCs or male BMDCs were washed, irradiated and plated at 100µl/well in 96 well flat-bottom plates at 2.5×10^4 - 1×10^5 /ml in triplicate. B9 and CTL-10 clones were harvested, washed and added at $1-2 \times 10^5$ /ml (100µl/well). The B9 clone had rIL-2 added at 1 I.U./ml (final concentration). Assays were incubated for 48 hours at 37°C, pulsed with ³H-Thymidine and harvested 16 hours later as previously described.

2.15.3: *In vivo* experiments

C57BL/6 male or female BMDCs were cultured for 7 days in the presence or absence of 10^{-6} M dexamethasone. After 7 days in culture they were harvested and washed three times before resuspending in 1ml RPMI. Female DCs were then pulsed for 2 hours with excess A^{b-Dby} or D^{b-Uty} peptides before washing twice and resuspending in an appropriate volume in PBS.

Male DCs or DEXDCs were washed and resuspended in PBS and 10^7 injected into the tail vein of B6 female mice. In addition, other mice received 10^7 or 5×10^6 fresh male bone marrow that had been harvested as described and resuspended in PBS. 14 days later the female mice were grafted with male tail-skin as previously described.

In a separate experiment, male BMDCs or DEXDCs, female DCs or female DEXDCs in the presence or absence of the individual peptides were injected at 5×10^6 cells/mouse into the footpad. 21 days later they were grafted with B6 male skin as described (James 2001).

2.16: Thymocyte apoptosis time-course

To examine the optimum dose of dexamethasone needed to induce apoptosis in thymocytes, B6 male thymocytes were harvested, washed and cultured in RPMI at 3.3×10^6 cells/ml (3ml/well). Dexamethasone was added at 1pM-10nM or 1-10000nM in two separate experiments and cultured for 24 hours. The thymocytes were harvested, washed and stained as described with Annexin V-FITC, PI and CD8-PE.

In time-course experiments, B6 male thymocytes were treated as described. Dexamethasone was added from 1-100nM and cultured at 37°C. After 15 and 30 minutes, 1, 2, 4 and 7 hours, the cells were harvested, washed three times in RPMI and put back into culture overnight. Other thymocytes were treated overnight. After the culture, thymocytes were harvested, washed and analysed by flow cytometry as previously described.

2.17: Thymocyte treatments to induce tolerance

Separate experiments were performed to examine different parameters. The first was to determine the optimum dose of thymocytes used for treatment. C56BL/6 female mice received 10^5 , 10^6 or 10^7 male or female thymocytes depleted of MHC class II positive cells as described in section 2.4 injected i.v., +/- 0.1mg dexamethasone i.p. on day (-21). On day 0, half of the mice received a further injection with the same treatments, while all were grafted with C57BL/6 male skin.

The second experiment compared the effects of dexamethasone treatment *in vivo* and *in vitro*. On days (-21) and (-1), C57BL/6 female mice were treated with 10^7 thymocytes i.v. Groups received male/female untreated thymocytes +/-0.1mg dexamethasone i.p. Other groups received male/female thymocytes that had been cultured for 4-5 hours in the presence of 100nM dexamethasone, then washed three times before injection (without dexamethasone injection). On day 0 they were grafted with male skin.

In a third experiment, the effects of different apoptotic stimuli were compared. In this experiment C57BL/6 or CBA/Ca female mice received 1×10^7 C57BL/6 male or female thymocytes i.v. either: untreated, dexamethasone *in vitro*, dexamethasone *in vitro* with 0.1mg dexamethasone i.p., or the calcium ionophore Calcimycin (Mixed Calcium-Magnesium Salt, stock dissolved to 10mM in DMSO- Sigma) treated *in vitro*.

Treatments were performed on days (-29), (-15), (-1), grafted on day 0 with C57BL/6 male skin, and those with surviving grafts treated again on day 14. Calcimycin treated thymocytes were cultured for 20 minutes in the presence of 10 μ M Calcimycin then washed three times before injection.

2.18: Adoptive transfer

Splenocytes were taken from C57BL/6 female mice that either accepted a B6 male graft (day 128 after initial grafting), had rejected on day 28-30, or from naïve females. The splenocytes were washed and resuspended in PBS and 3 $\times$ 10⁷ cells injected i.v. into young adult C57BL/6 females. 10⁶ of each splenocyte population were analysed by flow cytometry for tetramer positive cells. 3 days later the mice were grafted with male skin as described.

Chapter 3.

T cell selection mediated by limited MHC class II expression

3.1: Background

A number of theories have been postulated for the processes of thymic positive and negative selection. During thymocyte development, the vast majority of thymocytes (95-97%) do not survive the stringent selection (Huesmann, Scott et al. 1991; Shortman, Vremec et al. 1991). Insufficient interaction with self-MHC/peptide complexes on thymic epithelial cells leads to death through neglect. Conversely, high affinity interaction with MHC/peptides, possibly on thymic APCs, leads to active negative selection. This censorship allows removal of 'useless' thymocytes that cannot interact, deletion of 'dangerous' thymocytes expressing TCRs with high affinity for self that are potentially autoreactive, and the survival of a potentially 'useful' thymocyte pool (Robey and Fowlkes 1994).

Two principal theories have been proposed to explain differential survival of thymocytes dependent on interaction with MHC. The 'Alternate Ligand Hypothesis' (Marrack and Kappler 1988) suggests that positive and negative selection occur in distinct locations or with different peptide pools, inducing differential TCR signalling, determining thymocyte survival. The second is the 'Differential Avidity Model' (Ashton-Rickardt, Bandeira et al. 1994; Ashton-Rickardt and Tonegawa 1994) suggesting that positive and negative selection are regulated through a combination of the affinity of the interaction between TCR/MHC and the number of interactions occurring, known as avidity. This suggests that two thresholds exist, a lower threshold under which thymocytes die through neglect and an upper threshold over which thymocytes are deleted because of high avidity TCR/MHC interactions. In principal, the appropriate avidity window can be achieved in distinct ways. If the affinity of a TCR/MHC interaction is moderate, the number of interactions occurring will be moderate. If the number of interactions were low, the affinity of TCR/MHC interactions would need to be high and *vice versa*.

B-Asp mice express a single, mixed haplotype MHC class II molecule, expressed at around 10% of the parental MHC levels. If T cell selection were based on avidity, the affinity range of TCR/MHC interactions needed for positive selection, would be shifted in order to compensate for the reduced number of interactions.

Figure 3.1. Backcross breeding and potential genotypes of B-Asp and BN mice. NOD-Asp mice were bred with MHC class I and II knockout, then the F₁ progeny were backcrossed onto MHC class I and II^{-/-}. Coat colours of the mice are indicated in brackets, while the given name used in this thesis are noted in bold. Figure adapted from the thesis of D. Schoendorf, Transplantation Biology 2000.

$A\alpha^d A\beta^{g7}$	β_{2m}	$A\beta^{g7(asp)}$ transgene
$A\alpha^d A\beta^{g7}$	β_{2m}	$A\beta^{g7(asp)}$ transgene

NOD-Asp (white)

X

$A\alpha^b A\beta^o$	β_{2m^o}
$A\alpha^b A\beta^o$	β_{2m^o}

MHC class I and II knockout (black)



$A\alpha^d A\beta^{g7}$	β_{2m}	$A\beta^{g7(asp)}$ transgene
$A\alpha^b A\beta^o$	β_{2m^o}	

X

$A\alpha^b A\beta^o$	β_{2m^o}
$A\alpha^b A\beta^o$	β_{2m^o}

F₁ (agouti)

MHC class I and II knockout (black)

$A\alpha^d A\beta^{g7}$	β_{2m}
$A\alpha^b A\beta^o$	β_{2m^o}

BN MHC class I⁺

$A\alpha^d A\beta^{g7}$	β_{2m}	$A\beta^{g7(asp)}$ transgene
$A\alpha^b A\beta^o$	β_{2m^o}	

BN- Asp MHC class I⁺

$A\alpha^b A\beta^o$	β_{2m}	$A\beta^{g7(asp)}$ transgene
$A\alpha^b A\beta^o$	β_{2m^o}	

B-Asp MHC class I⁺

$A\alpha^b A\beta^o$	β_{2m}
$A\alpha^b A\beta^o$	β_{2m^o}

MHC class II^{-/-} Class I^{+/-}

Backcross
(black or
agouti)

$A\alpha^d A\beta^{g7}$	β_{2m^o}
$A\alpha^b A\beta^o$	β_{2m^o}

BN MHC class I⁻

$A\alpha^d A\beta^{g7}$	β_{2m^o}	$A\beta^{g7(asp)}$ transgene
$A\alpha^b A\beta^o$	β_{2m^o}	

BN-Asp MHC class I⁻

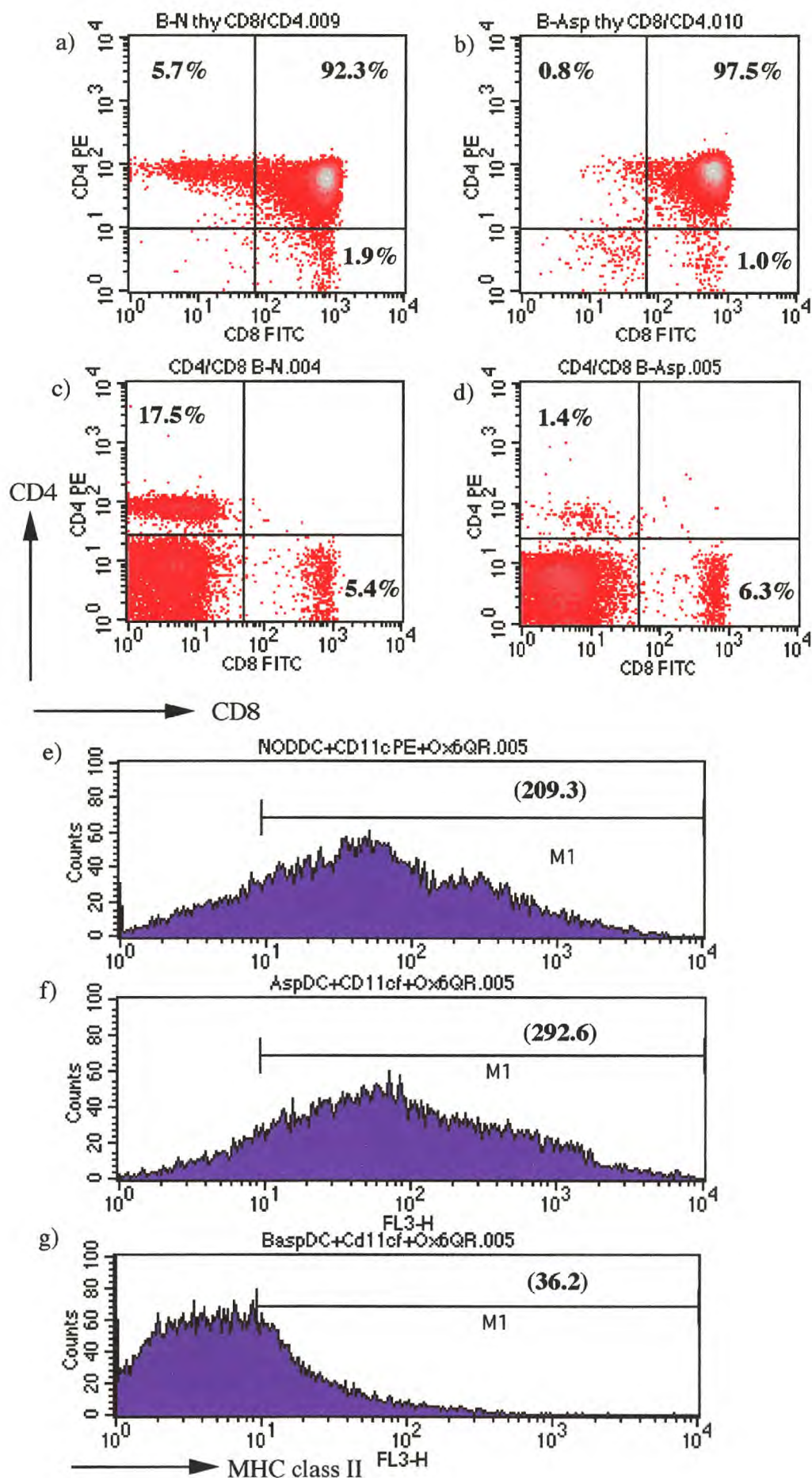
$A\alpha^b A\beta^o$	β_{2m^o}	$A\beta^{g7(asp)}$ transgene
$A\alpha^b A\beta^o$	β_{2m^o}	

B-Asp MHC class I⁻

$A\alpha^b A\beta^o$	β_{2m^o}
$A\alpha^b A\beta^o$	β_{2m^o}

MHC class II and I^{-/-}

Figure 3.2. a)- d) Thymic and splenic profiles from B-Asp and BN MHC class I+ve mice, CD4/CD8 FACS profiles showing decreased CD4 SP thymocytes in B-Asp mice. Thymocytes or splenocytes were stained with CD8-FITC and CD4-PE and analysed. Numbers indicate the percentage of total cells. e)- f) Flow cytometry histograms show the level of MHC class II expression on the surface of BMDCs cultured from NOD, NOD-Asp and B-Asp mice respectively. Cells were stained with CD11c-PE and OX-6 biotin (anti MHC class II g⁷(asp) beta-chain)+SA-QR, and gated on CD11c positive cells. Numbers indicate the mean fluorescence intensity for the MHC class II expression (contained within M1).



Increased TCR/MHC affinity may be apparent in the structure of the selected receptors through selection of a modified TCR V α / β repertoire, or alteration in the TCR/MHC interaction sites, particularly the CDR3 β region. From MHC class II/peptide/TCR structures (Reinherz, Tan et al. 1999), it is clear that the variable regions of both α - and β -chains interact closely with the MHC class II molecule, and form the basis for positive selection. Similarly, the CDR3 β is known to interact with the peptide in the binding groove of the MHC and may be influenced by the level of MHC class II expression in selection. The CDR3 α may be affected to a similar extent, but due to inefficient allelic exclusion, some T cells express more than one α -chain, making analysis more difficult. B-Asp mice were used to examine the pre and post-selection TCR repertoire in terms of CDR3 β sequence as well as V α / β usage to determine whether decreased MHC class II expression has altered the TCRs selected.

3.2: Effects of decreased MHC class II expression in thymus and periphery

A backcross of mice was generated between the NOD-Asp transgenic strain that expresses an A $\beta^{g7(asp)}$ transgene, leading to formation of the A $\alpha^d\beta^{g7(asp)}$ MHC class II molecule in addition to A $\alpha^d\beta^{g7}$, and MHC class I/II knockout (MHC $^{-/-}$) mice. F₁ mice were backcrossed onto MHC $^{-/-}$. 1/4 of the mice expressed just the mixed haplotype MHC class II molecule $\alpha^b\beta^{g7(asp)}$ (B-Asp) in the presence or absence of $\beta 2m$. Figure 3.1 shows the potential phenotypes obtained in this backcross and definitions that are used in this thesis. The $\alpha^b\beta^{g7(asp)}$ heterodimer is expressed at approximately 10% of the level of the parental wild-type MHC molecules on thymic epithelium and peripheral APCs. This has been shown using flow cytometry with antibodies to both the α and β -chains (Schoendorf 2000). In figure 3.2 a)-d), the thymic and peripheral profiles of B-Asp CD4 and CD8 expression are shown compared to normal littermate controls. The B-Asp thymus shows a dramatic decrease in the proportion of CD4 SP thymocytes from 5.7% in normal littermate controls, to around 1% in B-Asp, similar to that previously shown in MHC class II-deficient mice (Schoendorf 2000). An increase in the DP population from 92% to 97% is observed, indicating a block in T cell selection at the transition between DP to CD4 SP. In the periphery, it is clear that while the CD8 $^{+}$ T cells in the spleen make up around 5-6% of total cells in both B-Asp and controls, the CD4 $^{+}$ population decreases from 17.5% to 1.4%. A similar picture is also seen with CD4/CD8

in peripheral lymph nodes. Further analysis of B-Asp CD4⁺ cells revealed that they express CD44 at a higher level than that observed in controls (Schoendorf 2000), an activation/memory marker (Mackall, Hakim et al. 1997) although this is likely to be due to homeostatic expansion, as they do not express activation markers such as CD69 or CD62L. Similarly, in MHC^{-/-} mice, CD4⁺ cells that develop are all CD44^{hi} (Cosgrove, Gray et al. 1991).

The mixed haplotype MHC class II molecule was expressed at approximately 10% of wild-type levels on peripheral APCs. This had previously been shown in peripheral B cells, macrophages and lymph node DCs. Figure 3.2 e)-f) shows FACS analysis of bone marrow-derived DCs from NOD, NOD-Asp and B-Asp respectively. Bone marrow was cultured for 7 days with GM-CSF and stained with CD11c (expressed at high levels on DCs and low levels on macrophages) and OX-6, an anti-rat MHC class II antibody that cross reacts with murine A^{g7/g7(asp)} MHC class II β -chains. Figure 3.2 e)-f) shows MHC surface expression levels on gated CD11c^{hi} DCs and it is clear that on NOD and NOD-Asp DCs there is a broad range of MHC class II surface expression (common with cultured BMDCs) while those from B-Asp express lower levels of class II. If the MFI of the R1 gates are compared, NOD and NOD-Asp are 209.3 and 292.6 respectively while for B-Asp BMDCs it is only 36.2, approximately 12% of that seen in NOD-Asp. Previous work has shown that this level of expression is higher than the background staining seen in MHC class II-deficient mice (Schoendorf 2000). It is important to note in figure 3.1 that mice classified as BN express both A α^d A β^{g7} as well as A α^b A β^o . This means that BN mice can also express a mixed haplotype molecule A α^b A β^{g7} . This may influence to a minor degree the T cells that are selected; however as a mixed haplotype molecule, this will be expressed at low levels on the cell surface compared to A α^d A β^{g7} and will therefore have negligible impact on the repertoire selection process.

3.3: T cell receptor V α analysis shows decreased expression of V α 8

In order to examine whether the selected T cell peripheral repertoire had been influenced by selection on low expression MHC class II molecules, the CD4⁺ T cells were examined both for V α and V β expression by FACS analysis.

Figure 3.3 a)-f) shows representative FACS histograms comparing CD4⁺ mesenteric lymph node T cells from NOD, B-Asp and BN littermate control mice, and

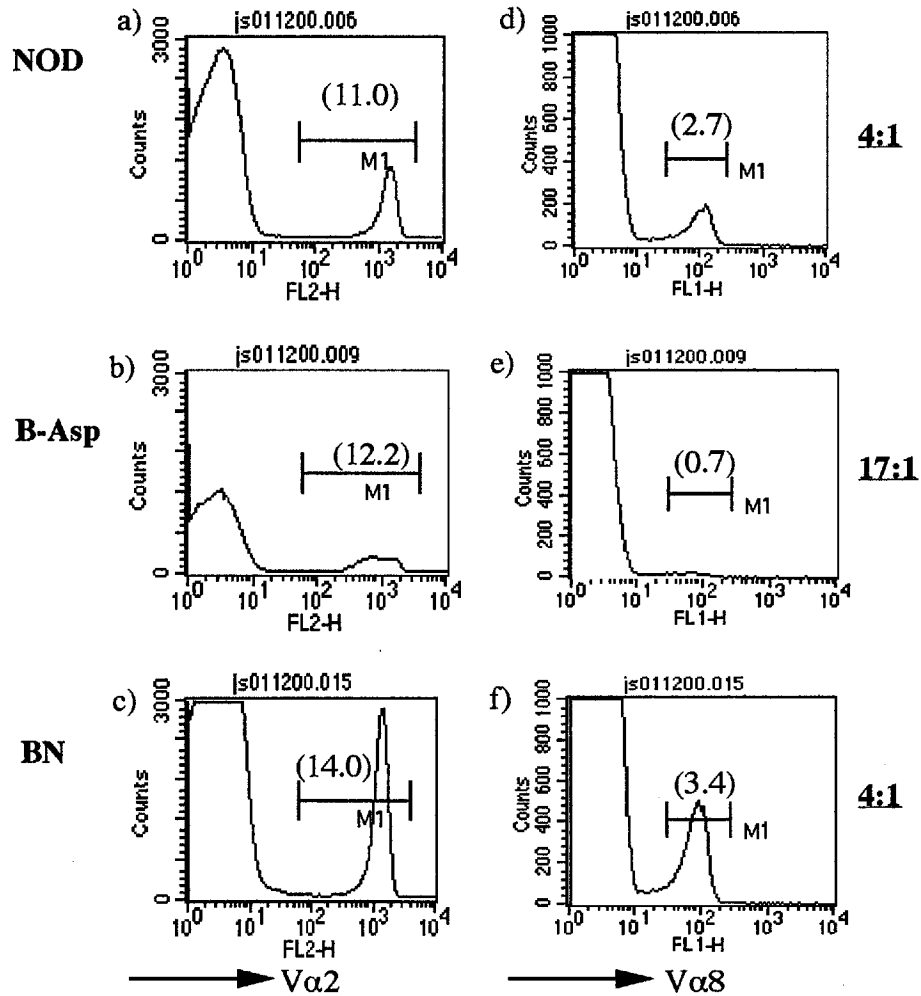


Figure 3.3. Flow cytometry histograms showing that $V\alpha 8^{+} CD4^{+}$ cells are not selected in B-Asp mice when compared to NOD and BN controls. Mesenteric LN cells were removed, fixed and stained with CD4-PerCP and appropriate $V\alpha$ -specific antibodies. Cells were gated on $CD4^{+}$ cells and displayed as histograms for expression of each $V\alpha$. a)-c) expression of $V\alpha 2$ on NOD, B-Asp and BN, d)-f) expression of $V\alpha 8$ on NOD, B-Asp and BN. Numbers in brackets indicate % of $CD4^{+}$ cells that are also $V\alpha$ positive. Numbers in bold indicate the ratio of $V\alpha 2$ positive to $V\alpha 8$ positive $CD4^{+}$ T cells.

shows V α 2 (Figure 3.3 a)-c) and V α 8 (Figure 3.3 d)-f) expression on gated CD4⁺ cells. Staining with three different V α - specific antibodies, V α 2, 3.2 (data not shown) and 8 with CD4, the proportion of CD4⁺ T cells expressing particular V α gene segments was calculated. While V α 2 is expressed on 11-14% of CD4⁺ in all three mouse strains (Figure 3.3 a)-c), V α 8 is expressed on 2.7% of NOD (3.3 d) and 3.4% of BN (3.3 f) CD4⁺ T cells but only 0.7% of B-Asp (3.3 e). Similar profiles were seen regardless of whether the B-Asp or BN were MHC class I⁺ or class I⁻. These experiments were repeated on at least three occasions with one NOD and two B-Asp and BN mice being analysed on each occasion. At least 10⁶ cells were collected in the gate for each sample. Interestingly, the ratio of V α 2 to V α 8 positive CD4⁺ cells was 4:1 in both BN and NOD controls, but 17:1 in B-Asp. This suggests that selection of V α 8 by MHC class II is unfavourable and that decreased levels of MHC class II makes the interaction less likely.

V α expression on CD4⁺ T cells in B-Asp mice was compared, not only with NOD and BN, but also with other strains of mice expressing different MHC class II molecules including: C57BL/6 (H2^b), BALB/c (H2^d), FVB (H2^q), B10.BR (H2^k), SJL (H2^s), B10.E α (transgenic H2-E α) and B10.GD (H2^{d^b}). Between 2 and 10 mice were examined for each strain and these data are summarised in figure 3.4 a). It is clear that there is some variability between the different mice examined in selection of V α regions, dependent on the MHC class II molecule involved. BALB/c, FVB and SJL mice select V α 2 least efficiently among the different strains, although this is clearly the most common V α region used among those tested, ranging from <10% in SJL to around 15% in C57BL/6, B10.E α and B10.GD mice. While none of the strains select V α 3.2 efficiently, B-Asp selects more than others although the standard deviation is larger in this sample. However the absence of V α 8 is most striking in B-Asp compared to all the other strains. It appears that in all strains examined the selection of V α 8 is poor, but in the B-Asp strain with low expression of MHC class II, this phenomenon becomes more extreme.

3.4: Examination of TCR V β gene segment usage selected on decreased MHC class II expression

While differences were seen when the V α selection profile was examined, it was also interesting to examine the TCR β -chain selection, to see whether the level of MHC

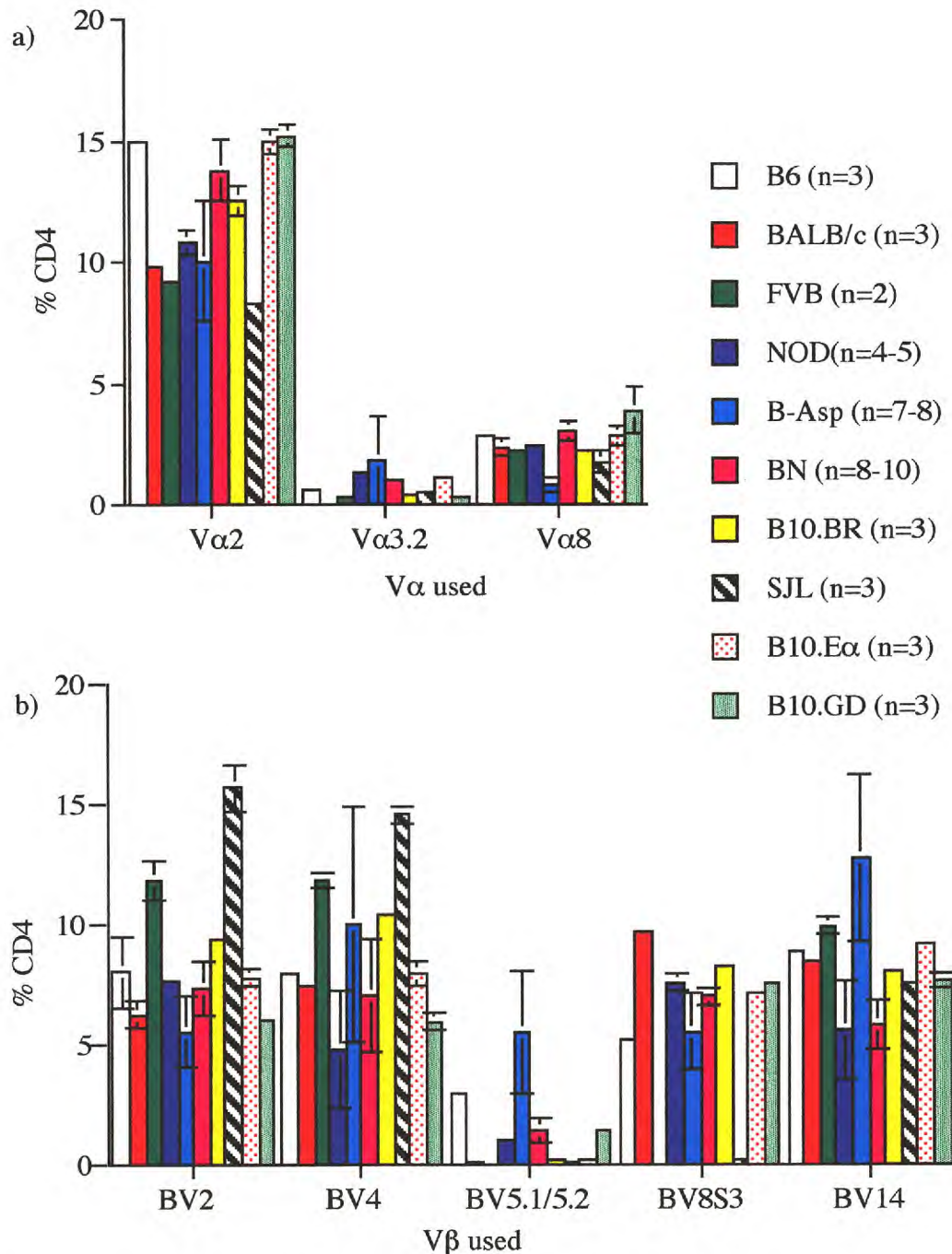


Figure 3.4. A comparison of a) V α and b) V β -gene usage by CD4⁺ T cells from a number of mouse strains (C57BL/6, BALB/c, FVB, NOD, B-Asp, BN, B10.BR, SJL, B10.E α and B10.GD) shows that B-Asp mice have more BV5.1/5.2 and BV14 expressing CD4⁺ T cells than other strains of mice and less V α 8. These figures show the % of CD4⁺ cells that are positive for various V α /V β segments within the TCR, using a panel of different V α /V β -specific antibodies (+/- SD). Mesenteric LN were removed from the mice and immediately fixed, then stained with CD4-PerCP and various V α /V β antibodies conjugated to either FITC or PE.

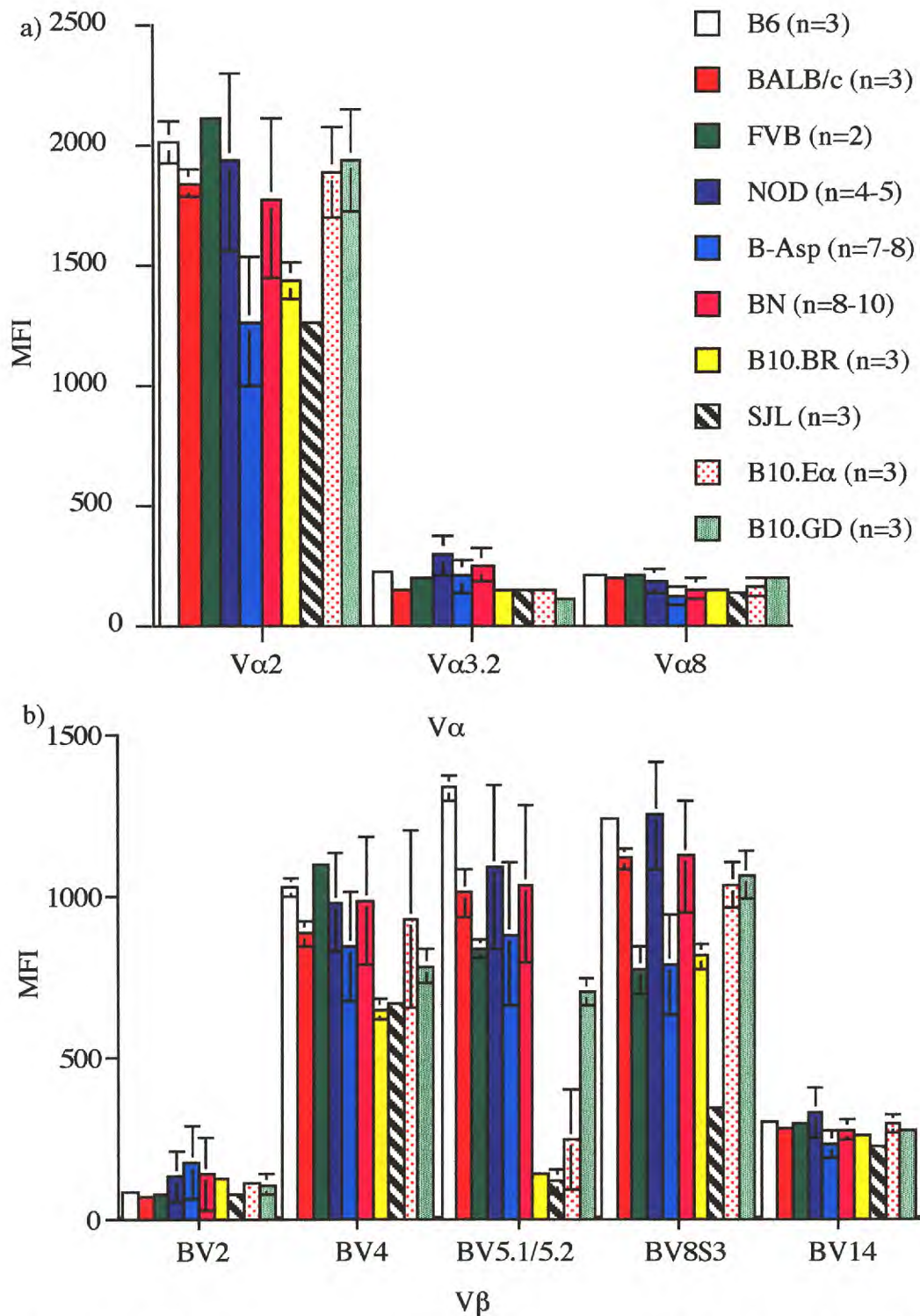


Figure 3.5. Mean fluorescence intensity of a) Vα and b) Vβ on the surface of B-Asp CD4⁺ T cells is lower in most cases than other mice strains tested. Using the flow cytometry profiles described previously, with CD4-PerCP and either FITC or PE- conjugated Vα or Vβ specific antibodies, cells were gated on CD4⁺ T cells and the MFI calculated for the various Vα and Vβ (+/- SD). Strains tested were C57BL/6, BALB/c, FVB, NOD, B-Asp, BN, B10.BR, SJL, B10.Eα and B10.GD. (n= number of mice analysed in each group).

class II expression influenced only the α -chain, or whether V β usage was also affected. Using a number of V β -specific antibodies (not all data shown), in combination with CD4-PerCP, the percentage of CD4⁺ cells expressing different V β was examined in B-Asp compared with the strains used above. While many of the V β tested showed similar expression patterns in all the mice tested (data not shown), figure 3.4 b) shows a striking over-representation of V β 5.1/2 and, to a lesser extent, V β 14 and V β 4 in the periphery, particularly when compared with NOD and BN. Again B-Asp and BN class I⁺ and class I⁻ mice were examined and little difference was seen between them. At least 500,000 cells were collected for each sample and the percentage of CD4⁺ cells expressing each V β calculated.

Figure 3.4 b) shows that B-Asp is among the strains that utilize V β 2 the least. V β 2 represents around 15% of CD4⁺ T cells in SJL mice while in B-Asp it is approximately 6%, similar to that seen both in NOD and BN mice. V β 4 use in B-Asp is increased compared to both NOD and BN, although when compared with FVB and SJL it is selected less commonly. Unfortunately the standard deviation for V β 4 is large, showing larger variation than other strains. V β 8.3 usage in all mice strains appears relatively consistent, with between 5-10% of CD4⁺ except for SJL in which it is absent. SJL mice have a genomic deletion, encoding both V β 5 and V β 8, explaining the differences between SJL and the other strains examined (Kappler, Pullen et al. 1989). Both V β 5.1/2 and V β 14 appear more commonly in B-Asp CD4⁺ T cells than any of the other strains tested. However the profiles differ markedly between these two V β . V β 5.1/2 shows almost complete deletion in most strains of mice, with expression being between 0-2.5%, while approximately 6% of B-Asp CD4⁺ express V β 5.1/2. V β 5.1/2 is, to a variable extent, deleted from the peripheral repertoire in in-bred mice, while strikingly in B-Asp the deletion is absent. V β 5.1/2 is eliminated from CD4⁺ T cells by negative selection following interaction with viral superantigens encoded by: *Mtv*3, 8, 9 and 11 as shown by use of single *Mtv* H2^k mice (Scherer, Ignatowicz et al. 1995). Interestingly, deletion of V β 5.1/2 occurs more efficiently if the superantigen is presented by H2-E, for example B6 mice only partially delete, while B10.E α delete almost completely. It is unlikely that the absence of deletion is due to the absence of appropriate *Mtv* as both parental strains show deletion, while BN would have the same *Mtv* as B-Asp. In comparison V β 14 is expressed at higher levels (5-10%) in most strains examined, with the lowest being NOD and BN with approximately 5% of CD4⁺ cells. Again B-Asp mice appear to preferentially select V β 14 expressing T cells with

13% CD4⁺/Vβ14⁺. It is possible that this may again be an *Mtv*-mediated effect as Vβ14⁺ cells are deleted in the periphery by *Mtv*-2 and could be partially deleted by other *Mtvs* (Nakano, Yoshimoto et al. 1996).

These data suggest that both the selecting MHC molecule, as well as the level of MHC class II expression involved in T cell selection may play an important role in influencing both the Vα and Vβ genes selected into the peripheral repertoire. It appears as though inefficient TCR/MHC interactions such as Vα8 that are selected poorly to the CD4⁺ T cell pool in a number of strains are less efficiently selected still when the level of MHC class II expression is decreased. Conversely, Vβ5.1/2 CD4⁺ T cells are not eliminated from the repertoire through negative selection mediated by *Mtv*-encoded vSAG when MHC class II expression is low. Since the MHC class II molecules of both parental strains can delete (A^b and A^{g7}), as can all others analysed, the failure of the mixed haplotype molecule to delete is very likely to be due to the level of its expression, suggesting that the level of MHC expression in the thymus is influencing selection of TCR Vα/Vβ regions and therefore playing a significant role in determination of the structure of selected TCRs.

3.5: Mean fluorescence intensity of Vα/Vβ expression in B-Asp

Because alterations in the Vα/β usage were observed it was also informative to examine the level of cell surface expression for each Vα/Vβ using the mean fluorescence intensity (MFI) of antibody staining. It is possible that being selected on low surface expression MHC class II could influence the level of expression of the TCR at the cell surface. Figure 3.5 a) compares the levels of expression of the different Vα regions tested on the cell surface by examining the MFI for each staining. It is clear that the antibodies used have different levels of staining and consequently it is more relevant to compare the expression levels in a Vα than between different Vα. There is little difference between the mouse strains in Vα3.2 and 8, although with such low surface expression it is difficult to draw conclusions from this. Vα2 has higher expression on all strains examined, but it is clear that SJL and B-Asp have the lowest surface expression levels of Vα2 of all the mice tested, including NOD and BN.

Figure 3.5 b) shows the MFI levels of five out of twelve of the Vβ tested. It is clear that the different Vβ regions stain with different efficiency as was seen in the Vα analysis. There are striking differences between some of the strains particularly B10.BR

and B10.E α in V β 5.1/2 which compared to the other strains have very low MFI. SJL have no V β 5.1/2 as previously described, while B10.BR and B10.E α have very few V β 5.1/2 cells remaining due to the presence of H2-E. When examining B-Asp MFI, it was clear that 11/12 V β examined showed a decrease of between 17% and 42% when compared with that on NOD controls (data not shown). This suggests that there may be a trend towards decreased surface expression of the TCR compared to that on NOD and BN control mice, perhaps as a result of the low expression selecting MHC class II. This result was a surprise as it might be expected that CD4⁺ cells selected on low MHC class II levels would have increased TCR expression to compensate during selection. However as previously described, CD4⁺ T cells from B-Asp express high levels of CD44 indicating a degree of chronic activation, which may result in TCR downregulation compared to CD4⁺ T cells selected on normal levels of MHC class II expression.

3.6: Sequence analysis of CDR3 β reveals minor alterations in the B-Asp repertoire

A variety of methods are available to study the TCR repertoire including RNase protection (Singer, Balderas et al. 1990), random sequencing, V β analysis by monoclonal antibodies as used above, and different PCR-based methods including Immunoscope (Pannetier, Cochet et al. 1993; Pannetier, Even et al. 1995), using fluorescently labelled primers to examine CDR3 length and J β gene usage. From the V β expression profiles, we decided to further examine two V β that were not affected in selection to determine whether the structure of these receptors had been altered in a subtler manner than frequency of selection. V β specific primers were used as described (Pannetier, Cochet et al. 1993) in conjunction with a C β specific primer to amplify the CDR3 region of the β -chain.

By purifying mRNA from thymocytes and CD4⁺ peripheral T cells taken from B-Asp and BN MHC class I mice, it was possible to examine the TCRs containing V β 2, V β 8.2 and V β 8.3 in the pre- and post-selection repertoires. Figure 3.6 a)-d) shows FACS profiles of B220/CD8 staining comparing MHC class I-deficient BN (a) and B-Asp (b) splenocytes before depletion of any remaining CD8⁺ T cells using CD8⁺ Dynabeads, with BN (c) and B-Asp (d) after depletion. Figure 3.6 e)- h) show similar profiles comparing CD4/CD8 stains showing that whether used for mRNA or for

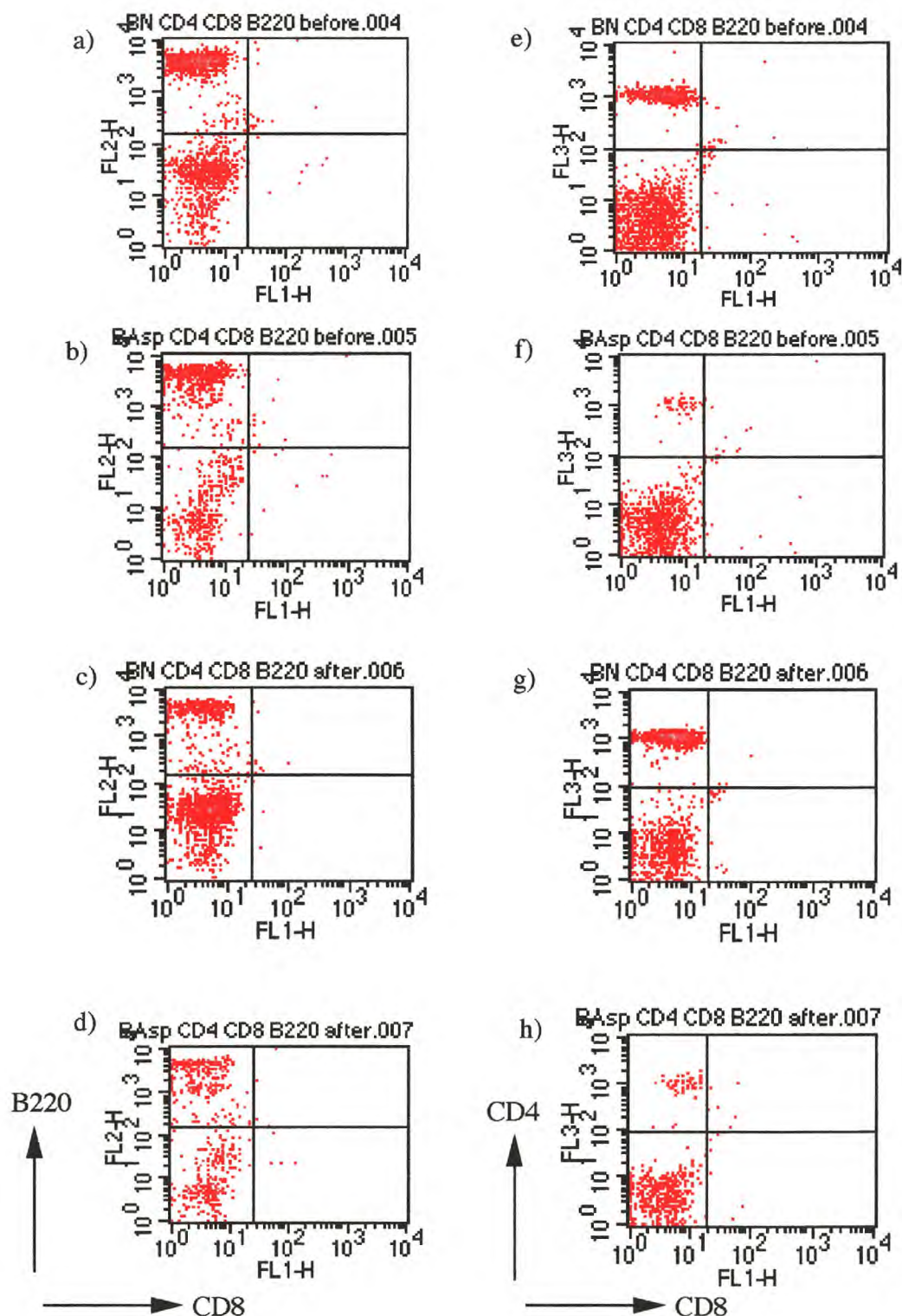


Figure 3.6. B-Asp and BN (MHC class I⁺) splenocytes were enriched for CD4⁺ T cells before making hybridomas or mRNA, depleting of CD8⁺ T cells and B cells using CD8 and anti-mouse IgG dynabeads. Flow cytometry profiles are shown as follows: B220/CD8 a) BN before purification, b) B-Asp before purification, c) BN after, d) B-Asp after. CD4/CD8 e) BN before purification, f) B-Asp before purification, g) BN after, h) B-Asp after.

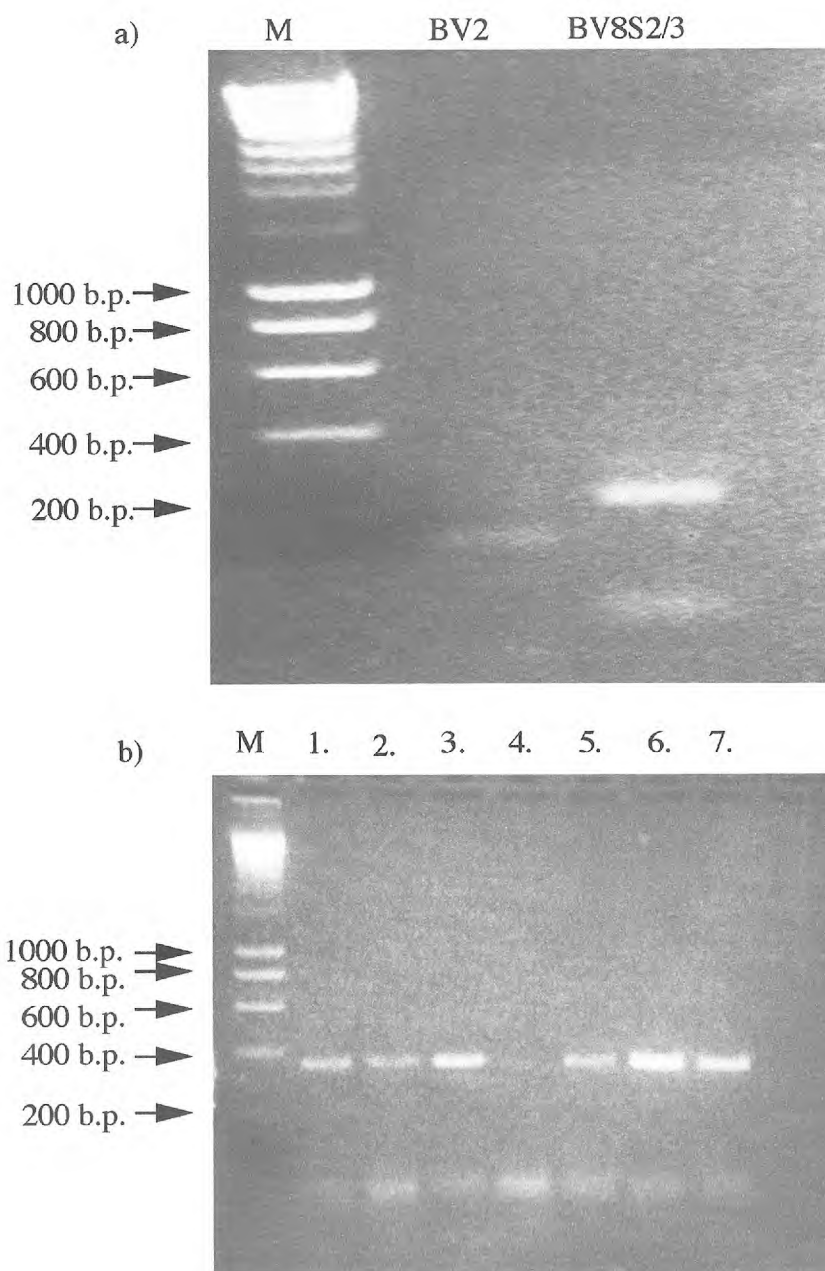


Figure 3.7. 2% agarose gel images: a) Optimization of the High-Fidelity Taq PCR reaction for BV2 and BV8S2/BV8S3 as described in materials and methods. Optimisation was performed using cDNA from splenocytes from a C57BL/6 mouse. 10 μ l of the PCR product was loaded into the wells. In a) and b) the first lane marked M is SmartLadder from Eurogentec. Figure b) shows a 2% agarose gel of HPRT control PCR reactions using cDNA made from 1. BN thymus, 2. and 3. BN CD4⁺ splenicT cells, 4. and 5. B-Asp thymus, and 6. and 7. B-Asp CD4⁺ splenic T cells. 10 μ l of PCR product were loaded into the wells.

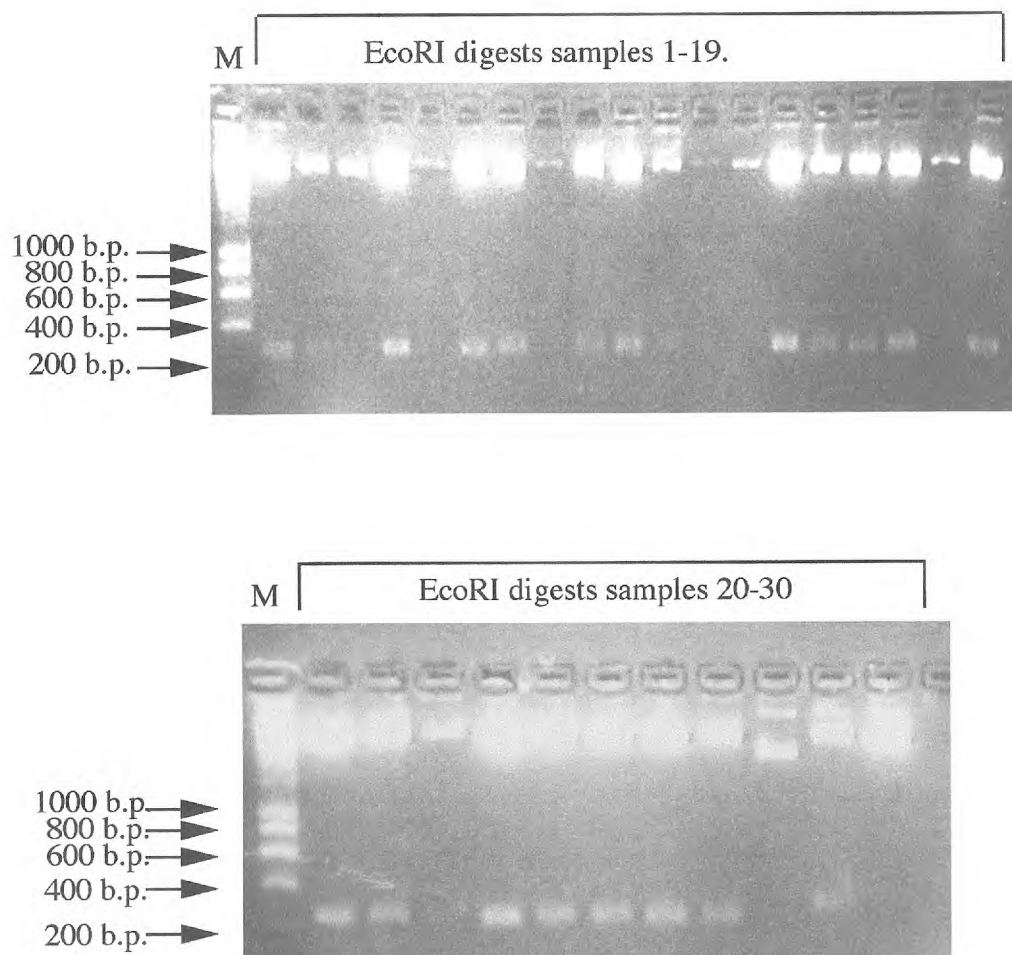


Figure 3.8. Example gels of EcoRI digests from B-Asp Thymus BV8S2/8S3CDR3 region. EcoRI digestions of 30 individual clones from the TA cloning procedure were cloned with purified PCR product containing B-Asp Thymus BV8S2/8S3. INV α F bacteria were grown overnight at 37°C on LB-Agar + Ampicillin + X-Gal, white (positive) colonies were picked and cultured overnight at 37°C in LB-broth + Ampicillin, after which the plasmid DNA was isolated using miniprep columns. DNA from each sample was digested with EcoRI for 4-5 hours and run on a 2% agarose gel to determine the presence of an appropriately sized (approximately 230 b.p.) insert. M = molecular weight markers (SmartLadder- Eurogentec).

a) TCRBV translated sequences (C-terminal)

BV2S1:

FTLRSPGDKEVKSLPGADYLATRVTDELRLQVANMSQGR--TLYCTCS

BV8S1:

HYSYVADSTEKGDIPDGYKASRPSQENFSLILELASLSQTAVYFCASSD

BV8S2A1:

HYSYGAGSTEKGDIPDGYKASRPSQENFSLILELATPSQTSVYFCASGD

BV8S2A2:

HYSYGAGSTEKGDIPDGYKASRPSQENFSLILELATPSQTSVYFCASG

BV8S2A3:

HYSYGADSTEKGDIPDGYKASRPSQKEFSLILELATPSQTSVYFCASS

BV8S3:

HYSYGAGNLQIGDVPDGYKATRTTQEDEFLLLELASPSQTSLYFCASSD

b)TCRBJ1S and BJ2S translated sequences (N-terminal).

TCRBJ1S1 NTEVFFGKGTRLTVV

TCRBJ1S2 NSDYTFGSGTRLLVM

TCRBJ1S3 SGNTLYFGEGSRLIVV

TCRBJ1S4 SNERLFFGHGTKLSVL

TCRBJ1S5 NNQAPLFGEGTRLSVL

TCRBJ1S6 SYNPLYFAAGTRLTVT

TCRBJ2S1 NYAEQFFGPGTRLTVL

TCRBJ2S2 NTGQLYFGEGSKLTVL

TCRBJ2S3 SAETLYFGSGTRLTVL

TCRBJ2S4 SQNTLYFGAGTRLSVL

TCRBJ2S5 NQDTQYFGPGTRLLVL

TCRBJ2S6 (pseudogene)

TCRBJ2S7 SYEQYFGPGTRLTVL

Figure 3.9. a) Translated germ-line sequences of the C-terminal region of BV2, BV8S1, BV8S2A1-3 and BV8S3. Sequences were taken from HGMP databases and translated using GCG. Regions underlined are identical between 8S1, 8S2A1-3 and 8S3. Marked in red is the cysteine residue found in all V β gene segments from which the CDR3 β region is measured. b) N-terminal translated germ-line sequences of TCRBJ1S and 2S joining region genes. These were used for sequence analysis.

making CD4⁺ T cell hybridomas (discussed later), there were few contaminating CD8⁺T cells. To increase accuracy, RT-PCR with high-fidelity Taq polymerase was used to amplify the CDR3 β , after which the gel-purified PCR products were cloned. Figure 3.7 a) shows an example of a 2% agarose gel loaded with PCR products from optimised reactions with V β 2 and V β 8.2/3. Optimisation was performed using cDNA from CD4⁺ splenocytes isolated from a C57BL/6 mouse. In each case, the size of the observed band correlated with the predicted length of approximately 140 b.p. for V β 2 and 200 b.p. for V β 8.2/3 (Barth, Kim et al. 1985; Arden, Clark et al. 1995).

After optimisation, cDNA was made from mRNA isolated from BN and B-Asp thymocytes and CD8⁺-depleted splenocytes (from MHC class I-deficient mice). The quality of the cDNA was examined using HPRT control primers (a ubiquitously expressed house-keeping gene) as described. Figure 3.7 b) shows a 2% agarose gel with distinct bands around 380 b.p., the expected size for HPRT in 6/7 samples tested from BN/B-Asp thymocytes and CD4⁺ T cells. The best quality cDNA from each sample was used for cloning, using the TA cloning kit into INV α F' *E. coli*. After cloning and incubating overnight on selecting agar, individual colonies were picked, grown, plasmid DNA isolated using Miniprep columns, examined for presence of an appropriately sized insert by EcoRI digestion, and sequenced using the Thermo Sequenase II dye terminator cycle sequencing kit (DNA Core Laboratory, MRC Clinical Sciences Centre). Figure 3.8 shows example gels from EcoRI digestions using B-Asp thymus V β 8.2/3 containing clones. If an insert of around 230 b.p. was present, the DNA was sequenced. It can be seen that in this example gel, 19/30 samples contained appropriately sized inserts.

On sequencing the V β 8.2 containing TCR regions, it was discovered that due to high sequence similarity between family members, the V β 8.2 primer used for this analysis (Pannetier, Cochet et al. 1993) amplified the related V β 8.3 preferentially to V β 8.2 (compare Figure 3.9 a). The initial aims were to characterise CDR3 regions from V β 2 and 8.2, but as approximately 60% of the sequences derived from the V β 8.2 pool were actually V β 8.3, both were examined. It can also be seen that V β 8.2 is divided into the three V β 8.2 gene segments (V β 8S2A1, 8S2A2 and 8S2A3 as seen in figure 3.9 a). V β 8.2 was treated as a single category in these analyses and the data are not separated according to subfamilies. In figures 3.9)-3.11) and table 3.1, the V β regions are classified according to the newer nomenclature BV2, BV8S2 and BV8S3 used in sequence databases (Arden, Clark et al. 1995), but for ease in the text these are

described interchangeably with older nomenclature as V β 2, V β 8.2 and V β 8.3 respectively. Similarly figure 3.9 b) and Table 3.1 describes J β regions according to the newer nomenclature (for example BJ1S1).

The DNA sequences were translated into amino acid sequences and those that were in frame through the CDR3 region were analysed for a series of parameters. As the start sequence and length of V β gene segments vary, the definition for the CDR3 region used here is from XCXX---FGXG (or FAAG) where XC are at positions 91 and 92 respectively (Kabat, Wu et al. 1991) and X is any amino acid. Figure 3.9 a) shows the C-terminal amino acid sequences for a number of different V β regions including those examined: V β 2, 8.2 and 8.3, as well as the start sequence for the CDR3 β region. Figure 3.9 b) shows the N-terminal amino acid sequences for the translated J β gene segments that were used in these analyses. The J β genes are separated into two families, associated with the two different C β regions. The first family contains 6 gene segments 1S1-1S6, while the second contains 7 segments, one of which (2S6) is a pseudogene and is not functionally expressed. The consensus 'CDR3-end' sequence of FGXG (or FAAG) can clearly be seen.

3.6.1: Comparison of CDR3 lengths

Between 59 and 140 individual clones were analysed from each pre and post selection sample. Comparing CDR3 length distributions from the B-Asp pre- and post-selection repertoires, it can be seen that there are some differences from wild-type littermate controls.

While some of the profiles (Figure 3.10) appear to be slightly different from one another comparing the pre and post selection samples, further analysis shows that the differences seen are minor. The commonest length of CDR3 used in all samples, both pre and post selection is 10 amino acids, except for: BN thymus V β 2, when both 10 and 11 are found in the same proportion, BN thymus V β 8.2 where 11 amino acids is most common and BN thymus and spleen CD4⁺ T cells V β 8S3 where respectively, 11 and 9 amino acids are most common. While the range of lengths varies slightly between samples, one consistent factor is a trend towards a tightening of the range from thymus into periphery, such that the range of CDR3s in the periphery is narrower than in the thymus, similar to previous observations (Candeias, Waltzinger et al. 1991).

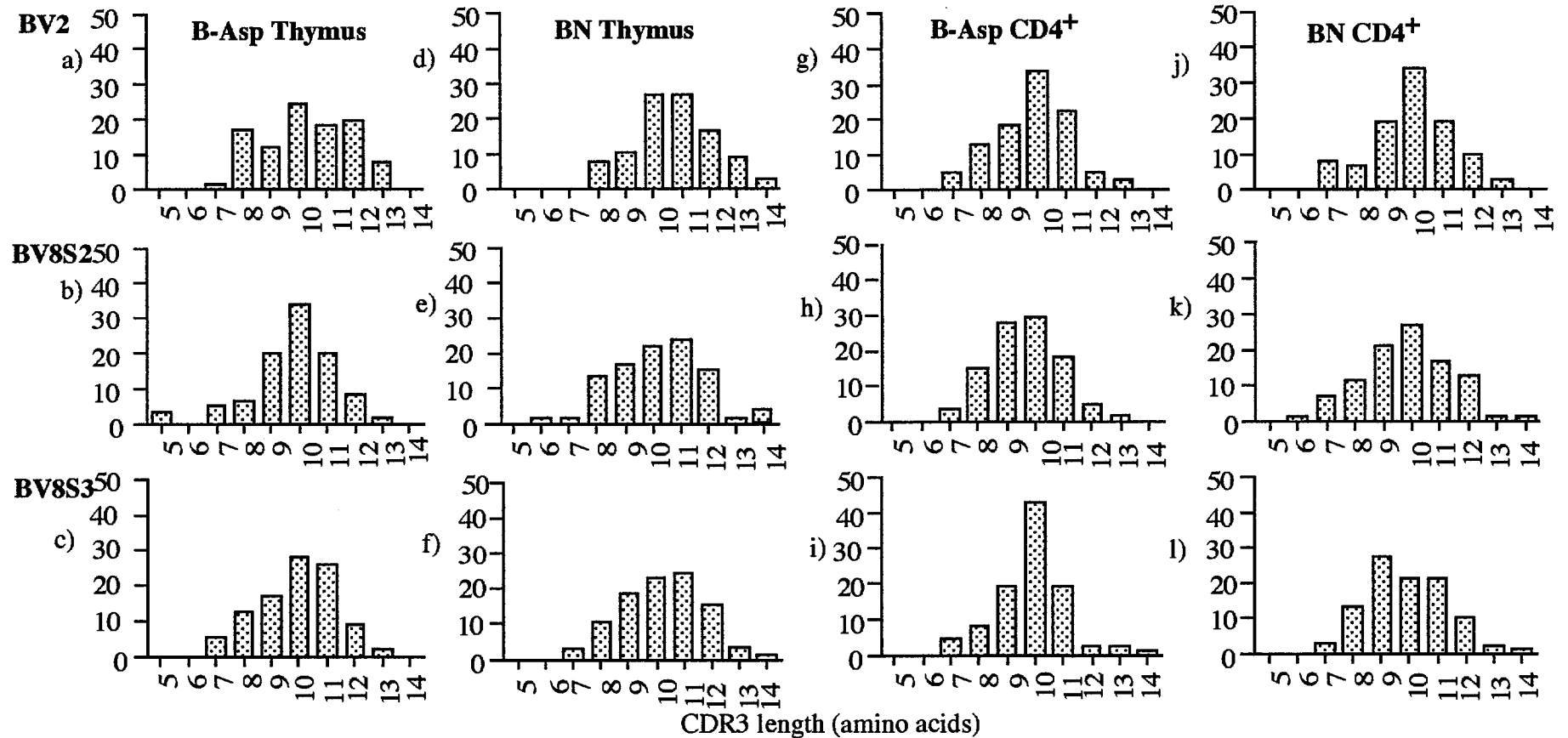
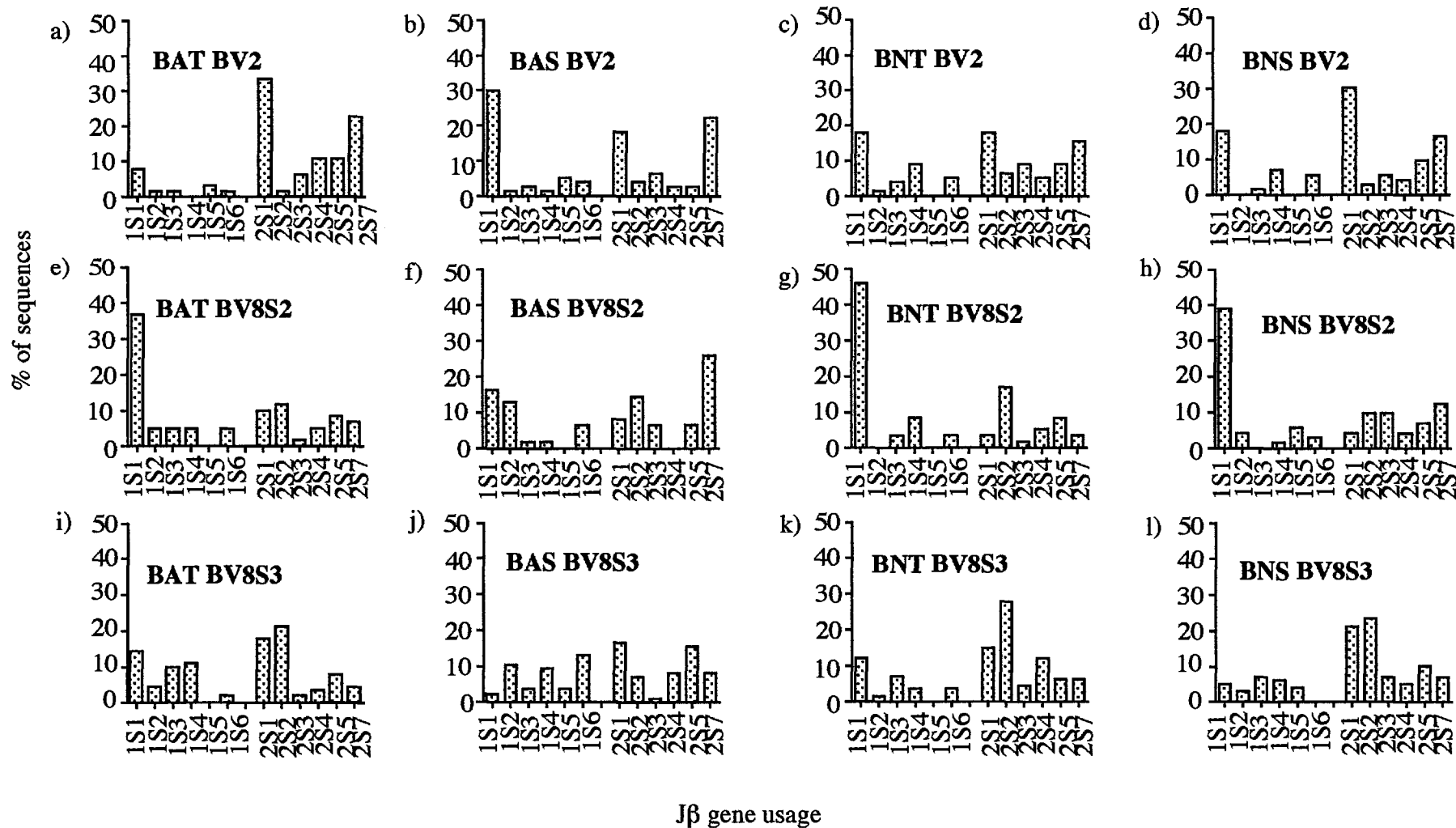


Figure 3.10. Comparison of the CDR3 β lengths in amino acids from B-Asp and BN reveals alterations in the selected repertoire between thymus and periphery. Using BV2 and 8S2-specific and C β primers, PCR products containing the CDR3 β region were made from B-Asp and BN (MHC class I⁻) thymocytes and CD4⁺ T cells. Individual clones were sequenced and sequences translated into in-frame protein sequence using GCG (HGMP-Resources) and analysed for CDR3 length. a)- c) B-Asp Thymus BV2, BV8S2 and BV8S3 respectively, d)- f) BN Thymus, g)- i) B-Asp peripheral CD4⁺ and j)- l) BN peripheral CD4⁺.

Figure 3.11. J β gene usage within pre- and post-selection BV2, BV8S2 and BV83. All values are expressed as a percentage of the sequences in the sample. Number of sequences = 59-130/sample. BJ2S6 is a pseudogene and is not expressed. a)-d) BV2 TCRs from B-Asp thymus (BAT), B-Asp peripheral CD4⁺ T cells (BAS), BN thymus (BNT) and BN peripheral CD4⁺ T cells (BNS). e)-h) BV8S2 TCRs from B-Asp thymus, B-Asp peripheral CD4⁺ T cells, BN thymus and BN peripheral CD4⁺ T cells. i)-l) BV8S3 B-Asp thymus, B-Asp peripheral CD4⁺ T cells, BN thymus and BN peripheral CD4⁺ T cells.



Another interesting point previously raised was that long CDR3 loops (≥ 12 amino acids) were more frequent in CD8 compared to CD4 in the periphery (Candeias, Waltzinger et al. 1991). While these data cannot confirm this directly, they show that during selection into the CD4 compartment, CDR3 loops ≥ 12 amino acids become less frequent, decreasing by up to 3.5 fold from thymus into the periphery. It also appears, at least in the three V β examined, that this phenomenon may be more pronounced in the periphery of B-Asp mice compared to BN controls (Figure 3.10 g-l). Further evidence of this is seen if the mean CDR3 length is examined. In all samples, a decrease is seen in the mean CDR3 length of between 0.1 and 0.8 amino acids from thymus to periphery, except B-Asp BV8S3 in which the difference is smaller.

If however the parameters of CDR3 length are examined in comparison with other data (Candeias, Waltzinger et al. 1991), the changes observed between receptors undergoing selection on low surface expression MHC class II and normal levels of expression, do not appear to be greater than those observed between selection on different MHC class II molecules.

3.6.2: J β gene usage

A second parameter, commonly discussed in CDR3 analysis is J β gene segment usage. During the gene rearrangement process, the TCR V β can recombine to any of the twelve J β gene segments. These are divided into two groups depending upon whether they are linked to C β 1 (BJ1S1- BJ1S6) or 2 (BJ2S1-2S7) of which BJ2S6 is a pseudogene. In this analysis, the J β usage in the thymic pool represents the pre-selected repertoire that have undergone recombination, while the splenic CD4⁺ pool represents samples that have undergone both recombination and subsequent selection. Previous analysis has shown that the process of J β gene selection is not random. Figure 3.11 shows the J β gene usage profiles for B-Asp and BN thymocytes and peripheral CD4⁺ T cells. As previously shown (Candeias, Waltzinger et al. 1991), the selection process is not random, with BJ1S1 being over-represented, occurring in 29.9% of B-Asp spleen V β 2 positive CD4⁺ CDR3s and is commonly found in most of the samples tested. This is quite variable between different V β , being particularly prevalent in both B-Asp and BN thymus V β 8.2. The preference for BJ regions shown by Candeias *et al* (Candeias, Waltzinger et al. 1991) in V β 17 appears to be distinct between particular V β containing TCRs. In this analysis, V β 2 CDR3 regions have a preference for BJ2, (particularly

BJ2S1 and 2S7) with 55.9-84.8% of sequences having BJ2 gene segments rather than BJ1. For V β 8.2, the picture is less clear, with the distribution more even between BJ1 and BJ2. In V β 8S3 the distribution is different again. In B-Asp thymus and spleen CD4 samples, BJ1 occurs in 42.6-42.9% of sequences. However in the normal control samples, J β usage is skewed towards the BJ2 segments with 72.1-74.4% of CDR3 containing BJ2S1-2S7 segments.

There is evidence for selection occurring between thymus and spleen, as would be expected. In figure 3.11, it is clear that a number of J β genes have undergone changes in expression between thymus and periphery. While BJ1S1 appears to be selected preferentially for survival into the periphery in V β 2 from B-Asp, increasing from 7.6% to 29.9%, both 2S4 and 2S5 decrease from 10.6% to 2.6%. In the BN controls, preferential selection of BJ1S1 is not seen, although the thymic population is considerably higher than in B-Asp.

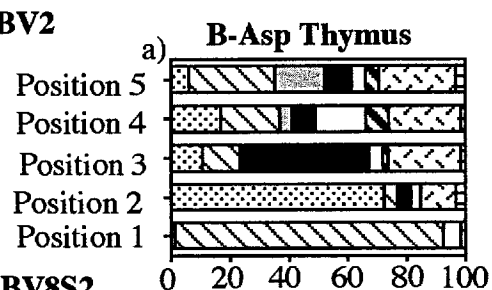
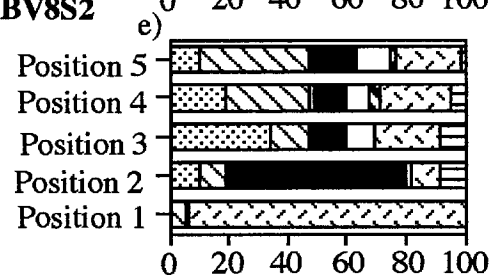
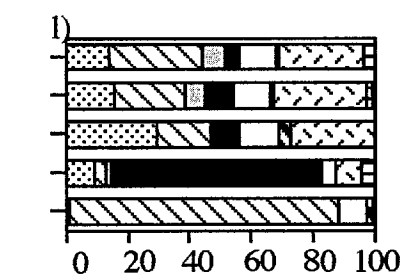
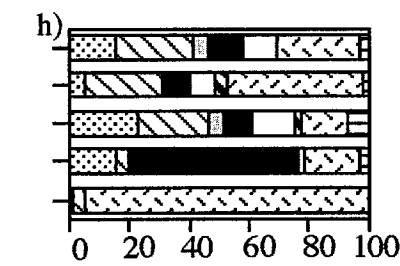
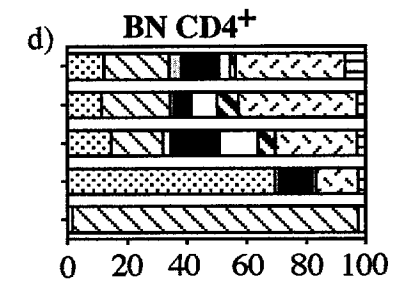
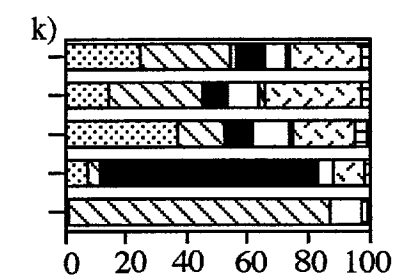
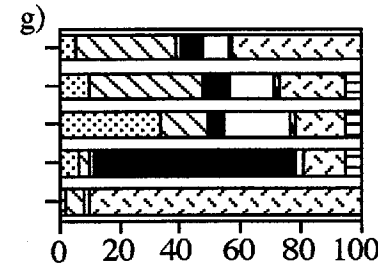
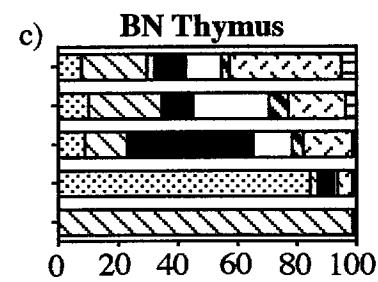
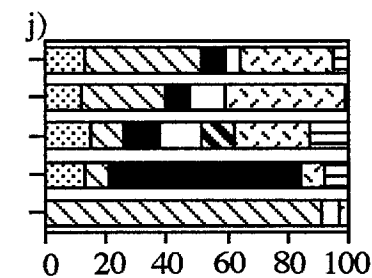
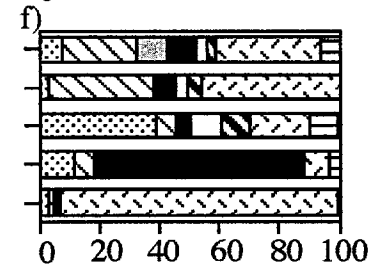
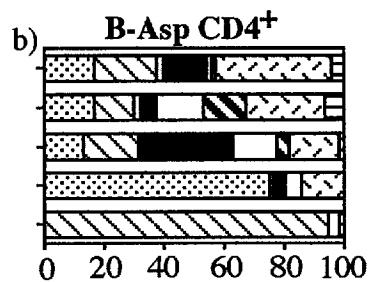
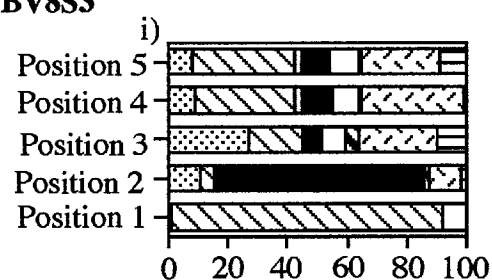
V β 8.2 also shows evidence of preferential selection into the periphery of particular J β regions. In the thymus of B-Asp, BJ1S1 accounts for 36.7% of CDR3 regions sequenced. In this case, the occurrence in the periphery is decreased to 16.1%. While a process of negative-selection also occurs in BN, it is less marked than in B-Asp. Decreased frequencies from thymus to periphery were also noted in 1S3, 1S4 and 2S4, while there is nearly a fourfold increase from 6.7% to 25.8% in BJ2S7 and from 1.7 to 6.5% in BJ2S3. In the case of BJ2S7 and 2S3 however, similar increases are also seen in BN.

When B-Asp V β 8.3 sequences are examined, expansions are seen in BJ1S2, 1S5, 1S6 and 2S4, while deletions are seen in BJ1S3 and 2S2. This differs somewhat from the pattern observed in the BN, although some of the expansion/deletions are occurring in the same J β gene segments.

3.6.3: CDR3 amino acid frequencies

In figure 3.12, the amino acid usage for the first five amino acids of the CDR3 region has been plotted and grouped according to their characteristics. As would be expected in the V β 2 sequences, position one is dominated by polar residues particularly serine, while position two is mainly hydrophobic residues such as alanine. The 3' end of the V β 2 segment encodes these, with contribution from N nucleotide additions. Positions 3, 4 and 5 become increasingly variable, with position 3 in both thymic

Figure 3.12. Amino acid usage within the first five positions of the CDR3 β region are comparable within a particular V β . Grouped amino acid usage is presented as a percentage. Amino acids are categorised as follows: Hydrophobic (L, I, F, M, V, A), Polar (Q, N, T, S), Tyrosine (Y), Acidic (D, E), Basic (R, K, H), Tryptophan (W), Glycine (G) or Proline (P). Figures are: a) B-Asp Thymus BV2, b) B-Asp spleen BV2, c) BN Thymus BV2, d) BN spleen BV2, e) B-Asp Thymus BV8S2, f) B-Asp spleen BV8S2, g) BN Thymus BV8S2, h) BN spleen BV8S2, i) B-Asp Thymus BV8S3, j) B-Asp spleen BV8S3, k) BN Thymus BV8S3, l) BN spleen BV8S3. 59-130 sequences were analysed for each sample.

BV2**BV8S2****BV8S3**

Hydrophobics
(L,I,F,M,V,A)

Polar (Q,N,T,S)

Y

Acidics (D,E)

Basic (R,K,H)

W

G

P

% of sequences

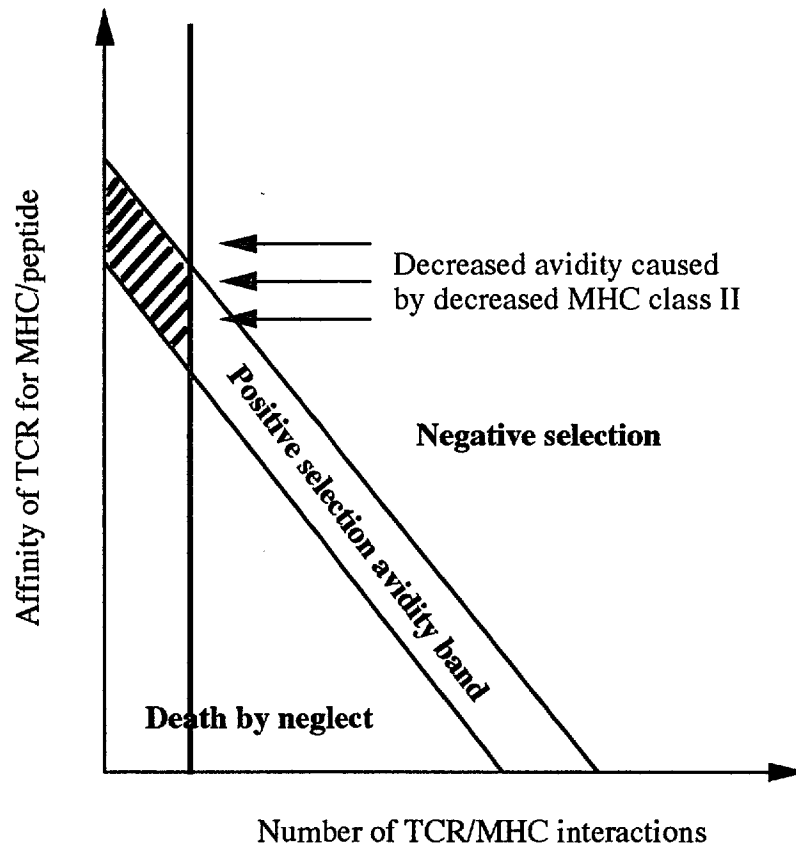


Figure 3.13. The avidity model of T cell selection in the B-Asp. A notional avidity band leading to positive selection is represented. By reducing MHC class II, the number of TCR/MHC interactions available will decrease, thus shifting the positively selected pool of cells to the higher affinity/lower number of interaction end of the avidity band.

samples being dominated by acidic residues, particularly aspartic acid, with decreased occurrence in periphery. Glycine is commonly found in all V β 2 samples, particularly in the periphery at positions 3-5. This was also seen in the previous study.

V β 8.2 and 8.3, despite being from the same gene family of sequences show different profiles at position one, although this can be accounted for by a different 3' sequence of the gene segments. V β 8.2 is mainly glycine at position one, while in V β 8.3 serine is the main contributor. Position two in both members of the family are mainly acidic residues, particularly aspartic acid. Positions 3-5 are again highly variable although large proportions are again glycine and polar residues, while tyrosine, tryptophan, proline, basic and acidic residues are in the minority. However in this case again, the differences between B-Asp and BN are minor, rather than gross.

3.7: Summary of the influences of altering MHC class II expression on TCR repertoire selection

The avidity model of T cell selection suggests that the affinity of interaction between TCR and MHC in the thymus, and the number of interactions that are occurring, combine to produce the avidity. Avidity thresholds determine whether or not a developing thymocyte will pass through positive and negative selection. If this model were correct, then decreasing the level of MHC class II would lead to the selection of a cohort of T cells expressing high-affinity TCRs (Figure 3.13).

This work has examined the V α repertoire in the periphery of B-Asp mice and shown decreased selection of V α 8⁺ CD4⁺ T cells. In figure 3.3, it is seen that compared to V α 2 and V α 3.2, the occurrence of V α 8⁺ CD4⁺ cells is decreased compared to either NOD or normal littermate controls. Between 2.7 and 3.4% of CD4⁺ cells are V α 8 positive in the control mice, while in B-Asp it is less than 1%. However both V α 2 and 3.2 have very similar profiles in all experiments. This suggests that V α 8 cells are preferentially under-represented in the periphery of B-Asp mice and higher in all the inbred strains examined. Many groups have shown skewing of V α or V β to CD4⁺ or CD8⁺ populations (Liao, Maltzman et al. 1990; Singer, Balderas et al. 1990; Munthe, Sollien et al. 1995; Sim, Aftahi et al. 1998; Sim, Wung et al. 1998). It has been shown that V α 8 and 3.2 are preferentially skewed towards the CD8⁺ population in B6 mice, although skewing of particular V α regions is dependent on the TCR α haplotype (Sim, Aftahi et al. 1998). This fits with our data, suggesting that the interaction between V α 8 and MHC class II during selection is unfavourable compared to other V α in the

potential repertoire. Even with normal levels of class II, it can be seen that V α 8 are poorly selected, with NOD and BN only having around 3% of CD4⁺ V α 8⁺. By lowering the level of MHC class II it appears that the interaction in the thymus has become so unfavourable that it almost no longer occurs.

In examining the V β repertoire in these mice, it is clear that several of the V β s are expanded in B-Asp mice in comparison to the controls. The percentage of V β 4⁺ CD4⁺ T cells is only slightly elevated when compared to both NOD and BN, while both V β 5.1 and 14 are expanded two fold or greater. This is interesting, although not too surprising as this suggests that the repertoire is being influenced not only in respect of the α , but also the β chain. Work by Liao *et al* (Liao, Maltzman et al. 1990), on a range of mouse strains found that V β 5.1 is predominantly skewed towards the CD8⁺ population, while in the presence of H2-E molecules V β 5.1 is deleted from both CD4 and CD8 subsets. Sarukhan *et al* (Sarukhan, Gombert et al. 1994) used semi-quantitative PCR and antibody staining to examine expression of various V β in the spleen and thymus of NOD mice. Similar to figure 3.4 b), it was found that V β 2, 12 and 14 are relatively abundant in the spleen, and that both V β 2 and V β 14 appear to be skewed towards the CD4⁺ population. It was suggested that these V β are preferentially expanded from the thymus to the spleen. It may be that if the interaction between V β 14 and MHC class II is particularly favourable, this could explain the relative expansion of V β 14 when MHC class II is reduced, allowing thymocytes expressing V β 14 to preferentially survive selection.

A number of *Mtv* have been implicated in mediating deletion of V β 5.1/2 including *Mtv*3, 8, 9 and 11 (Scherer, Ignatowicz et al. 1995), while deletion of V β 14⁺ cells is also thought to be influenced by *Mtv* (Penninger, Wallace et al. 1994) including *Mtv*2 (Nakano, Yoshimoto et al. 1996). It is likely that the overexpression of V β 5.1/2 seen in B-Asp mice is due to inefficient deletion by *Mtv*. However it is unlikely to be through lack of the *Mtv* as both NOD and littermate BN mice with related MHC molecules undergo deletion of V β 5.1/2. The low level of MHC class II expression in B-Asp mice will result in defective presentation of *Mtv*. Since both A^b and A^{g7} are known to present *Mtv* (Dyson, Elliott et al. 1998), it would be expected that the mixed haplotype molecule would also be capable of interaction with *Mtv*. Similarly it is also possible that partial *Mtv*-mediated deletion of V β 14 is occurring in a number of the different mice strains examined, but not in B-Asp.

While altering the level of MHC class II involved in selection appears to influence the repertoire of TCRs selected at a gross level in terms of V α and V β selection, the decision was made to examine some expressed V β gene segments that did not appear to be affected at this gross level. It was possible that the TCR sequences selected by low MHC class II may be structurally distinct, reflecting increased TCR affinity. V β 2 and V β 8.2 were examined, as these did not appear to be differentially expressed between B-Asp, BN and NOD, or were shown to be differentially skewed in NOD (McDuffie, Schweiger et al. 1992; Sarukhan, Gombert et al. 1994). V β 8.3 was also examined due to cross-reactivity of the primers occurring between V β 8.2 and V β 8.3. Some minor differences between the B-Asp and BN control sequences were shown in terms of different CDR3 parameters: CDR3 length, J β gene usage and amino acid usage in the first five positions of the CDR3.

In order to draw comparisons between analyses of CDR3 data, it is essential that the same definition of the CDR3 loop be used. Due to variable amino acids at the C-terminal region of the V β gene products, the definition described by Candéias *et al* (Candeias, Waltzinger et al. 1991), from Kabat *et al* (Kabat, Wu et al. 1991) was used. By comparing these data with that described by (Candeias, Waltzinger et al. 1991), it has been possible to add further supportive evidence to some of the points discussed by these authors. During the process of selection, the CDR3 length in CD4⁺ TCRs appears to undergo selection from the range available in the pre-selected repertoire, with an emphasis on shorter CDR3 β . By comparing the range of lengths, overall mean length and the percentage of CDR3 regions longer than 12 amino acids, we have shown that each of these parameters either decreases or remains stable, but never increases from thymus to spleen, irrespective of the level of MHC class II selection. There may be a structural reason for the relative absence of long CDR3 loops and there are two possibilities for this. It may be that long CDR3 loops sterically hinder interaction between TCR and MHC class II, such that they are less able to interact. The second is that the CD4 co-receptor is less able to interact with the TCR/peptide/MHC complex with long CDR3 loops such that the interaction and thus selection is less efficient. Further structural modelling using various long and short CDR3 β loops in the presence of CD4 and CD8 and MHC class I or II may shed further light on this phenomenon.

However, differences between CDR3 lengths in thymus and periphery comparing between TCRs selected on low MHC and those selected on normal levels do not appear to be of any greater magnitude than the comparison between thymus and

periphery between different MHC class II molecules A and E (Candeias, Waltzinger et al. 1991).

J β gene usage in the CDR3s selected on low MHC class II was compared to those undergoing normal selection. The process of J β gene selection appears to be dependent on the V β in question. Kato *et al* (Kato, Suzuki et al. 1994) analysed the J β gene usage in C57BL/6 spleen in three different V β containing TCRs: V β 2, 8.2 and 14 and showed again that J β usage was not random, that the profiles varied between V β segments. In C57BL/6, J β 2.6 was most commonly used, between 19.2 and 21.2% in the three different V β . This compares well with data for V β 2 in both B-Asp and BN, in that 2S7 is expressed at 22.7% and 16.4% in the spleen. It also coincides well with observations for V β 8.2 in that more than a four fold expansion from thymus to spleen of 2S7 containing CDR3s in both B-Asp and BN was seen. High frequencies of J β 2S1 were seen both in C57BL/6 and also in this study, while low frequencies of J β 1S3, 1S5 and 1S6 were commonly seen in both. It is also interesting to note that in V β 2 and V β 8.2, there is preferential use of the J β 2 cluster in comparison to J β 1. A similar profile is seen in figure 3.11 in V β 2, but in V β 8S2 it appears to be less skewed towards a gene cluster. This could be due to the difference in the selecting class II molecule. J β cluster skewing also appears to be highly dependent on the V β , with V β 2 showing strong J β 2 skewing and V β 8.2 is evenly distributed. V β 8.3 shows preferential J β 1 usage by the B-Asp CDR3s, while the normal controls preferentially select J β 2.

Summarising the work by Candéias (Candeias, Waltzinger et al. 1991) and Kato (Kato, Suzuki et al. 1994) together with the data presented here, strongly suggests that selection of J β genes is strongly dependent on the V β being examined, and that J β usage in a V β can be biased, although the process by which this bias occurs is unclear. Making a comparison between the selection of J β between B-Asp and BN is therefore difficult, although it is noticeable that there are some differences in the J β profiles. While in B-Asp V β 2, almost a 3-fold expansion of BJ1S1 CDR3s from 7.6% to 29.9% is seen, no comparable expansion is seen in BN. In the 2S1 selection in B-Asp V β 2 there is a decrease from 33.3% to 18.2%, an expansion is seen in the BN controls from 17.9% to 30.1%. Examining the V β 8.2 data also reveals similar occurrences, with 1S1 occurring less commonly in the spleen than the thymus in both B-Asp and BN. However the magnitude of the change is larger in the B-Asp compared to BN. One important factor to note is that in the BN thymus sequences there is likely to be some

contamination with mature CD4 SP thymocytes, which may be over-represented in the sequences, as they will have stable TCR mRNA with in-frame CDR3 rearrangements. This is less of a problem with the B-Asp as there are very few CD4 SP thymocytes.

Expansions are seen in 1S2, 2S3 and 2S7, but these are also observed in both samples. These data suggest that during the process of selection in the thymus, the J β gene used in the CDR3 plays an important part in determining whether the TCR is selected for survival. Structural models have indicated that the CDR3 β region interacts closely with the peptide-binding groove of the MHC class II molecule, so it may not be too surprising that the J β gene product, which forms the N-terminal section of the CDR3 loop, as well as extending beyond the defined CDR3, has such a strong influence on the TCR selected. These data also suggest that the J β usage is determined by the V β used. This implies that the V β region is able to influence, maybe sterically, the J β segment that is used in the CDR3 region.

The amino acid usage in the first 5 positions of the CDR3 loop has also been examined in detail. As previously described, the first two positions are dominated by the germ-line encoded 3'-terminal sequence of the V β gene being used, while further into the loop, at positions 3-5, the amino acids used are more variable. However there are minor variations in the amino acid usage that vary, from V β to V β , and between B-Asp and BN samples. In most of the samples tested, tyrosine, tryptophan, proline and both acidic and basic residues are rarely seen unless they are part of the V β region as in BV8S2 and 8S3. One caveat to this is B-Asp V β 2, where acidic residues form a large proportion in both thymus and spleen, while in BN acidic residues are only common in the thymus. Candéias *et al* (Candeias, Waltzinger et al. 1991) also noted the virtual absence of tryptophan in CD4⁺ CDR3 regions compared to CD8⁺.

Tryptophan is a large amino acid, normally found buried in hydrophobic regions of a protein, which suggests that the interaction between MHC class II and TCR is less amenable to such a bulky residue in the CDR3 than MHC class I/TCR. In most cases, positions 3-5 are mainly either hydrophobic or polar residues or glycine, even in the thymus. These suggest that residues that may have a destabilising effect, or form salt-bridges, are formed rarely even before selection. Comparison between B-Asp and BN reveal that in the V β s tested, differences in amino acid usage between thymus and spleen are few, but it would be difficult to attribute them to a change in the level of MHC class II selection alone.

Altogether these data have shown that by altering the level of expression of selecting MHC class II ligand, the process of thymocyte development is dramatically affected quantitatively and qualitatively. The level of MHC class II plays an important role in the structure of the TCRs that are able to survive selection and move into the periphery, influencing both the V α and V β chain usage. On close inspection, the CDR3 β sequence is not altered dramatically by the decreased levels of MHC class II beyond the changes seen between different selecting MHC class II ligands. Further confirmatory information has been added to the role of MHC class II restriction in influencing the CDR3 region. These data are consistent with the avidity model of T cell selection since the strongest effects seen (V α 8 under-representation and V β 5.1/5.2 over-representation) can be interpreted within the avidity model. The loss of positive selection seen in the presence of reduced MHC class II, differentially affects V α 8 in comparison to the other V α s analysed. V α 8 and V α 3.2 are used differently in BN, but V α 8 becomes under-represented in B-Asp relative to V α 2. It could be that the CDR1/2 regions of V α 8 are particularly important in determination of its reactivity to MHC class II, hence the reduction in MHC class II density is closely related to MHC/peptide affinity. For V α 2, reactivity to MHC class II may not be as dependent on CDR1/2 as CDR3 and hence its representation is not as affected by the drop in MHC class II expression. For V β 5.1/5.2, the clear escape from *Mtv*-mediated negative selection is consistent with a drop in avidity, through reduction in available MHC class II for presentation.

3.8: Alloreactivity of selected TCRs

In order that T cells are selected on low surface expression MHC class II molecules, it is possible that TCRs that are selected, as well as being altered structurally in terms of V α / β usage and possibly CDR3 parameters, may be functionally altered. It has been suggested that if TCRs are of higher affinity as may be expected, there is increased opportunity for autoreactive and alloreactive T cells to be selected (Speir, Garcia et al. 1998).

Previous work (Schoendorf 2000), as well as data not shown has revealed that B-Asp CD4⁺ T cells are functional in that they proliferate in response to allogeneic APCs including SJL (H2^s), C58 (H2^k) and B10.GD (H2^{g2}). In order to examine the role of the TCR in the absence of other influences, a number of T cell hybridomas were

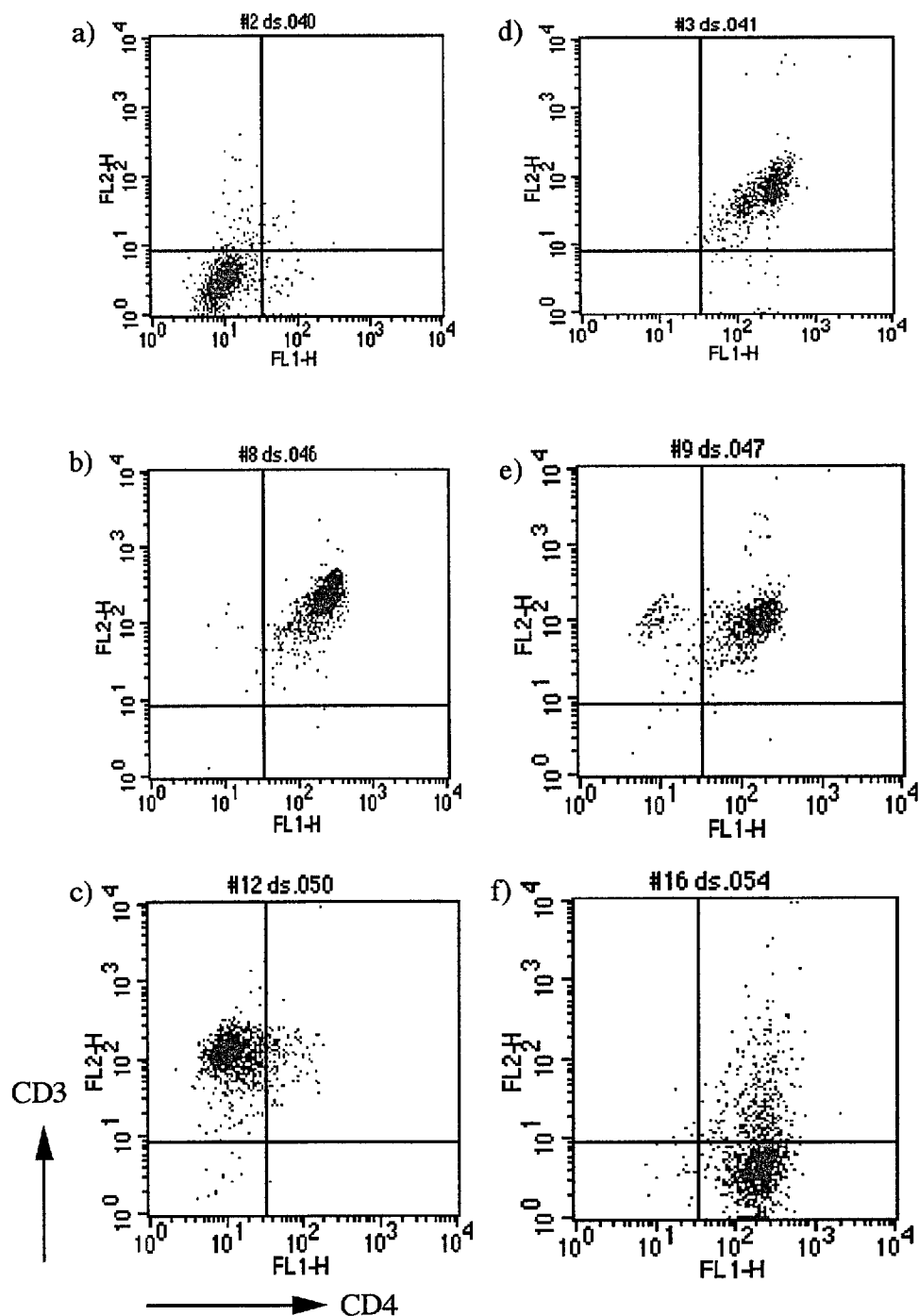


Figure 3.14. Hybridomas made from purified $CD4^+$ splenocytes from B-Asp mice. T cells were fused with BW7CD4 cells and cultured over a period of weeks in selection media. During selection some hybridomas lost expression of either CD4 or TCR. When in normal RPMI hybridomas were analysed by flow cytometry for expression of both markers (CD4-FITC and CD3-PE) after which double positive cells were used in alloreactivity proliferation assays.

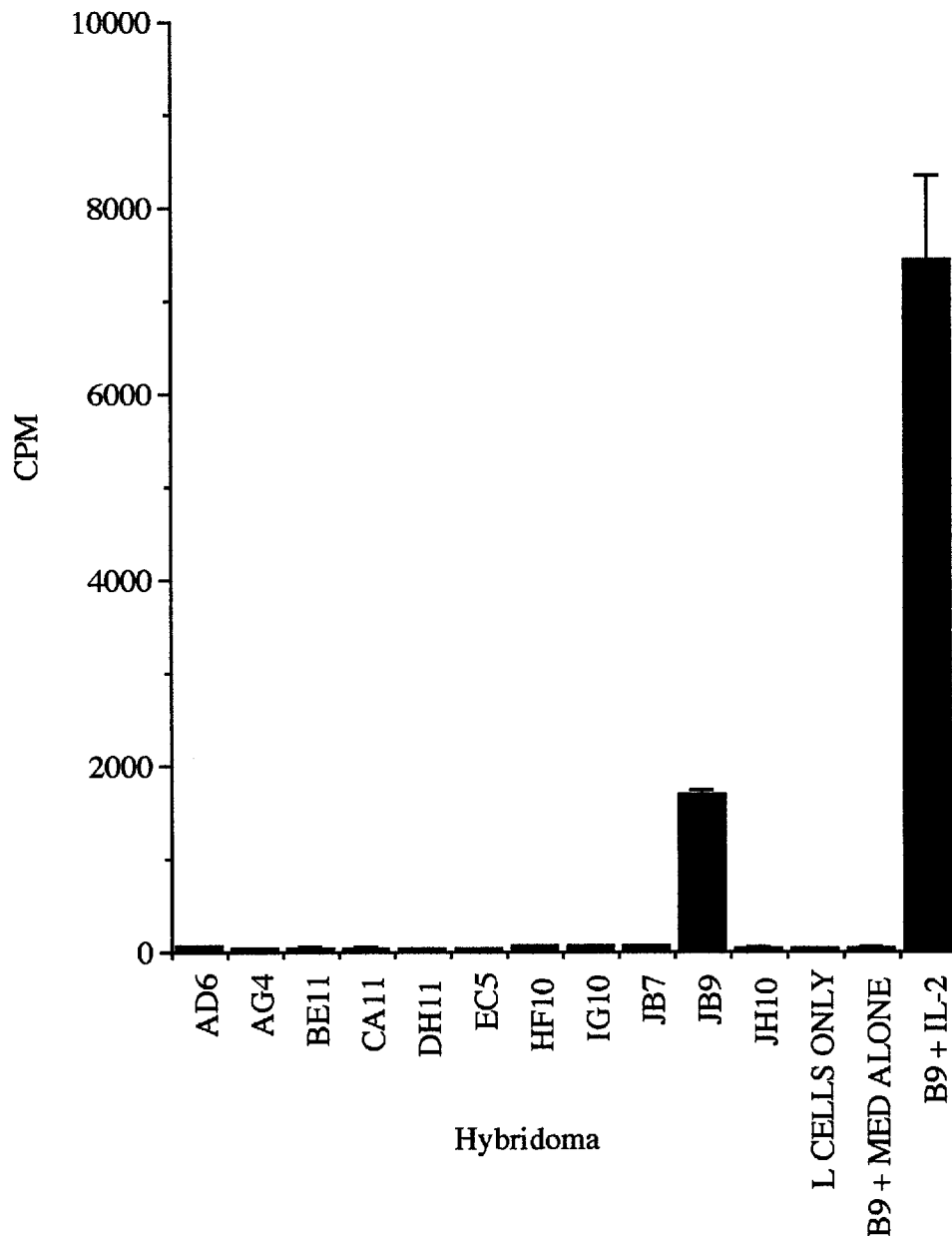


Figure 3.15. One hybridoma out of eleven tested displayed alloreactivity to L cells expressing non-self MHC molecules (A^b). IL-2 production assay using 11 hybridomas made from B10.A(4R) splenocytes fused with BW7CD4 cells. Also tested as APCs were L cells expressing A^d , A^k and A^s , but no alloreactivity was observed above background. L cells were cultured in the presence of the hybridomas for 24 hours. Supernatants were frozen and thawed out, after which they were added to the IL-2 responsive B9 T cell clone with known IL-2 concentration (1 I.U./ml) as control. This was then used in a proliferation assay.

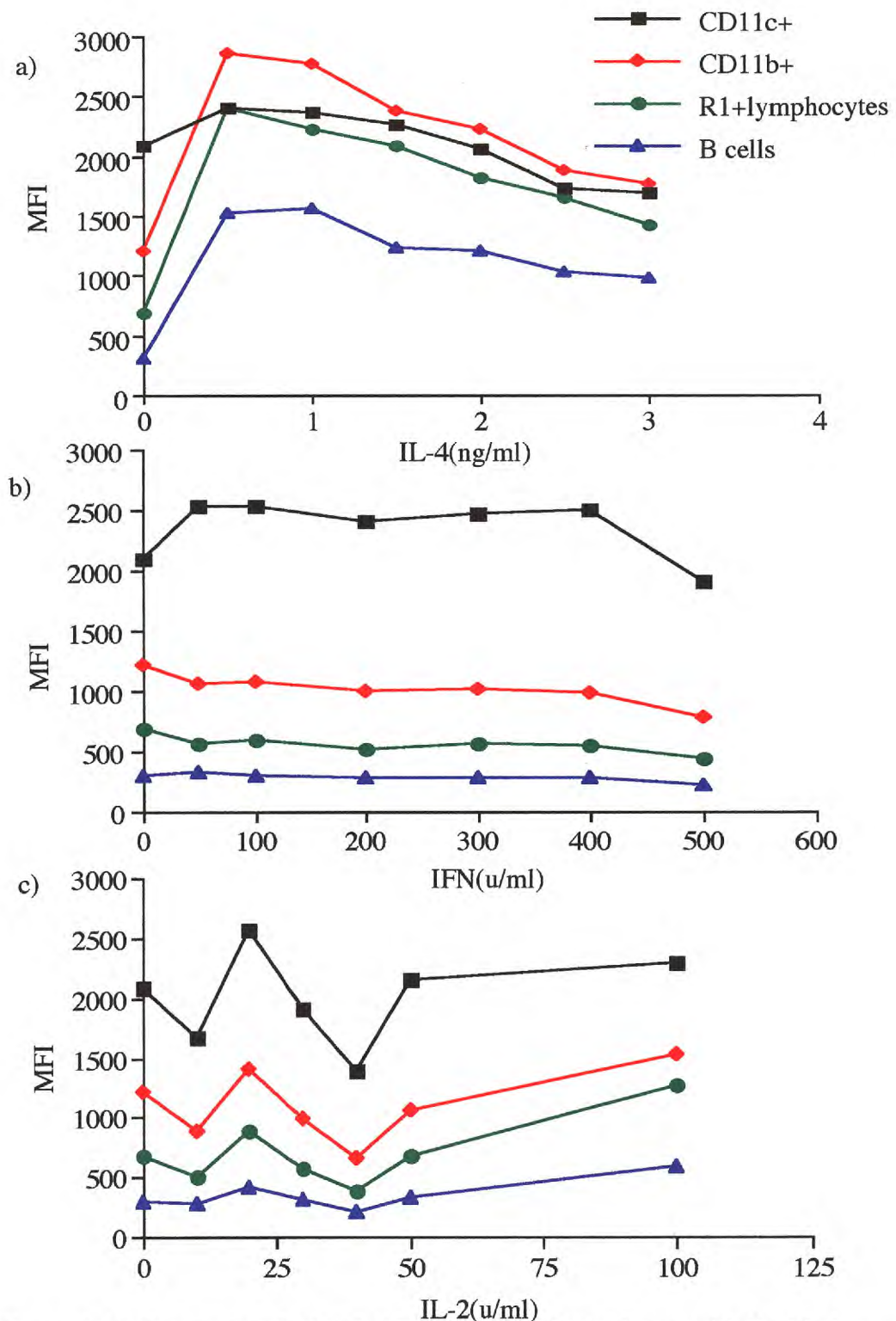


Figure 3.16. Treatment of C57BL/10 splenocytes for 24 hours with IL-4 induces upregulation of MHC class II expression on the cell surface, particularly on B cells and macrophages. Cells were cultured in the presence of a) IL-4, b) IFN- γ and c) IL-2 for 24 hours prior to analysis by flow cytometry. Antibodies used were CD11c-PE, CD11b-FITC and biotinylated Y3P (anti H2^b α -chain) + SA-QR. Cells were gated as follows: R1 = all APCs, (CD11c hi = DCs, CD11b hi = Macrophages), MHC class II positive lymphocytes = B cells. R1 + lymphocytes = all APCs + lymphocyte gate.

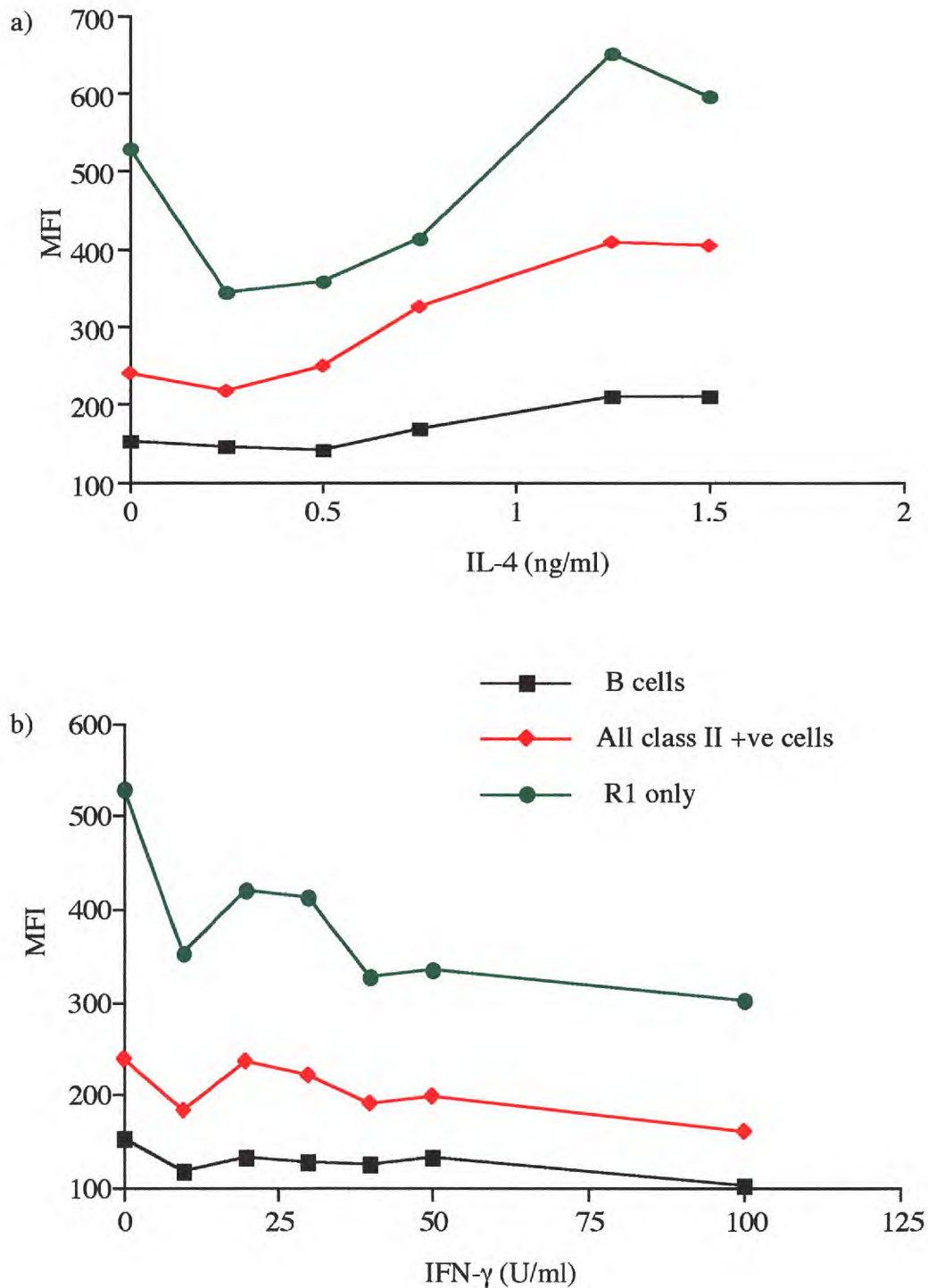


Figure 3.17. Treatment with IL-4 but not IFN- γ for 24 hours causes upregulation of MHC class II on the surface of B-Asp APCs. B-Asp splenocytes were cultured in the presence of a) IL-4 or b) IFN- γ for 24 hours prior to analysis by flow cytometry for CD11c-PE, CD11b-FITC and biotinylated Y3P + SA-QR. Cells are gated as follows: B cells = MHC class II positive lymphocytes, All MHC class II positive cells = R1 (DCs/Macrophages) and lymphocytes, APCs = R1.

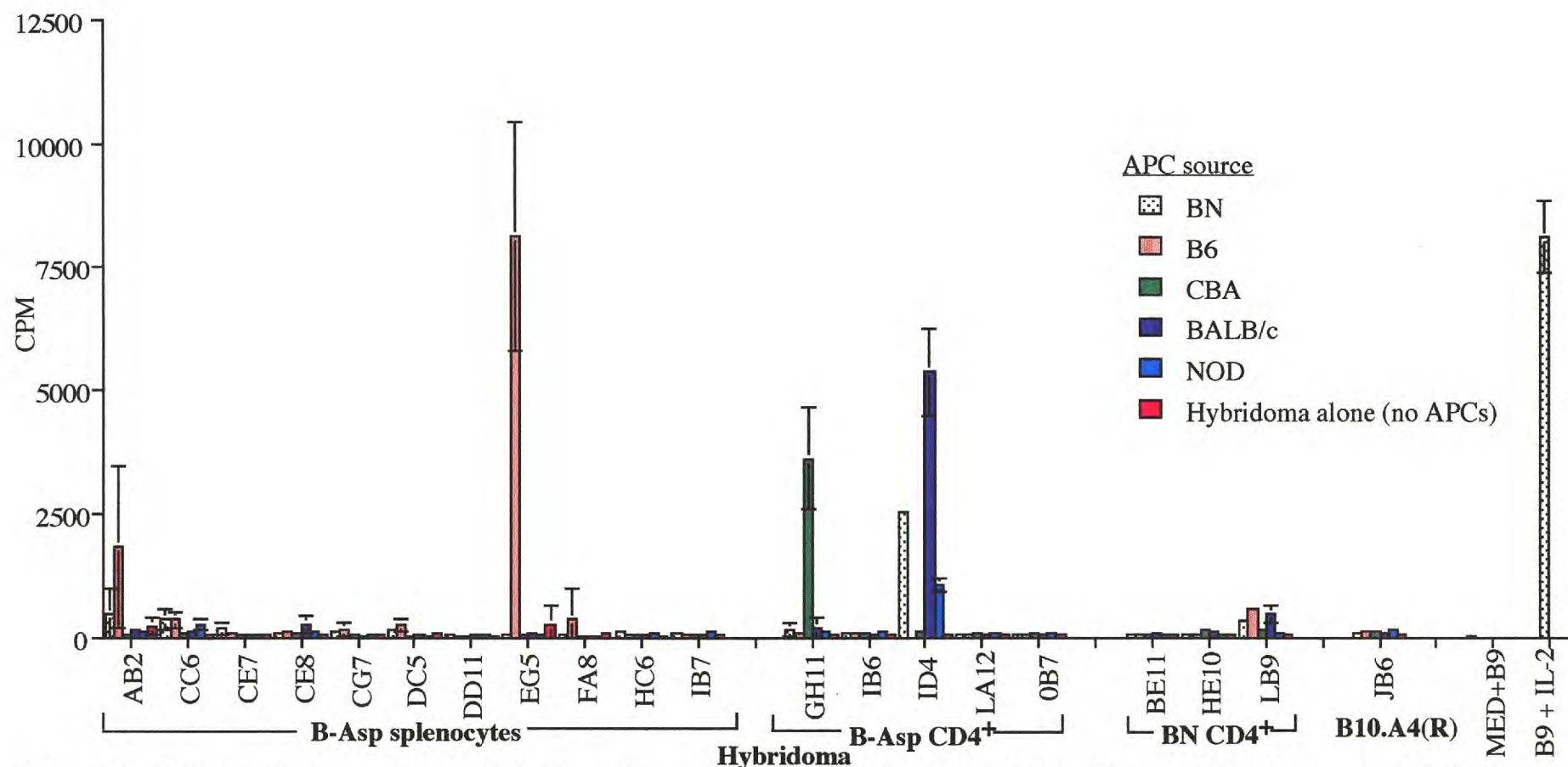


Figure 3.18. Alloreactivity is seen in some hybridomas from B-Asp mice. Hybridomas made from B-Asp whole spleen, purified B-Asp and BN CD4⁺ T cells and B10.A4(R) spleen were co-cultured with IL-4 treated, irradiated splenocytes for 24 hours from BN, B6, CBA, BALB/c and NOD mice. Supernatants were removed, frozen and thawed, then used to stimulate the IL-2 responsive B9 T cell clone. ³H-Thymidine was added after 48 hours in culture, then harvested. Controls include hybridoma alone (no APCs), B9 with and without 1 IU/ml rIL-2.

made to determine the level of alloreactivity of B-Asp TCRs compared to normal controls. T cell hybridomas were produced by fusing the immortal thymoma cell-line BW7CD4 (CD4⁺ TCR⁺) (Letourneur and Malissen 1989), and either unpurified splenocytes or CD4⁺ T cells from B-Asp or BN MHC class I mice. Initially, hybridomas were made from unsorted splenocytes, but it was thought that CD4⁺/TCR⁺ hybridomas could be produced from CD8⁺ T cells due to the presence of the CD4 co-receptor in the fusion partner and it would be difficult to determine the source of the TCR. Further hybridomas were then produced using CD8-depleted, MHC class I-deficient mice. After fusion, hybridomas are cloned in selection media, preventing growth of non-fused cells of either T cell or BW7CD4 origin. After selection, hybridomas are maintained in normal RPMI. However, as hybridomas are a fusion between two cell types, they have an unstable genome due to chromosome polyploidy. Unfortunately this means that hybridomas often have an unstable phenotype and after fusion, can 'lose' expression of either CD4 or the TCR in culture. Figure 3.14 shows FACS analysis of a panel of 6 hybridomas made from purified CD4⁺ T cells from B-Asp mice, staining with CD4-PE and CD3ε-FITC as a marker of the TCR complex. It can be seen that while hybridomas b), d) and e) express both TCR and CD4, hybridomas a), c) and f) have lost expression of both markers, CD4 or CD3 respectively. Hybridomas that express both CD4 and TCR were then used in proliferation assays to determine the incidence of alloreactivity.

Figure 3.15 shows a proliferation assay using a panel of 11 different hybridomas made from B10.A(4R) (H2^k) splenocytes to optimise the protocol initially. In this experiment, L cells transfected with different MHC molecules were used as APCs and it can be seen that in normal mice a degree of alloreactivity is observed, with one of the hybridomas (JB9) responding to L cells expressing A^b. Subsequently, splenocytes were used as APCs in further analysis of hybridomas due to concerns that peptides from FCS may be presented by the transfected MHC class II molecules which may be affecting the responses observed. In addition, L cells do not have MHC class II-processing machinery and therefore may express atypical class II molecules.

Initial assays using B-Asp and BN hybridomas yielded little information concerning alloreactivity (data not shown). In order to enhance reactivity, splenocytes were treated with a number of different cytokines *in vitro* for 24 hours and then examined for upregulation of MHC class II expression on the surface of APCs. A number of different cytokines are known to upregulate MHC expression both *in vitro*

and *in vivo* including GM-CSF, IFN- γ , and IL-4 (Ransohoff, Tuohy et al. 1992; Frandji, Tkaczyk et al. 1995; Akbar, Inaba et al. 1996; Fan and Wuthrich 1997).

Figure 3.16 a)-c) shows the mean fluorescence intensity for MHC class II expression using C57BL/10 splenocytes cultured overnight with different doses of a) rIL-4, b) IFN- γ and c) IL-2. With a dose of 0.5ng/ml IL-4, MHC class II expression is increased almost three-fold particularly on B cells and macrophages while little effect is seen with IFN- γ or IL-2. Further analysis of class II expression using B-Asp splenocytes shows that 1.25ng/ml IL-4 (Figure 3.17 a) induced maximal upregulation of MHC class II on a number of cell types while IFN- γ did not (Figure 3.17 b). Subsequent assays used APCs cultured in 1.25ng/ml IL-4 for 24 hours.

Previously it was shown that purified CD4⁺ T cells from B-Asp underwent alloresponses when stimulated by non-self MHC and the production of T cell hybridomas allowed quantitation of alloreactivity. Hybridomas were made from B-Asp splenocytes and purified CD4⁺ T cells, or BN CD4⁺ T cells and were used as responders in an IL-2 production assay. Hybridomas were cultured with irradiated, IL-4 treated APCs for 24 hours and the supernatant removed and freeze/thawed. Alloreactivity was indicated by the presence of IL-2 in the supernatant and was measured by proliferation of the B9 IL-2 responsive clone.

Figure 3.18 shows an example of an assay, using IL-4 treated splenocytes as APCs to examine alloreactivity including: BN (H2^{b/g7}), C57BL/6 (H2^b), CBA/Ca (H2^k), BALB/c (H2^d) and NOD (H2^{g7}). IL-2 production was detected by hybridomas from unsorted B-Asp splenocytes and B-Asp CD4⁺ T cells but little from BN CD4⁺ or the previously described B10.A(4R) hybridoma. 2/11 B-Asp splenocyte hybridomas responded above background to C57BL/6 splenocytes expressing one of the parental haplotype MHC (H2^b) while 2/5 B-Asp CD4⁺ hybridomas responded to CBA/Ca, BN, BALB/c or NOD APCs and 0/3 BN hybridomas responded above background.

Unfortunately, due to the instability of the hybridomas it was difficult to generate reproducible responses in these assays. It is unclear whether a lack of response is due to lack of alloreactivity or due to loss of either CD4 or TCR expression from the hybridoma during preparation for the assay. These data do suggest that there is a trend towards increased alloreactivity by the B-Asp TCR, looking at those made both from unpurified splenocytes and purified CD4⁺ T cells when compared to those made from normal mice. However the number of hybridomas assessed was small and further analysis would need to be done to confirm that this was the case.

3.9: Antigen-specific responses of B-Asp using a model antigen

Previously it was shown that B-Asp mice are able to mount immune responses. On exposure to keyhole limpet haemocyanin (KLH) *in vivo*, B-Asp mice were able to induce anti-KLH IgM responses and subsequently induce antibody class switching of B cells to IgG1, although a little delayed when compared with BN controls, possibly as a result of the low numbers of CD4⁺ T cells present, able to provide B cell help. However B-Asp mice were incapable of inducing IgG2a responses unlike BN controls, so although they are able to induce responses, these are delayed or defective (Schoendorf 2000). Interestingly IgG1-type responses are normally associated with Th2-type cytokines such as IL-4, and it has been suggested that the TCR/MHC avidity can influence the skewing of T cells to Th1 or Th2 phenotypes (Pfeiffer, Stein et al. 1995; Constant and Bottomly 1997). It is thought that low avidity interactions may favour a Th2-type response while high avidity interactions skew towards Th1. While this has been shown in the B-Asp model through antibody production *in vivo* (Schoendorf 2000), it had not been examined by testing cytokine secretion.

Using B-Asp and BN mice that had previously been immunised with KLH, KLH-specific CD4⁺ cell lines were made. Over a period of several weeks, the lines were stimulated repeatedly with syngeneic APCs and KLH *in vitro*. 72 hours after each stimulation, supernatant was taken from the cultures and tested for the presence of IL-4, IL-10 and IFN- γ with cytokine-specific ELISAs. B-Asp T cells secreted IL-4, IL-10 and little IFN- γ , while BN lines produced a similar profile (Figure 3.19 b) as would be expected from Th2 cells. At all times, both cell lines produced all cytokines, although the BN cells produced little IFN- γ (Figure 3.19 a). It is clear that neither cell-line remains with a stable phenotype over the period of time, although the BN line never produces significant amounts of IFN- γ in comparison to B-Asp. It is possible as the lines are non-clonal that with repeated stimulation, different T cell clones are being preferentially expanded compared to others such that the cytokine profiles do not remain steady. It is also possible that B-Asp T cells are less 'fixed' in their phenotype and are unable to maintain a phenotype while BN remains more constant in comparison.

The variability in phenotype of the lines makes it difficult to draw any meaningful conclusions from them. Dependent on the stimulation number it may appear that at one time B-Asp cells displayed a strongly Th2 phenotype, producing IL-4 and

IL-10 while at another time they appear more skewed to Th1, producing IFN- γ and little IL-4 and IL-10.

3.10: *In vivo* functional responses of B-Asp mice by skin graft

In the B-Asp backcross, mice are generated that express three distinct levels of MHC class II (Figure 3.1). BN mice express normal levels of MHC class II, B-Asp express around 10% of wild-type levels, and MHC class II-deficient mice have undetectable MHC class II. These can all occur in the presence or absence of $\beta 2m$ (MHC class I). In order to determine whether B-Asp mice can generate functional immune responses *in vivo*, mice were grafted with skin expressing MHC class II at three different levels. BN, B-Asp and MHC^{-/-} mice (all MHC class I^{-/-} and therefore CD8⁺-deficient) were grafted with C57BL/6 (complete allogeneic mismatch expressing both MHC class I and II), B-Asp (low MHC class II, no MHC class I) or MHC^{-/-} skin.

Figure 3.20 a) shows the nine possible donor/recipient combinations and highlights the degree of mismatch between them as well as the median survival time (MST) for the grafts in days. Figures 3.20 b)-d) show the graft rejection profiles for BN, B-Asp and MHC^{-/-} mice respectively. BN MHC class I-deficient mice (Figure 3.20 b) reject allogeneic grafts expressing both MHC class I and II with a MST of 13 days. This suggests that the absence of CD8⁺ T cells from these mice does not hinder the rejection of allogeneic grafts. BN mice reject B-Asp grafts slightly slower than B6 grafts. Although the MST for B-Asp grafts is 19 days, 4/7 mice rejected grafts quickly, with the remaining grafts surviving to between 40 and 70 days. When given MHC^{-/-} grafts, they reject the grafts with a similar speed to B-Asp grafts, with MST of 16 days. Again, a proportion of the grafts survived for longer than 16 days, and out until 68 days, similar to B-Asp. The B-Asp grafts are allogeneic for MHC class II, yet due to lower expression of MHC class II on the graft, the BN mice are less able to reject via the direct pathway of graft rejection, consequently much of the rejection in BN with B-Asp graft will be through minor antigens presented directly or indirectly. Interestingly, despite the absence of MHC expression on the graft, BN mice are able to reject MHC-deficient grafts with surprising rapidity, presumably via an indirect mechanism, showing the importance of the indirect pathway operating in the BN mice.

When B-Asp mice were grafted, again they were able to reject C57BL/6 grafts rapidly with MST of 13 days (Figure 3.20 c). This suggests that the small population of

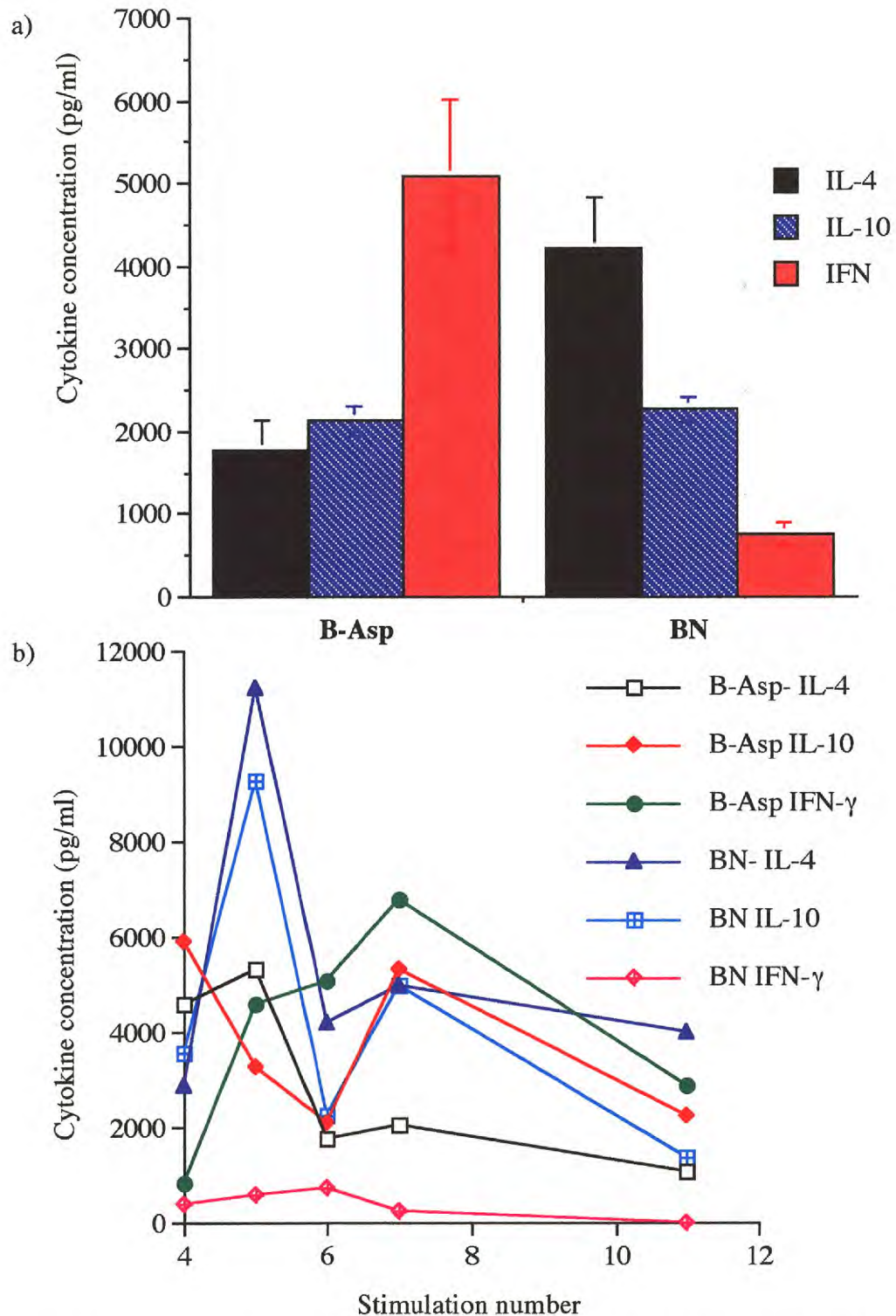


Figure 3.19. KLH specific cell lines from B-Asp and BN show skewed cytokine profiles 72 hours after stimulation with KLH and syngeneic APCs. However over repeated stimulation the profiles of cytokine secretion are altered. a) B-Asp KLH-line secretes little IL-4 and IL-10, with high IFN- γ , while BN KLH-line secretes IL-4, less IL-10 and little IFN- γ . b) Repeated stimulation of the cell lines shows alterations in cytokine profile of B-Asp and BN. Cytokine concentration was determined by taking supernatants 72 hours after stimulation, then using Quantikine ELISA kits for IL-10, IFN- γ and IL-4.

a)

Recipient	Donor	Disparity				MST
		MHC I	MHC II	mH I	mH II	
BN	<i>B6</i>	✓	✓	✓	✓	13
	<i>B-Asp</i>	-	✓ (low)	-	✓ (low)	19
	<i>MHC^{-/-}</i>	-	-	-	✓(**)	16
B-Asp	<i>B6</i>	✓	✓	✓	✓	13
	<i>B-Asp</i>	-	-	-	✓ (low)	47
	<i>MHC^{-/-}</i>	-	-	-	✓(**)	>160
MHC^{-/-}	<i>B6</i>	✓	✓	✓	✓	15
	<i>B-Asp</i>	-	✓ (low)	-	✓ (low)	39.5
	<i>MHC^{-/-}</i>	-	-	-	-	*

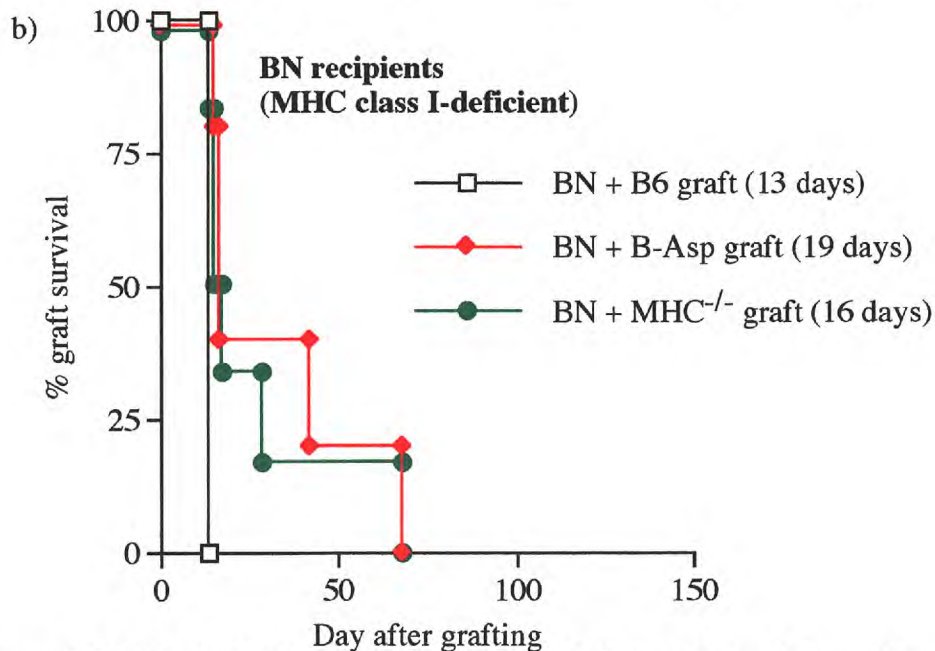
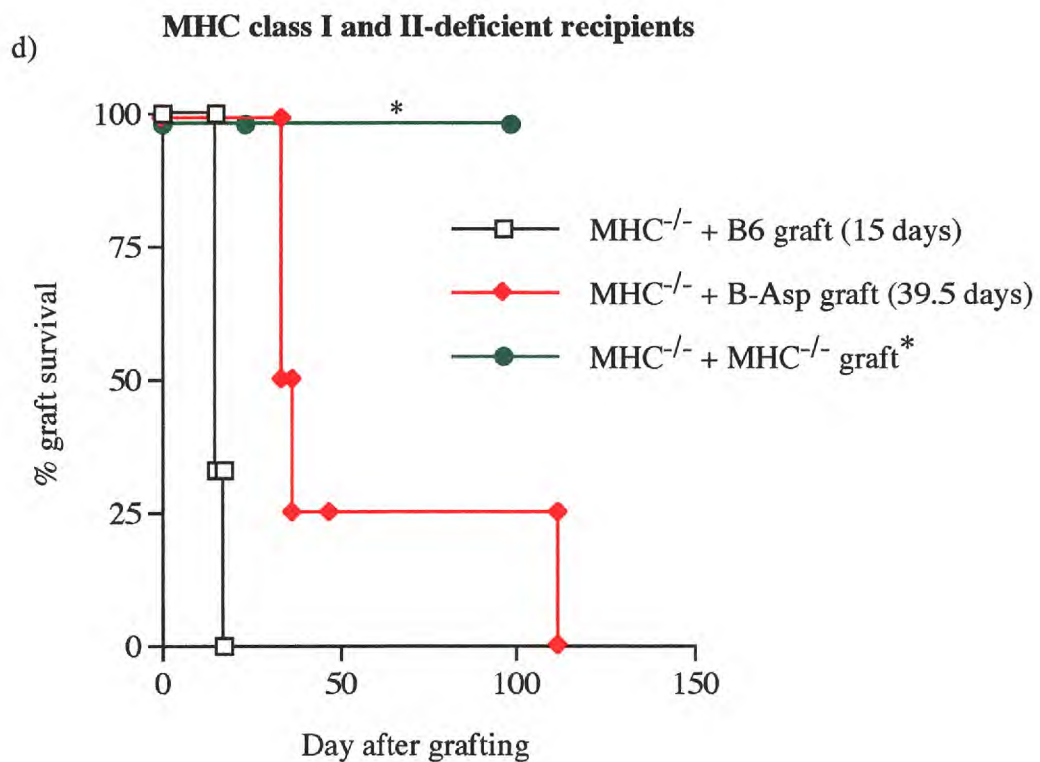
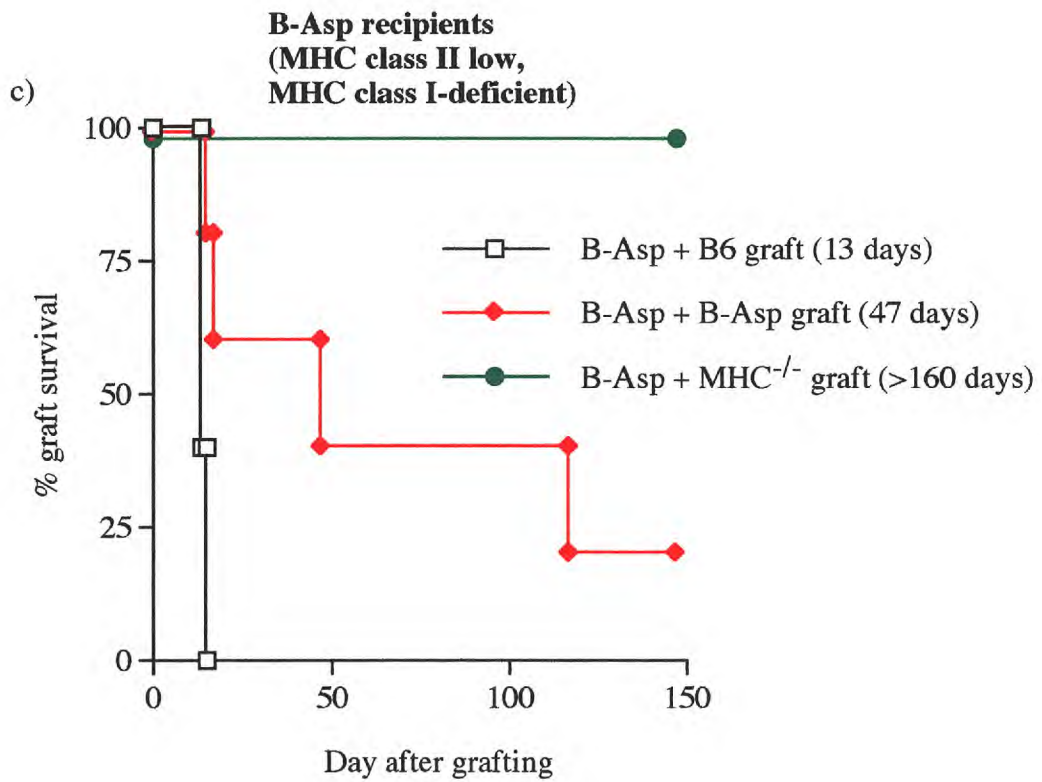


Figure 3.20. Graft rejection is dependent on MHC class II levels in recipient and graft. BN, B-Asp or MHC^{-/-} mice were grafted with B6, B-Asp or MHC^{-/-} skin. a) Table showing the degree of mismatch and median survival times (MST) for each combination. All recipients lack CD8⁺ T cells and are MHC class I-deficient. MHC I/II- MHC class I/II mismatched, mH I/mH II- minor antigens mismatched. (*) MHC^{-/-} with MHC^{-/-} grafts- This group was comprised of two mice, both of which died before the end of the experiment. The grafts were intact at time of death (day 23 and 98). (**) indirect presentation of minors is possible. b)BN, c) B-Asp (MHC low) and d) MHC^{-/-} rejection profiles of B6, B-Asp and MHC^{-/-} grafts with MST as shown.



B-Asp CD4⁺ cells are able to mediate graft rejection in the absence of CD8⁺ T cells. The graft expressed both allogeneic MHC class I and II, targets for alloreactive B-Asp CD4⁺ T cells, although MHC class I is likely to be indirectly recognised by CD4⁺ T cells due to the absence of CD8⁺ T cells. When grafted with B-Asp skin, B-Asp mice reject the skin over a longer time period with MST of 47 days, while a number of the grafts were maintained beyond 100, and one to 150 days. The rejection of B-Asp skin by B-Asp mice was not surprising as the backcross does not produce inbred mice and consequently the mice are different outside of the MHC locus, generating potential multiple minor mismatches in the context of low expression of MHC class II. This survival profile is reminiscent of unprimed, minor mismatched graft rejection as seen in the HY system. In the absence of both MHC class I and II from the graft, all the grafts were maintained by B-Asp mice to beyond 160 days. This suggests that due to the combined absence of MHC class I/II from the graft, as well as the low numbers of CD4⁺ T cells, the B-Asp mice are no longer able to induce rejection of the grafts indirectly.

MHC^{-/-} mice reject B6 grafts quickly with MST of 15 days (Figure 3.20 d). This was surprising and suggested that MHC^{-/-} mice are able to reject complete allogeneic mismatched grafts expressing both MHC class I and II in the near absence of both CD8⁺ and CD4⁺ T cells. This was similar to B6 rejection both by BN and B-Asp mice, although slightly slower. B-Asp grafts were again rejected surprisingly quickly with MST of around 40 days, while one of the grafts survived past 100 days. Unfortunately MHC-deficient mice did not survive particularly well, partly due to the time delays between birth, typing and transfer prior to grafting and then grafting, as well as deaths under anaesthesia. As a consequence, the MHC^{-/-} recipient group receiving MHC^{-/-} grafts only contained four mice, two of which died before removing the plaster, one died after about 25 days and the remaining mouse died around 100 days after grafting. However in each case the grafts were intact at time of death.

A number of groups have examined the different roles of CD4⁺ and CD8⁺ T cells, as well as MHC deficient mice in grafting models. Interestingly, corneal transplants are known to be deficient of MHC class II⁺ APCs and it is thought that rejection of minor mismatched corneal grafts occurs via the indirect pathway. Using BALB/c mice grafted with C57BL/6 cornea, it was found that CD4⁺ T cells activated by the indirect pathway were involved in the rejection process (Boisgerault, Liu et al. 2001). Recently Borenstein *et al* (Borenstein, Graham et al. 2000) used a number of HLA-transgenic mice to examine the role of CD8⁺ T cells in skin graft rejection and concluded that CD8⁺ T cells were needed to mediate graft rejection when mismatched

alleles were used, while if differences were locus- or xeno-mismatched, they were not necessary. CD4⁺ T cells injected into SCID and rag-deficient mice were able to mediate cardiac allograft rejection in the absence of CD8⁺, while CD4⁺ T cells injected into rag-deficient/MHC class II-deficient mice could also reject. These suggested that indirect presentation and MHC class II expression on the graft in this example was less important (Pietra, Wiseman et al. 2000).

MHC class II-deficient mice have been carefully studied and are deficient in clearing some viral and protozoal infections, while the role of the presence/absence of MHC class II has also been examined in a number of autoimmune disease models (Grusby and Glimcher 1995). MHC class II-deficient mice have been previously examined both as donors and recipients in transplantation. When MHC class II-deficient skin was grafted onto normal recipients, the graft is rejected with similar rapidity to grafts expressing MHC class II (Auchincloss, Lee et al. 1993), demonstrating the importance of the indirect pathway. The rejection of MHC^{-/-} skin has also been shown to be dependent both on CD4 and CD8 T cells while MHC^{-/-} mice grafted with normal allogeneic skin are able to reject with similar rapidity to mice with MHC molecules, despite the absence of CD4/CD8 T cells (Grusby, Auchincloss et al. 1993). This is consistent with the rather surprising responses seen in Figure 3.20. More recently, MHC class II-deficient mice were used to examine the role of blocking co-stimulatory molecules such as CD40L and CTLA-4 (Yamada, Chandraker et al. 2001). Mice were also used expressing MHC class II only on thymic epithelium and could therefore generate only CD4⁺ T cells. Co-stimulatory blockade has been previously shown to induce graft survival, yet in MHC class II-deficient mice, survival was prevented in cardiac allografts while it was less effective in skin graft models. These data suggest that for co-stimulatory blockade to be effective at preventing allograft rejection, the indirect pathway of antigen recognition needs to be functional.

The results shown in figure 3.20, although surprising, are not without precedent, revealing that in the absence of CD8⁺ T cells but with normal numbers of CD4⁺ T cells as a result of normal MHC class II expression (BN recipients), grafts can be rejected with alacrity. When B6 grafts are used, both the direct and indirect pathways are operational and grafts are rapidly rejected. As the 'amount of recognition' available to the direct pathway decreases with decreasing MHC class II expression on the grafts, the rejection occurs slightly slower, while rejection is still able to occur as described previously (Auchincloss, Lee et al. 1993).

If the level of MHC class II expression is decreased in recipients (B-Asp) they are also able to reject complete allogeneic grafts (B6) rapidly in the absence of CD8⁺ T cells and with similar rapidity to BN mice. This suggests that despite the low number of CD4⁺ T cells, there are functional allogeneic T cells (as shown previously (Schoendorf 2000) and in this chapter), able to reject grafts by the direct and indirect pathways. As the level of MHC class II expression on the graft decreases, the efficiency of the direct pathway decreases. Minor antigenic differences (B-Asp with B-Asp graft) stimulate a slower rejection involving the indirect pathway while the absence of MHC class I and II expression from the surface of the graft, completely preventing direct allorecognition, effectively inhibits the ability of the small population of CD4⁺ T cells to mediate rejection.

Finally as previously shown (Grusby, Auchincloss et al. 1993) mice lacking both MHC class I and II reject completely allogeneic mismatched grafts (MHC^{-/-} with B6 grafts) with surprising rapidity, and as the level of expression of MHC class II on the surface of the graft decreases, the potential antigen available for the direct pathway decreases and rejection is slower. MHC class II-deficient mice are able to select small populations of T cells, possibly restricted by non-classical MHC molecules such as CD1 (Cardell, Tangri et al. 1995), although it is unclear whether these are involved in graft rejection responses.

3.11: Summary

Mice expressing low levels of a mixed haplotype MHC class II molecule $\alpha^b\beta^{g7(asp)}$ in the absence of other MHC class II have been characterised and shown to select a small population of CD4⁺ T cells into the periphery. Analysis of the V α /V β gene usage in the TCR of the CD4⁺ T cells show differences when compared with those selected on normal levels of MHC class II, suggesting that the structure of the TCR has been influenced directly by the level of MHC expression. This is consistent with the avidity theory of T cell selection, suggesting that TCRs selected on decreased levels of MHC class II are structurally distinct from those selected on higher MHC class II expression, possibly due to selection of increased affinity TCRs in order to survive. The repertoire was examined in a more detailed fashion by studying the CDR3 β region from a number of V β -regions revealing minor differences in the selected repertoire compared

to normal. However this effect could not be directly attributed to decreased MHC class II expression.

T cells selected on low MHC class II expression have been studied functionally to determine the degree of alloreactivity observed and whether this selection pressure influences Th1/2 phenotype. Although a few hybridomas were examined, splenocytes treated with IL-4 to upregulate MHC class II were shown to stimulate proliferation in a number of the B-Asp hybridomas, while little was detected in normal hybridomas indicating possible increased alloreactivity in those selected on low MHC class II. Analysis of cytokine secretion by antigen-specific cell lines yielded little information, although it suggested that initially the lines selected in B-Asp were skewed to a Th2 phenotype secreting IL-4 and IL-10 as had been predicted, this phenotype was not stable and altered with repeated stimulation.

The aim of these experiments was to indirectly determine whether a population of high-affinity TCR-bearing T cells are selected in the presence of low expression, mixed haplotype MHC class II molecules. Using T cell hybridomas has proved difficult, partly due to the instability of the hybridomas while it is also unclear as to whether the surviving hybridomas from B-Asp and BN are representative of true T cell populations *in vivo*. Other experiments that may provide further evidence for high affinity TCRs could include proliferation assays using anti-CD3 antibodies to stimulate T cells from B-Asp to respond. If the dose response curves were different between B-Asp and BN, this may indicate an increase in TCR affinity of the TCRs. Similarly blocking CD4 antibodies could also be used to determine the dependence of the proliferative response on the presence of the CD4 interaction. In terms of functionality, it might also be interesting to directly compare *ex-vivo* cytokine production by B-Asp and BN CD4⁺ T cells using intracellular cytokine staining rather than using *in vitro* restimulated T cell lines.

Finally the role of CD4⁺ T cells and the level of MHC class II expression on skin grafts has been examined and shown that graft rejection can occur in the absence of CD8⁺ T cells and with few CD4⁺ T cells. The rate of graft rejection appears to correlate with the level of MHC class II expression on the graft indicating the importance of the direct pathway of antigen presentation, although interestingly MHC-deficient mice are capable of rejecting allogeneic grafts with remarkable rapidity.

Chapter 4.

Antigen transfer and direct/indirect antigen presentation

4.1: Background

A number of mechanisms mediating the transfer of antigens have been described including vesicular transfer (Raposo, Nijman et al. 1996; Knight, Iqbal et al. 1998; Bedford, Garner et al. 1999; Gardella, Andrei et al. 2001; Knight and Bedford 2001; Thery, Boussac et al. 2001) from B cells and DCs, while Knight *et al* (Knight and Bedford 2001) have suggested that DCs act as efficient acceptors of vesicular antigen transport. Antigen can be transferred from the surface of live APCs particularly onto DCs by a direct contact ingestion process (Harshyne, Watkins et al. 2001) and that this process can lead to CTL stimulation. Several papers have shown that antigen transfer is not a one-way process, but that APCs can transfer antigens to the surface of T cells (Arnold, Davidian et al. 1997; Arnold and Mannie 1999; Patel, Arnold et al. 1999). These processes add complexity to antigen presentation, allowing transfer of antigen between APCs in an immune response.

In transplantation there are two pathways of antigen presentation, direct and indirect presentation. Direct presentation is the recognition of non-self MHC molecules on the surface of donor APCs by recipient T cells. The indirect pathway, which despite the name is equally important, involves the uptake of cells and antigen from the graft by host APCs, followed by presentation in the context of self-MHC molecules (Liu, Sun et al. 1993; Gould and Auchincloss 1999; Womer, Sayegh et al. 2001).

The transgenic NOD-Asp mouse model and the HY system were used to examine methods of antigen transfer and the importance of direct and indirect presentation in graft rejection. We have been in the unique position to investigate a different type of antigen presentation, the transfer of class II molecules between cells and re-assortment of the chains into functional molecules. The effects of providing the different α - and β -chains of a MHC class II molecule on different APCs have been examined. The two chains were provided separately by: mixed haplotype MHC class II mice (B-Asp), described in detail in chapter 3, expressing $\alpha^b\beta^{g7(asp)}$ MHC class II at low levels; while NOD mice express the $\alpha^d\beta^{g7}$ MHC class II molecule (Figure 4.1). If this mechanism of transfer were possible, this would imply that transferred MHC class II molecules were being fed into the class II processing pathway and would constitute a novel type of indirect presentation.

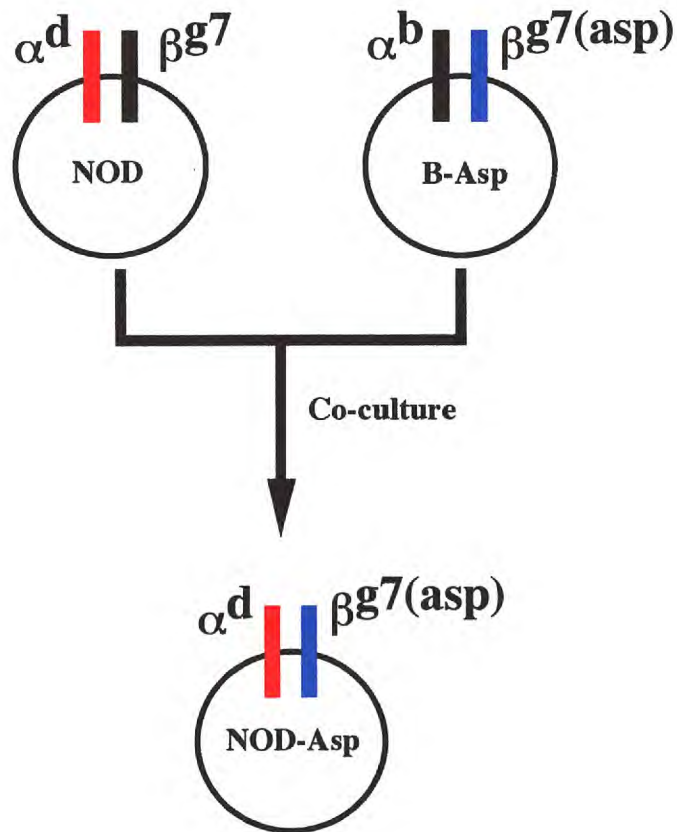


Figure 4.1. Possible mechanism for the transfer of MHC class II molecules. Co-culture of B-Asp ($\alpha^b\beta^{g7(asp)}$) and NOD ($\alpha^d\beta^{g7}$) splenocytes prior to use as APCs enables the two different haplotype MHC molecules to provide one chain each to form the NOD-Asp ($\alpha^d\beta^{g7(asp)}$) MHC molecule.

NOD mice can recognise the single mutation in the MHC class II NOD-Asp β -chain and thereby reject NOD-Asp expressing skin grafts (Antoniou, Elliott et al. 1998). NOD mice can also mount anti-HY responses with female mice rejecting male grafts, particularly when previously primed (Chandler, Fairchild et al. 1988). It is thought that the HY antigen can be presented both through the direct and indirect pathways, similar to other minor antigens. Conversely, NOD-Asp allo-MHC presentation is different. It is known that the mutation from Ser to Asp at position 57 of the MHC β -chain influences the shape of the peptide-binding groove and presumably the peptide repertoire bound, and therefore in the NOD/NOD-Asp system the majority of the allogeneic response is thought to be through the direct pathway. Previous data from this laboratory support this idea by showing that two T cell clones (1F8 and 2E4), isolated from NOD mice that have rejected NOD-Asp grafts, respond to NOD-Asp ($\alpha^d\beta^{g7(asp)}$) in the absence of $\alpha^d\beta^{g7}$ (NOD) (Antoniou, Elliott et al. 1998). It is possible that NOD rejection of NOD-Asp grafts is partially indirect, although the only difference is the mutated position 57(asp) of the β -chain. For indirect presentation to be functioning, peptides including the mutated residue would have to be presented by NOD or NOD-Asp MHC molecules. Presentation of a synthetic peptide encompassing the mutated residue by $\alpha^d\beta^{g7}$ did not stimulate either of the two T cell clones, suggestive of direct presentation, although it does not entirely exclude the possibility of indirect presentation. The NOD/NOD-Asp HY system has therefore been used to examine the effects of non-professional APCs on the direct and indirect pathways in graft rejection.

4.2: A novel mechanism of MHC transfer?

4.2.1: Stimulation of NOD-Asp specific clones *in vitro*

Two CD4⁺ T cell clones 1F8 and 2E4, specific for NOD-Asp were generated from NOD mice that had rejected NOD-Asp skin grafts (Antoniou, Elliott et al. 1998). Although the peptide specificity of the two clones is unknown, they proliferate when stimulated with NOD-Asp splenocytes *in vitro*. Figure 4.2 shows proliferation assays using irradiated NOD, NOD-Asp and B-Asp splenocytes as APCs. The clones were re-stimulated every two-three weeks with NOD-Asp splenocytes and approximately two weeks after stimulation were used as responders in a proliferation assay. Increasing numbers of splenocytes were plated out in triplicate in 96 well flat-bottom plates with 1-

2×10^4 of the clone in each well. The cultures were pulsed with ^3H -thymidine after 48 hours, then harvested and counted 12-15 hours later. Proliferation is measured by the uptake of ^3H -thymidine into the DNA of dividing cells. Significant proliferation of both 1F8 and 2E4 was seen in response to NOD-Asp splenocytes, but not NOD or B-Asp splenocytes (Figure 4.2). This shows that both clones are specific for the NOD-Asp MHC class II ($\alpha^d\beta^{g7(\text{asp})}$) molecule and associated peptide, but do not respond to either α^d paired with β^{g7} (NOD) or $\beta^{g7(\text{asp})}$ paired with α^b (B-Asp). This confirms and extends earlier observations, showing that 1F8 and 2E4 respond specifically to NOD-Asp and not to NOD or another mixed haplotype MHC molecule $\alpha^q\beta^{g7(\text{asp})}$ (Antoniou, Elliott et al. 1998).

4.2.2: Stimulation by co-cultured APCs

The next question to be examined was whether providing the α - and β -chains of the MHC class II molecule on different APCs would cause specific stimulation. This was done by co-culture of splenocytes or bone marrow DCs from NOD and B-Asp prior to use as stimulators in a proliferation assay. Splenocytes or day 7 BMDCs from NOD and B-Asp were harvested and co-cultured for 24 hours in 12 well plates at 1:1 or 1:3 ratio (NOD: B-Asp) prior to harvesting and irradiation. The higher ratio of B-Asp to NOD was used to partially compensate for the low level of cell surface expression of MHC class II on the surface of B-Asp cells. Figure 4.3 shows an example of a proliferation assay using co-cultured splenocytes as APCs. Co-cultures of DCs with splenocytes or DCs with DCs did not stimulate proliferation of either clone (data not shown), while B-Asp and NOD splenocytes when co-cultured either at 1:1 or 3:1 stimulated proliferation of both clones to about 10% of the level seen when using cultured NOD-Asp splenocytes. Neither chain in the absence of the other caused proliferation. This result was a surprise, but was reproduced in three independent experiments to a similar extent. These data suggested that NOD-Asp MHC class II/peptide complexes were being formed at the surface of the APCs.

A number of groups have described the isolation of exosomes, membrane-derived vesicles, enriched for a subset of cell surface proteins including MHC class I and II and costimulatory molecules from DCs (Thery, Boussac et al. 2001). Raposo *et al* (Raposo, Nijman et al. 1996) isolated vesicles from the supernatant of cultured B cell blasts. When pulsed with peptide, they stimulated antigen-specific proliferation *in vitro*. It was suggested that BMDCs were capable of donating, but not receiving MHC, while

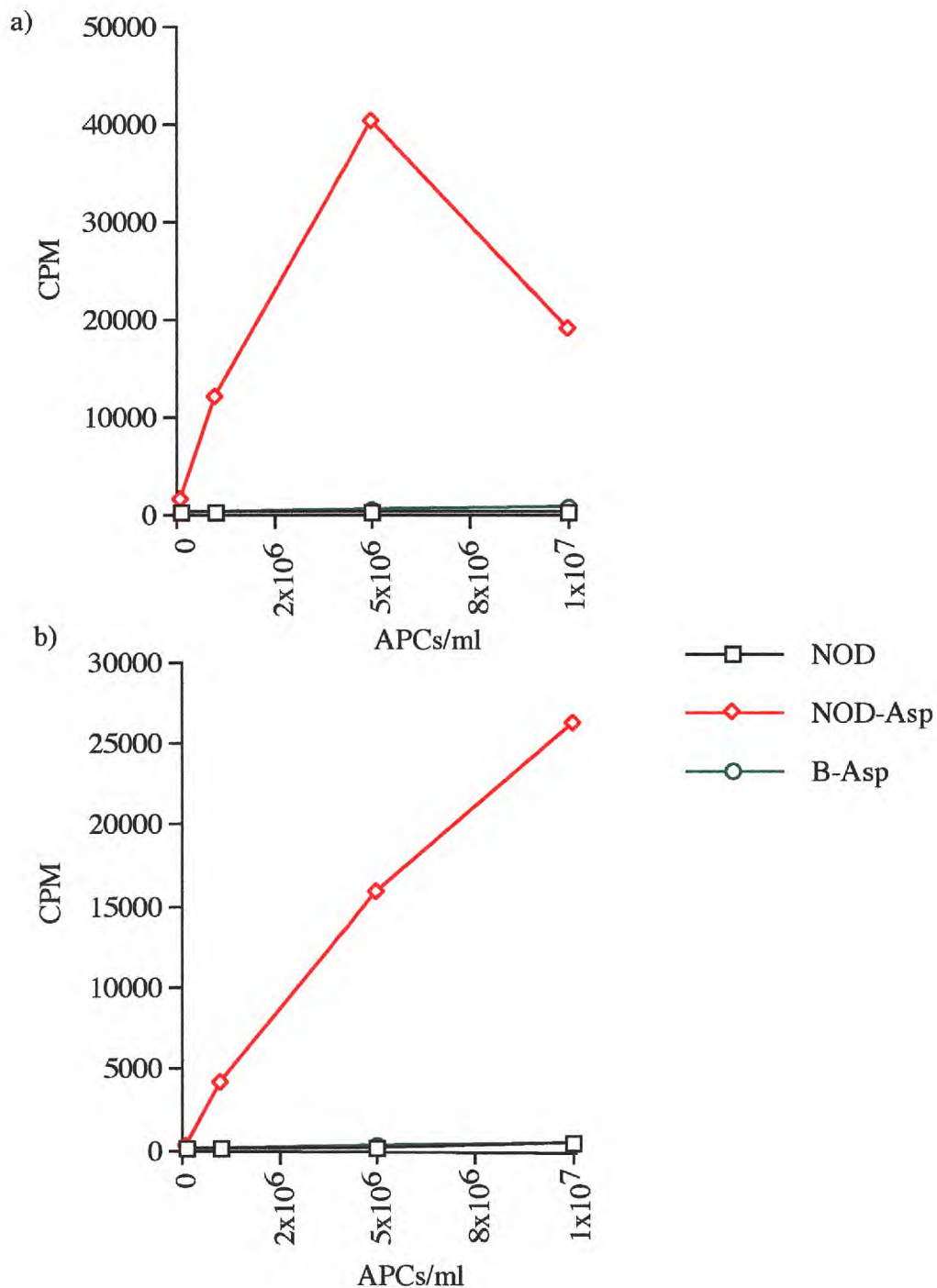


Figure 4.2. a) 1F8 and b) 2E4 T cell clones do not proliferate in response to NOD or B-Asp APCs, but are specific for the NOD-Asp MHC class II molecule. The NOD-Asp clones were cultured in 96 well plates with different numbers of irradiated splenocytes as APCs from either NOD, NOD-Asp or B-Asp mice. After 48 hours in culture, ³H-Thymidine was added overnight and the cultures harvested.

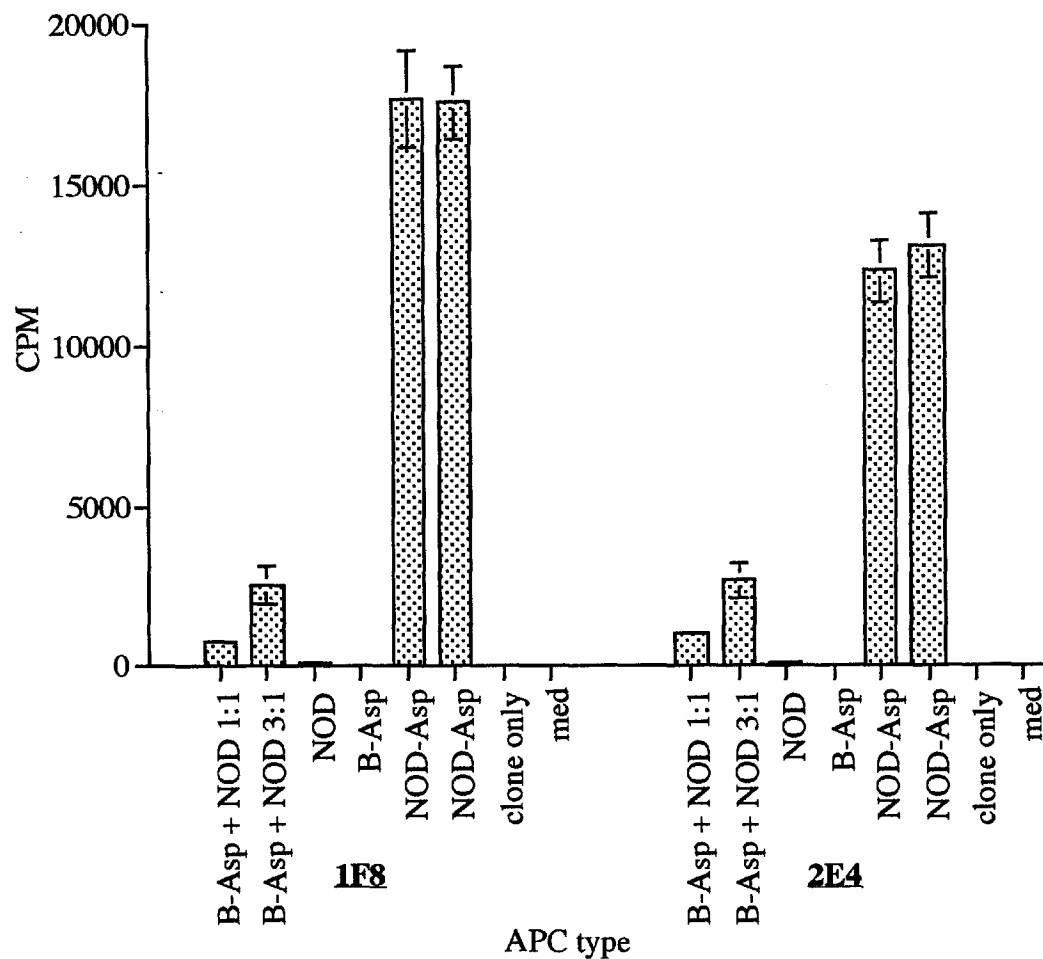


Figure 4.3. NOD-Asp specific clones 1F8 and 2E4 proliferate in response to splenocytes from B-Asp and BN that were co-cultured for 24 hours prior to use as APCs. B-Asp and NOD splenocytes were co-cultured at both 1:1 and 3:1 ratios in 12 well plates. After 24 hours they were irradiated and used as APCs in a 96 well proliferation assay. 0.5 μ Ci ^{3} H-Thymidine was added to each well after 48 hours in culture and then harvested after an overnight culture.

splenic and LN DCs were capable both of receipt and donation (Knight and Bedford 2001). It is possible that if vesicular transport is the mode of action in this transfer model, this may explain why co-cultures of BMDCs from B-Asp and NOD failed to induce proliferation of either clone, and why co-cultures of the two splenocytes induced a greater response. However this does not explain why co-cultures of BMDCs and splenocytes were incapable of stimulating, as splenic DCs are thought to be capable of both receipt and donation of MHC class II.

Harshyne *et al* (Harshyne, Watkins et al. 2001) proposed an alternative mechanism whereby immature DCs were able to acquire plasma membrane and associated proteins from a number of other cell types including T cells, B cells and macrophages in a cell-cell contact-dependent process. To examine whether stimulation of the clones by hybrid MHC class II molecules were dependent on cell-cell contact, trans-well experiments were performed. This allows separation of two cell populations by a membrane with pores of 0.4µm, preventing cell-cell contact but allowing diffusion of soluble factors or small particles across the membrane. B-Asp splenocytes were added to the bottom well, with NOD in the top well, either at 1:1 or 3:1. However in the trans-well experiments, the cells were left in co-culture for 48 hours at higher density prior to harvesting, irradiation and use as APCs in an attempt to encourage transfer. As controls, B-Asp and NOD cells were cultured together and apart under the same conditions. Figure 4.4 shows a representative proliferation assay from trans-well cultured APCs. Both clones show responses to co-cultured B-Asp and NOD splenocytes both at 1:1 and 3:1 as seen in figure 4.3. While 2E4 responds to a minimal extent with co-cultured APCs from trans-wells, 1F8 still responds, although to a lesser extent than in the co-culture. As the responses are not completely abrogated, this suggests that while a large part of the response is dependent on cell-cell contact, some of the response still occurs in the absence of cell-cell contact. Exosomes from DCs are known to be small, between 50-90nm in diameter (Thery, Boussac et al. 2001) and would cross the membrane.

These data suggested that while the majority of the response is dependent on cell-cell contact, some of the transfer might be mediated by vesicular transport. This would be a novel mechanism of MHC-pair formation, suggesting that either through vesicular transport or direct contact, MHC chains are being transferred between splenocytes, internalised and passed into the MHC biosynthetic and peptide-loading pathway. It is possible that as the B-Asp MHC class II molecule is very unstable

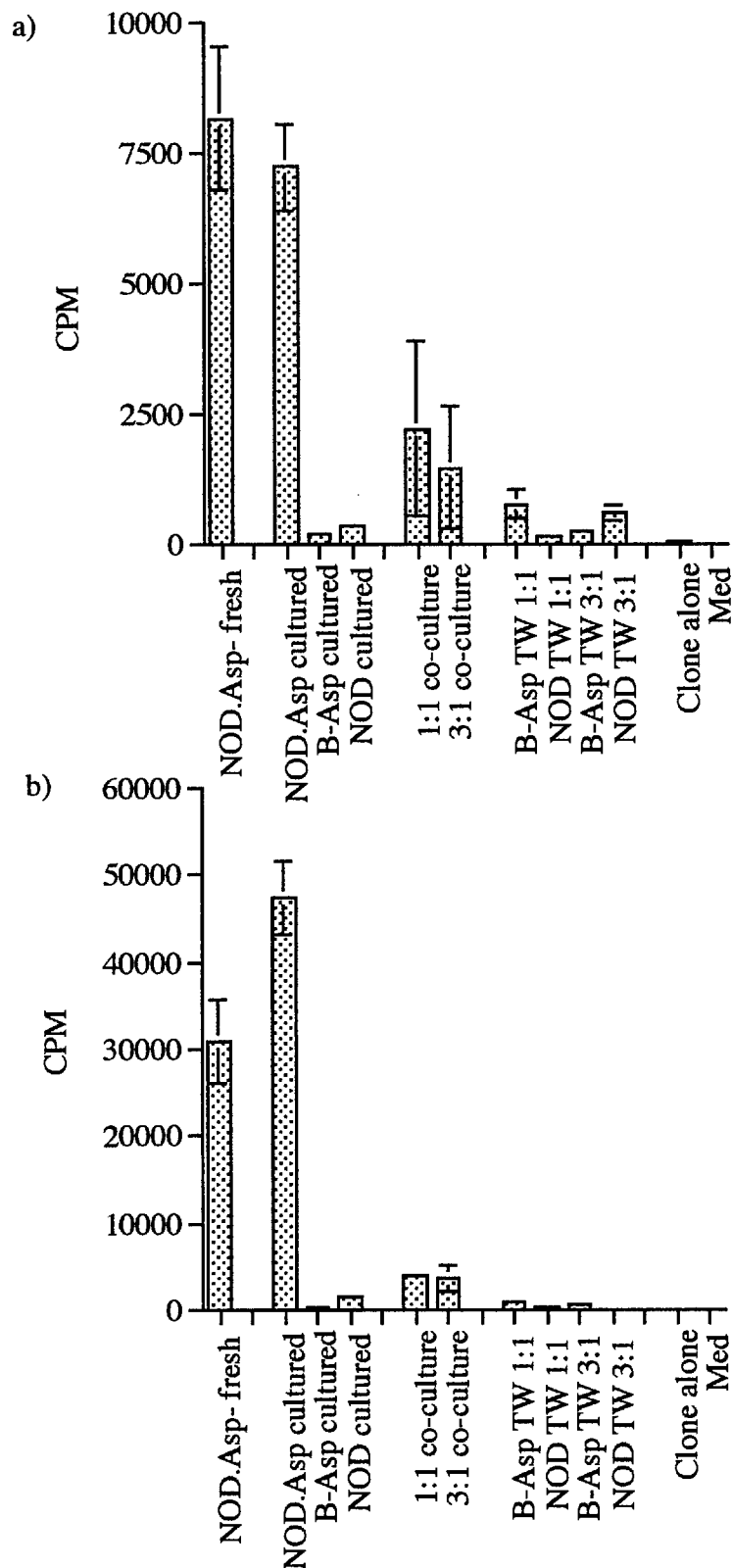


Figure 4.4. a) 1F8 and b) 2E4 NOD-Asp specific T cell clones were used in a proliferation assay. Stimulators were B-Asp and NOD splenocytes that had been co-cultured for 48 hours prior to the assay in 24 well plates at a ratio of either 3:1 or 1:1 in the presence or absence of transwells. The proliferation, although small is not completely abrogated by co-culturing in transwells. TW = cells from either the bottom or top of the transwell.

(Schoendorf 2000), pairing may occur in recipient cells, forming the more stable NOD-Asp MHC class II molecule.

4.2.3: Non-specific stimulation by co-cultured splenocytes

To determine whether transfer of MHC class II was the basis for the data described above, an allogeneic pair of splenocytes were co-cultured which do not provide the chains to produce $\alpha^d\beta^{g7(asp)}$. As in previous experiments, B-Asp and NOD splenocytes were co-cultured together, as well as with BALB/c ($H2^d$) and B10.BR ($H2^k$) as providers of MHC chains. Splenocytes were co-cultured for 48 hours prior to irradiation and use as APCs. Figure 4.5 shows one of two proliferation assays performed using these combinations of APCs. In figure 4.5 a) it can be seen that while B-Asp co-cultured with NOD stimulates 1F8 to a similar extent as NOD-Asp splenocytes, similar or even greater proliferation is seen when one of the chains is absent from the co-culture as seen in NOD with BALB/c or B10.BR, or BALB/c with B10.BR. A similar pattern is seen in figure 4.5 b). In both cases the B-Asp/NOD co-culture proliferation is at similar level with NOD-Asp splenocytes, while other combinations also induce proliferation. However in this experiment, NOD, B10.BR and BALB/c also stimulate both clones to some extent, making analysis difficult. Disappointingly, this suggests that the stimulation of the NOD-Asp clones by co-cultured splenocytes is unrelated to formation of the stimulatory class II molecule. This may be due to a mutual allogeneic stimulation of T cells/APCs in the co-culture (both before and after irradiation) and subsequent production of stimulatory cytokines such as IL-2. This has not been tested by cytokine ELISA, although this would be a useful experiment to do, and may show that for example NOD with BALB/c or B10.BR produces more IL-2 than other combinations inducing non-specific proliferation of the clones. However, this does not explain the limited proliferation observed in the transwells, as it would be expected that a transwell would prevent direct cell-cell contact and thus the allogeneic interaction and IL-2 production. Interestingly, responses of both clones to NOD and B10.BR or BALB/c are higher than that seen with the appropriate B-Asp combinations suggesting that B-Asp splenocytes induce allogeneic responses of lesser magnitude, possibly due to decreased expression of MHC class II at the cell surface and fewer T cells in the culture to respond. To see if the effect is completely explained by MLR, the co-culture experiments will be repeated using T cell depleted spleen cells. In addition, it does not explain the lack of responses using co-cultured

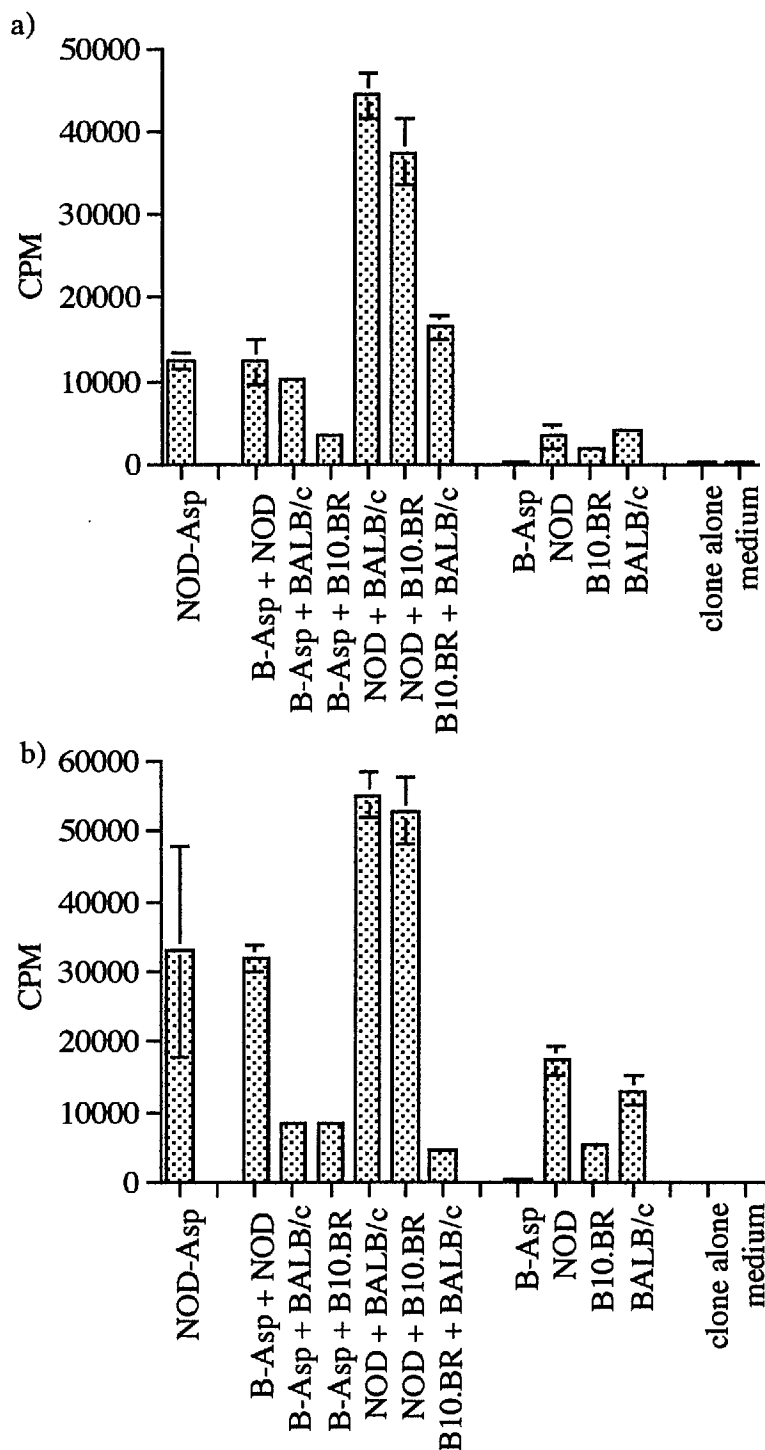


Figure 4.5. Non-specific stimulation of the NOD-Asp specific clones a) 1F8 and b) 2E4 occurs in the absence of one MHC class II chain. B-Asp, NOD, BALB/c and B10.BR splenocytes were co-cultured for 48 hours in 24 well plates prior to irradiation and use as APCs in a 96 well proliferation assay. APCs were cultured with the clones for 48 hours, then pulsed with ^3H -Thymidine and left in culture overnight.

splenocytes and BMDCs, as these would also be expected to produce cytokines in a similar manner.

4.3: B cells, tolerance and direct presentation

4.3.1: Comparison of anti NOD-Asp and anti HY responses in NOD female mice

DCs are able to stimulate naïve T cells while other non-professional APCs such as B cells do not share this characteristic (Lechler, Ng et al. 2001). Resting B cells are incapable of stimulating proliferation of resting T cells (Lassila, Vainio et al. 1988), while other work in the HY system showed that presentation of antigen by B cells induces tolerance in naïve T cells (Fuchs and Matzinger 1992). In HY-mismatched graft rejection, it is thought that both direct and indirect pathways are functional, such that antigen is not only presented by B cells, but also by recipient DCs and macrophages. To test whether presentation of antigen on B cells was immunogenic or tolerogenic when rejection occurs mainly by the direct pathway, the NOD/NOD-Asp system was used. HY was used as a control with rejection both through direct and indirect pathways.

60% of NOD male grafts are rejected spontaneously by NOD female mice with a median survival time (MST) of 60-70 days. Immunisation of NOD females with NOD male splenocytes in the footpad, intravenously (i.v.), intraperitoneally (i.p.) or through skin grafting led to the generation of HY-specific CTL as shown by ⁵¹Cr release assays *in vitro*. If NOD females are given an injection of 10⁷ male splenocytes two weeks before grafting with NOD male skin, they display accelerated graft rejection with a MST of 25 days, typical of a primed response to minor antigens (Chandler, Fairchild et al. 1988).

As previously shown (Antoniou, Elliott et al. 1998), if NOD female mice are given a NOD-Asp female skin graft, most are rejected with MST of between 36 and 70 days. Interestingly, rapid rejection of primary grafts by female mice correlated with rapid onset of autoimmune diabetes. However if NOD male mice are given NOD-Asp male grafts, they are rejected less vigorously, such that only ~30% of male mice rejected NOD-Asp male grafts and most grafts survived for longer than 100 days. In contrast if either NOD male or female mice are immunised with sex-matched NOD-Asp splenocytes prior to grafting with NOD-Asp skin, both are able to induce rapid graft rejection with a MST of around 24 days.

In order to establish a baseline for the rapidity of NOD/NOD-Asp and HY graft rejection, the following protocol was used. NOD female mice were immunised with 10^7 NOD-Asp male splenocytes intravenously as previously described. Three days later, the mice were grafted with NOD-Asp male, NOD-Asp female, NOD male or both NOD male and NOD-Asp female in the same graft bed. In this protocol, both HY and NOD-Asp antigens were present on the immunising cells, yet in the grafts either one or both antigens were present.

When NOD female mice are injected with NOD-Asp male splenocytes then given NOD-Asp male grafts, both Asp and HY antigens are present in the immunisation and the graft is rapidly rejected with a MST of 13 days (Figure 4.8 c). This particular combination was not used by Antoniou *et al* (Antoniou, Elliott et al. 1998), who examined secondary NOD/NOD-Asp interactions in sex-matched graft/donor pairings. In comparison, NOD-Asp female grafts (absence of HY antigens) are rejected slightly slower with MST of 26 days (Figure 4.8 b), similar to that observed previously (Antoniou, Elliott et al. 1998). NOD male grafts (absence of major mismatched antigens) are rejected with MST of 83 days (Figure 4.8 a) compared to >100 days seen previously, showing that NOD-Asp male splenocytes prime a response to both the HY and Asp antigens, and that the primed response to Asp leads to more rapid graft rejection than that to HY.

If injected with NOD-Asp male splenocytes, then given both NOD male and NOD-Asp female grafts in the same graft bed, such that both antigens are present on different grafts, the rejection times did not differ significantly from that described above. NOD male grafts (Figure 4.8 d) were rejected with the same MST of 83 days as in figure 4.8 a), while NOD-Asp female grafts were rejected slightly faster with MST of 13 days compared with 26 days in figure 4.8 b).

In summary, it appears that NOD-Asp male grafts with both antigens (Asp and HY) are rejected slightly faster, through the direct and indirect pathways than NOD-Asp female grafts, when only one antigen (Asp) is present, presumably rejecting through the direct pathway alone. NOD male grafts with only the HY antigen are rejected slowest in these primed mice, through both direct and indirect, but in the absence of major mismatched antigens. This is consistent with that previously described comparing major and minor mismatched allograft survival (Gould and Auchincloss 1999). However, compared with previous data, NOD-Asp male splenocytes are able to prime NOD female mice to both major and minor mismatched antigens.

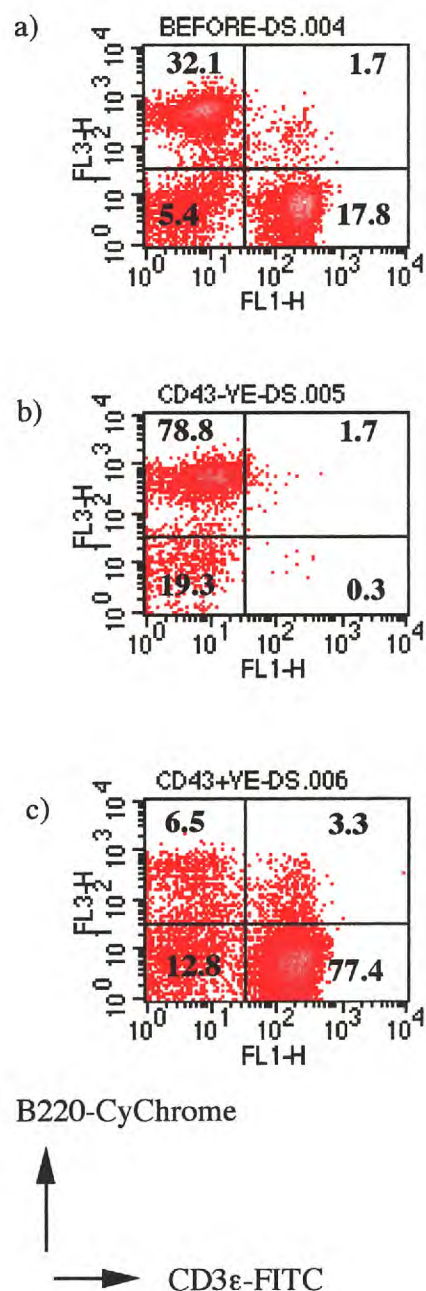


Figure 4.6. CD43- MACS depletion allows purification of naive B cells from spleen of NOD-Asp male mice for injection into NOD females. Flow cytometry of splenocytes, stained with B220-CyChrome and CD3ε-FITC a) before purification, b) in the CD43 negative fraction and c) in the CD43 positive fraction.

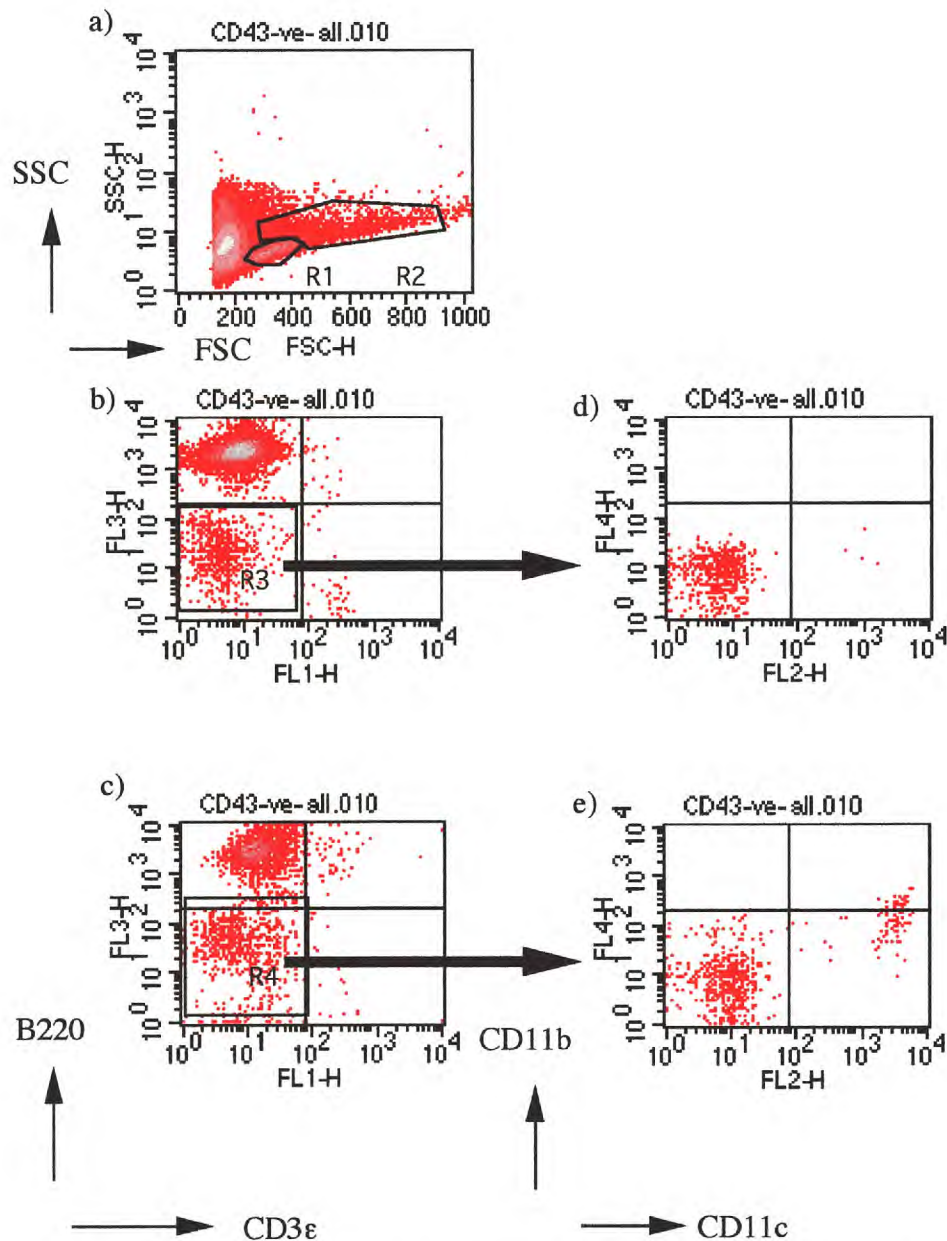


Figure 4.7. Analysis of CD43 negative purification products from NOD-Asp male spleen. Cells were stained with CD3ε-FITC, CD11c-PE, B220-CyChrome and CD11b biotin+ SA-APC. Cells were gated according to a) size and granularity into lymphocytes (R1) and non-lymphocytes (R2). Cells within b) R1 and c) R2 were analysed for B220 and CD3ε expression and showed little contamination with T cells. However a population of double negative cells exists. d) DN cells from within R1 were analysed for CD11b and CD11c expression and expressed neither marker. e) DN cells within the R2 were also examined for CD11b/CD11c and a population of 0.7% of the total gated cells (R1+R2) were found to express CD11c or CD11b. 100000 cells in total were analysed.

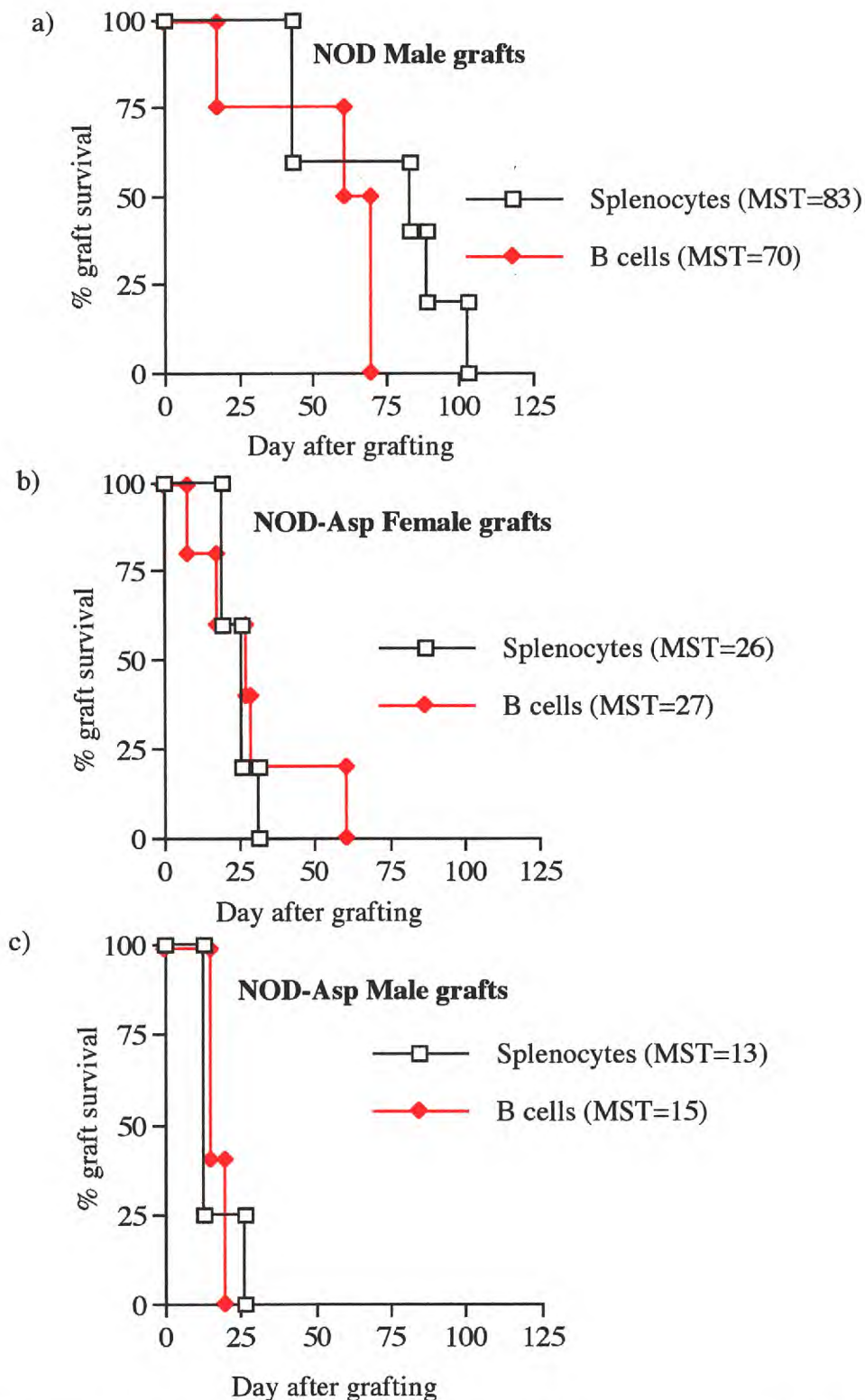
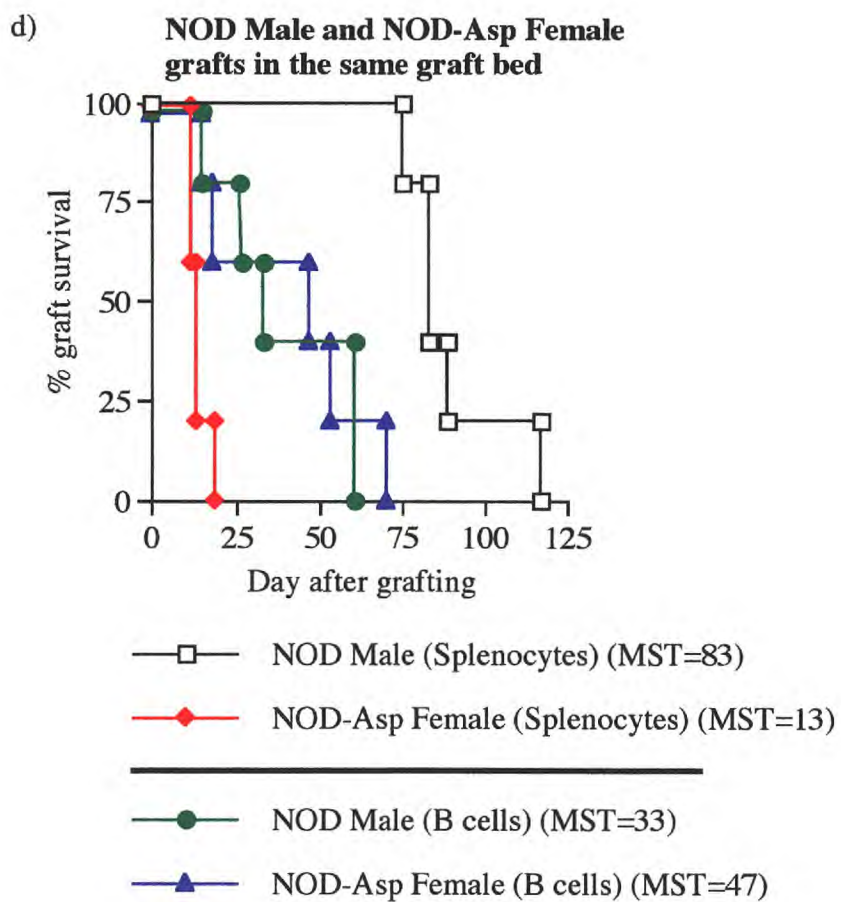


Figure 4.8. Comparison of the effectiveness of splenocytes or purified B cells for inducing tolerance by direct and indirect pathways. NOD female mice were injected with either 10^7 NOD-Asp male splenocytes or twice with 5×10^6 purified NOD-Asp male B cells (CD43 negative) i.v. prior to skin grafting with a) NOD male, b) NOD-Asp female, c) NOD-Asp male or d) NOD male and NOD-Asp female in the same graft bed. MST = Median Survival Time (days).



4.3.2: Purification of naïve B cells

In order to examine whether antigen presentation by B cells can be tolerogenic when graft rejection is mainly through the direct pathway, purified NOD-Asp male B cells were injected into NOD female mice.

CD43 (leukosialin) is a signal transduction/adhesion molecule, expressed on the surface of a number of cell types including plasma cells, macrophages, DCs and T cells (Hardy, Kishimoto et al. 1987; Gulley, Ogata et al. 1988; Hardy, Carmack et al. 1991). However it is not expressed on resting, peripheral B cells, allowing discrimination of resting B cells from other B cell and APC populations (Wells, Kantor et al. 1994). This property allowed enrichment of untouched resting B cells from the spleen of NOD-Asp male mice for injection into NOD-Asp female mice. Figure 4.6 a) shows a FACS plot of NOD-Asp male splenocytes stained with B220-CyChrome (a B cell marker) and CD3ε-FITC (a chain from the CD3 signalling complex expressed on T cells). In this plot, 32.1% of cells are B220 positive, principally resting and activated B cells while nearly 20% are CD3 positive T cells. CD43-conjugated MACS beads were used to magnetically separate splenocytes into CD43 positive and negative fractions, which were stained. Figure 4.6 b) shows the CD43 negative fraction, highly enriched for resting B cells such that now 78.8% of cells stain with B220. A small population of double negative cells remains, although there are less than 1% T cells remaining. In comparison the CD43 positive fraction (Figure 4.6 c) has a small population of 6.5% B220 positive, presumably activated B cells, as well as 77.4% T cells. These data show that this method of purification removes most cells apart from resting B cells.

Curiously, a population of nearly 20% B220/CD3 double negative cells remain in the CD43 negative population (Figure 4.6 b). These were further examined by four colour FACS to determine the identity of the cells. When splenocytes are examined by flow cytometry, they are gated according to forward (FSC) and side (SSC) scatter, measurements of size and granularity respectively. Lymphocytes are generally categorised by size and are contained in gate R1 shown in figure 4.7 a), while DCs and macrophages tend to be larger and more granular and are generally contained in gate R2. CD43 negative splenocytes from the MACS purification were stained with B220-CyChrome, CD3ε-FITC, CD11c-PE and CD11b biotin with streptavidin-APC and gated on R1 and R2 respectively. Figure 4.7 b) shows a CD3ε/B220 plot gated on the R1 lymphocyte gate. When the double negative (DN) cells were examined for CD11c (DC)

and CD11b (Macrophage) expression (Figure 4.7 d), they were negative for both markers. However when gated on the APC gate (R2), the CD3ε/B220 DN population (Figure 4.7 c) were mainly negative for CD11c/CD11b (Figure 4.7 e) but with a small population of CD11c/CD11b positive cells (0.7% of the gated cells R1+R2), which may indicate a contaminating DC/macrophage population. However the largest population is still DN for both APC markers. It is unclear what the CD3ε/B220/CD11c/CD11b-negative population is, although in discussions with others using CD43 MACS for depletion purposes, this appears to be a common occurrence.

4.3.3: Treatment of NOD female mice with resting NOD-Asp male B cells

In mouse spleen, B cells account for approximately 30-50% of the cells and consequently the immunisation protocol was altered to account for this. NOD female mice were injected on two occasions day -14 and day -1 with $3-4 \times 10^6$ purified, resting NOD-Asp male B cells and subsequently grafted one day later as previously described. This induced slightly different graft rejection responses compared to whole male splenocytes. When given NOD male grafts (Figure 4.8 a), the grafts were rejected at a similar rate to the splenocyte treatment (MST of 70 days compared to 83 days). Likewise NOD-Asp female and male grafts were rejected by NOD female mice similarly for B cell and splenocyte treatments respectively, 27 days compared to 26 days MST for NOD-Asp female (Figure 4.8 b) and 15 days compared to 13 days for NOD-Asp male (Figure 4.8 c). These data suggest that B cells are as able to prime a response to both major and minor antigens compared to whole unpurified spleen, when graft rejection occurs both through the direct and indirect pathways. This is contrast to that previously shown with the HY system (Fuchs and Matzinger 1992), showing that immunisation with purified B cells could induce tolerance. However it is possible that the small population of CD11b/CD11c positive cells remaining in the CD43 negative fraction (Figure 4.7 e) are either macrophages or more likely DCs, that are able to prime a naïve response, overcoming the tolerogenic effect of B cell immunisation. Fuchs *et al* (Fuchs and Matzinger 1992) suggested that the presence of contaminating DCs in the B cell preparation could cause priming, inducing an anti-HY response.

Where the two antigens are present on different grafts in the same graft bed, the picture is somewhat different. When whole NOD-Asp male splenocytes were used, NOD male grafts were rejected with MST of 83 days when in the same bed as NOD-

Asp female grafts, while NOD-Asp female grafts were rejected with MST of 13 days (Figure 4.8 d), similar to rejection of a solo graft. While B cell injections induce graft rejection similarly in the absence of other grafts, in the presence of another graft the rejection pattern is altered. NOD male grafts are rejected more quickly with a MST of 33 days (compared to 83 days when whole splenocytes were used) while NOD-Asp female grafts were rejected over a longer time than previously, with a MST of 47 days (compared to 13 days). It is unclear why this should be, although it does suggest that in this experiment, injection of B cells has prolonged survival of the major mismatched graft, while causing quicker rejection of the minor graft compared to priming with spleen cells. This could mean that when antigen presentation is principally via the direct pathway, B cells are less immunogenic, as in the NOD/NOD-Asp rejection, while if both direct and indirect pathways are functional, B cells are more potent at priming the response. These data contrast with Fuchs *et al* (Fuchs and Matzinger 1992) showing that B cells are tolerogenic in a HY-mismatched setting. However this experiment has only been performed once and it is difficult to draw any meaningful conclusions, and the possible contaminating DC population may also be affecting these results. It would be interesting to repeat these experiments using alternative methods of B cell purification, to determine whether in the absence of contamination the results were any different. It would also be interesting to examine the effect of one injection with B cells, compared with one injection of splenocytes. In these experiments, one injection of splenocytes or two of B cells were used prior to grafting, which may have influenced the outcome, particularly with the enhanced rejection of NOD male grafts when B cells were used compared with splenocytes (Figure 4.8 a).

These data show that in the presence of single grafts, purified resting B cells prime for rejection of the Asp and HY mismatched grafts, similar to whole splenocytes. These data suggest that i.v. injected splenocytes and purified B cells, traffic to spleen or lymph nodes where they are capable of priming responses, directly through interaction with recipient T cells and through cross-presentation of antigens by phagocytosis and presentation of donor cells by recipient APCs.

On challenge with grafts containing major and minor mismatches, these data have shown that unsurprisingly, major mismatched grafts are rejected more quickly than minor mismatched grafts (Gould and Auchincloss 1999). Interestingly it has been suggested that the direct pathway is mainly involved in early acute graft rejection while the indirect pathway is involved more in long-term chronic graft rejection and for peripheral tolerance to occur, both pathways will have to be modulated (Womer,

Sayegh et al. 2001). Recent work using HY-specific TCR transgenic mice (Braun, Grandjean et al. 2001) found that naïve CD4⁺ T cells were primed to respond to allografts solely through the indirect pathway. A^b-restricted transgenic TCR were not alloreactive to A^k, while A^k male, but not female skin was rejected by female mice. *In vitro* primed T cells secreted IL-4 and IFN-γ on stimulation by A^k male cells while if APCs were depleted from the responder population, this no longer occurred. Conclusions drawn from this work were that the graft rejection observed in these mice was due to direct recognition, but through shedding of HY antigens from the male graft and subsequent presentation by recipient APCs.

It is clear from these data that when both pathways are functioning simultaneously, graft rejection is rapid as seen when both the major (NOD-Asp) and minor (HY) differences are expressed in the same graft. Presumably when antigens are expressed on different grafts as in the double graft experiments, despite prior co-immunisation, responses to the antigens are independent. This implies that despite both antigens being present in the immunising cells and then in the same mouse (on the double grafts) they are maintained as distinct antigens and both grafts are rejected at different rates. This suggests that possibly different populations of APCs are responsible for presentation/recognition of the two antigens and shows the degree of control, regulation and specificity that is maintained in rejection responses.

4.4: Summary

These experiments have enabled examination of two aspects of antigen presentation in NOD mice. Initially it appeared as though a novel method of MHC class II processing and presentation had been discovered. Early work with NOD-Asp restricted T cell clones showed that while both clones proliferated specifically in response to NOD-Asp but not B-Asp or NOD splenocytes. If B-Asp and NOD splenocytes were co-cultured prior to use, they stimulated proliferation of both clones, although less efficiently than normal stimulation. This suggests that if an alternative method of presentation occurred it was less efficient than normal. This process was shown to be mainly dependent on cell-cell contact with almost complete abrogation of a response when co-cultured in trans-wells. Vesicular transport/transfer was initially investigated as a potential mechanism although the work was not continued. Co-culturing unrelated splenocytes induced proliferation in a non-specific manner suggesting alloreactivity between the co-cultured splenocytes, with subsequent

production of IL-2, was leading to non-specific responses. However these experiments were not repeated, and as responses were detected of the clones to unrelated splenocytes in the absence of either MHC chain this would need to be further investigated, depleting the splenocytes of T cells prior to co-culture.

Secondly, NOD mice were examined for graft rejection in response to major (NOD-Asp) and minor (HY) mismatched grafts after injection with splenocytes or purified B cells containing both antigen types. This allowed examination of the tolerogenic effects of B cells on the direct pathway of rejection, with NOD female mice rejecting NOD-Asp male grafts through the direct pathway only and NOD male grafts through both direct and indirect pathways. These data suggest that both splenocytes and resting B cells are able to prime graft rejection responses to a similar extent, although contaminating DCs may have played a role. Presumably both splenocytes and B cells are indirectly presented by recipient APCs as well as inducing alloreactive T cell responses *in vivo* prior to grafting. On grafting, direct presentation allows rapid graft rejection of NOD-Asp male grafts by NOD female mice while NOD-Asp female grafts are rejected slightly slower. In HY responses, rejection is slower as consistent with the difference between major and minor mismatched antigens. However it is clear that NOD females can be immunised to respond to male antigens by both splenocytes and B cells and this is quicker than the previously shown unimmunised response.

Chapter 5.

Effects of immunosuppressive treatment on dendritic cell function and development

5.1: Background

Glucocorticoids are commonly used immunosuppressive steroids, for treatment of inflammatory and autoimmune conditions. Glucocorticoids such as the artificial steroid dexamethasone and its derivatives exhibit many effects on different cell types. Dexamethasone has been extensively used to induce apoptosis in immature thymocytes, as well as being thought to influence dendritic cell maturation and development. Dendritic cells are the most potent antigen-presenting cell type in the immune system, uniquely able to prime naïve T cells and consequently play a central role in the regulation of T cell function *in vivo*.

A number of immunomodulatory compounds influence DC development, maturation and activation *in vitro* and *in vivo*. Treatments such as DNA containing bacterial CpG motifs, bacterial lipopolysaccharide and monoclonal CD40 antibody treatment are commonly used to induce DC maturation/activation. Other compounds including aspirin (Hackstein, Morelli et al. 2001), the immunosuppressive cytokine IL-10 (Steinbrink, Wolfl et al. 1997; Steinbrink, Jonuleit et al. 1999), vitamin D₃ (Piemonti, Monti et al. 2000; Gregori, Casorati et al. 2001; Griffin, Lutz et al. 2001) and dexamethasone (DEX) (Moser, De Smedt et al. 1995; Piemonti, Monti et al. 1999; Piemonti, Monti et al. 1999; Matyszak, Citterio et al. 2000; Woltman, de Fijter et al. 2000; Griffin, Lutz et al. 2001) have been shown to inhibit DC function, mainly by inhibiting maturation. This work has been performed using both human and murine DC cultures. Treated DCs were less capable of stimulating T cell proliferation *in vitro* and *in vivo*. A number of groups have shown that treatment with immature DCs can induce transplantation tolerance, particularly Steinbrink *et al* (Steinbrink, Wolfl et al. 1997) who used IL-10 treated DCs to induce T cell anergy.

Here, I have used murine BMDCs to examine the effects of dexamethasone treatment in the HY minor transplantation model system, first using HY-peptide specific T cell clones, and secondly *in vivo* using skin grafts as a readout.

5.2: Comparison of different immunomodulatory treatments on BMDC function *in vitro*

As previously described, many compounds have been suggested to have activating or inhibitory effects on DCs. Decreased stimulatory capacity is commonly correlated with reduced costimulatory molecule and MHC expression at the DC surface, induced or inhibited by treatment.

The HY-specific T cell clones B9 and CTL-10, were used in this study. B9 is CD4⁺, restricted by H2-A^b and recognises the peptide NAGFNSNRANSSRSS encoded the *Dby* gene on the Y-chromosome (Scott, Addey et al. 2000), while CTL-10 is CD8⁺, H2-D^b restricted and recognises the peptide WMHHNMDLI encoded by the *Uty* gene (Greenfield, Scott et al. 1996). They provide a useful model for examining the effects of various treatments on DC function and subsequent T cell responses.

Murine bone marrow (BM) cells were cultured for 7 days in the presence of GM-CSF to promote differentiation of DCs and were treated with different doses of IL-10, DEX, LPS and combinations of each to determine their inhibitory effects *in vitro*. C57BL/6 female BMDCs were treated on days 5-7, 6-7 or 5-8, washed, pulsed with the appropriate peptide, irradiated and used as APCs in proliferation assays (only LPS data shown). While modest inhibition of proliferation was observed using DEX or IL-10 treatment from day 5-7, the effect observed was minimal. Surprisingly, in these experiments, LPS appeared to have little positive effect. BM was cultured from day 6 with doses of LPS added for either 24 or 48 hours before assay. With 48 hours treatment, most BMDCs in the culture had died in a dose-dependent manner as assessed by Eosin staining. While treatment with LPS for 24 hours did not cause as much death as 48 hours, the cells were not consistently able to stimulate to a greater extent than untreated DCs. This was surprising, as many groups have used LPS to activate DCs, enhancing immune responses. Following discussion with other groups it was apparent that LPS induced DC death is not unknown. Gallucci *et al* (Gallucci, Graham et al. 2001) found that while BMDCs cultured for 24 hours appeared activated, treatment for 48-72 hours induced vast cell death.

Figure 5.1 shows a representative proliferation assay using the CTL-10 clone as responder cells. B6 female BMDCs were cultured for 7 days, then different doses (1.7µg-0.0001ng/ml) of LPS were added for 24 hours prior to use as APCs. Controls in this assay include untreated female DCs in the absence of peptide and peptide pulsed,

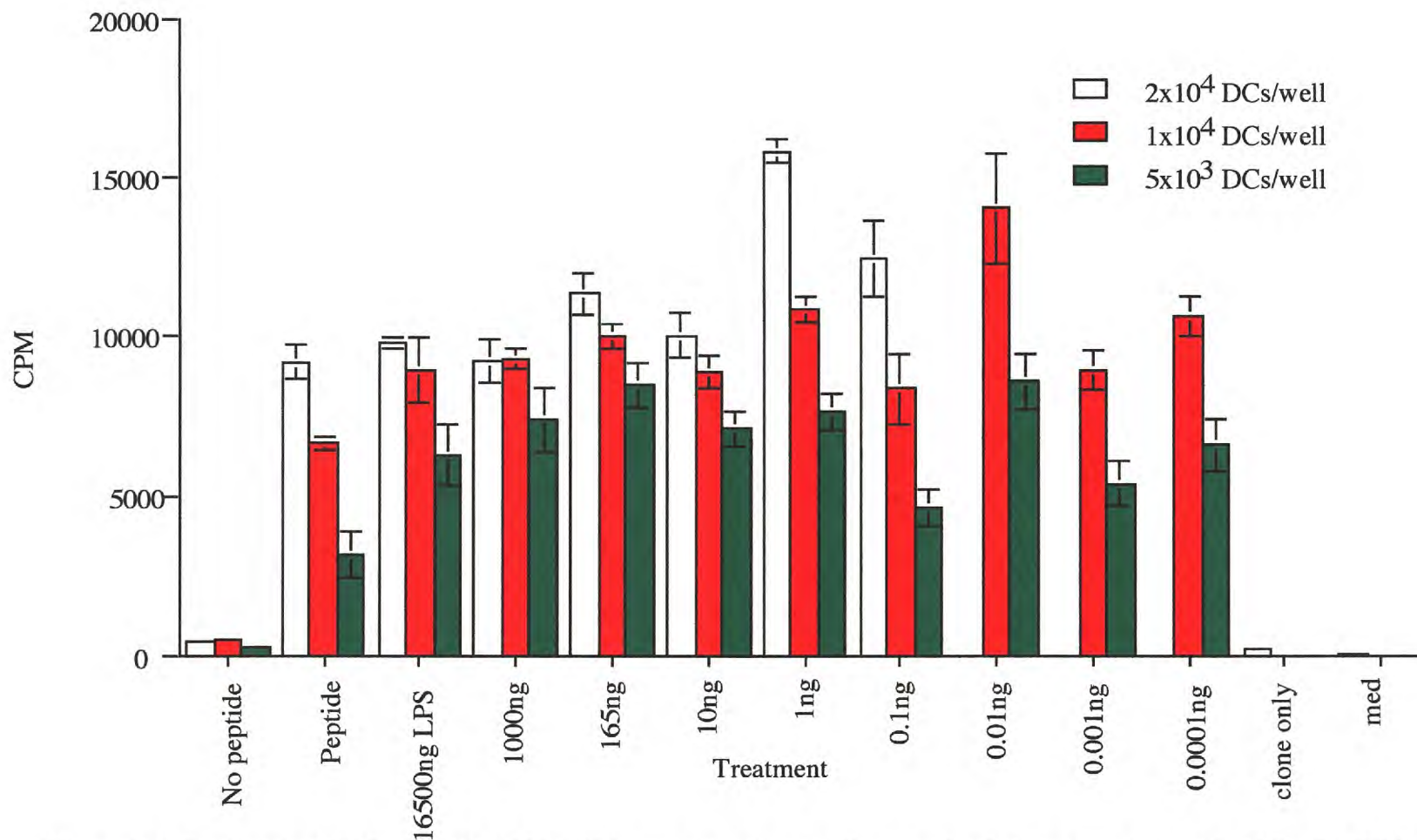


Figure 5.1. Treatment of BMDCs with LPS for 24 hours prior to use enhances stimulatory capacity for the CTL-10 CD8⁺ T cell clone to a limited extent. Female BMDCs were cultured for 7 days, then LPS was added overnight. DCs were washed, pulsed with peptide and used in a proliferation assay to stimulate the CTL-10 clone.

untreated DCs. Although high doses of LPS do not enhance DC function above controls, doses from 0.01-1ng/ml appear to enhance function of the DCs. However this effect is not consistent. LPS is generally used at doses of up to 10µg/ml, however this dose was found to give variable results (data not shown). Common methods for generating murine BMDCs involve culturing bone marrow for 7 days in the presence of recombinant GM-CSF and sometimes IL-4, while the method described in chapter 2 uses unpurified GM-CSF supernatant produced from a hybridoma (a kind gift from Dr. D. Gray) transfected with the murine GM-CSF gene. It is possible that the lack of reproducible responses to LPS may be an inherent characteristic of the method of DC generation. Variation in the preparation of LPS is unlikely to be the explanation for the inconsistency, as a number of different sources of LPS have been tested. Recent work described decreased expression of costimulatory molecules on the surface of BMDCs cultured with apoptotic cells. The BMDCs were cultured using the same GM-CSF supernatant described above and IL-10 was detected in the culture supernatant (Stuart, Lucas et al. 2002). If IL-10 is also secreted in the supernatant, this could explain the lack of responses to LPS compared with DCs cultured in the presence of recombinant GM-CSF/IL-4.

Low doses of IL-10 or dexamethasone did not significantly affect DC function when added late in maturation (data not shown). However high doses (10^{-7} - 10^{-6} M) DEX gave modest suppression of function. Figure 5.2 shows a representative proliferation assay using (a) B9 and (b) CTL-10 as responder cells and B6 female DCs pulsed with the *Dby* and *Uty* peptides as APCs. DCs were cultured for 6 days with GM-CSF and dexamethasone added from day 6-7. In order to minimise potential effects of dexamethasone on the T cell clones, DCs were washed three times before pulsing with peptides, incubated for 4-5 hours and then washed twice more before use in the assay. Figure 5.2 shows that in the absence of peptides neither clone is stimulated, while on addition of appropriate peptides both clones proliferate. In this experiment the same population of DCs was treated as described, pulsed with both peptides and used as APCs for both clones.

Comparing figure 5.2 a) and b) it can be seen that treatment with LPS overnight from day 6 to 7 before use in the assay marginally enhances proliferation of the CD8⁺ CTL-10 clone while it has no effect on the CD4⁺ B9 clone. However as previously described, this effect of LPS is not consistent from assay to assay. DC treatment with 10^{-6} and 10^{-7} M dexamethasone from day 6-7 only partially inhibits stimulation of B9

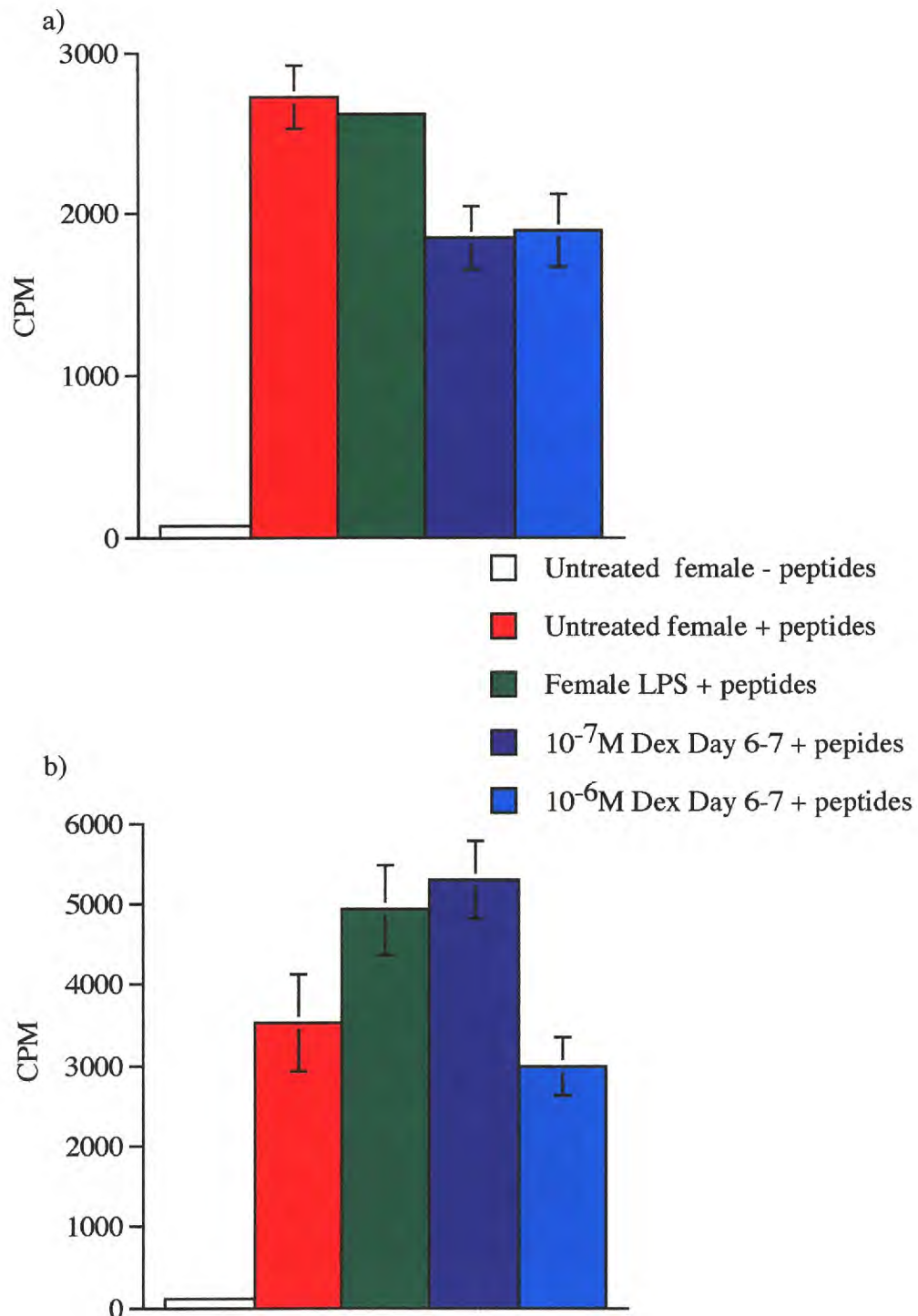


Figure 5.2. a) B9, b) CTL-10. Treatment of C57BL/6 female BMDCs with 10^{-6} or 10^{-7} M dexamethasone from day 6 of culture does not prevent stimulation of CD4⁺ (B9) or CD8⁺ (CTL-10) clones as much as from day 0. Female BMDCs were cultured for 6 days, before LPS or dexamethasone was added and recultured overnight. Cells were harvested, pulsed with A^b-Dby and D^b-Uty peptides prior to washing, irradiation and use as APCs in a proliferation assay.

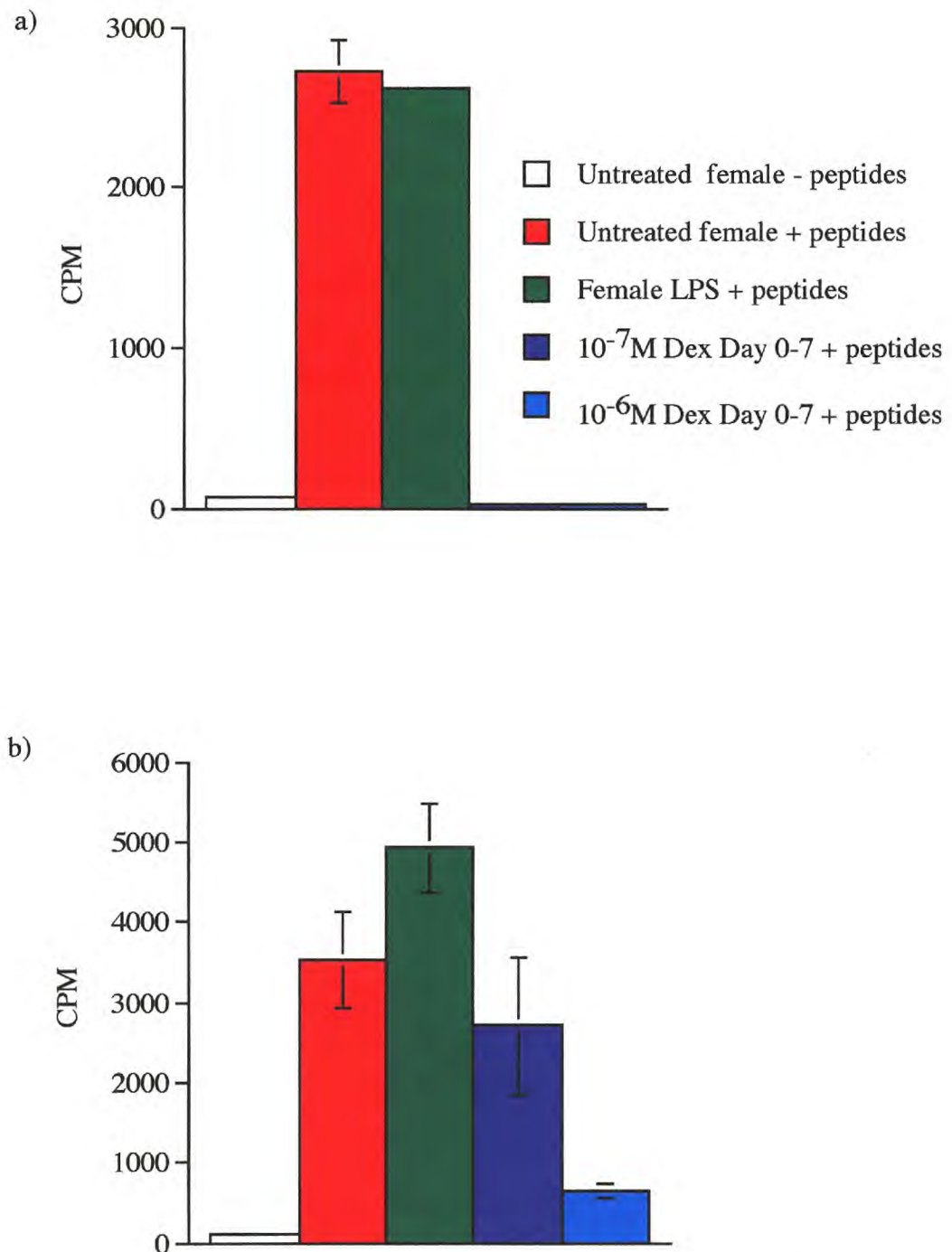


Figure 5.3. a) B9 (CD4⁺) and b) CTL-10 (CD8⁺). Treatment of C57BL/6 female BMDCs with 10^{-6} or 10^{-7} M dexamethasone from day 0 of culture prevents stimulation of CD4 but not CD8 T cell clones. BMDCs were cultured +/- DEX from day 0. Untreated DCs were either left untreated or 10 μ g LPS was added on day 6-7. Cells were harvested and pulsed with A^b-Dby and D^b-Uty peptides for 3-4 hours then used in a proliferation assay to stimulate T cell clones.

but not CTL-10. 10^{-6} M dexamethasone has little effect on inhibiting CTL-10 stimulation by DCs while 10^{-7} M appears to enhance stimulation of CTL-10.

Treatment with 10^{-6} or 10^{-7} M dexamethasone from day 0 through to day 7 significantly inhibits proliferation of the B9 CD4⁺ clone (Figure 5.3 a), while less of an effect is observed on the CTL-10 CD8⁺ clone (Figure 5.3 b). Both 10^{-6} and 10^{-7} M dexamethasone decrease the stimulation of B9 to below the negative control. In contrast, 10^{-7} M dexamethasone partially inhibits stimulation by DCs of CTL-10, while less proliferation is observed at 10^{-6} M dexamethasone. This is interesting as it suggests that dexamethasone may be preferentially reducing the stimulatory capacity of DCs from activating CD4⁺ T cells. These data imply that dexamethasone acts early in DC differentiation, blocking complete maturation. Another possibility is that high concentrations of dexamethasone may be toxic to DCs. Observation of treated and untreated DC cultures suggests that while dexamethasone may inhibit proliferation and growth of DCs in culture, it does not appear to be toxic. During the 7-day culture, nutrient components are utilised and the media turns yellow due to a pH change, while with high-dose dexamethasone this does not occur. When examined under the microscope the morphology of the DCs differs, with untreated DCs appearing larger with 'thicker' membranes due to membrane ruffles characteristic of DCs. DEXDCs appear smaller with fewer ruffles although the yield of cells between treatments does not appear to differ significantly. These findings are consistent with previous observations that dexamethasone appears to act as a block to DC development, preventing complete maturation *in vitro* and *in vivo*. A number of different models have used DC cell lines, *in vitro* cultured DCs and examination of effects *in vivo* (Kitajima, Ariizumi et al. 1996; Piemonti, Monti et al. 1999; Sacedon, Vicente et al. 1999; Matyszak, Citterio et al. 2000; Woltman, de Fijter et al. 2000; Griffin, Lutz et al. 2001). The mechanism of immune inhibition by dexamethasone has been examined, and TCR signalling (Van Laethem, Baus et al. 2001), production of IL-2 (Bianchi, Meng et al. 2000) and GM-CSF are reduced, consequently inhibiting DC maturation (Smith, Cousins et al. 2001). DEXDCs have decreased expression of MHC class I and II, as well as costimulatory molecules (Moser, De Smedt et al. 1995; Piemonti, Monti et al. 1999). Human DEXDCs show increased uptake of two different model antigens by phagocytosis and pinocytosis (Piemonti, Monti et al. 1999), again suggesting that the DCs are immature. Immature DCs *in vivo* are efficient at uptake of antigen, while after maturation and migration to lymph nodes they upregulate molecules involved in antigen presentation and costimulation and downregulate antigen uptake. However it has been

suggested that glucocorticoid treatment may inhibit uptake and possibly processing of antigens but not presentation, with airway-derived DCs (Holt and Thomas 1997).

If dexamethasone is able to inhibit maturation of DCs, this could be useful in inducing tolerance to transplantation antigens as has previously been described. A number of different mechanisms have been proposed for how treatment with immature DCs can induce peripheral tolerance. Matyszak *et al* (Matyszak, Citterio et al. 2000) found that immature DCs did not induce an efficient immune response through the relative decrease in antigen presentation/costimulation capacity, and that they were unable to prime Th1 cells specifically, leading to decreased numbers of TNF- α producing cells. With repeated restimulation, a population of what were described as Tr1 regulatory T cells producing IL-10 were generated. Interestingly the DCs described were unable to mature when treated with maturation stimuli. Other work used DEX-treated thymic DCs to stimulate MLR, which showed decreased secretion of IL-1 β and TNF- α in cultures (Sacedon, Vicente et al. 1999). Others used CD40L to activate DCs in the presence of dexamethasone and shown that after activation, the DCs could no longer secrete IL-12 (involved in inflammation) while IL-10 was upregulated (Rea, van Kooten et al. 2000). Both IL-10 and D $_3$ are thought to inhibit DC development in a similar manner, and use of treated, immature DCs is thought to induce tolerance or non-responsiveness either through inducing a population of CD4⁺ T cells that secrete IL-10 (Jonuleit, Schmitt et al. 2000) or through inducing anergic T cells (Steinbrink, Wolfi et al. 1997). Dhodapkar *et al* (Dhodapkar, Steinman et al. 2001) showed that immature DCs inhibited effector T cell function in an antigen-specific manner in humans. It has also been suggested that while IL-10 can inhibit DC maturation, it also acts in a feedback loop such that immature DCs secrete IL-10 thus inhibiting further DC maturation (Corinti, Albanesi et al. 2001).

5.3: Treatment with dexamethasone only partially prevents subsequent activation with LPS

In order for such immature DCs to be useful therapeutic reagents it may be important that maturation is terminally inhibited, such that after removal of the inhibitory agent used *in vitro*, the DCs can no longer differentiate. A number of groups have shown that treatment with such inhibitors renders DCs incapable of responding to maturation stimuli such as CD40L or LPS (Matyszak, Citterio et al. 2000; Griffin, Lutz et al. 2001). In order to examine this, B6 female DCs were cultured for 6 days in the

presence of 10^{-6} or 10^{-7} M dexamethasone, washed thoroughly and recultured from day 6-7 with GM-CSF and 10 μ g/ml LPS. Figure 5.4 shows representative proliferation assays of a) B9 and b) CTL-10. In this figure, DCs were used at three different concentrations from 1×10^4 /well to 2×10^3 /well and it is clear that treatment with LPS has enhanced proliferation of both clones above untreated DCs with peptide. As previously shown in figure 5.3, there is no proliferation of B9 when stimulated with DCs treated with either 10^{-7} or 10^{-6} M dexamethasone, while CTL-10 shows decreased but incomplete inhibition of stimulation. When the DCs were recultured in the absence of dexamethasone but with LPS, there was still no proliferation observed in the B9 clone but CTL-10 proliferates at an intermediate level between treatment with dexamethasone from days 0-7 and the untreated control. This suggests that unlike previous work with MLR, dexamethasone treatment only partially prevents activation with LPS once the dexamethasone has been removed, allowing partial recovery of responses from CD8⁺ but not CD4⁺ T cells.

Figure 5.5 a)-h) shows FACS analysis of the dendritic cells used as APCs in the assay shown in figure 5.4. Cells were stained with a) CD11c-PE together with b) CD80-FITC, c) CD40-FITC, d) MHC class II-biotin + SA-APC, e) Annexin V-FITC, f) CD86-biotin + SA-APC, g) MHC class I-FITC or h) CD11b-FITC. Untreated cells have a normal profile of CD11c expression together with CD80^{lo}, CD40⁻, broad MHC class II expression, CD86^{lo}, some MHC class I and are CD11b^{lo}. In comparison, LPS treatment as expected shows some upregulation of CD80/86 as well as MHC class II. Both 10^{-6} M and 10^{-7} M dexamethasone treatments reduce CD11c, CD86 and MHC class II expression to almost background, while CD80 is partially downregulated and MHC class I/CD11b are little affected. This is consistent with the absence of stimulation of the B9 CD4⁺ clone and the partial decrease in stimulation of the CTL-10 CD8⁺ clone shown in figures 5.3 and 5.4. Interestingly treatment with LPS after removing dexamethasone has little effect on MHC class II or CD86 expression, while a slight shift in CD11c and a moderate increase in CD80 expression compared to the dexamethasone treated cells is observed.

Using Annexin V as a marker for apoptosis, figure 5.5 e) examines the levels of phosphatidyl serine on the surface of the cells. Flipping of phosphatidyl serine to the outer surface of the plasma membrane and subsequent loss in membrane asymmetry is an early event in apoptosis and is used to determine the degree of apoptosis in these cultures. Untreated DCs show a bimodal distribution with a proportion of the cells Annexin V low and a smaller proportion with slightly higher staining indicating

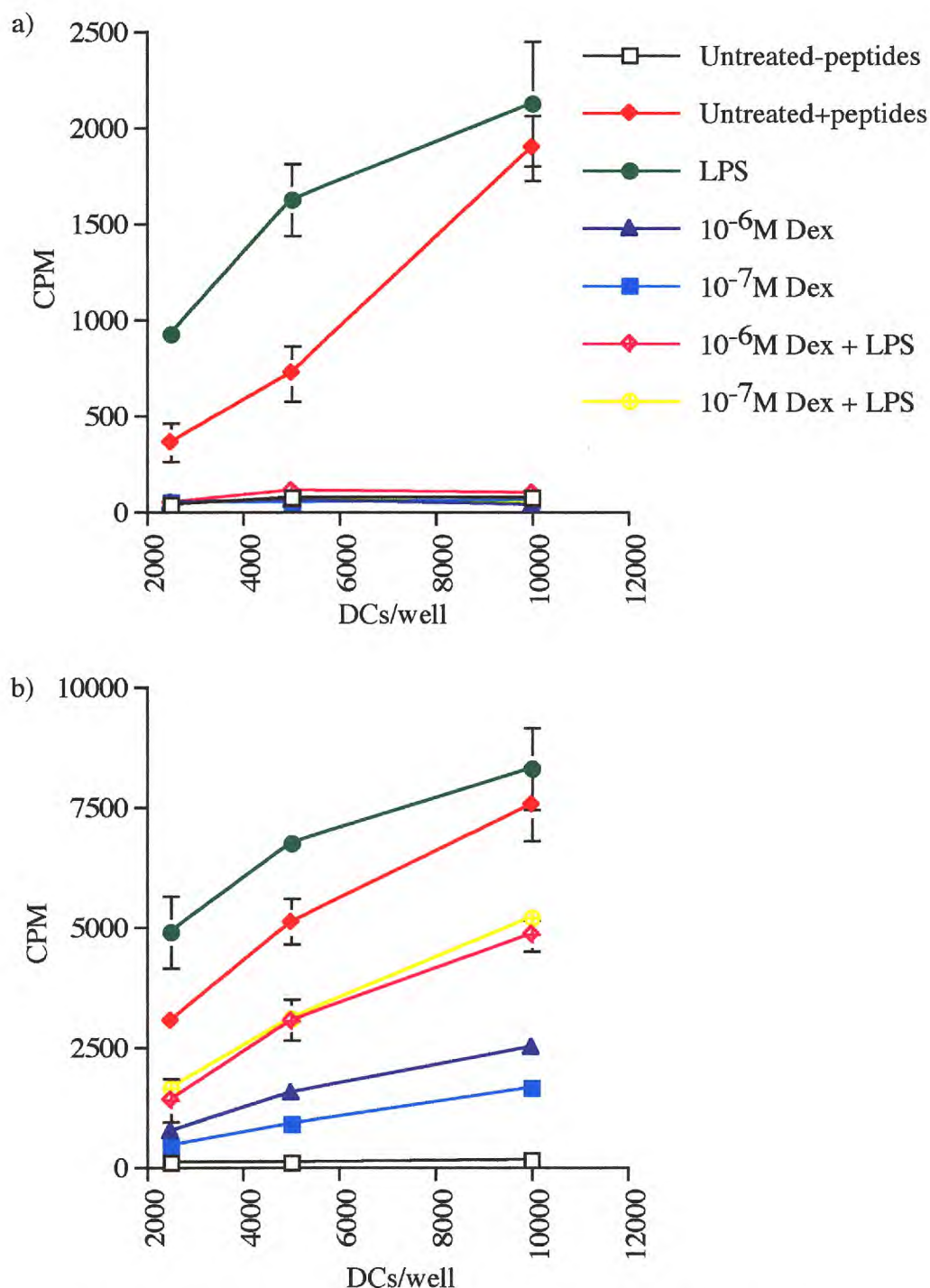


Figure 5.4. a) B9, b) CTL-10. Treatment of DEXDCs from day 6-7 with LPS does not overcome the non-stimulation of CD4⁺ clones. C57BL/6 female BMDCs were cultured for 6 days in the presence of 10⁻⁷ or 10⁻⁶M dexamethasone. After 6 days they were harvested, washed twice and recultured in the presence of 10µg/ml LPS overnight. They were harvested, pulsed with peptides, washed and irradiated, then used as APCs at three different doses to stimulate B9 (CD4⁺) and CTL-10 (CD8⁺) T cells clones.

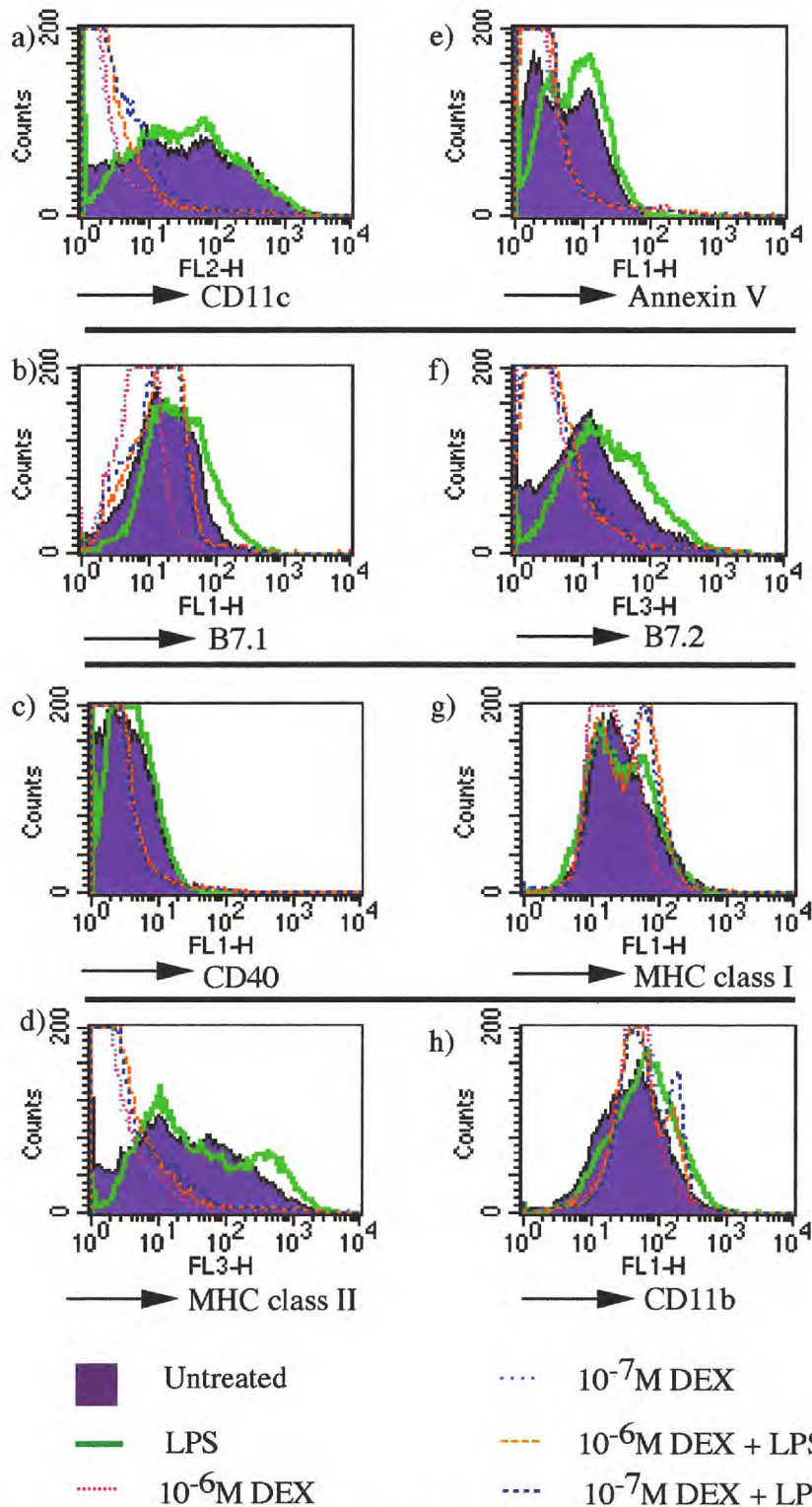


Figure 5.5. C57BL/6 female BMDCs were cultured for 6 days $\pm$ 10^{-6} or 10^{-7} M dexamethasone. They were washed twice, plated out and cultured overnight $\pm$ $10 \mu\text{g/ml}$ LPS. Cells were washed and analysed by flow cytometry for various markers: a) CD11c, b) B7.1 (CD80), c) CD40, d) MHC class II, e) Annexin V, f) B7.2 (CD86), g) MHC class I and h) CD11b. Cells treated with dexamethasone show decreased expression of CD11c, B7.2 and MHC class II and less significant decrease in B7.1 and MHC class I. These markers are not restored to untreated levels by addition of LPS for 24 hours.

apoptosis. This suggests that even in untreated cells a proportion undergo apoptosis *in vitro*. Treatment with LPS for 24 hours kills some of the DCs, with an increase in the proportion of Annexin V high cells and a slight increase in the staining of Annexin V low cells. However treatment of DCs with dexamethasone, or dexamethasone and then LPS appears to show decreased Annexin V staining and thus less apoptosis.

These data are partially consistent with that shown previously, that treatment with dexamethasone inhibits DC maturation *in vitro*, downregulating expression of molecules involved in antigen presentation and costimulation, as well as explaining the absence of CD4⁺ T cell responses while CD8⁺ T cell responses are only partially affected. Interestingly it can be seen in figure 5.5 a) that CD11c expression is almost completely ablated with dexamethasone treatment, suggesting that DC maturation is inhibited before CD11c upregulation, while CD11b (expressed at low levels on BMDCs) is not affected. The lack of CD11c expression on DEXDCs suggests that the cultures are not differentiating from BM into DCs at all.

5.4: Treatment with dexamethasone does not permanently inhibit DC function

While treatment with LPS is partially able to restore T cell responses and some markers associated with antigen presentation after one day in the absence of dexamethasone, it is interesting to investigate whether removal of dexamethasone allows complete maturation over a longer period of time. In order to study this, B6 female BM was cultured for 7 days in the presence of dexamethasone, washed thoroughly as before and returned to culture in new 6 well plates with GM-CSF for a further 7 days. At the same time more female DCs were set up with or without dexamethasone as controls.

Figure 5.6 a) and b) shows a representative proliferation assay using the DCs described above as APCs stimulating B9 and CTL-10 clones respectively. Again it is clear that day 0-7 10^{-6} and 10^{-7} M dexamethasone-treated DCs do not stimulate B9, while they only partially stimulate CTL-10 when compared with untreated DCs. Interestingly when 7 day DEXDCs were washed and recultured for a further 7 days with GM-CSF and used as APCs, they show enhanced stimulation compared to the untreated DCs. The DCs treated with 10^{-7} M dexamethasone and recultured show the highest stimulatory capacity, followed by those treated with 10^{-6} M. In previous work using cultured DCs this was not examined (Moser, De Smedt et al. 1995; Piemonti, Monti et

al. 1999). These data suggest that dexamethasone may act as a temporary inhibitor of maturation, but on removal the DCs are fully able to undergo maturation. An alternative explanation for the enhanced stimulatory capacity of the recultured cells is that through reculturing, the most 'potent' or longest surviving DCs are selected while other less robust DCs do not survive. It is possible that if this positive selection process is occurring, the DCs will be more immunogenic, stimulating clones more efficiently than normal DC cultures containing a mixture of DCs with different stimulatory capabilities.

When the treated DCs were examined by FACS analysis as before, figure 5.7 a)-g) shows that the profiles differ somewhat from those observed in figure 5.5 a)-h). The expression of CD11c (Figure 5.7 a) on the cultured, untreated DCs is similar to that observed previously in figure 5.5 a) with a broad spread of expression levels. Again on treatment with dexamethasone from day 0-7, CD11c is not expressed. In comparison, if DEXDCs are recultured for 7 days in the absence of dexamethasone, they show a distinct profile of increased CD11c expression, higher than DEXDCs and with a narrower distribution than untreated cells. CD80 b) and CD86 e) show lower expression on DEXDCs compared to untreated controls as before, while recultured DCs show an increase in CD86 and a small increase in CD80 expression. CD40 expression shows slight upregulation on recultured DCs compared to both untreated and DEXDCs. It is commonly observed in BMDCs cultured in this manner that CD40 expression is low (James 2001) and it is interesting that a shift in expression is observed in recultured DCs. Comparing levels of MHC class II expression (d), again there is a broad spread of class II expression when untreated, and when treated with dexamethasone for 0-7 days, expression is ablated as before. However when the DCs are recultured for a further 7 days in the absence of dexamethasone expression of MHC class II is upregulated. This is not surprising because of the increased stimulation of the CD4⁺ clone observed in figure 5.6 a). MHC class I expression on untreated DCs (Figure 5.7 f) shows a narrow range, similar to that seen in figure 5.5 g), while treatment with dexamethasone reduces surface expression of class I. Surprisingly this is not restored to untreated levels by reculturing for 7 days in the absence of dexamethasone as may be expected by the enhanced stimulation of the CD8⁺ clone observed in figure 5.6 b). Finally when comparing the levels of expression of CD11b (Figure 5.7 g), untreated cultures have a higher level of expression than in previous experiments, although this is not unknown (James 2001). Treatment of dexamethasone reduces expression of CD11b marginally, while reculturing for a further 7 days in the absence of dexamethasone promotes enhanced expression of CD11b compared with untreated controls.

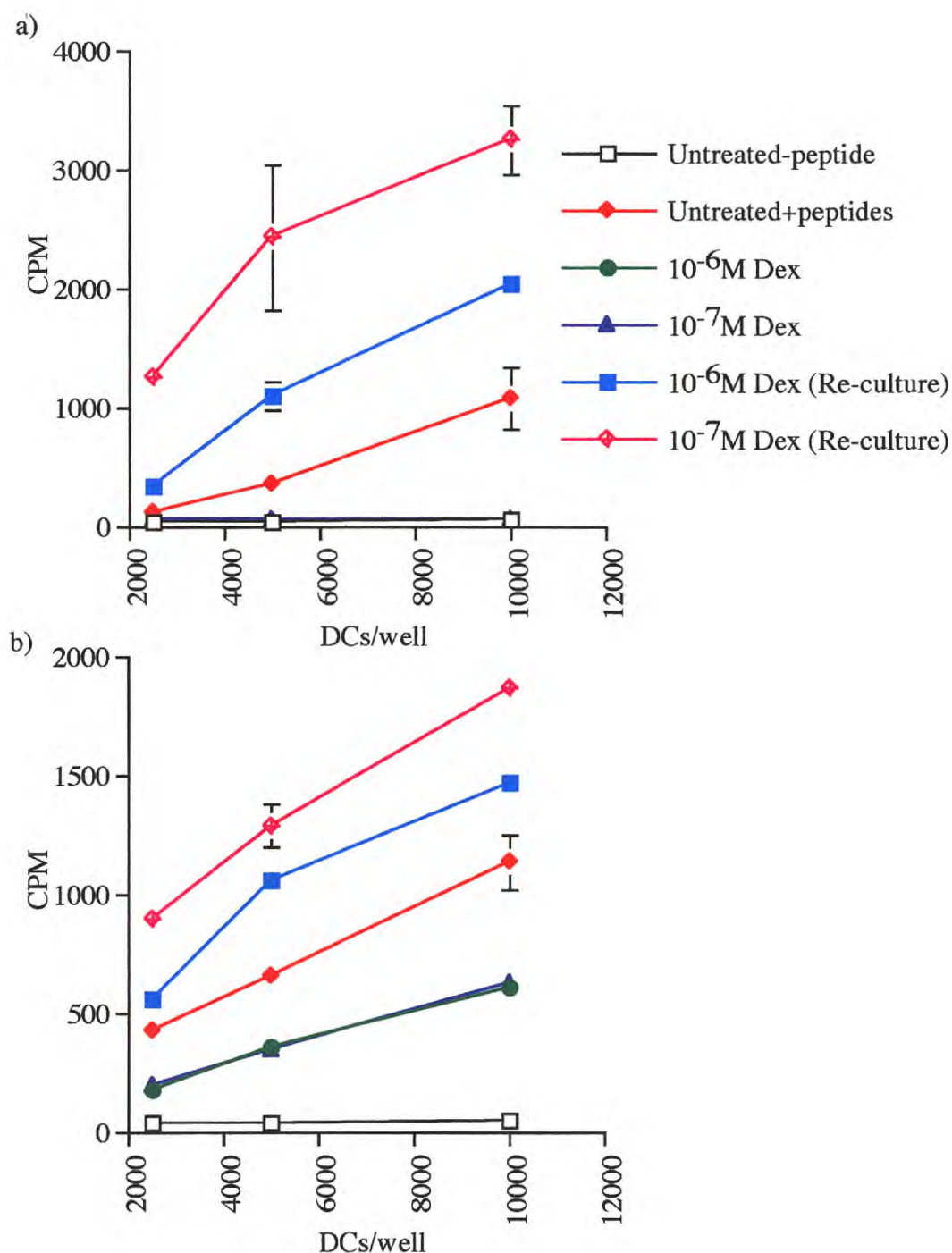


Figure 5.6. a) B9, b) CTL-10. Re-culturing DEXBMDCs for a further 7 days in the absence of dexamethasone restores their stimulatory capacity for CD4⁺ and CD8⁺ T cell clones B9 (CD4) and CTL-10 (CD8). Female BMDCs were cultured with 10^{-6} or 10^{-7} M dexamethasone for 7 days, harvested and washed twice, then plated out and recultured for a further 7 days in RPMI + 5% GM-CSF as normal. Cells were then harvested, pulsed with peptides, washed and irradiated, then used as APCs in a proliferation assay as described.

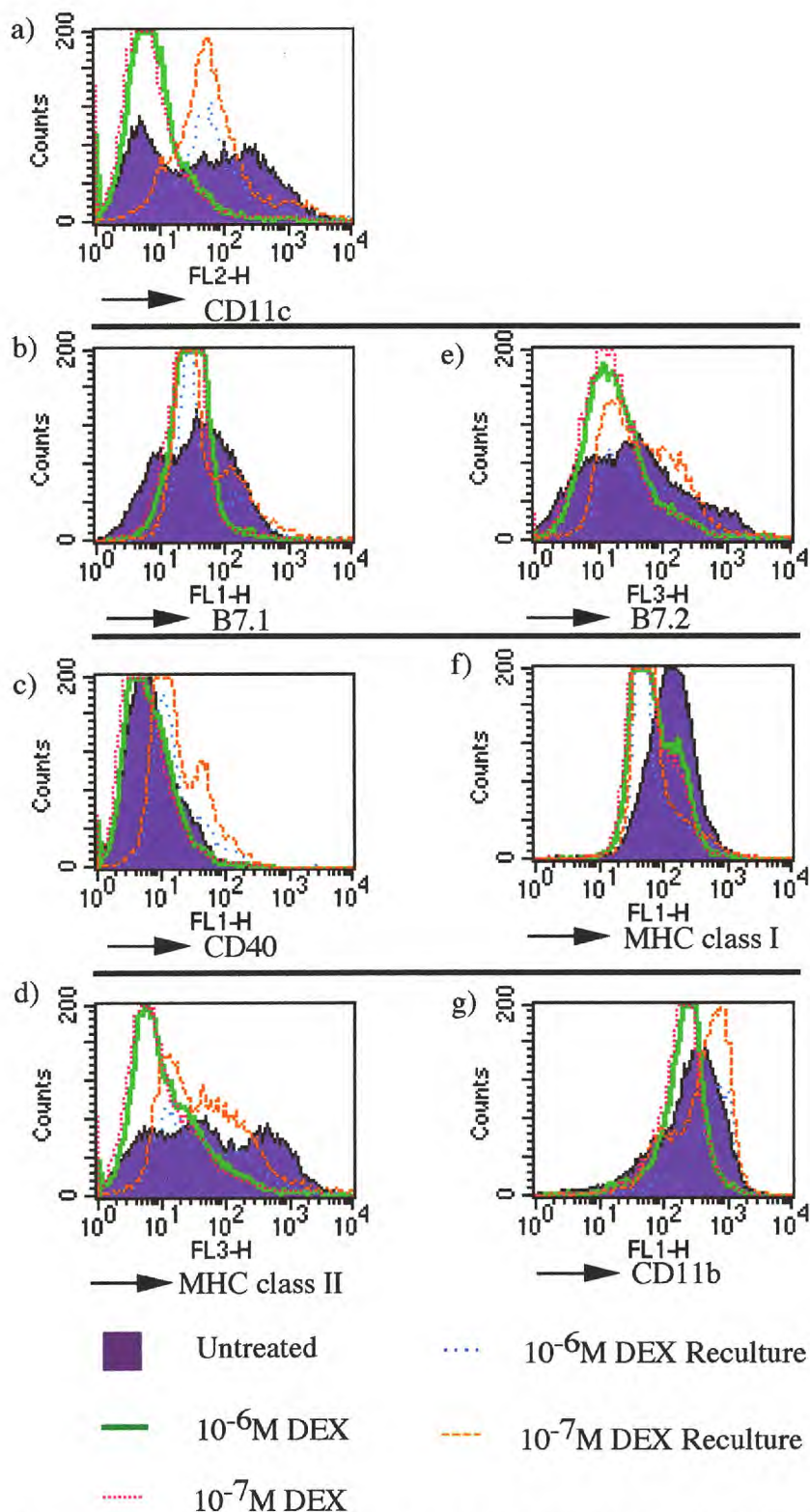


Figure 5.7. Flow cytometry profiles of C57BL/6 female BMDCs cultured for 7 days +/- 10⁻⁶ or 10⁻⁷M dexamethasone, then washed and recultured for a further 7 days in RPMI + 5% GM-CSF as before. These cells have regained their ability to stimulate both CD4 and CD8 T cell clones to a greater extent than untreated day 7 BMDCs. Cells are stained as follows: a) CD11c, b) CD80, c) CD40, d) MHC class II, e) CD86, f) MHC class I and g) CD11b.

It is clear that dexamethasone in DC cultures appears to inhibit differentiation from bone marrow-derived precursors early in development producing a population of immature DCs or DC precursors that are unable to stimulate T cell clones, particularly CD4⁺ T cell clones as efficiently as untreated DCs. Interestingly, dexamethasone treatment appears to inhibit stimulation more of a CD4⁺ clone than CD8⁺, and this has not been previously reported. In previous work, immature DEXDCs are commonly examined *in vitro* as APCs in MLR containing both CD4⁺ and CD8⁺ T cells, and shown to be unable to efficiently stimulate responses. The lack of response of the B9 clone may be explained by the almost complete absence of MHC class II expression on DEXDCs in comparison to untreated controls, while MHC class I shows less of an effect, confirmed by decreased, but not completely abrogated response of the CTL-10 clone. The limited expression of MHC class II means that although pulsed with *Dby* peptide, presentation is poor. This method of presentation does not allow examination of the phagocytic and antigen processing capacity of the DCs, as the peptide is pre-processed and does not need to enter into the phagocytic and degradative pathway in order to be presented.

It is unclear whether the cells that develop are 'true' DCs as they express slightly lower levels of CD11c than untreated DCs (although significantly higher than DEXDCs) and slightly higher CD11b expression than both untreated DCs and DEXDCs. It is possible that DEXDC treatment, subsequent washing and reculturing with GM-CSF allow a macrophage-like DC population to develop that are capable of stimulating both clones to a greater extent than observed with untreated BMDCs. It is perhaps not surprising that the proliferation is greater in recultured DCs than untreated DCs, as it has been shown that manipulations such as cell harvesting (and presumably washing) are capable of upregulating co-stimulatory molecules and inducing maturation/activation of DC populations (Gallucci, Lolkema et al. 1999; James 2001). This would correlate with the increased expression of CD40 on recultured DCs compared to previous experiments, suggesting that by harvesting, washing and reculturing the DEXDCs may have inadvertently activated the population of cells in culture. Addition of GM-CSF to DEX treated DCs partially restored the stimulatory capacity of the DCs, suggesting that dexamethasone may act as an indirect antagonist to GM-CSF (Moser, De Smedt et al. 1995). *In vivo*, dexamethasone may inhibit DC maturation by preventing T cells from releasing GM-CSF by binding to and inhibiting the NFAT-AP1 complex from binding to an enhancer region in the GM-CSF gene control region (Smith, Cousins et al. 2001).

5.5: Effects of dexamethasone-treated DCs on HY-skin graft survival

It has been shown that either dexamethasone-treated, or otherwise immature DCs are able to induce tolerance or non-responsiveness of T cells *in vitro* or *in vivo* including induction of graft survival and transferable tolerance (Steinbrink, Wolfl et al. 1997; Jonuleit, Schmitt et al. 2000; Lutz, Kukutsch et al. 2000; Lutz, Suri et al. 2000; Dhodapkar, Steinman et al. 2001; Griffin, Lutz et al. 2001).

In order to examine whether dexamethasone-treated BMDCs are able to induce tolerance in a HY-mismatched skin graft model, C57BL/6 male BM was cultured for 7 days with GM-CSF to form DCs, either in the presence or absence of 10^{-6} M dexamethasone. C57BL/6 females were injected intravenously with 10^7 male BMDCs or 10^7 DEXDCs 14 days prior to skin grafting with B6 male skin. To determine the immunogenicity of BM cells prior to any treatment, 10^7 freshly harvested B6 male BM cells were injected into a third group 14 days prior to skin grafting. Unfortunately 4/5 of the female mice that had received fresh bone marrow died within 7 days of receiving the injection. Subsequently another group of B6 female mice were injected intravenously with 5×10^6 male bone marrow cells 14 days before skin grafting and all of these mice survived.

Figure 5.8 a) shows the graft rejection profile comparing rejection of male skin grafts by female mice that had received either 10^7 male BMDCs, DEXDCs or 5×10^6 male BM cells 14 days prior to grafting. It can be seen that male BMDCs are highly immunogenic, with all grafts being rejected in less than 10 days, indicative of a primed immune response as would be expected. Both male bone marrow and male DEXDCs are also immunogenic, although slightly less than that seen with male BMDCs. The male bone marrow treated mice reject their grafts slightly slower than those receiving male DCs although this is likely to be due to the fact that only 5×10^6 BM cells were used for immunisation compared with 10^7 DCs. This result was not surprising, as it had previously been observed that male DCs prime an HY response (James 2001) and while others had shown that DEXDCs were unable to prime a response *in vivo*, figures 5.4 and 5.6 had shown that DEXDCs were still able to stimulate peptide-specific responses of a CD8⁺ clone *in vitro*, although at reduced levels. These data do not allow comparison of the immunogenicity or otherwise of different stages of DC development as bone marrow, 'immature' DEXDCs and BMDCs appear to be equally immunogenic.

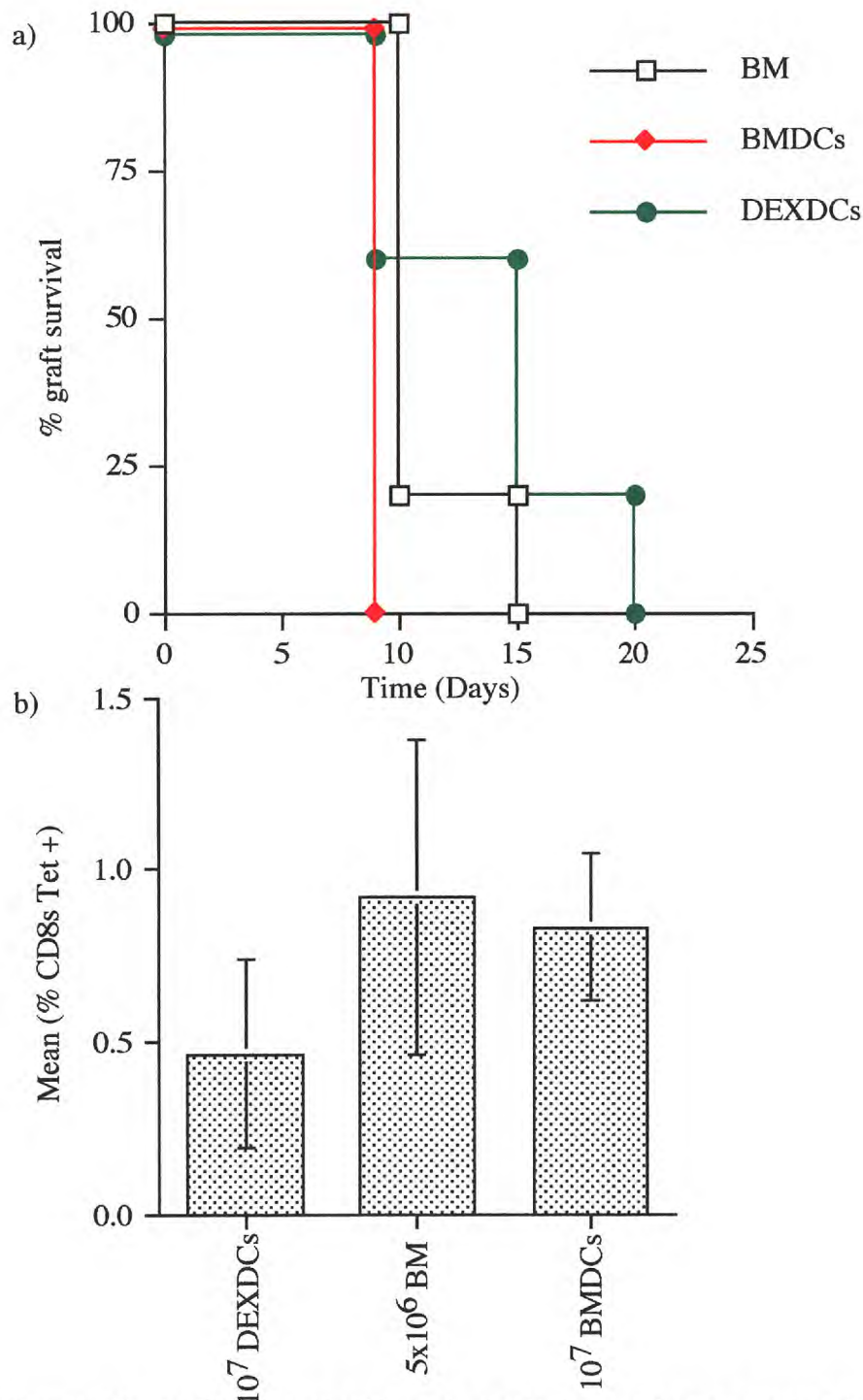


Figure 5.8. a) Mice treated with male day 7 dexamethasone treated BMDCs reject male skin in a primed manner, similar to that seen with male BMDCs or fresh male bone-marrow. C57BL/6 female mice were given 5×10^6 male bone-marrow, 1×10^7 BMDCs or 1×10^7 DEXDCs i.v. 14 days prior to grafting with male skin. Grafts were scored and data plotted as % graft survival. b) Mice that had been treated with male bone marrow, BMDCs or dexamethasone treated DCs were analysed after graft rejection for the presence of $D^b\text{-Uty}$ tetramer positive cells. Data shown is the mean percentage ($\pm$ SD) of $CD8^+$ cells that were tetramer positive, and that mice treated with DEXDCs appear to have less $D^b\text{-Uty}^+$ cells.

CD8⁺ T cell responses can be detected *in vivo* and *in vitro* using multimeric MHC class I/peptide complexes known as tetramers. A D^b-*Uty*-specific tetramer has been developed in this laboratory, known to bind specifically to the CTL-10 clone and has been used to examine HY specific responses (Millrain, Chandler et al. 2001). Shortly after the mice from figure 5.8 a) had rejected male skin grafts, splenocytes were stained with CD8 and D^b-*Uty* tetramer and examined for the presence of specific T cells. Figure 5.8 b) shows the mean percentage of CD8⁺ T cells specific for the tetramer (+/- standard deviation) comparing the three different pre-treatments (n = 5 mice per group). It can be seen that within a week of rejecting male grafts, mice pre-treated with 5x10⁶ male bone marrow cells have approximately 0.9% of tetramer⁺/CD8⁺ cells. Interestingly mice receiving BMDCs prior to grafting have a similar level of tetramer positive cells, although the mean is marginally lower. The third group receiving 10⁷ DEXDCs prior to grafting had approximately 0.5% CD8⁺ tetramer⁺ cells. Although the standard deviations in each group are relatively large, the percentages correlate well with that previously seen (Millrain, Chandler et al. 2001), such that in the absence of antigen the percentage of CD8⁺/tetramer⁺ cells decreases quickly, presumably through activation-induced cell death. In addition it appears that the rapidity of graft rejection correlated with the percentage of tetramer positive cells. It is possible as suggested in figures 5.6-5.7 that the effects of dexamethasone on BMDCs are not permanent, but that on removal of dexamethasone through washing, a population of cells develop that are capable of stimulating antigen-specific responses of both CD4⁺ and CD8⁺ T cells *in vitro* and this also happens *in vivo*. In figure 5.6-5.7, DCs were recultured in the absence of dexamethasone for 7 days and effectively stimulated antigen-specific responses. In the i.v. treatment experiment (Figure 5.8), 14 days passed between injection of the DCs and challenge with the graft. It is possible that in this time, the injected DCs have undergone maturation in the spleen/LN and stimulated proliferation of anti-HY clones *in vivo* that are able to induce graft rejection. It is also likely that unless a cell-type actively induces suppression, the antigens will prime indirectly.

Previous work in this laboratory has examined the role of different routes of administration of DCs and tolerance induction. Injection of 5x10⁶ female DCs pre-pulsed with D^b-*Uty* into the footpad 21 days prior to grafting with male skin induced tolerance in a proportion of C57BL/6 female mice, while DCs pre-pulsed with the A^b-*Dby* peptide or male DCs immunise, leading to rapid graft rejection (James 2001). To examine whether dexamethasone treated DCs could induce tolerance when administered by the same route, C57BL/6 female mice were injected in the footpad with

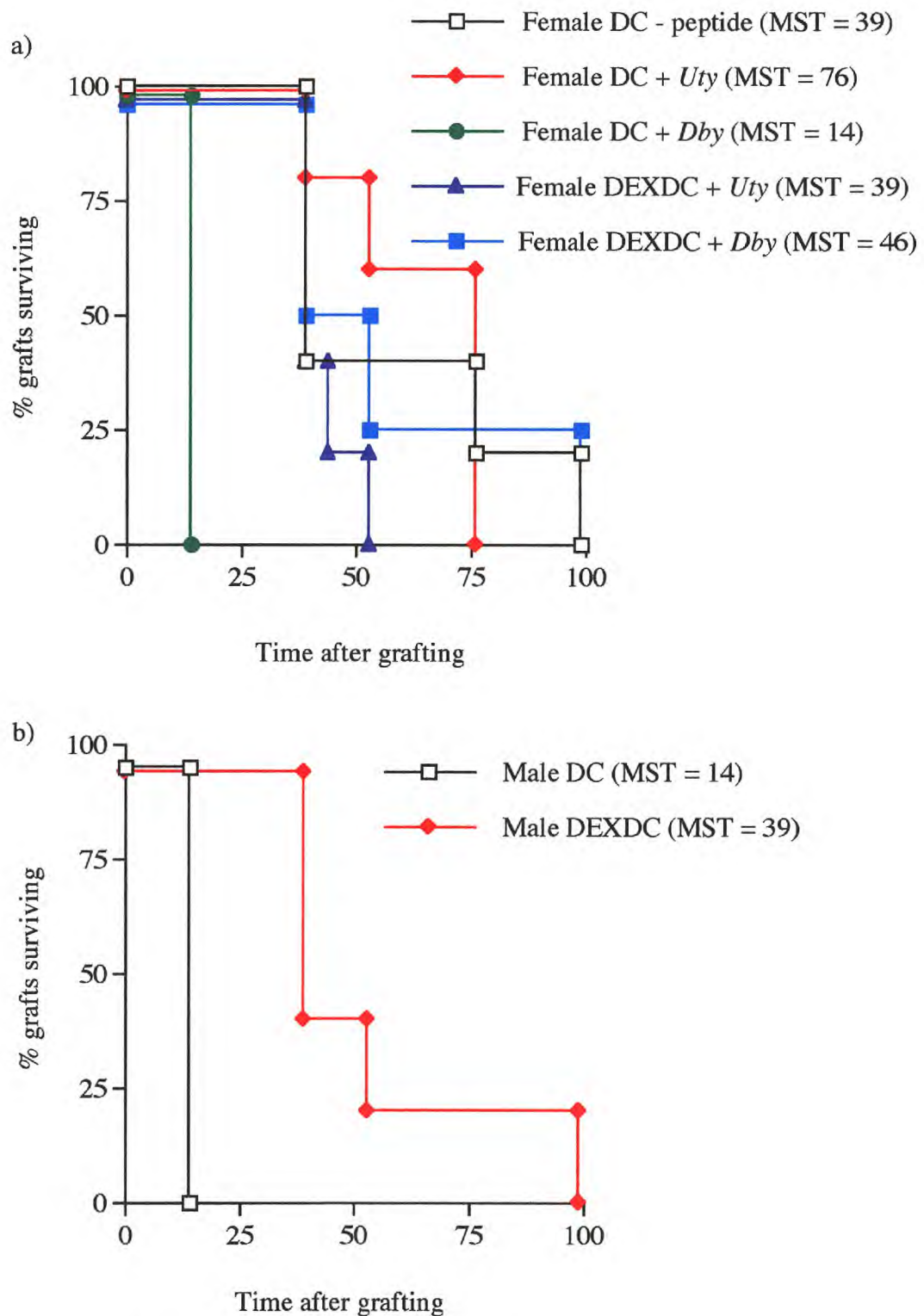


Figure 5.9. Different graft rejection profiles of C57BL/6 mice injected in the footpad with DEXDCs compared to untreated DCs. a) B6 female mice were injected with B6 female DCs or DEXDCs +/- A^b-*Dby* or D^b-*Uty* peptides. b) B6 female mice were injected with B6 male DCs or DEXDCs and grafted with B6 male skin.

5×10^6 male BMDCs or male DEXDCs 21 days prior to grafting with B6 male skin. A further group was treated with 5×10^6 female B6 DCs or female DEXDCs pulsed with either the class I or class II peptides to determine whether dexamethasone treatment of female DCs with different peptides would induce a different outcome to that previously shown (James 2001).

As expected (Figure 5.9 a), female DCs do not immunise and give an MST of 39 days, although 2/5 grafts survived to day 75 and 100 respectively and is typical of an unprimed HY response. In comparison, when treated with female DCs pre-pulsed with the A^b-*Dby* peptide, priming is seen and all male grafts are rejected by day 14. The difference between untreated female DCs and A^b-*Dby* pulsed female DCs is significant, with a P-value of less than 0.005 when analysed using the Log Rank test. This result is consistent with that previously shown in this laboratory (James 2001).

When pulsed with the *Uty* peptide however, the profile is different. Although the MST for male grafts when females were treated with female DCs with D^b-*Uty* is 76 days as compared with 39 days when treated with female DCs, the P-value is 0.95 and is therefore not significant.

In the protocol used in this work, bone marrow was harvested and the cells washed once by centrifuging for 7 minutes at 1500 rpm before resuspending and culturing. It may be that minor differences in the preparation protocol may influence the phenotype of the DCs. It has previously been shown that stress, such as through resuspending cultured DCs, can induce activation and upregulation of surface expression of a number of costimulatory molecules, to a similar extent to that seen on treatment with LPS (Gallucci, Lolkema et al. 1999). Although this may not be surprising, it does pose significant problems for the use of *in vitro* cultured DCs for therapeutic regimes currently being examined, at least in terms of tolerance versus immunity as it limits the amount of manipulation, and related stresses that can be performed with cultured APCs. Rigorous quality controls would be necessary before DCs could be useful as a tolerogenic therapy. This may affect the degree of comparison that can be made between studies from different laboratories or with different DC sources/preparations.

When female DCs are treated with 1 μ M dexamethasone from day 0-7, they have decreased surface expression of MHC class I and little MHC class II (Figures 5.5 and 5.7). When female mice were pre-treated in the footpad with 5×10^6 female DEXDCs with *Uty*, all of the mice rejected a male skin graft by day 53 with a MST of 39 days compared to 76 days in the absence of dexamethasone. Statistical comparison showed

that while the difference was not significant between female DEXDCs with *Uty* and female DCs without peptide ($P=0.27$), there was a significant difference when compared with female DCs with *Uty* ($P=0.03$). Together with the decreased MST (39 days compared with 76 days), this suggests that treatment with dexamethasone has not enhanced the tolerogenic properties of the DCs with *Uty*, but has made them less tolerogenic. Compared with the data using the CTL-10 T cell clone *in vitro* (Figure 5.2 b) this shows that the *in vitro* results do not correlate with the *in vivo* outcome.

Female DCs plus *Dby* peptide strongly primes an anti-HY immune response (Figure 5.9 a), while female DEXDCs plus *Dby in vitro* have limited MHC class II expression (Figure 5.5) and are unable to stimulate proliferation of the *Dby*-specific T cell clone B9 (Figure 5.2 a). The final group was B6 female mice pre-treated with DEXDCs plus *Dby*, this treatment did not prime the HY response since MST = 46 days was seen, although 2/5 grafts remained viable beyond 50 days. When compared with untreated female DCs without peptide, the difference is not significant ($P=0.96$), but if compared with untreated female DCs with *Dby* (MST= 14 days) the difference is significant ($P<0.01$). These data show that treatment with dexamethasone reduces the priming capacity of female DCs with *Dby* peptide compared with untreated DCs, although it has not been able to induce long-term tolerance to HY antigen. As previously shown, dexamethasone treated DCs have almost no MHC class II, CD11c and CD86, reduced expression of CD80 while MHC class I and CD11b are similar to untreated DCs. It is therefore possible that the reduced stimulatory capacity observed both *in vitro* and *in vivo* with dexamethasone treated DCs with *Dby* peptide can be accounted for by the relative absence of MHC class II at the cell surface. Treatment with dexamethasone inhibits DC maturation such that DEXDCs have enhanced phagocytic capacity and reduced antigen presentation capability (Piemonti, Monti et al. 1999; Piemonti, Monti et al. 1999; Matyszak, Citterio et al. 2000). It appears in this work that dexamethasone has different effects on MHC class I and class II presentation, severely limiting MHC class II expression, but having less of an effect on MHC class I. In addition, MHC class I is expressed on almost all somatic cells and is likely to be present on the bone marrow cells at the start. It is possible that dexamethasone may inhibit development of BMDCs in culture, but allow development of other populations of cells such as neutrophils (Strickland, Kisich et al. 2001) or macrophages which may not be sensitive to dexamethasone.

Figure 5.9 b) shows the graft rejection responses of B6 female mice treated with either 5×10^6 male BMDCs or male DEXDCs in the footpad. 21 days later the mice

were grafted with B6 male skin. Interestingly, while the mice given untreated male BMDCs reject their grafts rapidly with MST=14 days as was previously shown with i.v. administration (Figure 5.8 a), mice treated with male DEXDCs do not appear to be primed, showing a slower rejection profile with MST=39 days. While 3/5 of the mice rejected male grafts earlier than 50 days, one graft was maintained to almost 100 days and the difference between male DCs and DEXDCs was significant ($P=0.014$). This result was interesting as it suggested that the route of administration plays an important role in the effect of the DC treatment such that i.v. administration with either BMDCs or DEXDCs induces a primed response and rapid rejection (Figure 5.8 a), while in the footpad the effects are different (Figure 5.9 b). A reason for the different outcome could be due to the time between DC administration and grafting. Figure 5.8 a) shows rejection profiles when mice were grafted 14 days after treatment with DCs i.v., while in figure 5.9 b) there were 21 days between footpad DC treatment and grafting. Previous work in this laboratory has shown that both the route of DC administration, as well as the time between treatment and grafting can play a crucial role in whether tolerance is generated or not (James 2001). Material delivered intravenously will be systemic, mainly localised to the spleen and mesenteric lymph nodes, while when delivered into the footpad, the DCs are thought to migrate to the local draining popliteal lymph nodes. It is possible that the method of presentation may differ between these environments, either through the populations of cells that phagocytose/present antigen indirectly and the local environment of the lymph node or through the development of the administered cells and direct presentation. A related but alternative possibility is that intravenous administration is more efficient at delivering antigen for cross-priming than in the footpad. *In vivo*, there may be signals that induce maturation of the administered immature DCs if reversible, and it could therefore be important to reduce the time between administration and grafting, such that the DCs remain immature. On the other hand if regulatory cells are expanded (Shevach 2001), as has been suggested on treatment with immature DCs (Jonuleit, Schmitt et al. 2000; Matyszak, Citterio et al. 2000; Rea, van Kooten et al. 2000; Corinti, Albanesi et al. 2001; Gregori, Casorati et al. 2001; Hara, Kingsley et al. 2001), it is likely that the time between treatment and subsequent re-challenge with the graft is important for the outcome due to the rapidity of development and maintenance of the regulatory population.

5.6: Summary

These data (figures 5.2-5.5) suggest that as previously shown, (Moser, De Smedt et al. 1995; Kitajima, Ariizumi et al. 1996; Piemonti, Monti et al. 1999; Piemonti, Monti et al. 1999; Sacedon, Vicente et al. 1999; Matyszak, Citterio et al. 2000; Rea, van Kooten et al. 2000), maturation of DCs *in vitro* as examined by cell surface markers such as MHC class I and II, is inhibited by dexamethasone. In addition when used *in vitro*, DEXDCs have inhibited stimulatory capacity similar to that previously described when used as APCs in MLR. However this work has revealed by flow cytometry that rather than having an effect on both MHC class I and II as has previously been suggested, expression of MHC class II is almost completely abrogated while effects on MHC class I are limited (Figure 5.5-5.6). This was also detected functionally, as peptide-pulsed DEXDCs were used to stimulate CD4⁺ and CD8⁺ T cell clones. The CD4⁺ clone did not respond, while the CD8⁺ clone had a partially abrogated response.

Almost all of the previous studies with DEXDCs used MLR as an *in vitro* readout of DC stimulatory capacity, so in further experiments it would be interesting to compare different T cell clones when stimulated by DEXDCs, both CD4 and CD8, Th1 and Th2 to determine whether the effects are different on different T cell populations. It would also be interesting to examine in this model whether repeated stimulation with DEXDCs was able to induce T cell anergy or regulatory cells as has previously been shown with immature DCs (Jonuleit, Schmitt et al. 2000; Dhodapkar, Steinman et al. 2001; Griffin, Lutz et al. 2001).

Examination of the effects of dexamethasone on antigen processing and presentation would be interesting, as use of peptide-specific T cell clones does not test the need for uptake and presentation, as the peptides are pre-processed. Previous work has shown different effects on antigen uptake/processing with one group showing that glucocorticoids enhanced uptake and phagocytosis (Piemonti, Monti et al. 1999) (as would be expected if DEXDCs are truly immature), while use of airway DCs found that steroid treatment inhibits uptake and processing of antigen but not presentation (Holt and Thomas 1997). It would be interesting to examine the uptake of fluorescent antigens such as Lucifer Yellow or FITC-dextran (Piemonti, Monti et al. 1999), while processing of whole proteins such as KLH or HEL into peptide antigens could also be examined.

Further analysis of DEXDCs showed that washing and reculturing with LPS overnight did not promote stimulatory capacity or activate the DCs, further suggestive of an immature phenotype of DEXDCs compared with untreated BMDCs. However if washed after 7 days and recultured with 5% GM-CSF for a further 7 days, a population of cells developed, capable of stimulating proliferation of both CD4⁺ and CD8⁺ clones to a greater extent than untreated BMDCs. Recultured cells were found to have a different cell-surface phenotype to both BMDCs and DEXDCs with slightly elevated levels of CD11b as well as increased CD40, MHC class II and possibly CD80/86. In addition, the level of CD11c was higher than DEXDCs and had a narrower distribution than untreated DCs. It would be interesting to further characterise both DEXDCs and recultured/washed DEXDCs to examine the cell surface phenotype in more detail and compare gene-expression profiles between BMDCs and DEXDCs by microarray technology.

When administered *in vivo* prior to challenge with a male skin graft, different effects were seen depending on the route of administration or the time between treatment and grafting. Male bone marrow, BMDCs and DEXDCs injected i.v. 14 days before grafting efficiently primed anti-HY responses as seen by accelerated graft rejection in all mice. One week after graft rejection, mice treated with DEXDCs showed a slightly decreased proportion of splenic D^b-*Uty* tetramer-positive cells compared with those given bone marrow or BMDCs. If treated with female DCs +/- A^b-*Dby* or D^b-*Uty* peptides in the footpad 21 days prior to grafting the results are different. The A^b-*Dby* peptide was strongly immunogenic, as expected, when given on female BMDCs, while if given on female DEXDCs the male grafts survived longer. It is likely that this is due to the relative absence of class II from the DCs, so the DCs will have very little peptide bound at the cell surface. In comparison, the tolerogenic D^b-*Uty* peptide showed slightly accelerated graft rejection when given on DEXDCs when compared to treatment with BMDCs. Finally, male DCs in the footpad primed as well as when given i.v., yet DEXDCs were not as effective compared to i.v. injection, although it is unclear whether this is due to a difference in time-lapse between injection and grafting or the route of administration.

Further grafting experiments would be necessary to determine whether treatment with either male or female +/-peptides DEXDCs could be a potentially useful tolerogenic protocol.

Chapter 6.

Can apoptosis induce tolerance?

6.1: Background

There are two main processes of cell death, characterised by distinct morphological features and cellular processes. Necrosis is unprogrammed cell death, often triggered by tissue damage, injury or infection and is associated with the triggering of immune responses *in vivo*. Cells undergoing necrosis swell, after which the cell bursts releasing cytoplasmic contents including proteases, heat shock proteins and degraded DNA. In contrast, apoptosis is programmed cell death, constituting an essential part of development in multi-cellular organisms. In apoptosis, cell death is regulated and characterised by shrinkage, condensation of the nucleus and cleavage of chromatin into fragments in a process known as DNA laddering (Rathmell and Thompson 1999). During this process of shrinkage, apoptotic bodies are formed; membrane bound vesicles containing and isolating the cellular contents preventing their release into the local environment. Induction of apoptosis in a cell is marked by a loss in plasma membrane asymmetry such that phospholipids such as phosphatidyl serine, normally maintained at the cytoplasmic face of the membrane are flipped to the extracellular surface, acting as ligands for phagocytic cells (Platt, da Silva et al. 1998). In order to prevent inappropriate immune responses to apoptotic bodies, phagocytic cells particularly macrophages quickly internalise the apoptotic bodies in a receptor-mediated process, degrading the contents in internal lysosomes. In addition to macrophages, DCs are capable of phagocytosing apoptotic cells. A number of receptors have been characterised as being involved in uptake of apoptotic cells by macrophages including class A macrophage scavenger receptor (SR-A) (Platt, Suzuki et al. 1996), MER, a member of the receptor tyrosine kinase family (Scott, McMahon et al. 2001) and the phosphatidyl serine receptor, while DCs express a number of others including CD36 (Albert, Pearce et al. 1998).

Necrosis and apoptosis are thought to be immunologically distinct in that necrosis induces localised inflammation, while apoptosis is either immunologically 'silent' or induces tolerance, depending on the APC involved in uptake and presentation. A number of groups have compared the effects of apoptosis and necrosis on APC function, particularly on DCs and macrophages with regard to cross-presentation of antigen, in which antigen from cells is taken up by APCs and presented

in the context of MHC class I/II molecules (Heath and Carbone 2001). Sauter *et al* (Sauter, Albert et al. 2000) compared uptake of both necrotic and apoptotic tumour cells by immature DCs and showed that necrotic cells induced DC maturation, capable of stimulating *in vitro* CTL, while apoptotic cells were unable to induce such a response. Gallucci *et al* (Gallucci, Lolkema et al. 1999) have reported similar data. Macrophages were shown to respond differently to apoptotic and necrotic cells by producing inflammatory cytokines on exposure to lysed, but not apoptotic cells, and it is thought that intracellular proteases released from necrotic cells play a role in this differential response (Fadok, Bratton et al. 2001). Similarly, it has been shown that exposure of both macrophages and DCs to genomic double-stranded DNA released from necrotic cells, can induce maturation and upregulation of MHC class I/II (Ishii, Suzuki et al. 2001). It has also been suggested that apoptotic cells can have an immunosuppressive effect, influencing IL-10 and TNF- α secretion by APCs (Voll, Herrmann et al. 1997).

A number of groups have postulated that apoptosis is involved in peripheral tolerance to a number of antigens, either through direct tolerance with short-lived DCs containing apoptotic bodies, and/or through cross-tolerisation and transfer of the antigen to tolerogenic, immature DCs in the lymph nodes (Steinman, Turley et al. 2000). This suggests that uptake of antigen from apoptotic cells leads to presentation of antigen in the absence of inflammatory stimuli such as cytokines consistent with the 'danger model' (Matzinger 2001). A number of groups have suggested that it is possible to induce tolerance *in vivo* by administration of γ -irradiated, apoptotic or UV-B irradiated peripheral blood cells (Kao 1996; Bittencourt, Perruche et al. 2001).

Several groups have shown that APC uptake and cross-presentation of antigen induces functional immune responses. Inaba *et al* (Inaba, Turley et al. 1998) showed that DCs phagocytosed either necrotic or apoptotic B cell blasts and efficiently presented specific peptides in the context of MHC class II molecules. Similarly Bellone *et al* (Bellone, Iezzi et al. 1997) showed that uptake of apoptotic bodies by macrophages yielded T cell epitopes, presented by MHC class I molecules, then undergoing specific lysis by CD8⁺ T cells *in vitro*. Macrophages when 'fed' apoptotic cells secrete a number of inflammatory cytokines including macrophage inflammatory protein-2 and IL-8, although in this model, human macrophages were treated with PMA prior to addition of apoptotic cells (Kurosaka, Watanabe et al. 1998). More recently human DCs were shown to ingest apoptotic cells and cross-prime antigen-specific CTL, although this was dependent on DC maturation signals such as CD40L (Propato, Cutrona et al. 2001). Similarly others have shown that uptake of apoptotic cells by DCs can stimulate

antigen-specific CTL (Albert, Sauter et al. 1998; Larsson, Fonteneau et al. 2001) such that it has also been possible to use killed tumour cells fed to DCs as a potential anti-tumour therapy (Berard, Blanco et al. 2000; Kugler, Stuhler et al. 2000; Banchereau, Palucka et al. 2001).

It appears that cross-presentation of antigen by APCs and subsequent immunity or tolerance is strongly dependent on the type of cell death, whether necrotic or apoptotic and also whether other maturation stimuli are present. It is possible that phagocytosis of apoptotic cells and cross-presentation of antigen in the absence of inflammatory stimuli does not induce an immune response partly due to maintenance of an immature DC phenotype. It is suggested (as discussed in chapter 5) that immature DCs are capable of inducing peripheral tolerance to antigens, and this has been investigated in this study. A commonly used model for apoptosis is dexamethasone (discussed in detail in chapter 5) to induce apoptosis in thymocytes. This was applied to examine the effects of administration of apoptotic thymocytes on tolerance in the mouse HY skin graft system.

6.2: Dexamethasone can be used to induce apoptosis of thymocytes *in vitro*

Many groups have used dexamethasone to induce apoptosis, particularly in double positive thymocytes *in vitro* and *in vivo* (Screpanti, Morrone et al. 1989; Donner, Becker et al. 1999; Komoriya, Packard et al. 2000). Figure 6.1 shows the summary of two experiments comparing the levels of apoptosis induced with different doses of dexamethasone. C57BL/6 thymocytes were cultured in 12 well plates overnight at $3.3 \times 10^6/\text{ml}$ +/- dexamethasone from 0.1pM- 10nM or from 1nM- 10μM. Thymocytes were harvested the following day and stained with CD8-PE and Annexin V-FITC to assess the degree of apoptosis induction. Annexin V is commonly used to determine apoptosis by binding specifically to phosphatidyl serine exposed at the outer surface of the cell on induction of apoptosis. Double positive thymocytes are sensitive to dexamethasone-induced apoptosis, and so a CD8 antibody was used to minimise the observed contribution of single positive cells. Cells were gated on CD8⁺ thymocytes and the percentage of Annexin V⁺/CD8⁺ cells calculated. In the absence of dexamethasone, spontaneous apoptosis occurs in each experiment with approximately 25% of cells undergoing apoptosis. The process of spontaneous thymocyte apoptosis (STA) *in vitro* may mimic death-by-neglect observed *in vivo* and has been previously

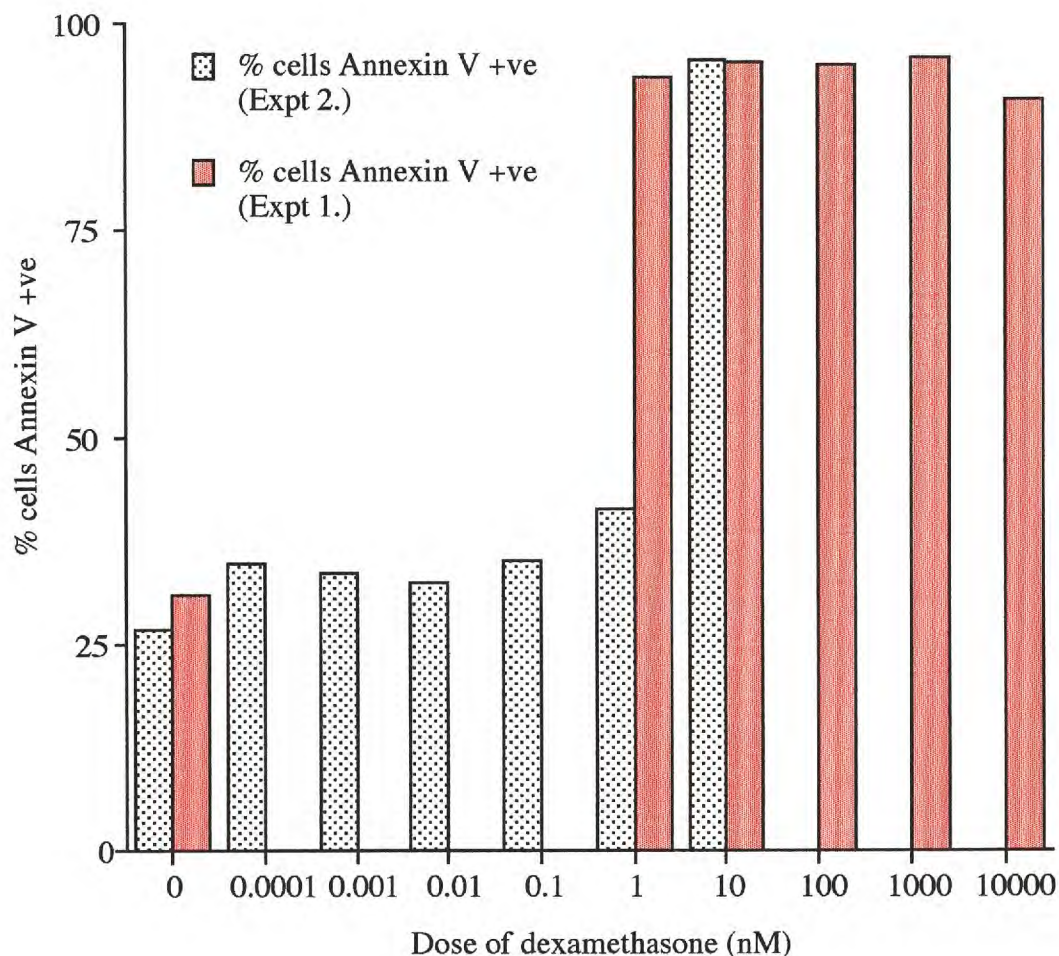


Figure 6.1. Induction of thymocyte apoptosis by dexamethasone. Male thymocytes were cultured with different doses of dexamethasone overnight *in vitro*, then washed and analysed by flow cytometry for CD8, Annexin V. Values represent % of CD8⁺ cells that are Annexin V positive after overnight culture. Two separate experiments (0-10000nM and 0-10nM) are shown.

characterised and is thought to be mediated by the release of cytochrome C from mitochondria (Zhang, Mikecz et al. 2000). In figure 6.1, experiment 1, it can be seen that while untreated thymocytes have around 25-30% apoptosis overnight, treatment with 1nM dexamethasone and above causes significantly more apoptosis such that approximately 90% of the CD8⁺ cells have become apoptotic. It is possible that a large proportion of the Annexin V-negative cells are single positive thymocytes and therefore insensitive to dexamethasone-induced apoptosis. In order to examine whether lower doses of dexamethasone could induce apoptosis in a similar manner, an overlapping dose response curve was used, treating with 10nM and lower doses of dexamethasone as before. Figure 6.1, experiment 2 shows that while 10nM dexamethasone causes around 90% apoptosis as before, doses of 1nM and lower do not induce more apoptosis than untreated controls. 1nM dexamethasone appears to be at the threshold of apoptosis induction since it has a marked effect in some experiments, but not in others. However it is clear that doses of 10nM and above will reliably induce apoptosis in the vast majority of double positive thymocytes during an *in vitro* overnight treatment.

It was important to determine for later experiments whether a shorter exposure to dexamethasone could be used to commit the thymocytes to apoptosis. Thymocytes were cultured as before and dexamethasone (1-10 and 10-100nM) added to the culture. At various time points after addition, the wells were harvested, the cells washed thoroughly to remove dexamethasone and plated out into fresh wells, to determine the effects of dose and exposure time and determine an effective dose for rapid commitment to apoptosis. After overnight incubation the thymocytes were harvested and stained with CD8 and Annexin V and the percentage of CD8⁺/Annexin V positive cells calculated. In figure 6.2 a) it can be seen that with increasing doses of dexamethasone (10-100nM), increasing apoptosis is induced as before. However in this experiment the maximum apoptosis induced was approximately 85%. It can be seen that depending on the dose of dexamethasone, an exposure time of at least five hours is required to induce maximum apoptosis, such that with 100nM over 80% of thymocytes are apoptotic by five hours with little subsequent increase. Experimental variation can again be seen in figure 6.2 b), treating with 1-10nM dexamethasone for increasing time. Treatment with 10nM dexamethasone induces over 80% apoptosis within 7 hours and that treatment for longer does not further increase apoptosis. It is also clear that less apoptosis occurs if lower doses of dexamethasone are used, with 5nM inducing apoptosis in 60-70% of thymocytes and 1nM only 50-60%. These data suggest that it is possible to induce maximum apoptosis at 24 hours in thymocytes with 5-7 hours

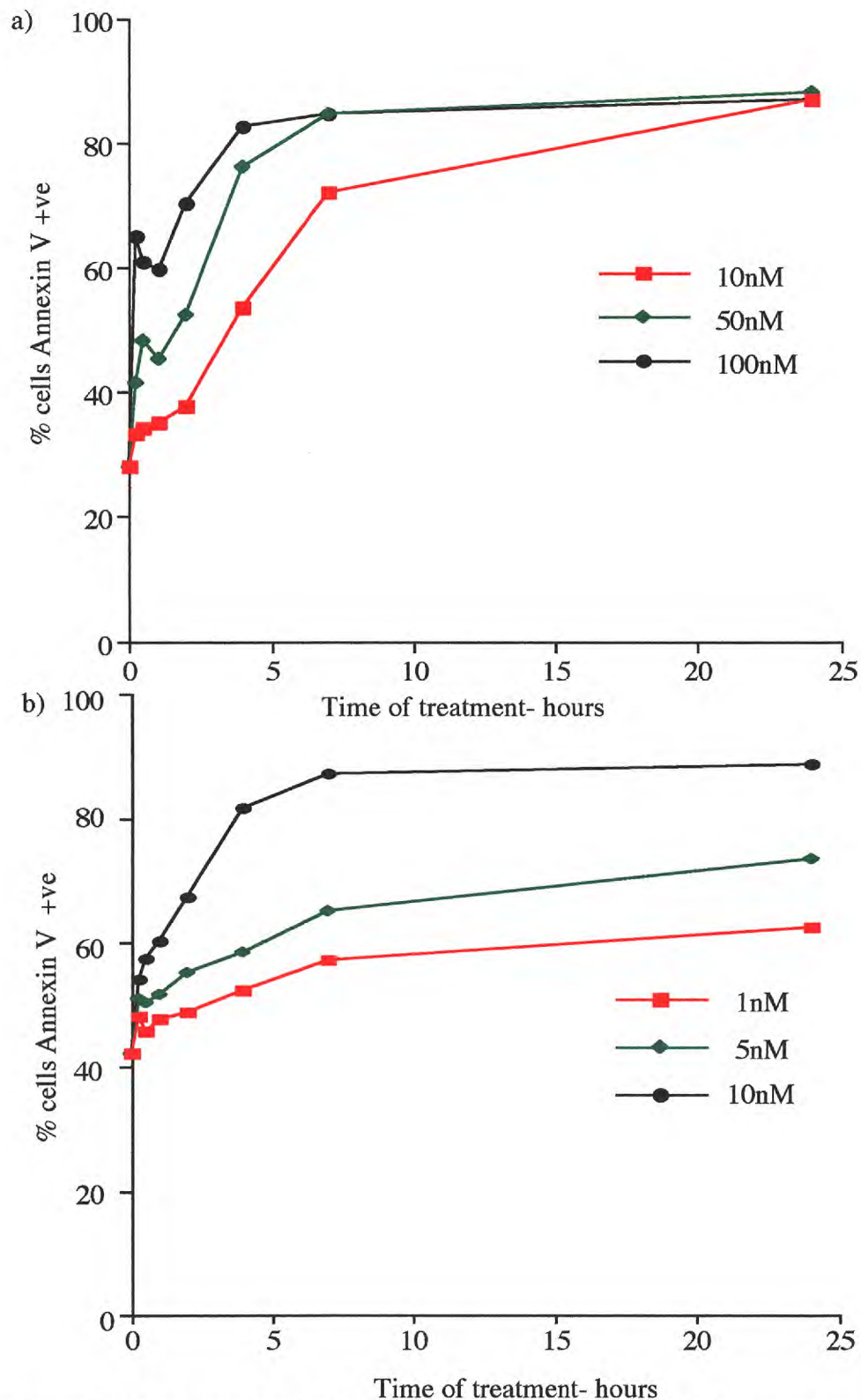


Figure 6.2. Treatment with 10-100nM dexamethasone will induce maximum apoptosis in cultured thymocytes. Two separate experiments a) 10-100nM, b) 1-10nM. Male thymocytes were treated *in vitro* for various lengths of time with different doses of dexamethasone. They were washed and recultured overnight before being analysed by flow cytometry for CD8 and Annexin V. Values represent the % of CD8⁺ cells that are Annexin V positive after the overnight culture.

exposure to 100nM dexamethasone. This is slightly quicker than that previously described by Donner *et al* (Donner, Becker et al. 1999) who used a number of methods to assess apoptosis induction in thymocytes by dexamethasone including 7-AAD, annexin, and other assays, and showed that after 4 hours apoptosis was detectable by annexin V staining but other assays showed maximum apoptosis around 12 hours of treatment.

6.3: Treatment with male thymocytes and dexamethasone *in vivo* prolongs survival of male skin grafts on female mice

The HY system involves the rejection of male grafts by syngeneic female mice due to polymorphisms in genes located on the Y-chromosome comparing female with male. In this model the only differences are the polymorphisms on the Y-chromosome and constitute minor histocompatibility antigens. A number of different peptide antigens have been characterised as being involved in HY graft rejection, both MHC class I and II-restricted epitopes from genes including *Smcy*, *Dby* and *Uty* (Greenfield, Scott et al. 1996; Simpson, Scott et al. 1997; Scott, Addey et al. 2000). Male graft rejection by female mice occurs in a process mediated by both CD4⁺ and CD8⁺ T cells. Typically an unprimed B6 female mouse will reject a syngeneic male graft within 40-80 days, while if previously immunised with male antigen, rejection is more rapid, less than 20 days after grafting (Figure 6.3 c).

Many groups have shown that it is possible to induce apoptosis of thymocytes *in vivo* by intraperitoneal (i.p.) injection of dexamethasone with many different doses. While the induction of apoptosis was being examined *in vitro*, other experiments were done to determine whether simultaneous introduction of male thymocytes and dexamethasone into female C57BL/6 mice could affect male skin graft survival. On day -21, female mice received 1×10^5 - 1×10^7 male thymocytes i.v. plus 0.1mg dexamethasone i.p. Control groups received either 1×10^7 male or female thymocytes in the absence of dexamethasone. 21 days later some of the mice received a repeat dose of thymocytes and dexamethasone and all were grafted with C57BL/6 male skin. Prior to administration, thymocytes were depleted of MHC class II positive cells such as thymic epithelia and DCs using anti-MHC class II antibodies and dynabeads. Although expressing MHC class I, thymocytes do not express MHC class II and should be unable to prime a response directly. Consequently the HY antigen is likely to be indirectly processed and presented by recipient APCs.

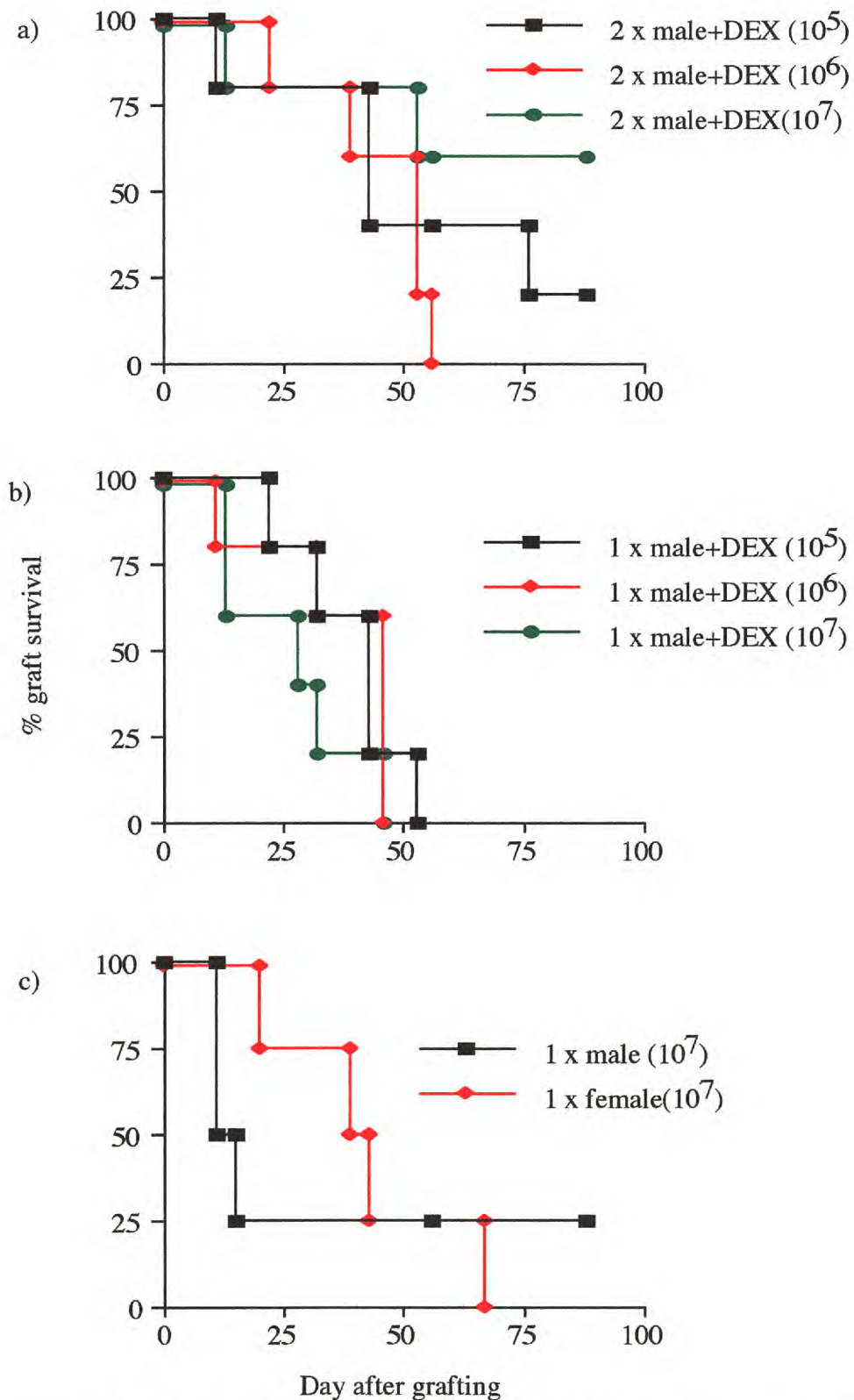


Figure 6.3. Two injections with 1×10^7 male thymocytes + dexamethasone prolongs male skin graft survival in female mice. Thymocyte tolerance induction experiment 1. Female B6 mice were given 1 or 2 injections i.v. with between 1×10^5 - 1×10^7 male or female thymocytes +/- 0.1mg DEX i.p. They were then grafted with B6 male skin.

Figure 6.3 c) shows the rejection responses of mice injected with 1×10^7 untreated male or female thymocytes 21 days prior to receiving a B6 male skin graft. As previously described, mice given female thymocytes have not been exposed to HY antigen and subsequently display a naïve phenotype, typically rejecting a graft between days 40-80. In comparison, female mice injected with untreated male thymocytes have been primed to the HY antigen and 4/5 mice reject a male graft before day 25. In contrast figure 6.3 a) shows the graft rejection responses of female mice that had received two injections of 10^5 , 10^6 or 10^7 male thymocytes and dexamethasone and it can be clearly seen that the grafts on 3/5 of the mice that received 10^7 thymocytes survived beyond day 75 while none of the mice showed a primed anti-HY response. This suggests that either apoptosis of the thymocytes is being induced *in vivo* and this is inducing tolerance, or that the dexamethasone itself is inducing immunosuppression. Figure 6.3 b) shows the responses of mice receiving only one injection of dexamethasone and thymocytes 21 days before grafting and it can be seen again that while the graft survival is not as good as those that received two treatments, the mice do not show a primed response.

These data show that administration of male thymocytes to female mice prior to challenge with a male skin graft is strongly immunogenic and causes priming of an anti-HY response. It is likely that *in vivo* in the absence of dexamethasone, thymocytes die through a mixture of either primary or secondary necrosis and apoptosis, which induces uptake of the cellular material by APCs in the periphery, accompanied by release of heat shock proteins (Feng, Zeng et al. 2001; Somersan, Larsson et al. 2001) and genomic double-stranded DNA (Ishii, Suzuki et al. 2001). Uptake of antigen released from necrotic cells induces maturation of DCs and subsequent cross-presentation (Inaba, Turley et al. 1998; Sauter, Albert et al. 2000). In comparison, mice treated with both male thymocytes and dexamethasone, do not prime a response. It is possible that the dexamethasone is suppressing immune responses, although this is likely to be transient as shown (Figure 6.4 c), while another alternative is that apoptosis of thymocytes is induced *in vivo* by the dexamethasone as previously described, and antigen is taken up in a non-inflammatory environment and incomplete activation of the APC occurs. Responses of mice given one treatment with thymocytes/dexamethasone appear to be less effective than mice receiving two treatments, such that graft survival is enhanced on receiving two injections (Figure 6.3 a and b).

6.4: Comparison of treatment with dexamethasone *in vivo* and *in vitro*

In order to determine whether the prolongation of male graft survival was dependent on administration with dexamethasone *in vivo* and subsequent immunosuppressive effects or whether the effects observed were due to thymocyte apoptosis *in vivo*, the following experiment was performed. Figure 6.3 shows that two treatments with thymocytes and dexamethasone was more effective at modulating the HY response than a single treatment, and that 1×10^7 thymocytes worked better than 1×10^6 . In the second experiment C57BL/6 female mice were injected with 1×10^7 male or female thymocytes intravenously prior to skin grafting on days -21 and -1. The thymocytes were either untreated, untreated but with an injection of 0.1mg dexamethasone (i.p.) or had been cultured *in vitro* for 5 hours with 100nM dexamethasone and washed thoroughly before injection.

Figure 6.4 a) shows a comparison of female mice treated with male or female thymocytes ($n = 7$ mice/group). It is clear that male thymocytes prior to skin grafting induce rapid graft rejection, typical of an immunised response in all mice. This shows that thymocytes themselves are immunogenic, causing graft rejection in approximately 15 days, similar to responses detected after immunisation with male DCs previously shown (Chapter 5, Figure 5.8). In comparison, similar treatment with female thymocytes before a male skin graft does not lead to accelerated graft rejection because of the absence of HY antigens.

Figure 6.4 b) compares the rejection responses when female mice were given *in vitro* treated- apoptotic thymocytes that were cultured with 100nM dexamethasone for 5 hours. It is clear that as before that female thymocytes do not prime and the recipients display a normal graft rejection course. 3/7 mice given two injections with 1×10^7 apoptotic male thymocytes rejected grafts quickly within 20 days as in figure 6.4 a). However the remaining mice reject grafts much more slowly with 2/7 grafts surviving to day 128 when the experiment was terminated. This suggests that induction of apoptosis of male thymocytes prior to administration significantly reduces their immunogenicity and in a proportion of the mice induces long-term graft survival.

Figure 6.4 c) compares the treatment of female mice with two injections of 1×10^7 untreated male or female thymocytes i.v. and 0.1mg dexamethasone i.p. as in figure 6.3. Again, treatment with female thymocytes has no effect on rejection of a male skin graft. Unlike that shown in figure 6.4 a) and b) only 1/7 mice given male

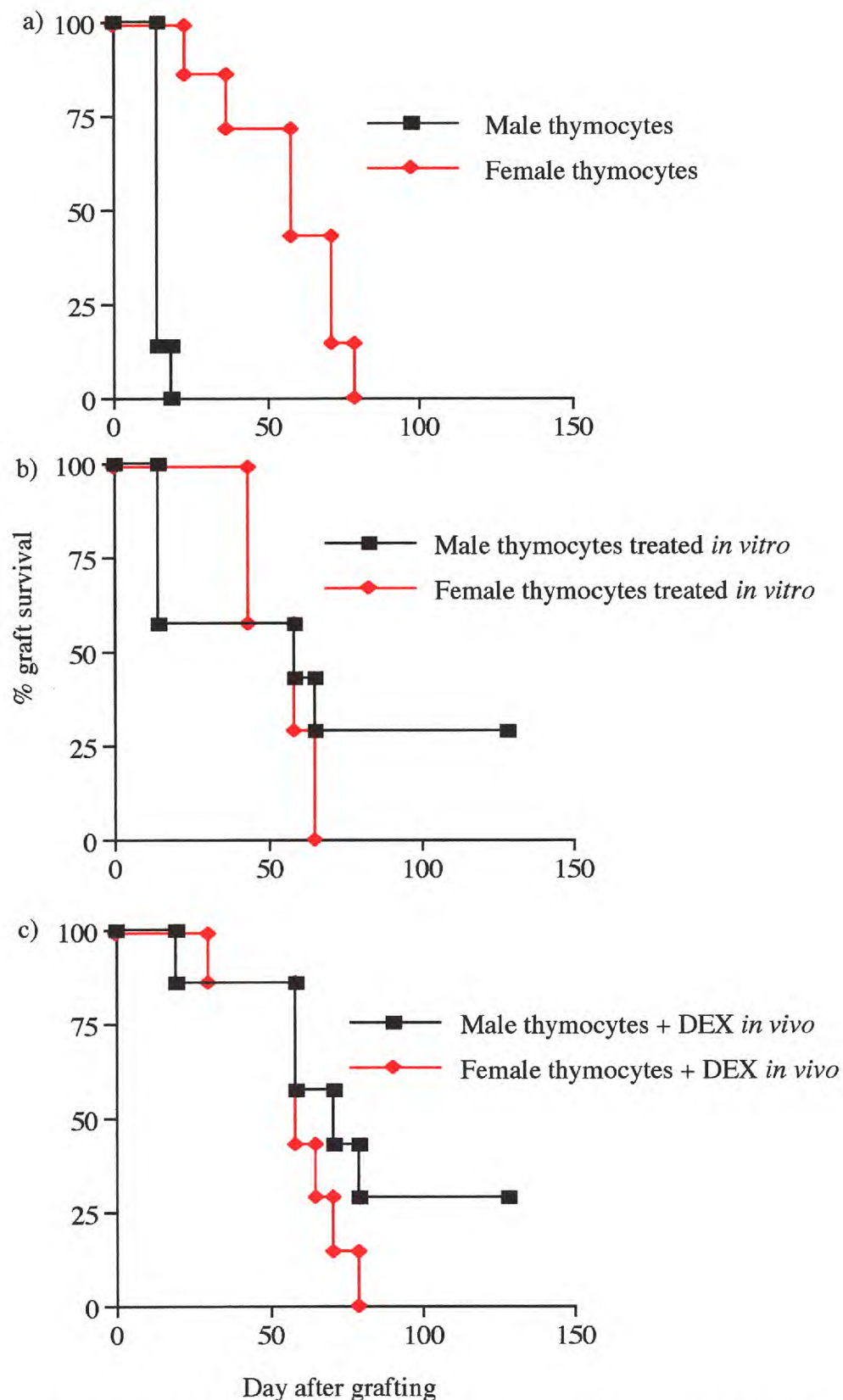


Figure 6.4. Both dexamethasone treatments b) *in vitro* and c) *in vivo* (0.1mg DEX i.p.) cause prolongation of graft survival compared to a) untreated male thymocytes . All B6 female mice received 2 injections of 1×10^7 male or female thymocytes i.v. and were then grafted with B6 male skin.

thymocytes and dexamethasone rejected a male graft in 20 days (MST = 71 days) compared to 3/7 when *in vitro* treated male thymocytes were used (MST = 58 days) and 7/7 mice with untreated male thymocytes (MST = 14 days). Over time a number of grafts are rejected, such that by the termination of the experiment on day 128, 2/7 grafts are surviving when treated *in vivo*, one of which was beginning to display early signs of rejection. At the termination of the experiment, both *in vitro* and *in vivo* treatments had 2/7 grafts remaining intact. Using the statistical analysis package Prism, P-values were calculated for graft survival data using the Log-rank test, comparing different treatments. The difference between untreated male thymocytes and *in vitro* apoptotic male thymocytes is significant with a P-value of less than 0.03, while between untreated male thymocytes and *in vivo* dexamethasone administration is less than 0.001. Only the untreated male thymocyte group differ significantly when compared with female thymocyte treatments. These data suggest that treatment with dexamethasone *in vitro* and *in vivo* induce similar graft prolongation although *in vivo* treatment may cause slightly more enhanced effects.

These data are consistent with other work, showing that if leukocytes (Bittencourt, Perruche et al. 2001) or mononuclear cells (Kao 1996) are irradiated to become apoptotic prior to administration *in vivo*, this promotes tolerance induction. The mechanism of tolerance induction when using apoptotic mononuclear cells was examined. Kao *et al* (Kao, Huang et al. 2001) found that depletion of CD4⁺CD25⁺ cells from splenocytes prior to adoptive transfer inhibited transfer of tolerance suggesting a regulatory T cell mechanism and it is possible that tolerance through uptake of apoptotic thymocytes by APCs *in vivo* has induced a similar regulatory T cell population.

6.5: Treatment with apoptotic thymocytes does not induce prolonged graft survival across MHC barriers or when using calcimycin to induce apoptosis

In order to determine whether addition of further apoptotic material can enhance the tolerogenic effect, a further experiment was performed. It was noted in figure 6.3, that two injections of thymocytes together with dexamethasone *in vivo* was required for partial tolerance induction. Other work using transfusion of UV-B irradiated, PBL cells i.v. into allogeneic recipients showed that tolerance could be induced in a dose-dependent manner by four weekly injections (Kao 1996). In order to examine whether

additional treatment with thymocytes could enhance tolerance induction, female mice were injected on four separate occasions, three times every 14 days prior to skin grafting and then 14 days after grafting in the mice that had surviving grafts.

A number of other agents are known to be pro-apoptotic including the calcium ionophore- calcimycin, thought to induce apoptosis by altering calcium flux across the plasma membrane and thus affecting the membrane potential. Thymocytes were treated with calcimycin and apoptosis induction measured by an increase in Annexin V and subsequent necrosis by propidium iodide staining (data not shown), showing that apoptosis could be induced in less than 20 minutes *in vitro*. In order to examine whether calcimycin could induce tolerance similar to that seen using dexamethasone, male and female thymocytes were treated *in vitro* for 20 minutes prior to injection of 1×10^7 i.v. every two weeks, three times before grafting.

A third interesting factor to examine is the effectiveness of apoptotic cells at inducing tolerance across major histocompatibility barriers. CBA/Ca mice rapidly reject H2-mismatched C57BL/6 grafts, while in the work from Bittencourt *et al* (Bittencourt, Perruche et al. 2001), bone marrow engraftment was enhanced by administration of apoptotic cells independent of the source of material. Different sources of apoptotic material were used in this work including donor, recipient and third-party splenocytes as well as human PBL and each appeared to enhance engraftment. To examine an allogeneic response, CBA/Ca female mice were treated with three doses of 1×10^7 normal or apoptotic C57BL/6 male or female thymocytes +/- dexamethasone i.p. every two weeks prior to grafting with B6 male skin.

Figure 6.5 does not show any of the data for the CBA/Ca female mice as all rejected B6 male skin within two days of removing the plaster, irrespective of the treatment. Interestingly, all grafts were intact and looked healthy immediately after removing the plaster, but in 5-6 hours the grafts were no longer healthy but red in colour, appearing inflamed. Interestingly this contrasts somewhat with results previously described (Bittencourt, Perruche et al. 2001) showing that tolerance induction occurred across MHC barriers. Introduction of apoptotic B6 male thymocytes +/- dexamethasone was unable to induce tolerance in CBA/Ca mice to C57BL/6 skin, suggesting that the polymorphism present in major mismatched grafts are too strong. It is clear that some of the rejection response in these mice will be through direct recognition of MHC class I and II molecules on the surface of the graft. It is unlikely that thymocytes would be able to tolerise both to direct and indirect MHC class II, as they do not express MHC class II molecules. It is also unlikely that tolerance to directly

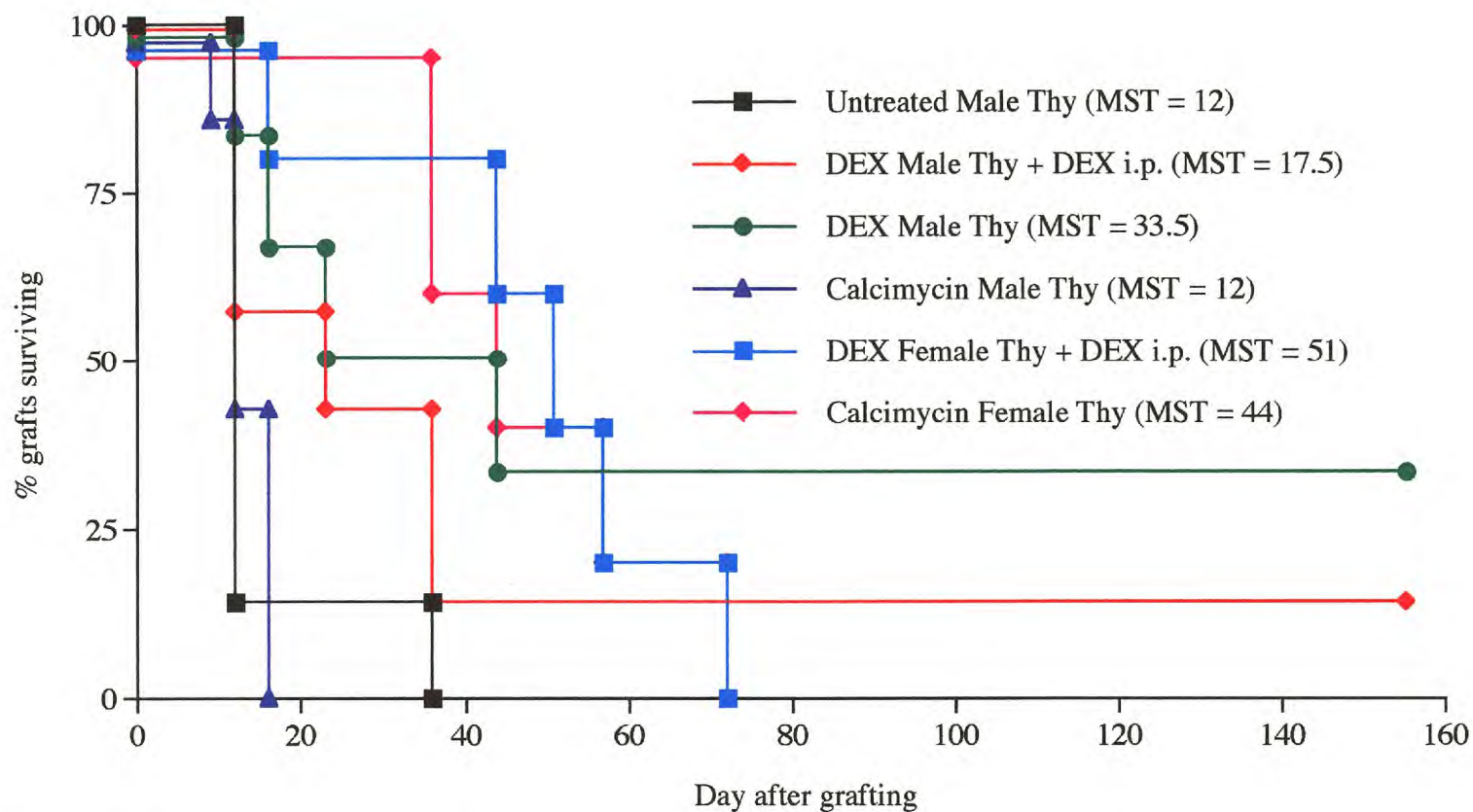


Figure 6.5. Female C57BL/6 or CBA/Ca mice were injected with 10^7 treated or untreated male or female B6 thymocytes i.v. +/- 0.1mg DEX i.p. every two weeks, 3 times before grafting with B6 male skin. Those with surviving grafts were given the same treatment 14 days after grafting. All CBA/Ca mice rejected their grafts within two days of unplastering and are not shown on this figure.

presented MHC class I is occurring with apoptotic thymocytes, as the antigens are being phagocytosed and re-presented indirectly. It is therefore likely in this model that the tolerance being mediated is against the indirect response to multiple minor differences and as well as to indirect MHC class I recognition. Further experiments to examine whether this was true could examine tolerance to multiple minor mismatched grafts for example using C57BL/6 and BALB/B (H2^b) or CBA and C3H (H2^k) mice.

It is thought that the direct pathway of antigen recognition is a major cause of acute (and hence rapid) graft rejection, while the indirect pathway is a cause of chronic long-term graft rejection (Womer, Sayegh et al. 2001). When major mismatched grafts are given, the direct pathway is strongly active due to mismatched MHC molecules on the graft and the presence of anti-donor alloreactive T cells in the recipient. These data suggest that while treatment with apoptotic cells is able to induce non-responsiveness or tolerance across minor mismatched antigens, it is unable to do the same for major mismatches. This is in contrast to that previously described, although it is possible that tolerance to bone marrow engraftment and a tissue graft such as skin are different, due to accessibility or immunogenicity *in vivo*. It would be interesting to compare the efficacy of treatment with apoptotic thymocytes on rejection of other graft-types such as cardiac allografts, as well as comparing the effect of different methods of apoptosis induction with other cell types such as splenocytes or bone marrow. This would allow determination of whether tolerance is dependent on the cell-type, apoptosis inducing agent or graft-type used.

Figure 6.5 shows the rejection responses of the C57BL/6 female mice from this experiment. Similar to that previously shown in figures 6.3 and 6.4, female mice injected with three doses of untreated male thymocytes display a primed response, rejecting most of the grafts in less than 20 days, although in this group one mouse maintained the graft to nearly day 40. Injection with three doses of untreated thymocytes does not enhance rejection compared to two treatments, such that male grafts are rejected with similar rapidity in both experiments.

Injection of *in vitro* induced apoptotic male thymocytes, together with a direct injection of dexamethasone does not prime as well as untreated cells. Prolonged graft survival was seen in a number of the mice and long-term tolerance in 1/7. This is a similar outcome to that previously shown in figure 6.4. It is also clear that further treatment with apoptotic thymocytes and dexamethasone (3 prior to grafting and 1 after, compared with 2 before grafting in a previous experiment) has not enhanced tolerance. In figure 6.4 b), over 50% of the mice maintained grafts beyond 50 days, while in figure

6.5, the MST for apoptotic thymocytes with dexamethasone was only 17.5 days. This treatment is slightly different from that used in figure 6.4 b), in that thymocytes were treated *in vitro* for 4-5 hours before injection and then the mice received an injection of dexamethasone. It is possible that the slightly decreased efficacy of the treatment compared to treatment with apoptotic cells in the absence of dexamethasone is due to further effects of dexamethasone on thymocytes *in vivo*. It may be that dexamethasone *in vivo* affects the apoptotic thymocytes such that they become late apoptotic and therefore more likely to release heat shock proteins and induce effective immune responses, whereas treatment *in vitro* alone is sufficient to initiate early apoptosis, without inducing the late stage apoptosis *in vivo*.

In contrast, female mice treated with *in vitro*-cultured apoptotic female thymocytes with dexamethasone i.p. do not display a primed response to the male antigens and subsequently reject their grafts with MST = 51 days, although in this group all grafts have been lost before day 80 as has previously been seen in figures 6.3 and 6.4. This suggests that the presence of dexamethasone in these mice is not affecting rejection of the male skin grafts to any great extent.

As a comparison, figure 6.5 also shows treatment with apoptotic male thymocytes in the absence of dexamethasone. In this example the MST is 33.5 days, with 2/7 mice maintaining their grafts beyond day 150 at the termination of the experiment. These data are similar to those in figure 6.4 and confirm that treatment with *in vitro* treated apoptotic male thymocytes induces prolonged male skin graft survival and in a proportion of the mice, long-term tolerance. Further, treatment with four injections of thymocytes (three before grafting and one 14 days afterwards) does not further enhance prolongation of graft survival compared to two injections as seen in figure 6.4 b). Kao *et al* (Kao 1996) used four weekly injections of irradiated splenocytes to induce tolerance and shown that this occurred in a dose-dependent manner. Restrictions on treatment timing meant that it was impossible to examine the effect of weekly doses of apoptotic thymocytes on tolerance induction in this model. However it is clear that while induction is better with two injections than one (compare figure 6.3 a and b), four treatments do not further enhance the protective effect *in vivo*.

In order to examine the effect of other apoptotic agents on thymocytes and tolerance induction, the calcium ionophore calcimycin was used. *In vitro* calcimycin treated thymocytes rapidly enter apoptosis as assessed by Annexin V staining, followed by necrosis associated with staining of the nucleus with propidium iodide (data not shown).

Male or female thymocytes were treated *in vitro* for 20 minutes with 10 μ M calcimycin to induce apoptosis, washed thoroughly and then 1 $\times$ 10⁷ injected i.v. three times into C57BL/6 females, every 14 days prior to grafting with B6 male skin. Not surprisingly, treatment with apoptotic female thymocytes (figure 6.5) has no effect on male skin graft survival as controls with MST=44 days, similar to dexamethasone treated female thymocytes with dexamethasone i.p. (MST=51 days). In contrast, when injected with calcimycin-treated male thymocytes, unlike that seen with dexamethasone-treated thymocytes, tolerance is not induced. Female mice injected with calcimycin-treated male thymocytes reject grafts with MST=12 days, very similar to that seen with untreated male thymocytes. This result was somewhat surprising and suggests that delivery of apoptotic thymocytes is not necessarily a tolerogenic event, but rather is critically dependent on the manner in which apoptosis is triggered.

The difference in effect may be explained by calcimycin and dexamethasone inducing apoptosis in different ways or at different rates. Time course experiments have shown that a 5-hour treatment with dexamethasone, followed by overnight culture induces maximal apoptosis in thymocytes, suggesting that dexamethasone-induced apoptosis is not immediate. Conversely, using calcimycin in a time course experiment, Annexin V staining was detectable in less than 30 minutes *in vitro*, rapidly followed by propidium iodide staining of DNA. This indicated that calcimycin rapidly induces thymocyte apoptosis (data not shown). It is possible that treatment *in vitro* with calcimycin, followed by washing and injection allows apoptosis to progress too far, such that secondary necrosis occurs. It has been previously shown that apoptotic and necrotic cells have differential effects on immune responses such that necrotic or untreated cells induce DC maturation and upregulation of antigen presentation capability, while this is not seen with apoptotic cells (Gallucci, Lolkema et al. 1999). It may be that *in vitro* dexamethasone-treated thymocytes are rapidly phagocytosed by APCs *in vivo* and do not induce maturation unlike cells in the later stages of apoptosis. This would mimic normal clearance of apoptotic cells *in vivo* that is rapid after release of the dying cell from the surrounding extracellular matrix and tissue. Incomplete clearance of apoptotic material induces autoimmune lupus via anti-DNA antibodies, so presumably to prevent this, clearance of apoptotic cells is a rapid process.

Collectively these data suggest that it is possible to induce tolerance or at least reduce immunogenicity of male thymocytes by inducing apoptosis prior to administration. It would be interesting to examine this model further. Other experiments could include use of MHC-deficient mice as thymocyte donors. DP thymocytes are

particularly sensitive to dexamethasone-induced apoptosis compared with SP thymocytes. Using normal thymocytes inevitably means that a population of thymocytes exist (SP) that are insensitive to dexamethasone effects and may therefore undergo necrosis *in vivo*, thus reducing the efficacy of the treatment. In figures 6.1 and 6.2, the maximum percentage of Annexin V⁺ CD8⁺ thymocytes attained *in vitro* was approximately 90%. MHC-deficient mice have a block in thymocyte maturation at the DP stage due to the absence of selecting MHC molecules and consequently have less SP thymocytes (Cosgrove, Gray et al. 1991; Grusby, Auchincloss et al. 1993; Grusby and Glimcher 1995) that are dexamethasone-insensitive. It may be that if thymocytes from MHC-deficient mice are treated, a larger population will undergo apoptosis and this may enhance the effect of treatment on graft survival. In addition to this, it will obviate the need for depletion of MHC class II positive cells prior to administration possibly able to prime a response, thus simplifying the protocol and limiting the handling time and washing steps that may play a role in non-apoptotic cell death *in vitro*. Experiments involving the use of MHC-deficient mice as thymocyte donors are currently underway.

6.6: Adoptive transfer of splenocytes from tolerant mice

An important aim of *in vivo* tolerance induction is to determine the underlying mechanism. T cell anergy, clonal deletion and regulatory cells have been implicated in tolerance (Li, Wells et al. 2000; Lechler, Chai et al. 2001; Shevach 2001). In some transplant models it is possible to transfer tolerance to either naïve adult or neonatal recipients by transferring populations of splenocytes from tolerant animals, suggesting that a population of cells exists, capable of mediating tolerance. Transfer of tolerance to naïve adults is thought to be more difficult than to neonatal animals due to the presence of a fully developed peripheral repertoire, while the immune system is not fully developed in neonates, allowing homeostatic proliferation and expansion of transferred cells.

Tetramers are multimeric MHC class I/peptide complexes, capable of binding to the TCR of T cells, allowing detection of specific T cell clones *ex vivo*. A number of tetramers have been produced in this laboratory with previously characterised HY-peptides and were used to compare the presence of antigen-specific CD8⁺ T cells in the spleen of mice that were tolerant or had rejected a male skin graft. Figure 6.6 a)-d) shows FACS analysis comparing CD8 and D^b-Uty tetramer staining on splenocytes

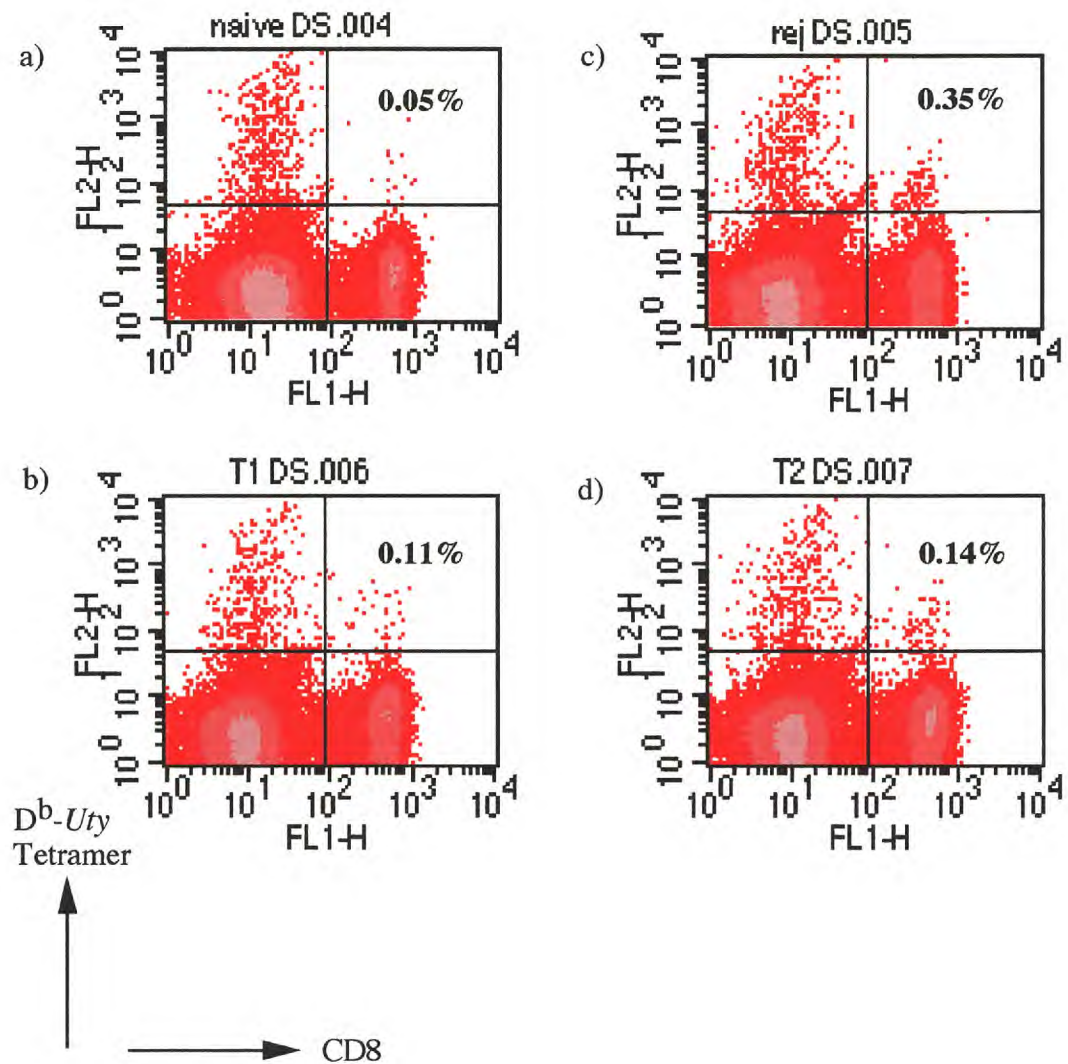


Figure 6.6. D^b -*Uty* tetramer analysis of a) naive, c) rejected, b) and d) tolerant C57BL/6 female splenocytes. Numbers in brackets indicate the percentage of $CD8^+$ T cells that are D^b -*Uty* positive.

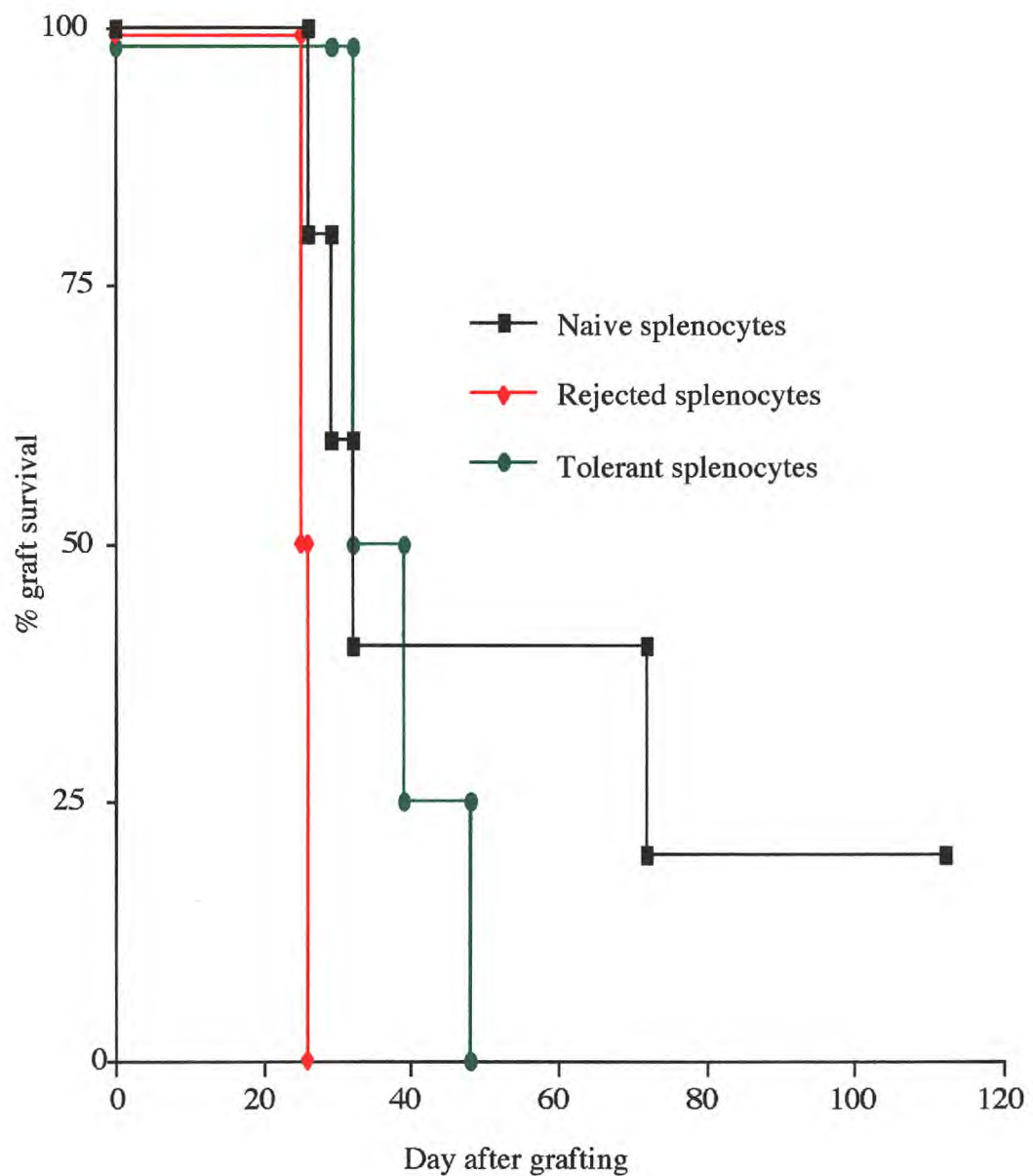


Figure 6.7. Adoptive transfer of 3×10^7 unsorted splenocytes from tolerant female C57BL/6 mice into naive adult females does not transfer tolerance. B6 female mice were given 3×10^7 splenocytes i.v. from naive females, females that had rejected a male skin graft at day 20 or females that had intact male skin grafts at time of sacrifice (day 128).

from C57BL/6 female mice. Figure 6.6 a) shows that in a naïve female there are less than 0.1% CD8⁺/tetramer⁺ cells as has been previously described (Millrain, Chandler et al. 2001). Mice were taken at day 128 post skin graft and figure 6.6 c) shows splenocytes from a female mouse that had rejected a male skin graft after treatment with male thymocytes, rejecting around day 28-30 after grafting. As a consequence, the population of tetramer positive cells has decreased after rejection of the graft to 0.35%.

Figures 6.6 b) and d) show analyses of splenocytes taken from two different mice that at day 128 had a surviving skin graft. It can be seen that the populations of tetramer positive cells are 0.11 and 0.14% of CD8⁺ T cells, slightly higher than that seen in 6.6 a), but less than a mouse that had rejected a male graft. This suggests that the mechanism of tolerance in these mice may be failure of the CD8⁺ population to expand, although a small population remains and is unable to respond to the presence of the male graft. It is possible that the small population detected could be either a regulatory population or background.

In order to examine whether tolerance was transferable and whether a regulatory population exists, 3×10^7 splenocytes were adoptively transferred into naïve adult female mice i.v., either from naïve female mice, female mice that had previously rejected a male graft (as in figure 6.6 c) or from females with a surviving male graft (tolerant-figure 6.6 b) and d)). Three days after injection, the mice were grafted with C57BL/6 male skin. Figure 6.7 shows the rejection responses of mice receiving adoptively transferred splenocytes. It is clear the mice receiving splenocytes from a mouse that had previously rejected a male graft, reject grafts between 20-30 days, indicating an accelerated rejection response. This suggests that within the transferred splenocytes was a memory population of T cells, capable of transferring a memory response to previously unchallenged mice. However when either tolerant splenocyte or naïve splenocytes are transferred, rejection occurs in 30-50 days, although two mice receiving naïve splenocytes maintained grafts to around 75 and 115 days respectively. Both groups are indicative of an unprimed response, suggesting that while graft rejection is not accelerated as in those receiving splenocytes from mice that have rejected male grafts, either a memory response is not present or it is being inhibited. However the tolerance does not appear to be transferred in this experiment and is consistent with the failure of CD8⁺ T cells to expand as previously discussed.

6.7: Tracing the fates of apoptotic thymocytes *in vitro* and *in vivo*

To gain insight into the mechanism of tolerance using apoptotic thymocytes, it is important to determine which APCs are involved in uptake of antigen and cross-presentation. DCs, particularly immature DCs and macrophages are capable of phagocytosing, processing and presenting antigen derived from apoptotic cells (Platt, Suzuki et al. 1996; Bellone, Iezzi et al. 1997; Albert, Pearce et al. 1998; Albert, Sauter et al. 1998; Inaba, Turley et al. 1998; Platt, da Silva et al. 1998; Harshyne, Watkins et al. 2001; Heath and Carbone 2001; Scott, McMahon et al. 2001).

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a commonly used fluorescent dye that intercalates into the plasma membrane and is used to examine cell migration and proliferation (Graziano, St-Pierre et al. 1998; Parish 1999). To determine which APCs phagocytose apoptotic thymocytes *in vitro*, thymocytes were cultured as before with 100nM dexamethasone for 5 hours before washing and labelling with CFSE. Equal numbers of CFSE-labelled apoptotic thymocytes or untreated thymocytes +/- 100nM dexamethasone were co-cultured overnight with T cell-depleted splenocytes (enriching for B cells), day 7 cultured BMDCs, splenic DCs or peritoneal macrophages. The following day the cultures were harvested and stained with B220, CD11c or CD11b respectively as APC markers. As controls, the samples were also stained with CD4 to mark unphagocytosed thymocytes.

Figure 6.8 a), c) and e) shows peritoneal macrophages that have been co-cultured overnight with CFSE-labelled untreated, apoptotic thymocytes, or untreated thymocytes with 100nM dexamethasone. With untreated thymocytes 5.1% of the CFSE labelled cells are positive for CD11b expression, suggesting uptake of CFSE-labelled membrane by macrophages in the culture (Figure 6.8 a). In comparison, if the thymocytes were pre-treated (Figure 6.8 c) with dexamethasone to induce apoptosis, 15% of the CFSE positive cells are now CD11b positive, indicating increased uptake by macrophages. However if thymocytes were untreated but were co-cultured in the presence of dexamethasone (Figure 6.8 e), the degree of uptake increases to 20.3%, suggesting that the presence of dexamethasone in culture does not inhibit uptake of antigen. The relative absence of CD11b positive cells from these figures can be explained by the gating of the FACS analysis. In this experiment, the figures were gated using a lymphocyte gate to examine the degree of CD11b staining on CFSE positive cells. This would exclude the majority of CD11b positive macrophages except for those that were CFSE positive. If this experiment were reanalysed, it would also be useful to

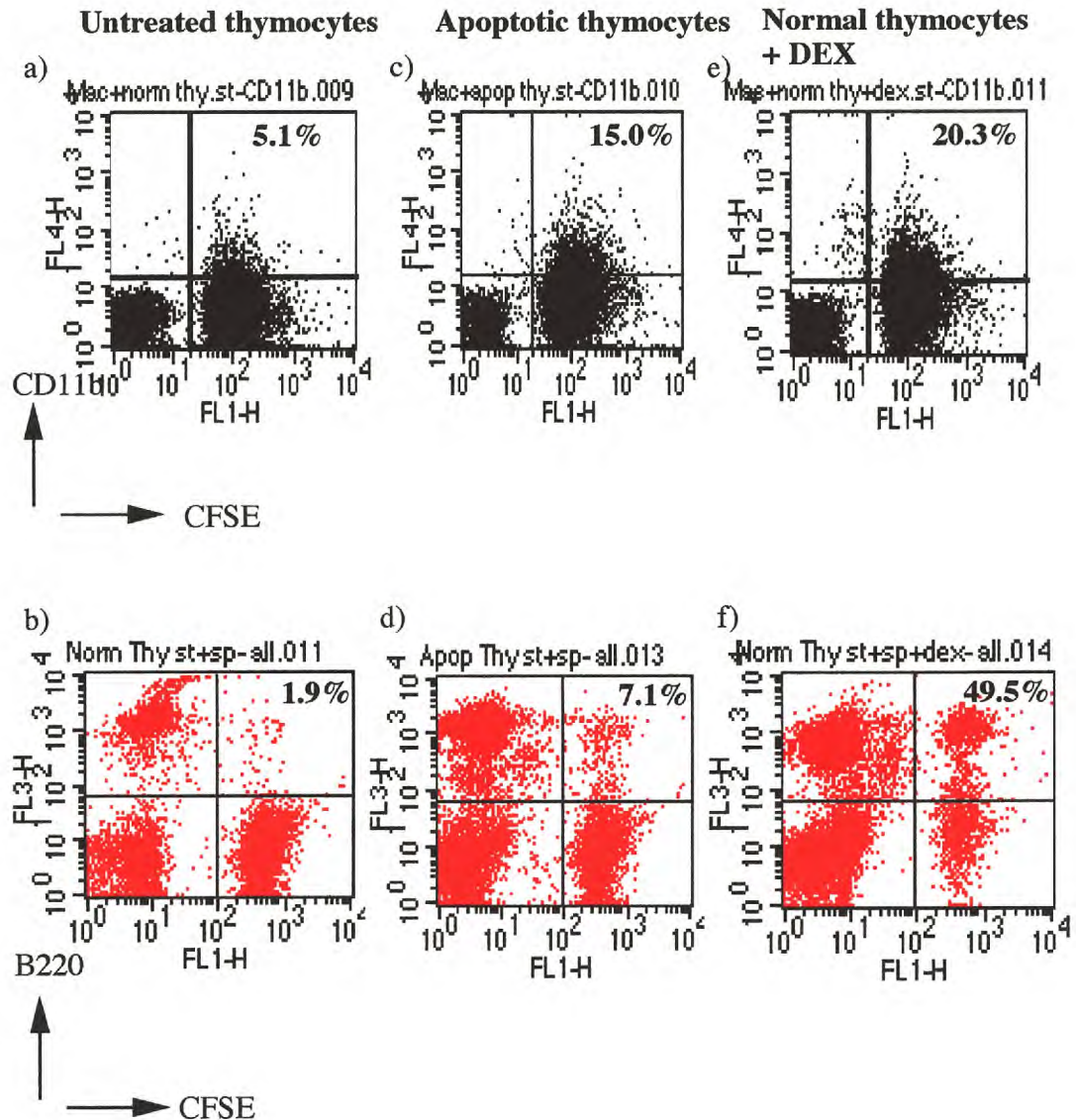


Figure 6.8. Overnight culture of CFSE labelled thymocytes (untreated, apoptotic or untreated + dexamethasone overnight) with either peritoneal macrophages or B cells. Numbers indicate the % of CFSE labelled cells (thymocytes) that have become either CD11b+ or B220+. Thymocytes were cultured overnight with a), c), e): peritoneal macrophages or b), d), f): T cell depleted splenocytes, then stained with either B220 or CD11b and analysed by flow cytometry.

examine the CD11b positive population and look for the degree of CFSE staining within that population.

Figure 6.8 b), d) and f) shows T cell-depleted splenocyte/thymocyte co-cultures. If thymocytes are untreated prior to co-culture, only 1.9% of thymocytes are B220 positive with overnight co-culture, indicating potential uptake by B cells *in vitro* (6.8 b). If thymocytes were pre-treated with dexamethasone prior to co-culture, 7.1% of CFSEpositive cells have become B220 positive suggesting more than a three-fold increased uptake compared to untreated thymocytes. Finally if dexamethasone was added to the co-culture overnight, nearly 50% of the CFSE positive thymocytes stain for B220, indicating increased uptake by B220 positive cells *in vitro*. Again the increased uptake observed in the presence of dexamethasone suggests that dexamethasone is not inhibiting phagocytosis or pinocytosis.

Using either BMDCs or splenic DCs in co-cultures yielded little information about uptake *in vitro*. Although the experiment was repeated 3-4 times, it proved difficult to show that DCs were becoming CFSE positive during the overnight co-culture. Cultured BMDCs and splenic DCs are highly autofluorescent making gating and compensation in FACS analysis difficult. Commonly, CD11c-PE fluorescence 'spills' over into the other fluorescent colours such that it became difficult to determine increases in a double-positive population. It was possible to show that CFSE-positive, CD11c-negative cells (thymocytes) disappear in culture with DCs, but it was difficult to show that DCs had become CFSE positive (data not shown).

These data suggest that at least *in vitro*, macrophages and B cells are involved in uptake of thymocytes, although it has been difficult to draw any conclusions about the role of DCs. It is established that macrophages and DCs are involved in uptake and clearance of apoptotic cells *in vivo* and *in vitro* (Platt, Suzuki et al. 1996; Albert, Sauter et al. 1998; Fadok, Bratton et al. 2001; Leverrier, Lorenzi et al. 2001; Scott, McMahon et al. 2001), but there is little information on the role of B cells. B cells are inefficient at fluid-phase pinocytosis but in compensation, have antigen-specific receptors for uptake of foreign antigens. Alternatively it has been shown that B cells can take up antigen coated with complement components such as C3dg, in a process mediated by complement receptor type-2 (CD21) (Hess, Schwendinger et al. 2000).

Although these data suggest that both macrophages and B cells play an important role in uptake of apoptotic cells *in vitro*, it is unclear whether this occurs *in vivo* or is simply an *in vitro* artefact. In order to examine this, a time-course experiment was performed. C57BL/6 male thymocytes were treated *in vitro* overnight with 100nM

dexamethasone, washed and then labelled with CFSE before injecting 1×10^7 cells i.v. into a group of C57BL/6 female mice. Other mice received untreated, labelled thymocytes i.v. +/- 0.1mg dexamethasone i.p. as in the tolerance-inducing experiments. 1, 3, 6, 24 hours and 1 week after injection, two mice from each group were sacrificed and the spleen, thymus and mesenteric LN removed. The samples were then stained with CD11c, CD11b and B220 or CD4 to examine the cell populations involved in uptake of apoptotic and untreated thymocytes *in vivo*. The percentages of CFSE-positive, APC-marker positive cells were calculated and the means plotted against time. An untreated mouse was used as a control to determine background. Figure 6.9 a) shows the percentage of splenic CD11b positive (high) cells (macrophages) that have become CFSE positive with increasing time in the spleen. Interestingly, while both untreated male thymocytes and untreated thymocytes together with an injection of dexamethasone show around 20% of CD11b positive cells have become CFSE positive within the first hour, there are no CFSE stained CD11b positive cells when apoptotic thymocytes were used. In this experiment the background is approximately 6%. With increasing time, the percentage of double positive cells decays suggesting that the CFSE is being degraded or diluted out by influx or efflux of macrophages from the spleen.

To examine whether CFSE-labelled cells can be detected in the LN having trafficked from the spleen, the mesenteric LN were also examined. Figure 6.9 b) shows CD11b⁺CFSE⁺ cells in the mesenteric LN when treated with apoptotic, normal or normal with dexamethasone thymocytes. In this figure, the degree of CFSE found in the LN is much lower than that in the spleen, suggesting as may be expected that when given i.v., thymocytes are preferentially carried to the spleen, possibly by migrating macrophages, rather than to the LN where they are in such small numbers that they are almost undetectable.

These data are interesting, as they suggest that while macrophages may be involved in the uptake of untreated thymocytes *in vivo*, either apoptotic thymocytes do not efficiently label with CFSE, are more rapidly phagocytosed and digested in macrophages, or are less efficiently phagocytosed by macrophages when apoptotic. The first possibility is unlikely, as apoptotic thymocytes have previously been stained with CFSE and examined *in vitro* (Figure 6.8 and data not shown) and were shown to stain effectively. Figure 6.8 suggests that apoptotic thymocytes are intact after overnight incubation *in vitro*, while other data has shown that the overnight treatment does not kill the cells and that they stain effectively. Macrophages are capable of phagocytosing apoptotic granulocytes rapidly (Fadok, Bratton et al. 2001), while murine macrophages

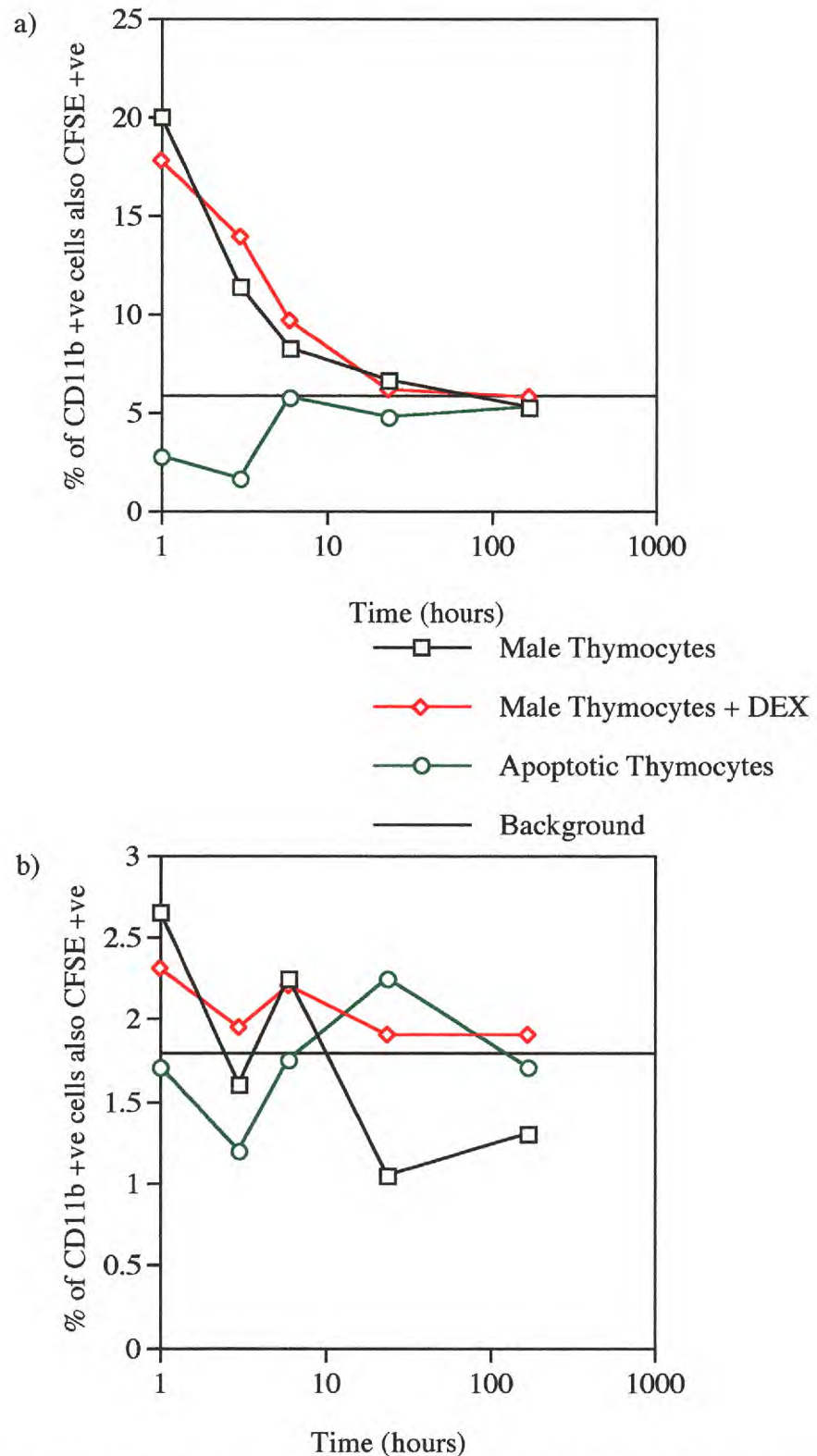


Figure 6.9. Macrophage time course experiment. CFSE-labelled male thymocytes were injected and two mice sacrificed at each time-point. Spleen and LN were harvested, fixed and stained with CD11c, CD11b and B220. Mean % of CD11b+ (Macrophages) CFSE +ve after injection with 10^7 thymocytes (2 mice/group). a) Spleen, b) Mesenteric lymph nodes. Background is a mouse not treated with thymocytes.

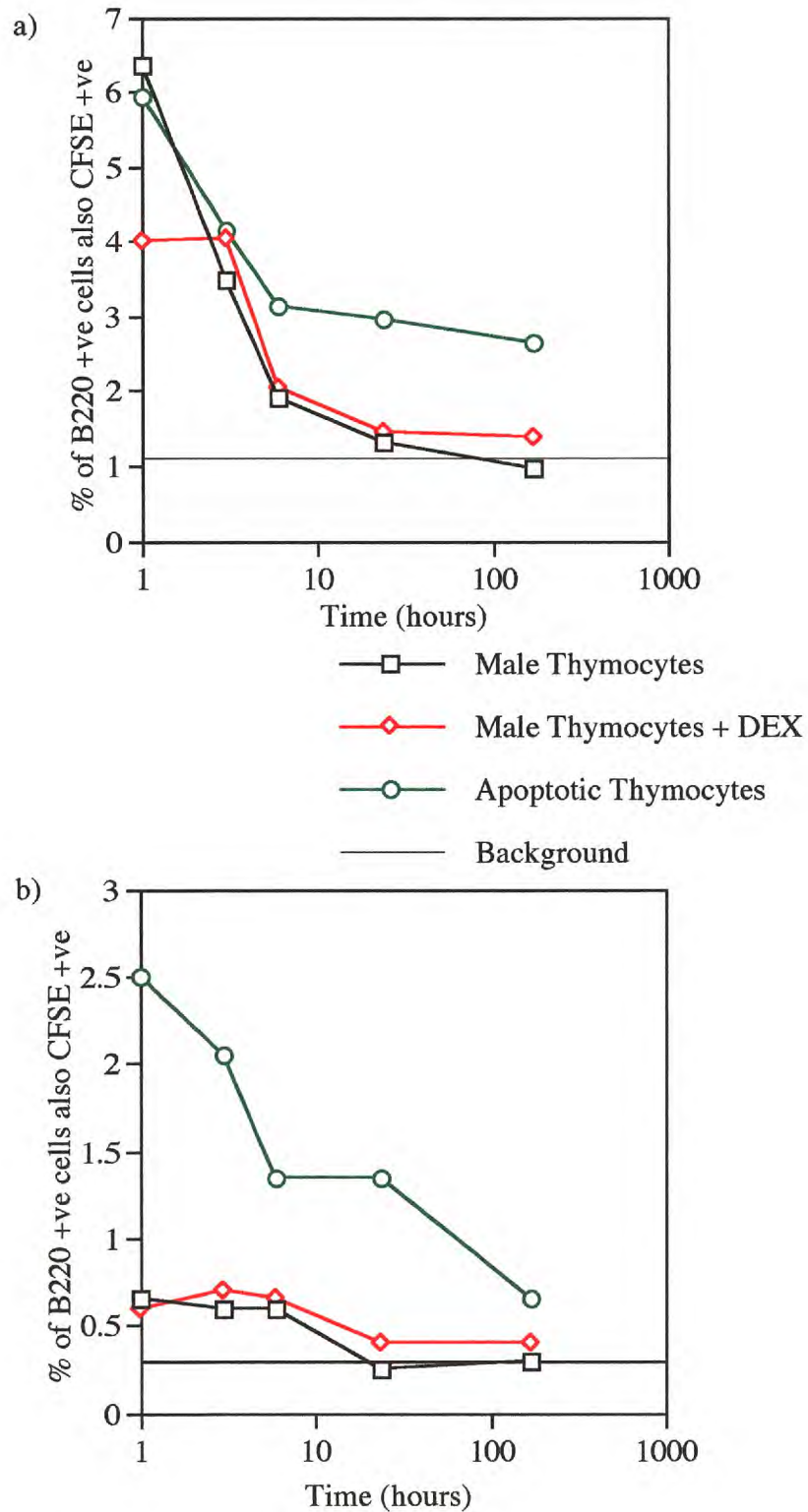


Figure 6.10. B cell time course. CFSE labelled male thymocytes were injected into female mice i.v. Two mice were sacrificed at each time-point, spleen and LN removed, fixed and stained with CD11c, CD11b and B220. Mean % of B220+ (B cells) CFSE +ve after injection with 10^7 thymocytes (2 mice/group). a) Spleen, b) Mesenteric lymph nodes. Background is an untreated mouse.

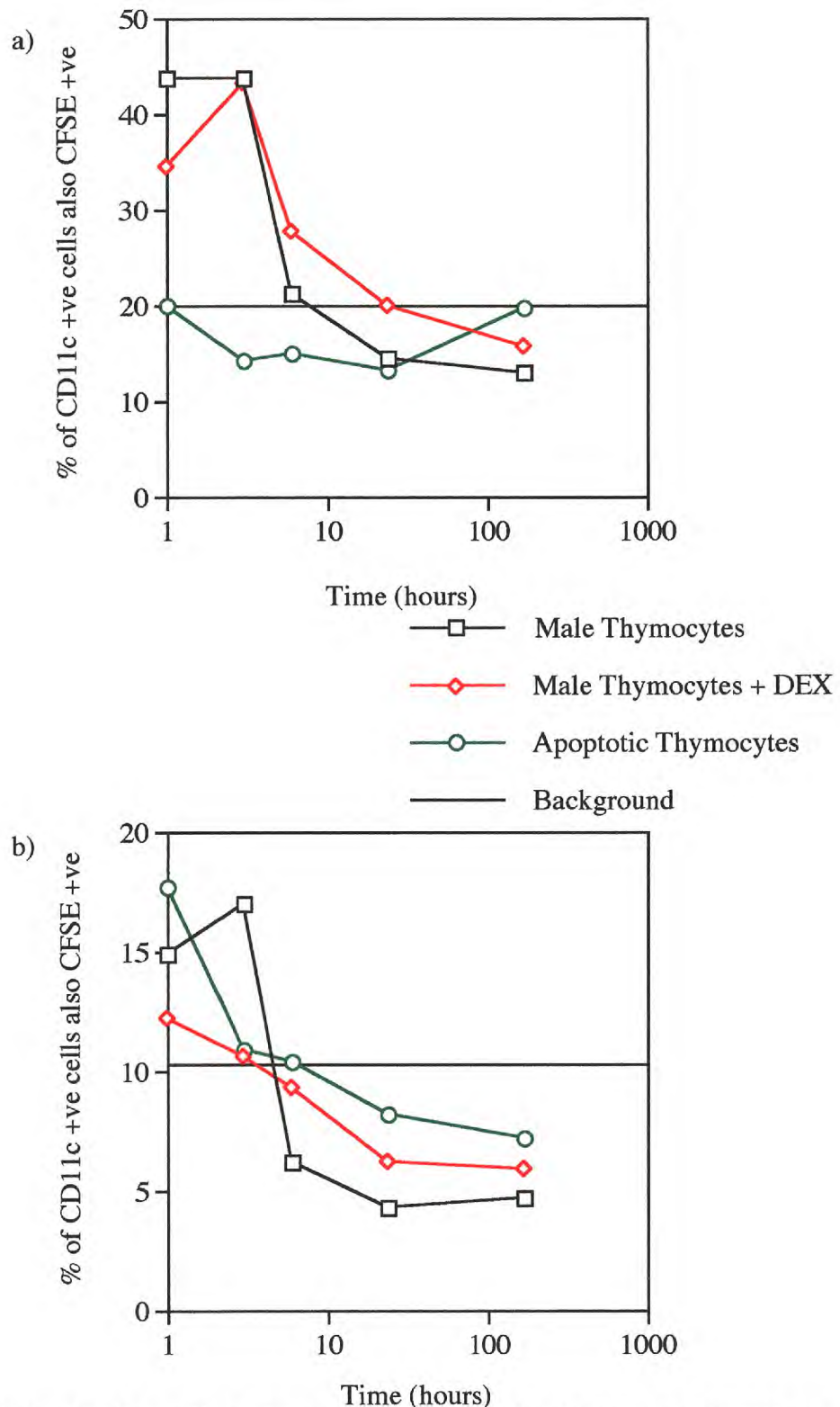


Figure 6.11. Dendritic cell time course experiment. C57BL/6 male thymocytes were taken and either used untreated or cultured with 100nM dexamethasone for 4-5 hours prior to injection. CFSE labelled thymocytes were injected i.v.(+/- 0.1mg DEX i.p.) and two mice sacrificed at each timepoint. Spleen and LN were harvested, fixed and stained with CD11c, CD11b and B220. Mean % of CD11c+ve cells (DCs) also CFSE +ve after injection with 10^7 thymocytes. a) Spleen, b) Mesenteric lymph nodes.

efficiently phagocytose apoptotic human THP-1 cells (a macrophage line) (Kurosaka, Watanabe et al. 1998). Platt *et al* (Platt, Suzuki et al. 1996) showed that steroid-treated apoptotic thymocytes were phagocytosed *in vitro* in a scavenger receptor A (SRA)-mediated pathway that was blocked by addition of an anti-SRA antibody or in mice in which the SRA gene had been disrupted. The macrophages examined by Platt were thymic macrophages, mimicking *in vitro* the uptake of apoptotic thymocytes that have died during positive/negative selection. It is possible that thymic macrophages may have peculiar thymocyte-tropic properties, compared with splenic macrophages. It is possible that macrophages in the spleen may phagocytose and degrade apoptotic thymocytes more rapidly than if they are untreated or treated with dexamethasone *in vivo*. While untreated (or *in vivo* treated) CFSE-labelled cells can be detected in macrophages in the spleen, it is possible that they are phagocytosed more slowly than those that are already apoptotic and are consequently degraded more slowly. *In vivo* treatment with dexamethasone is unlikely to have induced apoptosis of the thymocytes in one hour and so those thymocytes can essentially be considered untreated. However it is also likely that dexamethasone may also be having other immunosuppressive effects *in vivo*. It is also possible that as in this experiment the thymocytes were not depleted of MHC class II positive cells prior to injection, contaminating thymic macrophages are being detected, although this would also apply to the apoptotic thymocytes.

Figure 6.10 a) shows analysis of CFSE⁺B220⁺ cells in the spleen and although the percentages of CFSE⁺B220⁺ cells are lower than those seen in figure 6.9 a), populations of double positive cells are detectable with all treatments in one hour. When treated with apoptotic or untreated thymocytes, approximately 6% of B220 positive cells are CFSE positive one hour after treatment. If the female mice are simultaneously injected with untreated thymocytes i.v. and 0.1 mg dexamethasone i.p., the percentage is around 4%. Comparing the three treatments both untreated and untreated with dexamethasone decay to almost background by the end of 1 week *in vivo*. However if given apoptotic thymocytes, although the initial percentage of double positive cells is similar to that seen with untreated cells, the decay of CFSE staining occurs more slowly. This is interesting as it suggests that B cells may be capable of phagocytosis or receptor-mediated uptake of CFSE from apoptotic thymocytes *in vivo*, similar to that previously shown *in vitro* (Figure 6.8). When mesenteric LN were examined in these mice (Figure 6.10 b) a clear difference can be seen between apoptotic and untreated thymocytes. Comparing the degree of staining with that seen from CD11b⁺ cells (Figure

6.9 b), the percentage of double stained cells are similar but in figure 6.10 b) the background is much lower, less than 0.5% compared to approximately 1.75% of CD11b⁺ cells. In figure 6.10 a), while both untreated and untreated plus dexamethasone thymocytes are detectable at around 0.6%, decaying to background in 1 week, apoptotic thymocytes are detectable at 2.5% after 1 hour, only decaying to background by 1 week. Although similar percentages of double positive cells were observed in figure 6.9 b), the background was significantly higher, such that there was no difference between treated and untreated control mice. However as the background is much lower in figure 6.10 b), although the percentage of B220⁺CFSE⁺ cells when apoptotic is similar, compared to background there is a large difference. These data suggest that B cells staining with CFSE from both untreated and apoptotic thymocytes can be detected in female spleen in one hour, while apoptotic thymocyte CFSE can be detected up to a week after introduction. In the mesenteric LN, CFSE can only be detected when apoptotic thymocytes were introduced, suggesting either trafficking of B cells containing apoptotic material from spleen to LN or preferential transfer of apoptotic material to the mesenteric LN compared to untreated thymocytes. This is consistent with previous data *in vitro* (figure 6.8 b, d, f) showing that B cells appear to be capable of preferentially phagocytosing apoptotic as opposed to untreated thymocytes, although the mechanism of uptake is unclear. It would be interesting to examine the uptake of apoptotic thymocytes in CD21 or CD91 (α2-macroglobulin)-deficient mice as it has they have been shown to be involved in antigen uptake by B cells and macrophages respectively (Hess, Schwendinger et al. 2000; Ogden, deCathelineau et al. 2001), although CD21-mediated uptake by B cells is dependent on coating of the antigen before uptake with complement components. Similarly it would also be interesting to examine B cells for the surface expression of other phagocytic markers such as SR-A and CD91 to further examine other potential molecules that may be involved in thymocyte uptake.

Figure 6.11 a) shows the analysis of CD11c⁺CFSE⁺ cells in the spleen of treated female mice as described. Compared to both figures 6.9 and 6.10, the percentage of double positive cells is much higher, with the maximum detected using untreated male thymocytes at approximately 45% while the background is also higher at around 20%. In DCs, as in macrophages (Figure 6.9 a), CFSE can be detected in splenic DCs after one hour when the mice were given untreated thymocytes or untreated thymocytes plus dexamethasone *in vivo*. CFSE was detectable in CD11c high cells above background up to 6 hours after injection but not further into the time-course. After 24 hours and 1 week, no CFSE above background was detected. Similar to that seen in macrophages,

CD11c⁺ cells are not CFSE positive when apoptotic thymocytes are injected, although the background is higher in CD11c⁺ cells. With increasing time *in vivo*, the level of CFSE decreases. Flow cytometry of the mesenteric LN (Figure 6.11 b) again has higher background than the other stains at approximately 10%. However in the LN it can be seen that in one hour, CFSE from all thymocyte treatments can be detected, although this was higher with apoptotic and untreated thymocytes than when given dexamethasone *in vivo*. The CFSE decreases with increasing time *in vivo* in all samples tested. CFSE staining can be detected above background for the first two hours in all three curves, although CFSE from untreated male thymocytes peaks three hours after thymocyte administration, while with the other two treatments, CFSE is close to background at this point. These data suggest that either apoptotic thymocytes are phagocytosed more quickly than untreated thymocytes *in vivo* by DCs or are not phagocytosed detectably in the spleen. Compared to CD11b and B220, CD11c⁺ cells have higher levels of CFSE stain in all cases, suggesting better phagocytosis of all cell types. In the LN, CFSE is also detected in CD11c⁺ cells more than CD11b⁺ or B220⁺ cells, suggesting phagocytosis by DCs. It is possible that thymocytes were phagocytosed *in situ* by LN DCs or underwent phagocytosis in the spleen and were subsequently trafficked to the LN.

To determine whether staining and non-specific binding of antibody to thymocytes may have influenced any of the above results, thymocytes were treated with dexamethasone for 5 hours as before and stained with CD11c, CD11b and B220 and compared with untreated thymocytes. Figure 6.12 a) compares unstained apoptotic thymocytes and shows that they do not have inherent autofluorescence for any of the three wavelengths tested. However when treated with dexamethasone for 5 hours, a population of 1.8% of thymocytes stain positive for B220. Untreated thymocytes show a population of 1.5% of thymocytes that are B220 positive when stained with the three antibodies, similar to that seen with untreated thymocytes co-cultured *in vitro* (Figure 6.8 b). It is possible that this may be due to contaminating B cells from peripheral blood or B cells in the thymus and suggests that some of the B220 positive cells observed in the previous figures may have been of thymic rather than splenic or LN origin. While this may have influenced, to a limited extent, the flow cytometry analysis of the mechanism of apoptosis and tolerance due to non-depletion of MHC class II positive cells from the thymocytes, in the tolerance induction/grafting experiments thymocytes were depleted of MHC class II positive cells prior to introduction.

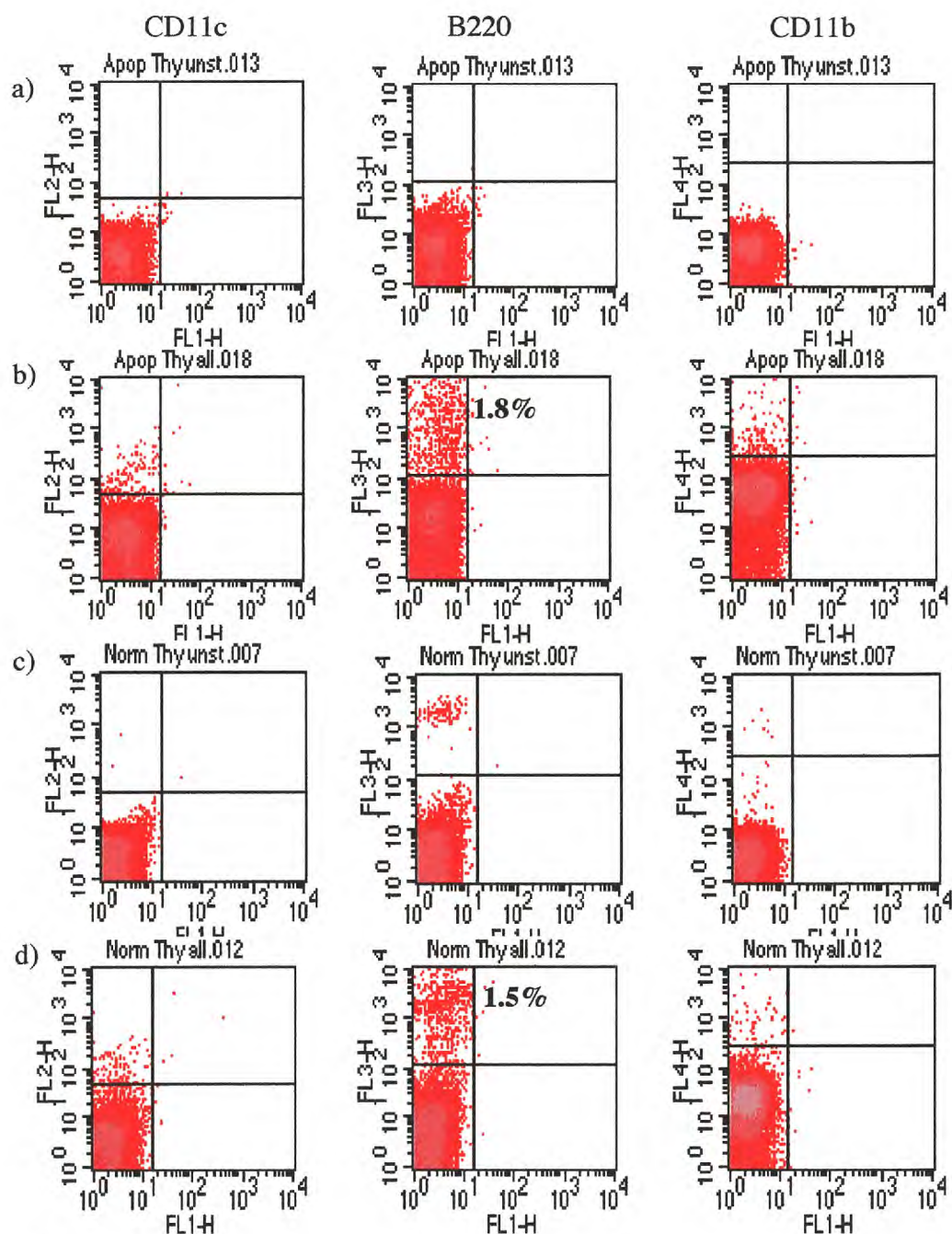


Figure 6.12. Flow cytometry of a) apoptotic male thymocytes- unstained, b) apoptotic thymocytes stained for CD11c, B220 and CD11b, c) untreated male thymocytes- unstained and d) untreated thymocytes stained for CD11c, B220, and CD11b. This shows that a small population of thymocytes, both untreated and apoptotic stain for B220, which may indicate contaminating B cells, and to a lesser extent CD11b.

Many groups have shown that DCs are capable of phagocytosis of apoptotic material and presentation of antigen (Albert, Pearce et al. 1998; Albert, Sauter et al. 1998; Inaba, Turley et al. 1998), however it is controversial whether uptake of apoptotic cells allows a functional immune response to develop. Uptake, processing and presentation of antigen from apoptotic cells is capable of stimulating CTL responses (Bellone, Iezzi et al. 1997; Albert, Pearce et al. 1998; Albert, Sauter et al. 1998; Propato, Cutrona et al. 2001), while others have shown that on phagocytosis of apoptotic cells, macrophages produce proinflammatory cytokines (Kurosaka, Watanabe et al. 1998). In contrast others have suggested that treatment with, or phagocytosis of apoptotic cells may be immunosuppressive or tolerogenic (Kao 1996; Voll, Herrmann et al. 1997; Steinman, Turley et al. 2000; Bittencourt, Perruche et al. 2001; Fadok, Bratton et al. 2001; Kao, Huang et al. 2001; Magnus, Chan et al. 2001). One possible reason for this dichotomy is potential differences in induction protocols and characterisation of apoptotic phenotype. Many assays can be used to determine apoptosis including Annexin V staining for flipped phosphatidyl serine, TUNEL staining and examination of DNA laddering. It is likely that if different criteria are used to define apoptosis, the cell populations will not behave equivalently. Further, if apoptosis proceeds rapidly, secondary necrosis may commence *in vivo*.

Interestingly, of the studies showing that apoptosis could induce immune responses, one used influenza-infected, apoptotic monocytes fed to DCs, to stimulate influenza-specific CTL (Albert, Sauter et al. 1998). Although CTL were stimulated, the presence of virus may have provided DC maturation signals and cell adhesion by expression of haemagglutinin. While the authors did not discount the release of heat shock proteins (HSPs) by the apoptotic cells inducing DC maturation, a further study showed that stressed-apoptotic tumour cells released HSPs, causing subsequent anti-tumour responses (Feng, Zeng et al. 2001).

It is clear that different kinds of cell death have different effects on immune responses, with necrotic death releasing heat shock proteins (Gallucci, Lolkema et al. 1999; Feng, Zeng et al. 2001; Somersan, Larsson et al. 2001) or intracellular proteases (Fadok, Bratton et al. 2001), reminiscent of 'danger-signals' (Matzinger 2001) initiating release of inflammatory cytokines, induction of DC maturation and activation (Somersan, Larsson et al. 2001) and induction of an immune response. On the other hand, apoptosis is thought to be immunosuppressive (Voll, Herrmann et al. 1997) or tolerogenic (Steinman, Turley et al. 2000) and uptake of antigen in a non-inflammatory environment does not induce DC maturation. Many groups have shown that immature

DCs are highly phagocytic (Albert, Pearce et al. 1998), while they are also tolerogenic (Steinbrink, Wolfl et al. 1997; Jonuleit, Schmitt et al. 2000; Lutz, Kukutsch et al. 2000; Matyszak, Citterio et al. 2000; Corinti, Albanesi et al. 2001; Dhodapkar, Steinman et al. 2001), by altered cytokine secretion, induction of anergy, apoptosis or regulatory T cell populations such as $CD4^+CD25^+$ (Shevach 2001). It has also been suggested that apoptosis may be a mechanism for maintaining unresponsiveness to environmental antigens in the intestine (Bu, Keshavarzian et al. 2001).

It is possible that treatment with apoptotic cells involves phagocytosis of antigen and presentation by DCs while not inducing their activation and maturation and that subsequent presentation of the antigen by immature DCs can induce tolerance to HY antigen. On the other hand, if as is suggested by the *in vitro* and *in vivo* tracking experiments, apoptotic cells are preferentially phagocytosed and maintained in B cells in the spleen and LN, these may be unable to induce an effective immune response, being unable to prime a naïve response and thereby be tolerogenic. It is possible that by introducing HY antigen in an apoptotic, non-inflammatory environment in a 'Trojan-Horse' mechanism, this allows uptake of antigen prior to challenge with a skin graft in the absence of 'danger' inducing a degree of tolerance in female mice.

This study is not complete and other work will be done to further examine the mechanisms of tolerance being induced by introduction of apoptotic thymocytes. Previous work has used irradiated apoptotic PBMCs to affect bone marrow engraftment, inhibiting GvHD although this was into sublethally irradiated recipients (Bittencourt, Perruche et al. 2001) and interestingly was shown to function independently of the source of the apoptotic cells as well as the method of tolerance induction. Others showed that irradiated PBMCs could induce tolerance (lack of antibody responses) across major histocompatibility barriers (Kao 1996) through development of transferable regulatory $CD4^+$ T cells, although it was unclear whether they were $CD25^+$ (Kao, Huang et al. 2001). It would be interesting to repeat the tolerance transfer experiments with larger groups of mice, comparing of the ability of different populations of splenocytes to transfer, whether $CD4^+$ ($CD25^+$, $CD25^-$ or both).

Further work addressing mechanism would involve introduction of CFSE-labelled apoptotic thymocytes, followed by microscopic analysis of spleen sections, using confocal microscopy. This would allow sections to be stained with different fluorescent antibodies and identify CFSE-labelled apoptotic bodies or fragments inside APCs. A number of groups have shown that it is possible to visualise uptake of fluorescent material by APCs including Harshyne *et al* (Harshyne, Watkins et al. 2001)

who labelled various cell types with two different fluorescent markers, either labelling the plasma membrane or the cytoplasm and showed uptake of antigen by immature DCs by flow cytometry. Subsequently, confocal microscopy was used to examine the phagocytic cells and determine that the APCs had ingested fluorescently labelled material from live cells and that after ingestion, the donor cells remained viable.

It would be interesting to determine whether the degree of tolerance induction could be further improved by examining closely the degree of apoptosis undergone by thymocytes prior to administration. It is possible to perform flow cytometry time-course experiments to ascertain the length of time necessary to initiate phosphatidyl serine flipping and subsequent propidium iodide staining in thymocytes after treatment with a number of different apoptotic agents. In this study dexamethasone and calcimycin have been used and it is possible that agents such as anti-Fas antibodies could be used to induce apoptosis. Other experiments that could be of interest would be to examine the liver as the potential site of clearance for apoptotic thymocytes. It is known that T cells undergoing apoptosis at the end of an immune response migrate to the liver, either to die or to be killed (Crispe, Dao et al. 2000). It is also known that administration of antigen into the hepatic portal vein can be tolerogenic, which may be another possible explanation for the tolerogenic effect seen on administration of apoptotic thymocytes intravenously. It would also be of interest to examine cytokine production on administration of apoptotic thymocytes compared with untreated thymocytes. It may be that immunosuppressive cytokines are secreted by T cells that reduce the effectiveness of APCs at stimulating an immune response through indirect presentation of antigen. This could be done both by *ex vivo* cytokine analysis and either use of neutralising anti-cytokine antibodies or various cytokine knockout animals. Together these experiments may provide more detail about the mechanism of tolerance being induced in this context.

While this particular protocol using apoptotic thymocytes prior to a graft could not be applied in a clinical setting directly, it is important to underline the potential applications of this work. Further work using apoptosis induction prior to a transplant could be beneficial if other cell types could be used. In previous work irradiated, apoptotic splenocytes were injected prior to a bone marrow graft (Bittencourt, Perruche et al. 2001). It would be useful to examine the potency of immunosuppression of different types of cells as well as different methods of apoptosis induction. In a clinical setting it may be possible to improve transplant survival by transfusing a patient with

apoptotic T cells or whole PBL from a donor and thus inducing a degree of tolerance, some time prior to transplantation, be it of solid organs or bone marrow.

6.8: Summary

The synthetic glucocorticoid dexamethasone has a number of immunomodulatory effects including suppression of responses, inhibition of DC maturation and also induction of thymocyte apoptosis both *in vitro* and *in vivo*. Through Annexin V staining, it has been possible to determine the dose of dexamethasone necessary as well as the length of time needed culturing *in vitro* in order to induce maximum apoptosis of male thymocytes in the shortest possible time. Treatment of C57/BL6 female mice with untreated B6 male thymocytes immunises, leading to rapid rejection of subsequent male skin grafts. However if male thymocytes are treated *in vitro* or *in vivo* to induce apoptosis, this leads to prolongation of graft survival and in a number of mice, tolerance. Treatment of female CBA mice with B6 male thymocytes prior to a B6 male graft does not enhance graft survival. Use of another apoptosis-inducing agent, calcimycin, does not induce tolerance but in fact appears to prime to a similar extent to that seen with untreated male thymocytes. It is possible that calcimycin treatment induces rapid apoptosis and subsequent secondary necrosis, before administration *in vivo*, such that maturation stimuli for recipient APCs are present at the time of phagocytosis.

Initial studies to determine the mechanisms of tolerance have suggested that it cannot be transferred to naïve adult females while tracking experiments have shown that in the spleen and to some extent the mesenteric LN, CFSE from apoptotic thymocytes can be found particularly in B cells but also in DCs. It is possible that the mechanism of tolerance is through the uptake and presentation of antigen by B cells or immature DCs in the absence of a maturation signal, although further work would allow this to be characterised in greater detail.

Chapter 7.

General conclusions

In this thesis, I have analysed a number of factors potentially controlling the development and regulation of T cells. T cell development and regulation involves many complex and interlinked factors. Firstly during thymocyte development and TCR selection, secondly, as naïve T cells in the periphery, thirdly, during activation by APCs to become effector T cells and finally, cell death and removal.

During the early stages of T cell development in the thymus, one of the major control points is the selection of a functional TCR enabling interaction with self-MHC/peptide complexes with sufficient avidity to survive positive selection, but with low enough avidity not to be deleted in negative selection. The 'avidity window' of selection has been examined to determine whether altering avidity thresholds can influence the structural and possibly functional outcomes of T cell selection. The B-Asp mouse expresses a mixed-haplotype MHC class II molecule at low levels, in the absence of other matched-haplotype molecules, offering a unique opportunity to examine the repertoire of the small population of CD4⁺ T cells selected. In out-bred human populations, mixed-haplotype MHC molecules occur naturally, but it has not been possible to determine whether they are capable of selecting T cells

The TCR repertoire selected on mixed-haplotype, low surface expression MHC class II molecules has been examined in two ways, first by flow cytometry of V α and V β gene segments found on peripheral T cells, and secondly by detailed sequence analysis of the CDR3 β region of both thymocytes and post-selection peripheral CD4⁺ T cells. Comparisons of V α and V β usage, with normal littermate controls and NOD mice have revealed a number of differences in V α and V β usage that could be attributed to low levels of MHC class II being involved in selection. V α 8, which is poorly selected into the CD4⁺ compartment under normal circumstances, is scarcely selected by decreased MHC class II molecules; suggesting that an unfavourable interaction has been made more unfavourable by decreasing the levels of MHC class II. V β analysis has revealed a number of V β segments that have altered expression profiles between the strains, one of which appears to be due to inefficient clonal deletion by *Mtv*, adding further evidence that decreasing the level of MHC class II in selection has structural implications in terms of the TCR repertoire. This may be to compensate for low numbers of interactions by increasing the affinity of the TCRs in order to maintain

moderate avidity and survive selection. Interestingly, reducing MHC class II expression has led to impaired positive selection (V α 8) and impaired negative selection (V β 5.1/5.2).

Sequence analysis of CDR3 β regions from a large set of TCRs containing particular V β regions has provided detailed information about the region of the TCR thought to closely interact with the peptide in the binding groove of the MHC molecule. Analysis has shown that while a number of CDR3 β parameters are altered dramatically comparing the pre-selected TCR pool with that in the periphery, these alterations are not enhanced compared to previously published work between different selecting MHC molecules. Interestingly the length of the CDR3 β regions appears to shorten between thymus and CD4 $^{+}$ peripheral T cells, similar to that previously shown with shorter CDR3 regions in CD4 $^{+}$ than CD8 $^{+}$ T cells (Candeias, Waltzinger et al. 1991).

Together, these analyses suggest that by decreasing the levels of MHC class II involved in selection, structural changes are selected in the TCR repertoire in order to compensate and attain sufficient avidity, as predicted by the avidity theory of T cell selection. Further analyses of B-Asp mice have provided information about the functionality of T cells selected on a mixed haplotype background. Hybridomas were made and these suggested that selection on decreased MHC class II may affect the degree of alloreactivity in the T cell repertoire, while analysis of the cytokine profiles produced by B-Asp and BN KLH-specific cell-lines provided some useful information, however Th1/2 skewing was unclear due to the non-clonal nature of the lines.

Functional analysis of the CD4 $^{+}$ T cells by skin grafting provided useful data concerning the roles of CD8 $^{+}$ and CD4 $^{+}$ T cells as well as the level of MHC class I/II expression in the direct and indirect graft rejection pathways. In the absence of MHC class I and CD8 $^{+}$ T cells, BN, B-Asp and MHC $^{-/-}$ mice are capable of rejecting fully allogeneic grafts expressing both class I and II. Decreasing the level of MHC class II (and hence CD4 $^{+}$ T cells) in the recipient appears to have little effect on survival of fully allogeneic grafts. By decreasing the level of MHC class II on the surface of the grafts, the immunogenicity also decreases in a dose-dependent manner, such that B-Asp grafts are rejected more slowly than grafts with normal levels of MHC class II. It appears that there is close correlation between the level of MHC class I/II on the surface of the graft and the rapidity of rejection, highlighting the importance of both direct and indirect pathways. MHC $^{-/-}$ mice are able to reject fully allogeneic grafts with surprising alacrity, as previously described.

From these experiments it is clear that B-Asp CD4⁺ T cells are functionally active using a number of criteria. T cells selected on mixed haplotype MHC class II molecules are able to proliferate *in vitro* in response to allogeneic stimuli, produce cytokines in response to antigen and are able to mediate graft rejection of both major and also minor mismatched grafts despite their low numbers and the low level of MHC class II expression in the recipient. Importantly this shows the effectiveness of the immune system in responding to non-self, that in the absence of 90% of CD4⁺ and all of CD8⁺ as well as reduced antigen presentation capability, the remaining CD4⁺ T cells are capable of orchestrating functional immune responses.

Use of NOD-Asp-restricted T cell clones has enabled examination of a possible mechanism of MHC class II transfer between cells and formation of hybrid molecules. Although this was complicated by an element of non-specific stimulation, further work using T cell depleted APC populations may shed more light on whether this mechanism operates *in vitro* and *in vivo*. The importance of context, with regard to antigen presentation and T cell function was also examined using the NOD/NOD-Asp and HY systems to compare the relative roles of the direct and indirect pathways in graft rejection. HY recognition can occur both by the direct and indirect pathways of antigen presentation, while recognition of NOD-Asp is thought to be mainly through the direct pathway and therefore independent of presentation by recipient APCs. Injection of NOD female mice with NOD-Asp male splenocytes primed the host T cells to both antigens, such that one treatment induced relatively rapid rejection of grafts containing either antigen in the absence of the other, while when both were combined on the same graft, the process was slightly enhanced.

In an attempt to tolerise, resting purified NOD-Asp male B cells were injected into NOD female mice. FACS analysis showed that the purified B cell population contained less than 1% CD11b or CD11c positive cells. Interestingly, while B cells were clearly able to prime for both HY and NOD-Asp, the efficiency of priming was qualitatively different to spleen. While spleen cell immunisation led to Asp graft rejection with a quicker tempo than male grafts, B cell immunisation led to an enhanced tempo for HY graft rejection, but a reduced tempo for Asp rejection. This effect was most clearly seen when the grafts shared a common bed. Together these data reveal more about the importance of both direct and indirect pathways of graft rejection and also highlight the need for development of therapeutic regimes capable of tackling both pathways in transplantation.

The importance of the APC in regulating T cell responses has been examined using the HY system. The immunosuppressive glucocorticoid, dexamethasone was used to treat dendritic cells *in vitro* and shown to partially inhibit their ability to stimulate a CD8⁺ clone and completely inhibit stimulation of a CD4⁺ T cell clone when used early in culture. However if added later on in the culture, little effect was observed, suggesting as had previously been described that dexamethasone is able to inhibit development and maturation of DCs. Interestingly, the lack of a CD4⁺ response was likely to be due to the absence of MHC class II and a partial decrease in the expression of costimulatory molecules, while MHC class I was little affected. These data suggest that dexamethasone functions by inhibiting DC maturation as previously stated, but showed that one arm of the adaptive T cell immune response was affected more than the other, at least *in vitro*. While removal of dexamethasone and addition of LPS for one day did not completely restore proliferative responses, culturing the cells for longer allowed development of cells capable of stimulating both CD4⁺ and CD8⁺ clones. This suggests that while dexamethasone may inhibit maturation, it is not a permanent block and that on its removal cells are able to develop or differentiate.

It is thought that immature DCs are unable to effectively prime an immune response and this is involved in maintenance of tolerance in the periphery, possibly through inducing populations of regulatory or anergic T cells. Treatment of female mice with dexamethasone-treated 'immature' male DCs i.v. or in the footpad; or female DCs with HY peptides in the footpad was unable to induce long-term tolerance and survival of male allografts. It is possible that removal of dexamethasone from the DCs before injection allows development/maturation *in vivo* and development of functional T cell responses leading to graft rejection.

Finally, apoptosis has been used to manipulate T cell function *in vivo*. It is currently unclear as to whether apoptotic death *in vivo* is stimulatory or inhibitory, as evidence for both has been published. Dexamethasone has been used to induce apoptosis in male thymocytes *in vitro*; and while untreated male thymocytes prime, leading to rapid rejection of a male skin graft by female mice, treatment with apoptotic male thymocytes causes prolongation of graft survival and in some cases long-term tolerance. Treatment with dexamethasone *in vivo* at the time of thymocyte administration has a similar effect, possibly through induction of apoptosis *in vivo*. Perhaps surprisingly, another apoptotic agent Calcimycin does not have the same effect, although it is possible that cell death is more rapid, such that cells may be post-apoptotic on injection.

It is possible that apoptotic cells are immunosuppressive by modulating APC maturation. It was suggested that APCs involved in apoptotic uptake remain immature due to the absence of other activating signals such as inflammation and as previously suggested are tolerogenic. Examination of the mechanism of tolerance induction using apoptotic thymocytes has suggested that it is not transferable to intact adult mice, although whether neonatal or transfer post-irradiation is possible remains to be determined. Uptake of thymocytes *in vitro* suggested that both B cells and macrophages phagocytose apoptotic thymocytes, while DCs have not been shown to. When examined *in vivo* at various time points after treatment, B cells were implicated in the uptake of apoptotic thymocytes, both in spleen and LN while CD11c and CD11b positive cells phagocytose untreated thymocytes. B cells are not thought to be able to prime a naïve response and it may be that if phagocytosed by B cells, antigen is presented on a non-professional APC in the absence of inflammation inducing tolerance. Alternatively it is still possible that the absence of maturation stimuli on apoptotic antigen uptake by other APCs causes antigen presentation by immature DCs and thereby tolerance.

Together, the strands of this project illustrate the complexity and multi-layered regulation of T cell function. T cell responses are modulated by many different factors including: the level of MHC class II involved in T cell selection and subsequent effects on the TCRs selected as well as the APC presenting the antigen, whether professional or non-professional, mature or immature. Related to the maturity of the APC is also the source of the antigen. If provided in an inflammatory environment with activating cytokines and other 'danger' signals as during an infection, the APC will mature and induce appropriate T cell responses, while if there are no activating signals or possibly inhibitors of maturation at the time of phagocytosis, tolerant or non-responsive T cells may be produced.

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