Analysis of the role of CD8β in co-receptor function

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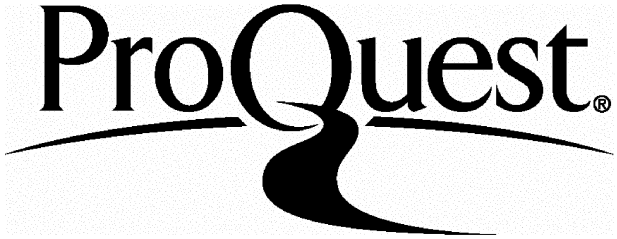

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Abstract

The T cell receptor is found on the surface of the T cell in association with a complex of invariant proteins that are essential for functional signal transduction. One such molecule, CD8, is a co-receptor that binds to invariant parts of the MHC Class I molecule. CD8 comprises a disulphide linked α and β chain. Although the α chain has been shown to interact with the MHC as a CD8αα homodimer, the β chain is implicated in this interaction. The majority of CD8 molecules exist as αβ heterodimers rather than αα homodimers. Previous studies have identified biochemical alterations in CD8β upon T cell activation, predicted to occur in the hinge region between the Ig like domain and the transmembrane domain. In order to study the nature of this region specifically, a transgenic mouse was created expressing an altered CD8β chain. The mutated CD8β chain comprises the Ig like, transmembrane and cytoplasmic domain of CD8β, but the hinge region of CD8α. By comparing functional and biochemical data from these mice with wild type data, the project aims to define how TcR-CD8/ MHC interactions regulate T cell differentiation and activation.

In this thesis it is shown that the mutated CD8β chain cannot restore selection in the thymus when expressed as a transgene on a CD8β knockout background, similarly to mice lacking CD8β altogether. Polyclonal mice were backcrossed onto the *Rag*$^{-/-}$ F5 TcR monoclonal background, where many characteristic maturation markers are comparable with wild type in the mutated mice. However certain phenotypic markers suggest there is a defect in the cells at the double positive stage, where they fail to advance through positive selection. There is a corresponding drop in the percentage and overall numbers of CD8^{+} T cells in the periphery in mice expressing the mutated transgene, similar to that of mice lacking CD8β. Antibodies specific for the Ig-like domain of CD8 were able to bind the chimeric molecule, the stoichiometry of the molecule was comparable to wild type, and that it was able to associate with the signaling molecule LCK. Like wild type, the mutated molecule became a faster

migrating species on activation, probably due to de-sialylation, and data collected using lectins showed that in post translational modification, glycans are attached to the mutated molecule via the same specific linkages as wild type.

Nevertheless there were striking differences between cells expressing the mutated molecule and those expressing wild type CD8αβ. Double positive thymocytes were significantly less able to bind cognate MHC, while the calcium flux in response to TcR/ co-receptor crosslinking was also severely reduced in lymphocytes expressing the mutated molecule compared to wild type. When challenged with antigen peptide the response of lymphocytes expressing the mutated molecule is slightly different to that of wild type cells. This difference in response is also demonstrated when blocking antibodies are included in the assay. Cells expressing the mutated molecule go through more cell cycles and have a higher percentage of $CD25^{hi}$ cells than wild type controls, and blocking antibodies have little effect on this response.

These data suggest that the glycosylation of CD8β has a very specific influence on development and selection in the thymus, and that CD8β could be acting in a regulatory fashion in the periphery.

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Abbreviations

2ME	2-MercaptoEthanol
Ab	Antibody
AICD	Activation Induced Cell Death
AID	Activation-Induced cytidine Deaminase
APCs	Antigen Presenting Cells
APC	AlloPhycoCyanin
APS	Ammonium persulphate
Azide	Sodium azide
β2M	β2-Microglobulin
BcR	B cell Receptor
Bio	Biotin
BM	Bone Marrow
BSA	Bovine Serum Albumin
C	Constant segment
Ca^{++}	Calcium
CD40L	CD40 Ligand
CFSE	5(6)-CarboxyFluorescein diacetate, Succinimidyl-Ester
CLIP	Class II-associated Invariant chain Peptide
CLP	Common Lymphoid Precursor
cSMAC	central SupraMolecular Activation Cluster
C-terminal	Carboxy-terminal
CTL	Cytotoxic T Lymphocyte
D	Diversity segment

DAG	DiAcyl Glycerol
DC	Dendritic cell
DD	Death Domain
dH_20	Distilled water
DMSO	DiMethyl SulfOxide
DN	Double Negative thymocyte
DNA	DeoxyriboNucleic Acid
DP	Double Positive thymocyte
DTT	DiThioThreitol
ECA	*Erythrina Cristagalli* lectin
ECL	Enhanced ChemiLuminescence
Endo H/F	Endoglycosidase H/F
ER	Endoplasmic Reticulum
ERK	Extracellular signal-Regulated Kinase
FACS	Fluorescent Activated Cell Sorter
FCS	Foetal Calf Serum
FITC	Fluorescein IsoThioCyanate
FLICE	Fas-associating protein with DD-like IL-1β converting enzyme
FLIP	FLICE-like Inhibitory Protein
FYN	$p59^{fyn}$
GAP	GTPase Activating Protein
GC	Germinal Centre
GDP	Guanine DiPhosphate
GEF	Guanine nucleotide Exchange Factor
GTP	Guanine TriPhosphate
GTPase	Guanine TriPhosphatase

H	Heavy chain
HEV	High Endothelial Venule
HSC	Hematopoietic Stem Cell
ICAM-1	InterCellular Adhesion Molecule 1
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
Il-	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IP3	Inositol 1,4,5 triPhosphate
IS	Immunological Synapse
ITAM	Immunoregulatory Tyrosine Activation Motif
J	Joining segment
kB	kiloBase pairs
kDa	kiloDaltons
L	Light chain
LAT	Linker for Activation of T cells
LCK	$p56^{lck}$
LCR	Locus Control Region
LFA-1	Leukocyte Functional Antigen 1
LPS	LipoPolySaccharide
mAb	Monoclonal antibody
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
MKK	MAP Kinase Kinase

MKKK	MAP Kinase Kinase Kinase
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor κB
NK	Natural Killer cell
N-terminal	Amino-terminal
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	PhycoErythrin
PI	PhosphoInositol
PIP2	PhosphatidylInositol-4,5 biPhosphate
PI3K	PhosphoInositol-3 Kinase
PKC	Protein Kinase C
PLC	PhosphoLipase C
pMHC	peptide bound to MHC complex
PNA	Peanut Agglutinin
pSMAC	peripheral Supra Molecular Activation Cluster
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatase
RAG	Recombinase Activating Genes
rER	rough Endoplasmic Reticulum
RIg	Rat Ig
RNA	RiboNucleic Acid
RT	Room Temperature
S/A	StreptAvidin
SDS-PAGE	Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis
SH	Src Homology

SHP-1	SH2 domain containing tyrosine phosphatase
SLAP	SLP-76-Associated Protein
SLP-76	SH2 domain-containing Leukocyte Protein of 76 kDa
SNA	*Sambucus Nigra* bark protein
SP	Single Positive thymocyte
SPI	Small Protease Inhibitors
SR	Scavenger Receptor
TAP	Transporter associated with Antigen Processing
Tc	Cytotoxic T cell
TcR	T cell Receptor
TGF-β	Transforming Growth Factor β
T_H	Helper T cell
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
T_{reg}	regulatory T cell
Tris	Tris(hydrocymethyl)aminomethane
Tween-20	polyxyethylene-sorbitan monolaurate
V	V segment
Y	Tyrosine
ZAP-70	Zeta chain-Associated Protein of 70kDa

1. Introduction

1.1 Innate vs. adaptive immunity

Innate immunity

In an organism's constant battle with pathogens there are two lines of defence. Innate immunity is provided by pre-existing, non-specific defence mechanisms and adaptive immunity provides long lasting, specific protection against subsequent infection as a consequence of the adaptive immune response to an invading pathogen.

The innate immune system is responsible for the initial defence mounted by the organism upon infection and does not discriminate between pathogens. An innate response does not depend upon prior exposure to the pathogen and does not generate immunological memory. Unlike the adaptive response, cells involved in an innate immune response react in the same way when a second exposure to the same antigen takes place.

The first response of the innate immune system is provided by the alternative pathway of complement activation, which provides the same antimicrobial protection as the classical complement pathway but without the 5-7 day wait needed for this to function. Pathogens lack the complement regulatory proteins that host cells express, and become targets for opsonisation. Not all microbial surfaces are vulnerable to complement attack however, and the innate immune system has other methods of defence. Macrophages and neutrophils work by recognizing invariant products of non-self microbes, either by recognizing the pathogen surface directly or by recognition of circulating microbial products, which trigger the cells to englulf the bacteria and cause inflammation of the host tissues. Phagocytes release a variety of other molecules in response to infectious agents including cytokines, toxic oxygen radicals, peroxides, nitric oxide and lipid mediators of inflammation. The most important cytokines

released by phagocytes include IL-1, IL-8, TNF-α, IL-6 and IL-12 which mediate a variety of local and systemic effects. The combined local effects of the cytokines released by the phagocytes result in an inflammatory response, characterised by pain, redness, heat and swelling at the site of infection. This is caused by an increase in vascular diameter and concomitant decreased blood flow, the increased adhesion of circulating leukocytes to endothelial blood cells and also the increased permeability of blood vessels.

Natural killer cells (NK cells) are activated in response to cytokines secreted by macrophages, especially IL-12, and as such the wave of NK cells appears slightly later in the innate immune response. NK cells express two forms of receptors on the cell surface; activating receptors and inhibitory receptors. Activating receptors mediate the cytolytic activity of NK cells while inhibitory receptors are specific for MHC Class I alleles and prevent the NK cell from targeting normal host cells. In the absence of MHC Class I or abnormal expression of MHC Class I in an infected cell, the inhibitory receptor cannot bind and the activating receptor initiates the killing response. NK cells kill an infected cell in the same way as cytotoxic T cells in the adaptive immune response, by releasing cytotoxic granules onto the surface of the target cell which penetrate the cell membrane and initiate apoptosis.

Adaptive immunity

Whereas the innate immune system acts within minutes of infection, the adaptive response takes about 4-7 days to be effective. Both responses employ similar techniques for elimination of pathogen; cells of the adaptive immune response also activate microbicidal and cytokine-secreting properties of macrophages, and antibodies activate the classical complement pathway, act as direct opsonins for phagocytes and stimulate NK cells to kill infected cells. The cells of the adaptive response also secrete cytokines and chemokines to induce responses. The main difference is that the adaptive immune response can target a pathogen with far greater precision than the innate response, and this requires clonal expansion meaning a latent

period after infection before the effector cells are capable of eliminating infection. The mechanistically similar way in which the adaptive and innate response function has led to the conclusion that the adaptive immune response evolved from the evolutionarily ancient innate system. The most important feature of the adaptive response in comparison to the innate response is that an adaptive response to invading pathogen results in immunological memory which enables the organism to mount a stronger response on re-infection. The specificity of the adaptive response is provided by a range of cell surface receptors that have an almost limitless capacity for variation, namely the B cell receptor (BcR) and the T cell receptor (TcR).

1.2 Adaptive immunity – B cells

B cells originate in the bone marrow and are found in secondary lymphoid tissues and in the blood. In the bone marrow the B cell rearranges its genes to produce a cell surface receptor (BcR) of unique specificity (Cooper 1987), and a circulating B cell encountering cognate antigen will proliferate and eventually differentiate into an antibody-producing plasma cell or a memory cell. The antibody that a B cell produces is a soluble form of the membrane bound antibody which makes up the BcR, and antibodies bind specifically to the pathogen eliciting the immune response and recruiting other cells to neutralize the infection.

The BcR is part of the immunoglobulin (Ig) superfamily and comprises two identical light chains bound non-covalently to two identical heavy chains, which are joined by two disulphide bonds. Each of these four chains consists of a constant region (C), which determines the functional properties of the antibody, and a variable region (V), which provides the specificity for binding to different antigens. The V region can be further subdivided into gene segments known as variable (V), joining (J) and, unique to the heavy chain, diversity (D) segments. There are multiple varients of each gene segment and any one can join to any other, creating the potential for the enormous diversity needed to neutralize the variety of invading pathogens. This process is

known as somatic V(D)J recombination and occurs during B cell development in the bone marrow, driven by enzymes encoded by recombination-activating genes, *Rag-1* and *Rag-2*. The *Rag* genes are lymphoid specific and mice lacking these genes do not have a functional mature immune system (Mombaerts *et al.* 1992; Shinkai *et al.* 1992). Like T cells, self-reactive B cells are deleted from the repertoire. Allelic exclusion after a non-autoreactive BcR has been formed ensures that B cells express only one specificity of receptor.

B cells express IgM on the cell surface as they mature in the bone marrow but while circulating in the periphery express IgM and IgD. A mature circulating B cell can be activated by encounter with their specific foreign antigen in the secondary lymphoid organs, causing proliferation of the activated cell and terminal differentiation into an antibody-secreting plasma cell or a memory cell. Antigen is bound by B cells, internalized, processed and returned to the cell surface in association with MHC Class II molecules, which are recognized by activated T cells carrying a specific TcR (Lanzavecchia 1985). The interacting T cell initially triggers B cell proliferation, but another T cell surface molecules, CD40 ligand (CD40L) interacts with CD40 on the B cell and, with cytokine help from IL-4, IL-5 and IL-6, induces B cell differentiation into plasma cells and isotype switching (Jabara *et al.* 1990).

The BcR is non-covalently associated with two invariant chains, Igα and Igβ, which are required for transportation of the BcR complex to the cell surface as well as initiation of signaling when the receptor binds to an extracellular ligand. The cytoplasmic domains of Igα and Igβ contain signaling motifs called immunoreceptor tyrosine-based activation motifs (ITAMs), which become phosphorylated on B cell activation by receptor associated Src family kinases BLK, LYN or FYN. This allows the phosphorylated ITAMS to bind with high affinity to other protein tyrosine kinases (PTKs) in order to continue downstream signaling. The activity of BLK, LYN and FYN is tightly controlled by the ratio of CSK, which prevents their activation, and CD45,

which restores the potential of these proteins for being activated. Phosphorylation of ITAMs results in the recruitment of the PTK SYK, which is activated by auto-phosphorylation. Activated SYK phosphorylates the adapter B cell linker protein (BLNK), which recruits signaling molecules to the BcR complex and propagates the signal from the cell membrane to the nucleus. Two of the main signaling cascades are propagated by the activation of PLCγ by Tec family kinases recruited to BLNK, and the third is generated by the activation of the small G protein RAS. PLCγ cleaves membrane associated PIP_2 to generate intracellular second messengers IP_3 and DAG, which in turn release Ca^{++} into the cytosol and activate PKC respectively. RAS is activated by the action of guanine-nucleotide exchange factors (GEFs), which in turn activates the MAP kinase cascade. All signaling cascades result in the translocation of transcription factors from the cytoplasm to the nucleus culminating in changes in gene expression leading to cell proliferation and differentiation. B cell receptor signaling is enhanced between 1000- and 10 000-fold by the presence of the B cell co-receptor, which is phosphorylated on cytosolic tyrosine domains when clustering of the co-receptor and BcR takes place. Other Src family kinases are able to bind the phosphorylated B cell co-receptor and enhance signaling through the BcR.

B cells achieve diversity by rearrangement of genes, random pairing of heavy and light chains and finally by a process of somatic hypermutation which introduces point mutations to the BcRs expressed by activated B cells. This is mediated by the enzyme activation-induced cytidine deaminase (AID) (Muramatsu *et al.* 2000) and occurs in the germinal centers of secondary lymphoid organs, where the antigen is trapped on the surface of specialized follicular dendritic cells allowing the BcRs of the surrounding cells to compete for recognition (Liu *et al.* 1992). Point mutations having a deleterious effect on the affinity of the BcR for antigen will not be selected and result in the death of the B cell, but mutations giving rise to improved antigen binding will expand and preferentially become antibody secreting cells. As well as being important for somatic hypermutation, the enzyme AID is thought to be involved in class switch

recombination, also seen after B cell activation (Muramatsu *et al.* 2000). The first antibodies produced in a humoral response are IgM but activated B cells undergo isotype switching and secrete IgG, IgA and IgE antibodies. Isotype switching has no effect on specificity of antibody, as the V region is not targeted, but the change in C region associated with isotype switching alters the effector function of the antibody.

Circulating antibodies produced by a plasma cell protect the host organism in three ways. They can bind an invading pathogen and prevent it adhering to host cells, neutralizing the infection. Secondly they can target the pathogen for opsonisation by binding and the antibodies themselves becoming targets for the Fc-recognising phagocytes which engulf the pathogen. Finally the antibodies can activate the classical complement pathway, which enhances opsonisation and lyses some bacteria.

1.3 Adaptive immunity – T cells

Like B cells, T cells also originate in the bone marrow but instead of maturing there they migrate to the thymus where development takes place before they are exported to the periphery. A population of extra-thymically derived T cells is present in the adult gut mucosa, but the majority of T cells mature in the thymus.

Unlike the BcR, T cell receptors (TcR) do not recognize cognate antigen directly, instead recognizing peptide fragments of antigen bound to MHC molecules (Davis and Bjorkman 1988; Bjorkman *et al.* 1987). MHC proteins are highly polymorphic cell surface glycoproteins of which the two main classes, I and II, bind intracellularly generated peptides and present them on
the cell surface. The MHC Class I molecule structure has been resolved (Stern and Wiley 1994) and consists of a tri-globular membrane associated heavy chain and a non-covalently attached light chain, known as β_2-microglobulin (β_2m). MHC Class II molecules are structurally similar to Class I, but differ since they consist of a

heterodimer of an α and β chain, both of which are membrane bound. The TcR is able to recognize peptide fragments presented by MHC molecules and can distinguish self from non-self-peptides due to selection processes during differentiation in the thymus. The TcR is also a heterodimeric glycoprotein formed by an α chain disulphide bonded to a β chain, but a small population of T cells express a γδ heterodimer (Winoto and Baltimore 1989). T cells expressing the γδ TcR are preferentially localized in the epidermis and intestinal epithelium (Itohara *et al.* 1989; Haas and Tonegawa 1992) and recognize antigen in a similar way to B cells; that is they recognize intact protein antigens (Hayday 2000). γδ T cells have been implicated in several immunological roles, including immediate responses to pathogen invasion and long-term modulation of inflammation (Carding and Egan 2000). The αβ TcR is overwhelmingly favoured however and is present on 95% of peripheral T cells in mice. Like the BcR, the TcR consists of variable and constant regions, and achieves specificity for antigen by rearranging TcR gene segments in the α and β chains (Davis and Bjorkman 1988). Each chain of the TcR comprises an Ig-like domain, a transmembrane section and a short cytoplasmic tail, which has no known signaling capability. Instead the signaling capacity of the TcR is by association with the CD3 complex, which is non-covalently bound to the TcR and consists of three pairs of di-sulphide linked molecules. The CD3 complex possesses cytoplasmic tails which contain conserved motifs known as immunoreceptor tyrosine-based activation motifs (ITAMs) (Reth 1989; Cambier 1995). The protein tyrosine kinases $p56^{lck}$ (LCK) and $p59^{fyn}$ (FYN) mediate ITAM phosphorylation in T cells after activation (Appleby *et al.* 1992; Straus and Weiss 1992) allowing the recruitment of the syk-family kinase ZAP-70 (Wange and Samelson 1996). Activation of ZAP-70 allows the phosphorylation of multiple substrates involved in critical signaling events, including LAT, SLP-76, PLCγ-1, PYK2 and CBP (Zamoyska *et al.* 2003). Each αβ T cell expresses a co-receptor which binds to the same MHC molecule as the TcR. Both the CD4 and CD8 co-receptors are invariant proteins which restrict the class of MHC to which the TcR is able to bind, and are essential for mature helper- and cytotoxic-T cell maturation, as well as activation of T cells in the periphery.

CD4 T cells

$CD4^+$ T cells, also called helper T cells (T_H) recognize antigen in the context of MHC Class II molecules which are expressed constitutively by professional APCs like DCs and B cells. The cytokine profile of effector $CD4^+$ T cells allows further subdivision into T_H1, T_H2 and T regulatory (T_{reg}) cells. T_H1 cells are important for the eradication of intracellular pathogens and viruses via the activation of macrophages, T_H2 cells target extracellular parasites and soluble toxins by activating B cells to make antibody while T_{reg} cells are reported to inhibit immune responses (Sakaguchi 2000; Shevach 2000). The APCs internalize the invading antigen in endosomal vesicles and later in lysosomes. MHC Class II molecules are synthesised as a complex in the endoplasmic reticulum (ER) with the invariant chain (Ii) polypeptide which prevents endogenously derived peptides being presented on the cell surface (Roche and Cresswell 1990). Instead of being transported directly from the Golgi to the cell surface, the MHC Class II is diverted to an acidic lysosomal compartment where it encounters the degraded antigenic protein. The Class II-associated invariant chain peptide (CLIP), derived from the Ii, is removed from the MHC complex in the class II loading compartment, allowing the molecule to translocate to the cell surface bound to antigenic peptide. The processing pathway which results in the presentation of antigen in MHC Class II proteins on the cell surface is essentially conserved throughout the APCs interacting with helper T cells, but there is variation with regard to the location and extent of degradation of protein antigens and the site of peptide binding to MHC Class II, even when processing a single antigen (Griffin *et al.* 1997; Fernandes *et al.* 2000).

CD8 T cells

$CD8^+$ T cells, also called cytotoxic T cells (CTLs), recognize antigen in the context of MHC Class I molecules, which are expressed on every nucleated cell. Class I molecules are synthesized and, in contrast to MHC Class II molecules, loaded with peptide in the ER. Cytosolic pathogens are degraded by the cytosolic proteosome (Rock *et al.* 1994) and transported into the lumen of the ER by the Transporter associated with Antigen

Processing (TAP) (Neefjes *et al.* 1993). TAP associates with the MHC Class I complex in the ER whereupon the fully formed peptide/ MHC complex (pMHC) is released from TAP and transported to the cell surface via the Golgi (Pamer and Cresswell 1998). Unassembled or misfolded Class I molecules are not transported to the cell surface and are degraded in the ER (Hammond and Helenius 1995).

Pathogen-derived cytosolic proteins are thus presented to $CD8^+$ T cells which, upon recognition of cognate antigen, kill the infected cell by apoptosis. Activated CD8 T cells are able to induce cytolysis of infected cells in two distinct molecular pathways; by perforin lysis or Fas-mediated apoptosis. During the perforin lytic mechanism, perforin is released by the $CD8^+$ effector cells which creates perforin channels in the membrane of the target cell. Enzymes including granzyme B, also released by the $CD8^+$ cell, pass through the channels causing DNA fragmentation and lysis of the target cell (Podack *et al.* 1991). In Fas-mediated killing, the Fas ligand on the $CD8^+$ effector cell interacts with the Fas receptor expressed on the target cell, triggering activation of target cell proteases which cleave nucleoproteins causing DNA fragmentation and apoptosis (Van Parijs and Abbas 1996).

1.4 T cell development

T cells are derived from common lymphoid precursor (CLPs) and mature in the thymus, where they differentiate into mature T cells which are then exported to the periphery. CLPs can mature into a B cell or a T cell, and the transmembrane receptor Notch-1 is thought to play an important role in this lineage decision (Pui *et al.* 1999; Radtke *et al.* 1999). A small population of extra-thymically derived T cells is present in the adult gut mucosa (Saito *et al.* 1998).

Double negative thymocytes

The overall process of T cell development can be summarized using the expression of the co-receptors CD4 and CD8 as reference points, and is represented schematically in figure 1.1. Immature T cells enter the thymus as $CD4^-CD8^-$ double negative cells (DN) which express CD25 and rearrange their β, γ or δ TcR genes. Successful rearrangement will either lead to γδ TcR expression and maturation down this lineage, or in the vast majority of cases the expression of successfully rearranged TcRβ with the invariant preTcRα (pTα) chain. The mechanism by which a T cell commits to expressing either αβ TcR or γδ TcR is still poorly understood, although the cytokine receptor IL-7R could be important in this decision (Kang *et al.* 2001). The preTcR of the αβ TcR lineage is expressed on the cell surface and allows the T cell to progress to the double positive stage of development, as well as being a marker for commitment to the αβ TcR lineage. Thymocytes unable to rearrange their TcRβ genes are arrested at the DN stage of development (Mombaerts *et al.* 1992; Shinkai *et al.* 1992). Expression of the preTcR on the DN cell causes the loss of CD25 expression, cessation of β-chain rearrangement, cell proliferation and the expression of the co-receptors CD4 and CD8. The expression of these two cell surface markers denotes the entry of the maturing thymocyte to the $CD4^+CD8^+$ double positive (DP) stage of maturation, where the cell undergoes various selection events.

Double positive thymocytes

After the extensive expansion triggered by the expression of the preTcR and subsequent expression of CD4 and CD8 on the cell surface, the developing DP T cell rearranges the TcRα genes to produce the mature TcR, whereupon the DP thymocyte is ready to undergo selection. The selection process comprises positive selection, where DP cells are selected for their ability to recognize self-MHC, followed by negative selection, where DP cells recognizing self-peptide in self-MHC with overtly high affinity are eliminated from the repertoire. Only 2% of DP thymocytes make it through this rigorous process, with 98% dying by apoptosis.

During positive selection, DP thymocytes interact with MHC molecules on the thymic cortical epithelial cells (Cosgrove *et al.* 1992). DP thymocytes that are able to recognize the self-MHC molecules with sufficient affinity receive TcR-mediated signals which allow cell survival (Huesmann *et al.* 1991; Jameson and Bevan 1998), although the nature of these survival signals is still poorly understood. Those cells unable to recognize self-MHC, or doing so with low affinity, receive no survival signal and die of neglect (Huesmann *et al.* 1991). As well as ensuring the T cell repertoire is able to recognize antigen in self-MHC, positive selection also determines the cell surface phenotype and effector function of the mature naïve T cell by selecting the appropriate co-receptor for efficient MHC binding. It is the specificity of the TcR for self-MHC that determines which co-receptor the mature T cell will express; that is a TcR that is able to bind MHC Class I molecules will end up as $CD4^{-}CD8^{+}$ whereas a TcR that recognizes MHC Class II molecules will become $CD4^{+}CD8^{-}$ (von Boehmer *et al.* 1989). The developing T cell requires signals from both the TcR and the co-receptor, as mice lacking CD8 develop no MHC Class I restricted T cells and mice lacking CD4 are deficient in MHC Class II restricted T cells (Locksley *et al.* 1993; Bachmann *et al.* 1995), but the precise nature of these signals remains unclear.

How the commitment to one lineage or the other is made is poorly understood, and the two mechanisms that are posited are the instructive model or the stochastic model (Jameson *et al.* 1995; von Boehmer 1996). The instructive model proposes that the interaction of a TcR and co-receptor with MHC instructs the cell by a unique biochemical event to down-regulate and cease transcribing one or the other co-receptor. The stochastic model proposes the random down-regulation of one co-receptor, with cell survival depending on the randomly selected co-receptor complimenting the TcR. TcR signaling initially leads to some loss of both co-receptors irrespective of the recognized MHC class, followed by subsequent upregulation of CD4 then CD8, and finally the selective loss of either CD4 or CD8 (Lucas and Germain 1996). Recent evidence favours the instructive model of selection (Itano and Robey

2000), and further reports suggest lineage fate is largely decided by the signal transmitted by the co-receptor, rather than the class of MHC recognized (Seong *et al.* 1992; Itano *et al.* 1994; Itano *et al.* 1996).

Data showing that MHC Class II restricted thymocytes became $CD8^+$ single positive (SP) T cells in the absence of CD4 (Matechak *et al.* 1996) suggested that a weak signal led to differentiation into the CD8 lineage. This led to the idea that the 'strength' of the signal could also be important for lineage commitment; that is a weak signal leads to a CD8 fate whereas a strong signal leads to a CD4 fate. This idea is consistent with data showing that crosslinking TcR with either co-receptor – a strong signal – gives rise to CD4 T cells whereas crosslinking CD3 alone – a weak signal – gives rise to the CD8 lineage (Bommhardt *et al.* 1997; Basson *et al.* 1998a; Basson *et al.* 1998b). More LCK is associated with CD4 than CD8 in DP thymocytes (Veillette *et al.* 1988) and the contribution of LCK is thought to be important in lineage commitment (Basson *et al.* 1998b; Sharp and Hedrick 1999; Hernandez-Hoyos *et al.* 2000). This is consistent with the report of Bommhardt *et al* (1997) who showed that crosslinking TcR with CD8 produced T cells of the CD4 lineage, but less efficiently than crosslinking TcR with CD4, presumably due to the smaller amount of LCK associated with CD8. The strength of signal hypothesis cannot fully explain lineage commitment, and the duration of the signal is also thought to be important (Ohoka *et al.* 1997; Basson *et al.* 1998a). Again LCK activation is central to this idea, and sustained activation of LCK has been shown to be associated with CD4 commtiment while weaker, transient activation of LCK favours the CD8 lineage (Legname *et al.* 2000).

In contrast to mature naïve T cells, the response of immature T cells to stimulation by antigen is death by apoptosis, and this is the basis of negative selection in the thymus. Thymocytes that have passed positive selection and recognize self-MHC will be deleted at the next stage of development, negative selection, if they recognize self-peptides in self-MHC which would cause autoimmunity in the periphery (Kisielow *et al.* 1988; Zal *et al.* 1994). Negative selection is mediated by bone marrow derived DCs

and macrophages at the interface between the thymus cortex and medulla as the thymocyte matures (see Figure 1), and more recent evidence suggests cells in the medullary epithelium are also implicated in the process (Klein *et al.* 1998). The result of negative selection is that the TcR repertoire circulating in the periphery is largely not autoreactive. Tolerance is also thought to be mediated by T_{reg} cells which also undergo interactions with self-antigens in the thymic epithelium (Shevach 2000).

Single positive thymocytes

DP thymocytes that survive positive and negative selection mature into single positive thymocytes (SP) expressing only one of CD4 or CD8 co-receptors. Although this means T cell effector function is already finalized (reviewed in (Kioussis and Ellmeier 2002)), SP cells undergo changes in the expression of cell surface molecules (Gabor *et al.* 1997) and several rounds of cell division (Ernst *et al.* 1995) before being exported to the periphery. The total time between a T cell progenitor entering the thymus and the export of a mature SP naïve T cell is approximately three weeks in mice. In a monoclonal environment the formation of mature cells is 10-20 times more efficient than a polyclonal environment, yet the frequency of SP cells is not 100% (Huesmann *et al.* 1991). The reason for this discrepancy is thought to be due to the limiting number of selection niches on the thymic stroma, and the limited amount of time (3-5 days) a DP cell has to be selected or die of neglect (Huesmann *et al.* 1991; Merkenschlager *et al.* 1994). In the mouse, the thymus exports approximately 1-2 x 10^6 SP cells to the periphery every day.

1.5 Peripheral T cells

Thymic output and clonal expansion of lymphocytes are balanced by the death of cells competing for limited survival factors, and as such the peripheral T cell pool is maintained at a constant number (Freitas and Rocha 2000). The thymus generates a T cell repertoire that is capable of recognizing almost any antigen due to the rearranging of TcR genes giving rise to a vast array of receptors of different specificities.

Necessarily though, this means that for any given antigen there will be very few T cells bearing the specific receptor needed for recognition. In order to overcome this the adaptive immune response relies on the clonal expansion of antigen-specific lymphocytes in response to antigenic stimulation. Expansion leads to the creation of a population of effector T cells, which mediate the neutralization of the invading pathogen by utilizing their effector functions. Some effector T cells differentiate into long-lived memory T cells at the conclusion of an adaptive immune response, increasing the frequency of this specificity above that of the naïve pool, and upon secondary infection with the same pathogen will respond more rapidly than naïve T cells, a key difference between the innate and the adaptive responses.

Naïve T cells

Naïve T cells constantly circulate the peripheral lymphoid tissues but have never encountered cognate antigen. The survival of naïve T cells depends on the interaction of the TcR with self-peptide/ self-MHC complexes and cytokine signals, the signaling pathways of which synergise to provide the cell with the necessary signals for persistence in the periphery, and neither signal alone is sufficient for survival (Seddon and Zamoyska 2002b). Interestingly these synergenistic signals are also the requirement for homeostatic proliferation of naïve T cells when in conditions of lymphopenia (Seddon and Zamoyska 2002a). Homeostatic expansion cannot entirely regenerate the enormous diversity of TcR specificities exported from the thymus, but it provides a wider set of TcR specificities than expanding the memory pool. The survival signals required by naïve T cells are distinct for those required by memory T cells. This is probably so that rapidly acting memory cells are maintained in the face of new exports from the thymus, and also so that the diverse naïve T cell pool is not compromised by the proliferation of the memory pool, whose range of specificity is limited to antigen already encountered (Arstila *et al.* 1999). As well as receiving survival signals from MHC expressed on APCs in the T cell zones of secondary lymphoid organs, naïve T cells are constantly screening the APCs they encounter for

cognate antigen. Upon recognizing cognate antigen, the naïve T cell is activated and becomes an effector T cell.

Effector T cells

Engagement of the TcR on mature naïve T cells initiates multiple intracellular signals which lead to cellular proliferation and the acquisition of complex effector functions. One of the initial effects of antigen recognition is massive expansion to create a pool of effector cells. Once the effector cell has completed differentiation in the lymphoid tissue it migrates to the point of infection. Binding of the TcR to the pMHC complex on the target cell (figure 1.2) causes the clustering of TcR which results in the effector molecules being focused on release towards the target cell. The directing of effector molecules towards the target cell is further helped by the organization of non specific adhesion molecules, e.g. LFA-1 and ICAM-1 which form a tight ring around the TcR cluster. The different types of effector cell – cytotoxic T cell, T_H1 and T_H2 cells – release distinct effector molecules that are targeted to the appropriate target cell. Cytotoxic T cells release perforin and granzyme proteases, which will destroy the target cell and are non-specific, and both cytotoxic and helper T cells release cytokines, which are synthesized *de novo* after T cell/ target cell interaction and are specifically directed at precise receptors on a target cell. The main cytokine released by cytotoxic T cells is IFNγ which blocks viral replication. T_H1 cells also release IFNγ, which activates macrophages and TNF-β, which activates macrophages and inhibits B cells. The cytokines released by T_H1 and T_H2 are mutually exclusive, with T_H2 cells secreting IL-4 and IL-5, which activate B cells, and IL-10, which inhibits macrophage activity. All three effector cells express membrane-associated proteins, which are structurally related to tumour necrosis factor (TNF). These bind TNF receptors on the target cell and can affect the activity of the target cell or stimulate death by apoptosis.

Unlike naïve and memory cells however, effector cells do not persist in the peripheral T cell pool as this could lead to autoimmunity and chronic inflammation as well as to

effector cells occupying naïve and memory cell niches, thus compromising the broad specificity of the repertoire. After the invading pathogen has been neutralized, the effector cells are removed in two ways. Effector cells can be eliminated by activation induced cell death (AICD) or by cytokine withdrawal. In *in vivo* AICD, the IL-2 secreted by all effector cells causes the downregulation of FLIP (Algeciras-Schimnich *et al.* 1999), a protein which inhibits the FAS apoptosis pathway, rendering the effector cell sensitive to AICD. Low levels of TGF-β may also increase AICD susceptibility because T cells deficient in TGF-β have increased sensitivity to FAS mediated death, possibly due to a failure in elevating FLIP levels (Chen *et al.* 2001). Cytokine withdrawal was described far earlier than AICD (Duke and Cohen 1986) but paradoxically is less well understood. It is thought that BCL-2, an anti-apoptotic protein, is important in this response as BCL-2 levels in activated cells are decreased in comparison to resting T cells (Marrack *et al.* 1999). These two regulatory pathways are entirely separate, as over-expression of BCL-2 does not protect against FAS-mediated cell death and the absence of FAS does not protect cells from death which was otherwise prevented by BCL-2 over-expression.

Memory T cells

The mechanism by which some effector T cells escape death and go on to become long-lived memory cells is still unresolved. Although it is well established that naïve T cells need to undergo several rounds of division before long-term memory arises (Swain 1994; Garcia *et al.* 1999; Opferman *et al.* 1999), and there is evidence that memory cells arise from effector cells (Opferman *et al.* 1999; Saparov *et al.* 1999), this does not discount the possibility that memory cells could also arise from partially differentiated naïve cells. All memory T cells have higher levels of CD44 than naïve T cells. $CD8^+$ memory cells express higher levels of BCL-2, which prevents apoptosis. When identifying $CD4^+$ memory T cells, other key cell surface markers are L-selectin, which affects the migration of cells into tissues and lymphoid organs and has very low expression in memory cells, and CD45. In naïve $CD4^+$ T cells, it is the high molecular

weight isoform of CD45, - CD45RA - that is predominantly expressed, whereas in memory cells it is CD45RO, a protein of lower molecular weight due to differential splicing of the gene (Michie *et al.* 1992).

As an organism experiences many infections over a lifetime, quantitative deletion and qualitative alteration of specificity of the memory pool occurs (Selin *et al.* 1999; Appay *et al.* 2002). Memory cell survival does not seem to require the interaction of the TcR with self-peptide/ MHC complexes (Murali-Krishna *et al.* 1999; Swain *et al.* 1999; Kassiotis *et al.* 2002), although the functional ability of $CD4^+$ memory cells is impaired without MHC-derived signals (Kassiotis *et al.* 2002). Memory T cell survival is regulated by specific cytokines (Ku *et al.* 2000). The cytokines necessary for survival differ between $CD8^+$ and $CD4^+$ memory T cells. There is now strong evidence that IL-15 acts directly on $CD8^+$ memory cells (Kennedy *et al.* 2000; Nishimura *et al.* 2000), yet it is a poor stimulator of $CD4^+$ memory T cells (Zhang *et al.* 1998b; Tan *et al.* 2002), probably due to the comparatively low levels of IL-15 receptor expressed on $CD4^+$ memory cells in comparison to $CD8^+$ cells (Ku *et al.* 2000). Both naïve and memory T cells display a requirement for the cytokine IL-7 in survival (Seddon and Zamoyska 2002b; Seddon *et al.* 2003). Immunological memory can be transferred, which is demonstrated by adoptive transfer of memory cells to a naïve individual. On re-infection with the original antigen, the naïve cells of the host do not proliferate and instead the transferred memory cells clear the infection. The key feature of immunological memory is that on secondary infection the memory cells are hyper-responsive to antigen and release large quantities of cytokines (Lanzavecchia and Sallusto 2000) in comparison to naïve cells.

1.6 Antigen presentation

Antigen presenting cells (APCs) take up incoming protein antigens, break them down into peptide fragments intracellularly, form complexes between the peptides and MHC molecules (pMHC), and display the complexes on the cell surface. In this form antigen

is recognized and responded to by T cells bearing receptors specific for the pMHC initiating various types of immune response. APCs also express other co-stimulatory molecules on the cell surface and secrete cytokines according to the nature of the invading pathogen, both of which are necessary for correct T cell activation (Fearon and Locksley 1996; Ito *et al.* 2002). The three main types of APC recognized by T cells are macrophages, B cells and dendritic cells.

Macrophages

Macrophages are often able to clear an infection without needing an adaptive immune response. Pathogens that can evade an innate immune response are still phagocytosed by macrophages, which results in the upregulation of MHC Class II bound to pathogen peptide fragments and B7, which provides an essential co-stimulatory signal, on the cell surface of the APC. The uptake of antigen by the macrophage is mediated by Fcγ-receptors (Ravetch and Bolland 2001), complement receptors and scavenger receptors (Hughes *et al.* 1995). However the process of endocytosis is clearly very complicated, as recent data showed that purified macrophage phagosomes contained more than 140 proteins (Garin *et al.* 2001). In order to become fully functional, macrophages need to be activated by IFNγ, secreted by activated T cells, or micro-organism derived components (Celada and Nathan 1994). Macrophages can be myeloid-derived, becoming residential in tissue and self-renewing, or monocyte-derived which are short lived and have no capacity for proliferation (Naito *et al.* 1996).

B cells

Unlike macrophages, which scavenge pathogens, modified host molecules and apoptotic cells, B cells are highly specific in their uptake of antigen due to the unique specificity of the BcR. Also in contrast to macrophages, B cells constitutively express MHC Class II molecules on the cell surface meaning ingested antigen is presented to T cells extremely efficiently. B cells can ingest other, non specific proteins but presentation on the cell surface is less efficient. B cell antigen presentation can serve

two purposes; recognition of antigen bound in MHC Class II by T cells can drive B cell differentiation and B cells can also stimulate naïve T cells if they are induced to express co-stimulatory molecules, which happens when the BcR is triggered (Lenschow *et al.* 1994). These co-stimulatory molecules are only induced by microbial components (Hathcock *et al.* 1994), which explains why B cells presenting self-antigen are not targeted.

T cells in secondary lymphoid organs are restricted to T cell areas in the lymph nodes (paracortex) and spleen (Peyer's patches and periarteriolar lymphoid sheath). Although T cells are largely confined to secondary lymphoid organs shortly after T cell activation (Reinhardt *et al.* 2001), B cells are restricted to their own B cell follicles in lymphoid tissues, raising the question of how the two cells meet after activation. This question was resolved by experiments showing that T cells migrated to the border region to make contact with B cells (Gulbranson-Judge and MacLennan 1996; Garside *et al.* 1998; Reinhardt *et al.* 2001), suggesting the importance of B cells as APCs is relatively minor, and that B cells are largely the beneficiaries of T cell help.

Dendritic cells

Dendritic cells (DCs) are also bone marrow-derived and differentiate from the common myeloid progenitor. The only known function of DCs is the uptake of antigen in peripheral sites and subsequent antigen presentation in the lymph nodes. Since they are widely distributed throughout the tissues, they have a central role in antigen capture and the initiation of an immune response (Steinman *et al.* 1997). DCs emerging from the bone marrow circulate in the blood and peripheral tissues and have an immature phenotype, expressing low levels of MHC proteins and no co-stimulatory molecules. As such these immature DCs are not able to stimulate naive T cells. Like macrophages, immature DCs are also highly phagocytotic and can recognize non-specific microbial surfaces via receptors expressed on their cell surface, as well as ingesting extracellular antigens by a process called macropinocytosis (Sallusto *et al.* 1995).

Immature DCs are highly phagocytic and upon antigen uptake following infection they rapidly migrate to secondary lymphoid tissues. This results in loss of ability to ingest and process antigen, but a concomitant increase in ability to present antigens and expression of co-stimulatory and adhesion molecules. These mature DCs in the lymphoid tissues also secrete a chemokine, DC-CK, which attracts naïve T cells (Adema *et al.* 1997).

The signals causing an immature DC to migrate to lymphoid tissue can be triggered either by direct interaction with pathogen or by cytokine stimulation. Pathogen receptors include the toll-like receptor (TLR) which recognizes conserved pathogenic compounds (Visintin *et al.* 2001), receptors for complement or the mannose receptor. Cytokine stimuli include TNFα, IL-1β and prostaglandin E2 (Banchereau and Steinman 1998). Both methods of antigen detection cause the upregulation of co-stimulatory molecules which directs the maturing DC to lymphoid tissue. DCs present antigen from fungal and bacterial pathogens and are thought to be especially important in viral infection, and produce different effector molecules according to the type of infection. In this way the DC is able to tailor the differentiation of the responding T cell. DCs are also thought to have a role in T cell tolerance in the periphery (Jonuleit *et al.* 2001), possibly by interaction with T_{reg} cells (Kurts *et al.* 1997; Adler *et al.* 1998; McGuirk *et al.* 2002).

1.7 T cell activation

Recognition of cognate MHC by the TcR on a mature naïve T cell, along with a co-stimulatory signal provided by the APC, inititates T cell activation and subsequent T cell proliferation and differentiation to effector function. The recognition of MHC by TcR is represented schematically in figure 1.2.

The TcR

As described previously, the TcR comprises an α:β heterodimer stably associated with the CD3 complex. The extracellular TcR re-arranges its genes to provide diversity but has very short cytoplasmic domain that has no known signaling capability. The CD3 complex comprises the non-polymorphic CD3ε, CD3γ, CD3δ and TcRζ chains which are all essential for receptor assembly, cell-surface expression and signaling (Ashwell and Klusner 1990). In total, the TcR has ten immunoreceptor tyrosine-based activation motifs (ITAMs) (Cambier 1995) which have no inherent kinase activity but are phosphorylated by protein tyrosine kinases (PTKs) of the Src family upon ligand binding (Letourneur and Klausner 1992). ITAMs are semi-conserved and consist of the sequence YxxL/Ix_{6-8}YxxL/I where x is any residue except proline. The ITAMs provide the crucial link between extracellular antigen recognition and intracellular signal transduction mediated by intracellular PTKs. There are two possible mechanisms concerning the recruitment of Src family PTKs to the TcR, known as the raft hypothesis and the immunological synapse.

CD4 and CD8 co-receptors

The co-receptors CD4 and CD8 bind the same MHC as the TcR and as such are termed co-receptors (Janeway 1992). Both co-receptors are associated with LCK and bring this signaling molecule into proximity with the TcR (Parnes 1989). CD8 is crucial for the development of cytotoxic T cells (Fung-Leung *et al.* 1991) and mice lacking CD4 develop only a few double negative helper T cells (Rahemtulla *et al.* 1991), indicating the importance of the co-receptor for development. In spite of the similar effector function of the co-receptors, they are very different structurally.

Murine CD4 is a single polypeptide of molecular weight 55kDa, expressed as a monomer on the cell surface. It is folded into four external Ig-like domains (D1 – D4) and has a hydrophobic transmembrane segment and a highly basic and conserved cytoplasmic tail (Maddon *et al.* 1985; Parnes 1989). The D1 and D2 domains, distal to

the cell membrane, have been shown to interact with the β2 domain of the MHC Class II molecule (Konig *et al.* 1992), while the D3 and D4 domains contact the TcR (Vignali *et al.* 1996). CD8 is a di-sulphide bonded heterodimer comprising an α and a β chain, both of which contain a single Ig-like domain, hinge region, hydrophobic transmembrane region and cytosolic region (Parnes 1989). The Ig-like domain of CD8α has been shown to interact with the α3 portion of the MHC Class I molecule (Salter *et al.* 1990), and although CD8 interaction with the TcR has not been demonstrated directly, it has been suggested that CD8β could interact with TcR without MHC involvement (Kwan Lim *et al.* 1998; Wheeler *et al.* 1998). The interaction of both co-receptors with their respective MHC molecules is represented schematically in figure 1.2. Antibody crosslinking of the TcR with either CD4 or CD8 leads to LCK-mediated tyrosine phosphorylation of various intracellular substrates including the ITAMs of the TcRζ chain (Veillette *et al.* 1988; Gilliland *et al.* 1991), as well as enhanced Ca^{++} mobilization (Ledbetter *et al.* 1988) and cellular proliferation (Eichmann *et al.* 1989).

One important difference between CD4 and CD8 is that CD8 can exist in a number of isoforms. The CD8α gene encodes two polypeptide chains, CD8α and CD8α', as a result of differential splicing of the gene. CD8α' is identical to CD8α but lacks the cytoplasmic domain, and so is impaired in its ability to bind LCK (Zamoyska *et al.* 1985). Furthermore CD8α' is developmentally regulated and can be readily detected on the surface of thymocytes but not in peripheral T cells, although it can be found intracellularly (Zamoyska and Parnes 1988). CD8 can exist as a CD8αβ heterodimer or a CD8αα homodimer, but the heterodimer is implicated in efficient co-receptor function given that mice expressing only CD8αα have an 80% drop in thymocytes being selected (Crooks and Littman 1994). Recent reports have focused on addressing the question of why and how the CD8αβ co-receptor functions more efficiently than CD8αα. The Ig-like, transmembrane, and cytoplasmic domains of CD8β have all been shown to enhance TcR-MHC binding (Wheeler *et al.* 1998; Bosselut *et al.* 2000; Arcaro *et al.* 2001). Interestingly, soluble CD8αβ has the same affinity for MHC as soluble

CD8αα, yet cell bound CD8αβ has been shown to bind MHC Class I more avidly than CD8αα (Garcia *et al.* 1996; Kern *et al.* 1998; Bosselut *et al.* 2000; Arcaro *et al.* 2001; Moody *et al.* 2001b). One possible reason for this is that CD8β is palmitoylated and targets CD8αβ but not CD8αα to lipid rafts (Arcaro *et al.* 2000). Since efficient association of CD8 and LCK takes place in rafts, and signaling is inhibited by the disruption of rafts (Xavier *et al.* 1998; Doucey *et al.* 2001), this is thought to be a possible explanation for the preference of CD8αβ over CD8αα (Arcaro *et al.* 2000; Doucey *et al.* 2001).

Raft Hypothesis

Cell membranes are comprised of a complex mixture of cholesterol, glycerophospholipids and sphingolipids. These lipids have a tendency to behave in distinct ways when forming a bilayer, resulting in areas rich in cholesterol and glycosphingolipids known as lipid rafts. Lipid rafts have been visualized in living cells (Friedrichson and Kurzchalia 1998; Varma and Mayor 1998; Schutz *et al.* 2000) and are defined as dispersed liquid-ordered phase domains to which specific proteins are sequestered but from which others are excluded (Simons and Toomre 2000).

The TcR/ CD3 complex is thought to be excluded from lipid rafts (Montixi *et al.* 1998) under resting conditions, whereas the CD8αβ co-receptor is not (Arcaro *et al.* 2000). Activation of the T cell causes the translocation of the TcR to lipid rafts, where co-receptor associated LCK is localized (Montixi *et al.* 1998; Doucey *et al.* 2001). This membrane compartmentalization seems to be a pre-requisite for TcR signal transduction, as disruption of raft domains abrogates T cell activation (Xavier *et al.* 1998). The conditions under which a protein is targeted to lipid rafts seem to rely on post-translational addition of lipids by myristolation, palmitoylation and prenylation. LCK is targeted to membrane rafts by dual acetylation of its N-terminus (Resh 1994) and LCK lacking this modification is not associated with raft domains and cannot signal properly (Kabouridis *et al.* 1997). Similarly, the CD8αβ co-receptor is targeted to

lipid rafts via palmitoylation of the cytoplasmic domain of the β-chain (Arcaro *et al.* 2000), which is thought to be important for efficient association with LCK (Arcaro *et al.* 2001). Furthermore, CD8 molecules lacking this modification are excluded from raft domains and unable to associate with LCK or mobilize Ca^{++} in response to binding of MHC (Arcaro *et al.* 2001).

Raft domains in resting cells are dynamic structures estimated to be small, but may fuse on ligand binding and subsequent receptor clustering to contribute to the immunological synapse (IS) (Janes *et al.* 1999).

The immunological synapse

In contrast to circulating T cells that display uniform radial distribution of membrane domains and molecules (Sanchez-Madrid and del Pozo 1999), a T cell interacting with an APC polarizes actin filaments at the site of cell-cell contact, at which specific ligands and receptor molecules trigger and sustain the T cell activation process. This site of interaction is termed the immunological synapse (Grakoui *et al.* 1999), and is viewed as a concentric structure which can be subdivided into the central supramolecular activation cluster (cSMAC) and the peripheral SMAC (pSMAC). The cSMAC is characterised by the enriched presence of TcR, CD2 and CD28 and the pSMAC contains cell adhesion molecules such as leukocyte function-associated antigen-1 (LFA-1), whereas proteins with large extracellular domains such as CD45 and CD43 are initially excluded from the IS (Johnson *et al.* 2000); indeed elongated forms of CD2 can also impair T cell activation (Wild *et al.* 1999). TcR/ pMHC interactions are thought to be of low affinity and have fast off rates (Davis *et al.* 1998), and this apparent inconsistency with sustained T cell activation can be explained if clusters of TcRs, localised in the cSMAC create a stable interaction sustained by other accessory molecules and dynamic serial engagement of MHC by TcR (Valitutti *et al.* 1995).

T cell signaling and actin remodeling are closely linked for optimal T cell activation. IS formation and cytoskeletal rearrangement is associated with increased tyrosine

phosphorylation of TcRζ and LCK, and recruitment of zeta chain associated protein of 70kDa (ZAP-70). Furthermore, actin regulatory molecules like Wiskott Aldrich Syndrome protein (WASP), Fyn-binding protein (FYB), Src homology (SH)2-domain-containing leukocyte protein of 76kDa (SLP-76) and VAV play important roles in actin remodeling at the synapse (Krause *et al.* 2000). Nevertheless, the recent report of Lee *et al* (2002), challenged the precise role of the IS in TcR signaling. These data showed that the TcR is initially confined to the pSMAC, and migrates to the centre of the IS upon mature synapse formation. Furthermore, LCK can only ever be detected in the pSMAC of the immature IS, and is undetectable in the mature synapse. It is possible from these data that the initiation of TcR signaling preceded synapse formation, although another report has suggested that the IS provides a point of activation for receptors other than the TcR (van Der Merwe and Davis 2002).

Initiation of T cell signaling

TcR triggering, which leads to signal transduction inside the cell, is still poorly understood. It was thought for a long time that TcR signaling could only be initiated by multimerised TcR ligands since cross-linking of TcR/ CD3 complexes is stimulatory, as is triggering with multimeric pMHC complexes in both $CD4^+$ and $CD8^+$ T cells (Symer *et al.* 1992; Boniface *et al.* 1998; Cochran *et al.* 2000; Daniels and Jameson 2000). While these data are not in dispute, more recent reports have shown that T cells can be stimulated with very few (Delon *et al.* 1998a) or even monomeric (Sykulev *et al.* 1996; Delon *et al.* 1998b; Irvine *et al.* 2002) pMHC complexes, confounding the multimerization model. The responses to monomeric pMHC were strictly dependent on the presence of either CD4 (Irvine *et al.* 2002) or CD8 (Delon *et al.* 1998b). Two models have been proposed to account for these findings, called the heterodimerisation model and the pseudodimer model (van der Merwe 2002).

The heterodimerisation model broadly accounts for $CD8^+$ T cell signaling, and suggests that signaling can be initiated in these cells by the heterodimerisation of TcR with LCK-associated CD8. This brings LCK into proximity with ITAMs on the TcR complex, the

phosphorylation of which initiates TcR signaling. This model was challenged on the basis that stimulation could be coming not from the monomeric pMHC but from T cell/ T cell interactions after peptide transfer (Schott *et al.* 2002). This proved to be irrelevant in initial signaling, as adding free peptide could not re-create data gathered using monomeric pMHC. The pseudodimer model was proposed to explain data gained using $CD4^+$ T cells, and suggests that the foreign pMHC-binding TcR can be crosslinking via CD4 to a different TcR which is binding self-pMHC (Irvine *et al.* 2002). This hypothesis is based on the ability of MHC Class II molecules to form dimers or 'dimers of dimers' (Brown *et al.* 1993; Fremont *et al.* 1996).

Other data has shown that adherent T cells are more responsive to monomeric pMHC stimulation than T cells in suspension, which is consistent with data showing that a single pMHC is stimulatory when a CTL adheres to its target (Sykulev *et al.* 1996) and that in an IS, a single pMHC can trigger Ca^{++} mobilization (Irvine *et al.* 2002). Furthermore, T cells in suspension are unresponsive to monomeric pMHC (Doucey *et al.* 2003; Randriamampita *et al.* 2003). The exact mechanism by which adhesion-induced T cell priming exerts effect is not understood, but could involve increased Ca^{++} stores and PIP_2 accumulation in the cell membrane, leading to amplification of Ca^{++} influx (Randriamampita *et al.* 2003). Another way in which adhesion could be important for T cell priming is through the interaction of adhesion molecules on the T cell, e.g. LFA-1 and CD2, with their ligands on the APC, e.g ICAM-1. As well as enhancing adhesion, these molecules are important for delivering co-stimulatory signals to the T cell (Carrera *et al.* 1988; van Noesel *et al.* 1988; Bierer *et al.* 1989; Van Seventer *et al.* 1990; Beyers *et al.* 1992). Adhesion also leads to the phosphorylation of LCK and FYN, as well as activating the MAPK pathway which is known to have a positive effect on T cell signaling (Stefanova *et al.* 2003). Some or all of these effects could combine to lower the threshold needed for T cell activation. Another recent report suggests that 'self-peptide' priming could reduce the threshold needed for antigenic stimulation, as freshly isolated $CD4^+$ T cells, where self-pMHC recognition is on-going *in vivo*, are significantly more responsive to antigenic stimulus than $CD4^+$ T cells cultured for as

little as 30 minutes (Stefanova *et al.* 2002). Bearing all these data in mind, the heterodimerisation model is more consistent, suggesting that the T cell primed by adhesion (and possibly recognition of self-pMHC) forms a heterotrimeric structure between TcR/ co-receptor/ MHC.

Proximal T cell signaling

Once the naïve T cell has received the specific ligand and co-stimulatory signals required for TcR signaling from the APC, proliferation and differentiation are initiated. One of the first stages of activation involves the entry of the T cell into the G_1 phase of the cell cycle, expression of a high-affinity form of the IL-2 receptor and release by the T cell of IL-2, which has a proliferative effect on the cell (Cerdan *et al.* 1995). Synthesis of IL-2 requires the co-stimulatory signal provided by CD28, and binding of IL-2 to the high-affinity IL-2 receptor triggers progression through the rest of the cell cycle.

The early events following TcR triggering are similar to those involved in BcR signaling (Weiss and Littman 1994). Initial binding of antigen by TcR and co-receptor brings co-receptor associated LCK into proximity with the TcR signaling complex, causing the ITAMs on the accessory chains of the T cell receptor complex to become phosphorylated by activated LCK and TcRζ-associated FYN (Veillette *et al.* 1988; Barber *et al.* 1989). As in B cells, the activity of the Src family kinases LCK and FYN is regulated by ratio of the inhibitory protein CSK to the activating phosphatase CD45. Also in common with BcR signaling, co-receptors in T cells enhance proximal TcR signaling events significantly in comparison to signaling via the TcR alone. One of the principle targets of activated LCK is the ζ-chain-associated protein (ZAP-70) (van Oers *et al.* 1996; Wiest *et al.* 1996), which itself phosphorylates the adapter molecule LAT, which is palmitoylated and targeted to lipid rafts in the membrane (Zhang *et al.* 1998a). Phosphorylated LAT is subsequently bound by downstream signaling molecules including Grb2, SLP-76, VAV, CBL, PLCγ and PI3 kinase, propagating the TcR-derived signal away from the membrane and towards the nuclues. Like BcR signaling,

activated PLCγ and RAS initiate three signaling pathways culminating in the translocation of transcription factors to the nucleus and gene induction leading to cell proliferation and differentiation.

Active PLCγ cleaves a membrane lipid, PIP_2, to generate IP_3 and DAG, both second messengers. IP_3 binds to Ca^{++} channels in the ER allowing the release of Ca^{++} into the cytosol and also facilitates an influx of Ca^{++} from the extracellular fluid. The increase in Ca^{++} concentration in the cytoplasm activates calcineurin, which activates the transcription factor NFAT. NFAT translocates to the nucleus where the initial signal from the plasma membrane is translated into changes in gene expression. DAG, aided by the raised Ca^{++} levels in the cytosol, activates protein kinase C (PKC), which activates another transcription factor, NFκB.

A third downstream signaling cascade is initiated by the activation of guanine nucleotide exchange factors (GEFs) which themselves activate the small G protein RAS. RAS activates the MAP kinase cascade which culminates in the translocation of activated MAPK to the nucleus and the induction and activation of Fos, a component of the AP-1 transcription factor. Another MAP kinase cascade is activated via a signal received from the co-stimulatory molecule CD28.

After 4-5 days of rapid growth, activated T cells differentiate into armed effector T cells that no longer require co-stimulation for activity when recognizing antigen (Gudmundsdottir *et al.* 1999). The cell adhesion molecules of an effector cell are different to those expressed by a naïve T cell, with loss of L-selectin, higher expression of LFA-1 and CD2, and *de novo* synthesis of VLA-4. These changes reflect the different circulation pathway of an effector T cell compared to a naïve T cell, as effector cells must be able to enter the site of infection.

In contrast to $CD8^+$ naïve T cells, which can only differentiate into cytotoxic effector cells after TcR triggering, $CD4^+$ immature effector T cells are termed T_H0 reflecting the fact that they have the potential to differentiate into either T_H1 or T_H2 cells, which have different effector functions. The signals governing this lineage choice are not fully understood. Differentiation into the T_H1 lineage results in cell-mediated immunity and is critically dependent on transducer and activator of transcription (STAT) 4, class II transcription factor (CIITA) T-bet and IL-12 (O'Garra and Arai 2000; Asnagli and Murphy 2001). T_H2 cells elicit a humoral response, and their development is dependent on STAT6, GATA-3, JunB, c-Maf NFAT45 and IL-4 (O'Garra and Arai 2000; Asnagli and Murphy 2001). The MAPK pathway regulates most transcription factors, suggesting differential activation of this pathway is also important for T_H1 or T_H2 lineage choice. JNK2 has been reported to be an important factor for the polarization of CD4 T cells towards a T_H1 phenotype (Yang *et al.* 1998), and JNK1 is capable of blocking the development of a T_H2 response (Dong *et al.* 1998), whereas GATA-3 expression is crucial for the differentiation of T_H2 cells (reviewed in (O'Garra and Arai 2000)). JNK1 acts by decreasing the nuclear abundance of NFATc, which binds the IL-4 promoter. Thus the ratio between transcription factors is important in determining the outcome of CD4 differentiation – large amounts of NFATc will promote IL-4 production and differentiation into the T_H2 lineage, whereas a reversal of this ratio will promote cytokines associated with T_H1 commitment. Furthermore, there is evidence to suggest that sustained Ca^{++} release leads to T_H1 development (Leitenberg and Bottomly 1999) while transient Ca^{++} mobilization favours the maturation of T_H2 cells (Sloan-Lancaster and Allen 1996; Leitenberg and Bottomly 1999).

1.8 Modification of T cells by glycosylation

T cell development is marked by changes in the expression of cell surface glycoproteins (Despont *et al.* 1975; Reisner *et al.* 1976; Pink 1983; Toporowicz and Reisner 1986; Lefrancois 1987). Post-translational glycosylation of proteins can have many effects on

the cell, both for regulation and effector function. If the glycosylation of a protein is incorrect, the protein may fail to fold correctly and be unable to exit the ER, resulting in degredation (Suzuki *et al.* 1995). The glycosylation can often affect the intracellular trafficking of protein through the cell, allowing cell surface expression or retention in the Golgi complex (Gleeson *et al.* 1994). Clustered areas of glycosylation can rigidify short stretches of protein and forcing functional globular domains away from the membrane, allowing them to interact with their ligands (Hounsell *et al.* 1996), or can act as ligands themselves to enhance protein interactions or cell-cell adhesion. Glycans can be attached to a protein by two specific linkages, denoted N- and O-linked. N-linked glycans are linked via asparagine residues and can be classified into three subgroups; complex, high-mannose or hybrid, which respond differently to endoglycosidase enzymes and represent differing additions to a trimannosyl core. N-glycans are often modified by the further addition of sialic acid, which carries a negative charge, just before transport to the cell surface in the Golgi. O-linked sugars are attached to a protein via a serine or threonine side chain (Goochee *et al.* 1991). They have no common core structure and can be categorized into eight groups according to the core structure expressed, but all O-linked glycans have as a starting point a GalNac moiety attached to the serine or threonine residue (Van den Steen *et al.* 1998). The glycoprotein is modified as the molecule is transported through the ER-plasma membrane to the cell surface, with further modification by addition of sialic acid in the Golgi (Pascale *et al.* 1992a; Pascale *et al.* 1992b). Almost all of the key molecules involved in antigen recognition and subsequent effector function are glycoproteins, and some of the most heavily glycosylated cell surface proteins on T cells are CD43, CD45 and CD8.

Glycosylation of CD43

CD43 is an abundant, highly glycosylated T cell surface protein which exists in a lower-molecular weight form in resting cells and a higher-molecular weight form in activated cells. There is data showing it functions as both an adhesion and an anti-adhesion molecule, as well as having roles in T cell trafficking, co-stimulation and

apoptosis (Manjunath *et al.* 1995; Sperling *et al.* 1995; Ostberg *et al.* 1998; Sperling *et al.* 1998; Stockton *et al.* 1998; He and Bevan 1999; Kishimoto and Sprent 1999; Onami *et al.* 2002). The glycosylation of CD43 is developmentally regulated, with the higher-molecular weight protein produced after activation due to enzymatic changes causing differences in the core structure of the O-glycans (Higgins *et al.* 1991; Ellies *et al.* 1994; Jones *et al.* 1994; Kishimoto and Sprent 1999). These developmental changes in CD43 glycosylation may regulate function, as ligands recognizing specific glycan structures on the protein, e.g. Siglec-1 (van den Berg *et al.* 2001), will be affected by changes in core structure and sialylation.

Glycosylation of CD45

CD45 is a transmembrane protein tyrosine phosphatase (PTP) which is important for removing negatively regulating phosphates on Src family kinases, implicating the protein in the selection of both T and B lymphocytes (Byth *et al.* 1996; Cyster *et al.* 1996; Penninger *et al.* 2001). The ectodomain, like CD43, is highly N- and O-glycosylated and contains multipe helix-breaking amino acids, suggesting a rod-like structure (Neel 1997). Differential splicing of three exons results in up to eight isoforms of CD45, each differing in structure, glycosylation content and overall charge. The splice variants are expressed on particular cell lineages, and the alternative glycosylation of each isoform has been suggested to be important for developmentally regulated functioning of the molecule, with dimerisation of the molecule strictly controlled by the presence of particular O-linked glycans and sialic acid (Xu and Weiss 2002). The glycan moieties of CD45 can be ligands for other molecules, an effect also regulated by changes in glycosylation observed during T cell maturation (Wu *et al.* 1996; Priatel *et al.* 2000; Baldwin and Ostergaard 2001; Nguyen *et al.* 2001).

Glycosylation of CD8

CD43 and CD45 have extended length and heavy sialylation, and as such are likely to be excluded from the IS (Shaw and Dustin 1997), but in contrast CD8 is known to bind the same MHC molecule as the TcR (Janeway 1992). As such, the developmentally

regulated glycosylation of CD8 is likely to have important effects for T cell/ APC interaction.

Both the α- and the β-chain of CD8 are subject to modification by N- and O-linked glycosylation. CD8α has three sites of N-linked glycosylation and CD8β one, all of which are located in the Ig-like domain of the molecule (Rothenberg and Triglia 1983; Luscher *et al.* 1985). Both chains are also modified by the addition of O-linked glycans, which are then further modified by the addition of sialic acid, giving the molecule an overall negative charge. Human and rat CD8α have multiple sites of O-linked glycosylation clustered in the hinge region (Classon *et al.* 1992; Pascale *et al.* 1992b), and murine CD8α, which has not been mapped, is predicted to have a similar pattern of O-linked glycans. Murine CD8β was recently mapped and showed five sites of O-linked glycosylation clustered distal to the cell membrane, near the Ig-like domain (Moody *et al.* 2003). A map of known and predicted sites of O- and N-linked glycosylation is presented in figure 4.1

Developmentally regulated changes in CD8 glycans have been previously noted both in thymocytes and activated T cells (Casabo *et al.* 1994; Wu *et al.* 1996). Previous work in the lab also showed that on T cell activation, CD8β, but not CD8α becomes hyposialylated, and that CD8 changes the core structure of its O-glycans after T cell activation (Casabo *et al.* 1994; McNeill 1999). Recent studies have shown that developmental changes in CD8 binding to MHC Class I are also regulated by the addition of sialic acid (Daniels *et al.* 2001; Moody *et al.* 2001a).

1.9 Aim of the thesis

The CD8β polypeptide has long been implicated in selection in the thymus, since mice lacking CD8β and expressing only CD8αα show an 80% drop in peripheral T cell numbers (Crooks and Littman 1994). Also, it is CD8β that uniquely becomes

hyposialylated after T cell activation (Casabo *et al.* 1994). The O-linked glycans of CD8β have been mapped to the hinge region of the molecule, near the Ig-like domain (Moody *et al.* 2003), and a mouse expressing a mutated hinge region of CD8β was created to better understand the role of the glycans in T cell differentiation and activation. The mutated CD8β polypeptide has the Ig-like, transmembrane and cytosolic region of wild type CD8β, but the hinge region of CD8α and is therefore termed CD8βα hinge ($CD8\beta^{\alpha h}$) (figure 1.3). For a description of the generation of $CD8\beta^{\alpha h}$ expressing mice, see Chapter 2, section 2.2. Surprisingly, when expressed on a polyclonal, $Cd8\beta^{-/-}$ background, the mutant molecule failed to restore selection above levels seen in mice lacking CD8β (McNeill 1999). To address the question of why the mutated molecule was failing to restore selection, the mice were back-crossed with $Rag^{-/-}$, F5 transgenic TcR expressing mice. The only cells which are able to develop are $CD8^{+}$ cells expressing the F5 TcR, allowing selection conditions to be more easily manipulated.

The aim of this thesis is to investigate why the $CD8\beta^{\alpha h}$ molecule is no more efficient as a co-receptor than CD8αα. The question was approached in a variety of ways. Phenotypic data compared cell surface maturation markers on the thymus in order to try and establish where selection was failing. The ability of cells expressing $CD8\beta^{\alpha h}$ to bind cognate and self-pMHC, in comparison to cells expressing CD8αβ or CD8αα was assessed, both in thymus and in peripheral cells. Signalling capability of the cells was assessed biochemically, by co-immunoprecipitation of key signaling molecules, as well as *in vitro*, using cross-linking of antibodies to generate a Ca^{++} flux. The response of peripheral cells to cognate peptide provided more insight into $CD8\beta^{\alpha h}$ binding MHC, and threshold of activation, and sensitivity to CD8-specific antibodies addressed the question of T cell dependence on CD8 in order to respond. Finally, the glycan structures associated with $CD8\beta^{\alpha h}$ were assessed in resting and activated cells.

Figure 1.1 - T cell development in the thymus.

Common lymphoid progenitors migrate from the bone marrow to the thymus, where they are termed double negative thymocytes (DN) due to the lack of expression of CD4 and CD8. DN thymocytes progress through four distinct developmental stages, DN1 to DN4, characterized by the expression of cell surface maturation markers CD25 and CD44. DN thymocytes do not express TcR, but express pre-TcR at the DN3 stage. Successful expression causes the cell to proliferate during the DN4 stage, and progress to the double positive stage (DP), where the developing thymocytes expresses CD4 and CD8 as well as the mature TcR, and undergo strict selection criteria. Positive and negative selection occur when the maturing thymocytes interact with self-MHC/ self-peptide complexes expressed on cortical epithelial cells, and successful negotiation of both processes results in $CD8^+$ single positive (SP) thymocytes that are MHC Class I restricted or $CD4^+$ SP thymocytes that are specific for MHC Class II binding. SP cells proliferate and develop further in the thymus medulla before being exported to the periphery as mature naïve T cells. Derived from (Germain 2002).

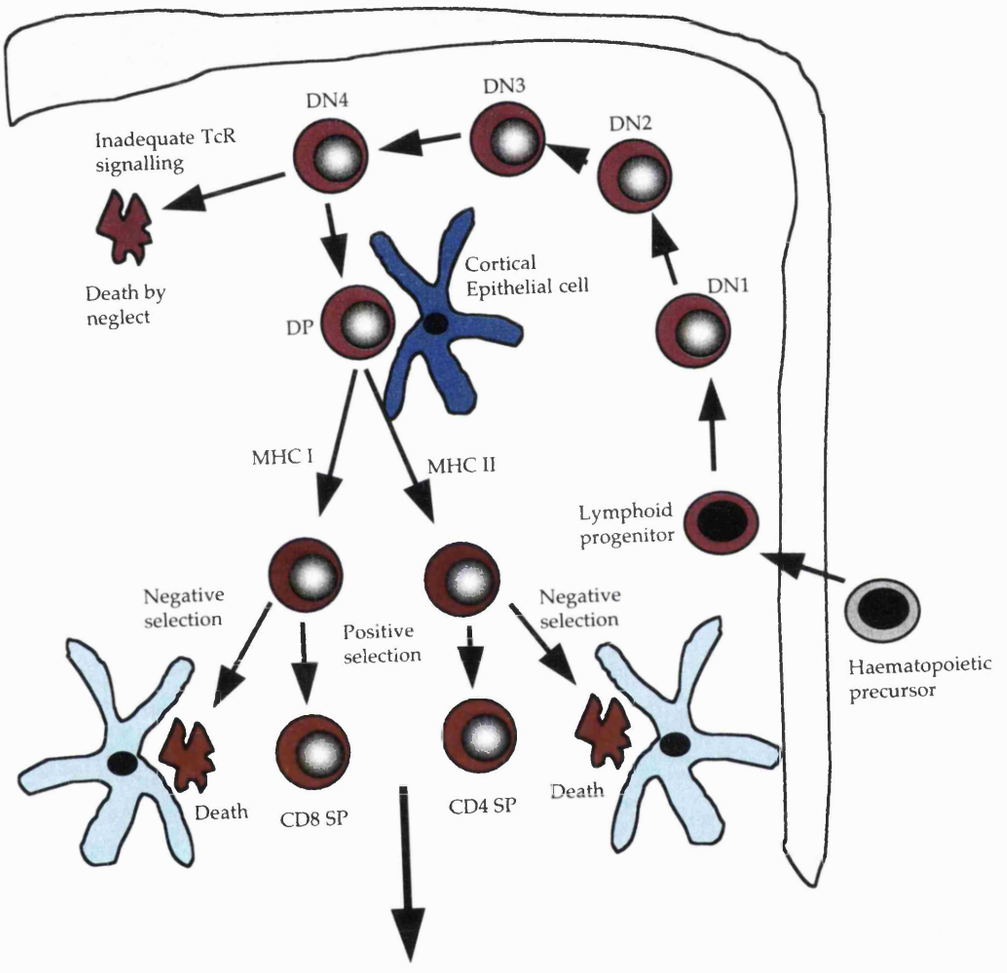
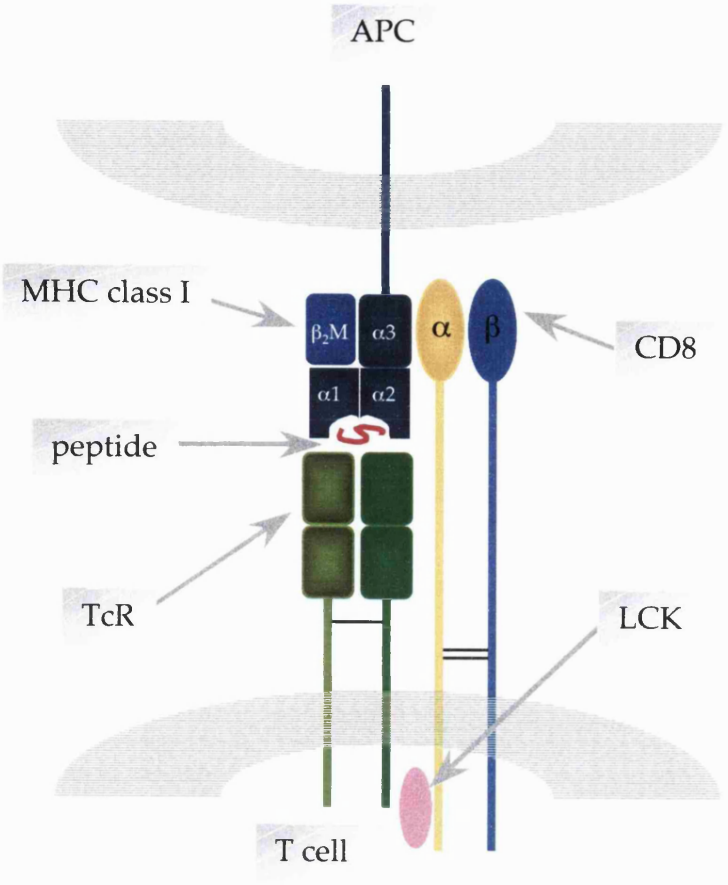

DN4
DN3
DN2
Inadequate TcR signalling
Death by neglect
Cortical Epithelial cell
DP
DN1
MHC I
MHC II
Lymphoid progenitor
Negative selection
Positive selection
Negative selection
Haematopoietic precursor
Death
CD8 SP
CD4 SP
Death

Figure 1.2 - Interaction between pMHC and the TcR complex.

Schematic showing the interaction of the MHC Class I/ peptide complex with the TcR and CD8 co-receptor on the cytotoxic T cell (top), and the interaction of the MHC Class II/ peptide complex with the TcR and CD4 co-receptor on the helper T cell (below). CD8α interacts with the α3 domain of MHC Class I, while the D1 and D2 domains of CD4 interact with the β2 domain of MHC Class II.


APC
MHC class I
β_2M
α3
α
β
CD8
α1
α2
peptide
TcR
LCK
T cell

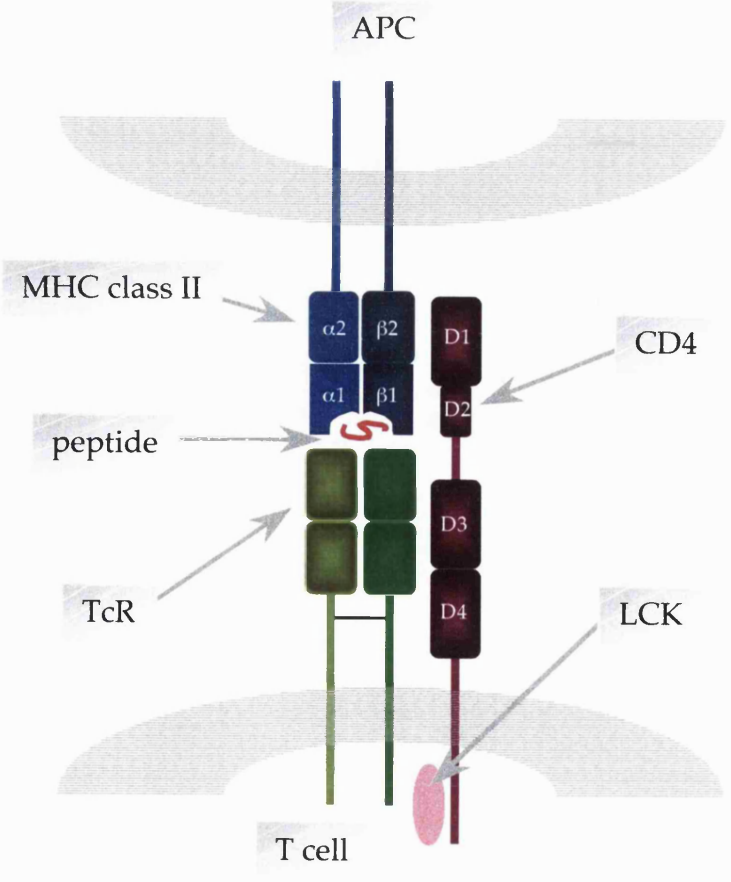
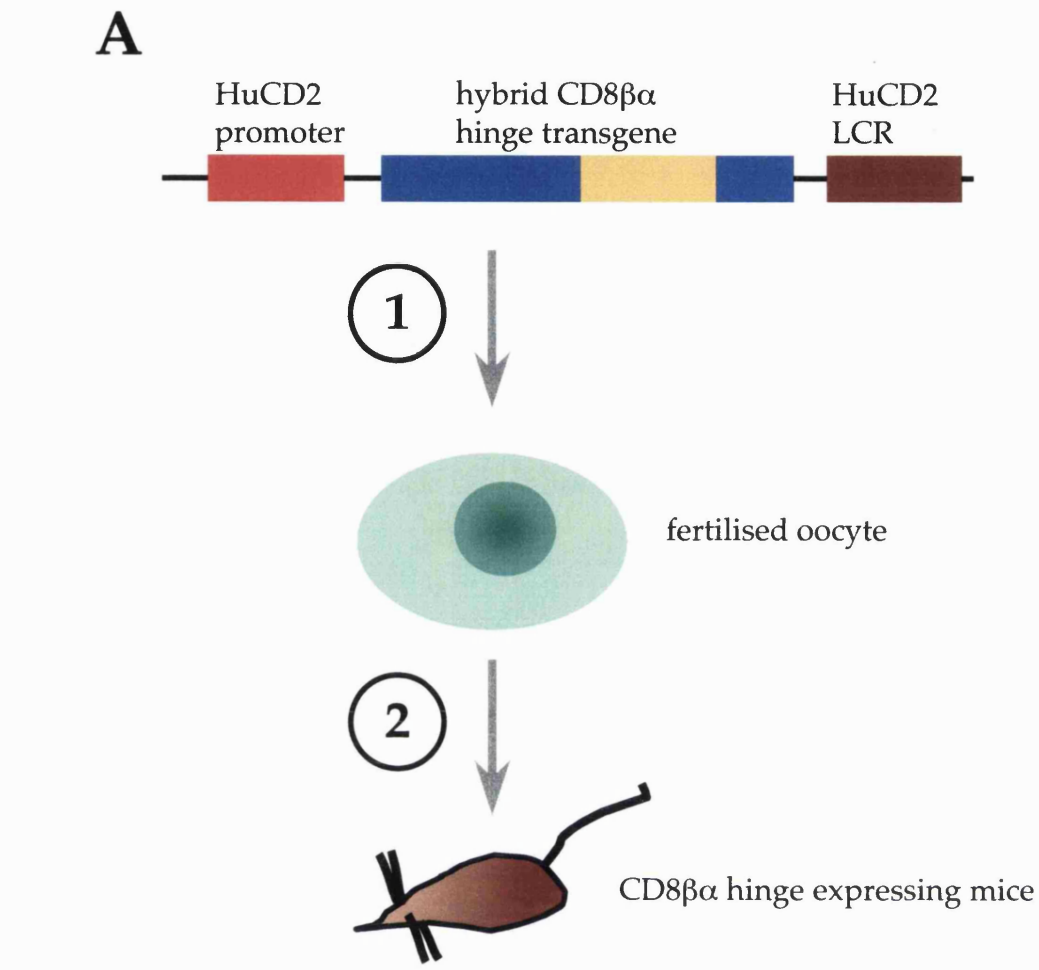
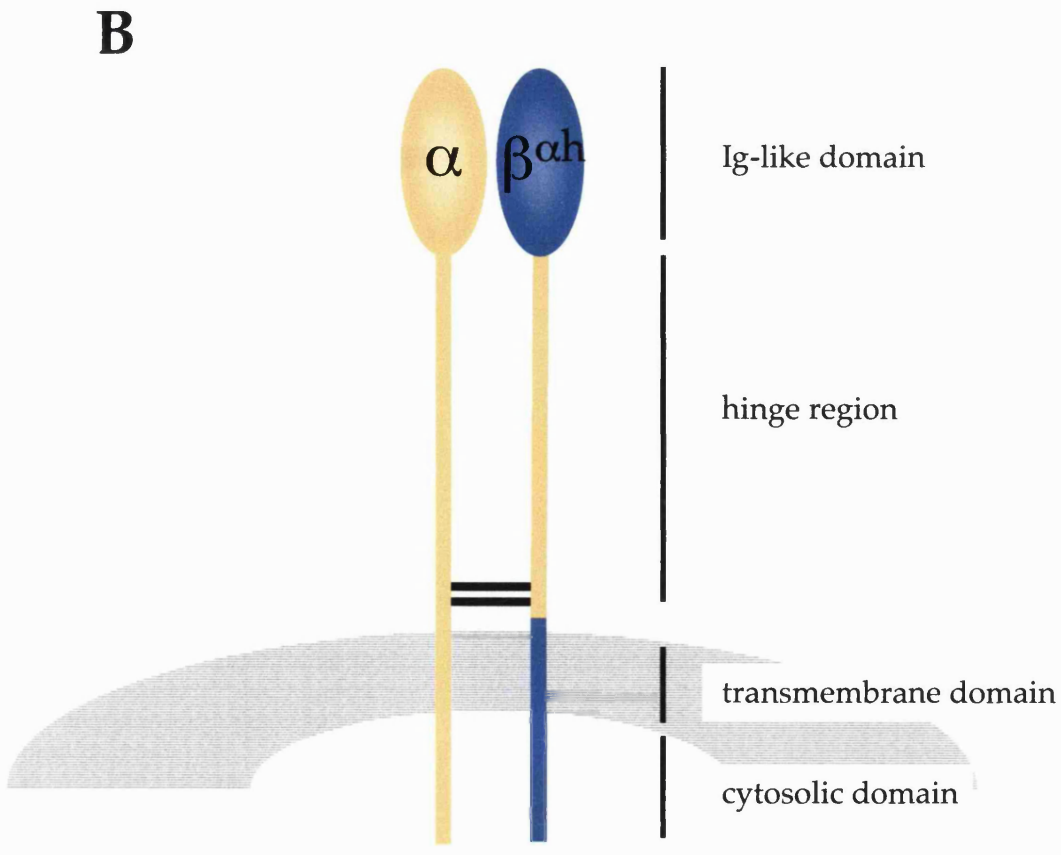

APC
MHC class II
α2
β2
D1
CD4
α1
β1
D2
peptide
D3
TcR
D4
LCK
T cell

Figure 1.3 - Creation of CD8β$^{\alpha h}$ expressing mice.

Panel A – DNA containing the hybrid molecule is injected into fertilized oocytes from (CBA x B10) F2 mice (1). Yellow represents CD8α, blue represents CD8β. Founder mice are generated (2) and screened for the presence of the transgene.

Panel B – the structure of the CD8β$^{\alpha h}$ molecule. CD8α (yellow) is disulphide bonded to the CD8β$^{\alpha h}$ polypeptide (blue/ yellow). The CD8β$^{\alpha h}$ polypeptide contains Ig-like, transmembrane and cytosolic CD8β domains (blue), and the hinge region of CD8α (yellow).


A
HuCD2
promoter
hybrid CD8βα
hinge transgene
HuCD2
LCR
1
fertilised oocyte
2
CD8βα hinge expressing mice


B
α
βαh
Ig-like domain
hinge region
transmembrane domain
cytosolic domain

2. Materials & methods

2.1 Materials

Handling medium

Air-buffered Iscove's Modified Dulbecco's Medium (IMDM), (Sigma)/ 2% fetal calf serum (FCS)

Culture medium

IMDM/ 10% FCS/ 1% glutamine/ 1% antibiotics/ 5 x 10^{-5}M 2-ME

Protease inhibitors

PEFA block (Sigma) 1mM in dH_2O

SPI – Antipain/ Chymostatin/ Leupeptin/ Pepstatin A (all Sigma) 5μg/ml in DMSO

FACS buffer

0.5% BSA/ 0.05% azide in PBS

Lysis buffer

1% Triton X-100 (Sigma)/ 150mM NaCl/ 10mM Tris pH7.5 in dH_2O

Lower gel buffer

1.5M Tris pH 8.8/ 0.4% SDS in dH_2O

Upper gel buffer

0.5M Tris pH 6.8/ 0.4% SDS in dH_2O

SDS running buffer

400mM glycine/ 0.1% SDS/ 50mM Tris pH8.3 in dH_2O

Reducing sample buffer

2% SDS/ 10% glycerol/ 6.25mM Tris pH6.8/ 5% 2-ME/ 0.01% bromophenol blue in dH_2O

Western blot buffer

200mM glycine/ 25mM Tris in dH_2O

Western blot blocking buffer

5% dry milk/ 1% Tween-20 (Sigma)/ 0.05% azide in PBS

Western blot washing buffer

1% Tween-20 (Sigma) in PBS

Western blot stripping buffer

100mM 2-ME/ 2% SDS/ 62.5mM Tris pH6.7

Antibody reagents

Name	Specifity	Purification	Reference
YTS169.4V	anti-CD8α	Mono S	Cobbold *et al* (1984)
53.6.7	anti-CD8α	Commercial (Pharmingen)	Ledbetter *et al* (1979)
YTS156.7	anti-CD8β	Mono S	Qin *et al* (1989)
KT112	anti-CD8β	Mono S	Tomonari *et al* (1990)
53-5.8	anti-CD8β	Commercial (Pharmingen)	Ledbetter *et al* (1979)
H57-597	anti-TcRβ	Commercial (Pharmingen)	Gascoigne (1990)
53-7.3	anti-CD5	Commercial (Pharmingen)	Ledbetter *et al* (1979)
RM4-5	anti-CD4	Commercial (Pharmingen)	Nakamura (1992)

PNA	core-1 glycan	Commercial (EY Laboratories, Inc.)	Lotan *et al* (1975)
ECA	core-2 glycan	Commercial (EY Laboratories, Inc.)	Teneberg *et al* (1994)
SNA	sialic acid	Commercial (EY Laboratories, Inc.)	Shibuya *et al* (1987)

2.2 Methods

Generation of $CD8\beta^{\alpha h}$ expressing mice

The hybrid $CD8\beta^{\alpha h}$ transgene was constructed by ligation of three separate PCR fragments formed by two PCR reactions. In the first reaction, two DNA products were separately amplified from an SK plasmid containing cDNA encoding the murine CD8β gene. One PCR product encoded the Ig-like domain of CD8β, the second PCR product spanned the transmembrane and cytoplasmic domains of CD8β. In the second PCR reaction, one DNA product was amplified from an SK plasmid containing the murine CD8α gene. This third PCR product spanned the membrane proximal domain or hinge region of CD8α. The fragments were three part ligated to form the transgene construct and cloned into the SK plasmid. The sequence of the DNA was confirmed by DNA sequencing. In order to obtain expression of the $CD8\beta^{\alpha h}$ transgene in transgenic mice, the hybrid molecule was prepared in the VA expression vector containing the human CD2 control region, which gives expression in DP and SP thymocytes and mature CD4 and CD8 cells (Zhumabekov *et al.* 1995). The $CD8\beta^{\alpha h}$ construct was subcloned into the VA vector and the plasmid containing the construct transformed into competent *E. coli* by the heat shock method. Positive bacteria grown in selecting medium were picked and one correctly orientated clone was identified, grown up and DNA prepared for microinjection of fertilized oocytes (figure 1.3). DNA was screened from founder mice for the presence of the transgene and mice positive for transgene expression were back-crossed with $Cd8\beta^{-/-}$ mice to exclude the effect of endogenous CD8β. Control mice were

created expressing a wild type CD8β construct also in the VA vector, screened for the presence of the transgene and mice positive for transgene expression also back-crossed with *CD8β*$^{-/-}$ mice. All mice were on a B10 background, and were backcrossed to a monoclonal background.

Mice for analysis in the thesis were, unless otherwise stated, all homozygous for the monoclonal F5 transgenic TcR and on a *Rag1*$^{-/-}$ background (Townsend *et al.* 1984). Mice expressing transgenes were F5 TcR homozygous, *Rag1*$^{-/-}$ *CD8β*$^{-/-}$ to exclude the effect of endogenous CD8β. Mice were bred in a conventional colony free of Pathogens at the National Institute for Medical Research, and phenotyping analysed at 4-6 weeks old.

Flow cytometric analysis

Ex vivo cells were teased to a single cell suspension in handling medium and counted. Cells were pelleted at 4°C, 1200rpm for 5 minutes and resuspended in handling medium. 1 x 10^6 cells were removed to tubes or a 96 well plate, pelleted as before and resuspended in 50μl fluorescently labeled antibodies. The dilutions of each antibody were determined by antibody titration prior to analysis, and the antibodies were diluted in FACS buffer. The cells with antibody mix were incubated at 4°C for 45 minutes, then washed with FACS buffer at 4°C and pelleted.

If biotinylated antibody had been used, a second layer of 50μl fluorescently labelled streptavidin in FACS buffer at a concentration of 2μg/ml was added to the cells. The cells were incubated at 4°C for 15 minutes then washed and pelleted.

After the final wash the cells were resuspended in 100μl FACS buffer and analysed using a FACS Calibur (Becton Dickinson) and CellQuest software. In all figures, fluorescent intensity is represented on a log scale from 10^0 to 10^4.

Preparation of recombinant MHC dimers for flow cytometric analysis

If cells were to be stained using Dimer X I (BD Biosciences Pharmingen), the recombinant dimers were prepared one day prior to analysis. Peptide was incubated with Dimer X product diluted in PBS pH7.2 overnight at 37°C. The amount of peptide to be incubated was worked out according to the following calculation:

$$Mp = MD^b \times R \times Dp / DD^b$$

Where

Dp = molecular weight of peptide to be added

DD^b = molecular weight of Dimer X (250 kDa)

R = desired excess molar ratio, typically 160

Mp = micrograms of peptide to be added

MD^b = micrograms of Dimer X in the reaction, typically 0.25μg per million cells.

After overnight incubation at 37°C, 50μl of undiluted dimer solution was incubated with 1 x 10^6 test cells at 4°C for 30 minutes. Cells were washed and resuspended in 50μl of fluorescently labeled antibodies diluted in FACS buffer and treated as in *Flow cytometric analysis*.

Preparation of cell lysates

Ex vivo cells to be lysed were teased to a single cell suspension, pelleted at 4°C, 1200rpm for 5 minutes and the supernatant discarded. The pellet was resuspended in lysis buffer with protease inhibitors and incubated at 4°C for 15 – 30 minutes.

Lysates were microfuged at 13 000 rpm for 3 minutes at 4°C to pellet nuclei and post-nuclear supernatants used or stored at –20°C until needed.

Immunoprecipitation

Lysates were firstly precleared by incubation with 30µl of RIg (or appropriate antibody control for specific immunoprecipitation) coupled to sepharose beads, BSA coupled to sepharose beads and beads alone to remove any non-specific binding. Lysates were pre-cleared for 2 hours at 4°C then microfuged at 6500rpm for 3 minutes at 4°C to pellet the sepharose beads. The supernatant was removed either for further preclearing or for specific immunoprecipitation by incubating with antibody coupled to sepharose beads at 4°C for between 2 and 12 hours, depending on the antibody used. After precipitation the beads were microfuged and the pellet washed 3 times in lysis buffer with protease inhibitors to remove any trace of lysate, then used or stored at –20°C until required.

SDS/ Polyacrylamide gel electrophoresis

12.5% gels were made as follows, according to the method described by (Laemmli 1970): 7.5ml lower gel buffer, 12.5 ml of 30% (w/v) acrylamide (National Diagnositcs) and 10ml dH_2O. After 10-15 minutes' degassing, 100µl of 10% freshly made ammonium persulfate (APS) and 10µl Temed (BioRad) were added, the gel was cast and left to polymerize for at least 45 minutes. The polymerizing gel was covered with a layer of butanol to aid polymerisation. The stacking gel was prepared as follows: 2.5ml upper gel buffer, 1.5 ml 30% acrylamide, 6ml dH_2O, 50µl 10% ammonium persulfate and 10µl Temed. The lower gel was rinsed to remove butanol, air dried and the stacking gel poured on top. The gel comb was set in place and the gel left to polymerize, then the comb was removed and the wells washed with SDS running buffer.

In some experiments pre-cast gels were used (Invitrogen).

Proteins were immunoprecipitated as described. Samples were prepared for gel electrophoresis by adding 50µl of 2x reducing sample buffer, boiling for 5 minutes, cooling on ice and finally microfuged at 6500 rpm, 4°C for 3 minutes. Molecular weight markers or total cell lysate (10^6 cell equivalents) were boiled with 30µl of 2x reducing

sample buffer and cooled on ice. 30µl of supernatant, lysate or molecular weight markers were loaded onto the gel and the electrophoresis run at 30mA (day) or 7mA (overnight) until completion.

Western blotting

The blotting membrane was soaked in 100% methanol for 15 minutes, then the methanol was diluted out with dH_2O. The SDS gel to be blotted was equilibriated in Western blot buffer (10% blot buffer, 30% methanol) or CAPS buffer (Sigma). The blot was made up as follows: negative electrode, sponge, 2 sheets of filter paper, SDS gel, blotting membrane, 2 sheets of filter paper, sponge, positive electrode, all equilibriated for 10 minutes in Western blot buffer. The proteins were blotted overnight onto the membrane at 120mA at 4°C.

After transfer the blot was briefly rinsed with PBS several times to remove any remaining blot buffer. The blot was then blocked for non-specific binding with blocking buffer for at least 2 hours at 4°C. The specific antibody was added at an appropriate dilution in blocking buffer and incubated with the membrane at 4°C overnight.

The blot was rinsed several times with PBS to remove any blocking buffer, and washed in a large volume of washing buffer with several changes for about 6 hours. The HRP-conjugated detection antibody was diluted in washing buffer and incubated with the blot at room temperature for 1 hour in the dark. The blot was washed as described above for another hour with several changes of wash buffer, then dried between 2 filter papers.

Proteins were visualized using ECL kit from Amersham International. After 1 minute's incubation with ECL solution, the blot was dried between filter papers and placed in plastic. The blot was then exposed to x-ray film.

In order to probe with another antibody, some blots were stripped for 15 minutes at 55°C in stripping buffer. The blot was rinsed several times with PBS to remove all trace of stripping buffer, then incubated with blocking buffer as described above. The blot was then washed and probed with a different specific antibody diluted in washing buffer as described above.

In vitro *culturing and analysis of cells*

Cells to be analysed *in vitro* were removed from mice under sterile conditions into sterile handling medium. All further work was done in a tissue culture hood to prevent contamination. A single cell suspension was made and cells washed in handling medium. Cells were resuspended at a concentration of 0.5×10^6 cells/ ml in culture medium. Cells were incubated at 37°C for 72 hours unless otherwise stated. Cells were activated with NP68 peptide - amino acids 366-374 of influenza nuclear protein from 1968 flu strain (Townsend and Skehel 1984) - the agonist peptide for the F5 TcR.

For CFSE labeled cells, the dye was added to cells at a concentration of 2nM in PBS, 30×10^6 cells/ ml at room temperature for ten minutes. The cells were washed twice in PBS then resuspended in culture medium. After culture the cells were analysed by flow cytometry.

Data from the frequency of CFSE labeled cells (F) that had undergone division rounds (d) were used to calculate the adjusted frequencies (AF) by dividing F by 2^d. The average number of cell divisions of the starting cell population was then calculated according to the formula $\Sigma(AF \times d)/\Sigma F$.

Transfer of cells to immunodeficent hosts

Lymphocytes were removed from donor mice into handling medium and teased to form a single cell suspension. Cells were washed once in handling medium and centrifuged at 1200 rpm for 5 minutes. Cells were resuspended in PBS containing CFSE at a concentration of 2nM and labeled at a concentration of 30×10^6 cells/ ml at room

temperature for ten minutes. The cells were washed twice in PBS, resuspended in PBS/ 0.5% BSA and re-counted. The CFSE labeled cells were transferred via tail vein injections into RAG deficient hosts at a concentration of 2×10^6 cells per animal.

After two weeks the lymph nodes and spleens of the host animals were removed into handling medium and teased to form a single cell suspension. The cells were washed and counted and all cells resuspended in FACS buffer containing appropriate dilutions of fluorescently labeled antibodies. Cells were then treated as described in *Flow cytometric analysis*.

Inducing Ca^{++} flux by crosslinking antibodies

At all stages of this procedure the cells were kept at room temperature.

Thymocytes or lymph nodes were teased into handling medium. Cells were washed in PBS and resuspended in PBS containing 2μM Indo-1 AM at a concentration of 2.5×10^7 cells/ ml. After incubation at room temperature in the dark for 30 – 45 minutes, cells were washed in PBS and resuspended in PBS containing appropriate dilutions of fluorescently labeled or biotinylated antibodies and incubated at room temperature for 30 minutes. Cells were washed once more and resuspended in 400μl PBS. Cells were run through an LSR (Becton Dickinson) and analysed using CellQuest software. After 1 minute's running to establish a base line, 2μl of streptavidin was added to cross link biotinylated antibodies and the cells returned to the LSR for a further 5 minutes.

3. Phenotype and physical characteristics of mice expressing CD8β$^{\alpha h}$ in the thymus.

Introduction

The CD8 co-receptor is critical for the differentiation of MHC class I restricted T cells, and mice lacking CD8 have none of these cells in the periphery (Fung-Leung *et al.* 1991). CD8 enhances T cell responses via recognition and direct binding of MHC (Salter *et al.* 1990; Gao *et al.* 1997), and also by association with the signaling molecule LCK (Veillette *et al.* 1988) which is essential for downstream signaling in T cell activation (Weiss and Littman 1994; van Oers *et al.* 1996).

CD8 molecules comprise two disulphide linked chains and can exist as CD8αβ heterodimers or CD8αα homodimers. The interaction between MHC and CD8 has been visualized in the crystal structure using CD8αα homodimers (Gao *et al.* 1997), and these molecules are able to associate with and signal via LCK (Irie *et al.* 1995; Irie *et al.* 1998). Yet thymocytes almost exclusively express CD8αβ heterodimers, and CD8β has been shown to greatly enhance association with LCK and subsequent signaling events (Bosselut *et al.* 2000; Arcaro *et al.* 2001), suggesting an important role in thymocyte selection for the CD8β chain. Moreover, when the gene for CD8β is disrupted, mice show an 80% reduction in normal numbers of CD8$^+$ SP cells, even though the cells are expressing CD8αα homodimers on the cell surface (Crooks and Littman 1994). The drastic reduction in cell numbers of these mice suggests that a significant role is played by the CD8β chain in thymus selection.

The availability of MHC tetramers has allowed many questions to be asked of the role of CD8β in the recognition of MHC, both for differentiation and activation. Daniels et al. (2000) showed that TcR binding to cognate MHC Class I molecules is CD8

dependent. Other reports indicate that cell bound CD8αβ, but not CD8αα or soluble CD8αβ substantially increases the avidity of TcR-ligand binding (Bosselut *et al.* 2000; Arcaro *et al.* 2001). These differences are thought to occur as a result of the targeting of cell bound CD8αβ to lipid rafts due to palmitoylation of the cytoplasmic region, as well as assuming an optimal conformation by way of glycosylation. CD8αα is glycosylated differently and is not targeted to lipid rafts, whereas soluble CD8 proteins are unglycosylated and also not associated with the cell membrane, which may account for the inability of these molecules to enhance binding to cognate MHC.

MHC tetramers have also elucidated another potentially important recognition event in thymic development. DP cells can bind self, non-cognate MHC molecules in the thymus in a CD8 dependent, but TcR and peptide independent manner (Moody *et al.* 2001b). Recognition is highest in DP thymocytes and decreases throughout the cell's ontogeny (Daniels *et al.* 2001; Moody *et al.* 2001a). Studies using mice with differing efficiencies of positive selection indicated that the extent of binding correlates inversely with the extent of positive selection (Daniels *et al.* 2001).

In contrast to CD8αβ molecules, CD8αα molecules on DP thymocytes have not been demonstrated to bind in a non-cognate fashion to any specificity of MHC (Bosselut *et al.* 2000; Moody *et al.* 2001a). This recognition step has been suggested to promote a successful interaction between the DP cell and the thymus epithelium at a developmental stage where TcR levels are comparatively low on the cell (Daniels *et al.* 2001). Non-cognate recognition depends on the expression of CD8β and is another possible reason for the $CD8^+$ T cells to overwhelmingly favour expression of CD8αβ heterodimers. Non-cognate recognition of MHC by DP thymocytes is developmentally regulated and has been shown to be directly related to the extent of glycosylation of the CD8 molecule (Daniels *et al.* 2001; Moody *et al.* 2001a). As the cell undergoes selection and becomes SP, the CD8αβ molecules become more heavily sialylated and non-cognate binding of MHC diminishes as a direct consequence of this modification.

Previous work in the lab reported reduction in sialylation of CD8 in activated peripheral T cells (Casabo *et al.* 1994) although, interestingly, this does not seem to restore binding of non-cognate MHC (Harrington *et al.* 2000; Priatel *et al.* 2000). One reason for this could be the finding that it is the CD8β chain only, and not the CD8α chain, that becomes hyposialylated, in contrast to the glycosylation state of DP cells where both chains are hyposialylated. Sialic acid can be attached to O- and N-linked glycans by two different linkages, known as α2,3 and α2,6, which require different enzymes and are distinguishable with carbohydrate specific plant lectins. The nature of the linkage of sialic acid to CD8 has been shown to be an α2,6 linkage in thymocytes, resting and activated T cells (McNeill 1999), suggesting that it is not an inherent difference in the sialylation state of the glycans that is causing this inconsistency. Nevertheless, even removal of sialic acid with neuraminidase from mature $CD8^+$ cells does not restore non-cognate MHC binding ability (Daniels *et al.* 2001).

The hinge region of CD8β is highly conserved across species, and has been shown to have five sites of O-linked glycosylation clustered in the hinge region near the Ig-like domain of the polypeptide (Moody *et al.* 2003). The O-linked glycans are themselves modified by the addition of sialic acid. In order to test the relevance of the hinge region and the glycosylation of the molecule, a mouse was created expressing a mutated form of CD8. The mutant mouse expresses endogenous CD8α and transgenic CD8β on a CD8β knockout background, negating any effect that endogenous CD8β would have. The transgenic CD8β gene, called 'CD8βα hinge' – $CD8\beta^{\alpha h}$ – expresses the Ig-like, transmembrane and cytoplasmic domains of the wild type protein, but the hinge region of the CD8α protein (figure 1.3). In this way it was hoped to assess the impact of the substitution without substantially altering the overall structure of the molecule.

When expressed on the B10 polyclonal, $Cd8\beta^{-/-}$ background, the $CD8\beta^{\alpha h}$ transgene failed to restore selection in the thymus, and the percentage of cells selected was

similar to that of mice lacking CD8β altogether (McNeill 1999). In order to address the question of why and where the block in differentiation occurred, the mice were crossed onto the F5 *Rag1*$^{-/-}$ monoclonal background expressing a single TcR specificity. In order to address the question of whether this failure to restore selection was a consequence of the protein being expressed by the huCD2 promoter, a second control transgenic mouse was created expressing wild type CD8β, CD8β^{tg}, under the same promoter as CD8β$^{\alpha h}$ and also on a CD8β knockout background.

Thymocyte differentiation can be followed by the characteristic expression patterns of a variety of maturation markers. This chapter compares a number of these markers between the different mouse strains in order to characterize at which stage of differentiation thymocytes were failing to mature.

3.1 Mice expressing CD8β$^{\alpha h}$ on the F5 background do not restore selection, but this is not due to lower expression levels of CD8β.

Mice lacking CD8β have a drastic reduction in the percentage of cells being selected (Crooks and Littman 1994), implicating CD8β as important in this process. As already demonstrated on a polyclonal background CD8β$^{\alpha h}$, when expressed as a transgene, cannot restore selection (McNeill 1999). Surface levels of CD8β have been shown to be critical for selection and function (Nakayama *et al.* 1994), and so this was the first surface level marker to be analysed in developing thymocytes.

Thymocytes from CD8β^{wt} (shaded histogram), CD8β^{tg} (grey line), CD8β$^{\circ}$ (dotted line) and CD8β$^{\alpha h}$ (black line) mice were stained with antibodies specific for CD4 and CD8α. DP and CD8^{+} SP cells were gated and analysed for expression of CD8β. Figure 3.1 shows that mice bearing the CD8β$^{\alpha h}$ transgene do express CD8β on the cell surface. It also confirms lack of CD8β expression in CD8β$^{\circ}$ mice. In DP cells, levels of expression

of CD8β$^{\alpha h}$ on the F5 TcR background are similar to wild type levels. In cells expressing CD8β^{tg} however expression of CD8β in DP cells is about 50% of wild type levels.

As seen in figure 3.1 and enumerated in figure 3.2, in mice lacking CD8β, the percentage of SP cells in thymus drops from wild type levels of approximately 20% on the F5 TcR background to approximately 3%. Expression of CD8β as a transgene can restore selection to roughly 10%, a two-fold decrease in wild type levels. The expression level of CD8β has been shown to be critical for selection in thymus (Nakayama *et al.* 1994), which is a possible explanation for the reduced selection of CD8$^+$ cells in CD8β^{tg} transgenic mice expressing wild type CD8β at approximately half normal levels. In contrast, expression level of the β-chain cannot be responsible for the lack of selection in DP cells expressing CD8β$^{\alpha h}$. Given that selection of CD8$^+$ thymocytes in control CD8β^{tg} mice occurs efficiently for the level of CD8β expressed on the cell surface, this lack of selection in the thymus of animals expressing CD8β$^{\alpha h}$ is unlikely to be a consequence of changes resulting from the protein being expressed as a transgene.

In SP cells levels of CD8β expression are again comparable between mice expressing CD8β^{wt} and CD8β$^{\alpha h}$ (figure 3.1). Levels of CD8β expression in SP cells of CD8β^{tg} mice are lower than that of CD8β^{wt} or CD8β$^{\alpha h}$ mice, approximately 70-80% of wild type levels, compared to 50% in DP cells. CD8β is not upregulated from DP to SP cells in wild type mice, but the expression level of CD8β has been shown to be critical for positive selection. The data of Nakayama *et al* (1994) described mice expressing a transfected copy of the CD8β gene, with lower expression of CD8β in DP cells in these animals compared to wild type. This study found that only thymocytes expressing CD8β over a certain threshold were positively selected, resulting in peripheral T cells expressing amounts of CD8β nearer to wild type levels. This may also be the case for CD8β^{tg} mice since the level of CD8β is higher in SP than in DP cells, suggesting only those expressing the higher levels of CD8β are being positively selected.

3.2 Thymocytes expressing CD8β$^{\alpha h}$ are able to bind antibodies specific for the Ig-like domain of CD8β.

It would be predicted that substituting the hinge region of CD8β with that of CD8α would have no adverse impact on the structure of the Ig domain. However in order to discount the possibility that a structural change in the recognition domain of the CD8β$^{\alpha h}$ molecules was in some way responsible for the lack of selection in thymus, the integrity of the Ig-like domain was examined by looking at the binding of a number of antibodies which bind the Ig-like domain and are conformationally dependent.

Thymocytes from mice expressing wild type CD8β (CD8β^{wt}), wild type CD8β transgene (CD8β^{tg}), no CD8β (CD8β^{-}) or CD8β$^{\alpha h}$ transgene (CD8β$^{\alpha h}$) were labeled using antibodies specific for CD4, CD8α and CD8β. CD4^{+} CD8α^{+}DP and CD4^{-}CD8α^{+} SP cells were gated and analysed for CD8β-specific binding using antibodies YTS156.7, KT112 and 53-5.8 (Ledbetter and Herzenberg 1979; Qin *et al.* 1989; Tomonari and Spencer 1990).

Figure 3.3 shows that both DP (figure 3.3, left histograms) and SP (figure 3.3, right histograms) thymocytes bind all CD8β-specific antibody clones tested. Since these antibodies rely on correct folding of the Ig-like domain for efficient binding, this result indicates that the Ig-like domain of CD8β$^{\alpha h}$ molecules is conformationally intact. Binding of antibody in CD8β^{tg}-expressing thymocytes is about 70% of wild type levels for all clones tested, probably due to lower levels of CD8β on the cell surface. This is consistent with the lower levels of selection seen in these mice, as levels of cell-surface CD8β have been shown to be important for selection (Nakayama *et al.* 1994). However, there are subtle differences in binding between the clones tested in cells expressing CD8β$^{\alpha h}$. Whereas clone 53-5.8 binds the chimeric molecule with the same affinity as wild type CD8αβ, clone YTS156.7 binds the mutated molecule with slightly lower

affinity than wild type, and clone KT112 binds at approximately 70% of wild type levels. This could be due to slight conformational changes in the epitopes, and antibody mapping experiments could help determine if there are important structural differences between the variant CD8 molecules.

3.3 Levels of TcR in mice expressing CD8β^{ah} suggest the block in selection occurs early in DP development.

As the TcR is also essential for selection, and mice deficient in TcR or CD3 components show a block in T cell development (Malissen and Malissen 1996), it was important to determine that TcR expression by CD8β^{ah} mice was also comparable to control CD8β^{wt} and CD8β^{tg} mice. Analysis of TcR levels on DP cells showed considerable heterogeneity in the MFI between the genotypes. Figure 3.4 shows that in CD8β^{-} mice, DP TcR MFI is about 60% of wild type levels. However it is apparent that distinct populations of TcR^{dull} and TcR^{int} (boxed area on histograms) thymocytes are present in CD8β^{wt} and CD8β^{tg} mice whereas only the TcR^{dull} population is seen in CD8β^{-} mice, which do not select as well as the CD8β^{wt} and CD8β^{tg} controls. This is also the case for CD8β^{ah} mice, which have a large population of TcR^{dull} DP cells and very few TcR^{int} cells, and are also impaired in selection in comparison to controls.

Levels of TcR in DP cells are lower compared to SP cells, and TcR expression levels are upregulated as cells mature from DP to SP (Petrie *et al.* 1990; Shortman and Wu 1996). It is possible that the TcR profiles seen in Figure 3.4 reflect the heterogeneity of maturation states of cell subpopulations in the thymus. Various studies have shown that most DP thymocytes express low levels of functional TcR (TcR^{dull}) and that they mature into SP thymocytes with higher levels of TcR via an intermediate transitional stage, where levels of TcR are somewhere between the two (TcR^{int}) (Davis 1990; Guidos *et al.* 1990; Petrie *et al.* 1990). In polyclonal wild type mice, approximately 20% of DP cells are TcR^{int} (Havran *et al.* 1987). In the monoclonal mice analysed in this study, this

figure is approximately 26% for wild type, probably due to it being a monoclonal environment. This figure drops to 20% for CD8β^{tg} expressing mice, 3% for CD8β° mice and 7% for CD8β$^{\alpha h}$ expressing mice. There is a strong correlation between the extent of selection seen in the mice analysed, and the percentage of TcR^{int} DP cells. Evidence suggests that the maturation to this intermediate, TcR^{int} stage occurs as a result of positive selection, and that in the absence of positive selection differentiation is arrested at the TcR^{dull} stage (Guidos *et al.* 1990; Petrie *et al.* 1990).

The TcR^{int} population probably reflects the selection potential of the DP cells in each strain, and the lower proportion of DP cells in this subpopulation in mice expressing CD8β$^{\alpha h}$ and lacking CD8β could be accounted for in two ways. It is likely that the lack of TcR^{int} DP cells in CD8β$^{\alpha h}$ and CD8β° expressing mice is a manifestation of the cells' inability to be selected, and the failure in selection can be visualized as a reduction in TcR^{int}. Alternatively it is possible that the TcR is unable to be upregulated from TcR^{dull} to TcR^{int}, and this directly results in the lower percentage of SP cells in strains CD8β$^{\alpha h}$ and CD8β°. The analysis of other maturation markers associated with the development of DP to SP thymocytes can be used to distinguish between these two possibilities.

In CD8^{+} SP cells (Figure 3.4), strains CD8β^{tg}, CD8β° and CD8β$^{\alpha h}$ have slightly higher levels of TcR than wild type mice, and these three strains all have impaired selection in comparision to CD8β^{wt}. Both TcR and CD8 participate in selection, and lower levels of surface CD8αβ expression in the case of CD8β^{tg} mice, or the apparent absence of functional CD8 in the case of CD8β° and CD8β$^{\alpha h}$ mice could result in a greater reliance on TcR for successful selection. Thus, only DP cells with relatively higher levels of TcR are able to have a successful interaction with the thymus stroma during positive selection, resulting in those cells that are selected having high levels of TcR.

3.4 Mice expressing CD8β$^{\alpha h}$ show normal glycosylation of cell surface molecules but lower levels of CD5 in the thymus.

The lack of TcR^{int} DP cells in comparison to TcR^{dull} DP cells in strains CD8β$^{\alpha h}$ and CD8β$^{\circ}$ strongly suggests that development is arrested at the positive selection stage (Petrie *et al.* 1990). In order to distinguish whether this finding illustrates aborted thymic development, or if it is an inherent defect in TcR upregulation, post-translational glycosylation of CD8^{+} cells and the maturation marker CD5 were analysed. It is unlikely that there would be an inherent defect in the upregulation of these maturation markers as well as an inherent defect in TcR upregulation from DP to SP cells, so this will help distinguish between the two possibilities.

Thymocytes from mice expressing CD8β^{wt}, CD8β^{tg}, CD8β$^{\circ}$ and CD8β$^{\alpha h}$ were incubated with antibodies specific for CD4 and CD8α. DP and SP cells were gated and analysed for expression of core 1 O-linked glycans and expression of CD5. Figure 3.5 compares the binding of PNA, a plant lectin that binds to a specific O-linked glycan which is a post translational modification of CD8 as well as other molecules, e.g. CD43. The binding of this lectin is developmentally regulated, and decreases from DP to SP cells due to the further addition of sialic acid to the glycans, masking the binding site for PNA (Reisner *et al.* 1976; Brown and Williams 1982). In CD8β^{wt} wild type mice and mice expressing CD8β^{tg}, there is greater binding of PNA in DP cells than CD8^{+} SP cells. In CD8β$^{\circ}$ mice and those expressing CD8β$^{\alpha h}$ (figure 3.5), there is also a decrease in the binding of PNA from DP cells to SP cells, indicating that these strains also undergo post-translational modification and further addition of sialic acid. When the strains are compared (Figure 3.5, lower panel) there is little difference in PNA binding in the DP or SP cells, except for SP cells from CD8β^{tg} mice which show consistently lower binding of PNA. This could be due to the lower levels of CD8β being expressed in comparison to both CD8β^{wt} and CD8β$^{\alpha h}$ expressing mice – there is less CD8 available on the cell surface to be glycosylated, resulting in lower binding of PNA. Two other

cell surface molecules, CD45 and CD43, are also heavily glycosylated and sialylated and it is possible that the lower levels of PNA binding are as a consequence of unknown differences in the expression pattern of these molecules on SP CD8β^{tg} cells. Certainly it is not because of a higher level of sialylation, thus masking the binding site of PNA more than controls, because data from the next chapter will show that CD8β from CD8β^{tg} thymus lysates runs comparably with CD8β from CD8β^{wt} thymus lysates in SDS gel (discussed in more detail in Chapter 4). Apart from the lower binding of PNA in SP CD8β^{tg} cells in comparison to controls, these data indicate that the post-translational modification of CD8 as well as other molecules occurs comparably in all of the strains.

CD5 is expressed on all mature T cells and most thymocytes and is thought to be involved in signal transduction (Tarakhovsky *et al.* 1995). In DP cells, CD5 levels are maintained by low affinity MHC interactions, and the precise level of expression is determined by the nature of this interaction, with stronger TcR/MHC interactions driving higher levels of expression (Azzam *et al.* 1998; Azzam *et al.* 2001; Wong *et al.* 2001). On a monoclonal background then, it would be expected that CD5 levels would be comparable throughout the test mouse strains. Since CD5 is upregulated during positive selection as the cells develop from DP to SP, it is possible that the block in maturation seen in mice expressing CD8β^{ch} and CD8β$^{\circ}$ could be visualized as lower levels of CD5 in DP cells. Nevertheless, there could still be a range of expression levels in each strain due to differing states of maturity in the thymus.

Figure 3.6 shows varying amounts of CD5 expression in DP cells amongst the strains. CD8β^{wt} DP cells have the highest expression level compared to the other test strains. Levels of expression in DP CD8β^{tg} cells are slightly lower than wild type, which could be reflecting the reduced level of selection in these animals. CD5 expression in DP cells in CD8β$^{\circ}$ mice and mice expressing CD8β^{ch} is lower still in comparison to control

strains, and this is again reflected in the lower percentage of cells selected in these strains.

The lower levels of CD5 in CD8β° and CD8β^{ah} DP cells indicate that, in comparison to cells expressing wild type CD8β there is a difference in the selection of the DP cells. However the overlayed profiles of all four strains of SP cells are more similar (Figure 3.6, lower right panel), which suggests that CD5 is upregulated on selection, as happens in wild type. As with TcR expression level, there is heterogeneity between the different strains which could be a manifestation of differing selection potential between them. Together with TcR data, both suggest early failure of differentiation at the DP stage but once the cells are selected they regulate these molecules in the normal way.

CD5 is phosphorylated on tyrosine residues on T cell activation (Raab *et al.* 1994). Although the mechanism by which CD5 generates intracellular signals is unclear, the lower levels of CD5 in DP cells expressing CD8β° or CD8β^{ah} could have an impact on the signaling ability of the cells and thus the ability to be selected. Alternatively the heterogeneous profiles of CD5 expression could be reflecting the different maturation states of the DP cells in each strain. The similar profiles of SP cells suggest that those cells that are able to make the transition seem to be compensating in some way for the lower expression of CD5 at the DP stage.

3.5 CD8β^{ah} molecules are less able than wild type to recognize cognate pMHC.

Recognition of MHC by TcR in the thymus is a key event leading to a DP cell being selected and maturing to a SP and both TcR and the CD8 co-receptor are essential for this process. Mice lacking any of TcR, CD8 or MHC Class I do not develop mature CD8$^+$ T cells, indicating the importance of these three proteins for T cell development (Zinkernagel *et al.* 1978; Fung-Leung *et al.* 1991; Raulet 1994; Malissen and Malissen

1996). T cells must recognize peptide bound in self-MHC in order to mature (Zinkernagel *et al.* 1978), and it is possible that the block in selection seen in CD8β° mice and mice expressing $CD8\beta^{\alpha h}$ is due to a reduced ability of TcR and co-receptor to bind peptide/ self-MHC complexes. This was tested by examining if DP cells were able to bind MHC.

The F5 TcR recognizes complexes of MHC D^b and NP68 peptide (Townsend *et al.* 1984). The recently introduced bivalent MHC/ Ig recombinant protein was incubated with NP68 peptide overnight to load the MHC molecules. Freshly isolated thymocytes from mice expressing $CD8\beta^{wt}$, $CD8\beta^{tg}$, CD8β° or $CD8\beta^{\alpha h}$ were incubated with the recombinant, peptide loaded MHC (pMHC), washed and resuspended in fluorescently labeled secondary antibodies specific for the recombinant protein, together with anti-CD8α and anti-CD4. Thymocytes from wild type B10 mice and F5/ $Cd8\alpha^{-/-}$ mice were stained and included as controls.

Figure 3.7 panel A shows the binding of the wild type B10 DP cells (shaded histogram) and F5/ $Cd8\alpha^{-/-}$ $CD4^+$ cells (grey line) when incubated with the MHC protein. There is no detectable binding of the recombinant MHC protein to B10 DP cells, indicating that the MHC binding is specific for the F5 TcR. CD8 is also essential for binding, as there is no binding in selecting cells lacking CD8 even though the TcR is specific for this particular peptide/ MHC complex (grey line). There is no binding of the MHC protein to the SP cells in the thymi of these control mice also (panel B).

Figure 3.7 panel C shows the binding of the MHC dimers to DP cells from $CD8\beta^{wt}$ mice (shaded histogram), $CD8\beta^{tg}$ mice (grey line), CD8β° mice (dotted line) and $CD8\beta^{\alpha h}$ mice (black line). This pattern of binding is very similar to the pattern of binding of anti-TcR antibodies (section 3.3). In mice expressing $CD8\beta^{wt}$, there is a population that is dull for MHC binding (62%) and a population that is intermediate for MHC binding (38%, panel C, grey box). In mice expressing $CD8\beta^{tg}$ these two populations are more

pronounced, with a greater percentage of cells in the MHC^{dull} population compared to the MHC^{int} population (72% and 28% respectively). In mice expressing $CD8\beta^{\alpha h}$ almost all of the DP cells are in the MHC^{dull} population (90% compared to 10% MHC^{int}), and in $CD8\beta^{\circ}$ mice this percentage is even greater (96% MHC^{dull} compared to 4% MHC^{int}). The ability of a cell to become MHC^{int} correlates with the extent of selection in each strain; that is in comparison to $CD8\beta^{wt}$ mice there are fewer cells being selected in mice expressing $CD8\beta^{tg}$, and even fewer being selected in $CD8\beta^{\circ}$ mice and mice expressing $CD8\beta^{\alpha h}$. It is likely that this inability of cells expressing $CD8\beta^{\alpha h}$ or cells lacking CD8β altogether to bind MHC is due to inefficient co-receptor function of $CD8\beta^{\alpha h}$ and CD8αα respectively.

In order to determine whether the reduced pMHC binding was simply a reflection of lower TcR expression between the strains, the pMHC binding was normalized for TcR expression. The results for each subpopulation of thymocytes are shown in table 3.1.

Table 3.1 – binding of MHC in thymus

Strain	**Ratio of MHC:TcR binding**		
	DP^{dull}	DP^{int}	SP
$CD8\beta^{wt}$	0.75	2.04	3.26
$CD8\beta^{\circ}$	0.31	1.16	1.46
$CD8\beta^{tg}$	0.61	2.01	2.36
$CD8\beta^{\alpha h}$	0.39	1.19	1.75

These data demonstrate that in $CD8\beta^{wt}$ and $CD8\beta^{tg}$ mice, binding of pMHC to DP cells is roughly twice as efficient per TcR molecule expressed compared to that observed with cells from $CD8\beta^{\circ}$ or $CD8\beta^{\alpha h}$ mice. This is true for TcR^{dull} and TcR^{int} DP cells, showing that the impaired ability of $CD8\beta^{\circ}$ and $CD8\beta^{\alpha h}$ expressing thymocytes to bind cognate MHC can be observed at an early stage of development and making it more

likely that this is causing the block in selection seen in these animals. In the absence of positive selection, differentiation is arrested at the TcR^{dull} stage (Guidos *et al.* 1990; Petrie *et al.* 1990). If TcR^{dull} DP cells from each strain were able to bind MHC comparably, this might suggest that the impaired ability to bind MHC at the TcR^{int} stage was a consequence of the defect in positive selection, rather than the reason for it.

Panel D shows the binding of MHC to SP cells in each strain. Cells that are able to progress from the DP to the SP stage are able to bind MHC at high affinity although there are still differences in the ratio of MHC:TcR binding, as shown in table 3.1 above. Even though there is more binding of MHC in SP cells expressing $CD8\beta^{ah}$ or $CD8\beta^{\circ}$ compared to DP cells from the same animals, SP cells are still significantly less able to bind MHC for the same amount of TcR on the cell surface as $CD8\beta^{wt}$ or $CD8\beta^{tg}$ control mice. Surprisingly, SP cells expressing $CD8\beta^{tg}$ do not bind MHC quite as well as $CD8\beta^{wt}$, in contrast to the situation in DP cells where both bind comparably for the same level of TcR on the cell surface. This could be a consequence of the lower levels of CD8β expression, which has been shown to be important for selection (Nakayama *et al.* 1994) and binding MHC (Janeway 1992), and the higher level of TcR seen in SP $CD8\beta^{tg}$ cells is a way of compensating for the lack of co-receptor help available. This has the effect of lowering the ratio of MHC:TcR binding when comparing with $CD8\beta^{wt}$ expressing cells. Again both $CD8\beta^{\circ}$ and $CD8\beta^{ah}$ expressing cells have approximately a two-fold reduction in ability to bind MHC for the same level of TcR as $CD8\beta^{wt}$ control in SP cells. This could have important consequences for recognition of MHC antigen complexes once mature naïve T cells are circulating in the periphery.

Interestingly, there is little difference in the increasing amount of MHC bound by the T cell as it matures in each strain; there is approximately a four-fold increase in the amount of MHC binding per TcR expressed on the cell surface as the cell progresses from MHC^{dull} to MHC^{hi}, regardless of strain. This developmental consistency further suggests that the important difference between those strains able to select and those

that are impaired, is directly related to how well the T cell can bind MHC at key developmental stages.

Discussion

CD8 molecules are post-translationally modified by the addition of N- and O-linked glycans which are themselves further modified by the addition of sialic acid (Luscher *et al.* 1985; Casabo *et al.* 1994). Previous work in the lab had shown that CD8β, but not CD8α, becomes hyposialylated on T cell activation (Casabo *et al.* 1994), and mice lacking CD8β have an 80% reduction in cell numbers in the periphery (Crooks and Littman 1994), indicating an important role for this molecule.

Sites of O-linked glycosylation have been mapped in the human and the rat CD8α polypeptide and have been shown to be clustered in the hinge region of the molecule (Classon *et al.* 1992; Pascale *et al.* 1992). Given that the hinge region of CD8 is highly conserved across species (Hansen *et al.* 1997), and also by examining predictive computer programmes based on the amino acid sequence of the molecule, most of the O-linked sugars are predicted to be attached to the hinge region of mouse CD8α. The O-glycans of murine CD8β were recently mapped, and showed five sites of O-linked glycosylation clustered in the hinge region of the molecule near the Ig-like domain (Moody *et al.* 2003).

In order to investigate the role of the hinge region of CD8β, the hybrid molecule $CD8\beta^{\alpha h}$ was constructed with the hinge region of CD8β swapped with the corresponding region of CD8α. Another possible strategy would have been to have alanine point mutations in specific residues in the hinge region of CD8β that are predicted to be the sites of O-linked glycosylation, but given that glycosylation is thought to protect the molecule from proteolysis (Hounsell *et al.* 1996), such a construct may have had a more disruptive effect on the molecule.

When expressed on a polyclonal, CD8β knock out background, CD8β$^{\alpha h}$ failed to overcome the block in selection seen in these mice. In order to better assess the question of where and why selection was failing, the transgene was expressed on a monoclonal TcR, CD8β knock out background. This chapter shows that on the F5 *Rag*$^{-/-}$ background, CD8β$^{\alpha h}$ still fails to restore selection. The block in selection in CD8β$^{\alpha h}$ mice is not due to expression levels of CD8β which have been shown to be critical for the differentiation of CD8^{+} cells (Nakayama *et al.* 1994). When using conformationally dependent antibodies to the Ig-like recognition domain of CD8β, subtle differences were seen between antibody clones when analyzing binding in CD8β$^{\alpha h}$ molecules. This could indicate slight changes in conformation in the recognition domain of the chimeric molecule compared to wild type CD8αβ, and the possibility that the conformation adopted by CD8β$^{\alpha h}$ molecules due to glycosylation of the hinge region is unable to promote optimal interaction with MHC. Furthermore, the differences in binding of each CD8 antibody clone could have consequences when CD8-specific antibodies are used for blocking stimulation (see section 6.6).

Post translational modification of thymocytes expressing CD8β$^{\alpha h}$ by the addition of glycans did appear to be broadly normal, as demonstrated by the reduction in binding of PNA lectin as the thymocytes develop which is consistent with wild type development. It is unlikely that a mutation in CD8 would have a global effect on the glycosylation of other molecules expressed on developing T cells, and the normal pattern of PNA binding suggested these were unaffected.

However, analysis of TcR levels in DP cells expressing CD8β$^{\alpha h}$ showed altered representation of thymocyte subsets in these mice. In mice expressing CD8β^{wt}, there are clear populations of TcR^{dull} and TcR^{int} DP cells, which are more mature. This distinction is also visible in mice expressing CD8β^{tg}. In contrast, in mice expressing CD8β$^{\alpha h}$ and also CD8β$^{\circ}$ mice, both of which have impaired selection as measured by percentage of

SP cells in the thymus, there are very few TcR^{int} DP cells and almost all are TcR^{dull}. The percentage of cells in the TcR^{int} subset correlates with the extent of selection seen in these mice, consistent with evidence suggesting that this TcR^{int} subset of DP cells is the immediate precursor of mature thymocytes, and that this population is not seen in the absence of positive selection (Guidos *et al.* 1990; Petrie *et al.* 1990).

The absence of TcR^{int} DP cells suggested that cells expressing $CD8\beta^{ah}$ were failing at an early positive selection stage of development. Expression levels of CD5, another maturation marker, on DP cells seemed to corroborate this idea. High levels of CD5 expression are an early indicator of TcR engagement and are associated with the progression to positive selection (Azzam *et al.* 1998; Azzam *et al.* 2001; Wong *et al.* 2001). Furthermore, the transition from TcR^{dull} to TcR^{int} has been shown to be accompanied by a concomitant increase in the amount of CD5 on the cell surface (Lanier *et al.* 1986). In mice expressing $CD8\beta^{ah}$, levels of CD5 are significantly lower in DP cells than in wild type mice. There is a correlation between the extent of selection and the expression of CD5 on DP cells among the four strains tested, however levels of CD5 in SP cells are approximately equivalent in all strains, suggesting that cells that are selected are upregulating these molecules normally. Analysis of other activation markers such as CD69 may further clarify at which stage selection is occurring.

The block in selection suggested by the lack of a TcR^{int} population as well as reduced levels of CD5 in DP thymocytes expressing $CD8\beta^{ah}$ raised the question of whether these mutated molecules can recognize MHC. Events leading to the selection of T cells are still not well understood, but recognition of MHC is essential. CD8 is also necessary (Fung-Leung *et al.* 1991) and CD8β has been specifically implicated, since mice lacking CD8β have reduced numbers of T cells being selected (Crooks and Littman 1994). CD8αα homodimers have been crystalised binding to MHC and binding studies of these molecules showed that it is the α3 domain that is making contact with the CD8 molecule (Salter *et al.* 1990; Gao *et al.* 1997). Nevertheless CD8β has been shown in

hybridomas to be capable of independent interaction with MHC Class I (Wheeler *et al.* 1998) suggesting that the presence of CD8β enhances the affinity of the T cell for MHC. When thymocytes from all mouse strains under investigation were analysed with cognate MHC and cognate peptide, there was a correlation between the level of TcR expression and binding of pMHC and the extent of selection seen in those strains as measured by the percentage of SP cells. When the amount of MHC binding was compared for the same level of TcR on the cell surface, again there was a correlation between the amount of MHC able to bind and the extent of selection seen in each mouse strain. Thymocytes from $CD8\beta^{wt}$ had the highest ratio of MHC:TcR binding in all thymocyte subpopulations. In $CD8\beta^{tg}$ expressing mice, which have slightly impaired selection compared to $CD8\beta^{wt}$ expressing mice, the ratio is slightly lower in both TcR^{dull} and SP cells, but interestingly the ratio is comparable to $CD8\beta^{wt}$ binding in TcR^{int} DP cells. Cells that fail positive selection are arrested at the TcR^{dull} stage of maturation (Guidos *et al.* 1990; Petrie *et al.* 1990), and it is likely that the slightly reduced ratio of MHC:TcR seen in $CD8\beta^{tg}$ DP cells at the TcR^{dull} stage is reflected in the lower percentage of $CD8^+$ SP cells being selected. The reduced ratio in these animals could also reflect the comparative lack of CD8αβ on the surface of $CD8\beta^{tg}$ expressing cells, which express CD8β at approximately 60% of the levels seen in $CD8\beta^{wt}$ expressing mice. Expression level of CD8β is implicated in selection (Nakayama *et al.* 1994), and TcR and CD8αβ have been suggested to contribute to selection in a cumulative way (Moody *et al.* 2001b). The lower levels of expression of CD8β in $CD8\beta^{tg}$ expressing mice compared to $CD8\beta^{wt}$ mice could mean there is less co-receptor help available for the same amount of TcR expressed on the surface, which therefore could mean that fewer MHC:TcR interactions are being stabilized in these mice, resulting in a lower ratio of MHC:TcR binding.

In $CD8\beta^{\circ}$ mice, which show an 80% reduction in percentage of SP cells being selected (Crooks and Littman 1994), the ratio of MHC:TcR binding is more than two fold lower than $CD8\beta^{wt}$ expressing mice at the TcR^{dull} stage. In the case of $CD8\beta^{ah}$ expressing mice,

TcR^{dull} thymocytes are two fold less able to bind MHC than $CD8\beta^{wt}$ expressing mice. Like the data of Wheeler *et al* (1998), this suggests that wild type CD8β enhances the affinity of the T cell for MHC and suggests that CD8αα and $CD8\beta^{\alpha h}$ complexes are less able to do so. In SP cells expressing CD8αα or $CD8\beta^{\alpha h}$ binding to MHC is again approximately two fold lower than the binding seen in $CD8\beta^{wt}$ SP cells, which could have important consequences for MHC recognition and binding when mature naïve T cells are circulating in the periphery.

Recent data shows that the cytoplasmic tail of CD8β is palmitoylated and targets the CD8αβ molecules to lipid rafts in the plasma membrane, where the majority of TcR-MHC interactions are predicted to take place (Arcaro *et al.* 2000). More specifically, the transmembrane and cytoplasmic regions of CD8β have been shown to greatly enhance TcR-ligand binding (Arcaro *et al.* 2001). It is all the more curious that $CD8\beta^{\alpha h}$ should fail to restore selection when it has wild type recognition domain, transmembrane region and cytoplasmic domain, all of which are essential for MHC binding. Nevertheless the variation in binding of antibodies specific for the Ig-like recognition domain of CD8β seen in thymocytes expressing $CD8\beta^{\alpha h}$ could indicate conformational changes in the binding epitopes, which could have important effects on MHC binding.

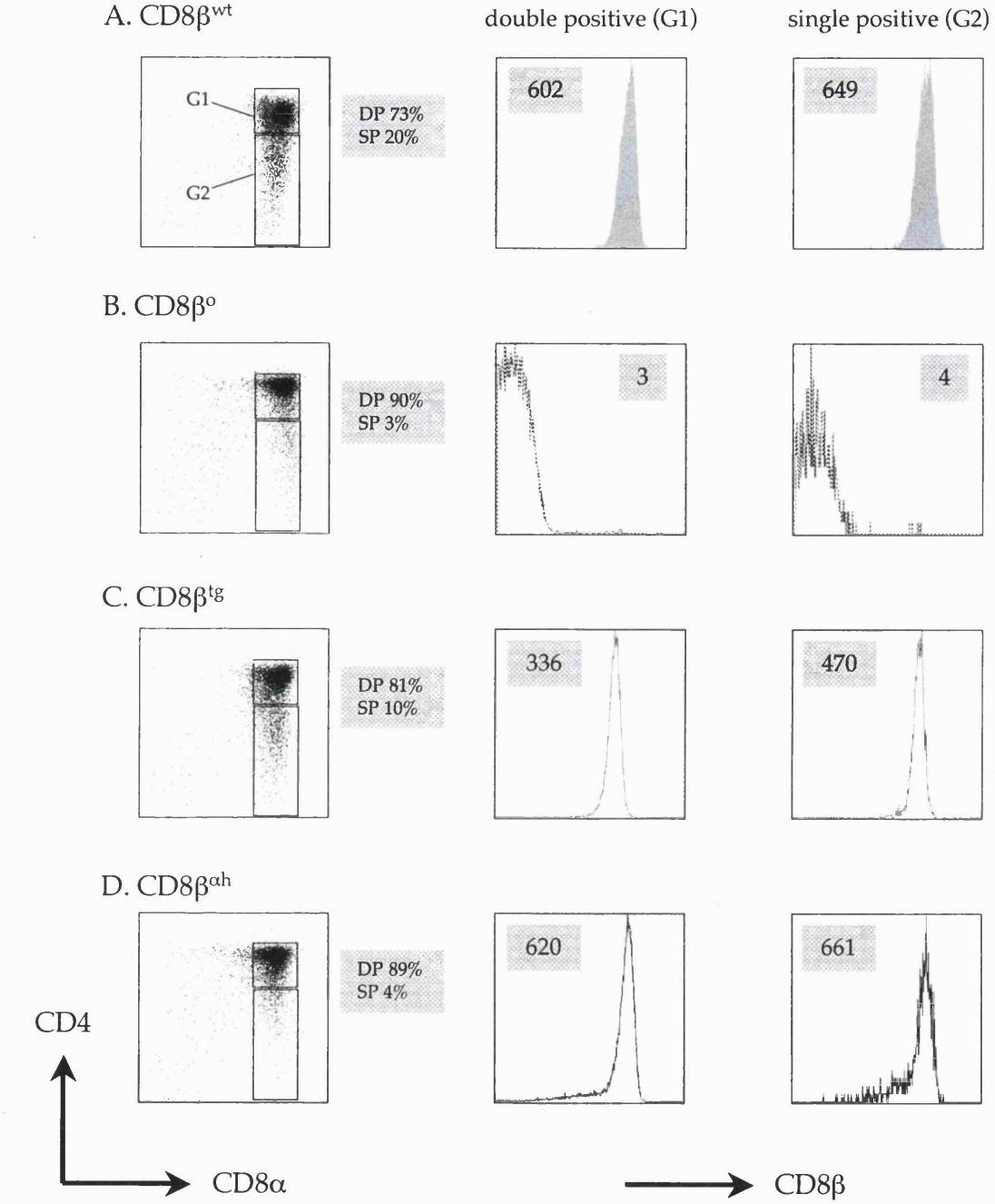
It should be remembered however that in the thymus a DP cell will not encounter cognate peptide in cognate MHC – this would result in the deletion of the cell. Experiments using NP34, an antagonist peptide would better re-create selection conditions found in the thymus as this peptide has been shown to induce efficient positive selection in F5 mice (Smyth *et al.* 1998), and these experiments are planned. Nevertheless, if $CD8\beta^{\alpha h}$ is less able to bind cognate peptide:MHC, it is likely to have lower binding to a weaker agonist and may therefore fall below the threshold for selection.

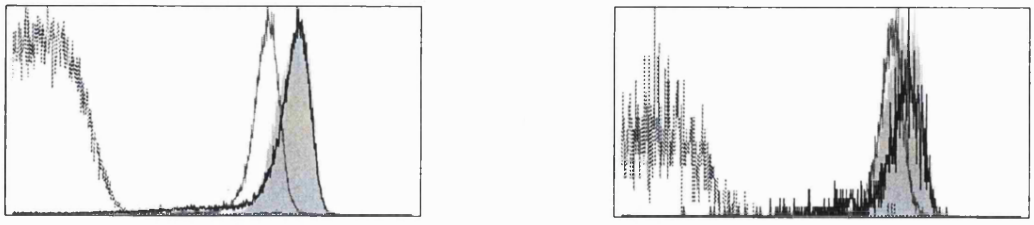
Many recent reports have focused on the importance of CD8β in the recognition of non-cognate MHC in DP cells. This effect is developmentally regulated by glycosylation but also varies between TcR and MHC specificities, and has been suggested to be important for thymus selection (Bosselut *et al.* 2000; Daniels *et al.* 2001; Moody *et al.* 2001a; Moody *et al.* 2001b). It remains to be determined if this effect is intact in mice expressing $CD8\beta^{ah}$, as in spite of using an MHC specificity that had shown a high degree of binding in other systems, experiments in the lab were inconclusive. Both TcR and CD8 are essential for selection, and current thinking suggests that TcR and CD8 contribute to selection in a cumulative way (Moody *et al.* 2001b); that is a TcR with a high affinity for self-MHC requires less help from CD8 than a TcR of lower affinity.

When mice are transgenic for a form of CD8β that lacks the cytoplasmic domain, some specificities of TcR show impaired positive selection as measured by number of $CD8^+$ SP cells but the F5 TcR does not (Itano *et al.* 1994), suggesting that it requires less co-receptor help than other monoclonal specificities. It may be that the F5 TcR transgenics are not the ideal model in which to investigate non-cognate MHC binding. However experiments conducted in the lab on mice which had a polyclonal background also failed to give a conclusive result, suggesting the experimental system needs refining. It will be interesting to determine if this effect can be demonstrated in mice expressing $CD8\beta^{ah}$ as well as $CD8\beta^{-}$ mice, both of which have impaired selection, in order to ascertain the importance of non-cognate MHC binding of DP cells in selection.

Figure 3.1 - Impaired selection of thymocytes expressing CD8β$^{\alpha h}$ is not due to lower expression levels of the transgene.

Thymocytes from mice expressing CD8β^{wt} (A), CD8β$^{\circ}$ (B), CD8β^{tg} (C) and CD8β$^{\alpha h}$ (D) were stained with fluorescently labeled antibodies specific for CD4 (RM4-5), CD8α (53.6.7) and CD8β (53.5.8) at 4°C for 30 minutes. Live cells were gated for expression of CD4 and CD8α, and expression of CD8β analysed by FACS Calibur in DP cells (left histograms) and SP cells (right histograms). The MFI and percentage of DP and SP cells for each strain is shown in grey boxes. CD8β expression in all strains is compared in overlaid histograms from DP cells (lower left panel) and SP cells (lower right panel). Data are representative of nine experiments, and fluorescent intensity is shown on log scale from 10^0 to 10^4. Notations are the same in upper and lower panels.



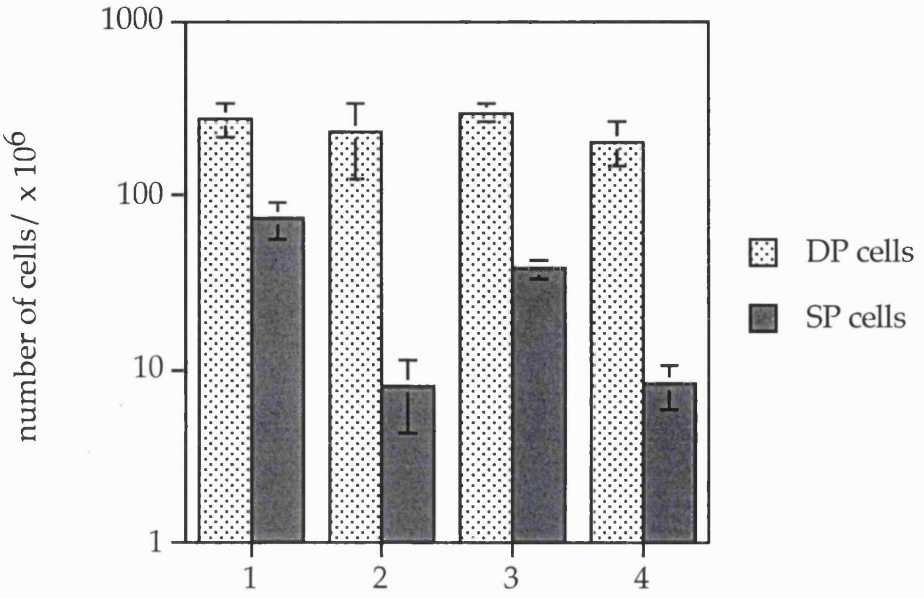


double positive

single positive

Figure 3.2 - CD8β^{ah} is not able to restore selection of F5 TcR transgenic mice on a CD8β° background.

Thymocytes from mice expressing CD8β^{wt} (1), CD8β° (2), CD8β^{tg} (3) and CD8β^{ah} (4) were stained with fluorescently labeled antibodies specific for CD4 (RM4-5) and CD8α (53.6.7) at 4°C for 30 minutes, then analysed by FACS. Live cells were gated for expression of CD4 and CD8α, and the number of DP cells (light bars) and SP cells (dark bars) recorded. Data are representative of nine experiments.



1. CD8β^{wt}

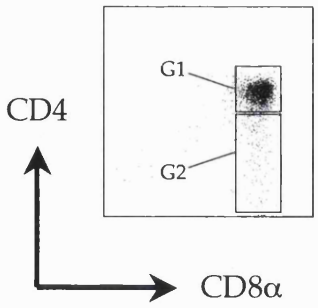
2. CD8β^{0}

3. CD8β^{tg}

4. CD8β$^{\alpha h}$

Figure 3.3 - Cells expressing CD8β^{αh} bind CD8β antibodies specific for the Ig-like domain.

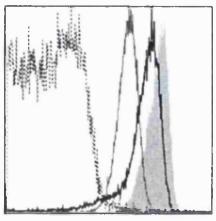
Thymocytes from CD8β^{wt} (shaded histogram), CD8β^{tg} (grey line), CD8β^{-} (dashed line) and CD8β^{αh} (black line) expressing mice were incubated at 4°C for 30 minutes with antibodies specific for CD4 (RM4-5), CD8α (53.6.7) and CD8β (A – YTS156.7; B – KT112; C – 53-5.8). Cells were analysed by FACS Calibur and results are representative of three experiments. Fluorescent intensity is shown on log scale from 10^0 to 10^4.

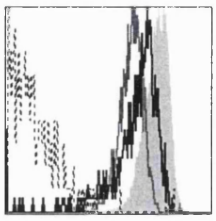


shaded histogram	CD8β^{wt}
grey line	CD8β^{ts}
dashed line	CD8β^{0}
black line	CD8β^{ch}

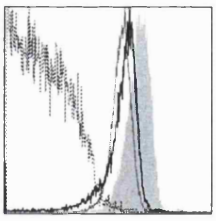
double positive G1 single positive G2

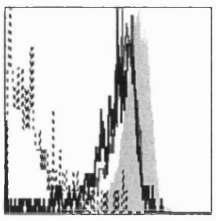
A. YTS 156.7



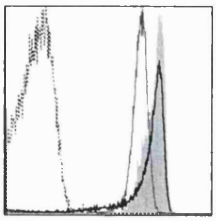


B. KT112





C. 53-5.8



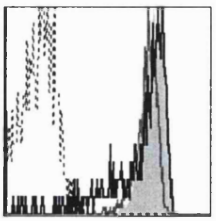


Figure 3.4 - TcR expression levels in DP cells suggest an early block in positive selection in thymocytes expressing CD8β$^{\alpha h}$.

Thymocytes from mice expressing CD8β^{wt} (A), CD8β$^{\circ}$ (B), CD8β^{tg} (C) and CD8β$^{\alpha h}$ (D) were stained with fluorescently labeled antibodies specific for CD4 (RM4-5), CD8α (53.6.7) and TcR (H57) at 4°C for 30 minutes. Live cells were gated for expression of CD4 and CD8α, and expression of TcR analysed by FACS Calibur in DP cells (left histograms) and SP cells (right histograms). The MFI of TcR for each population is shown in grey boxes. In DP cells the population of TcR^{hi} cells is highlighted and is smaller in thymocytes expressing CD8β$^{\alpha h}$ and CD8β$^{\circ}$ in comparison to wild type. Percentage of TcR^{hi} cells is shown to right of DP panels. TcR expression in all strains is compared in overlaid histograms from DP cells (lower left panel) and SP cells (lower right panel). Data are representative of nine experiments, and fluorescent intensity is shown on log scale from 10^0 to 10^4.

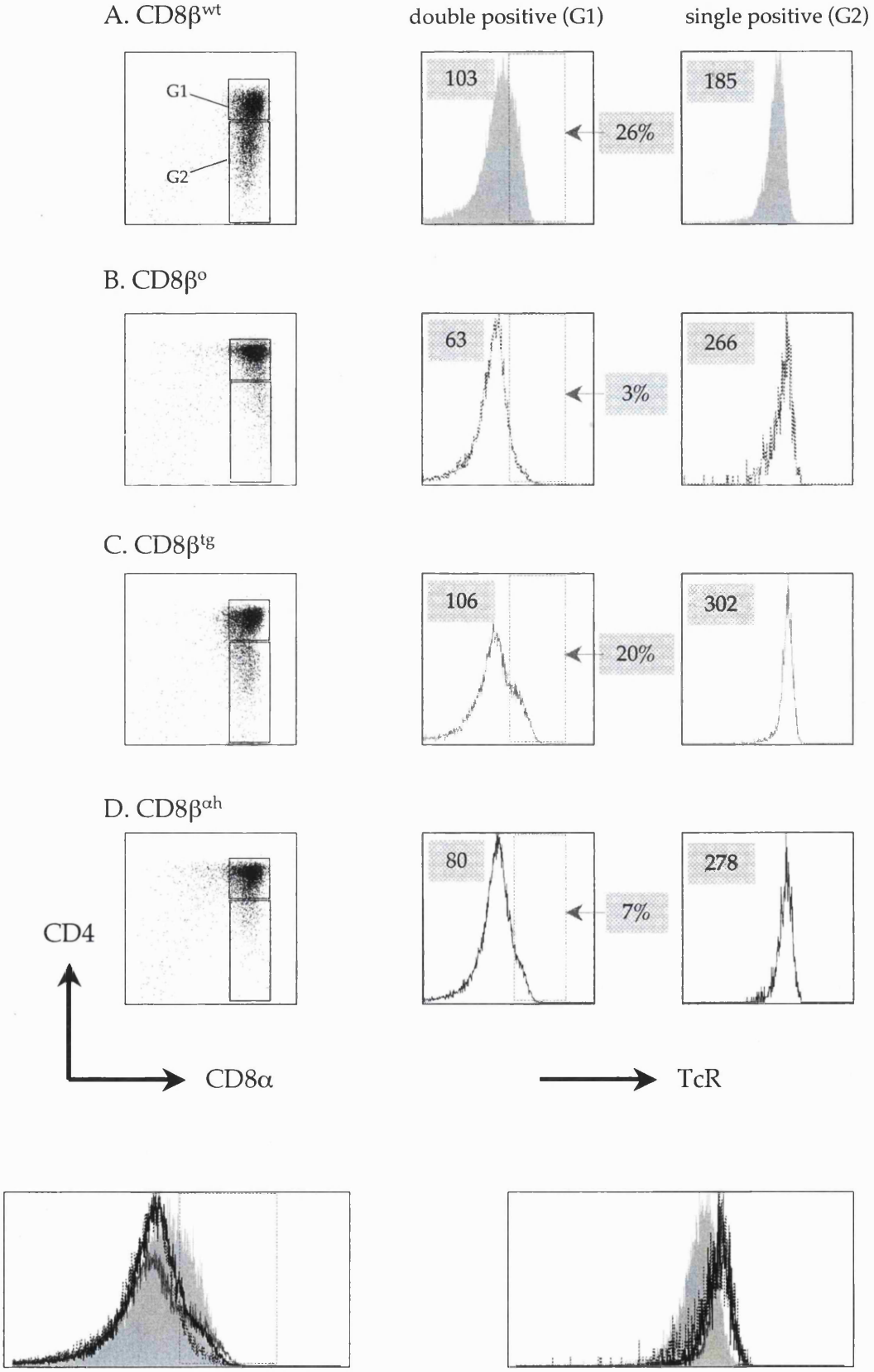

A. CD8β^wt
double positive (G1)
single positive (G2)
G1
G2
103
26%
185
B. CD8β^o
63
3%
266
C. CD8β^tg
106
20%
302
D. CD8β^αh
80
7%
278
CD4
CD8α
TcR
double positive
single positive

Figure 3.5 - Binding levels of PNA in thymocytes expressing CD8β^{ah} are comparable to wild type controls, suggesting post translational modification of cell surface proteins in these animals is unaffected.

Thymocytes from mice expressing CD8β^{wt} (A), CD8β$^{\circ}$ (B), CD8β^{tg} (C) and CD8β^{ah} (D) were stained with fluorescently labeled antibodies specific for CD4 (RM4-5) and CD8α (53.6.7), and PNA lectin at 4°C for 30 minutes. Live cells were gated for expression of CD4 and CD8α, and binding of PNA analysed by FACS Calibur in DP cells (left histograms) and SP cells (right histograms). The MFI of PNA binding for each population is shown in grey boxes. Binding of PNA in all strains is compared in overlaid histograms from DP cells (lower left panel) and SP cells (lower right panel). Data are representative of nine experiments, and fluorescent intensity is shown on log scale from 10^0 to 10^4.

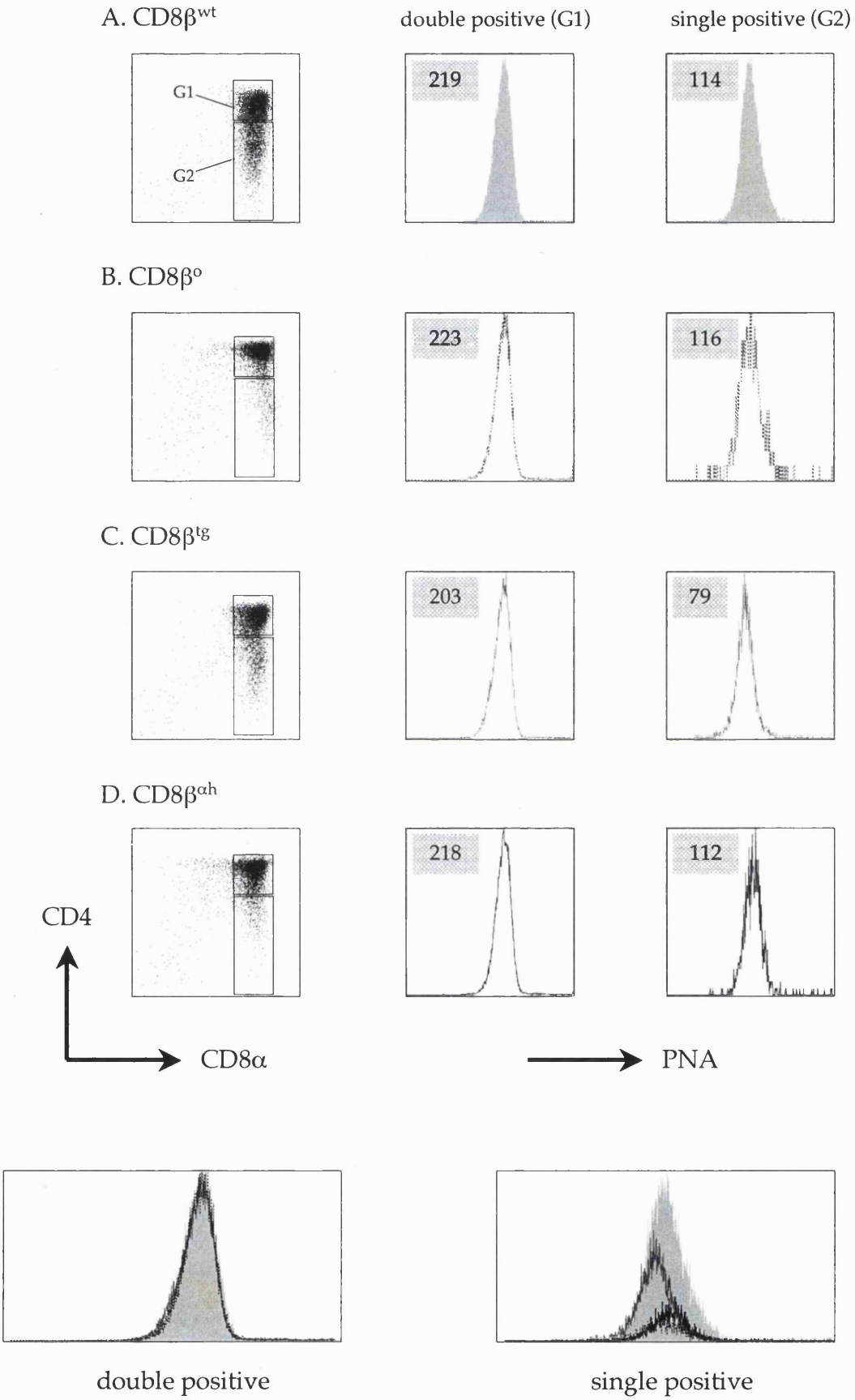

A. CD8β^wt
double positive (G1)
single positive (G2)
G1
G2
219
114
B. CD8β^o
223
116
C. CD8β^tg
203
79
D. CD8β^αh
218
112
CD4
CD8α
PNA
double positive
single positive

Figure 3.6 - Expression levels of CD5 in DP thymocytes expressing CD8β$^{\alpha h}$ suggest positive selection is impaired in these animals in comparison to wild type controls.

Thymocytes from mice expressing CD8β^{wt} (A), CD8β$^{\circ}$ (B), CD8β^{tg} (C) and CD8β$^{\alpha h}$ (D) were stained with fluorescently labeled antibodies specific for CD4 (RM4-5), CD8α (53.6.7) and CD5 (53-7.3) at 4°C for 30 minutes. Live cells were gated for expression of CD4 and CD8α, and expression of CD5 analysed by FACS Calibur in DP cells (left histograms) and SP cells (right histograms). The MFI of CD5 for each population is shown in grey boxes. CD5 expression in all strains is compared in overlaid histograms from DP cells (lower left panel) and SP cells (lower right panel). Data are representative of nine experiments, and fluorescent intensity is shown on log scale from 10^0 to 10^4.

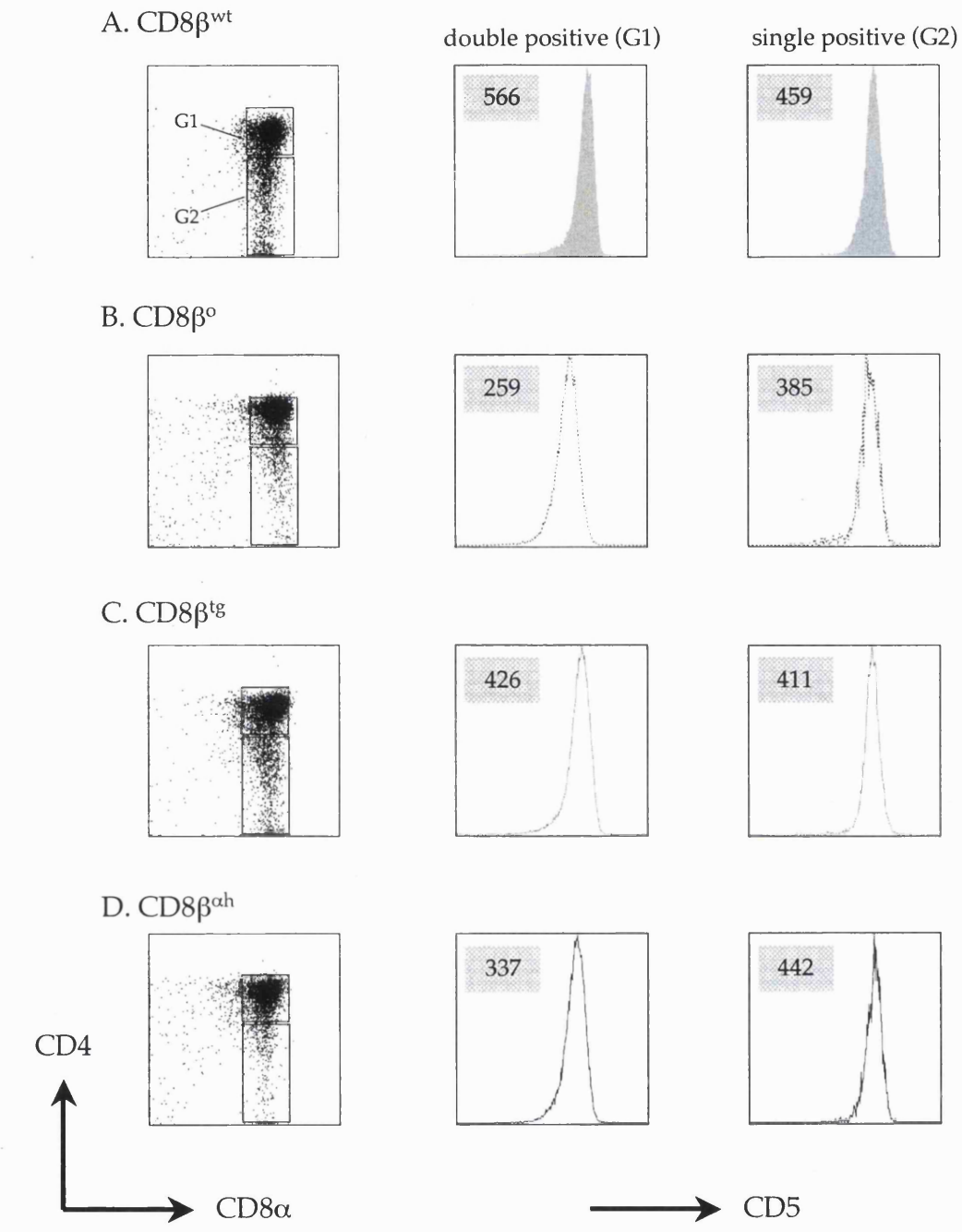

A. CD8β^wt
double positive (G1)
single positive (G2)
G1
G2
566
459
B. CD8β^o
259
385
C. CD8β^tg
426
411
D. CD8β^αh
337
442
CD4
CD8α
CD5
double positive
single positive

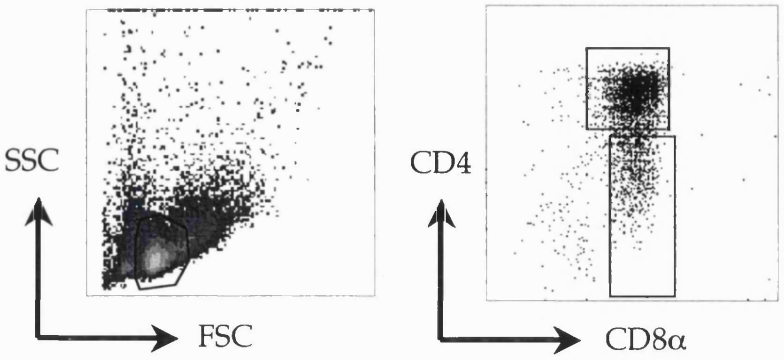
Figure 3.7 - Differences in MHC binding in DP populations in mice expressing $CD8\beta^{\alpha h}$.

MHC recombinant dimers (Pharmingen) were incubated with cognate NP68 peptide at 37°C overnight. Thymocytes were incubated with peptide loaded MHC dimers at 4°C for one hour at a concentration of 0.25μg dimers per 10^6 cells. Cells were washed and resuspended with fluorescently labeled antibodies specific for CD4 (RM4-5) and CD8α (53.6.7). Cells were washed again and analysed by FACS Calibur.

(A and B) Thymocytes from B10 (shaded histogram) and $CD8^0$ mice (grey line) were stained as above, showing that binding of cognate MHC is both TcR specific and CD8 dependent. This is true for $CD4^+$ cells (A) and $CD4^-$ (B) cells.

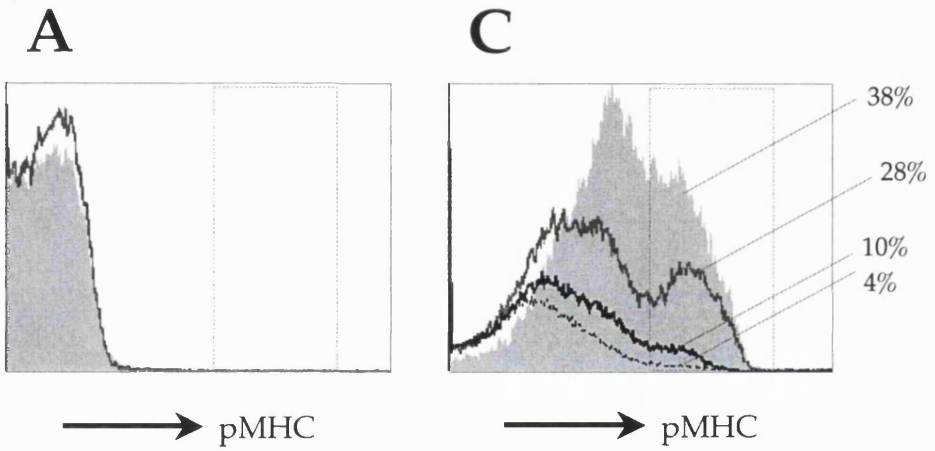
(C and D) Thymocytes from $CD8\beta^{wt}$ (shaded histogram), $CD8\beta^{tg}$ (grey line), $CD8\beta^\circ$ (dashed line) and $CD8\beta^{\alpha h}$ (black line) expressing mice were stained as above. There is a lower percentage of MHC^{hi} binding cells in $CD4^+$ cells in $CD8\beta^\circ$ and $CD8\beta^{\alpha h}$ mice (shaded box, C), suggesting a defect in positive selection at the DP stage. All $CD4^-$ SP cells (D) are able to bind MHC, however there is approximately two-fold lower binding of MHC by SP cells from $CD8\beta^\circ$ and $CD8\beta^{\alpha h}$ expressing mice.

Cells were analysed by FACS Calibur and results are representative of four experiments, and fluorescent intensity is shown on log scale from 10^0 to 10^4.

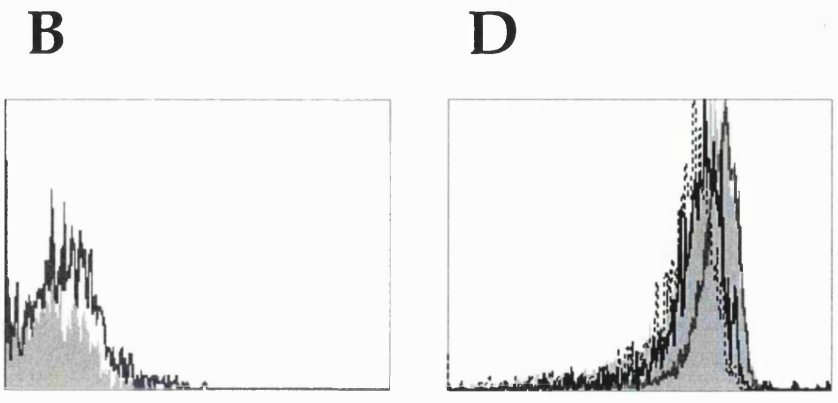


shaded histogram	$CD8\beta^{wt}$
grey line	$CD8\beta^{tg}$
dashed line	$CD8\beta^{0}$
black line	$CD8\beta^{ah}$

$CD4^+CD8^+$



$CD8^+$



4. Biochemistry of DP cells expressing CD8β$^{\alpha h}$ molecules.

Introduction

Data from Chapter 3 showed that mice expressing CD8β$^{\alpha h}$, a mutated form of CD8β, were unable to restore selection in the thymus when expressed as a heterodimer with CD8α on a *Cd8β*$^{-/-}$ background. This is in spite of the CD8β$^{\alpha h}$ polypeptide having the Ig-like domain, transmembrane domain and cytoplasmic domain of wild type CD8β, all of which have been shown to enhance TcR-ligand binding (Wheeler *et al.* 1998; Arcaro *et al.* 2000; Bosselut *et al.* 2000; Arcaro *et al.* 2001). Phenotypic analysis showed that a high percentage of CD8β$^{\alpha h+}$ DP cells were arrested at the TcR^{dull} maturation point, suggesting a failure in early development at the positive selection stage (Guidos *et al.* 1990; Petrie *et al.* 1990). This was further supported by data showing that DP cells expressing CD8β$^{\alpha h}$ were impaired in their ability to bind MHC for the same number of TcR molecules on the cell surface in comparison with selecting controls.

This chapter uses biochemical techniques to address the question of why and how DP cells are failing to select in the thymus. Experiments were designed to ask if the impairment in selection is due to different processing of the CD8β$^{\alpha h}$ molecules, or a difference in signaling ability in comparison with CD8β^{wt} mice.

The CD8 complex is expressed on the cell surface of mature αβTcR T cells almost exclusively as a disulphide linked heterodimer of CD8α and CD8β (Panaccio *et al.* 1987; Shiue *et al.* 1988), but other species of CD8 have been described. On the cell surface of thymocytes there are approximately equal populations of CD8αβ and CD8α'β, but in peripheral T cells very little CD8α' is detectable on the cell surface although it is found intracellularly (Zamoyska and Parnes 1988). CD8αα and CD8α'α homodimers also exist but are retained intracellularly in αβTcR T cells (Ledbetter *et al.* 1981), although

CD8αα molecules are expressed on the surface of γδTcR T cells, intestinal intraepithelial gut cells (IEL), NK cells and dendritic cells (Torres-Nagel *et al.* 1992; Gapin *et al.* 1999), leading to recent suggestions that the CD8αα homodimer may be functionally distinct from the CD8αβ heterodimer. This was demonstrated by showing that CD8αα mediates the activation of IELs by binding to TL antigen, an MHC-related molecule expressed on the IEL cell surface (Leishman *et al.* 2001). Although CD8β cannot generally be expressed on the cell surface unless it is associated with CD8α (DiSanto *et al.* 1988; Gorman *et al.* 1988), human CD8β has been expressed in COS –7 cells and mouse lymphocytes as a CD8ββ homodimer (Devine *et al.* 2000). As these different species of CD8 have differing ability to bind MHC and to transmit TcR mediated signals, it was important to check whether $CD8\beta^{\alpha h}$ was behaving inefficiently as a co-receptor due to aberrant association with CD8α.

Another reason for the impairment in selection seen in mice expressing $CD8\beta^{\alpha h}$ could be found in the post-translational modification of CD8. CD8 molecules are extensively modified by the addition of post-translational glycans. CD8β is modified by the addition of one N-linked glycan in the Ig-like domain (Rothenberg and Triglia 1983) as well as having five sites of O-linked glycosylation in the hinge region of the molecule, all of which are clustered near the Ig-like domain of the protein (Moody *et al.* 2003). Murine CD8α has three sites of N-linked glycosylation (Luscher *et al.* 1985) but has not been mapped for sites of O-linked glycosylation, although it is predicted to have at least six potential sites. Known and potential sites of glycosylation in CD8 molecules are outlined in figure 4.1. O-linked glycans are attached to glycoproteins through a serine or threonine side chain via an initial GalNAc residue (Van den Steen *et al.* 1998). Modification by O-glycosylation is highly regulated during T cell maturation and is dependent on the primary, secondary and tertiary structure of the molecule to which the glycans are attached. Attachment of O-glycans occurs post-translationally as the molecule is transported through the ER-plasma membrane pathway to the cell surface, with many regulatory enzymes localized in the Golgi complex (Pascale *et al.* 1992a;

Pascale *et al.* 1992b). In the case of CD8 molecules, developmentally regulated changes in O-glycosylation have previously been reported during differentiation and activation (Casabo *et al.* 1994; Wu *et al.* 1996; McNeill 1999; Daniels *et al.* 2001; Moody *et al.* 2001a), and the CD8β^{ah} molecule was created to address the question of why and how these changes were important for T cell differentiation and activation. O-glycosylation affords a glycoprotein stability and resistance to proteases, and many O-glycans are also ligands for other molecules at specific points in the cell's maturation. Aberrant modification of CD8β^{ah} molecules by glycosylation enzymes could be affecting any of the roles that O-glycans play in T cell development, and this could be another reason for the impairment in selection in mice expressing CD8β^{ah}.

The finding in Chapter 3 that CD8β^{ah} molecules cannot restore selection in the thymus on a *Cd8β*$^{-/-}$ background also raised questions about the signaling capability of cells expressing CD8β^{ah}. LCK is a src-family kinase associated with CD8 molecules via a CXCP binding motif in the cytoplasmic domain of CD8α (Shaw *et al.* 1990) and is one of the first signaling molecules to be activated downstream of the TcR. LCK is implicated in the transduction of signals from the preTcR (Levelt and Eichmann 1995) as well as the mature αβTcR (Weiss and Littman 1994; van Oers *et al.* 1996) and is also implicated in the decision of thymocytes to adopt the CD4 or CD8 lineage (Bommhardt *et al.* 1997; Basson *et al.* 1998a; Basson *et al.* 1998b). One of the ways in which CD8 functions as a co-receptor is by recruiting LCK to the TcR signaling complex when CD8 binds MHC, thus enhancing signal transduction. Aberrant association of LCK with CD8β^{ah} molecules could abrogate TcR mediated signaling in mice expressing the mutated molecule, and could be a reason for the block in development seen at the positive selection stage of maturation in mice expressing CD8β^{ah}. Furthermore, LCK is absolutely required for downstream mobilization of Ca^{++} after stimulation (Trobridge and Levin 2001), and this chapter discusses experiments comparing the Ca^{++} flux seen in cells expressing CD8β^{ah} to that seen in selecting controls as a method for testing the functionality of LCK in CD8β^{ah} expressing mice.

4.1 The CD8β$^{\alpha h}$ protein is more heavily sialylated and glycosylated than the primary structure predicts.

The first step taken in the biochemical analysis of CD8β$^{\alpha h}$ was to assess the size of the CD8β$^{\alpha h}$ polypeptide in comparison to wild type CD8β. Given that glycosylation has been shown to play a key role in immune regulation (Daniels *et al.* 2002), it is important to know the extent and composition of the glycan moieties of the CD8β$^{\alpha h}$ protein in order to assess the impact on T cell response. The CD8α polypeptide is 235 amino acids in length and has a predicted size of 26kDa, yet runs at around 36-38kDa in SDS PAGE. This extra 10-12kDa size is accounted for by the addition of three 3kDa N-linked glycans, and 1-3 kDa of O-linked glycans, which are smaller than N-linked. Wild type CD8β is 193 amino acids long and runs at approximately 28-30kDa. The predicted molecular weight of the mature unglycosylated protein is 22kDa, and the remaining 6-8kDa is contributed by one 3kDa N-linked glycan, and 3-5kDa of O-linked glycans. Treatment with O-glycanase to remove O-glycans results in a band 1-2 kDa smaller in SDS-PAGE in the case of both CD8α and CD8β, possibly because some O-glycans are more resistant to removal. The 10 amino acid increase in the CD8β$^{\alpha h}$ polypeptide gives a predicted molecular weight of 23kDa, and if the glycan adducts are equivalent to wild type this gives a predicted size in SDS gel of 30-34kDa. In order to examine the extent of glycosylation on the CD8β$^{\alpha h}$ protein in comparison to wild type controls it was run on SDS PAGE. Therefore, thymocytes from mice expressing CD8β^{wt}, CD8β^{tg} and CD8β$^{\alpha h}$ were lysed and immunoprecipitated with CD8β-specific beads. The precipitates were resolved in SDS-PAGE under reducing conditions. The gel was western blotted and probed with rabbit serum specific for CD8β, with total thymus lysates from CD8β^{wt} and CD8β$^{\circ}$ mice included as positive and negative controls respectively (figure 4.2, lanes 1 and 2).

Figure 4.2 shows that both CD8β^{wt} (lane 3) and CD8β^{tg} (lane 4) run at approximately 29kDa, the predicted size for mature glycosylated wild type CD8β. The CD8β$^{\alpha h}$ polypeptide is 203 amino acids in length and contains an extra N-linked glycosylation site compared to wild type CD8β, increasing the predicted molecular weight to approximately 33kDa, therefore the mature protein would be predicted to be slightly larger than wild type CD8β but smaller than wild type CD8α. However as seen in figure 4.2, lane 5 the CD8β$^{\alpha h}$ molecule runs substantially higher than wild type CD8β and wild type CD8α (not shown) at about 40kDa. In order to determine what was responsible for the increased size of the protein, further glycan analysis was carried out. The CD8β$^{\alpha h}$ polypeptide has an extra site of N-linked glycosylation in comparison to wild type CD8β, but previous work showed that this extra 3kDa alone did not account for the increased size in the protein (McNeill 1999). Removal of sialic acid and O-linked sugars in wild type CD8 causes both the α and the β-chain to shift by 1-2 kDa to a faster migrating species. When sialic acid and O-linked sugars are removed from the CD8β$^{\alpha h}$ polypeptide however, there is a 4-5kDa shift to a faster migrating species, suggesting that the glycosylation of the mutant chain is not only different to that predicted, but distinct from wild type CD8α and CD8β entirely, and confirming that the increased size is due to the addition of O-linked sugars. It is possible that CD8β$^{\alpha h}$ has a greater number of the predicted glycosylation sites utilized, or that the modification of the predicted sites is discrete from either wild type chain. Further analysis of the glycan moieties of the CD8β$^{\alpha h}$ polypeptide is discussed in Chapter 5.

4.2 CD8β$^{\alpha h}$ polypeptide forms a heterodimer with CD8α.

Biochemical analysis of the CD8β$^{\alpha h}$ molecule established that the CD8β$^{\alpha h}$ polypeptide was larger than predicted due to an additional N-linked glycan and heavier O-linked glycosylation and sialylation than wild type CD8β, leading to the question of whether the assembly of the molecule was also different to wild type, possibly as a consequence of the altered glycosylation.

Surface composition of the CD8 heterodimer is regulated in a developmental fashion, with CD8α'β and CD8αβ heterodimers found on the surface of thymocytes in approximately equal amounts. In contrast, in mature T cells, only CD8αβ molecules are expressed on the cell surface while CD8α'β molecules are held intracellularly (Walker *et al.* 1984; Zamoyska and Parnes 1988). Very few CD8αα or CD8α'α homodimers are expressed on the cell surface of thymocytes or mature T cells, and clearly this molecule is very poor at promoting selection as $Cd8\beta^{-/-}$ mice, which express only these molecules, demonstrate (Crooks and Littman 1994). CD8αα homodimers can be easily detected intracellularly (Walker *et al.* 1984) suggesting there is tight control of the species which are transported to the cell surface.

In order to examine whether $CD8\beta^{\alpha h}$ polypeptides formed similar heterodimers to wild type, thymus cells expressing $CD8\beta^{wt}$, CD8β° or $CD8\beta^{\alpha h}$ were lysed and the CD8 complex immunoprecipitated sequentially with CD8β-specific beads to precipitate all CD8αβ heterodimers. The lysate was then immunoprecipitated with one further round of CD8α-specific beads to ask whether there were any remaining CD8αα homodimers. The precipitates were resolved in SDS PAGE under reducing conditions, and western blotted with anti-serum specific for CD8β. Figure 4.3, panel A shows that in thymocytes expressing $CD8\beta^{wt}$, all CD8αβ heterodimers are removed from the lysate in two rounds of immunoprecipitation ($CD8\beta^{wt}$, lanes 1-3). As expected, no CD8 was precipitated from cells negative for CD8β (CD8β°, lanes 5-7) showing that the anti CD8β serum is specific. All $CD8\beta^{\alpha h}$ heterodimers are removed from the lysate in the first round of immunoprecipitation ($CD8\beta^{\alpha h}$, lanes 9-11), suggesting there are fewer heterodimers expressed in these cells. The immunoprecipitation is efficient and all of the CD8αβ heterodimers are removed by the CD8β-specific beads, as no heterodimers can be detected when the lysate is further precipitated for remaining CD8α. The western blot was re- probed for the presence of CD8α, and figure 4.3, panel B shows that in cells expressing $CD8\beta^{wt}$ CD8αα homodimers can be detected (lane 4).

Thymocytes from CD8β° mice only express CD8αα homodimers and these can be detected when immunoprecipitated using beads specific for CD8α (lane 8). The faint signal detected in lane 5 is likely to be spillage from lane 4 in $CD8\beta^{wt}$ immunoprecipitation, but the experiment must be repeated to confirm this. In cells expressing $CD8\beta^{\alpha h-}$, CD8αα homodimers can also be detected (lane 12). This experiment indicates that, like $CD8\beta^{wt}$ animals, mice expressing $CD8\beta^{\alpha h}$ express CD8 both as CD8αβ heterodimers and CD8αα homodimers.

4.3 Normal LCK association with $CD8\beta^{\alpha h}$ proteins.

Although the size of the $CD8\beta^{\alpha h}$ polypeptide was found to be larger than wild type CD8β due to an extra N-linked glycan and heavier O-linked glycosylation and sialylation, the stoichiometry of the molecule was shown to be the same as wild type, with CD8αβ heterodimers and a population of CD8αα homodimers. The next question was to ask if $CD8\beta^{\alpha h}$ molecules were capable of contributing to TcR delivered signals which are essential for positive selection in the thymus.

An important function of the CD8 molecules is to participate in cell signaling through its association with an intracellular tyrosine kinase. LCK is runs at 56kDa when resolved in SDS-PAGE. It is associated with CD8 via cysteine residues of a CXCP motif in the cytoplasmic domain of CD8α, and cells lacking this domain are impaired in their ability to interact with LCK (Zamoyska *et al.* 1989; Shaw *et al.* 1990). CD8β lacks a binding motif for LCK, and is not directly associated with this polypeptide (Zamoyska *et al.* 1989), yet the presence of the cytoplasmic domain of CD8β has been shown to enhance the association of LCK with CD8 (Irie *et al.* 1998; Arcaro *et al.* 2000; Arcaro *et al.* 2001). The cytoplasmic domains of CD8α and CD8β are intact in $CD8\beta^{\alpha h}$ mice, so it could be expected that LCK will associate normally with the molecule.

In order to see if LCK was associated with CD8, the latter was immunoprecipitated and the presence of LCK assessed by Western blotting. Total cell lysates from $CD8\beta^{wt}$ and *Lck*$^{-/-}$ thymocytes were included as a positive and negative control respectively for the presence of LCK (figure 4.4, lanes 1 and 2). Lanes 3, 5, 7 and 9 show no LCK protein in control immunoprecipitations with Rat Ig coupled to beads. There is a background band that is visible which is probably attributable to the light chains of the Rat Ig molecules, since it is also visible in the precipitates from $CD8\beta^{\circ}$ mice. There is no LCK precipitated from $CD8\beta^{\circ}$ mice with anti-CD8β antibodies (lane 8). In contrast, CD8β precipitates from $CD8\beta^{wt}$, $CD8\beta^{tg}$ and $CD8\beta^{\alpha h}$ expressing mice all show the presence of co-precipitating LCK (lanes 4, 6 and 10). Figure 4.4 panel B is a loading control and shows that immunoprecipitates from mice expressing $CD8\beta^{\alpha h}$ are slightly underloaded in comparison to wild type. This could be the reason for the apparently smaller amount of LCK associated with $CD8\beta^{\alpha h}$ seen in panel A.

The blot was stripped and reprobed for the presence of LAT, but none could be detected. LAT is palmitoylated and is tightly associated with lipid rafts (Zhang *et al.* 1998), and it is possible that the detergent used is not able to dissociate LAT from these rafts.

4.4 Ca^{++} response is not impaired in thymus cells expressing $CD8\beta^{\alpha h}$.

The previous section showed that LCK associates normally with $CD8\beta^{\alpha h}$ molecules demonstrating that the mutation in these molecules has not affected the association of LCK, but this alone does not confirm that mice expressing $CD8\beta^{\alpha h}$ are communicating downstream signals via LCK in a comparable fashion to mice expressing wild type CD8. Ca^{++} mobilization is an early activation event that can be easily studied in real time with the use of fluorescent dyes. The activation of LCK is critical for Ca^{++} mobilisation (Trobridge and Levin 2001), and experiments comparing the Ca^{++} flux of $CD8\beta^{\alpha h}$ expressing cells with $CD8\beta^{wt}$ cells in response to TcR-co-receptor cross linking

will test whether the LCK associated with the CD8β^{ah} co-receptor is functional. Other studies have shown that Ca^{++} mobilization is greatest when TcR and CD8$\alpha\beta$ are co-engaged, as opposed to CD8$\alpha\alpha$ or engaging TcR alone (Arcaro *et al.* 2001), indicating the importance of CD8β for this effect.

Thymocytes from mice expressing CD8β^{wt}, CD8β^{tg}, CD8β° and CD8β^{ah} were isolated at room temperature and incubated in the dark with Indo-1 AM. Cells were washed and resuspended in PBS containing antibodies specific for CD4 (coupled to APC) and varying combinations of TcR and CD8 specific biotinylated antibodies at room temperature for 30 minutes. After washing, cells were run on an LSR and gated on $CD4^+$ DP cells to establish the ratio of resting to activated Indo-1 AM in unstimulated cells. After one minute streptavidin was added in order to cross link the biotinylated antibodies and the cells returned to the LSR for analysis. Crosslinking of TcR alone results in a weak Ca^{++} flux (figure 4.5, panel A). The response is the same in all strains, suggesting that differences seen when crosslinking TcR with the co-receptor will be directly attributed to the influence of the CD8 molecule rather than inherent differences between strains.

Panels B and C show that cross-linking the TcR with the CD8 co-receptor significantly enhances the response as measured by release of Ca^{++} in comparison to that of TcR cross-linking alone (panel A). The response to cross-linking with a co-receptor is both stronger and occurs more rapidly. In panel B, the TcR is crosslinked with antibodies specific for CD8α. In cells expressing CD8β^{wt}, CD8β^{tg}, and CD8β^{ah} Ca^{++} is mobilized with the same time course and magnitude after cross linking. In contrast, there is a slight delay in release and a less robust mobilization in CD8β° cells.

Similar results were obtained when CD8β-specific rather than CD8α-specific antibodies were used for cross-linking with TcR. Again the response of cells expressing CD8β^{wt}, CD8β^{tg} and CD8β^{ah} is comparable. As predicted, CD8β° cells show greatly reduced

response compared to the other test cells, as this control is equivalent to crosslinking TcR alone and shows that anti-CD8β antibodies do not have a negative, non-specific influence on Ca^{++} mobilisation. Panels B and C show how much more robust the response is to crosslinking when CD8 is involved, suggesting that close proximity of the co-receptor to the TcR is a more favourable conformation for signal transduction than clustering of TcR alone.

Another interesting question was to pre-bind CD8 molecules with non-biotinylated antibodies followed by crosslinking biotinylated TcR antibodies, to see if this would have an inhibitory effect on the Ca^{++} response. The binding of unbiotinylated CD8 antibodies has two effects – preventing crosslinking of CD8 with the TcR by the introduction of streptavidin and blocking the downstream proliferative signaling from the TcR complex. Panel D shows the response of all strains tested is similar to that of crosslinking TcR alone indicating that binding of CD8 independently from the TcR has no influence on the Ca^{++} flux. Panel E shows that crosslinking CD8 molecules alone does not stimulate a Ca^{++} flux. This demonstrates that TcR clustering is absolutely required for signal transduction, as crosslinking the co-receptor alone has no effect on the cell.

Discussion

The Ig like domain, transmembrane domain and cytoplasmic domain of CD8β have all been shown to enhance TcR-MHC binding (Wheeler *et al.* 1998; Bosselut *et al.* 2000; Arcaro *et al.* 2001). Yet the $CD8\beta^{ah}$ molecule, which has all of these domains intact, has a severe block in selection. In Chapter 3, analysis of maturation markers in the thymus suggested that the block occurred in early DP development, and data gathered using cognate peptide/ MHC complexes also suggested a defect in the binding affinity of $CD8\beta^{ah}$ molecules to MHC. This chapter addresses whether $CD8\beta^{ah}$ molecules are

processed differently to wild type CD8 molecules, or show reduced ability to transmit TcR mediated signals, which are essential for selection.

O-linked glycosylation can give a glycoprotein extended conformation and provide the molecule with rigidity and protection from proteases, and certain O-linked glycans act as ligands at specifically controlled checkpoints in T cell maturation (Lowe 2001). In the case of CD8 molecules, O-linked glycosyation is predicted to give the molecule an extended conformation as well as orienting the Ig-like domains for optimal binding of MHC (Zamoyska 1994; Moody *et al.* 2001a). With this in mind, the glycosylation of $CD8\beta^{\alpha h}$ was examined by resolution of the molecule in SDS PAGE, which showed that the molecule was larger than the primary structure would predict. This was partly due to the inclusion of another N-linked sugar, but also due to greater O-linked glycosylation and heavier sialylation than would be expected from the amino acid sequence (McNeill 1999).

Cell bound CD8αβ has been shown to bind MHC Class I more avidly than CD8αα (Renard *et al.* 1996; Wheeler *et al.* 1998; Bosselut *et al.* 2000). When using soluble, unglycosylated CD8αβ and CD8αα protein, there is little difference in binding affinity to MHC, indicating that either being membrane-bound and/ or the glycosylation of the molecules is important for the differences in MHC binding observed between CD8αα and CD8αβ when expressed on the cell surface (Garcia *et al.* 1996; Kern *et al.* 1998; Arcaro *et al.* 2001; Moody *et al.* 2001b). It is possible that the altered glycosylation found in $CD8\beta^{\alpha h}$ molecules is preventing the optimal conformation for MHC binding that has been suggested for membrane bound, glycosylated wild type CD8αβ. This is consistent with results from the previous chapter showing that CD8αα and $CD8\beta^{\alpha h}$ have reduced capacity to bind cognate MHC in the thymus. The altered glycosylation seen in thymocytes from $CD8\beta^{\alpha h}$ expressing cells could have a profound effect on cell-cell, cell-matrix or *cis* inter-molecule interactions, and could in some way be responsible for the block in selection seen in these animals.

The first approach taken to address this question was to ask if the CD8β$^{\alpha h}$ polypeptide was capable of forming a heterodimer with endogenous CD8α. In thymocytes there are approximately equal amounts of CD8α'β and CD8αβ expressed on the cell surface, but in peripheral cells CD8α' is held intracellularly (Zamoyska and Parnes 1988). Although CD8 can form CD8αα and CD8αα' homodimers, the majority of CD8 molecules on the cell surface of MHC Class I restricted T cells is in the CD8αβ heterdimer form. The presence on the surface of CD8β$^{\alpha h}$ expressing cells of molecules not usually associated with particular stages of development could have important consequences for T cell interactions. This question was addressed by analysis of the stoichiometry of CD8 molecules. Analysis of total CD8 (intracellular and extracellular) showed CD8αβ heterodimers, as well as CD8αα homodimers, in mice positive for the CD8β$^{\alpha h}$ transgene. However these experiments did not address species of CD8 expressed on the cell surface only, which will be the molecules acting as co-receptors in MHC recognition. Future experiments will utilize cell surface protein labeling techniques in order to establish quantitively and qualitively precisely the nature of the CD8 molecules on the surface of T cells in CD8β$^{\alpha h}$ expressing mice. Using anti-sera specific for CD8α or CD8α' could quantitively establish if the CD8 species expressed on the cell surface in thymocytes are comparable to wild type, as well as establishing which species are held intracellularly in mature cells expressing CD8β$^{\alpha h}$. This question is important because if the species of CD8 found on the cell surface of thymocytes differ in CD8β$^{\alpha h}$ mice in comparison to CD8β^{wt} mice at specific developmentally controlled points in the cell's maturation, possibly as a result of the altered glycosylation found on the molecule, this could have an impact on MHC binding, and possible consequences for selection.

Any difference in the ratio of CD8 species on the cell surface in CD8β$^{\alpha h}$ mice could also have an effect on numbers of CD8αβ molecules found in the lipid rafts, and consequently have implications for T cell selection and activation. CD8αβ molecules

are targeted to lipid rafts via palmitoylation of the cytoplasmic domain of CD8β, but CD8αα molecules are not (Arcaro *et al.* 2000). TcR signaling kinases are concentrated in lipid rafts making them privileged sites for phosphorylation, and crosslinking TcR with raft-associated CD8/LCK promotes the translocation of the TcR complex to lipid rafts and enhancement of TcR signaling (Doucey *et al.* 2001).

LCK, a src family protein tyrosine kinase which is essential for the induction of TcR signalling, is also targeted to lipid rafts (Arcaro *et al.* 2000; Yasuda *et al.* 2000; Arcaro *et al.* 2001; Doucey *et al.* 2001) and has been shown to associate with CD8 (Veillette *et al.* 1988). LCK associates with CD8 via a recognition domain in CD8α, but not CD8α', and is not associated with CD8β (Zamoyska *et al.* 1989). Nevertheless there is a wealth of data showing that CD8β positively influences the interaction of CD8α with LCK (Irie *et al.* 1995; Irie *et al.* 1998; Arcaro *et al.* 2000; Bosselut *et al.* 2000; Arcaro *et al.* 2001). The cytoplasmic domain of the $CD8\beta^{\alpha h}$ polypeptide is identical to that found in wild type, and, consistent with this, the $CD8\beta^{\alpha h}$ molecule was shown to associate with LCK. It is likely therefore that $CD8\beta^{\alpha h}$ is also targeted to lipid rafts as the palmitoylation site of CD8β that has been shown to target CD8αβ to rafts (Arcaro *et al.* 2000) is intact in the molecule, and also because LCK itself is targeted to rafts through palmitoylation (Brown and London 1997; Simons and Ikonen 1997) and appears to be associated normally with $CD8\beta^{\alpha h}$. Future experiments could confirm the presence of $CD8\beta^{\alpha h}$ molecules in lipid rafts by separating the detergent insoluble membrane fraction from lysates. This method could also be used to quantify the amount of LCK associated with $CD8\beta^{\alpha h}$ molecules within and outwith the lipid rafts in comparison to $CD8\beta^{wt}$ control.

If there were any changes in the amount of $CD8\beta^{\alpha h}$ associated with lipid rafts and/or the association of LCK with $CD8\beta^{\alpha h}$, this could have a profound effect on TcR activation. Crosslinking of raft-associated CD8/ LCK with TcR/ CD3 optimises T cell activation, and inhibition of entry for raft associated molecules has a deletrious effect on TcR-mediated cell activation, suggesting that it is important for molecules involved

in TcR-MHC interactions to be located in lipid rafts (Ilangumaran *et al.* 1997; Doucey *et al.* 2001). The TcR/ CD3 complex is only weakly associated with lipid rafts, and engagement of the TcR by its ligand promotes translocation to the raft domain (Montixi *et al.* 1998). Only once LCK activity and CD3 phosphorylation have exceeded a certain threshold will other signaling molecules be recruited to the TcR signaling complex.

The experiment showing association of LCK with $CD8\beta^{\alpha h}$ molecules was limited to showing that the initial signaling machinery associated with TcR signals is intact in cells expressing $CD8\beta^{\alpha h}$. Other reports have shown that LCK kinase activity and downstream phosphorylation in T cells is enhanced by the presence of the CD8β cytoplasmic domain (Irie *et al.* 1995; Irie *et al.* 1998; Arcaro *et al.* 2000; Bosselut *et al.* 2000). Decreased CD8-associated LCK kinase activity correlates with impaired T cell development, so future experiments could assess the kinase activity of cells expressing $CD8\beta^{\alpha h}$ in comparison to $CD8\beta^{wt}$. There is also a correlation between surface expression of wild type CD8β and CD8-associated LCK activity (Irie *et al.* 1998), so another interesting experiment would be to compare the activity of LCK in $CD8\beta^{wt}$ mice and $CD8\beta^{tg}$ mice, which express CD8β at only 50% of wild type levels.

Downstream of LCK is Linker for Activation of T cells (LAT) which is an adaptor protein phosphorylated by ZAP-70 that has also been shown to associate with CD8 molecules (Bosselut *et al.* 1999). Like LCK, LAT is also targeted to lipid rafts (Zhang *et al.* 1998), and LAT association with CD8 is decreased in thymocytes lacking the cytoplasmic domain of CD8β (Bosselut *et al.* 2000). If LAT could be shown to be associated with $CD8\beta^{\alpha h}$ molecules this would help to demonstrate that the requisite signaling machinery is in place in these mice, and that the block in selection is not due to lack of association of the correct signaling molecules. When the same Western blot that showed LCK association was re-probed for the presence of LAT, there was no signal detected. Other reports show that LAT can only be detected very weakly when

co-immunoprecipitated with CD8 (Arcaro *et al.* 2000; Doucey *et al.* 2001), or not at all (Arcaro *et al.* 2001), so it is possible that the assay was not sensitive enough. Also, lipid rafts are insoluble in Triton X-100 at 4°C (Zhang *et al.* 1998), so it is likely that the detergent used for the initial lysis was unsuitable to answer the question. Future experiments will utilize a detergent that has been shown to solublise lipid rafts, e.g. Brij58.

Another indication that the LCK associated with CD8$\beta^{\alpha h}$ is functioning normally is that Ca^{++} mobilization can be induced by crosslinking TcR with or without co-receptor in DP cells expressing the molecule. Activation of LCK is critical for Ca^{++} flux and LCK deficient cells fail to mobilize Ca^{++} after anti-CD3 stimulation (Trobridge and Levin 2001). LCK was previously shown to associate with CD8$\beta^{\alpha h}$ molecules, and this chapter also showed that Ca^{++} flux occurs when TcR and CD8 are crosslinked on DP cells expressing CD8$\beta^{\alpha h}$, suggesting that the LCK is being activated normally. Other reports have found that CD8$\alpha\beta$, but not CD8$\alpha\alpha$ promotes efficient Ca^{++} flux when T cell hybridomas are stimulated (Arcaro *et al.* 2000; Arcaro *et al.* 2001). This effect is mediated by the cytoplasmic domain of CD8β and is dependent on the palmitoylation of this domain and the partitioning into lipid rafts that this modification affords (Arcaro *et al.* 2000). CD8$\beta^{\alpha h}$ shows Ca^{++} flux similar to wild type when stimulated by crosslinking antibodies specific for TcR and CD8. This shows that the signaling machinery in CD8$\beta^{\alpha h}$ expressing cells can be functional under these specific circumstances, but does not show whether the signaling cascade is fully functional during selection, or which signaling molecules are taking part in the cascade.

Curiously, although crosslinking CD8$\alpha\alpha$ and TcR in *Cd8β*$^{-/-}$ cells enhances the response in comparison to crosslinking TcR alone, this enhancement is less than seen with the other strains. Arcaro *et al* (2000) showed no Ca^{++} mobilization in hybridomas expressing CD8$\alpha\alpha$, suggesting impaired co-receptor function for CD8$\alpha\alpha$ compared to

CD8αβ. This is similar but more extreme than the results shown here for CD8αα compared to CD8αβ in DP thymocytes.

Using Ca^{++} mobilization as a readout, it appears that $CD8\beta^{ah}$ expressing cells are capable of transmitting signals after cross-linking with antibodies. Yet data from the previous chapter showed that DP cells expressing $CD8\beta^{ah}$ were arrested at an early stage of maturation, suggesting that the DP cell is not receiving signals appropriate selection signals. This is not due to an inability of $CD8\beta^{ah}$ molecules to associate with LCK, or an inability of the associated LCK to become activated, which is essential for Ca^{++} flux (Trobridge and Levin 2001). Cross linking antibodies shows that the signaling machinery associated with $CD8\beta^{ah}$ can work, but is restricted as a method for asking questions of the selecting capability of DP cells because in the thymus a very strong stimulus like this would lead to the DP cell being deleted. A more physiological way of approaching the question of why DP cells expressing $CD8\beta^{ah}$ are failing to select would be to take advantage of the recombinant MHC/Ig protein described in Chapter 3. MHC loaded with NP34 peptide, an antagonist for the F5 TcR, has been shown to induce positive selection in $F5^{+}$ thymocytes (Smyth *et al.* 1998). Repeating the experiments using the recombinant MHC protein loaded with NP34 peptide as a stimulus to induce Ca^{++} mobilization in DP cells would better re-create selection conditions in the thymus and help address the question of whether DP cells expressing $CD8\beta^{ah}$ are selecting in the same fashion as $CD8\beta^{wt}$ controls.

Data from this chapter suggests that DP thymocytes are capable of transmitting signals, but in contrast, data from Chapter 3 shows a block in selection at an early stage in DP development. CD8β has been shown to be capable of interacting with the TcR/ CD3 complex (Wheeler *et al.* 1998), and if it is the Ig-like domain that contacts the MHC molecule, the region most likely to be interacting with TcR/ CD3 is the hinge region (Arden 1998). Another approach to answering the question of why the block in selection occurs in mice expressing $CD8\beta^{ah}$ would be to perform experiments looking

for co-immunoprecipitation of CD8 with CD3 and TcR (Gallagher *et al.* 1989; Beyers *et al.* 1992; Suzuki *et al.* 1992), which would confirm if CD8β$^{\alpha h}$ molecules are capable of the same close interaction with TcR as wild type molecules. The report of Arden *et al* (1998) suggested that the interaction between CD8 and TcR was mediated by the sialylated O-linked glycans, which have been shown in this chapter and (McNeill 1999) to be different in CD8β$^{\alpha h}$ molecules in comparison to wild type CD8β. It is possible that the difference in glycosylation is affecting any interaction between CD8β$^{\alpha h}$ and TcR, and this question can be answered by quantitively showing how much CD8 co-immunoprecipitates with TcR in mice expressing CD8β$^{\alpha h}$. Furthermore, confocal studies have suggested an interaction between the TcR and CD8β which allows CD8β antibodies to co-cap the TcR more efficiently than antibodies specific for CD8α (Kwan Lim *et al.* 1998). The question of whether the interaction between CD8β and TcR has been affected by the replacement of the hinge region can be assessed by repeating the confocal co-capping studies using cells expressing CD8β$^{\alpha h}$. If the interaction between TcR and CD8β$^{\alpha h}$ is impaired, this could affect the interaction of the T cell with MHC and possibly help elucidate why there is a block in selection in mice expressing CD8β$^{\alpha h}$.

Figure 4.1 - Sites of glycosylation in CD8 molecules.

Known (red) and predicted (green) sites of O-linked (circle) and N-linked (triangle) glycosylation for wild type CD8αβ molecules (panel A) and CD8β$^{\alpha h}$ molecules (panel B). O-linked glycans can be further modified by the addition of sialic acid. The two predicted O-glycan sites marked with a star (*) are less likely to be modified in this way due to the presence of the bulky N-glycan close by.

The CD8α polypeptide is shown in yellow; the CD8β polypeptide is shown in blue with the CD8β$^{\alpha h}$ polypeptide coloured accordingly. Also shown is the disulphide bond (black) and the T cell membrane (grey).

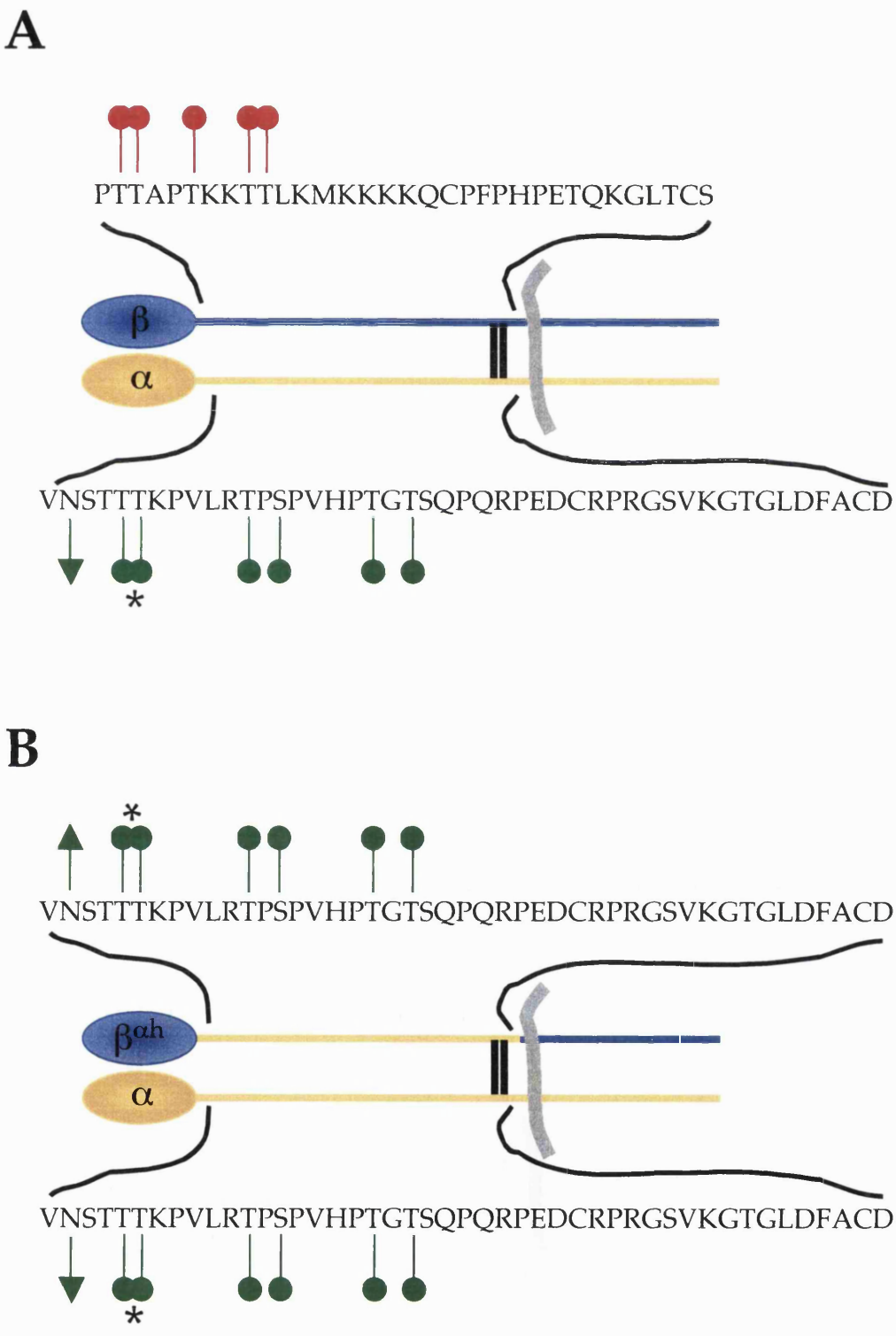

A
PTTAPTKKTTLKMKKKQCPFPHPETQKGLTCS
β
α
VNSTTTKPVLRTPSPVHPTGTSQPQRPEDCRPRGSVKGTGLDFACD
*
B
*
VNSTTTKPVLRTPSPVHPTGTSQPQRPEDCRPRGSVKGTGLDFACD
βαh
α
VNSTTTKPVLRTPSPVHPTGTSQPQRPEDCRPRGSVKGTGLDFACD
*

Figure 4.2 - CD8β^{ah} runs at a higher molecular weight in SDS PAGE than predicted by the primary structure.

Thymocytes (1 x 10^8) from CD8β^{wt}, CD8β^{tg} and CD8β^{ah} expressing mice were lysed for 30 minutes at 4°C in 1% triton and lysates precleared with RIg Abs for 90 minutes at 4°C. Thymus lysates were then immunoprecipitated with anti-CD8β mAb YTS156.7 for 2 hours at 4°C. Total cell lysates from CD8β^{wt} mice (lane 1) and CD8β° mice (lane 2), and immunoprecipitates from CD8β^{wt} (lane 3), CD8β^{tg} (lane 4) and CD8β^{ah} (lane 5) lysates were analysed by SDS PAGE and Western blotting with anti-CD8β antiserum. Molecular weight markers are shown.

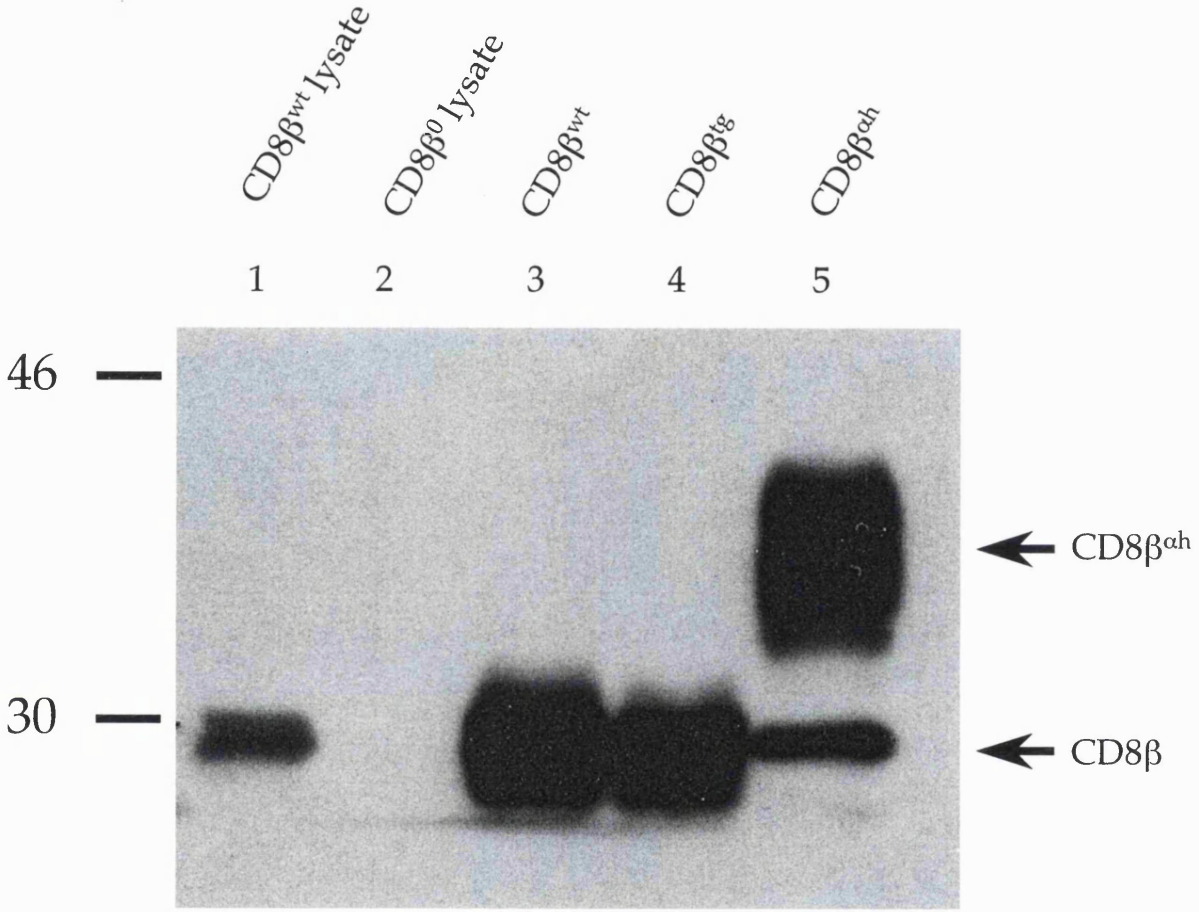
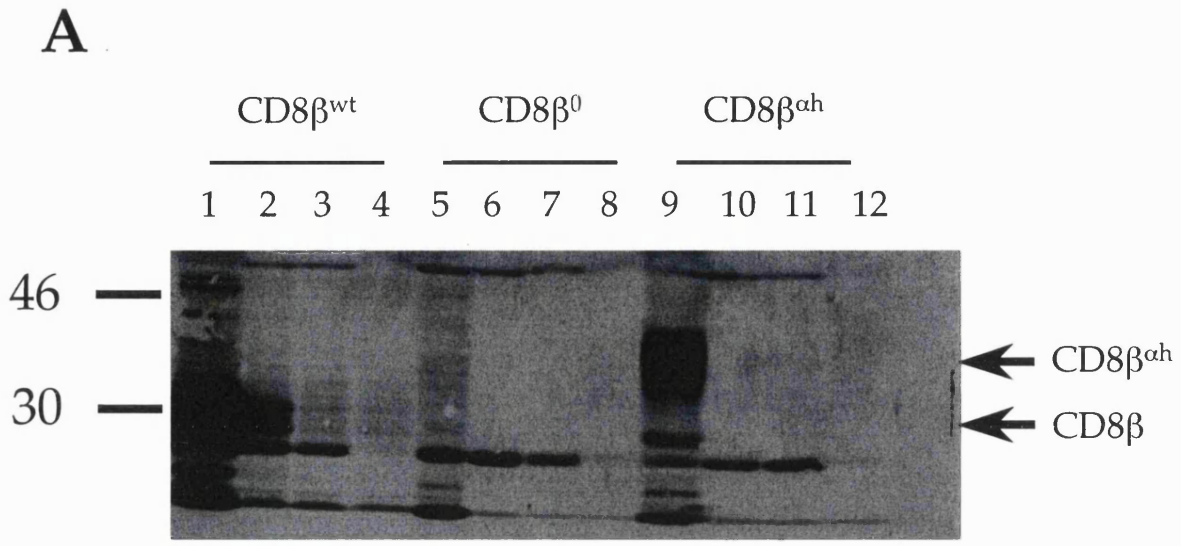

CD8β^wt lysate
CD8β^0 lysate
CD8β^wt
CD8β^tg
CD8β^αh
1
2
3
4
5
46
30
CD8β^αh
CD8β

Figure 4.3 - Mice expressing CD8β$^{\alpha h}$ express a population of CD8αα homodimers as well as CD8αβ heterodimers.

Thymocytes (1 x 10^8) from CD8β^{wt}, CD8β° and CD8β$^{\alpha h}$ expressing mice were lysed at 4°C for 30 minutes in 1% triton and precleared for 90 minutes at 4°C with RIg Abs. Thymus lysates were then immunoprecipitated three successive times for 2 hours at 4°C with anti-CD8β mAb YTS156.7, then one further immunoprecipitation with anti-CD8α mAb YTS169.4. Immunoprecipitates were washed twice in 1% triton. The immunoprecipitates from CD8β^{wt} (lanes 1 - 4), CD8β° (lanes 5 - 8) and CD8β$^{\alpha h}$ (lanes 9 - 12) thymus lysates were analysed by SDS PAGE and Western blotting with anti-CD8β (A) and anti-CD8α (B) antiserum. For each strain analysed, the three lanes furthest left are successive CD8β-specific immunoprecipitations, and the right hand lane is the CD8α-specific immunoprecipitation. Molecular weight markers are shown.


A
CD8β^wt
CD8β^0
CD8β^αh
1 2 3 4 5 6 7 8 9 10 11 12
46
30
CD8β^αh
CD8β

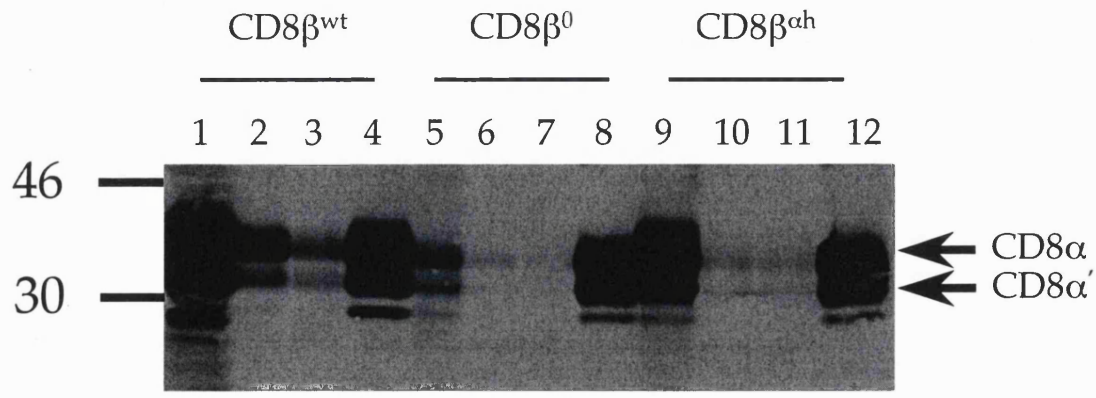

B
CD8β^wt
CD8β^0
CD8β^αh
1 2 3 4 5 6 7 8 9 10 11 12
46
30
CD8α
CD8α'

Figure 4.4 - LCK co-immunoprecipitates with CD8β^{ah} molecules.

Thymocytes (1 x 10^8) from CD8β^{wt}, CD8β^{tg}, CD8β° and CD8β^{ah} expressing mice were lysed for 30 minutes at 4°C in 1% triton and lysates immunoprecipitated with RIg Abs for 90 minutes (lanes 3, 5, 7 and 9) followed by anti-CD8β mAb YTS156.7 (lanes 4, 6, 8 and 10) for 2 hours, both at 4°C. Immunoprecipitates were washed twice with 1% triton at 4°C. Total cell lysates from CD8β^{wt} (lane 1) and *lck*$^{-/-}$ (lane 2) expressing thymocytes, and immunoprecipitates from CD8β^{wt} (lanes 3 and 4), CD8β^{tg} (lanes 5 and 6), CD8β° (lanes 7 and 8) and CD8β^{ah} (lanes 9 and 10) thymus lysates were analysed by SDS PAGE and Western blotting with anti-lck (A) and anti-CD8β (B) antiserum. Lanes 3, 5, 7 and 9 are RIg immunoprecipitates, lanes 4, 6, 8 and 10 are YTS156.7 immunoprecipitates. Molecular weight markers are shown.

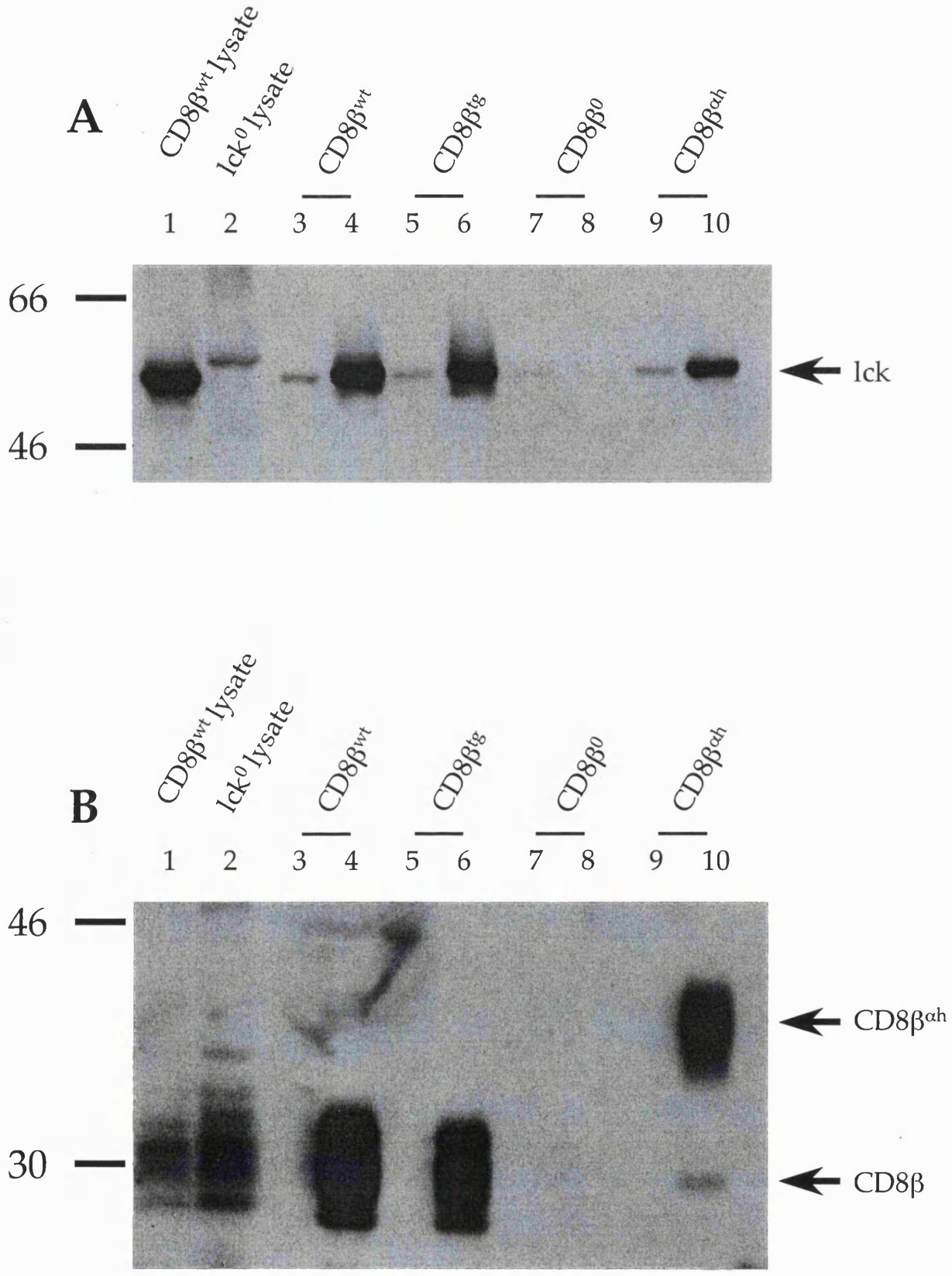
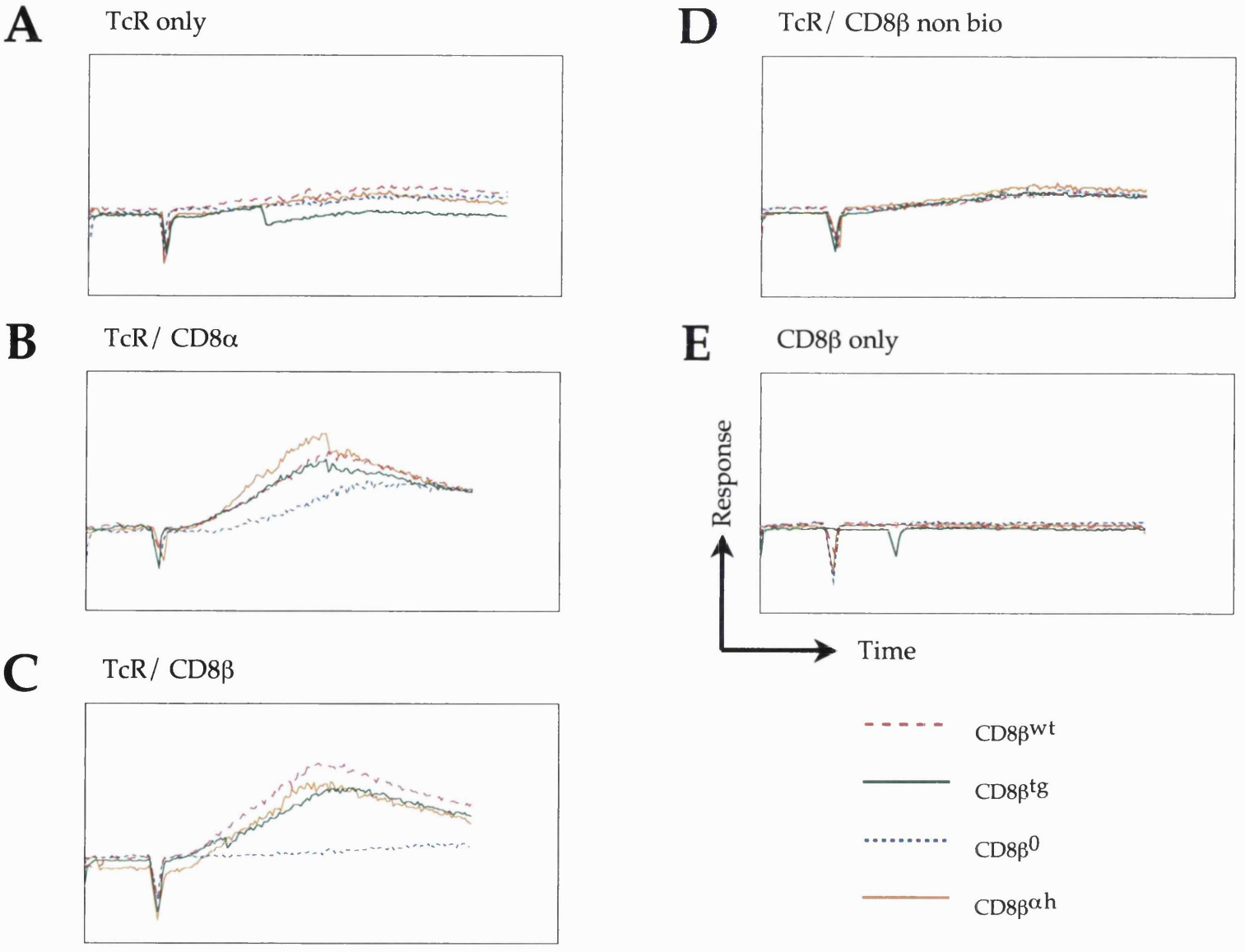

A
CD8β^wt lysate
lck^0 lysate
CD8β^wt
CD8β^tg
CD8β^0
CD8β^αh
1 2 3 4 5 6 7 8 9 10
66
46
lck
B
CD8β^wt lysate
lck^0 lysate
CD8β^wt
CD8β^tg
CD8β^0
CD8β^αh
1 2 3 4 5 6 7 8 9 10
46
30
CD8β^αh
CD8β

Figure 4.5 - Ca^{++} flux response in thymocytes induced by crosslinking antibodies specific for TcR or CD8 is comparable for all strains.

Ca^{++} response in $CD8\beta^{wt}$ (red line), $CD8\beta^{tg}$ (green line), $CD8\beta^{\circ}$ (blue line) and $CD8\beta^{\alpha h}$ (gold line) thymocytes treated with biotinylated antibodies specific for TcR only (A), TcR and CD8α (B), TcR and CD8β (C), TcR and non-biotinylated CD8β (D) or CD8β only (E). Cells were kept at room temperature, labeled with Indo-1AM in darkness for 45 minutes and pretreated with the above antibodies as well as antibodies specific for CD4. The streptavidin crosslinker was added after analyzing cells on LSR for 1 minute, and cells were analysed for a further 5 minutes. Antibodies used were H57 (TcR) YTS169.4 (CD8α) YTS156.7 (CD8β) and RM4-5 (CD4). All traces are the median of the responding population of DP cells as measured by change in fluorescence of the Indo-1AM dye. Axes are labeled, the Y-axis range is identical in each case.


A
TcR only
D
TcR/ CD8β non bio
B
TcR/ CD8α
E
CD8β only
Response
Time
C
TcR/ CD8β
CD8β^wt
CD8β^tg
CD8β^0
CD8β^αh

5. The effect of altered glycosylation and sialylation on CD8β^{ah} molecules.

Introduction

CD8 molecules are heavily post-translationally modified by the addition of N- and O-linked glycans, which are themselves further modified by the addition of sialic acid (Rothenberg and Triglia 1983; Luscher *et al.* 1985). Detailed analysis of O-linked carbohydrates has been greatly facilitated by the used of lectins, which recognize specific glycan residues attached to glycoproteins (Sharon 1983). Lectins can be used to separate cell subsets (Fowlkes *et al.* 1980), precipitate glycoproteins and identify the type of glycosylation present. Changes in specific glycan moieties, mediated by regulatory enzymes, can affect protein-protein interactions as the cell develops. For example, in a wild type situation, PNA binds to DP thymocytes by recognition of a particular glycan moiety, the core-1 O-glycan (table 5.1). This binding is lost as the DP thymocyte matures to SP, due to the upregulation of the enzyme ST3Gal-1 which attaches sialic acid to core-1 glycans via an α(2, 3) linkage ((Reisner *et al.* 1976; Fowlkes *et al.* 1980; Toporowicz and Reisner 1986; Gillespie *et al.* 1993; Baum *et al.* 1996; Priatel *et al.* 2000) and table 5.1). T cell maturation also results in the sialylation of a specific N-linked glycan, which can be recognized specifically by the lectin SNA ((Baum *et al.* 1996; Martin *et al.* 2002) and table 5.1). The core-1 O-glycan is targeted by two competing enzymes, ST3Gal-1 which attaches sialic acid, and C2GnT, which attaches a GlcNAc glycan creating a core-2 glycan that is recognized by another lectin, ECA. Core-2 O-glycan expression decreases with T cell maturation (Baum *et al.* 1995; Ellies *et al.* 1996; Tsuboi and Fukuda 2001).

Table 5.1 – Specificities of lectin binding to glycans

Lectin	recognizes glycan	common name
PNA	Galβ(1,3)GalNAc	core-1
ECA	Galβ1-3(GlcNAcβ1-6)GalNAc	core-2
MAA	α-NeuNAc(2,3)Gal	α2-3 sialic acid
SNA	α-NeuNAc(2,6)GalNAc	α2-6 sialic acid

There is much evidence demonstrating that desialylation of the T cell or APC significantly enhances the activation of T cells (Knop 1980; Cullen *et al.* 1981; Cowing and Chapdelaine 1983; Hunig 1983; Powell *et al.* 1987; Boog *et al.* 1989; Sprent and Schaefer 1990). Other reports have shown that T cells in a developmentally low state of sialylation correlate with enhanced sensitivity to TcR ligands (Pihlgren *et al.* 1996; Curtsinger *et al.* 1998; Davey *et al.* 1998; Lucas *et al.* 1999), and T cells that carry a mutation for the endogenous sialidase Neu-1, which is upregulated and removes sialic acid on activation, show aberrant T cell response (Taira and Nariuchi 1988; Chen *et al.* 1997).

Developmentally regulated changes in CD8 glycans have been previously noted both in thymocytes and activated T cells (Casabo *et al.* 1994; Wu *et al.* 1996), and recent reports have shown that developmental changes in CD8 binding to MHC Class I are also regulated by the addition of sialic acid (Daniels *et al.* 2001; Moody *et al.* 2001). Previous work in the lab also showed that on T cell activation, CD8β, but not CD8α becomes hyposialylated, and that CD8 changes from having predominantly core-1 O-glycan adducts to predominantly core-2 O-glycans (Casabo *et al.* 1994; McNeill 1999).

The O-linked glycosylation sites have been mapped in the human and rat CD8α polypeptide and found to be clustered in the hinge region of the molecule (Classon *et al.* 1992; Pascale *et al.* 1992). The sites of O-linked glycosylation in murine CD8α are not

mapped, but are also predicted to occur in the hinge region (Casabo *et al.* 1994; Hansen *et al.* 1997). Recent data showed that the O-linked glycans of murine CD8β were clustered in five sites on the hinge of the molecule near the Ig-like domain, distal to the membrane (Moody *et al.* 2003). This has been suggested to orientate the Ig like domains of CD8αβ to facilitate optimal binding with the MHC Class I α3 domain in desialylated DP cells (Moody *et al.* 2001). In contrast, the addition of sialic acid to the O-linked glycans, facilitated by increased ST3Gal-1 sialyltransferase activity as thymocytes mature (Despont *et al.* 1975), is suggested to weaken the interaction of $CD8^+$ SP cells with MHC, again by re-orientating the Ig-like domains (Kern *et al.* 1998; Moody *et al.* 2001). Moody *et al* show that binding of non-cognate MHC to thymocytes is CD8 dependent and decreases with the maturation of the cell as a direct consequence of increased sialylation of CD8. However another group found that lymphocyte binding to cognate MHC is also CD8 dependent, in spite of CD8 being highly sialylated in these cells (Daniels and Jameson 2000), in direct contrast to the binding of non-cognate MHC in thymocytes. Furthermore, de-sialylation of the O-linked glycans on lymphocytes only occurs sometime after the peak of the T cell response (Priatel *et al.* 2000). Interestingly, mice lacking the ST3Gal-1 sialyltranferase have no sialic acid adducts in $CD8^+$ SP T cells but no impairment in thymocyte development or viability, although peripheral cells are more susceptible to apoptosis (Priatel *et al.* 2000).

The role of developmentally regulated glycolysis of CD8 is complicated and still unclear, and mice expressing a mutated CD8 molecule, $CD8\beta^{\alpha h}$, were created to help answer the question of why these changes in glycosylation are important for T cell development and co-receptor function. Thymocytes expressing $CD8\beta^{\alpha h}$ have been shown in chapter 3 to be arrested at the positive selection stage of maturation, and data in chapter 4 showed that the $CD8\beta^{\alpha h}$ molecule runs higher in an SDS gel due to being more heavily glycosylated and sialylated than the primary structure would predict. Given the importance for cell-cell interactions of highly specific glycan moieties for T cell maturation and activation, a closer investigation of the glycans on $CD8\beta^{\alpha h}$ was

undertaken to answer the question of whether the altered glycosylation seen in these molecules has significance for the cell, and could explain the block in selection.

5.1 Lectin analysis of thymus lysates from mice expressing CD8β$^{\alpha h}$ reveals the glycan structures of CD8.

Data from the previous chapter and (McNeill 1999) showed that in thymocytes expressing CD8β$^{\alpha h}$, the CD8β$^{\alpha h}$ polypeptide had more O-linked glycans and was more heavily sialylated than the primary structure would predict, causing the protein to run higher in SDS gel than expected. The precise regulation of oligosaccharide synthesis during T cell development suggests that the different glycan moieties have unique functions at specific points in thymocyte maturation. The arrest of development of thymocytes expressing CD8β$^{\alpha h}$ at an early DP stage shows impairment in the maturation of these cells, which could be due to changes in the specific glycan moieties attached to the molecule.

In order to answer the question of whether aberrations in developmentally regulated glycan moieties were causing the block in selection seen in mice expressing CD8β$^{\alpha h}$, thymocytes from mice expressing CD8β^{wt} and CD8β$^{\alpha h}$ were lysed and precleared with beads coupled to streptavidin. Lysates were filtered and immunoprecipitated with biotinylated lectins specific for particular glycan structures and streptavidin beads. Precipitates were resolved in SDS PAGE under reducing conditions, the gel Western blotted and probed with serum specific for CD8β.

Total cell lysates from CD8β^{wt} and CD8β$^{\circ}$ thymi were included as a positive and negative control respectively for the presence of CD8β (Figure 5.1, lanes 1 and 2). In lysates from CD8β^{wt} expressing thymocytes, CD8 molecules can be immunoprecipitated with biotinylated lectins PNA and SNA, but not ECA (Figure 5.1, lanes 3, 4 and 5), which is in agreement with previous studies (Wu *et al.* 1996; McNeill

1999). In mice expressing CD8β$^{\alpha h}$, like CD8β^{wt} thymus lysates, CD8 molecules can also be immunoprecipitated with biotinylated lectins PNA and SNA, and not ECA (Figure 5.1, lanes 6, 7 and 8). This indicates that although the amount of post-translational O-glycan adducts is greater in CD8β$^{\alpha h}$ expressing mice in comparison to CD8β^{wt} mice (Chapter 4 and (McNeill 1999)), the nature of the glycans as visualized by binding to the core structures with lectins is the same as that found in wild type CD8 molecules. This suggests that the regulation of the enzymes involved in glycosylation is not grossly different in thymocytes expressing CD8β$^{\alpha h}$ compared to CD8β^{wt}, but this does not explain why the CD8β$^{\alpha h}$ polypeptide is larger in SDS gel than the primary structure predicts, due to additional O-linked glycosylation and sialylation, especially given that the wild type CD8α chain with which the CD8β$^{\alpha h}$ polypeptide dimerises shows the same glycosylation as in wild type (McNeill 1999). Mass spectrometry analysis of the glycans would help identify precisely the amino acid residues of the CD8β$^{\alpha h}$ polypeptide that are modified by the addition of O-linked glycosylation and sialic acid, and possibly help answer why the CD8β$^{\alpha h}$ polypeptide is larger than predicted.

5.2 The β-chain of CD8β$^{\alpha h}$ becomes a faster migrating species in SDS gel after stimulation *in vitro* for 72 hours.

Upon activation, CD8β but not CD8α becomes hyposialylated (Casabo *et al.* 1994), and the molecule as a whole undergoes a change in the core structure of the glycans on activation (McNeill 1999). De-sialylation lessens the overall negative charge of the molecule and changing glycan structures can either affect cell-cell interactions or provide developmentally regulated ligands at tightly controlled points in the T cell's ontogeny (Tsuboi and Fukuda 2001). In the case of CD8, the functional significance of these changes for the cell is not yet clear, and answering this question was the rationale for the creation of the CD8β$^{\alpha h}$ expressing mice.

A change in the content of sialic acid in CD8 molecules on activation can be visualized as a change in the mobility of the molecule through an SDS gel. Sialic acid has an overall negative charge, as does SDS, and a reduction in the negative charge of sialic acid due to hyposialylation on activation causes the individual chains of CD8 to become faster migrating species through the gel. The different sialylation states of CD8β can be clearly seen as the appearance of individual faster migrating bands after stimulation.

In order to assess how substituting a CD8α hinge region for the CD8β hinge region might affect hyposialylation upon T cell activation, lymphocytes from mice expressing $CD8\beta^{wt}$, $CD8\beta^{tg}$, CD8β° or $CD8\beta^{\alpha h}$ were cultured *in vitro* for 72 hours with stimulating peptide. After this time, fresh lymphocytes were isolated from all strains and resting and activated lymphocytes lysed and immunoprecipitated with CD8β-specific beads, or CD8α-specific beads in the case of CD8β° lysates. Precipitates were resolved on SDS PAGE under reducing conditions, Western blotted and probed with serum specific for CD8β or CD8α.

Figure 5.2, panel A shows resting and activated CD8β. As demonstrated previously (Casabo *et al.* 1994), in cells expressing $CD8\beta^{wt}$, the CD8β chain from activated lymphocytes (lane 2) largely converts to a faster migrating species in SDS PAGE compared to resting lymphocytes (lane 1), due to the removal of sialic acid. This is also true for activated lymphocytes from $CD8\beta^{tg}$, which express wild type CD8β polypeptide as a transgene (panel A, lanes 3 and 4). There is no CD8β detectable for resting or activated lymphocytes from CD8β° mice (lanes 5 and 6). In lymphocytes expressing $CD8\beta^{\alpha h}$, activated CD8β also becomes faster migrating in SDS PAGE after activation in comparison to resting lymphocytes, suggesting that this polypeptide also becomes hyposialylated on activation (lanes 7 and 8). In contrast, there is no change in mobility in CD8α between resting and activated cells in any of the strains (figure 5.2,

panel B). The lower intensity of CD8 bands in resting cells in panels A and B is a reflection of lower starting numbers of cells.

These results show that the hinge region of CD8α, when placed in the context of the CD8β polypeptide, behaves differently from the wild type CD8α hinge region. In contrast to wild type CD8α, it is more heavily glycosylated and sialylated and becomes hyposialylated upon activation.

Discussion

CD8 is heavily post-translationally modified with three sites of N-linked glycosylation and several potential sites of O-linked glycosylation (Rothenberg and Triglia 1983; Luscher *et al.* 1985; Hansen *et al.* 1997; Moody *et al.* 2003). The O-linked glycans are further modified by the addition of sialic acid (Reisner *et al.* 1976; Toporowicz and Reisner 1986). Developmentally regulated changes in the glycan adducts of CD8 have been described previously, both in thymus and on activation (Casabo *et al.* 1994; Wu *et al.* 1996). In the case of CD8 on activation, these changes seem to be specific for CD8β.

In spite of having the wild type Ig-like domain, transmembrane domain and cytoplasmic domain of CD8β, all of which have been demonstrated to be important for MHC recognition (Wheeler *et al.* 1998; Arcaro *et al.* 2000; Bosselut *et al.* 2000; Arcaro *et al.* 2001), selection in the thymus of mice expressing $CD8\beta^{\alpha h}$ is arrested at an early DP stage of maturation. Previous data showed that the $CD8\beta^{\alpha h}$ polypeptide ran higher in an SDS gel than the primary structure predicts, and that this was due to heavier O-linked glycosylation and sialylation than wild type molecules. The precise regulation of glycan synthesis during T cell maturation suggests that different glycan moieties have unique functions at specific points in thymocyte development, so lectins were used to analyse the precise glycoforms found in thmyocytes expressing $CD8\beta^{\alpha h}$. In thymus lysates from mice expressing $CD8\beta^{\alpha h}$, CD8 molecules could be

immunoprecipitated with peanut agglutinin (PNA) and *Sambucus Nigra* bark (SNA) lectins, but not *Erythrina Cristagalli* (ECA). This shows that immature T cells in the thymus of mice expressing CD8β$^{\alpha h}$ are, like wild type, modified by the addition of core-1 O linked glycans, recognized by PNA. Furthermore, CD8β$^{\alpha h}$ molecules are modified by the addition of sialic acid in an α2,6 linkage, recognized by SNA. Another lectin, MAA, recognizes sialic acid attached by an α2,3 linkage, and could not be detected in CD8β$^{\alpha h}$ molecules from thymus lysates (table 5.1, (McNeill 1999)). ST3Gal-1 is upregulated as thymocytes develop (Priatel *et al.* 2000), and is uniquely specific for the core-1 O-glycan structure recognized by PNA (Kono *et al.* 1997). ST3Gal attaches sialic acid in an α2,3 linkage, yet MAA did not precipitate CD8 or CD8β$^{\alpha h}$ molecules from thymus lysates. Given that thymocytes have been shown to bind MAA (Baum *et al.* 1996), this indicates either that ST3Gal is specifically not targeting CD8 molecules, or that the product of ST3Gal-1 sialylation is itself further modified, as suggested by Moody *et al* (2003). This is possibly achieved by the enzyme ST6GalNAc-III, which modifies the MAA ligand by attaching sialic acid in an α2,6 linkage and would be recognized by SNA. This further addition represents a synthetic 'dead end' for glycan modification, indicating that the addition of glycans to CD8β$^{\alpha h}$ molecules follows the same synthetic path as wild type CD8 molecules, and is not arrested at a precursor stage.

ECA, which recognizes and binds core-2 glycans (table 5.1), did not immunoprecipitate CD8 from thymus lysates of CD8β^{wt} or CD8β$^{\alpha h}$. Core-2 O-glycans are detectable on DP thymocytes, and expression of these glycans decreases as the cells matures to SP (Baum *et al.* 1995; Ellies *et al.* 1996). The attachment of core-2 O-glycans in DP cells seems to be principally confined to the CD43 glycoprotein, which is so rich in potential sites of O-glycan attachment that the majority of all T cell O-glycans are associated with this molecule (Andersson *et al.* 1978; Carlsson and Fukuda 1986). Attachment of core-2 O-glycans is controlled by the upregulation of C2GnT enzyme at the DP stage and subsequent downregulation of this enzyme as the cell is selected and becomes SP.

Core-2 O glycans are required for the apoptosis seen in DP thymocytes, but the survival of SP cells depends on the enzyme C2GnT being switched off and no core-2 O-glycans being expressed (Baum *et al.* 1995; Ellies *et al.* 1996). Interestingly, although these data show that wild type CD8 molecules, as well as CD8β$^{\alpha h}$ molecules, do not appear to have core-2 O-glycan adducts at any stage of thymocyte development, the reduction in attachment of core-2 O-glycans to CD43 that is associated with positive selection is specific for MHC Class I restricted cells, but not MHC Class II cells (Ellies *et al.* 1996).

A simplified version of the glycan adduct pathway is shown in figure 5.3, demonstrating that the enzymes ST3Gal-1 and C2GnT compete for the same core-1 O-glycan structure. Other reports have shown that that the sialylation of the core-1 O-glycans actively inhibits core-2 biosynthesis *in vivo* (Priatel *et al.* 2000), and that ST3Gal-1 can inhibit C2GnT activity *in vitro* (Schachter and Brockhausen 1989). Both CD43 and CD8 are substrates for ST3Gal-1 (Casabo *et al.* 1994; Wu *et al.* 1996; Priatel *et al.* 2000). Yet the C2GnT enzyme in DP cells will attach core-2 O-glycans to CD43, but not CD8, raising the question of how the enzymes achieve this specificity. Furthermore, activation of T cells is associated with the re-appearance of core-2 O glycans on CD43 (Piller *et al.* 1988) as well as their novel appearance on CD8 (McNeill 1999).

These data show that the O-linked glycan structures associated with CD8β$^{\alpha h}$ molecules in thymocytes are of the same core structure and linkage as those associated with wild type CD8 molecules, but data from the previous chapter and (McNeill 1999) indicates that the O-linked glycans of CD8β$^{\alpha h}$ are more abundant. Core-2 O-glycans are bulkier than core-1 O-glycans and this may cause a reduction in cell-cell interactions by making T cell-APC interaction inefficient, thereby regulating immune response (Tsuboi and Fukuda 1997; 1998). It is possible that the extra bulk of the core-1 O-glycans seen in CD8β$^{\alpha h}$ molecules is having a similar effect, inhibiting the interaction between DP thymocytes and the thymic epithelial cells.

The CD8β$^{\alpha h}$ polypeptide contains an extra potential site of N-linked glycosylation, near the Ig like domain and previous work in the lab has shown that there is a second N-linked glycan attached (McNeill 1999). The importance of this extra glycan should not be dismissed when considering the question of why mice expressing CD8β$^{\alpha h}$ show a severe impairment in selection. A typical N-glycan is approximately the length of one Ig domain (Rudd *et al.* 1999), and the impact on the CD8β$^{\alpha h}$ polypeptide of having two N-linked glycans instead of one as in wild type could be important for protein-protein interaction. Other reports have suggested that O-linked glycosylation of the hinge region of CD8αβ holds the hinge region in a specific extended conformation, thereby creating an optimal conformation of the Ig-like domains to interact with MHC (Moody *et al.* 2001). It is possible that the presence of the extra N-linked glycan near the Ig-like domain on the CD8β$^{\alpha h}$ polypeptide is disrupting this optimal conformation by steric hindrance or by forcing the Ig-like domains to adopt a different conformation to wild type CD8 molecules. Previous work showed that this extra N-linked glycan could be removed with endoglycosidase-H, showing it was of high mannose type and thus unprocessed and immature (McNeill 1999), consistent with the equivalent N-linked glycan in CD8α molecules. The second N-linked glycan attached to the CD8β$^{\alpha h}$ chain, also found in wild type CD8β, was resistant to treatment (McNeill 1999), indicating this N-glycan was more mature. Future experiments could address the importance of the extra N-linked glycan for MHC interaction by comparing the response of cells expressing CD8β$^{\alpha h}$ with or without endoglycosidase-H treatment.

Previous data from the lab showed that they glycosylation of CD8β seemed to be correlated with T cell activation due to the fact that it, but not CD8α, becomes hyposialylated (Casabo *et al.* 1994). Data from this chapter shows that the CD8β$^{\alpha h}$ polypeptide also becomes hyposialylated on activation, in spite of having altered glycans in comparison to wild type CD8β. It is interesting that a CD8α-hinge in the context of a CD8β molecule will become hyposialylated, presumably due to the

upregulation of the endogenous sialidase Neu-1 (Chen *et al.* 1997; Galvan *et al.* 1998; Chen *et al.* 2000), again raising the question of how the enzymes associated with the regulation of cell surface glycosylation achieve such specificity.

Like wild type CD8α, the CD8α chain from $CD8\beta^{\alpha h}$ molecules does not become hyposialylated on T cell activation. Interestingly, this is also true for the CD8αα molecules from CD8β° mice, which suggests that hyposialylation is specific for CD8β. It has been shown using crystallography techniques that binding of CD8αα molecules to MHC is asymmetric even though both chains are the same (Gao *et al.* 1997). Figure 5.4, reproduced from Gao *et al* (1997) shows the asymmetric binding of CD8αα represented by CD8α-1 being red and CD8α-2 being blue. Binding of CD8αβ to MHC is predicted to be similarly asymmetric (Gao *et al.* 1997) but it is not known whether CD8β is the 'red' or the 'blue' chain. It was possible that the hyposialylation of one chain of CD8 (e.g. figure 5.4, red chain) but not the other (e.g. figure 5.4, blue chain) on binding of MHC and subsequent T cell activation was dependent on the position of the chain with respect to the asymmetric binding. The finding that CD8αα does not become hyposialylated on T cell activation suggests this is not the case, and that the hyposialylation is peculiar to CD8β.

A further change associated with CD8 on T cell activation is a change in the core structure of the glycan adducts from predominantly core-1 O-glycans to predominantly core-2 O-glycans (McNeill 1999). Currently it is not known if this is specific for one or both chains, and future experiments will determine if this change is common to both chains of CD8 or, like the hyposialylation, associated only with CD8β. In the case of CD43, whose glycans also shift from a core-1 to a core-2 structure after T cell activation, this is attributable to the upregulation of the C2GnT enzyme after T cell activation (Higgins *et al.* 1991; Ellies *et al.* 1994). A correlation between upregulation of C2GnT and the appearance of core-2 O-glycans on wild type CD8 molecules would

suggest this enzyme is responsible for this change in CD8 molecules also, and future experiments can address this question.

The change in core structure of the O-glycans of CD8 in activated cells could provide the answer to surprising data describing the binding of non-cognate MHC by CD8. As discussed in Chapter 3, DP cells are able to bind non-cognate MHC but SP and mature naïve T cells are not (Daniels *et al.* 2001; Moody *et al.* 2001). The lack of binding seen in more mature T cells is directly due to their greater sialylation in comparison to DP cells (Daniels *et al.* 2001; Moody *et al.* 2001). On T cell activation however, when the T cell becomes hyposialylated and could be thought of as re-creating the DP phenotype, or when mature naïve T cells are treated with neuraminidase, binding of non-cognate MHC is not restored (Harrington *et al.* 2000; Priatel *et al.* 2000). An important difference between the DP state and activated T cells, in spite of both being hyposialylated, is that in DP cells the glycan adducts of CD8 are core-1 O-glycans, but in activated T cells they are predominantly core-2 O-glycans (McNeill 1999). It is possible that the non-cognate recognition of MHC is specific for core-1 O-glycans and their relative absence from activated T cells could be the reason that the binding seen in thymus is not restored. Given that non-cognate binding of MHC has also been shown to be CD8β dependent (Bosselut *et al.* 2000; Moody *et al.* 2001), it is all the more interesting to determine if the change in core structure observed in activated CD8 molecules is chain specific.

There was not time to investigate if the core structure of O-glycans associated with $CD8\beta^{\alpha h}$ molecules changes from predominantly core-1 to predominantly core-2, and future experiments will address this question. Given that the appearance of core-2 O-glycans has important consequences for cell-cell adhesion (Tsuboi and Fukuda 1997), apoptosis of activated cells (Priatel *et al.* 2000) and the differentiation and survival of memory cells (Mukasa *et al.* 1999; Harrington *et al.* 2000), this will be important to assess.

Figure 5.1 - Lectin analysis of thymus lysates from CD8β^{ah} expressing mice reveals the glycan structures of CD8.

Thymocytes (3 x 10^8) from CD8β^{wt} and CD8β^{ah} expressing mice were lysed for 30 minutes at 4°C in 1% triton, the lysate split into three and each aliquot precleared with RIg Abs at 4°C for 90 minutes. Thymus lysates were then immunoprecipitated with one of biotinylated lectins PNA, ECA and SNA at 4°C overnight. PNA is specific for core-1 glycans, ECA for core-2 glycans and SNA for sialic acid. Immunoprecipitates were washed twice in 1% triton at 4°C. Total cell lysates from CD8β^{wt} mice (lane 1) and CD8β° mice (lane 2), and immunoprecipitates from PNA lectin (lanes 3 and 6), ECA lectin (lanes 4 and 7) and SNA lectin (lanes 5 and 8) were analysed by SDS PAGE and Western blotting with anti-CD8β antiserum. Molecular weight markers are shown.

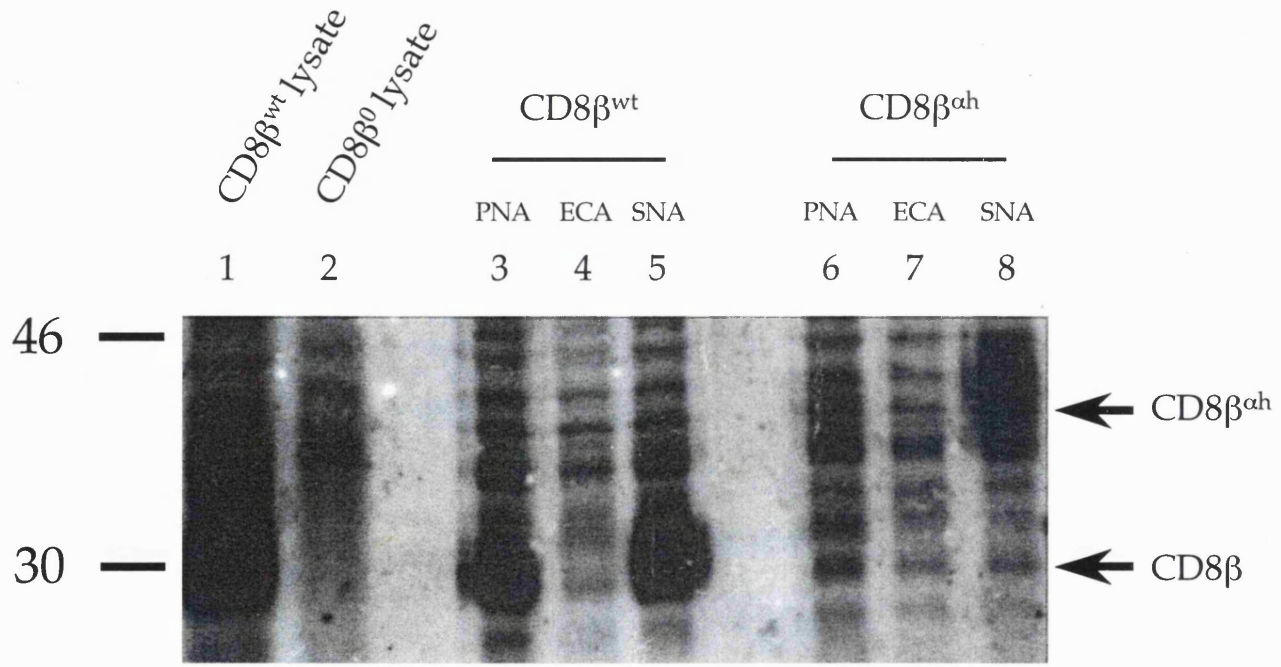
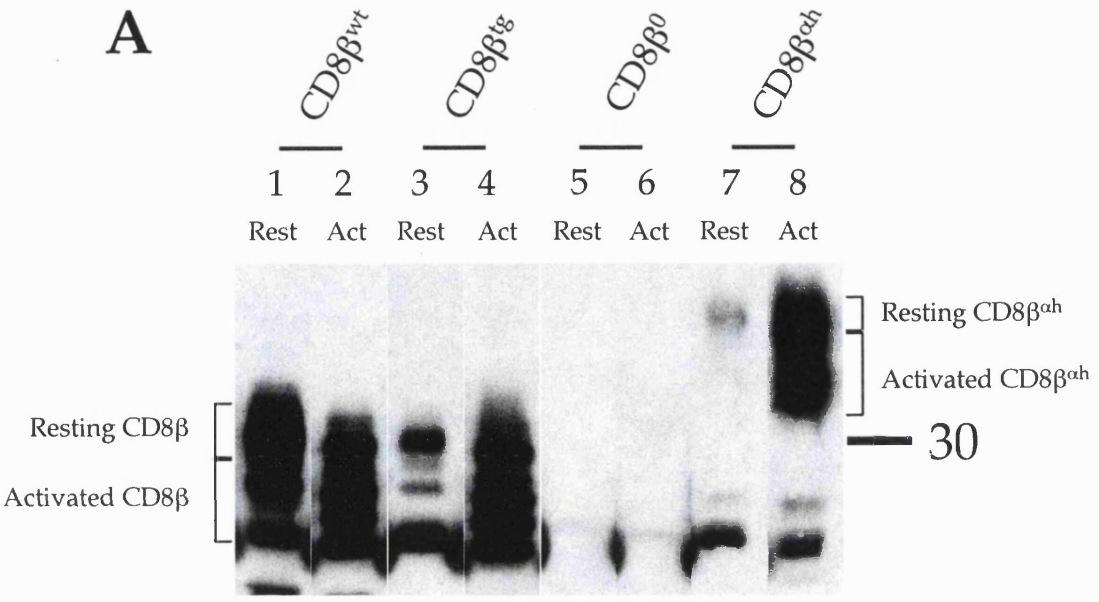

CD8β^wt lysate
CD8β^0 lysate
CD8β^wt
CD8β^αh
PNA
ECA
SNA
PNA
ECA
SNA
1
2
3
4
5
6
7
8
46
30
CD8β^αh
CD8β

Figure 5.2 - The β-chain of CD8β$^{\alpha h}$ becomes a faster migrating species in SDS gel after stimulation *in vitro* for 72 hours.

Lymphocytes from CD8β^{wt}, CD8β^{tg}, CD8β° and CD8β$^{\alpha h}$ expressing mice were incubated at 37°C with soluble NP68 peptide antigen (0.1μM) for 72 hours in full culture medium (1 x 10^6 cells/ ml). Activated lymphocytes and resting lymphocytes from each strain were lysed at 4°C in 1% triton for 30 minutes and lysates precleared with RIg Abs at 4°C for 90 minutes. Thymus lysates were immunoprecipitated for 2 hours at 4°C with either anti-CD8β mAb YTS156.7, or anti-CD8α mAb YTS169.4 in the case of lysates from CD8β° mice. Immunoprecipitates were washed twice at 4°C in 1% triton. The immunoprecipitates from CD8β^{wt} (lanes 1 and 2), CD8β^{tg} (lanes 3 and 4), CD8β° (lanes 5 and 6) and CD8β$^{\alpha h}$ (lanes 7 and 8) thymus lysates were analysed by SDS PAGE and Western blotting with anti-CD8β (A) and anti-CD8α (B) antiserum. Lanes 1, 3, 5 and 7 are resting lymphocyte immunoprecipitates, lanes 2, 4, 6 and 8 are activated lymphocyte immunoprecipitates. Molecular weight markers are shown.


A
CD8βwt
CD8βtg
CD8β0
CD8βαh
1 2 3 4 5 6 7 8
Rest Act Rest Act Rest Act Rest Act
Resting CD8β
Activated CD8β
Resting CD8βαh
Activated CD8βαh
30

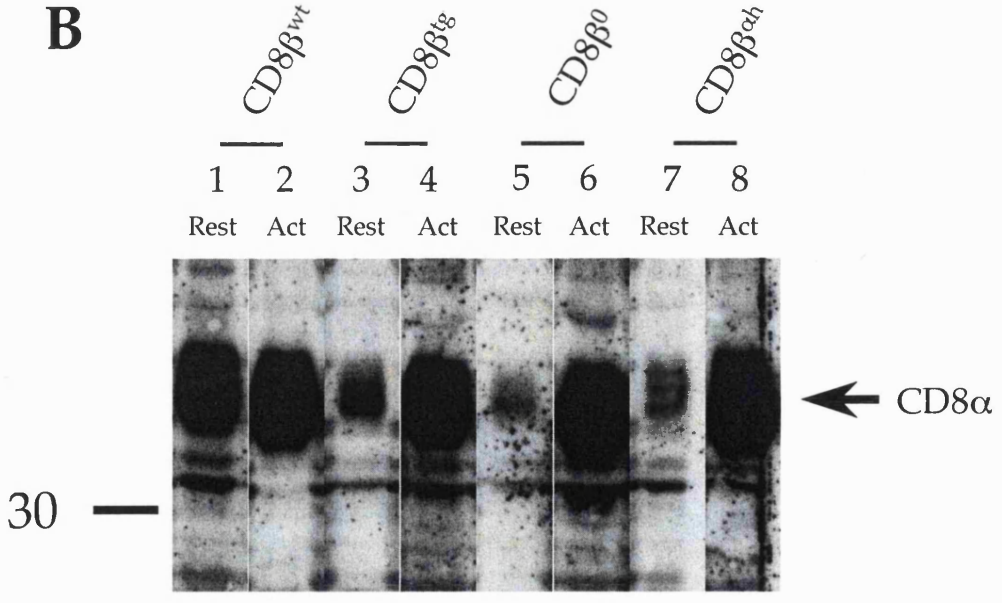

B
CD8βwt
CD8βtg
CD8β0
CD8βαh
1 2 3 4 5 6 7 8
Rest Act Rest Act Rest Act Rest Act
30
CD8α

Figure 5.3 - Biosynthetic pathways for the addition of O-glycans in T cells.

Serine and threonine residues are subject to modification by the addition of O-linked glycans. A GalNAc glycan is attached to a serine or threonine residue, and is itself further modified by the addition of a galactose residue. This structure (*) is targeted specifically by PNA (outlined in red). The PNA ligand is a substrate for two enzymes, ST3Gal-1 (1) and C2GnT (2). ST3Gal-1 will outcompete C2GnT *in vivo*, resulting in no core-2 structures, recognizable by the lectin ECA (green), on CD8 cells in the thymus. Core-2 structures are detectable in activated CD8 molecules. ST3Gal-1 attaches sialic acid in an α(2,3) linkage recognized by the lectin MAA (blue), but MAA cannot bind CD8 from thymocytes. This suggests that the MAA ligand is further modified by the addition of sialic acid in an α(2,6) linkage recognized by the lectin SNA (yellow), which is detectable in CD8 molecules in the thymus. Adapted from (Moody *et al.* 2003).

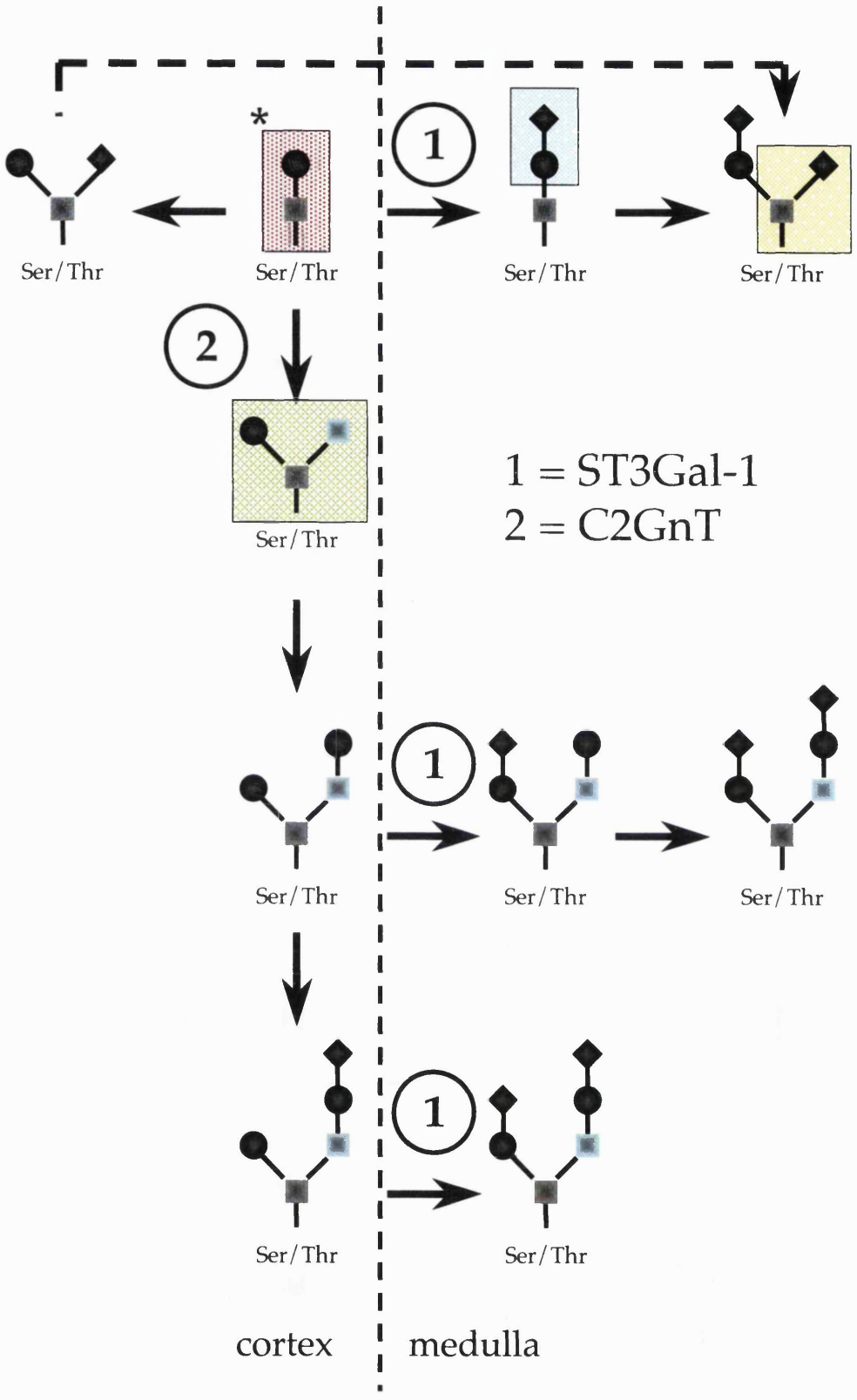
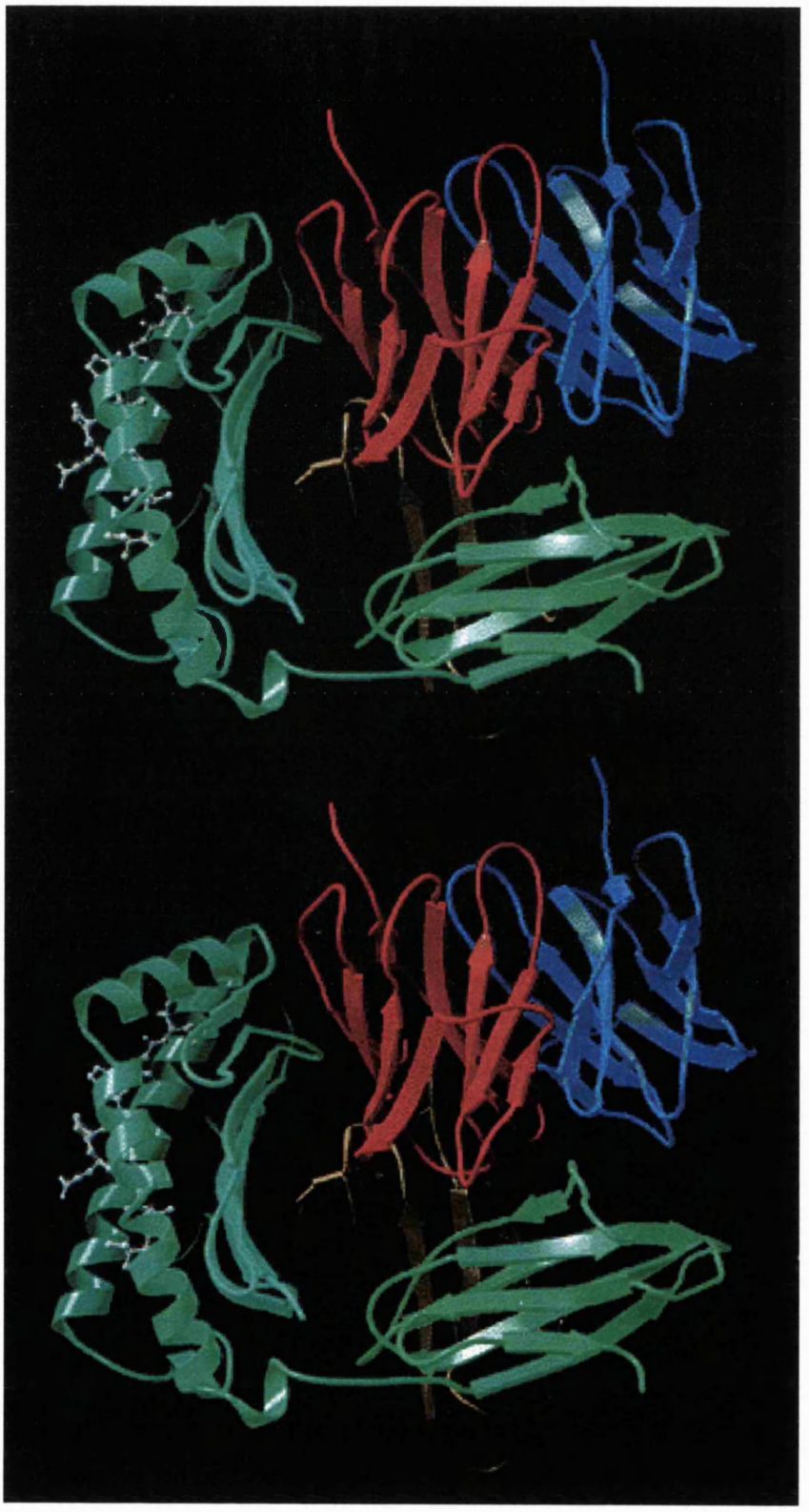

-
*
1
Ser/Thr
Ser/Thr
Ser/Thr
Ser/Thr
2
Ser/Thr
1 = ST3Gal-1
2 = C2GnT
Ser/Thr
Ser/Thr
Ser/Thr
1
Ser/Thr
Ser/Thr
cortex
medulla
sialic acid
galactose
GalNAc
GlcNAc
binds PNA
binds ECA
binds MAA
binds SNA

Figure 5.4 - CD8αα binds asymmetrically to HLA-A2/ peptide complex.

Each chain of CD8αα contributes asymmetrically to binding of HLA-A2/ peptide complex. Shown are HLA heavy chain (green), β_2m chain (gold), peptide (white), CD8α-1 chain (red) and CD8α-2 chain (blue). 73% of the surface area buried by CD8αα on complex formation is from the CD8α-1 (red) chain. CD8αβ is predicted to bind in a similarly asymmetric fashion, but it is not known which of the roles CD8β would adopt, that of the 'red' or the 'blue' chain. Stereo image, reproduced from Gao *et al*, (1997).



6. Response of peripheral cells expressing CD8β$^{\alpha h}$ to antigen.

Introduction

Data from previous chapters showed that mice expressing CD8β$^{\alpha h}$ exhibit a block in thymus maturation at the positive selection stage of development. Further investigation revealed more extensive O-linked glycosylation and sialylation on the CD8β$^{\alpha h}$ polypeptide than predicted by the primary structure, as well as reduced capability to bind cognate MHC compared to CD8αβ. Chapter 5 showed that the glycan structures found in wild type CD8 molecules could also be found in CD8β$^{\alpha h}$ molecules, and became hyposialylated upon activation of mature CD8β$^{\alpha h+}$ T cells, in an equivalent fashion to wild type CD8β hinge (Casabo *et al.* 1994). This raised the question of whether the few cells that are being selected in mice expressing CD8β$^{\alpha h}$ were functionally competent once in the periphery. In this chapter a comparison is made of the response of CD8β$^{\alpha h+}$ peripheral cells to CD8αβ^{+} peripheral cells when challenged with either self-pMHC or cognate pMHC. The signaling capability and participation of CD8 in the response of CD8β$^{\alpha h+}$ mature T cells compared to wild type CD8αβ^{+} T cells was also assessed, by blocking with CD8-specific antibodies.

Positive selection, which is impaired in mice expressing CD8β$^{\alpha h}$, relies on the recognition of self-pMHC by the DP thymocyte (Jameson *et al.* 1995). T cell survival and homeostatic proliferation also depend on TcR mediated signals after the recognition of self-pMHC (Kirberg *et al.* 1997; Tanchot *et al.* 1997; Ernst *et al.* 1999; Goldrath and Bevan 1999; Kieper and Jameson 1999; Viret *et al.* 1999), although the nature of these signals differs (Seddon *et al.* 2000). Although the efficiency of CD8 as a co-receptor has not been assessed in the context of expansion in a lymphopenic host, the interaction with self-pMHC complexes is thought to be similar to selection

conditions in the thymus (Viret *et al.* 1999), and was used to address the question of whether CD8$\beta^{\alpha h}$ is less able to recognize self-pMHC than CD8$\alpha\beta$. Homeostatic proliferation in a lymphopenic host is dependent on TcR mediated signals via the kinase LCK (Seddon and Zamoyska 2002), and so this system can also be used to ask questions of the signaling capability of CD8$\beta^{\alpha h}$ expressing lymphocytes in comparison to CD8$\alpha\beta$ expressing lymphocytes.

The block in positive selection seen in mice expressing CD8$\beta^{\alpha h}$ suggested impaired co-receptor ability of these molecules when recognizing self-pMHC, and CD8$\beta^{\alpha h}$ molecules were also demonstrated in Chapter 3 to be two-fold less able to bind cognate pMHC for the same amount of TcR on the surface of DP thymocytes. It has long been known that co-ligating CD8 with the TcR enhances T cell responses compared to ligating TcR alone (Boyce *et al.* 1988; Jonsson *et al.* 1989). Later it was shown that CD8 binds the same cognate pMHC as the TcR (Janeway 1992), augmenting the affinity of TcR for MHC as well as recruiting LCK to the signaling complex. Differences in cognate pMHC binding between CD8$\beta^{\alpha h}$ and CD8$\alpha\beta$ could have a profound effect on both co-receptor functions when mature naïve T cells expressing the different co-receptors are responding to antigen. In mice lacking CD8β, the CD8$\alpha\alpha$ co-receptors expressed on the cell surface are poor at promoting selection in the thymus and there is an 80% drop in numbers of cells being selected, yet peripheral cells expressing CD8$\alpha\alpha$ were able to mount a cytolytic response comparable to CD8$\alpha\beta$ expressing cells (Crooks and Littman 1994). This suggested that CD8$\alpha\alpha$ was inefficient as a co-receptor in the thymus when recognizing self-pMHC, but that CD8$\alpha\alpha$ expressing peripheral cells were not grossly disadvantaged when mature naïve T cells were recognizing cognate pMHC, and this could be true for the CD8$\beta^{\alpha h}$ co-receptor also.

Antibody blocking of TcR mediated signals by the use of CD8-specific antibodies acts in two ways, by preventing the CD8 molecule binding MHC and also by generating an inhibitory or negative signal (Janeway 1992). Some specificities of TcR have been

shown to be less sensitive to the effects of CD8-specific antibodies and are thought of as co-receptor independent. This is probably related to the TcR affinity for antigen since expressing TcR without co-receptor in hybridomas or T cells clones showed that co-receptor independent TcR expressing cells could generally respond to antigen, while co-receptor dependent TcRs expressing cells could not (Kwan-Lim *et al.* 1993; Miceli and Parnes 1993). However the dependence on co-receptor or otherwise is also related to the efficiency of signal transduction, with co-receptor independent hybridomas being more sensitive to stimulation by CD3-specific antibodies than co-receptor dependent cells (Kwan-Lim *et al.* 1993). Data from Chapter 4 showed that when antibodies bound to TcR and CD8$\beta^{\alpha h}$ were crosslinked in DP thymocytes, comparable Ca^{++} mobilization was observed to that seen with CD8$\alpha\beta$ molecules. LCK activation is absolutely required for a Ca^{++} flux to occur (Trobridge and Levin 2001), demonstrating that signal transduction in DP thymocytes expressing CD8$\beta^{\alpha h}$ was not inherently inefficient compared to DP thymocytes expressing CD8$\alpha\beta$. Differences in sensitivity to CD8-specific antibodies between different CD8 co-receptors could however be indicative of differences in the efficiency of signal transduction (Kwan-Lim *et al.* 1993), and so the signaling capacity of CD8$\beta^{\alpha h}$ expressing peripheral cells was also assessed by comparing Ca^{++} flux with mature T cells expressing CD8$\alpha\beta$. Ca^{++} flux after T cell activation has been shown in certain systems to be CD8 dependent (Daniels and Jameson 2000). Furthermore, it is the cytoplasmic and transmembrane domains of CD8β that confer increased Ca^{++} mobilization efficiency, possibly as a result of the lipid raft association that these domains confer (Arcaro *et al.* 2001). Both of these domains are intact in CD8$\beta^{\alpha h}$ and so any differences in signaling efficiency are more likely to be attributable to the altered glycosylation seen in the hinge region of the CD8$\beta^{\alpha h}$ polypeptide.

6.1 In spleen, mice expressing CD8$\beta^{\alpha h}$ show reduced numbers of CD8$^+$ T cells, decreased levels of CD8β and increased levels of TcR.

Data from Chapter 3 showed that there were fewer cells being selected in the thymus of CD8$\beta^{\alpha h}$ expressing mice in comparison to CD8β^{wt} expressing mice, as measured by the percentage of SP cells. Also, SP thymocytes from mice expressing CD8$\beta^{\alpha h}$ had slightly elevated expression levels of TcR but normal levels of CD8β in comparison to CD8β^{wt} expressing controls. The first question to ask of peripheral T cells was whether the phenotypic traits of CD8$\beta^{\alpha h}$ expressing cells were similar to those encountered in the thymus, as phenotypic differences in cell surface markers associated with T cell activation could account for any variability in peripheral CD8$\beta^{\alpha h+}$ T cell response to antigen in comparison to CD8β^{wt} controls.

Spleen cells from mice expressing CD8β^{wt}, CD8β^{tg}, CD8β° or CD8$\beta^{\alpha h}$ were stained using antibodies specific for CD4, CD8α, TcR and CD8β. Mice lacking CD8β have 80% reduced numbers of $CD8^+$ cells in the periphery, all of which are CD8αα (figure 6.1, panel B and figure 6.2, column 2 and (Crooks and Littman 1994)). This defect is largely restored in mice that are expressing CD8β^{tg}, as can be seen in figure 6.1, panel C and figure 6.2, column 3. In mice transgenic for CD8$\beta^{\alpha h}$ however this defect is not restored and these mice also have approximately 80% reduced numbers of $CD8^+$ cells in the periphery. This is seen in figure 6.1, panel D and figure 6.2, column 4.

When analysed for the presence of CD8β, (figure 6.1) there is no binding of antibody to CD8β° cells confirming there is no expression of CD8β in peripheral T cells (figure 6.1, panel B). Levels of expression of CD8β in mice positive for transgenic CD8$\beta^{\alpha h}$ (figure 6.1, panel D) are approximately 75% of those seen in CD8β^{wt} expressing mice (figure 6.1, panel A). Expression of CD8β in mice expressing CD8β^{tg} (figure 6.1, panel C) is lower than either CD8β^{wt} or CD8$\beta^{\alpha h}$ expressing cells at approximately 50% of CD8β^{wt} levels, consistent with data from the thymus. The slightly reduced levels of cell surface CD8β in CD8$\beta^{\alpha h}$ expressing cells compared to CD8β^{wt} expressing cells could be important for peripheral T cell response. However, any effect that is directly attributable to the slightly reduced levels of co-receptor on the cell surface in CD8$\beta^{\alpha h}$

expressing cells in comparison to CD8β^{wt} cells will be more pronounced in CD8β^{tg} expressing cells, which express CD8β at approximately 50% of CD8β^{wt} levels.

TcR levels on CD8$^+$ cells are all approximately 30% higher in CD8β^{tg}, CD8β$^\circ$ and CD8β$^{\alpha h}$ expressing mice than in CD8β^{wt} mice, possibly due to the higher expression levels of TcR seen in selecting DP thymocytes. Given that DP thymocytes that fail positive selection are arrested at the TcR^{dull} stage (Guidos *et al.* 1990; Petrie *et al.* 1990), it is reasonable to suppose that TcR^{int} DP cells are those that will be positively selected and a proportion exported to the periphery. In CD8β^{wt} expressing mice there are more selecting DP cells, with a lower average TcR expression level in comparison to other strains tested (Chapter 3, section 3.3). In CD8β^{tg}, CD8β$^\circ$ and CD8β$^{\alpha h}$ expressing mice the population is more discrete and tends towards higher expression level of TcR, which is also reflected in the periphery. TcR is essential for T cell response to antigen so it is important to know that any differences in this response can be directly attributed to the contribution of the co-receptor, and not to differing levels of TcR on the cell surface. Differences between TcR levels on CD8β^{wt} and CD8β$^{\alpha h}$ expressing cells could possibly account for differential response to antigen, but the CD8β^{tg} expressing control provides a direct comparison for CD8β$^{\alpha h}$ expressing cells as levels of TcR expression are equivalent.

6.2 In lymph nodes, mice expressing CD8β$^{\alpha h}$ show reduced percentage of cells, slightly reduced levels of CD8β and slightly increased levels of TcR.

When examining the response of peripheral cells expressing CD8β$^{\alpha h}$ to cognate antigen, lymph node cells were used in all experiments due to ease of purification and higher percentage of CD8$^+$ cells in comparison to splenocytes. Therefore, it was necessary to examine cellularity, CD8β and TcR expression level on lymphocytes from lymph nodes as differences in these cell surface markers between strains could have important effects on T cell response to antigen.

Lymph node cells from mice expressing CD8β^{wt}, CD8β^{tg}, CD8β° and CD8β^{αh} were isolated and stained with antibodies specific for CD4, CD8α, TcR and CD8β. In a polyclonal situation, approximately 15% of lymph node cells are CD8$^+$. In mice lacking CD8β this drops to approximately 5%. In a polyclonal situation, CD8β° mice that express the CD8β^{αh} transgene also have only 5% CD8$^+$ cells in lymph nodes, the same percentage as mice lacking CD8β altogether (McNeill 1999).

When crossed onto the monoclonal background expressing the MHC Class I restricted F5 TcR, and with RAG genes inactivated to prevent gene rearrangement (CD8β^{wt}), the percentage of CD8$^+$ T cells in the lymph nodes of these animals is approximately 90% (figure 6.3, panel A and figure 6.4, column 1). On the same background but in mice lacking CD8β (CD8β°), the percentage of CD8$^+$ T cells in lymph nodes drops to approximately 65% (figure 6.3 panel B and figure 6.4 column 2). In mice expressing CD8β^{tg} on an F5, *Cd8β*$^{-/-}$ background, the percentage of CD8$^+$ T cells in lymph nodes is largely restored to CD8β^{wt} levels, at approximately 90% (figure 6.3 panel C and figure 6.4 column 3). However in cells expressing CD8β^{αh} on an F5, *Cd8β*$^{-/-}$ background, frequency is not restored and the percentage of CD8$^+$ T cells in lymph nodes is the same as that of mice lacking CD8β, at approximately 65% (figure 6.3 panel D and figure 6.4 column 4). Furthermore, the number of cells recovered from lymph nodes mirrors the absolute reduction seen in spleen with approximately 4-6 x 10^6 cells recovered from CD8β° and CD8β^{αh} mice compared to approximately 8-9 x 10^6 from CD8β^{wt} and CD8β^{tg} mice.

There is no binding of CD8β specific antibodies to CD8β° cells, confirming the lack of protein expressed on the cell surface in these animals (figure 6.3, panel B). Expression of CD8β in mice expressing CD8β^{αh} (figure 6.3, panel D) is slightly reduced in comparison to CD8β^{wt} levels (figure 6.3, panel A). In mice expressing CD8β^{tg} there is a two-fold decrease in expression level of CD8β (figure 6.3, panel C) in comparison to

CD8β^{wt} expressing mice. This pattern of expression closely follows that of SP thymocytes, however there seems to be a slightly lower level of CD8β expression in lymphocytes from CD8β$^{\alpha h}$ mice in comparison to CD8β^{wt} expressing mice.

CD8β^{tg} mice were created as a control, not only to account for any differences that can be directly attributed to the expression of the CD8 protein as a transgene but also as an expression level control. If any change in response to antigen by cells expressing CD8β$^{\alpha h}$ were to be attributed to the slightly lower levels of CD8β expressed on the cell surface, this change in response should be more exagerrated in mice expressing CD8β^{tg}, which have lower surface expression of CD8β than do mice expressing CD8β$^{\alpha h}$. As seen in the spleen, levels of TcR in lymph nodes of mice expressing CD8β^{tg} (figure 6.3, panel C), CD8β$^{\circ}$ (panel B) and CD8β$^{\alpha h}$ (panel D) are approximately 30% higher than those on CD8β^{wt} mice (figure 6.3, panel A).

6.3 Lymphocytes expressing CD8β$^{\alpha h}$ undergo homeostatic proliferation in lymphopenic hosts.

In order to see if naïve T cells expressing CD8β$^{\alpha h}$ were capable of recognizing MHC, experiments were designed asking questions about recognition of self-pMHC in a lymphopenic environment, which is close to recreating selection conditions in the thymus. In a lymphopenic environment, lymphocytes will expand to fill the available space (Ernst *et al.* 1999). This process is reliant on TcR mediated signals which are received on engagement with self-peptide bound to MHC (Goldrath and Bevan 1999; Kieper and Jameson 1999) and the cytokine IL-7 (Schluns *et al.* 2000; Tan *et al.* 2001).

Lymphocytes from mice expressing CD8β^{wt}, CD8β^{tg}, CD8β$^{\circ}$ and CD8β$^{\alpha h}$ were isolated and labeled using CFSE dye. Excess dye was washed away and equivalent numbers of cells transferred to lymphopenic hosts, *Rag*$^{-/-}$ animals. Two weeks later, lymphocytes from the host animals were isolated and stained with antibodies specific for TcR, CD8α

and CD44, to follow expansion specifically in the naïve population (figure 6.5, panel A). The average number of divisions of the T cell population from each donor mouse was calculated according to the formula in *materials and methods* and is shown in figure 6.5, panel B.

Figure 6.5, panel B shows that expansion of donor cells in a lymphopenic host reflects the efficiency of selection seen in the thymus for the different strains. Lymphocytes expressing CD8β^{wt} (panel B, column 1) go through more divisions than any other donor strain. Cells expressing CD8β^{tg} (panel B, column 3) go through slightly fewer divisions than CD8β^{wt}. In CD8β° and CD8$\beta^{\alpha h}$ expressing donor cells (panel B, columns 2 and 4), the cells go through approximately half as many divisions as those expressing CD8β^{wt}. The pattern of behaviour in cells expressing CD8$\beta^{\alpha h}$ more closely resembles that of CD8β° cells than CD8β^{tg} cells. These data show that, like selection in thymus, CD8$\alpha\alpha$ and CD8$\beta^{\alpha h}$ expressing cells can recognise self-pMHC, but respond less efficiently than cells expressing CD8$\alpha\beta$ co-receptors.

6.4 Lymphocytes expressing CD8$\beta^{\alpha h}$ bind cognate MHC dimers with reduced affinity in comparison to lymphocytes expressing CD8$\alpha\beta$.

In view of the reduced response to self-pMHC from peripheral CD8$\beta^{\alpha h}$ expressing cells, the ability of these cells to bind cognate pMHC dimers was analysed. Bivalent MHC/ Ig recombinant protein was incubated with NP68 peptide overnight to load the MHC molecules. Freshly isolated lymphocytes from mice expressing CD8β^{wt}, CD8β^{tg}, CD8β° and CD8$\beta^{\alpha h}$ were incubated with the recombinant, peptide loaded MHC (pMHC), washed and resuspended in fluorescently labeled antibodies specific for the recombinant protein, together with anti-CD8α and anti-CD4. Lymphocytes from wild type B10 mice and F5/ *Cd8*$\alpha^{-/-}$ mice were stained and included as controls.

Figure 6.6, panel A shows that there is no binding of pMHC to either B10 lymphocytes which express CD8 but are polyclonal (shaded histogram) or lymphocytes bearing the F5 TcR but lacking CD8 (grey line). This shows that the binding of the NP68 peptide complexed with D^b MHC is dependent on the presence of both the F5 TcR and CD8 on the cell surface, and is consistent with results from the thymus (chapter 3, section 3.6).

Figure 6.6, panel B shows the binding of pMHC to lymphocytes from mice expressing $CD8\beta^{wt}$ (shaded histogram), $CD8\beta^{tg}$ (grey line), $CD8\beta^{\circ}$ (dotted line) and $CD8\beta^{\alpha h}$ (black line). All strains are able to bind the pMHC at high affinity in comparison to DP thymocytes (chapter 3, section 3.6). Nevertheless, and consistent with data from the thymus (chapter 3, section 3.6), there are differences in MHC binding when normalized for the same amount of TcR expressed on the cell surface (table 6.1).

Table 6.1 – binding of MHC to lymphocytes

Strain	Ratio of MHC:TcR binding
$CD8\beta^{wt}$	0.39
$CD8\beta^{\circ}$	0.18
$CD8\beta^{tg}$	0.31
$CD8\beta^{\alpha h}$	0.21

Binding of MHC per TcR is slightly lower in lymphocytes expressing $CD8\beta^{tg}$ compared with $CD8\beta^{wt}$, in spite of the mean fluorescence intensity being equivalent. This is probably due to the higher levels of TcR expression seen in $CD8\beta^{tg}$ mice, as discussed in section 6.2. In lymphocytes expressing $CD8\beta^{\alpha h}$ or $CD8\beta^{\circ}$, there is consistently two-fold lower binding of pMHC (table 6.1). Again these results are consistent with data from the thymus showing a defect in MHC binding in both of these strains (chapter 3, section 3.6).

6.5 Lymphocytes expressing CD8β$^{\alpha h}$ respond to NP68 peptide *in vitro* differently to wild type controls.

Even though selection in the thymi of mice expressing CD8β$^{\alpha h}$ molecules is severely blocked, some CD8^{+} cells do exit the thymus into the periphery. Knowing how these cells respond to peptide in comparison to CD8β^{wt} controls is essential in determining the role of the hinge region in CD8β when responding to antigen. Therefore, proliferation of lymph node cells was analysed. Equal numbers of lymphocytes from CD8β^{wt}, CD8β^{tg}, CD8β$^{\circ}$ and CD8β$^{\alpha h}$ mice were labeled with CFSE dye and cultured *in vitro* with NP68. After 72 hours cells that were labeled with CFSE were analysed by flow cytometry. Proliferation was analysed at both 48 and 72 hours, and the data from 72 hours is shown as this was the optimal time. FACS profiles showing the CFSE content of the population at peptide concentration 10^{-4}nM are shown in figure 6.7, panel A. In order to present an accurate representation of the response to peptide the data are calculated as the average number of divisions of the starting population as described in *materials and methods*.

Figure 6.7, panel B shows that CD8β^{wt} (red line) and CD8β^{tg} (green line) control have a very similar dose response to peptide, in spite of CD8β^{tg} cells having 50% lower CD8β and 30% higher TcR on the cell surface in comparison to CD8β^{wt} expressing cells. In contrast, CD8β$^{\circ}$ (blue line) and CD8β$^{\alpha h}$ (gold line) cells, the two mouse strains with reduced numbers of CD8 cells in periphery are less responsive to peptide at lower concentrations as indicated by a shift in the dose response curve. At higher peptide concentrations cells expressing CD8β$^{\circ}$ and CD8β$^{\alpha h}$ reach higher numbers of absolute divisions than the positive controls. Both strains appear to reach a plateau by the highest peptide concentration considered, in contrast to CD8αβ expressing cells which still show a dose response at higher concentrations.

It appears that cells from CD8β° and CD8β$^{\alpha h}$ mice have a higher activation threshold than those from CD8β^{wt} and CD8β^{tg} control mice. The activation threshold for CD8β° mice seems higher than all other strains in the analysis, as seen from the FACS profiles (panel A), showing a distinct population of undivided cells compared to all cells going through division in all other strains at a peptide concentration of 10^{-4}nM. The activation threshold for cells expressing CD8β$^{\alpha h}$ is between that of CD8β° cells and those expressing CD8αβ. At peptide concentration 10^{-3}nM, all cells from CD8β° and CD8β$^{\alpha h}$ mice have gone into division (data not shown). Curiously, once triggered into division, cells lacking wild type CD8αβ may be more responsive that their wild type counterparts, and this is reflected in the increased burst size of CD8β$^{\alpha h}$ proliferative cells, suggesting that wild type CD8αβ molecules may also have a role in moderating an ongoing immune response. In order to investigate this, the influence of CD8-specific antibodies in inhibiting the response of these cells was analysed.

6.6 The response of lymphocytes expressing CD8β$^{\alpha h}$ to NP68 peptide *in vitro* can be partially blocked by antibodies specific for CD8α and CD8β.

The experiments showing that lymphocytes expressing CD8β$^{\alpha h}$ are able to respond to cognate peptide *in vitro* are not able to answer the question of the extent of CD8β$^{\alpha h}$ co-receptor involvement in this response, so to address this question experiments were devised taking advantage of the action of anti-CD8 specific antibodies. Antibodies to CD8 are able to block the response of CD8$^+$ T cells in two ways, by hindering CD8 binding to MHC, which is known to be impaired in cells expressing CD8β$^{\alpha h}$, and by blocking downstream proliferative signaling from the TcR complex (Janeway 1992). In order to address whether the response of cells expressing CD8β$^{\alpha h}$ could be blocked by antibodies specific for CD8α or CD8β, cells from mice were cultured with peptide as described in section 6.5 in the presence or absence of anti-CD8α or anti-CD8β antibodies.

From the results in figure 6.8 it can be seen that in CD8β^{wt} mice (panel A) the response to peptide can be blocked equally efficiently by either CD8α- or CD8β-specific antibodies. Both antibodies produce approximately a 2-log decrease in activation for the same peptide concentration. In CD8β^{tg} mice (panel C), the response without antibody is similar to that of CD8β^{wt} mice, and the level of blocking achieved by anti-CD8α antibodies is in the same order of magnitude. However, blocking with CD8β-specific antibodies is considerably less efficient than in CD8β^{wt} controls, producing only a 10x reduction in response. This is probably due to the lower expression of CD8β in these animals, resulting in the presence of many more CD8αα homodimers on the cell surface. In CD8β° mice (panel B), the anti-CD8β antibody has no effect on response in comparison to conditions with no antibody blocking. This is to be expected since CD8β° mice do not express CD8β; nevertheless it provides a useful negative control and demonstrates that anti-CD8β antibody does not have a non specific anti-proliferative effect. Surprisingly, the anti-CD8α has little effect on the proliferation, also suggesting that CD8αα participation in the response is not equivalent between these strains. In CD8β^{αh} expressing mice (panel D) the pattern of blocking is between that of CD8β^{tg} and CD8β° mice. Blocking with anti-CD8β antibodies produces a 7x reduction in response, and there is a similar level of reduction in response with CD8α-specific antibodies. It is striking that the decreased response with anti-CD8α antibodies is similar to that of anti-CD8β, in contrast to the CD8β^{tg} control cells where blocking with anti-CD8α produces a 50-fold reduction in response. This result suggests that cells expressing CD8β^{αh} are relying less on the co-receptor to stabilize the TcR/ MHC interaction compared to CD8αβ expressing cells, but to a greater extent than cells expressing CD8αα. This indicates the importance of the hinge region of CD8β for the correct functioning of CD8αβ molecules in T cell activation,

6.7 The Ca^{++} flux in response to antibody crosslinking is impaired in lymphocytes expressing $CD8\beta^{\alpha h}$.

Data in Chapter 4, section 4.4 showed that when TcR was crosslinked with CD8α or CD8β using biotinylated antibodies, thymocytes expressing $CD8\beta^{\alpha h}$ were able to respond in a comparable way to CD8αβ expressing controls when using Ca^{++} mobilization as a measure of activity. Data from this chapter however showed that lymphocytes expressing $CD8\beta^{\alpha h}$ responded less well to antigenic stimulation and were inhibited less by CD8 antibodies than CD8αβ expressing lymphocytes. In order to compare further the signaling processes of lymphocytes expressing $CD8\beta^{\alpha h}$ with those expressing wild type CD8αβ, Ca^{++} mobilization on antibody crosslinking was used as a marker for LCK-mediated signaling (Trobridge and Levin 2001).

Lymphocytes from mice expressing $CD8\beta^{wt}$, $CD8\beta^{tg}$, CD8β° and $CD8\beta^{\alpha h}$ were isolated at room temperature and incubated in the dark with Indo-1 AM. Cells were washed and resuspended in PBS containing antibodies specific for varying combinations of TcR and CD8 specific biotinylated antibodies at room temperature for 30 minutes. After washing, cells were run on an LSR and a ratio established for resting to activated Indo-1 AM in unstimulated, live cells. After one minute streptavidin was added in order to cross link the biotinylated antibodies and the cells returned to the LSR for analysis.

Figure 6.9, panel A shows the result when TcR is crosslinked alone. In contrast to the results seen with DP thymocytes, cells expressing $CD8\beta^{\alpha h}$ (gold line) and CD8αα (blue line) show a severely impaired response when compared to cells expressing CD8αβ (red line and green line). In lymphocytes expressing $CD8\beta^{wt}$ (red line), there is a delayed and reduced response to TcR crosslinking in comparison to cells expressing $CD8\beta^{tg}$ (green line). These data are representative of only one experiment, and it is possible that this is an anomalous result, given that the proliferative response of

$CD8\beta^{wt}$ is comparable with $CD8\beta^{tg}$ expressing lymphocytes. However there are higher amounts of TcR in peripheral cells from $CD8\beta^{tg}$ mice compared to $CD8\beta^{wt}$ mice, so it is also possible that it is a genuine result. This can be addressed by repetition of the experiment. Cross-linking both $CD8\beta^{wt}$ and $CD8\beta^{tg}$ expressing cells with TcR-specific antibodies and either anti-CD8α or anti-CD8β gives equivalent and stronger Ca^{++} responses than cross-linking TcR alone (figure 6.9, panels B and C), in spite of the lower levels of expression of CD8β on the surface of cells from $CD8\beta^{tg}$ mice. However there is a drastically reduced response in CD8β° (blue line) and $CD8\beta^{\alpha h}$ (gold line) expressing lymphocytes when CD8α and TcR are crosslinked (figure 6.9, panel B).

Figure 6.9, panel C shows the result when TcR is crosslinked with CD8β. In the case of CD8β° animals (blue line), since no CD8β is expressed on the cell surface, this gives the same result as crosslinking the TcR alone (panel A). Consistent with the results from DP thymocytes (chapter 4, section 4.4), the response to crosslinking as measured by Ca^{++} mobilization is comparable whether the TcR is crosslinked with CD8α or CD8β. Again, the response in $CD8\beta^{wt}$ cells is equitable with that of $CD8\beta^{tg}$ cells. Again, there is a drastically reduced response in CD8β° and $CD8\beta^{\alpha h}$ cells, which show a similar response pattern.

Discussion

In this chapter a closer analysis of the response of peripheral lymphocytes expressing the $CD8\beta^{\alpha h}$ molecule to cognate stimuli as well as the ability to proliferate in a lymphopenic environment was undertaken to address the question of how the mutated hinge region was affecting the interaction of the T cell with APC, and if the impairment seen in selection in mice expressing the mutated transgene would have any bearing on cells that had been exported to the periphery.

The first point addressed was the phenotypic analysis of TcR and CD8 expression on peripheral cells, as both of these molecules contribute to T cell/ APC interaction (Janeway 1992; Davis *et al.* 1998; Garcia *et al.* 1999). These data were broadly consistent with data from the thymus, showing that the TcR level in $CD8\beta^{tg}$, $CD8\beta^{\circ}$ and $CD8\beta^{\alpha h}$ expressing mice was approximately 30% higher than $CD8\beta^{wt}$ expressing mice. This increase in TcR expression in comparison to $CD8\beta^{wt}$ is probably as a result of the altered selection in the thymus due to lower expression of CD8β. As seen in thymocytes, levels of CD8β in peripheral T cells from $CD8\beta^{tg}$ expressing mice were approximately 50% of $CD8\beta^{wt}$ expressing mice, with levels in $CD8\beta^{\alpha h}$ mice approximately 90% of $CD8\beta^{wt}$ levels. Given that the CD8 co-receptor is required to bind the same MHC as the TcR (Janeway 1992), any reduction in CD8 level on the cell surface could affect this interaction. However given that the control $CD8\beta^{tg}$ cells had both lower CD8β than $CD8\beta^{\alpha h}$ expressing cells and similar levels of TcR, any effects due to changes in expression of these molecules should be more exaggerated in $CD8\beta^{tg}$ expressing mice, and this was not the case.

The ability of naïve T cells expressing $CD8\beta^{\alpha h}$ to recognize self-pMHC was addressed by transferring peripheral cells expressing $CD8\beta^{\alpha h}$ to lymphopenic host animals and analysing proliferation. This process relies on the recognition of self-pMHC, and is thought to be similar to the recognition step needed to progress through selection in the thymus (Ernst *et al.* 1999; Goldrath and Bevan 1999; Kieper and Jameson 1999; Viret *et al.* 1999). Data gathered after transferring lymphocytes bearing the different CD8 co-receptors seemed to show dependence on functional CD8β for efficient expansion in a T cell deficient host. Cells from $CD8\beta^{\circ}$ mice divide half as much as cells from $CD8\beta^{wt}$ mice. An intermediate number of divisions was observed by $CD8\beta^{tg}$ expressing cells, which have less CD8β on the cell surface and this correlates well with the efficiency of selection seen in the thymus. Transfer of $CD8\beta^{\alpha h}$ or $CD8\beta^{\circ}$ lymphocytes to a lymphopenic host showed equivalent expansion which was approximately half that of lymphocytes from control $CD8\beta^{wt}$ mice. These data show that expression of wild type

CD8αβ molecules enhances expansion of peripheral T cells in response to lymphopenia and furthermore suggest that the CD8β$^{\alpha h}$ molecule is non-functional in this context.

Given that there are two signals that drive proliferation of naïve T cells to lymphopenia: one mediated through the TcR and the second driven by cytokine IL-7 (Seddon and Zamoyska 2002), it is not possible to assess directly the contribution of CD8αα molecules in this experiment. If CD8αα molecules do assist in recognition of MHC for homeostatic expansion, but in a sub-optimal way, it appears that CD8β$^{\alpha h}$ molecules contribute no more than a CD8αα homodimer, since the pattern of behaviour is very similar when cells of both strains are transferred to a lymphopenic host. It may be possible to assess the contribution of the co-receptor directly by transferring cells to *IL-7*$^{-/-}$ *Rag*$^{-/-}$ recipients where the only signal for homeostatic expansion is via the TcR/ co-receptor combination.

Interestingly, the lymph nodes of CD8β° and CD8β$^{\alpha h}$ expressing mice are smaller and have a lower percentage of CD8^{+} T cells than those from CD8β^{wt} or CD8β^{tg} expressing mice. The prolonged survival of naïve T cells also requires TcR interaction with self-MHC ligands (Kirberg *et al.* 1997; Tanchot *et al.* 1997; Viret *et al.* 1999), although the TcR-mediated signals necessary for survival differ to those needed for homeostatic proliferation (Seddon *et al.* 2000). Nevertheless, the defect seen in homeostatic proliferation could indicate an impairment in recognizing self-MHC ligands, and could be negatively affecting the survival of CD8β° and CD8β$^{\alpha h}$ expressing naïve T cells. The impairment in recognition of self-pMHC suggested by the decreased expansion in a lymphopenic host in lymphocytes expressing CD8β$^{\alpha h}$ could be as a result of the increased glycosylation of CD8β$^{\alpha h}$ in comparison to CD8αβ. The extra bulk of core-2 O-glycans in comparison to core-1 O-glycans makes the T cell/ APC interaction inefficient (Tsuboi and Fukuda 2001). It is possible that the extra bulk of the glycans of CD8β$^{\alpha h}$ in comparison to the glycans of CD8αβ is having the same effect, limiting the interactions needed for T cell survival and expansion, as well as homeostatic

proliferation. Homeostatic proliferation in a lymphopenic host is also dependent on signals transmitted via LCK (Seddon *et al.* 2000). These data indicate that LCK mediated signals are being transduced in lymphocytes from CD8β° and CD8β^{ah} expressing mice.

Consistent with data from the thymus was the finding that cells expressing CD8αα and CD8β^{ah} co-receptors were two-fold less efficient at binding antigenic pMHC complexes for the same amount of TcR on the cell surface. TcR/ ligand binding has been shown in many reports to be substantially strengthened by CD8αβ but not CD8αα (Luescher *et al.* 1994; Luescher *et al.* 1995a; Luescher *et al.* 1995b; Renard *et al.* 1996), which may account for this result. The importance of CD8 to be both cell bound and glycosylated for optimal co-receptor function is demonstrated by other studies showing that soluble recombinant, unglycosylated CD8αα and CD8αβ molecules bind MHC with similar affinities (Garcia *et al.* 1996; Kern *et al.* 1999). CD8-mediated increase in binding of TcR to MHC can be attributed to the transmembrane and/ or cytoplasmic regions of CD8β (Arcaro *et al.* 2001), both of which are the same as wild type CD8β in mice expressing CD8β^{ah}. This suggests the reason for the reduced ability of CD8β^{ah} molecules to assist in binding of T cells to MHC lies in the altered glycosylation of these molecules in comparison to CD8αβ. Experiments comparing the binding of soluble recombinant CD8αβ and CD8β^{ah} would help clarify this hypothesis.

The data reported so far all suggest that the CD8αβ^{ah} heterodimer behaves no differently to a CD8αα homodimer for recognition of MHC *in vivo*, and yet the CD8αβ^{ah} heterodimer becomes hyposialylated in the same way as wild type CD8αβ molecules when lymphocytes expressing the CD8β^{ah} co-receptor are activated. Furthermore, data from this chapter showed that lymphocytes expressing CD8β^{ah} were able to proliferate *in vitro* when challenged with cognate antigen.

Importantly, in the thymus, the strength of agonist encountered by the cell during selection is not the same as that which activates a naïve T cell in the periphery. In the thymus, the cell must recognize self-peptide bound to self-MHC with intermediate affinity so that it is not deleted (Hogquist *et al.* 1997). Expression of functional CD8β is clearly important for selection in the thymus, which is equivalent to a response to antagonist, but in periphery where the cell encounters a strong agonist the cell may or may not require CD8 co-receptor help to initiate a response. This question can be addressed by analyzing the response of lymphocytes expressing $CD8\beta^{\alpha h}$ molecules to a range of agonist and antagonist peptides. These different ligands may differentiate the requirement for co-receptor help more readily than the response to a strong agonist.

Interestingly, the reduced expression level of CD8β in $CD8\beta^{tg}$ mice compared to $CD8\beta^{wt}$ mice does not affect the dose response to peptide and the response is comparable at each peptide concentration between these two strains. It is known that just a few pMHC complexes on a DC can be sufficient to trigger T cells (Valitutti *et al.* 1995; Sykulev *et al.* 1996), and it is likely that the lower levels of CD8β expressed on $CD8\beta^{tg}$ mice are nonetheless sufficient to stabilize this small number of TcR/ pMHC interactions in a comparable way to $CD8\beta^{wt}$ mice. Lymphocytes from $CD8\beta^{\circ}$ and $CD8\beta^{\alpha h}$ mice show a different dose response to peptide, and are less responsive than lymphocytes from $CD8\beta^{wt}$ and $CD8\beta^{tg}$ at lower peptide concentrations but go through more absolute divisions at higher peptide concentrations. It is likely that the lack of response at low peptide concentrations in lymphocytes expressing either CD8αα or $CD8\beta^{\alpha h}$ is due to the impaired capability of these two co-receptors to bind cognate pMHC in comparison to CD8αβ expressing lymphocytes. At higher peptide concentrations however there will be more TcR/ pMHC interactions taking place and a greater likelihood of signal transduction, even with inefficient co-receptor help. This evidence seems to suggest that cells with impaired co-receptor function in the thymus are not grossly disadvantaged once they have made it through selection and into the periphery.

It is also possible that CD8β is delivering a regulatory signal to limit the degree of activation, which would account for the increased division number at high peptide concentration in mice lacking CD8β and mice expressing CD8β$^{\alpha h}$. If wild type CD8β is delivering a regulatory signal, this could also help explain the block in selection in the thymus. In CD8β$^{\circ}$ and CD8β$^{\alpha h}$ expressing mice, the lack of wild type CD8β and the associated negative signal could cause an overtly strong signal which in turn causes the cell to apoptose. In effect, the lack of functional CD8β unbalances the delicate equilibrium that allowed normal selection.

Furthermore, there is evidence that the F5 TcR specifically requires less co-receptor help than other TcR specificities for thymus selection (Itano *et al.* 1994). It is all the more likely then that CD8β$^{\alpha h}$/ F5^{+} lymphocytes that have made it through selection in the thymus require less co-receptor help than other TcR specificities, making a wild type response to a strong agonist more predictable, even in the presence of relatively inefficient CD8β$^{\alpha h}$. This is consistent with data showing striking differences between lymphocytes expressing CD8αβ and those expressing either CD8αα or CD8β$^{\alpha h}$ when antibodies specific for CD8 were included in the *in vitro* proliferation assay. The response of lymphocytes expressing CD8αβ to peptide antigen was effectively blocked when either CD8α- or CD8β-specific antibodies were included in the assay. In lymphocytes expressing CD8β$^{\alpha h}$, antibodies of either specificity had a reduced effect compared to the response seen in CD8αβ-expressing cells, and the effect of the antibodies was abrogated entirely in CD8αα expressing cells. These data suggest that cells expressing a variant form of CD8 are relying on co-receptor help to a lesser extent than those expressing wild type CD8αβ. Importantly, the choice of CD8β antibody clone could have an effect on this assay as it was shown in section 3.2 that there was variation in binding between clones in thympcytes expressing CD8β$^{\alpha h}$. The antibody clone chosen in these experiments, YTS156.7, binds the β-chain of CD8β$^{\alpha h}$ with lower affinity compared to CD8αβ, possibly due to subtle changes in the epitope

conformation. These experiments could be repeated using clone 53-5.8, which binds $CD8\beta^{\alpha h}$ at 100% of wild type levels. Further functional assays are planned in order to elucidate the contribution of each of the variant CD8 molecules to the dynamic TcR/ MHC interaction, as these data suggest differences in functional response between $CD8\alpha\beta$, $CD8\alpha\alpha$ and $CD8\beta^{\alpha h}$.

Given that one of the ways that CD8 functions as a co-receptor is to bring signaling molecule LCK into proximity with the TcR signaling complex, these data raised the question of whether LCK is as involved in the response by $CD8\alpha\alpha$ and $CD8\beta^{\alpha h}$ expressing lymphocytes to antigen, and Ca^{++} mobilization was again used to address this question.

Interestingly however, Ca^{++} responses were quite different in peripheral T cells of these mice compared to thymocytes. The role of CD8 in inducing Ca^{++} flux is still controversial. Conflicting data have shown that monomeric, soluble pMHC complexes can trigger a Ca^{++} flux in primed $CD8^+$ CTLs (Delon *et al.* 1998) but not in naïve $CD8^+$ T cells (Daniels and Jameson 2000), and that dimeric pMHC complexes could induce Ca^{++} mobilization even in the absence of CD8 in T cell hybridomas (Abastado *et al.* 1995) in contrast to data showing an absolute requirement for CD8 in naïve T cells (Daniels and Jameson 2000). The situation is further complicated by data suggesting that co-receptor involvement could vary depending on the affinity of the TcR for the pMHC ligand, and is possibly not generalisable (Alam *et al.* 1996; Lyons *et al.* 1996; Daniels and Jameson 2000). In the case of the mice under investigation in this study however, the TcR specificity and hence affinity for pMHC alone is identical, and any discrepancies between responses should be directly attributable to the contribution of the co-receptor. Nevertheless the system described in this study uses crosslinking of antibodies to stimulate cells, and future experiments will concentrate on stimulation using pMHC.

Ca^{++} mobilization following mature primary T cell stimulation is dependent on the activation of LCK (Trobridge and Levin 2001). Data showing that Ca^{++} flux is severely impaired after TcR cross-linking in lymphocytes expressing CD8αα or CD8β^{ah} co-receptors in comparison to CD8αβ co-receptors, might suggest that recruitment of LCK to TcR is impaired in these mice. This would be consistent with the finding that lymphocytes expressing CD8αα and CD8β^{ah} are less sensitive to the effects of blocking CD8 specific antibodies, since CD8 engagement gives a negative signal which is thought to be mediated by LCK (Weiss and Littman 1994). This conclusion is in contrast however to data from Kwan-Lim *et al* (1993), who showed that CD8-independent hybridomas had markedly increased sensitivity to stimulation with anti-CD3 antibodies compared to CD8-dependent hybridomas. Instead of demonstrating an inverse correlation between CD8 dependence and the ease with which the T cell can be activated as shown by Kwan-Lim *et al* (1993), these data seem to indicate the opposite. Nevertheless, it cannot be the case that mice expressing CD8αα or CD8β^{ah} have developed an entirely CD8 independent pathway for development, since mice lacking CD8 altogether, which presumably would be similarly able to take advantage of such a signaling pathway, do not develop MHC Class I restricted T cells (Fung-Leung *et al.* 1991).

These data raise interesting questions on how lymphocytes expressing CD8αα and CD8β^{ah} are able to proliferate, given that both co-receptors are inefficient at binding MHC in comparison to CD8αβ and seem unable to target LCK to the TcR signaling complex, and future experiments will be aimed at elucidating which signaling molecules are actually involved in this response.

Figure 6.1 - Expression of CD8β in splenocytes expressing the $CD8\beta^{\alpha h}$ transgene is slightly reduced compared to wild type levels, but TcR expression is higher.

Splenocytes from mice expressing $CD8\beta^{wt}$ (A), CD8β° (B), $CD8\beta^{tg}$ (C) and $CD8\beta^{\alpha h}$ (D) were stained with fluorescently labeled antibodies specific for CD4 (RM4-5), CD8α (53.6.7), CD8β (53.5.8) and TcR (H57) at 4°C for 30 minutes. Live cells were gated for expression of CD4 and CD8α, and expression of CD8β (left histograms) and TcR (right histograms) analysed by FACS Calibur. The MFI and percentage of $CD8^+$ cells for each strain is shown in grey boxes. Expression of CD8β (lower left panel) and TcR (lower right panel) is compared between strains. Data are representative of nine experiments, and fluorescent intensity is shown on log scale from 10^0 to 10^4. Notations are the same in upper and lower panels.

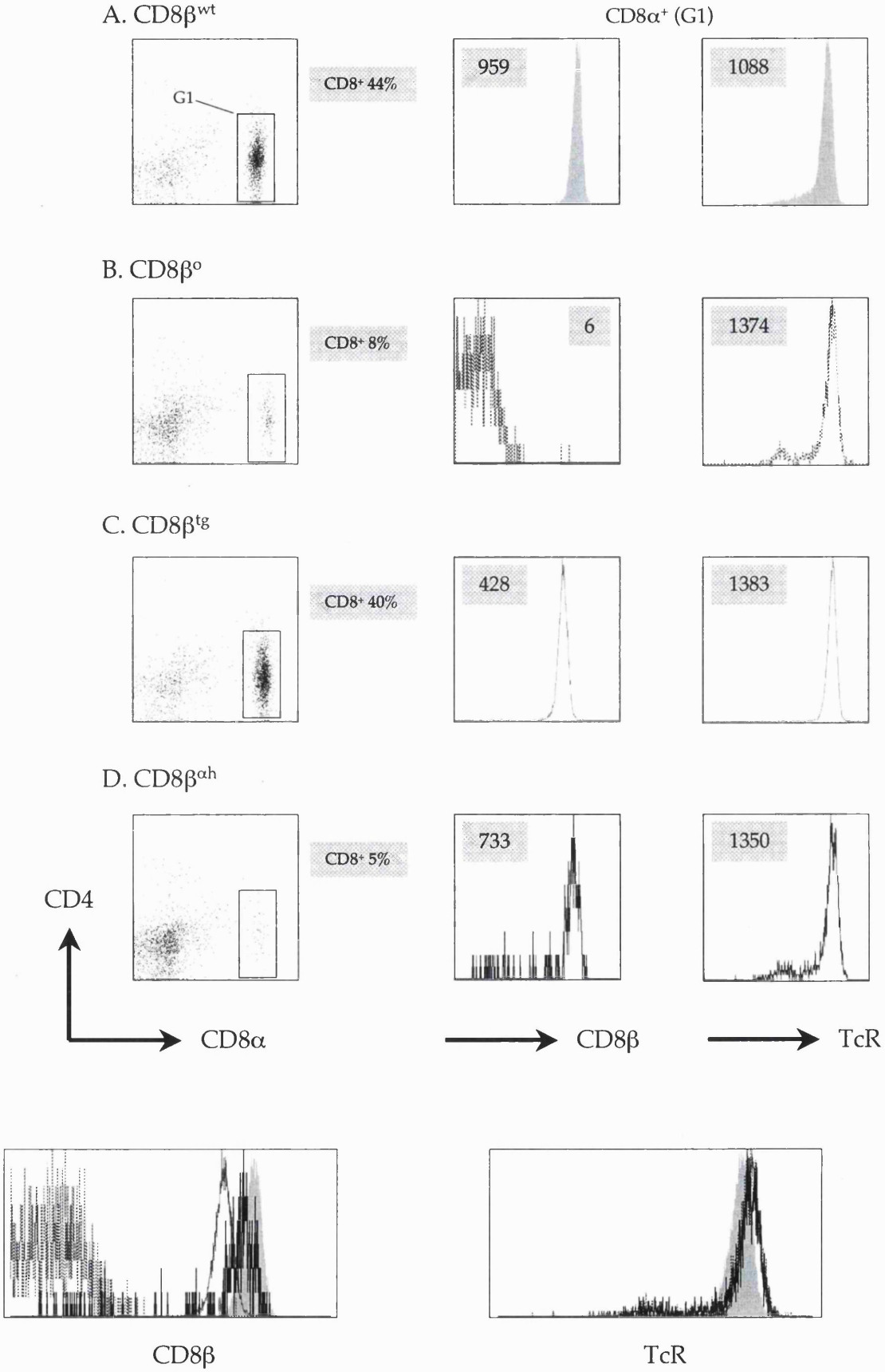

A. CD8β^wt
CD8α+ (G1)
G1
CD8+ 44%
959
1088
B. CD8β^o
CD8+ 8%
6
1374
C. CD8β^tg
CD8+ 40%
428
1383
D. CD8β^αh
CD8+ 5%
733
1350
CD4
CD8α
CD8β
TcR
CD8β
TcR

Figure 6.2 – Mice expressing CD8β$^{\alpha h}$ have lower numbers of CD8^{+} cells in spleen than wild type controls.

Splenocytes from mice expressing CD8β^{wt} (1), CD8β$^{\circ}$ (2), CD8β^{tg} (3) and CD8β$^{\alpha h}$ (4) were stained with fluorescently labeled antibodies specific for CD4 (RM4-5) and CD8α (53.6.7) at 4°C for 30 minutes, then analysed by FACS. Live cells were gated for expression of CD4 and CD8α, and numbers of CD8^{+} cells recorded. Data are representative of nine experiments.

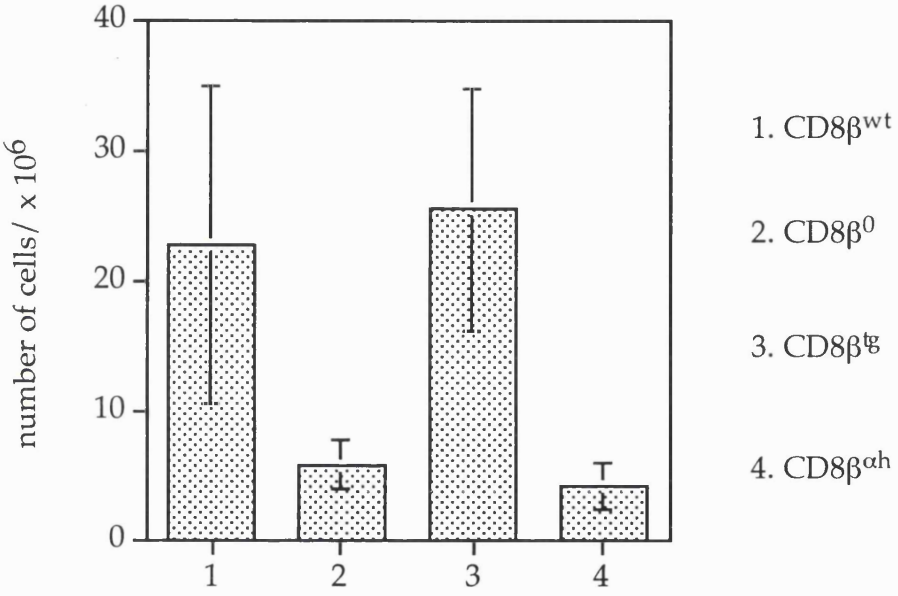

40
30
20
10
0
number of cells/ x 10^6
1
2
3
4
1. CD8β^{wt}
2. CD8β^{0}
3. CD8β^{tg}
4. CD8β$^{\alpha h}$

Figure 6.3 - Expression of CD8β in lymphocytes expressing the $CD8\beta^{\alpha h}$ transgene is comparable to wild type levels, but TcR expression is higher.

Lymphocytes from mice expressing $CD8\beta^{wt}$ (A), $CD8\beta^{\circ}$ (B), $CD8\beta^{tg}$ (C) and $CD8\beta^{\alpha h}$ (D) were stained with fluorescently labeled antibodies specific for CD4 (RM4-5), CD8α (53.6.7), CD8β (53.5.8) and TcR (H57) at 4°C for 30 minutes. Live cells were gated for expression of CD4 and CD8α, and expression of CD8β (left histograms) and TcR (right histograms) analysed by FACS Calibur. The MFI and percentage of $CD8^+$ cells for each strain is shown in grey boxes. Expression of CD8β (lower left panel) and TcR (lower right panel) is compared between strains. Notations are the same as upper panels. Data are representative of nine experiments, and fluorescent intensity is shown on log scale from 10^0 to 10^4.

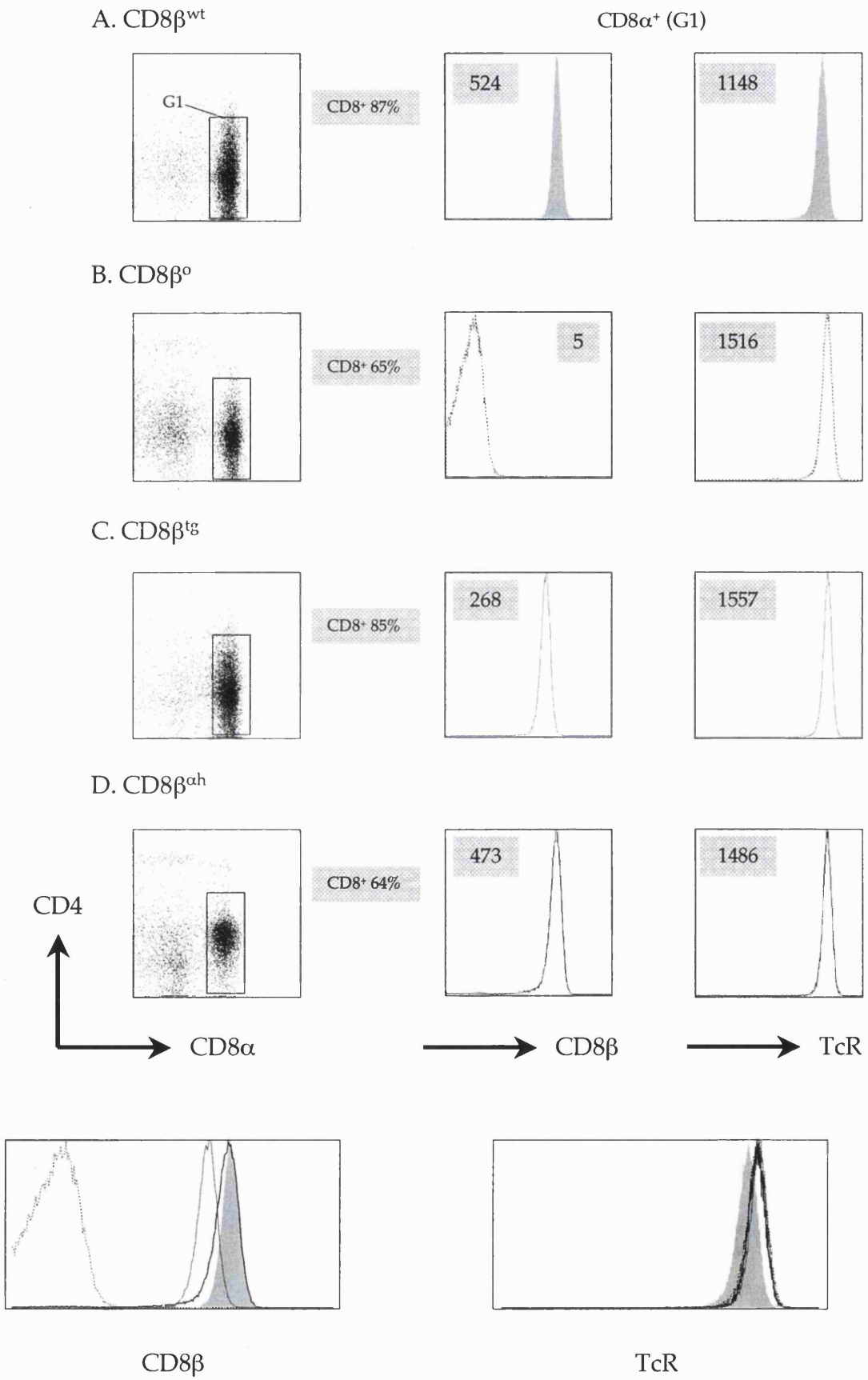
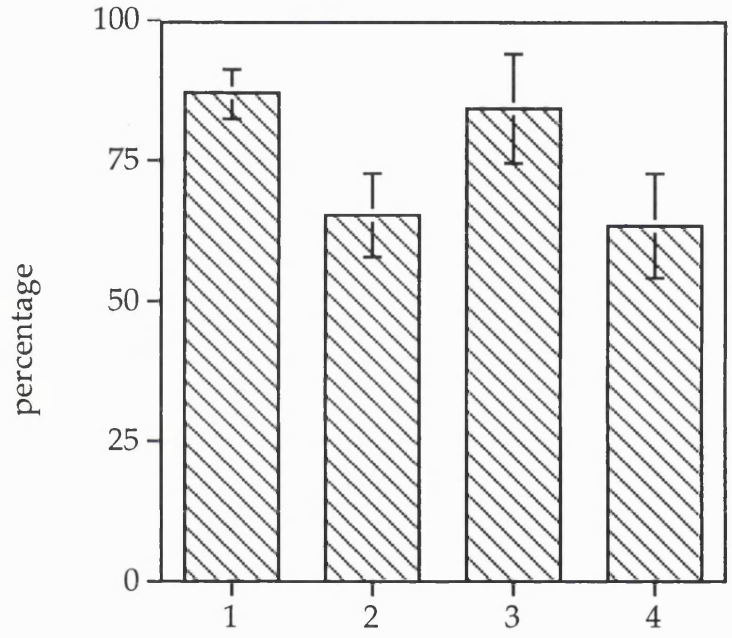

A. CD8β^wt
CD8α+ (G1)
G1
CD8+ 87%
524
1148
B. CD8β^o
CD8+ 65%
5
1516
C. CD8β^tg
CD8+ 85%
268
1557
D. CD8β^αh
CD8+ 64%
473
1486
CD4
CD8α
CD8β
TcR
CD8β
TcR

Figure 6.4 - Mice expressing CD8β$^{\alpha h}$ have a lower percentage of CD8$^+$ cells in lymph nodes than wild type controls.

Lymphocytes from mice expressing CD8β^{wt} (1), CD8β$^{\circ}$ (2), CD8β^{tg} (3) and CD8β$^{\alpha h}$ (4) were stained with fluorescently labeled antibodies specific for CD4 (RM4-5) and CD8α (53.6.7) at 4°C for 30 minutes, then analysed by FACS. Live cells were gated for expression of CD4 and CD8α, and the percentage of CD8$^+$ cells recorded. Data are representative of nine experiments.



1. CD8β^{wt}

2. CD8β^{0}

3. CD8β^{tg}

4. CD8β$^{\alpha h}$

Figure 6.5 - Lymphocytes expressing CD8β$^{\alpha h}$ are able to proliferate when transferred to a lymphopenic host.

Lymphocytes from CD8β^{wt} (1), CD8β^{o} (2), CD8β^{tg} (3) and CD8β$^{\alpha h}$ (4) expressing mice were labeled with 1uM CFSE dye in PBS at room temperature for 10 minutes and washed twice. Labelled cells were transferred into lymphopenic recipients via tail injection and the mice housed in pathogen free conditions. After 14 days lymphocytes were removed from the host mice and cells were incubated with fluorescently labeled antibodies specific for CD8α (53.6.7) and TcR (H57) at 4°C for 30 minutes. CD8α^{+} TcR^{+} CD44^{lo} cells were analysed for binding of CFSE dye (panel A) and the average number of divisions calculated for each population of cells according to the formula in *materials and methods* (panel B). Data are representative of 4 experiments, with two mice in each group, and fluorescent intensity is shown on log scale from 10^0 to 10^4.

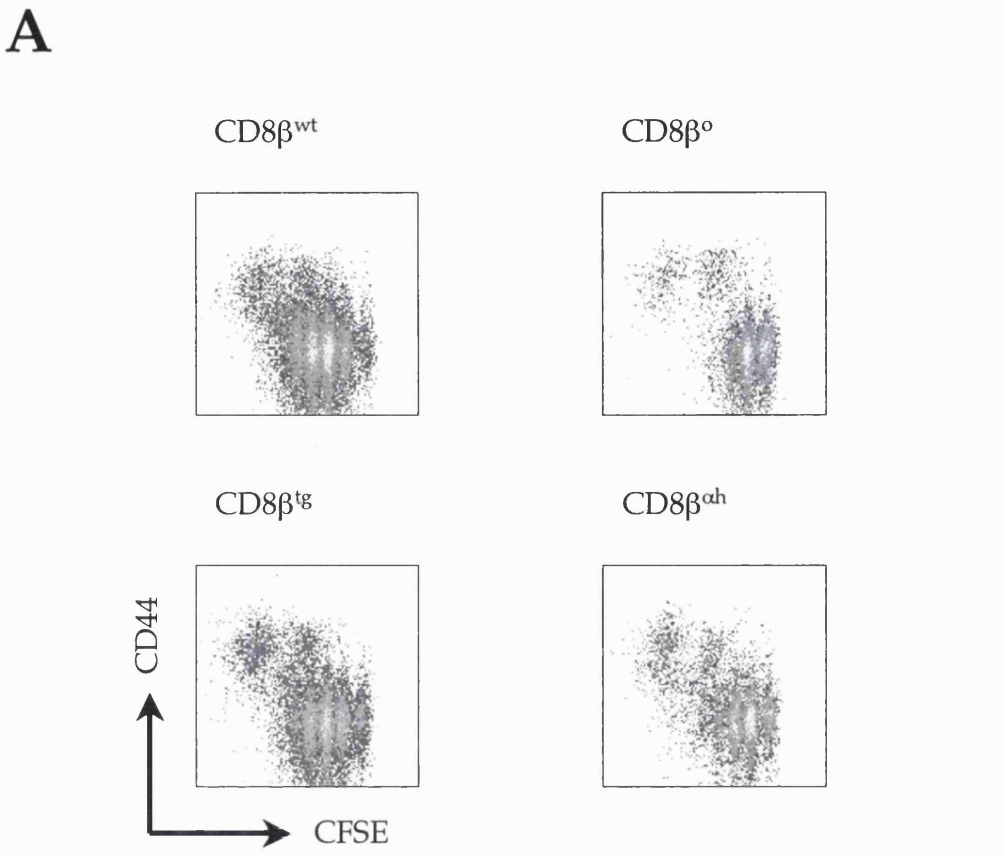

A
CD8β^wt
CD8β^o
CD8β^tg
CD8β^αh
CD44
CFSE
B
average divisions
2
1.5
1
0.5
0
1
2
3
4
strain
1. CD8β^wt
2. CD8β^0
3. CD8β^tg
4. CD8β^αh

Figure 6.6 - Peripheral cells from mice expressing CD8β^{ah} are less able to bind cognate pMHC.

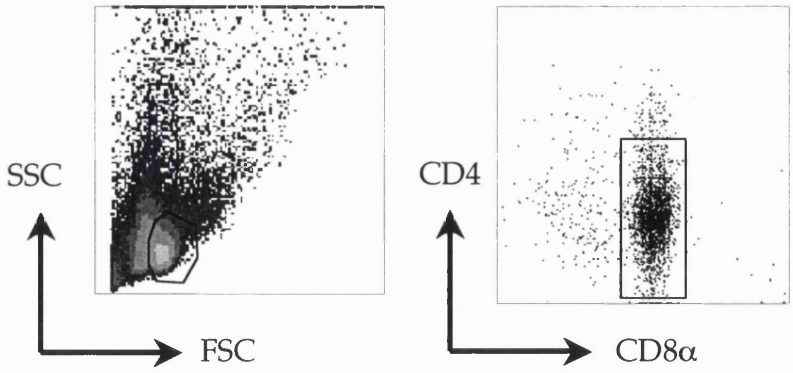
MHC recombinant dimers (Pharmingen) were incubated with NP68 peptide at 37°C overnight. Lymphocytes were incubated with peptide loaded MHC dimers at 4°C for one hour at a concentration of 0.25μg dimers per 10^6 cells. Cells were washed and resuspended with fluorescently labeled antibodies specific for CD8α (53.6.7). Cells were washed again and analysed by FACS Calibur.

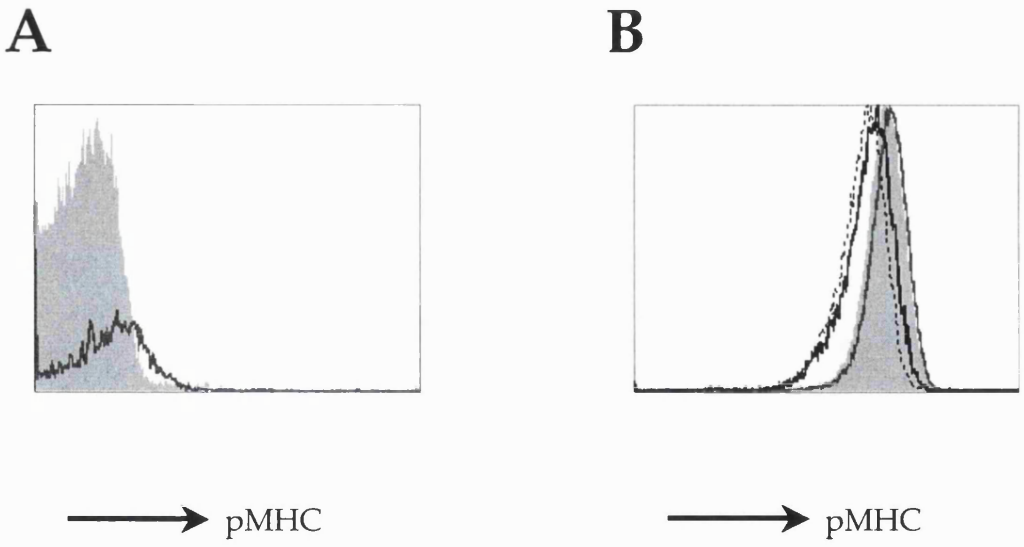
Top panels: cross-stain showing gating.

(A) Lymphocytes from B10 (shaded histogram) and CD8^0 mice (grey line) were stained as above, showing that binding of cognate pMHC is both TcR specific and CD8 dependent.

(B) Lymphocytes from CD8β^{wt} (shaded histogram), CD8β^{tg} (grey line), CD8β° (dashed line) and CD8β^{ah} (black line) expressing mice were stained as above. CD8$^+$ cells in all strains are able to bind cognate pMHC. There is approximately two fold less binding in lymphocytes expressing CD8β° and CD8β^{ah} in comparison to wild type controls.

Cells were analysed by FACS Calibur and results are representative of four experiments, and fluorescent intensity is shown on log scale from 10^0 to 10^4.

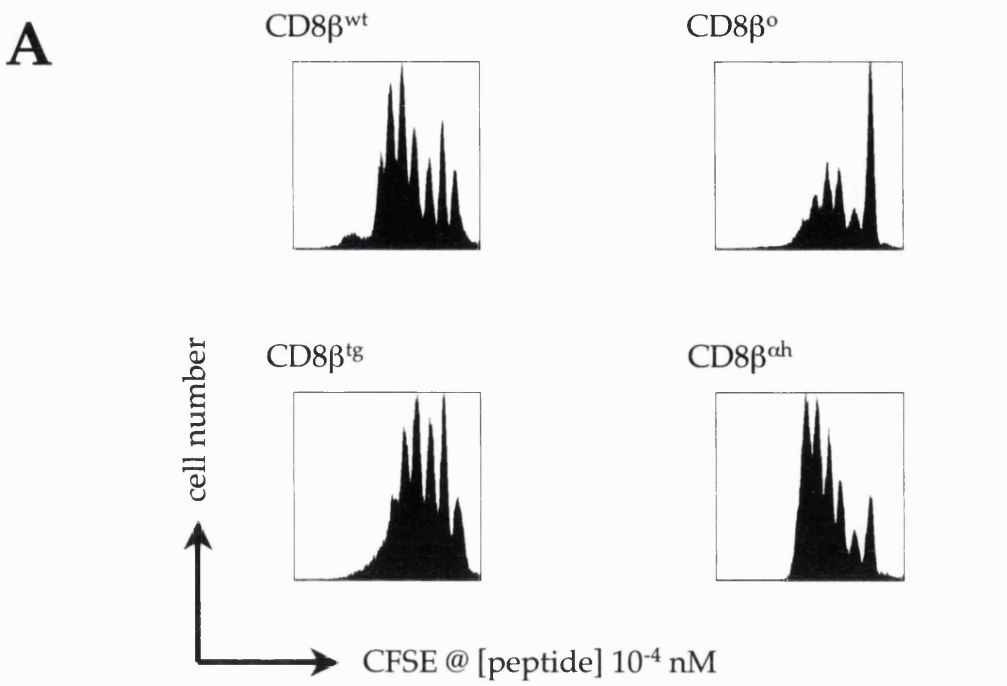




shaded histogram	$CD8\beta^{wt}$
grey line	$CD8\beta^{tg}$
dashed line	$CD8\beta^{0}$
black line	$CD8\beta^{\alpha h}$

Figure 6.7 - Lymphocytes expressing CD8β$^{\alpha h}$ have altered dose response to antigen stimulation after 72 hours *in vitro*.

Lymphocytes from CD8β^{wt} (red line), CD8β^{tg} (green line), CD8β° (blue line) and CD8β$^{\alpha h}$ (gold line) expressing mice were labeled with 1uM CFSE dye in PBS at room temperature for 10 minutes and washed twice. Labelled cells were incubated at 37°C with titrated soluble NP68 peptide antigen for 72 hours in full culture medium (1 x 10^6 cells/ ml). After 72 hours cells were incubated with fluorescently labeled antibodies specific for CD8α (53.6.7) and CD8β (YTS156.7) at 4°C for 30 minutes. CD8β$^+$ (or CD8α$^+$ in CD8β° populations) cells were analysed for binding of CFSE dye (panel A) and the average number of divisions calculated for each population of cells according to the formula in *materials and methods* (panel B). Panel A shows cell division profile as measured with CFSE when peptide concentration is 10^{-4} nM. Data are representative of 6 experiments, and fluorescent intensity is shown on log scale from 10^0 to 10^4.


A
CD8β^wt
CD8β^o
CD8β^tg
CD8β^αh
cell number
CFSE @ [peptide] 10^-4 nM

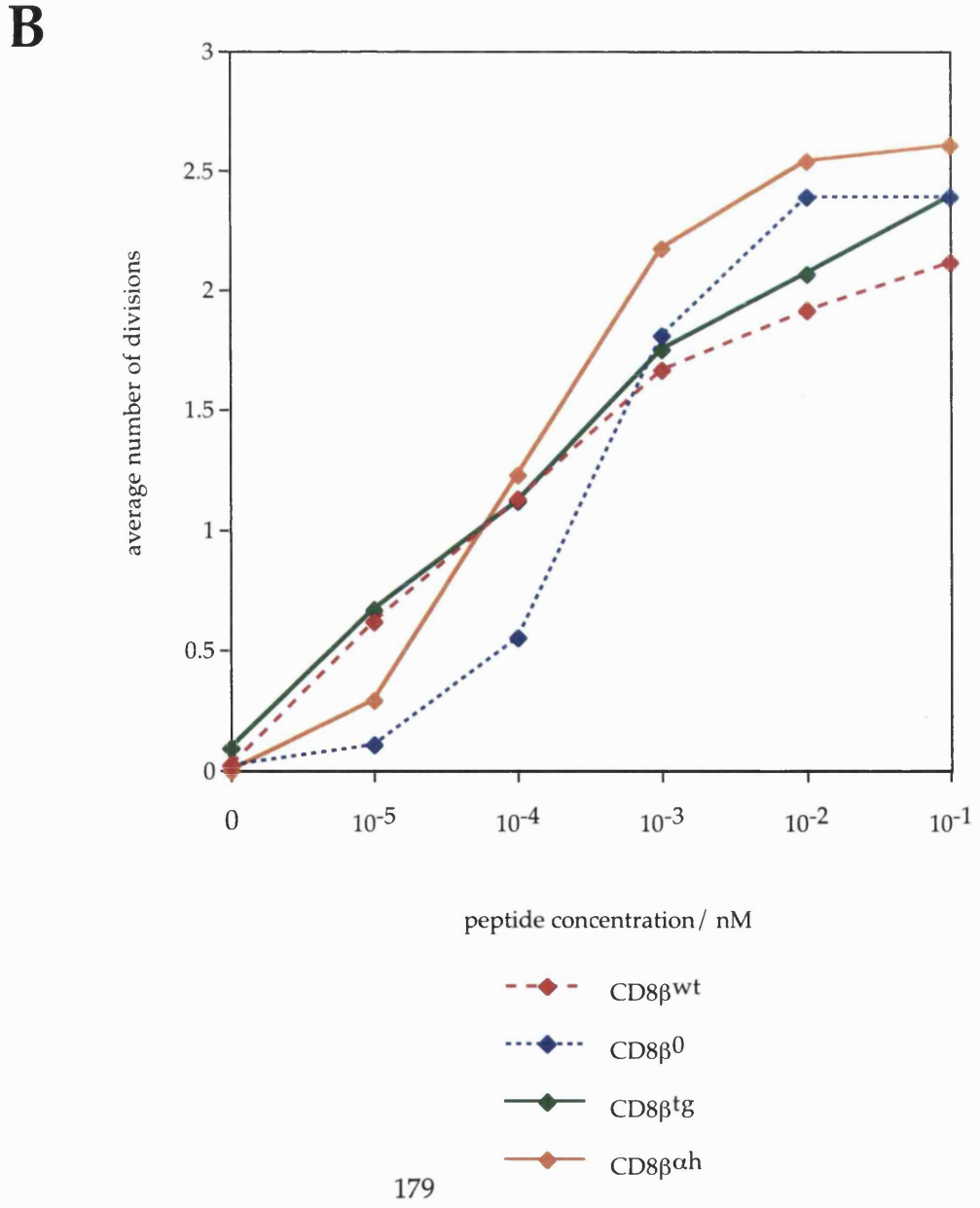

B
average number of divisions
3
2.5
2
1.5
1
0.5
0
0
10^-5
10^-4
10^-3
10^-2
10^-1
peptide concentration / nM
CD8β^wt
CD8β^0
CD8β^tg
CD8β^αh

Figure 6.8 - Anti-CD8 antibodies are less inhibitory for the response of CD8β$^{\alpha h}$ lymphocytes to peptide.

Lymphocytes from CD8β^{wt} (A), CD8β^{tg} (B), CD8β$^{\circ}$ (C) and CD8β$^{\alpha h}$ (D) expressing mice were labeled with 1uM CFSE dye in PBS at room temperature for 10 minutes and washed twice. Labelled cells were incubated at 37°C with a titration of soluble NP68 peptide antigen for 72 hours in full culture medium (1 x 10^6 cells/ ml) either with peptide alone (red line), peptide and α-CD8α (YTS169.4, green line) or peptide and α-CD8β (YTS156.7, blue line). After 72 hours cells were incubated with fluorescently labeled antibodies specific for CD8α (53.6.7) and CD8β (YTS156.7) at 4°C for 30 minutes. CD8β^{+} (or CD8α^{+} in CD8β$^{\circ}$ populations) cells were analysed for binding of CFSE dye and the average number of divisions calculated for each population of cells according to the formula in *materials and methods*. Data are representative of 6 experiments.

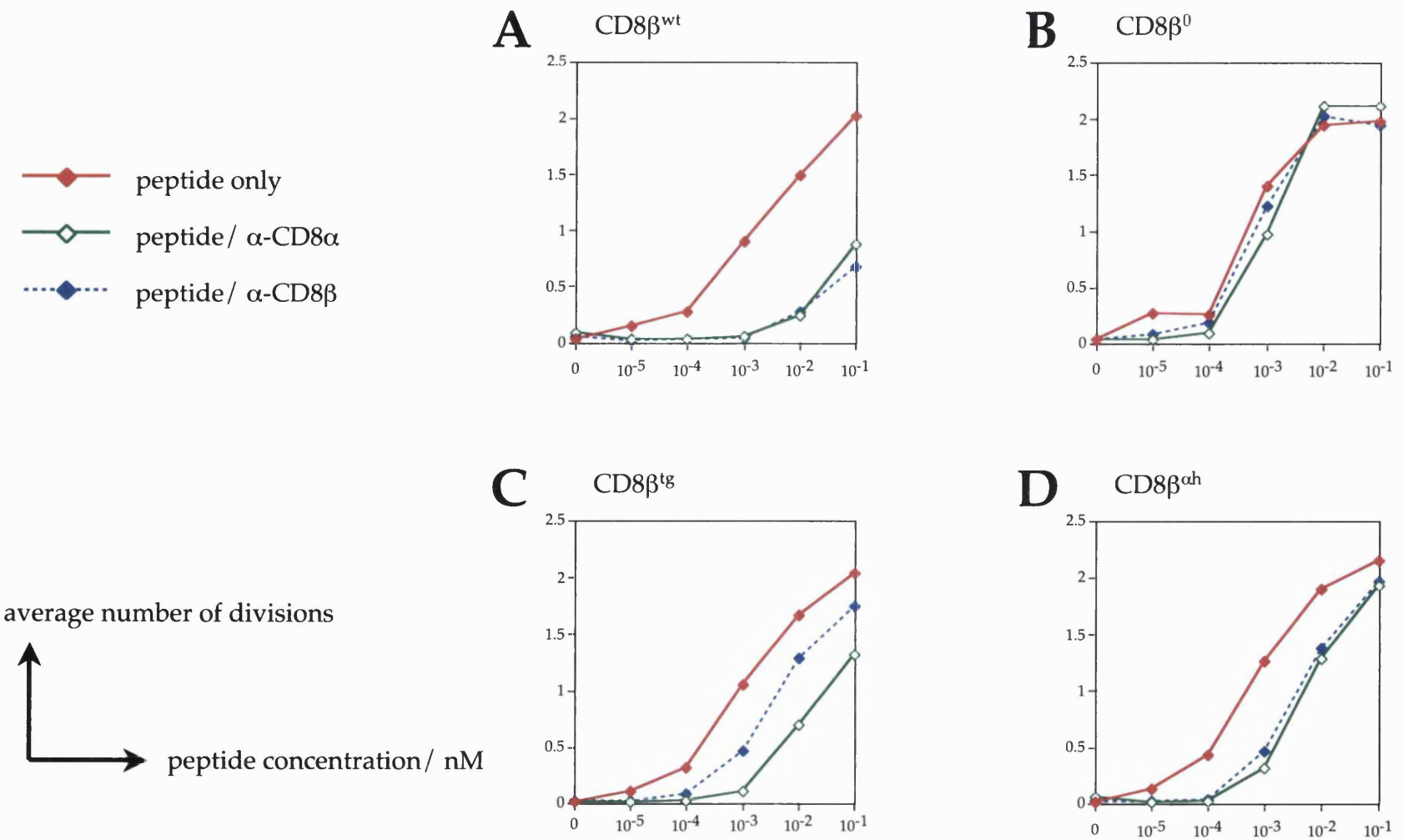
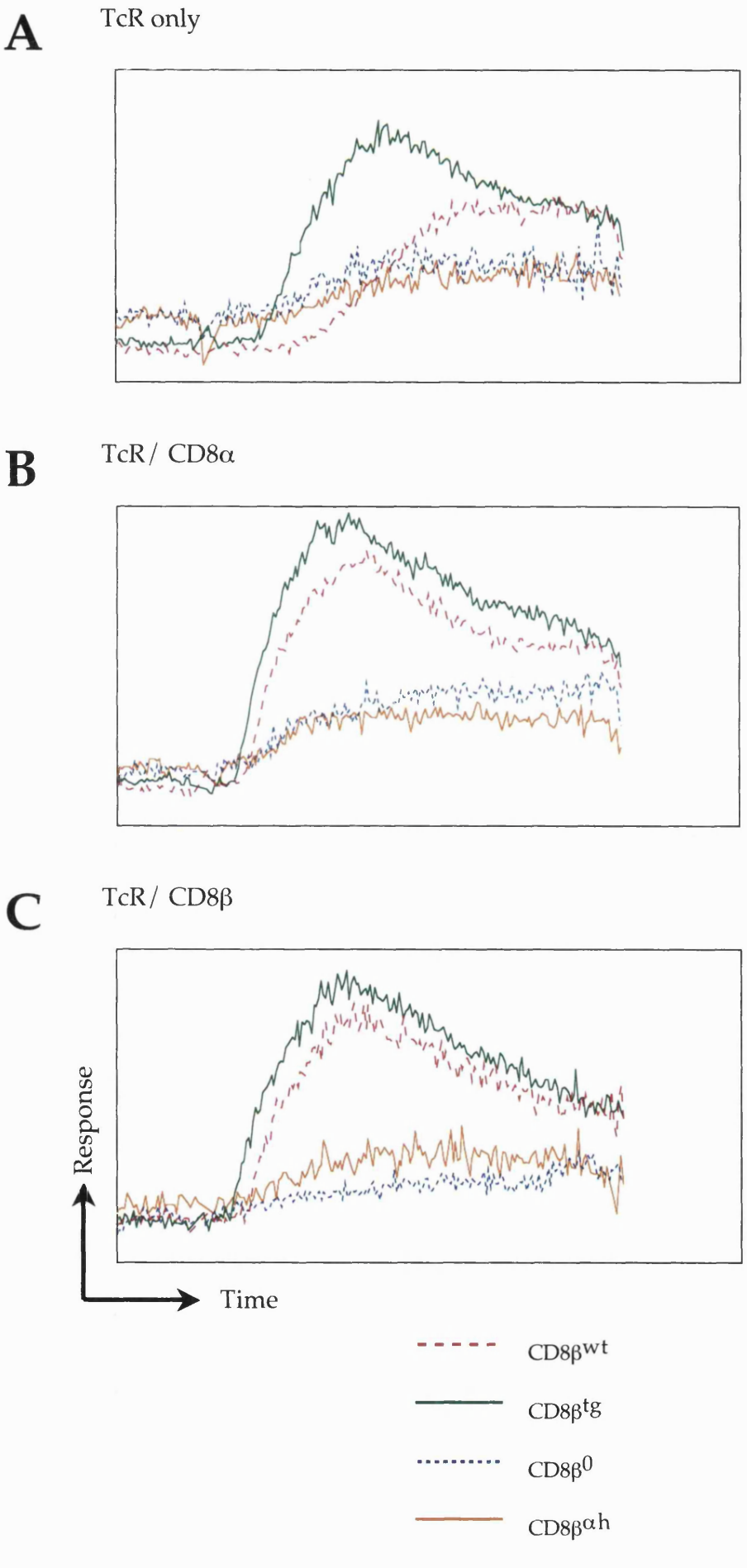

peptide only
peptide / α-CD8α
peptide / α-CD8β
A
CD8β^wt
B
CD8β^0
C
CD8β^tg
D
CD8β^αh
average number of divisions
peptide concentration / nM
2.5
2
1.5
1
0.5
0
0
10^-5
10^-4
10^-3
10^-2
10^-1

Figure 6.9 - Ca^{++} flux response induced by crosslining antibodies specific for TcR or CD8 is reduced in lymphocytes expressing $CD8\beta^{\alpha h}$.

Ca^{++} response in $CD8\beta^{wt}$ (red line), $CD8\beta^{tg}$ (green line), $CD8\beta^{\circ}$ (blue line) and $CD8\beta^{\alpha h}$ (gold line) lymphocytes treated with biotinylated antibodies specific for TcR only (A), TcR and CD8α (B) or TcR and CD8β (C). Cells were kept at room temperature, labeled with Indo-1AM in darkness for 45 minutes and pretreated with the above antibodies. The streptavidin crosslinker was added after analyzing cells on LSR for 1 minute, and cells were analysed for a further 5 minutes. Antibodies used were H57 (TcR) YTS169.4 (CD8α) and YTS156.7 (CD8β). All traces are the median of the responding population of lymphocyte cells as measured by change in fluorescence of the Indo-1AM dye. Axes are labeled, the Y-axis range is identical in each case.


A
TcR only
B
TcR/ CD8α
C
TcR/ CD8β
Response
Time
CD8β^wt
CD8β^tg
CD8β^0
CD8β^αh

Concluding remarks

The aim of the thesis was to investigate the influence of the glycosylation in the hinge region of CD8β on the function of the CD8β polypeptide. To do this, a relatively conservative substitution of the CD8α hinge in place of the CD8β hinge was performed. Surprisingly, this resulted in a heterodimer which behaves more like CD8αα as a co-receptor than CD8αβ.

Initial phenotype analysis showed that in cells expressing CD8αα or CD8β$^{\alpha h}$, there was a marked decrease in numbers of DP thymocytes with the phenotype TcR^{int} compared to cells expressing CD8αβ. This was further reflected in the CD5 profile seen for DP thymocytes bearing each co-receptor, in that DP thymocytes expressing CD8αβ had higher levels of CD5 than either CD8αα or CD8β$^{\alpha h}$ expressing cells. The small population of TcR^{int} thymocytes expressing CD8αα or CD8β$^{\alpha h}$ was indicative of a block in maturation at an early stage in positive selection (Guidos *et al.* 1990; Petrie *et al.* 1990). Positive selection requires a developing thymocyte to recognize self-pMHC complexes and receive signals via the TcR (Jameson *et al.* 1995), so the failure of CD8β$^{\alpha h}$ expressing thymocytes to progress through positive selection suggested that there was impaired recognition of MHC or impaired signaling in these animals.

CD8αβ has been demonstrated to be more effective than CD8αα at facilitating recognition of the same peptide antigen by TcR (Renard *et al.* 1996; Witte *et al.* 1999), and the extracellular, transmembrane and cytosolic regions of CD8β have all been suggested to enhance MHC binding (Bosselut *et al.* 2000; Arcaro *et al.* 2001). In spite of expressing all of these domains, data from this thesis shows that CD8β$^{\alpha h}$ is less able to bind MHC than CD8αβ, and the pattern of behaviour of cells expressing CD8β$^{\alpha h}$ is more similar to that of cells bearing CD8αα. The block in positive selection suggested an impaired ability to recognize self pMHC, and this was confirmed when lymph node cells expressing each co-receptor were transferred to a lymphopenic host and the

expansion analysed. Homeostatic proliferation in a lymphopenic environment relies on recognition of self pMHC, and is thought to be similar to the recognition step needed to progress through selection in the thymus (Ernst *et al.* 1999; Goldrath and Bevan 1999; Kieper and Jameson 1999; Viret *et al.* 1999). Cells expressing CD8αα or CD8β^{ah} go through approximately half as many divisions as those expressing CD8αβ, showing that CD8αβ enhances expansion of peripheral cells in response to lymphopenia and suggesting a role for wild type CD8β in this process. Another process that is reliant on the recognition of self-pMHC is the survival of naïve T cells circulating in the periphery (Kirberg *et al.* 1997; Tanchot *et al.* 1997; Viret *et al.* 1999), although the signals necessary for survival are different to those required for proliferation in response to lymphopenia (Seddon *et al.* 2000). A defect in recognition of self-pMHC, suggested by the response of cells to conditions of lymphopenia, could be adversely affecting the survival of peripheral T cells expressing CD8αα or CD8β^{ah}, which is consistent with the observation that the lymph nodes of mice expressing either of these co-receptors are smaller and have fewer CD8$^+$ T cells than those of mice expressing CD8αβ. Positive selection for the F5 TcR specificity can also be driven by an antagonist peptide (Smyth *et al.* 1998), and future experiments could take advantage of this to better re-create selection conditions found in the thymus and directly test the ability of each CD8 co-receptor to enhance positive selection through recognition of self-pMHC.

The ability of cells expressing each co-receptor to bind antigenic pMHC was assessed more directly, with the cognate peptide for the F5 TcR bound to D^b MHC dimers. In this way it was shown that lymphoid cells expressing CD8αα or CD8β^{ah} were less able to bind cognate pMHC than cells expressing CD8αβ at all stages of maturation. This was consistent with the finding that when incubated with cognate antigen *in vitro*, lymph node cells expressing CD8αα or CD8β^{ah} were unable to respond at low concentrations of antigen in comparison to the response of lymph node cells expressing CD8αβ. At higher antigen concentration however cells expressing CD8αα or CD8β^{ah}

co-receptors were able to proliferate, and in fact went through higher numbers of divisions than cells expressing CD8αβ.

Surprisingly, lymph node cells expressing CD8αα or CD8β$^{\alpha h}$ do not seem to be signalling via LCK in the same way as cells expressing CD8αβ, which was shown by measuring Ca^{++} mobilisation in response to antibody cross-linking. Activation of LCK is required for a Ca^{++} flux (Trobridge and Levin 2001), and lymph node cells expressing CD8αα or CD8β$^{\alpha h}$ showed greatly reduced response to antibody cross-linking compared to the enhanced response seen in lymph node cells expressing CD8αβ. LCK kinase activity and downstream phosphorylation in T cells is enhanced by the presence of the CD8β cytoplasmic domain (Irie *et al.* 1995; Irie *et al.* 1998; Bosselut *et al.* 2000; Arcaro *et al.* 2001), which is intact in CD8β$^{\alpha h}$ molecules, yet LCK signalling seems to be impaired in lymph node cells expressing this co-receptor. TcR-mediated signals via LCK are essential for selection in the thymus, and it is possible that in addition to the impairment in MHC recognition suggested by the above data, another reason for the block in selection could be due to a failure of signalling cascades in thymocytes expressing CD8β$^{\alpha h}$.

LCK is associated with CD8 via a conserved motif on the cytoplasmic domain of CD8α (Shaw *et al.* 1990), which is intact in the CD8αβ$^{\alpha h}$ heterodimer and accordingly LCK was shown to associate with the mutated molecule. Furthermore, cross-linking of antibodies specific for the TcR and CD8 produced comparable Ca^{++} fluxes in thymocytes expressing all forms of the CD8 co-receptor, indicating that there was no inherent signalling defect in cells expressing CD8β$^{\alpha h}$, but the response of cells to antibody cross-linking is clearly not identical to the response when recognizing MHC *in vivo*.

One striking difference between the CD8β$^{\alpha h}$ co-receptor and either CD8αβ or CD8αα lay in the nature of the post-translational addition of glycans to the molecule. O-linked

glycosylation has been shown to be associated with the hinge region of CD8β and is predicted to lie in the hinge region of CD8α (Hansen *et al.* 1997; Moody *et al.* 2003), and previous work in the lab showed that the nature of the O-linked glycans on the $CD8\beta^{\alpha h}$ polypeptide was distinct from that observed either in wild type CD8β or, more surprisingly, CD8α. In contrast to wild type CD8α, the extent of O-linked glycosylation and sialylation observed was greater than predicted by the primary structure of the molecule, although the β-chain of the molecule did become hyposialylated after T cell activation, and the same core structure of O-glycans could be detected on CD8 molecules in thymocytes. Developmentally regulated glycosylation of CD8 has been observed in thymocytes as well as activated T cells (Casabo *et al.* 1994; Wu *et al.* 1996), and recent reports have focused on the importance of differential glycosylation of CD8 for non-cognate binding of MHC in the thymus, which has been suggested to be important for selection (Daniels *et al.* 2001; Moody *et al.* 2001). Additionally, non-cognate binding of MHC in the thymus cannot be observed in thymocytes expressing CD8αα (Bosselut *et al.* 2000; Moody *et al.* 2001), and mice expressing this co-receptor show an 80% drop in numbers of thymocytes being selected (Crooks and Littman 1994). Since mice expressing $CD8\beta^{\alpha h}$ show a similar behaviour pattern to that of mice expressing CD8αα in terms of selection and MHC recognition, and given that non-cognate recognition of MHC depends on the expression of wild type CD8β and is thought to promote positive selection (Daniels *et al.* 2001), it is possible that the different glycosylation observed on $CD8\beta^{\alpha h}$ molecules is affecting this interaction and causing a block in selection.

The altered glycosylation of $CD8\beta^{\alpha h}$ molecules could be affecting T cell function in other ways. Other reports have suggested an interaction between TcR and CD8 that is facilitated by CD8β (Kwan Lim *et al.* 1998; Wheeler *et al.* 1998), and since it is the Ig-like domain that is binding MHC, it is predicted to be the hinge region of CD8 that needs to abut the TcR (Arden 1998). A change in the glycans of CD8β could be affecting this interaction negatively and causing inefficient co-receptor function, possibly as a result

of the greater abundance of glycans associated with the CD8β$^{\alpha h}$ polypeptide. The change in core structure of O-linked glycans associated with T cell activation results in bulkier glycans that have the predicted effect of reducing cell-cell interactions and making T cell-APC interaction inefficient (Tsuboi and Fukuda 1997; 1998). The glycans of CD8β$^{\alpha h}$, although identical in core structure, are nevertheless bulkier than the wild type CD8αβ molecule, and could be similarly affecting T cell-APC interaction. Given that CD8 glycosylation and sialylation is increased as the T cell matures, this could explain why cross-linking of TcR- and CD8-specific antibodies results in Ca^{++} mobilization in the less mature thymocytes expressing CD8β$^{\alpha h}$, but is severely impaired in lymph nodes expressing this co-receptor, possibly as a result of bulkier glycans. The association of CD8β$^{\alpha h}$ with TcR could be tested by repeating experiments which showed that CD8 co-caps with TcR when incubated with antibodies specific for TcR and CD8β. If the bulkier glycans of CD8β$^{\alpha h}$ are preventing efficient localization of the co-receptor with TcR, this effect could be reduced or abrogated in cells bearing the mutated molecule.

Replacing the CD8β hinge region with that of CD8α resulted in a more dramatic phenotype than expected, and data from this thesis suggests a role for wild type CD8β glycosylation in correct co-receptor function. Expressing the mutated protein in mice was advantageous compared to transfecting cells lines with the CD8β$^{\alpha h}$ protein, as there were developmentally regulated differences in signaling in cells expressing CD8β$^{\alpha h}$ and the signaling capacity of an immortal cell line is unlikely to reflect accurately the signaling associated with an intact animal system. The system was limited however when assessing precisely the impact of the altered glycosylation associated with CD8β$^{\alpha h}$. The addition of glycans to a molecule can affect conformation and charge of the modified protein as well as providing ligands for cell-cell interactions. The O-glycans of CD8β have been suggested to hold the molecule in such a conformation as to achieve optimal association with MHC (Moody *et al.* 2001), and the de-sialylation associated with CD8β on T cell activation reduces the overall

negative charge of the molecule (Casabo *et al.* 1994), although the importance of this for the T cell has not been established. It was not possible with this system to establish if the altered glycosylation is affecting conformation, charge or the interaction of potential ligands, and it could be adversely affecting all three of these effects.

Point mutagenesis of specific residues of the CD8β hinge region could result in a less dramatic phenotype, and help isolate which, if any, O-linked glycans are specifically important for co-receptor function. This approach could have a less severe effect on overall charge of the molecule and may target potential ligand sites more specifically, but could also result in a conformational change in the molecule which could have a more global effect on the protein.

Interesting ways to develop the work from this project could be to employ mass spectrometry to isolate precisely the residues of the CD8β$^{\alpha h}$ polypeptide that are O-glycosylated and sialylated, in a similar way as Moody *et al* (2003) showed for wild type CD8β. They demonstrated not only which residues were modified by the addition of O-linked glycans, but also that some O-glycans were further modified by the addition of sialic acid whereas others were not. By following this approach it would be possible to establish if more residues of the CD8β$^{\alpha h}$ polypeptide are glycosylated than predicted, as well as if a higher percentage of those O-glycans are sialylated in comparison to wild type CD8β. Another possible way of addressing the question of the impact of CD8β$^{\alpha h}$ on co-receptor function could be to use crystallography techniques in order to establish more precisely the way in which the Ig-like domains of the CD8β$^{\alpha h}$ co-receptor interact with MHC. Gao *et al* (1997) crystallized the complex formed between the Ig-like domains of CD8αα and MHC, and being able to compare the CD8αα, CD8αβ and CD8β$^{\alpha h}$ interactions with MHC could show important differences in this association relating to efficiency of co-receptor function. However, based on the data from Gao *et al* (1997), CD8αβ is predicted to bind MHC in the same way as CD8αα. Data from this thesis has shown that CD8αα and CD8αβ assist the TcR to bind

cognate MHC with differing affinities, suggesting this may not be the case. Since the Ig-like domains of CD8αβ and CD8$\beta^{\alpha h}$ are the same, crystallography may also predict that these two co-receptors bind with similar affinity, yet data from this study again shows that CD8$\beta^{\alpha h}$ is impaired in comparison to CD8αβ when binding MHC. This could be due to differences in glycosylation between the different forms of CD8, which will not be present in the purified protein used in crystallography. Furthermore, the inefficient co-receptor function of CD8$\beta^{\alpha h}$ could be as a result of the difference in secondary structure of the molecule, and it is possible that the altered glycosylation associated with the protein could be having little effect. Crystallography could be useful in addressing this question, if an unglycosylated section of the hinge region of each of the co-receptors could be purified and the effect on binding assessed. Future experiments are planned to address the questions raised by this work.

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