

T cell development and lineage commitment :

studies based on differential gene expression in thymocyte subsets

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To my parents, Eduardo and Lúdia

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Abstract

T lymphocytes develop primarily in the thymus, where lymphoid progenitors imported from foetal liver or adult bone marrow go through a series of differentiation events that produce mature T cells bearing antigen-specific T cell receptors (TCR).

Early thymocyte development (before TCR expression) is marked by two crucial events: $\alpha\beta$ *versus* $\gamma\delta$ T cell lineage commitment; and " β -selection". The two T cell lineages derive from a common thymic progenitor and are defined by the TCR isotype expressed on the cell surface, $\alpha\beta$ or $\gamma\delta$. " β -selection" consists of a checkpoint in the differentiation of the $\alpha\beta$ lineage, in which only precursors that receive signals from the pre-TCR – a complex made of a newly synthesised TCR β chain and the invariant protein pT α – are selected for further maturation.

Since very little was known about the genetic programme that accompanies these two processes, we used cDNA-RDA (Representation Difference Analysis) to identify genes differentially expressed in thymocyte subsets representative of distinct lineages or developmental stages.

The ICER isoform of the CREM gene was identified as differentially expressed between lineage-committed thymic $\alpha\beta$ and $\gamma\delta$ T cell populations. Although thymic development was unperturbed in CREM/ICER^{-/-} mice, we demonstrate that subsequent to the DN4 stage of thymocyte differentiation, ICER is a robust marker of the $\gamma\delta$ T cell lineage. ICER expression is not observed in $\alpha\beta$ -committed DP or SP thymocytes, or in $\alpha\beta$ T cells from the lymph node and spleen. Furthermore, we show that ICER expression is a characteristic of developmental lineage rather than the type of TCR that is expressed, which supports a non-instructive mechanism for the lineage divergence. In addition, the analysis of ICER expression in subsets of less well characterised intestinal intraepithelial lymphocytes (IELs) allowed us to propose a refinement to the conventional $\alpha\beta/\gamma\delta$ classification of T cells that incorporates TCR $\alpha\beta$ (+)CD8 $\alpha\alpha$ (+) IELs as having a " $\gamma\delta$ -like" profile.

Surprisingly (for a $\gamma\delta$ lineage marker), ICER expression is severely impaired in the thymus of pT α ^{-/-} and TCR β ^{-/-} mice, suggesting a genetic link between pre-TCR

and ICER expression. Moreover, ICER expression can be induced in pre-TCR-deficient pre-T cells by CD3 signalling, and this induction is dependent on an intact MAPK pathway. These data suggest that ICER is a downstream target of pre-TCR signalling in pre-T cells. Consistent with this, ICER(+) pre-T cells have the phenotype and developmental behaviour of β -selected thymocytes.

However, in the $\gamma\delta$ lineage, ICER expression is not a direct consequence of pre-TCR signalling, since this complex is absent from the vast majority of $\gamma\delta$ cells. We provide evidence for the existence, in a normal thymus, of a *trans*-induction mechanism by which β -selected thymocytes (DP cells in particular) influence gene expression and the physiology of $\gamma\delta$ cells. This novel mechanism constitutes the first cross-talk reported for the $\alpha\beta$ and $\gamma\delta$ lineages during their thymic differentiation.

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Table of contents

Abstract	2
Acknowledgements	4
List of figures and tables	11
List of abbreviations	14
Chapter I : Introduction	16
1. T cells and the thymus in the immune system	17
1.1. Pioneering work on thymectomy	17
1.2. The T cell lineage within haematopoiesis	19
1.3. T cell receptor, the hallmark of T cells	23
2. T cell development	26
2.1. Thymus organogenesis	26
2.2. Thymic microenvironment	28
2.3. Developmental stages of murine thymocytes	30
2.4. The role of pre-TCR in thymocyte development	36
2.4.1. <i>pTα as part of pre-TCR</i>	36
2.4.2. <i>"β-selection"</i>	37
2.4.3. <i>Components of pre-TCR signalling pathway(s)</i>	38
2.4.4. <i>Transcription factors involved in "β-selection"</i>	43
2.5. The role of TCR $\alpha\beta$ in thymocyte development	48
2.5.1. <i>Positive and negative selection</i>	48
2.5.2. <i>CD4/CD8 lineage commitment</i>	51
2.5.3. <i>Signalling pathways and TFs downstream of TCR$\alpha\beta$</i>	53
2.6. TCR-independent signalling pathways in T cell development	58
2.6.1. <i>Pro- and anti-apoptotic pathways</i>	58
2.6.2. <i>Interleukin-7/IL-7R signalling</i>	61
2.6.3. <i>Wnt signalling</i>	63
2.6.4. <i>Notch signalling</i>	65

3. T cell lineage commitment: $\alpha\beta$ versus $\gamma\delta$	69
3.1. $\gamma\delta$ T cell biology	69
3.2. Models for the $\alpha\beta/\gamma\delta$ lineage split	76
3.3. Analysis of TCR rearrangements in T cell subsets	81
3.4. Analysis of TCR transgenic and gene-deficient mice	85
3.5. TCR-independent mechanisms in $\alpha\beta$ vs. $\gamma\delta$ cell differentiation	91
3.5.1. <i>IL-7 / IL-7R signalling</i>	91
3.5.2. <i>Notch signalling</i>	93
4. Objectives of the studies presented in this thesis	95
Chapter II : Methods	96
1. Cellular biology - general methods	97
1.1. Preparation of murine cells	97
1.2. Depletion of CD4(+)/CD8(+) T cells	98
1.3. Cell staining with antibodies and chemicals	98
1.4. Flow cytometry analysis and cell sorting	101
1.5. Foetal thymic organ cultures	102
2. Molecular biology - general methods	103
2.1. Protein	103
2.1.1. <i>Protein extraction</i>	103
2.1.2. <i>SDS-polyacrylamide gel electrophoresis</i>	103
2.1.3. <i>Western blotting</i>	104
2.2. DNA	105
2.2.1. <i>Extraction of genomic DNA</i>	105
2.2.2. <i>Preparation of plasmid DNA</i>	105
2.2.3. <i>Polymerase chain reaction</i>	106
2.2.4. <i>Agarose gel electrophoresis</i>	107
2.2.5. <i>Purification and radio-labelling of DNA probes</i>	108
2.2.6. <i>Restriction fragment length polymorphism -PCR</i>	108
2.2.7. <i>Cloning - general procedures</i>	111
2.2.8. <i>CD2-ICER construct for generation of transgenic mice</i>	113

2.3. RNA	115
2.3.1. RNA extraction and DNase treatment	115
2.3.2. Reverse transcription and RT-PCR	116
2.3.3. Real time (quantitative) PCR	117
2.3.4. Northern and virtual northern blotting	119
2.3.5. Probing of Atlas cDNA array	121
2.3.6. Probing of cDNA library filter array	122
3. Representation difference analysis	123
3.1. Synthesis of double-stranded cDNA	125
3.2. Generation of tester and driver representations	126
3.3. First subtractive hybridisation	127
3.4. Second subtractive hybridisation	129
3.5. Isolation, sequencing and identification of differentially expressed genes	130
 Chapter III : Results	 132
 1. Genes differentially expressed in $\alpha\beta$ versus $\gamma\delta$ thymocytes	 133
1.1. RDA analysis of DP vs. $\gamma\delta$ thymocytes	133
1.2. Differential expression of candidate genes	139
1.3. Pattern of expression of candidate genes	141
1.4. Preliminary studies on candidate genes	145
1.4.1. <i>Ly-49A</i>	148
1.4.2. <i>Sugano EST</i>	151
2. Identification of pre-TCR responsive genes	155
2.1. RDA analysis of TCR β (+) vs. TCR β (-) pre-T cells	155
2.2. RDA analysis of RAG(-) pre-T cells unstimulated vs. stimulated with anti-CD3 ϵ antibody	158
2.3. Expression of candidate genes: induction and dependence on pre-TCR signalling	163
2.4. Studies on a candidate gene: IL-7 receptor	166
2.4.1. <i>Pre-TCR dependence of IL-7R expression in DN4 cells</i>	166
2.4.2. <i>Requirement for IL-7R signalling in DN to DP transition</i>	168
2.4.3. <i>Requirement for IL-7R signalling for survival of DN4 cells</i>	170

3. ICER in $\alpha\beta$ versus $\gamma\delta$ T cell lineage commitment	174
3.1. Identification of CREM isoforms expressed in $\gamma\delta$ thymocytes	174
3.2. Pattern of expression of ICER in the thymus	177
3.3. ICER expression and the status of TCR gene rearrangements in pre-T cells	177
3.4. Analysis of the thymus of CREM/ICER deficient mice	182
3.5. Role of cyclic-AMP signalling in ICER expression during thymocyte development	182
3.6. Generation and analysis of CD2-ICER transgenic mice	184
3.7. Pattern of expression of ICER in peripheral lymphoid tissues	186
3.8. ICER expression in mouse mutants for TCR	192
3.9. Lineage potential of pre-T cells expressing different levels of ICER	194
4. ICER, β-selection and $\gamma\delta$ thymocyte development	198
4.1. ICER expression in pre-T cells undergoing β -selection	198
4.2. ICER expression in pre-T cells with impaired pre-TCR signalling	200
4.3. Induction of ICER expression by signalling through the CD3 complex	200
4.3.1. <i>ICER expression in response to anti-CD3ϵ antibody</i>	200
4.3.2. <i>Involvement of the MAPK pathway</i>	204
4.4. Analysis of the DN compartment of ICER deficient mice	206
4.5. Analysis of pre-T cells expressing different levels of ICER	207
4.5.1. <i>Developmental potential</i>	207
4.5.2. <i>Phenotypic analysis</i>	210
4.6. Gene expression in $\gamma\delta$ thymocytes developing in the absence of β -selected cells	213
4.7. Requirement of a normal (β -selected) composition of the thymus for ICER expression.	215
4.8. Cross-talk between $\alpha\beta$ and $\gamma\delta$ lineages during $\gamma\delta$ thymocyte differentiation	218

Chapter IV : Discussion	220
1. Genes differentially expressed between $\alpha\beta$ and $\gamma\delta$ T cells	222
2. Pre-TCR responsive genes	228
2.1. Role of IL-7 receptor in the DN to DP transition	229
3. ICER as a marker for the $\gamma\delta$ T cell lineage	233
4. Pre-TCR dependent expression of ICER in pre-T cells	239
5. Cross-talk between $\alpha\beta$ and $\gamma\delta$ T cell differentiation	243
6. Conclusion	249
References	252

List of figures and tables

Figures

1	Model for murine haematopoiesis.	22
2	Early thymocyte development.	32
3	Proximal molecules associated with pre-TCR signalling.	39
4	T cell developmental blocks caused by gene deletion.	47
5	Models for the $\alpha\beta/\gamma\delta$ T cell lineage split.	80
6	Structure of the human CD2 expression cassette.	114
7	Outline of representation difference analysis.	124
8	FACS sorting of TCR $\alpha^{-/-}$ thymocytes for RDA analysis.	134
9	Summary of RDA analysis of $\gamma\delta$ vs. DP thymocytes.	136
10	RT-PCR for candidate genes in wild type $\gamma\delta$ and DP thymocytes.	140
11	FACS profiles of WT thymocytes in cell sorting experiments.	142
12	RT-PCR for candidate genes in WT haematopoietic lineages.	143
13	Ly49A protein surface expression in NK and T cells.	149
14	Sugano EST: sequence and linkage to IL-2R β transcript.	152
15	IL-2R β protein surface expression in thymic subsets.	154
16	FACS plots (CD25 vs. CD44) for WT and TCR $\beta^{-/-}$ DN thymocytes.	155
17	FACS plots (CD25 vs. CD44) used in the purification of cells for RDA analysis of anti-CD3 ϵ mAb stimulated vs. unstimulated RAG(-) cells.	159
18	FACS profiles of sorted populations used for RDA analysis: CD25, CD69 and CD2 surface expression; forward scatter.	160
19	Cell cycle status of sorted populations used for RDA analysis.	161
20	Induction of gene expression in RAG(-) pre-T cells by anti-CD3 ϵ mAb.	164
21	Expression of candidate genes in WT and pre-TCR deficient T cells.	165
22	Surface expression of IL-7R α in pre-TCR(+) and pre-TCR(-) DN3 and DN4 thymocytes.	167
23	Effect of blocking antibodies to IL-7R complex on DN4 to DP transition.	169
24	Comparison of WT and IL-7R α -deficient DN4 thymocytes: cell cycle status, cell death and intracellular TCR β expression.	171
25	Effect of blocking antibodies to IL-7R complex on proliferation and death of DN4 thymocytes.	173

26	Identification of CREM isoforms expressed in $\gamma\delta$ thymocytes.	175
27	Real-time PCR expression profile for ICER in the thymus.	178
28	Pre-TCR levels and ICER expression in WT pre-T cells.	180
29	Pre-TCR levels and TCR gene rearrangement status of WT pre-T cells.	181
30	FACS analysis of thymocyte development in CREM ^{-/-} mice.	183
31	Effect of cyclic-AMP on thymocyte development (<i>in vitro</i>) and ICER expression.	185
32	Generation and screening of CD2-ICER transgenic mice.	187
33	FACS analysis of thymocyte development in CD2-ICER transgenic mice.	188
34	Real-time PCR expression profile for ICER in lymph nodes and spleen.	190
35	Real-time PCR expression profile for ICER in intra-epithelial lymphocytes.	191
36	ICER expression in TCR α -deficient and TCR δ -deficient mice.	193
37	ICER expression in TCR $\alpha\beta$ transgenic and TCR β -deficient mice.	195
38	Lineage potential of pre-T cells expressing different levels of ICER.	197
39	ICER-LacZ protein expression in pre-T cells of CREM/ICER-LacZ mice.	199
40	ICER mRNA expression in pre-T cells of mice with deficient pre-TCR signalling.	201
41	Induction of ICER expression by anti-CD3 ϵ mAb stimuli.	203
42	Effect of MAPK pathway inhibitors on the induction of ICER-LacZ protein expression by anti-CD3 ϵ mAb stimuli.	205
43	FACS plots (CD25 <i>vs.</i> CD44) of WT and CREM ^{-/-} DN thymocytes.	206
44	Developmental potential of pre-T cells expressing different levels of ICER-LacZ protein.	208
45	Phenotypic analysis of DN3 cells expressing different levels of ICER-LacZ protein.	211
46	Gene expression in pre-T cells expressing different levels of ICER-LacZ protein.	212
47	Gene expression in $\gamma\delta$ cells of TCR β ^{-/-} and pT α ^{-/-} mice.	214
48	ICER expression in foetal and adult pro-T and $\gamma\delta$ thymocytes.	215
49	ICER expression in the thymus of WT \rightarrow RAG ^{-/-} bone marrow chimera.	217
50	Induction of ICER expression in $\gamma\delta$ thymocytes mediated by DP cells.	219
51	Comparison of proliferation and effector functions of WT and TCR β ^{-/-} $\gamma\delta$ cells.	245
52	Model for ICER expression during T cell differentiation.	250

Tables

1	Genes isolated from the RDA subtractive hybridisation of $\gamma\delta$ and DP thymocytes.	137
2	Candidate gene expression (mRNA) in haematopoietic lineages.	141
3	cDNA sequences with no matches in GenEMBL databases, obtained from the RDA subtraction DP - $\gamma\delta$ cells.	145
4	Genes isolated from the RDA subtraction of TCR β (+) and TCR β (-) DN3 thymocytes.	156
5	Genes isolated from the RDA analysis of RAG(-) thymocytes stimulated for 3 hours <i>in vivo</i> with anti-CD3 ϵ mAb <i>versus</i> unstimulated cells.	162

List of abbreviations

APC	antigen presenting cell
BCR	B cell receptor
CD	cluster of differentiation
cDNA	complementary DNA
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CRE	cyclic-AMP response element
CREM	cyclic-AMP response element modulator
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytosine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
DC	dendritic cell
DETC	dendritic epidermal T cell
DN	double negative (CD4 ⁻ CD8 ⁻) thymocyte
DN1	DN thymocyte subset 1 (CD44 ⁺ CD25 ⁻)
DN2	DN thymocyte subset 2 (CD44 ⁺ CD25 ⁺)
DN3	DN thymocyte subset 3 (CD44 ⁻ CD25 ⁺)
DN4	DN thymocyte subset 4 (CD44 ⁻ CD25 ⁻)
DNA	deoxyribonucleic acid
DP	double positive (CD4 ⁺ CD8 ⁺) thymocyte
E	embryonic day
ECL	enhanced chemiluminescence
FACS	fluorescence-activated cell sorting
FTOC	foetal thymic organ culture
g	gravitational constant ($\approx 9.8 \text{ m/s}^2$)
GAPDH	glyceraldehyde 3-phosphate
GFP	green fluorescent protein
HSC	haematopoietic stem cell
ic	intracellular
ICER	inducible cyclic-AMP early repressor
IEL	intra-epithelial lymphocyte
IL	interleukin

ISP	immature single positive (CD8 ⁺) thymocyte
ITAM	immunoglobulin family tyrosine-based activation motif
kb	kilobase
kD	kilodalton
KO	"knock-out"
-L	ligand
LCR	locus control region
loxP	locus of cross-over in P1 bacteriophage
lg	immunoglobulin
Mφ	macrophage
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
mRNA	messenger RNA
n	number of replicate experiments
NK	natural killer cell
NT	no treatment
PCR	polymerase chain reaction
PI	propidium iodide
pTα	pre-TCRα chain
PAGE	polyacrilamide gel electrophoresis
PTK	protein tyrosine kinase
-R	receptor
RAG	recombination activating gene
RDA	representation difference analysis
RSS	recombination signal sequence
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
SCID	severe combined immunodeficiency
SH	Src-homology domain
SP	single positive (CD4 ⁺ or CD8 ⁺) thymocyte
Tg	transgenic
tRNA	transfer RNA
TCR	T cell receptor
V-D-J	variable-diversity-junctional (rearrangement of TCR genes)
WT	wild type

Chapter I :

INTRODUCTION

1 T cells and the Thymus in the Immune System

“The outstanding feature of the development of immunobiology in the last 10 years has been the recognition of the function of the lymphocyte and of the importance of the thymus in the immune process”.

M. Burnet (Nobel Prize of Medicine and Physiology), 1966

Nowadays, T cells are one of the most well known cell types with the general public. This is the result of the extensive coverage in the lay press of AIDS (acquired immunodeficiency syndrome), one of the most devastating diseases of modern times. In stark contrast, before 1960, neither T cells nor their preferential producing organ, the thymus, were considered to play any role in immunity. In fact, only 45 years ago MacLean and collaborators concluded from their research that “the thymus gland does *not* participate in the control of the immune response” (MacLean et al., 1957). Even though the thymus was already known to be a lymphocyte-producing organ, its relevance was not recognised by the community of immunologists. Peter Medawar (Nobel Prize laureate himself) suggested in 1963 that “we shall come to regard the presence of lymphocytes in the thymus as an evolutionary *accident* of *no* very great significance” (Medawar, 1963).

What then was the turning point?

1.1 Pioneering work on thymectomy

As so often happens in science, it was an unpredictable result that caused a revolution in this field. While studying the relationship between a viral infection and the onset of leukaemia, Jacques Miller was surprised to see that neonatally thymectomized mice died prematurely from causes unrelated to leukaemia induction. He proposed that “the thymus at birth may be essential to life” (Miller, 1961). Further work by Miller demonstrated that newborn mice thymectomized

not later than a few days after birth were very susceptible to infections, had a marked deficiency of lymphocytes in the blood and in lymphoid organs, and were unable to reject foreign skin grafts. In contrast, it had been known for a long time that thymectomy of adult mice did *not* have such dramatic consequences - which was why the thymus had, until then, been disregarded as a vital lymphoid organ. Miller then showed that if adult thymectomized mice were exposed to total body irradiation, the recovery of the lymphoid system was thymus-dependent (Miller, 1962). Indeed, thymus implants allowed the development of a normal immune (lymphoid) system in both neonatally thymectomized and irradiated adult thymectomized mice. Importantly, if the thymus tissue used for the implant was obtained from a foreign strain of mice, the neonatally thymectomized recipients became specifically immunologically tolerant to the histocompatibility antigens of the donor, implying that the thymus was the site where self tolerance is established.

Another striking phenotype of neonatally thymectomized mice was that lymphocyte deficiency was seen in areas associated with cellular immune responses, and not in areas where antibody-producing cells resided (Parrott et al., 1966). Subsequently, the introduction of genetically marked cells into neonatally thymectomized mice established beyond doubt and for the first time that antibody-producing cells (B cells), derived from the bone marrow, were different from thymus-derived cells (T cells). It also demonstrated that T cell communication with B cells was essential for antibody production (Mitchell, 1968).

The existence of two different lymphocyte lineages was initially regarded with surprise and skepticism (reviewed by Miller, 1995). Some were puzzled by the fact that T cell precursors ultimately also had a bone marrow origin. Eventually, clarification came from extensive studies that demonstrated the existence of the hematopoietic stem cell, from which all blood cell types originate.

1.2 The T cell lineage within haematopoiesis

All cellular components of the blood, from leukocytes of the immune system to erythrocytes of the respiratory system, derive from haematopoietic stem cells (HSC). These special cells are pluripotent (generate many different cell types) and are also capable of self-renewal, unlike the cells they generate.

The production of all blood cell types from HSC is termed haematopoiesis. This process is characterised by a continuous loss of potency, as immature pluripotent cells give rise to more mature cells with more limited potential.

In adult mammals, HSC are present in the bone marrow. However, in foetal life they initially localise to the yolk sac blood islands, and only produce cells of primitive erythroid lineages (required for oxygen transport). This is called "primitive haematopoiesis". Later, HSC appear in the liver and spleen of the embryo, and finally in the bone marrow. During this second phase of haematopoiesis, termed "definitive", cells of erythroid, myeloid and lymphoid lineages are formed.

The breakthrough in the field of HSC happened in the 1970s, with the experiments of Moore and Metcalf (Metcalf and Moore, 1971), first to address the different origins of primitive and definitive haematopoiesis, and Dieterlen-Lievre (Dieterlen-Lievre, 1975), who used quail-chick chimeras (of yolk sac-embryo, respectively) to investigate those origins. An updated view was provided by Yoder (Yoder and Hiatt, 1997; Yoder et al., 1997a; Yoder et al., 1997b) and by Cumano (Cumano et al., 2001; Cumano and Godin, 2001). They have collectively demonstrated, by *in vivo* reconstitution (grafting) experiments in mice, that HSC localise to the yolk sac at E7.5 (embryonic day 7.5), when gastrulation starts, but are only capable of generating erythrocytes (and some myeloid cells, but no lymphocytes). This corresponds to primitive haematopoiesis. From E9 onwards, HSC are found in the embryo, in the spanchnopleura, an embryonic tissue of mesoderm associated with endoderm that is a developmental precursor of the aorta-gonad-mesonephros (AGM). (HSC are not found in the liver before E11.5) These cells are now capable of multilineage (including lymphocytes) long term

reconstitution (of lymphopaenic recipients), unlike the HSC of the yolk sac. Definitive haematopoiesis therefore seems to derive from the embryo, and not from the yolk sac.

During their maturation, HSC first lose the ability to self-renew while still maintaining their full developmental potential. These cells isolated from murine bone marrow are termed short-term (ST)-HSC, because they self-renew for only 6 weeks in transplantation experiments (reviewed in Kondo, 2001). This stage precedes the generation of distinct blood cell types.

There have been several descriptions of the developmental relationships between haemopoietic cell lineages (reviewed in Keller, 1999) - **Figure 1**.

On receiving differentiation signals, HSC commit to either the lymphoid or the myeloid lineage, thereby losing their pluripotency. Whereas lymphoid cells (T, B and NK cells) play a role in both adaptive and innate immunity, myeloid cells (i.e., macrophages and granulocytes) essentially belong to the innate immune system.

Recently, clonogenic lymphoid- and myeloid-lineage committed progenitors have been identified in adult mouse bone marrow. Common lymphoid progenitors (CLP) are the most immature lymphoid-committed precursors identified to date. These differentiate into T, B and NK lineages but not into myeloid lineages. Their phenotype is IL-7R α (+) Lin(-) Sca-1(low) c-kit(low), where Lin represents mature lineage markers, IL-7R α is the α chain of the IL-7 receptor, Sca-1 is stem cell antigen 1 and c-kit is stem cell factor receptor (reviewed in Akashi, 2000).

The cytokine IL-7 is indispensable for both T and B cell development. The IL-7 receptor is composed of a specific α chain and the common cytokine receptor γ chain (γ_c) (Kondo et al., 1994). Mice that are genetically deficient for IL-7 or IL-7R have a severe reduction in their T and B cell compartments (Peschon et al., 1994). Mice deficient for γ_c also lack NK cells (Cao et al., 1995) due to an impairment in the formation of IL-15 receptor, for which γ_c is also necessary.

Commitment of a HSC to the CLP pathway is regulated by a variety of transcription factors including c-myb, PU.1, GATA-2 and Ikaros (reviewed in Rothenberg, 1999). Proteins of the Ikaros family (Ikaros, Helios, Aiolos) are

lymphoid-restricted zinc-finger transcription factors (TFs). In their functional absence, mice are devoid of all lymphoid lineages (Georgopoulos et al., 1994).

The next step in T cell differentiation is the commitment of CLPs to the T lineage and involves transcription factor GATA-3 (reviewed in Kuo, 1999). GATA-3 belongs to the GATA family of zinc-finger TFs, of which members -1/-2/-3 are highly expressed in haematopoietic cells, whereas members -4/-5/-6 predominate in heart, gut and muscle. GATA-3 is expressed in both T and NK lineages, and was first identified as a TF that binds to the T cell receptor α chain gene enhancer. Targeted disruption of the GATA-3 gene in mice resulted in embryonic lethality at E11 (Pandolfi et al., 1995), which precluded an analysis of its role in T cell development. To overcome this difficulty, Ting *et al.* (Ting et al., 1996) complemented RAG-2^{-/-} blastocysts (Chen et al., 1993), from which no T or B cells can be generated, with GATA-3-deficient ES cells. The resultant RAG-2^{-/-} GATA-3^{-/-} chimeric mice had normal B cell populations, but completely lacked T cells. Detailed analysis of the thymus of these animals demonstrated that GATA-3 is necessary for the survival and development of the earliest T cell committed CD4(-) CD8(-) thymocytes or their precursors, defining it as the earliest known TF required specifically for T cell lineage commitment.

T cell development occurs preferentially in the thymus, from CLP precursors imported from the bone marrow. It is still unknown whether CLPs home directly to the thymus *in vivo*. The earliest identified thymic progenitors are CD4(low) CD8(-) CD44(+) CD25(-) c-kit(+). Although the majority of these cells seem to be T cell-committed, there are also some that can differentiate into B, NK and lymphoid dendritic cells (DC) at a low frequency (Ardavin, 1993; Wu, 1991). Consequently, it is possible that the earliest thymic progenitor population contains a small number of CLP that have homed to the thymus. Because the thymic microenvironment (see 2.2) is best suited for T cell differentiation, CLP may preferentially develop into T cells in the thymus (Akashi et al., 2000). This contrasts with what happens to CLP in the bone marrow, where the environment favours B cell development.

The definitive molecular marker of commitment to the T cell lineage is rearrangement of the gene loci that encode the T cell receptor.

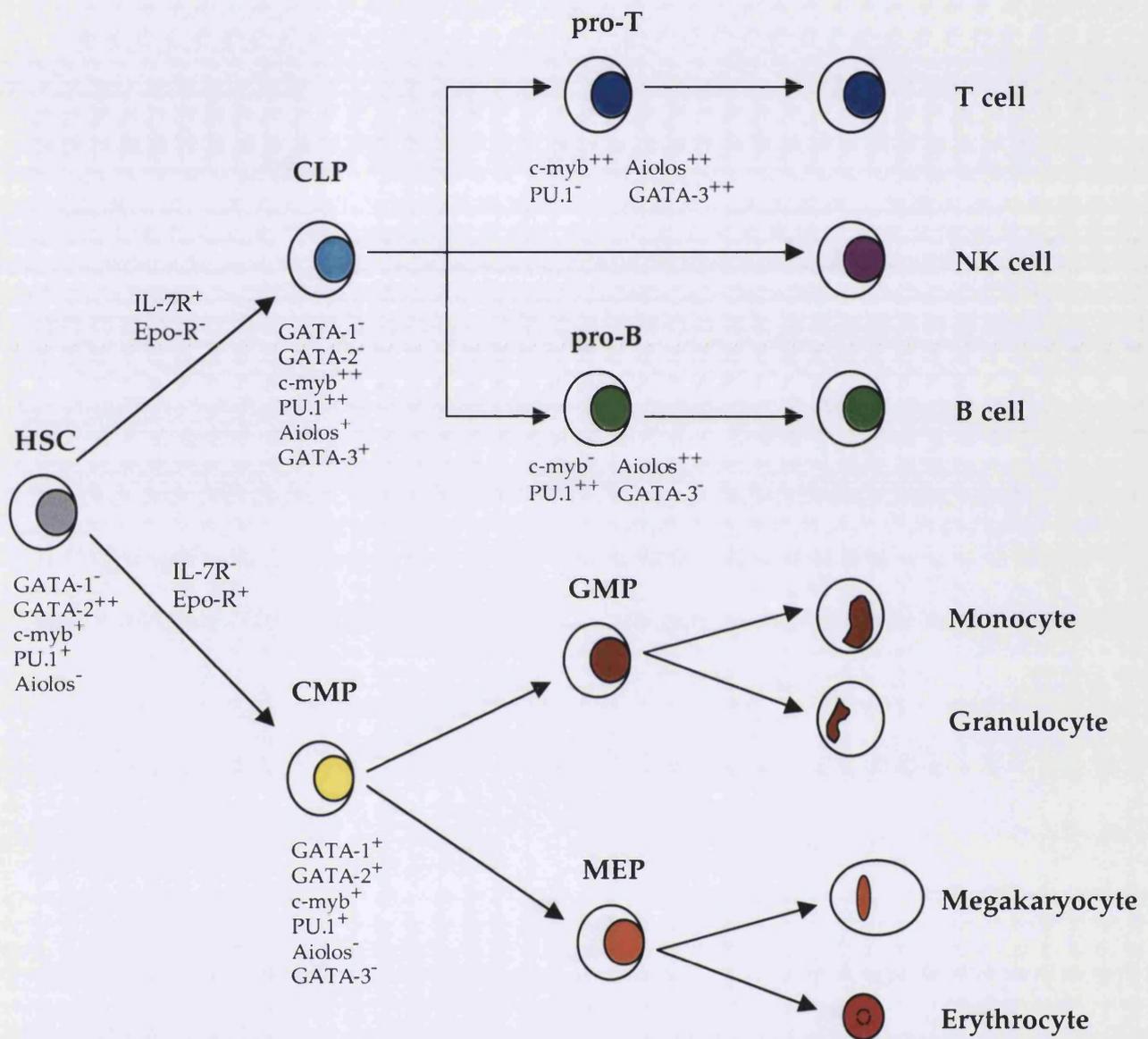


Figure 1 : Model for murine haematopoiesis (adapted from Akashi, 2000).

Expression levels of commitment factors are indicated. For abbreviations see text.

1.3 T cell receptor, the hallmark of T cells

The T cell receptor genes were initially identified as cDNAs differentially expressed between T and B cells, that respected two criteria: were encoded by gene segments that had undergone rearrangement only in T cells; and had sequence similarity to immunoglobulin (Ig), which had been previously identified as the B cell receptor (Kronenberg et al., 1986).

The TCR and Ig genes are indeed structurally related: each is composed of multiple variable (V), diversity (in some cases) (D) and joining (J) gene segments that are subjected to rearrangement during lymphocyte development. This process is mediated by the same enzymes (RAG – recombination activation gene - recombinases) in both cell types, but is tightly regulated in a lineage-specific manner (the TCR only rearranging in T cells, and Ig only in B cells) (Yancopoulos, 1986).

Murine TCR genes are spread between three loci: β , which comprises 800 kb on chromosome 6; γ , 205 kb on chromosome 13; and α/δ , a common locus to TCR α and TCR δ , 1000 kb on chromosome 14. Within the α/δ locus, J and C (and D) segments are specific for either TCR α or TCR δ , whereas the majority of V segments can be incorporated in both chains (reviewed in Grawunder, 1998).

Somatic recombination involves V (to D) to J rearrangements that proceed between gene segments marked by “recombination signal sequences” (RSS). These are DNA patterns consisting of a palindromic heptamer (CACAGTG) and a nonamere (ACAAAACC) spaced by either 12 or 23 bp (termed RSS-12 and RSS-23, respectively). Rearrangements follow the “12/23” rule, which imposes the involvement of one RSS-12 and one RSS-23 gene segments. The process is catalysed by RAG (recombination activation gene) enzymes (-1 and -2), and is facilitated in terms of DNA locus accessibility by HMG (high mobility group) proteins, which are capable of modifying the conformation of DNA (“DNA bending proteins”) (reviewed in Grawunder, 1998).

The TCR protein is a heterodimer composed of two polypeptide chains linked by a disulfide bond. Of the four possible TCR chains (α , β , δ , γ), two are used by

each T cell to assemble its TCR, depending on the lineage it commits to: $\alpha\beta$ T cells use α and β ; $\gamma\delta$ T cells use γ and δ chains (Kronenberg, 1986; Marrack, 1987; Raulet, 1989; Strominger, 1989; Davis, 1988).

Both chains of the TCR have, similarly to Ig, an amino-terminal variable (V) region, a constant (C) region, and a short hinge region with a cysteine residue that forms the inter-chain disulfide bond. Each chain spans the cell membrane lipid bilayer by a hydrophobic transmembrane domain, the notable feature of which is the presence of positively charged amino acid residues, important for the association with the signalling CD3 subunits (see ahead). Finally, each chain ends with a short cytoplasmic domain (Clevers et al., 1988). Consistent with these analogies, the X-ray crystal structures obtained so far for particular Ig and TCR molecules have shown that these proteins fold in a similar way. In particular, each domain is a globular structure in which several strands of polypeptide chain come together to form two anti-parallel β -sheets, held together by an intra-chain disulfide bond. This type of 3D structure is called an "Ig-like domain" and is present in many proteins involved in cell-cell recognition, especially in the immune and nervous systems (Novotny et al., 1986). There are, though, particular differences in alignments, angles and points of contact between domains of Ig and TCR molecules (reviewed in Wilson, 2001).

More strikingly, though, TCR differs from Ig in that TCR is monovalent, whereas Ig is bivalent (two antigen binding sites) and the TCR is never secreted, unlike Ig (which is secreted after activation of the B cell).

In order to signal upon antigen recognition, the TCR stably associates with invariant proteins of the **CD3 signalling complex**. There are four CD3 proteins: γ , δ , ϵ and ζ . The first three are encoded by linked genes (Saito et al., 1987), and have extracellular domains with weak homology to Ig domains and small intracellular domains. Their transmembrane regions are characterised by an acidic (negatively charged) residue that forms a salt bridge with the basic (positively charged) amino acids of the transmembrane region of the TCR. CD3 ζ is a small disulfide-linked dimer, most of which lies in the cytoplasm (Clevers et al., 1988).

The cytoplasmic domains of all the CD3 subunits contain sequences called immunoreceptor tyrosine-based activation motifs (ITAM) that allow them to associate with cytosolic protein tyrosine kinases following receptor stimulation. Particularly important for this signal transduction process are CD3 ϵ and CD3 ζ (Terhorst et al., 1995). CD3 proteins are also required for the assembly and cell-surface expression of the TCR complex. Therefore, it is not surprising that mice lacking these proteins (due to genetic manipulation) suffer from immunodeficiency.

The TCR $\alpha\beta$ complex also contains CD4 or CD8 “co-receptor” molecules, which co-operate with the TCR in antigen recognition (Zuniga-Pflucker et al., 1991). CD4 is a single-chain molecule composed of four Ig-like domains. Its cytoplasmic domain interacts strongly with Src family tyrosine kinase p56Lck, promoting signal transduction from the TCR. Indeed, the presence of CD4 has been estimated to lower by 100-fold the dose of antigen required for T cell activation (reviewed in Zamoyska and Travers, 1995). CD8, in contrast with CD4, is a disulfide-linked heterodimer comprising α and β chains (although a CD8 $\alpha\alpha$ homodimer also exists in some less abundant T cell subsets), each containing a single Ig-like domain (Zamoyska and Travers, 1995).

CD4 and CD8 can be expressed in the same cell during thymocyte development (indeed, about 80% of all thymocytes are CD4(+) CD8(+)), but mature $\alpha\beta$ T lymphocytes only express one or the other co-receptor, depending on the lineage they commit to, CD4+ or CD8+. In the periphery, CD4+ (helper) T cells recognise antigen (via their TCR $\alpha\beta$ -CD4 complex) presented by MHC class II molecules, whereas CD8+ (cytotoxic) T cells do it (via their TCR $\alpha\beta$ -CD8 complex) in the context of MHC class I molecules.

2 T cell development

The vertebrate thymus is responsible for the production of self-restricted, self-tolerant T (thymus-dependent) cells. In the thymus, immature T cells (thymocytes) proliferate and differentiate, passing through a series of discrete phenotypic stages that can be identified by particular patterns of expression of various cell-surface proteins. During differentiation, T cells undergo gene rearrangements, commit to a T cell lineage ($\alpha\beta$ or $\gamma\delta$), express an appropriate T cell receptor, and are then submitted to developmental checkpoints such as “positive” and “negative” selection. Cells that fail these selection processes die by apoptosis or by neglect, whereas the selected cells survive and leave the thymus to seed the peripheral lymphoid organs.

2.1 Thymus organogenesis

Thymus organogenesis requires interactions between cells of all three embryonic germ layer origins: endoderm-derived epithelium, neuroectoderm-derived neural crest mesenchyme, and mesoderm-derived haematopoietic cells and endothelial cells of blood vessels (Le Douarin and Jotereau, 1975; Moore and Owen, 1967; Owen and Ritter, 1969). For convenience, this entire process can be divided into three main stages: early organogenesis (E9.5-E11.5 in the mouse embryo), late organogenesis (E12-E15) and late foetal development (E15.5-birth) (Manley, 2000).

Early organogenesis occurs prior to the import of haematopoietic cells. The murine thymus forms by an epithelial-mesenchymal interaction between the third pharyngeal pouch endoderm and neural crest mesenchyme from the third and fourth pharyngeal arches. Chick-quail chimera experiments showed that, in chick, ectopically transplanted prospective pharyngeal pouch endoderm was sufficient to induce the formation of a thymic rudiment (Le Douarin and Jotereau, 1975). Nevertheless, further growth and differentiation of the rudiment requires an interaction with the neural crest mesenchyme (LeLievre and LeDouarin, 1975). This

is particularly obvious in mice lacking migrating neural crest cells, either by experimental ablation (Bockman et al., 1989) or due to a genetic defect, in Pax3^{-/-} mice (Conway et al., 1997). Mesenchymal cells appear to be required for epithelial growth and differentiation (including induction of MHC class II expression) by providing both secreting factors and extracellular matrix (Owen et al., 2000; Suniara et al., 2000).

At least three transcription factors have been clearly implicated in this early phase of murine thymus organogenesis: Hoxa3, Pax9 and Whn. Mutant mice for each of these genes show an early failure in thymus formation (reviewed in Manley, 2000). Hoxa3^{-/-} mice are athymic, this being one of several defects in the pharyngeal region. The best characterised mutation affecting early thymic epithelium development is the Whn gene, which results in the nude mouse (so called due to its absence of hair). The thymic rudiment of the nude mouse has deficient epithelium and is not populated by lymphoid progenitor cells.

At this stage there are effectively two bilateral rudiments which proliferate and bud off from the pharynx. They then move medially, ventrally and caudally until they join at the midline above the heart by E12.5. This migration seems to be regulated independently of differentiation, as a normal migration can be seen in the nude mouse.

At E11.5, lymphoid progenitor cells start colonising the thymus (Owen and Ritter, 1969). From E12 onwards (late organogenesis), differentiation requires interaction between these incoming cells and the epithelium. Indeed, it is now clear that epithelial differentiation (at the stage of cortical *versus* medullary specification) and thymocyte development are interdependent. Thus, transgenic and mutant mice in which thymocyte development is blocked at specific stages show blocks in epithelial differentiation and cortical and medullary compartment formation (reviewed in Naquet, 1999). Complete cortical development appears to require interactions with thymocytes committed to the T cell lineage, while complete medullary development is dependent on the presence of mature TCRαβ⁺ thymocytes (reviewed in Manley, 2000).

2.2 Thymic microenvironment

The stromal compartment of the thymus is heterogeneous, consisting of cortical and medullary epithelium, mesenchymal fibroblasts, dendritic cells and macrophages (Boyd et al., 1993). These contribute to thymocyte development via cell-cell interactions and the production of soluble factors.

Lymphoid precursors start colonising the thymus prior to vascularization of the organ (which occurs at E14). Colonising cells need to leave the pharyngeal vessels and traverse the peri-thymic mesenchyme before penetrating the basement membrane surrounding the embryonic thymic rudiment. It is therefore likely that this process involves chemotactic factors. Indeed, *in vitro* transfilter migration assays have shown that alymphoid thymuses consisting only of thymic stroma attract precursors from donor lymphoid tissues (foetal liver or normal thymic lobes) (Fontaine-Perus et al., 1981; Jenkinson et al., 1982). Furthermore, chemokines such as MIP (macrophage inflammatory protein) -1α , -1β , -1γ and -2 , SDF-1 (stromal cell-derived factor -1), TECK (thymus-expressed chemokine) and TARC (thymus activation-regulated chemokine), and respective receptors - CCR5, CCR7, CCR9, CCR4 and CCR8 - are expressed in the thymus (reviewed in Anderson, 2000).

Consistent with a chemokine-dependent mechanism of colonisation, this process can be inhibited *in vitro* by pertussis toxin, an inhibitor of G protein-mediated chemokine receptor signalling (Wilkinson et al., 1999). However, lymphoid progenitors colonise only the thymus and not other chemokine-expressing organs. Therefore, due to its thymus-restricted expression, TECK was initially seen as the best candidate for providing thymic-specific homing (Anderson et al., 2000a). But further studies have shown that, not only is TECK expressed in tissues that do not attract lymphoid precursors, but also neutralising anti-TECK antibodies cannot inhibit *in vitro* thymic colonisation (Wilkinson et al., 1999). Thus, the molecular basis of a chemokine-dependent colonisation of the thymus is still to be clarified.

Chemokines may also be important for cell migration within the thymus, in particular between the cortical and the medullary areas. TECK has also been

implicated in this process. TECK receptor (CCR9) is upregulated during thymocyte development in the transition between immature CD4(-)CD8(-) and CD4(+)CD8(+) stages, and it has been shown to be a chemoattractant for the more mature single positive thymocytes that reside in the medulla (Norment et al., 2000).

Besides the stromal cell network, the thymus also contains mesenchymal fibroblasts that interact with thymocytes and play a role in their development. These fibroblasts provide extracellular matrix (ECM) components such as fibronectin, laminin, collagen and vimentin to thymocytes. Thymocytes, in their turn, express a variety of ECM-receptors: VLA-4, VLA-5 and CD44, which are particularly abundant in early immature cells, suggesting a developmentally regulated role for ECM components in thymocyte maturation (Anderson et al., 2000a).

The relevance of mesenchymal fibroblasts and their ECM components to early T cell development has been shown in reaggregate thymus organ cultures (RTOC) (Hare et al., 1999). A combination of both epithelial cells and fibroblasts was found to be required for the maturation of CD4(-)CD8(-) precursors to the CD4(+)CD8(+) stage (Anderson et al., 1993), and pre-treatment of these fibroblasts with hyaluronidase, an ECM disrupting enzyme, abrogated their ability to support this developmental transition (Anderson and Jenkinson, 2000b).

The mechanism by which ECM components influence T cell development is still unclear. One hypothesis is that they act as a 'scaffold' to concentrate and localise growth and survival factors for presentation to thymocytes. It has been shown (Banwell et al., 2000) that mesenchymal fibroblasts contribute to IL-7 cytokine presentation to thymocytes (which express IL-7 receptor in a developmentally regulated manner). IL-7 is extremely important for T cell development (see 2.6) and lineage commitment (see 3.5). It is produced by MHC class II (+) thymic epithelial cells, but mesenchymal fibroblasts also participate in its presentation to thymocytes. This presentation is ECM-dependent, as it is blocked by pre-treatment of the RTOC with heparitinase, an enzyme that selectively disrupts ECM component heparan sulphate (Banwell et al., 2000).

Cell-cell interactions between thymocytes and stromal cells are also extremely important for triggering of receptor-associated signalling pathways in thymocytes. In particular, thymic epithelial cells seem to provide ligands (of Jagged and Delta families) for Notch (Anderson et al., 2001) and Wnt family ligands (Wnt -4, -7) for Frizzled receptor signalling (Jenkinson, 2002) (see 2.6).

Finally, thymic selection processes also require interactions between CD4(+)CD8(+) thymocytes and thymic epithelial cells (Anderson et al., 1995; Anderson et al., 1994a; Anderson et al., 1994b; Hare et al., 2001; Wilkinson et al., 1995). *In vitro*, these two cell types form a structure known as 'rosette' where one thymocyte is surrounded by an average of three epithelial cells. Incubation of 'rosettes' in foetal thymic organ cultures (FTOC) allows maturation of the thymocytes to the single-positive stage. This 'rosette' structure is abrogated if the epithelial cells are MHC-deficient, or if the thymocytes are TCR-deficient, indicating that the association between the two cell types is dependent on TCR-MHC interaction. The TCR-MHC contact point is known as a "synapse", and it produces a re-distribution of cell surface molecules including co-receptors CD4 and CD8, CD3 and CD45, integrin LFA-1, and signalling molecules p56Lck and LAT.

Thus, in summary, the thymic microenvironment provides a series of soluble factors and cell-cell interactions that are fundamental for T cell development. These include chemokines that attract lymphoid progenitors to enter the thymus, and that help direct thymocyte migration within the thymus; growth and survival factors produced and presented by epithelial cells and fibroblasts; and selection and differentiation signals that are essential at certain stages of T cell development.

2.3 Developmental stages of murine thymocytes

The programmed succession of events that lead to the generation of a mature T cell population with a diverse TCR repertoire can be divided into two phases: an early phase, that precedes the expression of a mature TCR, and a late phase, in which further differentiation is dependent on the cell surface expression of a functional TCR (Fehling and von Boehmer, 1997).

The *early phase* includes the expansion of early thymic immigrants, their commitment to the T lineage, rearrangement of the TCR γ , δ and β loci, commitment to the $\alpha\beta$ or $\gamma\delta$ lineage and isotypic exclusion (i.e., the expression of only one type of TCR, $\alpha\beta$ or $\gamma\delta$, per cell), and allelic exclusion (expression of only one variant of TCR chain; silencing of the other allele) (Fehling and von Boehmer, 1997).

The *late phase* is marked by: positive selection of the cells expressing a TCR capable of effective (intermediate signal strength) interaction with MHC; negative selection of the cells in which that interaction is too strong (due to recognition of "self" antigen); commitment to one of the single-positive lineages, CD4+ (helper lymphocytes) or CD8+ (cytotoxic lymphocytes); and final export of mature T cells to the periphery (Kisielow and von Boehmer, 1995).

These differentiation events occur in a tightly regulated developmental sequence, which can be followed by analysing the expression of certain cell surface molecules (*developmental markers*). The availability of antibodies that selectively recognise each of those markers allows a rapid purification of thymocyte subsets and thus greatly facilitates the study of differentiation processes.

Two of such markers are CD4 and CD8 (see 1.3). On the basis of their expression, developing thymocytes are sub-divided into four populations of different maturity: "double negative", "double positive" and each "single positive" subset (reviewed in Kisielow, 1995).

The most immature subset is CD4(-)CD8(-), "double negative" (DN), and also lacks the expression of a mature TCR-CD3 complex; it constitutes only about 2% of all thymocytes.

The *DN compartment* of the thymus can be further sub-divided into four subsets, according to the expression of *c-kit* (stem cell factor receptor), CD25 (IL2 receptor α chain) and CD44 (phagocytic glycoprotein -1). Based on the cytofluorometric detection of these markers, the following description of successive developmental stages within the DN population has emerged (Figure 2) (Godfrey et al., 1993).

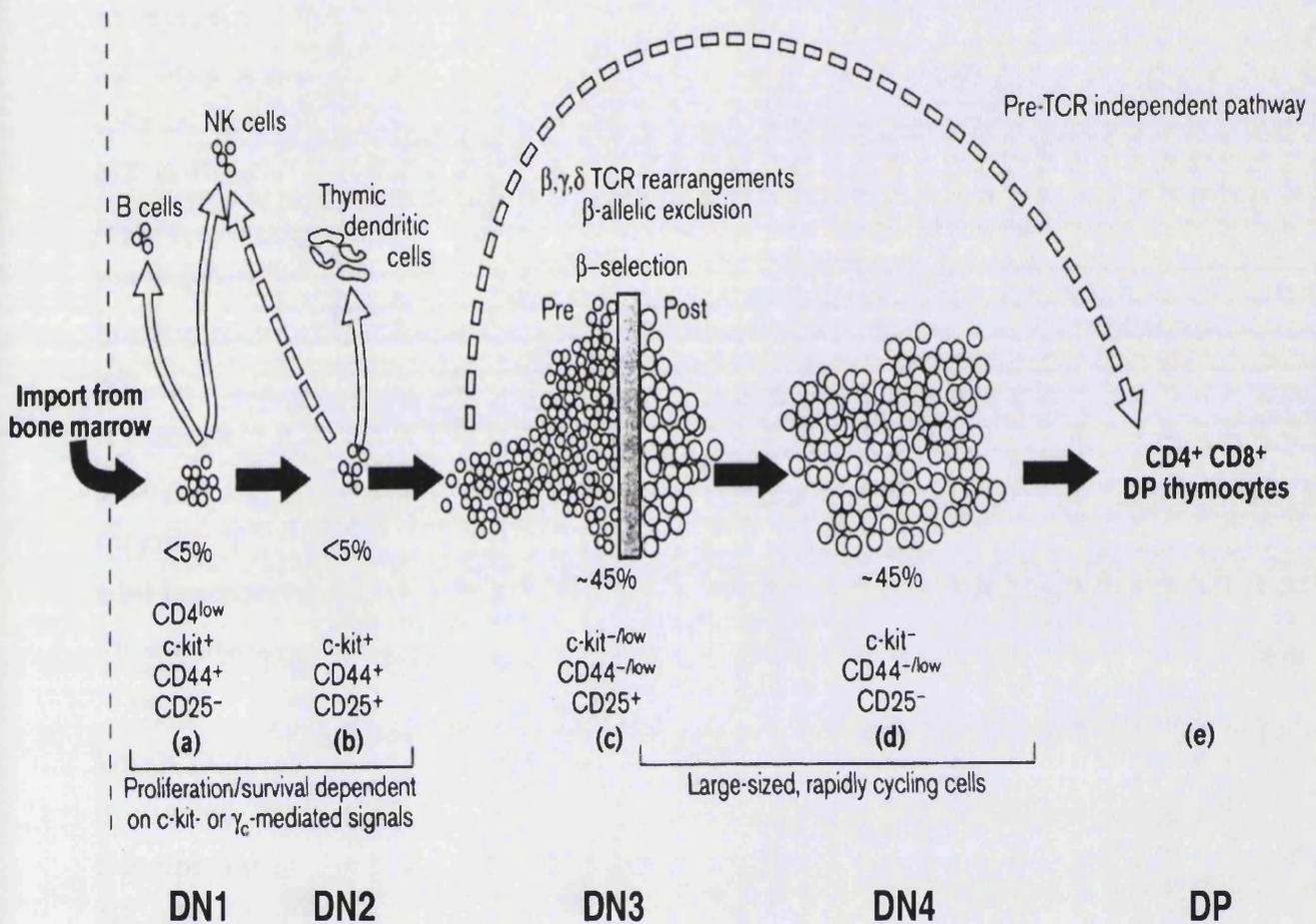


Figure 2 : Early thymocyte development

Schematic illustration of thymic differentiation of T cells up to DP stage (e) (Fehling and von Boehmer, 1997). The DN compartment is subdivided into four subsets, DN1-4, (a-d). Percentages of the approximate proportion of each subset (in an adult thymus) are given. Thick shaded arrows define the major developmental pathway; thin open arrows define minor, alternative pathways. The straight vertical dashed line on the left indicates the boundary of the thymus. The shaded bar in the middle of the DN3 (c) population represents a developmental checkpoint known as " β -selection" (see below and 2.4.2).

The most immature DN subset (DN1) is c-kit(+)CD44(+)CD25(-). These cells tend to express a small amount of CD4 on their cell surface and are therefore not strictly DN thymocytes (Wu et al., 1991a). Although they are lymphoid-restricted, DN1 cells are not yet fully committed to the T lineage, as they still can develop into B, NK or thymic dendritic cells (DC) (Ardavin et al., 1993; Wu et al., 1991b). In this regard, it is important to stress the importance of c-kit as a marker for DN1 cells, as the majority of CD44(+)CD25(-) thymocytes are not T cell precursors. CD44 is a homing molecule for cells that colonise the thymus (Wu et al., 1993), whether or not they are T cell progenitors. Indeed, only about 3% of all CD44(+)CD25(-) thymocytes express high levels of c-kit and are, therefore, "true" DN1. This problem can be also avoided by pre-gating on Thy1(+) cells, which excludes the majority of CD44(+) "contaminants". (Note: after the DN1 stage, the expression of c-kit becomes very similar to that of CD44, which justifies the use of only one of these markers in the analysis of the DN compartment.)

Upon up-regulation of CD25, thymocytes progress to the c-kit(+)CD44(+)CD25(+), DN2 stage of development. The first detectable DN2 cells during mouse foetal development occur at E13.5. DN2 cells can no longer generate B lymphocytes, but some still retain the potential to produce DC (Wu et al., 1996) or NK cells (Moore and Zlotnik, 1995). DN2 cells that commit to the T lineage (**pro-T** cells) begin to rearrange their TCR γ , δ and β loci (Capone et al., 1998). This is possible due to the initiation of expression of RAG-1/2 enzymes (Capone et al., 1998; Wilson et al., 1994). TCR β rearrangements occur only in the D (diversity) and J (junctional) regions; no full V-DJ rearrangements have been detected at this stage of development (Godfrey et al., 1994; Capone et al., 1998). DN2 is a population of actively proliferating cells (approximately five-fold more so than DN1 (Moore and Zlotnik, 1995)), suggesting the existence of a cell cycle activator mechanism. Signals from IL-7 receptor and c-kit (stem cell factor receptor) have been suggested to play a role in this mechanism (Rodewald et al., 1997).

Defined as c-kit(-)CD44(-)CD25(+), DN3 is the most abundant DN subset in a normal thymus. The first detectable DN3 cells during mouse foetal development occur at E14.5. All DN3 cells are committed to the T lineage (Wu et al., 1996), as they

can only give rise either to $\gamma\delta$ or $\alpha\beta$ T cells. Full V-DJ rearrangements of TCR β genes are now detectable in this population, and if *in frame* they enable the synthesis of a TCR β protein. Cells that successfully rearrange and express a functional TCR β chain are selected for further maturation (survival, proliferation and differentiation), a process termed “ β -selection” (Dudley et al., 1994; Mallick et al., 1993). The basis of this selection event is the emergence of a new signalling receptor, the pre-TCR (Saint-Ruf et al., 1994), a heterodimer consisting of a TCR β chain and an invariant chain, pT α (Bruno et al., 1995). As is the case of the TCR complex (see 1.3), the pre-TCR also associates with CD3 molecules, which are the active signalling components of the complex. Signals triggered by the pre-TCR are fundamental for maturation of $\alpha\beta$ cells beyond the DN3 stage; in contrast, $\gamma\delta$ cell development seems to be largely pre-TCR independent (Fehling et al., 1995a).

The final DN subset is DN4, characterised by a c-kit(-)CD44(-)CD25(-) surface phenotype. These cells, like DN3 thymocytes, first appear at E14.5 of foetal thymic development. DN4 cells start to express CD4 and CD8 at the mRNA level, and if cultured for 24 hours in medium without added differentiation factors they spontaneously develop into DP thymocytes. Their most striking feature is a high rate of proliferation, a consequence of β -selection (Hoffman et al., 1996). Indeed, this population is highly enriched for productive TCR β rearrangements, as shown by RFLP-PCR (restriction fragment length polymorphism -PCR) (Dudley et al., 1994), and shows relatively high levels of surface TCR β protein expression, as assayed by liposome staining (Bruno et al., 1999). Nevertheless, except for DN4 expressing the highest levels of pre-TCR on the surface, these pre-T cells can still generate cells of both $\gamma\delta$ and $\alpha\beta$ lineages (Bruno et al., 1999). In fact, Wilson and MacDonald have identified a subset (8%) of DN4 cells that is intracellular TCR $\gamma\delta$ (+), although extracellular TCR $\gamma\delta$ (-), and have suggested that these are $\gamma\delta$ precursors in the DN4 population (Wilson et al., 1999).

Immature DN thymocytes differentiate into either $\gamma\delta$ or $\alpha\beta$ T cells. If they commit to the $\gamma\delta$ lineage (1-2%), expressing TCR $\gamma\delta$, they basically remain negative for the expression of both CD4 and CD8 markers. In $\alpha\beta$ development, however, the next stage is CD4(+)CD8(+). Commitment between $\alpha\beta$ / $\gamma\delta$ lineages, and $\gamma\delta$

cells themselves, will be the focus of part 3 of this introduction; for the remaining of part 2 we will concentrate on $\alpha\beta$ T cell development.

The vast majority of cells in the thymic cortex, and about 80% of all thymocytes, are CD4(+)CD8(+), DP ("double positive"), progressing from the DN4 stage via CD8(+) immature single positive (ISP) intermediates. During foetal murine development, DP are first detectable at E15.5, one day later than DN4 thymocytes. In contrast, in the adult thymus, differentiation from the late DN to DP stages takes 2-3 days. This difference suggests different kinetics between foetal and adult thymocyte development (Manley, 2000). TCR α rearrangements are completed at the DP stage, which allows the expression of a TCR $\alpha\beta$ complex. Most DP cells express TCR $\alpha\beta$ at a low level, but around 30% have no detectable surface expression, while another 5% express it at a maximum level, identical to the one of SP thymocytes and peripheral T cells. DP cells are less proliferative than DN4 cells, 20% being in cycle. This subset is also very susceptible to cell death, either by neglect (lack of survival signal) or by apoptosis (programmed cell death). Only a minority of DP cells are able to survive both negative and positive selection (see 2.5.1).

DP cells which are positively selected (and survive negative selection) go through a lineage commitment decision, between CD4+ and CD8+ "single positive" (SP) lineages (see 2.5.2). These constitute the most mature thymic subsets. Approximately 10% of all thymocytes are CD4+ SP, whereas CD8+ SP account for 5% of the thymocytes.

SP cells are located in the thymic medulla, from where they finally leave the thymus to seed peripheral lymphoid organs. SP thymocytes also become functionally competent: CD8+ SP reproduce the functions of cytotoxic CD8+ peripheral T cells, and CD4+ SP cells function like helper CD4+ lymphocytes (reviewed in Crispe, 1995).

2.4 The role of pre-TCR in thymocyte development

The importance of a functional TCR β chain for early thymocyte differentiation became obvious through the analysis of mice deprived of such entity. The first informative mouse mutant was the naturally occurring SCID (severe-combined immunodeficiency), which cannot efficiently rearrange its TCR (and BCR) genes due to a defect in a DNA-dependent protein kinase that participates in somatic recombination (Blunt et al., 1995). Further evidence was gathered from the analysis of RAG (recombination activation gene)-deficient mice (Mombaerts et al., 1992a) and, decisively, from TCR β 'knockout' mice (Mombaerts et al., 1992b). In these murine models, T cell differentiation is severely impaired, with complete (SCID, RAG $^{-/-}$) or partial (TCR $\beta^{-/-}$) developmental blocks at the DN3 stage, and therefore none (SCID, RAG $^{-/-}$) or very few (TCR $\beta^{-/-}$) DP thymocytes produced. Since it was accepted that the TCR would not be expressed in immature DN thymocytes, an active search for a "pre-TCR" was initiated.

2.4.1 pT α as part of pre-TCR

By 1992, TCR $\beta^{-/-}$ and TCR $\alpha^{-/-}$ mice had been generated, and it was obvious that the two chains that composed the TCR had distinct roles in T cell development. Whereas TCR $\beta^{-/-}$ differentiation was severely blocked at the DN3 stage, TCR $\alpha^{-/-}$ thymocytes were able to mature much further, producing a large DP compartment (but no SP cells) (Mombaerts et al., 1992b). This led to a puzzling question, "how could a TCR β chain promote T cell development at the immature DN3 stage, in the absence of a functional TCR α chain?"

The key information that solved this mystery came from the analysis of a T cell line derived from immature SCID thymocytes, which was shown to express on the cell surface substantial amounts of a TCR β -CD3 complex that clearly lacked a conventional TCR α chain (Groettrup et al., 1993). The biochemical characterisation of this complex revealed the presence of a novel 33 kDa type I transmembrane

protein that was covalently bound to TCR β ; it was named pre-TCR α (pT α) (Saint-Ruf et al., 1994).

pT α belongs to the immunoglobulin superfamily and is encoded by a non-rearranging gene (Fehling et al., 1995b). Its expression pattern has been analysed in detail using RT-PCR (Bruno et al., 1995) and the results suggest that expression is confined to immature cells of the T cell lineage. Interestingly, pT α message has also been detected in mouse bone marrow, although B cells (including pre-B cells) and all other hematopoietic lineages do not express the gene. This might suggest that bone marrow harbours very early T lineage-committed precursors, and that pT α could be used as a marker for their identification (Fehling and von Boehmer, 1997).

In the thymus, pT α is expressed in all DN subsets (although at a very low level in DN1) and also in DP cells, but not in SP thymocytes. At the DP stage, pT α is competitively displaced by TCR α , thus allowing mature TCR to take the place of pre-TCR at the cell surface (Trop et al., 2000).

The generation and analysis of pT α ^{-/-} mice (Fehling et al., 1995a) has provided conclusive evidence for pT α being *the* partner of TCR β in immature DN thymocytes. The similarity between the phenotypes of pT α ^{-/-} and TCR β ^{-/-} mice is striking. In both cases, a 10-fold reduction in thymic cellularity is observed, due to severely decreased numbers of DP and SP thymocytes. The DN compartment is devoid of DN4 cells, whereas DN3 thymocytes are twice as abundant as in normal mice; this results in a developmental block at the DN3 stage, where pre-TCR expression and function is required for differentiation. Interestingly, development of $\gamma\delta$ cells is not impaired in these mice (in fact, their absolute numbers are higher than in normal mice), suggesting that the $\gamma\delta$ lineage does not depend on “ β -selection” for maturation (Fehling et al., 1995a).

2.4.2 “ β -selection”

To develop beyond the DN3 stage, thymocytes must traverse a checkpoint known as “ β -selection”, contingent on successful rearrangement of TCR β . The pre-TCR therefore acts as the molecular sensor that allows cells to pass this checkpoint.

As a consequence, there is intense proliferation and rapid progression to the DP stage. Cells that are not selected, because they fail to generate a pre-TCR complex, will die (unless they become $\gamma\delta$ cells) (Fehling and von Boehmer, 1997).

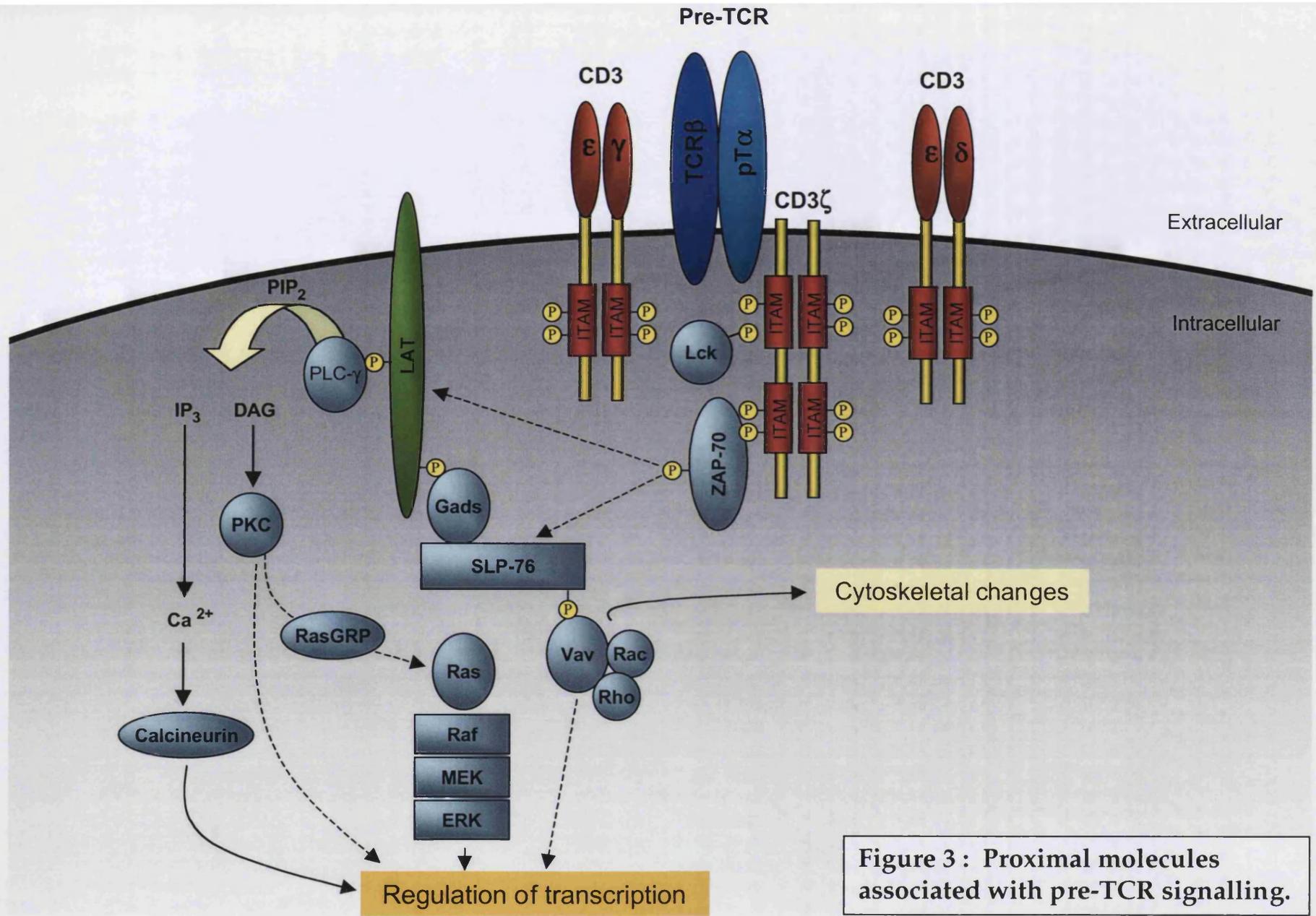
Cell survival, proliferation and differentiation at the DN3 \rightarrow DN4 (\rightarrow DP) transition are consequences of signals triggered by the pre-TCR. As with the mature TCR, the pre-TCR also relies on association with CD3 - γ , δ , ϵ , ζ - molecules for signal transduction. In fact, pre-TCR and TCR complexes have very comparable subunit compositions, the major difference being pT α in the place of TCR α (reviewed in Kruisbeek, 2000). However, unlike the TCR, the pre-TCR does not seem to require a ligand for initiation of signalling. In particular, the extracellular domains of pT α and TCR β are not required for signalling (Irving et al., 1998).

It is important to note that pT α ^{-/-} and TCR β ^{-/-} thymocytes, which express partial CD3 complexes on their cell surfaces, fail to signal and to differentiate, unless triggered by antibody-mediated cross-linking (Levelt et al., 1993). Also, it seems that pre-TCR complexes do not simply serve to increase the density of CD3 subunits at the cell surface, since transgenes used to restore development in pre-TCR deficient mice do not cause any detectable change in CD3 expression (Irving et al., 1998).

Therefore, pre-TCR seems to have an unique capacity to transduce β -selection signals, even when expressed at low levels and when not engaged by a surface ligand. Such properties of the pre-TCR have led to the suggestion that it acts as a constitutively active signalling complex. This ability could be conferred upon the pre-TCR by some unique property of pT α , a matter still under investigation (reviewed in Kruisbeek, 2000).

2.4.3 Components of pre-TCR signalling pathway(s)

The signalling molecules working downstream of the pre-TCR and their interactions are represented in **Figure 3**.



Regardless of how pre-TCR signalling is initiated, its association with CD3 subunits (γ , δ , ϵ , ζ) guarantees efficient signal transduction. As previously mentioned (1.3.1), CD3 molecules contain immuno-receptor tyrosine-based activation motifs (ITAM) within their cytoplasmic domain. As in the TCR, these ITAM are phosphorylated by activated Src-family protein tyrosine kinases (PTK). Based on the phenotype of individual 'knockout' mice for each of the four chains (γ , δ , ϵ , ζ), the most important for pre-TCR signalling seem to be CD3 ϵ and CD3 γ . Both CD3 ϵ ^{-/-} (Malissen et al., 1995) and CD3 γ ^{-/-} (Haks et al., 1998) mice display a severe block at the DN3 stage of development. In contrast, CD3 δ ^{-/-} (Dave et al., 1997) and CD3 ζ ^{-/-} (Love et al., 1993) mice are more permissive to thymocyte development. In CD3 δ ^{-/-} mice, for example, differentiation is only blocked at the late DP stage, beyond the influence of the pre-TCR complex. (For a thorough review on CD3 molecules, see Malissen, 1999)

Several lines of evidence document the involvement of lymphocyte-specific tyrosine kinase p56Lck in the transmission of pre-TCR signals. Both p56Lck-deficient (Molina et al., 1992) and dominant negative p56Lck transgenic (Levin et al., 1993) mice have a profound block in thymocyte development, similar (although less severe) to that of pre-TCR-deficient mice (TCR β ^{-/-}, pT α ^{-/-}). In addition, a constitutively active Lck transgene is capable of rescuing DP production in RAG-deficient (Mombaerts et al., 1994) and in pT α -deficient mice (Fehling et al., 1997b). The proximal promoter of p56Lck is one of two promoters (together with CD2 promoter) used for T cell-restricted expression of particular transgenes. It seems to be active from the DN1 stage of development, and mRNA levels for Lck are similar (varying by less than 2-fold) in all DN subsets (Buckland et al., 2000a). Lck protein, though, is dramatically (20-fold) up-regulated in DN2 → DN3 transition, exposing a post-transcriptional mechanism that regulates Lck in accordance with pre-TCR expression (Buckland et al., 2000a).

It is not yet clear whether another Src-PTK, p59Fyn, functions in the pre-TCR signalling cascade. Fyn-deficiency alone had no major impact on pre-T cell development (Appleby, 1992). However, the combined deficiency of Lck and Fyn clearly augmented the Lck^{-/-} block, since no DP cells were detected in the double

'knockout' mouse (Groves et al., 1996; van Oers et al., 1996), suggesting that Fyn plays a largely redundant role in pre-TCR signalling.

Src-PTKs are activated by the phosphatase **CD45**, which dephosphorylates their C-terminal regulatory tyrosine residues (Src-PTKs are inactive in the phosphorylated state). CD45-deficient mice show an accumulation of DN3 cells and a reduction in DP and SP numbers (Byth et al., 1996; Kishihara et al., 1993).

Src-kinase activity is negatively regulated by carboxy-terminal Syk kinase, **Csk**. Consistent with this, Csk-deficient mice have an "opposite" phenotype to Lck-deficient animals. Csk^{-/-} mice generate TCR(-) DP and SP cells, thus bypassing the need for pre-TCR and TCR in T cell development (Schmedt et al., 1998).

Phosphorylation of ITAMs of CD3 subunits by Src-PTKs creates docking sites for SH2-domain containing PTKs **ZAP-70** and **Syk**. Similar to Lck and Fyn, there seems to be a marked redundancy in the function of ZAP-70 and Syk downstream of the pre-TCR. Whereas single 'knock-out' mice exhibit none (Syk^{-/-}) or only very small (ZAP-70^{-/-}) defects in T cell differentiation (Cheng et al., 1995; Negishi et al., 1995; Turner et al., 1995), double 'knockout' animals show a complete arrest at the DN3 stage of development (Cheng et al., 1997a).

Several adaptors, exchange factors and GTPases previously known to be involved in mature TCR signalling have recently been implicated in transducing pre-TCR signals. Adaptor proteins **SLP-76** and **LAT** (linker for activation of T cells), which are substrates for TCR-induced PTK activity, are crucial for pre-TCR signalling. In both mouse 'knockout' models, thymocyte development is completely blocked at the DN3 stage (Clements et al., 1998; Pivniouk et al., 1998; Zhang et al., 1999). The fact that this block is not rescued by anti-CD3ε Ab cross-linking demonstrates the importance of these adaptor proteins as "common platforms" for the recruitment of effectors of pre-TCR signalling.

While LAT is located at the cell membrane, SLP-76 is recruited to LAT by another adaptor protein, **Gads**, which binds phospho-LAT via a SH2 domain, and SLP-76 (constitutively) via a SH3 domain (Zhang et al., 2000). Upon phosphorylation, SLP-76 acts as a platform for activation of multiple effectors, including phospholipase-Cγ (PLCγ), GTPase p21ras, and G-nucleotide exchange factor Vav.

Mice deficient for **Vav-1** display a partial defect at the DN → DP transition, with an accumulation of DN3 thymocytes (Fischer et al., 1995; Tarakhovsky et al., 1995; Turner et al., 1997; Zhang et al., 1995). Vav activates GTPases of the Rho family. The inactivation of **Rho** in the thymus caused a severe reduction in survival of pro-T cells and in cell cycle progression of pre-T cells (Galandrini et al., 1997; Henning et al., 1997). The pro-T cell defect may be related to a role in IL-7R signalling (see 2.6.2), but in the case of pre-T cells, Rho seems to control a p53-dependent survival checkpoint downstream of the pre-TCR (Costello et al., 2000). Besides Rho, another small GTPase, **Rac**, has been implicated in β -selection. A constitutively active Rac-1 mutant can partially substitute for the pre-TCR complex, and can fully complement defects of Vav-deficient pre-T cells (Gomez et al., 2000).

LAT/SLP-76 also activate GTPase **Ras** (p21ras) via guanine nucleotide exchange factor RasGRP, recruited to the membrane by DAG (diacylglycerol), which in turn is a second messenger of the PLC γ pathway (also activated by LAT) (reviewed in Leo, 2001). Constitutively activated Ras is able to reconstitute the DN → DP transition in RAG-deficient mice (Swat et al., 1996). Since Ras signalling feeds into the **MAPK** pathway (via Ras → Raf → MEK → ERK) in many cellular systems, where this pathway is responsible for cell growth and differentiation, its role in “ β -selection” has been intensely investigated. Initial studies produced contradictory results. On one hand, the expression of components of this pathway seemed to promote DP production in mice with deficient β -selection (Crompton et al., 1996; Swat et al., 1996). On the other, transgenic mice expressing catalytically inactive forms of Ras or **Raf** or ERK in thymocytes showed no defect in maturation to the DP stage (Alberola-Ila, 1995; O’Shea, 1996). However, further studies have supported the participation of MAPKs in maturation of DN thymocytes to the DP stage, as both constitutively active Ras (Gartner et al., 1999) and Raf (Iritani et al., 1999) rescue thymocyte maturation in the absence of the pre-TCR. Indeed, and perhaps more convincingly, a novel reporter plasmid system has demonstrated that **ERK** is strongly activated within the developing thymus (Michie et al., 1999).

2.4.4 Transcription factors involved in “ β -selection”

The nuclear targets of the signalling pathways triggered by the pre-TCR are still largely unknown. Nevertheless, gene targeting and transgenic studies, and gene manipulation experiments in foetal thymic organ culture, have suggested crucial roles for a few transcription factors in β -selection and DN \rightarrow DP transition. It has to be said, though, that the putative targets of these TFs in this process remain to be defined.

EGR-1 (early growth response gene -1) is a zinc-finger-containing TF, whose transcription correlates with β -selection. Moreover, its enforced expression (via a transgene) in a RAG-deficient background rescues thymocytes from developmental arrest at DN3, allowing maturation to proceed to the ISP (immature single positive, CD8⁺) stage (Miyazaki, 1997). However, development to the DP stage requires irradiation of the EGR-1 Tg / RAG KO mice. Thus, these data provided evidence for a two-step progression from DN3 to DP: the first step, DN3 to ISP, being promoted by EGR-1; and the second step, ISP to DP, relying on transcriptional induction of additional genes (Miyazaki, 1997). An independent group has also shown a similar rescue (to the ISP stage) of CD3 γ -deficient foetal thymocytes retrovirally transduced with any of the EGR family members, -1, -2 or -3 (Carleton et al., 2002). They also showed that dominant negative versions of Egr-1 interfered with the development of wild type foetal thymocytes, causing an accumulation of DN3 cells. The authors further demonstrated that ectopic expression of EGR-1 in a SCID cell line caused down-regulation of pT α and up-regulation of TCR α messages, a pattern associated with β -selection (Carleton et al., 2002).

E2A, **E2-2** and **HEB** are members of the **E** family of basic helix-loop-helix (**bHLH**) transcription factors. E-box sites are present in several important T cell-specific gene enhancers, including those of TCR α , TCR β and CD4 genes. Targeted disruption of **HEB** results in a 5-10 fold reduction in thymic cellularity due to a block in DN \rightarrow DP transition (Barndt et al., 1999). Unlike developmental blocks associated pre-TCR deficiency (which occur at DN3 stage), **HEB**^{-/-} thymi show a dramatic increase in ISP

thymocytes. In addition, this developmental arrest is not rescued by CD3 cross-linking. It is therefore still not clear whether HEB functions downstream of the pre-TCR, or if it is part of an unidentified pathway required for ISP → DP progression.

E2A proteins (E47 and E12) are initially expressed at the DN2 stage, before the pre-TCR. However, although E2A 'knockout' mice show a developmental block at that stage, stimuli that mimic pre-TCR signalling (anti-CD3 ϵ mAb) lead to a severe *reduction* of E2A proteins activity (Engel et al., 2001). Such stimuli also induce the bHLH inhibitor Id3 through a MAPK-dependent pathway. Strikingly, crossing E2A-deficient with RAG-deficient mice rescued the RAG-specific block at DN3 stage (Engel et al., 2001). Therefore, E2A proteins seem to initiate T cell differentiation at the DN2 stage but then inhibit further development in the absence of pre-TCR expression at DN3. In the later case, E2A proteins would be essential components of the "β-selection machinery", although not direct targets of pre-TCR. Efficient pre-TCR signalling would inhibit E2A activity possibly through a sequential effect on the following transcription factors: EGR-1 (induction) → Id3 (induction) → E2A (inhibition) (Engel et al., 2001).

A recent report has suggested the importance of E2A-HEB heterodimers in T cell development. These dimers are abundant in thymocyte extracts, and their role might have not been exposed in the single 'knockout' mice due to compensation by homodimers of the other (not disrupted) bHLH protein. A dominant negative allele of HEB was shown to form non-functional heterodimers with E2A proteins, and mice carrying this mutation displayed a stronger and earlier block in T cell differentiation than HEB^{-/-} mice: cells accumulated at the DN2 stage, before pre-TCR expression, and they could not be rescued by a functional TCR transgene (Barndt et al., 2000). This phenotype is similar to that of E2A-deficient mice, and could therefore be due to sequestration of E2A.

CREB (cyclic-AMP response element binding protein) is a basic/leucine zipper TF that binds CRE sequences present in regulatory regions of many genes, including TCR α , TCR β , CD3 δ and CD8 α . Initial studies with transgenic mice expressing a dominant-negative form of CREB showed normal thymocyte development, even though activated thymocytes and T cells failed to proliferate efficiently or produce appropriate cytokine responses (Barton et al., 1996). More

recently though, CREB null mice were generated and their thymi have a severe reduction in $\alpha\beta$ T cell number caused by a partial block in the DN to DP transition (Rudolph et al., 1998). The previous studies had failed to identify a T cell developmental role for CREB probably because the mutation did not inactivate one of the three major isoforms of CREB (CREB β), which in fact was over-expressed in those transgenic mice as part of a compensatory mechanism for the loss of the other isoforms (α , δ) (Rudolph et al., 1998).

The **Ikaros** family of TFs (Ikaros, Helios, Aiolos) are collectively essential for lymphoid lineage commitment (see 1.2). Ikaros-deficient mice have a 5-fold reduction in early thymocyte precursors of DN1 and DN2 subsets, and a 10-fold reduction of DN3 cells, but surprisingly have normal absolute numbers of late DN4 cells (Winandy et al., 1999). When these mice were crossed onto a RAG^{-/-} background, the deficiency in Ikaros allowed thymocytes to progress from DN to DP (and CD4⁺ SP) stages, rescuing the RAG^{-/-} block without need of pre-TCR signalling. However, this developmental transition was not accompanied by the proliferation that is usually associated with β -selection. It therefore seems that Ikaros has a regulatory role in DN to DP differentiation, inhibiting the progression of cells that lack a functional pre-TCR. Interestingly, this suggests that β -selection, as a checkpoint, is not regulated simply by the presence or absence of positive signals (generated by pre-TCR), but also involves negative modulators of differentiation that set thresholds that need to be overcome by pre-TCR signalling.

Tcf-1 (T cell factor 1) and **Lef-1** (lymphoid enhancer-binding factor 1) are members of the high-mobility group (HMG) of proteins, first identified because of their selective binding to CD3 ϵ and TCR α gene enhancers, respectively. They are essential components of the Wnt signalling pathway (see 2.6.3). Foetal thymic organ cultures of Tcf^{-/-}/Lef^{-/-} double KO thymocytes (the mice die at birth) showed a complete block at the ISP stage, with an earlier accumulation (3-fold in comparison with wild type FTOCs) at the DN3 stage (Okamura et al., 1998). After 7 days in FTOC, Tcf^{-/-}/Lef^{-/-} ISP cells showed very low levels of DNA rearrangements and TCR α transcripts, in contrast with wild type or single KO FTOCs. These data suggest that, together, Tcf and Lef play an important role in the DN \rightarrow DP transition.

Sox-4 is the third member of the HMG box family of T cell TFs. In adult mice, its expression is restricted to immature B and T cells and the gonads. In the embryo, Sox-4 is also expressed in the heart, and thus Sox-4 KO mice died at E14.5 from defects in heart development (Schilham et al., 1996). T cell development of Sox-4 deficient E13.5 thymocytes was studied *in vitro* by FTOC. These experiments highlighted a 10- to 50-fold reduction in DP and SP production as compared with age-matched wild type cells (Schilham et al., 1997). Consistent with these results, Sox-4 deficient foetal liver progenitors also displayed a reduced ability to compete with wild type cells in reconstituting thymic populations after injection into sub-lethally irradiated hosts (Schilham et al., 1997).

As illustrated by the data review in this section, insights into the components of the pre-TCR signalling cascade(s) have been largely obtained through genetic manipulation of mice. The major developmental blocks observed in those mouse mutants are represented in **Figure 4**.

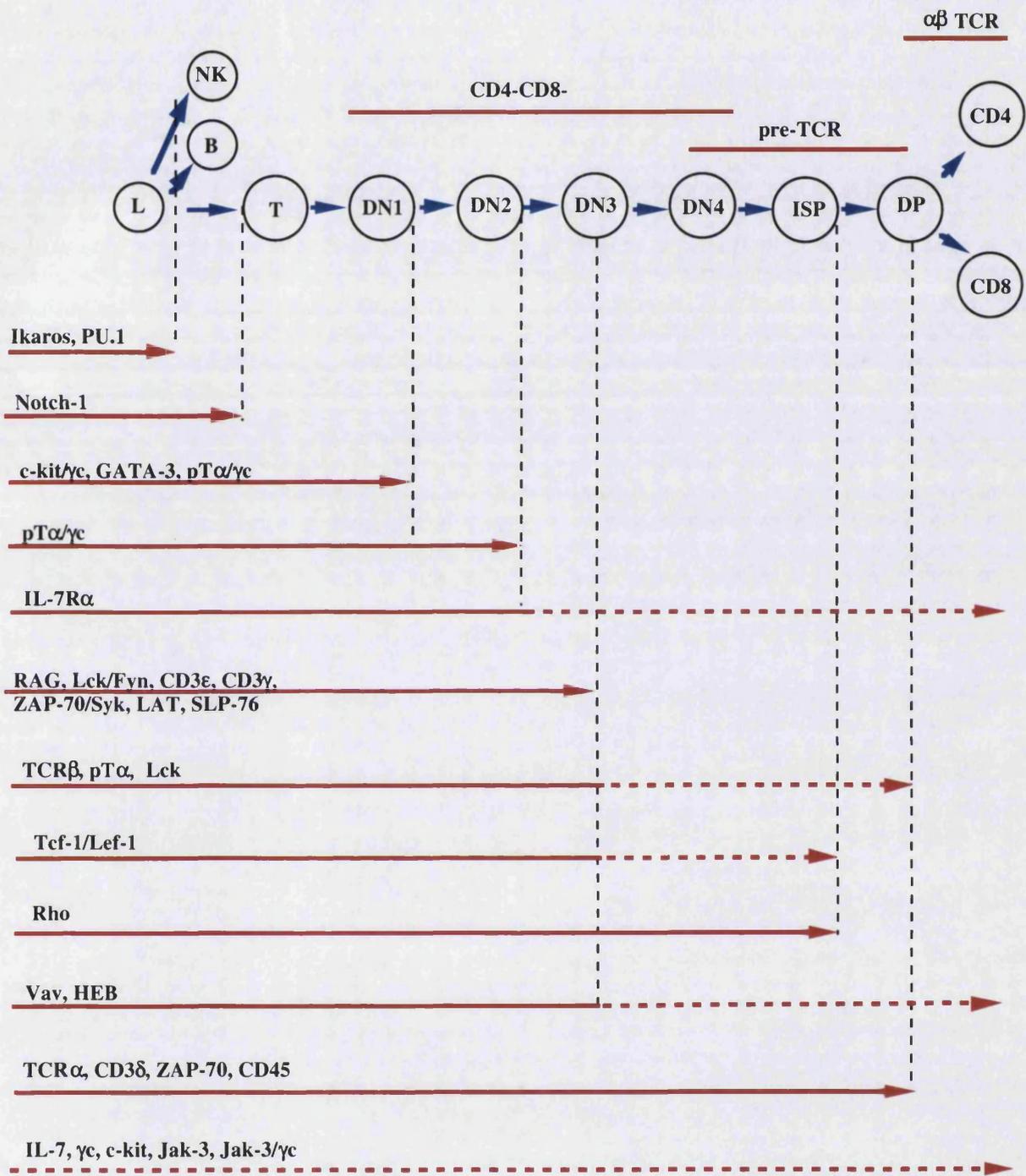


Figure 4 : T cell developmental blocks caused by gene deletion (adapted from Buckland, 2000).

2.5 The role of TCR $\alpha\beta$ in thymocyte development

The pre-TCR is responsible for the intense proliferation of pre-T cells and for their differentiation along the DN3 > DN4 > ISP > DP pathway. As a result, a large pool of DP cells expressing TCR β are generated. The completion of TCR α rearrangements at the DP stage finally allows the expression of the TCR $\alpha\beta$ complex (in the place of the pre-TCR). The TCR $\alpha\beta$ participates in three crucial events during late thymocyte development: positive selection of cells that are self-MHC restricted, negative selection (deletion) of potentially auto-reactive cells, and lineage commitment between the CD4+ and CD8+ SP lineages.

2.5.1 Positive and negative selection

The TCR $\alpha\beta$ recognises antigen associated with MHC molecules. Positive selection guarantees that only DP thymocytes whose TCRs binds peptide-MHC effectively mature any further. Negative selection ensures that DP cells bearing TCRs that interact too strongly with peptide-MHC, and hence could recognise self peptides, are eliminated.

Thymus grafting experiments (Fink and Bevan, 1978; Zinkernagel et al., 1978a; Zinkernagel et al., 1978b) demonstrated that MHC-restriction is imposed in the thymus. The bone marrow-derived components of the thymus (T cells and APCs) have little influence on positive selection. In contrast, thymic epithelial cell grafts are fully competent to impose MHC-restriction on T cells (reviewed in Crispe, 1995). Thus, thymocytes with the potential to recognise exogenous antigens in the context of self-MHC molecules expressed on the thymus epithelium are selected to complete their maturation.

The thymus is also responsible for induction of the so-called "central T cell tolerance", which consists of physical elimination of immature self-reactive thymocytes by clonal deletion. Support for the concept of clonal deletion came

from the finding that mice expressing the MHC class II molecule, H-2E, lacked T cells utilising the variable element V β 17, in contrast with H-2E negative mice (Kappler et al., 1987a; Kappler et al., 1987b). Furthermore, in TCR HY transgenic mice, transgenic T cells that are specific for a male antigen are present in female mice, but not in male siblings (The et al., 1990; von Boehmer, 1990).

Unlike positive selection, negative selection appears to be primarily a result of the interaction of thymocytes with bone marrow-derived cells, in particular APCs (such as dendritic cells) (Matzinger and Guerder, 1989). Reports that the thymic epithelium induces positive selection, while APCs stimulate negative selection inspired the "altered ligand model". According to it, the two selection events are induced by mutually exclusive cell populations (epithelial cells *versus* APCs), which display a different array of self-peptides bound to their MHC molecules. Although this does explain how the same TCR can be involved in both types of selection, a considerable amount of data argues against it (reviewed in Sebzda et al., 1995). In particular, cell type does not seem to be critical. For example, transfected fibroblasts are capable of mediating positive selection (Hugo et al., 1993; Pawlowski et al., 1993) and thymic epithelial cell lines can induce negative selection (Hugo et al., 1994). In addition, the repertoire of peptides bound to MHC in thymic cortical epithelial cells, supposed to be restricted to the thymus for induction of positive selection, is very similar to the subset present in the spleen (Marrack et al., 1993).

The current and widely accepted model to explain positive and negative selection is the affinity/avidity model. This hypothesis argues that positive selection is the result of low/intermediate avidity binding of thymocytes to peptide-MHC, whereas negative selection is triggered by high avidity interactions. The model does not distinguish between cell types involved in such interactions. Ultimately, T cells with intermediate avidity for self-peptide-MHC are allowed to exit the thymus. Below the threshold required for positive selection (null interaction), thymocytes are not selected and eventually die. It should also be said that in the periphery, T cell activation has been shown to require a higher avidity interaction than the one necessary for negative selection in the thymus (Pircher et al., 1991) - which is important to understand how a positively selected thymocyte becomes an

activated peripheral lymphocyte without the activation signal being perceived intracellularly as a deletion signal.

To link this hypothesis with the data demonstrating critical roles for thymic epithelial cells in positive selection and for APCs in negative selection, variation MHC density in these cell types, and of TCR density in developing thymocytes, has been postulated. For example, TCR density increases as thymocytes mature, and as they move from the cortex to the medulla. Moreover, thymic cortical epithelial cells express lower levels of MHC molecules than APCs, which are found primarily in the medulla. These differences explain how the same TCR can be positively selected in the cortex and then possibly negatively selected in the medulla.

The affinity/avidity model is supported by much experimental data in which avidity thresholds were supposedly modified either by mutations in MHC molecules or by variations in co-receptor (CD4, CD8) expression levels (reviewed in Sebzda et al., 1995). In particular, experiments have demonstrated how a particular peptide influences thymic selection of thymocytes expressing a specific TCR (as in TCR transgenic mice). These studies examined the effects of peptide agonists (variants of wild type peptide capable of inducing response) and antagonists (which contain subtle amino acid variations from the wild type peptide and functionally inhibit responses) on selection, and showed there is no correlation between the nature of the peptide, in terms of it being an agonist or an antagonist, and the degree of positive or negative selection. Rather, by manipulating the levels of the peptide, certain avidity thresholds seemed to be surpassed and this led to either positive (at low/medium avidity) or negative (at high avidity) selection (Sebzda et al., 1994; Hogquist, 1994 ; reviewed in Sebzda et al., 1999).

How do avidity thresholds translate themselves into selection of thymocytes, i.e., how does the cell distinguish between positive and negative selection? Are there distinct intracellular pathways downstream of the TCR that lead to either positive or negative selection? If so, where do these pathways diverge – proximally or distally? Or, are avidity thresholds translated purely as a 'strength of signal' response? The evidence collected thus far suggests that unique signalling aspects of positive versus negative selection do exist, and a point of divergence has been identified at the level of MAPK pathways. However, experiments in which the

intensity of individual signalling pathways was manipulated showed that there is also a component of signal strength that modifies T cell selection (reviewed in Mariathasan, 1999).

Regarding the MAPK pathway, dominant negative versions of Ras, Raf and Mek-1 have been shown to inhibit positive selection, while not affecting negative selection (Alberola-Ila et al., 1995; Alberola-Ila et al., 1996; O'Shea et al., 1996; Swan et al., 1995). These findings have been supported by retroviral transduction of activated forms of these molecules, and by studies with specific inhibitors of the pathway. Conversely, the p38 (an alternative MAPK) pathway seems to favour negative selection (Sugawara et al., 1998) (see 2.5.3).

2.5.2 CD4 / CD8 lineage commitment

TCR $\alpha\beta$ specificity is co-ordinated with MHC (class I or II) binding and co-receptor expression during CD4/CD8 lineage commitment. This thymic event is crucial for the establishment of a competent immune system, as TCR-co-receptor-MHC matching is translated into the peripheral physiology of T cells. Cells that commit to the CD4+ SP lineage in the thymus display helper functions in the periphery, whereas CD8+ SP thymocytes become cytotoxic lymphocytes (reviewed in Singer, 1999).

Experiments in TCR transgenic mice demonstrated that the MHC-specificity of the TCR dictated which co-receptor would be expressed on mature T cells (Kisielow et al., 1988; Scott et al., 1989; Sha et al., 1988a; Sha et al., 1988b; Teh et al., 1988). Thus, expression of MHC class I specific TCR transgenes resulted in the generation of CD8+ T cells, whereas expression of MHC class II TCR transgenes produced CD4+ T cells. These observations inspired the "*instructive model*" of CD4/CD8 lineage commitment, which postulated that simultaneous co-engagement of peptide-MHC complexes by TCR and a particular co-receptor instructed the DP cell to selectively terminate the expression of the other, non-engaged, co-receptor molecule.

Further support for this model came from experiments involving chimeric CD8/CD4 molecules in which the cytosolic tail of CD8 was switched for that of

CD4 (Itano et al., 1996; Seong et al., 1992; Seong and Parnes, 1992). Expression of the chimeric CD8/CD4 molecule in MHC class I-specific TCR transgenic mice generated significant numbers of CD4 T cells that otherwise would not arise, suggesting that the tail of CD4 signalled commitment to the CD4 lineage. Conversely, mutations or removal of the CD8 tail impaired the production of CD8 T cells (Fung-Leung et al., 1993; Itano et al., 1994). However, since the only signalling pathway known to be downstream of both the CD4 and CD8 co-receptors involved p56Lck, it was not clear how thymocytes distinguished between tyrosine kinase activation via CD4/MHC-II or CD8/MHC-I co-engagements.

Studies on the interaction between Lck and CD4 or CD8 revealed that the PTK binds to the cytosolic tail of CD4 with greater affinity than to the tail of CD8 (reviewed in Singer, 1999). Consequently, TCR/CD4 co-engagement by MHC-II generates a stronger intracellular signal than TCR/CD8 co-engagement by MHC-I. These considerations were formulated into a refinement of the instructive model, the "*strength of signal*" hypothesis. According to it, strong Lck signals in DP thymocytes led to commitment to the CD4 lineage, whereas weak Lck signals induced CD8-commitment. This hypothesis provided a plausible explanation for the results with the chimeric CD8/CD4 molecule, and was further supported by the generation of MHC-II-restricted CD8 cells in CD4^{-/-} mice (Matechak et al., 1996) and by the preferential generation of CD4⁺ SP (to the detriment of CD8⁺ SP) in Lck^{-/-} mice in which Lck expression was restored by an inducible transgene (Legname et al., 2000). However, it was not consistent with the findings that a tail-less CD4 molecule was able to promote CD4⁺ SP differentiation (Killeen and Littman, 1993) and that mice transgenic for a dominant negative Lck showed no perturbation in CD4 *vs.* CD8 lineage distributions (Hashimoto et al., 1996; Levin et al., 1993).

Although the analysis of MHC-I and MHC-II deficient mice (Grusby et al., 1991; Zijlstra et al., 1990) confirmed the importance of class specificity in determining the co-receptor phenotype (SP cells were practically absent), the detection of 'transitional' populations, CD4^{low}CD8⁺ cells in MHC-I^{-/-}, CD4⁺CD8^{low} thymocytes in MHC-II^{-/-}, was difficult to conciliate with the instructive model. Indeed, this data

implied that the instruction to shut off expression of the “unwanted” co-receptor had been given in the absence of MHC. This led to the proposal of *non-instructive* models for CD4/CD8 commitment, which stated that lineage decision was made independently of TCR/MHC interactions, the latter serving only as survival/selection signals for pre-committed thymocytes. The lineage decision was postulated to either depend on TCR-independent factors or to occur randomly, as in the “*stochastic*” model (Davis et al., 1993).

The major objective of current research on CD4 *vs.* CD8 lineage commitment is the identification of factors that can be the main players in such non-instructive models. Interestingly, very recent results have shown that CD83-deficient mice have a specific block in CD4+ SP differentiation (down to 20% of their normal numbers), without increased DP or CD8+ SP (Fujimoto et al., 2002). The lack of this surface molecule (expressed on thymic epithelial and dendritic cells) in host mice did not allow donor WT cells to develop into CD4+SP thymocytes. The molecular basis of this phenotype could provide a key insight to the mechanism(s) of CD4/CD8 lineage fate determination.

2.5.3 Signalling pathways and transcription factors

downstream of TCR $\alpha\beta$

The TCR $\alpha\beta$ complex shares many similarities to the pre-TCR complex, with major differences being TCR α in place of pT α , and the presence of co-receptors CD4 or CD8. Importantly, they share many signalling components. In fact, most of the factors mentioned for pre-TCR signal transduction (see 2.4.3) were initially identified as components of the TCR $\alpha\beta$ pathway and then demonstrated to be also part of pre-TCR signalling cascades.

An important feature of TCR $\alpha\beta$ signalling is **signal strength**. While DN cells express very low levels of surface TCR β , the majority of DP cells express intermediate levels, and SP cells express the highest levels (10-fold higher than an average DP). Obviously, the respective complexes containing CD3 molecules will produce overall signals of quite different intensities. In addition, the incorporation

of CD4 and/or CD8, which bind *Lck* molecules via their cytosolic tails, into TCR $\alpha\beta$ complexes will also influence signalling potential. Therefore, TCR $\alpha\beta$ signals are of higher magnitude than pre-TCR signals. As such, they can produce dramatically different results such as cell differentiation (pre-T cells) or cell death (mature T cells) (reviewed in van Oers, 1999).

a) Signalling molecules

The PTK *Fyn* is expressed at 10-fold higher levels in SP cells than in DP thymocytes (Olszowy et al., 1995). *Fyn*-deficient SP cells show impaired proliferation in response to TCR signals (Appleby et al., 1992; Stein et al., 1992). A similar peripheral phenotype is seen in *Fyb*^{-/-} mice. *Fyb* is an additional substrate for *Fyn*-mediated phosphorylation in mature T cells (but not in pre-T cells) (da Silva et al., 1997). *Fyb* seems to be involved in cell adhesion (via the LFA-1/ICAM system), which is required for T cell activation, but does not play a significant role in T cell development, as thymus and spleen of *Fyb*^{-/-} mice are normal (Geng et al., 2001; Griffiths et al., 2001).

ZAP-70 is also up-regulated in SP thymocytes, and *ZAP-70*^{-/-} mice are deficient in these cells, although earlier T cell development is not impaired (Kadlecek et al., 1998; Wiest et al., 1997). This might reflect the fact that *Syk* is down-regulated after β -selection (Kadlecek et al., 1998; Wiest et al., 1997), and therefore DP thymocytes, unlike pre-T cells, cannot rely on its compensating role in the absence of ZAP-70.

Itk is a member of the Tec family of PTKs that localise to the cell membrane by interacting with phospholipid PIP₃. Mice deficient in *Itk* have a small reduction in SP thymocytes, an effect more obvious in a TCR transgenic background (Liao and Littman, 1995). Moreover, mature *Itk*^{-/-} T cells are hypo-responsive to TCR signals, with impaired IL-2 production and cell proliferation.

Vav deficiency seems to have a more dramatic effect on TCR than on pre-TCR signalling. *Vav*^{-/-} mice show a 90% block in SP production, whereas DP production via β -selection is only reduced by a factor of 50% (Fischer et al., 1995; Turner et al., 1997). A critical role for *Vav* downstream of the TCR $\alpha\beta$ is also suggested by the

fact that SP production in $RAG^{-/-}/Vav^{-/-}$ mice cannot be rescued by a TCR transgene (F5, a MHC class I restricted TCR), unlike in the single $RAG^{-/-}$ background, where significant numbers of CD8+ SP are produced.

MAPK pathways may play an important role in discriminating signals for positive or negative selection. Current data suggest that whereas the Ras / Raf / MEK / ERK pathway is needed for positive selection, the alternative Ask / MKK / p38 pathway is unique to negative selection (reviewed in Mariathasan, 1999). Briefly, dominant negative transgenes of both Ras and MEK-1 inhibit positive selection, while not affecting negative selection (Alberola-Ila et al., 1995; Alberola-Ila et al., 1996; O'Shea et al., 1996; Swan et al., 1995). Furthermore, expression (via retroviral transduction) of constitutively active MEK-1 in FTOC rescues positive selection in $TCR\alpha^{-/-}$ cells, and inhibition of MEK-1 with the drug PD98059 impairs positive, but not negative, selection in WT thymocytes (Sugawara et al., 1998). Similar experiments using constitutively active MKK6 (which activates p38) and specific p38 inhibitor SB203580 suggested that the p38 pathway was important for negative, but not positive, selection (Sugawara et al., 1998).

b) Transcription factors

EGR-1 is rapidly up-regulated after TCR stimulation, and this induction is dependent on Ras / MAPK activation. In MHC-deficient thymi, EGR-1 expression is dramatically reduced, but can be induced *in vitro* by anti-CD3 ϵ antibody treatment (Shao et al., 1997). Transgenic mice over-expressing EGR-1 were able to positively select CD8+ SP cells on a non-selecting background and even on a MHC-deficient background (Miyazaki and Lemonnier, 1998). Although these data imply a role for EGR-1 in positive selection of CD8+ SP cells, recent reports (using pharmacological inhibition of EGR-1) have suggested that its expression is not required for CD8 lineage commitment and selection (Basson et al., 2000). In summary, EGR-1 is one of the earliest downstream targets of TCR signalling, and thus may potentially regulate gene transcription during selection, but does not seem to be essential for the selection process itself.

Nurr77, a member of the nuclear orphan receptor family of TFs, appears to be involved in negative selection. Three lines of evidence support this role for Nurr77: its activity in TCR-induced apoptosis (Liu et al., 1994; Woronicz et al., 1994), the fact that a dominant negative isoform blocks negative selection (Lee et al., 1995; Zhou et al., 1996), and the observation that a constitutively active version of the molecule induces apoptosis (Lee et al., 1995). However, Nurr77^{-/-} mice show no gross selection abnormalities, suggesting that redundant factors (possibly other nuclear orphan receptors, such as NOR-1) may function in these processes.

IRF-1 (interferon regulatory factor 1) appears to play a role in both positive and negative selection. IRF-1^{-/-} mice have reduced numbers of thymic and peripheral CD8⁺ T cells, and positive selection of MHC class I -restricted transgenes is impaired on an IRF-1^{-/-} background (Penninger et al., 1997). This is not due to a thymic stroma defect, as normal development of WT thymocytes can be supported by IRF-1-deficient stroma, both in FTOC and in bone marrow chimeras. In terms of negative selection, a 1,000-fold increase in the amount of selecting peptide was required to delete TCR transgenic thymocytes on an IFR-1^{-/-} background (Penninger et al., 1997). IRF-1 only seems to play a role in late T cell development, as its expression is not detectable in immature thymocytes before TCR $\alpha\beta$ expression.

One of the crucial functions of TCR signalling is the induction of expression of cytokines, especially IL-2, which is vital for T cell physiology. IL-2 expression is regulated by several transcription factors that are downstream effectors of TCR signalling cascades, namely NFAT, NF- κ B and CREB.

NFAT (nuclear factor of activated T cells) was initially identified as an inducible protein complex that could bind a regulatory element in the IL-2 promoter, but in fact NFAT binding sites are also present in the IL-3, IL-4 and TNF- α promoters. TCR signalling activates NFAT via the PLC- γ /IP₃/Ca²⁺/calcineurin pathway. Calcineurin dephosphorylates inactive NFAT in the cytosol, thereby unmasking a nuclear localisation signal. This change leads to rapid translocation to the nucleus, where NFAT pairs with AP1 complexes composed of Fos and Jun dimers (reviewed in Kuo, 1999). Over-expression of Vav also leads to a marked increase in NFAT activity and IL-2 expression (Wu et al., 1995). Importantly, NFAT-4^{-/-} mice show a

reduction in SP cells (Oukka et al., 1998). This defect seems to be due to increased sensitivity of DP thymocytes to apoptosis, as the expression of survival gene Bcl-2 is reduced.

NF- κ B transcription factors include NF- κ B-1/-2, Rel-A/-B and c-Rel. Their activity is controlled at the post-translational level by association with inhibitory I κ B proteins. TCR signalling induces degradation of I κ B, which releases NF κ B and allows it to translocate to the nucleus. Although NF κ B-1^{-/-} mice showed no abnormality in T cell development, this could be due to functional redundancy between the several NF κ B proteins (Sha et al., 1995; Snapper et al., 1996). To circumvent this, transgenic mice expressing a "super-inhibitory" form of I κ B under the control of T cell specific promoters were generated (Boothby et al., 1997; Esslinger et al., 1997). Since this mutant I κ B cannot be phosphorylated and degraded in response to TCR engagement, the function of all NF κ B proteins are inhibited. These mice displayed significantly decreased numbers of peripheral CD8 T cells, and a severe proliferative defect in response to TCR cross-linking (among other activation defects).

CREB is a basic/leucine zipper TF that binds CRE sequences present in regulatory regions of many genes, including TCR α , TCR β , CD3 δ and CD8 α (see 2.4.4). CREB is responsive to both TCR-dependent and TCR-independent signals. Among the latter, CREB activity is particularly sensitive to variations in intracellular cyclic-AMP levels. Treatment of foetal lobes with cyclic-AMP analogues, in FTOC, resulted in a major loss of DP cells, presumably due to apoptosis (Lalli et al., 1996). On the other hand, upon TCR engagement, CREB is rapidly phosphorylated and activated via a single pathway that involves Lck/Ras/Raf/MEK/RSK-2 (Muthusamy and Leiden, 1998). Peripheral T cells from transgenic mice expressing a dominant-negative CREB failed to proliferate efficiently or to produce appropriate cytokine (namely IL-2) responses (Barton et al., 1996). These defects correlated with a decreased induction of transcription factors Fos and Jun (AP1 complex). CREB(-) cells underwent apoptosis in response to a variety of stimuli that activated CREB(+) T cells (Barton et al., 1996).

2.6 TCR-independent signalling pathways in T cell development

T cell development, in its full extent, is not a cell autonomous process, as it requires the input of the thymic microenvironment (see 2.2). In addition to pre-TCR/TCR signals, both soluble factors and cell-cell interactions are also important in determining the fate of developing thymocytes.

2.6.1 Pro- and anti-apoptotic pathways

Apoptosis (programmed cell death) is a dominant feature of T cell differentiation. At the major checkpoints of early (β -selection) and late (positive and negative selection) development, apoptosis is responsible for the removal of non-selected cells. Apoptosis is characterised by cytoplasmic blebbing, chromatin condensation, exposure of phosphatidyl-serine residues on the outside of the cell, DNA fragmentation and, as a consequence, loss of cell viability (reviewed in Cory, 1995).

DP thymocytes are particularly sensitive to apoptosis, which can be triggered both by TCR engagement (Smith et al., 1989) and by TCR-independent stimuli, such as γ -radiation (Sellins and Cohen, 1987) and glucocorticoids (Cohen and Duke, 1984).

Glucocorticoids (GC) been known for a long time to induce thymocyte death at high concentrations. Blomberg and Andersson showed in 1971 that 100% of cortical and 50% of medullary thymocytes were depleted after 48 hours of administration of GCs (Blomberg and Andersson, 1971). However, recently, a more complex role for GCs in the thymus has been proposed, in which GC signalling modulates TCR signalling during thymocyte selection (reviewed in Vacchio, 2000; Ashwell, 2000).

The major source of GC (corticosterone in mice, cortisol in humans) in the body are the adrenal glands. Although circulation via the blood is probably sufficient for GC function in the thymus (reviewed in Vacchio, 2000), the production of GC in

the thymus has been investigated. The results are controversial, as some reported the presence of functional cholesterol-metabolising enzymes (which are involved in GC production) in subsets of thymic epithelial cells (Vacchio et al., 1994), whereas others reported their absence (Jenkinson et al., 1999).

Being lipophilic, GCs are able to diffuse through the plasma membrane and bind to receptors (GR) in the cytosol. This interaction promotes nuclear translocation of the receptor, and binding to glucocorticoid response elements in gene regulatory regions. GRs can also affect gene transcription indirectly, by interacting with CREB, AP-1 and NF κ B (reviewed in Ashwell, 2000).

Death is not necessarily the only consequence of GR activation. Indeed, while GC and TCR signalling taken individually induce apoptosis of DP cells, their collective action results in cell survival (Iwata et al., 1991; Zacharchuk et al., 1991; Zacharchuk et al., 1990). This “mutual antagonism” has been proposed to be an important modulator of TCR signalling, producing the low/intermediate signal intensities required for positive selection. In this model, which is a refinement of the signal strength hypothesis, if TCR avidity > GC signal or if TCR avidity < GC signal, death is the outcome, due to TCR-induced apoptosis in the first case and GC-induced apoptosis in the second. However, if the signals are of similar intensities (which would be the case for intermediate TCR avidities), they cancel each other out, allowing cell survival and differentiation.

There is some evidence supporting this “mutual antagonism” model. Using metyrapone, an inhibitor of an enzyme involved in GC synthesis, a significant increase in TCR-induced cell death of wild type E17 DP cells was observed in FTOC (Vacchio and Ashwell, 1997). Also using a model for positive selection (transgenic HY female thymi), metyrapone was shown to cause a large loss in FTOC-cell recovery that was directly attributable to enhanced DP apoptosis, as measured by TUNEL. Thus, cells that would normally have undergone positive selection were deleted in the absence of GC. Since the utilised drug blocks synthesis of GC *in situ*, these experiments also established a role for endogenous, non-circulating, GCs in thymic selection.

One of the best described apoptotic pathways is the one mediated by **Fas / Fas-L** (*L, ligand*). Although it has been for long known to play a crucial role in peripheral deletion of activated mature T cells (at the end of an immune response), its function in the thymus is less clear. Fas is a member of the TNF (tumour necrosis factor) receptor family, which also includes TNF-R1, DR (death receptor) -3/-4/-5. Upon binding of Fas-L to Fas, apoptosis of Fas-bearing cells is induced. Fas is expressed at low levels in DN thymocytes, but it is highly expressed in DP and SP cells. Indeed, DP cells have been observed to selectively undergo apoptosis upon treatment with an anti-Fas antibody (Ogasawara et al., 1995).

Naturally occurring Fas mutant mice (*lpr* mice) accumulate thymocytes with a CD4(-)CD8(-)CD3(+) phenotype and suffer from auto-immunity and lymphadenopathy. Additionally, both positive and negative selection seem to be impaired in these mice (Kishimoto et al., 1998; Kurasawa et al., 1999). Another TNF receptor family member, DR3, has also been reported to play a limited role in negative selection, as seen by the defective phenotype of TCR HY transgenic / DR3 KO male mice (Wang et al., 2001).

The **Bcl** (B cell lymphoma factors) family of regulators of apoptosis includes both pro-apoptotic (Bad, Bax, Bik) and anti-apoptotic (Bcl-2, Bcl-x_L) genes. The first member to be identified was Bcl-2, which was mutated in neoplastic B cells. Later, Bcl-2 was shown to inhibit apoptosis in selected haematopoietic cell lines following cytokine deprivation (Vaux et al., 1988).

Bcl-2 is expressed at high levels in DN thymocytes, low levels in DP cells and is again up-regulated in SP cells (Veis et al., 1993). Over-expression of Bcl-2 within the thymus results in protection of immature thymocytes from a variety of death stimuli including DNA damage and glucocorticoid-induced apoptosis (Strasser et al., 1991). These thymocytes remain sensitive to Fas (CD95)/Fas-L induced cell death, indicating that these are distinct apoptotic pathways (Strasser et al., 1995). Interestingly, these Bcl-2 transgenic mice show a marked skewed commitment toward the CD8 lineage, even on a MHC-II selecting background (Linette et al., 1994).

Bcl-x_L, unlike Bcl-2, is expressed at high levels in DP cells (Grillot et al., 1995). Thymocytes over-expressing Bcl-x_L are also resistant to apoptosis induced by GC and γ -radiation, and even to Fas/Fas-L induced cell death (Zhang et al., 1996). Their expression patterns suggest that Bcl-2 is down-regulated and Bcl-x_L is up-regulated during the DN \rightarrow DP transition.

The anti-apoptotic action of Bcl-2 and Bcl-x_L is antagonised by Bad and Bax. These pro-apoptotic proteins seem to bind and inhibit the anti-apoptotic members of the family, with Bad binding preferentially to Bcl-x_L. Expression of Bad and Bax is low in thymocytes, increasing greatly with apoptosis (Mok et al., 1999). Over-expression of either of the two pro-apoptotic proteins in T cells (driven by CD2 promoter) results in a marked reduction of mature T cells (Brady et al., 1996; Mok et al., 1999), which are particularly sensitive to apoptosis.

The anti-apoptotic Bcl family members seem to be common mediators of cell survival, and Bcl-2 transgenic mice have become a widely used tool to rescue survival phenotypes of other mouse models (see example below).

2.6.2 Interleukin-7 / IL-7R signalling

IL-7 was originally identified as a factor produced by bone marrow stromal cells that could support the proliferation of B cell precursors, but it plays a similar role in thymopoiesis. IL-7 mediates cellular responses by interacting with the IL-7R α chain in association with the common cytokine receptor gamma (γ_c) chain, that together form the IL-7 Receptor (IL-7R) (Sudo et al., 1993; Kondo et al., 1994). IL-7R is expressed on the surface of immature DN thymocytes, but not on DP cells; later SP thymocytes re-express IL-7R (Sudo et al., 1993). Such a pattern of expression suggested distinct roles for IL-7 in different thymocyte subsets.

To investigate the potential roles of IL-7/IL-7R signalling, two approaches were adopted: the administration of neutralising IL-7 or IL-7R α antibodies *in vivo*, and the generation of IL-7 and IL-7R α 'knockout' mice. These studies demonstrated the requirement for IL-7/IL-7R signalling in early thymocyte development, specifically in survival and proliferation of T cell precursors (Kim et al., 1998).

Mice deficient for IL-7R α displayed a dramatic reduction (10-10,000 fold) in the absolute number of both thymocytes and peripheral T cells (Peschon et al., 1994). A detailed analysis of thymocyte development in these animals revealed a clear role for IL-7/IL-7R in the early pro-T cell subsets (DN1 and DN2) (Kim et al., 1998). IL-7/IL-7R signalling was demonstrated to protect pro-T cells from apoptosis, at least in part by the upregulation of Bcl-2 (von Freeden-Jeffry, 1997), since crossing IL-7R KO mice with Bcl-2 transgenic mice partially rescued the thymic phenotype (Maraskovsky et al., 1997).

A similar, although less severe, phenotype was also seen in IL-7^{-/-} (*cytokine-deficient*) (von Freeden-Jeffry et al., 1995) and γ_c ^{-/-} mice (DiSanto et al., 1995). Thymocyte levels were reduced by 10-fold, despite the CD4 / CD8 distribution (DN, DP, SP) being relatively normal. This suggested that the role of IL-7 signalling in thymocyte development was the survival and expansion of early precursors (pro-T cells), which is required for normal thymic cellularity, but not the promotion of differentiation *per se*.

The receptor for TSLP (thymic stroma derived lymphopoietin), which was recently cloned (Park et al., 2000), also contains an IL-7R α chain, but no γ_c chain. Therefore, the more severe phenotype of IL-7R α ^{-/-} mice (compared to IL-7^{-/-}) might be due to the contribution of TSLP.

IL-7R deficient *mature* T cells also display dramatic defects in both survival and proliferation following a variety of *in vitro* activation stimuli, such as phorbol esters and ionomycin (Maraskovsky et al., 1996). This suggests that T cells developing in the absence of an IL-7R signal are functionally impaired.

Several downstream targets of IL-7/IL-7R signalling have been identified, including the pathways mediated by PI-3-kinase (Dadi and Roifman, 1993; Pallard et al., 1999), Jak3 and STAT5 (Nosaka et al., 1995; Pallard et al., 1999). Jak3^{-/-} mice (Nosaka et al., 1995; Thomis et al., 1995) exhibit similar defects in lymphoid development to those observed in IL-7^{-/-} and γ_c ^{-/-} mice, demonstrating a primary role for this tyrosine kinase in IL-7 signal transduction.

To address the developmental relationship between γ_c -dependent cytokine and pre-TCR signalling, $\gamma_c/pT\alpha$ double KO mice were generated (Di Santo et al., 1999). These animals showed a striking thymic phenotype, with a complete block at the DN2 stage of development. This result suggested that the pre-TCR could provide a survival/differentiation signal that would rescue IL-7/ γ_c signalling-deficient pro-T cells, implying a critically overlapping function for cytokine (IL-7) and pre-TCR receptor signals in early thymocyte development (Di Santo et al., 1999).

As the absence of a functional IL-7/IL-7R signal results in early defects at the pro-T cell stage of development, it has been difficult to determine whether subsequent stages are also dependent on signalling via this cytokine receptor pathway. Indirect evidence suggests that IL-7 could be functionally relevant for stages of thymocyte development after the β -selection checkpoint. For example, it is known that IL-7R α is expressed in the DN4 population (Porter and Malek, 1999), and recently a role for IL-7/IL-7R signalling has been identified at the DP to SP transition (Hare et al., 2000). Furthermore, intra-thymic injection of wild type DN3 or DN4 thymocytes into lethally irradiated mice in the presence of a blocking anti- γ_c antibody led to a decrease in T cell reconstitution when compared with non-treated controls (Malek et al., 1998). Nevertheless, the relationship between the pre-TCR and the IL-7/IL-7R signalling pathway and the precise role, if any, of IL-7/IL-7R signalling at the DN to DP transition are yet to be fully determined.

2.6.3 Wnt signalling

Wnt signalling is an excellent example of how the same signalling pathway can be used for the differentiation of diverse cell types – from tissues such as brain, muscle, sperm or thymus (reviewed in Wodarz, 1998). Moreover, this pathway is conserved and plays as important roles in invertebrates: Wnt-1 homologue, Wg – *wingless* – was originally identified as a mutation in *Drosophila* that caused the absence of wings. Furthermore, when dysregulated this pathway is involved in cell transformation and tumour formation (especially in colon cancer).

In what thymocytes are concerned, the importance of Wnt signalling can be easily inferred from the block in differentiation at the DN stage caused by selective inhibition of the pathway (Staal et al., 2001).

Wnt signalling is initiated by binding of Wnt ligands to Frizzled transmembrane receptors. These activate Dsh (*dishevelled*), which in turn inactivates GSK3 (glycogen synthase kinase 3), a negative regulator of the pathway. In the absence of Wnt signals, GSK3 phosphorylates β -catenin and tags it for degradation via the ubiquitin-proteasome process. Upon Wnt signalling, inhibition of GSK3 allows stabilisation of β -catenin, which translocates to the nucleus and provides a trans-activation domain to transcription factors Tcf-1 (T cell factor 1) and Lef-1 (lymphoid enhancer-binding factor 1) (reviewed in Wodarz, 1998).

Tcf-1 and Lef-1 were initially identified by their ability to bind to CD3 δ and TCR α enhancers, respectively. The two proteins share nearly identical HMG DNA-binding domains and thus can bind to the same gene regulatory elements – including the ones present in TCR β and TCR δ enhancers, besides the previously mentioned. The two genes also display overlapping patterns of expression during T cell development, being expressed in all T cell subsets from early DN cells to mature peripheral SP cells (Oosterwegel et al., 1993; Verbeek et al., 1995).

Surprisingly, Tcf-1^{-/-} and Lef-1^{-/-} mice showed very mild (Tcf-1) or no (Lef-1) defects in T cell development (van Genderen et al., 1994; Verbeek et al., 1995). However, Lef-1^{-/-} mice died post-natally with multiples abnormalities. Recently, Tcf-1 has been reported to control survival of DP thymocytes, as Tcf-1^{-/-} DP cells seem very prone to apoptosis; this correlates with very low expression of anti-apoptotic protein Bcl-x_L, and can be rescued by expression of a Bcl-2 transgene (Ioannidis et al., 2001).

The lack of a significant lymphoid phenotype in those mice raised the possibility, supported by their overlapping DNA binding sites and expression patterns, that Tcf and Lef function in a redundant manner in T cell development. To address this question, Tcf-1 / Lef-1 double KO mice were generated (Okamura et al., 1998). These mice lacked DP and mature SP cells. To circumvent the post-natal lethality of the Lef mutation, T cell differentiation in these mice was further analysed *in vitro* by

FTOC. A partial block at the DN3 stage, and a complete block at the ISP stage, were observed (Okamura et al., 1998). These experiments demonstrated that Tcf and Lef, downstream effectors of Wnt pathway, together played a crucial role in the DN → DP transition.

Recently, the role of Wnt signalling in thymic selection processes was examined by concentrating on the other main effector of the pathway, β -catenin. Mice were genetically manipulated so that the phosphorylation sites that usually target β -catenin for degradation were flanked by loxP sites (recognition sites for Cre recombinase, which specifically removes the intervening DNA). By crossing the altered β -catenin mice with Lck-Cre transgenic mice, in which the Cre enzyme is under the control of T cell specific promoter Lck, a mutant β -catenin was generated in T cells - and *only* in T cells (Gounari et al., 2001). As this mutant β -catenin could not be targeted for degradation, it was stabilised and acted as a constitutively active Wnt signal. These β -catenin mutant thymocytes developed in the absence of pre-TCR-mediated and TCR-mediated selection. Indeed, there was a marked reduction of the intracellular TCR β (+) subsets of DN3 and DN4 cells. In addition, these thymocytes produced SP cells that lacked TCR $\alpha\beta$, with 40% of CD4+ SP and 85% of CD8+ SP being negative for surface expression of TCR. Although such differentiation was induced, it was not accompanied by the high proliferation and survival rates usually associated with pre-TCR and TCR signalling (Gounari et al., 2001). These data suggested that a constitutively active Wnt signal is able to bypass pre-TCR signalling (β -selection) and TCR selection, but does not fully rescue T cell survival and proliferation.

Recent data regarding the expression of Wnt ligands (Wnt-4, -7) on thymic epithelial cells and of Frizzled receptors (Fzd-6, -7) on thymocytes suggest a developmental regulation of the expression of members of the family during the DN → DP transition (Jenkinson, 2002; Staal et al., 2001). It will be interesting to find out if different versions of Wnt signalling are important at different stages of thymocyte development, and how these stimuli are integrated with pre-TCR / TCR signals.

2.6.4 Notch signalling

Notch proteins are a family of highly conserved transmembrane receptors that regulate cell fate choices during the development of many cell lineages in both vertebrates and invertebrates (reviewed in Artavanis-Tsakonas, 1999). The first Notch gene was initially identified in *Drosophila* as a regulator of lineage determination during neuronal and epidermal cell differentiation.

Notch proteins (-1, 2, 3, 4 in mammals) interact with ligands of the Jagged (-1, 2) and Delta-like (-1, 3) families. As a result of ligand binding, Notch is proteolytically cleaved and its intracellular domain (icNotch) is released. icNotch then translocates to the nucleus where it converts the CBF1 transcription factor from a repressor to an activator of gene expression (reviewed in Deftos, 2000b).

Determination of cell fate by Notch can occur via two mechanisms: either lateral inhibition or inductive signalling. In lateral inhibition, a positive feed-back loop is created between two adjacent cells that accentuates differences in levels of expression of Notch and its ligand in opposite directions, until one cell becomes Notch-Ligand(+)Notch(-) and adopts one cell fate, whereas the other cell becomes Notch-Ligand(-)Notch(+) and adopts the alternative cell fate. In inductive signalling, Notch and its ligand are expressed separately on neighbouring cells. The cell that is Notch-Ligand(+)Notch(-) adopts one fate in the absence of Notch signalling, while it induces (via ligand binding) the differentiation of the Notch-Ligand(-)Notch(+) cell into the alternative fate, dependent on Notch signalling.

Expression of Notch in the thymus (on thymocytes, and Jagged ligands on thymic epithelial cells) and its identification as a T cell oncogene (Ellisen, 1991) prompted the investigation of a function for Notch in normal T cell development.

Recent experiments have indicated that Notch signalling plays a critical role in committing CLPs to the T lineage. Using the Cre-loxP system to inducibly inactivate Notch-1 in newborn mice or in bone marrow stem cells, a severe block of T cell development at DN1 was observed (Radtke et al., 1999). While the majority of these thymocytes phenotypically resembled immature bone marrow B cells, there was no effect on the development of other haematopoietic lineages.

Complementary to these findings are studies suggesting that Notch signalling may be sufficient to induce T cell lineage commitment. When bone marrow expressing a constitutively active Notch-1 was transferred into irradiated hosts, it gave rise to a thymus-independent population of cells expressing T cell markers (Thy1, CD4, CD8) in the bone marrow (Pui et al., 1999). In some cases, a proportion of these cells also expressed CD3 and even TCR β . In addition, differentiation of stem cells along the B cell lineage was completely inhibited. These results suggest that Notch signalling in CLPs favours a T cell fate over a B cell fate.

It is important to note, in this context, that the expression of Notch ligands in both the thymus and the bone marrow (Li et al., 1998; Luo et al., 1997) contradicts the hypothesis of T *vs.* B lineage commitment being determined by the differential availability of ligands in these two sites. However, it is interesting that downstream targets of Notch signalling, Deltex and Hes-1, are not expressed in foetal liver T cell precursors, but begin to be expressed in the thymus at the earliest DN1 stage (Jenkinson, 2002). *In vitro* culture of such foetal liver T cell precursors results in the expression of both genes after 5 days in RTOC, where they interact with thymic epithelial cells.

Differential availability of ligands for Notch in various developmental compartments has been suggested to be important for thymic positive *vs.* negative selection. Indeed, whereas thymic epithelial cells (major players in positive selection) express both Jagged and Delta-like ligands, thymic dendritic cells (involved in negative selection) do not (Hare et al., 2001).

The involvement of Notch signalling in CD4 *vs.* CD8 lineage commitment was first suggested by a 10-fold increase in CD8+ SP thymocytes (and slight decrease in CD4+ SP cells) observed in icNotch transgenic mice, in which icNotch expression was driven by *Lck* promoter (Robey et al., 1996). BrdU labelling experiments showed a 3-fold increase in the rate of production of CD8+ SP, and a 5-fold decrease in the rate of CD4+ SP generation. However, lymph nodes of these mice had a normal CD4/CD8 ratio and a 4-fold reduction in total T cell numbers. This suggested that the excess of CD8+ thymocytes might not correspond to mature SP cells, but to ISPs (accordingly, they expressed lower levels of TCR than normal CD8+ SP cells), and that the transgene was affecting the survival of mature T cells.

Indeed, recent data demonstrate that, rather than regulating lineage commitment between the two lineages, Notch-1 signalling is involved in the maturation of both CD4+ and CD8+ SP thymocytes (Deftos et al., 1998; Deftos et al., 2000a). icNotch was shown to confer resistance to glucocorticoid-induced and TCR-induced apoptosis of DP cells. In addition, maturation phenotypes were also induced by icNotch – but without any lineage bias (Deftos et al., 1998). A different line of *Lck*-icNotch transgenic mice was generated to that of Robey *et al.* (1996), and in contrast with the later, CD4/CD8 lineage commitment was not perturbed in favour of the CD8 lineage. The new line of transgenic mice showed an excess of mature thymocytes of *both* SP lineages, which could develop in the absence of MHC expression on thymic epithelium (Deftos et al., 2000a). The reason for the differences between Robey's and Deftos's mice remains unclear, but it may be due to the regions of Notch1 used as a transgene, or to transgene levels of expression.

During the DP → SP transition icNotch induces the up-regulation of TCR $\alpha\beta$ and Bcl-2 (Deftos et al., 1998), and Deltex, a positive regulator of Notch signalling that is now taken as a indicator of Notch signalling (reviewed in Deftos, 2000b). Deltex expression mimics that of Notch: it is high in DN and SP cells, and low in DP thymocytes. Importantly, Deltex expression is equivalent in both CD4+ and CD8+ SP cells, supporting a role for Notch in the maturation of both lineages.

Besides Deltex, Hes-1 has also been identified as a downstream target of Notch signalling. Hes-1 KO mice die shortly after birth with severe defects of the neural tube (Ishibashi et al., 1995). Over 90% of these mice either lack or have a very small thymus, with no mature T cells. When injected into RAG-deficient hosts, Hes-1 deficient bone marrow cannot generate T cells (differentiation is blocked at DN1), although B and myeloid lineages are generated normally (Tomita et al., 1999).

One important goal in this field is the identification of (other) genes regulated by Notch signalling, an area that has already begun with the RDA (representation difference analysis) studies of Deftos and Bevan (Deftos et al., 2000a).

3 T cell lineage commitment: $\alpha\beta$ versus $\gamma\delta$

Following Miller's thymectomy experiments and the realisation that the thymus was the primary lymphoid organ responsible for the generation of T cells, it was assumed that there was only one lineage of T cells. With the discovery that T lymphocytes were clearly part of the adaptive immune system, it seemed obvious that, like B cells, they would also express clonally distributed antigen receptor molecules. However, the fact that, unlike antibodies, T cell receptor molecules were not secreted meant that their identification was delayed and required the advent of more sophisticated approaches such as the ones developed in the 1980s in molecular biology (Hedrick et al., 1984) and biochemistry (Allison and Lanier, 1987). These technical advances finally allowed the identification of the genes (Hayday et al., 1985; Saito et al., 1984) and of the receptor proteins (reviewed in Raulet, 1989) of the TCR $\gamma\delta$, expressed by an independent (from $\alpha\beta$) T cell lineage.

Since then, it has become clear that all jawed vertebrates possess $\alpha\beta$ and $\gamma\delta$ lineages of T cells. Such a subdivision of T lymphocytes has apparently been conserved for 450 million years. It is also known that both lineages develop from a common thymic precursor, but the mechanism of their divergence is still unclear.

3.1 $\gamma\delta$ T cell biology

$\gamma\delta$ cells are defined by the surface expression of TCR $\gamma\delta$. In fact, there is no other unequivocal marker for $\gamma\delta$ cells (reviewed in Hayday, 2000). Some $\gamma\delta$ cells also express, independently, the following surface molecules: CD28, the ligand for B7 and the major co-stimulator for $\alpha\beta$ lymphocytes; CD40L; NK inhibitory receptors; NK activation receptors such as NKG2D; CD2 and CD5 (these vary between species). Unlike $\alpha\beta$ cells, most $\gamma\delta$ cells are CD4(-)CD8(-), both in the thymus and in the periphery, which reflects their MHC-independent selection and recognition of

antigen. Indeed, the development and the physiology of the $\gamma\delta$ lineage is not impaired in MHC deficient mice (Grusby et al., 1993).

The TCR $\gamma\delta$ shares a number of features with TCR $\alpha\beta$: RAG-dependent gene rearrangements (V-J for the γ chain, similar to the α chain; V-D-J for the δ chain, similar to the β chain); and the three-dimensional organisation of each V and C domain into “Ig folds” (seven β strands packed face to face in two anti-parallel β sheets), constrained by intra-domain disulphide bonding. However, many signatory features of TCR $\gamma\delta$ clearly distinguish it from its $\alpha\beta$ counterpart, from DNA sequence motifs, particularly in their CDRs (complementarity determining regions), to conformation patterns (reviewed in Hayday, 2000). In addition, TCR $\gamma\delta$, as an antigen receptor, is characterised by a very limited repertoire of specificities (Janeway, 1988).

$\gamma\delta$ cells account for only 1 – 5% of adult murine or human T cells. However, this is *not* invariably the case. They are more abundant in chickens, and in newborn lambs, $\gamma\delta$ comprise more than 80% of peripheral T cells, although this decreases to 30% in adult sheep (reviewed in Hayday, 1995). Interestingly, there is an approximate correlation between the complexity of TCR γ/δ genes and the representation of $\gamma\delta$ cells: for example, there are 3 TCR γ genes in the mouse, but more than 30 in the chicken. Also strikingly, this further correlates inversely with the complexity of TCR α/β genes: there are 75 V α and 23 V β genes in the mouse, but only two sub-families of chicken TCR α and β genes (reviewed in Hayday, 2000).

One notable feature of $\gamma\delta$ cells is their unique **anatomical distribution**. $\gamma\delta$ cells localise to tissues. Whereas they are rare in the thymus and in peripheral lymphoid organs (spleen, lymph nodes), they are disproportionately abundant in the skin, intestines, tongue, lung, mammary gland and uterine and vaginal epithelia. In humans, for example, $\gamma\delta$: $\alpha\beta$ ratios vary from 1:50 in the lymph nodes to 1:5 in the intestines. In mice, essentially all of the intraepithelial lymphocytes (IELs) of the skin are $\gamma\delta$ cells (Kuziel et al., 1987).

This distribution of $\gamma\delta$ cells may explain the **restricted diversity** of antigen specificities for the TCR $\gamma\delta$. Unlike $\alpha\beta$ lymphocytes, they do not reside in lymph

nodes or T cell areas of the spleen, where an immense diversity of antigens is constantly delivered (from body tissues) by “professional” APCs. Rather, they home to the body tissues, where they may recognise antigens directly, without relying on APCs (Mosley, 1992). Because most tissues are not anatomically compatible with the presentation and sampling of tens of thousands of antigens, this would appear to limit the diversity of antigens to which $\gamma\delta$ cells could be exposed, and therefore the useful diversity of TCR $\gamma\delta$ (Hayday, 2000).

This phenomenon is particularly evident in IEL repertoires. A single TCR (V γ 5V δ 1) is expressed by more than 90% of murine skin IELs (also called dendritic epidermal T cells (DETCs) because of their morphology), whereas a similar proportion of uterine IELs expresses a single V γ 6V δ 1, which differs from the DETC receptor only in the germline-encoded V γ sequences (Itohara, 1990). The extremely limited diversity of $\gamma\delta$ cells of epithelia provoked the “first line of defense” hypothesis (Janeway, 1988), proposing that $\gamma\delta$ cells respond not to a diversity of antigens, but to unique patterns associated with cell infection or transformation. In this context, Allison *et al.* suggested that members of the heat shock protein family, typical stress antigens, would be attractive candidates for $\gamma\delta$ ligands (Asarnow *et al.*, 1988).

Probably the most extraordinary feature of $\gamma\delta$ cell biology is their **antigen recognition**. They do *not* recognise ‘classical’ ligands (i.e., peptides) and they do *not* depend on MHC-presentation of antigen. This clearly distinguishes them from $\alpha\beta$ lymphocytes. The mechanism of antigen presentation to $\gamma\delta$ cells is still a complete mystery, almost 20 years after the identification of $\gamma\delta$ cells.

In terms of **antigen specificities**, the effort of trying to identify the unorthodox ligands for TCR $\gamma\delta$ has provided the following results:

- *Human systemic* $\gamma\delta$ cells recognise small, non-peptidic products, in particular phosphoantigens. Monoalkyl (pyro)phosphates of less than 5 carbons are recognised by V γ 2V δ 2 cells (Pfeffer *et al.*, 1990; Tanaka *et al.*, 1995; Tanaka *et al.*, 1994). These compounds are primarily found in the cytoplasm of mycobacteria, being metabolites of pathways specific for prokaryotes (Constant *et al.*, 1994).

- *Human IEL* $\gamma\delta$ cells recognise 'non classical' MHC class I related (MIC) antigens (Groh et al., 1998; Li et al., 1999; Steinle et al., 1998). MICA and MICB are located approximately 50kb from HLA-B in the MHC locus. Importantly, although also polymorphic (16 alleles) and sharing some homology (circa 30%) with conventional MHC class I, MICA is β_2 -microglobulin-independent. MICA is a ligand also for NKG2D, a NK receptor expressed in $\gamma\delta$ cells. It's not clear if there is cross-talk between TCR $\gamma\delta$ and NKG2D in binding to MICA. Interestingly, MICA and MICB expression in intestinal epithelial cells is driven by heat shock promoters (Groh et al., 1996), an ideal scenario for the "first line of defense" hypothesis.

- *Mouse systemic* $\gamma\delta$ cells recognise native, non-processed, proteins. A V γ 2(+) clone (LBK5) recognises MHC class II proteins (IE^k, IE^b, IE^s) (Weintraub et al., 1994), whereas another clone (TgI4.4) recognises glycoprotein I (gI) of herpes simplex virus (HSV) (Johnson et al., 1992; Sciammas et al., 1994), all independently of peptide. Two independent $\gamma\delta$ cell hybridomas, G8 and KN6, recognise two closely related (94% identity) 'non classical' MHC class IB proteins, T10 and T22, respectively, also independently of antigen processing (Crowley et al., 1997).

The most remarkable aspect of above examples is that *no* human $\gamma\delta$ specificities seem to be conserved in mice. No murine reactivities to low molecular mass antigens have been described. Furthermore, the region encoding MICA is deleted in the mouse genome, and no functional equivalent has yet been clearly characterised (Hayday, 2000). These surprising facts have precluded the application of animal systems to establish the importance of $\gamma\delta$ responses to the ligands identified in the human system. This topic thus requires extensive research.

$\gamma\delta$ cells also interact with a broad spectrum of other cell types. For IELs, the epithelial cell is an obvious candidate for both an antigen presenting cell and the target of $\gamma\delta$ cell effector function. Several studies have shown that DETCs can be stimulated to release IL-2 and IFN- γ , or to become cytolytic, by keratinocytes, in a TCR-dependent fashion (Havran et al., 1991). Systemic $\gamma\delta$ lymphocytes interact with 'professional' APCs, such as macrophages and dendritic cells. These produce IL-1, IL-12 and IL-15, which, alongside IL-7 released by several cell types, are very effective at driving murine $\gamma\delta$ proliferation (Skeen and Ziegler, 1993).

$\gamma\delta$ cells, macrophages and NK cells have been proposed to co-operate in the early “innate” phases of immune responses. Macrophages activate both $\gamma\delta$ cells and NK cells via secreting IL-12/IL-1/TNF- α , and these two latter cell types produce IFN- γ , which activates macrophages to release further amounts of those cytokines (Skeen, 1995; Balaji, 1995). $\gamma\delta$ cells also seem to provide help to B cells in germinal centre formation and class-switching (Horner et al., 1995). Finally, cross-talk between $\gamma\delta$ and $\alpha\beta$ T cell types also seems to occur, by which they positively or negatively regulate one another (reviewed in Hayday, 2000). It’s still not clear if $\gamma\delta$ cells can work as APCs, although they do commonly express CD1 (Wen et al., 1998), the only known function of which is to present antigens to T cells.

$\gamma\delta$ cells are activated by **infection**, which triggers oligoclonal expansion, change of surface markers, and effector functions such as cytolysis and cytokine release ($\gamma\delta$ cells can display both Th1 and Th2 phenotypes, similar to $\alpha\beta$ cells, although, more often, they are associated with Th1 responses). This has been demonstrated to be the case for *in vivo* challenges with many different pathogens: *Mycobacteria*, influenza virus, Epstein-Barr virus, *Listeria*, *Plasmodium*, *Leishmania*, *Salmonella* and *Toxoplasma* (reviewed in Hayday, 2000). It is still not clear, though, since none of these responses have been unequivocally attributed to a specific antigen, whether the $\gamma\delta$ cells were activated by foreign antigens or by “stress” self-antigens expressed due to a “danger” signal, i.e. the infection (as proposed by the “first line of defense” hypothesis).

However, there are several examples of essential and non-redundant $\gamma\delta$ cell contributions in *primary* immune responses, and were exposed in mice completely lacking $\gamma\delta$ cells (TCR $\delta^{-/-}$) (Itohara et al., 1993):

- Lung infection with *Nocardia asteroides*, an intracellular bacterium, is normally cleared in WT mice, but caused mortality of 100% of TCR $\delta^{-/-}$ mice (Mombaerts et al., 1993). These mice failed to develop inflammatory infiltrates of macrophages and neutrophils.

- Infection with *Listeria monocytogenes*, whose titers, after 8 days of infection, were 1,000-fold higher in TCR $\delta^{-/-}$ mice (Ladel et al., 1996; Nishimura et al., 1995), and persisted beyond the usual period of clearance. The mice showed reduced

levels of IFN- γ and TNF- α , consistent with the known interaction of $\gamma\delta$ cells with NK cells and macrophages, respectively.

- Infection with *Candida albicans*, to which TCR $\delta^{-/-}$ mice were particularly susceptible and exhibited abnormally low production of nitric oxide (NO), an important effector of the collective anti-microbial action of macrophages and $\gamma\delta$ cells (Jones-Carson et al., 1995).

Other reported examples include infections with *M.tuberculosis* (Ladel, 1995; Balaji, 1995) and with viruses, such *Vaccinia* (Welsh et al., 1997).

Despite the above susceptibilities to infections, TCR $\delta^{-/-}$ mice are for the most part immuno-competent. This might well indicate that $\alpha\beta$ lymphocytes can substitute for $\gamma\delta$ cells (either directly or via interactions with other cell types), as it has been demonstrated, notably in the case of HSV-1 infection, that although both TCR $\beta^{-/-}$ and TCR $\delta^{-/-}$ were able to clear the pathogen, TCR $\beta^{-/-}$ /TCR $\delta^{-/-}$ (double KO) mice were *not* (Sciammas et al., 1997).

In terms of secondary immune responses, immunity to re-challenge with pathogens develops normally in TCR $\delta^{-/-}$ mice, in stark contrast with the immunodeficiency displayed by TCR $\beta^{-/-}$ mice. It is still unclear if $\gamma\delta$ cells, following their initial proliferation (triggered by antigen), develop a specific component of immunological memory, even if they can express CD45R0, normally considered to be a memory marker.

$\gamma\delta$ cells have also been proposed to play a **regulatory role** in several non-infectious process, such as **inflammation** or **autoimmunity**. Indeed, $\gamma\delta$ cell populations expand significantly under non-infectious conditions. For example, during pregnancy, there is a 100-fold increase in the numbers of $\gamma\delta$ cells in the reproductive tract, and during inflammatory processes, $\gamma\delta$ cells have been seen to infiltrate damaged tissues and participate in the regulation of subsequent events (reviewed in Hayday, 2000).

More strikingly, $\gamma\delta$ cells have been implicated in the regulation of graft *vs.* host disease, since they suppressed the auto-reactive attack of an $\alpha\beta$ T cell clone to skin Langerhans cells (Kikuchi et al., 1992; Peng et al., 1996). Perhaps even more

impressive was the increase in the autoimmune disease systemic lupus erythematosus detected in MRL.lpr mice (model system for the disease) when they were crossed onto a $\gamma\delta$ cell-deficient background (Seymour et al., 1998). These mice displayed dramatic increases in the titres of auto-antibodies, the amount of Ig deposition in the kidneys and the numbers of CD4(+) lymphocytes, when compared with "normal" MRL.lpr mice. Importantly, the TCR δ^{-} /MRL.lpr mice showed a 3-fold increase in mortality at 6 months, from 23% to 68%. These data provide clear evidence that, in a particular genetic context, immuno-regulation by $\gamma\delta$ cells can have a major impact on life expectancy.

What are the selective forces responsible for the evolutionary conservation of $\gamma\delta$ cells across half a billion years, in a scenario where $\alpha\beta$ cells seem to do "more and better"? According to Hayday (Hayday, 2000), possibilities include:

- Immuno-regulation, particularly regarding epithelial tissue repair since this affects reproductive fitness. In addition, ruminants such as cattle (in which $\gamma\delta$ cells are particularly abundant) would need to avoid the chronic inflammation that would otherwise accompany their constant exposure to antigens.

- Immuno-protection in cases where $\alpha\beta$ lymphocytes are not effective. These would include viral infections that down-regulate MHC class I synthesis in host cells (for which a MHC-independent immune response is particularly important), and early life, when $\alpha\beta$ responses are less effective (especially Th1 responses).

Immunoprotection in young animals may be a particularly crucial aspect of $\gamma\delta$ cell biology. $\gamma\delta$ cells are disproportionately abundant in young individuals (across many species), and their early waves of production populate peripheral epithelia (skin, tongue, gut), creating a barrier against foreign pathogens. On the other hand, they are known to co-operate with potent anti-microbial cells, such as macrophages and NK cells. Finally, unlike $\alpha\beta$ cells, $\gamma\delta$ cells require neither the complicated antigen processing and presentation machinery, nor the sophisticated antigen sampling, which may not be fully developed at birth. Therefore, $\gamma\delta$ cells might have

been evolutionary conserved because they are essential for primary immunity at the body surfaces of young animals (Hayday, 2000).

A final remark regarding this topic: since the adaptive immune response is characterised by the capacity to clonotypically expand and delete cells with different specificities, and by the creation of immunological memory, the failure to identify/clarify these processes in $\gamma\delta$ cells has tempted some to regard them as part of the innate immune system. It should be stressed, though, that $\gamma\delta$ cells, like $\alpha\beta$ T cells and B lymphocytes, are capable of somatic rearrangement that can develop novel antigen specificities. Furthermore, these antigen specificities can be enriched by antigen-driven selection in the periphery.

3.2 Models for the $\alpha\beta$ / $\gamma\delta$ lineage split

The two T cell lineages, $\alpha\beta$ and $\gamma\delta$, are derived from a common precursor. Late DN thymocytes (including DN4) can give rise to cells of both types in FTOC, whereas they can no longer generate cells of any other haematopoietic lineages. The similarity of surface phenotypes and patterns of gene rearrangement further demonstrates the close relationship between the two branches of T cells. In fact, *no single marker*, besides the TCR itself, has been found to date that unequivocally distinguishes both types of lymphocytes (reviewed in Fehling, 1999).

Many studies using adoptive cell transfer (*in vivo*) and re-population assays (in FTOC) have tried to identify the developmental stage at which the two lineages diverge (Bruno et al., 1999; Dudley et al., 1995; Godfrey and Zlotnik, 1993; Petrie et al., 1992). The overall conclusion was that even late DN4 thymocytes are bipotential, suggesting that the split takes place just prior to the surface expression of CD4 and CD8. These experiments have one important caveat: they are unable to establish the clonality of precursor-product relationships. Therefore, it cannot be excluded that lineage divergence occurs at a relatively early stage and that pre-committed cells follow separate developmental pathways, which (at present) are phenotypically indistinguishable until after the expression of the appropriate TCR.

An alternative approach, consisting of the analysis of TCR gene rearrangements in immature DN thymocytes, was not able to provide the clear results that were expected, since those rearrangements are promiscuous, occurring in cells of both lineages (reviewed in Fehling, 1999).

The apparent difficulty in demarcating a specific stage for $\alpha\beta/\gamma\delta$ split may reflect a very gradual process of lineage commitment involving several developmentally successive populations that become increasingly unable to change their fate. In the end, the lineage choice becomes irreversible via isotypic exclusion, which guarantees that only one type of TCR (either $\alpha\beta$ or $\gamma\delta$) is expressed at the cell surface of a T cell.

In spite of the difficulties mentioned, several models have been proposed to explain how the bipotential precursor becomes either an $\alpha\beta$ or $\gamma\delta$ T cell:

- **Sequential rearrangement model.** This model was inspired by the defined temporal order of TCR isotype expression in foetal thymocytes, with $\gamma\delta$ cells first detectable at E14.5 and $\alpha\beta$ cells only at E17.5. Allison and Pardoll suggested that γ and δ gene rearrangements are attempted first and, if successful (both in-frame), the thymocyte becomes a $\gamma\delta$ cell. If one of those two genes is not rearranged productively (on either allele) the thymocyte then rearranges its β and α loci to become an $\alpha\beta$ cell (Allison and Lanier, 1987; Pardoll et al., 1987).

- **Competitive rearrangement model.** Based on subsequent studies that showed a temporal coincidence (same developmental stage) of TCR rearrangements in adult thymocytes (Godfrey et al., 1994; Livak et al., 1999), this model defended that γ , δ and β gene rearrangements occur simultaneously in uncommitted thymocytes. If γ and δ are rearranged first, the cell becomes a $\gamma\delta$ cell, while if a productive β gene is rearranged first, the cell commits to the $\alpha\beta$ lineage (initially expressing a pre-TCR complex).

- **Separate lineages model(s).** They state that the $\alpha\beta$ and $\gamma\delta$ lineages are independent, diverging before the expression of the TCR isotype, which is a consequence (rather than a cause) of lineage commitment. This decision is taken independently of the outcome of TCR gene rearrangements (Winoto and Baltimore, 1989b), and is brought about by some other mechanism.

These models are depicted schematically in **Figure 5**.

Importantly, both the sequential and the competitive models are *instructive* - assume that (pre-)TCR-mediated processes dictate lineage commitment -, whereas the separate lineages model is *non-instructive*.

Until recently, the separate lineages model was seen as unsatisfactory, because it did not provide a precise explanation for how the lineage split occurs, it merely pointed out how it does *not* occur (Fehling et al., 1999). But novel data regarding TCR-independent pathways has erased this criticism, by providing some evidence that DN precursors can be sorted into discrete subsets with indistinguishable TCR gene rearrangement status but exclusive developmental potential (see 3.5).

Whereas the separate lineages model considers the outcome of TCR gene rearrangements irrelevant for the lineage choice itself, the other two models make particular predictions concerning the status of gene rearrangements in $\alpha\beta$ and $\gamma\delta$ thymocytes:

- According to the *sequential rearrangement* model, $\alpha\beta$ cells should bear signs of failed γ and δ rearrangements, which should be extensively but non-productively rearranged. The frequency of out-of-frame γ/δ rearrangements should be higher than that expected for a random process, since those rearrangements would have been attempted, but unsuccessfully, before TCR α rearrangement occurred. Conversely, $\gamma\delta$ cells should essentially be devoid of V-(D)J α/β rearrangements, as they should have become lineage-committed before these loci became available for recombination.

- In line with the *competitive rearrangement* model, at least some $\alpha\beta$ cells should contain γ/δ rearrangements, and vice-versa for $\gamma\delta$ cells, since the TCR loci would have been rearranging simultaneously when commitment occurred due to productive completion of either γ/δ or β rearrangements. In both cases, at the population level, there would be a selection against (below random probability) in-frame rearrangements of the loci pertaining to the opposite lineage.

Predictions of rearrangement status can be extended to phenotypes of TCR transgenic or 'knock-out' mice, where lineage commitment is supposedly perturbed (see 3.3 and 3.4).

Lineage commitment requires irreversibility of the cell phenotype, in this case by isotypic exclusion, and two theories ("lineage maintenance mechanisms") have tried to explain how that occurs when thymocytes adopt an $\alpha\beta$ lineage fate.

The first, put forward by Hockett and de Villartay (1988), states that $\alpha\beta$ -committed cells go through a programmed excision event that deletes the TCR δ locus (D, J and C regions), thus permanently preventing the formation of TCR $\gamma\delta$. This is supported by the identification of a recombination mechanism for TCR α genes that indeed involves the excision of TCR δ segments, which are located within the TCR α locus (reviewed in Fehling, 1999).

The second, proposed by Haas and Tonegawa (1992), suggests that $\alpha\beta$ -committed cells activate a putative TCR γ -specific silencer which then prevents the expression of rearranged TCR γ genes. This is in line with the phenotype of TCR γ transgenic mice (see 3.4).

Analogously, a silencer element has been identified in the TCR α enhancer (3' of the C α gene) that is engaged in $\gamma\delta$, but not in $\alpha\beta$, cells (Winoto and Baltimore, 1989b; Winoto and Baltimore, 1989c). This silencer inactivates the TCR α enhancer in $\gamma\delta$ cells, thus preventing both rearrangement and transcription of TCR α genes.

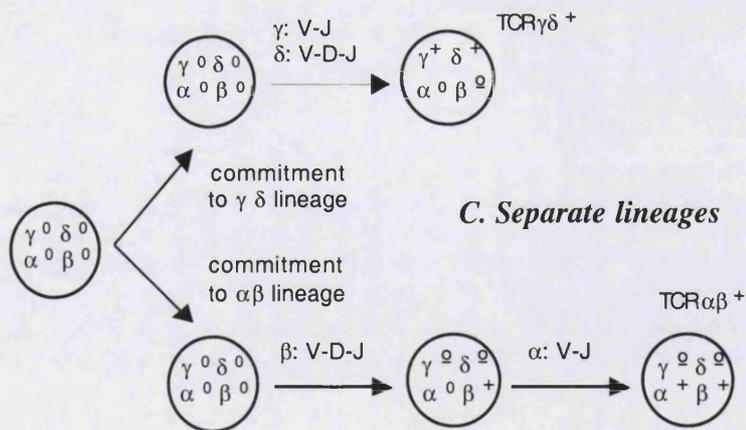
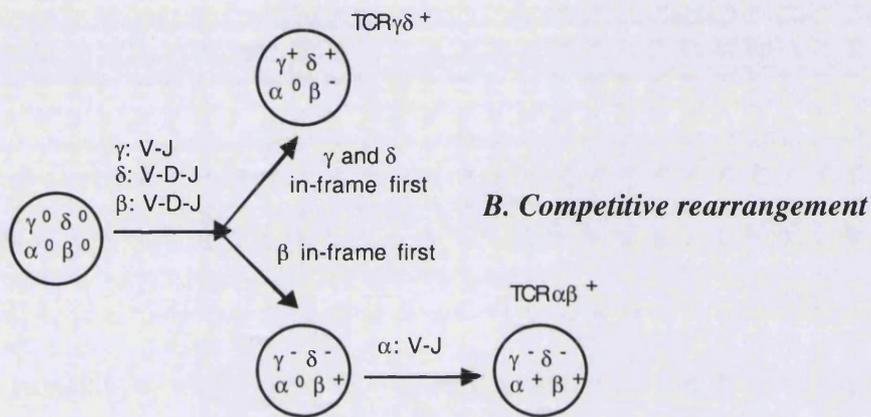
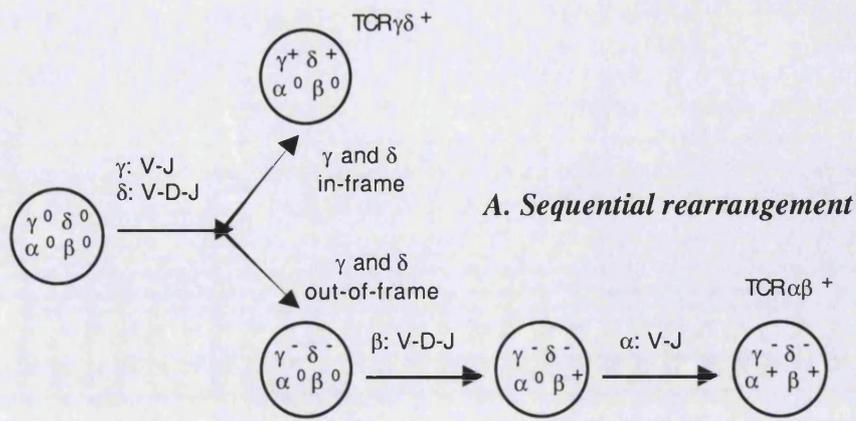


Figure 5 : Models for the $\alpha\beta$ / $\gamma\delta$ lineage split

Beginning with a precursor thymocyte, the rearrangement status of TCR loci is represented by: $^{\circ}$, un-rearranged (germline); $^+$, productively rearranged; $^-$, non-productively rearranged; $^{\circ}$, un- or non-productively rearranged (irrelevant). Cells unable of any productive rearrangement die.

3.3 Analysis of TCR rearrangements in T cell subsets

Two methods have been extensively used to determine the status of TCR gene rearrangements in thymocyte and lymphocyte subsets. First, *Southern blotting* of genomic DNA, where DNA fragments hybridising with defined regions of particular TCR loci serve as probes to visualise potential rearrangements. This method suffers from relative low sensitivity (only rearrangements expressed in at least 10% of a population of cells are detectable), but has the advantage of not relying on 'biased' amplification approaches (such as polymerase chain reaction, PCR). Second, *restriction fragment length polymorphism - PCR* (RFLP-PCR), which reflects the ratio of coding (in-frame) to non-coding (out-of-frame) sequences in a population after amplification of rearranged gene segments. The ratio is determined by densitometry of bands on a sequencing gel corresponding to labelled fragments digested with restriction enzymes. In-frame rearrangements are represented as bands spaced by 3 nucleotides (a codon). Alternatively, some authors have amplified and sequenced individual rearrangements, collecting hundreds of sequences to make the analysis statistically significant.

• *γ rearrangements in αβ cells*

V-J rearrangements of γ genes are very abundant in $\alpha\beta$ cells. This suggests that either commitment occurs after those genes are rearranged, or such rearrangements are not suppressed upon commitment. However, some $\alpha\beta$ cells carry TCR γ in germline configuration (reviewed in Fehling, 1999).

According to RFLP-PCR analysis (Dudley et al., 1995), there is selection against in-frame V γ 1.1-J γ 4 rearrangements in $\alpha\beta$ cells. Both DP thymocytes (19% in-frame) and peripheral $\alpha\beta$ cells (18%) show ratios below that predicted for a random event (33%). This was supported by an independent experiment (Kang et al., 1995) where a transgenic TCR γ mini-locus (encompassing V γ 2/3/4, J γ 1 and C γ 1) was introduced into mice. Only 18% of transgenic sequences were productively rearranged in $\alpha\beta$ cells, supporting selection against in-frame TCR γ gene rearrangements in these cells.

- *δ rearrangements in $\alpha\beta$ cells*

TCR δ rearrangements are not trivial to study in $\alpha\beta$ cells, since recombination in the α loci (usually both alleles are used by $\alpha\beta$ cells) deletes the δ genes from the chromosomes. However, the by-products of this excision event are circular DNA molecules that are largely retained in the cells (even in some peripheral T cells). These circles have been purified and used to construct “circle DNA libraries”, allowing the analysis of TCR δ rearrangements.

Although initial results using probes flanking the J δ 1 segment (by far the most frequently used in $\gamma\delta$ cells) argued that these circles contained mostly un-rearranged δ genes (Winoto and Baltimore, 1989a), they were contradicted by later reports. Livak *et al.* (Livak et al., 1995) showed by detailed Southern blot analysis that numerous circles (estimated 40%) had V(D)J δ rearrangements (involving predominantly the J δ 1 segment). Of these, only 20% were productive (shown by collection of sequences). Similar results were obtained by RFLP-PCR: only 19% - 24% of δ rearrangements of DP and SP cells were in-frame (Dudley et al., 1995).

A separate study using Northern blotting (Wilson et al., 1996) demonstrated for the first time that V(D)J δ rearrangements could be completed before the V α -J α excision event in committed $\alpha\beta$ thymocytes. Abundant full-length TCR δ transcripts were detected in CD8(+) ISP cells. Although the authors reported a value (29%) close to the expected in absence of selection (33%) for the quantification of productive rearrangements (contrasting with Livak and Dudley) this estimate is based on the analysis of RNA rather than DNA, and could therefore be biased in favour of in-frame rearrangements due to preferential stability of productive message.

The studies on TCR δ rearrangements also established that both the silencing and the excision of the TCR δ locus was not required for commitment to the $\alpha\beta$ lineage, as had originally been proposed by Winoto & Baltimore (1989), and Hockett & de Villartay (1988), respectively. These two mechanisms are now considered “lineage maintenance” rather than “lineage commitment” (Fehling et al., 1999).

• *β rearrangements in $\gamma\delta$ cells*

Initial reports involving a limited number of $\gamma\delta$ T cell clones and hybridomas suggested that D-J β rearrangements were very common, whereas complete V-DJ rearrangements were quite rare in $\gamma\delta$ cells (reviewed in Haas, 1992). However, recent studies have clearly shown this not to be correct. For example, Dudley *et al.* analysed lymph node, splenic and IEL $\gamma\delta$ cells from both TCR α -deficient (Dudley *et al.*, 1994) and wild type mice (Dudley *et al.*, 1995), and showed that, in both cases, besides almost all cells containing D-J β rearrangements, at least 50% had completed V-DJ β rearrangements. RFLP-PCR analysis of these (V β 13-J β 2.2 and V β 4-J β 2.2) revealed that 70% of them were in-frame - as much as in $\alpha\beta$ cells! This suggested that the peripheral $\gamma\delta$ cells had been selected (supposedly in the thymus) for functional TCR β chains.

Burtrum *at al.* (Burtrum *et al.*, 1996) examined thymic $\gamma\delta$ cells of wild type mice by quantitative Southern blotting, and determined that 20% of all TCR β alleles had V-DJ rearrangements (compared with 75% in mature $\alpha\beta$ cells). RFLP-PCR with V β 2-J β 2.6 and V β 4-J β 2.6 primers showed that 51-55% of such rearrangements in $\gamma\delta$ thymocytes were in-frame. Although lower than Dudley's (for peripheral $\gamma\delta$ cells), these values were still well above the expected for a random (non-selected) event (33%).

Taken together, these results showed that $\gamma\delta$ cells are probably " β -selected". The enrichment of productive rearrangements from thymus to periphery could suggest that these recombination events at the β locus occur after lineage commitment in the thymus, as the $\gamma\delta$ cells mature. Nevertheless, the important questions raised are: a) do these productive β rearrangements encode functional TCR β chains in $\gamma\delta$ cells? b) If so, do these TCR β chains form a pre-TCR (since pT α protein is not detected in $\gamma\delta$ cells (Bruno *et al.*, 1995))? c) Does a productively rearranged TCR β chain confer any physiological (survival, proliferation, effector functions) advantage to the $\gamma\delta$ cells that express it?

Despite these striking findings, subsequent studies have suggested that β -selection in $\gamma\delta$ cells is not as common as previously implied: only 42% of V β 6-J β 2.5

rearrangements were productive in both foetal and adult $\gamma\delta$ thymocytes (Mertsching and Ceredig, 1996; Mertsching et al., 1997). It is not clear whether these conflicting reports are due to the analysis of different V-J gene segment rearrangements, or with the existence of distinct $\gamma\delta$ sub-populations, or with technical inconsistency (in this regard, one should note that Mertsching's experiments were done by collecting individual sequences, and maybe the sampling - total of 43 - was not sufficient to make it statistically significant).

Nonetheless, more conclusive evidence for β -selection of $\gamma\delta$ cells was presented by Wilson and MacDonald (Wilson and MacDonald, 1998), who showed that 14-17% of wild type $\gamma\delta$ thymocytes and splenocytes express intracellular TCR β protein (icTCR β). They also showed that TCR β expression conferred a selective proliferation advantage to $\gamma\delta$ cells.

- ***α rearrangements in $\gamma\delta$ cells***

It has been shown that the α locus is both recombination- and transcriptionally silent in $\gamma\delta$ cells, due to the lack of activity of the TCR α enhancer (Capone et al., 1993; Diaz et al., 1994; Lauzurica and Krangel, 1994). This regulatory element is located 3' of the C α gene and contains a silencer region that is engaged in $\gamma\delta$ cells, but not in $\alpha\beta$ T cells (Winoto and Baltimore, 1989b; Winoto and Baltimore, 1989c). The absence of V-J α rearrangements is often as an useful molecular marker for the identification of $\gamma\delta$ lineage cells in situations where the determination of the TCR isotype is either not possible or not informative, e.g., in TCR transgenic mice (for an example, see Bruno, 1996).

- ***Implications for the lineage divergence models***

The data discussed above do not support the *sequential rearrangement* model, as frequent V(D)J rearrangements of TCR β genes in $\gamma\delta$ cells, and TCR γ and TCR δ genes in $\alpha\beta$ cells argues that TCR rearrangements are not lineage-specific. Moreover, the detection of un-rearranged TCR γ and TCR δ genes in many $\alpha\beta$ cells shows that these cells did not attempt γ/δ rearrangements first.

The *competitive rearrangement* model is supported by selection against in-frame γ/δ rearrangements in $\alpha\beta$ cells, *but* is not consistent with the apparent selection for

productive β rearrangements in $\gamma\delta$ cells. To salvage this model, an additional assumption is required, that $\gamma\delta$ -committed cells are able to subsequently extensively rearrange their TCR β genes (although this would presumably arrive too late to influence the lineage choice). Also difficult to conciliate with the simultaneous rearrangement of γ , δ , and β TCR loci proposed by this model is the fact that considerable numbers of $\alpha\beta$ cells bear un-rearranged TCR γ and TCR δ genes. In fact, a more recent report (Livak et al., 1999) has demonstrated that, if anything, TCR γ and TCR δ rearrangements precede (at DN2 stage) TCR β rearrangements (at DN3 stage), which makes it difficult for the competitive model to explain how $\alpha\beta$ cells are devoid of TCR γ/δ rearrangements.

Since the *separate lineages* model did not make any predictions regarding the status of TCR rearrangements in developing thymocytes (it considered them irrelevant for the lineage decision), it was neither supported nor contradicted by these data. It did, in fact, gain supporters because of the downfalls of the other two models. However, the selection against TCR γ/δ rearrangements in $\alpha\beta$ cells shows that these are not neutral events in thymocyte development, and should therefore be incorporated in any viable model for the $\alpha\beta/\gamma\delta$ lineage split.

3.4 Analysis of TCR transgenic and gene-deficient mice

The generation of mouse models in which TCR genes were either provided ectopically (as functional transgenes) or disrupted endogenously seemed a very good tool for studying the mechanism of the $\alpha\beta/\gamma\delta$ lineage split. Indeed, the above models made clearly distinct predictions regarding such experiments. For the TCR transgenic studies, for example, the two rearrangement models predicted that a functionally rearranged receptor should direct all developing thymocytes into the corresponding lineage, and prevent the formation of cells of the opposite lineage. By contrast, according to the separate lineages model, functional transgenes should have little impact on the balance between the two lineages. However, as is so often the case in biology, the results turned out to be both highly variable and not straightforward to interpret.

- *TCR $\alpha\beta$ transgenic mice*

Initial analysis of HY transgenic mice, in which the TCR $\alpha\beta$ transgene recognises a male-specific peptide in the context of H-2D^b (Fenton et al., 1988; Kisielow et al., 1988) seemed to support the competitive model. Endogenous V γ 2-J γ 1 rearrangements were suppressed and $\gamma\delta$ cells were absent in the thymus and lymph nodes. However, detailed analysis of the very unusual CD4(-)CD8(-), TCR transgene(+), lymphocytes present in the lymph nodes of these mice provided an alternative explanation for the absence of $\gamma\delta$ cells, which supported the separate lineages model (Bruno et al., 1996). Such lymphocytes had all the characteristics of $\gamma\delta$ cells, apart from TCR $\gamma\delta$ expression. Specifically, they were CD4(-)CD8(-), they were not dependent on positive selection (since they developed normally in a non-selecting MHC background), or on negative selection (since they accumulated in the LN of male mice, in which the conventional transgenic(+) $\alpha\beta$ thymocytes were deleted), and they did not rearrange their endogenous TCR α genes, thereby retaining their TCR δ alleles in both chromosomes (unlike $\alpha\beta$ lymphocytes). Moreover, these cells were absent in a γ_c -deficient background (DiSanto et al., 1996), which specifically blocks the development of $\gamma\delta$ cells and were able to co-express a TCR $\gamma\delta$ when on a pT α -deficient background (Bruno et al., 1996). Therefore, it seems that $\gamma\delta$ lineage cells are not really absent in HY transgenic mice, but they are rather disguised as cells expressing the transgenic TCR $\alpha\beta$ (Fehling et al., 1999).

Similar results were also obtained by Capone *et al.* (Capone et al., 1995) using a distinct TCR $\alpha\beta$ transgene. They also found the unusual CD4(-)CD8(-), transgene(+), cells with identical characteristics to those described for HY transgenic mice. Furthermore, they demonstrated that an artificial rearrangement substrate (mini-locus) under the control of the TCR α enhancer was not rearranged in those DN cells, whereas it was extensively rearranged in SP cells from the $\alpha\beta$ lineage.

Taken together, these data suggest that early expression of a TCR $\alpha\beta$ transgene does not prevent the formation of $\gamma\delta$ lineage cells. Rather, the transgenic TCR $\alpha\beta$ seems to be able to functionally replace TCR $\gamma\delta$ in promoting $\gamma\delta$ development, despite the absence of the 'correct' receptor. As such, these results support the separate lineages model for $\alpha\beta/\gamma\delta$ divergence.

• *TCR $\gamma\delta$ transgenic mice*

TCR $\gamma\delta$ transgenic mice should, in theory, provide a clear test for the above models. The two rearrangement (instructive) models predicted a block in $\alpha\beta$ development, which would not be expected according to the separate lineages model. Unfortunately, the results have been inconsistent and difficult to interpret. For example, the phenotype of mice expressing a V γ 3J γ 1/V δ 1DJ δ 2 transgene (coding for a receptor characteristic of $\gamma\delta$ DETCs) was strain-dependent. The presence of some negative selection factor led to severe thymocyte depletion (and thus a striking phenotype) in B6 mice (Bonneville et al., 1989), whereas thymocytes (including the $\alpha\beta$ lineage) developed normally on a C3H background (Iwashima et al., 1991).

The scenario got even more confusing with the study of Sim *et al.* (Sim et al., 1995), which made it obvious that the biggest challenge regarding TCR $\gamma\delta$ transgenic mice was to determine which of the many observed phenotypes reflected a physiologically relevant situation. Sim *et al.* generated nine transgenic V γ 4J γ 1/V δ 1DJ δ 2 lines, which displayed a wide range of phenotypes. For example, $\gamma\delta$ cell frequencies in the thymus varied between 2% and 93%! Two of the lines had normal numbers of $\alpha\beta$ splenocytes, whereas seven of them showed a 25%-75% reduction. Moreover, the extent of $\alpha\beta$ suppression did not correlate either with transgene copy number, or with the frequency of thymic $\gamma\delta$ cells. Interestingly, in two of the lines, a distinct population of lymphocytes co-expressed endogenous TCR $\alpha\beta$ and transgenic TCR $\gamma\delta$. These results were interpreted as a demonstration that expression of TCR $\gamma\delta$ is not incompatible with $\alpha\beta$ development. On the other hand, the expression of TCR $\gamma\delta$ seemed insufficient to direct precursor differentiation into the $\gamma\delta$ lineage. These conclusions contradicted the predictions of the rearrangement models. However, the variability of the phenotypes raised many doubts to the validity of the conclusions drawn from them.

Mice generated in Hedrick's lab (Dent et al., 1990; Kersh et al., 1995) also suffered from a range of variable phenotypes (reviewed in Fehling, 1999). In this case though, the severity of phenotypes at least correlated with the transgene copy

number. In particular, some of the V γ 1.1J γ 4/V δ 6D δ 2J δ 1 transgenic mice expressed extremely high levels of the transgene and exhibited a block in V-DJ β rearrangements and very few TCR $\alpha\beta$ (+) cells (Kersh et al., 1995). However, even though V-DJ β rearrangements were almost completely blocked, these mice had virtually normal numbers of DP thymocytes, which were CD25(-) and transgenic TCR $\gamma\delta$ (low). These cells had deleted their endogenous TCR δ loci and expressed full length TCR α transcripts, strongly suggesting they belonged to the $\alpha\beta$ lineage. However, $\alpha\beta$ T cell development was blocked at the DP stage, probably because the DP cells could not be positively selected by the transgenic TCR $\gamma\delta$, in the absence of TCR $\alpha\beta$.

Taken together, these data implied that a transgenic TCR $\gamma\delta$ expressed at high levels in early thymocytes of the $\alpha\beta$ lineage did not block their development, but in fact was able to substitute for the pre-TCR in promoting their differentiation along the $\alpha\beta$ pathway. Indeed, as down-regulation of CD25, a burst of proliferation, differentiation into DP subset, and inhibition of complete rearrangements of the endogenous TCR β locus (analogous to β allelic exclusion), were all observed.

• *TCR-deficient mice*

Mice lacking TCR $\alpha\beta$ or TCR $\gamma\delta$ were generated by targeted disruption of the constant region involved in the assembly of each type of receptor. The phenotypes of these mice are clear. TCR α null (Mombaerts et al., 1992b; Philpott et al., 1992) and TCR β null (Mombaerts et al., 1992b) mice are devoid of mature $\alpha\beta$ cells (SP thymocytes and peripheral lymphocytes), but maintain a full complement of $\gamma\delta$ cells. Conversely, TCR δ null mice (Itohara et al., 1993) have no $\gamma\delta$ cells but generate normal numbers of $\alpha\beta$ cells. Although this shows that the two lineages can develop independently of each other, it does not provide any further insight into the mechanism of lineage commitment.

Interestingly though, TCR β -deficient mice contain significant numbers of DP thymocytes, about 5% of the absolute number found in wild type mice. Importantly, these DP cells are almost absent (0.05% of WT) in TCR β ^{-/-} x TCR δ ^{-/-},

double deficient mice. This implies that the DP thymocytes of $\text{TCR}\beta^{-/-}$ mice are largely dependent on $\text{TCR}\delta$ for their generation. The ability of $\text{TCR}\gamma\delta$ to support $\text{DN} \rightarrow \text{DP}$ transition had also been suggested by the phenotype of $\text{TCR}\gamma\delta$ transgenic mice (see above). In both cases, the DP thymocytes displayed characteristics of cells of the $\alpha\beta$ lineage (reviewed in Fehling, 1999).

To further investigate the role of $\text{TCR}\gamma\delta$ in the production of DP thymocytes, Passoni *et al.* and Livak *et al.* (Livak *et al.*, 1997; Passoni *et al.*, 1997) analysed the quality of $\text{TCR}\delta$ (and $\text{TCR}\gamma$) gene rearrangements in $\text{TCR}\beta^{-/-}$ DP cells. Both groups showed by RFLP-PCR that, as predicted, those cells had been selected for in-frame (75%) $\text{TCR}\delta$ (and $\text{TCR}\gamma$) rearrangements. Furthermore, they expressed $\text{TCR}\delta$ transcripts at much higher levels than wild type DP thymocytes. These data indicated that, in the absence of a $\text{TCR}\beta$ chain, a sizeable fraction of DP cells were generated from precursor cells expressing a functional $\text{TCR}\gamma\delta$.

However, it is important to note that, in the presence of a $\text{TCR}\beta$ chain, this alternative pathway is insignificant, as the overall DP population seems to be selected against productive $\text{TCR}\gamma/\delta$ rearrangements (see previous section). This is probably due to the inefficiency of this alternative, $\text{TCR}\gamma\delta$ -dependent pathway, when compared with the usual pre-TCR-mediated process.

• *pT α -deficient mice*

Since $\text{TCR}\alpha$ genes are rearranged during late thymocyte development (ISP/DP), the outcome of this process should not influence the $\alpha\beta/\gamma\delta$ lineage decision. Accordingly, no change of $\alpha\beta/\gamma\delta$ ratio can be detected in $\text{TCR}\alpha^{-/-}$ mice, and $\alpha\beta$ T cell development proceeds normally up to the DP stage (Mombaerts *et al.*, 1992b; Philpott *et al.*, 1992). Therefore, a more informative model to study lineage divergence is the $\text{pT}\alpha^{-/-}$ mouse, due to the association of $\text{pT}\alpha$ and $\text{TCR}\beta$ in the formation of the pre-TCR.

As expected, $\text{pT}\alpha^{-/-}$ mice (Fehling *et al.*, 1995a) have a similar phenotype to $\text{TCR}\beta^{-/-}$ mice: $\alpha\beta$ development is severely impaired (with DP thymocytes

accounting for not more than 20% of the 10-fold smaller than WT thymus), but $\gamma\delta$ cells differentiate normally. In fact, the $\gamma\delta$ cell compartment is over-represented both in relative (10-25% of all thymocytes) and absolute (3-5 fold increase) numbers (Fehling et al., 1997b; Fehling et al., 1995a). Indeed, this has been interpreted as providing evidence for the competitive rearrangement model, since the absence of a pre-TCR seems to divert thymocyte precursors from the $\alpha\beta$ to the $\gamma\delta$ lineage. However, as in the case of $\text{TCR}\beta^{-/-}$ mice, the presence of significant numbers of DP cells in $\text{pT}\alpha^{-/-}$ (and also in $\text{pT}\alpha^{-/-}$ x $\text{TCR}\alpha^{-/-}$) mice demonstrates that the pre-TCR (and the $\text{TCR}\alpha\beta$) does not play an obligatory role in lineage commitment.

• *Implications for the lineage divergence models*

The data reviewed above contradicted a number of predictions from the rearrangement models: First, early expression of $\text{TCR}\alpha\beta$ does not block $\gamma\delta$ development, since “ $\gamma\delta$ -like” cells are found in the periphery of $\text{TCR}\alpha\beta$ transgenic mice. Second, and conversely, early expression of $\text{TCR}\gamma\delta$ does not block $\alpha\beta$ development (up to DP stage), as events associated with β -selection, including the production of DP thymocytes, occur in $\text{TCR}\gamma\delta$ transgenic mice. Third, expression of $\text{TCR}\gamma\delta$ is capable of promoting the progression to the DP stage, as shown by the studies of $\text{TCR}\beta^{-/-}$ and $\text{TCR}\beta^{-/-}$ x $\text{TCR}\delta^{-/-}$ mice.

Nevertheless, the competitive rearrangement model is somewhat salvaged by the fact that this $\text{TCR}\gamma\delta$ -dependent pathway for DP production is very inefficient, and practically insignificant in a normal ($\text{TCR}\beta$ -proficient) thymus (and it may even be an artifact of the “non-physiological” $\text{TCR}\beta^{-/-}$ mouse model).

The only piece of data that supports the competitive rearrangement model is the increased frequency of $\gamma\delta$ cells in $\text{pT}\alpha^{-/-}$ (and $\text{TCR}\beta^{-/-}$) mice. However, this could also be explained by the “space” in the thymus created by the severe reduction in $\alpha\beta$ cell numbers (i.e., a homeostatic effect). In this scenario, the function of the pre-TCR would be limited to the expansion of $\alpha\beta$ -committed thymocytes, and would not influence the lineage decision itself (Fehling et al., 1999).

Taken together, these data and the results of the rearrangement analyses (3.3) have convinced many scientists of the flaws of the rearrangement models, and encouraged them to look for TCR-independent mechanisms of $\alpha\beta/\gamma\delta$ lineage commitment.

3.5 TCR-independent mechanisms in $\alpha\beta$ vs. $\gamma\delta$ cell

differentiation

The search for TCR-independent mechanisms of $\alpha\beta/\gamma\delta$ lineage commitment is still in its early stages, and as yet, only the signalling pathways involving IL-7R and Notch receptors, have been identified as potential candidates.

3.5.1 IL-7 / IL-7R signalling

IL-7 is the first extracellular agent identified that appears to modulate directly and specifically transcription and rearrangement of the TCR (and BCR) genes (Berg and Kang, 2001). The physiological targets of IL-7R signalling are the TCR γ (and IgH) genes. Such effects are the basis of a selective role of the IL-7/IL-7R pathway in $\gamma\delta$ cell differentiation.

In the IL-7R deficient thymus, whereas $\alpha\beta$ thymocytes are present (although in reduced numbers, due to the effect on early DN precursors), $\gamma\delta$ cells are completely absent (Maki et al., 1996). Furthermore, TCR γ rearrangements are undetectable in IL-7R null thymocytes, while other TCR rearrangements seem to proceed normally (Durum et al., 1998; Kang et al., 1999). Conversely, enforced expression of a rearranged TCR γ transgene can partially rescue $\gamma\delta$ lineage development in the absence of IL-7R signalling (Kang et al., 1999). In IL-7^{-/-} and γ_c ^{-/-} mice, residual TCR γ gene recombination is detectable, which may result from the activity of TSLP, the other cytokine that uses the IL7R α chain in the assembly of its receptor (Park et al., 2000), as previously discussed.

The mechanism behind the effect of IL-7/IL-7R signalling on TCR γ rearrangements is still unclear. The fact that the TCR γ locus is hypermethylated in IL-7R deficient thymocytes (Durum et al., 1998) implies that the locus is inaccessible and inactive, and thus suggests a role for IL7R pathway in control of locus accessibility. In accordance with this, IL-7R^{-/-} thymocytes are not susceptible to *in vitro* cleavage of J γ 1 gene segment by RAG enzymes (Schlissel et al., 2000).

Indirect evidence supports the involvement of STAT5, a downstream mediator of IL-7R signalling, in the regulation of the transcriptional activity of the TCR C γ 1 locus. For example, the presence of several consensus STAT5-binding sites in its regulatory regions, and, more convincingly, the generation of $\gamma\delta$ cells in FTOC of IL-7R^{-/-} thymocytes expressing a constitutively active (but not wild type) STAT5 (Ye et al., 1999). However, STAT5^{-/-} mice exhibit near normal numbers of both $\alpha\beta$ and $\gamma\delta$ cells (Teglund et al., 1998), suggesting that other STATs (or other, unidentified, signalling mediators) are involved in this aspect of IL-7R signalling.

The involvement of the IL-7R pathway in $\alpha\beta$ *vs.* $\gamma\delta$ differentiation (in the context of a separate lineages model) were recently highlighted by Kang and Raulet (Kang et al., 2001). These authors showed they could sub-divide pro-T cells (DN2 subset) into two populations with distinct biased $\alpha\beta/\gamma\delta$ developmental potentials based on IL-7R α (CD127) surface expression levels. They sorted DN2 thymocytes expressing high *vs.* low levels of CD127, and assessed their ability to generate $\alpha\beta$ and $\gamma\delta$ cells after intra-thymic injection or FTOC. In both assays, the CD127(low) subset was biased towards $\alpha\beta$ development, whereas the CD127(high) subset preferentially produced $\gamma\delta$ cells. Since these outcomes did not correlate with level of TCR rearrangements of the sorted cells (note that pro-T cells are devoid of pre-TCR and TCR expression), Kang and Raulet concluded that $\alpha\beta/\gamma\delta$ cell fate determination is (at least in part) independent of TCR signals.

Although these results are not definitive in terms of a separate lineages model (each subset was not restricted to one lineage, it was just biased towards one of them), they provided the first evidence that precursor thymocytes with indistinguishable TCR rearrangement status can have distinct lineage potentials, a specific requirement of the separate lineages hypothesis.

3.5.2 Notch signalling

Notch signalling has been previously described in section 2.6.4. As mentioned there, this pathway has been implicated in all lineage decisions that take place in the thymus, including $\alpha\beta/\gamma\delta$ (Robey and Fowlkes, 1998).

The first evidence suggesting a role for Notch in the $\alpha\beta/\gamma\delta$ split came from mixed haematopoietic stem cell chimeras using Notch-1 $+/+$ and $+/-$ donors (Washburn et al., 1997). While mice with a single copy of the gene displayed normal T cell development, in mixed chimeras the $+/-$ cells gave rise to reduced numbers of $\alpha\beta$ T cells compared to $+/+$ cells developing in the same irradiated host, whereas $\gamma\delta$ frequency was less affected.

These studies were followed by the generation of transgenic mice expressing constitutively active Notch-1 (icNotch-1) under the control of the *Lck* promoter (Washburn et al., 1997). However, overall $\alpha\beta/\gamma\delta$ ratios were unperturbed, even if $\gamma\delta$ thymocytes expressed CD4 and CD8 at a higher frequency than normal. These mice were then crossed with $\text{TCR}\beta^{-/-}$ mice, and on such a gene-deficient background icNotch was able to promote $\alpha\beta$ development, as it was able to overcome the block in T cell development characteristic of $\text{TCR}\beta^{-/-}$ mice.

The majority of DP cells generated in $\text{TCR}\beta^{-/-}$ icNotch-1 mice had in-frame γ/δ rearrangements, suggesting that icNotch had diverted thymocytes from the $\gamma\delta$ to the $\alpha\beta$ lineage. On the other hand, icNotch did not bypass the developmental block of $\text{RAG-1}^{-/-}$ mice, which, together with the selection for in-frame γ/δ rearrangements in the previous case, shows that Notch is not sufficient to direct $\alpha\beta$ development. Indeed, Notch signalling appears to require co-ordination with TCR (at least $\text{TCR}\gamma\delta$) signals in order to exert its role in thymocyte development. According to the model proposed by Robey and collaborators (Robey and Fowlkes, 1998), normal T cell differentiation would involve 'shutting off' Notch signalling in thymocytes that would follow the $\gamma\delta$ pathway, whereas the $\alpha\beta$ cell fate would be controlled by both pre-TCR and Notch signals. Since the mechanism for 'shutting off' Notch in the $\gamma\delta$ lineage would probably arise from $\text{TCR}\gamma\delta$ itself, this

model is not properly TCR-independent, and does not fulfil the requirements of the separate lineages hypothesis.

Furthermore, even the positive effect of Notch-1 on $\alpha\beta$ development could simply be due to its anti-apoptotic activity (Hayday et al., 1999), as proved to be the case for the CD8 lineage (Deftos et al., 1998) (see 2.6.4). This role of Notch would be particularly obvious on DP cells, since they are the thymic subset more prone to apoptosis.

Finally, the model proposed by Robey and Fowlkes is completely inconsistent with the report of an independent group (Jiang et al., 1998) which showed that mice deficient for Jagged-2, a ligand for Notch-1 highly expressed in the thymus (Luo et al., 1997), exhibit an impaired (50% reduced) $\gamma\delta$ foetal development but normal $\alpha\beta$ cell numbers. These results have been dismissed by Robey and collaborators.

One interesting aspect of Notch (as a developmental pathway) still to be addressed is that of timing and specificity, particularly when this pathway has been implicated in all other thymic lineage decisions.

4 Objectives of the studies presented in this thesis

My PhD studies focused on two crucial aspects of T cell development: $\alpha\beta$ vs. $\gamma\delta$ lineage commitment; and β -selection.

Regarding the $\alpha\beta/\gamma\delta$ lineage choice, all currently available models are very unsatisfactory. In particular, although many believe in some kind of separate lineages model, the components of such a pathway are still unknown. Even the most convincing candidate, IL-7R, is not capable of sub-dividing precursor DN thymocytes into populations with restricted lineage potential.

Thus, one of the major objectives was to identify genes involved in this lineage divergence process. We therefore attempted to isolate genes differentially expressed between early $\alpha\beta$ and $\gamma\delta$ thymocytes, from which we hoped to select and test prime candidates for a role in a separate lineages model. At the same time, this approach had the potential to provide us with $\gamma\delta$ -specific markers, especially important since no other marker, besides the TCR $\gamma\delta$ itself, is yet known. In addition, these markers could be extremely useful for the definition of a $\gamma\delta$ -specific promoter, which would allow (for the first time) the selective expression of transgenes in the $\gamma\delta$ lineage.

Concerning β -selection, the objectives were to identify nuclear targets of the signalling pathways triggered by the pre-TCR (since those downstream effectors are still largely unknown), and to investigate their role in the DN to DP transition.

Both projects relied on a technique well established in the Owen laboratory, RDA (representation difference analysis), a subtractive hybridisation method that is used to compare gene expression between closely related cell populations.

The importance and the role (in T cell development) of the candidate genes obtained from the RDA was investigated in the murine system via *in vivo/ex vivo* (involving several genetic manipulated mice) and *in vitro* (in particular, FTOC) strategies.

Chapter II :

METHODS

1 Cellular biology - general methods

1.1 Preparation of murine cells

T cells were prepared from *ex vivo* murine organs: thymus, spleen, lymph nodes. The organs were extracted from lethally anaesthetised (5 minutes with carbon dioxide) animals. In general, 3-4 week old adults were used. For foetal studies, thymus was extracted (under light microscope) from E14-E18 embryos.

All animals were maintained in a barrier facility, at Cancer Research UK's animal house (Clare Hall, South Mimms) to ensure a specific pathogen free (SPF) health status. Mice imported from an outside source were kept in a separate unit. Mice were bred at sexual maturity (6-7 weeks) and samples of tissues (tail snip, tail bleed) for genotyping were taken at 10-20 days of age.

Cell suspensions were obtained by mashing the organs on 70 μm (nylon) cell strainers (Becton Dickinson) and flushing with PBSA. Cells were generally washed with FACS buffer, centrifuged at 1,600 \times g for 5 minutes and re-suspended in appropriate solutions.

PBSA (pH 7.4): 137 mM NaCl, 3.3 mM KCl, 1.7 mM KH_2PO_4 , 10 mM Na_2HPO_4 .

FACS buffer: 2% (v/v) foetal calf serum (FCS), heat inactivated, in PBSA.

When required, cells were counted on a haemocytometer / counting chamber (Weber Scientific International) under the light microscope. 5 μl of cell suspension were mixed with 5 μl of Tripan blue (Sigma) for exclusion of dead cells. Cell number was obtained by multiplying the total count (of the central, double-lined, square of the grid) by the conversion factor 10^4 cells/ml.

1.2 Depletion of CD4(+)/CD8(+) T cells

Studies on CD4(-)CD8(-) thymic precursors frequently required that they were enriched from the characteristic 2% abundance (in a wild type adult thymus) to 80-98%. This was accomplished by complement-mediated depletion of CD4(+)/CD8(+) thymocytes that were selected via antibody supernatants specific for CD4 (from hybridoma RL172) and CD8 (from hybridoma 31M). These supernatants were obtained from cells at the end of their exponential growth in fresh medium (Cancer Research UK antibody production department). The original hybridomas (stored in aliquots in liquid nitrogen) were a kind gift from Dr. Rhodri Ceredig (Universite Joseph Fourier, Grenoble, France).

Up to $2-4 \times 10^8$ thymocytes (corresponding to 2 wild type adult thymuses) were re-suspended in 7 ml DMEM (no additives) and incubated in 37°C water bath for the entire procedure. 1 ml of each supernatant (RL172 and 31M) was then added to the cell suspension. After 10 min, 1 unit (dissolved in 1 ml dH₂O) of low-tox-M rabbit complement (Cedarlane CL 3051) was added. Cells were incubated for 45 min, with occasional shaking (every 15 min) of the tube. 4 ml of Ficoll-paque plus (Amersham Pharmacia) were then deposited in the bottom of the tube, and this mixture was centrifuged for 20 min at 1,600xg, 25°C.

The interfase, containing living cells, was collected into a new tube and washed with FACS buffer. Cells of this DN preparation were finally re-suspended in appropriate solutions for the experiment that followed.

1.3 Cell staining with antibodies and chemicals

1.3.1 Extracellular (surface) staining

Cells were typically re-suspended either in 100-200 µl (for analysis) or in 400-1000 µl (for sorting) of FACS buffer, resulting in a final sample concentration that would not exceed 10^5 cells/µl. Antibodies were added (typically in 1:100 to 1:500 dilutions, according to individual titrations) and samples were shaken (vortex) and incubated on

ice for 15 minutes. Samples were washed (once or twice) with FACS buffer, centrifuged and re-suspended in appropriate volumes (minimum 200 μ l for analysis and 1000 μ l for sorting) of FACS buffer.

Fluorescent antibodies were purchased from Pharmingen, unless otherwise stated (those labelled with * were from Caltag). The conjugated fluorochromes were: fluorescein isothiocyanate (FITC), phycoerythrin (PE), Cy Chrome, Tricolor and allophycocyanin (APC).

Antibodies routinely used were specific for the following markers (-fluorochrome):

CD2 (-PE), clone T11	CD3 ϵ (-FITC, -PE, -Cy Chrome)
CD4 (-FITC, -PE, -Cy Chrome, -APC)	CD5 (-FITC, -PE)
CD8 α (-PE, -Cy Chrome, -APC)	CD8 β (-FITC)
CD23 (-PE)	CD24 / HSA (-FITC, -PE)
CD25 / IL-2R β (-FITC), clone 7D4	CD25 / IL-2R β (-APC), clone PC61
CD43 (-FITC, -PE)	CD44 (-FITC, -PE, -Cy Chrome)
CD45R / B220 (-FITC, -PE, -Cy Chrome)	CD69 (-PE)
CD90 / Thy1.2 (-FITC *, -APC)	CD117 / c-kit (-PE), clone 2B8
CD122 (-FITC), clone TM- β 1	CD127 / IL-7R α (-PE)
TCR $\alpha\beta$, H57-597 (-FITC, -PE, -APC)	TCR $\gamma\delta$, GL3 (-FITC, -PE, -Tricolor *)
NK1.1 (-PE)	IgD (-FITC)

1.3.2 Extracellular (surface) liposome staining

For detection of the very low levels of surface TCR β expression on DN thymocytes, we used a sensitive liposome technique developed by Bruno and Radbruch (Bruno et al., 1999). The liposomes (Scheffold et al., 1995) contain fluorescein (green fluorochrome) and are coupled to anti-digoxigenin antibody fragments (Boehringer). They were a kind gift from Dr. Andreas Radbruch (DRFZ Berlin, Germany).

After depletion of CD4(+)/CD8(+) cells (see above), DN-enriched 2×10^6 thymocytes (equivalent to 1 wild type adult thymus, after depletion) were re-suspended in 100 μ l PBA.

PBA: 0.5% bovine serum albumine (Sigma), 0.5% NaN_3 , in PBSA.

5 μ l of anti-hamster IgG and 5 μ l anti-CD16 (anti-F_c-R) were added to reduce unspecific binding of the antibody used for liposome-mediated amplification of signal (H57-597). After 5 min on ice, all the antibodies required for definition of thymocyte subsets, plus 10 μ g/ml of anti-TCR β -digoxigenin, were added in an additional 100 μ l of PBA. Cells were incubated for 15 min on ice, and then washed twice (5 min at 1,600xg, 4°C).

200 μ l of diluted (typically 1:50) anti-digoxigenin-liposomes were then mixed with the cell suspension. This sample was incubated with permanent shaking (500 rpm) for 30 min in the cold room (4°C). Cells were finally washed three times and re-suspended in (400/1000 μ l) PBA for FACS analysis / cell sorting.

1.3.3 Intracellular staining

- For intracellular staining with antibodies, cells were first stained for cell surface markers on ice, as described above, and subsequently fixed in 1% paraformaldehyde for 10 min at room temperature (RT).

Cells were pre-incubated (to reduce unspecific staining) with 5 μ g purified hamster IgG2 λ , isotype standard (Ha4/8, Pharmingen) in 0.3% saponin buffer (for cell permeabilisation) for 10 min at RT.

Saponin buffer (0.3%): 0.3% saponin, 5% FCS, 10mM HEPES pH7.4, in PBSA.

0.2 μ g of PE-conjugated antibody, either the specific one, TCR β (H57-597) in particular, or the isotype control, IgG2 λ (Pharmingen), was then added to the cell suspension. After 30 min at RT, cells were washed with 0.1% saponin buffer and analysed.

- For evaluation of the DNA content of cells (cell cycle analysis), these were incubated with 7-AAD (20 μ g/ml) diluted in FACS buffer containing 0.03% saponin for 1 hour at 37°C.

- To measure cell proliferation, BrdU Flow Kit (Pharmingen) was used (following the manufacturer's protocol) to pulse cells for 6 hours, followed by the analysis of incorporated BrdU.

- For intracellular staining of β -galactosidase (LacZ) products, thymocyte preparations of CREM-LacZ mice were incubated for 30 min at 25°C in 33 μ M C₁₂FDG (Molecular Probes). This C₁₂-version of FDG (fluorescein di-galactopyranoside) is lipophilic and therefore permeates cells directly. Samples were then transferred to ice and stained with antibodies for the desired extracellular markers, as mentioned above.

- To identify apoptotic cells, samples stained for cell-surface markers were washed twice in Annexin-V buffer (Pharmingen) and subsequently incubated with FITC-conjugated Annexin-V (1:200) in Annexin-V buffer at RT for 30 min. TO-PROTM-3 iodide (Molecular Probes) was added to a final concentration of 50nM, 5 min before cells were analysed.

1.4 Flow cytometry (FACS) analysis and cell sorting

Flow cytometric analysis of samples was performed on a FACScalibur machine (Beckton Dickinson) using CellQuest software. Starting parameter voltage values for detection of T cells: forward scatter (FSC) E00, gain 1, linear scale; side scatter (SSC) 450, linear; fluorescence channels (FL) FL1 at 705, FL2 at 670, FL3 at 720 and FL4 at 800 Volt, logarithmic scale. Fluorochromes and their FL channels: FITC on FL1 (green), PE on FL2 (red), Cy Chrome and Tricolor on FL3 (purple), APC on FL4 (blue). Compensations were set using single-colour controls for each channel.

Florescence-associated cell sorting was performed on either a MoFlo (Cytomation) or Vantage (Beckton Dickinson) machine, by staff of Cancer Research UK's FACS laboratory. Cells of >95% purity were collected on solutions appropriate for the experiments that were to follow.

1.5 Foetal thymic organ cultures

Foetal thymic organ cultures (FTOC) and reaggregate thymic organ cultures (RTOC) were used to study T cell development *in vitro*, following the protocols of Jenkinson *et al.* (Jenkinson *et al.*, 1992).

FTOCs and RTOCs were cultured in standard RPMI 1640 medium supplemented with 10% FCS, 50 μ M 2-ME, L-Glutamine, non-essential amino acids, 10mM HEPES, penicillin and streptomycin.

Hanging drop FTOCs were established in Terysaki plates by placing 5×10^4 to 5×10^5 thymocytes with a 5-day 2-deoxyguanosine-treated (1.35mM) E14-15 foetal wild type lobe in 25 μ l of culture medium. The plates were upturned and incubated in a humidified chamber. The cultures were fed with fresh medium every 2 days.

RTOCs were established by first generating a single cell suspension of wild type thymic stroma by digesting 5-day 2-deoxyguanosine-treated E14-15 thymic lobes with trypsin (0.05% trypsin in Versene) for 2x10 min at 37°C. Stromal cells were filtered, washed and counted. Reaggregates were then formed by centrifuging 7.5×10^5 stromal cells with $1-2 \times 10^4$ FACSsorted thymocytes (see above), at 2,800xg for 2 min, and pipetting the pellet (re-suspended in about 10 μ l) onto a 0.8 μ m nucleopore filter (Millipore) placed in standard medium. When required, antibodies (such as anti-CD3 ϵ) or chemicals (such as cyclic-AMP analogues) were also added to the medium (see results).

The cultures were then incubated for 2-7 days, after which they were stained and analysed by FACS.

2 Molecular biology - general methods

Protein, DNA and RNA were normally stored at -20°C, unless otherwise stated.

2.1 Protein

2.1.1 Protein extraction

Cells were re-suspended in 1% Triton lysis buffer and incubated on ice for 15 min. Samples were then centrifuged at 16,000xg for 5 min (at 4°C) and supernatants were collected (pellets were discarded). These extracts were stored at -20°C until they were loaded onto polyacrylamide gels for electrophoresis. Then, they were diluted in 4x SDS sample buffer and boiled (95 °C) for 5 min.

1% Triton lysis buffer: 20 mM Tris-HCl (pH 7.4), 160 mM NaCl, 1 mM EDTA, 1mM EGTA, 1 mM PMSF (added fresh), 5 mM NaF, 10 mM β-glycerolphosphate, 10% glycerol, 1% Triton X 100, 1 µg/ml protease inhibitors (added fresh).

4x SDS sample buffer: 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.05% bromophenol blue.

2.1.2 SDS-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was carried out in a vertical gel apparatus (Hoefer) using two 18x16 cm glass plates assembled with 1.5 mm spacers. 30 ml of 15% resolving gel was prepared just before use and poured into the assembly, followed by a thin layer of 0.25% SDS. After solidifying, the mixture was overlaid with stacking gel and a comb was inserted.

15% resolving gel: 15 ml acrylamide 30% (37.5:1), 7.5 ml Tris-HCl 1.5 M pH 8.8, 7.4 ml dH₂O, 150 µl SDS 20%, 300 µl APS 10%, 30 µl TEMED.

Stacking gel: 1.7 ml acrylamide 30% (37.5:1), 2.5 ml Tris-HCl 0.5 M pH 6.8, 5.75 ml dH₂O, 50 µl SDS 20%, 100 µl APS 10%, 10 µl TEMED.

After loading of protein samples (pre-boiled) and 15 μ l Rainbow markers (Amersham Pharmacia), the gel was run at 20 mA until the samples had passed through the stacking gel, and then at 30 mA for approximately 4-5 hours, until the markers had separated and the blue dye front had just come off the gel.

1x running buffer: 3 g/l Tris, 14.4 g/l glycine, 1g/l SDS (5 ml SDS 20%), in dH₂O.

2.1.3 Western blotting

Gels were transferred using a Hoefer Semi-Phor blotting apparatus. The stacking gel was discarded. Six pieces of 3MM blotting paper and a piece of ImmobilonTM-P PVDF 0.45 μ m membrane were cut to fit the gel size. The membrane was pre-wet in methanol, rinsed with dH₂O and then soaked, together with the blotting paper, in transfer buffer for 2 min.

Transfer buffer: 10 mM CAPS pH 10.

A mylar mask with an opening 2 mm smaller than the gel in width and length was placed over the electrode. Three sheets of blotting paper, followed by the membrane, then the gel and three more paper pieces were centred over the opening of the mask. The edges of the 'sandwich' were rolled out to ensure there were no air bubbles in it (which would block the transfer). A current of 0.8 mA/cm² of gel, during 1 hour, was applied to transfer the proteins from the gel onto the membrane.

The membrane was then rinsed with dH₂O and placed in blocking solution for 1 hour at RT.

Blocking solution: 5% milk powder in 1x TBS-Tween-20.

1x TBS-Tween-20: 2.42 g/l Tris, 8 g/l NaCl, 380 μ l/l HCl 10M, 1ml/l Tween, in dH₂O.

The membrane was incubated with primary antibody (anti-CREM, diluted 1:500) in blocking solution (in small plastic bag) for 2 hours at RT or overnight at 4°C, on an orbital shaker.

Membrane was washed 3x10 min in 1x TBS-Tween-20, and then incubated with secondary antibody (anti-rabbit Ig coupled to horseradish peroxidase), diluted 1:5,000 in 10 ml blocking solution for 30 min at RT (on shaker).

The membrane was then washed 3x10 min in 1x TBS-Tween-20. Protein-antibody complexes were detected by chemio-luminescence (ECL) using "ECL kit" (Amersham): 2 ml solution A and 2 ml solution B over the membrane for 30 seconds, followed by autoradiograph (Kodak film) exposure for 1-10 min. Autoradiographs were developed on a RG II X-ray film processor (Fuji).

2.2 DNA

2.2.1 Extraction of genomic DNA

Cells were boiled in PBSA for 5 min at 95°C. 10 µg/ml of proteinase K (Roche) was added and the samples were incubated for 3 hours (or overnight) at 55°C.

DNA was extracted with phenol: chloroform: isoamylol (24:25:1) mixture, which (1x volume) was added to samples, they were vortexed and left at RT for 5 min, after which they were centrifuged at 16,000xg for 5 min. Upper (aqueous) phase was collected and DNA was precipitated with 2 volumes of ethanol and 100 µM sodium acetate. The pellet was re-suspended in dH₂O (at approximately 1-2x10³ cell equivalents/µl).

2.2.2 Preparation of plasmid DNA

2.2.2.1 *Small scale ("mini-prep")*

Ampicillin-resistant single colonies (grown on agar plate with 50 µg/ml ampicillin) were picked into 5 ml LB (Cancer Research UK) plus 50 µg/ml ampicillin (Sigma) and incubated overnight at 37°C on shaker.

LB (lauria-broth): 0.5% bacto-yeast extract, 1% bacto-tryptone, 1% NaCl, pH adjusted to 7.6 with KOH 10 M.

Lauria-agar: 15 g/l bacto-agar, in LB.

After centrifugation at 16,000xg for 3 min, bacterial pellets were re-suspended in 100 µl solution I, followed by the addition of 200 µl solution II and incubation at 4°C for 5 min. 150 µl of solution III was then added, lysates were vortexed, incubated at 4°C for a further 5 min and centrifuged at 16,000xg for 10 min. The supernatants were collected and DNA was precipitated with 2 volumes of ice-cold ethanol and re-suspended in dH₂O.

Solution I: 50 mM glucose, 10 mM EDTA pH 8.0, 25 mM Tris-HCl pH 8.0.

Solution II: 0.2 M NaOH, 1% SDS.

Solution III: 3 M potassium / 5 M acetate, pH 4.8 (60 ml potassium acetate 3M plus 11.5 ml glacial acetic acid, per 100 ml solution).

2.2.2.2 Large scale (“maxi-prep”)

Bacteria (*E.coli*) were grown overnight (800 ml inoculated with 2 ml of mini-prep) on LB + ampicillin. After centrifugation, the pellet was treated with 30 ml solution I, 45 ml solution II and 45 ml solution III, and centrifuged again. The supernatant was filtered and DNA was precipitated with 1 volume of isopropanol. The DNA pellet was re-suspended in 50 mM TE pH 8.0 and 1.05 g/ml caesium chloride and 600 µl ethidium bromide 10 mg/ml were added to the solution, which was transferred to ultracentrifuge tubes. After 18-24 hours of ultracentrifugation at 45,000 rpm (at 18°C), the ethidium bromide band containing the DNA was extracted (with a needle) and precipitated with 2 volumes of ethanol. The pellet was re-suspended in dH₂O.

DNA was quantified on a LKB spectrophotometer, using the absorbance at 260 nm. The optical coefficient (ϵ) for DNA at 260 nm is 50 (and for RNA, it is 40). The absence of contaminating proteins was verified by the absorbance reading at 280 nm: the ratio A₂₆₀/A₂₈₀ was within the range 1.7-1.95.

2.2.3 Polymerase chain reaction (PCR)

PCR was used to amplify DNA (genomic or cloned) and cDNA. Short oligonucleotide primers (20-30mers) were designed to have roughly 50% G/C content (including the last 3' nucleotides, either G or C) and to span 200-400 bp. They were

synthesised in the Oligonucleotide department of Cancer Research UK (Clare Hall, South Mimms).

Template DNA was mixed with 50 pM of sense (forward) and anti-sense (reverse) primers and 200 μ M of each dATP, dCTP, dGTP and dTTP (Amersham Pharmacia), in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 4 mM MgCl₂ and 0.1 % Triton X-100. 2.5 units of either stock Taq polymerase (Cancer Research UK) or Advantage 2 DNA polymerase (Clontech) was added to the mixture.

Amplification reactions were carried out in a Peltier thermal cycler (MJ Research). Typical conditions were:

Denaturation: 3 min at 94°C.

30-35 cycles of: 30 sec at 94°C (denaturation);

30 sec at primers annealing temperature ($T_m = n(A+T) \times 2 + n(C+G) \times 4$);

30 sec at 72°C (extension).

Final extension: 5 min at 72°C.

2.2.4 Agarose gel electrophoresis

Agarose gels (usually 1-2%) were prepared by dissolving agarose (Novara) in 1x TAE buffer.

50x TAE buffer (stock): 2 M Tris base pH 8.0, 1 M glacial acetic acid, 50 mM EDTA pH 8.0.

50 μ g ethidium bromide were added per 100 ml gel for DNA band visualisation under UV light. Boiling gel mixtures were poured onto gel trays (Jencons). After solidifying, gels were loaded with samples (supplemented with loading buffer) and run at 100 V for a typical time of 1 hour (or longer).

6x loading buffer: 0.25% bromophenol blue, 0.25% xylene-cyanole, 30% glycerol.

DNA bands were visualised and recorded on an ImaGO machine (B&L systems).

2.2.5 Purification and radio-labelling of DNA probes

PCR-amplified DNA product was run on agarose gel. The band of interest (100-600 bp in size) was visualised on a UV transilluminator (GRI Ltd.) under low intensity UV light, and excised from the gel.

The gel slice was placed on an eppendorf tube with 100 µl capture buffer of "GFX gel band purification kit" (Amersham Pharmacia) per 100 mg of gel. Tubes were incubated at 60°C for 15 min and then, after gel had melted, samples were transferred to GFX columns placed inside collection tubes.

Following 1 min incubation at RT, samples were centrifuged at 16,000xg for 30 sec and then a further 30 sec with 500 µl wash buffer. All the liquid collected was discarded. The columns, retaining the DNA in its resin, were placed inside fresh eppendorf tubes, where 30 µl of elution buffer was laid on the resin, and tubes were centrifuged at 16,000xg for 1 min. The eluted DNA was either used directly or precipitated with ethanol and 10% (v/v) sodium acetate 3M pH 5.2.

With the exception of RFLP-PCR experiments, probes were radio-labelled using Stratagene's "Prime IT II random primer kit" (Stratagene). DNA (50-100 ng) was re-suspended in 24 µl dH₂O and 10 µl of random primers were added. After 5 min at 100°C, 10 µl of 5x buffer (for dCTP), 5 µl of ³²P-α-dCTP (ICN biomedical) and 1 µl Klenow DNA polymerase were added to the cooled solution. The reaction mixture was incubated at 37°C for 15 min. The excess of dCTP was removed by gel filtration on a Sephadex G50 column.

2.2.6 Restriction fragment length polymorphism (RFLP) -

PCR

Template genomic DNA was submitted to 2 rounds of PCR-amplification of the following TCR gene rearrangements: Vδ5-Jδ1; Vβ2-Jβ2.2; and Vβ5-Jβ2.5. In the *first* round, *long* (37-39mers) reverse primers were used in 30 µl reaction mixtures, each containing 3 µl DNA template. In the *second* round, 3 µl of the first round product

were amplified in each of five 30 μ l reaction mixtures, now using *shorter* (24-26mers) reverse primers.

V δ 5-long, 5'-AGGATGATTCTTCCCGCGACC

J δ 1-long, 5'-AGTCACTTGGGTTTCCTTGTCCAAAGACGAGTTTGTTCGTT

V δ 5-short, 5'-AACTGCAGACCCTTACCCTTC

J δ 1-short, 5'-CGGGATCCCCAAAGACGAGTTTGTTCG

V β 2-short, 5'-ATCCCTGGATGAGCTGGTAT

J β 2.2-short, 5'-TACTTTGGTGAAGGCTCAAAGCTG

V β 5-short, 5'-GGGGTTGTCCAGTCTCCAAG

J β 2.5-short, 5'-TACTTTGGGCCAGGCACTCGGCTC

These primers and their use in RFLP-PCR had been previously described (Dudley et al., 1995; Mallick et al., 1993; Passoni et al., 1997).

Each round was of 30 cycles of 1 min at each temperature: 94°C / 60-65°C / 72°C.

The contents of the five tubes (per rearrangement) of the 2nd round PCR were pooled and run on 1% agarose gels (with particularly large wells). Gel bands were excised according to the predicted product size (500 bp for V δ 5-J δ 1; 200 bp for V β 2-J β 2.2; and 300 bp for V β 5-J β 2.5) and DNA fragments were purified as described.

DNA fragments were digested with specific restriction enzymes (Roche): Hae III for V δ 5-J δ 1; Hinf I and Alu I for V β 2-J β 2.2; and Mae I and Hae III for V β 5-J β 2.5. 50 μ l reaction mixtures, containing 1 μ l of each enzyme and 10% buffer 2, were incubated overnight at 37°C.

Digested DNA segments were precipitated with ethanol and 10% (v/v) ammonium acetate 10M, and re-suspended in 30 μ l dH₂O. These samples were radio-labelled with ³²P- γ -dATP (ICN) in T₄ kinase (New England Biolabs) -mediated reactions. γ ³²-dATP (5 μ l), T₄ kinase (2 μ l), T₄ kinase buffer (5 μ l) and dH₂O (8 μ l) were added to each sample. After 30 min at 37°C, EDTA (2 μ l of 0.5M solution) was added to stop the reaction. Labelled DNA fragments were purified on a G50 Sephadex column and then

precipitated (ethanol and ammonium acetate). Each sample was re-suspended in 10 μ l of dH₂O with loading dye (for sequencing gel).

Because RFLP-PCR relies on the identification of rearrangements spaced by 3 bp, an internal reference (*sequencing ladder*) was required. This was provided by running a proper sequencing reaction for a plasmid, pBluescript II KS, *in parallel*. For this reaction, which involved a distinct radioisotope (α -³⁵S) to that of the samples, we made use of "T7 sequenase (version 2.0 DNA) kit" (Amersham Pharmacia).

Plasmid DNA (5 μ g) was denatured with 10% (v/v) NaOH 2 N/EDTA 2 mM, for 5 min at RT. DNA was precipitated with 75% (v/v) ethanol and 5% (v/v) sodium acetate, for 30 min at -70°C.

The plasmid pellet was re-suspended in 7 μ l dH₂O, 2 μ l sequenase buffer 5x and 1 μ l T7 sequencing primer 5 pM, and the primer was annealed at 65°C for 2 min. After cooling down the product on ice, 2 μ l labelling mix (diluted 1:5), 0.5 μ l α -³⁵S-dATP, 1 μ l DTT 0.1 M and 2 μ l polymerase mix were added and the sample was incubated at room temperature for 5 min.

Sequenase polymerase mix (10 μ l): 1 μ l T7 sequenase polymerase, 0.5 μ l pyrophosphatase, in 6.5 μ l enzyme dilution buffer.

The plasmid DNA sample was split into 4 aliquots of 3.5 μ l, and each reaction was terminated independently, one for each nucleotide (A, C, G, T) of the sequencing ladder. This was done by adding 2.5 μ l of each termination mix (ddATP, ddCTP, ddGTP, ddTTP), pre-warmed at 37°C, and 4 μ l of stop-dye solution, to each tube.

Labelled genomic DNA and plasmid DNA samples were denatured at 80°C for 3 min, and then loaded onto a Biorad sequencing gel.

The polyacrylamide sequencing gel was prepared with 50 ml "Easigel" (Scotlab) and 500 μ l APS 10% and 25 μ l TEMED.

"Easigel": 6% acrylamide : 0.3% bis-acrylamide (19:1 ratio), 7M urea.

Gel was run in 1x TBE at 50°C until the loading dye reached the bottom (3-4 hours) and then it was dried for 1 hour at 80°C.

10x TBE (stock): 0.89 M Tris base pH 7.4, 0.89 M boric acid, 10 mM EDTA pH 8.0.

Signals were detected on a Phosphoimager system (Molecular Dynamics) and analysed (by densitometry) using the accompanying ImageQuant software.

2.2.7 Cloning – general procedures

2.2.7.1 *Dephosphorylation of vector*

Calf intestinal phosphatase (CIP) treatment was employed to reduce the possibility of vector self-ligation. After restriction digestion, vector DNA was diluted to 50 µg/ml and mixed with 1 unit of CIP (Boehringer) and 1x CIP buffer for 1 hour. Vectors with blunt ends or 3' overhanging ends were incubated at 50°C, whereas those with 5' overhanging ends were treated at 37°C. Addition of 2 µl proteinase K (10 mg/ml) in appropriate buffer, at 56°C for 30 min, terminated the reaction (and destroyed any remaining proteins). DNA was extracted with phenol:chlorophorm:isoamylol (24:25:1), precipitated with ethanol and re-suspended in dH₂O.

Proteinase K buffer: 100 mM Tris pH 8.0, 200 mM NaCl, 5 mM EDTA, 0.2% SDS.

2.2.7.2 *End-filling with Klenow DNA polymerase I*

Restriction enzyme-digested DNA (2-6 µg) was end-filled with 4 units Klenow DNA polymerase I (Boehringer) in a volume of 50-100 µl containing 33-66 µM dNTPs and 1x restriction buffer (with MgCl₂). After 30 min at room temperature, the reaction was stopped by adding EDTA to a final concentration of 10 mM and by heating at 75°C for 10 min.

2.2.7.3 *Ligation reactions*

Ligation reactions consisted of a mixture of 10-50 ng of linearised and gel-purified vector and 30-150 ng of restriction-digested and gel-purified insert (in a total volume of 8 µl), to which 1 µl of T₄ DNA ligase (400 U/ml) 1 µl of 10x ligase buffer were added. Vector and insert were ligated usually in a 1:1 ratio. The samples were incubated overnight at 16°C.

2.2.7.4 *Preparation of competent bacteria*

Electrocompetent bacteria were prepared from *E.coli* derivative XL-1 Blue (Stratagene) which possesses tetracycline resistance. 10 ml of overnight-grown, saturated bacterial culture (prepared from an individual colony) were used to inoculate 400 ml LB plus 50 µg/ml tetracycline (Sigma). After growth up to an optical density (at 600 nm) of 0.5, cultures were centrifuged and cell pellets were washed with dH₂O and finally resuspended in 20% glycerol, aliquoted and stored at -70°C.

Heat-shock competent bacteria were prepared from *E.coli* derivative JM109 (Stratagene) which is ampicillin-resistant. After growth up to an optical density (at 600 nm) of 0.5, cultures were centrifuged and gently resuspended in 1/4 volume of ice-cold MgCl₂ 0.1 M. After centrifugation, cells were resuspended in 1/4 volume ice-cold CaCl₂ 0.1 M and incubated on ice for 20 min. After centrifugation, the pellet was finally resuspended in 26.5 ml CaCl₂ 0.1 M plus 3.5 ml glycerol and aliquot samples were snap frozen in liquid nitrogen.

2.2.7.5 *Bacterial transformation*

Ligation reactions were incubated at 70°C for 10 min to inactivate ligase activity. The products were then put onto millipore filters (0.8 µm) floating on dH₂O and dialysis proceeded for 15 min.

Electro-competent bacteria were thawed on ice and 40 µl were mixed with 2.5 µl ligation product. **Electroporation** was performed on a 0.1 cm cuvette of a Bio-Rad *E. coli* Pulser, at 1.8 kV pulse, 200 Ohm resistance and 25 µF capacitance. 1 ml of LB was then added and the culture was shaken at 37°C for 1 hour, after which agar-tetracyclin plates were inoculated and incubated overnight at 37°C.

Alternatively, transformation by **heat-shock** was achieved by mixing 90 µl of thawed competent bugs with 1 µl ligation product and placing the sample: 30 sec on ice, followed by 90 sec on 42°C waterbath, followed by 3 min on ice. 1 ml of LB was then added and bugs were grown for 1 hour at 37°C (shaker).

Agar plates supplemented with L-ampicillin (100 µg/ml) were inoculated with 100 µl transformed bugs and incubated overnight at 37°C. Blue-white selection was also provided by pre-coating agar plates with 20 µl X-Gal (40 µg/ml) and 4 µl IPTG (200

$\mu\text{g}/\mu\text{l}$). Single resistant white colonies were picked and grown overnight in 3 ml LB (+ampicillin), at 37°C (shaker).

2.2.8 CD2-ICER construct for generation of transgenic

mice

The generation of transgenic mice involves the introduction of a foreign gene into the mouse germline. By using a specific promoter, transgene expression can be restricted to a particular lineage of cells.

The human SVA(+) CD2 expression vector (Zhumabekov et al., 1995) is commonly employed for selective expression in the T cell lineage. The activity of this promoter seems to be detected as early as in the DN2 stage of thymocyte development (Cleverley et al., 1999). This agrees with the normal expression of endogenous CD2 glycoproteins, first detected by FACS analysis in DN2 cells (our data). Although some murine B cells also express CD2, the human CD2 promoter follows the human pattern, i.e., its activity is restricted to the T lineage.

The human CD2 (hCD2) expression vector possesses a locus control region (LCR) that confers position-independent and copy number-dependent expression of the integrated transgene. The LCR consists of a strong T cell specific enhancer and additional regulatory elements responsible for establishing an active chromatin domain in T cells (Festenstein et al., 1996).

The human CD2 coding sequences have been reduced in the hCD2 expression cassette, creating a mini-gene comprising the promoter (5 kb) and the first exon and first intron of the hCD2 gene; exon 2 has been interrupted by the introduction of a poly-linker with multiple cloning sites, and the rest of the hCD2 coding sequence has been removed (Figure 6). The inclusion of intron 1 has been shown to greatly enhance the level of transgene expression (Festenstein et al., 1996). The ATG residues in exon 1 have all been mutated to prevent formation of fusion proteins between hCD2 and transgene of interest. The cloned transgenes have their own initiation and termination codons, but they do not contain a poly-adenylation signal. This is therefore provided by a poly(A) signal in the hCD2 mini-gene.

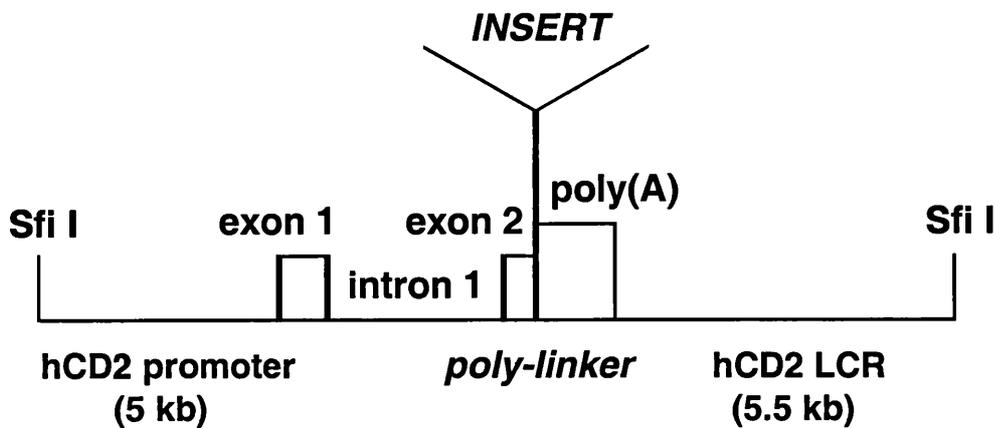


Figure 6 : Structure of the human CD2 expression cassette.

The transgene of interest, ICER, was initially cloned in pBluescript KS after PCR amplification from sorted murine $\gamma\delta$ thymocytes. After restriction enzyme (Kpn I) digestion, the resulting fragment was sub-cloned into the KpnI cloning site of the poly-linker of hCD2 cassette.

The transgene was linearised and agarose gel-purified. DNA was precipitated and resuspended in transgenic injection buffer to a final concentration of 2-4 ng/ μ l (in 30 μ l aliquots) and sent off to be micro-injected at ICRF (Cancer Research UK) Biological Resources Unit (Clare Hall, South Mimms).

Transgenic injection buffer: 10 mM Tris, 0.1 mM EDTA, in ultra-pure dH₂O.

The transgene was micro-injected into the male pronucleus of day 1 fertilised F1 x F1 hybrids (CBA x C57BL/6) mouse oocytes by Ian Rosewell (Cancer Research UK). Injected fertilised oocytes were transferred into day 1 pseudo-pregnant foster mothers. Three founder lines were established in a specific pathogen free (SPF) environment and mice were bred at sexual maturity (6-7 weeks). Mice were sacrificed for analysis at 2-4 weeks of age and levels of ICER transgene expression were measured by real-time PCR (see below, 2.3.3).

2.3 RNA

RNA samples were handled with gloves, transferred with sterile instruments and diluted with fresh milliQ dH₂O. Visualisation of centrifugated pellets was facilitated by addition of 10µg glycogen (Boehringer) in the RNA precipitation step.

2.3.1 RNA extraction and DNase treatment

Total RNA was extracted with TRIZOL reagent (GibcoBRL). Up to 5×10^5 cells were treated with 500 µl TRIZOL. After 10 min at room temperature, 100 µl chloroform was added, mixed, incubated at room temperature for 5 min and centrifuged at 16,000xg for 15 min (at 4°C). The upper (aqueous) phase was collected and transferred to new eppendorf tubes, where RNA was precipitated with equal volume of isopropanol, for 15 min at room temperature (not on ice, to avoid excessive salt precipitation). After centrifugation at 16,000xg for 15 min (at 4°C), the pellet was either stored in 70% ethanol at -20°C, or re-suspended in 34µl RNase-free dH₂O for immediate DNase treatment.

DNase treatment of RNA samples is very important as residual contaminating genomic DNA must be removed. A high quality DNase, RQ1 (Promega), was used. To each RNA sample, 4µl DNase buffer (10x) and 2µl Dnase were added and the mixture was incubated at 37°C for 30 min.

Each sample was diluted with 160 µl RNase-free dH₂O and two phenol:chloroform:isoamylol extractions plus one chloroform extraction were performed (equal volumes, 200 µl). To the final aqueous phase, 20µl NaOAc 2M pH 5.4 and 500µl ethanol were added and RNA was precipitated for 15 min on ice. After centrifugation at 16,000xg for 15min (at 4°C), the pellet was usually resuspended in 4µl RNase-free H₂O and immediately submitted to reverse transcription.

2.3.2 Reverse transcription and RT-PCR

The SMART cDNA Synthesis kit (Clontech) was used to generate single-stranded cDNA. To the 4 μ l RNA sample, 1 μ l oligo-dT (100ng/ μ l) was added, and this was incubated at 72°C for 2 min, then cooled to RT.

The reaction mixture was completed with 2 μ l of first-strand buffer (5x), 1 μ l dNTP mix (5mM), 1 μ l of DTT (100mM) and 1 μ l Powerscript reverse transcriptase, and it was incubated at 42°C for 2hr. The enzyme was then inactivated at 72°C for 7 min.

The cDNA product was diluted with dH₂O to a final concentration of approximately 2,000 cell equivalents/ μ l.

For RT-PCR, cDNA templates were submitted to the PCR procedure described in 2.2.3. All primers had annealing temperatures 56-64°C and produced amplified products of 200-400 bp.

List of primers used for PCR amplification of cDNA messages:

CREM fwd primer,	5'-GGAAGTGTATCCTGATGACATGACG
CREM rev primer,	5'-TGAAATGGCCCAGTATTTGCAAGGC
ICER fwd primer,	5'-GCTAGTTGGTACTGCCATGGTAGC
ICER rev primer,	5'-AGCCCAACATGGCTGTAAGTGGAG
NOR-1 fwd primer,	5'-AGGATACACTTCCTGTGTCAAGGG
NOR-1 rev primer,	5'-CCATTCATAGCATGACTGCCTCC
Ly49A fwd primer,	5'-GATTGGGCATGGATTGACAATCGC
Ly49A rev primer,	5'-TGCACTGCAGACTAAGTCCAATGG
Leukocystatin fwd primer,	5'-CTGCTTACCAGCGAGCAGATTAGC
Leukocystatin rev primer,	5'-AGTCTGATGGTAGCAGACCAGACG
Sugano EST fwd primer,	5'-AGCCAAGTAGGGTGTCTCTCAACC
Sugano EST rev primer,	5'-GCCCAGGATACACAGTGAAGAACG
Myeloblastin fwd primer,	5'-AGCAGGACCAGACTCTGTCCCAGG
Myeloblastin rev primer,	5'-CCGGGAAGAAATCAGGGACTG

PD-1 fwd primer,	5'-CTGGAGTCCTCACTTCTACCC
PD-1 rev primer,	5'-GATGGCCCCACAGAGGTAGATGCC
Mac-2 fwd primer,	5'-GATATGGGTGCATGGGGACC
Mac-2 rev primer,	5'-CTCGAGGCAAGGGCAGGTCATAGG
Mg11 fwd primer,	5'-TGGGAACCGGAGGACGTGTGCTCC
Mg11 rev primer,	5'-GCAGCCTGCTAGGTACCCCACTCC
Laminin-R 1 fwd primer,	5'-CGTGATCCGGAAATCGGGGGATCC
Laminin-R 1 rev primer,	5'-ACCACTTTGCCCCCTGGACCTTGG
"Novo-1" fwd primer,	5'-CCAAACCTTTGTAAACCAGCTGGG
"Novo-1" rev primer,	5'-TCTGTAATGTCGTCCCTCCATAGG
"Novo-2" fwd primer,	5'-AGATGAGCCTTGGAGAAGAGCTGC
"Novo-2" rev primer,	5'-TTAGTGGATGAACCAGCCACTGGC
IL-7Rα fwd primer,	5'-TTAAAGCCGAGGCTCCCTCTGACC
IL-7R α rev primer,	5'-TTGGACTCCACTCGCTCCAGAAGC

In parallel to the PCR for the gene of interest, an additional PCR for the house-keeping gene β -actin was run. This allowed direct comparison between the signals obtained for the gene of interest in different samples, once they had been normalised using the house-keeping gene PCR (semi-quantitative PCR).

β-actin fwd primer,	5'-CTGACGGCCAGGTCATCACTA
β -actin rev primer,	5'-CCGGACTCATCGTACTCCTGC

2.3.3 Real-Time (quantitative) PCR

Real-Time PCR is a quantitative technique that monitors the kinetics of accumulation of a specific PCR product, which is recognised by a specific fluorescent probe. By plotting the increase in fluorescence versus cycle number, the system constructs amplification plots that provide a more complete picture of the PCR

process than methods involving fixed numbers of cycles. Quantitation can be obtained by several procedures; we used the 'standard curve method'. In this method, serial dilutions of a reference cDNA (batch of SCB129 cell line cDNA in our case) are used to establish a calibration curve, from which concentrations of unknown samples are obtained by intrapolation.

Real-Time PCR was performed on an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). Parallel (separate tubes) PCR reactions were run for ICER (gene of interest) and GAPDH (normalisation control).

Probe and primers for ICER were designed according to the criteria imposed by PE Applied Biosystems.

ICER probe, *FAM-CGGGCAGCTTCCCTGTTTTTCATCA-TAMRA*

(*FAM* is the 5' reporter dye; *TAMRA* is the 3' quencher dye)

ICER fwd primer, *5'-ATATTCTTTCTTCTTCCTGCGACT*

ICER rev primer, *5'-CCAGCAACTAGCAGAAGAAGCA*

Optimal concentrations of these reagents were estimated by titration (using the reference cDNA) over the following range: 50, 300 and 900 nM for primers (all combinations were tested); 25, 50, 75, 100, 125, 150, 175, 200 and 225 nM for probe.

Based on those titrations, the composition of the reaction mixture for ICER amplification was: 125nM ICER fluorescent (FAM - labelled) probe, 300nM forward primer, and 900nM reverse primer, which were diluted 1:2 in TaqMan Master Mix (containing dNTPs, AmpliTaq polymerase and MgCl₂); (PE Applied Biosystems).

For GAPDH quantification, "TaqMan Rodent GAPDH control reagent kit - VIC labelled" (PE Applied Biosystems) was used. Composition of the reaction mixture: 100nM GAPDH fluorescent (VIC - labelled) probe, 200nM forward primer, and 200nM reverse primer, diluted 1:2 in TaqMan Master Mix.

Reactions were run for 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 sec at 95°C and 1 min at 60°C.

2.3.4 Northern and virtual Northern blotting

2.3.4.1 *Agarose gels (for RNA) and transfer to membrane*

To avoid RNA degradation, gel tray and tank were washed with NaOH 0.1 M, rinsed well with dH₂O, and placed in the fume cupboard. The normal agarose gel preparation was supplemented with MOPS 10x and 5% (v/v) formaldehyde (Fluka).

10x MOPS: 0.2 M MOPS, 50 mM NaOAc, 10 mM EDTA, in sterile dH₂O.

The samples were loaded and the gel was run in 1x MOPS buffer.

Formaldehyde was washed away from the gel with 10x SSC for 2x20 min, and the gel was then blotted onto a nitrocellulose Hybond N membrane (Amersham) pre-soaked in 20x SSC.

20x SSC (stock): 3 M NaCl, 0.3 M sodium citrate, pH adjusted to 7 with NaOH.

The transfer occurred in the following set up: gel covered with membrane, 3 pieces of soaked 3MM paper (in 20x SSC), 20 pieces of dry 3MM paper, a 5 cm stack of dry paper towels, a glass plate and a weight over 0.5 kg. After 12-16 hours, the system was dismantled and the position of the wells was marked on the membrane. The blot was rinsed in 10x SSC and UV cross-linked (Stratagene UV cross-linker) to immobilise the RNA.

2.3.4.2 *Membrane hybridisation with radioactive cDNA probe*

RNA filters were pre-wetted in 2x SSC and placed in pyrex bottles (Hybaid). The filters were pre-hybridised in 2x20 ml hybridisation solution (without probe), at 42°C for 2x2 hours.

Hybridisation solution: 5x SSC, 1% SDS, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA, 60% formamide, 10 mg/ml polyA, 7% dextran sulphate, 20 mM sodium phosphate buffer pH 6.8, 100 µg/ml yeast tRNA.

The pre-boiled radio-labelled probe was added to 15 ml fresh hybridisation solution supplemented with 50 µg/ml sheared salmon testes DNA (Sigma), pre-denatured at 100°C for 5 min. This replaced the pre-hybridisation solution in the

pyrex bottle, and membrane and probe were left hybridising overnight at 42°C (rotating at 7 rpm in oven).

The membrane was washed for 2x20 min with 2x SSC / 0.1% SDS at RT, and then for 2x20 min with 0.1x SSC / 0.1% SDS at 52°C (on shaker). After removal of excess liquid and wrapping in plastic film (Saranwrap), the blot was exposed to X-ray film (Kodak) for 1-5 days at -70°C, and then the film was developed on a RG II X-ray film processor (Fuji).

2.3.4.3 Virtual Northern blots

Virtual Northern blots were introduced by Franz *et al.* (Franz *et al.*, 1999) to minimise the amount of RNA required for analysing gene expression in a population of cells.

Instead of running the RNA pool on an agarose gel, this is converted into large amounts of cDNA by reverse transcription plus PCR amplification. Therefore much more material becomes available for the membrane transfer (similar to Southern blot transfer, as this is DNA rather than RNA) and hybridisation. Indeed, with the amount of RNA used for one conventional Northern blot you can perform almost 100 virtual Northern blots!

To achieve the required amplification of the cDNA pool, we used the Cap-finder method, which depends on the ability of reverse transcriptase to add nucleotides to the 3'-end of the newly synthesised cDNA. The cap-finder (3'-end) and the oligo-dT (5'-end) sequences are adapted to design primers for the amplification step.

To 3µl of total RNA, 1µl CF-oligo-dT (100ng/µl) and 1µl of Capfinder oligo (100ng/µl) were added.

CF-oligo-dT - 5'-AAGCAGTGGTAACAACGCAGAGTACT₍₃₀₎N₋₁N

(N = A, C, G, or T; N₋₁ = A, G or C)

Capfinder - 5'-AAGCAGTGGTAACAACGCAGAGTACGCGGG

The mixture was incubated at 72°C for 2 min, then cooled to RT and supplemented with: 2µl of first-strand buffer (5x) (Clontech), 1µl dNTP mix (5mM), 1µl of DTT (100mM) and 1µl RT enzyme - either Powerscript (Clontech) or Superscript II

(GibcoBRL). The RT reaction proceeded at 42°C for 2hr. At the end, the enzyme was inactivated at 72°C for 7 min. The sample was diluted with dH₂O to 2,000 cell equivalents/ μ l.

The RT product was diluted to 40 μ l, of which 2 μ l were used as template per PCR amplification. Primers (2 μ l each) were similar to those above, but without the homopolymeric stretches. The 50 μ l reaction mixture also contained: 2 μ l dNTP mix (5 mM), 5 μ l MgCl₂ (25mM), 5 μ l PCR buffer 10x and 1 μ l Advantage 2 DNA polymerase (Clontech).

Reactions followed the scheme:

n. cycles	95°C	60°C	68°C
1 x	1 min	1 min	12 min
7 x	30 sec	30 sec	12 min
7 x	30 sec	30 sec	14 min
7 x	30 sec	30 sec	16 min

The amplified cDNA product was run on 1% agarose gel and then transferred to a nylon membrane and hybridised with a radioactive labelled cDNA probe as described above for conventional Northern blots, except in that hybridisation temperature was now 65°C.

2.3.5 Probing of Atlas cDNA arrays

Pools of cDNA were used as probes on an Atlas mouse cDNA expression array (Clontech, cat. 7741-1), which covered 588 mouse genes. In particular, we used this method to identify messages present in products of subtractive hybridisations (RDA, see section 3).

The cDNA probes were radio- labelled as described in 2.2.5. They were purified on a G50 Sephadex column and their specific activity (usually 2-5x10⁶ cpm) was measured on an emission counter.

The Atlas array was pre-hybridised with 10 ml of ExpressHyb solution (Clontech) containing 1.5 mg of denatured (5 min at 100°C) sheared salmon testes DNA (Sigma), for 30 min at 68°C, rotating at 7 rpm (bottle in hybridisation oven).

ExpressHyb solution: 5x SSC, 1% SDS, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA, 60% formamide, 10 mg/ml polyA, 7% dextran sulphate, 20 mM sodium phosphate buffer pH 6.8, 100 µg/ml yeast tRNA.

The probe was denatured with 100 mM NaOH / 1 mM EDTA, for 20 min at 68°C. The solution was neutralised with 500 mM sodium bi-phosphate pH 7.0. After 10 min at 68°C, this mixture was diluted with 5 ml of ExpressHyb solution and was poured into the hybridisation bottle to replace the pre-hybridisation solution.

Hybridisation occurred overnight at 68°C, with constant agitation (7 rpm).

The array was washed for 3x20 min with 200 ml of 2x SSC / 1% SDS, and then 2x20 min with 200 ml of 1x SSC / 0.5% SDS, under 35 rpm (at 68°C).

The array was exposed to X-ray film (Kodak) for 1-5 days at -70°C, and then the film was developed on a RG II X-ray film processor (Fuji).

Probes were stripped from the array by placing it in boiling 0.5% SDS for 5-10 min, and then rinsing with 2x SSC / 1% SDS. The array was stored at -20°C.

2.3.6 Probing of cDNA library filter arrays

In order to identify particular cDNA clones contained in a C57BL6 (wild type) adult mouse thymus cDNA library, the library no. 400 from the Resource Center of German Human Genome Project (RZPD, Berlin, Germany) was used.

Probes for the cDNAs of interest were hybridised with the three high density filter arrays that constituted the library, in a similar way to that described for Atlas arrays (2.3.5), with the following differences: incubations were done at 65°C; Church buffer was used for (pre-)hybridisations; sodium bi-phosphate pH 7.2 (200 or 40 mM)/SDS (0.5% or 0.1%) buffer was used for stringent washing; and E.coli DNA labelled with α -³⁵S-dATP (0.5 µl) was included in the hybridisation (in order to light up the background grid, facilitating the identification of the signals).

Church buffer : 0.5 M sodium bi-phosphate pH 7.2, 7% SDS, 1 mM EDTA.

3 Representation Difference Analysis (RDA)

Alternative gene expression is responsible for distinct phenotypes between otherwise similar populations. The identification of critical “divergence” genes is greatly facilitated by rapid reduction of the number of expressed genes to a few candidates which differ between the isolates. Representation Difference Analysis is a rapid and effective method by which this can be achieved. While differential display amplifies fragments from all represented mRNA species, RDA eliminates those fragments present in both populations, leaving only the differences.

RDA is a PCR-based method of subtractive hybridisation that therefore allows the identification of genes that are differentially expressed in cell population A (the tester population) when compared to cell population B (the driver population). The basic procedure is summarised in **Figure 7**.

In a brief overview, total RNA from the two cell subsets was converted to double stranded cDNA and then digested with a restriction endonuclease to produce a cDNA representation. The two representations were then hybridised at a 1:100 tester:driver ratio over three days. Fragments of genes that were differentially expressed in the tester population were amplified by a series of PCR reactions to generate difference product 1 (DP1). A second hybridisation at a DP1:driver ratio of 1:1000 was then performed followed by the PCR-mediated generation of difference product 2 (DP2). The fragments from the DP2 were sub-cloned into a convenient vector, sequenced, and compared to the various cDNA and expressed sequence tag (EST) databases.

I used an adaptation of the cDNA-RDA protocol of Hubank and Schatz (Hubank and Schatz, 1994), which was modified by Daniel J. Pennington in the host laboratory.

This modified version of the original RDA protocol has been developed to facilitate the identification of differentially expressed genes from small numbers of cells. As a rough guide, the cDNA representation from 5×10^3 tester cells can be subtracted with the cDNA representation from 5×10^4 driver cells. However, if possible it is recommended that $1-3 \times 10^5$ tester and driver cells are used for optimal results.

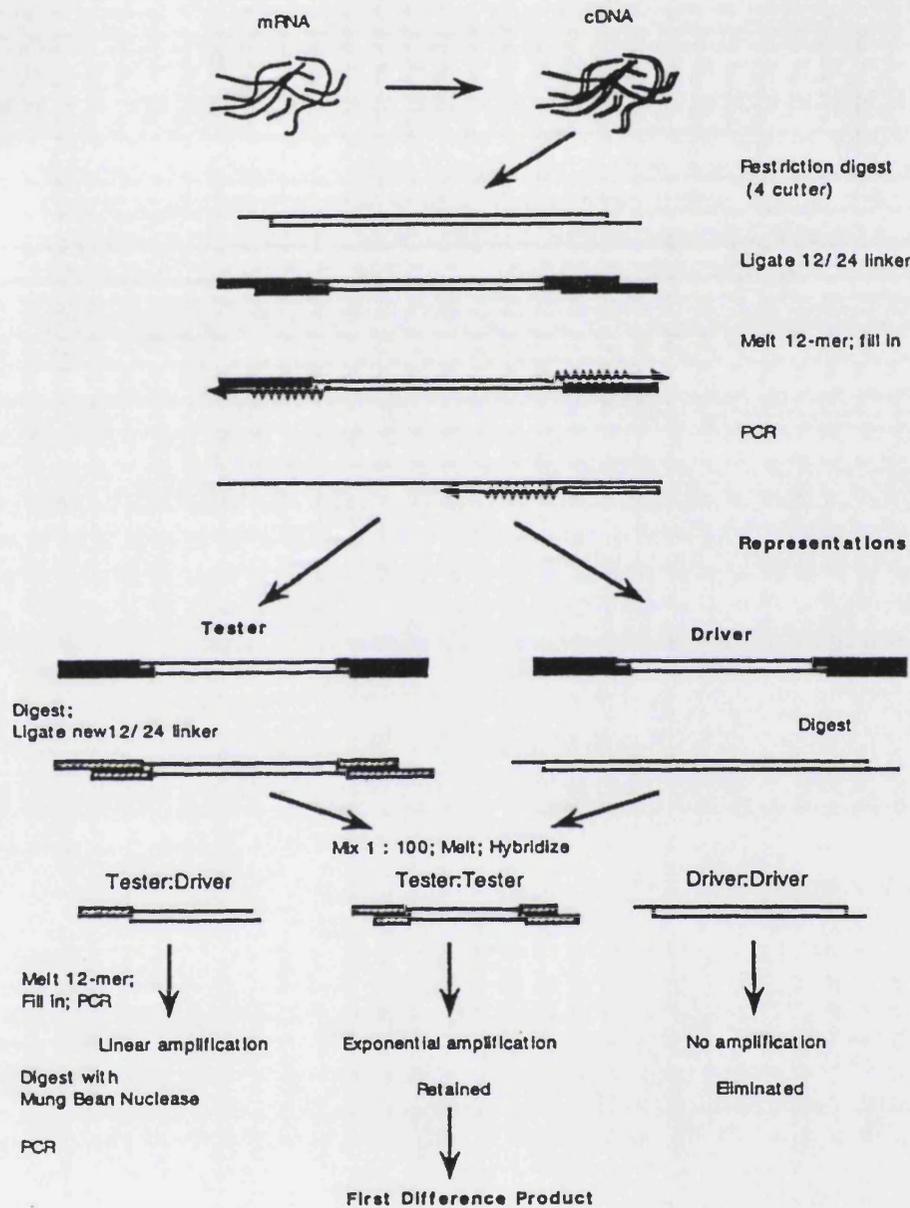


Figure 7 : Outline of Representation Difference Analysis.

3.1 Synthesis of double-stranded cDNA

Total RNA was extracted with TRIZOL (GibcoBRL) and DNase-treated, as described above (2.3.1). RDA requires the synthesis of double-stranded cDNA (ds-cDNA), in contrast with the single-stranded cDNA used for RT-PCR (2.3.2). SMART cDNA Synthesis kit (Clontech) provides all the reagents necessary for double-stranded cDNA production.

The generation of first-strand cDNA uses the Cap-finder method, described above for virtual Northern blots (2.3.4.3). Previous methods of generating ds-cDNA relied on RNaseH, a DNA polymerase and a DNA ligase, resulting in relatively poor yields. However, with the CapFinder technique, the second cDNA strand can be synthesised by PCR. In addition, the cDNA can be amplified further by a low cycle number PCR reaction resulting in workable amounts of cDNA from limiting starting material.

To generate ds-cDNA for RDA, the cycle number for the PCR reaction (below) must be titrated. A cycle number between 16 and 22 is usually required, with the correct cycle number being the one that gives a clear cDNA smear on an ethidium bromide staining agarose gel from 6 kb-200 bp, while still displaying a linear amplification of material when compared to the products obtained from a PCR using the previous cycle number. For most tissues, distinct bands should be visible in the smear, representing abundant mRNA transcripts.

PCR samples contained: 5 µl PCR buffer (10x), 41 µl dH₂O, 1 µl dNTPs (10 mM), 1 µl EXT-primer (100 ng/µl), 1 µl ss-cDNA and 1 µl Advantage 2 DNA polymerase (Clontech)

EXT-primer, 5'-AAGCAGTGGTAACAACGCAGAGT

Reactions were run on a PTC-225 DNA engine (MJ Research), for 2 min 95°C and then 16-21 cycles of: 15 sec at 95°C, 30 sec at 65°C and 6 min 68°C.

To create a tester representation, 4 reactions were done; to generate a driver representation, 24 reactions were set up.

Each 4 PCR reactions were pooled (total volume = 200 µl) and subjected to one phenol/chloroform extraction, followed by one chloroform extraction. For cDNA

precipitation, 20 μ l of NaOAc 2 M and 220 μ l of isopropanol were added to the final aqueous phase and this was incubated at RT for 20 min. (Note: it is critical that isopropanol and not ethanol is used at this stage.) After centrifugation at 16,000xg for 10 min, the pellet was washed with 70% ethanol, dried, and resuspended in 34 μ l dH₂O.

3.2 Generation of tester and driver representations

In order to perform effective hybridisations, the full-length ds-cDNA must be digested into smaller fragments, known as **representations**. This is done using a four-base cutting **restriction endonuclease** that cleaves the DNA, on average, every 256 bp. One of two enzymes can be used; Dpn II (New England Biolabs) - cutting at GATC (37°C), and Tsp 509I (NEB) - cutting at AATT (65°C). In these RDA studies, **Dpn II** was used.

To the 34 μ l of full-length cDNA, 4 μ l of Dpn II buffer (10x) and 2 μ l of Dpn II were added and the sample was incubated at 37°C for 3 hr.

The digest was diluted with 160 μ l dH₂O, and subjected to 2x phenol/chloroform and 1x chloroform extractions. 20 μ l of NaOAc and 220 μ l of isopropanol were added to the final aqueous phase, and cDNA was precipitated at RT for 20 min. The pellet was washed with 70% ethanol, dried, and then resuspended in 10 μ l dH₂O (for every 4 PCR reactions that were performed above).

In order to prepare the tester representation for the hybridisation step, linkers must be added so that at later stages the **tester** DNA can be readily distinguished from the driver DNA. For historical reasons these linkers are termed **J-linkers**, and comprise of a 24-mer and 12-mer. It is important to note that the 12-mer is specific for use with a particular restriction enzyme, and must be changed if a different enzyme is used to cleave the cDNA.

J-24 5'-ACCGACGTCGACTATCCATGAACA

J-12 (Dpn II) 5'-GATCTGTTTCATG

(note: J-12 (Tsp) 5'-AATTTGTTTCATG)

For both tester and driver representations (i.e. cut cDNA), 1 μl and 0.5 μl were run on an ethidium bromide stained 1.5% agarose gel. After a sufficient period, the intensity of the DNA "smears" was checked and recorded on an ImaGO machine (B&L systems). The smear of the driver was used this as a standard reference, taken as one unit (1U) of DNA. The volume of tester would give a smear of equal intensity to 1 U of driver was estimated and used to set up the following ligation: 1 U of tester cDNA, 12 μl ligase buffer 5x (GIBCO BRL), 6 μl of J-24 primer (1 $\mu\text{g}/\mu\text{l}$), 3 μl of J-12 primer (1 $\mu\text{g}/\mu\text{l}$), dH₂O to 57 μl .

Primers were annealed in PCR machine by heating to 50°C then cooling 1°C/min to 15°C. Then, 3 μl ligase (GIBCO BRL) were added and the reaction mixture was incubated overnight at 4°C.

3.3 First subtractive hybridisation

The following components were mixed in a 0.5 ml eppendorf tube: 12 μl (0.2U) of ligated tester representation, 20 U of driver representation, and dH₂O to 120 μl . After 1x phenol/chloroform and 1x chloroform extractions, the final aqueous phase was collected and DNA was precipitated (on ice, 10-15 min) with 30 μl of 10 M NH₄ acetate and 150 μl isopropanol. The pellet was washed twice with 70% EtOH. (The pellet should be glassy in appearance, it should not be big and white, which would imply too much salt in the procedure).

The pellet was resuspended in 4 μl of EEx3 buffer and transferred to a 0.2 ml thin-walled tube and cover with a drop of mineral oil.

EEx3 buffer: 30 mM EPPS (Sigma), 3 mM EDTA pH 8.0.

The tube was placed in a PCR machine and incubated for 6 min at 95°C. After cooling to 72°C, 1 μl of 5M NaCl was added directly to the 4 μl of DNA without removing tube from the block.

Hybridisation was promoted by cooling 0.2°C every hour to 67°C, then 67°C for 24 hr, then cooling 0.2°C every hour to 63°C.

In the end, mineral oil was removed (as much as possible) and 156 μl dH_2O were added by vigorous pipetting. Then four PCR reactions were set up as follows; 20 μl of hybridisation product, 5 μl PCR buffer (10x), 1 μl dNTPs (10mM) and 22 μl dH_2O .

Tubes were incubated in the PCR block for 10 min at 72°C (note - without enzyme or primer). This melted away the J-12 primer, which was not covalently linked to any fragment as it lacked a 5'-phosphate in the ligase reaction.

After 3 min of the 10 min incubation, 1 μl of Pic Taq polymerase (Cancer Research UK) was added (note - this must be a Taq enzyme that does not need to be activated at 95°C before it is active). The Taq filled in the ends of hybridised DNA, making a complementary copy of the J-24 primer which was ligated to the 5'-ends of the tester DNA.

After 8 min of the 10 min incubation, 1 μl of J-24 primer was added to each tube.

10 cycles of 1 min 95°C and 3 min 70°C followed.

The 4 PCR products were pooled and supplemented with 200 μl dH_2O and 1 μl (1 $\mu\text{g}/\mu\text{l}$) glycogen. After 1x phenol/chloroform and 1x chloroform extractions, 40 μl of NaOAc 2 M (pH 5.4) and 450 μl isopropanol were added to the final aqueous phase. Precipitation occurred on ice for 20 min, and was followed by centrifugation (10 min at 16,000xg).

The pellet was resuspended in 34 μl dH_2O . To this, 4 μl Mung Bean Nuclease Buffer 10x (GIBCO BRL) and 2 μl Mung Bean Nuclease (20 U) (GIBCO BRL) were added, and the mixture was incubated at 30°C for 30 min. This digestion removed all single strand amplification products from the sample. To stop the reaction, 120 μl of Tris-HCl 50 mM (pH 8.9) were added and incubated at 98°C for 5 min, followed by immediate transfer onto ice.

4 PCR reactions were set up as follows: 20 μl of sample (from above), 5 μl PCR buffer (10x), 1 μl dNTPs (10mM), 1 μl J-24 primer, 22 μl H_2O , 1 μl advantage 2 Taq polymerase (Clontech). These were subjected to 18 cycles of: 1 min at 95°C, 3 min at 70°C.

The 4 PCR products were pooled and 200 μl dH_2O were added. After 1x phenol/chloroform and 1x chloroform extractions, the final aqueous phase was

supplemented with 40 μ l of NaOAc 2M (pH 5.4), and 450 μ l isopropanol. DNA was precipitation on ice for 20 min, then centrifuged and the pellet was resuspend in 40 μ l dH₂O. This was the **first differential product (DP1)**, of which 4 μ l and 2 μ l were run on a 1.5% agarose gel. 1U of driver was run next to the DP1 to get a good estimation of the concentration (U/ μ l) of DP1.

3.4 Second subtractive hybridisation

A second subtractive hybridisation involved the **removal of J-linkers** from DP1, allowing their substitution for N-linkers. The following digest was set up and incubated at 37°C for 3 hr: 2 U of DP1, 4 μ l of Dpn II buffer (10x), 2 μ l of Dpn II and dH₂O to 40 μ l.

To the digest, 160 μ l dH₂O were added and 2x phenol/chloroform and 1x chloroform extractions were performed. The final aqueous phase was supplemented with 20 μ l NaOAc 2 M, 1 μ g glycogen and 220 μ l isopropanol. After 20 min at RT, the sample was centrifuged (10 min at 16,000xg) and the pellet was washed with 70% EtOH, dried, and then resuspended in 20 μ l dH₂O.

0.2 U of digested DP1 were mixed with 12 μ l ligase buffer 5x (GIBCO BRL), 6 μ l of N-24 primer (1 μ g/ μ l), 3 μ l of N-12 primer (1 μ g/ μ l) and dH₂O to 57 μ l. **N-linkers** were annealed in PCR machine by heating to 50°C and then cooling 1°C/min to 15°C. 3 μ l ligase (GIBCO BRL) were then added and the reaction mixture was incubated overnight at 4°C.

N-24 5'-AGGCAACTGTGCTATCCGAGGGAA

N-12 (Dpn II) 5'-GATCTTCCCTCG

(note: N-12 (Tsp) 5'-AATTTTCCCTCG)

The **second hybridisation** (1 : 1,000 ratio) was set up with: 6 μ l (0.02 U) of N-ligated DP1, 20 U of driver representation, and H₂O to 120 μ l. This mixture was subjected to 1x phenol/chloroform and 1x chloroform extractions, and DNA from the final aqueous phase was precipitated with 30 μ l of ammonium acetate 10 M and 150 μ l isopropanol (on ice, 10-15 min). The centrifuged pellet was washed twice with 70% ethanol, and

resuspended in 4µl of EEx3 buffer. After transfer to a 0.2ml thin-walled tube, the sample was covered with a drop of mineral oil and placed in a PCR machine, where it was incubated at 95°C for 6 min. After cooling to 72°C, 1µl NaCl 5 M was added directly to the 4µl of DNA without removing tube from the block.

Hybridisation was promoted by cooling 0.2°C every hour to 67°C, then 67°C for 24 hr, then cooling 0.2°C every hour to 63°C.

The **second differential product (DP2)** was generated identically to DP1, except in that the N-24 primer now took the place of the J-24 primer and the extension temperature of the PCR reaction was 72°C rather than 70°C.

3.5 Isolation, sequencing and identification of differentially expressed genes

N-linkers were removed from DP2 by setting up the following digest: 5 U of DP2, 4µl of Dpn II buffer (10x), 2 µl of Dpn II, dH₂O to 40 µl. This was incubated at 37°C for 3 hours.

The final product was run on a 2% agarose gel. The bands were carefully excised (as described in 2.2.5) and sub-cloned into BamHI-cut (note: EcoRI-cut for Tsp generated fragments) Bluescript KS plasmid (see 2.2.7.3). After transformation of competent bacteria by heat-shock (see 2.2.7.5), single resistant white colonies were picked up, grown, and DNA was extracted (by mini-prep, see 2.2.2.1) and tested for fragment insertion.

The presence of insert was analysed by restriction enzyme digestion of the bacterial DNA. Since the cDNA fragments were sub-cloned into the BamHI site of the poly-linker of the vector, the usage of two restriction enzymes that cut, one upstream, and the other downstream of the BamHI site allowed the visualisation of the insert on an agarose gel. The following digestion (3 hours at 37°C) was performed: 8 µl (out of 50 µl) DNA sample, 3 µl NEB buffer 1 (New England Biolabs), 3 µl BSA 10x, 1 µl SacI, 1 µl KpnI, 14 µl dH₂O.

The DNA samples which included an insert were amplified by PCR in preparation for automatic **sequencing**. 3 μ l DNA sample, 8 μ l BDT fluorochrome mixture, 2 μ l T7 primer and 7 μ l dH₂O were mixed and subjected to the following PCR: 3 min at 96°C and 25 cycles of 10 sec at 96°C, cooling at 1°C/sec to 46°C, 5 sec at 46°C, heating at 1°C/sec to 60°C, 4 min at 60°C. Products were diluted to 150 μ l with dH₂O and DNA was precipitated (on ice, 10-15 min) with 1/10 volume sodium acetate 2 M and 3 volumes ethanol. The pellet was air-dried for 1 hour and delivered to Cancer Research UK's Equipment Park / Sequencing lab (directed by Graham Clark) for automatic sequencing.

The retrieved sequences were run against gene/expression sequence tags (EST) DNA databases (GenEMBL, Heidelberg) using **BLAST homology searches**.

Alternatively, the differential product DP2 was radioactively labelled and used to probe an Atlas mouse cDNA expression array (Clontech, cat. 7741-1), as described in 2.3.5.

The genes obtained by RDA should be expressed in the tester mRNA but not in the driver mRNA, which was confirmed by RT-PCR or Northern blotting.

Chapter III :

RESULTS

1 Identification of genes differentially expressed in $\alpha\beta$ versus $\gamma\delta$ thymocytes

1.1 RDA analysis of DP vs. $\gamma\delta$ thymocytes

Our objective was to identify genes involved in $\alpha\beta/\gamma\delta$ lineage commitment, or in the selective development of one of the lineages. We therefore decided to compare gene expression in thymocytes representative of the two lineages, but developmentally as close as possible to the commitment step.

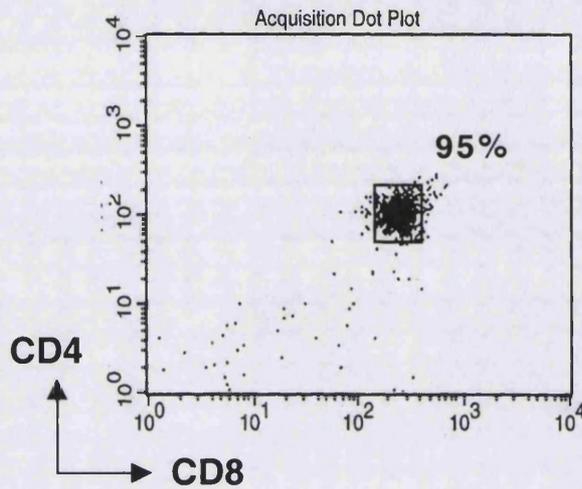
For the $\gamma\delta$ lineage, there is no other reliable marker besides TCR $\gamma\delta$ itself; furthermore, no discrete stages of differentiation within the TCR $\gamma\delta(+)$ compartment have been described. Nevertheless, newly generated thymic $\gamma\delta$ cells are known to be HSA(+), in contrast with circulating $\gamma\delta$ lymphocytes. Thus FACSsorted TCR $\gamma\delta(+)$ HSA(+) thymocytes were used as the $\gamma\delta$ population.

For the $\alpha\beta$ lineage, the co-expression of CD4 and CD8 is the earliest event that occurs in $\alpha\beta$ -committed cells, and therefore DP thymocytes were the obvious candidates. However, the DP subset is very heterogeneous, and once they express the mature TCR $\alpha\beta$ they undergo complex mechanisms of selection and late lineage decisions (CD4 *vs.* CD8), which are bound to involve dramatic changes in gene expression. Since those changes would mask transcripts involved in the earlier $\alpha\beta/\gamma\delta$ lineage commitment step, we FACSsorted DP cells from TCR α -deficient thymuses. As mentioned in the introduction, $\alpha\beta$ T cell development in TCR α KO mice is completely blocked at the DP stage due to the lack of mature TCR $\alpha\beta$, whereas $\gamma\delta$ development proceeds normally.

In order to have the same genetic background, both populations ($\gamma\delta$ and DP) were isolated from TCR α -deficient thymuses, as depicted in **Figure 8**. Total RNA was extracted from the purified (>97% purity) subsets and used for RDA analysis.

TCR $\alpha^{-/-}$ thymus

DP (CD4⁺ CD8⁺) cells



$\gamma\delta$ cells

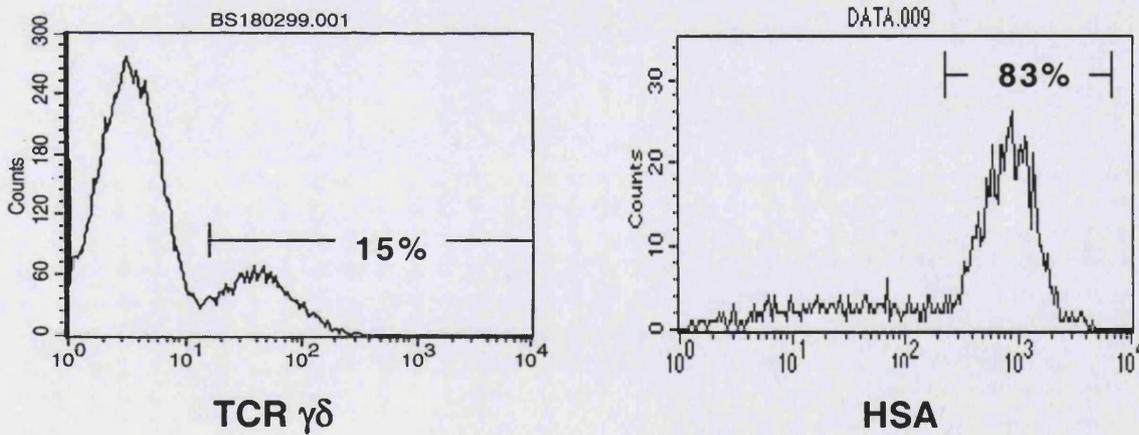


Figure 8 : FACSsorting of TCR $\alpha^{-/-}$ thymocytes for RDAnalysis.

TCR $\gamma\delta$ profile was pre-gated on CD4(-)CD8(-) cells, and HSA profile was pre-gated on TCR $\gamma\delta$ (+) thymocytes.

RDA experiments were conducted using both populations as either tester or driver: $\gamma\delta$ - DP and DP - $\gamma\delta$ subtractions. An outline of the experiment is presented in **Figure 9** and the results are summarised in **Table 1**.

(Note: house-keeping genes, circa 1/4 of all retrieved sequences, were discarded).

Five of the identified surface proteins actually functioned as internal controls of the RDA experiment, as they were already known to be differentially expressed between $\gamma\delta$ and DP thymocytes:

* IL7-R α expression was known to be down-regulated in the DN \rightarrow DP transition (and later up-regulated in the SP stage), whereas it is maintained in $\gamma\delta$ cells, which are completely dependent on IL-7 signals. IL-2R β chain, part of the IL2-R complex, was also known to be expressed in a subset of $\gamma\delta$ thymocytes, while most DP thymocytes are devoid of IL-2R (which comes up in later stages of development).

* CD4 and CD8 protein expression defines the DP population, whereas $\gamma\delta$ cells are fundamentally CD4(-)CD8(-); therefore those molecules were expected products of the DP- $\gamma\delta$ subtraction. Likewise for TCR β , the primary marker of β -selected/ $\alpha\beta$ -committed cells such as DP.

In the same line of thought, also RAG-1 was predicted to come out of the DP- $\gamma\delta$ subtraction, as DP thymocytes are actively rearranging their TCR α locus, while $\gamma\delta$ cells have completed gene rearrangements and should in fact have down-regulated RAG-1 as part of the isotypic exclusion mechanism.

Unexpectedly, the probing of an Atlas cDNA array with a $\gamma\delta$ - DP differential product (after two rounds of subtractive hybridisation) resulted in the identification of many protease-related genes (see Table 1).

The biggest challenge with any subtractive hybridisation study is the selection of candidate genes from the entire set of results obtained directly from the differential analysis.

As our first criterion we decided to look at the relative levels of expression of the identified genes in the two populations under comparison, using semi-quantitative RT-PCR.

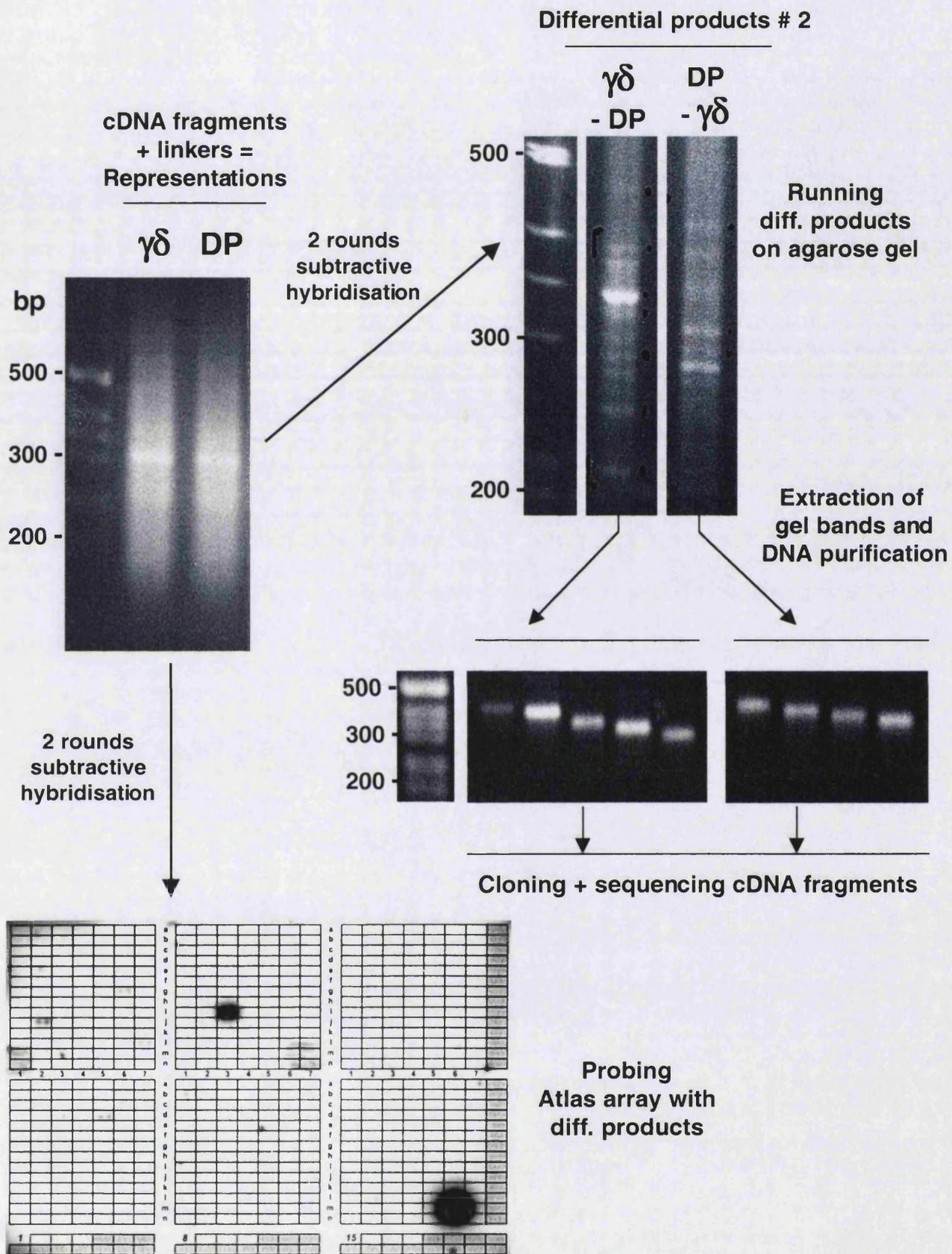


Figure 9 : Summary of RDA analysis of $\gamma\delta$ vs. DP thymocytes.

TABLE 1

$\gamma\delta$ - DP subtraction	DP - $\gamma\delta$ subtraction
Transcription Factors	New genes (no database matches)
Nuclear Orphan Receptor NOR-1	"Novo-1"
cyclic-AMP Response Element modulator (CREM)	"Novo-2"
Myogenic Factor 5 (Myf-5) *	Transcription Factor
Cell Surface Proteins	RAG-1 (Recombination Activation Gene 1)
Ly49A	Cell Surface Proteins
IL2 Receptor β chain	CD4
IL7 Receptor α chain	CD8 (both α and β chains)
Mac-2 (Galectin-3)	TCR β chain
PD-1 (Programmed death gene 1)	Laminin Receptor 1
Proteases and their modulators	Protease
Cytotoxic cell protease 2 *	Cathepsin L (Cys protease)
T-cell specific Ser protease CTLA-1 *	Other known genes
Insulin-growth factor BP 4 (IGFBP-4) *	Schlafen-2
Myeloblastin (Ser protease) *	T-cell specific protein Tcl-30
Leukocystatin (Ser protease inhibitor)	Expression Sequence Tag (EST)
Urokinase-plasminogen activator surface receptor CD87 *	Soares 2NbMT cDNA clone

$\gamma\delta$ - DP subtraction**Other known genes**IFN- γ induced Mg11Regulator of G-protein Signalling
(RGS-2)**Expression Sequence Tags (EST)**

Sugano cDNA clone

Knowles Solter blastocyst cDNA

Table 1 : Genes isolated from the RDA subtractive hybridisation of $\gamma\delta$ and DP thymocytes. Genes marked with (*) were obtained by probing a mouse cDNA expression Atlas array with the RDA differential products; all others were gel-isolated from the differential products, sequenced, and identified by BLAST homology searches.

1.2 Differential expression of candidate genes

Although we had used $\gamma\delta$ and DP cells from TCR α -deficient mice for the RDA experiment, the semi-quantitative RT-PCR analysis was done in wild type thymocyte subsets. This served three purposes: 1st, it provided additional evidence of the relevance of the differential expression (conserved between WT and TCR α KO mice); 2nd, it excluded any “false positives” obtained - by technical artefacts - from the RDA; 3rd, it exposed the degree of differential expression of each gene (relative intensity of PCR products in the two populations).

The genes which produced more striking results, i.e., a clear difference in expression between $\gamma\delta$ and DP cells, are shown in **Figure 10** (Ly49A, NOR-1, PD-1, CREM, Leukocystatin, Sugano EST, “Novo-1” and “Novo-2”). Identical results were also obtained for myeloblastin, Mg-11 and laminin receptor (data not shown in Figure 10), as it can be inferred from Figure 12 (see ahead). These 11 genes were thus selected for further studies.

In contrast, some genes (from the RDA) revealed a very insignificant differential expression (evidencing the high sensitivity of RDA as a subtractive method): Myf-5, Mac-2, RGS-2 and Knowles Solter EST, from the $\gamma\delta$ - DP subtraction; and Schlafen-2 (an inhibitor of cell growth), Tcl-30 and Soares EST, from the DP - $\gamma\delta$ subtraction. In all these cases, RT-PCR signals were found in both cell subsets after the same number of amplification cycles. Because we were interested in genes with a clear differential expression, potentially indicating a selective effect on one of the two lineages or/and on lineage commitment, the genes mentioned were not considered for any further analysis.

The second criterion used to judge the 11 candidate genes that passed this initial RT-PCR test was their pattern of expression in several haematopoietic lineages. We were looking for restriction to the T cell lineage (and their late precursors), rather than an unspecific expression in all haematopoietic lineages.

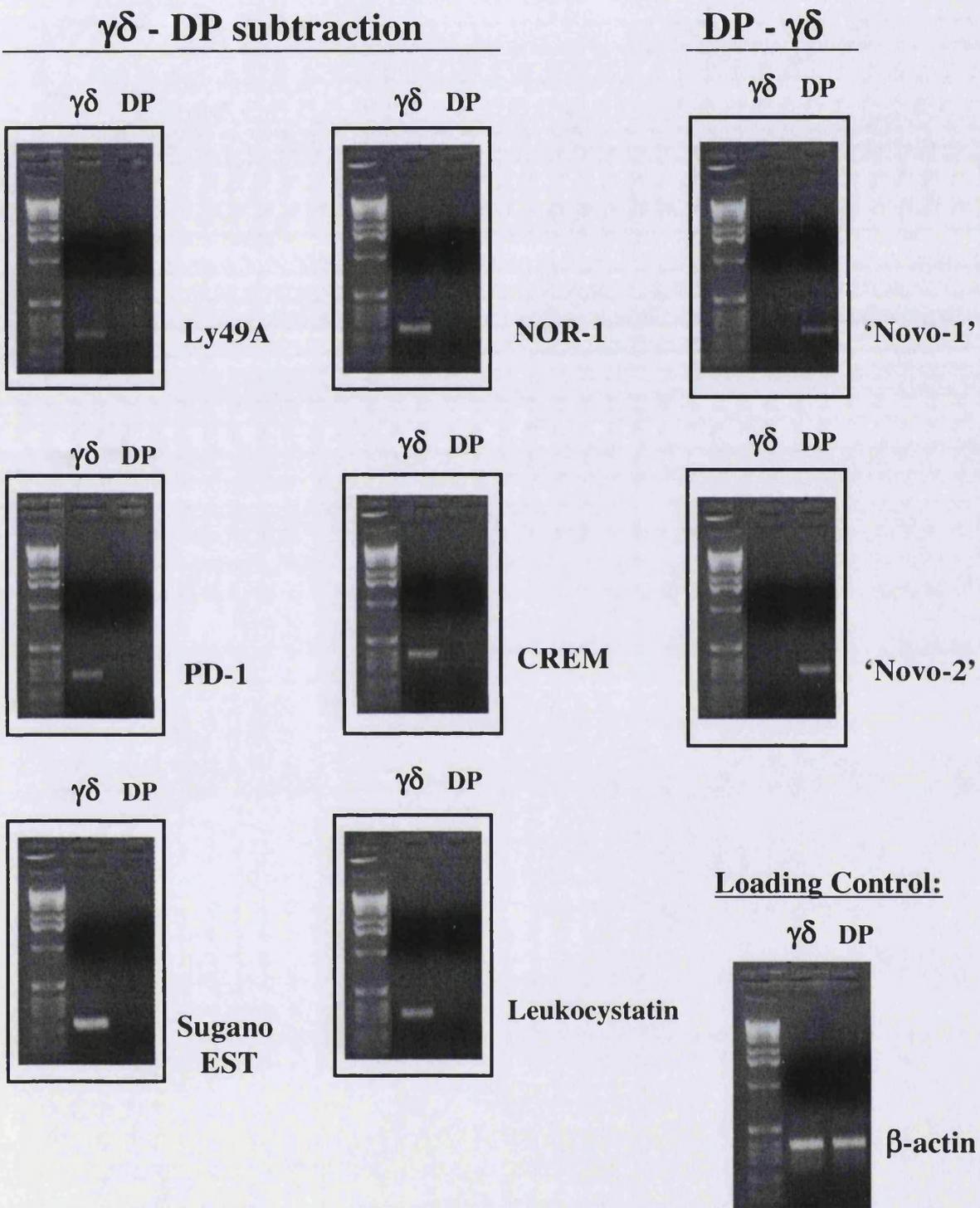


Figure 10 : RT-PCR for candidate genes (from RDA) in wild type $\gamma\delta$ and DP thymocytes.

1.3 Pattern of expression of candidate genes

Gene expression was assessed by RT-PCR on FACSorted macrophages, NK, B and T cells. Thymocytes were further subdivided into populations representative of distinct developmental stages or lineages, as depicted in Figure 11.

The RT-PCR results are illustrated in Figure 12 and summarised in Table 2.

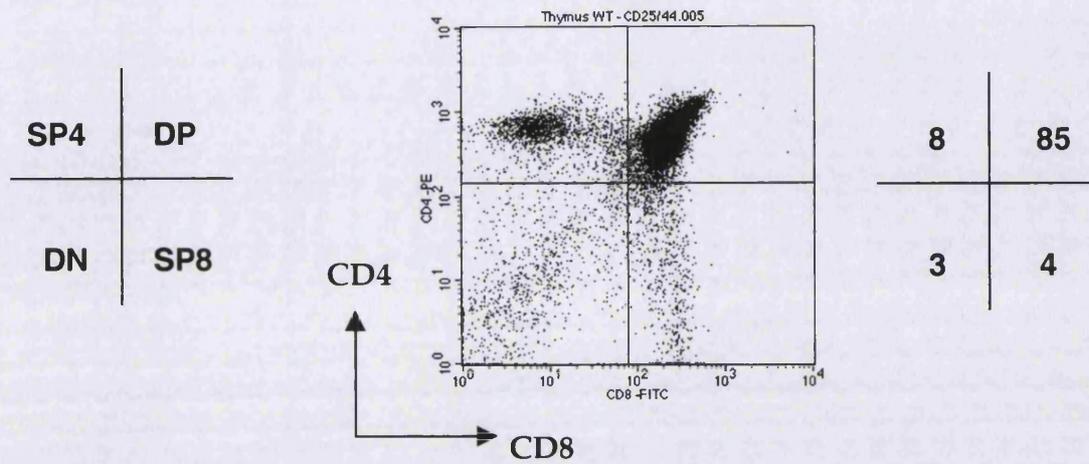
TABLE 2

	M ϕ	NK	B	DN1	DN2	DN3	DN4	$\gamma\delta$	DP	SP4
Ly49A	-	+	-	-	+	+	+	+	-	-
NOR-1	-	-	-	-	-	+	+	+	-	-
Sugano EST	-	+	-	-	-	-	-	+	-	-
Leukocystatin	+	+	-	+	+	+	+	+	-	-
CREM	-	-	-	-	+	+	+	+	-	-
Mg-11	+	+	-	+	-	-	+	+	-	+
PD-1	-	-	-	-	-	-	-	+	-	+
Myeloblastin	-	+	+	+	-	-	-	+	-	-
Laminin-R 1	-	-	-	-	-	-	-	-	+	+
'Novo-1'	-	-	-	-	-	-	-	-	+	-
'Novo-2'	-	-	-	-	-	-	-	-	+	-

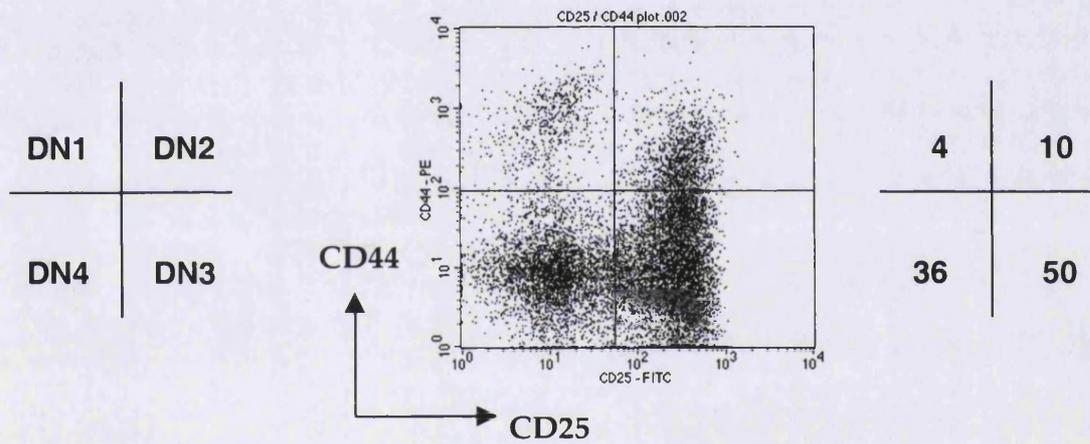
Table 2 : Gene expression (mRNA) in haematopoietic lineages.

RT-PCR results (n=3). Levels of expression: - (low), + (high), + (very high).

DN, DP and SP populations (*) :



DN subsets () :**



$\gamma\delta$ thymocytes (*) :**

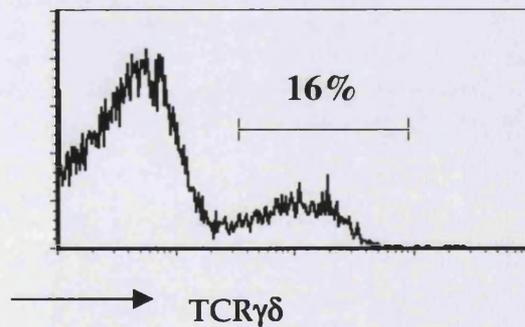


Figure 11 : FACS profiles of WT thymocytes in cell sorting experiments.

Pre-gating: (*) Thy1⁺ ; (**) Thy1⁺ CD4⁻ CD8⁻ TCR $\gamma\delta$ ⁻ B220⁻ NK1.1⁻ ;

(***) Thy1⁺ CD4⁻ CD8⁻ cells. Percentages of gated subsets are indicated.

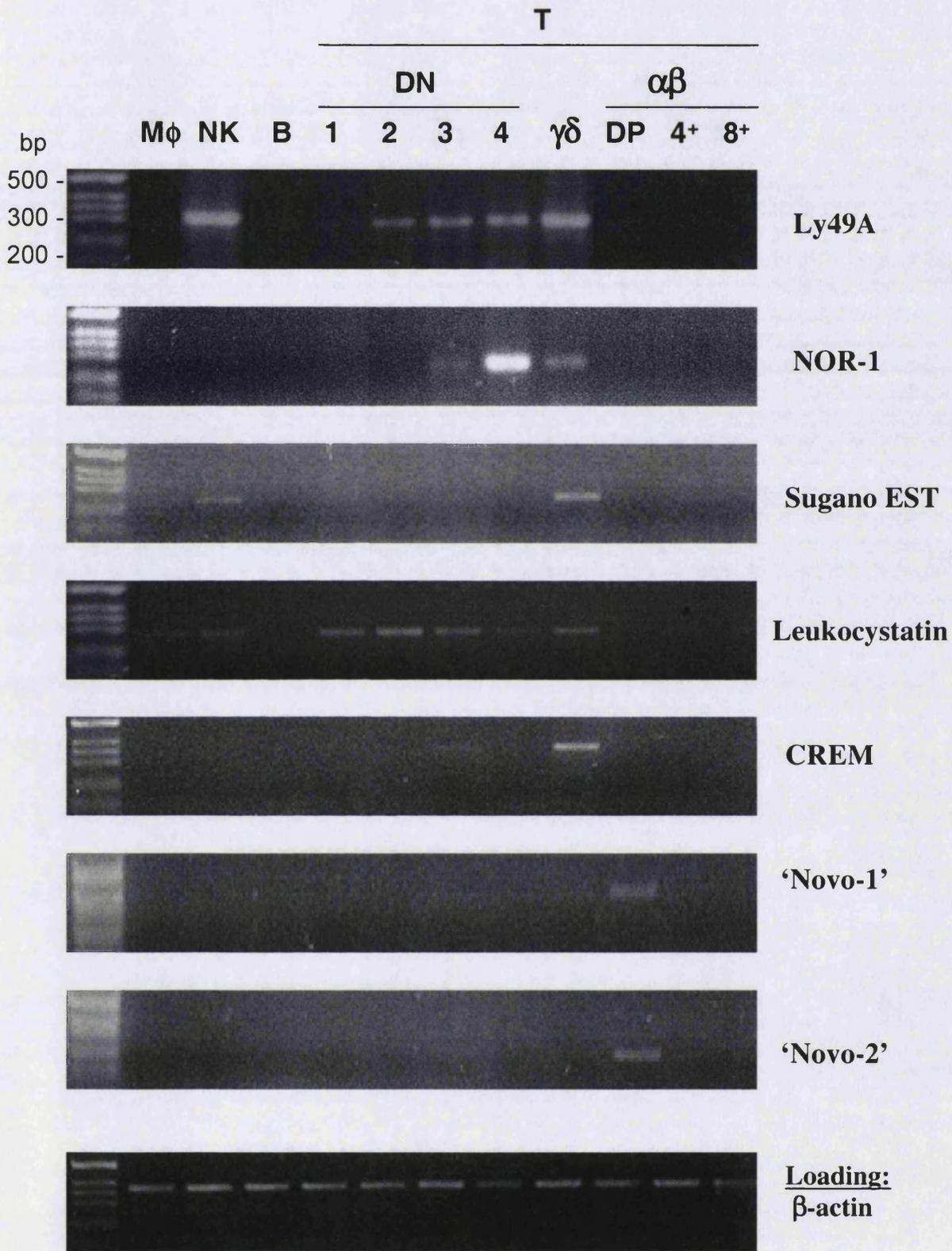


Figure 12: RT-PCR for candidate genes in WT haematopoietic lineages: Macrophages, NK cells, B cells; and Double-Negative, Double-Positive, CD4+ and CD8+ SP Thymocytes.

The expression of an interesting candidate would be fundamentally restricted to the T cell lineage and, ideally, would be developmentally regulated within the thymic DN precursor compartment. This would be consistent with a potential participation in the $\alpha\beta/\gamma\delta$ lineage commitment process.

The selection criterion mentioned above was met by 5 out of the 11 candidate genes: NOR-1 and CREM, for “ $\gamma\delta$ lineage genes”; and “Novo-1”, “Novo-2” and Laminin receptor 1, for “ $\alpha\beta$ lineage genes”. In contrast to “Novo-1/2”, the expression of Laminin receptor 1 increased between DP and SP cells, suggesting such expression to be related to $\alpha\beta$ cell late development or function rather than to $\alpha\beta / \gamma\delta$ lineage commitment.

Two other candidates, Ly49A and Sugano EST, had a similar pattern of expression to NOR-1 and CREM, with the difference of being also expressed in NK cells (isolated from the thymus). Since NK and T cells seem to share a late thymic precursor (Rodewald et al., 1992), such pattern could be relevant, and therefore those genes were also selected.

Leukocystatin, Mg-11 and Myeloblastin seemed to have an unspecific expression in haematopoietic lineages, as their mRNAs were also clearly detectable in macrophages and/or B cells.

Although PD-1 did not suffer from such caveat, its differential expression between $\gamma\delta$ and $\alpha\beta$ cells was not maintained in later stages of development: SP ($\alpha\beta$) cells also expressed PD-1, in levels comparable to $\gamma\delta$ thymocytes. Mg-11 expression, too, was up-regulated during DP \rightarrow SP transition.

Due to the characteristics of their expression pattern in haematopoietic lineages, five of the eleven candidates (from 1.2) were not selected for further studies: Leukocystatin, Mg-11, Pd-1, Myeloblastin and Laminin receptor 1.

For the other six genes, additional studies were performed to assess their relevance in the $\alpha\beta / \gamma\delta$ lineage split. Such preliminary studies are described in the following paragraph (1.4), except in the case of CREM, which was the protagonist of an extensive series of experiments presented ahead in section 3.

1.4 Preliminary studies on candidate genes

We initially tried to obtain as much information as possible about the candidate genes from studies carried out by other groups, or by using tools (antibodies, mice) created by them.

Regarding "Novo-1" and "Novo-2", no useful information was available, since genes comprising those partial transcripts had yet to be cloned. The sequences (5' to 3') of the partial transcripts (cDNA representations) obtained from the RDA analysis are presented in Table 3.

TABLE 3

Novo-1	GATCTAAGTGGGATTCCCCAAACCTTTGTAAACCAGCTGGGT GTGATATCCCATTCTTGTAATCTCAGCACCAGGGAGAAAGAG GTCTTTGGGGCTTACTGGACAGTCAGCGATGATATCACTGTTA GCAGGAGAGGGTGAGTCAATCAGGGGAGTGCATGGGTTACA GTTAGAGCTGACAAGAAATGAGCTGCTGCTGAGCTTTCTAGA CACCCAAGGTTACTGATGGTATCTCACTCTAAGCCACAGTCT TGTCTCGCTTGTCGATATCACCCAGGCTGGTTTCCCACAGAG TCCATGGCCTATGGAGGGACGACATTACAGACACGGTGGTAG ATGCTAACAATTCCAAGGTGATGCCGCTGACTGGGATAGATC
Novo-2	GATCCAGATGAGCCTTGGAGAAGAGCTGCATGCTGGGTGCTG GATGCCGATGGAGCACCAACTACAACGGGTCTACCCTACCCC CACTCAACCCCCCAGCTGCTTAGAGGAGAGACAGAGGCTT GGAGAAGTGCTGAGACCTGCCCCAAGTCAGCCCAATTGCTCA TCACCCAGCAGTTCCTCAGCAGCTCCCTCAGCCAGTGGCTGG TTCATCCACTAATCAACACGGACTCTCAGCCCACCTTGTGAGAC TTTGTGATC

Table 3 : cDNA sequences with no matches in GenEMBL databases, obtained from the RDA subtraction DP - $\gamma\delta$, named "Novo-1" and "Novo-2".

The possibility of both cDNA sequences being part of the same full-length transcript was investigated by RT-PCR, using the specific primers designed for each sequence (previously employed in Figures 10 and 12) in combination: "Novo-1" forward primer + "Novo-2" reverse primer, and vice-versa. No signal was detected in DP thymocytes (data not shown), suggesting that the two sequences are not linked in the same transcript. (They could still correspond to the same gene, if its mRNA was alternatively spliced.)

In order to isolate full-length transcripts for "Novo-1/2", a cDNA library from C57BL6 (wild type) adult mouse thymus (Resource Center of German Human Genome Project / RZPD, Berlin, Germany) was screened. Such library (RZPD reference 400) consisted of three filters, which were hybridised with radio-labelled probes for "Novo-1" and "Novo-2".

No hybridisation signals were detected for "Novo-1", whereas two signals, in distinct filters, were obtained for "Novo-2". The corresponding cDNA clones were requested to RZPD, and were then tested for the presence of the original "Novo-2" sequence. One of the clones (RZPD reference ICRFp400L0658Q4, filter 150-2-229, probe 9305) was positive for the sequence, whereas the other (ICRFp400L1697Q4, filter 150-1-252, probe 9304) was not ("false positive").

The sequence obtained from this clone was run against the Celera mouse genome database. The results showed that "Novo-2" maps to a region located approximately 1.5 kb upstream (5') of the RAG-1 gene. It is still not clear whether it represents a separate gene, an alternative splicing form of RAG-1, or a sterile transcript that is produced when the RAG-1 locus is open. Its proximity with RAG-1 suggests a similar regulation of expression, which is reflected in the fact that both RAG-1 and "Novo-2" were isolated as being differentially expressed between DP and $\gamma\delta$ thymocytes. Since "Novo-2" does not contain an obvious open reading frame, we do not know if it codes for a protein. These are questions to be addressed in future investigations.

In contrast with the "Novo" candidates, **NOR-1** (neuron-derived orphan receptor - 1) was being studied by several groups and much more information and tools were available. In particular, NOR-1 had been constitutively expressed in murine thymocytes under the control of the p56Lck proximal promoter (Cheng et al., 1997b).

The result was a 25-fold reduction in thymic cellularity due to programmed cell death. Especially sensitive to apoptosis were DP thymocytes (which constituted only 28% of the mutant thymus, in contrast to 87% of control WT littermates). Apoptosis seemed to be Fas-independent, as the cells did not express Fas Ligand. The same study showed that NOR-1 expression is induced to a very high level upon TCR stimulation (anti-CD3 mAb). This was similar to Nur-77, with whom NOR-1 seemed to share DNA binding sites and cellular functions.

Since we isolated NOR-1 as a $\gamma\delta$ - DP differential product, we were interested in investigating whether it played a role in $\gamma\delta$ cell lineage commitment and / or development. For that we contacted Alexandra Bras in Carlos Martinez's group (Madrid, Spain), who analysed the thymus of NOR-1 'knock-out' mice for us. The analysis revealed no differences between NOR-1 deficient and WT mice: in particular, absolute numbers of $\gamma\delta$ thymocytes were $(2.6\pm 0.4)\times 10^5$ for NOR-1(-) and $(2.3\pm 0.4)\times 10^5$ for NOR(+), whereas DP cell numbers were $(8.0\pm 0.8)\times 10^7$ for NOR(-) and $(8.6\pm 0.8)\times 10^7$ for NOR(+) mice.

These data suggested that NOR-1 does not play a crucial role in thymocyte development and lineage commitment. There is the possibility of redundancy between NOR-1 and Nur77, as previously described by Cheng, Winoto *et al.* (Cheng *et al.*, 1997b), which could be important in $\gamma\delta$ thymocytes since they also express Nur77 (our data; data not shown). However, the fact that the expression of both genes is induced by a CD3 signal raises the possibility of a TCR $\gamma\delta$ -mediated mechanism accounting for the differential expression between $\gamma\delta$ and DP thymocytes.

Interestingly, the thymocyte subset that expresses NOR-1 at highest levels is DN4. Since the thymic expression of NOR-1 begins at DN3 stage and is clearly up-regulated upon DN3 \rightarrow DN4 transition (Figure 12), it suggests the involvement of pre-TCR signalling. This hypothesis is currently under investigation. Regarding the $\alpha\beta$ / $\gamma\delta$ lineage split, though, NOR-1 was not selected for further studies due to the previous considerations.

1.4.1 Ly-49A

Ly-49 proteins are NK inhibitory receptors, which deliver inhibitory signals to NK cells upon MHC class I (MHC I) recognition. Ly-49A, in particular, interacts with two allelic forms of H-2D, H-2D^d and H-2D^k. This is seen as a general mechanism of 'NK cell tolerance', as it guarantees that only cells with a deficient MHC I expression (due to viral infection, for example) are lysed by NK lymphocytes.

The murine Ly-49 receptors are type II receptors belonging to the C-type lectin family, whilst killer inhibitor receptors (KIR), another class of NK inhibitory receptors, belong to the Ig superfamily.

Additionally to NK cells, small subsets of T cells were known to express Ly-49 family members (Wong et al., 1991). In our RDA experiment, we identified Ly-49A as being differentially expressed between $\gamma\delta$ and $\alpha\beta$ thymocytes. We therefore wanted to analyse this differential expression in more detail.

We found Ly49A to be expressed at the mRNA level in NK, $\gamma\delta$ and pre-T cells (Figure 12). Using a commercial mAb specific for Ly49A, the surface expression of the protein was analysed in thymic subsets - **Figure 13**.

Although the differential expression of Ly49A between the $\gamma\delta$ and $\alpha\beta$ lineages was extended to the protein level, only 5% of all $\gamma\delta$ thymocytes displayed high levels of surface expression (Figure 13 B). Importantly, most cells of the positive population had a NK1.1(+) phenotype, implying they were NK T cells.

These results regarding $\gamma\delta$ and $\alpha\beta$ NK T cells might suggest a closer relationship between $\gamma\delta$ T and NK cells than between $\alpha\beta$ T and NK cells, eventually at the level of T/NK precursors. This is consistent with the common expression, in $\gamma\delta$ T cells, of other molecules typical of NK cells (such as NKG2D). Such a parallel between these two lymphocyte lineages could reflect their involvement in innate immune responses, in contrast with the prototypical adaptive immunity of $\alpha\beta$ T cells.

However, these results suggest that in terms of the two T cell lineages *per se*, Ly49A does not seem to be preferentially expressed in NK1.1(-) $\gamma\delta$ thymocytes. We therefore did not consider it for further studies on $\alpha\beta$ / $\gamma\delta$ lineage commitment.

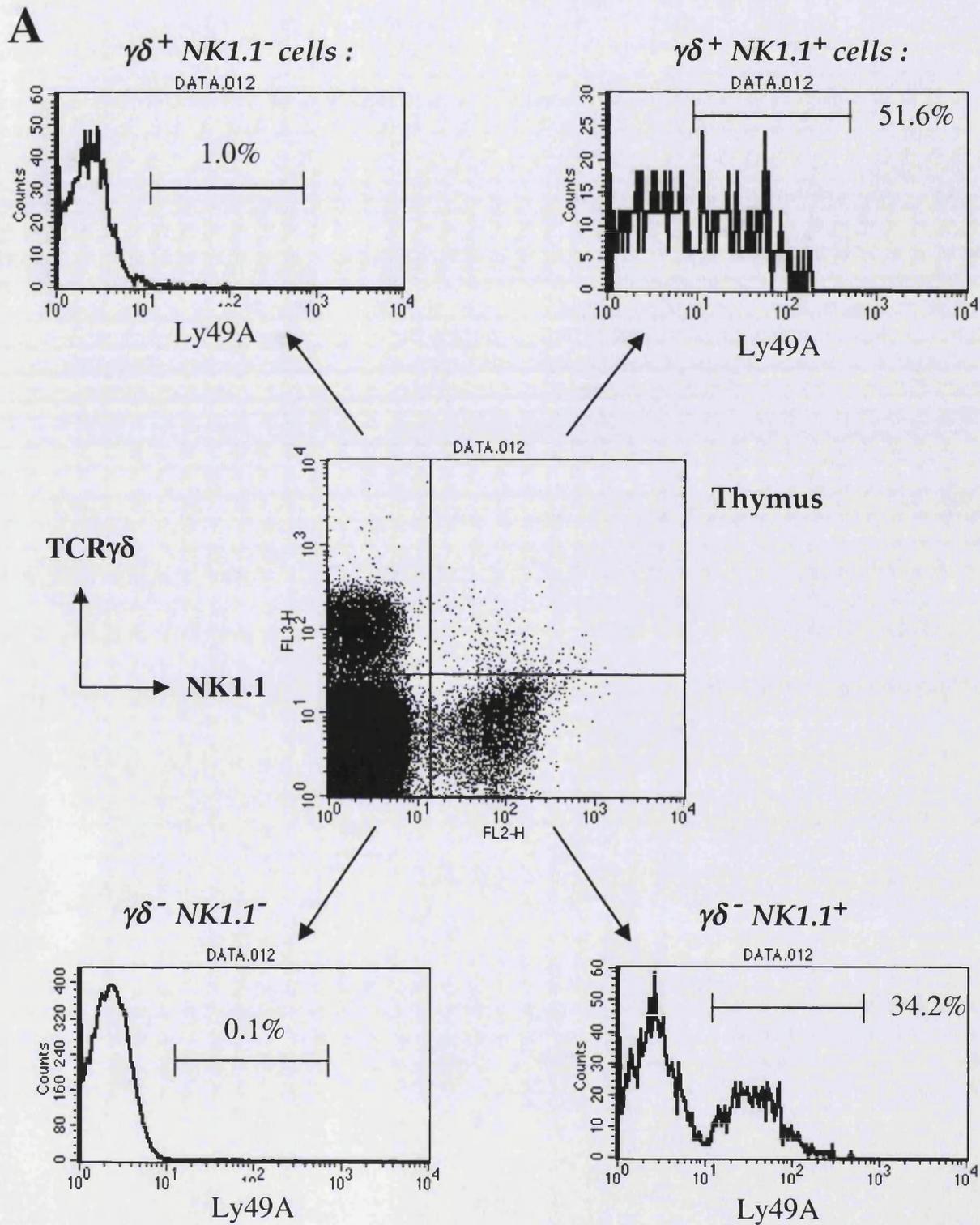


Figure 13 : Ly49A protein surface expression in NK and T cells.
(A) FACS profiles of thymic populations stained with anti-Ly49A mAb.
 (Thymocyte suspension was previously enriched for CD4⁻ CD8⁻ cells.)

B

NK	$\gamma\delta$		DN	$\alpha\beta$		
33 %	5 %		0.1 %	0.2 %		
	NK1.1			DP	SP4	SP8
	+	-		0.2 %	0.6 %	0.0%
	47 %	0.8 %				

Figure 13 : Ly49A protein surface expression in NK and T cells.

(B) Summary table (n=6) for thymic populations stained with anti-Ly49A mAb. (Percentage of Ly49A expressing cells is indicated.)

1.4.2 Sugano EST

One other sequence isolated from the $\gamma\delta$ - DP subtraction corresponded to an expression sequence tag (from GenEMBL database, AI790276), initially obtained from a murine kidney cDNA library created by Dr. Sumio Sugano (1999) – “Sugano EST”. As shown in Figure 12, this EST is a transcription product in $\gamma\delta$ and NK thymocytes, but not in other haematopoietic lineages. Unlike Ly49A, the expression of Sugano EST is mainly attributable to NK1.1(-) $\gamma\delta$ thymocytes (data not shown).

A more detailed study of this EST required more sequence data. In order to isolate a full-length transcript that would comprise it, we used the C57BL6 adult mouse thymus cDNA library (Resource Center of German Human Genome Project / RZPD, Berlin, Germany), previously employed for “Novo-1/2”. One hybridisation signal was detected with a radioactive probe of the sequence presented in Figure 14(A), and the corresponding clone (RZPD reference ICRFp400F06107Q4, filter 150-1-252, probe 9276) was obtained from RZPD.

The cDNA clone was indeed positive for Sugano EST (data not shown). Upon 5'-sequencing (RACE) of the cDNA, a homology to IL-2R β was identified. In order to investigate whether this was an artefact introduced in the cDNA library, or if it illustrated a real link between Sugano EST and IL-2R β , the experiment represented in Figure 14 (B) was performed. Using a combination of IL-2R β forward primer and Sugano EST reverse primer, a RT-PCR reaction was run in $\gamma\delta$ thymocytes. A clear band of the size (2.6 kb) expected from the sequencing data (of the cDNA clone) was obtained, suggesting an effective linkage between Sugano EST and IL-2R β transcripts.

Furthermore, when the “Sugano EST” sequence was run against the Celera mouse genome database, it mapped to a region less than 100 bp downstream of the 3'-end of the IL-2 β gene, consistent with the proposed linkage between the transcripts.

The previous results prompt us to investigate whether the IL2-R β /Sugano transcript was translated into a protein expressed on the cell surface (of $\gamma\delta$ thymocytes), where the conventional IL-2R β chain localises. Previous data (Takeuchi et al., 1992; Tanaka et al., 1991) suggested that the IL-2R β protein is expressed in very few (1-4%) thymocytes, whereas it is more frequent among splenic CD8+ T cells and NK cells.

A

5'- GATCTAGCC AAGTAGGGTGTCTCTCAA CCAGATGTC ATGGGAA CAGGTGC
 TTGTGGCAA GGCCATTG TTAGCAAG AGGAGATGAGCCAG TGCAGAC GGGAT
 TCAGAA CCCATGCAAGGGTTCTCTTGC CCATCCCC TCCTGCCAC CGGATGCTG
 CCTGCCA CCAGCCTCCTCTAACTCAGCAATGAGTGCAGACCTGAGCTCTGCATT
 CACGTTCTTCACTGTGTATCCTGGGCAAATGGACAAACCTTCAGGATTTTCTT
 GCCTCGG TTCCCAAATAGGAATACA ACTTATTTCCA CAGCTGAAAAGGTC TG
 AGAAGGATTCAGTTCTCTTTCAATGATC -3'

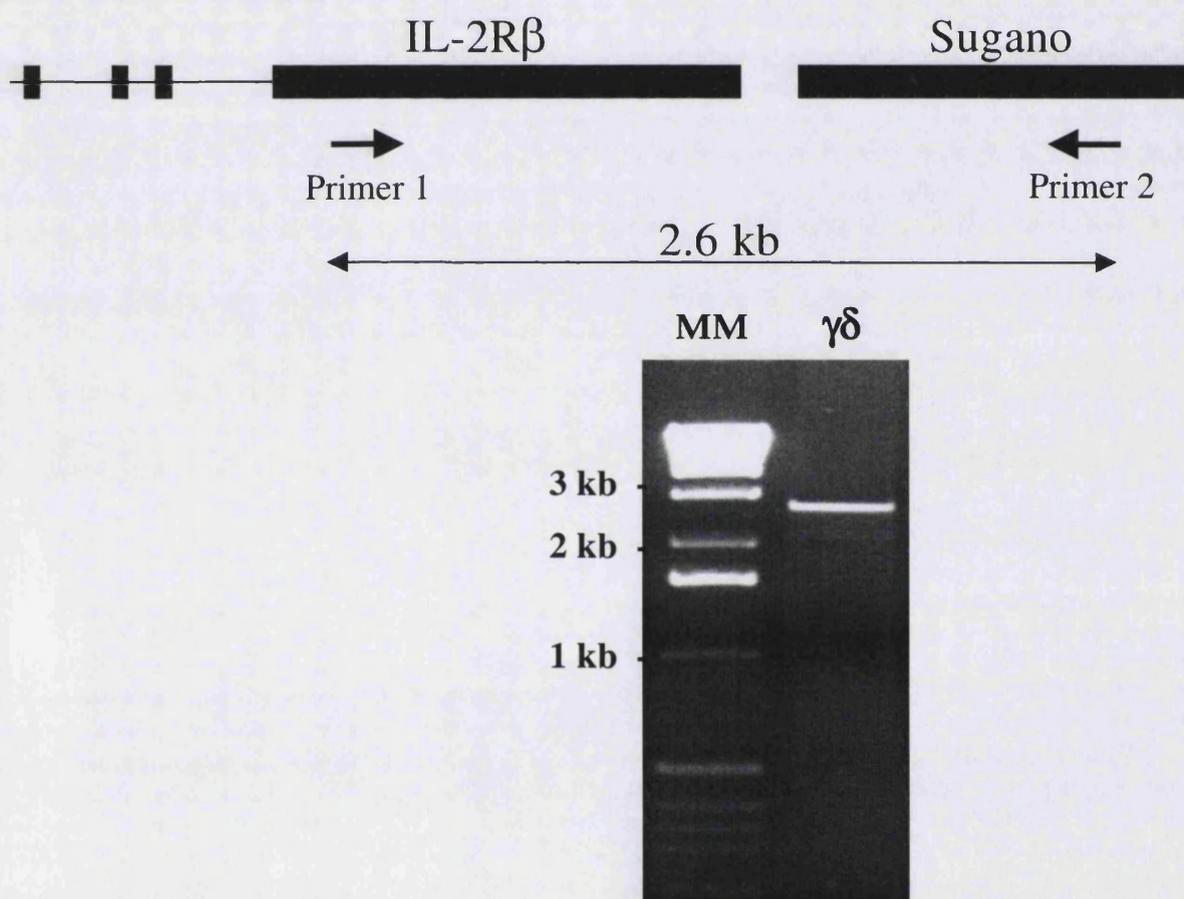
B

Figure 14: Sugano EST : sequence and linkage to IL-2R β transcript.

(A) Original sequence (10 hits) obtained from $\gamma\delta$ -DP differential product, 99% identical to Sugano mouse cDNA clone (GenEMBL EST database, AI 790276).

(B) RT-PCR on $\gamma\delta$ cells using combined primers for IL-2R β and Sugano EST.

In mature lymphocytes, IL-2R β is one component of the IL-2 receptor (together with α and γ chains), which plays a crucial role in T cell proliferation. Moreover, the ligand, IL-2, triggers an up-regulation of IL-2R β expression. Not much was known about the levels of expression of IL-2R β in particular thymocyte subsets. Two commercial antibodies specific for IL-2R β (also known as CD122) were available: clone 5H4, which did not block binding of the ligand (IL-2) to the receptor; and clone TM- β 1, which blocked high affinity binding of the cytokine to its receptor. Both were used to stain thymocyte populations; the results for clone TM- β 1 are presented in **Figure 15**. Similar results were obtained with clone 5H4.

In contrast to NK cells, $\gamma\delta$ thymocytes did not express IL-2R β protein on the cell surface. The same was true for precursor DN cells.

Therefore, the IL-2R β /Sugano mRNA transcribed in $\gamma\delta$ cells is not expressed as a IL-2R β -related protein on the cell surface. Although there is the possibility that the mAbs available do not recognise the protein product of the alternative transcript, the fact that they (at least the clone TM- β 1) are capable of abrogating IL-2 binding suggests that the IL2-R β /Sugano transcript expressed in $\gamma\delta$ thymocytes is not relevant in terms of IL-2 signalling.

Thus, the biological relevance of the IL2-R β /Sugano transcript, in which the Sugano sequence represents an alternative 3'-end for the IL-2R β gene, is still under investigation.

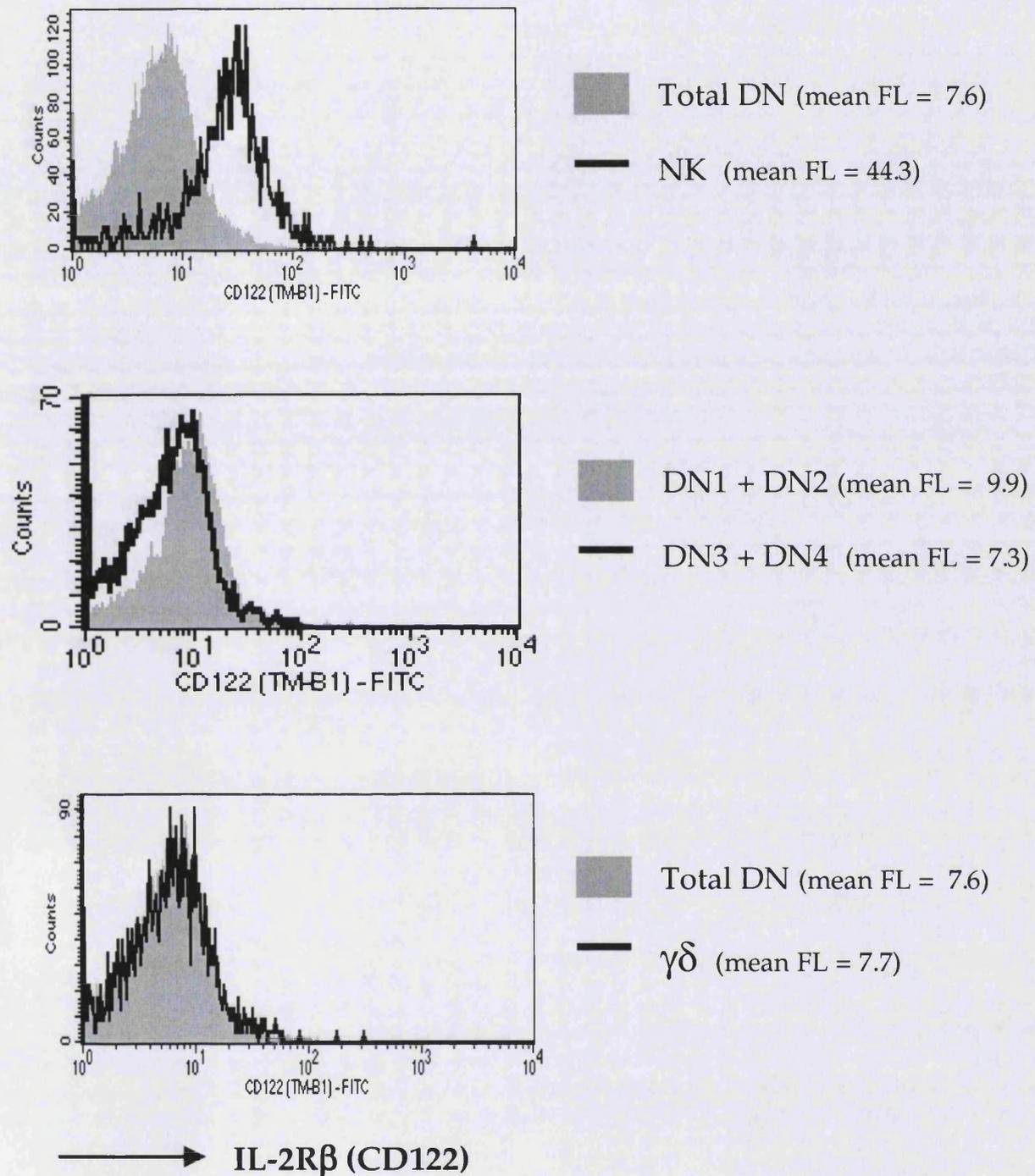


Figure 15: IL-2R β protein surface expression in thymic subsets.

FACS profiles for wild type DN, NK and $\gamma\delta$ thymocytes, using mAb clone TM- β 1.

(Mean fluorescence intensity is indicated.)

2 Identification of pre-TCR responsive genes

(Collaborative work with Cesar Trigueros and Daniel J. Pennington)

2.1 RDA analysis of TCR β (+) vs. TCR β (-) pre-T cells

With the objective of identifying genes whose expression is induced by pre-TCR signalling, we initially compared (by RDA) wild type pre-T cells with pre-TCR deficient thymocytes. Since the pre-TCR is firstly detected on the surface of thymocytes at the DN3 stage of development, DN3 populations were isolated from TCR β (+) and TCR β (-) mice. As seen on **Figure 16**, these comprise 50% and 80% of the thymic DN compartments, respectively. The accumulation of DN3 cells in the TCR β KO thymus (block in DN3 \rightarrow DN4 transition) is due to the absence of “ β -selection”.

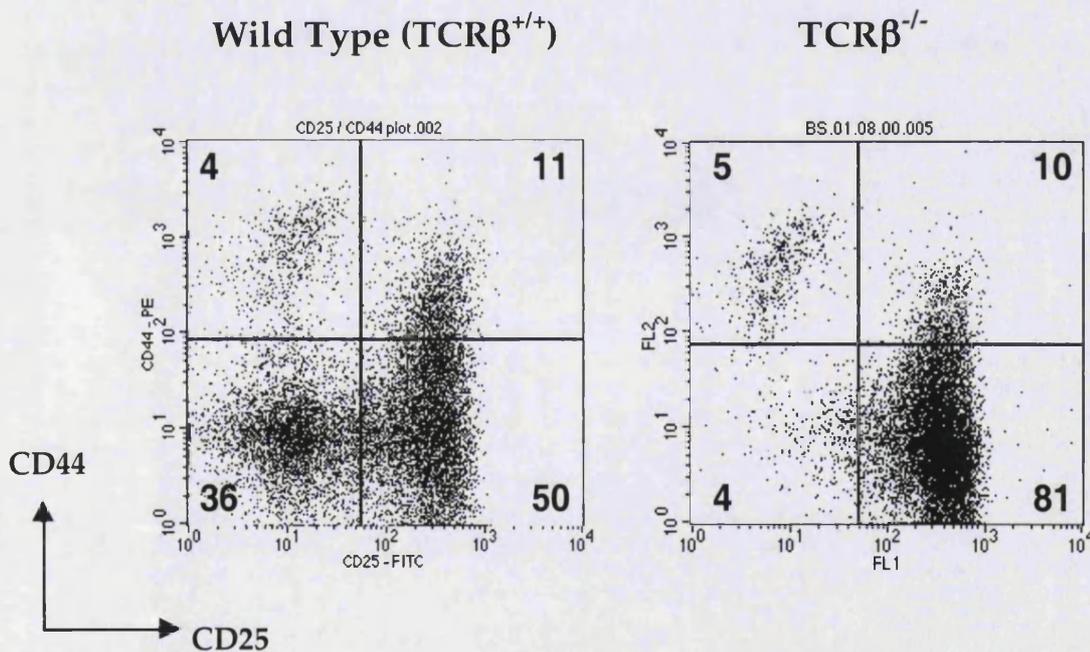


Figure 16 : FACS plots (CD25 vs. CD44) for WT and TCR $\beta^{-/-}$ DN thymocytes. (Pre-gated on Thy1 $^{+}$ CD4 $^{-}$ CD8 $^{-}$ TCR $\gamma\delta^{-}$ NK1.1 $^{-}$ B220 $^{-}$ cells).

We were therefore assuming that the differences in gene expression between the two populations, as identified by a TCR β (+) DN3 - TCR β (-) DN3 subtractive hybridisation (Table 4), would be a direct consequence of pre-TCR signalling.

TABLE 4

Protein family	Gene
Structural proteins	β -Tubulin
	M- α -Tubulin
	Crk
	Ribosomal proteins 18S ; 57 L18 ; L19
Mitogen activated proteins	MNK2 (mitogen-activated kinase)
	EIF4E (transcription initiation factor)
Chromosomal proteins	Histone H2A.Z
	HMG-17 (high-mobility group protein)
Regulators of signalling	FRAT-1 (regulator of GSK-3)
	SAM-9 (phospholipase D homologue)
Regulators of protein folding	Calnexin
Transcription factors	CREM (cyclic-AMP responsive TF)
	Nur-77 (nuclear orphan receptor)

Table 4 : Genes isolated from the RDA subtraction of TCR β (+) and TCR β (-) DN3 thymocytes.

By using, in both driver and tester populations, an equivalent thymocyte subset (DN3), characterised by specific developmental markers, we hoped to minimise all differences in gene expression due to other, pre-TCR unrelated, differentiation processes.

However, the results of that RDA analysis (Table 4) suggested that the presence of pre-TCR in WT DN3 cells had such dramatic effects on the proliferation status of the thymocytes that most of the products of the subtractive hybridisation could be related to cell proliferation. This prevented an efficient identification of genes whose expression is directly controlled by pre-TCR signalling, since these were diluted in a pool of many transcripts accumulated in cells which were induced to proliferate. Theoretically, the genes with a less direct link between their expression and cell proliferation status were transcription factors ICER and Nur-77. But the fact they were isolated in the same experiment with all the other, proliferation-related, results was not a good indicator of them being direct targets of pre-TCR signalling.

These RDA results suggested that the analysis of thymocytes in a given developmental stage might not be the most informative of experiments. Such populations were probably still too heterogeneous to give rise to differential products which reflected their most significant distinct properties. We therefore decided to adopt a new strategy.

2.2 RDA analysis of RAG(-) pre-T cells unstimulated *versus* stimulated with anti-CD3 ϵ antibody

We decided to use a system in which the effects of pre-TCR signalling on gene transcription would be simulated in an inducible way, so that the kinetics of those events could be followed.

In RAG-1^{-/-} mice, thymocyte differentiation is blocked at the β -selection checkpoint. Treatment of 3-week-old RAG-1^{-/-} mice with anti-CD3 ϵ monoclonal antibodies *in vivo* results in rapid thymocyte expansion, down-regulation of cell-surface CD25, and onset of CD4 and CD8 expression. This procedure reproduces many characteristics of the DN3 \rightarrow DN4 \rightarrow DP transition and is recognised as a tractable model for pre-TCR-driven β -selection.

Using this methodology, thymocytes were sorted from a group of animals sacrificed at different time points, as illustrated in **Figure 17**. CD25 down-regulation, the phenotypic marker of DN3 \rightarrow DN4 transition, is first detected 12 hours after mAb injection. By the 48 hour stage, 90% of thymocytes are CD25 negative and approximately 10-20% of cells express CD4 and CD8 (data not shown), showing that the system allows an efficient generation of DP cells.

The model system is further characterised in **Figure 18**, where changes in surface levels in CD25, CD69 and CD2, and in cell size, are shown. After 12 hours, CD25 down-regulation is accompanied by up-regulation of CD2 and CD69, which are well-established signatures of β -selection. An increase in the mean cell size of the population is only detected after 24 hours. This increase is only transient, as by 48 hours the mean cell size has already decreased. This later change, together with a reduction in CD69 expression, correlate with the appearance of small, CD69(-), DP thymocytes (data not shown).

The evolution of the cell cycle status in this system is depicted in **Figure 19**. Once again, the 12 hour time point is the first to show a significant difference: twice as many cells (25%) are in cycle (S/G2/M phases). This percentage of cycling cells duplicates in the following 12 hours (47% at the 24 hour stage).

RAG-1^{-/-} thymus

Time after injection of anti-CD3 mAb:

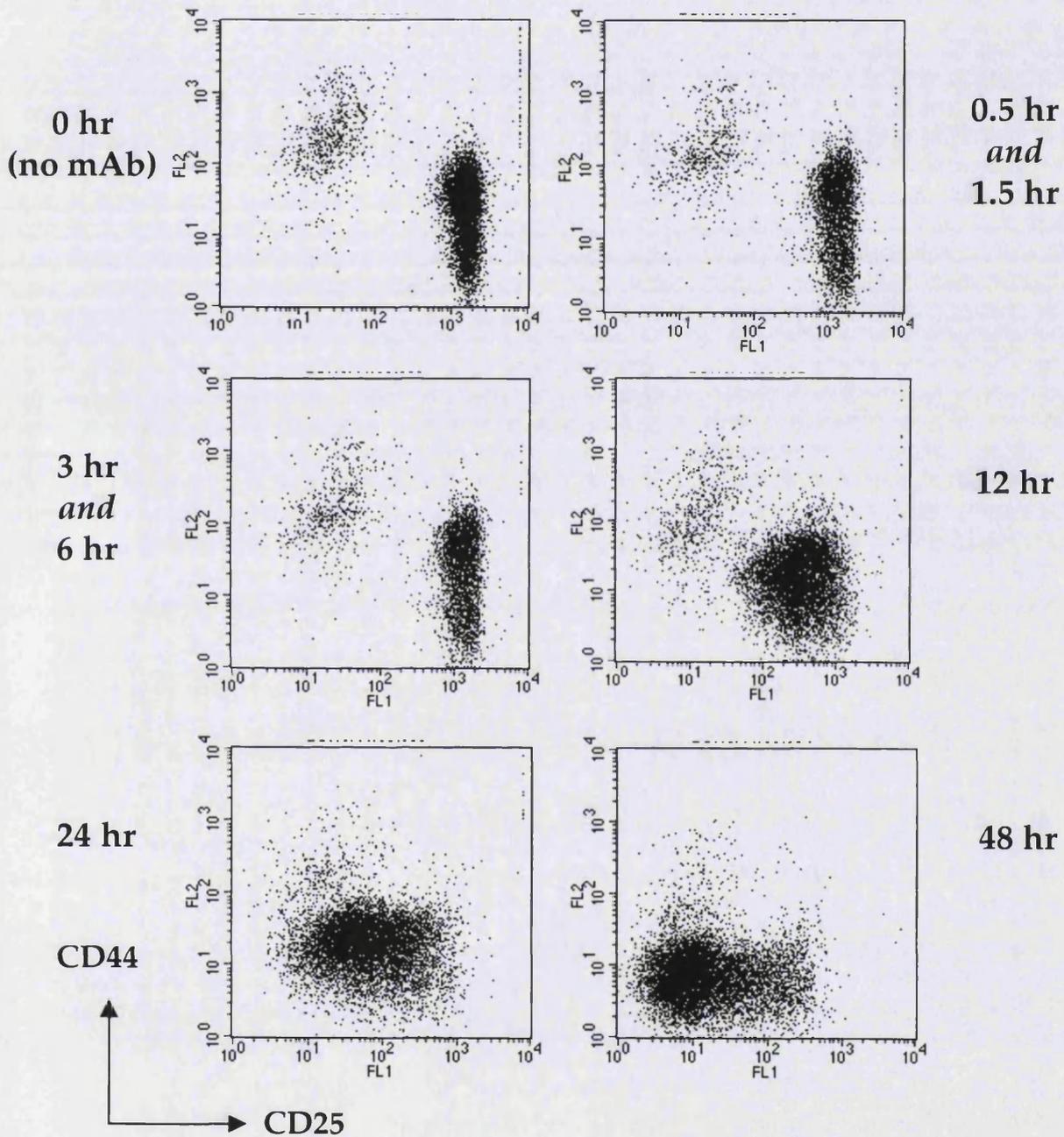


Figure 17: FACS plots (CD25 / CD44) used in the purification of cells for RDA analysis of anti-CD3 mAb stimulated *vs.* unstimulated RAG(-) pre-T cells . At each time point, the major CD44⁺ population was sorted.

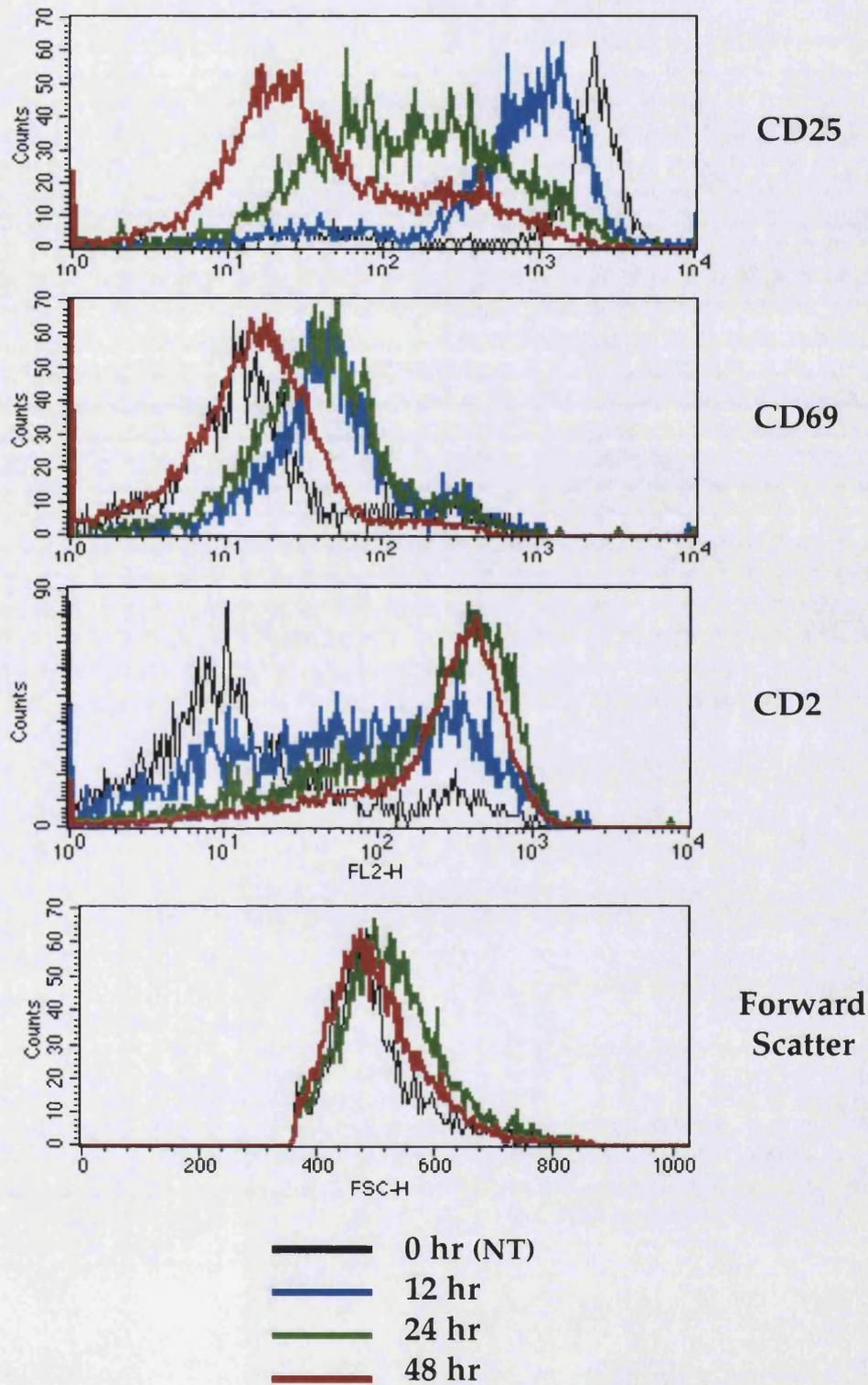


Figure 18: FACS profiles of sorted populations used for RDA analysis:
 CD25, CD69 and CD2 levels of surface expression;
 Forward Scatter (cell size).

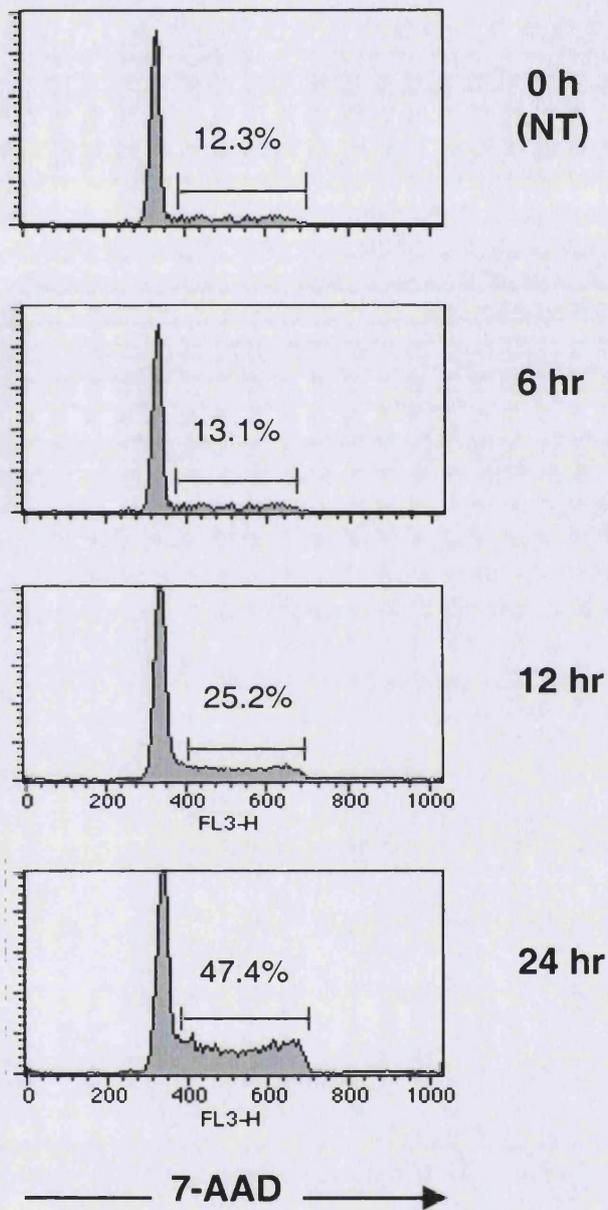


Figure 19: Cell cycle status of sorted populations used for RDAnalysis .

In order to identify genes which would be *direct* targets of pre-TCR-like signalling, an **RDAnalysis** was performed between stimulated and unstimulated (no mAb treatment control, "0 hours") thymocytes. The time point chosen to represent the CD3-stimulated cells was **3 hours**. This was seen as the ideal compromise in terms of duration of the antibody stimulus: long enough to allow transcriptional changes to occur, but short enough to represent direct targets of signalling - well before any phenotypical changes were detected. In particular, that time point preceded CD25 down-regulation and the increase in the number of cells in the S/G2/M phases of the cell cycle, both of which characterise the DN3 to DN4 transition.

The results of this RDAnalysis (3 hr - 0 hr subtraction) are presented in **Table 5**.

TABLE 5

Protein family	Gene
Transcription factors	ICER (cyclic-AMP responsive TF)
	Nur-77 (nuclear orphan receptor)
	Egr-1 (early growth response gene)
	c-Maf
	ZFP-36 (zinc finger protein)
	Shp-1 (protein tyrosine phosphatase)
Regulators of signalling	
Membrane receptors	IL-7R α

Table 5 : Genes isolated from the RDAnalysis of RAG(-) thymocytes stimulated (for 3 hours *in vivo*) with anti-CD3 ϵ mAb *versus* unstimulated cells.

2.3 Expression of candidate genes: induction and dependence on pre-TCR signalling

The seven candidate genes were initially tested for their differential expression between the samples used in the RDA analysis: 3 hour stimulation and NT. All of them showed a clear up-regulation between the "0 hr" and the "3 hr" time points, except Shp-1, which was highly expressed even in the NT sample (data not shown) and was therefore disregarded for posterior studies. The expression of the remaining six candidates was evaluated (by semi-quantitative RT-PCR) in all time points of the antibody injection experiment. The results are presented in **Figure 20**.

The mRNA expression of all six candidate genes is strongly induced by anti-CD3 antibody treatment, with all of them up-regulated after 3 hours. Four of them (EGR-1, c-Maf, Nur-77 and ZFP-36) exhibit their highest levels of expression after 6 hours, whereas ICER peaks earlier (3 hours) and IL-7R α does it later (12 hours). As expected, induction of CD4 expression is a late consequence of CD3-signalling, only obvious after 48 hours (when DP cells appear).

These data showed how a stimulus that mimics pre-TCR signalling was sufficient to induce the expression of the six candidate genes. We then investigated whether pre-TCR signalling was indeed necessary for such expression.

For that we analysed gene expression in DN3 thymocytes isolated from WT and pre-TCR deficient mice. Since pre-TCR starts being expressed at the DN3 stage of development, its absence in mutant thymuses should impair the expression of downstream genes in DN3 cells. Four different mouse models for pre-TCR deficiency were used: RAG-1^{-/-}, TCR β ^{-/-} and pT α ^{-/-}, all lacking one component of the complex; and p56Lck^{-/-}, in which pre-TCR signals are not efficiently transduced (Introduction, 2.4.3). The results (**Figure 21**) demonstrated that the expression of ICER and Nur-77 in DN3 thymocytes is pre-TCR dependent. In contrast, the expression of the other four candidate genes in pre-TCR(-) DN3 thymocytes was only slightly diminished, or not perturbed at all, suggesting that although they can be up-regulated by CD3-signalling, such a process is not relevant in normal DN3 cells.

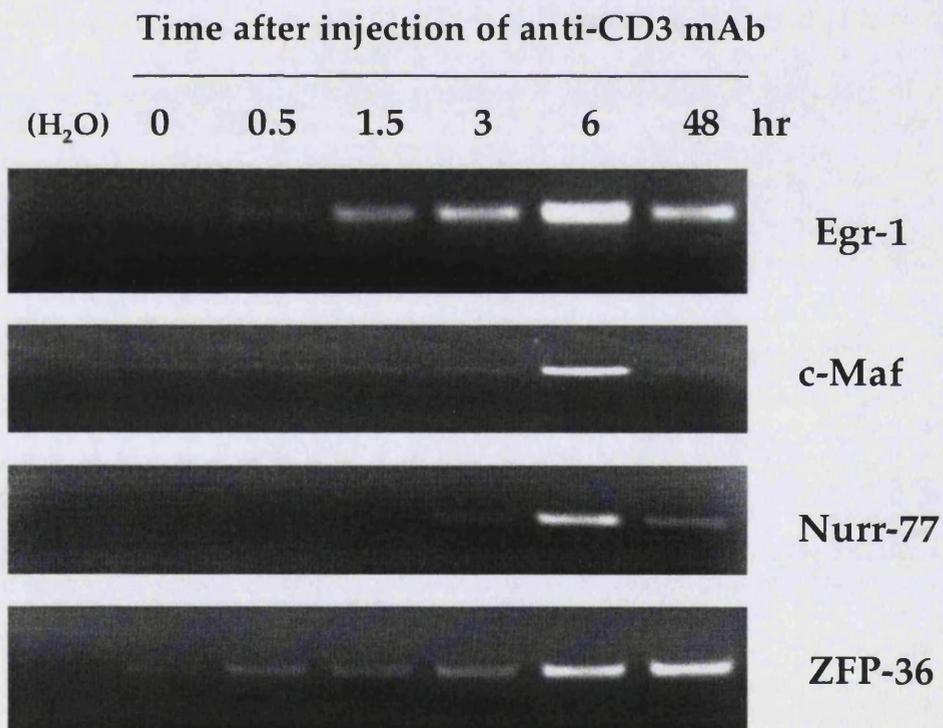
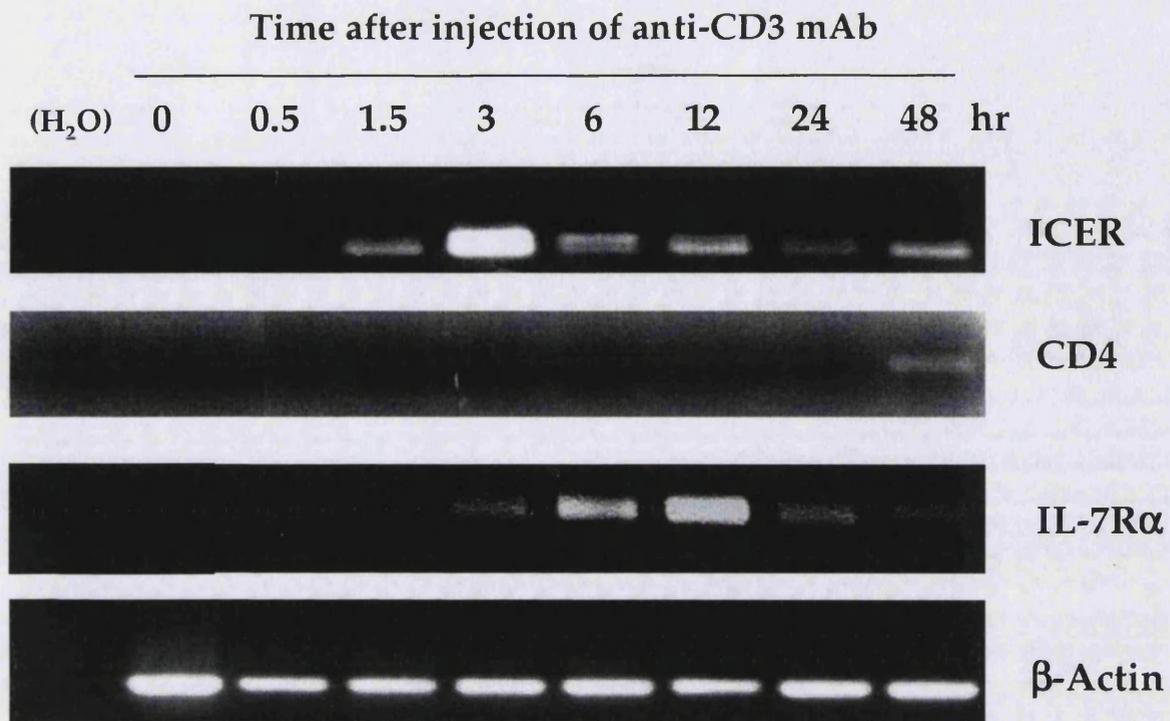


Figure 20: Induction of gene expression in RAG(-) pre-T cells by anti-CD3 ϵ mAb stimulus. RT-PCR for candidate genes on cell populations sorted at each time point of the RDA experiment.

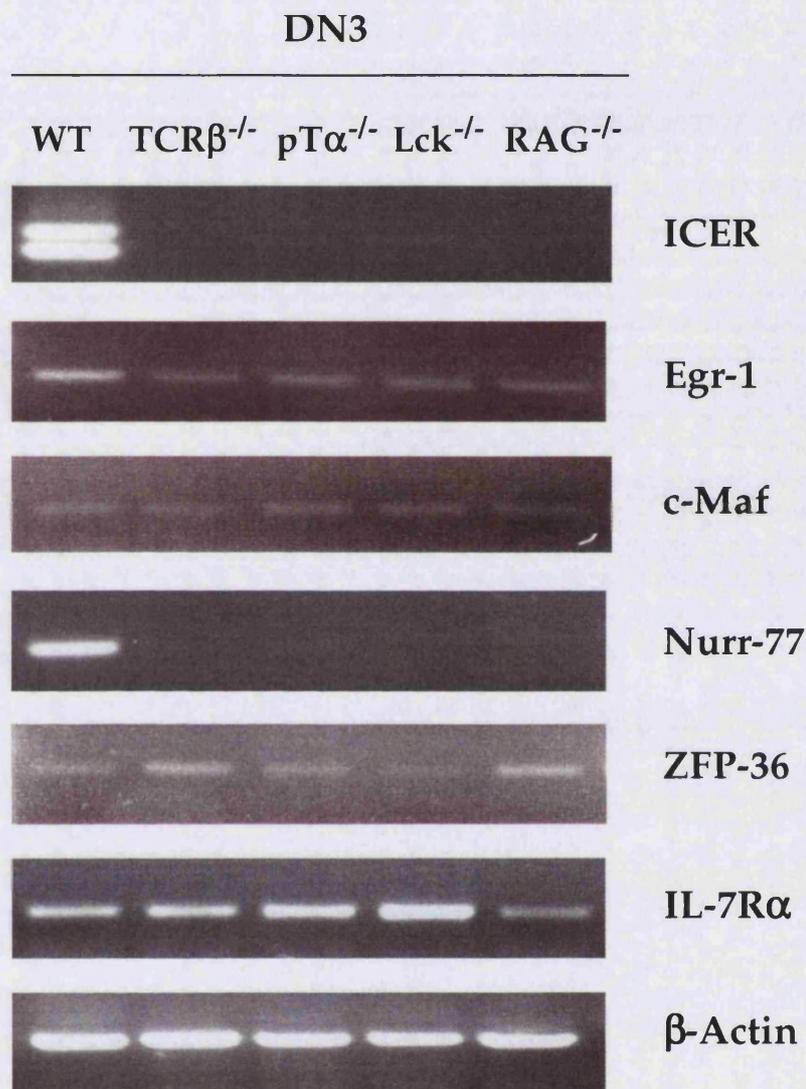


Figure 21: Expression of candidate genes in WT and pre-TCR deficient pre-T cells. RT-PCR for candidate genes on DN3 thymocytes sorted from mice with either normal or impaired pre-TCR signalling.

2.4 Studies on a candidate gene: IL-7 receptor

2.4.1 Pre-TCR dependence of IL-7R expression in DN4 cells

Our previous data had shown IL-7R α expression to be inducible in RAG(-) DN3 thymocytes by an anti-CD3 stimulus (Figure 20), but the endogenous expression of IL-7R α was not compromised in pre-TCR deficient DN3 cells (Figure 21). However, a parallel experiment carried out with Cesar Trigueros suggested that, at the subsequent developmental stage (DN4), IL-7R α expression was indeed pre-TCR dependent. As shown on **Figure 22 A**, mice lacking a component of the pre-TCR, either TCR β or pT α , fail to express normal levels of surface IL-7R in DN4 thymocytes.

This later effect of pre-TCR signalling on the endogenous IL-7R levels of WT thymocytes was consistent with the slower induction kinetics of induction of IL-7R α mRNA (when compared to the other candidate genes), as seen in Figure 20. The induced expression of IL-7R α peaked at 12 hours after antibody injection, whereas all other candidate genes peaked at 3-6 hours. The 12 hour time point was the first to exhibit a down-regulation of surface CD25 (Figures 17 and 18), which phenotypically marks DN3 \rightarrow DN4 transition. Moreover, at that time point cells up-regulated surface CD2 and CD69 (Figure 18) and became a more proliferative population (Figure 19), all of which are also events associated with transition to the DN4 stage.

In accordance with the previous considerations, IL-7R α protein expression was also clearly up-regulated in RAG(-) thymocytes 12 hours after injection of anti-CD3 mAb, as seen in **Figure 22 B**. Surface protein did not return to initial levels until the 48 hour stage (data not shown). At this point 90% of thymocytes were CD25 negative and approximately 10-20% of cells expressed CD4 and CD8 (data not shown). Thus, CD3-mediated signalling in RAG-1^{-/-} thymocytes enhanced surface IL-7R expression at the DN3 to DN4 transition, resulting in sustained IL-7R levels until differentiating cells reached the DP stage. Therefore, although IL-7R expression is independent of the pre-TCR before the DN4 stage, the lack of pre-TCR signalling at the DN3 to DN4 transition results in DN4 thymocytes that fail to sustain IL-7R expression.

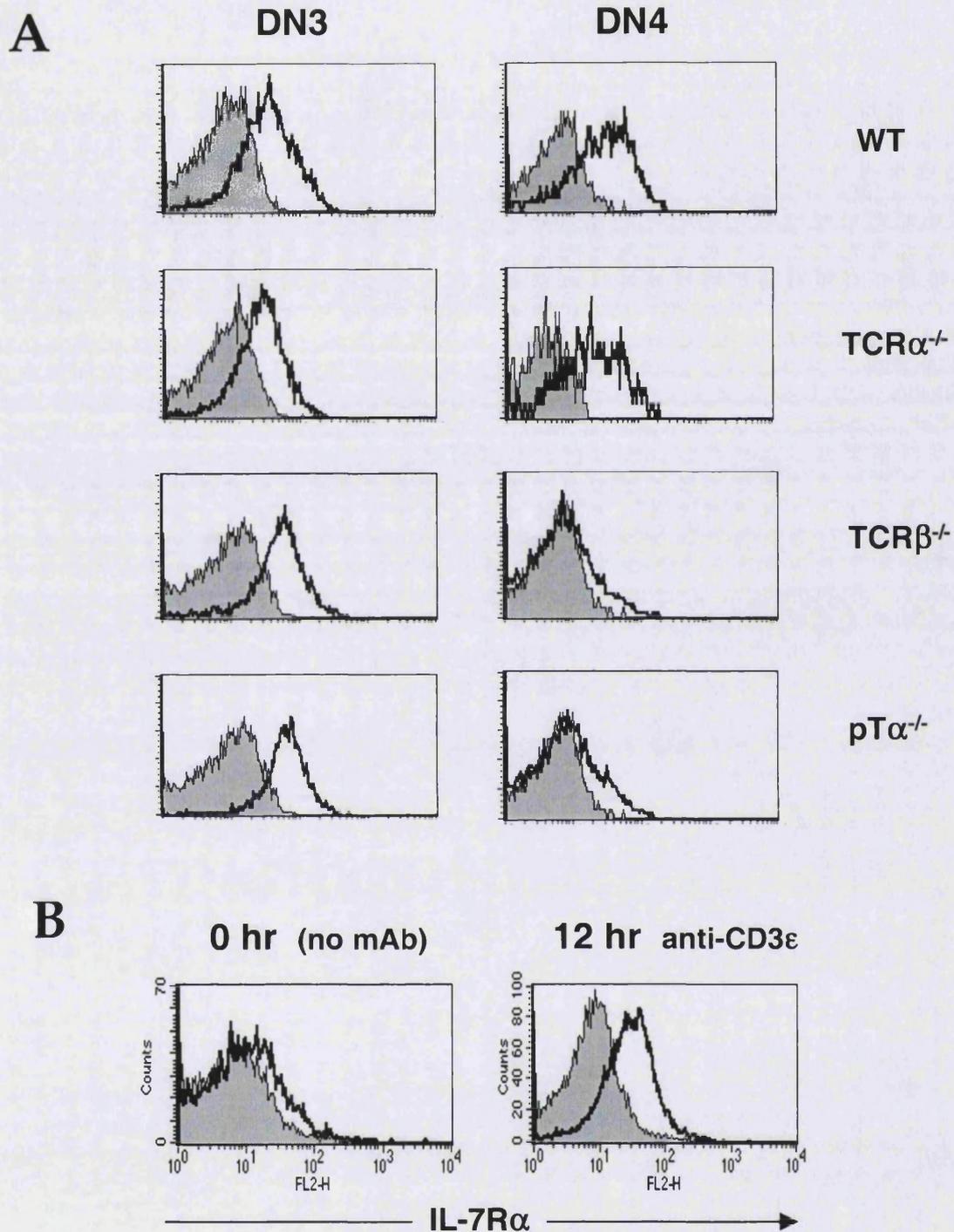


Figure 22 : Surface expression of IL-7R α : (A) in pre-TCR(+) and pre-TCR(-) DN3 and DN4 thymocytes; (B) in RAG(-) pre-T cells unstimulated *vs.* stimulated (12 hr *in vivo*) with anti-CD3 ϵ mAb. Staining for IL-7R α (unshaded) is compared to IgG2 α isotype control (shaded).

2.4.2 Requirement for IL-7R signalling in the DN → DP

transition

As pre-TCR signalling appears to maintain IL-7R α expression in the DN3 to DN4 transition, one may postulate that IL-7/IL-7R signalling could mediate effects associated with β -selection at the DN4 stage. In particular, β -selection is seen as responsible for survival, proliferation and differentiation of DN4 thymocytes, events that had not been linked to IL-7R signalling before.

To examine the relevance of IL-7R signalling at the DN4 stage, (1×10^4) sorted WT DN4 thymocytes isolated from day 16 embryos (E16) were placed in 6-day reaggregate thymic organ culture (RTOC) with WT stromal cells (**Figure 23A**) in the presence of blocking monoclonal antibodies (mAbs) for either IL-7R α or γ_c , or IgG2a and IgG2b isotype controls.

In the presence of control antibodies, generation of DP (and SP) thymocytes and a 10-fold increase in total cell number (to $\sim 1 \times 10^5$) were observed (**Figure 23B**), as expected. In comparison, cultures treated with one of the two mAbs (anti-IL-7R α or anti- γ_c) expanded less, to $\sim 7-8 \times 10^4$ cells (data not shown). Furthermore, when both antibodies were used together, total cell number reached only $\sim 20\%$ ($\sim 2 \times 10^4$) of that observed in control cultures, a synergistic effect that has been reported previously (Jenkinson et al., 1992).

This decrease in total cell number was not evenly distributed between all thymocyte subsets as DN4 and (CD4-CD8+) immature single positive (ISP) thymocytes were more severely affected than DP cells (a ~ 15 -fold and ~ 18 -fold reduction compared with a ~ 2.5 -fold reduction, respectively).

Thus, these data indicate that IL-7R signalling is critical at the DN4 stage for efficient generation of mature thymocyte populations. As the expansion of thymocyte number subsequent to β -selection is characterised both by promotion of cell survival and by rapid proliferation, we were interested in investigating the role of IL-7/IL-7R signalling in these two distinct processes.

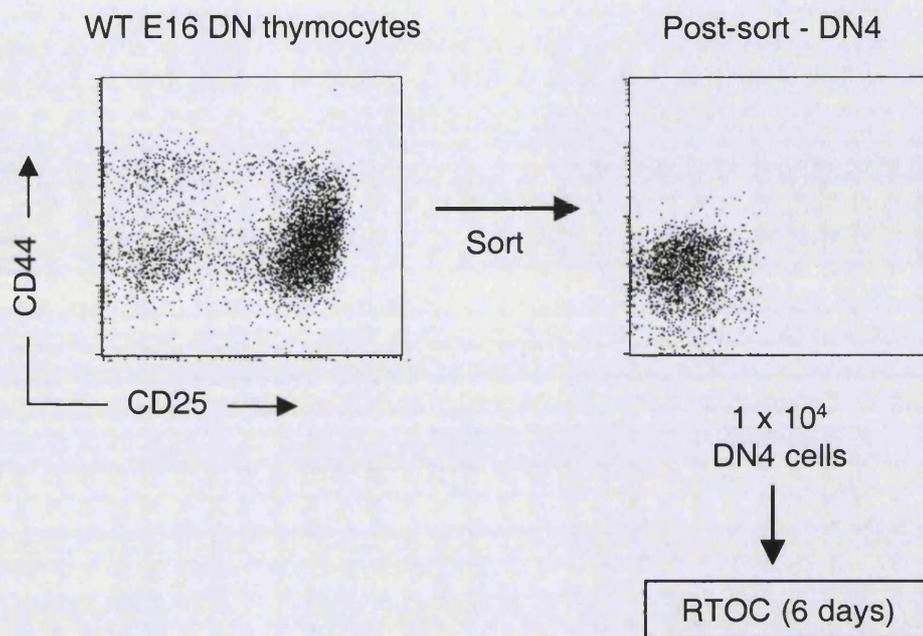
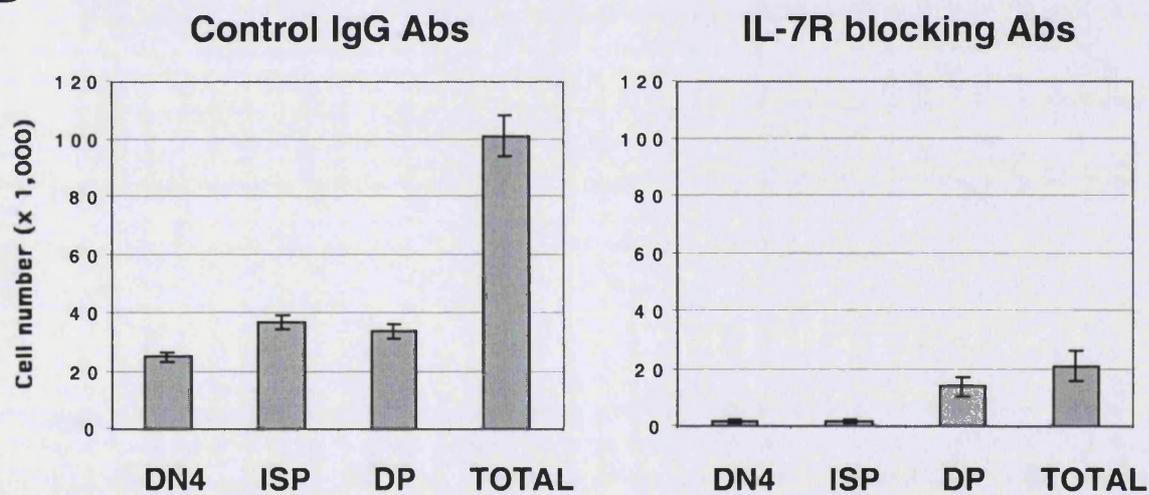
A**B**

Figure 23 : Effect of blocking antibodies to IL-7R complex on DN4 → DP transition. Anti-IL7R α and anti- γ_c mAbs (or isotype controls) used in RTOC.
(A) FACS profiles of foetal thymocytes used for RTOC (pre- and post-sort);
(B) Absolute cell number of total thymocytes and subsets after 6 days in RTOC.

2.4.3 Requirement for IL-7R signalling for the survival of DN4 thymocytes

To assess the role of IL-7/IL-7R signalling in cell survival *versus* proliferation, cell cycle and apoptosis profiles for DN4 cells from IL-7R $\alpha^{-/-}$ mice were compared to those from WT and TCR $\beta^{-/-}$ animals – **Figure 24**.

Consistent with previous reports (**ref. 9 paper**), the percentage of WT DN4 cells in S/G2/M phases was ~45%, while that of TCR $\beta^{-/-}$ DN4 cells was only ~11%. However, such a reduction in percentage of cycling cells was not observed for IL-7R $\alpha^{-/-}$ DN4 thymocytes as the proportion of cells in S/G2/M was comparable to WT at ~41%.

To analyse cell death, DN4 thymocytes were stained with Annexin-V in the presence of propidium Iodide (PI). In WT and TCR $\beta^{-/-}$ DN4 subsets, ~80% of thymocytes were PI/Annexin-V negative, a pattern indicative of live cells. However, in contrast the IL-7R $\alpha^{-/-}$ DN4 subset had only ~50% PI/Annexin-V negative cells.

Thus, IL-7R $\alpha^{-/-}$ DN4 thymocytes have a cell cycle profile consistent with WT DN4 cells, but display a marked increase in their susceptibility to cell death.

Since it had been suggested that the IL-7R might regulate TCR β gene rearrangements in early DN thymocyte differentiation (**ref. 38 paper**), we had to consider that hypothesis. If this were true, the IL-7R $\alpha^{-/-}$ (DN3 and) DN4 population(s) could be relatively TCR β deficient and hence cell death may reflect an increased failure of β -selection.

To test this, intracellular TCR β expression was analysed in DN3 and DN4 thymocytes from WT and IL-7R $\alpha^{-/-}$ mice. In the DN3 subset ~40% of WT cells were intracellular TCR β (+) compared to ~50% from IL-7R $\alpha^{-/-}$ mice (data not shown). Likewise, comparable levels of intracellular TCR β (88% *vs.* 77%) were also observed for WT and IL-7R $\alpha^{-/-}$ DN4 cells (Figure 24, 3rd row). Furthermore, expression of CD2, a marker associated with β -selection, was similar in DN4 subsets from both WT and IL-7R $\alpha^{-/-}$ mice (data not shown). Thus, failure to express a productively rearranged TCR β gene is not observed in IL-7R $\alpha^{-/-}$ mice and hence cannot explain the increased level of cell death observed in IL-7R $\alpha^{-/-}$ DN4 cells.

DN4 cells

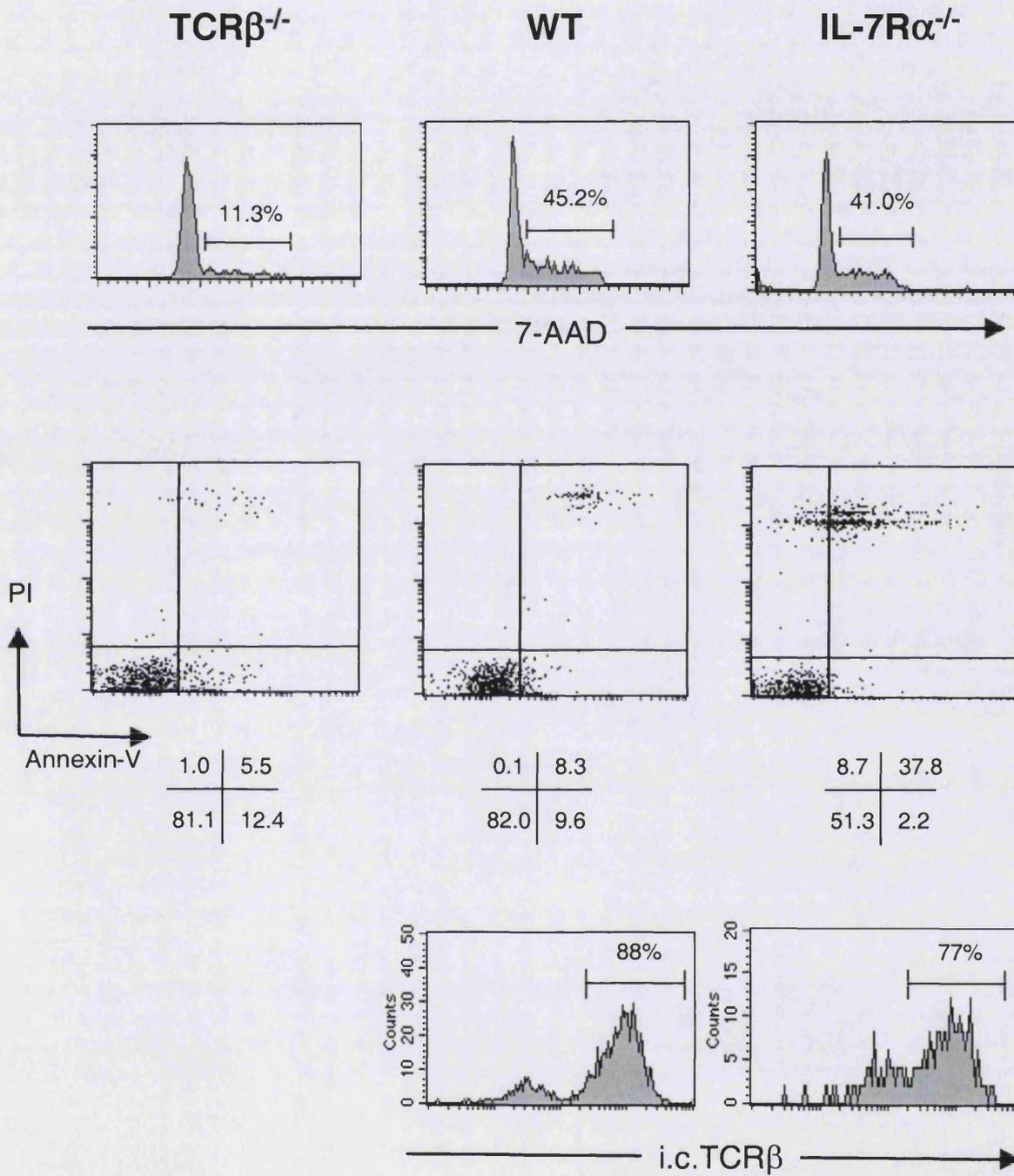


Figure 24 : Comparison of WT and IL-7R α deficient DN4 thymocytes: cell cycle status, cell death and intracellular TCR β expression. Also shown are plots for cell cycle and death of TCR β -deficient DN4 cells.

Although DN4 thymocytes from IL-7R α ^{-/-} mice showed increased susceptibility to cell death, it was possible that lack of a functional IL-7R at earlier stages of thymic differentiation in these mice could have primed thymocytes for death at the DN4 stage. To exclude this possibility, cell cycle and apoptosis profiles were assessed after 10⁴ WT DN4 thymocytes had developed in 5-day WT RTOC in the presence or absence of anti-IL-7R α and anti- γ c blocking antibodies – **Figure 25**.

Again, total cell number increased ~8-fold in RTOC with IgG2a/IgG2b isotype controls, while only a ~2-fold increase was observed in the presence of anti-IL-7R α and anti- γ c antibodies (data not shown). Cell cycle status was analysed by 6-hour BrdU incorporation and 7-AAD staining. In both the control and anti-IL-7R α /anti- γ c RTOCs, ~30% of DN4 cells had incorporated BrdU, with a slightly higher percentage of thymocytes from the control culture (31% vs. 26%) in the S/G2/M phase of the cell cycle. Therefore, proliferation of DN4 thymocytes was not affected by inhibition of IL-7R signalling.

However, when cell death was analysed, only ~38% of DN4 cells from the anti-IL-7R α /anti- γ c RTOC were in the live TO-PROTM-3/Annexin-V negative quadrant, compared with ~79% of DN4 cells from the control culture.

Thus, IL-7/IL-7R signalling is required to protect β -selected DN4 cells from cell death, thereby promoting the efficient generation of mature thymocyte subsets. This consists of a novel mechanism by which the pre-TCR controls cytokine signalling in order to promote the survival of β -selected thymocytes.

DN4 cells in RTOC

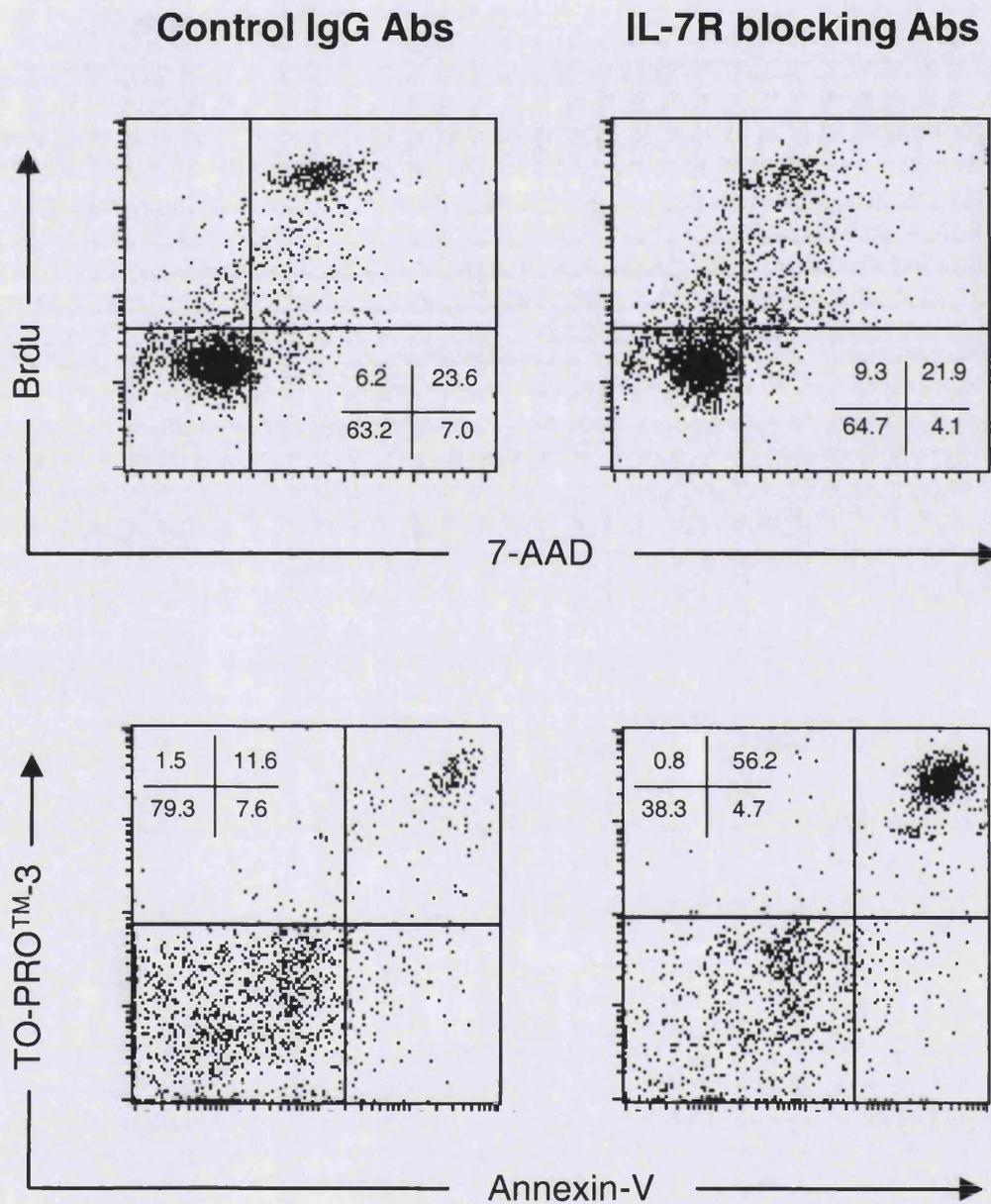


Figure 25 : Effect of blocking antibodies to IL-7R complex on proliferation and death of DN4 thymocytes. Anti-IL7R α and anti- γ_c mAbs (or isotype controls) used in 5 day- RTOC.

3 ICER in $\alpha\beta$ versus $\gamma\delta$ T cell lineage commitment

3.1 Identification of CREM isoforms expressed in $\gamma\delta$

thymocytes: ICER

Cyclic-AMP response element modulator (CREM) was one of the transcription factors identified in the $\gamma\delta$ - DP subtraction (1.1). According to our RT-PCR results (Figure 12), CREM is expressed in $\gamma\delta$ and pre-T cells, but not in $\alpha\beta$ thymocytes. Its restricted expression to the $\gamma\delta$ - and not other haematopoietic - lineages made it an attractive candidate in the context of $\gamma\delta$ / $\alpha\beta$ lineage commitment.

The CREM gene comprises of two differentially regulated promoters and a number of exons that can be alternatively spliced to generate at least eleven proteins (Figure 26A). As the fragment cloned from the RDA corresponded to a 3' untranslated region of the gene that is common to all the CREM splice variants, an RT-PCR strategy using multiple primers was devised to identify the CREM isoforms that were present in thymic $\gamma\delta$ T cells. Figure 26B demonstrates that in thymic $\gamma\delta$ cells the major CREM-specific transcripts code for ICER I and ICER I γ . PCR bands specific for the other CREM and ICER isoforms were not detected at significant levels with this degree of sensitivity. Furthermore, this expression of predominantly ICER I and ICER I γ was observed (data not shown) in all subsequent T cell populations that were studied by Real Time PCR (see later).

At the protein level, also, ICER was confirmed to be the only CREM variant present in $\gamma\delta$ thymocytes - Figure 26C. The antibody used for Western blot analysis recognises all CREM isoforms, as illustrated with a mixture of recombinant CREM proteins (right lane).

We therefore concentrated our subsequent studies on ICER (inducible cyclic-AMP early repressor).

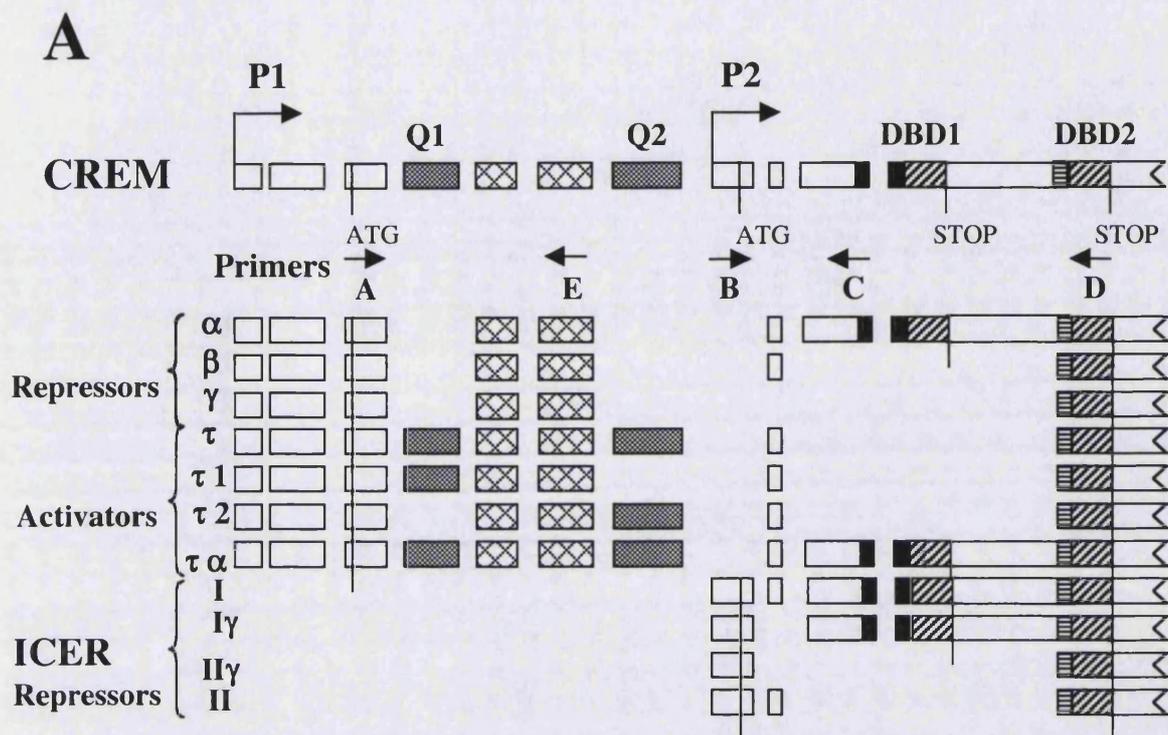


Figure 26 : Identification of CREM isoforms expressed in $\gamma\delta$ thymocytes.

(A) Schematic of the CREM gene showing the exons that are alternatively spliced to generate CREM and ICER transcripts. P1 and P2 are promoters; Q1/2 are activation domains; DBD1/2 are DNA-binding domains.

(B) RT-PCR for CREM/ICER transcripts in $\gamma\delta$ thymocytes and in total brain (control), using combinations of the primers (A-E) presented above.

C

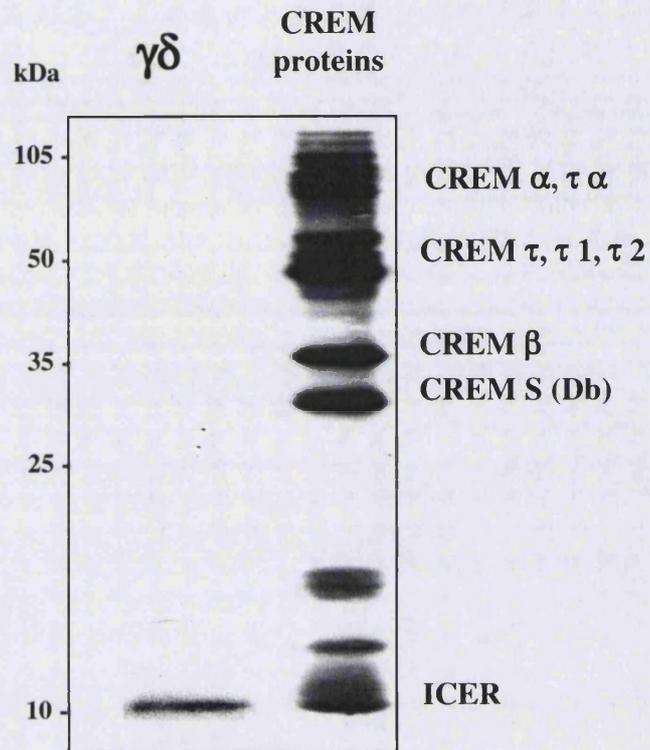


Figure 26 : Identification of CREM isoforms expressed in $\gamma\delta$ thymocytes.
(C) Western blot for CREM/ICER in thymic $\gamma\delta$ cells. Anti-CREM mAb provided by P. Sassone-Corsi. Mixture of recombinant CREM/ICER proteins run as control.

3.2 Pattern of expression of ICER in the thymus

As ICER is differentially regulated between thymic $\gamma\delta$ and DP ($\alpha\beta$) T cells, we quantified its mRNA expression in multiple thymic subsets by real-time PCR. This technique provides precise quantification of the levels of a particular transcript by use of a specific fluorogenic probe, which hybridises with new copies of the transcript as they are produced by PCR-amplification ("real-time"). All samples were normalised according to their GAPDH expression levels.

As seen in Figure 27, ICER transcripts are abundant in double negative (DN) thymocytes and thymic $\gamma\delta$ T cells, but not in NK cells or in thymic DP or single positive (SP) thymocytes that represent committed $\alpha\beta$ lineage T cells. Overall, ICER expression in $\gamma\delta$ thymocytes is at least 20-fold higher than in $\alpha\beta$ cells.

When ICER expression was analysed in the DN subset in more detail, it was observed to be low in the DN1 subset, but significantly higher in DN2, DN3 and DN4 cells. The average level of expression in these DN subsets is around 1/3 of that of thymic $\gamma\delta$ cells.

These results indicate that although ICER is expressed in early thymic DN subsets, its expression is restricted to the $\gamma\delta$ lineage after the DN4 stage. This is particularly significant as the DN4 subset is the last thymocyte population that has been shown to retain the potential to generate both $\alpha\beta$ and $\gamma\delta$ T cells. Furthermore, DN4 thymocytes express less ICER than their precursor populations (DN2/3), suggesting a progressive down-regulation of ICER in $\alpha\beta$ thymocyte development, if we take into account that most DN4 cells commit to the $\alpha\beta$ rather than the $\gamma\delta$ lineage (Bruno et al., 1999).

3.3 ICER expression and the status of TCR gene

rearrangements in pre-T cells

To gain insight of the degree of commitment of DN thymocytes (expressing variable levels of ICER) to a particular T cell lineage, we looked at the status of TCR gene rearrangements, usually taken as molecular indicator of lineage commitment.

ICER / GAPDH

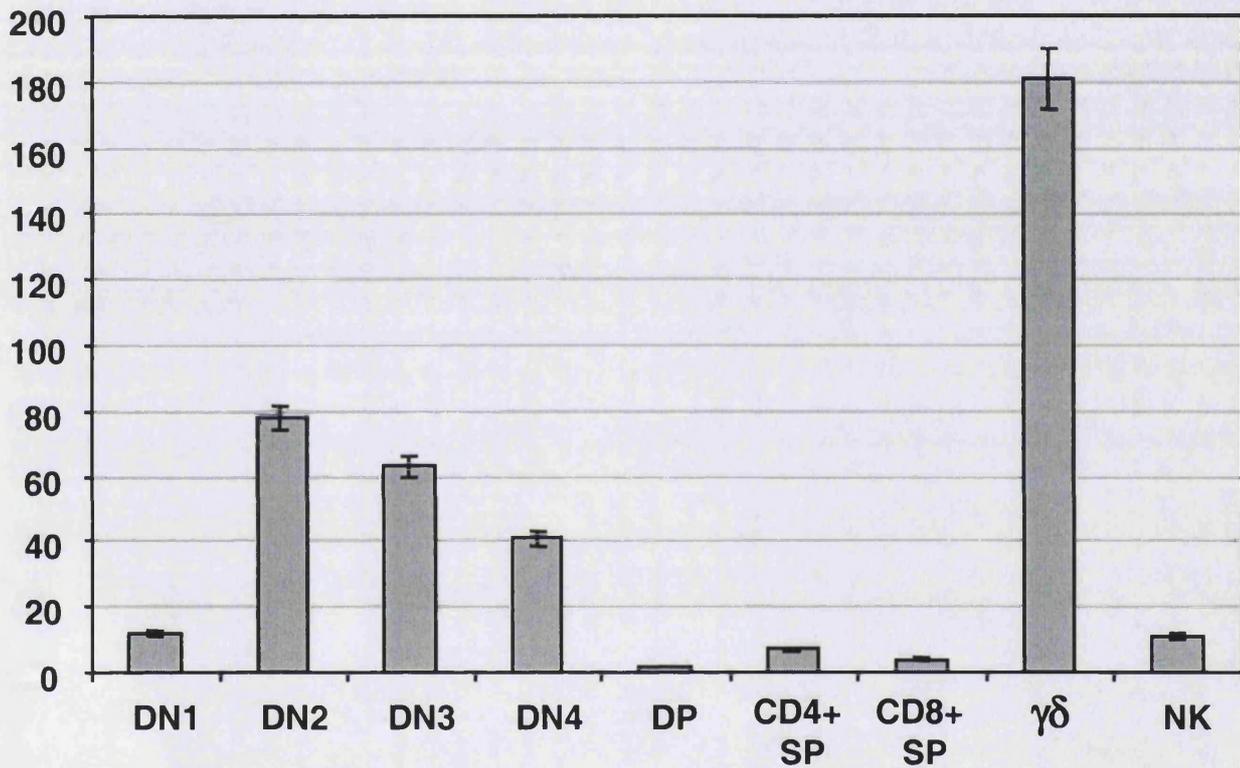


Figure 27 : Real-time PCR expression profile for ICER in the thymus.

ICER expression was quantified in each FACSorted thymocyte subset. (n=6)

Parallel real-time PCR reactions for GAPDH were used for normalisation.

In order to obtain informative subsets of DN cells for the analysis of gene rearrangements and ICER expression, we took advantage of a technique previously developed in the lab by Ludovica Bruno (Bruno et al., 1999). This relies on fluorescent liposomes which, by virtue of being conjugated to anti-digoxigenin antibodies, bind digoxigenin-labelled H57 (pan anti-TCR β) antibodies (**Figure 28A**). Such association allows the amplification (by approximately 100 x) of the H57 signals on pre-T cells, which are undetectable by more conventional staining methods (due to the very low levels of expression of the pre-TCR on the cell surface).

DN3 and DN4 thymocytes were thus sorted according to their levels of pre-TCR expression (**Figure 28B**). Using this strategy, the previous study by Bruno *et al.* had shown that DN4 cells expressing high levels of surface TCR β were essentially restricted to the $\alpha\beta$ lineage, as they did not generate $\gamma\delta$ thymocytes in RTOC. The results were not so clear for DN3 cells.

In terms of ICER mRNA expression, we observed a down-regulation of ICER expression between DN4-low and DN4-high subsets (**Figure 28C**). In DN3 cells, the phenomenon was not significant.

To establish the degree of lineage commitment of the populations in which ICER expression had been analysed, their TCR gene rearrangements were studied by restriction length polymorphism – PCR (RFLP-PCR). This method allows the quantification of in-frame *versus* out-of-frame rearrangements by amplifying the DNA of particular TCR gene segments, digesting them with convenient restriction enzymes and running the (radio-labelled) fragments on a sequencing gel. In-frame rearrangements are characterised by a 3 bp spacing (an amino-acid anti-codon) and can be quantified by densitometry.

Our results (**Figure 29**) showed a selection for (>71.4%) productive TCR β rearrangements and a selection against (approx. 20%) in-frame TCR δ rearrangements in DN4-high cells; these are typical of $\alpha\beta$ -committed cells. In contrast, the percentages of in-frame rearrangements for both TCR loci in DN4-low thymocytes were close to the ones expected for a random event (without selection) – as in uncommitted cells.

These results suggest that late DN4 cells that have already committed to the $\alpha\beta$ lineage down-regulate ICER expression, towards the low levels subsequently exhibited by DP and SP thymocytes.

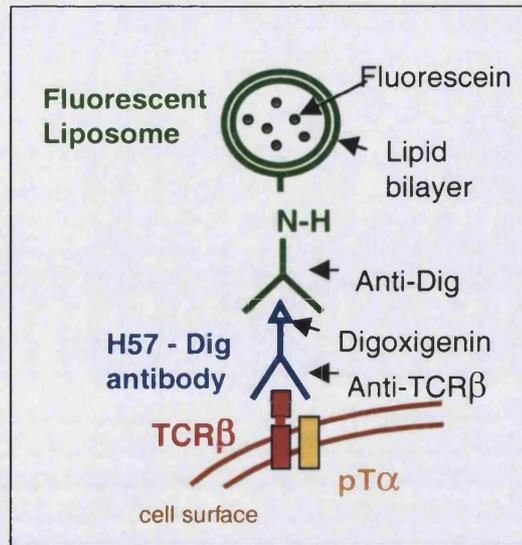
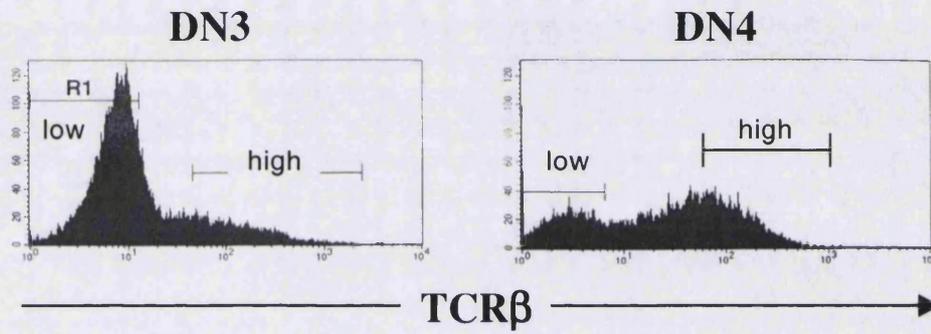
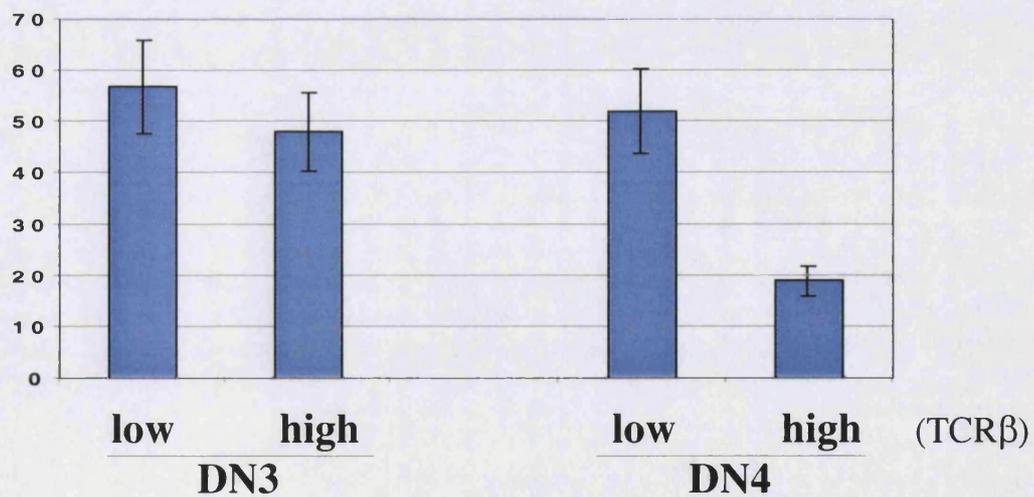
A**B****C**

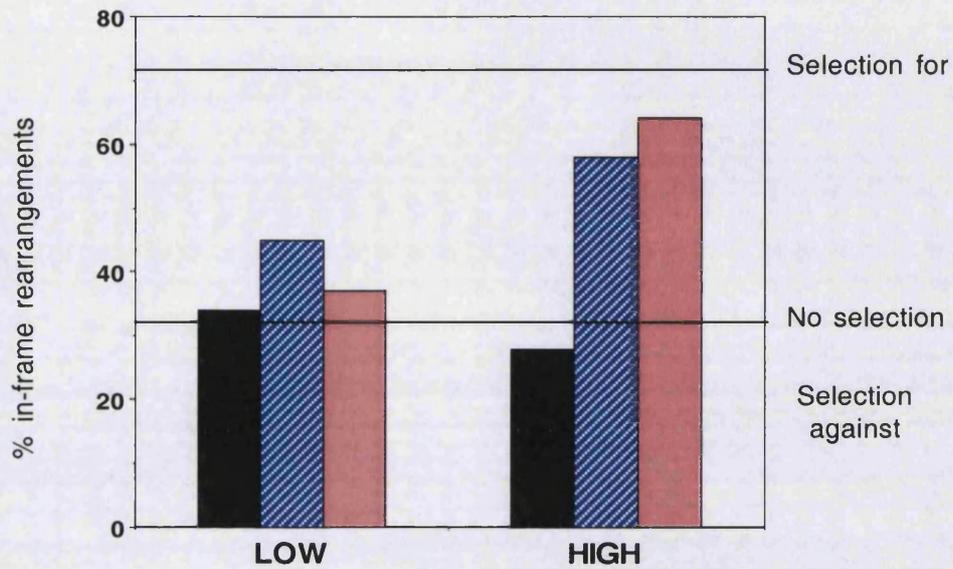
Figure 28 : Pre-TCR levels and ICER expression in WT pre-T cells.

(A) Schematic of the liposome staining method for surface TCR β detection.

(B) Pre-TCR levels (by liposome staining) in DN3 and DN4 thymocytes.

(C) Real-time PCR for ICER in DN3 and DN4 thymocytes expressing low / high levels of pre-TCR.

DN3



DN4

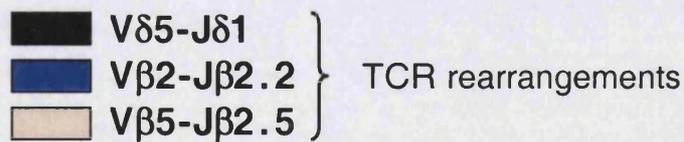
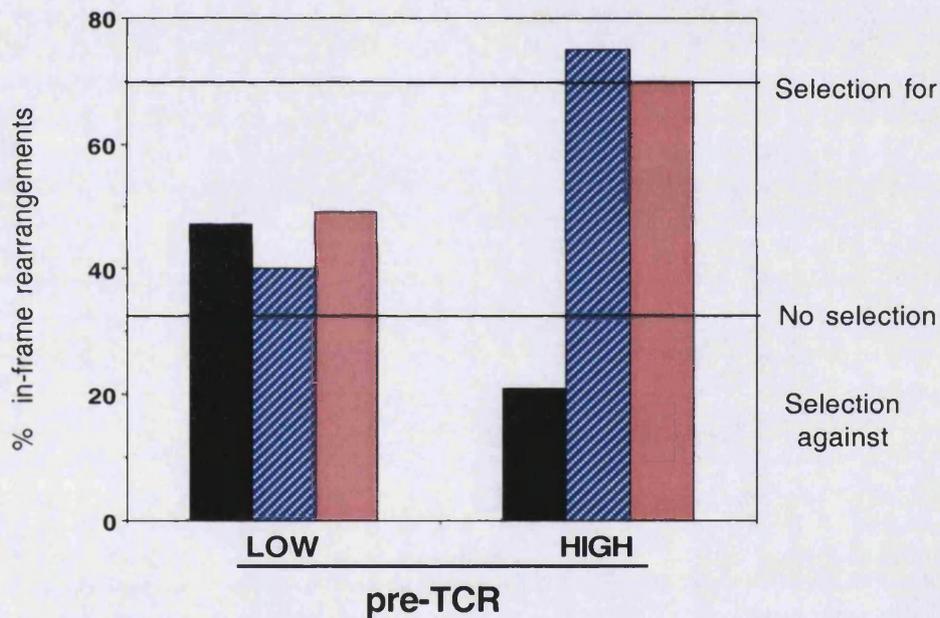


Figure 29: Pre-TCR levels and TCR gene rearrangement status of WT pre-T cells. RFLP-PCR results for TCR δ and TCR β rearrangements in DN3 and DN4 thymocytes sorted according to their levels of pre-TCR expression.

3.4 Analysis of the thymus of CREM/ICER deficient mice

As the expression of ICER in the thymus appeared to segregate with the $\gamma\delta$ and not the $\alpha\beta$ T cell lineage, we reasoned that ICER could play a role in thymocyte development and more specifically in $\alpha\beta/\gamma\delta$ T cell lineage divergence.

To investigate this role, mice deficient for all isoforms of the CREM gene were obtained from Prof. Paolo Sassone-Corsi (Nantel et al., 1996), and various T cell subsets were analysed – **Figure 30**.

CREM^{-/-} animals showed no defects in either absolute thymocyte number or in the percentage of specific thymic subsets. Importantly, ICER-deficient $\gamma\delta$ T cells developed to normal numbers (Figure 30B).

This data demonstrates that CREM, and more relevantly ICER, does not have a non-redundant role in thymocyte development / lineage commitment.

3.5 Role of cyclic-AMP signalling in ICER expression during thymocyte development

ICER is a cyclic-AMP (cAMP) responsive gene; on the other hand, cAMP had been shown to affect T cell development *in vitro* (Lalli et al., 1996). We therefore investigated the relationship between the effects of cAMP on thymocytes with their expression of ICER.

As previously reported by Ceredig *et al.*, DN → DP differentiation in FTOC was severely affected by the administration of a version of cAMP to which the cell membrane is permeable (di-butyl-cAMP). Our data (**Figure 31A**) showed that in the presence of cAMP, WT foetal lobes constituted exclusively by DN cells (at E15) gave rise to DP cells that were less than 2% of the number that would normally develop in a non-treated FTOC over 3 days. The total cellularity of cAMP-treated lobes was under 1/5 of the NT control, and only 5% of the cells treated with cAMP were DP, in contrast with 42% of the NT control.

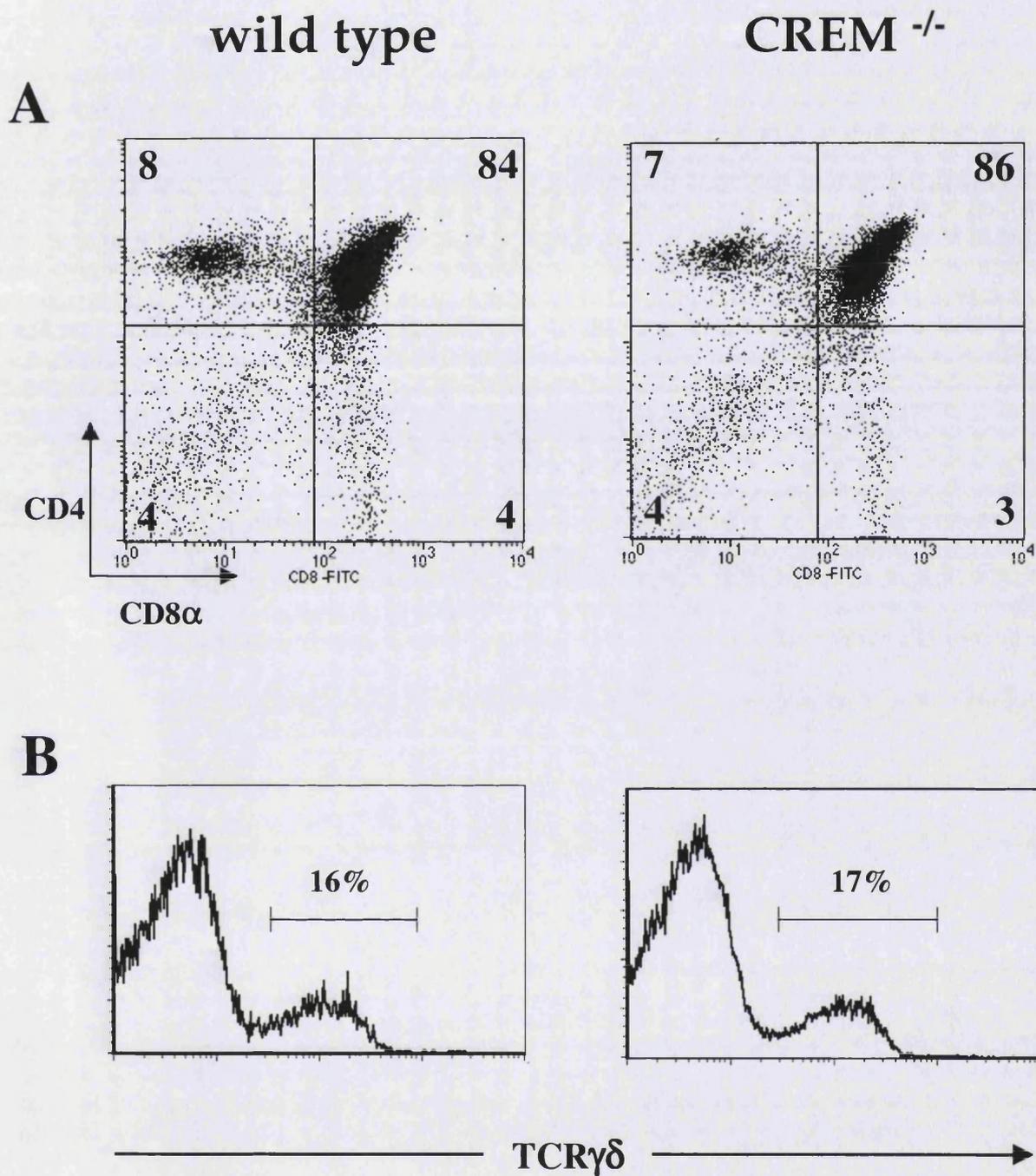


Figure 30 : FACS analysis of thymocyte development in CREM^{-/-} mice.

(A) CD8 *versus* CD4 plots for thymocytes from CREM^{-/-} and normal mice.

(B) TCR $\gamma\delta$ histograms, pre-gated on CD4⁻ CD8⁺ thymocytes from CREM^{-/-} and wild type control mice.

Contrary to $\alpha\beta$ lineage cells, $\gamma\delta$ thymocytes differentiated normally in the presence of cAMP: $1,800\pm 600$ cells were generated in both cAMP-treated and control FTOCs (data not shown).

The inhibitory effect on $\alpha\beta$ thymocyte differentiation *in vitro* was specific for cAMP, as it was not reproduced by a similar analogue of cGMP (di-butyl-cGMP).

When the mRNA expression of ICER was analysed in DN and DP cells sorted from the cultures of Figure 31A, an abnormally high level of ICER in DP thymocytes was detected in the cAMP-treated FTOC (Figure 31B). Unlike the control DP cells or wild type (adult) DP thymocytes (Figure 27), the DP cells that had developed in the presence of cAMP expressed ICER well above background levels: approximately 50 units (ICER/GAPDH), comparable to the amounts usually found in DN thymocytes.

This correlation between impaired generation of DP cells (*in vitro*) and their over-expression of ICER raised the possibility that ICER expression was detrimental to DP differentiation.

3.6 Generation and analysis of CD2-ICER transgenic mice

Since the high levels of ICER in normal $\gamma\delta$ thymocytes did not translate into a perturbed $\gamma\delta$ development in ICER-deficient mice, we decided to investigate whether the low levels of ICER in normal DP cells were crucial for $\alpha\beta$ differentiation, and in particular if an enforced expression of ICER in DP thymocytes would disturb $\alpha\beta$ development – which would agree with the cyclic-AMP data (see above). For that we generated mice over-expressing ICER selectively in the thymus, under the control of the CD2 promoter.

The structure of the CD2-ICER construct is shown in Figure 32A. The transgene was inserted into the human CD2 cassette (described in Methods, 2.2.8) for expression in pre- and mature T cells. The construct was micro-injected into blastocysts which were implanted in female mice, from which three litters were obtained.

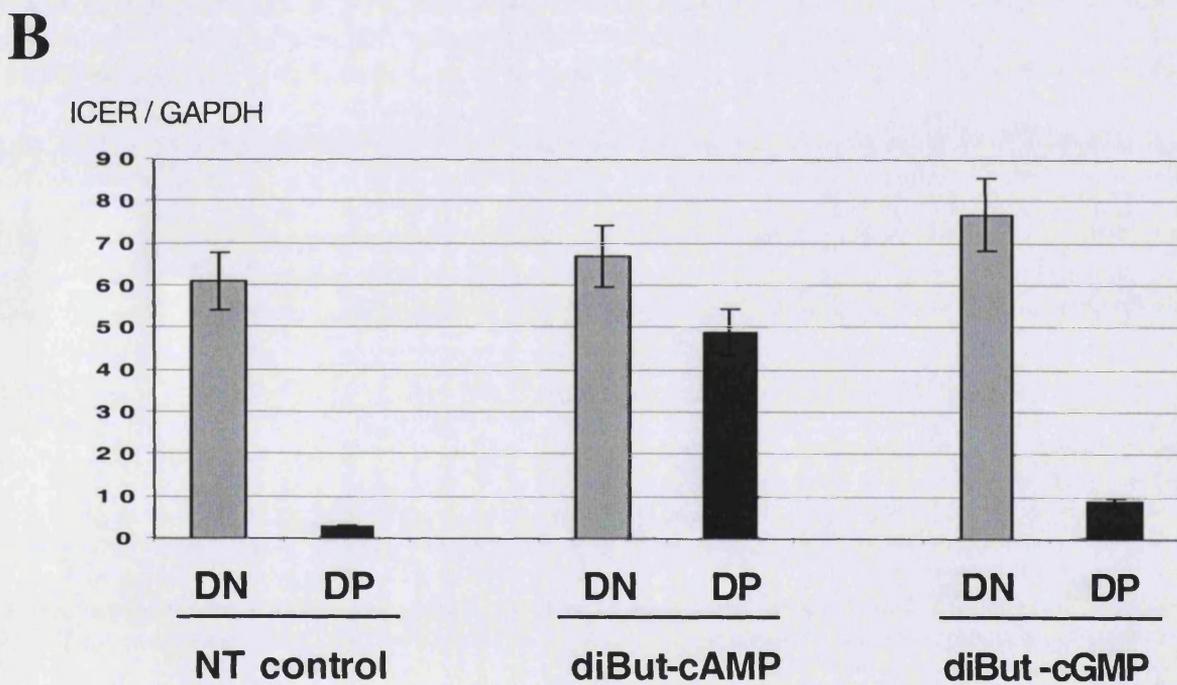
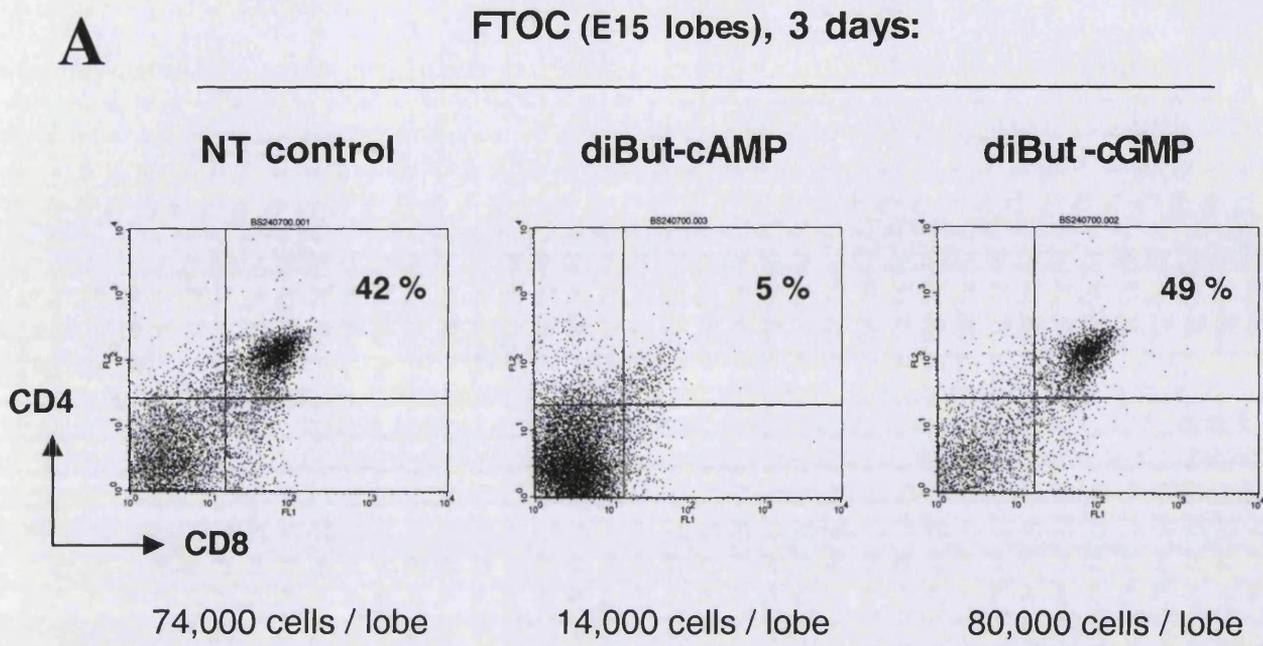


Figure 31 : Effect of cyclic-AMP on thymocyte development (*in vitro*) and ICER expression.

(A) CD4/CD8 plots and total cell number of WT foetal thymic lobes (E15) after 3 days in FTOC with (or without) 0.1 μ M di-butyl-cAMP/cGMP.

(B) Real-time PCR for ICER expression in DN and DP cells sorted from (A).

As the ultimate parameter that reflected the success of this experiment was the expression level of ICER in DP thymocytes, we screened the mice according to it. Real-time PCR was used to measure ICER mRNA levels in FACSsorted DP thymocytes, whereas thymocyte development was analysed (by FACS) in parallel in the same mice. **Figure 32B** illustrates the levels of expression of ICER that were achieved, including the lowest (non-transgenic controls) and highest levels (CD2-ICER transgenic in litter 1). The FACS analysis of T cell development in the mice of litter 1 is presented in **Figure 33**.

The maximum level of ICER obtained in DP cells of CD2-ICER mice was around 60 real-time PCR units (ICER/GAPDH). This is the average level found endogenously in normal pre-T cells (DN3 stage in particular, see Figure 27). Even if this is only 1/3 of the typical expression in $\gamma\delta$ thymocytes, it still represents a 30-fold increase in the usual levels found in normal DP thymocytes. However, such over-expression did not cause any phenotypical change in the subset distribution or cellularity of the thymus (Figure 33). Importantly, DP thymocytes over-expressing ICER still developed in normal numbers and efficiently generated SP cells.

Thus, over-expression of ICER does not disturb $\alpha\beta$ development, at least at the mRNA levels achieved in these CD2-ICER transgenic mice.

3.7 Pattern of expression of ICER in peripheral lymphoid tissues

Although ICER did not seem to play a non-redundant role in $\alpha\beta/\gamma\delta$ lineage commitment, it could still be a novel and reliable marker of the $\gamma\delta$ T cell lineage.

In particular, the dramatic contrast in ICER expression between thymic $\gamma\delta$ and $\alpha\beta$ T cell subsets after the point at which the $\gamma\delta$ and $\alpha\beta$ T cell lineages diverge, led us to investigate whether this differential lineage-specific ICER expression was rigidly maintained in peripheral T cell populations.

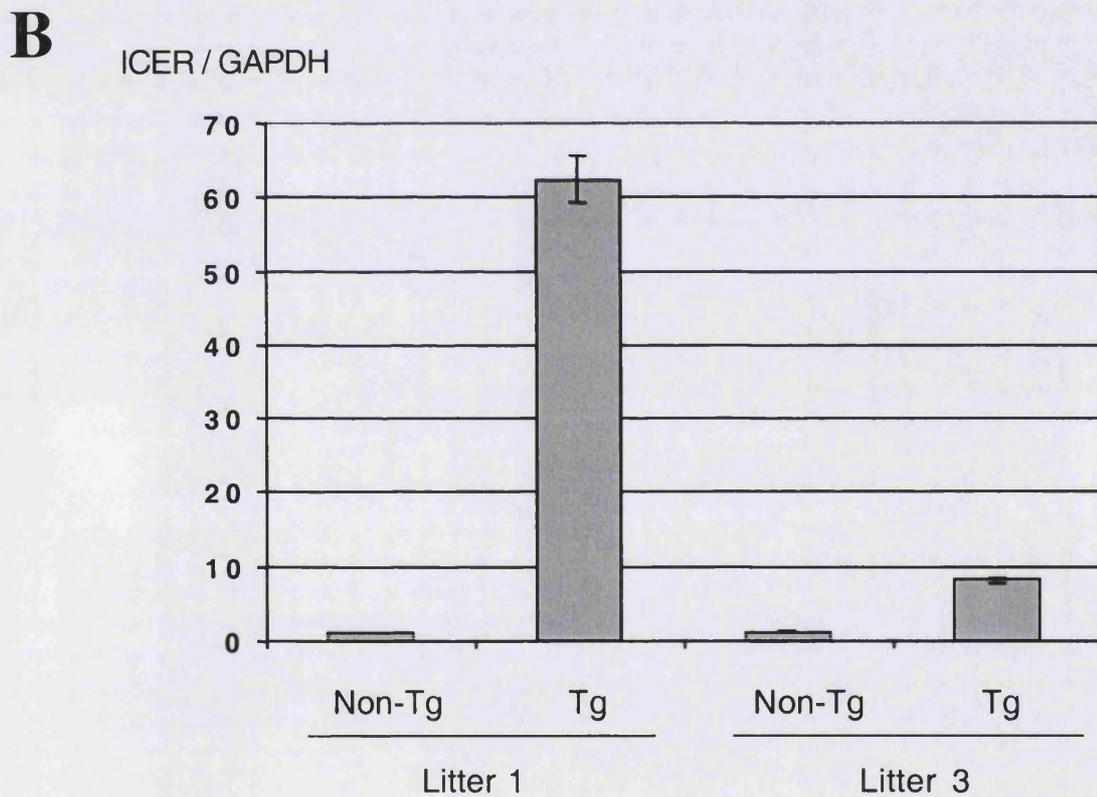
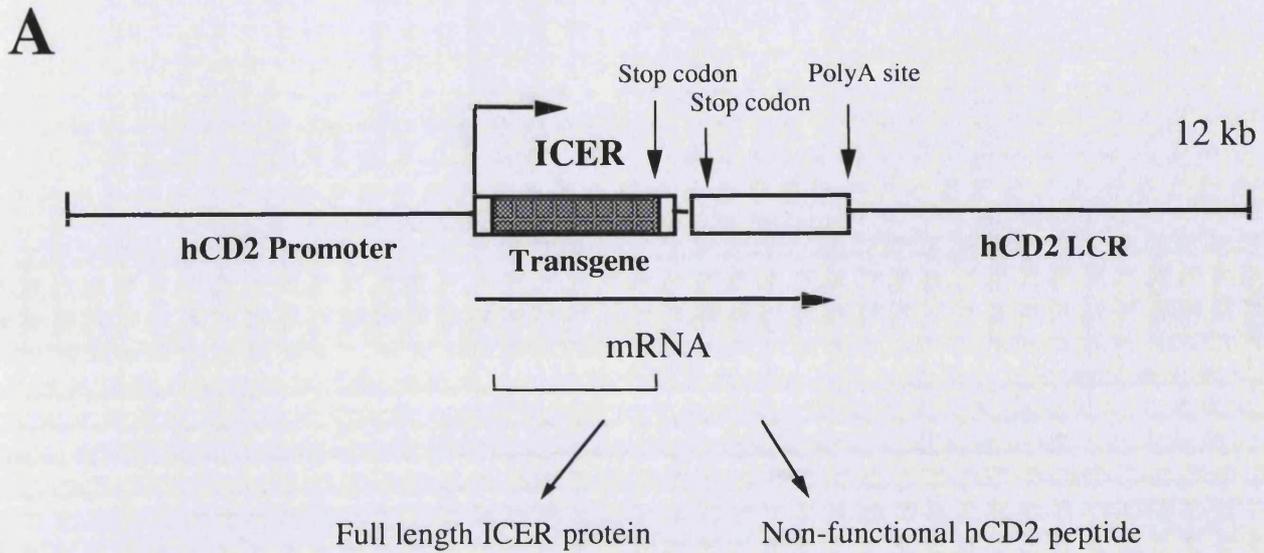


Figure 32 : Generation and screening of CD2-ICER transgenic mice.

(A) Schematic of the CD2-ICER construct micro-injected into murine blastocysts.

(B) Real-time PCR for ICER expression in DP thymocytes of littermates of the CD2-ICER transgenic (Tg) mouse lines.

Non-Tg control
(1.5 U ICER/GAPDH)
 $1.6 \pm 0.2 \times 10^8$ cells

CD2-ICER Tg
(62.5 U ICER/GAPDH)
 $1.8 \pm 0.4 \times 10^8$ cells

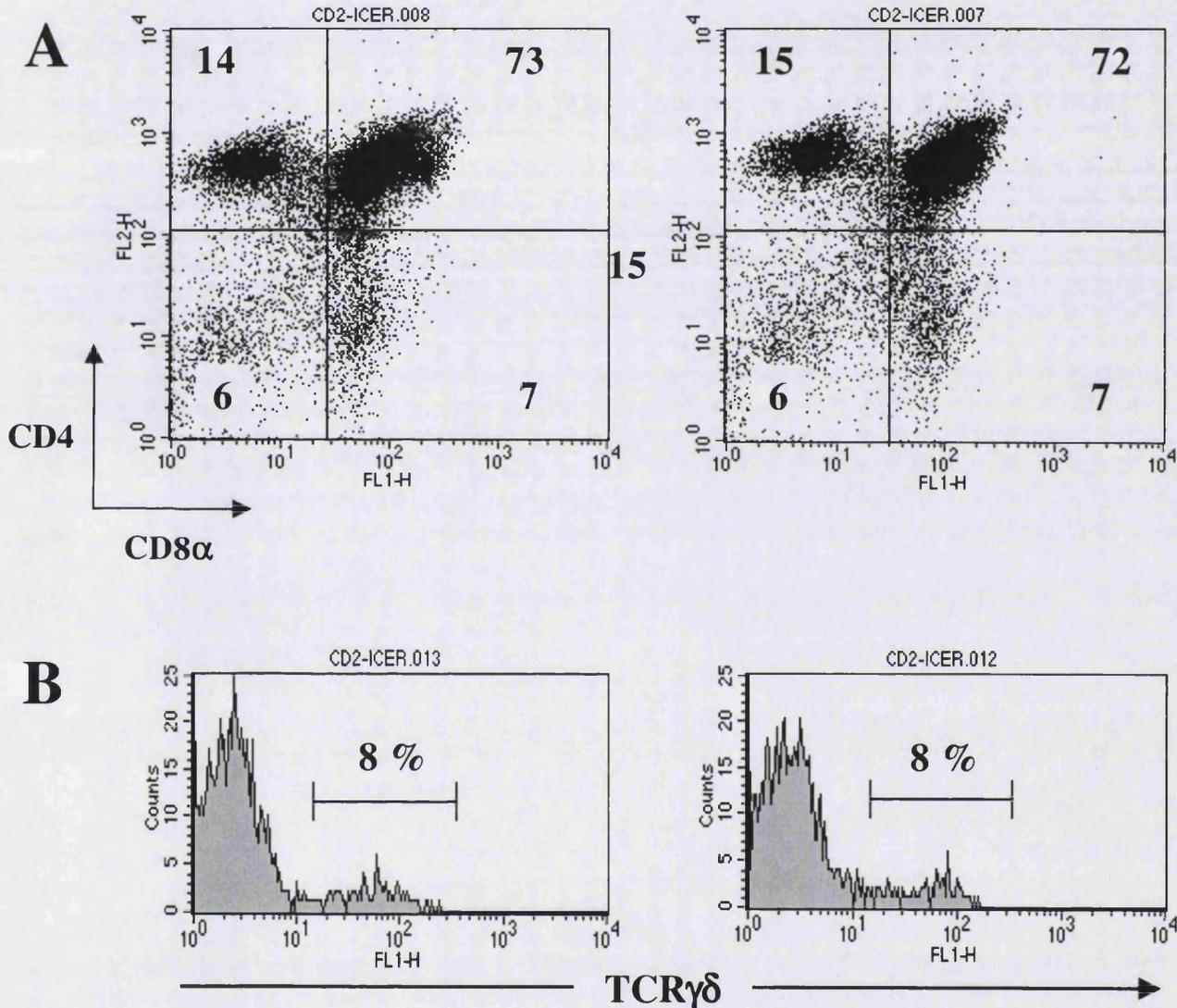


Figure 33 : FACS analysis of thymocyte development in CD2-ICER transgenic mice.

(A) CD8 *versus* CD4 plots for thymocytes from CD2-ICER transgenic (Tg) and non-transgenic (control) mice.

(B) TCR $\gamma\delta$ histograms, pre-gated on CD4⁻ CD8⁻ thymocytes from CD2-ICER transgenic (Tg) and non-transgenic (control) mice.

$\gamma\delta$ T cells from both the lymph nodes and the spleen expressed high levels of ICER; in contrast, CD4+ SP or CD8+ SP $\alpha\beta$ cells from those tissues did not express ICER to any significant level - **Figure 34**.

A slight increase in ICER expression was observed in activated $\alpha\beta$ T cells but this level was still at least six-fold lower than that observed in peripheral $\gamma\delta$ subsets. B cells and macrophages from the lymph nodes also failed to express ICER above background levels.

These data confirm that after the DN4 stage of thymocyte development ICER expression is restricted to the $\gamma\delta$ T cell lineage in both the thymus and in the peripheral lymphoid organs.

We extended our analysis of ICER expression to less-well characterised peripheral $\alpha\beta$ and $\gamma\delta$ T cell populations. Specifically, we examined various subsets of intestinal intraepithelial lymphocytes (IELs).

Traditionally, IEL subsets have been divided into those that express either $\alpha\beta$ or $\gamma\delta$ TCR. Analysis of the TCR $\gamma\delta$ (+) subset has identified cells that are either not expressing CD8 α and CD8 β (TCR $\gamma\delta$ DN) or that express CD8 α as a homodimer (TCR $\gamma\delta$ CD8 $\alpha\alpha$). Likewise, TCR $\alpha\beta$ (+) IELs can be sub-divided into (TCR $\alpha\beta$ CD8 $\alpha\alpha$) cells or cells that express CD8 α as a heterodimer with CD8 β (TCR $\alpha\beta$ CD8 $\alpha\beta$).

The analysis of ICER transcripts in these four IEL populations - **Figure 35** - revealed that consistent with $\gamma\delta$ T cells from the lymph node and spleen, both the (TCR $\gamma\delta$ DN) and the (TCR $\gamma\delta$ CD8 $\alpha\alpha$) IEL subsets expressed a significant amount of ICER transcript. However, unlike all other mature $\alpha\beta$ T cell populations, the (TCR $\alpha\beta$ CD8 $\alpha\alpha$) IEL subset had an expression level of ICER comparable to that observed for all the various $\gamma\delta$ T cell populations that were studied. In contrast, (TCR $\alpha\beta$ CD8 $\alpha\beta$) IELs only expressed ICER at very low levels.

These data demonstrate that $\gamma\delta$ IEL populations express ICER at a level consistent with $\gamma\delta$ T cells from the thymus, lymph node and spleen. Moreover, according to the data presented in the next paragraph (3.8), which establishes that ICER expression characterises the "lineage" of a T cell rather than the type of TCR that it may express, they suggest that the (TCR $\alpha\beta$ CD8 $\alpha\alpha$) IEL subset is " $\gamma\delta$ -like" with respect to ICER expression.

ICER / GAPDH

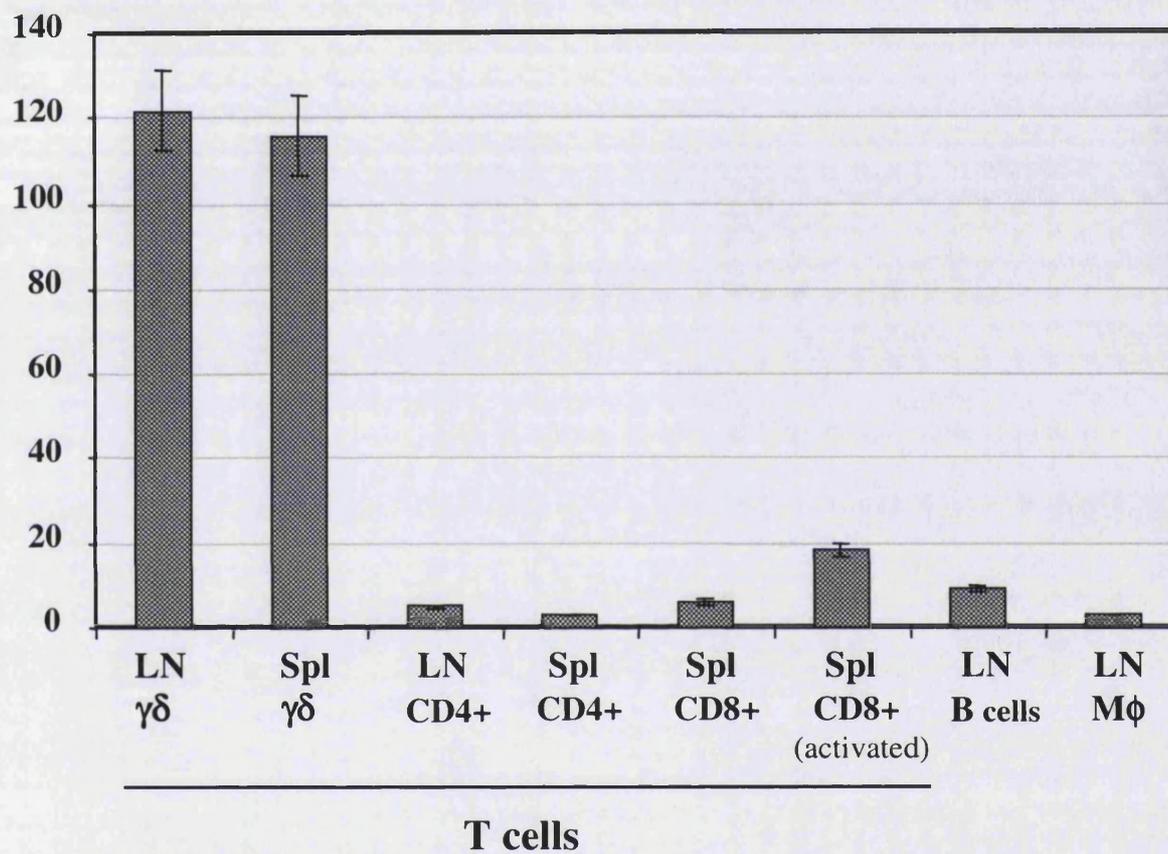


Figure 34: Real-time PCR expression profile for ICER in lymph nodes (LN) and spleen (Spl) of WT mice.

ICER mRNA was quantified in each FACSsorted lymphocyte subset. (n=3)
Parallel real-time PCR reactions for GAPDH were used for normalisation.

ICER / GAPDH

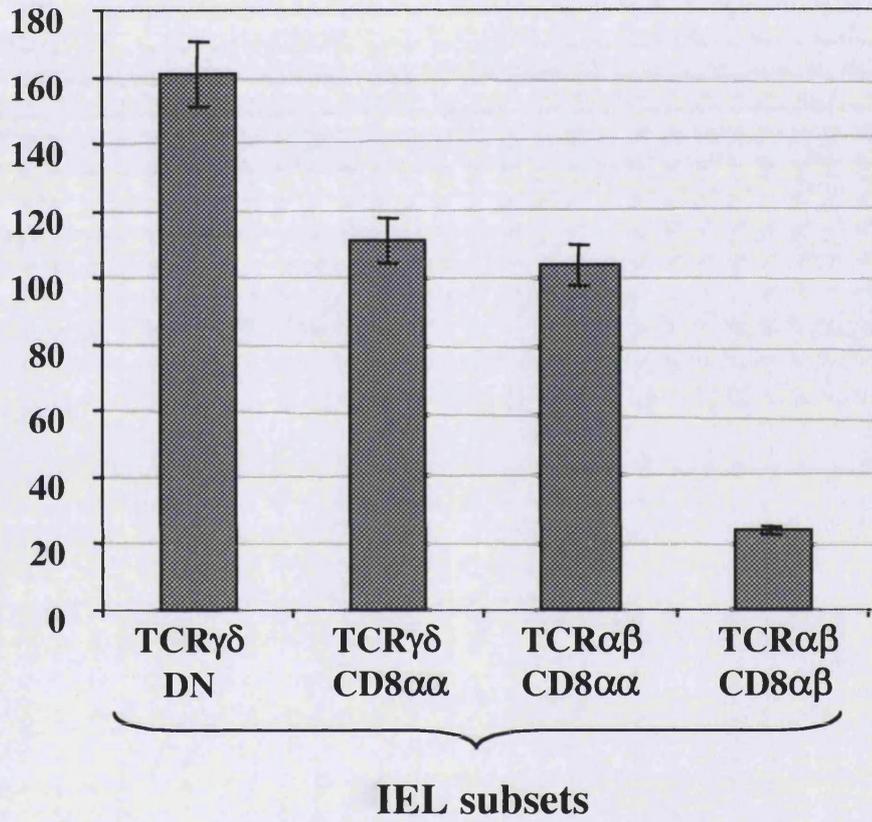


Figure 35 : Real-time PCR expression profile for ICER in intra-epithelial lymphocytes (IEL) of WT mice.

3.8 ICER expression in mouse mutants for TCR

The results presented before demonstrated that after the DN4 stage of thymocyte differentiation ICER expression is regulated in a $\gamma\delta$ T cell lineage-specific manner, both in the thymus and in peripheral lymphoid tissues. To investigate whether this expression was dependent on the TCR isotype, we analysed ICER expression in mouse mutants for TCR.

On one hand, we examined $\text{TCR}\delta^{-/-}$ and $\text{TCR}\alpha^{-/-}$ mice, which completely lack $\text{TCR}\gamma\delta$ and $\text{TCR}\alpha\beta$, respectively. On the other hand, we studied models where the development of either the $\alpha\beta$ or $\gamma\delta$ T cell lineage has been demonstrated to be directed by the opposite type of TCR (TCR HY transgenic and $\text{TCR}\beta^{-/-}$ mice).

ICER expression profiles in the thymus of both $\text{TCR}\delta^{-/-}$ and $\text{TCR}\alpha^{-/-}$ mice are presented in Figure 36. They are largely comparable to the WT thymus profile (Figure 27), if we obviously exclude the populations that are absent in these mutant mice ($\gamma\delta$ thymocytes in $\text{TCR}\delta^{-/-}$ and SP cells in $\text{TCR}\alpha^{-/-}$, see Figure 33A). These similarities imply that: 1) $\text{TCR}\gamma\delta$ is not involved in ICER expression in pre-T cells (*a priori*, one could imagine a low, almost undetectable by FACS, level of $\text{TCR}\gamma\delta$ being responsible for ICER expression in DN subsets); 2) $\text{TCR}\alpha\beta$ is not required to down-regulate ICER expression in DP/SP thymocytes.

Therefore, ICER expression seems to be TCR-independent. Moreover, the analysis of TCR HY transgenic and $\text{TCR}\beta^{-/-}$ mice provided evidence that ICER expression segregates with the lineage, rather than with the TCR isotype.

TCR HY transgenic male mice have a mixed population of $\alpha\beta$ TCR transgene positive T cells in their lymph nodes. Those that express CD8 are considered to be conventional MHC class I restricted $\alpha\beta$ lineage T cells. However, the smaller population of CD8(-) $\text{TCR}\alpha\beta$ (+) cells have been shown to display characteristics of the $\gamma\delta$ T cell lineage (see Introduction, 3.4).

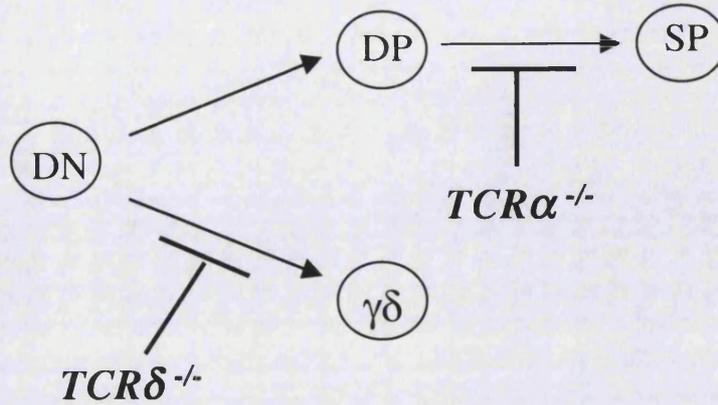
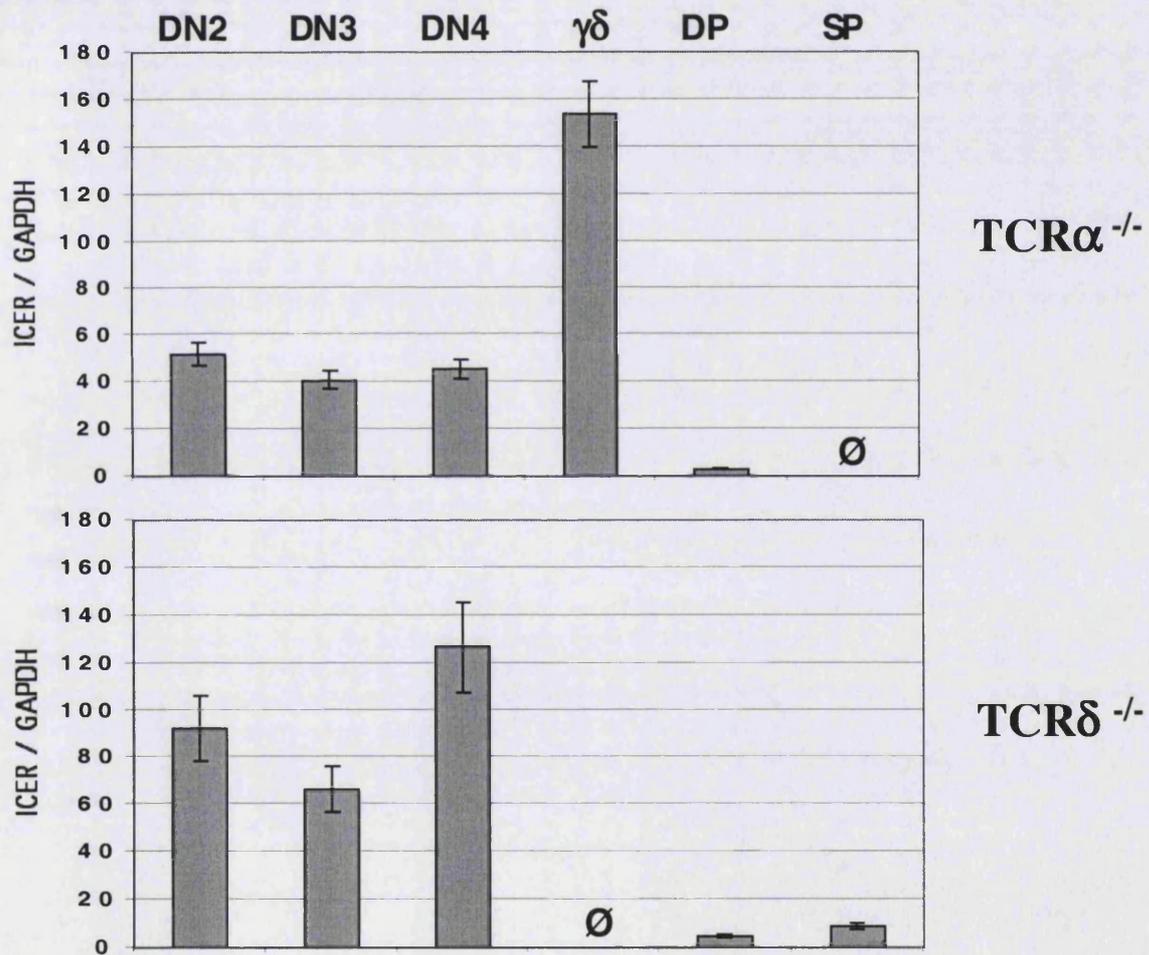
A**B**

Figure 36 : ICER expression in $TCR\alpha$ deficient and $TCR\delta$ deficient mice.

(A) Representation of T cell developmental blocks in $TCR\alpha^{-/-}$ and $TCR\delta^{-/-}$ mice.

(B) Real-time PCR for ICER in thymocyte subsets of $TCR\alpha^{-/-}$ and $TCR\delta^{-/-}$ mice.

When the expression of ICER was analysed in these $\gamma\delta$ lineage T cells that express an “inappropriate” $\alpha\beta$ TCR, a high level of transcript, almost comparable to that expressed in conventional $\gamma\delta$ T cells, was observed – **Figure 37A**. This was in contrast to the low level of ICER expression that was seen in the CD8(+)TCR $\alpha\beta$ (+) $\alpha\beta$ lineage cells.

In TCR $\beta^{-/-}$ mice, $\alpha\beta$ lineage-committed DP thymocytes are observed in significant numbers even though a pre-TCR or TCR $\alpha\beta$ cannot be formed. These β -negative DP cells are thought to have been selected by TCR $\gamma\delta$ as they are lost in TCR $\beta^{-/-}$ x TCR $\delta^{-/-}$ animals (see Introduction, 3.4).

As seen in **Figure 37B**, when ICER expression was analysed in these $\alpha\beta$ lineage cells that had received signals only from TCR $\gamma\delta$, a negligible level of transcript, comparable to that seen in wild type DP cells, was observed.

Taken together, these results demonstrate that ICER expression is not regulated by the expression of either the $\gamma\delta$ or the $\alpha\beta$ TCR. In contrast, ICER expression appears to be a characteristic of cells that have adopted a “ $\gamma\delta$ -like” T cell fate, regardless of whether they have employed either the $\gamma\delta$ or $\alpha\beta$ TCR to achieve this.

3.9 Lineage potential of pre-T cells expressing different levels of ICER

ICER is expressed in DN thymocytes, which are progenitors of both $\alpha\beta$ and $\gamma\delta$ lineages, and then becomes restricted to mature $\gamma\delta$ cells. We therefore explored the possibility of the expression of ICER specifically marking $\gamma\delta$ -precursors within the DN compartment. We took advantage of previously generated CREM-LacZ mice (Blendy et al., 1996), which we obtained from Prof. Gunther Schutz (Heidelberg, Germany). In these mice, the expression of reporter gene LacZ, coding for the enzyme β -galactosidase, is driven by the promoter of CREM/ICER (**Figure 38A**).

Since we had shown (**Figure 26**) that the dominant isoform of CREM in the thymus is ICER, we could use β -galactosidase expression as an indirect measurement of ICER protein expression. (We’ll refer to it as “CREM/ICER-LacZ protein”).

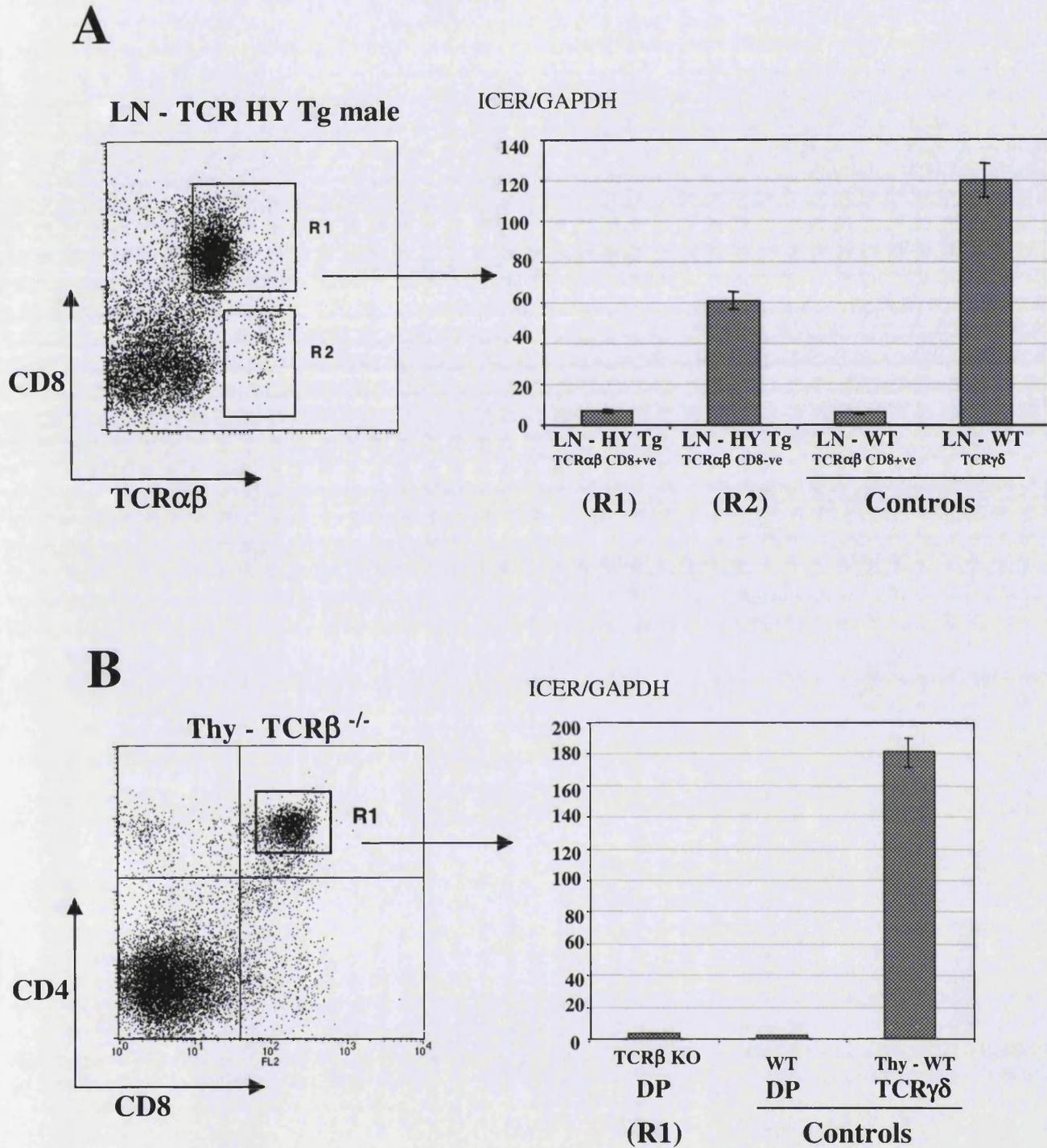


Figure 37 : ICER expression in TCR $\alpha\beta$ transgenic and TCR β KO mice.

FACS plots and real-time PCR for ICER in T cells isolated from:

(A) lymph nodes of TCR HY transgenic male mice (or WT controls).

(B) thymus of TCR $\beta^{-/-}$ mice (or WT controls).

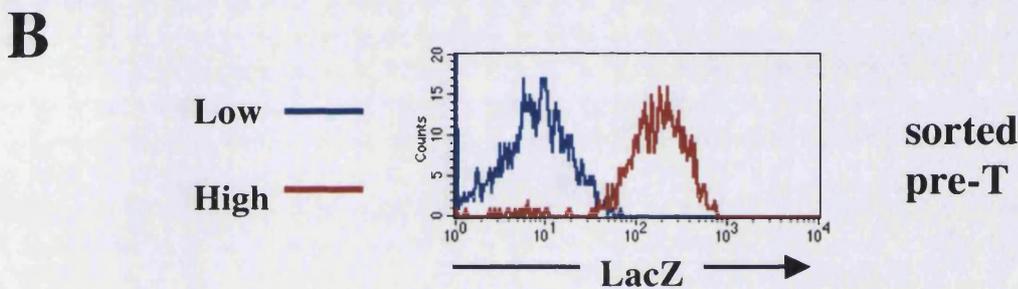
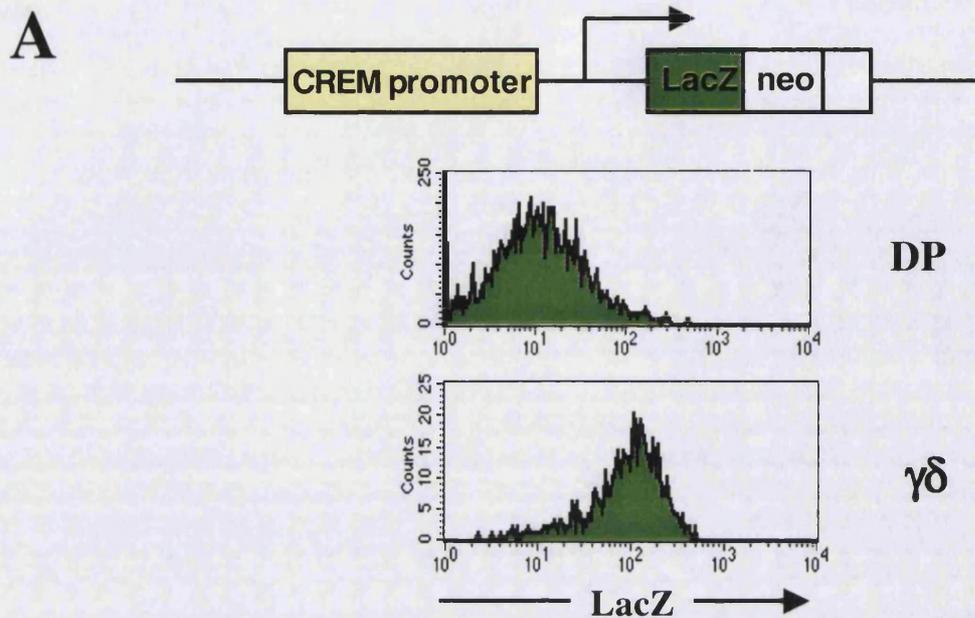
The activity of reported gene LacZ was assessed by intracellular staining with fluorescein di-galactopyranoside, a substrate for β -galactosidase which emits fluorescence once cleaved. The FACS profiles for DP and $\gamma\delta$ thymocytes from CREM-LacZ mice are shown in Figure 38A. As expected (from 3.1), $\gamma\delta$ cells are essentially positive for "CREM/ICER-LacZ protein", in contrast with DP cells.

To address the relationship between ICER expression in pre-T cells and their lineage potential, DN thymocytes were sorted as either "low" or "high" expressors of CREM/ICER-LacZ protein (Figure 38B). These distinct populations were used to colonise foetal thymic stroma in RTOC systems. If the initial hypothesis was correct, ICER-high cells should be biased towards the $\gamma\delta$ lineage.

After 6 days in RTOC, both early (DN2) and late (DN4) pre-T cells produced surprising results: cells expressing high ICER-LacZ levels generated a bigger proportion of $\alpha\beta$ lineage cells (DP) than their ICER-low counterparts - Figure 38C. The ratio $\alpha\beta/\gamma\delta$ (absolute numbers) increased from 2.9 to 5 (DN2 RTOC) and from 5.4 to 7 (DN4 RTOC) between low and high expressors of CREM/ICER-LacZ. However, this increase mirrored almost perfectly the increase in total cellularity of the RTOCs (see last row of tables of Figure 38C). This suggests that the variations in the ratio $\alpha\beta/\gamma\delta$ are due to the differences in the specific proliferation rates of $\alpha\beta$ and $\gamma\delta$ cells (the first being more proliferative than the second; see Introduction, 2.3). Therefore, the lineage potential of ICER-low and ICER-high cells seems to be intrinsically identical.

This conclusion contradicts the initial hypothesis; ICER expression in DN thymocytes does not mark $\gamma\delta$ -precursors and is likely to be governed by a distinct mechanism to the one operating in mature T cells (responsible for the restricted expression in the $\gamma\delta$ lineage).

The extensive generation of $\alpha\beta$ cells by ICER-high pre-T cells, although maybe paradoxical in the context of this section, becomes clearer in that of section 4, where the relationship between ICER expression and β -selection is addressed.



C

DN2 (RTOC, 6 days)			DN4 (RTOC, 6 days)		
CREM-LacZ level	Ratio $\alpha\beta$ / $\gamma\delta$	RTOC cellularity	CREM-LacZ level	Ratio $\alpha\beta$ / $\gamma\delta$	RTOC cellularity
Low	2.93	95,000	Low	5.42	144,000
High	5.04	156,000	High	6.99	177,000
Ratio High / Low	1.72	1.64	Ratio High / Low	1.29	1.23

Figure 38 : Lineage potential of pre-T cells expressing different levels of ICER. (A) LacZ protein staining for DP and $\gamma\delta$ thymocytes of CREM-LacZ mice. (B) LacZ levels for pre-T cells sorted as low / high expressors of CREM/ICER. (C) Analysis of 6 day-RTOCs of sorted DN2 and DN4 cells expressing low / high levels of CREM/ICER-LacZ.

4 ICER, β -selection and $\gamma\delta$ thymocyte development

ICER was one of the genes identified as induced in RAG-1^{-/-} pre-T cells by an anti-CD3 mAb stimulus (section 2.2, Table 5). Posterior data suggested that ICER expression in WT pre-T cells is pre-TCR dependent (2.3). These findings were very interesting as a complement to the studies on ICER and $\alpha\beta$ *vs.* $\gamma\delta$ lineage commitment, in particular in light of the results of 3.9, which showed that pre-T cells expressing high levels of ICER (protein) could generate large amounts of $\alpha\beta$ thymocytes. The connection between ICER expression and β -selection is further examined in this section.

4.1 ICER expression in pre-T cells undergoing β -selection

Signalling through the pre-TCR in DN3 thymocytes leads to CD25 down-regulation, which defines progression to the following stage of development, DN4.

In DN3 and DN4 thymocytes, like in $\gamma\delta$ cells (Figure 26), ICER is the major isoform of CREM expressed (data not shown). This validates the use of the activity of the LacZ reporter gene as a measurement of ICER expression in CREM-LacZ mice.

The very low surface levels of pre-TCR in these DN subsets can nevertheless be detected by the liposome technique described in 3.3. Using that procedure on DN thymocytes of CREM-LacZ mice, the profiles shown in **Figure 39B** were obtained. As expected, the majority (85%) of DN3 cells express low levels of surface TCR β , whereas most (65%) of DN4 thymocytes show high levels of pre-TCR expression.

Interestingly, similar distributions were detected for the ICER-LacZ protein (by staining with FDG) – **Figure 39A**. (Note: the ‘positive’ gate for LacZ was defined by comparing negative and positive controls; data not shown).

Thus, the expression of ICER-LacZ protein correlates with that of pre-TCR over the DN3 → DN4 transition.

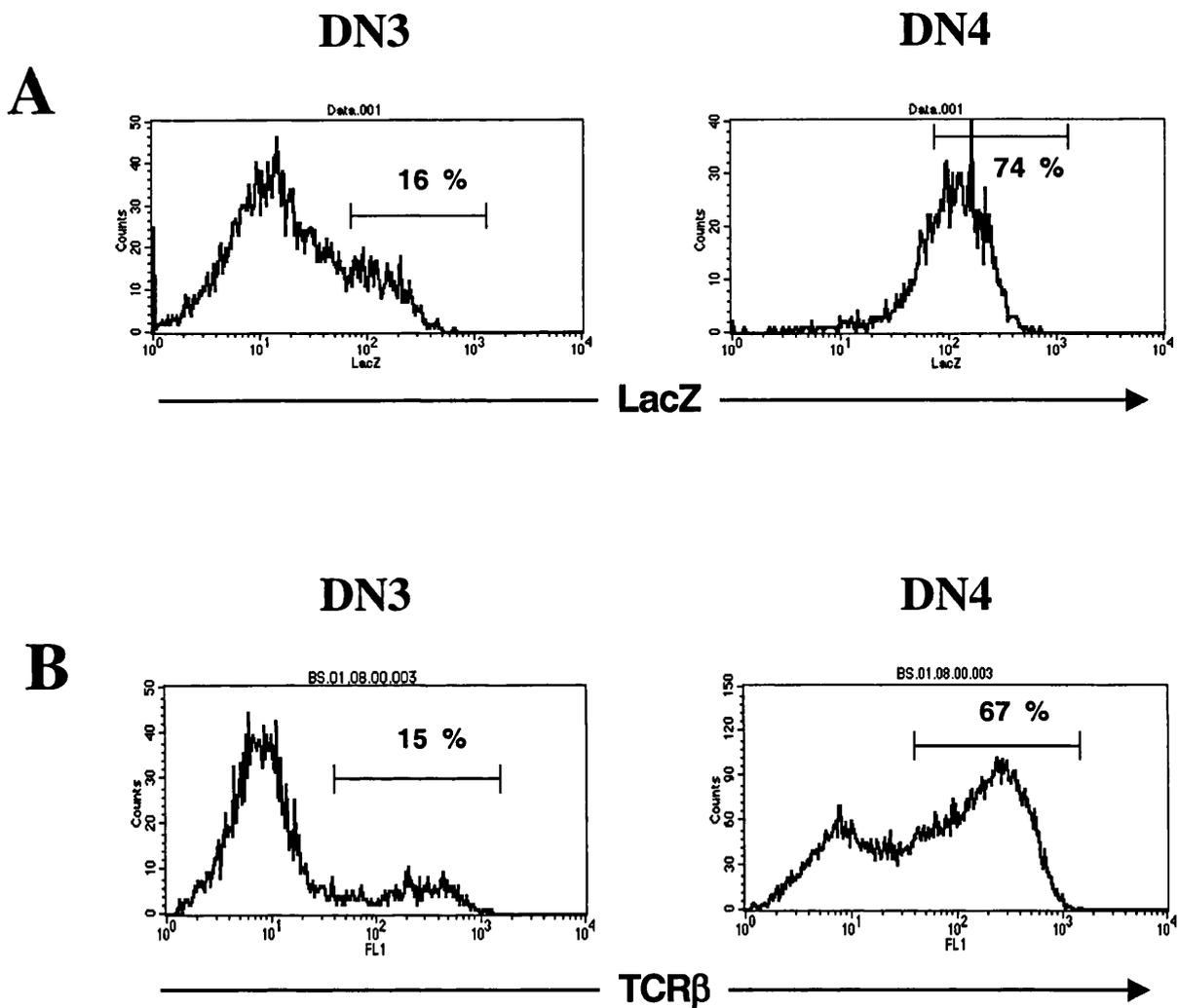


Figure 39: ICER-LacZ protein expression in pre-T cells of CREM/ICER-LacZ mice.

- (A) LacZ protein staining for DN3 and DN4 cells of CREM-LacZ mice.
- (B) Surface TCRβ-liposome staining for DN cells of CREM-LacZ mice.

4.2 ICER expression in pre-T cells with impaired pre-TCR signalling

As a first approach to investigate the role of the pre-TCR in ICER expression in pre-T cells, the transcription of ICER was analysed in the thymus of WT and several mutant mice with deficient pre-TCR signalling. The semi-quantitative results of Figure 20 were re-assessed by real-time (quantitative) PCR.

As shown in Figure 40, DN3 thymocytes (first population to express the pre-TCR in a normal thymus) from $\text{TCR}\beta^{-/-}$, $\text{pT}\alpha^{-/-}$, $\text{p56Lck}^{-/-}$ and $\text{RAG-1}^{-/-}$ mice, all of which lack essential components of the pre-TCR signalling machinery, express ICER at levels at least 10-fold lower than WT DN3 thymocytes. This genetic evidence suggests that full expression of ICER in pre-T cells is dependent on effective pre-TCR signalling.

This effect on ICER expression is specific to the pre-TCR as a signalling complex, as DN3 cells isolated from mice deficient for IL-7R or $\text{TCR}\gamma\delta$ express normal (or slightly higher, in the case of $\text{TCR}\delta^{-/-}$) levels of ICER mRNA.

4.3 Induction of ICER expression by signalling through the CD3 complex

4.3.1 ICER expression in response to anti-CD3 ϵ mAb

The original piece of data that put ICER in the context of β -selection was the induction of its expression in $\text{RAG-1}^{-/-}$ pre-T cells by anti-CD3 mAb stimuli (Figure 20), which mimic pre-TCR triggering. That *in vivo* induction was quantified by real-time PCR (Figure 41A).

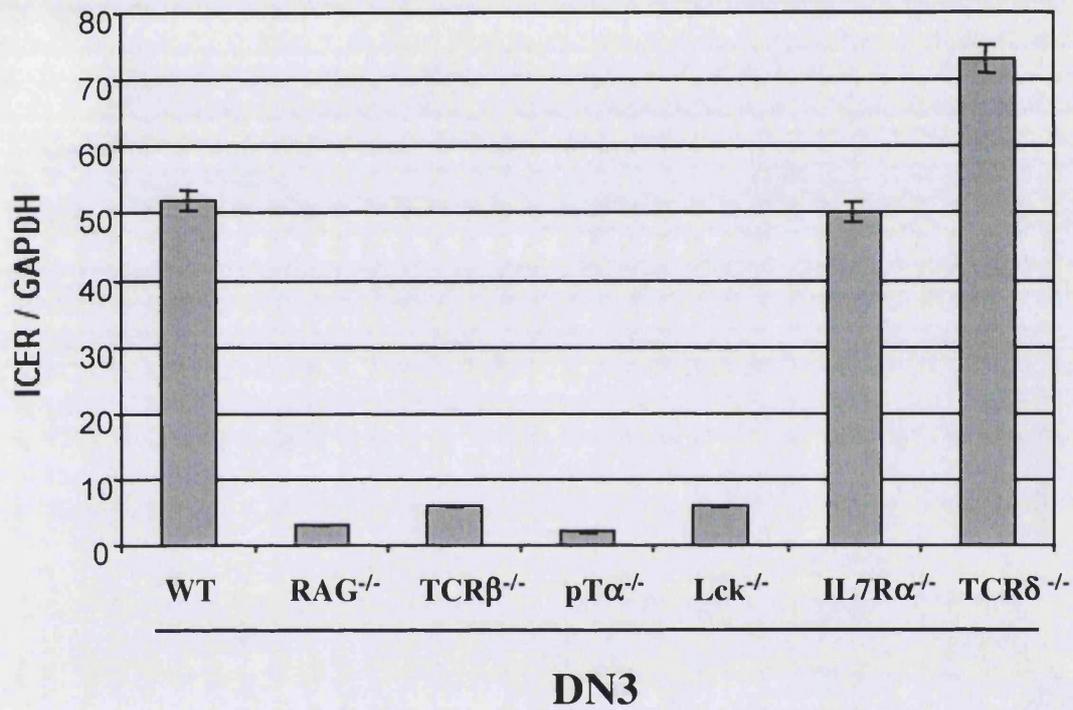


Figure 40 : ICER mRNA expression in pre-T cells of mice with deficient pre-TCR signalling. Real-time PCR for ICER in DN3 thymocytes isolated from RAG-1^{-/-}, TCRβ^{-/-}, pTα^{-/-} and Lck^{-/-} mice. Results for WT control, IL7Rα^{-/-} and TCRδ^{-/-} DN3 cells are also shown.

ICER mRNA, which was not expressed in untreated RAG(-) cells, came up after 1.5 hr (Figure 20) and peaked after 3 hr of stimulation, when it achieved a level approximately 25-fold higher than in the NT control (Figure 41A). After 24 hr, the expression was down to 1/4 of its 3 hr-peak. This is consistent with the early kinetics of induction described for ICER in other systems, such as the nervous system (reviewed in Sassone-Corsi, 1998).

As mentioned in 2.3, ICER's kinetics of induction was faster than EGR-1, a transcription factor previously implicated in "β-selection". Such a rapid induction (much more immediate than any phenotypic cellular changes) suggests that ICER is a direct target of pre-TCR / CD3 signalling.

The treatment of RAG-1^{-/-} foetal thymocytes *in vitro* with the same monoclonal antibody produced similar results (Figure 41B). After 5 days in FTOC, the RAG-1^{-/-} DN thymocytes had produced a mixed culture (approximately 1:1) of DN and DP cells (data not shown). The DN thymocytes of this final (stimulated) stage showed a notable expression of ICER (79 units of ICER / GAPDH), in stark contrast with the untreated cells (3 units). Unlike the stimulated DN cells, the DP thymocytes produced after 5 days did not express ICER at significant levels (5 units). This highlights the transient character of the induction of ICER, as well as the difference between immature and mature cells of the αβ lineage (addressed in section 3).

The up-regulation of CREM-LacZ expression during DN3 → DN4 transition could also be simulated *in vitro* (RTOC) by treating sorted DN3 thymocytes isolated from CREM-LacZ mice with anti-CD3ε antibody (Figure 41C). After 15 hr of stimulation, 62% of the cells expressed high levels of CREM-LacZ, contrasting with 16% of the initial (t=0 hr) sorted DN3 cells and 18% of untreated control DN3 cells (also cultured for 15 hr, but in the absence of antibody). This result further strengthens the link between pre-TCR signalling and ICER expression.

Collectively with the observations of 4.2 (Figure 40), these findings demonstrate that pre-TCR signalling is both sufficient and necessary for ICER expression in DN3/4 thymocytes.

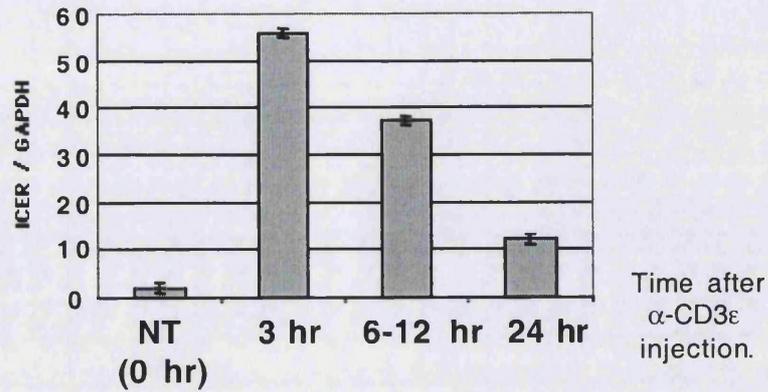
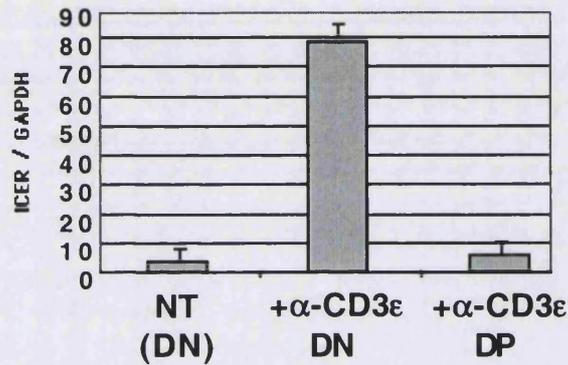
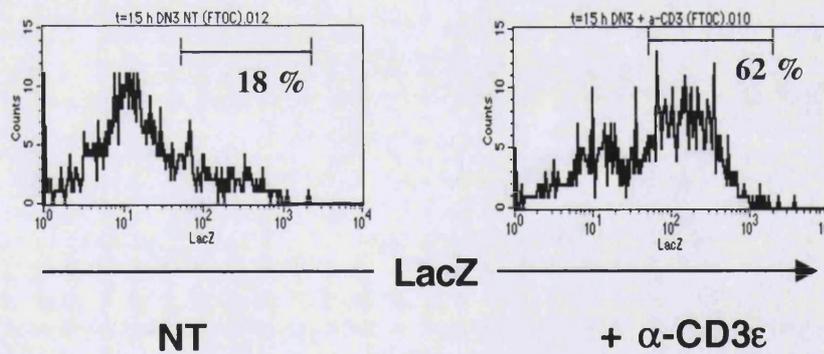
A**B****C**

Figure 41 : Induction of ICER expression by anti-CD3 mAb stimuli.

(A) Real-time PCR for ICER on adult RAG-1^{-/-} thymocytes treated *in vivo* with anti-CD3 ϵ mAb (experiment of Figures 17-20, samples of Figure 20).

(B) Real-time PCR for ICER on foetal RAG-1^{-/-} thymocytes treated *in vitro* with anti-CD3 ϵ mAb. After 5 days in FTOC, DN and DP cells were FACSsorted.

(C) LacZ protein staining for sorted DN3 thymocytes of CREM/ICER-LacZ mice, treated (or not, NT) *in vitro* (15 hour RTOC) with anti-CD3 ϵ mAb.

4.3.2 Involvement of the MAPK pathway

Since the results presented before exposed a link between the pre-TCR and the induction of ICER expression in DN3 thymocytes, we tried to dissect the signalling pathway(s) downstream of the pre-TCR necessary for such induction.

For that we used specific chemical inhibitors of known pathways, in a similar system to the one presented involving anti-CD3 ϵ stimulation, but this time in cell suspension rather than in organ culture.

DN3 thymocytes sorted as expressing low levels of CREM-LacZ (0% above threshold depicted in **Figure 42A**) were incubated for 6 hr with (40 μ g/ml) or without anti-CD3 ϵ antibody. Untreated cells showed only 2% of spontaneous up-regulation of CREM-LacZ protein, whereas 36% of stimulated cells were now "positive" for LacZ staining. Taking this induction as reference (100%) for the system, the pre-incubation of cells for 2 hr with specific drugs had the following effects: 39% inhibition with 10 μ M MEK1 inhibitor PD98059, 61% with 25 μ M of the same compound, and no effect with 5 μ M of the PI-3 kinase inhibitor Ly294002 (**Figure 42B**). (Concentrations of chemicals were chosen according to Davies *et al.* (Davies *et al.*, 2000).)

The particular effect of the PD inhibitor was not due to cell death, as it can be inferred from **Figure 42C**.

Roche compound RO-8220, which blocks both MAPK and PKC signalling pathways, also had an inhibitory effect (53% at 2.5 μ M) on anti-CD3 ϵ - mediated induction of CREM-LacZ expression (data not shown). Furthermore, substitution of anti-CD3 ϵ antibody by phorbol ester PDBu (20 ng/ml), which activates both PKC (directly) and MAPK (via RasGRP) pathways, was able to induce LacZ expression up to 72% of the level achieved by anti-CD3 ϵ .

The extent of the contribution of the PKC pathway to this induction is difficult to assess due to lack of specific inhibitors that do not affect the MAPK pathway. The results with PD98059, though, being a MEK1 inhibitor with no cross-effect with PKCs, suggest that the major input to the CREM-LacZ induction by CD3 triggering comes from the MAPK pathway.

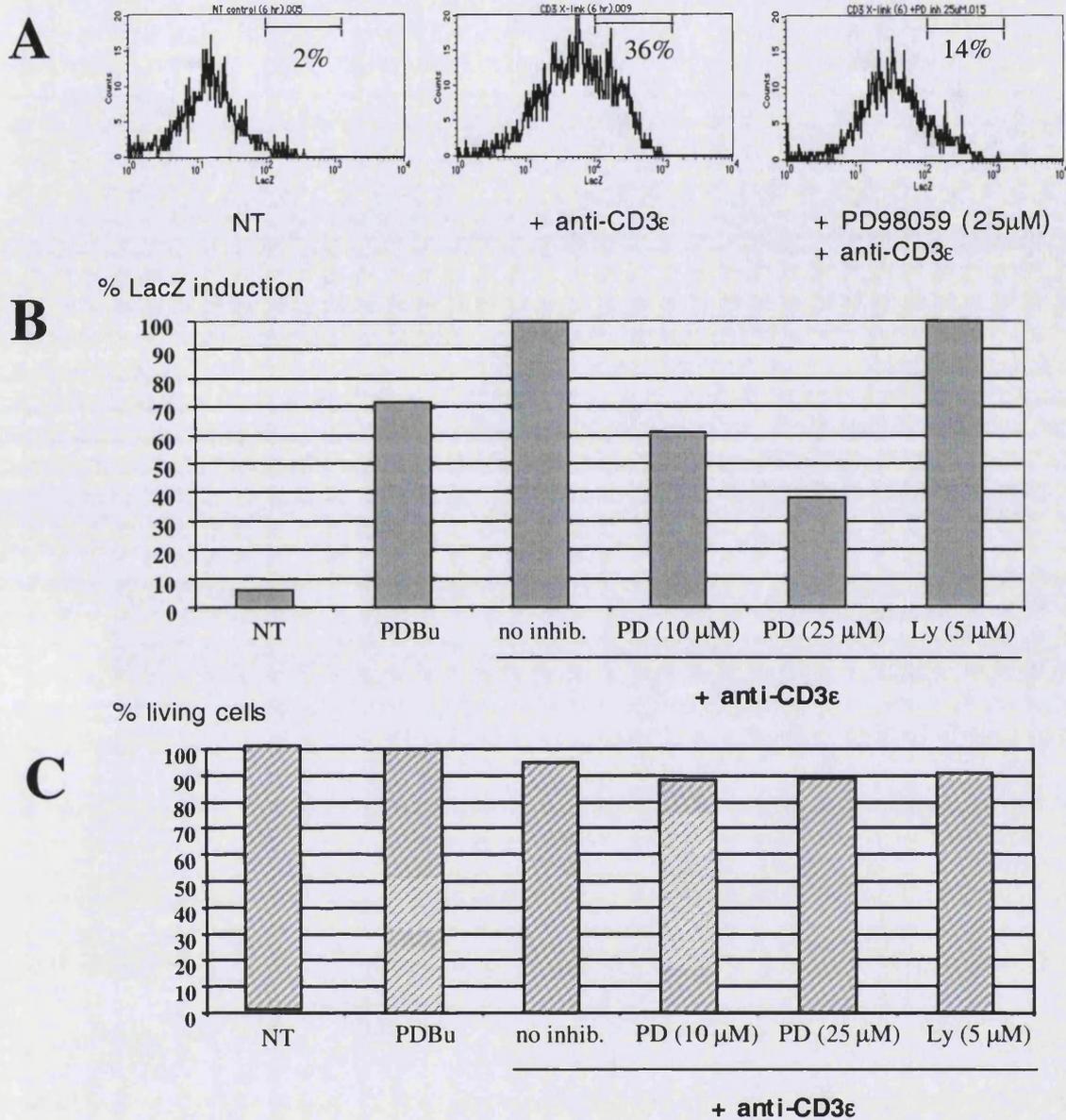


Figure 42 : Effect of MAPK pathway inhibitors on the induction of ICER-LacZ protein expression by anti-CD3 mAb stimuli.

DN3 thymocytes of CREM-LacZ mice were FACSsorted as LacZ negative (0% in gate of Fig. A), pre-incubated for 2 hr with (or without) inhibitors, and cultured for 6 hr in the presence (or absence) of either a PDBu or anti-CD3 ϵ mAb stimulus.

(A) Effect of MEK1 inhibitor PD98059 on CREM-LacZ induction by α -CD3 Ab.

(B) Quantification of the induction with/without inhibitors, considering the induction by α -CD3 ϵ mAb in the absence of inhibitors as 100%.

(C) Control for cell survival, as seen on FSC/SSC plots, in samples from (B).

4.4 Analysis of the DN compartment of CREM / ICER deficient mice

Since ICER expression in DN3 thymocytes is a product of pre-TCR signalling, one could hypothesise that ICER could play an active role in β -selection, that is, it could be required for DN3 \rightarrow DN4 transition.

To assess the impact of ICER on this developmental process, we analysed the DN compartment of CREM / ICER - deficient mice (obtained from Prof. Paolo Sassone-Corsi). As presented in Figure 43, the mutant mice showed a normal distribution of DN subsets, and in particular, no perturbation of the DN3/DN4 ratio was observed.

This suggests that ICER does not play a non redundant role in β -selection. Such conclusion is consistent with the normal development of $\alpha\beta$ cells in ICER(-) mice (3.4).

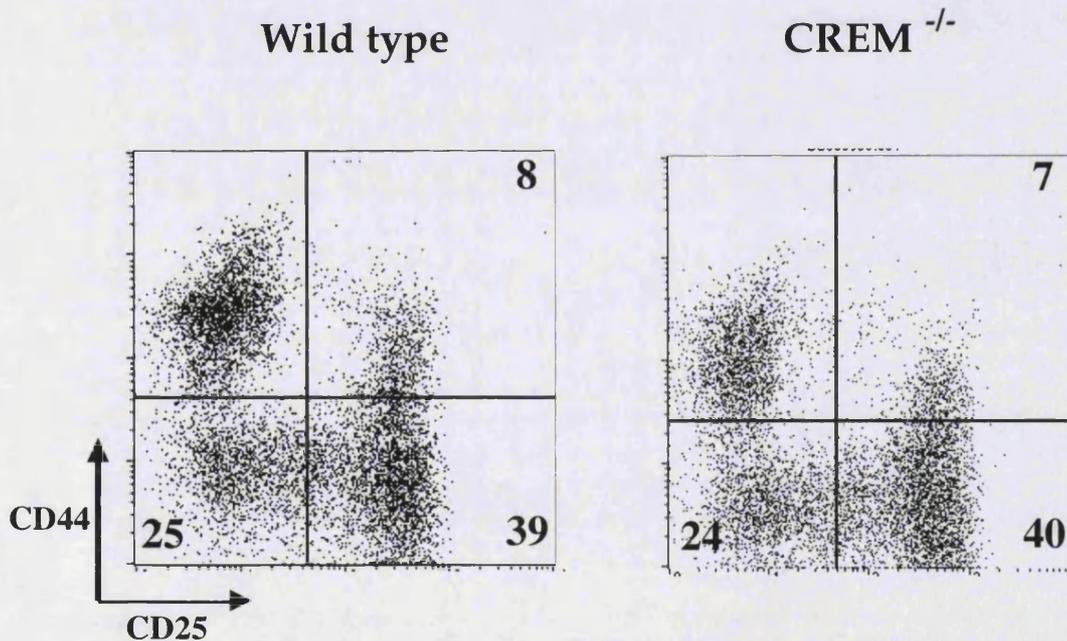


Figure 43 : FACS plots (CD25 vs. CD44) of WT and CREM-deficient DN thymocytes. (Pre-gated on CD4⁻ CD8⁻ TCR $\gamma\delta$ ⁻ cells.)

4.5 Analysis of pre-T cells expressing different levels of ICER-LacZ protein

4.5.1 Developmental potential

The correlation between ICER-LacZ protein and surface pre-TCR levels in DN3/DN4 thymocytes of CREM/ICER-LacZ mice raised the possibility of ICER marking β -selected cells. To address this hypothesis, DN3 and DN4 thymocytes were sorted from CREM-LacZ mice, according to their intensities of LacZ staining, and they were allowed to develop in FTOC/ROTC.

The “low” and “high” populations were defined as the 25% lowest / highest intensities of LacZ staining. If ICER-high thymocytes were indeed biased towards β -selected cells, they should differentiate faster / more extensively to the DP stage.

In 6 days, 20,000 sorted (ICER) low-expressing DN3 thymocytes generated 71,000 cells, a 3.5-fold increase in cellularity. Of these later cells, the majority remained in the DN compartment (57%), and only very few (7%) ended up in the more mature DP stage (Figure 44A, upper panel).

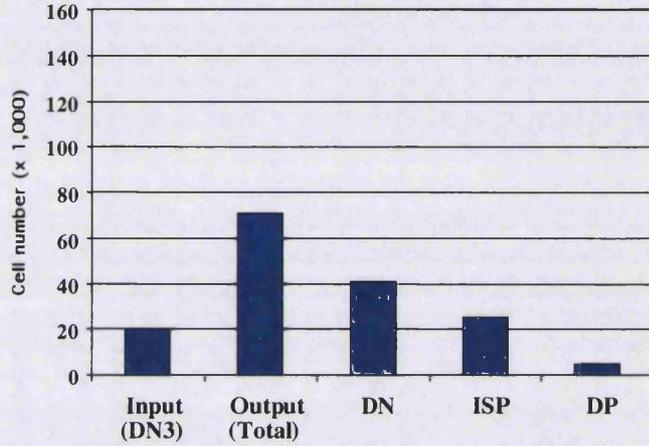
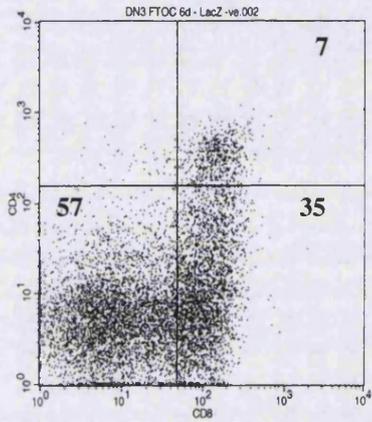
During the same time, the same amount of high-expressing DN3 cells produced a 8-fold increase in cellularity. This difference was fundamentally accounted by the DP subset, which was dominant in this case (45%) (Figure 44A, lower panel)

With the DN4 subset, after 3 days in ROTC, the difference was even more striking: the output / input ratio was 0.7 for low-expressing cells, and 5.9 for high-expressing DN4 thymocytes. (Figure 44B). The most dramatic results with DN4 cells were expected, since cells in that developmental stage should have already been β -selected.

These data suggests immature thymocytes with a considerable difference in the levels of expression of ICER also have distinct developmental potentials, with those cells in which ICER transcription has been up-regulated being faster in differentiating into more mature subsets.

A

DN3 LacZ low



DN3 LacZ high

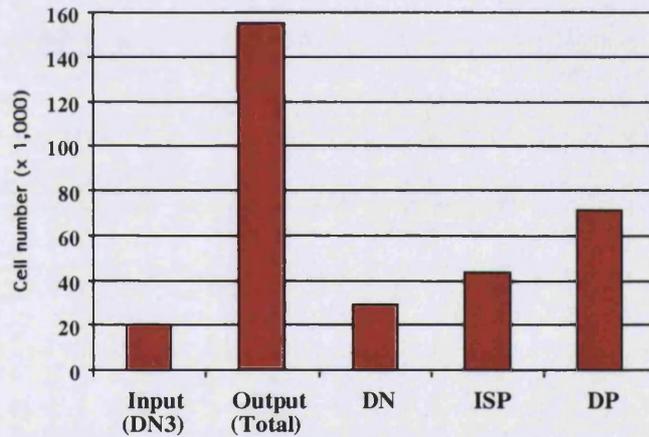
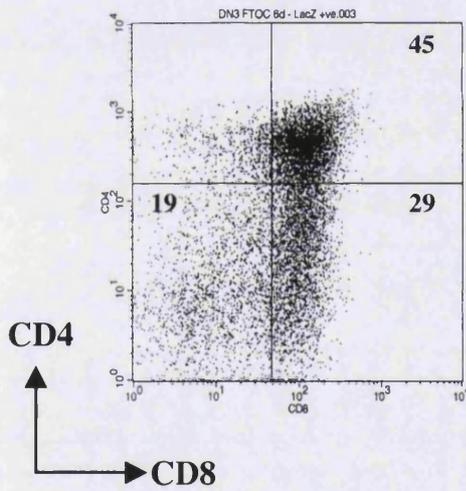
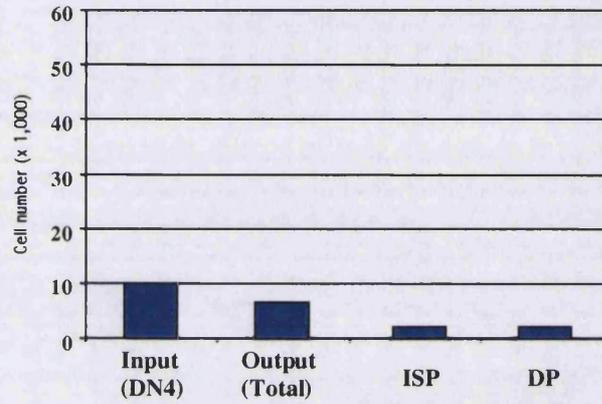
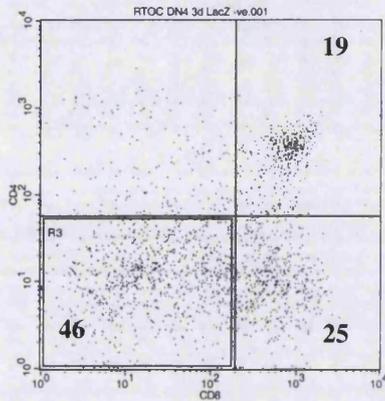


Figure 44 : Developmental potential of pre-T cells expressing different levels of CREM /ICER-LacZ protein.

(A) CD4/CD8 plots and absolute numbers of individual thymocyte subsets in 6-day FTOCs of DN3 cells sorted from CREM-LacZ mice according to their levels of LacZ expression.

B

DN4 LacZ low



DN4 LacZ high

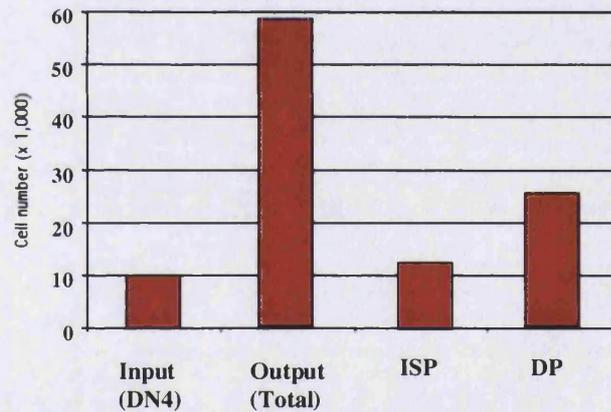
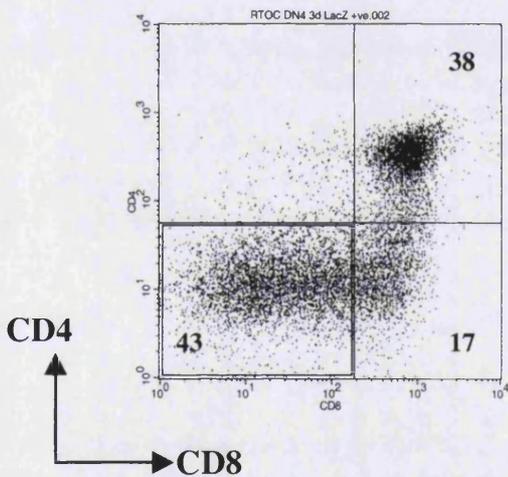


Figure 44 : Developmental potential of pre-T cells expressing different levels of CREM /ICER-LacZ protein.

(B) CD4/CD8 plots and absolute numbers of individual thymocyte subsets in 3-day RTOCs of DN4 cells sorted from CREM-LacZ mice according to their levels of LacZ expression.

4.5.2 Phenotypic analysis

To understand the difference in developmental potential of ICER-low *vs.* high pre-T cells, they were sorted from CREM-LacZ mice and analysed for some cell properties and for the expression of particular proteins and transcripts.

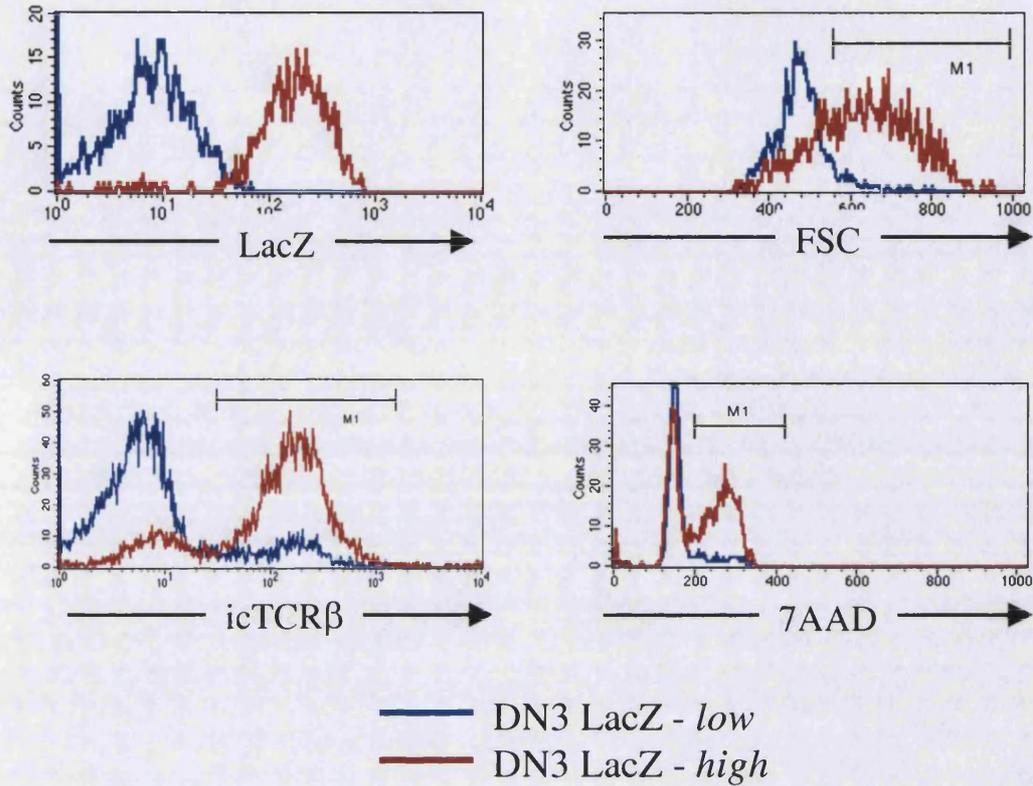
Cell size (forward scatter), cell cycle (7AAD), intracellular TCR β content and surface CD2 expression were estimated by flow cytometry – **Figure 45**. There was a marked contrast between the two populations regarding all those properties: whereas “low” DN3 thymocytes were essentially small, non-cycling, intra-cellular TCR β -negative cells, “high”-expressing thymocytes were large (63%), cycling (52%), intra-cellular TCR β -positive (84%) cells that had up-regulated CD2. All of these characteristics of ICER “high” thymocytes are typical of “ β -selected” cells.

Consistent such interpretation of the previous data, RT-PCR done on mRNAs extracted from the two populations revealed that high-expressing cells were significantly richer in EGR-1 and cyclin D3 transcripts – **Figure 46**.

Thus, the potential of pre-T cells expressing high levels of ICER to develop faster to the DP stage can be accounted by their high levels of pre-TCR expression and consequent up-regulation of genes involved in cell proliferation and differentiation.

Interestingly, whereas ICER mRNA expression increased from DN3-low to DN3-high cells, the opposite scenario was observed in DN4 thymocytes: ICER was in fact down-regulated from “low” to “high” cells. This implies that CREM/ICER-LacZ protein does not follow the same kinetics of ICER mRNA; ICER-LacZ protein down-regulation seems to be delayed (compared to the mRNA), since it only occurs in the DP stage (Figure 38; Figure 39).

In fact, in paragraph 3.3 we had reported that DN4 thymocytes expressing high levels of surface TCR β expressed less ICER transcripts; since LacZ “high” cells are also intracellular TCR β -high (Figure 45, also valid for DN4 cells; data not shown), this is in agreement with their lower levels of ICER mRNA. DN4-high cells are in their majority $\alpha\beta$ -committed thymocytes (see 3.3), which, as such, should down-regulate ICER (in line with the results of section 3).

A**B**

	Forward Scatter (Size)	7AAD	icTCR β	CD2
DN3 LacZ low (3 %)	465.9 (3 %)	155.3 (6 %)	34.8 (13 %)	6.6
DN3 LacZ high (63 %)	610.2 (63 %)	210.1 (52 %)	195.8 (84 %)	14.1

Figure 45 : Phenotypic analysis of DN3 cells expressing different levels of CREM/ICER-LacZ protein .

(A) FACS profiles for DN3 thymocytes expressing low *vs.* high levels of ICER-LacZ: LacZ (re-analysis), forward scatter (size), intracellular TCR β and 7AAD (cell cycle).

(B) Mean intensity / fluorescence for the properties of (A) plus surface CD2 expression, and percentage of cells inside "M1" gates of (A).

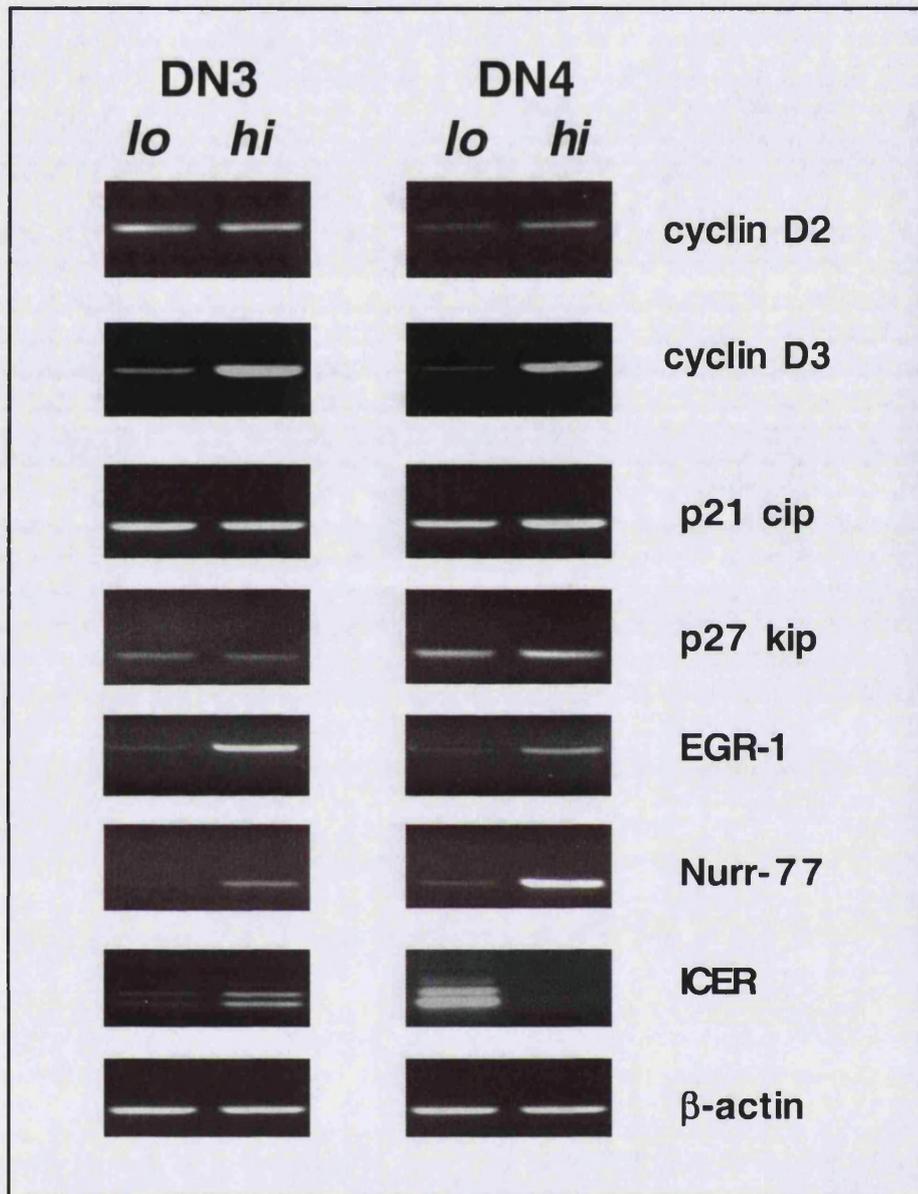


Figure 46: Gene expression in pre-T cells expressing different levels of CREM/ICER-LacZ protein.

RT-PCR for cell cycle genes cyclins D2 and D3, p21 cip and p27 kip; and for transcription factors EGR-1, Nurr-77 and ICER, in DN3 and DN4 cells expressing low *vs.* high levels of CREM/ICER-LacZ protein.

4.6 Gene expression in $\gamma\delta$ thymocytes developing in the absence of β -selected cells

We had established before that ICER acts as a $\gamma\delta$ cell lineage marker, both in the thymus and in the periphery (section 3). The data presented above suggests that ICER expression in pre-T cells is downstream of the pre-TCR. Since $\gamma\delta$ cells derive from pre-T cells, we were interested to investigate if ICER expression in $\gamma\delta$ thymocytes was conditioned by previous expression in their DN precursors. For that, we examined ICER expression in $\gamma\delta$ cells of pre-TCR deficient mice ($\text{TCR}\beta^{-/-}$, $\text{pT}\alpha^{-/-}$), whose pre-T cells are ICER(-) (see Figure 40). We observed a ten-fold reduction in ICER expression between WT and pre-TCR deficient $\gamma\delta$ cells - **Figure 47A**. This was the case in both the thymus and the spleen of $\text{TCR}\beta^{-/-}$ and $\text{pT}\alpha^{-/-}$ mice.

ICER is not the only gene whose expression in $\gamma\delta$ cells is affected by the loss of β -selection. As shown in **Figure 47B**, nuclear orphan receptor NOR-1 follows a very similar expression pattern, as does myeloblastin (and, to a lesser extent, Nur-77; not shown). Such is not the general case in these $\gamma\delta$ cell populations, as illustrated with transcription factor c-Maf, an important factor for $\gamma\delta$ development (Laurie Glimcher, unpublished data) and controls β -actin and V δ 2. This suggests a specific effect of pre-TCR signalling in the expression of genes such as ICER or NOR-1 in $\gamma\delta$ cells, underlying a novel role for β -selection in $\gamma\delta$ thymocyte differentiation.

However, two lines of evidence suggest that the effect of pre-TCR on $\gamma\delta$ differentiation is not in *cis*. Firstly, the distribution of ICER-LacZ protein in $\gamma\delta$ thymocytes is uni-modal (close to normal distribution) (Figure 38A). Since only circa 15% of $\gamma\delta$ cells express icTCR β (Wilson and MacDonald, 1998), endogenous pre-TCR cannot account for ICER expression in the vast majority of ICER(+) cells. Secondly, and more strikingly, also pro-T cells (DN2 thymocytes) express ICER (Figure 27). Since DN2 cells are known not to express pre-TCR (Capone et al., 1998; Godfrey et al., 1994), this is incompatible with a *cis*-mechanism for ICER induction in these cells.

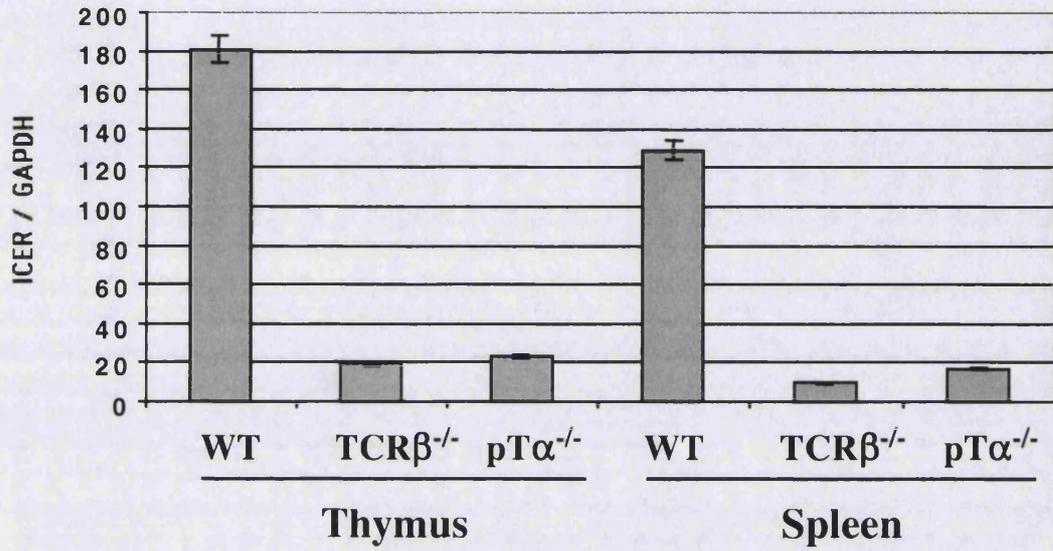
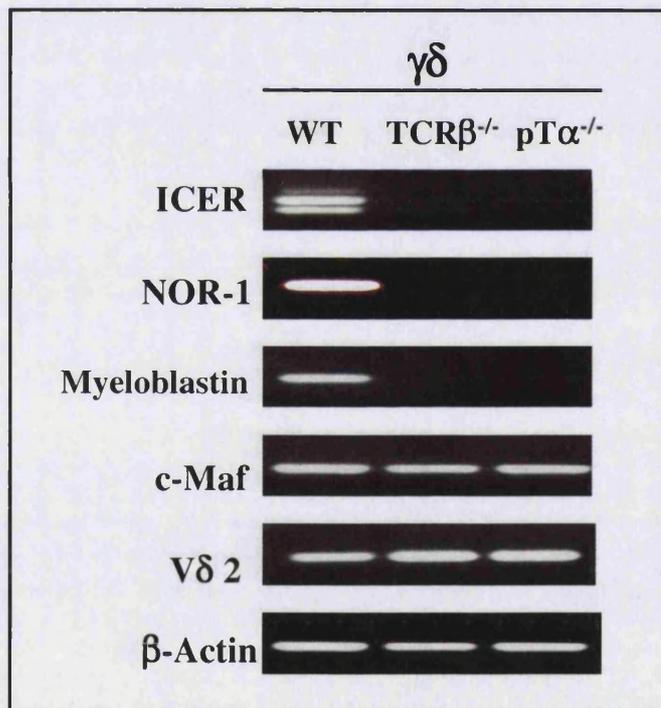
A**B**

Figure 47 : Gene expression in $\gamma\delta$ cells of TCRβ^{-/-} and pTα^{-/-} mice:

(A) Real-time PCR for ICER in thymic and splenic $\gamma\delta$ cells.

(B) RT-PCR for various genes in thymic $\gamma\delta$ cells.

To be certain that ICER(+) cells in the DN2 subset were not a consequence of contamination with more mature cells, single ICER(+) DN2 thymocytes were sorted and analysed for the expression of rearranged TCR β gene segments by single-cell PCR. No amplification signal for TCR β transcripts was detected in many ICER(+) pro-T cells (data not shown), excluding an endogenous pre-TCR-mediated induction of ICER expression.

Since ICER expression in $\gamma\delta$ cells is severely compromised in pre-TCR-deficient mice, but this does not seem to be due to a direct (pre-TCR) effect, we hypothesised the existence of a pre-TCR dependent *trans*-mechanism responsible for ICER induction in these cells.

4.7 Requirement of a normal (β -selected) composition of the thymus for ICER expression

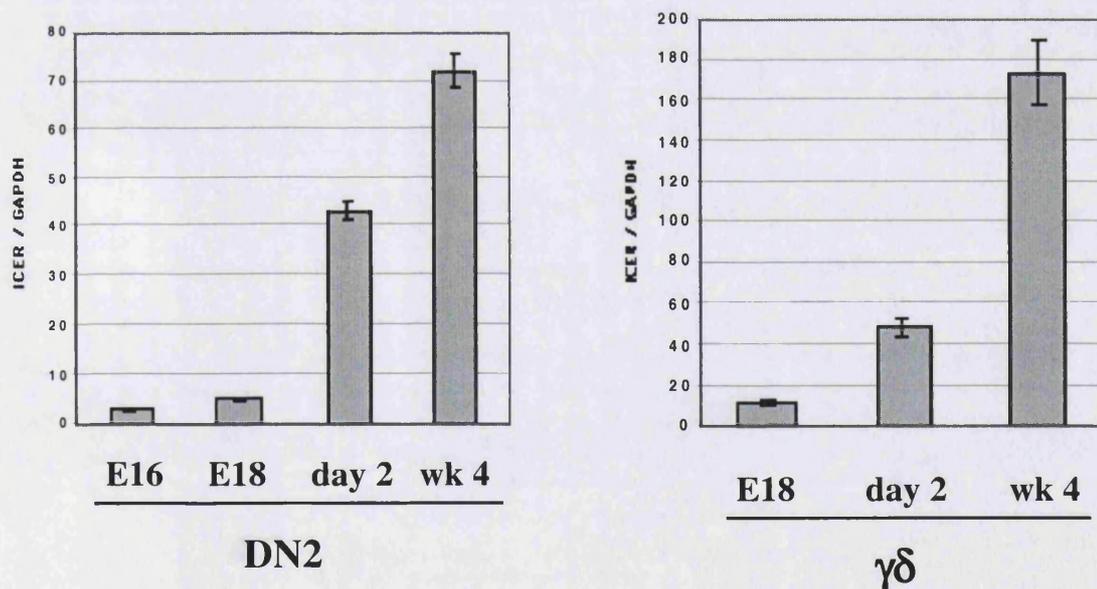


Figure 48 : ICER expression in foetal and adult pro-T and $\gamma\delta$ thymocytes. Real-time PCR on sorted WT subsets.

More data supporting the role of a *trans*-induction mechanism in the context of $\gamma\delta$ T cell differentiation was obtained from ICER's pattern of expression during murine development. ICER expression in DN2 and $\gamma\delta$ thymocytes was only significant after birth, but not during embryonic life (E16 or E18) – **Figure 48**.

Although the pre-TCR is expressed on the surface of foetal thymocytes from E15 onwards (Bruno et al., 1999), E16-E18 thymocytes do not express ICER. Therefore, this expression pattern suggests that a particular composition of the thymus is necessary for ICER expression. The major change in the composition of the thymus over this developmental time frame is an accumulation of DP and SP cells of the $\alpha\beta$ lineage, which is obviously dependent on β -selection.

To further address the relevance of a normal cellular composition of the thymus for ICER expression in pro/pre-T cells, we obtained stable (6 weeks after injection) bone marrow chimeras of WT donor cells and RAG-1^{-/-} host cells from Roman Spoerri and Caetano Reis-e-Sousa (CR UK). As shown in **Figure 49**, the use of congenic markers CD45.1 and CD45.2 allowed us to distinguish cells of each origin in the chimeric thymuses. Whereas the host-derived population had a homogeneous CD4(-)CD8(-) phenotype, characteristic of RAG^{-/-} thymocytes, the donor-derived population differentiated normally into DP and SP cells. (SP cells of donor origin were also observed in peripheral blood.)

Real time PCR quantification of ICER expression in thymocyte subsets isolated from these chimeric thymuses (Figure 49B) showed that DN2/DN3 cells of donor (WT) origin did not express significant levels of ICER, in contrast with WT non-chimeric cells (reference). All cell subsets of donor origin expressed ICER at similar levels to host RAG-1^{-/-} thymocytes, suggesting that normal ICER expression in WT thymocytes requires a normal cellular composition of the thymus. According to the data presented before, such is strictly dependent on effective " β -selection".

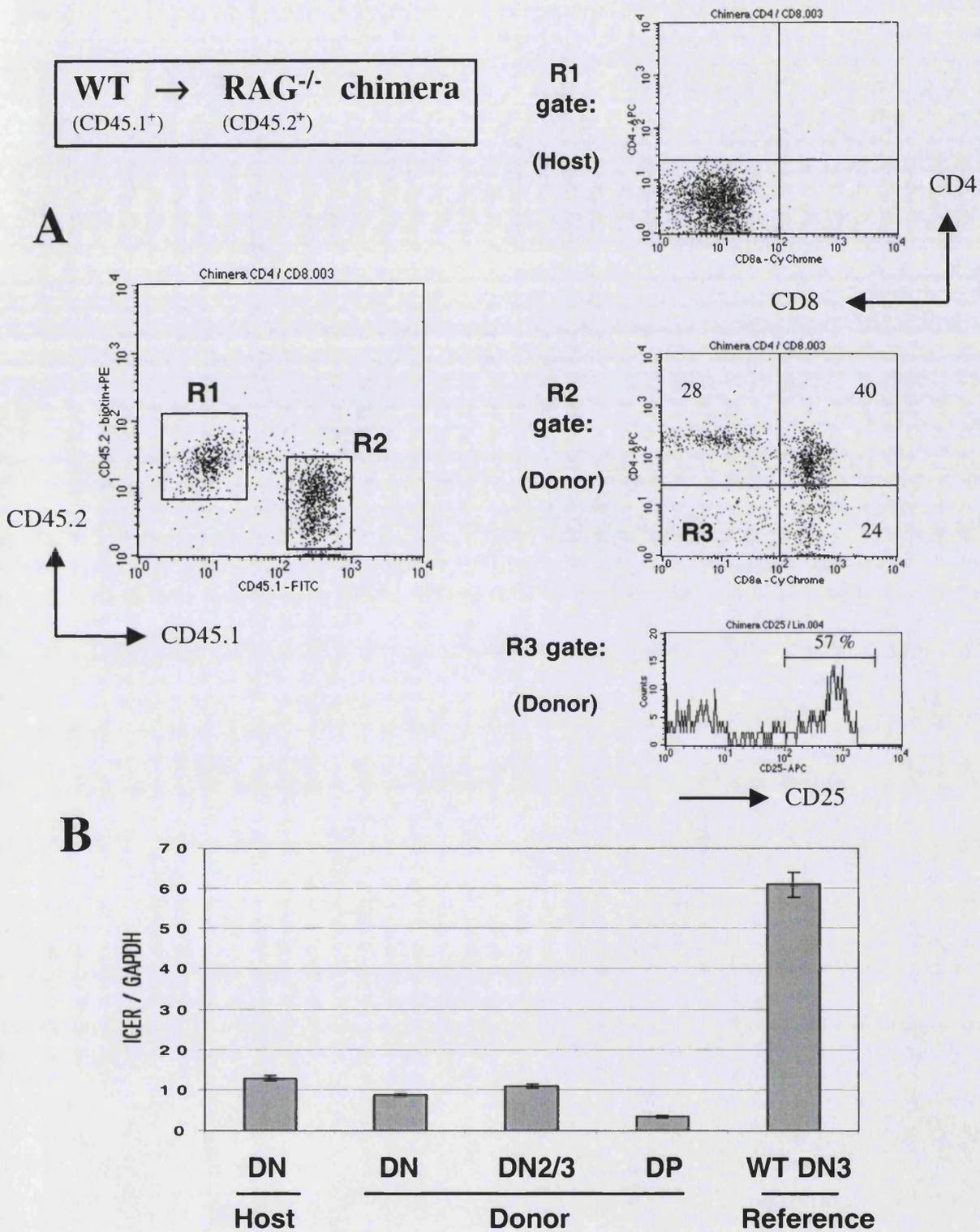


Figure 49 : ICER expression in WT → RAG^{-/-} bone marrow chimera:

(A) FACS profiles of the chimeric thymus (see text for details). (n=5)

(B) Real-time PCR for ICER in subsets isolated from the chimeric thymus.

4.8 Cross-talk between $\alpha\beta$ and $\gamma\delta$ lineages during $\gamma\delta$ thymocyte differentiation

Since ICER expression is impaired in pre-TCR deficient mice, which lack DP and SP thymocytes, but not in $\text{TCR}\alpha^{-/-}$ mice (Figure 36), which only lack SP cells, we hypothesised that the critical link in the *trans*-induction mechanism is the DP population.

To test this hypothesis, we set up an RTOC system in which $\gamma\delta$ thymocytes (targets of the *trans*-induction mechanism) were subjected to the presence of a large excess of DP thymocytes (the effector cells). The target $\gamma\delta$ cells had a pre-TCR-deficient background, so that they did not express ICER at the start of the experiment. In more detail, 1.5×10^4 $\text{TCR}\beta^{-/-}$ $\gamma\delta$ thymocytes were mixed with 1.5×10^6 DP cells (isolated from $\text{TCR}\delta^{-/-}$ mice, to avoid any contamination with extra $\gamma\delta$ cells) and cultured for 9 days. (Note: the ratio 1 $\gamma\delta$: 100 DP is similar to the one found in a normal thymus.) As controls, we established RTOCs in which $\gamma\delta$ thymocytes isolated from either WT or $\text{TCR}\beta^{-/-}$ mice were cultured in the absence DP cells. After 9 days, $\gamma\delta$ cells of each RTOC were re-sorted and analysed for ICER expression.

As seen in Figure 50, the $\text{TCR}\beta^{-/-}$ $\gamma\delta$ thymocytes incubated with DP cells showed a 4-fold up-regulation of ICER expression, in contrast with “non-treated” control cells. These results are consistent with the requirement of a normal DP thymocyte population for ICER expression in $\gamma\delta$ cells. Thus, DP cells seem to be the likely effectors of the *trans*-induction mechanism responsible for ICER expression in $\gamma\delta$ and pro-T cells.

We therefore propose that β -selection is important for $\gamma\delta$ cell differentiation since it provides a large pool of DP thymocytes that act on $\gamma\delta$ thymocytes and induce them to express a subset of cellular genes, including $\gamma\delta$ lineage marker ICER.

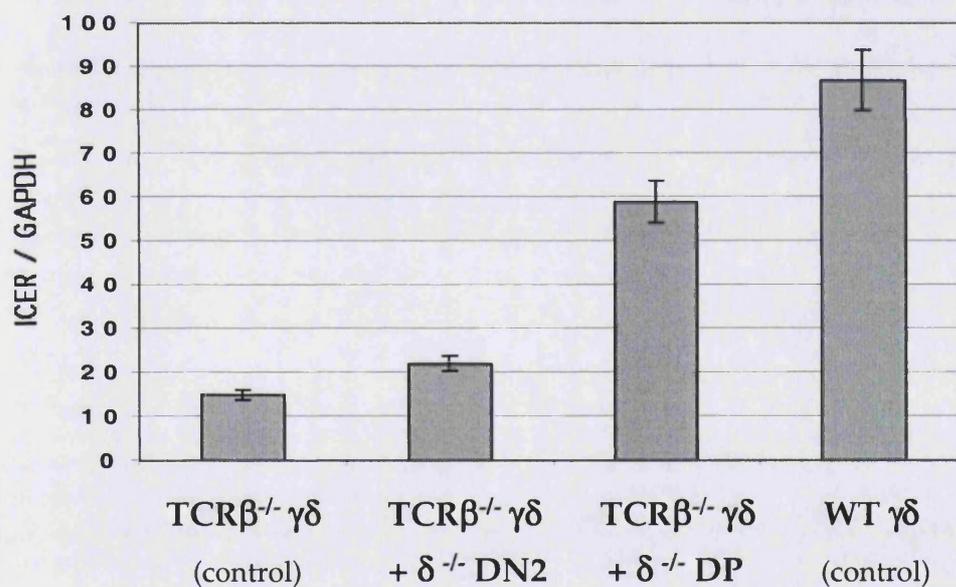
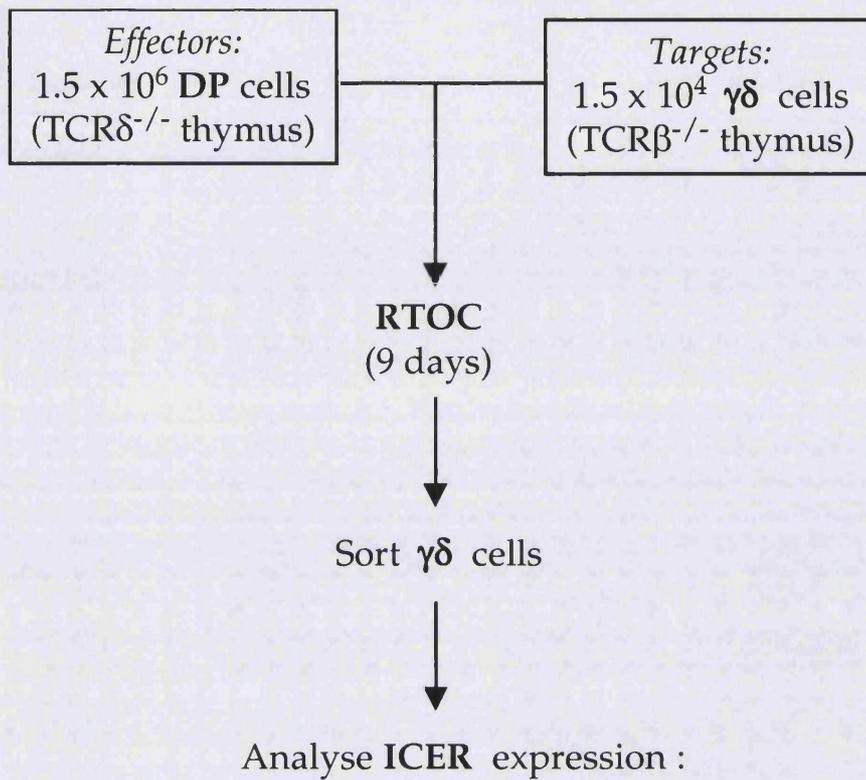


Figure 50 : Induction of ICER expression in $\gamma\delta$ thymocytes mediated by DP cells. Real-time PCR for ICER in $\gamma\delta$ cells purified after incubation (9-day RTOCs) in the presence or absence of DP thymocytes.

Chapter IV :

DISCUSSION

The vertebrate immune system relies on T lymphocytes as the cellular basis of adaptive immune responses. T cells are produced in the thymus from bone marrow-derived lymphoid progenitors, following a tightly regulated co-ordination of signals for maturation, proliferation and survival.

The fundamental characteristic of a T cell is the expression of a clonotypic T cell receptor. Thus, successful TCR gene rearrangements, transcription, surface expression and selection can be seen as the primary objectives of T cell development.

Thymocyte differentiation is marked by lineage commitment and cell selection events. In particular, pre-T cells split between $\alpha\beta$ and $\gamma\delta$ lineages; and $\alpha\beta$ -committed cells go through "β-selection", a process mediated by the pre-TCR. Although extensive amounts of data on the phenotype and behaviour of these cells have been accumulated, the genetic changes behind those processes are still largely unknown.

Our research on thymocyte differentiation resulted in the identification of genes whose expression is differentially regulated between T cell developmental stages and lineages. In chapter III we presented our results regarding: the differential analysis of $\alpha\beta$ and $\gamma\delta$ cells (section 1), the identification of pre-TCR responsive genes (section 2), and the studies on a candidate gene, ICER, in the context of $\alpha\beta$ / $\gamma\delta$ lineage commitment (section 3) and β-selection (section 4). Here we discuss the implications of those results.

Although differing in some aspects (developmental markers, for example), human and murine T cell differentiation share the same fundamental processes and events, making the mouse thymus the most widely used system for such studies.

In our case, the use of mice greatly facilitated the isolation and manipulation (in foetal thymic cultures) of particular T cell populations. Furthermore, the analysis of genetically modified mice allowed us to establish important genetic links, which would have been difficult to dissect in the human system.

1 Genes differentially expressed between $\alpha\beta$ and $\gamma\delta$ T cells

The precise mechanism of $\alpha\beta/\gamma\delta$ lineage commitment is still unclear. Nevertheless, it is known that DN thymocytes must productively rearrange and express a TCR isotype in order to survive and mature. At the DN2 to DN3 transition, the TCR γ , TCR δ and TCR β chains begin to rearrange. Thymocytes that successfully rearrange TCR γ and TCR δ express a TCR $\gamma\delta$ and can proceed to the $\gamma\delta$ T cell lineage. Similarly, cells that successfully rearrange TCR β express a pre-TCR (the TCR β chain in association with the invariant pT α chain) allowing them to adopt an $\alpha\beta$ T cell fate.

The extent to which either the pre-TCR or the TCR $\gamma\delta$ directly influence lineage determination remains controversial. On the one hand, instructive models propose that the pre-TCR and TCR $\gamma\delta$ provide distinct signals that directly determine the lineage fate adopted by a bipotential progenitor. Alternatively, selective models suggest that $\alpha\beta/\gamma\delta$ lineage determination is made independently of TCR expression. Subsequent to this, $\alpha\beta$ -committed progenitors must then express a pre-TCR, and $\gamma\delta$ -committed progenitors must express a TCR $\gamma\delta$ in order for the cells to survive and mature along the pre-determined lineage pathway.

We used a differential analysis technique, RDA, to compare the gene expression in thymocytes that we considered the best representatives of cells “recently committed” to either lineage. We thus sorted CD4(+)CD8(+) and TCR $\gamma\delta$ (+)HSA(+) thymocytes from TCR α -deficient mice. Besides expressing the earliest markers known to identify $\alpha\beta$ and $\gamma\delta$ -committed cells, respectively, the genetically modified background of these cells eliminated transcriptional changes due to TCR $\alpha\beta$ signalling.

The RDA protocol had been modified (by Daniel J. Pennington in the lab) to allow the use of limited amounts of starting material (mRNA), an important aspect when analysing less abundant cell subsets (such as the TCR $\gamma\delta$ (+)HSA(+) one). For this reason, RDA was also a more practical technical approach than micro-chip

technology (which was still in its infancy in 1998, when this project began). Furthermore, RDA had the advantage of permitting the identification of novel genes. Such drawbacks of the micro-chip technology will soon be resolved once the mouse genome has been completely sequenced.

The genes identified by RDA (Table 1) included some that were previously known to be differentially expressed between $\gamma\delta$ and DP thymocytes: IL-2R β and IL-7R α in $\gamma\delta$ cells; CD4, CD8, TCR β and RAG-1 in DP cells. Therefore, they confirmed the success of the subtractive hybridisation procedure, acting as “internal controls” of the RDA experiment. More importantly, the differential expression of the identified genes was confirmed (by RT-PCR) in most of the cases (Figure 10 and data not shown).

A consequence of the RDA methodology (isolation of cDNAs from gel bands; Figure 9) is the retrieval of a relatively limited number of genes (Table 1). In order to maximise the number of cDNAs identified in the differential products, we coupled the subtractive hybridisation procedure of RDA to the Atlas array method of identification of cDNAs (Figure 9). However, this modification was not particularly successful, as it only allowed the identification of six additional genes (see “*” labels in Table 1), five of which belonging to the same family (proteases and their modulators). This number was surprisingly low if we take into account that the Atlas array covered 588 genes. As RDA is a very sensitive method (effectively amplifying differences of 2% between tester and driver), including in our hands (see results with Ly49A), the low amount of retrieved genes may reflect a striking similarity between the populations analysed, $\gamma\delta$ and early DP thymocytes. This would suggest that lineage divergence at the level of the common DN precursor would only require the differential expression of a limited number of genes. Differences would presumably be multiplied later on as cells mature along each lineage.

Overall, RDA proved to be a reliable method of differential analysis, as it produced very few “false positives”. Its major limitation, the low number of retrieved genes, can also be seen as an advantage, as it concentrates the effort of the investigator onto a few reliable candidates. In cases where such restrictions are not desirable, a more appropriate approach would be SAGE (serial analysis of gene

expression), which has its own drawback of requiring a large amount of nucleotide sequencing for the identification of the cDNAs.

The pattern of mRNA expression of some of the candidate genes was consistent with a potential involvement in the $\alpha\beta/\gamma\delta$ lineage split: restriction to the T (or at least T/NK) lineage and maintenance of the differential expression in more mature subsets (in particular, SP cells of the $\alpha\beta$ lineage). This was the case for CREM, NOR-1, Ly-49A and Sugano EST, among “ $\gamma\delta$ genes”; and “Novo-1” and “Novo-2”, among “ $\alpha\beta$ genes” (Table 2).

CREM (ICER) was the gene selected for extensive studies in the context of the $\alpha\beta/\gamma\delta$ split, and it is discussed later (section 3). Regarding the other candidates, we performed some preliminary experiments to assess their relevance for the lineage commitment process.

NOR-1 (neuron-derived orphan receptor -1), isolated from a $\gamma\delta$ - DP differential product, was an interesting candidate, in particular since its over-expression had been shown to provoke a 25-fold reduction in thymic cellularity (Cheng et al., 1997b). This effect was primarily due to programmed cell death of DP cells (which constituted only 28% of the mutant thymus, in contrast to 87% of control WT littermates).

The pattern of expression of NOR-1 mRNA (Figure 12) was also appealing in terms of the $\alpha\beta / \gamma\delta$ lineage split, as it was abundant in the DN3 and DN4 thymocyte subsets, which contain precursors of both lineages.

However, analysis of NOR-1 deficient mice revealed no perturbation in the distributions of $\gamma\delta$ and $\alpha\beta$ populations, suggesting that the gene does not play a non-redundant role in lineage commitment. In that regard, it is important to note the redundancy between NOR-1 and Nurr-77 (Cheng et al., 1997b). Since Nurr-77 is also expressed in $\gamma\delta$ thymocytes, it is possible that the two members of the nuclear orphan receptor family play overlapping functions in that process and can compensate for the absence of the other gene in its single ‘knock out’ mouse model.

Interestingly, the thymocyte subset that expresses NOR-1 at highest levels is DN4. Since the thymic expression of NOR-1 begins at DN3 stage and is clearly up-regulated in the DN3 → DN4 transition (Figure 12), it would be consistent with the involvement of pre-TCR signalling. This hypothesis is currently under investigation. In this context, it is important to note our data on Nurr-77 (Results, section 2): its expression in DN3 cells is dependent on pre-TCR signalling and can be induced by a pre-TCR-type stimulus (anti-CD3 mAb). Therefore, once again, these two nuclear orphan receptors seem to behave very similarly and a proper examination of their biological roles may require simultaneous inactivation of both genes.

Ly-49A was also identified as being expressed in $\gamma\delta$, but not $\alpha\beta$, thymocytes. Since Ly49 molecules are an important family of NK inhibitory receptors, we were interested in assessing the relevance of this differential expression.

NK inhibitory receptors deliver inhibitory signals to NK cells upon MHC class I (MHC I) recognition. This is seen as a general mechanism of 'NK cell tolerance', as it ensures that only cells with deficient MHC I expression (due to viral infection, for example) are lysed by NK lymphocytes. Ly-49A, in particular, interacts with two allelic forms of H-2D, H-2D^d and H-2D^k.

The murine Ly-49 receptors are type II receptors belonging to the C-type lectin family, whilst killer inhibitor receptors (KIR), another class of NK inhibitory receptors, belong to the Ig superfamily.

In addition to NK cells, small subsets of T cells were also known to express Ly-49 family members (Wong et al., 1991). We found Ly49A to be expressed at the mRNA level in NK, $\gamma\delta$ and pre-T cells (Figure 12).

Although the differential expression of Ly49A between the $\gamma\delta$ and $\alpha\beta$ lineages was extended to the protein level, only 5% of all $\gamma\delta$ thymocytes displayed high levels of surface expression (Figure 13). Importantly, most cells of the positive population had a NK1.1(+) phenotype, implying they were NK T cells.

These results regarding $\gamma\delta$ and $\alpha\beta$ NK T cells might suggest a closer relationship between $\gamma\delta$ T and NK cells than between $\alpha\beta$ T and NK cells, possibly at the level of T/NK precursors. This is consistent with the expression, in $\gamma\delta$ T cells, of other molecules typical of NK cells, such as NKG2D. NKG2D is a NK cell activating receptor expressed by a spectrum of cytotoxic cells. Interestingly, it binds non-classical MHC class I molecules such as MICA (in humans) and T22 (in mice), which are seen as candidate ligands for TCR $\gamma\delta$ (see Introduction, 3.1). It is currently being investigated whether NKG2D plays a co-receptor role in $\gamma\delta$ cells, similar to that of CD8 in $\alpha\beta$ lymphocytes. In that case, NKG2D would facilitate of the interaction between TCR $\gamma\delta$ and its non-classical MHC I ligand.

In our RDA analysis, we also came across other genes expressed selectively in both $\gamma\delta$ and NK cells: leukocystatin, Mg-11, myeloblastin and the "Sugano" EST (Table 2).

Such parallels between these two lymphocyte lineages could reflect their involvement in innate immune responses, in contrast with the prototypical adaptive immunity of $\alpha\beta$ T cells.

However, these results suggest that in terms of the two T cell lineages *per se*, Ly49A does not seem to be preferentially expressed in NK1.1(-) $\gamma\delta$ thymocytes. Therefore, while Ly49A provided a good validation of the RDA analysis, we did not consider this gene to be relevant for further studies on $\alpha\beta$ / $\gamma\delta$ lineage commitment.

The "Sugano EST", an expression sequence tag (from GenEMBL database, AI790276) initially obtained from a murine kidney cDNA library created by Dr. Sumio Sugano (1999), was detected by RT-PCR in $\gamma\delta$ and NK thymocytes, but not in other haematopoietic lineages (Figure 12). Unlike Ly49A, the expression of Sugano EST in $\gamma\delta$ thymocytes is not limited to NK1.1(+) cells.

The full-length transcript containing this EST, obtained by screening a mouse thymus cDNA library, included a 5' sequence homologous to IL-2R β . The existence of a IL2-R β /Sugano transcript in $\gamma\delta$ thymocytes was confirmed by its amplification

(2.6 kb product) via a combination of primers specific for either Sugano EST or IL-2R β . Since the Sugano EST was located 3' of the IL-2R β sequence, this could represent an alternative transcript under the control of the IL-2R β promoter.

Previous data (Takeuchi et al., 1992; Tanaka et al., 1991) suggested that the IL-2R β protein is expressed in very few (1-4%) thymocytes, whereas it is more frequent among splenic CD8⁺ and NK lymphocytes. In mature lymphocytes, IL-2R β is one component of the IL-2 receptor (together with α and γ chains), which plays a crucial role in T cell proliferation. Moreover, the ligand, IL-2, triggers an up-regulation of IL-2R β expression.

However, using monoclonal antibodies specific for IL-2R β , including a blocking mAb (clone TM- β 1) that disrupts IL-2 signalling, we could not detect any signal in $\gamma\delta$ thymocytes, implying that if the IL-2R β /Sugano transcript is translated into a protein, it is not involved in IL-2R signalling.

Thus, the biological relevance of the IL-2R β /Sugano transcript, in which the Sugano sequence represents an alternative 3'-end for the IL-2R β gene, is still under investigation. It will be important to establish if this transcript can be alternatively spliced, generating mRNAs with distinct functions; and what type of protein containing the Sugano EST is translated from that mRNA. This information will probably suggest its function - if any - in $\gamma\delta$ thymocytes.

2 Pre-TCR responsive genes

“ β -selection” is a process by which signalling events originated at the pre-TCR / CD3 complex promote proliferation and differentiation (DN3 \rightarrow DN4 \rightarrow DP) of pre-T cells. “ β -selected” DN3 thymocytes receive survival signals and expand massively, while they begin a differentiation program culminating in the generation of DP thymocytes.

Pre-TCR / CD3 signalling is known to involve tyrosine kinases such as p56Lck and p59Fyn and adaptor molecules such as LAT and SLP-76, upstream of PLC- γ / PKC, Ras / MAPK and Vav / Rho signalling pathways. However, very little is known of downstream targets of these pathways, including the actual effector genes responsible for the differentiation programme initiated by the pre-TCR.

Our first strategy to address this issue, an RDA analysis of WT and TCR β -deficient DN3 thymocytes (Results, 2.1), suffered from many intrinsic differences between the two cell populations: pre-TCR signalling induces such changes in the proliferation status (of WT cells) that the comparison with pre-TCR(-) cells basically reflects those, rather than specific alterations in gene expression related to differentiation.

Our second strategy, the *in vivo* stimulation of RAG-deficient cells with anti-CD3 mAbs (Results, 2.2), proved to be much more successful. Not only did it reproduce many of the phenotypic changes associated with DN3 \rightarrow DN4 (\rightarrow DP) transition (for example, down-regulation CD25; up-regulation of CD2, CD69, CD4 and CD8), but it also retrieved a panel of genes not directly associated with altered cell cycle status (Table 5). By concentrating on genes induced after a short time (3 hours) of antibody injection, we hoped to identify direct targets of pre-TCR signalling.

Following expression studies on the kinetics of gene induction by anti-CD3 mAb (Figure 20) and the dependence on pre-TCR signalling in DN3 thymocytes (Figure 21), two genes were selected for further studies: ICER (Results, section 4; see paragraph 4 ahead) and IL-7R (Results, 2.4).

2.1 Role of IL-7 receptor in the DN to DP transition

IL-7R α mRNA expression was clearly up-regulated in DN3 thymocytes after 3 hours of treatment with anti-CD3 mAb *in vivo*, and peaked after 12 hours (Figure 20). Its expression in pre-TCR deficient thymocytes, although not compromised at the DN3 stage, was significantly impaired at the DN4 stage (Figure 22).

IL-7/IL-7R signalling had been previously demonstrated to protect pro-T cells (DN1-DN2 stages) from apoptosis, at least in part by the upregulation of the survival factor Bcl-2 (Kim et al., 1998; von Freeden-Jeffry et al., 1997). Furthermore, a role in the induction of pro-T cell proliferation had also been suggested.

However, as the absence of a functional IL-7/IL-7R signal results in such a severe phenotype at the pro-T cell stages of T cell development, it had been difficult to determine whether subsequent stages are also dependent on signalling via this cytokine receptor pathway. Indirect evidence suggested that IL-7 could be functionally relevant for stages of thymocyte development after the β -selection checkpoint. For example, it was known that IL-7R α is expressed in the DN4 population, and recently a role for IL-7/IL-7R signalling had been identified at the later DP \rightarrow SP transition (Hare et al., 2000). Nevertheless, the precise role, if any, of IL-7/IL-7R signalling at the DN \rightarrow DP transition had yet to be fully determined.

Whether the pre-TCR is able to directly control survival, proliferation and differentiation is a subject of lively debate. For example, it has been suggested that the formation of a pre-TCR may only provide a signal for thymocyte survival (Petrie et al., 2000). This rescue of DN3 cells would then facilitate the onset of an independent program of differentiation and proliferation regulated by factors and signals unrelated to the pre-TCR. Such signals may include those that are mediated by HES-1, HEB, Hedgehog, Tcf-1/Lef-1, or CREB (see Introduction, 2.4.4).

The full repertoire of signals involved in regulating the DN to DP transition and the relationship of these pathways to pre-TCR signalling have remained unclear. In particular, no (direct) relationship between the pre-TCR and the IL-7/IL-7R pathway had been previously demonstrated.

Our data showed that pre-TCR signalling is required to maintain IL-7R α expression on the surface of developing thymocytes as they progress from the DN3 to the DN4 stage. In mice that are deficient for a component of the pre-TCR, thymocytes that develop to the DN4 stage lack expression of the IL-7R α chain.

As the induction of surface IL-7R α protein expression was observed at just 6 hours after the cross-linking of CD3 ϵ on RAG-1-deficient thymocytes, before either the downregulation of CD25 or the onset of an increase in the number of cells in the S/G₂/M phases of the cell cycle, this suggests a direct link between the nuclear events activated by the formation of a pre-TCR and the up-regulation of IL-7R α expression. Such a direct relationship would ensure that only β -selected thymocytes would retain the ability to respond to IL-7 at subsequent developmental stages.

Interestingly, a comparable relationship has recently been observed in the IL-7-dependent early stages of B cell development (Fleming and Paige, 2001). Pre-B cell receptor (pre-BCR) mediated signalling via the ERK map kinase pathway is required for the optimal response of pre-B cells to low or limiting concentrations of IL-7.

Since cross-linking of CD3 complexes in RAG-1^{-/-} thymocytes may not entirely reproduce all aspects of pre-TCR signalling, Cesar Tigueros followed the expression of surface IL-7R α during FTOC of E16 RAG-1^{-/-} thymocytes retrovirally transduced with a rearranged TCR β gene (V β 11-specific). The pMX-TCR β construct also included an IRES-GFP cassette to facilitate identification of infected cells by FACS analysis. After 3-6 days in FTOC, DP cells were abundant and GFP positive thymocytes expressing rearranged TCR β showed a significant increase in IL-7R α expression (data not shown). Thus, this experiment formally showed that generation of a pre-TCR in RAG-1^{-/-} thymocytes results in an increase in surface IL-7R α expression.

Additionally, our results also define a precise role for IL-7/IL-7R signalling at the DN to DP transition. We show by RTOC that the treatment of β -selected wild type DN4 thymocytes with monoclonal antibodies that inhibit the function of the IL-7 receptor results in a massive reduction in the generation of DP thymocytes due to an increased level of cell death at the DN4 stage.

Consistent with these observations, increased apoptosis was also observed in DN4 thymocytes of IL-7R α ^{-/-} mice. Although such an anti-apoptotic role has not previously been documented for IL-7/IL-7R signalling at this stage of thymocyte differentiation, this pathway has been implicated in survival of DN1-DN2-DN3 populations, via the up-regulation of the anti-apoptotic gene Bcl-2 (Kim et al., 1998; von Freeden-Jeffry et al., 1997). Accordingly, IL-7R α ^{-/-} or γ c^{-/-} mice expressing a Bcl-2 transgene showed a significant increase in total thymocyte numbers (Akashi et al., 1997; Maraskovsky et al., 1997). However, two groups have reported a failure to observe such a Bcl-2-mediated rescue (Di Santo and Rodewald, 1998), and Bcl-2-deficient mice do not display a loss of thymic output comparable to that observed in either IL-7R α ^{-/-} or γ c^{-/-} animals (Veis et al., 1993). Therefore, the precise mechanism by which IL-7 can promote cell survival is still unclear.

With regard to the IL-7-mediated protection of DN4 thymocytes, it is very unlikely that Bcl-2 is involved, as the expression of Bcl-2 dramatically decreases as wild type thymocytes progress from the DN3 to the DN4 stage (Voll et al., 2000). Moreover, recent work has demonstrated that NF- κ B activation can provide a selective survival signal for β -selected thymocytes at a stage when Bcl-2 expression is being downregulated (Voll et al., 2000). It was suggested that pathways that activate NF- κ B, such as the pre-TCR, could protect β -selected DN4 thymocytes from apoptosis. In this context, IL-7/IL-7R signalling may directly or indirectly interact with components of the NF- κ B pathway, thereby promoting cell survival at the DN4 stage.

In addition to a role in cell survival, the IL-7/IL-7R pathway has also been implicated in the regulation of T cell proliferation. However, in both the antibody-treated RTOCs and in the analysis of DN4 cells from IL-7R α ^{-/-} mice the number of DN4 cells in the S/G₂/M phase of the cell cycle appears largely comparable to controls. This would suggest that the major role of IL-7/IL-7R signalling at the DN4 stage is one of cell survival rather than the promotion of proliferation.

Nevertheless, it is unlikely that survival and proliferation are completely independent processes. For example, in mice deficient for a component of the pre-TCR, the IL-7R α chain is largely absent from DN4 cells, but these cells do not

display the high incidence of cell death that is associated with either DN4 cells from IL-7R α ^{-/-} mice or with wild type DN4 cells treated with anti-IL-7R α /anti- γ c mAbs in RTOC. Importantly, DN4 cells from pre-TCR-deficient animals are not in a highly proliferative state, in contrast to both wild type DN4 cells or β -selected thymocytes from IL-7R α ^{-/-} mice. This would therefore imply that IL-7/IL-7R signalling is required to maintain cell viability *specifically* in the context of the extended phase of pre-TCR-mediated proliferation that occurs in β -selected thymocytes at the DN4 stage.

Taken together, our results identify a direct relationship between pre-TCR signalling and expression of the IL-7R α chain as thymocytes progress through the β -selection checkpoint. They also demonstrate that IL-7/IL-7R signalling is critically necessary for thymocyte survival during the massive burst of proliferation that is observed at the DN4 stage, thus mediating an efficient DN \rightarrow DP transition. Therefore, pre-TCR-regulated expression of a functional IL-7 receptor provides a selective survival advantage by which β -selected thymocytes progress to the DP stage.

3 ICER as a marker for the $\gamma\delta$ T cell lineage

The ICER (inducible cyclic-AMP early repressor) isoform of the CREM (cyclic-AMP response element modulator) gene was identified by RDA as being expressed in thymic $\gamma\delta$ T cells but not in DP thymocytes of the $\alpha\beta$ T cell lineage. CREM/ICER belongs to the cyclic-AMP response element binding protein (CREB) family of basic-domain leucine zipper transcription factors that have been implicated in numerous biological systems (Sassone-Corsi, 1998). Several endocrine and neuronal functions are governed by the cAMP-dependent pathway. CREM, in particular, plays a key physiological and developmental role in the hypothalamic-pituitary-gonadal axis. CREM-deficient mice suffer from a complete block in early spermatogenesis, leading to infertility of the male mice (Nantel et al., 1996).

ICER has four splice variants (I, I γ , II, II γ) transcribed from an internal promoter in the CREM gene (Molina et al., 1993). These proteins incorporate one of the two cAMP responsive element (CRE)-specific DNA-binding domains of CREM, but do not include the activation domains responsible for the recruitment of co-activators such as CREB-binding protein (CBP) and p300. Therefore, ICER proteins act as potent repressors of CREB family (CREB, AFT-1, CREM activators) - mediated transcription (Molina et al., 1993).

CREM/ICER proteins are cAMP responsive because they are activated by phosphorylation via the cAMP-dependent protein kinase PKA. PKA itself is directly activated by binding to the cAMP produced by adenylyl cyclase as a result of the stimulation of G protein-coupled receptors. Upon PKA-mediated phosphorylation (of serine residues), CREM/ICER proteins bind CRE sites in the regulatory regions of many genes, thus regulating their expression. CREM/ICER proteins can also be phosphorylated by Cam kinase IV and Rsk-2 (link with MAPK pathway) (reviewed in Sassone-Corsi, 1998).

ICER is the only inducible CRE-binding protein. ICER expression is tissue specific and developmentally regulated (reviewed in Sassone-Corsi, 1998). By sequentially repressing distinct sets of CRE-containing genes, ICER can modulate cell fate

(Ruchaud et al., 1997). The kinetics of ICER expression are characteristic of an early response gene. ICER is involved in auto-regulatory feedback loops of transcription that govern the down-regulation of early response genes, such as the proto-oncogene *c-fos* (Molina et al., 1993; Servillo et al., 2002).

The importance of the regulation of ICER expression is highlighted in the pineal gland (site of production of the hormone melatonin), where it follows a circadian (night-day) oscillation driven by the endogenous biological clock located in the suprachiasmatic nucleus. In this system, ICER expression transcription peaks at night, induced by adrenergic input to the pineal gland from the suprachiasmatic nucleus. This induction is transient because ICER represses its own transcription (via CRE sites located in its promoter).

Previous reports on ICER had suggested a 'dual' physiology for this gene. On one hand, it seemed to act as a tumour suppressor by repressing the expression of cell growth related genes. Indeed, not only were ICER levels reduced in cancer cells, but its over-expression was also able to revert the transformed phenotype of tumour cell lines (Razavi et al., 1998; Yehia et al., 2001). But on the other hand, ICER expression was induced by the proliferation promoter noradrenaline in brown adipocytes (Thonberg et al., 2001), and its *in vitro* over-expression in neurons caused the re-activation of a latent herpes virus. Therefore, ICER's action seems to be cell context-dependent.

It was not previously known how ICER behaves in the thymus. Our data showed that ICER is expressed in thymic $\gamma\delta$ T cells, but not in DP thymocytes of the $\alpha\beta$ T cell lineage. As well as being observed in $\gamma\delta$ cells, significant ICER expression is also detected in DN2, DN3, and DN4 thymocytes. In contrast, ICER is not expressed in DP, CD4+ SP or CD8+ SP cells (Figure 27). Such regulated expression could implicate ICER in both thymocyte differentiation and in the $\alpha\beta/\gamma\delta$ lineage divergence. However, our analysis of thymi from *CREM/ICER*^{-/-} mice failed to demonstrate any perturbation in the development of either $\alpha\beta$ or $\gamma\delta$ T cells, implying that ICER does not have a non-redundant role in the thymus.

ICER competes with activated CREB for binding to CREs in the promoters of various genes. *CREB*^{-/-} thymi have a severe reduction in $\alpha\beta$ T cell number caused

by a partial block in the DN to DP transition (Rudolph et al., 1998). In contrast, $\gamma\delta$ T cell development appears normal. As CREM/ICER^{-/-} mice show no thymic defects, it is unlikely that ICER regulates CREB in the thymus.

The treatment of FTOCs with activators of the cAMP pathway results in a block in DN to DP thymocyte differentiation similar to that observed in CREB^{-/-} mice. We observed an increase in ICER expression on administration of cAMP analogues in FTOC, in particular in the DP subset, the one that seems to be most affected by the treatment (Figure 31). This up-regulation of ICER is unlikely to be part of an apoptotic programme, since transfection of cell lines with ICER (constitutively expressed at high levels) was seen to block apoptosis (Ruchaud et al., 1997).

On the other hand, we observed that ICER expression in pre-T cells (at DN4 stage) decreases as they become committed to the $\alpha\beta$ lineage (Figures 28-29). Thus, since ICER expression is down-regulated at the DN \rightarrow DP transition, and the cAMP-induced block of that process correlates with an over-expression of ICER, we investigated whether an enforced expression of ICER in pre-T and DP cells would be detrimental to the $\alpha\beta$ lineage. The existence of CRE sites in both the TCR β promoter and the TCR α enhancer provided a potential mechanism for such an effect: ICER could repress TCR $\alpha\beta$ expression, thus blocking $\alpha\beta$ development.

To address this hypothesis, we generated CD2-ICER transgenic mice, which specifically over-express ICER in the thymus. The mice showed normal thymocyte development. In particular, DP and SP cells of the $\alpha\beta$ lineage differentiated normally (Figure 33). As a criticism to our experiment, the levels of expression of the transgene in DP cells were not particularly high (Figure 32) and might have been insufficient to induce cellular changes. In addition, we did not test if it was translated into a functional protein (for example, by band-shift with CRE sequences). Meanwhile, Gress and collaborators reported the generation of Lck-ICER transgenic mice (Bodor et al., 2000; Bodor et al., 2001), which showed a suppression of $\alpha\beta$ T lymphocyte function: activated cells failed to proliferate efficiently or produce appropriate cytokine (IL-2) responses. However, the mice lacked a thymic phenotype. (In our CD2-ICER mice, we did not even observe the peripheral T cell deficiencies reported by Bodor *et al.* (data not shown)).

Thus, the absence of a thymic phenotype in the two transgenic mice models mentioned above make it unlikely that ICER plays a major role in the cAMP-mediated effects on DN → DP transition. Moreover, transgenic mice expressing a dominant-negative CREB gene also displayed normal thymocyte development (Barton et al., 1996).

Although ICER does not appear to have a non-redundant role in thymocyte development, we observed that its expression robustly differentiates between the $\alpha\beta$ and $\gamma\delta$ T cell lineages. The fact that ICER expression is not critical for T cell differentiation, but that it segregates with particular subsets, make it an “ideal” lineage marker. In fact, ICER is the first $\gamma\delta$ lineage marker, besides TCR $\gamma\delta$ itself, ever described.

The DN4 stage of thymic ontogeny represents the most mature thymocyte population that can still generate $\alpha\beta$ and $\gamma\delta$ T cells. After this stage of development, subsets of the $\alpha\beta$ T cell lineage from the thymus, lymph nodes and spleen do not express a significant level of ICER transcripts. In contrast, thymic $\gamma\delta$ T cells and $\gamma\delta$ T cells from the peripheral lymphoid organs express a level of ICER that is at least ten-fold greater than the maximum observed in any $\alpha\beta$ T cell subset from the lymph node or spleen (Figure 34).

ICER does not seem to be a downstream target of mature TCR signalling, since mice deficient for either TCR $\gamma\delta$ (potentially a positive effector) or TCR $\alpha\beta$ (potentially a negative effector) do not show perturbations in ICER expression. In particular, ICER expression is neither lost in pre-T cells of TCR $\delta^{-/-}$ mice, nor is it increased in DP cells of TCR $\alpha^{-/-}$ mice (Figure 36).

Furthermore, our data demonstrate that ICER expression is a characteristic of cells that have adopted a “ $\gamma\delta$ -like” T cell fate, regardless of whether they have developed as a consequence of differentiation and/or survival signals from either a $\alpha\beta$ or $\gamma\delta$ TCR. This is evident in the lymph nodes of male HY-TCR transgenic mice, where CD4(-) CD8(-) TCR $\alpha\beta$ (+) TCR $\gamma\delta$ (-) cells that have “ $\gamma\delta$ -like” characteristics (Bruno et al., 1996) express a level of ICER consistent with that seen in conventional lymphoid $\gamma\delta$ T cells (Figure 37). To compliment these data, we also failed to observe

ICER expression in DP cells from TCR $\beta^{-/-}$ mice. These cells have characteristics of the $\alpha\beta$ T cell lineage even though they have been shown to be dependent on the TCR $\gamma\delta$ for development (Livak et al., 1997).

Importantly, the observation that a marker for the $\gamma\delta$ lineage segregates independently of TCR $\gamma\delta$ strongly supports a non-instructive, separate lineage model for $\alpha\beta$ / $\gamma\delta$ lineage commitment.

As ICER expression is a marker of developmental lineage rather than the particular TCR that is expressed *per se*, it provides a unique tool to investigate the nature of less well characterised T cell populations such as intestinal intraepithelial lymphocytes (IELs).

Traditionally, the major subdivision of IELs is based on the expression of either the $\alpha\beta$ or the $\gamma\delta$ TCR. Furthermore, the expression of CD8 α and CD8 β defines four major populations that are referred to as TCR $\gamma\delta$ DN, TCR $\gamma\delta$ CD8 $\alpha\alpha$, TCR $\alpha\beta$ CD8 $\alpha\alpha$ and TCR $\alpha\beta$ CD8 $\alpha\beta$ (Shires et al., 2001). ICER expression in both the TCR $\gamma\delta$ DN and TCR $\gamma\delta$ CD8 $\alpha\alpha$ IEL populations was comparable to that observed in $\gamma\delta$ T cell populations from the thymus, lymph nodes and spleen. However, although TCR $\alpha\beta$ CD8 $\alpha\beta$ IELs did not express ICER, a result consistent with their $\alpha\beta$ lineage classification, TCR $\alpha\beta$ CD8 $\alpha\alpha$ IELs expressed a level of ICER similar to that seen in $\gamma\delta$ T cells (Figure 35), suggesting that the TCR $\alpha\beta$ CD8 $\alpha\alpha$ IEL subset is a “ $\gamma\delta$ -like” population.

It has previously been demonstrated that TCR $\alpha\beta$ CD8 $\alpha\alpha$ IELs are not MHC class-I restricted and use TCR β chains that would normally be deleted in the thymus (reviewed in Hayday, 2001). Taken together, these results demonstrate that the conventional classification of T cells into $\alpha\beta$ and $\gamma\delta$ is not fully appropriate for all T cell populations.

Furthermore, the data suggest that a refined classification of T cells could be employed that includes, on the one hand, conventional $\alpha\beta$ T cells and, on the other, both TCR $\gamma\delta(+)$ T cells and non-conventional TCR $\alpha\beta(+)$ T cells. In such a revised classification ICER expression would be a lineage marker for the latter cell type.

Since ICER marks mature $\gamma\delta$ cells (in both the thymus and the periphery), we considered the hypothesis of it also segregating with DN precursors committed to the $\gamma\delta$ lineage. In this context, it is interesting to note that $\text{TCR}\delta^{-/-}$ pre-T cells expressed ICER at levels consistently higher than in WT DN populations (Figure 36). The $\text{TCR}\delta^{-/-}$ DN4 subset, in particular, expressed abnormally high levels of ICER transcripts, evocative of “ $\gamma\delta$ -like” cells. Since these pre-T cells are incapable of expressing $\text{TCR}\gamma\delta$, which defines the $\gamma\delta$ lineage, they could be accumulating $\gamma\delta$ -committed precursors (particularly at the DN4 stage).

This hypothesis was tested in RTOC by analysing the lineage potential of pre-T cells expressing high *vs.* low levels of ICER, as indicated by LacZ staining of $\text{CREM}/\text{ICER-LacZ}$ thymocytes (Figure 38). However, neither early (DN2) nor late (DN4) pro/pre-T cells expressing high levels of ICER-LacZ protein appeared to be biased towards the $\gamma\delta$ lineage. The major difference between low and high expressors of ICER was their proliferation rates, which accounted for the higher RTOC cellularity with ICER-high cells. This difference in proliferation rate was reflected in the $\alpha\beta/\gamma\delta$ ratios, as expected as $\alpha\beta$ lineage cells expand much more than $\gamma\delta$ thymocytes.

Thus, ICER expression in DN thymocytes does not mark $\gamma\delta$ -precursors and is likely to be governed by a distinct mechanism to the one operating in mature T cells (responsible for the restricted expression in the $\gamma\delta$ lineage).

The extensive generation of $\alpha\beta$ cells by pre-T cells expressing high levels of ICER-LacZ protein prompted us to investigate the relationship between ICER expression and β -selection.

4 Pre-TCR dependent expression of ICER in pre-T cells

The fact that ICER was also expressed in thymic DN precursors but was down-regulated during DN → DP transition led us to investigate the role of the pre-TCR in controlling its expression. Contrary to our initial prediction that the pre-TCR signalling would decrease ICER expression, leading to its absence in DP thymocytes, the data obtained revealed that the pre-TCR actually induces ICER expression in pre-T cells.

ICER expression is lost in pre-T cells deficient for pre-TCR signalling (from TCRβ^{-/-}, pTα^{-/-}, p56Lck^{-/-}, RAG-1^{-/-} mice; Figure 40). Furthermore, it can be rescued by a pre-TCR-type of stimulus. Indeed, ICER was one of the genes obtained from the RDA analysis of RAG-deficient pre-T cells stimulated *vs.* non-stimulated with anti-CD3ε mAb (Table 5).

The kinetics of ICER induction by CD3ε stimulation *in vivo* are consistent with the early response and transient characteristics of the expression of this gene. Up-regulation of ICER mRNA is notable after 1.5 hours and peaks at 3 hours (Figure 41). Of all genes from this RDA analysis, ICER was the one with fastest kinetics.

At the DN3 stage, ICER expression can be induced by pre-TCR-like signals: purified RAG-deficient DN3 cells show a 25-fold up-regulation of ICER levels upon administration of anti-CD3ε mAb *in vitro* (FTOC); the same treatment of purified WT DN3 cells leads to an increased expression of ICER-LacZ protein, both in RTOC (Figure 41) and in cell suspension (Figure 42).

As ICER mRNA expression in DN3 thymocytes is dependent on pre-TCR signalling and is induced very rapidly by pre-TCR-like stimuli, ICER is genetically downstream of the pre-TCR. Furthermore, the fact that CD3-mediated induction of ICER can occur in purified DN3 thymocytes in cell suspension implies that this is a cell-autonomous process (direct signalling event).

Our results with specific inhibitors of signalling pathways suggest that the MAPK pathway has an important role in pre-TCR-mediated induction of ICER

expression (Figure 42). MEK1 inhibitor PD98059 specifically blocks ICER-LacZ up-regulation by anti-CD3 mAb. At a concentration of 25 μM of this drug, a 62% inhibition was observed. At higher concentrations (50 μM) of the inhibitor, effects can be less specific and a higher incidence of cell death occurs (data not shown).

The MAPK pathway has been previously implicated as a transducer of pre-TCR signalling essential for the DN \rightarrow DP transition (Crompton, 1996), with proliferation and survival of " β -selected" cells being dependent on ERK-1/2 activation.

A complete inhibition of pre-TCR mediated ICER induction might also require the impairment of the PKC signalling pathway, as inferred from the effect of Roche compound RO-8220. This drug is a PKC inhibitor, but it also perturbs MAPK signalling. Treatment of DN3 thymocytes with RO-8220 produced a 53% inhibition of ICER induction following anti-CD3 mAb treatment. The use of both PD98059 and RO-8220 inhibitors simultaneously was inconclusive because it decreased cell viability significantly (data not shown).

The extent of the contribution of the PKC pathway to this induction is difficult to assess due to lack of specific inhibitors that do not affect the MAPK pathway. However, the results with PD98059, a MEK1 inhibitor with no cross-effect with PKCs, suggest that the major input to the CREM-LacZ induction by CD3 triggering comes from the MAPK pathway.

Moreover, substitution of anti-CD3 ϵ antibody by phorbol ester PDBu, which activates both PKC (directly) and MAPK (via RasGRP) pathways, was able to induce LacZ expression up to 72% of the level achieved by anti-CD3 ϵ .

These data further support a direct signalling mechanism from the pre-TCR, via the MAPK pathway, to induce ICER expression at the DN3 stage of thymocyte development. This is one of very few cAMP-independent processes of ICER induction so far described. The first was reported by Monaco *et al.* (Monaco and Sassone-Corsi, 1997) and involved nerve growth factor (NGF). Interestingly, the MAPK pathway was also implicated in that system.

The mechanism responsible for ICER induction in pre-T cells operates in a short developmental window, since DP thymocytes do not express the gene. This

transient characteristic is consistent with an event mediated by pre-TCR signalling, as this complex is only present on the surface of DN3 and DN4 thymocytes.

The analysis of ICER-deficient mice showed that ICER does not play a non redundant role in β -selection, as the DN3 \rightarrow DN4 (\rightarrow DP) transition proceeds normally in its absence (Figure 43).

However, ICER-LacZ protein expression proved to be a good indicator of the DN3 \rightarrow DN4 transition, which is mediated by the pre-TCR. ICER-LacZ protein expression correlates with pre-TCR levels in this transition (Figure 39), and ICER-LacZ(+) cells show characteristics typical of “ β -selected” thymocytes: they are large, cycling, icTCR β (+) thymocytes that have up-regulated expression of CD2 and EGR-1, genes previously implicated in this selection process (Figures 45-46).

Nurr-77 and cyclin D3 were also up-regulated in ICER-LacZ (+) pre-T cells (Figure 46). Nurr-77 was another gene that we identified as pre-TCR responsive (Figures 20-21), whose involvement in β -selection is currently being investigated. Cyclin D3 has been reported to be a crucial factor in the rapid proliferation of memory T lymphocytes. Memory T cells showed a 6-fold increase in cyclin D3 content when compared to naïve cells (Veiga-Fernandes et al., 2000). This has been suggested to represent an advance in cell cycle (“late G1 stage”), thus allowing cells to proliferate faster (lag time of 12 hours, in contrast with 27 hours for naïve T cells). Following the same rationale, higher levels of cyclin D3 in ICER-LacZ(+) pre-T cells might also favour their rapid proliferation.

Consistent with the hypothesis of being “ β -selected” thymocytes, ICER-LacZ(+) pre-T cells differentiate (*in vitro*) to the DP stage much faster than ICER-LacZ(-) thymocytes (Figure 44). Whereas ICER(+) 6-day FTOCs show a subset distribution in which DP > ISP > DN (in absolute numbers), the opposite is observed in ICER(-) FTOCs (hanging drop system). To minimise differences of the two thymocyte types in the colonisation of the thymic stroma, the experiments were also done in RTOC (in which circa 20, instead of 1, thymic lobes were used as source of stromal cells). The results were identical, suggesting an intrinsic difference in developmental potential of the thymocyte populations.

Since ICER-LacZ expression correlates with β -selection, it should become more critical at the DN4 stage of development, which is largely dependent on that process. In agreement with this, the *in vitro* differentiation potential of DN4-low thymocytes (output/input ratio = 0.7) is much more compromised than that of DN3-low cells (output/input ratio = 3.6) (Figure 44 A/B).

Interestingly, ICER mRNA expression was lower in DN4-high than in DN4-low cells (for levels of ICER-LacZ protein). Thus, the rapid down-regulation of ICER mRNA following its induction (possibly via an auto-regulatory feedback loop (Molina et al., 1993)) is not accompanied by a decrease in ICER-LacZ protein expression. ICER-LacZ protein down-regulation seems to be delayed (compared to the mRNA), since it only occurs at the DP stage (Figures 38-39). This is probably due to the intrinsic kinetics (synthesis / degradation) of the β -galactosidase protein (coded by the LacZ gene under the control of ICER promoter).

In fact, since "DN4-high" cells are mainly intracellular icTCR β (+), they should express high levels of surface TCR β and, therefore, express less ICER mRNA (Figure 28). This is in line with the fact that $\alpha\beta$ -committed DN4 thymocytes tend to express low levels of ICER mRNA (Figures 28-29), even if they still retain a high ICER-LacZ protein content (due to the mentioned delay in its down-regulation).

Arrival at the DP stage promotes pre-TCR disassembly by displacement of pre-T α by newly synthesised TCR α (Trop et al., 2000). Theoretically, the new antigen receptor complex, TCR $\alpha\beta$, could actively down-regulate ICER in the $\alpha\beta$ lineage. However, our data from TCR $\alpha^{-/-}$ thymocytes showed this is not the case, as ICER (low) expression in DP cells is not altered by the absence of TCR $\alpha\beta$ (Figure 36).

Thus, it is more likely that pre-TCR signalling produces a transient induction of ICER transcription at the DN3 stage, which is extinguished at the DN4 stage. This is supported by the data regarding the *in vivo* stimulation with anti-CD3 mAb, where a marked decrease was observed 24 hours after mAb injection (Figure 41), the same time-frame required to down-regulate CD25 (which defines the DN4 stage) (Figure 18). As previously mentioned, transience is a characteristic of ICER induction in various systems (Sassone-Corsi, 1998).

5 Cross-talk between $\alpha\beta$ and $\gamma\delta$ T cell differentiation

Unlike $\alpha\beta$ cells, $\gamma\delta$ thymocytes express ICER, and in fact their levels of expression are three-fold higher than those of pre-T cells (Figure 27). Having established ICER as a $\gamma\delta$ lineage marker, it was surprising to realise that its expression could be induced by the pre-TCR, whose signalling consequences have been suggested to favour commitment to the $\alpha\beta$ lineage, in detriment of the $\gamma\delta$ lineage (Fehling et al., 1995a; von Boehmer et al., 1999).

While examining these apparently contradictory results, we observed that ICER expression was severely reduced in pre-TCR deficient mice ($pT\alpha^{-/-}$, $TCR\beta^{-/-}$), not only in pre-T cells, but also in $\gamma\delta$ thymocytes and splenocytes (Figure 47). Moreover, this effect was not restricted to ICER but was a specific phenomenon for a few “ $\gamma\delta$ genes” studied, including NOR-1 and myeloblastin.

This observation prompts us to revisit the much debated role of the pre-TCR in $\alpha\beta$ / $\gamma\delta$ lineage commitment. Different groups working on this topic have obtained contradictory data.

On one hand, pre-TCR deficient mice, in addition to their low numbers of $\alpha\beta$ cells, have increased absolute numbers of $\gamma\delta$ cells. This would suggest that the pre-TCR not only is not necessary for $\gamma\delta$ development, but also seems to divert cells from the $\alpha\beta$ to the $\gamma\delta$ pathway of differentiation.

On the other hand, detailed analysis of TCR β rearrangements by three independent groups (Burtrum et al., 1996; Dudley et al., 1995; Mertsching et al., 1997) has evidenced a selection for in-frame rearranged V β gene segments in $\gamma\delta$ cells. Moreover, Wilson *et al.* have detected intracellular TCR β protein in a significant (17%) subset of $\gamma\delta$ cells, and showed that $\gamma\delta$ cells of pre-TCR deficient mice proliferate less than wild type $\gamma\delta$ cells (Wilson and MacDonald, 1998). They concluded there was a “limited role” for the pre-TCR in $\gamma\delta$ cell physiology. This role would not be essential for $\gamma\delta$ development since, as mentioned, pre-TCR-deficient mice have normal (or even increased) numbers of $\gamma\delta$ cells.

In this context, it is interesting to note the results obtained by Bruno *et al.* (1999) regarding thresholds of pre-TCR expression and $\alpha\beta$ / $\gamma\delta$ lineage commitment. They observed that DN3 and even DN4 thymocytes that clearly expressed pre-TCR on the cell surface still generated $\gamma\delta$ cells in RTOC. Only DN4 cells expressing the very highest pre-TCR levels seemed to be committed to the $\alpha\beta$ lineage (Bruno *et al.*, 1999).

According to those results, it is possible that a pre-TCR(+) subset of $\gamma\delta$ thymocytes exists. The major obstacle to such an assumption is the fact that pT α protein has not been detected in the cytoplasm of $\gamma\delta$ cells (von Boehmer *et al.*, 1999).

However, even if such a subset exists, pre-TCR signalling cannot account for ICER expression in $\gamma\delta$ cells: whereas only 17% of $\gamma\delta$ thymocytes express TCR β intracellularly (Wilson and MacDonald, 1998), the wide majority (>90%) of that population express ICER-LacZ protein (Figure 38).

Furthermore, pro-T cells (DN2 thymocytes) also express ICER (Figure 27). Since DN2 cells are known not to express the pre-TCR (Capone *et al.*, 1998; Godfrey *et al.*, 1994), this is incompatible with a *cis*-mechanism for ICER induction in these cells.

To be certain that ICER(+) cells in the DN2 subset were not a consequence of contamination with more mature cells, single ICER(+) DN2 thymocytes were sorted and analysed for the expression of rearranged TCR β gene segments by single-cell PCR. No amplification signal was detected in many ICER(+) pro-T cells (data not shown), excluding an endogenous pre-TCR-mediated induction of ICER expression.

Since ICER expression in $\gamma\delta$ and pro-T cells is severely compromised in pre-TCR-deficient mice, but this does not seem to be due to a direct (pre-TCR) effect, we hypothesised the existence of a pre-TCR dependent *trans*-induction mechanism responsible for ICER induction in these cells. This mechanism could also account for the expression of other genes (NOR-1, myeloblastin) whose expression is impaired in TCR β ^{-/-} $\gamma\delta$ cells (Figure 47).

Such a mechanism could be the explanation for the data first obtained by Wilson *et al.* (Wilson and MacDonald, 1998) and Kohyama *et al.* (Kohyama *et al.*, 1999) that showed that $\text{TCR}\beta^{-/-}$ $\gamma\delta$ cells have impaired proliferation and effector functions (when compared to WT $\gamma\delta$ cells) - **Figure 51**.

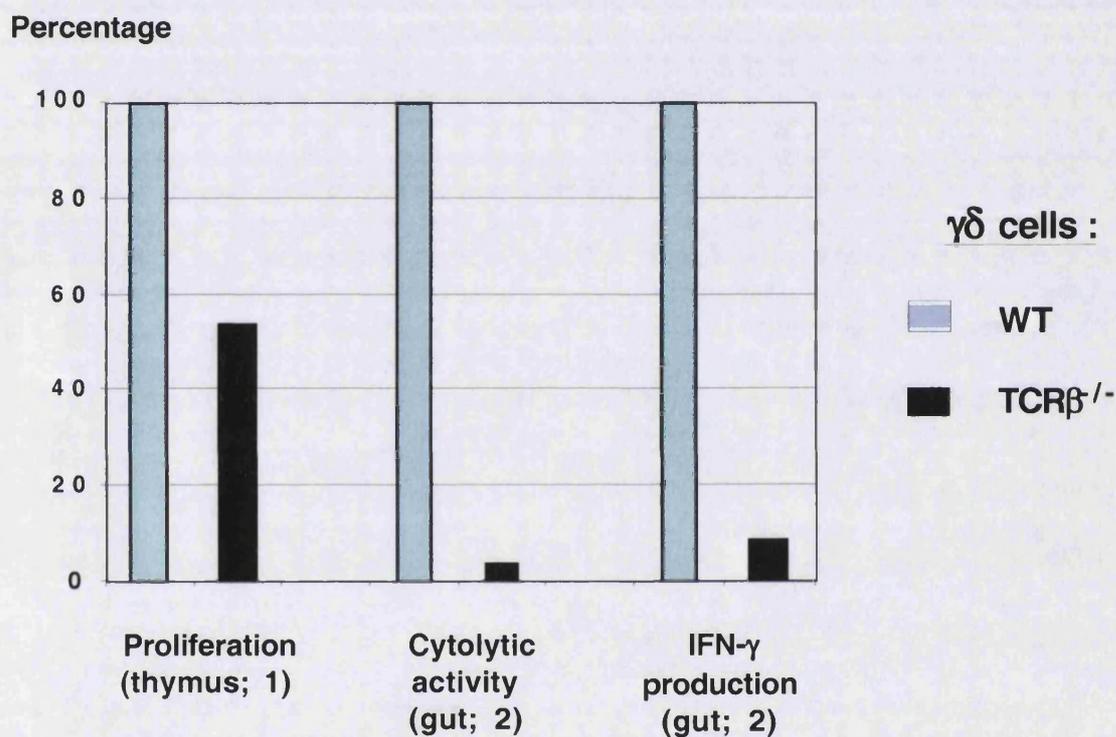


Figure 51 : Comparison of proliferation and effector functions of WT and $\text{TCR}\beta$ -deficient $\gamma\delta$ cells. Proliferation was estimated by PI staining in (1) Wilson *et al.* (Wilson and MacDonald, 1998). Cytolytic activity of target (^{51}Cr -labelled) cells and IFN- γ production (measured by ELISA) were reported in (2) Kohyama *et al.* (Kohyama *et al.*, 1999).

More data supporting the role of a *trans*-induction mechanism in the context of $\gamma\delta$ T cell differentiation was obtained from ICER's pattern of expression during murine development (Figure 48). ICER expression in DN2 and $\gamma\delta$ thymocytes was only significant after birth, but not during embryonic life (E16 or E18). However, the pre-TCR is expressed on the surface of foetal thymocytes from E15 onwards (Bruno et al., 1999). Therefore, this pattern suggests that a particular, β -selection dependent, composition of the thymus (as found in adult mice) is necessary for ICER expression. Consistent with this view, we observed that WT pro/pre-T cells developing in a $RAG^{-/-}$ host thymus (bone marrow chimera, Figure 49) failed to express normal levels of ICER (as found in a WT thymus).

The required components of the *trans*-induction mechanism could, *a priori*, belong to either the stromal or the thymocyte compartments of the organ. However, our results with pre-TCR deficient thymuses ($pT\alpha^{-/-}$, $TCR\beta^{-/-}$), for which no stromal deficiencies have been reported, suggest that it is the absence of a thymocyte population that causes the loss of the proposed induction mechanism.

The major change in the composition of the thymus over that developmental time frame is an accumulation of DP and SP cells of the $\alpha\beta$ lineage. Since the loss of ICER expression is not observed in $TCR\alpha$ -deficient mice (Figure 36), which specifically lack SP cells, we hypothesised that the critical link is the DP population.

The overwhelming abundance of DP cells in the cortex of a normal thymus could explain how this *trans*-induction mechanism is able to affect the other rarer subsets on a population scale (normal distributions of ICER-LacZ protein). Interestingly, according to the data of Petrie and collaborators regarding the localisation of cell subsets within the thymus, DN2 thymocytes are ideally localised for a direct interaction with DP cells (Lind et al., 2001).

To test this hypothesis, we set up an RTOC system in which target cells of the *trans*-mechanism, $\gamma\delta$ thymocytes in particular, were subjected to the presence of a large excess of β -selected DP thymocytes (the effector cells). In more detail, 1.5×10^4 $TCR\beta^{-/-}$ $\gamma\delta$ thymocytes were mixed with 1.5×10^6 DP cells and re-sorted after 9 days

in culture. The target cells had a pre-TCR-deficient background, so that they did not express ICER at the beginning of the experiment. The results showed a 4-fold upregulation of ICER expression in the $\text{TCR}\beta^{-/-}$ $\gamma\delta$ thymocytes that had been incubated with DP cells, when compared to “non-treated” $\text{TCR}\beta^{-/-}$ $\gamma\delta$ cells (Figure 50).

These data are consistent with the requirement of a normal (β -selected) cellular composition of the thymus for ICER expression, and pinpoint DP thymocytes as the effectors of the *trans*-induction mechanism responsible for ICER expression in $\gamma\delta$ and pro-T cells. Furthermore, they clarify the role of β -selection in $\gamma\delta$ cell development, which had been previously postulated (Kohyama et al., 1999; Wilson and MacDonald, 1998) but never examined in detail.

Thus, we propose that β -selection is important for $\gamma\delta$ cell differentiation because it provides a large pool of highly metabolic DP thymocytes (known, for example, to produce large amounts of cytokines) that act on $\gamma\delta$ thymocytes and induce them to express a subset of genes, including the $\gamma\delta$ lineage marker ICER. Some of these genes are most probably important for $\gamma\delta$ cell physiology, as attested by the deficient proliferation of $\text{TCR}\beta^{-/-}$ $\gamma\delta$ thymocytes (Wilson and MacDonald, 1998) and the impaired cytokine production and cytotoxicity of $\text{TCR}\beta^{-/-}$ $\gamma\delta$ lymphocytes (Kohyama et al., 1999).

The nature of the proposed interaction between DP cells and $\gamma\delta$ thymocytes is still unknown. It may be direct (cell-cell) or indirect (via soluble molecule). Interestingly, we have observed (by RT-PCR) that DP cells express higher levels of Notch receptors (-1 and -3) than do $\gamma\delta$ thymocytes, whereas the reverse is true for Notch ligand Jagged-1 (data not shown; collaborative work with Katsuto Hozumi). Since Notch signalling has been suggested to be important for cell fate determination, including $\alpha\beta/\gamma\delta$ lineage commitment (Introduction, 3.5.2), these molecules could be regarded as attractive candidates for the *trans*-induction mechanism. However, Notch receptors are expressed on thymocytes much before the DP stage, and no link between their expression and β -selection has been observed, as it would be required for the main players in this mechanism.

In terms of soluble molecules, one obvious candidate would be IL-7, since the expression of its receptor mirrors that of ICER: is high in $\gamma\delta$ and DN2 thymocytes, but not in DP cells. Moreover, IL-7/IL-7R signalling is known to play a crucial role in $\gamma\delta$ cell differentiation (Introduction, 3.5.1). However, such hypothesis requires DP cells to secrete IL-7, which has not been reported. Furthermore, it is difficult to envisage how that eventual cytokine production by DP cells would be determinant for $\gamma\delta$ thymocyte differentiation, since thymic epithelial cells are known to actively produce IL-7 (see Introduction, 2.2). All these aspects have to be considered when trying to dissect the components of the *trans*-induction mechanism.

6 Conclusion

We have performed a detailed study on transcription factor ICER in the context of T cell development. The complex pattern of ICER expression during T cell differentiation is summarised in **Figure 52**.

On one hand, we have shown that ICER is a nuclear target of pre-TCR signalling. The facts that ICER expression is so rapidly (90 minutes) up-regulated *in vivo* and that it is inducible in isolated DN3 thymocytes in cell suspension (*in vitro*) via the MAPK pathway, suggest that it can be induced by a *cis*-operating mechanism in pre-TCR(+) cells.

On the other hand, in pre-TCR(-) thymocytes, such as pro-T and $\gamma\delta$ cells, ICER expression seems to be regulated by a *trans*-induction mechanism that arises from "β-selected" DP cells and, as such, is dependent on the pre-TCR. In Figure 52, we attribute this effect of DP cells on pro-T and $\gamma\delta$ cells to the expression of an unidentified ligand and receptor pair (named "X") in effector and target cells, respectively. However, we have no data that proves that cell-cell contact is required for the induction; it's equally plausible that DP cells secrete a soluble factor that acts on target cells.

Several aspects of this model still require clarification: first, the identification of the precise components of the DP/ $\gamma\delta$ interaction (ligand? soluble factor?); second, the signalling details associated with the gene induction (MAPK pathway? cyclic-AMP pathway?); third, the justification for the lack of ICER induction in DP and SP cells (lack of receptor?). As it is so often the case, the results obtained have provided more new questions than definitive answers.

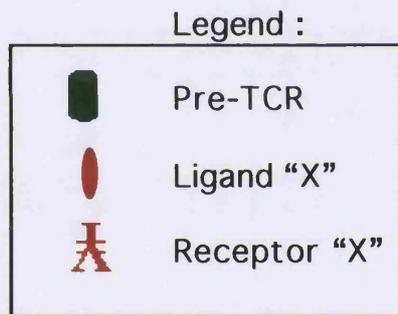
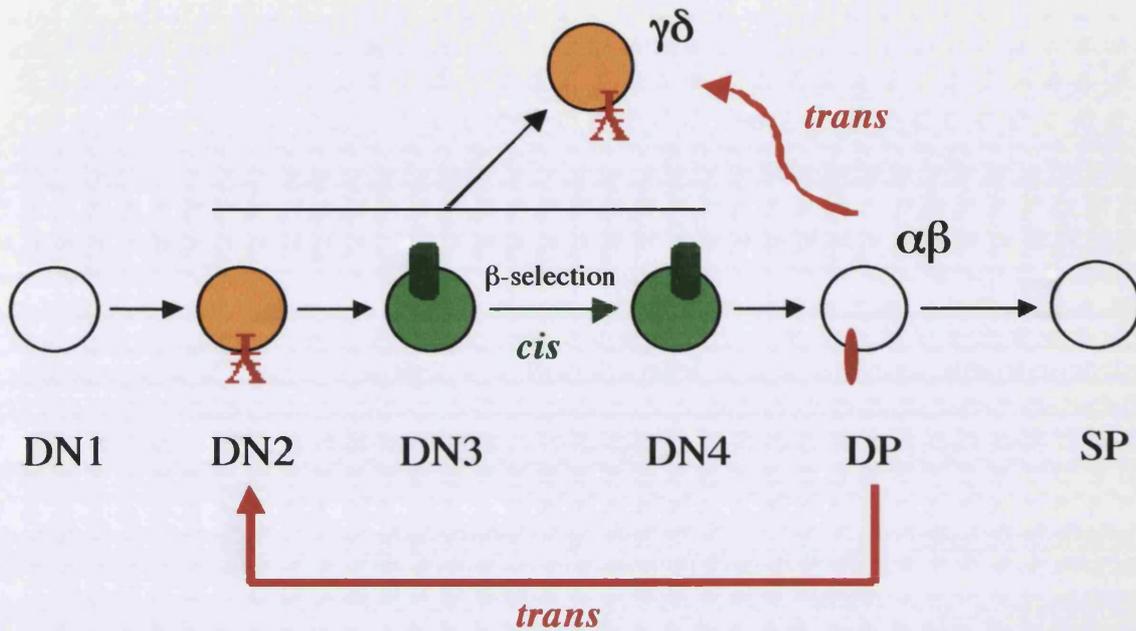


Figure 52 : Model for ICER expression during T cell differentiation. ICER expression in T cells is dependent on β -selection by two mechanisms: a direct (*cis*) signalling process mediated by the pre-TCR on DN3 and DN4 thymocytes (represented in green); and an indirect (*trans*) effect of DP cells (product of β -selection) on DN2 and $\gamma\delta$ cells (represented in red). We speculate that DP cells express a ligand "X" that interacts with a receptor "X" expressed on the surface of DN2 and $\gamma\delta$ cells. (Coloured circles represent ICER-expressing subsets).

The relative contributions of the pre-TCR dependent *cis and trans*-induction mechanisms are not clear for pre-T cells (DN3/DN4 stages). Although our data clearly showed that pre-TCR signalling can directly (*cis*) induce ICER expression in pre-TCR(+) cells, and that there is a strong correlation between the expression of pre-TCR and ICER-LacZ protein, it is still possible that the *trans*-mechanism is dominant also in pre-T cells (as suggested by the bone marrow chimera data).

The full extent of the gene alterations caused by the *trans*-induction mechanism also need to be examined. These experiments are currently under way. Ideally, some of those alterations should correlate with the phenotype of TCR $\beta^{-/-}$ $\gamma\delta$ cells: reduced proliferation, impaired cytokine production and cytotoxicity.

It is most interesting that ICER, being a $\gamma\delta$ lineage marker, depends on cells of the $\alpha\beta$ lineage for its expression. This is the first time such a mechanism is proposed for thymocyte development, and it demands a re-examination of T cell lineage relationships, both in terms of their differentiation and their physiology. In this context, it is important to note that TCR $\beta^{-/-}$ mice, traditionally taken as a model for $\gamma\delta$ lymphocyte physiology, may be far from producing "normal" $\gamma\delta$ cell populations. Therefore we suggest that all the data interpreted following that assumption should be re-evaluated.

In conclusion, our research led to the identification of multiple genes differentially expressed between developmental stages and lineages of T cells. The importance of some of them was further assessed in *in vitro* and *in vivo* systems.

In particular, we showed that IL-7R expression was maintained by pre-TCR signalling in the DN4 stage of development and was important for further maturation to the DP stage.

We identified ICER as a $\gamma\delta$ lineage marker, the first to be described besides the TCR $\gamma\delta$ itself. Expression studies provided evidence supporting a separate lineages model for $\alpha\beta$ / $\gamma\delta$ lineage divergence. They also suggested the existence of a mechanism by which $\alpha\beta$ lineage cells (dependent on β -selection) influence the fate of $\gamma\delta$ lineage thymocytes. This is a novel insight into lineage relationships during T cell development.

References

- Aifantis, I., O. Azogui, J. Feinberg, C. Saint-Ruf, J. Buer, and H. von Boehmer. 1998. On the role of the pre-T cell receptor in alphabeta versus gammadelta T lineage commitment. *Immunity*. 9:649-655.
- Aifantis, I., J. Buer, H. von Boehmer, and O. Azogui. 1997. Essential role of the pre-T cell receptor in allelic exclusion of the T cell receptor beta locus. *Immunity*. 7:601-607.
- Aifantis, I., J. Feinberg, H.J. Fehling, J.P. Di Santo, and H. von Boehmer. 1999a. Early T cell receptor beta gene expression is regulated by the pre-T cell receptor-CD3 complex. *J Exp Med*. 190:141-144.
- Aifantis, I., F. Gounari, L. Scorrano, C. Borowski, and H. von Boehmer. 2001. Constitutive pre-TCR signaling promotes differentiation through Ca²⁺ mobilization and activation of NF-kappaB and NFAT. *Nat Immunol*. 2:403-409.
- Aifantis, I., V.I. Pivniouk, F. Gartner, J. Feinberg, W. Swat, F.W. Alt, H. von Boehmer, and R.S. Geha. 1999b. Allelic exclusion of the T cell receptor beta locus requires the SH2 domain-containing leukocyte protein (SLP)-76 adaptor protein. *J Exp Med*. 190:1093-1102.
- Akashi, K., M. Kondo, S. Cheshier, J. Shizuru, K. Gandy, J. Domen, R. Mebius, D. Traver, and I.L. Weissman. 1999. Lymphoid development from stem cells and the common lymphocyte progenitors. *Cold Spring Harb Symp Quant Biol*. 64:1-12.
- Akashi, K., M. Kondo, U. von Freeden-Jeffry, R. Murray, and I.L. Weissman. 1997. Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. *Cell*. 89:1033-1041.
- Akashi, K., M. Kondo, and I.L. Weissman. 1998. Role of interleukin-7 in T-cell development from hematopoietic stem cells. *Immunol Rev*. 165:13-28.
- Akashi, K., T. Reya, D. Dalma-Weiszhausz, and I.L. Weissman. 2000. Lymphoid precursors. *Curr Opin Immunol*. 12:144-150.
- Alberola-Ila, J., K.A. Forbush, R. Seger, E.G. Krebs, and R.M. Perlmutter. 1995. Selective requirement for MAP kinase activation in thymocyte differentiation. *Nature*. 373:620-623.
- Alberola-Ila, J., K.A. Hogquist, K.A. Swan, M.J. Bevan, and R.M. Perlmutter. 1996. Positive and negative selection invoke distinct signaling pathways. *J Exp Med*. 184:9-18.
- Allison, J.P., and L.L. Lanier. 1987. Structure, function, and serology of the T-cell antigen receptor complex. *Annu Rev Immunol*. 5:503-540.
- Alt, F.W., G.D. Yancopoulos, T.K. Blackwell, C. Wood, E. Thomas, M. Boss, R. Coffman, N. Rosenberg, S. Tonegawa, and D. Baltimore. 1984. Ordered rearrangement of immunoglobulin heavy chain variable region segments. *Embo J*. 3:1209-1219.
- Amsen, D., and A.M. Kruisbeek. 1998. Thymocyte selection: not by TCR alone. *Immunol Rev*. 165:209-229.

- Amsen, D., C. Revilla Calvo, B.A. Osborne, and A.M. Kruisbeek. 1999. Costimulatory signals are required for induction of transcription factor Nur77 during negative selection of CD4(+)CD8(+) thymocytes. *Proc Natl Acad Sci U S A.* 96:622-627.
- Anderson, G., K.L. Anderson, L.A. Conroy, T.J. Hallam, N.C. Moore, J.J. Owen, and E.J. Jenkinson. 1995. Intracellular signaling events during positive and negative selection of CD4+CD8+ thymocytes in vitro. *J Immunol.* 154:3636-3643.
- Anderson, G., B.C. Harman, K.J. Hare, and E.J. Jenkinson. 2000a. Microenvironmental regulation of T cell development in the thymus. *Semin Immunol.* 12:457-464.
- Anderson, G., and E.J. Jenkinson. 1995. The role of the thymus during T-lymphocyte development in vitro. *Semin Immunol.* 7:177-183.
- Anderson, G., and E.J. Jenkinson. 2000b. Review article: thymus organ cultures and T-cell receptor repertoire development. *Immunology.* 100:405-410.
- Anderson, G., E.J. Jenkinson, N.C. Moore, and J.J. Owen. 1993. MHC class II-positive epithelium and mesenchyme cells are both required for T-cell development in the thymus. *Nature.* 362:70-73.
- Anderson, G., J.J. Owen, N.C. Moore, and E.J. Jenkinson. 1994a. Thymic epithelial cells provide unique signals for positive selection of CD4+CD8+ thymocytes in vitro. *J Exp Med.* 179:2027-2031.
- Anderson, G., J.J. Owen, N.C. Moore, and E.J. Jenkinson. 1994b. Characteristics of an in vitro system of thymocyte positive selection. *J Immunol.* 153:1915-1920.
- Anderson, G., K.M. Partington, and E.J. Jenkinson. 1998. Differential effects of peptide diversity and stromal cell type in positive and negative selection in the thymus. *J Immunol.* 161:6599-6603.
- Anderson, G., J. Pongracz, S. Parnell, and E.J. Jenkinson. 2001. Notch ligand-bearing thymic epithelial cells initiate and sustain Notch signaling in thymocytes independently of T cell receptor signaling. *Eur J Immunol.* 31:3349-3354.
- Antica, M., L. Wu, K. Shortman, and R. Scollay. 1993. Intrathymic lymphoid precursor cells during fetal thymus development. *J Immunol.* 151:5887-5895.
- Antica, M., L. Wu, K. Shortman, and R. Scollay. 1994. Thymic stem cells in mouse bone marrow. *Blood.* 84:111-117.
- Apostolou, I., A. Cumano, G. Gachelin, and P. Kourilsky. 2000. Evidence for two subgroups of CD4-CD8- NKT cells with distinct TCR alpha beta repertoires and differential distribution in lymphoid tissues. *J Immunol.* 165:2481-2490.
- Appleby, M.W., J.A. Gross, M.P. Cooke, S.D. Levin, X. Qian, and R.M. Perlmutter. 1992. Defective T cell receptor signaling in mice lacking the thymic isoform of p59fyn. *Cell.* 70:751-763.
- Ardavin, C., L. Wu, C.L. Li, and K. Shortman. 1993. Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature.* 362:761-763.

- Artavanis-Tsakonas, S., M.D. Rand, and R.J. Lake. 1999. Notch signaling: cell fate control and signal integration in development. *Science*. 284:770-776.
- Asarnow, D.M., W.A. Kuziel, M. Bonyhadi, R.E. Tigelaar, P.W. Tucker, and J.P. Allison. 1988. Limited diversity of gamma delta antigen receptor genes of Thy-1+ dendritic epidermal cells. *Cell*. 55:837-847.
- Ashton-Rickardt, P.G., A. Bandeira, J.R. Delaney, L. Van Kaer, H.P. Pircher, R.M. Zinkernagel, and S. Tonegawa. 1994. Evidence for a differential avidity model of T cell selection in the thymus. *Cell*. 76:651-663.
- Ashton-Rickardt, P.G., and S. Tonegawa. 1994. A differential-avidity model for T-cell selection. *Immunol Today*. 15:362-366.
- Ashton-Rickardt, P.G., L. Van Kaer, T.N. Schumacher, H.L. Ploegh, and S. Tonegawa. 1993a. Peptide contributes to the specificity of positive selection of CD8+ T cells in the thymus. *Cell*. 73:1041-1049.
- Ashton-Rickardt, P.G., L. Van Kaer, T.N. Schumacher, H.L. Ploegh, and S. Tonegawa. 1993b. Repertoire-determining role of peptide in the positive selection of CD8+ T cells. *Immunol Rev*. 135:157-182.
- Ashwell, J.D., F.W. Lu, and M.S. Vacchio. 2000. Glucocorticoids in T cell development and function*. *Annu Rev Immunol*. 18:309-345.
- Bain, G., I. Engel, E.C. Robanus Maandag, H.P. te Riele, J.R. Volland, L.L. Sharp, J. Chun, B. Huey, D. Pinkel, and C. Murre. 1997. E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas. *Mol Cell Biol*. 17:4782-4791.
- Bain, G., E.C. Maandag, D.J. Izon, D. Amsen, A.M. Kruisbeek, B.C. Weintraub, I. Krop, M.S. Schlissel, A.J. Feeney, M. van Roon, and et al. 1994. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell*. 79:885-892.
- Bandeira, A., S. Itohara, M. Bonneville, O. Burlen-Defranoux, T. Mota-Santos, A. Coutinho, and S. Tonegawa. 1991. Extrathymic origin of intestinal intraepithelial lymphocytes bearing T- cell antigen receptor gamma delta. *Proc Natl Acad Sci U S A*. 88:43-47.
- Banwell, C.M., K.M. Partington, E.J. Jenkinson, and G. Anderson. 2000. Studies on the role of IL-7 presentation by mesenchymal fibroblasts during early thymocyte development. *Eur J Immunol*. 30:2125-2129.
- Barcena, A., A.H. Galy, J. Punnonen, M.O. Muench, D. Schols, M.G. Roncarolo, J.E. de Vries, and H. Spits. 1994. Lymphoid and myeloid differentiation of fetal liver CD34+lineage- cells in human thymic organ culture. *J Exp Med*. 180:123-132.
- Barcena, A., M.O. Muench, A.H. Galy, J. Cupp, M.G. Roncarolo, J.H. Phillips, and H. Spits. 1993. Phenotypic and functional analysis of T-cell precursors in the human fetal liver and thymus: CD7 expression in the early stages of T- and myeloid-cell development. *Blood*. 82:3401-3414.

- Barndt, R., M.F. Dai, and Y. Zhuang. 1999. A novel role for HEB downstream or parallel to the pre-TCR signaling pathway during alpha beta thymopoiesis. *J Immunol.* 163:3331-3343.
- Barndt, R.J., M. Dai, and Y. Zhuang. 2000. Functions of E2A-HEB heterodimers in T-cell development revealed by a dominant negative mutation of HEB. *Mol Cell Biol.* 20:6677-6685.
- Baron, A., K. Hafen, and H. von Boehmer. 1994. A human CD4 transgene rescues CD4-CD8+ cells in beta 2-microglobulin- deficient mice. *Eur J Immunol.* 24:1933-1936.
- Barton, K., N. Muthusamy, M. Chanyangam, C. Fischer, C. Clendenin, and J.M. Leiden. 1996. Defective thymocyte proliferation and IL-2 production in transgenic mice expressing a dominant-negative form of CREB. *Nature.* 379:81-85.
- Basson, M.A., T.J. Wilson, G.A. Legname, N. Sarnier, P.D. Tomlinson, V.L. Tybulewicz, and R. Zamoyska. 2000. Early growth response (Egr)-1 gene induction in the thymus in response to TCR ligation during early steps in positive selection is not required for CD8 lineage commitment. *J Immunol.* 165:2444-2450.
- Berg, L.J., and J. Kang. 2001. Molecular determinants of TCR expression and selection. *Curr Opin Immunol.* 13:232-241.
- Berger, M.A., M. Carleton, M. Rhodes, J.M. Sauder, S. Trop, R.L. Dunbrack, P. Hugo, and D.L. Wiest. 2000. Identification of a novel pre-TCR isoform in which the accessibility of the TCR beta subunit is determined by occupancy of the 'missing' V domain of pre-T alpha. *Int Immunol.* 12:1579-1591.
- Blendy, J.A., K.H. Kaestner, G.F. Weinbauer, E. Nieschlag, and G. Schutz. 1996. Severe impairment of spermatogenesis in mice lacking the CREM gene. *Nature.* 380:162-165.
- Blom, B., M.H. Heemskerk, M.C. Verschuren, J.J. van Dongen, A.P. Stegmann, A.Q. Bakker, F. Couwenberg, P.C. Res, and H. Spits. 1999a. Disruption of alpha beta but not of gamma delta T cell development by overexpression of the helix-loop-helix protein Id3 in committed T cell progenitors. *Embo J.* 18:2793-2802.
- Blom, B., P. Res, E. Noteboom, K. Weijer, and H. Spits. 1997. Prethymic CD34+ progenitors capable of developing into T cells are not committed to the T cell lineage. *J Immunol.* 158:3571-3577.
- Blom, B., P.C. Res, and H. Spits. 1998. T cell precursors in man and mice. *Crit Rev Immunol.* 18:371-388.
- Blom, B., M.C. Verschuren, M.H. Heemskerk, A.Q. Bakker, E.J. van Gastel-Mol, I.L. Wolvers-Tettero, J.J. van Dongen, and H. Spits. 1999b. TCR gene rearrangements and expression of the pre-T cell receptor complex during human T-cell differentiation. *Blood.* 93:3033-3043.
- Blomberg, H., and B. Andersson. 1971. Characterisation of the immunocompetent cells in the mouse thymus: cell population changes during cortisone-induced atrophy and subsequent regeneration. *Cell. Immunol.* 1:545-560.
- Blunt, T., N.J. Finnie, G.E. Taccioli, G.C. Smith, J. Demengeot, T.M. Gottlieb, R. Mizuta, A.J. Varghese, F.W. Alt, P.A. Jeggo, and et al. 1995. Defective DNA-dependent protein

- kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. *Cell*. 80:813-823.
- Bluthmann, H., P. Kisielow, Y. Uematsu, M. Malissen, P. Krimpenfort, A. Berns, H. von Boehmer, and M. Steinmetz. 1988. T-cell-specific deletion of T-cell receptor transgenes allows functional rearrangement of endogenous alpha- and beta-genes. *Nature*. 334:156-159.
- Bodor, J., J. Bodorova, and R.E. Gress. 2000. Suppression of T cell function: a potential role for transcriptional repressor ICER. *J Leukoc Biol*. 67:774-779.
- Bodor, J., L. Feigenbaum, J. Bodorova, C. Bare, M.S. Reitz, Jr., and R.E. Gress. 2001. Suppression of T-cell responsiveness by inducible cAMP early repressor (ICER). *J Leukoc Biol*. 69:1053-1059.
- Bonneville, M., I. Ishida, P. Mombaerts, M. Katsuki, S. Verbeek, A. Berns, and S. Tonegawa. 1989a. Blockage of alpha beta T-cell development by TCR gamma delta transgenes. *Nature*. 342:931-934.
- Bonneville, M., K. Ito, E.G. Krecko, S. Itohara, D. Kappes, I. Ishida, O. Kanagawa, C.A. Janeway, D.B. Murphy, and S. Tonegawa. 1989b. Recognition of a self major histocompatibility complex TL region product by gamma delta T-cell receptors. *Proc Natl Acad Sci U S A*. 86:5928-5932.
- Bonneville, M., C.A. Janeway, Jr., K. Ito, W. Haser, I. Ishida, N. Nakanishi, and S. Tonegawa. 1988. Intestinal intraepithelial lymphocytes are a distinct set of gamma delta T cells. *Nature*. 336:479-481.
- Boothby, M.R., A.L. Mora, D.C. Scherer, J.A. Brockman, and D.W. Ballard. 1997. Perturbation of the T lymphocyte lineage in transgenic mice expressing a constitutive repressor of nuclear factor (NF)-kappaB. *J Exp Med*. 185:1897-1907.
- Borgulya, P., H. Kishi, U. Muller, J. Kirberg, and H. von Boehmer. 1991. Development of the CD4 and CD8 lineage of T cells: instruction versus selection. *Embo J*. 10:913-918.
- Borgulya, P., H. Kishi, Y. Uematsu, and H. von Boehmer. 1992. Exclusion and inclusion of alpha and beta T cell receptor alleles. *Cell*. 69:529-537.
- Boyd, R.L., C.L. Tucek, D.I. Godfrey, D.J. Izon, T.J. Wilson, N.J. Davidson, A.G. Bean, H.M. Ladyman, M.A. Ritter, and P. Hugo. 1993. The thymic microenvironment. *Immunol Today*. 14:445-459.
- Brady, H.J., G. Gil-Gomez, J. Kirberg, and A.J. Berns. 1996. Bax alpha perturbs T cell development and affects cell cycle entry of T cells. *Embo J*. 15:6991-7001.
- Brockner, T., M. Riedinger, and K. Karjalainen. 1997. Targeted expression of major histocompatibility complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes in vivo. *J Exp Med*. 185:541-550.
- Bruno, L., H.J. Fehling, and H. von Boehmer. 1996a. The alpha beta T cell receptor can replace the gamma delta receptor in the development of gamma delta lineage cells. *Immunity*. 5:343-352.

- Bruno, L., J. Kirberg, and H. von Boehmer. 1995a. On the cellular basis of immunological T cell memory. *Immunity*. 2:37-43.
- Bruno, L., P. Res, M. Dessing, M. Cella, and H. Spits. 1997. Identification of a committed T cell precursor population in adult human peripheral blood. *J Exp Med*. 185:875-884.
- Bruno, L., B. Rocha, A. Rolink, H. von Boehmer, and H.R. Rodewald. 1995b. Intra- and extra-thymic expression of the pre-T cell receptor alpha gene. *Eur J Immunol*. 25:1877-1882.
- Bruno, L., A. Scheffold, A. Radbruch, and M.J. Owen. 1999. Threshold of pre-T-cell-receptor surface expression is associated with alphabeta T-cell lineage commitment. *Curr Biol*. 9:559-568.
- Bruno, L., H. von Boehmer, and J. Kirberg. 1996b. Cell division in the compartment of naive and memory T lymphocytes. *Eur J Immunol*. 26:3179-3184.
- Buckland, J. 2000b. T cell development: studies on p56Lck and CBF. University of London.
- Buckland, J., D.J. Pennington, L. Bruno, and M.J. Owen. 2000a. Co-ordination of the expression of the protein tyrosine kinase p56(lck) with the pre-T cell receptor during thymocyte development. *Eur J Immunol*. 30:8-18.
- Buer, J., I. Aifantis, J.P. DiSanto, H.J. Fehling, and H. von Boehmer. 1997a. Role of different T cell receptors in the development of pre-T cells. *J Exp Med*. 185:1541-1547.
- Buer, J., I. Aifantis, J.P. DiSanto, H.J. Fehling, and H. von Boehmer. 1997b. T-cell development in the absence of the pre-T-cell receptor. *Immunol Lett*. 57:5-8.
- Burtrum, D.B., S. Kim, E.C. Dudley, A.C. Hayday, and H.T. Petrie. 1996. TCR gene recombination and alpha beta-gamma delta lineage divergence: productive TCR-beta rearrangement is neither exclusive nor preclusive of gamma delta cell development. *J Immunol*. 157:4293-4296.
- Byth, K.F., L.A. Conroy, S. Howlett, A.J. Smith, J. May, D.R. Alexander, and N. Holmes. 1996. CD45-null transgenic mice reveal a positive regulatory role for CD45 in early thymocyte development, in the selection of CD4+CD8+ thymocytes, and B cell maturation. *J Exp Med*. 183:1707-1718.
- Cao, X., E.W. Shores, J. Hu-Li, M.R. Anver, B.L. Kelsall, S.M. Russell, J. Drago, M. Noguchi, A. Grinberg, E.T. Bloom, and et al. 1995. Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. *Immunity*. 2:223-238.
- Capone, M., J. Curnow, G. Bouvier, P. Ferrier, and B. Horvat. 1995. T cell development in TCR-alpha beta transgenic mice. Analysis using V(D)J recombination substrates. *J Immunol*. 154:5165-5172.
- Capone, M., R.D. Hockett, Jr., and A. Zlotnik. 1998. Kinetics of T cell receptor beta, gamma, and delta rearrangements during adult thymic development: T cell receptor rearrangements are present in CD44(+)/CD25(+) Pro-T thymocytes. *Proc Natl Acad Sci U S A*. 95:12522-12527.
- Capone, M., P. Romagnoli, F. Beermann, H.R. MacDonald, and J.P. van Meerwijk. 2001. Dissociation of thymic positive and negative selection in transgenic mice expressing

- major histocompatibility complex class I molecules exclusively on thymic cortical epithelial cells. *Blood*. 97:1336-1342.
- Capone, M., F. Watrin, C. Fernex, B. Horvat, B. Krippel, L. Wu, R. Scollay, and P. Ferrier. 1993. TCR beta and TCR alpha gene enhancers confer tissue- and stage- specificity on V(D)J recombination events. *Embo J*. 12:4335-4346.
- Carding, S.R., S. Kyes, E.J. Jenkinson, R. Kingston, K. Bottomly, J.J. Owen, and A.C. Hayday. 1990. Developmentally regulated fetal thymic and extrathymic T-cell receptor gamma delta gene expression. *Genes Dev*. 4:1304-1315.
- Carleton, M., M.C. Haks, S.A. Smeele, A. Jones, S.M. Belkowsky, M.A. Berger, P. Linsley, A.M. Kruisbeek, and D.L. Wiest. 2002. Early growth response transcription factors are required for development of CD4(-)CD8(-) thymocytes to the CD4(+)CD8(+) stage. *J Immunol*. 168:1649-1658.
- Chandler, P., A.J. Frater, D.C. Douek, J.L. Viney, G. Kay, M.J. Owen, A.C. Hayday, E. Simpson, and D.M. Altmann. 1995. Immune responsiveness in mutant mice lacking T-cell receptor alpha beta+ cells. *Immunology*. 85:531-537.
- Chen, J., R. Lansford, V. Stewart, F. Young, and F.W. Alt. 1993. RAG-2-deficient blastocyst complementation: an assay of gene function in lymphocyte development. *Proc Natl Acad Sci U S A*. 90:4528-4532.
- Cheng, A.M., I. Negishi, S.J. Anderson, A.C. Chan, J. Bolen, D.Y. Loh, and T. Pawson. 1997a. The Syk and ZAP-70 SH2-containing tyrosine kinases are implicated in pre-T cell receptor signaling. *Proc Natl Acad Sci U S A*. 94:9797-9801.
- Cheng, A.M., B. Rowley, W. Pao, A. Hayday, J.B. Bolen, and T. Pawson. 1995. Syk tyrosine kinase required for mouse viability and B-cell development. *Nature*. 378:303-306.
- Cheng, L.E., F.K. Chan, D. Cado, and A. Winoto. 1997b. Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis. *Embo J*. 16:1865-1875.
- Chu, D.H., H. Spits, J.F. Peyron, R.B. Rowley, J.B. Bolen, and A. Weiss. 1996. The Syk protein tyrosine kinase can function independently of CD45 or Lck in T cell antigen receptor signaling. *Embo J*. 15:6251-6261.
- Clements, J.L., N.J. Boerth, J.R. Lee, and G.A. Koretzky. 1999. Integration of T cell receptor-dependent signaling pathways by adapter proteins. *Annu Rev Immunol*. 17:89-108.
- Clements, J.L., B. Yang, S.E. Ross-Barta, S.L. Eliason, R.F. Hrstka, R.A. Williamson, and G.A. Koretzky. 1998. Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development. *Science*. 281:416-419.
- Cleverley, S., S. Herring, and D. Cantrell. 1999. Inhibition of Rho at different stages of thymocyte development gives different perspectives on Rho function. *Curr Biol*. 9:657-660.
- Clevers, H., B. Alarcon, T. Wileman, and C. Terhorst. 1988. The T cell receptor/CD3 complex: a dynamic protein ensemble. *Annu Rev Immunol*. 6:629-662.
- Cohen, J.J., and R.C. Duke. 1984. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J Immunol*. 132:38-42.

- Collins, C., S. Norris, G. McEntee, O. Traynor, L. Bruno, H. von Boehmer, J. Hegarty, and C. O'Farrelly. 1996. RAG1, RAG2 and pre-T cell receptor alpha chain expression by adult human hepatic T cells: evidence for extrathymic T cell maturation. *Eur J Immunol.* 26:3114-3118.
- Constant, P., F. Davodeau, M.A. Peyrat, Y. Poquet, G. Puzo, M. Bonneville, and J.J. Fournie. 1994. Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands. *Science.* 264:267-270.
- Conway, S.J., D.J. Henderson, and A.J. Copp. 1997. Pax3 is required for cardiac neural crest migration in the mouse: evidence from the splotch (Sp2H) mutant. *Development.* 124:505-514.
- Cory, S. 1995. Regulation of lymphocyte survival by the bcl-2 gene family. *Annu Rev Immunol.* 13:513-543.
- Costello, P.S., S.C. Cleverley, R. Galandrini, S.W. Henning, and D.A. Cantrell. 2000. The GTPase rho controls a p53-dependent survival checkpoint during thymopoiesis. *J Exp Med.* 192:77-85.
- Costello, P.S., A.E. Walters, P.J. Mee, M. Turner, L.F. Reynolds, A. Prisco, N. Sarner, R. Zamoyska, and V.L. Tybulewicz. 1999. The Rho-family GTP exchange factor Vav is a critical transducer of T cell receptor signals to the calcium, ERK, and NF-kappaB pathways. *Proc Natl Acad Sci U S A.* 96:3035-3040.
- Crispe, I.N., and D.G. Schatz. 1995. Development of T cells in the thymus. In T cell receptors. J.I. Bell, M.J. Owen, and E. Simpson, editors. Oxford University Press, New York. 15-45.
- Cristanti, A., A. Colantoni, R. Snodgrass, and H. von Boehmer. 1986. Expression of T cell receptors by thymocytes: in situ staining and biochemical analysis. *Embo J.* 5:2837-2843.
- Crompton, T., K.C. Gilmour, and M.J. Owen. 1996. The MAP kinase pathway controls differentiation from double-negative to double-positive thymocyte. *Cell.* 86:243-251.
- Crompton, T., S.V. Outram, J. Buckland, and M.J. Owen. 1997. A transgenic T cell receptor restores thymocyte differentiation in interleukin-7 receptor alpha chain-deficient mice. *Eur J Immunol.* 27:100-104.
- Crompton, T., S.V. Outram, J. Buckland, and M.J. Owen. 1998. Distinct roles of the interleukin-7 receptor alpha chain in fetal and adult thymocyte development revealed by analysis of interleukin-7 receptor alpha-deficient mice. *Eur J Immunol.* 28:1859-1866.
- Crowley, M.P., Z. Reich, N. Mavaddat, J.D. Altman, and Y. Chien. 1997. The recognition of the nonclassical major histocompatibility complex (MHC) class I molecule, T10, by the gammadelta T cell, G8. *J Exp Med.* 185:1223-1230.
- Cumano, A., F. Dieterlen-Lievre, and I. Godin. 1996. Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. *Cell.* 86:907-916.

- Cumano, A., F. Dieterlen-Lievre, and I. Godin. 2000. The splanchnopleura/AGM region is the prime site for the generation of multipotent hemopoietic precursors, in the mouse embryo. *Vaccine*. 18:1621-1623.
- Cumano, A., J.C. Ferraz, M. Klaine, J.P. Di Santo, and I. Godin. 2001. Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. *Immunity*. 15:477-485.
- Cumano, A., and I. Godin. 2001. Pluripotent hematopoietic stem cell development during embryogenesis. *Curr Opin Immunol*. 13:166-171.
- Cumano, A., B.L. Kee, D.A. Ramsden, A. Marshall, C.J. Paige, and G.E. Wu. 1994. Development of B lymphocytes from lymphoid committed and uncommitted progenitors. *Immunol Rev*. 137:5-33.
- da Silva, A.J., Z. Li, C. de Vera, E. Canto, P. Findell, and C.E. Rudd. 1997. Cloning of a novel T-cell protein FYB that binds FYN and SH2-domain-containing leukocyte protein 76 and modulates interleukin 2 production. *Proc Natl Acad Sci U S A*. 94:7493-7498.
- Dadi, H.K., and C.M. Roifman. 1993. Activation of phosphatidylinositol-3 kinase by ligation of the interleukin-7 receptor on human thymocytes. *J Clin Invest*. 92:1559-1563.
- Dave, V.P., Z. Cao, C. Browne, B. Alarcon, G. Fernandez-Miguel, J. Lafaille, A. de la Hera, S. Tonegawa, and D.J. Kappes. 1997. CD3 delta deficiency arrests development of the alpha beta but not the gamma delta T cell lineage. *Embo J*. 16:1360-1370.
- Davies, S.P., H. Reddy, M. Caivano, and P. Cohen. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J*. 351:95-105.
- Davis, C.B., N. Killeen, M.E. Crooks, D. Raulet, and D.R. Littman. 1993. Evidence for a stochastic mechanism in the differentiation of mature subsets of T lymphocytes. *Cell*. 73:237-247.
- Davis, M.M., and P.J. Bjorkman. 1988. T-cell antigen receptor genes and T-cell recognition. *Nature*. 334:395-402.
- de Vries, J.E., H. Yssel, and H. Spits. 1989. Interplay between the TCR/CD3 complex and CD4 or CD8 in the activation of cytotoxic T lymphocytes. *Immunol Rev*. 109:119-141.
- Deftos, M.L., and M.J. Bevan. 2000b. Notch signaling in T cell development. *Curr Opin Immunol*. 12:166-172.
- Deftos, M.L., Y.W. He, E.W. Ojala, and M.J. Bevan. 1998. Correlating notch signaling with thymocyte maturation. *Immunity*. 9:777-786.
- Deftos, M.L., E. Huang, E.W. Ojala, K.A. Forbush, and M.J. Bevan. 2000a. Notch1 signaling promotes the maturation of CD4 and CD8 SP thymocytes. *Immunity*. 13:73-84.
- Del Porto, P., L. Bruno, M.G. Mattei, H. von Boehmer, and C. Saint-Ruf. 1995. Cloning and comparative analysis of the human pre-T-cell receptor alpha-chain gene. *Proc Natl Acad Sci U S A*. 92:12105-12109.

- Delaney, J.R., Y. Sykulev, H.N. Eisen, and S. Tonegawa. 1998. Differences in the level of expression of class I major histocompatibility complex proteins on thymic epithelial and dendritic cells influence the decision of immature thymocytes between positive and negative selection. *Proc Natl Acad Sci U S A.* 95:5235-5240.
- Dembic, Z., W. Haas, R. Zamoyska, J. Parnes, M. Steinmetz, and H. von Boehmer. 1987. Transfection of the CD8 gene enhances T-cell recognition. *Nature.* 326:510-511.
- Dent, A.L., L.A. Matis, F. Hooshmand, S.M. Widacki, J.A. Bluestone, and S.M. Hedrick. 1990. Self-reactive gamma delta T cells are eliminated in the thymus. *Nature.* 343:714-719.
- Di Santo, J.P., I. Aifantis, E. Rosmaraki, C. Garcia, J. Feinberg, H.J. Fehling, A. Fischer, H. von Boehmer, and B. Rocha. 1999. The common cytokine receptor gamma chain and the pre-T cell receptor provide independent but critically overlapping signals in early alpha/beta T cell development. *J Exp Med.* 189:563-574.
- Di Santo, J.P., H.J. Fehling, B. Malissen, H. Spits, and J. Borst. 1998. Antigen receptors in lymphoid cell development and lymphocyte activation. *Res Immunol.* 149:868-871.
- Di Santo, J.P., F. Radtke, and H.R. Rodewald. 2000. To be or not to be a pro-T? *Curr Opin Immunol.* 12:159-165.
- Di Santo, J.P., and H.R. Rodewald. 1998. In vivo roles of receptor tyrosine kinases and cytokine receptors in early thymocyte development. *Curr Opin Immunol.* 10:196-207.
- Dianda, L., A. Gulbranson-Judge, W. Pao, A.C. Hayday, I.C. MacLennan, and M.J. Owen. 1996. Germinal center formation in mice lacking alpha beta T cells. *Eur J Immunol.* 26:1603-1607.
- Diaz, P., D. Cado, and A. Winoto. 1994. A locus control region in the T cell receptor alpha/delta locus. *Immunity.* 1:207-217.
- Dieterlen-Lievre, F. 1975. On the origin of haemopoietic stem cells in the avian embryo: an experimental approach. *J Embryol Exp Morphol.* 33:607-619.
- Dieterlen-Lievre, F., and A. Cumano. 1998. Cellular and molecular events that govern the development of the hematopoietic and immune system in the embryo. *Dev Comp Immunol.* 22:249-252.
- DiSanto, J.P., D. Guy-Grand, A. Fisher, and A. Tarakhovsky. 1996. Critical role for the common cytokine receptor gamma chain in intrathymic and peripheral T cell selection. *J Exp Med.* 183:1111-1118.
- DiSanto, J.P., W. Muller, D. Guy-Grand, A. Fischer, and K. Rajewsky. 1995. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain. *Proc Natl Acad Sci U S A.* 92:377-381.
- Douagi, I., I. Andre, J.C. Ferraz, and A. Cumano. 2000. Characterization of T cell precursor activity in the murine fetal thymus: evidence for an input of T cell precursors between days 12 and 14 of gestation. *Eur J Immunol.* 30:2201-2210.
- Douagi, I., F. Colucci, J.P. Di Santo, and A. Cumano. 2002. Identification of the earliest prethymic bipotent T/NK progenitor in murine fetal liver. *Blood.* 99:463-471.

- Douglas, N.C., H. Jacobs, A.L. Bothwell, and A.C. Hayday. 2001. Defining the specific physiological requirements for c-Myc in T cell development. *Nat Immunol.* 2:307-315.
- Dudley, E.C., M. Girardi, M.J. Owen, and A.C. Hayday. 1995. Alpha beta and gamma delta T cells can share a late common precursor. *Curr Biol.* 5:659-669.
- Dudley, E.C., H.T. Petrie, L.M. Shah, M.J. Owen, and A.C. Hayday. 1994. T cell receptor beta chain gene rearrangement and selection during thymocyte development in adult mice. *Immunity.* 1:83-93.
- Durum, S.K., S. Candeias, H. Nakajima, W.J. Leonard, A.M. Baird, L.J. Berg, and K. Muegge. 1998. Interleukin 7 receptor control of T cell receptor gamma gene rearrangement: role of receptor-associated chains and locus accessibility. *J Exp Med.* 188:2233-2241.
- Eberl, G., H.J. Fehling, H. von Boehmer, and H.R. MacDonald. 1999. Absolute requirement for the pre-T cell receptor alpha chain during NK1.1+ TCRalphabeta cell development. *Eur J Immunol.* 29:1966-1971.
- Egerton, M., R. Scollay, and K. Shortman. 1990a. Kinetics of mature T-cell development in the thymus. *Proc Natl Acad Sci U S A.* 87:2579-2582.
- Egerton, M., K. Shortman, and R. Scollay. 1990b. The kinetics of immature murine thymocyte development in vivo. *Int Immunol.* 2:501-507.
- Elliott, J.F., E.P. Rock, P.A. Patten, M.M. Davis, and Y.H. Chien. 1988. The adult T-cell receptor delta-chain is diverse and distinct from that of fetal thymocytes. *Nature.* 331:627-631.
- Ema, H., A. Cumano, and P. Kourilsky. 1997. TCR-beta repertoire development in the mouse embryo. *J Immunol.* 159:4227-4232.
- Ema, H., I. Douagi, A. Cumano, and P. Kourilsky. 1998. Development of T cell precursor activity in the murine fetal liver. *Eur J Immunol.* 28:1563-1569.
- Engel, I., C. Johns, G. Bain, R.R. Rivera, and C. Murre. 2001. Early thymocyte development is regulated by modulation of E2A protein activity. *J Exp Med.* 194:733-745.
- Esslinger, C.W., A. Wilson, B. Sordat, F. Beermann, and C.V. Jongeneel. 1997. Abnormal T lymphocyte development induced by targeted overexpression of IkappaB alpha. *J Immunol.* 158:5075-5078.
- Ewing, T., M. Egerton, A. Wilson, R. Scollay, and K. Shortman. 1988. Subpopulations of CD4- CD8- murine thymocytes: differences in proliferation rate in vivo and proliferative responses in vitro. *Eur J Immunol.* 18:261-268.
- Eynon, E.E., F. Livak, K. Kuida, D.G. Schatz, and R.A. Flavell. 1999. Distinct effects of Jak3 signaling on alphabeta and gammadelta thymocyte development. *J Immunol.* 162:1448-1459. [frame.html](#).
- Falk, I., C.N. Levelt, and K. Eichmann. 1993. Lineage relationships of the fetal thymocyte subset that expresses the beta chain of the interleukin-2 receptor. *Eur J Immunol.* 23:3373-3376.

- Farr, A., S. Hosier, A. Nelson, S. Itohara, and S. Tonegawa. 1990. Distribution of thymocytes expressing gamma delta receptors in the murine thymus during development. *J Immunol.* 144:492-498.
- Fehling, H.J., S. Gilfillan, and R. Ceredig. 1999. Alpha beta/gamma delta lineage commitment in the thymus of normal and genetically manipulated mice. *Adv Immunol.* 71:1-76.
- Fehling, H.J., B.M. Iritani, A. Krotkova, K.A. Forbush, C. Laplace, R.M. Perlmutter, and H. von Boehmer. 1997b. Restoration of thymopoiesis in pT alpha-/- mice by anti-CD3epsilon antibody treatment or with transgenes encoding activated Lck or tailless pT alpha. *Immunity.* 6:703-714.
- Fehling, H.J., A. Krotkova, C. Saint-Ruf, and H. von Boehmer. 1995a. Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. *Nature.* 375:795-798.
- Fehling, H.J., C. Laplace, M.G. Mattei, C. Saint-Ruf, and H. von Boehmer. 1995b. Genomic structure and chromosomal location of the mouse pre-T-cell receptor alpha gene. *Immunogenetics.* 42:275-281.
- Fehling, H.J., and H. von Boehmer. 1997. Early alpha beta T cell development in the thymus of normal and genetically altered mice. *Curr Opin Immunol.* 9:263-275.
- Fenton, R.G., P. Marrack, J.W. Kappler, O. Kanagawa, and J.G. Seidman. 1988. Isotypic exclusion of gamma delta T cell receptors in transgenic mice bearing a rearranged beta-chain gene. *Science.* 241:1089-1092.
- Festenstein, R., M. Tolaini, P. Corbella, C. Mamalaki, J. Parrington, M. Fox, A. Miliou, M. Jones, and D. Kioussis. 1996. Locus control region function and heterochromatin-induced position effect variegation. *Science.* 271:1123-1125.
- Fine, J.S., and A.M. Kruisbeek. 1991. The role of LFA-1/ICAM-1 interactions during murine T lymphocyte development. *J Immunol.* 147:2852-2859.
- Fink, P.J., and M.J. Bevan. 1978. H-2 antigens of the thymus determine lymphocyte specificity. *J Exp Med.* 148:766-775.
- Fischer, K.D., A. Zmuldzinas, S. Gardner, M. Barbacid, A. Bernstein, and C. Guidos. 1995. Defective T-cell receptor signalling and positive selection of Vav- deficient CD4+ CD8+ thymocytes. *Nature.* 374:474-477.
- Fischer, M., I. MacNeil, T. Suda, J.E. Cupp, K. Shortman, and A. Zlotnik. 1991. Cytokine production by mature and immature thymocytes. *J Immunol.* 146:3452-3456.
- Fleming, H.E., and C.J. Paige. 2001. Pre-B cell receptor signaling mediates selective response to IL-7 at the pro-B to pre-B cell transition via an ERK/MAP kinase-dependent pathway. *Immunity.* 15:521-531.
- Fontaine-Perus, J.C., F.M. Calman, C. Kaplan, and N.M. Le Douarin. 1981. Seeding of the 10-day mouse embryo thymic rudiment by lymphocyte precursors in vitro. *J Immunol.* 126:2310-2316.
- Franz, O., I.I. Bruchhaus, and T. Roeder. 1999. Verification of differential gene transcription using virtual northern blotting. *Nucleic Acids Res.* 27:e3.

- Fry, A.M., L.A. Jones, A.M. Kruisbeek, and L.A. Matis. 1989. Thymic requirement for clonal deletion during T cell development. *Science*. 246:1044-1046.
- Fujihashi, K., J.R. McGhee, M.N. Kweon, M.D. Cooper, S. Tonegawa, I. Takahashi, T. Hiroi, J. Mestecky, and H. Kiyono. 1996. gamma/delta T cell-deficient mice have impaired mucosal immunoglobulin A responses. *J Exp Med*. 183:1929-1935.
- Fujimoto, Y., T. LiLi, A.S. Miller, C. Bock, M. Fujimoto, C. Doyle, D.A. Steeber, and T.F. Tedder. 2002. CD83 expression influences CD4+ T cell development in the thymus. *Cell*. 108:755-767.
- Fujise, S., G. Matsuzaki, K. Kishihara, T. Kadena, T. Molina, and K. Nomoto. 1996. The role of p56lck in the development of gamma delta T cells and their function during an infection by *Listeria monocytogenes*. *J Immunol*. 157:247-254.
- Fukui, Y., T. Ishimoto, M. Utsuyama, T. Gyotoku, T. Koga, K. Nakao, K. Hirokawa, M. Katsuki, and T. Sasazuki. 1997. Positive and negative CD4+ thymocyte selection by a single MHC class II/peptide ligand affected by its expression level in the thymus. *Immunity*. 6:401-410.
- Fung-Leung, W.P., M.C. Louie, A. Limmer, P.S. Ohashi, K. Ngo, L. Chen, K. Kawai, E. Lacy, D.Y. Loh, and T.W. Mak. 1993. The lack of CD8 alpha cytoplasmic domain resulted in a dramatic decrease in efficiency in thymic maturation but only a moderate reduction in cytotoxic function of CD8+ T lymphocytes. *Eur J Immunol*. 23:2834-2840.
- Galandrini, R., S.W. Henning, and D.A. Cantrell. 1997. Different functions of the GTPase Rho in prothymocytes and late pre-T cells. *Immunity*. 7:163-174.
- Galy, A.H., and H. Spits. 1992. CD40 is functionally expressed on human thymic epithelial cells. *J Immunol*. 149:775-782.
- Gartner, F., F.W. Alt, R. Monroe, M. Chu, B.P. Sleckman, L. Davidson, and W. Swat. 1999. Immature thymocytes employ distinct signaling pathways for allelic exclusion versus differentiation and expansion. *Immunity*. 10:537-546.
- Geng, L., S. Pfister, S.K. Kraeft, and C.E. Rudd. 2001. Adaptor FYB (Fyn-binding protein) regulates integrin-mediated adhesion and mediator release: differential involvement of the FYB SH3 domain. *Proc Natl Acad Sci U S A*. 98:11527-11532.
- Geng, L., M. Raab, and C.E. Rudd. 1999. Cutting edge: SLP-76 cooperativity with FYB/FYN-T in the Up-regulation of TCR-driven IL-2 transcription requires SLP-76 binding to FYB at Tyr595 and Tyr651. *J Immunol*. 163:5753-5757.
- Georgopoulos, K., M. Bigby, J.H. Wang, A. Molnar, P. Wu, S. Winandy, and A. Sharpe. 1994. The Ikaros gene is required for the development of all lymphoid lineages. *Cell*. 79:143-156.
- Gibbons, D., N.C. Douglas, D.F. Barber, Q. Liu, R. Sullo, L. Geng, H.J. Fehling, H. von Boehmer, and A.C. Hayday. 2001. The biological activity of natural and mutant pTalpha alleles. *J Exp Med*. 194:695-703.
- Girardi, M., D.E. Oppenheim, C.R. Steele, J.M. Lewis, E. Glusac, R. Filler, P. Hobby, B. Sutton, R.E. Tigelaar, and A.C. Hayday. 2001. Regulation of cutaneous malignancy by gammadelta T cells. *Science*. 294:605-609.

- Godfrey, D.I., J. Kennedy, P. Mombaerts, S. Tonegawa, and A. Zlotnik. 1994. Onset of TCR-beta gene rearrangement and role of TCR-beta expression during CD3-CD4-CD8-thymocyte differentiation. *J Immunol.* 152:4783-4792.
- Godfrey, D.I., J. Kennedy, T. Suda, and A. Zlotnik. 1993. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8-triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *J Immunol.* 150:4244-4252.
- Godfrey, D.I., and A. Zlotnik. 1993. Control points in early T-cell development. *Immunol Today.* 14:547-553.
- Godin, I., F. Dieterlen-Lievre, and A. Cumano. 1995. Emergence of multipotent hemopoietic cells in the yolk sac and paraaortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus. *Proc Natl Acad Sci U S A.* 92:773-777.
- Godin, I., J.A. Garcia-Porrero, F. Dieterlen-Lievre, and A. Cumano. 1999. Stem cell emergence and hemopoietic activity are incompatible in mouse intraembryonic sites. *J Exp Med.* 190:43-52.
- Gomez, M., D. Kioussis, and D.A. Cantrell. 2001. The GTPase Rac-1 controls cell fate in the thymus by diverting thymocytes from positive to negative selection. *Immunity.* 15:703-713.
- Gomez, M., V. Tybulewicz, and D.A. Cantrell. 2000. Control of pre-T cell proliferation and differentiation by the GTPase Rac-I. *Nat Immunol.* 1:348-352.
[taf/DynaPage.taf?file=/ni/journal/v341/n344/full/ni1000_1348.html](http://www.nature.com/taf/DynaPage.taf?file=/ni/journal/v341/n344/full/ni1000_1348.html)
[taf/DynaPage.taf?file=/ni/journal/v1001/n1004/abs/ni1000_1348.html](http://www.nature.com/taf/DynaPage.taf?file=/ni/journal/v1001/n1004/abs/ni1000_1348.html).
- Gounari, F., I. Aifantis, K. Khazaie, S. Hoeflinger, N. Harada, M.M. Taketo, and H. von Boehmer. 2001. Somatic activation of beta-catenin bypasses pre-TCR signaling and TCR selection in thymocyte development. *Nat Immunol.* 2:863-869.
- Grassi, F., E. Barbier, S. Porcellini, H. von Boehmer, and P.A. Cazenave. 1999. Surface expression and functional competence of CD3-independent TCR zeta-chains in immature thymocytes. *J Immunol.* 162:2589-2596.
- Grawunder, U., R.B. West, and M.R. Lieber. 1998. Antigen receptor gene rearrangement. *Curr Opin Immunol.* 10:172-180.
- Griffiths, E.K., C. Krawczyk, Y.Y. Kong, M. Raab, S.J. Hyduk, D. Bouchard, V.S. Chan, I. Kozieradzki, A.J. Oliveira-Dos-Santos, A. Wakeham, P.S. Ohashi, M.I. Cybulsky, C.E. Rudd, and J.M. Penninger. 2001. Positive regulation of T cell activation and integrin adhesion by the adapter Fyb/Slap. *Science.* 293:2260-2263.
- Grillot, D.A., R. Merino, and G. Nunez. 1995. Bcl-XL displays restricted distribution during T cell development and inhibits multiple forms of apoptosis but not clonal deletion in transgenic mice. *J Exp Med.* 182:1973-1983.
- Groettrup, M., A. Baron, G. Griffiths, R. Palacios, and H. von Boehmer. 1992. T cell receptor (TCR) beta chain homodimers on the surface of immature but not mature alpha, gamma, delta chain deficient T cell lines. *Embo J.* 11:2735-2745.

- Groettrup, M., K. Ungewiss, O. Azogui, R. Palacios, M.J. Owen, A.C. Hayday, and H. von Boehmer. 1993. A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor beta chain and a 33 kd glycoprotein. *Cell*. 75:283-294.
- Groettrup, M., and H. von Boehmer. 1993a. A role for a pre-T-cell receptor in T-cell development. *Immunol Today*. 14:610-614.
- Groettrup, M., and H. von Boehmer. 1993b. T cell receptor beta chain dimers on immature thymocytes from normal mice. *Eur J Immunol*. 23:1393-1396.
- Groh, V., S. Bahram, S. Bauer, A. Herman, M. Beauchamp, and T. Spies. 1996. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proc Natl Acad Sci U S A*. 93:12445-12450.
- Groh, V., M. Fabbi, F. Hochstenbach, R.T. Maziarz, and J.L. Strominger. 1989a. Double-negative (CD4-CD8-) lymphocytes bearing T-cell receptor alpha and beta chains in normal human skin. *Proc Natl Acad Sci U S A*. 86:5059-5063.
- Groh, V., S. Porcelli, M. Fabbi, L.L. Lanier, L.J. Picker, T. Anderson, R.A. Warnke, A.K. Bhan, J.L. Strominger, and M.B. Brenner. 1989b. Human lymphocytes bearing T cell receptor gamma/delta are phenotypically diverse and evenly distributed throughout the lymphoid system. *J Exp Med*. 169:1277-1294.
- Groh, V., A. Steinle, S. Bauer, and T. Spies. 1998. Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. *Science*. 279:1737-1740.
- Gross, J.A., M.W. Appleby, S. Chien, S. Nada, S.H. Bartelmez, M. Okada, S. Aizawa, and R.M. Perlmutter. 1995. Control of lymphopoiesis by p50csk, a regulatory protein tyrosine kinase. *J Exp Med*. 181:463-473.
- Groves, T., P. Smiley, M.P. Cooke, K. Forbush, R.M. Perlmutter, and C.J. Guidos. 1996. Fyn can partially substitute for Lck in T lymphocyte development. *Immunity*. 5:417-428.
- Grusby, M.J., H.J. Auchinloss, R. Lee, V.E. Papaioannou, and L.H. Glimcher. 1993. Mice lacking major histocompatibility complex class I and class II molecules. *Proc. Natl. Acad. Sci. USA*. 90:3913-3917.
- Grusby, M.J., R.S. Johnson, V.E. Papaioannou, and L.H. Glimcher. 1991. Depletion of CD4+ T cells in major histocompatibility complex class II- deficient mice. *Science*. 253:1417-1420.
- Haas, W., P. Pereira, and S. Tonegawa. 1993. Gamma/delta cells. *Annu Rev Immunol*. 11:637-685.
- Haas, W., and S. Tonegawa. 1992. Development and selection of gamma delta T cells. *Curr Opin Immunol*. 4:147-155.
- Haks, M.C., T.A. Cordaro, J.H. van den Brakel, J.B. Haanen, E.F. de Vries, J. Borst, P. Krimpenfort, and A.M. Kruisbeek. 2001. A redundant role of the CD3 gamma-immunoreceptor tyrosine-based activation motif in mature T cell function. *J Immunol*. 166:2576-2588.
- Haks, M.C., P. Krimpenfort, J. Borst, and A.M. Kruisbeek. 1998. The CD3gamma chain is essential for development of both the TCRalpha and TCRgammadelta lineages. *Embo J*. 17:1871-1882.

- Haks, M.C., P. Krimpenfort, J.H. van den Brakel, and A.M. Kruisbeek. 1999a. Pre-TCR signaling and inactivation of p53 induces crucial cell survival pathways in pre-T cells. *Immunity*. 11:91-101.
- Haks, M.C., M.A. Oosterwegel, B. Blom, H.M. Spits, and A.M. Kruisbeek. 1999b. Cell-fate decisions in early T cell development: regulation by cytokine receptors and the pre-TCR. *Semin Immunol*. 11:23-37.
- Hare, K.J., E.J. Jenkinson, and G. Anderson. 1999. In vitro models of T cell development. *Semin Immunol*. 11:3-12.
- Hare, K.J., E.J. Jenkinson, and G. Anderson. 2000. An essential role for the IL-7 receptor during intrathymic expansion of the positively selected neonatal T cell repertoire. *J Immunol*. 165:2410-2414.
- Hare, K.J., E.J. Jenkinson, and G. Anderson. 2001. Specialisation of thymic epithelial cells for positive selection of CD4+8+ thymocytes. *Cell Mol Biol (Noisy-le-grand)*. 47:119-127.
- Hashimoto, K., S.J. Sohn, S.D. Levin, T. Tada, R.M. Perlmutter, and T. Nakayama. 1996. Requirement for p56lck tyrosine kinase activation in T cell receptor-mediated thymic selection. *J Exp Med*. 184:931-943.
- Havran, W.L., Y.H. Chien, and J.P. Allison. 1991. Recognition of self antigens by skin-derived T cells with invariant gamma delta antigen receptors. *Science*. 252:1430-1432.
- Hayday, A., and D. Gibbons. 2001. Regulated T-cell development: a victim of multiple conspiracies. *Immunology*. 104:8-10.
- Hayday, A., E. Theodoridis, E. Ramsburg, and J. Shires. 2001. Intraepithelial lymphocytes: exploring the Third Way in immunology. *Nat Immunol*. 2:997-1003.
- Hayday, A.C. 1995. Gamma-delta T cell specificity and function. In T cell receptors. J.I. Bell, M.J. Owen, and E. Simpson, editors. Oxford University Press, New York. 70-91.
- Hayday, A.C. 2000. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol*. 18:975-1026.
- Hayday, A.C., D.F. Barber, N. Douglas, and E.S. Hoffman. 1999. Signals involved in gamma/delta T cell versus alpha/beta T cell lineage commitment. *Semin Immunol*. 11:239-249.
- Hayday, A.C., D.J. Diamond, G. Tanigawa, J.S. Heilig, V. Folsom, H. Saito, and S. Tonegawa. 1985a. Unusual organization and diversity of T-cell receptor alpha-chain genes. *Nature*. 316:828-832.
- Hayday, A.C., H. Saito, S.D. Gillies, D.M. Kranz, G. Tanigawa, H.N. Eisen, and S. Tonegawa. 1985b. Structure, organization, and somatic rearrangement of T cell gamma genes. *Cell*. 40:259-269.
- Hedrick, S.M., D.I. Cohen, E.A. Nielsen, and M.M. Davis. 1984. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature*. 308:149-153.

- Heemskerck, M.H., B. Blom, G. Nolan, A.P. Stegmann, A.Q. Bakker, K. Weijer, P.C. Res, and H. Spits. 1997. Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. *J Exp Med.* 186:1597-1602.
- Heid, C.A., J. Stevens, K.J. Livak, and P.M. Williams. 1996. Real time quantitative PCR. *Genome Res.* 6:986-994.
- Heilig, J.S., L.H. Glimcher, D.M. Kranz, L.K. Clayton, J.L. Greenstein, H. Saito, A.M. Maxam, S.J. Burakoff, H.N. Eisen, and S. Tonegawa. 1985. Expression of the T-cell-specific gamma gene is unnecessary in T cells recognizing class II MHC determinants. *Nature.* 317:68-70.
- Heilig, J.S., and S. Tonegawa. 1986. Diversity of murine gamma genes and expression in fetal and adult T lymphocytes. *Nature.* 322:836-840.
- Heilig, J.S., and S. Tonegawa. 1987. T-cell gamma gene is allelically but not isotypically excluded and is not required in known functional T-cell subsets. *Proc Natl Acad Sci U S A.* 84:8070-8074.
- Henning, S.W., R. Galandrini, A. Hall, and D.A. Cantrell. 1997. The GTPase Rho has a critical regulatory role in thymus development. *Embo J.* 16:2397-2407.
- Hilyard, K., and J.L. Strominger. 1995. The immune recognition unit: the TCR-peptide-MHC complex. In T cell receptors. J.I. Bell, M.J. Owen, and E. Simpson, editors. Oxford University Press, New York. 403-423.
- Hoffman, E.S., L. Passoni, T. Crompton, T.M. Leu, D.G. Schatz, A. Koff, M.J. Owen, and A.C. Hayday. 1996. Productive T-cell receptor beta-chain gene rearrangement: coincident regulation of cell cycle and clonality during development in vivo. *Genes Dev.* 10:948-962.
- Hogquist, K.A., and M.J. Bevan. 1996. The nature of the peptide/MHC ligand involved in positive selection. *Semin Immunol.* 8:63-68.
- Hogquist, K.A., M.A. Gavin, and M.J. Bevan. 1993. Positive selection of CD8+ T cells induced by major histocompatibility complex binding peptides in fetal thymic organ culture. *J Exp Med.* 177:1469-1473.
- Hogquist, K.A., S.C. Jameson, W.R. Heath, J.L. Howard, M.J. Bevan, and F.R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell.* 76:17-27.
- Holland, G.D., K. Ito, D.A. Kaehler, S. Tonegawa, and R. Risser. 1991. Thymic targets for Abelson murine leukemia virus are early gamma/delta T lymphocytes. *Proc Natl Acad Sci U S A.* 88:3700-3704.
- Holland, S.J., X.C. Liao, M.K. Mendenhall, X. Zhou, J. Pardo, P. Chu, C. Spencer, A. Fu, N. Sheng, P. Yu, E. Pali, A. Nagin, M. Shen, S. Yu, E. Chan, X. Wu, C. Li, M. Woisetschlager, G. Aversa, F. Kolbinger, M.K. Bennett, S. Molineaux, Y. Luo, D.G. Payan, H.S. Mancebo, and J. Wu. 2001. Functional cloning of Src-like adapter protein-2 (SLAP-2), a novel inhibitor of antigen receptor signaling. *J Exp Med.* 194:1263-1276.
- Horner, A.A., H. Jabara, N. Ramesh, and R.S. Geha. 1995. gamma/delta T lymphocytes express CD40 ligand and induce isotype switching in B lymphocytes. *J Exp Med.* 181:1239-1244.

- Howard, F.D., H.R. Rodewald, J.P. Kinet, and E.L. Reinherz. 1990. CD3 zeta subunit can substitute for the gamma subunit of Fc epsilon receptor type I in assembly and functional expression of the high-affinity IgE receptor: evidence for interreceptor complementation. *Proc Natl Acad Sci U S A.* 87:7015-7019.
- Hubank, M., and D.G. Schatz. 1994. Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res.* 22:5640-5648.
- Huesmann, M., B. Scott, P. Kisielow, and H. von Boehmer. 1991. Kinetics and efficacy of positive selection in the thymus of normal and T cell receptor transgenic mice. *Cell.* 66:533-540.
- Hughes, D.P., A. Hayday, J.E. Craft, M.J. Owen, and I.N. Crispe. 1995. T cells with gamma/delta T cell receptors (TCR) of intestinal type are preferentially expanded in TCR-alpha-deficient lpr mice. *J Exp Med.* 182:233-241.
- Hugo, P., J.W. Kappler, D.I. Godfrey, and P.C. Marrack. 1994. Thymic epithelial cell lines that mediate positive selection can also induce thymocyte clonal deletion. *J Immunol.* 152:1022-1031.
- Hugo, P., J.W. Kappler, J.E. McCormack, and P. Marrack. 1993. Fibroblasts can induce thymocyte positive selection in vivo. *Proc Natl Acad Sci U S A.* 90:10335-10339.
- Hugo, P., G.A. Waanders, R. Scollay, K. Shortman, and R.L. Boyd. 1990. Ontogeny of a novel CD4+CD8-CD3- thymocyte subpopulation: a comparison with CD4- CD8+ CD3- thymocytes. *Int Immunol.* 2:209-218.
- Hunter, A.J., N. Ottoson, N. Boerth, G.A. Koretzky, and Y. Shimizu. 2000. Cutting edge: a novel function for the SLAP-130/FYB adapter protein in beta 1 integrin signaling and T lymphocyte migration. *J Immunol.* 164:1143-1147.
- Hussey, R.E., L.K. Clayton, A. Diener, D.J. McConkey, F.D. Howard, H.R. Rodewald, L. D'Adamio, F. Dallenbach, H. Stein, E.V. Schmidt, and et al. 1993. Overexpression of CD3 zeta during thymic development does not alter the negative selection process. *J Immunol.* 150:1183-1194.
- Ikuta, K., D.E. Ingolia, J. Friedman, S. Heimfeld, and I.L. Weissman. 1991. Mouse hematopoietic stem cells and the interaction of c-kit receptor and steel factor. *Int J Cell Cloning.* 9:451-460.
- Ikuta, K., T. Kina, I. MacNeil, N. Uchida, B. Peault, Y.H. Chien, and I.L. Weissman. 1990. A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. *Cell.* 62:863-874.
- Ikuta, K., and I.L. Weissman. 1992. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc Natl Acad Sci U S A.* 89:1502-1506.
- Ioannidis, V., F. Beermann, H. Clevers, and W. Held. 2001. The beta-catenin--TCF-1 pathway ensures CD4(+)CD8(+) thymocyte survival. *Nat Immunol.* 2:691-697.
- Iritani, B.M., J. Alberola-Ila, K.A. Forbush, and R.M. Perimutter. 1999. Distinct signals mediate maturation and allelic exclusion in lymphocyte progenitors. *Immunity.* 10:713-722.

- Irving, B.A., F.W. Alt, and N. Killeen. 1998. Thymocyte development in the absence of pre-T cell receptor extracellular immunoglobulin domains. *Science*. 280:905-908.
- Ishibashi, M., S.L. Ang, K. Shiota, S. Nakanishi, R. Kageyama, and F. Guillemot. 1995. Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. *Genes Dev*. 9:3136-3148.
- Ishida, I., S. Verbeek, M. Bonneville, S. Itohara, A. Berns, and S. Tonegawa. 1990. T-cell receptor gamma delta and gamma transgenic mice suggest a role of a gamma gene silencer in the generation of alpha beta T cells. *Proc Natl Acad Sci U S A*. 87:3067-3071.
- Ishikawa, H., Y. Li, A. Abeliovich, S. Yamamoto, S.H. Kaufmann, and S. Tonegawa. 1993. Cytotoxic and interferon gamma-producing activities of gamma delta T cells in the mouse intestinal epithelium are strain dependent. *Proc Natl Acad Sci U S A*. 90:8204-8208.
- Itano, A., D. Cado, F.K. Chan, and E. Robey. 1994. A role for the cytoplasmic tail of the beta chain of CD8 in thymic selection. *Immunity*. 1:287-290.
- Itano, A., P. Salmon, D. Kioussis, M. Tolaini, P. Corbella, and E. Robey. 1996. The cytoplasmic domain of CD4 promotes the development of CD4 lineage T cells. *J Exp Med*. 183:731-741.
- Ito, K., M. Bonneville, Y. Takagaki, N. Nakanishi, O. Kanagawa, E.G. Krecko, and S. Tonegawa. 1989. Different gamma delta T-cell receptors are expressed on thymocytes at different stages of development. *Proc Natl Acad Sci U S A*. 86:631-635.
- Itohara, S., A.G. Farr, J.J. Lafaille, M. Bonneville, Y. Takagaki, W. Haas, and S. Tonegawa. 1990. Homing of a gamma delta thymocyte subset with homogeneous T-cell receptors to mucosal epithelia. *Nature*. 343:754-757.
- Itohara, S., P. Mombaerts, J. Lafaille, J. Iacomini, A. Nelson, A.R. Clarke, M.L. Hooper, A. Farr, and S. Tonegawa. 1993. T cell receptor delta gene mutant mice: independent generation of alpha beta T cells and programmed rearrangements of gamma delta TCR genes. *Cell*. 72:337-348.
- Itohara, S., N. Nakanishi, O. Kanagawa, R. Kubo, and S. Tonegawa. 1989. Monoclonal antibodies specific to native murine T-cell receptor gamma delta: analysis of gamma delta T cells during thymic ontogeny and in peripheral lymphoid organs. *Proc Natl Acad Sci U S A*. 86:5094-5098.
- Itohara, S., and S. Tonegawa. 1990. Selection of gamma delta T cells with canonical T-cell antigen receptors in fetal thymus. *Proc Natl Acad Sci U S A*. 87:7935-7938.
- Iwashima, M., M.M. Davis, and Y.H. Chien. 1991a. A gamma/delta cell receptor heterodimer induces the expression of CD4 and CD8 in thymocytes. *J Exp Med*. 174:293-296.
- Iwashima, M., A. Green, M. Bonyhadi, M.M. Davis, J.P. Allison, and Y.H. Chien. 1991b. Expression of a fetal gamma delta T-cell receptor in adult mice triggers a non-MHC-linked form of selective depletion. *Int Immunol*. 3:385-393.
- Iwata, M., S. Hanaoka, and K. Sato. 1991. Rescue of thymocytes and T cell hybridomas from glucocorticoid-induced apoptosis by stimulation via the T cell receptor/CD3

- complex: a possible in vitro model for positive selection of the T cell repertoire. *Eur J Immunol.* 21:643-648.
- Izon, D.J., L.A. Jones, E.E. Eynon, and A.M. Kruisbeek. 1994. A molecule expressed on accessory cells, activated T cells, and thymic epithelium is a marker and promoter of T cell activation. *J Immunol.* 153:2939-2950.
- Jacobs, H., J. Iacomini, M. van de Ven, S. Tonegawa, and A. Berns. 1996. Domains of the TCR beta-chain required for early thymocyte development. *J Exp Med.* 184:1833-1843.
- Jacobs, H., P. Krimpenfort, M. Haks, J. Allen, B. Blom, C. Demolliere, A. Kruisbeek, H. Spits, and A. Berns. 1999. PIM1 reconstitutes thymus cellularity in interleukin 7- and common gamma chain-mutant mice and permits thymocyte maturation in Rag- but not CD3gamma-deficient mice. *J Exp Med.* 190:1059-1068.
- Jaleco, A.C., B. Blom, P. Res, K. Weijer, L.L. Lanier, J.H. Phillips, and H. Spits. 1997. Fetal liver contains committed NK progenitors, but is not a site for development of CD34+ cells into T cells. *J Immunol.* 159:694-702.
- Janeway, C.A., and P. Travers. 1997. Immunobiology. Current Biology, London.
- Jenkinson, E. 2002. Control of T cell development by the thymic microenvironment. Cancer Research UK seminar.
- Jenkinson, E.J., G. Anderson, and J.J. Owen. 1992. Studies on T cell maturation on defined thymic stromal cell populations in vitro. *J Exp Med.* 176:845-853.
- Jenkinson, E.J., L.L. Franchi, R. Kingston, and J.J. Owen. 1982. Effect of deoxyguanosine on lymphopoiesis in the developing thymus rudiment in vitro: application in the production of chimeric thymus rudiments. *Eur J Immunol.* 12:583-587.
- Jenkinson, E.J., P. Jhittay, R. Kingston, and J.J. Owen. 1985a. Studies of the role of the thymic environment in the induction of tolerance to MHC antigens. *Transplantation.* 39:331-333.
- Jenkinson, E.J., R. Kingston, and J.J. Owen. 1985b. Stromal cell populations in the developing thymus of normal and nude mice. *Adv Exp Med Biol.* 186:245-249.
- Jenkinson, E.J., R. Kingston, and J.J. Owen. 1987. Importance of IL-2 receptors in intrathymic generation of cells expressing T-cell receptors. *Nature.* 329:160-162.
- Jenkinson, E.J., R. Kingston, and J.J. Owen. 1990. Newly generated thymocytes are not refractory to deletion when the alpha/beta component of the T cell receptor is engaged by the superantigen staphylococcal enterotoxin B. *Eur J Immunol.* 20:2517-2520.
- Jenkinson, E.J., R. Kingston, C.A. Smith, G.T. Williams, and J.J. Owen. 1989. Antigen-induced apoptosis in developing T cells: a mechanism for negative selection of the T cell receptor repertoire. *Eur J Immunol.* 19:2175-2177.
- Jenkinson, E.J., and J.J. Owen. 1990. T-cell differentiation in thymus organ cultures. *Semin Immunol.* 2:51-58.
- Jenkinson, E.J., S. Parnell, J. Shuttleworth, J.J. Owen, and G. Anderson. 1999. Specialized ability of thymic epithelial cells to mediate positive selection does not require expression of the steroidogenic enzyme p450scc. *J Immunol.* 163:5781-5785.

- Jiang, R., Y. Lan, H.D. Chapman, C. Shawber, C.R. Norton, D.V. Serreze, G. Weinmaster, and T. Gridley. 1998. Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev.* 12:1046-1057.
- Johnson, R.M., D.W. Lancki, A.I. Sperling, R.F. Dick, P.G. Spear, F.W. Fitch, and J.A. Bluestone. 1992. A murine CD4-, CD8- T cell receptor-gamma delta T lymphocyte clone specific for herpes simplex virus glycoprotein I. *J Immunol.* 148:983-988.
- Jones, L.A., D.J. Izon, J.D. Nieland, P.S. Linsley, and A.M. Kruisbeek. 1993. CD28-B7 interactions are not required for intrathymic clonal deletion. *Int Immunol.* 5:503-512.
- Jones, L.A., and A.M. Kruisbeek. 1992. What is the mechanism of induction of intrathymic tolerance? *Res Immunol.* 143:291-294.
- Jones-Carson, J., A. Vazquez-Torres, H.C. van der Heyde, T. Warner, R.D. Wagner, and E. Balish. 1995. Gamma delta T cell-induced nitric oxide production enhances resistance to mucosal candidiasis. *Nat Med.* 1:552-557.
- Kadlecek, T.A., N.S. van Oers, L. Lefrancois, S. Olson, D. Finlay, D.H. Chu, K. Connolly, N. Killeen, and A. Weiss. 1998. Differential requirements for ZAP-70 in TCR signaling and T cell development. *J Immunol.* 161:4688-4694.
- Kang, J., J. Baker, and D.H. Raulet. 1995. Evidence that productive rearrangements of TCR gamma genes influence the commitment of progenitor cells to differentiate into alpha beta or gamma delta T cells. *Eur J Immunol.* 25:2706-2709.
- Kang, J., M. Coles, and D.H. Raulet. 1999. Defective development of gamma/delta T cells in interleukin 7 receptor- deficient mice is due to impaired expression of T cell receptor gamma genes. *J Exp Med.* 190:973-982.
- Kang, J., A. Volkmann, and D.H. Raulet. 2001. Evidence that gammadelta versus alphabeta T cell fate determination is initiated independently of T cell receptor signaling. *J Exp Med.* 193:689-698.
- Kappes, D.J., C.P. Browne, and S. Tonegawa. 1991. Identification of a T-cell-specific enhancer at the locus encoding T- cell antigen receptor gamma chain. *Proc Natl Acad Sci U S A.* 88:2204-2208.
- Kappes, D.J., and S. Tonegawa. 1991. Surface expression of alternative forms of the TCR/CD3 complex. *Proc Natl Acad Sci U S A.* 88:10619-10623.
- Kappler, J.W., N. Roehm, and P. Marrack. 1987a. T cell tolerance by clonal elimination in the thymus. *Cell.* 49:273-280.
- Kappler, J.W., T. Wade, J. White, E. Kushnir, M. Blackman, J. Bill, N. Roehm, and P. Marrack. 1987b. A T cell receptor V beta segment that imparts reactivity to a class II major histocompatibility complex product. *Cell.* 49:263-271.
- Keller, G., G. Lacaud, and S. Robertson. 1999. Development of the hematopoietic system in the mouse. *Exp Hematol.* 27:777-787.
- Kelley, C.M., T. Ikeda, J. Koipally, N. Avitahl, L. Wu, K. Georgopoulos, and B.A. Morgan. 1998. Helios, a novel dimerization partner of Ikaros expressed in the earliest hematopoietic progenitors. *Curr Biol.* 8:508-515.

- Kersh, G.J., F.F. Hooshmand, and S.M. Hedrick. 1995. Efficient maturation of alpha beta lineage thymocytes to the CD4+CD8+ stage in the absence of TCR-beta rearrangement. *J Immunol.* 154:5706-5714.
- Kikuchi, G.E., K. Roberts, E.M. Shevach, and J.E. Coligan. 1992. Gene transfer demonstrates that the V gamma 1.1C gamma 4V delta 6C delta T cell receptor is essential for autoreactivity. *J Immunol.* 148:1302-1307.
- Killeen, N., and D.R. Littman. 1993. Helper T-cell development in the absence of CD4-p56lck association. *Nature.* 364:729-732.
- Kim, K., C.K. Lee, T.J. Sayers, K. Muegge, and S.K. Durum. 1998. The trophic action of IL-7 on pro-T cells: inhibition of apoptosis of pro-T1, -T2, and -T3 cells correlates with Bcl-2 and Bax levels and is independent of Fas and p53 pathways. *J Immunol.* 160:5735-5741.
- Kirberg, J., A. Baron, S. Jakob, A. Rolink, K. Karjalainen, and H. von Boehmer. 1994. Thymic selection of CD8+ single positive cells with a class II major histocompatibility complex-restricted receptor. *J Exp Med.* 180:25-34.
- Kirberg, J., W. Swat, B. Rocha, P. Kisielow, and H. von Boehmer. 1993. Induction of tolerance in immature and mature T cells. *Transplant Proc.* 25:279-280.
- Kishihara, K., J. Penninger, V.A. Wallace, T.M. Kundig, K. Kawai, A. Wakeham, E. Timms, K. Pfeffer, P.S. Ohashi, M.L. Thomas, and et al. 1993. Normal B lymphocyte development but impaired T cell maturation in CD45- exon6 protein tyrosine phosphatase-deficient mice. *Cell.* 74:143-156.
- Kishimoto, H., C.D. Surh, and J. Sprent. 1998. A role for Fas in negative selection of thymocytes in vivo. *J Exp Med.* 187:1427-1438.
- Kisielow, P., H. Bluthmann, U.D. Staerz, M. Steinmetz, and H. von Boehmer. 1988a. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes. *Nature.* 333:742-746.
- Kisielow, P., and A. Miazek. 1996. Thymic selection and tolerance. *Transplant Proc.* 28:3429-3430.
- Kisielow, P., H.S. Teh, H. Bluthmann, and H. von Boehmer. 1988b. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature.* 335:730-733.
- Kisielow, P., and H. von Boehmer. 1990. Negative and positive selection of immature thymocytes: timing and the role of the ligand for alpha beta T cell receptor. *Semin Immunol.* 2:35-44.
- Kisielow, P., and H. von Boehmer. 1991. Kinetics of negative and positive selection in the thymus. *Adv Exp Med Biol.* 292:31-42.
- Kisielow, P., and H. von Boehmer. 1993. Role of the thymus in the acquisition of immunologic self-tolerance. *Exp Nephrol.* 1:112-119.
- Kisielow, P., and H. von Boehmer. 1995. Development and selection of T cells: facts and puzzles. *Adv Immunol.* 58:87-209.

- Klug, C.A., S.J. Morrison, M. Masek, K. Hahn, S.T. Smale, and I.L. Weissman. 1998. Hematopoietic stem cells and lymphoid progenitors express different Ikaros isoforms, and Ikaros is localized to heterochromatin in immature lymphocytes. *Proc Natl Acad Sci U S A*. 95:657-662.
- Kohyama, M., M. Nanno, M. Kawaguchi-Miyashita, S. Shimada, M. Watanabe, T. Hibi, S. Kaminogawa, and H. Ishikawa. 1999. Cytolytic and IFN-gamma-producing activities of gamma delta T cells in the mouse intestinal epithelium are T cell receptor-beta-chain dependent. *Proc Natl Acad Sci U S A*. 96:7451-7455.
- Komano, H., Y. Fujiura, M. Kawaguchi, S. Matsumoto, Y. Hashimoto, S. Obana, P. Mombaerts, S. Tonegawa, H. Yamamoto, S. Itoharu, and et al. 1995. Homeostatic regulation of intestinal epithelia by intraepithelial gamma delta T cells. *Proc Natl Acad Sci U S A*. 92:6147-6151.
- Kondo, M., D.C. Scherer, A.G. King, M.G. Manz, and I.L. Weissman. 2001. Lymphocyte development from hematopoietic stem cells. *Curr Opin Genet Dev*. 11:520-526.
- Kondo, M., D.C. Scherer, T. Miyamoto, A.G. King, K. Akashi, K. Sugamura, and I.L. Weissman. 2000. Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. *Nature*. 407:383-386.
- Kondo, M., T. Takeshita, M. Higuchi, M. Nakamura, T. Sudo, S. Nishikawa, and K. Sugamura. 1994. Functional participation of the IL-2 receptor gamma chain in IL-7 receptor complexes. *Science*. 263:1453-1454.
- Kondo, M., I.L. Weissman, and K. Akashi. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell*. 91:661-672.
- Koning, F., A.M. Kruisbeek, W.L. Maloy, S. Marusic-Galesic, D.M. Pardoll, E.M. Shevach, G. Stingl, R. Valas, W.M. Yokoyama, and J.E. Coligan. 1988. T cell receptor gamma/delta chain diversity. *J Exp Med*. 167:676-681.
- Kosugi, A., J.C. Zuniga-Pflucker, S.O. Sharrow, A.M. Kruisbeek, and G.M. Shearer. 1989. Effect of cyclosporin A on lymphopoiesis. II. Developmental defects of immature and mature thymocytes in fetal thymus organ cultures treated with cyclosporin A. *J Immunol*. 143:3134-3140.
- Krangel, M.S., H. Yssel, C. Brocklehurst, and H. Spits. 1990. A distinct wave of human T cell receptor gamma/delta lymphocytes in the early fetal thymus: evidence for controlled gene rearrangement and cytokine production. *J Exp Med*. 172:847-859.
- Kranz, D.M., H. Saito, C.M. Distech, K. Swisshelm, D. Pravtcheva, F.H. Ruddle, H.N. Eisen, and S. Tonegawa. 1985. Chromosomal locations of the murine T-cell receptor alpha-chain gene and the T-cell gamma gene. *Science*. 227:941-945.
- Krimpenfort, P., R. de Jong, Y. Uematsu, Z. Dembic, S. Ryser, H. von Boehmer, M. Steinmetz, and A. Berns. 1988. Transcription of T cell receptor beta-chain genes is controlled by a downstream regulatory element. *Embo J*. 7:745-750.
- Kronenberg, M., G. Siu, L.E. Hood, and N. Shastri. 1986. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. *Annu Rev Immunol*. 4:529-591.
- Kronin, V., H. Hochrein, K. Shortman, and A. Kelso. 2000. Regulation of T cell cytokine production by dendritic cells. *Immunol Cell Biol*. 78:214-223.

- Krotkova, A., H. von Boehmer, and H.J. Fehling. 1997. Allelic exclusion in pTalpha-deficient mice: no evidence for cell surface expression of two T cell receptor (TCR)-beta chains, but less efficient inhibition of endogeneous Vbeta--> (D)Jbeta rearrangements in the presence of a functional TCR-beta transgene. *J Exp Med.* 186:767-775.
- Kruisbeek, A. 1997. T-cell tolerance. *Res Immunol.* 148:420-424.
- Kruisbeek, A., and U. Storb. 1994. Lymphocyte development. *Curr Opin Immunol.* 6:199-202.
- Kruisbeek, A.M. 1993. Development of alpha beta T cells. *Curr Opin Immunol.* 5:227-234.
- Kruisbeek, A.M. 1999. Introduction: regulation of T cell development by the thymic microenvironment. *Semin Immunol.* 11:1-2.
- Kruisbeek, A.M., and D. Amsen. 1996. Mechanisms underlying T-cell tolerance. *Curr Opin Immunol.* 8:233-244.
- Kruisbeek, A.M., M.C. Haks, M. Carleton, A.M. Michie, J.C. Zuniga-Pflucker, and D.L. Wiest. 2000. Branching out to gain control: how the pre-TCR is linked to multiple functions. *Immunol Today.* 21:637-644.
- Kruisbeek, A.M., J.D. Nieland, and L.A. Jones. 1992. Mechanism of tolerance induction. *Adv Exp Med Biol.* 323:101-109.
- Kruisbeek, A.M., J. Zuniga-Pflucker, S. Marusic-Galesic, M.A. Weston, L. Tentori, and D.L. Longo. 1988. Thymic selection of the T-cell repertoire. *Immunol Res.* 7:318-328.
- Kuo, C.T., and J.M. Leiden. 1999. Transcriptional regulation of T lymphocyte development and function. *Annu Rev Immunol.* 17:149-187.
- Kurasawa, K., Y. Hashimoto, and I. Iwamoto. 1999. Fas modulates both positive and negative selection of thymocytes. *Cell Immunol.* 194:127-135.
- Kuziel, W.A., A. Takashima, M. Bonyhadi, P.R. Bergstresser, J.P. Allison, R.E. Tigelaar, and P.W. Tucker. 1987. Regulation of T-cell receptor gamma-chain RNA expression in murine Thy- 1+ dendritic epidermal cells. *Nature.* 328:263-266.
- Kyes, S., E. Carew, S.R. Carding, C.A. Janeway, Jr., and A. Hayday. 1989. Diversity in T-cell receptor gamma gene usage in intestinal epithelium. *Proc Natl Acad Sci U S A.* 86:5527-5531.
- Kyes, S., W. Pao, and A. Hayday. 1991. Influence of site of expression on the fetal gamma delta T-cell receptor repertoire. *Proc Natl Acad Sci U S A.* 88:7830-7833.
- Ladel, C.H., C. Blum, A. Dreher, K. Reifenberg, and S.H. Kaufmann. 1995a. Protective role of gamma/delta T cells and alpha/beta T cells in tuberculosis. *Eur J Immunol.* 25:2877-2881.
- Ladel, C.H., C. Blum, and S.H. Kaufmann. 1996. Control of natural killer cell-mediated innate resistance against the intracellular pathogen *Listeria monocytogenes* by gamma/delta T lymphocytes. *Infect Immun.* 64:1744-1749.
- Ladel, C.H., J. Hess, S. Daugelat, P. Mombaerts, S. Tonegawa, and S.H. Kaufmann. 1995b. Contribution of alpha/beta and gamma/delta T lymphocytes to immunity against

- Mycobacterium bovis bacillus Calmette Guerin: studies with T cell receptor-deficient mutant mice. *Eur J Immunol.* 25:838-846.
- Lafaille, J.J., A. DeCloux, M. Bonneville, Y. Takagaki, and S. Tonegawa. 1989. Junctional sequences of T cell receptor gamma delta genes: implications for gamma delta T cell lineages and for a novel intermediate of V-(D)-J joining. *Cell.* 59:859-870.
- Lafaille, J.J., W. Haas, A. Coutinho, and S. Tonegawa. 1990. Positive selection of gamma delta T cells. *Immunol Today.* 11:75-78.
- Lalli, E., P. Sassone-Corsi, and R. Ceredig. 1996. Block of T lymphocyte differentiation by activation of the cAMP- dependent signal transduction pathway. *Embo J.* 15:528-537. [abs.html](#).
- Lanier, L.L., C. Chang, H. Spits, and J.H. Phillips. 1992a. Expression of cytoplasmic CD3 epsilon proteins in activated human adult natural killer (NK) cells and CD3 gamma, delta, epsilon complexes in fetal NK cells. Implications for the relationship of NK and T lymphocytes. *J Immunol.* 149:1876-1880.
- Lanier, L.L., H. Spits, and J.H. Phillips. 1992b. The developmental relationship between NK cells and T cells. *Immunol Today.* 13:392-395.
- Lantz, O., L.I. Sharara, F. Tilloy, A. Andersson, and J.P. DiSanto. 1997. Lineage relationships and differentiation of natural killer (NK) T cells: intrathymic selection and interleukin (IL)-4 production in the absence of NKR-P1 and Ly49 molecules. *J Exp Med.* 185:1395-1401.
- Lauzurica, P., and M.S. Krangel. 1994. Temporal and lineage-specific control of T cell receptor alpha/delta gene rearrangement by T cell receptor alpha and delta enhancers. *J Exp Med.* 179:1913-1921.
- Le Douarin, N.M., and F.V. Jotereau. 1975. Tracing of cells of the avian thymus through embryonic life in interspecific chimeras. *J Exp Med.* 142:17-40.
- Le Douarin, N.M., F.V. Jotereau, E. Houssaint, and M. Belo. 1976. Ontogeny of the avian thymus and bursa of Fabricius studied in interspecific chimeras. *Ann Immunol (Paris).* 127:849-856.
- Lee, S.L., R.L. Wesselschmidt, G.P. Linette, O. Kanagawa, J.H. Russell, and J. Milbrandt. 1995. Unimpaired thymic and peripheral T cell death in mice lacking the nuclear receptor NGFI-B (Nur77). *Science.* 269:532-535.
- Legname, G., B. Seddon, M. Lovatt, P. Tomlinson, N. Sarnar, M. Tolaini, K. Williams, T. Norton, D. Kioussis, and R. Zamoyska. 2000. Inducible expression of a p56Lck transgene reveals a central role for Lck in the differentiation of CD4 SP thymocytes. *Immunity.* 12:537-546.
- LeLievre, C.S., and N.M. LeDouarin. 1975. Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos. *J Embryol Exp Morph.* 34:125-154.
- Leo, A., and B. Schraven. 2001. Adapters in lymphocyte signalling. *Curr Opin Immunol.* 13:307-316.
- Levelt, C.N., R. Carsetti, and K. Eichmann. 1993a. Regulation of thymocyte development through CD3. II. Expression of T cell receptor beta CD3 epsilon and maturation to the

- CD4+8+ stage are highly correlated in individual thymocytes. *J Exp Med.* 178:1867-1875.
- Levelt, C.N., A. Ehrfeld, and K. Eichmann. 1993b. Regulation of thymocyte development through CD3. I. Timepoint of ligation of CD3 epsilon determines clonal deletion or induction of developmental program. *J Exp Med.* 177:707-716.
- Levelt, C.N., E. Mizoguchi, X. Huang, R. Zacks, A.K. Bhan, and S. Tonegawa. 1998. Inhibition of intrathymic T cell development by expression of a transgenic antagonist peptide. *Proc Natl Acad Sci U S A.* 95:14349-14354.
- Levelt, C.N., P. Mombaerts, A. Iglesias, S. Tonegawa, and K. Eichmann. 1993c. Restoration of early thymocyte differentiation in T-cell receptor beta- chain-deficient mutant mice by transmembrane signaling through CD3 epsilon. *Proc Natl Acad Sci U S A.* 90:11401-11405.
- Levelt, C.N., P. Mombaerts, B. Wang, H. Kohler, S. Tonegawa, K. Eichmann, and C. Terhorst. 1995a. Regulation of thymocyte development through CD3: functional dissociation between p56lck and CD3 sigma in early thymic selection. *Immunity.* 3:215-222.
- Levelt, C.N., B. Wang, A. Ehrfeld, C. Terhorst, and K. Eichmann. 1995b. Regulation of T cell receptor (TCR)-beta locus allelic exclusion and initiation of TCR-alpha locus rearrangement in immature thymocytes by signaling through the CD3 complex. *Eur J Immunol.* 25:1257-1261.
- Levin, S.D., S.J. Anderson, K.A. Forbush, and R.M. Perlmutter. 1993. A dominant-negative transgene defines a role for p56lck in thymopoiesis. *Embo J.* 12:1671-1680.
- Li, L., L.A. Milner, Y. Deng, M. Iwata, A. Banta, L. Graf, S. Marcovina, C. Friedman, B.J. Trask, L. Hood, and B. Torok-Storb. 1998. The human homolog of rat Jagged1 expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch1. *Immunity.* 8:43-55.
- Li, P., S.T. Willie, S. Bauer, D.L. Morris, T. Spies, and R.K. Strong. 1999. Crystal structure of the MHC class I homolog MIC-A, a gammadelta T cell ligand. *Immunity.* 10:577-584.
- Li, Z., D.I. Dordai, J. Lee, and S. Desiderio. 1996. A conserved degradation signal regulates RAG-2 accumulation during cell division and links V(D)J recombination to the cell cycle. *Immunity.* 5:575-589.
- Liao, X.C., and D.R. Littman. 1995. Altered T cell receptor signaling and disrupted T cell development in mice lacking Itk. *Immunity.* 3:757-769.
- Lind, E.F., S.E. Prockop, H.E. Porritt, and H.T. Petrie. 2001. Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development. *J Exp Med.* 194:127-134.
- Linette, G.P., M.J. Grusby, S.M. Hedrick, T.H. Hansen, L.H. Glimcher, and S.J. Korsmeyer. 1994. Bcl-2 is upregulated at the CD4+ CD8+ stage during positive selection and promotes thymocyte differentiation at several control points. *Immunity.* 1:197-205.

- Liu, Z.G., S.W. Smith, K.A. McLaughlin, L.M. Schwartz, and B.A. Osborne. 1994. Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene *nur77*. *Nature*. 367:281-284.
- Livak, F., D.B. Burtrum, L. Rowen, D.G. Schatz, and H.T. Petrie. 2000. Genetic modulation of T cell receptor gene segment usage during somatic recombination. *J Exp Med*. 192:1191-1196.
- Livak, F., H.T. Petrie, I.N. Crispe, and D.G. Schatz. 1995. In-frame TCR delta gene rearrangements play a critical role in the alpha beta/gamma delta T cell lineage decision. *Immunity*. 2:617-627.
- Livak, F., M. Tourigny, D.G. Schatz, and H.T. Petrie. 1999. Characterization of TCR gene rearrangements during adult murine T cell development. *J Immunol*. 162:2575-2580.
- Livak, F., A. Wilson, H.R. MacDonald, and D.G. Schatz. 1997. Alpha beta lineage-committed thymocytes can be rescued by the gamma delta T cell receptor (TCR) in the absence of TCR beta chain. *Eur J Immunol*. 27:2948-2958.
- Love, P.E., E.W. Shores, M.D. Johnson, M.L. Tremblay, E.J. Lee, A. Grinberg, S.P. Huang, A. Singer, and H. Westphal. 1993. T cell development in mice that lack the zeta chain of the T cell antigen receptor complex. *Science*. 261:918-921.
- Lucas, K., D. Vremec, L. Wu, and K. Shortman. 1998. A linkage between dendritic cell and T-cell development in the mouse thymus: the capacity of sequential T-cell precursors to form dendritic cells in culture. *Dev Comp Immunol*. 22:339-349.
- Lundberg, K., W. Heath, F. Kontgen, F.R. Carbone, and K. Shortman. 1995. Intermediate steps in positive selection: differentiation of CD4+8int TCRint thymocytes into CD4+8+TCRhi thymocytes. *J Exp Med*. 181:1643-1651.
- Lundberg, K., and K. Shortman. 1994. Small cortical thymocytes are subject to positive selection. *J Exp Med*. 179:1475-1483.
- Luo, B., J.C. Aster, R.P. Hasserjian, F. Kuo, and J. Sklar. 1997. Isolation and functional analysis of a cDNA for human Jagged2, a gene encoding a ligand for the Notch1 receptor. *Mol Cell Biol*. 17:6057-6067.
- MacDonald, H.R., F. Radtke, and A. Wilson. 2001. T cell fate specification and alphabeta/gammadelta lineage commitment. *Curr Opin Immunol*. 13:219-224.
- MacDonald, H.R., and A. Wilson. 1998. The role of the T-cell receptor (TCR) in alpha beta/gamma delta lineage commitment: clues from intracellular TCR staining. *Immunol Rev*. 165:87-94.
- MacLean, L.D., S.J. Zak, R.L. Varco, and R.A. Good. 1957. The role of the thymus in antibody production: an experimental study of the immune response in thymectomized rabbits. *Transplant Bull*. 41:21-22.
- Maki, K., S. Sunaga, Y. Komagata, Y. Kodaira, A. Mabuchi, H. Karasuyama, K. Yokomuro, J.I. Miyazaki, and K. Ikuta. 1996. Interleukin 7 receptor-deficient mice lack gammadelta T cells. *Proc Natl Acad Sci U S A*. 93:7172-7177.

- Malek, T.R., R.B. Levy, B. Adkins, and Y.W. He. 1998. Monoclonal antibodies to the common gamma-chain as cytokine receptor antagonists in vivo: effect on intrathymic and intestinal intraepithelial T lymphocyte development. *J Leukoc Biol.* 63:643-649.
- Malissen, B., L. Ardouin, S.Y. Lin, A. Gillet, and M. Malissen. 1999. Function of the CD3 subunits of the pre-TCR and TCR complexes during T cell development. *Adv Immunol.* 72:103-148.
- Malissen, B., and M. Malissen. 1995. Allelic exclusion of T cell antigen receptor genes. In *T cell receptors*. J.I. Bell, M.J. Owen, and E. Simpson, editors. Oxford University Press, New York. 352-365.
- Malissen, M., A. Gillet, L. Ardouin, G. Bouvier, J. Trucy, P. Ferrier, E. Vivier, and B. Malissen. 1995. Altered T cell development in mice with a targeted mutation of the CD3-epsilon gene. *Embo J.* 14:4641-4653.
- Malissen, M., P. Pereira, D.J. Gerber, B. Malissen, and J.P. DiSanto. 1997. The common cytokine receptor gamma chain controls survival of gamma/delta T cells. *J Exp Med.* 186:1277-1285.
- Mallick, C.A., E.C. Dudley, J.L. Viney, M.J. Owen, and A.C. Hayday. 1993. Rearrangement and diversity of T cell receptor beta chain genes in thymocytes: a critical role for the beta chain in development. *Cell.* 73:513-519.
- Mallick-Wood, C.A., W. Pao, A.M. Cheng, J.M. Lewis, S. Kulkarni, J.B. Bolen, B. Rowley, R.E. Tigelaar, T. Pawson, and A.C. Hayday. 1996. Disruption of epithelial gamma delta T cell repertoires by mutation of the Syk tyrosine kinase. *Proc Natl Acad Sci U S A.* 93:9704-9709.
- Mancini, S., S.M. Candeias, H.J. Fehling, H. von Boehmer, E. Jouvin-Marche, and P.N. Marche. 1999. TCR alpha-chain repertoire in pTalpha-deficient mice is diverse and developmentally regulated: implications for pre-TCR functions and TCRA gene rearrangement. *J Immunol.* 163:6053-6059.
- Manley, N.R. 2000. Thymus organogenesis and molecular mechanisms of thymic epithelial cell differentiation. *Semin Immunol.* 12:421-428.
- Maraskovsky, E., L.A. O'Reilly, M. Teepe, L.M. Corcoran, J.J. Peschon, and A. Strasser. 1997. Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant rag-1^{-/-} mice. *Cell.* 89:1011-1019.
- Maraskovsky, E., M. Teepe, P.J. Morrissey, S. Braddy, R.E. Miller, D.H. Lynch, and J.J. Peschon. 1996. Impaired survival and proliferation in IL-7 receptor-deficient peripheral T cells. *J Immunol.* 157:5315-5323.
- Mariathasan, S., S.S. Ho, A. Zakarian, and P.S. Ohashi. 2000. Degree of ERK activation influences both positive and negative thymocyte selection. *Eur J Immunol.* 30:1060-1068.
- Mariathasan, S., R.G. Jones, and P.S. Ohashi. 1999. Signals involved in thymocyte positive and negative selection. *Semin Immunol.* 11:263-272.
- Mariathasan, S., A. Zakarian, D. Bouchard, A.M. Michie, J.C. Zuniga-Pflucker, and P.S. Ohashi. 2001. Duration and strength of extracellular signal-regulated kinase signals are altered during positive versus negative thymocyte selection. *J Immunol.* 167:4966-4973.

- Marrack, P., L. Ignatowicz, J.W. Kappler, J. Boymel, and J.H. Freed. 1993. Comparison of peptides bound to spleen and thymus class II. *J Exp Med.* 178:2173-2183.
- Marusic-Galesic, S., D.A. Stephany, D.L. Longo, and A.M. Kruisbeek. 1988. Development of CD4-CD8+ cytotoxic T cells requires interactions with class I MHC determinants. *Nature.* 333:180-183.
- Matechak, E.O., N. Killeen, S.M. Hedrick, and B.J. Fowlkes. 1996. MHC class II-specific T cells can develop in the CD8 lineage when CD4 is absent. *Immunity.* 4:337-347.
- Matzinger, P., and S. Guerder. 1989. Does T-cell tolerance require a dedicated antigen-presenting cell? *Nature.* 338:74-76.
- Mazel, S., D. Burtrum, and H.T. Petrie. 1996. Regulation of cell division cycle progression by bcl-2 expression: a potential mechanism for inhibition of programmed cell death. *J Exp Med.* 183:2219-2226.
- McCarthy, S.A., A.M. Kruisbeek, I.K. Uppenkamp, S.O. Sharrow, and A. Singer. 1988. Engagement of the CD4 molecule influences cell surface expression of the T-cell receptor on thymocytes. *Nature.* 336:76-79.
- Medawar, P.B. 1963. The role of the thymus in the origin of immunological competence. In *The immunologically competent cell.* G.E. Wolstenholme and J. Knight, editors. Churchill, London. 70.
- Mee, P.J., M. Turner, M.A. Basson, P.S. Costello, R. Zamoyska, and V.L. Tybulewicz. 1999. Greatly reduced efficiency of both positive and negative selection of thymocytes in CD45 tyrosine phosphatase-deficient mice. *Eur J Immunol.* 29:2923-2933.
- Mertsching, E., and R. Ceredig. 1996. T cell receptor-gamma, delta-expressing fetal mouse thymocytes are generated without T cell receptor V beta selection. *Eur J Immunol.* 26:804-810.
- Mertsching, E., A. Wilson, H.R. MacDonald, and R. Ceredig. 1997. T cell receptor alpha gene rearrangement and transcription in adult thymic gamma delta cells. *Eur J Immunol.* 27:389-396.
- Metcalf, D., and M.A.S. Moore. 1971. Embryonic aspects of haemopoiesis. In *Haematopoietic cells.* A. Neuberger and E.L. Tatum, editors. North Jollan Publisher Co., Amsterdam. 173-271.
- Michie, A.M., S. Trop, D.L. Wiest, and J.C. Zuniga-Pflucker. 1999. Extracellular signal-regulated kinase (ERK) activation by the pre-T cell receptor in developing thymocytes in vivo. *J Exp Med.* 190:1647-1656.
- Miller, J.F.A.P. 1961. Analysis of the thymus influence in leukaemogenesis. *Nature.* 191:248-249.
- Miller, J.F.A.P. 1962. Immunological significance of the thymus of the adult mouse. *Nature.* 191:248-249.
- Miller, J.F.A.P. 1995. Introduction: historical overview. In *T cell receptors.* J.I. Bell, M.J. Owen, and E. Simpson, editors. Oxford University Press, New York. 1-8.

- Miyazaki, T. 1997. Two distinct steps during thymocyte maturation from CD4-CD8- to CD4+CD8+ distinguished in the early growth response (Egr)-1 transgenic mice with a recombinase-activating gene-deficient background. *J Exp Med.* 186:877-885.
- Miyazaki, T., and F.A. Lemonnier. 1998. Modulation of thymic selection by expression of an immediate-early gene, early growth response 1 (Egr-1). *J Exp Med.* 188:715-723.
- Mok, C.L., G. Gil-Gomez, O. Williams, M. Coles, S. Taga, M. Tolaini, T. Norton, D. Kioussis, and H.J. Brady. 1999. Bad can act as a key regulator of T cell apoptosis and T cell development. *J Exp Med.* 189:575-586.
- Molina, C.A., N.S. Foulkes, E. Lalli, and P. Sassone-Corsi. 1993. Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. *Cell.* 75:875-886.
- Molina, T.J., K. Kishihara, D.P. Siderovski, W. van Ewijk, A. Narendran, E. Timms, A. Wakeham, C.J. Paige, K.U. Hartmann, A. Veillette, and et al. 1992. Profound block in thymocyte development in mice lacking p56lck. *Nature.* 357:161-164.
- Mombaerts, P., S.J. Anderson, R.M. Perlmutter, T.W. Mak, and S. Tonegawa. 1994. An activated lck transgene promotes thymocyte development in RAG-1 mutant mice. *Immunity.* 1:261-267.
- Mombaerts, P., J. Arnoldi, F. Russ, S. Tonegawa, and S.H. Kaufmann. 1993a. Different roles of alpha beta and gamma delta T cells in immunity against an intracellular bacterial pathogen. *Nature.* 365:53-56.
- Mombaerts, P., A.R. Clarke, M.A. Rudnicki, J. Iacomini, S. Itoharu, J.J. Lafaille, L. Wang, Y. Ichikawa, R. Jaenisch, M.L. Hooper, and et al. 1992b. Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature.* 360:225-231.
- Mombaerts, P., J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, and V.E. Papaioannou. 1992a. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell.* 68:869-877.
- Mombaerts, P., E. Mizoguchi, M.J. Grusby, L.H. Glimcher, A.K. Bhan, and S. Tonegawa. 1993b. Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. *Cell.* 75:274-282.
- Mombaerts, P., C. Terhorst, T. Jacks, S. Tonegawa, and J. Sancho. 1995. Characterization of immature thymocyte lines derived from T-cell receptor or recombination activating gene 1 and p53 double mutant mice. *Proc Natl Acad Sci U S A.* 92:7420-7424.
- Monaco, L., and P. Sassone-Corsi. 1997. Cross-talk in signal transduction: Ras-dependent induction of cAMP- responsive transcriptional repressor ICER by nerve growth factor. *Oncogene.* 15:2493-2500.
- Moore, M.A., and J.J. Owen. 1967. Experimental studies on the development of the thymus. *J Exp Med.* 126:715-726.
- Moore, T.A., and A. Zlotnik. 1995. T-cell lineage commitment and cytokine responses of thymic progenitors. *Blood.* 86:1850-1860.
- Mori, S., K. Shortman, and L. Wu. 2001. Characterization of thymus-seeding precursor cells from mouse bone marrow. *Blood.* 98:696-704.

- Morita, C.T., S. Verma, P. Aparicio, C. Martinez, H. Spits, and M.B. Brenner. 1991. Functionally distinct subsets of human gamma/delta T cells. *Eur J Immunol.* 21:2999-3007.
- Morrison, S.J., N. Uchida, and I.L. Weissman. 1995. The biology of hematopoietic stem cells. *Annu Rev Cell Dev Biol.* 11:35-71.
- Morrison, S.J., A.M. Wandycz, H.D. Hemmati, D.E. Wright, and I.L. Weissman. 1997. Identification of a lineage of multipotent hematopoietic progenitors. *Development.* 124:1929-1939.
- Muthusamy, N., and J.M. Leiden. 1998. A protein kinase C-, Ras-, and RSK2-dependent signal transduction pathway activates the cAMP-responsive element-binding protein transcription factor following T cell receptor engagement. *J Biol Chem.* 273:22841-22847.
- Myung, P.S., N.J. Boerthe, and G.A. Koretzky. 2000. Adapter proteins in lymphocyte antigen-receptor signaling. *Curr Opin Immunol.* 12:256-266.
- Nantel, F., L. Monaco, N.S. Foulkes, D. Masquillier, M. LeMeur, K. Henriksen, A. Dierich, M. Parvinen, and P. Sassone-Corsi. 1996. Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice. *Nature.* 380:159-162.
- Naquet, P., M. Naspetti, and R. Boyd. 1999. Development, organization and function of the thymic medulla in normal, immunodeficient or autoimmune mice. *Semin Immunol.* 11:47-55.
- Negishi, I., N. Motoyama, K. Nakayama, S. Senju, S. Hatakeyama, Q. Zhang, A.C. Chan, and D.Y. Loh. 1995. Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature.* 376:435-438.
- Neubauer, H., A. Cumano, M. Muller, H. Wu, U. Huffstadt, and K. Pfeffer. 1998. Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. *Cell.* 93:397-409.
- Nishimura, H., M. Emoto, K. Hiromatsu, S. Yamamoto, K. Matsuura, H. Gomi, T. Ikeda, S. Itohara, and Y. Yoshikai. 1995. The role of gamma delta T cells in priming macrophages to produce tumor necrosis factor-alpha. *Eur J Immunol.* 25:1465-1468.
- Norment, A.M., L.Y. Bogatzki, B.N. Gantner, and M.J. Bevan. 2000. Murine CCR9, a chemokine receptor for thymus-expressed chemokine that is up-regulated following pre-TCR signaling. *J Immunol.* 164:639-648.
- Nosaka, T., J.M. van Deursen, R.A. Tripp, W.E. Thierfelder, B.A. Witthuhn, A.P. McMickle, P.C. Doherty, G.C. Grosveld, and J.N. Ihle. 1995. Defective lymphoid development in mice lacking Jak3. *Science.* 270:800-802.
- Novotny, J., S. Tonegawa, H. Saito, D.M. Kranz, and H.N. Eisen. 1986. Secondary, tertiary, and quaternary structure of T-cell-specific immunoglobulin-like polypeptide chains. *Proc Natl Acad Sci U S A.* 83:742-746.
- O'Shea, C.C., T. Crompton, I.R. Rosewell, A.C. Hayday, and M.J. Owen. 1996. Raf regulates positive selection. *Eur J Immunol.* 26:2350-2355.

- Ogasawara, J., T. Suda, and S. Nagata. 1995. Selective apoptosis of CD4+CD8+ thymocytes by the anti-Fas antibody. *J Exp Med.* 181:485-491.
- Okamura, R.M., M. Sigvardsson, J. Galceran, S. Verbeek, H. Clevers, and R. Grosschedl. 1998. Redundant regulation of T cell differentiation and TCRalpha gene expression by the transcription factors LEF-1 and TCF-1. *Immunity.* 8:11-20.
- Olszowy, M.W., P.L. Leuchtman, A. Veillette, and A.S. Shaw. 1995. Comparison of p56lck and p59fyn protein expression in thymocyte subsets, peripheral T cells, NK cells, and lymphoid cell lines. *J Immunol.* 155:4236-4240.
- Oosterwegel, M., M. van de Wetering, J. Timmerman, A. Kruisbeek, O. Destree, F. Meijlink, and H. Clevers. 1993. Differential expression of the HMG box factors TCF-1 and LEF-1 during murine embryogenesis. *Development.* 118:439-448.
- Oosterwegel, M.A., M.C. Haks, U. Jeffry, R. Murray, and A.M. Kruisbeek. 1997. Induction of TCR gene rearrangements in uncommitted stem cells by a subset of IL-7 producing, MHC class-II-expressing thymic stromal cells. *Immunity.* 6:351-360.
- Osborne, B.A. 2000. Transcriptional control of T cell development. *Curr Opin Immunol.* 12:301-306.
- Oukka, M., I.C. Ho, F.C. de la Brousse, T. Hoey, M.J. Grusby, and L.H. Glimcher. 1998. The transcription factor NFAT4 is involved in the generation and survival of T cells. *Immunity.* 9:295-304.
- Owen, J.J., D.E. McLoughlin, R.K. Suniara, and E.J. Jenkinson. 2000. The role of mesenchyme in thymus development. *Curr Top Microbiol Immunol.* 251:133-137.
- Owen, J.J., and M.C. Raff. 1970. Studies on the differentiation of thymus-derived lymphocytes. *J Exp Med.* 132:1216-1232.
- Owen, J.J., and M.A. Ritter. 1969. Tissue interaction in the development of thymus lymphocytes. *J Exp Med.* 129:431-442.
- Pallard, C., A.P. Stegmann, T. van Kleffens, F. Smart, A. Venkitaraman, and H. Spits. 1999. Distinct roles of the phosphatidylinositol 3-kinase and STAT5 pathways in IL-7-mediated development of human thymocyte precursors. *Immunity.* 10:525-535.
- Palmer, D.B., J.L. Viney, M.A. Ritter, A.C. Hayday, and M.J. Owen. 1993. Expression of the alpha beta T-cell receptor is necessary for the generation of the thymic medulla. *Dev Immunol.* 3:175-179.
- Pandey, A., H. Duan, and V.M. Dixit. 1995. Characterization of a novel Src-like adapter protein that associates with the Eck receptor tyrosine kinase. *J Biol Chem.* 270:19201-19204.
- Pandolfi, P.P., M.E. Roth, A. Karis, M.W. Leonard, E. Dzierzak, F.G. Grosveld, J.D. Engel, and M.H. Lindenbaum. 1995. Targeted disruption of the GATA3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis. *Nat Genet.* 11:40-44.
- Pao, W., L. Wen, A.L. Smith, A. Gulbranson-Judge, B. Zheng, G. Kelsoe, I.C. MacLennan, M.J. Owen, and A.C. Hayday. 1996. Gamma delta T cell help of B cells is induced by repeated parasitic infection, in the absence of other T cells. *Curr Biol.* 6:1317-1325.

- Pardoll, D.M., B.J. Fowlkes, J.A. Bluestone, A. Kruisbeek, W.L. Maloy, J.E. Coligan, and R.H. Schwartz. 1987a. Differential expression of two distinct T-cell receptors during thymocyte development. *Nature*. 326:79-81.
- Pardoll, D.M., B.J. Fowlkes, A.M. Lew, W.L. Maloy, M.A. Weston, J.A. Bluestone, R.H. Schwartz, J.E. Coligan, and A.M. Kruisbeek. 1988. Thymus-dependent and thymus-independent developmental pathways for peripheral T cell receptor-gamma delta-bearing lymphocytes. *J Immunol*. 140:4091-4096.
- Pardoll, D.M., A.M. Lew, W.L. Maloy, B.J. Fowlkes, A. Kruisbeek, J.A. Bluestone, R.H. Schwartz, and J.E. Coligan. 1987b. Analysis of T-cell receptor gamma chain expression in the thymus. *Adv Exp Med Biol*. 225:241-246.
- Park, I.K., Y. He, F. Lin, O.D. Laerum, Q. Tian, R. Bumgarner, C.A. Klug, K. Li, C. Kuhr, M.J. Doyle, T. Xie, M. Schummer, Y. Sun, A. Goldsmith, M.F. Clarke, I.L. Weissman, L. Hood, and L. Li. 2002. Differential gene expression profiling of adult murine hematopoietic stem cells. *Blood*. 99:488-498.
- Park, L.S., U. Martin, K. Garka, B. Gliniak, J.P. Di Santo, W. Muller, D.A. Largaespada, N.G. Copeland, N.A. Jenkins, A.G. Farr, S.F. Ziegler, P.J. Morrissey, R. Paxton, and J.E. Sims. 2000. Cloning of the murine thymic stromal lymphopoietin (TSLP) receptor: Formation of a functional heteromeric complex requires interleukin 7 receptor. *J Exp Med*. 192:659-670.
- Parrott, D.M.V., M.A.B. de Sousa, and J. East. 1966. Thymus-dependent areas in the lymphoid organs of neonatally thymectomized mice. *J Exp Med*. 123:191-204.
- Passoni, L., E.S. Hoffman, S. Kim, T. Crompton, W. Pao, M.Q. Dong, M.J. Owen, and A.C. Hayday. 1997. Intrathymic delta selection events in gammadelta cell development. *Immunity*. 7:83-95.
- Pawlowski, T., J.D. Elliott, D.Y. Loh, and U.D. Staerz. 1993. Positive selection of T lymphocytes on fibroblasts. *Nature*. 364:642-645.
- Pearse, M., P. Gallagher, A. Wilson, L. Wu, N. Fisticaro, J.F. Miller, R. Scollay, and K. Shortman. 1988. Molecular characterization of T-cell antigen receptor expression by subsets of CD4- CD8- murine thymocytes. *Proc Natl Acad Sci U S A*. 85:6082-6086.
- Pearse, M., L. Wu, M. Egerton, A. Wilson, K. Shortman, and R. Scollay. 1989. A murine early thymocyte developmental sequence is marked by transient expression of the interleukin 2 receptor. *Proc Natl Acad Sci U S A*. 86:1614-1618.
- Peault, B., I.L. Weissman, A.M. Buckle, A. Tsukamoto, and C. Baum. 1993. Thy-1-expressing CD34+ human cells express multiple hematopoietic potentialities in vitro and in SCID-hu mice. *Nouv Rev Fr Hematol*. 35:91-93.
- Peng, S.L., M.P. Madaio, D.P. Hughes, I.N. Crispe, M.J. Owen, L. Wen, A.C. Hayday, and J. Craft. 1996. Murine lupus in the absence of alpha beta T cells. *J Immunol*. 156:4041-4049.
- Penninger, J., K. Kishihara, T. Molina, V.A. Wallace, E. Timms, S.M. Hedrick, and T.W. Mak. 1993. Requirement for tyrosine kinase p56lck for thymic development of transgenic gamma delta T cells. *Science*. 260:358-361.

- Penninger, J.M., C. Sirard, H.W. Mittrucker, A. Chidgey, I. Kozieradzki, M. Nghiem, A. Hakem, T. Kimura, E. Timms, R. Boyd, T. Taniguchi, T. Matsuyama, and T.W. Mak. 1997. The interferon regulatory transcription factor IRF-1 controls positive and negative selection of CD8⁺ thymocytes. *Immunity*. 7:243-254.
- Pereira, P., D. Gerber, S.Y. Huang, and S. Tonegawa. 1995. Ontogenic development and tissue distribution of V gamma 1-expressing gamma/delta T lymphocytes in normal mice. *J Exp Med*. 182:1921-1930.
- Pereira, P., J.J. Lafaille, D. Gerber, and S. Tonegawa. 1997. The T cell receptor repertoire of intestinal intraepithelial gammadelta T lymphocytes is influenced by genes linked to the major histocompatibility complex and to the T cell receptor loci. *Proc Natl Acad Sci U S A*. 94:5761-5766.
- Pereira, P., M. Zijlstra, J. McMaster, J.M. Loring, R. Jaenisch, and S. Tonegawa. 1992. Blockade of transgenic gamma delta T cell development in beta 2- microglobulin deficient mice. *Embo J*. 11:25-31.
- Peschon, J.J., P.J. Morrissey, K.H. Grabstein, F.J. Ramsdell, E. Maraskovsky, B.C. Gliniak, L.S. Park, S.F. Ziegler, D.E. Williams, C.B. Ware, and et al. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med*. 180:1955-1960.
- Petrie, H.T., P. Hugo, R. Scollay, and K. Shortman. 1990a. Lineage relationships and developmental kinetics of immature thymocytes: CD3, CD4, and CD8 acquisition in vivo and in vitro. *J Exp Med*. 172:1583-1588.
- Petrie, H.T., F. Livak, D.G. Schatz, A. Strasser, I.N. Crispe, and K. Shortman. 1993. Multiple rearrangements in T cell receptor alpha chain genes maximize the production of useful thymocytes. *J Exp Med*. 178:615-622.
- Petrie, H.T., M. Pearse, R. Scollay, and K. Shortman. 1990b. Development of immature thymocytes: initiation of CD3, CD4, and CD8 acquisition parallels down-regulation of the interleukin 2 receptor alpha chain. *Eur J Immunol*. 20:2813-2815.
- Petrie, H.T., R. Scollay, and K. Shortman. 1992. Commitment to the T cell receptor-alpha beta or -gamma delta lineages can occur just prior to the onset of CD4 and CD8 expression among immature thymocytes. *Eur J Immunol*. 22:2185-2188.
- Petrie, H.T., M. Tourigny, D.B. Burtrum, and F. Livak. 2000. Precursor thymocyte proliferation and differentiation are controlled by signals unrelated to the pre-TCR. *J Immunol*. 165:3094-3098.
- Pfeffer, K., B. Schoel, H. Gulle, S.H. Kaufmann, and H. Wagner. 1990. Primary responses of human T cells to mycobacteria: a frequent set of gamma/delta T cells are stimulated by protease-resistant ligands. *Eur J Immunol*. 20:1175-1179.
- Philpott, K.L., J.L. Viney, G. Kay, S. Rastan, E.M. Gardiner, S. Chae, A.C. Hayday, and M.J. Owen. 1992. Lymphoid development in mice congenitally lacking T cell receptor alpha beta-expressing cells. *Science*. 256:1448-1452.
- Pircher, H., U.H. Rohrer, D. Moskophidis, R.M. Zinkernagel, and H. Hengartner. 1991. Lower receptor avidity required for thymic clonal deletion than for effector T-cell function. *Nature*. 351:482-485.

- Pivniouk, V., E. Tsitsikov, P. Swinton, G. Rathbun, F.W. Alt, and R.S. Geha. 1998. Impaired viability and profound block in thymocyte development in mice lacking the adaptor protein SLP-76. *Cell*. 94:229-238.
- Porter, B.O., and T.R. Malek. 1999. IL-2Rbeta/IL-7Ralpha doubly deficient mice recapitulate the thymic and intraepithelial lymphocyte (IEL) developmental defects of gammac^{-/-} mice: roles for both IL-2 and IL-15 in CD8alphaalpha IEL development. *J Immunol*. 163:5906-5912.
- Pui, J.C., D. Allman, L. Xu, S. DeRocco, F.G. Karnell, S. Bakkour, J.Y. Lee, T. Kadesch, R.R. Hardy, J.C. Aster, and W.S. Pear. 1999. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity*. 11:299-308.
- Radtke, F., I. Ferrero, A. Wilson, R. Lees, M. Aguet, and H.R. MacDonald. 2000. Notch1 deficiency dissociates the intrathymic development of dendritic cells and T cells. *J Exp Med*. 191:1085-1094.
- Radtke, F., A. Wilson, G. Stark, M. Bauer, J. van Meerwijk, H.R. MacDonald, and M. Aguet. 1999. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity*. 10:547-558.
- Randall, T.D., and I.L. Weissman. 1998. Characterization of a population of cells in the bone marrow that phenotypically mimics hematopoietic stem cells: resting stem cells or mystery population? *Stem Cells*. 16:38-48.
- Raulet, D.H. 1989. The structure, function, and molecular genetics of the gamma/delta T cell receptor. *Annu Rev Immunol*. 7:175-207.
- Raulet, D.H., R.D. Garman, H. Saito, and S. Tonegawa. 1985. Developmental regulation of T-cell receptor gene expression. *Nature*. 314:103-107.
- Razavi, R., J.C. Ramos, G. Yehia, F. Schlotter, and C.A. Molina. 1998. ICER-IIgamma is a tumor suppressor that mediates the antiproliferative activity of cAMP. *Oncogene*. 17:3015-3019.
- Res, P., and H. Spits. 1999. Developmental stages in the human thymus. *Semin Immunol*. 11:39-46.
- Res, P.C., F. Couwenberg, F.A. Vyth-Dreese, and H. Spits. 1999. Expression of pTalpha mRNA in a committed dendritic cell precursor in the human thymus. *Blood*. 94:2647-2657.
- Robey, E., D. Chang, A. Itano, D. Cado, H. Alexander, D. Lans, G. Weinmaster, and P. Salmon. 1996. An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. *Cell*. 87:483-492.
- Robey, E., and B.J. Fowlkes. 1994. Selective events in T cell development. *Annu Rev Immunol*. 12:675-705.
- Robey, E., and B.J. Fowlkes. 1998. The alpha beta versus gamma delta T-cell lineage choice. *Curr Opin Immunol*. 10:181-187.
- Robey, E.A., B.J. Fowlkes, J.W. Gordon, D. Kioussis, H. von Boehmer, F. Ramsdell, and R. Axel. 1991. Thymic selection in CD8 transgenic mice supports an instructive model for commitment to a CD4 or CD8 lineage. *Cell*. 64:99-107.

- Robinson, J.H., and J.J. Owen. 1977. Generation of T-cell function in organ culture of foetal mouse thymus. II. Mixed lymphocyte culture reactivity. *Clin Exp Immunol.* 27:322-327.
- Rodewald, H.R. 1995. Pathways from hematopoietic stem cells to thymocytes. *Curr Opin Immunol.* 7:176-187.
- Rodewald, H.R., T. Brocker, and C. Haller. 1999. Developmental dissociation of thymic dendritic cell and thymocyte lineages revealed in growth factor receptor mutant mice. *Proc Natl Acad Sci U S A.* 96:15068-15073.
- Rodewald, H.R., and H.J. Fehling. 1998. Molecular and cellular events in early thymocyte development. *Adv Immunol.* 69:1-112.
- Rodewald, H.R., K. Kretzschmar, W. Swat, and S. Takeda. 1995. Intrathymically expressed c-kit ligand (stem cell factor) is a major factor driving expansion of very immature thymocytes in vivo. *Immunity.* 3:313-319.
- Rodewald, H.R., K. Kretzschmar, S. Takeda, C. Hohl, and M. Dessing. 1994. Identification of pro-thymocytes in murine fetal blood: T lineage commitment can precede thymus colonization. *Embo J.* 13:4229-4240.
- Rodewald, H.R., P. Moingeon, J.L. Lucich, C. Dosiou, P. Lopez, and E.L. Reinherz. 1992. A population of early fetal thymocytes expressing Fc gamma RII/III contains precursors of T lymphocytes and natural killer cells. *Cell.* 69:139-150.
- Rodewald, H.R., M. Ogawa, C. Haller, C. Waskow, and J.P. DiSanto. 1997. Pro-thymocyte expansion by c-kit and the common cytokine receptor gamma chain is essential for repertoire formation. *Immunity.* 6:265-272.
- Rodewald, H.R., C. Waskow, and C. Haller. 2001. Essential requirement for c-kit and common gamma chain in thymocyte development cannot be overruled by enforced expression of Bcl-2. *J Exp Med.* 193:1431-1437.
- Rosat, J.P., F. Conceicao-Silva, G.A. Waanders, F. Beermann, A. Wilson, M.J. Owen, A.C. Hayday, S. Huang, M. Aguet, H.R. MacDonald, and et al. 1995. Expansion of gamma delta+ T cells in BALB/c mice infected with *Leishmania major* is dependent upon Th2-type CD4+ T cells. *Infect Immun.* 63:3000-3004.
- Rothenberg, E.V., J.C. Telfer, and M.K. Anderson. 1999. Transcriptional regulation of lymphocyte lineage commitment. *Bioessays.* 21:726-742.
- Ruchaud, S., P. Seite, N.S. Foulkes, P. Sassone-Corsi, and M. Lanotte. 1997. The transcriptional repressor ICER and cAMP-induced programmed cell death. *Oncogene.* 15:827-836.
- Rudolph, D., A. Tafuri, P. Gass, G.J. Hammerling, B. Arnold, and G. Schutz. 1998. Impaired fetal T cell development and perinatal lethality in mice lacking the cAMP response element binding protein. *Proc Natl Acad Sci U S A.* 95:4481-4486.
- Ruggiero, G., E.M. Caceres, A. Voordouw, E. Noteboom, D. Graf, R.A. Kroccek, and H. Spits. 1996. CD40 expressed on thymic epithelial cells provides costimulation for proliferation but not for apoptosis of human thymocytes. *J Immunol.* 156:3737-3746.

- Rytkonen-Nissinen, M., J.L. Hurwitz, S. Pelkonen, C. Levelt, and J. Pelkonen. 1999. Early activation of TCR alpha gene rearrangement in fetal thymocytes. *Eur J Immunol.* 29:2288-2296.
- Saint-Ruf, C., O. Lechner, J. Feinberg, and H. von Boehmer. 1998. Genomic structure of the human pre-T cell receptor alpha chain and expression of two mRNA isoforms. *Eur J Immunol.* 28:3824-3831.
- Saint-Ruf, C., M. Panigada, O. Azogui, P. Debey, H. von Boehmer, and F. Grassi. 2000. Different initiation of pre-TCR and gammadeltaTCR signalling. *Nature.* 406:524-527.
- Saint-Ruf, C., K. Ungewiss, M. Groettrup, L. Bruno, H.J. Fehling, and H. von Boehmer. 1994. Analysis and expression of a cloned pre-T cell receptor gene. *Science.* 266:1208-1212.
- Saito, H., T. Koyama, K. Georgopoulos, H. Clevers, W.G. Haser, T. LeBien, S. Tonegawa, and C. Terhorst. 1987. Close linkage of the mouse and human CD3 gamma- and delta-chain genes suggests that their transcription is controlled by common regulatory elements. *Proc Natl Acad Sci U S A.* 84:9131-9134.
- Saito, H., D.M. Kranz, Y. Takagaki, A.C. Hayday, H.N. Eisen, and S. Tonegawa. 1984. Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. *Nature.* 309:757-762.
- Sassone-Corsi, P. 1998. Coupling gene expression to cAMP signalling: role of CREB and CREM. *Int J Biochem Cell Biol.* 30:27-38.
- Satyanarayana, K., S. Hata, P. Devlin, M.G. Roncarolo, J.E. De Vries, H. Spits, J.L. Strominger, and M.S. Krangel. 1988. Genomic organization of the human T-cell antigen-receptor alpha/delta locus. *Proc Natl Acad Sci U S A.* 85:8166-8170.
- Saunders, D.J., H.M. Georgiou, L. Wu, and K. Shortman. 1995. Induction of limited growth and differentiation of early thymic precursor cells by thymic epithelial cell lines. *Immunol Lett.* 47:45-51.
- Scheffold, A., S. Miltenyi, and A. Radbruch. 1995. Magnetofluorescent liposomes for increased sensitivity of immunofluorescence. *Immunotechnology.* 1:127-137.
- Schilham, M.W., P. Moerer, A. Cumano, and H.C. Clevers. 1997. Sox-4 facilitates thymocyte differentiation. *Eur J Immunol.* 27:1292-1295.
- Schilham, M.W., M.A. Oosterwegel, P. Moerer, J. Ya, P.A. de Boer, M. van de Wetering, S. Verbeek, W.H. Lamers, A.M. Kruisbeek, A. Cumano, and H. Clevers. 1996. Defects in cardiac outflow tract formation and pro-B-lymphocyte expansion in mice lacking Sox-4. *Nature.* 380:711-714.
- Schilham, M.W., A. Wilson, P. Moerer, B.J. Benaissa-Trouw, A. Cumano, and H.C. Clevers. 1998. Critical involvement of Tcf-1 in expansion of thymocytes. *J Immunol.* 161:3984-3991.
- Schlissel, M.S., S.D. Durum, and K. Muegge. 2000. The interleukin 7 receptor is required for T cell receptor gamma locus accessibility to the V(D)J recombinase. *J Exp Med.* 191:1045-1050.

- Schmedt, C., K. Saijo, T. Niidome, R. Kuhn, S. Aizawa, and A. Tarakhovsky. 1998. Csk controls antigen receptor-mediated development and selection of T-lineage cells. *Nature*. 394:901-904.
- Sciammas, R., R.M. Johnson, A.I. Sperling, W. Brady, P.S. Linsley, P.G. Spear, F.W. Fitch, and J.A. Bluestone. 1994. Unique antigen recognition by a herpesvirus-specific TCR-gamma delta cell. *J Immunol*. 152:5392-5397.
- Sciammas, R., P. Kodukula, Q. Tang, R.L. Hendricks, and J.A. Bluestone. 1997. T cell receptor-gamma/delta cells protect mice from herpes simplex virus type 1-induced lethal encephalitis. *J Exp Med*. 185:1969-1975.
- Scott, B., H. Bluthmann, H.S. Teh, and H. von Boehmer. 1989. The generation of mature T cells requires interaction of the alpha beta T-cell receptor with major histocompatibility antigens. *Nature*. 338:591-593.
- Sebzda, E., S. Mariathasan, T. Ohteki, R. Jones, M.F. Bachmann, and P.S. Ohashi. 1999. Selection of the T cell repertoire. *Annu Rev Immunol*. 17:829-874.
- Sebzda, E., and P.S. Ohashi. 1995. The role of peptides in positive and negative thymocyte selection. In *T cell receptors*. J.I. Bell, M.J. Owen, and E. Simpson, editors. Oxford University Press, New York. 133-147.
- Sebzda, E., V.A. Wallace, J. Mayer, R.S. Yeung, T.W. Mak, and P.S. Ohashi. 1994. Positive and negative thymocyte selection induced by different concentrations of a single peptide. *Science*. 263:1615-1618.
- Sellins, K.S., and J.J. Cohen. 1987. Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes. *J Immunol*. 139:3199-3206.
- Seong, R.H., J.W. Chamberlain, and J.R. Parnes. 1992. Signal for T-cell differentiation to a CD4 cell lineage is delivered by CD4 transmembrane region and/or cytoplasmic tail. *Nature*. 356:718-720.
- Seong, R.H., and J.R. Parnes. 1992. Alteration of T cell lineage commitment by expression of a hybrid CD8/CD4 transgene. *Adv Exp Med Biol*. 323:79-87.
- Servillo, G., M.A. Della Fazio, and P. Sassone-Corsi. 2002. Coupling cAMP signaling to transcription in the liver: pivotal role of CREB and CREM. *Exp Cell Res*. 275:143-154.
- Seymour, B.W., L.J. Gershwin, and R.L. Coffman. 1998. Aerosol-induced immunoglobulin (Ig)-E unresponsiveness to ovalbumin does not require CD8+ or T cell receptor (TCR)-gamma/delta+ T cells or interferon (IFN)-gamma in a murine model of allergen sensitization. *J Exp Med*. 187:721-731.
- Sha, W.C., H.C. Liou, E.I. Tuomanen, and D. Baltimore. 1995. Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. *Cell*. 80:321-330.
- Sha, W.C., C.A. Nelson, R.D. Newberry, D.M. Kranz, J.H. Russell, and D.Y. Loh. 1988a. Positive and negative selection of an antigen receptor on T cells in transgenic mice. *Nature*. 336:73-76.

- Sha, W.C., C.A. Nelson, R.D. Newberry, D.M. Kranz, J.H. Russell, and D.Y. Loh. 1988b. Selective expression of an antigen receptor on CD8-bearing T lymphocytes in transgenic mice. *Nature*. 335:271-274.
- Shao, H., D.H. Kono, L.Y. Chen, E.M. Rubin, and J. Kaye. 1997. Induction of the early growth response (Egr) family of transcription factors during thymic selection. *J Exp Med*. 185:731-744.
- Shires, J., E. Theodoridis, and A.C. Hayday. 2001. Biological insights into TCRgammadelta+ and TCRalphabeta+ intraepithelial lymphocytes provided by serial analysis of gene expression (SAGE). *Immunity*. 15:419-434.
- Shizuru, J.A., I.L. Weissman, R. Kernoff, M. Masek, and Y.C. Scheffold. 2000. Purified hematopoietic stem cell grafts induce tolerance to alloantigens and can mediate positive and negative T cell selection. *Proc Natl Acad Sci U S A*. 97:9555-9560.
- Shortman, K. 1992. Cellular aspects of early T-cell development. *Curr Opin Immunol*. 4:140-146.
- Shortman, K., M. Egerton, G.J. Spangrude, and R. Scollay. 1990. The generation and fate of thymocytes. *Semin Immunol*. 2:3-12.
- Shortman, K., R. Scollay, P. Andrews, and R. Boyd. 1986. Development of T lymphocytes within the thymus and within thymic nurse cells. *Curr Top Microbiol Immunol*. 126:5-18.
- Shortman, K., H. Von Boehmer, J. Lipp, and K. Hopper. 1975. Subpopulations of T-lymphocytes. Physical separation, functional specialisation and differentiation pathways of sub-sets of thymocytes and thymus-dependent peripheral lymphocytes. *Transplant Rev*. 25:163-210.
- Shortman, K., D. Vremec, L.M. Corcoran, K. Georgopoulos, K. Lucas, and L. Wu. 1998. The linkage between T-cell and dendritic cell development in the mouse thymus. *Immunol Rev*. 165:39-46.
- Shortman, K., and L. Wu. 1996. Early T lymphocyte progenitors. *Annu Rev Immunol*. 14:29-47.
- Shortman, K., L. Wu, K.A. Kelly, and R. Scollay. 1991. The beginning and the end of the development of TCR gamma delta cells in the thymus. *Curr Top Microbiol Immunol*. 173:71-80.
- Sim, G.K., C. Olsson, and A. Augustin. 1995. Commitment and maintenance of the alpha beta and gamma delta T cell lineages. *J Immunol*. 154:5821-5831.
- Singer, A., R. Bosselut, and A. Bhandoola. 1999. Signals involved in CD4/CD8 lineage commitment: current concepts and potential mechanisms. *Semin Immunol*. 11:273-281.
- Skeen, M.J., and H.K. Ziegler. 1993. Intercellular interactions and cytokine responsiveness of peritoneal alpha/beta and gamma/delta T cells from Listeria-infected mice: synergistic effects of interleukin 1 and 7 on gamma/delta T cells. *J Exp Med*. 178:985-996.
- Skeen, M.J., and H.K. Ziegler. 1995. Activation of gamma delta T cells for production of IFN-gamma is mediated by bacteria via macrophage-derived cytokines IL-1 and IL-12. *J Immunol*. 154:5832-5841.

- Smith, C.A., G.T. Williams, R. Kingston, E.J. Jenkinson, and J.J. Owen. 1989. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature*. 337:181-184.
- Smith, L.G., I.L. Weissman, and S. Heimfeld. 1991. Clonal analysis of hematopoietic stem-cell differentiation in vivo. *Proc Natl Acad Sci U S A*. 88:2788-2792.
- Snapper, C.M., P. Zelazowski, F.R. Rosas, M.R. Kehry, M. Tian, D. Baltimore, and W.C. Sha. 1996. B cells from p50/NF-kappa B knockout mice have selective defects in proliferation, differentiation, germ-line CH transcription, and Ig class switching. *J Immunol*. 156:183-191.
- Snodgrass, H.R., Z. Dembic, M. Steinmetz, and H. von Boehmer. 1985a. Expression of T-cell antigen receptor genes during fetal development in the thymus. *Nature*. 315:232-233.
- Snodgrass, H.R., P. Kisielow, M. Kiefer, M. Steinmetz, and H. von Boehmer. 1985b. Ontogeny of the T-cell antigen receptor within the thymus. *Nature*. 313:592-595.
- Sosinowski, T., N. Killeen, and A. Weiss. 2001. The Src-like adaptor protein downregulates the T cell receptor on CD4+CD8+ thymocytes and regulates positive selection. *Immunity*. 15:457-466.
- Spaner, D., K. Migita, A. Ochi, J. Shannon, R.G. Miller, P. Pereira, S. Tonegawa, and R.A. Phillips. 1993. Gamma delta T cells differentiate into a functional but nonproliferative state during a normal immune response. *Proc Natl Acad Sci U S A*. 90:8415-8419.
- Spangrude, G.J., L. Smith, N. Uchida, K. Ikuta, S. Heimfeld, J. Friedman, and I.L. Weissman. 1991. Mouse hematopoietic stem cells. *Blood*. 78:1395-1402.
- Spits, H. 1991. Human T cell receptor gamma delta + T cells. *Semin Immunol*. 3:119-129.
- Spits, H. 1994. Early stages in human and mouse T-cell development. *Curr Opin Immunol*. 6:212-221.
- Spits, H., F. Couwenberg, A.Q. Bakker, K. Weijer, and C.H. Uittenbogaart. 2000. Id2 and Id3 inhibit development of CD34(+) stem cells into predendritic cell (pre-DC)2 but not into pre-DC1. Evidence for a lymphoid origin of pre-DC2. *J Exp Med*. 192:1775-1784.
- Spits, H., X. Paliard, V.H. Engelhard, and J.E. de Vries. 1990. Cytotoxic activity and lymphokine production of T cell receptor (TCR)- alpha beta+ and TCR-gamma delta+ cytotoxic T lymphocyte (CTL) clones recognizing HLA-A2 and HLA-A2 mutants. Recognition of TCR-gamma delta+ CTL clones is affected by mutations at positions 152 and 156. *J Immunol*. 144:4156-4162.
- Spits, H., H. Yssel, C. Brockelhurst, and M. Krangel. 1991. Evidence for controlled gene rearrangements and cytokine production during development of human TCR gamma delta+ lymphocytes. *Curr Top Microbiol Immunol*. 173:47-55.
- Staal, F.J., J. Meeldijk, P. Moerer, P. Jay, B.C. van de Weerd, S. Vainio, G.P. Nolan, and H. Clevers. 2001. Wnt signaling is required for thymocyte development and activates Tcf-1 mediated transcription. *Eur J Immunol*. 31:285-293.
- Steele, C.R., D.E. Oppenheim, and A.C. Hayday. 2000. Gamma(delta) T cells: non-classical ligands for non-classical cells. *Curr Biol*. 10:R282-285.

- Stein, P.L., H.M. Lee, S. Rich, and P. Soriano. 1992. p59fyn mutant mice display differential signaling in thymocytes and peripheral T cells. *Cell*. 70:741-750.
- Steinle, A., V. Groh, and T. Spies. 1998. Diversification, expression, and gamma delta T cell recognition of evolutionarily distant members of the MIC family of major histocompatibility complex class I-related molecules. *Proc Natl Acad Sci U S A*. 95:12510-12515.
- Storb, U., and A.M. Kruisbeek. 1996. Lymphocyte development. *Curr Opin Immunol*. 8:155-159.
- Strasser, A., A.W. Harris, and S. Cory. 1991. bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell*. 67:889-899.
- Strasser, A., A.W. Harris, D.C. Huang, P.H. Krammer, and S. Cory. 1995. Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. *Embo J*. 14:6136-6147.
- Strasser, A., A.W. Harris, H. von Boehmer, and S. Cory. 1994. Positive and negative selection of T cells in T-cell receptor transgenic mice expressing a bcl-2 transgene. *Proc Natl Acad Sci U S A*. 91:1376-1380.
- Strominger, J.L. 1989a. Developmental biology of T cell receptors. *Science*. 244:943-950.
- Strominger, J.L. 1989b. The gamma delta T cell receptor and class Ib MHC-related proteins: enigmatic molecules of immune recognition. *Cell*. 57:895-898.
- Sudo, T., S. Nishikawa, N. Ohno, N. Akiyama, M. Tamakoshi, and H. Yoshida. 1993. Expression and function of the interleukin 7 receptor in murine lymphocytes. *Proc Natl Acad Sci U S A*. 90:9125-9129.
- Sugawara, T., T. Moriguchi, E. Nishida, and Y. Takahama. 1998. Differential roles of ERK and p38 MAP kinase pathways in positive and negative selection of T lymphocytes. *Immunity*. 9:565-574.
- Suniar, R.K., E.J. Jenkinson, and J.J. Owen. 2000. An essential role for thymic mesenchyme in early T cell development. *J Exp Med*. 191:1051-1056.
- Swan, K.A., J. Alberola-Ila, J.A. Gross, M.W. Appleby, K.A. Forbush, J.F. Thomas, and R.M. Perlmutter. 1995. Involvement of p21ras distinguishes positive and negative selection in thymocytes. *Embo J*. 14:276-285.
- Swat, W., M. Dessing, A. Baron, P. Kisielow, and H. von Boehmer. 1992. Phenotypic changes accompanying positive selection of CD4+CD8+ thymocytes. *Eur J Immunol*. 22:2367-2372.
- Swat, W., M. Dessing, H. von Boehmer, and P. Kisielow. 1993. CD69 expression during selection and maturation of CD4+8+ thymocytes. *Eur J Immunol*. 23:739-746.
- Swat, W., Y. Shinkai, H.L. Cheng, L. Davidson, and F.W. Alt. 1996. Activated Ras signals differentiation and expansion of CD4+8+ thymocytes. *Proc Natl Acad Sci U S A*. 93:4683-4687.

- Takagaki, Y., A. DeCloux, M. Bonneville, and S. Tonegawa. 1989. Diversity of gamma delta T-cell receptors on murine intestinal intra- epithelial lymphocytes. *Nature*. 339:712-714.
- Takahama, Y., K. Sugaya, S. Tsuda, T. Hasegawa, and Y. Hashimoto. 1995. Regulation of early T cell development by the engagement of TCR-beta complex expressed on fetal thymocytes from TCR-beta-transgenic scid mice. *J Immunol*. 154:5862-5869.
- Takeuchi, Y., T. Tanaka, K. Hamamura, T. Sugimoto, M. Miyasaka, H. Yagita, and K. Okumura. 1992. Expression and role of interleukin-2 receptor beta chain on CD4-CD8- T cell receptor alpha beta+ cells [corrected]. *Eur J Immunol*. 22:2929-2935.
- Tanaka, T., M. Tsudo, H. Karasuyama, F. Kitamura, T. Kono, M. Hatakeyama, T. Taniguchi, and M. Miyasaka. 1991. A novel monoclonal antibody against murine IL-2 receptor beta-chain. Characterization of receptor expression in normal lymphoid cells and EL- 4 cells. *J Immunol*. 147:2222-2228.
- Tanaka, Y., C.T. Morita, E. Nieves, M.B. Brenner, and B.R. Bloom. 1995. Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. *Nature*. 375:155-158.
- Tanaka, Y., S. Sano, E. Nieves, G. De Libero, D. Rosa, R.L. Modlin, M.B. Brenner, B.R. Bloom, and C.T. Morita. 1994. Nonpeptide ligands for human gamma delta T cells. *Proc Natl Acad Sci U S A*. 91:8175-8179.
- Tang, J., S. Sawasdikosol, J.H. Chang, and S.J. Burakoff. 1999. SLAP, a dimeric adapter protein, plays a functional role in T cell receptor signaling. *Proc Natl Acad Sci U S A*. 96:9775-9780.
- Tarakhovsky, A., M. Turner, S. Schaal, P.J. Mee, L.P. Duddy, K. Rajewsky, and V.L. Tybulewicz. 1995. Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. *Nature*. 374:467-470.
- Teglund, S., C. McKay, E. Schuetz, J.M. van Deursen, D. Stravopodis, D. Wang, M. Brown, S. Bodner, G. Grosveld, and J.N. Ihle. 1998. Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. *Cell*. 93:841-850.
- Teh, H.S., H. Kishi, B. Scott, P. Borgulya, H. von Boehmer, and P. Kisielow. 1990. Early deletion and late positive selection of T cells expressing a male- specific receptor in T-cell receptor transgenic mice. *Dev Immunol*. 1:1-10.
- Teh, H.S., P. Kisielow, B. Scott, H. Kishi, Y. Uematsu, H. Bluthmann, and H. von Boehmer. 1988. Thymic major histocompatibility complex antigens and the alpha beta T- cell receptor determine the CD4/CD8 phenotype of T cells. *Nature*. 335:229-233.
- Teh, H.S., B. Motyka, and S.J. Teh. 1997. Influence of the affinity of selecting ligands on T cell positive and negative selection and the functional maturity of the positively selected T cells. *Crit Rev Immunol*. 17:399-410.
- Tentori, L., D.L. Longo, J.C. Zuniga-Pflucker, C. Wing, and A.M. Kruisbeek. 1988. Essential role of the interleukin 2-interleukin 2 receptor pathway in thymocyte maturation in vivo. *J Exp Med*. 168:1741-1747.

- Terhorst, C., S. Simpson, B. Wang, K. Eichmann, C. Levelt, and M. Exley. 1995. Plasticity of the TCR-CD3 complex. *In* T cell receptors. J.I. Bell, M.J. Owen, and E. Simpson, editors. Oxford University Press, New York. 370-402.
- Thomis, D.C., C.B. Gurniak, E. Tivol, A.H. Sharpe, and L.J. Berg. 1995. Defects in B lymphocyte maturation and T lymphocyte activation in mice lacking Jak3. *Science*. 270:794-797.
- Thonberg, H., E.M. Lindgren, J. Nedergaard, and B. Cannon. 2001. As the proliferation promoter noradrenaline induces expression of ICER (induced cAMP early repressor) in proliferative brown adipocytes, ICER may not be a universal tumour suppressor. *Biochem J*. 354:169-177.
- Ting, C.N., M.C. Olson, K.P. Barton, and J.M. Leiden. 1996. Transcription factor GATA-3 is required for development of the T-cell lineage. *Nature*. 384:474-478.
- Tomita, K., M. Hattori, E. Nakamura, S. Nakanishi, N. Minato, and R. Kageyama. 1999. The bHLH gene Hes1 is essential for expansion of early T cell precursors. *Genes Dev*. 13:1203-1210.
- Tonegawa, S. 1983. Somatic generation of antibody diversity. *Nature*. 302:575-581.
- Tonegawa, S. 1985. The molecules of the immune system. *Sci Am*. 253:122-131.
- Tonegawa, S. 1988a. Antibody and T-cell receptors. *Jama*. 259:1845-1847.
- Tonegawa, S. 1988b. Nobel lecture in physiology or medicine--1987. Somatic generation of immune diversity. *In Vitro Cell Dev Biol*. 24:253-265.
- Tonegawa, S. 1988c. Somatic generation of immune diversity. *Biosci Rep*. 8:3-26.
- Tonegawa, S., A. Berns, M. Bonneville, A.G. Farr, I. Ishida, K. Ito, S. Itohara, C.A. Janeway, Jr., O. Kanagawa, R. Kubo, and et al. 1991. Diversity, development, ligands, and probable functions of gamma delta T cells. *Adv Exp Med Biol*. 292:53-61.
- Tourigny, M.R., S. Mazel, D.B. Burtrum, and H.T. Petrie. 1997. T cell receptor (TCR)-beta gene recombination: dissociation from cell cycle regulation and developmental progression during T cell ontogeny. *J Exp Med*. 185:1549-1556.
- Travers, H., G. Anderson, D. Gentle, E. Jenkinson, and J. Girdlestone. 2001. Protocols for high efficiency, stage-specific retroviral transduction of murine fetal thymocytes and thymic epithelial cells. *J Immunol Methods*. 253:209-222.
- Trobridge, P.A., K.A. Forbush, and S.D. Levin. 2001. Positive and negative selection of thymocytes depends on Lck interaction with the CD4 and CD8 coreceptors. *J Immunol*. 166:809-818.
- Trop, S., M. Rhodes, D.L. Wiest, P. Hugo, and J.C. Zuniga-Pflucker. 2000. Competitive displacement of pT alpha by TCR-alpha during TCR assembly prevents surface coexpression of pre-TCR and alpha beta TCR. *J Immunol*. 165:5566-5572.
- Tsuji, M., P. Mombaerts, L. Lefrancois, R.S. Nussenzweig, F. Zavala, and S. Tonegawa. 1994. Gamma delta T cells contribute to immunity against the liver stages of malaria in alpha beta T-cell-deficient mice. *Proc Natl Acad Sci U S A*. 91:345-349.

- Turner, M., P.J. Mee, P.S. Costello, O. Williams, A.A. Price, L.P. Duddy, M.T. Furlong, R.L. Geahlen, and V.L. Tybulewicz. 1995. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature*. 378:298-302.
- Turner, M., P.J. Mee, A.E. Walters, M.E. Quinn, A.L. Mellor, R. Zamoyska, and V.L. Tybulewicz. 1997. A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. *Immunity*. 7:451-460.
- Uchida, N., and I.L. Weissman. 1992. Searching for hematopoietic stem cells: evidence that Thy-1.1lo Lin- Sca-1+ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. *J Exp Med*. 175:175-184.
- Uematsu, Y., S. Ryser, Z. Dembic, P. Borgulya, P. Krimpenfort, A. Berns, H. von Boehmer, and M. Steinmetz. 1988. In transgenic mice the introduced functional T cell receptor beta gene prevents expression of endogenous beta genes. *Cell*. 52:831-841.
- Vacchio, M.S., and J.D. Ashwell. 1997. Thymus-derived glucocorticoids regulate antigen-specific positive selection. *J Exp Med*. 185:2033-2038.
- Vacchio, M.S., and J.D. Ashwell. 2000. Glucocorticoids and thymocyte development. *Semin Immunol*. 12:475-485.
- Vacchio, M.S., V. Papadopoulos, and J.D. Ashwell. 1994. Steroid production in the thymus: implications for thymocyte selection. *J Exp Med*. 179:1835-1846.
- Van Ewijk, W., P. Kisielow, and H. Von Boehmer. 1990. Immunohistology of T cell differentiation in the thymus of H-Y-specific T cell receptor alpha/beta transgenic mice. *Eur J Immunol*. 20:129-137.
- van Genderen, C., R.M. Okamura, I. Farinas, R.G. Quo, T.G. Parslow, L. Bruhn, and R. Grosschedl. 1994. Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes Dev*. 8:2691-2703.
- Van Kaer, L., P.G. Ashton-Rickardt, H.L. Ploegh, and S. Tonegawa. 1992. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4-8+ T cells. *Cell*. 71:1205-1214.
- Van Kaer, L., M. Wu, Y. Ichikawa, K. Ito, M. Bonneville, S. Ostrand-Rosenberg, D.B. Murphy, and S. Tonegawa. 1991. Recognition of MHC TL gene products by gamma delta T cells. *Immunol Rev*. 120:89-115.
- van Oers, N.S. 1999. T cell receptor-mediated signs and signals governing T cell development. *Semin Immunol*. 11:227-237.
- van Oers, N.S., B. Lowin-Kropf, D. Finlay, K. Connolly, and A. Weiss. 1996. alpha beta T cell development is abolished in mice lacking both Lck and Fyn protein tyrosine kinases. *Immunity*. 5:429-436.
- van Oers, N.S., H. von Boehmer, and A. Weiss. 1995. The pre-T cell receptor (TCR) complex is functionally coupled to the TCR-zeta subunit. *J Exp Med*. 182:1585-1590.
- Vaux, D.L., S. Cory, and J.M. Adams. 1988. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c- myc to immortalize pre-B cells. *Nature*. 335:440-442.

- Veiga-Fernandes, H., U. Walter, C. Bourgeois, A. McLean, and B. Rocha. 2000. Response of naive and memory CD8+ T cells to antigen stimulation in vivo. *Nat Immunol.* 1:47-53.
http://www.nature.com/ni/journal/v41/n41/full/ni0700_0747.html
http://www.nature.com/ni/journal/v0701/n0701/abs/ni0700_0747.html.
- Veillette, A., J.C. Zuniga-Pflucker, J.B. Bolen, and A.M. Kruisbeek. 1989. Engagement of CD4 and CD8 expressed on immature thymocytes induces activation of intracellular tyrosine phosphorylation pathways. *J Exp Med.* 170:1671-1680.
- Veis, D.J., C.L. Sentman, E.A. Bach, and S.J. Korsmeyer. 1993a. Expression of the Bcl-2 protein in murine and human thymocytes and in peripheral T lymphocytes. *J Immunol.* 151:2546-2554.
- Veis, D.J., C.M. Sorenson, J.R. Shutter, and S.J. Korsmeyer. 1993b. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell.* 75:229-240.
- Verbeek, S., D. Izon, F. Hofhuis, E. Robanus-Maandag, H. te Riele, M. van de Wetering, M. Oosterwegel, A. Wilson, H.R. MacDonald, and H. Clevers. 1995. An HMG-box-containing T-cell factor required for thymocyte differentiation. *Nature.* 374:70-74.
- Voll, R.E., E. Jimi, R.J. Phillips, D.F. Barber, M. Rincon, A.C. Hayday, R.A. Flavell, and S. Ghosh. 2000. NF-kappa B activation by the pre-T cell receptor serves as a selective survival signal in T lymphocyte development. *Immunity.* 13:677-689.
- von Boehmer, H. 1990. Developmental biology of T cells in T cell-receptor transgenic mice. *Annu Rev Immunol.* 8:531-556.
- von Boehmer, H. 1991. Positive and negative selection of the alpha beta T-cell repertoire in vivo. *Curr Opin Immunol.* 3:210-215.
- von Boehmer, H. 1992a. T cell development and selection in the thymus. *Bone Marrow Transplant.* 9:46-48.
- von Boehmer, H. 1992b. Thymic selection: a matter of life and death. *Immunol Today.* 13:454-458.
- von Boehmer, H. 1994. Positive selection of lymphocytes. *Cell.* 76:219-228.
- von Boehmer, H. 1995. T cell differentiation: control by the pre-TCR and alpha beta TCR. *Clin Immunol Immunopathol.* 76:S145-150.
- von Boehmer, H. 1996. CD4/CD8 lineage commitment: back to instruction? *J Exp Med.* 183:713-715.
- von Boehmer, H. 1997a. Aspects of lymphocyte developmental biology. *Immunol Today.* 18:260-262.
- von Boehmer, H. 1997b. T-cell development: is Notch a key player in lineage decisions? *Curr Biol.* 7:R308-310.
- von Boehmer, H. 1999. T-cell development: What does Notch do for T cells? *Curr Biol.* 9:R186-188.

- von Boehmer, H. 2000. T-cell lineage fate: instructed by receptor signals? *Curr Biol.* 10:R642-645.
- von Boehmer, H. 2001. Coming to grips with Notch. *J Exp Med.* 194:F43-46.
- von Boehmer, H., I. Aifantis, O. Azogui, J. Feinberg, C. Saint-Ruf, C. Zober, C. Garcia, and J. Buer. 1998. Crucial function of the pre-T-cell receptor (TCR) in TCR beta selection, TCR beta allelic exclusion and alpha beta versus gamma delta lineage commitment. *Immunol Rev.* 165:111-119.
- von Boehmer, H., I. Aifantis, O. Azogui, C. Saint-Ruf, and F. Grassi. 1999a. The impact of pre-T-cell receptor signals on gene expression in developing T cells. *Cold Spring Harb Symp Quant Biol.* 64:283-289.
- von Boehmer, H., I. Aifantis, J. Feinberg, O. Lechner, C. Saint-Ruf, U. Walter, J. Buer, and O. Azogui. 1999b. Pleiotropic changes controlled by the pre-T-cell receptor. *Curr Opin Immunol.* 11:135-142.
- von Boehmer, H., M. Bonneville, I. Ishida, S. Ryser, G. Lincoln, R.T. Smith, H. Kishi, B. Scott, P. Kisielow, and S. Tonegawa. 1988. Early expression of a T-cell receptor beta-chain transgene suppresses rearrangement of the V gamma 4 gene segment. *Proc Natl Acad Sci U S A.* 85:9729-9732.
- von Boehmer, H., and H.J. Fehling. 1997. Structure and function of the pre-T cell receptor. *Annu Rev Immunol.* 15:433-452.
- von Boehmer, H., and K. Hafen. 1993. The life span of naive alpha/beta T cells in secondary lymphoid organs. *J Exp Med.* 177:891-896.
- von Boehmer, H., and P. Kisielow. 1993. Lymphocyte lineage commitment: instruction versus selection. *Cell.* 73:207-208.
- von Boehmer, H., and K. Rajewsky. 1997. Lymphocyte development essential features. *Curr Opin Immunol.* 9:213-215.
- von Boehmer, H., W. Swat, and P. Kisielow. 1993. Positive selection of immature alpha beta T cells. *Immunol Rev.* 135:67-79.
- von Freeden-Jeffry, U., N. Solvason, M. Howard, and R. Murray. 1997. The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression. *Immunity.* 7:147-154.
- von Freeden-Jeffry, U., P. Vieira, L.A. Lucian, T. McNeil, S.E. Burdach, and R. Murray. 1995. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med.* 181:1519-1526.
- Wallace, V.A., K. Kawai, C.N. Levelt, K. Kishihara, T. Molina, E. Timms, H. Pircher, J. Penninger, P.S. Ohashi, K. Eichmann, and et al. 1995. T lymphocyte development in p56lck deficient mice: allelic exclusion of the TcR beta locus is incomplete but thymocyte development is not restored by TcR beta or TcR alpha beta transgenes. *Eur J Immunol.* 25:1312-1318.
- Wang, B., C. Levelt, M. Salio, D. Zheng, J. Sancho, C.P. Liu, J. She, M. Huang, K. Higgins, M.J. Sunshine, and et al. 1995. Over-expression of CD3 epsilon transgenes blocks T lymphocyte development. *Int Immunol.* 7:435-448.

- Wang, E.C., A. Thern, A. Denzel, J. Kitson, S.N. Farrow, and M.J. Owen. 2001. DR3 regulates negative selection during thymocyte development. *Mol Cell Biol.* 21:3451-3461.
- Washburn, T., E. Schweighoffer, T. Gridley, D. Chang, B.J. Fowlkes, D. Cado, and E. Robey. 1997. Notch activity influences the alphabeta versus gammadelta T cell lineage decision. *Cell.* 88:833-843.
- Weintraub, B.C., M.R. Jackson, and S.M. Hedrick. 1994. Gamma delta T cells can recognize nonclassical MHC in the absence of conventional antigenic peptides. *J Immunol.* 153:3051-3058.
- Weissman, I.L. 1996. From thymic lineages back to hematopoietic stem cells, sometimes using homing receptors. *J Immunol.* 156:2019-2025.
- Welsh, R.M., M.Y. Lin, B.L. Lohman, S.M. Varga, C.C. Zarozinski, and L.K. Selin. 1997. Alpha beta and gamma delta T-cell networks and their roles in natural resistance to viral infections. *Immunol Rev.* 159:79-93.
- Wen, L., D.F. Barber, W. Pao, F.S. Wong, M.J. Owen, and A. Hayday. 1998. Primary gamma delta cell clones can be defined phenotypically and functionally as Th1/Th2 cells and illustrate the association of CD4 with Th2 differentiation. *J Immunol.* 160:1965-1974.
- Wen, L., W. Pao, F.S. Wong, Q. Peng, J. Craft, B. Zheng, G. Kelsoe, L. Dianda, M.J. Owen, and A.C. Hayday. 1996. Germinal center formation, immunoglobulin class switching, and autoantibody production driven by "non alpha/beta" T cells. *J Exp Med.* 183:2271-2282.
- Wiest, D.L., J.M. Ashe, T.K. Howcroft, H.M. Lee, D.M. Kemper, I. Negishi, D.S. Singer, A. Singer, and R. Abe. 1997. A spontaneously arising mutation in the DLAARN motif of murine ZAP-70 abrogates kinase activity and arrests thymocyte development. *Immunity.* 6:663-671.
- Wilkinson, B., J.J. Owen, and E.J. Jenkinson. 1999. Factors regulating stem cell recruitment to the fetal thymus. *J Immunol.* 162:3873-3881.
- Wilkinson, R.W., G. Anderson, J.J. Owen, and E.J. Jenkinson. 1995. Positive selection of thymocytes involves sustained interactions with the thymic microenvironment. *J Immunol.* 155:5234-5240.
- Wilson, A., M. Capone, and H.R. MacDonald. 1999. Unexpectedly late expression of intracellular CD3epsilon and TCR gammadelta proteins during adult thymus development. *Int Immunol.* 11:1641-1650.
- Wilson, A., J.P. de Villartay, and H.R. MacDonald. 1996. T cell receptor delta gene rearrangement and T early alpha (TEA) expression in immature alpha beta lineage thymocytes: implications for alpha beta/gamma delta lineage commitment. *Immunity.* 4:37-45.
- Wilson, A., I. Ferrero, H.R. MacDonald, and F. Radtke. 2000. Cutting edge: an essential role for Notch-1 in the development of both thymus-independent and -dependent T cells in the gut. *J Immunol.* 165:5397-5400.

- Wilson, A., W. Held, and H.R. MacDonald. 1994. Two waves of recombinase gene expression in developing thymocytes. *J Exp Med.* 179:1355-1360.
- Wilson, A., and H.R. MacDonald. 1995. Expression of genes encoding the pre-TCR and CD3 complex during thymus development. *Int Immunol.* 7:1659-1664.
- Wilson, A., and H.R. MacDonald. 1998. A limited role for beta-selection during gamma delta T cell development. *J Immunol.* 161:5851-5854.
- Wilson, A., H.R. MacDonald, and F. Radtke. 2001. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. *J Exp Med.* 194:1003-1012.
- Wilson, A., H.T. Petrie, R. Scollay, and K. Shortman. 1989. The acquisition of CD4 and CD8 during the differentiation of early thymocytes in short-term culture. *Int Immunol.* 1:605-612.
- Wilson, A., H. Pircher, P. Ohashi, and H.R. MacDonald. 1992. Analysis of immature (CD4-CD8-) thymic subsets in T-cell receptor alpha beta transgenic mice. *Dev Immunol.* 2:85-94.
- Wilson, I.A., and R.L. Stanfield. 2001. Unraveling the mysteries of gammadelta T cell recognition. *Nat Immunol.* 2:579-581.
- Winandy, S., L. Wu, J.H. Wang, and K. Georgopoulos. 1999. Pre-T cell receptor (TCR) and TCR-controlled checkpoints in T cell differentiation are set by Ikaros. *J Exp Med.* 190:1039-1048.
- Winoto, A., and D. Baltimore. 1989a. Separate lineages of T cells expressing the alpha beta and gamma delta receptors. *Nature.* 338:430-432.
- Winoto, A., and D. Baltimore. 1989b. Alpha beta lineage-specific expression of the alpha T cell receptor gene by nearby silencers. *Cell.* 59:649-655.
- Winoto, A., and D. Baltimore. 1989c. A novel, inducible and T cell-specific enhancer located at the 3' end of the T cell receptor alpha locus. *Embo J.* 8:729-733.
- Wodarz, A., and R. Nusse. 1998. Mechanisms of Wnt signaling in development. *Annu Rev Cell Dev Biol.* 14:59-88.
- Wolfer, A., T. Bakker, A. Wilson, M. Nicolas, V. Ioannidis, D.R. Littman, P.P. Lee, C.B. Wilson, W. Held, H.R. MacDonald, and F. Radtke. 2001. Inactivation of Notch 1 in immature thymocytes does not perturb CD4 or CD8T cell development. *Nat Immunol.* 2:235-241.
- Wong, S., J.D. Freeman, C. Kelleher, D. Mager, and F. Takei. 1991. Ly-49 multigene family. New members of a superfamily of type II membrane proteins with lectin-like domains. *J Immunol.* 147:1417-1423.
- Woronicz, J.D., B. Calnan, V. Ngo, and A. Winoto. 1994. Requirement for the orphan steroid receptor Nur77 in apoptosis of T- cell hybridomas. *Nature.* 367:277-281.
- Wu, J., S. Katzav, and A. Weiss. 1995. A functional T-cell receptor signaling pathway is required for p95vav activity. *Mol Cell Biol.* 15:4337-4346.

- Wu, L., M. Antica, G.R. Johnson, R. Scollay, and K. Shortman. 1991. Developmental potential of the earliest precursor cells from the adult mouse thymus. *J Exp Med.* 174:1617-1627.
- Wu, L., M. Antica, G.R. Johnson, R. Scollay, and K. Shortman. 1991b. Developmental potential of the earliest precursor cells from the adult mouse thymus. *J Exp Med.* 174:1617-1627.
- Wu, L., A. D'Amico, H. Hochrein, M. O'Keeffe, K. Shortman, and K. Lucas. 2001. Development of thymic and splenic dendritic cell populations from different hemopoietic precursors. *Blood.* 98:3376-3382.
- Wu, L., P.W. Kincade, and K. Shortman. 1993. The CD44 expressed on the earliest intrathymic precursor population functions as a thymus homing molecule but does not bind to hyaluronate. *Immunol Lett.* 38:69-75.
- Wu, L., C.L. Li, and K. Shortman. 1996. Thymic dendritic cell precursors: relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny. *J Exp Med.* 184:903-911.
- Wu, L., R. Scollay, M. Egerton, M. Pearse, G.J. Spangrude, and K. Shortman. 1991a. CD4 expressed on earliest T-lineage precursor cells in the adult murine thymus. *Nature.* 349:71-74.
- Wu, L., and K. Shortman. 2000. Isolation and characterization of murine early intrathymic precursor populations. *Methods Mol Biol.* 134:25-35.
- Yamazaki, T., H. Arase, S. Ono, H. Ohno, H. Watanabe, and T. Saito. 1997. A shift from negative to positive selection of autoreactive T cells by the reduced level of TCR signal in TCR-transgenic CD3 zeta-deficient mice. *J Immunol.* 158:1634-1640.
- Yannoutsos, N., P. Wilson, W. Yu, H.T. Chen, A. Nussenzweig, H. Petrie, and M.C. Nussenzweig. 2001. The role of recombination activating gene (RAG) reinduction in thymocyte development in vivo. *J Exp Med.* 194:471-480.
- Ye, S.K., Y. Agata, H.C. Lee, H. Kurooka, T. Kitamura, A. Shimizu, T. Honjo, and K. Ikuta. 2001. The IL-7 receptor controls the accessibility of the TCRgamma locus by Stat5 and histone acetylation. *Immunity.* 15:813-823.
- Ye, S.K., K. Maki, T. Kitamura, S. Sunaga, K. Akashi, J. Domen, I.L. Weissman, T. Honjo, and K. Ikuta. 1999. Induction of germline transcription in the TCRgamma locus by Stat5: implications for accessibility control by the IL-7 receptor. *Immunity.* 11:213-223.
- Yehia, G., R. Razavi, E. Memin, F. Schlotter, and C.A. Molina. 2001. The expression of inducible cAMP early repressor (ICER) is altered in prostate cancer cells and reverses the transformed phenotype of the LNCaP prostate tumor cell line. *Cancer Res.* 61:6055-6059.
- Yoder, M.C., and K. Hiatt. 1997. Engraftment of embryonic hematopoietic cells in conditioned newborn recipients. *Blood.* 89:2176-2183.
- Yoder, M.C., K. Hiatt, P. Dutt, P. Mukherjee, D.M. Bodine, and D. Orlic. 1997a. Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. *Immunity.* 7:335-344.

- Yoder, M.C., K. Hiatt, and P. Mukherjee. 1997b. In vivo repopulating hematopoietic stem cells are present in the murine yolk sac at day 9.0 postcoitus. *Proc Natl Acad Sci U S A*. 94:6776-6780.
- Zacharchuk, C.M., M. Mercep, and J.D. Ashwell. 1991. Thymocyte activation and death: a mechanism for molding the T cell repertoire. *Ann N Y Acad Sci*. 636:52-70.
- Zacharchuk, C.M., M. Mercep, P.K. Chakraborti, S.S. Simons, Jr., and J.D. Ashwell. 1990. Programmed T lymphocyte death. Cell activation- and steroid-induced pathways are mutually antagonistic. *J Immunol*. 145:4037-4045.
- Zamoyska, R., and P. Travers. 1995. The function of T cells and the role of the co-receptor molecules CD4 and CD8. In T cell receptors. J.I. Bell, M.J. Owen, and E. Simpson, editors. Oxford University Press, New York. 46-69.
- Zerrahn, J., W. Held, and D.H. Raulet. 1997. The MHC reactivity of the T cell repertoire prior to positive and negative selection. *Cell*. 88:627-636.
- Zhang, R., F.W. Alt, L. Davidson, S.H. Orkin, and W. Swat. 1995. Defective signalling through the T- and B-cell antigen receptors in lymphoid cells lacking the vav proto-oncogene. *Nature*. 374:470-473.
- Zhang, W., C.L. Sommers, D.N. Burshtyn, C.C. Stebbins, J.B. DeJarnette, R.P. Tribble, A. Grinberg, H.C. Tsay, H.M. Jacobs, C.M. Kessler, E.O. Long, P.E. Love, and L.E. Samelson. 1999. Essential role of LAT in T cell development. *Immunity*. 10:323-332.
- Zhang, W., R.P. Tribble, M. Zhu, S.K. Liu, C.J. McGlade, and L.E. Samelson. 2000. Association of Grb2, Gads, and phospholipase C-gamma 1 with phosphorylated LAT tyrosine residues. Effect of LAT tyrosine mutations on T cell antigen receptor-mediated signaling. *J Biol Chem*. 275:23355-23361.
- Zhang, X., L. Li, J. Choe, S. Krajewski, J.C. Reed, C. Thompson, and Y.S. Choi. 1996. Up-regulation of Bcl-xL expression protects CD40-activated human B cells from Fas-mediated apoptosis. *Cell Immunol*. 173:149-154.
- Zhou, T., J. Cheng, P. Yang, Z. Wang, C. Liu, X. Su, H. Bluethmann, and J.D. Mountz. 1996. Inhibition of Nur77/Nurr1 leads to inefficient clonal deletion of self-reactive T cells. *J Exp Med*. 183:1879-1892.
- Zhumabekov, T., P. Corbella, M. Tolaini, and D. Kioussis. 1995. Improved version of a human CD2 minigene based vector for T cell-specific expression in transgenic mice. *J Immunol Methods*. 185:133-140.
- Zijlstra, M., M. Bix, N.E. Simister, J.M. Loring, D.H. Raulet, and R. Jaenisch. 1990. Beta 2-microglobulin deficient mice lack CD4-8+ cytolytic T cells. *Nature*. 344:742-746.
- Zinkernagel, R., and P. Doherty. 1974. Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. *Nature*. 251:547-548.
- Zinkernagel, R.M., G.N. Callahan, A. Althage, S. Cooper, P.A. Klein, and J. Klein. 1978a. On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition? *J Exp Med*. 147:882-896.

- Zinkernagel, R.M., G.N. Callahan, J. Klein, and G. Dennert. 1978b. Cytotoxic T cells learn specificity for self H-2 during differentiation in the thymus. *Nature*. 271:251-253.
- Zuniga-Pflucker, J.C., L.A. Jones, L.T. Chin, and A.M. Kruisbeek. 1991. CD4 and CD8 act as co-receptors during thymic selection of the T cell repertoire. *Semin Immunol*. 3:167-175.
- Zuniga-Pflucker, J.C., L.A. Jones, D.L. Longo, and A.M. Kruisbeek. 1989a. Both TCR/MHC and accessory molecule/MHC interactions are required for positive and negative selection of mature T cells in the thymus. *Cold Spring Harb Symp Quant Biol*. 54:153-158.
- Zuniga-Pflucker, J.C., L.A. Jones, D.L. Longo, and A.M. Kruisbeek. 1990a. CD8 is required during positive selection of CD4-/CD8+ T cells. *J Exp Med*. 171:427-437.
- Zuniga-Pflucker, J.C., D.L. Longo, and A.M. Kruisbeek. 1989b. Positive selection of CD4-CD8+ T cells in the thymus of normal mice. *Nature*. 338:76-78.
- Zuniga-Pflucker, J.C., S.A. McCarthy, M. Weston, D.L. Longo, A. Singer, and A.M. Kruisbeek. 1989c. Role of CD4 in thymocyte selection and maturation. *J Exp Med*. 169:2085-2096.
- Zuniga-Pflucker, J.C., K.A. Smith, L. Tentori, D.M. Pardoll, D.L. Longo, and A.M. Kruisbeek. 1990b. Are the IL-2 receptors expressed in the murine fetal thymus functional? *Dev Immunol*. 1:59-66.