Generation and immunoresponsiveness of transgenic mice expressing T cell localised MHC class II

A thesis submitted to

Imperial College of Science, Technology and Medicine,

University of London

in partial fulfillment for the Degree of

Doctor of Philosophy

by

Deborah Palliser

B.Sc.

For My Parents

ABSTRACT

In this thesis the generation, analysis and immunocompetence of transgenic mice expressing MHC class II on their T cells is described. Murine T cells do not normally express MHC class II, whereas in other species such as man, MHC class II molecules are present on activated T cells. From *in vitro* studies of human T cell clones it is established that MHC class II molecules on T cells are required for the induction of specific anergy, although in a few cases such antigen presentation has been found to induce T cell activation. Therefore to mimic the human situation and assess the regulatory role of T cell MHC class II *in vivo* transgenic mice expressing MHC class II of the I-A^b haplotype on their T cells were generated.

The molecular biology required for the preparation of these MHC class II constructs and their subsequent analysis is described. To obtain T cell specific expression a plasmid containing the human CD2 promoter and enhancer was used. Due to the presence of this enhancer expression is not dependent on the site of integration and the level of transgene expression is directly proportional to the number of copies integrated. Once the transgenic line had been derived the offspring were analysed for genomic integration of the transgene by Southern blotting and polymerase chain reaction. Protein expression was detected by cytofluorimetry.

Activated and resting T cells from various lymphoid tissues were analysed for expression of MHC class II and other cell surface molecules. The pattern of expression of cell surface molecules indicative of the presence of a population of T cells that were tolerised, or were more susceptible to tolerisation. The ability of the MHC class II+ T cells to present antigen was examined using T cell hybridomas. Furthermore, antibody, CD4+ and CD8+ T cell responses elicited by transgenic and control mice were compared. The results of these experiments suggest that antibody and CD8+ T cell responses are similar in the transgenic and control mice, however, CD4+ T cell responses exhibit altered cytokine production. Preliminary experiments on peptide-mediated T cell unresponsiveness are also reported. Initial observations suggest that on administration of intranasally delivered peptide, the transgenic T cells are able to downregulate their responses more efficiently and effectively than their control counterparts.

ACKNOWLEDGEMENTS

Firstly I must thank Professor Jonathan Lamb not only for allowing me to embark on this project, but for his help and supervision throughout.

I am also extremely grateful to Dr. Richard Lake for his help and patience in guiding me through arguably the most labour intensive part of this project, as well as for his continual insistence of my ability to finish even during the bleakest times.

Thanks also to my former colleagues at St. Mary's where I started this work, especially to the members of lab.106, Dr. Claerwen Jones, Dr. John Hayball and in particular to Dr. Mark Larché who taught me many of the techniques I needed.

I would also like to thank the many people in the Immunology department at Imperial College who helped me at various times, in particular Dr. Gerry Hoyne for his many pragmatic ideas concerning the direction of the project at various stages and Sandra Hirschberg and Nelofer Syed not only for their technical help and advice but also for their friendship throughout.

Of course my greatest thanks must go to my parents for all their encouragement not just over the last three and a half years but for the duration of my studies. And not forgetting Michael for his endless help and encouragement and generally just for putting up with me.

TABLE OF CONTENTS

Chapter 2 Materials and Methods

LIST OF FIGURES AND TABLES

ABBREVIATIONS

ABC	ATP binding cassette
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
APL	Altered peptide ligand
ATP	Adenosine triphosphate
dATP	Deoxyadenosine 5'-triphosphate
ddATP	Dideoxyadenosine 5'-triphosphate
β2m	β-2 microglobulin
BiP	Ig heavy chain binding protein
BSA	Bovine serum albumin
Ca^{2+}	Intracellular calcium
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CDR	Complementary determining region
CFA	Complete Freund's adjuvant
CLIP	Class II associated invariant chain peptide
CNS	Central nervous system
Con A	Concanavalin A
CR	Coreceptor
CsA	Cyclosporin A
CIITA	MHC class II transactivator
CTL	Cytotoxic T lymphocytes
dCTP	Deoxycytosine 5'-triphosphate
ddCTP	Dideoxycytosine 5'-triphosphate
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
Der p	*Dermatophagoides pteronyssinus*
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
DP	Double positive
DTH	Delayed type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum

ERK	Extracellular signal regulated kinase
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FDA	Fluorescein diacetate
FITC	Fluorescein isothiocyanate
β-Gal	β-galactosidase
GDP	Guanosine 5'-diphosphate
GI	Gastrointestinal
GM-CSF	Granulocyte macrophage colony stimulating factor
GTP	Guanosine 5'-triphosphate
dGTP	Deoxyguanosine 5'-triphosphate
ddGTP	Dideoxyguanosine 5'-triphosphate
GVHD	Graft versus host disease
HA	Hemagglutinin
HDM	House dust mite
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ddH_2O	Double distilled water
HSA	Heat stable antigen
ICAM	Intercellular adhesion molecule
IEL	Intestinal epithelial lymphocytes
IFA	Incomplete Freund's adjuvant
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
IN	Intranasal
IP	Intraperitoneal
IP3	Inositol 1,4,5-trisphosphate
IPTG	Isopropylthio-β-D-galactosidase
ITAM	immunoreceptor tyrosine-based activation motif
IV	Intravenous
JAM	Just another method
JNK	Jun N-terminal kinase
LCMV	Lymphocytic choriomeningitis virus
LCR	Locus control region
LFA	Lymphocyte function-associated antigen
LMP	Low molecular weight proteins
LPS	Lipopolysaccharide
mAb	Monoclonal antibody

MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MIIC	MHC class II compartment
MFI	Mean fluorescence intensity
MHC	Major Histocompatibility Complex
MLR	Mixed lymphocyte reaction
MS	Multiple sclerosis
NK	Natural killer cell
NOD	Nonobese diabetic mice
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PI	Propidium iodide
PKC	Protein kinase C
PLC	Phospholipase C
PLP	Proteolipid protein
PTK	Protein-tyrosine kinase
QR	Quantum red
RAG	Recombinase-activating gene
RNA	Ribonucleic acid
mRNA	Messenger RNA
RNase	Ribonuclease
SC	Subcutaneous
SCID	Severe combined immune deficiency
SDS	Sodium dodecyl sulphate
SEB	Stapylococcal enterotoxin B
SEM	Standard error of the mean
SP	Single positive
T1	Type 1 T cell
T2	Type 2 T cell
TAP	Transporters associated with antigen processing
TBS	Tris buffered saline
TcR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
Tg	Transgene
TGF	Transforming growth factor
Th	T helper cell
TLP	Thymic lymphoid progenitor
TN	Triple negative

TNF	Tumour necrosis factor
TSST1	Toxic shock syndrome toxin 1
dTTP	Deoxythymidine 5'-triphosphate
ddTTP	Dideoxythymidine 5'-triphosphate
UV	Ultraviolet light
Y	Tyrosine

CHAPTER 1
General Introduction

1.1 Antigen processing and presentation

The first step in the generation of a T cell response is presentation of peptide antigen by MHC molecules to the T lymphocyte. Unlike B cells that are able, by virtue of their immunoglobulin (Ig) receptor, to recognise antigen in its native conformation, T cells are only able to recognise short lengths of antigen (peptide) in conjunction with MHC expressed on the surface of another cell. The genetic loci of MHCs of different species vary in size from 2.5 to 4 megabases. Within this area, two families of genes; MHC classes I and II, have been shown to be involved in recognition of foreign antigen and subsequent activation of T lymphocytes. The MHC antigens were defined by Gorer and Snell in the 1930's and '40's, based on work looking at the success of transplanting tumours into inbred strains of mice (reviewed in (Klein J 1975)). It was not until 1974, however, that work by Zinkernagel *et al.* looking at the interactions between LCMV-infected macrophages and LCMV-immune T cells indicated that this association was specific and restricted (Zinkernagel RM and Doherty PC 1974). Around the same time Shevach and Rosenthal discovered the phenomenon of MHC class II-restricted T cell responses whilst looking at guinea pig T cell responses to antigen presented by macrophages (Shevach EM and Rosenthal AS 1973).

Antigen derived from intracellular sources in the cytosol binds to MHC class I molecules whereas antigen acquired from a distinct compartment in the endocytic/lysosomal pathway binds MHC class II. MHC class I antigens are expressed on all nucleated cells, whereas MHC class II is normally only expressed on cells of the immune lineage. Due to universal expression and acquisition of antigen from the cytosol, MHC class I molecules make ideal tools for immune surveillance of intracellular pathogens such as viruses. CD8+ T cells recognise antigen in the context of MHC class I, and therefore constitute one of the main lines of defence against viruses and pathogens with a similar lifecycle. CD8+ T cells kill infected cells directly, whereas CD4+ T cells recruit other cells of the immune system via cytokines on encountering antigen in the context of MHC class II molecules.

Structural Studies

1.1a MHC class I

Crystallisation of the MHC class I molecule provided new insights into the process of MHC restriction and T cell recognition at a molecular level. In particular this work was able to dispell hypotheses such as the suggestion that viral proteins were to

be found on APCs, adjacent to MHC class I molecules thereby allowing joint recognition. In 1987 when HLA-A2.1 was crystallised (Bjorkman PJ *et al.* 1987), it was shown that the peptide lay within a cleft formed at the surface of the molecule.

MHC class I molecules are made up of two polypeptide chains, termed alpha (α) or heavy and beta or beta-2 microglobulin (β or β2m). The α chain is about 44kD and is encoded within the MHC locus. The β chain of about 12kD is non-MHC encoded. The α chain has its carboxy terminal located in the cytoplasm, there is a short (25 amino acids) transmembrane section, with the remainder extending into the extracellular environment. Amino acid analysis reveals the extracellular part of the α chain to be made up of three domains α1, α2 and α3. α1 and α2 are each made up of a long α helix and four strands of β-pleated sheet (**Figure 1.1**).

It is the α1 and α2 domains that combine to form the peptide binding groove. This groove is composed of an eight stranded antiparallel β-sheet flanked by two α helices. It is here that the polymorphic residues are located, with their side chains either pointing into the cleft (thus affecting peptide binding) or towards the top of the helices (affecting TcR binding). The TcR thus recognises the surface of the peptide in the context of the surrounding α-helices.

The α3 domain has an immunoglobulin (Ig)-like disulphide loop, composed of two antiparallel β-sheets. Residues of the α3 domain have been shown to interact with the TcR coreceptor CD8 (Rosenstein Y *et al.* 1989, Salter RD *et al.* 1989).

The β2m displays no polymorphism. Like the α3 domain it contains an Ig-like disulphide loop. These two domains interact with each other in a non-covalent fashion. The β2m also interacts with the β-pleated sheet of the peptide-binding platform. These interactions appear critical for maintaining the MHC class I in its native conformation. It is, therefore, not surprising that *in vitro* studies suggest that β2m is absolutely necessary for peptide binding (Jackson MR *et al.* 1992). However, *in vivo* studies looking at β2m deficient mice show peptide binding can take place in the absence of β2m (Lehman-Grube F *et al.* 1994).

Peptides that bind MHC class I are typically 8-10 amino acids long (Falk K *et al.* 1990, Jardetzky TS *et al.* 1991). Peptide elution studies have shown that one MHC haplotype is capable of binding many peptides displaying sequence and length heterogeneity (Hunt DF *et al.* 1992) and crystallographic studies have confirmed this observation (**Figure 1.2**). MHC class I molecules bind a subset of available peptides. Which bind depends on the length and sequence of the peptide. Elution studies have shown that the peptides obtained are enriched for certain amino acids at certain positions. Two positions that seem to be of particular importance are known as anchors. The amino acids at these positions bind in specificity pockets (polymorphic residues that determine peptide binding ability) in the binding cleft (Garrett TP *et al.* 1989). Despite these observations, other groups have identified peptides that bind

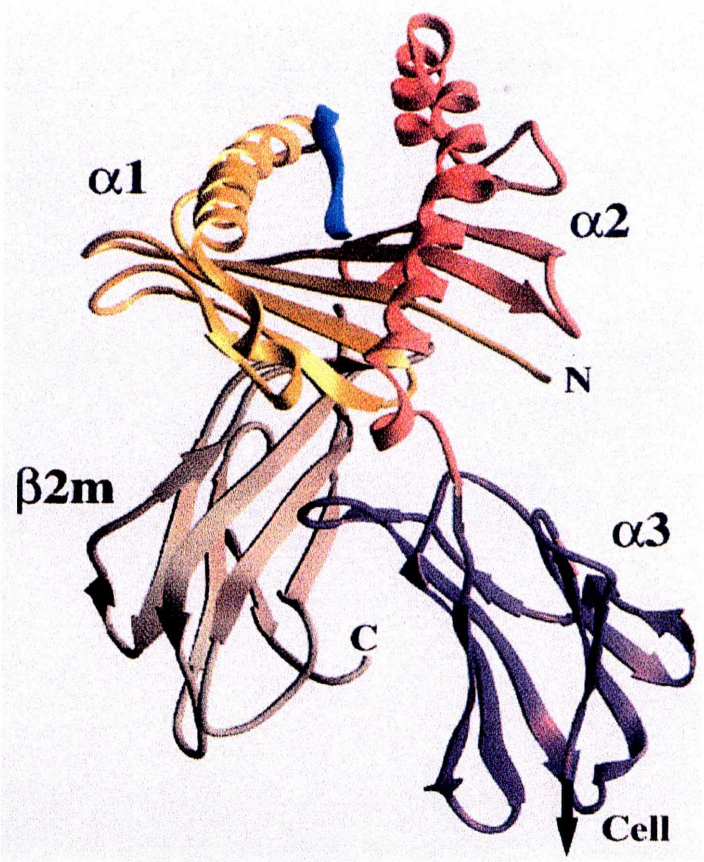


Figure 1.1 Ribbon diagram of the extracellular portion of MHC class I

The three domains of the heavy chain (α1, α3 and α3) are shown in gold, red and purple respectively. β2M is shown in brown.
Reproduced from: *MHC Molecules. Expression, assembly and function* by R.G Urban & R.M Chicz.

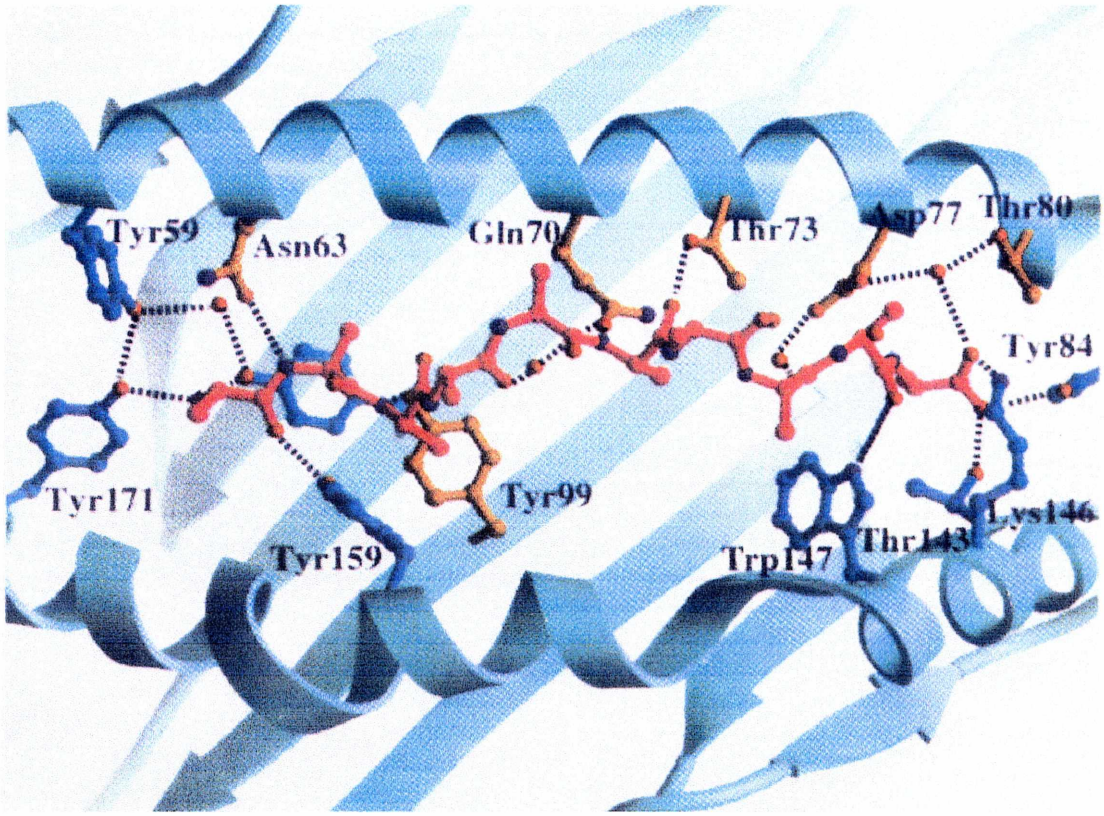


Figure 1.2 Peptide binding to MHC class I

Binding of the peptide **EVAPPEYHRK** to human class I histocompatibility antigen HLA-Aw68. Polymorphic residues are orange, conserved residues are blue.

Reproduced from: *MHC Molecules. Expression, assembly and function* by R.G Urban & R.M Chicz.

MHC class I alleles but are missing the important motif elements (Huczko EL *et al.* 1993). However, other studies found peptides unable to bind despite the presence of the optimal motifs (Ruppert J *et al.* 1993). Taken together, these studies suggest sequence analysis predictions of binding give a general indication as to peptide binding ability but more subtle aspects of peptide selection are clearly involved. The study by Ruppert *et al* also reports the binding of over 400 peptides to purified HLA-A2. Peptides eluted from HLA-A2 were found to be derived from an HLA-A2-like protein (Shimizu Y and DeMars R 1989). The peptides bind the MHC class I molecule in such a way that a 'kink' or arch is found approximately one third of the way from the N-terminus. This lifts the main chain up away from the floor of the cleft (Madden DR 1995).

1.1b MHC class II

It was not until 1993 that an MHC class II molecule was crystallised. Up to this point predictions regarding its structure and peptide binding capacity were made based on the MHC class I crystal. Once DR1 had been crystallised, it was apparent that its structure was indeed similar to that of MHC class I. The two α chain domains, α1 and α2, of DR1 superimpose closely on the corresponding α1 domain and β2m subunit, respectively, of MHC class I. The two β-chain domains, β1 and β2, of DR1 superimpose on the α2 and less closely on the α3 domains, respectively (Brown JH *et al.* 1993).

As with MHC class I the two polypeptide chains are non-covalently associated. The α chain (32 to 34kD) is slightly larger than the β chain (29 to 32kD), due to more extensive glycosylation and both chains have their carboxy termini located in the cytoplasm. Again, they possess a short transmembrane domain (25 amino acids), with the remaining portion located extracellularly (**Figure 1.3**). Both chains are encoded by genes within the MHC. Site-directed mutagenesis studies show that the residues at position 137 and 142 within the β2 domain are absolutely required for the interaction with the CD4 coreceptor (Konig R *et al.* 1992).

The peptide binding groove is formed by the α1 and β1 domains. Like MHC class I, it is made up of eight strands of antiparallel β-sheet as a floor and two antiparallel α-helical regions as the sides. Similarities between the two structures are even evident in the characteristic 'bulges' in the β-sheet and 'kinks' in the α-helices found in the MHC class I molecule. These superimpose almost exactly onto the MHC class II molecule. Clusters of polymorphic residues are located here, and as for MHC class I, those with their side chains pointing into the cleft are involved in peptide binding, whereas those pointing towards the top of the helices are involved in TcR binding.

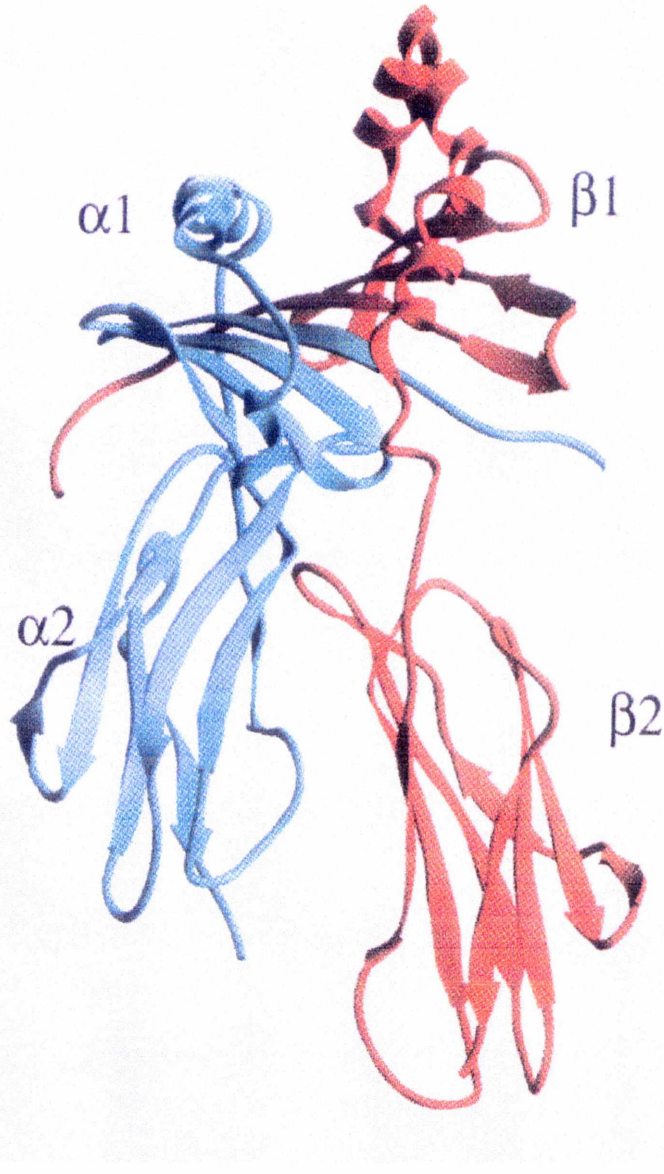


Figure 1.3 Ribbon drawing of MHC class II

The α1 and α2 domains are shown in blue, β1 and β2 domains in red.
Reproduced from: *MHC Molecules. Expression, assembly and function* by R.G Urban & R.M Chicz.

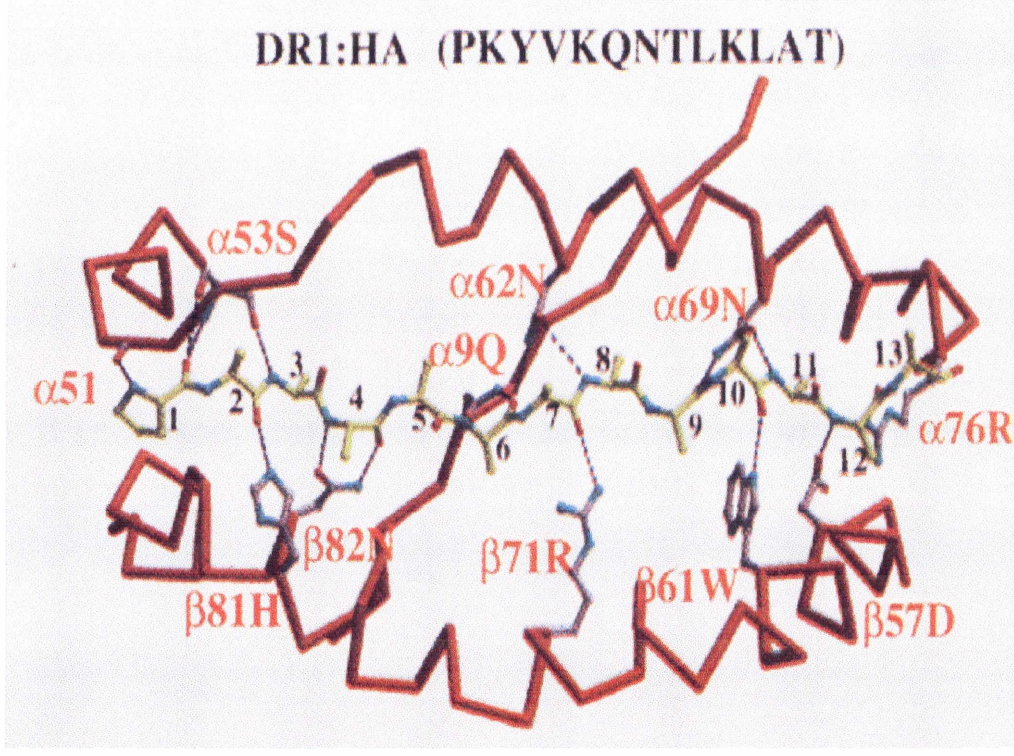


Figure 1.4 Peptide binding to MHC class II

Binding of the HA peptide PK**Y**VK**Q**N**TL**K**L**AT to the MHC class II antigen DR1 (putative anchor residues are underlined and in bold). Conserved residues are numbered.

Reproduced from: *MHC Molecules. Expression, assembly and function* by R.G Urban & R.M Chicz.

One of the fundamental differences between the structures of MHC class I and MHC class II arises due to the MHC class II binding longer peptides (15-24 residues) **(Figure 1.4)**. Whereas in the MHC class I molecule there is a helical region at the amino terminal end of the α1 domain, this is replaced by a stretch of extended chain in MHC class II. The carboxy terminal end of the α1 domain also bends more towards the floor of the peptide binding groove. Additionally, amino acid side chains present in the MHC class I molecule that have been noted to contribute to 'closing off' the ends of the MHC class I site are not present in MHC class II. These differences are responsible for 'opening out' the MHC class II molecule in order to accommodate longer peptides that extend out of the cleft at both ends (Brown JH *et al.* 1993).

Peptide elution studies show that, as is the case for MHC class I, MHC class II molecules are able to bind a large variety of peptides. For each allele of HLA-DR2, DR3, DR4, DR7 and DR8 more than 200 unique peptides have been identified. These peptides were found to be derived mainly from endogenous proteins, even though MHC class II is associated with presentation of exogenously derived foreign peptide (Chicz RM *et al.* 1993).

1.2 MHC class I processing pathways

MHC class I peptides are known to be derived mainly from cytosolic proteins. Over the past few years the importance of the proteasome and the transporter associated with antigen processing (TAP) in the process of peptide delivery to MHC class I has been elucidated.

1.2a The Proteasome

The eucaryotic proteasome is made up of 14 different subunits, each derived from a separate gene. The outer rings are termed α subunits, the inner rings β subunits. It is known that the peptidase activities of the mammalian proteasome reside in the β subunits **(Figure 1.5a)**. Access to the proteasome is by a channel, and only completely unfolded proteins are able to enter (Wenzel T and Baumeister W 1995). Site-directed mutagenesis studies show the importance of a threonine in the proteolytic activity of the proteasome. Use of peptide aldehyde inhibitors indicate sequestering of the proteolytic sites within the proteasome central channel, thus preventing indiscriminate hydrolysis of cytosolic peptides (Seemüller E *et al.* 1995). These inhibitors were also used to show that the proteasome is the major protease responsible for the generation of MHC class I associated peptides. However, these preliminary studies were not conclusive as the inhibitors are not absolutely specific for the proteasome, inhibiting other proteases also (Rock KL *et al.* 1994). Recently, Cerundolo *et al.* have used a proteasome specific inhibitor, lactacystin, to demonstrate a

block in presentation of influenza antigens to influenza-specific CTL. In this study they also found that by expressing the viral protein in the lumen of the endoplasmic reticulum (ER) presentation to CTLs was observed, confirming the specificity of lactacystin for cytosolic proteases (Cerundolo V *et al.* 1997).

The role played by the MHC-encoded subunits LMP2 and LMP7 remains controversial. Upon induction with IFN-γ the LMP2 and the LMP7 are incorporated into the 20s proteasome (Früh K *et al.* 1994). These conditions are reported to favour the generation of peptides with hydrophobic or basic carboxy-termini (Gaczynska M *et al.* 1993). Following translocation by the transporter associated with antigen processing (TAP) these peptides would be predicted to bind preferentially to the MHC class I. These findings have been disputed by other groups who maintain they do not find such preferential peptide generation (Boes B *et al.* 1994). More recently, further studies on LMP2 and LMP7 suggest either preferential production of peptides containing hydrophobic or basic carboxy-terminal residues (Gaczynska M *et al.* 1994), or reduced production of peptides acidic carboxy-terminal residues (Kuckelhorn U *et al.* 1995).

In vivo studies give the most direct evidence for a role of LMP2 and LMP7 in antigen processing. Deletions have been made of both LMP2 (Van Kaer L *et al.* 1994) and LMP7 (Fehling HJ *et al.* 1994). LMP2-/- mice showed no decrease in MHC class I expression, but a 60-80% reduction in CD8+ T cells. LMP7-/- mice had normal numbers of CD8+ T cells, but a 10-45% decrease in MHC class I expression. Concerning peptide generation, both sets of knockout mice displayed a deficiency in the generation of a subset of antigenic peptides. The LMP2-/- yielded five fold lower influenza virus specific CTL precursor frequencies. In the LMP7-/- gave a 50% reduction in response to the HY antigen.

Other IFN-γ inducible genes have been identified as activators of the 20s proteasome. PA28 (11s regulator) is made up of two subunits which bind to the end of the 20s proteasome (Gray CW *et al.* 1994). Studies show that transfection of the PA28α subunit into a murine fibroblast line expressing the murine cytomegalovirus protein pp89, leads to enhancement of recognition by pp89-specific CTLs (Groettrup M *et al.* 1996). The 19s regulator also binds the 20s proteasome, forming the 26s proteasome. *In vivo*, the 20s proteasome exists in at least these two forms.

It is known that most proteins degraded by the 26s proteasome require covalent conjugation to ubiquitin. The requirement of protein ubiquitination in the generation of MHC class I epitopes remains controversial. Ubiquitin is bound by the action of the E1 ubiquitin-activating enzyme. Two groups, using an identical cell line with temperature-sensitive E1 ubiquitin-activating enzymes, have generated conflicting results. Michalek *et al.* found a reduction in presentation of cytosolically loaded ovalbumin at the non-permissive temperature (Michalek MT *et al.* 1993). Whereas Cox *et al.* using the same

cell line, as well as a newly characterised one, found the capacity to ubiquitinate protein at non-permissive temperatures was maintained (Cox JH *et al.* 1995).

These studies therefore suggest that ubiqutination of proteins does seem to be important for degradation by the proteasome. Other approaches have involved looking at proteins modified by addition of destabilising amino-terminal residues. In this case not only was the rate of MHC class I presentation enhanced, but this enhancement could be blocked by proteasome inhibition or by blocking of potential ubiquitination sites on the protein (Grant EP *et al.* 1995).

1.2b Transporters associated with antigen processing (TAPS)

Once peptides are generated by the proteasome they must be translocated into the ER where they combine with the MHC class I complex. Endogenous antigens gain access to MHC class I molecules within the ER via a TAP-dependent pathway and from signal sequences by a TAP-independent mechanism (Wei ML and Cresswell P 1992). Studies have demonstrated that the proteasome is able to interact with the ER membrane (Rivett AJ *et al.* 1992) and may therefore interact, transiently, with the TAP molecules, thus allowing peptide delivery. In the absence of such an interaction, studies have suggested roles for chaperones such as the heat shock protein Gp96 (Suto R and Srivastava PK 1995), which has been reported to bind antigenic peptides.

TAP is a member of the superfamily of ATP binding cassette (ABC) transporters (Deverson EV *et al.* 1990) and is located in the ER/*cis*-Golgi membrane (Kleijmeer MJ *et al.* 1992). TAP is made up of two non-covalently linked protein chains, TAP1 and TAP2 both of which contain an ATP binding domain and a domain predicted to span the membrane 6 to 10 times (DeMars R and Spies T 1992). Peptide binding to TAP is ATP-independent, although ATP is required for peptide translocation (Shepherd JC *et al.* 1993). Use of photoactivatable peptide cross-linkers have revealed that only when the TAP exists as the heterodimer, TAP1/TAP2, are peptides able to bind (Androlewicz MJ *et al.* 1994).

TAP has been shown to bind preferentially to peptides of 8-13 amino acids in length (Androlewicz MJ *et al.* 1993), although it is able to translocate longer peptides, albeit at a lower efficiency (Momburg F *et al.* 1994). Translocation of these longer peptides has lent support to the idea, originally proposed by Rammensee *et al.* that peptide trimming may occur in the ER (Falk K *et al.* 1990). Recently, it has been shown that peptides can be trimmed at both the amino and carboxyl termini within the ER (Snyder HL *et al.* 1994). The efficiency and physiological relevance of this process, however, remains unclear. Studies looking at the translocation rates of optimal length peptides have found varying efficiencies, depending on the peptide sequence. Schumacher *et al.* found that the C-terminal amino acid of the peptide to be of critical importance in rat TAP2^{u} and mouse TAP2 allelic products (Schumacher TNM *et al.*

1994). They found that a hydrophobic C-terminal was required for efficient translocation. By contrast, translocation efficiencies were hardly affected by the nature of the C-terminal amino acid by transporters containing the human TAP2 or rat TAP2^a allelic product (Momburg F *et al.* 1994). Various studies have continued to try to elucidate the determinants of peptide binding to the TAP complex. Recently, Nijenhuis *et al.* identified a peptide binding site composed of both the human TAP1 and TAP2 domains. They provided experimental evidence indicating that the peptide was bound over most of its length, with the major contact site for the peptide found on TAP1 (Nijenhuis M *et al.* 1996).

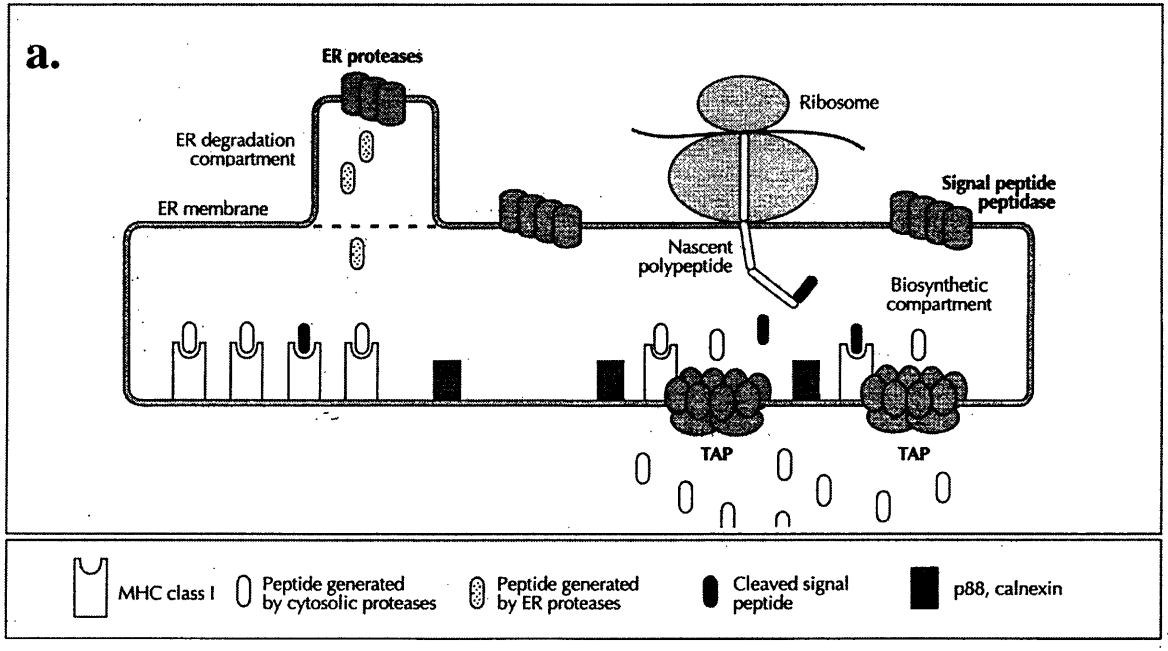
Once the peptide has gained access to the ER it binds to the heavy chain-β2m complex, which is able to interact with the TAP complex. Formation of these ternary complexes results in dissociation from the TAP complex, transit from the ER to the Golgi and finally egress to the cell surface (Ortmann B *et al.* 1994).

Prior to binding peptide, nascent MHC class I heavy chains associate with the ER resident chaperone calnexin. Calnexin mediates disulphide bond formation in MHC class I heavy chains (Tector M and Salter RD 1995), and may promote dimerisation of MHC class I with β2m (Sugita M and Brenner M 1994). It has been shown that β2m is required for binding to TAP, and is able to do so whether or not it is bound to MHC class I heavy chain (Carreno BM *et al.* 1995). During its progression through the ER the MHC class I heavy chains are modified by addition of N-linked high mannose oligosaccharides. As they exit the ER and enter the Golgi, these are converted to a more complex form (Abbas AK *et al.* 1991). An overview of peptide traffic and loading in the ER is shown in **Figure 1.5b**.

Recently, studies have demonstrated the ability of exogenously as well as endogenously derived antigens to generate MHC class I restricted cytotoxic responses. One study reports that following phagocytosis of ovalbumin-coated beads, macrophages were able to present ovalbumin to CD8+ T cells. The process was TAP-dependent, chloroquine-insensitive and proteasome inhibitor sensitive (Kovacsovics-Bankowski M and Rock KL 1995).

1.3 MHC class II processing pathways

In contrast to MHC class I, MHC class II molecules bind mainly exogenously derived peptide. Factors such as mechanism of antigen uptake and degradation affect peptide availability and binding. Furthermore, two additional molecules, the invariant chain (Ii) and HLA-DM influence peptide availability by modulating ligand access to the binding groove during maturation of MHC class II molecules.



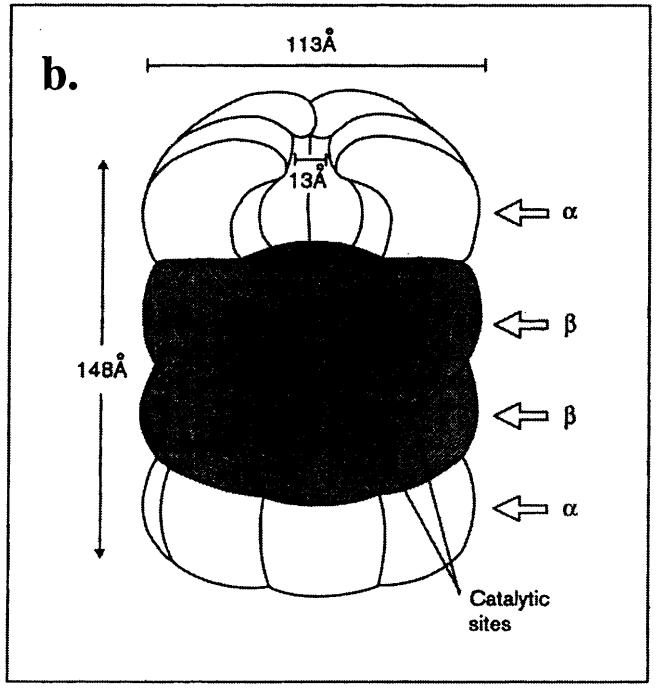


Figure 1.5a Peptide traffic and loading in the ER

Peptides generated by cytosolic proteasomes are delivered to TAP molecules, possibly via a direct interaction between the proteasome and the TAP molecule. TAP and to a lesser extent signal peptide peptidase contribute peptides directly into the biosynthetic compartment where they load effectively into class I molecules that are physically tethered to TAP molecules. Calnexin associates transiently with newly formed class I molecules before they associate with TAP. Peptides generated or modified in the downstream ER degradative compartment are ignored because class I molecules passing near this compartment are already loaded with peptide. Reproduced from: Howard, 1995.

Figure 1.5b Longitudinal and transverse section through 20S proteasome

The 20S proteasome is composed of two outer and two inner rings. The two outer rings each comprise seven copies of the 25.9kDa α subunit. The two inner rings each comprise seven copies of the 22.3kDa β subunit. The rings form three chambers; two antechambers flank a central chamber. The inner chamber contains the 14 catalytic cites (dark grey). The shortest distance between two active sites is 20Å, which may be spanned by a 7-mer or 8-mer peptide in extended conformation. Access to the channel is controlled by four narrow gates, with an entrance of only 13Å, allowing only unfolded proteins access to the active sitres of the protease. Reproduced from: Lehner & Cresswell, 1996.

1.3a Invariant Chain

Nascent MHC class II α and β chains transiently bind an immunoglobulin heavy chain binding protein (BiP), forming high molecular weight aggregates, prior to binding to calnexin (Marks MS *et al.* 1995). Ii chain binds at this stage, and a nonameric $(\alpha\beta)_3Ii_3$ complex is formed that is unable to bind peptide. Calnexin is released, and the nonamer is transported out of the ER, across the Golgi and trans-Golgi network to the endocytic compartments (Anderson KS and Cresswell P 1994). The inability of MHC class II molecules to bind peptides at this stage, has lent support to the idea of Ii preventing cytosolic peptides from binding whilst the MHC class II is still in the ER. *In vitro* studies show that in the absence of Ii, MHC class II is able to bind antigenic peptides in ER microsomes (Bijlmakers M-J *et al.* 1994), and that expression of Ii inhibits the presentation of a cytosolically expressed peptide (Long EO *et al.* 1994). Ii is also believed to play an important role in the maintenance of MHC class II conformation. The observation that in Ii-negative cells MHC class II αβ dimers aggregate in the ER (Marks MS *et al.* 1995) gives support to this idea. Studies using Ii knockout mice and Ii-negative transfectants show the presence of SDS-resistant αβ dimers, that are able to bind a heterogeneous set of polypeptides (Bikoff EK *et al.* 1995, Busch R *et al.* 1996). These complexes are generated in the ER, and Bodmer *et al.* have reported that MHC class II molecules also bind antigens expressed in the secretory pathway in Ii knockout mice (Bodmer H *et al.* 1994). At this stage, Ii therefore acts to prevent formation of MHC class II αβ dimer aggregates, and precludes binding of endogenously derived peptide in the ER.

MHC class II transit to the endosome is also regulated by Ii. Ii contains at least two motifs within its cytosolic tail that are involved in targeting to the endosome (Pieters J *et al.* 1993). These are leucine-based signals found within the α-helical structures (Motta A *et al.* 1995). On arrival in the endosome, the Ii of the $(\alpha\beta)_3Ii_3$ complex undergoes sequential proteolysis initiating from its lumenal carboxy terminus. This gives rise to 22kDa and subsequent 10kDa fragments that remain associated with the MHC class II (Amigorena S *et al.* 1995). Aspartic proteases have been implicated in the initial stages of proteolysis and thiol proteases in the latter (Maric MA *et al.* 1994, Xu M *et al.* 1994). The final product of Ii chain degradation is a nested set of class II-associated Ii peptides (CLIPs), which span residues 81-104 of Ii (Avva RR and Cresswell P 1994). CLIPs, like intact Ii, act to block the peptide binding groove of the αβ dimer, and must be removed prior to antigenic peptide loading. Studies of CLIP associated and disassociated with the Ii, show that the CLIP region of Ii is disordered, whereas the remainder of the molecule consists of an α-helical coil (Jasanoff A *et al.* 1995, Park S-J *et al.* 1995). CLIP could inactivate the groove either by binding directly to the groove itself, or by attaching itself to the outside resulting in a conformational change that would inhibit binding. Binding studies making use of substituted peptides

reveal CLIP anchor residues interact with similar specificity pockets within the binding groove as do antigenic peptides (Geluk A *et al.* 1995, Malcharek G *et al.* 1995). The kinetics and structural requirements for CLIP binding have also been shown to be affected by MHC class II polymorphism, although to a lesser extent than for antigenic peptide binding (Geluk A *et al.* 1995, Sette A *et al.* 1995). In 1995, X-ray crystallography provided confirmation that CLIP occupies the peptide-binding groove when Ghosh *et al.* demonstrated that CLIP bound within the groove of HLA-DR3 (Ghosh P *et al.* 1995). Compared to the X-ray structure of influenza peptide HA307-319 binding HLA-DR1 (Stern LJ *et al.* 1994), the locations of the side-chains/pockets, hydrogen bonds and buried surface area are similar. The CLIP anchor residues, however, are methionines which would confer the greater flexibility needed in binding multiple MHC class II alleles. This work also identified a 'core' region of CLIP (residues 89-101) which was the region that contacted the groove. The remaining part of CLIP appeared to protrude from the groove. This region, together with some CLIP flanking Ii peptides bind to the outside of the MHC class II molecule and inhibit binding of certain anti-class II antibodies, as well as the superantigens staphylococcal enterotoxin B (SEB) and toxic shock syndrome toxin 1 (TSST1) (Ericson ML *et al.* 1994, Romagnoli P and Germain RN 1994).

Despite the plethora of evidence pointing towards a role in MHC class II processing for Ii, there is also evidence for Ii-independent MHC class II transport. In one such study, the presentation of immunodominant epitopes from inactivated influenza virus and myelin basic protein were shown to correlate with recycling of surface HLA-DR molecules. Truncation of either α or β cytoplasmic tails led to an almost total abrogation of peptide presentation. However, in cells transfected with the Ii no effect on peptide presentation was observed (Pinet V *et al.* 1995). This study therefore not only proposes that there is a second pathway by which exogenously derived peptide is able to bind MHC class II, but that this pathway is also Ii-independent.

1.3b HLA-DM

The involvement of another subsidiary molecule in MHC class II processing was proposed in 1990, when Mellins *et al.* made a mutant B cell line that had no defect in its MHC class II genes or Ii (Mellins E *et al.* 1990). This cell line failed to present native exogenous protein, but was able to present immunogenic peptides derived from these proteins. The mutation seemed to be affecting the conformation of mature MHC class II dimers. This defect was mapped to a region within the MHC locus (Mellins E *et al.* 1991) and was termed DM (Kelly AP *et al.* 1991).

HLA-DM (H2-M in the mouse), is a relatively non-polymorphic, heterodimeric glycoprotein with weak sequence similarity to MHC class II (Karlsson L *et al.* 1994).

Expression of HLA-DM genes is coregulated with MHC class II genes (Kelly AP *et al.* 1991) although HLA-DM protein levels are much lower than MHC class II, possibly suggesting a catalytic role for DM (Denzin LK and Cresswell PA 1995). Further work on the mutant cells revealed that lack of DM resulted in the majority of MHC class II molecules remaining associated with CLIP, with a subsequent accumulation of the MHC class II-CLIP complexes observed. However, when *DMA* and *DMB* were transfected back into these lines, CLIP was exchanged for immunogenic peptides, thereby restoring the antigen presenting function of the cell (Denzin LK *et al.* 1994).

Studies using either DM expressed as a recombinant soluble molecule (sDM) (Sloan VS *et al.* 1995), purified from mammalian cells (Denzin LK and Cresswell PA 1995) or reconstituted in membranes (Sherman MA *et al.* 1995), have demonstrated that DM mediates the rapid release of CLIP from a variety of MHC class II molecules at endosomal pH. This results in accelerated binding of immunogenic peptides to MHC class II molecules. As well as enhancing the dissociation of CLIP, DM has been shown to induce dissociation of the 22kDa Ii species, but it has no effect on the full-length Ii (Denzin LK and Cresswell PA 1995). DM can also enhance dissociation of peptides, unrelated to CLIP, such as myelin basic protein (Sloan VS *et al.* 1995) and it also has the capacity to induce the formation of SDS-stable MHC class II-peptide complexes in the absence of Ii (Karlsson L *et al.* 1994). DM, however, is not able to cause dissociation of all peptides from MHC class II. One study showed that DM was capable of removing CLIP from DR3, but failed to remove the influenza derived peptide HA307-319 from DR1 (Sloan VS *et al.* 1995). The crystal structures of DR3-CLIP and DR1-HA307-319 are very similar however, the spontaneous dissociation rates are considerably different, that of DR3-CLIP being 11 hours, compared with 6 days for DR1-HA307-319. This implies that even in the absence of DM, the DR3-CLIP complex is more likely to exist as an open, exchange-prone structure. In the presence of DM, this conformation may be augmented and CLIP itself has been implicated in contributing to this open structure. Studies have shown that the amino-terminal CLIP segment Ii81-89 may interact with a site adjacent to the MHC class II groove to induce an open MHC class II conformation (Kropshofer H *et al.* 1995). Allelic variation has also been shown to be a decisive factor on DM dependence. I-A^k MHC class II molecules are able to present immunogenic peptides almost as well in DM-negative lines as in those containing DM (Brooks AG *et al.* 1994). In this case DM independence is suggested to result from the ability of CLIP to spontaneously release from the MHC class II molecule in less than 1 hour, well within the endosomal residence time of the MHC class II molecules. This explanation is not true in all cases, however. The I-A^d allele, which has a much higher affinity for CLIP, is also able to present a set of peptides via a DM-independent mechanism (Stebbins CC *et al.* 1995).

In this case the stability of empty MHC class II heterodimers, due to allelic variation may influence dependence on DM (Bikoff EK *et al.* 1995).

DM appears to act in a catalytic capacity. This hypothesis is supported by the observations that the kinetics, but not the affinity of peptide binding is affected by DM (Sloan VS *et al.* 1995), and that small amounts of DM facilitate the removal of CLIP from MHC class II, thus suggesting that it is released from peptide-loaded cells and is subsequently re-used (Denzin LK and Cresswell PA 1995). DM dissociation from MHC class II has been attributed to a conformational change in the MHC class II molecule on peptide binding (Sadegh-Nasseri S *et al.* 1994). Alternatively DM binding to MHC class II may be pH sensitive, in which case DM would dissociate from MHC class II on export to the cell surface and would have to be transported back to the endosomal compartment. This mechanism for DM removal gains support from the observation that low levels of DM have been detected at the cell surface (Karlsson L *et al.* 1994), and the existence of a retrieval pathway for DM, dependent on the amino acids Tyr-Thr-Pro-Leu in the DMβ chain cytoplasmic chain (Lindstedt R *et al.* 1995).

Recent studies have elucidated the cellular compartments where the various stages of MHC class II processing take place. The MHC class II α and β chains are synthesised in the ER. Here they form high molecular weight aggregates, firstly binding BiP, and then calnexin (Marks MS *et al.* 1995). Ii also associates at this time, and calnexin is released on egress from the ER, the MHC class II/Ii complexes then traverse the Golgi and *trans*-Golgi network and subsequently arrive at the endocytic compartments.

Peptide-MHC class II complexes form in endosomal/prelysosomal compartments known as a class II MHC-rich compartment (MIIC). These have a multivesicular morphology and are thought to be specialised for antigen presentation (Amigorena S *et al.* 1994, West MA *et al.* 1994). Ii chain proteolysis and CLIP release are also believed to take place here. HLA-DM has also been detected in this compartment, however, studies looking at murine B cells have observed HLA-DM and peptide-MHC class II complexes more broadly distributed throughout the endocytic pathway (Castellino F and Germain RN 1995). The characteristics of these compartments, therefore, appear to vary depending on the cell type used. Availability of antigenic peptides in these compartments is dependent on factors such as method of antigen uptake, selective delivery of antigen to the MIIC and action of proteolytic enzymes (Lanzavecchia A 1995). Once peptide loading has occurred, the complex is transported to the cell surface via an unknown mechanism. This could involve fusion of part of the MIIC directly with the cell surface or by making use of the vesicles utilised in the recycling pathway. Transferrin receptors that are known to recycle between the endosomes and cell surface, have been detected in the MIIC, thus raising the possibility

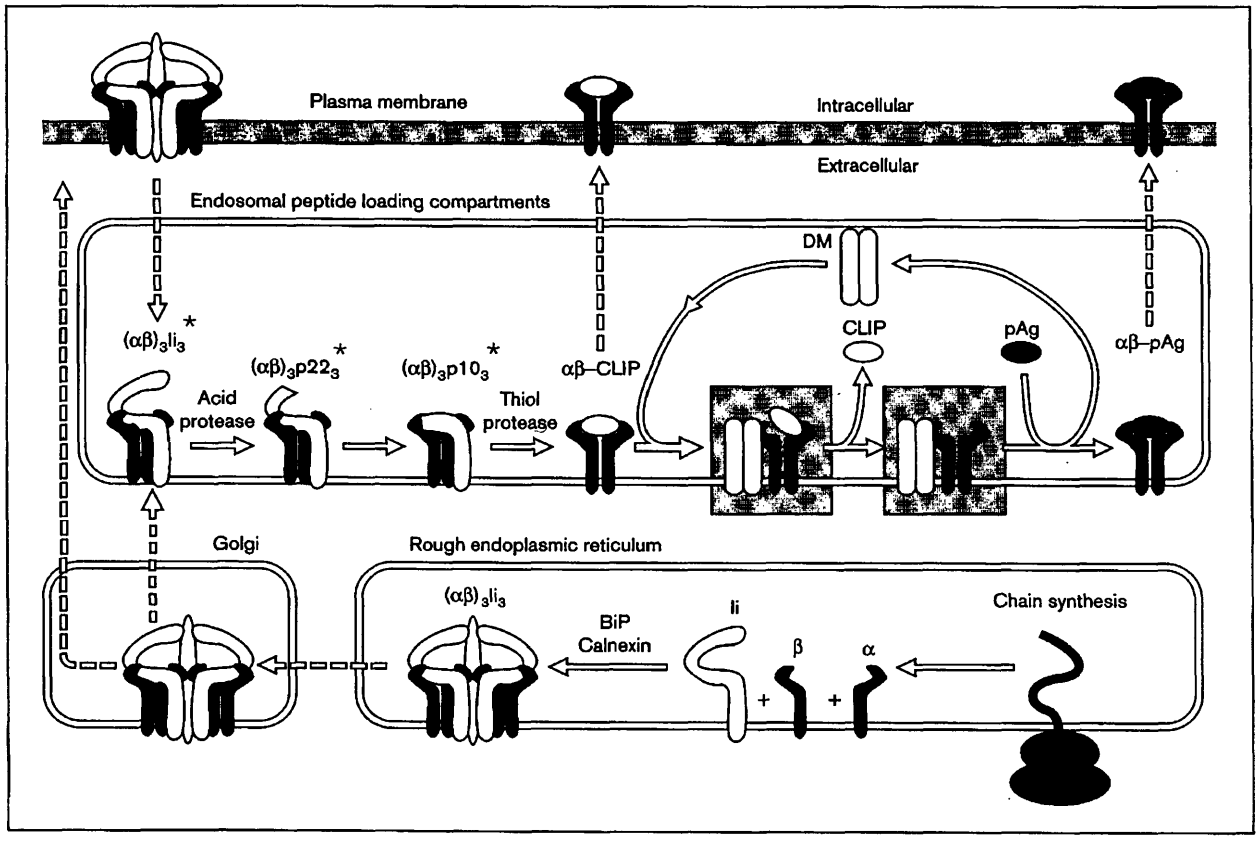


Figure 1.6 Intracellular transport and maturation of MHC class II molecules

The fate of newly synthesized class II α, β and Ii chains (bottom right) is followed through the cell. Changes in non-covalent associations and processing steps are shown in continuous arrows; intracellular transport steps are shown in broken arrows. At branch points the thicker arrow denotes the more abundant pathway in normal EBV-transformed B cells. Shaded boxes indicate hypothetical intermediates in DM-catalyzed CLIP removal. The point at which DM dissociates from αβ-peptide complexes and the export pathway from peptide loading compartments to the plasma membrane are unknown. Ii has two trimerization domains: one amino-terminal to the CLIP region and the other close to the carboxyl terminus of Ii.
* For clarity, nonameric Ii processing intermediates in endosomes are drawn as heterodimers.
Reproduced from: Busch & Mellins, 1996,

that the recycling pathway may be used (Mellman I *et al.* 1995). An overview of the transport and maturation of MHC class II is depicted in **Figure 1.6**.

Just as exogenously derived antigens are able to be presented by MHC class I, endogenous antigen can also be presented by MHC class II. One study, looking at processing of live influenza virus, shows presentation of matrix protein (M1 17-31), in a chloroquine-insensitive, brefeldin A sensitive manner. This would suggest the antigen binding in the ER, due to absence of acidic endosomal compartments (Nuchtern JG *et al.* 1990). A second study looked at presentation of the influenza virus transmembrane antigen H3. Two pathways were identified, both chloroquine sensitive. One involved transport of short peptides in a TAP-dependent manner, the other pathway was TAP-independent, possibly involving the further processing of the H3 peptides in an endosomal compartment (Malnati MS *et al.* 1992).

1.4 T lymphocyte development

T lymphocytes differentiate from hematopoietic stem cells into antigen-reactive T lymphocytes within the microenvironment of the thymus. Population of the murine thymus by these stem cells begins on day 11 of foetal life, with differentiation also beginning at this time (Zlotnik A and Moore TA 1995). During foetal life these stem cells derive from the yolk sac and liver, in the adult from the bone marrow. In the foetus thymic ontogeny proceeds in an ordered fashion, with stem cells maturing in waves. The first set of T cells to appear are those expressing the γδ T cell receptor (TcR). After a series of γδ waves, αβ TcR+ cells appear. Studies looking at development of γδ and αβ T cells by deleting α, β, or δ TcR genes show that the γδ T cell population develops independently of the αβ T cells, as ablation of one subset does not lead to disappearance of the other (Itohara S *et al.* 1993, Mombaerts P *et al.* 1992, Philpott KL *et al.* 1992).

1.5 γδ T cells

γδ T cells represent a set of T cells with specificity and function distinct from that of αβ T cells. Two subtypes of γδ T cells have been identified. Those present in lymphoid tissue appear to originate in the thymus, whereas γδ T cells in epithelial tissue seem to be of a thymic-independent origin.

The earliest waves of γδ T cells display a complete absence of diversity due to lack of expression of the enzyme TdT, responsible for catalysing N-linked glycosylation patterns (Allison JP and Havran WL 1991). These precursor cells do, however, undergo intrathymic differentiation prior to emigration into the periphery, as measured by differential expression of TcR and heat stable antigen (HSA) (Kelly KA *et al.* 1993, Leclercq G *et al.* 1993). Subsequent γδ T cell waves exhibit restricted γ and δ

gene segment usage, but extensive junctional diversity due to initiation of TdT expression is now achieved (Asarnow DM *et al.* 1993, Itohara S *et al.* 1993). In contrast epithelial γδ T cells express a restricted set of tissue-specific V-region genes with no junctional diversity (Havran WL and Boismenu R 1994).

Studies using knockout mice have been carried out to assess the factors required for normal γδ T cell development. CD3ζ- and ζ/η-deficient mice established that ζ was critical for αβ T cell development, but was not required for development of γδ T cells present in either lymphoid or epithelial tissues (Malissen M *et al.* 1993, Ohno H *et al.* 1993). Epithelial γδ T cells have subsequently been shown to use the Fc receptor γ chain as a component of the CD3 complex instead of ζ (Ohno H *et al.* 1994). Other work looking at $p56^{lck}$ -deficient mice revealed a block in the thymic development of γδ T cells. Appearance of thymic-independent γδ intestinal epithelial lymphocytes (IELs) would suggest a differential requirement between these two γδ T cell populations for $p56^{lck}$, and therefore possibly for other, as yet undetermined, developmental or signal transduction molecules (Penninger J *et al.* 1993).

Studies of β2m mutant mice report normal γδ T cell development, with no abnormalities in their distribution or V gene usage (Correa I *et al.* 1992). Similar studies looking at MHC class II-knockout mice, once again indicate there is no role for classical MHC molecules in γδ T cell development or expression in either lymphoid or epithelial tissue (Bigby M *et al.* 1993). γδ T cell clones and hybridomas that do not require MHC gene products for antigen recognition have also been reported (Havran WL *et al.* 1991, O'Brien RL and Born W 1991). A notable exception being some $V\gamma2^+$ T cell clones that appear to undergo αβ-like development and are selected on MHC class I (Pereira P *et al.* 1992, Wells FB *et al.* 1993).

γδ T cells appear to recognise a wide spectrum of antigens. Candidates most likely to play such a role are keratinocyte antigens, found in the skin (Havran WL *et al.* 1991), and mycobacterial heat shock proteins (Haas W *et al.* 1993). Vγ3/Vδ1, a dendritic epidermal T cell, has been shown to recognise self antigens expressed on stressed neighbouring keratinocytes (Havran WL *et al.* 1991), murine Vγ1/Vδ6 hybridoma cells recognise mycobacterially-derived peptides (O'Brien RL *et al.* 1992). Molecular modelling of the complementary determining region 3 (CDR3), suggests that the γδ TcRs are more similar to immunoglobulin than to αβ TcR (Rock EP *et al.* 1994), leading to the hypothesis that αβ and γδ T cells recognise antigen in a different way. Further evidence supporting this theory came from the observation that MHC class I and class II antigen processing pathways were not needed for antigen recognition by some MHC class I and class II molecule restricted γδ T cell clones. In this work it was also reported that the γδ T cell clones did not appear to be recognising a peptide bound within the MHC, but that the TcR may be contacting the MHC in a region distinct from the peptide-binding groove (Schild H *et al.* 1994).

1.6 γδ T cell function

γδ T cells appear to collaborate with αβ T cells to mount effective immune responses *in vivo* against certain pathogens. Mombaerts *et al.* selectively depleted mice of either γδ or αβ T cells. They found that either population was capable of eliciting a primary response to *Listeria monocytogenes*, but only αβ could provide resistance to secondary infection (Mombaerts P *et al.* 1993). Studies looking at malaria responses found increased numbers of γδ T cells, this expansion appearing to require activated CD4+ T cells. Resolution of blood-stage malaria also appeared to require the presence of both T cell populations (Van Der Heyde HC *et al.* 1993). These studies suggest that γδ T cells play a role in conventional T cell functions, indeed studies have demonstrated that the majority of γδ T cells display cytolytic activity upon activation (Spaner D *et al.* 1993). Despite the absence of CD4 or CD8 molecules, γδ T cells display a similar phenotype to αβ T cells (Leclercq G *et al.* 1993, Zorbas M and Scollay R 1993). Some γδ T cells require co-stimulation through CD28-B7 interactions (Sperling AI *et al.* 1993), and the lymphokines produced on stimulation through the antigen-receptor appear to be similar to those produced by αβ T cells (Yamamota M *et al.* 1993). However, γδ T cells, such as those found in the epidermis have been found to release unique cytokines, upon activation these epidermal γδ T cells produce a keratinocyte-specific growth factor (Havran WL *et al.* 1991).

In conclusion, γδ T cells collaborate with αβ T cells in the development of protective immune responses. Under these conditions they seem to respond in a similar way to αβ T cells regarding production of lymphokines or induction of cytolytic responses. However, γδ T cells, also appear to be able to produce unique cytokines, dependent on their location, supporting the hypothesis that resident γδ T cells play a role in local immune surveillance (Havran WL and Boismenu R 1994).

1.7 Development of αβ T cells

The fate of a hematopoietic stem cell arriving in the thymus is dependent on its interactions with the thymic stroma. The earliest thymic immigrants are known as thymic lymphoid progenitors (TLPs). These cells express the thymic homing receptor CD44 and CD117, which together with the IL-7 receptor promote thymocyte survival and growth. Low levels of Thy-1 and heat stable antigen (HSA) are also expressed (Zlotnik A and Moore TA 1995). TLPs lack expression of CD4, CD8 and CD3 leading to Shortman and colleagues terming these cells 'triple negatives' (TNs) (Wu L *et al.* 1991). They found that these cells gave rise to all the other thymocyte subsets, suggesting that these were the earliest progenitor cells. They also observed differences between progenitors recovered from adults compared with those from a foetus. Progenitors from adult mice were found to express low levels of CD4, whereas those

from foetal mice did not. It was also found that upon intravenous injection of adult progenitors TLPs gave rise not only to T cells, but to B cells and CD8α+ dendritic cells, additionally, TLPs from a foetal thymus gave rise to natural killer cells.

Induction of CD25 expression occurs after approximately one day in the thymus, defining entry of the thymocytes into the pro-T cell stage (Amagai T *et al.* 1995, Moore TA and Zlotnik A 1995). At this stage the thymocytes begin to proliferate, and the induction of gene products such as NF-κB, NF-AT and AP-1 as well as the appearance of cell surface markers such as CD54 and CD59 occurs (Zúñiga-Pflücker JC *et al.* 1993). Despite the acquisition of this 'activated' phenotype, the TCR-β and TCR-γ genes remain in their germline configurations (Dudley EC *et al.* 1994). Commitment to the T cell lineage occurs during the transition from the TLP to the pro-T cell. Production of the cytokines IL-1α and TNF-α occurs at this stage, and these cytokines are believed to push the thymocytes into their 'activated' state by causing induction of CD25 (Zúñiga-Pflücker JC *et al.* 1995).

The next stage of thymocyte differentiation is characterised by the loss of CD44 and CD117. The thymocytes are now known as early pre-T cells, 60% of TN thymocytes are at this stage. Proliferation slows down, and TCR-β gene rearrangement begins. It is at this stage that thymocyte maturation in mice with severe combined immune deficiency (SCID) or with a mutated recombinase-activating gene (RAG) stops (Pénit C *et al.* 1995). The TCR-β protein is now known to form a complex (pre-TcR) with a 33kD glycoprotein (gp33) termed 'pre-Tα'. Pre-Tα is a transmembrane protein, with a single extracellular immunoglobulin-like structural domain, and an intracellular portion that contains potential signalling motifs (Saint-Ruf C *et al.* 1994). The pre-TcR complex transduces a selective signal through CD3ε and $p56^{lck}$, thereby allowing differentiation of the thymocytes to the CD4+CD8+ or double positive (DP) stage. It is believed this signal may act by inhibiting further V(D)J recombination and establish allelic exclusion at the TCR-β locus (Anderson SJ and Perlmutter RM 1995, Levelt CN and Eichmann K 1995).

Differentiation to the late pre-T cell stage is characterised by the loss of CD25 expression and further proliferation. Few early pre-T cells make it to this stage, probably due to aberrant rearrangement at the β chain locus (Pénit C *et al.* 1995). The TcR-α locus begins to rearrange at this stage (Zlotnik A and Moore TA 1995), and these newly synthesised α chains displace the pre-Tα complex, with the αβ complex replacing the pre-TcR complex. At this point expression of the pre-Tα gene switches off and proliferation ceases, resulting in the large DP thymocytes evolving into small, resting DP thymocytes (Saint-Ruf C *et al.* 1994). An overview of the stages of lymphocyte development described so far is depicted in **figure 1.7**.

The pre-TcR complex is thought to act as a checkpoint, ensuring survival only of those thymocytes that are immunologically useful. Prior to the next stages of positive

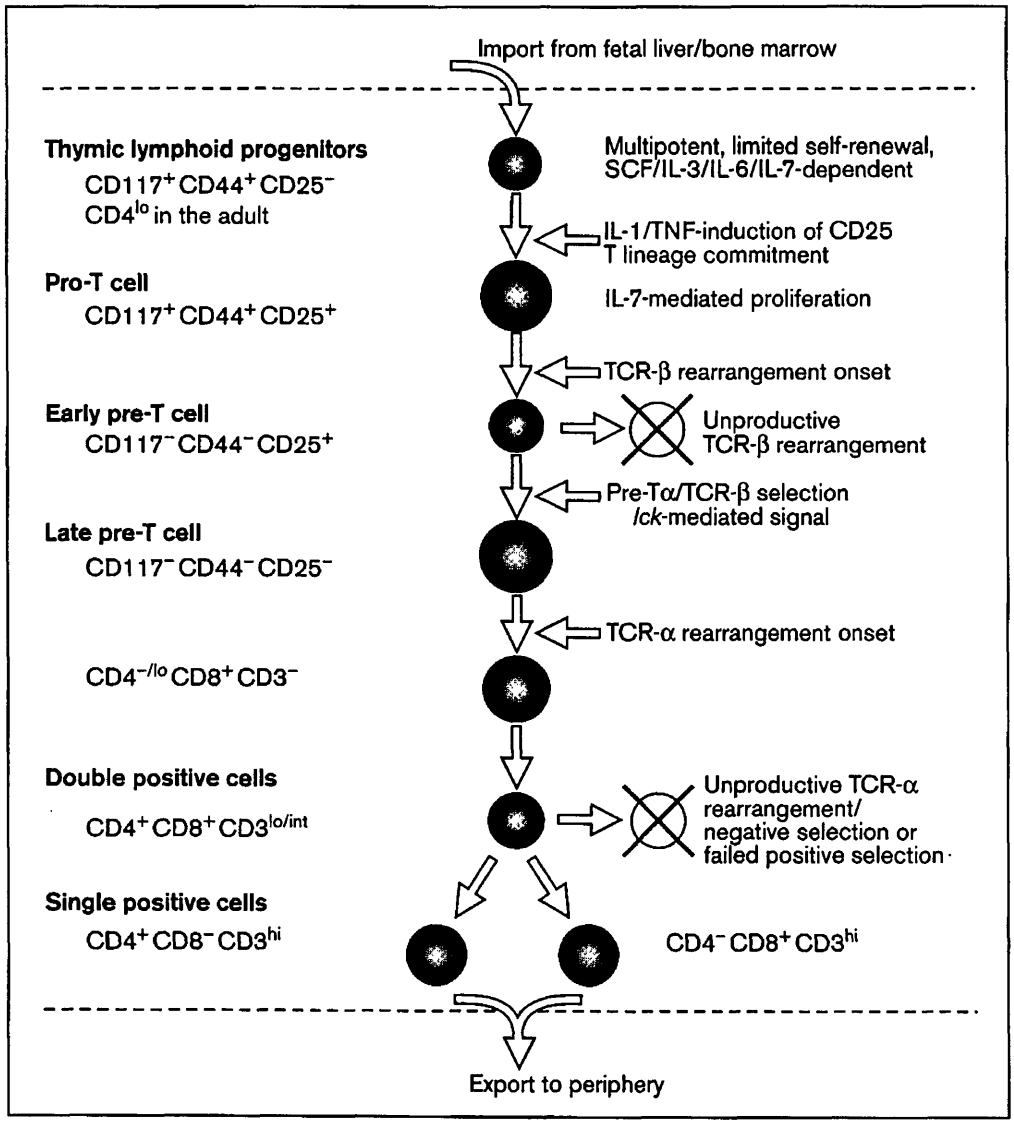


Figure 1.7 Early stages of murine αβ T cell differentiation

Simplified scheme of the stages of mouse αβ T cell differentiation that precede the TCR+CD4+CD8+ (DP) stage. The relative size of the thymocyte symbols indicate their relative proliferative status.

Reproduced from: Zuñiga-Pflücker & Lenardo, 1996.

and negative selection, thymocytes are reported to die by apoptosis if successful β or α chain rearrangements do not occur.

1.8 Positive and Negative selection.

The TcRαβ complexes on the developing DP thymocytes must be able to recognise self MHC-peptide complexes on the thymic stroma. This recognition event gives the thymocyte a survival signal, and allows it to differentiate into a TcR^{hi} CD4 or CD8 single positive (SP) T cell. This stage is termed positive selection. It has been shown that most DP thymocytes express TcRαβs that are unable to recognise self MHC-peptide complexes, and these are destined to die by apoptosis within 3-4 days (Robey E and Fowlkes BJ 1994). The DP thymocytes that are positively selected express only low densities of the self MHC-restricted TcRαβ. If, however, the TcRαβ complex ligates a higher density of self MHC-peptide complexes, or the signal is somehow qualitatively different, these thymocytes are then deleted in the thymus. This process is termed negative selection (Janeway Jr. CA 1994).

1.8a Positive selection.

Positive selection *in vivo* is associated with a down regulation of RAG expression, although complete loss does not occur until the mature SP stage (Kouskoff V *et al.* 1995). *In vitro* studies looking at the earliest stages of positive selection show that cross-linking of TcR on TcR^{lo} DP thymocytes causes upregulation of TcRα as well as CD5 and Bcl-2 (Kearse K *et al.* 1995). Intrathymic adoptive transfer studies suggest continued engagement of the TcR is required for further maturation and differentiation of the resulting $TcR^{int/hi}$ DP thymocytes into mature SP cells (Kisielow P and Maizek A 1995). Use of a reaggregation culture system lends support to this hypothesis, showing that $TcR^{int/hi}$ DP thymocytes generated 10-20 fold more SP thymocytes when cultured in the presence of thymic epithelial cells than when cultured alone (Wilkinson RW *et al.* 1995).

$TcR^{int/hi}$ DP thymocytes are thought to undergo lineage commitment at this stage. $TcR^{int/hi}$ CD4+8^{lo} and $TcR^{int/hi}$ $CD4^{lo}$8+ or coreceptor (CR) transitional thymocyte populations were identified several years ago (Guidos CJ *et al.* 1990).Two theories have been put forward to try to explain the mechanism of positive selection. The first, the instructive model, suggests that differential signalling by CD4 and CD8 causes DP thymocytes to extinguish expression of the other irrelevant CR (Robey EA *et al.* 1991). The stochastic model, proposes that TcR engagement by the DP thymocytes leads to stochastic loss of one of the coreceptors (Von Boehmer H 1986). Studies have shown that $CD4^{lo}$8+ develop independently of MHC class I, whilst the CD4+$CD8^{lo}$ develop independently of MHC class II (Davis CB *et al.* 1993). This evidence therefore

supports the stochastic model of lineage commitment. The results of experiments reported by Dyall and Nikolic-Zugic have caused them to question the concept of CD4+CD8^{lo} thymocytes being committed to the CD4 lineage. Using intrathymic transfer they give evidence that a small number of these thymocytes are able to differentiate into CD8+ T cells (Dyall R and Nikolic-Zugic J 1995). Further studies have demonstrated that CD4+CD8^{lo} phenotype does indeed represent a population that contains a mixture of CD4- and CD8-committed thymocytes. However, the CD4^{lo}CD8+ population seems to be made up of CD8-committed cells alone, thus suggesting lineage commitment at this stage for this set of thymocytes (Lundberg K *et al.* 1995, Suzuki H *et al.* 1995). Suzuki *et al.* also observed that the appearance of CD8-committed cells from CD4+8^{lo} or CD4^{lo}8+ was dependent on prior TcR-MHC class I interactions, whereas appearance of CD4-committed cells from CD4+8^{lo} cells did not depend on interactions between TcR and MHC class II (Suzuki H *et al.* 1995). This observation led to the hypothesis that development of the CD8 lineage is governed via an instructional mechanism, whereas CD4 lineage development occurs via a stochastic mechanism.

Positive selection requires not only recognition of the MHC by the DP thymocyte, but also of self peptide contained within the binding groove. Studies show that mice lacking TAP-1 positively select a diverse repertoire of functional CD8+ T cells only when crossed with a mouse expressing the human transgene for β2m (Van Santen HM *et al.* 1995). This may suggest that selection is occurring either on empty MHC class I molecules or that the peptides utilised are derived from a TAP-independent source. Alternatively, it has been shown that the non-classical MHC class Ib molecule, CD1, is dependent on β2m, but independent of TAP for its expression (Brutkiewicz RR *et al.* 1995). The β2m transgene may therefore have restored expression of CD1, which was then used as the selective molecule. It has, therefore, been hypothesised that CD1 and other MHC class Ib molecules may have some role to play in the selection of CD8+ T cells.

Evidence is emerging that selection of CD4 SP T cells also relies on peptide recognition. Ii chain knockout mice were crossed with three different MHC class II-specific TcR transgenics. Positive selection did not occur in mice expressing two of the TcR specificities, however, selection did take place in those expressing the third TcR (Tourne S *et al.* 1995). These results indicating peptide playing a part in the selection of these cells, although as seen in the CD8 selection studies some CD4 cells can be selected by MHC class II-peptide complexes generated independently of Ii. Another study inserted the transgenes for the Ii chain isoforms p31 and p41 into an Ii knockout mouse. They were expressed at levels too low to observe normal peptide loading or cell surface expression of MHC class II, however, efficient selection of CD4+ T cells was observed (Naujokas MF *et al.* 1995). It has been proposed that the MHC class II-CLIP

complex may be responsible for mediating the efficient positive selection seen in these mutant mice (Guidos CJ 1996).

This work therefore suggests a direct role for Ii in CD4+ T cell positive selection, as opposed to its requirement merely to restore MHC class II and peptide loading to normal levels.

1.8b Negative Selection

As mentioned above, any DP thymocyte expressing a TcRαβ complex specific for self MHC-peptide may be deleted under certain circumstances within the thymus. This process must occur to enable the deletion of T cells with receptors specific for self-peptides and self-MHC, thus preventing self-reactive SP T cells emigrating from the thymus resulting in the potential for autoimmune disease. Two mechanisms have been proposed to explain the phenomenon of negative selection. The first, termed the affinity model predicts that the ligation of a low number of self MHC-peptide complexes by the TcRαβ on the DP thymocyte results in positive selection with ligation of a larger number of self MHC-peptide complexes resulting in negative selection. Alternatively, the differential signalling model suggests that it is the nature of the peptide-MHC complex that decides the fate of a specific TcRαβ DP thymocyte. In this case ligation of the TcR with a certain peptide-MHC complex would result in a signal being generated resulting in positive selection, however, interaction with other peptide-MHC complexes would result in a qualitatively different signal leading to negative selection.

One requirement for thymic deletion is for any autoantigenic peptide derived from self proteins to be presented at sufficiently high density to allow a threshold of receptor occupancy to be reached. For peptides with low affinities for their MHC molecules, the density of peptide-MHC complexes formed may not be sufficiently high to result in deletion. Several groups have observed this lack of deletion resulting from low-affinity autoantigenic peptides interactions (Joosten I *et al.* 1994, Liu GY *et al.* 1995). Other groups have shown that T cells with low affinity/avidity for MHC expressing cells in the thymus are also able to escape intrathymic deletion (Kawai K and Ohashi PS 1995). This study showed that in the periphery these cells could function as immunocompetent T cells if triggered by cross-reactive antigens through high-affinity interactions.

Increasing evidence has emerged that the costimulatory molecules responsible for determining the fate of a mature peripheral T cell on ligation of its TcR, also play a role during thymic development. Punt *et al.* have shown that T cell apoptosis driven via the CD3 molecule requires costimulation through CD28 (Punt JA *et al.* 1994). Studies looking at TcR transgenic mice showed that cross-linking CD28 on DP thymocytes complemented peptide agonist-induced deletion. If these thymocytes were cultured together with peptide presented on APCs expressing only B7-1 or B7-2, T cell deletion

could be blocked on addition of anti-B7-1 or anti-B7-2 antibodies (Kruisbeek AM and Amsen D 1996). Additionally, the ligand for CD40, gp39 is also purported to play a role in the T cell thymic education. gp39 deficient mice display a lack of B7-2 expression in the cortical medullary region, which correlates with their strongly reduced deletion of thymocytes specific for endogenously expressed self-antigens. Within the thymus gp39 is expressed on T cells, CD40 on thymic epithelial and dendritic cells. Ligation of CD40 by gp39 is reported to increase expression of B7-1 and B7-2 on peripheral B cells (Roy M *et al.* 1995), and it is therefore hypothesised that gp39 may be affecting B7 expression on thymic APCs (Foy TM *et al.* 1995).

In summary, these mechanisms are designed to produce T cells capable of responding to pathogens and to prevent any potentially autoreactive cells from escaping into the periphery. In reality, the process of intra-thymic negative selection is not fail-safe and some self-reactive T cells do evade this checkpoint and escape into the periphery. Here, further mechanisms are employed, such as induction of antigen-specific nonresponsiveness, immune deviation and elimination to avoid induction of autoimmune diseases.

1.9 The TcR complex

On leaving the thymus mature T cells enter the bloodstream from which they migrate through peripheral lymphoid organs, returning to the bloodstream to recirculate until they encounter antigen. Antigen is presented to T cells by three types of professional antigen presenting cell (APC). There are the dendritic cells, which capture antigen at peripheral sites and migrate to secondary lymphoid organs where they trigger naive T cells (Steinman RM 1991). Macrophages, that are distributed throughout the lymph node and are able to actively ingest particulate antigens, and B cells, present in the lymphoid follicles are particularly efficient at taking up soluble antigen. It has been reported that as few as 100 peptide-MHC complexes (Demotz S *et al.* 1990) are needed to engage and trigger as many as 1800 TcRs (Valitutti S *et al.* 1995).

The TcR-MHC interaction has been studied extensively over the past few years. The TcR has been shown to recognise only MHC-peptide complexes, and fails to bind either in isolation (Davis MM and Bjorkman PJ 1988). Jorgensen *et al.* have shown that the first and second complementarity determining regions (CDR1 and CDR2) recognise the MHC molecule, whilst CDR3 binds the peptide (Jorgensen JL *et al.* 1992). Studies of the rotational orientation of the MHC-TcR interaction have resulted in two opposing models. The first by Jorgensen *et al.* suggests that the TcR-Vα domain binds to the MHC class II β chain α-helix (Jorgensen JL *et al.* 1992), whereas Hong *et al.* propose that the TcR-Vα domain binds to the MHC class II α chain (Hong S-C *et al.* 1992). These two groups looked at different TcRs and MHC molecules, which may explain why the radical differences in binding were seen. A third model suggested by

Janeway *et al.* proposes that the TcRα chain straddles the amino terminal end of the MHC class II-bound peptide, while the TcRβ chain covers the carboxy terminal end. They also suggest that the TcR-CDR2 loop may be involved in peptide binding, along with CDR3 (Janeway Jr. CA *et al.* 1995). It has been argued that the topology of the TcR-MHC can indeed vary, and that it is probably determined by such factors as the nature of the bound peptide and whether the TcR is interacting with an MHC class I or MHC class II molecule (**figure 1.8**) (Chein Y-H and Davis MM 1993, Garcia KC *et al.* 1996).

As mentioned previously, as few as 100 MHC-peptide complexes are needed to trigger a T cell response. It has also been reported only 0.8% (out of ~4000 molecules per cell) of H-2A^k molecules on B cells contain the immunodominant peptide from a pulsed antigen. To achieve this sensitivity and specificity, it would be predicted that, like antibodies, the TcR has a high affinity for its ligand. However, the affinity of the TcR for its ligand is extremely broad, between 10^{-4} and 10^{-7}M, whereas for an antibody that has undergone affinity maturation it is in the range of 10^{-9} to 10^{-10}M (Davis MM 1990, Sykulev Y *et al.* 1994). Additionally, the TcR has also been found to have a very rapid kinetic dissociation rate of between 3-9x10^{-2} seconds^{-1} (Corr M *et al.* 1994).

These findings therefore raise the intriguing question, how can the TcR bind its ligand with such low affinity and short duration, and yet exhibit such high sensitivity and specificity? Two theories have been suggested. The first, proposes that the signalling mechanism of the TcR-MHC complex is extremely efficient, such that even low TcR occupancy would lead to T cell activation. Ligation of the TcR complex leads to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs), present in the cytoplasmic region of the TcR complex chains, thereby initiating the signalling cascade. ITAM multimers are involved in the signalling process, suggesting that they may possess the capacity to amplify the signal from the TcR (Irving BA *et al.* 1993). The second theory, proposes that high receptor occupancy is achieved by the serial ligation of many TcRs by relatively few MHC-peptide complexes (Valitutti S *et al.* 1995). This group found that within the first 2-3 hours of T cell activation, large numbers of the TcR complexes were downregulated, with irrelevant receptors remaining unaffected. This effect was most dramatic when looking at low densities of MHC-peptide complexes. In such a situation it was observed that ~100 MHC-peptide complexes could trigger up to 1800 TcRs.

What triggers the signalling cascade has, as yet, not been fully elucidated. Some groups propose that on TcR-MHC dimerisation, provided a preset threshold is achieved, signal transduction will be triggered (Davis MM 1995, Emmrich F 1988). Others believe this view is too simplistic, and that MHC-peptide complexes induce a

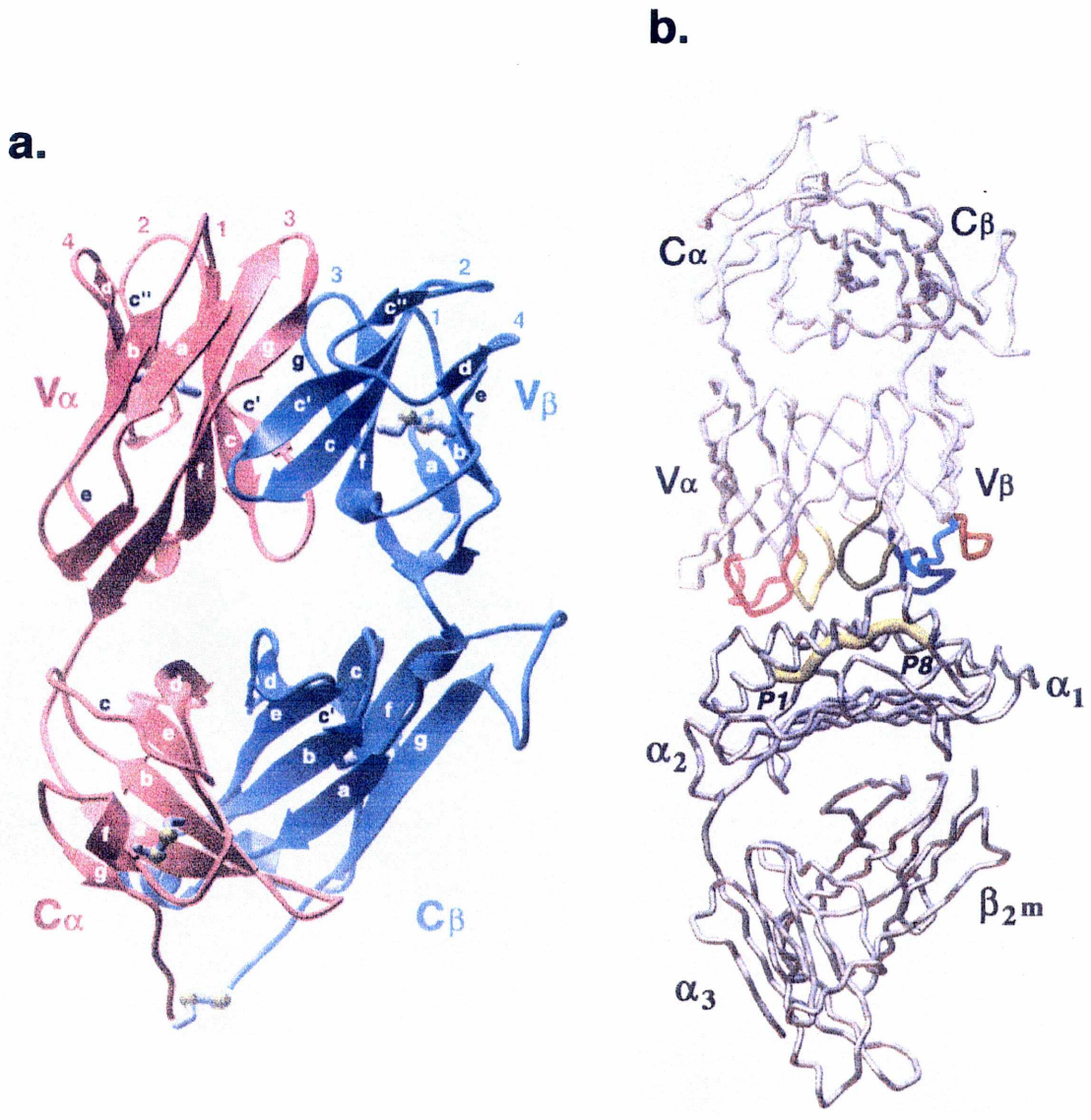


Figure 1.8a Three-dimensional structure of an αβ TCR
2C TCR is a murine receptor of a cytotoxic T cell with specificity for MHC class I H-2K^b molecules. The α chain is in pink (residues 1-213), with the β chain in blue (residues 3-247). The β strands are represented as arrows and labelled according to the standard convention used for Immunoglobulin folds. The disulphide bonds (yellow balls for sulphur atoms) are shown within each domain and for the COOH-terminal interchain disulphide. The CDRs are numerically labelled (1 to 4) for each chain.

Figure 1.8b The TCR-peptide-MHC interaction
Backbone tube representation of the orientated 2C-H-2K^b-peptide complex. The peptide-MHC is below with the octamer peptide (ova peptide EQYKFYSV) shown as a large tube in yellow. The 2C TCR is above with the α1 and α2 CDRs coloured pink; αHV4, white; CDRs β1 and β2, blue; βHV4, orange; CDR3s, yellow.

Reproduced from: Garcia *et al*, 1997.

conformational change in the TcR that results in T cell activation (Karjalainen K 1994, Vignali DAA and Strominger JL 1994). Dimerisation and clustering of the TcR complex does occur under certain circumstances. Studies using T cells specific for haptens, which bind in the absence of MHC molecules, show that activation only occurs when multimerised haptens are used (Symer DE *et al.* 1992). Furthermore, the MHC class II molecule has been shown to exist in a dimeric form when crystallised (Brown JH *et al.* 1993), these dimers were also identified by immunoprecipitation from murine B cells (Schafer PH and Pierce SK 1994). Davis has proposed that cocapping of the surface molecules between the T cell and the antigen presenting cell may occur (Davis MM 1995). This would provide a mechanism by which MHC class II dimerisation could take place, bearing in mind that a small number of MHC-peptide complexes must be able to dimerise from within a sea of irrelevant cell surface molecules. Alternatively, Janeway and others propose that monovalent interactions result in the TcR undergoing a conformational change thereby triggering T cell activation (Janeway Jr. CA *et al.* 1995, Valitutti S *et al.* 1995). This phenomenon is well documented for antibodies binding short peptides. They have been shown to alter their V_H:V_L alignment by as much as 10Å (Stanfield RL *et al.* 1993). Assembly of the complement C1 complex is also accompanied by conformational changes (Arlaud GJ *et al.* 1993). Interestingly, on binding its receptor, the monomeric ligand epidermal growth factor (EGF) induces a conformational change, this subsequently leads to receptor dimerisation (Fantl WJ *et al.* 1993).

1.10 Signal Transduction in T lymphocytes.

The TcR$\alpha\beta$ and TcR$\gamma\delta$ chains are associated with the non-polymorphic chains CD3ε, CD3γ, CD3δ as well as with either the TcR$\zeta\zeta$ or TcR$\zeta\eta$ dimers. These non-polymorphic chains are required for signalling as well as for TcR assembly and expression at the cell surface (Ashwell JD and Klausner RD 1990). Each TcR can contain one TcRζ dimer and two CD3 dimers ($\varepsilon\delta$ and $\varepsilon\gamma$). As mentioned above, regions in the cytoplasmic domains of these chains contain ITAMs, three in the TcRζ chain and one in each of the CD3 chains (Cambier JC 1995). The TcR can therefore contain a total of ten ITAMs. It is known that these motifs are necessary and sufficient for coupling of the TcR to the intracellular signalling machinery. On TcR activation, the tyrosine residues within the ITAMs become phosphorylated by the Src family protein-tyrosine kinases (PTKs) (Van Oers NS *et al.* 1996). This phosphorylation event is achieved in two ways. Firstly, receptor cross-linking mediated by Lck and Fyn and secondly interactions between the antigen receptors and PTKs associated with co-receptor or accessory molecules result in ITAM phosphorylation. It has been proposed that ITAM phosphorylation by kinases directly associated with the ITAM may be

distinct from phosphorylation mediated via co-receptor molecules. Support for this hypothesis has come from signalling studies in thymocytes.

In CD4+CD8+ thymocytes the TcRζ chains are constitutively phosphorylated, however, these thymocytes are poorly responsive to TcR cross-linking. ZAP-70 has been shown to be associated with these phosphorylated ζ chains, but in an inactive and unphosphorylated state (Madrenas J *et al.* 1995). Studies by Wiest *et al.* have shown low levels of Lck associated with CD4 in thymocytes that are engaged to MHC class II molecules. They propose that upon disengagement of the thymocyte from the MHC class II, assembly of CD4-Lck complexes takes place, resulting in acquisition of responsiveness to TcR cross-linking (Wiest DL *et al.* 1993). In this case the CD4-Lck complex may not be acting by phosphorylating the ITAM complex itself, but rather the ZAP-70 molecule, constitutively bound to the TcRζ chain.

Regulation of the catalytic activity of the Src-family PTKs is mediated via tyrosine phosphorylation. Autophosphorylation of tyrosine at position 416 (Y416) within the Src catalytic domain upregulates Src activity, whereas phosphorylation of Y527 inhibits Src function. Y527 phosphorylation is regulated by the protein tyrosine phosphatase CD45. It is proposed that the balance between kinases and phosphatases acts in this way to set the threshold for antigen receptor signalling (Chan AC and Shaw AS 1996). Studies have demonstrated that Src PTKs binding to ITAMs, leads to the competing off of the negative regulatory tyrosine present in the SH2 domain of the Src PTK. This results in stimulating the catalytic activity of the Src PTK (Flaswinkel H and Reth M 1994, Johnson SA *et al.* 1995).

Phosphorylation of both tyrosine residues within the ITAMs by the Src kinases mediates ZAP-70 binding via its SH2 domains to the phosphorylated sites. Lck or Fyn subsequently phosphorylate the Y493 residue, located within the kinase domain of ZAP-70, resulting in ZAP-70 activation (Chan AC *et al.* 1995, Wange RL *et al.* 1995). ZAP-70 activation has an absolute requirement for phosphorylation of the Y493 residue. Studies show that ZAP-70 is constitutively associated with the phosphorylated TcR in both resting thymocytes and peripheral T cells (Madrenas J *et al.* 1995, Van Oers N *et al.* 1994). Furthermore, binding of the ZAP-70 to the ITAM alone has no effect on its catalytic activity (Neumeister EN *et al.* 1995).

Syk, a PTK belonging to the same family as ZAP-70, also binds the doubly phosphorylated ITAMs. It is unclear whether Src kinases are needed for Syk activation as studies indicate that Syk can be activated by binding to the phosphorylated ITAMs alone (Couture C *et al.* 1994).

Substrates of ZAP-70 and Syk have proved difficult to identify, and it now appears that they themselves are one of their most important substrates. Studies show that activation of ZAP-70 and Syk results in the generation of multiple phosphorylation

sites that probably act as binding sites for downstream effector molecules (Chan AC *et al.* 1995, Neumeister EN *et al.* 1995). The auto-phosphorylated receptors are now able to recruit SH2-containing signalling proteins, and Lck, Fyn, ras-GAP, abl, vav and cbl have all been identified associated with ZAP-70 (Fournel M *et al.* 1996, Katzav S *et al.* 1994, Neumeister EN *et al.* 1995). Other proteins that have been found to undergo tyrosine phosphorylation following TcR cross-linking include LNK and SLP-76. These two proteins associate with Grb2 and phospholipase C (PLC) after TcR stimulation, and may be involved in ZAP-70 gaining access to the Ras and calcium pathways (Huang X *et al.* 1995, Jackman JK *et al.* 1995).

Activation of PLC leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate yielding the secondary messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG activates protein kinase C (PKC), whereas IP_3 increases intracellular calcium concentration (Weiss A and Littman DR 1994). Activation of calcineurin also leads to increase in intracellular calcium levels. Ras is a 21kD GTP-binding protein. On TcR activation, Ras is converted from the GDP to GTP state (Downward J *et al.* 1992, McCormick F 1993). PTKs may mediate this change via guanine nucleotide exchange proteins such as sos and vav (Cantrell D 1994). Downstream effectors of Ras include Raf-1, MEK and MAP-kinase (Crews CM and Erikson RL 1993, Nel AE *et al.* 1990).

Downstream of Ras, mitogen-activated protein kinases (MAPKs), a group of serine/threonine specific protein kinases are activated. Several MAPKs have been identified and it has become clear that they are able to transduce the signals they receive in qualitatively different ways. At least three MAPK cascades that result in gene expression have been identified. These cascades are the ERK1/2 (extracellular signal regulated kinase) MAPK cascade, the JNK (Jun N-terminal kinase) MAPK cascade and the p38 MAPK cascade. Activation of the ERK1/2 and JNK cascades results in induction of c-*fos* transcription (Cavigelli M *et al.* 1995, Price MA *et al.* 1995), in addition JNK activation induces transcription of c-*jun* (Price MA *et al.* 1995). The genes affected by activation of the p38 MAPK cascade are as yet unknown.

Both c-*fos* and c-*jun* are known as 'immediate-early' genes, meaning their transcription is rapidly induced following cell activation, independently of *de novo* protein synthesis. Transcription of c-*fos* and c-*jun* via the ERK and JNK pathways, results in the formation of c-Fos and c-Jun which dimerise forming AP-1 heterodimers (Su B and Karin M 1996). AP-1 heterodimers mediate their effect by binding the IL-2 gene, resulting in its transcription. AP-1 must bind to the IL-2 gene in conjunction with NFAT-1, a transcription factor belonging to the NF-κB/rel family, for IL-2 mRNA transcription to proceed (Jain J *et al.* 1992). A brief schematic diagram of how the signal transduction pathways interconnect is shown in **figure 1.9**.

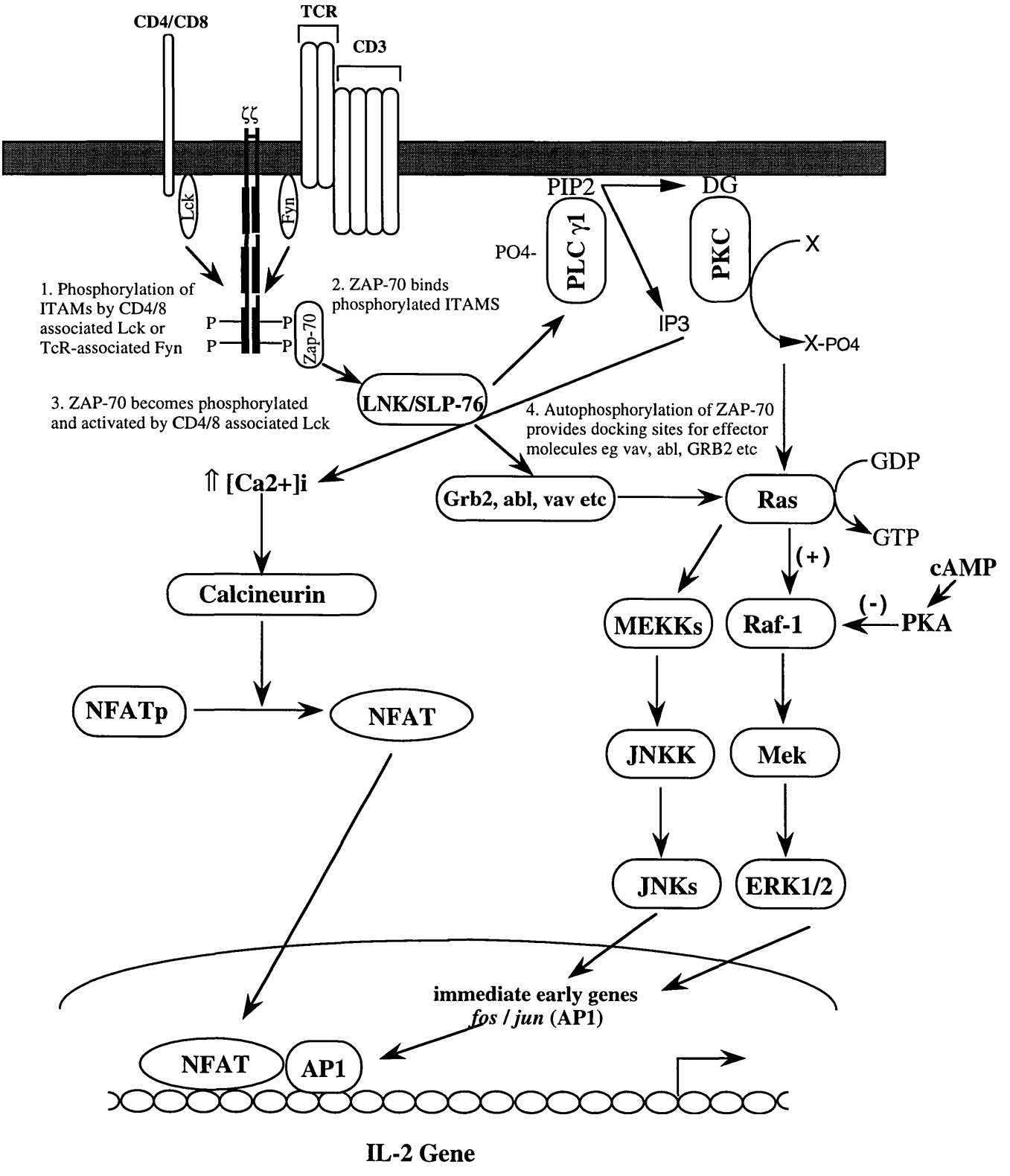


Figure 1.9 **Signal transduction cascade induced in T lymphocytes following TCR engagement.**

Adapted from Keogh, 1995

The MAPK cascades serve an important function in both T and B lymphocyte activation. Ligation of the antigen receptors results in rapid activation of the Ras/Raf/ERK1 kinase cascade, and inhibition of these pathways using dominant negative Ras or Raf has been shown to block T cell activation (Izquierdo M *et al.* 1994). However, stimulation of the ERK1/2 cascade alone is not sufficient to activate T cells, this observation therefore supporting the finding that T cell activation requires co-stimulatory signals, in addition to antigen receptor occupancy (Schwartz RH 1992). Studies show that the ERK1/2 cascade is activated by ligation of the TcR/CD3 complex alone. The JNK cascade, however, requires two stimuli, one coming from antigen receptor occupancy, the other from the CD28 coreceptor (Su B *et al.* 1994). JNK activation can be achieved by simultaneous treatment of phorbol ester and Ca^{2+} ionophore. It has also been observed that the JNK cascade is inhibited on treatment with cyclosporin A (CsA) (Su B *et al.* 1994). The target of CsA is calcineurin, thus suggesting its possible involvement in JNK activation. JNK therefore seems to act in concert with signalling pathways other than Ras. To this end, cAMP has been shown to inhibit the JNK pathway, whilst not affecting the ERK1/2 cascade (Hsueh YP and Lai MZ 1995).

1.11 The role of costimulation.

As mentioned in the previous section T cell activation is dependent on two distinct signals; one coming from the TcR, the other from the interaction of cell surface molecules providing costimulatory signals. This concept was first put forward by Lafferty *et al.* who showed that foreign tissues depleted of 'passenger leukocytes' were unable to induce a productive immune response (Lafferty KJ *et al.* 1993). Further studies showed that stimulation of T cells with anti-T-cell receptor antibodies, mitogenic lectins in the absence of viable antigen-presenting cells or fixed antigen-presenting cells resulted in neither clonal expansion nor interleukin-2 (IL-2) production (Mueller DL *et al.* 1989, Schwartz RH 1992). CD28 is expressed constitutively on both resting and activated CD4+ and CD8+ T cells (Harding FA *et al.* 1992, Schwartz RH 1992) and on activation T cells express a second coreceptor, CTLA-4. CTLA-4 is primarily found intracellularly, and its limited cell surface expression is regulated by TcR engagement (Linsley PS *et al.* 1996). Work by Krummel and Allison has demonstrated that by varying experimental conditions anti-CTLA-4 mAbs could either inhibit or stimulate T cell activation, suggesting that the outcome of the T cell receptor engagement is ultimately determined by integration of signals provided by both the CTLA-4 and CD28 molecules (Krummel M and Allison J 1995). Both receptors bind to the B7 family of molecules, which includes at least two ligands, B7-1 (CD80) and B7-2 (CD86) (June CH *et al.* 1994). Blocking CD28-B7 interactions with monovalent 'Fab' fragments of anti-CD28 or B7-specific antibodies has also been shown to prevent

activation of CD4+ T cells (Boussiotis VA *et al.* 1994c, Linsley PS and Ledbetter JA 1993). B7-1 and B7-2 both have low affinities for CD28 and high affinities for CTLA-4, however their binding kinetics differ, with B7-2 having faster dissociation kinetics (Linsley PS *et al.* 1994). On stimulation of the antigen presenting cell, B7-2 expression is induced more rapidly than B7-1 (Lenschow DJ *et al.* 1994). However, B7-1 expression predominates following antigen exposure or during inflammatory conditions (Lenschow DJ *et al.* 1996). These findings have led to the hypothesis that the early expression of B7-2 may be indicative of its role in initiating an immune response, thereby playing a pivotal role in the decision between T cell activation and anergy. B7-1, on the other hand, being expressed later, may function by amplifying or regulating an ongoing immune response (Tivol EA *et al.* 1996).

The differences in binding affinities that the B7 ligands have for CD28 and CTLA-4 may determine which interactions i.e. B7-CD28 or B7-CTLA-4 predominate at different stages of the immune response. In this scenario, low levels of B7 would engage the high affinity inhibitory receptor CTLA-4. Whereas on B7 upregulation, for example after antigen encounter, the predominant interaction would be with the low affinity CD28 receptor and B7.1, resulting in T cell proliferation and differentiation. It would be envisaged that this immune response would in turn be regulated, as a result of the upregulation of CTLA-4 following T cell activation. B7 would now, once again, predominantly bind the CTLA-4 receptor thereby terminating the T cell response (Tivol EA *et al.* 1996).

The first insight that stimulation of the CTLA-4 molecule resulted in a negative signal came from *in vitro* studies looking at the effects of anti-CTLA-4 monoclonal antibodies (mAbs) on T cell stimulation. It was found that in the absence of anti-CD28 mAbs, anti-CTLA-4 mAbs were unable to provide a costimulatory signal to purified T cells activated with anti-CD3 mAbs. When CTLA-4 monovalent Fab fragments were used to block B7-CTLA-4 interactions, proliferation of T cells that had been activated using anti-CD3 mAbs and costimulated with anti-CD28 mAbs was augmented. Furthermore, under conditions of Fc cross-linking, CTLA-4 mAbs inhibited T cell proliferation (Kearney ER *et al.* 1995). Direct evidence for the role of CTLA-4 in negatively regulating T cell activation came from the phenotype of CTLA-4 gene knockout mice (CTLA-4-/-). These mice developed a spontaneous lymphoproliferative disease with multiorgan lymphocytic infiltration and tissue destruction. The mice also displayed severe myocarditis and pancreatitis and died by 3-4 weeks of age. The peripheral T cells from these mice were activated and proliferated spontaneously *in vitro* producing abundant cytokines. It is hypothesised that the lymphoproliferation observed in these mice may be due to a failure to delete autoreactive cells in the thymus, a failure to terminate antigen-specific responses or a failure of antigen-specific apoptosis of activated T cells (Tivol EA *et al.* 1995, Waterhouse P *et al.* 1995).

The phenotype of these mice also suggests, contrary to the hypothesis that TcR signalling in the absence of costimulation results in peripheral tolerance, that costimulation plays an essential role in maintaining tolerance to self antigens, with negative signalling via CTLA-4 regulating autoreactive T cells. A recent study lends support to this hypothesis. Transgenic mice expressing soluble CTLA-4-Ig were found to respond comparably to wild type mice on administration of SEB. Vβ8+ T cells expanded and were subsequently deleted. However, whereas the remaining Vβ8+ T cells in the wild type mouse were tolerant on SEB stimulation *in vitro*, the remaining Vβ8+ T cells from the transgenic mice were found to be responsive to the superantigen *in vitro*. As the CTLA-4-Ig will have bound any B7 ligands, it will have prevented interactions of the B7 family members with CD28 and CTLA-4. These findings thereby suggesting that a B7-dependent mechanism is needed for tolerance induction (Lane P *et al.* 1996).

Gene knockout technology has also provided insights into the roles played by the CD28 and B7 molecules. Development of T and B cells in CD28 deficient mice appeared normal. However, T cell proliferative responses to the lectin concanavalin A were significantly reduced. Additionally, reduced levels of IL-2 were secreted, IL-2 receptor expression was decreased and addition of exogenous IL-2 only partially restored the proliferative response. Basal immunoglobulin levels were found to be about one-fifth of those found in wild type controls. Activity of T helper cells was shown to be reduced, and immunoglobulin class switching impaired after vesicular stomatitis virus infection. However, cytotoxic T cell function could still be induced as well as a delayed-type hypersensitivity reaction (Shahanian A *et al.* 1993). This study therefore indicated that cell-mediated responses appear dependent on the CD28 costimulation pathway, whereas humoral responses may utilise an alternate costimulatory pathway.

At around the same time, with the existence of the B7-2 molecule still unknown, a B7-1 deficient mouse was made. The B7-1-/- mice possessed normal numbers of thymocytes which appeared to mature normally. They also had normal numbers of CD3+, B220+, CD4+ and CD8+ cells in the lymph node and spleen. The concentrations of immunoglobulins in their serum was similar, and spleen cells responded similarly to the B cell mitogen lipopolysaccharide and the T cell mitogen concanavalin A. Response to alloantigens was, however, decreased by 70% compared with wild type mice, with the residual response being blocked on administration of CTLA-4-Ig (Freeman GJ *et al.* 1993b). This lack of a dramatic phenotype as seen for the CD28 and CTLA-4 knockout mice was explained by the existence of additional CTLA-4 counter-receptors. Freeman *et al.* and others, then going on to clone the B7-2 receptor (Azuma M *et al.* 1993, Freeman GJ *et al.* 1993a, Freeman GJ *et al.* 1993).

Manipulation of the B7-CD28/CTLA-4 pathway in models of autoimmunity using mAbs have shown that the initiation of an autoimmune response as well as an ongoing autoimmune process can be prevented. CTLA-4-Ig protected against experimental autoimmune encephalomyelitis (EAE) induced by either immunisation with activated myelin basic protein (MBP)- or proteolipid protein (PLP)-specific T cells. An effective long-term suppression of the clinical and histological symptoms of EAE was observed, even after cessation of CTLA-4-Ig treatment (Cross AH *et al.* 1995). Similar observations have been made in female nonobese diabetic (NOD) mice. Here, CTLA-4-Ig treatment of 2 week old mice led to a greatly reduced incidence of diabetes, with administration of the CTLA-4-Ig at the onset of diabetes resulting in reduced insulitis (Lenschow DJ *et al.* 1995). In depth studies of the EAE model, however, reveal the difficulties in predicting disease outcome using mAb therapies. Racke *et al.* found that a single injection of CTLA-4-Ig two days post immunisation could reduce the disease, whereas multiple CTLA-4-Ig injections (from day -1 to day 17) led to disease enhancement. The timing of the treatment therefore appears critical. This may be related to the distinct temporal expression of the receptors and ligands in the pathway and their complex interactions determining whether the T cell receives a positive or negative signal (Racke MK *et al.* 1995, Tivol EA *et al.* 1996).

The mechanism by which CTLA-4-Ig mediates protection in EAE is still unknown. Splenocytes derived from CTLA-4-Ig treated mice were still able to proliferate in response to PLP *in vitro*, ruling out induction of tolerance to the antigen (Cross AH *et al.* 1995). However, immunohistological studies of the central nervous system (CNS) in CTLA-4-Ig treated Lewis rats, indicated that there was an inhibition of inflammatory Th1 cytokines and an upregulation of Th2 cytokines, suggesting that CTLA-4-Ig may mediate protection by immune deviation (Khoury SJ *et al.* 1995).

In vivo studies looking at anti-B7-1 and anti-B7-2 mAbs suggest they may play distinct roles in autoimmune diseases. Treatment of actively induced EAE with anti-B7-1 mAbs was found to ameliorate the disease, with anti-B7-2 treatment resulting in disease exacerbation. Once again the mechanism appears to be one of immune deviation. T cells were still found to be responsive to the immunising antigen, but displayed an altered cytokine profile. Th1 activation seems to be prevented by the anti-B7-1 mAb, resulting in the predominant generation of Th2 clones (Kuchroo VK *et al.* 1995).

Use of CTLA-4-Ig as a therapy for abrogation of disease in autoimmune models has shown the difficulty in predicting the outcome of disease when using mAbs. Studies utilising the B7-1 and B7-2 mAbs show that they also display anomalies in their responses. In the murine model of lupus, both anti-B7-1 and anti-B7-2 mAbs are needed to prevent the development and progression of lupus, with B7-2 playing a more critical role in inhibition of autoantibody production (Nakajima A *et al.* 1995). In the

NOD mouse, anti-B7-2 mAbs were found to block the development of diabetes, whereas treatment with anti-B7-1 mAbs was found to exacerbate the disease (Lenschow DJ *et al.* 1995).

The different outcomes of anti-B7 treatment seen in these models may relate to distinct strengths of signalling resulting from differences in the manner of antigen exposure and/or from involvement and timing of expression of B7-1 and B7-2 on distinct cell types (Tivol EA *et al.* 1996). Further studies are therefore needed to look at the mechanism of T cell activation via the B7-CD28 pathway, as well as the ability of the CTLA-4-B7 pathway to downregulate activated T cells.

Other molecules are known to play key roles in determining the outcome of an encounter by a T cell with an APC. These molecules are depicted in **figure 1.10**. As mentioned in **section 1.10** the CD4/8 coreceptors play a pivotal role in signal transduction in T cells. Waldmann and colleagues exploited this knowledge to try to manipulate the outcome of tissue transplantation. Donor skin grafts (B10.BR) that varied at multiple minor histocompatibility antigens were grafted onto a recipient mouse (CBA/Ca). After grafting, mice were treated with a short course of non-depleting mAbs to CD4 and CD8 on days 0, 2 and 4. Five weeks after transplantation, spleen cells were removed from the recipient CBA/Ca mouse and transferred to a second CBA/Ca mouse. The transferred cells behaved as if tolerant, in so far as all secondary recipients accepted B10.BR skin grafts, while rejecting third party grafts (Scully R *et al.* 1994). The mechanism for this state of tolerance was postulated to be either one of immune response deviation, thereby selecting a Th2 response which would become dominant over time and thus suppress a Th1-like graft rejection response, or one of facilitating the induction of a state of anergy. The same group were also able to achieve tolerance to MHC-mismatched skin grafts by using a combination of blocking and depleting mAbs to CD4 and CD8 (Cobbold S *et al.* 1990).

Similar studies have been performed by Isobe *et al.* They used non-depleting mAbs to LFA-1 and ICAM-1 to establish long-term tolerance to cardiac allografts. An initial reduction in LFA-1/ICAM-1 levels was observed, although normal expression was restored after 40 days. The mechanism of tolerance induction was not elucidated, but a lack of alloreactive CTLs was noted (Isobe M *et al.* 1992). Taking into account the more recent report by Scully *et al.*, it could be perceived that this observation may signify a mechanism involving immune deviation from a Th1 to Th2 type response.

Recently the role of CD2 in regulation of T cell responses has come under scrutiny. Boussiotis *et al.* induced alloantigen-specific anergy in DR7-specific T cell clones by blockade of the B7 family of molecules. They found that reversal of anergy could be achieved by culturing the cells in the presence of IL-2, followed by restimulation with the alloantigen in the presence of LFA-3. In this model of anergy it is known that the CD2 molecule undergoes a conformational change following anergy

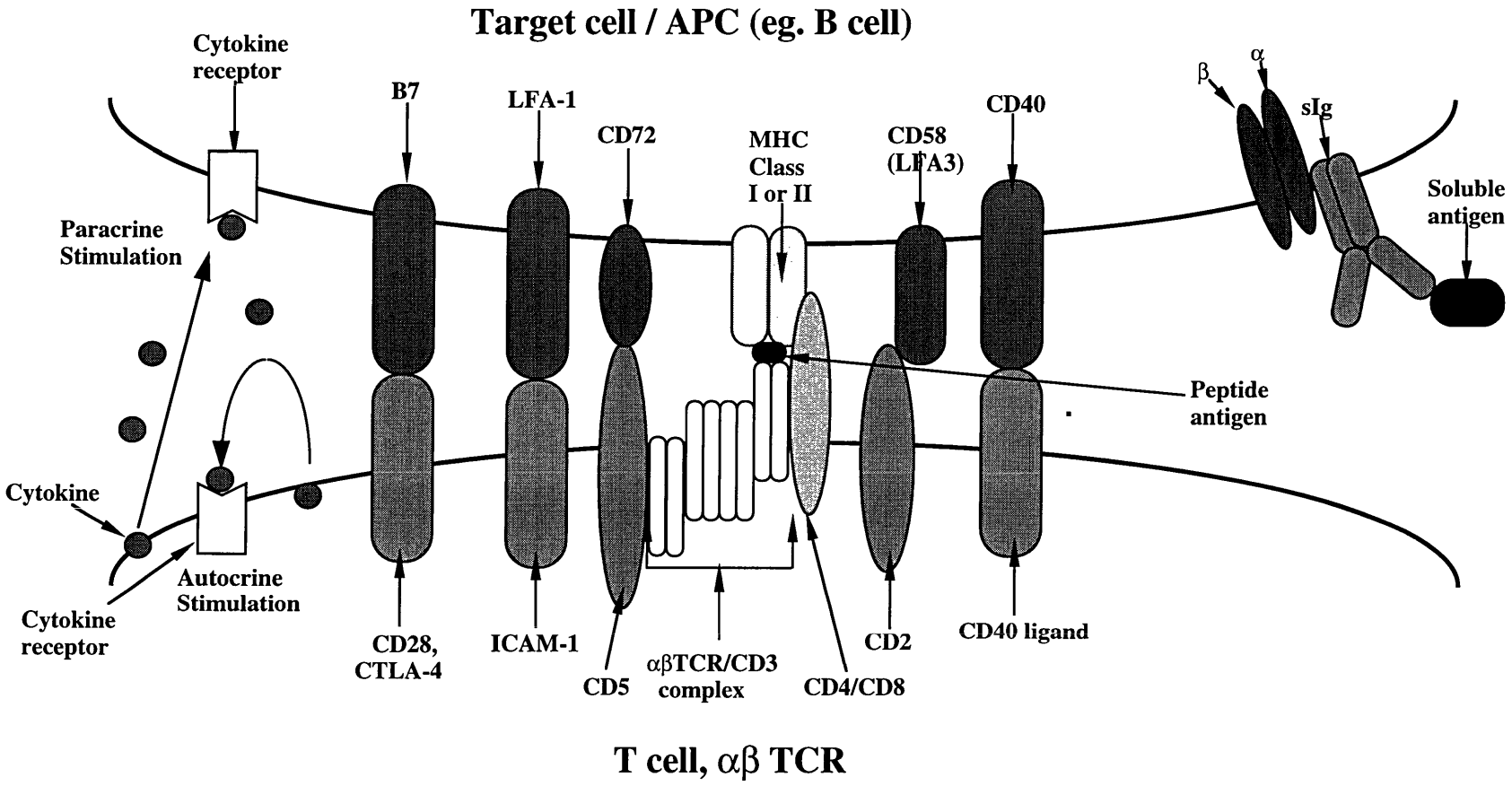


Figure 1.10 Receptor ligand interactions for T cells and APCs/Targets

The view shown is stylistic and not representative of receptor structure or ligand distribution - not all interacting cells will support all the ligand-pairings depicted. The CD3 complex is composed of the γ, δ, ε, ζ and η chains. Autocrine and paracrine methods of cytokine stimulation are shown.

Key: LFA, Lymphocyte functional antigen; ICAM, intracellular adhesion molecule; CD, cluster of differentiation; TCR, T cell receptor; sIg, surface immunoglobulin.

Adapted from: Keogh, 1995

induction, resulting in the loss of the epitope required to transduce positive signals to the T cell. The observation that mAbs directed at other CD2 epitopes are still able to bind CD2 confirms this hypothesis. This change renders the CD2 molecule unable to deliver stimulatory signals. Following exposure to IL-2, the epitope required for LFA-3 binding and the delivery of a positive signal is re-expressed resulting in the reversal of the anergic state (Boussiotis VA *et al.* 1994b).

1.12 CD4+ T helper cell responses.

In 1972, Parish and Liew made the observation that cell-mediated and humoral immunity alternated in reciprocal dominance over each other across a wide dose response of immunisation (Parish CR and Liew FY 1972). In 1986, Mosmann *et al.* found that long-term cultured murine CD4+ T helper (Th) cell clones produced two distinct patterns of cytokines. These were found to provide help for different arms of the immune response and displayed profound counter-regulatory effects (Mosmann TR *et al.* 1986). *In vivo* evidence for the existence of these subsets came from the mouse model of leishmaniasis (Scott P *et al.* 1988) and in humans with leprosy (Yamamura M *et al.* 1991). As discussed in the previous section, disease may be exacerbated or ameliorated depending on which arm of the immune response is activated. Prior to terminal differentiation, T cells are described as Th0 cells. These cells secrete mixtures of both types of cytokines until they are signalled into polarising their response and commit to either the Th1 or Th2 pathway. The Th1 subset is defined as producing IL-2, IFN-γ and TNF-α/β cytokines. This cytokine profile being associated with the DTH response, macrophage activation and limitation of B cell help resulting in production of antibodies of the IgG_{2a} isotype alone. Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, resulting in macrophage, eosinophil and basophil deactivation, mast cell stimulation and B-cell help. Ig production is switched to produce the IgG_1 and IgE isotypes **(summarised in Table 1.1)** (Carter LL and Dutton RW 1996).

The major controlling factor in the development of Th1 and Th2 populations from naive T cells in model systems, is the presence of either IL-12 or IL-4 respectively. IL-12 induces IFN-γ production by NK cells, but the mechanism of IL-12 action is unclear. In the IL-12 knockout mouse, Th1 responses are diminished, but not abolished, lending support to the hypothesis that IL-12 is important in determining the magnitude of the Th1 response but may, in some cases, have a redundant role in initiation of a Th1 response (Carter LL and Dutton RW 1996). Dependence on IL-12 for immune response initiation is observed in studies of toxoplasmosis. Here, neutralisation of IL-12 early in infection leading to ablation of IFN-γ production and disease exacerbation (Gazzinelli RT *et al.* 1994). IL-12 may also act directly on T cells, as well as mediating its effect through IFN-γ. TcR transgenic T cells cultured in the presence of rIL-12 were shown to differentiate into Th1 cells, even in the presence of

Table 1.1 A summary of the spectrum of cytokines produced by Type 1 and Type 2 T cells and their subsequent effects on immune responses.

	Type 1	Type 2
Characteristic cytokines	IL-2 IFN-γ TNF-α/β	IL-4 IL-5 IL-6 IL-10 IL-13
Major functions	DTH Macrophage activation Cytotoxicity Limited B cell help/ B cell inhibition	B cell help Eosinophil stimulation Mast cell stimulation Macrophage deactivation
Associated isotypes	IgG_{2a}	IgG_1 IgE
Beneficial responses in	Leishmaniasis Leprosy Virus Allergy	Arthritis Autoimmunity Helminths Pregnancy
Detrimental responses in	Arthritis Autoimmunity Helminths	Leishmaniasis Leprosy Virus Allergy

Key: DTH, delayed type hypersensitivity.
Reproduced from Carter & Dutton, 1996.

IFN-γ-neutralising antibodies, thereby supporting this hypothesis (Seder RA *et al.* 1993). However, IFN-γ gene knockout mice display a default to the Th2 phenotype when challenged with *L.major* (Wang Z-E *et al.* 1994), suggesting that IFN-γ is required for induction of a Th1 response in this case. In contrast to the ability of IL-12 to mediate its action on T cells directly in initiating a Th1 response, its ability to inhibit initiation of a Th2 response appears to be mediated through IFN-γ (Morris SC *et al.* 1994).

IL-10 has been defined as an inhibitor of Th1 cytokine production, and studies indicate that its mechanism of action may be through regulation of IL-12 production (Kennedy MK *et al.* 1994). In many *in vivo* systems rIL-12 has been shown to induce IL-10 production (Romani L *et al.* 1994, Wang Z-E *et al.* 1994). This may seem paradoxical, however, the complementary induction of IL-10 production may serve as a homeostatic mechanism, controlling a Th1 response, possibly by ameliorating pro-inflammatory effects that a prolonged response could otherwise elicit (Reiner SL and Seder RA 1995). In contrast, IFN-γ has been shown to provide a positive feedback loop for IL-12 induction, *in vivo* studies showing that there is a reduction in IL-12 production when mice are treated with antibodies to IFN-γ (Wynn TA *et al.* 1994). A summary of the role played by the various cytokines in directing Th development is depicted in **figure 1.11**.

Adoptive transfer studies in IL-4 gene knockout mice indicate that CD4+ cells act as a priming source of IL-4 for Th2-mediated responses (Schmitz J *et al.* 1994). NK1.1 CD4+ cells, as well as CD4+ αβ T cells being implicated as potential sources of IL-4 (Yoshimoto T and Paul WE 1994). γδ T cells (Ferrick DA *et al.* 1995) and CD8+ T cells (Croft M *et al.* 1994) have also been found to be IL-4 producers. The predominant source of IL-12 appears to come from macrophages, although dendritic cells which primarily appear to be the actual initiators of an *in vivo* immune response also produce IL-12 (Macatonia SE *et al.* 1995). IL-10 is reported to be produced by an undefined accessory cell population. Studies show that on restimulation of a purified CD4+ T cell population no IL-10 transcripts are obtained (Oswald IP *et al.* 1994). IL-10 production in the SCID mouse has also been reported, suggesting that IL-10 is unlikely to be produced by T cells (Wang Z-E *et al.* 1994).

Recently, a number of groups have shown that both human and murine CD8+ T cells are able to produce Th1- or Th2-like cytokine profiles, necessitating a change in the nomenclature from Th1/Th2 to type1/type2 (T1/T2), thereby reflecting the cytokine profile generated, rather than the cell type that produced it. Horvat *et al.* demonstrated that murine CD8+ T cells were able to produce IL-4 in an anti-tumour response (Horvat B *et al.* 1991). Fong and Mosmann demonstrated that a panel of alloreactive CD8+ clones secreted a Th1-like cytokine profile on rechallenge with Con A (Fong TA and

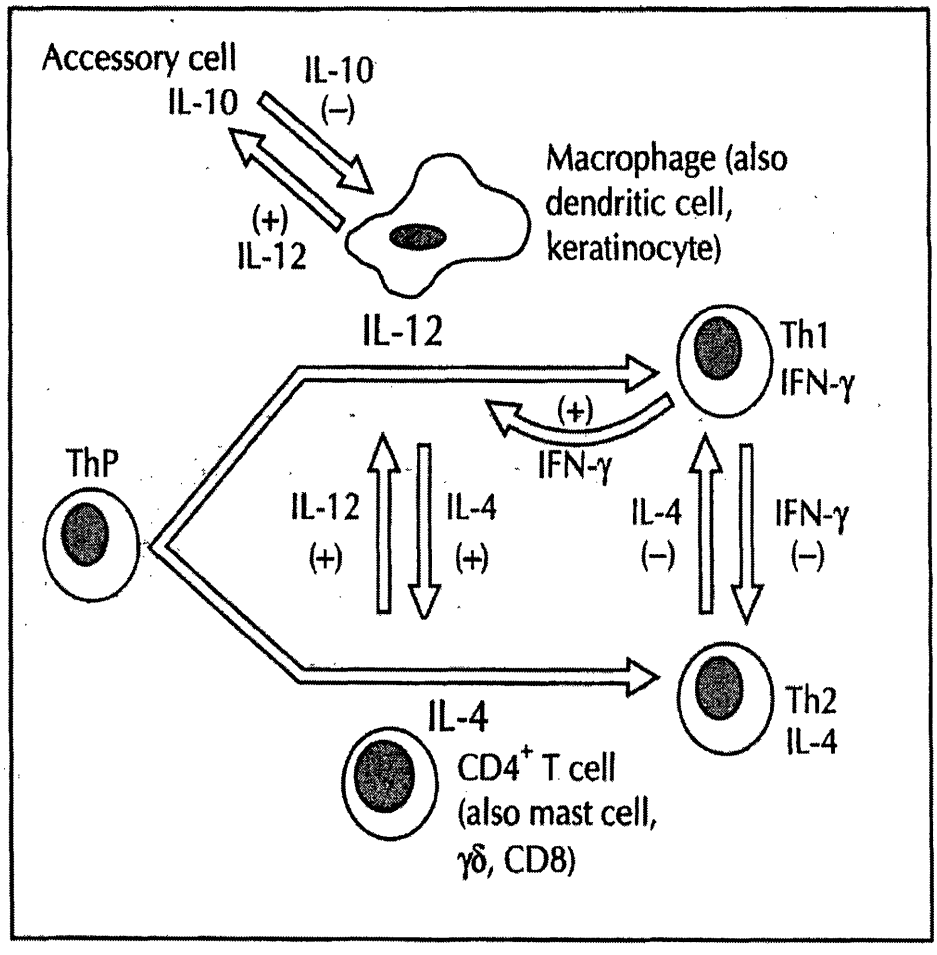


Figure 1.11 Relationship between key Cytokines during T helper development

IL-2 and IL-4 are the most potent factors for priming Th1 and Th2 responses by acting directly on the T cells to induce IFNγ and IL-4 production, respectively. IL-12 inhibition of IL-4 production appears, under many circumstances, to be indirect (through induction of IFNγ). In some instances, IL-12 is able to act directly on a population of early IL-4 producing cells that may have a distinct lineage from the naive Th precursors (ThP). IL-10 is derived from accessory cells (and possibly Th cells); although it acts to downregulate IL-12 production / Th1 augmentation. IL12 has been found to induce IL-10 production thus providing a potential homeostatic feedback loop.

Reproduced from: Reiner & Seder, 1995

Momann TR 1990). Later studies showed that when CD8+ cells were primed with anti-CD3 in the presence of IL-4, they produced IL-4 at levels comparable to those seen with CD4+ T cells (Seder RA *et al.* 1992). Similar observations were made on treating CD8+ cells with IL-12 in both human and mouse systems (Croft M *et al.* 1994). As was seen for CD4+ cells, polarisation of CD8+ cells is not merely an *in vitro* phenomenon, having also been isolated in various human and murine disease states and models. T2, IL-4 secreting CD8+ clones have been isolated from the blood of patients with lepromatous leprosy, with T1 clones isolated from patients with the tuberculoid form producing IFN-γ (Salgame P *et al.* 1991). In allergy, where CD4+ Th2 cells have a clear role (Anderson GP and Coyle AJ 1994), CD8+ cells isolated from drug-allergic patients responded to drug stimulation by proliferation and production of high levels of IL-5, with normal IL-2, IL-4 and TNF-α levels (Mauri-Hellweg D *et al.* 1995).

The cytolytic activity of CD8+ T cells is well documented as their major function, and their ability to produce cytokines has resulted in studies investigating the cytolytic capacity of T1 and T2 CD8 cells. Horvat *et al.* reported that IL-4 producing CD8+ T cells were also cytolytic (Horvat B *et al.* 1991). Sad *et al.* showed that both their allospecific T1 and T2 CD8+ populations were highly effective killers (Sad S *et al.* 1995). In contrast, Erard *et al.* have described a non-cytolytic IL-4-secreting CD8+ cell (Erard F *et al.* 1993). Similarly a reduction in CTL activity by T2 CD8+ cells has been demonstrated in some AIDS patients, which has been hypothesised to be indicative of disease progression (Maggi E *et al.* 1994).

Interestingly, CD8+ T cells are now being implicated as regulator cells. Weiner *et al.* showed that oral administration of antigen to rats and mice induces TGF-β and IL-4 production from CD8+ T cells and prevents EAE development (Weiner HL *et al.* 1994). IL-4-secreting CD8+ T cells from lepromatous leprosy patients have also been shown to suppress the proliferation of leprosy-specific CD4+ clones in an IL-4-dependent manner (Salgame P *et al.* 1991). Similarly, in a murine model of graft-versus-host disease (GVHD), injection of T2 T cells (CD4+ or CD8+) together with allogeneic bone marrow does not result in GVHD, whereas injection with T1 cells does. Moreover, co-injection of both T1 and T2 also prevents disease development indicating that T2 cells are able to suppress the inflammatory actions of the T1 cells (Krenger W *et al.* 1995).

In summary, αβCD4+ and αβCD8+ T cells, as well as γδ T cells, have been shown to be capable of secreting significant amounts of cytokines. This cytokine production can be polarised into distinct subsets, T1 and T2. The functions of these subsets, their role in disease and their interplay with each other remain to be fully elucidated.

1.13 The role of MHC-peptide:T cell interactions in allergy.

Allergic disorders affect between 10-20% of the population in the western world and are on the increase. The antigens (or allergens) responsible for eliciting these inappropriate immune responses are prevalent throughout the environment. Common sources include pollens, mites and animal danders. Patients suffering from atopic allergic diseases have high levels of allergen-specific circulating IgE which occurs as a result of a previous encounter with a specific antigen. On subsequent encounters with this antigen, tissue mast cells and basophils are activated. The specific antigen cross-links IgE molecules bound to mast cells and basophils via FcεRI molecules, resulting in a rapid release of inflammatory mediators such as histamine. This gives rise to the clinical symptoms of allergy such as hayfever, asthma and eczema (O'Hehir RE *et al.* 1991c).

CD4+ T cells play an important role in the antigen-specific and nonspecific effector mechanisms of the allergic immune response. They are involved in induction of IgE synthesis and two mechanisms have been implicated. Firstly by their production of the cytokine IL-4 and secondly by their direct interaction (either through antigen recognition or by CD40 ligation) with B cells (Vercelli D *et al.* 1989). CD4+ T cells are also able to produce two defined patterns of cytokines (as discussed in the previous section), IL-4 production inducing B cell proliferation and isotype switch to IgE production, whilst IFN-γ acts by inhibiting B cells as well as proliferation of any IL-4-secreting T cells (Snapper CM and Paul WE 1987, Vercelli D *et al.* 1989). Human allergen-reactive T cell clones derived from non-atopic individuals, have been shown to secrete elevated levels of IFN-γ and IL-2, whereas those derived from atopic patients secreted elevated levels of IL-4 and IL-5 on *in vitro* rechallenge (Kapsenberg ML *et al.* 1991). *In vivo* observations also provided evidence for the existence of dominant populations of polyclonal human Th2 cells in allergic patients (Field EH *et al.* 1993).

Effective treatment for some allergic disorders has been achieved using intact native allergens, with evidence that allergen desensitisation may be accompanied by the induction of Th1-derived cytokines (Jutel MP *et al.* 1995). However, due to the risk of anaphylaxis, which may occur due to the presence of potential immunoglobulin binding sites, the use of allergen derivatives such as peptides is favoured (Muller U *et al.* 1995). Nonstimulatory peptides could be used as antagonists to compete with allergen-derived peptides for MHC occupancy. *In vitro* studies show that a nonstimulatory analogue of the influenza T cell epitope HA307-319 that binds MHC class II with high affinity, is able to inhibit the proliferation of house dust mite (HDM)-specific polyclonal and monoclonal T cell populations derived from allergic patients by 65-90% (O'Hehir RE *et al.* 1991b). Furthermore, in the murine model of multiple sclerosis, EAE induction is prevented if the mouse is first immunised with nonimmunogenic peptides with a high affinity for MHC class II (Lamont AG *et al.* 1990). This approach,

however, may not prove feasible for a myriad of reasons. Firstly, it would involve the identification of determinant T cell epitopes, and evidence suggests that multiple T cell epitopes are present in a single allergenic protein. Furthermore, *in vitro* studies and a lack of HLA disease association demonstrate that HDM allergens can be presented by a wide variety of MHC alleles. Finally, exogenous peptide would have to compete for MHC occupancy with a constant supply of HDM allergens, the quantity of the nonstimulatory peptide required may, therefore, exceed physiologically acceptable levels (Hetzel C *et al.* 1996).

Deviation of Th1/Th2 phenotype of the responder population in mice has shown to be dependent on conditions during priming such as type of APC encountered, presence and type of adjuvant used, nature of any costimulatory action, cytokine presence and the nature of the TcR-MHC interaction. As discussed in **section 1.11** , costimulatory signals may determine the cytokine profile secreted by T cell populations. Evidence suggests that B7-1 and B7-2 do not deliver identical signals, as ligation of B7-2 on naive T cells induces higher levels of IL-4 (Freeman GJ *et al.* 1995). Further evidence supporting this hypothesis coming from the work, discussed previously, by Kuchroo *et al.* They show in their model of EAE that treatment with anti-B7-1 mAbs leads to amelioration of the disease with generation of Th2 clones predominating, whereas treatment with anti-B7-2 mAbs favours Th1 development and disease exacerbation (Kuchroo VK *et al.* 1995).

Modulating the TcR-MHC interaction by using analogues of immunogenic peptides (termed altered peptide ligands or APLs) has also been looked at as a way of deviating the immune response, either by Th1/Th2 deviation or by tolerance induction. *In vitro* studies show APLs are able to prevent murine Th2 proliferation without affecting IL-4 production (Evavold BD and Allen PM 1991). Treatment of house dust mite reactive T cells with APLs has been shown to deviate the response from IL-4 to IFN-γ secretion (Tsitoura D *et al.* 1996). Futhermore, when some APLs were presented to T cells on live APCs a form of anergy was induced (Sloan-Lancaster J *et al.* 1993). Comparison of anergy induction in Th1 Vs Th2 clones of identical specificity using APLs, shows that anergy seems to be induced under similar conditions, i.e., on stimulation with fixed APCs or with live APCs together with APL. The state of anergy was long lasting, and unlike the Th1 clones, the Th2 clones retained the ability to secrete IL-4 (Sloan-Lancaster J *et al.* 1994a). Windhagen *et al.* have observed that production of IL-2, IL-4, IL-10 and IFN-γ is abrogated when human Th0 clones are stimulated with APLs, with only TGF-β1 being secreted (Windhagen A *et al.* 1995).

In vivo studies lend support to the *in vitro* findings. Soloway *et al.* have shown that changes in either the MHC class II allele or the bound peptide can change the response from one that induces Th1 proliferation, to one that results in IgE secretion (Soloway P *et al.* 1991). Renz *et al.* analysed Vβ restriction in mice responding to

ragweed allergen. They found that allergen-specific Vβ8.2 T cells stimulated IgE and IgG_1 production, whereas antigen-specific T cells expressing other Vβs induced IgG_{2a} and IgG_3 production (Renz H *et al.* 1993). Furthermore, mice injected with human collagen IV selectively induced a Th1 or a Th2 response depending on the MHC background of the immunised mice (Murray JS *et al.* 1993).

It is, therefore, apparent that many approaches are being studied, not only to try to elucidate the molecular mechanisms of allergy induction, but also to try to utilise this knowledge to obtain successful and long lasting immunotherapies. One possible approach that has been mentioned briefly is that of anergising allergic T cells, and in this way inducing a long lasting nonresponsiveness to a particular allergen. Many models of T cell anergy exist and this is discussed in the next section.

1.14 The role of T cells in anergy

1.14a *In vitro* models of T cell anergy

An anergised lymphocyte is a viable cell that fails to display certain functional responses when optimally stimulated through both its antigen-specific receptor and any other receptors normally required for full activation (Schwartz RH 1996). *In vitro* studies of anergy can generally be classed as falling into one of four types or models, with three of these four models seemingly sharing a common molecular mechanism of induction of the anergic state. **Figure 1.12** depicts the possible mechanisms by which anergy may be achieved in the models discussed below.

Anergy induction due to an absence of costimulation

The first model is that of murine or human T cell anergy induced by TcR occupancy in the absence of costimulation. Two hypotheses have been suggested to explain these observations. One is that costimulation would normally deliver a signal suppressing the production of molecular inhibitors that are purported to cause anergy, in the absence of costimulation, therefore, the state of anergy ensues. Studies using human clones have reported that B7 engagement of CD28 does indeed block the production of Nil-2a, a negative regulatory factor that has been shown to block AP-1-induced transactivation of reporter constructs (Becker JC *et al.* 1995, Williams TM *et al.* 1991). IL-2 production on T cell activation in the presence of costimulation (approx 30-100 fold more than in its absence) provides the basis for the second hypothesis. This increase in IL-2 levels is thought to prevent the Nil-2a from being produced by signal transduction through the IL-2 receptor (IL-2R), block its action once it has been made, or dilute out any effect it may have had by stimulating multiple rounds of division. Studies have shown that signalling through the γ chain of the IL-2R prevents anergy in human T cell clones (Boussiotis VA *et al.* 1994a). Induction of anergy in murine clones was observed if antibodies to IL-2 or IL-2R were added on normal

stimulation of the cells, or if any IL-2 produced after 12 hours of stimulation was washed out (Beverly B *et al.* 1992, DeSilva DR *et al.* 1991).

This model of anergy has been well characterised and has been shown to be caused by a block in IL-2 production. IL-2 gene transcription is decreased about 8-fold, its secretion 20-fold (Kang S-M *et al.* 1992). Recently, this transcriptional inhibition has been attributed to a failure in $p21^{ras}$ activation following TcR occupancy, resulting in inhibition of the ERK and JNK pathways and subsequent block in AP-1 transcription, which itself is critical for IL-2 gene transcription (Fields PE *et al.* 1996, Li W *et al.* 1996). Additionally, increased amounts of Nil-2a, a negative regulatory factor have been found in anergic human T cells (Becker JC *et al.* 1995). Nil-2a has been shown to block AP-1-induced transactivation in reporter constructs (Williams TM *et al.* 1991). As well as a decrease in IL-2 production, IL-3 and GM-CSF secretion is reduced 10-fold. IFN-γ production has been shown to be unaffected, as has IL-4 in murine studies looking at Th0 cells (Beverly B *et al.* 1992).

The *in vitro* anergy model described above is able to reverse its anergic phenotype if stimulated in the presence of IL-2 (Beverly B *et al.* 1992). This occurs as the anergised cells express very low levels of the high affinity IL-2R, and IL-2 production has been demonstrated at both the level of cytokine production and transcriptional activation of the IL-2 gene on anergy reversal (Beverly B *et al.* 1992, Kang S-M *et al.* 1992). However, in one study of anergised human T cell clones, reversal of anergy only occurred if an additional stimulus of anti-CD2 antibodies was given (Boussiotis VA *et al.* 1994b).

The *in vitro* phenomenon of anergy reversal raises questions regarding its possible physiological relevance. It could be argued that localised pockets of IL-2, produced by T cells responding to foreign antigen, may reactivate anergised cells tolerant to self, resulting in autoimmunity. However, studies looking at superantigens and adoptive transfer of T cells from TcR-transgenic mice, have demonstrated that anergy can be induced *in vivo*. Immunisation of superantigens induced expansion and deletion phases in T cells expressing certain Vβs, however, a residual population of Vβ+ T cells was observed. In a subsequent proliferation assay, these remaining T cells were refractory to restimulation, and were found to produce only small amounts of IL-2. It was also noted that tyrosine phosphorylation events, indicative of the anergic state also occurred. Intriguingly, the proliferative block was only partially reversed if IL-2 was added back into the restimulation cultures, an event that has never been seen in *in vitro* studies using murine T cell clones (Bhandoola A *et al.* 1993, Kearney ER *et al.* 1994, Sundstedt A *et al.* 1996). The possibility of anergic T cells in the periphery being able to respond to IL-2 *in vivo*, has led to speculation that these autoreactive cells may take place in normal immune responses, thereafter reverting to a quiescent form until being eventually deleted (Janeway Jr. CA 1992, Matzinger P 1994).

Anergy induction in the presence of IL-10

As discussed in **section 1.12**, IL-10 has been shown to exert major effects on macrophages and dendritic cells. Studies show IL-10 is able to convert activated macrophages to a resting monocyte-like state, in which cytokines such as IL-1, IL-12 and TNF-α as well as any costimulatory molecules are downregulated (Ding L and Shevach EM 1992). Exposure of freshly isolated Langerhans cells to IL-10 overnight can convert them from stimulators of proliferation to inducers of anergy in murine T cell clones (Macatonia SE *et al.* 1993). Groux *et al.* purified human CD4+ T cells and stimulated them with anti-CD3 mAbs coated onto a plate in the presence of IL-10. Anergy induction ensued, characterised by a block in IL-2 and GM-CSF as seen in the previous model, but unexpectedly IFN-γ production was also blocked (Groux H *et al.* 1996). As for the absence of costimulation model, signal transduction studies revealed an intact calcium mobilisation pathway, and the ability of phorbol esters and ionomycin to bypass the anergic block. However, cells in which anergy was induced using IL-10 failed to reexpress the high affinity IL-2R, and therefore could not be stimulated using exogenous IL-2, thus this anergic state appears not be reversible (Fields PE *et al.* 1996, Groux H *et al.* 1996, Li W *et al.* 1996).

As a consequence of this study the belief that anergy may exist at different levels has arisen. Studies by Sundstedt *et al.* lend support to this idea. They repeatedly immunised mice with superantigens to create an anergic T cell population, and found that on restimulation *in vitro*, NF-AT, NF-κB p65/p50 and AP-1 binding to IL-2 enhancer response elements was impaired (Sundstedt A *et al.* 1996). The *in vivo* studies mentioned earlier involving TcR-transgenic mice, as with the IL-10 anergy model, found that the anergic T cells failed to reexpress the high affinity IL-2R (Bhandoola A *et al.* 1993). This observation suggesting that these anergic states may reflect a more profound block in T cell responsiveness than that seen in the *in vitro* absence of costimulation model.

Altered peptide ligand anergy induction

As discussed in **section 1.13**, some APLs have been shown to possess the ability to induce a state of unresponsiveness in murine T cell clones (Sloan-Lancaster J *et al.* 1993). On restimulation, IL-2 production was blocked, although upregulation of the IL-2Rα chain still occurred. In this case, however, anergy induction could still be achieved in the presence of functional APCs. Phosphorylation patterns compared to stimulation with full agonists revealed that Zap-70 was not phosphorylated and that there was a marked reduction in CD3ε chain phosphorylation and in 23kD ζ chain phosphorylation (Sloan-Lancaster J *et al.* 1994b). Initial interpretation of these findings suggested that the partial signalling pattern was responsible for the anergic state.

However, more recent studies by Madrenas *et al.* show that this unusual phosphorylation pattern is not observed when fixed APC and full agonist peptide are used to induce anergy. They therefore suggest that either the partial signalling is not indicative of the anergic state or that the two unresponsive states are not equivalent (Madrenas J *et al.* 1996). The observation that IL-2 can prevent anergy induction in this model, and that its addition does not alter the unusual tyrosine phosphorylation pattern, suggests that this event is indeed not indicative of anergy induction in this case.

The ability of IL-2 to reverse the anergic state in this model leads to the conclusion that, as for the absence of costimulation model, downstream events directly controlling IL-2 production, such as inhibition of the Ras signal transduction pathway determine whether T cell anergy or proliferation follow stimulation using APLs.

Anergy induction in the presence of high concentrations of soluble peptides

Studies looking at the effects of high dose peptide antigens on human CD4+ T cell clones, led to the first observation of proliferative nonresponsiveness in purified T cells (Lamb JR *et al.* 1983). Subsequent blocking studies showed the involvement of MHC class II molecules expressed on T cells, anergy induction being attributed to T cells presenting peptide to each other (Lamb JR and Feldman M 1984). As seen with the APL model, presence of APCs does not inhibit the induction of anergy. Indeed, APC presence leads to a large proliferative response with significant amounts of IL-2 being produced. Thus despite the presence of large quantities of IL-2, anergy induction still ensues. As with the IL-10 model, the nonresponsive state is profound with IL-2, IL-4 and IFN-γ mRNA and IL-2 cytokine secretion undetectable (LaSalle JM *et al.* 1992). Downmodulation of CD28 and the TcR also occurs, further studies showing the rapid decay of CD28 mRNA transcripts on anergy induction (Lake RA *et al.* 1993). Molecular studies indicate impaired calcium responses and diminished binding of NF-AT, but not AP-1, to the IL-2 response element (Wotton D *et al.* 1995). This model of anergy is reversible on addition of exogenous IL-2 or on addition of both PMA and ionomycin (Essery G *et al.* 1988, Lamb JR and Feldman M 1984, LaSalle JM *et al.* 1992). Prevention of anergy induction on addition of IL-2 to the tolerance-inducing culture has also been reported (Essery G *et al.* 1988).

This model therefore appears to differ from the other three in that the mechanism of anergy induction seems to be via a block in the calcium/calcineurin pathway. For the other models described the mechanism has either been shown to be due to a block in the Ras pathway (anergy in the absence of costimulation) or is speculated to be induced by such a block (Schwartz RH 1996).

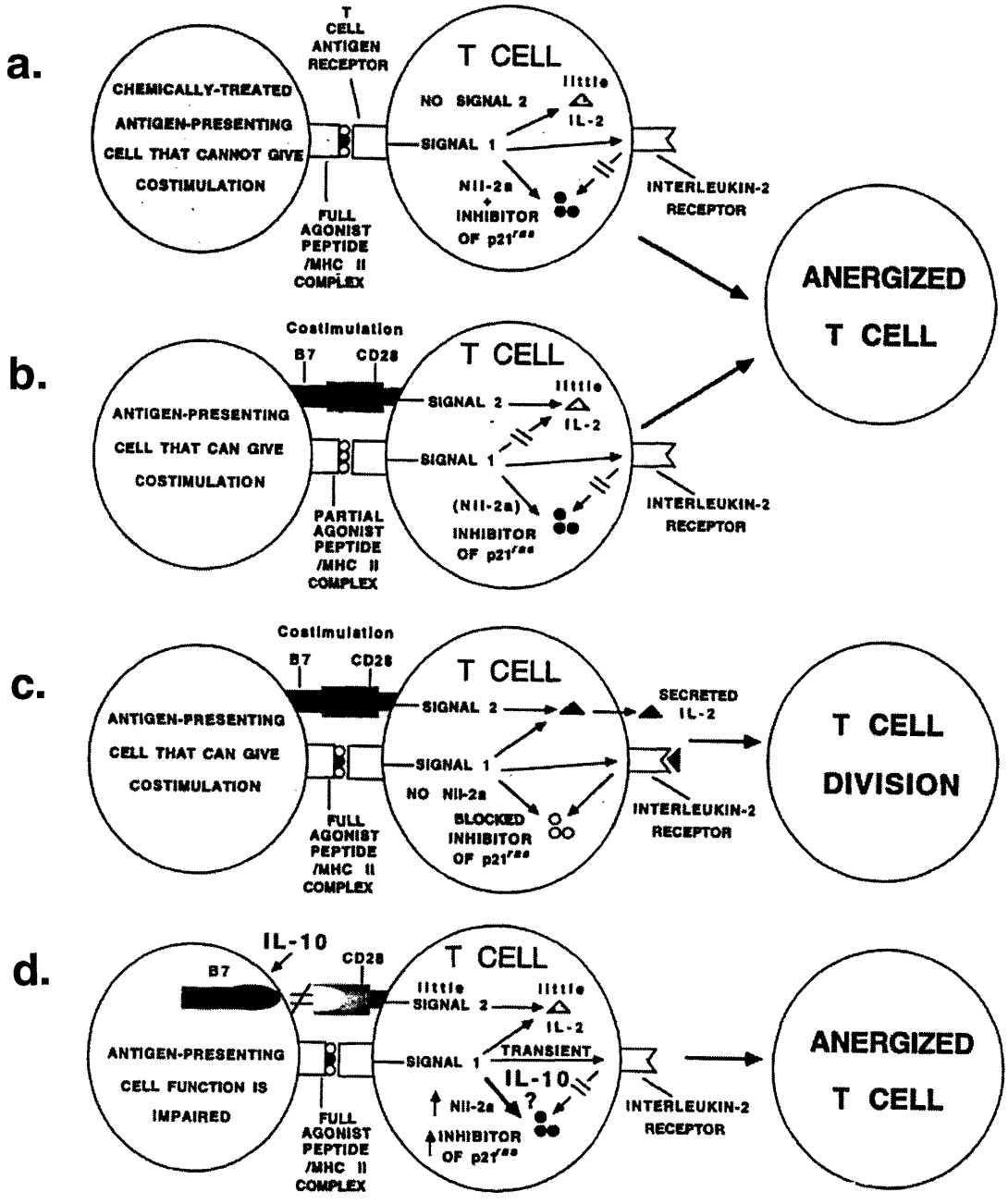


Figure 1.12 Potential outcome of TCR occupancy

A method of unifying anergy induced by TCR occupancy in the absence of costimulation with anergy induced by altered peptide ligands (APLs) through a common underlying molecular mechanism. A speculative model for how IL-10 may augment anergy induction is also shown.

a. TCR occupancy in absence of costimulation
b. anergy induced by APLs
c. optimal ligation leading to full cell activation
d. Anergy induction in the presence of IL-10

Reproduced from: Schwartz, 1996

In general all these models of anergy can be characterised as performing the function of blocking the cell's ability to produce or respond to proliferative signals. Anergic Th0 cells do not produce IL-2, they do secrete IL-4, but are unable to use it as a proliferative signal (Chiodetti L and Schwartz RH 1992, Mueller DL *et al.* 1991). Th2 cells have been shown to behave similarly on anergy induction (Sloan-Lancaster J *et al.* 1994a). Anergy induction in Th1 cells has been shown to lead to a loss in response to IL-12 and an inability to induce B cell proliferation due to a block in CD40 ligand expression (Bowen F *et al.* 1995, Quill H *et al.* 1994). Finally, CD8+ T cells have been reported to display a block in IL-2 secretion, but are able to continue to function effectively as CTLs (Otten GR and Germain RN 1991).

1.14b *In vivo* systems of T cell anergy

The *in vivo* immune response to an antigen is dependent on the dose and physicochemical nature of the antigen, as well as the route, duration and conditions of its administration (Hetzel C *et al.* 1996). Studies indicate that delivery of moderate to high doses of soluble protein or peptide antigen via mucosal routes (Hoyne GF *et al.* 1993), subcutaneously (SC) (Briner TJ *et al.* 1993), intraperitoneally (IP) (Burnstein HJ *et al.* 1992) or intravenously (Peterson JD *et al.* 1993) result in nonresponsiveness to subsequent challenge with antigen in adjuvant. Although these studies report selective tolerance induction in the Th1 subset, immunisation using chemically modified antigen via the SC, IP or intranasal (IN) routes report a partial or substantial downregulation of antigen-specific human IL-4 and IL-5, with a concomitant increase in IFN-γ production (Jutel MP *et al.* 1995, Secrist H *et al.* 1993). Murine studies by Briner *et al.* found that SC immunisation of a peptide (IPC-2) from the cat allergen *Fel d* I, resulted in decreased T cell responses on subsequent challenge with IPC-2 in both naive and primed mice. Additionally, pretreatment using two dominant peptides led to nonresponsiveness against the whole recombinant protein (known as 'intramolecular epitope suppression'). Production of IL-2, IL-4 and IFN-γ was diminished, suggestive of either nonresponsiveness in both the Th1 and Th2 populations and/or possibly in the Th0 population (Briner TJ *et al.* 1993).

Tolerance induction via mucosal routes (i.e. respiratory and G.I. tracts) has been documented by several groups. Weiner *et al.* attempted to tolerise MBP-specific responses in multiple sclerosis (MS) patients by feeding spinal cord as a source of MBP antigens, some patients experiencing limited disease amelioration (Weiner HL *et al.* 1993). However, in the murine model of MS no significant impact on EAE incidence, onset or severity was seen when H-2^u mice were fed encephalitogenic peptide analogues. When the same peptide was inhaled prior to EAE induction, however, disease development was markedly reduced (Metzler B and Wraith DC 1993).

Work by Holt and colleagues has begun to elucidate the mechanism of tolerance to inhaled antigen. They have shown that inhaled OVA antigen was poorly presented by lung dendritic cells under the regulatory influence of alveolar macrophages in the respiratory tract (Holt PG *et al.* 1993). Further studies indicated that acquired IgE hyporesponsiveness on peptide inhalation, may be due to the stimulation of CD8+, IFN-γ producing cells, which would deviate the response away from a Th2 type. Furthermore, these cells were reported to secrete, and be responsive to IL-2. Their IL-2 production was measured as being in the range of 6-fold more than that observed for antigen-specific CD4+ T cells (McMenamin C and Holt PG 1993). This IgE regulation has since been found to be mediated by a population of γδ T cells (McMenamin C *et al.* 1994). This effect of antigen inhalation is therefore more accurately described as immune deviation as opposed to tolerance per se. As discussed previously, other groups have found that *in vivo* tolerance can be induced by peptides administered via the oral or intranasal routes.

Hoyne *et al.* have used low concentrations of dominant and subdominant epitopes from the house dust mite allergen *Der p* I, and observed that after intranasal exposure to either epitope mice became profoundly unresponsive to subsequent SC challenge with whole *Der p* I in adjuvant. Lymph node cells from these mice failed both to secrete IL-2 upon stimulation *in vitro* with *Der p* I, and to provide cognate help to primed B cells. The tolerised cells also displayed the ability to suppress the effector function of primed CD4+ T cells in the spleen. Furthermore, peptide administered intranasally was reported to induce tolerance not only in naive mice, but also in mice that had been previously sensitised to *Der p I* via an immunogenic route (Hoyne GF *et al.* 1993).

1.15 MHC Class II on T cells and its role in anergy induction

Studies, to date, indicate that a proportion of human (Lamb JR *et al.* 1983), rat (Reizis B *et al.* 1994), guinea pig (Burger R *et al.* 1984) and ovine (Dutia BM *et al.* 1993) peripheral T cells synthesise and express MHC class II molecules. In humans and rats this proportion has been shown to be about 10% of peripheral T cells, with an increased frequency observed upon activation (Reizis B *et al.* 1994, Yu DTY *et al.* 1980).

1.15a Human T cell studies

Activated human T cells have been shown to express MHC class I, ICAM-1, LFA-3 and B7.1, although at lower levels than on a professional APC such as a B cell (Pichler WJ and Wyss-Coray T 1994). Few resting T cells express MHC class II, but the majority begin to express HLA-DR, DP and DQ molecules upon activation. *In vivo* studies have revealed that B7.1 expression varies between MHC class II+ T cells.

Further work has indicated that B7.1 is preferentially found on activated T cells infiltrating tissues, but not on circulating T cells (Barnaba V *et al.* 1994). Furthermore, repetitive stimulation of T cells has been hypothesised to be a requirement for induction of B7.1 expression. This idea was supported by studies of HIV-1-infected-T cell lines that were shown to express around 9-fold more B7.1 than uninfected T cell lines, as well as about 1.5 times more B7.1 than a representative B cell line (Haffar OK *et al.* 1993).

Human T cell clones have been shown to secrete the same pattern of cytokines when stimulated by either peptide presented on T cells or peptide presented on B cells, the cytokine profiles produced being representative of their functional phenotype (Wyss-Coray T *et al.* 1993). However, if these clones are stimulated with either superantigen or peptide presented on T cells they become unresponsive to further antigenic stimuli, whereas activation with B cell-presented peptide does not alter their function. As discussed in the previous section the anergised T cells fail both to respond to anti-CD3- or anti-CD2-mediated signals and to secrete cytokines, but are able to proliferate in response to exogenous IL-2 (LaSalle JM *et al.* 1992, O'Hehir RE *et al.* 1991). Pichler *et al.* also investigated the effect of T cell clones if used as stimulators in an alloresponse, with resting T cells as responders. They found that such a mixed lymphocyte reaction (MLR) yielded antigen-specific, CD4+ cytotoxic T lymphocytes, whereas if they used B cells as stimulators CD4+ T cells with no cytotoxic activity were induced (Pichler WJ and Wyss-Coray T 1994). In contrast, Satyaraj *et al.* found that an MLR set up using freshly isolated T cells as responders were anergised in coculture with anti-CD3-activated T cells (Satyaraj E *et al.* 1994).

Barnaba *et al.* have reported that activated human T cells can take up antigen and present it via their MHC class II molecules. It was also observed that synthesis of new MHC class II molecules only occurs on antigen-specific stimulation via a TcR/CD3-mediated signal, suggesting that the T cells may preferentially present the stimulatory antigen (Barnaba V *et al.* 1994). In contrast, LaSalle *et al.* found that their T cell clones were only able to present and respond to peptide or degraded antigen, but were unable to process intact protein (LaSalle JM *et al.* 1992). The reason for these different observations may lie in differences in the T cell clones themselves, or possibly in the nature of antigen used.

1.15b Rat T cell studies

Reizis *et al.* have shown that around 10% T cells found in the spleen and lymph nodes were MHC class II+. They found that in the thymus small, TcR^{lo} cells and about 20% of the mature single positive (SP) T cells also expressed MHC class II, and at higher levels than seen in the periphery. They were able to isolate mRNA transcripts from these cells, but were only able to detect newly synthesised MHC polypeptides

from the mature population. Induction of an *in vivo* immune response using MBP, led to an increase in the number of T cells in the periphery expressing MHC class II, to a maximum of about 20% on day 5, before decreasing back to initial levels on day 9. Once again, immunoprecipitation indicated that at least some of this MHC class II had been synthesised by the T cells. However, when long term T cell lines were set up, it was observed that if these T cells were activated in the presence of bystander thymocytes or APCs passive acquisition of MHC class II occurred. This observation was confirmed by the finding that mRNA was present at low levels that increased only slightly on activation, and by immunoprecipitation experiments that detected the synthesis of only low levels of MHC class II molecules (Reizis B *et al.* 1994).

Short-term rat T cell lines grown in the absence of additional APCs, were able to present native MBP as effectively as pepsin digested MBP to MBP-specific responder lines. Pulsing these T cells with the native MBP protein in the presence of chloroquine, resulted in a loss in ability to activate responder cells, thereby providing further evidence that processing is actually taking place. Finally, this group incubated their MBP-specific line for 72 hours, together with MBP but in the absence of additional APCs. They then transferred these cells to syngeneic recipients which resulted in induction of EAE with such severe clinical manifestations that all the recipients had to be euthanised (St. Louis JM *et al.* 1994).

Finally, Mannie *et al.* looked at two CD4+ T cell clones, both specific for MBP. One (GP2) recognised MBP as a partial agonist and mediated mild EAE, whereas the R2 clone recognised MBP with full efficacy and mediated severe EAE. They found that addition of an anti-CD4 antibody, together with LRTC1 (an antibody that neutralised an undefined rat costimulatory activity without blocking antigen recognition), fully blocked MBP-stimulated proliferation of GP2 cells, but only partially inhibited R2 responses. They also observed a higher susceptibility to anergy in the GP2 clones, which they suggested was due to the MBP interacting with this T cell in a partial agonistic/antagonistic capacity. Data was also presented showing the superior APC activity of the anergic T cells, compared with activated MHC class II+ T cells. MHC class II restricted presentation of MBP by anergic GP2 cells to R2 cells, led to anergy induction in the R2 population. This anergic state could also be transferred to a separate population of R2 cells. Finally, adoptive transfer experiments showed that immunisation of anergic GP2 T cells led to the establishment of tolerance, as measured by acquired resistance to active induction of EAE (Mannie MD *et al.* 1996).

1.15c Murine T cell studies

Despite the fact that several groups have reported the expression of MHC class II both *in vitro* and *in vivo* (Gautam SC *et al.* 1991, Kira J-I *et al.* 1989), others have maintained, and it is now generally accepted that any MHC class II present on murine T

cells is passively absorbed from antigen-presenting stimulator cells (Lorber MI *et al.* 1982).

An exception to this rule seems to be in the case of some T cell clones. Reske-Kunz and colleagues were able to establish an OVA-specific T cell clone that expresses the IL-2R at low density and synthesises and expresses MHC class II molecules constitutively. Furthermore, they showed that this clone was able to present various protein antigens to antigen-specific CD4+ T cell lines. Presentation of antigen to the T cell lines by the clone resulted in production of lymphokines, including IL-2, although no upregulation of the IL-2R occurred (Reske-Kunz AB and Diamantstein T 1987, Reske-Kunz AB *et al.* 1986). Compared with the more traditional APCs, such as whole spleen or bone marrow-derived macrophages, the T cell clones required 10-fold higher antigen concentrations in order to activate the antigen-specific T cells. Further work by the group used a system of OVA coupled to transferrin and showed that when this conjugate was used in their presentation assays, 240-fold less OVA was sufficient to induce a proliferative response equivalent to that seen when normal OVA was used. A similar shift in the dose response curve was seen if B lymphoma cells were used as APCs, whereas no shift was seen if bone marrow-derived macrophages were used (Tschoetschel U *et al.* 1996). These results were interpreted to suggest that the T cell clones normally take up antigen by the method of pinocytosis, and that this method is relatively inefficient at targeting the antigen to the processing compartment. Coupling the antigen to transferrin, and thereby entering the cell via the transferrin receptor allows targeting mainly to the early endosomes, in which processing is reported to occur (Dickson RB *et al.* 1983, McCoy KL *et al.* 1993).

The findings that MHC class II is expressed on both human and rat T cells, and can play a role in the cell mediated immune response, has led to studies looking into the reasons for the absence of this molecule in murine T cells at a molecular level. Steimle *et al.* described the gene CIITA which was found to be responsible for expression of the MHC class II genes (Steimle V *et al.* 1993). Cells derived from patients with bare lymphocyte syndrome were found to lack CIITA expression, and CIITA was subsequently found to closely correlate with both constitutive and inducible expression of MHC class II molecules (Chang C-H *et al.* 1994, Steimle V *et al.* 1993). Chang *et al.* transfected a murine T cell hybridoma with human CIITA cDNA, and found that high levels of I-A and I-E MHC class II molecules were expressed on the cell surface. Antigen presentation experiments showed that the transfectants were able to present peptide to each other in an MHC class II restricted manner, and secreted IL-2 as a consequence. They were also shown to be able to present peptide antigens to other responder T cells, however, they were unable to respond to whole protein antigen. This may be due to inefficient uptake of the whole antigen, or possibly some additional molecules required for normal antigen processing are missing from the cell (Chang C-H

et al. 1995). It may also be, as suggested by Tschoetschel *et al.*, that the antigen may not be being targeted to the correct compartment for antigen processing (Tschoetschel U *et al.* 1996) and that the molecules that would normally facilitate this process are absent in this model. CIITA has been shown to facilitate expression of invariant chain and HLA-DM (Chang C-H *et al.* 1995).

Taken together, these various studies strongly suggest that the MHC class II found on T cells in humans and rats does have an integral role to play in regulation of the cell mediated immune response. The overall picture emerging from these studies suggests that these cells, on stimulation, may act either by generating populations of cytotoxic T cells, or by inducing anergy in the responder cell population, thereby possibly contributing to homeostasis in the immune system. The differences and similarities observed in the functionality and phenotype of MHC class II expressing T cells from humans, rats and mice are summarised in **table 1.2**.

Table 1.2 Summary of the similarities and differences observed in human, rat and mouse MHC class II expression and its possible functions.

	Human	Rat	Mouse
T cell MHC class II	+	+	clonal (+/-)
Peripheral expression	10% resting 100% activation	10% resting 20% activation	0% resting 0% activation
T cell B7 family expression	+	ND	ND
Ag presenting ability	conflicting results: peptide ++ Native Ag +/-	peptide ++ Native Ag ++	Positive clones: peptide ++ Native Ag +/-
Cytokine profile on T-T presentation	IL-2 IFN-γ IL-4 TNF-α	IL-2	IL-2 IL-3
Result of T-T presentation	CTL development (CD4 and CD8) Development of anergy	T cell response depends on peptide and presence of costimulatory molecules: Activation or anergy	ND

Key: Ag, Antigen; T-T, T cell to T cell presentation; ND, not done.

CHAPTER 2
Materials and Methods

2.1 Buffers and Solutions

The composition of all solutions and reaction buffers is listed in **Appendix 1**. All chemicals used were purchased from BDH unless stated otherwise.

2.2 Cell lines

Cell lines were obtained from a variety of sources: The European Collection of Animal Cell Cultures (ECACC), the American Type Culture Collection (ATCC), and as gifts from other researchers. Lines were grown in either RPMI 1640 medium (human lines) or Dulbecco's modified eagle medium (DMEM, murine cells and lines), supplemented with 10% fetal calf serum (FCS) (Gibco/BRL), penicillin (100 IU/ml), streptomycin (100µl/ml), glutamine (2mM) and $5x10^{-5}$M β-mercaptoethanol (2-ME) in a water saturated atmosphere of 5% CO_2 in air at 37°C unless specified otherwise. All steps involving the continued culture of cells were performed aseptically in a laminar flow hood using sterile equipment. Cells were maintained in exponential phase by diluting the culture 1:10 with complete RPMI at periodic intervals chosen dependent on the growth characteristics of the cell line. All cell lines were periodically tested for the presence of mycoplasma, using a standard hybridisation assay (Genprobe).

2.2.1 Cell lines used

Jurkat (J6)	-	human T cell leukaemic line
EL4	-	murine T cell lymphoma line, H-2^b haplotype
HB99	-	murine B cell leukaemic line
HT2	-	murine IL-2 dependent helper T-cell line
P815	-	murine mastocytoma line, H-2^d haplotype
MF2.2D9	-	T-T hybridoma, H-2^b haplotype (kind gift of Dr. K.L. Rock)

2.3 Bacterial strain

XL-1 blue - genotype: *sup*E44, *hsd*R17, *rec*A1, *end*A1, *gyr*A46, *thi*, *rel*A1, *lac*$^-$, F'[*pro*AB$^+$ *lac*^{q} *lac*ZΔM15, Tn10(tet^r)]

The strain (a derivative of K12 *E.coli*) carries *lac*ZΔM15 and *lac*^{q} on an F' episome allowing for blue-white colour selection when transformed with plasmids

carrying the β-Gal gene. In addition, the strain contains an uncharacterised mutation which enhances the α-complementation to give a more intense blue colour on plates containing 5-bromo, 4-chloro, 3-indolyl, β-D-galactoside (X-gal), a chromogenic substrate, and Isopropylthio-β-D-galactoside (IPTG), an inducer of the β-Gal promoter.

2.4 Preparation of chemically competent *E.coli*

The protocol used was a modification of that previously described (Hanahan D 1983). An inoculum of *E.coli* XL1-blue was transferred aseptically from a frozen glycerol stock to 50mls 2xYT/tetracycline (12.5μg/ml) and grown overnight at 37°C with shaking. The overnight culture was sub-inoculated 1:50 into 1000mls prewarmed 2xYT. The $OD_{\lambda 600}$ was assayed (blanking on 2xYT) (T_o) and the flask incubated at 37°C with shaking until the culture reached mid-log ($OD_{\lambda 600} \approx 0.45$ - 0.6). The culture was aseptically transferred to pre-chilled sterile centrifuge flasks. These were incubated on ice for 15 minutes and spun at 4000rpm for 15 minutes in a pre-chilled rotor (all manipulations from this point were carried out on ice). The supernatant was removed and the bacterial pellet resuspended in 40mls ice cold TFB1, incubated on ice for 10 minutes and centrifuged as above. The supernatant was removed by decanting and the pellet drained thoroughly and resuspended in 4mls ice-cold TFB2. The mixture was dispensed in 200μl aliquots into prechilled microfuge tubes and snap-frozen in liquid N_2. The aliquots were stored at -70°C until required. Typical competent cells obtained with this method had a transformation efficiency of $5x10^{6}$-10^{7} transformants per μg supercoiled DNA.

2.5 Transformation of chemically competent *E.coli*

Competent *E.coli* were slowly thawed on ice and 40μl added to 4μl of a ligation reaction or 1ng supercoiled plasmid in a 1.5ml microfuge tube. Bacteria and DNA were incubated on ice for 20 minutes, heat shocked at 42°C for 90 seconds, returned to ice for 2 minutes and added to 250μl 2xYT medium in a flat bottomed 5ml tube. Transformations were incubated at 37°C for 45 minutes with shaking and ~150μl spread onto an ampicillin treated (50μg/ml) 9cm 2xYT agar plate. Plates were incubated overnight at 37°C in an inverted position.

One of the vectors used in this study, BSIIKS+, contains part (*lac*Z) of the bacterial β-galactosidase gene which can complement the *lac* deletion in the XL1-blue strain. In the presence of a chromogenic substrate, X-Gal (Sigma) and an inducer of the plasmid β-galactosidase gene (IPTG; Sigma) bacteria transformed with these plasmids will form blue colonies. If a fragment of DNA is cloned into the polylinker of this

plasmid, the *lac*Z gene is disrupted and the resulting colonies are white due to their inability to make β-Gal and act on the chromogenic substrate. For colour selection 40µl X-Gal (20mg/ml in dimethyl formamide) and 32µl IPTG (25mg/ml) were spread on the plate before the addition of the transformed *E.coli.*

2.6 Isolation of plasmid DNA

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

A single bacterial colony was inoculated into 5mls 2xYT medium (containing 50µg/ml ampicillin) and incubated with shaking at 37°C overnight. The overnight culture was centrifuged at 3000rpm for 10 minutes and the supernatant discarded. The bacterial pellet was resuspended by vigorous pipetting in 100µl Solution 1, transferred to a 1.5ml microfuge tube, and 200µl Solution 2 (alkaline SDS) was added to lyse the bacteria. Following gentle mixing and a 5 minute incubation on ice 150µl Solution 3 was added. This step complexes the genomic DNA and a high percentage of the bacterial proteins allowing them to be removed easily. The lysate was vortexed for 5 seconds and spun at 6000rpm for 2 minutes. The supernatant was transferred to a fresh tube containing 150µl of phenol:chloroform:propan-2-ol (PhOH/$CHCl_3$/IPA) (25:24:1), vortexed for 10 seconds and spun at 12000rpm for 5 minutes to remove the remaining protein. The aqueous phase was transferred to a fresh microfuge tube and 1/10 of a volume of 3M sodium acetate (NaOAc) pH 5.2 and 2.5 volumes of ethanol (-20°C) were added to precipitate the plasmid DNA. The tube was vortexed, incubated on ice for 10 minutes and spun at 12000rpm for 5 minutes. The resulting DNA pellet was drained and washed with 200µl 70% ethanol. The liquid was aspirated and the DNA pellet dried and resuspended in 50µl TER.

2.6.2 CsCl gradient preparation (maxi-prep)

A single bacterial colony was inoculated into 500ml 2xYT medium (ampicillin 50µg/ml) and incubated, with shaking, overnight at 37°C. The overnight culture was centrifuged at 4000rpm for 20 minutes in 500ml centrifuge tubes (Du Pont) in a Sorvall RC-28S centrifuge. The bacterial pellet was drained, resuspended in 5mls Solution 1 by vortexing and transferred to a 50ml falcon tube on ice. 10mls Solution 2 was added and the bacterial lysate gently mixed for 30 seconds and incubated on ice for 10 minutes. 7.5mls Solution 3 was added, mixed vigorously and the lysate was centrifuged at 3000rpm for 10 minutes at 4°C in a benchtop centrifuge. The supernatant was transferred to a fresh 50ml tube containing 10mls PhOH/$CHCl_3$/IPA (25:24:1), vortexed for 30 seconds and centrifuged for 10 minutes at 3000rpm. The aqueous layer was removed to a fresh 50ml tube and the DNA precipitated by the addition of 15mls of

propan-2-ol followed by vortexing and centrifugation at 3000rpm for 10 minutes. The supernatant was discarded and the pellet washed in 10mls 70% ethanol, drained and resuspended in 4mls TE pH 8.0. 8.4g CsCl was added and the solution made to 14g with TE pH8.0. 500µl ethidium bromide (10µg/ml) was added and the solution vortexed and spun at 3000rpm for 10 minutes and 25°C to remove any protein precipitate. The clear solution was transferred to a 16mm x 75mm heat-sealable ultracentrifuge tube (Beckman). The sample was then overlaid with a CsCl solution (ρ=1.48g/L) and the tube heat-sealed and spun in an ultracentrifuge rotor (70iTi - Beckman) in a Beckman LM-8 ultracentrifuge at 60000rpm and 25°C for 16 hours. Following ultracentrifugation, the tube was clamped over a 600ml beaker and the top of the tube pierced using an 18G needle. A second needle (18G) was inserted into the tube just above the lower ethidium bromide stained DNA band which was removed with a 5ml syringe (the lower band contained the supercoiled plasmid DNA, the upper band contained the circular and linearised plasmid DNA). Ethidium bromide was extracted from the DNA by mixing with three changes of 5mls of water saturated butan-1-ol which was removed from the aqueous (DNA-containing) layer after the two phases had separated. After the final extraction an equal volume of 1M ammonium acetate and six volumes of room temperature ethanol was added, mixed and centrifuged at 3000rpm for 20 minutes. The DNA pellet was rinsed with 70% ethanol, drained, air-dried in a laminar flow hood and resuspended in 500µl sterile TE pH8.0.

2.7 Determination of nucleic acid concentration

DNA solutions were diluted in the appropriate buffer by a factor of 50-100 and placed in a quartz cuvette of 100µl capacity. The optical density (OD) of the solution was measured, relative to the diluent buffer at a wavelength of 260nm ($OD_{\lambda 260}$) using a UV/vis spectrophotometer (Pharmacia). The original concentration in µg/ml of DNA was determined by multiplying the absorbance by the appropriate dilution factor and, for DNA dividing by 20 or, for single stranded DNA oligonucleotides, dividing by 30. Thus, an absorbance reading of 1.0 corresponds to a concentration of 50µg/ml for DNA or 33µg/ml for an oligonucleotide. To assay the purity of the sample the OD at 280nm was determined. A ratio ($OD_{260/280}$) of between 1.8-2.0 indicates that the sample is free from contaminating protein.

2.8 Restriction enzyme digests

Plasmids and amplified PCR fragments **(Section 2.14)** were tested for authenticity by restriction enzyme digestion. 4µl of miniprep DNA, 4µl of purified PCR fragment (both ≈ 1µg), or 1µg of maxiprep DNA were digested in a final volume of 20µl with 2-10U restriction enzyme (Boehringer, NBL, Promega). For purification

of restriction fragments, 10-20µl of the miniprep or 5-20µg of maxiprep DNA were digested in a final volume of 40µl with 20-30U restriction enzyme. Digestion was carried out in the appropriate restriction buffer at the appropriate incubation temperature according to the manufacturer's instructions for 1-3 hours. Double digests were carried out according to the manufacturer's tables of optimal enzyme performance in each buffer.

2.9 Agarose gel electrophoresis

The resolution of DNA fragments for analytical or preparative purposes was carried out by horizontal agarose gel electrophoresis. High melting temperature, electrophoresis grade agarose (Appligene), 0.8 - 2.0% (w/v) in 0.5xTBE was dissolved in a microwave oven, cooled to 50°C and ethidium bromide was added to 0.1µg/ml. Agarose was poured into a horizontal gel forming tray with the ends sealed with masking tape. Wells for sample loading were formed using a plastic comb. One sixth of a volume of sample loading buffer (6xGLB) was added to the sample before loading. 1Kb or 100 base pair markers (Gibco BRL) were electrophoresed, as appropriate, on all gels as size standards. Agarose gels were run in 0.5xTBE at a fixed voltage of 1-5V/cm. Ethidium bromide stained DNA fragments were visualised on a UV light box in a dark room and gels were photographed using polaroid film. Resolution of small DNA fragments (less than ~1Kb) was achieved by running DNA on agarose composed of a mixture of high melting temperature agarose (1%) combined with 2% nusieve agarose gel (Appligene).

2.10 Purification of DNA fragments from agarose gels

DNA was electrophoresed through 1 or 2% agarose in 0.5xTBE until clear separation of the desired fragment was achieved. The fragment was visualised using a UV light source and excised from the gel using a scalpel. The gel slice was added to a microfuge tube containing 300µl extraction buffer (Biorad) and incubated at 55°C for 5 minutes until the gel dissolved. 1ml of Wizard PCR resin (Promega) was added and the DNA sample treated according to the manufacturer's instructions. DNA was eluted from the resin using 50µl TE pH8 at 65°C.

2.11 Wizard method of DNA purification (Promega)

Commercially available DNA affinity resins (Promega) were used to purify plasmid DNA from agarose gels (**Section 2.10**) or enzyme reactions prior to further treatments or ligations. To a 50-500µl sample 1ml of the appropriate resin (PCR or DNA clean-up resin) was added. The sample was then transferred to a 3ml syringe barrel attached to a wizard minicolumn (Promega). After a 3 minute incubation at room

temperature, the syringe plunger was inserted and the resin slowly forced through the mini-column. The syringe was removed from the mini-column and the plunger removed. The barrel was then reattached to the mini-column (this prevents disruption of the resin bed) and 2mls of 80% IPA were added. The plunger was again inserted into the syringe and the solution pushed through the minicolumn. The syringe barrel was discarded and the mini-column transferred to a 1.5ml microcentrifuge tube. This was centrifuged at 12000rpm for 2 minutes to dry the resin. The mini-column was transferred to a new microcentrifuge tube and 30-50µl of TE pH8.0 (prewarmed to 65°C) was added. After a 2 minute incubation, the mini-column was centrifuged at 12000rpm for 20 seconds to elute the DNA. The minicolumn was then discarded and the DNA used or stored in the microcentrifuge tube.

2.12 Blunt-ending of purified restriction fragments

When attempting to clone fragments of DNA in cases where no enzyme sites were available which were compatible with the cloning vector, the ends of the fragment were blunted. Purified restriction fragments with 5' protruding ends were blunt-ended using the Klenow fragment of *E.coli* DNA Polymerase (Promega). 0.1-1.0µg of DNA fragment was resuspended in a total volume of 30µl containing 3µl of 10x Klenow buffer, 1µl of deoxynucleotides (2mM each of dATP, dCTP, dGTP and dTTP) and 4U Klenow. The reaction was allowed to proceed at 37°C for 30 minutes. The reaction was stopped by heating to 70°C for 15 minutes to denature the polymerases and the DNA was purified through a Wizard column (Promega) prior to being used in a ligation reaction.

2.13 PCR amplification of DNA fragments

For the PCR amplification of DNA fragments, 1-2ng of target DNA was made up to a total volume of 40µl containing 5µl of 10x PCR buffer, 5µl of nucleotides (2mM each dATP, dTTP, dGTP, dCTP) and 5µl of 5' and 3' primers (2.5µM each). A drop of mineral oil was then added to the sample to prevent evaporation during amplification. The reaction mixture was heated to 95°C for 10 minutes and then chilled on ice for 2 minutes. 1U of Taq polymerase (Promega) was added to this reaction in a volume of 10µl. The PCR reaction then proceeded using the following reaction conditions: T1; 94°C for 1 minute, T2; 50-60°C for 1 minute, T3; 72°C for 2 minutes for 35 cycles with a two second per cycle extension on the extension step, T3. The appropriate annealing temperature (AT) (T2) was chosen based on the characteristics of the primers chosen according to the formula AT = [(4 x (G + C)) + (2 x (A + T)) - 3]. In all PCR amplifications a tube containing no target DNA was included as a control. Amplified PCR fragments were run on a 1-3% agarose gel, depending on expected fragment size prior to further treatment. To ensure that the fragment amplified up was

indeed the correct piece of DNA, the PCR gel could be blotted onto Hybond N+ and probed with a cDNA probe (**section 2.21**).

2.14 Kinase treatment of purified PCR fragments

Prior to subcloning, some amplified PCR fragments or oligonucleotides need to be kinased in order to add on 5' phosphate groups since PCR primers being chemically synthesized do not begin with 5' phosphates. In this study, as the PCR fragments that were synthesised for subcloning incorporated restriction enzyme sites, on digestion 5' phosphate groups were generated and kinasing was not needed in this case (**section 3.2.3**). However, the oligonucleotide which was subcloned into the pTexIIL vector lacked 5' phosphate groups, thus kinasing was required here. The annealed oligonucleotides (~100pg) were resuspended in a volume of 20µl containing 2µl of 10x T4 Kinase buffer, 1µl of 0.1M dATP and 2U T4 kinase (Promega). The reactions were incubated at 37°C for 45 minutes. Reactions were stopped by heating to 65°C for 10 minutes. The oligonucleotides were then purified with phenol:chloroform (1:1), followed by reprecipitation using 1/10 3M NaOAc and 2-3 volumes ethanol.

2.15 Preparation of cloning vectors

5µg of plasmid vector was digested with a restriction enzyme to leave ends compatible with those of the DNA fragment to be ligated. Following digestion, 1ml of wizard DNA clean up resin (Promega) was added to the digest and the sample purified according to the manufacturer's instructions and eluted in TE (pH 8.0) in a volume of 50µl. The concentration of the DNA was assayed by spectrophotometry and made to 100ng/µl.

To minimize non-recombinant background when cloning fragments into a single restriction enzyme cut vector, the digested vector was treated with calf intestinal alkaline phosphatase (CIAP, Promega) to remove the 5' phosphate groups and thereby prevent self-ligation. 5µg of single cut vector was made up to 30µl containing 3µl of 10 x CIAP buffer and 1U of CIAP. The reaction was incubated at 37°C for 30 minutes. The CIAP was inactivated by the addition of 4µl of 0.5M EDTA followed by heating to 75°C for 15 minutes. The vector was purified by the addition of 1ml of wizard DNA clean-up resin and eluted in 50µl of TE pH8.0. The concentration of the DNA was assayed by spectrophotometry and made to 100ng/µl.

2.16 Ligation of DNA inserts into plasmid vectors

Ligations were carried out in a final volume of 10µl containing 1µl plasmid vector (100ng), 1-7µl of DNA fragment, 1µl of 10x ligase buffer and 2U DNA ligase (Promega). An estimated molar ratio of DNA fragment to plasmid of ~1:1 was usually used. The ligation reaction was carried out from 15 minutes to overnight at 22°C. 4µl

of the ligation mix was used to transform 50µl of competent *E.coli*. If possible DNA fragments were ligated into vectors cut with two restriction enzymes which generate internally incompatible ends to prevent religation of the vector. If a single cut vector, or blunt ended vector was used, the vector was first treated with alkaline phosphatase.

2.17 PCR screening of recombinant plasmids

Religated plasmids were tested for the presence of test insert by the use of PCR. Following ligation, transformation and incubation test colonies were examined. If colour selection was employed white colonies, indicating a recombination event, were tested further. In the absence of colour selection colonies were picked at random. Test colonies were inoculated into a mix of 30µl containing 3µl of nucleotides (2mM each of dATP, dTTP, dCTP and dGTP), 3µl of 10 x PCR buffer, 3µl of the appropriate primer pair (2.5µM each), and 1U Taq polymerase (Promega), using a 10µl pipette tip and then stabbed into an agar plate and numbered for further testing if required. In all amplifications two control samples were included - one containing no template and the other containing 10ng of empty plasmid as a standard to assay non-recombinant events. A drop of mineral oil was added to the reactions to prevent evaporation during amplification. Amplification conditions chosen were T1; 94°C 60 seconds, T2; 50°C 60 seconds, T3; 72°C 90 seconds for 35 cycles with a one second per cycle increase on the extension step, T3. Following amplification, 10µl of each reaction was mixed with 2µl of 6x sample loading buffer and resolved on a 1% agarose gel in 0.5xTBE at 5V/cm for 1 hour. 1Kb marker fragments (Gibco BRL) were electrophoresed on all gels as size standards.

Positive recombination events were indicated by the size of the PCR fragment - if the appropriate recombination event occurred, the size of the PCR fragment equalled the size of the insert plus the size of the fragment amplified from an empty vector (for pTexIIIa plus I-Aα ~1.4Kb). Colonies indicating a positive recombination were mini-prepped and restriction enzyme digest analysed to confirm this result.

2.18 Preparation of radiolabelled probes

Probes were radiolabelled using the random prime method (Feinberg AP and Vogelstein B 1984). The Megaprime labelling system (Amersham) was used for radiolabelling. DNA was diluted to a concentration of 5ng/ml in distilled water, 5µl DNA was added to 5µl of a random nonamer primer solution provided, and denatured by heating to 100°C for 5 minutes on a heat block. 10µl labelling buffer and 2µl Klenow were added to the tube at room temperature. 5µl of α-^{32}P-dCTP, specific activity 3000Ci/mmol, was added and the reaction mixture was incubated at 37°C for 10-30 minutes. Labelled probe was separated from unincorporated radionuclide by spin

column chromatography through a 1ml resin bed of sephadex G-50. The resin was placed in a 1ml syringe barrel packed with a 0.1ml volume of glass wool, which prevents the gel leaving the syringe barrel but allows liquid to pass through. This was placed in a 15ml falcon tube and centrifuged at 1100rpm for 1 minute to pack the resin. The spin column was washed once with TEN. 150µl of TEN was added to the labelling reaction which was transferred to the spin column and centrifuged at 1500rpm for 2 minutes. Labelled probe was collected in a microcentrifuge tube in the bottom of the 15 ml tube, and denatured by boiling for 5 minutes prior to use. In standard reactions it is reported that >70% of the radionuclide is incorporated into the probe (Amersham).

2.19 Southern blotting

Following the resolution of DNA fragments by agarose gel electrophoresis the gel was photographed, UV treated for 90 seconds to break the DNA backbone thereby allowing transfer to take place and pressure blotted onto Hybond N+ membrane (Amersham) using 0.4M NaOH as the transfer buffer in a modification of the standard protocol (Southern E 1975). Following overnight transfer, the apparatus was carefully disassembled, the membrane washed in 5xSSC and air dried. As the NaOH acts to crosslink the DNA to the membrane, UV crosslinking was not necessary.

2.20 Isolation of genomic DNA

Genomic DNA was prepared using Qiagen genomic tips (Qiagen). Cells were resuspended at a concentration of 10^7/ml in 2mls. 2mls of ice-cold buffer C1 (cell lysis buffer) and 6mls of ice-cold ddH_2O were added and the tube was inverted several times to mix. The mixture was then incubated for 10 minutes on ice. The lysed cells were centrifuged at 4°C for 15 minutes at 1300xg and the supernatant discarded leaving a small nuclear pellet visible. 1ml ice-cold buffer C1 and 3mls ice-cold ddH_2O were added and the pellet was resuspended by vortexing. The mixture was then centrifuged, again at 4°C for 15 minutes at 1300xg, after which the supernatant was discarded. 5mls of buffer G2 was added to the pellet and the nuclear pellet was resuspended by vigorous vortexing. 95µl of freshly prepared Proteinase K (20mg/ml) was added and the mixture was incubated at 50°C for 1 hour.

A Qiagen genomic-tip was equilibrated with 4mls of buffer QBT and the column was allowed to empty by gravity flow. Following the 1 hour incubation, the sample was vortexed, applied to the equilibrated column and was allowed to pass through the resin by gravity flow. The column was washed twice with 7.5mls of buffer QC. The genomic DNA was then eluted with 5mls buffer QF. 3.5mls isopropanol (equilibrated to room temperature) was added to the genomic DNA and the sample centrifuged at 5000xg at 4°C for 30 minutes. The supernatant was carefully poured off and the DNA was washed in 2mls ice-cold 70% ethanol, following which it was briefly

air dried and resuspended in 200-500µl TE pH8 depending on the size of the pellet. The concentration of the DNA was determined using a spectrophotometer (**section 2.7**) and the sample was stored at 4°C.

2.21 Preparation of RNA

All manipulations of RNA were carried out with clean sterile pipettes, tubes and tips, and buffers and solutions were prepared with double distilled sterile DEPC-treated water. Great care was taken with all steps since RNA is a very labile molecule, easily degraded by RNases and heavy metals. Thus gloves were used when handling all equipment and disposables. Any surface likely to come into contact with RNA was treated to remove contaminating RNases by thorough cleaning, soaking in 3% H_2O_2 and rinsing in DEPC-treated water.

Total cellular RNA was prepared using RNAzol B (Biotecx laboratories Inc). $2x10^7$ cells were washed twice in DEPC-PBS in a microfuge tube and resuspended in 200µl DEPC-PBS. 400µl of RNAzol B was added and the sample vortexed and stored on ice for 5 minutes. 200µl chloroform (Appligene) was added and the sample vortexed and centrifuged at 15000rpm for 10 minutes. The aqueous top layer was removed and transferred to a fresh microfuge tube containing 400µl isoproponal and the sample was stored at 4°C for 15 minutes. The RNA was pelleted by centrifugation at 15000rpm for 10 minutes at 4°C and the RNA pellet washed in 200µl 75% ethanol. RNA was resuspended in 5µls DEPC-H_2O for further manipulations.

2.22 Northern analysis of RNA

Total RNA isolated from $2x10^7$ cells was electrophoresed through 1.2% agarose-formaldehyde gels. 2.2g of electrophoresis grade agarose (Kodak IBI) was added to 140mls of DEPC-H_2O and melted in a microwave. The gel was cooled to 60°C and 44mls 5x formaldehyde gel running buffer and 40mls of formaldehyde (Sigma) were added and the gel poured into a thoroughly cleaned sealed horizontal gel casting tray with a gel comb in place (RNase treated by soaking in 3% H_2O_2 for 30 minutes followed by rinsing in DEPC treated water). The set gel was placed into a horizontal gel tank containing 1x formaldehyde gel running buffer.

To 5µl total RNA isolated as above, a cocktail of 15.5µl containing 10µl formamide, 3.5µl formaldehyde and 2µl 5x formaldehyde gel running buffer was added and the RNA denatured by heating to 65°C for 10 minutes. 4µl sample loading buffer was added and the samples were loaded onto the gel and run at 4V/cm for 4 hours. An extra lane containing J6 RNA was run on the gel to use as a marker track. After running the gel, the marker track was cut off and soaked in a solution of 1mg/ml ethidium bromine in water for 20 minutes. The marker track was then photographed under UV onto polaroid film and the 18S and 28S ribosomal RNA bands used as size

controls. The RNA was then transferred from gel to membrane by pressure-blotting overnight onto Hybond N using 20xSSC as the transfer buffer using a modification of the standard protocol (Southern E 1975). Following transfer, the membrane was air dried and the RNA UV-crosslinked onto the membrane.

2.23 Filter hybridisation

Hybond N or N+ membranes were separated using nylon sheets, rolled up and placed in hybridisation bottles (Hybaid UK) in 20-40mls of Church and Gilberts (C&G) solution. For membranes containing genomic DNA the C&G solution was supplemented with sheared salmon sperm DNA, boiled for 5 minutes on a hot block, at a final concentration of 100µg/ml. Filters were pre-hybridised for 1-3 hours at 65°C. Following prehybridisation, the buffer was replaced with 4-8mls fresh pre-heated C&G, denatured probe was added and the filters were hybridised overnight at 65°C. Following hybridisation, the probe was removed and the filters washed for 20 minutes at 65°C in successive changes of solutions containing 0.1% SDS and, in order, 2xSSC, 1xSSC, 0.5xSSC and 0.1xSSC. Following the 0.5xSSC / 0.1% SDS wash, the membrane was monitored and, if the radioactive counts were low, the 0.1xSSC wash was omitted. After washing, membranes were sealed in saran wrap and exposed to Fuji XR film and stored at -70°C for 1 hour to one week as necessary.

2.24 Restriction digestion of genomic DNA

20µg of genomic DNA was dispensed into a 1.5ml microfuge tube and made up to 200µl with deionised sterile water. Spermidine (Sigma) was added to 4mM and the tube incubated at 37°C for 30 minutes. A three-fold excess (60U) of the restriction enzyme of choice and 22µl of the appropriate 10x restriction buffer were then added. The DNA was incubated overnight at 37°C following which another 20U of the restriction enzyme was added and the sample was left for a further 3 hours. 200µl of PhOH/$CHCl_3$/IPA (25:24:1) was then added and the sample vortexed for 15 seconds and spun for 10 minutes at 15000rpm in a microcentrifuge. The aqueous layer was removed and transferred to a new microcentrifuge tube and the DNA was precipitated by the addition of 1/10 volume 3M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol followed by spinning in a microcentrifuge at 15000rpm for 10 minutes at 4°C. The supernatant was carefully decanted and the pellets washed in 200µl 70% ethanol. The pellet was air dried and resuspended in 30µl TE pH8. 6µl sample loading buffer (6x) was then added. Samples were loaded onto a 0.8% agarose gel and run at 100V for 1 hour and 30V overnight using 0.5x TBE as the running buffer. Gels contained a marker track of 1Kb ladder (Gibco BRL) as a size standard. Following electrophoresis,

the gel was photographed under UV and transferred onto Hybond N+ using pressure blotting with 0.4M NaOH as the transfer buffer.

2.25 DNA sequencing of double stranded templates

A modified version of the standard dideoxy chain termination sequencing method (Sanger F *et al.* 1977) was used with modified T7 Sequenase, v2.0 (USB) according to the manufacturer's instructions. 10µg maxiprep DNA or 10µl miniprep DNA was made up to 18µl in a microfuge tube with ddH_2O. 2µl 2M NaOH was added and the reaction incubated at room temperature for 5 minutes. The denaturation reaction was neutralised by the addition of 8µl of 5M ammonium acetate pH4.6 and the DNA precipitated by the addition of 200µl of ethanol at -20°C for 15 minutes. The tube was centrifuged at 15000rpm for 10 minutes at 4°C and the DNA pellet washed with 100µl 70% ethanol. The pellet was air dried and dissolved in 7µl ddH_2O and 10ng of the appropriate primer was then added to the sample.

Primers were annealed to the DNA by heating the mixture to 65°C for 2 minutes on a heat block, after which the block was removed from the heat source, the samples cooled slowly to 35°C and then stored on ice. 2.5µl of termination mixes (ddA, ddT, ddG, ddC; each at 8mM, and dA, dT, dG, dC at 80mM) were added to four separate 500µl PCR tubes. A mixture was made containing (per sequencing reaction): 1µl of 100mM DTT, 2µl of a five fold dilution in water of labelling mix (7.5mM each of dGTP, dATP and dTTP), 0.5µl of ^{35}S-dATP and 2µl of a five-fold dilution of Sequenase (T7 polymerase v2.0) in enzyme dilution buffer. 5.5µl of this mixture was added to each primer/template sample and after 5 minutes on ice, 3.5µl of the mix was transferred to the PCR tubes containing the termination mixes. After 5 minutes at room temperature, 4µl stop solution was added to each tube and the tubes were heated to 100°C for 2 minutes immediately prior to loading onto the gel. Sequencing reactions were electrophoresed through a 6% poly-acrylamide sequencing gel.

90cm x 750cm sequencing plates (Kodak IBI) were washed thoroughly with detergent, rinsed in ddH_2O and wiped clean with 90% ethanol. Plates, separated with 0.3mm spacers, were clipped together and the bottom sealed with pressure tape. 75mls of gel was added to 750µl 10% ammonium persulphate (APS) and 21µl TEMED (Sigma) and the gel poured between the plates. The flat edge of a 32 well sharkstooth comb (Kodak IBI) was inserted into the top of the gel to form a well and the gel was left in a horizontal position for at least 1 hour to polymerise. After polymerisation, the tape at the bottom of the plates was removed, the comb removed and the gel apparatus assembled according to the manufacturer's instructions. The well was rinsed in running buffer and the sharkstooth comb inserted so that 32 wells were available for loading. The gel was prerun at 40W in 1xTBE running buffer for 1 hour until the gel

temperature reached 50-55°C. Samples were electrophoresed for 2-4 hours at 40W, the plates separated and the urea was removed from the gel by soaking in denaturing buffer for 10 minutes. The gel was transferred to 3MM paper, vacuum dried for 1 hour at 80°C and exposed overnight at room temperature to Kodak X-AR5 film.

2.26 Transfection of eukaryotic cells by electroporation

2.26.1 Optimisation of conditions

Optimal electroporation conditions for Jurkat (J6) and EL4 cells were obtained using an FDA/PI analysis assay. Cells were washed twice in TBS and resuspended at a concentration of 10^7/ml. 500µl of the resuspended cells were electroporated over a wide range of voltages (200-400V) at varying capacitancies (250-960µF). After addition of PI (4µg/ml final) to a 200µl aliquot the electroporated cells was incubated on ice for 10 minutes. The cells were then made up to 1ml with culture medium and incubated at 37°C for 30 minutes. Finally the cells were washed in PBS and resuspended in 1ml of PBS containing 1ng/ml FDA. Cells staining positively for PI on subsequent FACS analysis indicated cell permeabilisation, whilst FDA staining was indicative of cell viability. Thus cells dual staining for PI and FDA were both permeabilised and viable. Therefore it was this subset of cells that needed to be optimised to ensure not only good levels of transfection but also good cell recovery rates.

2.26.2 Control transfections: β-galactosidase assays

10µg of pSV-β-Galactosidase control vector (Promega) was transfected into EL4 and J6 cells by electroporation. Optimal conditions as assessed by FDA/PI analysis were used, and 48 hours later a standard assay was performed.

The transfected cells were washed twice in PBS, and 100µl of 1x reporter lysis buffer (Promega) was added. The cells were incubated at room temperature for 15 minutes, vortexed for 15 seconds and spun in a microcentrifuge for 2 minutes at 4°C. The supernatant was transferred to a fresh tube. 50µl of the supernatants were placed into the wells of a 96 well plate and 50µl of 2x assay buffer (Promega) was added. The samples were mixed by pipetting, the plate covered and incubated at 37°C for 30 minutes or until a yellow colour appeared. The reaction was stopped by addition of 150µl 1M sodium carbonate and the absorbance was read at 410nm. Negative control wells containing mock transfected EL4 and J6 cells were also set up. A further well containing only reporter lysis buffer and 2x assay buffer to blank the spectrophotometer was also included.

2.26.3 Transient transfections

$5x10^6$ cells per transfection were pelleted by centrifuging at 1100rpm for 5 minutes, washed twice with TBS, and resuspended in 500µl TBS in a transfection cuvette (Hoefer) with a 0.4cm gap. 10-40µg DNA was added and the cuvette was placed on ice for 5 minutes. The cuvette was wiped dry, placed in the electroporation chamber (Hoefer) and a single charge delivered. Cells were left on ice for 5 minutes, transferred to a $25cm^2$ flask containing 10mls complete RPMI (10% FCS) and incubated at 40-48 hours at 37°C. Electroporation conditions chosen were based on experimental determination of the optimal transfection conditions using the FDA/PI analysis assay:

Jurkat (J6)	-	300V, 500µF, 1 second charge delivery
EL4	-	280V, 500µF, 1 second charge delivery

2.26.4 Stable transfection

Treatment of the cells was similar to the above protocol except for the following: the DNA used was linearised to allow for easier insertion into the genome. If no eukaryotic selection marker was present on the transfected DNA a second linearised plasmid containing this marker was co-transfected in at a 1/10 molar ratio. Following electroporation and overnight incubation the selective agent at the appropriate concentration was added and the cells were incubated for a further 24 hours. The culture was split at periodic intervals over the following 10 days and then tested for expression of the transfected gene. If appropriate expression was demonstrated, cell stocks were frozen down and the derived cell line was cultured as normal. Selection was continued for a further week and then applied at periodic intervals during long term culture. Selective agents were chosen from the following, dependent on the selection marker resistance gene utilised:

Hygromycin B	-	200µg/ml final concentration
Puromycin	-	2µg/ml final concentration
Geneticin	-	1mg/ml final concentration

2.27 Freezing and storage of eukaryotic cell stocks

10^7 cells of an exponential culture were pelleted by centrifugation and washed with serum free RPMI. The cells were resuspended in 1ml of 0.22µM filter sterilised 15% DMSO (Sigma) in FCS (Gibco BRL) and placed in a cryovial (Nunc). The vial was placed in a freezing box (Nunc) and slowly cooled to -70°C overnight. The following day the vial was transferred to liquid N_2 for long term storage. For thawing, frozen vials were removed from liquid N_2 and rapidly thawed over 1-2 minutes in a

37°C water bath. The cell suspension was added dropwise to a universal containing 10mls of complete medium with gentle mixing. The cells were pelleted by centrifuging at 1100rpm for 5 minutes and resuspended in 10mls of complete medium in a $25cm^2$ culture flask and cultured at 37°C.

2.28 Viability of eukaryotic cells by trypan blue exclusion

The ability to exclude trypan blue (Gibco BRL) was used to assay the viability of cells in culture. Cells were either counted directly, or washed and resuspended in PBS. 20µl of the cell suspension was mixed with 20µl of the trypan blue solution and applied to a haemocytometer (Improved Neubauer, depth 0.1mm; Weber) under a cover slip. The cells were counted under 40x magnification and the viability determined as the percentage of cells excluding the dye (which appear clear) compared to the cell total. The cell count was calculated by the following formula: number of cells in 25 squares x 2×10^4 = number of cells/ml.

2.29 Cytofluormetric analysis

Fluorescence analysis of cells for cell surface expression of proteins was carried out on a FACScan (Becton Dickinson). 10^6 cells per analysis were washed twice in PBS / 0.5% BSA, pelleted by centrifugation and resuspended in a solution of PBS containing 10% normal mouse serum (Sigma) in a volume of 50µl. Following a 15 minute incubation on ice, 50µl of the desired antibody, at the appropriate concentration, was added and labelling was carried out on ice for a further 15 minutes. The cells were then washed twice in 150µl of PBS / 0.5% BSA and either analysed directly, or an appropriate second layer was added and the labelling steps repeated as above. Following labelling the cells were resuspended in a volume of 500µl and analysed on the FACScan according to the manufacturer's instructions with the appropriate gatings and colour compensation. If cells were to be stored and analysed at a later stage they were fixed by addition of paraformaldeyde at a 1% final concentration.

2.30 T cell purification

Lymph nodes were collected from mice and cells released from the organ by crushing them between the frosted ends of two glass slides. Cells were washed once in culture medium without serum and resuspended in a final volume of 5mls. Sheep anti-mouse IgG dynabeads (Dynal) were washed twice in serum-free media, resuspended in 1ml and left on ice until required. The resuspended cells were added to the dynabeads and placed on an end-to-end rotor at 4°C for 15 minutes. The cell/bead mixture was then placed on a magnet for 5 minutes and the cells remaining in suspension were

transferred to a new tube. The beads were washed once with serum-free media and after 5 minutes on the magnet any cells remaining in suspension were removed.

For optimal T cell purification a bead:cell ratio of 2:1 is required. This method depletes all B cells and any other cells expressing Fcγ receptors such as macrophages, and populations of T cells in the range of 92-95% purity are obtained.

2.31 Lymphocyte isolation from tail blood

100µl mouse tail blood was collected in a 1.5ml eppendorf tube containing 1ml of Alserver's solution. This mixture was then layered onto 1ml of Ficoll (Sigma: Ficoll 1083) in a 5ml FACS tube and spun at 2200rpm at room temperature for 45 minutes. The interface, comprising lymphocytes alone was then removed, transferred to a 1.5ml eppendorf and washed with PBS. A small pellet containing between 10^5 and $5x10^5$ lymphocytes would be visible at the bottom of the tube.

2.32 Proliferation assays

Cells were plated at varying cell densities and under varying conditions, as required, in a final volume of 200µl in 96 well flat-bottomed tissue culture plates (Nunc). Treatments were performed in triplicate and at the appropriate time point cultures were pulsed with 10µl (1µCi) of tritiated thymidine ([^{3}H]TdR) (Amersham) per well 6 hours prior to harvesting. Incubations were performed in 95% humidity and 5% CO_2 at 37°C. Cells were lysed and harvested onto glass fibre filters (Wallac) using a commercial harvesting apparatus (Tomtec). Proliferation was measured by [^{3}H]TdR incorporation as assayed by liquid scintillation spectroscopy in a 1205 betaplate counter (Wallac). Results were expressed as mean counts per minute (+/- SEM) of the triplicate cultures.

2.33 IL-2 production assays

The IL-2 dependent HT2 indicator cell line was maintained in culture in exponential growth phase until needed. This required splitting the cells every 3 days and reculturing in the presence of IL-2 (20ng/ml final). For the assay the cells were pelleted by centrifugation at 1200rpm for 5 minutes and washed twice in complete medium to ensure any residual IL-2 was removed. Cells were counted and resuspended at a concentration of 10^5/ml. 50µl of supernatants were collected in 96 well flat-bottomed plates from cell cultures to be assayed, typically after 24 hours culture, and 50µl of the HT2 cells were added. A standard IL-2 titration was added as a control starting at 20ng/ml and titrating down in 5-fold dilutions. Cells were incubated at 95% humidity, 5% CO_2 , 37°C for 18 hours at which point 10µl (1µCi) [^{3}H]TdR was added per well and after a further 6 hours the cells were harvested.

2.34 The JAM assay for measuring T cell-mediated cytotoxicity

The JAM assay (Matzinger P 1991) measures DNA degradation resulting from apoptosis. Proliferating target cells, labelled with [^{3}H]TdR are incubated with cytotoxic T cells (CTLs), harvested onto filters and counted in an identical manner to proliferation assays. If target cell degradation has occurred the fragmented DNA will be washed through the filter, whereas the intact labelled DNA from undegraded cells will remain bound to the filter.

14 days before the assay female C57BL6/J mice were immunised intraperitoneally with 10^7 C57BL6/J male spleen cells. 9 days later their spleens were removed and $4x10^6$ were co-cultured with either $2x10^6$ irradiated spleen cells from C57BL6/J male to generate an anti-HY response or with BALB/c spleens to generate an allogeneic response. For each mouse 4 wells of a 24 well plate were set up.

Two days before the assay $30x10^6$ spleen cells from male and female C57BL6/J mice were stimulated with 50µg/ml LPS in 30mls complete medium in 75cm^2 flasks.

On the day of the assay LPS blasts were labelled with 5µCi/ml [^{3}H]TdR for 6 hours, following which the cell suspension was ficolled (Sigma: Ficoll 1083) and the blasts were harvested from the interface and washed twice. The tumour cell lines P815 (H-2^d) and EL4 (H-2^b) were labelled for 3 hours with 5µCi/ml [^{3}H]TdR, following which they were washed twice. At this point the LPS blasts and tumour cells were counted and resuspended at 10^5/ml. The 4 wells of effector cells set up 5 days previously were pooled, washed twice and resuspended in 1.6mls. They were plated out in duplicate at 100µl/well in doubling dilutions down a 96 well flat-bottomed plate. To each well 100µl of targets was added and the assay was incubated for 3-4 hours in a 37°C, 5% CO_2 humidified incubator. Control wells containing spleen cells from naive mice and co-cultures of C57BL6/J spleen with the EL4 cell line showed non-specific killing at very low levels (<3%). After the 3-4 hour incubation period the plates were harvested and counted and CTL activity calculated as:

$$[(T-S)/T] \times 100 = \% \text{ cytotoxicity}$$

where T=targets alone
S=targets + effectors

Targets alone were harvested at the beginning and end of the assay to assess spontaneous release. Spontaneous degradation of up to 25% had no effect on the assay.

2.35 Immunisations

2.35.1 Generation of a Th2 response

The first immunisation was administered on day 0. Mice were each immunised with 2µg *Der p* I in alum, subcutaneously at the base of the tail, in a total of 200µl.

2μg *Der p* I was combined with 4mg alum and left for 20 minutes at room temperature on a shaker. The volume was then made up to 200μl with PBS and the mice immunised. Any excess was stored at -20°C. 2 weeks later the immunisations were repeated except this time they were administered intraperitoneally. 10 days later a second boost was given, again via the intraperitoneal route, followed by a third boost 10 days after that. 10 days later tail blood was removed and serum analysed for Th2 and Th1 associated antibodies.

2.35.2 Generation of a Th1 response

On day 0 mice were immunised with 10nmoles of the *Der p* I peptide 110-131 in complete Freund's adjuvant (CFA), subcutaneously at the base of the tail in a total of 200μl.

100μl of the *Der p* I peptide 110-131 was mixed with 100μl CFA. An emulsion was made by forcing the two solutions trough a two-way stopcock which had a syringe attached at either exit point. This emulsion was then immunised. 2 weeks later the immunisation was repeated except this time incomplete Freund's adjuvant (IFA) was used. 7 days later a 2nd boost was given, followed by removal of tail blood 10 days later and analysis of the serum for Th1 and Th2 associated antibodies.

2.36 Measuring IgG_1 and IgG_{2a} levels

Mice were tail bled in the absence of anti-coagulants and the blood stored at 4°C for 30 minutes. The tubes were spun at 1200rpm at 4°C for 15 minutes after which the serum was removed. The serum was stored at -70°C until assayed by ELISA.

The day before the ELISA assay was performed, 96 well ELISA plates (Sterilin) were coated with 5μg/ml of either *Der p* I or the *Der p* I peptide 110-131 made up in an ELISA coating buffer (dw scientific), previously diluted 1:10. 50μl of this solution was added to each well and left at 4°C overnight. The following day the coating solution was removed and 200μl of a 10% FCS blocking solution in TBS was added to each well and left for 1 hour at 37°C. The plates were then washed 5 times using a TBS+0.05% Tween 20 solution. The serum was then diluted in TBS, for IgG_1 1/100, 1/1000, 1/10000 and 1/100000 dilutions were made up, for IgG_{2a} the 1/100000 dilution was omitted. 150μl of each dilution was added per well and was incubated overnight at 4°C. The following day the plates were washed 5 times, as before, and a 1/1000 dilution of either the anti-IgG_1 or IgG_{2a} antibodies (Southern Biotechnologies) was made up in TBS and 50μl was added per well. The plates were then incubated for 2 hours at 37°C. Following this incubation cells were again washed 5 times and 50μl of a 1/1000 dilution of streptavidin alkaline phosphatase conjugate (Amersham), made up in TBS was added to each well. The plates were incubated at 37°C for 1 hour, after

which they were washed 5 times, as before. The substrate, nitrophenylphosphate (Sigma) was diluted to a final concentration of 1mg/ml in ELISA substrate buffer (dw scientific) and 50µl was added to each well. The plates were placed in the dark and the OD_{450} was read using an ELISA plate reader once a colour change was observed, this could take from 15 minutes to 1 hour.

CHAPTER 3

The generation and characterisation of a mouse expressing MHC class II on its T cells

3.1 Introduction

3.1.1 A possible role for MHC class II on human and rat T cells

The human *in vitro* work discussed in **section 1.15** together with the rat *in vivo* studies point towards an immunoregulatory role for MHC class II molecules on T cells. *In vitro* evidence suggests induction of anergy or cytotoxicity in T cells that have been presented with antigen in the context of MHC class II by other T cells. A comprehensive *in vivo* study looking at rat responses undertaken by Mannie *et al.*, observed that of two rat MBP-specific T cell lines one mediated severe EAE (termed R2), whilst the other mediated a mild form of the disease (termed GP2). The authors suggested that this was due to the R2 T cell line recognising rat MBP as a full agonist, whereas the GP2 T cell line recognised it only as a partial agonist. Experimental models have proposed that T cells recognise self predominantly as antagonistic ligands, with agonistic ligands largely representing nonself. The basis of this model being that interaction of partial or antagonistic MHC/peptide complexes with the TcR/CD3 complex results in a partial or complete inability of the T cell to become activated. The result of such an interaction would be anergy, and this formed the basis of experiments performed by Mannie *et al.* These experiments have been discussed in **section 1.15b**. the main findings of this report can be summarised as being:

1. The observation that the GP2 T cell line exhibited a higher susceptibility to tolerance than the R2 T cell line. As discussed above this was attributed to GP2 recognising rat MBP as a partial agonist.

2. Anergic GP2 T cells displayed antigen presenting abilities via MHC class II superior to those observed in activated MHC class II+ T cells.

3. Co-culturing of the R2 T cells with anergised GP2 T cells resulted in the transfer of anergy to the R2 line.

4. Adoptive transfer of anergised GP2 T cells into rats subsequently challenged with guinea pig MBP in complete Freund's adjuvant (CFA), resulted in a decreased intensity of EAE. The authors attributed this to infectious tolerance, mediated via MHC class II restricted T-T antigen presentation by the anergic GP2 T cells (Mannie MD *et al.* 1996).

The induction of nonresponsiveness in T cells, *in vitro*, was first observed in human CD4+ T cell clones specific for the influenza haemagglutinin peptide 306-329. On pretreatment with high dose peptide (50μg/ml), in the absence of APCs, the T cell

clone (HA1.7) was rendered nonresponsive to antigenic restimulation. Furthermore, the requirement for MHC class II was demonstrated by addition of anti-MHC class II antibodies into the tolerance-inducing cultures, thereby inhibiting the development of anergy. This observation, therefore, suggested that anergy induction may occurr due to the T cells presenting peptide antigen to each other in an MHC class II restricted fashion (Lamb JR and Feldman M 1984). In an extension of this study, O'Hehir *et al.* demonstrated that treatment of T cell clones with high dose peptide in the presence of professional APCs still resulted in the induction of T cell nonresponsiveness (O'Hehir RE *et al.* 1991). Further studies observed that on treatment of human Th2-like cell clones specific for the *Der p* I peptide 101-119 with high doses of a peptide analogue derived from the influenza HA peptide 307-319 (100μg/ml), the T cells were rendered unresponsive to a subsequent immunogenic challenge (Higgins JA *et al.* 1992). This study presented the possibility of such peptides being of therapeutic use in certain diseases such as localised allergic responses. This peptide-mediated anergy was associated with downmodulation of CD3 expression and upregulation of CD2 and CD25 expression (O'Hehir RE *et al.* 1991a, O'Hehir RE and Lamb JR 1990). Co-modulation of CD4, CD11a/18 and CD44 was found to vary between individual T cell clones (Yssel H *et al.* 1994). Expression of CD80 and CD86 remained unchanged on anergy induction, with CD28 expression being downregulated (Lake RA *et al.* 1993, Yssel H *et al.* 1994). As discussed in **section 1.14a** this model of anergy is reversible on addition of exogenous IL-2 or PMA and ionomycin to the restimulatory cultures (Lamb JR and Feldman M 1984, LaSalle JM *et al.* 1992).

From the observations that T cell clones derived from allergic patients could be tolerised *in vitro*, and that this anergy induction was associated with peptide presentation in the context of MHC class II expressed on T cells, the question arose as to whether antigen presentation under such conditions could be used as a therapy for allergic individuals. The limited *in vivo* work carried out to date looking at the possible role of T cell expressed MHC class II in the rat, suggests it may have a regulatory role, with anergy induction being observed in adoptive transfer experiments under certain conditions (Mannie MD *et al.* 1996, St. Louis JM *et al.* 1994). Based on the preliminary studies discussed, the rat may seem an ideal model in which to dissect out an *in vivo* role of T cell expressed MHC class II. However, the work by Reizis *et al.* demonstrated that on activation rat T cells expressed MHC class II only in the region of 20%, compared with 100% on activated human T cells (Reizis B *et al.* 1994). Therefore, a more appropriate *in vivo* model to address the question of T cell expressed MHC class II being involved in some form immune regulation, possibly by induction of anergy, was by generation of a mouse that expressed MHC class II as a transgene on its T cells alone.

3.1.2 The human CD2 expression vector

The I-Aα^b and I-Aβ^b cDNA constructs were kindly supplied by Drs. C. Benoist and D. Mathis (Landais D *et al.* 1986). In order to direct expression to the T cell compartment alone, the I-Aα^b and I-Aβ^b cDNA were cloned into pTex II (kind gift of Dr. D. Wotton), an expression vector containing human CD2 promoter and enhancer regions known to be required for copy number-dependent and position-independent expression of any heterologous genes under its control (Wotton D 1993). Human CD2 is known to be expressed on thymocytes, peripheral T cells and the majority of NK cells, but is not expressed on B cells (Diamond DJ *et al.* 1988, Sayre PH *et al.* 1987). Transgenic mouse studies have shown that a 5Kb region at the 5' end of the CD2 gene locus and a 7.5Kb region at its 3' end are sufficient to control correct tissue-specific and temporal expression (Lang G *et al.* 1988). Within the 5' region, a weak promoter has been identified (Wotton D 1993). Enhancers vary in their ability to up-regulate transcription, and additional DNA core sequences that act as binding sites for transcription factors have been identified that lie outside the enhancer core. These additional sequences are known as locus control regions (LCRs), and they act in conjunction with the promoter and enhancer regions to confer position-independent, copy-number dependent expression on transgenes (Orkin SH 1990). The 3' region has been found to contain an enhancer and an LCR (Wotton D 1993). Within the 3' region, a 2Kb *Hind* III fragment was found to display enhancer ability. This site has been identified in all T cell lines, whether they express CD2 or not, and thus indicates a region of lineage specific transcription factor binding. Within this region an 'enhancer core' has been mapped. This 531bp region has been shown to contain full enhancer activity, and six regions within this core have been identified as containing consensus motifs to which transcription factors can bind (Lake RA *et al.* 1990). The LCR was also found to be contained within this 2Kb *Hind* III fragment. Analysis of this fragment showed that deletions from the 3' end resulting in a 1.5Kb fragment retained full LCR activity. A deletion resulting in the generation a 1.1Kb fragment, retained all defined enhancer activities, but loss of some LCR activity. A study of transgenic mice carrying this 1.1Kb fragment linked to the human CD2 gene found that some of the mice retained tissue-specific transgene expression but did not demonstrate copy-number dependence. As the LCR is located within the same region as the enhancer, this 2Kb *Hind* III fragment can therefore, by itself, control the tissue-specific, copy number-dependent and position-independent expression of either the CD2 gene, or any other gene under its control (Greaves DR *et al.* 1989).

The expression vector pTexII is comprised of a 5Kb fragment of the 5' sequence that contains the promoter region, terminating a few base pairs upstream from the ATG site. An *Xho* I cloning site has been incorporated, downstream of which is a 2.5Kb fragment encompassing the 3' untranslated flanking region that contains a poly

A sequence, followed by the 2Kb *Hind* III fragment that contains the enhancer and LCR regions, as well as an additional poly A sequence located within the first *Hind* III site **(Figure 3.1a)**. Use of this expression vector therefore ensured that gene expression would be restricted to T cells, and that due to the presence of the enhancer and LCR regions the inserted heterologous genes would be expressed in a copy number-dependent and position-independent manner.

3.1.3 Choice of MHC class II allele.

The decision as to which H-2 haplotype was to be used in the generation of these mice was based on the epitope mapping studies of *Der p* I carried out by Hoyne *et al.* (Hoyne GF *et al.* 1993). This work showed that C57BL/6J mice (H-2^b haplotype), were high responders to the house dust mite allergen *Der p* I. T cells were found to recognise three different epitopes located within the following sequences: 110-131, 78-100 and 21-49, with the immunodominant determinant identified as lying within 110-131. Mice of the H-2^k haplotype were classed as medium responders compared with the H-2^b mice, whilst H-2^d mice were found to be low responders. It was therefore decided that the high responder strain (C57BL/6J) would be used to generate the transgenic mouse. Furthermore, the C57BL/6J strain only express the MHC class II I-A molecules, therefore generation of the transgenic mouse was simplified to the extent that MHC class II I-E molecule transgenes were not needed.

Once the I-Aα^b and I-Aβ^b cDNA had been cloned into the human CD2 expression vector, the two constructs had to be analysed to confirm that cloning had been successful. Sequencing was carried out to ensure appropriate religation and orientation of the inserts. In addition, both stable and transient transfections systems were set up, to determine expression of MHC class II at the cell surface, at the RNA level and within the genome of stably transfected cells. The biological function of any cell surface MHC class II detected was tested in a mixed lymphocyte reaction (MLR) system.

3.1.4 Practical applications of transgene technology.

The method of introducing additional genes into the genome of a developing mouse such that a stable integration event occurs, resulting in the transmission of these extra genes to any progeny, was first described by Gordon *et al.* in 1981 (Gordon JW and Ruddle FH 1981). This method, known as transgenesis, is now used routinely as one way of identifying possible functional and/or regulatory roles that a gene/protein may have. Many examples of the extensive use of transgenesis within the field of immunology have already been mentioned. The creation of the transgenic mice described above, expressing human CD2 under the control of a 1.1Kb fragment derived from the enhancer region but lacking some LCR activity, provided definitive *in*

vivo evidence of the importance of this region with respect to retaining tissue specificity, copy-number-dependence and position-independent gene expression.

Several groups have appreciated the need for transgenic mice expressing elements of the HLA class II loci to facilitate the analysis of the human immune system. This appraisal in part compensates for the inadequacies of *in vitro* assay systems used to determine human T cell function. These groups have all used HLA-DR or -DQ molecules under control of their own transcriptional control regions to create these transgenic mice. In many instances these animals have been used to study human diseases such as autoimmunity and acquired immune deficiency syndrome (AIDS). The problem of the ability of murine CD4 to interact with human HLA molecules was addressed. Some groups found that a transgene of human CD4 gave equivalent responses to those generated in the presence of murine CD4 (Altmann DM *et al.* 1995, Yamamoto K *et al.* 1994), others circumvented this possible problem by creating MHC class II chimeras that possessed the peptide-binding domain of HLA-DR4, but the $\alpha 2\beta 2$ domain ($\beta 2$ domain involved in CD4-binding) (Konig R *et al.* 1992) of murine origin (Woods A *et al.* 1994). MFIs, giving some indication of HLA class II expression levels, where given, were in the range of 30-300. A comparison of HLA expression levels on transgenic cells with cells that normally express HLA was not made. Therefore it could not be ascertained whether the levels of HLA expressed on the transgenic cells fell within the physiological range. Yamamoto *et al.* found that the transgenic mouse they had created expressed a mixed MHC isotype DRA-B6 together with I-Eβ^b (Yamamoto K *et al.* 1994). The other groups discussed either circumvented this problem by using genetically altered mice to minimise such molecular interactions, or made no mention of this issue (Altmann DM *et al.* 1995, Woods A *et al.* 1994, Yeung RSM *et al.* 1994). Yeung *et al.* created a transgenic line in which they reconstituted a CD4-/CD8- gene knockout mouse with human CD4 and HLA-DQw6. They found that the human CD4 was able to rescue T lymphocyte development and restore the CD4+ peripheral T cell compartment. This T cell population was able to provide effective help to B cells on vesicular stomatitis virus challenge, and HLA-DQw6 was able to present the Streptococcus-derived peptide M6C2 (Yeung RSM *et al.* 1994).

A recent report by Neeno *et al.* describes the responses observed to whole body extract of *Der p* when immunised into I-Aβ^0 knockout mice expressing the transgenes for HLA-DQ8. They demonstrated that the *in vitro* responses to the *Der p* extract were HLA-DQ8 restricted and mediated by CD4+ T cells. *In vitro* rechallenge in the presence of *Der p* 2 overlapping synthetic peptides resulted in the identification of three immunodominant epitopes (Neeno T *et al.* 1996). The aim of this study was to investigate the efficacies of peptide-based immunotherapies for allergy. Having created

this 'humanised' murine model they state that they are attempting to induce *Der p* 2-specific IgE responses and airway hyper-responsiveness following allergen inhalation.

These transgenic lines therefore differ from that which I describe in many ways. The transgenic mice discussed above been reconstituted with transgenes encoding human molecules in an attempt to directly study human disease mechanisms in an *in vivo* system. Furthermore, the HLA molecules are expressed under the control of their endogenous transcriptional elements, with the exception of the chimeric HLA model, which was under the control of the murine MHC class II transcriptional elements. This may result in HLA molecules being expressed on T cells (possibly more noticeably upon activation) as well as on the professional antigen presenting cells such as B cells, dendritic cells and macrophages. From the studies, to date, it would be predicted that due to the lack of the transcription factor CIITA in the murine T cells HLA would not be expressed (Chang C-H *et al.* 1995, Rigaud G *et al.* 1996, Siegrist C-A *et al.* 1995). However, as it is known that many factors contribute to HLA expression, that the overall importance of CIITA is not known, and of the possible role played in immune regulation by T cell expressed HLA, it would seem erudite to analyse these mice for its presence.

3.1.5 Generation of a transgenic mouse.

The technique of transgenesis involves the intricate task of manipulating a newly fertilised oocyte by introducing extra genes into its nucleus in such a way that they become incorporated into the germline of the resulting progeny. F2 mice are typically used for microinjection to circumvent problems such as poor reproductive performance and poor maternal instinct that may be encountered in certain inbred mouse strains (Brinster RL *et al.* 1985). Superovulated females are used for the production of fertilised eggs. Typically, six week old females are immunised with pregnant mare's serum, which mimics the effect of follicle stimulating hormone and human chorionic gonadotrophin, which mimics the effect of leutinising hormone. On average, this treatment yields 20-30 eggs, whereas natural ovulation yields merely ~10 (Hogan B *et al.* 1986). 48 hours after hormone immunisation the female is placed in a cage with a male who is known to be fertile. 4 days after mating, the female is sacrificed and the ovulated fertilised oocytes are collected. Any fertilised oocytes will be seen to contain two pronuclei, one from the ovum, the other from the sperm. The male pronucleus appears larger and closer to the cell membrane, and it is into this pronucleus that the DNA construct(s) is microinjected (Gordon JW and Ruddle FH 1981). The fertilised oocytes are then transferred into a pseudopregnant female. Pseudopregnant mice are prepared by mating females in natural oestrus with sterile males (usually vasectomised). After mating, the reproductive tract of the female becomes receptive for transferred embryos, her own unfertilised eggs subsequently degenerating. Stable chromosomal

integration of the microinjected DNA occurs in ~10-40% of the surviving embryos, ~10% of these being expected to develop to term (Constantini F and Lacy E 1981). The resulting pups are tested for successful integration of the transgene using methods such as Southern blotting or PCR analysis, any mice that develop from microinjected eggs being termed 'founder' mice. Mice determined to be positive for the transgene are mated to establish a transgenic line.

In most cases, prior to microinjection, the cDNA is linearised by restriction enzyme (RE) digest, multiple copies of the gene usually concatamerise by virtue of their RE-derived compatible ends, and would be expected to integrate at a single site in a tandem array (Jaenisch R 1988, Palmiter RD *et al.* 1982). Random chromosome breaks, possibly caused by DNA repair enzymes induced by the free ends of the microinjected DNA, serve as integration sites for the foreign DNA (Brinster RL *et al.* 1985). Rearrangements, deletions, duplications and translocations of the host sequences have all been observed at the integration site (Mahon KA *et al.* 1988, Mark WH *et al.* 1985). Some instances of multiple insertion sites have also been recorded (Wagner EF *et al.* 1983).

3.1.6 Characterisation of the transgenic line.

Once the transgenic line was derived, the animals were characterised. The presence of the transgene within the genome was verified by Southern blotting and PCR, with an estimate of gene copy number derived from Southern blots. FACS analysis was routinely used to screen pups for T cell MHC class II expression, and cells were assayed to determine whether expression of MHC class II on T cells had any effect on other cell surface molecules. The functional ability of these T cells was also investigated. Their ability to present peptides to T cell hybridomas, to act as CTLs and to provide T cell help was examined and compared with non-transgenic littermates. Antibody responses on immunisation of antigen using standard protocols designed to skew responses either towards a Th1 or Th2 profile (i.e. IgG_{2a} or IgG_1 respectively), were examined and compared with those of non-transgenic littermates. The ability of intranasally delivered peptide to modulate ongoing immune responses in transgenic and non-transgenic littermates was examined. Intranasal peptide was also administered to transgenic and non-transgenic mice prior to an immunogenic antigenic challenge and the subsequent responses were compared.

3.2 Making and testing the human CD2-I-Aα and CD2-I-Aβ constructs.

3.2.1 Modification of the pTexII expression vector: Deletion of a 2.5Kb *Cla* I-*Bgl* II fragment.

From the work previously discussed in **section 3.1.2**, it is evident that the transcriptional elements of the human CD2 gene that are essential for transcription initiation of heterologous genes under its control have been elucidated (Greaves DR *et al.* 1989, Lake RA *et al.* 1990, Wotton D 1993). These studies indicate that a 2.5Kb *Cla* I to *Bgl* II fragment, at the 5' end of the promoter has no discernible effect on expression vector function. For this reason it was decided to remove this fragment, and to clone in a polylinker at the *Xho* I site, to create additional unique sites into which the I-Aα and I-Aβ cDNAs could be cloned **(Figure 3.1b)**.

For removal of the *Cla* I-*Bgl* II fragment 2μg of the pTexII vector was digested with *Cla* I and *Bgl* II under optimal conditions as derived from the manufacturers tables of appropriate buffer conditions **(section 2.8)**. The digests were then resolved on a 1% horizontal agarose gel and photographed under UV transillumination **(section 2.9)**. The pTexII *Cla* I/*Bgl* II digest resolving into one 2.5Kb band (the *Cla* I/*Bgl* II fragment), and a larger 8-9Kb band, which comprised of the remainder of the expression vector that was to be religated **(Figure 3.2)**. The 8-9Kb fragment was cut out of the gel, solubilised with perchlorate binding buffer (BioRad), and subsequently purified through a wizard minicolumn (Promega) **(section 2.10)**. The linearised vector was flushed to blunt and a ligation reaction was set up **(section 2.12 and 2.16)**. Ligation success was determined by transforming the DNA into competent *E.coli*, and assaying colonies that grew on the ampicillin treated plates **(section 2.5)**. Restriction enzyme digest analysis shows the pattern observed in colonies containing DNA that has successfully religated **(Figure 3.3)**. *Sac* I digestion of the religated pTexII shows the complete absence of one band of ~2Kb, compared with the original pTexII (lanes 2 and 5 compared with lane 8). This arises due to the loss of a *Sac* I site that lies within the *Cla* I-*Bgl* II fragment that has been removed. The absence of any bands in the *Cla* I/*Bgl* II digests in the religated pTexII, other than the 8-9Kb band that constitutes undigested pTex II, gives clear evidence that the *Cla* I-*Bgl* II fragment has been successfully removed (lanes 3 and 6), a 2.5Kb band clearly visible in the digest with the original vector (lane 9). This modified vector is termed pTexIIL.

Addition of a multiple cloning site.

2μg of the pTexIIL was digested under the appropriate conditions with *Xho* I. A sample was run on a 1% agarose gel to verify linearisation of the vector and the

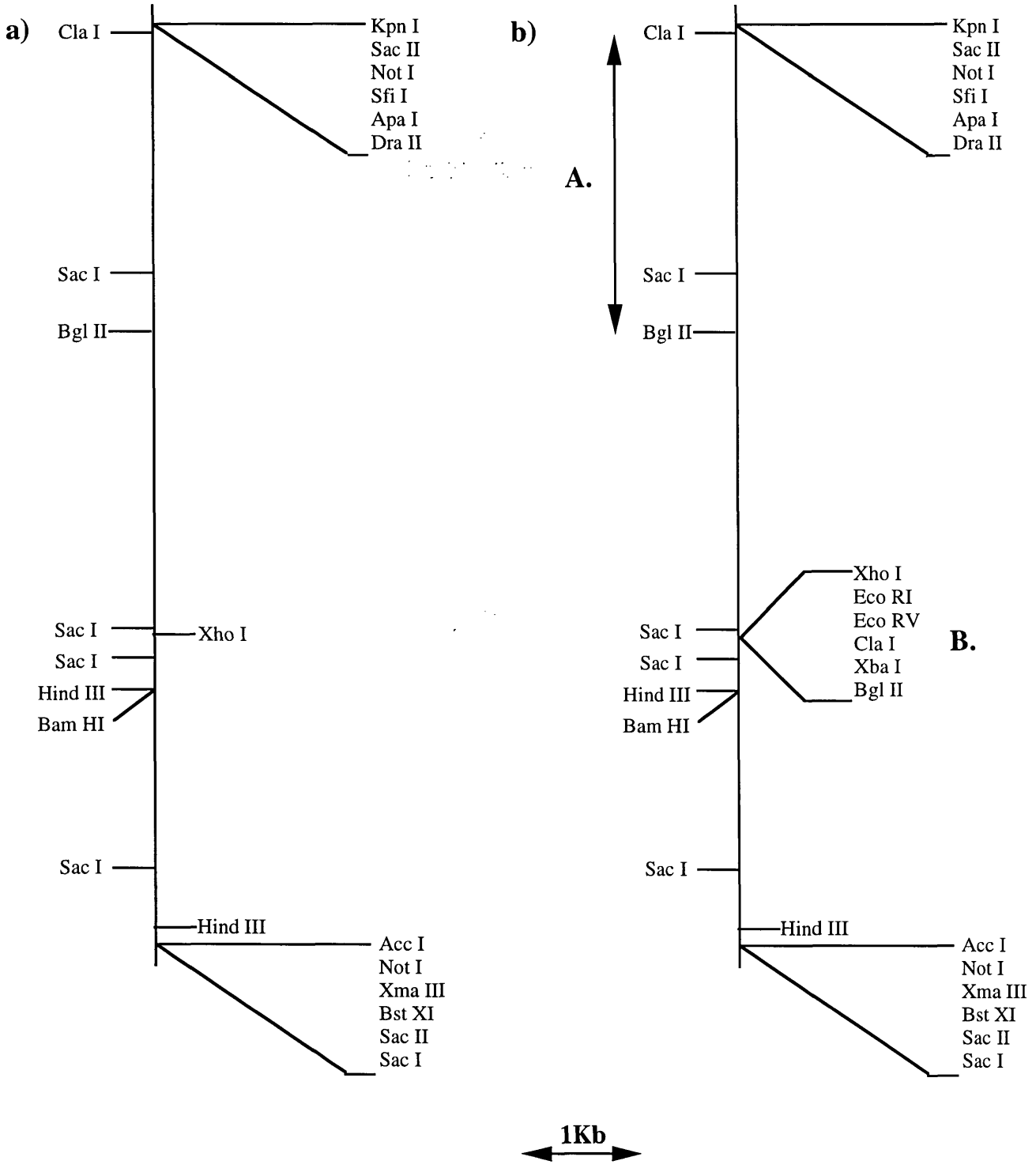


Figure 3.1 Vector maps of pTexII
Map indicates **A.** *Cla* I - *Bgl* II fragment deleted and **B.** polylinker incorporated into the *Xho* I cloning site giving the orientation as seen in the pTexIIIa construct.

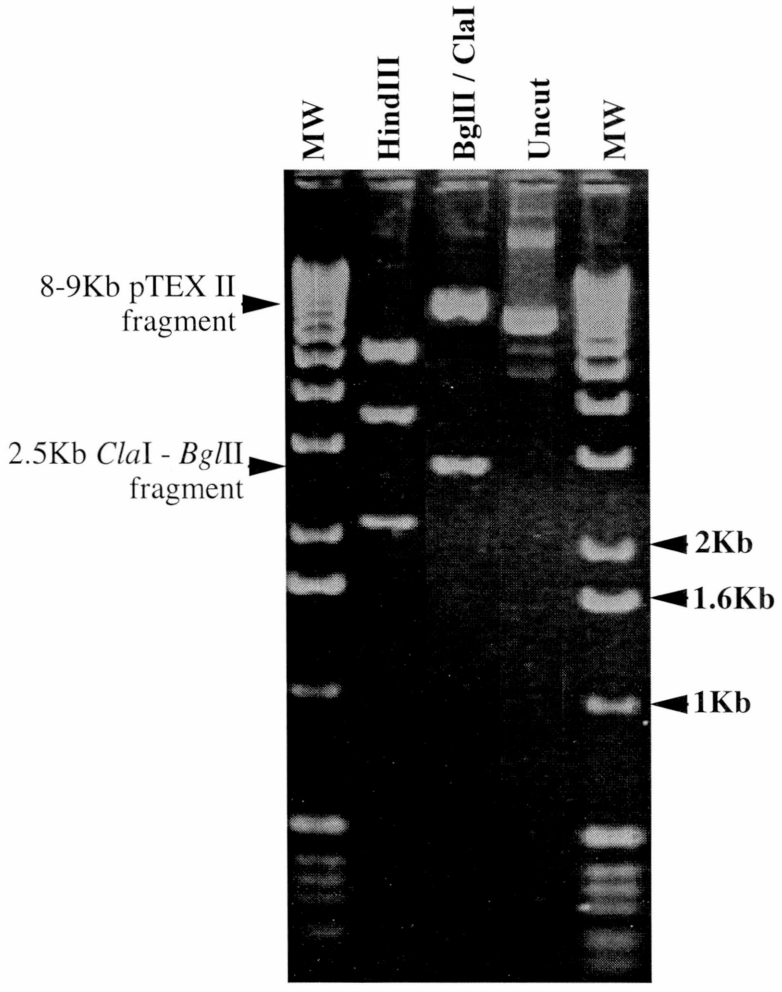


Figure 3.2 Restriction enzyme digestion of pTEXII expression vector

Key: MW, Molecular weight markers - 1Kb ladder.

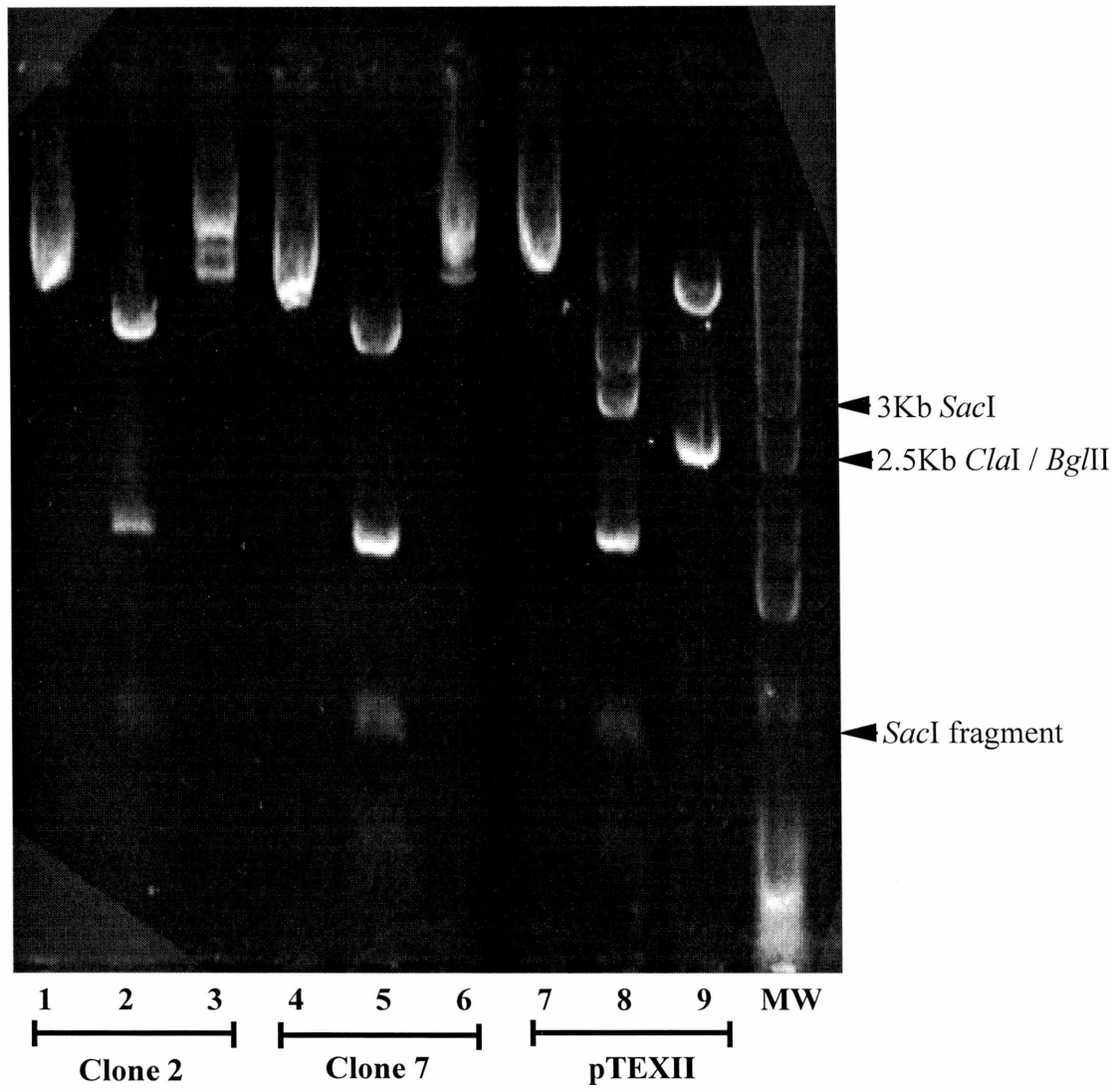


Figure 3.3 Restriction enzyme digection showing successful religation of the pTEXII expression vector with 2.5kB *Cla*I - *Bgl*II fragment deleted

Lanes 1, 4, 7 - Uncut; Lanes 2, 5, 8 - *Sac*I; Lanes 3, 6, 9 - *Bgl*II / *Cla*I
Note the absence of the 3Kb *Sac*I fragment and the 2.5Kb *Cla*I / *Bgl*II fragments in clones 2 & 7 relative to pTEXII.

Key: MW, Molecular weight markers - 1Kb ladder.

linearised pTexIIL was treated with calf intestinal alkaline phosphatase (CIAP, Boeringer) **(section 2.15)**. The complementary strands of the polylinker that was to be cloned in were annealed, treated with T4 kinase (Promega) and cleaned up using a standard phenol/chloroform method **(section 2.14)**. A ligation reaction was set up, and the following day competent *E.coli* transformed. Mini preps were double digested with *Xho* I/*Sac* I, and run on a 3% agarose gel **(Figure 3.4a)**. The gel shows that the polylinker was cloned in in both orientations. The DNA yielding two fragments, one of ~260 base pairs, the other of ~60 base pairs contains the polylinker in the orientation: *Bgl* II, *Xba* I, *Cla* I, *Eco* RV, *Eco* RI, *Xho* I (termed pTexIIIb). Only one fragment is seen in the preps containing the polylinker in the opposite orientation. This fragment runs at ~310 base pairs, indicative of the polylinker being cloned in in the orientation: *Xho* I, *Eco* RI, *Eco* RV, *Cla* I, *Xba* I, *Bgl* II (termed pTexIIIa). Although a second fragment of 25 base pairs was generated, it was too small to be visible on the gel. To check that the information derived from the restriction enzyme digests of the vector was indeed correct, sequencing was also carried out **(section 2.25)** **(Figure 3.4b)**.

3.2.2 Preparation of I-Aα and I-Aβ for ligation into the pTexIIIa expression vector.

To increase the efficiency of cloning the I-Aα and I-Aβ chains into pTexIIIa, PCR was used to amplify the DNA **(section 2.13)**, and the primers were designed with restriction enzyme sites incorporated into either end:

I-Aα:	5'-GCCG ATCGAT CTC CCA GAG ACC AGG ATG-3'	
(coding primer)	*Cla* I	Methionine
I-Aα:	5'-GGCC GGATCC GGG ACA GCC TCA GGG CC-3'	
(non-coding primer)	*Bam* HI	
I-Aβ:	5'-GCCG ATCGAT ACC TGT GCC TTA GAG ATG-3'	
(coding primer)	*Cla* I	Methionine
I-Aβ:	5'-GGCC GGATCC CAA AAC ACT CTG AGT CAC TG-3'	
(non-coding primer)	*Bam* HI	

The I-Aα coding primer incorporates bases 9-26; the non-coding primer bases 949-933. The I-Aβ coding primer incorporates bases 1-18; the non-coding primer bases 827-808. The PCR products were purified using a standard phenol/chloroform protocol, and then digested with *Cla* I/*Bam* HI under appropriate conditions. The digests were run on a 1% agarose gel and the bands cut out and purified through a wizard column **(Figure 3.5).**

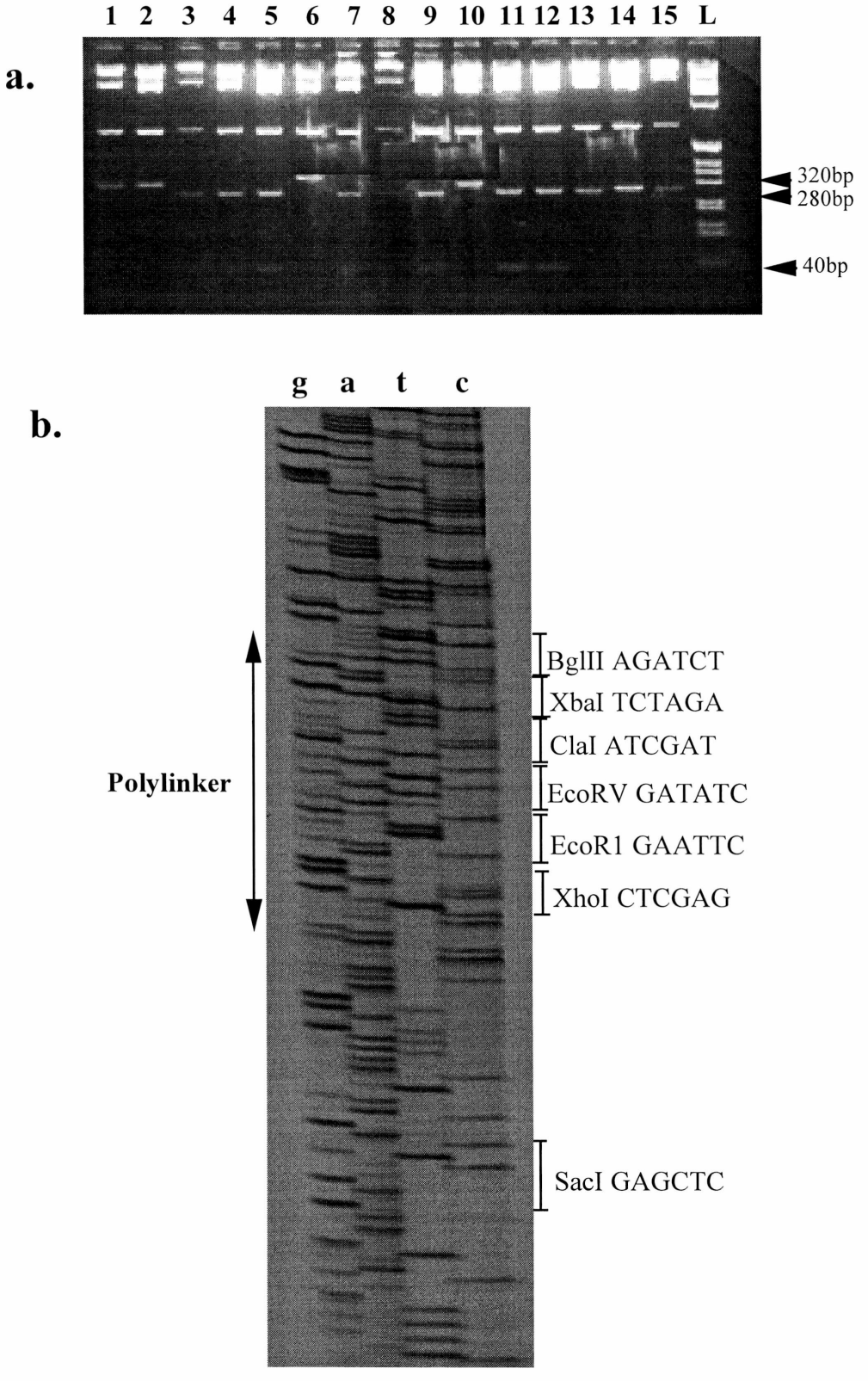


Figure 3.4 Successful incorporation of a polylinker into the *Xho*I site of the pTEXIIL vector

a. *Xho*I / *Sac*I restriction enzyme digests of a number of putative clones showing appropriate insertion, in both orientations, of the polylinker.

b. Sequencing of pTEXIIIa vector confirming polylinker orientation

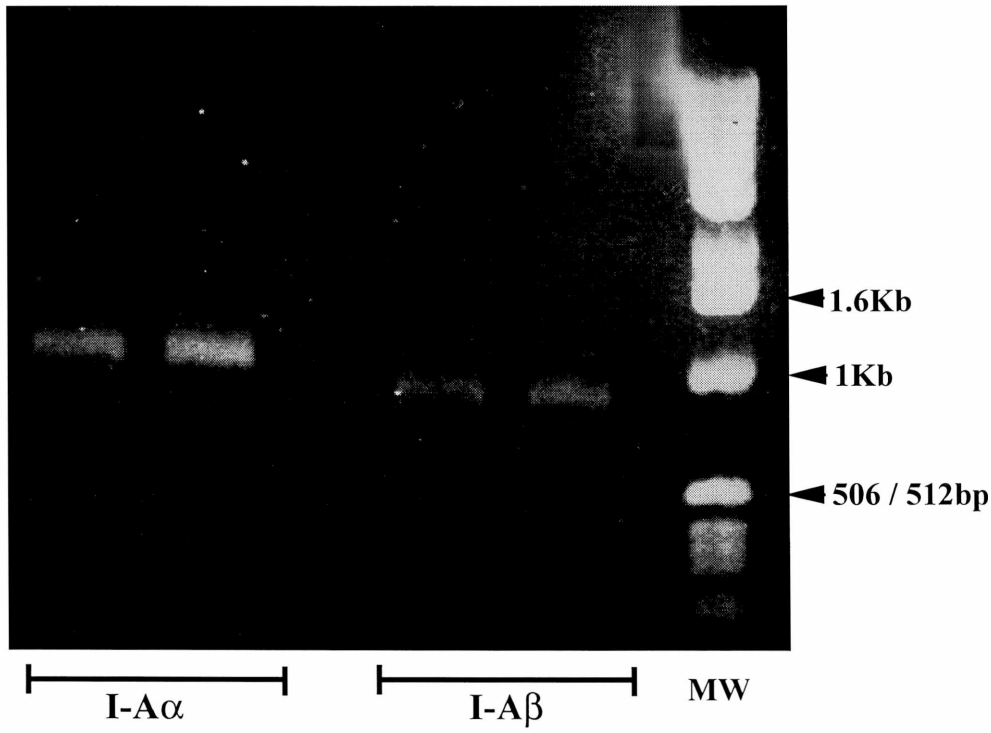


Figure 3.5 **PCR amplified I-Aα and I-Aβ chains for cloning into pTEXIIIa expression vector**

Key: MW, Molecular weight markers - 1Kb ladder.

3.2.3 Cloning of I-Aα and I-Aβ into the pTexIIIa expression vector.

2μg of pTexIIIa was double digested with *Cla* I/*Bgl* II under appropriate conditions, and a sample of the digest run on a 1% agarose gel to ensure linearisation. The remainder was then purified through a wizard column, and ligation reactions were set up with either the I-Aα or I-Aβ chain which had been previously digested with *Cla* I/*Bam* HI. The restriction enzymes *Bgl* II and *Bam* HI cut at different sites but produce compatible ends explaining why these two enzymes could be used in this ligation reaction. The ligation products were transformed into competent *E.coli* and any colonies that grew on the ampicillin treated plates were assayed. Restriction enzyme digest with *Xho* I/*Eco* RV shows the appearance of a ~770 base pair band in only one lane (32β), this band representing a probable β chain insert into the *Cla* I/*Bgl* II site. *Hind* III digestion of a successfully inserted α chain, would yield a band of ~1.2Kb, as a *Hind* III site is found at position 260 of I-Aα. This band appears in the preps from 8α, 16α and 41α. I-Aα also contains a *Sac* I site at position 41. A prep containing I-Aα would therefore yield a band of ~1.2Kb. In addition to 8α, 16α and 41α, other preps also appear to give this band, this may indicate the incorporation of a piece of unrelated DNA into the *Cla* I/*Bgl* II site in these preps. I-Aβ chain incorporation into pTexIIIa results in the appearance of a ~1.2Kb band on *Sac* I digestion, which is seen in the 32β and 35β preps **(Figure 3.6).**

3.2.4 Sequencing of I-Aα and I-Aβ.

Clones 8α and 32β (termed IIIAα and IIIAβ) were sequenced to ensure that there were no mutations within either of the chains due to PCR errors **(section 2.25)**, and that the cloning event itself had not resulted in any deletions or additions of bases. **Figure 3.7** shows the successful ligation of the α and β chains, at their 5' and 3' ends, into the pTexIIIa expression vector. No restriction enzyme sites have been lost and no addition or deletion of bases observed. At the 3' end, a hybrid *Bam* HI/*Bgl* II (aGATCc) is seen, where ligation occurred.

3.2.5 Testing the IIIAα and IIIAβ expression cassettes-Southern blots of stable transfectants.

IIIAα and IIIAβ expression cassettes were linearised with *Not* I and stably transfected into Jurkat (J6) cells by electroporation **(section 2.26.4)**. *Pvu* II linearised neomycin was simultaneously electroporated into the J6 cells as a selection marker. The transfected population was cloned by limiting dilution and to ensure gene cassette integration into the genome, southern blot analysis was performed. Genomic DNA was isolated using Qiagen genomic tips (Qiagen) **(section 2.20)**. 20μg genomic DNA from untransfected J6 cells, a stable clone or HB99 (murine B cell line)

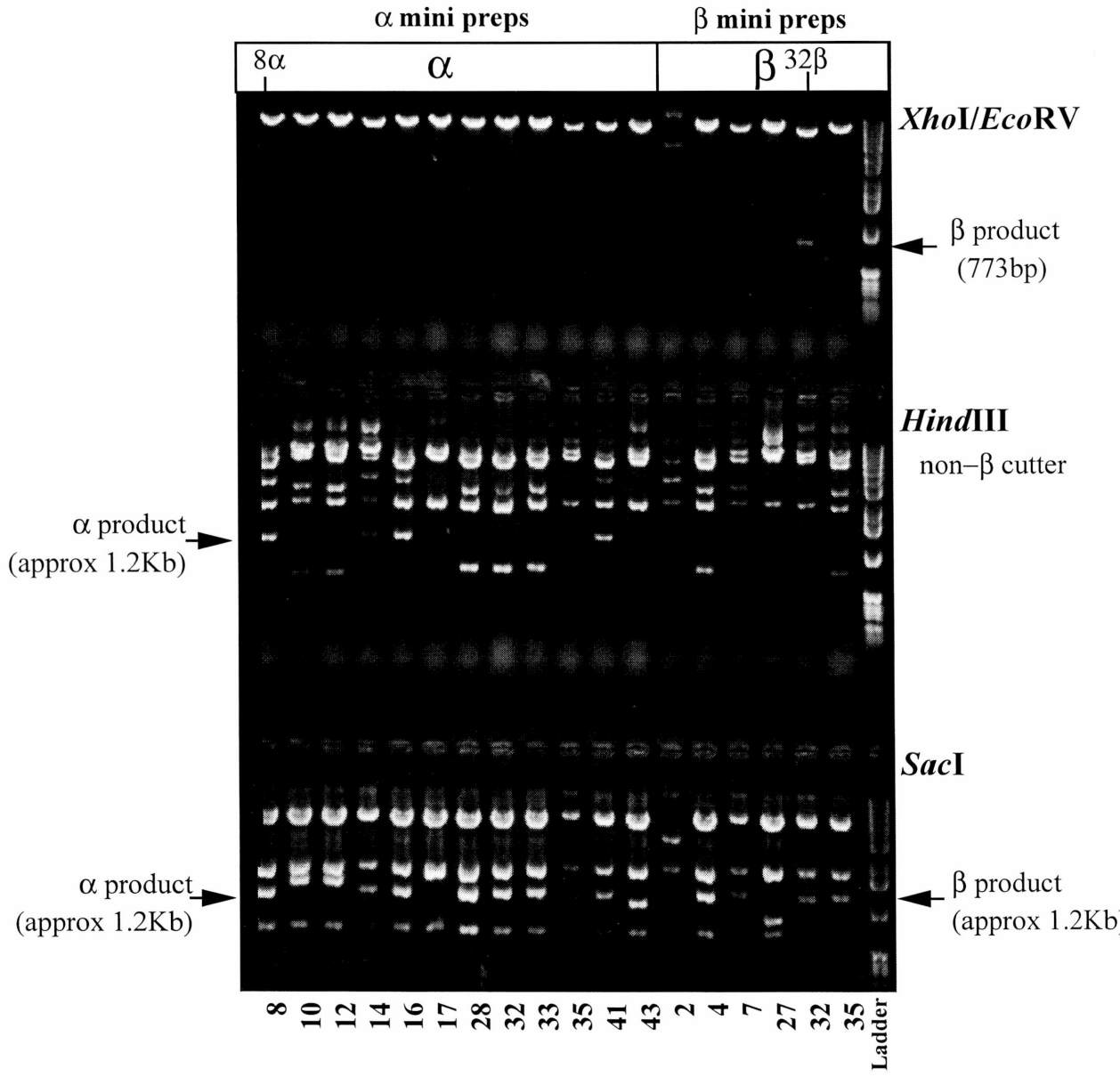


Figure 3.6 Restriction enzyme digestion of putative I-Aα & I-Aβ clones in pTEXIIIa

Clones 8α and 32β were used for derivation of transgenic mice.

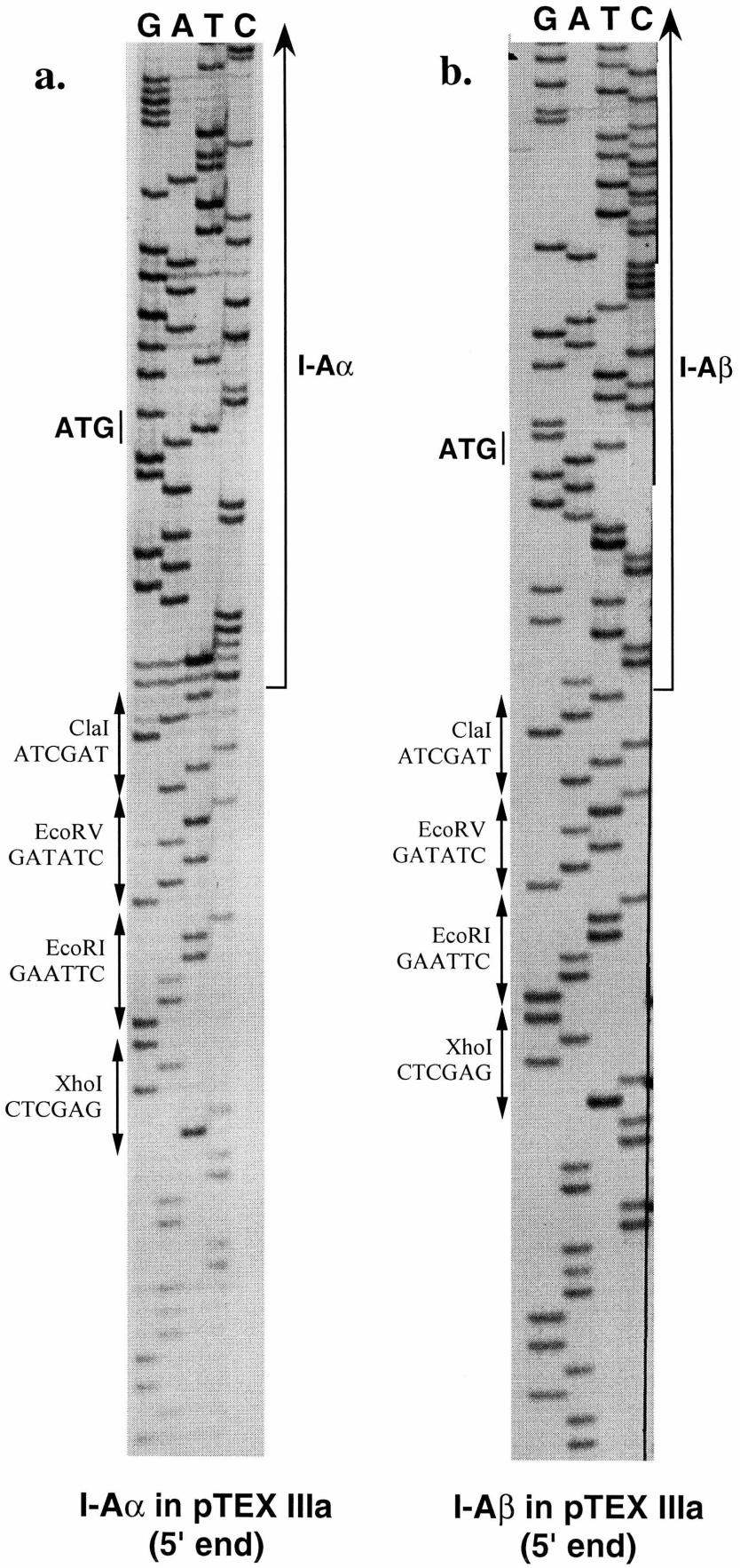


Figure 3.7a I-Aα and I-Aβ in pTEX IIIa vector, 5' end

a. I-Aα; **b.** I-Aβ;

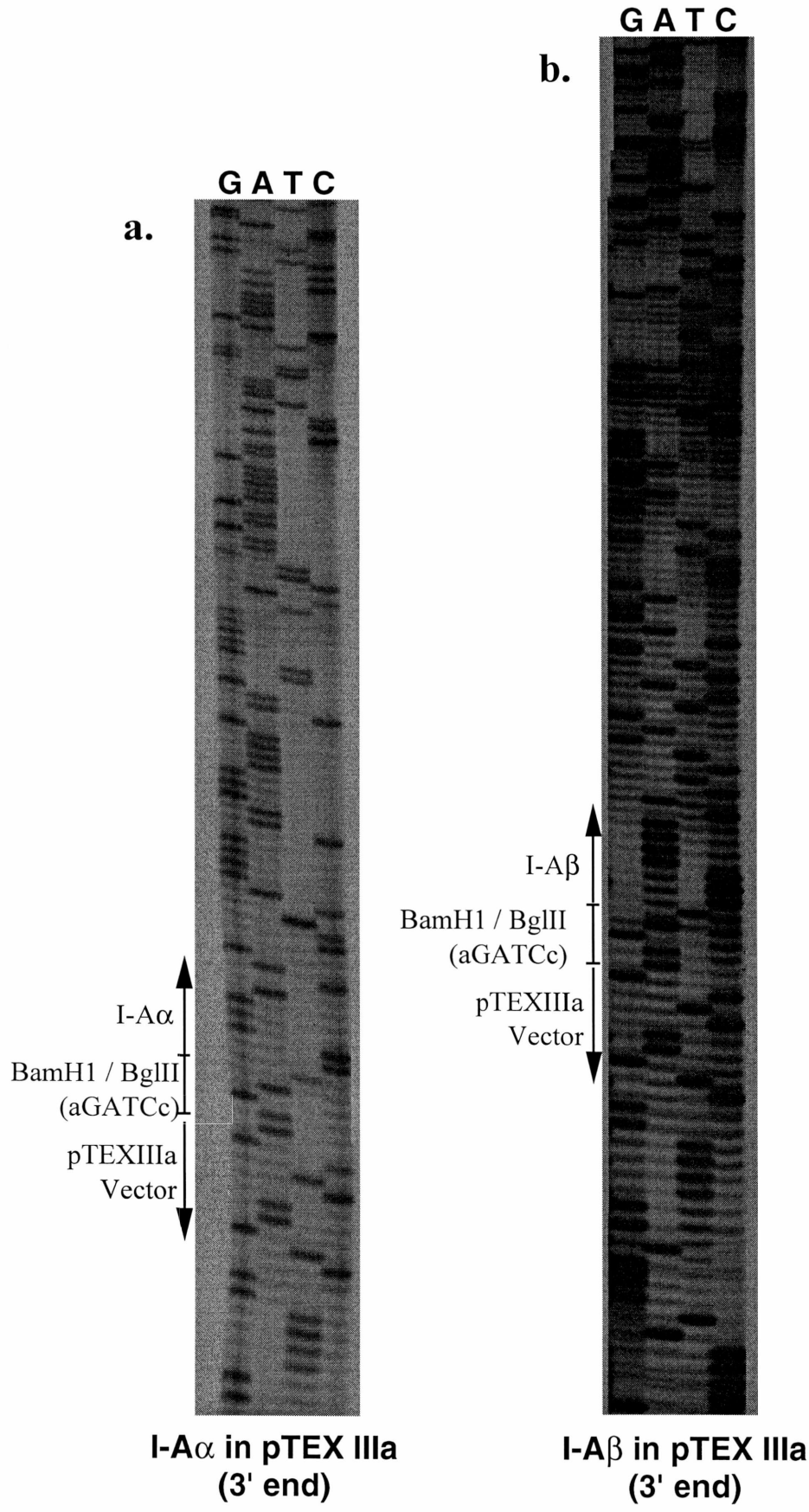


Figure 3.7b **I-Aα and I-Aβ in the pTEX IIIa vector, 3' end**

a. I-Aα; **b.** I-Aβ

was digested with *Hind* III. The untransfected J6 was used as a negative control, the HB99 line as a positive control. The digests were electrophoresed, blotted and probed with I-Aα cDNA (**section 2.19**). **Figure 3.8** shows the bands observed on probing with the I-Aα probe. In the lane containing the stably transfected clone B12, the band corresponding to 1.2Kb represents an I-Aα *Hind* III fragment, also seen in the restriction enzyme digest analysis (**Figure 3.6**). The 2Kb fragment is representative of the 2Kb *Hind* III fragment encompassing the enhancer region of human CD2 and appears due to the probe containing a partial CD2 sequence. This band is also seen in **Figure 3.6.** The heavier bands are probably representative of partial digests. Two bands appear in the HB99 lane, one band at ~1.2Kb, the other running slightly heavier at 1.3Kb. The 1.3Kb band arising due to the probable presence of a *Hind* III site within the intronic sequence of the murine endogenous I-Aα. The 900 base pair band that appears in the empty J6 and B12 clone lanes is of unknown origin, but may possibly be representative of endogenous HLA-DQα. *Hind* III is a non cutter of I-Aβ cDNA, therefore these blots were not probed for I-Aβ cDNA presence.

The Southern data therefore verifies that integration of the I-Aα chain has occurred, but the presence or absence of the I-Aβ chain cannot be determined due to the absence of the *Hind* III site. However, from the studies discussed in **section 3.1.5** it is possible that the I-Aα and I-Aβ concatamarised due to linearisation of both using *Not* I, and subsequently integrated at a single site in a tandem array.

3.2.6 Transient transfection assays- optimisation of conditions.

To ascertain the ability of the pTexIIIa expression vector to drive I-Aα and I-Aβ transcription transient transfection assays were performed. J6 and EL4 were used in these studies as representative human and murine T cell lines. FDA/PI analysis was carried out to obtain optimal electroporation conditions which were then used for subsequent studies (**section 2.26.1**) (**Figure 3.9**). FDA staining indicates cell viability, whilst PI is indicative of the cell having been permeabilised. The double stained cell population has therefore undergone permeabilisation, but is still viable. 500μF and 300V were judged to be the optimal conditions to use for J6 cells, whilst 500μF and 280V were optimal in the case of EL4. Under these conditions 15% of EL4 cells double stained for FDA and PI. In the case of J6, only ~7% of cells double stained at both 280V and 320V, but a distinct shift was observed for FDA staining, with almost 50% cells being FDA+ at 280V, decreasing to only 25% FDA+ at 320V. 300V was therefore chosen as the optimal voltage for electroporation at 500μF.

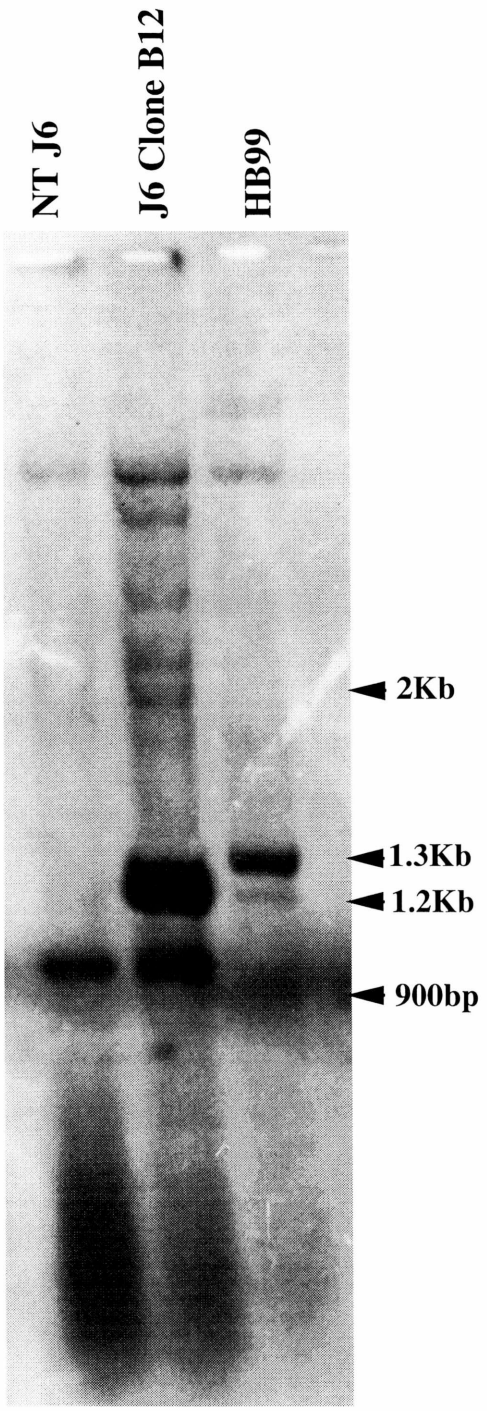
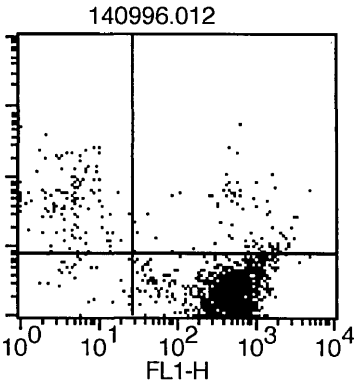


Figure 3.8 Genomic Southern showing I-Aα in J6 Clone B12

Southern blot showing incorporation of I-Aα chain in J6 clone B12. The I-Aα runs at 1.2Kb and is present in both Clone B12 and HB99 (positive control), but not in non-transfected J6 (NT).

a)

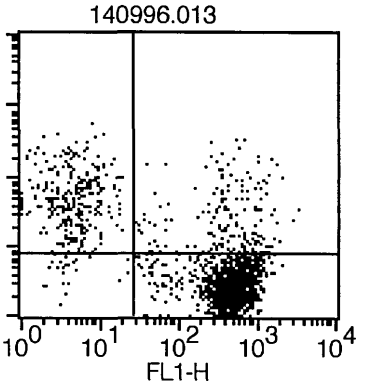


Quadrant Statistics

File: 140996.012 Total Events: 5000
Quad Location: 26, 7

Quad	Events	% Gated	% Total
UL	401	8.02	8.02
UR	240	4.80	4.80
LL	90	1.80	1.80
LR	4269	85.38	85.38

b)

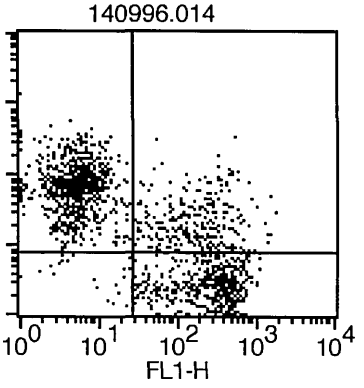


Quadrant Statistics

File: 140996.013 Total Events: 5000
Quad Location: 26, 7

Quad	Events	% Gated	% Total
UL	916	18.32	18.32
UR	542	10.84	10.84
LL	71	1.42	1.42
LR	3471	69.42	69.42

c)



Quadrant Statistics

File: 140996.014 Total Events: 5000
Quad Location: 26, 7

Quad	Events	% Gated	% Total
UL	2613	52.26	52.26
UR	800	16.00	16.00
LL	71	1.42	1.42
LR	1516	30.32	30.32

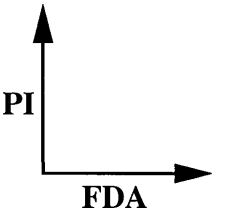
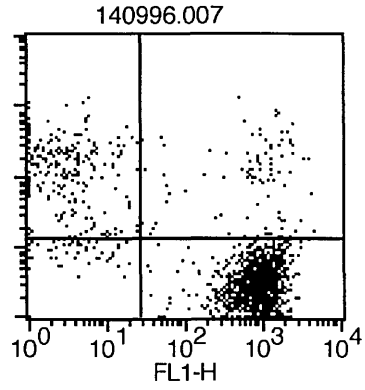


Figure 3.9: FDA/PI analysis of EL4 and J6 cells following electroporation under various conditions

a) EL4: 500µF, 200V; **b)** EL4: 500µF, 240V; **c)** EL4: 500µF, 280V
d) J6: 500µF, 240V; **e)** J6: 500µF, 280V; **f)** J6: 500µF, 320V

d)



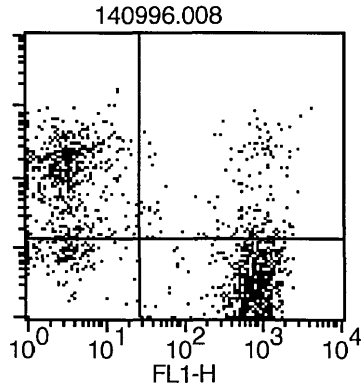
Quadrant Statistics

File: 140996.007 Total Events: 5000

Quad Location: 26, 13

Quad	Events	% Gated	% Total
UL	686	13.72	13.72
UR	252	5.04	5.04
LL	139	2.78	2.78
LR	3923	78.46	78.46

e)



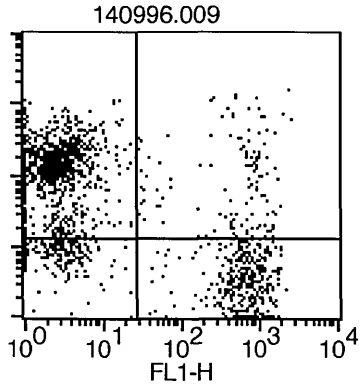
Quadrant Statistics

File: 140996.008 Total Events: 5000

Quad Location: 26, 13

Quad	Events	% Gated	% Total
UL	1724	34.48	34.48
UR	508	10.16	10.16
LL	475	9.50	9.50
LR	2293	45.86	45.86

f)



Quadrant Statistics

File: 140996.009 Total Events: 5000

Quad Location: 26, 13

Quad	Events	% Gated	% Total
UL	2832	56.64	56.64
UR	395	7.90	7.90
LL	635	12.70	12.70
LR	1138	22.76	22.76

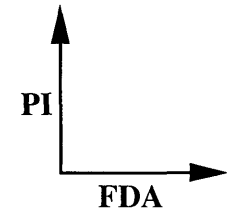


Figure 3.9 Continued

3.2.7 Expression of transiently transfected MHC class II on J6 and EL4 cell lines.

J6 and EL4 cell lines were transiently transfected with 10μg each of both the IIIAα and IIIAβ constructs (**section 2.26.2 and.3**). After 48 hours cells were stained with M5/114-biotin (anti-MHC class II) antibody (**appendix II**), with strepavidin-FITC used as the second layer. Cells electroporated in the absence of DNA were used as negative controls. **Figure 3.10a** shows the staining pattern observed. No MHC class II staining was seen in either the J6 or EL4 MHC class II-transfected cells. Control transfections using the reporter construct β-galactosidase were also performed to check that expression of plasmids in this transient transfection system is possible. **Figure 3.10b** gives the results of these studies, and confirms that in both the J6 and EL4 cell lines the β-galactosidase construct was successfully expressed.

3.2.8 Ability of stable transfectants to induce allo-responses.

Despite the absence of detectable MHC class II on the transiently transfected J6 and EL4 cell lines, it was thought possible that there may be MHC class II on the cell surface, but at levels below the sensitivity of FACS analysis (sensitivity >10,000 molecules/cell). It has been previously shown that low levels of transfected MHC class II, undetectable by FACS, are indeed functional (Higgins and Lamb, unpublished observations). Therefore the ability of 2, 4 and $8x10^5$ stably transfected J6 clones B2 and B12 to induce an alloresponse in 2 and $4x10^5$ H-2^d spleen cells was examined. B2, B12, untransfected J6 (negative control) and the murine H-2^b B cell line HB99 (positive control), were Mitomycin C (Sigma) treated, to block DNA synthesis and cell division, and were co-cultured with spleen cells derived from BALB/c (H-2^d) mice. Three days later proliferation levels were assayed. **Figure 3.11** shows that proliferation of the H-2^d spleen cells was only induced in the wells containing the HB99 cells, the B2 and B12 clones giving proliferative responses equivalent to the untransfected J6 cells. This data suggests the absence of functional MHC class II on the cell surface of the clones B2 and B12. Northern analysis was therefore performed to verify the presence of IIIAα and IIIAβ transcripts.

3.2.9 Northern analysis of J6 clone B12.

mRNA from the clone B12, untransfected J6 and HB99 cell lines was isolated using the RNAzol B method (**section 2.21**). The mRNA was run on a formamide gel, blotted and subsequently probed with I-Aα and actin cDNA probes (**section 2.22**). **Figure 3.12** shows that only the HB99 cell line transcribed the I-Aα mRNA. These results suggest that despite the successful incorporation of the I-Aα and I-Aβ chains into the genome, mRNA transcription is not occurring.

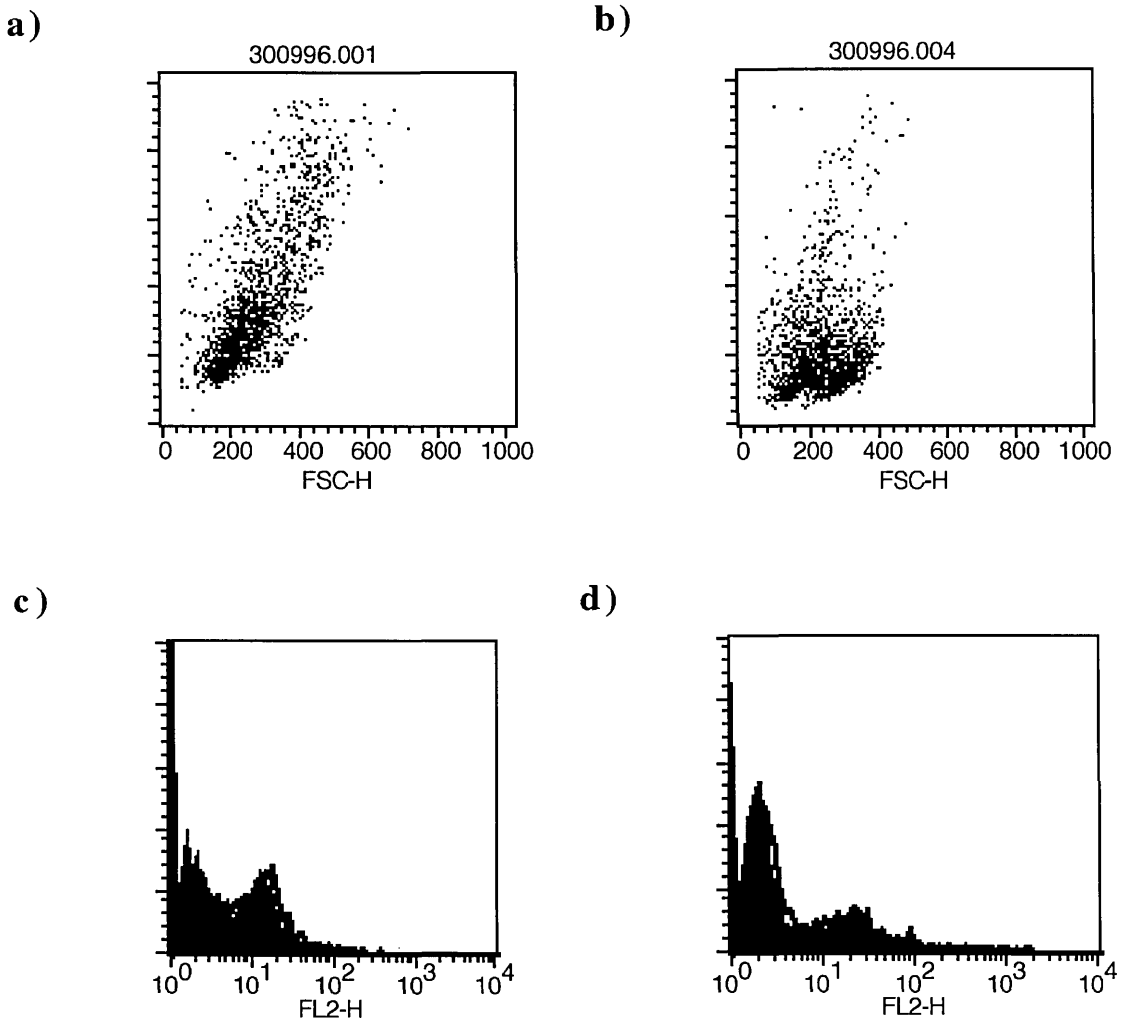


Figure 3.10a MHC class II expression following transient transfection with I-Aα and I-Aβ cDNA constructs

FACS analysis showing MHC class II staining of untransfected and transiently transfected EL4 and J6 cell lines .
a) & b) FSC/SSC profiles of transfected EL4 and J6 respectively
c) & d) MHC class II expression on transfected EL4 and J6 respectively
(filled lines represent transfected cells)

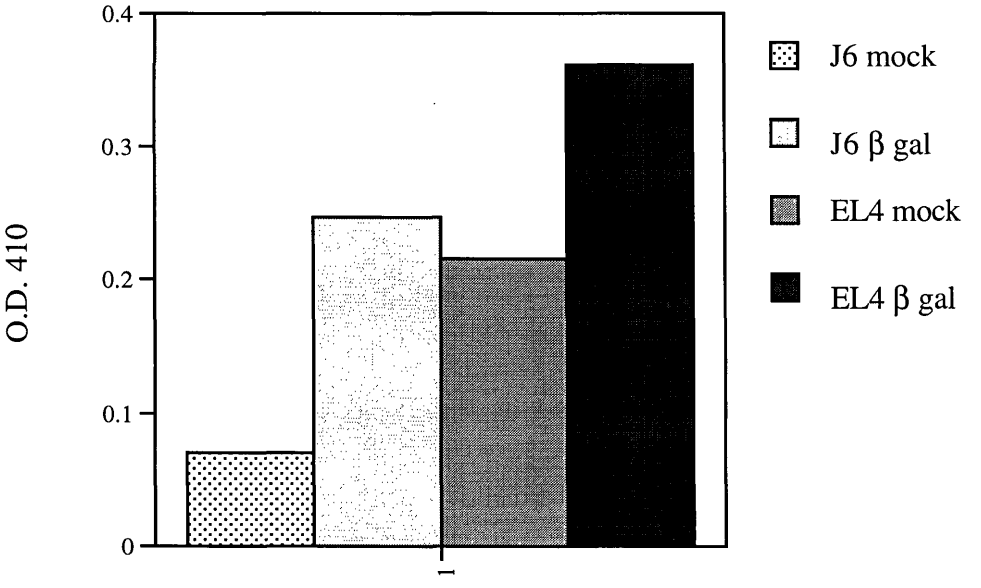
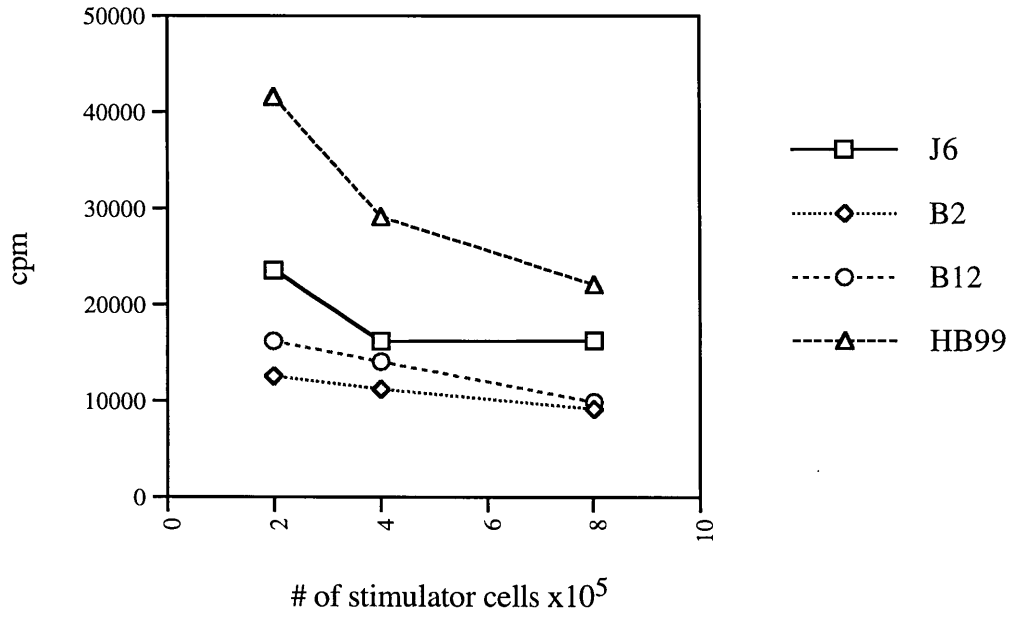


Figure 3.10b. Expression of the β-galactosidase construct in a transient transfection study.

$OD\lambda_{410}$ = β-Galactosidase enzymatic activity of cell lysates derived from mock and transfected J6 and EL4 cells

a)



b)

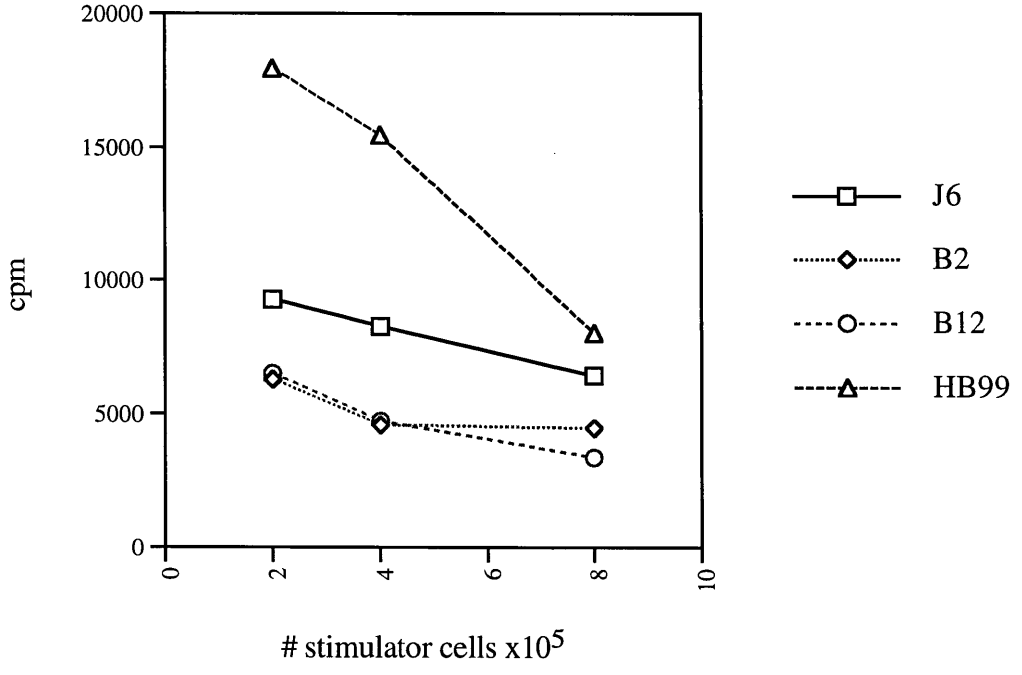


Figure 3.11. Ability of B2 and B12 clones to induce an alloreactive reponse in H-2^d spleen cells.

a) 4x10^5 and **b)** 2x10^5 H-2^d responder cells.

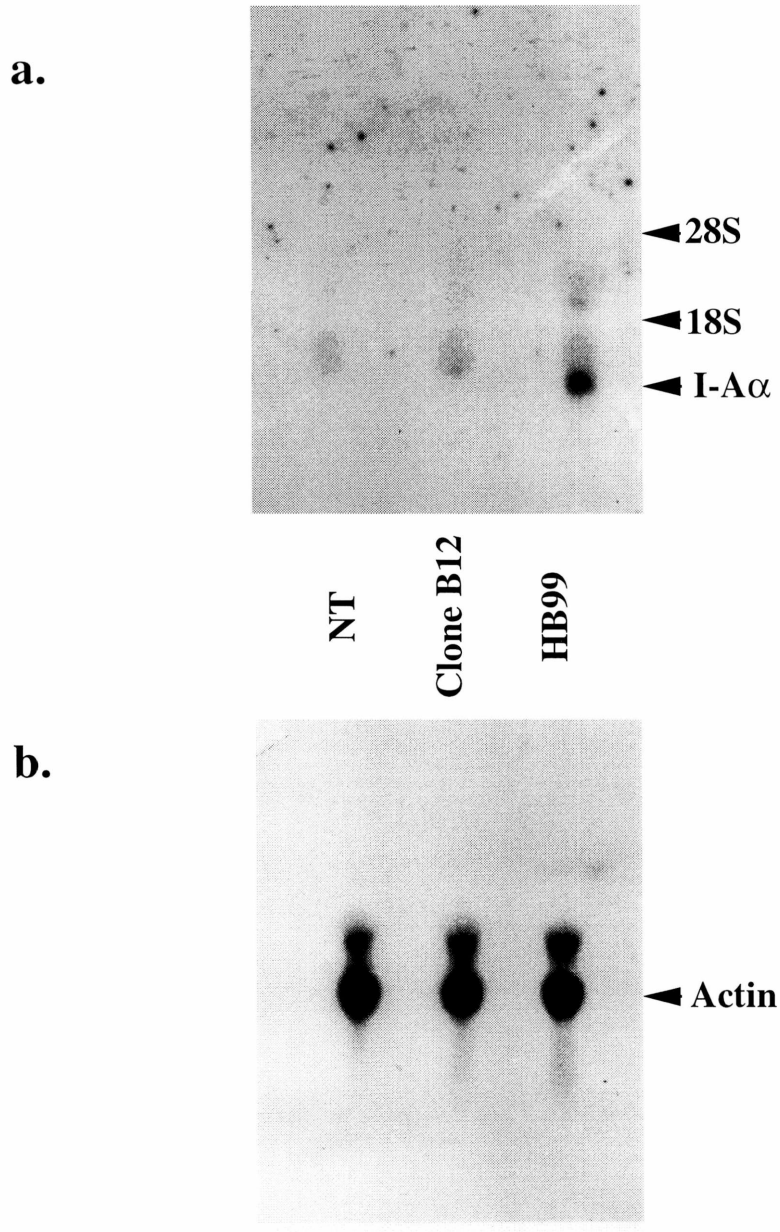


Figure 3.12 Northern analyses of J6 Clone B12

Tested lines were non-transfected (NT) J6 (negative control), J6 Clone B12 and HB99 (positive control).

a. probed with I-Aα cDNA
b. probed with Actin showing equivalent loading in each lane

3.3 Characterisation and functional responses of the IIIAα/IIIAβ transgenic line.

3.3.1 Testing the IIIAα/IIIAβ transgenic mice- Southern Blot analysis.

Genomic DNA was isolated from spleen cells taken from IIIAα/IIIAβ transgenic and non-transgenic littermates using Qiagen genomic tips (Qiagen) (**section 2.20**). The DNA was digested with *Hind* III, thereby only allowing probing for the I-Aα chain, as no *Hind* III site is present in the I-Aβ cDNA. As it is probable that the α and β chains will have concatamerised, and thus inserted into the same site within the genome, as discussed in **section 3.2.6**, presence of I-Aα should be indicative of presence of I-Aβ. Figure **3.13** shows the presence of two bands in the lane containing digested DNA from the transgenic mouse, but only one band in the lane containing DNA from the non-transgenic mouse. The bands correspond to DNA of ~1.2Kb and ~1.3Kb, the ~1.3Kb band seen in both lanes representing endogenous I-Aα. The 1.2Kb band was also seen in the stable J6 clone transfectant, with both the 1.2Kb and 1.3Kb bands observed in the lane containing DNA from the HB99 cell line (**section 3.2.6**), supporting the evidence that these two bands are indeed representative of the I-Aα transgene and endogenous I-Aα . Using the endogenous α chain as an indicator of differences in DNA loading between the two lanes, an estimate of I-Aα transgene copy number can be made.

3.3.2 Detection of I-Aα and I-Aβ cDNA in genomic DNA from IIIAα/IIIAβ transgene positive and negative mice.

PCR was used to detect the presence of the I-Aα and I-Aβ transgenes within the genome of the transgenic mice (**section 2.13**). The primers used were as follows:

I-Aα: (coding primer)	5'-GAC CAC CAT GCT CAG CCT CTG-3'	position 65-85
I-Aα: (non-coding primer)	5'-TGT GCC AGG TCA CCC AGC AC-3'	position 863-844
I-Aβ: (coding primer)	5'-TGT ACC AGT TCA TGG GCG AGT G-3'	position 116-137
I-Aβ: (non-coding primer)	5'-TGA GCA GAC CAG AGT GTT GTG G-3'	position 450-429

Figure 3.14a shows the presence of a PCR product of ~800 base pairs in the lane in which genomic DNA from a transgenic mouse was used as a template together with the I-Aα primers. **Figure 3.14b** shows the appearance of a PCR product of ~350 base

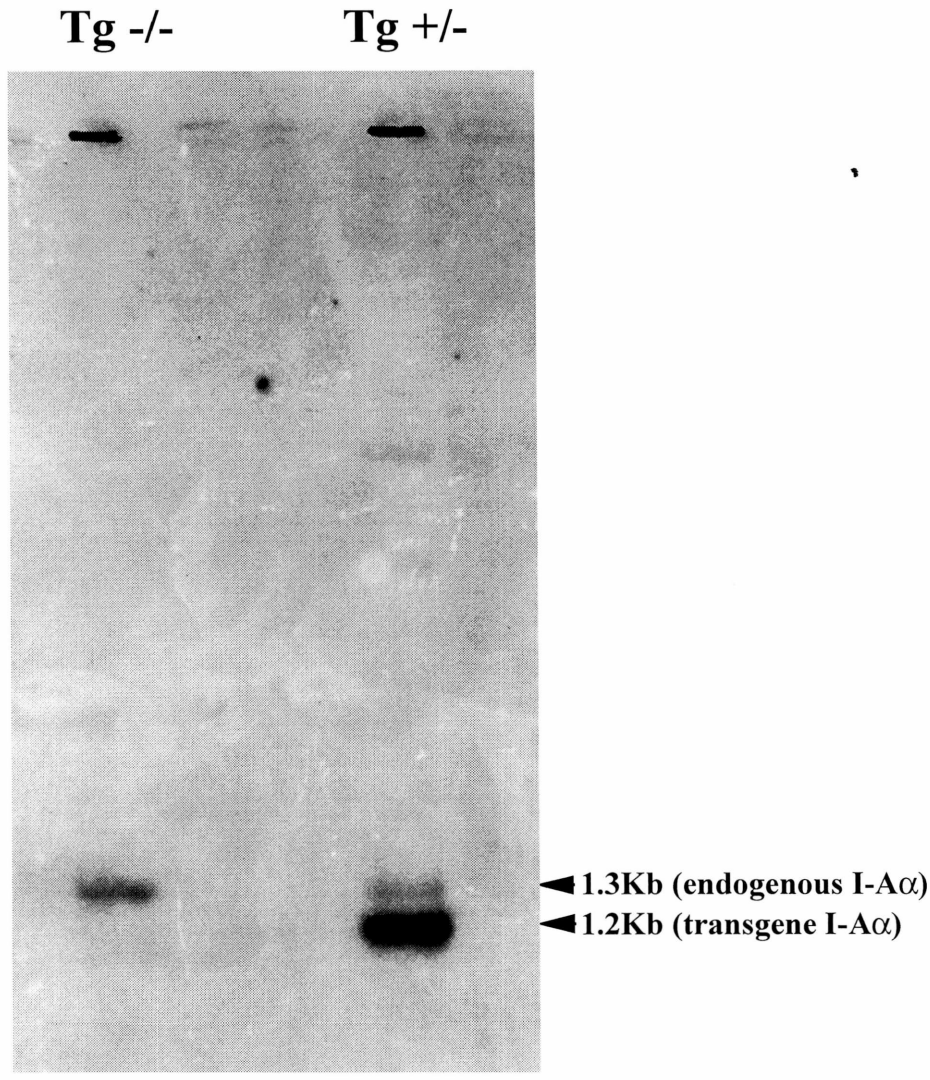


Figure 3.13 Tg founder is I-Aα positive

Southern blot analysis of *Hind*III digested genomic DNA isolated from spleens of Tg+/- and Tg -/- mice probed with I-Aα cDNA.

Key: Tg, transgenic

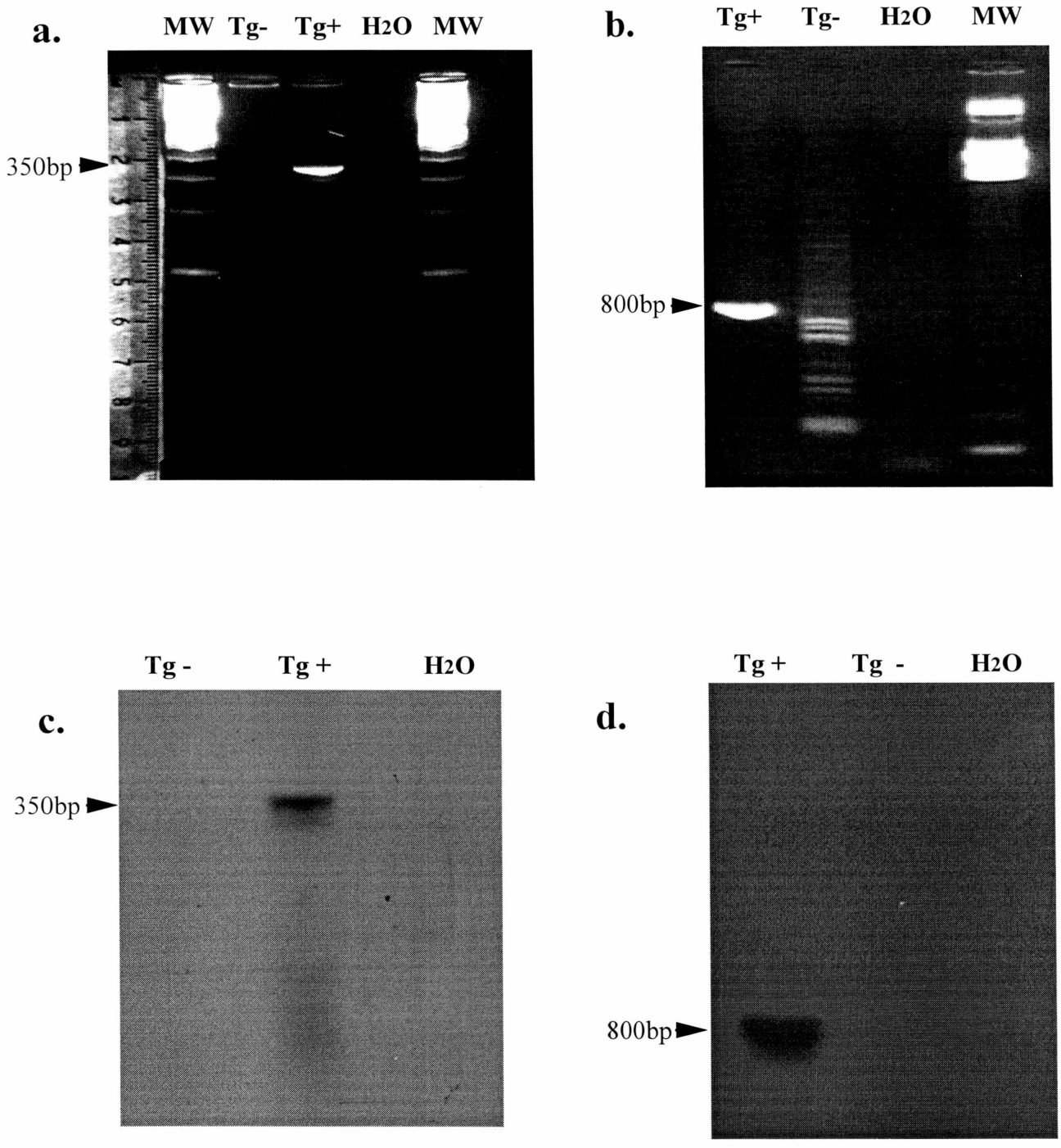


Figure 3.14 PCR screening of Tg- and Tg+ mouse genomic tail DNA

a. MHC class II I-Aβ primers; **b.** MHC class II I-Aα primers;
c. PCR gel probed with I-Aβ cDNA; **d.** PCR gel probed with I-Aα cDNA

Key: MW, Molecular weight markers; Tg, transgenic

pairs in the equivalent lane, in this case when using I-Aβ primers. No bands are seen in the lanes in which non-transgenic DNA or water were added, except in **figure 3.14a** in which some bands appear in the non-transgenic DNA lane. Therefore, in order to verify that these bands represent non-specific priming events, both gels were blotted onto membranes, and probed with either I-Aα or I-Aβ cDNA. **Figures 3.14c and d** indicate that no α- or β-specific bands appeared in either of the PCR reactions using non-transgenic DNA or water as a template, whether I-Aα or I-Aβ, respectively, had been used as primers. These results indicate the presence of both I-Aα and I-Aβ chains in the genome of the transgenic mice.

3.3.3 Screening F3 generation mice for expression of H-2^b allele.

The IIIAα/IIIAβ transgenic mice were derived from C57BL/6 x CBA (I-A^b x I-A^k) parents to maximise breeding efficiency **(section 3.1.5)**. The founder mouse was therefore crossed with C57BL/6 females and the resulting pups termed the F1 generation. After two further crosses with C57BL/6 mice, the third generation IIIAα/IIIAβ Tg-/+ mice were tested for the presence of the I-A^b allele, as well as absence of I-A^k. **Figure 3.15** shows the staining pattern observed when mouse tail blood was analysed **(section 2.31)**. The OX-6 (Sigma) antibody stains I-A^k- but not I-A^b-positive cells, whereas Y3P stains I-A^b- but not I-A^k-positive cells **(appendix II)**. **Figure 3.15c** indicates a complete absence of the I-A^k allele in all the mice tested with exclusive expression of the I-A^b allele. These mice were subsequently used to set up breeding colonies to generate IIIAα/IIIAβ homozygous mice.

3.3.4 Expression levels of MHC class II in IIIAα/IIIAβ transgenic mice.

Tail blood from transgene negative (Tg-/-), heterozygous (Tg-/+) and homozygous (Tg+/+) IIIAα/IIIAβ transgenic mice was separated on a Ficoll gradient, stained with M5/114-biotin and TcR-FITC (Sigma), with strepavidin-PE used as the second layer **(section 2.31)**. **Figure 3.16** shows typical histogram plots obtained. **Figure 3.16a** compares MHC class II expression levels on T cells from Tg-/-, Tg-/+ and Tg+/+ mice. No MHC class II is detectable on T cells from Tg-/- mice, T cells from Tg-/+ mice express MHC class II with a mean fluorescence intensity (MFI) of 23, Tg+/+ T cells display an MFI of 46 indicative that they do express two copies of the IIIAα/IIIAβ transgenes. **Figure 3.16b** compares the levels of T cell expressed MHC class II in the transgenic mice with those found on B cells. As discussed in **section 1.9** B cells, alongside macrophages and dendritic cells are collectively known as professional antigen presenting cells. These cell types expressing high levels of MHC class II at certain stages of their life cycle. The B cells expressed MHC class II at levels

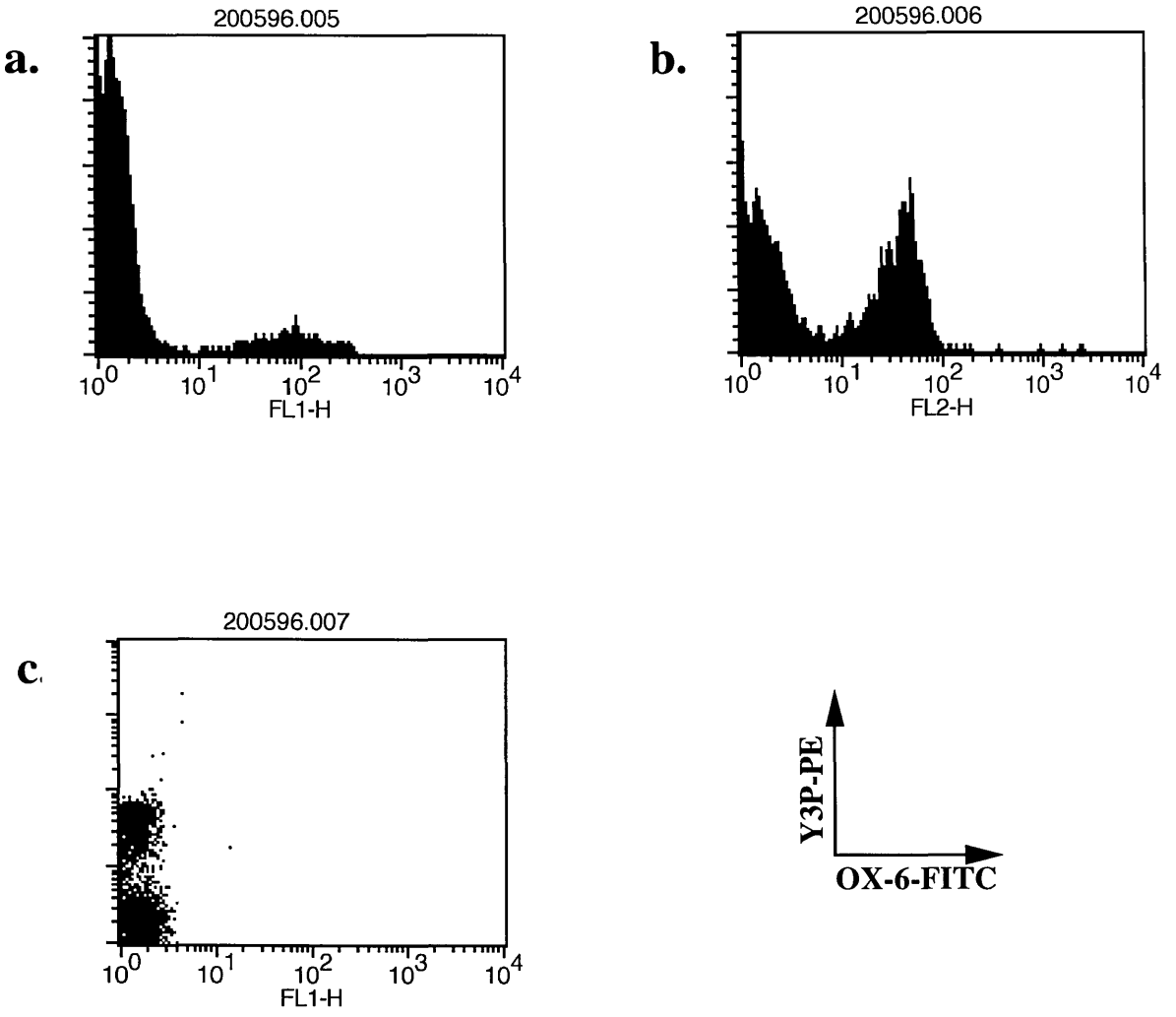
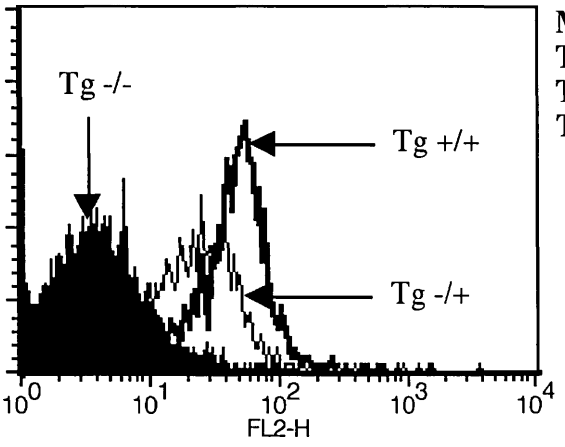


Figure 3.15 Histogram and dot plot analysis indicating the absence of I-Ak MHC class II in F3 generation MHC Class II transgenic mice

a. OX-6-FITC (I-Ak) staining - CBA control mouse
b. Y3P-PE (I-Ab) staining - C57BL6 control mouse
c. Profile observed on double staining tail blood derived from transgene positive F3 generation mice

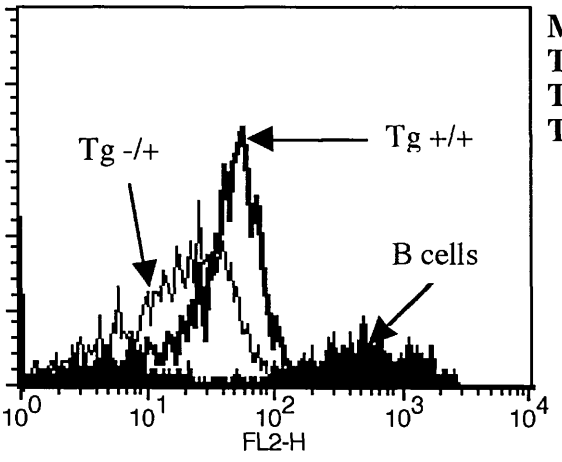
CBA mice did not stain for MHC class II when the Y3P antibody was used. Conversely, C57Bl6 mice did not stain with the OX-6 antibody (data not shown).

a.



MFIs:
Tg -/- = 7
Tg -/+ = 23
Tg +/+ = 46

b.



MFIs:
Tg -/+ = 23
Tg +/+ = 46
Tg -/- (B cells) = 207

Figure 3.16 Relative Class II expression levels in:

a. Tg -/-, Tg -/+ and Tg+/+ T cells
b. Tg -/+ and Tg +/+ T cells relative to Tg -/- B cells

This analytical method was used routinely to screen for Tg -/-, Tg -/+ and Tg+/+ mice

MFI = mean fluorescent intensity

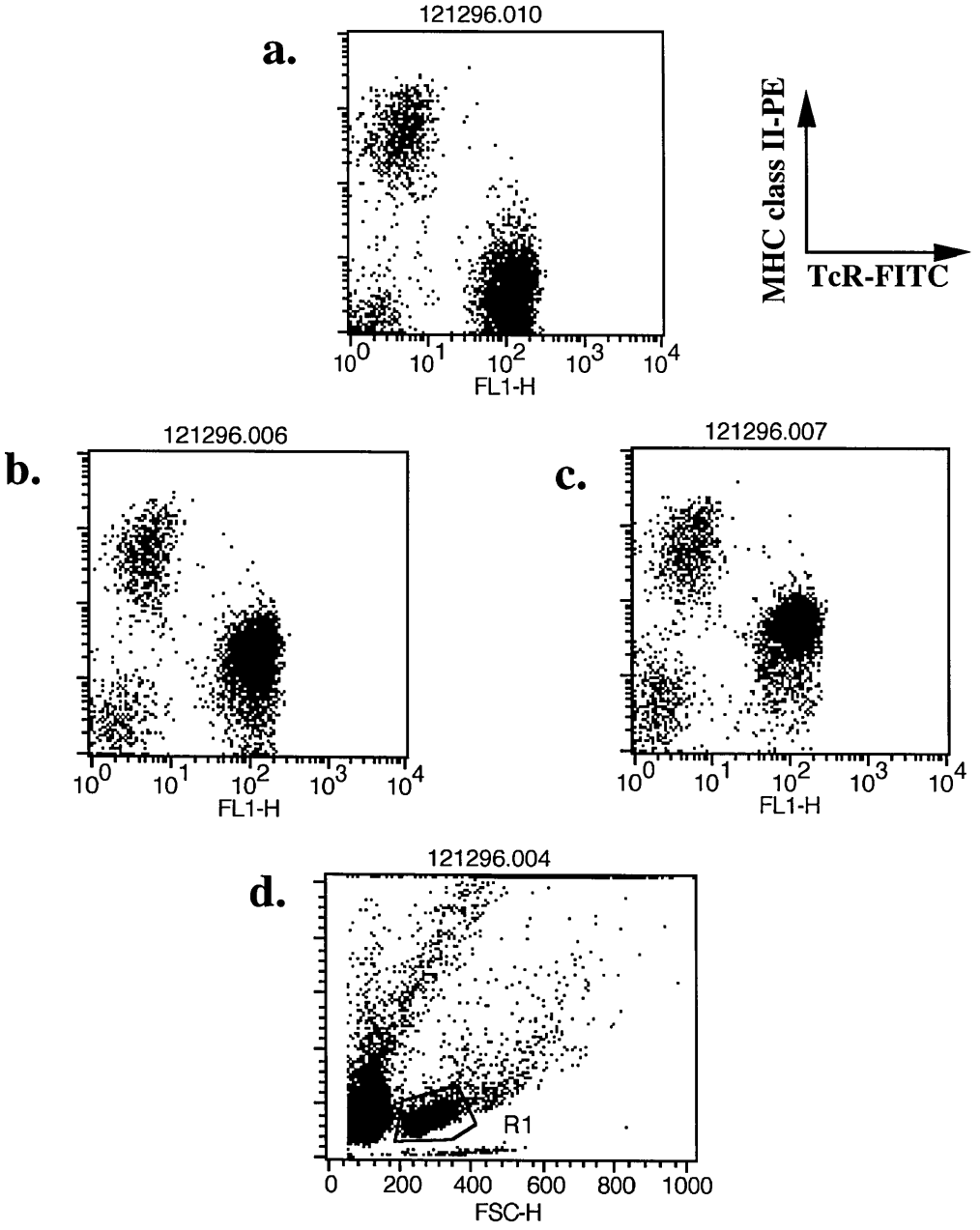


Figure 3.17 Transgene MHC class II expression on T cells of:

a. Tg -/- mice; **b.** Tg -/+ mice; **c.** Tg+/+ mice
d. Gate R1 indicates the cell population analysed, representative of all lymphocytes

of approximately one log greater than that seen for the Tg+/+ mice (MFI=407). The transgenic T cells, therefore, do express membrane levels of MHC class II at relatively high levels, but at significantly lower levels than that seen on professional antigen presenting cells such as B cells. **Figure 3.17** shows dot plot analysis of the tail blood lymphocytes. The differences in levels of T cell expressed MHC class II between Tg-/-, Tg-/+ and Tg+/+, as well as for B cell expressed MHC class II are clearly seen.

3.3.5 Effects of transgene expression on other T cell surface molecules.

12 and 4 week old mice were analysed to ascertain what effect, if any, expression of the IIIAα/IIIAβ transgene had on the expression of other T cell surface molecules **(section 2.29)**. **Figure 3.18** shows the levels of various surface molecules on T cells from the lymph nodes of 4 week old mice. These graphs indicate that the majority of these molecules are expressed at equivalent levels on Tg-/-, Tg-/+ and Tg+/+ T cells. B7.1, B7.2, CD28, ICAM-1, CD2, IL-2R, LFA-1, Thy-1, HSA, CD69 and CD40L were all assayed and only IL-2R staining gave a subtly different profile. The 12 week old mice gave similar profiles, and a representative panel is shown in **Figure 3.19.** CD2 and LFA-1 levels are equivalent in transgene negative and positive mice, whereas IL-2R staining highlights distinct differences between the three mouse types. The 4 week old Tg+/+ mice showed weak positive staining for IL-2R **(Figure 3.18f)**, whereas the Tg-/- and Tg-/+ were completely IL-2R negative. The Tg-/+ and Tg+/+ 12 week old mice, however, gave increased levels of staining compared with that seen for the Tg-/- mice.

3.3.6 Relative numbers of B- and T-cells in lymph nodes of IIIAα/IIIAβ transgenic and non-transgenic mice.

Lymph node cells from 4 and 12 week old mice were stained with TcR-FITC and B220-PE antibodies. **Figure 3.20** shows typical FACS profiles obtained. 4 week old mice give similar staining patterns, B cells accounting for ~20% of the total cell compartment, T cells for ~70%. The staining patterns observed in the 12 week old mice show distinct differences in B- and T-cell numbers when comparing the transgene positive and negative mice. B cell levels are reduced from 19% in the Tg-/- mice to 11% in the Tg-/+ mice with a further decrease to 8% in the Tg+/+ mice. The T cell compartment shows an equivalent rise in cell numbers with Tg-/- containing 64% T cells, Tg-/+ 76% and Tg+/+ 81%.

3.3.7 CD4:CD8 T cell ratios in lymph nodes of IIIAα/IIIAβ transgenic and non-transgenic mice.

Lymph node T cells from transgene positive and negative mice were stained with the antibodies CD4-SAQR and CD8-PE to determine what effect expression of the

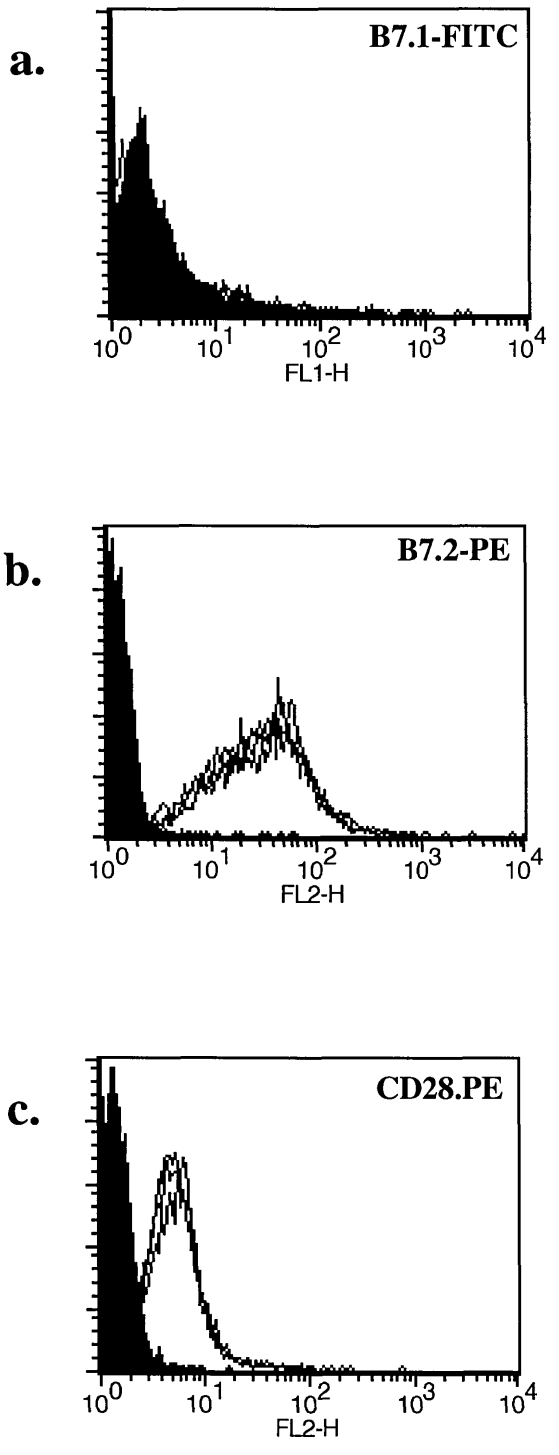


Figure 3.18 Expression level of various T cell surface molecules isolated from lymph-nodes of 4 week old Tg -/-, Tg -/+ and Tg +/+ mice

a. B7.1; **b.** B7.2; **c.** CD28; **d.** ICAM-1; **e.** CD2; **f.** IL2R; **g.** LFA1; **h.** Thy1; **i.** HSA; **j.** CD69; **k.** CD40L

Note: Isotype matched antibodies were used as negative controls (filled lines)

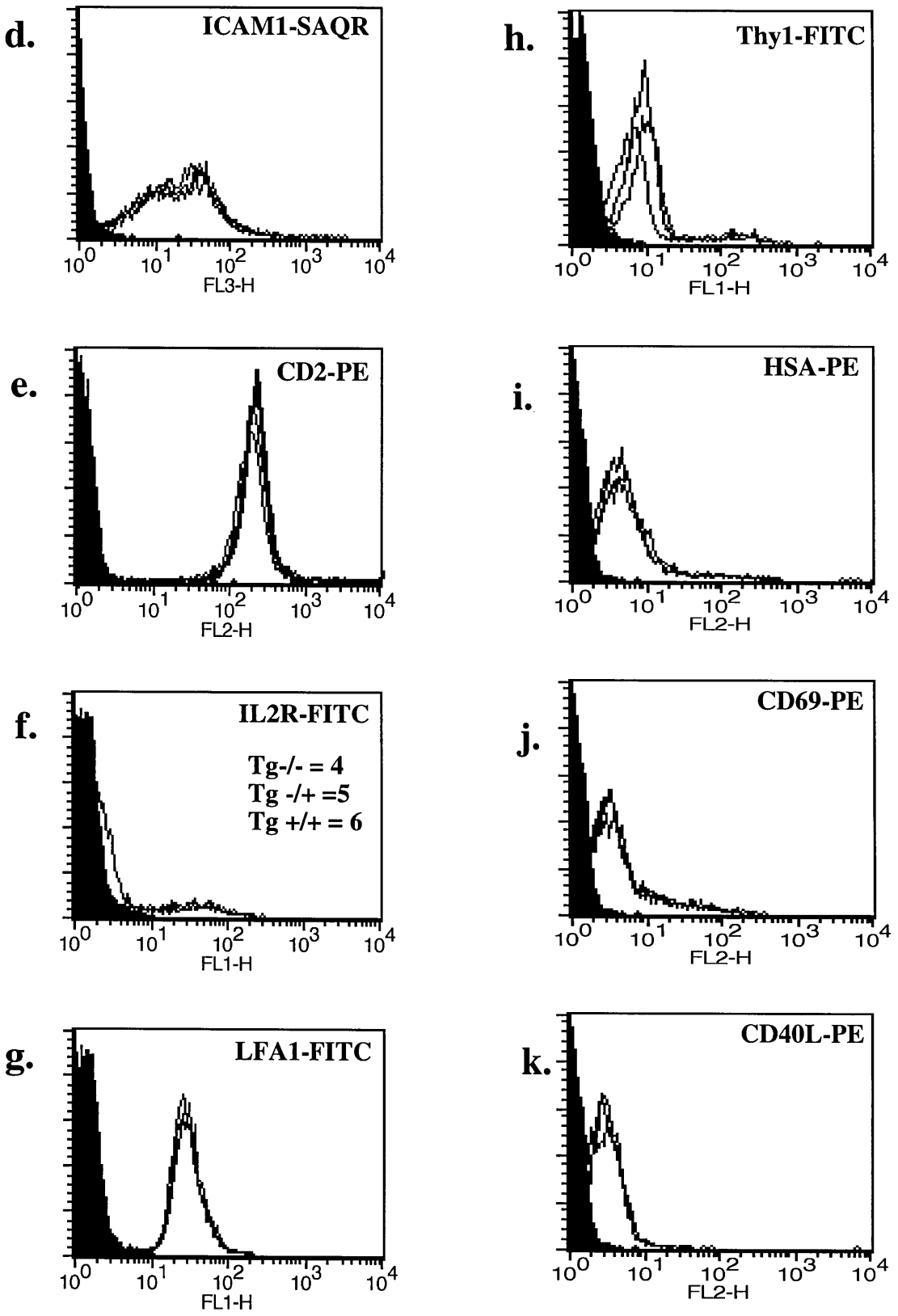


Figure 3.18 Continued

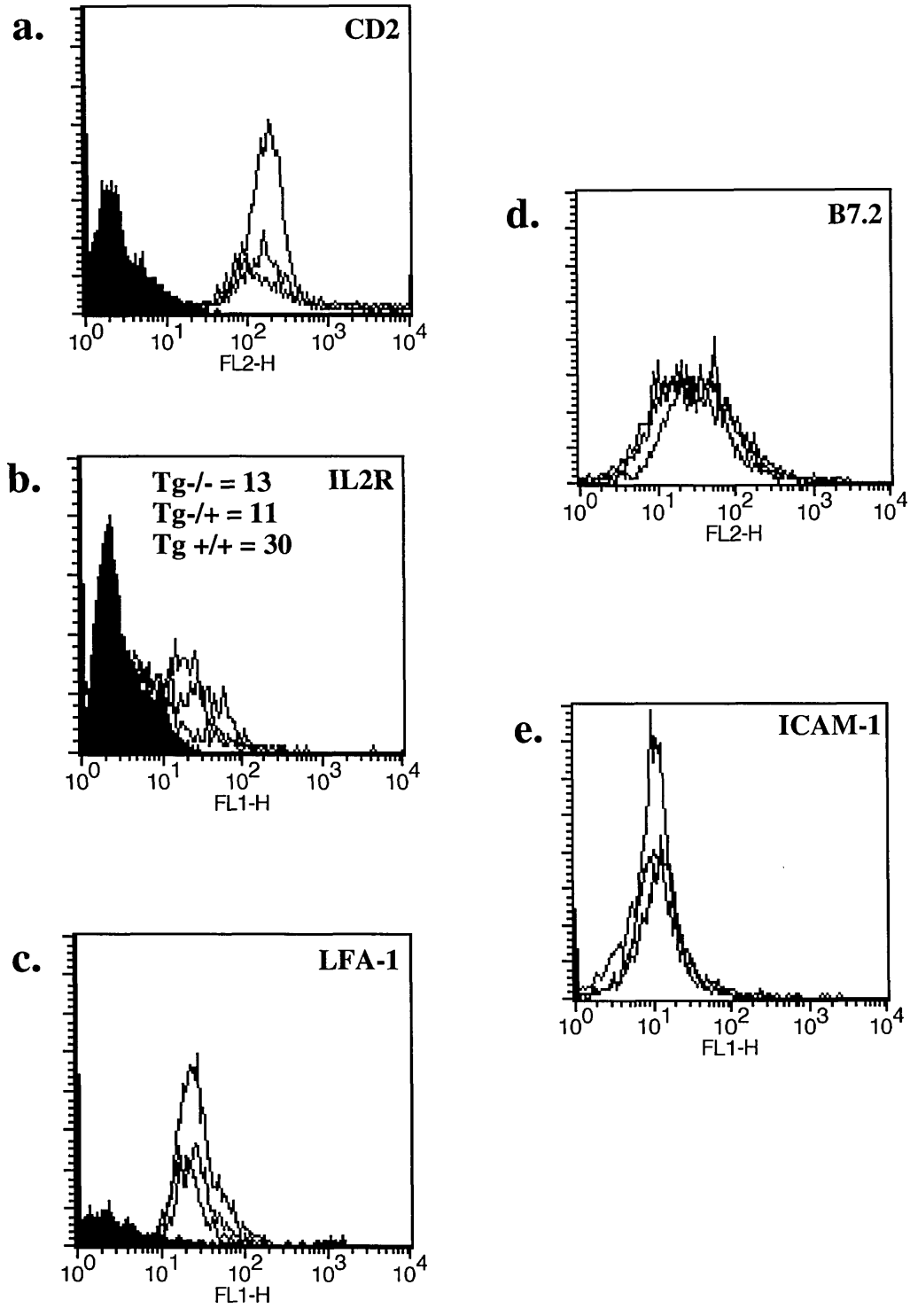
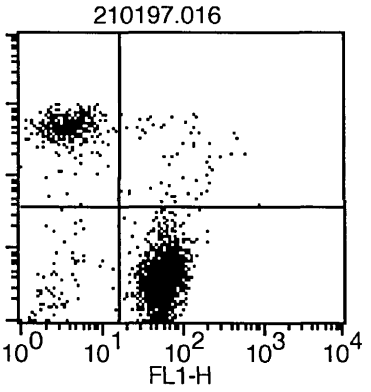


Figure 3.19 12 week old mice lymph node T cell expression of:

a. CD2; **b.** IL2R; **c.** LFA-1; **d.** B7.2; **e.** ICAM-1

a.

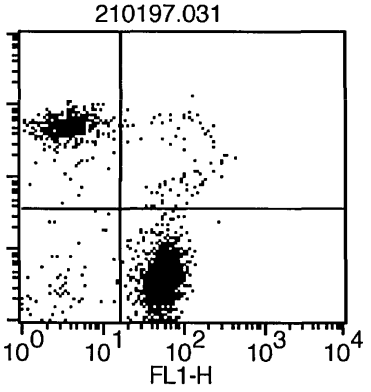


Quadrant Statistics

File: 210197.016 Gated Events: 7729
Total Events: 10000 X Parameter: FL1-H (Log)
Y Parameter: FL2-H (Log) Quad Location: 16, 37

Quad	Events	% Gated	% Total
UL	1244	16.10	12.44
UR	178	2.30	1.78
LL	206	2.67	2.06
LR	6101	78.94	61.01

b.

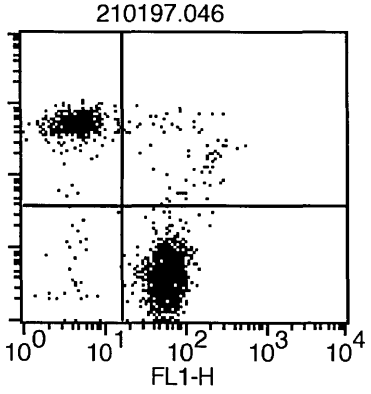


Quadrant Statistics

File: 210197.031 Gated Events: 7028
Total Events: 10000 X Parameter: FL1-H (Log)
Y Parameter: FL2-H (Log) Quad Location: 16, 37

Quad	Events	% Gated	% Total
UL	1564	22.25	15.64
UR	234	3.33	2.34
LL	135	1.92	1.35
LR	5095	72.50	50.95

c.



Quadrant Statistics

File: 210197.046 Gated Events: 7072
Total Events: 10000 X Parameter: FL1-H (Log)
Y Parameter: FL2-H (Log) Quad Location: 16, 37

Quad	Events	% Gated	% Total
UL	1513	21.39	15.13
UR	191	2.70	1.91
LL	119	1.68	1.19
LR	5249	74.22	52.49

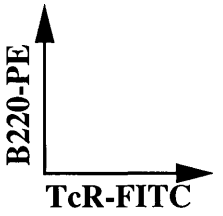
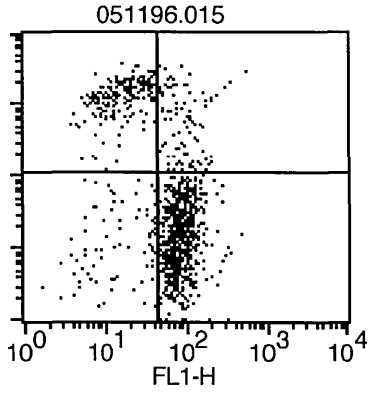


Figure 3.20 Dot plots showing relative numbers of B- and T- cells in lymph nodes of:

a. Tg -/-; **b.** Tg -/+; **c.** Tg+/+ in 4 week old mice
d. Tg -/-; **e.** Tg -/+; **f.** Tg+/+ in 12 week old mice

d.

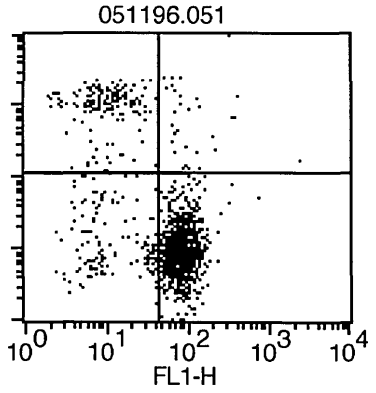


Quadrant Statistics

File: 051196.015
Total Events: 10000
Y Parameter: FL2-H (Log)
Gated Events: 3196
X Parameter: FL1-H (Log)
Quad Location: 44, 113

Quad	Events	% Gated	% Total
UL	604	18.90	6.04
UR	309	9.67	3.09
LL	246	7.70	2.46
LR	2037	63.74	20.37

e.

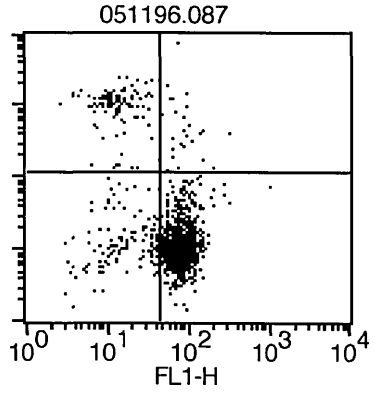


Quadrant Statistics

File: 051196.051
Total Events: 10000
Y Parameter: FL2-H (Log)
Gated Events: 5054
X Parameter: FL1-H (Log)
Quad Location: 44, 113

Quad	Events	% Gated	% Total
UL	562	11.12	5.62
UR	103	2.04	1.03
LL	543	10.74	5.43
LR	3846	76.10	38.46

f.



Quadrant Statistics

File: 051196.087
Total Events: 10000
Y Parameter: FL2-H (Log)
Gated Events: 4254
X Parameter: FL1-H (Log)
Quad Location: 44, 113

Quad	Events	% Gated	% Total
UL	356	8.37	3.56
UR	72	1.69	0.72
LL	370	8.70	3.70
LR	3456	81.24	34.56

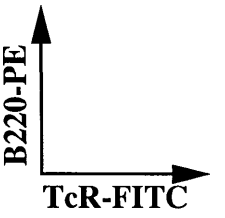


Figure 3.20 continued

IIIAα/IIIAβ transgene may have on the CD4:CD8 ratio. **Figure 3.21** shows typical FACS profiles obtained. Despite an overall rise in T cell levels as discussed in **section 3.3.6**, 4 and 12 week old mice whether transgene positive or negative exhibit CD4:CD8 ratios of approximately 2:1. These results, together with those presented in **section 3.3.6** suggest that as the IIIAα/IIIAβ transgenic mice get older increasing numbers of T cells appear in the periphery, but that the CD4:CD8 ratio remains unaffected. The development of the T cells in the thymus may, therefore, be altered due to the expression of MHC class II on the T cells, and this seems to be influencing the cellular composition of the lymph node cells only later in the life of the mouse.

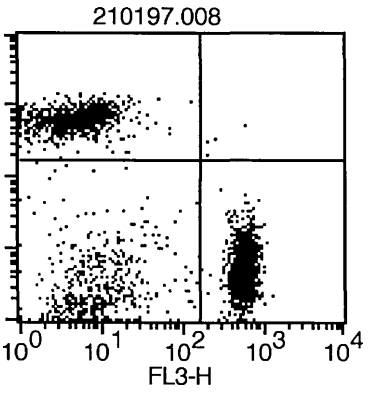
3.3.8 CD4 and CD8 profiles in thymic T cells from 4, 8 and 12 week old mice.

Thymi from 4, 8 and 12 week old mice were stained with the antibodies CD4-SAQR and CD8-PE. FACS profiles obtained are presented in **Figure 3.22**. Once again the age of the mouse seemed to be a major determining factor in the profile obtained. CD4 and CD8 T cell levels in 4 week old mice were comparable in the transgene positive and negative mice (**Figure 3.22a,b,c**). The pattern observed in 8 week old mice revealed differences emerging between the transgene negative and positive mice and was reflected in the appearance of a $CD8^{int}CD4^{hi}$ population, which was more distinct in Tg+/+ than in the Tg-/+ mice. The double positive cell population was slightly reduced in the Tg-/+ mice from 80% to 66% as a result, with the $CD4^{hi}CD8^{lo}$ cell population together with the novel $CD4^{hi}CD8^{int}$ population making up 27% of total thymocytes. The Tg+/+ mice showed a more dramatic shift with only 49% DP cells observed, and a combined $CD4^{hi}CD8^{lo}$ and $CD4^{hi}CD8^{int}$ compartment of 43% (**Figure 3.22d,e,f**). By the age of 12 weeks this population had become more distinct. The DP compartment in the Tg-/+ thymocytes makes up only 65% of the total cell population with 31% thymocytes found in the combined $CD4^{hi}CD8^{lo}$/$CD4^{hi}CD8^{int}$ compartment. Again the Tg+/+ mice exhibited the most extreme phenotype with only 38% thymocytes found in the DP compartment and 58% observed to be of the $CD4^{hi}CD8^{lo}$/$CD4^{hi}CD8^{int}$ phenotype (**Figure 3.22g,h,i**).

3.3.9 The effect of Concanavalin A treatment on IIIAα/IIIAβ transgenic mice.

Spleen cells were cultured in the presence of concanavalin A (Con A) for 48 hours at which point cells were examined for the presence of lymphoblasts, counted and stained with various cell surface markers. Microscopic examination revealed that whereas the Tg-/- appeared to be blasting, indicated by the presence of many large clumps of cells, the Tg-/+ and Tg+/+ showed little signs of blasting with the majority

a.

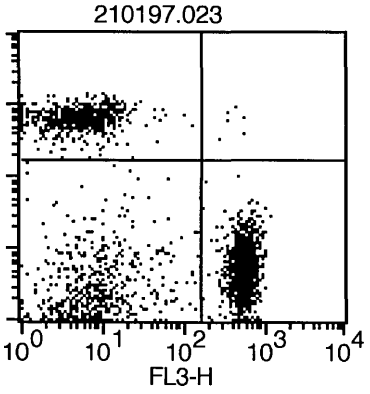


Quadrant Statistics

File: 210197.008
Total Events: 10000
Y Parameter: FL2-H (Log)
Gated Events: 8163
X Parameter: FL3-H (Log)
Quad Location: 164, 175

Quad	Events	% Gated	% Total
UL	2543	31.15	25.43
UR	7	0.09	0.07
LL	1517	18.58	15.17
LR	4096	50.18	40.96

b.

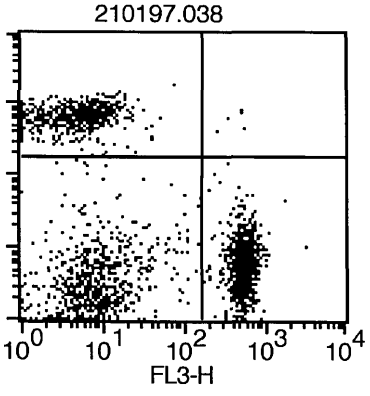


Quadrant Statistics

File: 210197.023
Total Events: 10000
Y Parameter: FL2-H (Log)
Gated Events: 7631
X Parameter: FL3-H (Log)
Quad Location: 164, 175

Quad	Events	% Gated	% Total
UL	2138	28.02	21.38
UR	13	0.17	0.13
LL	1698	22.25	16.98
LR	3782	49.56	37.82

c.



Quadrant Statistics

File: 210197.038
Total Events: 10000
Y Parameter: FL2-H (Log)
Gated Events: 7476
X Parameter: FL3-H (Log)
Quad Location: 164, 175

Quad	Events	% Gated	% Total
UL	2026	27.10	20.26
UR	9	0.12	0.09
LL	2113	28.26	21.13
LR	3328	44.52	33.28

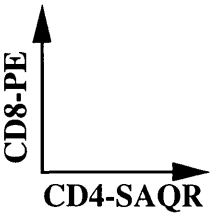
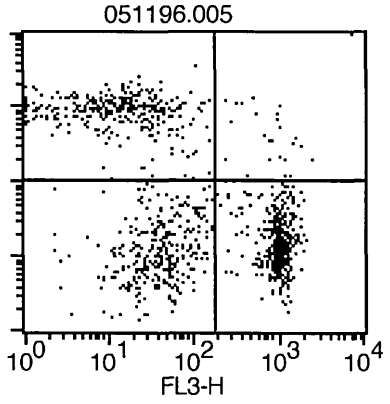


Figure 3.21 Dot plots showing relative numbers of CD4+ and CD8+ cells in lymph nodes of:

a. Tg -/-; **b.** Tg -/+; **c.** Tg+/+ in 4 week old mice
d. Tg -/-; **e.** Tg -/+; **f.** Tg+/+ in 12 week old mice

d.

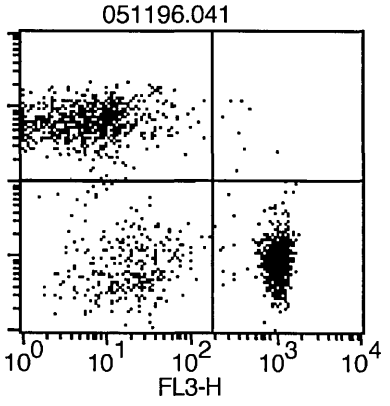


Quadrant Statistics

File: 051196.005 Gate: G3
Gated Events: 3333 Total Events: 10000
Quad Location: 173, 104

Quad	Events	% Gated	% Total
UL	948	28.44	9.48
UR	85	2.55	0.85
LL	983	29.49	9.83
LR	1317	39.51	13.17

e.

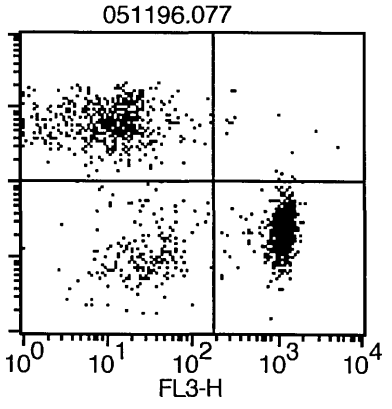


Quadrant Statistics

File: 051196.041 Gate: G3
Gated Events: 6647 Total Events: 10000
Quad Location: 173, 104

Quad	Events	% Gated	% Total
UL	2422	36.44	24.22
UR	29	0.44	0.29
LL	844	12.70	8.44
LR	3352	50.43	33.52

f.



Quadrant Statistics

File: 051196.077 Gate: G3
Gated Events: 5406 Total Events: 10000
Quad Location: 173, 104

Quad	Events	% Gated	% Total
UL	1900	35.15	19.00
UR	55	1.02	0.55
LL	580	10.73	5.80
LR	2871	53.11	28.71

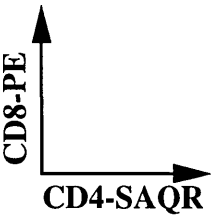
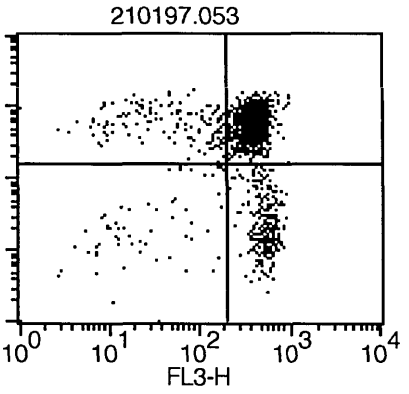


Figure 3.21 continued

a.

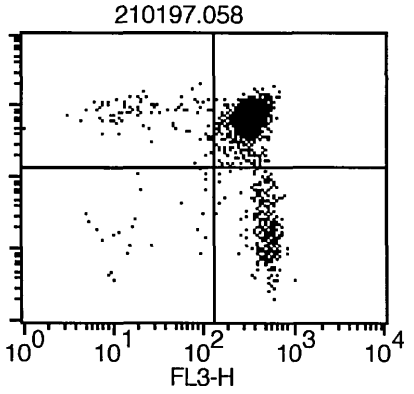


Quadrant Statistics

File: 210197.053 Gate: G1
Gated Events: 6913 Total Events: 10000
Quad Location: 193, 160

Quad	Events	% Gated	% Total
UL	422	6.10	4.22
UR	5456	78.92	54.56
LL	186	2.69	1.86
LR	849	12.28	8.49

b.

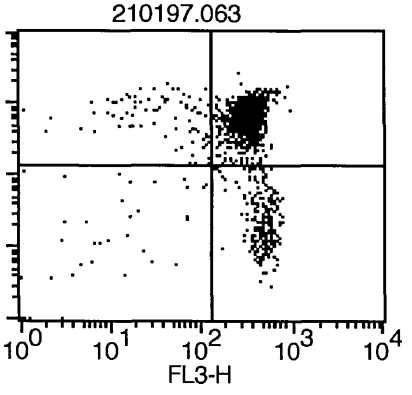


Quadrant Statistics

File: 210197.058 Gate: G1
Gated Events: 6960 Total Events: 10000
Quad Location: 126, 135

Quad	Events	% Gated	% Total
UL	288	4.14	2.88
UR	5581	80.19	55.81
LL	82	1.18	0.82
LR	1009	14.50	10.09

c.



Quadrant Statistics

File: 210197.063 Gate: G1
Gated Events: 7084 Total Events: 10000
Quad Location: 126, 135

Quad	Events	% Gated	% Total
UL	238	3.36	2.38
UR	5929	83.70	59.29
LL	110	1.55	1.10
LR	807	11.39	8.07

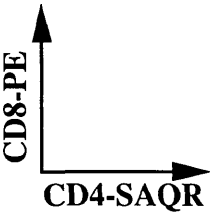
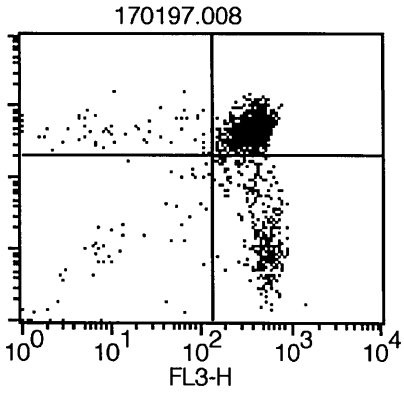


Figure 3.22 Levels of CD4 and CD8 expressed in the thymi of :

a. Tg -/-; **b.** Tg -/+; **c.** Tg+/+ in 4 week old mice
d. Tg -/-; **e.** Tg -/+; **f.** Tg+/+ in 8 week old mice
g. Tg -/-; **h.** Tg -/+; **i.** Tg+/+ in 12 week old mice

d.

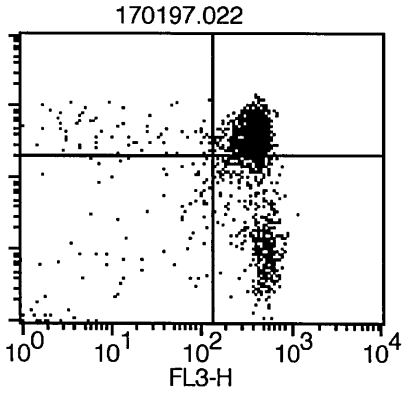


Quadrant Statistics

File: 170197.008 Gate: G1
Gated Events: 7516 Total Events: 10000
Quad Location: 126, 207

Quad	Events	% Gated	% Total
UL	216	2.87	2.16
UR	6025	80.16	60.25
LL	184	2.45	1.84
LR	1091	14.52	10.91

e.

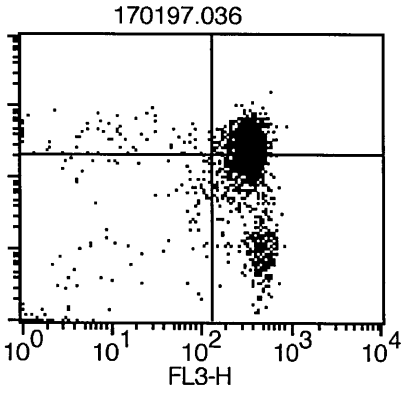


Quadrant Statistics

File: 170197.022 Gate: G1
Gated Events: 7174 Total Events: 10000
Quad Location: 126, 207

Quad	Events	% Gated	% Total
UL	224	3.12	2.24
UR	4728	65.90	47.28
LL	272	3.79	2.72
LR	1950	27.18	19.50

f.



Quadrant Statistics

File: 170197.036 Gate: G1
Gated Events: 7398 Total Events: 10000
Quad Location: 126, 207

Quad	Events	% Gated	% Total
UL	183	2.47	1.83
UR	3641	49.22	36.41
LL	401	5.42	4.01
LR	3173	42.89	31.73

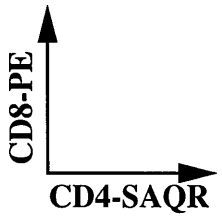
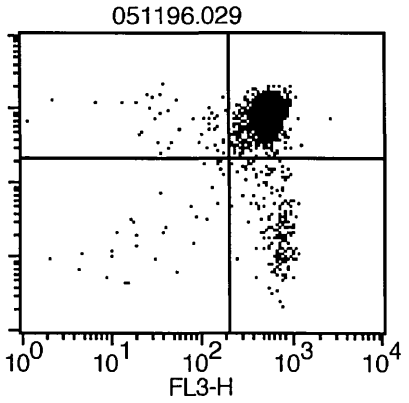


Figure 3.22 continued

g.

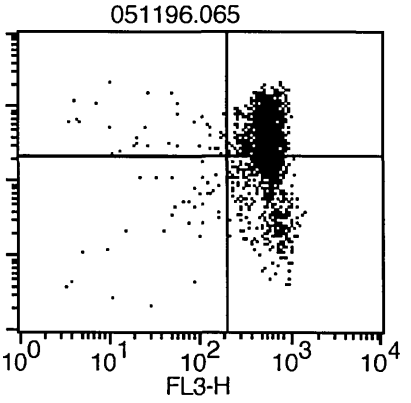


Quadrant Statistics

File: 051196.029 Gate: G1
Gated Events: 5817 Total Events: 10000
Quad Location: 193, 219

Quad	Events	% Gated	% Total
UL	135	2.32	1.35
UR	4903	84.29	49.03
LL	75	1.29	0.75
LR	704	12.10	7.04

h.

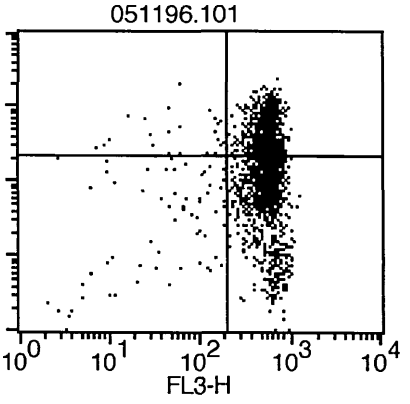


Quadrant Statistics

File: 051196.065 Gate: G1
Gated Events: 6443 Total Events: 10000
Quad Location: 193, 219

Quad	Events	% Gated	% Total
UL	103	1.60	1.03
UR	4239	65.79	42.39
LL	105	1.63	1.05
LR	1996	30.98	19.96

i.



Quadrant Statistics

File: 051196.101 Gate: G1
Gated Events: 6563 Total Events: 10000
Quad Location: 193, 219

Quad	Events	% Gated	% Total
UL	54	0.82	0.54
UR	2526	38.49	25.26
LL	181	2.76	1.81
LR	3802	57.93	38.02

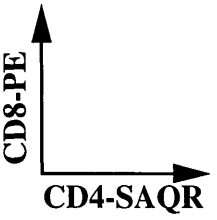


Figure 3.22 continued

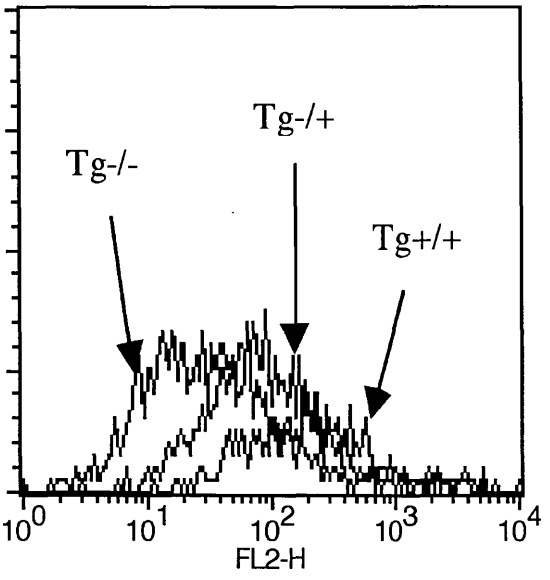
of cells seen as small, single cells and few blasts observed. Furthermore Tg-/+ and Tg+/+ cell numbers were reduced by between 1/3 and 2/3 compared with Tg-/- (data not shown).

Staining of the Con A stimulated cells with the MHC class II antibody M5/114 revealed that the level of T cell expressed MHC class II in the Tg-/+ and Tg+/+ mice was similar to that seen on unstimulated T cells isolated from tail blood (**figures 3.23, 3.16 and 3.17**). The levels were slightly increased on the Con A treated T cells, but it is unlikely that this is due to an increase in transcription of the transgenes as it is known that CD2 is not regulated at the transcriptional level (Alberola-ila J *et al.* 1991), therefore the pTexIIIa expression vector will not be able to modify the transcription rate of any heterologous genes under its control. The most likely explanation for this slight increase being the ability of murine T cells to passively absorb MHC class II molecules onto their cell surface after activation. This phenomenon has been observed by other groups, and initially led to the assumption that murine T cells were able to express MHC class II (Gautam SC *et al.* 1991, Kira J-I *et al.* 1989). It is now generally accepted, however, that murine T cells do not synthesise MHC class II molecules and its presence is due solely to passive absorption (Lorber MI *et al.* 1982). The ability to passively acquire MHC class II molecules is evident from the FACS profile of the activated Tg-/- mouse. In this case the level of M5/114 staining has increased dramatically giving an MFI of 74 (**figure 3.23**).

3.3.10 Evidence for loss of T cells in Concanavalin A stimulated spleen cultures.

As mentioned in **section 3.3.9** Con A does not appear to induce the formation of T lymphoblasts in Tg-/+ and Tg+/+ spleen cell cultures as potently as observed for the Tg-/- cultures. Viable cell numbers are also decreased in the transgenic cultures. The Con A stimulated populations were therefore stained with antibodies directed at the B220 and TcR epitopes to establish the cellular distribution of the remaining cells. **Figure 3.24.1** shows the FACS profiles obtained. The profile of the control Tg-/- Con A stimulated spleen cells indicates the presence of 72% T cells and 18% B cells (**figure 3.24.1a**). The profiles of both the Tg-/+ and Tg+/+ cultures show a reduction in the number of T cells present when compared with the Tg-/- mice, with 68% and 53% T cells observed in the Tg-/+ and Tg+/+ mice, respectively (**figures 3.24.1b and c**).

Staining of Con A stimulated spleens with other cell surface markers was also performed. IL2R profiles indicate a significant loss in membrane associated IL2R in Tg-/+ (MFI=65) and Tg+/+ (MFI=41) T cells compared with Tg-/- controls (MFI=140) (**figure 3.24.2**). CD69 (**figure 3.24.3**) and CD28 (**figure 3.24.4**) levels

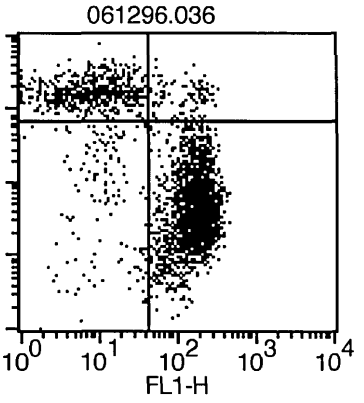


MFIs:
Tg -/- = 74
Tg -/+ = 155
Tg +/+ = 179

Figure 3.23 Relative T Class II expression levels following Concanavalin A stimulation of spleen cells

MFI = mean fluorescent intensity

a.

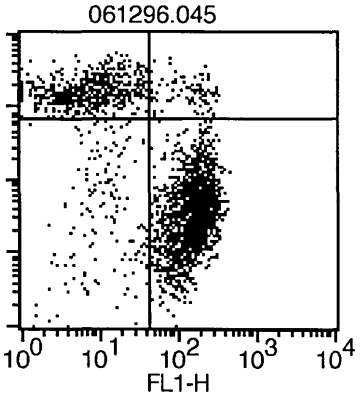


Quadrant Statistics

File: 061296.036 Gated Events: 2987
Total Events: 15000 Quad Location: 41, 679

Quad	Events	% Gated	% Total
UL	552	18.48	3.68
UR	105	3.52	0.70
LL	157	5.26	1.05
LR	2173	72.75	14.49

b.

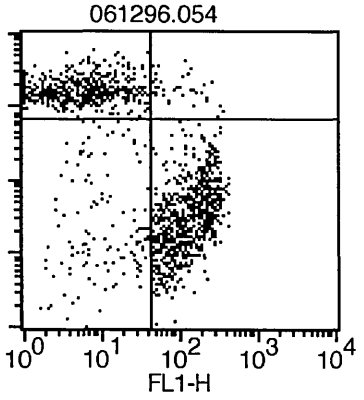


Quadrant Statistics

File: 061296.045 Gated Events: 2833
Total Events: 15000 Quad Location: 41, 679

Quad	Events	% Gated	% Total
UL	641	22.63	4.27
UR	101	3.57	0.67
LL	162	5.72	1.08
LR	1929	68.09	12.86

c.



Quadrant Statistics

File: 061296.054 Gated Events: 1489
Total Events: 15000 Quad Location: 41, 679

Quad	Events	% Gated	% Total
UL	522	35.06	3.48
UR	39	2.62	0.26
LL	133	8.93	0.89
LR	795	53.39	5.30

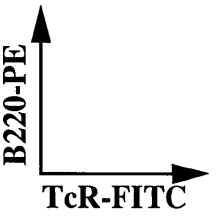
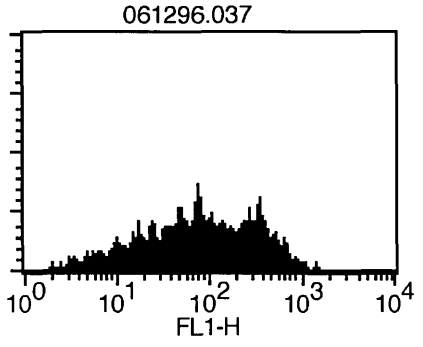


Figure 3.24.1 Relative levels of T- and B- cells as seen in Concanavalin A stimulated spleens:

a. Tg -/-; **b.** Tg -/+; **c.** Tg+/+

a.

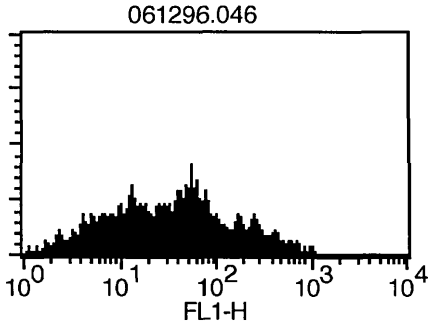


Histogram Statistics

File: 061296.037 Gated Events: 1917
Total Events: 15000

Marker	Events	% Gated	% Total	Mean
All	1917	100.00	12.78	140.80

b.

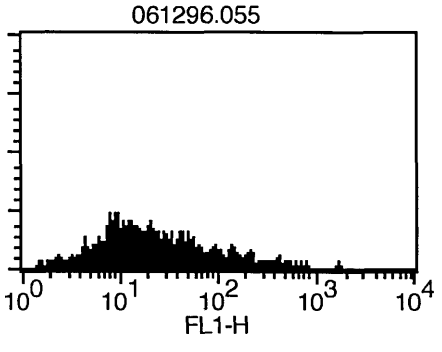


Histogram Statistics

File: 061296.046 Gated Events: 2152
Total Events: 15000

Marker	Events	% Gated	% Total	Mean
All	2152	100.00	14.35	64.81

c.



Histogram Statistics

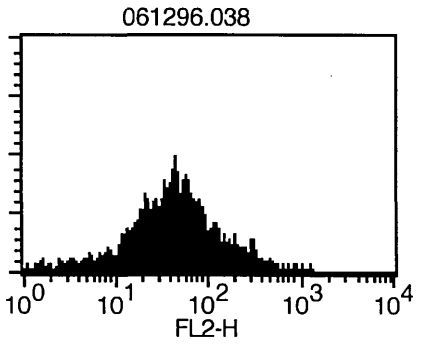
File: 061296.055 Gated Events: 1108
Total Events: 15000

Marker	Events	% Gated	% Total	Mean
All	1108	100.00	7.39	40.95

Figure 3.24.2 IL-2R expression in Concanavalin A stimulated spleens

a. Tg -/-; **b.** Tg -/+; **c.** Tg+/+

a.

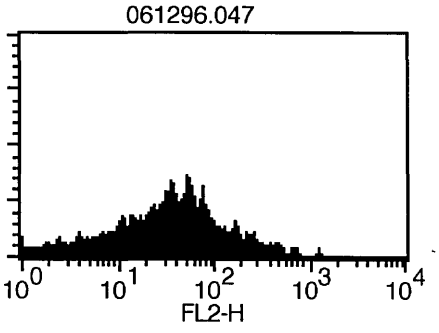


Histogram Statistics

File: 061296.038 Gated Events: 2195
Total Events: 15000

Marker	Events	% Gated	% Total	Mean
All	2195	100.00	14.63	60.70

b.

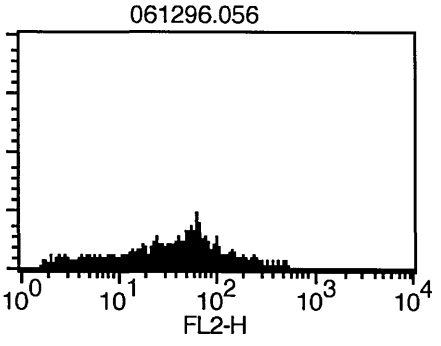


Histogram Statistics

File: 061296.047 Gated Events: 1720
Total Events: 15000

Marker	Events	% Gated	% Total	Mean
All	1720	100.00	11.47	55.17

c.



Histogram Statistics

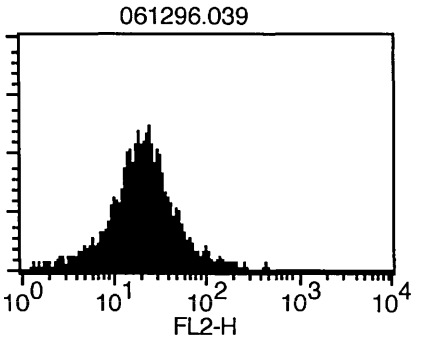
File: 061296.056 Gated Events: 599
Total Events: 15000

Marker	Events	% Gated	% Total	Mean
All	599	100.00	3.99	56.17

Figure 3.24.3 CD69 expression in Concanavalin A stimulated spleens

a. Tg -/-; **b.** Tg -/+; **c.** Tg+/+

a.

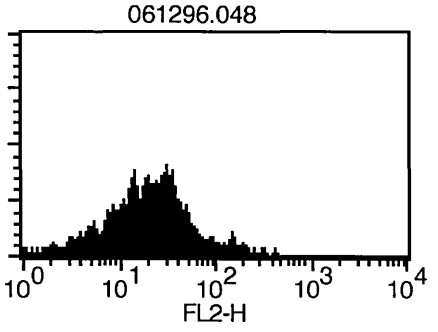


Histogram Statistics

File: 061296.039 Gated Events: 2454
Total Events: 15000

Marker	Events	% Gated	% Total	Mean
All	2454	100.00	16.36	24.15

b.

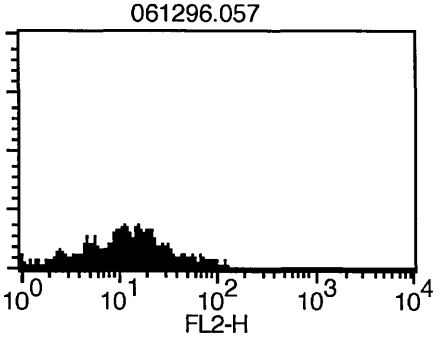


Histogram Statistics

File: 061296.048 Gated Events: 1842
Total Events: 15000

Marker	Events	% Gated	% Total	Mean
All	1842	100.00	12.28	26.48

c.



Histogram Statistics

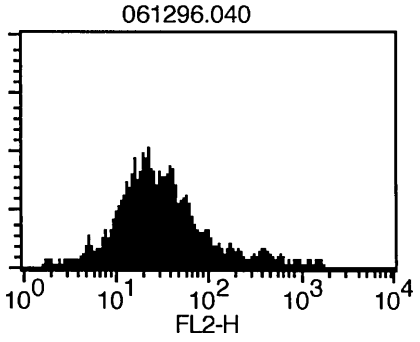
File: 061296.057 Gated Events: 638
Total Events: 15000

Marker	Events	% Gated	% Total	Mean
All	638	100.00	4.25	14.69

Figure 3.24.4 CD28 expression in Concanavalin A stimulated spleens

a. Tg -/-; **b.** Tg -/+; **c.** Tg+/+

a.

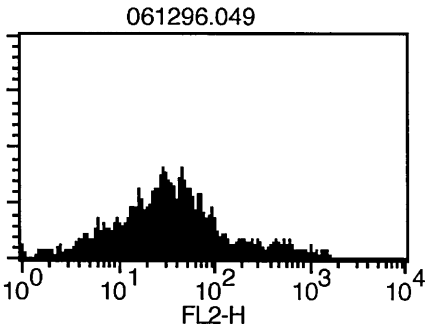


Histogram Statistics

File: 061296.040 Gated Events: 2360
Total Events: 15000

Marker	Events	% Gated	% Total	Mean
All	2360	100.00	15.73	48.30

b.

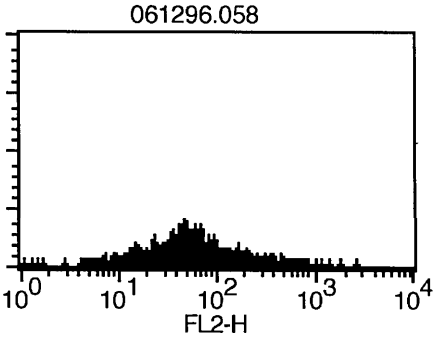


Histogram Statistics

File: 061296.049 Gated Events: 1989
Total Events: 15000

Marker	Events	% Gated	% Total	Mean
All	1989	100.00	13.26	60.39

c



Histogram Statistics

File: 061296.058 Gated Events: 706
Total Events: 15000

Marker	Events	% Gated	% Total	Mean
All	706	100.00	4.71	92.23

Figure 3.24.5 B7.2 expression in Concanavalin A stimulated spleens

a. Tg -/-; **b.** Tg -/+; **c.** Tg+/+

remain essentially similar, and a distinct increase in B7.2 expression levels is observed **(figure 3.24.5)**.

This data indicates a loss in the T cell compartment on culturing transgenic spleen cells in the presence of Con A, as well as subsequent changes in the phenotype of the T cells. B7.2 expression levels in the Tg-/-, Tg-/+ and Tg+/+ vary, with a significant increase associated with transgene expression. Conversely, expression levels of IL2R decrease in Tg-/+ and Tg+/+ compared with the Tg-/- control.

3.3.11 Presentational ability of T cell localised MHC class II.

Lymph nodes from Tg-/-, Tg-/+ and Tg+/+ mice were isolated and purified in a one-step procedure using sheep anti-mouse IgG dynabeads (Dynal) **(section 2.30)**. This isolation method typically yielded T cells of >92% purity, as determined by FACS analysis **(figure 3.25b)**. A control Con A assay was also set up to ascertain the purity of the T cells with regard to their functionality. After 48 hours stimulation a proliferation assay was performed and **figure 3.25c** shows the results obtained. Unpurified lymph node cells gave equivalent counts, however, after the purification step all three groups showed increased proliferation in response to Con A with the Tg-/- cells giving the highest counts. This response increased further on addition of Mitomycin C (Sigma) treated spleen cells, indicating that the purification procedure has not affected the viability of the T cells. The Con A results suggest that despite the purity of the T cells being >92% enough viable APCs remain to elicit a strong proliferative response to mitogens.

For the assay itself, Mitomycin C treated, purified T cells were co-cultured with the T-T hybridoma MF2.2D9 (kind gift of Dr. Ken Rock) which recognises the ovalbumin peptide 258-276. T cell numbers were titrated against peptide concentration and co-cultured with $5x10^4$ MF2.2D9 hybridoma cells /well. After 24 hours an IL-2 assay was performed on the supernatants **(section 2.33)**. The results obtained are seen in **figure 3.25a**. Titration of cell number produced a very similar overall picture. As expected from the Con A control assay, **(figure 3.25c)** the background counts were relatively high which was due to the presence of <8% APCs in the purified T cell population. Control spleen cells consistently gave the highest read outs at ~70000 cpm, with the Tg+/+ T cells giving only slightly lower counts. Tg-/+ T cells are also able to present the peptide efficiently, but again at a slightly lower level than that seen for Tg+/+.

3.3.12 Comparative ability of Tg-/-, Tg-/+ and Tg+/+ CD4+ T cells to develop antigen-specific responses *in vivo*.

Tg-/-, Tg-/+ and Tg+/+ mice were immunised subcutaneously with 20 nmoles of the *Der p* I peptide 110-131 in CFA. After 7 days local lymph nodes were removed

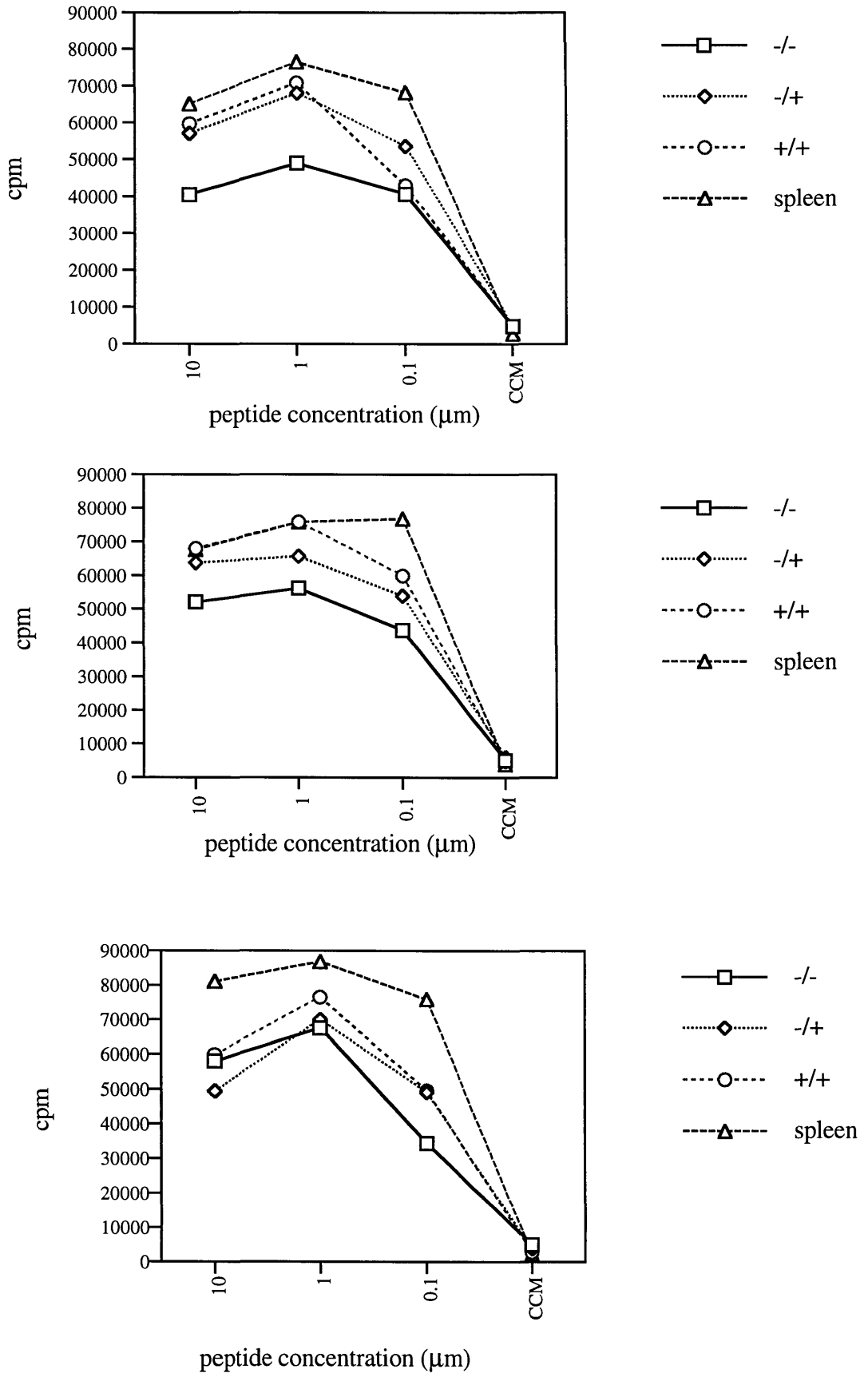
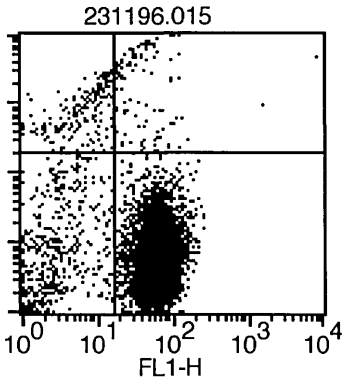


Figure 3.25a. Dose response curves showing the ability of a) $6x10^5$ b) $4x10^5$ c) $2x10^5$ transgenic and non-transgenic T cells to present D65 peptide to the hybridoma MF2 2D9.

a.

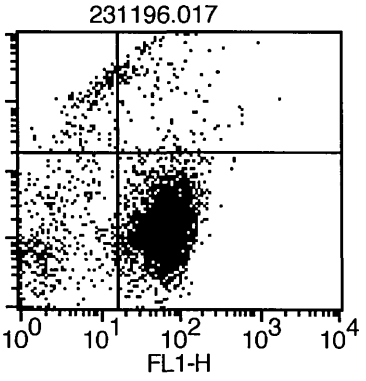


Quadrant Statistics

File: 231196.015 Gate: No Gate
Gated Events: 10000 Total Events: 10000
Quad Location: 16, 195

Quad	Events	% Gated	% Total
UL	199	1.99	1.99
UR	129	1.29	1.29
LL	489	4.89	4.89
LR	9183	91.83	91.83

b.

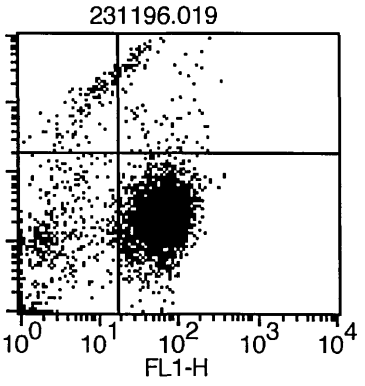


Quadrant Statistics

File: 231196.017 Gate: No Gate
Gated Events: 10000 Total Events: 10000
Quad Location: 16, 195

Quad	Events	% Gated	% Total
UL	145	1.45	1.45
UR	156	1.56	1.56
LL	481	4.81	4.81
LR	9218	92.18	92.18

c.



Quadrant Statistics

File: 231196.019 Gate: No Gate
Gated Events: 10000 Total Events: 10000
Quad Location: 16, 195

Quad	Events	% Gated	% Total
UL	122	1.22	1.22
UR	122	1.22	1.22
LL	494	4.94	4.94
LR	9262	92.62	92.62

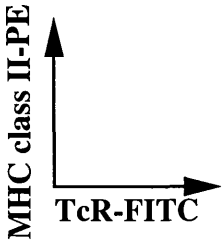
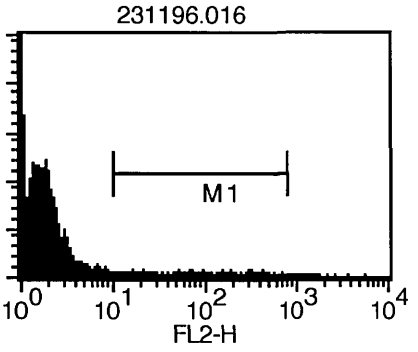


Figure 3.25b FACS analysis indicating purity of dynabead isolated lymph-node derived T-cells

a. Tg -/-; **b.** Tg -/+; **c.** Tg+/+ (TcR and M5/114 stained)
d. Tg -/-; **e.** Tg -/+; **f.** Tg+/+ (B220 stained)

d.

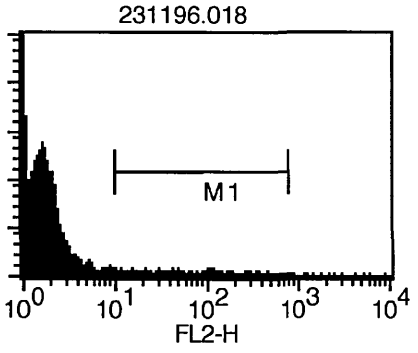


Histogram Statistics

File: 231196.016 Gate: No Gate
Gated Events: 10000 Total Events: 10000

Marker	Events	% Gated	% Total
All	10000	100.00	100.00
M1	707	7.07	7.07

e.

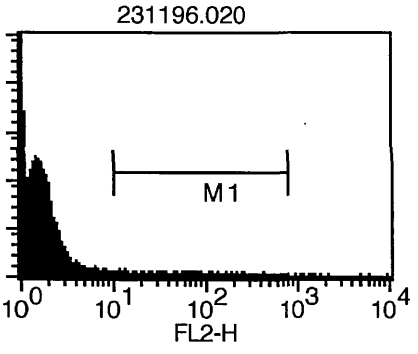


Histogram Statistics

File: 231196.018 Gate: No Gate
Gated Events: 10000 Total Events: 10000

Marker	Events	% Gated	% Total
All	10000	100.00	100.00
M1	601	6.01	6.01

f.



Histogram Statistics

File: 231196.020 Gate: No Gate
Gated Events: 10000 Total Events: 10000

Marker	Events	% Gated	% Total
All	10000	100.00	100.00
M1	479	4.79	4.79

Figure 3.25b continued

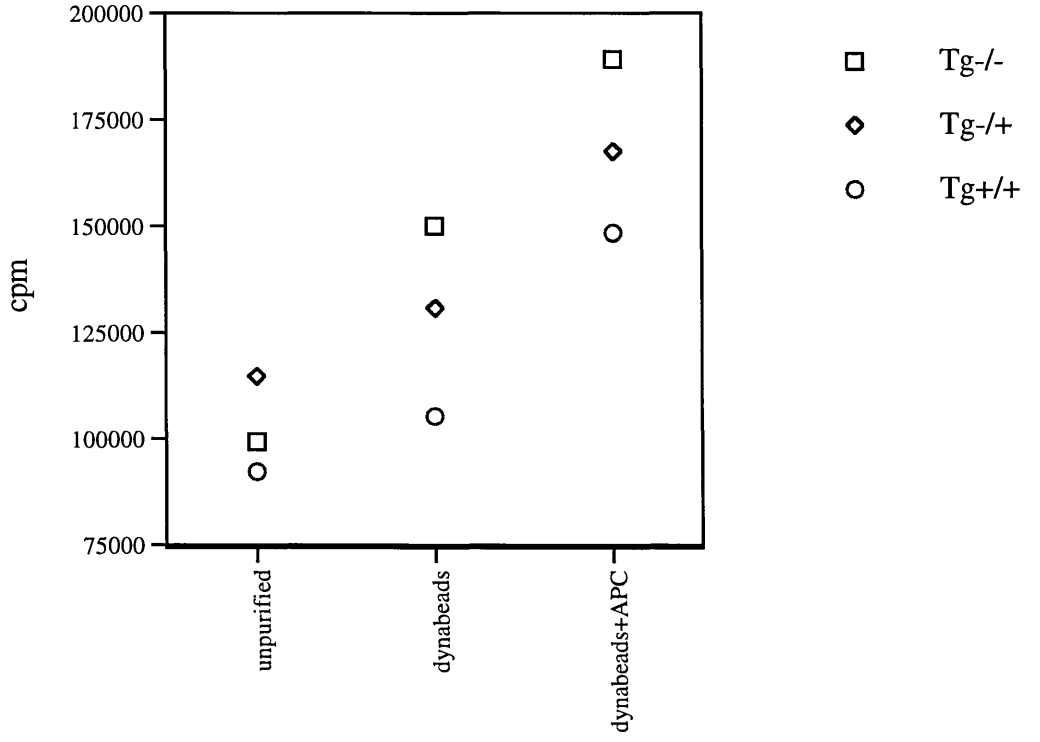


Figure 3.25c. Concanavalin A stimulation of purified lymph node derived T cells from Tg-/, Tg-/+ and Tg+/+ mice.

Key: Tg, transgenic mouse

and standard proliferation and IL-2 assays were set up **(sections 2.32 and 2.33)**. IL-2 production was measured after 24 hours and proliferative responses at 72 hours. The results obtained are seen in **figure 3.26**. Proliferative responses indicate that the Tg-/- mice give much higher responses compared with the transgenics except for one Tg+/+ mouse that gave responses equivalent to the Tg-/- mice. At the highest concentration of peptide (40μm) responses from the transgenic mice were approximately half that seen in the Tg-/- mice (~150,000cpm). By 0.4μm responses of the transgenic mice had reached almost background levels whereas the Tg-/- mice were still proliferating at relatively high levels of ~50,000cpm **(figure 3.26a)**.

The results of the IL-2 production assay for these mice were inconclusive. **Figure 3.26b** shows that only one mouse (Tg-/-) produced significant levels of IL-2, with the Tg+/+ mouse that had displayed similar prolific activity to both the Tg-/- mice producing a small amount that fell to background levels very rapidly (at 0.13μm).

These results show that the transgenic T cells do not proliferate so vigorously to peptide *in vitro* compared with their non-transgenic littermates, and that the responses observed in the transgenic mice titrate down to background levels much more rapidly than is seen in the non-transgenics. IL-2 production is seen in only one mouse, a Tg-/-, although one Tg+/+ mouse produces some IL-2 but only at the highest peptide concentrations. Taking into account the work by Mannie *et al.* and Lamb *et al.* (Lamb JR and Feldman M 1984, Lamb JR *et al.* 1983, Mannie MD *et al.* 1996) this preliminary data may infer that the transgenic T cells are somehow acting to downregulate the response to the *Der p* I peptide. This downregulation manifests itself by way of a decreased proliferative response as well as an almost total abrogation in IL-2 production.

3.3.13 Comparative ability of Tg-/- and Tg-/+ CD8+ T cells to develop cytolytic capacity.

Female responses against the male HY antigen were used as a measure of CD8+ T cells to act in a cytolytic capacity. On day 0 female Tg-/- and Tg-/+ mice were each given intraperitoneal immunisations of 10^7 male spleen cells. On day 17 a JAM assay was performed to assess CTL activity **(section 2.34)**. The results obtained are seen in **figure 3.27**. Differences are observed between individual mice, but these differences appear to be random with both groups displaying equivalent cytolytic anti-HY activity **(figure 3.27a)**. As a positive control alloantigenic responses were assayed using the P815 (I-A^d) T cell line as the target population **(figure 3.27b)**. The results obtained suggest that once again the transgenic and non-transgenic mice give similar responses and that differences observed between individual mice represent only random fluctuation patterns with no distinct inter-group variations seen.

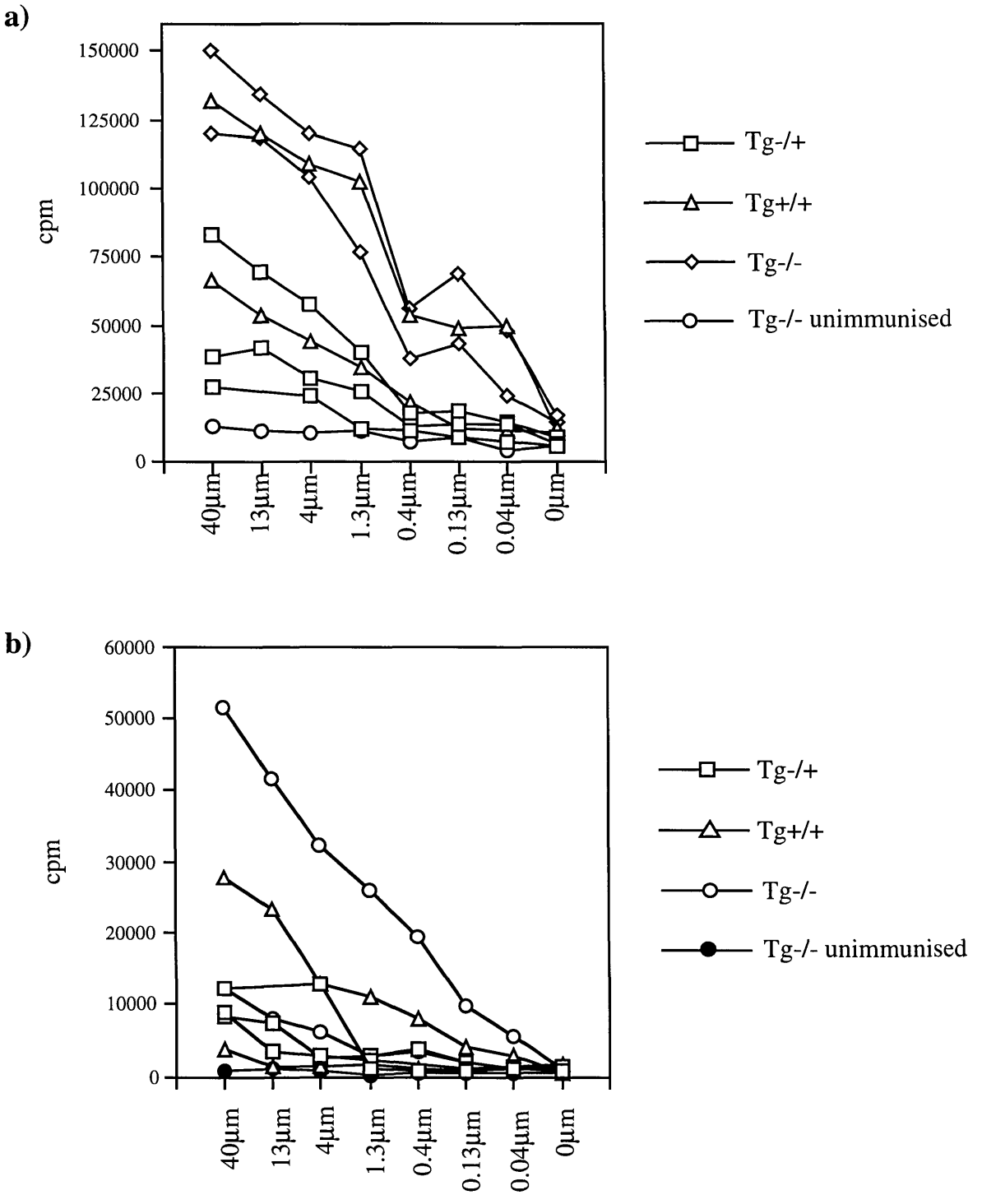


Figure 3.26. Lymph node recall responses in transgenic and non-transgenic mice to the Der pI peptide 110-131

a) proliferative responses; b) IL-2 production.

Each line is representative of one mouse, with different symbols indicating different transgenic status (see Key)

Tg, transgenic mouse

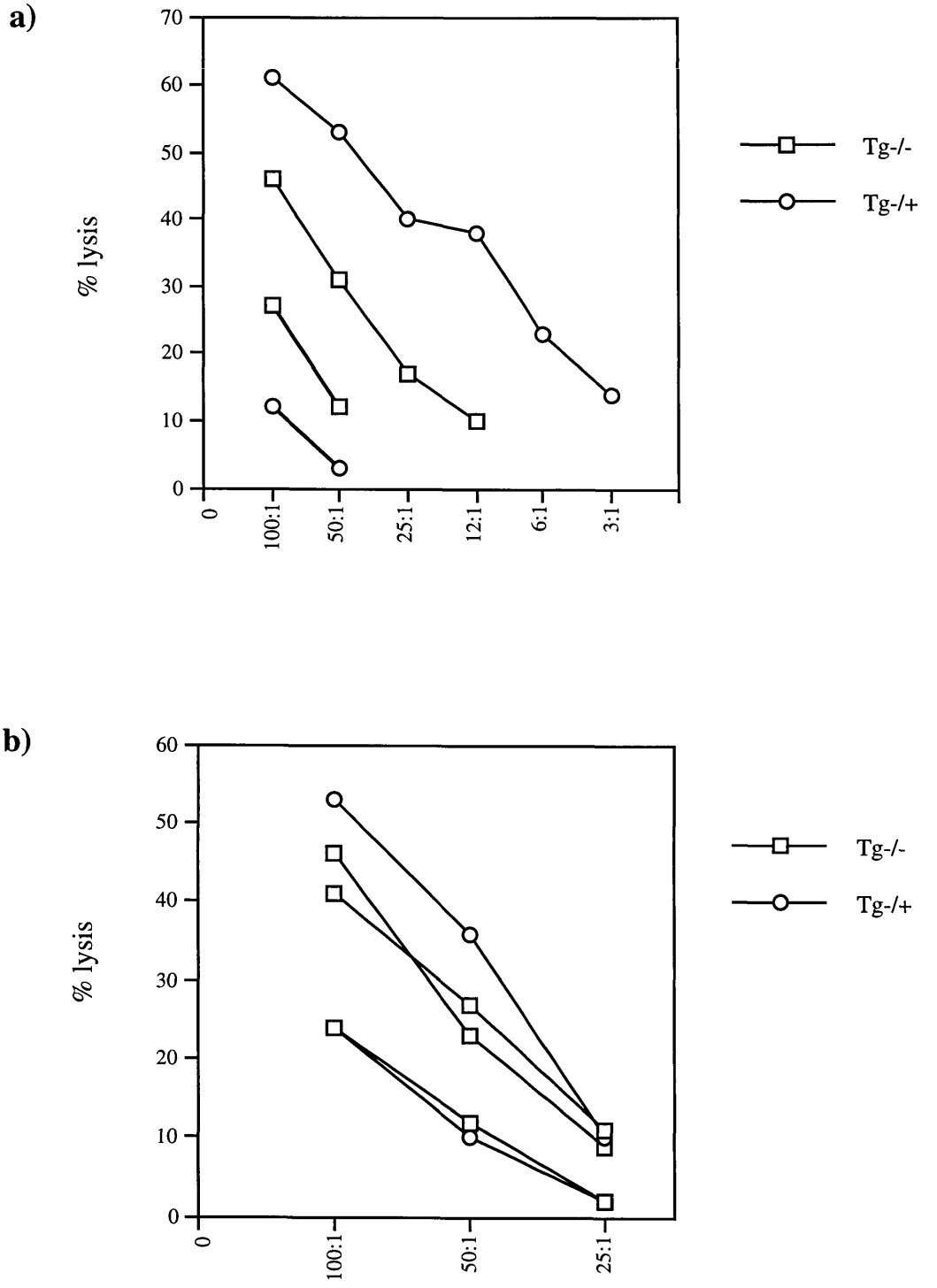


Figure 3.27. CTL responses in Tg-/- and Tg-/+ mice in response to

a) HY antigen
b) BALB/c.

3.3.14 Ability of Tg-/- and Tg-/+ mice to elicit antibody responses characteristic of a Th1 or a Th2 response.

Mice were immunised according to protocols designed to generate either a Th1- or a Th2-type response (**section 2.35**). Tail blood was taken from the mice on day 0 (preimmune antibody levels) and after different boosting regimes the mice were bled again and specific antibody levels to *Der p* I (Th2) and the *Der p* I peptide 110-131 (Th1) were measured by ELISA. **Figure 3.28** shows the results obtained. Both the transgenic and non-transgenic mice primed to elicit a Th2 response gave similar antibody profiles. Both groups gave high titers of the antibody associated with Th2 responses IgG_1, with IgG_{2a} titers remaining at background levels. Mice immunised to elicit a Th1-like response also produced antibodies at similar levels. Unlike the Th2-priming protocol the Th1-priming method generates antibodies associated with both types of response. Therefore in addition to eliciting high titers of the Th1-associated antibody IgG_{2a}, significant levels of IgG_1 were also observed.

Therefore it appears that the transgenic mice do not differ in their ability to generate antibody responses just as they were shown not to differ in their ability to elicit cytolytic responses compared with their non-transgenic littermates (**section 3.3.13**).

3.3.15 Modulation of an ongoing *Der p* I specific immune response on administration of an immunodominant determinant derived from *Der p* I (residues 110-131) via the intranasal route.

The observation that inhalation of certain peptides can result in downregulation of ongoing immune responses (Hoyne GF *et al.* 1993) led to the continuation of the experiment set up in **section 3.3.14**. The Tg-/- and Tg-/+ mice were divided into two groups. One group was given 200μg of the *Der p* I peptide 110-131 in PBS via the intranasal (i.n.) route, the second group acted as a control receiving PBS alone i.n. 7 days later the mice were tail bled and *Der p* I specific antibody levels determined by ELISA. The results obtained are seen in **figure 3.29**. As was observed in **figure 3.28b** no IgG_{2a} antibodies were detected in mice immunised via a Th2-priming protocol and subsequently given i.n. 110-131 peptide or PBS alone (**figure 3.29b**). IgG_1 titers indicate that modulation of the ongoing response is variable in the non-transgenic mice, with one mouse showing a downregulation on 110-131 treatment and another continuing to give relatively high IgG_1 titers. In contrast both the transgenic mice that received the 110-131 peptide downregulated their responses with regard to IgG_1 levels, with the immune sera still giving relatively high IgG_1 titers (**figure 3.29a**).

The observation that IgG_{2a} titers in Th1-primed Tg-/- mice are almost down to background levels indicates that the Th1 response has not been sustained, therefore i.n. administration of the 110-131 peptide has had no effect with regard to shutting off the

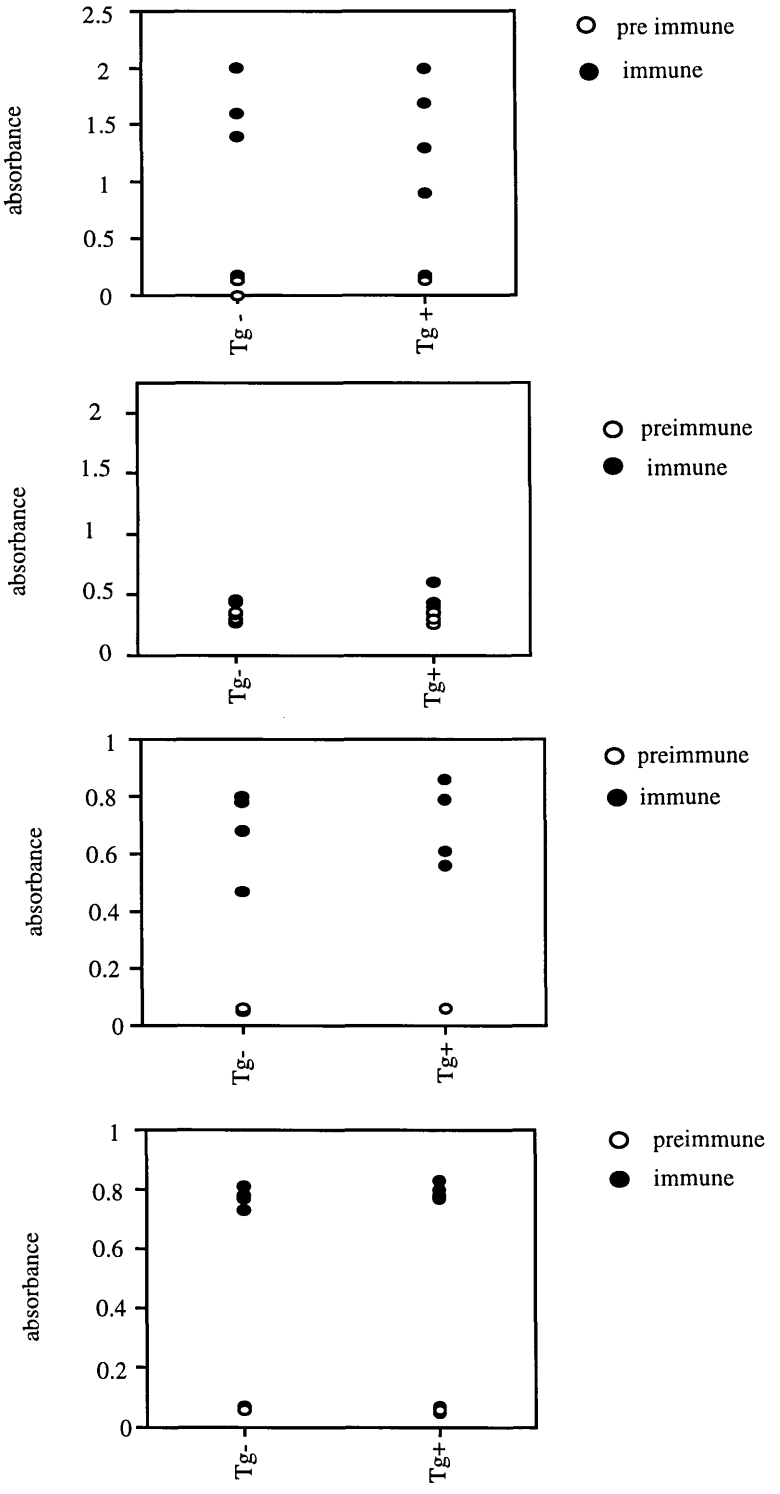


Figure 3.28. ***Der p*** **I specific antibody levels in Tg-/- and Tg-/+ mice after immunisation designed to elicit either a Th2- (a) and (b) or Th1-type (c) and (d) response:**

(a) IgG1, **(b)** IgG2a, **(c)** IgG2a and **(d)** IgG1.

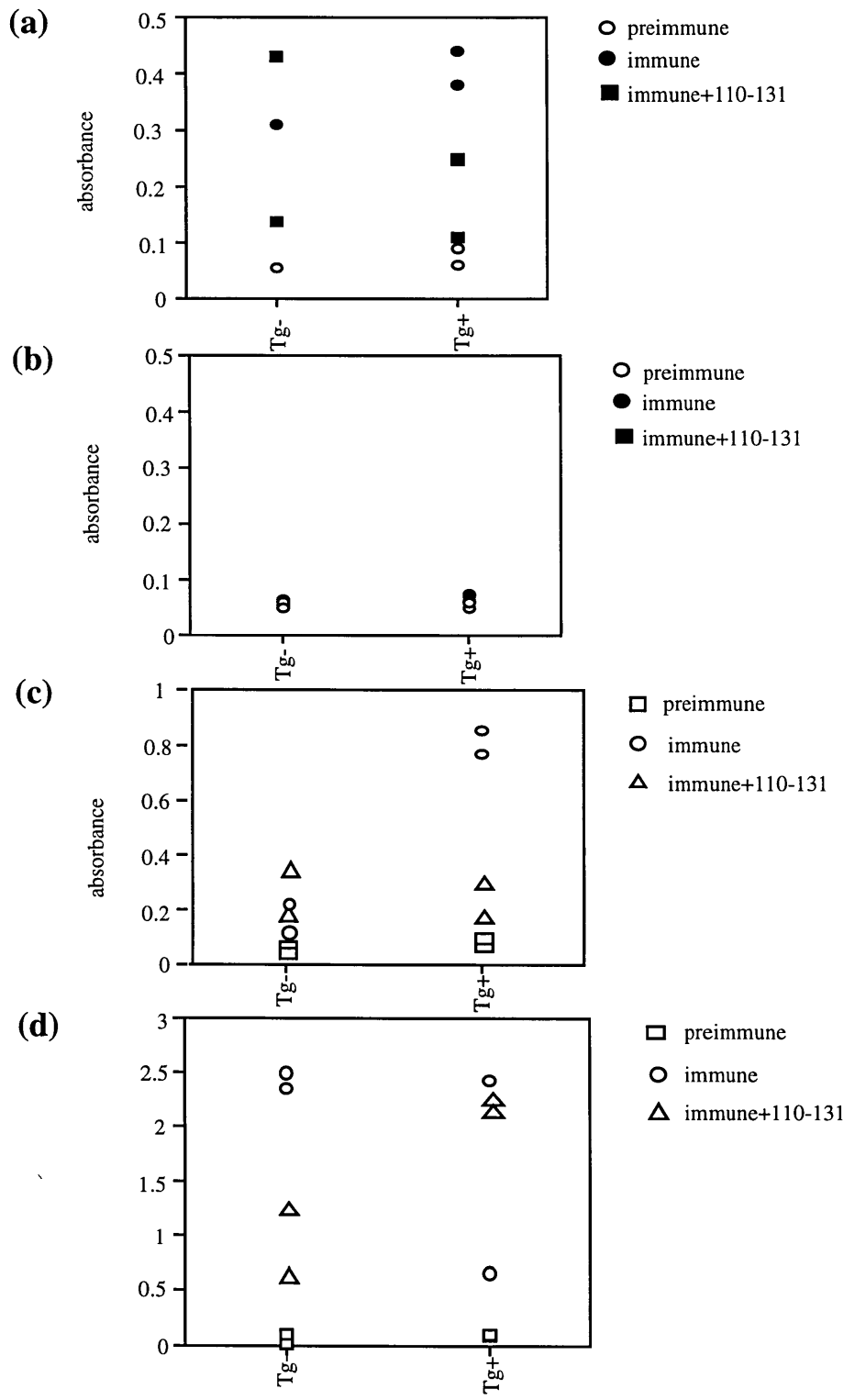


Figure 3.29. ***Der p*** **I specific antibody levels in Tg-/- and Tg-/+ mice after immunisation designed to elicit either a Th2- (a) and (b) or Th1-type (c) and (d) response and subsequently receiving 200μg** ***Der p*** **I peptide 110-131 intranasally**

(a) IgG1, **(b)** IgG2a, **(c)** IgG2a and **(d)** IgG1.

response in these mice. However, the Tg-/+ mice are not only able to sustain a Th1 antibody response but on inhalation of the 110-131 peptide they are able to shut down this response (**figure 3.29c**). IgG_1 levels in these mice are also affected. Interestingly only the Tg-/- mice seem able to partially downregulate IgG_1 levels on peptide inhalation, with Tg-/+ mice showing sustained high antibody titers (**figure 3.29d**).

3.3.16 Responses of Tg-/- and Tg-/+ on intranasal immunisation with the *Der p* I peptide 110-131 followed by subcutaneous immunisation of *Der p* I 110-131.

Mice were given either 200µg of the *Der p* I peptide 110-131 in PBS or PBS alone intranasally. 14 days later the mice were given a subcutaneous immunisation of 10 nmoles *Der p* I 110-131 in CFA. 7 days later the local lymph nodes (periaortic) were removed and recall responses were assayed. **Figure 3.30** shows the results obtained. All the immunised mice elicited strong proliferative responses giving counts in the region of 80000cpm. Some variation was seen in the mice that had received the 110-131 peptide. One Tg-/- mouse out of three exhibited downregulated its responses, with two Tg-/+ showing a large decrease in responsiveness whilst a third was seen to downregulate its responses, but to a lesser extent (**figure 3.30a**).

IL-2 was only produced in the Tg-/- mice that had not received intranasal peptide. Of the Tg-/+ mice that had not received the peptide only one produced IL-2, and the amount was small compared with that seen for the Tg-/- mice. All mice, whether Tg-/- or Tg-/+, that were treated with intranasal peptide produced very little IL-2 (**figure 3.30b**).

These results suggest that immune responses from Tg-/- mice can be downregulated with regards to IL-2 production if the mice are initially treated with intranasal peptide. It does seem, however that the cells still maintain their ability to proliferate. The proliferative abilities of the Tg-/+ mice, however, are not conclusive. Of the five mice initially given intranasal peptide, two gave strong proliferative responses, two showed greatly downregulated responses with a fifth mouse showing some decrease in its response. All three mice treated with PBS showed strong proliferative responses. The IL-2 data, however, suggests that regardless of whether the mice were treated with intranasal peptide or not the transgene seems to confer these cells with the ability to inhibit IL-2 production.

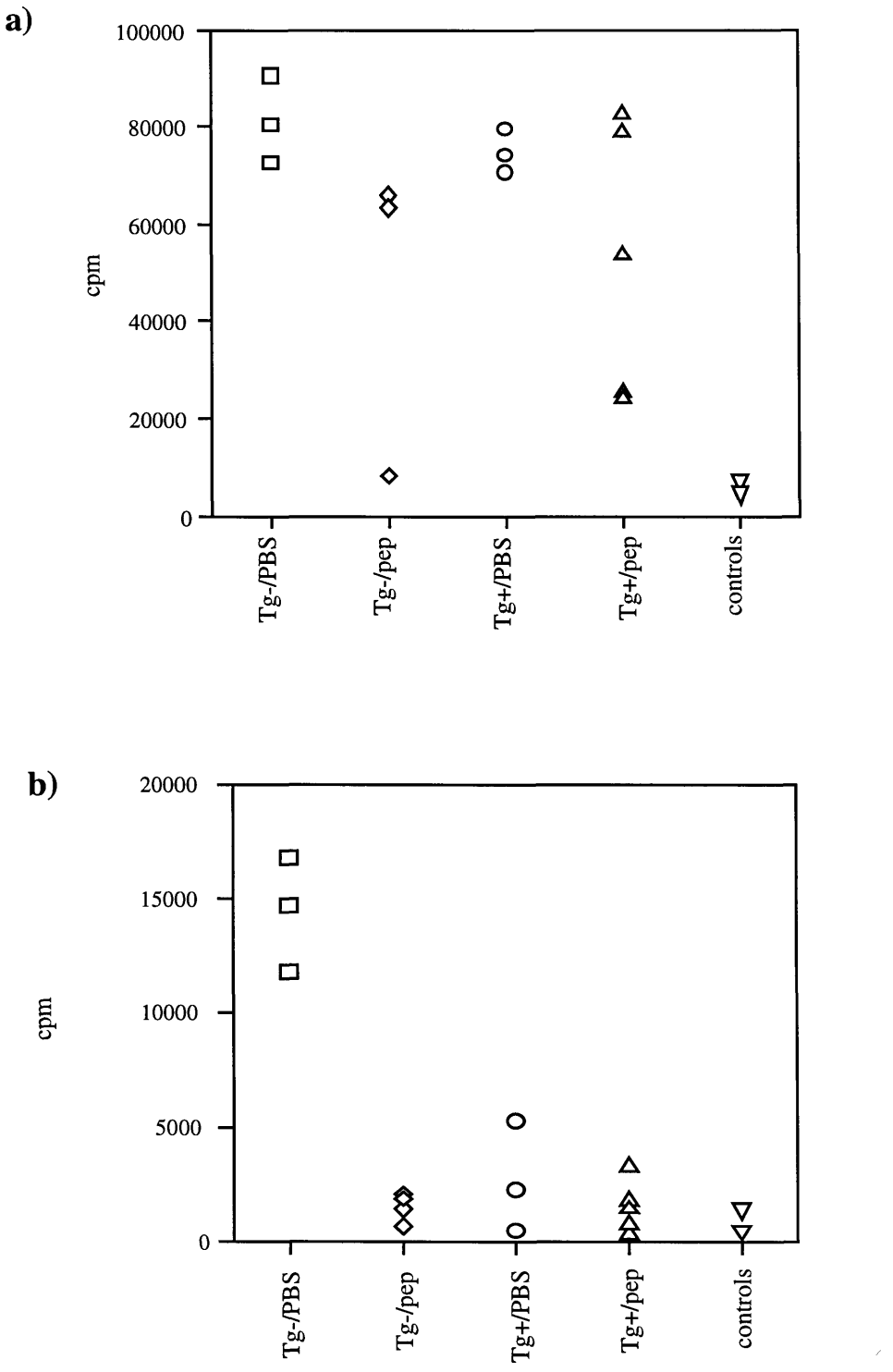


Figure 3.30. Lymph node responses to 1μm *Der p* I peptide 110-131 after intranasal immunisation with 200μg 110-131 followed by subcutaneous immunisation with 10nmoles 110-131 in CFA:

a) proliferative responses
b) IL-2 production.

CHAPTER 4
Discussion

The results presented in this thesis describe the generation and characterisation of a mouse that expresses the transgene for MHC class II on its T cells. The aim being to partially 'humanise' the mouse and, thereby, create a model to allow the potential roles of T cell expressed MHC class II in immune regulation to be determined. As discussed in **section 3.1.4** several groups have generated similar mice using elements of HLA class II, under the control of their endogenous transcriptional elements (Altmann DM *et al.* 1995, Neeno T *et al.* 1996, Yeung RSM *et al.* 1994). Although these models also represent 'humanised' mice, they differ in one fundamental way. The aim of all these groups can be summarised as being to recreate elements of the human immune system within the mouse in order to study responses restricted by those particular HLA class II gene products that previously had only been possible *in vitro*. The aim of the mouse that I generated was to investigate specifically the *in vivo* role of T cell expressed MHC class II with regard to immune regulation.

In vitro human studies gave the first indication that T cell expressed HLA may play a role in immune responses. Lamb *et al.* demonstrated the ability of human T cell clones to present peptide antigen to each other and that this interaction was HLA restricted (Lamb JR and Feldman M 1984, Lamb JR *et al.* 1983). Since then the possible function of T cell expressed MHC class II has come under the scrutiny of many groups, but controversy concerning its role still remains. Most of the published studies argue that the function of T cell expressed MHC class II is one of negative immune regulation. These groups have shown that on restimulation of T cell clones in the presence or absence of professional APCs the T cells become anergised (LaSalle JM and Hafler DA 1994, O'Hehir RE *et al.* 1991). However, Lanzavecchia *et al.* demonstrated the ability of T cell clones derived from HIV+ individuals to present antigens such as gp120 to each other in an MHC class II restricted manner resulting in a potent proliferative response (Lanzavecchia A *et al.* 1988). The majority of the studies looking at human T cell clones do suggest, however, that anergy induction is the most common outcome when peptide or superantigen is presented to T cells by T cell expressed MHC class II. One of the drawbacks of these studies is that they all use T cell clones. T cell clones, although useful, represent an unusual cell type more similar to a memory cell, than a naive T cell. Memory T cells have been reported to require less stringent stimuli to bring about activation compared with naive T cells. It is also questionable whether extrapolating data derived from long-term T cell clones to predict the behaviour of T cells *in vivo* is entirely erudite. A more physiological approach came

from Satyaraj *et al.* Freshly isolated CD4+ T cells from a healthy donor were stimulated using anti-CD3. They were then irradiated and used as stimulator APCs in an alloresponse in which purified CD4+ T cells from a second donor (who differed at multiple HLA loci) were used as responders. No proliferative responses were observed in cultures in which activated T cells were used as stimulators. This study therefore is more suggestive that T-T encounters *in vivo* result in the induction of nonresponsiveness compared with the studies looking at T cell clones (Satyaraj E *et al.* 1994).

The discovery that a population of rat T cells express MHC class II on activation, presented the first opportunity to study its function *in vivo*. However, although the limited studies to date have provided useful information on the function of these T cells in a polyclonal situation (discussed in **section 3.1.1**), there remains one main drawback (Mannie MD *et al.* 1996, St. Louis JM *et al.* 1994). As mentioned earlier, only a small subset (~20%) of the activated T cells express MHC class II therefore its role in the rat may differ from that in the human where, upon activation, 100% HLA expression is observed.

A progression in these studies can thus be seen. T cell clones providing the first glimpses of a role for T cell expressed MHC class II, with allogeneic studies using freshly isolated T cells backing up the observations that T-T presentation results in nonresponsiveness. *In vivo* studies looking at rat T cell expressed MHC class II giving further credence to this work. The reasons, and therefore the advantages, for creating the T cell expressing MHC class II transgenic mouse thus become clear. Not only does this provide an *in vivo* model, thereby enabling polyclonal responses to be studied, but all the T cells express MHC class II therefore it should reflect the human *in vivo* situation more accurately than the rat.

Derivation of the IIIAα/IIIAβ transgenic line initially involved cloning the I-Aα and I-Aβ chains into the pTexIIIa expression vector. Once this had been achieved prior to microinjection of the DNA, the constructs were tested to determine that successful cloning had occurred. Initial restriction enzyme digest analysis of the constructs resulted in the appearance of bands of the size expected if cloning had occurred (**section 3.2.3**). Subsequent sequencing analysis revealed that the cloning had indeed been successful, no restriction enzyme sites having been lost, no addition or deletion of bases being seen and no PCR errors observed (**section 3.2.4**). However, the transfection studies, unexpectedly, yielded no positive results with the reasons for the failure to obtain transgene expression in this system remaining elusive. Southern blot analysis of the human T cell line J6, that had been stably transfected with both the IIIAα/IIIAβ expression cassettes, verified that the IIIAα cassette had integrated into the genome and as both cassettes were linearised with the same restriction enzyme (*Not* I), it was highly likely that the IIIAβ cassette had integrated in tandem (**section 3.2.5**).

Staining of this stably transfected clone with the anti-MHC class II antibody M5/114 gave the surprising result that no detectable membrane-associated MHC class II was observed (data not shown). Transient transfections of the IIIAα/IIIAβ cassettes into J6 or the murine T cell line EL4 also gave the same result **(section 3.2.7)**, with subsequent Northern blot analysis of the B12 stable clone demonstrating that this was due to a lack of IIIAα/IIIAβ mRNA transcription **(section 3.2.9)**.

The reasons for this lack of expression of the IIIAα/IIIAβ construct in these transfection systems are enigmatic. The pTexII vector has been used successfully by other groups in similar transfection systems to drive expression of other heterologous genes (Dr. M. Owen, personal communication). Control β-galactosidase transfections demonstrated that there was not an inherent failure in the transfection system employed itself **(section 3.2.7)**, and lack of expression in both transient and stable transfection systems argues against such trivial reasons as linearisation of the DNA at an inappropriate place or degradation of the transfected DNA. The knowledge that transgenic mice expressing these constructs appropriately (i.e. expressing T cell localised MHC class II) have been derived adds further mystery to these results. Two possible explanations concerning the failure of these transfections remain. One concerns the nature of the cells involved. Both J6 and EL4 are transformed cell lines, they may therefore influence expression of transfected genes in a different way to genes introduced into fertilised oocytes by microinjection. As the pTexIIIa expression vector is known to contain all the regions required to drive tissue-specific, copy-number dependent and position-independent transgene expression, it could be conceived that the J6 and EL4 cell lines may in some way be inhibiting gene expression by some unknown mechanism. An alternative explanation being that the removal of the *Cla* I-*Bgl* II fragment from the pTexII vector is somehow affecting expression of the IIIAα/IIIAβ in the transfection system, but has no discernible effect on transgenic expression **(section 3.2.1)**.

Southern blotting and PCR screening of the IIIAα/IIIAβ transgenic mice confirmed the presence of both the I-Aα and I-Aβ chains integrated into the genome **(sections 3.3.1 and .2)**. From the Southern blot it is apparent that many more copies of the I-Aα transgene are present within the genome compared to endogenous I-Aα (~100 copies of the transgene). Despite the presence of multiple copies of the gene, MHC class II levels on T cells derived from mouse tail blood are approximately one log lower than is observed on B cells **(section 3.3.4)**. This could be due to the pTexIIIa expression vector being unable to drive I-Aα and I-Aβ gene expression as efficiently as endogenous MHC class II transcriptional elements would. It may also be due to an inability of the T cell to support the system of MHC class II molecule synthesis and progression through the cell as efficiently as a professional APC.

Phenotypic studies of the IIIAα/IIIAβ transgenic mice uncovered changes in expression levels of certain cell surface molecules in an age-related manner. Analysis of T cells derived from lymph nodes of 4 week old mice showed that levels of all cell surface markers assayed was broadly similar in transgenic mice compared with non-transgenic littermates, with the exception of IL2R expression which was slightly elevated in the transgenic mice (**section 3.3.5**). This elevation was more distinct in lymph node T cells derived from 12 week old mice, suggesting that as the mice get older there is a change in the phenotype of the peripheral T cells. Additionally a rise in the number of peripheral CD4+ and CD8+ T cells was observed in the lymph nodes of 12 week old mice, with no change seen in the 4 week old mice compared with their non-transgenic littermates (**section 3.3.6 and .7**). These T cells expressed the αβTcR. Whether γδ T cells contribute to this increase in T cell numbers has not been determined. These results therefore suggest that for some reason as the mouse ages an increase is observed in the total number of peripheral T cells and that these T cells are altered phenotypically compared with their non-transgenic littermates. Increase in IL2R expression is observed on both activation of T cells and on anergy induction, therefore the ability of these T cells to participate in an immune response was of particular interest.

Parallels can be drawn between the resting phenotype of these transgenic mice with the studies of superantigen-induced peripheral anergy performed by Rammensee *et al.* and Bhandoola *et al.* Rammensee *et al.* were the first to demonstrate that immunisation of Mls-1^a specific T cells into adult Mls-1^b mice resulted in specific unresponsiveness to Mls-1^a (Rammensee H-G *et al.* 1989). It was also observed that these anergic T cells displayed levels of TcR and CD4 similar to normal T cells. On *in vitro* restimulation of these cells with Mls-1^a, IL2R was expressed but no IL-2 produced. Despite this group claiming that the anergy they observed was exclusively due to induction of nonresponsiveness as opposed to clonal deletion, later studies demonstrated that this hypothesis did not represent the full picture. The *in vivo* response to superantigens, such as Mls-1^a involves an initial clonal expansion of the antigen-reactive CD4+ T cells, followed by peripheral deletion of some of these T cells. A cohort of Vβ+ cells do however remain, and it is these cells that appear refractory to restimulation in subsequent *in vitro* proliferation assays (Bhandoola A *et al.* 1993).

The phenotypic observations made by Rammensee *et al.* therefore clearly mirror those seen in the resting lymph node T cells of the IIIAα/IIIAβ transgenic mice. The inability of the restimulated T cells to produce IL-2 thus also reflects the studies looking at recall responses of the IIIAα/IIIAβ mice to *Der p* I (**sections 3.3.12 and .16**). The Rammensee study also demonstrated that addition of exogenous IL-2 to the restimulation cultures only partially reversed the proliferative block in these T cells.

This observation contrasting with the human *in vitro* studies in which anergy induced in T cell clones was fully reversible on addition of exogenous IL-2 (Essery G *et al.* 1988). This, again is of interest in our studies, as repeated attempts at deriving a T cell line from the IIIAα/IIIAβ mice consistently failed, addition of exogenous IL-2 to try to 'rescue' the unresponsive cells having no effect (data not shown). The work of Bhandoola *et al.* also demonstrated that on restimulation of the superantigen-anergised T cells, an altered tyrosine phosphorylation pattern was observed that has been recently confirmed as being indicative of the anergic state (Bhandoola A *et al.* 1993, Sundstedt A *et al.* 1996). This observation therefore evokes the question as to whether the IIIAα/IIIAβ mice also display such an altered tyrosine phosphorylation pattern on restimulation.

The phenotype of the thymocytes of the transgenic mice also showed age-related distinctions. As the transgenic mice became older a population of $CD4^{hi}CD8^{int}$ cells appeared, this population first seen in 8 week old mice with 12 week old mice exhibiting a more dramatic shift **(section 3.3.8)**. This T cell population could be representative of a set of thymocytes that have escaped negative selection, this observation implying that they may exhibit autoreactivity. Alternatively, they may have interacted with the MHC class II present on other T cells, as opposed to MHC class II on the thymic epithelium and this interaction may result in cell anergy as opposed to apoptosis.

Studies looking at the thymic development of αβ T cells have established that the process of positive and negative selection occur once the thymocyte has reached the DP stage, indicative that it has successfully rearranged its α and β TcR chains (Zúñiga-Pflücker JC and Lenardo MJ 1996). In a normal thymus, DP thymocytes are positively selected by interacting with MHC class II expressed on the thymic epithelium. It is proposed that only thymocytes expressing low densities of self-restricted TcR are positively selected. Those recognising self-MHC with too great an affinity, or possibly in a qualitatively different way are deleted (Janeway Jr. CA 1994). Recent studies have provided evidence that T cell apoptosis in the thymus is influenced by interactions between endogenous steroids and signalling via the TcR. Zacharchuk *et al.* demonstrated that simultaneous signalling via the TcR and exposure to steroids resulted in cell survival (Zacharchuk CM *et al.* 1990). In this scenario thymic deletion is hypothesised to be a result of this steroid/TcR antagonism. The gene *nur77* has been implicated in mediating apoptosis resulting from negative selection (Calnan BJ *et al.* 1995). Until recently it had been a commonly held belief that the role of the thymus in terms of generation of the T cell repertoire was purely to delete any cells reactive to self. A report by Saoudi *et al.* challenges this theory. They have found that they are able to reverse induction of autoimmune diabetes in a rat model on immunising low numbers ($6x10^5$) of CD4+CD8- thymocytes into a thymectomised, lymphopenic animal. They

conclude that these thymocytes had been shaped by intrathymic selection events to act as a cell population that regulates the autoimmune potential of peripheral T cells that have either not undergone thymic deletion or been rendered anergic in the periphery (Saoudi A *et al.* 1996). It is known that rat thymocytes express high levels of MHC class II, with 85% of the small DP cells expressing MHC class II (Reizis B *et al.* 1994). It is therefore possible that this regulatory population (which was also shown not to provide high levels of B cell help, compared with peripheral CD4+ T cells) is undergoing positive selection on T cell expressed MHC class II and therefore represents an anergised population, as is hypothesised for the IIIAα/IIIAβ thymocytes. It would therefore be interesting to see whether induction of autoimmunity in syngeneic recipients could be prevented if the $TcR^{int/hi}CD4+CD8^{lo}$ thymocyte population was adoptively transferred.

Positive selection in the thymus is accompanied by CD4/CD8 lineage commitment. As discussed in **section 1.8a**, lineage-committed intermediates have been identified. In the normal thymus they are present at low frequencies (1-5% of total thymocytes) and display activation and maturation markers similar to that of $TcR^{int/hi}$ DP cells, suggesting they have begun the process of positive selection (Guidos CJ 1996). These cells have been phenotypically identified as $TcR^{int/hi}CD4^{lo}$ 8+ and $TcR^{int/hi}CD4+CD8^{lo}$. This phenotype is therefore similar to that seen in the thymus of the IIIAα/IIIAβ, the IIIAα/IIIAβ thymocytes showing an increased number in the $TcR^{int/hi}CD4+CD8^{lo}$ population. If the IIIAα/IIIAβ population did represent the $TcR^{int/hi}CD4+CD8^{lo}$ population it would express markers such as CD69, CD5 and Bcl-2 as well decreased levels of RAG-1 and -2. It would therefore be of interest to look at the cell surface markers expressed by these cells.

In vitro Con A stimulation of spleen cells from IIIAα/IIIAβ mice resulted in a drastic reduction in T cell numbers, with remaining T cells showing increased levels of B7.2 expression and decreased levels of IL2R expression **(section 3.3.9 and .10)**. All other membrane-associated molecules looked at were expressed at levels similar to those found on non-transgenic littermates. The observation that not only do T cell numbers decrease, but those remaining exhibit altered levels of activation-associated molecules such as B7.2 and IL2R suggests that a change in the responsiveness of the T cells is taking place.

A simple explanation may account for the decreased levels of IL2R expression. Although CD4+ T cells have been shown not to produce IL-2 in *in vitro* recall responses to the *Der p* I peptide 110-131, mitogen stimulation, which probably activates the cells independently of the TcR and may therefore bypass the anergic block, may induce IL-2 production. It could therefore be envisaged that the IL-2 binds the

IL2Rs resulting in their internalisation. This may account for the few lymphoblasts that were observed in the cultures.

The observation that B7.1 expression levels remained at similarly low levels in the *in vitro* Con A activated transgenic mouse T cells compared with non-transgenic littermates could have been predicted (data not shown). As the phenotype of these cells was assessed 48 hours following Con A activation, it would be unlikely that expression of the B7.1 molecule would be observed. Normal cells have been found to express low levels of B7.1 48 hours after activation, with maximal levels observed at 72 hours. Furthermore, B7.1 has been reported to be involved in the amplification of an ongoing immune response, studies by Miller *et al.* demonstrating that B7.1 was responsible for the maintenance and progression of murine EAE (Miller SD *et al.* 1995).

Increased levels of B7.2 on Con A treatment of these cells is especially interesting in the light of recent evidence that T cells not only normally express B7.2, but that it seems to be involved in inhibition of T cell responses. A report by Greenfield *et al.* studied EL4 cells transfected with either B7.1 and B7.2. They demonstrated that the B7.1 expressing cells induced T cell costimulation as well as tumour regression, the B7.2 expressing cells failing to induce either response. When they looked at the binding capabilities of both molecules they found that whereas the B7.1 expressing cells bound both CTLA4-Ig and CD28-Ig, the B7.2 transfectants preferentially bound the CTLA4-Ig. Freshly isolated murine T cells which have been shown to constitutively express B7.2 showed similar binding capabilities (Greenfield EA *et al.* 1997). If the prediction that a population of T cells present in the periphery of the IIIAα/IIIAβ mice being anergised is indeed correct, then the increased levels of B7.2 expression on these T cells may reflect a mechanism by which this state of anergy is being maintained.

Despite the persistence of a population of T cells following *in vitro* Con A stimulation, it was evident from microscopic examination that fewer cells were present in the transgenic cultures compared with their non-transgenic littermates. FACS analysis of the remaining cells showing a selective decrease in the T cell compartment (**section 3.3.10**). It was hypothesised that these 'disappearing' T cells were undergoing apoptosis. Induction of T cell death in the periphery has been demonstrated to occur through the interaction of T cell expressed Fas with Fas ligand (FasL). Activated T cells are induced to die in the periphery to prevent continued secretion of potentially harmful amounts of cytokines, thereby maintaining cellular homeostasis. The Fas-FasL pathway of apoptosis has been demonstrated *in vitro* using T cell hybrids that die in response to TcR ligation, as well as *in vivo* using *lpr/lpr* or *gld/gld* mice that are defective in Fas or Fas ligand, respectively (Osborne BA 1996). These mice exhibit severe lymphoproliferative disorders due to their inability to delete activated T cells. This pathway of apoptosis together with apoptosis induced by TNF receptor binding TNF, are reported to be the two mechanisms of apoptosis utilised in the periphery for

removal of activated T cells (Schwartz RH 1996). Whether this mechanism is also used on deletion of the IIIAα/IIIAβ peripheral Con A activated T cells remains to be investigated. Initial studies will have to establish that apoptosis is indeed taking place, if this is the case staining of cells looking for expression of Fas and TNF receptor would also be useful.

It is therefore possible that at least two populations of cells are present in the IIIAα/IIIAβ Con A activated cell cultures. One cell cohort undergoing apoptosis on mitogen activation, a second cohort downregulating its IL2R expression and upregulating B7.2 possibly as a mechanism of maintaining anergy. These two populations may therefore have also been at differing stages of anergy prior to mitogen stimulation, with the cohort that underwent apoptosis representative of the deepest state of tolerance achievable prior to deletion. Such a situation has been described by Arnold *et al.* They propose that the level to which a T cell is tolerised is dependent on such factors as the affinity of the TcR, the presence of accessory molecules, the developmental stage of the T cell when it encounters antigen, the properties of the tissue bearing the antigen and the frequency, length and intensity of the contacts between the T cell and the target antigen. They describe the distinct levels of anergy phenotypically, proposing that coreceptor expression is lost at the initial step of tolerance induction, followed by loss of TcR expression if further tolerogenic signals are received. The final level being apoptosis of the T cell. They also assessed the resistance of these cells to reactivation *in vitro*, thus finding that T cells that had downregulated their coreceptor were more easily activated compared with T cells that had downregulated both their coreceptor and TcR (Arnold B *et al.* 1993).

This model may not reflect the situation in the IIIAα/IIIAβ mice, phenotypic data showing that TcR expression levels were not reduced. However, CD4 and CD8 levels were not determined. Taking into account that the deepest state of anergy according to Arnold *et al.*, prior to cell apoptosis, exhibits downregulated TcR as well as coreceptor, it does seem that this model may not explain the phenotypic observations in the IIIAα/IIIAβ transgenic mouse. A variation of this model has been proposed by Schwartz. He suggests that whereas Arnold *et al* consider anergy as a single stage from which cells can be rescued, with other stages characterised by TcR downmodulation and deletion, anergy itself may have different levels. He proposes that this may be characterised by the degree to which *ras* activation is blocked. A partial block preventing transactivation of AP-1 bound to phorbol ester response elements, whereas a complete block may prevent AP-1 from participating with NF-AT at other sites in the enhancer (Schwartz RH 1996). It would be more difficult to test the possibility that this is occurring in the IIIAα/IIIAβ mice as no phenotypic differences between these levels of anergy have been described.

Experiments performed to assay the presentational ability of the IIIAα/IIIAβ transgenic T cells were described in **section 3.3.11**. Purified transgenic T cells were able to present peptide to the T-T hybridoma MF2.2D9 almost as efficiently as the control spleen cells, with the T cells derived from the homozygous mice being able to present peptide at higher levels than that seen for the heterozygous mice. Although these studies give strong evidence for the T cell expressed MHC class II presenting peptide to the hybridoma, confirmation of this would be derived by performing these experiments in the presence of anti-MHC class II blocking antibodies. The antigen processing abilities of the transgenic T cells could also be tested within this system, and in the light of conflicting reports of the ability of murine MHC class II+ T cells to process and present native antigen in various *in vitro* systems more information would be gained by looking at these transgenic T cells (Chang C-H *et al.* 1995, Tschoetschel U *et al.* 1996).

Analysis of the functional activity of CD4+ and CD8+ T cells in the transgenic mice revealed that the CD8+ T cell compartment of these mice appeared to be unaffected by the presence of the transgene with regard to development of cytolytic function (**section 3.3.13**). CD4+ T cell responses did, however, differ between transgenic mice and their non-transgenic littermates. Proliferation in a recall response to the *Der p* I peptide 110-131 appeared to be slightly lower in the transgenic mice, although comparing this result with the PBS controls from **section 3.3.15**, the significance of this result will only become apparent as more mice are investigated. What was distinctive, however, was the failure to detect IL-2 in the transgenic mice. These mice may be producing levels of IL-2 equivalent to their non-transgenic littermates, however, due to the elevated levels of IL-2R expression on the transgenic T cells, IL-2 levels may become undetectable. Alternatively, these T cells may be producing other cytokines, and if, as suggested, these T cells are anergic they could be producing factors such as IFN-γ and IL-4, both cytokines that are reported to be unaffected by anergy induction (Mueller DL *et al.* 1991).

The transgenic mice exhibited similar abilities to elicit *Der p* I specific antibody responses characteristic of either a Th1 or a Th2 response compared with non-transgenic littermates (**section 3.3.14**). However, the transgenic mice did differ in their capacity to sustain antibody levels and to actively downregulate these responses on administration of peptide via the intranasal (i.n.) route. Six weeks following the final boost the non-transgenic mice that had not been given the *Der p* I peptide i.n. displayed slightly lower levels of IgG1 compared to their transgenic littermates. Only one out of the two non-transgenic mice given the 110-131 peptide i.n. was able to downregulate its responses, whereas both the transgenic mice could do so. A similar situation was observed for the Th1 primed mice, with non-transgenics unable to sustain IgG_{2a} levels

4 weeks after receiving the final boost, and i.n. administration resulting in transgenics downregulating their responses. The high levels of IgG_1 antibody that accompany this priming protocol appeared to be unaffected by peptide inhalation. These results suggest that the IIIAα/IIIAβ mice are able to sustain antibody levels for longer periods of time than conventional mice. They are also able to downregulate them on inhalation of peptide antigen.

The observations regarding the ability of the transgenic and non-transgenic mice to elicit equivalent antibody responses is puzzling in the light of the hypothesis that the transgenic T cells exhibit some form of anergy. Indeed, if the transgenic T cells are determined to exist in an anergic state these findings would be in contrast to published studies which report the inability of tolerised T cells to provide help to B cells regarding antibody production (Holt PG *et al.* 1993, Hoyne GF *et al.* 1993, Schwartz RH 1996). The possibility that distinct transgenic T cell populations may exist at different levels of anergy may provide an answer to these observations. Depending on the level of tolerance the transgenic T cells may exhibit differing abilities to provide B cell help, with only those populations existing in the deepest form(s) of anergy being unable to provide such help.

In contrast to the published studies mentioned above, a recent report by Akdis *et al.* found that following the treatment of patients with whole bee venom for two months allergen-specific tolerance in T cells, as indicated by suppression of T cell proliferation together with abolition of type 1 and type 2 cytokine secretion, was observed. However, serum levels of IgE and IgG_4 were slightly elevated during immunotherapy indicating that somehow B cells were still receiving proliferative signals. Two explanations were proposed. One suggested that tolerance was restricted to CD4+ T cells, thereby enabling CD8+ T cells to provide cytokine help to B cells. Alternatively, this group found that by culturing the tolerised cells *in vitro* with IL-2 or IL-15 type 1 cytokines were secreted, with treatment using IL-4 inducing secretion of type 2 cytokines. These observations were proposed to be indicative of the importance of microenvironmental cytokines in determining the success of immunotherapy (Akdis CA *et al.* 1996). Either of these scenarios could be perceived as occurring in the IIIAα/IIIAβ transgenic mice. The data proposing that the functionality of the CD8+ T cell population in the transgenic mice was unaffected may suggest a role for cytokine production by these CD8+ T cells in providing B cell help.

Finally the ability of i.n. administered peptide to prevent the induction of an immune response was also addressed (**section 3.3.16**). Proliferative responses of the transgenic and non-transgenic animals that had not received i.n. peptide were similar, whereas both groups gave mixed responses on immunisation with i.n. peptide. IL-2 responses were more uniform, with only the non-transgenic mice that had not received the i.n. peptide producing significant quantities of IL-2. These results suggest

that the transgenic T cells are able to proliferate in a similar capacity to non-transgenic T cells, although their ability to remain unresponsive to a subsequent immunogenic challenge is unclear. The suggestion that these T cells are producing cytokines other than IL-2 appears to be highly probable, and this will have to be determined.

These results, yet again, contrast with published data. Studies carried out by other groups using an immunodominant *Fel d* I peptide or the *Der p* I peptide 110-131 propose that subcutaneous immunisation of the *Fel d* I peptide in PBS or i.n. immunisation of the *Der p* I peptide in PBS results in secretion of low levels of IL-2, poor proliferation and the inability to provide cognate help to stimulate specific antibody production (Briner TJ *et al.* 1993, Hoyne GF *et al.* 1993). Why the non-transgenic control mice have not shown similar decreased responses as observed in those mice treated in an identical fashion by Hoyne *et al.* is unclear (Hoyne GF *et al.* 1993). However, if the observation that the IIIAα/IIIAβ mice are more susceptible to downregulation of their cytokine responses compared with their non-transgenic littermates is borne out, it would appear likely that MHC class II restricted T-T presentation of the peptide is playing a role. Further work looking at cytokine and antibody production should provide some answers to these questions.

In summary the experiments discussed present evidence that a mouse expressing T cell localised MHC class II is able to present peptide efficiently to T-T hybridomas. It is able to develop cytolytic responses equivalent to those observed in non-transgenic littermates, and although CD4+ T cell proliferative responses also appear equivalent to those of non-transgenic mice the cytokines produced are altered. Antibody production is equivalent to non-transgenic mice, although their production is sustained over a longer time period. Antibody production can also be downregulated by inhalation of peptide antigen in these transgenic mice.

Phenotypic data suggest that as these mice get older an unusual set of $CD4^{hi}$ $CD8^{int}$ T cells appears in the thymus. This population may represent T cells that have escaped negative selection, instead becoming anergised, due to their interaction with T cell expressed MHC class II as opposed to MHC class II present on the thymic epithelium. Once these T cells enter the periphery they are more susceptible to mitogen-induced apoptosis, as was observed on activation with Con A. Any cells that remain after such treatment displaying a phenotype possibly suggestive of anergy induction (upregulation of B7.2, downregulation of IL2R). This scenario being likened to the multiple levels of tolerance model described by Arnold *et al.* and Schwartz.

These mice therefore present a potentially useful new model of the influence that T cell localised MHC class II may have on immune modulation, and as such should provide a model for human conditions in which T cell expressed MHC class II is believed to play a role, for example in certain allergic diseases. The preliminary data presented suggests that these cells may well be anergised, or may be more susceptible

to anergy in the periphery compared with non-transgenic littermates. Comprehensive studies of the molecular mechanisms involved in the induction of tolerance in this model would clarify the function of T cell expressed HLA *in vivo*. This model could also be used to test the efficacies of possible therapies for diseases thought to involve T cell expressed HLA, such as certain allergic conditions and autoimmune disease.

APPENDIX I

Buffers and solutions used

2xYT medium (per/L):	bacto-yeast extract bacto-tryptone NaCl pH adjusted to 7.0 with 10M NaOH
2xYT Agar:	2xYT medium plus 15g/L bacto-agar Ampicillin as required (50μg/ml final)
Alserver's solution: (Tri-sodium citrate buffer)	Glucose 20.5g Citric acid 0.55g tri-sodiumcitrate-dehydrate 8g NaCl 4.2g make up to 1L in ddH2O, adjust to pH 6.1, filter sterilise through 0.22μm filter
Assay buffer, 2x: (Promega)	200mM sodium phosphate buffer, pH7.3 2mM $MgCl_2$ 100mM β-mercaptoethanol 1.33mg/ml ONPG
C1 buffer: (Qiagen)	320mM Saccharose 5mM $MgCl_2$ 10mM Tris.HCl 1% Triton X-100, pH7.5
Church & Gilberts:	0.5M Na_2HPO_4 0.5M NaH_2PO_4 7% SDS
Denaturing buffer:	10% methanol 10% glacial acetic acid 80% ddH_2O

DEPC-treatment:	Solutions: 1ml DEPC per litre. Incubate overnight with gentle shaking at 37°C. Autoclave and stored in a clean dry place. All solutions handled aseptically with clean gloves.
Enzyme dilution buffer (T7 Sequenase v2.0):	10mM Tris.HCl pH7.5 5mM DTT 0.5mg/ml BSA
Formaldehyde gel running buffer, 5x:	100mM MOPS 25mM sodium acetate 5mM EDTA, pH 8.0
G2 buffer: (Qiagen)	800mM GuHCl 30mM EDTA, pH8.0 30mM Tris HCl, pH8.0 5% Tween-20 0.5% Triton X-100, pH8.0
Klenow buffer, 10x:	500mM Tris.HCL pH7.4 100mM $MgCl_2$ 10mM dithiothreitol (DTT)
Ligase buffer, 10x:	500mM Tris.HCL, pH 7.5 100mM $MgCl_2$ 10mM dATP 10mM DTT
PCR buffer, 10x: (Promega formulation)	500mM Tris.HCL pH9.0 100mM $MgCl_2$
PBS:	137mM NaCl 2.7mM KCl 1.47mM KH_2PO_4 8mM Na_2HPO_4 pH adjusted to 7.4 with conc. HCl

QBT: (Qiagen)	750mM NaCl 50mM MOPS 15% ethanol 0.15% Triton X-100, pH7.0
QC: (Qiagen)	1.0M NaCl 50mM MOPS 15% ethanol, pH7.0
QF: (Qiagen)	1.25M NaCl 50mM Tris HCl 15% ethanol, pH8.5
Sample loading buffer: (6xGLB)	30% glycerol 0.1% bromophenol blue 0.1% xylene cyanol FF
Sequencing gel mix:	210g urea (Analar grade) 100ml 30% acrylamide (Appligene) 100ml 5x TBE Make to 500ml with ddH_2O Filter through a 0.22µM filter and degas
Solution 1:	10mM Tris.HCl, pH 8.0 1mM EDTA, pH 8.0
Solution 2:	0.1M NaOH 0.5% SDS
Solution 3:	60mls 5M potassium acetate 11.5mls glacial acetic acid 28.5mls H_20 Final is 3M with respect to potassium and 5M with respect to acetate.
SSC, 20x:	per litre; 175.3g NaCl 88.2g $Na_3C_6H_2O_5.2H_20$ pH adjusted to 7.0 with 10M NaOH

Sequencing stop solution:	95% formamide 20mM EDTA, pH 8.0 0.05% bromophenol blue 0.05% xylene cyanol FF
T4 kinase buffer, 10x:	500mM Tris.HCl pH 9.0 100mM $MgCl_2$ 100mM DTT 500µg/ml BSA
TBE, 5X:	per litre; 54g Tris base (0.89M) 27.5g Boric Acid (0.89M) 20ml 0.5M EDTA, pH 8.0
TBS:	25mM Tris-HCl, pH 7.4 137mM NaCl 5mM KCl 0.7mM $CaCl_2$ 0.5mM $MgCl_2$ 0.6mM NaH_2PO_4
TE:	10mM Tris.HCl (pH as required) 1mM EDTA, pH 8.0
TEN:	10mM Tris HCl, pH 8.0 1mM EDTA, pH 8.0 100mM NaCl
TER:	10mM Tris.HCl, pH 8.0 1mM EDTA, pH 8.0 10µg/ml RNase A

TFB1:	30mM potassium acetate 50mM $MgCl_2$ 100mM KCl 10mM $CaCl_2$ 15% (v/v) glycerol
TFB2:	10mM MOPS, pH7.0 75mM $CaCl_2$ 10mM KCl 15% (v/v) glycerol
1M Tris.HCl:	124.89g Tris base per litre pH adjusted as required with conc. HCl (pH Stocks; 7.4, 7.6, 8.0)
Tris / NH_4Cl lysis solution:	10mls 0.17M Tris.HCl, pH 7.65 90mls 0.16M NH_4Cl

APPENDIX II

Origin and specificity of antibodies used in phenotypic studies.

Antibody	Hybridoma	Species/Isotype	Supplier
B7.1	1G10	Rat IgG2a	Pharmingen
CD28	37.51.1	Syrian Hamster IgG	Serotec
TcR	H57-597	Syrian Hamster IgG	Sigma
Y3P	HB-183	Mouse IgG2a	ATCC
M5/114.15.2	TIB-120	Rat IgG2b	ATCC
Ia	OX6	Mouse IgG	Sigma
B7.2	GL1	Rat IgG2a	Pharmingen
CD4	H129.19	Rat IgG2a	Pharmingen
CD2	RM2-5	Rat IgG2b	Pharmingen
CD8a	53-6.7	Rat IgG2a	Pharmingen
CD45R/B220	RA3-6B2	Rat IgG2a	Pharmingen
CD69	H1.2F3	Armenian Hamster IgG	Pharmingen
CD40L	MR1	Armenian Hamster IgG	Pharmingen
HSA/CD24	J11d	Rat IgM	Pharmingen
IL-2R	7D4	Rat IgM	Dr. A. Livingstone
ICAM-1	Y9.1/1.7.4	Rat IgG2b	Dr. A. Livingstone
LFA-1	M117/4	Rat IgG2a	Dr. A. Livingstone
IgG	F6258	Goat	Sigma

Antibodies used as negative controls:

Armenian Hamster IgG (Serotec)

Rat IgG1 (Serotec)

Rat IgG2a (Serotec)

Mouse IgM (Sigma)

(The specificity of these antibodies was not given by the supplier).

BIBLIOGRAPHY

Abbas, AK, Lichtman, AH and Pober, JS. Cellular and molecular immunology. Philadelphia: W.B. Saunders, 1991.

Akdis, CA, Akdis, M, Blesken, T, Wymann, D, Alkan, SS, Müller, U and Blaser, K. (1996) Epitope-specific T cell tolerance to phospholipase A_2 in bee venom immunotherapy and recovery by IL-2 and IL-15 *in vitro*. *J Clin Invest* **98**: 1676-1683.

Alberola-ila, J, Places, L, de la Calle, O, Romero, M, Yague, J, Gallart, J, Vives, J and Loano, F. (1991) Stimulation through the TCR/CD3 complex up-regulates the CD2 expression on human T lymphocytes. *J Immunol* **146**: 1085-1092.

Allison, JP and Havran, WL. (1991) The immunobiology of T cells with invariant γδ antigen receptors. *Ann Rev Immunol* **9**: 679-705.

Altmann, DM, Douek, DC, Frater, AJ, Hetherington, CM, Inoko, H and Elliott, JI. (1995) The T cell response of HLA-DR transgenic mice to human myelin basic protein and other antigens in the presence and absence of human CD4. *J Exp Med* **181**: 867-875.

Amagai, T, Itoi, M and Kondo, Y. (1995) Limited development capacity of the earliest embryonic murine thymus. *Eur J Immunol* **25**: 757.

Amigorena, S, Drake, JR, Webster, P and Mellman, I. (1994) Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes. *Nature* **369**: 113-120.

Amigorena, S, Webster, P, Drake, J, Newcomb, J, Cresswell, P and Mellman, I. (1995) Invariant chain cleavage and peptide loading in major histocompatibility complex class II vesicles. *J Exp Med* **181**: 1729-1741.

Anderson, GP and Coyle, AJ. (1994) TH2 and 'TH2-like' cells in allergy and asthma: pharmacological perspectives. *Trends Pharmacol Sci* **15**: 324-332.

Anderson, KS and Cresswell, P. (1994) A role for calnexin (IP90) in the assembly of class II molecules. *EMBO J* **13**: 675-682.

Anderson, SJ and Perlmutter, RM. (1995) A signalling pathway governing early thymocyte maturation. *Immunol Today* **16**: 99.

Androlewicz, MJ, Anderson, KS and Cresswell, P. (1993) Evidence that transporters associated with antigen processing translocate a major histocompatibility complex class I-binding peptide into the endoplasmic reticulum in an ATP-dependent manner. *Proc Natl Acad Sci USA* **90**: 9130-9134.

Androlewicz, MJ, Ortmann, B, Van Endert, PM, Spies, T and Cresswell, P. (1994) Characteristics of peptide and class I/β2m binding to the transporters associated with antigen processing (TAP.1 and TAP.2). *Proc Natl Acad Sci USA* **91**: 12716-12720.

Arlaud, GJ, Thielens, NM and Illy, C. (1993) Assembly of the C1 complex. *Behring Inst Mit* **93**: 189-195.

Arnold, B, Schönrich, G and Hämmerling, G. (1993) Multiple levels of peripheral tolerance. *Immunol Today* **14**: 12-14.

Asarnow, DM, Cado, D and Raulet, DH. (1993) Selection is not required to produce invariant T cell receptor γ gene junctional sequences. *Nature* **362**: 158-160.

Ashwell, JD and Klausner, RD. (1990) Genetic and mutational analysis of the T-cell antigen receptor. *Annu Rev Immunol* **8**: 139-167.

Avva, RR and Cresswell, P. (1994) *In vivo* and *in vitro* formation and dissociation of HLA-DR complexes with invariant chain-derived peptides. *Immunity* **1**: 763-774.

Azuma, M, Ito, D, Yagita, H, Okumura, K, Phillips, JH, Lanier, LL and Somoza, C. (1993) B70 antigen is a second ligand for CTLA-4 and CD28. *Nature* **366**: 76-79.

Barnaba, V, Watts, C, de Boer, M, Lane, P and Lanzavecchia, A. (1994) Professional presentation of antigen by activated human T cells. *Eur J Immunol* **24**: 71-75.

Becker, JC, Brabletz, T, Kirchner, T, Conrad, CT, Brocker, E-B and Reisfeld, RA. (1995) Negative transcriptional regulation in anergic T cells. *Proc Natl Acad Sci USA* **92**: 2375-2378.

Beverly, B, Kang, S-M, Lenardo, MJ and Schwartz, RH. (1992) Reversal of *in vitro* T cell clonal anergy by IL-2 stimulation. *Int Immunol* **4**: 661-671.

Bhandoola, A, Cho, EA, Yui, K, Saragovi, HU, Greene, MI and Quill, H. (1993) Reduced CD3-mediated protein tyrosine phosphorylation in anergic CD4+ and CD8+ T cells. *J Immunol* **151**: 2355-2367.

Bigby, M, Markowitz, JS, Bleicher, PA, Grusby, MJ, Simha, S, Siebrecht, M, Wagner, M, Nagler-Anderson, C and Glimcher, LH. (1993) Most γδ T cells develop normally in the absence of MHC class II molecules. *J Immunol* **151**: 4465-4475.

Bijlmakers, M-J, Benaroch, P and Ploegh, HL. (1994) Assembly of HLA-DR1 molecules translated *in vitro*: binding of peptide in the endoplasmic reticulum precludes association with invariant chain. *EMBO J* **13**: 2699-2707.

Bikoff, EK, Germain, RN and Robertson, EJ. (1995) Allelic differences affecting invariant chain dependency of MHC class II subunit assembly. *Immunity* **2**: 301-310.

Bjorkman, PJ, Saper, MA, Samraoui, WS, Bennett, JL and Wiley, DC. (1987) Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* **329**: 509-512.

Bodmer, H, Viville, S, Benoist, C and Mathis, D. (1994) Diversity of endogenous epitopes bound to MHC class II molecules limited by invariant chain. *Science* **263**: 1284-1286.

Boes, B, Hengel, H, Ruppert, T, Multhaup, G, Koszinowski, UH and Kloetzel, PM. (1994) Interferon γ stimulation modulates the proteolytic activity and cleavage site preference of 20S mouse proteasomes. *J Exp Med* **179**: 901-909.

Boussiotis, VA, Barber, DL, Nakarai, T, Freeman, GJ, Gribben, JG, Bernstein, GM, D'Andrea, AO, Ritz, J and Nadler, LM. (1994a) Prevention of T cell anergy by signalling through the gamma c chain of the IL-2 receptor. *Science* **266**: 1039-1042.

Boussiotis, VA, Freeman, GJ, Griffen, JD, Gray, GS, Gribben, JG and Nadler, LM. (1994b) CD2 is involved in maintenance and reversal of human alloantigen-specific clonal anergy. *J Exp Med* **180**: 1665-1673.

Boussiotis, VA, Gibben, JC, Freeman, GJ and Nadler, LM. (1994c) Blockade of the CD28 costimulatory pathway: a means to induce tolerance. *Curr Opin Immunol* **6**: 797-807.

Bowen, F, Haluskey, J and Quill, H. (1995) Altered CD40 ligand induction in tolerant T lymphocytes. *Eur J Immunol* **25**: 2830-2834.

Briner, TJ, Kuo, M-C, Keating, KM, Rogers, BL and Greenstein, JL. (1993) Peripheral T cell tolerance induced in naive and primed mice by subcutaneous injection of peptides from the major cat allergen *Fel d* I. *Proc Natl Acad Sci USA* **90**: 7608-7612.

Brinster, RL, Chen, HY, Trumbauer, ME, Yagle, MK and Palmiter, RD. (1985) Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc Natl Acad Sci USA* **82**: 4438-4442.

Brooks, AG, Campbell, PL, Reynolds, P, Gautam, AM and McCluskey, J. (1994) Antigen presentation and assembly by mouse I-A^k class II molecules in human APC containing deleted or mutated HLA-DM genes. *J Immunol* **153**: 5382-5392.

Brown, JH, Jardetzky, TS, Gorga, JC, Stern, LJ, Urban, RG, Strominger, JL and D.C, W. (1993) Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* **364**: 33-39.

Brutkiewicz, RR, Bennink, JR, Yewdell, JW and Bendelac, A. (1995) TAP-independent, β2m-dependent surface expression of functional mouse CD1.1. *J Exp Med* **182**: 1913-1919.

Burger, R, Scher, I, Sharrow, SO and Shevach, EM. (1984) Non-activated guinea-pig T cells and thymocytes express Ia antigens: FACS analysis with alloantibodies and monoclonal antibodies. *Immunology* **51**: 93-102.

Burnstein, HJ, Shea, CM and Abbas, AK. (1992) Aqueous antigens induce *in vivo* tolerance selectively in IL-2- and IFN-γ-producing (Th1) cells. *J Immunol* **148**: 3687-3691.

Busch, R, Cloutier, I, Sékaly, R-P and Hämmerling, GJ. (1996) Invariant chain protects class II histocompatibility antigens from binding intact polypeptides in the endoplasmic reticulum. *EMBO J* **15**: 418-428.

Calnan, BJ, Szychowski, S, Chan, FK-M, Cado, D and Winoto, A. (1995) A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection. *Immunity* **3**: 273-282.

Cambier, JC. (1995) Antigen and Fc receptor signalling: the awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). *J Immunol* **155**: 3281-3285.

Cantrell, D. (1994) G proteins in lymphocyte signalling. *Curr Opin Immunol* **6**: 380-384.

Carreno, BM, Solheim, JC, Harris, M, Stroynowski, I, Connolly, JM and Hansen, TH. (1995) TAP associates with a unique class I conformation, whereas calnexin associates with multiple class I forms in mouse and man. *J Immunol* **155**: 4726-4753.

Carter, LL and Dutton, RW. (1996) Type 1 and type 2: a fundamental dichotomy for all T-cell subsets. *Curr Opin Immunol* **8**: 336-342.

Castellino, F and Germain, RN. (1995) Extensive trafficking of MHC class II-invariant chain complexes in the endocytic pathway and appearance of peptide-loaded class II in multiple compartments. *Immunity* **2**: 73-88.

Cavigelli, M, Dolfi, F, Claret, FX and Karin, M. (1995) Induction of c-*fos* expression through JNK-mediated TCF/Elk-1 phosphorylation. *EMBO J* **14**: 5957-5964.

Cerundolo, V, Benham, A, Braud, V, Mukherjee, S, Gould, K, Macino, B, Neefjes, J and Townsend, A. (1997) The proteasome-specific inhibitor lactacystin blocks presentation of cytotoxic T lymphocyte epitopes in human and murine cells. *Eur J Immunol* **27**: 336-341.

Chan, AC, Dalton, M, Johnson, R, Kong, GH, Wang, T, Thoma, R and Kurosaki, T. (1995) Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. *EMBO J* **14**: 2499-2508.

Chan, AC and Shaw, AS. (1996) Regulation of antigen receptor signal transduction by protein tyrosine kinases. *Curr Opin Immunol* **8**: 394-401.

Chang, C-H, Fontes, J, Peterlin, M and Flavell, RA. (1994) Class II transactivator (CIITA) is sufficient for the inducible expression of major histocompatibility complex class II genes. *J Exp Med* **180**: 1367.

Chang, C-H, Hong, S-C, Hughes, CCW, Janeway Jr., CA and Flavell, RA. (1995) CIITA activates the expression of MHC class II genes in mouse T cells. *Int Immunol* **7**: 1515-1518.

Chein, Y-H and Davis, MM. (1993) How αβ T cell receptors 'see' peptide/MHC complexes. *Immunol Today* **14**: 597-602.

Chicz, RM, Urban, RG, Gorga, JC, Vignali, DAA, Lane, WS and Strominger, JL. (1993) Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J Exp Med* **178**: 27-47.

Chiodetti, L and Schwartz, RH. (1992) Induction of competence to respond to IL-4 by CD4+ Th1 cells requires costimulation. *J Immunol* **149**: 901-910.

Cobbold, S, Martin, G and Waldmann, H. (1990) The induction of skin graft tolerance in major histocompatibility complex-mismatched or primed recipients: primed T cells can be tolerised in the periphery with anti-CD4 and anti-CD8 antibodies. *Eur J Immunol* **20**: 2747-2755.

Constantini, F and Lacy, E. (1981) Introduction of a rabbit β-globin gene into the mouse germ line. *Nature* **294**: 92-94.

Corr, M, Slanetz, AE, Boyd, LF, Jelonek, MT, Khilko, S, al-Ramadi, BK, Kim, YS, Maher, SE, Bothwell, AL and Margulies, DH. (1994) T cell receptor-MHC class I peptide interactions: affinity, kinetics and specificity. *Science* **265**: 946-948.

Correa, I, Bix, M, Liao, N-S, Zijlstra, M, Jaenisch, R and Raulet, D. (1992) T cells bearing γδ T cell receptors develop normally in β2-microglobulin-mutant mice. *Proc Natl Acad Sci USA* **89**: 653-657.

Couture, C, Baier, G, Altman, A and Mustelin, T. (1994) p56lck-independent activation and tyrosine phosphorylation of p72syk by T-cell antigen receptor/CD3 stimulation. *Proc Natl Acad Sci USA* **91**: 5301-5305.

Cox, JH, Galardy, P, Bennink, JR and Yewdell, JW. (1995) Presentation of endogenous and exogenous antigens is not affected by inactivation of E1 ubiquitin-activating enzyme in temperature-sensitive cell lines. *J Immunol* **154**: 511-519.

Crews, CM and Erikson, RL. (1993) Extracellular signals and reversible protein phosphorylation: what to Mek of it all. *Cell* **74**: 215-217.

Croft, M, Carter, L, Swain, SL and Dutton, RW. (1994) Generation of polarised antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *J Exp Med* **180**: 1715-1728.

Cross, AH, Girard, TJ, Giacoletto, KS, Evans, RJ, Keeling, RM, Lin, RF, Trotter, JL and Karr, RW. (1995) Long-term inhibition of murine experimental autoimmune encephalomyelitis using CTLA-4-Fc supports a key role for CD28 costimulation. *J Clin Invest* **95**: 2783-2789.

Davis, CB, Kileen, N, Crooks, MEC, Raulet, D and Littman, DR. (1993) Evidence for a stochastic mechanism in the differentiation of mature subsets of T lymphocytes. *Cell* **73**: 237-247.

Davis, MM. (1990) T cell receptor gene diversity and selection. *Annu Rev Biochem* **59**: 475-496.

Davis, MM. (1995) Serial engagement proposed. *Nature* **375**: 104.

Davis, MM and Bjorkman, PJ. (1988) T cell antigen receptor genes and T cell recognition. *Nature* **334**: 395-402.

DeMars, R and Spies, T. (1992) New genes in the MHC that encode proteins for antigen processing. *Trends Cell Biol* **2**: 81.

Demotz, S, Grey, HM and Sette, A. (1990) The minimal number of class II MHC-antigen complexes needed for T cell activation. *Science* **249**: 1028-1030.

Denzin, LK and Cresswell, PA. (1995) HLA-DM induces CLIP dissociation from MHC class II αβ dimers and facilitates peptide loading. *Cell* **82**: 155-165.

Denzin, LK, Robbins, NF, Carboy-Newcomb, C and Cresswell, PA. (1994) Assembly and intracellular transport of HLA-DM and correction of the class II antigen-processing defect in T2 cells. *Immunity* **1**: 1-20.

DeSilva, DR, Urdahl, KB and Jenkins, MK. (1991) Clonal anergy is induced *in vitro* by T cell receptor occupancy in the absence of proliferation. *J Immunol* **147**: 3261-3267.

Deverson, EV, Gow, IR, Coadwell, WJ, Monaco, JJ, Butcher, GW and Howard, JC. (1990) MHC class II region encoding proteins related to the multidrug resistance family of transmembrane transporters. *Nature* **348**: 738-741.

Diamond, DJ, Clayton, LK, Sayre, PH and Reinherz, EL. (1988) Exon-intron organisation and sequence specific comparison of human and murine T11 (CD2) genes. *Proc Natl Acad Sci USA* **85**: 1615-1619.

Dickson, RB, Beguinot, L, Hanover, JA, Richert, ND, Willingham, MC and Pastan, I. (1983) Isolation and characterisation of a highly enriched preparation of receptosomes (endosomes) from a human cell line. *Proc Natl Acad Sci USA* **80**: 5335-5339.

Ding, L and Shevach, EM. (1992) IL-10 inhibits mitogen-induced T cell proliferation by selectively inhibiting macrophage costimulatory function. *J Immunol* **148**: 3133-3139.

Downward, J, Graves, J and Cantrell, D. (1992) The regulation and function of p21ras in T cells. *Immunol Today* **13**: 89-92.

Dudley, EC, Petrie, HT, Shah, LM, Owen, MJ and Hayday, AC. (1994) T cell receptor β chain gene rearrangement and selection during thymocyte development in adult mice. *Immunity* **1**: 83.

Dutia, BM, McConnell, I, Bird, K, Keating, P and Hopkins, J. (1993) Patterns of major histocompatibility complex class II expression on T cell subsets in different immunological compartments: Expression on resting T cells. *Eur J Immunol* **23**: 2882-2888.

Dyall, R and Nikolic-Zugic, J. (1995) The majority of post-selection CD4+ single-positive thymocytes requires the thymus to produce long-lived functional T cells. *J Exp Med* **181**: 235-245.

Emmrich, F. (1988) Cross-linking of CD4 and CD8 with the T cell receptor complex: quaternary complex formation and T cell repertoire selection. *Immunol Today* **9**: 296-300.

Erard, F, Wild, MT, Garcia-Sanz, JA and Le Gros, G. (1993) Switch of CD8 T cells to noncytolytic CD8-CD4- cells that make TH2 cytokines and help B cells. *Science* **260**: 1802-1805.

Ericson, ML, Sundstrøm, M, Sansom, DM and Charron, DJ. (1994) Mutually exclusive binding of peptide and invariant chain to major histocompatibility complex class II antigens. *J Biol Chem* **269**: 26531-26538.

Essery, G, Feldmann, M and Lamb, JR. (1988) Interleukin-2 can prevent and reverse antigen-induced unresponsiveness in cloned human T lymphocytes. *Immunology* **64**: 413-417.

Evavold, BD and Allen, PM. (1991) Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science* **252**: 1308-1310.

Falk, K, Rötzschke, O and Rammensee, HG. (1990) Cellular peptide composition governed by major histocompatibility complex class I molecules. *Nature* **348**: 248-251.

Fantl, WJ, Johnson, DE and Williams, LT. (1993) Signalling by receptor tyrosine kinases. *Annu Rev Biochem* **62**: 453-481.

Fehling, HJ, Swat, W, Laplace, C, Kühn, R, Rajewsky, K, Müller, U and Von Boehmer, H. (1994) MHC class I expression in mice lacking the proteasome subunit LMP-7. *Science* **265**: 1234-1237.

Feinberg, AP and Vogelstein, B. (1984) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **137**: 266-267.

Ferrick, DA, Schrenzel, MD, Mulvania, T, Hsieh, B, Ferlin, WG and Lepper, H. (1995) Differential production of interferon-γ and interleukin-4 in response to Th1- and Th2-stimulating pathogens by γδ T cells *in vivo*. *Nature* **373**: 255-257.

Field, EH, Noelle, RJ, Rouse, T, Goeken, J and Waldschmidt, T. (1993) Evidence for excessive Th2 CD4+ subset activity *in vivo*. *J Immunol* **151**: 48-59.

Fields, PE, Gajewski, TF and Fitch, FW. (1996) Blocked ras activation in anergic CD4+ T cells. *Science* **271**: 1276-1278.

Flaswinkel, H and Reth, M. (1994) Dual role of the tyrosine activation motif of the Ig-alpha protein during signal transduction via the B cell antigen receptor. *EMBO J* **13**: 83-89.

Fong, TA and Momann, TR. (1990) Alloreactive murine CD8+ T cell clones secrete the Th1 pattern of cytokines. *J Immunol* **144**: 1744-1752.

Fournel, M, Davidson, D, Veil, R and Veillette, A. (1996) Association of tyrosine protein kinase ZAP-70 with the protooncogene product p120-cbl in T lymphocytes. *J Exp Med* **183**: 301-306.

Foy, TM, Page, DM, Waldschmidt, TJ, Schoneveld, A, Laman, JD, Masters, SR, Tygrett, L, Ledbetter, JA, Aruffo, A and Claassen, E. (1995) An essential role for gp39, the ligand for CD40, in thymic selection. *J Exp Med* **182**: 1377-1388.

Freeman, GJ, Borriello, F, Hodes, RJ, Reiser, H, Gribben, JG, Ng, JW, Kim, J, Goldberg, JM, Hathcock, K, Laszlo, G, Lombard, LA, Wang, S, Gray, GS, Nadler, LM and Sharpe, AH. (1993a) Murine B7-2, an alternative CTLA-4 counter-receptor that costimulates T cell proliferation and interleukin-2 production. *J Exp Med* **178**: 2185-2192.

Freeman, GJ, Borriello, F, Hodes, RJ, Reiser, H, Hathcock, KS, Laszlo, G, McKnight, AJ, Kim, J, Du, L, Lombard, DB, Gray, GS, Nadler, LM and Sharpe, AH. (1993b) Uncovering of functional alternative CTLA-4 counter-receptor in B7-deficient mice. *Science* **262**: 907-909.

Freeman, GJ, Boussiotis, VA, Anumanthan, A, Bernstein, GM, Ke, XY, Rennert, PD, Gray, GS, Gribben, JG and Nadler, LM. (1995) B7-1 and B7-2 do not deliver identical costimulatory signals, since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4. *Immunity* **2**: 523-532.

Freeman, GJ, Gribben, JG, Boussiotis, VA, Ng, JW, Restivo, V, Lombard, L, Gray, GS and Nadler, LM. (1993) Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. *Science* **262**: 909-911.

Früh, K, Gossen, M, Wang, KN, Bujard, H, Peterson, PA and Yang, Y. (1994) Displacement of housekeeping proteasome subunits by MHC-encoded LMPs-a newly discovered mechanism for modulating the multicatalytic proteinase complex. *EMBO J* **13**: 3236-3244.

Gaczynska, M, Rock, KL and Goldberg, AL. (1993) Gamma-interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. *Nature* **365**: 264-267.

Gaczynska, M, Rock, KL, Spies, T and Goldberg, AL. (1994) Peptidase activities of proteasomes are differentially regulated by the major histocompatibility complex-encoded genes for LMP-2 and LMP-7. *Proc Natl Acad Sci USA* **91**: 9213-9217.

Garcia, KC, Degano, M, Stanfield, RL, Brunmark, A, Jackson, MR, Peterson, PA, Teyton, L and Wilson, IA. (1996) An αβ T cell receptor structure at 2.5Å and its orientation in the TCR-MHC complex. *Science* **274**: 209-219.

Garrett, TP, Saper, MA, Bjorkman, PJ, Strominger, JL and Wiley, DC. (1989) Specificity pockets for the side chains of peptide antigens in HLA-Aw68. *Nature* **342**: 692-696.

Gautam, SC, Matriano, JA, Chiccala, NF, Edinger, MG and Tubbs, RR. (1991) L3T4 (CD4+) cells that mediate contact sensitivity to trinitrochlorobenzene express I-A determinants. *Cell Immunol* **135**: 27-41.

Gazzinelli, RT, Wysocka, M, Hayashi, S, Denkers, EY, Hieny, S, Caspar, P, Trinchieri, G and Sher, A. (1994) Parasite-induced IL-12 stimulates early IFN-γ synthesis and resistance during acute infection with *Toxoplasma gondii*. *J Immunol* **153**: 2533-2543.

Geluk, A, Van Meijgaarden, KE, Drijfhout, JW and Ottenhoff, THM. (1995) CLIP binds to HLA class II using methionine-based, allele-dependent motifs as well as allele-independent supermotifs. *Mol Immunol* **32**: 975-981.

Ghosh, P, Amaya, M, Mellins, E and Wiley, DC. (1995) The structure of HLA-DR3 complexed with CLIP, an intermediate in peptide loading. *Nature* **387**: 457-462.

Gordon, JW and Ruddle, FH. (1981) Integration and stable germline transmission of genes injected into mouse pronuclei. *Science* **214**: 1244-1246.

Grant, EP, Michalek, MT, Goldberg, AL and Rock, KL. (1995) Rate of antigen degradation by the ubiquitin-protease pathway influences MHC class I presentation. *J Immunol* **155**: 3750-3758.

Gray, CW, Slaughter, CA and DeMartino, GN. (1994) PA28 activator protein forms regulatory caps on proteasome stacked rings. *J Mol Biol* **236**: 7-15.

Greaves, DR, Wilson, FD, Lang, D and Kioussis, D. (1989) Human CD2 3' flanking sequences confer high level T cell specific position-independent gene expression in transgenic mice. *Cell* **56**: 979-986.

Greenfield, EA, Howard, E, Paradis, T, Nguyen, K, Benazzo, F, McLean, P, Höllsberg, P, Davis, G, Hafler, DA, Sharpe, AH, Freeman, GJ and Kuchroo, VK. (1997) B7.2 expressed by T cells does not induce CD28-mediated costimulatory activity but retains CTLA4 binding. *J Immunol* **158**: 2025-2034.

Groettrup, M, Soza, A, Eggers, M, Kuehn, L, Dick, TP, Schild, H, Rammensee, HG, Koszinowski, UH and Kloetzel, PM. (1996) A role for the proteasome regulator PA28alpha in antigen presentation. *Nature* **381**: 166-168.

Groux, H, Bigler, M, de Vries, JE and Roncarolo, MG. (1996) Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *J Exp Med* **184**: 19-29.

Guidos, CJ. (1996) Positive selection of CD4+ and CD8+ T cells. *Curr Opin Immunol* **8**: 225-232.

Guidos, CJ, Danska, JS, Fathman, CG and Weissman, IL. (1990) T cell receptor-mediated negative selection of autoreactive T lymphocyte precursors occurs after commitment to the CD4 or CD8 lineage. *J Exp Med* **172**: 835-845.

Haas, W, Pereira, P and Tonegawa, S. (1993) Gamma/delta T cells. *Annu Rev Immunol* **11**: 637-685.

Haffar, OK, Smithgall, MD, Bradshaw, J, Brady, B, Damle, NK and P.S, L. (1993) Costimulation of T-cell activation and virus production by B7 antigen on activated CD4+ T cells from human immunodeficiency virus type 1-infected donors. *Proc Natl Acad Sci USA* **90**: 11904-11908.

Hanahan, D. (1983) Studies on transformation of *Escherischia coli* with plasmids. *J Mol Biol* **166**: 557-580.

Harding, FA, McArthur, JG, Gross, JA, Raulet, DH and Allison, JP. (1992) CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* **356**: 607-609.

Havran, WL and Boismenu, R. (1994) Activation and function of γδ T cells. *Curr Opin Immunol* **6**: 442-446.

Havran, WL, Chien, Y-H and Allison, JP. (1991) Recognition of self antigens by skin-derived T cells with invariant γδ antigen receptors. *Science* **252**: 1430-1432.

Hetzel, C, Hoyne, GF, Kristensen, NM, Bourne, T, Tsitoura, D and Lamb, JR. Peptide-mediated regulation of allergic diseases. MHC molecules. Chapman and Hall, 1996.

Higgins, JA, Lamb, JR, Marsh, SGE, Tonks, S, Hayball, JD, Rosen-Bronson, S, Bodmer, JG and O'Hehir, RE. (1992) Peptide-induced nonresponsiveness of HLA-DP restricted human T cells reactive with *Dermatophagoides* spp. (house dust mite). *J Allergy Clin Immunol* **90**: 749-756.

Hogan, B, Constantini, F and Lacy, E. Manipulating the mouse embryo- A laboratory manual. Cold Spring Harbour Laboratory, 1986.

Holt, PG, Oliver, J, Bilyk, N, McMenamin, C, McMenamin, PG, Kraal, G and Thepen, T. (1993) Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells *in vivo* by resident alveolar macrophages. *J Exp Med* **177**: 397-407.

Hong, S-C, Chelouche, A, Lin, R-H, Shaywitz, D, Braunstein, NS, Glimcher, L and Janeway Jr., CA. (1992) An MHC interaction site maps to the amino-terminal half of the T cell receptor α chain variable domain. *Cell* **69**: 999-1009.

Horvat, B, Loukides, JA, Anandan, L, Brewer, E and Flood, PM. (1991) Production of interleukin 2 and interleukin 4 by immune CD4-CD8+ and their role in the generation of antigen-specific cytotoxic T cells. *Eur J Immunol* **21**: 1863-1871.

Howard, JC. (1995) Supply and transport of peptides presented by class I MHC molecules. *Curr Opin Immunol* **7**: 69-76.

Hoyne, GF, O'Hehir, RE, Wraith, DC, Thomas, WR and Lamb, JR. (1993) Inhibition of T cell and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitised mice. *J Exp Med* **178**: 1783-1788.

Hsueh, YP and Lai, MZ. (1995) c-Jun N-terminal kinase but not mitogen-activated protein kinase is sensitive to cAMP inhibition in T lymphocytes. *J Biol Chem* **270**: 18094-18098.

Huang, X, Li, Y, Tanaka, K, Moore, KG and Hayashi, JI. (1995) Cloning and characterisation of Lnk, a signal transduction protein that links T-cell receptor activation signal to phospholipase Cγ1, Grb2, and phosphatidylinositol 3-kinase. *Proc Natl Acad Sci USA* **92**: 11618-11622.

Huczko, EL, Bodnar, WM, Benjamin, D, Sakaguchi, K, Zhu, NZ, Shabanowitz, J, Henderson, RA, Appella, E, Hunt, DF and Engelhard, VH. (1993) Characteristics of endogenous peptides eluted from the class I MHC molecule HLA-B7 determined by mass spectrometry and computer modelling. *J Immunol* **151**: 2572-2587.

Hunt, DF, Henderson, RA, Shabanowitz, J, Sakaguchi, K, Michel, H, Sevilir, N, Cox, AL, Appella, E and Engelhard, VH. (1992) Characterisation of peptides bound to the HLA class I molecule HLA-A2.1 by mass spectrometry. *Science* **255**: 1261-1263.

Irving, BA, Chan, AC and Weiss, A. (1993) Functional characterisation of a signal transducing motif present in the T cell antigen receptor ζ chain. *J Exp Med* **177**: 1093-1103.

Isobe, M, Yagita, K and Ihara, OA. (1992) Specific acceptance of cardiac allograft after treatment with antiodies to ICAM-1 and LFA-1. *Science* **255**: 1125-1128.

Itohara, S, Mombaerts, P, Lafaille, JJ, Iacomini, J, Nelson, A, Clarke, AR, Hooper, ML, Farr, A and Tonegawa, S. (1993) T cell receptor δ gene mutant mice: Independent generation of αβ T cells and programmed rearrangements of γδ TcR genes. *Cell* **72**: 337-348.

Izquierdo, M, Leevers, SJ, Williams, DH, Marshall, CJ, Weiss, A and Cantrell, D. (1994) The role of protein kinase C in the regulation of extracellular signal-regulated kinase by the T cell antigen receptor. *Eur J Immunol* **24**: 2462-2468.

Jackman, JK, Motto, DG, Sun, Q, Tanemoto, M, Turck, CW, Peltz, GA, Koretzky, GA and Findell, PR. (1995) Molecular cloning of SLP-76, a 76kDa tyrosine phosphoprotein associated with Grb2 in T cells. *J Biol Chem* **270**: 7029-7032.

Jackson, MR, Song, ES, Yang, Y and Peterson, PA. (1992) Empty and peptide-containing conformers of class I major histocompatibility complex molecules expressed in Drosophila melanogaster cells. *Proc Natl Acad Sci USA* **89**: 12117-12121.

Jaenisch, R. (1988) Transgenic animals. *Science* **240**: 1468-1474.

Jain, J, McCaffrey, PG, Valge-Archer, VE and Rao, A. (1992) Nuclear factor of activated T cells contains *fos* and *jun*. *Nature* **356**: 801-804.

Janeway Jr., CA. (1992) The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today* **13**: 11-16.

Janeway Jr., CA. (1994) Thymic selection: two pathways to life and two to death. *Immunity* **1**: 3-6.

Janeway Jr., CA, Medhzhitov, R and Pfeiffer, C. (1995) Altered peptide ligands: conformational changes in the TCR. *The Immunologist* **3**: 41-44.

Jardetzky, TS, Lane, WS, Robinson, RA, Madden, DR and Wiley, DC. (1991) Identification of self peptides bound to purified HLA-B27. *Nature* **353**: 326-329.

Jasanoff, A, Park, S-J and Wiley, DC. (1995) Direct observation of disordered regions in the MHC class II-associated invariant chain. *Proc Natl Acad Sci USA* **92**: 9900-9904.

Johnson, SA, Pleiman, CM, Pao, L, Schneringer, J, Hippen, K and Cambier, JC. (1995) Phosphorylated immunoreceptor signalling motifs (ITAMs) exhibit unique abilities to bind and activate Lyn and Syk tyrosine kinases. *J Immunol* **155**: 4596-4603.

Joosten, I, Wauben, MHM, Holewijn, MC, Reske, K, Pedersen, LF, Roosenboom, CFP, Hensen, EJ, Van Eden, W and Buus, S. (1994) Direct binding of autoimmune disease related T cell epitopes to purified Lewis rat MHC class II molecules. *Int Immunol* **6**: 751-759.

Jorgensen, JL, Reay, PA, Ehrich, EW and Davis, MM. (1992) Molecular components of T cell recognition. *Annu Rev Immunol* **10**: 835-873.

June, CH, Bluestone, JA, Nadler, LM and Thompson, CB. (1994) The B7 and CD28 receptor families. *Immunol Today* **15**: 321-331.

Jutel, MP, Pichler, WJ, Skrbic, D, Urwyler, A, Dahinden, C and Muller, UR. (1995) Bee venom immunotherapy results in decrease of IL-4 and IL-5 and an increase of IFN-γ secretion in specific allergen stimulated T cell cultures. *J Immunol* **154**: 4187-4194.

Kang, S-M, Beverly, B, Tran, A-C, Brorson, K, Schwartz, RH and Lenardo, MJ. (1992) Transactivation of AP-1 is a molecular target of T cell clonal anergy. *Science* **257**: 1134-1138.

Kapsenberg, ML, Wierenga, EA, Bos, JD and Jansen, HM. (1991) Functional subsets of allergen-reactive human CD4+ T cells. *Immunol Today* **12**: 392-395.

Karjalainen, K. (1994) High sensitivity, low affinity-paradox of T cell receptor recognition. *Curr Opin Immunol* **6**: 9-12.

Karlsson, L, Péléraux, A, Lindstedt, R, Liljedahl, M and Peterson, PA. (1994) Reconstitution of an operational MHC class II compartment in nonantigen-presenting cells. *Science* **266**: 1569-1573.

Katzav, S, Sutherland, M, Packham, G, Yi, T and Weiss, A. (1994) The protein tyrosine kinase ZAP-70 can associate with the SH2 domain of proto-Vav. *J Biol Chem* **269**: 32579-32585.

Kawai, K and Ohashi, PS. (1995) Immunological function of a defined T-cell population tolerised to low-affinity self antigens. *Nature* **374**: 68-69.

Kearney, ER, Pape, KA, Loh, DY and Jenkins, MK. (1994) Visualisation of peptide-specific T cell immunity and peripheral tolerance induction *in vivo*. *Immunity* **1**: 327-339.

Kearney, ER, Walunas, TL, Karr, AW, Morton, PA, Loh, DY, Bluestone, JA and Jenkins, MK. (1995) Antigen-dependent clonal expansion of antigen-specific CD4+ T cells *in vivo* is dependent on CD28 costimulation and inhibited by CTLA-4. *J Immunol* **155**: 1032-1036.

Kearse, K, Takahama, Y, Punt, J, Sharrow, S and Singer, A. (1995) Early molecular events induced by TcR signalling in immature CD4+CD8+ thymocytes. *J Exp Med* **181**: 193-202.

Kelly, AP, Monaco, JJ, Cho, S and Trowsdale, J. (1991) A new human HLA class II-related locus, *DM*. *Nature* **353**: 571-576.

Kelly, KA, Pearse, M, LeFrancois, L and Scollay, R. (1993) Emigration of selected subsets of γδ T cells from the adult murine thymus. *Int Immunol* **5**: 331-335.

Kennedy, MK, Picha, KS, Shanebeck, KD, Anderson, DM and Grabstein, KH. (1994) Interleukin-12 regulates the proliferation of Th1, but not Th2 or Th0 clones. *Eur J Immunol* **24**: 2271-2278.

Keogh, M-C. *Regulation and function of CD2 in the mouse.* PhD. Imperial College of Science, Technology and Medicine., 1995.

Khoury, SJ, Akalin, E, Chandraker, A, Turka, LA, Linsley, PS, Sayegh, MH and Hancock, WW. (1995) CD28-B7 costimulatory blockade by CTLA-4-Ig prevents actively induced experimental autoimmune encephalomyelitis and inhibits Th1 but spares Th2 cytokines in the central nervous system. *J Immunol* **155**: 4521-4524.

Kira, J-I, Itoyama, Y and Goto, I. (1989) Generation of CD4+ blastoid T cells showing marked upregulation of CD4, class I and II MHC, and IL2 receptor molecules is required for the expression of potent encephalitogenicity. *Cell Immunol* **123**: 264-275.

Kisielow, P and Maizek, A. (1995) Positive selection of T cells: rescue from programmed cell death and differentiation require continual engagement of the T cell receptor. *J Exp Med* **181**: 1975-1984.

Kleijmeer, MJ, Kelly, A, Geuze, HJ, Slot, JW, Townsend, A and Trowsdale, J. (1992) Location of MHC-encoded transporters in the endoplasmic reticulum and *cis*-Golgi. *Nature* **357**: 342-344.

Klein, J. Biology of the mouse histocompatibility-2 complex. Springer-Verlag, 1975.
Konig, R, Huang, L-Y and Germain, RN. (1992) MHC class II interactions with CD4 mediated by a region analagous to the MHC class I binding site for CD8. *Nature* **356**: 796-798.

Kouskoff, V, Vonesh, J-L, Benoist, C and Mathis, D. (1995) The influence of positive selection on RAG expression in thymocytes. *Eur J Immunol* **25**: 54-58.

Kovacsovics-Bankowski, M and Rock, KL. (1995) A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* **267**: 243-246.

Krenger, W, Snyder, KM, Byon, JC, Falzarano, G and Ferrara, JL. (1995) Polarised type 2 alloreactive CD4+ and CD8+ donor T cells fail to induce experimental acute graft-versus-host disease. *J Immunol* **155**: 585-593.

Kropshofer, H, Vogt, A-B, Stern, LJ and Hämmerling, GJ. (1995) Self-release of CLIP in peptide loading of HLA-DR molecules. *Science* **270**: 1357-1359.

Kruisbeek, AM and Amsen, D. (1996) Mechanisms underlying T cell tolerance. *Curr Opin Immunol* **8**: 233-244.

Krummel, M and Allison, J. (1995) CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* **182**: 459-466.

Kuchroo, VK, Das, MP, Brown, JA, Ranger, AM, Zamvil, SS, Sobel, RA, Weiner, HL, Nabavi, N and Glimcher, LH. (1995) B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* **80**: 707-718.

Kuckelhorn, U, Frentzel, S, Kraft, R, Kostka, S, Groettrup, M and Kloetzel, PM. (1995) Incorporation of major histocompatibility complex-encoded subunits LMP2 and LMP7 changes the quality of the 20S proteasome polypeptide processing products independent of interferon-γ. *Eur J Immunol* **25**: 2605-2611.

Lafferty, KJ, Prowse, SJ and Simeonovic, CJ. (1993) Immunobiology of tissue transplantation: A return to the passenger leukocyte concept. *Annu Rev Immunol* **11**: 143-173.

Lake, RA, O'Hehir, RE, Verhoef, A and Lamb, JR. (1993) CD28 mRNA rapidly decays when activated T cells are functionally anergised with specific peptide. *Int Immunol* **5**: 461-466.

Lake, RA, Wotton, D and Owen, MJ. (1990) A 3' transcriptional enhancer regulates tissue specific expression of the human CD2 gene. *EMBO J* **9**: 3129-3136.

Lamb, JR and Feldman, M. (1984) Essential requirement for major histocompatibility complex in recognition in T cell tolerance induction. *Nature* **308**: 72-74.

Lamb, JR, Skidmore, BJ, Green, N, Chiller, JM and Feldman, M. (1983) Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. *J Exp Med* **157**: 1434-1447.

Lamont, AG, Sette, A, Fujinami, R, Colon, SM, Miles, C and Grey, HM. (1990) Inhibition of experimental autoimmune encephalomyelitis induction in SJL/J mice by using a peptide with a high affinity for IA^S molecules. *J Immunol* **145**: 1687-1693.

Landais, D, Beck, BN, Buerstedde, J-M, Degraw, S, Klein, D, Koch, N, Murphy, D, Pierres, M, Tada, T, Yamamoto, K, Benoist, C and Mathis, D. (1986) The assignment of chain specificities for anti-Ia monoclonal antibodies using L cell transfectants. *J Immunol* **9**: 3002-3005.

Lane, P, Haller, C and McConnell, F. (1996) Evidence that induction of tolerance *in vivo* involves active signalling via a B7 ligand-dependent mechanism: CTLA-4-Ig protects Vβ8+ T cells from tolerance induction by the superantigen stapylococcal enterotoxin B. *Eur J Immunol* **26**: 858-862.

Lang, G, Wotton, D, Owen, MJ, Sewell, WA, Brown, MH, Mason, DY, Crumpton, MJ and Kioussis, D. (1988) The structure of the human CD2 gene and its expression in transgenic mice. *EMBO J* **7**: 1675-1682.

Lanzavecchia, A. (1995) How can cryptic epitopes trigger autoimmunity? *J Exp Med* **181**: 1945-1948.

Lanzavecchia, A, Roosnek, E, Gregory, T, Berman, P and Abrignani, S. (1988) T cells can present antigens such as HIV gp120 targeted to their own surface molecules. *Nature* **334**: 530.

LaSalle, JM and Hafler, DA. (1994) T cell anergy. *FASEB J* **8**: 601-608.

LaSalle, JM, Tolentino, PJ, Freeman, GJ, Nadler, LM and Hafler, DA. (1992) Early signalling defects in human T cells anergised by T cell presentation of autoantigen. *J Exp Med* **176**: 177-186.

Leclercq, G, Plum, J, Nandi, D, De Smedt, M and Allison, JP. (1993) Intrathymic differentiation of Vγ3 T cells. *J Exp Med* **178**: 309-315.

Lehman-Grube, F, Dralle, H, Utermohlen, O and Lohler, J. (1994) MHC class I molecule-restricted presentation of viral antigen in β2-microglobulin-deficient mice. *J Immunol* **94**: 595-603.

Lehner, PJ and Cresswell, P. (1996) Processing and delivery of peptides presented by MHC class I molecules. *Curr Opin Immunol* **8**: 59-67.

Lenschow, DJ, Ho, SC, Sattar, H, Rhee, L, Gray, G, Nabavi, N, Herold, KC and Bluestone, JA. (1995) Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse. *J Exp Med* **181**: 1145-1155.

Lenschow, DJ, Sperling, AI, Cooke, MP, Freeman, GJ, Rhee, L, Decker, DC, Gray, G, Nadler, LM, Goodnow, CC and Bluestone, JA. (1994) Differential up-regulation of the B7-1 and B7-2 costimulatory molecules following immunoglobulin receptor engagement by the antigen. *J Immunol* **153**: 1990-1997.

Lenschow, DJ, Walunas, TL and Bluestone, JA. (1996) CD28/B7 system of T cell costimulation. *Annu Rev Immunol* **14**: 233-258.

Levelt, CN and Eichmann, K. (1995) Receptors and signals in early thymic selection. *Immunity* **3**: 667.

Li, W, Whaley, CD, Mondino, A and Mueller, DL. (1996) Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4+ T cells. *Science* **271**: 1272-1276.

Lindstedt, R, Liljedahl, M, Péléraux, A, Peterson, PA and Karlsson, L. (1995) The MHC class II molecule H-2M is targeted to an endosomal compartment by a tyrosine-based targeting motif. *Immunity* **3**: 561-572.

Linsley, PS, Bradshaw, J, Greene, J, Peach, R, Bennett, KL and Mittler, RS. (1996) Intracellular trafficking of CTLA-4 and focal localisation towards sites of TCR engagement. *Immunity* **4**: 535-544.

Linsley, PS, Greene, JL, Brady, W, Bayorath, J, Ledbetter, JA and Peach, R. (1994) Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CLTA-4. *Immunity* **1**: 793-801.

Linsley, PS and Ledbetter, JA. (1993) The role of the CD28 receptor during T cell responses to antigen. *Annu Rev Immunol* **11**: 191-212.

Liu, GY, Fairchild, PJ, Smith, RM, Prowle, JR, Kioussis, D and Wraith, DC. (1995) Low avidity recognition of self-antigen by T cells permits escape from central tolerance. *Immunity* **3**: 407-415.

Long, EO, LaVaute, T, Pinet, V and Jaraquemada, D. (1994) Invariant chain prevents the HLA-DR-restricted presentation of a cytosolic peptide. *J Immunol* **153**: 1487-1494.

Lorber, MI, Loken, MR, Stall, AM and Fitch, FW. (1982) I-A antigens on cloned alloreactive murine T lymphocytes are acquired passivly. *J Immunol* **128**: 2798-2803.

Lundberg, K, Heath, W, Köntgen, F, Carbone, FR and Shortman, K. (1995) Intermediate steps in positive selection: differentiation of CD4+8^{int}TCR^{int} thymocytes into CD4-8+TCR^{hi} thymocytes. *J Exp Med* **181**: 1643-1651.

Macatonia, SE, Doherty, TM, Knight, SC and O'Garra, A. (1993) Differential effect of IL-10 on dendritic cell-induced T cell proliferation and IFN-gamma production. *J Immunol* **150**: 3755-3765.

Macatonia, SE, Hosken, NA, Litton, M, Vieira, P, Hsieh, C-S, Culpepper, JA, Wysocka, M, Trinchieri, G, Murphy, KM and O'Garra, A. (1995) Dendritic cells produce interleukin-12 and direct the development of Th1 cells from naive CD4+ T cells. *J Immunol* **154**: 5071-5079.

Madden, DR. (1995) The three-dimensional structure of peptide-MHC complexes. *Annu Rev Immunol* **13**: 587-622.

Madrenas, J, Schwartz, RH and Germain, RN. (1996) IL-2 production, not the pattern of early TcR-dependent tyrosine phosphorylation, controls anergy induction by both agonists and partial agonists. *Proc Natl Acad Sci USA* **93**: 9736-9741.

Madrenas, J, Wange, RL, Wang, JL, Isakov, N, Samelson, LE and Germain, RN. (1995) Zeta phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. *Science* **267**: 515-518.

Maggi, E, Giudizi, MG, Biagiotti, R, Annunziato, F, Manetti, R, Piccinni, MP, Parronchi, P, Sampognaro, S, Giannarini, L and Zuccati, G. (1994) Th2-like CD8+ T cells showing B cell helper function and reduced cytolytic activity in human immunodeficiency virus type 1 infection. *J Exp Med* **180**: 489-495.

Mahon, KA, Overbeek, PA and Westphal, H. (1988) Prenatal lethality in a transgenic mouse line is the result of a chromosonal location. *Proc Natl Acad Sci USA* **85**: 1165-1168.

Malcharek, G, Gnau, V, Jung, G, Rammensee, H-G and Melms, A. (1995) Supermotifs enable natural invariant chain-derived peptides to interact with many major histocompatibility complex-class II molecules. *J Exp Med* **181**: 527-536.

Malissen, M, Gillet, A, Rocha, B, Trucy, J, Vivier, E, Boyer, C, Kontgen, F, Brun, N, Mazza, G, Spanopoulou, E, Guy-Grand, D and Malissen, B. (1993) T cell development in mice lacking the CD3ζ/η gene. *EMBO J* **12**: 4347-4355.

Malnati, MS, Marti, M, La Vaute, T, Jaraquemada, D, Biddison, W, De Mars, R and Long, EO. (1992) Processing pathways for presentation of cytosolic antigen to MHC class II-restricted T cells. *Nature* **357**: 702-704.

Mannie, MD, Rendall, SK, Arnold, PY, Nardella, JP and White, GA. (1996) Anergy-associated T cell antigen presentation. *J Immunol* **157**: 1062-1070.

Maric, MA, Taylor, MD and Blum, JS. (1994) Endosomal aspartic proteinases are required for invariant-chain processing. *Proc Natl Acad Sci USA* **91**: 2171-2175.

Mark, WH, Signorelli, K and Lacy, E. (1985) An insertional mutation in a transgenic mouse line results in developmental arrest at day 5 of gestation. *Cold Spring Harbour Symp Quant Biol* **50**: 453-63.

Marks, MS, Germain, RN and Bonifacino, JS. (1995) Transient aggregation of major histocompatibility class II chains during assembly in normal spleen cells. *J Biol Chem* **270**: 10475-10481.

Matzinger, P. (1991) The JAM test. A simple assay for DNA fragmentation and cell death. *J Immunol Methods* **145**: 185-192.

Matzinger, P. (1994) Tolerance, danger, and the extended family. *Annu Rev Immunol* **12**: 991-1045.

Mauri-Hellweg, D, Bettens, F, Mauri, D, Brander, C, Hunziker, T and Pichler, WJ. (1995) Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulphonamides, phenytoin, and carbamazepine. *J Immunol* **155**: 462-472.

McCormick, F. (1993) How receptors turn Ras on. *Nature* **363**: 15-16.

McCoy, KL, Noone, M, Inman, JK and Stutzman, RJ. (1993) Exogenous antigens internalised through transferrin receptors activate CD4+ T cells. *J Immunol* **150**: 1691-1704.

McMenamin, C and Holt, PG. (1993) The natural immune response to inhaled soluble protein antigens involves major histocompatibility complex (MHC) class I-restricted CD8+ T cell-mediated but MHC class II-restricted CD4+ T cell-dependent immune deviation resulting in selective suppression of immunoglobulin E production. *J Exp Med* **178**: 889-899.

McMenamin, C, Pimm, C, McKersey, M and Holt, PG. (1994) Regulation of IgE responses to inhaled antigens in mice by antigen-specific γδ T cells. *Science* **265**: 1869-1871.

Mellins, E, Kempin, S, Smith, L, Monji, T and Pious, D. (1991) A gene required for class II restricted antigen presentation maps to the major histocompatibility complex. *J Exp Med* **174**: 1607-1615.

Mellins, E, Smith, L, Arp, B, Cotner, T, Celis, E and Pious, D. (1990) Defective processing and presentation of exogenous antigens in mutants with normal HLA class II genes. *Nature* **343**: 71-74.

Mellman, I, Pierre, P and Amigorena, S. (1995) Lonely MHC molecules seeking immunogenic peptides for meaningful relationships. *Curr Opin Cell Biol* **7**: 564-572.

Metzler, B and Wraith, DC. (1993) Inhibition of experimental autoimmune encephalomyelitis by inhalation but not oral administration of the encephalitogenic peptide: influence of MHC binding affinity. *Infect Immun* **5**: 1159-1165.

Michalek, MT, Grant, EP, Gramm, C, Goldberg, AL and Rock, KL. (1993) A role for the ubiquitin-dependent proteolytic pathway in MHC class I-restricted antigen presentation. *Nature* **363**: 552-554.

Miller, SD, Vanderlugt, CL, Lenschow, DJ, Dal Canto, MC and Bluestone, JA. (1995) Blockade of CD28/B7-1 interaction prevents epitope spreading and clinical relapses of murine EAE. *Immunity* **3**: 739.

Mombaerts, P, Arnoldi, J, Russ, F and Tonegawa, S. (1993) Different roles of αβ and γδ T cells in immunity against an intracellular bacterial pathogen. *Nature* **365**: 53-56.

Mombaerts, P, Clarke, AR, Rudnicki, MA, Iacomini, J, Itohara, S, Lafaille, JJ, Wang, L, Ichikawa, Y, Jaenisch, R, Hooper, ML and Tonegawa, S. (1992) Mutations in T cell antigen receptor genes α and β block thymocyte development at different stages. *Nature* **360**: 225-231.

Momburg, F, Roelse, J, Hämmerling, GJ and Neefjes, JJ. (1994) Peptide size selection by the major histocompatibility complex-encoded transporter. *J Exp Med* **179**: 1613-1623.

Moore, TA and Zlotnik, A. (1995) T-cell lineage commitment and cytokine response of thymic progenitors. *Blood* **86**: 1850.

Morris, SC, Madden, KB, Adamovicz, JJ, Gause, WC, Hubbard, BR, Gately, MK and Finkelman, FD. (1994) Effects of IL-12 on *in vivo* cytokine gene expression and Ig isotype selection. *J Immunol* **152**: 1047-1056.

Mosmann, TR, Cherwinski, H, Bond, MW, Giedlin, MA and Coffman, RL. (1986) Two types of murine helper T cell clone: Definition according to profiles of lymphokine activitoes and secreted proteins. *J Immunol* **136**: 2348-2357.

Motta, A, Bremmes, B, Castiglione Morelli, MA, Frank, RW, Saviano, G and Bakke, O. (1995) Structure-activity relationship of the leucine-based sorting motifs in the cytosolic tail of the major histocompatibility complex-associated invariant chain. *J Biol Chem* **270**: 27165-27171.

Mueller, DL, Chiodetti, L, Bacon, PA and Schwartz, RH. (1991) Clonal anergy blocks the response to IL-4, as well as the production of IL-2 in dual-producing helper T cell clones. *J Immunol* **147**: 4118-4125.

Mueller, DL, Jenkins, MK and Schwartz, RH. (1989) Clonal expansion versus clonal inactivation: A costimulatory pathway determines the outcome of T cell antigen receptor occupancy. *Annu Rev Immunol* **7**: 445-480.

Muller, U, Fricker, M, Caraballido, J and Blaser, K. Successful immunotherapy with T-cell epitopes of bee venom phospholipase A2 in two patients with bee venom allergy. Vol. IV of New trends in allergy. Hamburg: 1995.

Murray, JS, Madri, J, Pasqualini, T and Bottomly, K. (1993) Functional CD4 T cell subset interplay in an intact immune system. *J Immunol* **150**: 4270-4276.

Nakajima, A, Azuma, M, Kodera, S, Nuriya, S, Terashi, A, Abe, M, Hirose, S, Shirai, T, Yagita, H and Okumura, K. (1995) Preferential dependence of autoantibody production in murine lupus on CD86 costimulatory molecule. *Eur J Immunol* **25**: 3060-3069.

Naujokas, MF, Arneson, LS, Fineschi, B, Peterson, ME, Sitterding, S, Hammond, AT, Reilly, C, Lo, D and Miller, J. (1995) Potent effects of low levels of MHC class II-associated invariant chain on CD4+ T cell development. *Immunity* **3**: 359-372.

Neeno, T, Krco, CJ, Harders, J, Baisch, J, Cheng, S and David, CS. (1996) HLA-DQ8 transgenic mice lacking endogenous class II molecules respond to house dust allergens. *J Immunol* **156**: 3191-3195.

Nel, AE, Hanekom, C, Rheeder, A, Williams, K, Pollack, S, Katz, R and Landreth, GE. (1990) Stimulation of MAP-2 kinase activity in T-lymphocytes by anti-CD3 or anti-Ti MAb is partially dependent on PKC. *J Immunol* **144**: 2683-2689.

Neumeister, EN, Zhu, Y, Richard, S, Terhorst, C, Chan, AC and Shaw, AS. (1995) Binding of ZAP-70 to phosphorylated T-cell receptor zeta and eta enhances its autophosphorylation and generates specific binding sites for SH2 domain-containing proteins. *Mol Cell Biol* **15**: 3171-3178.

Nijenhuis, M, Schmitt, S, Armandola, EA, Obst, R, Brunner, J and Hämmerling, GJ. (1996) Identification of a contact region for peptide on the TAP1 chain of the transporter associated with antigen processing. *J Immunol* **156**: 2186-2195.

Nuchtern, JG, Biddison, WE and Klausner, RD. (1990) Class II MHC molecules can use the endogenous pathway of antigen presentation. *Nature* **343**: 74-76.

O'Brien, RL and Born, W. (1991) Shock proteins as antigens for γδ T cells. *Semin Immunol* **3**: 81-87.

O'Brien, RL, Fu, YX, Cranfill, R, Dallas, A, Ellis, C, Reardon, C, Lang, J, Carding, SR, Kubo, R and Born, W. (1992) Heat shock protein HSP60-reactive γδ cells: a large diversified T lymphocyte subset with highly focused specificity. *Proc Natl Acad Sci USA* **89**: 4348-4352.

O'Hehir, RE, Aguilar, BA, Schmidt, TJ, Gollnick, SO and Lamb, JR. (1991a) Functional inactivation of *Dermatophagoides* spp. (house dust mite) reactive human T-cell clones. *Clin Exp Allergy* **21**: 209-215.

O'Hehir, RE, Busch, R, Rothbard, JB and Lamb, JR. (1991b) An *in vitro* model of peptide-mediated immunomodulation of the human T cell response to *Dermatophagoides* spp. (house dust mite). *J Allergy Clin Immunol* **87**: 1120-1127.

O'Hehir, RE, Garman, RD, Greenstein, JL and Lamb, JR. (1991c) The specificity and regulation of T-cell responsiveness to allergens. *Annu Rev Immunol* **9**: 67-95.

O'Hehir, RE and Lamb, JR. (1990) Induction of specific clonal anergy in human T lymphocytes by *Staphylococcus aureus* enterotoxins. *Proc Natl Acad Sci USA* **87**: 8884-8888.

O'Hehir, RE, Yssel, H, Verma, S, de Vries, JE, Spits, H and Lamb, JR. (1991) Clonal analysis of differential lymphokine production in peptide and superantigen induced T cell anergy. *Int Immunol* **3**: 819-826.

Ohno, H, Aoe, T, Taki, S, Kitamuru, D, Ishida, Y, Rajewsky, K and Saito, T. (1993) Development and functional impairment of T cells in mice lacking CD3ζ chain. *EMBO J* **12**: 4357-4366.

Ohno, H, Ono, S, Hirayama, N, Shimada, S and Saito, T. (1994) Preferential usage of the Fc receptor γ chain in the T cell antigen receptor complex by γδ T cells localised in the epithelia. *J Exp Med* **179**: 365-369.

Orkin, SH. (1990) Globin gene regulation and switching: circa 1990. *Cell* **55**: 4251-4261.

Ortmann, B, Androlewicz, MJ and Cresswell, P. (1994) MHC class I/β2-microglobulin complexes associate with TAP transporters before peptide binding. *Nature* **368**: 864-867.

Osborne, BA. (1996) Apoptosis and the maintenance of homeostasis in the immune system. *Curr Opin Immunol* **8**: 245-254.

Oswald, IP, Caspar, P, Jankovic, D, Wynn, TA, Pearce, EJ and Sher, A. (1994) IL-12 inhibits Th2 cytokine responses induced by eggs of *Schistosoma mansoni*. *J Immunol* **153**: 1707-1713.

Otten, GR and Germain, RN. (1991) Split anergy in a CD8+ T cell receptor-dependent cytolysis in the absence of interleukin-2 production. *Science* **251**: 1228-1231.

Palmiter, RD, Chen, HY and Brinster, RL. (1982) Differential regulation of metallothionene- thymidine kinase fusion genes in transgenic mice and their offspring. *Cell* **29**: 701-710.

Parish, CR and Liew, FY. (1972) Immune response to chemically modified flagellin. *J Exp Med* **135**: 298-311.

Park, S-J, Sadegh-Nasseri, S and Wiley, DC. (1995) Invariant chain made in *Escherichia coli* has an exposed N-terminal segment that blocks antigen binding to HLA-DR1 and a trimeric C-terminal segment that binds empty HLA-DR1. *Proc Natl Acad Sci USA* **92**: 11289-11294.

Pénit, C, Lucas, B and Vasseur, F. (1995) Cell expansion and growth arrest phases during the transition from precursor (CD4-8-) to immature (CD4+8+) thymocytes in normal and genetically modified mice. *J Immunol* **154**: 5103.

Penninger, J, Kishihara, K, Molina, T, Wallace, VA, Timms, E, Hedrick, SM and Mak, TW. (1993) Requirement for tyrosine kinase $p56^{lck}$ for thymic development of transgenic γδ T cells. *Science* **260**: 358-361.

Pereira, P, Ziljstra, M, McMaster, J, Loring, JM, Jaenisch, R and Tonegawa, S. (1992) Blockade of transgenic γδ T cell development in β2-microglobulin deficient mice. *EMBO J* **11**: 25-31.

Peterson, JD, Karpus, WJ, Clatch, RJ and Miller, SD. (1993) Split tolerance of Th1 and Th2 cells in tolerance to Theiler's murine encephalomyelitis virus. *Eur J Immunol* **23**: 46-55.

Philpott, KL, Viney, JL, Kay, G, Rastan, S, Gardiner, EM, Chae, S, Hayday, AC and Owen, MJ. (1992) Lymphoid development in mice congenitally lacking T cell receptor αβ-expressing cells. *Science* **256**: 1448-1452.

Pichler, WJ and Wyss-Coray, T. (1994) T cells as antigen-presenting cells. *Immunol Today* **15**: 312-315.

Pieters, J, Bakke, O and Dobberstein, B. (1993) The MHC class II-associated invariant chain contains two endosomal targeting signals within its cytoplasmic tail. *J Cell Sci* **106**: 831-842.

Pinet, V, Vergelli, M, Martin, R, Bakke, O and Long, EO. (1995) Antigen presentation mediated by recycling of surface HLA-DR molecules. *Nature* **375**: 603-603.

Price, MA, Rogers, AE and Treisman, R. (1995) Comparitive analysis of the ternary complex factors Elk-1, SAP-Ia and SAP-2 (ERP/NET). *EMBO J* **14**: 2589-2601.

Punt, JA, Osborne, BA, Takaham, Y, Sharrow, SO and Singer, A. (1994) Negative selection of CD4+CD8+ thymocytes by T cell receptor-induced apoptosis requires a costimulatory signal that can be provided by CD28. *J Exp Med* **179**: 709-713.

Quill, H, Bhandoola, A, Trinchieri, G, Haluskey, J and Pritt, D. (1994) Induction of interleukin 12 responsiveness is impaired in anergic T lymphocytes. *J Exp Med* **179**: 1065-1070.

Racke, MK, Scott, DE, Quigley, L, Gray, GS, Abe, R, June, CH and Perrin, PJ. (1995) Distinct roles for B7-1 (CD80) and B7-2 (CD86) in the initiation of experimental autoimmune encephalomyalitis. *J Clin Invest* **96**: 2195-2203.

Rammensee, H-G, Kroschewski, R and Frangoulis, B. (1989) Clonal anergy induced in mature Vβ6+ T lymphocytes on immunising Ml-1^b mice with Mls-1^a expressing cells. *Nature* **339**: 541-544.

Reiner, SL and Seder, RA. (1995) T helper cell differentiation in immune response. *Curr Opin Immunol* **7**: 360-366.

Reizis, B, Schramm, C, Cohen, IR and Mor, F. (1994) Expression of Major histocompatibility complex class II molecules in rat T cells. *Eur J Immunol* **24**: 2796-2802.

Renz, H, Saloga, J, Bradley, KL, Loader, JE, Greenstein, JL, Larsen, G and Gelfand, EW. (1993) Specific Vβ T cell subsets mediate the immediate hypersensitivity response to ragweed allergen. *J Immunol* **151**: 1907-1917.

Reske-Kunz, AB and Diamantstein, T. (1987) Evaluation of antigen presentation by a murine Ia+ T cell clone, BK-BI-2.6. *Immunology* **61**: 489-495.

Reske-Kunz, AB, Reske, K and Rüde, E. (1986) Cloned murine Ia+ BK-BI-2.6 T cells function as accessory cells presenting protein antigens to long-term-cultured antigen-specific T cell lines. *J Immunol* **136**: 2033-2040.

Rigaud, G, De Lerma Barbaro, A, Nicolis, M, Cestari, T, Ramarli, D, Riviera, A-P and Accolla, RS. (1996) Induction of CIITA and modification of *in vivo* HLA-DR promoter occupancy in normal thymic epithelial cells treated with IFN-γ. *J Immunol* **156**: 4254-4258.

Rivett, AJ, Palmer, A and Knecht, E. (1992) Electron microscopic localisation of the multicatalytic proteinase complex in rat liver and in cultured cells. *J Histochem Cytochem* **40**: 1165-1172.

Robey, E and Fowlkes, BJ. (1994) Selective events in T cell development. *Annu Rev Immunol* **12**: 675-705.

Robey, EA, Fowlkes, BJ, Gorden, JW, Kioussis, D, Von Boehmer, H, Ramsdell, F and Axel, R. (1991) Thymic selection in CD8 transgenic mice supports an instructive model for commitment to a CD4 or CD8 lineage. *Cell* **64**: 99-107.

Rock, EP, Sibbald, PR, Davis, MM and Chien, Y-H. (1994) CDR3 length in antigen-specific immune receptors. *J Exp Med* **179**: 323-328.

Rock, KL, Gramm, C, Rothstein, L, Clark, K, Stein, R, Dick, L, Hwang, D and Goldberg, AL. (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* **78**: 761-771.

Romagnoli, P and Germain, RN. (1994) The CLIP region of invariant chain plays a critical role in regulating major histocompatibility complex class II folding, transport, and peptide occupancy. *J Exp Med* **180**: 1107-1113.

Romani, L, Mencacci, A, Tonnetti, L, Spaccapelo, R, Cenci, E, Puccetti, P, Wolf, SF and Bistoni, F. (1994) IL-12 is both required and prognostic *in vivo* for T helper type 1 differentiation in murine candidiasis. *J Immunol* **152**: 5167-5175.

Rosenstein, Y, Ratnofsky, S, Burakoff, SJ and Herrmann, SH. (1989) Direct evidence for binding of CD8 to HLA class I antigens. *J Exp Med* **169**: 149-160.

Roy, M, Aruffo, A, Ledbetter, J, Linsley, P, Kehry, M and Noelle, R. (1995) Studies on the interdependence of gp39 and B7 expression and function during antigen-specific immune responses. *Eur J Immunol* **25**: 596-603.

Ruppert, J, Grey, HM, Sette, A, Kubo, RT, Sidney, J and Celis, E. (1993) Prominent role of secondary anchor residues in peptide binding to A2.1 molecules. *Cell* **74**: 929-937.

Sad, S, Marcotte, R and Mosmann, TR. (1995) Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. *Immunity* **2**: 271-279.

Sadegh-Nasseri, S, Stern, LJ, Wiley, DC and Germain, RN. (1994) MHC class II function preserved by low-affinity peptide interactions preceding stable binding. *Nature* **370**: 647-650.

Saint-Ruf, C, Ungewiss, K, Groettrup, M, Bruno, L, Fehling, HJ and Von Boehmer, H. (1994) Analysis and expression of a cloned pre-T cell receptor gene. *Science* **266**: 1208.

Salgame, P, Abrams, JS, Clayberger, C, Goldstein, H, Convit, J, Modlin, RL and Bloom, BR. (1991) Differing lymphokine profiles of functional subsets of human CD4+ and CD8+ T cell clones. *Science* **254**: 279-282.

Salter, RD, Norment, AM, Chen, BP, Clayberger, C, Kresky, AM, Littman, DR and Parham, P. (1989) Polymorphism in the α3 domain of HLA-A molecules affects binding to CD8. *Nature* **338**: 345-347.

Sanger, F, Nicklen, S and Coulsen, AR. (1977) DNA-sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* **74**: 5463-5467.

Saoudi, A, Seddon, B, Fowell, D and Mason, D. (1996) The thymus contains a high frequency of cells that prevent autoimmune diabetes on transfer into prediabetic recipients. *J Exp Med* **184**: 2393-2398.

Satyaraj, E, Rath, S and Bal, V. (1994) Induction of tolerance in freshly isolated alloreactive CD4+ T cells by activated T cell stimulators. *Eur J Immunol* **24**: 2457-2461.

Sayre, PH, Chang, HC, Hussey, RE, Brown, NR, Richardson, NE, Spagnoli, G, Clayton, LK and Reinherz, EL. (1987) Molecular cloning and expression of T11 cDNAs reveal a receptor-like structure on human T lymphocytes. *Proc Natl Acad Sci USA* **84**: 2941-2945.

Schafer, PH and Pierce, SK. (1994) Evidence for dimers of MHC class II molecules in B lymphocytes and their role in low affinity T cell responses. *Immunity* **1**: 699-707.

Schild, H, Mavaddat, N, Litzenberger, C, Ehrich, EW, Davis, MM, Bluestone, JA, Matis, L, Draper, RK and Chien, Y-H. (1994) The nature of major histocompatibility recognition by γδ T cells. *Cell* **76**: 29-37.

Schmitz, J, Thiel, A, Kühn, R, Rajewsky, K, Müller, W, Assenmacher, M and Radbruch, A. (1994) Induction of interleukin 4 (IL-4) expression in T helper (Th) cells is not dependent on IL-4 from non-Th cells. *J Exp Med* **179**: 1349-1353.

Schumacher, TNM, Kantesaria, DV, Heemels, M-T, Ashton-Rickardt, PG, Shepherd, JC, Fruh, K, Yang, Y, Peterson, PA, Tonegawa, S and Ploegh, HL. (1994) Peptide length and sequence specificity of the mouse TAP1/TAP2 translocator. *J Exp Med* **179**: 533-540.

Schwartz, RH. (1992) Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell* **71**: 1065-1068.

Schwartz, RH. (1996) Models of T cell anergy: Is there a common molecular mechanism? *J Exp Med* **184**: 1-8.

Scott, P, Natovitz, P, Coffman, RL, Pearce, E and Sher, A. (1988) Immunoregulation of cutaneous leishmaniasis. *J Exp Med* **168**: 1675-1684.

Scully, R, Qin, S, Cobbold, S and Waldmann, H. (1994) Mechanisms in CD4 antibody-mediated transplantation tolerance: kinetics of induction, antigen dependency and role of regulatory T cells. *Eur J Immunol* **24**: 2383-2392.

Secrist, H, Chelen, CJ, Wen, Y, Marshall, JD and Umetsu, DT. (1993) Allergen immunotherapy decreases interleukin 4 production in CD4+ T cells from allergic individuals. *J Exp Med* **178**: 2123-2130.

Seder, RA, Boulay, JL, Finkelman, F, Barbier, S, Ben-Sasson, SZ, Le Gros, G and Paul, WE. (1992) CD8+ T cells can be primed *in vitro* to produce IL-4. *J Immunol* **148**: 1652-1656.

Seder, RA, Gazzinelli, RT, Sher, A and Paul, WE. (1993) Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon γ production and diminishes interleukin 4 inhibition of such priming. *Proc Natl Acad Sci USA* **90**: 10188-10192.

Seemüller, E, Lupas, A, Stock, D, Löwe, J, Huber, R and Baumeister, W. (1995) Proteasome from *Thermoplasma acidophilum*: a threonine protease. *Science* **268**: 579-582.

Sette, A, Southwood, S, Miller, J and Appella, E. (1995) Binding of major histocompatibility complex class II to the invariant chain-derived peptide, CLIP, is regulated by allelic polymorphism in class II. *J Exp Med* **181**: 677-683.

Shahanian, A, Pfeffer, K, Lee, KP, Kündig, TM, Kishihara, K, Wakeham, A, Kawai, K, Ohashi, PS, Thompson, CB and Mak, TW. (1993) Differential T cell costimulatory requirements in CD28-deficient mice. *Science* **261**: 609-612.

Shepherd, JC, Schumacher, TNM, Ashton-Rickardt, PG, Imaeda, S, Ploegh, HL, Janeway Jr., CA and Tonegawa, S. (1993) TAP1-dependent peptide translocation *in vitro* is ATP dependent and peptide selective. *Cell* **74**: 577-584.

Sherman, MA, Weber, DA and Jensen, PE. (1995) DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. *Immunity* **3**: 197-205.

Shevach, EM and Rosenthal, AS. (1973) Function of macrophages in antigen recognition by guinea pig T lymphocytes. II. Role of the macrophage in the regulation of genetic cotrol of the immune response. *J Exp Med* **138**: 1213-1229.

Shimizu, Y and DeMars, R. (1989) Production of human cells expressing individual transferred HLA-A, -B, -C genes using an HLA-A, -B, -C null human cell line. *J Immunol* **142**: 3320.

Siegrist, C-A, Martinez-Soria, E, Kern, I and Mach, B. (1995) A novel antigen-processing-defective phenotype in major histocompatibility complex class II-positive CIITA transfectants is corrected by interferon-γ. *J Exp Med* **182**: 1793-1799.

Sloan, VS, Cameron, P, Porter, G, Gammon, M, Amaya, M, Mellins, E and Zaller, DM. (1995) Mediation of HLA-DM of dissociation of peptides from HLA-DR. *Nature* **375**: 802-806.

Sloan-Lancaster, J, Evavold, BD and Allen, PM. (1993) Induction of T cell anergy by altered T cell-receptor ligand on live antigen-presenting cells. *Nature* **363**: 156-159.

Sloan-Lancaster, J, Evavold, BD and Allen, PM. (1994a) Th2 cell clonal anergy as a consequence of partial activation. *J Exp Med* **180**: 1195-1205.

Sloan-Lancaster, J, Shaw, AS, Rothbard, JB and Allen, PM. (1994b) Partial T cell signalling: altered phospho-zeta and lack of zap 70 recruitment in APL-induced T cell anergy. *Cell* **79**: 913-922.

Snapper, CM and Paul, WE. (1987) Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* **236**: 944-947.

Snyder, HL, Yewdell, JW and Bennink, JR. (1994) Trimming of antigenic peptides in an early secretory compartment. *J Exp Med* **180**: 2389-2394.

Soloway, P, Fish, S, Passmore, H, Gefter, M, Coffee, R and Manser, T. (1991) Regulation of the immune response to peptide antigens: Differential induction of immediate-type hypersensitivity and T cell proliferation due to changes in either peptide structure or major histocompatibility complex haplotype. *J Exp Med* **174**: 847-858.

Southern, E. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**: 503-517.

Spaner, D, Migita, K, Ochi, A, Shannon, J, Miller, RG, Pereira, P, Tonegawa, S and Philips, RA. (1993) γδ T cells differentiate into a functional but non-proliferative state during a normal immune response. *Proc Natl Acad Sci USA* **90**: 8415-8419.

Sperling, AI, Linsley, PS, Barrett, TA and Bluestone, JA. (1993) CD28-mediated co-stimulation is necessary for the activation of T cell receptor-γδ+ T lymphocytes. *J Immunol* **151**: 6043-6050.

St. Louis, JM, Pasick, JM, Stein, C, Freeman, D, Singh, B, Dales, S and Strejan, GH. (1994) Lewis rat T cells can reutilise, process, and present myelin basic protein to antigen-specific T cell lines. *Cell Immunol* **156**: 36-53.

Stanfield, RL, Takimoto-Kamimura, M, Rini, JM, Profy, AT and Wilson, IA. (1993) Major antigen-induced domain rearrangements in an antibody. *Structure* **1**: 83-93.

Stebbins, CC, Loss Jr., GE, Elias, CG, Chervonsky, A and Sant, AJ. (1995) The requirement for DM in class-II restricted antigen presentation and SDS-stable dimer formation is allele and species dependent. *J Exp Med* **181**: 223-234.

Steimle, V, Otten, LA, Zufferey, M and Mach, B. (1993) Complementation cloning of an MHC class II transactivator mutated in hereditary MHC class II deficiency (or bare lymphoctye syndrome). *Cell* **75**: 135-146.

Steinman, RM. (1991) The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* **9**: 271-296.

Stern, LJ, Brown, JH, Jardetzky, TS, Gorga, JC, Urban, RG, Strominger, JL and Wiley, DC. (1994) Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature* **368**: 215-221.

Su, B, Jacinto, E, Hibi, M, Kallunki, T, Karin, M and Ben-Neriah, Y. (1994) JNK is involved in signal integration during costimulation of T lymphocytes. *Cell* **77**: 727-736.

Su, B and Karin, M. (1996) Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr Opin Immunol* **8**: 402-411.

Sugita, M and Brenner, M. (1994) An unstable β2 microglobulin: major histocompatibility complex class I heavy chain intermediate dissociates from calnexin and then is stabilised by binding peptide. *J Exp Med* **180**: 2163-2171.

Sundstedt, A, Sigvardsson, M, Leanderson, T, Hedlund, G, Kalland, T and Dohlsten, M. (1996) *In vivo* anergised CD4+ T cells express perturbed AP-1 and NF-Kappa B transcription factors. *Proc Natl Acad Sci USA* **93**: 979-984.

Suto, R and Srivastava, PK. (1995) A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science* **269**: 1585-1588.

Suzuki, H, Punt, J, Granger, L and Singer, A. (1995) Asymmetric signalling requirements for thymocyte commitment to the CD4+ versus CD8+ T cell lineages: a new perspective on thymic commitment and selection. *Immunity* **2**: 413-425.

Sykulev, Y, Brunmark, A, Jackson, M, Cohen, RJ, Peterson, PA and Eisen, HN. (1994) Kinetics and affinity of reactions between an antigen-specific T cell receptor and peptide-MHC complexes. *Immunity* **1**: 15-22.

Symer, DE, Dintzis, RZ, Diamond, DJ and Dintzis, HM. (1992) Inhibition or activation of human T cell receptor transfectants is controlled by defined, soluble antigen arrays. *J Exp Med* **176**: 1421-1430.

Tector, M and Salter, RD. (1995) Calnexin influences folding of human class I histocompatibility proteins but not their assembly with β2-microglobulin. *J Biol Chem* **270**: 19638-19642.

Tivol, EA, Borriello, F, Schweitzer, AN, Lynch, WP, Bluestone, JA and Sharpe, AH. (1995) Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue detruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* **3**: 541-547.

Tivol, EA, Schweitzer, AN and Sharpe, AH. (1996) Costimulaton and autoimmunity. *Curr Opin Immunol* **8**: 822-830.

Tourne, S, Nakano, N, Viville, S, Benoist, C and Mathis, D. (1995) The influence of invariant chain on the positive selection of single T cell receptor specificities. *Eur J Immunol* **25**: 1851-1856.

Tschoetschel, U, McCoy, KL and Reske-Kunz, AB. (1996) Augmented antigen presentation by mouse Ia+ T clone cells BK-BI-2.6.04.1 mediated by transferrin receptors. *Scand J Immunol* **43**: 500-506.

Tsitoura, D, Verhoef, A and Lamb, JR. (1996) Altered T cell ligands derived from a major house dust mite allergen enhance IFN-gamma but not IL-4 production by human CD4+ T cells. *J Immunol* **157**: 2160-2165.

Valitutti, S, Müller, S, Cella, M, Padovan, E and Lanzavecchia, A. (1995) Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* **375**: 148-151.

Van Der Heyde, HC, Manning, DD and Weidanz, WP. (1993) Role of CD4+ T cells in the expansion of the CD4-, CD8- γδ T cell subset in the spleens of mice during blood stage malaria. *J Immunol* **151**: 6311-6317.

Van Kaer, L, Ashton-Rickardt, PG, Eichelberger, M, Gaczynska, M, Nagashima, K, Rock, KL, Goldberg, AL, Doherty, PC and Tonegawa, S. (1994) Altered peptidase and viral-specific T cell response in LMP2 mutant mice. *Immunity* **1**: 533-541.

Van Oers, N, Killeen, N and Weiss, A. (1994) ZAP-70 is constitutively associated with tyrosine-phosphorylated TcR zeta in murine thymocytes and lymph node T cells. *Immunity* **1**: 675-685.

Van Oers, NS, Killeen, N and Weiss, A. (1996) Lck regulates the tyrosine phosphorylation of the T cell receptor subunits and ZAP-70 in murine thymocytes. *J Exp Med* **183**: 1053-1062.

Van Santen, HM, Woolsey, A, Ashton Rickardt, PG, Van Kaer, L, Baas, EJ, Berns, A, Tonegawa, S and Ploegh, HL. (1995) Increase in positive selection of CD8+ T cells in TAP-1 mutant mice by human β2m transgene. *J Exp Med* **181**: 787-792.

Vercelli, D, Jabara, HH, Arai, K and Geha, RS. (1989) Induction of human IgE synthesis requires interleukin 4 and T/B interactions involving the T cell receptor/CD3 complex and MHC class II antigens. *J Exp Med* **169**: 1295-1307.

Vignali, DAA and Strominger, JL. (1994) Co-receptor function and the characteristics of MHC-bound peptides: a common link? *The Immunologist* **2**: 93-99.

Von Boehmer, H. (1986) The selection of the αβ heterodimeric T cell receptor for antigen. *Immunol Today* **7**: 333-336.

Wagner, EF, Covarrubias, L, Stewart, TA and Mintz, B. (1983) Prenatal lethalities in mice homozygous for human growth hormone gene sequences integrated in the germ line. *Cell* **35**: 647-655.

Wang, Z-E, Zheng, S, Corry, DB, Dalton, DK, Seder, RA, Reiner, SL and Locksley, RM. (1994) Interferon γ-independent effects of interleukin 12 administered during acute or established infection due to *Leishmania major*. *Proc Natl Acad Sci USA* **91**: 12932-12936.

Wange, RL, Guitian, R, Isakov, N, Watts, JD, Aebersold, R and Samelson, LE. (1995) Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70. *J Biol Chem* **270**: 18730-18733.

Waterhouse, P, Penninger, JM, Timms, E, Wakeham, A, Shahinian, A, Lee, KP, Thompson, CB, Griesser, H and Mak, TW. (1995) Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. *Science* **270**: 985-988.

Wei, ML and Cresswell, P. (1992) HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. *Nature* **356**: 443-446.

Weiner, HL, Friedman, A, Miller, A, Khoury, SJ, al-Sabbagh, A, Santos, L, Sayegh, M, Nussenblatt, RB, Trentham, DE and Hafler, DA. (1994) Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annu Rev Immunol* **12**: 809-837.

Weiner, HL, Mackin, GA, Matsui, M, Orav, EJ, Khoury, SJ, Dawson, DM and Hafler, DA. (1993) Double-blind pilot trial of oral tolerisation with myelin antigens in multiple sclerosis. *Science* **259**: 1321-1324.

Weiss, A and Littman, DR. (1994) Signal transduction by lymphocyte antigen receptors. *Cell* **76**: 263-274.

Wells, FB, Tatsumi, Y, Bluestone, JA, Hedrick, SM, Allison, JP and Matis, LA. (1993) Phenotypic and functional analysis of positive selection in the γδ lineage. *J Exp Med* **177**: 1061-1070.

Wenzel, T and Baumeister, W. (1995) Conformational constraints in protein degradation by the 20S proteasome. *Struct Biol* **2**: 199-204.

West, MA, Lucocq, JM and Watts, C. (1994) Antigen processing and class II MHC peptide-loading compartments in human B-lymphoblastoid cells. *Nature* **369**: 147-151.

Wiest, DL, Yuan, L, Jefferson, J, Benveniste, P, Tsokos, M, Klausner, RD, Glimcher, LH, Samelson, LE and Singer, A. (1993) Regulation of T cell receptor expression in immature CD4+CD8+ thymocytes by p56lck tyrosine kinase: basis for differential signalling by CD4 and CD8 in immature thymocytes expressing both coreceptor molecules. *J Exp Med* **178**: 1701-1712.

Wilkinson, RW, Anderson, G, Owen, JJT and Jenkinson, EJ. (1995) Positive selection of thymocytes involves sustained interactions with the thymic microenvironment. *J Immunol* **155**: 5234-5240.

Williams, TM, Moolten, D, Burlein, J, Romano, J, Bhaerman, R, Godillot, A, Mellon, M, Rauscher III, FJ and Kant, JA. (1991) Identification of a zinc finger protein that inhibits IL-2 gene expression. *Science* **254**: 1791-1794.

Windhagen, A, Scholz, C, Hollsberg, P, Fukaura, H, Sette, A and Hafler, DA. (1995) Modulation of cytokine patterns of human autoreactive T cell clones by a single amino acid substitution of their peptide ligand. *Immunity* **2**: 373-380.

Woods, A, Chen, HY, Trumbauer, ME, Sirotina, A, Cummings, R and Zaller, DM. (1994) Human major histocompatibility complex class II-restricted T cell responses in transgenic mice. *J Exp Med* **180**: 173-181.

Wotton, D. *Regulation of transcription of the human CD2 and T cell receptor β chain genes.* PhD. Open University, 1993.

Wotton, D, Higgins, JA, O'Hehir, RE, Lamb, JR and Lake, RA. (1995) Differential induction of the NF-AT complex during restimulation and the induction of T cell anergy. *Human Immunol* **42**: 95-102.

Wu, L, Scollay, R, Egerton, M, Pearse, M, Spangrude, GJ and Shortman, K. (1991) CD4 expressed on earliest T-lineage precursor cells in the adult murine thymus. *Nature* **349**: 71.

Wynn, TA, Eltoum, I, Oswald, IP, Cheever, AW and Sher, A. (1994) Endogenous interleukin 12 (IL-12) regulates granuloma formation induced by eggs of *Schistosoma mansoni* and exogenous IL-12 both inhibits and prophylactically immunises against egg pathology. *J Exp Med* **179**: 1551-1561.

Wyss-Coray, T, Gallati, H, Pracht, I, Limat, A, Mauri, D, Frutig, K and Pichler, WJ. (1993) Antigen-presenting human T cells and antigen-presenting B cells induce a similar cytokine profile in specific T cell clones. *Eur J Immunol* **23**: 3350-3357.

Xu, M, Capraro, GA, Daibata, M, Reyes, VE and Humphreys, RE. (1994) Cathepsin B cleavage and release of invariant chain from MHC class II molecules follow a staged pattern. *Mol Immunol* **31**: 723-731.

Yamamota, M, Fujihashi, K, Beagley, KW, J.R, M and Kiyono, H. (1993) Cytokine synthesis by intestinal intraepithelial lymphoytes. *J Immunol* **150**: 106-114.

Yamamoto, K, Fukui, Y, Esaki, Y, Inamitsu, T, Sudo, T, Yamane, K, Kamikawaji, N, Kimura, A and Sasazuki, T. (1994) Functional interaction between human histocompatibility leucocyte antigen (HLA) class II and mouse CD4 molecule in antigen recognition by T cells in HLA-DR and DQ transgenic mice. *J Exp Med* **180**: 165-171.

Yamamura, M, Uyemura, K, Dean, RJ, Weinberg, K, Rea, TH, Bloom, BR and Modlin, R. (1991) Defining protective responses to pathogens: cytokine profiles in leprosy lesions. *Science* **254**: 277-279.

Yeung, RSM, Penninger, JM, Kündig, TM, Law, Y, Yamamoto, K, Kamikawaji, N, Burkly, L, Sasazuki, T, Flavell, R, Ohashi, PS and Mak, TM. (1994) Human CD4-major histocompatibility complex class II (DQw6) transgenic mice in an endogenous CD4/CD8-deficient background: reconstitution of phenotype and human-restricted function. *J Exp Med* **180**: 1911-1920.

Yoshimoto, T and Paul, WE. (1994) $CD4^{pos}$, $NK1.1^{pos}$ T cells promptly produce interleukin 4 in response to *in vivo* challenge with anti-CD3. *J Exp Med* **179**: 1285-1295.

Yssel, H, Fasler, S, Lamb, JR and De Vries, JE. (1994) Induction of non-responsiveness in human allergen-specific type 2 helper cells. *Curr Opin Immunol* **6**: 847-852.

Yu, DTY, Winchester, RJ, Fu, SM, Gibofsky, A, Ko, HS and Kunkel, HG. (1980) Peripheral blood Ia-positive T cells. Increases in certain diseases and after immunisation. *J Exp Med* **151**: 91-100.

Zacharchuk, CM, Mercep, M and Ashwell, JD. (1990) Programmed T lymphocyte death: cell activation- and steroid-induced pathways are mutually antagonistic. *J Immunol* **145**: 4037-4045.

Zinkernagel, RM and Doherty, PC. (1974) Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* **248**: 701-702.

Zlotnik, A and Moore, TA. (1995) Cytokine production and requirements during T-cell development. *Curr Opin Immunol* **7**: 206-213.

Zorbas, M and Scollay, R. (1993) Development of γδ T cells in the adult murine thymus. *Eur J Immunol* **23**: 1655-1660.

Zúñiga-Pflücker, JC, Jiang, D and Lenardo, MJ. (1995) Requirement for TNF-α and IL-1α in foetal thymocyte commitment and differentiation. *Science* **268**: 1906.

Zúñiga-Pflücker, JC and Lenardo, MJ. (1996) Regulation of thymocyte development from immature progenitors. *Curr Opin Immunol* **8**: 215-224.

Zúñiga-Pflücker, JC, Schwartz, HL and Lenardo, MJ. (1993) Gene transcription in differentiating immature T cell $receptor^{neg}$ thymocytes resembles antigen-activated mature cells. *J Exp Med* **178**: 1139.