T cell selection in the thymus

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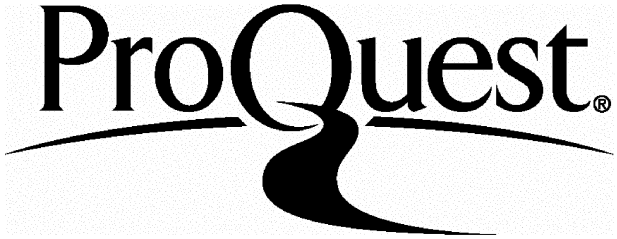

Abbreviations

αMEM	α minimum essential medium
β2m	β-2 microglobulin
BM	bone marrow
bp	base pairs
BSA	bovine serum albumin
CDR	complementality determining region
CFU-S	colony forming units of spleen
CTL	cytotoxic T lymphocyte
DMEM	Dulbecco's modified Eagle's essential medium
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
Eαts	Eα/tsA58
EDTA	ethylendiamine tetra-acetic acid
FACS	fluoresence activated cell sorter
FCS	fetal calf serum
FTOC	fetal thymic organ culture
g	gram(s)
H2ts	H-2K^b/tsA58
HSA	heat stable antigen
Hyb	hybridisation
IFN-γ	interferon-γ
IMDM	Iscove's modified Dulbecco's medium
MHC	major histocompatibility complex
ml	milli liter(s)
Mls	minor lymphocyte stimulating (antigen)
mM	milli molar
NP	nucleoprotein
Ntg	non-transgenic
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RAG	recombination activating gene
RNA	ribonucleic acid
RT	reverse transcription
SDS	sodium dodecyl sulfate
SP	single positive
SSC	3 M NaCl, 0.35 M sodium citrate
ssDNA	salmon sperm DNA
TAE	40 mM Tris-acetate, 1 mM EDTA
TBE	45 mM Tris-borate, 1 mM EDTA
TCR	T cell antigen receptor
TE	10 mM Tris, 1 mM EDTA
TES	10 mM Tris, 1 mM EDTA, 0.5 % SDS
TM	Thy-1/c-myc
TNE	50 mM Tris, 100 mM NaCl, 100 mM EDTA

Abstract

Expression of T cell antigen receptors (TCR) and their interaction with stromal ligands determine the fate of developing T cells in the thymus. To dissect molecular mechanisms involved in T cell selection, in vitro differentiation and selection system was developed using the thymocytes from $\alpha\beta$ TCR (F5) transgenic mice, thymic stromal cell lines derived from oncogene transgenic mice, and synthetic peptides presented by class I major histocompatibility complex (MHC).

Chapter 3 describes mice carrying a temperature sensitive SV40 large T antigen under the control of class I H-2K^b promoter (H2ts mice). These mice develop normally except for the enlargement of the thymus in the adults. Conditionally immortalised thymic stromal cell lines were established from such hyperplastic thymic tissues. To compare antigen presentation capacities, thymic cortical epithelial cell lines and freshly isolated thymic dendritic cells were co-cultured with immature thymocytes or mature T cells from F5 TCR transgenic mice in the presence or absence of cognate peptide. The results show that cortical epithelial cells are as efficient as dendritic cells in negative selection of F5 thymocytes, but not in activating mature F5 T cells. In an attempt to establish cell lines which support positive selection of T cells, adherent cells in a thymic tumour of an H2ts mouse were purified using magnetic beads coated with antibodies against CD45, class II MHC, and a medullary epithelial marker. Several epithelial cell lines expressing class II H-2A^b and a cortical marker ER-TR4 were established, and their function was assessed by reaggregate culture (chapter 5). Immature T cell lines were derived from mice which express c-myc proto-oncogene under the control of Thy1 gene promoter (TM mice). These mice develop thymic tumours consisting predominantly of CD4$^+$CD8$^+$ (DP) cells which are mono- or oligo-clonal. Overexpression of c-myc is associated with increased apoptosis of thymocytes in vivo, and DP cell lines derived from the tumours retained their abilities to undergo apoptosis upon TCR stimulation. However, it was not possible to induce differentiation of these DP cells to CD4 or CD8 single positive (SP) cells.

Chapter 4 describes development of T cells in mice transgenic for an $\alpha\beta$ TCR which was isolated from a cytotoxic clone F5 specific for a peptide from influenza nucleoprotein and class I H-2D^b. Ontogeny of T cells in F5 mice is largely similar to that in normal mice for expression of CD4, CD8, and TCR, except for slightly earlier appearance of immature CD8 SP cells and DP cells. The data suggest that expression of functional $\alpha\beta$ TCR enhances transition from CD4$^-$CD8$^-$ (DN) to DP cells. Addition of cognate peptide in F5 fetal thymic organ culture causes severe block at the stage between immature CD8 SP and DP cells. A significant number of DP cells which develop in the presence of cognate peptide do not express F5 TCR and are likely to escape from cell death by expression of endogenous TCR since these do not appear in F5/RAG1$^{-/-}$ thymic lobes.

Chapter 5 describes effects of thymic stromal cell lines and peptide analogues on F5 T cell development in vitro. F5 TCR transgenic mice were bred to non-selecting MHC backgrounds such as H-2^q or β-2 microgrobulin (β2m)-deficient mice, in which T cell development was arrested at DP stage. Total thymocytes, including T cell progenitors and stromal cells expressing non-selecting MHC, were then reaggregated with thymic cortical epithelial cell lines which express class I H-2^b. The data show that development of $F5^{q/q}$ or F5/$\beta 2m^{-/-}$ T cells can be restored by such epithelial cell lines. Further in an attempt to identify peptide/MHC ligands which mediate signals for positive selection of F5 T cells, peptide analogues were designed by introducing single amino acid substitutions in nonameric cognate peptide. The present study on F5 T cell development in fetal thymic organ culture confirms importance of side chains of residues at positions 4 and 7 of the peptide for interaction with TCR, as predicted from crystallographic data by others. Two of the peptides exhibit antagonistic activity and one of them appears to augment positive selection of F5 T cells if provided with suboptimal dose of cognate peptide. These data may imply significance of the presence of heterogeneous peptides in vivo for positive selection of T cells.

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Chapter 1 Introduction

1.1 Antigen recognition by T cells

T cells are derived from haematopoietic stem cells and differentiate in the thymus (Moore and Owen, 1967; Owen and Ritter, 1969). Being a subset of circulating blood cell, T cells play a crucial role in cell mediated immunity. T cells recognise antigens by their cell surface receptors (T cell antigen receptor, TCR), which are composed of variable chains (α/β or γ/δ TCR) and invariable CD3-$\gamma\delta\epsilon\zeta$ complexes (reviewed in Davis, 1990). The variable regions of α and β chains are thought to have immunoglobulin-like structure characterised by complementarity determining regions (CDR) looping out of constant domains of β sheet sandwiches (Chothia et al., 1988). The ligand recognised by TCR is peptide bound to membrane glycoprotein products of the major histocompatibility complex (MHC) genes (Townsend et al., 1985). The peptide binding pocket of MHC is made of two α helices on the floor of a β strand sheet (Davis and Bjorkman, 1988). Since MHC genes are highly polymorphic in amino acids facing inside the peptide binding groove, each MHC is thought to accommodate a different set of peptides. In addition, co-engagement of class I MHC with CD8 (expressed on cytotoxic T cells) and class II MHC with CD4 (helper T cells) co-receptors is required for antigen recognition by T cells.

TCR molecules are coupled with signal transduction mechanisms which are linked to cytotoxic functions in CD8 T cells, or helper functions in CD4 T cells. Effector functions of T cells are tightly regulated at multiple levels to prevent destruction of self entities. In essence, the ability to distinguish 'self' from 'non-self' lies at the heart of immune system. To understand how immune system achieves such self/non-self discrimination, it is important to know the structural basis of antigen recognition by T cells.

1.2 T cell antigen receptor

T cell receptor complexes consist of at least eight different transmembrane proteins, two of which are variable, determine specificity, and consist of either α/β or γ/δ heterodimers. Most of T cells in the blood and peripheral lymphoid organs are $\alpha\beta$ T cells. $\gamma\delta$ T cells are a distinct lineage of T cells of largely unknown functions and are mostly distributed in the epithelial tissues of the gut, uterus, and skin.

Variable chains of TCR are encoded by four genetic loci. Each locus consists of variable (V), diversity (D, only for β and δ genes), joining (J), and constant (C) regions. Each of these regions contains a number of homologous genes, and there are ~100 V_α, 50 J_α, and 1 C_α gene segments, and ~30 V_β, 2 D_β, 12 J_β, and 2 C_β genes in mice (Davis and Bjorkman, 1988). During early T cell development, these regions are rearranged in the order of D_β to

J_β, V_β to $D_\beta J_\beta$, and then V_α to J_α. Although mechanisms of V(D)J recombination remain largely unknown, the reaction is thought to involve recognition of conserved recombination signal (RS) sequences (heptamer-spacer-nonamer motifs) that flank germline V, D, and J segments, introduction of site-specific double-strand DNA breaks between the elements to be joined and the RS sequences, potential deletion or inversion, with or without addition of nucleotides at coding junctions, polymerization and ligation. Recombination activating genes RAG1 and RAG2 can introduce recombination activities in nonlymphoid cells (Schatz et al., 1989) and have been shown to be indispensable for V(D)J recombination in lymphoid cells (Mombaerts et al., 1992; Shinkai et al., 1992). Function of RAG1 appears to be important only for TCR and immunoglobulin gene rearrangements, although its role in the recombination mechanisms remains to be elucidated (Silver et al., 1993). Terminal deoxyribonucleotide transferase (TdT) may confer random insertion of oligo-nucleotides between coding joints (N regions, Gilfillan et al., 1993; Komori et al., 1993). Generation of coding joints in V(D)J recombination share common mechanisms with double-strand DNA break repair, since mutations in scid (Kirchgessner et al., 1995; Lees-Miller et al., 1995) and other genes (Pergola et al., 1993; Taccioli et al., 1993) affect both processes.

As lymphocytes are diploid cells, each T cell has two copies (alleles) of TCR genes and could potentially express more than one antigen receptors if both TCR alleles are functionally rearranged. Indeed TCR α loci, which require single recombination

events between V_α and J_α regions, are productively rearranged in both alleles in one third of T cells (Malissen et al., 1992), and a significant number of peripheral T cells express two α chains on cell surfaces (Padovan et al., 1993). On the other hand, T cells undergo only one productive $V_\beta D_\beta J_\beta$ rearrangement and the other allele either remains in germline configuration or only rearranges D_β-J_β but not V_β-D_β (Uematsu et al., 1988; Malissen et al., 1992). Although the mechanism of allelic exclusion is unknown, the fact that it operates in V-D but not in D-J (β chains) nor V-J (α chains) recombination may suggest mechanistic constraints associated with V-D recombination. Such a genetic model remains to be tested since recombination mechanisms are largely unknown. Alternatively, cellular mechanisms may eliminate possibilities of generating T cells with two functionally rearranged β chains. Since T cells rearrange TCR genes sequentially from D_β->J_β, V_β->D_β, and then V_α->J_α during development, signals from functionally rearranged $V_\beta D_\beta J_\beta$ gene products may shut down further recombination in the $_\beta$ loci and/or induce T cell differentiation to the next stage. In line with this hypothesis, RAG1 gene expressed in DN cells is down-regulated in their immediate progeny which have functionally rearranged TCR β genes (Spain et al., 1994). Such a feedback mechanism for regulation of V(D)J recombination is supported by data of RAG1 and RAG2 gene down-regulation in DP cells either in a process of positive selection <u>in vivo</u> (Borgulya et al., 1992; Brandle et al., 1992) or after cross-linking of TCR <u>in vitro</u> (Turka et al., 1991). Suppression of endogenous TCR β gene rearrangement in transgenic mice carrying TCR β genes is also

consistent with this model (Uematsu et al., 1988).

Products of functionally rearranged TCR genes are transported to the cell surface in complex with invariant CD3 chains consisting of γ/ϵ and δ/ϵ heterodimers and ζ chains as either homodimers or heterodimers with CD3-η or FcϵR1γ chains (reviewed in Weiss and Littman, 1994). Since TCR α and β subunits have only small cytoplasmic domains, those of CD3 chains are thought to link antigen-specific α/β subunits to intracellular signal transduction mechanisms. Two classes of protein tyrosine kinases (PTK), i.e. src and syk/ZAP-70 family members, interact with conserved (D/E)XXYXXL$(X)_{6-8}$ motifs of the CD3 subunits. Upon stimulation of TCR, co-receptor (CD4 or CD8)-associated p56lck is brought close to the CD3 complex and phosphorylates tyrosine residues of the CD3 cytoplasmic domains, providing binding sites for SH2 domains of other proteins. When bound to CD3-ζ, ZAP-70 is activated by phosphorylation and in turn phosphorylates down-stream target proteins, leading to activation of phospholipase C-γ1 (PLC-γ1), Phosphoinositide (PI) 3-kinase, and the ras pathway. PLC-γ1 can associate with p56lck and catalyses production of diacylglycerol which in turn activates protein kinase C. PI 3-kinase binds to p59fyn and generates inositol 1, 4, 5-triphosphate which causes mobilisation of intracellular Ca^{++}. TCR-induced actitivation of PTK and PKC also results in phosphorylation of guanine nucleotide exchange proteins (vav and grb2/sos) and GTPase activating proteins, leading to a cascade of activation events of raf-1, mekk, erk (mapk or mek), and jnk (jun kinases), which in turn will modify gene expression.

1.3 Antigen presentation

The class I and class II MHC molecules are two sets of devices which present antigens derived from either endogenous or exogenous proteins respectively (reviewed in Germain, 1994). In the class I pathway, peptide fragments are generated in the cytoplasm presumably by proteasome complexes, and then are transported into the endoplasmic reticulum (ER) by TAP1 and TAP2 gene products. After binding peptides, class I MHC molecules are released from chaperons, which anchor empty class I MHC molecules to the ER, and are transported to the cell surface. On the other hand, class II MHC molecules are initially in complex with invariant chains and are targeted to endosome-lysosome compartments, where peptide fragments are generated by proteolysis of endocytosed proteins. The acidic condition allows class II MHC molecules to dissociate with invariant chains and associate with peptide fragments. Peptide/class II MHC complexes are transported to the cell surface and afterwards recycled by endocytosis. Intracellular pathogens, such as virus, are mainly presented by the class I MHC pathway leading to the activation of cytotoxic T cells which destroy virus-infected cells. Extracellular pathogens, such as bacteria, are presented by the class II pathway which is linked to helper T cell activity resulting in secretion of neutralising antibodies by activated B cells. Several homologous genes encode different MHC molecules in an individual (H-2D, K, L, and H-2A, H-2E in mice). In addition, each MHC molecule is highly polymorphic and thus a

variety of MHC molecules exist in a population.

The class I MHC molecule is composed of a heavy chain and a smaller molecule called β-2 microglobulin (β2m). X-ray crystallographic studies on human leucocyte antigen (HLA)-A2 class I molecule first unravelled the structure of the peptide binding groove which consists of two antiparallel α helices on the floor of a β-strand sheet (Davis and Bjorkman, 1988). Subsequently, human HLA-Aw68 (Silver et al., 1992), HLA-B27 (Madden et al., 1992), mouse H-2K^b (Fremont et al., 1992; Zhang et al., 1992), and mouse H-2D^b (Young et al., 1994) class I molecules were crystallised in complex with single peptides. In addition, sequence analysis of acid-eluted endogenous peptides revealed that peptides bound to class I MHC molecules are 7-9 amino acids in length and have one or two conserved residue(s) (Falk et al., 1991). Peptides are accommodated in the groove of class I MHC molecule through interactions between conserved residues of the MHC and backbone or terminal residues of the peptide, as well as by interactions between polymorphic residues of the MHC and side chains of peptide anchor residues. Many interactions between peptides and MHC molecules are also mediated by water molecules present in the pocket. A large portion of the peptide is embedded in the groove in a stretched configuration, and only a limited number of specific amino acid residues of the peptide are directly accessible to TCR. However, binding of peptides to MHC can change the alignment between α helices and β sheets which could be recognised by TCR.

Class II MHC molecules are heterodimers of α and β transmembrane subunits and their crystal structure is similar to that of class I MHC (Brown et al., 1993). The peptide binding cleft of the class II MHC, however, is open at both ends and can accommodate variable length of peptides (13-18 amino acids). Sequence analysis of peptides eluted from class II MHC molecules also revealed allele-specific residues (Hammer et al., 1992).

Although it is largely unknown how the TCR interacts with the peptide/MHC complex, analogies could be drawn from the physical properties of antigen-antibody interactions (reviewed in Davies and Padlan, 1990). Studies on co-crystallised antigen-antibody complexes revealed that association between these macromolecules is generally mediated by 75 to 110 van der Waals interactions, 10 to 20 hydrogen bonds, and a few salt bridges. Almost all water molecules are excluded from the entire interacting surface of about 700 $Å^2$ due to close shape complementarity. Possible conformational changes in the backbone atoms of antigen and antibody molecules upon binding is still a matter of controversy, whereas some rearrangements of side chains do seem to occur. The affinity of TCR-peptide/MHC interaction is estimated to be in the range of Kd 10^{-5} to 10^{-7} M (Schneck et al., 1989; Matsui et al., 1991; Weber et al., 1992), which is very weak compared to binding of antibody to large protein surfaces (10^{-8} to 10^{-10} M). However, the contact surface area between TCR and peptide/MHC complex is similar to that of antigen-antibody interaction, judged by dimensions of the top surface of MHC which is about 40x20 $Å^2$. Whether activation through TCR involves any conformational

changes in TCR remains to be answered. Based on the fact that CDR3 loops are encoded by the most hypervariable regions of the TCR, i.e. V(D)J junctions and N insertions, it has been postulated that CDR3 loops make direct contact with the peptide whereas CDR1 and CDR2 loops interact with the top surface of MHC (Davis and Bjorkman, 1988). However, precise alignments between TCR and MHC molecules could differ from one TCR to the other (Hogquist et al., 1994; Spain et al., 1994).

1.4 T cell repertoire selection

The T cell repertoire is a dynamic population of various numbers of different clones, and can potentially respond to a diverse array of antigens. However, the mature T cell pool exhibits certain bias in reactivities against antigenic entities and MHC molecules; i.e. non-responsiveness to self-antigens and restriction to self-MHC. According to the clonal selection theory (Burnet, Talmage, Lederberg; see Matzinger, 1993), such functional bias is imposed on the repertoire by selecting individual clone upon its interaction with environmental ligands.

Negative selection

Minor lymphocyte stimulating (Mls) antigens where initially discovered by their abilities to activate a large proportion of peripheral T cells (Festenstein, 1973). Subsequently, mice

carrying certain alleles of Mls antigens were found to possess decreased numbers of T cells expressing particular Vβ genes. Such loss of specific $V\beta^+$ T cells occurs mainly in the thymus, and was taken as an indication of negative selection (reviewed in Kappler et al., 1988; Marrack and Kappler, 1990). Recently Mls antigens were found to be encoded by long terminal repeat open reading frames of mammary tumour viruses (reviewed in Kotzin et al., 1993). A group of bacterial enterotoxins can also activate a large fraction of T cells, and induce similar deletion of T cells of specific Vβ chains. The Mls and bacterial enterotoxins are collectively called superantigens, and are thought to directly cross-link between TCR β chains and class II MHC molecules regardless of peptides bound to the MHC.

A second line of evidence for the clonal selection came from studies in TCR transgenic mice (Table 1). Since most T cells in TCR transgenic mice express single TCR, it is possible to follow the developmental fate of clonotypic T cells under various conditions. These studies revealed that mechanisms of negative selection may differ between experimental systems depending on the amount, timing, and location of expression of TCR, antigen, and MHC molecules (reviewed in Nossal, 1994).

Positive selection

Intact thymus is required for the normal development of T cells, since congenitally athymic (nude) or neonatally thymectomised

Table 1 List of αβ TCR transgnic mice

Specificity	Clone	Origin	V region	Antibody	Reference
H-Y/H-2D^b	B6.2.16	C57BL/6^b	α3, β8.2	F23.1	Uematsu (1988)
/H-2L^d	2C	BALB.B^b	β8	1B2*	Sha (1988)
PCC/I-E^k	AN6.2 5C.C7	B10.A^k	α11, β3	1F2 KJ25	Kaye (1989)
LCMV$_{33-41}$/H-2D^b	P14	B10.BR^k ->C57BL/10^b	α2, β8.1	F23.1	Pircher (1989)
PCC/I-E^k	2B4	B10.A^k	β3	A2B5-2 KJ25	Berg (1989)
cOVA$_{323-229}$/I-A^d	DO-11.10	BALB/c^d	β8	KJI-26* F23.1	Murphy (1990)
/H-2K^b	KB5.C20	B10.BR^k		Desire-1*	Schoenrich(1991)
/H-2K^b	F3	C57BL/6-bm1	α8.1, β11	KT11	Morahan (1991)
SV40LT/H-2K^k	K	C3H/HeJ^k	α5, β8.1	KJ16	Geiger (1992)
INP$_{336-374}$/H-2D^b	F5	C57BL/6^b	α4, β11	KT11	Mamalaki (1992)
HA/I-E^d	vir-2-15				Swat (1992)
λ2$^{315}_{91-101}$/I-E^d	4B2A1	BALB/c^d	α1, β8.2	GB113 F23.1	Bogen (1992)
MBP$_{1-11}$/I-A^u	172.10	B10.PL^u	α2, β8.2	B20.1 F23.2	Goverman (1993)
ICA/I-A^{NOD}	BDC2.5	NOD/Lt^{g7}	α1, β4	KT4-10	Katz (1993)
OVA$_{257-264}$/H-2K^b	149.42		α2, β5	B20.1.1	Hogquist (1994)
/H-2K^b	BM3.3	CBA/J^k			Sponaas (1994)
HA$_{126-138}$/I-A^d	T2.5-5	BALB/c^d	α15		Lo (1994)
C5/I-E^k	A18	A/J^k	α11.1 β8.3	F23.1	Zal (1994)
/I-A^k	MS202	C3H^k			Kubo (1994)

(Miller, 1961) mice have severly reduced number of T cells. T cell development can be restored in these athymic mice by grafting irradiated or deoxyguanosine-treated thymic lobes (Lo and Sprent, 1986), suggesting a role for stromal cells. Recent experiments of reaggregate cultures using purified thymic stromal cells provide the most direct evidence that thymic cortical epithelial cells play an essential role for the T cell development (Jenkinson et al., 1992).

Antibody-treatment of neonatal mice in vivo or fetal thymus lobes in culture has shown that interactions between MHC (Kruisbeek et al., 1985; Marusic-Galesic et al., 1988), and CD4 (MacDonald et al., 1988) or CD8 (Zuniga-Pfluecker et al., 1990) are essential for the development of mature T cells. Similarly, T cell development is selectively affected in mice deficient for RAG1 (Mombaerts et al., 1992), RAG2 (Shinkai et al., 1992), TCR α (Mombaerts et al., 1992; Philpott et al., 1992), TCR β (Mombaerts et al., 1992), class I (Koller et al., 1990; Zijlstra et al., 1990) or class II (Cosgrove et al., 1991; Grusby et al., 1991) MHC, CD4 (Rahemtulla et al., 1991), CD8 (Fung-Leung et al., 1991; Killeen et al., 1992), and components of TCR-mediated signalling mechanisms such as CD3-ζ (Love et al., 1993; Malissen et al., 1993; Ohno et al., 1993), CD45 (Kishihara et al., 1993), p56lck (Molina et al., 1992), and ZAP-70 (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1994). These data provide evidence for the important role of TCR-associated signalling molecules in positive selection of T cells. It is unknown to what extent specificities of peptides and MHC are important. Several

arguments have been presented to account for the clonal specificity of positive selection.

1) It has been known that a large proportion of mature T cells (1 to 10%) recognise non-self (allo-) MHC. These reported frequencies of allo-reactive T cells are even underestimated given a limited number of allo-reactivities tested (discussed in Matzinger, 1993). Thus a large proportion T cells are likely to recognise both self-MHC and allo-MHC. The very fact that T cells can recognise non-self MHC suggests that either T cell are highly cross-reactive or positive selection of T cells is mediated by the invariable part of MHC.

2) Immune interactions of both cytotoxic and helper T cells are controlled by the host MHC. In F_1 -> P bone marrow chimaera, T cells preferentially respond to the antigens presented by host MHC (Bevan, 1977). In F_1 mice, which have been thymectomised, grafted with the parental thymus, and received the F_1 bone marrow cells, T cell responses are restricted by parental MHC (Zinkernagel et al., 1978). Although the degree of restriction varies considerably among reports, ranging from 2-6 fold to >50 fold differences between self and allogenic MHC, there is a bias towards self MHC (Stockinger et al., 1981). In these (axb -> a) bone marrow chimaera or (a -> axb) thymus graft experiments, MHC^a genes are expressed in both thymic and bone marrow derived cells, whereas MHC^b genes are expressed only in bone marrow derived cells. Thus, bias towards MHC^a implies that either those T cells which recognise MHC^a on thymic non-haematopoietic cells are

positively selected or thymic stromal cells (MHC^a) protect T cells from negative selection by bone marrow derived cells (MHC^b).

3) It has been shown that the development of TCR transgenic thymocytes is arrested at $CD4^+CD8^+$ stage in non-selecting MHC backgrounds (von Boehmer et al., 1989), suggesting that interaction between TCR and its restricting MHC is indispensable for the development of mature T cells.

4) Development of CD8 T cells in class I MHC-deficient mice is reconstituted by adding exogenous peptides. In $TAP1^{-/-}$ (van Kaer et al., 1992) or $\beta 2m^{-/-}$ (Zijlstra et al., 1990) mice, expression of class I MHC molecules is impaired and consequently the development of CD8 T cells is selectively blocked. Addition of peptides that can bind to the selecting MHC restores development of CD8 T cells (Ashton-Rickardt et al., 1993; Hogquist et al., 1993).

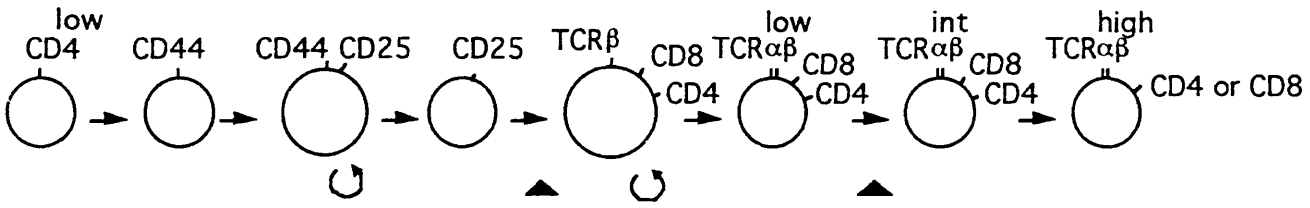
1.5 T cell turn over in the thymus

The development of T cells in the thymus is associated with proliferation and ordered expression of cell surface molecules (Havran and Allison, 1988, and reviewed in Scollay, 1991). As illustrated in figure 1, among $CD4^-CD8^-$ (DN) population of thymocytes those expressing CD44 (phagocytic glycoprotein-1) but not CD25 (α subunit of interleukin-2 receptor) are thought to be

Figure 1 T cell development in the thymus

A schematic representation of sequential changes in expression of cell surface markers during T cell development. Precursors for T cells are derived from fetal liver or bone marrow and express heat stable antigen (HSA) and CD44 (phagocyte glycoprotein-1, Pgp-1) but not CD25 (interleukin-2 receptor α). CD4 is also expressed at low levels at this immature stage. $CD4^{-/low}CD8^{-}CD44^{+}CD25^{-}$ cells give rise to $CD4^{-}CD8^{-}CD44^{+}CD25^{+}$ cells which contain blastic cells in cell cycle and subsequently down-regulate CD44. $CD44^{low/-}CD25^{+}$ cells in the $CD4^{-}CD8^{-}$ (DN) compartment contain a fraction of cells which have rearranged TCR β loci. At the next stage, CD8 and CD4 are up-regulated and TCR α loci are rearranged. Such intermediate cells between DN and $CD4^{+}CD8^{+}$ (DP) stages are also highly mitotic. Functionally rearranged TCR α and β chains are expressed on surface of DP cells initially at low levels. Subsequently TCR is up-regulated to intermediate levels either prior to or simultaneously with down-regulation of one of the co-receptors. CD4 or CD8 single positive cells then acquire higher levels of TCR.

A schematic model of T cell development



most immature (Petrie et al., 1990). These $CD44^{+}CD25^{-}$ DN cells give rise to $CD44^{+}CD25^{+}$ cells which then down-regulate CD44. Subsequently, CD44-CD25+ DN cells down-regulate CD25, up-regulate CD8 and CD4, and undergo rapid expansion (5-7 cell division cycles or 10^{2}-fold increase in cell number). It takes about 3 days after entry into the thymus that progenitor T cells reach $CD4^{+}CD8^{+}$ (DP) stage.

Transition from DN to DP stages is tightly regulated by functional rearrangement of TCR β genes which occurs at the $CD44^{-}$ $CD25^{+}$ DN stage. Introduction of transgenic TCR β genes into scid (Kishi et al., 1991), $RAG1^{-/-}$ (Mombaerts et al., 1992), and $RAG2^{-/-}$ (Shinkai et al., 1992) mice restores the arrest of T cell development at the DN stage in these recombination-deficient mice and allows thymocytes to differentiate to DP cells. TCR β chains are expressed at very low levels in DN cells, and are in complex with CD3-γ, δ, and ϵ chains and surrogate α chain gp33 (called pre-TCR, Groettrup et al., 1993). Recently, cross-linking of CD3-ϵ on $RAG1^{-/-}$ DN thymocytes has been shown to support their differentiation to DP cells in the absence of TCR α and β chains (Levelt et al., 1993).

Upon differentiation from DN to DP cells, thymocytes stop rearranging TCR β loci and initiate rearrangements at TCR α loci (Malissen et al., 1992). As a result, those DP thymocytes which achieved in-frame TCR α gene rearrangement can express TCR α/β heterodimers in complex with CD3-γ, δ, ϵ, and ζ chains initially at low levels. DP cells expressing slightly elevated levels of

TCR are thought to represent an intermediate stage between immature DP cells and mature SP cells. Generation of such TCR^{int} DP cells seems to be dependent on positive selection (Borgulya et al., 1991; Huesmann et al., 1991; Wu et al., 1991), and is accompanied with down-regulation of RAG2 gene (Turka et al., 1991; Brandle et al., 1992; Guy-Grand et al., 1992) and expression of T cell activation markers such as CD69 (Bendelac et al., 1992). Phenotypic maturation is completed after down-regulation of either one of CD4 or CD8 co-receptors. It takes about 8 to 12 days since their entry into the thymus that precursor T cells differentiate to SP cells (Kelly et al., 1993).

1.6 The thymus gland

The thymus gland consists of a large fraction of developing T cells and a few but heterogeneous populations of other stromal cells. Thymic stromal cells are derived from various embryonic sources and provide a microenvironment for differentiating T cells. In mice, early studies suggested that the thymic epithelial primordium is formed from the ectoderm of the third brachial cleft and the endoderm of the third pharyngeal pouch. Between day 11 and 12 of gestation, the thymus is first colonised with haematopoietic stem cells from the yolk sac and/or the fetal liver, which give rise to T cells, dendritic cells, macrophages and B cells in the thymus (Moore and Owen, 1967; Owen and Ritter, 1969). Subsequently a second wave of stem cells from the fetal liver colonises the thymus at day 18 of gestation. Then after

birth, bone marrow-derived stem cells gradually takes over (Wilson et al., 1989). The presence of intact thymic stromal cells is essential for the normal development of T cells, as demonstrated by severe T cell deficiencies in neonatally thymectomised mice (Miller, 1961) or congenitally athymic nude mice which are thought to have defects in thymic epithelial cells (van Vliet et al., 1985; Nehls et al., 1995). On the other hand, proper development of thymic stromal cells is dependent on the presence of T cells, as T cell-deficient mice exhibit abnormal thymic stromal architectures (van Ewijk et al., 1994).

The thymus consists of small lobules which are divided into subcapsular, cortical, and medullary areas (reviewed in van Ewijk, 1991). The subcapsular region contains immature DN thymocytes and epithelial cells (called thymic nurse cells) closely associated with them. The cortex has densely packed DP thymocytes and networks of cortical epithelial cells. In the medullary area, there are epithelial cells and a few mature SP T cells. Most dendritic cells and macrophages are in cortico-medullary junctions, and some macrophages are also found in the cortex. Large panels of monoclonal antibodies have been raised against thymic stromal cells and classified into six clusters according to their differential staining patterns (reviewed in Kampinga et al., 1989). Some of these antibodies recognise intracellular antigens, whereas others bind to cell surface molecules. The function of these antigens in the T cell development is under intensive scrutiny.

Previous studies on thymus graft-bone marrow chimaera experiments suggested a differential role for haematopoietic and epithelial components of thymic stroma in positive and negative selection of T cells. It has been proposed that bone marrow-derived cells (most importantly dendritic cells) cause negative selection (Sprent, 1989) and epithelial cells direct positive selection (Salaun et al., 1990). However, recent findings suggest that such functional partition is not absolute, since bone marrow-derived cells (Bix and Raulet, 1992) and fibroblasts (Hugo et al., 1993; Pawlowski et al., 1993) may support positive selection, and thymic epithelial cells cause negative selection in vivo (Carlow et al., 1992) and in vitro (Pircher et al., 1993 and this study).

Since the present study covers various experimental models of T cell development, most discussions were included in each result sections in order to avoid the information becoming fragmentary. The chapter for discussions thus describes only general implications of the current study in the context of other relevant investigations and future studies.

Chapter 2 Materials and methods

2.1 Chemicals, enzymes, and solutions

General chemicals and endonucleases were purchased from Sigma, Fluka, Boehringer Mannheim, and New England Biolab. Agarose, low melting point agarose, and bovine serum albumin (BSA, fraction V) were from Sigma. Bacto agar, Bacto tryptone, and Bacto yeast extract were from Gibco.

The following stock solutions were made at the institute: 1 M Tris/HCl (pH 7.5 and 8.0), 500 mM EDTA, 1 M NaCl, 1N NaOH, 1 M $MgCl_2$, 1 M $CaCl_2$, 20 % sodium dodecyl sulfate (SDS), 3 M potassium acetate (pH 4.8), 50 x TAE (2 M Tris-acetate, 50 mM EDTA), 10 x TBE (0.45 M Tris-borate, 10 mM EDTA), 20 x SSC (, 100 x Denhardt's solution (2 % Ficoll, 2 % BSA, 2 % Polyvinyl pyrollidone), RPMI 1640 complete medium (RPMI 1640, Gibco, supplemented with 2 g/l sodium bicarbonate, 10 mM HEPES, 1 mM Sodium Pyruvate, 0.06 g/l Penicillin, 0.1 g/l Streptomycin, and 1 % Monothioglycerol), Dulbecco's modified Eagle's essential medium (DMEM), phosphate buffered saline (PBS), PBS with 0.02 % azide.

Phenol (equilibrated with 10 mM Tris, pH 8.0), 10 mg/ml RNase (Sigma, boiled to inactivate contaminating DNase), 10 mg/ml proteinase K (Boehringer Mannheim), 10 mg/ml ampicillin, 10 mg/ml

kanamycin, 10 mg/ml ethidium bromide, LB medium, terrific medium, and LB plates with and without antibiotics were prepared as described (Maniatis et al., 1982).

2.2 Mice

2.2.1 Mouse strains

Inbred strains of C57BL/10, CBA/Ca, SWR, and nu/nu (H-2^b) mice were maintained in colonies in SPF unit at the institute. Thy-1/c-myc transgenic mice were generated by Dr. D. Kioussis at the institute (Spanopoulou et al., 1989). H2ts and Eαts transgenic mice were generated by Dr. D. Kioussis at the institute in collaboration with Drs. P. Jat, P. Ataliotis, and M. Noble (Ludwig Institute, London) (Jat et al., 1991). F5 TCR transgenic mice were generated by Dr. D. Kioussis at the institute (Mamalaki et al., 1992). RAG1-deficient mice were kindly provided by Dr. E. Spanopoulou et al. (Rockefeller University, USA) and were maintained as a colony at the institute (Silver et al., 1993). Mice deficient for β2m (Zijlstra et al., 1990) were from Dr. R. Jaenish's laboratory (Massachusettes Institute of Technology, USA). For timed matings, the morning when plugs were found was counted as day 0.

2.2.3 Tumour cell transplantation

For the test of malignancy of immortalised cell lines, 10^7 cells were injected intraperitoneally into syngeneic mice. For the passage of cultured cells, cells were transplanted subcutaneously into congenic mice.

2.2.4 Irradiation and bone marrow transfer

Bone marrow cells were isolated from femurs and tibia of H2ts, Eαts, TM25, and normal C57BL/10 mice, and were injected intravenously into sublethally irradiated (900 RAD) syngeneic sex-matched mice in collaboration with Dr. E. Dzierzak (NIMR). Haematopoietic reconstitution was confirmed by analysing spleen colony formation at day 12 and day 14.

2.2.5 Peptide injection

Nonameric oligo-peptides were synthesized and were purified by high pressure liquid chromatography (HPLC) at the institute. C57BL/10 or F5 TCR transgenic mice were injected intraperitoneally with 25 μg of peptides per 10 g of body weight everyday for 4 days.

2.3 Molecular techniques

2.3.1 Characterisation of DNA

Isolation of plasmid DNA

Solutions for bacterial culture and frozen stocks of competent bacteria were prepared as described (Maniatis et al.,). For fresh colony transformation, a colony from an LB-plate was resuspended in 200 µl of 100 mM $CaCl_2$ ice-cold in and were left on ice for several hours. After adding 50 ng DNA, competent bacteria were left on ice for 30 min, heat-shocked at 42 °C for 30 sec, placed on ice for 1 min, after which 800 µl SOC medium (2 % Bacto tryptone, 0.5 % Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added. Bacteria were pelleted by spinning for 16 sec, and resuspended in 100 µl SOC, and plated on selection plates.

The bacterial colony was grown in LB medium at 37 °C overnight in a shaker (180 rpm/min). For minipreps, the culture was centrifuged and the bacteria were resuspended in 200 µl of the glucose mix (25 mM Tris pH 8.0, 50 mM glucose, and 10 mM EDTA). The bacterial was lysed with 400 µl of 1 % SDS and 0.2 N NaOH on ice for 10 min. After adding 200 µl of potassium acetate pH 4.8, the solution was centrifuged for 10 min at room temperature. Aqueous phase was transferred to a fresh tube and 0.6 volume of isopropanol was added. After a spin, the pellet was rinsed with 70 % ethanol, resuspended in 100 µl TE with 100 mM NaCl, and treated with 20 µg/ml RNase at 37 °C for 30 min. The solution was

extracted with phenol:chloroform and with chloroform, and then DNA was precipitated with 2.5 volume of ethanol.

For maxipreps, bacterial culture in 1 to 2 litters of LB medium or 500 ml of terrific medium at 37 °C overnight was centrifuged at 6,000 rpm for 30 min in J6 rotor (Beckman). The pellet was resuspended in 50 ml of the glucose mix (as above), treated with 2 volumes of SDS/NaOH and 1 volume of potassium acetate as above. DNA was precipitated with isopropanol, rinsed with 70 % ethanol, and dissolved in 5 ml of TE. The solution was made up to 9 grams with H_2O, and 10 g CsCl and 1 ml ethidium bromide (5 mg/ml) were added. The solution was put in a quick seal tube, and centrifuged at 55,000 rpm overnight at 25 °C in 70.1 Ti rotor (Beckman). After the spin, the lower band containing supercoiled plasmid DNA was collected, diluted with 3 to 4 volumes of H_2O, and extracted with n-butanol. After adding 2.5 volumes of ethanol in 30 ml Corex tube, DNA was precipitated by spinning at 9,000 rpm for 20 min at room temperature. The pellet was dissolved in 500 μl TE with 100 mM NaCl, treated with RNase, phenol and chloroform extracted, ethanol precipitated, and resuspended H_2O in or TE.

The concentration and purity of DNA were analysed by measuring absorbances at 260 and 280 nm using a spectrophotometer (Hitachi).

Isolation of genomic DNA

Genomic DNA from mouse tails was extracted as described (Hogan et al., 1986). Briefly, tails (about 5 mm) were incubated in 500 to 700 μl of 50 mM Tris (pH 8.0), 100 mM EDTA, 100 mM NaCl, 1 % w/v SDS, and 20 μg/ml proteinase K (Boehringer Mannheim) in Eppendorf tubes at 55 °C overnight. Samples were treated with 10 μg/ml RNase (Sigma) at 37 °C for 1 hour, and were extracted twice with phenol:chloroform and once with chloroform, and DNA was precipitated with 0.6 volume of isopropanol. After centrifugation, pellet was rinsed with 70 % ethanol and DNA was dissolved in TE.

For isolation of large genomic DNA, tissue fragments were homogenised in TNE (50 mM Tris pH 8.0, 100 mM NaCl, and 100 mM EDTA) using Polytron. After adding equal volume of TNE containing 1 mg/ml proteinase K and 1 % SDS, the solution was incubated at 37 °C for overnight, and treated with 100 μl/ml RNase at 37 °C for 30 min. The solution was gently extracted with equal volume of phenol:chloroform in an Erlenmeyer flask for 5 to 10 min, transferred to 50 ml plastic tube (Falcon), and centrifuged at 3,000 rpm for 20 to 30 min in J6 rotor (Beckman). Aqueous phase was removed with a wide bore plastic pipette, and extracted with phenol:chloroform again and then with chloroform. Aqueous phase was transferred to an Erlenmeyer flask and 0.6 volume of isopropanol was added. Precipitating DNA was spooled with a glass rod which was dipped in 100 % ethanol, air dried, and rinsed in 70 % ethanol. DNA was allowed to dissolve in H_2O at 4 °C overnight.

Slot blots

Nitrocellulose filter (Schleicher and Scull) was wet in 1 M ammonium acetate and placed in the slot blot apparatus. Each slot was rinsed with 1 M ammonium acetate. Five μg DNA was diluted in 180 μl H_2O, 20 μl of 1 N NaOH was added. After incubation for up to 15 min at room temperature, equal volume of 2 M ammonium acetate was added to each sample, and the solution was applied in one or two slot(s) under vacuum. The filter was baked in an oven at 80 °C for 1 to 2 hours to immobilise DNA.

Southern blots

For genomic blots, 10 μg DNA was digested with 3- to 5-fold excess of restricting enzyme(s) in 50 μl of 1 x strength buffer provided by the manufacturer at 37 °C overnight. To check if digestion is complete, 0.3 μg uncut lambda DNA was mixed with 3 μl reaction solution and incubated in parallel. Samples were run in 20 x 20 cm agarose gel (0.5 to 0.7 %) at 40 V overnight. At the end of run, the gel was stained with ethidium bromide and a picture was taken including a ruler. The gel was treated with 0.25 N HCl for 30 min, with 0.5 N NaOH/1.5 M NaCl for 40 min, and then with 0.5 M Tris (pH 7.4)/1.5 M NaCl for 40 min at room temperature. The gel was blotted on the nitrocellulose filter using 2 x SSC for 3 to 5 hours at room temperature. After blotting, the filter was rinsed with 6 x SSC, air dried, and baked at 80 °C as above.

Preparation of DNA probes

Nick translation kit was purchased from Strategene and DNA fragments were labelled according to the manufacturer's instruction. Briefly, 100 to 200 μg DNA was dissolved in 4 μl H_2O, and 2 μl dNTP mix (dATP, dTTP, and dGTP), 3 μl ^{32}P-dCTP (30 μCi), and 1 μl enzyme mix (buffer and Klenow fragment) were added. The solution was incubated at 15 °C for 90 min.

For oligolabelling method (Hodgson and Fisk, 1987), the oligolabelling buffer (OLB) was made by mixing 100 μl solution A (1 ml 1.25 M Tris pH 8.0 and 0.125 M $MgCl_2$, 18 μl β-mercaptoethanol, 50 μl 10 mM cold dGTP, 50 μl 10 mM cold dTTP), 250 μl solution B (2 M Hepes pH 6.6), and 150 μl solution C (hexanucleotide primers p(dN)6, Pharmacia, 50 OD_{260} units in 555 μl TE). Fifty to 100 ng DNA was diluted in 11 μl H_2O, boiled for 3 min, and 4 μl OLB, 0.8 μl BSA (10 mg/ml), 2 μl ^{32}P-dATP (20 μCi), 2 μl ^{32}P-dATP (20 μCi), and 0.5 μl (2 units) Klenow fragment were added. The solution was incubated for 1.5 to 2.5 hours at room temperature.

To separate labelled DNA from hot dNTP, 100 μl TES was added to the reaction mixture and applied to a column set up with a pasteur pipette and G-50 (Pharmacia) pre-equilibrated with TES. DNA was eluted with TES, and 3 to 6 peak fractions of 10 drops each (approx. 50 μl) were collected. The radioactivity was measured using a beta counter.

Southern blot hybridisation

Salmon sperm DNA (ssDNA, Sigma) was dissolved in 0.2 N NaOH at 1 % w/v and boiled for 30 min. Tris was added to the solution to 100 mM, and pH was adjusted to 7.0 by adding 10 N HCl. NaCl was added to 0.4 M and the solution was phenol extracted three times. DNA was precipitated with ethanol and was dissolved in 10 mM Tris at 50 mg/ml concentration.

Pre-hyb mix contained 3 x SSC, 0.1 % SDS, and 10 x Denhardt's solution. Hyb mix was made by adding 10 % (w/v) dextran sulphate to the pre-hyb mix. Filter was wet with 2 x SSC and incubated in pre-hyb solution containing 50 μg/ml ssDNA (denatured by boiling for 5 min) at 65 °C for minimum 2 hours either in a hybridising box or cylinder. Filter was then hybridised with 100 to 200 ng of labelled probes in hyb mix containing 50 μg/ml ssDNA at 65 °C overnight. The probe and ssDNA were denatured by boiling for 5 min and rapidly placing on ice. The hyb mix containing the probe was re-used if necessary after denaturing it by boiling for 30 min. Subsequently, the filter was washed for 20 min at 65 °C twice with 3 x SSC, twice with 0.3 x SSC, and once with 0.1 x SSC, each containing 0.1 % SDS. After the final wash, the filter was rinsed with 2 x SSC, dried with 3 MM paper, and put in plastic membranes. The filter was exposed either on a film (Kodak, Fuji) or a screen for the phosphor-imager (Fuji). To strip the filter, it was incubated in 0.2 N NaOH at room temperature for 15 to 20 min, rinsed twice with 2 x SSC at room

temperature for 10 min, and air dried.

2.3.2 Characterisation of RNA

Preparation of total RNA

Total RNA was extracted from mouse tissues or cultured cells by one of the following methods. For a method adapted from Auffrey et al. (Auffrey and Rougeon, 1980), LiCl/urea solution was made with 126 g LiCl and 360 g urea per litter of sterile H_2O and was filtered through Whatmann paper. Frozen or fresh tissues or harvested cultured cells were homogenised in universal tubes containing 5 to 10 ml of 3 M LiCl/6 M urea per gram of tissue using Polytron at a maximum speed for 1 to 2 min on ice. Homogenate was further sonicated at a full power for 90 sec, kept at 4 °C overnight, and centrifuged at 4,000 rpm in HB-4 rotor (Beckman) for 30 min at 0 °C. The supernatant was poured off, half volume (approx. 2 ml) of cold 3 M LiCl/6M urea was added, and the solution was centrifuged again. The pellet was dissolved in 5 ml per gram tissue (approx. 400 μl in Eppendorf tubes) of TES (10 mM Tris pH 7.6, 1 mM EDTA, 0.5 % SDS) at room temperature for 10 min. The solution was extracted with phenol (saturated with TES):chloroform:isoamylalcohol (24:24:1) for 25 min, centrifuged at a top speed for 15 min. Aqueous phase was re-extracted with phenol:chloroform:isoamyl alcohol, and 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol were

added. RNA was precipitated at -20 °C overnight, centrifuged, rinsed once with 70 % ethanol, and dissolved in TE (10 mM Tris pH 7.6, 1 mM EDTA). For preparation of RNA from tissues rich in RNase, tissue fragments were sonicated in 5 ml of 50 g/dl Guanidium thiocyanate, 0.1 M Tris pH 7.6, and β-mercaptoehthanol for 2 min at room temperature using Polytron as described (Maniatis et al.,). After adding 1/20 volume of 10 % lauryl sarccosinate, the solution was centrifuged at 4,000 rpm for 10 min in HB-4. The supernatant was layered on 5 ml of 96 g/dl CsCl and 9 mM EDTA, and centrifuged at 32,000 rpm for 24 hours in SW41 rotor (Beckman). After removing supernatant, pellet was rinsed with 70 % ethanol, dried, re-dissolved in 300 μl TES, and extracted with phenol:chloroform and with chloroform, precipitated and re-dissolved in TE as above.

Northern blots

The denaturing gel was prepared with 4 g agarose in 156 ml H_2O, boiled until dissolved, cooled in 60 °C bath, mixed with 10 ml of 20 x MOPS and 34 ml of 37 % formaldehyde, poured and set up in 1 x MOPS. RNA dissolved in 10 μl TE was mixed with 2 μl 20 x MOPS (0.4 M MOPS pH7.0, 0.1 M sodium acetate, and 20 mM EDTA), 20 μl formamide, 7 μl formaldehyde, and 4 μl blue juice (50 % glycerol, 1 x MOPS, and 0.01 % Bromophenol blue), heat-shocked at 60 °C for 10 to 15 min, cooled on ice, and loaded on the gel. Electrophoresis was run at 60 to 70 V for 5 to 7 hours with occasional mixing of the buffer. The staining lane was cut off

after the run, washed twice with H_2O for 15 min, twice with 0.1 M ammonium acetate for 15 min, stained with 0.5 μg/ml ethidium bromide for 15 min, and destained twice with 0.1 M ammonium acetate for 25 min. For blotting the gel, the gel was soaked in 50 mM NaOH/0.1 M NaCl for 20 min, 0.1 M Tris pH 7.6 for 20 min, and 2 x SSC for 20 min successively. RNA was blotted to Genescreen or Genescreen plus filters with 20 x SSC overnight. After marking the lanes, the filter was rinsed with 2 x SSC, put in Saran wrap, immobilised using UV cross-linker (Strategene) and subsequently baking at 80 °C for 2 hours.

Northern hybridisation

The ssDNA was prepared as above. Pre-hyb mix contained 50 % (v/v) formamide (Fluka), 5 x SSC, 5 x Denhardt's solution, 50 mM sodium phosphate buffer (pH 6.8), 0.25 mg/ml denatured ssDNA, 0.1 mg/ml tRNA, 10 μg/ml poly A, poly C, and 0.2 % (w/v) SDS. Hyb mix was the same as pre-hyb except for 1 x Denhardt's, and 10 % (w/v) dextran sulphate. The filter was incubated in pre-hyb mix at 42 °C for 2 to 4 hours, and then hybridised with probe in hyb mix at 42 °C for overnight. The filter was washed in 3 x SSC, 0.1 % SDS twice for 15 min, 0.3 x SSC, 0.1 % SDS twice for 15 min, 0.1 x SSC, 0.1 % SDS once for 30 min, each at 52 °C. The filter was developed on films (Kodak) or phospho-imager screens (Fuji) as described above.

2.3.3 Polymerase chain reaction

Total RNA was extracted from cortical epithelial cells and WEHI-3 cells (grown at 37 °C for 3 days) by guanidium isothyanate method, and were reverse transcribed with Molony murine leukaemia virus reverse transcriptase (Cetus, Gibco BRL) according to the method described (Koopman et al., 1991). cDNA products were amplified by polymerase chain reaction (PCR) using a thermal cycler (Hybaid) and the following sets of primers (sense and anti-sense):

IL-1α; GGCTCACTTCATGACACTTGC, GCTGATACTGTCACCCGGC,

IL-1β; CGGACCCCAAAAGATGAAGGGC, GCCACAATGAGTGATACTGCC,

IL-2; GCACCCACTTCAAGCTCC, CCTGGGGAGTTTCAGGTTCC,

IL-3; GGGAAGCTCCCAGAACC, CGCAGATGTAGGCAGGC,

IL-4; CGGCACAGAGCTATTGATGGG, CGCCCCAGCAGTATCACCTGGG,

IL-5; GGAGAAATCTTTCAGGGGC, GCCTCAGCCTTCCATTGCCC,

IL-6; CCACTTCACAAGTCGGAGGC, CCAGGTAGCTATGGTACTCC,

IL-7; GGGCAGTATATAAACAGG, GCAGGAGGCATCCAGG,

IL-10; CCCAGTCGGCCAGAGCC, CCTGCATTAAGGAGTCGG,

IFN-γ; CCCACAGGTCCAGCGCC, CCCCACCCCGAATCAGCAGCG,

TNF-α; GGCAGGTCTACTTGGAGTCATTGC, ACATTCGAGGCTCCAGTGAATTCGG,

GM-CSF; GCCCTGAACCTCCTGG, GCCCCGTAGACCCTGCTCG,

TGF-β1; GCTTCTGCTCCCACTCCCGT, GGCTTGCGACCCACGTAGTA,

TAP1; GCCTCTGGGGCGCCCAGCGGC, GCGGCCCGTGAAGAAGGG,

TAP2; GGTTGCTACAAGGATCTCTG, TCAGTGTTCTGTTCTCCTGG,

β-actin; CATCACTATTGGCAACGAGC, ACGCAGCTCAGTAACAGTCC.

2.4 Immunological techniques

2.4.1 Immunohistochemistry

Cells grown on coverslips were stained with antibodies specific for cytokeratin 18 (LE61, a kind gift from Dr. P. Jat, London), cytokeratin 8 (RPN.1166, Amersham), pan cytokeratin (Lu5, Boehringer Mannheim), cytokeratin 8.13 (K8.13, Sigma), vimentin (V9, Sigma), Mac1α (M1/70.15.11.5.HL, ATCC), Mac2 (M3/38.1.2.8.HL.2, ATCC), H-2 (M1/42.3.9.8.HLK, ATCC), H-$2A^{b,d,q}$ (M5/114.15.2, ATCC), H-$2A^{b}$ (NIM-R4, a kind gift from Mr. G. Pearse, NIMR, London), H-$2E^{k}$ (ox-6), thymic epithelial cell markers (4F1 and IVC4 from Dr. M. Ritter, London, ER-TR4 and ER-TR5 from Dr. W. van Ewijk, Rotterdam),

2.4.2 Fluorescence activated cell sorter analysis

The thymus, spleen, and lymph nodes were gently teased with forceps and cells were resuspended in αMEM or air-buffered DMEM. Fetal thymic lobes and reaggregates were homogenised in Eppendorf tubes using a plastic micro-homogeniser (PolyLabo). Up to 1×10^{6} cells were transfered to 3 ml Falcon tubes and centrifuged at 1,200 rpm for 7 min. Cell pellets were resuspended in 50 μl staining solution containing first layer antibodies in PBS with 1 % BSA and 0.02 % azide. After incubation for 30 to 40 min on ice, cells were washed once with chilled PBS/azide. Cells were

stained with second layer antibodies in the same way, and were analysed by FACScan (Becton-Dickinson). FACS data were analysed using Lysis software (Becton-Dickinson) or FACSPLOT programme (Mr. J. Green, Computer division, NIMR). The following antibodies were used, CD4/phycoerythrin (L3T4, Becton-Dickinson, Boehringer Mannheim), CD4/RED123 (Gibco BRL), CD8/FITC (Lyt-2, Becton-Dickinson), CD8/FITC (YTS146.9), CD8/RD1 (Lyt-2, Coulter Immunology), Thy-1.2/FITC (Sigma), CD3ϵ/FITC and biotin-conjugated (2C11-145), TCR Vβ8/FITC or biotin-conjugated (F23.1), TCR Vβ11/FITC or biotinylated (KT11, a gift from Dr. K. Tomonari, CRC, London), TCR pan-$\alpha\beta$/FITC (H57.597, a gift from Dr. R. Zamoyska, NIMR), TCR pan-$\gamma\delta$/FITC (GL3F, a gift from Dr. R. Zamoyska), heat stable antigen (M1/69.16.11.HL, ATCC, and J11d), rat anti-goat IgG/phycoerythrin (Biogenesis), rat anti-hamster IgG/FITC, rat anti-mouse IgG/FITC, rat anti-mouse IgM/FITC, and streptavidin/phycoerythrin (Biogenesis) or TRICOLOUR (Coulter).

2.4.3 Immuno-magnetic beads separation

Thymic stromal cells were fractionated using magnetic beads coated with antibodies against CD45 (M1-9, ATCC), a medullary epithelial marker (A2B5, a gift from Dr. M. Raff), and H-2A^b (NIM-R4), according to the method described (Anderson et al., 1993). Day 14 fetal thymic lobes were treated with 1.35 mM deoxyguanosine for 7 days. Then, up to about 80 lobes were transfered to an Eppendof tube, washed twice with PBS, and digested with 600 μl of 0.125 % trypsin, 0.125 % versene solution

at 37 °C for 30 to 45 min. Thymic lobes were disaggregated with strong pipetting, and about 2 x 10^4 cells per lobe were recovered. Cells were washed once with ice cold complete medium, and were resuspended in 200 μl medium in a round bottom freezing vial. An aliquote of CD45-Dynabeads (50 μl, 2 x 10^7 beads, Dynal) was added, and cells were centrifuged at 1,000 rpm at 4 °C for 10 min. After removing cells bound to the beads with a magnetic separater (M-450, Dynal), more CD45 beads were added and cells were centrifuged as above twice. Subsequently, unbound cells were depleted twice with A2B5-Dynabeads (50 μl, 2 x 10^7 beads). From the remaining cells, class II MHC^+ cells were selected using NIM-R4-Dyanabeads (cell : beads ratio, 1 : 3). After a single spin for 10 min, bound cells were separated with the magnetic separator, and washed extensively with PBS.

2.4.4 Enzyme linked immunosorbent assay

Ninety-six well plates were coated with an antibody against IFN-γ (AN18, a kind gift from Dr. A. Sponaas, NIMR) at 10 μg/ml in 0.2 M borate buffer (pH 8.5) at 4 °C overnight. After washing with PBS, 0.05% Tween 20, wells were treated with 5 % fetal calf serum, 5 % horse serum in PBS for 1 hour at room temperature (RT). Wells were washed and 50 μl samples were added. After a 2-hour incubation at RT and washing, biotinylated second antibody against IFN-γ (R4-6A2, a kind gift from Dr. A. Sponaas) at 5 μg/ml was added. After 1-hour incubation at RT, wells were washed and streptavidin-horseradish peroxidase (Southern Biotechnology)

at 1 : 500 dilution was added. After 1-hour incubation at RT, wells were washed and substrate 2, 2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid, Sigma) at 27.4 mg/ml with 0.025 % H_2O_2 in 10 mM phosphate buffer (pH 6.2) was added. After developing for 5 to 30 min, absorbance at 414 nm was measured by Titertek Multiskan MCC/340. Unit concentrations of IFN-γ were calculated from standard curves obtained with a stock IFN-γ with a known concentration.

2.5 Tissue culture methods

2.5.1 Cell lines

Cells were cultured in RPMI 1640 complete medium containing 10 % fetal calf serum (Globe Farm, heat inactivated at 55 °C for 30 min) in a humidified chamber containing 5 % CO_2 at 37 °C. Thymic stromal cells expressing tsA58 were maintained at 33 °C in medium containing 10 or 100 IU/ml IFN-γ (Genzyme, a gift from Dr. B. Stockinger, NIMR). To subculture adherent cells, cells were rinsed once with PBS and treated with solution (0.5 ml/flask) containing 0.125 % trypsin and 0.125 % versene. Cells were resuspended in 10 ml complete medium, centrifuged at 1200 rpm for 7 min, and cell pellets were resuspended in fresh medium, and about one tenth cells were subcultured. Adherent cell lines were cloned either by plating at low densities and isolating individual colonies by creating a dry ring around the colony and

placing a drop of trypsin/EDTA solution, or by limiting dilution into 96 multi-well plates (Falcon). Growth characteristics of adherent cells were studied by plating at 1 x 10^4 cells/60 mm dish at either 33 °C or 37-39 °C with or without 10 IU/ml IFN-γ. Total cell numbers were counted on days 3, 7, and 14.

2.5.2 Suspension co-culture

Thymic epithelial cells were plated at 5 x 10^5 cells/well in 24 multi-well plates and were pre-conditioned for 3 days at 37 °C without IFN-γ (non-permissive condition). Epithelial cells were then incubated with various concentrations of the 1968 NP peptide at 37 °C without IFN-γ. After 2 hours, free 1968 NP peptide was thoroughly washed with PBS at 4 °C. Meantime, thymocytes were isolated from adult F5 TCR transgenic mice and were purified by passing through a Sephadex G-10 column to minimise contaminations with stromal cells (Ly and Mishell, 1974). Thymocytes were added to peptide-loaded epithelial cells at 1 x 10^6 cells/well, incubated for 12 hours at 37 °C, and were recovered by gentle pipetting.

Dendritic cells were prepared from the thymus of adult C57BL/10 mice in collaboration with Dr. B. Stockinger (NIMR). Briefly, thymic lobes were incubated with a cocktail of 1.6 mg/ml collagenase (Worthington CLS4) and 0.1 % DNase (Sigma, Fraction IX) in IMDM for 60 min at 37 °C. Thymic lobes were broken up by pipetting, and cells were pelleted and resuspended in 1.092 g/ml

Percoll solution. Percoll gradient was set up with 1.109 g/ml Percoll (bottom), the thymocyte suspension, 1.079 g/ml Percoll, 1.060 g/ml Percoll, and saline (top) in Falcon 2025 tubes. The gradient was centrifuged at 2,500 rpm for 20 min at room temperature. The top band was collected, and cells were washed twice with IMDM, 10 % FCS and plated in tissue culture dishes (60 mm diameter) in 5ml IMDM, 10 % FCS. After 3 hours, non-adherent cells were carefully removed and adherent cells were further incubated overnight. Cells detached from plastic were harvested and used as dendritic cells.

2.5.3 Fetal thymus organ culture

Fetal thymus organ culture was carried out as described (Jenkinson et al., 1982). Briefly, thymic lobes were dissected out of embryos at day 14 to 18 of gestation. Thymic lobes were cultured on Nucleopore polycarbonate filters, 13 mm diameter and 0.8 μ pore size (Costar), floating on the medium by surface tension.

For studies without peptide, thymic lobes were cultured in 4 ml complete medium (RPMI1640, 10 % FCS) in 60 mm dishes without change of medium. For peptide experiments, 1 ml of medium in 30 mm dishes were replaced with fresh medium everyday using new aliquotes of peptides (kept at -20 °C). Undiluted peptides were stored at -70 °C.

2.5.4 Reaggregate culture

Reaggregate cultures were set up according to the method described (Anderson et al., 1993). Fetal or newborn thymocytes from F5 TCR transgenic mice in non-selecting MHC ($H\text{-}2^{q/q}$) or of class I MHC-deficient ($\beta 2m^{-/-}$) mice were mixed with either normal thymic stromal cells prepared from deoxyganosine-treated fetal thymic lobes (B10) or various thymic stromal cell lines. Cells were mixed in 1.5 ml Eppendorf tubes and centrifuged at 1,200 rpm for 7 min at 4 °C (Beckman). Supernatant was removed leaving only about 50 μl medium, and were re-spun in a benchtop centrifuge (Heraeus) at 3,000 rpm for 10 sec. Supernatant was completely removed without disturbing the cell pellet on the wall of tube. Cells in slurry were placed on filter membranes using 5 μl fine tips and were cultured as standard organ culture.

Chapter 3 Transgenic oncogenesis

3.1 Introduction

3.1.1 Cell immortalisation

In studies of cellular and molecular biology when a large number of homogenous populations of cells are required, the availability of immortalised cell lines is often of a central importance. In animals, however, somatic cells are mortal; i.e. after a certain number of cell divisions, somatic cells cease to proliferate and become senescent (M1 arrest). M1 arrest can be bypassed by various oncogenes giving rise to transformed cells. However, transformed cells are not immortal, and soon face a second crisis, called M2, which is characterised by chromosome instability. Immortal cells arise from M2 crisis at a low frequency, apparently through additional mutation(s) of genes regulating the crisis. Although molecular mechanisms underlying immortality are poorly understood, viral and cellular oncogenes have been shown to facilitate immortalising cells when introduced *in vitro*. The delivery of oncogenes, however, has been a technical problem, since transfection and retrovirus-mediated gene transfer methods require a large number of dividing cells so that oncognes are integrated into the active genome. Transgenic technology, by which oncogenes can be stably

integrated into active regions of chromosomes, can overcome such a difficulty of introducing genes into scarce and/or non-dividing cells. The problem with transgenic animals, on the other hand, is a potential lethality caused by an unregulated expression of oncogenes. However, using tissue-specific gene regulatory elements, it is possible to direct the expression of oncogenes in selected cells at a specific developmental stage(s). Recent years have seen an increasing number of such targeted oncogenesis in transgenic mice (reviewed in Hanahan, 1986; Compere et al., 1988). An additional measure to control the activity of transgenic oncogene, although less explored in transgenic mice, is to use temperature-sensitive (ts) oncogene variants such as those of simian virus 40 (SV40) large T antigen (Tegtmeyer, 1975; Pintel et al., 1981, also p53, myb, src, abl, ros, rel, fos, and mos are known to have ts variants). In an attempt to immortalise cells contributing to the T cell development, we introduced a temperature sensitive SV40 large T antigen or the c-myc proto-oncogene into transgenic mice.

3.1.2 The SV40 large T antigen

Simian virus 40 (SV40) large tumour antigen can induce and maintain transformation of a wide range of cells either when transfected *in vitro* or in transgenic mice. Transformation by the SV40 large T antigen appears to be mediated by induction of cellular transcription, DNA synthesis, and its interaction with tumour suppressor genes p53 and pRB (product of the

retinoblastoma susceptibility gene RB1) (reviewed in Ludlow, 1993). pRB is thought to act as a checkpoint control at G1-S transition of the cell cycle. Inactivation of pRB (and probably its interaction with cellular transcription factor E2F) predisposes cells to neoplastic transformation (reviewed in Levine, 1993). The p53, on the other hand, is induced by a DNA damage and blocks entry into S phase of the cell cycle (probably through binding to a cellular oncogene mdm-2) allowing cells time to repair DNA, or otherwise induces apoptosis if DNA damage is more extensive (Clarke et al., 1993; Lowe et al., 1993). Thus, inactivation of p53 may disable cells to detect genetic damages associated with integration of viral genes, telomeric shortening in senescent cells in M1 arrest, or genetic mutations in M2 crisis.

A point mutation in the SV40 large T antigen at residue 438 from alanine to valine (tsA58) renders it temperature-sensitive (Tegtmeyer, 1971); tsA58 immortalises cells at a permissive temperature (32°C) but not at a restrictive temperature (39°C) (Tegtmeyer, 1975). tsA58 mutation is located in the α helix of a presumptive ATP-binding fold of the SV40 large T antigen (Loeber et al., 1989), although it is not clear whether temperature-sensitivity is due to a direct effect of a change in the structural domain or an indirect one by an increased turn over rate of the mutant protein. Cells immortalised by tsA58 at a permissive temperature revert to non-proliferating phenotype when brought to a restrictive temperature. One can reasonably expect that these non-dividing cells may have more physiological

function than permanently immortalised cells.

Using tsA58 in transgenic mice, difficulties assciated with transfecting the oncogene into rare cells *in vitro* and potentially lethal effects of the oncogene *in vivo* are avoided. In addition, it may help to maintain more physiological functions in conditionally immortalised cells. In order for tsA58 to be expressed in many different cells, a class I H-2K^b promoter was used to drive the expression of tsA58. Class I MHC is first detected at a midsomite stage of embryogenesis (around day 10 p.c.) and is constitutively expressed in most somatic cells in the adult at various levels (reviewed in David-Watine et al., 1990). In addition, interferon-γ (IFN-γ) induces the expression of class I MHC, even to a greater extent in cells with low basal levels. In the thymus, class I MHC antigens are constitutively expressed in cortical and medullary epithelial cells, macrophages, dendritic cells, and cortical and medullary thymocytes (reviewed in van Ewijk, 1989).

The transcriptional regulatory sequences of class I MHC have been identified in its 5′ flanking region which includes interferon response sequence (IRS), an enhancer element, cAMP responsive elements, and an upstream negative regulatory sequence (reviewed in David-Watine et al., 1990). Our transgenic mice carry a 4.2 kb fragment from the H-2K^b promoter encompassing all these regulatory elements. However, one can not usually predict the pattern and the level of transgene expression, because they are influenced by chromosome structures surrounding the site where

the transgene has integrated, unless dominant regulatory sequences such as locus control region (LCR) of human β-globin genes (Grosveld et al., 1987) are used. We generated several H-2K^b/tsA58 (H2ts) transgenic mouse lines some of which were successfully bred and gave rise to conditionally immortalised cell lines from various tissues.

3.1.3 The c-myc proto-oncogene

The proto-oncogene c-myc is thought to be involved in the control of G1-S transition in the cell cycle and is implicated in many neoplasias including sarcoma and leukaemia. Over-expression of the c-myc has also been shown to induce apoptosis of fibroblasts when transfected in vitro (Evan et al., 1992). The c-myc protein (Myc) belongs to a bHLH-Zip (basic-helix-loop-helix-leucine zipper) transcription factor family whose activity is regulated by homo- and/or hetero-dimerisation. The amino terminal basic region confers transcriptional activation and the carboxyl terminal HLH and Zip domains promote dimerisation. Myc is known to heterodimerise with Max (Blackwood and Eisenman, 1991), another member of the bHLH-Zip family. Max proteins can also homodimerise or form heterodimers with Mad (Ayer et al., 1993) or Mxi1 (Zervos et al., 1993). Myc is a very unstable protein whose synthesis is tightly regulated at both transcriptional and post-transcriptional levels. In contrast, its partner Max has a long half life (>18-24 hrs compared to 30 min of Myc) and is more abundantly expressed throughout the cell cycle. Myc-Max

heterodimers activate transcription from a consensus sequence CACGTG, whereas Max-Max homodimers and Max-Mad or Max-Mxi1 heterodimers do not exhibit such an activity despite their ability to bind to the same DNA motif. Thus in normal resting cells, the activity of Myc/Max heterodimers is regulated by levels of both Myc and counteracting Mad or Mxi1 proteins.

Since over-expression of c-myc is one of the most common genetic abnormalities associated with T cell leukaemia, it would be possible to immortalise T cells in vivo by targeting expression of c-myc to the T cell lineage in transgenic mice. To this end, T cell specific Thy-1 gene promoter sequences were used to direct expression of c-myc in transgenic mice (Spanopoulou et al., 1989). Thy-1/c-myc (TM) transgenic mice developed thymic tumours from which cell lines of both non-adherent (thymocytes) and adherent (epithelial cells) phenotypes were established (Spanopoulou et al., 1989). All thymocyte lines were $CD4^+CD8^+$ (double positive) and oligoclonal in origin. It was suggested that neoplastic transformation of thymocytes in TM mice occurs at a double positive stage in a stochastic manner. In an attempt to establish double positive cell lines expressing a known TCR, we further bred TM mice with those carrying a transgenic $\alpha\beta$ TCR which recognises a peptide from influenza virus nucleoprotein in the context of class I $H\text{-}2D^b$ (see Chapter 4). Subsequently, we established double positive T cell lines expressing the transgenic TCR and studied their function in vitro.

3.2 Temperature sensitive SV40 large T antigen (tsA58)

3.2.1 H-2K^b/tsA58 transgenic mice

A 2.7 kb fragment encoding the tsA58 early region was ligated to the 4.2 Kb 5′ flanking sequence of the H-2K^b promoter (figure 2-a) and was injected into one of the pronuclei of fertilised oocytes which were then placed in the oviduct of a pseudopregnant female and allowed to develop. In order to screen if the transgene was integrated into the genome, DNA was extracted from tails of founder mice, blotted on nitrocellulose membranes, and screened by a radiolabelled probe specific for tsA58. Mice transgenic for tsA58 were further bred to lines successfully.

The first series of H2ts mice were generated in a (C57BL/10 x CBA)F_1 background. Although class I MHC is known to be expressed as early as embryonic day 10, the tsA58 transgene did not affect the embryonic development in any of these lines. There was no macroscopic anomalies in organs of adult H2ts mice under dissection (data not shown), except for an enlargement of thymus to various digrees. All H2ts mice eventually developed thymomas causing airway obstruction in these mice. As a result, H2ts mice became dyspneic and died at 4-24 weeks of age. Thymomas were rarely associated with enlargement of spleens and lymph nodes.

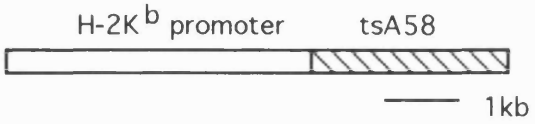
To examine the relationship between expression of tsA58 and thymic enlargement, genomic DNA and total RNA were extracted from

Figure 2 H-2K^b/tsA58 transgenic mice

a) The hybrid DNA construct used was made by ligating the early region of a temperature sensitive SV40 large T antigen to a 5′ promoter fragment of class I H-2K^b gene which contains interferon response elements and other regulatory sequences.

b) Genomic DNA and total RNA were extracted from the thymus of H2ts6, 12, 18, and 23 mice, and equal amounts of the samples were blotted and hybridised with a tsA58 probe. Ages of mice which developed dyspneic symptoms are listed as an indication for enlargement of the thymus.

a) H2-K^b/tsA58 construct



b) Co-relation of tsA58 expresssin with thymic tumourignesis

	Thymus		Age at the onset of dyspnea
	DNA	RNA	
H2ts6	++	-	12-24 wks
H2ts12	+++	++	6-11
H2ts18	++	+	4-6
H2ts23	++	+	7-13

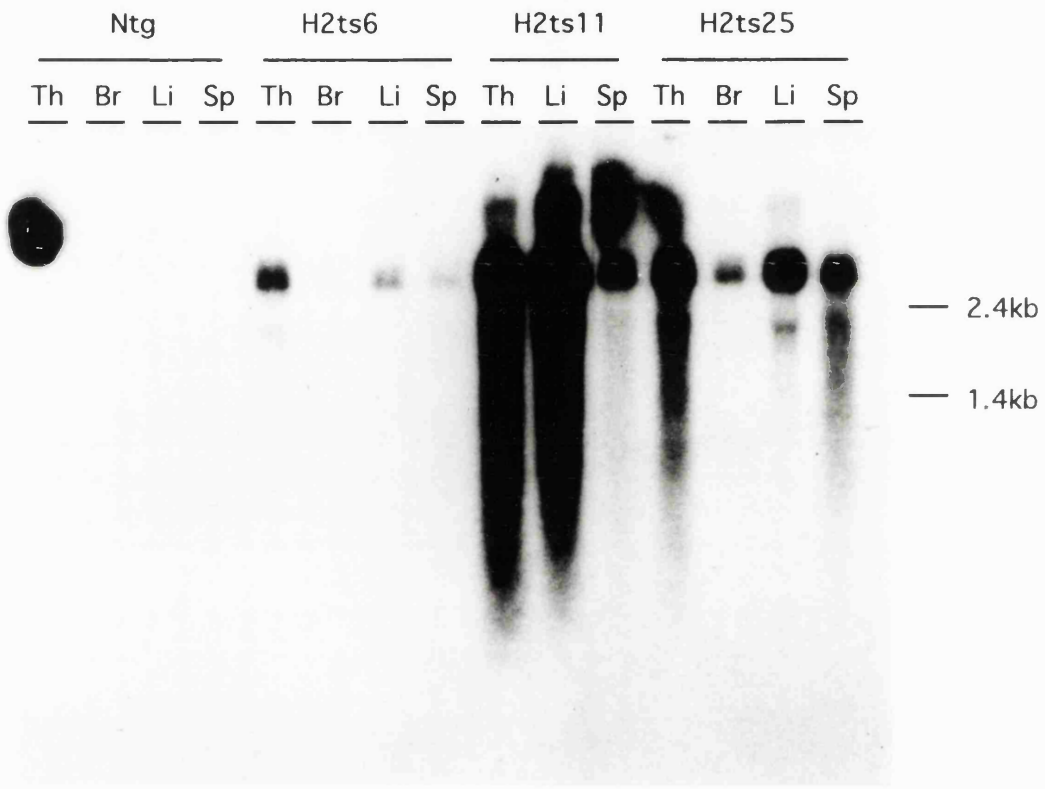
the thymus of four different lines of H2ts mice. Figure 2-b shows copy numbers and expression of tsA58 in the thymus. H2ts6 mice expressed the lowest level (RNA transcripts) of the oncogene and H2ts12 mice had the highest. The onset of dyspneic symptoms co-related with the expression of tsA58, the earliest developing in H2ts12 and the latest in H2ts6 mice. In addition, homozygous H2ts6 mice became dyspneic in about half time (7-10 weeks after birth) compared with heterozygous mice (12-24 weeks). These data demonstrate that higher expression of tsA58 leads to an earlier onset of thymic enlargement, suggesting direct cause-effect relationships. Although tsA58 is unstable at a normal body temperature of the mouse, probably a high enough level of expression allows tsA58 to exert its function even under a restrictive condition. Indeed, class I MHC is known to be most abundantly expressed in the thymic stroma. An alternative explanation is that thymic cells are more susceptible to the hyperplastic activity of the large T antigen.

To compare the levels of tsA58 expression in different tissues, total RNA was extracted from brain, liver, and spleen of three lines of H2ts mice. RNA samples were run in denaturing agarose gels, transferred to nitrocellulose membrane, and hybridised with a tsA58 specific probe (northern analysis). As shown in figure 3, these H2ts mice transcribed tsA58 at low (H2ts6), intermediate (H2ts25), and high (H2ts11) levels. The pattern of expression in different tissues looked similar between these mice, the thymus and the liver having the highest and the brain the lowest. In spite of an abundant expression of tsA58 in the liver,

Figure 3 Northern blot analysis of tsA58 expression in H2ts mice

Total RNA was extracted from the thymus (Th), brain (Br), liver (Li), and spleen (Sp) of non-transgenic (Ntg) and H2ts6, 11, and 25 mice. Samples were size fractionated in a denaturing agarose gel, blotted on a membrane, and hybridised with a tsA58 specific probe. Positions of ribosomal RNAs (2.4 kb, 1.4 kb) are indicated. A blot in a sample of non-transgenic thymus is nonspecific, as it is slightly off-lane and not of an expected size.

Northern analysis of tsA58 expression in H2ts mice.



macroscopically abnormal liver was rarely observed, suggesting an additional factor determining the growth of cells expressing tsA58.

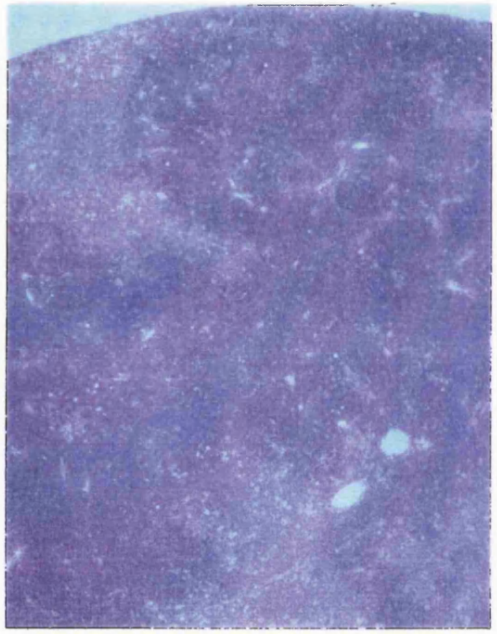
In order to identify which cells are expanded in H2ts mice, tissue sections from the thymus, liver, spleen, lymph node, intestine, and skin from H2ts6 mice were stained with haematoxylin and eosine. The only histological changes found were in the enlarged thymus as illustrated in figure 4 at different ages of H2ts6 mice. The thymus began to be hyperplastic at two months showing histological features of lympho-epithelial thymoma, i.e. a benign expansion of both lymphoid and epithelial compartments. At four months, the thymus was further enlarged and medulla-like structures were seen in subcapsular areas as depicted in the picture. At six months, the stromal compartment was more hyperplastic and there was a mild interstitial response resulting in thickened connective tissue. Malignant features, such as formation of tumour nodules, heterokaryotic changes in nuclei, hypervascularity, and necrosis were found only occasionally in very advanced stages of the tumour.
To further analyse subsets of cells in stromal and lymphoid compartments expanded in the enlarged thymus, immunohistochemical staining of cryostat sections using antibodies specific for various thymic stromal cells and T cells was performed in collaboration with Drs. M. Ritter and H. Ladyman (Hammersmith Hospital, London). As shown in figure 5, a dense network of cytokeratin positive epithelial cells was a predominant feature of the tumour. Thymic epithelial cell markers, 4F1 and IVC4, were

Figure 4 Histology of thymic tumours in H2ts6 mice

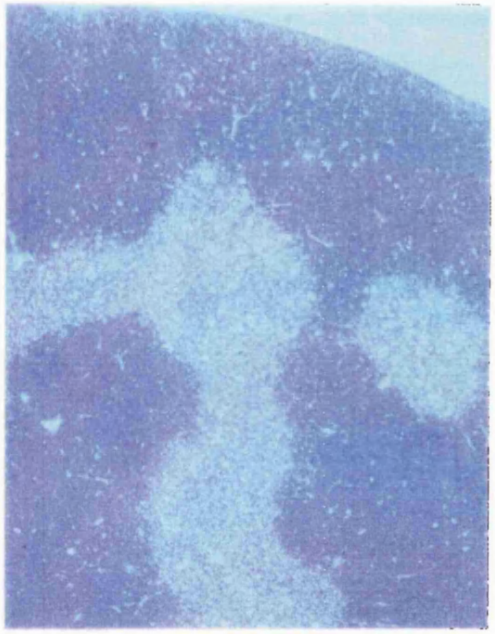
Sections of the thymus in H2ts6 mice at the age of 2, 4, and 6 months were stained with haematoxylin and eosin. Magnification x 120.

6 months

4 months



2 months

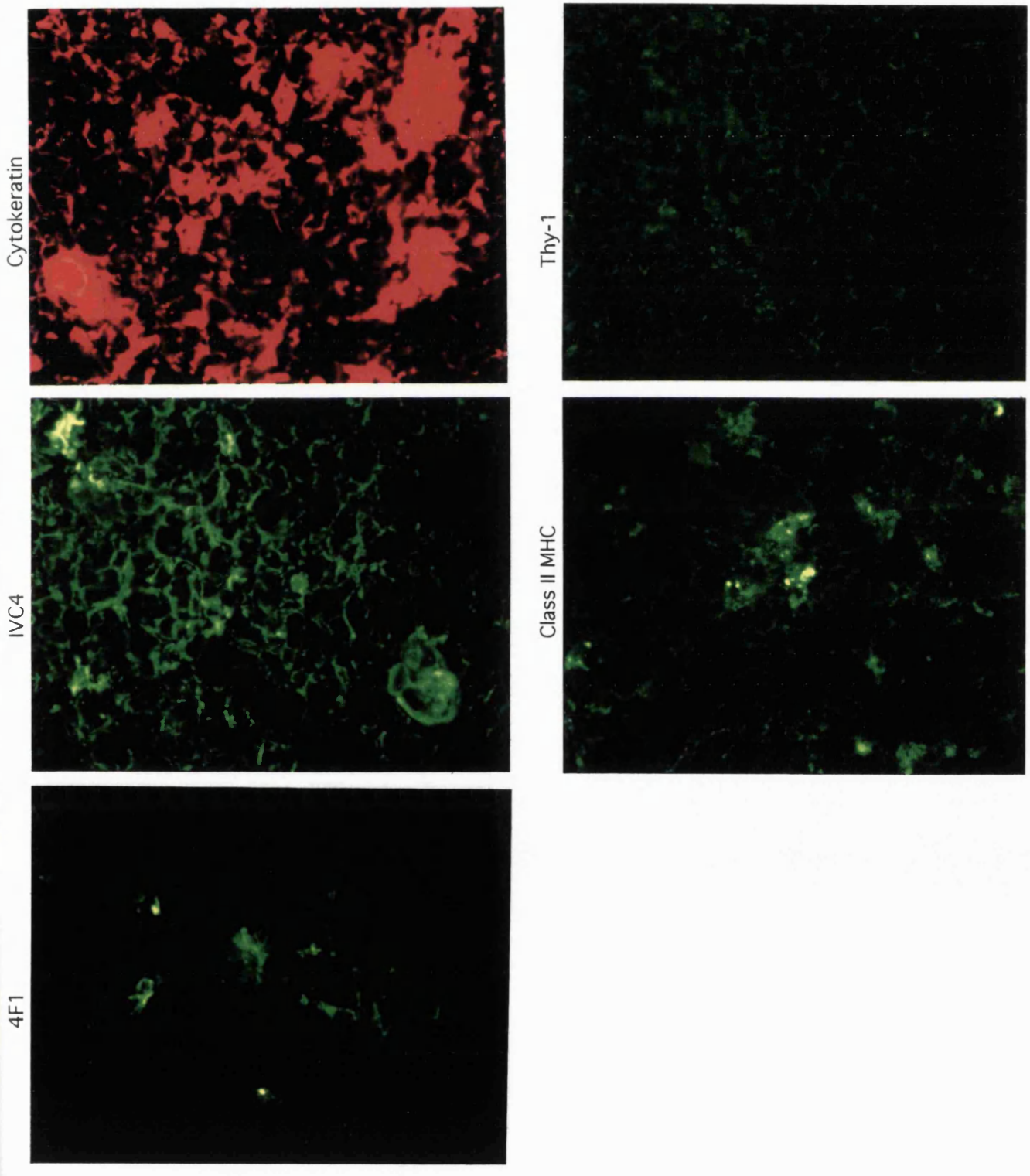


Thymus histology of H2ts6 mice

Figure 5 Immunohistochemistry of the thymus in CBA H2ts1 mouse

Cryostat sections of an enlarged thymus in CBA H2ts1 mouse were stained for cytokeratin, IVC4 (medullary marker), 4F1 (cortical marker), Thy-1, and class II MHC. (Collaboration with Drs. M. Ritter and H. Ladyman, Hammersmith Hospital, London) Magnification x 120.

Immunohistochemistry of the thymus in CBA H2ts1 mouse



found only sparsely suggesting that $4F1^{-}IVC4^{-}$ double negative cells, which are usually found in the medulla, were mainly expanded. T lymphocytes weakly staining for a Thy-1 antibody were interspersed throughout. These data demonstrate that both epithelial cells and thymocytes are expanded in these enlarged thymuses in tsA58 mice.

To examine if tsA58 expression affected the normal development of T cells, T cells in the thymus and lymph nodes of H2ts and non-transgenic littermates were stained for CD4 and CD8. As illustrated in figure 6, there was no difference in patterns of CD4 and CD8 expression between normal and H2ts mice. Table 2 also shows that normal and H2ts mice had similar proportions of DN, DP, and CD4 or CD8 SP thymocytes. In addition, expression of CD3ϵ and heat stable antigen (HSA) on thymocytes showed no difference, suggesting that tsA58 does not affect normal development of T cells.

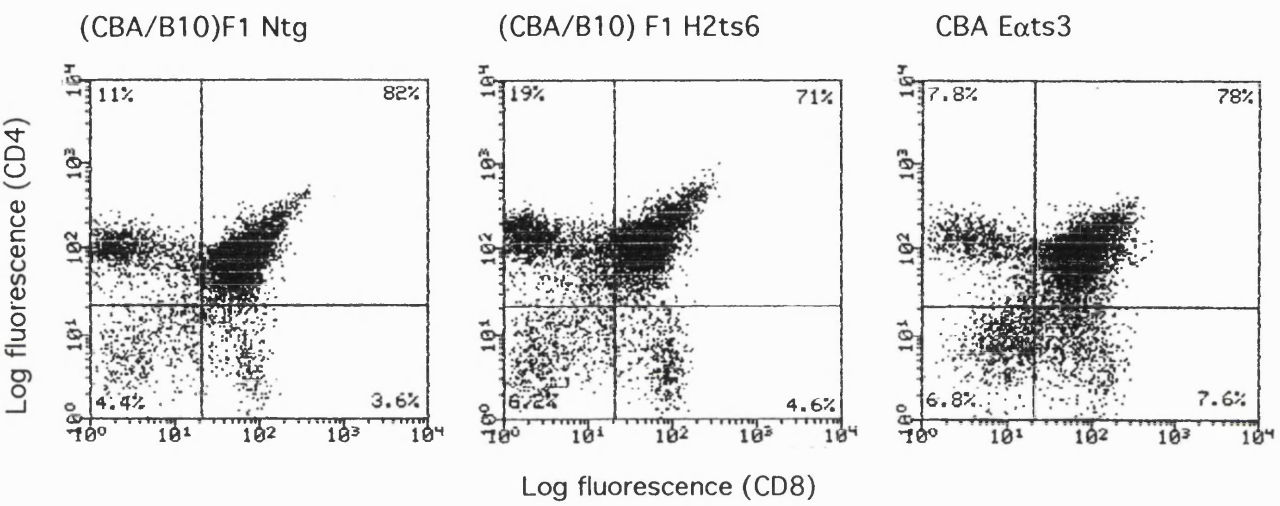
To study if the expansion of thymocytes in H2ts mice was caused by transformation of single or a few clones, the proportion of $V\beta8^{+}$ cells in the thymus was assessed. As shown in table 2, there were no increase or decrease of $V\beta8^{+}$ cells in H2ts mice compared with normal mice, suggesting that thymocytes in H2ts mice were polyclonally expanded. This was further supported by the Southern blot analysis of TCR β gene rearrangement. Thus, genomic DNA was extracted from the thymus or liver of non-transgenic, H2ts, and Thy-1/c-myc transgenic mice, and was digested with HindIII or PvuII restriction endonucleases. DNA

Figure 6 Thymic and lymph node T cells in tsA58 transgenic mice

a) Thymocytes from a non-transgenic mouse (Ntg), an H2ts6 transgenic littermate (F_1), and an Eαts3 transgenic mouse (CBA) were stained for CD4 and CD8.

b) Lymph node cells from the same mice as above were analysed for CD4 and CD8 expression.

a) Thymus



b)Lymph nodes

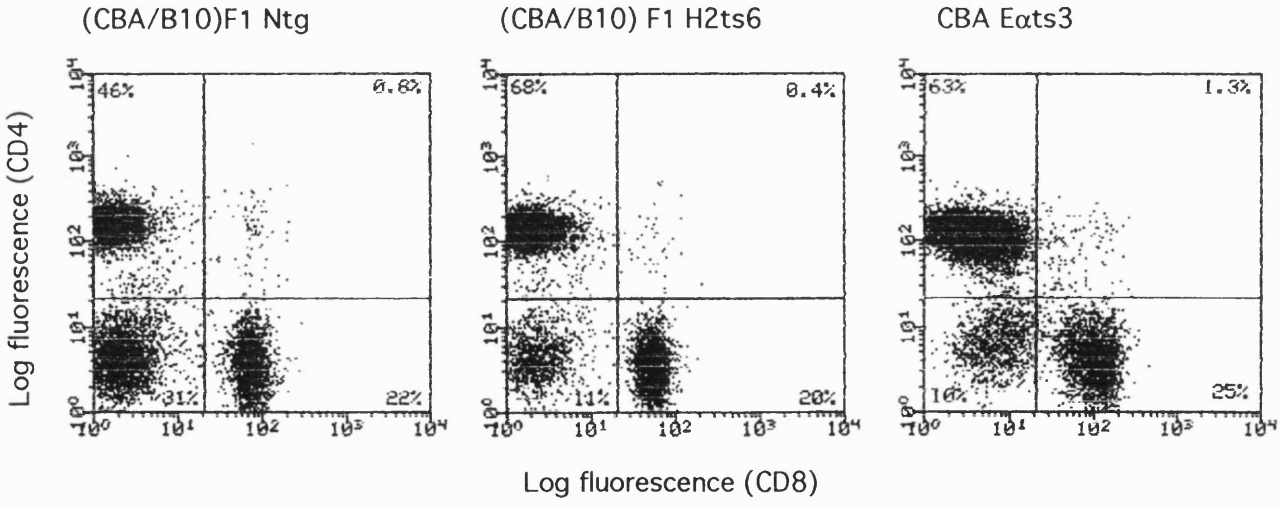


Table 2 Phenotype of thymocytes in H2ts mice†

Surface phenotype	Normal mice (n = 7)	H2ts6 mice (n = 4)
$CD4^-CD8^-$	11.3 ± 1.8	13.4 ± 0.7
$CD4^+CD8^+$	69.0 ± 2.8	62.2 ± 1.7
$CD4^-CD8^+$	3.7 ± 0.5	3.7 ± 0.5
$CD4^+CD8^-$	16.0 ± 1.5	20.0 ± 1.2
$CD3^+$	12.8 ± 3.6	10.8 ± 1.9
$V\beta8^+$	0.5 ± 0.3	0.9 ± 0.3
HSA^+	88.9 ± 1.1	85.9 ± 1.4

† Mean ± SD of percentage

samples were size fractionated by electrophoresis in agarose gels, blotted on filter membranes, and were hybridised with a probe specific for the TCR β constant region. As illustrated in figure 7-a, HindIII and PvuII digestion of unrearranged TCR β genes give rise to 9.4/3.1 kb and 6.2/6.0 kb bands respectively. Liver DNA from a non-transgenic mice has a germ line configuration of TCR β loci and contained predicted HindIII and PvuII restriction fragments (figure 7-b). HindIII digest of the thymic DNA from normal mice, on the other hand, contained only the lower 3.1 kb band and the upper band appeared as a smear of fragments of different sizes, suggesting that polyclonal rearrangements took place. Loss of the upper PvuII restriction fragment in the thymic DNA from normal mice was less evident because of a close molecular size to the lower band. All H2ts mice showed the same HindIII and PvuII restriction patterns as normal mice, suggesting that thymocytes in H2ts mice are polyclonally expanded. This was in contrast to a clonal expansion of thymocytes in Thy-1/c-myc transgenic mice, as judged by appearance in those mice of single rearranged HindIII and PvuII restriction fragments (Spanopoulou et al., 1989, and figure 7-b).

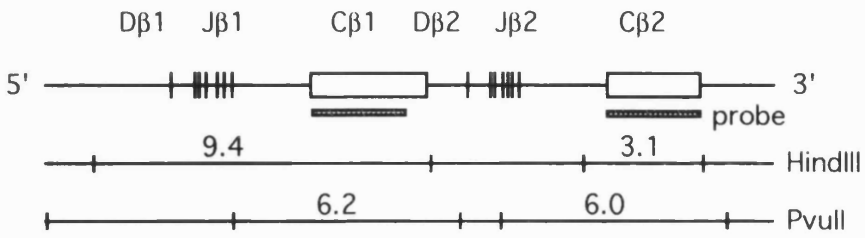
To tested if expansion of thymocytes in H2ts mice are due to neoplastic transformation, thymoma cells were transplanted in syngeneic animals. To this end, suspensions of $1x10^7$ cells were prepared from the enlarged thymus of 6 month-old H2ts6 mice, and were injected intraperitoneally into four syngeneic (CBAxB10)F_1 mice. Five months after injection, two of them were sacrificed to check tumour formation. One mouse had intraperitoneal tumour

Figure 7 Southern blot analysis of TCR β gene rearrangement in H2ts and TM mice

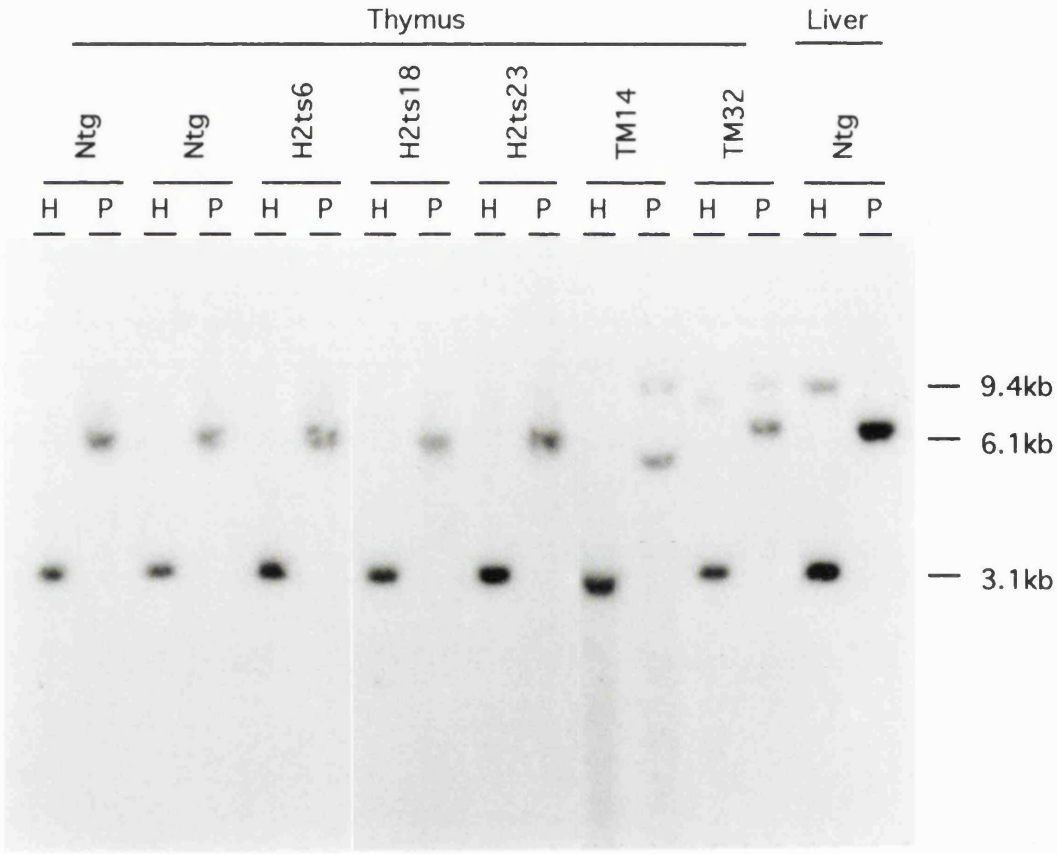
a) Restriction sites for HindIII and PvuII in the TCR β D-J-C locus. A probe from Cβ2 cross-hybridises with Cβ1 and detects 3.1 and 9.4 kb HindIII fragments or 6.0 and 6.2 kb PvuII fragments in germ line configurations. Gene rearrangements involving D1J1, D1J2, or D2J2 could alter sizes of restriction fragments which could be revealed by Southern blot analysis.

b) Total DNA was extracted from the thymus of a non-transgenic mouse, H2ts-6, -18, and -23 mice, and TM-14 and -32 mice, as well as from the liver of a non-transgenic (Ntg) mouse. Samples were digested with either HindIII (H) or PvuII (P) and were subjected to Southern blot analysis using a probe from Cβ2. The liver DNA shows restriction patterns of germ line configuration. In non-transgenic and H2ts mice, the thymus DNA does not have the 9.4 kb HindIII band and shows a smeary pattern indicating that polyclonal D-J rearrangements took place. Changes in PvuII restriction fragments are less evident, but the 6.2 kb band appears to be replaced with a smeary pattern. In contrast, thymic DNA from TM14 mouse has lost the 9.4 kb HindIII band suggesting a rearrangement across Cβ1 gene giving rise to a high molecular weight PvuII fragment. In TM32 mouse, there are rearranged HindIII and PvuII fragments suggesting a D1-J1 rearrangement.

a) Restriction fragment maps in TCR β locus



b) Sourthern analysis of TCR β lucus



on the peritoneal wall of 10 mm in diameter. The other had only smaller nodules on the mesenteric membrane. The remaining two mice were left up to nine months during which time no macroscopic tumour developed. Considering that malignant cells usually form tumours within a month after transplantation, we conclude that thymic cells in H2ts mice behave as benign cells in vivo.

3.2.2 Eα/tsA58 transgenic mice

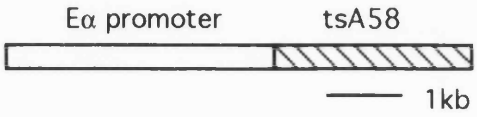
In contrast to the class I MHC, expression of class II MHC molecules is more restricted to lymphoid tissues. In the thymus, thymic cortical and medullary epithelial cells are among those which express the highest levels of class II MHC. Thymic dendritic cells and medullary macrophages (but not cortical macrophages) also express class II MHC (reviewed in van Ewijk, 1989). In order to selectively immortalise these cells, we generated transgenic mice carrying tsA58 under the control of class II Eα promoter in collaboration of Dr. R. A. Flavell (Yale Univ., USA). Eight Eα/tsA58 (Eαts) transgenic founder mice were generated as indicated by tail blots in figure 8. However, all Eαts mice developed thymic tumours at very early days of life (less than one month) allowing no sufficient time to breed. The reason why Eαts mice develop thymic tumours much early than H2ts mice remains to be answered. One explanation is that a constitutive activity of the Eα promoter is higher than that of the H-2K^b promoter in the thymus.

Figure 8 Eα/tsA58 transgenic mice

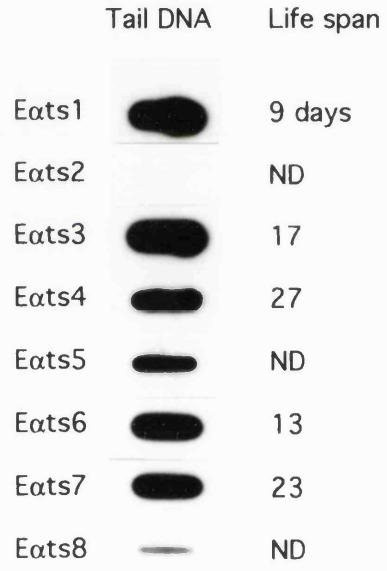
a) The hybrid DNA construct was made by ligating the early region of a temperature sensitive SV40 large T antigen to a 5′ promoter region of class II H-2E gene.

b) Tail DNA blot analysis of Eαts founder mice and their life span. ND: not determined when mice were sacrificed for analysis.

a) Eαts/tsA58 construct



b) Early death of Eαts transgenic mice



Macroscopically Eαts mice were normal except for the thymic tumours. Also, histological analysis by haematoxylin and eosin staining did not reveal any apparent abnormality (data not shown). However, when studied using antibodies against thymic stromal compartments (in collaboration with Drs. M. Ritter and H. Ladyman, London), large patches of cytokeratin positive cells were observed around perivascular areas (figure 9). These epithelial cells were $4F1^-$ and $IVC4^-$ double negative, which are found in the medulla in normal mice. The T cell development appeared normal in Eαts mice, since there were normal proportions of T cell subsets both in the thymus and lymph nodes (figure 6).

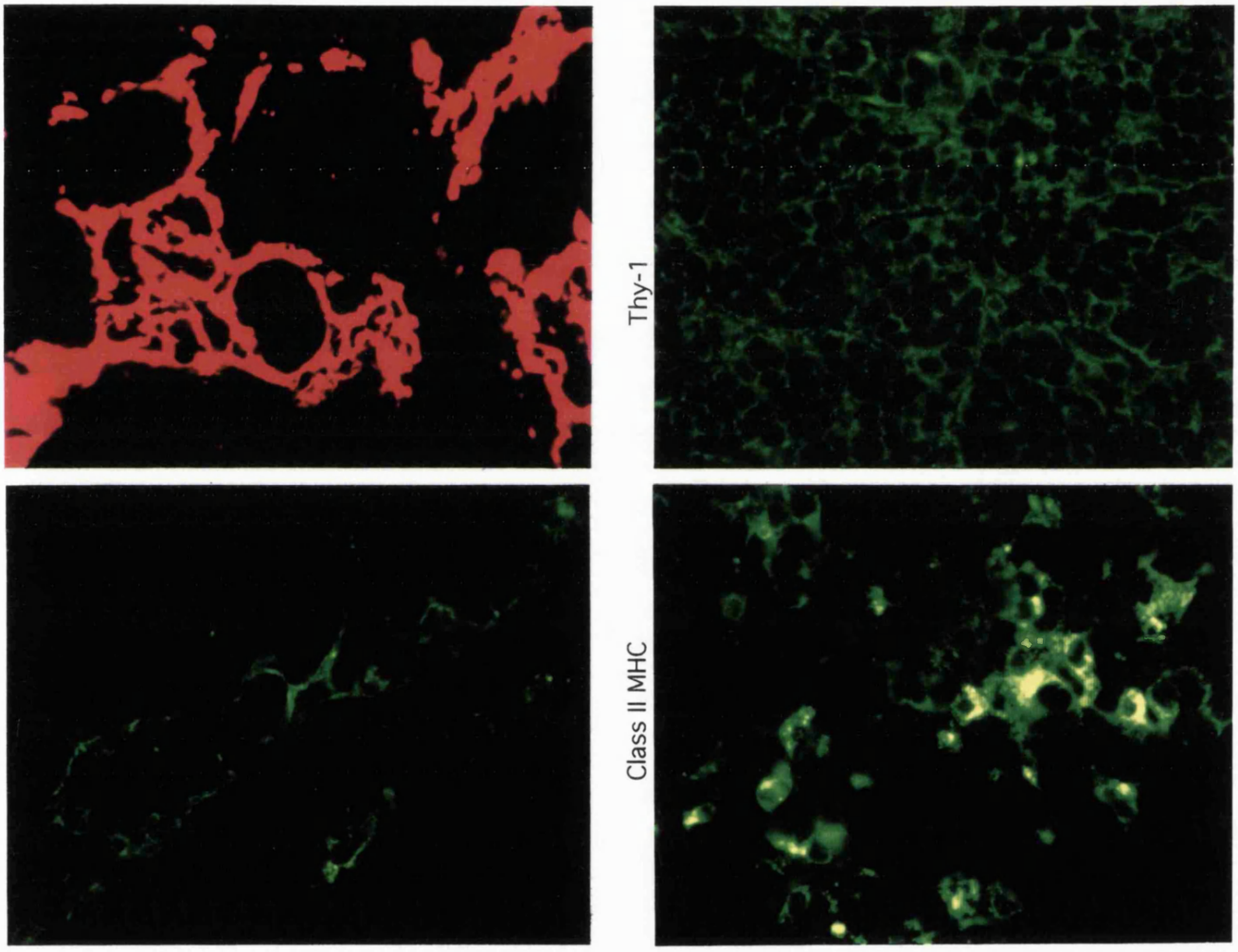
3.2.3 Cell lines derived from H-2K^b/tsA58 transgenic mice

As a first step to demonstrate the feasibility of H2ts mice as a source of conditionally immortalised cell lines, Drs. P. Jat, P. Ataliotis, and M. Noble (Ludwig Institute, London) established skin fibroblast cell lines from H2ts mice. The growth of fibroblasts from different founder mice were variously sensitive to temperature and IFN-γ, depending on the levels of tsA58 expression. Cell lines derived from H2ts6 mice were most strictly conditional in that they were sensitive to both temperature and IFN-γ (Jat et al., 1991). It was also possible to derive cell lines from other tissues of H2ts mice, including kidney, spleen, bone marrow, optic nerve, and ovary (Y.T., M. Noble, and B. Capelle, unpublished data).

Figure 9 Immunohistochemistry of the thymus in CBA Eαts12 mouse

Cryostat sections of an enlarged thymus in CBA Eαts12 mouse were stained for cytokeratin, IVC4 (medullary marker), 4F1 (cortical marker), Thy-1, and class II MHC. (Collaboration with Drs. M. Ritter and H. Ladyman, Hammersmith Hospital, London). Magnification x 120.

Immunohistochemistry of the thymus in CBA Eαts12 mouse



In order to dissect and reconstitute the T cell development in vitro, adherent cells were established from the thymus of H2ts mice in C57BL/10 (H-2^b) and CBA (H-2^k) backgrounds. The first series of thymic stromal cell lines were screened and selected by their immunohistochemical phenotype rather than function. Later, a procedure to purify a functional subpopulation of thymic stromal cells was introduced to maximise the chance to obtain functional thymic cortical epithelial cell lines.

Briefly, the thymic tissues were mechanically teased and put in culture in the presence of 10-100 IU/ml IFN-γ. Thymic stromal cells were allowed to adhere and grow (non-adherent lymphoid cells could not be immortalised under such conditions). Subsequently, adherent cells were trypsinised and subcultured at a limiting concentration after 2-4 weeks in culture by which time cells were vigorously proliferating. Individual colonies of adherent cells were then directly cloned. Since fibroblast-like cells (spindle-shaped) tend to proliferate much faster than epithelial type cells (cobblestone appearance), it was necessary to clone the cells at as earliest stage as possible. Several cell lines were established from H2ts1.9 (H-2^k), H2ts3 (H-2^b) and H2ts3F (H-2^b) mice, from which cells of epithelial phenotypes were selected. Thymic epithelial (Tep) cell lines derived from H2ts1.9 (Tep4) and H2ts3F (Tep1.2, Tep2) mice were further analysed for growth characteristics and function.

In order to test if the cell lines derived from H2ts mice are sensitive to temperature shift and induction by IFN-γ, Tep4 cells

were cultured under four conditions. Tep4 cells which have been subcultured at 33°C in the presence of 10 IU/ml IFN-γ were plated on day 0 at 33°C or 39°C and in the presence or absence of 10 IU/ml IFN-γ, and cell numbers were counted at day 3, 7, and 15. As shown in figure 10-a, Tep4 cells were sensitive to the temperature only when IFN-γ was absent, and continued to proliferate regardless of the temperature if IFN-γ was included in culture. Tep4 cells arrested their growth 3 days after they were placed under the non-permissive condition, i.e. at 39°C without IFN-γ, and became larger and flattened similarly to aging or senescent normal cells. When such cells were subcultured in the presence of IFN-γ for 4 to 8 days, they resumed proliferation again regardless of the temperature (figure 10-b). However, since Tep4 cells did not start proliferating in the absence of IFN-γ even at a permissive temperature, re-induction of tsA58 expression by IFN-γ was necessary for their growth. Thus, Tep4 cell line is temperature sensitive only in the absence of IFN-γ, and probably over-expression of tsA58 allows Tep4 cells to grow regardless of the temperature. Other cell lines, such as Tep1.1 and Tep2, showed more strict conditionality, as shown in figure 10-c, and were temperature sensitive even when tsA58 expression was induced with IFN-γ. These differences in temperature and IFN-γ dependency between cell lines probably reflect their basal and induced levels of tsA58, so that effect of shifting temperatures can be seen only when tsA58 expression is sufficiently low.

In order to identify their origin, thymic stromal cell lines were stained with antibodies which recognise different thymic stromal

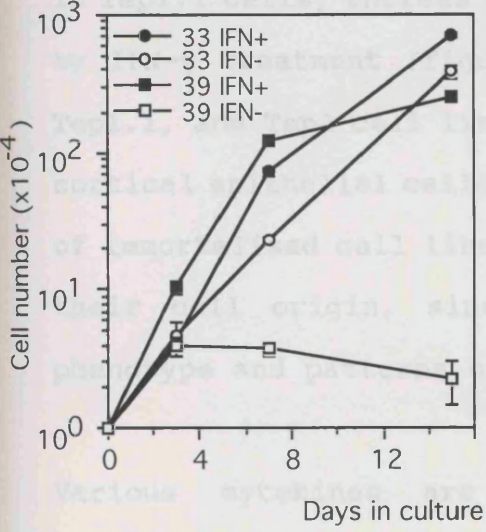
Figure 10 Growth profiles of thymic epithelial cell lines

a) Tep4 cell line were subcultured at 33°C in the presence of 10 IU/ml IFN-γ, and were plated at $1x10^4$ cells/dish (60 mm) at day 0, and were cultured either at 33°C with 10 IU/ml IFN-γ, 33°C without IFN-γ, 39°C with 10 IU/ml IFN-γ, or 39°C without IFN-γ. After culture for 3, 7, and 14 days, cells were trypsinised and numbers counted.

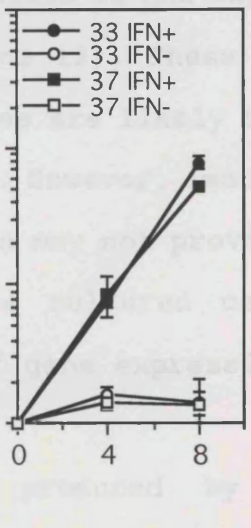
b) Tep4 cells were pre-cultured for 3 days in the absence of IFN-γ and at a restrictive temperature (39°C), were replated at $1x10^4$ cells/dish, and were cultured at 33 or 37°C in the presence or absence of 10 IU/ml IFN-γ. Cell numbers were counted at days 4 and 8.

c) Tep1.1 and Tep2 cell lines were plated at $1x10^4$ cells/dish and were cultured under different conditions as in (b), and cell numbers were counted at day 7.

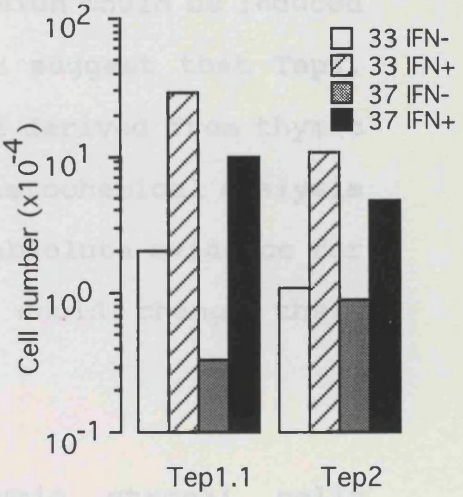
a) CBA Tep4 cell line



b) CBA Tep4 cell line



c) B10 Tep1.1 and Tep2 cell lines

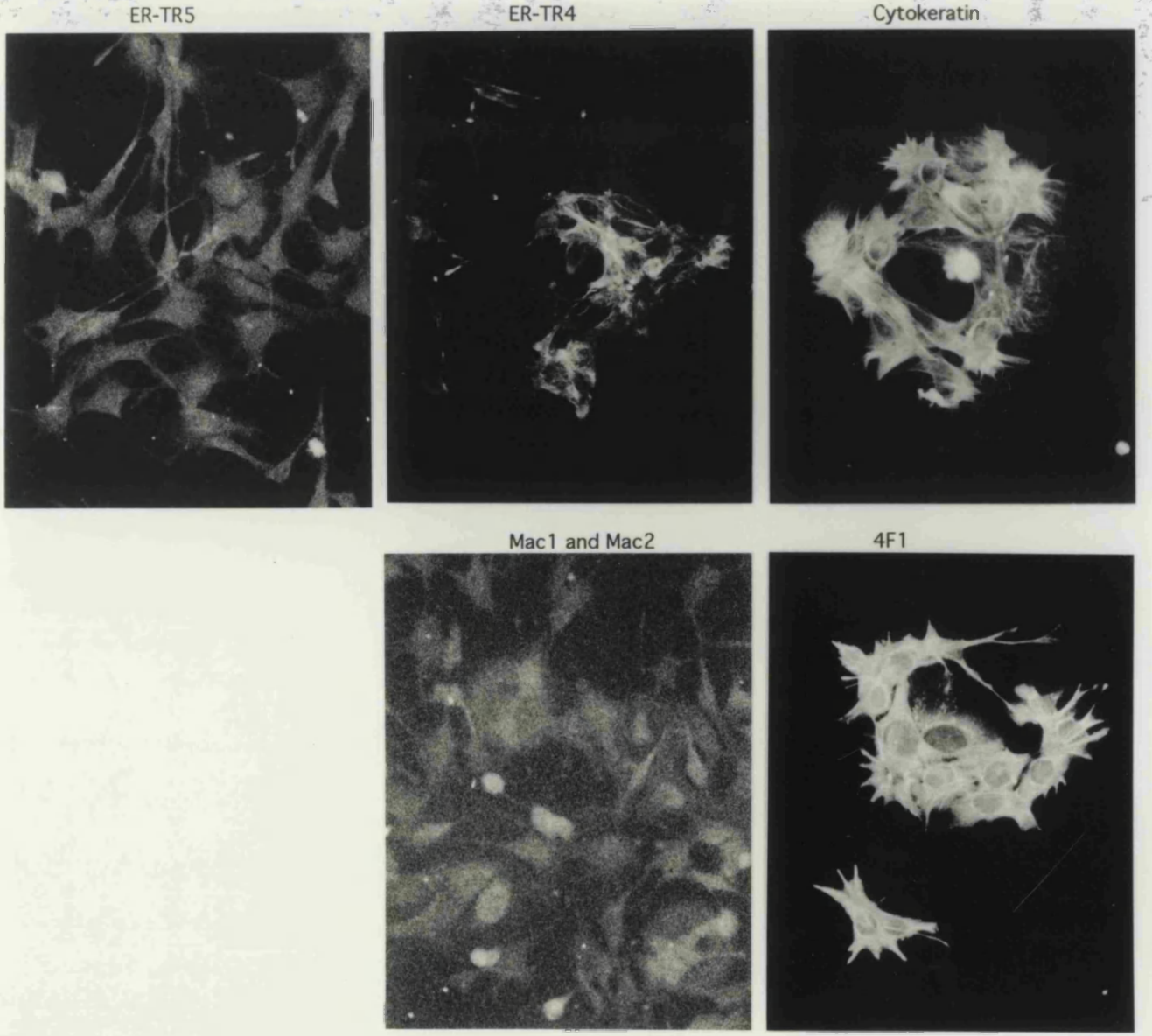


cell populations. A large panel of antibodies has been immunohistochemically characterised and classified in six groups according to the staining pattern (Kampinga et al., 1989). Among them, 4F1 and ER-TR4 are associated with cortical epithelial cells, whereas IVC4 and ER-TR5 antibodies recognise molecules expressed in medullary epithelial cells. Figure 11 shows that the phenotype of Tep1.1 cells is consistent with that of cortical epithelial cells since they express cytokeratin and two of epithelial markers (4F1 and ER-TR4), and are negative for medullary epithelial markers (IVC4, data not shown, and ER-TR5) and macrophage markers (Mac1 and Mac2). Tep4 and Tep2 cell lines have similar phenotypes. Class I MHC was expressed constitutively in Tep1.1 cells, whereas class II MHC expression could be induced by IFN-γ treatment (figure 12). These data suggest that Tep4, Tep1.1, and Tep2 cell lines are likely to be derived from thymic cortical epithelial cells. However, immunohistochemical analysis of immortalised cell lines may not provide absolute evidence for their cell origin, since cultured cells could change their phenotype and patterns of gene expression.

Various cytokines are produced by thymic stromal cells contributing to the microenvironment for T cell development. In order to test if thymic epithelial cell lines produce any cytokines, total RNA was extracted from Tep1.1 and Tep2 cells which had been pre-cultured for 4 days under a restrictive condition. Samples were reverse transcribed to cDNA, and were amplified by polymerase chain reaction (PCR) using primer sets specific for each cytokine. RNA samples from the myelomonocytic

Figure 11 Immunohistochemistry of Tep1.1 cell line

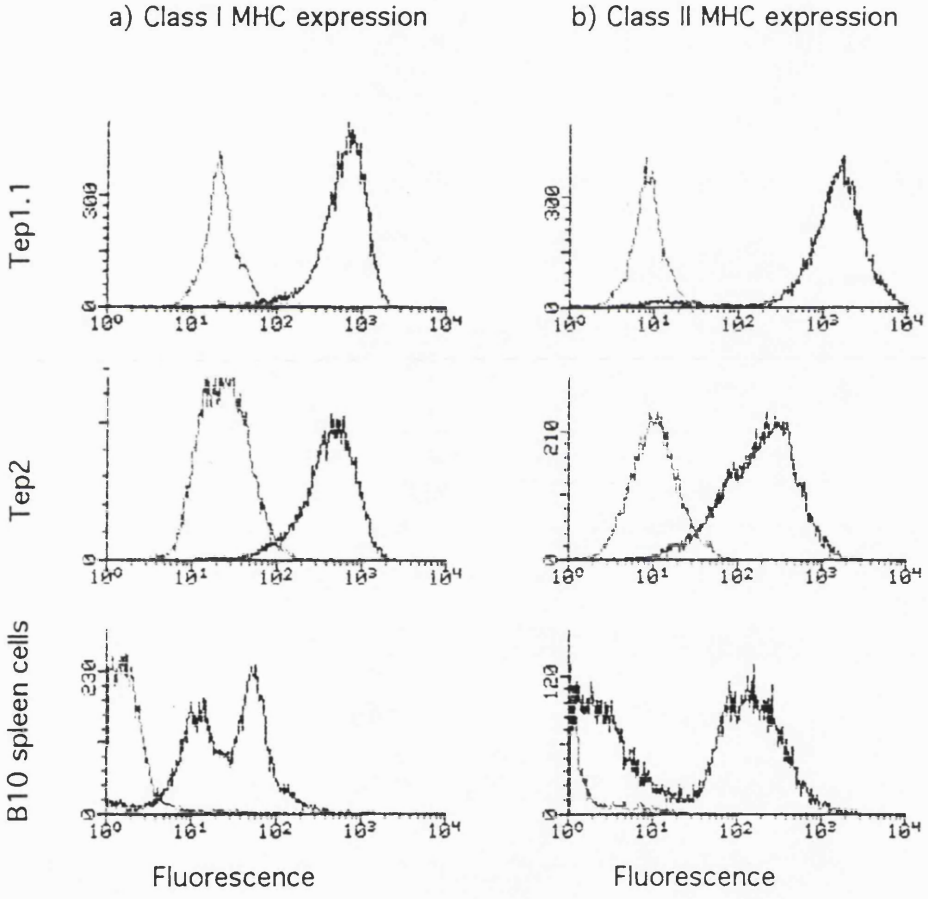
Tep1.1 cell line was grown on glass cover slips for 3 days at a non-permissive condition (at 33°C in the absence of IFN-γ), and fixed with cold methanol and stained for cytokeratin 18 (LE61), cortical epithelial markers (4F1, ER-TR4), medullary epithelial markers (ER-TR5), and macrophage markers (Mac1 and Mac2) which were revealed with FITC-conjugated anti-rat immunoglobulin antibody. A control using second layer only stained background levels. Magnification x 1200.



Immunohistochemistry of Tep1.1 cell line

Figure 12 Class I and II MHC expression on Tep cell lines

Tep1.1 and Tep2 cell lines ($H\text{-}2^b$) were cultured at 37°C in the presence of 10 IU/ml IFN-γ for 3 days trypsinised and stained with an anti-class I H-2 (M1/69) and anti-rat immunoglobulin antibody or with anti-H-2E (FITC-conjugated ox-6) antibodies as indicated by dark lines. Grey lines show second layer only or unstained controls. Total spleen cells from normal B10 mouse ($H\text{-}2^b$) were used as positive controls.


a) Class I MHC expression
b) Class II MHC expression
Tep1.1
Tep2
B10 spleen cells
300
210
230
120
0
10⁰
10¹
10²
10³
10⁴
Fluorescence
Fluorescence

cell line WEHI-3 were used as positive controls. House-keeping β-actin gene transcripts were also PCR-amplified, as an indication for the integrity of RNA preparation. As depicted in figure 13, Tep1.1 and Tep2 cells produced IL-1α and IL-7, whereas IL-6 was expressed only in Tep1.1 cells. No PCR products were detected for IL-2, IL-3, IL-4, IL-5, or IFN-γ (data not shown). Production of IL-7 strongly suggest that Tep1.1 and Tep2 cells are of cortical epithelial origin, since IL-7 is known to be most abundantly expressed in thymic cortical epithelial cells (Carding et al., 1991).

Since thymic cortical epithelial cells are in close contact with developing thymocytes in vivo, Tep1.1 cells were analysed for their ability to interact with thymocytes. Total thymocytes from adult mice were plated on monolayers of Tep1.1 cells and were incubated for 3 hours at 37°C. After culture floating thymocytes were recovered, and thymocytes bound to Tep1.1 cells were collected after a gentle wash. To assess if Tep1.1 cells interact with a specific population of thymocytes, the two fractions of thymocytes (bound and floating) were separately stained for CD4, CD8, and CD3. Figure 14-a shows patterns of CD4 and CD8 expression on thymocytes either bound to Tep1.1 cells or remaining in suspension. Although all four subsets of thymocytes appear to bind to Tep1.1 cells, DP cells are enriched in the bound fraction of thymocytes. SP cells on the other hand were found mainly in the floating fraction. The difference is further accentuated in patterns of CD3 expression as illustrated in figure 14-b. Thus, all subsets of thymocytes which bound to

Figure 13 RT-PCR analysis of cytokine and TAP expression

Total RNA was extracted from Tep1.1, Tep2, and myelomonocytic cell line WEHI-3. Samples were reverse transcribed, aliquoted, and were amplified by polymerase chain reaction using primer sets specific for IL-1α, IL-6, IL-7, TGF-β1, TAP1, TAP2, and β-actin. Mock controls were carried out without RNA. Preparation and reverse transcription of RNA samples were intact as judged by the presence of house keeping gene β-actin products.

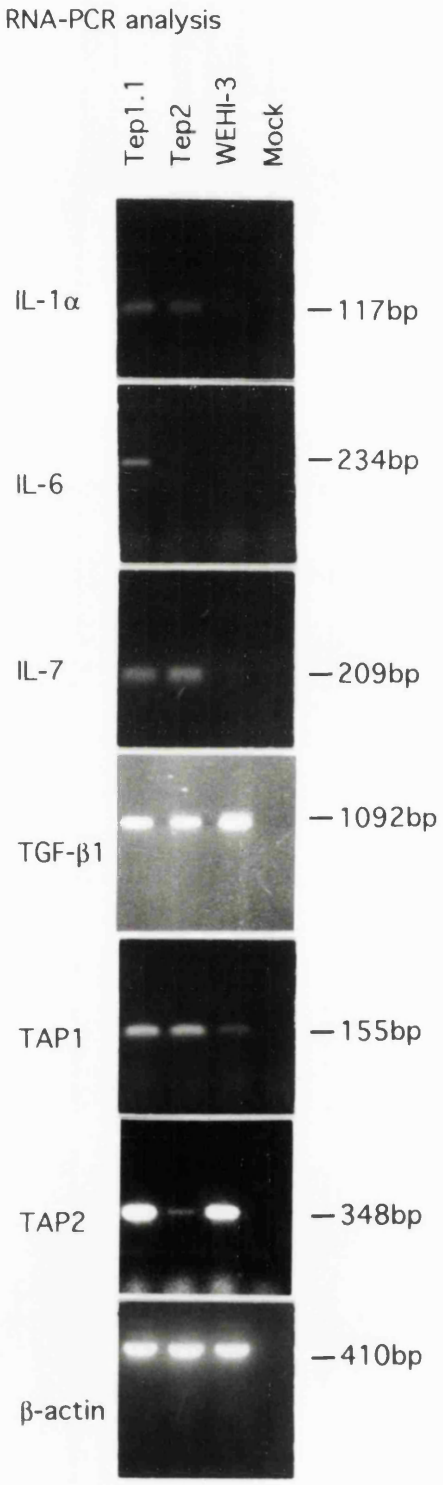
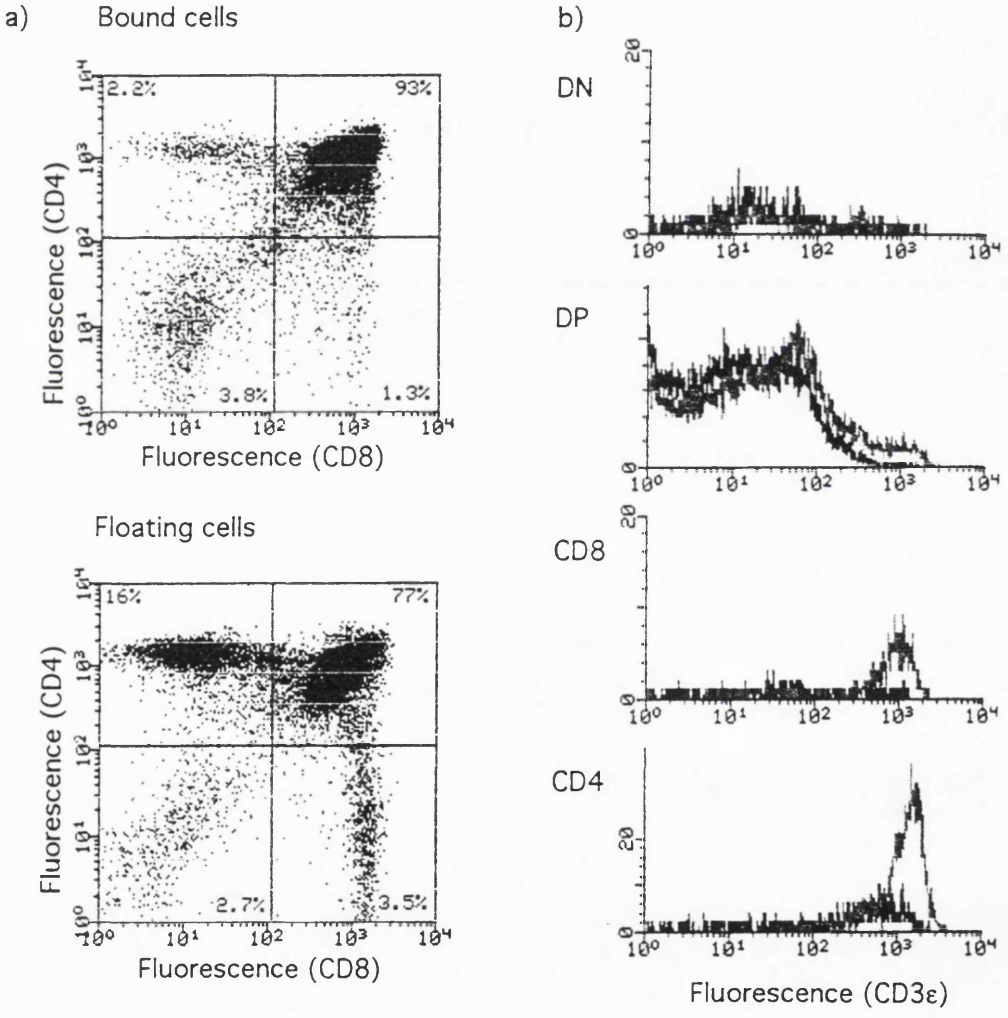

RNA-PCR analysis
Tep1.1
Tep2
WEHI-3
Mock
IL-1α
—117bp
IL-6
—234bp
IL-7
—209bp
TGF-β1
—1092bp
TAP1
—155bp
TAP2
—348bp
β-actin
—410bp

Figure 14 Lympho-epithelial binding assay

Tep1.1 cells were pre-cultured as a monolayer at a non-permissive condition for 3 days and were co-cultured with total thymocytes from adult non-transgenic mice. After 3 hours of incubation at 37°C, un-attached thymocytes were carefully removed (floating cells), remaining cells were gently washed, and thymocytes tightly bound to Tep1.1 cells were dissociated by vigorous pipetting (bound cells). Two populations of thymocytes were stained for CD4, CD8, and CD3ϵ and were analysed by FACS. a) Expression of CD4 and CD8 on non-transgenic thymocytes bound (upper) or un-bound (lower) to Tep1.1 cells. b) Levels of CD3ϵ on the four thymocyte subsets in bound (dark line) or un-bound (grey line) fractions.

Binding of non-transgenic thymocytes to Tep1.1 cell line



Tep1.1 cells expressed no or low level CD3, indicating that Tep1.1 cells specifically interact with immature thymocytes.

3.2.4 Antigen presentation by thymic cortical epithelial cell lines

Thymic cortical epithelial cells play a crucial role in generation of mature SP T cells by providing ligands for positive selection. On the other hand, cortical epithelial cells have been thought to be less efficient, compared with bone marrow-derived cells, in implementing tolerance for potentially auto-reactive T cells (Lo and Sprent, 1986). Since the ability of thymic cortical epithelial cells to process and present antigen was of an interest in this context, we analysed production of mRNA for TAP1 and TAP2 which are involved in transporting peptide fragments from cytoplasm into the endoplasmic reticulum in the class I antigen presenting pathway. As shown in figure 13, Tep1.1 and Tep2 cells transcribed both TAP1 and TAP2 genes. Taken together with the FACS analysis of class I MHC expression (figure 12), the class I antigen presentation pathway seems intact in Tep1.1 and Tep2 cells.

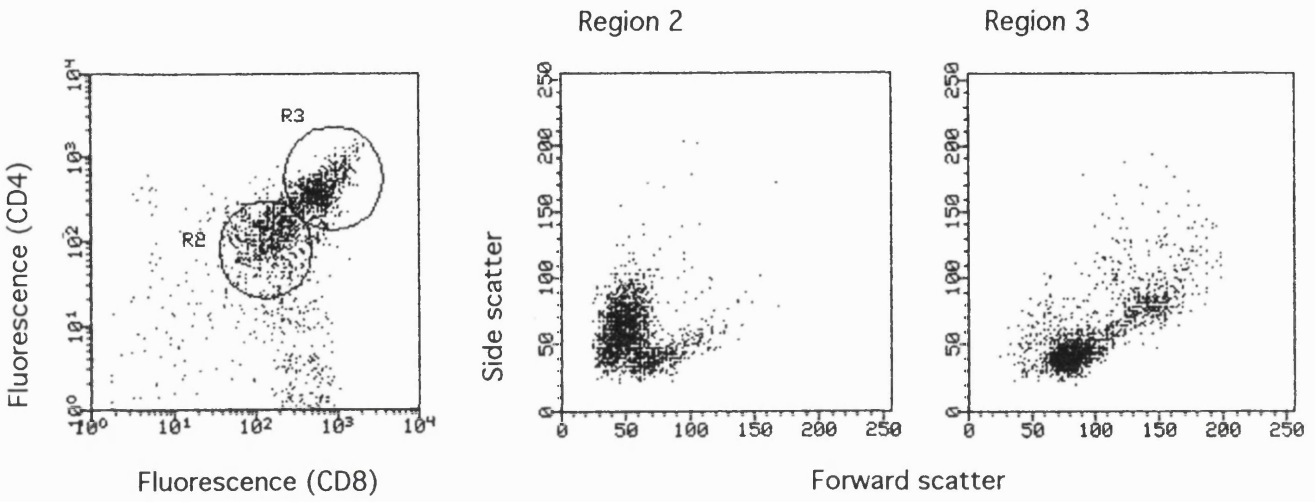
In order to assess function of the thymic epithelial cell lines in T cell development, we developed an *in vitro* assay using thymic epithelial cell lines together with H-2D^b-restricted F5 TCR transgenic thymocytes (see chapter 4.1). Thymic dendritic cells, which were freshly isolated from adult C57BL/10 mice, were

used as control antigen presenting cells. Preliminary experiments showed that culture of F5 thymocytes in the presence of cognate peptide (1968 NP) without additional antigen presenting cells accelerates cell death of F5 thymocytes, suggesting that thymocytes by themselves can induce negative selection (data not shown). Thus in order to analyse interactions between antigen presenting cells and F5 thymocytes, Tep1.1 cells ($H\text{-}2^b$) were pre-pulsed for 2 hours with 1μM 1968 NP, and were co-cultured with F5 thymocytes. After 12 hours thymocytes were analysed for their expression of CD4, CD8, and transgenic TCR β chains (Vβ11). As illustrated in figure 15-a, F5 thymocytes cultured with PBS-treated Tep1.1 cells contained two populations of DP cells, one expressing CD4 and CD8 at high levels (region R3) and the other at low levels (region R2). In cultures with peptide-treated Tep1.1 cells, the number of DP cells in R2 was proportionately increased compared with those in R3 (figure 15-b), implying an induction of negative selection of F5 thymocytes. This was supported by the fact that cells in R2 had lower forward scatter and higher side scatter compared with those in R3, as shown in the right two panels of figure 15, because thymocytes in the process of programmed cell death (apoptosis) have been known to shrink in size and become more granular (Wyllie, 1980). Similar dull staining DP population has been observed in F5 thymocytes treated with steroids <u>in vitro</u> (Y.T. data not shown) or in F5 mice after injection of the cognate peptide (Mamalaki et al., 1993). Further evidence of programmed cell death was provided by DNA analysis of two DP cell populations. F5 thymocytes were co-cultured with Tep1.1 cells, which had been pre-loaded with 100pM

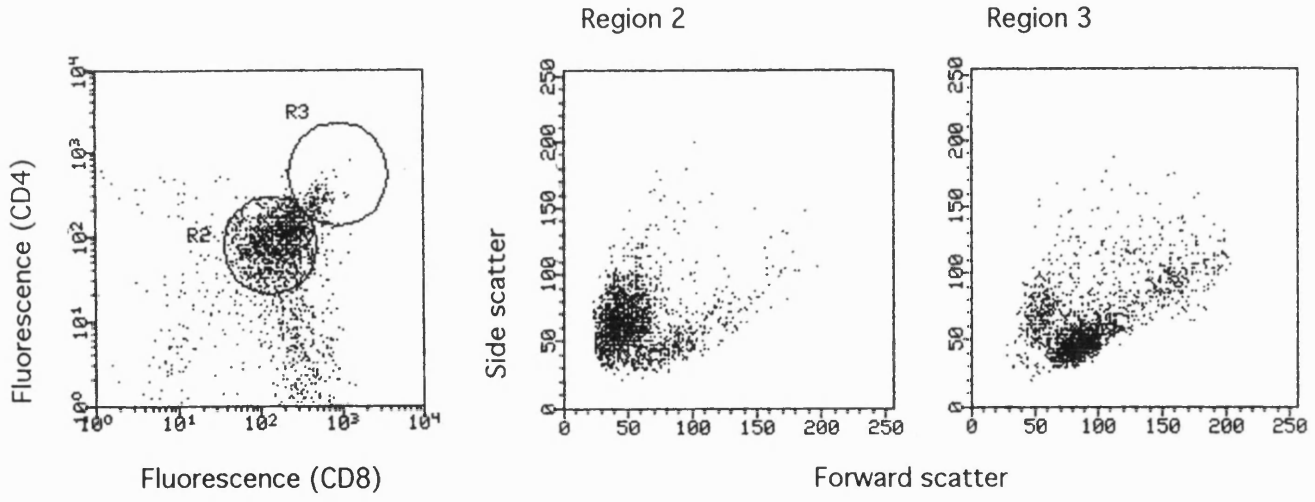
Figure 15 Programmed cell death of F5 thymocytes induced by peptide-loaded Tep1.1 cells

Tep1.1 cells were pre-cultured at 37°C in the absence of IFN-γ for 3 days. Trypsinised Tep1.1 cells were aliquoted in 96 multi-well plates at $4x10^5$ cells/well, and were incubated at 37°C in the presence or absence of 1 μM 1968 NP for 2 hours. After peptide treatment, Tep1.1 cells were extensively washed free of the peptide, and were co-cultured with $1x10^6$ total thymocytes freshly prepared from adult F5 TCR transgenic mice. After 12 hours, thymocytes were recovered by gentle pipetting, and were stained for CD4, CD8, and Vβ11 (transgenic TCR). The left panels show patterns of CD4 and CD8 expression in F5 thymocytes cultured in the presence of Tep1.1 cells pre-treated with PBS (a) or 1968 NP (b). Two populations in $CD4^+CD8^+$ cells expressing either high (R3) or low (R2) levels of CD4 and CD8 are indicated. Cell size (forward scatter) and granularity (side scatter) profiles of each double positive cell sub-populations are shown in the middle (R2) and right (R3) panels.

a) PBS



b) 1μM 1968 NP



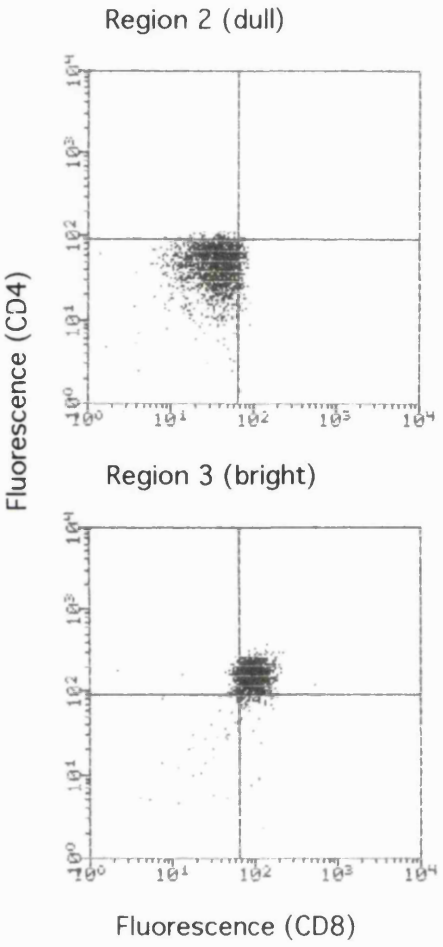
1968 NP, and were fractionated to DP dull (R2) and DP bright (R3) cells by FACS-sorting (figure 16-a). Total DNA was extracted from each population and was analysed by agarose gel electrophoresis. As shown in figure 16-b, DP cells in R3 contained intact high molecular weight genomic DNA, whereas those in R2 had a large amount of characteristic small DNA fragments generated by internucleosomal cleavages during the process of programmed cell death. These data demonstrate that Tep1.1 cells loaded with cognate peptide can cause negative selection of F5 thymocytes, causing down-regulation of CD4 and CD8 on DP cells.

In order to test dose-response relationships and cell- and MHC-specificities of this phenomenon, F5 thymocytes were co-cultured with Tep1.1 ($H\text{-}2^b$), Tep2 ($H\text{-}2^b$), Tep4 ($H\text{-}2^k$), or thymic dendritic cells ($H\text{-}2^b$), which had been pre-pulsed with various concentrations of 1968 NP. After 12 hours of culture, proportions of DP cells in regions R2 and R3 were measured, and relative numbers of intact (R3) DP cells were calculated. Relative numbers of intact DP cells were plotted against concentrations of 1968 NP. Figure 17 shows that $H\text{-}2^b$ thymic epithelial cell lines and dendritic cells induced negative selection of F5 thymocytes in a dose dependent manner. This phenomenon is MHC-restricted as thymic cortical epithelial cells of $H\text{-}2^k$ haplotype (Tep4 cells) had no effect on the number of intact DP thymocytes. Epithelial cells treated with 100 IU/ml of IFN-γ prior to co-culture expressed higher levels of class I MHC, but there was no difference in negative selection between epithelial cells whether or not they were pre-treated with IFN-γ (data not shown).

Figure 16 DNA fragmentation analysis of sorted F5 thymocytes

Total F5 thymocytes were co-cultured with Tep1.1 cells which were loaded with 100 nM 1968 NP for 12 hours as in figure 15. Thymocytes were stained for CD4 and CD8 and were sorted by into DP^{low} and DP^{high} populations. a) Levels of CD4 and CD8 in sorted DP^{low} (R2) and DP^{high} (R3) cells. b) Total DNA was extracted from sorted F5 thymocytes, and was size fractionated in an agarose gel. Lambda DNA digested with HinfI was used as a molecular size marker. DNA from region 2 (DP^{dull}) cells shows a DNA ladder characteristic of apoptotic cells.

a) Sorted DP thymocytes



b) DNA fragmentation assay

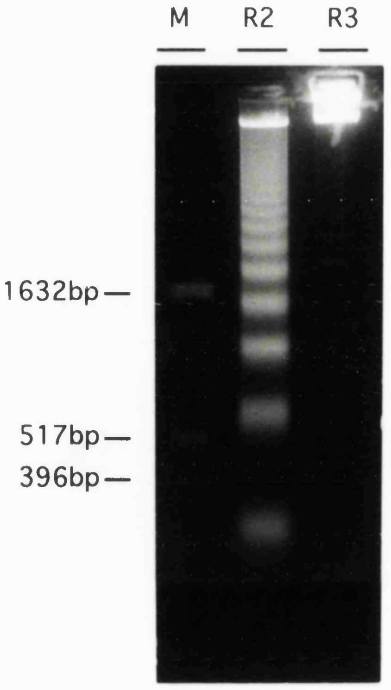
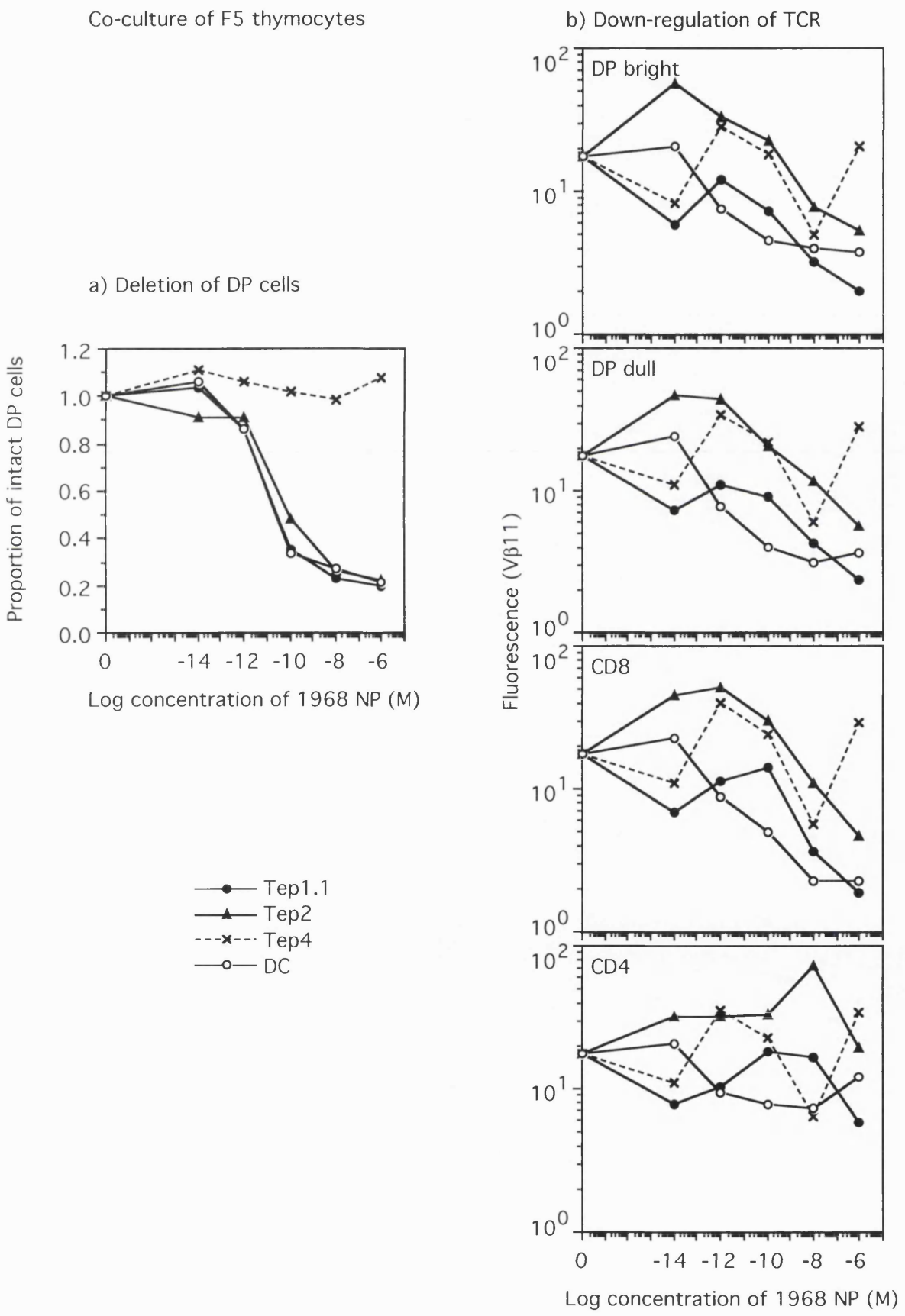


Figure 17 Negative selection of F5 thymocytes by Tep cells

Three thymic epithelial cell lines, Tep1.1 (H-2^b), Tep2 (H-2^b), and Tep4 (H-2^k) cells were pre-cultured at 37°C in the presence of 10 IU/ml IFN-γ for 3 days. Thymic dendritic cells (DC) were freshly isolated from normal B10 mice (H-2^b). The antigen presenting cells were incubated with different concentrations of 1968 NP for 2 hours, and were used for co-culture with F5 thymocytes. After 12 hours, thymocytes were stained for CD4, CD8, and Vβ11. a) The proportion of intact DP cells in cultures with different antigen presenting cells was calculated as follows: proportion of intact DP cells = (percentage of DP^{high} cells in cultures with 1968 NP) / (percentage of DP^{high} cells in cultures without peptide). b) Levels of F5 TCR expression in four thymocyte subsets are shown by mean fluorescence of Vβ11 staining at different concentrations of 1968 NP.

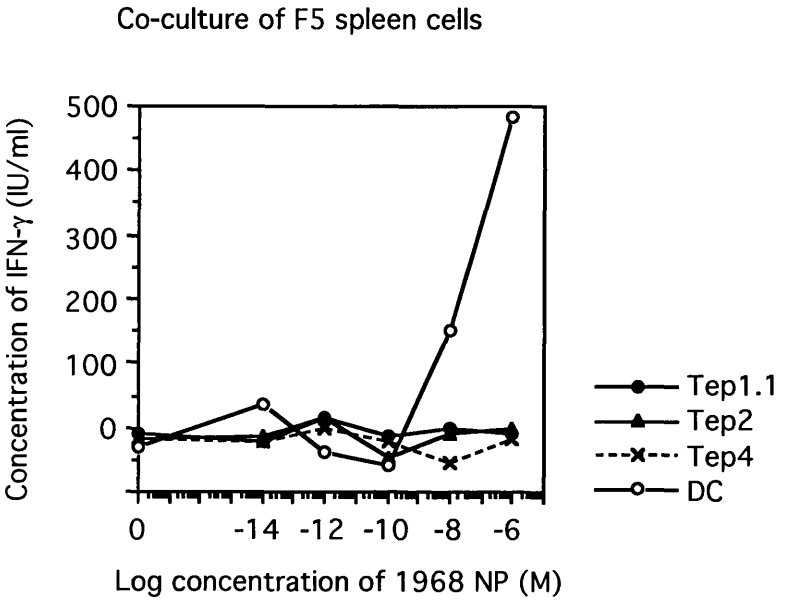

Co-culture of F5 thymocytes
a) Deletion of DP cells
Proportion of intact DP cells
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
-14 -12 -10 -8 -6
Log concentration of 1968 NP (M)
Tep1.1
Tep2
Tep4
DC
b) Down-regulation of TCR
DP bright
DP dull
CD8
CD4
Fluorescence (Vβ11)
10^2
10^1
10^0
0
-14 -12 -10 -8 -6
Log concentration of 1968 NP (M)

Levels of transgenic TCR expression on four thymocyte subsets were analysed after co-culture. As shown in figure 17-b, Vβ11 was down-regulated in DP and $CD8^+$ SP cells in co-cultures with either $H\text{-}2^b$ thymic epithelial cells or dendritic cells, but not with $H\text{-}2^k$ epithelial cells. Vβ11 on $CD4^+$ SP cells remained unaffected. In conclusion, thymic epithelial cells are as efficient as dendritic cells in causing negative selection of F5 thymocytes in suspension culture.

In order to test if the thymic epithelial cell lines can present antigen to mature F5 T cells, we studied the effect of peptide-loaded thymic epithelial cell lines and dendritic cells on F5 spleen cells. It has previously been shown that peripheral T cells in F5 mice can be activated by cognate peptide-loaded B10 spleen antigen presenting cells, resulting in cytolysis of target cells and production of IFN-γ (Mamalaki, 1992). After 3 days of co-culture of F5 spleen cells and various antigen presenting cells, amounts of IFN-γ in supernatants were quantified by enzyme-linked immunosorbent assay. Figure 18 shows that Tep1.1 and Tep2 cells failed to activate mature T cells with up to 1μM peptide, whereas dendritic cells could induce IFN-γ production with 10 nM peptide. The concentration of peptide required by dendritic cells to cause detectable deletion of double positive thymocytes (10-100 pM) was lower than that (10 nM) required to cause activation of mature T cells as measured by IFN-γ secretion, suggesting that negative selection of F5 thymocytes is more sensitive than activation of F5 mature T cells.

Figure 18 F5 CTL activation by thymic epithelial cell lines

Tep1.1, Tep2, Tep4, and thymic dendritic cells were treated with different concentrations of 1968 NP, and were co-cultured with total spleen cells from adult F5 TCR transgenic mice as in figure 17. After 3 days, culture supernatants were collected and IFN-γ concentration was measured by enzyme linked immunosorbent assay. IFN-γ concentration is indicated by IU/ml as obtained from a standard curve using IFN-γ of known concentration.


Co-culture of F5 spleen cells
Concentration of IFN-γ (IU/ml)
500
400
300
200
100
0
0
-14
-12
-10
-8
-6
Log concentration of 1968 NP (M)
Tep1.1
Tep2
Tep4
DC

3.2.5 Cell lines derived from purified cortical epithelial cells

Initial attempts to reconstitute T cell differentiation *in vitro* using immortalised thymic stromal cell lines were largely unsuccessful as described in chapter 5.1. Since these cell lines have been screened solely by their phenotype, there was a possibility that stromal cells which can support T cell differentiation were more difficult to be immortalised than others by unknown reasons. Such a problem could be overcome by purifying functional cell populations prior to their immortalisation. To this end, we fractionated thymic stromal cells from H2ts mice by a magnetic bead-based technique using different cell surface markers (Jenkinson et al., 1992). It was shown that thymic stromal cells expressing class II MHC but negative for CD45 (haematopoietic marker) or A2B5 (medullary epithelial marker) are mostly cortical epithelial phenotype and can support T cell development in reaggregate cultures *in vitro*. In order to isolate similar $CD45^-$, $A2B5^-$, and class II MHC^+ cells from a thymic tumour of an adult H2ts mouse, we first separated adherent cells and non-adherent (i.e. lymphoid) cells before depleting $CD45^+$ cells, as it was likely that many cortical epithelial cells are also removed by CD45-depletion since they are tightly bound to thymocytes. Thymic tumours were mechanically disrupted and grown in the presence of IFN-γ, allowing stromal cells to adhere on the plastic surface. After incubation for 7 days, non-adherent cells were removed, and $CD45^-$, $A2B5^-$, and class II MHC^+ cells were purified from trypsinised adherent cells. Purified cortical epithelial cells were subcultured at a

Figure 19 Colonies of purified thymic cortical epithelial cells

Thymic stromal cells from a tumour of an H2ts mouse were allowed to adhere to culture dishes for 7 days, and were fractionated using magnetic beads with antibodies against CD45, medullary epithelial marker (A2B5), and class II MHC. $CD45^{-}A2B5^{-}$class II MHC^{+} cells were cultured in a 24-multi well plate for 2 months. The top panel shows an isolated epithelial cell colony. The bottom panel depicts an epithelial colony overgrown by fibroblast-like cells. Magnification x 300.

Thymic epithelial cell colonies

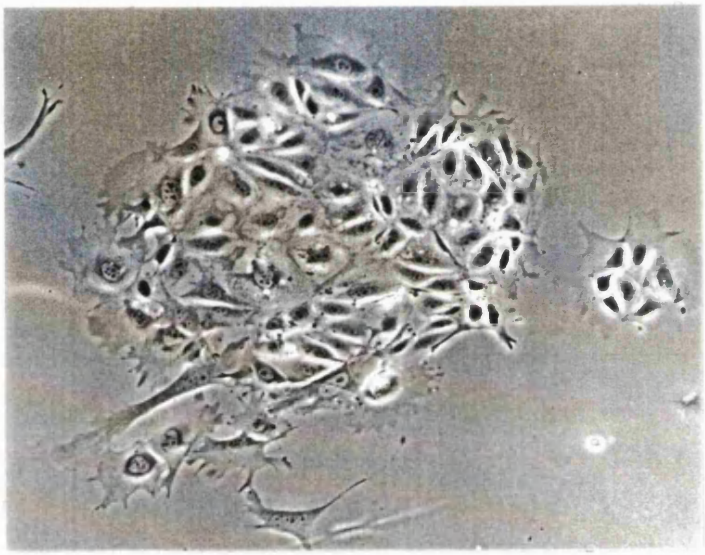
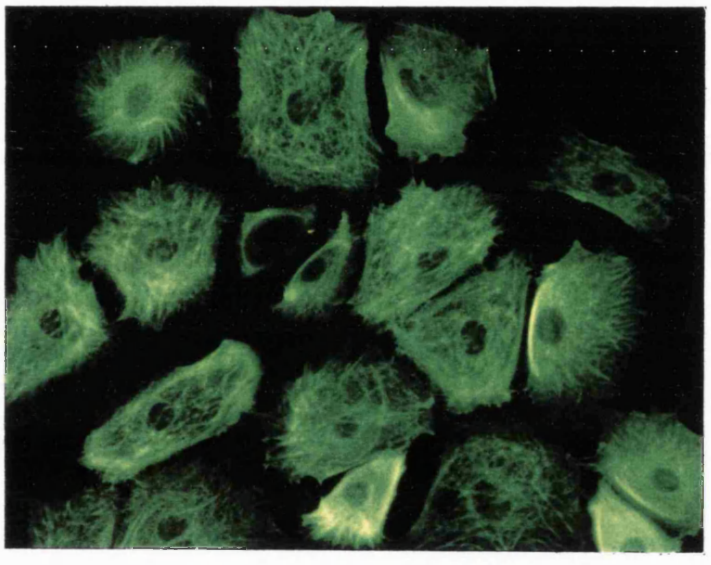


Figure 20 Immunohistochemistry of II-2 cell line

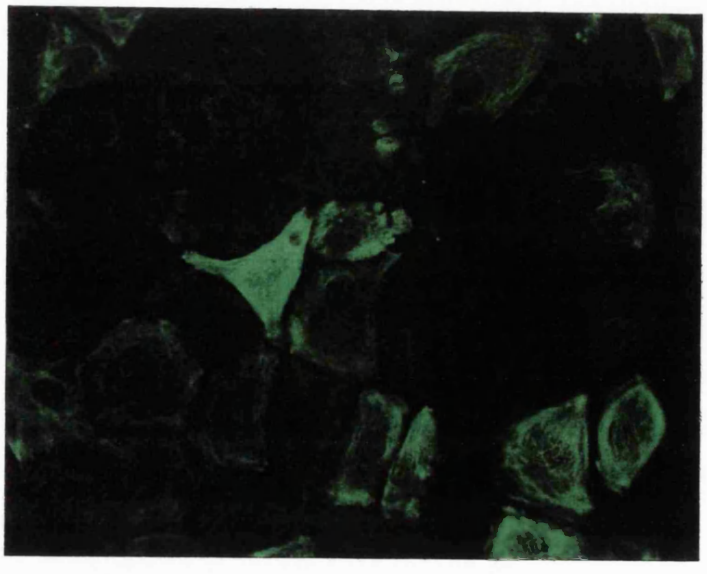
Thymic cortical epithelial cell lines were derived from a purified population of $CD45^-$, $A2B5^-$, and class II MHC^+ cells in the thymic tumour of an H2ts mouse. One of them were stained for cytokeratin (LE61) and a thymic cortical epithelial marker (ER-TR4). Magnification x 1200.

Immunohistochemistry of II-2 cell line

Cytokeratin



ER-TR4



permissive temperature in the presence of IFN-γ from which we obtained several cell lines.

Figure 19 shows various colonies observed after a secondary subculture two months after the thymic explantation. Although the initial population consisted of homogeneous epithelial cells, spindle-shaped cells and fibroblast-like cells gradually emerged and surrounded epithelial cell clusters. These fibroblast-like cells probably originate from an initial contamination which grew faster than epithelial cells. Spindle-shaped cells could be phenotypically altered epithelial cells. Epithelial cells growing as colonies were resistant to EDTA-treatment probably because they form tight junctions with each other, whereas spindle-shaped cells and fibroblasts readily came off after washing with Ca^{2+}, Mg^{2+}-free PBS. Subsequently epithelial cells were subcloned and were shown to express cytokeratin and a thymic cortical epithelial marker as depicted in figure 20. Function of these epithelial cell lines will be discussed in chapter 5.2.

3.3 c-myc

3.3.1 Thy-1/c-myc transgenic mice

In an attempt to establish immature T cell lines for *in vitro* studies, transgenic mice carrying a proto-oncogene c-myc have been generated in our laboratory as reported before (Spanopoulou

et al., 1989). In order to derive thymocyte lines from defined MHC backgrounds, the 13.0 kb hybrid construct, consisting of a 4.8 kb mouse c-myc fragment inserted into the first intron of mouse Thy-1.2 gene, was injected into fertilised oocytes from CBA and C57BL/10 inbred mice. Six CBA and 12 C57BL/10 transgenic founder mice were obtained. Mice were sacrificed at the onset of dyspneic symptoms, thymic tumours were gently teased and put in culture, and expression of CD4 and CD8 in thymocytes and peripheral T cells was analysed. All six CBA TM mice developed thymic tumours between 6 and 12 weeks of age (table 3), which consisted either predominantly of $CD4^+CD8^+$ cells (TM8 and TM13) or of a mixture of DP and $CD4^+CD8^-$ cells (TM2, TM11, and TM12) as illustrated in figure 21. Four out of six CBA TM mice had peripheral metastasis as judged by increased proportion of $CD4^+CD8^+$ cells in lymph nodes (table 3 and figure 21). In order to further assess the effect of c-myc on T cell development, thymic tumour cells were stained for HSA which in normal mice is expressed at high levels in DP cells and at low levels in SP cells. Thymocytes in three CBA TM mice expressed high level HSA, whereas thymocytes from other three expressed low level. It remains to be studied why DP thymocytes are preferentially expanded in most TM mice. Possible explanations are 1) c-myc is expressed at the highest levels in DP cells, 2) DP cells are more susceptible to transformation by c-myc, 3) overexpression of c-myc blocks T cell differentiation at the DP stage, or 4) because there are more DP cells than other subsets in the thymus. Given the fact that single positive cells exist in the thymus and periphery in TM mice, differentiation block by c-myc is unlikely.

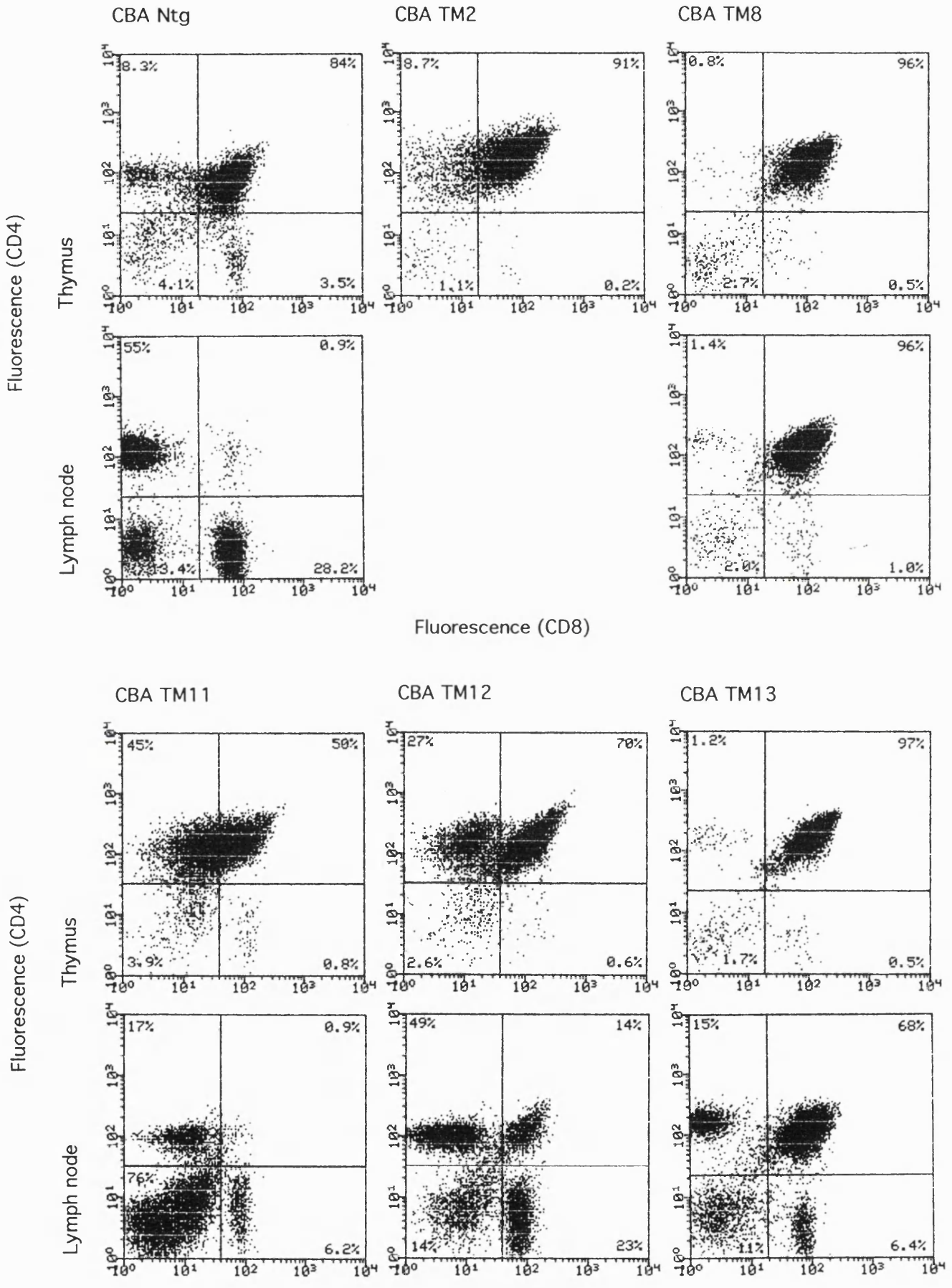
Table 3 Thy-1/c-myc mice

Mice	Age†	Vβ expression‡	HSA‡	Metastasis	Cell lines
CBA TM2	8	-	-	+ (20 %¶)	-
CBA TM7	12	-	-	-	-
CBA TM8	8	-	+	+ (96 %)	+
CBA TM11	10	-	-	-	-
CBA TM12	10	$V\beta8^+$ (37 %)	+	+ (15 %)	-
CBA TM13	6	$V\beta8^+$ (56 %)	+	+ (68 %)	+
B10 TM1	14	-		-	+
B10 TM2	9	$V\beta11^+$			+
B10 TM4	9				+
B10 TM5	15				
B10 TM15	11	-		-	-
B10 TM16	11	$V\beta8^+$	+	+	-
B10 TM17	11	-		-	-
B10 TM22	16				
B10 TM26	12				
B10 TM28	10				
B10 TM29	10				
B10 TM30	10				
F5/TM1	7	$V\beta11^+$		-	+
F5/TM2	9	$V\beta11^+$		-	+
F5/TM7	16	$V\beta11^+$ (46 %)		+ (25 %)	+
F5/TM25.1	12	$V\beta11^+$	+	-	+
F5/TM25.2	12				
F5/TM25.3	12				
F5/TM25.5	15				
F5/TM25.6	15				
F5/TM25.7	16				
F5/TM25.8	16				
F5/TM25.9	17				
F5/TM25.10	18				
F5/TM25.11	18				
F5/TM25.12	18				
F5/TM25.13	18				

† Age in weeks when mice were found dyspneic due to thymic tumour
‡ Thymocytes
¶ Proportions of $CD4^+CD8^+$ cells in lymph nodes or spleens

Figure 21 Phenotype of T cells in TM mice

Thymic tumour cells and lymph node cells from a non-transgenic CBA mouse and CBA Thy-1/c-myc (TM2, 8, 11, 12, and 13) transgenic founder mice were stained for CD4 and CD8. Peripheral metastasis is manifested with an appearance $CD4^{+}CD8^{+}$ cells in lymph nodes.


CBA Ntg
CBA TM2
CBA TM8
Thymus
Lymph node
8.3%
84%
4.1%
3.5%
8.7%
91%
1.1%
0.2%
0.8%
96%
2.7%
0.5%
55%
0.9%
3.4%
28.2%
1.4%
96%
2.0%
1.0%
Fluorescence (CD4)
Fluorescence (CD8)
CBA TM11
CBA TM12
CBA TM13
Thymus
Lymph node
45%
50%
3.9%
0.8%
27%
70%
2.6%
0.6%
1.2%
97%
1.7%
0.5%
17%
0.9%
76%
6.2%
49%
14%
14%
23%
15%
68%
11%
6.4%
Fluorescence (CD4)
Fluorescence (CD8)

As Thy-1 is expressed at higher levels in DP cells than in other subsets, probably these DP cells are more susceptible to transformation due to higher levels of transgenic c-myc.

In order to examine the clonal origin of thymic tumour cells, usage of TCR Vβ segments was analysed. In two (TM12 and TM13) out of six CBA TM mice, a large proportion of thymocytes expressed Vβ8, suggesting a mono- or oligo-clonal nature of the thymic tumour. This was further proven by southern blot analysis of TCR β gene rearrangement which demonstrated clonal TCR D_β-J_β rearrangement events as depicted in figure 7. These observations support the hypothesis that oncogenic transformation in TM mice takes place at the DP stage in a stochastic manner as suggested before (Spanopoulou et al., 1989). In spite of such (oligo-)clonal transformation of DP thymocytes <u>in vivo</u>, not all of expanded DP cells were able to survive in culture, and only two of CBA TM mice (TM8 and TM13) gave rise to continuously proliferating DP cell lines.

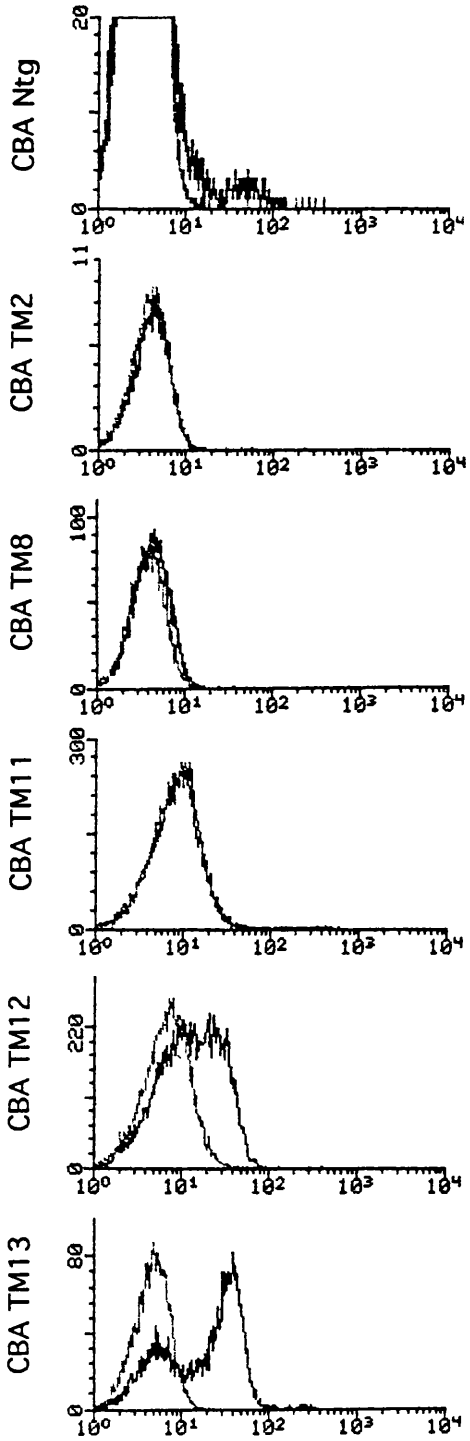
Twelve C57BL/10 (B10) TM founder mice also developed thymic tumours (table 3). Patterns of CD4, CD8, Vβ8, and HSA expression on T cells in the thymus and periphery were analysed in four B10 TM mice. The phenotype of thymocytes in B10 TM mice was similar to that of CBA TM mice and one of B10 TM thymic tumours (TM16) contained a high proportion of V$\beta 8^+$ cells.

Since DP cell lines established from TM mice were found to undergo spontaneous programmed cell death <u>in vitro</u> (data not

Figure 22 Clonal expansion of thymocytes in TM mice

Thymocytes from a non-transgenic CBA mouse and TM2, 8, 11, 12, and 13 mice were stained with an anti-Vβ8 antibody (F23.1) and were revealed with phycoerythrin-conjugated anti-hamster anti-immunoglobulin antibody (black line). Controls were stained only with the second layer antibody (grey line).

Vβ8 expression of thymocytes



shown), we analysed the presence of apoptotic DNA in vivo. Total genomic DNA was extracted from the thymus of a normal CBA mouse, thymic tumours of CBA H2ts, Eαts, or TM mice, or from the thymus and lymph node of a B10 TM mouse, and were size fractionated by electrophoresis in agarose gels. As shown in figure 23, normal thymus and H2ts or Eαts thymic tumours had no detectable amount of small size DNA fragments. In contrast, DNA from thymic tumours of TM mice contained the characteristic DNA ladder caused by internucleosomal fragmentation of DNA. In the lymph node of the B10 TM mouse, which did not have metastasis, there was no DNA fragmentation, suggesting that DP cells in the thymus were undergoing apoptosis. The presence of apoptotic cells in TM mice was further supported by histological examination of the thymic tumour, as depicted in the top panel of figure 24. Apoptotic cells are evident by a condensed nuclear staining pattern. The present data suggest that transformation of DP thymocytes in TM mice stand on a balance between opposing effects of the c-myc on cell growth and cell death.

3.4 Bone marrow chimaeras

The thymus consists of haematopoietic cells which are derived from migrating bone marrow (BM) stem cells, and non-haematopoietic resident stromal cells. In order to determine the primary cause of thymic tumour or hyperplasia in c-myc and tsA58 transgenic mice, BM stem cells from oncogene transgenic mice were transplanted into non-transgenic irradiated recipients in

Figure 23 Increased apoptosis in the thymus of TM mice

Total DNA was extracted from a non-transgenic CBA mouse, thymic tumours of CBA H2ts1, Eαts4, TM13, and B10 TM1 mice, and lymph nodes of B10 TM1 mouse. Samples were size fractionated by electrophoresis in agarose gels and stained with ethidium bromide. Positions of size markers (HinfI digested lambda DNA) are indicated.

Apoptosis in the thymus of TM mice

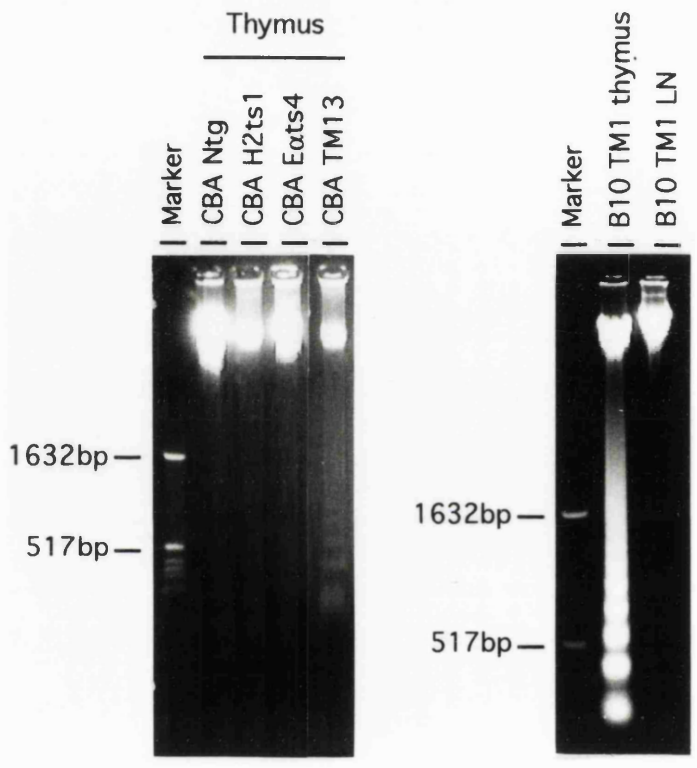
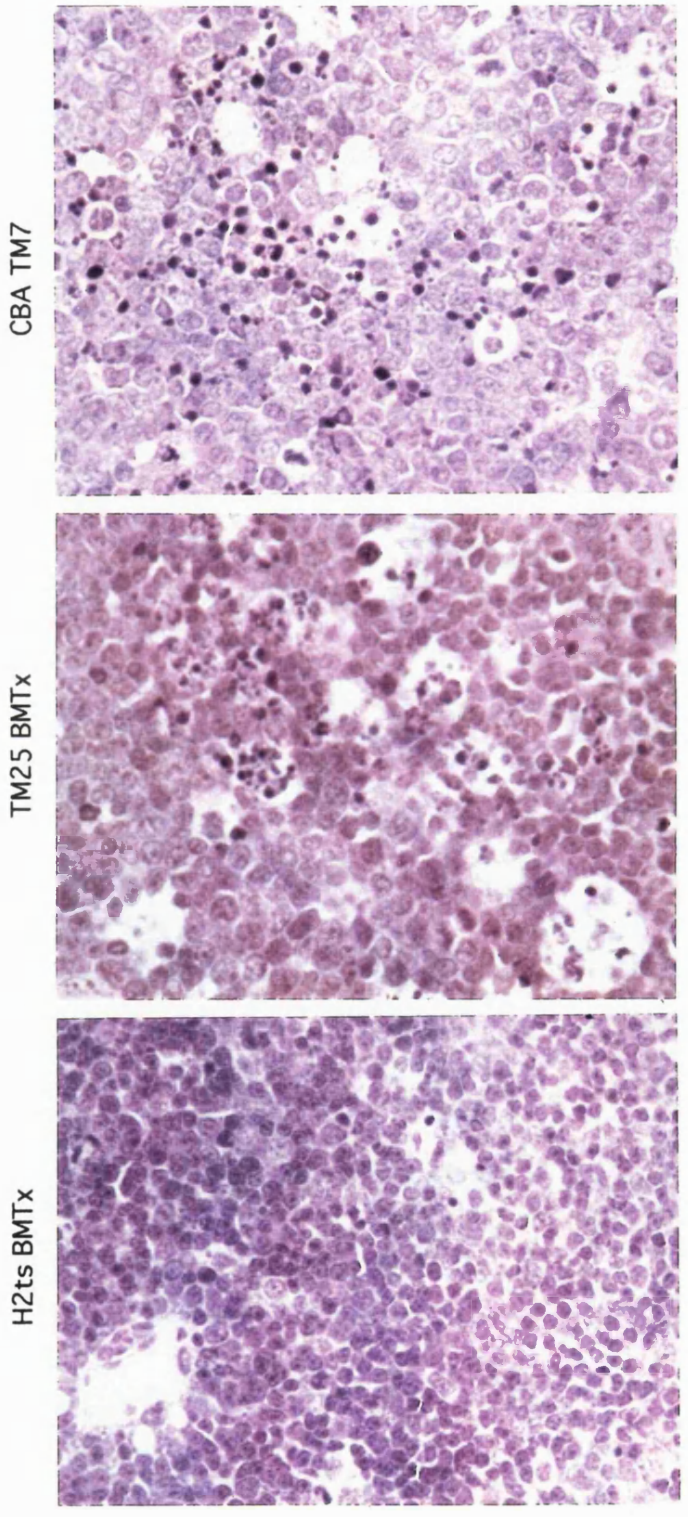


Figure 24 In situ apoptosis of thymocytes in TM mice and bone marrow chimeras

Sections of the thymus of a CBA TM7 mouse or chimeras which were irradiated and received bone marrow cells from either TM25 or H2ts mouse were stained with haematoxylin and eosin. Apoptotic cells have condensed nuclear staining patterns as seen in TM7 and TM25-chimaera.

Apoptosis of thymocytes in TM mouse and bone marrow chimaera



collaboration with Dr. E. Dzierzak (NIMR, London).

In the first experiment (table 4), normal (CBA/B10)F_1 mice were sublethally irradiated with 950 rad using a Cobalt source and were injected intravenously (i.v.) with either $1.1X10^7$ BM cells from normal mice (n = 3), $0.7X10^7$ BM cells from H2ts.CBA1.2 mouse (n = 2), or $1.2\text{-}1.0X10^7$ BM cells from TM25 mice (n = 8). On the day of BM transfer, donor H2ts and TM mice did not have thymic tumours. Each one of recipients which received normal or H2ts BM cells was sacrificed after 11 and 14 days for examination of haematopoietic reconstitution by spleen colony formation assay. As shown in figure 25-a, normal or H2ts BM chimeric mice had larger spleens at day 11 ($CFU\text{-}S_{11}$) than that of a non-reconstituted mouse, demonstrating a successful engraftment of BM stem cells. Haematopoietic colonies were further confirmed by histological examinations on these spleens (data not shown). Since $CFU\text{-}S_{11}$ reflects relatively committed stem cell activity, spleen colony formation was also examined at day 14 ($CFU\text{-}S_{14}$) in order to assess more immature and long-term reconstituting haematopoietic activity. Figure 25-b shows that both normal and H2ts BM chimaeras were successfully reconstituted as judged by spleen sizes.

The recipients sacrificed for $CFU\text{-}S_{14}$ assay were used as donors for secondary BM transfer experiments. Thus, either $6.5X10^6$ BM cells from the primary recipient of normal BM cells or $7.0X10^6$ BM cells from the primary recipient of H2ts BM cells were

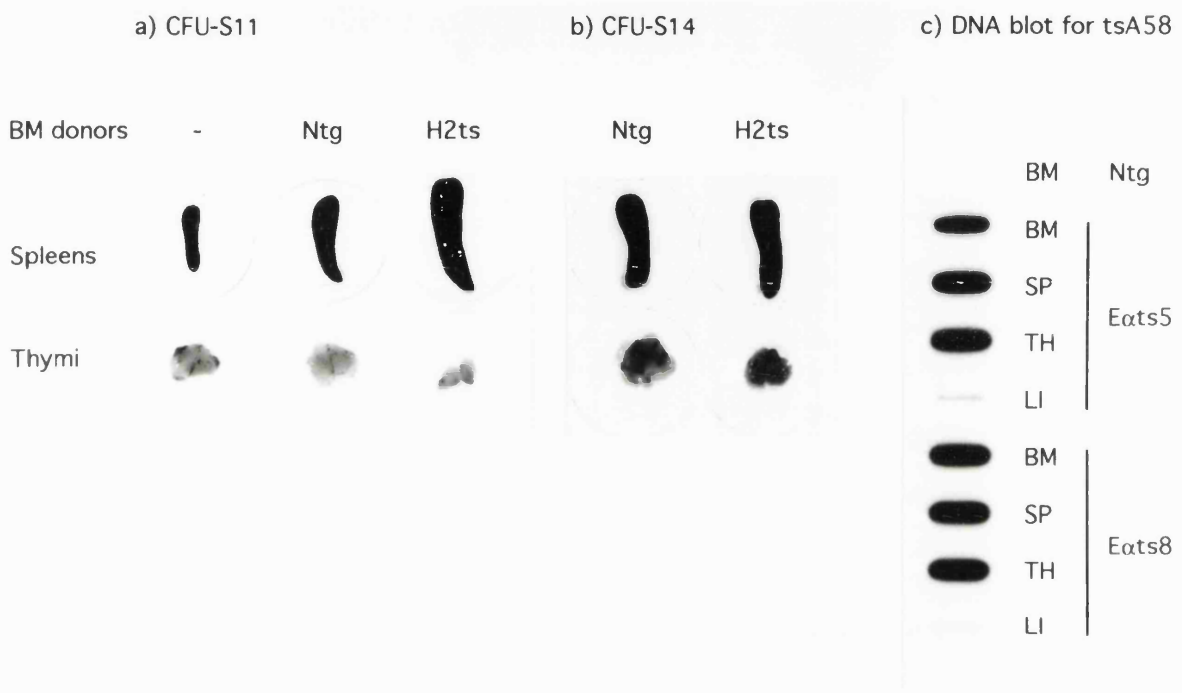
Table 4 Thymic tumorigenesis in bone marrow chimaera

Donors	Age	Tumour†	Latency	Tumour formation
Experiment 1				
(CBA/B10)F_1		-	> 6 months	0/3
- secondary‡			> 6 months	0/4
H2ts.CBA1.2	74 days	-	> 6 months	0/2
- secondary‡			> 6 months	0/4
TM25.1		-	86-97 days	4/4
TM25.2		-	85-97 days	4/4
Experiment 2				
(CBA/B10)F_1		-	> 60 days	0/5
Eαts.CBA5	35 days	+	> 60 days	0/5
Eαts.CBA8	31 days	+	> 60 days	0/5

† The presence of thymic tumours in donor mice
‡ Reconstituted bone marrow cells were transplanted into secondary recipients

Figure 25 Analysis of bone marrow chimerism

a) Recipient (CBAxB10)F_1 mice were sublethally irradiated with 900 Rad using a Co source, and were injected intravenously with or without bone marrow stem cells from either non-transgenic or H2ts F_1 mice. After 11 days, each one of recipient mice were sacrificed for examination of colony forming unit in the spleen (CFU-S11) which reflects activity of relatively differentiated haematopoietic stem cells. Spleens from recipients which received non-transgenic or H2ts bone marrow stem cells were bigger than that in a control mouse which received no bone marrow cells. Spleen colonies were confirmed by histological analysis (data not shown). b) Recipients as described above were analysed for colony forming unit in the spleen at day 14 (CFU-S14) after bone marrow transfer, which reflects activity of relatively un-differentiated haematopoietic stem cells. Large spleens in recipients of normal or H2ts bone marrow stem cells indicate that haematopoietic reconstitution was successful in these mice. c) Sublethally irradiated recipient F1 mice were i.v. injected with bone marrow cells from normal, Eαts5, or Eαts8 mice. After two months, chimeras were sacrificed and DNA was extracted from the bone marrow cells, spleen cells, thymocytes, and liver cells. DNA samples were blotted and were hybridised with a tsA58 probe. Haematopoietic tissues were highly reconstituted with Eαts-derived cells, whereas DNA from the liver showed low levels of tsA58 signals. Bone marrow DNA from the mouse reconstituted with non-transgenic (Ntg) bone marrow stem cells did not hybridise with tsA58 (a negative control). (Collaboration with Dr. E. Dzierzak, NIMR, London).


a) CFU-S11
b) CFU-S14
c) DNA blot for tsA58
BM donors
-
Ntg
H2ts
Ntg
H2ts
Spleens
Thymi
BM
Ntg
BM
SP
TH
LI
Eαts5
BM
SP
TH
LI
Eαts8

transferred to sublethally irradiated secondary recipients (n = 4 each). None of mice which received normal or H2ts BM cells developed thymic tumours within 6 months after BM transfer, whereas the parental H2ts mice developed thymic hyperplasia at 3 months of age. These data suggest that haematopoietic cells are not the primary cause of thymic enlargement in H2ts mice.

In contrast, all four recipient mice which received TM25 BM cells developed thymic tumours after approximately the same latency period as parental TM25 mice i.e. 3 months. In addition, the CD4/CD8 phenotype of thymocytes in TM25 chimaeras was very similar to that of parental TM25 mice, which is characterised by a large number of $CD4^+CD8^+$ and $CD4^+CD8^-$ cells (figure 26-a). In addition, one of two recipients of TM25 bone marrow cells which were analysed had an increased proportion of $V\beta8^+$ thymocytes (figure 26-b), suggesting a mono- or oligo-clonal origin of the tumour. Thymic tumour cells expressed intermediate levels of Thy-1 (figure 26-c), but were completely negative for HSA (figure 26-d) in spite of the fact that the parental TM25 mice frequently developed thymic tumours expressing high HSA. The present data suggest that tumorigenesis in TM mice is genetically programmed in developing thymocytes.

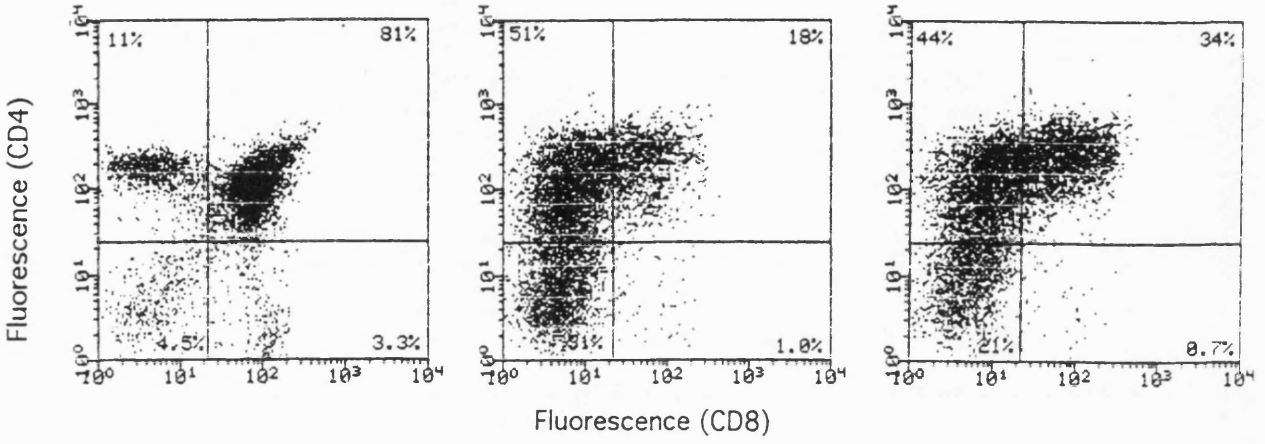
To further identify cells expanded in thymic tumours in TM25 BM chimaeras, thymic tissue sections were stained for Thy-1 and thymic epithelial cell markers in collaboration with Drs. M. Ritter and H. Ladyman (Hammersmith Hospital, London). As shown in figure 27, there were almost no cells expressing cytokeratin

Figure 26 Phenotype of thymocytes in bone marrow chimeras

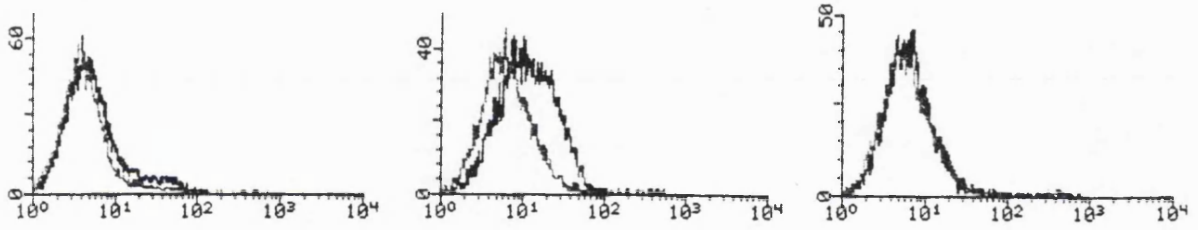
Sublethally irradiated recipient mice were reconstituted with bone marrow stem cells from non-transgenic (Ntg), TM25.1, or TM25.2 mice as in figure 25-a. After 3 months, mice were sacrificed and thymocytes were stained for CD4 and CD8 (a), Vβ8 (b), Thy-1 (c), and HSA (c).

BM donors Ntg TM25.1 TM25.2

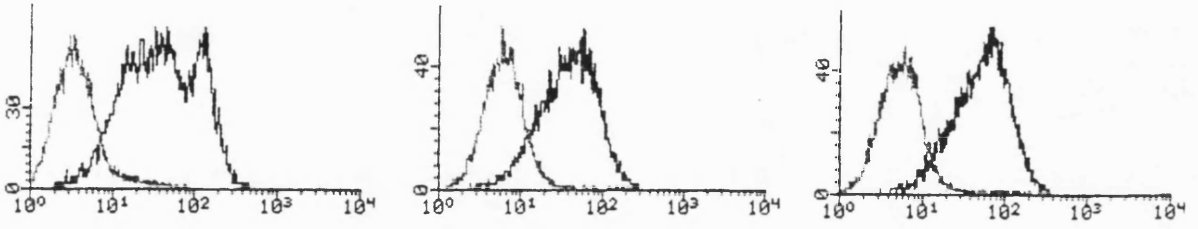
a) Thymus



b) Vβ8 expression



c) Thy-1 expression



d) HSA expression

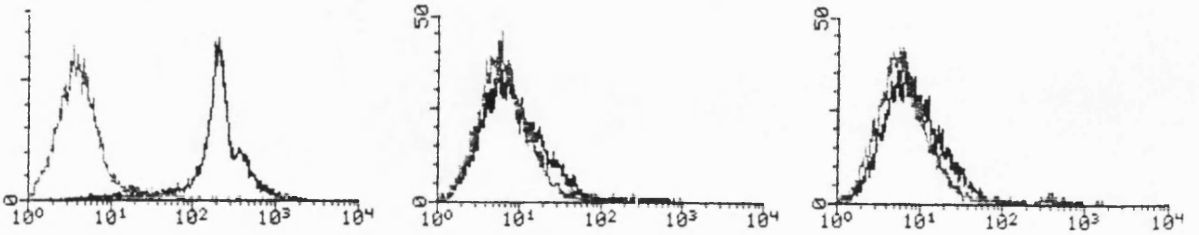
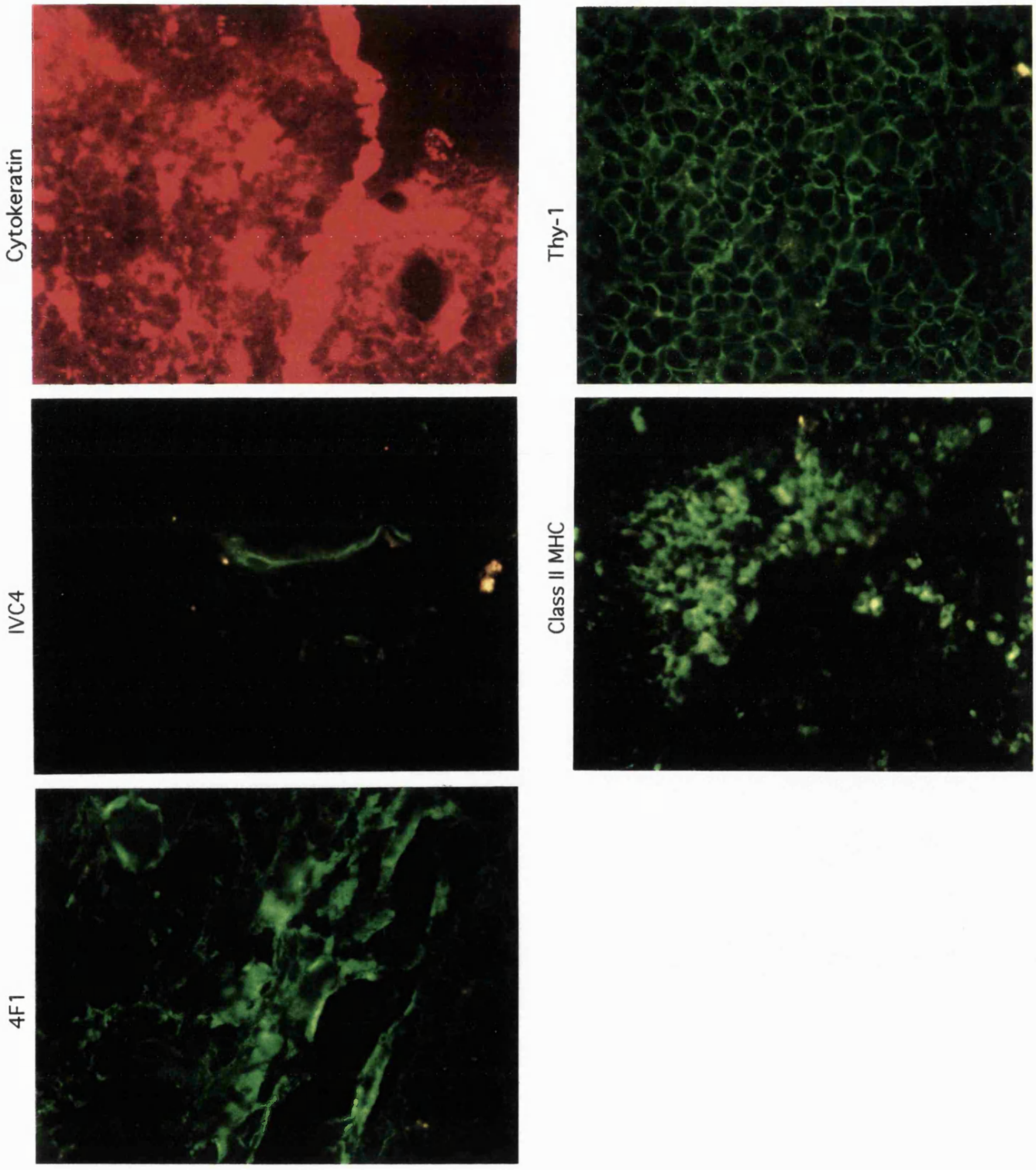


Figure 27 Immunohistochemistry of the thymus of TM25 bone marrow chimaera

TM25 bone marrow chimeras were generated as described in figure 26. Cryostat sections of a thymic tumour of one of the recipients were stained for cytokeratin, IVC4 (medullary marker), 4F1 (cortical marker), Thy-1, and class II MHC. (Collaboration with Drs. M. Ritter and H. Ladyman, Hammersmith Hospital, London).

In the cytokeratin staining, most cells were negative and the picture shows one of few clusters of positive cells.

Immunohistochemistry of the thymic tumour in TM25 bone marrow chimaera



except for a small group of cytokeratin+ cells as shown in the picture. Very occasionally patches of 4F1 or IVC4 (perivascular) staining cells were observed. Class II MHC$^+$ cells were present as islands or scattered positive cells, which were probably macrophages or dendritic cells. The tumour consisted mostly of large lymphocytes expressing high levels of Thy-1 with some variability. Selective expansion of thymocytes in TM25 BM chimaeras, in contrast to lympho-epithelial proliferation in original TM25 mice, suggests that thymic epithelial cells in TM25 mice were expanded autonomously, rather than due to secondary effect of over-produced thymocytes. This is in line with the ability of both cell types to be established in culture as reported before (Spanopoulou et al., 1989).

In the experiment 2 (table 4), either $1X10^6/1X10^7$ normal BM cells, or $5X10^6/1X10^7$ Eαts BM cells were i.v. injected into lethally irradiated mice (n = 5 each). Both donor Eαts mice had enlarged thymuses on the day of transfer. All recipient mice survived, and the haematopoietic reconstitution by Eαts BM stem cells was confirmed by slot blot analysis of DNA which showed tsA58 signals in the bone marrow, thymus, spleen, but not in the liver (figure 25-c). In spite of the fact that parental Eαts mice developed thymic hyperplasias within 1 month after birth, none of the Eαts BM recipient mice developed thymic tumours after 2 months. These data suggest that enlargement of the thymus in Eαts mice is primarily caused by non-haematopoietic cells, particularly those which express higher levels of class II MHC such as cortical and medullary epithelial cells.

In summary, thymic hyperplasia in H2ts and Eαts mice is caused primarily by proliferation of non-haematopoietic cells. Simultaneous expansion of thymocytes in these mice is probably due to increased capacity of the thymic stroma to support T cell development. In contrast, thymic tumorigenesis in TM mice is cell autonomous and primarily caused by a genetic programme in both immature T cells and thymic epithelial cells. It has recently been noted that thymic stromal cells and developing thymocytes are mutually dependent and lack of one compartment causes destruction of normal thymic architecture (reviwed in Ritter and Boyd, 1994; van Ewijk et al., 1994). Further analysis on cellular mechanisms of different thymic tumour models may provide information about signals involved in normal organogenesis of the thymus.

Chapter 4 TCR transgenic mice

4.1 Introduction

The cytotoxic T cell (CTL) clone F5 is derived from a C57BL/6 mouse primed by intranasal injection with live influenza virus A/NT/60/68 (H3N2), and recognises a nonameric peptide from the nucleoprotein ($NP_{366-374}$, ASNENMDAM, abbreviated 1968 NP) in the context of H-$2D^b$ (Townsend et al., 1984; Townsend et al., 1986). The F5 TCR utilises $V_{\alpha 4}J_{F5}$ and $V_{\beta 11}D_{\beta 2.1}J_{\beta 2.1}C_{\beta 2}$ gene segments (Palmer et al., 1989). Mamalaki et al. in our laboratory generated transgenic mice carrying the cDNA for the F5 TCR α and β chains inserted in the 5′ untranslated region of a human CD2 minigene cassette (Mamalaki et al., 1993). This expression vector has been shown previously to drive expression of transgenes in a T cell specific, copy number dependent, and integration site independent manner (Greaves et al., 1989). In F5 transgenic mice, T cell development is skewed towards $CD8^+$ cells in the thymus and in the periphery, most of which express the transgenic β chain as detected by an anti-Vβ11 antibody.

As discussed earlier, TCR is thought to have an immunoglobulin-like structure which is characterised by CDR loops hanging out of β-sheet sandwich domains. According to Cothia's alignment of TCR α and β gene families, the top surface of the F5 TCR could

be composed of CDR1α: 25-STTWYPT-31, CDR2α: 48-K^{+}VTTANNK^{+}G-55, CDR3α: 93-GD^{-}R^{+}QGGR^{+}ALIFG-104, CDR1β: 26-ISGH^{+}SA-31, CDR2β: 47-IYFR^{+}NQAPID^{-}D^{-}-57, and CDR3β: 95-SSR^{+}TGGH^{+}AE^{-}QFF-106, in which + and - symbols represent charges of side chains (figure 28). CDR1 and CDR2 loops are thought to make contact with H-2D^{b}, and the most variable CDR3 loops are expected to have the most important contribution to the contact with peptide (figure 28-a). However, precise locations of CDR loops do not go beyond speculation at present.

The H-2D^{b} molecule has recently been co-crystallised with an NP from influenza virus A/PR/8/34 (Young et al., 1994). The 1934 NP (ASNENMETM) differs from the 1968 NP at positions 7 and 8 and does not activate the F5 clone (Townsend et al., 1984). The top surface of the H-2D^{b} is composed of two α-helices; α1: 57-PEYWER<u>E</u>TQ<u>K</u>AKGQEQ<u>W</u>FRVSLR<u>N</u>LLG<u>Y</u>Y-85, and α2: 140-AQI<u>T</u>RRK<u>W</u>EQ<u>S</u>GAAE<u>HY</u>KA<u>Y</u>LEGECVEWLHR<u>Y</u>LK-173, in which underlined amino acids make hydrogen bonds with the peptide (figure 28-b). The 1934 NP peptide is held in the D^{b} cleft by 22 hydrogen bonds, five are indirect being mediated by ordered water molecules, ten are direct bonds between backbone atoms of the peptide and side chain atoms of residues of D^{b}, and seven are direct bonds between side chain atoms of the peptide and side chain atoms of D^{b} residues. Among side chains of the peptide amino acids, those of serine residue at position 2 (designated P2S), P3N, P5N, and P9M are buried in the groove, whereas those of P1A, P4E, P6M, P7E, P8T are exposed to the solvent. Since the movement of side chains

Figure 28 Interaction between F5 TCR and H-2D^b

a) Primary sequences of the F5 TCR α and β chains (GeneBank accession numbers X14387 and X14388 respectively) aligned with α chain of TA65 (Vα4) and β chain of AK1 (data from Chothia, 1988). Comlementarity determining regions are indicated by squares accoring to models described (Katayama, 1995). Letters in bold are conserved residues in immunoglobulin and TCR.

b) Primary sequences of α-helices of H-2D^b according to the crystallographic data (Bjorkman, 1989). Residues lining on the top surface are indicated by bold letters according to the data described (Young, 1994).

c) A model of alignment between F5 TCR and H-2D^b molecules. The two triangles represent CDR1, CDR2, and CDR3 loops of TCR α and β chains. Two alpha helices of H-2D^b are shown by cylinders. Charge distribution on the top surface of H-2D^b/1968 NP complex predicts that TCR α and β chains interact with α1 and α2 helices of H-2D^b respectively. Positively charged arginines in CDR3 loops of the F5 TCR may directly interact with negatively charged aspartate and/or glutamate in the 1968 NP.

a) F5 TCR

Vα

CDR1 CDR2

F5 DSVTQMQGQVTLSEDDFLFINCTYSTTWYPTLFWYVQYPGEFPQLLLKVTTANNKGI
TA65 DSVTQTEGQVALSEEDFLTIHCNYSASGYPALFWYVQYPGEGPQFLFRASRDKEKGS

CDR3

SRGFEATYDKGTTSFHLQKASVQESDSAVYYCVLGDRQGGRALIFGTGTTVSV
SRGFEATYDKGTTSFHLRKASVQESDSAVYYCALGTNTGYQNFYFGKGTSLTV

Vβ

CDR1 CDR2

F5 NAGVIQTPRHKVTGKGQEATLWCEPISGHSAVFWYRQTIVQGLEFLIYFRNQAPIDDSGMPK
AK1 NAGVIQTPRHKVTGKGNEATLWCEPISGHSAVFWYRQTSVQGLEFLTYFRNQAPIDDSGMPK

CDR3

ERFSAQMPNQSHSTLKIQSTQPQDSAVYLCASSSRTGGHAEQFFGPGTR
ERFSAQMPNQSHSTLKIQSTQPQDSAVYLCAS

b) MHC

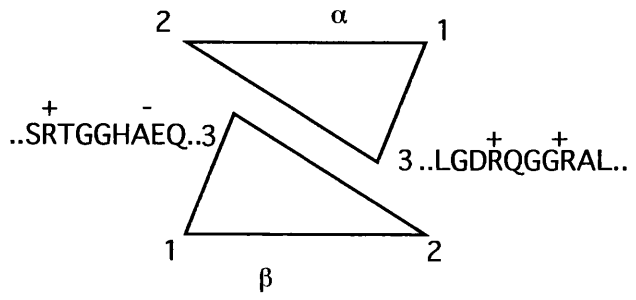
α 1 helix

Db RETQKAKGQEQWFRVSLRNLLGYY
Dq RITQIALGQEQWFRVNLRTLLGYY

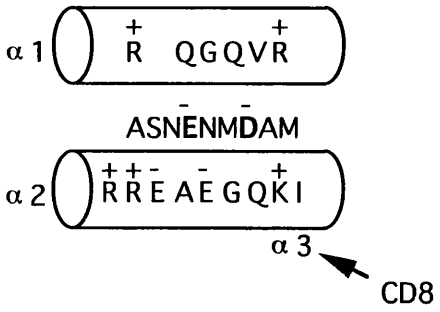
α 2 helix

Db ITRRKWEQSGAAEHYKAYLEGECVEWLHRYL
Dq ITRRLWEQAGAAEYYRAYLEGECVEWLHRYL

c) An alignment of F5 TCR and H-2Db/1968 NP



F5 TCR viewed from bottom



H-2Db viewed from top

of peptide residues at position 7 and 8 are not sterically restricted, the 1968 NP/D^b complex is expected to take an almost identical structure to that of the 1934 NP/D^b complex (figure 52-a). Thus, P1A, P4E, P6M, P7D, and P8A residues of the 1968 NP, which are accessible to the solvent, are likely to make direct contact with the F5 TCR. Assuming that CDR3 loops interact with the peptide, two negative charges in the NP antigen ($P4E^-$ and $P7D^-$) could make salt bridges with complementary positive charges in the CDR3α ($95R^+$ and $99R^+$) and CDR3β ($97R^+$ and $101H^+$). Among residues lining the top of $\alpha1/\alpha2$ helices of D^b are E^-58, R^+62, Q65, G69, Q72, V76, R^+79, L82, R^+145, K^+146, Q149, E^-154, H^+155, A158, E^-163, E^-166 (figure 28-b). Although all of these may not necessarily make salt bridges with CDR loops of TCR, the most reasonable alignment (to avoid electrostatic repulsion) seems to be obtained by CDR2α and CDR1α loops lying over $\alpha1$ helix of D^b, CDR2β and CDR1β loops over $\alpha2$ helix, CDR3β over the N-terminus and CDR3α over the C-terminus of the peptide (figure 28-c). These speculations of course are testable by point mutations on the TCR α and β chains to assess their ability to recognise H-$2D^b$ and NP 1968 molecules.

4.2 Kinetics of ontogeny and selection of T cells

4.2.1 In vivo ontogeny

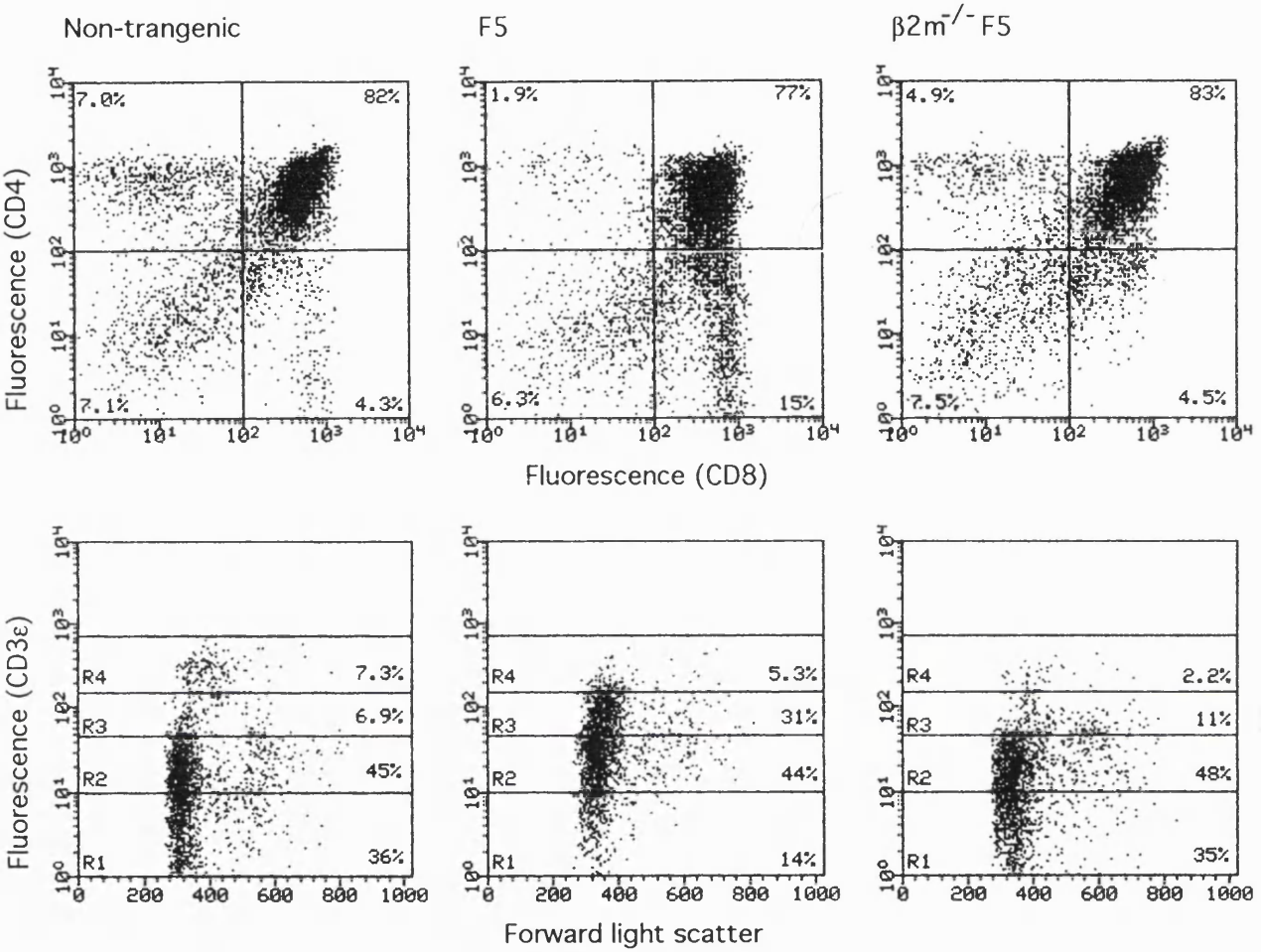
T cell development is associated with tightly regulated patterns of gene expression including CD4, CD8, and TCR. In order to determine impacts of transgenic TCR expression in F5 mice, different thymocyte subsets in fetal, neonatal, and adult mice were analysed in comparison with non-transgenic mice. Development of F5 T cells was also studied in a class I MHC-deficient ($\beta 2m^{-/-}$) background. Patterns of CD4 and CD8 expression on T cells in the thymus and spleen of normal, F5, and $\beta 2m^{-/-}$F5 mice are shown in figure 29. F5 thymocytes (a) and spleen T cells (b) contained more CD8 SP T cells than CD4 SP T cells in comparison to normal T cells, suggesting a preferential development of CD8 lineage in F5 mice as described before (Mamalaki et al., 1992). There was a defect in CD8 T cell development in $\beta 2m^{-/-}$F5 mice, demonstrating that development of F5 T cells requires the presence of class I MHC. The lower panel of figure 29-a shows cell size and levels of CD3ϵ on thymocytes. Four different levels of CD3 were observed in normal thymocytes, i.e. negative (R1), low (R2), intermediate (R3), and high (R4). In F5 mice, there was a decrease in the proportion of $CD3^-$ and $CD3^{high}$ cells and increase in $CD3^{int}$ cells compared with normal mice. The pattern of CD3 expression in $\beta 2m^{-/-}$ F5 mice was similar to that in normal mice. Figure 30 illustrates expression of CD4 and CD8 in four thymocyte subsets of normal, F5, and $\beta 2m^{-/-}$F5 mice which were gated by CD3 levels (R1 to R4 in figure 29-a). Thus, in normal and F5 mice, TCR^- cells consisted of DN ($CD4^-CD8^-$) and DP cells, and TCR^{low} cells consisted of intermediate $CD4^{dull}CD8^{dull}$ and DP cells. TCR^{int} cells in normal mice contained mostly DP cells, whereas those in F5 mice contained DP

Figure 29 Phenotype of thymus and spleen T cells in F5 mice

a) Thymocytes from a non-transgenic B10, an F5, and a $\beta 2m^{-/-}$F5 mice were stained for CD4, CD8, and CD3ϵ. Top panels show patterns of CD4 and CD8 expression. Bottom panels show cell size and levels of TCR (CD3ϵ) depicting gates of different levels of TCR as follows: $CD3^{-}$ (R1), $CD3^{low}$ (R2), $CD3^{int}$ (R3), and $CD3^{high}$ (R4).

b) Total spleen cells were stained as above, and expression of CD4 and CD8 is shown.

a) Thymus



b) Spleen

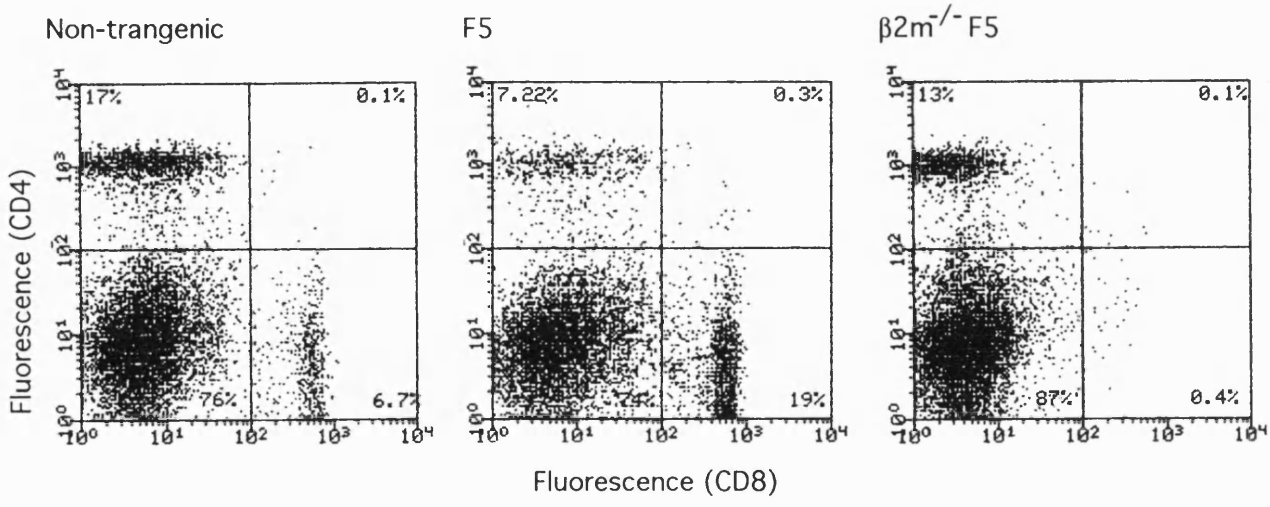
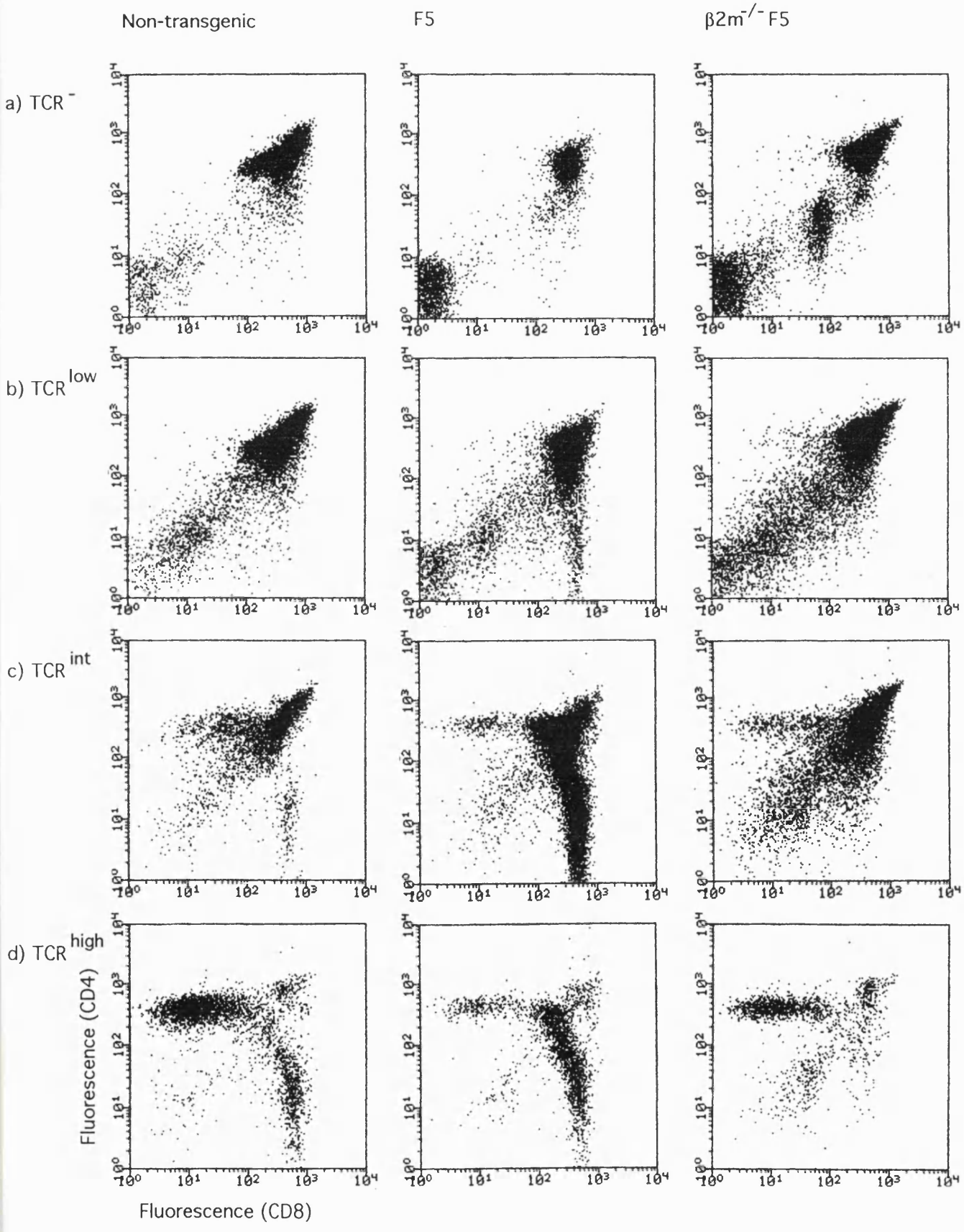


Figure 30 Thymocytes expressing different levels of TCR

Thymocytes from non-transgenic, F5, and $\beta 2m^{-/-}$F5 mice were stained as in figure 29, and were gated into four subsets. Patterns of CD4 and CD8 expression in TCR^{-} (a), TCR^{low} (b), TCR^{int} (c), and TCR^{high} (d) populations are shown.


Non-transgenic
F5
β2m-/- F5
a) TCR-
b) TCRlow
c) TCRint
d) TCRhigh
Fluorescence (CD4)
Fluorescence (CD8)

cells and also CD8 SP cells. CD4 or CD8 SP cells came in the fraction of TCR^{high} cells in normal mice as well as in F5 mice. Patterns of CD4 and CD8 expression in $\beta 2m^{-/-}$F5 mice were similar to normal mice except for the absence of CD8 SP cells. The observation that most CD8 SP cells in F5 mice express intermediate level TCR could be explained by limited transcription efficiency by the CD2 gene expression vector, or by an intrinsic property of the F5 TCR. Given the fact that CD8 T cells in homozygous F5 mice can express higher level TCR, as comparable to that in normal mice (C.M. unpublished data), the former explanation is likely.

The precursor-progeny relationships between different thymocyte subsets could be deduced from the order of their appearance during early thymus ontogeny. To this end, expression of CD4, CD8, and TCR on developing thymocytes was analysed in normal and F5 fetuses and neonates. At day 14 of gestation, fetal thymocytes consisted of $CD4^-CD8^-CD3^-$ cells in normal (data not shown) and F5 mice (figure 31). Triple negative cells in F5 mice up-regulated CD4, CD8, and CD3 Vβ11 in a sequential order during development (figure 32). Figure 33-a illustrates a slight difference between normal and F5 transgenic thymocytes at transition from DN to DP cells on day 15 to 16 of gestation. In both normal and F5 mice, DN cells started expressing low levels of CD4 and CD8 by day 15, and also became blastic in size (data not shown). During the following 24 hours, most of these DP^{low} cells in F5 mice further up-regulated CD4 and CD8 to higher levels, whereas most DP^{low} cells in normal mice remained so until after day 16. This

Figure 31 Ontogeny of CD4 and CD8 expression on F5 fetal thymocytes

Fetal thymocytes from F5 TCR transgenic mice at day 14, 15, 16, 17, and 18 of gestation, and thymocytes in an adult F5 mouse were stained for CD4, CD8, and Vβ11. The morning when plugs were found was counted as day 0 in the afternoons. Thus, day 14 in the figure corresponds to day 14.5 of gestation, and so on. Patterns of CD4 and CD8 expression are shown.

Fetal ontogeny in F5 mice

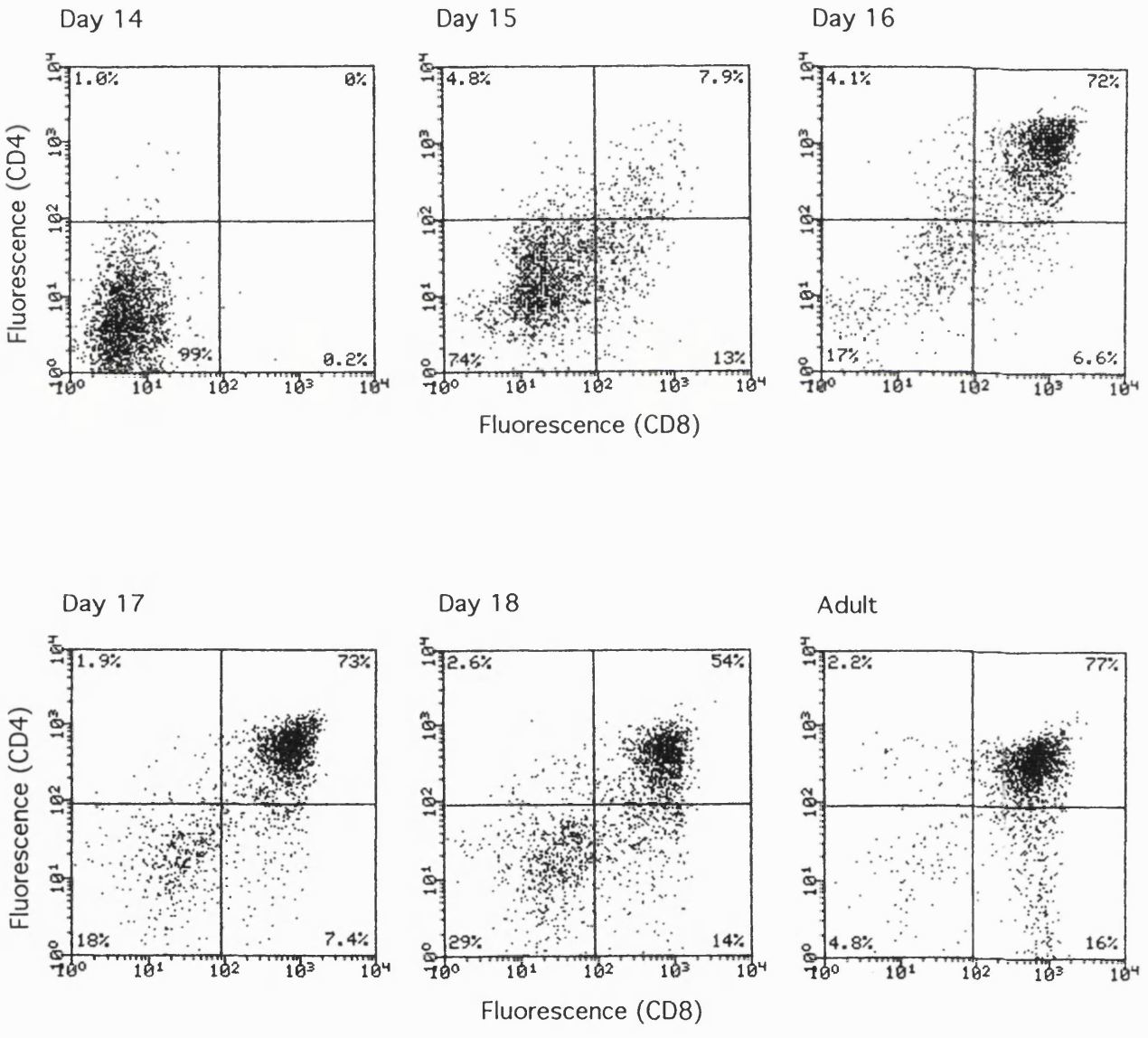


Figure 32 Ontogeny of Vβ11 expression on F5 fetal thymocytes

F5 fetal thymocytes were stained as in figure 31. Levels of Vβ11 expression are shown. F5 fetal thymocytes did not express Vβ11 until day 15, began to express low levels of Vβ11 at day 16, and further up-regulated their Vβ11 to higher levels at day 17. Day 18 F5 fetal thymocytes expressed low to intermediate levels (refer to figure 29 for TCR levels).

Fetal ontogeny in F5 mice

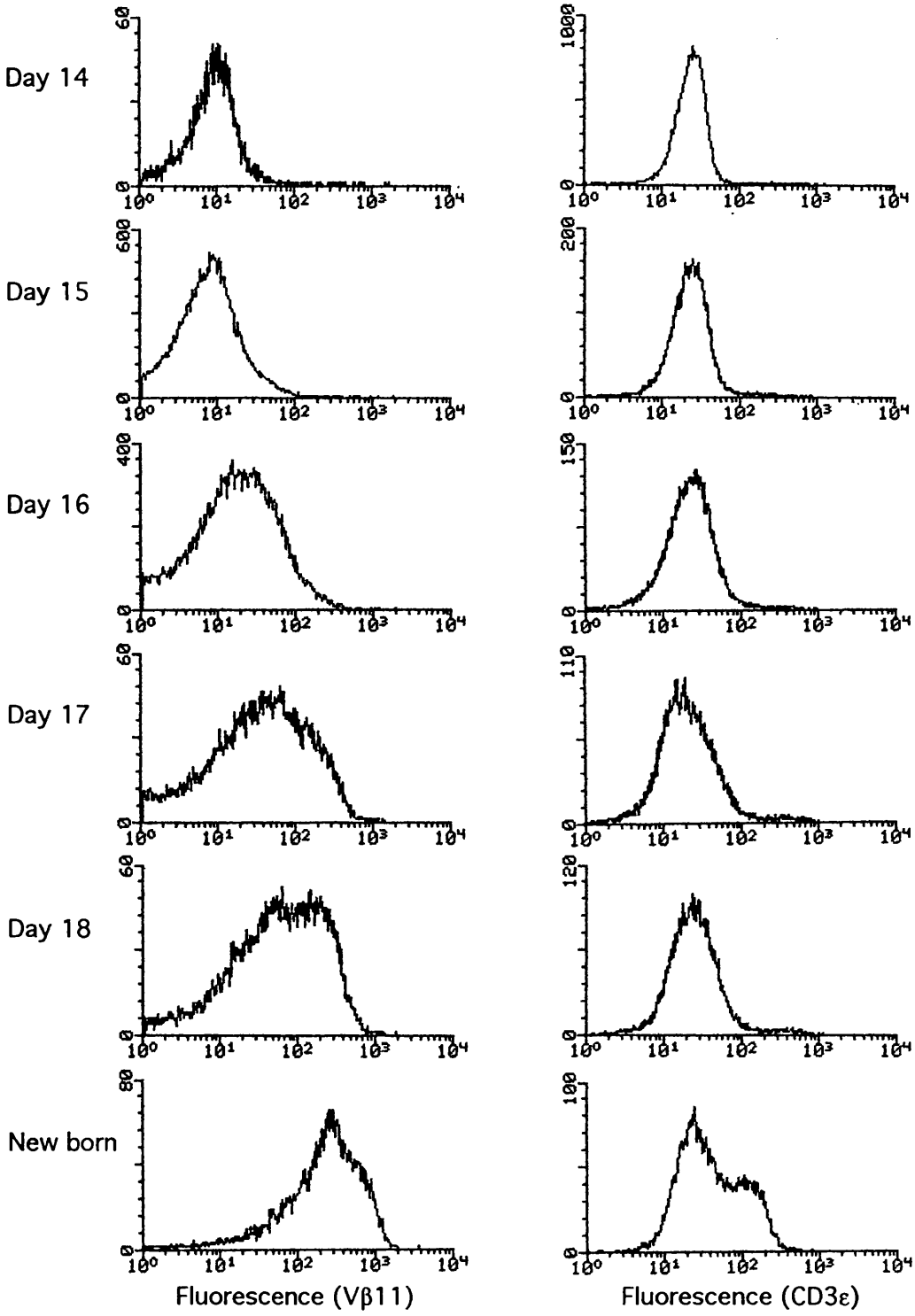
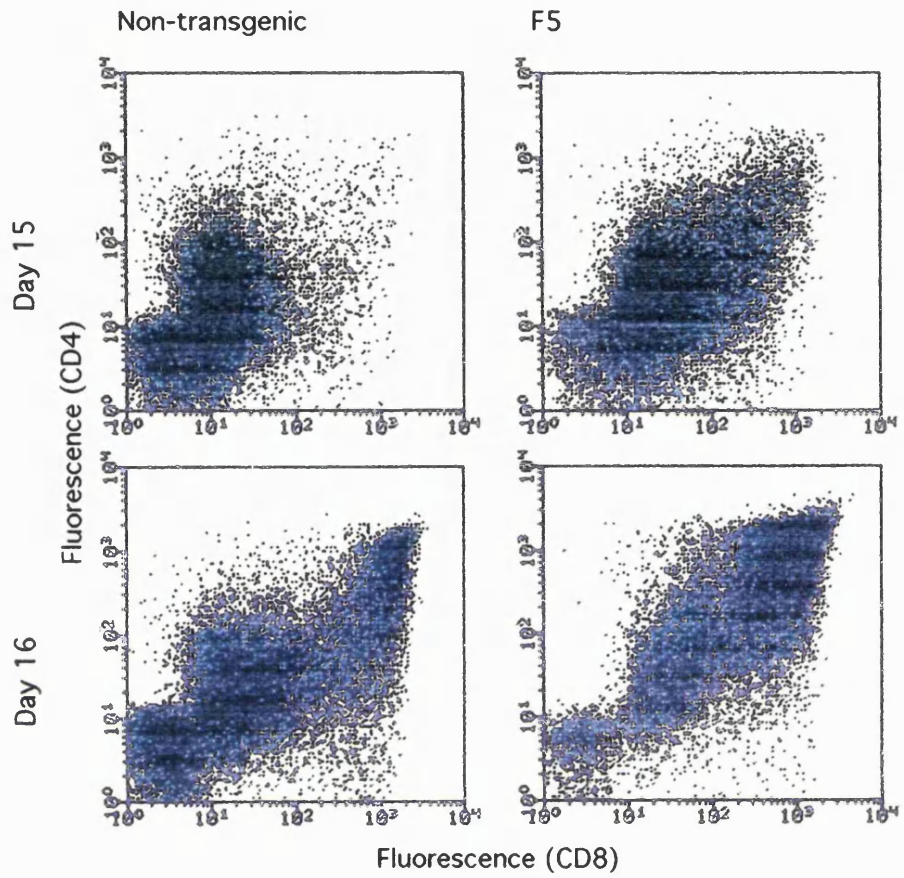


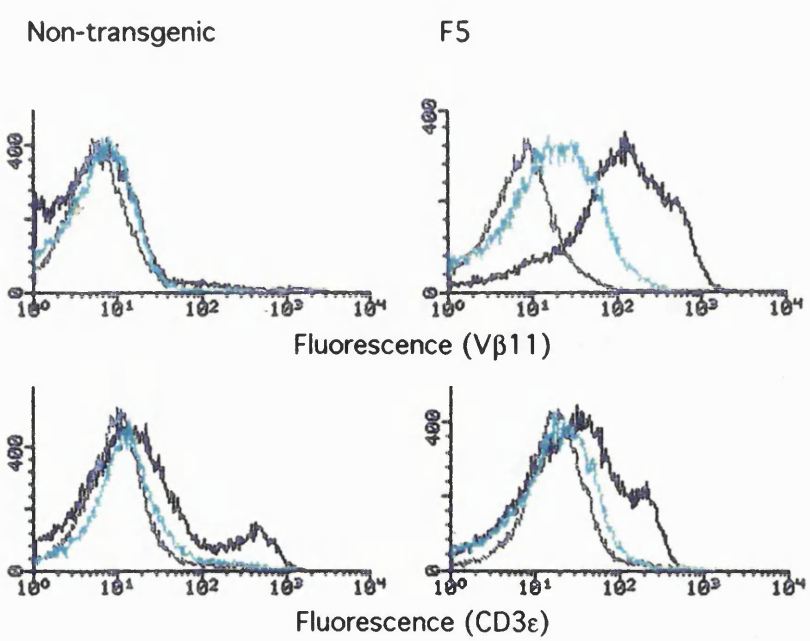
Figure 33 Transition from DN to DP cells in F5 mice

Thymocytes from non-transgenic and F5 transgenic mice at day 15 and 16 of gestation were stained for CD4, CD8, and Vβ11 or CD3ε. a) Expression of CD4 and CD8 on normal (left panels) and F5 (right) mice at day 15 (top) and 16 (bottom) of gestation are shown. b) Levels of Vβ11 and CD3ε expression in fetuses at day 15 (grey lines) and 16 (black lines), and in adult mice (green lines) are shown. F5 thymocytes at day 15 did not express Vβ11 and CD3 and began to up-regulate them at day 16, indicating that intermediate DP^{low} cells express bearly detectable levels of TCR (compare with the negative staining levels of Vβ11 in non-transgenic thymocytes).

a) Transition from DN to DP cells



b) TCR up-regulation



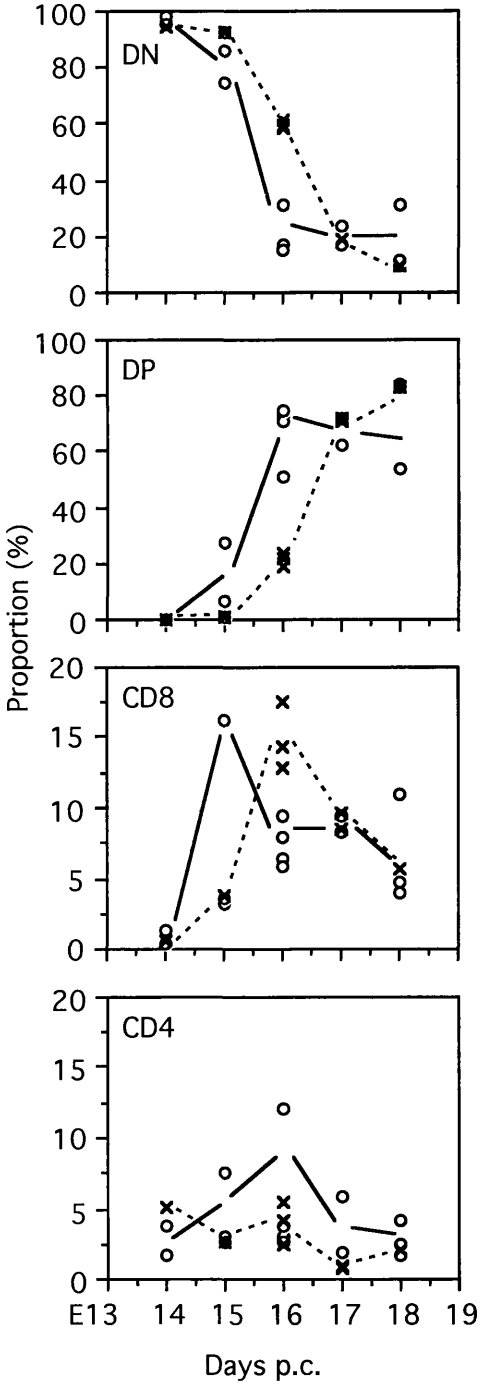
difference was significant as shown by kinetics study in figure 34-a, i.e. transition from DN (including DP^{low} population) to DP cells was about 24 hours earlier in F5 (solid lines) than in normal (dashed lines) mice. There are two explanations for this difference. One is that expression of functional TCR accelerates T cell development in F5 mice by inducing differentiation signals. Another possibility is that the presence of functional TCR in F5 thymocytes allows most of developing thymocytes to proceed to the DP stage by supporting their survival, whereas a majority of non-transgenic thymocytes fails to survive beyond the DN stage since functional TCR rearrangements are rare.

Transition from DN to DP^{low} in F5 mice at day 15 was not associated with changes in TCR expression, and DP^{low} cells remained largely TCR^- as shown by Vβ11 and CD3ϵ staining in figure 33-b. Up-regulation of TCR was first detected in F5 thymocytes at day 16, which coincided with their differentiation from DP^{low} to DP^{high} cells. Taken together, these kinetics data suggest an ordered development from TCR^-DN to TCR^-DP^{low} and then to $TCR^{low}DP^{high}$ cells, implying distinct signals involved in low and high expression of CD4 and CD8. High level expression of CD4 and CD8 appears to be associated with surface expression of TCR, whereas low level expression of CD4 and CD8 occurs before up-regulation of TCR and could potentially be induced by pre-TCR (Groettrup et al., 1993). Existence of DP^{low} cells has also been reported by others (Nikolic-Zugic et al., 1989; Petrie et al., 1990; Andjelic et al., 1993).

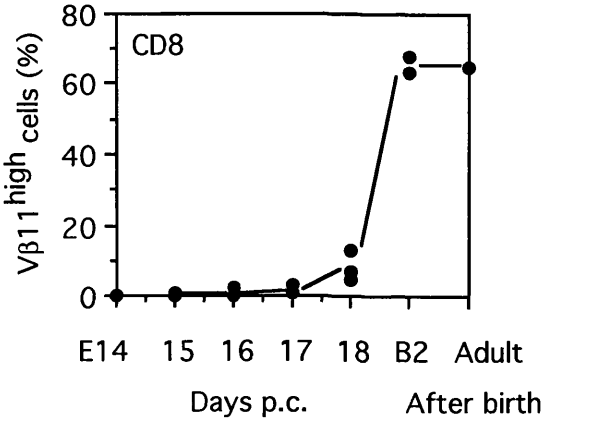
Figure 34 Kinetics of fetal ontogeny of F5 thymocytes

Fetal thymocytes from non-transgenic and F5 mice at day 14 to 18 of gestation were analysed as in figure 33. a) The proportion of DN, DP, and CD8 or CD4 SP cells is indicated for non-transgenic mice (x, dashed lines) and F5 mice (open circles, solid lines). b) Proportions of mature T cells among CD8 SP cells, expressing high Vβ11 at levels similar to those in adult F5 CD8 T cells, are shown from day 14 to 18 of gestation, 2 days and 2 months after birth.

a) Fetal ontogeny of thymic T cells



b) Proportion of mature T cells in F5 CD8^{+} cells



● F5 Vβ11^{+}
○ F5
× Ntg

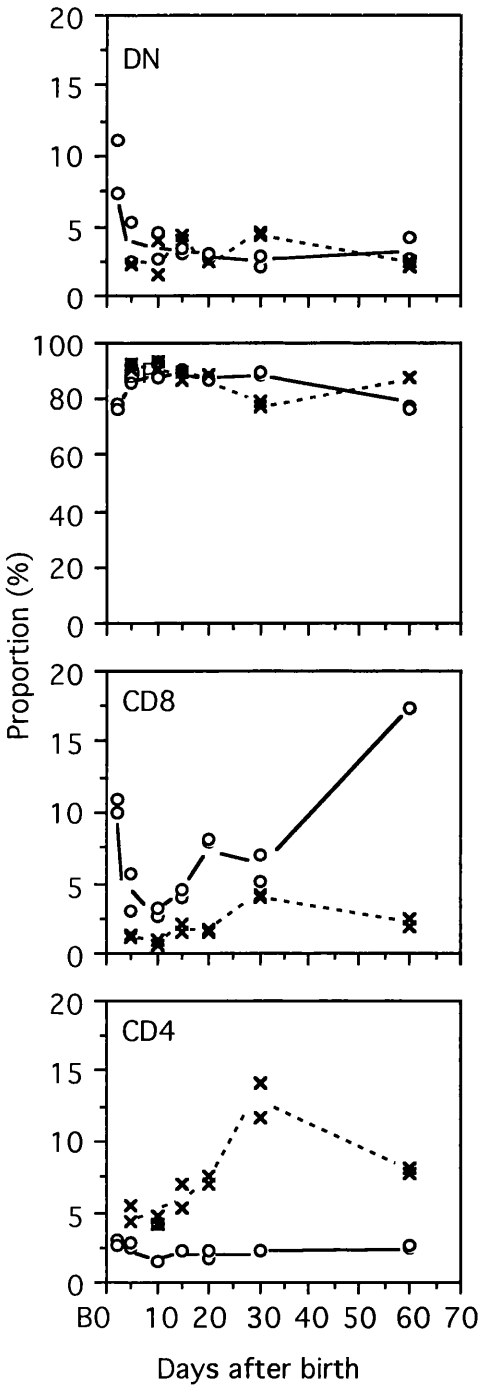
After day 16, F5 fetal thymocytes remained DP^{high}, but up-regulated their TCR from low to intermediate levels at day 17 (figure 32). F5 thymocytes at day 18 contained more TCR^{int} cells (figure 32). Thus, a large proportion of F5 fetal thymocytes differentiated as a single cohort giving rise to mature TCR^{high} CD8 T cells at around birth (figure 34-b). The proportion of CD8 SP cells in F5 thymus showed a transient increase at 2 days after birth, decreased at day 10, and afterwards gradually increased until 2 months (figure 35-a). Most of these CD8 SP cells expressed high levels of Vβ11 (see Mamalaki et al., 1992). These data suggest that the F5 T cell development occurs in two waves, one at around birth and another 20-30 days after birth, in agreement with a previous report that fetal thymus in normal mice is colonised by two waves of haematopoietic stem cells, one at day 11.5 and a second at day 18 of gestation (Havran and Allison, 1988). On the other hand, the proportion of CD4 SP cells in the F5 thymus remained low throughout. In normal mice, CD4 and CD8 SP cells accumulated in the thymus since 10 days after birth, but reached plateau at one month after birth (figure 35-a).

Similarly, in the periphery of F5 mice CD8 T cells appeared at 20 days after birth and continued to increase until 2 months, whereas increase in CD4 and CD8 T cells in normal mice stopped at one month after birth (figure 35-b). The observation that peripheral CD8 T cells develop at the same kinetics in F5 and normal mice until one month after birth suggests that the rate of T cell production at this stage is similar between F5 and normal mice. After one month, however, F5 mice continued to

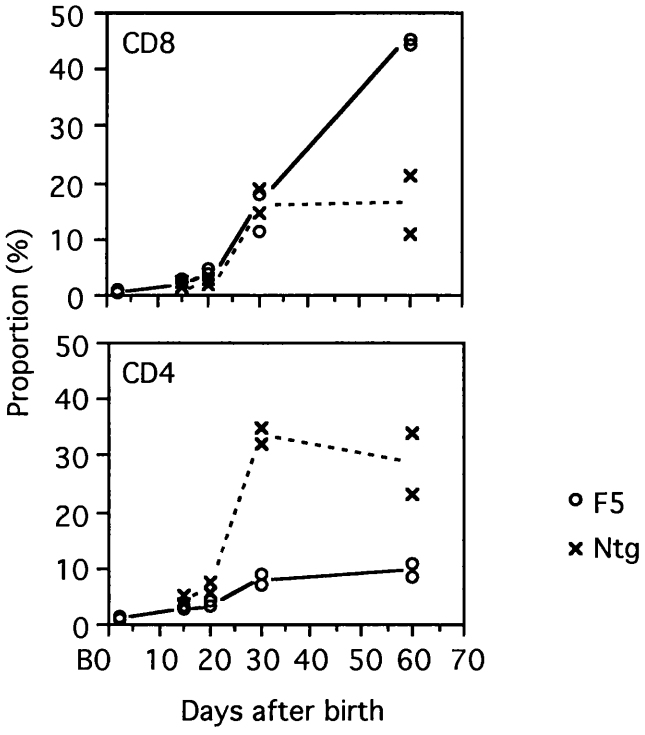
Figure 35 Kinetics of postnatal ontogeny of F5 thymocytes

a) Thymocytes from postnatal non-transgenic and F5 mice were analysed as in figure 34. The proportion of DN, DP, and CD8 or CD4 SP cells is indicated for non-transgenic mice (x, dashed lines) and F5 mice (open circles, solid lines). b) Spleen T cells from postnatal non-transgenic and F5 mice were analysed as above.

a) Postnatal ontogeny of thymic T cells



b) Postnatal ontogeny of peripheral T cells



accumulate more CD8 T cells than normal mice.

4.2.2 In vitro ontogeny

In order to study the effect of self-antigen on the kinetics of T cell development, day 14 fetal thymic lobes from F5 TCR transgenic mice were cultured in vitro in the presence or absence of cognate, non-antigenic, or unrelated peptides. After 3 to 11 days, expression of CD4, CD8, CD3, Vβ11, and HSA on thymocytes was analysed. At day 0 of culture, F5 thymic lobes consisted of $TCR^-CD4^-CD8^-$ cells (figure 36, left panels) which in the absence of cognate peptide proliferated from 0.86 - 1.62 x 10^4 to 17 - 35 x 10^4 cells (about 30-fold increase), and by day 4 differentiated to $CD4^+CD8^+$ cells expressing low level TCR (figure 36, left panels). There was no further increase in cell number after day 4, and most thymocytes remained as TCR^{low} double positive (DP) cells until day 7. Differentiation of F5 thymocytes was associated with successive up-regulation of TCR as depicted in histograms of figure 37. At day 4 in culture, CD8 SP cells expressed Vβ11 at a level between those of DN and DP cells (figure 37-a, dotted lines). Furthermore, high HSA expression on these CD8 SP cells (data not shown) suggest that they are immature. At around day 7, there was a decrease of DP cells concomitant with a reciprocal increase of $CD8^+$ cells (figure 36, left panels), most of which expressed high level TCR (figure 36, dashed lines in the left CD8 diagram, figure 37-b). About one third of DP cells completed differentiation by day 10 (5-9x10^4

Figure 36 Kinetics of <u>in vitro</u> ontogeny of F5 thymocytes in the presence or absence of cognate peptide

Fetal thymic lobes from F5 mice at day 14 of gestation (the day of plug was counted as day 0) were cultured with PBS (left panels) or 5 μM 1968 NP (right panels) for 3 to 11 days. Medium was replenished everyday using frozen stocks of peptide aliquots. Thymocytes were stained for CD4, CD8, and Vβ11, and numbers of each thymocyte subset were obtained from total cellularity and proportions of each subset. The figures were compiled from five separate series of experiments which consisted of one to six thymic lobes per sample. For CD8 SP cells, numbers of cells expressing high level Vβ11 (comparable to that of mature F5 CD8$^+$ cells) were also calculated and are indicated by dashed lines.

F5 FTOC E14

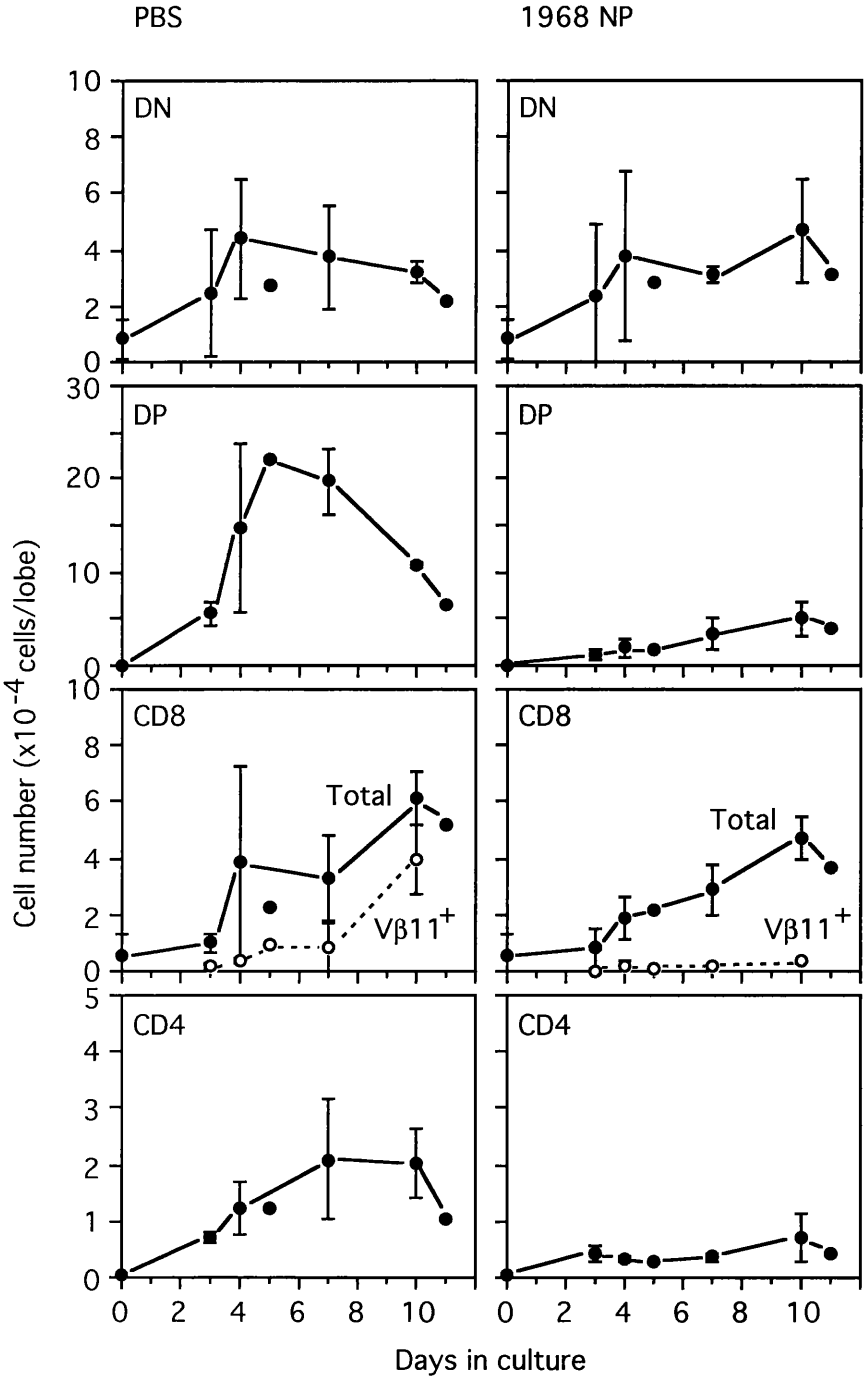
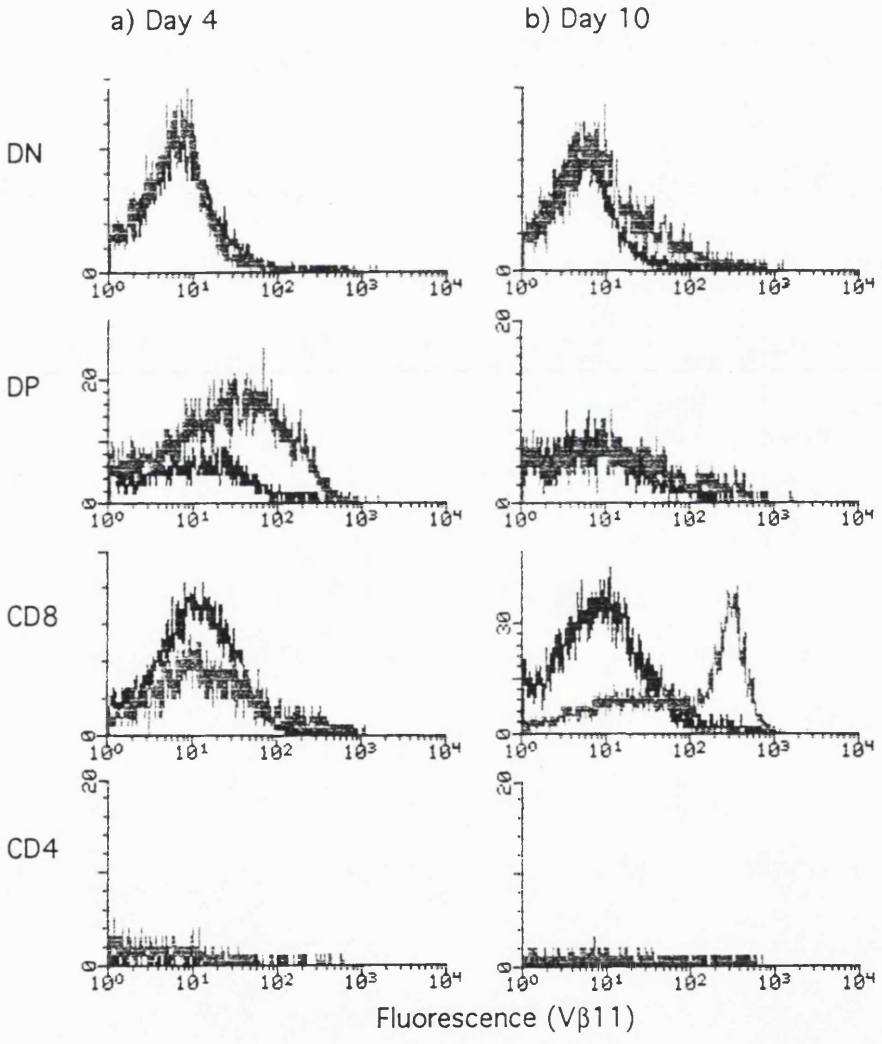

PBS
1968 NP
DN
DP
CD8
CD4
Total
Vβ11+
Cell number (x10-4 cells/lobe)
Days in culture

Figure 37 Effect of cognate peptide on Vβ11 expression on F5 fetal thymocytes

F5 fetal thymic lobes were cultured for 4 days (a) and 10 days (b), and were analysed as in figure 36. The histograms show expression of transgenic TCR (Vβ11) on DN, DP, and CD8 or CD4 SP cells cultured in the presence (solid lines) or absence (grey lines) of 1968 NP.

F5 FTOC E14



out of 18-25x10^4 cells). The kinetics of F5 T cell development in vitro was similar to that of normal T cells (data not shown), except that appearance of DP cells was earlier by one day in F5 thymic lobes than in normal lobes, as described above for in vivo development.

4.2.3 Kinetics of negative selection

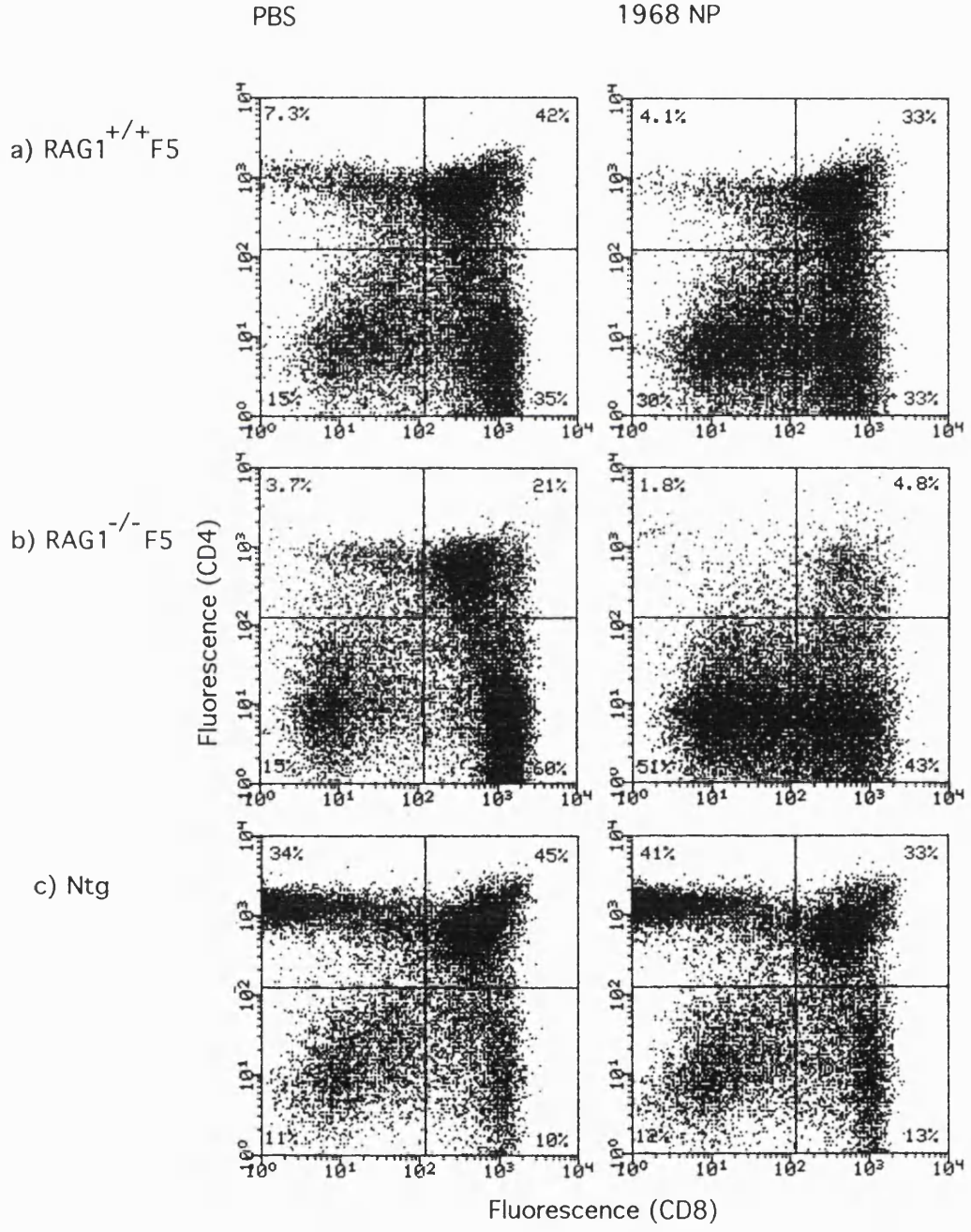
Next we analysed effects of high concentration (5 μM) of the 1968 NP on F5 T cell development as shown in right panels of figure 36. In the most immature DN compartment, there was not a significant difference in cell numbers between control PBS and 1968 NP cultures. This suggests that the cognate peptide does not block initial proliferation of DN cells. Also unaffected was the number (figure 36, right panels) and TCR expression (figure 37-a, solid lines) of CD8 SP cells during the first 7 days. On the other hand, generation of double positive cells was severely suppressed by the 1968 NP throughout the culture period (figure 36, right panels). At day 4, the number of DP cells was greatly reduced in the 1968 NP-treated thymic lobes compared with control cultures, and TCR expression on DP cells was lower in peptide-treated cultures (figure 37-a). After 10 to 11 days of culture, however, there was little difference in cell numbers (figure 36, right panels) and levels of TCR expression (figure 37-b) in DP thymocytes between control and the 1968 NP-treated cultures. However, generation of Vβ11$^+$ CD8 SP cells was completely suppressed by the cognate peptide as demonstrated by the absence of Vβ11$^+$ CD8 SP cells (figure 36, dashed lines in the right CD8

diagram) and low levels of Vβ11 expression (figure 37-b, solid lines).

The origin of DP cells in F5 thymic lobes treated with the cognate peptide for 11 days was not clear, since most DP thymocytes in adult F5 mice have been shown to undergo programmed cell death within 24 hours after injection of the peptide (Mamalaki et al., 1992). However, there is a report that, in TCR transgenic mice expressing a negatively selecting ligand, T cells which have lost the transgenic TCR and acquired endogenous TCR can survive and mature (Scott et al., 1989). To test the hypothesis that DP cells in the 1968 NP-treated F5 thymic lobes escaped negative selection by expressing endogenous TCR, F5 mice were crossed to RAG1-deficient mice so that only the F5 TCR can be expressed on developing thymocytes. Figure 38 illustrates that the effect of 1968 NP on developing T cells at day 11 was more pronounced in $RAG1^{-/-}$F5 thymic lobes (b) than in $RAG1^{+/+}$F5 lobes (a). Thus, generation of DP cells was only mildly suppressed in peptide-treated $RAG1^{+/+}$F5 lobes to 2.7×10^4 cells/lobe compared with 6.6×10^4 in control cultures, whereas the number of DP cells in peptide-treated $RAG1^{-/-}$F5 lobes was greatly reduced to 0.18×10^4 cells/lobe compared with 2.5×10^4 in un-treated lobes. Figure 38-c shows such an effect on DP cells was not due to non-specific cytotoxicity of the 1968 NP, since non-transgenic thymocytes were not affected. In a separate experiment using $RAG1^{-/-}$F5 thymic lobes and either 1968 NP or an unrelated HIV-gag peptide (table 5), HIV-gag peptide did not block T cell development, providing evidence that suppression of DP T cell

Figure 38 Negative selection in F5 and RAG1$^{-/-}$F5 thymic lobes

Day 14 fetal thymic lobes from RAG1$^{+/+}$F5 mice (a) and RAG1$^{-/-}$F5 mice (b), and day 15 fetal thymic lobes from non-transgenic (Ntg) mice (c) were cultured for 11 days as in figure 36, each sample consisting of two to four lobes. Thymocytes were stained for CD4, CD8, and Vβ11, and patterns of CD4 and CD8 expression in PBS (left panels) and 1968 NP (right panels) cultures are shown. Cell numbers were a) $1.5x10^5$ vs. $7.9x10^4$, b) $1.1x10^5$ vs. $3.0x10^4$, c) $2.3x10^5$ vs. $1.3x10^5$ cells/lobe in PBS or 1968 NP-treated cultures respectively. Two separate experiments on RAG1$^{-/-}$F5 thymic lobes, including one in table 5, gave identical results.


PBS
1968 NP
a) RAG1+/+F5
b) RAG1-/-F5
c) Ntg
7.3%
42%
15%
35%
4.1%
33%
30%
33%
3.7%
21%
15%
60%
1.8%
4.8%
51%
43%
34%
45%
11%
10%
41%
33%
12%
13%
Fluorescence (CD4)
Fluorescence (CD8)

development by the 1968 NP was peptide-specific. CD8 SP cells in the cognate peptide-treated RAG1$^{-/-}$F5 lobes were less affected in number (table 5). However, the level of CD8 co-receptor (figure 38-b) and TCR (figure 39, left panels) were notably reduced. In addition, these CD8 SP cells were mature since they expressed HSA at a level lower than that of DP cells (figure 39, right panels). These data suggest that clonal deletion in the F5 thymus is not complete, and that in the presence of high concentration of cognate antigen mature SP T cells which express low levels of TCR and co-receptor molecules can develop.

Table 5 Negative selection in $RAG1^{-/-}$F5 fetal thymus organ culture†

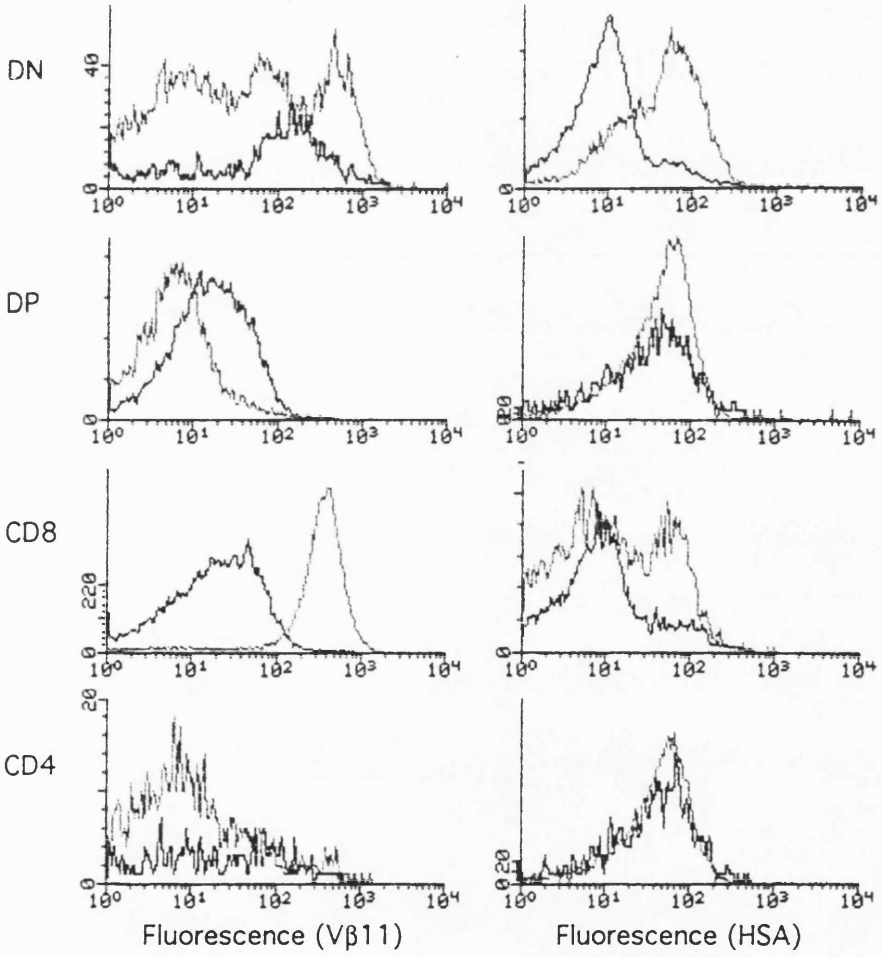
	DN	DP	$CD8^+$	$CD4^+$
PBS	1.75 ± 0.81	6.05 ± 2.32	2.08 ± 1.24	2.48 ± 0.99
1968 NP	3.87 ± 1.13	0.35 ± 0.08	1.11 ± 0.71	0.25 ± 0.10
HIV gag	1.66 ± 0.60	6.65 ± 3.36	2.73 ± 0.61	1.97 ± 0.74

† (Mean ± SD) x 10^{-4} cells/lobe

Figure 39 Levels of Vβ11 and HSA on $RAG1^{-/-}$F5 thymocytes cultured in the presence of cognate peptide

Day 14 $RAG1^{-/-}$F5 fetal thymic lobes were cultured for 11 days as in figure 38, and thymocytes were stained for CD4, CD8, and Vβ11 or HSA. Histograms show expression of Vβ11 (left panels) and HSA (right panels) in DN, DP, and CD8 or CD4 SP cell populations cultured with either PBS (grey lines) or 1968 NP (solid lines). In peptide-treated cultures, DP cells expressed no Vβ11 whereas CD8 SP cells had low levels of Vβ11 as comparable to those seen on DP cells in PBS-treated cultures. CD8 SP cells in 1968 NP cultures were HSA^{low}, whereas CD8 SP cells in control cultures consisted of HSA^{-} and HSA^{+} populations.

RAG1$^{-/-}$ F5 thymocytes



Chapter 5 Positive selection of T cells

5.1 Introduction

Progenitors of T cells undergo proliferation and differentiation events in the thymus over a period of 7-12 days before being exported to the periphery (Egerton et al., 1990). T cell differentiation is associated with a series of changes in expression of CD4, CD8, α/β TCR, and other cell surface molecules (figure 1). The most crucial step in T cell development is the acquisition of functionally rearranged TCR α and β chains and their expression on cell surface in complex with invariant CD3 chains. At an early stage of development, expression of TCR β chains allows DN cells to differentiate to DP cells (Kishi et al., 1991; Mombaerts et al., 1992; Shinkai et al., 1992). At a later stage, however, transition from DP to SP cells requires interaction between TCR α/β heterodimers with unknown thymic stromal ligands. This process is termed positive selection since only those T cells which express TCR that can interact with self-MHC can undergo full maturation. Despite the fact that MHC molecules are essential for positive selection of T cells, the precise role of MHC molecules and endogenous peptides bound to them are unknown. In addition, it is not clear whether positive selection is mediated by specific cells in the thymus and specific molecules expressed and/or secreted by thymic stromal cells.

Thymic stromal cells

The phenomenon of self-MHC restriction in mature T cells has been taken as a consequence of positive selection of those T cells which can recognise peptide antigens in complex with self-MHC. Thus, the fact that MHC restriction is imprinted on developing T cells by radiation-resistant or deoxyguanosine-resistant compartment in the thymus, which are largely epithelial cells, suggested that thymic epithelial cells are responsible for positive selection of T cells (Sprent, 1989). Thymic stromal cells remaining in deoxyguanosine-treated fetal thymic lobes have also been shown to support T cell development when co-cultured with immature T cells in hanging drops (Jenkinson et al., 1982). Taken together with the thymic architecture in which developing thymocytes are in close contact with a reticular network of cortical epithelial cells, the latter are most likely to be the one mediating positive selection. More direct evidence for involvement of thymic stromal cells in positive selection has come from reaggregate co-cultures of fractionated thymic stromal cells with immature T cells (Jenkinson et al., 1992). It was demonstrated that $CD45^-$, medullary epithelial marker$(A2B5)^-$, and class II MHC^+ cells isolated from deoxyguanosine-treated fetal thymic lobes were capable of supporting differentiation of DP thymocytes to CD4 or CD8 SP T cells. However, although most of such $CD45^-A2B5^-$class II MHC^+ stromal cells were shown to express a thymic cortical epithelial marker, it was still possible that these fractionated stromal cells consisted of heterogenous

populations. In addition, the number of cells recovered from fetal thymic lobes were in the order of 10^4/lobe which makes it difficult to carry out standard molecular analysis. In order to facilitate identification of specific cells and ligands for positive selection, thymic stromal cell lines were established from tsA58 transgenic mice and were analysed by in vitro T cell differentiation assays. Since cell-cell interactions between different thymic stromal cells could be important for maintaining their normal functions, a system was developed in which positive selection of TCR transgenic thymocytes could be measured in the presence of a mixture of a thymic stromal cell line expressing restricting MHC on one hand, and heterogenous populations of thymic stromal cells lacking selecting MHC on the other.

Peptide/MHC ligands

A possible role of endogenous peptides bound to self-MHC has been suggested by analysis of TCR transgenic mice in an MHC background which has mutations in residues facing the peptide binding groove which do not affect the structure of MHC molecule but would alter the kinds of peptides which can bind to the groove (Jacobs et al., 1990; Nikolic-Zugic and Bevan, 1990; Sha et al., 1990). However, it is not clear whether endogenous peptides play an active role in positive selection by inducing specific signals through TCR or they are required only for expression of MHC molecules and stabilising their 3-D structure.

Receptor-ligand interactions can theoretically involve two parameters, i.e. affinity and intrinsic activity (efficacy), which are independent from each other. Physical affinity can be measured directly, and intrinsic activity can be determined by concentration of ligand required to cause a given amount of signal, such that the higher the intrinsic activity, the lower the concentration required for activation. A ligand with high affinity and high intrinsic activity causes full activation of a receptor (agonist), whereas a ligand with either low affinity or low intrinsic activity may provoke only a part of activation signals (partial agonist). A class of ligands which has high affinity but low or no intrinsic activity could compete with agonists for interaction with a receptor without inducing activation signals and is called competitive antagonist. A ligand with no affinity can not interact with a receptor, but could reduce a concentration of agonists when the total amount of ligands is limited (non-competitive or non-specific antagonist). Recently, peptide analogues have been described which cause partial activation a Th2 clone (Evavold and Allen, 1991) or inhibition of CTL activation (De Magistris et al., 1992) suggesting that TCR could transduce qualitatively different signals.

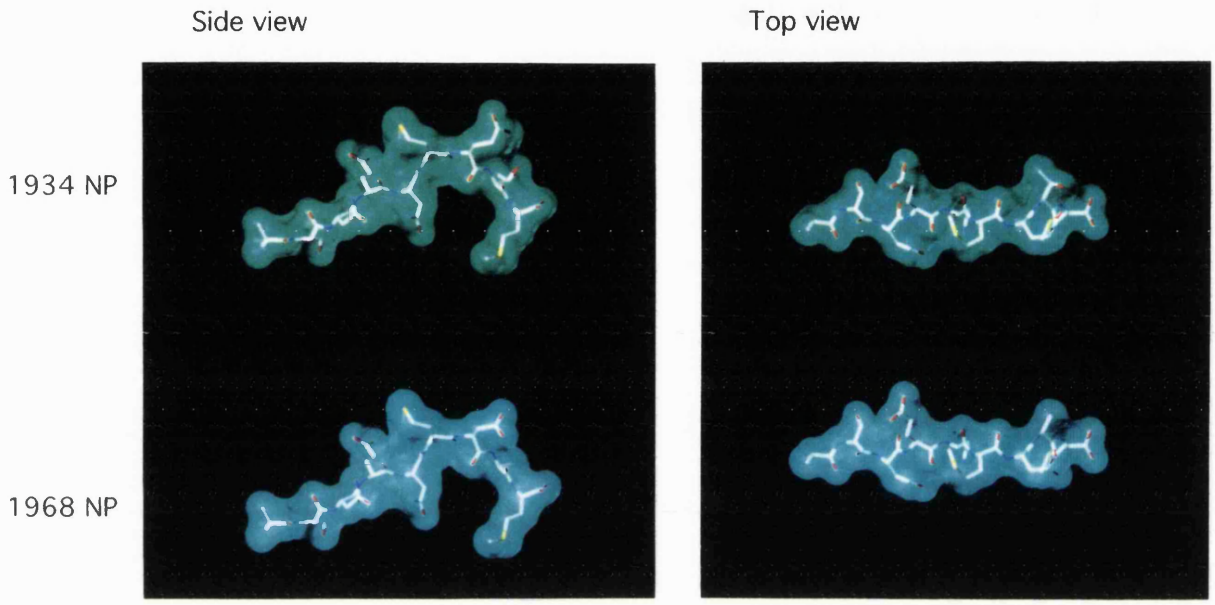
In search for peptides which can support positive selection of F5 T cells (described in chapter 4.1), a series of peptide analogues were designed by single amino acid substitutions in the antigenic peptide 1968 NP (figure 40-b). Previously it has been shown that peptides eluted from H-2D^b share common residues at

Figure 40 Peptide analogues

a) Structure of two peptides from influenza virus nucleoprotein (1968 and 1934 strains) are illustrated as viewed from the side (left panels) or the top (right panels) of peptide/MHC complexes. The structure of 1934 NP bound to H-2D^b has been published (Young, 1994), and the structure of 1968 NP bound to H-2D^b was calculated based on that of 1934 NP. (Courtesy of Dr. P. Travers, ICRF, London)

b) Synthetic peptides are listed with a summary of their characteristics. Single amino acid substitutions were introduced in the antigenic peptide 1968 NP at positions 4 (P4), 7 (P7), or 8 (P8), as shown for P4D, P4Q, P4K, P7E, P7N, P7K, and P8T. The 1934 NP has two amino acid substitutions at positions 7 and 8, and HIV-gag is an un-related peptide which binds to H-2D^b molecule with the same affinity as the 1968 NP. Characteristics of these peptide analogues in activation of mature F5 CTL and in negative selection of immature F5 thymocytes were measured using agonist (agn.) or antagonist (ant.) assays (see text). Symbols ++, +, and - indicate strong, weak, and no activities in each assay. Those which were not determined are left blank.

a) Structure of flu peptides bound to H-2D^{b}



b) Characteristics of peptide analogues

										Negative selection		CTL	
										agn.	ant.	agn.	ant.
1968 NP	A	S	N	E	N	M	D	A	M	++	-	++	-
P4D				D						++		++	-
P4Q				Q						+		+	-
P4K				K						+		-	-
P7E							E			-	+	-	+
P7N							N			++			
P7K							K			-			
P8T								T		++		++	-
1934 NP							E	T		-	+	-	+
HIV gag	S	Q	V	T	N	P	A	N	I	-		-	-

position 5 and 9 suggesting that these two amino acids are important for peptide binding to MHC (Falk et al., 1991). In addition, a non-antigenic peptide from naturally occurring viral mutant (1934 NP) has been known to possess two amino acid substitutions in 1968 NP at positions 7 and 8 (Townsend et al., 1985). Recently Mamalaki et al. analysed each single amino acid substitutions by injecting them into adult F5 transgenic mice (C. Mamalaki, unpublished data). An analogue with substitution at position 7 from aspartate to glutamate (P7E) had no effect on F5 T cells, whereas a mutant at position 8 from alanine to threonine (P8T) caused negative selection of F5 T cells. These data suggested that the side chain of residue at position 7 is facing toward the F5 TCR. The negatively charged side chain of glutamic acid at position 4 could also interact with TCR as it is located between anchoring residues.

Recently H-2D^b has been co-crystallised with the 1934 NP (Young et al., 1994), confirming the above predictions. Based on published information of the structure of 1934 NP bound to H-2D^b, a model of the 1968 NP bound to H-2D^b, was constructed in collaboration with Dr. P. Travers (ICRF, London). As shown in figure 40-a, these two peptides can bind to H-2D^b with the same backbone structure of peptides and the same configuration of MHC. A side chain at position 8 was fairly "buried" in the groove as has been expected by the fact that P8T was antigenic. On the other hand, carboxylate groups of residues at position 7 (aspartate in the 1968 NP and glutamate in the 1934 NP) had different orientations, so that only one of the oxygen atoms were

exposed to the solvent in the 1968 NP whereas both oxygen atoms were accessible to the solvent in the 1934 NP. A side chain of the amino acid at position 4 was also facing towards the TCR and was situated near the α1 helix of H-2D^b, whereas a side chain at position 7 was closer to the α2 helix of H-2D^b.

In an attempt to generate peptide analogues which can support positive selection of F5 T cells, single amino acid substitutions were made at positions 4 and 7 with relatively conserved changes in size and electrostatic charge. In addition to the above mentioned P7E and P8T, glutamate at position 4 was replaced with either aspartate (P4D), glutamine (P4Q), or lysine (P4K), whereas aspartate at position 7 was changed to either asparagine (P7N) or lysine (P7K). An unrelated peptide from HIV gag (p390-398) was chosen as a control since it has been shown to bind to H-2D^b with a similar affinity to that of the 1968 NP (Elvin et al., 1993). Effect of these peptide analogues on activation of mature F5 T cells and development of F5 thymocytes were studied as summarised in figure 40-b.

5.2 Reaggregate cultures

5.2.1 F5$^{q/q}$ and RAG1$^-$F5$^{q/q}$ mice

In order to test if thymic epithelial cell lines can support T cell development *in vitro*, we devised a functional reconstitution

assay using reaggregate cultures with F5 transgenic T cell precursors. To this end, F5 mice were backcrossed to either non-selecting H-2^q or class I MHC-deficient ($\beta 2m^{-/-}$) mice.

Figure 41 shows patterns of CD4 and CD8 expression on F5 thymocytes in different genetic backgrounds. Development of F5 T cells is affected by the presence of H-2^q molecules, since the proportion of selected CD8 SP cells were reduced in H-$2^{b/q}$ and H-$2^{q/q}$ compared with H-$2^{b/b}$ background. Similar reduction in proportion of CD8 SP cells were observed more clearly in the periphery as shown in figure 42 (compare proportions of CD8 SP cells in $F5^{b/b}$, $F5^{b/q}$, and $F5^{q/q}$ mice). Furthermore, generation of SP cells was completely blocked in a RAG1-deficient background ($RAG1^{-/-}F5^{q/q}$ in figure 41 and 42), suggesting that CD8 or CD4 SP cells in $F5^{q/q}$ mice are likely to be selected by expressing endogenous TCR. The defect of T cell development in $F5^{q/q}$ mice could be explained either by a lack of positive selection or by negative selection of F5 T cells by H-2^q. Comparison of transgenic TCR β expression may provide a clue to answer this question. Thus, in the thymus DP cells expressing intermediate levels of Vβ11 were severly reduced in H-$2^{b/q}$ and H-$2^{q/q}$ backgrounds (figure 43). In addition, CD8 SP cells in the thymus (figure 44) and in the periphery (figure 45) in H-$2^{q/q}$ mice expressed lower level Vβ11 than in H-$2^{b/b}$ mice. (An odd exception of spleen T cells in $RAG1^{-/-}F5^{q/q}$ mice which expressed high level Vβ11 in figure 45 could be due to non-T cells, since there was no significant population expressing normal level of CD8 in these

Figure 41 F5 thymocytes in different backgrounds (CD4/CD8)

Thymocytes from adult non-transgenic, F5/H-$2^{b/b}$, F5/H-$2^{b/q}$, F5/H-$2^{q/q}$, RAG1$^{-/-}$/F5/H-$2^{q/q}$, RAG1$^{-/-}$/F5/H-$2^{b/b}$, and β2m$^{-/-}$/F5/H-$2^{b/b}$ mice were stained for CD4, CD8, and Vβ11. Patterns of CD4 and CD8 expression are shown. Numbers in each quadrant indicate proportions of cells.

Thymus

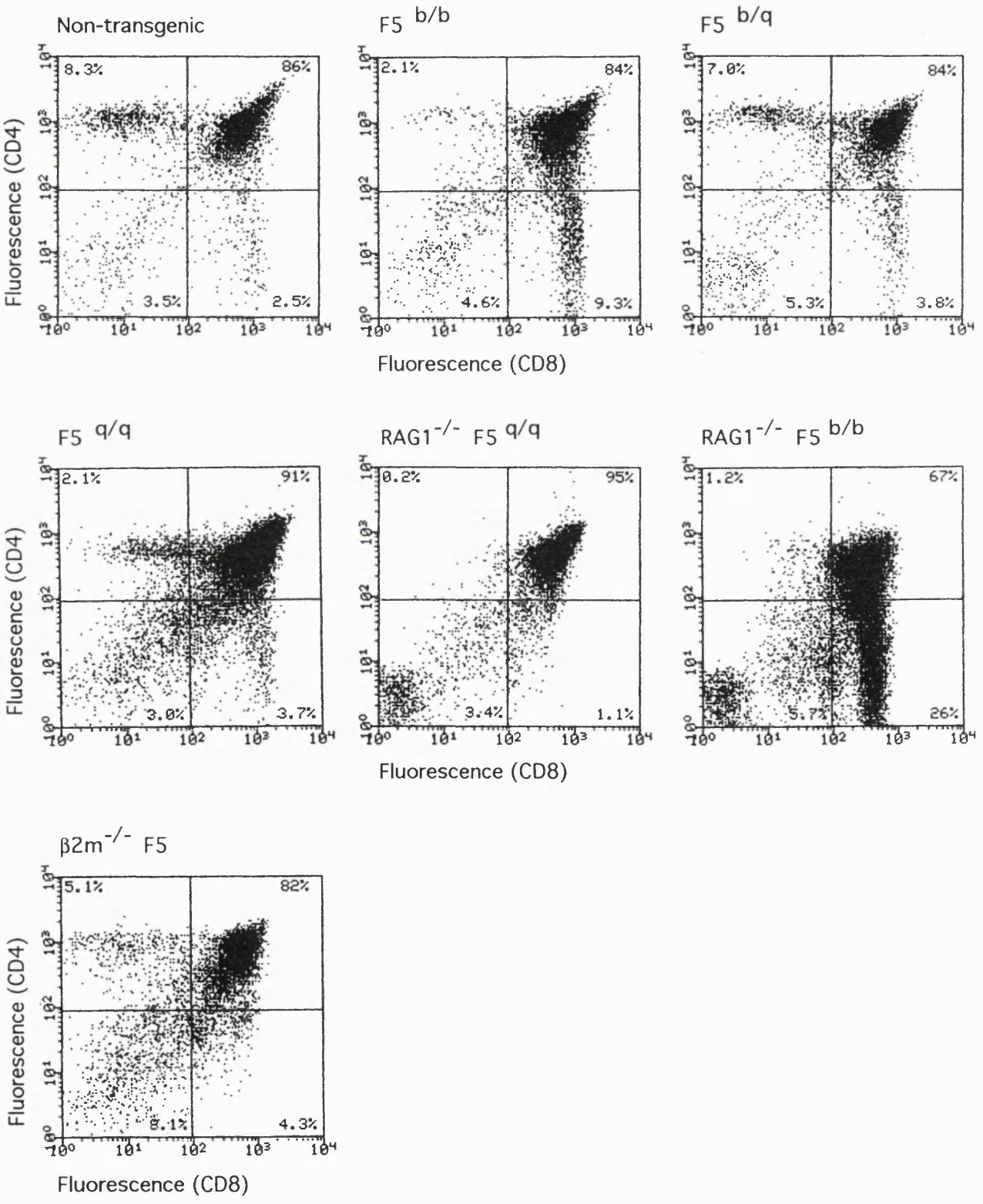

Non-transgenic
8.3%
86%
3.5%
2.5%
F5 b/b
2.1%
84%
4.6%
9.3%
F5 b/q
7.0%
84%
5.3%
3.8%
Fluorescence (CD4)
Fluorescence (CD8)
F5 q/q
2.1%
91%
3.0%
3.7%
RAG1-/- F5 q/q
0.2%
95%
3.4%
1.1%
RAG1-/- F5 b/b
1.2%
67%
5.7%
26%
β2m-/- F5
5.1%
82%
8.1%
4.3%

Figure 42 F5 spleen cells in different backgrounds (CD4/CD8)

Total spleen cells from adult non-transgenic, F5/H-$2^{b/b}$, F5/H-$2^{b/q}$, F5/H-$2^{q/q}$, RAG1$^{-/-}$/F5/H-$2^{q/q}$, RAG1$^{-/-}$/F5/H-$2^{b/b}$, and β2m$^{-/-}$/F5/H-$2^{b/b}$ mice were stained for CD4, CD8, and Vβ11. Patterns of CD4 and CD8 expression are shown. Numbers in each quadrant indicate proportions of cells.

Spleen

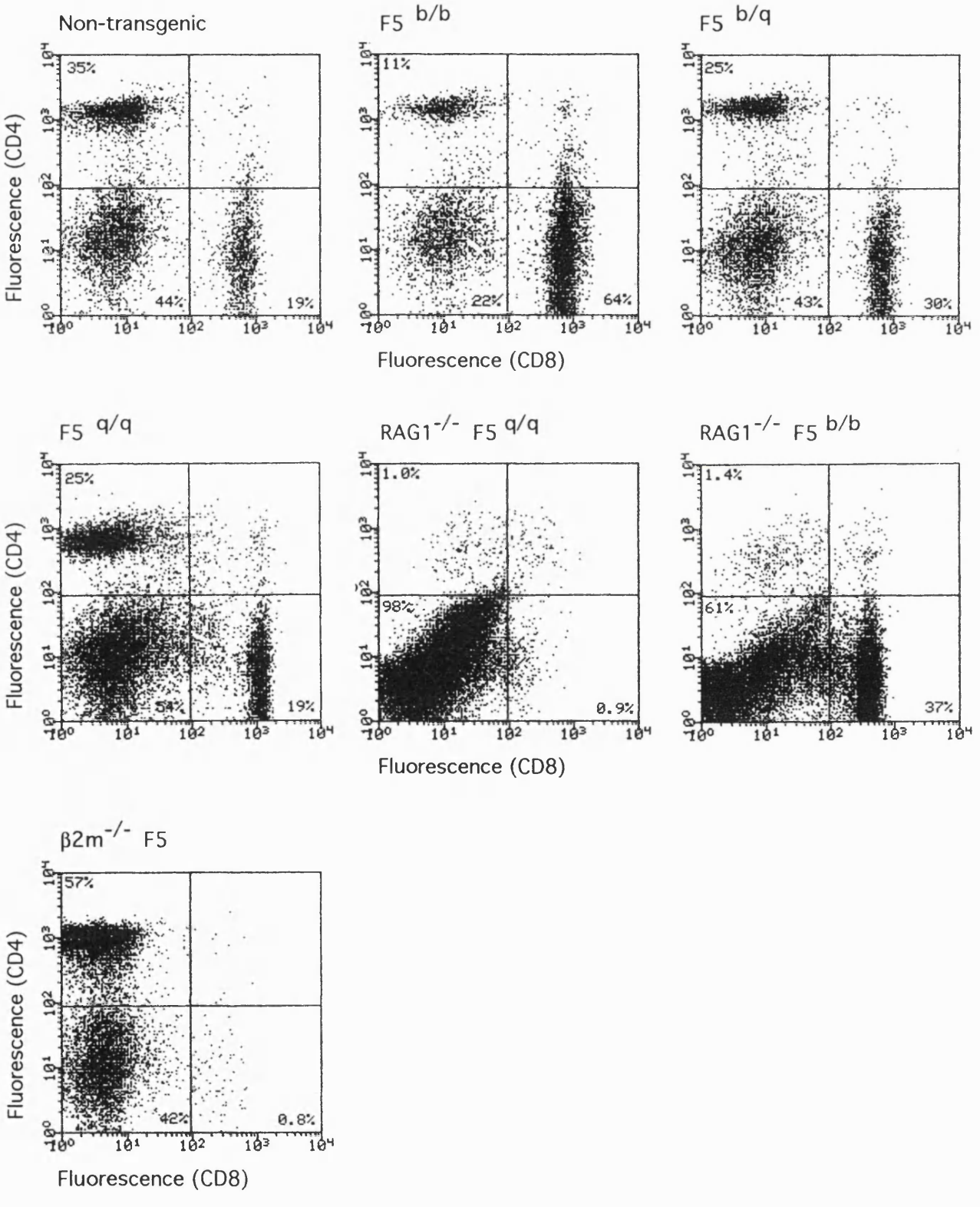

Non-transgenic
35%
44%
19%
F5 b/b
11%
22%
64%
F5 b/q
25%
43%
30%
F5 q/q
25%
54%
19%
RAG1-/- F5 q/q
1.0%
98%
0.9%
RAG1-/- F5 b/b
1.4%
61%
37%
β2m-/- F5
57%
42%
0.8%
Fluorescence (CD4)
Fluorescence (CD8)

Figure 43 F5 thymocytes in different backgrounds (Vβ11)

F5 thymocytes in different backgrounds were analysed as in figure 41, and levels of Vβ11 in DN and DP cells are shown by histograms.

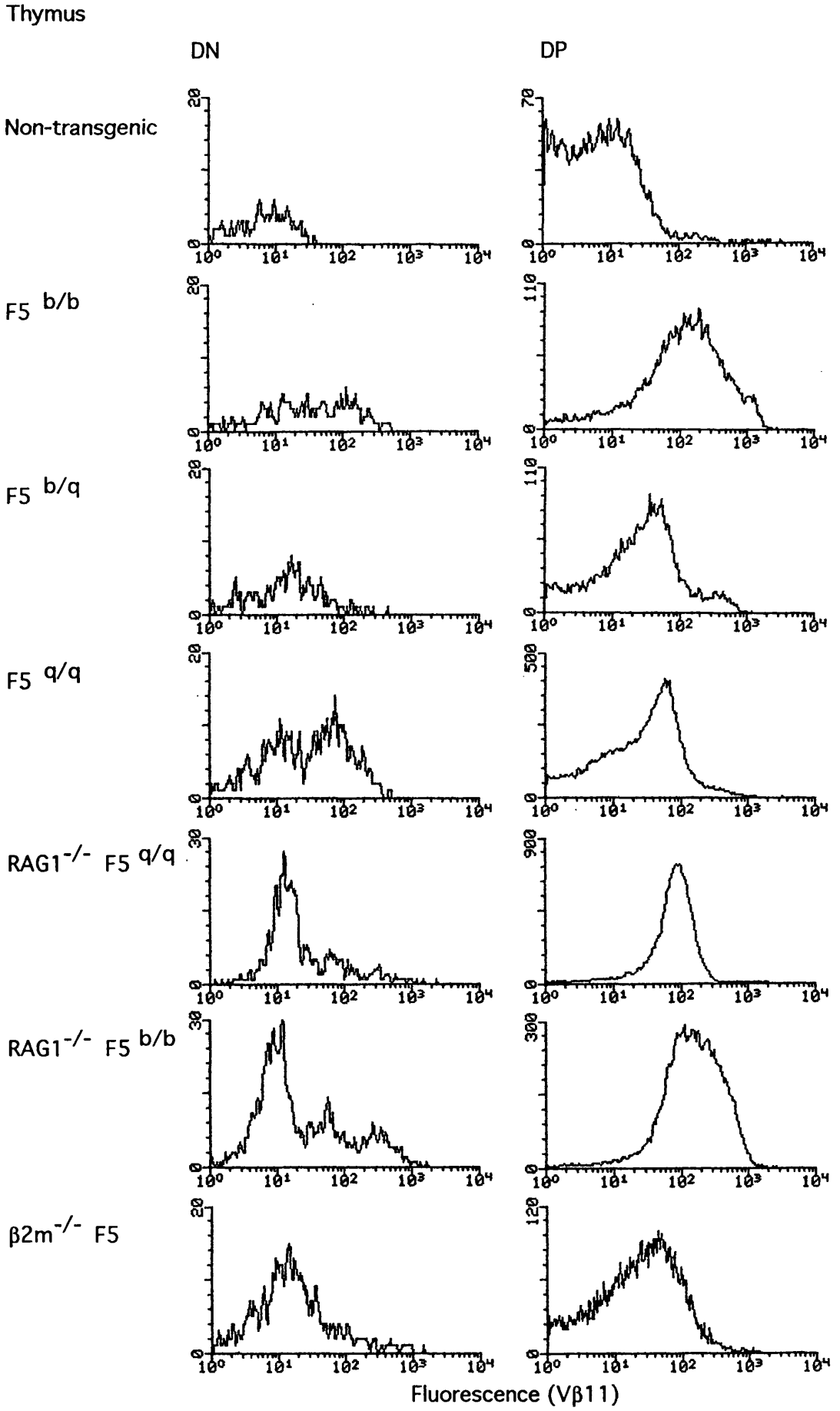

Thymus
DN
DP
Non-transgenic
F5 b/b
F5 b/q
F5 q/q
RAG1-/- F5 q/q
RAG1-/- F5 b/b
β2m-/- F5
Fluorescence (Vβ11)

Figure 44 F5 thymocytes in different backgrounds (Vβ11)

F5 thymocytes in different backgrounds were analysed as in figure 41, and levels of Vβ11 in CD4 and CD8 single positive cells are shown by histograms.

Thymus

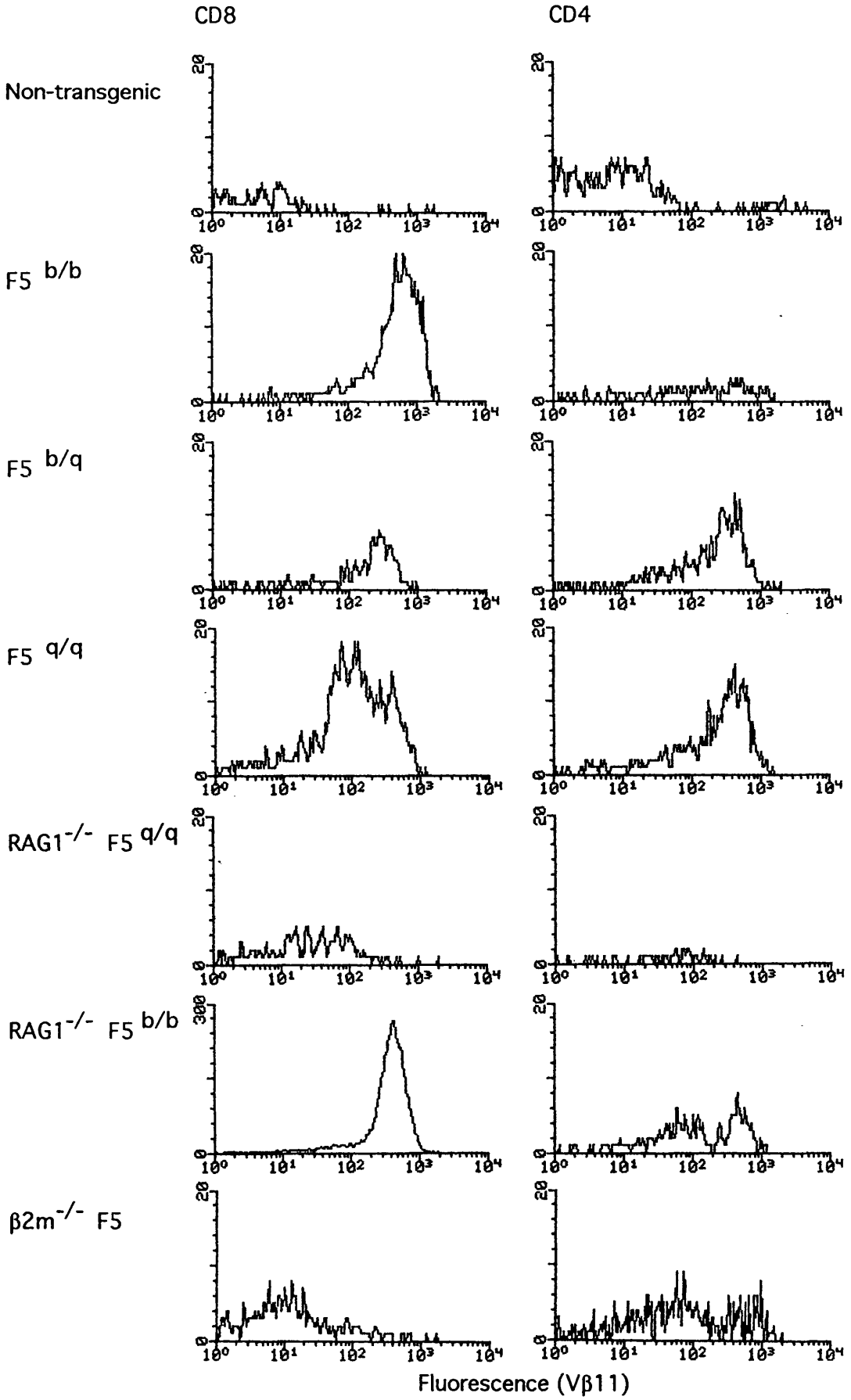
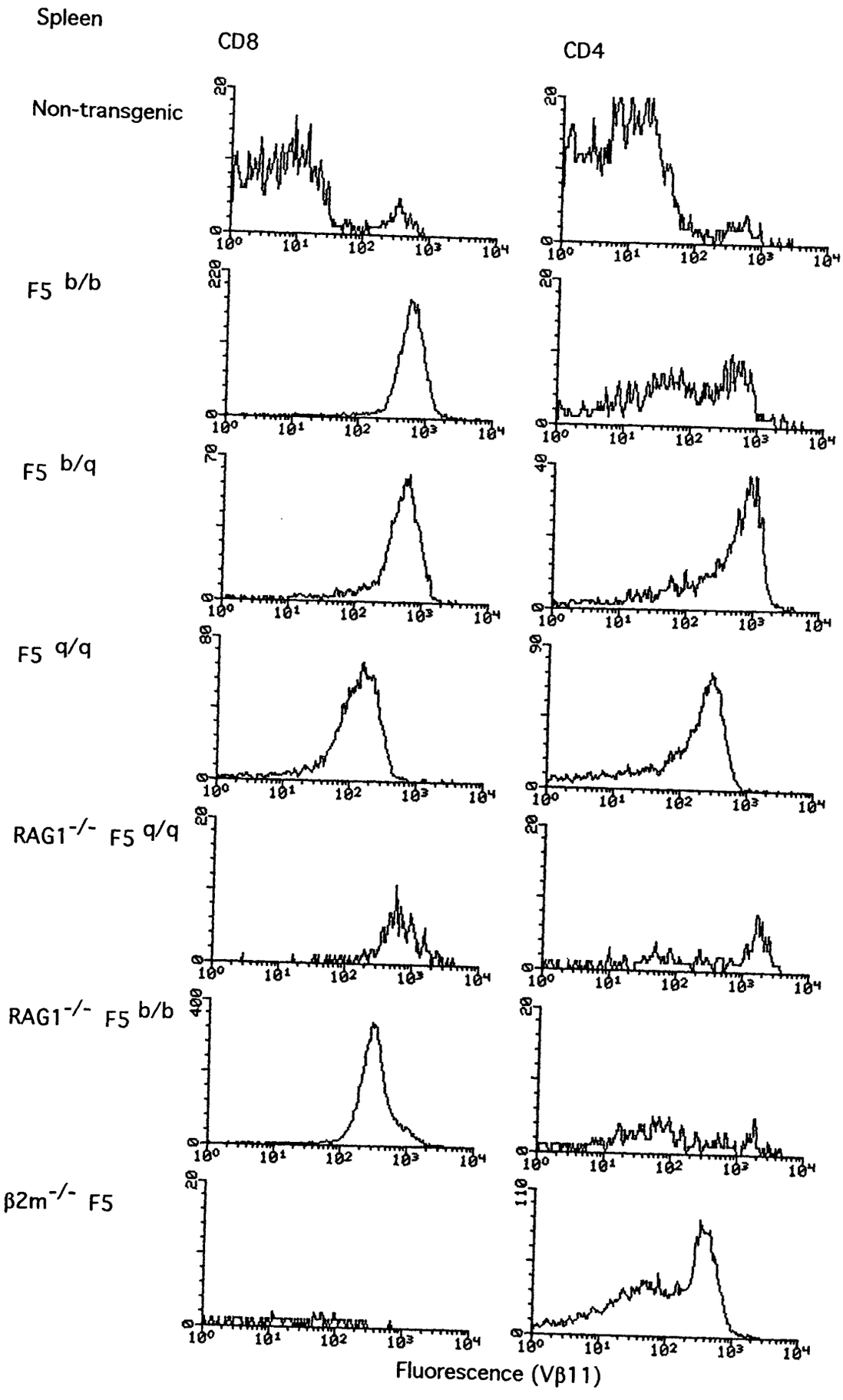

CD8
CD4
Non-transgenic
F5 b/b
F5 b/q
F5 q/q
RAG1-/- F5 q/q
RAG1-/- F5 b/b
β2m-/- F5
Fluorescence (Vβ11)

Figure 45 F5 spleen cells in different backgrounds (Vβ11)

Spleen cells from F5 mice in different backgrounds were analysed as in figure 44, and levels of Vβ11 in CD4 and CD8 single positive cells are shown by histograms.


Spleen
CD8
CD4
Non-transgenic
F5 b/b
F5 b/q
F5 q/q
RAG1-/- F5 q/q
RAG1-/- F5 b/b
β2m-/- F5
Fluorescence (Vβ11)

mice as shown in figure 42). These data are compatible with both models (lack of positive selection, or negative selection), since positive and negative selection can both operate at the $TCR^{int}DP$ stage. At present, it seems difficult to discriminate these possibilities.

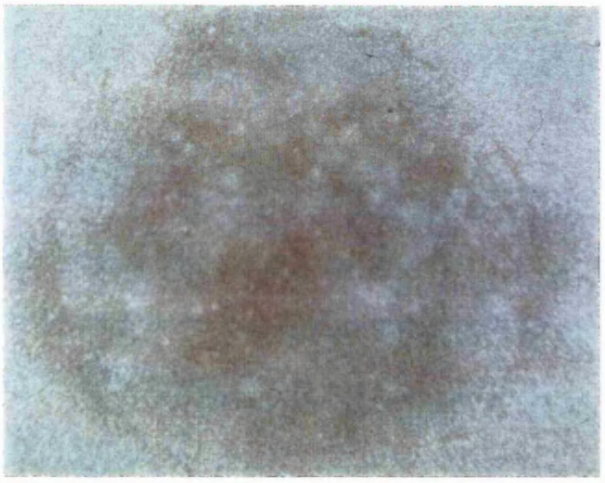
In an attempt to reconstitute normal T cell development in $F5^{q/q}$ background, we established new lines of thymic epithelial cells from a purified thymic stromal population (Jenkinson et al., 1992). Thus, a thymic tumour of an adult B10 H2ts mouse was mechanically disrupted and allowed to adhere to tissue culture flasks. Subsequently $CD45^-$, A2B5 (medullary marker)$^-$, and class II MHC^+ cells were purified from adherent cells using immune-magnetic beads (figure 19). Clones derived from such thymic stromal cells exhibited both epithelial and cortical characteristics as judged by expression of specific markers (figure 20). To test the functional ability of these thymic cortical epithelial cell lines, we employed a reaggregate co-culture system using day 14 fetal thymic lobes from $F5^{q/q}$ mice. Figure 46 depicts typical aggregates formed in the presence or absence of normal thymic stromal cells or epithelial cell clones. Thus, when reaggregated by themselves, day 14 thymocytes alone failed to reach a critical stromal cell density to form any shape and most thymocytes died. In the presence of additional thymic stromal cells which were freshly isolated from deoxyguanosine-treated fetal thymic lobes, successful reaggregates were formed and exibited lobular organisation under the inverted microscopy as reported before (Anderson et al., 1993). Reaggregates formed

Figure 46 Reaggregate formation

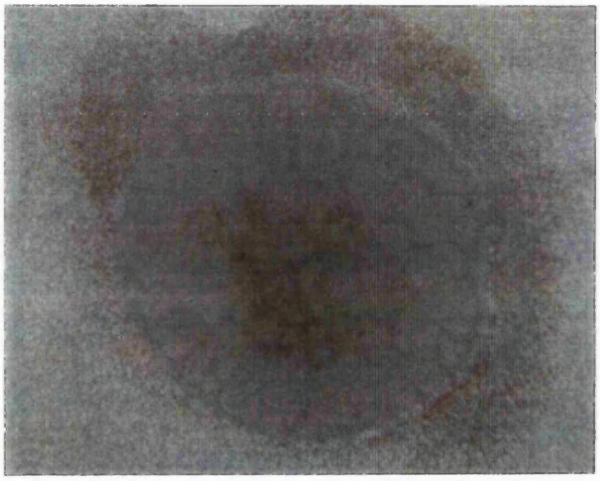
Day 14 fetal thymocytes were reaggregated in the absence or presence of normal thymic stromal cells freshly isolated from deoxyguanosine-treated fetal thymic lobes, II-2 cell line, or II-2 cell line and thymic stromal cells depleted of class II MHC^+ cells.

Reaggregate formation

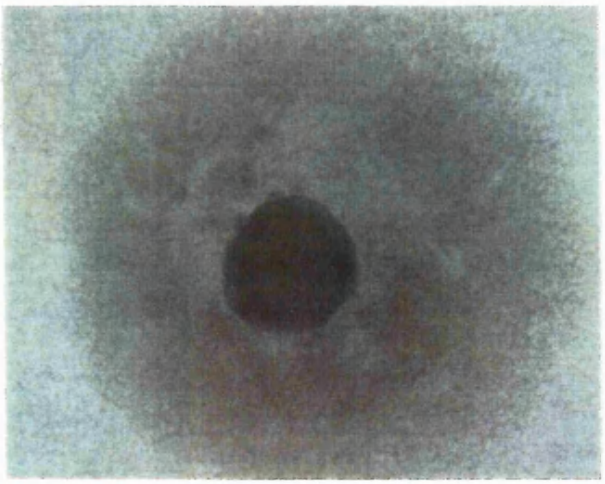
Thymocytes alone



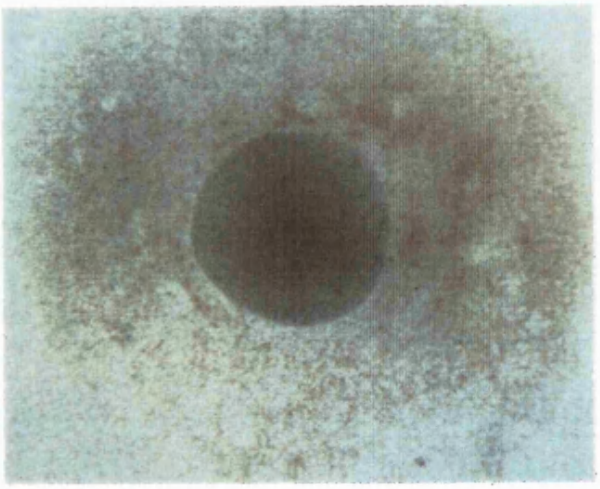
Thymocytes and B10 stromal cells



Thymocytes and Il-2 cells



Thymocytes, Il-2 cells, and fibroblasts



with epithelial cell clones, in contrast, exibited more compact and homogeneous appearance. Addition of class II^- thymic stromal cells partially restored the size of reaggregates but failed to produce lobulated structure. Results of five experiments using $F5^{q/q}$ thymocytes are summarised in table 6 which shows cell numbers recovered, proportion of CD8 SP cells, and frequency of $V\beta11^+$ cells among CD8 SP population. According to the cellularity, successful reaggregates were achieved in two experiments (exp. 2 and 5). In these cultures, neither the cell number nor the proportion of CD8 SP cells were affected by the thymic epithelial cell lines. However, the proportion of $V\beta11^+$ cells among $CD8^+$ cells were increased when $F5^{q/q}$ thymocytes were cultured in the presence of the II-2 cells compared with cultures of $F5^{q/q}$ cells alone, although this was not the case with another experiment (exp. 3). Figure 47-a illustrates a representative FACS profile of CD4 and CD8 expression in $F5^{q/q}$ reaggregate cultures in the presence or absence of II-2 cell line (exp. 2 in table 6). The II-2 cell line caused generation of a distinct CD8 SP population expressing high level CD8 and $V\beta11$. By contrast, most CD8 SP cells in control cultures were $V\beta11^{low/-}$. These data strongly suggest that II-2 cells can support phenotypic maturation of F5 T cells.

Nevertheless, a significant number of $CD8^+$ cells expressing high level $V\beta11$ were generated in $F5^{q/q}$ reaggregates in the absence of $H\text{-}2^b$ cells (figure 47-b) as well as in $F5/H\text{-}2^{q/q}$ mice <u>in vivo</u> (figure 41 and 44). Since $H\text{-}2^q$ can not, in principle, support

Table 6 $F5^{q/q}$ reaggregate cultures

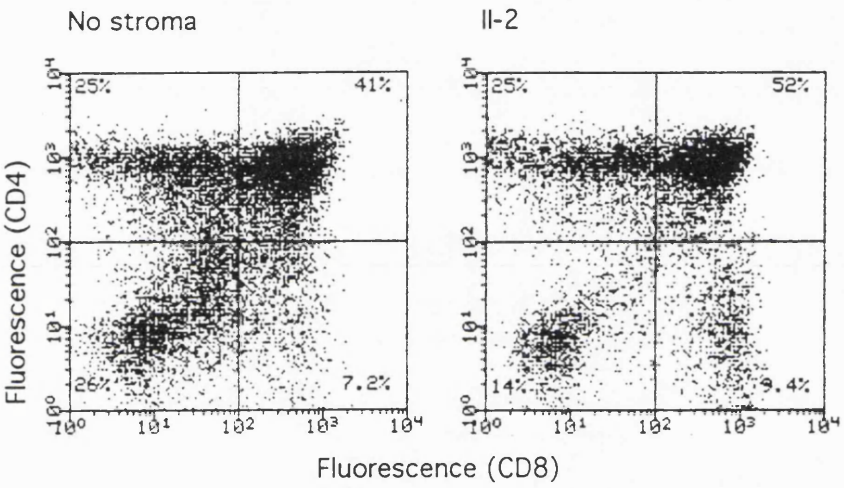
Stromal cells	Thymocyte input	Recovery	% CD8 (% $V\beta11^{+\dagger}$)
Exp. 1 (E14+d10)			
-	$3.3x10^5$	ND	15.4 (18.6)
II-2	$3.3x10^5$	ND	16.5 (53.0)
Exp. 2 (E14+d12)			
-	$3x10^5$	$8.3x10^5$	9.7 (17.0)
II-2	$3x10^5$	$3.9x10^5$	10.8 (73.5)
Exp. 3 (E14+d12)			
-	$3x10^5$	ND	25.7 (84.8)
II-1	$3x10^5$	ND	19.5 (85.3)
II-2	$3x10^5$	ND	22.5 (35.4)
Exp. 4 (E14+d11)			
-	$2.5x10^5$	$2.9x10^4$	22.3 (48.3)
B10	$2.5x10^5$	$3.3x10^4$	17.9 (50.1)
II	$2.5x10^5$	$0.11x10^4$	5.2 (86.5)
II+II-3	$2.5x10^5$	$0.13x10^4$	0.5
Exp. 5 (E14+d12)			
-	$2.4x10^5$	$5.3x10^5$	8.0 (23.8)
II-1	$2.4x10^5$	$3.6x10^5$	5.7 (39.2)
II-2	$2.4x10^5$	$3.5x10^5$	6.4 (52.0)

† Proportion of $V\beta11^{high}$ cells among CD8 SP cells
ND Not determined

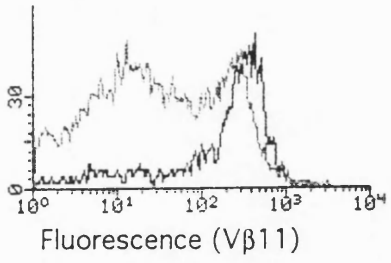
Figure 47 Reaggregate culture of $F5^{q/q}$ thymocytes with II-2 cell line

Thymic cortical epithelial cell lines were established from a thymic tumour of adult H2ts mouse after purification of $CD45^-$, $A2B5^-$, and class II MHC^+ thymic stromal cells. One of these, II-2 cells ($H\text{-}2^b$), were tested in reaggregate culture using day 14 $F5^{q/q}$ fetal thymocytes including lymphoid progenitors and stromal cells. After 12 day culture, reaggregates were mechanically disrupted and cells were stained for CD4, CD8, and Vβ11. a) Expression of CD4 and CD8 on $F5^{q/q}$ thymocytes cultured in the presence (right) or absence (left) of II-2 cells. b) Levels of Vβ11 expression on CD8 SP cells in $F5^{q/q}$ thymic lobes cultured in the presence (black line) or absence (grey line) of II-2 cells.

a) F5 $^{q/q}$ reaggregate culture



b) TCR expression in CD8 T cells



selection of F5 T cells, these $CD8^+V\beta11^+$ cells in $F5^{q/q}$ cultures were likely to be selected by endogenous TCR α chains in association with transgenic β. To eliminate such residual selection events, $RAG1^{-/-}F5^{q/q}$ thymic lobes were used for reaggregate co-cultures in subsequent experiments. Thus, total thymocytes from newborn $RAG1^{-/-}F5^{q/q}$ mice were co-cultured with thymic stromal cells freshly isolated from deoxyguanosine-treated B10 fetal thymic lobes. As shown in figure 48, culture of $RAG1^{-/-}F5^{q/q}$ thymocytes in the absence of $H\text{-}2^b$ stromal cells did not produce mature SP cells, whereas co-culture of $RAG1^{-/-}F5^{q/q}$ thymocytes with B10 stromal cells resulted in generation of CD8 SP cells expressing high level Vβ11 (data not shown) to different degrees. The data provide evidence that newborn $RAG1^{-/-}F5^{q/q}$ thymocytes are able to differentiate to SP T cells.

Subsequently, $H\text{-}2^b$ epithelial cells lines were used to reaggregate with $RAG1^{-/-}F5^{q/q}$ fetal thymocytes at day 15 of gestation. Most thymocytes in F5 fetuses were still DN at this stage as described above (figure 34). Table 7 summarises results of four such experiments. In the first experiment, freshly isolated B10 thymic stromal cells were also used as a control. As shown in figure 49, B10 stromal cells supported generation of CD8 SP cells in a $RAG1^{-/-}F5^{q/q}$ reaggregate culture. The level of Vβ11 on CD8 SP cells was higher than that on DP cells in this culture (figure 49, histograms), suggesting that these CD8 SP cells were mature. Similar reaggregate cultures using II-2 or II-4 cell lines resulted in generation of a large number of CD8 SP

Figure 48 Reaggregate culture of $RAG1^{-/-}F5^{q/q}$ newborn thymocytes and normal $H\text{-}2^{b}$ stromal cells

Thymocytes from $RAG1^{-/-}F5^{q/q}$ mice were cultured in the presence or absence of thymic stromal cells freshly isolated from C57BL/10 fetal thymic lobes treated with deoxyguanosine for 7 days. After 5 day culture, reaggregates were mechanically disrupted and cells were stained for CD4, CD8, and Vβ11.

New born RAG1$^{-/-}$F5$^{q/q}$ reaggregate cultures

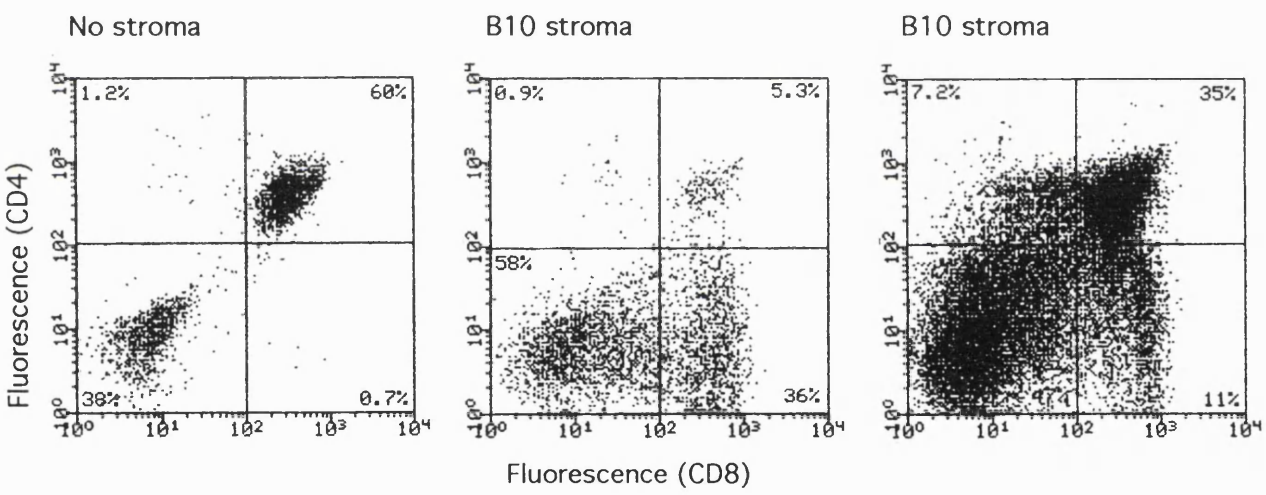


Table 7 $RAG1^{-/-}F5^{q/q}$ reaggregate cultures

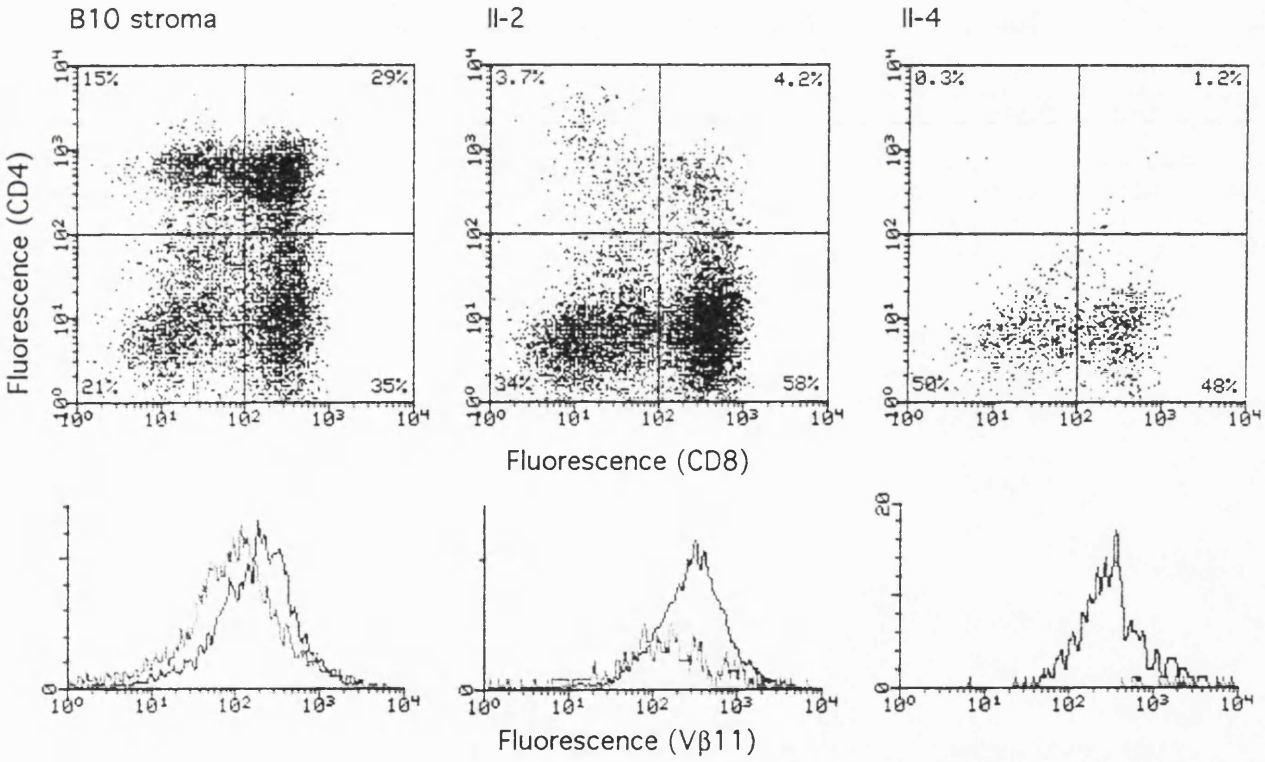
Stromal cells	Thymocyte input	Recovery	% CD8
Exp. 1 (E15+d7)			
-	$5x10^5$	ND	-
B10	$5x10^5$	ND	37.9
II-2	$5x10^5$	ND	61.6
II-4	$5x10^5$	ND	57.0
II-4	$5x10^5$	ND	47.2
Exp. 2 (E15+d7)			
-	$9x10^5$	$<10^3$	-
II-2	$9x10^5$	$2.5x10^4$	57.3
II-2†	$9x10^5$	$2.2x10^4$	47.9
Exp. 3 (E15+d8)			
-	$1x10^6$	$0.3x10^4$	-
II-2	$1x10^6$	$0.4x10^4$	-
Exp. 4 (E15+d7)			
-	$6x10^5$	$4.5x10^5$	2.0
II-2	$6x10^5$	$<10^3$	-
Kep1.2	$6x10^5$	$4.0x10^5$	6.6

† Class II MHC^+ cells were depleted from $RAG1^{-}F5^{q/q}$ thymocytes

Figure 49 Reaggregate culture of $RAG1^{-/-}F5^{q/q}$ fetal thymocytes with II-2 and II-4 cell lines

Thymocytes from $RAG1^{-/-}F5^{q/q}$ day 15 fetuses were cultured in the presence or absence of B10 thymic stromal cells, prepared as in figure 48, or II-2 and II-4 thymic cortical epithelial cell lines ($H\text{-}2^{b}$). After culture for 7 days, thymocytes were stained for CD4, CD8, and Vβ11. Top panels show expression of CD4 and CD8 on total population of cells, and bottom panels show levels of Vβ11 expression on DP (grey lines) and CD8 SP (black lines) cells.

RAG1 $^{-/-}$ F5 $^{q/q}$ reaggregate cultures



cells (figure 49, top panels) whose Vβ11 levels were comparable to those in CD8 SP cells in the B10 stromal culture (figure 49, bottom panels). These data provide the first evidence that cultured thymic cortical epithelial cell lines are able to support differentiation of immature T cells in vitro in the presence of non-selecting heterogeneous thymic stromal populations.

5.2.2 $\beta 2m^{-/-}$F5 mice

Since there was a formal possibility that H-2^q molecule causes negative selection rather than lacks positive selection, F5 thymocytes in a class I MHC-deficient ($\beta 2m^{-/-}$) background was used for reaggregate cultures with thymic cortical epithelial cell lines. The T cell development in $\beta 2m^{-/-}$F5 mice was arrested at the TCR^{low} DP stage as described before (figure 41). One of the reaggregate cultures was successful (exp.1 in table 8). Thus, co-culture of control B10 stromal cells with $\beta 2m^{-/-}$F5 thymocytes generated a large number of CD8 SP cells most of which expressed higher level Vβ11 than DP cells in the same culture (figure 50). Co-cultures of $\beta 2m^{-/-}$F5 thymocytes with II-4 cell line resulted in fewer cell numbers but proportions of CD8 SP cells and their Vβ11 expression was comparable to those in B10 stromal cultures. These data demonstrates that II-4 cell line is as efficient as freshly isolated thymic stromal cells in supporting positive selection of F5 T cells in theis system.

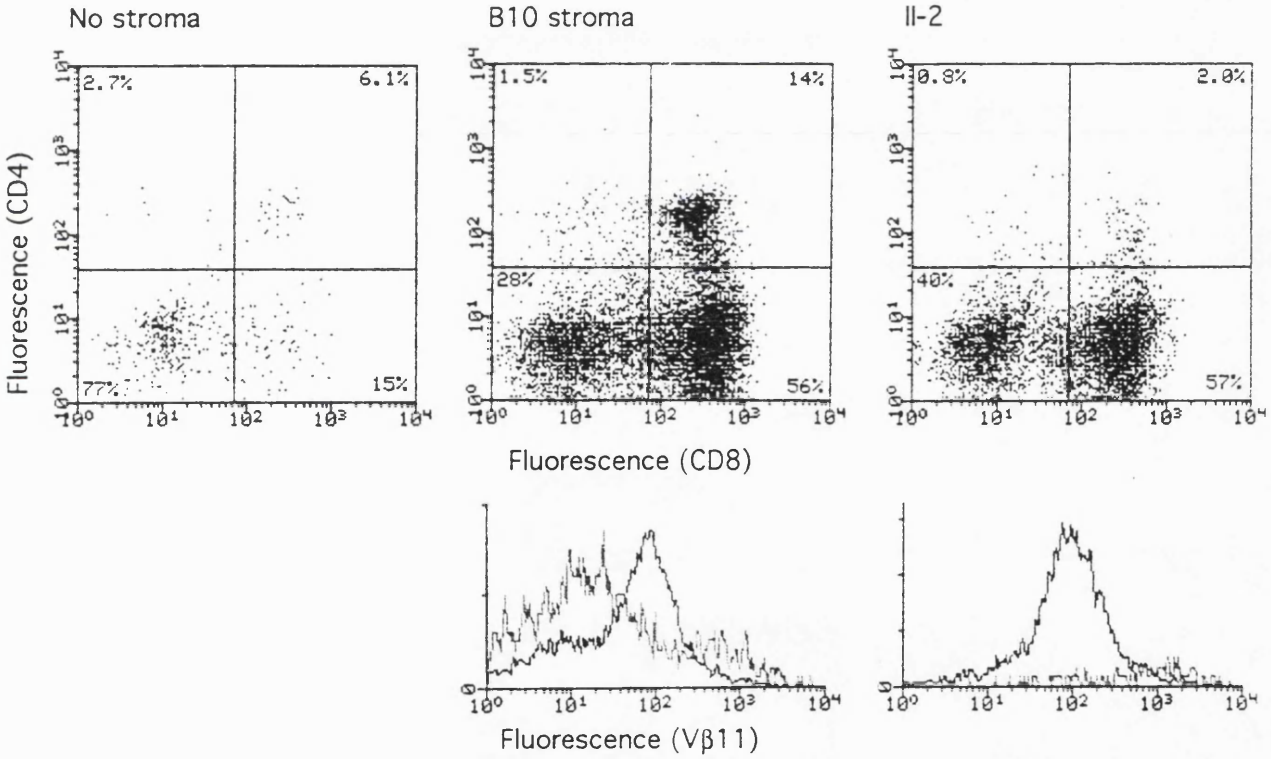
Table 8 $\beta 2m^{-/-}$F5 reaggregate cultures

Stromal cells	Thymocyte input	Recovery	% CD8
Exp. 1 (E16+d11)			
-	$1x10^6$	$0.14x10^5$	-
B10	$1x10^6$	$1.7x10^5$	58.1
B10	$1x10^6$	$1.8x10^5$	78.1
II-4	$1x10^6$	$0.3x10^5$	62.8
II-4	$1x10^5$	$0.9x10^5$	61.4
Exp. 2 (E15+d8)			
-	$1x10^6$	$0.11x10^4$	-
II-2	$1x10^6$	$0.65x10^4$	-

Figure 50 Reaggregate culture of $\beta 2m^{-/-}$F5 fetal thymocytes with II-2 cell line

Thymocytes from $\beta 2m^{-/-}F5^{b/b}$ day 16 fetuses were cultured in the presence or absence of B10 thymic stromal cells, prepared as in figure 48, or II-2 ($H\text{-}2^b$) thymic cortical epithelial cell lines ($H\text{-}2^b$). After culture for 11 days, thymocytes were stained for CD4, CD8, and Vβ11. Top panels show expression of CD4 and CD8 on total population of cells, and bottom panels show levels of Vβ11 expression on DP (grey lines) and CD8 SP (black lines) cells.

β2m$^{-/-}$ F5 reaggregate cultures



5.3 Peptide analogues

5.3.1 CTL activation

In order to study CTL activation by different peptide analogues, cytotoxic T cell lines were derived from spleen T cells of F5 mice in collaboration with Dr. M. Bix (UCSF, USA). Preliminary experiments using RMA-S cell line, which has defects in class I MHC presentation due to a mutation in one of the peptide transporter genes, showed that the 1968 NP, 1934 NP, P4D, P4Q, P4K, P7E, P8T, and HIV-gag peptides can restore the expression of class I MHC on RMA-S cells (data not shown). In order to test if peptide analogues were recognised by the F5 CTL, RMA-S cells were pre-pulsed with various concentrations of peptides, labelled with Cr^{51}, and were co-cultured with the F5 CTL. Specific cytolysis was measured according to the formula described in the legend.

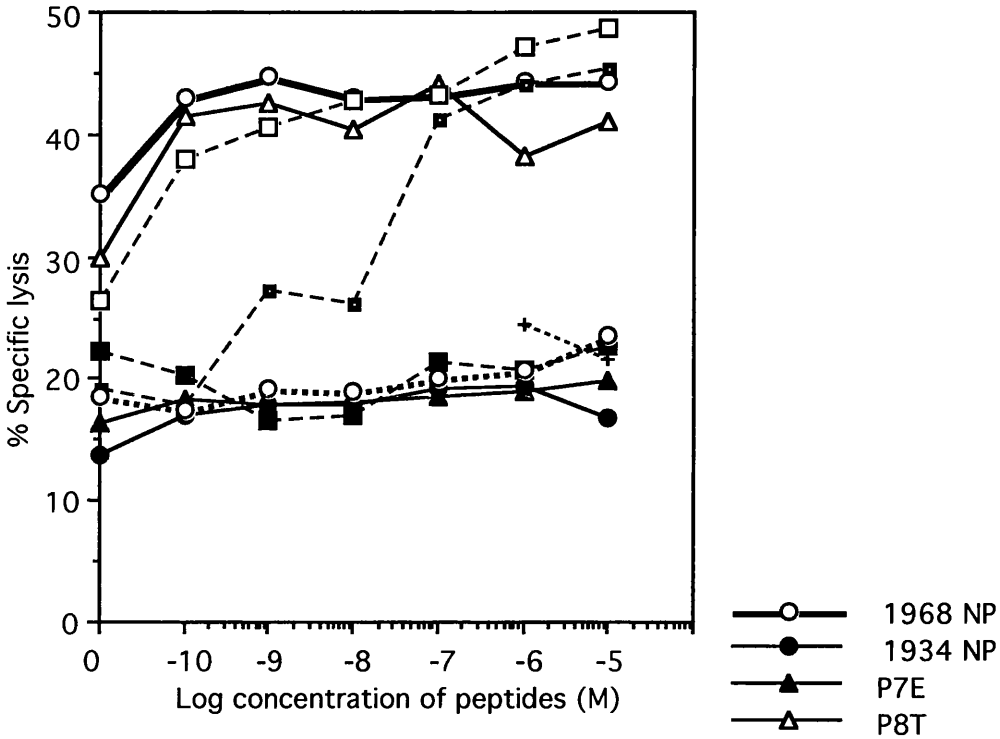
Figure 51-a shows proportions of specific cytolysis at different concentrations of peptide analogues. P4D and P8T were as potent as the 1968 NP as targets of cytolysis, P4Q had much reduced activity, and P4K, P7E, 1934 NP, and un-related HIV-gag did not cause any cytolysis above backgrounds. These data confirmed our previous observations that P8T is antigenic *in vivo* whereas P7E and 1934 NP are not. In addition, three different analogues at position 4 revealed different antigenic potencies (P4D > P4Q > P4K).

Figure 51 Agonist and antagonist assays for F5 CTL

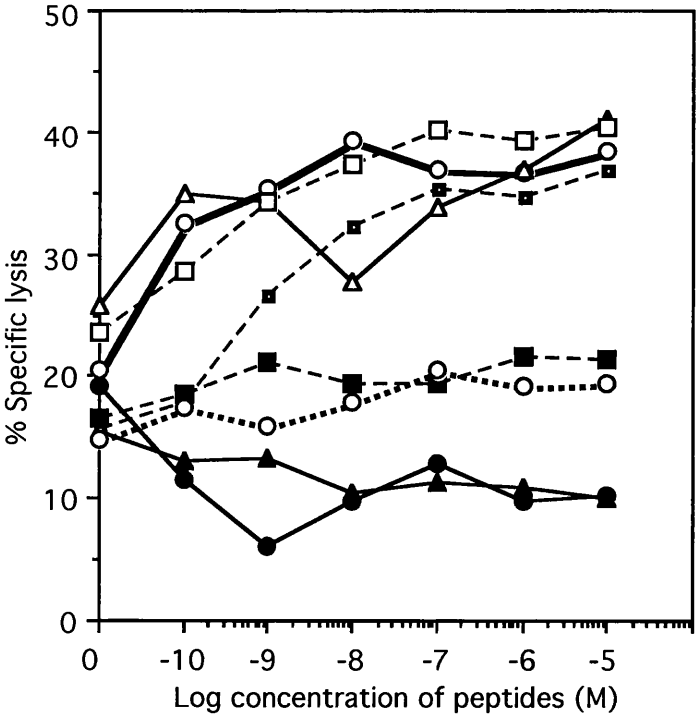
F5 CTLs were established from spleen T cells of adult F5 transgenic mice, and were maintained in culture with weekly stimulations with irradiated C57BL/10 spleen cells loaded with the cognate peptide 1968 NP. a) In agonist assays, RMA-S cells were pre-pulsed for 2 hours with Cr^{51} in the presence or absence of various concentrations of peptide analogues, and were co-cultured with F5 CTLs. After 3 hours, radiation activities of culture supernatants were measured by a γ-counter. Specific target lysis was measured by (Cr^{51} release of the sample - Cr^{51} release without F5 CTL clones) / (Cr^{51} release by detergent - Cr^{51} release without F5 CTL clones) x 100 (%). b) In antagonist assays, RMA-S cells were pre-pulsed for 2 hours with Cr^{51} and 50 nM 1968 NP, washed, and were co-cultured with F5 CTLs in the presence of different concentrations of peptide analogues. After 3 hours, Cr^{51} release and specific cell lysis were measured as above. (Collaboration with Dr. Mark Bix, UCSF, USA).

Activation of F5 CTL clone

a) Agonist assay



b) Antagonist assay



In order to examine the ability of peptide analogues to interact with the F5 TCR, competition for target recognition between sub-optimal doses of 1968 NP and various concentrations of peptide analogues was analysed (antagonist assay). To this end, RMA-S cells were first pre-pulsed with 50 pM concentration of 1968 NP, which caused half maximal activation of the F5 CTL (figure 51-a), and were labelled with Cr^{51}. The RMA-S cells were washed free of the peptide and Cr^{51}, and were co-cultured with F5 CTL in the presence of increasing amounts of analogues. After 3 hours, specific release of Cr^{51} was measured. As illustrated in figure 51-b, increasing amounts of agonistic peptides, such as 1968 NP, P4D, P8T, and P4Q, induced increasing lysis of target cells. On the other hand, the 1934 NP and P7E peptides caused decrease in specific cytolysis at a concentration of 10 to 100-fold excess to that of pre-pulsed 1968 NP. These effects were peptide specific since P4K and un-related HIV-gag peptides had no effect even at the highest concentration tested (10 μM), strongly suggesting that CTL inhibition is caused by the competition at the level of TCR-peptide/MHC interaction rather than at the level of peptide-MHC interaction. In other words, the 1934 NP and P7E peptides are capable of interacting with the F5 TCR but do not activate cytolytic pathways.

5.3.2 Negative selection

As the above set of peptide analogues exhibited various effects on mature F5 T cells, it was of interest to analyse if these

peptides can cause negative selection of immature F5 thymocytes. To this end, 1968 NP, P4D, P4K, or PBS (as a control) were injected intraperitoneally into adult F5 mice at 50 nmoles/20 g body weight and daily for 4 days, as described before (Mamalaki et al., 1992). The number of thymocytes was 2.2 x 10^8 in PBS, 0.09 x 10^8 in 1968 NP, 0.20 x 10^8 in P4D, and 2.3 x 10^8 in P4K-injcected mice respectively, clearly indicating that 1968 NP (also Mamalaki et al., 1992) and P4D, but not P4K, caused negative selection of F5 thymocytes. Analysis of CD4 and CD8 expression confirmed that DP cells were deleted in mice treated with 1968 NP or P4D but not P4K (data not shown). Also in mice treated with 1968 NP or P4D, peripheral CD8 T cells were selectively expanded and their CD8 levels were slightly down-regulated as described before (Mamalaki et al., 1992).

Effect of peptide analogues on T cell development was also analysed using fetal thymus organ culture. As described in chapter 4.2.3, organ culture of embryonic day 14 $RAG1^{-/-}$F5 thymic lobes in the presence of the 1968 NP caused deletion of most DP thymocytes and down-regulation of Vβ11 and CD8 in CD8 SP cells. Similar analysis using P4D, which is antigenic in a F5 CTL assay, showed that P4D causes deletion of DP thymocytes in $RAG1^{-/-}$F5 fetal thymus organ culture. On the other hand, P4K was found non-antigenic both in a CTL assay and an <u>in vivo</u> injection analysis as described above. The effect of P4K on F5 thymocytes was further studied using day 14 $RAG1^{-/-}$F5 fetal thymic lobes which had been pre-cultured for 7 days in the absence of peptide. After treatment with P4K at 5 μM for 3 days, thymocytes were stained

for CD4, CD8, and Vβ11 and the number of each thymocyte subset was calculated. Figure 52-d shows that the number of DP cells was selectively reduced in P4K-treated cultures, whereas that of CD8 SP cells was not affected. Expression of CD4 and CD8 in $V\beta11^{high}$ gated cells is also illustrated in figure 52-a, showing a selective deletion of DP cells. The remaining CD8 SP cells expressed down-regulated CD8 (figure 52-a) and Vβ11 (52-c), and were blastic in cell size (52-b). These data demonstrate that P4K causes deletion of DP thymocytes and down-modulation of TCR and co-receptors in CD8 SP cells. The <u>in vivo</u> finding that P4K had no effect on F5 thymocytes could be due to limited accessibility of the peptide to the thymus compared with <u>in vitro</u> culture. Taken together, P4K is not antigenic for the F5 CTL at 10 μM, but is able to cause negative selection of immature F5 T thymocytes at 5 μM.

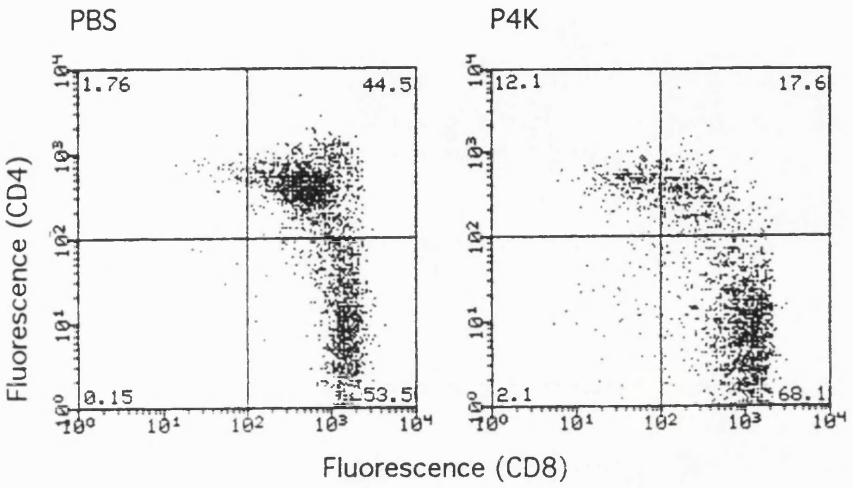
In order to test if CTL antagonists can also block negative selection of immature F5 T cells, $RAG1^{-/-}$F5 fetal thymic lobes were cultured in the presence of various concentrations of the 1968 NP and 1934 NP. After culture for 8 days, expression of CD4, CD8, and TCR was analysed for pools of 5 thymic lobes in each sample. Numbers of CD8 SP cells expressing high level Vβ11 (comparable to that of mature CD8 T cells in F5 mice) were measured as an indication of T cell selection. As illustrated in figure 53, a high concentration (10 μM) of 1968 NP suppressed generation of $V\beta11^{high}$ CD8 SP cells completely, whereas suboptimal doses of the 1968 NP at 10 pM or 100 pM caused about half maximal negative selection. In the presence of 10 pM 1968 NP, increasing

Figure 52 Negative selection of F5 T cells by P4K

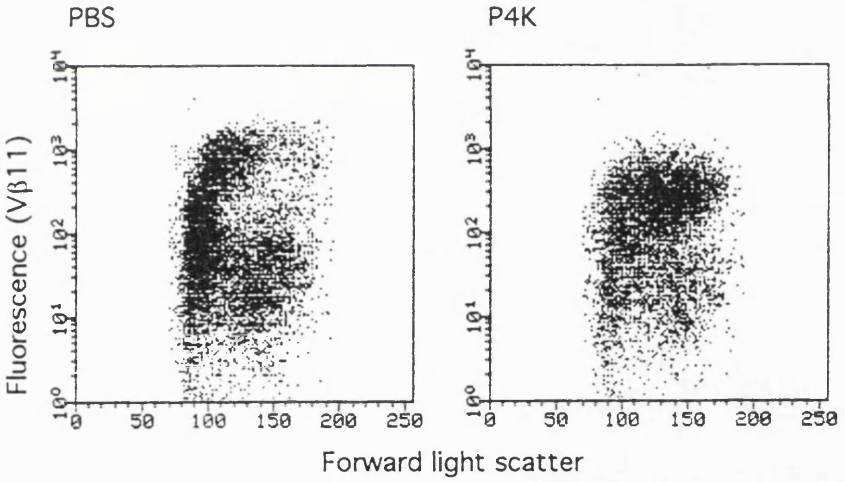
Day 14 RAG1$^{-/-}$F5 fetal thymic lobes were cultured for 7 days in the absence of peptide, and were subsequently treated with PBS or P4K for 3 days. Thymocytes were stained for CD4, CD8, and Vβ11. a) Patterns of CD4 and CD8 expression in Vβ11^{high} cell populations. b) Cell size and levels of Vβ11 expression. In P4K-treated lobes, a population of blastic cells expressing intermediate level Vβ11 are CD8 SP. c) Levels Vβ11 expression in DN, DP, and CD8 or CD4 SP cells are illustrated by histograms for PBS (black lines) and P4K (grey lines) cultures. d) Absolute numbers of DN, DP, and CD8 cells in cultures with either PBS (black) or P4K (shaded).

RAG1 $^{-/-}$ F5 FTOC E14+d7+d3

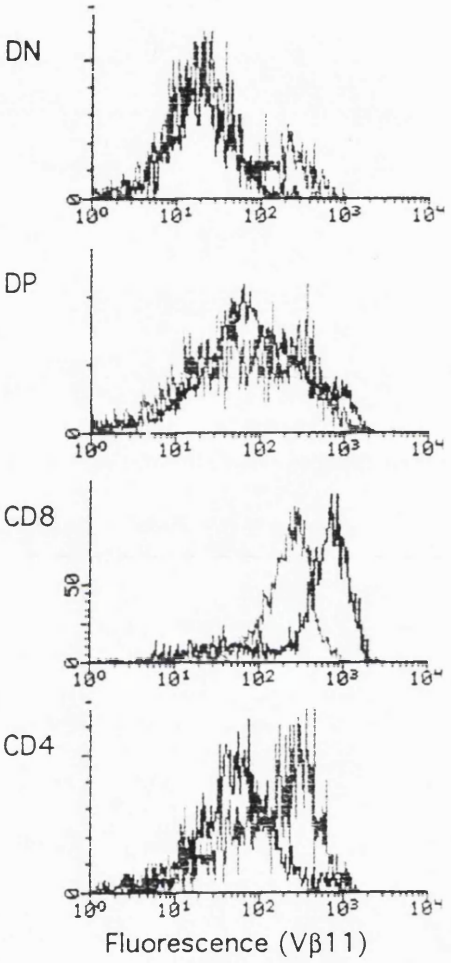
a) Vβ11 ^{high} gated cells



b) Cell size and Vβ11 expression



c) Levels of Vβ11



d) Cell numbers

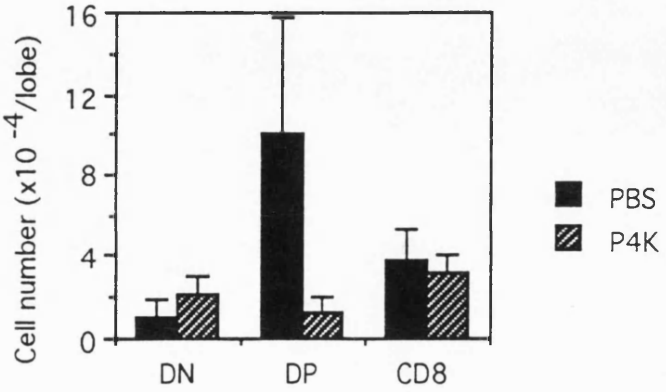
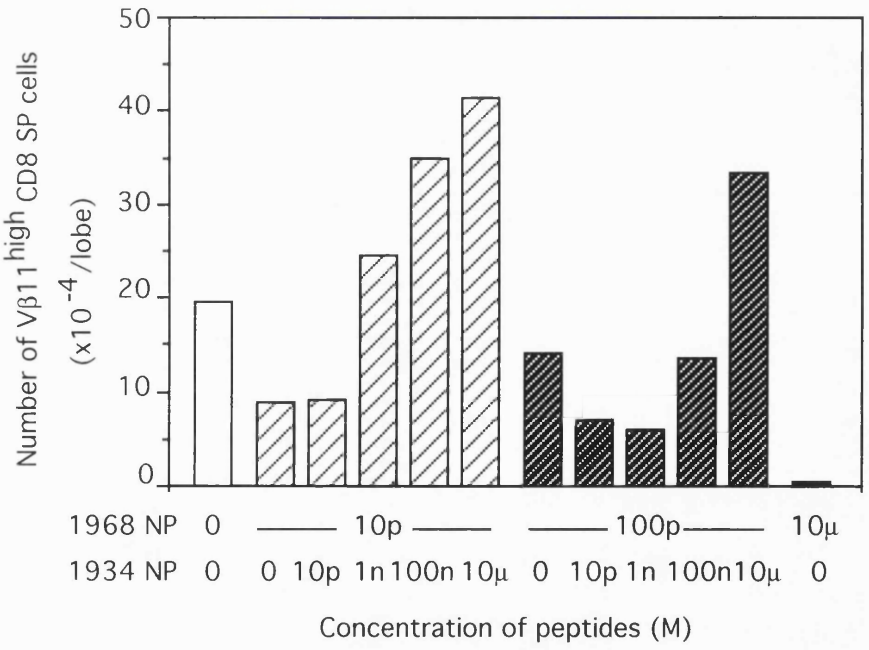


Figure 53 Competitive inhibition of negative selection

Day 15 RAG1$^{-/-}$F5 fetal thymic lobes were cultured for 8 days in the presence of different concentrations of 1968 NP and/or 1934 NP. Each sample consisted of five lobes which were pooled and were stained for CD4, CD8, and Vβ11. Numbers of cells expressing CD8 and high level Vβ11 (as comparable to that of mature F5 CD8 T cells) were calculated. The presence of 10 μM 1968 NP alone completely suppressed generation of CD8^{+}Vβ11^{high} cells. Sub-optimal concentrations of 1968 NP (10 pM and 100 pM) caused half maximal deletion of CD8^{+}Vβ11^{high} cells. Addition of increasing amounts of 1934 NP restored the number of CD8^{+}Vβ11^{high} cells.

RAG1$^{-/-}$ F5 FTOC E15+d8



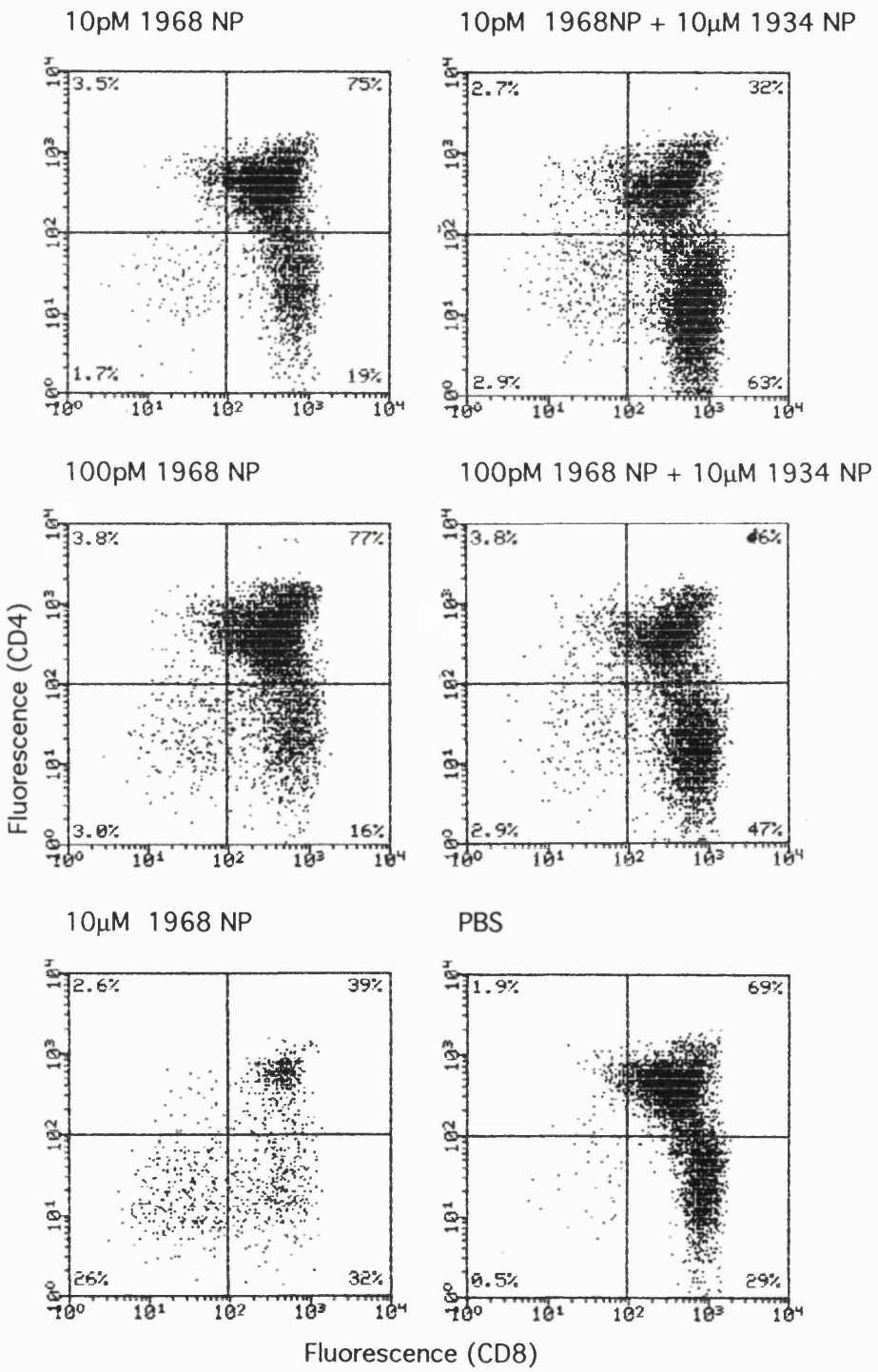
amounts of the 1934 NP restored the number of $V\beta11^{high}$ $CD8^+$ T cells even to a greater extent (about 2-fold) than PBS-treated cultures. Figure 54 illustrates patterns of CD4 and CD8 expression on $V\beta11^{high}$ gated cells. Suboptimal doses of 1968 NP caused reduction in the proportion of CD8 SP cells, whereas addition of high concentrations of 1934 NP to suboptimal 1968 NP resulted in largely increased CD8 SP cells. These data provide the first evidence for competitive inhibition of negative selection and enhancement of positive selection by a non-antigenic peptide analogue. Whether the blocking of negative selection was caused by TCR competition or MHC competition remains to be resolved. However, the relatively low dose of the 1934 NP required to antagonise the 1968 NP (about 10^2-fold excess) suggests a specific competition at the level of interaction between TCR and peptide/MHC complexes rather than competition for MHC binding, considering the fact that non-specific competition requires more than 10^5 order excess of competitors as reported by De Magistris et al. (De Magistris et al., 1992). A slightly higher concentration (100pM) of the 1968 NP required more excess of the competitor (>10^3) to block negative selection, suggesting that blocking of negative selection can operate only under limiting concentrations of cognate peptide.

In order to determine if the 1934 NP alone can promote F5 T cell differentiation, F5 thymic lobes from day 14 embryos were cultured in the presence of a high concentration (5 μM) of 1934 NP for 3 to 11 days. Thymocytes were stained for CD4, CD8, and

Figure 54 Competitive inhibition of negative selection

Day 15 RAG1$^{-/-}$F5 fetal thymic lobes were cultured and analysed as in figure 53. Cells expressing high level Vβ11 were gated and their CD4 and CD8 expression presented.

RAG1$^{-/-}$F5 FTOC E15+d8: Vβ11^{high} gated cells

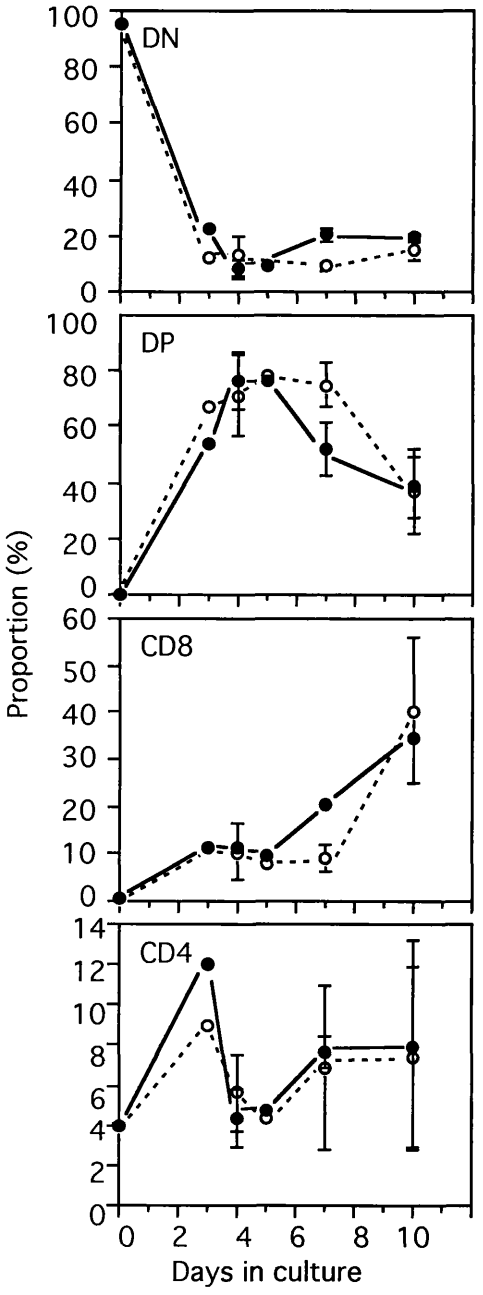


Vβ11, and the proportion of each thymocyte subset was measured. As illustrated in figure 55 (left panels), development of F5 T cells was largely unaffected by the 1934 NP. However, there were slightly less DP and more CD8 SP cells in 1934 NP-treated lobes compared with PBS-treated cultures at day 7. Since there was no difference in cellularity between PBS or 1934 NP-treated thymic lobes (right panel in figure 55), the 1934 NP alone had only a marginal effect, if any, on the F5 T cell development. Similar developmental analysis was carried out using another CTL antagonist P7E. Day 14 fetal thymic lobes from $RAG1^{-/-}$F5 mice were cultured in the presence or absence of 5μM P7E for 6, 8, or 11 days. As shown in figure 56, neither proportion (left panels) nor cell number (right panels) of DN, DP, and CD8 SP thymocyte subsets was affected by P7E. From these data, we conclude that antagonistic peptides alone have little effect on the kinetics of F5 T cell development. This contrasts to the above observation that the 1934 NP enhanced selection of F5 thymocytes in the presence of suboptimal doses of 1968 NP. Taken together, a synergistic effect between the agonistic (1968 NP) and antagonistic (1934 NP) peptides is suggested. Such a model predicts that positive selection in normal mice, in which heterogeneous peptides are expressed on thymic stromal cells, could be mediated by combinations of agonistic and antagonistic peptides, rather than single peptides.

Figure 55 Effect of 1934 NP on the F5 T cell development

Day 14 F5 fetal thymic lobes were cultured for 3 to 10 days in the presence or absence of 5 μM 1934 NP. Thymocytes were stained for CD4, CD8, and Vβ11, and proportions of DN, DP, and CD8 or CD4 SP cells were measured. The figure was compiled from seven experiments each consisting of one to six thymic lobes per sample. Occasional samples, in both PBS and 1934 NP-treated cultures, had very few cell numbers probably because accumulation of fluid on filter membranes prevented their development, and therefore were excluded from the analysis. Dashed lines with open circles represent cultures with PBS and solid lines with closed circles are of cultures with the 1934 NP.

F5 FTOC E14 with 1934 NP



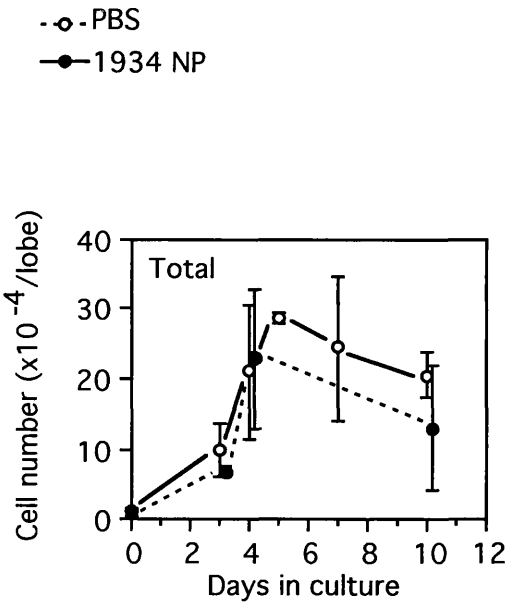
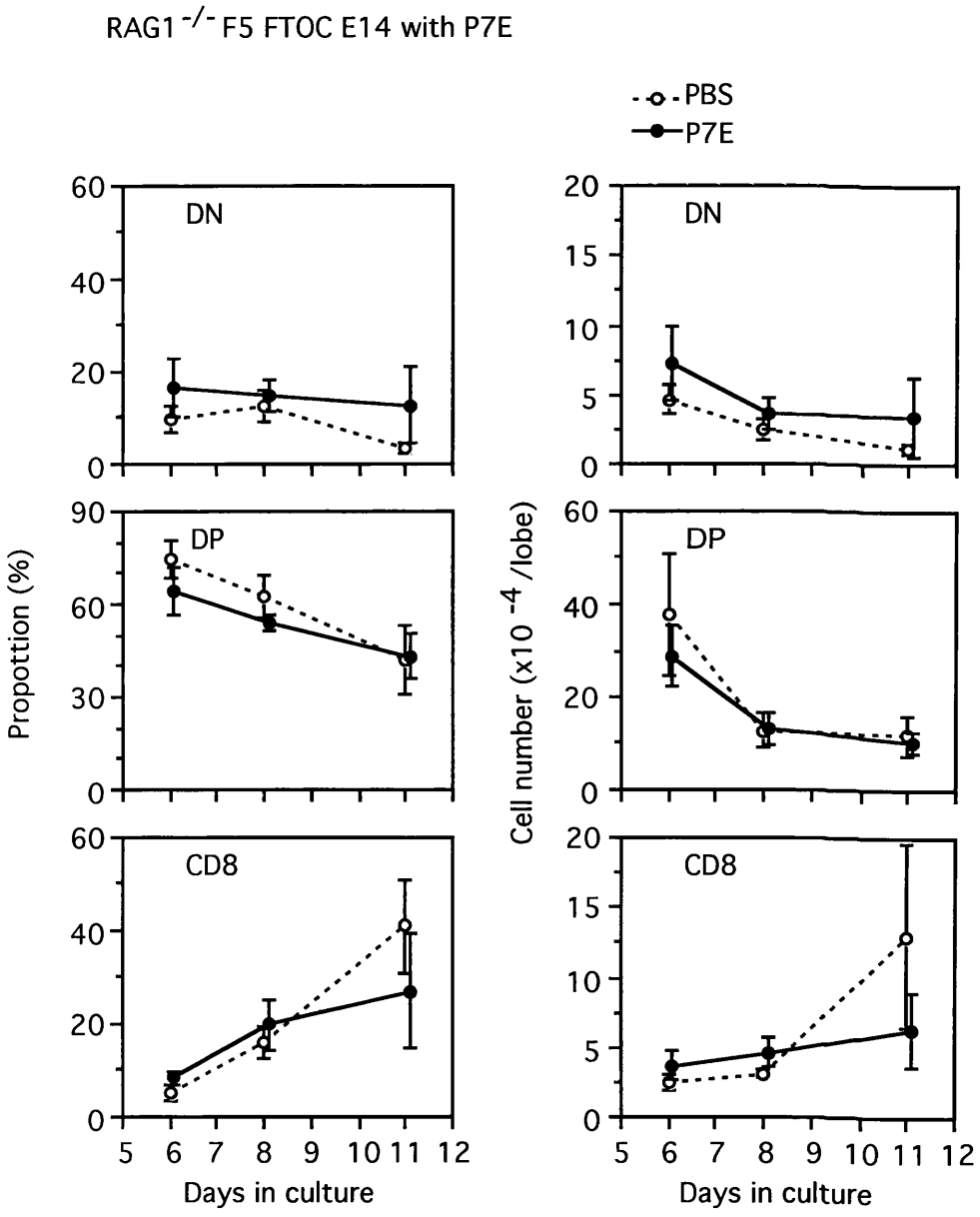


Figure 56 Effect of P7E on the F5 T cell development

Day 14 F5 fetal thymic lobes were cultured for 6, 8, and 11 days in the presence or absence of 5 μM P7E. Thymocytes were stained for CD4, CD8, and Vβ11, and proportions of DN, DP, and CD8 SP cells were measured and illustrated in the left panels. Absolute numbers of DN, DP and CD8 SP cells were calculated from their proportions and total cell numbers (the right panels). Dashed lines with open circles indicate PBS cultures, and solid lines with closed circles are of cultures with the 1934 NP. There were no difference between cultures with or without P7E.


RAG1 -/- F5 FTOC E14 with P7E
PBS
P7E
DN
DP
CD8
Propottion (%)
Cell number (x10 -4 /lobe)
Days in culture
Days in culture

Chapter 6 Discussion

As mentioned before, this section describes only general implications of the current investigation in the context of other relevant studies and future works, and most discussions were included in each result sections.

The central dogma of T cell repertoire selection is that it is based on clonal selection by specific antigen receptors. Interaction between self-reactive clones and their antigens causes their elimination from the repertoire either by physical deletion or functional inactivation. In addition, an unidentified interaction between TCR, co-receptors, and MHC molecules appear to be required for the development of DP cells to mature SP cells. In an attempt to identify specific TCR-ligand interactions mediating such positive selection processes, we studied the development of F5 TCR transgenic thymocytes in vivo and in vitro using peptide derivatives of cognate antigen and conditionally immortalised thymic stromal cell lines.

Effects of transgenic TCR expression on the T cell development

Expression of TCR plays a pivotal role in T cell development. It has been shown that expression of only TCR β chain is required for progression from DN to DP cells, whereas both α and β chains are necessary for generation of mature SP cells (Kishi et al.,

1991; Mombaerts et al., 1992; Shinkai et al., 1992). In the present study, we observed slightly earlier differentiation of DN cells to DP cells in F5 TCR transgenic thymocytes compared to normal thymocytes, consistent with the fact that a fraction (20 - 50 %) of DN cells in F5 mice express detectable levels of TCR (Mamalaki et al., 1993), whereas TCR on DN cells in normal mice is largely undetectable (Groettrup et al., 1993). Whether this increase in number of emerging DP cells is caused by induction of differentiation or by higher frequency of survival due to expression of F5 TCR remains to be studied.

Other studies have reported that expression of transgenic TCR causes a decrease in DP cells by reducing the rate of DN to DP progression. It was suggested that the selecting niche of the thymic stroma could be rate-limiting (Huesmann et al., 1991), or the number of precursor cells such as DP cells (Kelly et al., 1993) and $CD25^+$ DN and $CD4^{low}CD8^{low}$ cells (Nikolic-Zugic et al., 1993) were reduced. Thus, expression of a single TCR could result in positive effect by increasing the number of selectable T cells, or negative effect by competition for limited selecting niche or changes in the number of precursor cells. The net effect could be different from one TCR transgenic model to the other.

Development of SP cells is similar between F5 and normal mice as in other TCR transgenic models (Kelly et al., 1993). Thus, proportions of selected CD8 SP cells in the periphery of F5 mice show the same kinetic pattern as that in normal mice up to 1 month after birth. Subsequently, however, CD8 cells continue to

fill up the space of peripheral lymphoid organs in F5 mice, probably because there are less CD4 cells to compete with. In normal mice, proportions of CD8 and CD4 cells reach plateau after 1 month.

Thymic cortical epithelial cell lines

In an attempt to establish cell lines which can support positive selection of T cells, thymic cortical epithelial cells were purified from a thymic tumour of an H2ts mouse from which several cell lines were derived. To assess their function, mixed reaggregate cultures were devised using fetal thymocytes from F5 mice in non-selecting backgrounds such as $H\text{-}2^q$ or $\beta 2m^{-/-}$. Thus, two of the cell lines expressing $H\text{-}2^b$ molecules were shown to restore F5 T cell development in the presence of either $H\text{-}2^q$ or $\beta 2m^{-/-}$ stromal cells, providing evidence that cultured stromal cell lines can support positive selection of T cells. However, there is a possibility that cell lines contributed only $H\text{-}2D^b$/peptide ligand whereas the essential positive selection signal(s) was(were) supplied by residual thymic stromal cells. Such a mechanism could also be the case for positive selection caused by intrathymic injection of epithelial (Hugo et al., 1992; Vukmanovic et al., 1992) or fibroblast (Hugo et al., 1993; Pawlowski et al., 1993) cell lines. To resolve this issue, it is feasible in our system to deplete residual class II MHC^+ cells and make reaggregates with T cell precursors, class II^- thymic stromal cells, and class II MHC^+ cell lines (table 7, exp. 2).

Such a system allows epithelial cell lines to receive possible signals (van Ewijk et al., 1994) from developing thymocytes and other thymic stromal components to maintain or induce their normal function. A problem using F5 in H-2^q background is that it is impossible to discriminate negative selection and a lack of positive selection. In other words, the absence of $TCR^{high}DP$ cells and SP cells in $F5^{q/q}$ mice could be due to negative selection (cross-reactivity). For this reason, F5 mice in β2m-deficient background afford a better model for complementation experiments, and are being crossed to $RAG1^{-/-}$ for further analysis.

Selection of F5 thymocytes with peptide analogues

In order to analyse the effect of self-antigen on the kinetics of T cell development, fetal thymic lobes from F5 TCR transgenic mice were cultured in the presence or absence of a high concentration (5 μM) of cognate peptide. Our results show that generation of DP cells is selectively suppressed by cognate peptide, whereas development of immature $CD8^+$ cells is not affected. These data are largely consistent with a previous study using class II MHC-restricted TCR transgenic mice (Spain and Berg, 1992). Thus, as long as sufficient amounts of self-antigens are accessible to developing thymocytes, both class I and class II MHC-restricted thymocytes are deleted or arrest differentiation at the early DP TCR^{low} stage. In vivo, however, negative selection could take place at different stages of T cell development depending on the levels and geometry of self-antigen

expression (Pircher et al., 1989; Teh et al., 1990; Schneider et al., 1992).

Similar in vitro assays were performed using a series of peptide analogues with one or two amino acid substitutions from cognate peptide. As summarised in figure 40-b, potencies for negative selection of F5 thymocytes are reduced by mutations at position 4 in the order of

$$D = \mathbf{E} > Q > K$$

in which glutamate (M.W. 147), glutamine (M.W. 146), and lysine (M.W. 146) share almost identical molecular weights whereas charges of side chains vary from negative to neutral and positive. The fact that slightly smaller (M.W. 133) but negatively charged aspartate is as potent as glutamate suggests that these negatively charged carboxylate groups play a crucial role, possibly forming a salt bridge(s) with one of positively charged arginine or histidine residues in α/β CDR3 loops of the F5 TCR. It is predicted that a substitution with negatively charged methionine (M.W. 149) will also give an antigenic analogue. Such a hypothesis of TCR-peptide interaction could be tested by introducing mutations in each of positively charged residues in CDR3 loops of F5 TCR α and β chains. Recognition of a residue at position 7 by F5 TCR is slightly different, and antigenic potencies come in the order of

$$N = \mathbf{D} > E = K$$

in which aspartate and asparagine (M.W. 132) are almost identical in size whereas glutamic acid and lysine are slightly bigger. Thus, increase by one carbon unit results in a complete loss of antigenicity, whereas charges of side chains are less important since neutral asparagine is still antigenic. These data suggest that aspartic acid at position 7 is in van der Waals contact with the F5 TCR without intercalating water molecules, and its carboxylate group is likely to form a hydrogen bond. Thus, substitutions to leucine (M.W. 131) or isoleucine (M.W. 131) are predicted to have little effect on the antigenicity.

Effect of these peptide analogues on activation of mature F5 T cells were also studied in collaboration with Dr. M. Bix (UCSF, USA). As shown in figure 40-b, agonistic activities of peptides were similar in the negative selection assay and the CTL activation assay for most peptides. An exception was P4K which caused selective deletion of DP F5 thymocytes but did not activate F5 CTL at the same concentration, suggesting that negative selection of immature thymocytes is triggered at a lower threshold than activation of mature CTL. A peptide of similar property has recently been reported by others (Spain et al., 1994).

Using combinations of suboptimal doses of cognate peptide and excess of non-antigenic peptides, two peptides (1934 NP and P7E) were shown to block F5 CTL activation. Similar assays were carried out by measuring negative selection of F5 thymocytes in fetal thymus organ culture. We showed that negative selection can

be competitively inhibited by antagonistic peptides. It remains to be studied whether such blocking of negative selection is caused by either MHC competition or TCR competition. However, relatively low doses of competitors required to antagonise the effect of cognate peptide suggests a mechanism at the level of interaction between TCR and peptide/MHC complexes as described before (De Magistris et al., 1992). Since a slightly higher concentration of cognate peptide required much larger excess of non-antigenic peptide to be competed, such an antagonism of negative selection appears to operate only under limited concentrations of agonistic peptide. The present data also suggest that potentially auto-reactive T cells could escape clonal deletion if provided with sufficient amount of competitors.

Recently, two groups reported reconstitution of positive selection of CD8 T cells in class I MHC-deficient mice using peptides. Hogquist et al. demonstrated that several antagonistic peptides (Hogquist et al., 1994) and an agonistic peptide (Hogquist et al., 1994) could restore differentiation of CD8 SP cells in β2m-deficient mice. Ashton-Rickardt et al. reported that an agonistic peptide causes both positive selection at low concentrations and negative selection at higher concentrations (Ashton-Rickardt et al., 1994). However, in the $\beta2m^{-/-}$ system it remains to be studied if low levels of endogenous class I MHC on $\beta2m^{-/-}$ cells (Zijlstra et al., 1990) had played a role, since the apparent increase in single positive cells could be due to proliferation of T cells already pre-selected on endogenous

peptides. In addition, both studies left a possibility that positive selection was mediated by endogenous TCR α chains (in complex with transgenic β) rather than by transgenic TCR. It is also important to see if those CD8 SP cells generated in vitro are functionally competent and not anergic, especially when agonistic peptides were used, in order to demonstrate that these in vitro models represent faithfully in vivo positive selection.

In summary, there is not definitive evidence that a single peptide can mediate positive selection of a given TCR. The present study, in contrast, may suggest that combination of peptides could mediate positive selection. Thus, in the case of F5 TCR, antagonistic peptides augment the generation of mature T cells only when sub-optimal dose of agonistic peptide is present. Antagonistic peptides alone have little, if any, effect on F5 T cell development. Indeed, previous studies on class I MHC-deficient fetal thymic organ cultures in the absence of transgenic TCR demonstrated that mixture of peptides are more efficient than single peptide for positive selection (Ashton-Rickardt et al., 1993; Hogquist et al., 1993). It was interpreted that each clone needs specific peptide for positive selection and therefore many different peptides are required for the whole repertoire. However, these data could also indicate an importance of having multiple peptide/MHC ligands for positive selection. If this is the case, one explanation is that different ligands are required at different stages of T cell selection. Alternatively, agonistic and antagonistic ligands are recognised simultaneously and generate different signals. In this context,

it is intriguing that antagonistic or partially agonistic peptides cause phosphorylation of CD3ζ chains without activating ZAP-70 (Madrenas et al., 1995). It suggests that antagonistic peptides alone will not be sufficient (or efficient) for positive selection since there is accumulating evidence that ZAP-70 (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1994) and its down-stream signals such as p21ras (Swan et al., 1995) and MEK (Alberola-Ila et al., 1995) are involved in positive selection. Taken together, our data are consistent with a model that positive selection requires at least two signals, one mediated by agonistic interaction (which involves ZAP-70), and the other caused by antagonistic stimuli. It is tempting to speculate signalling molecules involved in the latter pathway, such as PI-3 kinase and shc which are know to bind to the phosphorylated CD3 ζ chain. A set of peptide analogues used in the present study provide an unique model to study biochemical events associated with T cell development in the thymus.

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Developmental Biology

Direct derivation of conditionally immortal cell lines from an *H-2K*^{b}-tsA58 transgenic mouse

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ABSTRACT **Studies on cell lines have greatly improved our understanding of many important biological questions. Generation of cell lines is facilitated by the introduction of immortalizing oncogenes into cell types of interest. One gene known to immortalize many different cell types *in vitro* encodes the simian virus 40 (SV40) large tumor (T) antigen (TAg). To circumvent the need for gene insertion *in vitro* to generate cell lines, we created transgenic mice harboring the SV40 TAg gene. Since previous studies have shown that TAg expression in transgenic mice is associated with tumorigenesis and aberrant development, we utilized a thermolabile TAg [from a SV40 strain, tsA58, temperature sensitive (ts) for transformation] to reduce the levels of functional TAg present *in vivo*. To direct expression to a broad range of tissues, we used the mouse major histocompatibility complex *H-2K*^{b} promoter, which is both widely active and can be further induced by interferons. tsA58 TAg mRNA was expressed in tissues of all animals harboring the hybrid construct. Development of all tissues was macroscopically normal except for thymus, which consistently showed hyperplasia. Fibroblast and cytokeratin$^+$ thymic epithelial cultures from these mice were readily established without undergoing crisis and were conditionally immortal in their growth; the degree of conditionality was correlated with the levels of tsA58 TAg detected. One strain of *H-2K*^{b}-tsA58 mice has been bred through several generations to homozygosity and transmits a functional copy of the transgene.**

Although the use of cell lines has been of central importance in the development of cellular and molecular biology, the limited number of available cell lines and the difficulty in obtaining new ones have impeded many areas of study. The increasing realization of the value of cell lines has been associated with a continual evolution in relevant technologies. Initially, cell lines were obtained only as tumor cells or as spontaneously immortalized variants of cells that grew readily in tissue culture (1). More recently, transfection and retroviral-mediated gene insertion of immortalizing genes have been used to facilitate the production of cell lines from various tissues (2–11). However, transfection requires a large number of target cells to ensure that some cells of interest stably integrate the chosen DNA in a position suitable for expression. Viral-mediated gene transfer can be carried out with fewer cells by cocultivation of target cells with virus-producing feeder layers; however, this method still requires that target cells are dividing to achieve integration of the selected DNA into the genome (2). Moreover, both of these technologies require the growth of cells for extended periods of time in culture, under selective pressure, to obtain sufficient numbers of cells expressing the immortalizing gene to allow experimentation. In addition, lines from putatively identical cells have different sites of gene integration and often express markedly different behaviors and levels of expression of the immortalizing gene.

An additional problem associated with the introduction of immortalizing genes into cells is that these genes can alter normal cellular physiology (1, 12), a problem that is also relevant to the isolation of cell lines from transgenic animals (e.g., refs. 28–30, 34, 35). This problem theoretically can be overcome through the use of conditional immortalizing genes, which allow the generation of continuously proliferating cell lines capable of differentiation after inactivation of the immortalizing gene. For example, the simian virus 40 (SV40) mutant temperature-sensitive (ts) strain tsA58, which encodes a thermolabile large tumor (T) antigen (TAg) capable of immortalization only at the permissive temperatures, has been used in the generation of a variety of conditionally immortal cell lines (13–17). However, introduction of conditional immortalizing genes *in vitro* still suffers from the problems discussed above for transfection and infection of wild-type genes.

To overcome some of the difficulties in the generation of cell lines, an approach was developed that facilitates and ensures the presence of a conditional oncogene in all of the cells of interest at a common integration site. Thus, transgenic mice were generated that harbor SV40 strain tsA58 early region coding sequences under the control of the mouse major histocompatibility complex *H-2K*^{b} class I promoter (18–21). This promoter is active at various levels in different tissues of the body but can be induced to higher levels of expression in almost all cells by exposure of the cells to interferons (IFNs) (21–23). Skin fibroblast cultures derived from these mice were conditional in their growth, as has been demonstrated for rat embryo fibroblasts immortalized by infection with a recombinant retrovirus that transduces the tsA58 TAg (15). Work with transfection and viral-mediated gene insertion has consistently indicated that techniques developed through the use of fibroblast populations can be transferred readily to other cell systems. This is also the case with the cells obtained from these transgenic mice, and cytokeratin$^+$ thymic epithelial cell lines that were also established readily from these animals.

MATERIALS AND METHODS

Construction of the Transgene. The 5′ flanking promoter sequences and the transcriptional initiation site of the mouse *H-2K*^{b} class1 gene were fused to the SV40 tsA58 early region coding sequences. The 4.2-kilobase (kb) *Eco*RI–*Nru* I fragment encompassing the *H-2K*^{b} promoter sequences was ligated to the 2.7-kb *Bgl* I–*Bam*HI fragment derived from the tsA58 early region gene and pUC19 double-digested with

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Abbreviations: TAg, large tumor (T) antigen; ts, temperature sensitive; IFN, interferon; mAb, monoclonal antibody; SV40, simian virus 40.

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*Eco*RI and *Bam*HI. The Bgl I site was blunted by using the Klenow fragment of *Escherichia coli* DNA polymerase I to allow fusion to the *Nru* I site. For microinjection, the *H-2K*^{b}-tsA58 DNA fragment was isolated free of vector sequences by digestion with *Eco*RI and *Sal* I (24). All DNA manipulations were carried out by standard procedures (25).

RNA Blot-Hybridization (Northern) Analysis. RNA was prepared and analyzed by hybridization to a ^{32}P-labeled SV40 early region fragment using standard procedures (25, 26).

Cloning and Proliferation Assays. Skin fibroblasts were prepared as described (27) and grown in Dulbecco's modified Eagle's medium supplemented with 100 units of penicillin, streptomycin, and recombinant murine γ interferon (IFN-γ, Genzyme) per ml. For colony assays, 10^3 cells derived from cultures grown at 33°C in the presence of IFN-γ were replated in 6-cm tissue culture dishes in the absence of IFN-γ at 33°C to allow adherence under identical conditions. Growth conditions were changed after 24 hr to indicated conditions (Fig. 2). Cultures were refed twice weekly for 14 days and stained with 2% methylene blue; colonies then were counted blind. For proliferation assays, 10^4 cells were similarly plated, and dishes were analyzed after 7 and 14 days. A single dish was also counted on day 1 to determine the number of adhering cells. All determinations were carried out in duplicate.

Immunoblot (Western Blot) Analysis. Preparation of protein extracts and their analysis with mAb PAb419, directed against TAg, were performed by standard procedures (26).

Immunofluorescence Analysis of Thymic Epithelial Cells. Cells grown on poly(L-lysine)-coated coverslips were stained with an antibody specific for keratin 8 (LE41; ref. 32) or an anti-TAg mAb, PAb412 (31).

RESULTS

Generation of *H-2K*^{b}-tsA58 Transgenic Mice. A hybrid construct containing the *H-2K*^{b} 5′ promoter sequences fused to the tsA58 early region gene, which encodes both TAg and the small tumor antigen (Fig. 1 *Upper*), was microinjected into fertilized oocytes from (CBA/Ca × C57BL/10) F_1 mice. After reimplantation, 88 mice were born, of which 34 carried one to five copies of the gene. RNA from a variety of tissues from one nontransgenic and three transgenic animals was analyzed by Northern blot analysis with an SV40 early region-specific probe (Fig. 1 *Lower*). RNA extracted from tissues of transgenic mice contained various amounts of a 2.5-kb RNA species, while no tsA58 TAg RNA was detected in tissues of the nontransgenic mouse; thymus and liver showed the highest level of expression, while brain showed the lowest.

Fibroblasts Derived from *H-2K*^{b}-tsA58 Transgenic Mice Are Conditionally Immortal. Skin fibroblasts from normal and founder transgenic animals 2–10 weeks old were placed in culture at 33°C, the permissive temperature for the tsA58 TAg, in the presence of IFN-γ (to increase expression from the *H-2K*^{b} promoter; refs. 21–23). Fibroblasts derived from nontransgenic mice stopped dividing *in vitro* within a small number of passages. This cessation of division, which has been termed both "senescence" and "crisis," occurs reproducibly in fibroblasts that do not express immortalizing genes. In contrast, fibroblasts derived from most transgenic mice continued to grow for as long as the cultures were maintained under appropriate conditions (see below).

Detailed analysis of skin fibroblast cultures for conditionality of growth revealed three families of cultures, depending upon the ability of cells to grow in fully permissive, semipermissive, and nonpermissive conditions. Permissive conditions were defined as growth at 33°C in the presence of IFN-γ; semipermissive conditions, growth at 33°C in the absence of IFN-γ or 39.5°C in the presence of IFN-γ; and nonpermissive conditions, growth at 39.5°C in the absence of IFN-γ (Fig. 2).

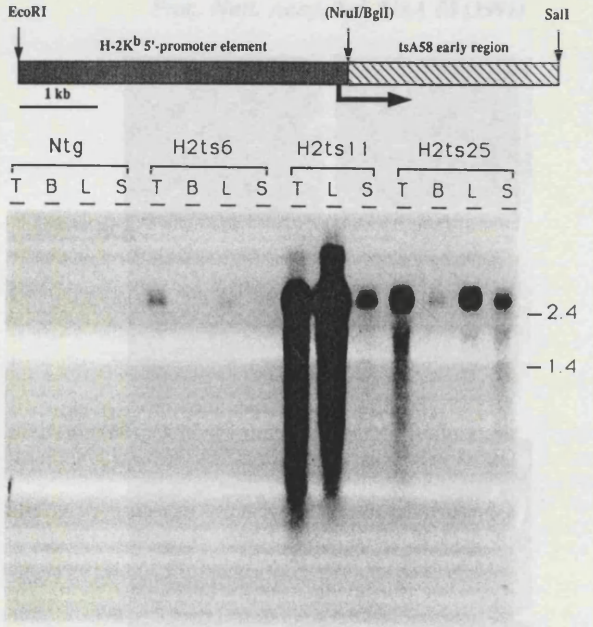

FIG. 1. (*Upper*) Schematic representation of the *H-2K*^{b}-tsA58 fragment. Size in kb is indicated. (*Lower*) Northern blot analysis shows TAg mRNA at various levels in thymus (lanes T), brain (lanes B), liver (lanes L), and skin (lanes S) of different *H-2K*^{b}-tsA58 transgenic mice. Loading of RNA was checked by hybridization of the same filter with an actin probe (not shown). Size in kb is indicated on the right. Ntg, nontransgenic.

In the first family of cultures, growth was fully conditional and only occurred under permissive conditions. If cells were grown at 39.5°C and/or were grown in the absence of IFN-γ, cell division did not occur either in standard growth assays or in colony-forming assays (Fig. 2). These fibroblasts thus behaved as expected from previous studies in which rat embryo fibroblasts were conditionally immortalized with tsA58 TAg by retroviral infection (15). All cultures derived from different individuals within this strain yielded identical results.

In a second family of cultures, optimal growth was obtained under fully permissive conditions, a lesser degree of growth was seen under semipermissive conditions, and no growth occurred under nonpermissive conditions. In the third family, cell growth did not completely cease even under nonpermissive conditions, although the best growth was seen under fully permissive conditions.

The conditionality of growth seen in fibroblasts derived from transgenic animals was correlated with the levels of tsA58 TAg (Fig. 2*e*). In all cultures, the level of tsA58 TAg was reduced by temperature increase and/or by removal of IFN-γ. Interestingly, when the most conditional cultures (those derived from progeny of mouse H2ts6) were grown at 33°C in the absence of IFN-γ, a condition where these cells did not grow, low levels of TAg were still detected (Fig. 2*e*).

Thymic Hyperplasia in *H-2K*^{b}-tsA58 Transgenic Mice. Enlarged thymuses occurred in all transgenic animals, a tissue-specific hyperplasia that previously has been observed in transgenic mice harboring wild-type TAg (33, 34); the time of onset of hyperplasia (2–20 weeks) was correlated with the levels of TAg mRNA (see Fig. 1 *Lower*). Despite the thymic enlargement, there was no evidence for malignant transformation of this tissue as judged by the following criteria: both lobes of the thymus were equally enlarged in all animals examined, and histological and immunohistochemical examination revealed

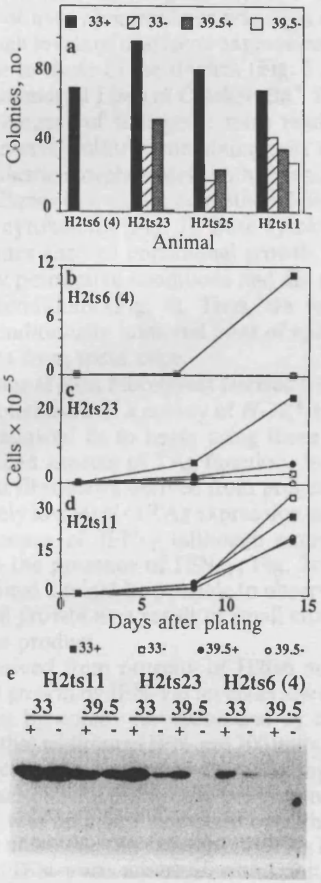

FIG. 2. (*a–d*) Cloning and proliferation analysis of skin fibroblasts from *H-2K*^{b}-tsA58 transgenic mice reveals three families of cells. In family 1 (mouse H2ts6-4), cloning and proliferation are fully conditional and only occur when cells are grown at 33°C in the presence of IFN-γ (33+) (*a* and *b*). In family 2 (mice H2ts23 and H2ts25), optimal results were obtained when cells were grown at 33°C in the presence of IFN-γ (33+), no growth occurred at 39.5°C in the absence of IFN-γ (39.5−), and intermediate levels of growth were seen in the semipermissive conditions of 33°C, IFN-γ$^-$ (33−) or 39.5°C, IFN-γ$^+$ (39.5+) (*a* and *c*). In family 3 (mouse H2ts11), growth occurred in all conditions but was most vigorous at 33°C in the presence of IFN-γ (*a* and *d*). A reduced cloning efficiency and rate of cell growth was seen in semipermissive conditions, and a still greater reduction was seen in fully nonpermissive conditions. (*e*) Western blot analysis of skin fibroblasts shows that the levels of TAg are correlated with the conditionality of *in vitro* growth. The most conditional cells (derived from progeny of H2ts6) contained the lowest levels of TAg, and the least conditional cells (derived from H2ts11) showed the highest levels of TAg. In all cases, the level of TAg present increased upon addition of IFN-γ to the cultures and decreased upon shift to 39.5°C.

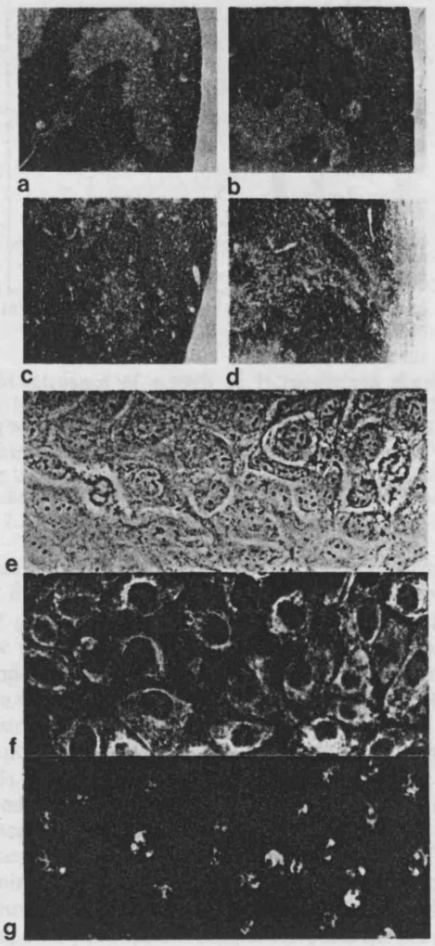

FIG. 3. (*a–d*) Histological analysis of thymic tissues from a nontransgenic mouse (*a*) and from H2ts6 mice 2 months (*b*), 4 months (*c*), and 6 months (*d*) old. The thymuses of H2ts6 mice up to 2 months old appeared to be identical to those of normal mice (*a* and *b*), exhibiting normal ratios between cortex (darkly stained tissue) and medulla (lightly stained tissue). The thymic architecture in 4-month-old mice (*c*) showed signs of disruption, while areas with cortical or medullary characteristics were still maintained. In thymic tissue of 6-month-old H2ts6 mice, extensive lightly staining areas were evident even in subcapsulary regions. However, even in these organs a clear demarcation between "cortical" and "medullary" areas was still maintained. (*e–g*) Immunofluorescent staining of thymic epithelial cells. Cells from an adherent cell line (7P) derived from the thymus of H2ts23 were photographed under: phase optics, showing flattened cells with tightly apposed borders (*e*); optics with fluorescein isothiocyanate, showing filamentous cytoplasmic staining characteristic of keratins (*f*); and optics with rhodamine isothiocyanate, indicating the presence and nuclear localization of TAg in virtually all cells (*g*). (×200.)

extensive growth of epithelial cells and the presence of apparently normal thymocyte populations, as determined by fluorometric cytometry (not shown). In addition, demarcation between cortical and medullary regions was still maintained even after prolonged hyperplastic growth (Fig. 3). Moreover, dissociated cells obtained from enlarged thymuses did not yield tumors in syngeneic recipients even when 10^7 cells were injected s.c. or i.p., and recipient animals were sacrificed after 3 months (unpublished observations). Finally, analysis of T-cell receptor β chain gene rearrangements by Southern blot of DNA from enlarged thymuses suggested polyclonal expansion of thymocyte populations (Y.T., unpublished observations), in contrast to the oligoclonal expansion observed in mice that harbor a hybrid *Thy-1-myc* gene (35). As it is possible that the large number of highly proliferative and hyperplastic cells in the thymus represents a target in which secondary cooperating mutations might occur, we cannot exclude the possibility that a very small number of cells within the hyperplastic thymus have undergone transformation.

In heterozygous progeny of one mouse (H2ts6), the thymus displayed normal development for extended periods, with the first histological appearance of hyperplasia seen at 4 months (Fig. 3). Homozygote offspring of H2ts6 developed thymic hyperplasia earlier (unpublished observations), in agreement with the view that the time of onset of this abnormality is correlated with TAg levels. Thymic hyperplasia was occasionally seen in conjunction with enlargement of peripheral lymphoid organs (spleen, lymph nodes), but these tissues

maintained their normal histological architecture. Macroscopic evidence of liver abnormalities was seen only in one animal even though levels of transgene expression in the liver were comparable to those in the thymus (Fig. 1 *Lower*).

Conditionally Immortal Lines of Cytokeratin$^+$ Thymic Epithelial Cells. Thymuses of transgenic mice readily yielded conditionally immortal cultures containing cells of both epithelial and fibroblastic morphologies, both of which could be readily cloned. Clones that exhibited epithelial-like morphologies expressed cytokeratin (Fig. 3). Both cytokeratin$^+$ and cytokeratin$^-$ clones showed conditional growth. Cells grew optimally in fully permissive conditions and did not grow in nonpermissive conditions (Fig. 4). Thus, we were able to readily derive conditionally immortal lines of epithelial cells and of fibroblasts from these mice.

Dose Dependence of Skin Fibroblasts Derived from H2ts6 to IFN-γ. The establishment of a colony of *H-2K^b*-tsA58 transgenic mice has allowed us to begin using these animals to study more detailed aspects of TAg function. In particular, observations that fibroblasts derived from progeny of H2ts6 showed a relatively low level of TAg expression at 33°C in the presence or absence of IFN-γ (although expression was clearly higher in the presence of IFN-γ; Fig. 2*e*) suggested that with this animal it might be possible to observe dramatic alterations in cell growth as a result of small changes in the level of this gene product.

Fibroblasts derived from progeny of H2ts6 mice showed promotion of cell growth by IFN-γ at levels as low as 1 unit/ml (Fig. 5). Analysis by colony formation and by cell number analysis showed that addition of IFN-γ at 100 units/ml to these cultures only increased the frequency of colony formation 3.5-fold in comparison with that seen in the presence of IFN-γ at 1 unit/ml and was only 40% increased over that achieved with IFN-γ at 10 units/ml. The difference in TAg levels at the different doses of IFN-γ was not large, with 1 unit/ml causing a 2.5-fold increase over basal levels of TAg and 100 units/ml causing an ≈6-fold increase over basal levels of TAg.

DISCUSSION

We have generated transgenic mice that have stably integrated the SV40 mutant strain tsA58 thermolabile TAg gene,

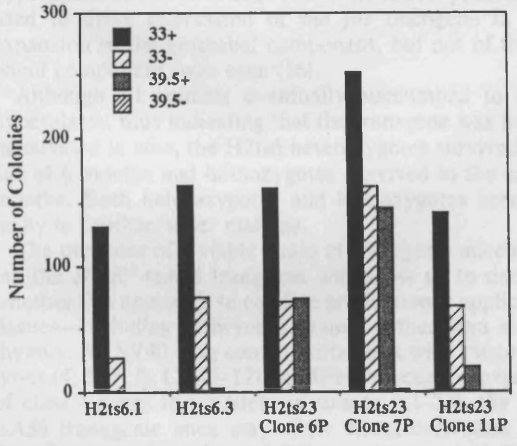

FIG. 4. Analysis of growth of thymic adherent cells by colony formation demonstrates that these cells exhibited conditional growth *in vitro*. Optimal growth occurred under the fully permissive condition of 33°C, IFN-γ^+ (33+) in all cases, and no colony formation occurred in nonpermissive conditions. Cultures of cells from H2ts6.1 and H2ts6.3 were derived from the thymuses of two separate progeny of founder mouse 6, and cultures of H2ts23 clones 6P, 7P, and 11P are three separate clonal cultures derived from the thymus cells of animal H2ts23; the clones 6P and 7P were morphologically epithelial, whereas clone 11P was fibroblastic. 33+, 33°C and IFN$^+$; 33−, 33°C and IFN-γ^-; 39.5+, 39.5°C and IFN-γ^+; 39.5−, 39.5°C and IFN-γ^-.

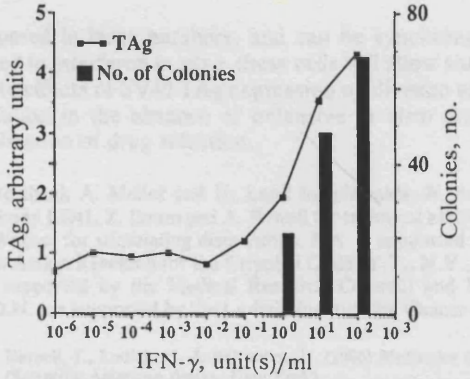

FIG. 5. Maintenance of growth of H2ts6-derived fibroblasts requires low levels of IFN-γ. No colonies were obtained in the absence of IFN-γ, but the presence of as little as 1 unit of IFN-γ per ml was sufficient to allow colony formation. Determination of the level of TAg by Western blot analysis coupled with densitometry showed that the increase in colony formation was associated with no more than a 2.5-fold increase in the level of TAg.

a conditional immortalizing gene, under the control of the inducible 5′ flanking promoter of the mouse *H-2K^b* gene. The tsA58 TAg gene product is functional at the permissive temperature of 33°C but is rapidly degraded at the nonpermissive temperature of 39.5°C (13, 15). The *H-2K^b* promoter is active in a wide variety of tissues at various levels (18–21), and expression can be increased above basal levels in most cells by exposure to IFN (21–23). Fibroblasts and thymic stromal cells derived from the *H-2K^b*-tsA58 transgenic mice showed conditional proliferation that could be modulated both with temperature and by application of IFN-γ; cells from all mice grew optimally at 33°C in the presence of IFN-γ. Founder animal H2ts6, whose progeny yielded fibroblast cultures whose growth in cloning assays was completely dependent upon the permissive temperature and the presence of IFN-γ, has bred successfully to homozygosity to yield a strain of *H-2K^b*-tsA58 transgenic mice.

The assay system used to examine conditionality of immortalization was based on results of previous studies in which tsA58 TAg was introduced into fibroblasts by retroviral infection (15). These conditionally immortalized fibroblasts grew indefinitely when maintained at 33°C but rapidly ceased proliferation when switched to 39.5°C. Cells derived from *H-2K^b*-tsA58 transgenic mice behaved similarly. Skin cells from these mice grown *in vitro* at 33°C in the presence of IFN-γ readily yielded fibroblast cultures from all transgenic animals. Shift to semipermissive conditions of growth (i.e., 33°C/IFN-γ^- or 39.5°C/IFN-γ^+) was sufficient to eliminate growth of cells derived from the H2ts6 strain of mice. In all other cases, shift to semipermissive conditions was associated with a reduction in cell growth but not a cessation of growth. Cultures from almost all animals ceased growth when shifted to nonpermissive conditions—i.e., 39.5°C, IFN-γ^-. Moreover, in the cultures (family 3 in Fig. 3) in which growth occurred after temperature increase to 39.5°C in the absence of IFN-γ, this growth was still less vigorous than that seen in semipermissive conditions. It should be noted that all cultures established from the same founder mouse, or strain of mice, exhibited identical characteristics.

Determination of the amount of TAg present in different cultures by Western blot analysis showed a direct correlation between the amount of TAg present and the growth potential of the cells. Cells in which only small amounts of TAg were produced showed stringent growth regulation, while cultures expressing high levels of TAg showed poor growth regulation. It was also clear that only small increases of TAg were needed to maintain immortalization, in that we saw only a

2.5-fold difference in levels of TAg between untreated cultures and those grown in the presence of IFN-γ at 1 unit/ml, yet only the cultures receiving the IFN-γ were able to generate colonies in a limiting dilution assay.

Conditional immortalization and the ability to readily generate rapidly growing cultures were also seen with cells derived from thymuses of transgenic mice. As with skin fibroblasts, optimal growth of the thymic cultures occurred at 33°C in the presence of IFN-γ, was reduced in semipermissive conditions, and was reduced still further in nonpermissive conditions. Interestingly, thymic cells derived from H2ts6 animals did not grow at 39.5°C in the presence of IFN-γ but did grow at 33°C in the absence of IFN-γ. This pattern of growth may reflect a higher constitutive level of transcription from the *H-2K*^{b} promoter in the thymic cells and/or a greater sensitivity of thymic cells to the action of TAg as compared with fibroblasts. The probable relevance of the first explanation is supported by observations that *in vivo* expression of the transgene in the thymus was generally higher than in other organs, while the relevance of the second explanation is supported by observations that the liver—the one organ in which transgene expression was similar to that of the thymus—rarely showed abnormal growth. The different effects of the transgene on thymus and liver *in vivo* suggest that cell types can differ in their susceptibility to the action of TAg.

Long-term survival of the transgenic mice was correlated with the level of conditionality of growth of the *in vitro* cultures. The only visible cause of physical distress found repeatedly was thymic enlargement. This enlargement seemed to represent hyperplastic growth rather than malignancy because thymic histology, T-cell repertoire, and T-cell clonality were all normal, and cells derived from enlarged thymuses did not generate tumors in syngeneic recipients. Although all populations of the thymus were expanded *in vivo*, only adherent cell cultures were readily obtained in long-term culture, in contrast to cells derived from *Thy-1-myc* mice (35). The generalized hyperplasia of thymic populations we have observed is similar to that seen in transgenic mice when wild-type TAg gene expression was regulated by its own early region promoter (35) or by the promoter from growth hormone-releasing factor gene (34). It differs from the hyperplasia observed in mice where the *H-2K*^{b} promoter was used to drive expression of the *fos* oncogene in which expansion of the epithelial component, but not of the lymphoid component, was seen (36).

Although all animals eventually succumbed to thymic hyperplasia, thus indicating that the transgene was not fully inactivated *in vivo*, the H2ts6 heterozygotes survived to the age of 6 months and homozygotes survived to the age of 3 months. Both heterozygotes and homozygotes breed normally in brother/sister matings.

The presence of a viable strain of transgenic mice harboring the *H-2K*^{b}-tsA58 transgene will allow us to determine whether this approach to cell line production is applicable to tissues—including embryonic tissues—other than skin and thymus. As SV40 TAg can immortalize a wide range of cell types (4, 5, 7, 9, 11, 15–17) and IFN induces the expression of class I genes in a variety of tissues (21–23), the *H-2k*^{b}-tsA58 transgenic mice may allow direct derivation of cell lines from a wide variety of different tissues and cell types. Moreover, the ability to remove the immortalizing function of the tsA58 TAg in cells derived from these transgenic mice by temperature shift up may allow us to generate cell lines that are not only conditional in their growth but also may be capable of differentiating into different types of end-stage cells (see, e.g., refs. 4 and 16). Finally, as cells prepared from these transgenic mice are genetically homogeneous, can be prepared in large numbers, and can be synchronously exposed to interferon *in vitro*, these cells will allow study of the acute effects of SV40 TAg expression on division and differentiation in the absence of extensive *in vitro* growth and application of drug selection.

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In vitro negative selection of αβ T cell receptor transgenic thymocytes by conditionally immortalized thymic cortical epithelial cell lines and dendritic cells*

We have established conditionally immortalized thymic cortical epithelial cell lines from transgenic mice carrying a temperature-sensitive SV40 large T antigen. One of these cell lines expresses cortical markers and produces IL-1α, IL-6, IL-7, and TGF-β1. These cells express class I major histocompatibility complex (MHC) constitutively and class II MHC upon induction with IFN-γ. The cells appear to have a normal class I antigen presenting pathway since messages for both peptide transporter genes (TAP1, TAP2) were detected.
The ability of these cortical epithelial cells to present peptide antigen was compared to that of thymic dendritic cells. In suspension culture with αβ T cell receptor (TcR) transgenic thymocytes, these epithelial cells and dendritic cells (pre-pulsed with peptide cognate for the transgenic TcR) caused down-regulation of CD4, CD8, and TcR in an antigen dose-dependent and MHC-restricted manner. $CD4^{dull}CD8^{dull}$ cells were taken as evidence for negative selection because these cells contained apoptotic DNA. Concentration of peptide required for negative selection of thymocytes was similar between dendritic cells and cortical epithelial cells. In contrast, αβTcR transgenic spleen cells were activated only by dendritic cells but not by cortical epithelial cells.

1 Introduction

Antigen recognition of T cells is mediated by the T cell receptor (TcR) which recognizes peptides in association with membrane glycoprotein products of the major histocompatibility complex (MHC). The specificity of the TcR not only determines reactivity of mature T cells to foreign antigens, but also influences the developmental fate of immature T cells. Thus, in contrast to the apparent randomness of TcR gene rearrangement [1], the peripheral T cell repertoire is comprised of those T cells which preferentially recognize self-MHC and do not react to self-peptides [2–4].

It has been shown that self-reactive thymocytes are negatively selected by various kinds of cells in the thymus including hematopoietic cells, such as dendritic cells, macrophages, B cells, thymocytes [5–9] and epithelial cells [10, 11]. On the other hand, thymic cortical epithelial cells are thought to confer positive selection [12–14]. Recent studies also show that bone marrow-derived cells can participate in positive selection to a certain degree [15]. It remains controversial, however, whether it is quantitative or qualitative differences in TcR-mediated signals, additional factors such as adhesion molecules and cytokines, or the developmental stage of thymocytes, that determine the consequences of engagement of TcR with MHC/peptide complexes.

In order to address such questions, we developed an *in vitro* assay system using thymic cortical epithelial cells and αβ TcR transgenic thymocytes. Conditionally immortalized cortical epithelial cell lines were established from transgenic mice carrying a temperature-sensitive SV40 large T antigen (H2ts mice, [16]). We show that the characteristics of these cortical epithelial cell lines (expression of cortical markers, MHC molecules, and cytokines) are very similar to that of freshly isolated cortical epithelial cells [17]. The function of these cells was studied in suspension co-cultures with αβ TcR (F5) transgenic thymocytes [18] in the presence or absence of the cognate antigenic peptide. Here we report that such thymic cortical epithelial cells can induce negative selection of thymocytes at very low concentrations of the antigen, as efficiently as thymic dendritic cells. They are, however, unable to stimulate mature T cells.

2 Materials and methods

2.1 Mice

Inbred CBA/Ca and C57BL/10 mice were maintained in colonies at the institute.

2.2 Cell culture

Thymic tissues obtained from H2ts mice were gently teased apart and cultured in tissue culture flasks (Falcon) in complete medium, *i.e.* RPMI 1640 (Gibco) supplemented with 10 mM Hepes, 1 mM sodium pyruvate, 0.06 mg/ml penicillin, 0.1 mg/ml streptomycin, 1% monothioglycerol,

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Abbreviations: **TGF:** Transforming growth factor

Key words: Cortical epithelium / Immortalization / TcR transgenic mice / Negative selection

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and 10% fetal calf serum (Globe Farm), in the presence of 10–100 IU/ml IFN-γ (Genzyme). Cell clones were obtained from individual colonies plated at low cell density. Cell lines were maintained in the complete medium and were subcultured by treating with solution containing 0.05% trypsin (Sigma) and 0.016% EDTA. Growth characteristics were studied by plating cells at 10^4 per 60-mm dish at either 33 °C or 37–39 °C with or without 10 IU/ml IFN-γ. Total cell numbers were counted on days 3, 7, and 14.

Dendritic cells were prepared from thymuses of adult C57BL/10 mice. Thymus lobes were incubated with a cocktail of 1.6 mg/ml collagenase (Worthington CLS4) and 0.1% DNase (Sigma, Fraction IX) for 60 min at 37 °C. Cell suspensions were washed twice and then centrifuged on Percoll gradient with densities of ϱ = 1.078, 1.062, and 1.05 kg/l for 20 min at 1200 × g. The low density fraction (1.062) was placed on 60-mm petris dishes and cultured for 2 h at 37 °C. Non-adherent cells were washed off and adherent cells were cultured overnight at 37 °C. After this incubation, collected floating cells and loosely bound cells were enriched for dendritic cells. To minimize contaminating thymocytes, the pooled cells were washed with PBS and treated with 5 mM EDTA for 10 min at room temperature and centrifuged on the same Percoll gradient as above. The low density fraction was used as enriched dendritic cells.

For co-culture experiments, epithelial cells were cultured for 3 days at 37 °C with or without 100 IU/ml IFN-γ. Trypsinized epithelial cells and freshly isolated dendritic cells were cultured with different concentrations of oligopeptide for 2 h at 37 °C. Cells were then washed thoroughly to remove free peptide. Thymocytes were prepared from adult F5 transgenic mice and were passed through G-10 column to minimize contamination with residual stromal cells as described [19]. Thymocytes were mixed with epithelial cells or dendritic cells at 10 : 1 ratio in 96-well plates, and were cultured for 12 h at 37 °C. Cells were stained for CD4, CD8, and Vβ11, and were analyzed by flowcytometry.

Spleen cells were prepared from adult F5 transgenic mice by gently teasing apart the tissue. These cells were co-cultured with epithelial cell lines and dendritic cells as described above for 4 days at 37 °C. IFN-γ secreted in culture supernatant was measured by enzyme linked immunosorbent assay (ELISA) as described below.

2.3 Immunohistochemistry

Immunohistochemical analysis of epithelial cells, grown on glass cover slips for 3 days at 37 °C without IFN-γ, was carried out as described [20]. We used antibodies against cytokeratin 18 (LE61) [21], cytokeratin 8 (Amersham), cytokeratin 1–19 (Lu5, Boehringer Mannheim), cytokeratin 1, 5–11, and 18 (K8.13, Sigma), class I MHC (M1/42.3.9.8.HLK), and class II MHC (FITC-conjugated NIM-R4 and FITC-conjugated OX-6), Mac1 (M1/70.15.11.5.HL), Mac2 (M3/38.1.2.8.HL.2), thymic stromal cell markers 4F1 and IVC4 [22] as well as ER-TR4 and ER-TR5 [23]. Second layer antibodies were FITC-conjugated goat anti-mouse IgG2A and FITC-conjugated anti-mouse IgG1 (Southern Biotechnology Associates Inc.), and FITC-conjugated goat anti-mouse IgG (DAKO Ltd.).

2.4 mRNA-PCR analysis

Total RNA was extracted from cortical epithelial cells and WEHI-3 cells (grown at 37 °C for 3 days) by guanidium isothiocyanate method [24] and was reverse-transcribed with Mo-MuLV reverse transcriptase (Cetus, Gibco BRL) according to procedures described [25]. Mock samples contained no RNA. Products were then polymerase chain reaction (PCR)-amplified by using sets of primers for cytokines, TAP1, TAP2, and β-actin on a thermal cycler (Hybaid) according to a method described [25]. The following sense and anti-sense primer sets were synthesized at NIMR:

IL-1α;	GGCTCACTTCATGAGACTTGC,
	GCTGATACTGTCACCCGGC,
IL-1β;	CGGACCCCAAAACATGAAGGGC,
	GCCACAATGAGTGATACTGCC,
IL-2;	GCACCCACTTCAAGCTCC,
	CCTGGGGAGTTTCAGGTTCC,
IL-3;	GGGAAGCTCCCAGAACC,
	CGCAGATGTAGGCAGGC,
IL-4;	CGGCACAGAGCTATTGATGGG,
	CGCCCCAGCAGTATCACCTGGG,
IL-5;	GGAGAAATCTTTCAGGGGC,
	GCCTCAGCCTTCCATTGCCC,
IL-6;	CCACTTCACAAGTCGGAGGC,
	CCAGGTAGCTATGGTACTCC,
IL-7;	GGGCAGTATATAAACAGG,
	GCAGGAGGCATCCAGG,
IL-10;	CCCAGTCGGCCAGAGCC,
	CCTGCATTAAGGAGTCGG,
IFN-γ;	CCCACAGGTCCAGCGCC,
	CCCCACCCCGAATCAGCAGCG,
TNF-α;	GGCAGGTCTACTTGGAGTCATTGC,
	ACATTCGAGGCTCCAGTGAATTCGG,
GM-CSF;	GCCCTGAACCTCCTGG,
	GCCCCGTAGACCCTGCTCG,
TGF-β1;	GCTTCTGCTCCCACTCCCGT,
	GGCTTGCGACCCACGTAGTA,
TAP1;	GCCTCTGGGCGCCCAGCGGC,
	GCGGCCCGTGAAGAAGGG,
TAP2;	GGTTGCTACAAGGATCTCTG,
	TCAGTGTTCTGTTCTCCTGG,
β-actin:	CATCACTATTGGCAACGAGC,
	ACGCAGCTCAGTAACAGTCC.

2.5 Flow cytometric analysis

Cells were stained with antibodies against CD4 (RED^{123}-CD4-conjugated, Gibco BRL), CD8 (FITC-conjugated Lyt2, Becton Dickinson), Vβ11 (biotinylated KT11, Dr. K. Tomonari, London). Phycoerythrin-conjugated Streptavidin (Biogenesis) was used as second layer. Stained cells were analyzed by FACScan and Lysis I or II software (Becton Dickinson).

2.6 DNA fragmentation assay

F5 thymocytes co-cultured with Tep1.1 cells in the presence or absence of peptide were stained for CD4/CD8 and sorted into double-positive dull and bright cells by flowcytometry.

Genomic DNA was extracted from 6.4×10^5 cells of both populations as described [26]. DNA was electrophoresed in a 2% agarose gel and was visualized by ethidium bromide staining.

2.7 ELISA for IFN-γ

Ninety-six well plates were coated with an antibody against IFN-γ (AN18), 10 μg/ml, in 0.2 M borate buffer (pH 8.5) at 4 °C for overnight. After washing with PBS with 0.05% Tween 20, wells were treated with PBS containing 5% fetal calf serum and 5% horse serum for 1 h at room temperature (RT). Wells were washed and 50 μl samples were added. After a 2-h incubation at RT and washing, biotinylated second antibody against IFN-γ (R4-6A2), 5 μg/ml, was added. After 1-h incubation at RT, wells were washed and streptavidin-horseradish peroxidase (Southern Biotechnology), 1 : 500 dilution, was added. After 1 h incubation at RT, wells were washed and substrate 2, 2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma), 27.4 mg/ml, with 0.025% H_2O_2 in 10 mM phosphate buffer (pH 6.2) was added. Absorbance at 414 nm was measured by Titertek Multiskan MCC/340 after developing for 5–30 min. Unit concentrations of IFN-γ were calculated from standard curves.

3 Results

3.1 Phenotypic analysis of cell lines established from H2ts mice

Thymic stromal cell lines were established from CBA/Ca (H-2^k) or C57BL/10 (H-2^b) H2ts transgenic mice and were maintained in culture in the presence of 10 IU/ml of IFN-γ. H2ts1.9 (CBA/Ca), H2ts3 (C57BL/10) and H2ts3F (C57BL/10) transgenic mice gave rise to several cortical epithelial cell lines whose phenotypes are summarized in Table 1. Cell lines which exhibited the cobblestone appearance, typical of epithelial type cells, were selected and further characterized by immunohistochemistry using monoclonal antibodies. As shown in Fig. 1, cells stained strongly with antibodies specific for cortical epithelial markers (4F1, ER-TR4) and cytokeratins but not with antibodies specific for medullary epithelial cells (IVC4; data not shown, ER-TR5) or with macrophage specific antibodies. All cell lines expressed class I MHC molecules constitutively. Class II MHC expression was low in the absence of IFN-γ but was induced by exposure to IFN-γ (data not shown).

Table 1. Characteristics of thymic epithelial cell lines

Founder mice Cell lines	CBA.H2ts1.9 Tep4	B10.H2ts3F Tep1.1	B10.H2ts3F Tep2
Cell growth at			
33 °C IFN$^-$	+	–	–
33 °C IFN$^+$	++	++	++
37 °C IFN$^-$	–	–	–
37 °C IFN$^+$	++	+	+
Markers			
Cytokeratin	+	+	+
ER-TR4[a]	ND	+	+
ER-TR5[b]	ND	–	–
4F1[a]	+	+	+
IVC4[b]	–	–	–
Class I MHC	+	+	+
Class II MHC[c]	+	+	+

a) Thymic cortical epithelial marker.
b) Thymic medullary epithelial marker.
c) Induced by IFN-γ.

3.2 Growth characteristics of established cell lines

Growth dependence on temperature and IFN-γ was studied using one line isolated from a H2ts1.9 transgenic mouse (Tep4) and two lines isolated from a H2ts3F transgenic

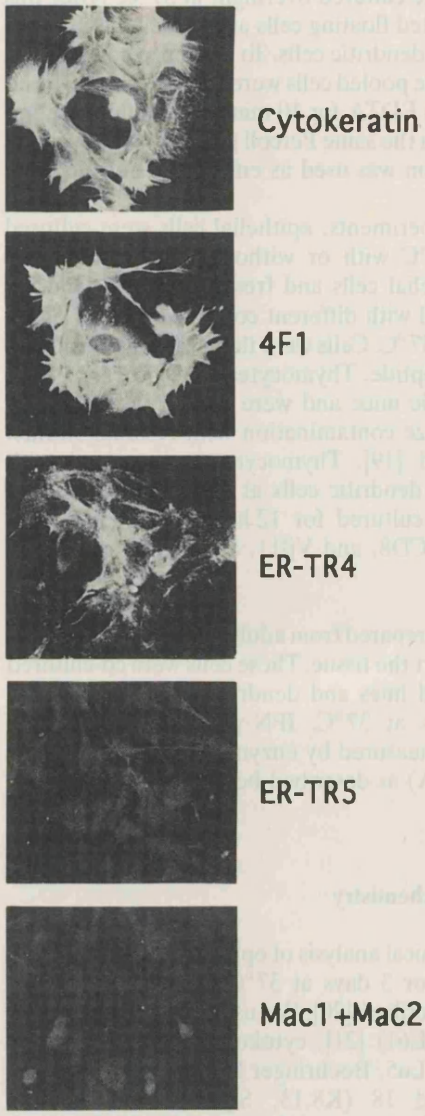

Figure 1. Immunohistochemical analysis of Tep1.1 cell line. Tep1.1 cells were grown on glass cover slips for 3 days at 37 °C in the absence of IFN-γ (non-permissive conditions), and were stained with the following antibodies: (a) LE61 (cytokeratin 18), (b) 4F1 (cortical epithelial marker), (c) ER-TR4 (cortical epithelial marker), (d) ER-TR5 (medullary epithelial marker), and (e) Mac1 and Mac2 (macrophage markers).

mouse (Tep1.1 and Tep2). As shown in Fig. 2 A, Tep4 cells stop growing 3 days after withdrawal of IFN-γ at a non-permissive temperature but continue to grow when either the temperature was 33 °C or IFN-γ was included in the medium, confirming the semi-conditional nature of the cell line. When growth was arrested the cells became large in size, a phenomenon usually associated with aging of cells in culture. When such arrested cells were returned to permissive culture conditions (in the presence of IFN-γ at 33 °C or 37 °C), they resumed their growing ability possibly due to re-induction and/or stabilization of the large T antigen (Fig. 2 B). Cell lines Tep1.1 and Tep2 showed more strict conditionality in their growth characteristics. Thus, they grew vigorously at 33 °C in the presence of IFN-γ but failed to grow in the absence of IFN-γ at either 37 °C or 33 °C. Their growth rate was reduced at 33 °C without IFN-γ (Fig. 2 C).

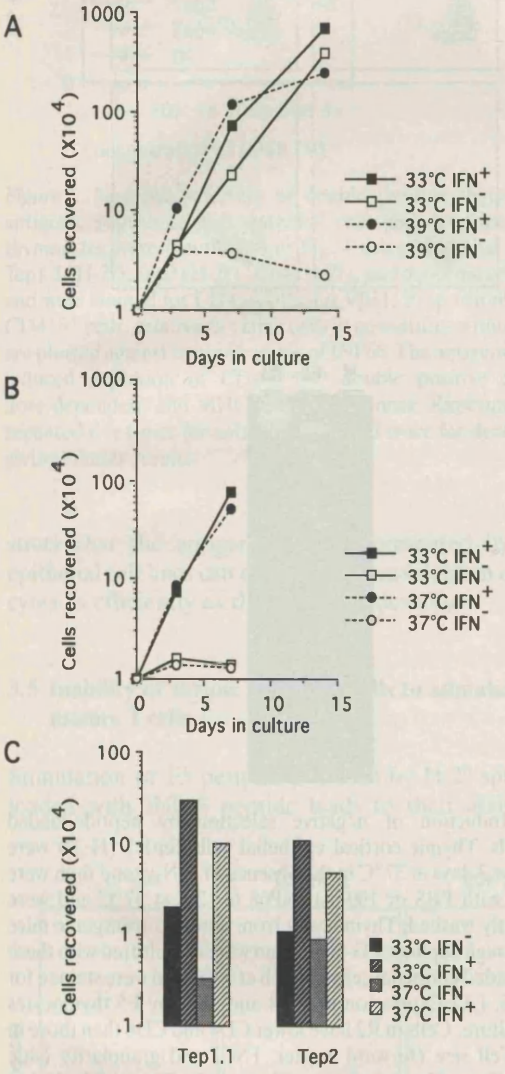

Figure 2. Growth profiles of thymic epithelial cell lines. (A) Tep4 cell line (H-2^k), which had been subcultured at 33 °C with 10 IU/ml IFN-γ, was plated at 1 × 10^4/60-mm dish and cultured under different conditions, *i.e.* at 33 or 39 °C, with or without 10 IU/ml IFN-γ. Cell numbers recovered per dish after 3, 7, and 14 days are shown. Tep4 cells stopped growing after 3 days at 39 °C in the absence of IFN-γ. (B) Tep4 cell line (H-2^k) was plated at 1 × 10^4/60-mm dish and cultured for 3 days at 39 °C in the absence of IFN-γ, by which time they stopped growing. Cells were then trypsinized and replated at 1 × 10^4/60-mm dish and were further cultured at either 33 or 37 °C with or without 10 IU/ml IFN-γ. (C) Tep1.1 and Tep2 cell lines (H-2^b) were plated at 1 × 10^4/60-mm dish and were cultured at various conditions. After 3 and 7 days, the number of cells per dish were counted.

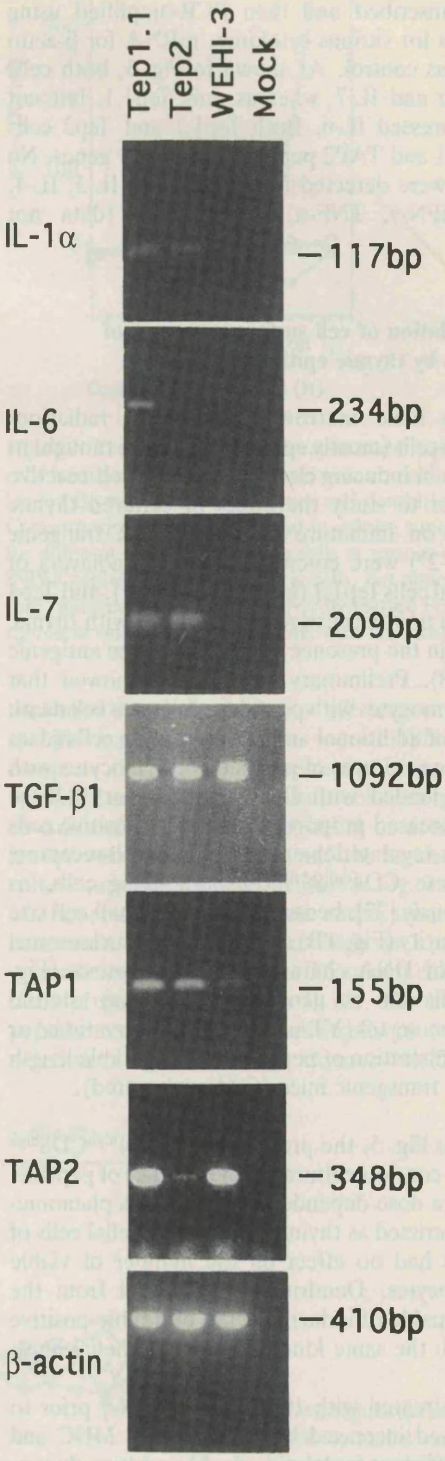

Figure 3. mRNA-PCR analysis of cytokine production by Tep1.1 and Tep2 cell lines. Total RNA (1 μg) from Tep1.1, Tep2, and WEHI-3 cell lines or water for mock control was reverse-transcribed, and was PCR-amplified as described in Sect. 2.4. Products using primers for IL-1α, IL-6, IL-7, TGF-β1, TAP1, TAP2, and β-actin were run in 1.75% agarose gel and were visualised by staining with ethidium bromide. The length of each product matched with that predicted from the sequence database (EMBL).

3.3 Expression of cytokines and peptide transporter genes by cortical epithelial cell lines

Production of cytokines by Tep1.1 and Tep2 cells was assessed by cDNA-PCR analysis. Total RNA from these cells, precultured for 4 days at 37 °C in the absence of IFN-γ, was reverse-transcribed and then PCR-amplified using pairs of primers for various cytokines. mRNA for β-actin was measured as control. As shown in Fig. 3, both cells produced IL-1α and IL-7, whereas only Tep1.1, but not Tep2, cells expressed IL-6. Both Tep1.1 and Tep2 cells expressed TAP1 and TAP2 peptide transporter genes. No PCR products were detected for IL-1β, IL-2, IL-3, IL-4, IL-5, IL-10, IFN-γ, TNF-α, or GM-CSF (data not shown).

3.4 Down-regulation of cell surface receptors of thymocytes by thymic epithelial cells

In contrast to bone marrow-derived cells, radiation-resistant thymic cells (mostly epithelial cells) are thought to be less effective in inducing clonal deletion of self reactive T cells. In order to study the effect of cultured thymic epithelial cells on immature T cells, F5 TcR trangenic thymocytes (H-2^b) were co-cultured with monolayers of thymic epithelial cells Tep1.1 (H-2^b), Tep2 (H-2^b), and Tep4 (H-2^k) grown in non-permissive conditions or with thymic dendritic cells, in the presence or absence of the antigenic peptide (INP68). Preliminary experiments showed that culturing F5 thymocytes with peptide accelerates cell death in the absence of additional antigen presenting cells (data not shown). In co-cultures of purified F5 thymocytes with epithelial cells loaded with INP68 peptide for 12 h we observed an increased proportion of double-positive cells which had down-regulated their CD4 and CD8 co-receptors (Fig. 4A). These $CD4^{dull}8^{dull}$ cells are dying cells, as reported previously [27], because they have small cell size and high granularity (Fig. 4B) and show inter-nucleosomal fragmentation of DNA characteristic of apoptosis (Fig. 4C). These cells can be generated by *in vitro* steroid treatment of thymocytes (Y.T. unpublished observations) or by *in vivo* administration of peptide for comparable length of time into F5 transgenic mice (C.M., submitted).

As illustrated in Fig. 5, the proportion of $CD4^{high}CD8^{high}$ cells, relative to controls cultured in the absence of peptide, was reduced in a dose-dependent manner. This phenomenon is MHC-restricted as thymic cortical epithelial cells of H-2^k haplotype had no effect on the number of viable $CD4^+8^+$ thymocytes. Dendritic cells derived from the thymus were capable of inducing death of double-positive thymocytes with the same kinetics as the epithelial lines.

Epithelial cells treated with 100 IU/ml of IFN-γ prior to co-culture showed increased levels of surface MHC and were found as efficient in deleting double-positive thymocytes as control cultures without IFN-γ and, therefore, lower levels of MHC (data not shown).

Using three-color fluorometric analysis, we assessed the levels of transgenic TcR on the different thymocyte subpopulations after co-culture with the different epithelial or dendritic cells. Fig. 6 shows that Vβ11 was down-regulated in double-positive cells and $CD8^+$ single-positive cells in co-cultures with H-2^b thymic and dendritic cells, but not with H-2^k thymic epithelial cells. Vβ11 on $CD4^+$ single-positive cells remained unaffected. Similar results were obtained using cell lines from another independent H2ts transgenic mouse (data not shown). These data demon-

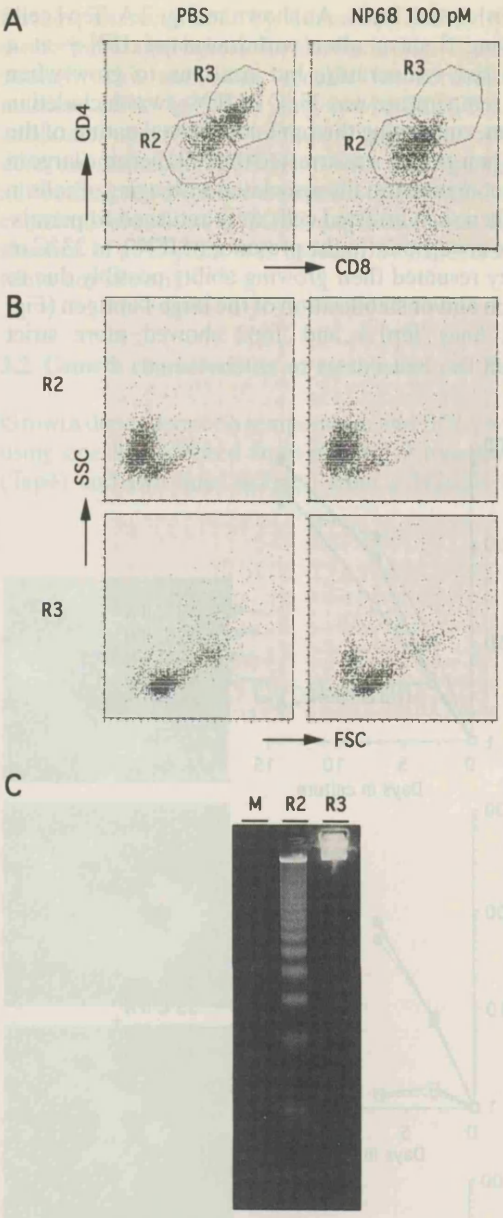

Figure 4. Induction of negative selection by peptide-loaded Tep1.1 cells. Thymic cortical epithelial cells Tep1.1 (H-2^b) were cultured for 3 days at 37 °C in the absence of IFN-γ, and then were incubated with PBS or 100 pM INP68 for 2 h at 37 °C and were subsequently washed. Thymocytes from adult F5 transgenic mice passed through Sephadex G-10 column were co-cultured with these peptide-loaded epithelial cells for 12 h at 37 °C and were stained for CD4, CD8. (A) Expression of CD4 and CD8 by F5 thymocytes after co-culture. Cells in R2 have lower CD4 and CD8 than those in R3. (B) Cell size (forward scatter, FSC) and granularity (side scatter, SSC) profile of double positive cells in R2 and R3. R2 has increased proportion of cells with small cell size and high granularity. (C) DNA fragmentation assay of cells in R2 and R3 from F5 thymocytes co-cultured with peptide-loaded Tep1.1 cells. Cells in R2 showed characteristic ladder caused by inter-nucleosomal fragmentation of DNA in apoptotic cells. Molecular size marker (M) was pBR322 plasmid digested with HinfI.

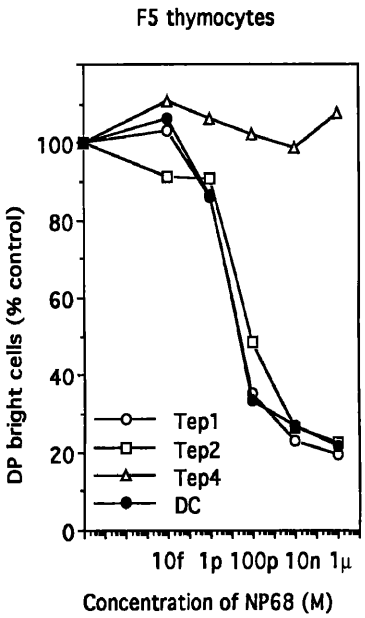

Figure 5. Negative selection of double positive thymocytes by antigenic peptide-loaded epithelial cells and dendritic cells. F5 thymocytes were cultured as in Fig. 4 using epithelial cell lines, Tep1.1 (H-2^b), Tep2 (H-2^b), Tep4 (H-2^k), and dendritic cells (H-2^b), and were stained for CD4, CD8, and Vβ11. Proportions of viable $CD4^+8^+$ cells, relative to viable cells in co-cultures without INP68, are plotted against concentrations of INP68. The antigenic peptide-induced depletion of $CD4^{high}8^{high}$ double positive cells in a dose-dependent and MHC-restricted manner. Experiments were repeated five times for epithelial cells and twice for dendritic cells giving similar results.

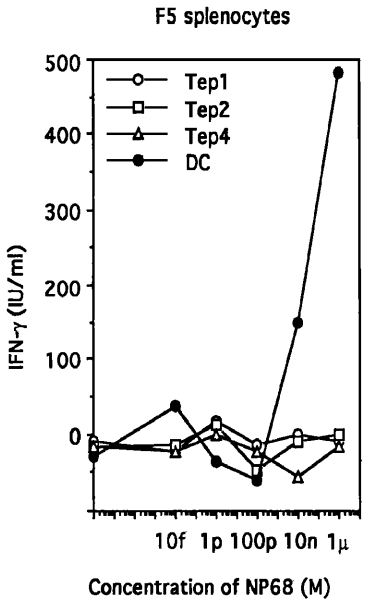

Figure 7. Production of IFN-γ by activated F5 spleen cells. Spleen cells from an F5 transgenic mouse were co-cultured with peptide-loaded thymic epithelial cell lines and dendritic cells for 4 days. Concentration of IFN-γ secreted in culture supernatant is shown for different antigen presenting cells at various concentrations of INP68 peptide. Thymic dendritic cells, but none of the epithelial cells, induced production of IFN-γ by mature F6 T cells. The data represent one of two experiments with identical results.

strate that the antigenic peptide presented by cortical epithelial cell lines can induce negative selection of thymocytes as efficiently as thymic dendritic cells.

3.5 Inability of thymic epithelial cells to stimulate mature T cells

Stimulation of F5 peripheral T cells by H-2^b spleen cells loaded with INP68 peptide leads to their activation as measured by their ability to kill targets in an antigen-specific manner [28] and to secrete IFN-γ. In order to compare the antigen presentation capacity by thymic epithelial cell lines and dendritic cells, F5 spleen cells were co-cultured with thymic epithelial or dendritic cells pre-loaded with INP68 peptide, and the levels of IFN-γ in the culture supernatant were measured. Fig. 7 shows that none of the epithelial cells could cause stimulation even at concentrations of peptide as high as 1 μM. In contrast, dendritic cells could activate F5 spleen cells at concentrations of peptide as low as 10 nM. The concentration of peptide required by dendritic cells to cause detectable deletion of double positive thymocytes (10–100 pM) was

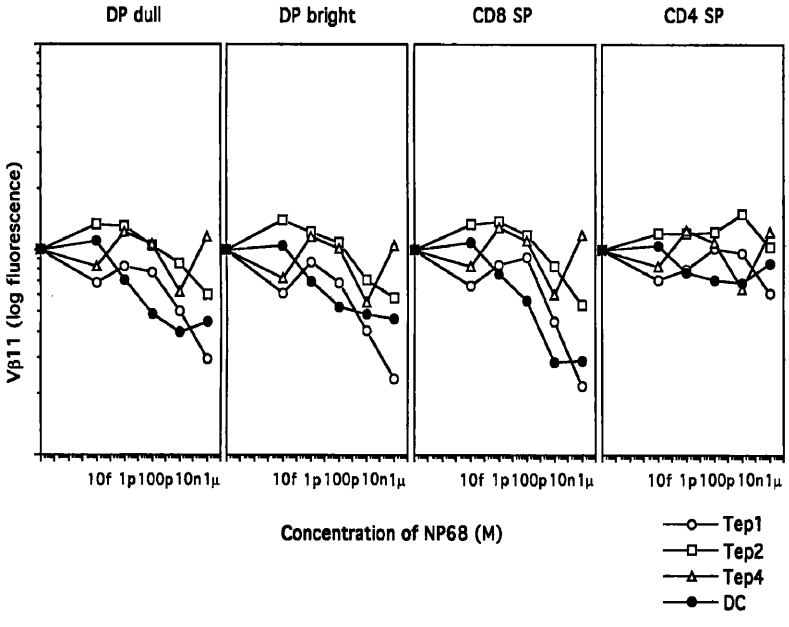

Figure 6. Down-regulation of TcR by antigenic peptide-loaded epithelial cells and dendritic cells. F5 thymocytes were cultured as in Fig. 5. Cells were gated according to the expression of CD4 and CD8, and mean fluorescence of Vβ11 in different thymocyte subpopulations, *i.e.* $CD4^{dull}8^{dull}$, $CD4^{high}8^{high}$, $CD4^-8^+$, and $CD4^+8^-$, are plotted against concentrations of INP68. The antigenic peptide induced down-regulation of Vβ11 in $CD8^+$ cells. Similar results were obtained by five experiments for epithelial cells and two for dendritic cells.

lower than that (10 nM) required to cause detectable stimulation of mature T cells as measured by IFN-γ secretion.

4 Discussion

In order to study lympho-epithelial interactions *in vitro*, we isolated conditionally growing thymic epithelial cells from mice transgenic for a temperature-sensitive SV40 large T-antigen (tsA58). Such cells lose their ability to proliferate under non-permissive culture conditions (37 °C in the absence of IFN-γ). It is reasonable to assume that the disappearance of T antigen in these cells results in a reversion to a physiological phenotype. Indeed, after some time under non-permissive conditions the cells acquire the expected appearance of senescent normal cells in culture. Cells grown under non-permissive conditions for 3 days can revert to their immortalized phenotype upon switching to permissive culture conditions [29].

The epithelial nature of the cultured cells was confirmed by their morphology and their abundant expression of cytokeratins. These cell lines were found positive for markers usually expressed on cortical epithelial cells of the thymus, whereas they did not seem to express any of the medullary markers. They express mRNA for the peptide transporter genes TAP1 and TAP2 and produced IL-Iα and IL-7. Only the Tep1.1 line expresses IL-6, which may reflect functional heterogeneity of thymic cortical epithelial cells. The significance of this profile of cytokine production in T cell development is under investigation at the moment. In conclusion, most of the phenotypic characteristics of these cells resemble those of freshly isolated thymic cortical stromal cells [17].

Co-culture of the established epithelial lines under non-permissive conditions with thymocytes isolated from mice transgenic for the F5 TcR, restricted by H-2D^b and the influenza peptide INP68, caused down-regulation of CD4 and CD8 on the double-positive cells, the first step to clonal elimination by apoptosis [27]. In addition, TcR levels were down-regulated in those populations carrying the CD8 molecule, in agreement with the finding that the F5 TcR is CD8 dependent for its function [30]. Absence of TcR down-regulation on CD4 single-positive cells can be explained by the fact that CD4$^+$8$^-$ cells in F5 transgenic thymus do not express both chains of the F5 TcR and have been selected using a heterodimer of Vβ transgenic and Vα endogenous chains.

Co-cultures of F5 thymocytes with epithelial cells from inappropriate MHC haplotype (H-2^k) in the presence or absence of antigenic peptide showed no effect on the levels of CD4 and CD8 or Vβ11. This is expected since the antigenic peptide does not bind H-2^k MHC molecules. It also eliminates the possibility that contaminating deleting elements in the thymocyte preparation are responsible for the deletion seen in this system. In addition, it shows that the down-regulation of CD4 and CD8 is not a nonspecific effect of epithelial cells on thymocytes, but rather that it strictly requires TcR/MHC-peptide interaction.

The concentration of peptide needed to down-regulate TcR levels was found to be approximately 10–100 times higher than that which caused the down-regulation of CD4 and CD8 in the double-positive thymocytes. This may indicate that the initial step towards clonal deletion of thymocytes is down-regulation of CD4 and CD8 co-receptors, and that this phenomenon is more sensitive than the down-regulation of TcR. The range of peptide concentration needed for negative selection in this system correlates roughly with those needed for activation [31]. Thymic epithelial cells and dendritic cells were shown to cause negative selection to the same extent within 1 pM and 100 pM concentrations of peptide. Using a different assay we found that peripheral T cells from F5 transgenic mice required more than 100 pM concentrations of peptide to be stimulated by thymic dendritic cells. This is in line with previous observations indicating that negative selection occurs at lower dose of antigen than activation [32]. It could be argued, however, that the read-out systems used (CD4/CD8 down-regulation **vs.** cytokine release) have different sensitivities.

Down-regulation of CD4, CD8 and TcR was also seen when F5 thymocytes were co-cultured with kidney epithelial cells of the appropriate haplotype (H-2^b) (data not shown). In recent studies comparing the capacity of different cells to induce clonal deletion in suspension cultures fibroblasts [33], B cells [34], thymic nurse cells [35], double-positive thymocytes [8, 9], thymic [7] and peripheral [27] APC were all able to cause apoptosis of double-positive thymocytes from mice transgenic for specific TcR genes. In those cases where cognate peptide was used the concentrations required to induce apoptosis were above 10 nM. It is not clear at the moment whether the higher sensitivity to peptide concentrations in our case (less than 100 pM) reflects higher affinity of the TcR for the MHC/peptide complex or of the peptide for the MHC or both.

Our results are not necessarily contradicting the general idea that cortical epithelial cells are responsible for positive selection and bone marrow-derived cells are responsible for negative selection. According to the affinity model, positive selection is mediated by a low-affinity interaction between TcR and MHC/endogenous self-peptide complex, whereas negative selection results from a higher affinity interaction of thymocytes with APC. In line with this interpretation, it has been shown that increased affinity interactions can lead to negative selection in transgenic mice. Thus, increased levels of antigen on selecting cells [36] or of CD8 on thymocytes [37] can influence the fate of αβ TcR thymocytes in transgenic mice. In the present study a high affinity interaction between transgenic TcR and epithelial cells loaded with the antigenic peptide caused negative selection. Alternatively, the antigenic peptide may induce a conformation of TcR which results in a negative signal, whereas the endogenous positively-selecting peptide may induce a qualitatively different signal [38, 39].

In this report, we described establishment of thymic cortical epithelial cell lines and demonstrated that these cells can cause negative selection of specific thymocytes given the appropriate antigenic peptide. Important questions concerning biochemical processes of positive and negative selection of thymocytes can be addressed *in vitro* using these cell lines.

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Note added in proof: After submitting this manuscript a paper describing *in vitro* deletion of $CD4^+8^+$ transgenic thymocytes was published by Pircher et al. [40]. Their data are in agreement with our findings.

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