The role of Zap70 in naïve T cell homeostasis

Ina Corinne Dorothea Schim van der Loeff

UCL

A thesis presented for the degree of Doctor of Philosophy, 2013
Declaration

I, Ina Schim van der Loeff confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Ina Schim van der Loeff

*Contributed equally (see Appendix).
Acknowledgements

To start, I would like to thank my supervisor Dr Benedict Seddon for giving me the opportunity to work on this project. This paragraph will not do justice to his support, advice and clarity of vision during discussions about experiments, data and the writing of thesis. I would also like to thank the members of my thesis committee Dr Andreas Wack, Dr Victor Tybulewicz and Dr Pavel Tolar for their advice and comments throughout my PhD. I would also like to thank the UCL MB PhD programme for allowing me to take time out of my medical training to pursue full-time scientific research.

Throughout my PhD I also benefited from working alongside Dr Charles Sinclair who taught me numerous experimental techniques and whose almost encyclopaedic knowledge of immunology I frequently consulted during my PhD. I would also like to thank Sim Tung and Dr Iren Bains for being great neighbours, as well as Dr Thea Hogan, Dr Jie Yang, Dr Ana Silva Daniel Marshall and Louise Webb who I have happily shared lab space with for the last 3 years.

Thanks also go to Victoria, Olivia, Pippa, Charlie and Louise from the various immunology divisions but especially to Elizabeth Natkanski for her friendship during many shared lunch and coffee breaks, and drinks in the NIMR bar.

Finally, I would like to thank my parents and 3 sisters, Lily, Sanne and Wendela for their love and support, and my mum, for proofreading this thesis. And to Dr Luke La Hausse de Lalouvière for your love, encouragement and insight, for proofreading this thesis and for always challenging all my ideas – scientific and otherwise.
Abstract

TCR signalling is crucial to both T cell development and naïve T cell homeostasis. Naïve T cell survival in the periphery is thought to depend on both cytokine signalling and constitutive TCR signalling. The nature of this TCR dependent survival signal remains controversial. The tyrosine kinase, Zap70, is essential for TCR signalling. The aim of this project is to investigate the role of Zap70 in the transduction of survival signals in naïve T cells. Using mice that conditionally express Zap70 by means of a tetracycline-inducible system, we found that Zap70 is absolutely required for the maintenance of naïve T cells in the periphery. Loss of Zap70 resulted in a dramatic and rapid reduction in mature naïve CD8 T cells in the blood and peripheral organs consistent with a naïve T cell survival defect. This survival defect could not be accounted for by cell-intrinsic differences in IL-7Rα or Bcl-2 expression. Analysis of T cell survival in vitro revealed no differences in responses to IL-7 between F5 T cells with or without Zap70. Survival in vitro was found to be enhanced by the presence of CD11c+ enriched splenocytes but not T or B cells. Survival of F5 T cells in these cultures was dependent on Zap70 expression and also required intact MEK signalling. In addition, we also tested the ability of previously described Zap70 mutants, Zap70^{SKG} and Zap70^{YYAA}, to transduce homeostatic TCR survival signals in vivo. Both mutants were unable to support the development, survival or antigen-induced expansion of F5 T cells. Additionally, we found evidence that Zap70^{YYAA} had a dominant negative effect on T cell development and reconstitution in F5 TetZap70 mice. In conclusion, we find that Zap70 is
essential for transmission of signals required for naive T cell survival and requires both full expression and functionality of Zap70.
# Table of contents

Declaration .............................................................................................................. 1
Publications arising from this thesis ................................................................. 2
Acknowledgements ............................................................................................ 3
Abstract ............................................................................................................... 4
Table of contents ............................................................................................... 6

List of figures ..................................................................................................... 10
List of tables ....................................................................................................... 12
List of abbreviations ......................................................................................... 13

**Chapter 1** Introduction .................................................................................. 23

1.1 The immune system ...................................................................................... 23
   1.1.1 Innate immunity .................................................................................. 24
   1.1.2 Adaptive immunity ............................................................................ 26

1.2 Signalling pathways involved in T cell homeostasis .................................... 29
   1.2.1 IL-7Rα signalling ............................................................................. 29
   1.2.2 Regulation of IL-7Rα expression ....................................................... 32
   1.2.3 TCR signalling .................................................................................. 34
      1.2.3.1 The regulation of Lck activity by CD45 and Csk ....................... 35
      1.2.3.2 The initiation of Zap70 signalling in T cells ............................... 37
      1.2.3.3 The roles of Y292, Y315 and Y319 during Zap70 signalling .... 39
      1.2.3.4 Downstream targets of Zap70 ................................................... 41
      1.2.3.5 Integrin signalling ................................................................... 45

1.3 T cell development ....................................................................................... 47
   1.3.1 Double negative stages of T cell development ................................. 47
   1.3.2 Positive and negative selection ......................................................... 50
   1.3.3 CD8/CD4 lineage decision ............................................................... 54
   1.3.4 Development of γδ T cells, T<sub>reg</sub> and NK T cells .................... 56
   1.3.5 Single positive thymocyte maturation and thymic egress ............... 59

1.4 Peripheral αβ T cell biology ....................................................................... 60
   1.4.1 Recent thymic emigrants ................................................................. 60
   1.4.2 Naïve CD4 and CD8 T cells ............................................................... 62
   1.4.3 T cell activation and proliferation .................................................... 64
   1.4.4 Effector αβ T cells ............................................................................ 67
   1.4.5 Memory T cells ................................................................................ 69

1.5 Peripheral αβ T cell homeostasis ............................................................... 70
   1.5.1 Homeostatic and lymphopaenia-induced proliferation .................... 71
   1.5.2 Regulation of apoptosis .................................................................... 72
   1.5.3 Cytokine regulation of naïve T cell survival .................................... 76
   1.5.4 Cytokine regulation of memory T cell survival ............................... 78
   1.5.5 TCR signals that regulate naïve T cell survival ............................... 79
   1.5.6 Cross-regulation of TCR and IL-7Rα signalling ............................. 83

1.6 Thesis aims ................................................................................................. 85
Chapter 2 Materials and methods

2.1 Mice

2.1.1 Experimental mice used in this study

2.1.2 Generation of F5 TetZap70, F5 TetZap70 Zap70\(^{Yyaa/}\) and F5 TetZap70 Zap70\(^{Skg/-}\) mice

2.1.3 Radiation bone marrow chimeras

2.1.3.1 Donor BM isolation

2.1.3.2 Host irradiation and bone marrow reconstitution

2.2 Media

2.3 Preparation of single cell suspensions

2.3.1 Thymus, lymph nodes and spleen

2.3.2 Peripheral blood

2.3.3 Splenocytes for CD11c\(^+\) enrichment

2.4 Flow cytometry

2.4.1 Antibodies and reagents used for flow cytometry

2.4.2 Surface staining

2.4.2.1 Primary staining and flow cytometry

2.4.2.2 Recombinant annexin-V staining

2.4.2.3 Intracellular flow cytometry

2.4.3 Calculation of absolute cell numbers and number of precursors

2.4.3.1 Calculation of absolute cell numbers

2.4.3.2 Calculation of number of precursors

2.5 In vivo experiments

2.5.1 In vivo T cell survival in intact chimeras and mice

2.5.2 CTV staining before adoptive transfer experiments

2.5.3 Adoptive transfer experiments – homing, survival and proliferation

2.6 Cell purification by FACS

2.7 In vitro experiments

2.7.1 In vitro T cell survival

2.7.2 CD11c\(^+\) enrichment

2.7.3 MACS\(^\circlearrowleft\) purification

2.7.4 Dynabead\(^\circledR\) purification

2.8 mRNAseq

2.8.1 RNA isolation

2.8.2 Sequencing

2.8.3 Quantification of gene expression

2.9 Calculations and statistical testing
Chapter 3  The role of Zap70 in naïve T cell survival in vivo .......................... 111

3.1  Introduction ........................................................................................................... 111
3.2  Results .................................................................................................................. 113
  3.2.1  Reconstitution of T cell development in F5 TetZap70 mice following transgene induction .................................................................................................................. 113
  3.2.2  Reconstitution of the peripheral T cell compartment in F5 TetZap70 mice following transgene induction ............................................................................................................. 117
  3.2.3  Kinetics of transgene ablation following dox-withdrawal ............................. 119
  3.2.4  F5 TetZap70 fail to persist in the absence of Zap70 induction ................. 119
  3.2.5  F5 TetZap70<sup>OFF</sup> T cells do not survive upon adoptive transfer ...... 121
  3.2.6  F5 TetZap70<sup>ON</sup> thymocytes are outcompeted in mixed F5 Rag<sup>1</sup>−/− F5 TetZap70 chimeras ................................................................. 122
  3.2.7  Periphery of F5 TetZap70<sup>ON</sup> is enriched for RTEs ....................... 123
  3.2.8  In vivo homing of F5 TetZap70<sup>OFF</sup> T cells to LN and spleen ............ 124
  3.2.9  F5 TetZap70<sup>OFF</sup> T cells survive but do not proliferate in vivo .... 125
  3.2.10  Bmf expression is upregulated in F5 TetZap70<sup>OFF</sup> T cells ............. 126
3.3  Discussion ............................................................................................................. 127

Chapter 4  The role of Zap70 in naïve T cell survival in vitro .......................... 158

4.1  Introduction ........................................................................................................... 158
4.2  Results .................................................................................................................. 160
  4.2.1  Sing in F5 TetZap70 mice ........................................................................... 160
  4.2.2  Zap70-deficiency does not affect the IL-7-induced survival of naïve CD8<sup>T</sup> T cells at saturating concentrations of IL-7 ..................................................... 162
  4.2.3  B6 Rag<sup>1</sup>−/− splenocytes enriched for CD11c<sup>+</sup> cells promote Zap70-dependent T cell survival .............................................................................................................. 163
  4.2.4  FACS-purified cDCs and pDCs alone do not promote substantial naïve T cell survival in vitro ............................................................. 164
  4.2.5  The survival signal provided by CD11c<sup>+</sup> splenocytes is β2m- and IL-7-independent ............................................................. 165
  4.2.6  The T cell survival signal provided by CD11c<sup>+</sup> splenocytes is impaired by MEK inhibition ..................................................... 166
4.3  Discussion ............................................................................................................. 167

Chapter 5  The importance of two Zap70 domains for the transduction of TCR survival signals ......................................................... 189

5.1  Introduction ........................................................................................................... 189
5.2  Results .................................................................................................................. 191
  5.2.1  F5 T cell development is blocked in F5 TetZap70<sup>unind</sup> Zap70<sup>Yyaa</sup>−/− and F5 TetZap70<sup>unind</sup> Zap70<sup>Skg</sup>−/− chimeras ................................................................. 191
  5.2.2  Peripheral reconstitution of constitutively dox-fed F5 TetZap70 Zap70<sup>Yyaa</sup>−/− and F5 TetZap70 Zap70<sup>Skg</sup>−/− chimeras .............................................. 194
  5.2.3  Regulation of Zap70 abundance in F5 TetZap70 Zap70<sup>Yyaa</sup>−/− and F5 TetZap70 Zap70<sup>Skg</sup>−/− chimeras ................................................................. 194
  5.2.4  Zap70<sup>YYAA</sup> and Zap70<sup>SKG</sup> cannot substitute for Zap70<sup>WT</sup> to promote Zap70-dependent survival of F5 T cells in F5 TetZap70 chimeras ........ 196
5.3  Discussion ............................................................................................................. 198
Chapter 6  The role of TCR signalling during a cognate immune response 210

6.1 Introduction .............................................................................................................. 210
6.2 Results ....................................................................................................................... 212
  6.2.1 F5 TetZap70\textsuperscript{ON} T cells expand in response to cognate antigen in B6
       \textit{Rag1}\textsuperscript{-/-} mice \textit{in vivo} .................................................................. 212
  6.2.2 F5 TetZap70\textsuperscript{ON} T cells expand in response to cognate antigen in
       C57Bl/6 mice \textit{in vivo} .................................................................................. 213
  6.2.3 Neither Zap70\textsuperscript{Yyaa} or Zap70\textsuperscript{Skg} can substitute for Zap70\textsuperscript{Wt} in F5
       TetZap70 cognate response ............................................................................ 214
  6.2.4 TCR signalling is required until 96-144 hours after influenza
       challenge for optimal CD8 T cell expansion ................................................. 216
6.3 Discussion ................................................................................................................. 217

Chapter 7  Final discussion ............................................................................................ 233

7.1 The role Zap70 signalling in T cell homeostasis .................................................... 234
7.2 The source and downstream targets of a Zap70-dependent survival
    signal for naïve F5 T cells ..................................................................................... 238
7.3 The TCR signalling requirements during antigen-induced T cell
    activation and proliferation ................................................................................... 240
7.4 Clinical perspectives ............................................................................................... 242

References .................................................................................................................... 244

Appendix ....................................................................................................................... 283
### List of figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>IL-7Rα signalling</td>
<td>86</td>
</tr>
<tr>
<td>1.2</td>
<td>TCR signalling</td>
<td>88</td>
</tr>
<tr>
<td>1.3</td>
<td>Structure of murine Zap70</td>
<td>90</td>
</tr>
<tr>
<td>1.4</td>
<td>The regulation of apoptosis</td>
<td>91</td>
</tr>
<tr>
<td>2.1</td>
<td>Dox-inducible expression of Zap70 in F5 TetZap70 mice</td>
<td>110</td>
</tr>
<tr>
<td>3.1</td>
<td>Reconstitution of T cell development in F5 TetZap70 mice following transgene induction</td>
<td>138</td>
</tr>
<tr>
<td>3.2</td>
<td>Reconstitution of the peripheral T cell compartment in F5 TetZap70 mice following transgene induction</td>
<td>140</td>
</tr>
<tr>
<td>3.3</td>
<td>Kinetics of transgene ablation following dox-withdrawal</td>
<td>142</td>
</tr>
<tr>
<td>3.4</td>
<td>F5 TetZap70 fail to persist in the absence of Zap70 induction</td>
<td>144</td>
</tr>
<tr>
<td>3.5</td>
<td>F5 TetZap70(^{OFF}) do not survive upon adoptive transfer</td>
<td>146</td>
</tr>
<tr>
<td>3.6</td>
<td>F5 TetZap70(^{ON}) thymocytes are outcompeted in mixed F5 (Rag1^{−/−}) F5 TetZap70 chimeras</td>
<td>148</td>
</tr>
<tr>
<td>3.7</td>
<td>Periphery of F5 TetZap70(^{ON}) is enriched for recent thymic emigrants</td>
<td>150</td>
</tr>
<tr>
<td>3.8</td>
<td>(In vivo) homing of F5 TetZap70(^{OFF}) T cells to LN and spleen</td>
<td>152</td>
</tr>
<tr>
<td>3.9</td>
<td>F5 TetZap70(^{OFF}) T cells survive but do not proliferate (in vivo)</td>
<td>154</td>
</tr>
<tr>
<td>3.10</td>
<td>Bmf expression is upregulated in F5 TetZap70(^{OFF}) T cells</td>
<td>156</td>
</tr>
<tr>
<td>4.1</td>
<td>Intact IL-7R signalling in F5 TetZap70 mice</td>
<td>177</td>
</tr>
<tr>
<td>4.2</td>
<td>Zap70-deficiency does not affect the IL-7-induced survival of naïve CD8 T cells at saturating concentrations of IL-7</td>
<td>179</td>
</tr>
<tr>
<td>4.3</td>
<td>B6 (Rag1^{−/−}) splenocytes enriched for CD11c(^{+}) cells promote Zap70-dependent T cell survival</td>
<td>181</td>
</tr>
<tr>
<td>4.4</td>
<td>The T cell survival signal provided by CD11c(^{+}) splenocytes is β2m- and IL-7-independent</td>
<td>185</td>
</tr>
<tr>
<td>4.5</td>
<td>The T cell survival signal provided by CD11c(^{+}) splenocytes is impaired by MEK inhibition</td>
<td>187</td>
</tr>
</tbody>
</table>
Figure 5.1 F5 T cell development is blocked in F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Yyaa/} and F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Skg/} chimeras ................................................................. 202

Figure 5.2 Peripheral reconstitution of constitutively dox-fed F5 TetZap70 Zap70\textsuperscript{Yyaa/} and F5 TetZap70 Zap70\textsuperscript{Skg/} chimeras ................................................................. 204

Figure 5.3 Regulation of Zap70 abundance in F5 TetZap70 Zap70\textsuperscript{Yyaa/} and F5 TetZap70 Zap70\textsuperscript{Skg/} chimeras ................................................................. 206

Figure 5.4 Zap70\textsuperscript{YYAA} and Zap70\textsuperscript{SKG} cannot substitute for Zap70\textsuperscript{WT} to promote Zap70-dependent survival of F5 T cells in F5 TetZap70 chimeras ................................................................. 208

Figure 6.1 F5 TetZap70\textsuperscript{ON} T cells expand in response to cognate antigen B6 Rag1\textsuperscript{-/-} mice \textit{in vivo} ........................................................................................................................................ 225

Figure 6.2 F5 TetZap70\textsuperscript{ON} T cells expand in response to cognate antigen in C57Bl/6 mice \textit{in vivo} ........................................................................................................................................ 227

Figure 6.3 Neither Zap70\textsuperscript{Yyaa} or Zap70\textsuperscript{Skg} can substitute for Zap70\textsuperscript{WT} in F5 TetZap70 cognate response ........................................................................................................ 229

Figure 6.4 TCR signalling is required until 96-144 hours after influenza challenge for optimal CD8 T cell expansion ........................................................................................................ 231
List of tables

Table 2.1 List of mice used in this study ................................................................. 93
Table 2.2 List of primary antibodies used in this study ................................. 98
Table 2.3 List of recombinant annexin-V conjugates used in this study ....... 100
Table 2.4 List of intracellular antibodies used in this study ............................ 100
List of abbreviations

Abbreviations used in this thesis:

A  Alanine
A1  Bcl-2-related protein A1
Adap  Adhesion and degranulation-promoting adaptor protein
Aire  Autoimmune regulator
Akt/PKB  Protein kinase B
AP1/c-Jun  Jun oncogene
Apaf1  Apoptosis protease activating factor 1
APC(s)  Antigen presenting cell(s)
B-CLL  B cell chronic lymphocytic leukemia
Bad  Bcl-2 associated antagonist of cell death
BAFF  B cell activating factor
BAFFR  B cell activating factor receptor
Bak  Bcl-2 antagonist/killer
Bax  Bcl-2-associated X protein
Bcl-2  B cell leukaemia/lymphoma 2
Bcl-xL  B cell leukaemia/lymphoma x
BCR  B cell receptor
BH3  Bcl-2 homology domain 3
Bid  BH3 interacting domain death agonist
Bim  Bcl-2 interacting mediator of cell death
BM  Bone marrow
Bmf  Bcl-2 modifying factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>c-Cbl</td>
<td>E3 ubiquitin-protein ligase Cbl</td>
</tr>
<tr>
<td>c-kit</td>
<td>Tyrosine protein kinase kit</td>
</tr>
<tr>
<td>C1q</td>
<td>Complement component 1, subcomponent q</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C motif chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C motif chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDC(s)</td>
<td>Conventional dendritic cell(s)</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cell division cycle 42</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-phosphate-guanine</td>
</tr>
<tr>
<td>CR3</td>
<td>Complement receptor 3</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>Csk</td>
<td>C-terminal Src kinase</td>
</tr>
<tr>
<td>cSMAC</td>
<td>Central supramolecular activation cluster</td>
</tr>
<tr>
<td>cTEC(s)</td>
<td>Cortical thymic epithelial cell(s)</td>
</tr>
<tr>
<td>CTV</td>
<td>Cell trace violet</td>
</tr>
<tr>
<td>CXCL</td>
<td>C-X-C motif chemokine ligand</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DC(s)</td>
<td>Dendritic cell(s)</td>
</tr>
<tr>
<td>DISC</td>
<td>Death inducing signalling complex</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>F</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>F5 TetZap70</td>
<td>F5 (Rag^{1-/-}) (Zap70^{Tre})rtTA.C(^{huCD2}) Zap70(^{-/-})</td>
</tr>
<tr>
<td>F5 TetZap70</td>
<td>F5 (Rag^{1-/-}) (Zap70^{Tre})rtTA.C(^{huCD2}) Zap70(^{Skg/-})</td>
</tr>
<tr>
<td>F5 TetZap70</td>
<td>F5 (Rag^{1-/-}) (Zap70^{Tre})rtTA.C(^{huCD2}) Zap70(^{Yyaa/-})</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FASL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallisable</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>Foxo</td>
<td>Forkhead box o</td>
</tr>
<tr>
<td>Foxp</td>
<td>Forkhead box p</td>
</tr>
<tr>
<td>FRC(s)</td>
<td>Follicular reticular cell(s)</td>
</tr>
<tr>
<td>Fyn</td>
<td>Fyn proto-oncogene</td>
</tr>
<tr>
<td>Gads</td>
<td>Grb2-related adaptor downstream of Shc</td>
</tr>
<tr>
<td>Gata3</td>
<td>GATA-binding protein 3</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Glucose transporter 1</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>hCD2</td>
<td>Human CD2</td>
</tr>
<tr>
<td>Hepes</td>
<td>Hydroxyethyl piperazineethanesulfonic acid</td>
</tr>
</tbody>
</table>
HEV(s)  High endothelial venule(s)
Hpk1  Haematopoietic progenitor kinase 1
HSA/CD24  Heat stable antigen
I-Aα and β  MHCII Aα and β alleles
I-Eα and β  MHCII Aα and β alleles
ICAM1  Intercellular adhesion molecule 1
IFN  Interferon
IKK  IkB kinase
IL  Interleukin
IL-15Ra /CD122  Interleukin 15 receptor α
IL-15Rβ  Interleukin 15 receptor β
IL-7Rα  Interleukin 7 receptor α
IMDM  Iscove’s modified Dulbecco’s medium
IP₃  Inositol triphosphate
IRAK  Interleukin-1 receptor-associated kinase
IRES  Internal ribosome entry site
ITAM(s)  Immunotyrosine activation motif(s)
Itk  IL-2 inducible T cell kinase
IV  Intravenous(ly)
IκB  Inhibitor of κB
Jak  Janus kinase
Jnk  c-jun N-terminal kinase
Klf2  Krueppel-like factor 2
Klrg1  Killer cell lectin-like receptor subfamily G 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>Ligand</td>
</tr>
<tr>
<td>LAT</td>
<td>Linker of activated T cells</td>
</tr>
<tr>
<td>Lck</td>
<td>Lymphocyte-specific protein tyrosine kinase</td>
</tr>
<tr>
<td>LFA1</td>
<td>Lymphocyte function associated antigen 1</td>
</tr>
<tr>
<td>LIP</td>
<td>Lymphopaenia-induced proliferation</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKK/MEK</td>
<td>MEK kinase</td>
</tr>
<tr>
<td>K</td>
<td>MEK kinase</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Myeloid cell leukemia sequence 1</td>
</tr>
<tr>
<td>MEK/MAPKK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilisation</td>
</tr>
<tr>
<td>mPDCA1</td>
<td>Mouse plasmacytoid dendritic cell antigen 1</td>
</tr>
<tr>
<td>mTEC(s)</td>
<td>Medullary thymic epithelial cell(s)</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>Nck</td>
<td>Non-catalytic region of tyrosine kinase adaptor protein</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NIMR</td>
<td>National Institute for Medical Research</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>Noxa</td>
<td>Phorbol-12-myristate-13-acetate-(PMA)-induced protein 1</td>
</tr>
<tr>
<td>Ova</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>p27&lt;sup&gt;Rip1&lt;/sup&gt;</td>
<td>Cyclin-dependent kinase inhibitor 1B</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>p38/Crk</td>
<td>Crk proto-oncogene</td>
</tr>
<tr>
<td>p85</td>
<td>Phosphatidylinositol 3-kinase regulatory subunit</td>
</tr>
<tr>
<td>Pak1</td>
<td>Cdc42/Rac-activated kinase 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>pDC(s)</td>
<td>Plasmacytoid dendritic cell(s)</td>
</tr>
<tr>
<td>Pdk1</td>
<td>Pyruvate dehydrogenase kinase 1</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Phosphatidylinositol 3,4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC&lt;sub&gt;θ&lt;/sub&gt;</td>
<td>Protein kinase C θ</td>
</tr>
<tr>
<td>PLCγ</td>
<td>Phospholipase C γ</td>
</tr>
<tr>
<td>pMHC</td>
<td>Antigenic peptide in the context of MHC</td>
</tr>
<tr>
<td>pSMAC</td>
<td>Peripheral supramolecular activation cluster</td>
</tr>
<tr>
<td>PTPn22</td>
<td>Protein tyrosine phosphatase non-receptor type 22</td>
</tr>
<tr>
<td>Qa2</td>
<td>Qa lymphocyte antigen 2 region</td>
</tr>
<tr>
<td>Rac1</td>
<td>Ras-related C3 botulinum substrate 1</td>
</tr>
<tr>
<td>Raf1</td>
<td>Raf proto-oncogene 1</td>
</tr>
<tr>
<td>Rag1/2</td>
<td>Recombination activation gene 1/2</td>
</tr>
<tr>
<td>Rap1</td>
<td>Ras-related protein 1</td>
</tr>
<tr>
<td>Ras</td>
<td>Ras proto-oncogene</td>
</tr>
<tr>
<td>RhoH</td>
<td>Ras homology gene family member H</td>
</tr>
<tr>
<td>RORγT</td>
<td>RAR-related orphan receptor γT</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute 1640</td>
</tr>
<tr>
<td>RSS(s)</td>
<td>Recombination signal sequence(s)</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTE(s)</td>
<td>Recent thymic emigrant(s)</td>
</tr>
<tr>
<td>rtTA.C</td>
<td>Reverse tetracyclin transactivator</td>
</tr>
<tr>
<td>Runx</td>
<td>Runt-related transcription factor</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>S1P₁</td>
<td>Sphingosine-1-phosphate receptor 1</td>
</tr>
<tr>
<td>Sca-1</td>
<td>Stem cell antigen 1</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology domain 2</td>
</tr>
<tr>
<td>Shp1/Ptpn6</td>
<td>Protein tyrosine phosphatase non-receptor type 6</td>
</tr>
<tr>
<td>SLO(s)</td>
<td>Secondary lymphoid organ(s)</td>
</tr>
<tr>
<td>S Hip76</td>
<td>SH2 domain containing leukocyte protein of 76kDa</td>
</tr>
<tr>
<td>Sos1</td>
<td>Son of sevenless 1</td>
</tr>
<tr>
<td>Sox</td>
<td>Sry-box containing gene</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive</td>
</tr>
<tr>
<td>spMHC</td>
<td>Self-peptide in the context of MHC</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>T-ALL</td>
<td>T cell acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>Tak1</td>
<td>TGFβ activated kinase 1</td>
</tr>
<tr>
<td>T&lt;sub&gt;CM&lt;/sub&gt;</td>
<td>Central memory T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>T&lt;sub&gt;EM&lt;/sub&gt;</td>
<td>Effector memory T cell</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TetZap70</td>
<td>$\text{Tet70}^{\text{Cre}} \text{rtTA.C}^{\text{huCD2}} \text{Zap70}^{\text{c}}$</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>Th-POK</td>
<td>T-helper-inducing POZ/Kruppel-like</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMRE</td>
<td>Tetramethylrhodamine ethyl ester</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>TRA(s)</td>
<td>Tissue-restricted antigen(s)</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor-associated factor 6</td>
</tr>
<tr>
<td>TRE</td>
<td>Tetracycline-response element</td>
</tr>
<tr>
<td>T_{reg}</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>Vav1</td>
<td>Vav1 proto-oncogene 1</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Zap70</td>
<td>$\zeta$-associated protein of 70kDa</td>
</tr>
<tr>
<td>β2m</td>
<td>$\beta_2$-microglobulin</td>
</tr>
<tr>
<td>Y_{c}</td>
<td>Common $\gamma$</td>
</tr>
</tbody>
</table>
Fluorescent antibody conjugates used in this thesis:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>APC eF780</td>
<td>Allophycocyanin E-Fluor 780</td>
</tr>
<tr>
<td>eF450</td>
<td>E-Fluor 450</td>
</tr>
<tr>
<td>eF780</td>
<td>E-Fluor 780</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PE Cy5</td>
<td>Phycoerythrin cyanine 5</td>
</tr>
<tr>
<td>PE Cy7</td>
<td>Phycoerythrin cyanine 7</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin-chlorophyll protein</td>
</tr>
<tr>
<td>PE TR</td>
<td>PE Texas red</td>
</tr>
<tr>
<td>PO</td>
<td>Pacific orange</td>
</tr>
</tbody>
</table>
Units used in this thesis:

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Unit Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>+/-</td>
<td>Heterozygous gene knockout</td>
</tr>
<tr>
<td>-/-</td>
<td>Homozygous gene knockout</td>
</tr>
<tr>
<td>d</td>
<td>Days</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>sec</td>
<td>Second</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>nRPKM</td>
<td>Normalised reads/kilobase of exon/ x10^6 mapped reads</td>
</tr>
<tr>
<td>pH</td>
<td>[H]</td>
</tr>
<tr>
<td>g</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometre</td>
</tr>
</tbody>
</table>
Chapter 1  Introduction

1.1  The immune system

Integrated and overlapping cell-mediated and molecular responses protect hosts from infection by pathogens and make up the immune system. To mount a successful immune response an organism must become aware that infection by a pathogen has taken place. This recognition is followed by the control or elimination of the pathogen by effector immune cells and molecules. Finally, the cellular and humoral responses to a pathogen need to be integrated and regulated to avoid damage to the host. The first response to infection is provided by the innate immune system, which is present in some form in all animals and plants – even bacteria have antiviral mechanisms to prevent infection by bacteriophages (reviewed in Labrie et al., 2010). The adaptive immune response develops after the innate immune response is unable to contain an infection and depends on the antigen-specific recognition of pathogens by lymphocytes. Adaptive immune cells also have the unique capacity to persist after an initial infection as memory cells, which mediate long-term antigen-specific protection against re-infection. Although adaptive immunity was thought to have arisen in jawed vertebrates recent studies show that many invertebrate immune systems possess characteristics similar to that of adaptive immunity, including antigen-specificity and immunological memory. This suggests that the innate and adaptive immune responses are closely linked and might have co-evolved (reviewed in Boehm, 2011).
1.1.1 Innate immunity

The innate immune system provides the first line of largely non-antigen-specific defence against infection by a pathogen. This is mediated in the first instance by epithelial surfaces, which provide a mechanical barrier as well as chemical and microbiological barriers to infecting viruses, bacteria, fungi and parasites. These barriers include the skin, gut, respiratory tract and eyes, nose and oral cavity. The tight junctions joining epithelial cells in these organs, and the mucous and tear production in the lungs and oral cavity seal the host off from its external environment and prevent adherence of pathogens to epithelial surfaces. Furthermore, most epithelial surfaces are colonised with non-pathogenic commensal bacteria, which compete with pathogenic bacteria for nutrients and attachment to epithelial cells. Epithelial cells and phagocytes also produce an array of anti-microbial enzymes and peptides that either kill pathogens or limit their growth. The anti-microbial enzymes lysozyme and phospholipase A\textsubscript{2} damage bacterial cell walls and are secreted into tears and saliva but are also produced by the gut epithelium. Epithelial cells and innate immune cells like neutrophils and mast cells also secrete antimicrobial peptides including α- and β-defensins, cathelicidins and lectins (reviewed in Gallo and Hooper, 2012). Defensins and cathelicidins bind to pathogen membranes through electrostatic interactions ultimately disrupting the membrane and leading to pathogen lysis (reviewed in Gallo and Hooper, 2012). C-type lectins depend on peptidoglycan recognition to kill pathogens although whether this killing is the direct result of cell wall disruption or an enzymatic attack remains unclear (reviewed in Gallo and Hooper, 2012).
Following breach of the epithelial surface pathogens next encounter the complement system, which cooperates with innate immune cells to mark pathogens for phagocytosis or cell lysis (Gros et al., 2008). Complement activation can be mediated by antibodies, C-reactive protein (CRP) and complement component 1 subcomponent q (C1q) (classical pathway), lectins (lectin pathway) or by the spontaneous hydrolysis of C3 to C3b known as tick-over (alternative pathway). Complement activation results in the initiation of a proteolytic cascade that generates complement fragments, which mediate the lysis, agglutination and opsonisation of pathogens and also induce a local inflammatory response. Epithelial damage by pathogens alone can induce local inflammation characterised by the release of prostaglandins and leukotrienes from endothelial cells and innate immune cells like polymorphonuclear leukocytes, macrophages and mast cells (reviewed in Funk, 2001). Acute inflammation is also characterised by vasodilation and increased vascular permeability promoting the transmigration and recruitment of innate and later adaptive immune cells. Innate immune cells express a number of invariant receptors that recognise specific pathogen-associated molecular patterns and can induce signalling via myeloid differentiation primary response gene 88 (MyD88), interleukin-1 receptor-associated kinase (IRAK), the adaptor TNF receptor-associated factor 6 (TRAF6) and the MAPK kinase (MEK) kinase (MAPKKK) transforming growth factor β (TGFβ) activated kinase 1 (Tak1) ultimately activating Jun oncogene (AP1) and the inhibitor of κB (IκB) kinase (IKK) complex (reviewed in Akira and Takeda, 2004). Phosphorylation and degradation of IκB and activation of nuclear factor κB (NFκB) signalling by pattern recognition receptors stimulate the transcription and expression of pro-
inflammatory genes like tumour necrosis factor α (TNFα), IL-1β, IL-6, IL-12p40, interferon-(IFN)-inducible genes and co-stimulatory molecules like cluster of differentiation (CD) 40, CD80 and CD86 (reviewed in Akira and Takeda, 2004). Innate immune cell pattern recognition receptors recognise a broad range of common pathogen motifs including fungal zymosan (complement receptor 3) (CR3), mannose or fucose on pathogen-infected cells (mannose receptor), bacterial peptidoglycan (Toll-like receptor 2, TLR2), lipopolysaccharide (LPS) (TLR4) and bacterial unmethylated cytosine-phosphate-guanine (CpG) dinucleotides (TLR9) (reviewed in Akira et al., 2001 and in Gordon, 2002). In addition to these pattern recognition receptors, scavenger receptors on macrophages, lectin receptors on macrophages and dendritic cells (DCs), and complement receptors on macrophages and neutrophils, promote the phagocytosis of both pathogens and senescent, apoptotic, and necrotic cells, which then allows antigen presenting cells (APCs) like macrophages and DCs to process and present potential antigens to T and B lymphocytes (reviewed in Gordon, 2002). Innate immune cells thus mediate the detection and clearance of broad groups of pathogens and can recruit and activate adaptive immune cells, which can mount highly antigen-specific cell-mediated and humoral responses.

1.1.2 Adaptive immunity

The adaptive immune response depends on the recognition of specific antigens from bacteria, viruses or parasites by specialised antigen receptors on B and T lymphocytes and becomes important when the innate immune response fails to completely eliminate an infection. Recirculating B and T lymphocytes first
encounter antigen on activated APCs in specialised lymphoid tissues, which in addition to co-stimulatory signals (reviewed in Lenschow et al., 1996 and in Acuto and Michel, 2003) induce antigen-specific lymphocytes to proliferate and differentiate into effector and memory T and B cells. Both B and T cells are of lymphoid origin and develop in the bone marrow (BM) and thymus, respectively. B cells mediate the humoral response to pathogens by differentiating into antibody-producing plasma cells while effector T cells can differentiate into cytotoxic T cells in response to CD8 and T cell receptor (TCR) interactions with antigenic peptide in the context of major histocompatibility complex I (pMHC\textsubscript{I}), or various types of helper T cell in response in response to CD4 and TCR interactions with pMHC\textsubscript{II}. Fundamental to the specific recognition of pathogens by T and B lymphocytes is the expression of polyclonal antigen-specific receptors which can recognise a variety of antigens. B cells express membrane-bound immunoglobulin on their surface, known as the B cell receptor (BCR), and immunoglobulins of the same antigen specificity as the BCR are secreted by differentiated plasma cells. These secreted antibodies agglutinate, neutralise and opsonise pathogens as well as activate complement. Interaction between the immunoglobulin fragment crystallisable (Fc) region and the Fc receptor on phagocytes is essential for phagocytosis, degranulation and the initiation of cytokine transcription and expression (reviewed in Daëron, 1997). Unlike B cells, T cells only express a membrane-bound antigen receptor, the TCR, which only recognises oligomer peptide fragments of foreign peptides in the context of MHC\textsubscript{I} or II molecules.

The generation of TCRs and immunoglobulins with a wide range of antigen-specificities is mediated primarily by somatic recombination in both T and B
cells. The V regions of both immunoglobulins and the TCR are encoded by separate gene segments, V, D and J, which are rearranged and brought together during somatic recombination to generate a complete V-region exon made up of different combinations of V, D and J segments (Fugmann et al., 2000). V(D)J recombination is mediated by the V(D)J recombinase complex, consisting of recombination activation gene (Rag) 1 and Rag2 as well as DNA ligase and DNA repair enzymes. The V(D)J recombinase is involved in the alignment and cleavage of sites of recombination, which are flanked by recombination signal sequences (RSSs) (Oettinger et al., 1990; Schatz et al., 1989). Rag1 and Rag2 might also be important for coupling the cleavage and non-homologous end joining stages of V(D)J recombination (reviewed in Fugmann et al., 2000). Additional variability arises at the joints between gene segments as a result of the variable insertion of palindromic- and non-template-encoded-nucleotides (reviewed in Fugmann et al., 2000). The importance of Rag-proteins and thus an adaptive immune system is clear from the severe primary immunodeficiencies in humans if either of the Rag genes are mutated (Sobacchi et al., 2006) and from Rag-deficient mice which lack any T or B cells and are severely immunocompromised (Mombaerts et al., 1992; Shinkai et al., 1992).

Antigen receptor signalling, in addition to being essential for the activation of T and B cells by their cognate antigen, also plays an important role in the regulation of lymphocyte development and maintenance. B lymphocytes have been shown to require tonic signalling through their BCR in addition to soluble signalling from B cell activating factor (BAFF) for their survival (reviewed in Rickert et al., 2011 and in Mackay et al., 2010). Similarly, T cell homeostasis
depends on tonic TCR signalling as well as cytokine signalling, in particular from interleukin-(IL)-7 (Seddon and Zamoyska, 2003). The regulation of T cell homeostasis by TCR and IL-7 signals is essential for the maintenance of a normal T cell repertoire that can respond and expand during an infection, as well as generate and maintain memory cells that can respond to re-infection with the same pathogen (Jameson, 2005).

1.2 Signalling pathways involved in T cell homeostasis

Homeostasis is defined as the maintenance of an equilibrium despite changes in the environment. In the case of the T cell compartment, both the development of new T cells in the thymus and the survival, proliferation and differentiation of peripheral naïve and memory T cells is under tight homeostatic control. This means that the size and composition of the T cell pool remain fairly stable throughout life (reviewed in Almeida et al., 2005 and in Freitas and Rocha, 2000). Both Interleukin 7 receptor α (IL-7Rα) signalling and non-antigenic signalling through the TCR have been shown to be important for the regulation this homeostasis (Seddon and Zamoyska, 2003).

1.2.1 IL-7Rα signalling

IL-7Rα signalling (summarised in Figure 1.1) is initiated by cross-linking of the extracellular domains of the IL-7Rα chain and the common γ (γc) chain by IL-7, a 25kDa glycoprotein first identified as growth factor that could stimulate the proliferation of B cell progenitors in vitro (Namen et al., 1988). IL-7 is mainly produced by non-haematopoietic stromal cells in the thymus (Namen et al., 1988; Wiles et al., 1992), spleen, kidney, liver (Namen et al., 1988) and skin
(Heufler et al., 1993) and the amount of available IL-7 is thought to be largely regulated by IL-7 consumption (reviewed in Mazzucchelli et al., 2007 and in Fry and Mackall, 2005). The cross-linking of the extracellular domains of the IL-7Rα chain and the γ_c chain of the IL-7R brings together Janus kinase 1 (Jak1) and Jak3, which are constitutively associated with the cytoplasmic tails of the IL-7Rα and the γ_c chain of IL-7R, respectively (Russell et al., 1994). Heterodimerisation brings the Jak1 and Jak3 in close proximity allowing phosphorylation of Jak1 and the IL-7Rα chain by Jak3, which creates a docking site for the Src homology domain 2 (SH2) domain of signal transducer and activator of transcription (STAT) 1, 3 and 5 (reviewed in Jiang et al., 2005). Jak3 appears to be the major transducer of γ_c chain signals since \( \text{Jak}3^{-/-} \) mice (Nosaka et al., 1995; Park et al., 2004b; Suzuki et al., 2000) resemble \( \gamma_c^{-/-} \) mice (Cao et al., 1995) exhibiting similar defects in T and B cell development and signalling as well as reduced B cell leukaemia/lymphoma 2 (Bcl-2) expression and IL-7-induced Bcl-2 synthesis (Suzuki et al., 2000). A subsequent study showed that in addition to reduced Bcl-2, Jak3-deficient thymocytes and T cells also had elevated Bcl-2-associated X protein (Bax) abundance (Wen et al., 2001). \( \text{Jak}1^{-/-} \) mice also have severely impaired T and B cell development, with reduced thymocyte numbers but not an absolute block of T cell development, similar to \( \text{IL}-7^{-/-} \) mice (Freeden-Jeffry et al., 1995).

Following the phosphorylation of Jak1 and IL-7Rα chain by Jak3, Jak1 and Jak3 phosphorylate the STATs recruited to IL-7Rα, which induces their dimerisation and translocation to the nucleus where they can activate specific gene transcription. In T cells, IL-7 predominantly induces the phosphorylation of two isoforms of STAT5, STAT5a and STAT5b, which can form homo- or
heterodimers (Foxwell et al., 1995; Rosenthal et al., 1997; Yu et al., 1998). As well as their tyrosine phosphorylation, IL-7 also stimulates the DNA binding activity of STAT5a/b (Foxwell et al., 1995). STAT5a and b are at least partially redundant in lymphocytes since deficiency of either STAT5a (Nakajima et al., 1997) or STAT5b (Imada et al., 1998) has little effect on T and B cell development. Mice lacking both Stat5a and Stat5b do show defects in both T and B cell development (Yao et al., 2006). In fact, similar to Jak3-deficiency, STAT5a/b-deficiency leads to a severe reduction in thymocyte numbers without a complete block of T cell development, as well a reduction in the proportion of B cells and peripheral CD8 T cells, in particular (Yao et al., 2006) reflecting the importance of STAT5 in the transduction of γc chain and Jak3 signals. Interestingly, unlike other STATs, STAT5 has mostly been implicated in the transduction of pro-survival signals by regulating the expression of Bcl-2 family members and caspases (reviewed in Debierre-Grockiego, 2004). In fact, STAT5 has been implicated in the regulation of IL-7-induced Bcl2 transcription (Jiang et al., 2004) and expression of Bcl2 was also impaired in the STAT5a/b-double-deficient mouse (Yao et al., 2006). Furthermore, Jak1, Jak3 and STAT5 activity are all implicated in IL-7-induced growth and proliferation signals (Foxwell et al., 1995) and STAT5 has been shown to induce CyclinD1 transcription in vitro (Matsumura et al., 1999) and maintain CyclinD2 expression in vivo (Yao et al., 2006). IL-7 has also been implicated in the regulation of c-myc expression (Crawley et al., 1996; Seckinger et al., 1994), another important regulator of cell growth and proliferation (reviewed in Dang, 2012).
Finally, it has been shown that IL-7 can induce the phosphorylation of the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (p85) in human thymocytes (Dadi et al., 1994) although the downstream targets of this signal remain poorly characterised (reviewed in Jiang et al., 2005). The p85α subunit has been shown to interact with a tyrosine residue on IL-7Rα, Y449, in mouse B cells (Venkitaraman and Cowling, 1994) and the same residue of IL-7Rα is also thought to be important for the phosphorylation of STAT5 (Jiang et al., 2004). In T cell acute lymphoblastic leukaemia (T-ALL) cells, PI3K was shown to be indispensable for IL-7-induced upregulation of Bcl-2, downregulation of the cell cycle inhibitor cyclin-dependent kinase inhibitor 1B (p27kip1) and expression of glucose transporter 1 (GLUT1) (Barata et al., 2004). Both STAT5 and protein kinase B (Akt/PKB) are also implicated in the regulation of glucose metabolism by IL-7 in T cells in vitro (Wofford et al., 2008). T cell development and proliferation, however, are normal in mice lacking the p85α subunit of PI3K (Fruman et al., 1999) calling into question the exact role of PI3K in IL-7 signalling in vivo.

1.2.2 Regulation of IL-7Rα expression

While IL-7 transcription is thought to occur at a constant constitutive rate, IL-7Rα transcription and expression is strictly controlled during development and modulated by various external stimuli on mature T cells (reviewed in Mazzucchelli and Durum, 2007). It is expressed on the most immature CD4− CD8− double negative (DN) thymocytes, downregulated on CD4+ CD8+ double positive (DP) thymocytes after which it is re-expressed on CD4+ and CD8+ single positive (SP) thymocytes and IL-7 responsiveness measured by STAT5
phosphorylation correlates well with IL-7Rα expression on each of these thymocyte populations (Van De Wiele et al., 2004). IL-7 signalling in DN thymocytes is important for their protection from apoptosis (Khaled et al., 2002) and has also been implicated in the rearrangement of the Tcrg gene (Candeias et al., 1997b). Naïve CD4 and CD8 T cells in the periphery express heterogeneous levels of IL-7Rα but are all able to upregulate their surface IL-7Rα after overnight culture without IL-7 (Park et al., 2004a), suggesting that naïve T cells receive signals that induce IL-7Rα downregulation in vivo. In fact, one such factor is IL-7 itself, which has been shown to induce the downregulation of IL-7Rα on naïve T cells and suppress Il7ra transcription (Park et al., 2004a). Interestingly, forced expression of IL-7Rα leads to a reduction in the size of the naïve T cell pool (Park et al., 2004a). These data are consistent with an altruistic model of IL-7Rα signalling whereby IL-7-induced downregulation of IL-7Rα expression prevents cells that have already received sufficient IL-7 survival signals to consume further IL-7 (Park et al., 2004a). Finally, while most effector CD8 T cells dramatically downregulate IL-7Rα expression, a proportion of virus-specific effector CD8 T cells retain high IL-7Rα expression and are thought to be the precursors for long-term memory CD8 T cells (Huster et al., 2004; Kaech et al., 2003). Other studies, however, have failed to find a relationship between IL-7Rα re-expression and CD8 memory formation (Lacombe et al., 2005).
1.2.3 TCR signalling

The TCR complex consists of a heterodimer of membrane-bound, TCRα and β (Kappes et al., 1983), associated with CD3δε and CD3γε heterodimers and a CD3ζ homodimer. Deficiency in any of the CD3 subunits leads to impaired TCR signalling and T cell development (Dave et al., 1998; Haks et al., 1998; Liu et al., 1993; Malissen et al., 1995) reflecting the importance of TCR signalling for T cell homeostasis. The TCR complex recognises antigenic peptides in the context of MHCI in the case of CD8 T cells, and MHCII in the case of CD4 T cells. The CD4 and CD8 co-receptors interact specifically with the non-polymorphic domains of MHCII (Doyle and Strominger, 1987) and MHCI (Norment et al., 1988), respectively. Phosphorylation of lymphocyte-specific protein tyrosine kinase (Lck), associated with the CD4 and CD8 co-receptors (Veillette et al., 1988), is one of the earliest events in TCR signalling (summarised in Figure 1.2) (reviewed in Brownlie and Zamoyska, 2013), and it has been proposed that the main function of the CD4 and CD8 co-receptors is to bring Lck in close proximity to its substrates, the CD3 immunotyrosine activation motifs (ITAMs) (Artyomov et al., 2010). The TCR complex, however, does not have any intrinsic kinase activity and so how pMHC interactions with TCR initiate the TCR signalling cascade remains unknown.

One model proposes that TCR binding of pMHC causes the TCR to undergo a conformational change to initiate signalling. In support of this model, the cytoplasmic domain of CD3ε is inserted in the plasma membrane in resting T cells, inaccessible to Lck and TCR ligation would need to induce dissociation of the CD3ε ITAM from the plasma membrane to render it accessible to Src family
kinases (Xu et al., 2008a). What might cause dissociation of the CD3ε ITAM from the plasma membrane remains unclear. In a second model, pMHC binding to the TCR segregates the TCR complex in areas of the plasma membrane rich in Lck but devoid of CD45, protecting it from dephosphorylation by CD45 (reviewed in van der Merwe and Dushek, 2010). Indeed, it was recently shown in an artificial receptor system that extracellular protein-protein interactions can generate binding energies that can induce segregation of membrane proteins without conformational change (James and Vale, 2012). Following the initiation of TCR signalling, Lck phosphorylates its targets, including the ITAMs of CD3γ, CD3δ, CD3ε, CD3ζ and ζ-associated protein of 70kDa (Zap70) which ultimately results in a conformational change in Zap70 (Brdicka et al., 2005) allowing it to phosphorylate its targets, linker of activated T cells (LAT) and SH2 domain containing leukocyte protein of 76kDa (Slp76) (Bubeck Wardenburg et al., 1996; Zhang et al., 1998a).

1.2.3.1 The regulation of Lck activity by CD45 and Csk

Src family kinase activity is regulated by the phosphorylation of an activating tyrosine in the catalytic domain and a C-terminal inhibitory tyrosine. It was recently shown that in resting T cells ~40% of total Lck is active and phosphorylated at Y394 and ~20-30% of total Lck is doubly phosphorylated at both the activating and inhibitory tyrosines, Y394 and Y505 (Nika et al., 2010). Interestingly, both Lck phosphorylated only at Y394 (pY394) and Lck doubly phosphorylated at Y394 and Y505 (pY394 pY505) showed very similar kinase activity in vitro (Nika et al., 2010) and were in the open, active conformation. It has also been shown that no additional activation of Lck is required for TCR
signalling (Nika et al., 2010). A subsequent study demonstrated that TCR triggering induces clustering of Lck in its active conformation but not in its closed inactive conformation (Rossy et al., 2013). The clusters of active Lck also excluded the phosphatase CD45 (Rossy et al., 2013). Thus, the local concentration of active Lck in an open conformation appears to be more important than the overall abundance of phosphorylated Lck in a cell (reviewed in Brownlie and Zamoyska, 2013).

While the activating tyrosine of Src family kinases is autophosphorylated, the inhibitory tyrosine is phosphorylated by C-terminal Src kinase (Csk), a ubiquitous kinase whose only substrate is the inhibitory tyrosine of Src family kinases (reviewed in Veillette et al., 2002). Once phosphorylated, the C-terminal inhibitory tyrosine of Lck, Y505, can bind to the SH2 domain, blocking the substrate binding domain and locking the Src family kinase into an auto-inhibited closed conformation (Xu et al., 2008b). Consistent with a negative regulatory role for Csk in TCR signalling, Csk-deficient thymocytes and mature T cells all show constitutive TCR signalling independent of TCR, Rag1 or MHC expression (Schmedt and Tarakhovsky, 2001; Schmedt et al., 1998). Furthermore, inhibition of analog-sensitive form Csk was sufficient to activate TCR signalling independent of TCR interactions with pMHC (Schoenborn et al., 2011).

The activating and inhibitory tyrosines of Src family kinases are also substrates for phosphatases including CD45, protein tyrosine phosphatase non-receptor type 6 (Shp1) and protein tyrosine phosphatase non-receptor type 22 (Ptpn22) (Rhee and Veillette, 2012). In Cd45−/− mice, T cell development and signalling is
severely impaired (Byth et al., 1996) and Lck and Fyn proto-oncogene (Fyn) are hyperphosphorylated at their inhibitory tyrosine residues (Stone et al., 1997). Expression of constitutively active Lck rescues T cell development in Cd45−/− mice suggesting CD45 mainly counteracts Csk by dephosphorylating Y505 and is thus a positive regulator of TCR signalling (Pingel et al., 1999; Seavitt et al., 1999). The kinase activity of Lck, however, is paradoxically increased in CD45-deficient thymocytes suggesting that CD45 might have both a positive and a negative role in TCR signalling (D’Oro and Ashwell, 1999). More recent work has shown that low CD45 abundance is sufficient to dephosphorylate the inhibitory pY505 residue, whereas more CD45 is required to dephosphorylate the activating pY394 residue (McNeill et al., 2007). Subsequent studies have suggested that at low doses, CD45 has predominantly positive regulatory role in TCR signalling while at higher doses CD45 inhibits TCR signalling (McNeill et al., 2007; Zikherman et al., 2010).

1.2.3.2 The initiation of Zap70 signalling in T cells

Following TCR engagement with pMHC, Zap70, is recruited to the TCR signalling complex. This was shown to involve Ras homology gene family member H (RhoH), a haematopoietic GTPase-deficient member of the Rho GTPase family, whose noncanonical ITAMs bind Zap70 (Gu et al., 2006). RhoH−/− mice show a block in T cell development leading to reduced peripheral T cells and reduced CD3ζ phosphorylation and Zap70 translocation following CD3 stimulation (Gu et al., 2006). A small fraction of Zap70 is constitutively associated with the tyrosine-phosphorylated CD3ζ (van Oers et al., 1994). Lck-mediated phosphorylation of CD3ζ following TCR triggering leads to further
recruitment of Zap70, which like its family member spleen tyrosine kinase (Syk), consists of a C-terminal kinase domain and two tandem N-terminal SH2 domains (Chan et al., 1992) (Figure 1.3). Importantly, two tyrosine residues in the domain linking the SH2 domains and the kinase domain of Zap70, Y315 and Y319, need to be phosphorylated by Lck to relieve the auto-inhibitory conformation of inactive Zap70 (Brdicka et al., 2005) enhancing its kinase activity (Deindl et al., 2009). Binding of the doubly phosphorylated ITAMs of CD3ζ and CD3ε by the tandem Zap70 SH2 domains (Hatada et al., 1995; Wange et al., 1993), is thought to further stabilise the open and active conformation of Zap70 as well as localise Zap70 near the TCR and Lck (reviewed in Au-Yeung et al., 2009). Zap70 kinase activity requires phosphorylation Y492 and Y493 in the kinase domain of Zap70 by Lck or Zap70 itself (reviewed in Au-Yeung et al., 2009), which allows Zap70 to phosphorylate its targets, LAT (Zhang et al., 1998a) and Slp76 (Bubeck Wardenburg et al., 1996).

The importance of the SH2 domains for the interaction between Zap70 and the CD3ζ ITAMs is very clear in SKG mice, which express a mutant Zap70 containing a missense mutation in the C-terminal SH2 domain (Sakaguchi et al., 2003). Reduced recruitment of Zap70^{SKG} to the TCR complex and CD3ζ chains in these mice results in impaired calcium (Ca^{2+}) mobilisation and impaired phosphorylation of LAT and phospholipase Cγ (PLCγ) following TCR stimulation in vitro (Sakaguchi et al., 2003). Unlike in Zap70^-/- mice (Negishi et al., 1995), T cell development is not completely blocked in SKG mice although the reduced TCR signalling does lead to impaired positive and negative selection (Sakaguchi et al., 2003). The weaker TCR signalling mediated by
Zap70\textsuperscript{SKG} is thought to lead to the selection of T cells with higher affinity for self-peptide in the context of MHC (spMHC). Potentially autoreactive T cells are thus selected, which might have been eliminated during negative selection in wildtype (WT) mice (Sakaguchi et al., 2003). This shift in the T cell repertoire leads to the spontaneous development of arthritis in SKG mice unless they are housed in a clean environment (Sakaguchi et al., 2003; Yoshitomi et al., 2005). Interestingly, while the number and \textit{in vitro} suppressive activity of CD4 regulatory T cell (T\textsubscript{reg}) from SKG mice is normal (Hsu et al., 2009), they appear less able to prevent arthritis if co-transferred with SKG CD4 T cells into immunodeficient BALB/c mice (Sakaguchi et al., 2006). Importantly, in addition to mediating attenuated TCR signalling, Zap70\textsuperscript{SKG} abundance is also significantly reduced compared to Zap70\textsuperscript{WT}, which is likely to contribute to the signalling and T cell development defects seen in SKG mice (Hsu et al., 2009).

\textbf{1.2.3.3 The roles of Y292, Y315 and Y319 during Zap70 signalling}

Three tyrosine residues in the interdomain B linking the two SH2 domains of Zap70 and its kinase domain are both known sites of phosphorylation and have been implicated in interactions with Zap70 targets such as E3 ubiquitin-protein ligase Cbl (c-Cbl), Vav1 proto-oncogene 1 (Vav1), Lck, Crk proto-oncogene (p38) and PLC\textgamma{} (reviewed in Au-Yeung et al., 2009). Y292 appears to negatively regulate Zap70 function since T cells from knock-in mice expressing Y292 mutated to phenylalanine (Y292F) showed hyperphosphorylation of LAT and SLP-76 (Magnan et al., 2001), both Zap70 substrates.
Y315 and Y319 are phosphorylated after TCR engagement (Chan et al., 1995) and are both thought to be positive regulators of Zap70 function (reviewed in Au-Yeung et al., 2009) in addition to being important for destabilising the auto-inhibited conformation of Zap70 once phosphorylated (Brdicka et al., 2005). Phosphorylated Y315 is thought to be a binding site for the SH2 domain of Vav1 (Wu et al., 1997) and the adaptor protein p38 (Gelkop et al., 2005), which might link TCR stimulation to reorganisation of the actin cytoskeleton (reviewed in Au-Yeung et al., 2009). Finally, Y315 has also been implicated in the activation of PLCγ and mutation of Y315 to phenylalanine (F) (Y315F) mutation lead to a small reduction in PLCγ phosphorylation as well as small reductions in downstream extracellular regulated kinase (Erk) phosphorylation and Ca^{2+} mobilisation (Magnan et al., 2001). Y319, meanwhile, binds the SH2 domain of Lck (Pelosi et al., 1999) and PLCγ (Williams et al., 1999). The Y319F mutation results in reduced Ca^{2+} mobilisation, Ras proto-oncogene (Ras) activation and CD69 expression as well as impaired phosphorylation of PLCγ and LAT (Williams et al., 1999).

T cell development and TCR signalling in mice expressing Zap70 with both Y315 and Y319 residues of Zap70 mutated to alanine (A) (YYAA hereon) were recently analysed (Hsu et al., 2009). Similar to the SKG mutation, the YYAA mutation leads to a hypomorphic allele of Zap70. Like in SKG mice, both positive and negative selection is impaired in YYAA mice leading to a shift in the T cell repertoire towards more autoreactive T cells (Hsu et al., 2009). Interestingly, despite showing defects in TCR signalling as assessed by Erk phosphorylation, Ca^{2+} mobilisation and CD5 and CD69 expression during T cell development, YYAA mice do not develop arthritis following innate immune cell
activation (Hsu et al., 2009). Despite SKG T cells having a much more severe defect in TCR signalling than YYAA T cells, T cell numbers in SKG and YYAA mice are similar (Hsu et al., 2009) contradicting the view that the absolute numbers of T cells correlate with Zap70 signalling strength (Siggs et al., 2007). In fact, evidence from the YYAA mice suggests that as well as quantitative differences in TCR signalling between Zap70 alleles, the specific functions impacted by the different Zap70 mutations lead to differential effects on T cell development and function (Hsu et al., 2009). Consistent with this, the YYAA mice showed a specific defect in T<sub>reg</sub> development and suppressive activity <i>in vitro</i> which did not affect the SKG mice (Hsu et al., 2009). Again, when considering the effect of Zap70<sup>YYAA</sup> on TCR signalling it is important to take into account that the abundance of Zap70 is reduced in YYAA compared to WT mice, especially in peripheral T cells (Hsu et al., 2009).

1.2.3.4 Downstream targets of Zap70

After TCR engagement, active Zap70 phosphorylates LAT (Zhang et al., 1998a) and Slp76 (Bubeck Wardenburg et al., 1996). LAT is also phosphorylated by Lck (Jiang and Cheng, 2007) and IL-2 inducible T cell kinase (Itk) (Perez-Villar et al., 2002). The tyrosine phosphorylation of LAT requires its constitutive association with lipid rafts (Zhang et al., 1998b). Slp76, meanwhile, is constitutively associated with growth-factor-receptor-bound-protein-2-(Grb2)-related adaptor downstream of Shc (Gads) and the Slp76-Gads complex is recruited to phosphorylated LAT within lipid rafts following TCR engagement (Liu et al., 1999). LAT and Slp76 lack any intrinsic enzymatic activity but contain protein-binding domains and play a crucial role in the transduction of TCR
signals by recruiting signalling and additional adaptor proteins into multiprotein signalling complexes, which couple TCR engagement of pMHC to downstream signalling pathways (reviewed in Koretzky et al., 2006 and in Balagopalan et al., 2010). Phosphorylated LAT has been shown to associate with Vav1, PLCγ, Slp76, c-Cbl, the p85 subunit of PI3K and various Grb2 family members including Gads, Grb2 and son of sevenless 1 (Sos1) (reviewed in Balagopalan et al., 2010). Phosphorylated Slp76 interacts with Vav1, non-catalytic region of tyrosine kinase adaptor protein (Nck), the p85 subunit of PI3K and Itk (reviewed in Koretzky et al., 2006). The proline-rich domain of Slp76 is important for interactions with Gads (Liu et al., 1999), PLCγ (Yablonski et al., 2001) and Lck (Sanzenbacher et al., 1999) while the SH2 domain is important for binding adhesion and degranulation-promoting adaptor protein (Adap) and haematopoietic progenitor kinase 1 (Hpk1) (reviewed in Koretzky et al., 2006).

The study of LAT-deficient and Slp76-deficient T cell lines has revealed the importance of the recruitment of these adaptors and their substrates to the TCR signalling complex for the activation of a number downstream signalling pathways (reviewed in Balagopalan et al., 2010 and in Koretzky et al., 2006). LAT-deficient T cells showed reduced TCR-induced Ca\(^{2+}\) mobilisation and suboptimal phosphorylation of Vav1, PLCγ and Slp76, both of which were rescued by LAT reconstitution (Zhang et al., 1999). In the same T cell line (Zhang et al., 1999), LAT was also shown to be important Erk activation and for CD69 upregulation, which are both downstream of Ras signalling (D'Ambrosio et al., 1994 and reviewed in Rincon, 2001). Finally, LAT is also required for TCR- and nuclear-factor-of-activated-T-cells-(NFAT)-mediated gene transcription (Zhang et al., 1999). Slp76-deficiency also lead to defects in Erk
activation and Ca\(^{2+}\) mobilisation and TCR-induced NFAT activation was completely abrogated (Yablonski et al., 1998). Phosphorylation of Zap70, LAT and Itk, however, was normal in Slp76-deficient T cells (Yablonski et al., 1998).

PLC\(\gamma\) is therefore an important downstream target of both LAT and Slp76. The recruitment of PLC\(\gamma\) to LAT is important for its tyrosine activation and to localise PLC\(\gamma\) near its substrate phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) and does not require Slp76 (Yablonski et al., 1998). The phosphorylation of PLC\(\gamma\), however is significantly impaired, in absence of Slp76 (Yablonski et al., 1998). PLC\(\gamma\) activation by Slp76 and LAT leads to the generation of inositol triphosphate (IP\(_3\)) and diacylglycerol (DAG) and the activation of protein kinase C (PKC\(\theta\)) as well as the mobilisation of intracellular Ca\(^{2+}\) (reviewed in Balagopalan et al., 2010 and in Koretzky et al., 2006). The rapid IP\(_3\)-dependent increase in intracellular Ca\(^{2+}\) activates a number of downstream signalling proteins, including the phosphatase calcineurin which dephosphorylates the NFAT family of transcription factors (reviewed in Hogan et al., 2010 and in Oh-hora, 2009), inducing them to translocate to the nucleus and initiate the transcription of NFAT-dependent genes (reviewed in Hogan et al., 2003). DAG, meanwhile, activates PKC\(\theta\) in T cells, which in turn activates the AP1 transcription factor complex (Baier-Bitterlich et al., 1996) and NF\(\kappa\)B signalling (Sun et al., 2000). PLC\(\gamma\) and DAG have also been shown to regulate the activity of RasGRP and downstream Ras signalling in thymocytes (Dower et al., 2000).

Ras and downstream mitogen-activated protein kinase (MAPK) signalling in T cells can also be activated by Sos1 (Buday et al., 1994), a well-know activator of Ras in multiple tissues which constitutively associates with Grb2 (Egan et al.,
Sos1 recruitment to LAT (Zhang et al., 1998a) and CD3ζ (Nel et al., 1995) following TCR stimulation is thought to depend on Grb2 interacting with LAT. Consistent with this, mutation of the Grb2 binding residues of LAT, Y171 and Y191, reduces the amount Sos1 that associated with LAT (Zhang et al., 1998a). The recruitment of the Grb2-Sos1 complex to LAT and the plasma membrane colocalises Sos1 with its substrate Ras (reviewed in Mor and Philips, 2006). Active Ras recruits Raf proto-oncogene 1 (Raf1) to the plasma membrane enhancing its enzymatic activity (reviewed in Morrison and Cutler, 1997) allowing Raf1 to phosphorylate and activate MEK which in turn phosphorylates and activates Erk1 and Erk2 which ultimately leads to the induction of AP1 (reviewed in Mor and Philips, 2006).

In addition to activating PLCγ, Slp76 also plays an important role in the TCR-induced reorganisation of the cytoskeleton, which is essential for productive engagements with APCs, formation of the immunological synapse at the T cell-APC interface and ultimately T cell activation (reviewed in Billadeau et al., 2007). Tyrosine phosphorylation of Slp76 has been shown to be required for the recruitment of Nck and WASP to the site of TCR engagement as well as Vav1 and cell division cycle 42 (Cdc42), key activators of WASP (Zeng et al., 2003). Consistent with this, overexpression of Slp76 or Vav1 lead to increased TCR-induced F-actin polymerisation while expression of a Slp76 mutant that fails to be tyrosine-phosphorylated results in reduced TCR-induced actin polymerisation (Bubeck Wardenburg et al., 1996). Furthermore, cells expressing mutant Slp76, Vav1 or Nck also fail to activate Cdc42/Rac-activated kinase 1 (Pak1), a downstream target of Cdc42 (Bubeck Wardenburg et al., 1998). Slp76-, LAT- and Nck-deficient T cells show normal TCR-induced Pak1
activation and activation of Ras-related C3 botulinum substrate 1 (Rac1), a potential activator of Ras-related protein 1 (Rap1), only requires Zap70 and LAT, but not Slp76 calling into question the role of Slp76 in actin polymerisation downstream of TCR activation (Ku et al., 2001).

Finally, LAT and Slp76 activate PI3K signalling and the p85 regulatory subunit of PI3K has been shown to bind directly to both LAT (reviewed in Balagopalan et al., 2010) and Slp76 (Shim et al., 2004). PI3K consists of 110kDa and an 85kDa subunit and promotes the conversion of PIP$_2$ to phosphatidylinositol 3,4,5-bisphosphate (PIP$_3$), which recruits pleckstrin homology (PH) domain containing proteins, like Akt and pyruvate dehydrogenase kinase 1 (Pdk1) (reviewed in Fruman and Bismuth, 2009). Akt activates mTOR signalling, which is essential for TCR-induced proliferation, but also phosphorylates forkhead box O (Foxo) proteins which are important regulators of T cell trafficking, proliferation, survival, tolerance and T$_{reg}$ induction (reviewed in Ouyang and Li, 2011).

1.2.3.5 Integrin signalling

Integrin signalling, predominantly mediated by the β2 integrin lymphocyte function associated antigen 1 (LFA1), plays an important role in T cell migration and arrest on surfaces that express the LFA1 ligand intercellular adhesion molecule 1 (ICAM1). It is also essential during T cell contact with ICAM1-expressing APCs leading to immunological synapse formation and T cell activation (reviewed in Hogg et al., 2011). At the immunological synapse, TCR-pMHC complexes and LFA1-ICAM1 complexes are organised and spatially separated into a central supramolecular activation cluster (cSMAC), containing
the TCR complex, and a peripheral supramolecular activation cluster (pSMAC), containg LFA1 and the cytoskeletal protein talin (Monks et al., 1998). While initial contacts between T cells and APCs are transient, TCR signalling leads to a conformational change in LFA1, increasing its affinity for ICAM1 allowing stable T cell-APC interactions, which are important for T cell activation (reviewed in Billadeau et al., 2007). The signals that mediate the increase in LFA1 affinity downstream of TCR signalling have not been fully characterised but a number of signalling and adaptor proteins have been implicated (reviewed in Billadeau et al., 2007). These include Zap70 and LAT (Epler et al., 2000; Goda et al., 2004), PLCγ (Katagiri et al., 2004), Vav1 and Itk (reviewed in Billadeau et al., 2007). Slp76 is also thought to be essential for integrin activation, both downstream of integrin signalling itself and downstream of TCR stimulation (Baker et al., 2009). Importantly, the C-terminal SH2 domain of Slp76 binds Adap (reviewed in Koretzky et al., 2006) and this interaction is important for integrin induced outside-in signals (Baker et al., 2009).
1.3 T cell development

Unlike B cell development, which occurs almost entirely in the BM, T cell development occurs at least partly in a specialised, dedicated organ in the superior mediastinum, the thymus (Miller, 1961). Pluripotent T cell progenitors from BM migrate to the thymus where they proliferate and initiate commitment to the T cell lineage leading to the generation of a polyclonal T cell repertoire that recognises spMHC but not so strongly as to lead to T cell activation. αβ T cells, which develop alongside γδ T cells in the thymus, also commit to either CD4 or CD8 T cell lineage and acquire effector potential before leaving the thymus and entering the naïve T cell pool as recent thymic emigrants (RTEs).

1.3.1 Double negative stages of T cell development

Early T cell progenitors are characterised by high expression levels of stem cell antigen 1 (Sca-1), tyrosine protein kinase kit (c-kit) and CD44 but lack expression of IL-7Rα and the CD4 and CD8 co-receptors and are referred to as DN thymocytes (reviewed in Weerkamp et al., 2006). DN thymocytes are typically subdivided into four phenotypically distinct subsets based on their relative expression of CD44 and CD25, including a CD44⁺CD25⁻ DN1, a CD44⁺CD25⁺ DN2, a CD44⁻CD25⁺ DN3 and a CD44⁻CD25⁻ DN4 subset and these subsets also represent sequential stages of DN thymocyte development as follows: DN1 → DN2 → DN3 → DN4 thymocytes (Godfrey et al., 1993). Intact TCR and IL-7Rα signalling are essential for progression beyond the DN stage of T cell development and induce TCR rearrangement and promote DN thymocyte survival, respectively.
The DN1 subset is a highly heterogeneous population and that only DN1 thymocytes with high c-kit expression are actually early T-cell progenitors (reviewed in Ceredig and Rolink, 2002). DN2 thymocytes can also be subdivided into DN2a and DN2b based on their expression of c-kit (Ceredig and Rolink, 2002). DN2a and DN2b thymocytes also represent two distinct developmental stages and DN2b thymocytes express GFP downstream of the proximal Lck promoter and have lost DC development potential (Masuda et al., 2007). Interestingly, the same study also showed that fully T-cell lineage committed DN2b thymocytes undergo several more rounds of proliferation before starting to somatically recombine their Tcrb or Tcrg and Tcrd loci (Masuda et al., 2007). DN3 thymocytes are commonly subdivided into DN3a and DN3b based on their size (Hoffman et al., 1996) and CD27 expression (Taghon et al., 2006) just before they undergo before and after β- or γδ-selection, respectively.

The differentiation of DN thymocytes involves the migration of DN thymocytes from the perimedullary cortex outward to the subcapsular zone through distinct microenvironments that provide specific differentiation, proliferation, survival and further migration signals (reviewed in Petrie and Zuniga-Pflucker, 2007). DN2 thymocytes first express Rag1 in the inner cortex (Wilson et al., 1994) when they start recombining their Tcrd and Tcrg loci (Capone et al., 1998; Livak et al., 1999). Despite expression of Rag, Tcrb recombination is not initiated until DN3 thymocytes migrate to the outer cortex and subcapsular zone (Petrie et al., 1995) and this delay is thought to reflect the accessibility of the Tcrb locus (reviewed in Petrie and Zuniga-Pflucker, 2007). IL-7 has been proposed to regulate Tcrb accessibility (Crompton et al., 1997; Muegge et al., 1993) and is
also known to play a role in Tcrγ recombination (Candeias et al., 1997b; Maki et al., 1996). Transgenic Bcl2 expression, however, successfully rescues αβ but not γδ T cell development in IL-7Rα-deficient mice (Akashi et al., 1997), suggesting IL-7 is dispensable for Tcrβ recombination but mainly acts to promote the survival of DN thymocytes. Thymocytes that fail to successfully recombine their Tcrb loci die (Falk et al., 2001) whereas thymocytes that successfully recombine their Tcrb loci express heterodimers of the recombined TCRβ chain and invariant pre-TCRα chain (Groettrup et al., 1993) (pre-TCR).

Pre-TCR expression is required for the dramatic increase in proliferation that follows β-selection (Pénit et al., 1995; Vasseur et al., 2001) and pre-TCR signalling then triggers the recombination and expression of Tcra and silences the expression of pre-TCRα (Koyasu et al., 1997). Both Cd4 and Cd8 transcription and co-receptor expression is also upregulated at this stage so that cells become CD4⁺ CD8⁺ DP thymocytes (Petrie et al., 1990). Src family kinases Lck and Fyn downstream of the pre-TCR are also partially redundant and only mice lacking both Fyn and Lck have a complete block in T cell development at the DN stage (Groves et al., 1996; van Oers et al., 1996). Zap70 and Syk downstream of Lck and Fyn are also partially redundant for pre-TCR signalling since early thymocyte maturation appears to be normal in Zap70-deficient (Negishi et al., 1995) and Syk-deficient mice (Cheng et al., 1997).
1.3.2 Positive and negative selection

In contrast to DN thymocytes, CD4\(^+\) CD8\(^+\) DP thymocytes migrate from the subcapsular cortex inward back to the central medulla and thus the same microenvironments that induced outward migration of DN thymocytes, induce the inward migration of DP thymocytes (reviewed in Petrie and Zuniga-Pflucker, 2007). The inward migration of DP thymocytes is accompanied by a number of changes including the termination of thymocyte proliferation (Egerton et al., 1990). Thymocytes only start proliferating again when they reach the medulla as mature CD4 and CD8 SP to undergo selection of functional non-autoreactive T cells by positive and negative selection (reviewed in Starr et al., 2003). Thymocytes that do not recognise spMHC die by neglect, while cells that recognise spMHC too strongly, and are thus potentially autoreactive, undergo receptor editing, deviate to another lineage or die by apoptosis (reviewed in Starr et al., 2003). Migration of DP thymocytes prior to positive selection is random and not directed towards the medulla, possibly to allow for interactions with MHC-expressing thymic epithelial cells, whereas positively selecting DP thymocytes migrate rapidly in the direction of the medulla (Witt et al., 2005).

The productive recombination of the Tcra alone is insufficient for the termination of recombination, allelic exclusion and further differentiation of αβ thymocytes (reviewed in Starr et al., 2003). Only interactions of TCRαβ heterodimers with spMHC leads to reduced Rag1 and Rag2 transcription and prevents further Tcra recombination (Borgulya et al., 1992; Brandle et al., 1992). Consistent with the important role of MHCI and MHCII for the maturation of thymocytes beyond the DP stage, β2-microglobulin\(^-/-\) (β2m\(^-/-\)) Aβ\(^-/-\) thymocytes are blocked at the
In B cells, the successful recombination and assembly of the BCR is sufficient to trigger allelic exclusion and differentiation (Nemazee, 2006). The checkpoint for MHC-recognition and MHC-restriction during T cell maturation fits with T cells only recognising antigenic peptides in the context of MHC. The positive selection of T cells that express a TCR with a low affinity for spMHC requires direct cell-to-cell contact with radio-resistant MHC+ cortical thymic epithelial cells (cTEC) in vitro (Anderson et al., 1994) and in vivo (Speiser et al., 1989). cTECs exclusively express a catalytic proteasome subunit β5t, instead of β5 or β5i which reduces the activity of the proteasome (Murata et al., 2007). The immunoproteasome processes antigenic peptides for presentation in the context of MHCI (reviewed in Murata et al., 2008). β5t-deficient mice have a specific defect in CD8 T cell development (Murata et al., 2007) and so the immunoproteasome in cTECs has been proposed to play a particularly important role in the generation of positive selection ligands for MHCI-restricted thymocytes. Cathepsin L, meanwhile, plays an important role in the processing and presentation of peptides in the context MHCII (Honey et al., 2002; Nakagawa et al., 1998). Firstly, it has an important role in the degradation of the invariant chain in cTECs, which binds the MHCII peptide binding groove to prevent inappropriate peptide binding (Nakagawa et al., 1998). Secondly, independently of its role in the processing of the invariant chain, cathepsin L is thought to be involved in the generation of peptide ligands for MHCII in cTECs (Honey et al., 2002).

The establishment of tolerance to self in the thymus is the result of clonal deletion or lineage deviation of potentially autoreactive T cells during negative selection in the thymic medulla by thymic DCs and medullary thymic epithelial
cells (mTEC) (reviewed in Anderson et al., 2007). Whereas positive selection of T cells is largely mediated by rare low-affinity spMHC, recognition of spMHC with high affinity either induces negative selection by apoptosis, lineage deviation or receptor editing (reviewed in Palmer and Naeher, 2009). Strong TCR signalling mediated by CD3 antibodies clearly causes the death CD4+ CD8+ DP thymocytes \textit{in vitro} (Smith et al., 1989) and the administration of antigenic peptide \textit{in vivo} also induces clonal deletion of TCR stimulated DP thymocytes (Murphy et al., 1990; Tarazona et al., 1998). Importantly, inhibition C-C-motif-chemokine-receptor-(CCR) 7-mediated migration of DP thymocytes from the cortex to the medulla leads to the development of autoreactive T cells suggesting that the medulla plays an important role in the negative selection of autoreactive thymocytes (Kurobe et al., 2006). Consistent with this, a subset of mTECs express \textit{autoimmune regulator} (Aire), a transcription factor involved in the expression of tissue-restricted antigens (TRAs) in mTECs and Aire-deficiency leads reduced TRA expression and autoimmunity in mice (Anderson et al., 2002). Recently, thymocytes about to undergo clonal deletion in TCR transgenic mice were shown to associate with cortical CD11c+ DCs which play an important role in their negative selection (McCaughtry et al., 2008).

Finally, the downstream targets of weak and strong TCR signaling leading to thymocyte survival and death during positive and negative selection, respectively (Alam et al., 1996) are thought to diverge at the level of Erk and c-jun N-terminal kinase (Jnk). Deletion of the B1 subunit of calcineurin in thymocytes \textit{in vivo} leads to inefficient Erk signalling whereas TCR-induced Jnk phosphorylation was normal (Neilson et al., 2004). Intriguingly, B1-deficiency completely abolishes positive selection but is dispensable for negative selection
TCR-induced Jnk and p38 activation, in contrast, is specifically impaired in Grb2−/− mice which show normal TCR-induced ERK activation but impaired negative selection (Gong et al., 2001). Furthermore, Erk has been shown to have a lower activation thresholds than Jnk and p38 in thymocytes (Gong et al., 2001). A more recent study characterised the selection potential of a range of ovalbumin-(Ova)-variants in foetal thymic organ cultures of OT-I TCR transgenic thymocytes and correlated the selection potential and TCR affinity of the ligands with downstream TCR signalling (Daniels et al., 2006). This study revealed that even very small differences in ligand affinity, as assessed by tetramer binding, can lead to differences in proximal TCR signalling events such as CD3ζ and Zap70 phosphorylation (Daniels et al., 2006). Furthermore, ligands that induced negative selection induced a greater concentration of phosphorylated Zap70 at the plasma membrane, enhanced phosphorylation of LAT, recruitment of Grb2-Sos1 to LAT ultimately leading to Ras and Erk signalling at the plasma membrane (Daniels et al., 2006). Positive selectors, on the other hand, did not recruit Grb2-Sos1 and activated Ras and then Erk at the Golgi resulting in slower and overall reduced phosphorylation of Erk (Daniels et al., 2006). This is consistent with previous work that suggested that activation of Erk at the plasma membrane is fast and transient, while activation at the Golgi is slower and more sustained (Chiu et al., 2002 and reviewed in Mor and Philips, 2006). Clearly, TCR signals of different strength can induce divergent downstream signalling pathways but exactly how these pathways induce thymocyte death or survival downstream of the TCR remains unclear.
Currently there are two models that describe commitment to the CD4 or CD8 T cell lineage based on MHC-I- and MHCII-restricted thymocytes receiving different signals following spMHC and TCR engagement. Since CD4 has a higher binding affinity for Lck than CD8 (Wiest et al., 1993), stronger TCR signalling mediated by CD4 in MHCII-restricted thymocytes was initially proposed to lead to commitment to the CD4 lineage mediated, while weaker signalling in MHC-I-restricted thymocytes would promote CD8 lineage commitment. Consistent with a quantitative instructive model of lineage commitment, increasing Lck activity promotes MHC-I-restricted thymocytes to differentiate into CD4 T cells while, reducing Lck activity promotes MHCII-restricted thymocytes to commit to the CD4 T cell lineage (Hernández-Hoyos et al., 2000). Furthermore, transgenic expression of a hybrid co-receptor with a CD8 extracellular domain and a CD4 cytoplasmic domain leads to the development of MHC-I-restricted CD4 T cells (Itano et al., 1996; Seong et al., 1992). MHC-I-restricted thymocytes were not redirected, however, when expression of the hybrid CD8.4 co-receptor was regulated by endogenous CD8α transcriptional control elements (Bosselut et al., 2001; Erman et al., 2006) suggesting that quantitative differences alone do not instruct CD4/CD8 lineage fate.

In an alternative instructive model the length rather than the strength of TCR signalling is proposed to determine CD4/CD8 lineage commitment. Intermediate CD4+CD8lo thymocytes which are thought to have undergone positive selection but have not yet committed to the CD4 or CD8 T cell lineage can give rise to
CD8 SP thymocytes (Brugnera et al., 2000). The termination of CD8 expression and interruption of TCR signalling in CD4^+CD8^{lo} DP thymocytes is proposed to promote commitment to the CD8 lineage in an IL-7-dependent manner (Brugnera et al., 2000) and is also consistent with the regulation of co-receptor expression being important for CD4/CD8 lineage commitment (Bosselut et al., 2001; Erman et al., 2006). Furthermore, when CD4 expression is regulated by CD8α transcriptional control elements (CD4^{E8III}) CD4 expression is prematurely terminated and this has been shown to promote CD8 T cell development in MHCI-deficient mice (Sarafova et al., 2005). Restricting Zap70 expression and thus TCR signalling to CD4^+ CD8^{+} DP thymocytes, however, leads to a block in CD4 and CD8 T cell development rather than redirection of MHCII-restricted to the CD8 lineage, suggesting that TCR signalling is required beyond an initial positive selection signal in CD4 and CD8 T cell development (Liu et al., 2003).

Recent work using mice that conditionally express Zap70 integrated aspects of the quantitative and kinetic signalling instructing models of CD4/CD8 lineage development (Saini et al., 2010). CD4 and CD8 T cells were shown to develop from phenotypically distinct subsets of DP thymocytes with different kinetics and CD8 T cell development was shown to require the temporal upregulation of Zap70 expression downstream of TCR-mediated positive selection signals (Saini et al., 2010).

While the proximal signalling events that regulate lineage commitment remain controversial a number of transcription factors have been shown to be essential for the development of CD4 and CD8 T cells. Runt-related transcription factor (Runx) proteins play an important role during CD8 T cell development by silencing Cd4 transcription but while the overexpression of Runx3 does
increase the proportion of developing CD8 SP thymocytes, it does not completely block CD4 T cell development (Kohu et al., 2005). It was recently shown that, transgenic expression of Runx3 in Zap70−/− thymocytes was insufficient for CD8 T cell development but that IL-7 could induce Runx3 expression and CD8 T cell development in absence of TCR signalling (Park et al., 2010). Interestingly, the ratio of developing CD4 and CD8 thymocytes in IL-7− an IL-7Rα-deficient mice is not dramatically altered compared to WT mice, despite an overall reduction in T cell development, calling into question the proposed non-redundant instructive role for IL-7 signalling for CD8 lineage commitment (Freeden-Jeffry et al., 1995; Peschon et al., 1994). Finally, Runx proteins are also implicated in the repression T-helper-inducing POZ/Krueppel factor (Th-POK) expression (Collins et al., 2009), an important regulator of CD4 lineage commitment (He et al., 2005; Sun et al., 2000). In addition to Th-POK, GATA-binding protein 3 (Gata3) has been shown to be an important regulator of CD4 T cell development and deletion of Gata3 in DP thymocytes completely abolishes CD4 T cell development but has no effect on CD8 thymocytes (Pai et al., 2003). A mutation in Th-POK, meanwhile, results in redirection of MHCII-restricted thymocytes to the CD8 lineage (He et al., 2005).

1.3.4 Development of γδ T cells, Treg and NK T cells

While αβ thymocytes undergo positive and negative selection based on their affinity for spMHC, it is less clear whether prospective γδ T cells also undergo positive selection (reviewed in Hayday and Pennington, 2007). In contrast to the positive and negative selection of αβ thymocytes, any selection of γδ thymocytes is likely MHC-independent since β2m−/− Aβ−/− mice have normal
numbers of γδ T cells (Grusby et al., 1993). Pre-TCR signalling is also completely dispensable for γδ T cell development (Fehling et al., 1995). Interestingly, there does seem to be a role for TCR signalling in γδ T cell development since attenuated TCR signalling in Lck-deficient γδ TCR transgenic mice diverted γδ to the αβ lineage (Haks et al., 2005). Furthermore, a reduction of CD5 expression, a negative regulator of TCR signalling (Azzam et al., 2001; Tarakhovsky et al., 1995), has also been shown to increase γδ lineage commitment (Hayes et al., 2005). In addition, IL-7R signalling plays an important role in γδ thymocyte development. IL-7Rα hi DN2 thymocytes give rise to more γδ than αβ cells, whereas IL-7Rα lo DN2 thymocytes give rise to more αβ than γδ cells (Kang et al., 2001). Finally, the transcription factor Sox-box containing gene 13 (Sox13) was recently identified as γδ lineage specific gene and consistent with this, Sox13-deficiency results in impaired γδ T cell development without affecting αβ T cell development, while transgenic Sox13 expression promotes γδ T cell development (Melichar et al., 2007). Despite a clear role for TCR signalling strength in γδ T cell development, it is less clear how γδ TCR ligand interactions instruct γδ T cell development in absence of a γδ TCR restricting element like MHC for αβ TCR (reviewed in Pang et al., 2012). Strong TCR signalling is also implicated in the development T_{reg} and natural killer (NK) T cells allowing potentially autoreactive thymocytes to differentiate into alternative αβ T cell lineages (reviewed in Moran and Hogquist, 2012). CD4 T_{reg} play an important regulatory role in the immune response by suppressing the activation of other immune cells in response to self-peptide or antigen (Sakaguchi et al., 1995). The current model for T_{reg} lineage differentiation in the thymus is that strong spMHC TCR signalling, over the threshold for positive
selection, induces the upregulation of CD25, increasing the sensitivity of the T\textsubscript{reg} precursor to IL-2, which plays an important role in the induction of the T\textsubscript{reg} marker forkhead box p (Foxp3) (Lio and Hsieh, 2008). The role of TCR signalling strength during T\textsubscript{reg} development and function was recently assessed using a TCR signalling reporter mouse (Moran et al., 2011). In this mouse, Nur77 expression, an immediate early gene upregulated following TCR stimulation, is reported by GFP (Nur77\textsuperscript{GFP}) (Moran et al., 2011). The study showed that T\textsubscript{reg} perceive stronger TCR signals during development than conventional T cells and continue to do so in the periphery (Moran et al., 2011).

NK T cells express a relatively invariant TCR chain, which recognises self-lipids in the context of the MHCI-related molecule CD1d, and they are also thought to perceive stronger TCR signals during their development and selection in the thymus (reviewed in Moran and Hogquist, 2012). The idea that NK T cells develop following strong, high affinity TCR signals was originally based on the activated phenotype of NK T cells, with high CD69 and CD44 expression, even in absence of intentional antigen-exposure (reviewed in Kronenberg, 2005). Interestingly, NK T survival and homeostasis in the periphery was shown to be largely CD1d-independent and thus, at least in the periphery, NK T cells are not thought to be autoreactive (McNab et al., 2005). Using the Nur77\textsuperscript{GFP} TCR signalling reporter mice, it was recently shown that NKT precursors do indeed perceive strong TCR signals and so are conceivably selected following interactions with a high affinity ‘agonist’ ligand (Moran et al., 2011). Peripheral NK T cells, on the other hand, did not express more GFP than conventional CD4 T cells suggesting they, unlike T\textsubscript{reg}, do not continuously encounter high affinity ligands in the periphery (Moran et al., 2011).
1.3.5 Single positive thymocyte maturation and thymic egress

After positive selection and CD4/CD8 lineage differentiation in the cortex and subsequently negative selection in the medulla, SP thymocytes achieve phenotypic and functional maturity in the central medulla before they leave the thymus at the cortico-medullary junction in a process called thymic egress (reviewed in Weinreich and Hogquist, 2008). After positive selection SP thymocytes downregulate heat stable antigen (HSA) (Bruce et al., 1981; Crispe and Bevan, 1987; Wilson et al., 1988) and CD69 expression, upregulate Qa lymphocyte antigen 2 region (Qa2) (Vernachio et al., 1989) and CD62-ligand (L) and also lose their susceptibility to apoptosis (Kishimoto and Sprent, 1997). Thymocytes leave the thymus via a strict ‘conveyor belt’ with more mature thymocytes emigrating before immature thymocytes (McCaughtry et al., 2007). Thymic egress occurs approximately 4-5 days after thymocytes become CD4 or CD8 SP as estimated by GFP-decay in mice with GFP expression under control of the Rag2 promoter (McCaughtry et al., 2007). Interestingly, in the same study (McCaughtry et al., 2007) it was also shown that CD8 SP take longer to mature and egress from the thymus than CD4 SP, consistent with data that CD8 development is delayed compared to CD4 development (Saini et al., 2010).

Thymic egress of the most mature thymocytes requires the Krueppel-like-factor-2-(Klf2)-mediated upregulation of sphingosine-1-phosphate receptor 1 (S1P₁) (Carlson et al., 2006; Matloubian et al., 2004), a receptor for sphingosine-1-phosphate (S1P) produced by neural crest derived pericytes that surround the blood vessels at the cortico-medullary junction (Zachariah and Cyster, 2010). Consistent with an important role for S1P₁ signalling in thymic egress, S1pr1⁻⁻
thymocytes fail to leave the thymus but interestingly, also failed to downregulate CD69 expression (Matloubian et al., 2004). Subsequently, CD69 has been shown to associate with S1P₁, in vitro, facilitating its internalisation and degradation (Bankovich et al., 2010). Since CD69 expression is regulated by TCR signalling in thymocytes (Swat et al., 1993), the reciprocal inhibition of CD69 and S1P₁ has been suggested to be a mechanism by which TCR signalling can regulate thymic export (Love and Bhandoola, 2011). Foxo1 has recently been shown to regulate both Il7ra and Klf2 transcription (Kerdiles et al., 2009), which in turn regulates S1pr1 and Cd62l transcription (Carlson et al., 2006). TCR signals via Pdk1 and Akt to Foxo1 might represent an additional way in which TCR signalling regulates thymic egress (reviewed in Love and Bhandoola, 2011). Consistent with thymic egress being regulated by TCR signalling via PI3K and Akt, activation of thymocytes by intrathymic antigenic challenge (Uldrich et al., 2006) or constitutive PI3K signalling in thymocytes (Barbee and Alberola-Ila, 2005) inhibit thymic emigration.

1.4 Peripheral αβ T cell biology

1.4.1 Recent thymic emigrants

Following thymic egress, RTEs undergo functional as well as phenotypic maturation as they integrate into the mature naïve T cell pool (reviewed in Fink and Hendricks, 2011). Recently, mice expressing GFP under control of the Rag2 promotor have been used to study this population of naïve T cells (Boursalian et al., 2004). In these, GFP is first expressed late during DN development after which GFP expression drops in DP and SP thymocytes (Boursalian et al., 2004). Nevertheless, GFP-expressing cells are still detectable
in the periphery. GFP^{hi} cells were shown to disappear 1 week post-thymectomy, while GFP^{lo} cells only disappeared 1-2 weeks post-thymectomy and so represent a more mature subset of RTE (Boursalian et al., 2004). Interestingly, GFP^{hi} CD4 and CD8 RTEs in these mice, appear to be phenotypically and functionally immature compared to more mature GFP^{-} naïve T cells (Boursalian et al., 2004). RTEs express more HSA and CD3 than mature naïve T cells, whereas surface expression of Qa2, IL-7Rα and CD45RB is lower on RTEs (Boursalian et al., 2004).

Both CD8 and CD4 RTEs also undergo functional maturation following thymic egress (Boursalian et al., 2004). CD8 RTEs give rise to fewer cytolytic CD8 T cells than mature naïve T cells in vitro (Boursalian et al., 2004) and generate fewer effector and memory cells than mature naïve T cells in vivo (Makaroff et al., 2009). Interestingly, secondary effectors generated by memory cells derived from RTEs or mature naïve T cells are functionally and phenotypically similar (Makaroff et al., 2009). CD4 RTE are also functionally immature and proliferate less than mature naïve T cells, produce less IL-2 and express less CD25, the high affinity receptor for IL-2 (Boursalian et al., 2004). Finally, CD4 RTEs also poorly differentiate into Th1, Th17 and Treg in vitro and are bias towards adopting Th2 lineage fate in vitro and in vivo, possibly as a result of reduced T-bet, an important regulator of Th1 differentiation (Hendricks and Fink, 2011). The signals that induce RTE maturation are not fully characterised although a full DC compartment and access to SLOs are required (Houston et al., 2008). Interestingly, despite the importance of IL-7Rα and TCR signalling for T cell development and peripheral homeostasis IL-7 and spMHC signals appear to be dispensable for the maturation of RTEs (reviewed in Fink and Hendricks, 2011).
1.4.2 Naïve CD4 and CD8 T cells

Naïve T cells continuously recirculate from blood to lymph via SLOs including the spleen and lymph node (LN) (Gowans and Knight, 1964) where antigen is collected and presented to T cells by APCs (reviewed in Masopust and Schenkel, 2013). Recently, LN entry, retention and egress of CD4 and CD8 T cells was analysed in detail using a combination of intravital imaging and mathematical modelling (Mandl et al., 2012). This study revealed substantial differences between the recirculation of CD4 and CD8 T cells with CD4 T cells spending far less time in LN than CD8 T cells. CD8 T cells, on the other hand, leave LNs much more slowly than CD4 T cells and, unlike CD4 T cells, LN retention is not affected by MHC-deficiency (Mandl et al., 2012).

In the blood, CD62L on T cells mediates the rolling on high endothelial venules (HEVs) in SLOs which allows T cell CCR7 to interact with C-C motif chemokine ligand (CCL) 21 immobilised on HEVs (reviewed in Masopust and Schenkel, 2013). Chemokine signalling activates LFA1 enabling it to bind to ICAM1, which ultimately results in T cell arrest within HEVs followed by migration into the T cell zone (reviewed in Hogg et al., 2011). T cells might subsequently enter LN downstream in the same chain of LNs via afferent lymph vessels (Braun et al., 2011). In addition to activation of LFA1, CCL19 and CCL21 signalling to CCR7 mediates T cell motility within the T cell zone, which is thought to facilitate T cell T scanning of APCs (Kaiser et al., 2005). Retention within the LN is also CCR7-dependent (Kaiser et al., 2005). CCL19 induces CCR7 desensitisation (Kohout et al., 2004) leading to a gradual reduction in the strength of CCR7-dependent retention within the T cell zone. Similarly, S1P, ubiquitous in lymph and blood,
induces the transient downregulation of S1P\(_1\) allowing T cells to enter LN against the S1P gradient (Lo et al., 2005). Only once T cells are sensitive to S1P again, and insensitive to CCL19 do they leave the LN and enter an efferent lymph vessel to ultimately return to the blood via the thoracic duct (reviewed in Cyster et al., 2012 and in Masopust and Schenkel, 2013).

Inflammation greatly increases the probability of naïve T cells encountering antigen by inducing an increase in the size of the arteriole that feeds the LN (Soderberg et al., 2005) but also by altering the expression of chemokine receptors in inflamed LNs (reviewed in Masopust and Schenkel, 2013). For instance, CD8 T cells in draining LN upregulate CCR5 allowing them to home to CD4 T cells interacting with antigen-specific DCs along local CCL3 and CCL4 gradients (Castellino et al., 2006). Meanwhile, activated CD4 T cells upregulate CXCR3 allowing them to home to C-X-C motif chemokine ligand (CXCL) 9 and CXCL10 expressed in draining LNs (Groom et al., 2012). Finally, upon antigen recognition and subsequent T cell activation both CD69 and CCR7 are transiently upregulated, inhibiting surface S1P\(_1\) expression (Bankovich et al., 2010; Shiow et al., 2006) and preventing SLO egress, which provides the antigen, co-stimulation and cytokine signals T cells need to become fully activated, to proliferate and differentiate into effector cells (reviewed in Mescher et al., 2006).
1.4.3 T cell activation and proliferation

Optimal T cell activation, proliferation and acquisition of effector and memory function requires antigen stimulation (Zehn et al., 2012) as well as co-stimulation (reviewed in Acuto and Michel, 2003) and cytokine signals. CD8 T cells, are additionally thought to require help from differentiated CD4 helper T cells to initiate effector responses or for memory generation, although the exact role of CD4 help during CD8 T cell differentiation remains controversial (reviewed in Mescher et al., 2006). T cells sample and interact with antigen-presenting DCs in 3 distinct stages in vivo (Mempel et al., 2004). Following ~8h of multiple, brief T cell encounters with pulsed DCs, T cell motility dropped and more stable T cell DC conjugates were formed as T cells upregulated activation markers such as CD44, CD25 and CD69 and started secreting IL-2 and IFNγ (Mempel et al., 2004). The final stage T cell priming by DCs involved a return to short T cell DC interactions, which coincided with the onset of proliferation (Mempel et al., 2004).

Interestingly, CD8 T cells were shown to require only 2h of exposure to antigen in vitro in order to proliferate and differentiate (Kaech and Ahmed, 2001; van Stipdonk et al., 2001), although longer exposure was required for subsequent proliferation in vivo (van Stipdonk et al., 2003). Subsequent studies have shown that memory cell differentiation (Kaech and Ahmed, 2001) and the onset and kinetics of CD8 T cell contraction (Badovinac et al., 2002) following antigen clearance are also programmed during the initial antigen exposure. More recently, it has been shown that the duration of antigen exposure in vivo might not affect CD8 T cell effector functionality but does determine the magnitude of
clonal expansion (Prlic et al., 2006). Shortening the duration of an infection also reduced the number of CD8 T cell memory cells forms but had no effect on the quality of the secondary response (Williams and Bevan, 2004). Finally, CD8 expansion and differentiation into effector and memory cells is significantly enhanced by inflammatory signals (Shaulov and Murali-Krishna, 2008). In contrast to CD8 T cells, optimal CD4 T cell activation, proliferation and differentiation is thought to require continuous antigen and MHC exposure (reviewed in Zehn et al., 2012) as shown in a recent study using mice that express an inducible MHCII-restricted T cell epitope (Obst et al., 2005). Furthermore, CD4 memory formation is also impaired if the duration of an infection is shortened but the quality of the secondary response is not (Williams and Bevan, 2004).

The majority of antigen-specific T cells that proliferate and differentiate into effector and memory cells have high affinity for antigenic pMHC yet weak TCR signalling can also induce T cell activation, proliferation and differentiation (reviewed in Zehn et al., 2009). In fact, the initial expansion and differentiation of OT-I TCR transgenic T cells was indistinguishable despite being stimulated with altered peptide ligands which bind the OT-I TCR with different affinities (Zehn et al., 2009). Higher affinity altered peptide ligands did promote more extensive and sustained proliferation, consistent with an accumulation of mainly high-affinity antigen-specific T cells during polyclonal immune responses (Zehn et al., 2009). Interestingly though, low and high affinity stimulated CD8 T cells were phenotypically and functionally similar expressing CD44, CD62L and granzyme B and IFNγ, although weakly stimulated CD8 T cells did express less CD25 and CCR7 (Zehn et al., 2009). Furthermore, weakly stimulated CD8
effector and memory cells are functional (Zehn et al., 2009). The ability of the same altered peptide ligands to mediate positive or negative selection of OT-I thymocytes in vitro has also been studied (Daniels et al., 2006). The ligands that only induce positive selection of thymocytes all induced OT-I T cell expansion and differentiation in the periphery (Zehn et al., 2009) suggesting that OT-I T cell proliferation and negative selection occur at different thresholds (reviewed in Zehn et al., 2010).

Finally, using sensitive imaging techniques it was recently shown that a single pMHCII complex is enough to elicit a measurable Ca$^{2+}$ response in CD4$^+$ T cells, although a full Ca$^{2+}$ response and immunological synapse formation requires at least 10 pMHCII complexes (Irvine et al., 2002). Blocking CD4 impairs T cell Ca$^{2+}$ responses but only if they are stimulated with fewer than 30 pMHCII complexes suggesting that CD4 enhances T cell sensitivity at low ligand density, in particular (Irvine et al., 2002). CD8 T cells can also detect single pMHCI complexes and need approximately 10 pMHCI complexes for a full Ca$^{2+}$ response and immunological synapse formation (Purbhoo et al., 2010). Interestingly, 3 pMHCI were sufficient to enable CD8 T cell target cell killing suggesting that immunological synapse formation and full T cell activation might be dispensable for cytotoxicity (Purbhoo et al., 2004). In addition to antigen-derived peptides, infected DCs also present spMHC to T cells and while a single cognate pMHC complex is insufficient for T cell activation, heterodimers of cognate and spMHC could activate T cells suggesting that in addition to their role in positive selection and T cell survival, spMHC contribute to T cell activation (reviewed in Stefanova et al., 2003).
1.4.4 Effector αβ T cells

Following T cell activation and expansion, CD4 and CD8 T cells differentiate into effector cells. CD4 effector cells help B cells make antibody, sustain CD8 responses, and modulate the magnitude and duration and immune response (reviewed in Zhu et al., 2010). Initially, two distinct populations of differentiated CD4 T cells were characterised based on their cytokine productions, namely T helper 1 (Th1) cells, which make IFNγ, IL-2 and some TNFα and Th2 cells, which are characterised by their IL-4, IL-5 and IL-13 production (Mosmann et al., 1986). The in vitro differentiation of these CD4 effector subsets was also shown to depend on differential cytokine signalling with Th1 differentiation depending on IL-12 and IFNγ and Th2 differentiation requiring IL-4 as well as IL-2 (reviewed in Zhu et al., 2010). Subsequently more CD4 effector subsets have been described including Th17 cells, which produce IL-17A, IL-17F and IL-22 and can be differentiated in vitro in the presence of TGFβ and IL-6 (reviewed in Zhu et al., 2010). Furthermore, Treg develop in the thymus (see section 1.3.5) but can also be derived from naïve T cells in the periphery, which this is thought to require TGFβ and IL-2 (reviewed Josefowicz et al., 2012). In addition to cytokine and TCR signals, the differentiation of naïve CD4 T cells into effector cells also requires the expression of specific transcription factors and STAT proteins (reviewed Zhu et al., 2010). Th1 cells express T-bet and STAT4, Th2 cells Gata3 and STAT5, Th17 cells RAR-related orphan receptor γT (RORγT) and STAT3 and iTreg Foxp3 and STAT5 and together these transcription factors collaborate within a complex network that positively and negatively regulates Th lineage commitment (reviewed in Zhu et al., 2010).
Activated CD8 T cells differentiate into cytotoxic T cells, which express granzyme B and perforin to kill antigen-expressing target cells and produce antiviral cytokines such as IFNγ and TNFα (reviewed in Parish and Kaech, 2009). IL-12 and IFNα/β are particularly important for their differentiation and expansion in vivo (Cousens et al., 1999; Thompson et al., 2006) but also play a role in the induction of IFNγ production (Nguyen et al., 2002). The transcription factors eomesodermin (Pearce et al., 2003) and T-bet (Sullivan et al., 2003) are both expressed in cytotoxic CD8 T cells and thought to be important for their full effector differentiation. Consistent with this, T-bet deficiency leads to impaired cytotoxicity and reduced IFNγ production (Sullivan et al., 2003). Naïve CD8 T cells appear to give rise to two phenotypically distinct subsets of effector cells based on their expression of IL-7Rα and the senescence marker killer cell lectin-like receptor subfamily G 1 (KlrG1) (reviewed in Zhang and Bevan, 2011). The majority of CD8 effector cells terminally differentiate and become short-lived effector cells, expressing low IL-7Rα and high KlrG1. Some effector cells express high IL-7Rα and low KlrG1 and these cells are thought to specifically represent the precursors to memory CD8 T cells (Kaecch et al., 2003). Interestingly, a single naïve CD8 T cell can give rise to both the short-lived effector cells and the memory precursors in vivo (Gerlach et al., 2010; Stemberger et al., 2007) and this is possibly the result of asymmetric T-bet distribution during cell division (Chang et al., 2011). There is also evidence, however, that CD8 effector cell heterogeneity is fixed before the first cell division takes place and that early IFNγ activation and strong pMHC signalling are linked to increased production of IL-2, IFNγ and TNFα as an effector CD8 T cell (Beuneu et al., 2010). Meanwhile, the anti-inflammatory cytokine IL-10 might
play a particularly important role in the differentiation of CD8 memory precursors and fewer IL-7Rαhi memory precursors are generated in IL-10-deficient mice in response to *Listeria monocytogenes* (Foulds et al., 2006).

### 1.4.5 Memory T cells

Following the resolution of an infection most CD4 and CD8 effector T cells die. The cells that survive this contraction phase become long-lived memory T cells which have enhanced functional capacity, can quickly proliferate and re-express effector genes when re-challenged (reviewed and in Parish et al., 2009 and in Pepper and Jenkins, 2011). Memory T cells can be divided into two subsets based on their expression of homing receptors CD62L and CCR7 and the resulting migration patterns (reviewed in Sallusto et al., 2004). Central memory cells (T_{CM}) are CD62L^{hi} CCR7^{hi}, produce IL-2 and recirculate to SLOs and BM whereas effector memory cells (T_{EM}) are CD62L^{lo} CCR7^{lo} and recirculate to non-lymphoid tissues (Sallusto et al., 1999). Furthermore, T_{EM} cells retain some of their effector function and CD4 T_{EM} continue to express cytokines such as IFNγ and TNFα and CD8 T_{EM} continue to express granzyme B and perforin (reviewed in Sallusto et al., 2004). Upon re-infection T_{EM} cells will immediately migrate to the site of inflammation and readily display effector function whereas T_{CM} cells retain little effector function but proliferate and differentiate into effector cells upon antigen stimulation (reviewed in Sallusto et al., 2004). T_{EM} cells can also become long-term tissue residents in the small intestine, skin, brain, lung and salivary glands where they are characterised by high expression of CD103, CD69 and granzyme B but express less IL-7Rα and IL-15Rβ than recirculating T_{EM} (reviewed in Masopust and Schenkel, 2013).
The enhanced ability of memory T cells to control re-infection is based on their longevity but is also the result of enhanced TCR signalling. Memory T cells have a greater phosphoprotein content before TCR ligation (Kersh et al., 2003). Proximal TCR induced signalling events are similar in memory and naïve T cells, however, memory T cells phosphorylate LAT, ERK, Jnk and p38 more efficiently following TCR stimulation resulting in enhanced TCR sensitivity (Kersh et al., 2003). Interestingly, despite these clear signalling and functional differences, very few genes are expressed at significantly different levels in naïve and memory T cells (reviewed in Weng et al., 2012). The kinetics of gene expression following activation, however, is different in naïve and memory T cells with certain genes being upregulated much faster in memory than naïve T cells. These differences are largely thought to be regulated epigenetically (reviewed in Weng et al., 2012).

1.5 Peripheral αβ T cell homeostasis

Peripheral T cell homeostasis involves the strict regulation of T cell development, on the one hand, and T cell proliferation and survival, on the other hand. Competition for limiting resources, including spMHC and cytokines like IL-7 and IL-15 shapes and regulates the overall size and composition of the peripheral T cell pool (Freitas and Rocha, 2000). Increased resources lead to enhanced T cell survival and proliferation while reduced resources limit T cell numbers (Freitas and Rocha, 2000). Naïve and memory T cell proliferation and survival are regulated largely independently although CD4 and CD8 T cells occupy the same niche (reviewed in Freitas and Rocha, 2000).
T cells proliferate even in the absence of antigen and, the factors that govern this lymphopaenia-induced proliferation (LIP) are also important regulators of naïve αβ T cell survival (reviewed in Surh and Sprent, 2008). Although most prominent following T cell depletion, homeostatic proliferation is thought to occur all the time as a mechanism to regulate the overall size of the naïve T cell pool. Despite the absence of antigen, homeostatically proliferating T cells gradually and irreversibly acquire a memory phenotype including increased CD44 expression and more rapid IFNγ and granzyme B production following TCR activation (Min et al., 2005). In fact, OT-I TCR transgenic memory cells generated during homeostatic proliferation provided similar protection against *Listeria monocytogenes* infection as conventional memory T cells (Hamilton et al., 2006). Both CD4 and CD8 naïve T cells undergo homeostatic proliferation and LIP although CD8 T cells proliferate more rapidly than CD4 T cells (reviewed in Surh and Sprent, 2008).

LIP depends on both IL-7 (Schluns et al., 2000; Tan et al., 2001), spMHC (Ernst et al., 1999; Goldrath and Bevan, 1999) and access to SLO where these signals are thought to be concentrated (Dai and Lakkis, 2001; Dummer et al., 2001). Enhanced homeostatic proliferation in lymphopaenic hosts is thought to be the result of increased availability of spMHC and IL-7. The exact mechanisms by which TCR and IL-7Rα regulate homeostatic proliferation are not fully characterised (reviewed in Surh and Sprent, 2008). Interestingly, similar spMHC ligands are thought to mediate positive selection in the thymus and peripheral homeostatic proliferation (Ernst et al., 1999; Goldrath and Bevan, 1999; Viret et
al., 1999). Furthermore, the TCR affinity for spMHC correlates with the rate of proliferation (Kassiotis et al., 2003; Kieper et al., 2004). The Y449 residue of the IL-7Rα is required for IL-7-induced proliferation and is also important for IL-7-induced Bcl-2 synthesis and recruitment of STAT5 to IL-7Rα (Jiang et al., 2004). Transgenic Bcl2 expression, however, rescues αβ T cell development in IL-7Rα-deficient mice (Akashi et al., 1997) but fails to rescue LIP (Tan et al., 2001) suggesting that IL-7-induced proliferation is regulated differently from IL-7-induced survival during T cell development. In fact, IL-7 regulation of proliferation is more likely mediated by the cyclin-dependent kinase inhibitor p27kip1, which is thought to be destabilised by IL-7 (Li et al., 2006). Consistent with this, p27kip1 overexpression induces cell cycle arrest in vitro in the presence of IL-7 whilst p27kip1 deficiency promotes cell cycle entry in vitro in absence of IL-7 (Li et al., 2006). Furthermore, p27kip1 deficiency allows CD4 and CD8 T cells to undergo some proliferation in IL-7-deficient hosts although to a lesser extent than WT cells in IL-7 sufficient lymphopaenic hosts (Li et al., 2006)

1.5.2 Regulation of apoptosis

The number of peripheral naïve T cells is a consequence of T cell development in the thymus and homeostatic proliferation in the periphery but is also the result of peripheral T cell survival, which critically depends on TCR and cytokine receptor signalling (reviewed in Surh and Sprent, 2008). In the absence of appropriate survival signals naïve and memory T cells die by apoptosis, characterised by caspase-induced DNA and cell fragmentation, nuclear shrinkage, cell blebbing and plasma membrane changes before the dying cell is removed by phagocytes (reviewed in Krammer et al., 2007). Initiator caspases
are activated downstream of mitochondrial outer membrane permeabilisation (MOMP), which leads to the release of soluble cytochrome C into the cytosol. Cytochrome C oligomerises with Apaf1 to form the apoptosome, which binds and activates initiator caspases like caspase-8 and -9 (reviewed in Tait and Green, 2010). Active caspase-8, in turn, activates effector caspases such as caspase-3 and -7 which ultimately induce apoptosis (reviewed in Tait and Green, 2010). Alternatively, caspases can be activated downstream of ligands including TNFα, CD95L and Fas ligand (FASL) resulting in the formation of a death-inducing signalling complex (DISC) which recruits and activates initiator caspases. In certain cells, this extrinsic pathway requires amplification involving caspase-8-mediated cleavage and activation of Bid to tBid leading to MOMP (reviewed in Krammer et al., 2007). Hepatocytes are known to require this amplification and Fas injection does not induce hepatocellular apoptosis in Bid-deficient mice in vivo (Yin et al., 1999). In fact, most vertebrate cells require MOMP for caspase activation and apoptosis (reviewed in Tait and Green, 2010).

MOMP activation is regulated by pro- and anti-apoptotic members of the Bcl-2 family, which interact with Bcl-2-homology-domain-3-(BH3)-only proteins, so named because they all contain a BH3 domain, although they share little further homology (summarised in Figure 1.4) (reviewed in Chipuk et al., 2010). Anti-apoptotic Bcl-2 proteins are typically located within the outer mitochondrial membrane and include Bcl-2, B cell leukaemia/lymphoma x (Bcl-xL), Bcl-2-related protein A1 (A1) and myeloid cell leukemia sequence 1 (Mcl-1), which mostly inhibit pro-apoptotic Bcl-2 proteins directly (reviewed in Chipuk et al., 2010). BH3-only proteins include Bcl-2 associated antagonist of cell death (Bad) and phorbol-12-myristate-13-acetate-(PMA)-induced protein 1 (Noxa), which
only interact with anti-apoptotic Bcl-2 proteins, and Bid, Bcl-2 interacting mediator of cell death (Bim) and Bcl-2 modifying factor (Bmf), which can interact both with the anti-apoptotic Bcl-2 proteins and the effector proteins Bcl-2 antagonist/killer (Bak) and Bax (reviewed in Chipuk et al., 2010). Activation and subsequent oligomerisation of Bak and Bax within the outer mitochondrial membrane is essential for the initiation of MOMP and cells lacking both Bak and Bax fail to undergo MOMP or apoptosis (Wei et al., 2001). Activation of Bak and Bax involves substantial conformational changes, which result in the targeting of Bax to the outer mitochondrial membrane and the oligomerisation of both Bak and Bax into pores within the outer mitochondrial membrane to promote MOMP (reviewed in Chipuk et al., 2010). Both Bim and Bid can interact with and activate Bax directly (Gavathiotis et al., 2008; Lovell et al., 2008) although it seems that additional mechanisms for activating Bak and Bax exist since Bak and Bax activation and subsequent apoptosis can also occur in absence of Bim or Bid (Willis et al., 2007). Other BH3-only proteins including Bad, Bik, Hrk and Puma induce apoptosis by interacting with anti-apoptotic Bcl-2 family members preventing their inhibition of direct activators like Bim and Bid (reviewed in Chipuk et al., 2010). In addition, to sensitisation and derepression, BH3-only protein neutralisation of anti-apoptotic Bcl-2 proteins might be sufficient for MOMP and apoptosis independently of Bid and Bim (Willis et al., 2007) although apoptosis is thought to be most efficient when it involves Bid, Bim as well as sensitisation and derepression (reviewed in Chipuk et al., 2010).

The binding of anti-apoptotic Bcl-2 proteins by BH3-only proteins mediated by their BH3 domain is essential for the initiation of apoptosis. Interestingly, the affinity of BH3-only proteins for anti-apoptotic Bcl-2 proteins varies substantially
Bim and Puma interact similarly with all anti-apoptotic Bcl-2 proteins but Bad only binds Bcl-2 and Bcl-xL strongly, A1 weakly and Mcl-1 not at all (Chen et al., 2005b). Noxa, meanwhile only binds Mcl-1 and A1 and thus cooperates with Bad to induce apoptosis (Chen et al., 2005b). The BH3 domain is thought to confer binding properties to BH3-only proteins and consistent with this, chimeric Bim expressing the BH3 domain of Puma, Bad or Noxa behaved like Puma, Bad or Noxa, respectively (Chen et al., 2005b). The binding properties of Bmf are similar to Bad and despite originally being discovered following co-immunoprecipitation with Mcl-1 (Puthalakath, 2001) Bmf only weakly binds Mcl-1 (Chen et al., 2005b). Both Bim and Bmf are associated with cytoskeletal components, microtubules (Puthalakath et al., 1999), and actin filaments (Puthalakath, 2001), respectively, and both Bim and Bmf co-immunoprecipitate with Bcl-2 following certain cellular stress (reviewed in Piñon et al., 2008).

Bcl-2 protein activity is regulated in a variety of ways. Bid activation requires cleavage to tBid by caspases or granzyme B. Meanwhile, Bad activity is regulated by phosphorylation of its BH3 domain by Akt following growth factor withdrawal, which promotes its sequestration in the cytoplasm by 14-3-3 proteins (reviewed in Chipuk et al., 2010). Both Bim and Bmf activity is regulated by Erk signalling (Ley et al., 2005; Shao and Aplin, 2012). Interestingly, while Erk-mediated serine phosphorylation of Bim promotes survival by promoting Bim degradation, Jnk-mediated threonine phosphorylation increases the pro-apoptotic potential of Bim possibly by releasing it from microtubules (Lei and Davis, 2003). This threonine residue is conserved in Bmf and is phosphorylated by Jnk in vitro and in vivo (Lei and Davis, 2003).
Cytokines like IL-7 play an essential role in naïve T cell homeostasis and in absence of IL-7 or IL-7Rα, T cell development is impaired resulting in severely reduced thymocytes and peripheral T cell numbers (Freeden-Jeffry et al., 1995; Peschon et al., 1994). IL-7 also regulates naïve T cell survival in the periphery and IL-7Rα-deficient OT-I *Rag1*<sup>-/-</sup> T cells disappear more rapidly than WT OT-I *Rag1*<sup>+/+</sup> T cells after transfer into C57Bl/6 hosts (Schluns et al., 2000). In the study, naïve OT-I T cells were also shown to require IL-7 to undergo homeostatic proliferation (Schluns et al., 2000). WT CD4 and CD8 T cells also required IL-7 for their survival and disappeared more rapidly after transfer into IL-7-deficient hosts than after transfer into C57Bl/6 hosts (Tan et al., 2001). The number of naïve T cells in thymectomised C57Bl/6 mice treated with anti-IL-7Rα antibodies also declines rapidly whereas naïve T cell numbers remain constant in untreated thymectomised control mice reflecting their long half-life (Vivien et al., 2001). Meanwhile, administration of recombinant IL-7 (Geiselhart et al., 2001) or IL-7 overexpression (Kieper, 2002), leads to an increase in naïve and memory T cell numbers as a result of an increase in their basal homeostatic proliferation.

IL-7 is produced by non-haematopoietic stromal and epithelial cells in the thymus (Namen et al., 1988; Wiles et al., 1992), spleen, kidney, liver (Namen et al., 1988) and skin (Heufler et al., 1993) at a fixed rate so that IL-7 levels are regulated by IL-7 consumption (reviewed in Mazzucchelli et al., 2007 and in Fry and Mackall, 2005) (see section 1.2.1). SLOs are thought to be an important source of IL-7 and naïve T cells require access to SLOs in order to undergo...
homeostatic proliferation (Dummer et al., 2001) and survive (Cinalli et al., 2005). Recently, follicular reticular cells (FRCs) in LN T cell zones were shown to produce IL-7 and only purified LN FRCs were able to promote CD4 and CD8 T cell survival \textit{in vitro} in an IL-7- and CCL19-dependent manner (Link et al., 2007). Furthermore, blocking IL-7Rα signalling or access to SLOs \textit{in vivo} lead to similar reductions in naïve T cell numbers in peripheral blood, which is consistent with the view that naïve T cells receive IL-7 survival signals in SLOs (Link et al., 2007).

Despite its clear role in promoting naïve T cell survival \textit{in vivo} the mechanism by which IL-7 prevents T cell death remains controversial. The defect in thymopoiesis in absence of IL-7 signalling is at least partially rescued by transgenic expression of Bcl-2 (Akashi et al., 1997; Maraskovsky et al., 1997) suggesting that IL-7 might regulate mitochondrial homeostasis. Furthermore, deficiency in either Bax (Khaled et al., 2002) or Bim (Pellegrini et al., 2004) also partially rescues T cell development in IL-7Rα-deficient mice. IL-7 induces the upregulation of Bcl-2 (Akashi et al., 1997; Armant et al., 1995; Freedeen-Jeffry et al., 1997; Graninger et al., 2000; Karawajew et al., 2000; Vella et al., 1997) as well as Mcl-1 (Opferman et al., 2003) \textit{in vitro}, which are both anti-apoptotic members of the Bcl-2 family (reviewed in Chipuk et al., 2010). Finally, Bcl-2 expression \textit{in vivo} is reduced in T cells from C57Bl/6 treated with anti-IL-7Rα antibodies (Vivien et al., 2001) and in IL-7Rα-deficient OT-I T cells following an infection (Schluns et al., 2000). In contrast, a number of studies have failed to observe a reduction in Bcl-2 in IL-7Rα-deficient T cells and IL-7 is still able to prevent apoptosis of Bcl-2-deficient T cells (Nakayama et al., 1995) suggesting that, at least in lymphoreplete mice, IL-7 survival signals might be Bcl-2
independent (Jacobs et al., 2010; Pearson et al., 2012). Consistent with this, Bcl-2 expression does not decrease following IL-7 withdrawal in vitro despite considerable cell death (Rathmell et al., 2001). Mitochondrial homeostasis is perturbed in IL-7Rα-deficient cells in lymphoreplete mice as evidenced by their reduced tetramethylrhodamine ethyl ester (TMRE) staining indicative of a reduced mitochondrial membrane potential (Pearson et al., 2011). IL-7 has also been shown to regulated T cell size and glucose metabolism in PI3K-dependent manner in vitro in C57Bl/6 T cells (Rathmell et al., 2001) and T-ALL cells (Barata et al., 2004). It has subsequently been shown that the Y449 residue of IL-7Rα, important for the IL-7-induced activation of STAT5 (Jiang et al., 2004), is required for IL-7-induced upregulation of Glut1 expression at the cell surface (Wofford et al., 2008). However, the PI3K-dependent regulation of glucose metabolism appears to be largely dispensable for the maintenance of T cell viability by IL-7 in vitro (Pearson et al., 2012; Rathmell et al., 2001) and so it remains unclear what the in vivo target is of IL-7-mediated T cell survival signals.

1.5.4 Cytokine regulation of memory T cell survival

In contrast to naïve T cell survival, the survival of CD4 and CD8 memory T cells does not require MHC (Murali-Krishna et al., 1999; Swain, 1999) or TCR (Polic et al., 2001) but like naïve T cells, memory T cells do depend on IL-7 and IL-15 for their survival (reviewed in Surh and Sprent, 2008). CD8 memory T cell homeostasis, in particular, depends on IL-15 signalling and IL-15-deficient mice have fewer memory phenotype CD8 T cells and NK cells but normal numbers of memory phenotype CD4 T cells (Kennedy et al., 2000). Unusually, IL-15 is presented at the cell surface bound to IL-15Rα (Bulanova et al., 2007) and as
such IL-15Rα-deficient mice show a reduction in memory phenotype CD8 T cells and NK cells similar to IL-15-deficient mice (Lodolce et al., 1998). Memory CD8 T cells and NK cells express higher levels of the IL-15R, composed of CD122 or IL-15Rβ and the γc-chain, than memory CD4 T cells (Zhang et al., 1998c) and are thus thought to outcompete them (Purton et al., 2007). Consistent with this, removal of CD8 T cells and NK cells significantly increases the homeostatic proliferation of memory CD4 T (Purton et al., 2007). Memory T cell homeostasis also depends on IL-7Rα signalling and IL-7 overexpression rescues memory phenotype CD8 T cells in IL-15 deficient mice (Kieper, 2002), while IL-7Rα-deficient OT-I memory T cells have a survival defect in vivo that is rescued by transgenic Bcl-2 expression (Carrio et al., 2007). Since memory CD4 T cells express low CD122 and high IL-7Rα, IL-7 signalling is proposed to play a particularly important role in the maintenance of memory CD4 T cells (reviewed in Surh and Sprent, 2008) and is essential for their homeostatic proliferation (Lenz et al., 2004; Purton et al., 2007).

### 1.5.5 TCR signals that regulate naïve T cell survival

In addition to cytokine signal, naïve T cells require TCR signals for their long-term survival in the periphery. That naïve CD4 T cells require spMHC TCR interactions for their survival was first shown by grafting WT thymi into either \(Rag1^{-/-}\) or MHCII α-chain-deficient \(Rag1^{-/-}\) mice (Takeda et al., 1996). Naïve CD4 T cell proliferation was equivalent regardless of MHCII Aα (\(I\-Aα\)) expression but the long-term survival of CD4 T cells was impaired in absence of \(I\-Aα\) (Takeda et al., 1996). Restoring thymic MHCII expression in \(I\-Aβ\)-deficient mice (Cosgrove et al., 1991) rescues CD4 T cell development while CD4 T cells
still rapidly decay in the Aβ-deficient periphery, confirming that contact with MHCII is important for naïve CD4 T cell survival (Rooke et al., 1997). Furthermore, naïve CD4 T cells accumulate in I-Aα-deficient mice that also expressed MHCII Eα (I-Eα) in CD11c+ but not in mice lacking only I-Aα expression (Kontgen et al., 1993) suggesting that MHCII-expressing DCs might provide naïve CD4 T cells with survival signals. Interestingly, the reduced half-life of H-2d-restricted TCR transgenic CD4 T cells transferred into H-2b mice compared to their relative persistence in H-2d mice suggests that TCR spMHC interactions are MHC-restricted (Kirberg et al., 1997). Similarly, H-2b restricted TCR transgenic CD4 T cells survive well after transfer into β2m-deficient mice or C57Bl/6 mice, but fail to persist in I-Aβ-deficient or Balb/c mice, which are H-2d-restricted (Viret et al., 1999). Like CD4 T cells, naïve CD8 T cell survival also depends on MHC-restricted TCR signals and H-2Db restricted HY TCR transgenic CD8 T cells do not persist after transfer into either H-2Db-deficient or β2m-H-2Db-double-deficient mice (Tanchot et al., 1997).

Other studies have suggested spMHC TCR interactions only have a minor role in the long-term survival of naïve CD4 T cells. In particular, the rapid decline of CD4 T cells in absence of MHCII has been suggested to be a failure to undergo homeostatic proliferation rather than a failure to survive (Clarke and Rudensky, 2000). In these studies, naïve CD4 T cells transferred into I-Aβ-deficient hosts show a small defect in survival after 56 days (Clarke et al., 2000) or no survival defect at all (Dorfman et al., 2000) if cell division is taken into account. It has subsequently been shown, however, that I-Aβ-deficient mice are not truly MHCII-deficient (Martin et al., 2003) and that hybrid AαEβ in I-Aβ-deficient mice can support both the homeostatic proliferation and survival of naïve CD4 T cells.
(Martin et al., 2003). Naïve CD4 T cells transferred into MHCII-deficient mice lacking not only I-Aβ but also I-Aα, I-Eα and I-Eβ (Madsen et al., 1999) survive poorly suggesting that naïve CD4 T cell survival is indeed MHC-dependent (Martin, 2006). Interestingly, when the MHCII-deficient mice were also lymphopaenic CD4 T cells fail to proliferate but survive well, suggesting that CD4 T cell survival in lymphopaenia might be MHC-independent (Martin, 2006).

Cre-mediated deletion of Tcra in naïve T cells resulted in reduced half-lives of both CD4 and CD8 naïve T cells (Polic et al., 2001) as did the Tet-inducible deletion of transgenic OT-1 TCRα (Labrecque et al., 2001) suggesting both CD4 and CD8 naïve T cells require TCR expression for their survival under lymphoreplete conditions. Src family kinase expression, was initially thought to be dispensable for naïve T cell survival since naïve CD4 and CD8 T cell survival was unaffected by Lck expression in mice expressing a conditional Lck allele (Seddon et al., 2000). In absence of both Lck and Fyn, however, CD4 and CD8 naïve T cells failed to survive suggesting that Fyn can compensate for Lck to maintain naïve T cell survival in Lck-deficient mice (Seddon and Zamoyska, 2002a). Conversely, the persistence of CD4 and CD8 naïve T cells in absence of Fyn, confirms that Lck alone is also sufficient for naïve T cell survival (Seddon and Zamoyska, 2002a). Interestingly, Lck-deficient naïve T cells failed to undergo homeostatic proliferation, suggesting that the homeostatic TCR signals that promote naïve T cell survival and proliferation are different (Seddon et al., 2000). Furthermore, Fyn alone appears unable to phosphorylate the downstream target of TCR signalling, Zap70 (Lovatt et al., 2006) and so it remains unclear whether Zap70 is required for naïve T cell survival.
The impaired long-term survival of naïve T cells in absence of MHC contact (Dorfman et al., 2000; Witherden et al., 2000) or in absence of Src family kinases (Seddon and Zamoyska, 2002a) correlates with a reduction in the basal phosphorylation of CD3ζ. TCR-associated CD3ζ is phosphorylated in T cells in absence of antigen (van Oers et al., 1993) and a fraction of Zap70 is constitutively associated with this basally phosphorylated CD3ζ (van Oers et al., 1994). Like phosphorylated CD3ζ, naïve T cell CD5 expression is modulated by homeostatic TCR signals and correlates well with constitutively phosphorylated CD3ζ (Smith et al., 2001). It has therefore been suggested that constitutively phosphorylated CD3ζ might represent the transduction of a TCR survival signal although other studies that although dependent on homeostatic TCR signals, constitutively phosphorylated CD3ζ enhances TCR signalling sensitivity upon antigen recognition (Stefanova et al., 2002). Interestingly, a recent study showed that DCs isolated by plastic adherence could both form immunological synapses with naïve T cells and promote their survival \textit{in vitro} in absence of antigen (Revy et al., 2001). Furthermore, this DC-mediated survival signal is at least partially dependent on MHC and requires Src family kinase activity and also correlates with the constitutive phosphorylation of CD3ζ (Feuillet et al., 2005) and so DCs might provide naïve T cells with an MHC-dependent TCR-mediated survival signal. The importance of DCs in promoting naïve T cell survival remains controversial, however, since a more recent study was unable to show any effect of fluorescence-activated-cell-sorting-(FACS)-purified DCs on naïve T cell survival \textit{in vitro} (Link et al., 2007).
1.5.6 Cross-regulation of TCR and IL-7Rα signalling

In view of the importance of TCR and IL-7Rα signalling for T cell homeostasis, it has been proposed that IL-7Rα and TCR signalling feedback mechanisms might regulate one another in T cells in a similar way to the regulation of B cell activating factor receptor (BAFFR) signalling by BCR signalling in B cells (Rickert et al., 2011). In one model, IL-7 and other γc cytokines are proposed to increase $Cd8a$ transcription thereby enhancing TCR spMHC interactions, which in turn block IL-7Rα signalling and thus reduce $Cd8a$ transcription. Evidence to support this cyclical model comes largely from *in vitro* experiments showing that CD8α expression is upregulated following culture with IL-7 (Park et al., 2007). The study also shows that IL-7Rα expression is higher on male HY TCR transgenic T cells, which receive strong TCR signalling *in vivo*, whereas CD8α expression is higher on female HY TCR transgenic T cells (Park et al., 2007). Ultimately, this cross-regulation of TCR and IL-7Rα is proposed to ensure that no CD8 T cell receives excessive TCR or IL-7Rα stimulation (Park et al., 2007).

In an extension of this cyclical model, IL-7R signalling needs to be interrupted by homeostatic TCR signals to prevent cytokine-induced-cell death of naïve CD8 T cells (Kimura et al., 2012). The authors found no evidence of CD4 being subject to a similar ‘co-receptor tuning’ by IL-7 signalling (Park et al., 2007).

Despite the evidence in favour of this model of IL-7Rα-mediated modulation of $Cd8$ transcription, the *in vivo* data correlating CD8, IL-7Rα and CD5 expression are largely derived from TCR transgenic mice, which are commonly lymphopaenic (Ge et al., 2004). Furthermore, HY TCR transgenic T cells from male mice receive substantially stronger TCR signals than HY TCR transgenic
T cells from female mice calling into question the biological significance of ‘co-receptor tuning’ in cells that receive weaker homeostatic TCR signals. Since ablation of Lck has no effect on IL-7Rα-expression in vivo (Seddon and Zamoyska, 2003) it seems likely that TCR signals do not regulate IL-7Rα expressions directly. Consistent with this, a recent study showed that TCR signals did not regulate IL-7Rα expression in naïve T cells, but that TCR signals received during T cell positive selection programmed the IL-7Rα expression that is subsequently maintained on naïve T cells (Sinclair et al., 2011). There is even evidence that TCR signalling, rather than inhibiting IL-7Rα signalling, enhances sensitivity to IL-7 by promoting the localisation of γc in lipid rafts (Cho et al., 2010). It has also been shown that IL-7Rα and CD5 are downregulated, while CD8α is upregulated, in absence of TCR signalling (Takada and Jameson, 2009) and so it remains controversial, whether and how TCR and IL-7Rα signalling are cross-regulated in peripheral CD8 T cells.
1.6 Thesis aims

TCR and IL-7Rα signalling are important for T cell development and naïve T cell survival. In contrast to IL-7Rα signalling the source and downstream targets of TCR survival signals in naïve T cells remain largely uncharacterised. Src family kinases Lck and Fyn are indispensable for naïve T cell survival although Fyn can compensate for Lck-deficiency and only when both Fyn and Lck are deleted is naïve T cell survival impaired (Seddon and Zamoyska, 2002a). Impaired naïve T cell survival also correlates with reduced phosphorylation of CD3ζ in absence of MHC contact (Dorfman et al., 2000; Witherden et al., 2000) or in absence of Src family kinases (Seddon and Zamoyska, 2002a) and Zap70 is constitutively associated with constitutively phosphorylated CD3ζ. TCR-associated CD3ζ is phosphorylated in T cells in absence of antigen (van Oers et al., 1993) and a fraction of Zap70 is constitutively associated with constitutively phosphorylated CD3ζ (van Oers et al., 1994). The role of Zap70 in the transduction of TCR survival signals has not been characterised and neither have the downstream pathways and targets of TCR survival signals in naïve T cells. Using mice that conditionally express Zap70 we hope to investigate the following aspects of Zap70 signalling in naïve T cells:

1) The role of Zap70 in the transduction of TCR survival signals in naïve T cells
2) The ability of two Zap70 mutants to transduce TCR survival signals
3) The downstream targets of TCR survival signals in naïve T cells
4) The temporal requirements of Zap70 signalling during T cell activation and expansion
Figure 1.1 IL-7Rα signalling

Schematic diagram of IL-7Rα signalling initiated by cross-linking of the IL-7Rα chain and the γc chain by IL-7, which induces the recruitment and phosphorylation of Jak1 and Jak3 to the IL-7Rα chain and γc chain, respectively. STAT5 is subsequently recruited to Jak1 and Jak3 and phosphorylated after which STAT5 dimerises and translocate to the nucleus to activate specific gene transcription thought to include a number of Bcl-2 family members. IL-7 also induces the phosphorylation and recruitment of the p85 subunit of PI3K to the plasma membrane where it promotes the conversion of PIP2 to PIP3, which recruits PH-domain-containing proteins, like Akt, to PIP3. Activation of Akt by Pdk1 subsequently regulates Foxo1 phosphorylation and subsequent degradation, which in turn regulates Ccr7, Il7ra and Klf2 transcription. Akt is also thought to regulate GLUT1 localisation and thus glucose uptake.
Figure 1.2 TCR signalling

Schematic diagram of TCR signalling initiated by TCRαβ engagement with spMHC or pMHC, which induces the phosphorylation of ITAMS of the CD3δ, CD3γ CD3ε and CD3ζ chains by Src family kinases Lck or Fyn. Phosphorylated ITAMs then recruit Zap70, which is autophosphorylated and phosphorylated by Lck allowing active Zap70 to phosphorylate its targets, LAT and Slp76. Phosphorylated LAT then associates with Vav1, Nck PLCγ, Slp76, Adap, Itk Gads, Grb2 and Sos1. Further downstream signals then include the activation MAPK and NFκB downstream of PLCγ-mediated conversion of PIP2 to IP3 and DAG. IP3 then induces an increase in intracellular Ca2+, which results in the calcineurin-dependent dephosphorylation and activation of NFAT. DAG activates PKCθ and RasGRP, which in turn lead to the activation of NFκB and AP1, respectively. The association of phosphorylated Slp76 with Vav1 and Nck leads to the recruitment of Cdc42, WASP and Pak, which are involved in the TCR-dependent reorganisation of the cytoskeleton. Finally, Zap70, LAT, Vav1 and PLCγ have also been implicated in the activation of integrins downstream of TCR signalling via the Slp76-mediated recruitment Adap and a number of other adaptor proteins to the plasma membrane.
Figure 1.3 Structure of murine Zap70

Schematic diagram of Zap70 domains showing a C-terminal kinase domain and two tandem N-terminal SH2 domains. Two tyrosine residues in the interdomain linking the SH2 domains and the kinase domain, Y315 and Y319, regulate the auto-inhibitory conformation of inactive Zap70 but are also thought to mediate the adaptor function of Zap70. Y315 and Y319 are mutated to alanine in YYAA mice. Y292 is implicated in the negative regulation of Zap70 function. The SH2 domains are essential for the recruitment of Zap70 to doubly phosphorylated ITAMs of CD3ζ chains and mutation of tryptophan 163 in the C-terminal SH2 domain to cysteine in the SKG mice results in hypomorphic TCR signalling. The tyrosine residues, Y492 and Y493, in the kinase domain, are phosphorylated by Lck or are autophosphorylated to activate Zap70, allowing it to phosphorylate downstream targets.
Figure 1.4 The regulation of apoptosis

Schematic diagram the intrinsic pathway of apoptosis resulting in MOMP and the subsequent release of soluble cytochrome C into the cytosol. Cytochrome C oligomerises with Apaf1 to form the apoptosome, which binds and activates initiator caspases like caspase-8 and -9, which in turn activate effector caspases like caspase-3 and -7. In most vertebrate cells both the extrinsic and intrinsic pathway of apoptosis require the activation of MOMP. MOMP occurs following the activation and oligomerisation of active Bak and Bax, which form mediate MOMP by forming pores in the outer mitochondrial membrane. Anti-apoptotic Bcl-2 proteins typically located within the outer mitochondrial membrane include Bcl-2, Bcl-xL and Mcl-1, which inhibit pro-apoptotic Bcl-2 proteins directly. BH3-only proteins include Bid, Bim and Bmf, which can interact both with the anti-apoptotic Bcl-2 proteins and the effector proteins Bak and Bax but also Bad and Noxa (not depicted), which only interact with anti-apoptotic Bcl-2 proteins,
Chapter 2  Materials and methods

2.1  Mice

2.1.1  Experimental mice used in this study

Table 2.1 List of mice used in this study

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57Bl/6 (Ly5.2)</td>
<td></td>
</tr>
<tr>
<td>Ly5.1 C57Bl/6</td>
<td></td>
</tr>
<tr>
<td>B6 Rag1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>(Mombaerts et al., 1992)</td>
</tr>
<tr>
<td>F5 Rag1&lt;sup&gt;−/−&lt;/sup&gt; (Ly5.2)</td>
<td>(Mamalaki et al., 1993)</td>
</tr>
<tr>
<td>F5&lt;sup&gt;+&lt;/sup&gt;/− Zap70&lt;sup&gt;−/−&lt;/sup&gt; Rag1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Ly5.1 F5 Rag1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>OT-I Rag1&lt;sup&gt;−/−&lt;/sup&gt; (Ly5.2)</td>
<td>(Hogquist et al., 1993)</td>
</tr>
<tr>
<td>IL-7&lt;sup&gt;−/−&lt;/sup&gt; Rag1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>(Freeden-Jeffry et al., 1995)</td>
</tr>
<tr>
<td>β2m&lt;sup&gt;−/−&lt;/sup&gt; Rag1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>(Koller et al., 1990)</td>
</tr>
<tr>
<td>SKG</td>
<td>(Sakaguchi et al., 2003)</td>
</tr>
<tr>
<td>YYAA</td>
<td>(Hsu et al., 2009)</td>
</tr>
<tr>
<td>F5 Rag1&lt;sup&gt;−/−&lt;/sup&gt; Zap70&lt;sup&gt;cre&lt;/sup&gt;rtTA.C&lt;sup&gt;huCD2&lt;/sup&gt;Zap70&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>(Saini et al., 2010)</td>
</tr>
<tr>
<td>F5 Rag1&lt;sup&gt;−/−&lt;/sup&gt; Zap70&lt;sup&gt;cre&lt;/sup&gt;rtTA.C&lt;sup&gt;huCD2&lt;/sup&gt;Zap70&lt;sup&gt;Skg&lt;sup&gt;−/−&lt;/sup&gt;&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>F5 Rag1&lt;sup&gt;−/−&lt;/sup&gt; Zap70&lt;sup&gt;cre&lt;/sup&gt;rtTA.C&lt;sup&gt;huCD2&lt;/sup&gt;Zap70&lt;sup&gt;YYAA&lt;sup&gt;−/−&lt;/sup&gt;&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Mice used in this study (Table 2.1) were bred in a conventional colony under SPF conditions at The National Institute for Medical Research (NIMR), London, UK. Animal experiments were performed according to institutional guidelines and Home Office regulations. Mice strains were genotyped by flow cytometry of blood lymphocytes and PCR analysis of tail DNA. All experimental mice were used at 6-16 weeks of age. C57Bl/6 or mixed mice are on an H-2<sup>b</sup> background.
2.1.2 Generation of F5 TetZap70, F5 TetZap70 Zap70\textsuperscript{Yyaa/} and F5 TetZap70 Zap70\textsuperscript{Skg/} mice

Mice with doxycycline-(dox)-inducible expression of Zap70 (Figure 2.1) were described previously (Saini et al., 2010; Sinclair et al., 2011) but briefly, mice that expressed a Zap70 transgene and downstream internal ribosomal entry site (IRES)-human CD2 (hCD2) tailless reporter under the control of a tetracycline-response element (TRE) (Zap70\textsuperscript{TRE}) (Gossen and Bujard, 1992) were crossed with mice that constitutively expressed a reverse tetracyclin TransActivator (rtTA.C) whose expression was directed to T cells by human CD2 regulatory elements (rtTA.C\textsuperscript{hCD2}) (Legname et al., 2000; Zhumabekov et al., 1995). These mice were further crossed onto an endogenous Zap70-deficient background (Negish et al., 1995). Finally, we crossed these mice with F5 \textit{Rag1}\textsuperscript{-/-} TCR transgenic mice (Mamalaki et al., 1993) generating F5 \textit{Rag1}\textsuperscript{-/-} Zap70\textsuperscript{Tre} rtTA.C\textsuperscript{hCD2} Zap70\textsuperscript{-/-} (intact F5 TetZap70 mice hereon). F5 T cells express an MHCI H-2D\textsuperscript{b} restricted TCR, which recognises αα 366-374 of the influenza nucleoprotein (NP366-374) of influenza A/NT/60/68. F5 TetZap70 were also crossed with YYAA and SKG mice, both on a C57Bl/6 background, to generate F5 TetZap70 Zap70\textsuperscript{Yyaa/-} and F5 TetZap70 Zap70\textsuperscript{Skg/-} mice, respectively.

2.1.3 Radiation bone marrow chimeras

2.1.3.1 Donor BM isolation

BM was extracted from the tibial and femoral bones of Ly5.1 F5 \textit{Rag1}\textsuperscript{-/-} and F5 TetZap70\textsuperscript{unind}, F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Skg/-} mice. Following removal of the epiphyses, BM was isolated by flushing handling media through the medullary canal with a 25-gauge needle and 5mL syringe.
The BM was washed, centrifuged (Multifuge 3 s-r, Heraeus) (300g for 4min at 4°C) and resuspended in handling media before being filtered through a 30μm cell strainer. Ly5.1 F5 \( \text{Rag}^{1/-} \) BM was T-cell-depleted by incubating cell suspensions with biotinylated TCR antibodies (1:100 dilution) at 4°C with rotation for 30 min in handling media (1mL/10⁸ cells) after which samples were washed and resuspended in 4mL phosphate-buffered saline (PBS) (GIBCO). Dynal® beads were added (80x10⁶/4mL) to the cell suspension and incubated at 4°C with rotation for 20 min. Labelled BM cells bound to the Dynal® beads were removed by placing the cell suspension in a magnetic Dynal® bead separator (Dynal®), first for 2min, then for 30sec. Finally, the cell suspension was washed and resuspended in freezing buffer (90% foetal calf serum, FCS, 10%DMSO) at 30x10⁶ cells/mL to be stored at -80°C until use or in handling media at 10-25x10⁶ cells/mL to be used immediately.

2.1.3.2 Host irradiation and bone marrow reconstitution

B6 \( \text{Rag}^{1/-} \) host mice were sub-lethally irradiated with 500 Rad (caesium source) and allowed to reconstitute for a minimum of 6-8hrs. BM cells were filtered through a 35μm-pore cell strainer (BD) and injected intravenously (IV) with \( \sim 200\mu\text{L} \) of 2-5x10⁶ cells into the lateral tail vein of irradiated B6 \( \text{Rag}^{1/-} \) hosts using a 500μL microfine insulin syringe. Hosts were treated with water supplemented with 0.02% (v/v) Baytril® for >6 weeks and were placed on food supplemented with the tetracycline derivative dox (3mg/g). Chimeras were left for at least 6 weeks to allow reconstitution of the haematopoietic compartment, which was confirmed by the presence of CD8⁺ TCR⁺ cells in the peripheral blood, assessed by flow cytometry as described in section 2.3.2 and 2.4.2.
2.2 Media

**ACK lysis buffer**
- dH$_2$O with 150mM NH$_4$Cl, 10mM KHCO$_3$, 0.1mM ethylenediaminetetraacetic acid (EDTA).

**Annexin-V binding buffer**
- Annexin-V binding buffer was made by diluting annexin-V binding buffer 10x concentrate (BD) (0.1M hydroxyethyl piperazineethanesulfonic acid (Hepes) (pH 7.4), 1.4M NaCl, and 25 mM CaCl$_2$).

**Complete medium**
- Roswell Park Memorial Institute 1640 (RPMI) medium (GIBCO) supplemented with 5% (v/v) FCS, 0.5% (w/v) bovine serum albumin (BSA) (Sigma), 4mM L-glutamine (Sigma), 50μM β-mercaptoethanol (Sigma), 100μg/mL penicillin G sodium (Sigma) and 100μg/mL streptomycin sulphate (Sigma).

**Digestion media**
- RPMI medium (GIBCO) supplemented with 1.67 Wünsch units Liberase (Roche) and 0.2mg/mL DNase (Roche).

**Dynabead buffer**
- PBS (GIBCO) supplemented with 0.1% BSA and 2mM EDTA.

**FACS buffer**
- PBS (made in-house) supplemented with 0.5% (v/v) sodium azide and 5% (v/v) FCS.
Handling media
Air-buffered Iscove’s Modified Dulbecco’s medium (AB-IMDM) (made in-house) supplemented with 0.2% (v/v) BSA.

MACS buffer
PBS (GIBCO) supplemented with 0.5% BSA and 2mM EDTA.

2.3 Preparation of single cell suspensions

2.3.1 Thymus, lymph nodes and spleen
The thymus, spleen, cervical, axillary, brachial, mesenteric and inguinal LN were dissected from mice and placed on ice. Single cell suspensions were prepared by gently crushing organs between two layers of 75μm nylon mesh in ice-cold handling media. After this, cells were washed in handling media, centrifuged (300g for 4min at 4°C), resuspended in ice-cold fresh handling media and kept on ice for the remainder of the assay. Cells were then counted using a Casy-1 cell counter (Scharfe System, Germany) after which they were stained as described in section 2.4.2 and 2.4.3.

2.3.2 Peripheral blood
Approximately 100μL of blood was collected from experimental mice by nicking the lateral tail vein with a scalpel blade. Blood was mixed with ~100μL of heparin (Sigma) after which it was lysed in 2mL of ACK lysis buffer for 3min at room temperature (RT) or until the solution appeared transparent (but no longer than 5min). Cells were then washed in 2mL ice-cold FACS buffer, centrifuged (300g for 4min at 4°C) and stained as described in sections 2.4.2 and 2.4.3.
2.3.3 Splenocytes for CD11c+ enrichment

Spleens were dissected and placed on ice after which they were injected with 800μL-1mL digestion media using a 25-gauge needle and a 2mL syringe and placed in a petri dish at 37°C and 5% carbon dioxide (CO₂) for 30min. Digested spleens were then gently crushed in a 70μm sieve using the end of a 1mL syringe and washed in ice cold handling media. Splenocytes were counted (Scharfe System, Germany) and then enriched for CD11c+ cells as described in section 2.7.2.

2.4 Flow cytometry

2.4.1 Antibodies and reagents used for flow cytometry

A list of primary antibodies used for flow cytometry in this study is shown in Table 2.2.

Table 2.2 List of primary antibodies used in this study

<table>
<thead>
<tr>
<th>Marker</th>
<th>Clone</th>
<th>Conjugate</th>
<th>Supplier</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220</td>
<td>RA3-6B2</td>
<td>PE</td>
<td>eBioscience</td>
<td>1μg/mL</td>
</tr>
<tr>
<td>CD11c</td>
<td>HL3</td>
<td>FITC</td>
<td>BD</td>
<td>2.5μg/mL</td>
</tr>
<tr>
<td>CD11c</td>
<td>N418</td>
<td>PE</td>
<td>eBioscience</td>
<td>1μg/mL</td>
</tr>
<tr>
<td>CD19</td>
<td>eBio1D3</td>
<td>PE Cy5</td>
<td>eBioscience</td>
<td>1μg/mL</td>
</tr>
<tr>
<td>CD4</td>
<td>RM4-5</td>
<td>PE TR</td>
<td>Invitrogen</td>
<td>1μg/mL</td>
</tr>
<tr>
<td>CD4</td>
<td>RM4-5</td>
<td>eF450, PE</td>
<td>eBioscience</td>
<td>2μg/mL</td>
</tr>
<tr>
<td>CD44</td>
<td>IM7</td>
<td>APC eF780</td>
<td>eBioscience</td>
<td>0.5μg/mL</td>
</tr>
<tr>
<td>CD45RB</td>
<td>C363.16A</td>
<td>PE</td>
<td>eBioscience</td>
<td>1μg/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td>------------------</td>
<td>---------</td>
<td>---------------</td>
</tr>
<tr>
<td>CD5</td>
<td>53-7.3</td>
<td>FITC, eF450,</td>
<td>eBioscience</td>
<td>1-1.25μg/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PE Cy7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8α</td>
<td>5H10</td>
<td>PO, PE TR</td>
<td>Invitrogen</td>
<td>1μg/mL</td>
</tr>
<tr>
<td>CD8α</td>
<td>SK1</td>
<td>PE Cy7</td>
<td>eBioscience</td>
<td>0.5μg/mL</td>
</tr>
<tr>
<td>hCD2</td>
<td>RPA-2.10</td>
<td>PE Cy5</td>
<td>eBioscience</td>
<td></td>
</tr>
<tr>
<td>HSA</td>
<td>M1/69</td>
<td>FITC</td>
<td>Biolegend</td>
<td>2.5μg/mL</td>
</tr>
<tr>
<td>HSA</td>
<td>M1/69</td>
<td>PE Cy7</td>
<td>eBioscience</td>
<td>0.5μg/mL</td>
</tr>
<tr>
<td>IL-7Rα</td>
<td>A7R34</td>
<td>PE</td>
<td>eBioscience</td>
<td>1μg/mL</td>
</tr>
<tr>
<td>Ly5.1</td>
<td>A20</td>
<td>APC</td>
<td>eBioscience</td>
<td>1μg/mL</td>
</tr>
<tr>
<td>Ly5.2</td>
<td>104</td>
<td>PE</td>
<td>eBioscience</td>
<td>1μg/mL</td>
</tr>
<tr>
<td>Ly5.2</td>
<td>104</td>
<td>FITC</td>
<td>eBioscience</td>
<td>0.5μg/mL</td>
</tr>
<tr>
<td>MHCII</td>
<td>M5/114.15.2</td>
<td>PE Cy7</td>
<td>eBioscience</td>
<td>0.5μg/mL</td>
</tr>
<tr>
<td>MHCII</td>
<td>M5/114.15.2</td>
<td>eF450</td>
<td>eBioscience</td>
<td>1μg/mL</td>
</tr>
<tr>
<td>mPDCA1</td>
<td>JF05-1C2.4.1</td>
<td>FITC</td>
<td>Miltenyi Biotec</td>
<td>9μg/mL</td>
</tr>
<tr>
<td>Qa2</td>
<td>69H1-9-9</td>
<td>FITC</td>
<td>eBioscience</td>
<td>2.5μg/mL</td>
</tr>
<tr>
<td>TCRβ</td>
<td>H57-597</td>
<td>PE Cy5, APC,</td>
<td>eBioscience</td>
<td>1μg/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>APC ef780</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCR Va2</td>
<td>B20.1</td>
<td>PE</td>
<td>eBioscience</td>
<td>1μg/mL</td>
</tr>
<tr>
<td>TCR Vβ11</td>
<td>RR3-15</td>
<td>FITC</td>
<td>BD</td>
<td>2.5μg/mL</td>
</tr>
</tbody>
</table>

A list of recombinant annexin-V conjugates used for flow cytometry in this study is shown in Table 2.3. A list of intracellular antibodies used for flow cytometry in this study is shown in in Table 2.4.
### Table 2.3 List of recombinant annexin-V conjugates used in this study

<table>
<thead>
<tr>
<th>Marker</th>
<th>Conjugate</th>
<th>Supplier</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin-V</td>
<td>FITC</td>
<td>eBioscience</td>
<td>1 in 10</td>
</tr>
<tr>
<td>Annexin-V</td>
<td>APC</td>
<td>BD</td>
<td>1 in 10</td>
</tr>
</tbody>
</table>

### Table 2.4 List of intracellular antibodies used in this study

<table>
<thead>
<tr>
<th>Marker</th>
<th>Clone</th>
<th>Conjugate</th>
<th>Supplier</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zap70</td>
<td>1E7.2</td>
<td>PE</td>
<td>eBioscience</td>
<td>1μg/mL</td>
</tr>
<tr>
<td>Bcl-2 and IgG isotype</td>
<td>3F11</td>
<td>PE</td>
<td>BD</td>
<td>0.1mg/mL</td>
</tr>
<tr>
<td>and IgG isotype</td>
<td>A19-3</td>
<td>PE</td>
<td>BD</td>
<td>0.1mg/mL</td>
</tr>
</tbody>
</table>

APC – Allophycocyanin; APC eF780 – Allophycocyanin e-Fluor 780; eF450 – e-Fluor 450; eF780 – e-Fluor 780; FITC – Fluorescein isothiocyanate; PE – Phycoerythrin; PE Cy5 – Phycoerythrin cyanine 5; PE Cy7 – Phycoerythrin cyanine 7; PerCP – Peridinin-chlorophyll protein; PE TR – PE Texas red; PO – Pacific orange.
2.4.2 Surface staining

2.4.2.1 Primary staining and flow cytometry

Antibodies were made up to concentrations as indicated in Table 2.2 in ice-cold FACS buffer. Cells were incubated for at least 40min at 4°C and in the dark in 100-200μL at a density of 1-5x10^6 with antibodies conjugated to fluorescent labels. Cells were then washed 1-2 times in ice-cold FACS buffer, centrifuged (300g for 4min at 4°C) and resuspended in 100-300μL of FACS buffer before being filtered through a 35μm-pore cell strainer (BD). Flow cytometry was performed on the CyAN ADP (Beckman Coulter), BD FACSCantoll (BD), BD LSRII (BD) and FACSCalibur (Becton Dickinson) and data analysis was performed using FlowJo software (Tree Star).

2.4.2.2 Recombinant annexin-V staining

Cells were first stained for surface antigens as detailed in section 2.4.2.1 after which cells were washed in annexin-V binding buffer, centrifuged (300g for 4min at 4°C) and resuspended in 100μL annexin-V binding buffer with recombinant annexin-V made up to concentrations as indicated in Table 2.3. Cells were then incubated for 15min at RT and in the dark at a density of 1-5x10^6 after which they were washed in annexin-V binding buffer, centrifuged (300g for 4min at 4°C) and resuspended in 100-300μL of FACS buffer before being filtered through a 35μm-pore cell strainer (BD) and analysed by flow cytometry.
2.4.2.3 Intracellular flow cytometry

Cells were first stained for surface antigens as detailed in section 2.4.2.1 after which they were washed in PBS (GIBCO), centrifuged (300g for 4min at 4°C) and fixed with IC fixation buffer (eBioscience) at RT for 10min. After this, cells were washed again in PBS (GIBCO), centrifuged (300g for 4min at 4°C) and resuspended in 0.1% NP40 (Igepal ca-360) (Sigma-Aldrich) in dH₂O (v/v) for 3min at RT. Cells were then washed, centrifuged (300g for 4min at 4°C) and resuspended in 100-200μL ice-cold FACS buffer with intracellular (or isotype control) antibodies made up to concentrations as indicated in Table 2.4. Cells were incubated overnight at 4°C and in the dark and were washed in FACS buffer, centrifuged (300g for 4min at 4°C) and resuspended in 100-300μL of FACS buffer before being filtered through a 35μm-pore cell strainer (BD) and analysed by flow cytometry.

2.4.3 Calculation of absolute cell numbers and number of precursors

2.4.3.1 Calculation of absolute cell numbers

In order to calculate T cell recoveries from different hosts the following calculation was used:

Total single live cell number (counted by Casy-1 cell counter)  \( a \)

Frequency of total single live lymphocytes  \( b \)

Frequency specified cell population  \( x \)

Absolute number of cells within specified cell population  \( x \)  \( a.b.c \)
2.4.3.2 Calculation of number of precursors

Analysis of cell division by CellTrace™ Violet (CTV) labeling involved the following calculations to determine the total number of precursors that generated the observed number of cells within a specific cell population $x$:

- Absolute number of cells within specified cell population $x$: $a \cdot b \cdot c$
- Frequency of cells in division $i$ within $x$: $f_i$
- Absolute number of cells in division $i$ (within $x$): $N_i = a \cdot b \cdot c \cdot f_i$

Number of precursors required to divide $i$ times to generate the observed number of cells within division $i$ (within $x$): 

$$n_i = \frac{N_i}{2^i}$$

Total number of precursors required to divide $i$ times to generate the total observed number of cells within all divisions (within $x$):

$$\sum_{i=0}^{\infty} n_i$$

2.5 In vivo experiments

2.5.1 In vivo T cell survival in intact chimeras and mice

In vivo survival of F5 T cells in F5 TetZap70, F5 TetZap70 Zap70$^{Yyaa^{-}}$ and F5 TetZap70 Zap70$^{Skg^{-}}$ chimeras and mice was assessed by following the frequency of peripheral blood lymphocytes in a cohort of chimeras maintained on dox in comparison to a cohort of chimeras taken off dox-supplemented food. Peripheral blood was obtained as detailed in section 2.3.3 and stained as described in section 2.4.1.
2.5.2 CTV staining before adoptive transfer experiments

Cells isolated as described in 2.3.1 were counted, centrifuged (300g for 4min at 4°C) and resuspended in PBS (GIBCO) pre-warmed at 37°C at a density of 20x10^6/mL. The cell suspension was then combined with an equal volume of 2.5-5μM CTV in pre-warmed PBS (GIBCO) and incubated for 10min at 37°C (final cell density 10x10^6/mL, final CTV concentration 1.25-2.5μM). Cells were washed twice in at least 4x more PBS (GIBCO), centrifuged (300g for 4min at 4°C) and re-counted before being resuspended at 5x10^6/mL in handling media and injected into donor mice as detailed in sections 2.5.3.1 and 2.5.3.2.

2.5.3 Adoptive transfer experiments – homing, survival and proliferation

Cells isolated as described in 2.3.1 were counted, centrifuged (300g for 4min at 4°C) and resuspended in handling media before being filtered through a 35μm-pore cell strainer (BD). Filtered cells were CTV-labelled as described in section 1.5.2 and pooled with control cells as indicated, after which they were injected by IV administration of ~200μL of 1x10^6 cells into the lateral tail vein of host mice using a 500μL microfine insulin syringe. To assess the proliferation response to influenza, mice were additionally injected IV with 100 haemagglutinating units (HAU) of virus (A/NT/60-68) provided by Dr Andreas Wack, as previously described (Saini et al., 2009). Where cells were pooled with control cells, the ratio of Ly5.1 to Ly5.2 cells was assessed before injection. Hosts were placed on dox-supplemented food, where indicated, and bled or sacrificed after the indicated number of days to assess the frequency or expansion of injected cells by flow cytometry as detailed in section 1.4.2.
2.6 Cell purification by FACS

Cells isolated as described in 2.3.1 were stained for surface antigens as detailed in section 2.4.2.1, using handling media instead of FACS buffer. Cells were then washed 1-2 times in ice-cold handling media, centrifuged (300g for 4min at 4°C) and resuspended at a density of ~50x10^7/mL of handling media before being filtered through a 35μm-pore cell strainer (BD). FACS sorting was performed on the MoFlo XDP (Beckman Coulter) or BD FACSaria (BD) cell sorters. Sorted cells were collected in ice-cold handling media and used in vitro as described in section 1.7 or for mRNA-sequencing as described in section 1.8.

2.7 In vitro experiments

2.7.1 In vitro T cell survival

Cells isolated as described in 2.3.1 were FACS-purified where indicated and cultured at 37°C in a Sanyo CO₂ incubator (Sanyo) at 5% CO₂ for 24, 48 or 27h as indicated in 24-well plates in 2mL of complete media at a density of 0.5x10^6/mL. Complete medium was supplemented with IL-7 at 10ng/mL unless otherwise specified. Where indicated FACS-purified CD4 T cells, B cells or DC subsets or splenocytes enriched for CD11c^+ cells as described in section 1.7.2 were added to the T cells in addition to inhibitors at the following concentrations: UO126 (10uM) (Sigma), rapamycin (20nM) (VWR) and LY294002 (10uM) (Callbiochem) or the corresponding volume of DMSO. Cells were harvested at the indicated time and analysed by flow cytometry as described in section 2.4.
2.7.2 CD11c⁺ enrichment

Splenocytes isolated as described in section 2.3.3 were enriched for CD11c⁺ cells either by MACS purification of CD11c PE⁺ cells or by negative depletion of CD11c⁻ cells using a Dynabead® Mouse DC Enrichment Kit (Invitrogen). Similar post-enrichment purity was achieved using either method.

MACS® purification

Splenocytes were washed in MACS buffer, centrifuged (300g for 4min at 4°C) and passed through an LS column, unlabelled, to remove the majority of dead and adherent cells. Cells were then washed again, centrifuged (300g for 4min at 4°C) and counted before being incubated with for CD11c conjugated to PE at 2µg/mL at a density of 1-10x10⁷/mL for 20-30min at 4°C and in the dark. After this, cells were washed in MACS buffer, centrifuged (300g for 4min at 4°C) and incubated with anti-PE MicroBeads (Myltenyi Biotec) (1:5) in MACS® buffer at a density of 10x10⁷/mL for 15min at 4°C with constant rotation. Cells were then washed again in MACS buffer, centrifuged (300g for 4min at 4°C) and resuspended at a density of 1-10x10⁷/mL after which they were passed through a second, fresh LS column in the magnetic field of a MACS® separator (Myltenyi Biotec). Labelled cells were collected in 5mL of MACS buffer, centrifuged (300g for 4min at 4°C) and counted to be used for in vitro experiments as described in section 2.7.1 or to be analysed by flow cytometry as described in section 2.4.
Dynabead® purification

Splenocytes were washed in Dynabead buffer, centrifuged (300g for 4min at 4°C) and counted before being incubated with the Dynabead® Mouse DC Enrichment Kit Antibody Mix (Invitrogen) (1:6) in Dynabead buffer at a density of 1-10^7/mL for 20min at 4°C with constant rotation. Cells were then washed in Dynabead buffer, centrifuged (300g for 4min at 4°C) and incubated with calibrated Depletion MyOne™ SA Dynabeads® (Invitrogen) (1:10) in Dynabead buffer at a density of 1-10x10^6/mL for 15min at 4°C with constant rotation. Labelled splenocytes bound to the Dynabeads were removed by placing the cell suspension in a magnetic Dynal® bead separator (Dynal), first for 2min, then for 30sec. Unlabelled cells were then counted to be used for in vitro experiments as described in section 2.7.1 or to be analysed by flow cytometry as described in section 2.4.

2.8 mRNAseq

2.8.1 RNA isolation

FACS-purified T cells as described in section 1.6 were washed in PBS (GIBCO), centrifuged (300g for 4min at 4°C) and counted before being lysed in 750uL Trizol® (Invitrogen) in Non-Stick RNase-free Microfuge Tubes (Invitrogen) at a density of 2-10x10^6/mL. Samples were mixed by repetitive pipetting and incubated at RT for 10min after which 200μL of phenol-chloroform was added. Samples were then shaken vigorously for 15sec and left for 2-3min at RT to allow phase separation. Samples were centrifuged at 12,000rp for 15mim at 4°C and the aqueous phase was transferred into a fresh microfuge tube with an
equal volume of isopropanol. Samples were left for 10min at RT and then centrifuged at 21,000g for 30min at 4°C before the supernatant was carefully removed. Pellets were washed in 300μL of 70% ethanol in dH₂O (v/v) and centrifuged at 21,000g for 5min at 4°C. The RNA pellet was then resuspended in 15uL UltraPure™ DEPC-Treated Water (Invitrogen) and RNA was quantified by Nanodrop and a Total RNA Nanochip.

2.8.2 Sequencing

Libraries for sequencing were prepared with the mRNA Seq 8-Sample Preparation Kit (Illumina) according to manufacturer's instructions. Briefly, >100ng RNA was first fragmented after which two strands of cDNA were synthesised. After this cDNA 3’ and 5’ overhangs were converted to blunt ends using T4 DNA polymerase and Klenow DNA polymerase. Finally, 3’ ends of blunt DNA fragments were adenylated and adapters were ligated to the ends of DNA fragments before the 200-300bp-long DNA fragments were purified using an E-Gel® SizeSelect Gel (Invitrogen). 80-100ng of enriched DNA was then normalised using Duplex-Specific Thermostable Nuclease (DSN) in the DSN Kit (Illumina) (Christodoulou et al., 2011). Samples were sequenced at the MRC NIMR High-Throughput Sequencing Facility and 40 base-pair single-end reads were obtained using an Illumina Genome Analyser IIm.

2.8.3 Quantification of gene expression

Reads were aligned to the Mus musculus genome in the CLC Genomics Bench (V5) (CLC Bio) with default settings and exported to Avadis NGS (V1.3.1) (Strand Scientific Intelligence) to be mapped to the Refseq database. Mapped reads were then normalised using DEseq, as described previously (Anders and
Huber, 2010) and displayed as normalised reads per kilobase of exon per million mapped reads (nRPKM) (Mortazavi et al., 2008).

2.9 Calculations and statistical testing

The statistical significance of differences between and within groups and time-points was assessed using one- and two-way analysis of variance followed by Bonferroni correction for multiple comparisons and were calculated in Prism 5 and 6 for Mac OS X (Graphpad). F5 T cell half-lives were estimated from time-course data fitted with non-linear regression one-phase decay curves in Prism 5 and 6 for Mac OS X (Graphpad).
Figure 2.1 Dox-inducible expression of Zap70 in F5 TetZap70 mice

Schematic diagram of dox-inducible Zap70 in F5 TetZap70 mice which constitutively express a reverse tetracyclin TransActivator (rtTA.C) directed to T cells by human CD2 regulatory elements (rtTA.C$^{hCD2}$) (1-2). They also express a Zap70 transgene (yellow) and downstream internal ribosomal entry site (IRES)-human CD2 (hCD2) tailless reporter (grey/green) under control of tetracycline-response elements (TRE) (Zap70$^{TRE}$). In the presence of dox or any other tetracycline-derivative in vivo or in vitro (3) rtTA is able to interact with the TRE (4), where it acts as a transcription factor inducing the transcription of Zap70 and hCD2 (5). T cells in F5 TetZap70 mice also express the F5 TCR and are Rag1$^{-/-}$ so that they all recognise influenza nucleoprotein (NP366-374). Finally, F5 TetZap70 mice are also Zap70$^{-/-}$ that the only Zap70 expressed is dox-inducible transgenic Zap70.
Chapter 3  The role of Zap70 in naïve T cell survival \textit{in vivo}

3.1 Introduction

Both cytokine and TCR signalling play a crucial role in the survival of naïve T cells and competition for these signals maintains the size of the peripheral T cell pool (Seddon and Zamoyska, 2003). IL-7 is essential for naïve T cell homeostasis \textit{in vivo} and mice lacking either IL-7 (Freeden-Jeffry et al., 1995) or its receptor, IL-7Rα (Maraskovsky et al., 1997; Peschon et al., 1994) have reduced number of peripheral T cells. Increasing IL-7 availability, leads to an increase in the size of the peripheral T cell pool and favours homeostatic proliferation suggesting that in normal lymphoreplete hosts IL-7 limits the size of the peripheral T cell pool (Geiselhart et al., 2001; Kieper, 2002). Meanwhile, naïve T cell survival \textit{in vivo} is impaired in absence of IL-7 signalling (Schluns et al., 2000; Seddon and Zamoyska, 2002b; Tan et al., 2001; Vivien et al., 2001).

In addition to cytokine signals, naïve T cells also require TCR interactions with spMHC for their survival signal and both CD4 (Bröcker, 1997; Kirberg et al., 1997; Martin, 2006; Rooke et al., 1997; Takeda et al., 1996; Viret et al., 1999; Witherden et al., 2000) and CD8 (Tanchot et al., 1997) T cell survival is impaired in absence of MHCII or MHCI, respectively. The spMHC TCR interactions required for survival are MHC-restricted and H-2\textsuperscript{d}-restricted TCR transgenic CD4 T cells persist after transfer into H-2\textsuperscript{d} mice but not after transfer into H-2\textsuperscript{b} mice (Kirberg et al., 1997). The same is true for CD8 T cells and H-
2D\textsuperscript{b}-restricted HY TCR transgenic CD8 T cells are not maintained after transfer into H-2D\textsuperscript{b}-deficient mice (Tanchot et al., 1997). TCR expression is also required for the prolonged survival of naive CD4 and CD8 T cells but is dispensable for memory T cell survival (Labrecque et al., 2001; Polic et al., 2001). Furthermore, transduction of this TCR survival signal also requires expression of Src family kinases (Seddon and Zamoyska, 2002a). Fyn or Lck alone are sufficient to transduce TCR survival signals but Lck is essential for TCR-mediated homeostatic proliferation (Seddon et al., 2000). Basal phosphorylation of CD3\(\zeta\) chains in T cells has been shown to correlate with T cell survival and depends on the expression of MHC (Dorfman et al., 2000; Witherden et al., 2000) and Src family kinases (Seddon and Zamoyska, 2002a). This basal CD3\(\zeta\) phosphorylation has also been shown to correlate with T cell surface CD5 expression (Smith et al., 2001) and is thought to represent the transduction of homeostatic TCR survival signals. Zap70 has been shown to associate with constitutively phosphorylated CD3\(\zeta\) (van Oers et al., 1994) yet while Fyn can transduce TCR survival signals, it cannot efficiently phosphorylate Zap70 (Lovatt et al., 2006).

In this chapter, we hope to investigate the requirement of Zap70 for the transduction of TCR survival signals using F5 \textit{Rag1}\textsuperscript{-/-} mice expressing dox-inducible Zap70 on an endogenous Zap70-deficient background. We anticipate that transgenic Zap70 expression will overcome the block in T cell development in Zap70\textsuperscript{-/-} mice (Negishii et al., 1995). Following dox-withdrawal we then hope to study the survival of Zap70-deficient F5 \textit{Rag1}\textsuperscript{-/-} T cells \textit{in vivo}.
3.2 Results

3.2.1 Reconstitution of T cell development in F5 TetZap70 mice following transgene induction

Since Zap70−/− mice have a block in T cell development and thus no peripheral T cells (Negishi et al., 1995), we used mice that expressed a conditional Zap70 transgene to study the role of Zap70 in mediating a TCR survival signal in naive T cells. Mice that expressed a Zap70 transgene and downstream IRES-hCD2 tailess reporter under the control of a TRE (Zap70TRE) (Gossen and Bujard, 1992; Saini et al., 2010; Sinclair et al., 2011) were crossed with mice that constitutively expressed rtTA directed to T cells by human CD2 regulatory elements (rtTA.ChCD2) (Legname et al., 2000; Zhumabekov et al., 1995). To ensure that all Zap70 expression was due to the inducible transgene, these mice were further crossed onto an endogenous Zap70-deficient background (Negishi et al., 1995). Finally, we crossed these mice with F5 Rag1−/− TCR transgenic mice (Mamalaki et al., 1993) generating F5 Rag1−/− Zap70TRE rtTA.ChCD2 Zap70−/− (intact F5 TetZap70 mice hereon). F5 Rag1−/− T cells express an MHC I H-2D^b restricted TCR, which is specific for influenza nucleoprotein (NP366-374) of influenza A/NT/60/68. Induction of transgenic Zap70 could then be achieved through oral administration of the tetracycline derivative dox (refer to Materials and Methods and Figure).

We initially bred and maintained intact F5 TetZap70 mice constitutively on dox. We had difficulty maintaining the strain with this breeding strategy and so we used BM from F5 TetZap70 mice to make BM chimeras in sub-lethally irradiated B6 Rag1−/− hosts. F5 TetZap70 chimeras were left for 6 weeks on dox to allow
reconstitution of their haematopoietic compartment. We observed no significant
differences in CD8 SP thymocyte number between either intact F5 TetZap70
mice and F5 TetZap70 chimeras or intact F5 Rag1−/− mice and F5 Rag1−/−
chimeras (Figure 3.1F) and so both were used in subsequent experiments.
Whether data comes from intact or chimeric mice will be specified in both the
relevant results section and figure legends.

Before characterising the peripheral T cell compartment in F5 TetZap70 mice
we wanted to describe T cell development in F5 TetZap70 mice. Firstly, we
wanted to establish whether, like in the polyclonal TetZap70 mice, the TetZap70
system could lead to dox-inducible transgene expression in F5 TetZap70 mice
(Saini et al., 2010). To do this we looked at the thymocyte phenotype of
constitutively dox-fed F5 TetZap70 mice (F5 TetZap70ON hereon) by flow
cytometry. Indeed, thymocytes from F5 TetZap70 chimeras expressed both
hCD2 and Zap70 while mice that had never been fed dox, did not (F5
TetZap70unind hereon) (Figure 3.1A). In fact, the Zap70 abundance in total
thymocytes from F5 TetZap70ON and F5 Rag1−/− chimeras was similar (Figure
3.1A). Consistent with previous data (Saini et al., 2010) we observed variegated
transgene expression with some thymocytes failing to express Zap70 or hCD2
in F5 TetZap70 chimeras (Figure 3.1A). The proportion of thymocytes that were
hCD2 positive was 75±6% in intact F5 TetZap70ON mice and 73±11% in the F5
TetZap70ON chimeras. This transgene variegation could be due to the absence
of a locus control region (LCR) from the Zap70Tre transgene (reviewed in
Festenstein and Kioussis, 2000). hCD2 expression correlated well with Zap70
abundance in F5 TetZap70ON thymocytes as a faithful reporter of Zap70
expression (Figure 3.1B). Taken together, these data show dox-inducible Zap70 expression in vivo in F5 Rag1−/− mice on an endogenous Zap70−/− background.

Next we wanted to characterise F5 TetZap70 T cell development in some more detail. We wanted to confirm that transgenic expression of Zap70 in F5 TetZap70 was sufficient to allow T cells to develop beyond the developmental block seen in Zap70−/− mice (Negishi et al., 1995). We also characterised the reconstitution of the DP and CD8 SP compartments in F5 TetZap70 and control F5 Rag1−/− thymi (Figure 3.1C). The F5 TCR is MHCI-restricted (Mamalaki et al., 1993) and so on a Rag1−/− background only CD8 T cells develop in F5 TCR transgenic mice. In F5 TetZap70On chimeras both DP and CD8 SP populations were readily detectable and the proportion of CD8 SP developing was comparable to F5 Rag1−/− (Figure 3.1C). CD8 SP were only present in the hCD2+ fraction of the F5 TetZap70On thymus and the hCD2− fraction of the thymus exhibited a block in T cell development similar to that seen in the thymic of Zap70−/− (Negishi et al., 1995) and F5 TetZap70unind mice (Figure 3.1C). To investigate T cell development in F5 TetZap70On chimeras in more detail we investigated CD5 and TCR expression within the DP subset. Both the TCR (Havran et al., 1987; Pénit et al., 1995; Shortman and Liu, 2002) and CD5 (Azzam et al., 1998; Tarakhovsky et al., 1995), a negative regulator of TCR signalling (Azzam et al., 2001) are upregulated during and after positive selection. Previously, three developmentally and phenotypically distinct DP thymocyte subsets were described based on CD5 and TCR expression, a CD5lo TCRlo DP1, a CD5hi TCRint DP2 and a CD5int TCRhi Dp3 population (Saini et al., 2010). We can readily observe these three DP subsets while the DP thymocytes in the F5 TetZap70unind and the hCD2− F5 TetZap70On fraction were
blocked at the CD5\textsuperscript{lo} TCR\textsuperscript{lo} DP1 stage of development (Figure 3.1C). Interestingly, the CD5 expression on F5 TetZap70\textsuperscript{ON} DP thymocytes does not seem to be upregulated to the same extent as on F5 \textit{Rag1}\textsuperscript{−/−} DP thymocytes (Figure 3.1D) while TCR expression on F5 TetZap70\textsuperscript{ON} DP thymocytes does reach the level of F5 \textit{Rag1}\textsuperscript{−/−} DP thymocytes (Figure 3.1D). Furthermore, unlike F5 \textit{Rag1}\textsuperscript{−/−} thymocytes, F5 TetZap70\textsuperscript{ON} thymocytes did not downregulate surface CD5 expression as they transited from the DP2 to DP3 subset (Figure 3.1C). Inducible expression of \textit{Zap70}, thus, was sufficient to allow normal DP and CD8 SP T cell development in F5 TetZap70\textsuperscript{unind} mice although the regulation of CD5 expression on F5 TetZap70\textsuperscript{ON} DP thymocytes was altered compared to F5 \textit{Rag1}\textsuperscript{−/−}.

We also wanted to characterise \textit{Zap70} and \textit{hCD2} transgene expression by the DP thymocyte subpopulations in F5 TetZap70\textsuperscript{ON} chimeras. Previously, it was shown that \textit{Zap70} abundance is upregulated during positive selection of DP thymocytes in WT C57Bl/6, with the highest DP \textit{Zap70} expression in the CD5\textsuperscript{int} TCR\textsuperscript{hi} DP3 subset (Saini et al., 2010). In fact, this \textit{Zap70} upregulation, was shown to be very important for CD8 T cell development in particular. In polyclonal TetZap70 mice, thymocytes fail to upregulate \textit{Zap70}, which leads to a specific defect in CD8 development (Saini et al., 2010). Like DP thymocytes in WT C57Bl/6 mice, F5 \textit{Rag1}\textsuperscript{−/−} thymocytes upregulate \textit{Zap70} as they mature but DP thymocytes in F5 TetZap70\textsuperscript{ON} chimeras do not (Figure 3.1E). In fact, \textit{Zap70} abundance dropped in the most mature DP3 subset compared to the DP1 and DP2 subsets (Figure 3.1E). This is not inconsistent with data from other models using the TRE-dependent transgene expression (Buentke et al., 2006; Seddon and Zamoyska, 2002a; 2002b). We wanted to examine whether this failure to
upregulate Zap70 during positive selection had any effect on T cell development in F5 TetZap70 chimeras. We did this by looking at the overall number of CD8 SP thymocytes in F5 TetZap70\textsuperscript{ON} compared to F5 TetZap70\textsuperscript{unind} and F5 \textit{Rag}1\textsuperscript{-/-} (Figure 3.1F). Indeed, F5 TetZap70\textsuperscript{ON} chimeras had significantly fewer CD8 SP thymocytes than F5 \textit{Rag}1\textsuperscript{-/-} mice (Figure 3.1F and (Saini et al., 2010)). Collectively, these data show that inducible Zap70 expression can rescue T cell development in F5 TetZap70 mice and chimeras but fewer CD8 SP develop in intact F5 TetZap70\textsuperscript{ON} mice and chimeras.

### 3.2.2 Reconstitution of the peripheral T cell compartment in F5 TetZap70 mice following transgene induction

We next wanted to characterise the reconstitution of the peripheral T cell compartment in F5 TetZap70\textsuperscript{ON} chimeras. We found that dox-feeding of F5 TetZap70 chimeras rescued the peripheral T cell compartment (Figure 3.2). Both intact F5 TetZap70\textsuperscript{ON} mice and chimeras had a readily detectable population of CD8\textsuperscript{+} TCR\textsuperscript{+} peripheral T cells in both the LN and spleen (Figure 3.2A and B). F5 TetZap70\textsuperscript{unind} mice had no mature peripheral CD8 T cells (Figure 3.2A and B). Interestingly, the reconstitution of the spleen in intact and chimeric F5 TetZap70\textsuperscript{ON} mice was poor and variable compared to the reconstitution of CD8 T cells in the LN (Figure 3.2A and B). We found again that there were no significant differences in peripheral T cell number between intact and chimeric F5 \textit{Rag}1\textsuperscript{-/-} and F5 TetZap70\textsuperscript{ON} mice (Figure 3.2B). Meanwhile, F5 TetZap70\textsuperscript{ON} mice had significantly more peripheral CD8 T cells than F5 TetZap70\textsuperscript{unind} mice (Figure 3.2B). Together these data show that constitutive
dox-feeding successfully reconstitutes the periphery of F5 TetZap70 mice but that F5 TetZap70\textsuperscript{ON} have fewer peripheral CD8 T cells than F5 \textit{Rag1}\textsuperscript{−/−} mice.

We next assessed peripheral Zap70 and hCD2 transgene expression. Naïve CD8 T cells from F5 TetZap70\textsuperscript{ON} chimeras expressed only slightly higher hCD2 than F5 \textit{Rag1}\textsuperscript{−/−} T cells (Figure 3.2C). Meanwhile, Zap70 abundance in F5 TetZap70\textsuperscript{ON} T cells was much lower than in F5 \textit{Rag1}\textsuperscript{−/−} T cells (Figure 3.2C). Low peripheral transgene expression has previously been described in other TRE-dependent transgene models (Buentke et al., 2006; Seddon and Zamoyska, 2002a; 2002b). Both hCD2 expression and Zap70 abundance were dox-dependent however, since we observed no transgene expression in F5 TetZap70 T cells from chimeras that had been taken off dox ≥5 days previously (F5 TetZap70\textsuperscript{OFF} chimeras hereon) (Figure 3.2C). Together, these data indicate that both \textit{Zap70} and \textit{hCD2} are expressed in a dox-dependent manner in T cells in F5 TetZap70\textsuperscript{ON} chimeras albeit at a lower level than in thymocytes.

Since surface CD5 expression on mature T cells has been linked to homeostatic TCR signalling in naïve T cells (Saini et al., 2009; Smith et al., 2001), we wanted to characterise CD5 expression on F5 TetZap70\textsuperscript{ON} T cells. Consistent with the view that F5 TCR transgenic T cells have low affinity for spMHC (Ge et al., 2004), peripheral F5 \textit{Rag1}\textsuperscript{−/−} T cells did indeed express lower CD5 than CD8 T cells from WT C57Bl/6 mice (Figure 3.2D) (Sinclair et al., 2011). F5 TetZap70\textsuperscript{ON} T cells expressed an even lower level of CD5 than F5 \textit{Rag1}\textsuperscript{−/−} T cells even though F5 TetZap70\textsuperscript{ON} and F5 \textit{Rag1}\textsuperscript{−/−} expressed only slightly different levels of TCR (Figure 3.2D). Interestingly, CD8 T cells in F5 TetZap70\textsuperscript{OFF} chimeras expressed even lower CD5 than F5 TetZap70\textsuperscript{ON} T cells.
(Figure 3.2D), suggesting that F5 TetZap70$^{\text{OFF}}$, F5 TetZap70$^{\text{ON}}$ and F5 $\text{Rag1}^{-/-}$ T cells receive different homeostatic TCR signals (Saini et al., 2009; Sinclair et al., 2011). It also suggests that despite the very low levels of Zap70 expression F5 TetZap70$^{\text{ON}}$ T cells receive more homeostatic TCR signals than F5 TetZap70$^{\text{OFF}}$ T cells.

### 3.2.3 Kinetics of transgene ablation following dox-withdrawal

Before studying the effect of Zap70 ablation on naïve T cell survival in F5 TetZap70 mice and chimeras, we wanted to characterise the kinetics of Zap70 and hCD2 decay after dox-withdrawal. We did this by analysing the expression of hCD2 and abundance of Zap70 in thymocytes and T cells from F5 $\text{Rag1}^{-/-}$, F5 TetZap70$^{\text{ON}}$ and F5 TetZap70$^{\text{OFF}}$ chimeras (Figure 3.3). hCD2 expression in F5 TetZap70$^{\text{OFF}}$ thymocytes dropped 1 day following dox-withdrawal (Figure 3.3A). By day 3 most, and by day 7 almost all thymocytes no longer expressed hCD2 (Figure 3.3A). We observed a similar but more rapid reduction of Zap70 abundance in F5 TetZap70$^{\text{OFF}}$ thymocytes following dox-withdrawal. In fact, most F5 TetZap70$^{\text{OFF}}$ thymocytes appeared to be Zap70-deficient by day 5, as assessed by flow cytometry (Figure 3.3A). In the LN we detected a loss of hCD2 expression by day 3 following dox-withdrawal (Figure 3.3B). Zap70 abundance in F5 TetZap70$^{\text{OFF}}$ T cells dropped by day 2 and was almost undetectable by day 5 following dox-withdrawal. Differences in protein turnover might account for the loss of Zap70 before hCD2 in both the LN and the thymus.

### 3.2.4 F5 TetZap70 fail to persist in the absence of Zap70 induction

Naïve T cell survival in lymphoreplete animals is dependent on TCR signals (Labrecque et al., 2001; Polic et al., 2001; Seddon and Zamoyska, 2002a;
Witherden et al., 2000). Despite its role in transducing TCR signals, Lck deficiency alone does not lead to a survival defect in naïve T cells (Seddon et al., 2000). Only when both Src family kinases, Fyn and Lck, are ablated, a defect in naïve T cell survival becomes apparent (Seddon and Zamoyska, 2002a). We wanted to investigate the role of Zap70, activated downstream of Lck and Fyn, in naïve T cell survival. Transgenic Zap70 expression rescued T cell development and reconstituted the peripheral T cell compartment in F5 TetZap70\textsuperscript{ON} mice and chimeras (Figure 3.1 and 2). We took a cohort of F5 TetZap70 mice off dox and followed the frequency of naïve CD8 T cells in the blood of these mice (Figure 3.4) to assess the effect of Zap70 ablation on naïve T cell survival. The frequency of CD8 T cells in the blood of intact F5 TetZap70\textsuperscript{ON} mice was lower than in F5 \textit{Rag1}\textsuperscript{-/-} mice. To be able to compare between groups, we normalised the frequency of blood T cells to the starting frequency at d0. Following dox-withdrawal we observed a dramatic drop in the frequency of CD8 T cells in intact F5 TetZap70\textsuperscript{OFF} mice and chimeras (Figure 3.4B and C). Interestingly, in both cases the drop in the frequency of CD8 T cells in the blood seemed to slow down and reach a plateau (Figure 3.4B and C). We performed non-linear one-phase decay fits to six independent time-courses using intact F5 TetZap70 mice and F5 TetZap70 chimeras to determine that the half-life of F5 TetZap70\textsuperscript{OFF} T cells was 9.4±3.4 days. The decrease in CD8 T cells in the blood was replicated in the LN (Figure 3.4B and D). Taken together these data show that Zap70 is crucial for F5 TetZap70 T cell maintenance \textit{in vivo}. 
3.2.5 F5 TetZap70\textsuperscript{OFF} T cells do not survive upon adoptive transfer

The loss of peripheral CD8 T cells in F5 TetZap70\textsuperscript{OFF} mice, in addition to the block in T cell development are likely to make F5 TetZap70\textsuperscript{OFF} mice and chimeras progressively more lymphopaenic the longer they are off dox. Since competition for limiting survival factors, like IL-7, is an important mechanism by which the peripheral T cell pool is maintained at a constant size (reviewed in Freitas and Rocha, 2000) we predicted that the survival of F5 TetZap70\textsuperscript{OFF} T cells might be altered in these lymphopaenic conditions. To characterise the survival of F5 TetZap70\textsuperscript{OFF} T cells in lymphoreplete conditions, we transferred (Ly5.2) F5 TetZap70\textsuperscript{OFF} and Ly5.1 F5 \textit{Rag1}\textsuperscript{-/-} T cells into Ly5.1 Ly5.2 F5 \textit{Rag1}\textsuperscript{-/-} hosts and assessed their relative survival 1, 7 and 14 days after transfer (Figure 3.5A and B). The ratio of donor Ly5.1\textsuperscript{+} to Ly5.2\textsuperscript{+} CD8 T cells dropped dramatically in the LN of Ly5.1 Ly5.2 F5 \textit{Rag1}\textsuperscript{-/-} hosts so that the overwhelming majority of donor T cells in Ly5.1 Ly5.2 F5 \textit{Rag1}\textsuperscript{-/-} hosts were Ly5.1\textsuperscript{+} F5 \textit{Rag1}\textsuperscript{-/-} T cells by day 14 (Figure 3.5A and B). These data clearly demonstrate a survival defect in F5 TetZap70\textsuperscript{OFF} T cells compared to WT F5 \textit{Rag1}\textsuperscript{-/-} T cells in lymphoreplete conditions.

Considering the clonal competition for spMHC in F5 \textit{Rag1}\textsuperscript{-/-} (Ge et al., 2004; Hao, 2006; Moses et al., 2003; Troy and Shen, 2003) we also wanted to examine the survival of F5 TetZap70 T cells after transfer into OT-I \textit{Rag1}\textsuperscript{-/-} hosts. We followed donor F5 \textit{Rag1}\textsuperscript{-/-} and F5 TetZap70 T cells in the blood of OT-I \textit{Rag1}\textsuperscript{-/-} mice by expression of V\textbeta\textit{11} by donor CD8 T cells and V\alpha2 by endogenous CD8 T cells. Interestingly, despite reduced clonal competition, both F5 \textit{Rag1}\textsuperscript{-/-} and F5 TetZap70 T cells disappeared rapidly in OT-I \textit{Rag1}\textsuperscript{-/-} hosts,
although F5 TetZap70 T cells disappeared more rapidly than F5 Rag1^− T cells (Figure 3.5C). It is thought that OT-I TCR transgenic T cells have higher affinity for spMHC than F5 TCR transgenic T cells (Ge et al., 2004; Sinclair et al., 2011), reflected in the difference in CD5 expression between F5 Rag1^− and OT-I Rag1^− T cells (Figure 3.5D and E). Meanwhile, the level of IL-7Rα expression on OT-I Rag1^− T cells is also higher than on F5 Rag1^− and F5 TetZap70 T cells (Figure 3.5D and E). F5 Rag1^− and F5 TetZap70 T cells are likely outcompeted by OT-I Rag1^− T cells for IL-7 survival signals. Clearly, F5 TetZap70^OFF T cells have a survival defect compared to F5 Rag1^− T cells but our data also confirm the non-redundant role of IL-7 signals for naïve CD8 T cell survival.

### 3.2.6 F5 TetZap70^ON thymocytes are outcompeted in mixed F5 Rag1^− F5 TetZap70 chimeras

To identify specific blocks in F5 TetZap70 development and survival throughout development, we generated competitive mixed BM chimeras, reconstituted with a mix of F5 Rag1^− and F5 TetZap70 BM. Sub-lethally irradiated B6 Rag1^− hosts were reconstituted with a 1:10 mix of Ly5.1 F5 Rag1^− and (Ly5.2) F5 TetZap70 BM and then left for 6 weeks on dox before we assessed the representation of the two sets of donor BM at various stages of T cell development (Figure 3.6). Despite reconstituting mice with 1:10 ratio of F5 Rag1^− and F5 TetZap70 BM, 77% of CD5^lo TCR^lo DP1 were Ly5.1^− (Figure 3.6B and C). The ratio of Ly5.1^− to Ly5.1^+ cells dropped further in the DP3 population where only 40% of cells were Ly5.1^− (Figure 3.6B and C). The representation of F5 TetZap70 cells dropped even more as cells became CD8 single positive
thymocytes and downregulated their HSA expression (Figure 3.6B and C). Ly5.1^+ F5 TetZap70 cells were barely detectable in the periphery of these mixed BM chimeras (Figure 3.6B and C). Analysis of RTE markers by flow cytometry revealed that, Ly5.1^+ F5 TetZap70^{ON} T cells in mixed BM chimeras expressed lower IL-7Rα and higher HSA than to F5 Rag1^{−/−} T cells in the same host (Figure 3.6D) suggesting that peripheral Ly5.1^+ F5 TetZap70 were largely RTEs.

### 3.2.7 Periphery of F5 TetZap70^{ON} is enriched for RTEs

In mixed F5 Rag1^{−/−} F5 TetZap70^{ON} BM chimeras the majority of peripheral Ly5.1^+ F5 TetZap70 T cells were phenotypically similar to RTEs suggesting that F5 TetZap70 RTEs failed to integrate into the mature naïve T cell pool. To assess whether F5 TetZap70 RTEs similarly failed to integrate into the mature naïve T cell pool in absence of competing F5 Rag1^{−/−} we assessed HSA, Qa2 and CD45RB expression on CD8 T cells in F5 TetZap70^{ON} chimeras (Figure 3.7). HSA is downregulated on thymocytes as they leave the thymus, whereas Qa2 and CD45RB are upregulated (Boursalian et al., 2004; Kelly and Scollay, 1990; Lee et al., 2001). CD8 T cells from F5 TetZap70^{ON} chimeras expressed lower Qa2 and CD45RB and higher HSA than F5 Rag1^{−/−} T cells (Figure 3.7A) suggesting that, compared to F5 Rag1^{−/−} mice, the peripheral T cell compartment of F5 TetZap70^{ON} chimeras, is enriched for immature RTEs.

To quantify and compare the enrichment of RTEs in F5 Rag1^{−/−} and F5 TetZap70^{ON} chimeras we defined HSA^{hi}, HSA^{int} and HSA^{lo} CD8 T cell populations. This analysis revealed a specific enrichment of HSA^{int} CD8 T cells in the LN of F5 TetZap70^{ON} chimeras (Figure 3.7B). Similar analysis of Qa2 expression showed that Qa2^{int} CD8 T cells were enriched in the LN of F5
TetZap70<sup>ON</sup> compared to F5 <i>Rag1</i>−/− chimeras (Figure 3.7B). Together, these data show that the peripheral compartment of F5 TetZap70<sup>ON</sup> chimeras is enriched for less mature HSA<sup>int</sup> Qa2<sup>int</sup> T cells compared to F5 <i>Rag1</i>−/−.

### 3.2.8 In vivo homing of F5 TetZap70<sup>OFF</sup> T cells to LN and spleen

Considering the evidence that many important survival signals for naïve T cells are provided in SLOs (Dai and Lakkis, 2001; Dummer et al., 2001; Link et al., 2007) and that Zap70 might be involved in the transduction of T cell migration signals independent of TCR signalling (Evans et al., 2011) we wanted to assess whether homing of F5 TetZap70<sup>OFF</sup> T cells was normal. To do this we co-transferred a mix of Ly5.1 F5 <i>Rag1</i>−/− and (Ly5.2) F5 TetZap70<sup>OFF</sup> T cells into a Ly5.1 L5.2 F5 <i>Rag1</i>−/− mouse and analysed the presence of Ly5.1<sup>+</sup> or Ly5.2<sup>+</sup> donor cells in the blood, LNs and spleen of recipients by flow cytometry after 60 and 150 minutes (Figure 3.8). Both Ly5.1<sup>+</sup> F5 <i>Rag1</i>−/− and Ly5.2<sup>+</sup> F5 TetZap70<sup>OFF</sup> T cells were readily detectable in the blood and spleen of Ly5.1 Ly5.2 F5 <i>Rag1</i>−/− recipients 60 min after transfer (Figure 3.10B, C and D). 150 min after transfer, we detected both Ly5.1<sup>+</sup> F5 <i>Rag1</i>−/− and Ly5.2<sup>+</sup> F5 TetZap70<sup>OFF</sup> T cells in the LN of recipients (Figure 3.10B). We detected no significant difference in the number of donor Ly5.1<sup>+</sup> and Ly5.2<sup>+</sup> CD8 T cells in the LN or spleen of recipients either 60 or 150 min after transfer (3.9D). Furthermore, the ratio of F5 <i>Rag1</i>−/− to F5 TetZap70<sup>OFF</sup> donor cells at 150 min was 0.94±0.30 in the LN and 1.49±0.17 in the spleen (Figure 3.10C) suggesting that F5 TetZap70<sup>OFF</sup> T cells homed to SLOs as efficiently as F5 <i>Rag1</i>−/− cells.
3.2.9 F5 TetZap70\textsuperscript{OFF} T cells survive but do not proliferate \textit{in vivo}

Next, we examined the ability of F5 TetZap70\textsuperscript{OFF} T cells to transduce IL-7 survival signals \textit{in vivo}. We did this by transferring (Ly5.2) F5 TetZap70 and Ly5.1 F5 \textit{Rag1}\textsuperscript{-/-} cells into B6 \textit{Rag1}\textsuperscript{-/-} mice. B6 \textit{Rag1}\textsuperscript{-/-} mice lack any B or T cells (Mombaerts et al., 1992) and as a consequence there should be little competition for T cell survival factors in these mice. F5 \textit{Rag1}\textsuperscript{-/-} T cells proliferate after transfer into lymphopaenic hosts (Buentke et al., 2006; Ge et al., 2004; Hogan et al., 2013; Saini et al., 2009; Seddon and Zamoyska, 2002b; Yates et al., 2008) and in order to differentiate between proliferation and survival of F5 TetZap70\textsuperscript{OFF} and F5 \textit{Rag1}\textsuperscript{-/-} donor cells we labelled cells with CTV (Lyons, 2000). As previously described, F5 \textit{Rag1}\textsuperscript{-/-} T cells proliferated in B6 \textit{Rag1}\textsuperscript{-/-} recipients as determined by their dilution of CTV (Figure 3.9A). None of the donor F5 TetZap70\textsuperscript{OFF} T cells proliferated, however (Figure 3.9A) reflecting the importance of intact TCR signalling for homeostatic proliferation (Seddon et al., 2000). Interestingly, Ly5.1\textsuperscript{-} F5 TetZap70\textsuperscript{OFF} T cells remained readily detectable in the LN of B6 \textit{Rag1}\textsuperscript{-/-} recipients up to 14 days after transfer (Figure 3.9B). Analysing precursor populations normalised for proliferation revealed little difference in survival between F5 \textit{Rag1}\textsuperscript{-/-} and F5 TetZap70\textsuperscript{OFF} T cells (Figure 3.9C). Consistent with the idea that reduced competition for IL-7 survival signals in B6 \textit{Rag1}\textsuperscript{-/-} would lead to F5 \textit{Rag1}\textsuperscript{-/-} and F5 TetZap70\textsuperscript{OFF} T cells receiving more IL-7 signals in B6 \textit{Rag1}\textsuperscript{-/-} we detected an increase in IL-7R\textalpha expression 7 days after transfer (Figure 3.9E). Together these data suggest that, in lymphopaenic conditions, when IL-7 is non-limiting, F5 TetZap70\textsuperscript{OFF} T cells are able to persist as well as F5 \textit{Rag1}\textsuperscript{-/-} cells.
To test whether the survival defect of naïve F5 TetZap70\textsuperscript{OFF} T cells was IL-7-dependent we co-transferred Ly5.1 F5 \textit{Rag1}\textsuperscript{−/−} and (Ly5.2) F5 TetZap70 T cells into \textit{IL-7}\textsuperscript{−/−} \textit{Rag1}\textsuperscript{−/−} mice to assess the relative importance for naïve F5 \textit{Rag1}\textsuperscript{−/−} T cells of losing both the IL-7Rα and the TCR survival signal (Figure 3.9D).

Interestingly, analysis of the ratio of Ly5.1\textsuperscript{+} to Ly5.1\textsuperscript{−} F5 \textit{Rag1}\textsuperscript{−/−} T cells revealed that Ly5.1\textsuperscript{−} F5 TetZap70\textsuperscript{OFF} T cells disappeared more rapidly than Ly5.1\textsuperscript{+} F5 \textit{Rag1}\textsuperscript{−/−} T cells (Figure 3.9D). Consistent with transferred T cells not receiving any IL-7 signals in \textit{IL-7}\textsuperscript{−/−} \textit{Rag1}\textsuperscript{−/−}, IL-7Rα expression on F5 \textit{Rag1}\textsuperscript{−/−} and F5 TetZap70\textsuperscript{OFF} T cells was increased 3 days after transfer (Figure 3.9F). Collectively, these data demonstrate that Zap70-deficiency causes a survival defect in naïve F5 \textit{Rag1}\textsuperscript{−/−} T cells that impairs their survival even more than IL-7-deficiency alone does.

3.2.10 \textit{Bmf} expression is upregulated in F5 TetZap70\textsuperscript{OFF} T cells

Naïve T cells require spMHC TCR interactions for their survival (Labrecque et al., 2001; Polic et al., 2001; Seddon and Zamoyska, 2002a; Seddon et al., 2000; Takeda et al., 1996; Tanchot et al., 1997; Viret et al., 1999; Wang et al., 2001; Witherden et al., 2000) but the downstream targets of this TCR survival signal have not been characterised. We used F5 TetZap70\textsuperscript{OFF} T cells to investigate TCR gene regulation in F5 \textit{Rag1}\textsuperscript{−/−} T cells by RNA-sequencing. We compared gene expression patterns in FACS-purified CD8\textsuperscript{+} TCR\textsuperscript{+} T cells from F5 \textit{Rag1}\textsuperscript{−/−} and F5 TetZap70\textsuperscript{OFF} chimeras. First, we characterised \textit{Zap70} and \textit{Cd5} mRNA levels, since protein levels of \textit{Zap70} and \textit{Cd5} were reduced in F5 TetZap70\textsuperscript{OFF} T cells compared to F5 \textit{Rag1}\textsuperscript{−/−} T cells (Figure 3.1-4). Indeed, \textit{Zap70} and \textit{Cd5}
mRNA levels were reduced in F5 TetZap70\textsuperscript{OFF} T cells compared to F5 \textit{Rag1}\textsuperscript{−/−} T cells (Figure 3.10A).

Next, we characterised mRNA expression of Bcl-2 family members. Bcl-2, Mcl-1 and Bax have been proposed to be downstream targets of IL-7 signalling. Furthermore, the ratio of pro- and anti-apoptotic members of the Bcl-2 family members is thought to be critical in the regulation of lymphocyte survival and death (reviewed in Opferman and Korsmeyer, 2003; Wojciechowski et al., 2007). Interestingly, both \textit{Bcl2} and \textit{Bcl2l1} mRNA expression were slightly increased in F5 TetZap70\textsuperscript{OFF} T cells compared to F5 \textit{Rag1}\textsuperscript{−/−} T cells (Figure 3.10B). Expression of most Bcl-2 family members, including \textit{Bcl2} and \textit{Bcl2l1} did not differ more than 2-fold between F5 TetZap70\textsuperscript{OFF} T cells and F5 \textit{Rag1}\textsuperscript{−/−} T cells (Figure 3.10B). Interestingly, \textit{Bmf} mRNA expression was increased 14-fold in F5 TetZap70\textsuperscript{OFF} T cells compared to F5 \textit{Rag1}\textsuperscript{−/−} T cells (Figure 3.10B). Collectively, these data show that Zap70 and \textit{Cd5} mRNA levels correlate with Zap70 and CD5 protein levels in F5 \textit{Rag1}\textsuperscript{−/−} and F5 TetZap70\textsuperscript{OFF} T cells. These data also reveal a putative downstream target for TCR survival signals in \textit{Bmf}, which is upregulated in F5 TetZap70\textsuperscript{OFF} T cells compared to F5 \textit{Rag1}\textsuperscript{−/−} T cells.

### 3.3 Discussion

Naïve CD8 T cells require signals via the TCR for their survival (Labrecque et al., 2001; Polic et al., 2001; Seddon and Zamoyska, 2002a; Seddon et al., 2000; Takeda et al., 1996; Tanchot et al., 1997; Viret et al., 1999; Wang et al., 2001; Witherden et al., 2000) but the exact nature and downstream targets of this survival signal remain unclear. In this chapter, we sought to characterise the role of Zap70 in naïve T cell survival. Since Zap70\textsuperscript{−/−} lack any peripheral T cells
due to a block in T cell development (Negishi et al., 1995) we used a transgenic mouse model where Zap70 expression was conditional upon dox-feeding. Dox-inducible expression of Zap70 in the thymus of F5 TetZap70 mice overcomes the block in T cell development seen in Zap70-deficient mice and leads to the generation of mature naïve F5 Rag1\textsuperscript{-/-} T cells in which Zap70 expression can be ablated by dox-withdrawal. Despite conditional expression of Zap70 at levels comparable to F5 Rag1\textsuperscript{-/-}, constitutively dox-fed F5 TetZap70 mice and chimeras had fewer developing CD8 SP thymocytes and reduced peripheral CD8 T cells compared to F5 Rag1\textsuperscript{-/-}. The defect in F5 TetZap70 T cell development was more obvious in mixed F5 Rag1\textsuperscript{-/-} F5 TetZap70 BM chimeras, where F5 TetZap70 thymocytes were almost completely outcompeted by developing F5 Rag1\textsuperscript{-/-} thymocytes by the time they left the thymus. Also, F5 TetZap70\textsuperscript{ON} T cells in the LN and spleen of mixed chimeras were almost all immature RTEs. Interestingly, peripheral F5 TetZap70 T cells in intact F5 TetZap70\textsuperscript{ON} chimeras were also consistently enriched for RTEs. We showed that Zap70 was required for naïve T cell survival both in intact F5 TetZap70 mice and chimeras as well as after transfer into F5 Rag1\textsuperscript{-/-} mice. Ablation of Zap70 in F5 TetZap70 T cells, correlated with a reduction in surface CD5 expression, which was already reduced on F5 TetZap70 compared to F5 Rag1\textsuperscript{-/-} T cells. IL-7R signalling was intact in F5 TetZap70\textsuperscript{OFF} T cells, which survived in conditions where IL-7 was non-limiting. The survival defect of F5 TetZap70\textsuperscript{OFF} T cells was also not secondary to a defect in IL-7R signalling since in absence of IL-7, F5 TetZap70\textsuperscript{OFF} T cells still died more rapidly than Zap70-sufficient cells. Finally, mRNA of the pro-apoptotic Bmf was upregulated in F5 TetZap70\textsuperscript{OFF} T cells making it a putative target for a Zap70 dependent TCR survival signal.
Impaired development of T cells in absence of endogenous regulation of Zap70 expression in polyclonal TetZap70 mice has been previously described (Saini et al., 2010). In WT C57Bl/6 mice, Zap70 abundance and expression is progressively upregulated as thymocytes mature and undergo positive selection. Failure of Zap70 upregulation in transgenic TetZap70 mice leads to a specific defect in CD8 T cell development (Saini et al., 2010). The defect in CD8 T cell development in F5 TetZap70 mice (Figure 3.1C and F) is consistent with the loss of endogenous upregulation of Zap70 in DP thymocytes. In fact, Zap70 abundance in F5 TetZap70 DP thymocytes first remains constant and then drops in the most mature DP thymocytes and CD8 SP thymocytes (Figure 3.1E).

We generated mixed F5 Rag1−/− F5 TetZap70 BM chimeras to compare the selection efficiency and survival of F5 Rag1−/− and F5 TetZap70ON thymocytes at various developmental stages. Analysing the representation of Ly5.1−/− F5 TetZap70 thymocytes revealed that developing Ly5.1−/− F5 TetZap70 T cells were outcompeted by F5 Rag1−/− thymocytes throughout development. The proportion of Ly5.1−/− F5 TetZap70 thymocytes dropped in the DP3, CD8 SP HSA<sup>lo</sup> and peripheral CD8 T cells, in particular (Figure 3.6B and C). These data fit with a model in which the developmental upregulation of Zap70 in DP2 and DP3 thymocytes and CD8 SP thymocytes is necessary for the efficient generation of CD8 SP thymocytes (Saini et al., 2010). We cannot exclude that, at least in the periphery of F5 TetZap70ON mice and chimeras, a defect in proliferation contributes to the observed lymphopaenia (Figure 3.2B) since F5 TetZap70ON T cells fail to proliferate after transfer into lymphopaenic hosts (data not shown) and are therefore unlikely to do so in more lymphoreplete F5 TetZap70ON mice and chimeras.
Like Zap70, CD5 and TCR expression are upregulated during and after positive selection and this developmental upregulation requires Zap70 (Negishi et al., 1995; Otsu et al., 2002). CD5 expression is further modulated by TCR signalling in the thymus and periphery and is thought to correlate with TCR avidity for spMHC (Azzam et al., 1998; 2001; Smith et al., 2013; Wong et al., 2001). Interestingly, F5 TetZap70 DP thymocytes did not upregulate CD5 to the same degree as F5 *Rag1*−/− DP thymocytes (Figure 3.1C and D). Furthermore, while surface CD5 expression is downregulated as thymocytes become DP3 thymocytes, F5 TetZap70ON DP2 and DP3 thymocytes expressed similar levels of CD5 (Figure 3.1C). Finally, naïve F5 TetZap70 T cells also expressed lower surface CD5 than did F5 *Rag1*−/− T cells. The reduction in CD5 expression on DP thymocytes and on peripheral T cells correlated with the reduction in Zap70 abundance on DP3 thymocytes and peripheral F5 TetZap70 T cells suggesting Zap70 might transduce the TCR signals that modulate CD5 expression during positive selection and in peripheral T cells. The modulation of CD5 expression by TCR signalling in the thymus as well as the periphery is known to require Lck, which is upstream of Zap70 in the TCR signalling cascade and so a role for Zap70 is likely.

Despite the partial block in CD8 T cell development and the low Zap70 abundance and CD5 expression by F5 TetZap70ON T cells, dox-inducible Zap70 expression in F5 TetZap70ON mice was able to restore T cell development to allow the generation of F5 TetZap70ON T cells (Figure 3.1 and 2). These CD8 T cells were then used to investigate the role of Zap70 in naïve T cell survival. Naïve T cells in intact F5 TetZap70OFF mice and chimeras disappeared with a half-life of 9.4±3.4 days (Figure 3.4A and C). We also showed an overall
reduction in the frequency and number of naïve T cells in F5 TetZap70^{OFF} mice (Figure 3.4B and D). It could be argued that the observed drop in naïve F5 TetZap70^{OFF} T cells was the result of loss of thymic input into the peripheral pool of naïve T cells in F5 TetZap70 mice lacking any Zap70 expression. However, naïve F5 Rag1^{-/-} T cells persisted for much longer than F5 TetZap70^{OFF} T cells after transfer into F5 Rag1^{-/-} hosts (Figure 3.5A and B).

This experiment allowed us to compare directly the survival of a cohort of Zap70-deficient and Zap70-sufficient F5 Rag1^{-/-} T cells in lymphoreplete conditions and in absence of any thymic input. The disappearance of F5 TetZap70^{OFF} T cells in F5 Rag1^{-/-} hosts confirmed that Zap70 was required for the maintenance of naïve F5 Rag1^{-/-} T cells. The persistence of naïve F5 Rag1^{-/-} T cells after transfer is consistent with the view that naïve T cells have a relatively long lifespan of ~90 days (Seddon and Zamoyska, 2002a). The relatively long lifespan further supports the idea that the 9.4-day average half-life of F5 TetZap70^{OFF} T cells is the result of impaired naïve T cell survival and not merely the consequence of the loss of thymic input. Interestingly, the 9.4-day half-life of naïve T cells in absence of Zap70 is shorter than previous estimates of naïve CD8 T cell lifespan in absence of TCR signalling – 16 (Polic et al., 2001) and 19 (Labrecque et al., 2001) days following TCR ablation and 18 days following Lck and Fyn ablation (Seddon and Zamoyska, 2002a). This variability is probably due to the different experimental systems used in these studies but might also mean that Zap70 transduces naïve T cell survival signals independent of MHC, TCR and Src family kinases.

Since both Zap70, and Src family kinases, Lck and Fyn upstream of Zap70, can be activated independently of TCR activation (Adunyah et al., 1997; Brenner et
al., 1996; Evans et al., 2011; Hatakeyama et al., 1991; Kobayashi et al., 1993; Minami et al., 1993; Parravicini et al., 2002; Raab et al., 1995; Schmedt et al., 1998; Taher et al., 1996; Wary et al., 1998) it is possible that the effect of Zap70-deficiency on naïve T cell survival is TCR-independent. Interestingly, we consistently detected less CD5 on F5 TetZap70^{ON} and F5 TetZap70^{OFF} T cells than on F5 $Rag1^{-/-}$ T cells (Figure 3.2D). CD5 expression on naïve T cells correlates with TCR affinity for spMHC (Azzam et al., 1998; Smith et al., 2001) and requires TCR expression (Polic et al., 2001), spMHC-contact (Saini et al., 2009; Sinclair et al., 2011; Smith et al., 2001) and Lck- and Fyn-expression (Seddon and Zamoyska, 2002a). The reduced CD5 on F5 TetZap70^{ON} and F5 TetZap70^{OFF} T cells, therefore, reflect reduced homeostatic TCR signalling. Furthermore, T cell CD5 expression in Lck- and Fyn-deficient mice correlates with survival (Seddon and Zamoyska, 2002a), as it does in our study. Finally, CD5 expression on naïve T cells was also found to correlate with the degree of CD3ζ-chain phosphorylation (Smith et al., 2001). Basal CD3ζ-chain phosphorylation is MHC-dependent in vitro (Smith et al., 2001) and in vivo (Dorfman et al., 2000; Witherden et al., 2000), it correlates with expression of Lck and Fyn (Seddon and Zamoyska, 2002a) and naïve T cell survival (Seddon and Zamoyska, 2002a; Witherden et al., 2000). There is also evidence that, like surface CD5 expression, basal CD3ζ-chain phosphorylation correlates with TCR avidity for spMHC suggesting the phosphorylation of CD3ζ arises from TCR interactions with spMHC (Dorfman et al., 2000). Interestingly, in this (Dorfman et al., 2000) and another study (Clarke and Rudensky, 2000), naïve CD4 T cell survival was unaffected after transfer into an $A\beta^{-/-}$ MHCII-deficient host and the partial phosphorylation of CD3ζ was proposed to be a mechanism
to increase sensitivity to TCR signalling rather than play a role in the transduction of a TCR survival signal (Stefanova et al., 2003). Subsequently, it was shown, however, that Aβ−/− mice are not truly MHCII-deficient (Martin et al., 2003) and hybrid AαEβ MHCII in Aβ−/− mice might be able to promote T cell turnover or survival. CD4 T cell survival was indeed impaired after transfer into mice that lack the α and β chains of both I-A and I-E MHCII alleles (Madsen et al., 1999; Martin, 2006). Overall, our data show that Zap70 is crucial for naïve T cell survival and they suggest that the effect of Zap70-deficiency on naïve F5 T cell survival is TCR-dependent and MHCI-dependent.

Although our data suggest that naïve F5 TetZap70OFF T cells home normally to peripheral lymphoid organs (Figure 3.10), there is evidence that Zap70 regulates β1-integrin expression (Epler et al., 2000; Goda et al., 2004) and plays an important role in regulating T cell adhesion and migration by regulating LFA1 expression TCR independently (Evans et al., 2011). In one study (Evans et al., 2011), inhibition or siRNA-mediated knockdown of Zap70 and Lck in vitro, impaired T cell motility and migration on ICAM1. Interestingly, another study could only find a role for Lck in mediating LFA1-dependent T cell-B cell conjugates (Morgan et al., 2001). More recent work revealed that the kinase function of Zap70 was dispensable for adhesion to ICAM1 in vitro but that its adaptor function was not (Au-Yeung et al., 2010). The regulation of LFA1 and β1-integrin expression are clearly important for naïve T cell homing and migration to SLOs and therefore for naïve T cell survival (Dai and Lakkis, 2001; Dummer et al., 2001; Link et al., 2011) as well as for T cell-APC interactions and, consequently, for T cell activation and expansion (reviewed in Dustin and de Fougerolles, 2001). One criticism of many of the studies investigating the
role of Zap70 and Src family kinases in the regulation of integrin expression and activity is the fact that they are all largely carried out in vitro. F5 TetZap70$^{\text{OFF}}$ T cells might be used to study the role of Zap70 in T cell adhesion, homing and migration in vivo.

The data in this chapter also suggest a potential role for Zap70 in regulating integration of RTE into the mature naïve T cell pool. We showed that in mixed F5 $Rag1^{-/-}$ and F5 TetZap70 BM chimeras, F5 TetZap70$^{\text{ON}}$ T cells were being outcompeted during positive selection but most especially after thymic egress, as they become mature naïve T cells (Figure 3.6B and 6). Peripheral Ly5.1$^+$ F5 TetZap70$^{\text{ON}}$ T cells in these mixed BM chimeras were RTEs as defined by their low IL-7Rα and high HSA expression. One explanation for these data is that the poor survival of mature naïve F5 TetZap70$^{\text{ON}}$ T cells relative to F5 $Rag1^{-/-}$ T cells in the periphery leads to the outcompetition of F5 TetZap70$^{\text{ON}}$ T cells by F5 $Rag1^{-/-}$ T cells. Furthermore, naïve F5 TetZap70$^{\text{ON}}$ T cells fail to homeostatically proliferate in lymphopaenic hosts (data not shown) and this proliferative defect is likely to contribute to the outcompetition of F5 TetZap70$^{\text{ON}}$ by F5 $Rag1^{-/-}$ naïve T cells in the periphery of mixed BM chimeras. This data also suggests that the maintenance of RTEs is less dependent on Zap70 than the survival of naïve F5 $Rag1^{-/-}$ T cells. Surprisingly, we also found that the peripheral compartment of host F5 TetZap70$^{\text{ON}}$ chimeras was enriched for RTEs (Figure 3.7). F5 TetZap70$^{\text{ON}}$ mice and chimeras consistently had a higher proportion of HSA$^{\text{int}}$ and Qa2$^{\text{int}}$ T cells and expressed lower CD45RB (Figure 3.7A and B). These data, again, support the idea that the maintenance of naïve T cells is Zap70-dependent, whereas the maintenance of RTEs is less so.

Using mice that express GFP under control of the $Rag2$ promotor, cells that
recently expressed Rag2 in the thymus were tracked and characterised as they entered the periphery as GFP\(^{hi}\) and GFP\(^{lo}\) RTEs (Boursalian et al., 2004; and reviewed in Fink and Hendricks, 2011). Interestingly, a more recent study revealed that the accumulation of RTEs in the periphery as well as the survival of RTEs was impaired in \(Egr1^{-/-}\) mice in a TCR-dependent way suggesting that Egr1 might be important for thymic egress and RTE survival (Schnell and Kersh, 2005). Interestingly, another study (Houston et al., 2011) showed that RTE integration into the mature naïve T cell pool can be enhanced by transgenic expression of IL-7R\(\alpha\) or Bcl-2. RTEs express reduced IL-7R\(\alpha\) and might be less able to compete for IL-7 survival signals in the periphery (Boursalian et al., 2004). In conclusion, it seems likely that the survival of RTE and mature naïve T cell survival are not equally dependent on Zap70-and TCR-mediated signals and that WT Zap70-abundance is important for RTE integration into the mature naïve T cell pool.

Despite impaired survival in lymphoreplete conditions, we found that F5 TetZap70\(^{OFF}\) T cells survived but did not proliferate, after transfer into lymphopaenic B6 \(Rag1^{-/-}\) mice (Figure 3.9A, B and D). There is reduced competition for survival factors like IL-7 in absence of other T cells in B6 \(Rag1^{-/-}\) mice (Mombaerts et al., 1992) and consistent with this, we showed that transferred F5 \(Rag1^{-/-}\) and F5 TetZap70\(^{OFF}\) T cells had downregulated their IL-7R\(\alpha\) expression indicative of having received IL-7 signals (Park et al., 2004a). This is entirely consistent with the view that naïve T cell survival in lymphopaenic mice can be MHC- and thus probably TCR-independent (Martin, 2006; Martin et al., 2003). In fact, the decay of CD8 T cell frequencies in the blood of F5 TetZap70 mice taken off dox appeared to slow as they became
increasingly lymphopaenic (Figure 3.4) and this might be the result of increased IL-7 signalling and thus survival in F5 TetZap70^{OFF} mice and chimeras. F5 TetZap70^{OFF} T cells failed to proliferate, however, in B6 $\textit{Rag}1^{-/-}$ mice, which fits with homeostatic TCR signalling being indispensable for LIP (Seddon et al., 2000; Takeda et al., 1996; Tanchot et al., 1997; Viret et al., 1999). We also transferred F5 $\textit{Rag}1^{-/-}$ and F5 TetZap70^{OFF} T cells into $IL-7^{-/-}$ $\textit{Rag}1^{-/-}$ to further elucidate the interplay between IL-7 and TCR survival signals. Both transferred F5 $\textit{Rag}1^{-/-}$ and F5 TetZap70^{OFF} T cells died rapidly after transfer into IL-7-deficient hosts but the survival of F5 TetZap70^{OFF} T cells was still impaired compared to F5 $\textit{Rag}1^{-/-}$ T cells. Firstly, the impaired survival of F5 TetZap70^{OFF} T cells in absence of IL-7 confirms the non-redundant role of IL-7 signals in naïve T cell survival in vivo (Schluns et al., 2000; Seddon and Zamoyska, 2002b). Secondly, the impaired survival of F5 TetZap70^{OFF} T cells relative to F5 $\textit{Rag}1^{-/-}$ T cells, despite all cells being deprived of IL-7 survival signals, also suggests that the Zap70-dependent survival signal is largely IL-7-independent.

Finally, gene expression analysis of F5 $\textit{Rag}1^{-/-}$ and F5 TetZap70^{OFF} T cells revealed a potential target for Zap70-mediated TCR survival signals in peripheral T cells. We found that F5 TetZap70^{OFF} T cells had increased Bmf mRNA, a pro-apoptotic BH3-only member of the Bcl-2 family of apoptotic regulators (Figure 3.10B). The physiological role of Bmf is much less well characterised than the role of its closest relative Bim (reviewed in Piñon et al., 2008) which is known to play an important role in the regulation of T cell homeostasis (Wojciechowski et al., 2007). The recent characterisation of $Bmf^{-/-}$ mice and comparison with $Bim^{-/-}$ mice revealed that Bmf has a largely redundant role in embryonic development (Hubner et al., 2009; Labi et al., 2008), although
T cell development was not studied in *Bmf*−/− mice in any great detail. *Bmf*−/− mice have significantly increased CD8 T cells (Labi et al., 2008) and Bmf-deficiency improves CD8 T cell viability *in vitro* (Hubner et al., 2009). Interestingly, a recent study in melanoma cells (Shao and Aplin, 2012), revealed that Bmf apoptotic activity *in vitro* is reduced following phosphorylation by Erk2 downstream of MEK while phosphorylation of Bmf by Jnk enhances Bmf apoptotic activity *in vitro* (Hubner et al., 2009; Lei and Davis, 2003). Little else is known about the regulation of *Bmf* expression and function *in vivo*. Since *Bmf* mRNA levels were dramatically upregulated in F5 TetZap70OFF T cells, we propose that TCR signalling might regulate *Bmf* expression *in vivo* and thus naïve T cell survival. We will confirm whether *Bmf* is indeed a target for TCR survival signals by investigating whether Bmf-deficiency can rescue the survival of naïve F5 TetZap70OFF T cells.
Figure 3.1 Reconstitution of T cell development in F5 TetZap70 mice following transgene induction

Thymocytes from F5 \(\text{Rag}^{1-}/\) (grey fill) and F5 TetZap70\textsuperscript{unind} mice (red) and constitutively dox-fed F5 TetZap70\textsuperscript{ON} mice and BM chimeras (F5 TetZap70\textsuperscript{ON}) (blue) were counted and analysed by flow cytometry. BM chimeras were made by reconstituting sub-lethally irradiated B6 \(\text{Rag}^{1-}/\) mice with F5 TetZap70 BM. (A) Histograms show hCD2 expression and Zap70 abundance on live thymocytes. (B) Dot plot shows hCD2 expression against Zap70 abundance on F5 TetZap70\textsuperscript{ON} live thymocytes. (C) Dot plots show CD4 against CD8 expression on live, hCD2\(^+\) or hCD2\(^-\)thymocytes and CD5 against TCR expression on CD4\(^+\) CD8\(^+\) DP thymocytes from intact F5 \(\text{Rag}^{1-}/\) and F5 TetZap70\textsuperscript{unind} mice and F5 TetZap70\textsuperscript{ON} chimeras. (D) Histograms show TCR and CD5 expression on CD4\(^+\) CD8\(^+\) DP thymocytes. (E) Histograms show Zap70 abundance on hCD2\(^-\) (grey fill), DP1 (red), DP2 (blue) and DP3 (green) thymocytes. (F) Box and whisker plot shows the hCD2\(^+\) CD5\(^{hi}\) TCR\(^{hi}\) CD8\(^+\) SP thymocyte number in intact (n=14) and host chimera (n=2) F5 \(\text{Rag}^{1-}/\) mice and intact (n=13) and host chimera (n=14) F5 TetZap70\textsuperscript{ON} mice; **** \(p\leq 0.0001\) and ns \(p>0.05\) (one-way analysis of variance followed by Bonferroni correction for multiple comparisons). Data are representative of \(\geq\)three independent experiments.
Figure 3.2 Reconstitution of the peripheral T cell compartment in F5 TetZap70 mice following transgene induction

LN and spleen lymphocytes from C57Bl/6 (black), F5 $Rag^{1-/-}$ (grey fill) and F5 TetZap70$^{\text{unind}}$ mice and F5 TetZap70$^{\text{ON}}$ (blue) mice and BM chimeras were counted and analysed by flow cytometry. Lymphocytes from F5 TetZap70$^{\text{OFF}}$ chimeras taken off dox $\geq 5$ days previously were used as Zap70-deficient controls (red) in (C) and (D). BM chimeras were made by reconstituting sub-lethally irradiated B6 $Rag^{1-/-}$ mice with F5 TetZap70 BM. (A) Dot plots show CD8 against TCR expression on live LN and spleen lymphocytes. (B) Box and whisker plot shows the pooled number of LN and spleen CD8$^+$ TCR$^+$ CD5$^{hi}$ TCR$^{hi}$ lymphocytes in F5 $Rag^{1-/-}$ mice (n=14) and chimeras (n=2), F5 TetZap70$^{\text{unind}}$ mice (n=7) and F5 TetZap70$^{\text{ON}}$ mice (n=11) and chimeras (n=14); *** $p \leq 0.001$, * $p \leq 0.05$ and ns $p > 0.05$ (one-way analysis of variance followed by Bonferroni correction for multiple comparisons). (C) Histograms show hCD2 expression and Zap70 abundance on CD8$^+$ TCR$^+$ CD5$^{hi}$ TCR$^{hi}$ Lymphocytes (LN). (D) Histograms show TCR and CD5 expression on CD8$^+$ TCR$^+$ CD5$^{hi}$ TCR$^{hi}$ Lymphocytes (LN). Data are representative of $\geq$three independent experiments.
Figure 3.3 Kinetics of transgene ablation following dox-withdrawal

Thymocytes and Lymphocytes (LN) from F5 $\textit{Rag1}^{-/-}$ mice (black), F5 TetZap70$^{\text{ON}}$ chimeras (blue) and F5 TetZap70 chimeras taken off dox (F5 TetZap70$^{\text{OFF}}$) for 1-5 days (d1-d5), as indicated, were analysed by flow cytometry. BM chimeras were made by reconstituting sub-lethally irradiated B6 $\textit{Rag1}^{-/-}$ mice with F5 TetZap70 BM. F5 TetZap70$^{\text{OFF}}$ mice taken off dox $\geq$7 days previously were used as Zap70-deficient controls (red). (A) Histograms show hCD2 expression and Zap70 abundance on live thymocytes. (B) Histograms show hCD2 expression and Zap70 abundance on CD8$^+$ TCR$^+$ CD5$^{\text{hi}}$ TCR$^{\text{hi}}$ lymphocytes.
A Th:

- hCD2

B LN:

- hCD2

- Zap70

- F5 TetZap70^{OFF} (7 days)
- F5 Rag1^{-/-}
- F5 TetZap70^{ON}
- F5 TetZap70^{OFF}
Intact F5 TetZap70\textsuperscript{ON} mice and chimeras were taken off dox and the frequency and number of CD8\textsuperscript{+} TCR\textsuperscript{+} CD5\textsuperscript{hi} TCR\textsuperscript{hi} lymphocytes in the blood and LN of F5 \textit{Rag1}\textsuperscript{-/-} (grey), F5 TetZap70\textsuperscript{ON} (blue) and F5 TetZap70 taken off dox (F5 TetZap70\textsuperscript{OFF}) (red) was assessed by flow cytometry. BM chimeras were made by reconstituting sub-lethally irradiated B6 \textit{Rag1}\textsuperscript{-/-} mice with F5 TetZap70 BM. Line graphs show the frequency of CD8\textsuperscript{+} TCR\textsuperscript{+} CD5\textsuperscript{hi} TCR\textsuperscript{hi} lymphocytes in blood of intact mice (A) and BM chimeras (C) as a percentage of the frequency at d0; **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$ and * $p \leq 0.05$ F5 TetZap70\textsuperscript{OFF} compared to F5 TetZap70\textsuperscript{ON} (two-way analysis of variance followed by Bonferroni correction for multiple comparisons). Each time-point represents $\geq 3$ replicate mice. Box and whiskers plots show the frequency or number of LN CD8\textsuperscript{+} TCR\textsuperscript{+} CD5\textsuperscript{hi} TCR\textsuperscript{hi} lymphocytes in intact mice (n\geq 5) (B) and BM chimeras (n\geq 3) (D); **** $p \leq 0.0001$ (one-way analysis of variance followed by Bonferroni correction for multiple comparisons). Data are representative of $\geq$three independent experiments.
Figure 3.5 F5 TetZap70\textsuperscript{OFF} do not survive upon adoptive transfer

Ly5.1 F5 \textit{Rag1}\textsuperscript{−/−} and F5 TetZap70\textsuperscript{OFF} (Ly5.2) lymphocytes (LN) were transferred intravenously into Ly5.1 Ly5.2 F5 \textit{Rag1}\textsuperscript{−/−} placed on dox or OT-I \textit{Rag1}\textsuperscript{−/−} mice that were placed on or off dox. F5 TetZap70\textsuperscript{OFF} lymphocytes were isolated from the LNs of F5 TetZap70 chimeras taken off dox ≥5 days previously. BM chimeras were made by reconstituting sub-lethally irradiated B6 \textit{Rag1}\textsuperscript{−/−} mice with F5 TetZap70 BM. Host and donor LN or blood lymphocytes were analysed by flow cytometry 1-14 days after transfer (d1-d14). (A) Dot plots show Ly5.1 against Ly5.2 expression on donor CD8\textsuperscript{+} TCR\textsuperscript{+} CD5\textsuperscript{hi} TCR\textsuperscript{hi} Lymphocytes (LN) in Ly5.1 Ly5.2 F5 \textit{Rag1}\textsuperscript{−/−} host LNs. (B) Line graph shows the ratio of donor Ly5.2\textsuperscript{+} to Ly5.1\textsuperscript{+} CD8\textsuperscript{+} TCR\textsuperscript{+} CD5\textsuperscript{hi} TCR\textsuperscript{hi} lymphocytes in Ly5.1 Ly5.2 F5 \textit{Rag1}\textsuperscript{−/−} host LNs. Each time-points represents ≥2 replicate mice. (C) Line graph shows the frequency of transferred V\textit{β}11\textsuperscript{+} CD8\textsuperscript{+} TCR\textsuperscript{+} CD5\textsuperscript{hi} TCR\textsuperscript{hi} F5 \textit{Rag1}\textsuperscript{−/−} (grey) and F5 TetZap70 lymphocytes in the blood of OT-I \textit{Rag1}\textsuperscript{−/−} hosts on (blue) and off (red) dox. Each time-point represents ≥3 replicate mice. Histograms show CD5 and IL-7R\textalpha{} expression on endogenous OT-I \textit{Rag1}\textsuperscript{−/−} and transferred F5 \textit{Rag1}\textsuperscript{−/−} (grey) lymphocytes (D) and transferred F5 \textit{Rag1}\textsuperscript{−/−} and F5 TetZap70 lymphocytes (E) 7 days after transfer into OT-I \textit{Rag1}\textsuperscript{−/−} hosts that were on (red) or off (blue) dox. Intact F5 \textit{Rag1}\textsuperscript{−/−} (grey fill) lymphocyte CD5 and IL-7R\textalpha{} expression are also shown as controls. Data are representative of ≥two independent experiments.
A F5 Rag1<sup>-/-</sup>:

B F5 Rag1<sup>-/-</sup>:

C OT-I Rag1<sup>-/-</sup>:

D LN:

E LN:
Figure 3.6 F5 TetZap70\(^{ON}\) thymocytes are outcompeted in mixed F5 \(\text{Rag1}^{-/-}\)

**F5 TetZap70 chimeras**

Mixed F5 \(\text{Rag1}^{-/-}\) F5 TetZap70 BM chimeras were made by reconstituting sub-lethally irradiated B6 \(\text{Rag1}^{-/-}\) with a 1:10 ratio of T cell depleted Ly5.1 F5 \(\text{Rag1}^{-/-}\) and F5 TetZap70 BM. Representation of Ly5.1\(^+\) F5 \(\text{Rag1}^{-/-}\) and Ly5.2\(^+\) F5 TetZap70 cells in the thymus and LN of mixed BM chimeras was analysed by flow cytometry. (A) Dot plots show CD4\(^+\) CD8\(^+\) DP and CD8\(^+\) SP gating of total thymocytes, DP1, DP2 and DP3 gating of DP thymocytes, HSA\(^{hi}\) and HSA\(^{lo}\) gating of CD8\(^+\) SP thymocytes and CD8\(^+\) CD5\(^+\) gating of lymphocytes (LN). (B) Representation of Ly5.1\(^+\) F5 \(\text{Rag1}^{-/-}\) and Ly5.2\(^+\) F5 TetZap70 cells in DP1, DP2, DP3, HSA\(^{hi}\) and HSA\(^{lo}\) CD8\(^+\) SP thymocytes and in CD8\(^+\) CD5\(^+\) lymphocytes (LN) of mixed BM chimeras. (C) Bar chart shows the average representation of Ly5.1\(^+\) F5 \(\text{Rag1}^{-/-}\) and Ly5.2\(^+\) F5 TetZap70 cells in DP1, DP2, DP3, HSA\(^{hi}\) and HSA\(^{lo}\) CD8\(^+\) SP thymocytes and in CD8\(^+\) CD5\(^+\) lymphocytes (LN) of mixed BM chimeras. Each bar represents \(\geq\)6 replicate mice; **** \(p \leq 0.0001\) and *** \(p \leq 0.001\) compared to DP1 (one-way analysis of variance followed by Bonferroni correction for multiple comparisons). Histograms show C57Bl/6 (black), Ly5.1\(^+\) F5 \(\text{Rag1}^{-/-}\) (grey fill) and Ly5.2\(^+\) F5 TetZap70 (blue) lymphocyte (LN) CD5 (D), IL-7R\(\alpha\) and HSA (E) expression. Data are representative of \(\geq\)three independent experiments.
Figure 3.7 Periphery of F5 Tet Zap70\textsuperscript{ON} is enriched for recent thymic emigrants

F5 \textit{Rag1}\textsuperscript{-/-} (grey fill) and F5 Tet Zap70\textsuperscript{ON} (blue) thymocyte and lymphocyte (LN) RTE marker expression was analysed by flow cytometry. (A) Histograms show CD8\textsuperscript{+} CD5\textsuperscript{+} lymphocyte (LN) HSA, Qa2 and CD45RB expression. (B) Dot plots show CD8 and CD5 expression on live lymphocytes from LNs. Histograms show F5 \textit{Rag1}\textsuperscript{-/-} and F5 Tet Zap70\textsuperscript{ON} CD4\textsuperscript{+} CD8\textsuperscript{+} DP thymocyte (grey fill) and CD8\textsuperscript{+} CD5\textsuperscript{+} lymphocyte (LN) (black) HSA and Qa2 expression. Data are representative of \geq three independent experiments.
Figure 3.8 *In vivo* homing of F5 TetZap70$^{\text{OFF}}$ T cells to LN and spleen

Ly5.1 F5 *Rag1*"/" and F5 TetZap70$^{\text{OFF}}$ (Ly5.2) Lymphocytes (LN) were transferred intravenously into Ly5.1 Ly5.2 F5 *Rag1*"/" hosts. F5 TetZap70$^{\text{OFF}}$ lymphocytes were isolated from the LNs of F5 TetZap70 chimeras taken off dox ≥5 days previously. BM chimeras were made by reconstituting sub-lethally irradiated B6 *Rag1*"/" mice with F5 TetZap70 BM. Host and donor LN or blood lymphocytes were analysed by flow cytometry 60 and 150 min after transfer (d1-d14). (A) Dot plots show CD8 against TCR expression on live Lymphocytes (LN) and Ly5.2 against TCR expression on CD8$^+$ TCR$^+$ Lymphocytes (LN) before transfer. (B) Dot plots show Ly5.2 against Ly5.1 expression on CD8$^+$ TCR$^+$ blood, LN and spleen lymphocytes 60 and 150 min after transfer. (C) Plots show the ratio of donor Ly5.2$^+$ to Ly5.1$^+$ CD8$^+$ TCR$^+$ lymphocytes in Ly5.1 Ly5.2 F5 *Rag1*"/" host LN and spleen at 60 and 150 min. (D) Plots show donor Ly5.2$^+$ (grey) and Ly5.1$^+$ (red) CD8$^+$ TCR$^+$ lymphocyte numbers in Ly5.1 Ly5.2 F5 *Rag1*"/" host LN and spleen at 60 and 150 min. Each time-points represents ≥4 replicate mice.
Figure 3.9 F5 TetZap70\textsuperscript{OFF} T cells survive but do not proliferate \textit{in vivo}

CTV-labelled Ly5.1 F5 \textit{Rag1}\textsuperscript{-/-} and F5 TetZap70\textsuperscript{OFF} (Ly5.2) Lymphocytes (LN) were transferred intravenously into B6 \textit{Rag1}\textsuperscript{-/-} or \textit{IL-7}\textsuperscript{-/-} \textit{Rag1}\textsuperscript{-/-} hosts. F5 TetZap70\textsuperscript{OFF} lymphocytes were isolated from the LNs of F5 TetZap70 chimeras taken off dox ≥5 days previously. BM chimeras were made by reconstituting sub-lethally irradiated B6 \textit{Rag1}\textsuperscript{-/-} mice with F5 TetZap70 BM. Host and donor LN or spleen lymphocytes were analysed by flow cytometry 1-14 days after transfer (d1-d14). (A) Histograms show CTV-dilution by donor Ly5.1\textsuperscript{+} F5 \textit{Rag1}\textsuperscript{-/-} (grey) and Ly5.2\textsuperscript{+} F5 TetZap70\textsuperscript{OFF} (red) CD8\textsuperscript{+} TCR\textsuperscript{+} lymphocyte in B6 \textit{Rag1}\textsuperscript{-/-} LN. (B) Dot plots show Ly5.1 expression against CTV dilution on donor CD8\textsuperscript{+} TCR\textsuperscript{+} lymphocytes in B6 \textit{Rag1}\textsuperscript{-/-} LN. (C) Line graph shows the ratio of donor Ly5.2\textsuperscript{+} to Ly5.1\textsuperscript{+} CD8\textsuperscript{+} TCR\textsuperscript{+} lymphocytes in B6 \textit{Rag1}\textsuperscript{-/-} LNs. Each time-point represents ≥2 replicate mice. (D) Line graph shows the ratio of donor Ly5.2\textsuperscript{+} to Ly5.1\textsuperscript{+} CD8\textsuperscript{+} TCR\textsuperscript{+} lymphocytes in \textit{IL-7}\textsuperscript{-/-} \textit{Rag1}\textsuperscript{-/-} LNs. Each time-point represents ≥2 replicate mice. Histograms show IL-7R\textalpha expression on donor Ly5.1\textsuperscript{+} F5 \textit{Rag1}\textsuperscript{-/-} (grey fill) and Ly5.2\textsuperscript{+} F5 TetZap70\textsuperscript{OFF} (red) CD8\textsuperscript{+} TCR\textsuperscript{+} Lymphocytes (LN) 7 days after transfer into B6 \textit{Rag1}\textsuperscript{-/-} hosts (E) and 3 days after transfer into \textit{IL-7}\textsuperscript{-/-} \textit{Rag1}\textsuperscript{-/-} hosts (F). Pre-transfer (grey fill) IL-7R\textalpha expression is also shown as a control. Data are representative of ≥two independent experiments.
mRNA was isolated from FACS-purified CD8⁺ TCR⁺ F5 $Rag^{1/-}$ (grey) and F5 TetZap70⁺⁻ lymphocytes (LN) (red). F5 TetZap70⁺⁻ lymphocytes were isolated from the LNs of F5 TetZap70 chimeras taken off dox ≥5 days previously. BM chimeras were made by reconstituting sub-lethally irradiated B6 $Rag^{1/-}$ mice with F5 TetZap70 BM. Total mRNA was sequenced by an Illumina Genome Analyser IIx. Bar charts show expression of $Hprt$, $Zap70$ and $Cd5$ (A) and of indicated apoptotic regulators (B). Data are mean±S.D. nRPKM from duplicates.
A

nRPKM

Hprt  Zap70  Cd5

F5 Rag1
t-/

F5 TetZap70 OFF

B

nRPKM

Bcl2  Bcl2l1  McI1  Bcl2l11  Bmf  Bad  Bax  Bid
Chapter 4  The role of Zap70 in naïve T cell survival *in vitro*

4.1 Introduction

The survival of naïve CD8 T cells *in vivo* depends on MHC-dependent TCR signalling (Labrecque et al., 2001; Polic et al., 2001; Seddon and Zamoyska, 2002a; Tanchot et al., 1997) and also requires Zap70, as we show in Chapter 3. Very little is known, though, about the downstream targets (discussed in section 3.3) or the potential source of this TCR survival signal.

In contrast, the downstream targets of IL-7 survival signals, although not fully characterised, have been extensively studied (introduced and discussed in section 3.1 and 3.3). IL-7 production occurs mainly in the stroma of the thymus, lymphoid organs, skin, intestine as well as the liver at a constitutive rate such that the amount of IL-7 available is mostly regulated by consumption by lymphocytes (Fry and Mackall, 2005). In a recent study the importance of IL-7 produced by non-haematopoietic stromal cells in the LN was investigated (Link et al., 2007). Consistent with previous studies (Cinalli et al., 2005), the study showed firstly, that blocking access to SLOs leads to a drop in the total number of peripheral naïve CD4 and CD8 T cells suggesting access to SLOs is important for naïve T cell survival (Link et al., 2007). Secondly, treating mice with an IL-7Rα-neutralising antibody has a similar effect on naïve T cell numbers as preventing access to SLOs does. Finally, isolated LN gp38+ CD31− CD35− FRCs both expressed and produced IL-7 and were able to promote CD4
and CD8 T cell survival \textit{in vitro} in an IL-7- and CCL19-dependent manner. Interestingly, access to SLOs has also been shown to be important for homeostatic proliferation of naïve T cells (Dai and Lakkis, 2001; Dummer et al., 2001) which, like naïve T cell survival, requires both IL-7 (Schluns et al., 2000; Tan et al., 2001) and TCR signals (Seddon et al., 2000; Takeda et al., 1996; Tanchot et al., 1997; Viret et al., 1999).

The source of spMHC for TCR-dependent survival signals is still unknown. Ablation of \textit{I-Aβ} expression in MHCII\textsuperscript{+} dendritic cells revealed that CD4 T cell survival depends on MHCII\textsuperscript{+} DCs (Bröcker, 1997). Furthermore, DCs and T cells can form synapses in the absence of antigen and these antigen-independent interactions induce T cell Ca\textsuperscript{2+} responses as well as tyrosine phosphorylation at the T cell-DC interface (Revy et al., 2001). DCs isolated by plastic adherence have been shown to enhance T cell viability if co-cultured with either naïve CD4 or CD8 T cell survival \textit{in vitro} and this effect was shown to be specific to isolated DCs since co-culturing T cells with B cells had no effect on T cell viability (Feuillet et al., 2005; Revy et al., 2001). The survival signal provided by DCs to CD4 T cells \textit{in vitro} was shown to be IL-7-independent but was impaired, although not completely abolished, if DCs did not express any MHCII molecules (Feuillet et al., 2005). Interestingly, basal CD3ζ-phosphorylation was also maintained by co-culture of WT DCs and T cells but disappeared if DCs did not express MHCII (Feuillet et al., 2005). Finally, and perhaps most importantly, the survival signal provided by DCs also required Src family kinases raising the possibility that the DCs were providing a spMHC-induced TCR-dependent survival signal transduced by Src family kinases and possibly Zap70. There is also evidence that suggests that DCs do not provide naïve T cells with survival
signals, since at least in vitro FACS-purified DCs co-cultured with T cells at a 1 to 40 DC to T cell ratio had no effect on T cell viability (Link et al., 2007).

In this chapter we sought to characterise the IL-7 and TCR signalling requirements for T cell survival in more detail in vitro. We wanted to assess whether the Zap70-dependent TCR survival signal acts via IL-7. We also hope to investigate the source and downstream targets of a TCR survival signals in an in vitro model of TCR- and Zap70-dependent naïve T cell survival.

4.2 Results

4.2.1 sing in F5 TetZap70 mice

Given the evidence that TCR and IL-7R signalling are cross-regulated (Kimura et al., 2012; Park et al., 2007) we wanted to investigate whether F5 TetZap70OFF CD8 T cells also had a defect in IL-7R signalling in vivo which contributed to the survival defect seen in naïve CD8 T cells in F5 TetZap70OFF mice. Interestingly, IL-7Rα surface expression on both F5 TetZap70ON and F5 TetZap70OFF T cells was reduced to a similar extent compared to F5 Rag1−/− T cells (Figure 4.1A). There was also no difference in surface CD8α expression between F5 Rag1−/−, F5 TetZap70ON and F5 TetZap70OFF T cells (Figure 4.1A).

Since surface IL-7Rα expression is regulated by IL-7 availability in vivo (Park et al., 2004a) we wanted to examine the IL-7Rα expression of F5 TetZap70 and F5 Rag1−/− in the absence of this repressive signal in vitro. We did this by culturing F5 Rag1−/− and F5 TetZap70ON cells in vitro without IL-7 and then analysed IL-7Rα expression after 24h (Figure 4.1B). Despite low IL-7Rα expression ex vivo, F5 TetZap70OFF T cells upregulated their IL-7Rα expression
to a greater extent than F5 Rat1−/− T cells (Figure 4.1B). Interestingly, though, there was no change in F5 Rat1−/− CD8α expression after overnight culture without IL-7 (Figure 4.1B). Together, these data show that, the lower IL-7Rα expression on F5 TetZap70 T cells ex vivo is not cell-intrinsic but likely due to IL-7 availability in vivo.

Since Bcl-2 expression is reduced in absence of IL-7 signalling in vivo (Schluns et al., 2000; Vivien et al., 2001) and transgenic Bcl-2 is able to rescue thymopoiesis in IL-7Rα-deficient mice (Akashi et al., 1997; Maraskovsky et al., 1997) we assessed Bcl-2 abundance in F5 TetZap70 T cells to assess their ability to transduce IL-7 signals. We looked initially at Bcl-2 abundance in thymocytes from F5 Rat1−/− and F5 TetZap70ON mice. Bcl-2 is upregulated on thymocytes following positive selection (Akashi et al., 1997; Freedeen-Jeffry et al., 1995; 1997; Linette et al., 1994). We found that Bcl-2 abundance was higher in CD8 SP thymocytes than in DP thymocytes from F5 Rat1−/− and F5 TetZap70ON mice (Figure 4.1C). Although it is less clear what the role of IL-7 is in maintaining Bcl-2 in naïve T cells (Pearson et al., 2011; Rathmell et al., 2001; Schluns et al., 2000; Vivien et al., 2001) Bcl-2 abundance was consistently greater in F5 TetZap70 T cells than in F5 Rat1−/− T cells (Figure 4.1D). The IL-7-induced upregulation of Bcl-2 in vitro, as previously described (Akashi et al., 1997; Armant et al., 1995; Freedeen-Jeffry et al., 1997; Graninger et al., 2000; Karawajew et al., 2000; Vella et al., 1997), was also comparable in F5 Rat1−/− and F5 TetZap70 T cells (Figure 4.1E). These data show that despite reduced IL-7Rα expression, F5 TetZap70 T cells are able to transduce IL-7 signals in vitro.
4.2.2 Zap70-deficiency does not affect the IL-7-induced survival of naïve CD8 T cells at saturating concentrations of IL-7

Given the important role IL-7 signals play in mediating naïve T cell survival (Pearson et al., 2011; Saini et al., 2009; Schluns et al., 2000) and the variable surface expression of IL-7Rα on F5 TetZap70 T cells we wanted to investigate the IL-7-induced survival of F5 TetZap70\textsuperscript{ON} and F5 TetZap70\textsuperscript{OFF} T cells. To do this, we isolated F5 \textit{Rag1}\textsuperscript{−/−}, F5 TetZap70\textsuperscript{ON} and F5 TetZap70\textsuperscript{OFF} T cells and cultured these T cells with or without saturating concentrations of IL-7 \textit{in vitro} (Figure 4.2). We assessed survival \textit{ex vivo} and at 24, 48 and 72h by staining cells with recombinant annexin-V which binds to phosphatidylserine when it is externalised on the outer layer of the plasma membrane of cells undergoing apoptosis (Brumatti et al., 2008). There were no significant differences in lymphocyte viability between F5 \textit{Rag1}\textsuperscript{−/−}, F5 TetZap70\textsuperscript{ON} and F5 TetZap70\textsuperscript{OFF} T cells \textit{ex vivo} (Figure 4.2A). In order to compare the survival of F5 \textit{Rag1}\textsuperscript{−/−}, F5 TetZap70\textsuperscript{ON} and F5 TetZap70\textsuperscript{OFF} T cells directly, we normalised the frequency of live cells at each time-point to the starting frequency of live cells. In absence of IL-7, most T cells died after 72h \textit{in vitro} (Figure 4.2B and C). In four independent experiments, the viability of F5 TetZap70\textsuperscript{OFF} T cells in absence of IL-7 was reduced compared to F5 \textit{Rag1}\textsuperscript{−/−} and F5 TetZap70\textsuperscript{ON} T cells. Interestingly, the viability of F5 \textit{Rag1}\textsuperscript{−/−}, F5 TetZap70\textsuperscript{ON} and F5 TetZap70\textsuperscript{OFF} T cells cultured with IL-7 was very similar at all time-points tested (Figure 4.2B and C). Collectively, these data show that IL-7-induced survival of T cells is unaffected by Zap70-deficiency but that F5 TetZap70\textsuperscript{OFF} were more prone to apoptosis.
To investigate the effect of Zap70-deficiency on IL-7 sensitivity in vitro, we co-cultured Ly5.1 F5 \(Rag1^{-/-}\) cells isolated from LN with F5 TetZap70\(^{ON}\) (Ly5.2) or F5 TetZap70\(^{OFF}\) (Ly5.2) T cells and with a titration of IL-7 (Figure 4.2D). We normalised the viability of T cells at 72h to the 0h viability of T cells as well as to the viability of F5 \(Rag1^{-/-}\) T cells in the same well. Interestingly, the viability of F5 TetZap70\(^{ON}\) T cells and Ly5.1 F5 \(Rag1^{-/-}\) T cells was similar at all IL-7 concentrations tested (Figure 4.2D). The viability of F5 TetZap70\(^{OFF}\) T cells, was very similar to the viability of F5 \(Rag1^{-/-}\) T cells at IL-7 concentrations greater than 1.25ng/mL but at lower IL-7 concentrations F5 TetZap70\(^{OFF}\) T cell viability was reduced compare to co-cultured F5 \(Rag1^{-/-}\) T cells (Figure 4.2D). Together, these data show that F5 TetZap70\(^{OFF}\) T cells respond less well to IL-7 survival signals at low, non-saturating concentrations of IL-7.

4.2.3 B6 \(Rag1^{-/-}\) splenocytes enriched for CD11c\(^{+}\) cells promote Zap70-dependent T cell survival

We also wanted to set up an in vitro survival assay to investigate TCR survival signalling. Since CD11c\(^{+}\) MHCI\(^{+}\) DCs enriched by plastic adherence improve CD4 and CD8 T cell viability in vitro (Feuillet et al., 2005; Revy et al., 2001) we first tested whether DCs could improve the viability of CD8 T cells in vitro. To do this we enriched B6 \(Rag1^{-/-}\) splenocytes for CD11c\(^{+}\) cells (Figure 4.3A). To ensure that all the non-T cells originated from the B6 \(Rag1^{-/-}\) CD11c\(^{+}\) enriched fraction we FACS-purified CD8\(^{+}\) TCR\(^{+}\) T cells from F5 \(Rag1^{-/-}\), F5 TetZap70\(^{ON}\) and F5 TetZap70\(^{OFF}\) LN (Figure 4.3B). We assessed the viability of T cells cultured in the presence or absence of CD11c\(^{+}\) enriched B6 \(Rag1^{-/-}\) splenocytes at a 1 to 10 ratio at 24, 48 and 72h by annexin-V staining. Addition of CD11c\(^{+}\)
enriched splenocytes consistently enhanced T cell viability at all time-points tested (Figure 4.3C and D). FACS-purified T cells cultured alone, died very rapidly (Figure 4.3D). Interestingly, the viability of T cells cultured with CD11c+ enriched splenocytes correlated with their level of Zap70 abundance as assessed by flow cytometry (Figure 3.2). The viability of F5 Rag1–/– T cells was greatest, while F5 TetZap70OFF T cells responded least to CD11c+ enriched splenocytes in vitro (Figure 4.3C and D). Taken together, these results show that CD11c+ enriched splenocytes can improve T cell viability in a Zap70-dependent manner.

4.2.4 FACS-purified cDCs and pDCs alone do not promote substantial naïve T cell survival in vitro

It has been shown that both purified DCs and stromal cells can promote T cell survival in vitro (Feuillet et al., 2005; Link et al., 2011). Therefore, we wanted to identify which fraction of the CD11c+ enriched B6 Rag1–/– splenocytes was responsible for the Zap70-dependent survival signal in vitro (Figure 4.4). To do this, we first characterised CD11c+ enriched B6 Rag1–/– splenocytes in more detail by flow cytometry. Conventional DCs (cDCs), which express the highest levels of CD11c and MHCII, represented 14.3±6.5% of all CD11c+ enriched B6 Rag1–/– splenocytes on average (Figure 4.4B). Within the MHCII+ fraction we also detected a substantial population of plasmacytoid dendritic cells (pDCs), which express lower CD11c than cDCs and additionally express the pDC-marker mouse plasmacytoid dendritic cell antigen 1 (mPDCA1) (Barchet et al., 2005; Krug et al., 2004). Furthermore, there was a substantial MHCII– fraction, which contained both CD45+ haematopoietic cells and CD45– non-
haematopoietic cells (Figure 4.4D). We went on to FACS-purify MHCII− cells, cDCs and pDCs from CD11c+ enriched B6 Rag1−/− splenocytes and tested each subset for its ability to promote F5 Rag1−/− T cell survival in vitro (Figure 4.4E and G). To account for the different abundance of MHCII− cells, cDCs and pDCs in the CD11c+ enriched B6 Rag1−/− spleen, we added the equivalent number of FACS-purified cells present in 1x10⁶ CD11c+ enriched B6 Rag1−/− spleen. We also tested whether WT T cells or B cells were able to promote naïve T cell survival in vitro and so C57Bl/6 CD4+ TCR+ T cells and B220+ CD19+ B cells were also purified (Figure 4.4F and G). T cell viability was assessed at 48h and compared to the viability of T cells cultured with unfractionated CD11c+ enriched splenocytes. Interestingly, purified cDCs and pDCs alone were poor at promoting the survival of F5 Rag1−/− T cells (Figure 4.4G). FACS-purified C57Bl/6 CD4 T or B cells were also unable to replicate the survival promoted by CD11c+ enriched spleen although the viability of T cells cultured with B cells was consistently better than the viability of T cells cultured with CD4 T cells (Figure 4.4G). Interestingly, only the viability of F5 Rag1−/− T cells cultured with MHCII− cells was similar to the viability of T cells cultured with CD11c+ enriched DCs (Figure 4.4G). Collectively, these data show that only MHCII− cells, not cDCs or pDCs, were able to account for the survival promoted by unfractionated CD11c+ enriched splenocytes.

4.2.5 The survival signal provided by CD11c+ splenocytes is β2m- and IL-7-independent

We next wanted to ask whether the survival signals provided by CD11c+ enriched splenocytes were dependent on intact spMHC or IL-7. To do this, we
enriched CD11c+ splenocytes from β2m−/− Rag1−/− (β2m-deficient hereon), which lack MHCI surface expression (Koller et al., 1990; Zijlstra et al., 2010) and IL-7−/− Rag1−/− (IL-7-deficient hereon) (Freeden-Jeffry et al., 1995) mice (Figure 4.5A). We tested the ability of CD11c+ enriched β2m- and IL-7-deficient splenocytes to promote the survival of naïve F5 Rag1−/− T cells in vitro (Figure 4.5B and C). Interestingly, the viability of T cells cultured with CD11c+ enriched β2m- and IL-7-deficient splenocytes was indistinguishable from the viability of T cells cultured with CD11c+ enriched splenocytes from B6 Rag1−/− mice (Figure 4.5B and C). Collectively, these data show that the T cell survival signal provided by CD11c+ enriched splenocytes is β2m- and IL-7-independent.

To investigate any cooperation between IL-7 survival signals and the survival signal provided by CD11c+ enriched B6 Rag1−/− splenocytes we cultured F5 Rag1−/− T cells with titrations of IL-7 and of CD11c+ enriched splenocytes (Figure 4.5D). In absence of IL-7 and at a saturating concentration of IL-7, the viability of T cells at 48h correlated with the added number of CD11c+ enriched splenocytes. At intermediate concentrations of IL-7, however, addition of DCs had no additive effect on T cell survival (Figure 4.5D). These data show that the survival signal provided by CD11c+ enriched splenocytes in vitro is dose-dependent and that IL-7 and CD11c+ enriched splenocytes signals are not additive.

4.2.6 The T cell survival signal provided by CD11c+ splenocytes is impaired by MEK inhibition

Finally, we wanted to use this system of IL-7 independent, Zap70-dependent in vitro T cell survival to investigate putative downstream signalling pathways of a
Zap70-dependent T cell survival signal. We co-cultured F5 Rag1\(^{-/-}\) T cells and B6 Rag1\(^{-/-}\) splenocytes enriched for CD11c\(^{+}\) cells with specific inhibitors for MEK, mTOR and PI3K and then assessed T cell viability at 48h. Interestingly, both inhibition of mTOR, by rapamycin and PI3K, by LY294002, respectively, lead to a modest reduction of the T cell survival at 48h induced by CD11c\(^{+}\) enriched splenocytes (Figure 4.6B). Addition of the MEK1/2 inhibitor UO126, meanwhile, completely abrogated the T cell survival signal provided by CD11c\(^{+}\) enriched splenocytes, such that there was no significant difference between the viability of FACS-purified T cells alone and T cells cultured with CD11c\(^{+}\) enriched splenocytes and UO126 (Figure 4.6B). Together, these data show that the survival signal provided by CD11c\(^{+}\) enriched splenocytes in vitro depends on intact PI3K/Akt and intact mTOR signalling but mostly on intact MAPK/ERK signalling.

4.3 Discussion

Having characterised the effect of Zap70-deficiency on T cell survival in vivo using dox-inducible expression of Zap70 in F5 TetZap70 mice and chimeras in Chapter 3, we next investigated the survival of F5 TetZap70\(^{OFF}\) T cells in more detail in vitro. We show that Zap70-deficiency did not affect IL-7-induced Bcl-2 upregulation or survival, despite F5 TetZap70 T cells expressing reduced IL-7R\(\alpha\) ex vivo. At lower concentrations of IL-7 F5 TetZap70\(^{OFF}\) T cells did show reduced survival compared to F5 TetZap70\(^{ON}\) and F5 Rag1\(^{-/-}\). Interestingly, the reduced Zap70 abundance in F5 TetZap70\(^{ON}\) T cell had no effect on IL-7-induced survival in vitro at any IL-7 concentration tested. Furthermore, we show that Zap70-deficiency did affect the survival induced by CD11c\(^{+}\) enriched
splenocytes *in vitro*. F5 TetZap70\textsuperscript{OFF} T cells consistently responded less than F5 TetZap70\textsuperscript{ON} T cells, which in turn responded less than F5 *Rag1\textsuperscript{-/-}* T cells suggesting that Zap70-abundance was directly affecting T cell survival signal provided by CD11c\textsuperscript{+} enriched splenocytes. Surprisingly, FACS-purifying DC and non-DC subsets from CD11c\textsuperscript{+} enriched splenocytes revealed that neither cDCs nor pDCs alone were able to replicate the survival induced by CD11c\textsuperscript{+} enriched splenocytes. We also show that the survival signals from CD11c\textsuperscript{+} enriched splenocytes were largely, β2m- and IL-7-independent. Finally, we show that the survival signals from CD11c\textsuperscript{+} enriched splenocytes were only partially dependent on PI3K/Akt signalling but almost entirely on MAPK/ERK signalling since the survival induced by CD11c\textsuperscript{+} enriched splenocytes was almost completely abolished in the presence of MEK-inhibitor UO126.

Surface IL-7R\textalpha expression on F5 TetZap70\textsuperscript{ON} and F5 TetZap70\textsuperscript{OFF} T cells was reduced compared to F5 *Rag1\textsuperscript{-/-}* T cells *ex vivo* but, interestingly, F5 TetZap70\textsuperscript{ON} and F5 TetZap70\textsuperscript{OFF} T cells upregulated their surface IL-7R\textalpha expression to a greater extent than F5 *Rag1\textsuperscript{-/-}* T cells in absence of IL-7 (Figure 4.1A and B). These data are consistent with the view that *Il7ra* transcription and IL-7R\textalpha surface expression is inhibited by IL-7 signalling (Park et al., 2004a), in contrast to other γc-chain cytokine receptors whose expression is upregulated by cytokine signalling (Depper et al., 1985). We also characterised the intracellular abundance of anti-apoptotic regulator Bcl-2, which has been proposed to be a target for IL-7 survival signals in thymocytes and peripheral T cells. We did not detect a defect in Bcl-2 abundance between F5 *Rag1\textsuperscript{-/-}* and F5 TetZap70\textsuperscript{ON} thymocytes or naïve T cells (Figure 4.1C and D). In fact, in contrast to IL-7R\textalpha expression, F5 TetZap70\textsuperscript{ON} and F5 TetZap70\textsuperscript{OFF} T cells consistently
had higher Bcl-2 abundance (Figure 4.1D) and Bcl2 expression (Figure 3.10) than F5 Rag1\(^{-}\) T cells and this might reflect the preferential survival of cells with more anti-apoptotic Bcl-2.

There is evidence that TCR and IL-7 signalling are cross-regulated in naïve CD8 T cells (Park et al., 2007) such that TCR signalling limits IL-7 signalling, while IL-7 signalling enhances surface CD8\(\alpha\) expression and thus TCR signalling (Park et al., 2007). In an extension of this model, it was recently shown that homeostatic TCR signalling is, in fact, essential to interrupt IL-7 signalling and thus to prevent ‘cytokine-induced T cell death’ (Kimura et al., 2012). In contrast, there is also evidence for an opposite relationship between TCR and IL-7 signalling, since TCR signalling has been shown to enhance IL-7 sensitivity in vitro and in vivo by promoting localisation of the \(\gamma_c\) to lipid rafts (Cho et al., 2010). In another study (Takada and Jameson, 2009), IL-7R\(\alpha\) and CD5 expression were downregulated while CD8\(\alpha\) expression was upregulated in absence of spMHC interactions. Meanwhile, Lck-ablation has no effect on IL-7R\(\alpha\)-expression in vivo (Seddon and Zamoyska, 2003) and more recent work found no evidence of peripheral modulation of IL-7R\(\alpha\) expression by TCR signalling (Sinclair et al., 2011). Instead, TCR signalling strength during positive selection of T cells in the thymus determines IL-7R\(\alpha\) expression on peripheral naïve T cells (Sinclair et al., 2011). It is clear that the potential cross-regulation of TCR and IL-7R signaling is complex and so is the cross-regulation of CD8\(\alpha\) and IL-7R\(\alpha\) expression.

The low IL-7R\(\alpha\) expression on F5 TetZap70 T cells could be consistent with the ‘co-receptor tuning model’ (Park et al., 2007), where weak TCR signalling in
CD8 T cells with low or no Zap70 would lead to enhanced IL-7 signalling and thus reduced IL-7Rα expression (Park et al., 2004a). Cell-intrinsic surface IL-7Rα expression on F5 TetZap70 T cells, however, was increased compared to F5 Rag1−/− T cells (Figure 4.1B), which is inconsistent with the ‘co-receptor tuning model’ (Park et al., 2007). We also observed no differences in surface CD8α expression between F5 TetZap70 or F5 Rag1−/− T cells ex vivo or after overnight culture in absence of IL-7 (Figure 4.1A and B). Furthermore, in vitro IL-7-induced Bcl-2 upregulation was comparable in F5 TetZap70 and F5 Rag1−/− T cells. Finally, IL-7-induced survival of F5 Rag1−/−, F5 TetZap70ON or F5 TetZap70OFF T cells at saturating IL-7 concentrations in vitro was comparable (Figure 4.2A, B and C) suggesting that IL-7 signalling is largely intact in F5 TetZap70 T cells. Zap70-deficiency, meanwhile, impaired IL-7-induced survival in vitro at low concentrations of IL-7 (Figure 4.2D), while the ‘co-receptor tuning model’ predicts enhanced IL-7-sensitivity of F5 TetZap70OFF CD8 T cells (Park et al., 2007). Our data suggest that TCR signalling might have a minor role in the regulation of T cell sensitivity to IL-7 at low concentrations. The variable surface expression of IL-7Rα on F5 TetZap70 T cells is, in fact, likely the result of the relative lymphopaenia of F5 TetZap70 chimeras rather than reduced Zap70-abundance.

Splenocytes enriched for DCs are able to provide both survival (Feuillet et al., 2005; Revy et al., 2001) and proliferation (Ge et al., 2002) signals for naïve T cells in vitro and so we tested the ability of DCs to promote the survival of naïve F5 Rag1−/− and F5 TetZap70ON T cells (Figure 4.3). Interestingly, naïve T cell death in vitro in absence of any growth factors was consistently greater if T cells had been FACS-purified than if other LN cells were present (Figure 4.2). This
suggests that non-T cells contributed to T cell viability in vitro. CD11c+ enriched splenocytes, meanwhile, were able to promote the in vitro survival of F5 Rag1-/-, F5 TetZap70ON as well as F5 TetZap70OFF T cells but to different degrees (Figure 4.3C, D and E). In fact, the ability of CD11c+ enriched splenocytes to induce T cell survival correlated with naïve T cell Zap70 abundance as assessed by flow cytometry (Figure 3.2). This is consistent with a previous study where DC-induced survival of CD4 T cells in vitro was abolished by addition of PP2, an inhibitor of Src family kinases, upstream of Zap70 in the TCR signalling cascade (Feuillet et al., 2005). In the same study, CD4 T cell phosphorylation of CD3ζ, a correlate of homeostatic TCR signalling (Dorfman et al., 2000; Seddon and Zamoyska, 2002a; Smith et al., 2001; Witherden et al., 2000), was maintained if CD4 T cells were co-cultured with WT DCs but not if co-cultured with MHC-deficient DCs (Feuillet et al., 2005). Taken together, our data confirms that splenocytes enriched for CD11c+ cells can provide naïve CD8 T cells with a survival signal in vitro and suggests that this survival signal is in part Zap70- and thus TCR-dependent.

We found, however, that the survival signal provided by CD11c+ enriched splenocytes did not require splenocytes to express β2m, since the viability of T cells cultured with either β2m-deficient and B6 Rag1-/- CD11c+ enriched splenocytes was similar (Figure 4.5B, C and D). Our data are consistent with an earlier study, which shows that both the formation of antigen-independent DC-T cell synapses and DC-induced CD4 and CD8 T cell survival in vitro is MHC-independent (Revy et al., 2001). A more recent study showed that DC-induced survival of CD4 T cells in vitro was unaffected by deficiency of I-Aβ and β2m, but that DCs lacking expression of all MHCII chains (Madsen et al., 1999;
Martin, 2006) were less able to provide CD4 T cells with a survival signal \textit{in vitro}. This raises the possibility that DCs expressing hybrid non-classical αEβ MHCII might be able to promote CD4 T cell survival \textit{in vitro} (Feuillet et al., 2005; Revy et al., 2001) as well as \textit{in vivo} (Martin, 2006; Martin et al., 2003). Furthermore, there is evidence that β2m-deficient mice still express H-2D\textsuperscript{b} (Allen et al., 1986) enough to mediate the deletion of H2-D\textsuperscript{b} restricted HY TCR transgenic T cells in male mice. Therefore we cannot exclude that β2m-deficient CD11c\textsuperscript{+} enriched splenocytes are providing naïve CD8 T cells with an MHCI-dependent survival signal. Intriguingly, however, even completely MHCII-deficient DCs are able to promote some survival of CD4 T cells \textit{in vitro} (Feuillet et al., 2005) suggesting that at least some of the survival signal provided by DCs is MHC-independent. Like DC MHC-deficiency, T cell Zap70-deficiency only partially impaired the survival signal provided by CD11c\textsuperscript{+} enriched splenocytes suggesting that some of the survival signals provided by CD11c\textsuperscript{+} enriched splenocytes is Zap70-independent (Figure 4.3E). There is evidence that MHC-independent DC survival signals, at least to CD4 T cells \textit{in vitro}, can be mediated by CD28/B7 interactions as well LFA1/ICAM1 (Feuillet et al., 2005). It remains to be examined how the Zap70-independent survival signal provided by CD11c\textsuperscript{+} enriched splenocytes to T cells \textit{in vitro} is mediated.

We characterized the B6 \textit{Rag1}\textsuperscript{-/-} CD11c\textsuperscript{+} enriched splenocytes in more detail and as well as mPDCA1\textsuperscript{-} CD11c\textsuperscript{+} MHCII\textsuperscript{+} cDCs and mPDCA1\textsuperscript{+} CD11c\textsuperscript{int} MHCII\textsuperscript{int} pDCs, a large frequency of the CD11c\textsuperscript{+} enriched splenocytes were MHCII\textsuperscript{+} (Figure 4.4D). Interestingly, only the FACS-purified MHCII\textsuperscript{-} subset was able to promote F5 \textit{Rag1}\textsuperscript{-/-} survival \textit{in vitro} and F5 \textit{Rag1}\textsuperscript{-/-} T cell co-culture with either cDCs or pDCs did not improve F5 \textit{Rag1}\textsuperscript{-/-} T cell viability compared to
culturung F5 Rag1\(^{-/}\) T cells alone (Figure 4.4G). F5 Rag1\(^{-/}\) T cell survival promoted by CD11c\(^{+}\) enriched splenocytes correlated with the frequency of CD11c\(^{+}\) cells after enrichment, but not with the frequency of CD11c\(^{+}\) MHCII\(^{+}\) cDCs after enrichment, suggesting that the cell population providing F5 Rag1\(^{-/}\) T cells with a survival signal is CD11c\(^{+}\) but not a CD11c\(^{+}\) MHCII\(^{+}\) cDC. These data contradict previous data suggesting DCs can promote T cell survival in vitro (Feuillet et al., 2005; Revy et al., 2001) and provide an MHC-dependent survival signal to CD4 T cells in vivo (Bröcker, 1997). In the studies that support a role for DCs promoting T cell survival in vitro, DCs were isolated by repeated rounds of plastic adherence and were co-cultured with T cells at a 1 to 1 ratio (Feuillet et al., 2005; Revy et al., 2001). It is conceivable that a non-DC population contributed to the survival of T cells in these studies. Furthermore, culturing F5 Rag1\(^{-/}\) T cells with more FACS-purified cDCs or alternatively with bone marrow derived DCs might improve F5 Rag1\(^{-/}\) T cell viability in vitro.

Consistent with DCs not being the source of a T cell survival signal provided by CD11c\(^{+}\) enriched splenocytes, a recent study also found that FACS-purified CD45\(^{+}\) CD11c\(^{+}\) CD11b\(^{lo}\) DCs were unable to improve the viability of co-cultured T cells at a 40 to 1 T cell to DC ratio (Link et al., 2007). A stromal CD45\(^{-}\) gp38\(^{+}\) cell population, however, was shown to promote T cell survival in vitro at the same 40 to 1 ratio (Link et al., 2007) and stromal splenocytes present after CD11c\(^{+}\) enrichment might thus contribute to F5 Rag1\(^{-/}\) T cell survival in vitro. It seems unlikely, however, that stromal cells alone are responsible for the survival signal provided by CD11c\(^{+}\) enriched splenocytes for two reasons. Firstly, the survival signal provided by CD11c\(^{+}\) enriched splenocytes correlated with the frequency of CD11c\(^{+}\) cells after enrichment yet the frequency of CD45\(^{-}\)
cells dropped after CD11c\(^+\) enrichment (Figure 4.4D). Secondly, the survival signal provided by FRC is inhibited by addition of IL-7R\(\alpha\) neutralising antibodies (Link et al., 2007), whereas the survival signal provided by CD11c\(^+\) enriched splenocytes was IL-7-independent. In fact, IL-7\(^-\) \(Rag1^{-/-}\) CD11c\(^+\) enriched splenocytes provided F5 \(Rag1^{-/-}\) T cells with a better survival signal than B6 \(Rag1^{-/-}\) CD11c\(^+\) enriched splenocytes and addition of non-saturating concentrations of IL-7 reduced the survival signal provided by B6 \(Rag1^{-/-}\) CD11c\(^+\) enriched splenocytes (Figure 4.5E). These data suggest a more complex relationship between the survival signals provided by IL-7 and CD11c\(^+\) enriched splenocytes. Neither FACS-purified cDCs, pDCs or MHCII\(^-\) cells were able to promote F5 \(Rag1^{-/-}\) T cell survival alone but, interestingly, the survival signal they did provide correlated was dose-dependent (Figure 4.5D). This raised the possibility that the improving F5 \(Rag1^{-/-}\) T cell viability \textit{in vitro} was not a feature of CD11c\(^+\) enriched splenocytes alone, but that contact with any cell might enhance F5 \(Rag1^{-/-}\) T cell survival \textit{in vitro}. Co-culturing F5 \(Rag1^{-/-}\) T cells with either FACS-purified C57Bl/6 B or CD4 T cells did not improve F5 \(Rag1^{-/-}\) T cell viability \textit{in vitro} to the same degree as CD11c\(^+\) enriched splenocytes (Figure 4.4G). Interestingly, culturing F5 \(Rag1^{-/-}\) T cells with FACS-purified B cells did consistently improve \textit{in vitro} F5 \(Rag1^{-/-}\) T cell viability compared to the viability of F5 \(Rag1^{-/-}\) T cells cultured alone (Figure 4.4G). This observation is consistent with other work that finds a minimal role for B cells promoting T cell survival \textit{in vitro} (Link et al., 2007; Revy et al., 2001). It seems likely therefore, that one or multiple specific cell subsets enriched in the CD11c\(^+\) enriched spleen promote F5 \(Rag1^{-/-}\) T cell survival \textit{in vitro}, although which cell population or combination
of cell populations mediate this survival signal is still unclear. The importance of cell contact and soluble factors for survival signals from CD11c+ enriched splenocytes, also requires further investigation.

Finally, we were able to demonstrate a role for PI3K and mTOR signalling, as well as a role for and MEK signalling, in the T cell survival signal provided by CD11c+ enriched splenocytes (Figure 4.6A and B). The regulation of both glucose and amino acid metabolism by PI3K, and its target Akt, and mTOR signalling have been implicated in IL-7-induced T cell growth (Pearson et al., 2012; Rathmell et al., 2001; Wofford et al., 2008) and T cell survival (Barata et al., 2004; Kelly et al., 2002; Wofford et al., 2008). The exact role of glucose metabolism for IL-7-induced T cell survival in vitro and in vivo remains controversial since glucose has recently been shown to be dispensable for IL-7-induced survival in vitro (Pearson et al., 2012) and glucose uptake of IL-7Rα-deficient T cells appears normal (Jacobs et al., 2010). Regardless of their role in IL-7-mediated T cell survival, PI3K and Akt signalling have been implicated in multiple cellular survival processes (Datta et al., 1999). The modest reduction of viable F5 Rag1−/− T cells in the presence of either rapamycin or LY294002 (Figure 4.6A and B) suggests that both PI3K and mTOR signalling are important for the T cell survival signal from CD11c+ enriched splenocytes in vitro. Inhibition of MEK signalling, meanwhile, completely abolished the enhanced survival of F5 Rag1−/− T cells co-cultured with CD11c+ enriched splenocytes (Figure 4.6A and B) and this finding was of particular interest for several reasons.
Firstly, Erk activation by MEK is a downstream target of TCR signalling (reviewed in Brownlie and Zamoyska, 2013). Intriguingly, TCR signalling to Erk via Ras and MAPK in thymocytes has been shown to be particularly sensitive to TCR signalling strength. Even small differences in positive selection affinity are transduced to differences in Zap70 phosphorylation ultimately leading to either positive selection and survival or negative selection and cell death (Daniels et al., 2006). Secondly, constitutive activation of MAPK signalling has been implicated in the development and progression of many cancers where signalling to Erk via Raf and MEK has been shown to prevent apoptosis and promote cell growth while inhibition of MEK can induce apoptosis and inhibit growth (reviewed in Platanias, 2003). Finally, and perhaps most intriguingly, MEK inhibition in melanoma cells *in vitro* results in enhanced transcription of pro-apoptotic Bmf (Shao and Aplin, 2010; VanBrocklin et al., 2009), which we show is downstream of Zap70 signalling in F5 *Rag1*−/− T cells *in vivo* (Figure 3.10). The MEK dependence of the survival signal from TCR via Zap70 is interesting since MEK and Erk have been shown to regulate *Bmf* transcription (Shao and Aplin, 2010) and Bmf activity (Shao and Aplin, 2012). How MEK-inhibition will affect the *in vitro* survival of Zap70- or Bmf-deficient F5 *Rag1*−/− T cells co-cultured with CD11c+ enriched splenocytes remains to be investigated.
Figure 4.1 Intact IL-7R signalling in F5 TetZap70 mice

F5 Rag1−/− (grey fill), F5 TetZap70ON (blue) and F5 TetZap70OFF (red) LN lymphocyte IL-7Rα and CD8 expression and intracellular Bcl-2 abundance were analysed by flow cytometry. F5 TetZap70OFF lymphocytes were isolated from the LNs of F5 TetZap70 chimeras taken off dox ≥5 days previously. Bone marrow chimeras were made by reconstituting sub-lethally irradiated B6 Rag1−/− mice with F5 TetZap70 bone marrow. (A) Histograms show ex vivo IL-7Rα and CD8 expression on F5 Rag1−/− (grey fill), F5 TetZap70ON (blue) and F5 TetZap70OFF (red) CD8+ TCR+ CD5hi TCRhi Lymphocytes (LN). (B) Histograms show IL-7Rα and CD8 expression on F5 Rag1−/− (black), F5 TetZap70ON (blue) and F5 TetZap70OFF (red) CD8+ TCR+ CD5hi TCRhi Lymphocytes (LN) after 24h in vitro in absence of IL-7; 0h F5 Rag1−/− (grey fill) IL-7Rα and CD8 expression are also shown as controls. (C) Histograms show intracellular Bcl-2 abundance in F5 Rag1−/− and F5 TetZap70ON DP1 (dark grey fill) and CD8+ SP thymocytes (black); thymocyte PE-conjugated IgG monoclonal isotype staining (light grey fill) is also shown as a control. (D) Histogram shows intracellular Bcl-2 abundance in CD8+ TCR+ CD5hi TCRhi Lymphocytes (LN); LN lymphocyte PE-conjugated IgG monoclonal isotype staining (light grey fill) is also shown as a control. (E) Histograms show intracellular Bcl-2 abundance in F5 Rag1−/−, F5 TetZap70ON and F5 TetZap70OFF CD8+ TCR+ CD5hi TCRhi Lymphocytes (LN) cultured with 10ng/mL IL-7 (solid) or medium alone (dark grey fill) with 10ng/mL IL-7. Data are representative of ≥three independent experiments.
Figure 4.2 Zap70-deficiency does not affect the IL-7-induced survival of naïve CD8 T cells at saturating concentrations of IL-7

F5 Rag1−/− (grey), F5 TetZap70ON (blue) and F5 TetZap70OFF (red) lymphocytes (LN) were cultured at 1x10⁶ cells/well with 10ng/mL IL-7 (+IL-7, filled squares) or medium alone (-IL-7, empty squares) for 24, 48 or 72 hours (h), as indicated, after which they were counted and analysed by flow cytometry. F5 TetZap70OFF lymphocytes were isolated from the LNs of F5 TetZap70 chimeras taken off dox ≥5 days previously. Bone marrow chimeras were made by reconstituting sub-lethally irradiated B6 Rag1−/− mice with F5 TetZap70 bone marrow. Dot plots show annexin-V staining against forward scatter (FS) of cultured CD8⁺ TCR⁺ lymphocytes at 0 (A) and 72 (B) hours. (C) Line graphs show the frequency of live annexin-V⁻ CD8⁺ TCR⁺ lymphocytes (LN) at 0, 24, 48 and 72 hours (h) as a percentage of the frequency at 0 hours. Each time-point represents ≥2 replicate wells (technical). (D) Line graph shows the frequency of live annexin-V⁻ CD8⁺ TCR⁺ F5 TetZap70ON (Ly5.2) or F5 TetZap70OFF (Ly5.2) lymphocytes (LN) at 48 hours as a percentage of the frequency at 0 hours. Data are also expressed as a percentage of the frequency of live annexin-V⁻ CD8⁺ TCR⁺ Ly5.1 F5 Rag1−/− lymphocytes in the same well at 48 hours. Each time-point represents ≥2 replicate wells (technical). Data are representative of ≥three independent experiments.
Figure 4.3 B6 *Rag1<sup>−/−</sup>* splenocytes enriched for CD11c<sup>+</sup> cells promote Zap70-dependent T cell survival

FACS-purified CD8<sup>+</sup> TCR<sup>+</sup> F5 *Rag1<sup>−/−</sup>* (grey), F5 TetZap70<sup>ON</sup> (blue) and F5 TetZap70<sup>OFF</sup> (red) lymphocytes (LN) were cultured at 1x10<sup>5</sup> cells/well with 1x10<sup>6</sup> CD11c<sup>+</sup> enriched B6 *Rag1<sup>−/−</sup>* splenocytes (+CD11c<sup>+</sup>, filled squares) or medium alone (-CD11c<sup>+</sup>, empty squares) for 24, 48 or 72 hours (h), as indicated, after which they were counted and analysed by flow cytometry. F5 TetZap70<sup>OFF</sup> lymphocytes were isolated from the LNs of F5 TetZap70 chimeras taken off dox ≥5 days previously. Bone marrow chimeras were made by reconstituting sub-lethally irradiated B6 *Rag1<sup>−/−</sup>* mice with F5 TetZap70 bone marrow. (A) Dot plots show MHCII and CD11c expression and histograms show CD11c expression on total live splenocytes pre- and post-CD11c<sup>+</sup>-enrichment. (B) Dot plots show CD8 and TCR expression on FACS-purified CD8<sup>+</sup> TCR<sup>+</sup> lymphocytes (LN). (C) Dot plots show annexin-V staining against forward scatter (FS) of cultured CD8<sup>+</sup> TCR<sup>+</sup> lymphocytes at 0 and 72 hours. (D) Line graphs show the frequency of live annexin-V<sup>−</sup> CD8<sup>+</sup> TCR<sup>+</sup> lymphocytes (LN) at 0, 24, 48 and 72 hours (h) as a percentage of the frequency at 0 hours. Each time-point represents ≥2 replicate wells (technical). Data are representative of ≥three independent experiments.
Figure 4.4 FACS-purified cDCs and pDCs alone are unable to promote substantial naïve T cell survival in vitro

FACS-purified CD8+ TCR+ F5 Rag1−/− were cultured at 1x10^5 cells/well with 1x10^6 CD11c+ enriched B6 Rag1−/− splenocytes, FACS-purified DC subsets, CD4 (LN) or B (spleen) lymphocytes or medium alone, as indicated, for 48 hours (h) after which they were counted and analysed by flow cytometry. Histogram shows CD11c expression (A) and dot plots show MHCII and CD11c expression (B) on total live splenocytes pre- and post-CD11c+ enrichment. (C) Dot plot shows MHCII and CD19/TCR (Lin) expression on total live splenocytes post-CD11c+ enrichment. (D) Dot plot shows mPDCA1 and CD11c expression and histogram shows CD45 expression on MHCII+ and MHCII− live CD11c+ splenocytes pre- and post-enrichment. Dot plots show MCHII and CD19/TCR (Lin) expression and, mPDCA1 and CD11c expression on FACS-purified MHCII+, cDC and pDC subsets (E) and CD4 and TCR expression and, B220 and CD19 expression on FACS-purified CD4 and B lymphocytes (F). (G) Bar chart shows the frequency of live annexin-V− CD8+ TCR+ F5 Rag1−/− lymphocytes (LN) after 48 hours of culture with medium alone (white), MHCII− (1), cDCs (2), pDCs (3), CD4 T (4) or B (5) lymphocytes as a percentage of the frequency at 0 hours and as a percentage of the frequency of live annexin-V− CD8+ TCR+ F5 Rag1−/− lymphocytes after 48 hours cultured with 1x10^6 CD11c+ enriched B6 Rag1−/− splenocytes. Each time-point represents ≥2 replicate wells (technical). Data are representative of ≥two independent experiments.
A B6 Rag1−/− spn

Pre
Post
33.1
76.1

→ CD11c

CD11c+

MHCII

C

MHCI

25.1

→ Lin

73.5

D

mPDCA1

10.4

→ CD11c

CD45

E

MHC−
cDCs

97.5

→ Lin

95.4

mPDCA1

pDCs

95.3

CD11c

F

CD4

99.9

β220

G

Live cells (%0h, %unsorted)

<table>
<thead>
<tr>
<th></th>
<th>Nil</th>
<th>6x10^5 Rag1−/− MHCII−</th>
<th>6x10^4 Rag1−/− cDCs</th>
<th>3x10^4 Rag1−/− pDCs</th>
<th>1x10^6 B6 CD4 T cells</th>
<th>1x10^6 B6 B cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

H

TCR

CD19
Figure 4.5 The T cell survival signal provided by CD11c\(^{+}\) splenocytes is β2m- and IL-7-independent

FACS-purified CD8\(^{+}\) TCR\(^{+}\) F5 \textit{Rag1}\(^{-/-}\) were cultured at 1x10\(^5\) cells/well with 1x10\(^6\) CD11c\(^{+}\) enriched B6 \textit{Rag1}\(^{-/-}\), \textit{b2m}\(^{-/-}\) \textit{Rag1}\(^{-/-}\) or \textit{IL-7}\(^{-/-}\) \textit{Rag1}\(^{-/-}\) splenocytes with IL-7 and medium alone, as indicated, for 48 hours (h) after which they were counted and analysed by flow cytometry. (A) Dot plot shows CD8 and TCR expression on FACS-purified CD8\(^{+}\) TCR\(^{+}\) lymphocytes (LN). (B) Dot plots show MHCII and CD11c on total live splenocytes post-CD11c\(^{+}\)-enrichment. (C) Dot plots show annexin-V staining against forward scatter (FS) of cultured CD8\(^{+}\) TCR\(^{+}\) lymphocytes (LN) at 48 hours. (D) Bar chart shows the frequency of live annexin-V\(^{-}\) CD8\(^{+}\) TCR\(^{+}\) F5 \textit{Rag1}\(^{-/-}\) lymphocytes (LN) after 48 hours of culture with 10ng/mL IL-7 (black), medium alone (white), 1x10\(^6\) CD11c\(^{+}\) enriched B6 \textit{Rag1}\(^{-/-}\) (1), \textit{b2m}\(^{-/-}\) \textit{Rag1}\(^{-/-}\) (2) or \textit{IL-7}\(^{-/-}\) \textit{Rag1}\(^{-/-}\) (3) splenocytes. Each bar represents ≥4 replicate wells (biological); ns p>0.05 (one-way analysis of variance followed by Bonferroni correction for multiple comparisons). (E) Line graph shows the frequency of live annexin-V\(^{-}\) CD8\(^{+}\) TCR\(^{+}\) F5 \textit{Rag1}\(^{-/-}\) lymphocytes (LN) after 48 hours of culture with 0.25x10\(^5\), 0.5x10\(^6\) or 1x10\(^6\) CD11c\(^{+}\) enriched B6 \textit{Rag1}\(^{-/-}\) lymphocytes and 10ng/mL, 2ng/mL, 0.4ng/mL, 0.08ng/mL, 0.016ng/mL or medium alone as a percentage of the frequency at 0 hours. Data are representative of ≥three independent experiments.
A 0h: $\text{Rag}^{-/-}$
CD11c$^+$

- $\text{b2m}^{-/-}$ $\text{Rag}^{-/-}$
CD11c$^+$

- $\text{IL}-7^{-/-}$ $\text{Rag}^{-/-}$
CD11c$^+$

- CD11c

B 48h: $\text{Nil}$

- $\text{Rag}^{-/-}$

- $\text{b2m}^{-/-}$ $\text{Rag}^{-/-}$

- $\text{IL}-7^{-/-}$ $\text{Rag}^{-/-}$

- Anninexin-V

- FS

C

- Live cells (%0h)

- 10ng/mL IL-7
- 0ng/mL IL-7

1 $\text{Rag}^{-/-}$ CD11c$^+$

2 $\text{b2m}^{-/-}$ $\text{Rag}^{-/-}$ CD11c$^+$

3 $\text{IL}-7^{-/-}$ $\text{Rag}^{-/-}$ CD11c$^+$

D

- Live cells (%0h)

- CD11c$^+$ (x10$^6$)

- 10ng/mL IL-7
- 0.08ng/mL IL-7
- 2ng/mL IL-7
- 0.016ng/mL IL-7
- 0.4ng/mL IL-7
- 0ng/mL IL-7
Figure 4.6 The T cell survival signal provided by CD11c⁺ splenocytes is impaired by MEK inhibition

FACS-purified CD8⁺ TCR⁺ F5 Rag1⁻/⁻ were cultured at 1x10⁵ cells/well with 10ng/mL IL-7 (black), medium alone (white) or 1x10⁶ CD11c⁺ enriched B6 Rag1⁻/⁻ splenocytes alone (dark grey) or with DMSO, UO126, rapamycin or LY294002 (grey), as indicated. (A) Dot plots show annexin-V staining against forward scatter (FS) of cultured CD8⁺ TCR⁺ lymphocytes (LN) at 48 hours. (B) Bar chart shows the frequency of live annexin-V CD8⁺ TCR⁺ F5 Rag1⁻/⁻ lymphocytes (LN) after 48 hours. Each bar represents ≥3 replicate wells (biological); *** p≤0.001, ** p≤0.01, * p≤0.05 and ns p>0.05 (one-way analysis of variance followed by Bonferroni correction for multiple comparisons). Data are representative of ≥two independent experiments.
**A** 48h: +Rag1\(^{-/-}\) CD11c\(^{+}\) +DMSO +UO126 +rapamycin +LY296002

Annexin-V

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Annexin-V</th>
<th>FS</th>
</tr>
</thead>
<tbody>
<tr>
<td>+DMSO</td>
<td>79.1</td>
<td>14.4</td>
</tr>
<tr>
<td>+UO126</td>
<td>95</td>
<td>1.84</td>
</tr>
<tr>
<td>+rapamycin</td>
<td>87.8</td>
<td>7.24</td>
</tr>
<tr>
<td>+LY296002</td>
<td>89.6</td>
<td>6.73</td>
</tr>
</tbody>
</table>

**B**

Live cells (%Oh)

- **Black bar**: 10ng/mL IL-7
- **White bar** (Nil)
- **Gray bars** for Rag1\(^{-/-}\) CD11c\(^{+}\)
- **Dark gray bars** for Rag1\(^{-/-}\) CD11c\(^{+}\) +DMSO or inhibitor

- ns: not significant
- **Star symbols** indicate statistical significance:
  - *: p < 0.05
  - **: p < 0.01
  - ***: p < 0.001
Chapter 5  The importance of two Zap70 domains for the transduction of TCR survival signals

5.1 Introduction

We characterised the importance of Zap70-mediated TCR signals for naïve F5 T cell survival in Chapter 3 and Chapter 4. In this chapter we will investigate how mutations in Zap70 leading either to the loss of Zap70 adaptor function or impaired recruitment of Zap70 to the TCR signalling complex, affect the transduction of TCR survival signals in naïve F5 T cells. Zap70, like its family member Syk, consists of a C-terminal kinase domain and two tandem N-terminal SH2 domains, which bind phosphorylated ITAMs of CD3 chains associated with the TCR complex (Figure 1.5 and introduced in section 1.3.2) (reviewed in Au-Yeung et al., 2009). Three tyrosine residues located in the interdomain B in between the two SH2 domains of Zap70, Y292, Y315 and Y319, are known sites of phosphorylation and interact with downstream signalling targets of Zap70. Interestingly, as well as mediating the adaptor function of Zap70, Y315 and Y319 have also been implicated in regulating the auto-inhibitory conformation of Zap70, thus regulating its activity (Brdicka et al., 2005).
Mutation of Y315 and Y319 to alanine and a missense mutation in the C-terminal SH2 domain of Zap70 in YYAA and SKG mice, respectively, both result in hypomorphic TCR signalling (Hsu et al., 2009; Sakaguchi et al., 2003). The mutation in the SH2 domain of Zap70 leads to defective recruitment and association of Zap70 with CD3ζ ITAMs following TCR activation (Sakaguchi et al., 2003). Consistent with this TCR stimulation of T cells from SKG mice in vitro results in impaired Ca\(^{2+}\) mobilisation and impaired phosphorylation of LAT and PLC\(\gamma\) (Sakaguchi et al., 2003). The YYAA mutation is thought to specifically disrupt Zap70 adaptor function without stabilising the autoinhibitory conformation regulated by Y315 and Y319 (Hsu et al., 2009). Similar to T cells from SKG mice, T cells from YYAA mice show impaired Erk phosphorylation and Ca\(^{2+}\) mobilisation following TCR stimulation, although the defects in TCR signalling are more severe in T cells from SKG than from YYAA mice (Hsu et al., 2009). Although Zap70\(^{yc}\) mice apparently have no impairment in TCR signalling or T cell development, it is important to bear in mind the reduced T cell abundance of Zap70\(^{Yyya}\) and Zap70\(^{Skg}\) in YYAA and SKG mice (Hsu et al., 2009).

Despite the more severe TCR signalling defect in SKG mice, T cell numbers in SKG and YYAA mice are similar (Hsu et al., 2009). Positive and negative selection is impaired in both SKG and YYAA mice resulting in the selection of autoreactive T cells which would be eliminated during negative selection in WT mice (Hsu et al., 2009; Sakaguchi et al., 2003). Consistent with a shift in the T cell repertoire and reduced numbers of T\(_{reg}\), both strains develop serum rheumatoid factor following zymosan challenge but only SKG mice develop rheumatoid arthritis (Hsu et al., 2009; Sakaguchi et al., 2003). Interestingly, the
in vitro suppressive activity of CD4 T_{reg} from SKG mice is normal (Hsu et al., 2009) but CD4 T_{reg} from YYAA mice are much less able to suppress the proliferation of naïve T cells in vitro (Hsu et al., 2009). Differences in T_{reg} function between SKG and YYAA mice thus fail to explain why only SKG mice develop rheumatoid arthritis. Instead, it has been proposed that the difference in TCR signalling defects as a result of the SKG and YYAA mutation in Zap70 lead to differences in the T cell repertoire so that only SKG mice develop rheumatoid arthritis following zymosan challenge (Hsu et al., 2009).

In this chapter we sought to investigate whether two hypomorphic Zap70 alleles might be able to transduce TCR survival signals in naïve F5 T cells. To do this we crossed F5 TetZap70 mice with YYAA and SKG mice resulting in mice, which expressed WT Zap70 under transgenic control and mutant Zap70^{YYAA} or Zap70^{SKG} under endogenous control. We anticipated that this would allow the development of F5 T cells expressing WT and mutant Zap70 in dox-fed mice after which we hope to assess the effect of Zap70^{YYAA} or Zap70^{SKG} on naïve F5 T cell survival following dox-withdrawal.

5.2 Results

5.2.1 F5 T cell development is blocked in F5 TetZap70^{unind} Zap70^{Yyaa/-} and F5 TetZap70^{unind} Zap70^{Skg/-} chimeras

To investigate whether two Zap70 mutants, Zap70^{YYAA} and Zap70^{SKG}, were able to transduce a Zap70-dependent TCR survival signal we crossed F5 TetZap70 mice with polyclonal C57Bl/6 mice expressing Zap70^{Yyaa} (Hsu et al., 2009) or Zap70^{Skg} (Sakaguchi et al., 2003). These mice express dox-inducible and T-cell
specific Zap70 and hCD2 (Zap70<sup>Tre rTA.C</sup>huCD2), are Rag1-deficient and express the F5 TCR. In addition, these mice express mutant Zap70<sup>Yyaa</sup> or Zap70<sup>Skg</sup> under endogenous control on one allele while the second allele is a null allele (F5 TetZap70 Zap70<sup>Yyaa</sup>/ or F5 TetZap70 Zap70<sup>Skg</sup>/, respectively, hereon). We anticipated that transgenic expression of Zap70<sup>WT</sup> in constitutively dox-fed mice would allow normal F5 T cell development while dox-withdrawal would lead to expression of only Zap70<sup>Yyaa</sup> or Zap70<sup>Skg</sup>. As with F5 TetZap70 mice, we generated host bone marrow chimeras using F5 TetZap70 YYAA bone marrow in sub-lethally irradiated B6 Rag1<sup>-/-</sup> mice, which were left to reconstitute for a minimum of 6 weeks.

In view of the defect in TCR signalling and resulting impaired T cell development in polyclonal YYAA and SKG we wanted to characterise the thymic development of F5 T cells in constitutively dox-fed and uninduced F5 TetZap70 Zap70<sup>Yyaa</sup>- and F5 TetZap70 Zap70<sup>Skg</sup>- chimeras and mice (F5 TetZap70<sup>ON</sup> Zap70<sup>Yyaa</sup>/, F5 TetZap70<sup>ON</sup> Zap70<sup>Skg</sup>/, F5 TetZap70<sup>unind</sup> Zap70<sup>Yyaa</sup>/ and F5 TetZap70<sup>unind</sup> Zap70<sup>Skg</sup>/, respectively, hereon) (Figure 5.1). Since F5 TetZap70<sup>unind</sup> Zap70<sup>Yyaa</sup>- and F5 TetZap70<sup>unind</sup> Zap70<sup>Skg</sup>- mice express only one allele of Zap70 under endogenous control and only one allele of F5 TCR, we used F5<sup>+/-</sup> Rag1<sup>-/-</sup> Zap70<sup>+/-</sup> chimeras as controls in addition to F5 Rag1<sup>-/-</sup> mice and chimeras (Figure 5.1A). We detected no gross differences in T cell development in F5<sup>+/-</sup> Rag1<sup>-/-</sup> Zap70<sup>+/-</sup> and F5 Rag1<sup>-/-</sup> chimeras by flow cytometry, other than a small reduction in the number of CD8 SP thymocytes developing in F5<sup>+/-</sup> Rag1<sup>-/-</sup> Zap70<sup>+/-</sup> chimeras compared to F5 Rag1<sup>-/-</sup> chimeras (Figure 5.1A, D and E). Similar to Zap70<sup>-/-</sup> mice (Negishi et al., 1995), F5 T cell development was blocked at the DP stage in F5 TetZap70<sup>unind</sup> Zap70<sup>Yyaa</sup>- and F5
TetZap70\textsuperscript{unind} Zap70\textsuperscript{Skg/-} mice (Figure 5.1A). The block in F5 T cell development in F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Skg/-} mice was overcome by constitutive dox-feeding, and F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Skg/-} chimeras had readily detectable CD8 SP thymocyte populations (Figure 5.1A). Interestingly, we consistently detected fewer CD8 SP in F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Yyaa/-} than in F5 TetZap70\textsuperscript{ON} chimeras, whereas the frequency and number of CD8 SP thymocytes in F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Skg/-} and F5 TetZap70\textsuperscript{ON} chimeras was similar (Figure 5.1D and E). Collectively, these data show that neither Zap70\textsuperscript{Yyaa} nor Zap70\textsuperscript{Skg} can mediate the development of F5 T cells but that dox-feeding rescues T cell development in F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Skg/-} chimeras.

We looked at the block in T cell development in F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Skg/-} mice in some more detail by looking at CD5 and TCR expression within the DP subset by flow cytometry (Figure 5.1B). DP thymocytes can subdivided into 3 phenotypically and developmentally distinct DP subsets based on their CD5 and TCR expression (described in Chapter 3) and in both F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Skg/-} mice were blocked at the CD5\textsuperscript{lo} TCR\textsuperscript{lo} DP1 stage (Figure 5.1B). Thymocytes in Zap70\textsuperscript{+/} mice (Negishi et al., 1995) and F5 TetZap70\textsuperscript{unind} mice (Figure 3.1D and Figure 5.1B) are also blocked at the DP1 stage. Interestingly, while blocked DP1 thymocytes in F5 TetZap70\textsuperscript{unind} and F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Skg/-} mice express heterogeneous levels of CD5, all blocked DP1 thymocytes in F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Yyaa/-} mice had upregulated their CD5 expression (Figure 5.1B). Taken together, these data show firstly, that CD5 expression is modulated at the DP1 stage of T cell development and secondly, that the two
Zap70 mutants, Zap70\textsuperscript{YYAA} and Zap70\textsuperscript{SKG} have different effects on this modulation.

Finally, we also characterised transgene expression in F5 TetZap70 Zap70\textsuperscript{Yyaa/-} and F5 TetZap70 Zap70\textsuperscript{Skg/-} chimeras. In view of the difficulty of distinguishing between mutant and WT Zap70 we focussed mainly on transgenic hCD2 expression. Both thymocytes from F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Skg/-} chimeras expressed hCD2 upon dox-feeding (Figure 5.1C) although the overall abundance of Zap70 was reduced in thymocytes from F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Skg/-} chimeras compared to thymocytes from F5 TetZap70\textsuperscript{ON} chimeras (Figure 5.1C). The frequency of hCD2\textsuperscript{+} thymocytes was comparable in F5 TetZap70\textsuperscript{ON}, F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Skg/-}. Collectively, these data suggest that transgene expression reduced in F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Skg/-} chimeras consistent F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Skg/-} chimeras expressing only one inducible allele of Zap70\textsuperscript{Wt}.

5.2.2 Peripheral reconstitution of constitutively dox-fed F5 TetZap70 Zap70\textsuperscript{Yyaa/-} and F5 TetZap70 Zap70\textsuperscript{Skg/-} chimeras

Next we wanted to characterise the peripheral reconstitution of F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Skg/-} chimeras compared to F5 TetZap70\textsuperscript{ON}, F5 Rag\textsuperscript{-/-} and F5\textsuperscript{+/-} Rag\textsuperscript{-/-} Zap70\textsuperscript{+/-} chimeras (Figure 5.2). Despite the reduction in CD8 SP thymocytes in F5\textsuperscript{+/-} Rag\textsuperscript{-/-} Zap70\textsuperscript{+/-} chimeras compared to F5 Rag\textsuperscript{-/-} chimeras, F5 Rag\textsuperscript{-/-} and F5\textsuperscript{+/-} Rag\textsuperscript{-/-} Zap70\textsuperscript{+/-} chimeras had a similar frequency and number of peripheral CD8 T cells in the LN and
spleen (Figure 5.2A, B and C). We could not detect any peripheral CD8 T cells in F5 TetZap70<sup>unind</sup>, F5 TetZap70<sup>unind</sup> Zap70<sup>Yyaa</sup> and F5 TetZap70<sup>unind</sup> Zap70<sup>Skg</sup> mice (Figure 5.2A) consistent with the observed block in T cell development in these mice (Figure 5.1A). Peripheral CD8 T cells were readily detectable by flow cytometry in the LN of F5 TetZap70<sup>ON</sup> Zap70<sup>Yyaa</sup> and F5 TetZap70<sup>ON</sup> Zap70<sup>Skg</sup> chimeras (Figure 5.2A). In addition to fewer CD8 SP thymocytes, F5 TetZap70<sup>ON</sup> Zap70<sup>Yyaa</sup> chimeras also consistently had a reduced frequency and number of peripheral CD8 T cells in the LN and spleen. These data show that dox-feeding allows the peripheral reconstitution of F5 TetZap70<sup>ON</sup> Zap70<sup>Yyaa</sup> and F5 TetZap70<sup>ON</sup> Zap70<sup>Skg</sup> chimeras but that Zap70<sup>YYAA</sup> exerts a dominant negative effect on T cell homeostasis in F5 TetZap70<sup>ON</sup> Zap70<sup>Yyaa</sup> chimeras.

Since surface CD5 expression on peripheral CD8 T cells is modulated by homeostatic TCR signalling (Saini et al., 2009; Smith et al., 2001) and Zap70<sup>YYAA</sup> and Zap70<sup>SKG</sup> mediate reduced TCR signalling in polyclonal T cells we analysed CD5 expression on peripheral CD8 T cells from F5 TetZap70<sup>ON</sup>, F5 TetZap70<sup>ON</sup> Zap70<sup>Yyaa</sup> and F5 TetZap70<sup>ON</sup> Zap70<sup>Skg</sup> chimeras by flow cytometry (Figure 5.2D). CD8 T cells from F5<sup>+</sup>/<sup>−</sup> Rag1<sup>−/−</sup> Zap70<sup>++/−</sup> chimeras consistently expressed less CD5 than CD8 T cells from F5 Rag1<sup>−/−</sup> chimeras suggesting heterozygous Zap70 expression is sufficient for a reduction in T cell homeostatic TCR signalling (Figure 5.2D). Meanwhile CD8 T cells from F5 TetZap70<sup>ON</sup>, F5 TetZap70<sup>ON</sup> Zap70<sup>Yyaa</sup> and F5 TetZap70<sup>ON</sup> Zap70<sup>Skg</sup> chimeras all had the same low surface CD5 expression suggesting that CD8 T cells in these three mouse strains receive similar reduced homeostatic TCR signals.
5.2.3 Regulation of Zap70 abundance in F5 TetZap70 Zap70\(^{Yyaa/-}\) and F5 TetZap70 Zap70\(^{Skg/-}\) chimeras

In view of the expression defects of both Zap70\(^{Yyaa}\) and Zap70\(^{Skg}\) in polyclonal mice we wanted to characterise Zap70 abundance in F5 TetZap70 Zap70\(^{Yyaa/-}\) and F5 TetZap70 Zap70\(^{Skg/-}\) chimeras taken off dox at least 5 days previously (F5 TetZap70\(^{OFF}\) Zap70\(^{Yyaa/-}\) and F5 TetZap70\(^{OFF}\) Zap70\(^{Skg/-}\), respectively, hereon). Zap70 abundance in thymocytes from F5 TetZap70\(^{OFF}\) Zap70\(^{Yyaa/-}\) and F5 TetZap70\(^{OFF}\) Zap70\(^{Skg/-}\) chimeras was similar and higher than in thymocytes from F5 TetZap70\(^{OFF}\) chimeras but lower than in thymocytes from F5\(^{+/+}\) Rag1\(^{-/-}\) Zap70\(^{+/+}\) chimeras (Figure 5.3A). Interestingly, Zap70 abundance in CD8 T cells from F5\(^{+/+}\) Rag1\(^{-/-}\) Zap70\(^{+/+}\) and F5 TetZap70\(^{OFF}\) Zap70\(^{Yyaa/-}\) chimeras was similar, whereas Zap70 abundance in CD8 T cells from F5 TetZap70\(^{OFF}\) and F5 TetZap70\(^{OFF}\) Zap70\(^{Skg/-}\) chimeras was much lower. We also compared Zap70 abundance in CD8 T cells from F5 TetZap70\(^{ON}\) Zap70\(^{Yyaa/-}\) and F5 TetZap70\(^{OFF}\) Zap70\(^{Yyaa/-}\) chimeras and from F5 TetZap70\(^{ON}\) Zap70\(^{Skg/-}\) and F5 TetZap70\(^{OFF}\) Zap70\(^{Skg/-}\) chimeras (Figure 5.3B). CD8 T cell Zap70 abundance was very similar regardless of dox-feeding suggesting the majority of Zap70 in peripheral CD8 T cells comes from the endogenous Zap70 locus (Figure 5.3B). Together, these data show that Zap70 abundance is more severely affected in F5 TetZap70 Zap70\(^{Skg/-}\) chimeras than in F5 TetZap70 Zap70\(^{Yyaa/-}\) chimeras.

5.2.4 Zap70\(^{YyAA}\) and Zap70\(^{SKG}\) cannot substitute for Zap70\(^{WT}\) to promote Zap70-dependent survival of F5 T cells in F5 TetZap70 chimeras

Finally we wanted to assess the survival of CD8 T cells in F5 TetZap70 Zap70\(^{Yyaa/-}\) and F5 TetZap70 Zap70\(^{Skg/-}\) chimeras following dox-withdrawal to
investigate whether either Zap70$^{YYAA}$ or Zap70$^{SKG}$ can substitute for Zap70$^{WT}$ and promote the survival of naïve F5 T cells. We took a cohort of F5 TetZap70$^{ON}$, F5 TetZap70 Zap70$^{Yyaa/-}$ and F5 TetZap70 Zap70$^{Skg/-}$ chimeras off dox (F5 TetZap70$^{OFF}$ Zap70$^{Yyaa/-}$ and F5 OFF Zap70$^{Skg/-}$, respectively, hereon) and followed the peripheral frequency of CD8 T cells in the blood of these mice (Figure 5.4). To account for the different frequencies of CD8 T cells in the blood of F5 $Rag1^{-/-}$, F5$^{+/+}$ Zap70$^{-/-}$, F5 TetZap70, F5 TetZap70 Zap70$^{Yyaa/-}$ and F5 TetZap70 Zap70$^{Skg/-}$ chimeras at d0, we normalised the frequency of blood F5 T cells at each time-point to the starting frequency at d0. Interestingly, in both F5 TetZap70$^{OFF}$ Zap70$^{Yyaa/-}$ and F5 TetZap70$^{OFF}$ Zap70$^{Skg/-}$ chimeras, F5 T cells were not maintained (Figure 5.4A and B). The drop in CD8 T cells in F5 TetZap70$^{OFF}$ Zap70$^{Yyaa/-}$ and F5 TetZap70$^{OFF}$ Zap70$^{Skg/-}$ chimeras compared to F5 TetZap70$^{ON}$ Zap70$^{Yyaa/-}$ and F5 TetZap70$^{ON}$ Zap70$^{Skg/-}$ chimeras could not be explained by a reduction in Zap70 since the frequency of peripheral blood F5 T cells was maintained in F5$^{+/+}$ $Rag1^{-/-}$ Zap70$^{+/+}$ chimeras (Figure 3.5A). CD8 T cells had an average half-life of 21.1 ± 1.3 days in TetZap70$^{OFF}$ Zap70$^{Yyaa/-}$ chimeras and 18.2 days in F5 TetZap70$^{OFF}$ Zap70$^{Skg/-}$ chimeras. After 48 days the LN and spleen of F5 TetZap70$^{OFF}$ Zap70$^{Yyaa/-}$ and F5 TetZap70$^{OFF}$ Zap70$^{Skg/-}$ chimeras consistently had fewer CD8 T cells than the LN and spleen of F5 TetZap70$^{ON}$ Zap70$^{Yyaa/-}$ and F5 TetZap70$^{ON}$ Zap70$^{Skg/-}$ chimeras (Figure 5.3C and D). Taken together, these data show that neither Zap70$^{YYAA}$ nor Zap70$^{SKG}$ is able to substitute for Zap70$^{WT}$ in the transduction of a Zap70-dependent TCR survival signal in naïve CD8 T cells.
In this chapter we show that neither Zap70$^{YYAA}$ nor Zap70$^{SKG}$ are able to substitute for Zap70$^{WT}$ during F5 T cell positive selection or to mediate a TCR survival signal in peripheral naïve F5 T cells. We show that T cell development is blocked at the CD5$^{lo}$ TCR$^{lo}$ DP1 stage in F5 TetZap70$^{unind}$ Zap70$^{Yyaa/-}$ and F5 TetZap70$^{unind}$ Zap70$^{Skg/-}$ mice and that heterozygous dox-inducible expression of Zap70$^{Wt}$ rescues T cell development and reconstitutes the peripheral T cell compartment in F5 TetZap70$^{ON}$ Zap70$^{Yyaa/-}$ and F5 TetZap70$^{ON}$ Zap70$^{Skg/-}$ chimeras. Interestingly, blocked DP1 thymocytes F5 TetZap70$^{unind}$ Zap70$^{Yyaa/-}$ mice expressed higher CD5 than blocked DP1 thymocytes F5 TetZap70$^{unind}$ and F5 TetZap70$^{unind}$ Zap70$^{Skg/-}$ mice. F5 TetZap70$^{ON}$ Zap70$^{Yyaa/-}$ consistently had fewer CD8 SP thymocytes and peripheral CD8 T cells than F5 TetZap70$^{ON}$ Zap70$^{Skg/-}$ and F5 TetZap70$^{ON}$ chimeras, suggesting Zap70$^{YYAA}$ has a dominant negative effect on TCR signal transduction by Zap70$^{WT}$. Consistent with data from polyclonal YYAA and SKG mice, Zap70 abundance was lower in thymocytes and peripheral CD8 T cells from F5 TetZap70$^{unind}$ Zap70$^{Skg/-}$ than from F5 TetZap70$^{unind}$ Zap70$^{Yyaa/-}$ mice. CD5 expression was equally reduced on peripheral CD8 T cells from F5 TetZap70$^{ON}$, F5 TetZap70$^{ON}$ Zap70$^{Yyaa/-}$ and F5 TetZap70$^{ON}$ Zap70$^{Skg/-}$ chimeras.

In Chapter 3 we characterised the development and survival of CD8 T cells in F5 TetZap70$^{ON}$ and F5 TetZap70$^{OFF}$ chimeras and showed that CD8 T cell development and survival required dox-inducible Zap70$^{Wt}$ expression. Like in F5 TetZap70$^{unind}$ and F5 TetZap70$^{OFF}$ chimeras, T cell development was blocked at the DP1 stage in F5 TetZap70$^{unind}$ Zap70$^{Yyaa/-}$ and F5 TetZap70$^{unind}$ Zap70$^{Skg/-}$.
chimeras. Interestingly, blocked DP1 thymocytes in F5 TetZap70\textsuperscript{unind} \textit{Zap70}\textsuperscript{Yyaa/-} had all upregulated their CD5 expression in contrast to blocked DP1 thymocytes in F5 TetZap70\textsuperscript{unind} and F5 TetZap70\textsuperscript{unind} \textit{Zap70}\textsuperscript{Skg/-} chimeras (Figure 5.1B). Meanwhile, Zap70 abundance was similar in DP1 thymocytes from F5 TetZap70\textsuperscript{unind} \textit{Zap70}\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{unind} \textit{Zap70}\textsuperscript{Skg/-} chimeras (Figure 5.3A). These data suggested that, in addition to being modulated on thymocytes during positive selection (Azzam et al., 1998; Tarakhovsky et al., 1995), CD5 expression is regulated on thymocytes at the DP1 stage before positive selection. Furthermore, this regulation of CD5 expression appeared to be mediated by Zap70 and was preserved in absence of Zap70 adaptor function in thymocytes from F5 TetZap70\textsuperscript{unind} \textit{Zap70}\textsuperscript{Yyaa/-} chimeras. CD5 expression on DP thymocytes in polyclonal YYAA mice, is also higher than on DP thymocytes in SKG mice (Hsu et al., 2009). This and our data are consistent with the SKG mutation in Zap70 leading to a more severe impairment of Zap70 and TCR signalling. Interestingly, in the periphery CD5 expression on CD8 T cells from F5 TetZap70\textsuperscript{ON}, F5 TetZap70\textsuperscript{ON} \textit{Zap70}\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{ON} \textit{Zap70}\textsuperscript{Skg/-} chimeras was similar, suggesting that one allele of dox-inducible \textit{Zap70}\textsuperscript{Wt} is sufficient to transduce homeostatic TCR signals.

In this chapter we also show that \textit{Zap70}\textsuperscript{YYAA} exerts a dominant negative effect on T cell development and homeostasis in F5 TetZap70\textsuperscript{ON} \textit{Zap70}\textsuperscript{Yyaa/-} chimeras. F5 TetZap70\textsuperscript{ON} \textit{Zap70}\textsuperscript{Yyaa/-} chimeras consistently had fewer CD8 SP thymocytes and fewer peripheral CD8 T cells than F5 TetZap70\textsuperscript{ON} chimeras (Figure 5.1D and E and Figure 5.2D). F5 TetZap70\textsuperscript{ON} \textit{Zap70}\textsuperscript{Skg/-} and F5 TetZap70\textsuperscript{ON} chimeras, on the other hand had a similar frequency and number of CD8 SP thymocytes and peripheral CD8 T cells suggesting that \textit{Zap70}\textsuperscript{YYAA}, specifically,
exerts a dominant negative effect on T cell development and peripheral homeostasis in F5 TetZap70ON Zap70Yyaα^-/^- chimeras. F5^+/- Rag1^-/- Zap70^+/- chimeras had fewer CD8 SP thymocytes than F5 Rag1^-/- chimeras but had a similar number of peripheral CD8 T cells (Figure 5.1D and E and Figure 5.2D). Reduced Zap70 abundance alone, does not account for the defect in T cell development in F5 TetZap70ON Zap70Yyaα^-/^- chimeras since Zap70 abundance is similar in F5 TetZap70ON Zap70^Yyaα/- and F5^+/- Rag1^-/- Zap70^+/- thymocytes is similar (Figure 5.3A). The TCR-mediated upregulation of Zap70^YYAA during positive selection might be impaired in F5 TetZap70ON Zap70^Yyaα^-/^- chimeras. This seems unlikely, since peripheral CD8 T cells in F5 TetZap70ON Zap70^Yyaα^-/^- and F5^+/- Rag1^-/- Zap70^+/- chimeras have similar Zap70 abundance (Figure 5.3A) suggesting transgenic Zap70^WT can transduce the TCR signals that mediate the developmental upregulation of Zap70^Yyaα. Instead, it seems likely that Zap70^YYAA interferes with Zap70^WT signalling in F5 TetZap70ON Zap70^Yyaα^-/^- chimeras.

We generated F5 TetZap70 Zap70^Yyaα^-/^- and F5 TetZap70 Zap70^Skg^-/^- mice to investigate the effects of loss of Zap70 adaptor function and attenuated Zap70 signalling on naïve CD8 T cell survival in vivo. We show that neither Zap70^YYAA nor Zap70^Skg^- was sufficient for the transduction of TCR survival signals in naïve CD8 T cells. Furthermore, in absence of transgenic Zap70^WT expression, CD8 T cells are not maintained in F5 TetZap70OFF, F5 TetZap70OFF Zap70^Yyaα^-/^- or F5 TetZap70OFF Zap70^Skg^-/^- chimeras (Figure 5.4A and B). Reduced Zap70 abundance in F5^+/- Rag1^-/- Zap70^+/- chimeras had no effect on CD8 T cell half-life for 48 days (Figure 5.4A). Since Zap70 abundance in CD8 T cells from F5 TetZap70OFF Zap70^Yyaα^-/^- and F5^+/- Rag1^-/- Zap70^+/- chimeras was similar,
reduced Zap70-abundance does not account for the impaired maintenance of blood CD8 T cells in F5 TetZap70^{OFF} Zap70^{Yyaa/-}. Zap70 abundance in CD8 T cells from F5 TetZap70^{OFF} Zap70^{Skgl/-} and F5 TetZap70^{OFF} chimeras was comparable and so attenuated Zap70 signalling as well as reduced Zap70 abundance probably contributed to the impaired maintenance of blood CD8 T cells in F5 TetZap70^{OFF} Zap70^{Skgl/-} chimeras. In polyclonal mice the SKG mutation leads to a more severe defect in TCR signalling than the YYAA mutation (Hsu et al., 2009) and consistent with this the half-life of CD8 T cells was shorter in F5 TetZap70^{OFF} Zap70^{Skgl/-} chimeras than in F5 TetZap70^{OFF} Zap70^{Yyaa/-} chimeras. Importantly, F5 TetZap70^{ON} Zap70^{Yyaa/-} chimeras are relatively lymphopaenic compared to F5 TetZap70^{ON} Zap70^{Skgl/-} chimeras and F5 TetZap70^{ON} and it will be essential to characterise the maintenance CD8 T cells from F5 TetZap70^{OFF}, F5 TetZap70^{OFF} Zap70^{Yyaa/-} and F5 TetZap70^{OFF} Zap70^{Skgl/-} chimeras following transfer into F5 Rag1^{-/-} mice.
Figure 5.1 F5 T cell development is blocked in F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Skg/-} chimeras

Thymocytes from F5 Rag1\textsuperscript{-/-}, F5\textsuperscript{+/-} Rag1\textsuperscript{-/-} Zap70\textsuperscript{+/-}, F5 TetZap70\textsuperscript{unind} (red, solid), F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Yyaa/-} (red, dotted) and F5 TetZap70\textsuperscript{unind} Zap70\textsuperscript{Skg/-} (red, thin) mice and constitutively dox-fed F5 TetZap70\textsuperscript{ON} (blue, solid), F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Yyaa/-} (blue, dotted) and F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Skg/-} (blue, thin) bone marrow chimeras were counted and analysed by flow cytometry. Bone marrow chimeras were made by reconstituting sub-lethally irradiated B6 Rag1\textsuperscript{-/-} mice with F5 TetZap70 bone marrow. (A) Dot plots show CD4 against CD8 expression on live or hCD2\textsuperscript{+} thymocytes. (B) Dot plots show CD5 against TCR expression on CD4\textsuperscript{+} CD8\textsuperscript{+} DP thymocytes and histogram shows CD5 expression on CD4\textsuperscript{+} CD8\textsuperscript{+} DP thymocytes. (C) Histograms show hCD2 expression on total live thymocytes. Box and whisker plots show the frequency (D) and number (E) of hCD2\textsuperscript{+} CD5\textsuperscript{hi} TCR\textsuperscript{hi} CD8\textsuperscript{+} SP thymocytes F5 Rag\textsuperscript{/-} (n=2), F5\textsuperscript{+/-} Rag1\textsuperscript{-/-} Zap70\textsuperscript{+/-} (n=7), F5 TetZap70\textsuperscript{ON} (n=13), F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Yyaa/-} (n=13) and F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Skg/-} (n=5) chimeras; *p≤0.05 and ns p>0.05 (one-way analysis of variance followed by Bonferroni correction for multiple comparisons). Data are representative of ≥two independent experiments. CC)
Figure 5.2 Peripheral reconstitution of constitutively dox-fed F5 TetZap70

Zap70\(^{Yyaa^{-}}\) and F5 TetZap70 Zap70\(^{Skg^{-}}\) chimeras

Lymphocytes (LN) from F5 \(Rag^{1^{-}}\), F5\(^{+/-}\) \(Rag^{1^{-}}\) Zap70\(^{+/-}\), F5 TetZap70\(^{unind}\) (red, solid), F5 TetZap70\(^{unind}\) Zap70\(^{Yyaa^{-}}\) (red, dotted) and F5 TetZap70\(^{unind}\) Zap70\(^{Skg^{-}}\) (red, thin) mice and constitutively dox-fed F5 TetZap70\(^{ON}\) (blue, solid), F5 TetZap70\(^{ON}\) Zap70\(^{Yyaa^{-}}\) (blue, dotted) and F5 TetZap70\(^{ON}\) Zap70\(^{Skg^{-}}\) (blue, thin) bone marrow chimeras were counted and analysed by flow cytometry. Bone marrow chimeras were made by reconstituting sub-lethally irradiated B6 \(Rag^{1^{-}}\) mice with F5 TetZap70 bone marrow. (A) Dot plots show CD8 against TCR expression on live lymphocytes (LN). Box and whisker plots show the frequency (B) and number (C) of CD8\(^{+}\) TCR\(^{+}\) CD5\(^{hi}\) TCR\(^{hi}\) lymphocytes in F5 \(Rag^{1^{-}}\) (n=2), F5\(^{+/-}\) \(Rag^{1^{-}}\) Zap70\(^{+/-}\) (n=7), F5 TetZap70\(^{ON}\) (n=13), F5 TetZap70\(^{ON}\) Zap70\(^{Yyaa^{-}}\) (n=18) and F5 TetZap70\(^{ON}\) Zap70\(^{Skg^{-}}\) (n=5); **** \(p \leq 0.0001\) and ns \(p > 0.05\) (one-way analysis of variance followed by Bonferroni correction for multiple comparisons). (D) Histograms show CD5 expression on CD8\(^{+}\) TCR\(^{+}\) CD5\(^{hi}\) TCR\(^{hi}\) lymphocytes (LN). Data are representative of \(\geq\)two independent experiments.
Figure 5.3 Regulation of Zap70 abundance in F5 TetZap70 Zap70\textsuperscript{Yya\textasciitilde} and F5 TetZap70 Zap70\textsuperscript{Skg\textasciitilde} chimeras

Thymocytes and lymphocytes (LN) from F5 \textit{Rag1}\textsuperscript{\textasciitilde}, F5\textsuperscript{\textasciitilde} \textit{Rag1}\textsuperscript{\textasciitilde} Zap70\textsuperscript{\textasciitilde}, F5 TetZap70\textsuperscript{OFF} (red, solid), F5 TetZap70\textsuperscript{OFF} Zap70\textsuperscript{Yya\textasciitilde} (red, dotted) and F5 TetZap70\textsuperscript{OFF} Zap70\textsuperscript{Skg\textasciitilde} (red, thin) mice and constitutively dox-fed F5 TetZap70\textsuperscript{ON} (blue, solid), F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Yya\textasciitilde} (blue, dotted) and F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Skg\textasciitilde} (blue, thin) bone marrow chimeras were counted and analysed by flow cytometry. Bone marrow chimeras were made by reconstituting sub-lethally irradiated B6 \textit{Rag1}\textsuperscript{\textasciitilde} mice with F5 TetZap70 bone marrow. (A) Histograms show Zap70 abundance in total thymocytes and in CD8\textsuperscript{+} TCR\textsuperscript{+} CD5\textsuperscript{hi} TCR\textsuperscript{hi} lymphocytes (LN). (B) Histograms show Zap70 abundance in CD8\textsuperscript{+} TCR\textsuperscript{+} CD5\textsuperscript{hi} TCR\textsuperscript{hi} lymphocytes (LN). With the exception of the left panel of (A) and (B), data are representative of ≥three independent experiments.
Figure 5.4 Zap70<sup>YYAA</sup> and Zap70<sup>SKG</sup> cannot substitute for Zap70<sup>WT</sup> to promote Zap70-dependent survival of F5 T cells in F5 TetZap70 chimeras

A cohort of constitutively dox-fed F5 TetZap70<sup>ON</sup>, F5 TetZap70<sup>ON</sup> Zap70<sup>Yyaa</sup> and F5 TetZap70<sup>ON</sup> Zap70<sup>Skg</sup> bone marrow chimeras were taken off dox and the frequency and number of CD8<sup>+</sup> TCR<sup>+</sup> CD5<sup>hi</sup> TCR<sup>hi</sup> lymphocytes in the blood, LN and spleen of F5 <i>Rag1</i><sup>−/−</sup> (grey), F5<sup>+</sup> <i>Rag1</i><sup>−/−</sup> Zap70<sup>Yyaa</sup> (grey), F5 TetZap70<sup>ON</sup> (blue, solid), F5 TetZap70<sup>ON</sup> Zap70<sup>Yyaa</sup> (blue, dotted), F5 TetZap70<sup>ON</sup> Zap70<sup>Skg</sup> (blue, dotted), F5 TetZap70<sup>OFF</sup> (red, solid), F5 TetZap70<sup>OFF</sup> Zap70<sup>Yyaa</sup> (red, dotted), F5 TetZap70<sup>OFF</sup> Zap70<sup>Skg</sup> (red, dotted) was assessed by flow cytometry. Bone marrow chimeras were made by reconstituting sub-lethally irradiated B6 <i>Rag1</i><sup>−/−</sup> mice with F5 TetZap70 bone marrow. (A) and (B) Line graphs show the frequency of CD8<sup>+</sup> TCR<sup>+</sup> CD5<sup>hi</sup> TCR<sup>hi</sup> blood lymphocytes as a percentage of the frequency at d0; **** p≤0.0001, ** p≤0.01 and * p≤0.05 F5 TetZap70<sup>OFF</sup> compared to F5 TetZap70<sup>ON</sup>, F5 TetZap70<sup>OFF</sup> Zap70<sup>Skg</sup> compared to F5 TetZap70<sup>ON</sup> Zap70<sup>Skg</sup> or F5 TetZap70<sup>OFF</sup> Zap70<sup>Yyaa</sup> compared to F5 TetZap70<sup>ON</sup> Zap70<sup>Yyaa</sup> (two-way analysis of variance followed by Bonferroni correction for multiple comparisons). Each time-point represents ≥2 replicate mice. Box and whiskers plots show the frequency (C) and number (D) of LN CD8<sup>+</sup> TCR<sup>+</sup> CD5<sup>hi</sup> TCR<sup>hi</sup> lymphocytes in bone marrow chimeras (n≥2); *** p≤0.001 and ** p≤0.01 (one-way analysis of variance followed by Bonferroni correction for multiple comparisons). With the exception of (B) data are representative of ≥two independent experiments.
Chapter 6  The role of TCR signalling during a cognate immune response

6.1  Introduction

Naïve CD8 T cells are activated in SLOs by activated DCs presenting the correct pMHC ligand. Efficient T cell sampling of pMHC on DCs is mediated by transient T cell DC interactions (Gunzer et al., 2000) which become more stable following T cell recognition of a specific pMHC (Dustin and Springer, 1989; Shimizu et al., 1990; van Kooyk et al., 1989). Subsequent CD8 T cell activation, proliferation and differentiation into effector and memory cells requires CD28 co-stimulation (reviewed in Acuto and Michel, 2003), cytokine signals, CD4 T cell help (reviewed in Mescher et al., 2006) in addition to antigen stimulation (Zehn et al., 2012).

Following T cell activation, antigen-specific CD8 T cells expand for ~7-8 days, undergoing as many as 15-20 divisions (Butz and Bevan, 1998; Murali-Krishna et al., 1998; and reviewed in Williams and Bevan, 2007). After T cell expansion up to 90-95% antigen-specific T cells undergo apoptosis. The remaining T cells become long-lived memory cells (Kaech et al., 2002; and reviewed in Williams and Bevan, 2007). In addition to many other factors, the strength and duration of the initial TCR stimulus impact on T cell expansion and T cell differentiation during an immune response. A recent study investigated the impact of TCR signal strength on T cell expansion and differentiation using an OT-I TCR transgenic model (Zehn et al., 2009). Interestingly, in this model the initial T cell
expansion and differentiation response provoked by low and high-affinity TCR ligands is similar (Zehn et al., 2009). Stronger TCR signals ultimately promote more sustained proliferation explaining the accumulation of mainly high-affinity antigen-specific T cells during a polyclonal immune response (Zehn et al., 2009).

Interestingly, in this study (Zehn et al., 2009), even TCR stimulation mediated by low affinity ligands promoted the differentiation of CD8 T cells into effector and memory T cells.

Meanwhile, the impact of the duration of pMHC and TCR interactions has been studied extensively in vitro and in vivo (reviewed Williams et al., 2007 and in Masopust et al., 2004)). It seems that CD8 T cells only need very brief encounters with antigen after which the CD8 T cell enters a program of differentiation and expansion in vitro (Kaech and Ahmed, 2001; van Stipdonk et al., 2001; 2003). Even the onset of T cell contraction and differentiation into memory cells after the antigen is cleared, might be programmed very early on during an immune response (Badovinac et al., 2002; Kaech and Ahmed, 2001). Consistent with this, in vivo imaging of T cell DC interactions reveals that initial T cell and DC interactions in the LN are brief (Mempel et al., 2004). Once activated, T cell motility decreases and more stable T cell-DC conjugates form. Finally, proliferating T cells resume their brief encounters with DCs (Mempel et al., 2004). Recent in vivo experiments, however, show that removal of antigen does affect the degree of clonal expansion but that the functionality of generated effector cells does not depend on the duration of antigen exposure (Prlic et al., 2006).
In this chapter, we sought to examine both the strength and duration of TCR signals required to induce F5 T cell activation, expansion and differentiation in vivo. To do this, we challenged F5 $Rag^{1/-}$, F5 TetZap70$^{ON}$ and F5 TetZap70$^{OFF}$ T cells with live influenza in Ly5.1 C57Bl/6 hosts and assessed T cell activation and expansion 7 days after challenge. We also assessed the effect of two Zap70 mutants to transduce antigen-specific T cell activation signals and promote F5 T cell expansion. Finally, we assessed the temporal requirements of Zap70 and thus TCR signaling in vivo by withdrawing dox at various stages of the immune response from C57Bl/6 hosts who received F5 TetZap70$^{ON}$ T cells.

6.2 Results

6.2.1 F5 TetZap70$^{ON}$ T cells expand in response to cognate antigen in B6 $Rag^{1/-}$ mice in vivo

Naïve CD8 T cells from F5 TetZap70$^{ON}$ chimeras had low Zap70 abundance and low surface CD5 expression (Figure 3.2) consistent with receiving weak homeostatic TCR signals. We wanted to test whether CD8 T cells from F5 TetZap70$^{ON}$ chimeras, while less responsive to homeostatic TCR signalling, could respond to cognate antigen in vivo. We did this by challenging CD8 T cells from F5 TetZap70$^{ON}$ chimeras with A/NT/60/68 influenza virus after transfer into B6 $Rag^{1/-}$ hosts. We assessed CD8 T cell expansion and differentiation 7 days after transfer (Figure 6.1). Interestingly, we found that T cells from F5 TetZap70$^{ON}$ chimeras were able to proliferate when challenged with influenza in dox-fed B6 $Rag^{1/-}$ mice (Figure 6.1A and B). The number of expanded F5 TetZap70$^{ON}$ T cells in dox-fed hosts was 10-fold lower than the
number of expanded F5 $Rag1^{−/−}$ T cells 7 days after challenge (Figure 6.1B). We observed no significant expansion of F5 TetZap70$^{ON}$ T cells in B6 $Rag1^{−/−}$ hosts that were not fed dox (Figure 6.1A and B). Both F5 $Rag1^{−/−}$ and F5 TetZap70 T cells showed induction of the T cell activation marker CD44 (Budd et al., 1987) 7 days after challenge (Figure 6.1C). Interestingly, activated F5 TetZap70 and F5 $Rag1^{−/−}$ T cells had also upregulated surface expression of CD5 (Figure 6.1D). In fact, F5 TetZap70 CD5 expression 7 days after challenge exceeded CD5 expression of naïve F5 $Rag1^{−/−}$ T cells. Taken together these data show that CD8 T cells from F5 TetZap70$^{ON}$ chimeras can proliferate and become activated in vivo despite evidence for low homeostatic TCR signalling in naïve CD8 T cells from F5 TetZap70$^{ON}$ chimeras.

6.2.2 F5 TetZap70$^{ON}$ T cells expand in response to cognate antigen in C57Bl/6 mice in vivo

We next wanted investigate whether F5 TetZap70 would be able to respond to cognate antigen in a more physiological context. To do this we transferred F5 $Rag1^{−/−}$ and F5 TetZap70 T cells into lymphoreplete Ly5.1 C57Bl/6 hosts, challenged these mice with influenza and then analysed the expansion of Ly5.2$^+$ F5, F5 TetZap70$^{ON}$ and Zap70 deficient F5 T cells after 7 days of transfer (Figure 6.2). As we observed in B6 $Rag1^{−/−}$ hosts, CD8 T cells from F5 TetZap70$^{ON}$ chimeras proliferated in Ly5.1 C57Bl/6 hosts (Figure 6.2A and B). Again CD8 T cells from F5 TetZap70$^{ON}$ chimeras did not proliferate to the same extent as CD8 T cells from F5 $Rag1^{−/−}$ mice (Figure 6.2B and C). CD8 T cell proliferation required Zap70 expression, as CD8 T cells from of F5 TetZap70$^{OFF}$ chimeras did not expand to the same extent as CD8 T cells from F5
Tet Zap70ON chimeras (Figure 6.2B and C). T cell expansion was also influenza specific and F5 \textit{Rag1}⁻/⁻, F5 Tet Zap70ON and F5 Tet Zap70OFF T cells failed to proliferate in unchallenged Ly5.1 C57Bl/6 hosts (Figure 6.2B and C). Collectively, these data show that F5 Tet Zap70 T cell expansion is dependent on the presence of both antigen and Zap70.

We also characterised the expression of CD44 and IL-7Rα on challenged F5 \textit{Rag1}⁻/⁻, F5 Tet Zap70ON and F5 Tet Zap70OFF T cells. Activated T cells upregulate their expression of CD44 (Budd et al., 1987) while the re-expression of IL-7Rα during an immune response is thought to be important for the development of long-lived memory T cells (Buentke et al., 2006; Huster et al., 2004; Kaech et al., 2003). F5 \textit{Rag1}⁻/⁻, F5 Tet Zap70ON and F5 Tet Zap70OFF T cells had all upregulated CD44 7 days after being challenged (Figure 6.2D). This CD44 induction did not occur in animals that were not challenged (Figure 6.2D). IL-7Rα expression was also upregulated on influenza-challenged F5 \textit{Rag1}⁻/⁻, F5 Tet Zap70ON and F5 Tet Zap70OFF T cells as well as on endogenous Ly5.1⁺ CD8 T cells (Figure 6.2E). Taken together these data show that F5 \textit{Rag1}⁻/⁻ and F5 Tet Zap70ON but not F5 Tet Zap70OFF T cells become activated and expand if challenged with cognate antigen in lymphoreplete hosts in vivo.

6.2.3 Neither Zap70⁰⁰⁰Yyaa or Zap70⁰⁰⁰Skg can substitute for Zap70⁰⁰⁰Wt in F5 Tet Zap70 cognate response

We also wanted to investigate whether mutant Zap70⁰⁰⁰Yyaa or Zap70⁰⁰⁰Skg could substitute for Zap70⁰⁰⁰Wt in response to cognate antigen. Both Zap70 mutants have been shown to mediate weaker TCR signalling (Hsu et al., 2009; Sakaguchi et al., 2003) and neither is able to substitute for Zap70⁰⁰⁰Wt in the
transduction of the TCR survival signal in naïve T cells (Chapter 5). To study this, we challenged CD8 T cells from F5 TetZap70\textsuperscript{ON} \textit{Zap70}\textsuperscript{Yyaa/-}, F5 TetZap70\textsuperscript{ON} \textit{Zap70}\textsuperscript{Skg/-}, F5 TetZap70\textsuperscript{OFF} \textit{Zap70}\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{OFF} \textit{Zap70}\textsuperscript{Skg/-} chimeras with influenza in Ly5.1 C57Bl/6 hosts. We used F5 \textit{Rag1}\textsuperscript{-/-} and F5 TetZap70\textsuperscript{ON} T cells as controls and analysed hosts 7 days after challenge (Figure 6.3). Influenza-challenged F5 \textit{Rag1}\textsuperscript{-/-} and F5 TetZap70\textsuperscript{ON} T cells proliferated when in Ly5.1 C57Bl/6 (Figure 6.3B). CD8 T cells from F5 TetZap70\textsuperscript{ON} \textit{Zap70}\textsuperscript{Yyaa/-}, F5 TetZap70\textsuperscript{ON} \textit{Zap70}\textsuperscript{Skg/-}, F5 TetZap70\textsuperscript{OFF} \textit{Zap70}\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{OFF} \textit{Zap70}\textsuperscript{Skg/-} chimeras however, did not expand to the same extent as F5 TetZap70\textsuperscript{ON} or F5 \textit{Rag1}\textsuperscript{-/-} T cells (Figure 6.3C and D). These data show that neither of the two \textit{Zap70} mutants can substitute for \textit{Zap70}\textsuperscript{WT} signalling during T cell expansion. It also suggests that heterozygous expression of transgenic \textit{Zap70}\textsuperscript{WT} in F5 TetZap70\textsuperscript{ON} \textit{Zap70}\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{ON} \textit{Zap70}\textsuperscript{Skg/-} chimeras is insufficient to mediate antigen-induced proliferation.

To investigate whether CD8 T cells from F5 TetZap70\textsuperscript{OFF} \textit{Zap70}\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{OFF} \textit{Zap70}\textsuperscript{Skg/-} chimeras underwent any proliferation following an influenza challenge we labelled CD8 T cells with CTV prior to transfer (Figure 6.3E). Interestingly, F5 \textit{Rag1}\textsuperscript{-/-}, F5 TetZap70\textsuperscript{ON}, F5 TetZap70\textsuperscript{OFF} \textit{Zap70}\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{OFF} \textit{Zap70}\textsuperscript{Skg/-} T cells had all diluted out their CTV by day 7 despite differences in expansions (Figure 6.3E and G). CD8 T cells from F5 TetZap70\textsuperscript{OFF} \textit{Zap70}\textsuperscript{Yyaa/-} and F5 TetZap70\textsuperscript{OFF} \textit{Zap70}\textsuperscript{Skg/-} chimeras also showed evidence of activation and had upregulated CD44 (Figure 3.6F). Collectively, these data show that, CD8 T cells from F5 TetZap70\textsuperscript{OFF} \textit{Zap70}\textsuperscript{Yyaa/-} and F5
TetZap7\textsuperscript{OFF} Zap70\textsuperscript{Ski-} chimeras do not expand as much as F5 TetZap70\textsuperscript{ON} T cells but do show evidence of activation and significant cell division.

6.2.4 TCR signalling is required until 96-144 hours after influenza challenge for optimal CD8 T cell expansion

Finally, we also wanted to investigate the temporal requirements for TCR signalling during the activation and expansion of CD8 T cells challenged with influenza in vivo. It has been proposed that commitment of CD8 T cells to proliferate and differentiate into effector cells might require only a brief encounter with antigen and that expansion after this initial contact can occur without further antigenic stimulation (Kaech and Ahmed, 2001; van Stipdonk et al., 2001). To investigate the temporal requirements of TCR signalling throughout this response, we used F5 TetZap70 mice where Zap70 expression and thus TCR signalling could be controlled by dox-feeding. As before, we transferred F5 TetZap70 T cells into Ly5.1 C57Bl/6 hosts and challenged these hosts with influenza. We then took cohorts of C57Bl/6 mice off dox-supplemented food throughout the 7-day time-course to characterise how long F5 TetZap70\textsuperscript{ON} T cells required Zap70 expression and intact TCR signalling in order to expand maximally (Figure 6.4). It takes approximately 48-72 hours for Zap70 abundance to drop in F5 TetZap70 T cells following dox-withdrawal, as determined by intracellular flow cytometry staining (Figure 3.3). F5 TetZap70\textsuperscript{ON} T cells transferred into hosts not fed dox, failed to expand to the same degree as F5 Rag1\textsuperscript{+/} and F5 TetZap70\textsuperscript{ON} T cells in dox-fed hosts (Figure 6.4A and B). Interestingly, F5 TetZap70\textsuperscript{ON} T cells transferred into Ly5.1 C57Bl/6 hosts that were fed dox for 1 day did not expand substantially either (Figure 6.4A, B and
E). In hosts taken off dox 2 days after cell transfer and challenge we observed a range of responses. In some hosts, F5 TetZap70 T cells expanded, while we observed little expansion in other hosts (Figure 6.4A, B and E). Overall, the influenza-induced F5 T cell expansion in hosts that were fed dox for less than 2 days never matched the expansion of F5 TetZap70\textsuperscript{ON} T cells in hosts fed dox for 3 or more days (Figure 6.3E). Collectively, these data show that Zap70 expression was required until 96-144 hours after the initial IV administration of influenza, for maximal expansion of F5 TetZap70 T cells.

### 6.3 Discussion

In this chapter we investigated the temporal requirements of TCR signalling during a cognate immune challenge using F5 T cells from mice in which conditional Zap70 expression could be controlled by dox-feeding. We show that, despite much lower Zap70 abundance in F5 TetZap70\textsuperscript{ON} T cells compared to F5 \textit{Rag1}\textsuperscript{\textminus\textminus} T cells, F5 TetZap70\textsuperscript{ON} T cells could become activated and expand when challenged with live influenza, both in lymphopaenic B6 \textit{Rag1}\textsuperscript{\textminus\textminus} and in lymphoreplete Ly5.1 C57Bl/6 hosts. This expansion was dependent on continued Zap70 expression since F5 TetZap70\textsuperscript{ON} T cells transferred into dox-free B6 \textit{Rag1}\textsuperscript{\textminus\textminus} and Ly5.1 C57Bl/6 hosts did not expand. We confirmed that expansion of F5 \textit{Rag1}\textsuperscript{\textminus\textminus} and F5 TetZap70\textsuperscript{ON} T cells was antigen-dependent, as F5 T cells transferred into unchallenged hosts did not expand. Interestingly, the recovery of F5 TetZap70\textsuperscript{OFF} T cells from influenza-challenged Ly5.1 C57Bl/6 hosts was consistently greater than the recovery from unchallenged hosts. Furthermore, F5 TetZap70\textsuperscript{OFF} T cells also upregulated CD44, a sign of activation. We show that CD5 expression on influenza-challenged F5
TetZap70ON T cells is upregulated so that the CD5 on activated and expanded F5 TetZap70ON T cells was as high as on activated F5 Rag1\(^{−/−}\) T cells. Influenza-challenged F5 TetZap70ON T cells also upregulated surface IL-7R\(\alpha\) expression. Neither heterozygous expression of Zap70\(^{Yya}\) or Zap70\(^{Skg}\) was able to mediate F5 T cell expansion in response to a challenge with influenza. Finally, we transferred F5 TetZap70ON T cells into Ly5.1 C57Bl/6 hosts that we challenged with influenza and then took off dox to test the temporal requirements for TCR signalling during T cell activation and expansion. Interestingly, unless animals remained on dox for 2 or more days after the challenge with influenza, F5 T cell expansion was submaximal. In other words, F5 T cells appeared to require intact Zap70 signalling until 96-144 hours after the challenge with influenza in order to respond and expand maximally.

In view of the low Zap70 abundance in naïve F5 TetZap70ON T cells (Figure 3.2C), as well as their low surface CD5 expression (Figure 3.2D) and failure to proliferate in lymphopaenic B6 Rag1\(^{−/−}\) hosts placed on dox (data not shown) we were surprised to find that F5 TetZap70ON T cells were activated, expanded and adopted an activated phenotype following challenge with live influenza both in lymphopaenic and lymphoreplete hosts in vivo (Figure 6.1 and 6.2). The role of TCR signalling strength in promoting T cell activation and proliferation has been studied extensively using H2-K\(^{b}\)-restricted OT-I TCR transgenic CD8 T cells (Clarke et al., 2000), since so many ligands with varying binding affinity for the OT-I TCR exist (reviewed in Zehn et al., 2012). By generating recombinant L. monocytogenes strains that express Ova containing various altered peptide ligands, the role of TCR-ligand avidity in T cell activation and expansion has been studied in vivo. In this study revealed that even L. monocytogenes
expressing Ova containing altered peptide ligands that mediated very weak TCR-peptide interactions were able to promote T cell activation and clonal expansion in vivo (Zehn et al., 2009). While the initial T cell responses were very similar regardless of whether OT-I T cells had been strongly or weakly stimulated, strongly stimulated OT-I T cells proliferated longer and contracted later than more weakly stimulated OT-I T cells ultimately leading to greater OT-I T cell numbers accumulating in response to higher-affinity antigen (Zehn et al., 2009). Although we did not characterise the kinetics of F5 TetZap70ON expansion, we consistently detected fewer F5 TetZap70ON T cells than F5 Rag1+/− T cells 7 days after challenge (Figure 6.1B and 6.2C and D), which could fit with F5 TetZap70ON T cells receiving a weaker TCR signal than F5 Rag1+/− T cells as a result of reduced Zap70 abundance in F5 TetZap70ON compared to F5 Rag1+/− T cells. Indeed, both Lck and Zap70 are indispensable for T cell activation and proliferation (Au-Yeung et al., 2010; Tewari et al., 2006), although the importance of Zap70 abundance has not been specifically assessed. Interestingly, T cell development and TCR signalling are largely normal in polyclonal Zap70+/− (Hsu et al., 2009) and F5 Rag1+/− Zap70+/− (Figure 5.1, 2 and 3). Furthermore, Erk2 activation downstream of TCR signalling has also been implicated in regulating the survival of differentiated CD8 T cells after viral infection (D’Souza et al., 2008) and we can not exclude that impaired survival after T cell activation affected F5 TetZap70ON expansion and accumulation.

Data in this chapter also show that CD5 expression was upregulated on both F5 Rag1+/− and F5 TetZap70ON T cells such that 7 days after challenged F5 Rag1+/− and F5 TetZap70ON T cells expressed similar CD5. So far, the regulation of CD5 expression by spMHC TCR has largely been studied in developing thymocytes
and naïve T cells (Azzam et al., 1998; 2001; Smith et al., 2013; Wong et al., 2001). This has lead to a model where productive spMHC TCR interactions in the thymus and periphery modulate CD5 expression (Azzam et al., 1998; Smith et al., 2001). In turn, CD5 modulates TCR signalling and T cell responsiveness (Azzam et al., 2001; Tarakhovsky et al., 1995; Wong et al., 2001). Here we show that activated F5 Rag1−/− and F5 TetZap70ON T cell CD5 expression is increased by challenge with live influenza in vivo.

Interestingly, the effector phenotype of OT-I T cells stimulated by low- as well as high-affinity ligands was similar (Zehn et al., 2009). Consistent with this, both F5 TetZap70ON and F5 Rag1−/− had upregulated the activation marker CD44 7 days after transfer (Figure 6.1C and 6.2E). Although, we did not specifically assess F5 TetZap70ON memory or effector function in our model, activated and expanded F5 TetZap70ON T cells all expressed high levels of IL-7Rα 7 days after challenge (Figure 6.2F) which is known to be important for the development of long-lived memory T cells in lymphoreplete hosts (Buentke et al., 2006; Huster et al., 2004; Kaech et al., 2003). Interestingly, the frequency of endogenous CD44+ Ly5.1+ CD8 T cells was smaller in hosts that received F5 Rag1−/− T cells and largest in hosts that received F5 TetZap70OFF T cells and thus correlated with the expansion of F5 T cells (Figure 6.2E and 6.4D). These data suggest that the endogenous CD8 T cell response might be greater if the F5 T cell expansion is suboptimal or absent. Indeed, some endogenous C57Bl/6 CD8 T cells responded better than OT-I T cells in response to Ova containing weak affinity ligands (Zehn et al., 2009). The endogenous C57Bl/6 T cell response to influenza remains to be examined in more detail by tetramer staining in the influenza challenge model described in this chapter.
We also assessed the ability of two hypomorphic Zap70 mutants to mediate T cell activation and expansion signals in response to an influenza challenge. Interestingly, while CD8 T cells from F5 TetZap70\(^{OFF}\) Zap70\(^{Yyaa/-}\) and F5 TetZap70\(^{OFF}\) Zap70\(^{SKG/-}\) chimeras divided and became activated following influenza challenge (Figure 6.3B, C and D) their accumulation was reduced compared to the accumulation of influenza-challenged F5 TetZap70\(^{ON}\) T cells (Figure 6.3G). Furthermore, even the addition of heterozygous dox-inducible Zap70 expression to mutant Zap70\(^{YYAA}\) or Zap70\(^{SKG}\) did not lead to a greater influenza-induced CD8 T cell expansion (Figure 6.3C, D and G). Interestingly, influenza-challenged CD8 T cells from F5 TetZap70\(^{ON}\) Zap70\(^{SKG/-}\) and F5 TetZap70\(^{OFF}\) Zap70\(^{SKG/-}\) chimeras were consistently more readily detectable than CD8 T cells from F5 TetZap70\(^{ON}\) Zap70\(^{Yyaa/-}\) and F5 TetZap70\(^{OFF}\) Zap70\(^{Yyaa/-}\) chimeras (Figure 6.3G) despite Zap70 abundance being lower in F5 TetZap70\(^{ON}\) Zap70\(^{SKG/-}\) and F5 TetZap70\(^{OFF}\) Zap70\(^{SKG/-}\) T cells than in F5 TetZap70\(^{ON}\) Zap70\(^{Yyaa/-}\) and F5 TetZap70\(^{OFF}\) Zap70\(^{Yyaa/-}\) T cells (Figure 5.3). Furthermore, polyclonal SKG T cells both have a greater defect in TCR signalling and T cell proliferation than polyclonal YYAA T cells (Hsu et al., 2009). Although the altered TCR signalling sensitivity in F5 TetZap70 Zap70\(^{SKG/-}\) and F5 TetZap70 Zap70\(^{Yyaa/-}\) T cells remains to be examined in more detail in vitro, our data suggest the scaffolding function of Zap70, impaired in mutant Zap70\(^{YYAA}\), is crucial for CD8 T cell activation and expansion.

Intriguingly, despite expanding less than F5 \(Rag1^{-/-}\) T cells, influenza-challenged F5 TetZap70\(^{OFF}\), F5 TetZap70\(^{OFF}\) Zap70\(^{Yyaa/-}\) and F5 TetZap70\(^{OFF}\) Zap70\(^{SKG/-}\) T cells had all upregulated CD44 suggesting they had become activated (Figure 6.2D and 6.3F). In fact CTV-labelled F5 TetZap70\(^{OFF}\) Zap70\(^{Yyaa/-}\) and F5
TetZap70^OFF \text{Zap70}^{Skg/-} T cells had completely diluted out CTV 7 days following challenge with influenza (Figure 6.3E). Furthermore, influenza-challenged F5 TetZap70^OFF T cells were consistently more readily detectable than unchallenged F5 TetZap70^OFF T cells (Figure 6.2D). A small subset of F5 TetZap70^OFF T cells expressing sufficient Zap70 to become activated and divide could explain the apparent activation and improved recovery of influenza-challenged F5 TetZap70^OFF T cells. Alternatively, all F5 TetZap70^OFF T cells underwent some minimal influenza-induced proliferation independent of Zap70 signalling. One possibility is that influenza-challenged F5 TetZap70^OFF, F5 TetZap70^OFF \text{Zap70}^{Yyaa/-} and F5 TetZap70^OFF \text{Zap70}^{Skg/-} T cells became activated and proliferated as bystanders in response to cytokines and non-antigen-specific signals (Tough et al., 1996; Tripp et al., 1995; Yang et al., 1989) although this remains to be formally assessed in our model.

Finally, we used the ability to ablate Zap70 expression by removing hosts from dox to assess the temporal requirements of TCR signalling during an immune response in vivo. We show that F5 TetZap70^ON T cells in hosts fed dox for less than 2 days did not expand to the same degree as F5 TetZap70^ON T cells that were transferred into hosts fed dox for more than 2 days (Figure 6.4E). These data suggest that F5 TetZap70^ON T cells need intact Zap70 signalling until 96-144 days after the influenza challenge for maximal influenza-induced CD8 T cell expansion in vivo. One interpretation of our data is that CD8 T cells require \textit{continuous} Zap70 signalling for at least 96-144 hours following challenge with influenza to expand maximally in vivo. This contrasts with data from in vitro studies where activated T cells were shown to require only \textasciitilde2h of antigen exposure to divide and become effector CD8 T cells (Kaech and Ahmed, 2001;
van Stipdonk et al., 2001 and reviewed in Masopust et al., 2004). Longer in vitro antigen exposure was required for sustained CD8 T cell differentiation and expansion in vivo (van Stipdonk et al., 2003). Continuous Zap70 signalling might not only be required for optimal CD8 T cell activation and expansion. T cell sensitivity to foreign antigen is also thought to depend on spMHC contact and intact TCR signalling (reviewed in Stefanova et al., 2003) and so intact Zap70 signalling might be required to maintain F5 TetZap70 CD8 T cell sensitivity prior activation and expansion following challenge with influenza. Alternatively, intact Zap70 signalling might not be required continuously throughout the expansion phase of F5 TetZap70ON CD8 T cells n C57Bl/6 hosts but only for a discrete period during the 96-144 hours after challenge with influenza. In fact, intact Zap70 and TCR signalling might only be required for briefly in vivo as has been shown in vitro (Kaech and Ahmed, 2001; van Stipdonk et al., 2001 and reviewed in Masopust et al., 2004) and the 96-144 hours required for optimal T cell expansion might be the result of CD8 T cells migration and T cell APC interactions before Zap70 expression is required. We hope to investigate whether Zap70 signalling is required continuously throughout the expansion phase or for a brief and discrete period during the first 96-144 hours after challenge using F5 TetZap70 CD8 T cells exposed to doxycyline for different periods following their initial expansion.

Previous work has investigated the signalling requirements for T cell activation and expansion by studying ligand affinity and ligand exposure (reviewed Zehn et al., 2012). In this chapter we have used the F5 TetZap70 mice to directly study the duration and strength of signalling required for T cell activation and maximal F5 T cell expansion in response to cognate antigen. It is conceivable
that the temporal requirements for pMHC TCR engagement differ from the duration of TCR signalling necessary for maximal T cell expansion. Persistence of signalling downstream of the TCR beyond the exposure to antigen might explain why only brief contact with pMHC but much longer Zap70 signalling is required for T cell expansion. We propose that, as such, the expansion of F5 TetZap70ON T cells in C57Bl/6 mice in response to flu is a good model to study the TCR signalling requirements during primary and memory CD8 T cell responses directly.
Figure 6.1 F5 TetZap70ON T cells expand in response to cognate antigen B6 Rag1−/− mice in vivo

F5 Rag1−/− (grey fill/black) and F5 TetZap70ON lymphocytes (LN) were transferred intravenously into B6 Rag1−/− hosts placed on (blue) or off (red) dox and challenged with live influenza A/NT/60/68. Host and donor splenocytes were counted and analysed 7 days (d7) after challenge by flow cytometry. C (A) Density plots show CD8 against CD5 expression on live splenocytes 7 days after challenge. (B) Graph shows the number of CD8+ CD5+ splenocytes 7 days after challenge; *** p≤0.001 (one-way analysis of variance followed by Bonferroni correction for multiple comparisons). Histograms show CD44 (C) and CD5 (D) expression on donor CD8+ CD5+ splenocytes pre-challenge and 7 days after challenge. Data are representative of ≥two independent experiments.
A d7: F5 *Rag1*<sup>-/-</sup> in *Rag1*<sup>-/-</sup> OFF

F5 TetZap70<sup>ON</sup> in *Rag1*<sup>-/-</sup> ON

F5 TetZap70<sup>ON</sup> in *Rag1*<sup>-/-</sup> OFF

CD8

CD5

B

C

D

CD8 T cells spleen

***

10<sup>7</sup>

10<sup>6</sup>

10<sup>5</sup>

10<sup>4</sup>

10<sup>3</sup>

F5

ON

OFF

d0 F5 *Rag1*<sup>-/-</sup>

d0 F5 TetZap70<sup>ON</sup>

d7 F5 *Rag1*<sup>-/-</sup>

d7 F5 TetZap70<sup>ON</sup>
Figure 6.2 F5 TetZap70ON T cells expand in response to cognate antigen in C57Bl/6 mice in vivo

Ly5.2 F5 Rag1−/− (grey), F5 TetZap70ON (Ly5.2) (blue) and F5 TetZap70OFF (Ly5.2) (red) lymphocytes (LN) were transferred intravenously into Ly5.1 C57Bl/B6 hosts on or off dox and challenged with live influenza A/NT/60/68 (+flu, filled circles) or with medium (-flu, open circles). Host and donor splenocytes were counted and analysed 7 days (d7) after challenge by flow cytometry. F5 TetZap70OFF lymphocytes were isolated from the LNs of F5 TetZap70 chimeras taken off dox ≥5 days previously. Bone marrow chimeras were made by reconstituting sub-lethally irradiated B6 Rag1−/− mice with F5 TetZap70 bone marrow. (A) Density plots show Ly5.2 and CD5 expression on CD8+ TCR+ splenocytes 7 days after challenge. (B) Density plot shows CD8 against CD5 expression on Ly6.1 C57Bl/6 live splenocytes 7 days after challenge with live influenza A/NT/60/68. (C) Graphs show frequency and number of donor Ly5.2+ CD8+ CD5+ splenocytes 7 days after challenge. (D) Histograms show CD44 expression on host Ly5.1+ (grey fill) and donor Ly5.2+ CD8+ CD5+ splenocytes challenged with live influenza A/NT/60/68 (solid) or with medium (dotted); gate shows frequency (grey) of host Ly5.1+ CD44+ splenocytes. (E) Histograms show IL-7Rα expression on host Ly5.1+ (grey fill) and donor Ly5.2+ CD8+ CD5+ splenocytes pre-challenge (thin solid line) and 7 days after transfer (solid line). Data are representative of ≥two independent experiments.
A d7: F5 Rag1\(^{+/−}\) in C57Bl/6\(^{OFF}\)  
+flu 
22.9

-flu 
0.327

F5 TetZap70\(^{ON}\) in C57Bl/6\(^{ON}\)  
6.45

F5 TetZap70\(^{OFF}\) in C57Bl/6\(^{OFF}\)  
0.444

B d7: Ly5.1 B6+ 
CD8 
12.4

C 
Ly5.2 CD8 spn (% F5) C 

D d7: F5 Rag1\(^{+/−}\) in C57Bl/6\(^{OFF}\)  
F5 TetZap70\(^{ON}\) in C57Bl/6\(^{ON}\)  
F5 TetZap70\(^{OFF}\) in C57Bl/6\(^{OFF}\)  

E 
CD44 
IL-7Ra

d7 Ly5.1 B6 +flu 
solid d7 Ly5.2 +flu 
dotted d7 Ly5.2 -flu 
thin d0 Ly5.2
Figure 6.3 Neither Zap70\textsuperscript{Yya\textscript{a}} or Zap70\textsuperscript{Skg} can substitute for Zap70\textsuperscript{WT} in F5 TetZap70 cognate response

CTV-labelled Ly5.2 F5 \textit{Rag1}\textsuperscript{--} (grey), F5 TetZap70\textsuperscript{ON} (Ly5.2) (blue), F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Yya\textscript{a}/} (blue), F5 TetZap70\textsuperscript{ON} Zap70\textsuperscript{Skg/} (blue), F5 TetZap70\textsuperscript{OFF} (red), F5 TetZap70\textsuperscript{OFF} Zap70\textsuperscript{Yya\textscript{a}/} (red) and F5 TetZap70\textsuperscript{OFF} Zap70\textsuperscript{Skg/} (red) lymphocytes (LN) were transferred intravenously into Ly5.1 C57Bl/B6 hosts on or off dox and challenged with 100 HAU live influenza A/NT/60/68. Host and donor splenocytes were counted and analysed 7 days (d7) after challenge by flow cytometry. F5 TetZap70\textsuperscript{OFF}, F5 TetZap70\textsuperscript{OFF} Zap70\textsuperscript{Yya\textscript{a}/} and F5 TetZap70\textsuperscript{OFF} Zap70\textsuperscript{Skg/} lymphocytes were isolated from the LNs of chimeras taken off dox ≥5 days previously. Bone marrow chimeras were made by reconstituting sub-lethally irradiated B6 \textit{Rag1}\textsuperscript{--} mice with F5 TetZap70, F5 TetZap70 Zap70\textsuperscript{Yya\textscript{a}/} or F5 TetZap70 Zap70\textsuperscript{Skg/} bone marrow. (A) Density plot shows CD8 against CD5 expression on Ly6.1 C57Bl/6 live splenocytes 7 days after challenge. (B)-(C) Density plots show Ly5.2 and CD5 expression on CD8\textsuperscript{+} TCR\textsuperscript{+} splenocytes 7 days after challenge. Histograms show CTV-dilution (E) and CD44 expression (F) on host Ly5.1\textsuperscript{+} (grey fill) and donor Ly5.2\textsuperscript{+} CD8\textsuperscript{+} CD5\textsuperscript{+} splenocytes pre-challenge (thin solid line) and 7 days after transfer (solid line); gate shows frequency (grey) of host Ly5.1\textsuperscript{+} CD44\textsuperscript{+} splenocytes. (G) Graph shows number of donor Ly5.2\textsuperscript{+} CD8\textsuperscript{+} CD5\textsuperscript{+} splenocytes as a percentage of the number of donor Ly5.2\textsuperscript{+} CD8\textsuperscript{+} CD5\textsuperscript{+} F5 \textit{Rag1}\textsuperscript{--} splenocytes 7 days after challenge; **** p≤0.0001 (one-way analysis of variance followed by Bonferroni correction for multiple comparisons). Data are representative of ≥two independent experiments.
Figure 6.4 TCR signalling is required until 96-144 hours after influenza challenge for optimal CD8 T cell expansion

Ly5.2 F5 Rag1\(^{-}\) (grey) and F5 TetZap70\(^{ON}\) (Ly5.2) (blue) lymphocytes (LN) were transferred intravenously into Ly5.1 C57Bl/B6 hosts and challenged with live influenza A/NT/60/68. Ly5.1 C57Bl/B6 hosts were taken off dox 0-4 days after transfer as indicated (red and pink). (A) Density plot shows CD8 against CD5 expression on Ly6.1 C57Bl/6 live splenocytes 7 days after challenge. (B) and (C) Density plots show Ly5.2 and CD5 expression on CD8\(^{+}\) TCR\(^{+}\) splenocytes 7 days after challenge. (D) Histograms show CD44 expression on host Ly5.1\(^{+}\) (grey fill) and donor Ly5.2\(^{+}\) (solid) CD8\(^{+}\) CD5\(^{+}\) splenocytes 7 days after challenge; gate shows frequency (grey) of host Ly5.1\(^{+}\) CD44\(^{+}\) splenocytes. (E) Graph shows the number of donor Ly5.2\(^{+}\) CD8\(^{+}\) CD5\(^{+}\) splenocytes as a percentage of the number of donor Ly5.2\(^{+}\) CD8\(^{+}\) CD5\(^{+}\) F5 Rag1\(^{-}\) splenocytes 7 days after challenge; **** p≤0.0001, *** p≤0.001 and ns p>0.05 (one-way analysis of variance followed by Bonferroni correction for multiple comparisons). Data are representative of ≥two independent experiments.
Chapter 7  Final discussion

The aim of this thesis was to study the role of Zap70 in naïve T cell survival. The role of TCR signalling has been extensively studied but the source and downstream target of this TCR-mediated survival signal remain elusive. We studied naïve T cell survival in F5 TCR transgenic mice that conditionally express Zap70\textsuperscript{WT}, which allowed naïve T cells to develop normally. We were then able to ablate Zap70 expression. We characterised the survival of naïve CD8 T cells that lacked Zap70 both \textit{in vivo} and \textit{in vitro} in an attempt to identify the source and targets of TCR-mediated survival signals. We also studied the ability of two Zap70 mutants to substitute for Zap70\textsuperscript{WT} during the development and survival of F5 TCR transgenic T cells. In the final chapter we investigated the temporal requirements for TCR signalling during T cell activation and proliferation in response to cognate antigen. We show that Zap70 expression is indeed required for the maintenance of CD8 T cells \textit{in vivo} and that two previously described Zap70 mutants, Zap70\textsuperscript{YYAA} and Zap70\textsuperscript{SKG} were unable to substitute for Zap70\textsuperscript{WT} in the transduction of this survival signal. In absence of Zap70, the pro-apoptotic Bcl-2 family member Bmf mRNA is upregulated in naïve CD8 T cells raising the possibility that Bmf might be a target of a Zap70-dependent TCR survival signal. We also show that, CD8 T cells with low or no Zap70 fail to integrate properly into the mature naïve T cell pool, probably as a result of a survival defect. We found that CD8 T cells that no longer express Zap70 are less sensitive to IL-7 although the survival defect of CD8 T cells in absence of Zap70 is IL-7-independent. Unexpectedly, cDCs were unable to promote naïve T cell survival although CD11c\textsuperscript{+} enriched splenocytes improved
 naïve T cell survival in a MEK-dependent manner. Since MEK is an important regulator of Bmf transcription and activity we propose that a TCR-mediated survival signal transduced by Zap70 regulates Bmf via MEK.

7.1 The role Zap70 signalling in T cell homeostasis

In order to study the survival of naïve CD8 T cells that lacked Zap70 we used mice that conditionally express Zap70. This allowed the development of T cells in which could later be made Zap70-deficient. T cell development in polyclonal TetZap70 mice was previously studied and revealed that the TCR-mediated developmental upregulation of Zap70 during positive selection was crucial for CD8 T cell development in particular (Saini et al., 2010). Zap70 expression is not upregulated in TetZap70 or F5 TetZap70 mice. In fact, Zap70 abundance drops as T cells mature beyond the CD8 SP stage in F5 TetZap70 mice and chimeras. CD8 T cell development was also impaired in F5 TetZap70ON chimeras, which had fewer CD8 SP thymocytes and naïve CD8 T cells than control F5 Rag1−/− chimeras. F5 TetZap70 thymocytes were also outcompeted by F5 Rag1−/− thymocytes in mixed BM chimeras at the DP3 CD5int TCRhi and CD8 SP HSAlo, coinciding with the impaired upregulation of Zap70 and low abundance of transgenic Zap70. Furthermore, peripheral F5 TetZap70 T cells in these mixed BM chimeras were predominantly RTEs suggesting that naïve T cells require WT abundance of Zap70 for their integration into the mature naïve T cell pool. Interestingly, we found that the peripheral CD8 T cell compartment in F5 TetZap70 chimeras was also enriched for RTEs consistent with the idea that the survival of RTE is not TCR-dependent but that integration into the naïve T cell pool does require intact TCR signalling.
In absence of continued Zap70 expression CD8 T cells failed to be maintained in the SLOs of F5 TetZap70 chimeras. The half-life of CD8 T cells in absence of Zap70 was 9.4 days. The estimated half-life of WT naïve T cells is in excess of 90 days (Seddon and Zamoyska, 2002a) and so a half-life of 9.4 days is consistent with impaired survival and not just a block in T cell development in F5 TetZap70^{OFF} chimeras. Previous estimates of the half-life TCR-deficient naïve CD8 T cells and cells lacking Lck and Fyn ranged between 16 and 19 days (Labrecque et al., 2001; Polic et al., 2001; Seddon and Zamoyska, 2002a) and the shorter half-life of CD8 T cells in F5 TetZap70^{OFF} might reflect differences in the survival of polyclonal and F5 TCR transgenic naïve T cells, which express low IL-7Rα (Sinclair et al., 2011). The half-life of HY TCR transgenic T cells in MHCI-deficient hosts, is even lower than the half-life of CD8 T cells in F5 TetZap70^{OFF} chimeras and this variability is probably the result of the different experimental systems used.

TCR-independent Zap70 signals might also contribute to naïve T cell survival. Zap70 activation can occur downstream of LFA1 (Evans et al., 2011) and so T cell migration might be impaired in absence of Zap70. CD8 T cells from F5 TetZap70^{OFF} chimeras, however did not show any gross defects in homing to LN and spleen, which is where survival signals such as IL-7 and spMHC are proposed to be concentrated (Surh and Sprent, 2008). In fact, the impaired survival of CD8 T cells from F5 TetZap70^{OFF} chimeras correlated with reduced CD5 expression. CD5 expression is modulated by homeostatic MHC-dependent TCR signals on naïve T cells (Smith et al., 2001) and so it seems likely that the impaired survival of CD8 T cells in absence of Zap70 is largely the result of impaired TCR signalling.
Consistent with previous data (Seddon and Zamoyska, 2002a) CD8 T cells from F5 TetZap70\textsuperscript{OFF} chimeras failed to proliferate in lymphopaenic B6 \textit{Rag1}\textsuperscript{−/−} hosts, reflecting the importance of TCR signalling for homeostatic proliferation. Interestingly, CD8 T cells from F5 TetZap70\textsuperscript{OFF} chimeras survived as well as CD8 T cells from F5 \textit{Rag1}\textsuperscript{−/−} mice after transfer into lymphopaenic hosts. Our data fit with other studies, which show that survival in lymphopaenic conditions is MHC- and TCR-independent (Martin, 2006; Martin et al., 2003) and reduced competition for other survival factors, including IL-7, might keep naïve T cells alive in T-cell-deficient hosts independently of TCR.

Clearly, IL-7 signals are non-redundant for T cell survival and naïve CD8 T cells disappear rapidly after transfer into IL-7-deficient hosts. CD8 T cells from F5 TetZap70\textsuperscript{OFF} chimeras still disappeared more rapidly than CD8 T cells from F5 \textit{Rag1}\textsuperscript{−/−} mice and so the survival defect of Zap70-deficient naïve CD8 T cells is largely IL-7-independent. It has recently been shown that TCR signals regulate IL-7\textalpha expression in the thymus (Sinclair et al., 2011) but whether IL-7\textalpha and TCR signalling are crossregulated in naïve T cells remains controversial. There is evidence that IL-7\textalpha signalling downregulates its own signalling (Park et al., 2004a) while increasing the T cell sensitivity to spMHC by increasing \textit{Cd8a} transcription (Park et al., 2007). Other studies have failed to show that TCR signalling regulates IL-7\textalpha signalling in naïve T cells (Seddon et al., 2003; Sinclair et al., 2011). Interestingly, \textit{ex vivo} IL-7\textalpha expression on CD8 T cells from F5 TetZap70 chimeras was reduced compared to CD8 T cells from F5 \textit{Rag1}\textsuperscript{−/−} mice but after overnight culture without IL-7, IL-7\textalpha expression was higher on CD8 T cells from F5 TetZap70 chimeras than on control F5 \textit{Rag1}\textsuperscript{−/−} T cells. The higher IL-7\textalpha expression on CD8 T cells from F5 TetZap70 chimeras
could reflect impaired IL-7 signalling and in fact we do find that CD8 T cells from F5 TetZap70^{OFF} chimeras are less sensitive to low concentrations of IL-7 \textit{in vitro}. This is inconsistent with a ‘co-receptor tuning model’ of TCR and IL-7Rα crosstalk (Park et al., 2007) which would argue that reduced homeostatic TCR signals should instead enhance IL-7Rα signalling. Moreover, we observed no differences in CD8 expression or IL-7-induced Bcl-2 upregulation between naïve T cells from F5 TetZap70 and F5 \textit{Rag1^{-/}} chimeras and propose that the difference in IL-7Rα expression between CD8 T cells from F5 TetZap70 and F5 \textit{Rag1^{-/}} chimeras is largely the result of the relative lymphopaenia of F5 TetZap70 chimeras.

We also tested whether the Zap70 mutants, Zap70^{YYAA} and Zap70^{SKG}, were able to transduce this TCR-mediated survival signal. The TCR signalling and T cell development defects in polyclonal mice expressing these mutants were previously characterised (Hsu et al., 2009; Sakaguchi et al., 2003). Both Zap70^{YYAA} and Zap70^{SKG} are hypomorphic alleles of Zap70 and as a result the repertoire of T cells that are positively selected in polyclonal YYAA and SKG mice is shifted in favour of more auto-reactive T cells (Hsu et al., 2009; Sakaguchi et al., 2003). Neither Zap70^{YYAA} nor Zap70^{SKG} was able to support the development or the survival of F5 TCR transgenic T cells in F5 TetZap70^{OFF} Zap70^{Yyaa^{-/}} and F5 TetZap70^{OFF} Zap70^{Skg^{-/}} chimeras. The impaired survival of CD8 T cells in F5 TetZap70^{OFF} Zap70^{Skg^{-/}} chimeras could be attributed to a combined effect of reduced Zap70 abundance and attenuated TCR signalling mediated by Zap70^{SKG}. The abundance of Zap70^{YYAA} in CD8 T cells from F5 TetZap70^{OFF} Zap70^{Yyaa^{-/}} and F5^{+/} \textit{Rag1^{-/}} Zap70^{+/-} chimeras was equivalent and so the failure of CD8 T cells to survive in F5 TetZap70^{OFF} Zap70^{Yyaa^{-/}} chimeras
must reflect the importance of Zap70 adaptor function for the transduction of TCR survival signals. The tyrosine residues mutated in Zap70\(^{YYAA}\) have been implicated in the recruitment of Lck and PLC\(_\gamma\) and Ca\(^{2+}\) mobilisation, PLC\(_\gamma\) phosphorylation and Ras and Erk activation are impaired in mice expressing mutations of either of these residues (Magnan et al., 2001; Williams et al., 1999). The inability of Zap70\(^{YYAA}\) to transduce TCR-mediated survival signals F5 TetZap70\(^{OFF}\) Zap70\(^{Yyaa/-}\) chimeras probably reflects the importance of these pathways for naïve T cell survival.

**7.2 The source and downstream targets of a Zap70-dependent survival signal for naïve F5 T cells**

Despite extensive research characterising the requirement of interactions between spMHC and TCR for naïve T cell survival very little is known about the source or the downstream targets of TCR-mediated survival signals. In Chapter 4, we describe the Zap70-dependent survival of F5 TCR transgenic T cells cultured *in vitro* with CD11c\(^+\) enriched splenocytes. Despite evidence that cDCs are able to promote naïve T cell survival *in vitro* (Feuillet et al., 2005; Revy et al., 2001), we were unable to replicate the survival signal provided by CD11c\(^+\) enriched splenocytes using FACS-purified cDCs or pDCs. In the studies that support a role for DCs providing CD8 T cells with a survival signal *in vitro* DCs were not FACS-purified and contamination of other cell populations might explain the contrast with our results. In fact, a subsequent study also failed to show that FACS-purified cDCs were able to promote naïve T cell survival *in vitro* (Link et al., 2007) and so another CD11c\(^+\) cell population is probably the source of a Zap70-dependent survival signal. So far, only the FACS-purified
MHCI\(^{+}\) fraction of CD11c\(^{+}\) enriched splenocytes promoted the survival of F5 Rag1\(^{-/-}\) T cells \textit{in vitro} and we are currently investigating whether a specific cell type or combination of cell types is the source of this Zap70-dependent survival signal.

We were also able to show that the survival signal provided by CD11c\(^{+}\) enriched B6 Rag1\(^{-/-}\) splenocytes was completely dependent on MEK. This finding is particularly interesting since we also show that the pro-apoptotic Bcl-2 family member Bmf is upregulated in F5 TetZap70 T cells in absence of Zap70. Bmf transcription is upregulated in melanoma cells following MEK inhibition with UO126 (Shao and Aplin, 2010) and MEK signalling has also been shown to inhibit the pro-apoptotic activity of Bmf (Shao and Aplin, 2012). MAPK signalling downstream of the TCR is sensitive and enables thymocytes to distinguish between positive and negative selection despite very small differences in TCR affinity for the selecting ligand (Daniels et al., 2006). Bmf\(^{-/-}\) mice only have a small increase in the number of CD8 T cells (Labi et al., 2008) and Bmf is thought to be largely redundant during T cell development but the role of Bmf during T cell development and naïve T cell survival has not been studied in great detail. We propose that TCR signals via MEK regulate naïve T cell survival by inhibiting Bmf transcription and we plan to test whether Bmf-deficiency can rescue the survival defect of CD8 T cells in F5 TetZap70\(^{OFF}\) chimeras.
7.3 The TCR signalling requirements during antigen-induced T cell activation and proliferation

The temporal requirements for TCR signalling during CD8 T cell activation and expansion in response to cognate antigen have previously been studied in terms of the requirement for antigen exposure (Kaech and Ahmed, 2001; van Stipdonk et al., 2001; 2003). CD8 T cells from F5 TetZap70 allow us to study the signalling requirements for CD8 T cell activation and expansion. We show that despite very low Zap70 abundance, CD8 T cells from F5 TetZap70\textsuperscript{ON} chimeras become activated and expand substantially in response to cognate antigen. This is consistent with previous data showing that the strength of TCR stimulation has little impact on the early response to antigen and T cell activation but that it dictates the magnitude of the expansion (Zehn et al., 2009). Interestingly, CD8 T cells from F5 TetZap70 Zap70\textsuperscript{Yyaa/-} and F5 TetZap70 Zap70\textsuperscript{Skg/-} chimeras failed to expand to the same extent as F5 TetZap70\textsuperscript{ON} T cells in response to influenza regardless of whether they expressed transgenic Zap70\textsuperscript{WT}. Firstly, this suggests that heterozygous levels of Zap70\textsuperscript{WT} are insufficient to promote substantial CD8 T cell expansion in response to cognate antigen. Secondly, neither Zap70\textsuperscript{SKG} nor Zap70\textsuperscript{YYAA} are able to mediate CD8 T cell expansion. Interestingly, CD8 T cells from F5 TetZap70 Zap70\textsuperscript{Yyaa/-} and F5 TetZap70 Zap70\textsuperscript{Skg/-} chimeras did divide and showed signs of activation. Similarly, CD8 T cells from F5 TetZap70\textsuperscript{OFF} upregulated CD44 and were more readily detected in hosts challenged with influenza than in unchallenged hosts. It remains to be investigated whether this T cell activation is Zap70-independent.
or represents the activation and expansion of a small population of CD8 T cells that still express Zap70.

CD8 T cells have been shown to require a brief exposure to antigen in order initiate a program of CD8 T cell activation, expansion and differentiation (Kaech and Ahmed, 2001; van Stipdonk et al., 2001; 2003). We show that only if Zap70 expression was maintained for at least 96-144 hours, expansion of CD8 T cells in response to influenza was optima. This could reflect either a requirement for continuous Zap70 signalling throughout the expansion phase of F5 TetZap70 CD8 T cells, in contrast to data from in vitro studies where only exposure to MHC and antigen was sufficient for initiating CD8 T cell activation and differentiation (Kaech and Ahmed, 2001; van Stipdonk et al., 2001 and reviewed in Masopust et al., 2004). Alternatively, F5 TetZap70\textsuperscript{ON} CD8 T cells only required Zap70 expression briefly and the fact that maximal expansion only occurred in mice fed dox for more than 2 days reflects F5 TetZap70\textsuperscript{ON} CD8 T cells entering the peripheral lymphoid organs and interacting with APCs until they encounter their antigen. The expansion of CD8 T cells in which Zap70 expression was continued beyond 96-144 days broadly correlated with the duration of Zap70 induction. Similarly, antigen persistence has also been shown to affect the magnitude of T cell expansion rather than the functionality of the effector cells generated (Prlic et al., 2006). The response of CD8 T cells from F5 TetZap70 chimeras to influenza provides a good model to study the Zap70 and TCR signalling requirements of primary and secondary responses to antigen.
7.4 Clinical perspectives

Understanding the signalling pathways downstream of TCR that regulate the survival and proliferation of T cells in lymphoreplete and lymphopaenic conditions and in response to cognate antigen is important in the design of therapeutics. The restricted expression of Zap70, in T cells and NK cells (Chan et al., 1992) and its essential role in the transduction of TCR signals make it an interesting therapeutic target for immunosuppression following transplantation and in autoimmune disease. The two main immunosuppressants in common use, currently, tacrolimus and cyclosporin, have significant side effects as a result of their inhibition of calcineurin in the kidney (Dumont et al., 1992; Johnson et al., 1999; Wolf et al., 1990) which could be prevented by targeting Zap70 downstream of TCR engagement, specifically.

In this thesis we have clearly demonstrated that Zap70 plays an important role in transducing T cell survival signals, in a MEK-dependent manner. Inhibition of Zap70 signalling might thus prevent the survival and proliferation of malignant T cells as well as normal T cells, although the specificity and potency of Zap70 small molecule (Hirabayashi et al., 2009) and peptide (Nishikawa et al., 2000) inhibitors developed to date have not been tested in T cells in vivo. Interestingly, Zap70 is expressed in B cells in certain subtypes of B-CLL where it facilitates BCR signalling and enhances B cell migration (Calpe et al., 2011; Chen et al., 2005a). Zap70 expression in B-CLL also correlates with disease progression and poor survival (Crespo et al., 2003; Rassenti et al., 2004) and as such has been proposed to be an attractive target for B-CLL treatment. Meanwhile Raf, MEK and Erk kinases are important regulators of cell survival and proliferation.
in many non-malignant and malignant cells. Inhibition of this signalling cascade has been the subject of intensive research to design specific small molecule inhibitors for the treatment of cancer (Roberts and Der, 2007; Sebolt-Leopold and Herrera, 2004). Importantly, inhibition of MAPK signalling is toxic not only to malignant cells, but also to normal proliferating cells and so targeting Zap70 in T cells specifically might provide a more specific treatment of T cell malignancies alone or in combinations with other therapies. Whether specific T cell malignancies or T cell malignancy subtypes are sensitive to Zap70 inhibition, express increased Zap70 or hypermorphic mutants of Zap70, remains to be investigated. We hope, the important role Zap70 plays in the transduction of T cell survival and proliferation signals, as demonstrated in this thesis, might provide further incentive to develop a selective cell-permeable Zap70 kinase inhibitor.
References


Suzuki, K., Nakajima, H., Saito, Y., Saito, T., Leonard, W.J., and Iwamoto, I. (2000). Janus kinase 3 (Jak3) is essential for common cytokine receptor gamma chain (gamma(c))-dependent signaling: comparative analysis of gamma(c), Jak3, and gamma(c) and Jak3 double-deficient mice. Int Immunol 12, 123–132.


Vivien, L., Benoist, C., and Mathis, D.J. (2001). T lymphocytes need IL-7 but not IL-4 or IL-6 to survive in vivo. Int Immunol 13, 763–768.


Appendix


*Contributed equally.

Available online or in print.