T cell development and lineage commitment:

studies based on differential gene expression in thymocyte subsets

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A thesis submitted for the degree of
Doctor of Philosophy
at the University of London

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July 2002
To my parents, Eduardo and Lídia

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Programa Praxis XXI, BD/18594/98
Fundação para a Ciência e Tecnologia (Portugal)
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: αβ versus γδ 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, αβ or γδ. "β-selection" consists of a checkpoint in the differentiation of the αβ lineage, in which only precursors that receive signals from the pre-TCR – a complex made of a newly synthesised TCRβ chain and the invariant protein pTα – 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 αβ and γδ T cell populations. Although thymic development was unperturbed in CREM/ICER<sup>−/−</sup> mice, we demonstrate that subsequent to the DN4 stage of thymocyte differentiation, ICER is a robust marker of the γδ T cell lineage. ICER expression is not observed in αβ-committed DP or SP thymocytes, or in αβ 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 αβ/γδ classification of T cells that incorporates TCRαβ<sup>+</sup>CD8αα<sup>+</sup> IELs as having a "γδ-like" profile.

Surprisingly (for a γδ lineage marker), ICER expression is severely impaired in the thymus of pTα<sup>−/−</sup> and TCRβ<sup>−/−</sup> 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 γδ lineage, ICER expression is not a direct consequence of pre-TCR signalling, since this complex is absent from the vast majority of γδ 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 γδ cells. This novel mechanism constitutes the first cross-talk reported for the αβ and γδ lineages during their thymic differentiation.
Acknowledgements

Professional

I would like to thank Dr. Mike Owen for his supervision and support during our common time at ICRF (now CR UK); Dr. Doreen Cantrell, for her suggestions and advice throughout my PhD; Dr. Adrian Hayday, for his enthusiastic input to my work via our discussions and precious help regarding the related publications.

To Dan Pennington, I am extremely grateful for his guidance, both technical and intellectual. Our collaboration over these four years was the milestone of my PhD. Thank you so much, "Boss"!

I am also thankful to the other members of the Lymphocyte Molecular Biology Lab 1998-2001: Ludovica Bruno, for her important suggestions concerning my PhD project, and for introducing me to the "FACS world"; César Trigueros, for our collaborations, particularly in the IL-7R project; Diane Maurice, Juli Miller, Jenny Buckland, Anette Thern and Angela Denzel, for our discussions and - above all - our friendship, which made the lab such a pleasant place to be in; Jenny Dunne, Theresa Higgins, Eddy Wang, Katsuto Hozumi, Stéphane Mancini and Anthony Boureux, for their assistance with techniques and general help (in particular Anthony’s computer skills!).

To Dan Pennington and Jenny Buckland, I am also very grateful for their thorough and critical reading of this thesis.

Elsewhere at ICRF, I would like to thank: Roman Spoerri, Ulrica Marklund and Patrick Costello for their friendly collaborations; Dr. Caetano Reis-e-Sousa for all the initiatives of the “Immunology Supergroup”; Dr. Facundo Batista for his support during the writing of this thesis; and those responsible for the central services and facilities that kept my experiments running - Iain Goldsmith, Gary Martin, Tracy Crafton and Cheryl Young at Clare Hall; Gill Hutchinson and Julie Bee in the animal house; Graham Clark in the sequencing lab; and, last but not least, a very special thanks to all the FACS lab experts - Derek Davies, Cathy Simpson, Ayad Eddaoudi, Gary Warnes and Aaron Rae: without your cell sorting, this thesis would have been much thinner!

Outside ICRF, I have also collaborated with Dr. Enzo Lalli and Dr. Paolo Sassone-Corsi (Strasbourg), Dr. Gunther Schutz (Heidelberg), Dr. Alex Scheffold and Dr. Andreas Radbruch (Berlin) and Alexandra Brás and Dr. Carlos Martinez (Madrid).
Being part of the Gulbenkian PhD Programme for Biology and Medicine, I am grateful to its former director, Dr. António Coutinho, and former co-ordinator, Dr. Paulo Vieira, and my national supervisor, Dr. Ana Ponces Freire, for their support and advice. And to my colleagues of the 5th Programme - namely, Rita Nunes, Luís Graca, Mário Gomes-Pereira, Filipe Madeira, Susana Nery and Tiago Magalhães - for the great time we had together!

My work was funded by the Portuguese Government (Fundaçâo para a Ciência e Tecnologia, Programa Praxis XXI) and by Cancer Research UK (previously ICRF).

**Personal**

Science apart, these four years also meant that I left my Portuguese Motherland for England. Absolutely vital for the maintenance of my mental health and happiness were:

- In my new town London, my partners at home, who had to put up with me everyday after work: Neil Thorton (during the last year, which included this thesis!); and Jorge Vasconcelos (during the first three years). Thank you so much for your companionship!

- Also in London, my dear friends Rita Nunes (you, again?), Mónica Dias, Anita Gomes, Zé Leal, Paulo Pereira, Andrea Gaspar, Christian Dillon and Piero Bassu: your friendship made me "feel at home".

- Back in Lisbon, my faithful friends Maria de Lurdes (Mima!) Elias, Ana Pamplona, Pedro Lamosa, Rita Lemos, Patrícia Medeiros, Pedro Gomes, Isabel Abreu, Sofia Nunes, Agostinho Leite, David Benazulim and Paula Fareleira: thank you for making me feel as if I had never "left home"!

- Elsewhere in the World, I couldn't forget my precious friends Manuel Ostheider ("brotha"), Pedro Mattos and Cristina Santos.

Finally, this is my thesis - and I am who I am because of my family:

- my Parents (to whom this Thesis is dedicated), Eduardo and Lidia, who have always believed in me and given me so much strength and love. There are no words to express the extent of my gratitude, so I'll just say that I love you dearly.

- my brother Joao, with his constant presence in my life and his great personality.

- my aunt Helena (Lelé!) and my cousins Nuno and Luís, and young Francisco.

- my grandparents 'Avô Joao' and Nanda, my aunt Ana; and cousins Ricardo and Ana.

Thank you, obrigado! You've made me a very happy young scientist.
# 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. pTα as part of pre-TCR 36
      2.4.2. “β-selection” 37
      2.4.3. Components of pre-TCR signalling pathway(s) 38
      2.4.4. Transcription factors involved in “β-selection” 43
   2.5. The role of TCRαβ in thymocyte development 48
      2.5.1. Positive and negative selection 48
      2.5.2. CD4/CD8 lineage commitment 51
      2.5.3. Signalling pathways and TFs downstream of TCRαβ 53
   2.6. TCR-independent signalling pathways in T cell development 58
      2.6.1. Pro- and anti-apoptotic pathways 58
      2.6.2. Interleukin-7/IL-7R signalling 61
      2.6.3. Wnt signalling 63
      2.6.4. Notch signalling 65
3. T cell lineage commitment: αβ versus γδ

3.1. γδ T cell biology

3.2. Models for the αβ/γδ lineage split

3.3. Analysis of TCR rearrangements in T cell subsets

3.4. Analysis of TCR transgenic and gene-deficient mice

3.5. TCR-independent mechanisms in αβ vs. γδ cell differentiation
   3.5.1. IL-7/IL-7R signalling
   3.5.2. Notch signalling

4. Objectives of the studies presented in this thesis

Chapter II: Methods

1. Cellular biology - general methods
   1.1. Preparation of murine cells
   1.2. Depletion of CD4(+) / CD8(+) T cells
   1.3. Cell staining with antibodies and chemicals
   1.4. Flow cytometry analysis and cell sorting
   1.5. Foetal thymic organ cultures

2. Molecular biology - general methods
   2.1. Protein
      2.1.1. Protein extraction
      2.1.2. SDS-polyacrylamide gel electrophoresis
      2.1.3. Western blotting
   2.2. DNA
      2.2.1. Extraction of genomic DNA
      2.2.2. Preparation of plasmid DNA
      2.2.3. Polymerase chain reaction
      2.2.4. Agarose gel electrophoresis
      2.2.5. Purification and radio-labelling of DNA probes
      2.2.6. Restriction fragment length polymorphism - PCR
      2.2.7. Cloning - general procedures
      2.2.8. CD2-ICER construct for generation of transgenic mice
2.3. RNA

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
3.1. Synthesis of double-stranded cDNA 123
3.2. Generation of tester and driver representations 125
3.3. First subtractive hybridisation 126
3.4. Second subtractive hybridisation 127
3.5. Isolation, sequencing and identification of differentially expressed genes 129

Chapter III: Results 132

1. Genes differentially expressed in αβ versus γδ thymocytes 133
1.1. RDA analysis of DP vs. γδ 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. Ly-49A 148
1.4.2. Sugano EST 151

2. Identification of pre-TCR responsive genes 155
2.1. RDA analysis of TCRβ(+) vs. TCRβ(-) pre-T cells 155
2.2. RDA analysis of RAG(-) pre-T cells unstimulated vs. stimulated with anti-CD3ε 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. Pre-TCR dependence of IL-7R expression in DN4 cells 166
2.4.2. Requirement for IL-7R signalling in DN to DP transition 168
2.4.3. Requirement for IL-7R signalling for survival of DN4 cells 170
3. ICER in αβ versus γδ T cell lineage commitment

3.1. Identification of CREM isoforms expressed in γδ thymocytes

3.2. Pattern of expression of ICER in the thymus

3.3. ICER expression and the status of TCR gene rearrangements in pre-T cells

3.4. Analysis of the thymus of CREM/ICER deficient mice

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

3.6. Generation and analysis of CD2-ICER transgenic mice

3.7. Pattern of expression of ICER in peripheral lymphoid tissues

3.8. ICER expression in mouse mutants for TCR

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

4. ICER, β-selection and γδ thymocyte development

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

4.2. ICER expression in pre-T cells with impaired pre-TCR signalling

4.3. Induction of ICER expression by signalling through the CD3 complex

4.3.1. ICER expression in response to anti-CD3ε antibody

4.3.2. Involvement of the MAPK pathway

4.4. Analysis of the DN compartment of ICER deficient mice

4.5. Analysis of pre-T cells expressing different levels of ICER

4.5.1. Developmental potential

4.5.2. Phenotypic analysis

4.6. Gene expression in γδ thymocytes developing in the absence of β-selected cells

4.7. Requirement of a normal (β-selected) composition of the thymus for ICER expression.

4.8. Cross-talk between αβ and γδ lineages during γδ thymocyte differentiation
Chapter IV : Discussion

1. Genes differentially expressed between αβ and γδ T cells
2. Pre-TCR responsive genes
   2.1. Role of IL-7 receptor in the DN to DP transition
3. ICER as a marker for the γδ T cell lineage
4. Pre-TCR dependent expression of ICER in pre-T cells
5. Cross-talk between αβ and γδ T cell differentiation
6. Conclusion

References
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 αβ/γδ T cell lineage split. 80
6 Structure of the human CD2 expression cassette. 114
7 Outline of representation difference analysis. 124
8 FACSorting of TCRα^-/- thymocytes for RDA analysis. 134
9 Summary of RDA analysis of γδ vs. DP thymocytes. 136
10 RT-PCR for candidate genes in wild type γδ 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-2Rβ transcript. 152
15 IL-2Rβ protein surface expression in thymic subsets. 154
16 FACS plots (CD25 vs. CD44) for WT and TCRβ^-/- DN thymocytes. 155
17 FACS plots (CD25 vs. CD44) used in the purification of cells for RDA analysis of anti-CD3ε 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-CD3ε mAb. 164
21 Expression of candidate genes in WT and pre-TCR deficient T cells. 165
22 Surface expression of IL-7Rα 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-7Rα-deficient DN4 thymocytes: cell cycle status, cell death and intracellular TCRβ expression. 171
25 Effect of blocking antibodies to IL-7R complex on proliferation and death of DN4 thymocytes. 173
Identification of CREM isoforms expressed in γδ thymocytes. 175
Real-time PCR expression profile for ICER in the thymus. 178
Pre-TCR levels and ICER expression in WT pre-T cells. 180
Pre-TCR levels and TCR gene rearrangement status of WT pre-T cells. 181
FACS analysis of thymocyte development in CREM−/− mice. 183
Effect of cyclic-AMP on thymocyte development (in vitro) and ICER expression. 185
Generation and screening of CD2-ICER transgenic mice. 187
FACS analysis of thymocyte development in CD2-ICER transgenic mice. 188
Real-time PCR expression profile for ICER in lymph nodes and spleen. 190
Real-time PCR expression profile for ICER in intra-epithelial lymphocytes. 191
ICER expression in TCRα-deficient and TCRδ-deficient mice. 193
ICER expression in TCRαβ transgenic and TCRβ-deficient mice. 195
Lineage potential of pre-T cells expressing different levels of ICER. 197
ICER-LacZ protein expression in pre-T cells of CREM/ICER-LacZ mice. 199
ICER mRNA expression in pre-T cells of mice with deficient pre-TCR signalling. 201
Induction of ICER expression by anti-CD3e mAb stimuli. 203
Effect of MAPK pathway inhibitors on the induction of ICER-LacZ protein expression by anti-CD3e mAb stimuli. 205
FACS plots (CD25 vs. CD44) of WT and CREM−/− DN thymocytes. 206
Developmental potential of pre-T cells expressing different levels of ICER-LacZ protein. 208
Phenotypic analysis of DN3 cells expressing different levels of ICER-LacZ protein. 211
Gene expression in pre-T cells expressing different levels of ICER-LacZ protein. 212
Gene expression in γδ cells of TCRβ−/− and pTα−/− mice. 214
ICER expression in foetal and adult pro-T and γδ thymocytes. 215
ICER expression in the thymus of WT → RAG−/− bone marrow chimera. 217
Induction of ICER expression in γδ thymocytes mediated by DP cells. 219
Comparison of proliferation and effector functions of WT and TCRδ−/− γδ cells. 245
Model for ICER expression during T cell differentiation. 250
### Tables

1. Genes isolated from the RDA subtractive hybridisation of γδ 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 - γδ cells.  
   145

4. Genes isolated from the RDA subtraction of TCRβ (+) and TCRβ(-) DN3 thymocytes.  
   156

5. Genes isolated from the RDA analysis of RAG(-) thymocytes stimulated for 3 hours *in vivo* with anti-CD3ε mAb *versus* 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 (=9.8 m/s²)
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
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ISP</td>
<td>immature single positive (CD8&lt;sup&gt;+&lt;/sup&gt;) thymocyte</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoglobulin family tyrosine-based activation motif</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KO</td>
<td>&quot;knock-out&quot;</td>
</tr>
<tr>
<td>-L</td>
<td>ligand</td>
</tr>
<tr>
<td>LCR</td>
<td>locus control region</td>
</tr>
<tr>
<td>loxP</td>
<td>locus of cross-over in P1 bacteriophage</td>
</tr>
<tr>
<td>lg</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>Mφ</td>
<td>macrophage</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>n</td>
<td>number of replicate experiments</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NT</td>
<td>no treatment</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>pTα</td>
<td>pre-TCRα chain</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrilamide gel electrophoresis</td>
</tr>
<tr>
<td>PTK</td>
<td>protein tyrosine kinase</td>
</tr>
<tr>
<td>-R</td>
<td>receptor</td>
</tr>
<tr>
<td>RAG</td>
<td>recombination activating gene</td>
</tr>
<tr>
<td>RDA</td>
<td>representation difference analysis</td>
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<tr>
<td>RSS</td>
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<td>ribonucleic acid</td>
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<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SH</td>
<td>Src-homology domain</td>
</tr>
<tr>
<td>SP</td>
<td>single positive (CD4&lt;sup&gt;+&lt;/sup&gt; or CD8&lt;sup&gt;+&lt;/sup&gt;) thymocyte</td>
</tr>
<tr>
<td>Tg</td>
<td>transgenic</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>V-D-J</td>
<td>variable-diversity-junctional (rearrangement of TCR genes)</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
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</table>
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-7Rα(+) Lin(-) Sca-1(low) c-kit(low), where Lin represents mature lineage markers, IL-7Rα 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 (γc) (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 γc also lack NK cells (Cao et al., 1995) due to an impairment in the formation of IL-15 receptor, for which γc 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 TCRα and TCRδ, 1000 kb on chromosome 14. Within the α/δ locus, J and C (and D) segments are specific for either TCRα or TCRδ, 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 (ACAAAAACC) 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: αβ T cells use α and β; γδ 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. CD3ζ 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 CD3ε and CD3ζ (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 TCRαβ 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 CD8αα 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 αβ 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 TCRαβ-CD4 complex) presented by MHC class II molecules, whereas CD8+ (cytotoxic) T cells do it (via their TCRαβ-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 (αβ or γδ), 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).
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 TCR\(\alpha\beta^+\) 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 \( \gamma, \delta \) and \( \beta \) 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 \( \alpha \) 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 Thyl(+) 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). TCRβ 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 γδ or αβ T cells. Full V-DJ rearrangements of TCRβ genes are now detectable in this population, and if in frame they enable the synthesis of a TCRβ protein. Cells that successfully rearrange and express a functional TCRβ 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 TCRβ chain and an invariant chain, pTα (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 αβ cells beyond the DN3 stage; in contrast, γδ 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 TCRβ rearrangements, as shown by RFLP-PCR (restriction fragment length polymorphism -PCR) (Dudley et al., 1994), and shows relatively high levels of surface TCRβ 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 γδ and αβ lineages (Bruno et al., 1999). In fact, Wilson and MacDonald have identified a subset (8%) of DN4 cells that is intracellular TCRγδ(+) , although extracellular TCRγδ(-), and have suggested that these are γδ precursors in the DN4 population (Wilson et al., 1999).

Immature DN thymocytes differentiate into either γδ or αβ T cells. If they commit to the γδ lineage (1-2%), expressing TCRγδ, they basically remain negative for the expression of both CD4 and CD8 markers. In αβ development, however, the next stage is CD4(+)CD8(+). Commitment between αβ / γδ lineages, and γδ
cells themselves, will be the focus of part 3 of this introduction; for the remaining of part 2 we will concentrate on αβ 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). TCRα rearrangements are completed at the DP stage, which allows the expression of a TCRαβ complex. Most DP cells express TCRαβ 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 that 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 TCRβ 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 TCRβ- knock-out mice (Mombaerts et al., 1992b). In these murine models, T cell differentiation is severely impaired, with complete (SCID, RAGβ−/−) or partial (TCRβ−/−) developmental blocks at the DN3 stage, and therefore none (SCID, RAGβ−/−) or very few (TCRβ−/−) 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 pTα as part of pre-TCR

By 1992, TCRβ−/− and TCRα−/− mice had been generated, and it was obvious that the two chains that composed the TCR had distinct roles in T cell development. Whereas TCRβ−/− differentiation was severely blocked at the DN3 stage, TCRα−/− 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 TCRβ chain promote T cell development at the immature DN3 stage, in the absence of a functional TCRα 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 TCRβ-CD3 complex that clearly lacked a conventional TCRα 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 TCRβ; it was named pre-TCRα (pTa) (Saint-Ruf et al., 1994).

pTa 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, pTa 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 pTa could be used as a marker for their identification (Fehling and von Boehmer, 1997).

In the thymus, pTa 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, pTa is competitively displaced by TCRα, thus allowing mature TCR to take the place of pre-TCR at the cell surface (Trop et al., 2000).

The generation and analysis of pTa-/- mice (Fehling et al., 1995a) has provided conclusive evidence for pTa being the partner of TCRβ in immature DN thymocytes. The similarity between the phenotypes of pTa-/- and TCRβ-/- 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 γδ cells is not impaired in these mice (in fact, their absolute numbers are higher than in normal mice), suggesting that the γδ 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 TCRβ. 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 γδ cells) (Fehling and von Boehmer, 1997).

Cell survival, proliferation and differentiation at the DN3 → DN4 (→ 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 pTα in the place of TCRα (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 pTα and TCRβ are not required for signalling (Irving et al., 1998).

It is important to note that pTα−/− and TCRβ−/− 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 pTα, 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.
Figure 3: Proximal molecules associated with pre-TCR signalling.
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 CD3ε and CD3γ. Both CD3ε−/− (Malissen et al., 1995) and CD3γ−/− (Haks et al., 1998) mice display a severe block at the DN3 stage of development. In contrast, CD3δ−/− (Dave et al., 1997) and CD3ζ−/− (Love et al., 1993) mice are more permissive to thymocyte development. In CD3δ−/− 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 (TCRβ−/−, pTα−/−). In addition, a constitutively active Lck transgene is capable of rescuing DP production in RAG-deficient (Mombaerts et al., 1994) and in pTα-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-CD3e 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.

41
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 PLCγ 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 → 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 CD3γ-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 pTα and up-regulation of TCRα 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 TCRα, TCRβ and CD4 genes. Targeted disruption of HEB results in a 5-10 fold reduction in thymic cellularity due to a block in DN → 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-CD3e 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 TCRα, TCRβ, CD3δ and CD8α. 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$\beta$), which in fact was over-expressed in those transgenic mice as part of a compensatory mechanism for the loss of the other isoforms ($\alpha$, $\delta$) (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 $\beta$-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 $\beta$-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$\varepsilon$ and TCR$\alpha$ 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$\alpha$ 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 TCRαβ 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 TCRβ are generated. The completion of TCRα rearrangements at the DP stage finally allows the expression of the TCRαβ complex (in the place of the pre-TCR). The TCRαβ 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 TCRαβ 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 Vβ17, 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-Illa et al., 1995; Alberola-Illa 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

TCRαβ 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 selective 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<sup>-/-</sup> mice (Matechak et al., 1996) and by the preferential generation of CD4+ SP (to the detriment of CD8+ SP) in Lck<sup>-/-</sup> 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<sup>low</sup>CD8<sup>+</sup> cells in MHC-I<sup>-/-</sup>, CD4<sup>+</sup>CD8<sup>low</sup> thymocytes in MHC-II<sup>-/-</sup>, 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 TCRαβ

The TCRαβ complex shares many similarities to the pre-TCR complex, with major differences being TCRα in place of pTα, 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 TCRαβ pathway and then demonstrated to be also part of pre-TCR signalling cascades.

An important feature of TCRαβ signalling is signal strength. While DN cells express very low levels of surface TCRβ, 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 TCRαβ complexes will also influence signalling potential. Therefore, TCRαβ 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 TCRαβ 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 TCRα⁻/⁻ 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-CD3ε 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 IRF-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 immature thymocytes before TCRαβ 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-kB 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-κB** transcription factors include NF-κB-1/-2, Rel-A/-B and c-Rel. Their activity is controlled at the post-translational level by association with inhibitory IκB proteins. TCR signalling induces degradation of IκB, which releases NFκB and allows it to translocate to the nucleus. Although NFκB-1^-/-^ mice showed no abnormality in T cell development, this could be due to functional redundancy between the several NFκB proteins (Sha et al., 1995; Snapper et al., 1996). To circumvent this, transgenic mice expressing a “super-inhibitory” form of IκB under the control of T cell specific promoters were generated (Boothby et al., 1997; Esslinger et al., 1997). Since this mutant IκB cannot be phosphorylated and degraded in response to TCR engagement, the function of all NFκB 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 TCRα, TCRβ, CD3δ and CD8α (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 (API 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 NFκB (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 (Ipr mice) accumulate thymocytes with a CD4(-)CD8(-)CD3(+) phenotype and suffer from auto-immunity and lymphoadenopathy. 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-xL) 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<sub>L</sub>, unlike Bcl-2, is expressed at high levels in DP cells (Grillot et al., 1995). Thymocytes over-expressing Bcl-x<sub>L</sub> 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<sub>L</sub> is up-regulated during the DN → DP transition.

The anti-apoptotic action of Bcl-2 and Bcl-x<sub>L</sub> 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<sub>L</sub>. 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-7Rα chain in association with the common cytokine receptor gamma (γ<sub>γ</sub> 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-7Rα antibodies in vivo, and the generation of IL-7 and IL-7Rα '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-7Rα 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 γc^-/- 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-7Rα chain, but no γc chain. Therefore, the more severe phenotype of IL-7Rα^-/- 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 γc^-/- mice, demonstrating a primary role for this tyrosine kinase in IL-7 signal transduction.
To address the developmental relationship between γc-dependent cytokine and pre-TCR signalling, γc/pTα 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/γc 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-7Rα 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-γc 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 deregulated 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 CD3δ and TCRα 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 TCRβ and TCRδ 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 multiple 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-xL, 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 TCRβ(+) subsets of DN3 and DN4 cells. In addition, these thymocytes produced SP cells that lacked TCRαβ, 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 TCRβ. 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.

67
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 TCRαβ and Bcl-2 (Deftos et al., 1998), and DelteX, a positive regulator of Notch signalling that is now taken as an 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: αβ versus γδ

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 TCRγδ, expressed by an independent (from αβ) T cell lineage.

Since then, it has become clear that all jawed vertebrates possess αβ and γδ 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 γδ T cell biology

γδ cells are defined by the surface expression of TCRγδ. In fact, there is no other unequivocal marker for γδ cells (reviewed in Hayday, 2000). Some γδ cells also express, independently, the following surface molecules: CD28, the ligand for B7 and the major co-stimulator for αβ lymphocytes; CD40L; NK inhibitory receptors; NK activation receptors such as NKG2D; CD2 and CD5 (these vary between species). Unlike αβ cells, most γδ 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 γδ lineage is not impaired in MHC deficient mice (Grusby et al., 1993).

The TCRγδ shares a number of features with TCRαβ: 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 TCRγδ clearly distinguish it from its αβ counterpart, from DNA sequence motifs, particularly in their CDRs (complementarity determining regions), to conformation patterns (reviewed in Hayday, 2000). In addition, TCRγδ, as an antigen receptor, is characterised by a very limited repertoire of specificities (Janeway, 1988).

γδ 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, γδ 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 TCRγ/δ genes and the representation of γδ cells: for example, there are 3 TCRγ genes in the mouse, but more than 30 in the chicken. Also strikingly, this further correlates inversely with the complexity of TCRα/β genes: there are 75 Vα and 23 Vβ genes in the mouse, but only two sub-families of chicken TCRα and β genes (reviewed in Hayday, 2000).

One notable feature of γδ cells is their unique anatomical distribution. γδ 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, γδ : αβ 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 γδ cells (Kuziel et al., 1987).

This distribution of γδ cells may explain the restricted diversity of antigen specificities for the TCRγδ. Unlike αβ 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 γδ cells could be exposed, and therefore the useful diversity of TCRγδ (Hayday, 2000).

This phenomenon is particularly evident in IEL repertoires. A single TCR (Vγ5Vδ1) 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 Vγ6Vδ1, which differs from the DETC receptor only in the germline-encoded Vγ sequences (Itohara, 1990). The extremely limited diversity of γδ cells of epithelia provoked the "first line of defense" hypothesis (Janeway, 1988), proposing that γδ 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 γδ ligands (Asarnow et al., 1988).

Probably the most extraordinary feature of γδ 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 αβ lymphocytes. The mechanism of antigen presentation to γδ cells is still a complete mystery, almost 20 years after the identification of γδ cells.

In terms of antigen specificities, the effort of trying to identify the unorthodox ligands for TCRγδ has provided the following results:

- Human systemic γδ cells recognise small, non-peptidic products, in particular phosphoantigens. Monoalkyl (pyro)phosphates of less than 5 carbons are recognised by Vγ2Vδ2 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 γδ 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 β2-microglobulin-independent. MICA is a ligand also for NKG2D, a NK receptor expressed in γδ cells. It's not clear if there is cross-talk between TCRγδ 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 γδ cells recognise native, non-processed, proteins. A Vγ2(+) clone (LBK5) recognises MHC class II proteins (IE^k, IE^b, IE^b) (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 γδ 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 γδ 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 γδ responses to the ligands identified in the human system. This topic thus requires extensive research.

γδ 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 γδ 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 γδ 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 γδ proliferation (Skeen and Ziegler, 1993).
γδ cells, macrophages and NK cells have been proposed to co-operate in the early "innate" phases of immune responses. Macrophages activate both γδ 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). γδ cells also seem to provide help to B cells in germinal centre formation and class-switching (Horner et al., 1995). Finally, cross-talk between γδ and αβ 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 γδ 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.

γδ cells are activated by infection, which triggers oligoclonal expansion, change of surface markers, and effector functions such as cytolysis and cytokine release (γδ cells can display both Th1 and Th2 phenotypes, similar to αβ 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 γδ 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 γδ cell contributions in primary immune responses, and were exposed in mice completely lacking γδ cells (TCRδ−/−) (Itohara et al., 1993):

- Lung infection with Nocardia asteroides, an intracellular bacterium, is normally cleared in WT mice, but caused mortality of 100% of TCRδ−/− 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 TCRδ−/− 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 γδ cells with NK cells and macrophages, respectively.

- Infection with *Candida albicans*, to which TCRδ<sup>−/−</sup> 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 γδ 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, TCRδ<sup>−/−</sup> mice are for the most part immuno-competent. This might well indicate that αβ lymphocytes can substitute for γδ 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 TCRβ<sup>−/−</sup> and TCRδ<sup>−/−</sup> were able to clear the pathogen, TCRβ<sup>−/−</sup>/TCRδ<sup>−/−</sup> (double KO) mice were not (Sciammas et al., 1997).

In terms of secondary immune responses, immunity to re-challenge with pathogens develops normally in TCRδ<sup>−/−</sup> mice, in stark contrast with the immuno-deficiency displayed by TCRβ<sup>−/−</sup> mice. It is still unclear if γδ 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.

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

More strikingly, γδ cells have been implicated in the regulation of graft *vs.* host disease, since they suppressed the auto-reactive attack of an αβ 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 γδ 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 TCRδ⁻⁻ 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 γδ cells can have a major impact on life expectancy.

What are the selective forces responsible for the evolutionary conservation of γδ cells across half a billion years, in a scenario where αβ 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 γδ 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 αβ 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 αβ responses are less effective (especially Th1 responses).

Immunoprotection in young animals may be a particularly crucial aspect of γδ cell biology. γδ cells are disproportionally 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 αβ cells, γδ cells require neither the complicated antigen processing and presentation machinery, nor the sophisticated antigen sampling, which may not be fully developed at birth. Therefore, γδ 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 γδ cells has tempted some to regard them as part of the innate immune system. It should be stressed, though, that γδ cells, like αβ 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 αβ / γδ lineage split

The two T cell lineages, αβ and γδ, 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 αβ/γδ 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 αβ or γδ) 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 αβ or γδ T cell:

- **Sequential rearrangement** model. This model was inspired by the defined temporal order of TCR isotype expression in foetal thymocytes, with γδ cells first detectable at E14.5 and αβ 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 γδ 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 αβ 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 γδ cell, while if a productive β gene is rearranged first, the cell commits to the αβ lineage (initially expressing a pre-TCR complex).

- **Separate lineages** model(s). They state that the αβ and γδ 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 \(\gamma\) and \(\delta\) rearrangements, which should be extensively but non-productively rearranged. The frequency of out-of-frame \(\gamma/\delta\) rearrangements should be higher than that expected for a random process, since those rearrangements would have been attempted, but unsuccessfully, before TCR\(\alpha\) rearrangement occurred. Conversely, \(\gamma\delta\) cells should essentially be devoid of V-(D)J \(\alpha/\beta\) 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 \(\gamma/\delta\) 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 \(\gamma/\delta\) or \(\beta\) 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 αβ lineage fate.

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

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

Analogously, a silencer element has been identified in the TCRα enhancer (3' of the Ca gene) that is engaged in γδ, but not in αβ, cells (Winoto and Baltimore, 1989b; Winoto and Baltimore, 1989c). This silencer inactivates the TCRα enhancer in γδ cells, thus preventing both rearrangement and transcription of TCRα genes.
Figure 5: Models for the αβ / γδ lineage split

Beginning with a precursor thymocyte, the rearrangement status of TCR loci is represented by:

- \(^{0}\), un-rearranged (germline);
- \(^{+}\), productively rearranged;
- \(^{-}\), non-productively rearranged;
- \(^{\circ}\), un- or non-productively rearranged (irrelevant). Cells unable of any productive rearrangement die.

80
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 αβ cells. This suggests that either commitment occurs after those genes are rearranged, or such rearrangements are not suppressed upon commitment. However, some αβ cells carry TCRγ in germline configuration (reviewed in Fehling, 1999).

According to RFLP-PCR analysis (Dudley et al., 1995), there is selection against in-frame Vγ1.1-Jγ4 rearrangements in αβ cells. Both DP thymocytes (19% in-frame) and peripheral αβ 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 TCRγ mini-locus (encompassing Vγ2/3/4, Jγ1 and Cγ1) was introduced into mice. Only 18% of transgenic sequences were productively rearranged in αβ cells, supporting selection against in-frame TCRγ gene rearrangements in these cells.
δ rearrangements in αβ cells

TCRδ rearrangements are not trivial to study in αβ cells, since recombination in the α loci (usually both alleles are used by αβ 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 TCRδ rearrangements.

Although initial results using probes flanking the Jδ1 segment (by far the most frequently used in γδ 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 Jδ1 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 Vα-Jα excision event in committed αβ thymocytes. Abundant full-length TCRδ 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 TCRδ rearrangements also established that both the silencing and the excision of the TCRδ locus was not required for commitment to the αβ 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 γδ cells**

Initial reports involving a limited number of γδ T cell clones and hybridomas suggested that D-J β rearrangements were very common, whereas complete V-DJ rearrangements were quite rare in γδ 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 γδ cells from both TCRα-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 (Vβ13-Jβ2.2 and Vβ4-Jβ2.2) revealed that 70% of them were in-frame - as much as in αβ cells! This suggested that the peripheral γδ cells had been selected (supposedly in the thymus) for functional TCRβ chains.

Burtrum et al. (Burtrum et al., 1996) examined thymic γδ cells of wild type mice by quantitative Southern blotting, and determined that 20% of all TCRβ alleles had V-DJ rearrangements (compared with 75% in mature αβ cells). RFLP-PCR with Vβ2-Jβ2.6 and Vβ4-Jβ2.6 primers showed that 51-55% of such rearrangements in γδ thymocytes were in-frame. Although lower than Dudley’s (for peripheral γδ cells), these values were still well above the expected for a random (non-selected) event (33%).

Taken together, these results showed that γδ 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 γδ cells mature. Nevertheless, the important questions raised are: a) do these productive β rearrangements encode functional TCRβ chains in γδ cells? b) If so, do these TCRβ chains form a pre-TCR (since pTA protein is not detected in γδ cells (Bruno et al., 1995))? c) Does a productively rearranged TCRβ chain confer any physiological (survival, proliferation, effector functions) advantage to the γδ cells that express it?

Despite these striking findings, subsequent studies have suggested that β-selection in γδ cells is not as common as previously implied: only 42% of Vβ6-Jβ2.5
rearrangements were productive in both foetal and adult γδ 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 γδ 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 γδ cells was presented by Wilson and MacDonald (Wilson and MacDonald, 1998), who showed that 14-17% of wild type γδ thymocytes and splenocytes express intracellular TCRβ protein (icTCRβ). They also showed that TCRβ expression conferred a selective proliferation advantage to γδ cells.

### α rearrangements in γδ cells

It has been shown that the α locus is both recombination- and transcriptionally silent in γδ cells, due to the lack of activity of the TCRα enhancer (Capone et al., 1993; Diaz et al., 1994; Lauzurica and Krangel, 1994). This regulatory element is located 3’ of the Cα gene and contains a silencer region that is engaged in γδ cells, but not in αβ 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 γδ 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 TCRβ genes in γδ cells, and TCRγ and TCRδ genes in αβ cells argues that TCR rearrangements are not lineage-specific. Moreover, the detection of un-rearranged TCRγ and TCRδ genes in many αβ 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 αβ cells, but is not consistent with the apparent selection for
productive β rearrangements in γδ cells. To salvage this model, an additional assumption is required, that γδ-committed cells are able to subsequently extensively rearrange their TCRβ 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 αβ cells bear un-rearranged TCRγ and TCRδ genes. In fact, a more recent report (Livak et al., 1999) has demonstrated that, if anything, TCRγ and TCRδ rearrangements precede (at DN2 stage) TCRβ rearrangements (at DN3 stage), which makes it difficult for the competitive model to explain how αβ cells are devoid of TCRγ/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 TCRγ/δ rearrangements in αβ cells shows that these are not neutral events in thymocyte development, and should therefore be incorporated in any viable model for the αβ/γδ 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 αβ/γδ 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.
- **TCRαβ transgenic mice**

Initial analysis of HY transgenic mice, in which the TCRαβ 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\(γ2-Jγ1\) rearrangements were suppressed and γδ 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 γδ cells, which supported the separate lineages model (Bruno et al., 1996). Such lymphocytes had all the characteristics of γδ cells, apart from TCRγδ 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(+) αβ thymocytes were deleted), and they did not rearrange their endogenous TCRα genes, thereby retaining their TCRδ alleles in both chromosomes (unlike αβ lymphocytes). Moreover, these cells were absent in a γ\(λ\)-deficient background (DiSanto et al., 1996), which specifically blocks the development of γδ cells and were able to co-express a TCRγδ when on a pTα-deficient background (Bruno et al., 1996). Therefore, it seems that γδ lineage cells are not really absent in HY transgenic mice, but they are rather disguised as cells expressing the transgenic TCRαβ (Fehling et al., 1999).

Similar results were also obtained by Capone et al. (Capone et al., 1995) using a distinct TCRαβ 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 TCRα enhancer was not rearranged in those DN cells, whereas it was extensively rearranged in SP cells from the αβ lineage.

Taken together, these data suggest that early expression of a TCRαβ transgene does not prevent the formation of γδ lineage cells. Rather, the transgenic TCRαβ seems to be able to functionally replace TCRγδ in promoting γδ development, despite the absence of the 'correct' receptor. As such, these results support the separate lineages model for αβ/γδ divergence.
**TCRγδ transgenic mice**

TCRγδ transgenic mice should, in theory, provide a clear test for the above models. The two rearrangement (instructive) models predicted a block in αβ 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 Vγ3Jγ1/Vδ1DJδ2 transgene (coding for a receptor characteristic of γδ 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 αβ 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 TCRγδ transgenic mice was to determine which of the many observed phenotypes reflected a physiologically relevant situation. Sim et al. generated nine transgenic Vγ4Jγ1/Vδ1DJδ2 lines, which displayed a wide range of phenotypes. For example, γδ cell frequencies in the thymus varied between 2% and 93%! Two of the lines had normal numbers of αβ splenocytes, whereas seven of them showed a 25%-75% reduction. Moreover, the extent of αβ suppression did not correlate either with transgene copy number, or with the frequency of thymic γδ cells. Interestingly, in two of the lines, a distinct population of lymphocytes co-expressed endogenous TCRαβ and transgenic TCRγδ. These results were interpreted as a demonstration that expression of TCRγδ is not incompatible with αβ development. On the other hand, the expression of TCRγδ seemed insufficient to direct precursor differentiation into the γδ 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 Vγ1.1Jγ4/Vδ6Dδ2Jδ1 transgenic mice expressed extremely high levels of the transgene and exhibited a block in V-DJ β rearrangements and very few TCRαβ(+) 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 TCRγδ (low). These cells had deleted their endogenous TCRδ loci and expressed full length TCRα transcripts, strongly suggesting they belonged to the αβ lineage. However, αβ T cell development was blocked at the DP stage, probably because the DP cells could not be positively selected by the transgenic TCRγδ, in the absence of TCRαβ.

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

**TCR-deficient mice**

Mice lacking TCRαβ or TCRγδ 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. TCRα null (Mombaerts et al., 1992b; Philpott et al., 1992) and TCRβ null (Mombaerts et al., 1992b) mice are devoid of mature αβ cells (SP thymocytes and peripheral lymphocytes), but maintain a full complement of γδ cells. Conversely, TCRδ null mice (Itohara et al., 1993) have no γδ cells but generate normal numbers of αβ 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, TCRβ-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 TCRβ^-/- x TCRδ^-/-,
double deficient mice. This implies that the DP thymocytes of TCR\(\beta^{-/-}\) mice are largely dependent on TCR\(\delta\) for their generation. The ability of TCR\(\gamma\delta\) to support DN \(\rightarrow\) DP transition had also been suggested by the phenotype of 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 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 TCR\(\delta\) (and TCR\(\gamma\)) gene rearrangements in TCR\(\beta^{-/-}\) DP cells. Both groups showed by RFLP-PCR that, as predicted, those cells had been selected for in-frame (75%) TCR\(\delta\) (and TCR\(\gamma\)) rearrangements. Furthermore, they expressed TCR\(\delta\) transcripts at much higher levels than wild type DP thymocytes. These data indicated that, in the absence of a TCR\(\beta\) chain, a sizeable fraction of DP cells were generated from precursor cells expressing a functional TCR\(\gamma\delta\).

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

**pT\(\alpha\)-deficient mice**

Since 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 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 lineages divergence is the pT\(\alpha^{-/-}\) mouse, due to the association of pT\(\alpha\) and TCR\(\beta\) in the formation of the pre-TCR.

As expected, pT\(\alpha^{-/-}\) mice (Fehling et al., 1995a) have a similar phenotype to 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 γδ cells differentiate normally. In fact, the γδ 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 αβ to the γδ lineage. However, as in the case of TCRβ'' mice, the presence of significant numbers of DP cells in pTα'' (and also in pTα'' x TCRα'') mice demonstrates that the pre-TCR (and the TCRαβ) 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 TCRαβ does not block γδ development, since "γδ-like" cells are found in the periphery of TCRαβ transgenic mice. Second, and conversely, early expression of TCRγδ does not block αβ development (up to DP stage), as events associated with β-selection, including the production of DP thymocytes, occur in TCRγδ transgenic mice. Third, expression of TCRγδ is capable of promoting the progression to the DP stage, as shown by the studies of TCRβ'' and TCRβ'' x TCRδ'' mice.

Nevertheless, the competitive rearrangement model is somewhat salvaged by the fact that this TCRγδ-dependent pathway for DP production is very inefficient, and practically insignificant in a normal (TCRβ-proficient) thymus (and it may even be an artifact of the "non-physiological" TCRβ'' mouse model).

The only piece of data that supports the competitive rearrangement model is the increased frequency of γδ cells in pTα'' (and TCRβ'') mice. However, this could also be explained by the "space" in the thymus created by the severe reduction in αβ cell numbers (i.e., a homeostatic effect). In this scenario, the function of the pre-TCR would be limited to the expansion of αβ-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 αβ/γδ lineage commitment.

3.5 TCR-independent mechanisms in αβ vs. γδ cell differentiation

The search for TCR-independent mechanisms of αβ/γδ 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 TCRγ (and IgH) genes. Such effects are the basis of a selective role of the IL-7/IL-7R pathway in γδ cell differentiation.

In the IL-7R deficient thymus, whereas αβ thymocytes are present (although in reduced numbers, due to the effect on early DN precursors), γδ cells are completely absent (Maki et al., 1996). Furthermore, TCRγ 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 TCRγ transgene can partially rescue γδ lineage development in the absence of IL-7R signalling (Kang et al., 1999). In IL-7−/− and γc−/− mice, residual TCRγ gene recombination is detectable, which may result from the activity of TSLP, the other cytokine that uses the IL7Rα 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 TCRγ rearrangements is still unclear. The fact that the TCRγ 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 Jγ1 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 Cγ1 locus. For example, the presence of several consensus STAT5-binding sites in its regulatory regions, and, more convincingly, the generation of γδ 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 αβ and γδ 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 αβ vs. γδ 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 αβ/γδ developmental potentials based on IL-7Rα (CD127) surface expression levels. They sorted DN2 thymocytes expressing high vs. low levels of CD127, and assessed their ability to generate αβ and γδ cells after intra-thymic injection or FTOC. In both assays, the CD127(low) subset was biased towards αβ development, whereas the CD127(high) subset preferentially produced γδ 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 αβ/γδ 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 αβ/γδ (Robey and Fowlkes, 1998).

The first evidence suggesting a role for Notch in the αβ/γδ 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 αβ T cells compared to +/+ cells developing in the same irradiated host, whereas γδ 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 αβ/γδ ratios were unperturbed, even if γδ thymocytes expressed CD4 and CD8 at a higher frequency than normal. These mice were then crossed with TCRβ⁻⁻ mice, and on such a gene-deficient background icNotch was able to promote αβ development, as it was able to overcome the block in T cell development characteristic of TCRβ⁻⁻ mice.

The majority of DP cells generated in TCRβ⁻⁻ icNotch-1 mice had in-frame γ/δ rearrangements, suggesting that icNotch had diverted thymocytes from the γδ to the αβ lineage. On the other hand, icNotch did not bypass the developmental block of 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 αβ development. Indeed, Notch signalling appears to require co-ordination with TCR (at least TCRγδ) 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 γδ pathway, whereas the αβ cell fate would be controlled by both pre-TCR and Notch signals. Since the mechanism for 'shutting off' Notch in the γδ lineage would probably arise from TCRγδ 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 $\beta$-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 $\beta$-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 1,600xg for 5 minutes and re-suspended in appropriate solutions.

PBSA (pH 7.4): 137 mM NaCl, 3.3 mM KCl, 1.7 mM KH₂PO₄, 10 mM Na₂HPO₄.

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

When required, cells were counted on a haemacytometer / 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 10⁴ 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 2-4x10^6 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 dH_2O) 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 µl for analysis and 1000 µl 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):

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2 (-PE), clone T11</td>
<td>CD3ε (-FITC, -PE, -Cy Chrome)</td>
</tr>
<tr>
<td>CD4 (-FITC, -PE, -Cy Chrome, -APC)</td>
<td>CD5 (-FITC, -PE)</td>
</tr>
<tr>
<td>CD8α (-PE, -Cy Chrome, -APC)</td>
<td>CD8β (-FITC)</td>
</tr>
<tr>
<td>CD23 (-PE)</td>
<td>CD24 / HSA (-FITC, -PE)</td>
</tr>
<tr>
<td>CD25 / IL-2Rβ (-FITC), clone 7D4</td>
<td>CD25 / IL-2Rβ (-APC), clone PC61</td>
</tr>
<tr>
<td>CD43 (-FITC, -PE)</td>
<td>CD44 (-FITC, -PE, -Cy Chrome)</td>
</tr>
<tr>
<td>CD45R / B220 (-FITC, -PE, -Cy Chrome)</td>
<td>CD69 (-PE)</td>
</tr>
<tr>
<td>CD90 / Thy1.2 (-FITC *, -APC)</td>
<td>CD117 / c-kit (-PE), clone 2B8</td>
</tr>
<tr>
<td>CD122 (-FITC), clone TM-β1</td>
<td>CD127 / IL-7Rα (-PE)</td>
</tr>
<tr>
<td>TCRαβ, H57-597 (-FITC, -PE, -APC)</td>
<td>TCRγδ, GL3 (-FITC, -PE, -Tricolor *)</td>
</tr>
<tr>
<td>NK1.1 (-PE)</td>
<td>IgD (-FITC)</td>
</tr>
</tbody>
</table>

1.3.2 Extracellular (surface) liposome staining

For detection of the very low levels of surface TCRβ 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 2x10⁶ thymocytes (equivalent to 1 wild type adult thymus, after depletion) were re-suspended in 100 µl PBA.

PBA: 0.5% bovine serum albumine (Sigma), 0.5% NaN₃, in PBSA.

5 µl of anti-hamster IgG and 5 µl anti-CD16 (anti-Fc-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 µg/ml of anti-TCRβ-digoxigenin, were added in an additional 100 µl of PBA. Cells were incubated for 15 min on ice, and then washed twice (5 min at 1,600xg, 4°C).

200 µl 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 µl) 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 µg purified hamster IgG2λ, 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 µg of PE-conjugated antibody, either the specific one, TCRβ (H57-597) in particular, or the isotype control, IgG2λ (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 µg/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 µM 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.

Fluorescence-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 µM 2-ME, L-Glutamine, non-essential amino acids, 10mM HEPES, penicillin and streptomycin.

Hanging drop FTOCs were established in Terysaki plates by placing 5x10^4 to 5x10^5 thymocytes with a 5-day 2-deoxyguanosine-treated (1.35mM) E14-15 foetal wild type lobe in 25 µl 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 7.5x10^5 stromal cells with 1-2x10^4 FACSfected thymocytes (see above), at 2,800xg for 2 min, and pipetting the pellet (re-suspended in about 10 µl) onto a 0.8 µm nucleopore filter (Millipore) placed in standard medium. When required, antibodies (such as anti-CD3e) 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 16,000×g 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, 1 mM 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 μl 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 Immobilon™-P PVDF 0.45 μm 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 μl/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 spectophotometer, 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 µM 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 \(T_m=n(A+T)x2+n(C+G)x4\);

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 µg 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 biomedicals) 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: V85-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 µl reaction mixtures, now using shorter (24-26mers) reverse primers.

**Vδ5-long**, 5'-AGGATGATTCTTCCCCGCGACC

**Jδ1-long**, 5'-AGTCACCTTGCTTTCCTTGCTCCAAGACGAGTTTGTGTT

**Vδ5-short**, 5'-AATGCAGACCCTTACCCTTC

**Jδ1-short**, 5'-CGGGATCCCAAAGACGAGTTTGTGCG

**Vβ2-short**, 5'-ATCCCTGGATGAGCTGGTAT

**Jβ2.2-short**, 5'-TACTTTGGTGGAGCTCAAGCTG

**Vβ5-short**, 5'-GGGGTTGTCCAGTCTCCAAG

**Jβ2.5-short**, 5'-TACTTTGGGCCAGGCACTCGGTCTC

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 Vδ5-Jδ1; 200 bp for Vβ2-Jβ2.2; and 300 bp for Vβ5-Jβ2.5) and DNA fragments were purified as described.

DNA fragments were digested with specific restriction enzymes (Roche): Hae III for Vδ5-Jδ1; Hinf I and Alu I for Vβ2-Jβ2.2; and Mae I and Hae III for Vβ5-Jβ2.5. 50 µl reaction mixtures, containing 1 µl 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 µl dH₂O. These samples were radio-labelled with ³²P-γ-dATP (ICN) in T₄ kinase (New England Biolabs)-mediated reactions. γ³²-dATP (5 µl), T₄ kinase (2 µl), T₄ kinase buffer (5 µl) and dH₂O (8 µl) were added to each sample. After 30 min at 37°C, EDTA (2 µl 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 µl 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 µg) 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 µl dH₂O, 2 µl sequenase buffer 5x and 1 µl T7 sequencing primer 5 pM, and the primer was annealed at 65°C for 2 min. After cooling down the product on ice, 2 µl labelling mix (diluted 1:5), 0.5 µl α-³⁵S-dATP, 1 µl DTT 0.1 M and 2 µl polymerase mix were added and the sample was incubated at room temperature for 5 min.

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

The plasmid DNA sample was split into 4 aliquots of 3.5 µl, 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 µl of each termination mix (ddATP, ddCTP, ddGTP, ddTTP), pre-warmed at 37°C, and 4 µl 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 µl APS 10% and 25 µl 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* Pulsar, 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
μg/μl). 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 γδ 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/μl (in 30 μl 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 5x10⁵ 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 (aquaous) 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:isoamlylol extractions plus one chloroform extraction were performed (equal volumes, 200 µl). To the final aquous 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 µl RNA sample, 1 µl oligo-dT (100ng/µl) was added, and this was incubated at 72°C for 2 min, then cooled to RT.

The reaction mixture was completed with 2 µl of first-strand buffer (5x), 1 µl dNTP mix (5mM), 1 µl of DTT (100mM) and 1 µl 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/µl.

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'-AGCCCAACATGGCTGTAACTGGAG
- NOR-1 fwd primer, 5'-AGGATACACCTCCTGTGTAAGGG
- NOR-1 rev primer, 5'-CCATTTCATAGCATGACTGCCTCC
- Ly49A fwd primer, 5'-GATGCGACATGGGATTGCAATCGC
- Ly49A rev primer, 5'-TGCACTGCAGACTAAGTCCAATGG
- Leukocystatin fwd primer, 5'-CTGCTTACCAGCGAGCAGATTAGC
- Leukocystatin rev primer, 5'-AGTCTGATGGTGACAGACACAGAGC
- Sugano EST fwd primer, 5'-AGCCAAGTGGGTGCTCTCAACC
- Sugano EST rev primer, 5'-GCCCAGGATACACAGTGAAGAACG
- Myeloblastin fwd primer, 5'-AGCAGGACCAGACTCTGTCCAG
- Myeloblastin rev primer, 5'-CCGGGAAGAAATCAGGGACTG
PD-1 fwd primer, 5'-CTGGAGTCCTCCTTCTACCC
PD-1 rev primer, 5'-GATGGCCACAGGAGTAGATGCC
Mac-2 fwd primer, 5'-GATATGGGTGCATGGGGACC
Mac-2 rev primer, 5'-CTCGAGGCAAGGCAAGGTCATAGGG
Mg11 fwd primer, 5'-TGGGAACCGGAGCAGTGTGCTCC
Mg11 rev primer, 5'-GCAGCCTGCTAGGTACCCACTCC
Laminin-R 1 fwd primer, 5'-CTGTGATCCCGAATCGGGGATCC
Laminin-R 1 rev primer, 5'-ACCACCTTGCCCCCTGGACCTTGG
"Novo-1" fwd primer, 5'-CCAAACCTTTGTAAACCAGCTGGG
"Novo-1" rev primer, 5'-TGTGTAATGTCGTCCTCCATAGG
"Novo-2" fwd primer, 5'-AGATGAGCCTTGGAGAAGAGCTGC
"Novo-2" rev primer, 5'-TTAGTGGATGAACCAGCCACTGGC
IL-7Ra fwd primer, 5'-TTAAAGCCCGAGGCTCCCTCCTGCC
IL-7Ra rev primer, 5'-TTGGACTCCTCAGGCTCAGAACG

In parallel to the PCR for the gene of interest, an additional PCR for the housekeeping 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'-CTGACGGCCAGGATCATCACTTA
β-actin rev primer, 5'-CCGACTCATCGTACTCTCCTGC

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 interpolation.

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,  
\[ \text{FAM-CGGGCAGCTCCCTTTTCATCA-TAMRA} \]  
(FAM is the 5' reporter dye; TAMRA is the 3' quencher dye)

ICER fwd primer,  
\[ 5'-\text{ATATTCCTTCTTCTTCTGCGACACT} \]

ICER rev primer,  
\[ 5'-\text{CCAGCAACTAGCAGAAGCA} \]

Optimal concentrations of these reagents were estimated by titration (using the reference cDNA) over the following range: 50, 300 and 900 mM 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'-AAGCAGTGTAACAACGCAGAGTACT(N)N

(N = A, C, G, or T; N = A, G or C)

Capfinder - 5'-AAGCAGTGTAACAACGCAGAGTACGCGGG

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

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

Reactions followed the scheme:

<table>
<thead>
<tr>
<th>n. cycles</th>
<th>95°C</th>
<th>60°C</th>
<th>68°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x</td>
<td>1 min</td>
<td>1 min</td>
<td>12 min</td>
</tr>
<tr>
<td>7 x</td>
<td>30 sec</td>
<td>30 sec</td>
<td>12 min</td>
</tr>
<tr>
<td>7 x</td>
<td>30 sec</td>
<td>30 sec</td>
<td>14 min</td>
</tr>
<tr>
<td>7 x</td>
<td>30 sec</td>
<td>30 sec</td>
<td>16 min</td>
</tr>
</tbody>
</table>

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-³²S-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 5x10³ tester cells can be subtracted with the cDNA representation from 5x10⁴ driver cells. However, if possible it is recommended that 1-3x10⁵ 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 doubled-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'-AAGCAGTGTTAACAACGCAGAGT

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 µl of NaOAc 2 M and 220 µl 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 µl 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 µl of full-length cDNA, 4 µl of Dpn II buffer (10x) and 2 µl of Dpn II were added and the sample was incubated at 37°C for 3 hr.

The digest was diluted with 160 µl dH₂O, and subjected to 2x phenol/chloroform and 1x chloroform extractions. 20 µl of NaOAc and 220 µl 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 µl 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'-GATCTGTTCATG
(note: J-12 (Tsp) 5'-AATTTGTTCATG)
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 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 (1µg/µl), 3 µl of J-12 primer (1µg/µl), dH₂O to 57µl.

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 dH₂O 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 dH₂O.

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, 1µl 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 dH₂O and 1 µl (1µg/µl) 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 dH₂O. 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 H₂O, 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 dH₂O were added. After 1x phenol/chloroform and 1x chloroform extractions, the final aqueous phase was
supplemented with 40 µl of NaOAc 2M (pH 5.4), and 450 µl isopropanol. DNA was precipitation on ice for 20 min, then centrifuged and the pellet was resuspend in 40 µl dH₂O. This was the first differential product (DP1), of which 4 µl and 2 µl 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/µl) 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 µl of Dpn II buffer (10x), 2 µl of Dpn II and dH₂O to 40 µl.

To the digest, 160 µl dH₂O were added and 2x phenol/chloroform and 1x chloroform extractions were performed. The final aqueous phase was supplemented with 20 µl NaOAc 2 M, 1 µg glycogen and 220 µl 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 µl dH₂O.

0.2 U of digested DP1 were mixed with 12 µl ligase buffer 5x (GIBCO BRL), 6 µl of N-24 primer (1µg/µl), 3 µl of N-12 primer (1 µg/µl) and dH₂O to 57µl. N-linkers were annealed in PCR machine by heating to 50°C and then cooling 1°C/min to 15°C. 3 µl 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: 6µl (0.02 U) of N-ligated DP1, 20 U of driver representation, and H₂O to 120µl. This mixture was subjected to 1x phenol/chloroform and 1x chloroform extractions, and DNA from the final aqueous phase was precipitated with 30µl of ammonium acetate 10 M and 150 µl 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 µl DNA sample, 8 µl BDT fluorochrome mixture, 2 µl T7 primer and 7 µl 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 µl 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 αβ versus γδ thymocytes

1.1 RDA analysis of DP vs. γδ thymocytes

Our objective was to identify genes involved in αβ/γδ 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 γδ lineage, there is no other reliable marker besides TCRγδ itself; furthermore, no discrete stages of differentiation within the TCRγδ(+) compartment have been described. Nevertheless, newly generated thymic γδ cells are known to be HSA(+), in contrast with circulating γδ lymphocytes. Thus FACSorted TCRγδ(+) HSA(+) thymocytes were used as the γδ population.

For the αβ lineage, the co-expression of CD4 and CD8 is the earliest event that occurs in αβ-committed cells, and therefore DP thymocytes were the obvious candidates. However, the DP subset is very heterogeneous, and once they express the mature TCRαβ 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 αβ/γδ lineage commitment step, we FACSorted DP cells from TCRα-deficient thymuses. As mentioned in the introduction, αβ T cell development in TCRα KO mice is completely blocked at the DP stage due to the lack of mature TCRαβ, whereas γδ development proceeds normally.

In order to have the same genetic background, both populations (γδ and DP) were isolated from TCRα-deficient thymuses, as depicted in Figure 8. Total RNA was extracted from the purified (>97% purity) subsets and used for RDA analysis.
TCRα⁻/- thymus

DP (CD4⁺ CD8⁺) cells

Acquisition Dot Plot

CD4

CD8

γδ cells

BS180299.001

DAT.A009

TCR γδ

HSA

15%

95%

Figure 8: FACSorting of TCRα⁻/- thymocytes for RDAnalysis.

TCRγδ profile was pre-gated on CD4(-)CD8(-) cells, and HSA profile was pre-gated on TCRγδ(+) thymocytes.
RDA experiments were conducted using both populations as either tester or driver: γδ - DP and DP - γδ 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 γδ and DP thymocytes:

* IL7-Rα expression was known to be down-regulated in the DN→DP transition (and later up-regulated in the SP stage), whereas it is maintained in γδ cells, which are completely dependent on IL-7 signals. IL-2Rβ chain, part of the IL2-R complex, was also known to be expressed in a subset of γδ 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 γδ cells are fundamentally CD4(-)CD8(-); therefore those molecules were expected products of the DP-γδ subtraction. Likewise for TCRβ, the primary marker of β-selected/αβ-committed cells such as DP.

In the same like of thought, also RAG-1 was predicted to come out of the DP-γδ subtraction, as DP thymocytes are actively rearranging their TCRα locus, while γδ 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 γδ - 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.
cDNA fragments + linkers = Representations

Running diff. products on agarose gel

Extraction of gel bands and DNA purification

Cloning + sequencing cDNA fragments

Probing Atlas array with diff. products

Figure 9: Summary of RDA analysis of γδ vs. DP thymocytes.
TABLE 1

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

<table>
<thead>
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</thead>
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<tr>
<td>IFN-γ induced Mg11</td>
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<tr>
<td>Regulator of G-protein Signalling (RGS-2)</td>
</tr>
<tr>
<td>Expression Sequence Tags (EST)</td>
</tr>
<tr>
<td>Sugano cDNA clone</td>
</tr>
<tr>
<td>Knowles Solter blastocyst cDNA</td>
</tr>
</tbody>
</table>

**Table 1 : Genes isolated from the RDA subtractive hybridisation of γδ 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 γδ and DP cells from TCRα-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 TCRα 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 γδ 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 γδ - DP subtraction; and Schlafen-2 (an inhibitor of cell growth), Tcl-30 and Soares EST, from the DP - γδ 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 γδ and DP thymocytes.
1.3 Pattern of expression of candidate genes

Gene expression was assessed by RT-PCR on FACSsorted 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>
<thead>
<tr>
<th></th>
<th>MΦ</th>
<th>NK</th>
<th>B</th>
<th>DN1</th>
<th>DN2</th>
<th>DN3</th>
<th>DN4</th>
<th>γδ</th>
<th>DP</th>
<th>SP4</th>
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<tbody>
<tr>
<td>Ly49A</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NOR-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>-</td>
</tr>
<tr>
<td>Sugano EST</td>
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<td>+</td>
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<tr>
<td>Leukocystatin</td>
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<td>-</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>-</td>
<td>+</td>
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<td>-</td>
<td>+</td>
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<tr>
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<td>+</td>
<td>-</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Laminin-R 1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>‘Novo-1’</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

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, DP and SP populations (*):

**DN subsets (**) :**

γδ thymocytes (***) :

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

Pre-gating: (*) Thy1+; (**) Thy1+ CD4+ CD8- TCRγδ+ B220+ NKI.1- ; (***) Thy1+ CD4+ CD8+ cells. Percentages of gated subsets are indicated.
<table>
<thead>
<tr>
<th>bp</th>
<th>MΦ</th>
<th>NK</th>
<th>B</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>γδ</th>
<th>DP</th>
<th>4+</th>
<th>8+</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 αβ/γδ lineage commitment process.

The selection criterion mentioned above was met by 5 out of the 11 candidate genes: NOR-1 and CREM, for “γδ lineage genes”; and “Novo-1”, “Novo-2” and Laminin receptor 1, for “αβ 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 αβ cell late development or function rather than to αβ / γδ 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 γδ and αβ cells was not maintained in later stages of development: SP (αβ) cells also expressed PD-1, in levels comparable to γδ thymocytes. Mg-11 expression, too, was up-regulated during DP → 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 αβ / γδ 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 been cloned. The sequences (5' to 3') of the partial transcripts (cDNA representations) obtained from the RDAnalysis are presented in Table 3.

**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>GATCTAAGTGAGATTCCAAACCTTTGTAACCCAGCTGGGT</th>
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<table>
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</tr>
<tr>
<td></td>
<td>TTTGTGAC</td>
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</table>

Table 3: cDNA sequences with no matches in GenEMBL databases, obtained from the RDA subtraction DP - γδ, 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 γδ 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 γδ - DP differential product, we were interested in investigating whether it played a role in γδ 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 γδ thymocytes were $(2.6±0.4)x10^5$ for NOR-1(-) and $(2.3±0.4)x10^5$ for NOR(+) mice, whereas DP cell numbers were $(8.0±0.8)x10^7$ for NOR(-) and $(8.6±0.8)x10^7$ 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 γδ 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 TCRγδ-mediated mechanism accounting for the differential expression between γδ 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 → DN4 transition (Figure 12), it suggests the involvement of pre-TCR signalling. This hypothesis is currently under investigation. Regarding the αβ / γδ 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\(^a\). 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.)
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.)

<table>
<thead>
<tr>
<th>NK</th>
<th>γδ</th>
<th>DN</th>
<th>αβ</th>
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<tbody>
<tr>
<td>33%</td>
<td>5%</td>
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<td>0.2%</td>
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<table>
<thead>
<tr>
<th>NK1.1</th>
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<tbody>
<tr>
<td>+</td>
<td>0.2%</td>
<td>0.6%</td>
<td>0.0%</td>
</tr>
<tr>
<td>-</td>
<td>47%</td>
<td>0.8%</td>
<td></td>
</tr>
</tbody>
</table>
1.4.2 Sugano EST

One other sequence isolated from the γδ - 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 γδ and NK thymocytes, but not in other haematopoietic lineages. Unlike Ly49A, the expression of Sugano EST is mainly attributable to NK1.1(-) γδ 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-2RP 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-2RP, the experiment represented in Figure 14(B) was performed. Using a combination of IL-2RP forward primer and Sugano EST reverse primer, a RT-PCR reaction was run in γδ 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-2RP 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-2RP gene, consistent with the proposed linkage between the transcripts.

The previous results prompt us to investigate whether the IL2-RP/Sugano transcript was translated into a protein expressed on the cell surface (of γδ thymocytes), where the conventional IL-2RP chain localises. Previous data (Takeuchi et al., 1992; Tanaka et al., 1991) suggested that the IL-2RP protein is expressed in very few (1-4%) thymocytes, whereas it is more frequent among splenic CD8+ T cells and NK cells.
Figure 14: Sugano EST: sequence and linkage to IL-2Rβ transcript.

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

(B) RT-PCR on γδ cells using combined primers for IL-2Rβ and Sugano EST.
In mature lymphocytes, IL-2Rβ 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-2Rβ expression. Not much was known about the levels of expression of IL-2Rβ in particular thymocyte subsets. Two commercial antibodies specific for IL-2Rβ (also known as CD122) were available: clone 5H4, which did not block binding of the ligand (IL-2) to the receptor; and clone TM-β1, which blocked high affinity binding of the cytokine to its receptor. Both were used to stain thymocyte populations; the results for clone TM-β1 are presented in Figure 15. Similar results were obtained with clone 5H4.

In contrast to NK cells, γδ thymocytes did not express IL-2Rβ protein on the cell surface. The same was true for precursor DN cells.

Therefore, the IL-2Rβ/Sugano mRNA transcribed in γδ cells is not expressed as a IL-2Rβ-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-β1) are capable of abrogating IL-2 binding suggests that the IL2-Rβ/Sugano transcript expressed in γδ thymocytes is not relevant in terms of IL-2 signalling.

Thus, the biological relevance of the IL2-Rβ/Sugano transcript, in which the Sugano sequence represents an alternative 3′-end for the IL-2Rβ gene, is still under investigation.
Figure 15: IL-2Rβ protein surface expression in thymic subsets.

FACS profiles for wild type DN, NK and γδ thymocytes, using mAb clone TM-β1.

(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 TCRβ(+) vs. TCRβ(-) 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 TCRβ(+) and TCRβ(-) mice. As seen on Figure 16, these comprise 50% and 80% of the thymic DN compartments, respectively. The accumulation of DN3 cells in the TCRβ KO thymus (block in DN3 → DN4 transition) is due to the absence of "β-selection".

Figure 16 : FACS plots (CD25 vs. CD44) for WT and TCRβ+/− DN thymocytes. (Pre-gated on Thy1+ CD4+ CD8− TCRγδ− NK1.1− B220+ cells).
We were therefore assuming that the differences in gene expression between the two populations, as identified by a TCRβ(+) DN3 - TCRβ(-) DN3 subtractive hybridisation (Table 4), would be a direct consequence of pre-TCR signalling.

**TABLE 4**

<table>
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<tr>
<th>Protein family</th>
<th>Gene</th>
</tr>
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</tr>
<tr>
<td></td>
<td>M-α-Tubulin</td>
</tr>
<tr>
<td></td>
<td>Crk</td>
</tr>
<tr>
<td></td>
<td>Ribosomal proteins 18S ; 57 L18 ; L19</td>
</tr>
<tr>
<td><strong>Mitogen activated proteins</strong></td>
<td>MNK2 (mitogen-activated kinase)</td>
</tr>
<tr>
<td></td>
<td>EIF4E (transcription initiation factor)</td>
</tr>
<tr>
<td><strong>Chromosomal proteins</strong></td>
<td>Histone H2A.Z</td>
</tr>
<tr>
<td></td>
<td>HMG-17 (high-mobility group protein)</td>
</tr>
<tr>
<td><strong>Regulators of signalling</strong></td>
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<tr>
<td></td>
<td>SAM-9 (phospholipase D homologue)</td>
</tr>
<tr>
<td><strong>Regulators of protein folding</strong></td>
<td>Calnexin</td>
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<tr>
<td><strong>Transcription factors</strong></td>
<td>CREM (cyclic-AMP responsive TF)</td>
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<tr>
<td></td>
<td>Nur-77 (nuclear orphan receptor)</td>
</tr>
</tbody>
</table>

Table 4: Genes isolated from the RDA subtraction of TCRβ(+) and TCRβ(-) 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 RDAnalysis (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-CD3ε 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-CD3ε 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 → DN4 → 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 → 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).
Figure 17: FACS plots (CD25 / CD44) used in the purification of cells for RDAnalysis 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 RDAnalysis: CD25, CD69 and CD2 levels of surface expression; Forward Scatter (cell size).
Figure 19: Cell cycle status of sorted populations used for RDA analysis.
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**

<table>
<thead>
<tr>
<th>Protein family</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transcription factors</strong></td>
<td>ICER (cyclic-AMP responsive TF)</td>
</tr>
<tr>
<td></td>
<td>Nur-77 (nuclear orphan receptor)</td>
</tr>
<tr>
<td></td>
<td>Egr-1 (early growth response gene)</td>
</tr>
<tr>
<td></td>
<td>c-Maf</td>
</tr>
<tr>
<td><strong>Regulators of signalling</strong></td>
<td>ZFP-36 (zinck finger protein)</td>
</tr>
<tr>
<td><strong>Membrane receptors</strong></td>
<td>Shp-1 (protein tyrosine phosphatase)</td>
</tr>
<tr>
<td></td>
<td>IL-7Rα</td>
</tr>
</tbody>
</table>

*Table 5: Genes isolated from the RDAnalysis of RAG(-) thymocytes stimulated (for 3 hours in vivo) with anti-CD3ε 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 RDAnalysis: 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-7Rα 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<sup>−/−</sup>, TCRβ<sup>−/−</sup> and pTa<sup>−/−</sup>, all lacking one component of the complex; and p56Lck<sup>−/−</sup>, 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-CD3ε 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-7Rα expression to be inducible in RAG(-) DN3 thymocytes by an anti-CD3 stimulus (Figure 20), but the endogenous expression of IL-7Rα 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-7Rα expression was indeed pre-TCR dependent. As shown on Figure 22 A, mice lacking a component of the pre-TCR, either TCRβ or pTα, 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-7Rα mRNA (when compared to the other candidate genes), as seen in Figure 20. The induced expression of IL-7Rα 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 → 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-7Rα 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.

166
Figure 22: Surface expression of IL-7Rα: (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-CD3ε mAb. Staining for IL-7Rα (unshaded) is compared to IgG2a isotype control (shaded).
2.4.2 Requirement for IL-7R signalling in the DN → DP transition

As pre-TCR signalling appears to maintain IL-7Rα 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 x 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-7Rα or γc, 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 ~1 x 10^5) were observed (Figure 23B), as expected. In comparison, cultures treated with one of the two mAbs (anti-IL-7Rα or anti-γc) expanded less, to ~7-8 x 10^4 cells (data not shown). Furthermore, when both antibodies were used together, total cell number reached only ~20% (~2 x 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.
Figure 23: Effect of blocking antibodies to IL-7R complex on DN4 → DP transition. Anti-IL7Rα and anti-γc 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-7Rα−/− mice were compared to those from WT and TCRβ−/− 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 TCRβ−/− DN4 cells was only ~11%. However, such a reduction in percentage of cycling cells was not observed for IL-7Rα−/− 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 TCRβ−/− DN4 subsets, ~80% of thymocytes were PI/Annexin-V negative, a pattern indicative of live cells. However, in contrast the IL-7Rα−/− DN4 subset had only ~50% PI/Annexin-V negative cells. Thus, IL-7Rα−/− 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 TCRβ gene rearrangements in early DN thymocyte differentiation (ref. 38 paper), we had to consider that hypothesis. If this were true, the IL-7Rα−/− (DN3 and) DN4 population(s) could be relatively TCRβ deficient and hence cell death may reflect an increased failure of β-selection.

To test this, intracellular TCRβ expression was analysed in DN3 and DN4 thymocytes from WT and IL-7Rα−/− mice. In the DN3 subset ~40% of WT cells were intracellular TCRβ(+) compared to ~50% from IL-7Rα−/− mice (data not shown). Likewise, comparable levels of intracellular TCRβ (88% vs. 77%) were also observed for WT and IL-7Rα−/− 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-7Rα−/− mice (data not shown). Thus, failure to express a productively rearranged TCRβ gene is not observed in IL-7Rα−/− mice and hence cannot explain the increased level of cell death observed in IL-7Rα−/− DN4 cells.
**Figure 24**: Comparison of WT and IL-7Rα deficient DN4 thymocytes: cell cycle status, cell death and intracellular TCRβ expression. Also shown are plots for cell cycle and death of TCRβ-deficient DN4 cells.
Although DN4 thymocytes from IL-7Rα−/− 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-7Rα and anti-γc 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-7Rα and anti-γc 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-7Rα/anti-γc 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-7Rα/anti-γc 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.
Figure 25: Effect of blocking antibodies to IL-7R complex on proliferation and death of DN4 thymocytes. Anti-IL7Rα and anti-γc mAbs (or isotype controls) used in 5 day- RTOC.
3 ICER in αβ versus γδ T cell lineage commitment

3.1 Identification of CREM isoforms expressed in γδ thymocytes: ICER

Cyclic-AMP response element modulator (CREM) was one of the transcription factors identified in the γδ - DP subtraction (1.1). According to our RT-PCR results (Figure 12), CREM is expressed in γδ and pre-T cells, but not in αβ thymocytes. Its restricted expression to the γδ - and not other haematopoietic - lineages made it an attractive candidate in the context of γδ / αβ 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 γδ T cells. Figure 26B demonstrates that in thymic γδ cells the major CREM-specific transcripts code for ICER I and ICER Iγ. 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 Iγ 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 γδ 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 γδ 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 γδ thymocytes and in total brain (control), using combinations of the primers (A-E) presented above.
Figure 26: Identification of CREM isoforms expressed in γδ thymocytes. (C) Western blot for CREM/ICER in thymic γδ 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 γδ and DP (αβ) 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 γδ T cells, but not in NK cells or in thymic DP or single positive (SP) thymocytes that represent committed αβ lineage T cells. Overall, ICER expression in γδ thymocytes is at least 20-fold higher than in αβ 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 γδ cells.

These results indicate that although ICER is expressed in early thymic DN subsets, its expression is restricted to the γδ 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 αβ and γδ T cells. Furthermore, DN4 thymocytes express less ICER than their precursor populations (DN2/3), suggesting a progressive down-regulation of ICER in αβ thymocyte development, if we take into account that most DN4 cells commit to the αβ rather than the γδ 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.
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-TCRβ) 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 TCRβ were essentially restricted to the αβ lineage, as they did not generate γδ 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 TCRβ rearrangements and a selection against (approx. 20%) in-frame TCRδ rearrangements in DN4-high cells; these are typical of αβ-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 αβ lineage down-regulate ICER expression, towards the low levels subsequently exhibited by DP and SP thymocytes.
Figure 28: Pre-TCR levels and ICER expression in WT pre-T cells.

(A) Schematic of the liposome staining method for surface TCRβ 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.
Figure 29: Pre-TCR levels and TCR gene rearrangement status of WT pre-T cells. RFLP-PCR results for TCRδ and TCRβ 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 γδ and not the αβ T cell lineage, we reasoned that ICER could play a role in thymocyte development and more specifically in αβ/γδ 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 γδ 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) TCRγδ histograms, pre-gated on CD4+ CD8− thymocytes from CREM−/− and wild type control mice.
Contrary to αβ lineage cells, γδ thymocytes differentiated normally in the presence of cAMP: 1,800±600 cells were generated in both cAMP-treated and control FTOCs (data not shown).

The inhibitory effect on αβ thymocyte differentiation \textit{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 \textit{(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 γδ thymocytes did not translate into a perturbed γδ development in ICER-deficient mice, we decided to investigate whether the low levels of ICER in normal DP cells were crucial for αβ differentiation, and in particular if an enforced expression of ICER in DP thymocytes would disturb αβ 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 µM 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 FACSorted 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 γδ 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 αβ 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 αβ/γδ lineage commitment, it could still be a novel and reliable marker of the γδ T cell lineage.

In particular, the dramatic contrast in ICER expression between thymic γδ and αβ T cell subsets after the point at which the γδ and αβ 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.
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) TCRγδ histograms, pre-gated on CD4⁺ CD8⁻ thymocytes from CD2-ICER transgenic (Tg) and non-transgenic (control) mice.
γδ T cells from both the lymph nodes and the spleen expressed high levels of ICER; in contrast, CD4+ SP or CD8+ SP αβ cells from those tissues did not express ICER to any significant level - Figure 34.

A slight increase in ICER expression was observed in activated αβ T cells but this level was still at least six-fold lower than that observed in peripheral γδ 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 γδ 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 αβ and γδ T cell populations. Specifically, we examined various subsets of intestinal intraepithelial lymphocytes (IELs).

Traditionally, IEL subsets have been divided into those that express either αβ or γδ TCR. Analysis of the TCRγδ (+) subset has identified cells that are either not expressing CD8α and CD8β (TCRγδ DN) or that express CD8α as a homodimer (TCRγδ CD8αα). Likewise, TCRαβ (+) IELs can be sub-divided into (TCRαβ CD8αα) cells or cells that express CD8α as a heterodimer with CD8β (TCRαβ CD8αβ).

The analysis of ICER transcripts in these four IEL populations - Figure 35 - revealed that consistent with γδ T cells from the lymph node and spleen, both the (TCRγδ DN) and the (TCRγδ CD8αα) IEL subsets expressed a significant amount of ICER transcript. However, unlike all other mature αβ T cell populations, the (TCRαβ CD8αα) IEL subset had an expression level of ICER comparable to that observed for all the various γδ T cell populations that were studied. In contrast, (TCRαβ CD8αβ) IELs only expressed ICER at very low levels.

These data demonstrate that γδ IEL populations express ICER at a level consistent with γδ 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 (TCRαβ CD8αα) IEL subset is "γδ-like" with respect to ICER expression.
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.
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 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 TCRδ<sup>−/−</sup> and TCRα<sup>−/−</sup> mice, which completely lack TCRγδ and TCRαβ, respectively. On the other hand, we studied models where the development of either the αβ or γδ T cell lineage has been demonstrated to be directed by the opposite type of TCR (TCR HY transgenic and TCRβ<sup>−/−</sup> mice).

ICER expression profiles in the thymus of both TCRδ<sup>−/−</sup> and TCRα<sup>−/−</sup> 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 (γδ thymocytes in TCRδ<sup>−/−</sup> and SP cells in TCRα<sup>−/−</sup>, see Figure 33A). These similarities imply that: 1) TCRγδ is not involved in ICER expression in pre-T cells (<i>a priori</i>, one could imagine a low, almost undetectable by FACS, level of TCRγδ being responsible for ICER expression in DN subsets); 2) TCRαβ 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 TCRβ<sup>−/−</sup> 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 αβ TCR transgene positive T cells in their lymph nodes. Those that express CD8 are considered to be conventional MHC class I restricted αβ lineage T cells. However, the smaller population of CD8(-) TCRαβ(+) cells have been shown to display characteristics of the γδ T cell lineage (see Introduction, 3.4).
Figure 36: ICER expression in TCRα deficient and TCRδ deficient mice.

(A) Representation of T cell developmental blocks in TCRα⁻/⁻ and TCRδ⁻/⁻ mice.

(B) Real-time PCR for ICER in thymocyte subsets of TCRα⁻/⁻ and TCRδ⁻/⁻ mice.
When the expression of ICER was analysed in these γδ lineage T cells that express an "inappropriate" αβ TCR, a high level of transcript, almost comparable to that expressed in conventional γδ T cells, was observed – Figure 37A. This was in contrast to the low level of ICER expression that was seen in the CD8(+) TCR αβ(+) αβ lineage cells.

In TCRβ−/− mice, αβ lineage-committed DP thymocytes are observed in significant numbers even though a pre-TCR or TCRαβ cannot be formed. These β-negative DP cells are thought to have been selected by TCRγδ as they are lost in TCRβ−/− x TCRδ−/− animals (see Introduction, 3.4).

As seen in Figure 37B, when ICER expression was analysed in these αβ lineage cells that had received signals only from TCRγδ, 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 γδ or the αβ TCR. In contrast, ICER expression appears to be a characteristic of cells that have adopted a "γδ-like" T cell fate, regardless of whether they have employed either the γδ or αβ 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 αβ and γδ lineages, and then becomes restricted to mature γδ cells. We therefore explored the possibility of the expression of ICER specifically marking γδ-precursors within the DN compartment. We took advantage of previously generated CREM-LacZ mice (Blendy et al., 1996), which we obtained from Prof. Gunther Schütz (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 TCRαβ transgenic and TCRβ 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 TCRβ−/− 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 γδ thymocytes from CREM-LacZ mice are shown in Figure 38A. As expected (from 3.1), γδ 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 γδ 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 αβ lineage cells (DP) than their ICER-low counterparts - Figure 38C. The ratio αβ/γδ (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 αβ/γδ are due to the differences in the specific proliferation rates of αβ and γδ 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 γδ-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 γδ lineage).

The extensive generation of αβ 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.
Figure 38: Lineage potential of pre-T cells expressing different levels of ICER. (A) LacZ protein staining for DP and γδ 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 γδ 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 αβ vs. γδ 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 αβ 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 downregulation, which defines progression to the following stage of development, DN4.

In DN3 and DN4 thymocytes, like in γδ 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 TCRβ, 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 2 were re-assessed by real-time (quantitative) PCR.

As shown in Figure 4, DN3 thymocytes (first population to express the pre-TCR in a normal thymus) from TCRβ⁻/⁻, pTα⁻/⁻, p56Lck⁻/⁻ and 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 TCRγδ express normal (or slightly higher, in the case of TCRδ⁻/⁻) 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-CD3ε mAb

The original piece of data that put ICER in the context of β-selection was the induction of its expression in 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<sup>−/−</sup>, TCRβ<sup>−/−</sup>, pTα<sup>−/−</sup> and Lck<sup>−/−</sup> mice. Results for WT control, IL7Rα<sup>−/−</sup> and TCRδ<sup>−/−</sup> 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-CD3e 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.
Figure 41: Induction of ICER expression by anti-CD3 mAb stimuli.

(A) Real-time PCR for ICER on adult RAG-1⁻/⁻ thymocytes treated \textit{in vivo} with anti-CD3ε mAb (experiment of Figures 17-20, samples of Figure 20).

(B) Real-time PCR for ICER on foetal RAG-1⁻/⁻ thymocytes treated \textit{in vitro} with anti-CD3ε mAb. After 5 days in FTOC, DN and DP cells were FACSorted.

(C) LacZ protein staining for sorted DN3 thymocytes of CREM/ICER-LacZ mice, treated (or not, NT) \textit{in vitro} (15 hour RTOC) with anti-CD3ε 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-CD3ε 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 µg/ml) or without anti-CD3ε 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 µM MEK1 inhibitor PD98059, 61% with 25 µM of the same compound, and no effect with 5 µM 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 2.5µM) on anti-CD3ε - mediated induction of CREM-LacZ expression (data not shown). Furthermore, substitution of anti-CD3ε 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-CD3ε.

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 FACSorted 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-CD3ε 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-CD3ε 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 $\beta$-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 $\beta$-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 \( \beta \)-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/RTOC.

The "low" and "high" populations were defined as the 25% lowest / highest intensities of LacZ staining. If ICER-high thymocytes were indeed biased towards \( \beta \)-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 RTOC, 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 \( \beta \)-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.
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.
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 TCRβ 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 TCRβ-negative cells, “high”-expressing thymocytes were large (63%), cycling (52%), intra-cellular TCRβ-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 TCRβ expressed less ICER transcripts; since LacZ “high” cells are also intracellular TCRβ-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 αβ-committed thymocytes (see 3.3), which, as such, should down-regulate ICER (in line with the results of section 3).
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 TCRβ 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 γδ thymocytes developing in the absence of β-selected cells

We had established before that ICER acts as a γδ 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 γδ cells derive from pre-T cells, we were interested to investigate if ICER expression in γδ thymocytes was conditioned by previous expression in their DN precursors. For that, we examined ICER expression in γδ cells of pre-TCR deficient mice (TCRβ^−/−, pTα^−/−), whose pre-T cells are ICER(-) (see Figure 40). We observed a ten-fold reduction in ICER expression between WT and pre-TCR deficient γδ cells - Figure 47A. This was the case in both the thymus and the spleen of TCRβ^−/− and pTα^−/− mice.

ICER is not the only gene whose expression in γδ 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 γδ cell populations, as illustrated with transcription factor c-Maf, an important factor for γδ development (Laurie Glimcher, unpublished data) and controls β-actin and Vδ2. This suggests a specific effect of pre-TCR signalling in the expression of genes such as ICER or NOR-1 in γδ cells, underlying a novel role for β-selection in γδ thymocyte differentiation.

However, two lines of evidence suggest that the effect of pre-TCR on γδ differentiation is not in cis. Firstly, the distribution of ICER-LacZ protein in γδ thymocytes is uni-modal (close to normal distribution) (Figure 38A). Since only circa 15% of γδ cells express icTCRβ (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.
Figure 47: Gene expression in γδ cells of TCRβ⁻/⁻ and pTα⁻/⁻ mice:

(A) Real-time PCR for ICER in thymic and splenic γδ cells.

(B) RT-PCR for various genes in thymic γδ 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 TCRβ gene segments by single-cell PCR. No amplification signal for TCRβ 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 γδ 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 γδ thymocytes.](image)

Real-time PCR on sorted WT subsets.
More data supporting the role of a trans-induction mechanism in the context of γδ T cell differentiation was obtained from ICER's pattern of expression during murine development. ICER expression in DN2 and γδ 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 αβ 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 αβ and γδ lineages during γδ thymocyte differentiation

Since ICER expression is impaired in pre-TCR deficient mice, which lack DP and SP thymocytes, but not in TCRα−/− 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 γδ thymocytes (targets of the trans-induction mechanism) were subjected to the presence of a large excess of DP thymocytes (the effector cells). The target γδ cells had a pre-TCR-deficient background, so that they did not express ICER at the start of the experiment. In more detail, 1.5x10⁴ TCRβ−/− γδ thymocytes were mixed with 1.5x10⁶ DP cells (isolated from TCRδ−/− mice, to avoid any contamination with extra γδ cells) and cultured for 9 days. (Note: the ratio 1 γδ : 100 DP is similar to the one found in a normal thymus.) As controls, we established RTOCs in which γδ thymocytes isolated from either WT or TCRβ−/− mice were cultured in the absence DP cells. After 9 days, γδ cells of each RTOC were re-sorted and analysed for ICER expression.

As seen in Figure 50, the TCRβ−/− γδ 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 γδ cells. Thus, DP cells seem to be the likely effectors of the trans-induction mechanism responsible for ICER expression in γδ and pro-T cells.

We therefore propose that β-selection is important for γδ cell differentiation since it provides a large pool of DP thymocytes that act on γδ thymocytes and induce them to express a subset of cellular genes, including γδ lineage marker ICER.
**Figure 50**: Induction of ICER expression in γδ thymocytes mediated by DP cells. Real-time PCR for ICER in γδ 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 αβ and γδ lineages; and αβ-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 αβ and γδ cells (section 1), the identification of pre-TCR responsive genes (section 2), and the studies on a candidate gene, ICER, in the context of αβ / γδ 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 αβ and γδ T cells

The precise mechanism of αβ/γδ 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 TCRγ, TCRδ and TCRβ chains begin to rearrange. Thymocytes that successfully rearrange TCRγ and TCRδ express a TCRγδ and can proceed to the γδ T cell lineage. Similarly, cells that successfully rearrange TCRβ express a pre-TCR (the TCRβ chain in association with the invariant pTα chain) allowing them to adopt an αβ T cell fate.

The extent to which either the pre-TCR or the TCRγδ directly influence lineage determination remains controversial. On the one hand, instructive models propose that the pre-TCR and TCRγδ provide distinct signals that directly determine the lineage fate adopted by a bipotential progenitor. Alternatively, selective models suggest that αβ/γδ lineage determination is made independently of TCR expression. Subsequent to this, αβ-committed progenitors must then express a pre-TCR, and γδ-committed progenitors must express a TCRγδ 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 TCRγδ(+)HSA(+) thymocytes from TCRα-deficient mice. Besides expressing the earliest markers known to identify αβ and γδ-committed cells, respectively, the genetically modified background of these cells eliminated transcriptional changes due to TCRαβ 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 TCRγδ(+)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 γδ and DP thymocytes: IL-2Rβ and IL-7Rα in γδ cells; CD4, CD8, TCRβ 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, γδ 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 αβ/γδ 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 αβ lineage). This was the case for CREM, NOR-1, Ly-49A and Sugano EST, among "γδ genes"; and "Novo-1" and "Novo-2", among "αβ genes" (Table 2).

CREM (ICER) was the gene selected for extensive studies in the context of the αβ/γδ 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 γδ - 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 αβ / γδ 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 γδ and αβ 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 γδ 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 γδ, but not αβ, 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^ and H-2D^.

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, γδ and pre-T cells (Figure 12).

Although the differential expression of Ly49A between the γδ and αβ lineages was extended to the protein level, only 5% of all γδ 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 γδ and αβ NK T cells might suggest a closer relationship between γδ T and NK cells than between αβ T and NK cells, possibly at the level of T/NK precursors. This is consistent with the expression, in γδ 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 TCRγδ (see Introduction, 3.1). It is currently being investigated whether NKG2D plays a co-receptor role in γδ cells, similar to that of CD8 in αβ lymphocytes. In that case, NKG2D would facilitate the interaction between TCRγδ and its non-classical MHC I ligand.

In our RDA analysis, we also came across other genes expressed selectively in both γδ 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 αβ 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(-) γδ 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 αβ / γδ lineage commitment.

The "Sugano EST", an expression sequence tag (from GenEMBL database, Al790276) initially obtained from a murine kidney cDNA library created by Dr. Sumio Sugano (1999), was detected by RT-PCR in γδ and NK thymocytes, but not in other haematopoietic lineages (Figure 12). Unlike Ly49A, the expression of Sugano EST in γδ 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-2Rβ. The existence of a IL2-Rβ/Sugano transcript in γδ thymocytes was confirmed by its amplification
(2.6 kb product) via a combination of primers specific for either Sugano EST or IL2-Rβ. Since the Sugano EST was located 3' of the IL-2Rβ sequence, this could represent an alternative transcript under the control of the IL-2Rβ promoter.

Previous data (Takeuchi et al., 1992; Tanaka et al., 1991) suggested that the IL-2Rβ protein is expressed in very few (1-4%) thymocytes, whereas it is more frequent among splenic CD8+ and NK lymphocytes. In mature lymphocytes, IL-2Rβ 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-2Rβ expression.

However, using monoclonal antibodies specific for IL2-Rβ, including a blocking mAb (clone TM-β1) that disrupts IL-2 signalling, we could not detect any signal in γδ thymocytes, implying that if the IL2-Rβ/Sugano transcript is translated into a protein, it is not involved in IL-2R signalling.

Thus, the biological relevance of the IL2-Rβ/Sugano transcript, in which the Sugano sequence represents an alternative 3'-end for the IL2-Rβ 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 γδ 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 → DN4 → 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 RDAnalysis of WT and TCRβ-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 → DN4 (→ 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-7Ra 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-7Ra is expressed in the DN4 population, and recently a role for IL-7/IL-7R signalling had been identified at the later DP → SP transition (Hare et al., 2000). Nevertheless, the precise role, if any, of IL-7/IL-7R signalling at the DN → 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-7Rα 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-7Rα chain.

As the induction of surface IL-7Rα protein expression was observed at just 6 hours after the cross-linking of CD3ε 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-7Rα 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-7Rα during FTOC of E16 RAG-1−/− thymocytes retrovirally transduced with a rearranged TCRβ gene (Vβ11-specific). The pMX-TCRβ 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 TCRβ showed a significant increase in IL-7Rα 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-7Rα 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-7Rα<sup>−/−</sup> 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-7Rα<sup>−/−</sup> or γc<sup>−/−</sup> 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-7Rα<sup>−/−</sup> or γc<sup>−/−</sup> 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-κB 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-κB, 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-κB 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-7Rα<sup>−/−</sup> mice the number of DN4 cells in the S/G<sub>2</sub>/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-7Rα 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-7Rα−/− mice or with wild type DN4 cells treated with ant-IL-7Rα/anti-γc 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-7Rα−/− 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-7Rα 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→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 γδ 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 γδ T cells but not in DP thymocytes of the αβ 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, Iγ, II, Iγγ) 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 γδ T cells, but not in DP thymocytes of the αβ T cell lineage. As well as being observed in γδ 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 αβ/γδ lineage divergence. However, our analysis of thymi from CREM/ICER−/− mice failed to demonstrate any perturbation in the development of either αβ or γδ 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 αβ T cell number caused
by a partial block in the DN to DP transition (Rudolph et al., 1998). In contrast, γδ 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 αβ lineage (Figures 28-29). Thus, since ICER expression is down-regulated at the DN → 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 αβ lineage. The existence of CRE sites in both the TCRβ promoter and the TCRα enhancer provided a potential mechanism for such an effect: ICER could repress TCRαβ expression, thus blocking αβ 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 αβ 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 αβ 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 $\rightarrow$ 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 TCRβ⁻/⁻ mice. These cells have characteristics of the αβ T cell lineage even though they have been shown to be dependent on the TCRγδ for development (Livak et al., 1997).

Importantly, the observation that a marker for the γδ lineage segregates independently of TCRγδ strongly supports a non-instructive, separate lineage model for αβ / γδ 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 αβ or the γδ TCR. Furthermore, the expression of CD8α and CD8β defines four major populations that are referred to as TCRγδ DN, TCRγδ CD8αα, TCRαβ CD8αα and TCRαβ CD8αβ (Shires et al., 2001). ICER expression in both the TCRγδ DN and TCRγδ CD8αα IEL populations was comparable to that observed in γδ T cell populations from the thymus, lymph nodes and spleen. However, although TCRαβ CD8αβ IELs did not express ICER, a result consistent with their αβ lineage classification, TCRαβ CD8αα IELs expressed a level of ICER similar to that seen in γδ T cells (Figure 35), suggesting that the TCRαβ CD8αα IEL subset is a “γδ-like” population.

It has previously been demonstrated that TCRαβ CD8αα IELs are not MHC class-I restricted and use TCRβ 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 αβ and γδ 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 αβ T cells and, on the other, both TCRγδ(+) T cells and non-conventional TCRαβ(+) T cells. In such a revised classification ICER expression would be a lineage marker for the latter cell type.

237
Since ICER marks mature γδ cells (in both the thymus and the periphery), we considered the hypothesis of it also segregating with DN precursors committed to the γδ lineage. In this context, it is interesting to note that TCRδ−/− pre-T cells expressed ICER at levels consistently higher than in WT DN populations (Figure 36). The TCRδ−/− DN4 subset, in particular, expressed abnormally high levels of ICER transcripts, evocative of "γδ-like" cells. Since these pre-T cells are incapable of expressing TCRγδ, which defines the γδ lineage, they could be accumulating γδ-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 CREM/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 γδ 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 αβ/γδ ratios, as expected as αβ lineage cells expand much more than γδ thymocytes.

Thus, ICER expression in DN thymocytes does not mark γδ-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 γδ lineage).

The extensive generation of αβ 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⁻/⁻, pTcr⁻/⁻, 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 RDAnalysis 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 RDAnalysis, 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 → 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-CD3ε 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-CD3ε.

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 → DN4 (→ DP) transition proceeds normally in its absence (Figure 43).

However, ICER-LacZ protein expression proved to be a good indicator of the DN3 → 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, icTCRβ(+) 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 \textit{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 icTCRβ(+), they should express high levels of surface TCRβ and, therefore, express less ICER mRNA (Figure 28). This is in line with the fact that αβ-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-Tα by newly synthesised TCRα (Trop et al., 2000). Theoretically, the new antigen receptor complex, TCRαβ, could actively down-regulate ICER in the αβ lineage. However, our data from TCRαβ−/− thymocytes showed this is not the case, as ICER (low) expression in DP cells is not altered by the absence of TCRαβ (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 \textit{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 αβ and γδ T cell differentiation

Unlike αβ cells, γδ 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 γδ 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 αβ lineage, in detriment of the γδ 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 (pTα−/−, TCRB−/−), not only in pre-T cells, but also in γδ thymocytes and splenocytes (Figure 47). Moreover, this effect was not restricted to ICER but was a specific phenomenon for a few "γδ genes" studied, including NOR-1 and myeloblastin.

This observation prompted us to revisit the much debated role of the pre-TCR in αβ / γδ 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 αβ cells, have increased absolute numbers of γδ cells. This would suggest that the pre-TCR not only is not necessary for γδ development, but also seems to divert cells from the αβ to the γδ pathway of differentiation.

On the other hand, detailed analysis of TCRβ 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 Vβ gene segments in γδ cells. Moreover, Wilson et al. have detected intracellular TCRβ protein in a significant (17%) subset of γδ cells, and showed that γδ cells of pre-TCR deficient mice proliferate less than wild type γδ cells (Wilson and MacDonald, 1998). They concluded there was a "limited role" for the pre-TCR in γδ cell physiology. This role would not be essential for γδ development since, as mentioned, pre-TCR-deficient mice have normal (or even increased) numbers of γδ cells.
In this context, it is interesting to note the results obtained by Bruno et al. (1999) regarding thresholds of pre-TCR expression and αβ / γδ lineage commitment. They observed that DN3 and even DN4 thymocytes that clearly expressed pre-TCR on the cell surface still generated γδ cells in RTOC. Only DN4 cells expressing the very highest pre-TCR levels seemed to be committed to the αβ lineage (Bruno et al., 1999).

According to those results, it is possible that a pre-TCR(+) subset of γδ thymocytes exists. The major obstacle to such an assumption is the fact that pTα protein has not been detected in the cytoplasm of γδ cells (von Boehmer et al., 1999).

However, even if such a subset exists, pre-TCR signalling cannot account for ICER expression in γδ cells: whereas only 17% of γδ thymocytes express TCRβ 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 TCRβ 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 γδ 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 TCRβ−/− γδ 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 TCRβ⁻/⁻ γδ cells have impaired proliferation and effector functions (when compared to WT γδ cells) - Figure 51.

**Figure 51**: Comparison of proliferation and effector functions of WT and TCRβ-deficient γδ cells. Proliferation was estimated by PI staining in (1) Wilson et al. (Wilson and MacDonald, 1998). Cytolytic activity of target (⁵¹Cr-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 γδ T cell differentiation was obtained from ICER's pattern of expression during murine development (Figure 48). ICER expression in DN2 and γδ 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 (pTα−/−, TCRβ−/−), 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 αβ lineage. Since the loss of ICER expression is not observed in TCRα-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, γδ thymocytes in particular, were subjected to the presence of a large excess of β-selected DP thymocytes (the effector cells). In more detail, 1.5x10^4 TCRβ−/− γδ thymocytes were mixed with 1.5x10^6 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 TCRβ⁺/⁻ γδ thymocytes that had been incubated with DP cells, when compared to "non-treated" TCRβ⁺/⁻ γδ 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 γδ and pro-T cells. Furthermore, they clarify the role of β-selection in γδ 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 γδ 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 γδ thymocytes and induce them to express a subset of genes, including the γδ lineage marker ICER. Some of these genes are most probably important for γδ cell physiology, as attested by the deficient proliferation of TCRβ⁺/⁻ γδ thymocytes (Wilson and MacDonald, 1998) and the impaired cytokine production and cytotoxicity of TCRβ⁺/⁻ γδ lymphocytes (Kohyama et al., 1999).

The nature of the proposed interaction between DP cells and γδ thymocytes is still unknown. In 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 γδ 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 αβ/γδ 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 γδ and DN2 thymocytes, but not in DP cells. Moreover, IL-7/IL-7R signalling is known to play a crucial role in γδ 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 γδ 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 \textit{in vivo} and that it is inducible in isolated DN3 thymocytes in cell suspension \textit{(in vitro)} via the MAPK pathway, suggest that it can be induced by a \textit{cis}-operating mechanism in pre-TCR(+) cells.

On the other hand, in pre-TCR(-) thymocytes, such as pro-T and γδ cells, ICER expression seems to be regulated by a \textit{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 γδ 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/γδ 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 γδ 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 γδ 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 TCRβ−/− γδ cells: reduced proliferation, impaired cytokine production and cytotoxicity.

It is most interesting that ICER, being a γδ lineage marker, depends on cells of the αβ 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 TCRβ−/− mice, traditionally taken as a model for γδ lymphocyte physiology, may be far from producing "normal" γδ 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 than 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 γδ lineage marker, the first to be described besides the TCRγδ itself. Expression studies provided evidence supporting a separate lineages model for αβ / γδ lineage divergence. They also suggested the existence of a mechanism by which αβ lineage cells (dependent on β-selection) influence the fate of γδ lineage thymocytes. This is a novel insight into lineage relationships during T cell development.
References


kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. Cell. 80:813-823.


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


258


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.


274


Levett, 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


286


287


