Signals required for the induction of antigen-based therapeutic tolerance.

Joanne Elizabeth Konkel

A Thesis submitted for the Degree of Doctor of Philosophy
The University of Edinburgh

2009
DECLARATION

I declare that this thesis has been composed by myself, describes my own work and has not been submitted in any other application for a higher degree.

The experiments described in this thesis involving PD-1\(^{-}\) mice were performed in collaboration with Prof. A. Waisman; specifically they were designed by Prof S. Anderton and myself and performed by Dr. F. Frommer, University of Mainz, Germany.

Joanne Konkel

(November 2008)
ACKNOWLEDGEMENTS

Firstly, I would like to thank Steve, for putting up with me and all my “random” data and for helping me along my scientific path! I have thoroughly enjoyed my time in your lab.

Next my thanks fall on the members of Team Anderton; Mel, my sounding board for everything, Rich, for always giving a different perspective, and Katy, for always spilling my wine (?!?). Claire, thanks for your help since the beginning, Sarg, Chen, Cat, Antonio, Anne and everyone’s favourite surrogate lab member Sheila (spelt right?!?) muchas gracias to you all for wine, biscuits and crisps, for the Hollyoaks chats, and most importantly for always keeping me on track. I hope you will all forever remember my “special” peptide; I hope that I will never have to use the phrase “pant bag” ever again!!!!

Many thanks to those in the Maziels, MacDonald, and Grey labs for letting me steal reagents, and to everyone else in IIIR, Level 5 and the Ann Walker who has helped me along the way; specifically Bette and Martin.

Thanks to those outside the lab who has made Edinburgh fun for me, Naomi, Will, Moose, Matt, Vicky; special thanks must go to the running “team”!! Thanks to my family, who never complain when I fail to return calls, your support has been un-ending.

The Wellcome Trust must also receive thanks for making all this possible by funding me throughout my time in Edinburgh.

Finally, thanks to my best friend John.

I have enjoyed my time in Edinburgh, being part of team Anderton and doing my PhD….you have all made this so! Big Thanks!!
ABSTRACT

Despite the actions of central tolerance during thymic selection, it is clear that the peripheral T cell repertoire contains significant numbers of self-reactive T cells. The immune system needs to curtail the risk of autoimmune disease by controlling the activity of these self-reactive T cells. Various mechanisms are in place to achieve this control (peripheral tolerance).

Activation of CD4+ T cells requires two signals; engagement of the T cell receptor (TCR) with an appropriate peptide:MHC complex (signal 1), and the aggregate effect of multiple signals generated following ligation of costimulatory and coinhibitory molecules (signal 2). Both signals are required for the generation of a productive T cell response and both are provided by the professional antigen presenting cell, the dendritic cell (DC). T cells are fully activated upon receiving both signal 1 and 2, but are rendered tolerant when they receive only signal 1. This can be exploited therapeutically through the administration of peptides to induce tolerance in peptide-reactive T cells. Administration of peptide with an adjuvant provides both signal 1 and 2, and leads to a sustained T cell response against the administered peptide (immunity). However, if the same peptide is administered in soluble form, only signal 1 is provided, leading to the establishment of T cell tolerance. The studies in this thesis explore the role of both signal 1 and signal 2 in peptide-induced T cell tolerance.

Previous data from our laboratory have highlighted PD-1 and RANKL as costimulatory molecules which could play a role in peptide-induced T cell tolerance. Here we show that PD-1, an important coinhibitory molecule, plays a vital role in restraining peripheral T cell expansion under conditions leading to T cell immunity. However, in contrast to data from other studies, we demonstrate that PD-1 plays no role in the induction, establishment or maintenance of peptide-induced T cell tolerance. We show that the costimulatory receptor ligand pair RANK:RANKL plays a role in the balance between T cell tolerance and immunity; as administration of anti-RANKL was seen to potentiate both tolerance and immunity. We also explored the effect of altering the affinity of a peptide for MHC on the induction of peptide tolerance. We demonstrate that use of a peptide with a high-affinity for MHC induces tolerance via a novel, non-deletional mechanism of peptide-tolerance induction. Importantly, we show that the high-affinity peptide can form peptide-MHC complexes which persist in a biologically relevant form for fourteen days following peptide administration. We suggest that this leads to chronic stimulation of peptide-reactive T cells which promotes acquisition of a novel tolerant phenotype. Collectively the work described in this thesis demonstrates the important roles both signal 1 and 2 play in therapeutic-tolerance induction and how the qualitative and quantitative alteration of these signals can alter T cell fate and/or responsiveness.
Table of Contents

Acknowledgements...........................................................................................................ii
Abstract..........................................................................................................................iii
Abbreviations...................................................................................................................xiii

1. Introduction .......................................................................................................................1
  1.1. CD4+ T CELL BIOLOGY .........................................................................................1
  1.1.1. Requirements for T cell Activation .....................................................................2
  1.1.2. TCR Signalling ..................................................................................................5
  1.2. IMMUNE TOLERANCE; BASIC PRINCIPALS ......................................................7
  1.2.1. Central Tolerance ...............................................................................................7
  1.2.2. Peripheral Tolerance ........................................................................................7
    1.2.2.1. Deletion .........................................................................................................8
    1.2.2.2. Clonal Anergy and Adaptive Tolerance .......................................................8
    1.2.2.3. Regulatory T cells ........................................................................................10
  1.2.3. DC as APC in tolerance .....................................................................................13
  1.2.4. Autoimmunity ...................................................................................................14
  1.3. MOLECULAR MECHANISMS OF SURFACE DIALOGUE BETWEEN
       DC AND T CELLS.....................................................................................................15
    1.3.1. Ig Super family................................................................................................16
    1.3.2. PD-1; an introduction .....................................................................................16
      1.3.2.1. Expression of PD-1 and its Ligands ..........................................................17
      1.3.2.2. Role of PD-1 as a co-inhibitory molecule ...............................................18
      1.3.2.3. Signalling through PD-1:PD-L1/2 .............................................................19
      1.3.2.4. PD-1:PD-L in tolerance and autoimmunity .............................................19
      1.3.2.5. Role of PD-1 on Tregs ..............................................................................22
      1.3.2.6. PD-1 and chronic infection ......................................................................23
    1.3.3. TNF / TNFR family .........................................................................................24
    1.3.4. RANKL; an introduction .................................................................................26
      1.3.4.1. RANK:RANKL in the immune system ......................................................27
      1.3.4.2. RANKL in T cell responses .....................................................................28
        1.3.4.2.1. RANKL and the mucosal immune system ........................................29
1.3.4.2.2. RANKL and arthritis ................................................................. 29
1.3.4.2.3. RANKL and inflammation .................................................... 30
1.3.4.2.4. RANKL and tolerance ............................................................. 30
1.4. Ag-SPECIFIC THERAPEUTIC IMMUNE TOLERANCE .................. 31
1.4.1. Induction of Ag-induced T cell tolerance is an active process ....... 33
1.4.2. Altered Peptide Ligands (APL) ...................................................... 33
1.4.3. Oral Tolerance .............................................................................. 34
1.4.4. Mechanisms of Ag-induced T cell tolerance ............................... 35
1.4.5. Administration of soluble Ag as a therapy for autoimmune diseases 38
1.4.5.1. Ag-based therapy in animal models of disease ......................... 38
1.4.5.2. Ag-based therapy in humans ...................................................... 39
1.4.6. APC involved in the induction of Ag-based tolerance ................. 40
1.4.7. Imaging Ag-induced tolerance ...................................................... 41
1.5. EXPERIMENTAL MODELS ............................................................... 42
1.5.1. pOVA Reactive TCR transgenic; OT-II ........................................ 42
1.5.2. Experimental Autoimmune Encephalomyelitis (EAE) .................. 43
1.5.3. Ac1-9 Reactive TCR transgenic; Tg4 ............................................ 43
1.6. AIMS ............................................................................................... 46

2. Materials and methods ..................................................................... 52
2.1. MICE .............................................................................................. 52
2.2. GENERAL REAGENTS ................................................................... 52
2.3. ANTIGENS .................................................................................... 53
2.4. ANTIBODIES ................................................................................ 53
2.5. CELL PURIFICATIONS AND PREPARATIONS ............................ 54
2.6. IN VIVO ANTIGEN ADMINISTRATION ......................................... 57
2.7. ACTIVE EAE ................................................................................. 59
2.8. EX VIVO ASSESSMENT OF T CELL FUNCTION .............................. 59
2.9. IMMUNOFLUORESCENCE ............................................................. 66
2.10. EX VIVO ASSESSMENT OF T CELL FUNCTION ............................ 66
2.11. WESTERN BLOTTING OF CELLS ................................................ 67
2.12. STATISTICS ................................................................................ 68
3. The role of PD-1 mediated signalling in tolerance and immunity

3.1. INTRODUCTION ................................................................................................. 70

3.2. RESULTS ............................................................................................................ 72

  3.2.1. PD-L1 expression following tolerogenic or immunogenic treatment
differs to that of PD-L2. ............................................................................................ 72
  3.2.2. PD-1 signals are required to limit T cells responses in vitro. ...................... 73
  3.2.3. PD-1 signals are required to limit a productive immune response .......... 74
  3.2.4. Blockade of PD-1 signals does not convert a tolerogenic dose of peptide
to an immunogenic dose ......................................................................................... 75
  3.2.5. Blockade of PD-1 does not prevent the establishment of tolerance .......... 77
  3.2.6. Blockade of PD-1 does not overcome tolerance once it has been
established ................................................................................................................. 78
  3.2.7. PD-1 knockout peptide-reactive T cells can be rendered tolerant through
administration of soluble peptide ......................................................................... 79
  3.2.8. Different patterns of expression of PD-1 and PD-L1 on CD4+ and
CD8+ T cells ............................................................................................................ 80
  3.2.9. Anti-PD-1 enhances in vitro proliferative responses of CD8+ T cells to a
greater degree than CD4+ T cells. ......................................................................... 81
  3.2.10. Blockade of PD-1 does not prevent peptide-induced T cell tolerance in
CD4+ or CD8+ T cells .............................................................................................. 82
  3.2.11. Blockade of PD-1 does not affect the suppressive capacity of
CD4+CD25+foxp3+ Tregs ....................................................................................... 84

3.3. DISCUSSION .................................................................................................... 85

4. The role of RANKL mediated signalling in tolerance and immunity

4.1. INTRODUCTION .................................................................................................. 109

4.2. RESULTS ............................................................................................................ 112

  4.2.1. The role of RANKL in the induction of peptide-induced T cell
tolerance. ................................................................................................................. 112
  4.2.2. Administration of anti-RANKL can enhance peptide-induced T cell
tolerance. ................................................................................................................. 113
4.2.3. Administration of anti-RANKL with a non-tolerising dose of pOVA reduces responsiveness to pOVA ................................................................. 115
4.2.4. The effect of anti-RANKL on tolerance induction using the MBP Ac1-9 (4Lys) peptide. ............................................................... 116
4.2.5. Anti-RANKL does not alter the ability of Tregs to suppress naïve T cell proliferation ................................................................. 118
4.2.6. The role of RANKL in the LPS primed T cell immunity ............... 119
4.2.7. The role of RANKL in CFA primed T cell immunity .................... 120
4.2.8. The effect of anti-RANKL on in vitro T cell cultures .................... 121
4.3. DISCUSSION .................................................................................. 124

5. A variant myelin peptide induces T cell tolerance via a novel non-deletional mechanism ................................................................. 148
5.1. INTRODUCTION .............................................................................. 148
5.2. RESULTS ......................................................................................... 150
5.2.1. 4Tyr induces profound suppression of EAE in a TCR transgenic T cell transfer model ................................................................. 150
5.2.2. Systemic administration of 4Tyr induces tolerance via a novel mechanism .................................................................................. 151
5.2.3. Persisting 4Tyr tolerised Tg4 cells are not anergic ...................... 152
5.2.4. Only 4Tyr can maintain a population of tolerised Tg4 T cells ......... 152
5.2.5. Administration of soluble 4Tyr i.v. induces considerable proliferation of Tg4 T cells ........................................................................ 154
5.2.6. 4Tyr-MHC complexes persist for long periods of time in vivo following administration of 4Tyr .............................................................. 155
5.2.7. Induction of tolerance in Tg4 cells when 4Tyr is administered before Tg4 cell transfer ................................................................. 156
5.2.8. 4Tyr-MHC complexes are maintained by CD11c+CD4+ DC .......... 158
5.2.9. Tolerance induction following DC depletion ............................ 159
5.2.10. Proliferation of Tg4 cells following DC depletion ...................... 160
5.3. DISCUSSION .................................................................................. 161
6. Phenotype of 4Tyr tolerised Tg4 cells. ...................................................... 184

6.1. INTRODUCTION ....................................................................................... 184

6.2. RESULTS .................................................................................................... 186

6.2.1. Surface phenotype of 4Tyr tolerised Tg4 T cells............................. 186

6.2.2. 4Tyr tolerised Tg4 cells have a defect in TCR-induced calcium metabolism. ........................................................................................................... 188

6.2.3. 4Tyr tolerised Tg4 exhibit normal kinetics in the activation of ERK.... 189

6.2.4. 4Tyr tolerised Tg4 cells do not have altered sensitivities to secondary stimulation compared to naïve Tg4 cells. ........................................................ 190

6.2.5. 4Tyr treatment increases the expression of foxp3 in Tg4 cells during EAE .................................................................................................................. 191

6.2.6. 4Tyr treatment of Tg4 cells has no effect on foxp3 expression in a non-disease setting .............................................................. 193

6.2.7. 4Tyr tolerised Tg4 cells express T-bet when stimulated with peptide ex vivo. .................................................................................................................. 194

6.2.8. Cytokine profile of 4Tyr tolerised Tg4 cells after repeated doses of 4Tyr 195

6.2.9. Role of IL-2 in generating tolerant Tg4 cells following 4Tyr treatment. .......................................................................................................................... 197

6.2.10. The effect of 4Tyr treatment on the host CD4+ T cell compartment. 198

6.3. DISCUSSION ............................................................................................... 200

7. General Discussion ......................................................................................... 231

7.1 Signal 2 ........................................................................................................ 231

7.2 Signal 1 ........................................................................................................ 237

8. References ......................................................................................................... 241

9. Appendix ............................................................................................................ 267
Figures and Tables

Chapter 1: Introduction

Figure 1.1. Summary of costimulatory molecules known to be important in T cell activation. 47
Figure 1.2. Signalling events initiated by ligation of the TCR. 48
Figure 1.3. DC maturation states and their role in tolerance and immunity 49
Figure 1.4. PD-L1 and CD80 interact to mediate a negative signal to the T cell. 50
Figure 1.5. Schematic representation of the residues important for Ac1-9 binding to I-A<sup>U</sup> and the Tg4 TCR. 51

Chapter 2: Materials and methods

Table 2.1: ELISA reagents 61
Table 2.2: Antibodies used for flow cytometric analysis 65

Chapter 3: The role of PD-1 mediated signalling in tolerance and immunity

Figure 3.1. PD-L1 expression during the induction of tolerance and immunity. 93
Figure 3.2. PD-L2 expression during the induction of tolerance and immunity. 94
Figure 3.3. PD-L1/2 expression following priming with pOVA in CFA. 95
Figure 3.4. Anti-PD-1 enhances responses of in vitro T cells cultures. 96
Figure 3.5. Anti-PD-1 enhances LPS stimulated responses of OT-II cells. 97
Figure 3.6. Anti-PD-1 enhances CFA stimulated responses of OT-II cells. 98
Figure 3.7. Anti-PD-1 treatment does not convert a tolerogenic dose of pOVA to an immunogenic dose. 99
Figure 3.8. Experimental outline of experiments determining the role of PD-1 mediated signals in peptide-induced T cell tolerance. 100
Figure 3.9. Anti-PD-1 treatment does not prevent the establishment of peptide-induced tolerance in OT-II T cells. 101
Figure 3.10. Anti-PD-1 treatment does not overcome established tolerance in OT-II T cells. 102
Figure 3.11. Peptide-induced T cell tolerance can be induced in PD-1<sup>−/−</sup> 2D2 cells. 103
Figure 3.12. Expression of PD-1 and PD-L1 on \textit{in vitro} cultured CD4+ and CD8+ T cells.

Figure 3.13. Anti-PD-1 enhances \textit{in vitro} responses of CD8+ T cells to a greater extent than CD4+ T cells.

Figure 3.14. Anti-PD-1 treatment does not prevent peptide-induced T cell tolerance in pMOG-reactive CD4+ or CD8+ T cells.

Figure 3.15. Immunisation of PD-1 knockout mice leads to greater expansion of CD4+ and CD8+ T cells.

Figure 3.16. Anti-PD-1 does not affect the suppressive ability of Tregs in an \textit{in vitro} suppression assay.

\textbf{Chapter 4: The role of RANKL mediated signalling in tolerance and immunity.}

Figure 4.1. Role of RANK:RANKL interaction in the induction of tolerance.

Figure 4.2. Anti-RANKL enhances tolerance induced by 500\mu g of pOVA.

Figure 4.3. Anti-RANKL treatment does not overcome established tolerance in OT-II cells.

Figure 4.4. Administration of anti-RANKL with a non-tolerising dose of peptide enhances unresponsiveness to pOVA.

Figure 4.5. Administration of anti-RANKL with 4Lys does not potentiate tolerance induction in Tg4 cells.

Figure 4.6. The effect of anti-RANKL on EAE in B10.PLxC57BL/6 mice.

Figure 4.7. The effect of anti-RANKL on EAE in B10.PLxC57BL/6 mice.

Figure 4.8. The effect of anti-RANKL on regulatory T cell function.

Figure 4.9. Anti-RANKL has no effect on LPS primed immunity.

Figure 4.10. Anti-RANKL has no effect on LPS primed immunity.

Figure 4.11. Anti-RANKL enhances CFA primed T cell immunity.

Figure 4.12. Anti-RANKL has no effect on actively induced EAE.

Figure 4.13. The effect of anti-RANKL on peptide stimulated splenocyte cultures.

Figure 4.14. The effect of anti-RANKL on anti-CD3/anti-CD28 stimulated CD4+ T cell cultures.
Chapter 5: A variant myelin peptide induces T cell tolerance via a novel non-deletional mechanism

Figure 5.1. 4Tyr protects against 4Lys induced EAE. 169
Figure 5.2. Soluble 4Tyr induces tolerance via a different mechanism to soluble pOVA. 170
Figure 5.3. Persisting 4Tyr tolerised Tg4 T cells are not classically anergic. 171
Figure 5.4. Only 4Tyr can maintain a population of tolerised Tg4 cells. 172
Figure 5.5. The tolerant phenotype is established 4 days after 4Tyr treatment. 173
Figure 5.6. Tolerance induced with 4Val is different to that induced by 4Tyr. 174
Figure 5.7. 4Tyr induces considerable proliferation of transferred Tg4 cells. 175
Figure 5.8. 4Tyr:MHC complexes can be maintained for up to 14 days in vivo. 176
Figure 5.9. 4Tyr treatment 2 days before Tg4 T cell transfer induces tolerance. 177
Figure 5.10. 4Tyr treatment induces tolerance of Tg4 cells when administered less than 7 days prior to Tg4 cell transfer. 178
Figure 5.11.a. 4Tyr is presented predominately by DC to Tg4 T cells. 179
Figure 5.11.b-c. 4Tyr is presented to Tg4 cells predominantly by DC. 180
Figure 5.12. 4Tyr:MHC complexes are maintained on CD11c+ CD4+ DC following administration of 4Tyr i.v. 181
Figure 5.13. Tolerance can still be induced following DC depletion. 182
Figure 5.14. DC depletion after 4Tyr administration reduces the proliferation of Tg4 cells. 183

Chapter 6: Phenotype of 4Tyr tolerised Tg4 cells.

Figure 6.1. Surface phenotype of Tolerant Tg4 cells. 216
Figure 6.2. Surface phenotype of tolerant Tg4 cells following 4Lys+CFA immunisation. 217
Figure 6.3. Altered Ca^{2+} metabolism in tolerant Tg4 cells. 218
Figure 6.4. Altered Ca^{2+} metabolism in tolerant Tg4 cells. 219
Figure 6.5. Tolerant Tg4 cells do not display a Ca^{2+} signalling defect when stimulated in the presence of excess calcium. 220
Figure 6.6. Tolerant Tg4 cells do not have a defect in ERK phosphorylation. 221
Figure 6.7. Production of IL-2 by tolerant and naive Tg4 cells to 4Lys and 4Tyr stimulation. 222
Figure 6.8. 4Tyr treatment increases the expression of foxp3 in Tg4 cells during EAE. 223
Figure 6.9. 4Tyr treatment of Tg4 cells in vivo does not increase the expression of foxp3 in Tg4 cells. 224
Figure 6.10. Tolerant Tg4 cells express T-bet upon stimulation. 225
Figure 6.11. Cytokine production by tolerant Tg4 cells following repeated doses of 4Tyr. 226
Figure 6.12. Cytokine production by tolerant Tg4 cells following repeated doses of 4Tyr. 227
Figure 6.13. The effect of anti-IL-2 treatment on the induction of tolerance by 4Tyr. 228
Figure 6.14. Anti-IL-2 treatment alters the percent of foxp3+CD25+ cells. 229
Figure 6.15. Treatment with 4Tyr alters the percent of host foxp3+ cells. 230

Chapter 7: General Discussion
Figure 7.1. Anti-RANKL and anti-PD-1 in tolerance and immunity. 237
Figure 7.2. Quantitative Model: manifestations of tolerance following induction by administration of peptide. 240

Chapter 9: Appendix
Figure A.1. Anti-PD-1 enhances in vitro responses of CD8+ T cells to a greater extent than CD4+ T cells. 267
Figure A.2. Anti-RANKL enhances tolerance induced by 500μg pOVA. 268
Figure A.3. The effect of anti-RANKL on the molecular expression of cultured splenocytes. 269
Figure A.4. Anti-RANKL ablates LPS primed T cell immunity. 270
Figure A.5. DC depletion after 3 doses of DTx i.p. 271
Figure A.6. Tyrosine phosphorylation of stimulated tolerant and effector Tg4 cells. 272
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>APL</td>
<td>altered peptide ligand</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund's adjuvant</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T lymphocyte antigen-4</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DTx</td>
<td>diphtheria toxin</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorter</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL-</td>
<td>interleukin</td>
</tr>
<tr>
<td>i.n.</td>
<td>intranasal</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MACS</td>
<td>magnetically activated cell sorting</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility antigen</td>
</tr>
<tr>
<td>MOG</td>
<td>myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PD-1</td>
<td>program death-1</td>
</tr>
<tr>
<td>PD-L</td>
<td>program death ligand</td>
</tr>
<tr>
<td>p:MHC</td>
<td>peptide:MHC complex</td>
</tr>
<tr>
<td>pMOG</td>
<td>MOG peptide</td>
</tr>
<tr>
<td>pOVA</td>
<td>ovalbumin peptide</td>
</tr>
<tr>
<td>Ptx</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>RANK</td>
<td>receptor activator of NFκB</td>
</tr>
<tr>
<td>RANKL</td>
<td>RANK ligand</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>4Lys</td>
<td>wild-type Ac1-9 peptide of MBP</td>
</tr>
<tr>
<td>4Tyr</td>
<td>APL of Ac1-9 with a tyrosine residue at position 40</td>
</tr>
</tbody>
</table>
1. Introduction

T cells are an integral part of the adaptive immune response. These lymphocytes develop in the thymus and exit into the periphery where they have heterogeneity in phenotype and function. Lymphoid progenitors are generated in the bone marrow and enter the thymus via the blood. These cells then pass through a series of developmental stages, during which T cells will display functional T cell receptor chains, undergo positive and negative selection, and commit to either the CD4+ or CD8+ lineage before exiting to the periphery (Germain, 2002). Collectively these processes yield a population of T cells with a large diversity of T cell receptors (TCRs) which can contribute to a productive immune response and protect against invading pathogens.

A vast diversity of TCRs allows the T cell population to respond to a variety of peptide antigens. However this also includes peptides from our own tissues. In mice, it is estimated that the T cell repertoire consists of approximately $1 \times 10^8$ different TCRs, but in order for the T cell repertoire to recognise all possible presented peptides each TCR must recognise between $1 \times 10^4$–$1 \times 10^7$ different peptides (Mason, 1998). Thus there is a huge potential for TCRs to be generated which will recognise self-peptides.

In healthy individuals the immune system is able to remain unresponsive to self-peptides, however for roughly 5% of people in western populations this is not the case and inappropriate immune responses to self are mounted, resulting in the development of autoimmune diseases. In healthy individuals tolerance to self is maintained by two key processes; central and peripheral tolerance.

1.1. CD4+ T CELL BIOLOGY

T cells are mediators of the adaptive arm of the immune system, whereby they aid in the development of pathogen specific immune responses. This thesis will focus on CD4+ helper T cells which recognise peptides non-covalently complexed with major histocompatibility class II (MHC-II) antigens expressed on the surface of antigen presenting cells (APC). Generation of a productive CD4+ T cell response requires the T cells to receive two signals; engagement of the TCR with an appropriate
peptide:MHC (p:MHC) complex (signal 1), and provision of costimulation by an APC (signal 2). These signals activate the CD4+ T cells which subsequently undergo clonal expansion and differentiation into functional T helper cells. The interplay between CD4+ T cells and APC directs the fate of the T cells, such that this interaction drives the differentiation of the CD4+ T cell into specific effector cell subsets, each with specified functions. Moreover, it is the interaction between a T cell and APC that determines whether a T cell will be activated or rendered tolerant. In simplistic terms, provision of signal 1 and signal 2 leads to CD4+ T cell activation, whereas provision of signal 1 alone leads to tolerance.

1.1.1. Requirements for T cell Activation

Dendritic cells (DC) have been shown to be the best APC at activating naïve T cells. Compared to B cells, DC have demonstrated a fourfold greater ability to induce IL-2 in naïve T cells (Cassell and Schwartz, 1994). In fact, both B cells and macrophages have been shown to be poor stimulators of naïve T cells (Croft et al., 1992). Why are DC so efficient at priming naïve T cells? The reasons are most likely quantitative ones, with DC expressing 10-100 times more specific self-peptide:MHC complexes (Inaba et al., 1997) and expressing a plethora of costimulatory and adhesion molecules (Banchereau and Steinman, 1998). Moreover, expression of p:MHC and costimulatory molecules are further up-regulated following activation of DC. DC have thus been termed the professional APC (Lassila et al., 1988).

DC have been shown to be heterogeneous in both phenotype and function and can be distinguished based on their progenitors, tissues distribution and surface molecule expression. Specific populations of DC have specialised functions; CD8α+ DC have been shown to be optimised for cross-presentation of antigen (Ag) (Pooley et al., 2001) and CD103+ DC from the mesenteric lymph node have demonstrated a highly specialised role in the conversion of naive T cells into regulatory T cells (Coombes and Powrie, 2008).

Therefore the signals elicited from DC are vital in determining T cell activation and differentiation. It is the interaction between CD4+ T cells and DC on which this thesis will focus; determining the signals from DC to T cells that direct T cell fate. More specifically, the signals which are required for the induction of T cell tolerance.
**Signal 1: Antigen Presentation**

Ag capture, processing and presentation by DC is key to developing an adaptive immune response; TCR recognition of cognate p:MHC complexes is the initial step in T cell activation. DC exhibit morphology which is suited to these functions; they display fine dendrite processes which extend from the cell body allowing a large surface area for Ag uptake and T cell encounter. Immature DC are extremely good at Ag uptake and can endocytose Ag by phagocytosis, macropinocytosis and receptor-mediated endocytosis, for instance via the glycoprotein DEC205 (Trombetta and Mellman, 2005). Aiding this, immature DC have been shown to contain many MHC-II rich compartments, which are discharged onto the surface of the DC upon maturation (Banchereau and Steinman, 1998).

DC are located throughout the peripheral tissues, ideally placing them for surveillance; DC capture Ag from the periphery then migrate to lymphoid organs, spleen and lymph nodes where they can interact with T cells. This sentinel function of DC allows optimal Ag uptake and T cell encounter (Banchereau and Steinman, 1998). Collectively these processes allow DC to present Ag to, and activate, rare T cell clones.

**Signal 2: Costimulation**

Signal 2 is delivered through a variety of co-stimulatory molecules found on the surface of the DC. Binding of receptor-ligand pairs on the surface of the T cell and DC is the basis of costimulation. Ligation of these receptors initiates signalling events within the T cell. Costimulation was originally thought to simply enhance initial T cell activation, however different molecules have been shown to play roles in the promotion of effector function and cell survival. In contrast, some costimulatory molecules, here termed coinhibitory molecules, have a negative effect on T cell activation. An array of costimulatory receptor-ligand pairs have been identified, some are shown in Figure 1.1. Costimulatory molecules are discussed in greater detail in section 1.3.
Signal 3

Signal 3 is not required for T cell activation, but instead functions to direct the quality of the effector function of activated T cells. CD4+ T cells can differentiate into three main effector helper subsets; T helper 1 (Th1), Th2, and Th17 cells, each of which has a distinct cytokine-secretion pattern and as such can mediate distinct functions. Which subset arises following activation is subject to intense investigation and is thought to be dependent on the cytokine milieu present during CD4+ T cell activation by DC, the Ag dose, and the presence of certain costimulatory molecules (Kaiko et al., 2008).

The signature cytokine of a Th1 cell is interferon (IFN)-γ. Th1 cells are effective against intracellular infections, primarily through the activation of macrophages by IFN-γ. Presence of IL-12 promotes Th1 differentiation. Through the generation of a positive feed-back loop, IL-12 leads to IFNγ expression which in turn leads to the STAT1 mediated activation of the lineage specific transcription factor T-bet. T-bet expression defines a Th1 cell and leads to IFNγ production, remodelling of the IFNγ locus and expression of IL-12 receptor in T cells (Mullen et al., 2001).

The signature cytokines of a Th2 cell are IL-4, -5 and -13, which up-regulate antibody production, enhance mucus secretion and recruit/activate eosinophils. Th2 cells are therefore required for the clearance of parasitic extracellular organisms. IL-4 is important in the differentiation of Th2 cells, by inducing STAT6 expression. STAT6 causes expression of the Th2 lineage specific transcription factor, GATA-3, which drives epigenetic remodelling of the Th2 cytokine gene cluster (Zheng and Flavell, 1997). GATA-3 and T-bet act antagonistically on each another (Mullen et al., 2001; Ouyang et al., 1998).

A more recently described T helper subset are Th17 cells which secrete IL-17, IL-17F, IL-6, IL-22 and TNFα (Langrish et al., 2005). Th17 cells appear to play important roles in tissue inflammation and neutrophil recruitment to clear extracellular bacteria. The Th17 lineage specific transcription factor is RORγt (Ivanov et al., 2006). Both IL-4 and IFNγ inhibit the generation of the Th17 phenotype (Park et al., 2005). Reciprocal pathways of induction of Th17 and foxp3+ regulatory T cells (Tregs) have been demonstrated; in vitro IL-6 and TGFβ generates Th17 cells, whereas TGFβ alone generates foxp3+ cells (Bettelli et al., 2006). In
fact, foxp3 has been shown to interact with RORγt and inhibit its function (Zhou et al., 2008). The presence of proinflammatory cytokines, which would be indicative of infection, during T cell differentiation is therefore thought to play a major role in the decision between induction of Th17 cells versus Tregs.

“Signal 0”

Preceding signals 1, 2 and 3 is “signal 0”, mediated by the innate arm of the adaptive immune response. DC express Toll-like receptors (TLR), which recognise pathogen-associated molecular patterns (PAMPs). TLR ligands include a variety of pathogen derived products including lipopolysaccharide (LPS), double-stranded RNA, flagellin and unmethylated CpG DNA motifs (Kaisho and Akira, 2002). DC are exquisitely sensitive to PAMPs and TLR ligation induces pro-inflammatory cytokine production and the up-regulation of costimulatory molecules; thus TLR ligation triggers DC maturation and therefore the adaptive immune response.

LPS, an adjuvant frequently used in this thesis, is a constituent of the wall of gram-negative bacteria. Importantly, LPS can be released from bacteria and can exist in a soluble form. LPS is the ligand for TLR-4, however other host proteins are involved in the recognition of LPS; LPS is bound by LPS binding protein (LBP), this high-affinity complex then forms a ternary complex with CD14 (Palsson-McDermott and O'Neill, 2004). This allows LPS to be efficiently delivered to and stimulate TLR-4. Stimulation of TLR-4 causes the production of cytokines and up-regulation of costimulatory molecules on DC by MyD88-dependent and TRIF-dependent signalling pathways respectively (Hoebe et al., 2003). As such, LPS can act as an adjuvant; systemic application of LPS concomitant with Ag has been employed experimentally to generate an immune response to the administered Ag (Hochweller and Anderton, 2005).

1.1.2. TCR Signalling

The earliest biochemical event that can be seen in T cells following receipt of both signals 1 and signal 2 is an increase in phospho-tyrosine on many intracellular proteins (Kane et al., 2000). The intracellular chains of the TCR (TCRζ chains) do not possess any intrinsic tyrosine kinase activity. Instead signalling events depend
upon the recruitment of protein tyrosine kinases (PTKs) to the activated TCR complex. The intracellular domain of the TCR complex contains two immunoreceptor tyrosine-base activation motifs (ITAMs). PTKs, of the src-kinase family, known as Lck and Fyn, phosphorylate residues within ITAMs of the TCR complex. Phosphorylation of these two ITAMS allows recruitment of ZAP-70 via its tandem SH2 domains, which bind the phosphorylated ITAMs (Iwashima et al., 1994). ZAP-70 is then phosphorylated and activated. The main substrates downstream of Lck, Fyn and ZAP-70 are linker of activated T cells (LAT) and SH-2 domain containing lymphocyte protein of 76,000 MW (SLP-76) (Myung et al., 2000). These adaptors form scaffolds on which signalling molecules assemble and allow activation of intracellular signalling cascades and dissemination of the signal within the cell.

There are a number of important signalling pathways downstream of the TCR; one is mediated by phospholipase Cγ1 (PLCγ1). Activation of PLCγ1 causes the hydrolysis of phosphatidylinositol (PI)-4,5-P₂ to diacylglycerol (DAG) and inositol-1,4,5-P₃ (IP₃). These are second messengers which mediate the activation of other signalling pathways. DAG is involved in the activation of protein kinase C (PKC) and Ras, whereas IP₃ is responsible for the activation of calcium signalling (Huang and Wange, 2004). Another pathway is mediated by phosphatidylinositol-3 kinase (PI-3K). Active PI-3K generates (PI)-3,4,5-P₃ and (PI)-3,4P₂, which are bound by plexstrin homology (PH) domains of proteins, allowing other signalling proteins to be recruited to the plasma membrane where their activation can occur (Cantrell, 2002). For an overview of signalling events see Figure 1.2.

Alteration in gene expression is the ultimate result of TCR mediated signalling as the signal moves away from the plasma membrane to the nucleus of the cell. TCR-stimulated changes in gene expression are mediated by three key transcription factors; NF-κB, NF-AT and AP-1 (Huang and Wange, 2004).
1.2. IMMUNE TOLERANCE; BASIC PRINCIPALS

The ability to discriminate between self and non-self is key to developing a functional immune system. Inability to establish this will result in inappropriate immune responses to self-peptides and autoimmune disease. Thus the immune system is tasked with fighting invading pathogens and distinguishing a considerable array of non-self and self antigens.

1.2.1. Central Tolerance

The first level of discrimination between self and non-self occurs in the thymus and is referred to as central tolerance. The result of central tolerance is the irreversible deletion of self-reactive T cell clones, such that Harald von Boehmer stated that “the thymus selects the useful, neglects the useless and destroys the harmful” (von Boehmer et al., 1989). T cells bearing TCRs with a high affinity for self-peptides are deleted at the CD4+CD8+ stage (Palmer, 2003; Starr et al., 2003). This negative selection of thymocytes means that most self-reactive T cells are deleted before they enter the periphery, as are many self-Ag are expressed in the thymus. In fact, in the absence of negative selection the size of the single positive thymocyte pool almost doubles (van Meerwijk et al., 1997).

As a result of promiscuous gene expression co-ordinated by the transcription factor autoimmune regulator (AIRE), a wide variety of Ag are expressed in the thymus (Anderson et al., 2002). These are predominately expressed in thymic medullary epithelial cells. This plethora of Ag has been shown to consist of Ag from nearly all organs of the body, for example proteins from the CNS and pancreas have been detected in mTECs (Derbinski et al., 2001).

1.2.2. Peripheral Tolerance

Not all self-reactive T cells are deleted during the process of central tolerance, some escape and enter the periphery (Anderton and Wraith, 2002). This could occur because the self-Ag is not expressed in the thymus (Anderson et al., 2000), or because the self-Ag has such a low affinity for MHC that it cannot trigger negative selection (Liu et al., 1995). Altered Ag processing in the thymus compared to peripheral tissues could also allow escape of self-reactive T cell clones (Anderton et
al., 2002; Manoury et al., 2002). Accordingly the immune system has developed mechanisms by which these cells are permitted to persist in a non-aggressive, tolerant form. The three main mechanisms of peripheral CD4+ T cell tolerance are deletion, anergy/adaptation and regulation.

1.2.2.1. Deletion
Deletion is perhaps the most robust way of maintaining tolerance to self. Activation induced cell death (AICD) is an intrinsic T cell process whereby continual stimulation of a T cell clone by its cognate Ag leads to deletion of that clone by apoptosis. This is perhaps a somewhat misleading name as it implies that the process of death is linked to initial T cell activation. Instead, for AICD to occur a cycling T cell must simply be stimulated by Ag (Lenardo et al., 1999). As self-Ag are continuously present, only a self-reactive T cell clone is likely to continually encounter its cognate Ag, as such being repeatedly stimulated and therefore deleted. Fas-mediated apoptosis is the mechanism by which CD4+ T cells undergo AICD. The importance of Fas-mediated AICD is highlighted in lpr and gld strains of mice which have mutated Fas and Fas-Ligand (FasL) respectively. Both these strains suffer lymphoproliferative diseases and systemic autoimmunity (Sobel et al., 1993; Watanabe-Fukunaga et al., 1992). As well as a key role for Fas-FasL, IL-2 has also been shown to be vital for the induction of AICD (Lenardo, 1991).

1.2.2.2. Clonal Anergy and Adaptive Tolerance
Clonal anergy and adaptive tolerance are intrinsic Ag-induced T cell states of Ag-specific unresponsiveness.

Clonal Anergy
Clonal anergy was first produced in vitro as a result of TCR ligation in the absence of costimulation (Jenkins and Schwartz, 1987; Lamb et al., 1983). It is characterised by an inability of cells to proliferate and produce IL-2, but effector mechanisms of cells remain intact (Schwartz, 2003). The ligation of CD28 by CD80/CD86 is vital in preventing anergy induction (Harding et al., 1992). The signalling events which lead to this state of unresponsiveness have been explored in in vitro anergised T
cells. Primarily defects have been seen in Ras-mediated signalling pathways (Fields et al., 1996; Li et al., 1996). Also, a number of proteins have been seen to differ between normal and anergic T cells, most notably the cbl family of E3 ubiquitin ligases (Duan et al., 2004) and the transcription factor early growth response (Egr) 2 (Harris et al., 2004). The demonstration that treatment of T cells with cyclohexamide blocks anergy induction has led to the hypothesis that an “anergy factor” exists. A number of suggested candidates are gene related to anergy in lymphocytes (GRAIL) (Seroogy et al., 2004) and ITCH (Fathman and Lineberry, 2007), both E3 ligases, and the cell cycle inhibitor p27kip1 which is controlled by Egr-2 (Boussiotis et al., 2000). It is on the E3 ubiquitin ligases GRAIL, ITCH, cbl and cbl-b which research has mostly focused. E3 ubiquitin ligases attach ubiquitin to substrate proteins. This can target the substrate for degradation, alter protein trafficking, induce conformational changes or stabilise the protein (Fathman and Lineberry, 2007). These proteins attenuate peripheral T cell activation via different mechanisms. Cbl and cbl-b promote the destabilisation of the T cell synapse (Naramura et al., 2002). GRAIL has been shown to stabilise an inhibitor of Rho, a family of small GTP-binding proteins that regulate actin polymerisation; this results in failure to activate Rho and therefore inhibits the formation of the immunological synapse (Su et al., 2006). ITCH has been shown to be involved in the degradation of signalling intermediates downstream of the TCR, namely PLCγ and PKC0 (Heissmeyer et al., 2004).

**Adaptive Tolerance**

The adaptively tolerised T cell state is often referred to as *in vivo* anergy and is primarily characterised by a shift in the dose response. Unlike clonal anergy, adaptive tolerance requires T cells to proliferate (Schwartz, 2003). TCR transgenic models have allowed this state to be extensively examined *in vivo*, in hosts which constitutively express the cognate Ag. Rocha et al transferred H-Y Ag specific CD8+ T cells to athymic, nude male mice. Transferred cells initially proliferated before the population contracted leaving a small population of H-Y specific T cells which had become adapted to the constitutive level of Ag. Adaptated T cells were hyporesponsive to restimulation; they did not proliferate or make any IL-2 and
showed decreased levels of TCR and CD8 (Rocha and von Boehmer, 1991). Moreover, the continued presence of Ag was required to maintain T cells in the adaptively tolerised state, because on transfer to female mice the adapted phenotype was lost (Rocha et al., 1993). In a similar model transferring pigeon cytochrome c (PCC) specific CD4+ TCR transgenic T cells into mice which express PCC but lack T cells, it was shown that the adapted T cells failed to make any cytokines (Tanchot et al., 2001). Transfer of these cells to a host not expressing the Ag again reversed the adapted state, highlighting the importance of continued Ag persistence for the maintenance of adaptation.

1.2.2.3. Regulatory T cells

As early as the 1960s T cells had been shown to execute positive as well as negative affects on evolving immune responses (Germain, 2008). Later work demonstrated that certain T cells could dominantly inhibit or suppress helper T cells from functioning (Gershon and Kondo, 1971).

A series of seminal studies have since identified CD4+CD25+ T cells as capable of repressing conventional T cells. CD4+CD25+ cells protected mice from autoimmune diseases as removal of CD4+CD25+ cells caused autoimmune pathology and reconstitution of this population prevented autoimmune development (Asano et al., 1996; Sakaguchi et al., 1995). These regulatory T cells (Tregs) exit from the thymus and constitute about 10% of the peripheral T cell pool and are referred to as natural Tregs.

**Foxp3**

The forkhead transcription factor foxp3 is critical for the function and development of Tregs. Foxp3 is specifically expressed in naturally arising regulatory CD4+CD25+ T cells, moreover retroviral gene transfer of foxp3 confers naïve T cells with a regulatory phenotype (Hori et al., 2003). Generation of mixed bone marrow chimera consisting of wild-type and foxp3-/- or foxp3+/+ bone marrow demonstrated that foxp3-/- cells could not develop into CD4+CD25+ regulatory T cells (Fontenot et al., 2003). Foxp3 was also shown to be genetically defective in autoimmune inflammatory diseases in both humans and mice. The lymphoproliferative diseases
seen in the scurfy mouse and in immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) in humans are both due to mutations in foxp3 (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001). Moreover, mice in which foxp3 is specifically deficient in T cells have a phenotype identical to the scurfy mouse, reinforcing the role of this transcription factor specifically within T cells (Fontenot et al., 2005).

**Thymic development of natural Tregs**

Development of natural Tregs in the thymus is dependent on TCR interactions with p:MHC (Fontenot et al., 2005). Natural Tregs have been shown to be present at normal numbers in α/β-TCR transgenic mice, yet they are completely absent in the same TCR transgenic on the Rag2-/- background (Itoh et al., 1999). Thus endogenous rearrangement of TCRs in the thymus appears to be necessary for Treg development in this setting. Subsequently it was demonstrated that the proportion of Tregs that develop in the thymus of TCR-transgenic mice is enhanced when the cognate Ag is also expressed (Jordan et al., 2001). Jordan et al also demonstrated that selection of thymocytes to the regulatory phenotype required a TCR with high affinity for self peptide as thymocytes with low affinity did not become Tregs (Sakaguchi, 2001).

Little is known about how foxp3 expression is induced or regulated in the thymus. CD25, CD28, CD80/86, Cytotoxic T lymphocyte Ag-4 (CTLA-4) and IL-2 have all been shown to contribute to, but to be dispensable for, foxp3 induction in the thymus (Fontenot and Rudensky, 2005). Recently TGFβ has been shown to be critical for the development of foxp3+ Tregs in the thymus (Liu et al., 2008).

**Mechanisms of Suppression**

How Tregs mediate their suppressive activity is still a grey area, but a number of mechanisms have been described. The inhibitory cytokines IL-10 and TGFβ are thought to be important for both the mechanism of action and the generation of Treg cells (Gupta et al., 2008; Vignali et al., 2008). Increased IL-10 and TGFβ transcripts in CD4+CD25+ cells have been noted during suppression assays, however addition
of anti-IL-10 and anti-TGFβ did not overcome the suppressive effect of the Tregs (Takahashi et al., 1998).

*In vitro* suppression has been shown to be cell contact dependent as separation of cells by a transwell membrane alleviates suppression (Takahashi et al., 1998). This cell contact dependent mechanism was not due to direct killing mechanisms that involve Fas-FasL or TNF-TNFR. Recently a role has been suggested for membrane-bound TGFβ, which would mediate suppression via a cell contact dependent mechanism (Vignali et al., 2008).

CTLA-4 is constitutively expressed on the surface of CD4+CD25+ Tregs and is expressed to a higher degree on Tregs compared to effector T cells following stimulation (Takahashi et al., 2000). A number of studies have suggested that CTLA-4 plays an important role in contact mediated suppression by Tregs. Administration of anti-CTLA-4 to mice led to an autoimmune gastritis similar to that produced by Treg depletion (Takahashi et al., 2000), and abrogated the protection afforded by CD4+CD25+ Tregs in a mouse model of colitis (Read et al., 2000). A specific knockout of CTLA-4 on foxp3+ cells has highlighted that the expression of CTLA-4 on Tregs is vital in maintaining immunological self tolerance (Wing et al., 2008).

Tregs can also operate by modulating the maturation or function of DC. Tregs condition DC to make indoleamine 2,3-dioxygenase (IDO) through a CTLA-4 dependent mechanism (Fallarino et al., 2003). IDO production results in suppressed effector responses through production of pro-apoptotic metabolites from tryptophan. More recently it has been shown that cytokine deprivation-induced apoptosis is a prominent mechanism by which Tregs suppress effector T cell responses (Pandiyan et al., 2007). Thus consumption of cytokines, specifically IL-2, by Tregs abrogates effector T cell responses.

**Other regulatory subsets**

Tregs can be induced in the periphery from circulating CD4+CD25- T cells. TGFβ signalling, by promotion of foxp3 expression is critical for the peripheral conversion of CD4+CD25-foxp3- naïve cells into CD4+CD25+foxp3+ Tregs *in vitro* (Chen et al., 2003) and *in vivo* (Kretschmer et al., 2005).
Two further subsets of induced Tregs are Tr1 and Th3 cells, neither of which express foxp3. Tr1 cells mediate suppression through the secretion of IL-10 (Groux et al., 1997). Groux et al demonstrated that repetitive stimulation of TCRs with allo-Ags in the presence of IL-10 induced Tr1 cells, and that these Tr1 cells could suppress \textit{in vitro} and \textit{in vivo} in a model of colitis. As only self-Ag should be continually present and thus be able to chronically stimulate a T cell, \textit{in vivo}, such cells would be ideally suited to control inappropriate reactions to self. Th3 cells produce TGFβ and are predominately associated with the induction of oral tolerance (Weiner, 2001). Th3 cells not only suppress effector T cell responses, but due to the production of TGFβ, have been shown to drive the generation of foxp3+ Tregs (Carrier et al., 2007).

\textbf{1.2.3. DC as APC in tolerance}

DC are known to play a major role in the induction of tolerance in the periphery; inducing tolerance in self-reactive T cells. Thus, DC are the primary decision makers when it comes to the choice between the induction of tolerance or immunity (Banchereau and Steinman, 1998). But how exactly do DC mediate this immunity/tolerance decision? There are two schools of thought on the role of DC in the induction of peripheral T cell tolerance. Firstly, a specialised subset of regulatory DC could induce tolerance in all T cells to which they present. Secondly, the activation status of the DC could determine its ability to induce tolerance or immunity. The latter of these proposals is currently favoured.

Initially, tolerance was thought to be mediated by just immature DC, which reside in tissues expressing small amounts of MHC and no costimulatory molecules; ideal for anergy induction. In fact, administration of \textit{in vitro} generated immature bone-marrow derived DC has been shown to induce anergy in CD4+ T cells (Lutz et al., 2000). Moreover, as endocytosis does not induce DC maturation, targeting Ag to DC via conjugation to antibodies for DC specific markers has been shown to induce tolerance in Ag-specific T cells (Finkelman et al., 1996; Hawiger et al., 2001).

Although CD4+ T cell anergy and deletion can be induced by immature DC, it is thought that peripheral tolerance in the steady state can also be mediated by semi-mature DC. Importantly, semi-mature DC have migratory capacity, allowing them access to the lymph nodes where they can tolerise more self-reactive T cells (Lutz
Unlike immature DC, these semi-mature DC express higher levels of costimulatory molecules and display high levels of self-Ag on their surface (Inaba et al., 1997). Semi-mature DC have also been shown to induce T cells with a regulatory phenotype. Menges et al showed that transfer of semi-mature DC protected mice from a central nervous system (CNS) autoimmune disease, primarily through the induction of T cells which produce considerable amounts of IL-10 (Tr1 cells) (Menges et al., 2002).

The major difference between a semi-mature tolerogenic DC, and a mature immunogenic DC is their ability to produce cytokines. Fully mature DC can produce considerable amounts of IL-12p40, IL-12p70, TNFα, IL-1β, and IL-6 (Menges et al., 2002). Full maturation of a DC occurs upon microbial recognition and CD40 ligation, provided by CD154 on activated T cells. Thus immunity is induced by DC which have received “danger signals”. An overview of the role of immature, semi-mature and mature DC is shown in Figure 1.3.

1.2.4. Autoimmunity

Failure in the induction or maintenance of central or peripheral tolerance leads to the generation of immune responses against self-Ag. Diseases caused by these aberrant immune responses to self-Ag are referred to as autoimmune diseases. There are many examples of diseases resulting from the loss of immunological tolerance to self; of chief concern here is the disease multiple sclerosis.

**Multiple Sclerosis (MS) and Experimental Autoimmune Encephalomyelitis (EAE)**

MS is the most common disabling neurological condition in young adults (Compston and Coles, 2002). Early stages of MS are driven by an autoimmune reaction against components of the CNS and ultimately results in demyelination and axonal loss. It is defined as an autoimmune disease on the basis that susceptibility alleles associated with MS include immune receptors or accessory molecules, cytokines and their receptors and chemokines (Compston and Coles, 2002; Hafler et al., 2007). In fact, HLA class II genes confer the greatest genetic risk factor for MS in European populations, specifically two HLA-DRB* genes of the HLA-DR15 haplotype (Olsson et al., 2006). The critical epitopes targeted in MS remain undefined, but
there are various candidates against which the autoimmunity could develop; most notably in myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG).

Current therapies for MS aim to halt the pathogenesis of MS by suppressing the immune system non-specifically (Kappos et al., 2004). This may alleviate some disease symptoms, but it leaves the patient at increased risk of infection and cancer. No licensed therapy addresses the underlying cause of MS, namely the inappropriate activation of immune-responses to self.

Animal models of CD4+ T cell driven CNS autoimmune disease can be elicited upon immunisation with myelin Ags including MBP, PLP and MOG (Anderton and Wraith, 1998; Zamvil et al., 1986). EAE is the prototypic animal model used to investigate the activation and control of auto-reactive T cells and the breakdown of immune tolerance.

1.3. MOLECULAR MECHANISMS OF SURFACE DIALOGUE BETWEEN DC AND T CELLS.

As previously introduced, costimulatory signals are required for the full activation of CD4+ T cells and a considerable number of costimulatory pairs have been identified. The communication between DC and T cells determines tolerance versus immunity, and it is thought that costimulatory molecules play a major role in this. The evolution of the dialogue between DC and T cells is not fully defined. With many receptor-ligand pairs existing, it appears that redundancy exists between some costimulatory molecules. However, costimulatory molecules do show temporally distinct expression patterns, most probably reflecting the requirements for different signals at different times during the DC-T cell dialogue (these will be discussed in further detail below). Essentially each receptor-ligand pair forms a check-point during the induction of a T cell response.

Costimulatory receptor-ligand pairs can be divided into two families; the Immunoglobulin (Ig) super family and the TNF/TNFR family. This section will focus on a number of molecules from each family (Figure 1.1), with particular emphasis on PD-1 and RANKL.
1.3.1. Ig Super family

The best characterised member of this family is CD28, a molecule constitutively expressed on naïve T cells (Linsley and Ledbetter, 1993). CD28 binds to either CD80 or CD86, which were primarily thought of as being on DC (Greenwald et al., 2005), but which can also be expressed alongside CD28 on T cells (Azuma et al., 1993; Hakamada-Taguchi et al., 1998). Early in vitro experiments showed that agonistic anti-CD28 could stimulate T cell proliferation when antigen-specific T cells were cultured with costimulatory deficient APC (Jenkins et al., 1991). More recently it was shown that CD28−/− T cells fail to proliferate in vivo and in vitro (Howland et al., 2000). Furthermore, stimulation of T cells with anti-CD28 has been demonstrated to increase the production of IL-2, IFNγ and TNFα (Thompson et al., 1989). CD28 ligation has been shown to induce stabilisation of cytokine mRNA (Lindstein et al., 1989).

CTLA-4 is closely related to CD28 and is expressed on activated T cells. It also binds CD80 and CD86, but does so with a higher affinity (Collins et al., 2002). CTLA-4 functions to inhibit T cell activation as blockade of CTLA-4 on the T cell surface leads to enhanced T cell responses (Kearney et al., 1995). This inhibitory role of CTLA-4 is highlighted in the phenotype of CTLA-4 knockout mice which develop fatal lymphoproliferation (Tivol et al., 1995). The mechanism by which CTLA-4 ligation affects T cell activation is complex, with several pathways being involved (Scalapino and Daikh, 2008). As CTLA-4 has a higher affinity for CD80 and CD86, it can out-compete CD28 for binding. CTLA-4 can also mediate intracellular effects, recruiting SHP, a phosphotase, to the TCR machinery, and inhibiting Akt activation (Parry et al., 2005). In addition, CTLA-4 can also reverse-signal to an APC through CD80/CD86 to induce the production of IDO (Grohmann et al., 2002).

1.3.2. PD-1; an introduction

Programmed cell death 1 (PD-1) is encoded by the gene Pdcd1 and is also a CD28 homolog. PD-1 and its ligands form an important negative costimulatory pathway. The expression of this costimulatory molecule was identified as being enhanced in cells following apoptotic stimulation (Ishida et al., 1992). PD-1 has a single
extracellular IgV-like domain and a long cytoplasmic tail consisting of an immunoreceptor tyrosine-based switch motif (ITSM) and an immunoreceptor tyrosine-based inhibitory motif (ITIM). PD-1 shares 23% amino acid sequence homology with CTLA-4, and the ligands for PD-1 were identified by database searches based on their similarity with the CD80/CD86 (Freeman et al., 2000; Latchman et al., 2001). PD-L1 and PD-L2 are 38% identical at the amino acid level. The structural organisation of PD-L1 and 2 are similar to the other B7 molecules (CD80/86); they have IgV- and IgC-like domains, a trans-membrane domain and a short cytoplasmic tail (Latchman et al., 2001).

1.3.2.1. Expression of PD-1 and its Ligands

In mice, PD-1 is expressed upon activation on both CD4+ and CD8+ T cells, B cells, NKT cells and monocytes (Agata et al., 1996; Latchman et al., 2001). PD-1 is expressed on T cells within 24 hours of activation. Importantly it has been shown to be rapidly expressed on CD4+ T cells following both tolerogenic and immunogenic stimuli (Hochweller and Anderton, 2005). The two PD-1 ligands differ in their expression patterns, with PD-L1 being more widely expressed. PD-L1 is constitutively expressed on T cells, B cells, DC, macrophages and mast cells (Yamazaki et al., 2002). Interestingly, especially when considering peripheral tolerance mechanisms, PD-L1 is expressed on an array of non-haematopoietic cell types, and is found at sites of immune privilege including the brain, placenta and eye (Hori et al., 2006; Keir et al., 2008; Liang et al., 2003; Magnus et al., 2005; Rodig et al., 2003). PD-L2 expression is more restricted, it is expressed upon stimulation of DC, macrophages and bone-marrow derived mast cells (Latchman et al., 2001). The expression pattern of PD-1:PD-L is very different to that of other Ig super-family members, as the ligands for CTLA-4 and CD28 are lymphoid specific (Greenwald et al., 2005). As such, PD-1:PD-L interactions are thought to regulate T cell responses in both lymphoid and non-lymphoid compartments.
1.3.2.2. Role of PD-1 as a co-inhibitory molecule

Many lines of evidence have demonstrated that the interaction of PD-1 with PD-L leads to the inhibition of T cell activation. Initial *in vitro* studies showed that PD-1:PD-L1 interactions inhibited CD3-mediated T cell proliferation (Freeman et al., 2000). Incubation of both human and mouse CD4+ T cells with anti-CD3 and hPD-L1.Ig coated beads reduced proliferation and cytokine production compared to cells incubated with anti-CD3 control beads. Freeman et al also highlighted that PD-1 mediated signals are most effective at low levels of T cell stimulation. By titrating the concentration of anti-CD3 and anti-CD28 in cultures, it was demonstrated that the outcome of PD-1 ligation is dependent on the strength of TCR and CD28 mediated signals. Similar to PD-L1, ligation of PD-1 by PD-L2, along with TCR ligation, reduced proliferation and cytokine production by T cells (Latchman et al., 2001). PD-L2 mediated inhibition was again more pronounced at lower levels of TCR stimulation. Interestingly, the investigators showed that PD-L1 was still able to reduce T cell responsiveness at a level of TCR stimulation at which PD-L2 was no longer able to do so, suggesting PD-L1 was more effective at inhibition.

The PD-1:PD-L interaction is thought to function by inhibiting IL-2 production thereby reducing the number of cells that successfully enter into cell cycle (Carter et al., 2002).

Treatment of DC with IL-10 has been shown to reduce the stimulatory capacity of the DC and induce anergy in the responding T cell population (Jonuleit et al., 2001). Selenko-Gebauer et al reported that addition of anti-PD-L1 to cultures containing IL-10 treated DC restored the stimulatory capacity of these DC. If T cells initially stimulated by IL-10 treated DC are removed and restimulated with normal DC the T cells do not proliferate. This anergic T cell state was maintained by PD-L1 signalling, as addition of anti-PD-L1 to the re-stimulation cultures overcame the block in proliferation (Selenko-Gebauer et al., 2003).

Recently Butte et al demonstrated that PD-L1 interacts with CD80, and mediates a negative signal to the T cell (Butte et al., 2007). The authors found that PD-L1 and CD80 interact with an affinity intermediate to CD80:CTLA-4 and CD80:CD28. Using beads coated with anti-CD3 and CD80.Ig or PD-L1.Ig to stimulate either CD28-/-, CTLA-4-/- or PD-1-/- T cells they showed that the PD-L1:CD80 interaction
mediates a negative signal, reducing T cell proliferation and cytokine production (Figure 1.4).

1.3.2.3. Signalling through PD-1:PD-L1/2

Ligation of PD-1 by its ligands causes inhibition of TCR signalling. The negative signal is mediated by the cytoplasmic domain of PD-1, specifically the ITIM and ITSM. Phosphorylation of these motifs leads to the recruitment of the SHP phosphatase which directly dephosphorylates TCR signalling intermediates resulting in blockade of the ZAP-70:CD3ζ interaction (Sheppard et al., 2004) and inhibition of phosphatidylinositol-3-OH kinase (PI-3K) (Parry et al., 2005). This inhibits the early activation events that are positively regulated by CD28 and/or IL-2 (Carter et al., 2002). Both CD28 ligation and IL-2 overcome PD-1 mediated signals, fitting with data demonstrating that PD-1 inhibition is most effective at low levels of TCR stimulation. Although phosphorylation of the ITSM of PD-1 has been shown to be sufficient to mediate inhibition, this only occurs when PD-1 and the TCR are in cis (Bennett et al., 2003). When the two receptors are in trans no inhibition occurs, indicating that PD-1 must get recruited to the synapse and be close to the site of antigen receptor ligation.

Reverse signalling through PD-L1 and PD-L2 has also been demonstrated. Treatment of bone marrow-derived DC with soluble PD-1 (s-PD-1) inhibits DC activation and increases IL-10 production (Kuipers et al., 2006). Unlike the effect of CTLA-4 ligation, this effect is not mediated by IDO.

1.3.2.4. PD-1:PD-L in tolerance and autoimmunity

Many studies have shown PD-1:PD-L interactions are vitally important in peripheral tolerance. PD-1 knockout (PD-1−/−) mice neatly demonstrate this as they have an autoimmune prone phenotype (Nishimura et al., 1999; Nishimura et al., 2001). PD-1 deficiency is thought to function by exacerbating an underlying autoimmune susceptibility; such that PD-1−/− mice on the non-obese diabetic (NOD) background develop diabetes with 100% penetrance and without sex bias (Wang et al., 2005; Yoshida et al., 2008). PD-1 was shown to be expressed on double positive (DP) thymocytes and play a role in selection of the TCR repertoire, by contributing to both
positive (Keir et al., 2005) and negative (Blank et al., 2003) selection. These data demonstrate a role for PD-1 in central tolerance.

1.3.2.4.1. PD-1 in the induction of peripheral tolerance

PD-1 is rapidly up-regulated on T cell activation, and as such could play an important role in determining the outcome of the interaction between naïve T cell and APC. Despite this, the role of PD-1 signals in the induction of tolerance in CD4+ and CD8+ T cells has only been explored in a handful of studies.

Induction of tolerance in CD8+ T cells results following Ag encounter on resting “tolerogenic” DC. Loss of PD-1 from the responding CD8+ T cell turns this tolerogenic encounter into a priming one, resulting in activation of Ag-specific CD8+ T cells (Probst et al., 2005). PD-1:PD-L signals have been shown to play a vital role in the induction of peptide-induced T cell tolerance in CD8+ OT-I cells, bearing a transgenic TCR specific for a peptide of ovalbumin (OVA) (Tsushima et al., 2007). The PD-1:PD-L interaction is also vital for the induction of tolerance in OT-I cells transferred to mice in which OVA is constitutively expressed under the control of the rat insulin promoter (RIP-OVA) (Keir et al., 2007; Martin-Orozco et al., 2006).

Studies in the NOD mouse model of diabetes have highlighted a role for PD-1 signals in CD4+ T cell tolerance (Fife et al., 2006). Rapid onset diabetes was induced in NOD mice by adoptive transfer of activated CD4+ BDC2.5 TCR transgenic T cells which recognise an islet specific antigen. Disease can be prevented in these mice by transfer of peptide-loaded, chemically fixed splenocytes one day after T cell transfer. Administration of anti-PD-1 or anti-PD-L1 prevented the induction of tolerance in the CD4+ cells and disease resulted. Interestingly, PD-L1 expression was not needed on the APC population for tolerance to be induced, as PD-L1-/- peptide-loaded fixed splenocytes were equally as effective as wild-type splenocytes at tolerance induction.

Finally, PD-L2 has been shown to be critically important for the induction of oral tolerance (Zhang et al., 2006), as it has been reported that oral tolerance cannot be induced in a PD-L2 knockout mouse.
1.3.2.4.2. PD-1 in the maintenance of peripheral tolerance

PD-1 signals have been shown to influence T cell effector function and proliferation upon Ag re-encounter in lymphoid organs and peripheral tissues. Many studies have shown that blockade of PD-1:PD-L interactions enhances the onset and/or severity of a number of immune-driven conditions.

**NOD diabetes model**

In the NOD mouse, loss/blockade of PD-1 or PD-L1 leads to enhanced diabetes onset and severity due to enhanced T cell expansion and cytokine production (Ansari et al., 2003; Guleria et al., 2007; Keir et al., 2006; Wang et al., 2005). With PD-L1 known to be expressed on pancreatic islet cells (Liang et al., 2003), Keir et al went on to demonstrate a vital role for PD-L1 expression on non-lymphoid cells in the protection from diabetes. Excision of pancreatic lymph nodes from NOD mice of various ages, showed that PD-L1 mediated signals are important at different locations during disease development. During the pre-clinical phases of disease, PD-L1 plays an important role in regulating the activation of auto-reactive T cells in the pancreatic lymph node. At later time points; following T cell infiltration of the islets, PD-L1 has been shown to be more important within the tissue (Guleria et al., 2007). Therapeutic anti-CD3 treatment of young pre-diabetic NOD mice can prevent diabetes induction and reverse disease in already diabetic NOD mice (Chatenoud et al., 1994). Fife et al demonstrated that PD-L1 plays a vital role in the maintenance of this tolerance. Administration of anti-PD-L1 to anti-CD3 tolerised pre-diabetic and diabetic mice resulted in diabetes within 3 weeks and 2 days respectively (Fife et al., 2006).

**Experimental Autoimmune Encephalomyelitis (EAE)**

During EAE PD-L1 is expressed on CNS resident astrocytes, microglia and vascular endothelial cells (Liang et al., 2003; Magnus et al., 2005). Blockade of PD-1 during MOG-induced EAE enhanced both onset and severity of disease mediated by an increased frequency of activated, Ag-specific, cytokine producing T cells. Blockade/loss of either PD-L1 or PD-L2 in MOG-induced EAE, has been seen to enhance disease onset/severity in different strains of mice (Carter et al., 2007; Ortler
et al., 2008; Salama et al., 2003). Importantly, loss of the PD-1:PD-L interaction results in the induction of robust EAE in mouse strains considered to be EAE-resistant (Latchman et al., 2004; Zhu et al., 2006). The important role of PD-1:PD-L in limiting EAE was further highlighted by Hirata et al., who showed that transfer of embryonic stem cell-derived DC expressing the 35-55 peptide of MOG (pMOG) bound to MHCII and which co-expressed PD-L1, protected from pMOG induced EAE (Hirata et al., 2005). These engineered DC prevented both disease induction and reduced the severity of ongoing disease.

**Fetomaternal tolerance**

PD-1 ligands are expressed in the placenta, which prompted a number of studies to explore the role of PD-1:PD-L in fetomaternal tolerance. PD-L1 expression was shown to be restricted to the decidua basalis (Guleria et al., 2005). This is the maternal component of the placenta which is adjacent to the foetal trophoblast. Guleria et al demonstrated that blockade/genetic ablation of PD-L1, but not PD-L2, reduced foetal survival rates in allogenic matings. Consistent with other studies discussed above, blockade of PD-1:PD-L1 led to enhanced expansion of alloreactive T cells and increased rates of abortion. The effect of anti-PD-L1 on fetomaternal tolerance has been reported to be mediated by Tregs (Habicht et al., 2007). Depletion of Tregs via anti-CD25 treatment, abrogated the effect of anti-PD-L1 treatment on foetal survival.

**1.3.2.5. Role of PD-1 on Tregs**

The roles of PD-1 and PD-L1 on Tregs remains controversial. Early studies suggested expression of PD-1 was a means to differentiate activated T cells from Tregs, as effector T cells were CD4+CD25+PD-1+, whereas natural Tregs were CD4+CD25+PD-1- (Raimondi et al., 2006). However, PD-1 is up-regulated on Tregs upon their activation. It has been reported that PD-L1 is expressed on Tregs (CD4+CD25+ cells) to a slightly higher degree than on CD4+CD25- T cells (Sandner et al., 2005). When considering these data it is important to bear in mind that PD-L1 is up-regulated on T cells upon their activation. Whether expression of
PD-1 and PD-L1 is different on Tregs and effector T cells remains to be resolved, but both molecules are expressed on Tregs.

Some studies have implicated the PD-1:PD-L interaction in the mechanism of Treg action. Administration of anti-PD-L1 has been shown to lead to enhanced graft rejection and loss of fetomaternal tolerance (Habicht et al., 2007; Sandner et al., 2005). In these two studies depletion of Tregs abrogated the effect of anti-PD-L1, suggesting that Tregs are responsible for the anti-PD-L1 mediated effect. Administration of anti-PD-L1 to a suppression assay was shown to have no effect on the suppressive capacity of the Tregs (Baecher-Allan et al., 2001). Moreover administration of this antibody to NOD mice had no effect on the frequency of Tregs in the draining lymph node or spleen compared to control treated mice (Guleria et al., 2007).

Data differ in reports on whether loss of PD-1 from Tregs alters their ability to suppress. Polanczyk et al reported that PD-1−/− Tregs cannot suppress responders in a suppression assay (Polanczyk et al., 2007). In contrast, Wang et al show that PD-1−/− Tregs can suppress in a suppression assay (Wang et al., 2007). The reason for the different results is not clear, both studies used cells from the PD-1−/− on the C57BL6 background. The most notable difference between the studies was the concentration of anti-CD3 used to stimulate the cultures; the Polanczyk study used 0.5μg/ml, whereas the Wang study used double that amount.

PD-L1 has been reported to be involved in the generation of Tregs in vitro (Krupnick et al., 2005). Incubation of CD4+ T cells with activated vascular endothelium induced the generation of suppressive CD4+CD25+foxp3+ Tregs. Addition of anti-PD-L1 to these cultures prevented the induction Tregs suggesting PD-L1 on endothelium might play a role in the generation of Tregs in the periphery.

1.3.2.6. PD-1 and chronic infection

As PD-1:PD-L interactions help strike the balance between activating a T cell and switching it off, they may play a role in preventing immune-mediated damage of tissues. PD-L1−/− mice can clear an acute lymphocytic choriomeningitis virus (LCMV) as well as wild-type mice. However, when PD-L1−/− mice are infected with
A chronic LCMV clone they die due to massive immunopathological damage (Barber et al., 2006).

A number of chronic infections exploit the PD-1:PD-L pathway in order to avoid immune responses and facilitate chronic infections (Keir et al., 2008). During chronic viral infections the effector functions of virus-specific CD8+ T cells are impaired. Barber et al demonstrated that PD-1 is highly expressed by exhausted CD8+ T cells during chronic LCMV infection, and that virus-specific CD8+ T cell responses were enhanced upon anti-PD-L1 treatment (Barber et al., 2006). Similar studies have demonstrated increased expression of PD-1 during chronic human immunodeficiency virus (HIV), Hepatitis B virus (HBV) and HCV infection.

The data discussed above highlight the role of PD-1:PD-L interactions in tissues. As stated in the introduction, PD-L1 specifically is expressed on a variety of non-haematopoietic cell types. Manipulation of the PD-1:PD-L1 and, to a lesser extent, PD-1:PD-L2 interaction has been shown to enhance a plethora of immune-mediated conditions. Such data demonstrate that PD-1 signals are important in both the initial interaction of a T cell with APC, and subsequent T cell effector function within the target organ.

1.3.3. TNF / TNFR family

A key costimulatory pair within this family is CD40:CD154. CD40 is expressed on all types of APC, whereas CD154 expression is predominantly found on activated CD4+ T cells (Mackey et al., 1998). The CD40:CD154 interaction signals to the APC, licensing the APC to prime robust T cell responses (Mackey et al., 1998). Such data led to CD40 being termed the “master regulator” of costimulation. This signalling is critical for the priming of Th1 cells by stimulation of IL-12 production by CD40 bearing APC (Stuber et al., 1996). The CD40:CD154 interaction is vital for the production of a sustained T cell response, as CD40−/− DC fail to prime T cells (Hochweller et al., 2006a; Miga et al., 2001). CD154−/− T cells on the other hand can be activated but fail to sustain a response in vivo (Howland et al., 2000). In an EAE setting, lack of either molecule conferred a resistance to EAE (Hochweller et al., 2006a). Evidence now exists demonstrating CD154 signalling to the T cell occurs
following ligation by CD40 (van Essen et al., 1995). Importantly for the studies presented in this thesis, peptide-tolerance can be induced in CD154^{+} T cells (Hochweller et al., 2006a).

One consequence of CD40 ligation is the up-regulation of OX40L on DC (Fillatreau and Gray, 2003). OX40L:OX40 is thought to function downstream of CD40:CD154. OX40 is transiently expressed on activated T cells, but is up-regulated faster on the reactivation of effector T cells (Gramaglia et al., 1998). OX40L is expressed on DC upon activation and remains expressed for a lengthy period of time (Ohshima et al., 1997). The expression of OX40 and OX40L on T cells and DC has been explored in vivo following administration of tolerogenic and immunogenic stimuli. Administration of tolerogenic stimulus (soluble peptide alone) leads to OX40 expression which peaks at 24 hours, and is gone by 48 hours. Immunogenic stimulus (peptide plus anti-CD40) caused OX40 to be expressed for longer and to higher levels (Hochweller and Anderton, 2005). OX40L expression on DC was only seen 48 hours following immunogenic stimulus.

OX40 is required for T cell function (Chen et al., 1999; Ndhlovu et al., 2001). The OX40L:OX40 interaction functions to provide survival signals to newly activated T cells, through increased expression of the anti-apoptotic molecules Bcl-2 and Bcl-xL (Rogers et al., 2001). The OX40L:OX40 interaction is thought to be involved in the initiation of long-lived T cell responses, such that APC expressing OX40L can promote partial activation of naive T cells, however these APC can promote secretion of several cytokines from previously generated effector T cells (Gramaglia et al., 1998). These data are further supported by the demonstration that OX40L:OX40 interactions between T cells also contribute to CD4^{+} T cell longevity (Soroosh et al., 2006). Consistent with this, mice in which OX40L is constitutively expressed on T cells have an elevated number of memory T cells (Murata et al., 2002).

Previous work from this laboratory has demonstrated that exogenous ligation of CD40 can prevent peptide tolerance induction (Hochweller et al., 2006a). Stimulation of CD40 could be preventing tolerance by inducing OX40L, and thus Bcl-2 and Bcl-xL. As such, perhaps a defect in OX40L signalling might lead to
tolerance. This is supported by data showing that exogenous ligation of OX40 can prevent peptide tolerance (Hochweller et al., 2006a) and prevent anergy induction (Lathrop et al., 2004). Ligation of OX40 has also been shown to break an existing state of T cell tolerance (Bansal-Pakala et al., 2001).

1.3.4. RANKL; an introduction

Receptor activator of NFκB (RANK) interacts with RANK-Ligand (RANKL), also known as TNF-related activation induced cytokine (TRANCE), osteoprotegerin ligand (OPG-L) and osteoclast differentiation factor (ODF). Compared to other TNF/TNFR family members, little is known about the role RANK:RANKL in the immune system. The RANK:RANKL interaction is important in bone reabsorption, (Kong et al., 1999b), as RANKL binding to RANK expressed on haematopoietic progenitors induces osteoclastogenesis in the RANK bearing cell (Jones et al., 2002). RANKL is expressed on activated T cells, and as such T cells can directly control osteoclastogenesis (Kong et al., 1999a).

A second receptor for RANKL is osteoprotegerin (OPG) (Simonet et al., 1997), which is a secreted protein (Theill et al., 2002). OPG functions as a soluble decoy receptor, inhibiting the RANK:RANKL interaction. Consequently it was shown that the OPG knockout mouse suffered extreme osteoporosis (Bucay et al., 1998). As such the balance between RANK:RANKL signals and the level of soluble OPG finely control the activation of osteoclasts and bone reabsorption. In addition, OPG has also been shown to be expressed in DC (Yun et al., 1998). OPG expression on DC is regulated by CD40 stimulation, suggesting that it could also play a role in DC and T cell interactions.

Due to the importance of RANKL in bone metabolism, blockade of RANKL has been suggested as a therapy for bone loss conditions (Nakashima and Takayanagi, 2008). In models of collagen induced arthritis, administration of a blocking anti-RANKL antibody reduces bone loss during disease (Kamijo et al., 2006). A therapeutic anti-RANKL antibody has been developed for the treatment of osteoporosis in post menopausal women (Lewiecki et al., 2007; McClung et al., 2006). With the development of such therapies it is important to ascertain the role of RANK:RANKL interactions in T cell responsiveness and tolerance.
1.3.4.1. RANK:RANKL in the immune system

RANKL knockout mice suffer severe osteopetrosis and defective tooth eruption (Kong et al., 1999b). In terms of their immune system; these mice completely lack lymph nodes and have somewhat impaired B cell and thymocyte development. Similarly, the RANK knockout mouse lacks peripheral lymph nodes, and has defective B cell development (Dougall et al., 1999). Both strains of mice have normal splenic architecture and Peyer’s patches, suggesting RANK:RANKL plays an important role specifically in lymph-node organogenesis.

RANK and RANKL on T cells

Induction of RANKL expression on T cells is dependent on TCR ligation. RANKL induction is mediated by ERK1/2, PKC and calcium signalling pathways, and can be enhanced by addition of TGFβ (Wang et al., 2002) or by CD28 costimulation (Josien et al., 1999). RANKL can be expressed not only in a membrane bound form but also in a soluble form. Interestingly, RANKL has been shown to be expressed to a higher degree on Th1 cells compared to Th2 cells, as IL-4 has been reported to decrease RANKL expression (Josien et al., 1999; Wong et al., 1997a). Following prolonged stimulation of a T cell RANK expression is seen. Treatment of T cells with soluble forms of RANKL has been reported to have different effects. Josien et al showed treatment had no effect on the proliferation, activation or death of T cells (Josien et al., 1999), whereas Wong et al reported that a recombinant soluble form of RANKL could induce c-Jun N-terminal kinase (JNK) activation in T cells (Wong et al., 1997b). Reverse signalling through RANKL to the T cell has also been noted by others; RANKL mediates a p38-dependent reverse signal to Th1 cells stimulated by anti-TCRβ and RANK.Fc (Chen et al., 2001). This resulted in enhanced IFNγ production.

RANKL stimulation of DC

Most work concerning RANK:RANKL has focused on signals generated within the DC. RANK is up-regulated on DC upon ligation of CD40 (Anderson et al., 1997). A number of studies have shown that addition of soluble RANKL (sRANKL) to cultured DC increases the stimulatory capacity, however this was not achieved
through the up-regulation of any known costimulatory molecules (Anderson et al., 1997). Moreover, DC treatment with sRANKL increased their cytokine production, with increased levels of IL-1, IL-6, IL-12, IL-15 and IL-10 being seen depending on the source of the DC (Josien et al., 1999; Williamson et al., 2002). Ligation of RANK on DC has been shown to increase their survival (Cremer et al., 2002; Yu et al., 2003). This was shown to be due to up-regulation of Bcl-xL (Wong et al., 1997a) and protection of DC from FasL-mediated apoptosis (Chen et al., 2004a). Consequently, it was demonstrated that treatment of DC with sRANKL in vitro increases the number and persistence of these DC following their in vivo transfer (Josien et al., 2000). It has been suggested that RANKL functions as a positive feedback regulator of T cell responses, such that when a DC is activated and migrates to the lymph node, it dies if it fails to encounter an activated, and therefore RANKL bearing, T cell. Mature DC which have migrated to the lymph node have been shown to have short life-spans, following interaction with the T cell these DC are eliminated, avoiding excess immune activation (Ingulli et al., 1997). Thus, controlling the life-span of an activated DC could play an important role in the induction of immunity and tolerance.

Signals downstream of RANK within the DC have been investigated. Upon ligation of RANKL, RANK forms a complex with TRAF6 and c-Src, which then activates PI-3K and Akt (Wong et al., 1999). Cbl-b has also been shown to be a component of this signalling complex, and is required for the RANKL-induced activation of anti-apoptotic Akt and PI-3K (Arron et al., 2001).

1.3.4.2. RANKL in T cell responses

The outcome of the RANK:RANKL interaction on the T cell has yet to be fully elucidated, however some redundancy between CD40:CD154 and RANK:RANKL during the induction of CD4+ T cell responses has been shown. RANKL is expressed on activated T cells from CD154-/- mice and from patients with X-linked hyper IgM syndrome that have a CD154 deficiency (Lopez-Granados et al., 2007). Blockade of RANKL did not prevent priming of LCMV-specific T cells, but it did prevent the proliferation of these cells at later time points in infection (Bachmann et al., 1999). During late infection, RANK:RANKL interactions could yield a
productive anti-viral T cell response when CD40:CD154 interactions were missing. Such data suggest that RANK:RANKL interactions might be most important later in T cell responses.

1.3.4.2.1. RANKL and the mucosal immune system
A number of studies have highlighted a role for RANK:RANKL in the gut. The IL-2\(^{-}/-\) mouse develops systemic autoimmunity; studies utilising this mouse have demonstrated that bone loss and colitis were caused by increased levels of RANKL which enhanced bone turnover and increased intestinal DC survival (Ashcroft et al., 2003). Treatment of IL-2\(^{-}/-\) mice with recombinant Fc-OPG (to inhibit the RANK:RANKL interaction) increased bone densities, and mildly reduced inflammation in the bowel, by reducing the number of activated DC and macrophages in the colon. In humans, bone loss is a common consequence of IBD, and it has been hypothesised that inflammation, via the RANK:RANKL pathway, could directly cause bone loss (Moschen et al., 2005).

1.3.4.2.2. RANKL and arthritis
Considering the vital role played by RANK:RANKL in bone metabolism and its emerging role in the immune system, many studies have focused on RANK:RANKL in models of arthritis. With the demonstration that T cells could directly induce osteoclastogenesis (Horwood et al., 1999; Kong et al., 1999a), the importance of RANK:RANKL in osteo-immunology requires examination. In a T cell-dependent model of rat adjuvant-induced arthritis, blockade of RANKL by treatment with OPG at disease onset was shown to prevent bone and cartilage destruction but had no effect on inflammation (Kong et al., 1999a). A similar study in mice, using anti-RANKL, yielded comparable results (Kamijo et al., 2006). Pettit et al demonstrated that arthritis could be induced in RANKL\(^{-/-}\) mice by serum transfer (Pettit et al., 2001). In these mice, the level of inflammation was the same between knockout and wild-type mice but bone loss was reduced in the knockout. These studies suggest blockade of RANKL prevents bone loss but has no effect on inflammation. In contrast to these studies, blockade of RANKL has been shown to reduce both osteolysis and arthritis inflammation score in a model of arthritis induced by
antibodies against collagen administered with LPS (Seshasayee et al., 2004). Collectively, these data suggest that RANKL is the principal mediator of bone loss during arthritis.

Conversely, a number of studies have highlighted an inhibitory role of IFN\(\gamma\) on RANKL mediated osteoclastogenesis (Takayanagi et al., 2000). IFN\(\gamma\) caused destruction of TRAF6 and hence inhibited RANKL signalling. Thus, it has been suggested that IFN\(\gamma\) protects against bone destruction which would occur upon T cell activation. But how can inflammation enhance bone destruction when IFN\(\gamma\) potently inhibits osteoclastogenesis? This was recently solved when the role of Th17 cells on osteoclastogenesis was explored. Sato et al demonstrated that in an in vitro co-culture model of osteoclastogenesis, both Th1 and Th2 cells inhibit whereas Th17 cells enhanced osteoclastogenesis (Sato et al., 2006). This was due to IL-23 and IL-17, as addition of these cytokines to the cultures also enhanced osteoclast formation (Ju et al., 2008; Kotake et al., 1999). Ju et al also demonstrated that IL-23 increased RANKL mRNA in CD4+ T cells. Moreover administration of anti-IL-23 to rats with collagen-induced arthritis reduced both synovial tissue inflammation and bone destruction (Yago et al., 2007).

1.3.4.2.3. RANKL and inflammation

As drugs which block RANK:RANKL are being developed for bone disorders, the role of RANK:RANKL in inflammatory responses other than arthritogenic responses, needs to be determined. This has only been explored in a single study, Miller et al investigated the effect of RANK.Fc on anti-viral responses (Miller et al., 2007a). At doses which suppressed bone turnover, RANK.Fc had no detectable effect on the immune response to influenza infection.

1.3.4.2.4. RANKL and tolerance

A number of studies have highlighted an important role for RANK:RANKL in the generation of Tregs. In an accelerated model of diabetes (in mice termed tet-TNF\(\alpha\) mice), disease can be prevented by transfer of potent pancreatic lymph node derived Tregs. The ability of these Tregs to reduce disease was dependent on RANKL
mediated signals (Green et al., 2002). Blockade of RANK:RANKL reduced the frequency of CD4+CD25+ Tregs cells in the pancreas, and therefore permitted the rapid onset of diabetes. Similarly, transgenic mice which over-express RANKL on keratinocytes, have increased numbers of Tregs (Loser et al., 2006). Over-expression of RANKL in the skin was shown to alter epidermal DC function, in such a way that expansion of Tregs was supported. Recently it has emerged that RANKL could potentially play a role in the induction of T cell tolerance. During the induction of peptide-induced T cell tolerance in OT-II cells, Hochweller et al demonstrated that RANKL was expressed on T cells during tolerance induction (Hochweller and Anderton, 2005). RANKL was shown to be expressed more rapidly and to higher levels following tolerogenic (soluble peptide alone) compared to immunogenic (peptide plus LPS) stimulus. Expression on OT-II cells was not detected until 48 hours after peptide plus LPS, yet it was detected at 12 hours after the tolerogenic treatment. These data raise the interesting possibility that RANKL could be delivering a negative signal to DC via an alternative receptor which maintains the DC in a tolerogenic form. In keeping with this idea, application of sRANKL has been shown to potentiate oral tolerance (Williamson et al., 2002). Mechanistically, Williamson et al suggested this was due to increased IL-10 production from Peyer’s patch DC treated with sRANKL.

1.4. Ag-SPECIFIC THERAPEUTIC IMMUNE TOLERANCE

Diseases such as type I diabetes mellitus, MS, rheumatoid arthritis (RA) and myasthenia gravis (MG) are thought to be mediated by the inappropriate activation of tissue-specific T cells. Current therapies for autoimmune conditions are predominantly immunosuppressive drugs which aim to simply switch off or divert the immune system, therefore affecting both disease causing and non-disease causing T cells. Although such therapies are partially effective at reducing disease burden, they have considerable side effects and alter patient immunity to infections. The “holy grail” of therapeutics would specifically inactivate self-reactive, disease-causing T cells, but have no effect on overall immune function. As previously discussed, the generation of a productive T cell response requires T cells to receive
two signals; engagement of the TCR with the appropriate p:MHC complex (signal 1), and provision of costimulation by an APC (signal 2). This could be exploited therapeutically as it has long been established that the form in which an Ag is administered determines the outcome of the resultant immune response (Weigle, 1973). Initial studies by David Dresser highlighted that administration of Ag in a soluble form can lead to immune tolerance (Dresser, 1961; Dresser, 1962; Dresser and Gowland, 1964). Consequently, administration of Ag in an aggregated form or with the “danger” signals provided by an adjuvant, leads to the mounting of a robust T cell response. Yet, administration of that same Ag in a soluble form, in the absence of “danger”, leads to the induction of T cell tolerance. The paradigm: immunity results when a T cells receive both signal 1 and 2, and tolerance results when a T cell receives only signal 1.

A number of Ag-specific therapies are currently being investigated which exploit this paradigm, of chief interest in this thesis is the administration of soluble Ag. However, other protocols for tolerance induction include the administered of Ag in IFA (Daniel and Wegmann, 1996), through osmotic mini-pumps (Apostolou and von Boehmer, 2004), coupled to DC-specific antibodies (Hawiger et al., 2001), or encoded in DNA vaccines (Miller et al., 2007b). Ag-specific tolerance can also be induced by the transfer of Ag-loaded DC or ethylene carbodiimide (ECDI)-fixed APC (Hochweller and Anderton, 2004; Miller et al., 2007b). This latter therapy highlights the importance of route of administration in tolerance induction, as administration of cells intra-venously (i.v.) induces tolerance whereas immunity results when cells are administered subcutaneously (s.c.) (Tan et al., 1992), indicating systemic dissemination is required for tolerance induction.

Administration of soluble proteins via a variety of routes has proved highly effective at inducing tolerance in vivo. Identification of T cell epitopes within proteins (both self-proteins and model proteins), has allowed individual T cell epitopes (in the form of synthetic peptides) to be administered to induce tolerance. The administration of soluble proteins and peptides to induce tolerance in a specific set of T cells has been exploited therapeutically; administration of Ag has been shown to successfully prevent animal models of autoimmune disease. As such, administration of soluble
proteins and peptides is clinically applicable and holds potential as a future therapy in human immuno-pathological conditions (see section 1.4.5.2).

1.4.1. **Induction of Ag-induced T cell tolerance is an active process**

Initially, the study of tolerance induction by administration of soluble Ag was performed in wild-type mice; in these mice, the fraction of Ag-reactive T cells was small and very difficult to follow during tolerance induction. The advent of TCR-transgenic mice, in which all T cells recognise the same p:MHC, has allowed T cells to be followed during tolerance induction. Adoptive transfer of transgenic T cells to syngeneic recipients (Kearney et al., 1994), has allowed the mechanisms behind the induction of tolerance by administration of soluble Ag to be elucidated. Just how the form of Ag administrated influences tolerance induction remains to be fully explained. It is perhaps an oversimplification to suggest that T cell tolerance simply results from a complete lack of signal 2. In fact, because T cell tolerance is considered an active process (Hochweller et al., 2006b), this paradigm simply cannot explain Ag-tolerance induction.

A number of studies have shown that T cells undergo an initial activation and expansion phase on the way to tolerance (Kearney et al., 1994; Liblau et al., 1996). Consistent with this, T cell tolerance takes three days to become established following administration of soluble Ag (Liu and Wraith, 1995). Taken together these data suggest that T cell tolerance does not result from a failure to activate a T cell, instead the T cell is activated, but receives qualitatively or quantitatively different signals to that of a fully activated T cell. This causes the induction of a separate developmental path which results in T cell tolerance three days later.

1.4.2. **Altered Peptide Ligands (APL)**

Not only can proteins and peptides be used to induce Ag-specific T cell tolerance, altered peptide ligands (APL) can also be used. The term APL describes analogs of immunogenic peptide in which either TCR or MHC contact residues have been altered; originally, APLs were specifically peptides with altered residues at TCR contacts (Evavold et al., 1993). APL can be classified as agonists, weak agonists, partial agonists or antagonists, classification depends upon the type of response they
induce in the responding T cell. Specifically partial agonists and antagonists have been examined for their tolerogenic potential. Partial agonists activate some TCR-mediated functions, but not others; for example they may stimulate cytokine production and B cell help, but not T cell proliferation. Antagonists on the other hand require simultaneous presentation with the natural peptide and prevent T cell responsiveness. This is not due to competition for the TCR and involves TCR signalling events (Evavold et al., 1993). Thus both partial agonists and antagonists provide a mechanism by which the T cell response can manipulated.

Initially use of such APL provided much excitement in terms of development of a therapy (Nicholson et al., 1995), however, there is a major flaw in the use of altered TCR-contact APL. This was highlighted by the demonstration that an APL defined as antagonistic through its effects on clonal encephalitogenic T cell populations in vitro could induce EAE upon immunisation in vivo (Anderton et al., 1998). This was later emphasised by the need to halt clinical trails of an APL in MS due to evidence of disease exacerbation (Bielekova et al., 2000; Kappos et al., 2000). In these trials, hypersensitivity reactions developed, and worsening of the disease was noted which correlated with expansion of Ag-specific Th1 cells.

APL which do not provide this problem are ones which have altered residues at MHC-contacts. These APL should affect all Ag-specific T cell clones equally as they do not alter TCR binding to the peptide. Such peptides have proved to have improved tolerogenic properties in both clonal and polyclonal T cell populations (Burkhart et al., 1999; Ford and Evavold, 2003; Liu and Wraith, 1995).

1.4.3. Oral Tolerance

Oral tolerance reflects the need to tolerate dietary Ag. Early experiments to provide therapeutic tolerance made use of this by initially providing Ag via oral gavage prior to later immunisation to induce an (auto)-immune response (Mowat et al., 2004). The mechanisms by which oral administration of Ag can induce tolerance can be broadly split in two. Low doses of protein Ag are thought to favour tolerance via the induction of Ag-specific regulatory T cells, whereas high doses are thought to favour anergy (Friedman and Weiner, 1994) or deletion (Chen et al., 1995) of Ag-reactive T cells.
Oral tolerance to hen egg lysozyme (HEL) was induced in mice following feeding with a low dose of protein (Friedman and Weiner, 1994). Following tolerance induction HEL-reactive T cells produced increased amounts of TGFβ and were capable of suppressing other cells. Generation of TGFβ producing Th3 cells following Ag-feeding has been noted by a number of investigators, most notably myelin-reactive regulatory cells can be generated following feeding with myelin proteins in both mice (Chen et al., 1994) and humans (Fukaura et al., 1996). The generation of Ag-reactive Th3 cells has been shown to be enhanced by IL-4 (Inobe et al., 1998). Oral tolerance has also been associated with the induction of regulatory cells which not only make TGFβ but also produce IL-10 (Chen et al., 1994; Zhang et al., 2001).

Induction of oral tolerance to disease-relevant Ag appears an ideal therapy. Yet, such a therapy requires administration of whole proteins, disease-relevant peptides cannot be administered as they would be broken down. Also large amounts of proteins need to be administered. Administration of peptide via nasal mucosa is an alternative to Ag-feeding (Faria and Weiner, 2006). Repeated administration of peptides intra-nasally (i.n.) has been shown to induce Tr1 cells (Burkhart et al., 1999). Functionally, induction of cells with a regulatory phenotype following peptide treatment i.n. was indicated some time ago when Anderton et al demonstrated that tolerisation with a single peptide could suppress responses to other peptides both within the same myelin-derived protein and in other myelin proteins (Anderton and Wraith, 1998).

1.4.4. Mechanisms of Ag-induced T cell tolerance

Soluble peptides and proteins (and APL) can be administered via a variety of routes, other than oral, to elicit Ag-specific T cell tolerance. The mechanisms leading to T cell tolerance following administration of soluble Ag have undergone considerable investigation, and a number of different mechanisms have been reported. The consensus is that soluble Ag-induced tolerance can induce deletion, unresponsiveness (anergy), deviation or induction of regulatory cells in Ag-reactive T cells. Timing, route of soluble Ag administration, dose of Ag used, type of APC targeted, and type of Ag used can all affect the outcome of tolerance induction.
Importantly the involvement of one mechanism does not exclude the possible involvement of a second mechanism.

1.4.4.1. Deletion
Ag-reactive T cells have been shown to be susceptible to clonal deletion when exposed to Ag in vivo (Zhang et al., 1992). High doses of Ag administered intravenously (i.v.) (Critchfield et al., 1994; Liblau et al., 1996; Weishaupt et al., 1997) has been shown to induce deletion of Ag-reactive T cells. Deletion is most probably due to Fas-mediated apoptosis, as deletion of thymocytes but not peripheral T cells occurs after high dose peptide treatment of a TCR-transgenic LPR mouse (Singer and Abbas, 1994).

Similarly, Ag targeted to resting DC by conjugation to antibodies specific for DC (anti-DEC205) has also been shown to induce deletion of Ag-reactive T cells (Hawiger et al., 2001).

1.4.4.2. Induction of anergy in Ag-reactive T cells
In some systems it has been shown that, although most Ag-reactive T cells are deleted post administration of soluble Ag, a small number of Ag-reactive T cells remain and are hyporesponsive, often referred to as anergic. Pape et al reported that following administration of the 323-339 peptide from ovalbumin (pOVA) i.v. to a host, into which DO11.10 cells had been transferred, most DO11.10 cells were deleted but a small number survived in a functionally non-responsive state (Pape et al., 1998). The hyporesponsive cells neither proliferate nor make IL-2 or TNFα and last for approximately 45 days post peptide treatment. Tolerance induction through similar mechanisms have been noted in a number of other studies (Kearney et al., 1994), and has been associated with altered T cell calcium metabolism (Dubois et al., 1998; Srinivasan and Frauwirth, 2007) and with decreased levels of TCR and co-receptor (Mamalaki et al., 1993; Redmond et al., 2005; Rocha and von Boehmer, 1991).

In a model in which CD8+ HA-specific T cells were transferred to a host and then treated with increasing doses of HA peptide i.v., Redmond et al noted that, following one dose of either 100μg, 10μg, 1μg or 0.1μg HA peptide, tolerance was induced and...
most HA-reactive T cells were deleted. However, following all treatments, a population of cells persisted. Following the initial peptide treatment, further multiple treatments with lower doses of HA peptide led to efficient clonal deletion. However, repeated high dose treatment allowed a small population of anergic HA-specific T cells to persist (Redmond et al., 2005). The authors suggested that repeated high doses of peptide protected HA-specific cells from death but prevented them from being activated, as they showed reduced levels of TCR and phospho-ERK. In some cases this state of unresponsiveness or anergy has been shown to be overcome by addition of exogenous IL-2 (Liblau et al., 1996).

### 1.4.4.3. Deviation

A deviation of the immune response from a Th1 to a Th2 response has been noted following Ag treatment (Degermann et al., 1996; Forsthuber et al., 1996; Prakken et al., 2004). Intrinsic properties of the peptide administered may explain why some peptides induce deviation whereas others induce anergy and/or deletion. For example, immune deviation has been noted following administration of APL that alter the affinities within the trimolecular MHC-Peptide-TCR complex (Gaur et al., 1997; Pearson et al., 1997).

### 1.4.4.4. Generation of Ag-reactive regulatory T cells

Generation of Tregs following peptide treatment is generally associated with low dose peptide administration via mucosal routes. However, a limited number of studies have noted generation of T cells with a regulatory phenotype following Ag treatment via routes other than mucosal. Thorstenson et al transferred DO11.10 cells to hosts and then induced tolerance by i.v. treatment with a low dose of pOVA (5µg). They noted that peptide treatment resulted in the generation a population of CD25+CD4+ “Treg” cells that accounted for ~20% of the DO11.10 cell population (Thorstenson and Khoruts, 2001). Foxp3 expression was not assessed, but these “Treg cells” maintained CD25 post activation and were able to suppress the proliferation and IL-2 production of naïve T cells. A more recent study administered whole OVA protein to DO11.10 mice (Perruche et al., 2008), and by day 10 post OVA treatment an increase in the percentage of DO11.10 cells which were foxp3+.
was seen. Perruche et al suggested that this increase was due to increased levels of TGFβ in OVA treated mice, produced by immature DC or macrophages as they consume apoptotic DO11.10 cells induced to die following Ag treatment.

Apostolou et al employed a novel way to explore Treg induction following peptide treatment (Apostolou and von Boehmer, 2004). In this study TCR-transgenic HA-specific mice were connected to osmotic pumps which released constant low levels of HA (0.001-10μg) into the blood stream. This persistent level of Ag caused increased levels of CTLA-4 and foxp3 in the HA-specific T cells. Importantly these peripherally generated Tregs could prevent diabetes in a HA-peptide induced model. However, it remains to be determined whether administration of soluble Ag induces the conversion or expansion of foxp3+ cells. Determining the precise mechanism by which Tregs can be induced by Ag would be invaluable.

1.4.5. Administration of soluble Ag as a therapy for autoimmune diseases

The data outlined above demonstrate that administration of soluble Ag induces Ag-specific T cell tolerance via a variety of different mechanisms. Importantly, they highlight that administration of soluble Ag can specifically tolerise only disease causing, Ag-reactive T cells, and could therefore be employed to resolve autoimmune conditions.

1.4.5.1. Ag-based therapy in animal models of disease

Pre-treatment of mice with peptide and protein has been shown to prevent the induction of EAE (Anderton and Wraith, 1998; Critchfield et al., 1994; Hilliard et al., 2000; Meyer et al., 2001) and collagen-induced arthritis (Brand et al., 2002; Staines et al., 1996). Perhaps more importantly, peptide treatment has been shown to be effective against already established disease. This has been demonstrated in both actively induced EAE (Gaur et al., 1997; Leech et al., 2007; Samson and Smilek, 1995) and passively induced EAE (Brocke et al., 1996). Similarly, peptide therapy has proved effective at ameliorating experimental autoimmune MG (EAMG) which had been induced both passively (Katz-Levy et al., 1997) and actively (Paas-Rozner et al., 2000). Peptide administration has also been shown to inhibit the induction of a
rapid onset form of diabetes where previously activated and pathogenic BDC2.5 transgenic T cells are transferred to NOD mice (Judkowski et al., 2004). Strikingly, peptide-therapy has been shown to prevent the development of disease in spontaneous models of autoimmunity. Treatment of young pre-diabetic NOD mice with a peptide of β-insulin (Daniel and Wegmann, 1996) or a series of peptides from GAD65 (Tian et al., 1996) have been shown to protect mice from diabetes.

1.4.5.2. Ag-based therapy in humans

Administration of soluble Ag to induce tolerance has been trialled in a number of autoimmune conditions. However, as yet no peptide-therapy is routinely used to combat any disease. Administration of Ag via mucosal routes is an ideal therapy for autoimmune conditions due to the non-invasive administration and the induction of both Th3 and Tr1 cells. However, data from human trials conducted thus far have not been promising. Oral administration of MBP and PLP to MS patients with relapsing-remitting disease resulted in the development of MBP- and PLP-specific T cells secreting TGFβ (Fukaura et al., 1996). However, a controlled clinical trial feeding myelin to relapsing-remitting patients showed no differences between placebo and myelin fed groups (Weiner, 1997). In an RA study, patients orally given the peptide dnaJP1, derived from the bacterial heat shock protein dnaJ, showed reduced pro-inflammatory responses and increases in foxp3+ cells (Prakken et al., 2004). Despite these promising results no clinical efficacy of the treatment was determined but undoubtedly will be undertaken soon. Oral insulin has also been administered in a number of diabetes trials (Shoda et al., 2005), yet no delay or prevention of diabetes has been noted (Skyler et al., 2005).

A few trials have focused on the administration of peptide via other routes. Keller et al administered daily low doses of insulin subcutaneously (s.c.) to patients, identified to be in the pre-clinical stage of diabetes and every 9 months administered a 5-day course of i.v. insulin (Keller et al., 1993). This protocol caused some delay in the onset of diabetes. Similarly, Shah et al demonstrated that two weeks of i.v. insulin at the onset of clinical diabetes can improve beta-cell function for the subsequent year (Shah et al., 1989). Both treatments are similar to the high-dose therapy discussed above. A similar high dose tolerance has been anecdotally noted in haemophiliacs.
who develop immune responses to administered factor VIII (Lozier, 2005; Tamura et al., 2006). High dose i.v. administration of factor VIII, known as the “Bonn Protocol”, has been shown to restore tolerance to factor VIII.

Peptide therapy in humans has met with better success when administered for the treatment of allergies (Larche and Wraith, 2005). However, this therapy still holds massive potential for autoimmune conditions, highlighted by the large number of clinical trials still underway.

**Considerations for Ag-based therapy in humans**

As the potential of Ag-based therapies has yet to be fully realised, a number of considerations need to be taken when determining how best to proceed (Hochweller et al., 2006b; Larche and Wraith, 2005; Liblau et al., 1997). Firstly it is important to determine what type of tolerance, of those discussed above, is desirable. Secondly, what Ag or Ags are to be administered; which are the critical Ag to employ and how might these change over-time? The peptides to which a T cell response is directed at the beginning of a disease might not be the same as those once the disease is established. As such it is possible to conclude that the ideal Ag-based therapy would involve the administration of a single peptide but allow tolerance to other peptides by bystander suppressive effects (Anderton, 2001).

**1.4.6. APC involved in the induction of Ag-based tolerance**

An outstanding question concerning Ag-induced T cell tolerance, is which population of APC present Ag to T cells to induce tolerance? Both B cells and DC could play a role, and as such it has been demonstrated that both types of cell can present peptides after i.v. administration of soluble protein (Zhong et al., 1997). However, on a per cell basis, DC were better at presenting peptides following protein administration.

It is the DC that are generally considered to present peptides in a tolerogenic manner following systemic administration, as peptide induced tolerance can be achieved in a B cell deficient mouse (Vella et al., 1996). Consistent with this, a number of studies have shown that targeting Ag specifically to subsets of DC can induce T cell tolerance (Dudziak et al., 2007; Finkelman et al., 1996; Hawiger et al., 2001;
Mukhopadhaya et al., 2008). Moreover, the administration of Ag-loaded DC has been shown to induce tolerance in the EAE setting (Hochweller and Anderton, 2004).

Due to technical constraints, few studies have explored the DC subset to which short peptides bind following their systemic administration. However, a considerable number of studies have explored which population of DC can present peptides to T cells following administration of whole protein, as processed peptides can be presented up to 7 days after protein administration (Robinson et al., 1999). Many studies have administered proteins or cell preparations to mice then purified DC populations from treated mice and used these to stimulate CD4+ and CD8+ T cells. Such studies have highlighted a propensity for CD11c+ CD8α+ (DEC205+) DC to present to CD8+ T cells and for CD11c+ CD8α- (33D1+) DC to present to CD4+ T cells (Chung et al., 2007; Dudziak et al., 2007; Ingulli et al., 2002; McLellan et al., 2002; Pooley et al., 2001).

1.4.7. Imaging Ag-induced tolerance

Until relatively recently, the induction of tolerance or immunity in T cells could only be followed by taking snap-shots of these processes as they develop. Despite this, seminal work by Jenkins and colleagues followed adoptively transferred pOVA-reactive DO11.10 cells following immunogenic (pOVA in CFA s.c.) or tolerogenic (pOVA in PBS i.v.) stimulus (Kearney et al., 1994). Following pOVA in CFA, DO11.10 cells expanded and accumulated in the lymph node B cell follicles, a location permitting effective B cell help. In contrast, following soluble pOVA, DO11.10 cells initially proliferate in the paracortical regions of the lymph node but fail to enter the follicles. Similarly, following Ag feeding, Ag-reactive T cells were shown to be incapable of entering the B cell follicles, however upon challenge of mice with Ag, tolerant T cells could migrate to B cell follicles yet were unable to provide B cell help (Smith et al., 2002).

More recently, the use of two-photon microscopy has allowed dynamic cell interactions within tissues to be examined. The interactions between DC and T cells have been visualised during the induction of tolerance and immunity in both explanted lymph nodes or in surgically exposed lymph nodes of anesthetized mice.
Such studies have highlighted three phases to T cell priming; after exposure to DC bearing cognate p:MHC, peptide-specific T cells make multiple, dynamic DC interactions. After this, peptide-specific T cells slow and make prolonged DC contacts, causing DC-T cell clustering. Finally, T cells resume their highly mobile activity (Garside and Brewer, 2008). The question of how these processes are affected during the induction of tolerance has been asked by a number of investigators.

Tolerance induction in OT-1 CD8+ T cells has been examined following tolerogenic treatment with anti-DEC205 conjugated to OVA. This resulted in initial T cell proliferation, however by day 7 OT-I cells had been deleted. OT-I cells undergoing this process of tolerance induction failed to form long-lasting DC-T cell contacts, and remained motile through the imaged time-frame (Hugues et al., 2004).

This is not the case for tolerance induction in CD4+ T cells. In a similar system to Hugues et al, Shakhar et al transferred and followed OT-II (CD4+) T cells after anti-DEC205-OVA (Shakhar et al., 2005). OT-II cells heading towards tolerance were seen to form stable contacts with the DC, similar to those seen following priming conditions. The major difference between tolerance and priming, was that OT-II cells regained motility faster following tolerogenic versus immunogenic treatment (18 hours versus 24 hours). These studies were confirmed by Zinselmeyer et al, who observed tolerance induction in DO11.10 CD4+ T cells after OVA feeding (Zinselmeyer et al., 2005). Moreover, this study demonstrated that T cell-DC clusters were smaller and shorter lived during tolerance induction than during the induction of immunity.

1.5. EXPERIMENTAL MODELS
This project asked questions about the signals required for the establishment of peptide-induced immune tolerance using the experimental models outlined below.

1.5.1. pOVA Reactive TCR transgenic; OT-II
The OT-II mouse expresses a transgenic TCR recognising the 323-339 peptide of OVA in the context of the I-A^b MHC class II molecule (Barnden et al., 1998). The
OT-II has been crossed with Ly5.1+ mice generating congenically marked Ly5.1+ OT-II cells that can be followed in vivo. Previous studies have shown that soluble pOVA can induce tolerance (peptide tolerance) in adoptively transferred Ly5.1+ OT-II cells following systemic administration of a high dose of pOVA (Hochweller and Anderton, 2005). The induction of tolerance in this system has been shown to require an initial burst of proliferation, during which OT-II cells divide for up to three days after peptide treatment. However, this activation is transient and OT-II cells are ultimately deleted (Hochweller and Anderton, 2005). Hochweller et al also demonstrated that Ly5.1+ OT-II cells up-regulated RANKL and PD-1 during the induction of tolerance; experiments described in this thesis used this model to further probe these findings (Hochweller and Anderton, 2005).

1.5.2. Experimental Autoimmune Encephalomyelitis (EAE)

Active induction of EAE requires immunisation of mice with a CNS auto-antigen in CFA. Advances in understanding the antigens which drive MS and EAE have led to disease being induced by immunisation with individual myelin peptides in CFA. These include peptides from MBP, PLP and MOG. EAE is CD4+ T cell mediated and can be induced by the passive transfer of CNS-reactive pathogenic T cells to recipient mice (Zamvil et al., 1985). A number of TCR transgenics exist, providing T cells which can be followed during induction, peak and resolution of EAE disease.

1.5.3. Ac1-9 Reactive TCR transgenic; Tg4

Tg4 mice express a transgenic TCR which recognises a peptide of MBP; the immuno-dominant Ac1-9 (or 4Lys) peptide, in the context of I-A\textsuperscript{V}. Zamvil et al initially immunised mice with rat MBP and obtained Ac1-9 specific T cell clones (Zamvil et al., 1985). From these a hybridoma was later generated, from which the TCR was subsequently cloned for generation of the Tg4 transgenic (Liu et al., 1995). More recently the Tg4 has been crossed with Ly5.1+ mice (Ryan et al., 2005) allowing Tg4 cells to be followed in vivo.
**Ac1-9 and APL**

In wild-type mice, Ac1-9 reactive T cells are thought to escape central tolerance due to low affinity of the peptide for MHC (Liu et al., 1995). Binding studies have shown that Ac1-9 displays such a low affinity for the I-A\(^U\) that binding kinetics could not be determined because the interaction was so transient (Fairchild et al., 1993). Despite this poor binding, immunisation of mice with Ac1-9 can activate Ag-reactive T cells and lead to the induction of EAE (Zamvil et al., 1986).

Analysis of Ac1-9 binding to I-A\(^U\) have shown that residues 3 and 6 are vital for the peptide to interact with the TCR, whereas residues 4 and 5 are vital for binding to the of I-A\(^U\) (Wraith et al., 1992; Wraith et al., 1989), see Figure 1.5. The lysine residue at position 4 is thought to act unfavourably with a hydrophobic binding pocket in the I-A\(^U\) binding cleft. Replacing lysine with more hydrophobic amino acids increases the binding affinity of the peptide for MHC and stabilises binding (Pearson et al., 1999). A hierarchy of differing Ac1-9 APL exists which bind to the I-A\(^U\) with increasing affinity; 4Tyr binds with a higher affinity than 4Val>4Ala>wild-type Ac1-9 (Liu and Wraith, 1995). The higher affinity ligands are, unsurprisingly, better at stimulating Tg4 cells *in vitro*, and are known as super agonists (Anderton et al., 2001). As such, the 4Tyr peptide can stimulate Tg4 cells at femtomolar concentrations compared to the Ac1-9 which needs to be at nanomolar concentrations.

**Tolerance induction with 4Tyr**

The higher affinity position 4 APL were shown to be better at inducing tolerance in both thymocytes (Liu et al., 1995) and peripheral T cells (Liu and Wraith, 1995). Liu et al demonstrated that treatment of non-transgenic, wild-type mice with 100μg of the highest affinity APL, 4Tyr, intra-peritoneally (i.p.) was profoundly protective against Ac1-9 induced EAE. 4Tyr was protective when administered both before and after the onset of disease. Also in non transgenic, wild-type mice treatment with the 4Tyr peptide has been shown to protect against EAE induced not only with Ac1-9 but also with a separate MBP peptide, MBP 89-101 (Anderton and Wraith, 1998), and induce tolerance to whole MBP (Metzler and Wraith, 1996).
Previous experiments exploring tolerance induction using the 4Tyr peptide have predominantly induced tolerance via a mucosal surface; i.n. or orally. In wild-type mice, i.n. treatment with 4Tyr has been shown to be long-lasting, with responsiveness only being recovered after 8 weeks as new, non-tolerant cells, exit the thymus (Metzler and Wraith, 1999). Tolerance induction in the intact Tg4 mouse has been shown to require multiple i.n. doses. Burkhart et al demonstrated that $3 \times 10^7$ adoptively transferred Tg4 T cells can be tolerised following 2 doses of 100µg 4Tyr, yet it takes between 5 and 10 doses to tolerise a whole Tg4 mouse (Burkhart et al., 1999). In that study, repeated doses of 4Tyr i.n. were also associated with increased levels of IL-10 production. In fact, administration of anti-IL-10 following 4Tyr treatment, led to a reversal of the tolerant phenotype and resulted in 4Tyr no longer protecting against EAE.

More recent studies have shown that CD4+ T cells from intact Tg4 mice treated 10 times i.n. with 4Tyr, can reduce the proliferative response of naïve CD4+ T cells (Massey et al., 2002). This reduced proliferation of naïve cells correlates with an increased production of IL-10 from the peptide-treated cells. In fact, repeated i.n. treatment with 4Tyr is considered to induce T cells with a regulatory phenotype, termed peptide-induced Tregs (PI-Tregs). These PI-Tregs are neither foxp3+ or CD25+, but do have increased levels of CTLA-4, ICOS and CD38 (Sundstedt et al., 2003) and upon recall to Ag they produce IL-10 (Nicolson et al., 2006). The Wraith group have determined the biochemical phenotype of the PI-Treg following tolerance induction (Anderson et al., 2006; Anderson et al., 2005). However, little work has been done characterising the tolerant phenotype of adoptively transferred Tg4 cells following a single systemic dose of 4Tyr.

These data suggest that 4Tyr is an ideal tolerogen for Tg4 cells when administered via mucosal surfaces. Much needs to be considered when designing an APL for tolerisation purposes, but higher affinity ligands appear to be well suited to the task. Anderton et al introduced the avidity-based model of peripheral T cell clonal expansion in response to Ag (Anderton et al., 2001). In doing so, questions were raised concerning the ability of peptides to tolerise T cells with TCRs of differing affinity. Further studies demonstrated that in order to tolerise certain peptide-
reactive T cells, an APL with a higher affinity than the peptide should be used (McCue et al., 2004).

1.6. AIMS

The ultimate outcome of the DC-T cell dialogue can be immunity or tolerance. One aim of this project was to study the roles of two receptor:ligand pairs (RANK:RANKL and PD-1:PD-L1/2) in the decision between these processes. In essence, these studies addressed the role of “signal 2” and are addressed in chapters 3 and 4.

A further aim was to investigate how increasing the strength of “signal 1” can influence the form of tolerance produced. These studies used the superagonist MBP Ac1-9 (4Tyr) APL and are described in chapters 5 and 6.
Figure 1.1. **Summary of costimulatory molecules known to be important in T cell activation.**

To activate a T cell it needs to receive two signals; engagement of the TCR with its cognate peptide bound by MHC (signal 1), and provision of costimulation by an APC. Here a few of the most well characterised costimulatory receptor-ligand pairs are shown. These pairs fall into two families, the Immunoglobulin super family (CD28, CTLA-4 and PD-1) and the TNF/TNFR family (CD154, OX40, and RANKL).
Figure 1.2. Signalling events initiated by ligation of the TCR.
This figure provides an overview of key signalling events initiated by the correct ligation of TCR by p:MHC. Most signalling events involve the phosphorylation of substrates; phosphorylation is represented by small grey circles. Correct propagation of the signal within the cell results in the activation of the transcription factors NF-AT, AP-1 and NF-κB.
**Figure 1.3. DC maturation states and their role in tolerance and immunity**

Immature, tissue resident DC induce T cell anergy due to lack of costimulatory molecule expression and low levels of MHCII expression. In the steady state some DC are induced to migrate, these semi-mature DC mediate tolerance through the induction of T cells with regulatory potential. Immunity is only induced by fully mature DC which express costimulatory molecules and produce pro-inflammatory cytokines.
Figure 1.4. PD-L1 and CD80 interact to mediate a negative signal to the T cell. Recent data from Butte et al has demonstrated that PD-L1 and CD80 interact on a T cell and can deliver an inhibitory signal. This inhibitory signal can be initiated when either molecule is expressed on the responding T cell (a bidirectional signal). The PD-L1:CD80 interaction may not only deliver a negative signal but also function to sequester binding partners away from the previously identified receptors PD-1, CTLA-4 and CD28.
Affinity of APL for I-A<sup>U</sup>:
4Tyr > 4Val > 4Ala > wt Ac1-9

Figure 1.5. Schematic representation of the residues important for Ac1-9 binding to I-A<sup>U</sup> and the Tg4 TCR.
The Ac1-9 peptide of MBP is the immuno-dominant epitope in the context of I-A<sup>U</sup>. Residues 3 and 6 are important for peptide binding to the Tg4 TCR. Residues 4 and 5 are vital for the peptide to bind the I-A<sup>U</sup> molecule. The Ac1-9 binds extremely poorly to the I-A<sup>U</sup> molecule. APL of Ac1-9 have been created which bind to the I-A<sup>U</sup> with increasing affinities; 4Tyr binds with a higher affinity than 4Val > 4Ala > wild type Ac1-9.
2. Materials and methods

2.1. MICE
The following mice were bred under specific pathogen-free conditions at the Institute of Infection and Immunology Research, University of Edinburgh; C57BL/6, B10.PL, OT-II Ly5.1, Tg4 Ly5.1. Two transgenics were used extensively in these studies; OT-II Ly5.1 mice, expressing a transgenic pOVA-specific TCR (Barnden et al., 1998) were on the C57BL/6 (I-A<sup>b</sup>) background. Tg4 Ly5.1 mice, expressing a transgenic Ac1-9(MBP)-specific TCR (Liu et al., 1995), were on the B10.PL (I-A<sup>u</sup>) background. B10.PL mice were crossed with DOG mice (Hochweller et al., 2008) to generate B10.PLxDOG.

The following mice were bred under specific pathogen-free conditions at ZVTE, University of Mainz; PD-1<sup>-/-</sup> (Nishimura et al., 1999) and 2D2 Thy1.1. 2D2 Thy1.1 mice, expressing a transgenic pMOG-specific TCR (Bettelli et al., 2003) were on the C57BL/6 background. 2D2 mice were crossed with PD-1<sup>-/-</sup> to generate 2D2xPD-1<sup>-/-</sup> mice.

All mice were sex-matched within experiments and used between 6-10 weeks of age.

2.2. GENERAL REAGENTS

2.2.1. Wash Buffer
RPMI 1640 medium containing HEPES buffer was supplemented with 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin (Gibco), and 50nM 2-mercaptoethanol (all Gibco, UK).

2.2.2. RPMI-5 and RPMI-10 tissue culture medium
Wash buffer, as described in 2.2.1, with the addition of either 5% or 10% heat-inactivated foetal calf serum (HI-FCS) (Sigma, UK).

2.2.3. MACS Buffer
Hanks Balanced Salt Solution (Sigma) was supplemented with 2% HI-FCS (Sigma) and 100U/ml Penicillin and 100µg/ml streptomycin (Gibco).
2.2.4. FACS Buffer
PBS was supplemented with 2% heat-inactivated FCS (Sigma) and 0.05% sodium azide (Sigma).

2.2.5. X-VIVO tissue culture medium
X-VIVO 15 serum free medium (BioWhittaker, UK) was supplemented with 2mM L-glutamine and 50 nM 2-mercaptoethanol (Gibco).

2.2.6. Solutions for ELISA

10x Bicarbonate Buffer
0.1M Na₂CO₃ (Sigma) and 0.2M Na₂HCO₃ (Sigma) in ddH₂O adjusted to pH 9.6.

Phosphate-citrate Buffer
0.2M Na₂HPO₄ (27.5ml) and 0.1M anhydrous citric acid (24.3ml) were mixed and made up to 100ml final volume with ddH₂O, pH 5.

2.3. ANTIGENS
The peptide 323-339 of OVA (hereafter referred to as pOVA) (ISQAVHAHAHEINEAGR-COOH), the peptide 35-55 of MOG (hereafter referred to as pMOG) (MEVGWYRSPFSRVVHLRNYK-COOH), and the Ac 1-9 peptides of MBP; 4Lys (Ac-ASQKRPSQR-amide), were prepared at the Advanced Biotechnology Centre, Imperial College, UK. APL of the 4Lys peptide contained a substitution of the Lysine residue at position 4 for either alanine (4Ala), valine (4Val) or tyrosine (4Tyr).

2.4. ANTIBODIES
Anti-RANKL, clone IK22-5 (Kamijo et al., 2006), and anti-PD-1, clone RMP1-14 (Yamazaki et al., 2005) were a gift from Hideo Yagita (Juntendo University, Japan). Isotype controls for these antibodies were either the isotype control MAC-1 (IgG2a), or purified rat IgG from serum (Sigma) which had been run over a polymyxin B column (Pierce, USA). The MAC-1 control hybridoma (anti-glycoprotein of Chlamydomonas reinhardii, rat IgG2a), was obtained from the European Collection of Cell Culture (ECACC, Wiltshire, UK). The anti-CD40 (FGK-45) hybridoma and
the anti-IL-2 (S4B6) hybridoma were kindly provided by Prof D. Gray (University of Edinburgh, UK).

2.4.1. Production of antibodies in house
2.5x10⁷ hybridoma cells in 15mls of RPMI-10 were cultured in the cultivation chamber of an Integra CL1000 (Integra Biosciences, UK). One litre of nutrient rich media; RPMI 1640 media, 2mM L-Glutamine, 100U/ml Penicillin and 100μg/ml streptomycin, 5x10⁻⁵ M 2-mercaptoethanol, 1x non essential amino acids (Gibco), 2.5μg/ml glucose (Sigma) and 2μg/ml peptones (Sigma), was added to the nutrient chamber. On day 7, and every 3 days thereafter, the cells in the cultivation chamber were removed, diluted 1 in 5 in fresh RPMI-10 and replaced in the cultivation chamber. The remaining cultivation media was centrifuged for 5 minutes at 350 xg without the brake and the supernatant stored at -20°C until antibody purification. The nutrient media was also replaced. Antibodies from hybridomas were purified using an AktaPrime (Amersham Biosciences, USA) automated chromatography system using a High Trap protein G HP (Amersham Biosciences).

2.5. CELL PURIFICATIONS AND PREPARATIONS
2.5.1. Positive selection of naïve CD4+ T cells
CD4+ cells were purified from single cell suspensions of disaggregated spleen and peripheral lymph nodes. Erythrocytes were depleted from cell suspensions by re-suspending cells in 2ml of RBC lysis buffer (Sigma) for 2-3 minutes; subsequently 10ml of wash buffer was added to suspensions before centrifugation at 350g for 5 minutes. CD4+ T cells were isolated by positive selection using CD4 MACS microbeads (Miltenyi Biotec, UK) as per the manufacturer’s instructions. Cells were re-suspended in MACS buffer at 45μl per 1x10⁷ cells and incubated with 5μl of CD4 MACS beads per 1x10⁷ cells for 20 minutes at 4°C. After incubation cells were washed once in MACS buffer and put through a MACS LS or MS positive selection column (Miltenyi Biotec) on a MACS magnet (Vario MACS, Miltenyi Biotec). The cells retained in the column were collected after 3 washes. A consistent purity of ~95% was confirmed by flow cytometry.
For adoptive transfer experiments, purified transgenic CD4+ cells were re-suspended at 1-2x10^6 cells per 200μl of sterile PBS (Gibco). This cell suspension was passed through a fine gauze and 200μl injected intravenously (i.v.) into the tail vein of mice.

### 2.5.2. Negative selection of naive CD4+ T cells

Single cell suspensions prepared as described in section 2.5.1, were re-suspended at 2x10^8 cells/ml in a cocktail of the following antibodies; anti-CD8 (clone 53.6.72), anti-B220 (clone RAB832), anti-Mac-1 (clone M1/70), and anti-class II (clone M5/114.15.2), all home-grown and all rat IgG, each at 10μg/ml in MACS Buffer. Cells were incubated for 20-30 minutes on ice, washed and re-suspended at 1x10^8 cells/ml. An equivalent volume of washed sheep anti-rat IgG M450 Dynabeads (Dynal Biotech Ltd, UK) were added to the mixture, and incubated with rotation at 4°C for 30 minutes. The tube was placed in a magnetic field and the supernatant collected. Magnetic sorting was repeated twice to remove all contaminating beads. Negative selection routinely yielded an enriched population of ~70% CD4+ cells.

### 2.5.3. CFSE labelling of cell populations

Purified CD4+ T cells or splenocyte preparations at 5x10^7 cells/ml were incubated with 5μM CFSE (Sigma) for 6 minutes at 37°C. Unbound CFSE was neutralised by addition of an equal volume of heat inactivated FCS. Cells were then washed twice with wash buffer. Cells were either re-suspended in PBS for adoptive transfer as described in 2.5.1, or cultured.

Analysis of CFSE dilution was performed using FlowJo software (TreeStar, USA). The percentages of cells in daughter populations were calculated manually as per Current Protocols in Immunology; Supplement 49. Briefly, the geometric mean fluorescence was determined for the control CFSE stained cells. This was converted into its base 10 logarithm. The geometric mean fluorescence of daughter populations was then determined as these occur at 1/2, 1/4 etc. of the undivided cell value, and hence can be calculated by subtracting 0.3log10 units. The boundaries between the populations, where the gates would be drawn, are mid-way between successive peaks and so are therefore 0.15log10 units either side of the peak. These calculated values were manually inputted into the FlowJo software.
2.5.4. Isolation of splenic DC

Spleens were removed from animals and injected with ~100μl of 8mg/ml collagenase IV (Worthington, USA) made up in wash buffer. Spleens were incubated at 37°C for 20 minutes before manual disaggregation. Erythrocytes were depleted from cell suspensions as described previously, using RBC lysis buffer (Sigma). DC populations were characterised by flow cytometry or sorted by FACS.

2.5.5. CD4+CD25+ cell purification by FACS

MACS sorted CD4+ cells were stained with anti-CD4-PerCP and anti-CD25-FITC (clone 7D4, not PC-61) in MACS buffer for 20 minutes at 4°C. Cells were then washed and sorted on a Becton Dickinson FACS Aria. Purity of CD4+CD25+ cells was consistently above 95%. CD4+CD25- cells were also sorted from these preparations; purity of these cells was consistently above 95%. (See table 2.2 for all information in flow cytometry antibodies).

2.5.6. Purification of DC subsets by FACS

Cell suspensions were prepared as described in section 2.5.4, and depleted of T cells using anti-PE MACS microbeads (Miltenyi Biotec). Cell preparations were stained with anti-CD3-PE in MACS buffer for 20 minutes at 4°C. Cells were then washed in MACS buffer and re-suspended in 40μl of MACS buffer per 1x10^7 and incubated with 10μl of anti-PE MACS beads (Miltenyi Biotec) per 1x10^7 for 20 minutes at 4°C. After incubation, cells were washed once in MACS buffer and put through a MACS LS positive selection column (Miltenyi Biotec) on a MACS magnet (Vario MACS, Miltenyi Biotec). The negative fraction (column flow-through) was collected and washed. Cells were stained with anti-CD11c-FITC, anti-CD8-APC, anti-CD4-PE and anti-PDCA-1-biotin in MACS buffer for 20 minutes at 4°C. Cells were then washed and stained with streptavidin-PerCP in MACS buffer for 20 minutes at 4°C. Cells were washed and sorted into the following separate populations on a Becton Dickinson FACS Aria; CD11c+CD4+, CD11c+CD8+, CD11c+CD4-CD8-, PDCA-1+. Purity of sorted DC subsets ranged between 70-85%.
2.5.7. Preparation of mononuclear cell populations from the CNS
Mice were sacrificed by CO$_2$ asphyxiation, and perfused with PBS through either the left ventricle of the heart or through the retino-orbital plexus. Spinal cords were removed by intrathecal hydrostatic pressure, and brains were removed by dissection. Spinal cords and brains were cut into small pieces and digested at 37°C for 40-60 minutes in wash buffer containing 8mg/ml collagenase IV (Worthington), and 10mg/ml DNase (Sigma). After incubation a single cell suspension was prepared from tissue samples following manual disaggregation. Cells were washed once in wash buffer and re-suspended in 30% Percoll (Gibco), this was under laid with 70% Percoll. These gradients were spun at 850 xg for 20 minutes without a brake. The cells at the gradient interface were removed and washed before use.

2.5.8. Maintenance of T cell lines (TCL)
A Tg4 TCL previously generated in the laboratory, was maintained using a 14 day restimulation/expansion cycle (Anderton et al., 1998). The TCL was restimulated for 3 days with 4Lys in the presence of irradiated syngenic B10.PL splenocytes. T cell blasts were isolated using a NycoPrep 1.077 density gradient (Nycomed Pharma, Norway) and expanded in RPMI-5 containing 5% Con-A activated rat spleen supernatant. TCL were split every 2 days and used in proliferation assays once they had past their proliferative peak, which was between 7-11 days post stimulation. TCL were cultured in 96-well flat-bottomed plates at 4x10$^4$ T cells per well in RPMI-5 for proliferation assays.

2.6. IN VIVO ANTIGEN ADMINISTRATION
2.6.1. Induction of tolerance with soluble peptides
In the OT-II model of peptide induced tolerance, 500µg of pOVA in sterile PBS was administered i.v. one day after OT-II cell transfer to induce tolerance. In the C57BL/6 model of peptide induced tolerance in a heterogeneous population of pMOG-reactive T cells, 200µg of pMOG was administered i.v. In the Tg4 model of peptide induced T cell tolerance 200µg of 4Tyr was administered i.v. one day after Tg4 cell transfer to induce tolerance. In a number of
experiments repeated doses of 200μg 4Tyr were administered i.v. at indicated intervals.
All peptides were administered in a final volume of 200μl into the tail vein of mice. In some experiments mice were immunised with peptide in CFA 7 days after peptide treatment.

2.6.2. Administration of LPS to induce T cell immunity
LPS, *Escherichia coli* 026:B6 (Sigma), was used as an adjuvant to induce T cell priming. 30μg of LPS was given i.v. along with a peptide in a final volume of 200μl into the tail vein of mice.

2.6.3. Immunisations
Mice were immunised with peptide emulsified in complete Freund’s adjuvant (CFA) containing 1mg/ml of heat-killed *Mycobacterium tuberculosis* (Sigma). Mice were immunised with 50μl of peptide/CFA subcutaneously (s.c.) into each hind leg; 100μg of heat-killed *Mycobacterium tuberculosis* per mouse. The peptides used for immunisation were 20μg of pOVA, 100μg pMOG or 100μg 4Lys per mouse, all emulsified in CFA. Mice were sacrificed 10 days after immunisation and spleens and draining LN analysed.

2.6.4. Administration of antibodies
In experiments using anti-RANKL or anti-PD-1, 250μg of antibody (or isotype control) were administered at the indicated time-point in PBS. Antibodies were predominantly given intraperitoneally (i.p.) in a total volume of 500μl. There was one exception to this; when anti-RANKL was administered at the same time as induction of active EAE, 250μg of anti-RANKL was administered i.v. in a final volume of 200μl PBS.
In experiments using anti-IL-2, 200μg of antibody was administered i.p in a total volume of 500μl, at the same time as peptide treatment and two days later.
2.6.5. Diphtheria toxin (DTx) treatments
B10.PLxDOG mice were treated with ~22ng DTx (Sigma) /g body weight either once, or three times 2 days apart. DTx treatments were given i.p. in 500µl of PBS final volume.

2.7. ACTIVE EAE
2.7.1. pMOG induced EAE
C57BL/6 mice were immunised with 100µg pMOG emulsified in 100µl CFA containing 1mg/ml of heat-killed Mycobacterium tuberculosis. Mice were also given 200ng of Pertussis toxin (Heath Protection Agency, UK) in final volume of 500µl of PBS i.p. on the same day as immunisation and 2 days later.

2.7.2. Tg4 model of EAE
1-2x10^6 Tg4 Ly5.1 cells were transferred to B10.PLxC57BL/6 mice. Mice were given peptide treatments the following day. 7 days after peptide treatments mice were immunised with 100µg 4Lys in CFA. Mice were also given 200ng of Pertussis toxin in final volume of 500µl of PBS i.p. on the same day as immunisation and 2 days later.

2.7.3. EAE Scoring
Mice were monitored daily from day 5 after immunisation onwards. Mice were given a clinical score from 0-6 as follows: 0, no disease; 1, flaccid tail; 2, impaired gait and/or impaired righting reflex; 3, partial hind-limb paralysis; 4, total hind limb paralysis; 5, partial fore-limb paralysis; 6, moribund or dead. Mice scored as grade 5 for 2 consecutive days or that had lost more than 20% of their body weight were culled.

2.8. EX VIVO ASSESSMENT OF T CELL FUNCTION
2.8.1. Ex vivo recall proliferation assays
Single cell suspensions were prepared from experimental mice by disaggregation of LN and spleens through gauze. Erythrocytes were depleted from cell suspensions using RBC lysis buffer (Sigma). Cells were counted and plated out in 96-well flat-
bottomed micro-titre plates with X-VIVO tissue culture medium. Splenocyte preparations were plated out at 8x10^5 cells per well, LN cell preparations were plated out at 6x10^5 cells per well. Cultures were stimulated with a dose range of peptide; pOVA 0-100μM, pMOG 0-20μM, 4Lys 0-100μM or 4Tyr 0-100μM, for 48 hours. After 48 hours cells were pulsed with tritiated thymidine (\(^{3}\text{H}\)thymidine) (Amersham, UK) at 0.5μCi/well for the last 14-18 hours of culture. \(^{3}\text{H}\)thymidine incorporation was measured using a liquid scintillation β-counter (Wallac, UK). Results are expressed as mean counts per minute (CPM)±SEM.

### 2.8.2. Cytokine production assays

Single cell suspensions were prepared and plated out as in section 2.8.1. After 48 hours of culture, culture supernatant was removed for determination of cytokine levels by ELISA.

### 2.8.3. Enzyme-Linked Immunosorbent Assay (ELISA)

Capture antibodies (all BD Pharmingen, UK), outlined in Table 2.1, were diluted in 1x bicarbonate buffer. This solution was used to coat 96-well Maxi-sorp micro-titre plates (Nalge Nunc International, Denmark) at 50μl per well. Plates were incubated overnight at 4°C before being washed twice in PBS/0.1% Tween (Sigma). Non-specific binding was blocked with 200μl per well of PBS/1% BSA solution for 1 hour at 37°C. Plates were washed twice in PBS/0.1% Tween followed by two washes in PBS. Cytokine standards, diluted in PBS/1% BSA were added at 100μl per well in duplicate and two-fold dilutions performed to give a standard curve for each plate. All cytokine standards were recombinant mouse cytokines (BD Pharmingen), IL-2 and IL-4 top concentration: 1000ng/ml, IL-10 and IFNγ top concentration: 100ng/ml, IL-17 top concentration: 10ng/ml. Supernatant samples were added to wells at 100μl per well. Plates were incubated at room temperature for 2 hours or overnight at 4°C (IL-10 ELISA only). After 2 hours plates were washed 4 times in PBS/0.1% Tween and incubated with 100μl per well of biotinylated anticytokine detecting antibody (diluted as shown in Table 2.1), in PBS/1% BSA for 1 hour. Plates were washed 6 times with PBS/0.1% Tween before incubation with a 1/1000 dilution of extravidin peroxidase (Sigma) in PBS/1% BSA (gives a final
concentration of ~2μg/ml) for 30 minutes at room temperature. Plates were then washed 6 times in PBS/0.1% Tween before the ELISA was developed. The ELISA was developed by adding 100μl of TMB substrate solution: 100μl of a 10mg/ml Tetramethylbenzidine in DMSO to 9.9mls of phosphate-citrate buffer plus 3μl of 30% H₂O₂. Developing reactions were stopped by addition of 100μl of 2M sulphuric acid to each well. Absorbance values were read at 450nm using a Multi-scan plate reader (Labsystems, UK). Limits of detection of cytokine ELISAs were as follows; IL-2 and IL-4: 4-1000pg/ml, IL-10 and IFNγ: 0.4-100ng/ml, and IL-17: 0.02-10ng/ml.

<table>
<thead>
<tr>
<th>Capture antibody</th>
<th>Clone</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-IL-2</td>
<td>JES6-1A12</td>
<td>2μg/ml</td>
</tr>
<tr>
<td>Anti-IFNγ</td>
<td>R4-6A2</td>
<td>2μg/ml</td>
</tr>
<tr>
<td>Anti-IL-10</td>
<td>JES5-2A5</td>
<td>2μg/ml</td>
</tr>
<tr>
<td>Anti-IL-4</td>
<td>11B11</td>
<td>2μg/ml</td>
</tr>
<tr>
<td>Anti-IL-17</td>
<td>TC11-18H10</td>
<td>1μg/ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Detection antibody</th>
<th>Clone</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-IL-2</td>
<td>JES6-5H4</td>
<td>0.5μg/ml</td>
</tr>
<tr>
<td>Anti-IFNγ</td>
<td>XMG1.2</td>
<td>0.5μg/ml</td>
</tr>
<tr>
<td>Anti-IL-10</td>
<td>SXC-1</td>
<td>0.5μg/ml</td>
</tr>
<tr>
<td>Anti-IL-4</td>
<td>BCD6-24G2</td>
<td>0.5μg/ml</td>
</tr>
<tr>
<td>Anti-IL-17</td>
<td>TC11-8H4.1</td>
<td>0.25μg/ml</td>
</tr>
</tbody>
</table>

### 2.8.4. Intracellular cytokine staining (ICS)

Single cell suspensions were prepared from experimental animals and plated out as described in section 2.8.1. Cells were incubated overnight with 100μM 4Lys, 100μM pOVA, 5μM pMOG or 20μM pMOG depending on the experiment. After overnight stimulation Brefeldin A was added, as per manufacturer’s instructions (1 in 1000 dilution), to each well for 4-5 hours. After 4-5 hours, cells were centrifuged and washed in FACS Buffer plus 0.1% Saponin (Sigma). Intracellular cytokines were stained with anti-cytokine antibodies (see Table 2.2) for 40 minutes in a total volume of 50μl of FACS Buffer plus 0.1% Saponin in the dark at room temperature.
After intracellular staining, cells were washed once in FACS buffer and stained for
the expression of surface molecules as described in section 2.8.5.

**2.8.5. Flow cytometric analysis**

*Surface Stains*

Single cell suspensions were prepared as described in section 2.8.1. Cells were
stained with the indicated antibody (see Table 2.2 for antibody clones and
concentrations) in 50μl of FACS buffer for 15-20 minutes at 4°C. For biotinylated
antibodies, a streptavidin conjugated secondary antibody, either Streptavidin-APC or
Streptavidin-PerCP (both BD Pharmingen, UK), was added at 2-5μg/ml in 50μl of
FACS buffer for 15-20 minutes at 4°C. For PDCA-1 staining, cells were stained in
50μl of FACS buffer with a 1 in 15 dilution of anti-PDCA-1 (Miltenyi Biotec) as per
manufacturer’s instructions.

*Intracellular Stains*

For intracellular staining of foxp3, CTLA-4 or T-bet (see Table 2.2) single cell
susensions were first stained for the expression of surface molecules. Cells were
then fixed and permeabilised by re-suspension in 250μl of Fix/Perm solution
(eBiosciences, UK). Cells were incubated overnight at 4°C, washed once in PBS and
once in Permeabilisation buffer (eBiosciences). Cells were then stained with the
indicated antibody in 50μl of permeabilisation buffer for 20-30 minutes at 4°C. For
T-bet staining, cells were stained in 90μl FACS buffer plus 10μl of antibody as per
manufacturer’s instructions.

*FACS Analysis*

Cells were analysed using either a Becton Dickinson FACS Calibur with CellQuest
(Becton Dickinson, UK) or a Becton Dickinson LSRII with Diva software (Becton
Dickinson) for collection of data. All data was analysed using FlowJo (TreeStar).

**2.8.6. Annexin V and 7-AAD Staining**

Single cell suspensions were prepared from spleens as described in section 2.8.1.
1x10^7 cells were re-suspended in annexin-binding buffer (10mM HEPES, 140mM
NaCl₂, and 2.5mM CaCl₂, pH 7.4) (all Sigma) plus annexin V-PE at 1 in 20 dilution (BD Pharmingen), as per manufacturers instructions. Cells were incubated at room temperature in the dark for 15 minutes, and then washed twice in annexin-binding buffer. Immediately before collection cells were re-suspended in 300μl of annexin-binding buffer plus 4μl of 7-AAD (BD Pharmingen).

2.8.7. Cell stimulations and flow cytometric analysis of pERK

Single cell suspensions were prepared from spleens as described in section 2.8.1. Cells were re-suspended at 2x10⁷ cells per 500μl of RPMI-10 and plated out into a 24-well plate at 500μl per well. To control wells the pERK inhibitor, UO126 (Promega, UK) (specifically a MEK inhibitor), was added to give a final concentration of 10μM. Plates were incubated at 37°C for 30 minutes prior to addition of the following stimuli; 10μg/ml of phorbol 12-myristate 13-acetate (PMA) was added to inhibited (negative control) and un-inhibited wells (positive control), 100μM 4Tyr was added to experimental wells. Cells were incubated at 37°C for the indicated lengths of time before fixing by addition of 500μl of 4% Paraformaldehyde (Sigma). Cells were incubated at 37°C for at least 20 minutes before being re-suspended and centrifuged at 600 xg for 5 minutes. Cells were re-suspended in 2mls of ice-cold 90% Methanol (Fisher Scientific, UK) and stored overnight at -20°C.

1ml of cell suspension was washed twice in PBS/2% BSA. pERK antibody, clone 197G2 (Cell Signalling, UK) was used at a dilution of 1 in 50 as per manufacturers instructions. Cells were stained for pERK in 50μl of PBS/2% BSA for 30 minutes at room temperature in the dark. After incubation cells were washed in PBS/2% BSA before being stained with a secondary fluorochrome conjugated antibody, goat anti-Rabbit-FITC (BD Pharmingen) at a final concentration of 10μg/ml. Cells were stained in 50μl of PBS/2% BSA for 20 minutes at 4°C. After incubation cells were washed in PBS/2% BSA, before being stained with the following surface stains as described above; CD4-APC, Ly5.1-PE.
2.8.8. Indo-1 staining and flow cytometric analysis of calcium mobilisation

Single cell suspensions were prepared from spleens and $2 \times 10^7$ cells were stained for CD4 and Ly5.1 surface markers as described in section 2.8.1. At the same time as staining for surface markers, anti-CD3-biotin was also stained (clone 145-2C11) (BD Pharmingen) at 16μg/ml final concentration. Cells were washed after staining and re-suspended in RPMI-5 at $1 \times 10^7$ cells/ml. Indo-1 (Sigma) was added to the cell suspensions at a final concentration of 5μM and incubated at 37°C in the dark for 40 minutes with frequent agitation. After incubation, suspensions were washed with RPMI-5 and centrifuged at 350 xg for 8 minutes. Cells were then re-suspended in RPMI-5 for FACS analysis.

Indo-1 loaded cells were analysed on a Becton Dickinson LSRII with Diva software (Becton Dickinson). Experimental samples were collected on the LSRII for 1 minute 15 seconds before the sample was removed for 10 seconds for addition of streptavidin (Sigma) at a final of concentration of ~20μg/ml. Samples were replaced and run for a further 10 minutes. All data was analysed using FlowJo.

Measurement of Calcium flux in the presence of EGTA

Cells were surface stained and indo-1 loaded as described above. Before analysis on the LSRII cells were re-suspended in RPMI-5 contained 2mM EGTA (Sigma). Experimental samples were collected on the LSRII for 30 seconds before the sample was removed for 10 seconds for addition of Streptavidin at a final of concentration of ~20μg/ml. Samples were replaced and run for a further 40 seconds before the addition of CaCl$_2$ (Sigma) to give a final concentration of 3mM. Samples were replaced and run for a further 8 minutes.
Table 2.2: Antibodies used for flow cytometric analysis

<table>
<thead>
<tr>
<th>Target</th>
<th>Clone</th>
<th>Fluorescent conjugate</th>
<th>Concentration Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>RM4-5</td>
<td>FITC*, PE^3, PerCP^3</td>
<td>1-2.5µg/ml</td>
</tr>
<tr>
<td>CD4</td>
<td>GK1.5</td>
<td>APC^*</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>CD8</td>
<td>53-6.7</td>
<td>APC^*</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>CD3</td>
<td>14-2C11</td>
<td>FITC^*</td>
<td>2.5µg/ml</td>
</tr>
<tr>
<td>TCRβ</td>
<td>H57-597</td>
<td>PE^3, APC^*</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>Vα2</td>
<td>B20.1</td>
<td>PE^3</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>CD25</td>
<td>PC61</td>
<td>PE^3, APC^*</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>CD25</td>
<td>7D4</td>
<td>FITC^3</td>
<td>2.5µg/ml</td>
</tr>
<tr>
<td>CD5</td>
<td>53-7.3</td>
<td>PE^3</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>CD62L</td>
<td>MEL-14</td>
<td>PE^3, Biotin^*</td>
<td>1-2.5µg/ml</td>
</tr>
<tr>
<td>Ly5.1(CD45.1)</td>
<td>A20</td>
<td>PE^3, FITC^3</td>
<td>1-2.5µg/ml</td>
</tr>
<tr>
<td>CD44</td>
<td>IM7</td>
<td>PE^3</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>CD28</td>
<td>37.51</td>
<td>PE^3</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>PD-1</td>
<td>J43</td>
<td>PE^*</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>RANKL</td>
<td>IK22-5</td>
<td>PE^<em>, Biotin^</em></td>
<td>2-5µg/ml</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>UC10-4F10-11</td>
<td>PE^3</td>
<td>2µg/ml</td>
</tr>
<tr>
<td>OX40</td>
<td>OX-86</td>
<td>Biotin^5</td>
<td>5µg/ml</td>
</tr>
<tr>
<td>OX40-L</td>
<td>RM134L</td>
<td>Biotin^*</td>
<td>5µg/ml</td>
</tr>
<tr>
<td>CD154</td>
<td>MRI</td>
<td>Biotin^5</td>
<td>5µg/ml</td>
</tr>
<tr>
<td>ICOS</td>
<td>7E.17G9</td>
<td>Biotin^5</td>
<td>2.5µg/ml</td>
</tr>
<tr>
<td>CD103</td>
<td>2E7</td>
<td>PE^*</td>
<td>2µg/ml</td>
</tr>
<tr>
<td>CD69</td>
<td>H1.2F3</td>
<td>PE^*</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>CD80</td>
<td>16/10A1</td>
<td>PE^3</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>CD86</td>
<td>GL-1</td>
<td>PE^3</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>CD11c</td>
<td>HL3</td>
<td>FITC^3</td>
<td>5µg/ml</td>
</tr>
<tr>
<td>B220</td>
<td>RA3-6B2</td>
<td>PerCP^3</td>
<td>2µg/ml</td>
</tr>
<tr>
<td>PDCA-1</td>
<td>JF05.1C2.4.1</td>
<td>Biotin^†</td>
<td>NA</td>
</tr>
<tr>
<td>I-A^b</td>
<td>KH74</td>
<td>Biotin^*</td>
<td>2.5µg/ml</td>
</tr>
<tr>
<td>PD-L1</td>
<td>MH5</td>
<td>PE^*</td>
<td>2µg/ml</td>
</tr>
<tr>
<td>PD-L2</td>
<td>Ty25</td>
<td>PE^*</td>
<td>2µg/ml</td>
</tr>
<tr>
<td>IL-2</td>
<td>JES6-5H4</td>
<td>PE^3</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>IFNγ</td>
<td>XMG1.2</td>
<td>FITC^*</td>
<td>2.5µg/ml</td>
</tr>
<tr>
<td>IL-17</td>
<td>TC11-18H10.1</td>
<td>PE^3, APC^†</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>IL-10</td>
<td>JES5-16E3</td>
<td>PE^3</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>Foxp3</td>
<td>FJK-16s</td>
<td>PE^<em>, APC^</em>, FITC^*</td>
<td>4µg/ml</td>
</tr>
<tr>
<td>T-bet</td>
<td>4B10</td>
<td>FITC^*</td>
<td>NA</td>
</tr>
</tbody>
</table>

^* eBiosciences, USA
^5 BD Pharmingen, UK
^j Miltenyi Biotec, UK
^† BioLedgend, USA
2.9. **IMMUNOFLUORESCENCE**

CFSE-labelled Tg4 cells were transferred to mice which had received either PBS or 4Tyr one day prior to transfer. One day post transfer mice were sacrificed and spleens embedded in OCT (Sakura Finetek Europe, The Netherlands), snap-frozen on dry-ice and stored at -80°C. 5μm sections were prepared using a cryostat (Leica Microsystems Ltd, UK), mounted on super-frost slides (BDH, UK), air dried and stored at -80°C.

Slides were dried at room temperature for 30 minutes prior to fixing in ice-cold acetone for 5 minutes and re-hydration for 5 minutes in PBS. Slides were blocked with PBS/3% BSA for 1 hour at room temperature and stained with primary staining antibodies for 30 minutes at room temperature. Slides were washed 3 times in PBS before staining with fluorochrome conjugated secondary antibodies for 30 minutes at room temperature. Slides were washed 3 times and then stained with 1μg/ml DAPI (Invitrogen, USA) in PBS/3% BSA at room temperature for 5 minutes. Slides were washed a final 3 times in PBS before mounting with Mowiol mounting medium (Calbiochem, USA) plus 0.01% of p-phenylenediamine added before use. All incubations were carried out in a dark humidified staining chamber. Slides were imaged with a Leica SP5 microscope (CIR, University of Edinburgh) with the help of Mrs S. Johnson.

Primary antibodies used were; rat anti-mouse-CD19, clone 1D3 (BD Pharmingen), anti-mouse CD11c-AlexaFluor 647, clone N418 (eBioscience). The secondary antibody used was rabbit anti-rat-AlexaFluor 594 (Invitrogen).

2.10. **EX VIVO ASSESSMENT OF T CELL FUNCTION**

2.10.1. *Ex vivo* recall proliferation assays

96-well flat-bottom micro-titre plates were coated with 50μl of sterile PBS (Gibco) containing anti-CD3, clone 145.2C11 (eBiosciences), plus/minus anti-CD28, clone 37-51 (home-grown provided by Prof D. Gray), at indicated concentrations. Alternatively 24-well flat-bottom micro-titre plates were coated with 500μl of sterile PBS (Gibco) containing anti-CD3 plus/minus anti-CD28 at indicated concentrations. Plates were incubated at 37°C for 2 hours before being washed 3 times in sterile
PBS. CFSE-labelled or unlabelled splenocytes were plated out $8 \times 10^5$ cells per well (96 well plate), and proliferative responses or cytokine production determined at indicated time-points. Alternatively, MACS sorted CD4+ cells were plated out at $4 \times 10^4$ cells per well (96-well plate) or $5 \times 10^5$ cells per well (24-well plate), and proliferative responses or cytokine production determined at indicated time-points.

2.10.2. In vitro suppression assay
Sorted CD4+CD25- cells ($2 \times 10^4$ per well) were cultured with $1 \times 10^5$ irradiated splenocytes, $2 \mu$g/ml anti-CD3 or $10 \mu$M pOVA, with or without decreasing numbers of sorted CD4+CD25+ cells for 96 hours. For the last 18 hours of culture cells were pulsed with tritiated thymidine ($^3$H]thymidine) (Amersham, UK) at 0.5μCi/well.

2.10.3. Irradiation of APC for assays
Spleens received 30 Gy of γ-irradiation by a caesium isotope ($^{127}$Cs) source.

2.11. WESTERN BLOTTING OF CELLS
2.11.1. Sorting and stimulation of cells
CD4+ cells were prepared from experimental animals as described in section 2.5.2. CD4+ cells were stained with anti-CD4-PerCP, anti-Ly5.1-FITC (both at 2.5μg/ml), and anti-CD3-biotin (at 16μg/ml) in MACS buffer for 20 minutes at 4°C. Cells were washed and sorted on a Becton Dickinson FACS Aria. Purity of CD4+Ly5.1- and CD4+Ly5.1+ cells was consistently above 95%.

Sorted cells were re-suspended at $6.44 \times 10^6$ cells per 500μl RPMI-5 and 80μg/ml streptavidin (Sigma) was added to each cell suspension (some samples were left un-stimulated). Cell suspensions were cultured at 37°C for 5 minutes before being spun down at 600 xg for 5 minutes and washed 3 times in PBS. After the last wash as much of the liquid was removed as possible and cells were lysed in 100μl of 1x lysis buffer (Cell Signalling) plus 1mM PMSF (Sigma), on ice for 5 minutes. Cells were spun down at 16060 xg at 4°C for 20 minuets. The supernatant was then removed and stored at -80°C before analysis.
2.11.2. Gel Electrophoresis

20μl of sample supernatant (obtained as described in section 2.11.1) were heated at 95°C for 5 minutes in NuPAGE LDS sample buffer (Invitrogen) plus 0.5M 2-mercaptoethanol (Sigma), and separated on NuPAGE 4-12% Bis-Tris gels using Surelock Minicell system with NuPAGE MES SDS running buffer (all Invitrogen). Gels were run at a constant 200V for 40 minutes.

2.11.3. Western Blotting

Samples separated by gel electrophoresis were transferred to nitrocellulose membrane (BioRad, UK) using the Hoefer semi-dry transfer unit (Hoefer, USA) for 65 minutes at 45mAm. Membranes were blocked for 1 hour at room temperature in TBS/0.1% Tween (Sigma) plus 5% Milk (Marvel, UK). Membranes were probed with mouse anti-phospho-Tyr, P-Tyr-100 (Cell Signalling, USA), at a 1 in 1000 dilution in TBS/0.1% Tween plus 5% BSA overnight at 4°C, as per manufacturer’s instruction. Membranes were washed extensively in TBS/0.1% Tween, and incubated with a 1 in 1500 dilution of rabbit anti-mouse IgG-HRP (DakoCytomation, UK) for 1 hour at room temperature. Following further washes, blots were developed using ChemiGlow West (Alpha Innotech, USA) according to manufacturer’s instruction. Blots were imaged using a FluorChem SP (Alpha Innotech).

Blots were striped using 1x Strip solution (Chemicon, USA) for 15 minutes at room temperature, as per manufacturer’s instructions. Membranes were then re-blocked as above and re-probed for the house keeping protein β-actin. Antibodies used were; rabbit anti-β-actin, clone 13E5 (Cell signalling) used at 1 in 1000 and goat anti-Rabbit IgG-HRP (Cell Signalling) used at 1 in 1000. Both antibodies were used as per manufacturer’s instructions

2.12. STATISTICS

Statistical analysis of EAE disease score was performed using the Mann-Whitney test, comparing two experimental groups. Statistical analysis of other data were performed using either an unpaired Student’s T test, when comparing 2 experimental groups, or by an ANOVA using Tukey’s multiple comparison test, when comparing
3 or more experimental groups. Differences were regarded as significant when p values were <0.05.
3. The role of PD-1 mediated signalling in tolerance and immunity.

3.1. INTRODUCTION

T cell tolerance was thought to result from a lack of costimulation from the APC. However, it is now acknowledged that tolerance is an active process in which costimulation must play an important role. While specific ligands for the TCR define the target, it is the costimulatory signals, along with the cytokine milieu, which are required to tailor the response; generating the appropriate response to the pathogen depending on the context in which the TCR is ligated. As such it is likely that certain costimulatory molecules play an important role in the induction of tolerance in T cells.

Peptide-induced T cell tolerance has been well characterised using adoptive transfer models in which a population of TCR transgenic, peptide-reactive T cells can be monitored following peptide treatment. In the experiments described in this, and the subsequent chapter, OT-II CD4+ TCR transgenic cells were employed to discern the requirement for costimulation in pOVA-induced tolerance. A previous report examined the expression of various costimulatory pairs on OT-II T cells and dendritic cells (DC), following tolerogenic (pOVA) or immunogenic (pOVA+ LPS) stimulus (Hochweller and Anderton, 2005). Following either stimuli T cells up-regulated CD154, OX40, RANKL and PD-1. In this chapter the role of the coinhibitory molecule PD-1 in both the induction of tolerance and immunity is addressed.

PD-1 is an important coinhibitory molecule, which mediates a negative signal to the T cell upon co-ligation of the TCR. PD-1 mediated signalling has been shown to be most effective at low levels of TCR stimulus or in settings of reduced costimulation (Freeman et al., 2000); this latter situation would occur during peptide-induced T cell tolerance. The importance of PD-1 in dampening effector T cell functions and mediating tolerance in peripheral tissues is well documented. Experiments using
EAE (Salama et al., 2003), diabetes in the NOD mouse (Keir et al., 2006), allograft rejection (Wang et al., 2007) and numerous other immune-driven conditions (Keir et al., 2008) have all shown enhanced severity and/or onset upon disruption of the PD-1–PD-L interaction. Despite this, few studies have examined the role of PD-1 during the activation of a naïve cell, and as such in the induction of T cell tolerance. Those studies which have posed this question have shown that PD-1 signalling is vital for the induction of T cell tolerance. Probst et al demonstrated that resting, tolerogenic DC could no longer induce tolerance in CD8+ T cells when PD-1 knockout (PD-1<sup>−/−</sup>) T cells were examined (Probst et al., 2005). Two separate groups have highlighted the importance of PD-1 mediated signals in the induction of tolerance in CD8+ OT-I cells transferred to RIP-OVA mice; in this system, transfer of OT-I cells results in their deletion, yet loss/blockade of PD-1 prevents this and causes diabetes (Keir et al., 2007; Martin-Orozco et al., 2006). Perhaps most importantly, Tsushima et al have demonstrated a vital requirement for PD-1 mediated signals in the induction of peptide-induced T cell tolerance in OT-I cells (Tsushima et al., 2007).

In the experiments discussed in this chapter, a blocking antibody against PD-1, (Yamazaki et al., 2005), was administered with the aim of understanding the role of PD-1 signals in the OT-II model of peptide-induced T cell tolerance and activation.
3.2. RESULTS

3.2.1. PD-L1 expression following tolerogenic or immunogenic treatment differs to that of PD-L2.

Previous studies from this laboratory have shown that PD-1 is rapidly expressed on OT-II cells following administration of 500µg pOVA (tolerance) and 500µg pOVA plus 30µg LPS (immunity) (Hochweller and Anderton, 2005). These data suggested a role for PD-1 during the initial T cell-DC encounter, and as such it was necessary to determine the expression of PD-L1 and PD-L2 on DC and T cells during the induction of tolerance and immunity in OT-II cells. Similar to previous studies (Hochweller and Anderton, 2005), Ly5.1+OT-II cells were transferred to C57BL/6 hosts, which then received either a tolerising dose of pOVA (500µg in PBS), an immunogenic dose of pOVA (500µg pOVA plus 30µg LPS), or PBS. Expression of PD-L1 and PD-L2 was examined by flow cytometry on days 1, 2, and 3 post-peptide treatments (Figure 3.1a). Expression patterns on OT-II cells, CD11c+ CD4+ cells, CD11c+ CD8+ cells and plasmacytoid DC (pDC; defined by expression of PDCA-1) recovered from spleens were determined.

Consistent with other studies (Yamazaki et al., 2002), flow cytometric analysis showed un-stimulated naïve OT-II cells express PD-L1 (Fig. 3.1.d). PD-L1 expression on OT-II cells was strongly up-regulated at the 24-hour time-point, to a similar extent following either pOVA or pOVA+LPS treatment. Expression then declined almost to background by day 3.

PD-L1 was expressed on CD11c+ cells from naïve hosts (Figure 3.1e) consistent with previous reports (Yamazaki et al., 2002). The expression of PD-L1 was only significantly up-regulated on DC from pOVA+LPS treated mice. This enhanced level of expression was still present, but lower, at day 2, and by day 3 had almost returned to baseline levels. Expression of PD-L1 on DC showed similar kinetics on all the DC subsets examined. Figure 3.1e shows the expression of PD-L1 on all CD11c+ DC to best highlight this kinetic.
PD-L2 was not expressed on OT-II cells at any time-point or following any treatment (Figure 3.2a). PD-L2 was only expressed on DC following pOVA+LPS treatment (Figure 3.2b). Expression was transient on pOVA+LPS treated DC, being seen only on day 1, and being lost by day 2. Similarly to PD-L1, expression of PD-L2 on DC showed similar kinetics on all DC subsets examined, data presented in Figure 3.2b shows the expression on all CD11c+ DC to highlight this kinetic.

The expression of PD-L1 and PD-L2 was also examined on OT-II cells and CD11c+ DC following immunisation of mice with pOVA in CFA, a more potent immunogenic stimulus. Ly5.1+OT-II cells were transferred to C57BL/6 prior to immunisation with pOVA in CFA the following day. Mice were sacrificed at day 2, 6, and 10 post immunisation and spleens sampled. No expression of PD-L2 was seen on OT-II T cells (Figure 3.3a). PD-L1 expression was enhanced following immunisation, with greatest expression being seen at day 2 post immunisation; expression then declined by day 10 post immunisation (Figure 3.3a).

Expression of the PD-L was determined on all the DC subsets described above. Expression patterns were similar on all DC subsets examined with one exception; at day 10 post immunisation pDC could no longer be detected in the spleen and so expression of PD-Ls could not be determined. Figure 3.3b shows the expression of PD-L1 and PD-L2 on CD11c+ DC. PD-L1 expression was rapidly up-regulated following immunisation, expression then increased overtime with the highest expression being seen at day 10. PD-L2 expression was not seen at the later time points post immunisation, however, there was a suggestion of very low level expression at day 2 post immunisation; at this early time-point only 5.1±1% of DC were PD-L2+. Considering the rapid up-regulation of PD-L2 seen following pOVA+LPS treatment (Figure 3.2), these data suggest that the peak of PD-L2 expression on CD11c+ DC had perhaps been missed.

3.2.2. PD-1 signals are required to limit T cells responses in vitro.  
Ligation of PD-1 has been shown to inhibit T cell activation (Carter et al., 2002; Latchman et al., 2001). This was first demonstrated by Freeman et al, who showed
that incubation of T cells with anti-CD3 and hPD-L1.Ig coated beads led to a reduction in T cell proliferation and cytokine production (Freeman et al., 2000). Before in vivo experiments could be undertaken, it was necessary to determine the efficacy of the anti-PD-1 antibody. If ligation of PD-1 by PD-L1 in culture constrains T cell responses, then these should be enhanced by blockade of this interaction.

Splenocytes from OT-II mice were cultured with increasing concentrations of pOVA plus either 50μg/ml of anti-PD-1 or an isotype control for 72 hours, as per (Keir et al., 2005). Duplicates of cultures were established with or without addition of the LPS neutralising agent polymyxin B (PMB), to assure antibodies were free from contamination. Addition of anti-PD-1 to cultures enhanced the production of IFN\(\gamma\) (Figure 3.4a). This was true for cultures both with and without addition of PMB, however overall responses were slightly reduced in all PMB treated cultures. Proliferative responses were also enhanced upon the addition of anti-PD-1. Splenocytes from wild-type mice were stained with CFSE and cultured with increasing concentrations of anti-CD3 plus either 50μg/ml anti-PD-1 or an isotype control for 72 hours. Addition of anti-PD-1 to cultures significantly enhanced the proliferative response of T cells in splenocyte cultures at both high (8μg/ml) and lower (1.6μg/ml) concentrations of anti-CD3. Addition of anti-PD-1 had no effect on un-stimulated cultures (Figure 3.4b), demonstrating a requirement for co-ligation of TCR and PD-1 for PD-1 to mediate negative signalling.

3.2.3. PD-1 signals are required to limit a productive immune response

PD-1 and PD-L1 are expressed highly on OT-II cells on the path to immunity, Figure 3.1 and (Hochweller and Anderton, 2005). Moreover, both PD-L1 and PD-L2 were up-regulated on DC following immunogenic treatment. Considering these expression data, and the enhancement of in vitro T cell responses seen upon blockade of PD-1 signals, the role of PD-1 mediated signals in the induction of a productive immune response was determined. OT-II cells were transferred to C57BL/6 hosts, one day prior to treatment with 500μg of pOVA plus 30μg of the adjuvant LPS i.v. (pOVA+LPS). At the same time, mice received 250μg of either anti-PD-1 or an
isotype control i.p. Mice were sacrificed at day 7 post pOVA treatments, spleens sampled and \textit{ex vivo} responses assayed (Figure 3.5a).

Blockade of PD-1 signals following immunogenic stimuli increased \textit{ex vivo} recall responses (Figure 3.5). Administration of anti-PD-1 to LPS primed mice led to a significant enhancement in the percent of OT-II cells in the spleen at day 7 (Figure 3.5b), and \textit{ex vivo} recall responses to pOVA. IL-2 and proliferative responses were significantly increased (Figure 3.5c-d), most likely due to the increased expansion of OT-II cells seen upon treatment with anti-PD-1.

To further explore the role of PD-1 in active immune responses the effect of blocking PD-1 signals following immunisation of mice with Complete Freund’s Adjuvant (CFA) and peptide was determined. CFA is a powerful adjuvant which, unlike LPS, leads to a prolonged immune response against the co-administered peptide. OT-II cells were transferred to C57BL/6 hosts, one day prior to immunisation with pOVA in CFA. At the same time as immunisation, mice received either 250\(\mu\)g of anti-PD-1 or an isotype control i.p. Mice were sacrificed 10 days post-immunisation, spleens and LN sampled and \textit{ex vivo} recall assays performed (Figure 3.6a). Administration of anti-PD-1 was seen to enhance pOVA immunisation (Figure 3.6). \textit{Ex vivo} recall assays of splenocytes from anti-PD-1 treated mice produced higher levels of IL-2 (Figure 3.6c) and proliferated to a greater degree (Figure 3.6d). Enhanced \textit{ex vivo} recall responses were again due to an increased expansion of OT-II cells upon treatment with anti-PD-1 (Figure 3.6b). A similar pattern of responsiveness was seen in the LN (data not shown).

\textbf{3.2.4. Blockade of PD-1 signals does not convert a tolerogenic dose of peptide to an immunogenic dose.}

Tolerance can be induced in adoptively transferred OT-II cells following treatment with soluble pOVA in PBS. As previously discussed, such a tolerogenic protocol is well established and is termed peptide induced T cell tolerance or therapeutic tolerance. Following peptide treatment peptide-reactive T cells initially expand. However, this is only a transient activation and the cells are quickly deleted from the
system. Those which remain are highly unresponsive (Kearney et al., 1994; Liblau et al., 1996). Administration of anti-PD-1 with the tolerising dose of pOVA could potentially overcome the tolerance induction process and lead to full T cell activation. Probst et al demonstrated this when they showed that loss of PD-1 from CD8+ T cells prevented tolerance induction following T cell encounter with a resting, tolerogenic DC (Probst et al., 2005). More importantly, they showed that loss of PD-1 did not only prevent tolerance induction, but resulted in robust priming of the T cells; hence loss of PD-1 turned a tolerogenic signal into an immunogenic one. OT-II cells were transferred to C57BL/6 hosts one day prior to treatment with a tolerising dose of pOVA i.v. At the same time, mice received either 250µg of anti-PD-1 or an isotype control i.p. A cohort of mice were treated with 500µg of pOVA + 30µg LPS as an immunogenic control. Mice were sacrificed at day 7 post peptide treatments and \textit{ex vivo} responses of splenocytes assayed (Figure 3.7a).

Administration of anti-PD-1 with a tolerising dose of antigen did not convert tolerance to immunity. On the contrary, anti-PD-1 had no effect on the tolerant state induced in the OT-II cells (Figure 3.7). Similar levels of IL-2 production (Figure 3.7c) and proliferation (Figure 3.7d) were seen in splenocyte cultures from pOVA treated mice given anti-PD-1 and the isotype control. Tolerised groups both produced minimal levels of effector cytokine; IFNγ produced from cultures stimulated with 100µM pOVA was 0.18±0.04ng/ml, 0.8±1ng/ml and 80.3±34ng/ml for pOVA+isotype treated, pOVA+anti-PD-1 treated, and pOVA+LPS treated respectively. Hence, very low \textit{ex vivo} recall responses were seen from both pOVA-treated groups. Tolerance induction involves the deletion of peptide-reactive T cells following peptide treatment, leaving only a small number of peptide reactive T cells; this can be seen here (Figure 3.7b). Following administration of pOVA few OT-II cells persist in the spleen; administration of anti-PD-1 does not alter this. In all aspects examined, mice which received anti-PD-1 and a tolerising dose of pOVA responded exactly as tolerant control mice. This was significantly different to the responses seen in pOVA+LPS treated mice, in which OT-II cells expanded and showed robust \textit{ex vivo} recall responses to pOVA.
3.2.5. Blockade of PD-1 does not prevent the establishment of tolerance.

Data shown above demonstrated that anti-PD-1 could not convert tolerance to immunity (Section 3.2.4). However, these experiments did not confirm that tolerance had actually been induced in the OT-II cells following administration of anti-PD-1. As such it was necessary to determine whether tolerance had been established in OT-II cells following peptide treatment co-administered with anti-PD-1. Previously, Hochweller et al demonstrated that concomitant administration of anti-OX40 or anti-CD40 did not turn a tolerising dose of pOVA into an immunogenic dose (Hochweller et al., 2006a). However, when responsiveness of OT-II cells was examined after pOVA challenge, OT-II cells had not been rendered tolerant, i.e. ligation of OX40 or CD40 prevented the induction of tolerance.

The design of the next experiment is shown in Figure 3.8a; OT-II cells were transferred to C57BL/6 hosts one day prior to treatment with pOVA i.v. At the same time, mice received either 250μg of anti-PD-1 or isotype control i.p. At day 7 mice were immunised with pOVA in CFA, and ex-vivo recall responses of splenocytes and LN were examined 10 days later. A cohort of mice were treated with PBS following OT-II transfer, these are the immunogenic control mice which mount a robust immune response upon immunisation with pOVA in CFA.

The percent of OT-II cells persisting in the spleen (Figure 3.9a) and LN (data not shown) were similar between anti-PD-1 treated and isotype treated tolerant mice, with few OT-II cells remaining. This reduced number of Ag-specific OT-II cells resulted in limited ex vivo recall responses to pOVA; splenocyte (data not shown) and LN (Figure 3.9b-e) cultures from all pOVA treated mice produced no IL-2, IFNγ, or IL-17. All pOVA treated mice also failed to proliferate in response to pOVA stimulation (Figure 3.9e). These data demonstrate that tolerance was induced in pOVA treated mice upon concomitant administration of anti-PD-1. The recall responses of the pOVA treated mice were significantly reduced compared to the responses seen from PBS treated, control mice.
Collectively, these data demonstrate that anti-PD-1 treatment along with a tolerising dose of pOVA did not prevent the establishment of tolerance in pOVA-reactive OT-II cells.

3.2.6. Blockade of PD-1 does not overcome tolerance once it has been established.

The data shown above demonstrate that PD-1 mediated signals are not required for the induction or establishment of peptide-induced T cell tolerance. However, PD-1 mediated signals could be important in the maintenance of the tolerant state in OT-II cells. Data presented in Figures 3.5-3.6 demonstrate an important role of PD-1 signals in restraining the immune response. Therefore it is possible that PD-1 signals were important in maintaining tolerance in the OT-II cells upon immunisation with pOVA in CFA, by preventing their expansion. Thus ligation of PD-1, mediating negative signals within the T cells, could help maintain hyporesponsiveness in the small number of persisting OT-II cells. To explore this possibility, experiments were performed as outlined in Figure 3.8b; with anti-PD-1 or an isotype control being administered at the same time as mice were immunised with pOVA in CFA. Ex vivo recall responses were examined 10 days after immunisation.

Few OT-II cells persisted following pOVA treatment; administration of anti-PD-1 did not alter the percentage of OT-II cells in either the spleen (Figure 3.10a) or LN (data not shown). For all other parameters measured, anti-PD-1 treated mice responded similarly to tolerant control mice. Indicative of the low numbers of OT-II cells remaining following pOVA treatment, both pOVA treated groups produced no IL-2 (Figure 3.10b). Proliferative responses from tolerised groups were also minimal (Figure 3.10c). Intracellular cytokine staining was performed on splenocyte cultures stimulated overnight with 100μM pOVA. The few remaining OT-II cells were hyporesponsive, as they produced no IFN\(\gamma\) (Figure 3.10d) or IL-17 (Figure 3.10e). All responses from pOVA treated mice were significantly lower than those of the immunogenic control mice which mounted strong responses to pOVA ex vivo.
These data show that tolerance cannot be overcome by the administration of anti-PD-1 at the same time as pOVA immunisation in CFA. Thus, PD-1 signals are not required for the maintenance of the tolerant state in OT-II cells upon challenge.

3.2.7. PD-1 knockout peptide-reactive T cells can be rendered tolerant through administration of soluble peptide.

The observations discussed above suggest that PD-1 signalling plays no role in peptide-induced T cell tolerance in OT-II cells. These data are in contrast to other studies which suggest that signals mediated through PD-1 are vital for the establishment and maintenance of T cell tolerance (Keir et al., 2007; Martin-Orozco et al., 2006; Tsushima et al., 2007). In order to validate the data shown here, it was necessary to determine whether it was possible to induce peptide-induced T cell tolerance in PD-1 knockout (PD-1⁻/⁻) peptide-reactive T cells. The experiments in this section were performed using a different TCR transgenic; note they were performed by Dr. F Frommer, University of Mainz. The 2D2 TCR transgenic is a CD4+ T cell transgenic where all T cells are reactive to the 35-55 peptide of myelin oligodendrocyte glycoprotein (pMOG) (Bettelli et al., 2003). The susceptibility of 2D2 T cells to peptide-induced T cell tolerance was compared to that of PD-1⁻/⁻ 2D2 cells. Peptide-induced T cell tolerance can be induced in adoptively transferred 2D2 cells following treatment with 200μg of soluble pMOG administered either i.v. or i.p. As with the OT-II model, peptide treatment results in deletion of 2D2 cells, such that tolerance is manifest by the fact that few peptide-reactive T cells remain in the peripheral T cell pool. Wild type 2D2 or PD-1⁻/⁻ 2D2 cells were transferred to C57BL/6 hosts, one day prior to treatment with 200μg pMOG (tolerance) or PBS (immunity) i.p. and mice were immunised with pMOG in CFA 7 days later. Mice were sacrificed 10 days post-immunisation and spleen and LN sampled. Persistence of 2D2 cells was examined by flow cytometry; the prediction being that tolerance induction would be reflected by an absence of 2D2 cells.

PBS treatment prior to 2D2 transfer did not induce tolerance as 2D2 cells expanded upon immunisation with pMOG in CFA (Figure 3.11). Importantly, upon immunisation and after initial administration of PBS, 2D2 PD-1⁻/⁻ cells expanded to a
greater degree than wild-type 2D2 cells. This was seen in both the spleen (Figure 3.11a) and LN (Figure 3.11b). These data are consistent with that shown in section 3.2.3, where it was demonstrated that blockade of PD-1, through administration of anti-PD-1, during the induction of peptide-specific immunogenic responses, permits greater expansion of peptide-reactive T cells.

Tolerised, pMOG treated mice all exhibited deletion of 2D2 cells; this was true for both wild-type 2D2 and 2D2 PD-1 cells. At day 10 post immunisation few 2D2 cells persisted in any mouse treated with pMOG. The reduced numbers of peptide-reactive T cells following pMOG treatment demonstrated that peptide-induced T cell tolerance was effectively induced in PD-1/− 2D2 cells.

3.2.8. Different patterns of expression of PD-1 and PD-L1 on CD4+ and CD8+ T cells.

The data presented here demonstrated that PD-1 plays no role in peptide-induced T cell tolerance in either the OT-II or the 2D2 system. These data appear to directly contrast other studies, specifically a study by Tsushima et al who demonstrated that PD-1 signalling was vital for the induction, establishment and maintenance of tolerance in OT-I cells following pOVA administration via the i.v. route (Tsushima et al., 2007). Importantly, a major difference exists between the data presented here and that of Tsushima et al; here the role of PD-1 signals in peptide-induced tolerance in CD4+, not CD8+, T cells was examined. CD4+ and CD8+ T cells may have different requirements for activation, and PD-1 signals could play a more important role in CD8+ T cells than in CD4+ T cells. In order to begin to establish whether this was the case, the expression of both PD-1 and PD-L1 on CD4+ and CD8+ T cells was examined in vitro following stimulation with increasing concentrations of anti-CD3 after 1, 2 and 3 days of culture.

Following stimulation with anti-CD3, PD-1 expression was higher on CD8+ T cells compared to CD4+ T cells (Figure 3.12a). Maximal PD-1 expression on CD8+ T cells was reached after 2 days in culture, and remained high at day 3. PD-1 expression on CD4+ T cells was similar after 1 and 2 days in culture. However,
expression was notably decreased by day 3. Differences in the level of PD-1 expression on CD4+ and CD8+ cells were greatest following stimulation with higher concentrations of anti-CD3.

Expression of PD-L1 was very similar for both CD4+ and CD8+ T cells (Figure 3.12b). Consistent with data shown in Figure 3.1b, PD-L1 was expressed to a high degree on naive T cells, which then increased following stimulation (Figure 3.12b). CD4+ and CD8+ T cells showed similar patterns of expression at both day 1 and 2. PD-L1 expression at day 3 was different on CD4+ and CD8+ T cells; CD4+ T cells still showed high levels of expression, however expression did decline slightly with enhanced stimulation. In contrast, PD-L1 expression on CD8+ T cells at this time point had declined on T cells stimulated by low concentrations of anti-CD3, but remained at levels similar to naïve cells following stimulation with higher concentrations of anti-CD3.

Collectively, these data show that following in vitro culture with anti-CD3, CD8+ T cells express PD-1 to a greater degree than CD4+ T cells, however both express PD-L1 to a similar level.

3.2.9. Anti-PD-1 enhances in vitro proliferative responses of CD8+ T cells to a greater degree than CD4+ T cells.

Demonstration that PD-1 is expressed to a higher level on CD8+ T cells compared to CD4+ T cells, upon stimulation with anti-CD3, prompted the examination of in vitro CD4+ and CD8+ T cells responses upon blockade of PD-1 signalling. To compare CD4+ and CD8+ T cell responsiveness, splenocytes where stained with CFSE and stimulated for 72 hours with 1.6μg/ml anti-CD3 in vitro. Anti-PD-1 or an isotype control, were added to all culture wells. Proliferative responses of CD4+ and CD8+ T cells were examined separately by flow cytometry.

CFSE dilution indicated both CD4+ (Figure 3.13a) and CD8+ (Figure 3.13b) T cells had proliferated upon stimulation with anti-CD3. Addition of anti-PD-1 to cultures enhanced the proliferative responses of both CD4+ and CD8+ T cells; however, the
proliferation of CD8+ T cells was enhanced to a greater extent than that of CD4+ T cells (Figure 3.13c-d). When examining the fold difference and overall change caused by addition of anti-PD-1, the blocking antibody had the greatest effect on CD8+ T cells. More CD8+ T cells entered into cycle, as demonstrated by an increase in the percent of divided cells (Figure 3.13b), and those CD8+ T cells which did enter the cell cycle proliferated to a greater degree, as indicated by the significantly increased percent of CD8+ T cells in the later generations (Figure 3.13b).

As previously stated, PD-1 mediated inhibition of T cell responsiveness is most profound at low levels of TCR stimulation. Therefore this in vitro stimulation assay was also performed with greater TCR stimulation (8µg/ml anti-CD3), to determine whether blockade of PD-1 had differential effects on CD4+ and CD8+ T cells following higher TCR stimulation. Addition of anti-PD-1 to these culture wells only significantly enhanced the proliferation of CD8+ T cells, though to a lesser degree than seen in Figure 3.13 (Appendix Figure A.1). Addition of anti-PD-1 had no effect on the proliferation of CD4+ T cells in these cultures receiving greater TCR stimulation.

3.2.10. Blockade of PD-1 does not prevent peptide-induced T cell tolerance in CD4+ or CD8+ T cells.

The above data were suggestive of a more prominent role of PD-1 signalling for CD8+ T cells compared to CD4+ T cells. This could be tested by examining peptide-induced tolerance in CD4+ and CD8+ T cells in a system where PD-1 signalling was prevented. This needed to be performed in a model system where one peptide contained both CD4+ and CD8+ T cell epitopes. The MOG peptide 35-55 (pMOG) contains a CD4+ T cell epitope; 40-47 (Sweeney et al., 2007), and a CD8+ T cell epitope; 37-46 (Ford and Evavold, 2005). Moreover, tolerance can be induced in the whole T cell population following administration of this peptide i.v. (Hochweller et al., 2006a; Leech et al., 2007). Thus, this peptide could be employed to establish whether peptide-tolerance could be induced in CD4+ and CD8+ T cells following blockade of PD-1 mediated signals. C57BL/6 mice were treated with
200μg pMOG or PBS i.v., at the same time 250μg of anti-PD-1 or an isotype control were administered i.p. Mice were immunised 7 days later with pMOG in CFA, and ex vivo recall responses were examined 10 days after immunisation.

Spleocytes and LN cells were used for ex vivo CFSE proliferation assays and intracellular cytokine staining (ICS) to determine the responsiveness of CD4+ and CD8+ T cells separately. Figure 3.14 shows that tolerance was successfully induced in both CD4+ and CD8+ T cells following treatment with pMOG, with reduced IFNγ and proliferative responses seen compared to PBS treated mice. Strikingly, tolerance was successfully induced in both CD4+ and CD8+ T cells following administration of anti-PD-1.

These initial experiments highlighted that PD-1 signalling may not be as essential for peptide-induced tolerance induction in CD8+ T cells, as other investigators have suggested. Alternatively, PD-1 signalling could simply play a minimal role in tolerance induction in the particular model of tolerance used in the studies shown here, or the dose of antibody used (although already shown to be effective) may be ineffective for preventing tolerance in CD8+ T cells. As such, it was necessary to establish whether peptide-induced T cell tolerance could be induced in PD-1−/− CD8+ T cells. C57BL/6 mice and PD-1−/− mice were treated with 200μg pMOG or PBS i.v. and immunised 7 days later with pMOG in CFA. Ex vivo recall responses were examined 10 days after immunisation. Ex vivo readouts of tolerance have not yet been optimised for this experimental system, and as such, an appropriate readout of tolerance in both CD4+ and CD8+ T cells cannot be examined. However, these experiments did demonstrate an enhancement of T cell expansion when PD-1 signalling was absent following immunogenic challenge of mice (Figure 3.15). Robust pMOG-specific T cell responses were mounted in mice treated with PBS i.v., and subsequently immunised with pMOG in CFA. Therefore productive T cell responses could be followed in wild-type and PD-1−/− mice; enhancement of both CD4+ and CD8+ T cell percentages were seen in both the LN and spleen of PD-1−/− mice (Figure 3.15a-b). CD4+ and CD8+ T cells also exhibited a more activated phenotype (as assessed by CD44 and CD62L expression) when isolated from the PD-
1\(^+\) (Figure 3.15c-d). However, due to limited experimental mice, these experiments were unable to establish whether loss of PD-1 had differential effects on CD8+ T cell compared to CD4+ T cells.

3.2.11. Blockade of PD-1 does not affect the suppressive capacity of CD4+CD25+foxp3+ Tregs.

The data presented in this chapter established that PD-1 signalling plays an important role in limiting T cell responsiveness during the generation of a robust T cell response. However, PD-1 was found to play no role in peptide-induced T cell tolerance in CD4+ T cells. A contentious area is the role of PD-1 on Tregs. Different investigators have shown that blockade of PD-1 on Tregs either abrogates their *in vitro* suppressive capacity (Polanczyk et al., 2007) or has no effect (Wang et al., 2007). Here, the ability of purified CD4+CD25+ cells to suppress the proliferative response of naïve effector T cells was examined in the presence of anti-PD-1 or an isotype control (Figure 3.16). CD4+CD25+ Tregs were sorted to 90-95% purity and determined to express foxp3 to a high degree (~80% of cells were foxp3+). The ability of these Tregs to suppress the anti-CD3 stimulated responses of naïve T cells was not altered by addition of anti-PD-1.
3.3. DISCUSSION

3.3.1. Expression of PD-L1 and PD-L2.

Previous data from the Anderton laboratory have shown that PD-1 is rapidly up-regulated on T cells during the induction of tolerance and immunity (Hochweller and Anderton, 2005). Moreover, the functional effects of PD-1 ligation can be observed before 24 hours (Chemnitz et al., 2004). These data suggest an important role for PD-1 in early T cell fate decisions.

Expression studies have shown that PD-L1 and PD-L2 exhibit disparate expression patterns, with PD-L1 being more widely expressed. Here, these studies were extended by tracking the expression of PD-L1 and PD-L2 on OT-II cells and DC following immunogenic or tolerogenic stimulus. The constitutive level of PD-L1 expression on T cells was up-regulated following either form of stimulation. However, PD-L1 levels were only increased on DC following immunogenic stimulation (Figure 3.1). Expression of PD-L2 however was more restricted, being found only on DC, and only at 24 hours following immunogenic stimulus (Figure 3.2). In line with other studies, (Hochweller and Anderton, 2005), these data demonstrate that immunogenic stimulation predominately affects the phenotype of the DC. The expression patterns on *ex vivo* T cells and DC described here are consistent with the *in vitro* expression patterns noted by Yamazaki et al (Yamazaki et al., 2002). The expression of PD-1 ligands was also examined following immunisation of mice with pOVA in CFA; these experiments yielded similar results to pOVA+LPS treatment (Figure 3.3).

The data presented here continue to demonstrate the different expression patterns of the PD-1 ligands. The distinct expression pattern of these two ligands has been shown to be due to differential regulation at the level of transcription. Liang et al demonstrated that reduced NF-κB transcription drastically reduced PD-L2 expression but had no effect on the expression of PD-L1 (Liang et al., 2003). Several groups have begun to explore the functional outcomes of PD-1 ligation by PD-L1 compared to PD-L2. Many studies have shown that blockade or genetic ablation of PD-L1 has a greater effect on immune-driven conditions than loss of PD-L2. This has been
described in the NOD mouse model of diabetes (Ansari et al., 2003), in establishment of fetomaternal tolerance (Guleria et al., 2005), and in allograft tolerance (Wang et al., 2007). In fact, in the NOD model of diabetes, anti-CD3 induced expression of PD-L1 on CD3+ cells has been seen to decline with age, suggesting a reduction in this inhibitory pathway could contribute to susceptibility of these mice to diabetes onset (Ansari et al., 2003). The role of PD-L1 versus PD-L2 is not so clear in EAE, where disruption of PD-L1 or PD-L2 has been shown to enhance disease severity and onset in a strain dependent manner (Salama et al., 2003; Zhu et al., 2006); the reasons for these differences in EAE remain unclear. Collectively these data show a more important role for PD-1:PD-L1 interactions in maintaining tissue tolerance. The dominance of PD-L1 over PD-L2 in this role is indicative of its expression on non-lymphoid cells, PD-L1 is found to be expressed on a variety on non-lymphoid cell types and at sites of immune privilege (Hori et al., 2006; Liang et al., 2003; Rodig et al., 2003).

The results presented in section 3.2.1 demonstrate that PD-L1 and PD-L2 are rapidly up-regulated on the DC following immunogenic stimulation. These data suggest an important role for the PD-1:PD-L interaction in T cell activation and the initiation of a productive T cell response. However, this expression pattern does not preclude a role for PD-1:PD-L in tolerance. Due to the high constitutive expression of PD-L1 on DC and the enhanced expression of PD-L1 on T cells, the PD-1:PD-L1 interaction specifically could be important in the induction of peptide-tolerance. The expression data raise an important question of the role of PD-L1 in T cell immunity and tolerance. As PD-L1 is constitutively expressed on T cells it may well play an important role in both these processes. Moreover, ligation of PD-L1 by CD80 has also been shown to be mediate a negative signal (Butte et al., 2007); PD-L1 can therefore mediate a negative signal to the T cell by two receptors. These data highlight important avenues of research; it would be pertinent to establish the role of PD-L1 in peptide-induced T cell tolerance and immunity. Similar experiments to those discussed in this chapter could be performed with administration of anti-PD-L1 to ascertain this.
3.3.2. PD-1 plays an important role in limiting T cell responses.

The initial *in vitro* experiments presented in this chapter show that administration of anti-PD-1 to splenocyte cultures enhanced the cytokine production and proliferative response of T cells (Figure 3.4). These data are consistent with other studies showing that loss of PD-1 signals enhanced T cell responsiveness *in vitro* (Keir et al., 2006). Freeman et al have previously shown that co-ligation of CD3 and PD-1 led to decreased proliferation (Freeman et al., 2000). Most importantly they highlighted that PD-1 signals were most effective a low levels of TCR stimulus, causing the greatest inhibition of responsiveness at low concentrations of anti-CD3. Here, the proliferative responses of splenocytes in the presence of anti-PD-1 or an isotype control were compared at a high (8 µg/ml) and a lower (1.6 µg/ml) concentration of anti-CD3. The results of these experiments did not reflect the previously reported differential effect of PD-1 mediated signals at different levels of TCR stimulus. Anti-PD-1 enhanced the proliferative responses of T cells at both high and lower levels of TCR stimulus to an equal degree. However, when responsiveness of CD4+ and CD8+ T cells were examined separately (Figure 3.13) it was possible to see that addition anti-PD-1 could only enhance proliferation of CD4+ T cells at lower levels of TCR stimulus (Appendix Figure A.1).

The data in this chapter highlight an important role for PD-1 mediated signals in curtailing initial T cell activation, as administration of anti-PD-1 enhanced immunogenic T cell responses (Figure 3.5 and 3.6). This could have been due to an expanded number of OT-II cells or maintenance of OT-II cells in a heightened activation state capable of greater effector function, both have been reported by other authors (Martin-Orozco et al., 2006; Salama et al., 2003). Figures 3.5 and 3.6 show that the enhanced *ex vivo* responses seen from anti-PD-1 treated mice correlate with enhanced numbers of OT-II cells. Blockade of PD-1 permits greater OT-II cell expansion upon immunogenic treatment. Loss of PD-1 mediated signals to T cells has been shown to allow greater expansion of antigen-reactive T cells in EAE, diabetes, and allograft rejection (Ansari et al., 2003; Ito et al., 2005; Salama et al., 2003). Similar to experiments presented here, Salama et al transferred DO11.10 splenocytes to BALB/c mice and followed the Ag-reactive T cells following
immunisation with pOVA (Salama et al., 2003). Administration of anti-PD-1 led to a significant expansion of DO11.10 cells compared to administration of an isotype control.

This enhanced proliferation of T cells upon disruption of PD-1 signals is a T cell-intrinsic effect; loss of PD-1 mediated signals enhances TCR signalling. This was shown in critical experiments by Sharpe and colleagues, who transferred OT-I cells and PD-1−/− OT-I cells to mice expressing low levels of pOVA in the pancreas (RIP-OVA\textsubscript{low} mice). In these mice, the low level of Ag presentation was insufficient to activate wild-type OT-I cells. In contrast, PD-1−/− OT-I cells proliferated vigorously to this low level of Ag and caused diabetes in mice (Keir et al., 2007). Thus, loss of PD-1 allowed activation of T cells to a density of p:MHC which would usually have been too low to cause activation.

Taken together, these data demonstrate a vital role for PD-1 in limiting T cell activation upon encounter of cognate p:MHC. In an extension to previous studies, data presented here demonstrate that blockade of PD-1, specifically during initial T cell and APC encounters, is sufficient to allow greater expansion of stimulated T cells. Of interest, PD-1:PD-L1 interactions could mediate reciprocal regulation between T cells as both molecules are expressed by T cells. Although this would not play a role during initial T cell priming, as PD-1 needs to be recruited to the site of the TCR to mediate its effects (Bennett et al., 2003), PD-1:PD-L1 interactions between T cells at later time-points during the response could also limit T cell expansion. Importantly, loss of PD-1 mediated signals removes inhibitory signals to the T cell and permits greater expansion of T cells upon immunogenic stimulation. Thus, PD-1 signalling is critical in limiting effector T cell responses.
3.3.3. PD-1 plays no role in the induction of peptide-induced T cell tolerance in CD4+ T cells.

The first direct analysis of PD-1 involvement in peptide-induced CD4+ T cell tolerance has been performed here. The role of PD-1 mediated signals during T cell interactions with an APC during tolerance induction was determined. Previous reports have focused on the role of PD-1 signals in disease models, and as such these have included the role of PD-1:PD-L interactions from the non-lymphoid/tissue compartment. The data presented in this chapter exclude this possibility, tolerance is induced in OT-II cells through their interaction with host APC bearing peptide.

Previous data have shown that loss of PD-1 from a responding T cell can turn a tolerogenic stimulus into a priming one (Probst et al., 2005), however, this was not the case following administration of anti-PD-1 during the induction of peptide-induced OT-II cell tolerance (Figure 3.7). PD-1 signals were also dispensable for the induction and maintenance of T cell tolerance (Figure 3.9, 3.10, 3.11 and 3.14). This was most striking in the 2D2 PD-1\(^{-/-}\) studies where peptide-induced T cell tolerance was still induced (Figure 3.11), despite PD-1\(^{-/-}\) cells being capable of greater expansion, as shown in the non-tolerant mice. These data could appear to directly contrast other studies (Fife et al., 2006; Keir et al., 2007; Martin-Orozco et al., 2006; Tsushima et al., 2007). Here it is suggested that PD-1 mediated signals play no role in peptide-induced T cell tolerance in CD4+ T cells. Previous studies examining the role of PD-1 in tolerance induction have specifically focused on CD8+ T cells, identifying PD-1 to be critical for tolerance induction in these cells. Fife et al have, however, demonstrated an important role for PD-1:PD-L1 in the maintenance of CD4+ T cell tolerance (Fife et al., 2006). Transfer of peptide-coupled fixed APC prevented rapid onset diabetes caused by transfer of TCR transgenic BDC2.5 CD4+ T cells. Tolerogenic treatment, although preventing diabetes, did not completely prevent islet infiltration and BDC2.5 cells persisted in the pancreas post tolerance induction. Administration of anti-PD-1 or anti-PD-L1, but not anti-PD-L2, prevented tolerance and resulted in diabetes. Importantly, the PD-1:PD-L1 interaction was shown not to be required during the initial APC-T cell encounter, as peptide-coupled fixed PD-L1\(^{+/-}\) APC were equally capable of tolerance induction.
These data, together with the demonstration that anti-PD-1 or anti-PD-L1 treatment at later time points reversed tolerance, suggested an important role for PD-1 signals in maintaining tolerance within tissues. The authors also showed that PD-1 signals were important in maintaining tolerance following anti-CD3 treatment. These data would therefore suggest that in the studies presented in this chapter, following tolerance induction, administration of anti-PD-1 at the same time as immunising mice with pOVA in CFA, would overcome established tolerance and permit OT-II cell responsiveness (Figure 3.10). This did not occur, and instead tolerance to pOVA was maintained. Different mechanisms of tolerance induction can explain why anti-PD-1 treatment overcomes tolerance induced by peptide-coupled fixed APC but not peptide treatment. Following tolerance induction using peptide-coupled fixed APC, peptide-reactive T cells persist. This is not the case following peptide-induced T cell tolerance, hence anti-PD-1 administration at this later time-point could not overcome tolerance as there were few OT-II cell remaining for the antibody to act-upon.

3.3.4. A role for PD-1 signals in CD4+ versus CD8+ T cells.

The results presented in this chapter suggest PD-1 signalling to CD4+ T cells is more important in restraining a productive T cell response than in the induction of tolerance, yet PD-1:PD-L has been shown to be vital for tolerance induction in CD8+ T cells in a variety of tolerance models. In fact, the PD-1:PD-L interaction is critical for the induction of peptide-induced T cell in tolerance in CD8+ OT-I cells (Tsushima et al., 2007). A handful of studies have indicated a dichotomy between CD4+ and CD8+ T cells in their requirement for PD-1 mediated signals. Recently, Haspot et al demonstrated that the PD-1:PD-L1 interaction is required for deletional tolerance of CD8+ but not CD4+ T cells in a model of transplantation tolerance (Haspot et al., 2008). A differential requirement for PD-1 mediated signals between CD4+ and CD8+ T cells was also examined here.

Consistent with in vivo data (Hochweller and Anderton, 2005; Tsushima et al., 2007), CD4+ and CD8+ T cells show broadly similar kinetics of expression of PD-1 when stimulated with anti-CD3 in vitro (Figure 3.12). However, Figure 3.12a shows that PD-1 is expressed to a higher degree and for longer on CD8+ T cells than CD4+
T cells. Consistent with this, Figure 3.13 shows that anti-PD-1 treatment of *in vitro* cultures, enhances the proliferative responses of CD8+ T cells to a greater extent than CD4+ T cells. These results suggest that PD-1 mediated signalling is more important in limiting the responses of CD8+ T cells than CD4+ T cells. Supporting this, CD8+ T cells have been shown to be more sensitive to PD-1 mediated inhibition than CD4+ T cells (Carter et al., 2002). Carter et al proposed that this was due to CD4+ T cells producing higher levels of IL-2 upon stimulation. IL-2 can overcome PD-1 mediated inhibition of T cells, and therefore CD4+ T cells would be less sensitive to PD-1 signalling.

In contrast to Tsushima et al (Tsushima et al., 2007), data presented in Figure 3.14 demonstrate that peptide-induced T cell tolerance can be induced in CD8+ T cells upon blockade of PD-1 signals. Treatment of C57BL/6 mice with pMOG i.v. induced tolerance in CD4+ and CD8+ T cells when anti-PD-1 was administered along with the tolerising dose of peptide. It is of importance to determine whether this can still occur in the PD-1\(^{-/-}\), but experiments so far have proved inconclusive. These experiments have established that, consistent with the data presented in Figure 3.4 and 3.5, loss of PD-1 signals can lead to greater expansion and activation of T cells (Figure 3.15). These experiments need to be optimised in order to advance on the data presented in this chapter as two important questions remain outstanding; firstly, can peptide-tolerance be induced in CD8+ PD-1\(^{-/-}\) T cells? Secondly, are PD-1 mediated signals more important in curtailing CD8+ than CD4+ T cell responses?

### 3.3.5 Role of PD-1:PD-L on regulatory T cells.
Collectively, data shown in this chapter demonstrate that PD-1 signalling plays no role in the induction of T cell intrinsic tolerance in CD4+ T cells. There is much debate over whether PD-1 plays a role in the T cell extrinsic tolerance mediated by Tregs. *In vivo* studies have shown that Treg interactions via PD-1 are vital for the maintenance of fetomaternal (Habicht et al., 2007) and allograft (Sandner et al., 2005) tolerance. However, how the suppressive capacity of Tregs *in vitro* is altered upon inhibition of PD-1:PD-L interactions remains unknown. In conflicting studies Polanczyk et al and Wang et al demonstrated that PD-1\(^{-/-}\) Tregs are capable and
incapable of suppression respectively (Polanczyk et al., 2007; Wang et al., 2007). Figure 3.16 shows that blockade of PD-1 signals has no effect on the ability of Tregs to mediate suppression in an in vitro suppression assay. Examination of the phenotype of PD-1^-/- mice is consistent with lack of a Treg defect; these mice do not show the multi-organ autoimmunity diseases seen in mice lacking Tregs. Instead, PD-1^-/- mice develop organ specific autoimmunity which alters depending on the strain (Nishimura et al., 1999; Nishimura et al., 2001); essentially PD-1^-/- is thought to potentiate the underlying autoimmune susceptibility of mice strains.

3.3.6. Concluding remarks.

The outcome of the T cell-APC interaction during initial T cell activation is primarily dependent on the degree of costimulation. The data presented in these studies have explored the role of PD-1 mediated signals during this initial T cell-APC encounter. By administration of anti-PD-1 during priming and using PD-1^-/- T cells, the data show that PD-1 signals are important in curtailing immune responses. More interestingly, the data demonstrate that PD-1 signalling plays no role in the induction or maintenance of peptide-induced T cell tolerance in CD4+ T cells. As a number of studies have highlighted that PD-1 signals play a vital role in tolerance induction in CD8+ cells, these data highlight an emerging dichotomy of PD-1 function; PD-1 mediated inhibition is more effective on CD8+ T cells than CD4+ T cells. The studies presented here began to examine this possibility, and show that inhibition of PD-1 mediated signalling allows CD8+ T cells to proliferate to a greater degree than CD4+ T cells.

PD-1 mediated signalling is known to play a vital role in peripheral tolerance mechanisms, specifically in the maintenance of T cell tolerance within tissues. Data presented here show that this important coinhibitory molecule plays no role in peptide-induced T cell tolerance in CD4+ T cells.
**Figure 3.1. PD-L1 expression during the induction of tolerance and immunity.**

**a.** Experimental design; 1-2x10^6 Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior to treatment with PBS, 500μg pOVA or 500μg pOVA plus 30μg LPS i.v. Mice were sacrificed 1, 2, and 3 days later, and spleens sampled. Expression patterns on **a**, OT-II cells (CD4+Ly5.1+ cells) and **b**, all CD11c+ cells was determined via flow cytometry. Histograms show PD-L1 (black line) and isotype control (grey line) staining on **d**, OT-II cells and **e**, CD11c+ cells. Numbers on histograms represent the average percentage of cells which are PD-L1+. Results are representative of 2 experiments with 2-3 mice per group.
Figure 3.2. PD-L2 expression during the induction of tolerance and immunity. 1-2×10⁶ Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior to treatment with PBS, 500µg pOVA or 500µg pOVA plus 30µg LPS i.v. Mice were sacrificed 1, 2, and 3 days later, and spleens sampled. Expression of PD-L2 on a, OT-II cells (Ly5.1+ cells) and b, all CD11c+ cells was determined via flow cytometry. Histograms show PD-L2 (black line) and isotype control (grey line) staining. Numbers on histograms represent the average percentage of cells which are PD-L2+, no value has a standard deviation greater than ±0.07%. Results are representative of 2 experiments with 2-3 mice per group.
Results are representative of 1 experiment with 2-3 mice per group. CD11c+ cells no value has a standard deviation greater than ±3.5%, PD-L+. For OT-II cells no value has a standard deviation greater than ±3.66%, for staining. Numbers on histograms represent the average percentage of cells which are stained. Histograms show PD-L1 or PD-L2 (black line) and isotype control (grey line) expression of PD-L1/2 on CD11c+ cells was determined by flow cytometry. 

Figure 3.3. PD-L1/2 expression following priming with pOVA in CFA. 1-2x10^6 Ly5.1+OT-II cells were transferred to C57BL/6 mice. 1 day later mice were immunised with pOVA in CFA s.c. Mice were sacrificed 2, 6, and 10 days after immunisation, and spleens sampled. a, Expression of PD-L1/2 on OT-II cells and b, expression of PD-L1/2 on CD11c+ cells was determined by flow cytometry. Histograms show PD-L1 or PD-L2 (black line) and isotype control (grey line) staining. Numbers on histograms represent the average percentage of cells which are PD-L+. For OT-II cells no value has a standard deviation greater than ±3.66%, for CD11c+ cells no value has a standard deviation greater than ±3.5%. Results are representative of 1 experiment with 2-3 mice per group.
Figure 3.4. Anti-PD-1 enhances responses of *in vitro* T cells cultures.
Splenocytes from OT-II mice were cultured with increasing concentrations of pOVA. 50μg/ml of anti-PD-1 or isotype control were added to each culture well as was 20μg/ml Polymyxin B. a, IFNγ production from splenocyte cultures was determined by ELISA. Significant differences shown were determined by unpaired T test. Error bars represent SEM and data is representative of 3 experiments with 2-4 culture wells per condition.

Splenocytes from C57BL/6 mice were stained with CFSE and were cultured without stimulation (b) or with plate bound anti-CD3 at the concentrations shown (c-d). 50μg/ml of anti-PD-1 (black bar) or isotype control (white bar) were added to each culture well from the start of culture. 72 hours later CFSE dilution was determined by flow cytometry. The percent of T cells (TCRβ+ cells) in each generation was calculated manually as described in materials and methods. Asterisk represent significant differences; *p<0.04, **p<0.01, ***p<0.001, as determined by unpaired T test. Error bars represent SEM and data is representative of 2 experiments with 3 wells per culture condition.
Figure 3.5. Anti-PD-1 enhances LPS stimulated responses of OT-II cells.

**a,** Experimental outline; 1-2x10⁶ Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior to treatment with 500µg pOVA plus 30µg LPS i.v. At the same time as LPS treatment mice also received 250µg of anti-PD-1 or an isotype control i.p. Mice were sacrificed 7 days post LPS treatment and spleens sampled. **b,** Bar chart showing OT-II cells in the spleen as a percentage of all CD4+ cells. *Ex vivo* **c,** IL-2 production and **d,** proliferation of splenocytes to increasing concentrations of pOVA. Asterisk represent significant differences; *p<0.025 and **p<0.0071, as determined by unpaired T test.

Error bars represent SEM and data is representative of 2 experiments with 3 mice per group.
Figure 3.6. Anti-PD-1 enhances CFA stimulated responses of OT-II cells.

a, Experimental outline; 1-2x10^6 Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior to immunisation with pOVA in CFA s.c. At the same time as immunisation mice also received 250µg of anti-PD-1 or an isotype control i.p. Mice were sacrificed 10 days later and spleens sampled. b, Bar chart showing OT-II cells in the spleen as a percentage of all CD4+ cells. Ex vivo c, IL-2 production and d, proliferation of splenocytes to increasing concentrations of pOVA. Asterisk represent significant differences; *p<0.04 and **p<0.0082, as determined by unpaired T test.

Error bars represent SEM and data is representative of 2 experiments with 3 mice per group.
Figure 3.7. Anti-PD-1 treatment does not convert a tolerogenic dose of pOVA to an immunogenic dose.

a, Experimental outline; 1-2x10^6 Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior to treatment with 500μg pOVA i.v. (tolerance) or 500μg pOVA+LPS i.v. (immunity). At the same time as peptide treatment mice also received 250μg of anti-PD-1 or an isotype control i.p. Mice were sacrificed 7 days later. 
b, Bar chart showing OT-II cells in the spleen as a percentage of all CD4+ cells. Ex vivo c, IL-2 production and d, proliferation of splenocytes to increasing concentrations of pOVA.

Responses of both tolerant groups are significantly different to the pOVA+LPS, immunity control; tolerant groups are not significantly different to each other. Significant differences shown were determined by an ANOVA using Tukey’s multiple comparison test. Error bars represent SEM and data is representative of 2 experiments with 3 mice per group.
Figure 3.8. Experimental outline of experiments determining the role of PD-1 mediated signals in peptide-induced T cell tolerance.

a, Administration of anti-PD-1 or an isotype control at the same time as peptide treatment. These experiments are discussed in section 3.2.5.

b, Administration of anti-PD-1 or an isotype control at the same time as immunisation of mice with pOVA in CFA s.c. These experiments are discussed in section 3.2.6.
Figure 3.9. Anti-PD-1 treatment does not prevent the establishment of peptide-induced tolerance in OT-II T cells.

1-2x10^6 Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior to treatment with 500μg pOVA i.v. (tolerance) or PBS i.v. (immunity). At the same time as peptide treatment mice also received 250μg of anti-PD-1 or isotype control antibody i.p. Mice were immunised with pOVA in CFA s.c., 7 days later and sacrificed 10 days post immunisation. a, Bar chart showing OT-II cells in the spleen as a percentage of all CD4+ cells. Ex vivo b, IL-2, c, IFNγ, d, IL-17 production and e, proliferation of LN cells to increasing concentrations of pOVA.

Responses of both tolerant groups are significantly different to the PBS-treated, immunogenic control; tolerant groups are not significantly different to each other. Significant differences shown were determined by an ANOVA using Tukey’s multiple comparison test. Error bars represent SEM and data is representative of 2 experiments with 3 mice per group.
Figure 3.10. Anti-PD-1 treatment does not overcome established tolerance in OT-II T cells.
1-2x10^6 Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior to treatment with 500 μg pOVA i.v. (tolerance) or PBS i.v. (immunity). Mice were immunised with pOVA in CFA s.c. 7 days later. At the same time as immunisation, mice also received 250 μg of anti-PD-1 or isotype control antibody i.p. Mice were sacrificed 10 days post immunisation. a, Bar chart showing OT-II cells in the spleen as a percentage of all CD4+ cells. Ex vivo b, IL-2 production and c, proliferation of LN cells to increasing concentrations of pOVA. ICS on LN cells stimulated overnight with 100 μM pOVA; percentage of OT-II cells which are d, IFNγ+ and e, IL-17+. Responses of both tolerant groups are significantly different to the PBS-treated, immunogenic control; tolerant groups are not significantly different to each other. Significant differences shown were determined by an ANOVA using Tukey’s multiple comparison test.
Error bars represent SEM and data is representative of 2 experiments with 3 mice per group.
Figure 3.11. Peptide-induced T cell tolerance can be induced in PD-1\(^{-/-}\) 2D2 cells.
1-2x10^6 Thy1.1+2D2 cells (top row) or Thy1.1+2D2 PD-1\(^{+/}\) cells (bottom row) were transferred to C57BL/6 mice 1 day prior to treatment with 200\(\mu\)g pMOG or PBS i.p. Mice were immunised with pMOG in CFA s.c. 7 days later, and sacrificed 10 days post immunisation. Flow cytometric analysis was performed on a, spleen and b, LN samples stained for CD4 and Thy1.1. Numbers represent the percentage of Thy1.1+ (top) and Thy1.1- (bottom) cells as a percentage of all CD4+ cells. Figure shows representative plots from 2 consistent experiments with 2-3 mice per group. These experiments were carried out by F. Frommer and A. Waisman, Mainz, Germany.
Figure 3.12. Expression of PD-1 and PD-L1 on \textit{in vitro} cultured CD4+ and CD8+ T cells.

C57BL/6 splenocytes were cultured with increasing concentrations of soluble anti-CD3. Expression of \textbf{a}, PD-1 and \textbf{b}, PD-L1 on CD4+ (green line) and CD8+ (black line) T cells was determined after 1, 2, and 3 days of culture. *represents a significant difference of $p\leq0.025$, as determined by unpaired T test.

Error bars represent SEM and data is from 1 experiment with 3 culture wells per condition.
Figure 3.13. Anti-PD-1 enhances \textit{in vitro} responses of CD8$^+$ T cells to a greater extent than CD4$^+$ T cells.

Splenocytes from C57BL/6 mice were stained with CFSE and cultured on plates coated with 1.6\mu g/ml anti-CD3. 50\mu g/ml of anti-PD-1 (black bar) or isotype control (white bar) was added to each culture well. 72 hours later CFSE dilution was determined by flow cytometry. The percent of a, CD4$^+$ T cells or b, CD8$^+$ T cells in each generation was calculated manually as described in materials and methods. Asterisk represent significant differences; *p<0.05, **p<0.0074, ***p<0.0001, as determined by unpaired T test. c-d, Tables showing the percentages of divided or undivided cells and the fold difference (division of one value by the other) or $\Delta$ (subtraction of one value from the other) between anti-PD-1 and isotype treated groups. Error bars represent SEM and data is from 1 experiment with 3-4 wells per culture condition.
Figure 3.14. Anti-PD-1 treatment does not prevent peptide-induced T cell tolerance in pMOG-reactive CD4+ or CD8+ T cells. C57BL/6 mice were treated with 200μg pMOG (tolerance) or PBS (immunity) i.v. At the same time as peptide treatment mice received 250μg of anti-PD-1 or isotype control antibody i.p. Mice were immunised with pMOG in CFA s.c. 7 days later, and sacrificed 10 days post immunisation. a-b, Splenocytes were stimulated overnight with pMOG and IFNγ production by CD4+ and CD8+ T cells determined by ICS. c-d, Splenocytes were stained with CFSE and stimulated with 20μM pMOG (black bars) or unstimulated (open bars). CFSE dilution was examined after 72 hours by flow cytometry and bar charts show percentage of c, CD4+ or d, CD8+ T cells which had divided by this time point.

Responses from both tolerant groups are significantly different to immunogenic control; tolerant groups are not significantly different to each other. Significant differences shown were determined by an ANOVA using Tukey’s multiple comparison test.

Error bars represent SEM and data is from 1 experiment with 3 mice per group.
Figure 3.15. Immunisation of PD-1 knockout mice leads to greater expansion of CD4+ and CD8+ T cells.

C57BL/6 (WT) or PD-1−/− (KO) mice were immunised with 100μg pMOG in CFA s.c. Mice were sacrificed 10 days post immunisation and spleen and LN sampled. The percent of CD4+ and CD8+ cells was determined by flow cytometry and are shown as a percentage of all lymphocytes in the a, LN and b, spleen. Representative histograms show surface expression of c, CD62L and d, CD44 on splenocytes from WT (dotted red line) and KO (solid black line) mice.

Error bars represent SEM and results are representative of data from 2 experiments with 2 mice per group.

These experiments were carried out by F. Frommer and A. Waisman, Mainz, Germany.
Figure 3.16. Anti-PD-1 does not affect the suppressive ability of Tregs in an in vitro suppression assay.

OT-II splenocytes were CD4+ MACS sorted and purified by FACS into CD4+CD25+ and CD4+CD25- cells to a purity of 90-95%. CD4+CD25- cells were used as responders at 1x10^4 cells/well, with 1x10^5 irradiated APC and decreasing numbers of CD4+CD25+ Tregs. Into each well 10μg/ml of ant-PD-1 or isotype control antibody were added. Cultures were stimulated with 2μg/ml anti-CD3 for 96 hours. Graph shows the percentage of suppression of proliferation compared to cultures with no CD4+CD25+ Tregs added. Error bars represent SEM and data from 1 experiment with 3 wells per culture condition.
4. The role of RANKL mediated signalling in tolerance and immunity.

4.1. INTRODUCTION
A previous report from this laboratory had compared the kinetics of expression of various costimulatory molecular pairs on the surface of T cells and DC following tolerogenic versus immunogenic stimulation. This showed that the predominant differences in molecule expression between tolerance and immunity occurred on the surface of the DC (Hochweller and Anderton, 2005). The increased expression of CD40, RANK, CD80 and CD86 on DC was only seen following immunogenic treatment. These data would suggest that it is the signals provided by the DC that determine the outcome of the T cell-DC interaction. In contrast, the expression pattern of costimulatory molecules on the T cell was similar between tolerance and immunity. There was one striking exception; the expression of RANKL. RANKL was shown to be specifically up-regulated on T cells at early time-points during the induction of tolerance. This occurred in the absence of concomitant expression of RANK on the DC. These data could simply suggest that a signal from activated, immunogenic DC to T cells causes down-regulation of RANKL during T cell priming; during tolerance such a DC-derived signal cannot be provided. However, they also pose an interesting possibility, that RANKL mediated signals could provide a positive signal for tolerance via an alternative receptor. These data prompted the examination of the role of RANKL mediated signals in peptide-induced T cell tolerance.

RANKL is an important regulator of osteoclast differentiation. The role of RANKL in bone metabolism is well characterised. RANKL binds to RANK on osteoclastic precursor cells, this interaction is critical for the survival, activation and differentiation of these cells into osteoclasts which cause the break-down (or re-absorption) of bone (Khosla, 2001). This process needs to be highly regulated to maintain skeletal integrity and a soluble decoy receptor for RANKL called OPG also
exists, which inhibits the RANKL:RANK interaction and thus prevents bone re-absorption.

The roles of RANKL:RANK/OPG in the immune system remains to be fully characterised, but many studies have highlighted important roles for RANKL mediated signals in immune functions. A number of these reports have been contradictory. *In vitro* ligation of RANK on a bone-marrow derived DC (BMDC) by soluble RANKL (sRANKL) has been shown to enhance the life-span of the DC (Cremer et al., 2002; Wong et al., 1997a; Yu et al., 2003). Similarly, stimulation of T cells has been shown to cause up-regulation of RANK on the T cell, and ligation of RANK on a T cell with sRANKL led to enhanced T cell viability *in vitro* (Anderson et al., 1997). Moreover, *in vitro* treatment of BMDC with sRANKL permitted greater DC survival upon transfer to mice (Josien et al., 2000). In a model of systemic autoimmunity due to genetic ablation of IL-2 (IL-2−/−), blockade of RANKL mediated signals by addition of OPG.Fc has been shown to decrease inflammation specifically in the gut (Ashcroft et al., 2003). DC from an OPG−/− mouse are more effective at stimulating allo-reactive T cell responses than wild-type DC (Yun et al., 2001). Together, these data show that ligation of RANKL is important in promoting immunity.

In contrast, RANKL−/− mice produced increased levels of pro-inflammatory cytokines following systemic administration of LPS (Maruyama et al., 2006), suggesting a role for RANKL in inhibiting LPS primed immunity. Matuyama et al focused specifically on macrophages and showed that sRANKL treatment caused a decrease in their ability to produce pro-inflammatory cytokines. Adding further complexity are studies which suggest that blockade of RANKL mediated signals had no effect on immune responses (Kamijo et al., 2006; Kong et al., 1999a; Miller et al., 2007a; Pettit et al., 2001).

Collectively the data discussed above present a number of interesting questions concerning the role of RANKL in the immune system. In this chapter the following questions will be addressed:
1. What is the role of RANKL mediated signals in peptide-tolerance induction?

The data presented by Hochweller et al raise the interesting possibility that RANKL could provide a positive signal for tolerance (Hochweller and Anderton, 2005). As discussed, OPG is a second receptor for RANKL, as RANK is not expressed on DC during tolerance induction, then perhaps the RANKL:OPG interaction is important in tolerance induction. OPG is a soluble receptor, the secretion of which increases with DC maturation (Schoppet et al., 2007). However, serum levels of OPG in naïve mice have been shown to be ~2000ng/ml (Maruyama et al., 2006). Thus, this receptor could be present during induction of peptide tolerance. Despite this, other data suggest that RANK could play a role during peptide tolerance induction; Williamson et al showed low levels of constitutive expression of RANK on DC from the spleen, peripheral LN, gut-associated LN and Peyer’s patches (Williamson et al., 2002). Thus it could be possible that RANK could be important in the induction of peptide-induced T cell tolerance and that the methods employed by Hochweller et al were unable to detect its expression on DC.

2. What is the role of RANKL mediated signals in T cell immunity?

From the data presented to date, a role for RANKL mediated signals cannot be fully elucidated. This is important to determine, not only in better illuminating T cell biology, but because administration of anti-RANKL is being proposed as a new therapy for osteoporosis in post-menopausal women (Lewiecki et al., 2007; McClung et al., 2006). Therefore the influence of this drug on the immune system is essential to determine.

To determine the role of the RANKL:RANK/OPG interaction in T cell tolerance and immunity an antibody was employed to disrupt RANKL binding. The IK22-5 antibody has been shown to prevent RANK-Ig binding to RANKL transfected cells and to prevent RANKL mediated osteoclastogenesis (Kamijo et al., 2006). This chapter outlines a series of experiments in which this antibody was administered during the induction of T cell tolerance versus immunity to elucidate the role of the RANKL:RANK/OPG interaction in these processes.
4.2. RESULTS

4.2.1. The role of RANKL in the induction of peptide-induced T cell tolerance.

In order to ascertain the role of RANKL in peptide tolerance, a blocking antibody to RANKL was administered at various time-points during the induction and maintenance of peptide tolerance. Similar to the experiments discussed in chapter 3, Ly5.1+OT-II cells were transferred to C57BL/6 hosts which subsequently received a tolerising dose of pOVA (500μg). At the same time as peptide treatment mice also received either 250μg of anti-RANKL or isotype control. A third cohort of mice formed the immunogenic control; these received pOVA+LPS after adoptive transfer (Figure 4.1a). Spleens were sampled 7 days post peptide treatment and recall responses and OT-II cell numbers examined. If, as suspected, RANKL provides a positive signal for tolerance, then administration of the blocking anti-RANKL antibody should prevent tolerance induction, or inhibit induction of peptide tolerance to some degree.

Blockade of RANKL mediated signals during induction of peptide tolerance had no effect on the tolerance induced in the OT-II cells (Figure 4.1). Few OT-II cells persisted in the spleens of pOVA treated mice (Figure 4.1b) and very low levels of IL-2 and proliferation were seen in recall assays of splenocytes to pOVA (Figure 4.1c,e). Moreover, pOVA tolerised mice produced no IFNγ and this was not enhanced in the group that received anti-RANKL; following stimulation with 100μM pOVA, pOVA+isotype control treated mice produced 0.183±0.04ng/ml, pOVA+anti-RANKL treated mice produced 0±0ng/ml, and immunogenic control mice (pOVA+LPS) produced 80.3±34ng/ml IFNγ. Therefore, administration of anti-RANKL at this time point did not prevent tolerance. In fact, closer comparison of the two pOVA treated groups, suggested reduced responsiveness of splenocyte cultures from anti-RANKL treated mice (Figure 4.1d,f). Although, the difference between the two groups did not always reach statistical significance, this small reduction in responsiveness in anti-RANKL treated mice was consistently seen in repeated experiments.
4.2.2. Administration of anti-RANKL can enhance peptide-induced T cell tolerance.

The data presented above demonstrate that administration of anti-RANKL with a tolerising dose of pOVA, did not turn this dose into an immunogenic stimulus. However, it had not been determined whether anti-RANKL administration had actually prevented tolerance being established in the OT-II cells. In order to ascertain this, OT-II cells were transferred to C57BL/6 hosts which subsequently received a tolerising dose of pOVA (500μg). At the same time as peptide treatment mice also received either 250μg of anti-RANKL or isotype control, and 7 days after peptide treatment mice were immunised with pOVA in CFA. Mice were sacrificed 10 days post immunisation and spleens and LN sampled. A third group received PBS i.v. and responded robustly to pOVA upon immunisation (Figure 4.2a).

Following treatment with 500μg of pOVA, both the isotype control and anti-RANKL treated mice had a significantly smaller population of OT-II cells in the spleen, and significantly reduced ex vivo recall responses compared to PBS-treated control mice (Figure 4.2). Strikingly, comparison of the two tolerant pOVA treated groups revealed that anti-RANKL treated mice had significantly fewer OT-II cells persisting in the spleen (Figure 4.2b-c). Moreover, recall responses of splenocytes from anti-RANKL treated mice were also significantly reduced compared to isotype control treated tolerant mice (Figure 4.2e,g). This profound enhancement of tolerance was also seen in the lymph nodes of anti-RANKL treated mice (Appendix Figure A.2). These data demonstrate that administration of anti-RANKL does not prevent the induction of peptide tolerance; suggesting that RANKL does not function as a positive signal for tolerance. Instead, administration of anti-RANKL with a tolerising dose of pOVA led to a significant reduction in the number of OT-II cells resulting in significantly reduced ex vivo responses from anti-RANKL treated mice; suggesting that disruption of RANKL:RANK/OPG binding enhances pOVA induced tolerance.

The ability of anti-RANKL to potentiate tolerance when administered at a later time point was examined. As above, OT-II cells were transferred to C57BL/6 hosts which
then received a tolerising dose of pOVA (500µg). Mice were immunised with pOVA in CFA and given either 250µg of anti-RANKL or isotype control 7 days later. Mice were sacrificed 10 days post immunisation and spleens and LN sampled (see Figure 4.3a for experimental outline). A third control group received PBS i.v. after the adoptive transfer.

Tolerance was maintained in all pOVA treated mice (Figure 4.3). Following treatment with 500µg of pOVA, both isotype control and anti-RANKL treated mice had a smaller population of OT-II cells in the spleen, and significantly reduced ex vivo recall responses compared to immunogenic control mice (Figure 4.3). Comparison of the two tolerant pOVA treated groups showed similar responses from anti-RANKL and isotype control treated groups. Administration of anti-RANKL appeared to lead to a larger population of OT-II cells in the spleen, however this was not significant (Figure 4.3b). This remaining population of Ag-reactive cells produced little or no IFNγ or IL-17 as determined by intra-cellular cytokine staining (ICS) (Figure 4.3d-e). Moreover, splenocyte cultures from both of the tolerant, pOVA treated groups produced little IL-2 upon recall to pOVA when compared to PBS-treated control mice (Figure 4.3c). These data show two important points. Firstly, administration of anti-RANKL to pOVA treated mice at the same time as immunisation does not overcome the tolerance induced in the OT-II cells. Secondly, unlike the data presented in Figures 4.2 and A.1, administration of anti-RANKL at the same time as immunisation, does not enhance pOVA induced tolerance.

Collectively the data presented in this section demonstrate that RANKL does not deliver a positive signal for tolerance, as blockade of this signal by administration of a blocking anti-RANKL antibody does not prevent tolerance induction (Figure 4.1 and 4.2) or overcome tolerance once it had been induced (Figure 4.3). Strikingly, administration of anti-RANKL appears to potentiate peptide-induced tolerance in OT-II cells. Enhancement of tolerance was only seen when anti-RANKL was administered at the same time as tolerance induction, and led to reduced numbers of peptide-specific cells persisting and reduced responsiveness to peptide upon recall (Figure 4.2 and A.1).
4.2.3. Administration of anti-RANKL with a non-tolerising dose of pOVA reduces responsiveness to pOVA.

To further test the observation that anti-RANKL enhanced peptide-tolerance, anti-RANKL was administered along with a non-tolerising dose of pOVA to determine whether this would permit a non-tolerising dose of peptide to induce peptide tolerance, i.e. potentiate tolerance. Both in this thesis and elsewhere, 500μg of pOVA has been shown to be effective at tolerance induction in adoptively transferred OT-II cells (Hochweller and Anderton, 2005). Administration of only 100μg has been shown to be insufficient to induce peptide tolerance (S. Anderton, unpublished observations). Therefore, experiments were carried out to determine whether administration of anti-RANKL along with 100μg pOVA could permit this dose of peptide to induce tolerance in adoptively transferred OT-II cells.

Initial experiments were performed to establish whether RANKL was expressed on OT-II cells following treatment with this lower dose of pOVA. Here, OT-II cells were transferred to C57BL/6 hosts which then received a tolerising dose of pOVA (500μg), or a non-tolerising dose of pOVA (100μg). The expression of RANKL on OT-II cells in the spleen was examined 24 hours after peptide treatment. The expression of RANKL was lower on OT-II cells from mice treated with 100μg pOVA compared to mice treated with 500μg pOVA (Figure 4.4a). Importantly, treatment with a non-tolerising dose of pOVA caused the up-regulation of RANKL on OT-II cells.

OT-II cells were transferred to C57BL/6 hosts which subsequently received a non-tolerising dose of pOVA (100μg) i.v. a day later. At the same time as peptide treatment mice also received either 250μg of anti-RANKL or isotype control i.p. Mice were immunised with pOVA in CFA 7 days after peptide treatment. Mice were sacrificed 10 days post-immunisation and spleen and LN sampled.

Administration of anti-RANKL with 100μg pOVA resulted in a trend toward a reduced percentage of OT-II cells remaining in the spleen 10 days after immunisation (Figure 4.4b). Similar recall responses were seen for PBS-treated
control mice and 100µg pOVA+isotype control treated mice (Figure 4.4c-d); both produced considerable amounts of IL-2 and IFNγ. However, splenocytes from 100µg pOVA+anti-RANKL treated mice produced less IL-2 and IFNγ upon recall to pOVA compared to control groups. When comparing the two groups which received 100µg pOVA, the differences were not significant. However, the reduced responsiveness of OT-II cells following 100µg pOVA+anti-RANKL treatment was seen in a repeated experiment, and the trend was also continued in the lymph node cells (Figure 4.4e-g). Although not conclusive, these data suggest that administration of anti-RANKL can enhance the ability of 100µg pOVA to induce tolerance in adoptively transferred OT-II cells.

4.2.4. The effect of anti-RANKL on tolerance induction using the MBP Ac1-9 (4Lys) peptide.

Data presented in section 4.2.3, suggested that administration of anti-RANKL together with a non-tolerising dose of pOVA can potentiate unresponsiveness to pOVA upon recall (Figure 4.4). It was of interest to determine whether this was a general phenomenon, therefore the ability of anti-RANKL to potentiate tolerance in another system was tested. The Tg4 transgenic, introduced in Chapter 1, is a TCR transgenic in which the T cells are reactive to the Ac1-9 peptide of MBP (Liu et al., 1995). The wild-type Ac1-9 peptide (hereafter referred to 4Lys), is inefficient at inducing tolerance in Ac1-9 reactive T cells (Liu et al., 1995; Liu and Wraith, 1995), due to its weak affinity for the I-AU MHC molecule (Fairchild et al., 1993). Tg4 transgenic T cells were therefore used to determine whether anti-RANKL could improve the tolerance induced by the 4Lys peptide. This model of peptide tolerance could also potentially indicate a clinical relevance for anti-RANKL treatment.

Ly5.1+ Tg4 cells were transferred to B10.PL hosts that subsequently received 200µg 4Lys or PBS i.v. one day later. At the same time as peptide treatment, mice also received either 250µg of anti-RANKL or isotype control i.p. Mice were immunised with 4Lys in CFA 7 days after peptide treatment and mice were sacrificed 10 days post immunisation when spleens were sampled. Figure 4.5a shows that the Tg4 cell population was a similar size in the spleens of all treatment groups. Moreover, recall
responses of splenocytes to increasing concentrations of 4Lys, were similar when comparing all treatment groups (Figure 4.5b-d). These data demonstrate an inability of anti-RANKL to potentiate tolerance in this setting.

In the context of EAE, tolerance in Tg4 cells can give a profound read-out of disease versus no disease. Initial experiments were performed in B10.PLxC57BL/6 hosts, which develop EAE upon immunisation with Ac1-9 only when Tg4 T cells are first transferred (Ryan et al., 2005). Using this system, it was possible to determine whether co-administration of anti-RANKL with 4Lys could affect EAE course or severity. Tg4 cells were transferred to hosts and 1 day later mice were treated with either PBS or 200 μg 4Lys i.v. At the same time as peptide treatment mice received either 250 μg of anti-RANKL or isotype control antibody. EAE was induced 7 days later by immunisation with 4Lys in CFA and administration of pertussis toxin. The clinical course of EAE is shown in Figure 4.6a, and Figures 4.6b-e compare various disease parameters of experimental mice; note that day of onset and peak disease only consider mice which showed clinical signs of EAE. The clinical course of disease was not significantly different when comparing anti-RANKL treated mice and isotype control treated mice. However, treatment with 4Lys appeared to afford protection from EAE, reducing severity of disease compared to PBS treated control mice (Figure 4.6a). Closer examination of EAE parameters showed that 4Lys+anti-RANKL treated mice had a slightly delayed onset of disease compared to 4Lys+isotype control treated mice (Figure 4.6b). This difference was not significant, however the onset of disease in 4Lys+anti-RANKL treated mice was significantly delayed compared to onset in the PBS treated mice. At day 10 post immunisation the cumulative scores of isotype control treated mice were significantly greater than that of anti-RANKL treated mice (Figure 4.6a). However, for all other parameters examined 4Lys+anti-RANKL mice were similar to 4Lys+isotype control treated mice (Figure 4.6b-e).

To further examine the effect of anti-RANKL treatment on EAE course and severity, mice were sacrificed at day 37 after disease induction. This is a time-point at which most mice had recovered from disease; on the day of sacrifice 4 mice were score 0
and one had a score 2 in the anti-RANKL treated group (5 mice total), all were score 0 for the isotype control treated mice (2 mice total). No PBS treated mice survived to this time-point, and only 2 4Lys+isotype control treated mice remained as others had been sacrificed for another on-going experiment. Spleens, lymph nodes and CNS were all examined by flow cytometry and recall responses of splenocytes were examined. The major difference seen between anti-RANKL and isotype control treated mice was that anti-RANKL mice had a smaller CD4+ infiltrate in the CNS (Figure 4.7a). All other parameters examined were similar between anti-RANKL and isotype control treated mice. Similar populations of CD11c+, CD4+ and CD8+ cells were seen in the spleen and LN (Figures 4.7b-e). Moreover, splenocytes from both groups showed similar *ex vivo* recall responses to 4Lys (Figure 4.7f).

In sections 4.2.3 and 4.2.4 the potential of anti-RANKL to allow a non-tolerising treatment to induce tolerance has been examined. The experiments performed to establish this show no significant difference between isotype treated and anti-RANKL treated mice. However a pattern has emerged which, although not definitive, suggests that administration of anti-RANKL along with a non-tolerogenic peptide treatment results in reduced responsiveness in peptide-reactive T cells.

### 4.2.5. Anti-RANKL does not alter the ability of Tregs to suppress naïve T cell proliferation.

The mechanism by which anti-RANKL potentiates tolerance remains unaddressed, as such preliminary studies were undertaken to ascertain this. In initial experiments, the effect of anti-RANKL on the expression of a variety of T cell and DC molecules was examined. Stimulated or un-stimulated cultures were treated with anti-RANKL or control antibody. No differences in patterns of expression were seen between anti-RANKL and isotype treated cells (Appendix Figure A.3). Further experiments need to be performed to ascertain whether treatment with anti-RANKL alters the expression of these molecules *in vivo*, however, the *in vitro* experiments presented here would suggest this aspect of immune cell biology is not altered upon treatment with anti-RANKL.
Next, the effect of anti-RANKL on the functional capacity of Tregs was examined. Previous studies have suggested an important role of RANKL mediated signals in the generation of Tregs in the periphery. Loser et al showed that RANKL, expressed on keratinocytes, can influence epidermal DC (Loser et al., 2006). RANKL experienced DC can induce greater expansion of Tregs leading to greater peripheral Treg numbers. In a model of accelerated diabetes, CD4+CD25+ cells derived from the pancreatic lymph node are extremely potent at suppressing diabetes (Green et al., 2002). Green et al demonstrated that blockade of the RANK:RANKL interaction led to a specific depletion of these potent Tregs from the pancreatic lymph node. These data suggest a role for RANKL mediated signals in Treg generation and/or persistence. However, neither study, nor subsequent studies have examined the role of the RANKL:RANK/OPG interaction on the suppressive capacity of regulatory T cells. Here, the ability of purified CD4+CD25+ cells to suppress the proliferative response of naïve T cells was examined in the presence of anti-RANKL or an isotype control (Figure 4.8). CD4+CD25+ Tregs were sorted to 90-95% purity and determined to express foxp3 to a high degree (~80% of cells were foxp3+). Two suppression assays were established; one in which cultures were stimulated with 2μg/ml anti-CD3 (Figure 4.8a), and a second in which Ag-reactive T cells from OT-II mice were activated by 10μM pOVA (Figure 4.8b). The ability of CD4+CD25+foxp3+ cells to suppress either anti-CD3 stimulated responses, or pOVA-stimulated responses, was not effected by the addition of anti-RANKL to the suppression assay. These data indicate that the RANKL:RANK/OPG interaction plays no role in the suppressive capacity of CD4+CD25+foxp3+ regulatory T cells.

4.2.6. The role of RANKL in the LPS primed T cell immunity.

With the advent of the use of therapeutics which disrupt the RANKL:RANK interaction, it is important to determine how inhibition of RANKL mediated signals could influence immunity. Previous studies from this, and other laboratories, have shown RANK expression to be rapidly up-regulated on the DC following immunogenic stimulus (Hochweller and Anderton, 2005; Wong et al., 1997a). To examine the role of RANKL mediated signals in T cell immunity, OT-II cells were transferred to C57BL/6 hosts which subsequently received 500μg pOVA plus 30μg
LPS i.v. a day later. At the same time as peptide treatment mice also received either 250μg of anti-RANKL or isotype control i.p. Mice were sacrificed 7 days post peptide treatment and spleens sampled (Figure 4.9a). Administration of anti-RANKL did not influence the size of the OT-II cell population (Figure 4.9b). Ex vivo recall responses of splenocytes from anti-RANKL and isotype treated mice were also similar (Figure 4.9c-d). These data show that blockade of RANKL mediated signals during the induction of LPS primed T cell immunity had no effect on the T cell response mounted. Note: in one repeat of this experiment, treatment of mice with anti-RANKL at the same time as pOVA+LPS ablated responses to pOVA (Appendix Figure A.4).

The RANK:RANKL interaction has been shown to enhance the life-span of DC through the up-regulation of anti-apoptotic molecules (Wong et al., 1997a). Here, no differences in the DC populations of the spleen were seen when examined 7 days after anti-RANKL treatment (Figure 4.9e).

Addition of anti-RANKL at the same time as pOVA+LPS had no effect on the resulting T cell response when mice received a secondary stimulation of pOVA in CFA following LPS priming (Figure 4.10a). As shown in Figure 4.10b, anti-RANKL and isotype control treated animals had similar sized populations of OT-II cells in the spleen. Moreover, recall responses from anti-RANKL and isotype control treated mice were also similar (Figure 4.10c-e). Collectively, these data show that blockade of RANKL mediated signals during the induction of LPS primed T cell immunity had no effect on T cell responses.

4.2.7. The role of RANKL in CFA primed T cell immunity.

Next, T cells were provided with a greater priming stimulus and the effect of anti-RANKL administration was examined. OT-II cells were transferred to C57BL/6 hosts which were immunised with pOVA in CFA 1 day post transfer. At the same time as immunisation mice received 250μg of anti-RANKL or isotype control. Mice were sacrificed 10 days post immunisation and spleens and LN sampled. A significant enhancement of the OT-II cell population was seen in the spleen
following anti-RANKL treatment (Figure 4.11a). This gave a corresponding increase in \textit{ex vivo} recall responses to pOVA (Figure 4.11b-c), specifically IL-2 production and proliferation. No difference was seen in IFN\textsubscript{\textgamma} produced by splenocytes from anti-RANKL treated mice versus isotype control treated mice (Figure 4.11d). This pattern was reiterated in the LN; a slight increase in the population of OT-II cells was seen in anti-RANKL treated mice, and an enhancement of \textit{ex vivo} recall responses was also seen (Figure 4.11e-h). In the LN IFN\textsubscript{\textgamma} production was also enhanced following anti-RANKL treatment, although this was not significant (Figure 4.11h). These data suggest that administration of anti-RANKL effects the clonal expansion of peptide-reactive T cells.

To further test this enhancement of T cell responsiveness following anti-RANKL treatment, the effect of anti-RANKL on active EAE was determined. This was induced in C57BL/6 mice following immunisation with pMOG in CFA. At the same time as pMOG in CFA immunisation mice received 250\mu g of anti-RANKL or isotype control. As shown in Figure 4.12, administration of anti-RANKL had no effect on the EAE disease course or severity. Disease onset and recovery was synchronous between anti-RANKL and isotype control treated mice.

Together these data show that administration of anti-RANKL when priming T cells with peptide in CFA leads to enhanced numbers of peptide-specific T cells and increased \textit{ex vivo} IL-2 production and proliferation. However, this increase in T cell expansion was not sufficient to induce a more severe course of EAE in mice primed for disease in the presence of anti-RANKL.

\textbf{4.2.8. The effect of anti-RANKL on \textit{in vitro} T cell cultures.}\n
The data presented in sections 4.2.2-3 and 4.2.7 show that anti-RANKL can enhance both tolerance and immunity. These apparently contradictory results needed to be reconciled; therefore \textit{in vitro} T cell cultures were established to determine the effect of anti-RANKL on T cell function. Initially two culture systems were developed; one where OT-II splenocytes were stimulated with pOVA, and another where sorted CD4+ cells were stimulated with anti-CD3 and anti-CD28.
4.2.8.1. OT-II cultures.
Cultures were established in which OT-II splenocytes were stimulated with pOVA and the expression of RANKL was examined on CD4+ pOVA-reactive T cells every 24 hours (Figure 4.13a). RANKL expression was increased on a proportion of OT-II cells. Expression of RANKL was highest after 24 hours, and then declined over the next 48 hours, remaining slightly above baseline at 72 hours.

OT-II splenocytes were cultured with pOVA and either 10μg/ml of anti-RANKL, anti-CD40 (clone FGK-45) or isotype control antibody; concentration as per (Chen et al., 2001; Kamijo et al., 2006). Addition of agonistic anti-CD40 was used as a positive control. Addition of anti-CD40 to cultures led to a significant increase in both proliferation and IFNγ production (Figure 4.13b-c). Addition of anti-RANKL to these cultures did not significantly alter T cell responses compared to controls. These data suggested that the RANKL:RANK/OPG interaction was not required for proliferation or acquisition of effector function upon activation of naïve T cells. Thus, RANKL:RANK is not necessary for T cell activation. Moreover, addition of anti-RANKL to culture wells did not alter the amplitude of the T cell response, as would be suggested by Figure 4.11.

4.2.8.2. Sorted CD4+ T cell cultures.
A second culture system was employed in which MACS purified CD4+ T cells were stimulated with plate bound anti-CD3 and anti-CD28. RANKL was rapidly up-regulated on CD4+ T cells following stimulation with anti-CD3 plus anti-CD28 and with anti-CD3 alone (Figure 4.14a). Unlike in the previous culture system, RANKL was up-regulated on all CD4+ T cells. Expression was highest after 24 hours of stimulation and declined over the culture period, never returning to background levels. The levels of RANKL expression were different between the two culture systems; sorted CD4+ T cells all up-regulated RANKL expression. Expression of RANKL was seen to be highest following anti-CD3 plus anti-CD28 stimulation compared to anti-CD3 alone.

To determine whether blockade of RANKL mediated signals would affect T cell responses 10μg/ml of either anti-RANKL or isotype control was added to anti-CD3 plus anti-CD28 stimulated CD4+ T cell cultures. Due to the lack of DC, these
cultures allowed the interaction between RANKL and RANK on T cells to be examined. Anti-RANKL was added either directly to cultures in soluble form (Figure 4.14b) or plate bound (Figure 4.14c). Addition of anti-RANKL via either method did not influence T cell responses. Proliferative responses of cultures to which anti-RANKL had been added, were similar to control cultures. In these T cell only cultures very low levels of effector cytokines were produced; this was not altered by addition of anti-RANKL (data not shown).

Collectively these data demonstrate that in vitro the RANKL:RANK interaction was not required for T cell activation, and therefore that RANKL binding to either a T cell or DC was not necessary for induction of T cell responses.

In vitro data, unlike those shown in Figure 4.11, show that disruption of the RANKL:RANK interaction does not result in an enhancement of T cell responses. In fact, disruption of RANKL binding had no effect on either the proliferative response or IFNγ production from cultures (Figure 4.13-14). These data are consistent with those shown in Figures 4.9 and 4.10, in which anti-RANKL had no effect on LPS primed T cell responses. However, data presented in section 4.2.7 show that administration of anti-RANKL during T cell priming by CFA does lead to enhanced T cell responses (Figure 4.11). Taken together, these data suggest a highly specific role for the RANKL:RANK interaction; disruption of this interaction specifically enhances T cell responses primed by certain adjuvants. The major differences following adjuvant treatments would most probably be in DC phenotype. Thus, RANKL mediated signals may be important in regulating immunity induced by CFA primed, but not LPS primed, DC.
4.3. DISCUSSION

4.3.1. Anti-RANKL enhances peptide induced T cell tolerance.

The data presented in this chapter show that administration of anti-RANKL can enhance tolerance induction, including when an otherwise non-tolerising dose of peptide is used. To mediate this effect on tolerance, anti-RANKL needed to be administered at the same time as the tolerising dose of peptide, as administration at the time of immunisation did not achieve this (Figure 4.3).

As disruption of the RANKL:RANK/OPG interaction does not prevent peptide-tolerance induction, RANKL binding is not critical for tolerance induction as postulated by Hochweller et al (Hochweller and Anderton, 2005). Instead, the data presented here would suggest that RANKL binding is important in preventing tolerance induction, as in its absence tolerance is enhanced. This is in contrast to Williamson et al who demonstrated that administration of sRANKL (stimulation of RANKL mediated signalling) leads to better oral tolerance (Williamson et al., 2002). In a transfer model of oral tolerance induced by OVA feeding, that study showed sRANKL could specifically stimulate Peyer’s Patch DC to produce IL-10, and that the increased levels of IL-10 potentiated tolerance.

How could administration of anti-RANKL be mediating such an effect on tolerance? Data presented here demonstrate that anti-RANKL does not affect the suppressive capacity of Tregs (Figure 4.8), nor alter the expression of a variety of DC or T cell molecules (Appendix Figure A.3). No studies have specifically focused on the role of RANKL mediated signals in tolerance induction, and therefore the results presented here provide a novel insight. RANKL is expressed more rapidly on T cells during the induction of tolerance versus immunity (Hochweller and Anderton, 2005). This early up-regulation means that RANKL is expressed on a T cell heading for tolerance at a time-point when RANK, according to Hochweller et al, is not expressed on the DC. The enhancement of tolerance seen following blockade of RANKL mediated signals could be indicative of the following roles for RANKL mediated signals in T cell tolerance;
DC express RANK during tolerance induction and therefore blockade of RANKL mediated signals alters DC function.

Although Hochweller et al could not detect RANK expression on DC following tolerogenic treatment (Hochweller and Anderton, 2005), another study has shown that RANK is constitutively expressed at low levels on splenic DC (Williamson et al., 2002). Moreover, treatment of splenic DC with sRANKL led to increased levels of IL-12p40. This is in line with earlier studies which showed that sRANKL treated BMDC produced IL-12p40 and IL-12p35 (Bachmann et al., 1999). These data suggest that the RANKL:RANK interaction provides a positive costimulatory signal to the DC. Consistent with this a number of studies have demonstrated that ligation of RANK on DC by RANKL leads to enhanced DC survival (Cremer et al., 2002; Josien et al., 2000; Yu et al., 2003) through up-regulation of Bcl\textsubscript{Xl} (Wong et al., 1997a). Collectively these data suggest that loss of RANKL mediated signals to the DC could lead to better tolerance due to reduced DC activation, and specifically, reduction in IL-12p40 from the DC.

T cells express both RANKL and RANK following tolerogenic stimulus.

Anderson et al have shown that RANK is expressed on T cells following TCR ligation plus TGF\textbeta and IL-4 (Anderson et al., 1997). In a different model of peptide-induced T cell tolerance, IL-4 and TGF\textbeta, along with IFN\gamma, IL-10 and IL-2 have been shown to be produced in a cytokine burst 24 hours after treatment with a tolerogenic dose of peptide (Burkhart et al., 1999). Moreover, Josien et al showed low levels of RANK expression on T cells cultured in medium alone (Josien et al., 1999). Thus, it is conceivable that OT-II cells express both RANKL and RANK following tolerogenic pOVA treatment. Anderson et al demonstrated that blockade of RANKL:RANK specifically on T cells decreased the number of viable cells (Anderson et al., 1997). Following anti-RANKL plus peptide treatment, a smaller population of OT-II cells was consistently seen compared to isotype treated controls. Thus blockade of RANKL:RANK specifically between T cells could account for the enhanced tolerance seen.
**Ligation of RANKL allows reverse signalling to the T cell.**

Ligation of RANKL by either RANK or the soluble receptor OPG, could mediate a signal to the T cell. This reverse signalling has been noted for a number of TNF/TNFR family members (Sun and Fink, 2007), most notably for CD154 (van Essen et al., 1995). Of all the TNFRs, RANKL shares the highest homology with CD154 (28%) (Anderson et al., 1997). This reverse signal could be important for T cell survival and/or activation, in fact reverse signalling through RANKL has been suggested by two groups who showed it was mediated by JNK and/or MAPK p38 (Chen et al., 2001; Wong et al., 1997b). Thus, blockade of RANKL interaction with its receptors could lead to an overall decrease in the positive signals received by the T cell during tolerance induction and thus a more profound state of tolerance.

The studies presented have not determined which of these roles for RANKL mediated signals accounts for the enhancement of tolerance seen. To better understand which role RANKL mediated signals fulfil, the expression of RANK on T cells and DC following tolerogenic stimulus needs thorough examination. Whether RANKL can mediate a signal to the T cell could be determined through the use of soluble RANK, which would allow examination of signalling events within the RANKL+ T cell. As previous stated, studies have highlighted p38 and JNK as signalling intermediaries downstream of RANKL (Chen et al., 2001; Wong et al., 1997b) therefore examination of these signalling pathways would be an obvious starting point. The most striking and interesting result generated in this study was that administration of anti-RANKL potentiated tolerance. Further studies need to be done to conclusively show that administration of anti-RANKL can also do this when administered with non-tolerising doses of peptide; this could involve using higher or repeated dosing with anti-RANKL. Determining the mechanism by which anti-RANKL achieves this is important as it could provide clues as how to better create therapies for the clinic which induce Ag-specific tolerance.
4.3.2. **Anti-RANKL enhances CFA primed T cell immunity.**

The stimulus for examining the effect of anti-RANKL on T cell immunity was the development of anti-RANKL as a therapy for bone disorders. The role of the RANK:RANKL interaction in the immune system remains to be fully elucidated, and therefore must be thoroughly examined before such a treatment is widely available. Following immunogenic treatment, DC have been shown to up-regulate RANK expression (Hochweller and Anderton, 2005). More specifically both RANK and OPG are up-regulated by DC following CD40 ligation (Anderson et al., 1997; Yun et al., 1998). Thus the interaction of RANKL with either of its two receptors could play an important role in the induction, maintenance and/or resolution of T cell responses.

Administration of anti-RANKL has no effect on LPS primed T cell immunity (Figure 4.9-10), however administration of anti-RANKL at the same time as immunising mice with pOVA in CFA led to a larger population of pOVA-reactive T cells and increased ex vivo responses (Figure 4.11). Previous studies reported that ligation of RANK on a DC by sRANKL enhanced the stimulatory capacity of the DC by increasing the DC life-span (Anderson et al., 1997; Josien et al., 2000). Others have shown that sRANKL treatment of DC increases cytokine production (Josien et al., 1999; Williamson et al., 2002). Studies which have looked at the effect of anti-RANKL on immunity have predominantly been performed in arthritis models and have shown that anti-RANKL does not affect inflammation (Kamijo et al., 2006; Kong et al., 1999a; Pettit et al., 2001). Moreover, administration of RANK.Fc (blockade of RANKL binding) had no effect on the immune response to influenza infection in mice (Miller et al., 2007a).

Therefore, the results presented here were somewhat unexpected, as, if it is assumed that the anti-RANKL used is a blocking antibody, they highlight a role for the RANKL:RANK/OPG interaction in limiting T cell responses following priming with CFA. A key question raised in the studies presented here is why administration of anti-RANKL should specifically enhance CFA, but not LPS, primed T cell immunity? The answer could lie in the subsets of DC used to prime T cell responses.
Mice were immunised s.c. with pOVA in CFA, whereas, LPS priming treatments were given i.v; this could well lead to T cell priming by different DC subsets or DC from different anatomical locations. Williamson et al demonstrated that sRANKL had different effects on DC from different sources (Williamson et al., 2002). DC from the spleen, peripheral LN, mesenteric LN and Peyer’s patches, were shown to produce different patterns of cytokines upon ligation of sRANKL. Alternatively, the RANKL:RANK/OPG interaction may only be important following certain inflammatory stimuli. This could be quantitative; priming with CFA results in a greater and more prolonged stimulus compared to LPS, and as such the RANKL:RANK/OPG interaction may only be important when the T cell response reaches a certain magnitude. Alternatively, this could be qualitative; with the RANKL:RANK/OPG interaction only playing a role in specific inflammatory settings. In terms of TLRs ligated, LPS induces inflammation through the ligation of TLR-4 (Hoebe et al., 2003), however CFA, which contains heat-killed Mycobacterium tuberculosis, likely achieves this through ligation of a number of TLRs and other pathogen recognition receptors.

4.3.3. Administration of anti-RANKL enhances both tolerance and immunity.

Taken together the results presented in this chapter appear to be in direct contrast; how can administration of anti-RANKL enhance both tolerance and immunity? If the sole effect of anti-RANKL is blockade of the RANKL:RANK/OPG interaction then the answer could lie in the balance of expression of RANKL, RANK and OPG during tolerance and immunity. During the induction of immunity, the RANKL:RANK interaction between a T cell and DC would be the primary interaction. During tolerance induction this is less well defined, it is possible that RANKL:OPG could be the primary interaction. Alternatively, RANKL could interact with RANK on T cells or DC. Disruption of RANKL binding to RANK versus OPG could well mediate different effects, as could blockade of RANK from different cell types.
Alternatively, the anti-RANKL antibody could have agonistic properties and mediate a signal to the T cell upon ligation of RANKL. Therefore, a simple explanation is that anti-RANKL boosts signal 1. Upon ligation of RANKL, anti-RANKL could therefore mediate a signal to the T cell that enhances both tolerance and immunity. This could be mediated through a variety of different mechanisms; considering just a few options, the RANKL mediated signal could enhance TCR signalling by reducing inhibitory signals, strengthen the immunological synapse by enhanced TCR clustering or by preventing down-regulation of the TCR, or augment DC-T cell contacts. Irrespective of the mechanism, a greater signal 1 could lead to enhanced immunity through better activation of T cells and/or activation of T cells which would usually not be activated by the TCR stimulus used. During tolerance induction, as additional signals are not available to initiate immunity, an enhanced signal 1 could potentiate tolerance through more rapid induction of tolerance, a greater degree of tolerance being induced or by tolerance being induced in more cells. This boosting of signal 1 is further suggested by the data showing that administration of anti-RANKL does not appear to directly affect T cell effector function, rather it alters the number of Ag-reactive T cells; increasing the number of Ag-reactive T cells in the setting of immunity and decreasing the number in the tolerance setting. This leads to enhanced tolerance or immunity depending on the availability of signal 2.

A previous study has reported that cross-linking of RANKL along with the TCR enhances IFNγ production by Th1 cells (Chen et al., 2001). Preliminary experiments undertaken here to determine whether anti-RANKL could also achieve this, gave inconclusive results (data not shown). In vitro assays were established with the aim of elucidating the mechanism of action of anti-RANKL. In these simplistic systems the effects of anti-RANKL could be directly assessed. However, these failed to show any global effect of anti-RANKL on T cell responsiveness (Figure 4.13-14), and would require further optimisation. Other experiments showed that anti-RANKL treatment did not alter the expression of a variety of T cell and DC molecules (Appendix Figure A.3). Nevertheless, stimulation of RANKL may ultimately result in altered expression of molecules not examined in this study, or may mediate its
effects via another mechanism. Therefore, it remains possible that anti-RANKL antibody used in these studies is an agonistic antibody. If anti-RANKL has agonistic properties the contradictory results obtained in this chapter can be reconciled; anti-RANKL enhancing both tolerance and immunity. Moreover, the data presented in the immunity setting, whereby anti-RANKL enhances CFA primed immunity, can be reconciled with other studies in which the RANKL:RANK interaction has been shown to enhance the stimulatory capacity of DC (discussed above). Therefore, anti-RANKL mediates a signal to the T cell upon ligation of RANKL which enhances both tolerance and immunity through the promotion of signal 1.

For the results presented in this chapter to be fully explained the ability of anti-RANKL to mediate a signal to the T cell through RANKL needs to be determined. For anti-RANKL to mediate a signal to the T cell, RANKL must be involved in reverse signalling, as previously suggested. To determine whether anti-RANKL does stimulate RANKL, anti-RANKL treated T cells should be examined for p38 and JNK activation, molecules previously indicated to be downstream of RANKL (Chen et al., 2001; Wong et al., 1997b).

4.3.4. Concluding Remarks

The data shown in this chapter provide an interesting starting point to dissect the role of the RANKL:RANK interaction during the evolution of the dialogue between a DC and T cell during an immune response. Importantly, they suggest that the RANKL:RANK/OPG interaction is bidirectional. Previous work has shown that the RANKL:RANK interaction prolongs the life-span and/or cytokine production of p:MHC bearing RANK+DC. The data shown here suggest that a signal is mediated through RANKL (upon binding by anti-RANKL) which increases the TCR mediated signal to the T cell (signal 1). Thus a model can be suggested in which RANKL mediated signals, possibly via p38 and JNK, have a positive feedback effect on signal 1 and therefore enhance the induction of whichever process is occurring by boosting signal 1. Collectively these data highlight a novel role for the RANKL:RANK/OPG interaction in T cell biology, and suggest further work needs to be undertaken to fully elucidate the role of these molecules in the generation of T
cell tolerance and immunity. These data also provide a cautionary note for the use of anti-RANKL in the treatment of bone diseases; they show that anti-RANKL can affect the processes of both T cell tolerance and immunity. This may be due to agonistic properties of the antibody. Whether the clinical antibodies have such functions has not been reported.
Figure 4.1. Role of RANK:RANKL interaction in the induction of tolerance.

**a.** Experimental outline; 1-2x10⁶ Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior to treatment with 500µg pOVA i.v. (tolerance) or 500µg pOVA+LPS i.v. (immunity). At the same time as peptide treatment mice also received 250µg of anti-RANKL or isotype control i.p. Mice were sacrificed 7 days later. **b.** Bar chart showing OT-II cells in the spleen as a percentage of all CD4+ T cells. Ex vivo **c-d,** proliferation and **e-f,** IL-2 production from splenocytes to increasing concentrations of pOVA. Figures c and d are the same graph; d has a smaller scale and does not include the pOVA+LPS group. Figure e and f are the same graph; f has a smaller scale and does not include the pOVA+LPS group. Responses of pOVA+LPS group are significantly different from pOVA treated groups, as determined by an ANOVA using Tukey’s multiple comparison test. Asterisk represent significant differences as determined by unpaired T test, *p<0.01.

Error bars represent SEM and data is representative of 2 experiments with 3 mice per group.
b. c. p = 0.0202

Recall Assays

pOVA i.v. PBS i.v.

isotype control

anti-RANKL i.p.

isotype control i.p.

pOVA + CFA s.c.

% of CD4+ cells which are OT-II cells

total number of OT-II cells

LEGEND:

PBS

pOVA + isotype control

pOVA + anti-RANKL

d. e. f. g.

d 0

d 1

Transfer OT-II cells

PBS i.v.

pOVA i.v.

pOVA + CFA s.c.

anti-RANKL i.p.

isotype control i.p.

Recall Assays

pOVA + isotype control

pOVA + anti-RANKL

PBS

p<0.001

p<0.01

p<0.05

p<0.05

p<0.01

p<0.01

p<0.01

IL-2 (pg/ml)

pOVA (μM)

IL-2 (pg/ml)

pOVA (μM)

FN1 (ng/ml)

pOVA (μM)

FN1 (ng/ml)

pOVA (μM)
Anti-RANKL enhances tolerance induced by 500μg of pOVA.

**a.** Experimental Outline; 1-2x10^6 Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior to treatment with 500μg pOVA i.v. (tolerance) or PBS i.v. (immunity). At the same time as peptide treatment mice also received 250μg of anti-RANKL or isotype control i.p. Mice were immunised with pOVA in CFA s.c. 7 days later. Mice were sacrificed 10 days post immunisation. Bar chart showing **b,** OT-II cells in the spleen as a percentage of all CD4+ T cells and **c,** total number of OT-II cells in the spleen. *Ex vivo* **d-e,** IL-2 and **f-g,** IFNγ production from splenocytes to increasing concentrations of pOVA. Figure d and e are the same graph; e has a smaller scale and does not include the PBS treated group. Figure f and g are the same graph; g has a smaller scale and does not include the PBS treated group.

Responses of the PBS treated group are significantly different from pOVA treated groups, as determined by an ANOVA using Tukey’s multiple comparison test. Asterisk represent significant differences as determined by unpaired T test, *p<0.027 and **p<0.007. Error bars represent SEM and data is representative of 4 experiments with 3-4 mice per group.
a. Experimental outline; 1-2x10^6 Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior to treatment with 500µg pOVA i.v. (tolerance) or PBS i.v. (immunity). Mice were immunised with pOVA in CFA s.c. 7 days later. At the same time as immunisation mice also received 250µg of anti-RANKL or isotype control i.p. Mice were sacrificed 10 days post immunisation.  

b. Bar chart showing OT-II cells in the spleen as a percentage of all CD4+ T cells. Ex vivo responses of splenocytes were determine by ELISA and ICS.  

c. IL-2 production by splenocytes to increasing concentrations of pOVA. ICS on OT-II cells for IFNγ and e, IL-17. Statistical differences shown were determined by an ANOVA using Tukey’s multiple comparison test. 

Figure 4.3. Anti-RANKL treatment does not overcome established tolerance in OT-II cells. 

Error bars represent SEM and data is representative of 2 experiments with 3-4 mice per group.
a. PBS i.v.

b. SPLEEN

c. % of CD4+ cells which are OT-II cells

d. IL-2 (pg/ml)

e. LYMPH NODE

f. % of CD4+ cells which are OT-II cells

g. IL-2 (pg/ml)

LEGEND:
- PBS
- 100ug pOVA + isotype control
- 100ug pOVA + anti-RANKL

NS differences between isotype control and anti-RANKL treated groups.
Figure 4.4. Administration of anti-RANKL with a non-tolerising dose of peptide enhances unresponsiveness to pOVA.

1-2x10^6 Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior to treatment with 500µg pOVA (tolerising dose), 100µg pOVA (non tolerising dose), or PBS i.v. a, Expression of RANKL was determined on OT-II cells directly \textit{ex vivo} 24 hours after peptide treatment. Histograms show RANKL (black line) and isotype control (grey line) staining. Numbers represent the average percentage of cells which were RANKL+. Data is from one experiment.

1-2x10^6 Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior to treatment with 100µg pOVA (non-tolerising dose) or PBS (immunity) i.v. At the same time as peptide treatment mice also received 250µg of anti-RANKL or isotype control i.p. Mice were immunised with pOVA in CFA s.c. 7 days later, and sacrificed 10 days post immunisation. Bar chart showing OT-II cells as a percentage of all CD4+ T cells in the b, spleen and e, LN. \textit{Ex vivo} c, f, IL-2 and d, g, IFN\textgamma responses of splenocytes (c-d) and pooled LN cells (f-g) to increasing concentrations of pOVA were determined by ELISA. Error bars represent SEM, and data is representative of 2 experiments with 3 mice per group.
Figure 4.5. Administration of anti-RANKL with 4Lys does not potentiate tolerance induction in Tg4 cells.

1-2×10⁶ Ly5.1+Tg4 cells were transferred to B10.PL mice 1 day prior to treatment with 200μg 4Lys (non-tolerising) or PBS (immunity) i.v. At the same time as peptide treatment mice also received 250μg of anti-RANKL or isotype control i.p. Mice were immunised with 4Lys in CFA s.c. 7 days later, and sacrificed 10 days post immunisation. a, Bar chart showing Tg4 cells as a percentage of all CD4+ T cells in the spleen. Ex vivo b, proliferation, c, IL-2 and d, IFNγ responses of splenocytes to increasing concentrations of 4Lys. Error bars represent SEM and results are from 1 experiment with 3 mice per group.
Figure 4.6. The effect of anti-RANKL on EAE in B10.PLxC57BL/6 mice.
B10.PLxC57BL/6 mice received 4Lys or PBS i.v. 1 day after transfer of Ly5.1+ Tg4 T cells. At the same time as peptide treatment mice also received 250μg of anti-RANKL or isotype control i.p. Disease was induced in mice 7 days later by immunisation with 4Lys in CFA s.c. and administration of Ptx i.p. a, Clinical scores of mice from day of immunisation. Graphs show b, day of disease onset, c, day of peak disease, d, maximum EAE score and e, total disease burden (sum of all scores for individual mice). Total disease burden is calculated up to day 19, after which 4 4Lys+isotype control treated mice were sacrificed and so comparisons of disease burden after this time-point could not be done. Error bars represent SEM and lines show median values. Significant differences were determined by an ANOVA using Tukey's multiple comparison test, or the Mann-Whitney test (EAE disease course only). * represents a significant difference of p=0.0456 as determined by an unpaired T test. Data is from one experiment with 5-7 mice per group.
Figure 4.7. The effect of anti-RANKL on EAE in B10.PLxC57BL/6 mice.
In the same experiment described in Figure 4.6, remaining EAE mice were sacrificed at day 37 after immunisation, and spleen, LN and CNS sampled. a, CD4+ cells in the CNS as a percentage of all lymphocytes. Graphs showing CD11c+ cells in b, spleen and c, LN. Graphs showing CD4+ and CD8+ cells in d, spleen and e, LN. f, Ex vivo IL-2 (left axis) and IL-17 (right axis) production by splenocytes to increasing concentrations of 4Lys. Significant difference shown was determined by unpaired T test. Error bars represent SEM and lines show median values, data is from one experiment with 2-5 mice per group.
Figure 4.8. The effect of anti-RANKL on regulatory T cell function.

OT-II splenocytes were CD4+ MACS sorted and subsequently sorted by FACS into CD4+CD25+ and CD4+CD25- cells to a purity of 90-95%. CD4+CD25- cells were used as responders at 1x10^4 cells/well, with 1x10^5 irradiated APC and decreasing numbers of Tregs. 10 μg/ml of anti-RANKL or isotype control was added to each well. Cultures were stimulated with (a) 2 μg/ml anti-CD3 or (b) 10 μM pOVA for 96 hours. Proliferative responses were determined by 3H-thymidine incorporation. Graphs show the percentage of suppression of proliferation compared to cultures with no CD4+CD25+ Tregs added. Error bars represent SEM. Each assay was performed once with 3 wells per culture condition.
Figure 4.9. Anti-RANKL has no effect on LPS primed immunity.

a. Experimental outline; 1-2x10^6 Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior to treatment with 500µg pOVA+30µg LPS i.v. At the same time as peptide treatment mice also received 250µg of anti-RANKL or isotype control i.p. Mice were sacrificed 7 days post peptide treatment.

b. OT-II cells as a percentage of all CD4+ cells in the spleen. Ex vivo c, IL-2 and d, IFNγ production from splenocytes to increasing concentrations of pOVA. e, DC populations in the spleen as a percentage of all CD11c+ cells in anti-RANKL treated mice (black bars) and isotype control treated mice (white bars). Error bars represent SEM and data is representative of 2 experiments with 3 mice per group.
Figure 4.10. Anti-RANKL has no effect on LPS primed immunity.

a, Experimental outline; 1-2x10^6 Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior to treatment with 500μg pOVA+30μg LPS i.v. At the same time as peptide treatment mice also received 250μg of anti-RANKL or isotype control i.p. Mice were immunised with pOVA in CFA s.c. 7 days later, and sacrificed 10 days post immunisation. b, Bar chart showing OT-II cells in the spleen as a percentage of all CD4+ T cells. Ex vivo c, IL-2, d, IFNγ, and e, IL-17 production from splenocytes to increasing concentrations of pOVA. Error bars represent SEM and data is representative of 3 experiments with 3-4 mice per group.
Figure 4.11. Anti-RANKL enhances CFA primed T cell immunity.
1-2x10^6 Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior immunisation with pOVA in CFA s.c. At the same time as immunisation mice also received 250μg of anti-RANKL (black bars/symbols) or isotype control (white bars/symbols) i.p. Mice were sacrificed 10 days post immunisation. Bar chart showing OT-II cells as a percentage of all CD4+ cells in the a, spleen and e, LN. Ex vivo b,f, proliferation, c,g, IL-2 production and d,h, IFNγ production of splenocytes (b-d) and LN cells (f-h) to increasing concentrations of pOVA. Significant differences were determined by unpaired T test; *p=0.0059. Error bars represent SEM and data is representative of 2 experiments with 3-4 mice per group.
Figure 4.12. Anti-RANKL has no effect on actively induced EAE.
C57BL/6 mice were immunised with pMOG in CFA s.c. and given Ptx i.p at the same time and also 2 days later. At the same time as immunisation mice also received 250µg of anti-RANKL or isotype control i.v.  a, Clinical scores of mice from day of immunisation to day 37.  b, Table showing data on the EAE course of the two groups of mice. Error bars represent SEM and data is representative of 2 experiments with 6-7 mice per group.
Figure 4.13. The effect of anti-RANKL on peptide stimulated splenocyte cultures.

OT-II splenocytes were cultured with 10μM pOVA. a, Expression of RANKL was determined on Ly5.1+ OT-II cells after 24, 48, and 72 hours of culture. Histograms show RANKL (black line) and isotype control (grey line) staining, numbers represent the percentage of RANKL+ cells. Data is representative of 2 separate experiments.

OT-II splenocyte cultures were stimulated with pOVA, and to each well 10μg/ml of anti-RANKL, anti-CD40 or isotype control was added. Graphs showing b, proliferation and c, IFNγ production from splenocyte cultures. Significant differences were determined by an ANOVA using Tukey’s multiple comparison test. Error bars represent SEM and data is representative of 4 experiments.
Figure 4.14. The effect of anti-RANKL on anti-CD3/anti-CD28 stimulated CD4+ T cell cultures.

CD4+ cells were MACS sorted from splenocyte preparations and cultured with plate bound anti-CD3, anti-CD3 plus anti-CD28 or unstimulated (all antibodies at 2µg/ml). **a,** Expression of RANKL was determined on CD4+ cells after 24, 48, and 72 hours of culture. Histograms show RANKL (black line) and isotype control (grey line) staining, numbers represent the percentage of RANKL+ cells. Data is representative of 2 separate experiments.

MACS sorted CD4+ cells were cultured with plate bound anti-CD3, anti-CD3 plus anti-CD28 or unstimulated (all antibodies at 2µg/ml). To each well 10µg/ml of anti-RANKL or isotype control were **b,** added to the culture wells (soluble) or **c,** plate bound with anti-CD3 and anti-CD28 (plate bound). Proliferative responses of cultures were determined after 72 hours. Error bars represent SEM and data is representative of 5 consistent experiments.
5. A variant myelin peptide induces T cell tolerance via a novel non-deletional mechanism.

5.1. INTRODUCTION.

Peptide therapy holds great potential as a treatment for autoimmune conditions, as current therapies for autoimmune diseases are non-specific and do not target the underlying cause of the disease. This potential has not yet been fully realised, no consensus has been reached on how it is best to administer peptide therapy, and as such its application has not been fully translated to the clinic. Several issues need to be considered when employing peptides to treat autoimmune diseases, and as such it has been shown that route of peptide administration, dose of peptide, and the characteristics of peptide used can all influence the outcome of tolerance induction (Anderton, 2001; Liblau et al., 1997). The relative contribution of each of these aspects to tolerance induction remains to be fully elucidated. Despite this, it is well established that different types of tolerance can be induced by administration of peptide via different routes, for example systemic versus mucosal routes (Weiner, 1997). The effect of high dose tolerance versus low does tolerance has also been investigated (Apostolou and von Boehmer, 2004; Chen et al., 1995; Friedman and Weiner, 1994; Kretschmer et al., 2005). However, the effect of altering characteristics of the peptide on tolerance induction remains ill defined. As such an important question remains outstanding; what is the effect of peptide-MHC affinity on T cell tolerance?

A well established approach for peptide-induced tolerance in adoptively transferred TCR transgenic T cells is through the administration of a high dose of peptide. Tolerance induction via this protocol has been shown to require an initial burst of activation (Kearney et al., 1994; Liblau et al., 1996), followed by a crash in T cell numbers as most peptide-reactive T cells are deleted (Dubois et al., 1998; Pape et al., 1998). Those remaining are hyporesponsive to stimulation. The aim of the present study was to determine the effect of using a peptide with a high affinity for MHC as a tolerogen when administered systemically at a high dose. These studies were
performed using a TCR transgenic known as the Tg4. As previously introduced, this TCR transgenic is reactive to the Ac1-9 immunodominant peptide from MBP in the context of I-A\textsuperscript{U} (Liu et al., 1995). The wild type Ac1-9 binds to the I-A\textsuperscript{U} with a very low affinity; a hierarchy of Ac1-9 APL has been defined based on the affinity of peptide binding to the I-A\textsuperscript{U} (Figure 1.5). Administration of 4Tyr (the highest affinity peptide) intra-nasally (Burkhart et al., 1999; Metzler and Wraith, 1999) and systemically via the intra-peritoneal route (Liu and Wraith, 1995) has proved very effective at inducing tolerance both in Tg4 T cells and in heterogeneous T cell populations. However an important question outstanding from these studies is the mechanism of tolerance induction employed by 4Tyr.

The aim of the experiments discussed in this chapter was to characterise peptide-tolerance induced by the 4Tyr peptide. The approach was to transfer a traceable population of Tg4 cells (Ly5.1+) and assess their fate, examining Tg4 cell number, phenotype and function, after administering a high dose of 4Tyr (200\mu g) systemically.
5.2. RESULTS

5.2.1. 4Tyr induces profound suppression of EAE in a TCR transgenic T cell transfer model.

To follow a discrete population of T cells in a heterogeneous repertoire, Tg4 cells were transferred into non-transgenic I-A^U-expressing mice. As a clonotypic antibody to identify the Tg4 TCR is unavailable, transferred cells were followed by expression of the congenic marker Ly5.1 (CD45.1). Ly5.1+ Tg4 T cells were transferred into B10.PL or B10.PLxC57BL/6 hosts, both of which express Ly5.2 (CD45.2).

In initial EAE experiments B10.PLxC57BL/6 mice were used as hosts. These mice do not develop EAE upon immunisation with Ac1-9 unless Tg4 T cells are first transferred (Ryan et al., 2005). Therefore, in this setting the Tg4 cells play the major role in the pathogenesis of the disease. A single i.v. injection of 200\mu g 4Tyr gave protection against subsequent EAE induced by immunisation with the wild-type Ac1-9 (4Lys) in CFA (Figure 5.1b). In contrast to this profound tolerance, treatment with the 4Lys peptide was totally ineffective at inducing tolerance at the dose used. These data fit with previous observations in non-transgenic models that increased tolerogenic properties of the Ac1-9 APL correlate with increased MHC binding affinities (Liu and Wraith, 1995; McCue et al., 2004).

The first surprising observation came from the analysis of the percentage of Ly5.1+ Tg4 cells in mice protected from EAE. As peptide-induced T cell tolerance is most frequently associated with the deletion of the majority of peptide-reactive T cells, few, if any, Tg4 cells were expected to remain in mice protected from EAE by treatment with 4Tyr. In fact, Tg4 cells persisted following tolerance induction (Figure 5.1c-e). A population of Tg4 cells could be found in the spleens, draining LN and CNS of all protected mice examined. In the spleen and LN, the percentage of Tg4 cells in disease-free, 4Tyr treated mice was greater than in PBS, or 4Lys treated mice. Therefore a series of Tg4 transfer experiments into non-transgenic I-A^U expressing mice was undertaken to dissect this finding.
5.2.2. Systemic administration of 4Tyr induces tolerance via a novel mechanism.

To explore the mechanism of tolerance induction following administration of a high dose of 4Tyr, it was compared to a model known to operate through the deletion of peptide-reactive T cells. As shown in chapters 3 and 4, transfer of OT-II cells to congenic hosts and subsequent treatment with a high dose of pOVA i.v. results in deletion of most OT-II cells (Figure 5.2b). Upon challenge with pOVA in CFA, tolerised mice do not mount any responses to the immunised pOVA as determined by ex vivo recall assays performed on spleen and LN 10 days after immunisation (Figure 5.2c-d). Unresponsiveness is directly due to the few OT-II cells persisting.

Comparative experiments were performed, in which Tg4 cells were adoptively transferred to congenic hosts prior to treatment with 200μg 4Tyr i.v. Mice were challenged with 4Lys in CFA, sacrificed 10 days post immunisation and spleen and LN sampled (Figure 5.2a). Recall responses were determined and the percentages of Tg4 cells persisting examined. This is the experimental protocol which most experiments in this chapter will follow. Strikingly, analysis of spleens (Figure 5.2e) and LN (data not shown) revealed expanded populations of Tg4 cells in mice that had received 4Tyr. In vitro recall assays with 4Lys showed that these cells were unresponsive as measured by IFNg (Figure 5.2g) and proliferation (data not shown). No IL-4 or IL-10 were detected in splenocyte cultures from either PBS or 4Tyr treated mice (data not shown). However, these cells did produce a significant amount of IL-2 in recall assays, to a similar level as non-tolerant PBS treated control mice (Figure 5.2f).

High dose treatment with 4Tyr i.v. induces a profound degree of tolerance in transferred Tg4 cells, as demonstrated by complete protection against 4Lys induced EAE (Figure 5.1). However the mechanism by which 4Tyr achieves this is not one of deletion, as Tg4 cells persist and retain the capacity to produce IL-2 following 4Tyr treatment. This production of IL-2 by the tolerant Tg4 cells may explain the persistence of Tg4 cells following 4Tyr treatment.
5.2.3. Persisting 4Tyr tolerised Tg4 cells are not anergic.
Classically anergic cells lack the ability to make IL-2 but retain the ability to make effector cytokines (Schwartz, 2003). This T cell state can be overcome by the addition of IL-2 to cultures. To ascertain whether treatment of Tg4 cells with 4Tyr had altered their sensitivity to IL-2 and therefore, to some extent, be similar to anergic T cells, exogenous IL-2 was added to cultures of 4Tyr tolerised Tg4 T cells. Whether exogenous IL-2 was able to overcome the tolerant phenotype was determined; the ability of exogenous IL-2 to restore effector function was examined. Tg4 cells were transferred to congenic hosts and treated with 4Tyr or PBS i.v. Mice were immunised with 4Lys in CFA and recall responses determined 10 days later. As before, a population of Tg4 cells persisted in both the spleen (Figure 5.3a) and LN (data not shown) of 4Tyr treated mice. Exogenous IL-2 was added to recall splenocyte cultures and the IFNγ produced in these cultures was determined. Addition of exogenous IL-2 did not overcome the inability of 4Tyr tolerised Tg4 cells to make IFNγ (Figure 5.3b). 4Tyr tolerised Tg4 cells are not classically anergic. The phenotype of these persisting, tolerant Tg4 cells is the subject of Chapter 6, the remainder of this chapter will focus on how these tolerant Tg4 cells are produced and rendered tolerant following treatment with 4Tyr.

5.2.4. Only 4Tyr can maintain a population of tolerised Tg4 T cells.
Two mechanisms could account for the persisting population of Tg4 cells seen after 4Tyr tolerisation. First, Tg4 cells could expand strongly after soluble 4Tyr exposure and then remain at high numbers without any significant further expansion upon immunisation with 4Lys in CFA. Second, the Tg4 cells might be maintained at low numbers following 4Tyr treatment, with a significant expansion in response to immunisation. Analysis at 4 and 6 days after 4Tyr treatment (without immunisation) supported the first of these explanations. Tg4 cells were transferred to B10.PL hosts one day prior to treatment with either 200μg of 4Lys, 4Ala, 4Val 4Tyr, or PBS i.v. Tg4 T cells constituted a sizeable percentage of the whole splenic CD4+ T cell population in 4Tyr treated mice at both day 4 and day 6 (Figure 5.4.a). Expansion of the Tg4 population was only seen following 4Tyr treatment, and not following
administration of 4Lys, 4Ala or 4Val, despite all these peptides being able to induce Tg4 cell proliferation in this system (McCue et al., 2004).

Mice were also sacrificed 7 days post peptide treatment and recall responses to 4Lys determined. IL-2 was produced only in the cultures from mice which had been treated with 4Tyr (Figure 5.4b). IFN\(\gamma\) was not seen in any culture supernatants (Figure 5.4c), nor was the production of IL-4, or IL-10 (data not shown). These data do not indicate that the other peptides were in any way tolerogenic; the lack of responses seen were most likely a readout of low numbers of Tg4 cells in the splenocyte cultures. Importantly these data demonstrate that by day 4 following 4Tyr treatment, 4Tyr stimulation of T cells has set in motion a series of events which lead to, by day 7, the persisting Tg4 cells having the ability to make IL-2 but not IFN\(\gamma\). Thus, by day 7 the 4Tyr tolerised Tg4 cells had already adopted their novel tolerant phenotype. Previous studies have determined that tolerance induction following 4Tyr treatment takes 3 days to be established (Liu and Wraith, 1995), therefore analysis of Tg4 cell responsiveness was performed one day beyond this point, at day 4 following peptide treatment. Ex vivo recall responses of splenocytes from peptide treated mice were determined to increasing concentrations of 4Lys. As shown in Figure 5.5, 4 days after 4Tyr treatment tolerant Tg4 cells had the capacity to produce IL-2, but not IFN\(\gamma\) or IL-10. Some IFN\(\gamma\) was produced by 4Tyr tolerised Tg4 cells, but this was never significantly greater than that from other groups, and was consistently at a low level, never exceeding 1ng/ml (Figure 5.5b). The establishment of this cytokine profile 4 days after peptide treatment demonstrates the tolerance induction in Tg4 cells is complete by this early time point.

The 4Val APL shows the second highest affinity for I-A\(^U\) (the next APL down from 4Tyr in the hierarchy of affinity for I-A\(^U\)). 4Val binds to the I-A\(^U\) with only a 10-fold lower affinity than the 4Tyr peptide (Liu and Wraith, 1995). As this peptide differs the least in binding affinity compared to 4Tyr, its ability to induce tolerance compared to 4Tyr was examined. As shown in Figure 5.4, 4Val treatment does not lead to Tg4 T cell expansion at early time points after peptide treatment. Mice were immunised with 4Lys in CFA 7 days after peptide treatment and recall responses and
persisting Tg4 cells examined 10 days later. After immunisation, a trend of decreased percentages of Tg4 cells persisting following 4Val compared to 4Tyr treatment was seen (Figure 5.6a). Although repeated a number of times, this trend was continually seen but never became significant. Treatment with 4Val induced tolerance in the Tg4 cells, inhibiting the ‘normal’ production of IL-2 and IFNγ seen from the PBS treated groups (Figure 5.6b-c). Importantly, unlike 4Tyr tolerised groups, 4Val tolerised mice produced no IL-2 upon recall (Figure 5.6b). This experiment was carried out six times and in two experiments, treatment with 4Val failed to induce tolerance in the Tg4 cells. Nevertheless, these data demonstrate that tolerance induced with 4Tyr is different to that induced with 4Val, despite there being only a small, 10-fold difference in the binding affinity of the peptides for I-A^U. As such, only 4Tyr can induce the novel state of tolerance in the Tg4 T cells described here.

5.2.5. Administration of soluble 4Tyr i.v. induces considerable proliferation of Tg4 T cells.

Tg4 cells appear to undergo expansion following 4Tyr treatment (Figure 5.4a); this could account for the considerable population of tolerant Tg4 T cells persisting post tolerance induction. 4Tyr is a super-agonist of the Tg4 TCR, as in vitro it can induce proliferation of Tg4 cells at considerably lower concentrations compared to 4Lys (Anderton et al., 2001). The proliferation of transferred Tg4 cells in vivo following treatment of hosts with 200µg of 4Tyr was examined. The proliferation induced by the various position 4 APL has been examined before (McCue et al., 2004). The aim here was to compare the degree of proliferation induced by treatment with 200µg of 4Tyr to the maximal proliferation induced by 200µg 4Tyr administered with the adjuvant LPS, to assess the degree of proliferation induced by 4Tyr. CFSE-labelled Tg4 cells were transferred to hosts, which were subsequently treated with 4Tyr, 4Tyr+LPS or PBS i.v. CFSE dilution in Tg4 cells from the spleen was examined at day 2 and day 7 post peptide treatment.

As shown in Figure 5.7a-b, PBS treatment did not induce any proliferation in the Tg4 cells. Interestingly, 4Tyr appeared to induce a similar degree of proliferation in Tg4
cells compared to 4Tyr+LPS treatment. Closer examination of the percentage of cells in each generation revealed that 4Tyr treatment induces slightly, but significantly, reduced proliferation compared to 4Tyr+LPS treatment. At day 2 (Figure 5.7c), this was highlighted by a small, yet significantly reduced percentage of Tg4 T cells in the later generations and a small, but significant increase in the percentage of undivided Tg4 cells. By day 7 (Figure 5.7d), this was shown by looking at the percentages of undivided and divided cells which were significantly different between the two treatment groups. This resulted in greater expansion of the Tg4 cell population in the 4Tyr+LPS treated mice compared to those treated with 4Tyr alone. The percentage of Tg4 cells in the CD4+ splenic population at day 7 was 0.33±0.06% for PBS treated mice, 2.52±0.18% for 4Tyr treated mice, and 8.415±1.25% for 4Tyr+LPS treated mice. These data show that administration of 4Tyr i.v. induced a significant amount of proliferation in transferred Tg4 cells, but the addition of LPS caused more Tg4 cells to enter into cell cycle.

The degree of apoptosis induced in Tg4 cells following either 4Tyr or 4Tyr+LPS was examined. Annexin V and 7-AAD staining was employed to identify apoptotic cells; cells in the early stages of apoptosis are Annexin V+, cells in the later stages of apoptosis are Annexin V+, 7-AAD+. As shown in Figure 5.7e, few 4Tyr stimulated Tg4 cells were apoptotic. However, this was not the case for Tg4 cells stimulated by 4Tyr+LPS, where a considerable proportion of Tg4 cells had entered into apoptosis (Figure 5.7f).

5.2.6. 4Tyr-MHC complexes persist for long periods of time in vivo following administration of 4Tyr.

As the ability of the Ac1-9 position 4 APL to induce tolerance directly correlates with their affinity for I-A^U, it was important to ascertain how long 4Tyr-MHC complexes would be maintained in vivo. Few studies have been able to measure the display of peptide-MHC complexes after administration of soluble peptide and those which have been successful have noted peptide display to a maximum of 5 days following peptide injection (Pape et al., 1998). This most probably reflects the rapid clearance of peptides from the circulation.
This question could be answered via two approaches. First, the stability of 4Tyr-MHC complexes \textit{in vivo} could be tested by sampling spleens from mice treated with 4Tyr at different time points and using these to stimulate a Tg4 T cell line (TCL). This work had been previously performed in the laboratory, and it was shown that splenocytes from 4Tyr treated mice could activate a Tg4 TCL when taken up to 96 hours after peptide treatment. However activation of a Tg4 TCL by splenocytes from 4Val treated mice was only evident when spleens were taken two hours after peptide treatment, (Sweenie and Anderton, unpublished observations).

A second, and more sensitive, approach was to administer the peptide to the mice first, then after various time points inject CFSE-labelled Tg4 T cells and re-isolate them 3 days after transfer. The loss of CFSE by the Tg4 T cells would be indicative of \textit{in vivo} division, and therefore the display of 4Tyr-MHC complexes \textit{in vivo} in a form that was available for T cell stimulation. As shown in Figure 5.8a, this approach gave the remarkable result that the 4Tyr-MHC complexes were maintained \textit{in vivo} for up to 14 days after peptide treatment. As would be expected, Tg4 cell division was greatest when cells were given soon after peptide administration. However, the Tg4 cells could still enter mitosis when transferred to mice that had received the 4Tyr peptide either 14 or 10 days earlier, with only 27.4±6.7% and 10.3±1.2% of Tg4 cells respectively remaining undivided 3 days after transfer (Figure 5.8.b). This capacity of 4Tyr for long-term association with the MHC is a unique characteristic of this peptide and is likely the major driving force behind the novel mechanism of tolerance induced following 4Tyr treatment.

5.2.7. \textbf{Induction of tolerance in Tg4 cells when 4Tyr is administered before Tg4 cell transfer.}

4Tyr holds the striking ability to persist and influence the immune system for up to 14 days after its administration. This raised the possibility that administration of 4Tyr at time-points prior to Tg4 cell transfer would be able to induce tolerance in Tg4 cells. Immediately following peptide treatment, 4Tyr-MHC complexes would be present at a high density on APC. With time the number of 4Tyr-MHC would be
reduced, highlighted by the reduced proliferation seen when 4Tyr was given 14 and 10 days prior to Tg4 cell transfer (Figure 5.8). The ability of 4Tyr to induce tolerance when administered prior to Tg4 cells was addressed. These experiments would give an indication of the density of 4Tyr-MHC complexes needed to induce tolerance, and as such ascertain whether a high density and/or persistence of these complexes was needed for the novel mechanism of tolerance seen.

Mice were treated with 4Tyr or PBS 2 days prior to the transfer of Tg4 cells, 7 days post peptide treatment mice were immunised with 4Lys in CFA and recall responses determined a further 10 days later. Administration of 4Tyr 2 days prior to Tg4 cell transfer induced tolerance in the Tg4 cells (Figure 5.9). The phenotype of this tolerance was similar to that induced when 4Tyr was administered 1 day after Tg4 cells. A population of Tg4 cells persisted following tolerance induction, however, this population was significantly smaller than that from PBS treated mice (Figure 5.9a). Importantly, these persisting Tg4 cells did not proliferate or produce any IFNγ (Figure 5.9b-c). The persisting, tolerant Tg4 cells did however retain their ability to produce IL-2 upon recall to 4Lys (Figure 5.9d). These data suggest that tolerance had been induced in 4Tyr treated Tg4 cells via the novel non-deletional mechanism when 4Tyr was administered 2 days before Tg4 transfer.

A time course was established to determine the point at which tolerance was no longer induced when 4Tyr was administered prior to Tg4 cell transfer. In an experiment performed as above, 4Tyr was administered to mice 14, 7 or 4 days prior to the transfer of Tg4 cells (Figure 4.10a). Examination of recall responses 10 days after immunisation with 4Lys in CFA revealed that 4Tyr administration 14 days prior to cell transfer failed to induce tolerance in the Tg4 cells. A significant population of Tg4 cells was seen in the spleen (Figure 5.10b), and upon recall to 4Lys splenocytes from these mice produced significant amounts of IL-2, IFNγ and IL-17, Figure (5.10c-e). Tolerance was induced in the transferred Tg4 cells when peptide was administered either 7 days or 4 days prior to Tg4 cells transfer. Strikingly, the tolerance seen was not the same as the novel state of tolerance shown in Figure 5.2. Instead most of the Tg4 cells appeared to be deleted when 4Tyr was administered 7
days or 4 days before Tg4 cells transfer (Figure 5.10b). As a result, upon recall to 4Lys, splenocyte cultures produced little IL-2, IFNγ or IL-17 (Figure 5.10c-e). Therefore tolerance induced by 4Tyr administration at either day 7 or day 4 was more like the ‘normal’, deletional tolerance previously described.

5.2.8. 4Tyr-MHC complexes are maintained by CD11c+CD4+ DC.
An outstanding question concerning peptide-induced tolerance is which APC present the peptide to the T cell to induce tolerance? Several studies have attempted to address this question by quantifying in vitro readouts of p:MHC complexes on different APC following antigen administration (Chung et al., 2007; Pooley et al., 2001). Although these studies have proved successful and have demonstrated a propensity for CD11c+CD8+ DC to present to CD8+ T cells and CD11c+CD8−/CD11b+ DC to present to CD4+ T cells, these studies have administered whole protein Ags. Intact protein Ags require processing prior to formation of p:MHC complexes. Therefore such studies do not address which APC might preferentially bind soluble peptide, in a situation where no processing should be required. The persistence of 4Tyr:MHC complexes following 4Tyr administration provides an excellent tool to answer this question. Previous work in the laboratory has suggested that it is the DC which preferentially display 4Tyr-MHC complexes following 4Tyr administration i.v. (Sweenie and Anderton, unpublished observations). Here these studies were extended; immunofluorescent microscopy was performed looking at spleen sections from mice which had received 4Tyr or PBS, 1 day prior to transfer of CFSE labelled Tg4 cells. Sections were stained for CD11c, CD19 and DAPI and showed that the CFSE+ Tg4 cells localised to areas of CD11c staining rather than CD19 staining (Figure 5.11a). Following 4Tyr treatment, CFSE labelled Tg4 cells appeared to form more clusters. Importantly, following 4Tyr treatment, CFSE labelled Tg4 cells were seen to interact with CD11c+ cells (Figure 5.11b-c).

By FACS sorting DC populations from spleens following 4Tyr treatment it was possible to determine which DC subsets were maintaining the 4Tyr-MHC complexes. As shown in Figure 5.12a-b, four major subsets of DC are found in the spleen; CD11cmedPDCA-1+ plasmacytoid DC (pDC), CD11c+CD4+, CD11c+CD8+, CD11c−/CD11b−/CD11c+CD8+.
and CD11c+CD4-CD8- DC. These subsets were sorted and cultured with a Tg4 TCL at increasing DC number and proliferative responses determined. Only the CD11c+CD4+ DC could induce proliferation of the Tg4 TCL (Figure 5.12c) and only when purified from 4Tyr treated mice (Figure 5.12d). As a control to show that all sorted DC were capable of forming 4Tyr:MHC complexes and therefore stimulating the Tg4 TCL, 100μM exogenous 4Lys was added to culture wells containing the highest number of sorted DC. In all these cultures proliferation of the Tg4 TCL was seen (Figure 5.12.e). These data demonstrate that a population of DC specifically maintain the 4Tyr-MHC complexes; this population is the CD11c+CD4+ subset.

5.2.9. Tolerance induction following DC depletion.

The data presented above demonstrate that 4Tyr-MHC complexes are exclusively maintained on CD11c+CD4+ DC. It follows, therefore, that the depletion of this subset of DC should prevent the induction of peptide tolerance and also the proliferation of Tg4 cells. As yet the tools to deplete this specific DC subset do not exist, however it was possible to ask whether depletion of all CD11c+ DC from mice resulted in prevention of peptide-induced tolerance. CD11c.DOG mice are a bacterial artificial chromosome (BAC) transgenic, in which the BAC chromosome is under the control of the CD11c promoter and contains the diphtheria toxin receptor (DTR), eGFP and the ovalbumin protein (Hochweller et al., 2008). Injection of these mice with diphtheria toxin (DTx) has been shown to induce depletion of CD11c+ DC in the spleen, LN, thymus and bone marrow. CD11c.DOG mice were employed to ascertain the role of DC in peptide-induced tolerance, by crossing CD11c.DOG mice onto the B10.PL background (B10.PLxDOG). B10.PLxDOG mice were treated with either 4Tyr or PBS one day prior to DC depletion by administration of DTx (Figure 5.13a). The percentage of DC depleted is shown in Figure 5.13b, and shows that the pDC were the DC subset best depleted by DTx treatment. This was unexpected as this DC subset displays the lowest levels of CD11c. The CD11c+CD4+ subset was the least well depleted by DTx treatment, with only ~51% of these cells being depleted. As this is the DC subset of interest this limited depletion represents the major caveat of this experiment.
Determination of recall responses 10 days after immunisation with 4Lys demonstrated that depletion of DC did not prevent tolerance from being induced by 4Tyr treatment. Transferred Tg4 cells did not proliferate (Figure 5.13c) nor did they produce IFNγ (Figure 5.13d) upon recall to 4Lys. As such tolerance was successfully induced by 4Tyr treatment following the limited depletion of CD11c+ DC upon DTx treatment.

5.2.10. Proliferation of Tg4 cells following DC depletion

As 4Tyr induced tolerance could still be induced following CD11c+ DC depletion in B10.PLxDOG mice, the experimental approach was modified to determine the importance of DC in the display of 4Tyr:MHC complexes. The proliferative response of Tg4 cells to 4Tyr \textit{in vivo} was examined following DC depletion by DTx treatment. Proliferation could be explored at early time points after Tg4 cell transfer to determine if changes in DC number altered the proliferative capacity of the transferred Tg4 cells. As shown in Figure 5.14a, the DTx dose in these experiments was increased. This altered DTx dosing regime led to a greater depletion of CD11c+CD4+ DC (Figure 5.14b and Appendix Figure A.5).

Quantification of the number of Tg4 cells in the spleens of mice at each day is shown in Figure 5.14c. A clear reduction in Tg4 cell numbers was seen by day 3 post Tg4 cell transfer from mice given DTx. This was also true for the percent of Tg4 cells as a percent of all CD4+ cells in the spleen at day 3; 1.7±0.77% in PBS treated compared to 0.82±0.2% in DTx treated mice. CFSE dilution of Tg4 cells was determined at day 1, 2, and 3 post Tg4 cell transfer, as shown in Figures 5.14d-f. At day 1 no difference in the CFSE profile was noted between PBS and DTx treated mice. However, by day 2 a clear difference was observed, which was further exaggerated in the CFSE profiles from day 3; there was a significant reduction in the percentage of Tg4 cells in the later generations and a significant increase in the percentage of Tg4 cells in the earlier generations following DTx treatment. These data demonstrate that depletion of DC from mice reduces the availability of 4Tyr-MHC complexes and as such reduces the proliferation of transferred Tg4 cells.
5.3. DISCUSSION

5.3.1. 4Tyr induces a profound state of tolerance in Tg4 cells via a non-deletional mechanism.

Systemic administration of a high dose of 4Tyr was shown to induce a profound degree of tolerance in transferred Tg4 cells. 4Tyr treatment completely protected against EAE induction in a model of disease dependent on the presence of Tg4 cells (Figure 5.1). Tolerance is not mediated by the deletion of the peptide-reactive Tg4 cells, as they persist as a considerable percentage of the whole CD4+ population following establishment of tolerance to 4Lys. These data indicate that 4Tyr treatment of Tg4 cells induces tolerance via a novel mechanism. Tolerised Tg4 cells acquire a novel tolerant phenotype whereby they persist, but are incapable of initiating disease. Persisting tolerant Tg4 cells are not classically anergic as they retain the ability to make IL-2 but cannot make any effector cytokines upon recall to 4Lys (Figure 5.2 and 5.3), moreover, it takes only four days for this novel phenotype to be established (Figure 5.5). Thus, 4Tyr is a highly potent tolerogen and establishes tolerance via a novel mechanism. Although 4Tyr tolerised Tg4 cells do not become foxp3+ (discussed in chapter 6), it would be pertinent to ascertain whether they could regulate other immune cells. Continuing previous studies in the laboratory (Anderton and Wraith, 1998), it would be interesting to determine whether Tg4 cells tolerised by 4Tyr can mediate tolerance, by bystander suppression, to other CNS Ag.

The novel mechanism of tolerance employed by 4Tyr is most likely the result of the greatly increased I-A\textsuperscript{U} binding affinity of the 4Tyr APL which enhances stability of the p:MHC \textit{in vivo} (see section 5.3.2). This conclusion has been reached after examining tolerance induction following treatment with 4Val and examination of the Tg4 cell population following treatment with 4Lys, 4Ala, 4Val, and 4Tyr. Only the 4Tyr peptide can support the expansion of the Tg4 cell population following administration (Figure 5.4). Moreover, 4Val has only a ten-fold lower affinity for the I-A\textsuperscript{U} yet does not induce tolerance via the novel, non-deletional mechanism (Figure 5.6). The prediction would have been that 4Tyr and 4Val would share a similar mechanism of tolerance induction as, even though 4Tyr binds to the I-A\textsuperscript{U} with a 10-fold higher affinity, both peptides bind to the I-A\textsuperscript{U} with a 10\textsuperscript{6}-fold higher
affinity than 4Lys. However, previous data from the laboratory has shown that 4Val has a much faster *in vivo* off-rate when interacting with I-A\(^{U}\), compared to 4Tyr (Sweenie and Anderton, unpublished observations). Altering affinities within the trimolecular p:MHC:TCR complex has previously been shown to result in distinct mechanisms of tolerance induction. Skokos et al demonstrated that altering the potency of p:MHC for a TCR through use of APL, resulted in tolerance induction via difference mechanisms. High potency p:MHC complexes permitted formation of long-lived T cell-DC contacts and the induction of anergy via a calcineurin-dependent mechanism, which was not the case for lower potency p:MHC (Skokos et al., 2007).

Why are Tg4 cells not deleted during tolerance induction? As shown in Figure 5.7, 4Tyr induces a substantial degree of proliferation and little apoptosis in Tg4 cells. This initial burst of proliferation concomitant with minimal Tg4 cell deletion presumably allows tolerant Tg4 cells to persist. A non-deletional mechanism of tolerance raises questions over what is in fact the best mechanism of tolerance induction to employ following peptide treatment. Although most peptides induce a considerable degree of deletion following administration, is this really the best way for tolerance to be induced when considering this a therapy applicable to the clinic? The main disadvantage of deletion as a mechanism of tolerance is that it is exquisitely antigen specific and unlikely to cause tolerance to any other disease relevant peptide. Perhaps more importantly, Han et al demonstrated that deletion of all peptide-reactive T cells following peptide treatment was not the best way to protect against diabetes in the NOD mouse model (Han et al., 2005). This study showed that the peptides which were most protective against diabetes allowed low affinity T cells to escape deletion. These low affinity T cells occupied a niche in the T cell repertoire that would have been left empty following deletion and into which T cells, with different disease-relevant specificities, could expand. An alternative view of deletion as a mechanism of tolerance has recently been provided by Perruche et al who suggest that mass deletion of T cells following peptide treatment could induce Tregs (Perruche et al., 2008). They demonstrated that engulfment of apoptotic T cells by immature DC or macrophages caused the phagocytosing cell to produce
TGFβ and hence led to the generation of foxp3+ Tregs. These data highlight the continuing debate over the best mechanism of tolerance induction to employ when administering peptide therapy.

5.3.2. Cytokine profile of 4Tyr tolerised Tg4 cells.

No IL-10 was produced by tolerised Tg4 cells at any time point on recall to 4Lys. This is in contrast to the mechanism of 4Tyr-induced tolerance noted by other investigators (Burkhart et al., 1999; Massey et al., 2002). Studies have shown that 4Tyr tolerisation of Tg4 cells is primarily associated with the production of IL-10, as administration of anti-IL-10 overcomes the tolerant phenotype. These studies are different to those discussed here; 4Tyr was administered via the mucosal route and required repeated doses for tolerance to be induced (see section 5.3.4). In the experiments discussed here, a single high dose of 4Tyr delivered systemically was sufficient to induce tolerance in adoptively transferred Tg4 cells.

Although the persisting, tolerant Tg4 cells do not make IL-10, these cells produce significant amounts of IL-2 upon recall, Figure 5.2, 5.4, and 5.5. It appears somewhat counter-intuitive that disease-relevant cells could persist, retain the capacity to make IL-2 and yet not initiate disease, given the well characterised role of IL-2 in the generation and expansion of effector T cells. The question of whether inappropriate expression of IL-2 could mediate an autoimmune response has been addressed by others. Elliott et al generated RIP-IL-2 mice which developed diabetes due to islet infiltration and destruction of islet tissue mediated by non-Ag specific immune cells (Elliott and Flavell, 1994). Heath et al generated triple transgenic RIP-IL-2 x RIP-Kb crossed with a TCR transgenic specific for a Kb peptide (Heath et al., 1992). These mice rapidly developed diabetes mediated by the Kb-specific T cells, suggesting both locally produced IL-2 and a considerable pool of Ag-reactive T cells were needed for disease initiation. More recently, Waithman et al demonstrated tolerance induction in OT-I cells transferred to RIP-OVA mice was prevented and autoimmunity resulted when stimulatory IL-2 complexes (anti-IL-2+IL-2) were administered along with the OT-I cells (Waithman et al., 2008). These studies would suggest that the mechanism by which 4Tyr induces tolerance in Tg4 cells would be
insufficient for 4Tyr to protect against EAE, yet Figure 5.1 clearly demonstrates that 4Tyr profoundly protects against disease. Taken together, these studies suggest that production of only IL-2, without the necessary effector cytokines by the 4Tyr tolerised Tg4 cells is insufficient to induce disease. In addition, a considerable amount of data suggests that IL-2 plays a more important role in the development and function of Tregs compared to effector T cells (Malek, 2008). As such the enhanced IL-2 from tolerised Tg4 cells might be contributing to a more tolerogenic environment by enhancing the number and/or effectiveness of Tregs (see Chapter 6).

5.3.3. 4Tyr can persist in a biologically relevant form for 14 days.
4Tyr can induce tolerance in Tg4 cells via a mechanism not shared by any other Ac1-9 position 4 APL (Figure 5.4 and 5.6). Unlike the other APL, 4Tyr has the ability to persist in vivo, in a biologically relevant form bound to I-A\textsuperscript{U} for 14 days after administration (Figure 5.8). Persistence of 4Tyr would explain why this peptide induces tolerance by a novel mechanism. Previously, following systemic administration, peptide has been shown to remain available to the immune system for a maximum of 5 days (Pape et al., 1998). 4Tyr persistence for an extended period allows tolerance induction to occur when 4Tyr is administered to mice before the transfer of Tg4 cells (Figure 5.9 and 5.10). Tolerance induction occurs via the non-deletional mechanism when 4Tyr is administered 2 days before Tg4 cells, suggesting sufficient 4Tyr-MHC complexes persist at this time to stimulate Tg4 cells to adopt the novel tolerant phenotype. However, when 4Tyr is administered 4-7 days before Tg4 transfer, tolerance induction appears to occur through the deletion of Tg4 cells (Figure 5.10b). These data suggest that the mechanism of tolerance changes as the number of 4Tyr-MHC complexes is reduced. When 4Tyr-MHC complexes have been reduced below a certain level, shorter exposure to 4Tyr occurs and Tg4 cells are deleted on their way to tolerance. Significantly, tolerance could not be induced in Tg4 cells when 4Tyr was administered 14 days before Tg4 cell transfer (Figure 5.10).

Due to 4Tyr persistence, the tolerance model explored here is somewhat similar to that in which TCR-transgenic cells are transferred to mice in which their cognate Ag
is constitutively expressed. Transgenic mice expressing MBP1-10 covalently bound to I-A\textsuperscript{U} have been crossed with the Tg4 mouse (Kurschus et al., 2006). These mice experienced massive deletion of thymocytes. This is consistent with data from Liu et al showing that systemic administration of 4Tyr i.p. to TCR transgenic mice reactive to Ac1-9, results in thymic deletion of Ac1-9 reactive thymocytes (Liu et al., 1995). In the Kurschus et al study, Tg4 cells which did emerge from the thymus were refractory to stimulation \textit{in vitro}. Strikingly, the persisting Tg4 cells (both CD25+ and CD25- fractions) had the ability to suppress EAE progression when transferred to B10.PL mice. Such data propose an interesting hypothesis whereby continued Ag stimulation generates cells with a regulatory phenotype.

5.3.4. The effect of Ag persistence on CD4+ T cells.

It is generally accepted that in order to activate a CD4+ T cell continued presence of the cognate Ag is required throughout the priming events (Celli et al., 2007; Obst et al., 2005). Recently, Obst et al demonstrated that continued Ag persistence could lead to T cell priming similar to that induced by activated DC (Obst et al., 2007). Consequently it must be asked, why does continued 4Tyr presence not induce activation of Tg4 cells? Two factors could account for the difference; firstly Obst et al controlled Ag persistence at the genetic level, employing an inducible expression system to switch on presentation of a peptide from moth cytochrome c (MCC) which could potentially influence DC phenotype. Secondly, the number of p:MHC complexes on the surface of DC may well differ between the two experimental systems.

Repetitive Ag stimulation has been shown to alter the transcriptional program of Tg4 cells (Anderson et al., 2006). Administration of 10 doses of 4Tyr i.n. to a Tg4 transgenic mouse every 3-4 days resulted in the establishment of a different transcription program which caused suppression of proliferation and IL-2 production, and enhanced IL-10 production. Transcription factors found to be important for the tolerant state included T-bet and Erg-2. This group has also demonstrated that repeated Ag dosing is essential for acquisition of this tolerant state. The cytokine profiles of Tg4 cells were examined after 1, 2, 3, 5 and 10 doses of i.n. 4Tyr.
Anderson et al demonstrated that IFN\(\gamma\) is switched off and IL-10 switched on after 3 doses, and that it takes at least 5 doses to turn off IL-2 production (Anderson et al., 2005). As it is established that T cells can ‘count’ serial Ag encounters (Henrickson et al., 2008), the Tg4 cells studied here could have received enough TCR stimulation to switch off IFN\(\gamma\), but not yet received enough to switch IL-10 on or IL-2 off. Moreover, during the repeated dosing strategy the Ag load on the DC would continually be replenished, allowing repeated stimulation of Tg4 cells by a similar density of 4Tyr:MHC. Fittingly, Wells et al have demonstrated that the ability of a T cell to respond upon recall is dependent on the number of divisions the cell has previously gone through (Wells et al., 2000), and a number of studies have demonstrated that cell cycle determines cytokine expression with more cycles required to switch on IL-10 compared to IL-2 (Gett and Hodgkin, 1998). Such data could explain the difference in cytokine production by persisting tolerant T cells in this system compared to that seen by Wraith and colleagues (Anderson et al., 2005; Burkhart et al., 1999); the tolerant Tg4 cells studied here could have been stimulated enough to switch off IFN\(\gamma\) but not enough to switch IL-10 on or IL-2 off.

It would be interesting to ascertain whether Tg4-DC interactions last longer following 4Tyr treatment or whether Ag persistence allows for a single T cell to make more serial encounters with 4Tyr displaying DC. Such data would demonstrate whether the novel mechanism of tolerance induced in Tg4 cells following treatment with 4Tyr is due to longer lasting or more T cell-DC encounters. The persistence of 4Tyr:MHC complexes and the hierarchy of Ac1-9 peptide with increasing affinity for MHC could be employed to ask what drives a T cell and DC to dissociate during initiation of a T cell response. Data from a number of studies suggest this is mediated by break down of the p:MHC-TCR complex, in fact it is suggested that a T cell would disengage from DC once p:MHC complexes have fallen below a certain threshold (Celli et al., 2007). This could be investigated through use of 4Tyr and the other position 4 APL in the Tg4 system.
5.3.5. **CD11c+ CD4+ DC maintain the 4Tyr-MHC complexes.**

Previous data show that both B cells and DC can present peptides after i.v. administration of soluble protein, but that DC are superior (Zhong et al., 1997). Administration of whole protein requires internalisation of the protein and processing for presentation as peptides to T cells. Soluble peptides do not require such processing and would probably load onto MHC-II molecules in a compartment close to the cell surface, if not at the cell surface itself. In fact, soluble peptide has been shown to be correctly displayed on MHC-II 5 minutes after administration i.v. (Celli et al., 2007). Processing of proteins for display requires an extra level of complexity not required following administration of 4Tyr. Nevertheless, most studies exploring which DC subset present to CD4+ and CD8+ T cells have looked at T cell priming after administration of whole protein. Data have shown that CD11c+CD8α+ (DEC205+) DC present to CD8+ T cells whereas CD11c+CD8α- (33D1+, CD11b+, CD4+) DC present to CD4+ T cells. Dudziak et al demonstrated that these different populations of DC are biochemically programmed to be better at processing Ag in the MHC-I or MHC-II presentation pathways respectively (Dudziak et al., 2007).

Here it was determined which of the main DC subsets found in the spleen (Vremec et al., 2000), are the best at maintaining 4Tyr-MHC complexes. The data presented demonstrate that it is the CD11c+CD4+ DC which present 4Tyr to Tg4 cells *in vivo*. Depletion of CD11c+ cells from mice dramatically reduced the proliferation of Tg4 cells demonstrating the bulk, if not all, of the presentation is limited to DC (Figure 5.14). Moreover, isolation of DC populations from 4Tyr treated mice showed that only CD11c+CD4+ DC had the capacity to stimulate a Tg4 TCL (Figure 5.12). However, it was possible to induce tolerance in Tg4 cells upon DTx-mediated depletion of CD11c+ cells (Figure 5.13). This does not argue against a role for CD11c+CD4+ DC; instead it highlights a clear caveat of this experiment. Limited depletion of the CD11c+CD4+ DC resulted following DTx treatment; ~51% of this DC population was depleted, leaving a significant population of DC able to present 4Tyr to, and induce tolerance in, Tg4 cells.

Demonstration that CD11c+CD4+ DC maintain the 4Tyr-MHC complexes is consistent with previous data, highlighting the propensity of CD11c+CD4+ DC to...
present to CD4+ T cells. Why do CD11c+CD4+ DC present 4Tyr? This population of DC has been shown to have the longest half-life of the DC subsets found in the spleen ($t_{1/2}=2.9$ days) (Kamath et al., 2000), and could perhaps maintain the 4Tyr-MHC complexes for longer. However, it more likely that CD11c+CD4+ DC present 4Tyr-MHC complexes because of their location in the spleen. CD11c+CD4+ DC have been shown to be located mainly in the marginal zones and T cell areas of the spleen, CD11c+CD8+ and CD11c+CD4-CD8- DC are located mainly within the red pulp (McLellan et al., 2002). Such a location would place CD11c+CD4+ DC closer to the marginal sinus and therefore put them in a prime position to capture blood borne Ag. Therefore, it appears unlikely that these DC would bear any unique biochemical traits which would specifically endow them with the function of tolerance induction, as there ability to present peptide in a tolerogenic manner is dependent on their location. Nevertheless it would be interesting to determine whether targeting peptides specifically to this DC subset over others qualitatively alters peptide tolerance induction. The identification of CD11c+CD4+ DC as the subset of DC which maintain 4Tyr-MHC complexes raises a number of further questions. Firstly it is of importance to determine whether all peptides administered i.v. would preferentially bind to these DC. Here, it is suggested that they would, but such a question can be addressed through addition of FITC to other peptides and then examination of FITC+ cells following administration of peptide. Continuing studies should address whether specific depletion of CD11c+CD4+ DC prevents peptide-tolerance induction following systemic peptide administration.

5.3.6. Concluding Remarks.

Data presented here demonstrate that 4Tyr is an extremely potent tolerogen of Tg4 cells when administered i.v. at a high dose. Interestingly, induction of tolerance by 4Tyr is via a novel, non-deletional mechanism. Following 4Tyr treatment tolerant Tg4 cells persist; this has yielded a unique opportunity to explore the phenotype of these tolerant T cells, this is the focus of chapter 6.
Figure 5.1. 4Tyr protects against 4Lys induced EAE.

a, Experimental outline; B10.PLxC57BL/6 mice received PBS, 4Lys or 4Tyr i.v. 1 day after transfer of Ly5.1+ Tg4 T cells. Mice were immunised with 4Lys in CFA 7 days later and given Ptx i.p at the same time as immunisation and 2 days later. b, Clinical scores of mice from day of immunisation to day 27; p<0.005 represents a significant difference as determined by a Mann-Whitney test. At day 19 some mice were sacrificed and analysis of cell populations in the c, spleen, d, LN and e, pooled CNS performed. Error bars represent SEM, data is representative of 2 similar experiments with 5-8 mice per group.
Figure 5.2. Soluble 4Tyr induces tolerance via a different mechanism to soluble pOVA.

a, Experimental outline; b-d, 1-2x10^6 Ly5.1+OT-II cells were transferred to C57BL/6 mice. The following day mice received either 500μg pOVA or PBS iv, 7 days later mice were immunised with pOVA in CFA s.c. Mice were sacrificed 10 days post immunisation. Similarly, e-g, 1-2x10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice. The following day mice received either 200μg 4Tyr or PBS iv, 7 days later mice were immunised with 4Lys in CFA s.c. Mice were sacrificed 10 days post immunisation. b, e, Bar chart showing transgenic T cells as a percentage of all CD4+ cells in the spleen at time of harvest. Ex vivo cytokine production of splenocytes to increasing doses of pOVA (OT-II) or 4Lys (Tg4) were determined by ELISA. c, f, IL-2 and d, g, IFNγ production shown as a percentage of the maximum amount of cytokine produced.

Error bars represent SEM, data is representative of 7-9 consistent experiments with at least 3 mice per group.
Figure 5.3. Persisting 4Tyr tolerised Tg4 T cells are not classically anergic.
1-2x10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice 1 day prior to 200µg 4Tyr or PBS iv. Mice were immunised with 4Lys in CFA s.c. 7 days later and sacrificed 10 days post immunisation and recall responses of splenocytes determined. a, Representative plots of splenocytes from PBS or 4Tyr treated mice; percentages represent Tg4 cells as a percent of all cells in the lymphocytes gate. b, Bar chart showing IFNγ production from ex vivo splenocyte cultures with added exogenous IL-2; either stimulated with 10µM 4Lys (+) or unstimulated (-). Significant difference shown was determined by unpaired T test. Error bars represent SEM, data is representative of 1 experiment with 3-4 mice per group.
Figure 5.4. Only 4Tyr can maintain a population of tolerised Tg4 cells.

1-2x10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice one day prior to administration of 200µg of 4Lys, 4Ala, 4Val or 4Tyr or PBS iv. a, Mice were sacrificed at day 4 (open bars) or day 6 (closed bars) post peptide treatment and the percent of Tg4 cells in the spleen determined. * represents a significant difference of p<0.001 comparing 4Tyr treated groups to all other groups. Data is representative of 6 experiments with 2-4 mice per group.

In separate experiments, mice were sacrificed 7 days after peptide treatment and ex vivo recall responses assayed. b, IL-2 and c, IFNγ production from splenocytes to increasing concentrations of 4Lys were determined by ELISA. ** represents a significant difference of p<0.001 and data is representative of 3 experiments with 3 mice per group.

All significant differences were determined by an ANOVA using Tukey’s multiple comparison test comparing 4Tyr group to all other treatment groups. Error bars represent SEM.
Figure 5.5. The tolerant phenotype is established 4 days after 4Tyr treatment. 1-3×10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice one day prior to administration of 200μg of 4Lys, 4Tyr, or PBS i.v. Mice were sacrificed 4 days after peptide treatment and ex vivo recall responses determined. a, IL-2, b, IFNγ and c, IL-10 production by splenocytes to increasing concentrations of 4Lys were determined by ELISA. * represents a significant difference of at least p<0.05, as determined by an ANOVA using Tukey’s multiple comparison test comparing 4Tyr group to all other treatment groups. Error bars represent SEM, data is representative of 3 consistent experiments with 3 mice per group.
Figure 5.6. Tolerance induced with 4Val is different to that induced by 4Tyr.
1-2x10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice 1 day prior to administration of 200μg of 4Val, 4Tyr or PBS iv. Mice were immunised with 4Lys in CFA s.c. 7 days later and sacrificed 10 days after immunisation. 
a. Percent of Tg4 cells present in the LN 10 days post immunisation. 

b. IL-2 (pg/ml) production from splenocytes to increasing concentrations of 4Lys were determined by ELISA. Significant differences shown were determined by unpaired T test. Error bars represent SEM, data is representative of 4 consistent experiments with 3-4 mice per group.
Figure 5.7. 4Tyr induces considerable proliferation of transferred Tg4 cells. 1-2x10⁶ CFSE labelled Ly5.1+Tg4 cells were transferred to B10.PL mice 1 day prior to administration of 200µg 4Tyr, 200µg 4Tyr+30µg LPS or PBS iv. Mice were sacrificed 2 or 7 days later and spleens sampled. Representative plots of CFSE dilution in Tg4 cells at a, day 2, and b, day 7. The percentage of cells in each generation was calculated manually as described in materials and methods; c, Bar chart showing the percent of Tg4 cells in each generation at day 2. d, Bar chart showing the percent of Tg4 cells which had divided or remained undivided at day 7. *represents a significant difference of p≤0.0114 and **represents a significant difference of p≤0.0165 as determined by unpaired T test. Error bars represent SEM, data is representative of 4 similar experiments with 2-4 mice per group.

Representative plots from e, 4Tyr or f, 4Tyr+LPS treated mice showing Annexin V-PE staining against 7-AAD staining. Value in the upper left quadrant represents the percentage of cells in the early stages of apoptosis, data is representative of 2 experiments with 3 mice per group.
Figure 5.8. **4Tyr:MHC complexes can be maintained for up to 14 days in vivo.**
Mice received 4Tyr i.v. either 20, 18, 16, 14, 10, 7 or 2 days prior to the transfer of CFSE labelled Tg4 T cells. A further experimental group received PBS 2 days prior to transfer. Three days after transfer mice were sacrificed and flow cytometric analysis of CFSE dilution was determined for splenocytes. **a.** Representative plots of CFSE dilution. **b.** Percent of Tg4 cells which remained undivided at time of analysis. Data from groups treated with 4Tyr at day -14, -10, -7 and -2 were significantly different (p<0.001) to data from groups treated with 4Tyr at day -20, -18, -16 and with PBS at day -2; statistical differences were determined by an ANOVA using Tukey’s multiple comparison test. Error bars represent SEM, data is representative of two consistent experiments with 2-3 mice per group.
Figure 5.9. 4Tyr treatment 2 days before Tg4 T cell transfer induces tolerance.
B10.PL mice were treated with 4Tyr or PBS i.v. 2 days before the transfer of Tg4 T cells. Mice were immunised with 4Lys in CFA s.c. 7 days later and sacrificed 10 days post immunisation. Tg4 T cells were detected in the spleen (a) and LN (not shown) of both PBS and 4Tyr treated mice by flow cytometry. b, Ex vivo proliferative responses of LN cells to increasing doses of 4Lys. c, IFNγ and d, IL-2 production from splenocytes to increasing concentrations of 4Lys as determined by ELISA. * represents a significant difference of p<0.05, ** represents a significant difference of p=0.0017. Significant differences were determined by unpaired T test. Error bars represent SEM, data is representative of 2 consistent experiments with 3 mice per group.
Figure 5.10. 4Tyr treatment induces tolerance of Tg4 cells when administered less than 7 days prior to Tg4 cell transfer.

a, Experimental outline; B10.PL mice were treated with 4Tyr i.v 14, 7, 4 days before the transfer of Tg4 T cells. Mice were immunised with 4Lys in CFA s.c. 7 days after Tg4 transfer and sacrificed 10 days post immunisation. Tg4 T cells were detected in the spleen, b, by flow cytometry. c, IL-2, d, IFNγ and e, IL-17 production from splenocytes to increasing concentrations of 4Lys as determined by ELISA. Significant differences are; * p<0.01, **p<0.001. Significant differences were determined by an ANOVA using Tukey’s multiple comparison test. Error bars represent SEM, data is from 1 experiment with 3 mice per group.
Figure 5.11.a. 4Tyr is presented predominately by DC to Tg4 T cells. Mice were treated with PBS or 4Tyr 1 day prior to transfer of CFSE labelled Tg4 cells. One day after Tg4 transfer mice were sacrificed and spleens frozen. Sections were stained with DAPI (blue), CD11c (white), and CD19 (red). CFSE labelled Tg4 cells are shown in green. Images are of representative sections from 1 experiment with 3-4 mice per group.
Figure 5.11.b-c. 4Tyr is presented to Tg4 cells predominantly by DC. Mice were treated with b, PBS or c, 4Tyr 1 day prior to transfer of CFSE labelled Tg4 cells. One day after Tg4 transfer mice were sacrificed and spleens frozen. Sections were stained with DAPI (blue), CD11c (white), and CD19 (red). CFSE labelled Tg4 cells are shown in green, white arrowheads indicate CFSE+ Tg4 cells. Enlarged images are of representative sections from 1 experiment with 3-4 mice per group.
Figure 5.12. 4Tyr:MHC complexes are maintained on CD11c+ CD4+ DC following administration of 4Tyr i.v.
B10.PL mice were treated with 4Tyr or PBS i.v, 1 day later spleens were removed, DC populations sorted by FACS and plated out at increasing number with a Tg4 TCL.  

- a. Sorted DC populations; PDCA-1+ (pDC), CD11c+CD4+, CD11c+CD8+ and CD11c+CD4-CD8-.
- b. Purity of DC populations from 4Tyr and PBS treated mice.
- c. Proliferative responses of Tg4 TCL to increasing numbers of DC from 4Tyr treated mice.

- d. Bar graph of proliferative responses of TCL to 8x10⁴ sorted DC from 4Tyr treated (black bars) and PBS treated (white bars) mice; significant differences shown were determined by an ANOVA using Tukey’s multiple comparison test.
- e. Bar graph of proliferative responses of TCL to 8x10⁴ sorted DC from 4Tyr treated (black bars) and PBS treated (white bars) mice plus 100µM 4Lys. Error bars represent SEM, data from 1 experiment. Similar data was obtained from 2 experiments performed by a separate investigator in the laboratory.
Figure 5.13. Tolerance can still be induced following DC depletion.

a, Experimental outline; B10.PLxDOG mice were treated with 4Tyr or PBS i.v, 1 day prior to DC depletion by administration of ~22ng DTx /g of body weight. b, Bar chart showing percentage of DC depleted following DTx treatment. c, Proliferative responses of splenocytes to 0μM (unstimulated) or 0.1μM 4Lys (stimulated); significant differences are p<0.01 comparing all PBS treated groups to 4Tyr treated groups. There is no significant difference when comparing tolerant groups. d, IFNγ production by splenocytes to a dose range of 4Lys; *represents a significant difference of p<0.001 comparing all PBS treated groups to 4Tyr treated groups. There is no significant difference when comparing tolerant groups.

All significant differences were determined by an ANOVA using Tukey’s multiple comparison test. Error bars represent SEM, data is representative of 2 consistent experiments with 3 mice per group.
Figure 5.14. DC depletion after 4Tyr administration reduces the proliferation of Tg4 cells.

a, Experimental outline; B10.PLxDOG mice were treated with 4Tyr or PBS i.v. and DC depleted by administration of 3 doses of ~22ng DTx/g of body weight. b, Bar chart showing percentage of DC depleted following DTx treatment. c, Bar chart showing number of Tg4 cells in the spleen at days 1, 2, and 3 following Tg4 transfer; significant difference shown was determined by unpaired T test. CFSE dilution was monitored by flow cytometry, graphs show the percent of Tg4 cells in generations 0-8 at day 1 (d), day 2 (e), and day 3 (f). The percent of cells in each generation was calculated manually as described in materials and methods; statistical differences were determined by unpaired T test, *p≤0.05, **p≤0.003, Error bars represent SEM, data is representative of 2 experiments, with 4 mice per group.


6. Phenotype of 4Tyr tolerised Tg4 cells.

6.1. INTRODUCTION

The data presented in chapter 5 highlight a novel non-deletional mechanism of tolerance induction following administration of a peptide with a high affinity for MHC (4Tyr).

Persistence of T cells following tolerance induction has been described in a number of other studies (Pape et al., 1998; Redmond et al., 2005; Rocha and von Boehmer, 1991). Of these studies, most resulted in the generation of a very small population of tolerant T cells which could not be phenotypically examined (Kearney et al., 1994; Pape et al., 1998). Some studies have shown the generation of a large population of tolerant cells, but these were generated outside the therapeutic setting by transferring Ag-reactive T cells to host constitutively expressing the Ag (Rocha et al., 1993; Rocha and von Boehmer, 1991; Tanchot et al., 2001). Therefore the model discussed in chapter 5 presented a unique opportunity to examine the phenotype of tolerant T cells generated by administration of peptide.

Better characterisation of the tolerant phenotype would yield information on determinants of the tolerant state. A number of characteristics have previously been associated with a tolerant phenotype in T cells; namely altered calcium mobilisation (Dubois et al., 1998), and defects in MAPK signalling (Chiodetti et al., 2006; Fields et al., 1996). Tolerance in both CD4+ and CD8+ T cells is a state of unresponsiveness, shown to be dependent on altered TCR-induced signal transduction within the tolerant cell. Thus primary defects in calcium and MAPK mediated signalling could account for the phenotype of 4Tyr tolerised Tg4 cells. Other reported alterations in the biochemical make-up and/or functional capabilities of tolerant T cells include the enhanced expression of E3-ubquitin ligases (Fathman and Lineberry, 2007), the down-regulation of the TCR (Mamalaki et al., 1993), the up-regulation of coinhibitory molecules (Apostolou and von Boehmer, 2004), and the increased production of suppressive cytokines (Broere et al., 2008; Friedman and
Weiner, 1994). The studies presented in this chapter examined the characteristics of 4Tyr tolerised Tg4 cells by asking:

1. What is the surface phenotype of 4Tyr tolerised Tg4 cells?
2. What are the signalling capabilities of 4Tyr tolerised Tg4 cells?
3. Do 4Tyr tolerised Tg4 cells have a regulatory phenotype?
4. Can the phenotype of 4Tyr tolerised Tg4 cells be altered upon repeated treatment with 4Tyr?
6.2. RESULTS

6.2.1. Surface phenotype of 4Tyr tolerised Tg4 T cells.

Previous reports have ascertained that it takes 3-4 days for tolerance to be induced following peptide treatment in the Tg4 model (Liu and Wraith, 1995). Tg4 cells were therefore examined 4 days after peptide treatment once tolerance had been established. Having previously determined that the mechanism of tolerance induced following 4Val treatment was different to that induced following 4Tyr treatment (Figure 5.5), the surface phenotype of Tg4 cells following encounter with MHC bearing either peptide was examined.

Tg4 cells were transferred to B10.PL mice one day prior to treatment with a high dose of 4Tyr, 4Val or PBS i.v. Four days later spleens were sampled and the surface expression of a number of molecules examined via flow cytometry to determine whether there were any phenotypic differences between cells which had encountered 4Tyr compared to 4Val. At this time point, PBS treated mice had few Tg4 cells in the spleen, and as such 4Val treated Tg4 cells form the most reliable control.

As expected, following TCR ligation, Tg4 cells from peptide treated mice exhibited an activated phenotype, increasing CD44 expression and down-regulating CD62L, four days after peptide treatment (Figure 6.1). 4Tyr treated Tg4 cells exhibited slightly higher levels of CD44 expression and slightly reduced levels of CD62L compared to 4Val treated Tg4 cells. Also indicative of activated T cells, 4Tyr treated Tg4 cells showed a small down-regulation of TCR and up-regulation of CD28. As IL-2 production is maintained in tolerant Tg4 cells the expression of CD80 and CD86 on tolerant Tg4 cells was examined. Stabilisation of IL-2 mRNA is an important consequence of CD28 ligation by CD80 or CD86 (Lindstein et al., 1989). 4Tyr tolerised Tg4 cells showed a low level of CD80 expression, whereas 4Val treated Tg4 cells did not. However, Tg4 cells from all treatment groups expressed CD86 to a similar degree. Expression of CD69, CD5, ICOS, OX40, CD25, CD103, CD154 and RANKL was also examined and found to be similar between treatment groups (data not shown). Strikingly, 4Tyr treated Tg4 cells showed enhanced
expression of the negative costimulatory molecules CTLA-4 and PD-1 compared to 4Val treated Tg4 cells.

Surface expression of all molecules discussed above was also analysed 10 days after peptide treatment. At this later time point expression levels of most molecules examined were similar between 4Tyr and 4Val treated Tg4 cells. There were two exceptions; 4Tyr treated Tg4 cells still expressed slightly higher levels of PD-1 and had down-regulated CD62L to a greater degree (data not shown). These data suggest that 4Tyr treated Tg4 cells have an activated phenotype. 4Tyr treated Tg4 cells have higher expression of the negative costimulatory molecules PD-1 and, at the earlier time-point only, CTLA-4. The increased expression of these molecules could contribute to unresponsiveness of the Tg4 cell. However, both PD-1 and CTLA-4 are also up-regulated upon T cell activation, and as such the enhanced expression of these molecules on 4Tyr treated Tg4 cells could be indicative of better activation.

To further examine the expression of the molecules seen to differ the most between 4Tyr and 4Val treated Tg4 cells, expression of TCR, CD44, CD62L, CTLA-4 and PD-1 on Tg4 cells was examined following immunisation. Tg4 cells were transferred to B10.PL mice one day prior to treatment with 4Tyr or 4Val i.v. Seven days later mice were immunised with 4Lys in CFA, and spleen and LN taken 10 days after immunisation. Expression of CD44, CD62L and CTLA-4 was similar between treatment groups (data not shown). The down-regulation of the TCR is frequently associated with a tolerant phenotype, however, as Figure 6.2a shows, TCR expression was slightly increased on Tg4 cells from the spleen of 4Tyr treated mice compared to 4Val treated mice, and was similarly expressed on Tg4 cells from the LN of both groups. PD-1 was not expressed on Tg4 cells from the spleens of either treatment group. In contrast, PD-1 was expressed to a higher degree on Tg4 cells from LN of 4Tyr compared to 4Val treated mice (Figure 6.2b). Collectively these data highlight the activated phenotype of 4Tyr tolerised Tg4 cells, and suggest the potential for involvement of CTLA-4 and more specifically PD-1 in the maintenance of tolerance following 4Tyr treatment.
6.2.2. 4Tyr tolerised Tg4 cells have a defect in TCR-induced calcium metabolism.

The ability of tolerised Tg4 cells to flux calcium ($\text{Ca}^{2+}$) following TCR cross-linking was examined. Tg4 cells were transferred to B10.PL mice one day prior to treatment with 200 µg of 4Tyr, 4Tyr+LPS or PBS i.v. Four days later, Ca$^{2+}$ flux was examined by loading splenocytes with the Ca$^{2+}$-sensitive dye Indo-1 (Figure 6.3a). Indo-1 fluoresces at different wavelengths depending on whether it is bound to (398nm) or free from (481nm) Ca$^{2+}$, and therefore the ratio of these two wavelengths indicates changes in Ca$^{2+}$ concentration. Indo-1 loaded samples were stained with surface markers to distinguish host CD4+ cells from Tg4 cells, separated on the basis that Tg4 cells were Ly5.1+ (Figure 6.3b). The ability of cells to mobilise Ca$^{2+}$ upon TCR cross-linking was determined by staining cells with anti-CD3 biotin and then cross-linking the biotin by addition of streptavidin, as shown previously (Dubois et al., 1998).

At the time-point at which Ca$^{2+}$ signalling was examined few Tg4 cells could be found in the spleens of PBS treated mice. Due to the reduced number of Tg4 cells, PBS treated Tg4 cells yield a Ca$^{2+}$ flux plot which does not exhibit the averaged line seen for the other cell types, as fewer Tg4 cells are collected over time. Therefore the Ca$^{2+}$ flux of host cells from PBS and 4Tyr treated mice are also included as further naïve controls. As shown in Figure 6.3c, both naïve Tg4 cells (PBS treated) and host cells mobilised Ca$^{2+}$ efficiently following TCR stimulation by cross-linking. The Ca$^{2+}$ response was somewhat reduced in effector (4Tyr+LPS treated) Tg4 cells compared to naïve controls; this reduced Ca$^{2+}$ flux in effector compared to naïve T cells has been noted in other systems (Chiodetti et al., 2006). Strikingly, there was minimal Ca$^{2+}$ response by the tolerant Tg4 cells upon TCR cross-linking (Figure 6.3c-d). Tolerised Tg4 cells had a blunted Ca$^{2+}$ response, exhibiting a significantly reduced peak of indo-1 violet/blue ratio compared to both naïve and effector Tg4 cells (Figure 6.4a). Detailed analysis of Ca$^{2+}$ flux plots highlighted that tolerant Tg4 cells had significantly reduced areas under the curve (signifying magnitude of the response) (Figure 6.4b), mean indo-1 violet/blue ratios, and gradients of flux (data not shown), compared to both naïve and effector T cells. Basal Ca$^{2+}$ levels were
Ca\(^{2+}\) signalling in T cells is biphasic, TCR stimulation mediates the release of Ca\(^{2+}\) from intracellular stores contained in the endoplasmic reticulum (ER). Release of Ca\(^{2+}\) from the ER activates cell surface calcium release-activated calcium (CRAC) channels which causes a large influx of extra-cellular Ca\(^{2+}\). These two separate phases of Ca\(^{2+}\) release occur in quick succession, however, they can be resolved by chelating extra-cellular Ca\(^{2+}\) with EGTA. Cross-linking of the TCR under such conditions fails to induce a Ca\(^{2+}\) flux. When extra-cellular Ca\(^{2+}\) was restored, a substantial Ca\(^{2+}\) flux was seen in all Tg4 cells (Figure 6.5a). In fact, naïve, tolerant and effector Tg4 cells all exhibited similar values for peak and mean indo-1 violet/blue ratios, gradients of flux, and areas under the curve (data not shown). When extra-cellular Ca\(^{2+}\) was chelated, baseline differences in levels of free intracellular Ca\(^{2+}\) were obviously also abolished. Overlays of Ca\(^{2+}\) flux FACS plots from tolerant Tg4 cells and effector Tg4 cells highlights the very similar Ca\(^{2+}\) response patterns (Figure 6.5b).

**6.2.3. 4Tyr tolerised Tg4 exhibit normal kinetics in the activation of ERK.**

A second important signalling pathway downstream of the TCR is mediated by a MAPK signalling cascade. The ability of tolerant Tg4 cells to activate a key component of this signalling pathway, ERK, was therefore examined. Activation of ERK is controlled through the upstream sequential phosphorylation and activation of Ras, Raf and MEK (Huang and Wange, 2004). Here, the ability of tolerant Tg4 cells to phosphorylate ERK was determined by examining the kinetics of ERK phosphorylation following TCR stimulation. As before, Tg4 cells were transferred to B10.PL mice one day prior to treatment with a high dose of 4Tyr or 4Tyr+LPS i.v.
Four days later spleens were sampled and ERK phosphorylation examined by stimulating splenocyte cultures for specified lengths of time with 100μM 4Tyr.

As shown in Figure 6.6a, TCR stimulation of both effector (4Tyr+LPS treated) and tolerant (4Tyr treated) Tg4 cells resulted in an increased percentage of Tg4 cells which were phospho-ERK+ (pERK) over time. Importantly, splenocyte cultures treated with the pERK inhibitor U0126, and unstimulated Tg4 cells demonstrated no pERK staining (Figure 6.6b). Closer analysis of the kinetics of ERK phosphorylation, demonstrated that the rate of ERK activation was similar in both tolerant and effector Tg4 cells (Figure 6.6c). The levels of pERK were slightly reduced in tolerant compared effector Tg4 cells. This reduction was only significant at the earliest time point and most probably reflected the activated state of effector compared to tolerant Tg4 cells (note the higher basal level of pERK staining in effector Tg4 cells). Importantly the rate at which ERK was phosphorylated was similar for both Tg4 cells; gradients of increase in pERK over time for 4Tyr and 4Tyr+LPS treated samples were very similar, 2.02±0.25 and 2.18±0.12 respectively. Further examination of phosphorylation, specifically tyrosine phosphorylation, was performed following TCR cross-linking of sorted Tg4 cells from 4Tyr or 4Tyr+LPS treated hosts. Global levels of tyrosine phosphorylation were similar for Tg4 cells from mice treated with 4Tyr and 4Tyr+LPS following TCR cross-linking (Appendix Figure A.6).

6.2.4. 4Tyr tolerised Tg4 cells do not have altered sensitivities to secondary stimulation compared to naïve Tg4 cells.

In the periphery, T cells are conditioned to be tolerant of physiological levels of self-peptide MHC complexes. One mechanism by which this is maintained is through the adaptation of T cells. This involves the functional desensitisation of T cells at the single-cell level such that cells only respond to p:MHC complexes above a certain threshold. This leads to T cells that require stronger antigenic stimulus to be activated and acquire effector function, in other words a shift in the dose response is seen. Previous results from this and other laboratories have shown that adaptation correlates with increased levels of CD5 and decreased levels of TCR and co-receptor
(Rocha and von Boehmer, 1991; Ryan et al., 2005). Data discussed in section 6.2.1 showed that altered expression of TCR and CD5 is not a seen on 4Tyr tolerised Tg4 cells. Nevertheless, it was important to ascertain whether 4Tyr treatment of Tg4 cells resulted in adaptation. Adaptation of Tg4 cells would alter the concentrations of peptide need to induce a response. In order to ascertain whether Tg4 cells were adapted, an *ex vivo* readout of T cell responsiveness following tolerance induction needed to be followed. 4Tyr tolerised Tg4 cells do not proliferate or make any effector cytokines (Figure 5.2), however, their capacity for IL-2 production provided a read-out. Responses of naïve Tg4 cells to 4Lys and 4Tyr were determined in cultures of Tg4 and B10.PL splenocytes. In these cultures Tg4 cells constituted 3.5% of the whole culture; this was used because tolerant Tg4 cells constituted roughly 3.5% of spleens and hence naïve Tg4 cells were present in cultures at similar levels. Response curves were determined to both 4Lys and 4Tyr, as 4Tyr is a super-agonist it will induce responses at lower concentrations compared to 4Lys.

As shown in Figure 6.7, both naïve and tolerant Tg4 cells produce significant levels of IL-2 following stimulation with 4Tyr. However, naïve Tg4 cells produce more IL-2 compared to tolerant Tg4 cells. Unsurprisingly, lower levels of IL-2 were produced upon stimulation with 4Lys. Importantly, both naïve and tolerant Tg4 cells responded to 4Tyr and 4Lys within a similar dose range, suggesting that the tolerant Tg4 cells had not undergone a pronounced process of adaptation/desensitisation.

### 6.2.5. 4Tyr treatment increases the expression of foxp3 in Tg4 cells during EAE.

4Tyr treatment profoundly protects mice from developing EAE (Figure 5.1). Moreover, in the periphery of 4Tyr treated, disease free mice, there is a significant population of CD4+ antigen-reactive Tg4 cells persisting. A pertinent question arising is, do these persisting Tg4 T cells have a regulatory phenotype? A number of studies have shown induction of T cells with a regulatory phenotype following Ag treatment via mucosal routes (Broere et al., 2008; Chen et al., 1994; Friedman and Weiner, 1994). More importantly, some studies have highlighted the ability
specifically of peptide therapy via systemic routes to also induce Tregs (Perruche et al., 2008; Thorstenson and Khoruts, 2001).

Foxp3 expression was examined in transferred Tg4 cells from mice recovering from EAE. Using the mice shown in Figure 5.1, foxp3 expression was examined at day 19 following induction of EAE in recovering PBS and 4Lys treated mice and disease free 4Tyr treated mice. Foxp3 expression was seen in Tg4 cells from the LN and CNS of all mice (Figure 6.8). The percentage of cells which have foxp3 expression in the host compartment of LN cells was similar following all treatment regimens, however it was slightly enhanced following 4Tyr treatment; 9±1.28%, 10.6% and 12.05±1.9% following PBS, 4Lys and 4Tyr treatments respectively. In the host compartment of the CNS, the percentage of cells with foxp3 expression was very high in mice which had received either PBS (45.9% of all CD4+ cells) or 4Lys (47.4% of all CD4+ cells). This percentage was decreased slightly in the CNS from mice which had received 4Tyr (31.2% of all CD4+ cells), it is important to remember that 4Tyr treated mice are not sick and therefore have a reduced number of CD4+ cells in the CNS. A high expression of foxp3 was expected; previous work has shown that as mice recover from EAE the number of Tregs in the CNS increases (McGeachy et al., 2005).

Prior to transfer Tg4 cells contained between 3-6% foxp3+ cells. Following transfer, the highest percentage of foxp3 expression in Tg4 cells from the LN and CNS was seen following 4Tyr treatment (Figure 6.8a-c). This was most striking in the CNS, where foxp3+ Tg4 cells constituted almost 40% of all Tg4 cells present in the CNS following 4Tyr treatment. This was double that seen for PBS and 4Lys treated mice. When looking at the actual number of foxp3+ Tg4 cells in the LN and CNS following 4Tyr treatment, a similar pattern is seen (Figure 6.8d); the highest number of foxp3 Tg4 cells in the LN was seen following 4Tyr treatment. In the CNS, treatment with both 4Lys and 4Tyr led to an increased number of foxp3+ Tg4 cells. Taken together these data demonstrate there was an increased proportion of foxp3+ Tg4 cells in the CNS and LN following 4Tyr treatment, suggesting that 4Tyr treatment had some positive influence on either the induction of foxp3 in Tg4 cells or
the expansion of foxp3+ Tg4 cells. Strikingly, even in disease free mice the proportion of foxp3+ cells in the Tg4 cell compartment increased substantially following 4Tyr treatment.

6.2.6. 4Tyr treatment of Tg4 cells has no effect on foxp3 expression in a non-disease setting.

These data suggested that 4Tyr permits either the expansion or induction of foxp3+ Tg4 cells. To investigate this effect further, the expression of foxp3 in Tg4 cells was examined at early time points after peptide treatment. Tg4 cells were transferred to B10.PL mice one day prior to treatment with 4Lys, 4Tyr or PBS i.v. Four and six days later foxp3 expression was determined in persisting Tg4 cells, the percentage of Tg4 cells in the spleen at these time points is shown in Figure 5.4. As shown in Figure 6.9, foxp3 expression was similar between all treatment groups at day 4, with the percentage of foxp3+ Tg4 cells roughly 3.5%. By day 6 this percentage had increased slightly in all groups. However, there was no significant difference between any of the treatment groups, suggesting 4Tyr treatment at this time point had no effect on foxp3 expression.

It was plausible that tolerant Tg4 cells required a secondary challenge before foxp3+ Tg4 cells could either expand or be induced. To test this, mice were immunised with 4Lys in CFA 7 days after peptide treatment, and 10 days later foxp3 expression in the spleen and LN examined. The percentage of Tg4 cells persisting at this time point is shown in Figure 6.9b. Although the percentage of Tg4 cells present at this time point consistently showed a trend toward being increased following 4Tyr treatment compared to 4Val treatment, the percentage of Tg4 cells which were foxp3+ was similar across all treatment groups (Figure 6.9c). These data demonstrate that challenge of 4Tyr tolerised Tg4 cells did not alter the percentage of these cells which were foxp3+.

Previous studies have shown that repeated stimulation of peptide-reactive cells by peptide bearing APC induces a regulatory phenotype in the peptide-reactive T cells (Apostolou and von Boehmer, 2004). As such it was necessary to determine the
effect of continued peptide stimulation on foxp3 expression within the transferred Tg4 cell population. Tg4 cells were transferred to B10.PL mice, which received high dose 4Tyr treatment every three days; foxp3 expression was examined three days after each peptide treatment. As shown in Figure 6.9d, the percentage of Tg4 cells in the spleen increased dramatically when two 4Tyr treatments were given compared to only one. The percentage of Tg4 cells in the spleen plateaued after two treatments until it began to decline after five treatments. This most probably represents the ability of Tg4 cells to expand following repeated doses, and highlights the maximal point to which Tg4 cells can expand within homeostatic limits of the peripheral T cell pool, which is reached after two peptide treatments. Importantly the percentage of Tg4 cells that were foxp3+ was not altered with increasing 4Tyr treatment (Figure 6.9e), remaining constantly between 3-4%. These data suggest that simple treatment of Tg4 cells with 4Tyr, either singly or repeatedly, has no effect on foxp3 expression within the Tg4 cell population.

6.2.7. 4Tyr tolerised Tg4 cells express T-bet when stimulated with peptide ex vivo.

Previous studies have shown that repetitive stimulation of Tg4 cells results in the differentiation to a tolerant state with an altered transcriptional program (Anderson et al., 2006). The transcription factors shown to be induced in tolerant T cells included T-bet, Irf-1 and Egr-2. The expression of T-bet in Tg4 cells rendered tolerant by 4Tyr treatment four days prior to analysis was determined. T-bet expression in effector (4Tyr+LPS treated) and naïve (PBS treated) Tg4 cells was also determined. As was shown in the Anderson et al study, expression of T-bet was only seen following secondary stimulation of T cells. Examination of T-bet directly ex vivo, without secondary stimulation, showed no T-bet staining (data not shown). However, following overnight stimulation with 50µM 4Lys, expression of T-bet was seen in Tg4 cells but not host cells (Figure 6.10).

Tg4 cells from PBS and 4Tyr+LPS treated mice up-regulated T-bet to a similar extent. There was a trend toward a decrease in T-bet expression in Tg4 cells from 4Tyr treated mice. However, there was no significant difference between the
percentage of T-bet+ Tg4 cells or the mean fluorescence intensity (MFI) of T-bet staining, for any of the treatment groups. These data demonstrate that the transcription factor T-bet is not specifically associated with the tolerant phenotype in Tg4 cells.

6.2.8. Cytokine profile of 4Tyr tolerised Tg4 cells after repeated doses of 4Tyr

Data presented in chapter 5 demonstrated that tolerised Tg4 cells express high levels of IL-2, but no effector cytokines following tolerance induction (Figure 5.4), and that this cytokine profile was established four days after peptide treatment. IL-10 production has never been detected from tolerised Tg4 cells following recall to 4Lys; as previously discussed, this is in contrast to previous studies in which repeated dosing with 4Tyr induced a population of IL-10 producing cells in a Tg4 mouse (Burkhart et al., 1999). This group examined the cytokines produced by Tg4 cells after varying numbers of peptide stimulations and demonstrated that IL-10 production is switched on after three doses, and that it takes at least five doses to turn off IL-2 production (Anderson et al., 2005). In Chapter 5 it was proposed that continued systemic administration of 4Tyr i.v. in the system studied here might result in the production of tolerant Tg4 cells which had the capacity to make IL-10 but no longer retained the ability to make IL-2.

Tg4 cells were transferred to B10.PL mice one day prior to treatment with a high dose of 4Tyr i.v. Following this initial treatment, 4Tyr was administered at weekly intervals. Spleens were taken from mice after 1-3 treatments of 4Tyr, seven days after the final peptide treatment. Repeated doses of 4Tyr led to an increase in the percentage of Tg4 cells as a percentage of all CD4+ cells in the spleen; Tg4 cells constituted $2.83\pm0.38\%$, $5.86\pm0.88\%$ and $7.43\pm1.31\%$ of the whole CD4+ population after one, two and three doses respectively. Cytokine production by Tg4 cells was examined by intra-cellular cytokine staining (ICS) after overnight stimulation with 4Lys; cytokine production by transferred Tg4 cells could be specifically determined by gating on CD4+Ly5.1+ (Tg4) cells. ELISA of supernatants was also performed after 96 hours of culture of splenocytes with increasing concentrations of 4Lys. Both
read-outs showed an identical pattern of cytokine production (Figure 6.11). Repeated doses of 4Tyr caused a profound reduction in the production of IL-2 (Figure 6.11a-b), with IL-2 production being almost absent after treatment with 3 doses of 4Tyr. The expression of IL-10 was increased after repeated doses of 4Tyr, however it took three doses of 4Tyr for this to occur (Figure 6.11e-f). These data are consistent with the Anderson study (Anderson et al., 2005). In contrast, the expression of IFNγ was enhanced following repeated doses of 4Tyr (Figure 6.11c-d). These data could suggest that repeated doses of 4Tyr were permitting a reversal of the tolerant phenotype, as shown by the enhanced production of effector cytokines, specifically IFNγ. As such, the proliferative responses of splenocytes to increasing concentrations of 4Lys was determined. Proliferative responses seen were low, as described in Chapter 5 4Tyr treated Tg4 cells show reduced levels of proliferation. As such proliferative responses were calculated per Tg4 cell in each culture well (Figure 6.11g). The proliferative responses showed that repeated doses of 4Tyr enhanced the degree of unresponsiveness, with reduced levels of proliferation seen after two and three doses of 4Tyr. Collectively these data suggest that repeated dosing with 4Tyr does not generate Tg4 cells with a phenotype similar to that seen in the Anderson et al and Burkhart et al studies; namely IL-2-, IFNγ-, IL-10+ (Anderson et al., 2005; Burkhart et al., 1999). Instead, repeated dosing with 4Tyr generates Tg4 cells that proliferate less, produce less IL-2 and show increased expression of effector cytokines; a phenotype resembling that of classically anergic T cells.

To investigate this phenotype further, the number of 4Tyr doses administered was increased, giving 200μg 4Tyr every three days and removing spleens three days after peptide treatment. This is in line with the treatment regimen used in other studies (Anderson et al., 2005). Similar to before, repeated doses of 4Tyr led to an increase in the percentage of Tg4 cells in the spleen (Figure 6.9d). Repeated doses of 4Tyr decreased the percentage of Tg4 cells producing IL-2 and increased the percentage of Tg4 cells producing IFNγ (Figure 6.12a-b). IL-10 production was also increased with repeated doses of 4Tyr (Figure 6.12c). This only occurred after three 4Tyr treatments, at which point the percentage of Tg4 cells producing IL-10 had more
than doubled. However, if four or five doses of 4Tyr were administered, IL-10 production declined to below starting levels. Examination of proliferative capacity of Tg4 cells after each 4Tyr dose was also performed (Figure 6.12d). The ability of Tg4 cells to proliferate decreased with increased doses of 4Tyr. These data again suggest that repeated 4Tyr doses pushes Tg4 cells to a less responsive state resembling classical anergy. They also highlight a delicate balance between number of stimulations and the ability of Tg4 cells to produce IL-10.

6.2.9. Role of IL-2 in generating tolerant Tg4 cells following 4Tyr treatment.
IL-2 is the only cytokine produced by Tg4 cells rendered tolerant following one dose of 4Tyr. As IL-2 is important for T cell expansion, the continued expression of IL-2 following tolerance induction could be responsible for the persistence of a population of Tg4 cells. As such, it was important to ascertain the role of IL-2 in generating the population of tolerant Tg4 cells. Tg4 cells were transferred to B10.PL mice one day prior to treatment with a high dose of 4Tyr or 4Lys i.v. At the same time as peptide treatment, cohorts of mice also received 200 μg of anti-IL-2 i.p. Mice were sacrificed 4 days after peptide treatment, the number of Tg4 cells and their ex vivo recall responses were determined.

As seen previously 4Tyr treatment caused a population of Tg4 cells to persist following tolerance induction; this was not the case following 4Lys treatment (Figure 6.13a). Importantly, anti-IL-2 treatment caused an increase in the number of Tg4 cells persisting post tolerance induction for both peptide treatment groups; this increase was only significant for the 4Lys treatment groups. For the 4Tyr treatment groups a minor, but non-significant, increase was consistently seen in repeated experiments. Moreover, the Tg4 cells from 4Tyr+anti-IL-2 showed a more activated phenotype compared to Tg4 cells from 4Tyr only treated mice with increased CD44 and decreased CD62L expression (Figure 6.13b). Surface phenotype of Tg4 cells from 4Lys mice could not be reliably examined due to their low number. Ex vivo recall responses of splenocyte cultures were also examined.
Similar to previous data (Figure 5.5), neither 4Lys nor 4Tyr treated groups produced much IFNγ upon recall; this was not altered by anti-IL-2 treatment (Figure 6.13c-d). Anti-IL-2 treatment also had no effect on IL-2 produced from recall cultures (Figure 6.13e-f). Therefore, although anti-IL-2 treatment appeared to enhance Tg4 cells numbers there was no significant increase in cytokine production from recall splenocyte cultures (Figure 6.13c-f). Collectively these data show a minimal increase in Tg4 cell responsiveness following anti-IL-2 treatment; it was expected that antibody treatment would affect Tg4 cell expansion and therefore limit T cell responsiveness. However, IL-2 is also known to play a vital in Treg homeostasis, and as such the effect of anti-IL-2 on the Treg populations within 4Tyr treated mice was examined.

Figure 6.14a shows that anti-IL-2 treatment caused a decrease in the percentage of cells which were foxp3+; this was true for both Tg4 and host cells. However, this difference was not significant. CD4+foxp3+ T cells can be both CD25+ and CD25-. As CD25 is a component of the high affinity IL-2 receptor, foxp3+ CD25+ and CD25- cells were examined separately following anti-IL-2 treatment. There was a significant decrease in the percentage of foxp3+CD25+ cells following anti-IL-2 treatment for both host and Tg4 cells (Figure 6.14b-c). There was no change in the percentage of foxp3+CD25- cells following IL-2 treatment. Together, these data show that anti-IL-2 treatment enhances responses following administration with peptide. This enhancement is due to a decrease in the percentage of CD4+CD25+foxp3+ cells in the mouse creating a less well regulated T cell compartment and permitting enhanced T cell responsiveness.

6.2.10. The effect of 4Tyr treatment on the host CD4+ T cell compartment.

The data discussed in the previous section demonstrate that anti-IL-2 treatment does not affect the induction of peptide-tolerance but instead primarily affects Treg homeostasis (Figure 6.14). Tg4 cells tolerised with 4Tyr make a considerable amount of IL-2 upon secondary stimulation with 4Lys. 4Tyr treatment does not affect the foxp3+ compartment of transferred Tg4 cells. However, with the
considerable amount of IL-2 produced by Tg4 cells, it was important to determine whether 4Tyr treatment (through production of IL-2) affected the host foxp3+ compartment. Tg4 cells were transferred to B10.PL mice one day prior to treatment with a high dose of 4Tyr, 4Lys, or PBS i.v. Four or six days after peptide treatment mice were sacrificed and the percentage of foxp3+ cells in the host CD4+ compartment of the spleen determined.

As shown in Figure 6.15a-b, the percentage of host CD4+foxp3+ cells increased following 4Tyr treatment compared to 4Lys and PBS treatment. This increase was only significant by day 6, but an increase can be seen at the earlier time point of day 4.

To determine whether this increase was directly related to IL-2 production from Tg4 cells, the foxp3+ host cells were examined in a setting where IL-2 production by Tg4 cells has been shown to decline with time. Repeated doses of 4Tyr decreased the ability of persisting tolerant Tg4 cells to make IL-2 (Figures 6.11 and 6.12). The percent of CD4+foxp3+ cells in the host compartment of mice given multiple doses of 4Tyr at three day intervals was examined; note the percent of CD4+foxp3+ cells in PBS treated mice was 13.79±0.1%. As shown in Figure 6.15c, the percent of host foxp3+ cells declines with repeated doses of 4Tyr. Thus, as the ability of Tg4 cells to make IL-2 decreases, so does the population of host CD4+foxp3+ cells, suggesting that the IL-2 produced by 4Tyr tolerised Tg4 cells can influence the host compartment causing expansion of CD4+foxp3+ T cells.
6.3. DISCUSSION

Through the persistence of Tg4 cells following tolerance induction with the 4Tyr peptide, the phenotype of tolerant T cells has been examined. By comparing the phenotype of effector T cells, naïve T cells, differently tolerised Tg4 cells to 4Tyr tolerised Tg4 cells it has been possible to identify cellular modifications associated with the tolerant state. As such the signalling capabilities, surface phenotype, transcription factor expression and cytokine production from 4Tyr tolerised Tg4 cells has been demonstrated in this chapter.

6.3.1. Surface phenotype of 4Tyr tolerised Tg4 cells.

Expression of surface molecules is known to affect T cell responsiveness; changing levels of costimulatory and coinhibitory molecules as well as TCR and CD5 have been shown to correlate with altered T cell responsiveness. It was possible that the Tg4 T cell tolerance might have been regulated by the expression of surface molecules, and as such expression of a range of surface molecules was examined on 4Tyr tolerised Tg4 cells. It was important to establish levels of TCR following peptide tolerance induction as previous studies have demonstrated down-regulation of the TCR upon tolerance induction (Mamalaki et al., 1993; Pape et al., 1998; Redmond et al., 2005). Such a down regulation of TCR might explain the reduced Ca\(^{2+}\) signalling observed in tolerant Tg4 cells. Although TCR levels were initially decreased following 4Tyr treatment, by day 10 TCR levels were similar between all treatment groups (Figure 6.1 and not shown). Following subsequent immunisation with 4Lys in CFA, TCR levels were increased in the spleen following 4Tyr compared to 4Val treatment (Figure 6.2a). This may suggest that the 4Tyr tolerised Tg4 cells had not been triggered by immunisation because they had been adapted to only respond to a higher level of Ag. However, in the LN the expression of TCR was identical between 4Tyr and 4Val treated groups. Taken together, these data suggest that modulation of TCR expression was not involved in 4Tyr induced tolerance.

Tg4 cells tolerised by 4Tyr treatment had an activated surface phenotype. Compared to 4Val treated Tg4 cells, 4Tyr treated cells expressed both CD44 and CD28 to a
slightly higher degree and a larger proportion of 4Tyr treated cells showed reduced CD62L expression. As peptide-induced T cell tolerance is known to require an initial stage of activation and expansion (Kearney et al., 1994; Liblau et al., 1996), these data simply demonstrate that 4Tyr is better at activating Tg4 cells than 4Val; as would be expected as it is a greater super-agonist than 4Val.

Expression of CD80 and CD86 on 4Tyr tolerised Tg4 cells was also determined. Although primarily associated with the DC, both these molecules can be expressed on T cells (Azuma et al., 1993; Hakamada-Taguchi et al., 1998). 4Tyr tolerised Tg4 cells retain the ability to make IL-2, as the CD80/86:CD28 interaction is important in production of IL-2 through stabilisation of IL-2 mRNA (Lindstein et al., 1989), it was possible that increased levels of CD80/86 were mediating T cell-T cell interactions and permitting IL-2 production. However, expression levels of both CD80 and CD86 were similar between treatment groups.

Finally it was necessary to determine expression levels of coinhibitory molecules on tolerant Tg4 cells. Increased expression of negative costimulatory molecules could help maintain tolerant Tg4 cells in a state of reduced responsiveness. The most well characterised coinhibitory molecules are CTLA-4 and PD-1. Ligation of both molecules on the T cell surface has been shown to inhibit T cell activation (Freeman et al., 2000; Kearney et al., 1995; Parry et al., 2005). Moreover, increased expression of PD-1 is associated with the un-responsiveness of exhausted CD8+ T cells during viral infection; blockade of PD-1 on exhausted T cells enhances T cell responsiveness and permits viral clearance (Barber et al., 2006). Expression of both CTLA-4 and PD-1 was enhanced on 4Tyr treated Tg4 cells at day 4 following peptide treatment. This enhancement was maintained for PD-1 at day 10 and following immunisation with 4Lys in CFA. These data suggest one of two things. Firstly, CTLA-4 and PD-1 could play an important role in maintaining the tolerised state in 4Tyr treated Tg4 cells. Enhanced expression of both these negative costimulatory molecules would increase the negative signals received by the T cells upon stimulation and hence help maintain unresponsiveness in the tolerant cells. Signals generated following ligation of both CTLA-4 and PD-1 have been shown to
inhibit proximal TCR signalling, therefore up-regulation of these molecules could well be responsible for the tolerance seen in 4Tyr tolerised Tg4 cells. Alternatively, both CTLA-4 and PD-1 are up-regulated upon T cell activation (Agata et al., 1996; Scalapino and Daikh, 2008). The increased expression of CTLA-4 and PD-1 on 4Tyr tolerised Tg4 cells could therefore be indicative of greater Tg4 cell activation following 4Tyr treatment. Wraith and colleagues have previously explored the expression of CD28 and CTLA-4 on Tg4 cells from Tg4 mice given 4Tyr, 4Ala or 4Lys (Metzler et al., 1999), and demonstrated similar kinetics of expression for both CTLA-4 and CD28 following all treatments. However, 4Tyr treatment resulted in increased expression of both molecules compared to other peptide treatments. Although this would suggest that stimulatory capacity of peptides is responsible for the level of CTLA-4 and CD28 expression, Meltzler et al noted that following 4Tyr treatment CTLA-4 expression was enhanced to a greater degree than CD28 expression. Greater Tg4 cell activation following 4Tyr treatment, would not account for the enhanced expression of PD-1 seen in the LN following immunisation with 4Lys in CFA, Figure 6.2. As such it would be pertinent to administer blocking antibodies to both PD-1 and CTLA-4 upon challenge of tolerant Tg4 cells to ascertain whether these molecules play a role in maintenance of tolerance in the Tg4 cell.

6.3.2. Signalling events in 4Tyr tolerised T cells.

Ligation of the TCR by p:MHC initiates signalling pathways within the T cell which result in gene transcription and ultimately lead to T cell activation. An important pathway downstream of the TCR is mediated by Ca\(^{2+}\); released from intracellular stores through TCR-mediated activation of PLC\(_\gamma\) and the subsequent hydrolysis of PIP\(_2\) to DAG and IP\(_3\). IP\(_3\) is a secondary messenger and rapidly diffuses in the cytosol to bind specific receptors found on the endoplasmic reticulum (ER). IP\(_3\) receptor binding opens ER Ca\(^{2+}\) channels, permitting flow of Ca\(^{2+}\) from the ER to the cytosol. Upon depletion of ER Ca\(^{2+}\) stores, store-operated calcium entry (SOCE) occurs through activation of Ca\(^{2+}\)-release-activated Ca\(^{2+}\) (CRAC) channels. This results in increases in intracellular calcium concentrations, the magnitude and
duration of which contribute to the overall response to TCR ligation (Bootman et al., 2001; Feske, 2007).

Data presented here demonstrate that 4Tyr tolerised Tg4 cells display greatly reduced Ca\(^{2+}\) flux responses upon TCR stimulation (Figures 6.3. and 6.4). A number of other studies have highlighted defects in Ca\(^{2+}\) mobilisation in tolerant T cells (Dubois et al., 1998; Tanchot et al., 1998), in most cases mediated by defects in the activation of PLC\(\gamma\) (Chiodetti et al., 2006; Heissmeyer et al., 2004; Srinivasan and Frauwirth, 2007). Tanchot et al demonstrated the induction of tolerance in female CD8+ T cells specific for the male H-Y antigen upon transfer to T cell deficient male mice (Tanchot et al., 1998). Tolerance induction did not result in T cell deletion, instead tolerant CD8+ T cells persisted and had defects in their ability to mobilise Ca\(^{2+}\), such that they displayed a severely reduced amplitude of Ca\(^{2+}\) signal. CD8+ T cells rendered tolerant by this continual stimulation retained the ability to make IFN\(\gamma\) and some IL-2 but secreted high levels of IL-10 upon TCR stimulation. In terms of Ca\(^{2+}\) signalling capabilities, tolerant CD8+ T cells demonstrated defects in Ca\(^{2+}\) channels independent of TCR stimulation. Tanchot et al stated that CD8+ tolerant T cells had defective thapsigargin (TG)-induced Ca\(^{2+}\) responses. TG is a drug which depletes ER Ca\(^{2+}\) stores and therefore initiates SOCE and Ca\(^{2+}\) signalling separately from TCR stimulation; defective TG-induced Ca\(^{2+}\) response in tolerant cells highlights defective entry of Ca\(^{2+}\) from the extra-cellular space. There is the suggestion of such defects in 4Tyr tolerised Tg4 cells; calcium signalling defects are only see in normal media, when extracellular Ca\(^{2+}\) is chelated and then replaced in excess by CaCl\(_2\) no defects in Ca\(^{2+}\) signalling were observed in tolerant Tg4 cells (Figure 6.5).

These data could be interpreted in a number of ways. Firstly, 4Tyr tolerised Tg4 cells could have a reduced ability to allow Ca\(^{2+}\) entry through plasma membrane channels. This could be mediated by an altered plasma membrane potential, which would be abolished by addition of excess extracellular Ca\(^{2+}\). Secondly, 4Tyr tolerised Tg4 cells could have defects in release of Ca\(^{2+}\) from intracellular stores which would prevent or reduce SOCE. The former suggestion is favoured here, as addition of excess CaCl\(_2\) would not permit Ca\(^{2+}\) signalling in cells in which SOCE
had not been properly activated. Instead, defects in Ca\textsuperscript{2+} entry through plasma membrane channels would seem more likely and would explain the reduced intracellular Ca\textsuperscript{2+} levels in tolerant Tg4 T cells. Collectively, these data suggest a state of tolerance in which Ca\textsuperscript{2+} cannot be properly mobilised upon TCR stimulation due to altered resting Ca\textsuperscript{2+} concentrations within the cell, and thus an altered membrane potential. Such data are in line with studies showing different expression patterns of potassium channels on naïve and memory T cells. Potassium channels help establish the negative membrane potential of a T cell which is required for Ca\textsuperscript{2+} influx (Chandy et al., 2004). Recently, an inhibitor of the KCa3.1 potassium channel has been identified, knock-down of which causes enhanced Ca\textsuperscript{2+} signalling (Srivastava et al., 2008). Whether altered membrane potential is the cause of defective Ca\textsuperscript{2+} signalling in 4Tyr tolerised T cells could be tested by more precise measurements of intracellular Ca\textsuperscript{2+} concentration, determination of membrane potential, by observing Ca\textsuperscript{2+} mobilisation on addition of TG to tolerant Tg4 cells, and by examination of potassium channel expression.

What is the outcome of altered Ca\textsuperscript{2+} mobilisation in tolerant Tg4 cells? An obvious outcome is the inability to activate Ca\textsuperscript{2+}-dependent transcription factors and as such result in altered gene transcription. Srinivasan et al demonstrated that different isoforms of NFAT are responsive to different levels of cytosolic Ca\textsuperscript{2+} signals (Srinivasan and Frauwirth, 2007). As such, robust Ca\textsuperscript{2+} signalling activated NFAT 1, but reduced Ca\textsuperscript{2+} signals activated NFAT 2; the authors suggested that NFAT 2 might be important in transcribing genes involved in the maintenance of tolerance. Thus altered Ca\textsuperscript{2+} signalling could set a different transcription programme in place in tolerant T cells as well as prevent the induction of the activated effector T cell programme.

A second important signalling pathway downstream of TCR is the MAPK cascade. Many tolerant states have been suggested to have resulted from disruption of the TCR to MAPK signalling pathway, predominantly in clonally anergic T cells (Fields et al., 1996; Li et al., 1996). More relevant to the studies presented here, Choidetti et al demonstrated small defects in ERK activation in tolerant Ag-specific CD4+ T cells.
rendered tolerant by transfer to hosts constitutively expressing the Ag (Chiodetti et al., 2006). More recently, a study examining tolerant T cells at the single cell level showed reduced phosphorylation of ERK in tolerant compared to primed T cells (Morton et al., 2007). Importantly, Morton et al demonstrated these defects in Ag-specific T cells tolerised in vivo through oral administration of protein and, similar to here, systemic administration of peptides. In contrast, no defects in ERK activation were evident in 4Tyr tolerised Tg4 cells. Kinetic examination of phosphorylation of ERK following stimulation demonstrated similar rates of phosphorylation between effector (4Tyr+LPS treated) and tolerant (4Tyr treated) Tg4 T cells. Overall levels of pERK were consistently lower in tolerised compared to effector T cells (Figure 6.6). This reduction was only significant at early time points and is probably reflective of enhanced activation in the 4Tyr+LPS treated Tg4 cells, rather than defects in ERK activation in tolerant Tg4 cells. Also, the overall levels of phosphorylation within activated tolerant Tg4 cells were similar to activated effector cells (Appendix Figure A.6). These data highlight that although some pathways differ drastically between tolerant and effector Tg4 cells, most remain the same.

Together, the data presented here demonstrate the altered signalling capabilities of tolerant Tg4 cells compared to naïve and effector Tg4 cells. At the time point examined, tolerance has already been induced and so the altered signalling pathways shown are important in maintaining tolerance in the Tg4 cells. As 4Tyr tolerised Tg4 cells still retain the capacity to produce IL-2, severe defects in both Ca$$^{2+}$$ mobilisation and the MAPK signalling pathway were not expected. Previous data has primarily associated defects in MAPK signalling with clonal anergy and defects in Ca$$^{2+}$$ signalling with adaptation (Schwartz, 2003). As 4Tyr tolerised Tg4 cells bear none of the hallmarks of classically anergic T cells it is unsurprising that ERK activation remained intact. Similar to the state of tolerance induction explored here, adaptive tolerance requires the continued presence of Ag. Yet, 4Tyr tolerised Tg4 cells do not appear to be adapted; 4Tyr tolerised Tg4 cells do not have the surface phenotype indicative of an adapted T cell (Figure 6.1 and 6.2). Moreover, 4Tyr tolerised Tg4 cells do not exhibit an altered dose response to peptide stimulation when compared to naïve Tg4 cells (Figure 6.7). However, it is important to consider that the phenotype
of 4Tyr tolerised Tg4 cells was predominantly explored 4 days after peptide treatment; a time-point at which Tg4 cells had been shown to be tolerant (Figure 5.5). Tanchot et al have demonstrated that adaptation takes 7 days to be complete (Tanchot et al., 2001); by transferring PCC-specific T cells to a mouse consistently expressing the Ag, they demonstrated that PCC-specific T cells exhibited unresponsiveness by day 3, but that adaptation was not complete until day 7 after transfer. Thus, although 4Tyr tolerised Tg4 cells do not appear to be adapted at day 4, they may be undergoing adaptation and exhibit a more adapted phenotype at later time-points. This possibility remains to be explored, but taken together the data presented here suggest adaptation is perhaps the state of T cell unresponsiveness most similar to that seen in 4Tyr tolerised Tg4 cells compared to clonal anergy.

Further analysis of 4Tyr tolerised Tg4 cells should be undertaken to determine whether the cells express any molecules previously associated with the tolerant phenotype, for example any of the E3-ubquitin ligases (Fathman and Lineberry, 2007). Considering the unique cytokine production by 4Tyr tolerised Tg4 cells it would also be important to determine expression and activation of STAT proteins involved in IL-2 (STAT 5) and IFNγ (STAT 1) production/responsiveness. Importantly, it has clearly been demonstrated by the studies presented here that a key mechanism employed to dampen Tg4 cell responsiveness following 4Tyr tolerance induction is through the modification of Ca²⁺ mobilisation upon TCR ligation.

6.3.3. Induction of a regulatory phenotype in Tg4 cells following 4Tyr treatment.

The demonstration that Tg4 cells persist following tolerance induction, and that persistence of these Ag-reactive T cells in a disease setting is seen, raised the possibility that 4Tyr tolerised Tg4 cells could have a regulatory phenotype. Peptide treatment has been shown to induce peptide-reactive T cells with a regulatory phenotype. Generation of CTLA-4hi, foxp3- IL-10 producing regulatory Tg4 cells has been noted following multiple treatments of Tg4 mice with 4Tyr i.n. (Burkhart et al., 1999; Sundstedt et al., 2003). More importantly, studies have shown generation of T cells with a regulatory phenotype following systemic administration of peptide
(Judkowski et al., 2004). Apostolou et al continuously administered HA peptide to TCR transgenic mice specific for the peptide, through use of osmotic mini pumps (Apostolou and von Boehmer, 2004). This continuous Ag persistence caused an increase in peptide-specific foxp3+ cells, and, although more profound, is similar to that seen in this study. Thus it was possible that the persisting tolerant Tg4 T cells had adopted a regulatory phenotype.

Examination of foxp3 expression in persisting Tg4 cells in mice recovering from EAE demonstrated enhanced percentages of Tg4 cells which were foxp3+ in both the LN and CNS of 4Tyr treated mice (Figure 6.8). These data suggest that 4Tyr treatment was somehow influencing the foxp3+ compartment within the Tg4 population; this could be through the induction of foxp3 in foxp3- Tg4 cells or by allowing specific expansion of foxp3+ Tg4 cells. These observations led to a series of experiments in which 4Tyr was administered following adoptive transfer of Tg4 cells and the expression of foxp3 determined at time points after peptide treatment or following immunisation of mice with 4Lys in CFA (Figure 6.9). In no circumstances was foxp3 expression within the Tg4 cell population enhanced following treatment with 4Tyr.

Previous studies have shown induction a regulatory phenotype in peptide-specific T cells following chronic exposure to peptide. This has been noted following repeated dosing with peptide (Apostolou and von Boehmer, 2004; Broere et al., 2008; Paas-Rozner et al., 2003), and transfer of peptide-specific T cells into mice constitutively expressing the cognate Ag (Chen et al., 2004b; Kohyama et al., 2004). Importantly, most studies administered a low dose of Ag to induce a regulatory phenotype in Ag-reactive T cells; this could fundamentally account for lack of foxp3 induction following high dose 4Tyr treatment. In fact, Kretschmer et al demonstrated that proliferation was antagonistic to foxp3 induction (Kretschmer et al., 2005); Figure 5.7 shows 4Tyr induces considerable proliferation in Tg4 cells. Alternatively, more chronic stimulation of the TCR could be required to induce foxp3 expression. Data shown here demonstrate that 4Tyr is available to stimulate a T cell for up to 14 days after its administration (Figure 5.8). Adoption of a regulatory phenotype could
require persistence of p:MHC complexes for longer than 14 days, therefore permitting more chronic stimulation of the TCR. To determine if this was the case 4Tyr was administered one to five times at three day intervals, to mice following Tg4 cell transfer. Repeated 4Tyr treatment allowed Tg4 cells to persist as a large percentage of the CD4+ T cell pool, at peak Tg4 cells were 15.88±4.04% of the whole Tg4 cell population (Figure 6.9d). Importantly, the percentage of Tg4 cells which were foxp3+ remained constant following each peptide treatment (Figure 6.9e). These data suggest that repeated exposure of Tg4 cells to 4Tyr had no effect on foxp3 expression within the Tg4 cell population.

The Kretschmer et al study induced foxp3+ cells by targeting Ag to DEC205+ DC. Whether this DEC205+ DC subset was specifically tailored for foxp3 induction was not established (Kretschmer et al., 2005). A more recent study has asked this question and shown that CD8+DEC205+ splenic DC specialise in the induction of foxp3 (Yamazaki et al., 2008). This DC population specifically produced TGFβ, which was not the case for CD8-DEC205- DC. These data suggest that failure to induce foxp3 in Tg4 cells following peptide treatment could be indicative of presentation by a DC subset not suited to foxp3+ induction; 4Tyr:MHC complexes are specifically maintained by CD11c+CD4+ (CD8-) DC (Figure 5.12).

Together, these data suggest that 4Tyr treatment has a positive effect on foxp3 expression within the Tg4 population specifically within the EAE setting. This could be through induction or expansion of foxp3+ Tg4 cells. Neither proposal can be verified by the studies presented here, but previous work has shown Treg proliferation specifically within the CNS during recovery from passively induced EAE (O’Connor et al., 2007). Increases in foxp3+ Tg4 cells has only been noted in the EAE setting. As such, it appears that persisting tolerant Tg4 cells only show enhanced foxp3 expression following access to the environment of the CNS or exposure to the chronic inflammatory stimulus used for EAE induction. It is important to note that 4Tyr treated mice do not get sick, yet have a considerable CNS infiltrate of disease-relevant Tg4 cells, almost half of which are foxp3+. These data suggest that 4Tyr treatment allows Tg4 cells to persist in a non-aggressive state,
however when these cells gain access to the CNS environment an enhanced proportion of the cells will express foxp3.

A regulatory phenotype is not exclusively tied to expression of foxp3. The possibility remains that 4Tyr treatment has induced a regulatory phenotype in Tg4 cells not dependent on foxp3 expression. This has been noted in a number of studies in which production of IL-10 is the defining feature of the induced regulatory T cells (Broere et al., 2008; Burkhart et al., 1999; Massey et al., 2002). This is clearly not the case for 4Tyr tolerised Tg4 cells as no enhanced levels of IL-10 production have been detected. However, other mechanisms can be employed to suppress/regulate other immune cells and the possibility that 4Tyr tolerised Tg4 cells can mediate such suppression cannot be excluded. However preliminary experiments have been undertaken in which tolerant Tg4 cells have been sorted from 4Tyr treated mice and their ability to suppress naïve T cells in an in vitro suppression assay determined. Initial data suggest that 4Tyr tolerised Tg4 cells do not have the ability to suppress proliferation of naïve T cells and thus do not have a regulatory phenotype (data not shown). A recent study by Li et al showed that i.v. administration pMOG three times following induction of EAE prevented disease (Li et al., 2008). Importantly the authors reported that peptide treatment i.v. induced a population of tolerogenic DC; characterised as CD11c+CD11b+ and which secreted significantly more IL-10 and TGFβ and significantly less IL-12 and nitric oxide (NO) upon LPS stimulation. The ability of peptide treatment to specifically elicit DC with tolerogenic properties is yet to be explored in this system.

### 6.3.4. T-bet expression in tolerant Tg4 cells.

In the intact Tg4 mouse, repeated i.n. administration of 4Tyr has been shown to lead to a tolerant state characterised by increased IL-10 production and mediated by induction of a novel transcriptional program associated with enhanced levels of Egr-2 and T-bet (Anderson et al., 2006). Considering the Anderson et al paper, follow-on experiments explored the expression of T-bet in 4Tyr tolerised T cells (Figure 6.10). T-bet was only expressed in Tg4 cells upon ex vivo recall to 4Lys. Although not significant, there was a trend toward a decrease in T-bet expression in Tg4 cells
following 4Tyr treatment (Figure 6.10); this was true for both percentage of T-bet+ Tg4 cells and the MFI of T-bet staining. Anderson et al demonstrated that the enhanced expression of T-bet in tolerant Tg4 cells correlated with the suppression of IL-2 production. In fact, it is well characterised that T-bet expression is necessary for suppression of IL-2 production in developing Th1 cells (Hwang et al., 2005). As such it is not surprising that the Tg4 cells examined here, which produce significant levels of IL-2, do not exhibit the enhanced T-bet expression associated with the tolerant program in other tolerance systems.

Several studies have highlighted an important role for Egr-2 in enhancing expression of the cell-cycle inhibitor p21\textsuperscript{cip1} (Anderson et al., 2006; Zhu et al., 2008). A recent study by Zhu et al demonstrated that lack of Egr-2 expression in T cells causes enhanced expression of IFN\textgamma and IL-17 but not alter expression of IL-2 (Zhu et al., 2008). Thus it remains possible that Egr-2 could play an important role in maintenance of tolerance in 4Tyr tolerised Tg4 cells.

6.3.5. Cytokine production by 4Tyr tolerised Tg4 cells.

Tolerant Tg4 cells produced considerable levels of IL-2 but were unable to produce IFN\textgamma or IL-10. Importantly, this tolerant phenotype in Tg4 cells is established by four days after peptide treatment. Data shown in Chapter 5 suggest that this phenotype arises due to repeated stimulation of Tg4 cells following 4Tyr treatment, as 4Tyr-MHC complexes can remain for up to 14 days post peptide treatment. This phenotype is different to that seen by other investigators, specifically, repetitive Ag stimulation of T cells has been associated with increased production of IL-10 in various models of T cell tolerance (Broere et al., 2008; Burkhart et al., 1999; Kohyama et al., 2004; Tanchot et al., 1998). Moreover, Kohyama et al proposed that peripheral T cells become hyporesponsive when exposed to their cognate Ag without additional signals and this is not dependent on the level of Ag present. However, these hyporesponsive T cells only gain the ability to produce IL-10 when Ag is present above a certain threshold; this was determined by examining tolerised DO11.10 cells from RIP-OVA\textsuperscript{hi} and RIP-OVA\textsuperscript{low} mice (Kohyama et al., 2004). The hypothesis was proposed in the previous chapter that although 4Tyr tolerised Tg4
cells had received sufficient stimulation to turn off IFNγ, they had not yet been stimulated to a sufficient extent to turn off IL-2 and turn on IL-10 production; this hypothesis was tested here.

Repeated administration of 4Tyr at weekly intervals was sufficient to switch production of IL-2 off, and IL-10 production on. In contrast to what was anticipated, repeated doses of 4Tyr switched production of IFNγ on, as levels of IFNγ increased with increased number of doses (Figure 6.12). Increasing the number of doses and decreasing the time between doses, similar to the protocol of tolerance induction used by Wraith and colleagues, showed a similar pattern of increased IFNγ and decreased IL-2, as the number of 4Tyr doses was increased (Figure 6.13). IL-10 production was seen to increase after 3 doses of 4Tyr. However, after four peptide doses IL-10 production declined again. These data suggest that a delicate balance exists between the number of encounters with Ag and the ability of a T cell to make IL-10, as it is gained after three stimulations but then rapidly lost. The delicate pattern of cytokine expression highlighted in the data presented here cannot be seen in the studies by Wraith and colleagues (Anderson et al., 2005; Burkhart et al., 1999).

In the experiments performed here a cohort of cells were followed after synchronous encounter with peptide. When administering peptide to an intact transgenic, Tg4 cells will encounter peptide at different times. Thus the data presented here give a clearer kinetic picture of cytokine expression following repeated 4Tyr dosing. However, it is important to note that repetitive dosing tolerance protocols, as performed here and by Wraith and colleagues, led to a more profound level of tolerance in the Tg4 cells.

Following repeated 4Tyr treatments, tolerised Tg4 cells lost the ability to make IL-2 but gained the ability to produce effector cytokines; these data suggest that repeated doses of 4Tyr push Tg4 cells toward an anergic phenotype. In vivo anergy, or adaptation, requires both initial T cell expansion and persistence of the Ag to be induced (Rocha et al., 1993); resultant T cells have been shown to have defects in IL-2 production (Rocha and von Boehmer, 1991) and in the production of all cytokines (Tanchot et al., 2001). The 4Tyr tolerised Tg4 cells examined after multiple 4Tyr
treatments may well be have become adapted. The next logical step would be to
determine if *ex vivo* dose responses of Tg4 cells from mice which have received
multiple 4Tyr treatments are shifted compared to those from mice treated once with
4Tyr. Furthermore, Tg4 cells from mice treated multiple times with 4Tyr could be
left for long periods of time after the last 4Tyr treatment, allowing 4Tyr to be cleared
from the system, and then recall responses examined. In fact, it would be important
to examine responsiveness of Tg4 cells from mice which have received one or
multiple doses of 4Tyr in hosts not treated with 4Tyr; by transferring FACS sorted
4Tyr experienced Tg4 cells to naïve hosts. As 4Tyr forms long-lasting p:MHC
complexes, such experiments would determine whether 4Tyr tolerised Tg4 cells
retain their phenotype in the complete absence of 4Tyr:MHC complexes and,
importantly, how stable the tolerant phenotype is.

6.3.6. Role of IL-2 in generating the novel phenotype in 4Tyr tolerised
Tg4 cells.

IL-2 is the only detected cytokine produced by Tg4 cells tolerised by a single dose of
4Tyr. Moreover, it is produced from early time-points after peptide treatment. As it
is well established that IL-2 induces the proliferation of Ag-activated T cells (Malek,
2008), it was necessary to determine whether IL-2 production by tolerised Tg4 cells
aided the persistence of this Tg4 cell population. The effect of administration of
anti-IL-2 along with a tolerogenic dose of 4Tyr or 4Lys was determined (Figure
6.14). Anti-IL-2 had a small but significant effect on the number of Tg4 cells
persisting following 4Lys treatment, with increased numbers of Tg4 cells persisting
following 4Lys treatment. This translated to a trend toward increased *ex vivo*
responses from anti-IL-2 treated 4Lys tolerised mice. 4Lys induces tolerance
through deletion of Tg4 cells. Importantly IL-2 is needed for the process of
activation induced cell death (AICD) to occur, and administration of anti-IL-2
prevents Ag induced deletion (Critchfield et al., 1994; Lenardo, 1991); hence
increased numbers of Tg4 cells following 4Lys and anti-IL-2 treatment could be due
to reduced AICD.
Administration of anti-IL-2 had no significant effect on tolerance induction with 4Tyr; Tg4 cells were still able to persist following tolerance induction, and could produce significant amounts of IL-2 but minimal amounts of IFNγ upon recall. However there was a consistent trend toward an increase in Tg4 cell numbers and responsiveness upon administration of anti-IL-2, demonstrated by a more activated surface phenotype (as assessed by expression of CD44 and CD62L). The data presented in Figure 6.15 suggest that this slight enhancement of Tg4 cell numbers and responsiveness was due to specific depletion of CD4+CD25+foxp3+ from both the host and Tg4 cell compartment upon treatment with anti-IL-2. This could also account for the enhanced numbers of Tg4 cells following 4Lys treatment. A reduction in the number of CD4+CD25+foxp3+ Tregs would alter the regulatory environment of host animals and thus potentially permit greater expansion of Tg4 cells. Alternatively, Tg4 cells from anti-IL-2 treated mice could be expanding further to fill the niche emptied upon depletion of the Tregs.

The anti-IL-2 antibody used in the present study (clone S4B6) has been shown to bind IL-2 in such a way that it specifically prevents IL-2 binding to CD25 (Boyman et al., 2006). Previous reports have shown that IL-2 is critical for the peripheral maintenance of CD4+CD25+foxp3+ T cells and therefore the immune tolerance mediated by these cells. Setoguchi et al showed that administration of 1mg of anti-IL-2 led to a significant reduction in CD4+CD25+foxp3+ cells, but had no effect on the number of CD25- cells (Setoguchi et al., 2005). The reduced regulation caused by depletion of CD4+CD25+foxp3+ cells led to induction of a variety of autoimmune conditions in NOD mice, specifically autoimmune neuropathy, and autoimmune gastritis in BALB/c mice. These data highlight the important role IL-2 plays for peripheral Treg maintenance. Collectively, the data presented here demonstrate that significant reductions in CD4+CD25+foxp3+ Tregs following anti-IL-2 treatment permit greater expansion of Tg4 cells following peptide treatment.

6.3.7. 4Tyr treatment causes an increase in host foxp3+ cells.
The important role of IL-2 in maintaining T cell tolerance is highlighted by the severe autoimmunity suffered by IL-2, IL-2Rα and IL-2Rβ knockout mice (Malek,
As discussed above, removal of IL-2 incurs autoimmunity and decreases in Treg numbers (Setoguchi et al., 2005). However, if sequestering IL-2 away from Tregs can cause their depletion, can enhanced levels of IL-2 cause their expansion? This suggestion led to the examination of the foxp3+ compartment in host CD4+ cells following 4Tyr treatment.

Figure 6.16 shows increased percentages of CD4+foxp3+ cells following 4Tyr treatment specifically within the host compartment. The data suggest that this is due to enhanced levels of IL-2 caused by the production of IL-2 from persisting 4Tyr tolerised Tg4 cells. Supporting this is the demonstration that the percentage of host CD4+foxp3+ cells was enhanced the most following one dose of 4Tyr (Figure 6.16c) which causes Tg4 cells to produce the most IL-2. Administration of more doses of 4Tyr caused the number of host CD4+foxp3+ cells to steadily decrease. This was concomitant with a decrease in IL-2 production by Tg4 cells (Figure 6.12 and 6.13).

Initial experiments have been performed to detect IL-2 in the sera following Tg4 transfer and 4Tyr treatment. However, no IL-2 could be detected (data not shown), suggesting the IL-2 is produced in a local environment or the assay is insensitive to the levels of IL-2. The specific expansion of CD4+foxp3+ cells following increased IL-2 has been noted by other investigators. Boyman et al showed Treg expansion following administration of recombinant IL-2:anti-IL-2 complexes which specifically present IL-2 to CD25 bearing Tregs (Boyman et al., 2006). In a human study, enhanced percentages of CD4+CD25+foxp3+ cells were seen in cancer patients following IL-2 treatment (Ahmadzadeh and Rosenberg, 2006). Taken together, the data presented in this study suggest that the enhanced IL-2 from tolerised Tg4 cells might be contributing to a more tolerogenic environment by enhancing the number of host Tregs. Why a similar increase in foxp3+ Tg4 cells is not seen following 4Tyr treatment remains unresolved, but is probably reflective of the Tg4 TCR being ligated upon 4Tyr treatment; whereas host cells would just receive an IL-2 signal, Tg4 cells would receive both a TCR and IL-2 signal.

Collectively, in chapters 5 and 6, data has been presented examining tolerance induction in an adoptively transferred population via systemic administration of a peptide with high affinity for MHC. 4Tyr treatment induces a profound state of tolerance in adoptively transferred Tg4 cells which does not result in the deletion of Tg4 cells. This has allowed thorough analysis of the persisting tolerant Tg4 cells to be undertaken, with the result that their phenotype has been fully characterised in terms of signalling capabilities, surface molecule expression, transcription factor expression and cytokine production.
Figure 6.1. Surface phenotype of Tolerant Tg4 cells.

1-3x10^6 Ly5.1^+Tg4 cells were transferred to B10.PL or B10.PLxC57BL/6 mice 1 day prior to 200μg of 4Tyr, 4Val, or PBS i.v. Spleens were taken 4 days after peptide treatment, and the surface phenotype of Tg4 cells examined via flow cytometry. Filled histograms represent surface expression on Tg4 cells from PBS treated mice, dotted lines represent surface expression on Tg4 cells from 4Val treated mice, and solid lines represent surface expression on Tg4 cells from 4Tyr treated mice.

Results show representative plots from individual mice from 1 or 2 separate experiments with 3-4 mice per group.
Figure 6.2. Surface phenotype of tolerant Tg4 cells following 4Lys+CFA immunisation.

1-3x10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice 1 day prior to 200µg 4Tyr, or 4Val i.v. 7 days later mice were immunised with 4Lys in CFA s.c., and 10 days post-immunisation mice were sacrificed. Expression of a, TCR and b, PD-1 were examined on Tg4 cells from the spleen and LN via flow cytometry. Dotted lines represent surface expression on Tg4 cells from 4Val treated mice, and solid lines represent surface expression on Tg4 cells from 4Tyr treated mice. Results are representative of 1 experiment with 3 mice per group.
Figure 6.3. Altered Ca$^{2+}$ metabolism in tolerant Tg4 cells.

a, Experimental outline; 2x10$^6$ Ly5.1+Tg4 cells were transferred to B10.PL mice 1 day prior to 4Tyr, 4Tyr+LPS, or PBS i.v. Mice were sacrificed 4 days after peptide treatment and Ca$^{2+}$ flux of splenocytes examined by flow cytometry using the ratio of Indo-1 violet/blue fluorescence for host and Tg4 donor cells; separated by Ly5.1 expression (b). Calcium mobilisation was determined in response to cross-linking of CD3.

c, Cells were stimulated 1 minute after the beginning of the flow assay by adding 20μg/ml of streptavidin and fluorescence was followed for 11 minutes. Results are expressed as the geometric mean indo-1 violet/blue ratio of the host (black line) or Tg4 (blue line) population over time. d, Overlay of 4Tyr treated (red line) or 4Tyr+LPS treated (blue line) Tg4 cells. Results show representative plots of individual mice from 2 consistent experiments with 4 mice per group.
Figure 6.4. Altered Ca\(^{2+}\) metabolism in tolerant Tg4 cells.

1-3x10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice 1 day prior to 4Tyr, 4Tyr+LPS, or PBS i.v. Spleens were taken 4 days after peptide treatment and Ca\(^{2+}\) flux examined by flow cytometry using the ratio of Indo-1 blue to violet fluorescence. Calcium mobilisation was determined in response to cross-linking of CD3. The mean Indo-1 violet/blue emission ratio of a, Peak and c, Baseline intracellular calcium level in Tg4 cell. b, Bar graph showing the area under the curve determined from FACS plots of Ca\(^{2+}\) responses. Significant differences are shown, * p<0.05, ** p<0.01, ***p<0.001. Statistical analysis was determined by an ANOVA using Tukey’s multiple comparison test. Error bars represent SEM and data is representative of 2 experiments with 4 mice per group.
Figure 6.5. Tolerant Tg4 cells do not display a Ca$^{2+}$ signalling defect when stimulated in the presence of excess calcium.

1-3x10$^6$ Ly5.1+Tg4 cells were transferred to B10.PL mice 1 day prior to 4Tyr, 4Tyr+LPS, or PBS i.v. Mice were sacrificed 4 days after peptide treatment and Ca$^{2+}$ flux of splenocytes examined by flow cytometry using the ratio of Indo-1 violet to blue fluorescence. Calcium mobilisation was determined in response to cross-linking of CD3 in media containing 2mM EGTA. a. Cells were stimulated 30 seconds after the start of the flow assay by adding 20μg/ml of streptavidin. Two minutes after cross-linking, 3mM CaCl$_2$ was added and fluorescence followed for a further eight minutes. Results are expressed as the geometric mean indo-1 violet/blue ratio of the host (black line) or Tg4 (blue line) population over time. b. Overlay of 4Tyr treated (red line) or 4Tyr+LPS treated (blue line) Tg4 cells. Results show representative plots from individual mice from 2 experiments with 3-4 mice per group.
Figure 6.6. Tolerant Tg4 cells do not have a defect in ERK phosphorylation.

2x10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice 1 day prior to 4Tyr, 4Tyr+LPS, or PBS i.v. Mice were sacrificed 4 days after peptide treatment. a, Splenocytes from 4Tyr+LPS (top panel) and 4Tyr (bottom panel) treated mice were stimulated with 100μM 4Tyr for 5, 15 or 30 minutes in vitro before fix/permeabilisation. Flow cytometry was performed on cells stained with surface markers, to discriminate host from donor cells, and with pERK. Histograms show representative plots of Tg4 cells. b, Control samples were splenocyte cultures pre-treated with U0126 (a pERK inhibitor) 30 minutes prior to peptide stimulation, stimulated splenocytes stained with secondary antibody only, and an unstimulated sample. c, Graph showing increases in pERK staining over time in Tg4 cells. Results show representative plots of individual mice from 2 experiments with 3 mice per group.
Figure 6.7. Production of IL-2 by tolerant and naïve Tg4 cells to 4Lys and 4Tyr stimulation.
1-3x10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice 1 day prior to 4Tyr i.v. Spleens were taken 4 days after peptide treatment and ex vivo recall assays to increasing concentrations of 4Tyr and 4Lys performed. As a control Tg4 cells from naïve Tg4 mice were cultured with B10.PL splenocytes as 3.5% of the total number of cells in each well (similar to the expected ratio of tolerant Tg4 cells to splenocytes). IL-2 production was determined by ELISA. Error bars represent SEM, data is representative of 2 experiments with 3 tolerised mice per group and 2 naïve Tg4 culture wells per stimulation condition.
Figure 6.8. 4Tyr treatment increases the expression of foxp3 in Tg4 cells during EAE.
B10.PLxC57BL/6 mice received PBS, 4Lys or 4Tyr i.v. 1 day after transfer of Ly5.1+ Tg4 T cells. 7 days later EAE was induced by immunisation of mice with 4Lys in CFA s.c. Mice received Ptx i.p at the same time as immunisation and 2 days later. Foxp3 expression was examined by flow cytometry in Tg4 cells from a, CNS and b, LN of mice on day 19 after induction of EAE. c, Bar chart showing the percentage of foxp3+ Tg4 cells in the CNS and LN. d, Bar chart showing the total number of foxp3+ Tg4 cells in the CNS and LN.
Data is representative of one experiment.
Figure 6.9. 4Tyr treatment of Tg4 cells *in vivo* does not increase the expression of foxp3 in Tg4 cells.

**a.** 1-2x10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice 1 day prior to administration of 4Lys, 4Tyr or PBS i.v. Mice were sacrificed and the percent of Tg4 cells which were foxp3+ at day 4 (open bars) or 6 days (black bars) determined.

**b-c.** 1-2x10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice 1 day prior to administration of 4Val, 4Tyr or PBS i.v. 7 days later mice were immunised with 4Lys in CFA s.c. and mice sacrificed 10 days post immunisation. **b.** percent of Tg4 cells in the LN and **c.** percent of these Tg4 cells which are foxp3+.

**d-e.** 1-2x10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice and repeated doses of 4Tyr were given at 3 day intervals i.v. Mice were sacrificed 3 days after 1-5 doses of 4Tyr and **d.** the percent of Tg4 cells and **e.** the percent of Tg4 cells which were foxp3+ were determined.

For all experiments, error bars represent SEM and data is representative of 2 experiments with 3-4 mice per group.
Figure 6.10. Tolerant Tg4 cells express T-bet upon stimulation.
2x10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice 1 day prior to 4Tyr, 4Tyr+LPS, or PBS i.v. Mice were sacrificed 4 days post peptide treatment and splenocytes were stimulated overnight with 50μM 4Lys. Flow cytometry was performed on cells stained with surface markers to discriminate a, host and b, Tg4 cells. Filled histograms show T-bet expression in unstimulated cells and solid black lines show T-bet expression in stimulated cells. Numbers represent percent of Tg4 cells which are T-bet+ (top numbers) and the mean fluorescence intensity of T-bet (bottom numbers) ± standard deviation. Results show representative plots of individual mice from 2 experiments with 3-4 mice per group.
Figure 6.11. Cytokine production by tolerant Tg4 cells following repeated doses of 4Tyr i.v.
1-2×10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice and repeated doses of 4Tyr given at week intervals i.v. Mice were sacrificed 7 days after 1-3 doses of 4Tyr and spleens sampled. Cytokine production was determined by ICS following overnight stimulation with 100μM 4Lys or by ELISA of supernatants from 96 hour ex vivo recall cultures to increasing concentrations of 4Lys. a-b, IL-2, c-d, IFNγ and e-f, IL-10 production from Tg4 cells (a,c,e) or splenocyte cultures (b,d,f). g, Proliferative responses of splenocyte cultures to increasing concentrations of 4Lys determined as cpm per Tg4 cell. Significant differences are shown, * p<0.05, ** p<0.001. Statistical analysis was determined by an ANOVA using Tukey’s multiple comparison test. Error bars represent SEM, and lines show median values. Data is representative of 2 experiments with 3 mice per group.
Figure 6.12. Cytokine production by tolerant Tg4 cells following repeated doses of 4Tyr i.v.
2x10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice and repeated doses of 4Tyr given at 3 day intervals i.v. Mice were sacrificed 3 days after 1-5 doses of 4Tyr and spleens sampled. Cytokine production by Tg4 cells was determined by ICS following overnight stimulation with 100μM 4Lys for a, IL-2, b, IFNγ and c, IL-10. d, Proliferative responses of splenocyte cultures stimulated with 100μM 4Lys or unstimulated. Significant differences shown are; *p<0.05, **p<0.01, as determined by an ANOVA using Tukey’s multiple comparison test. Error bars represent SEM and line show median values. Data is representative of 1 experiment with 3 mice per group.
Figure 6.13. The effect of anti-IL-2 treatment on the induction of tolerance by 4Tyr.
1-2x10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice. The following day mice were treated with 4Lys or 4Tyr i.v. Cohorts of mice also received 200μg anti IL-2 i.p. at the same time as peptide treatment and 2 days later. Mice were sacrificed 4 days after peptide treatment. a. Tg4 cells as a percent of all CD4+ cells in the spleen. b, expression of CD44 (top panel) and CD62L (bottom panel) on Tg4 cells from mice treated with 4Tyr (dotted grey line) or 4Tyr+anti IL-2 (solid black line). c-d, IFNγ and e-f, IL-2 production from splenocyte cultures to increasing concentrations of 4Lys.
Error bars represent SEM and significant difference shown was determined by an unpaired T test. Data is representative of 2 experiments with 3 mice per group.
Figure 6.14.  Anti-IL-2 treatment alters the percent of foxp3+CD25+ cells.  
1-2x10^6 Ly5.1+Tg4 cells were transferred to B10.PL mice.  The following day mice were treated with 4Tyr i.v.  Groups of mice also received 200μg anti IL-2 i.p. at the same time as peptide treatment and 2 days later.  Mice were sacrificed 4 days after peptide treatment and spleens sampled.  a, Bar graph showing the percent of all Tg4 and host cells which are foxp3+.  Comparison of the percent of foxp3+CD25+ (white bars) and foxp3+CD25- (black bars) cells following 4Tyr or 4Tyr+anti-IL-2 treatment for b, Tg4 cells and c, Host cells.  
Error bars represent SEM, and significant differences shown were determined by unpaired T test.  Data is representative of 1 experiment with 3 mice per group.
**Figure 6.15.** Treatment with 4Tyr alters the percent of host foxp3+ cells.

1-2×10⁶ Ly5.1+Tg4 cells were transferred to B10.PL mice 1 day prior to 4Lys, 4Tyr or PBS i.v. The percent of host CD4+foxp3+ cells in the spleen was determined at **a**, day 4 and **b**, day 6 after peptide treatment. Significant differences shown are; *p<0.001, **p<0.01, as determined by an ANOVA using Tukey’s multiple comparison test. Error bars represent SEM and data is representative of 3-4 experiments with 3 mice per group.

1-2×10⁶ Ly5.1+Tg4 cells were transferred to B10.PL mice and repeated doses of 4Tyr given at 3 day intervals i.v. **c**, Mice were sacrificed 3 days after 1-5 doses of 4Tyr and the percent of host CD4+foxp3+ cells in the spleen determined by flow cytometry. Significant difference shown was determined by an ANOVA using Tukey’s multiple comparison test. Error bars represent SEM and data is representative of 1 experiment with 3 mice per group.
7. General Discussion

The induction of T cell tolerance or immunity is dependent on the dialogue between T cells and DC. Tolerance does not simply result from a lack of activating signals; it appears to be a choice of the immune system. As such, the dialogue between a DC and T cell must be qualitatively or quantitatively different to induce tolerance versus immunity. In this thesis these ideas have been investigated, and the result of altering either signal 1 or 2 on the induction of tolerance determined. When considering alterations in signal 2, data presented here have shown that PD-1 mediated signals are vital in limiting T cell responses, but play no role in the induction, establishment or maintenance of peptide-induced T cell tolerance (Chapter 3). RANKL mediated signals were shown to enhance signal 1, thereby enhancing tolerance or immunity depending on the context in which RANKL was ligated (Chapter 4). Changes to signal 1 considerably altered peptide-tolerance induction; by utilising a peptide with a high affinity for MHC, it was demonstrated that increasing the time a peptide is available to stimulate peptide-reactive T cells can profoundly influence the mechanism utilised to generate tolerance in the peptide-reactive T cells (Chapters 5 and 6). These data highlight an important factor concerning peptide tolerance induction; altering either signal 1 or signal 2 can have qualitative effects on tolerance induction. There is not an all or nothing approach to tolerance induction; rather the degree of tolerance induced can be altered depending on the signals received and either be enhanced, as shown by administration of anti-RANKL, or be phenotypically distinct, as shown following administration of 4Tyr.

7.1 Signal 2

*PD-1 in tolerance and immunity*

Disruption of the PD-1:PD-L interaction leads to enhanced EAE (Salama et al., 2003), diabetes in the NOD mouse (Ansari et al., 2003), and loss of allograft (Wang et al., 2007) and fetomaternal (Guleria et al., 2005) tolerance. Enhancement of these immune-driven conditions could indicate loss of tolerance in the pre-disease state or more vigorous effector T cell responses. The data presented in chapter 3 reflect the latter proposal. The studies presented here, and those of others, (Ansari et al., 2003; Salama et al., 2003), have shown expansion of Ag-specific T cells upon blockade of
PD-1. These data demonstrate a vital role for PD-1 in keeping T cell responses in check by limiting the amplitude of the response. The absence of PD-1 results in the outgrowth of T cells usually restrained by PD-1 mediated signalling. Consistent with this, Barber et al demonstrated that un-responsive (exhausted) CD8+ T cells express high levels of surface PD-1 (Barber et al., 2006). Blockade of PD-1 allowed full effector function of the CD8+ T cells to be regained. Taken together, these data indicate that anti-PD-1 could be used as a therapy in promoting immunity; potentially to promote anti-cancer responses. Moreover, expression of PD-1:PD-L1 is thought to contribute to the immunosuppressive microenvironment of tumours; in fact, expression of PD-L1 correlates with increased mortality in a variety of cancers (Keir et al., 2008). Thus, the administration of anti-PD-1 could well become an important anti-cancer therapy in both ablation of immunosuppressive environment and by promoting the expansion of cancer-specific effector T cells. Consequently a humanised anti-PD-1 antibody has been developed and recently performed well in phase 1 clinical trials against haematological malignancies (Berger et al., 2008).

Fundamental to the success of anti-PD-1 as a cancer therapy would be the demonstration that it has no effect on immunological self-tolerance. However, disruption of PD-1 signalling has been frequently shown to prevent induction of T cell tolerance, and/or to reverse it (Keir et al., 2007; Martin-Orozco et al., 2006; Tsushima et al., 2007). This has been most readily shown for CD8+ T cells. In the studies presented in Chapter 3, PD-1 was shown to be dispensable for peptide-induced T cell tolerance in CD4+ cells. Other investigators have determined that PD-1 mediated signals are more important in maintaining tolerance than in the induction of tolerance in CD4+ T cells (Fife et al., 2006). A model has been proposed for CD4+ T cells in which CTLA-4 is important for the induction of tolerance whereas PD-1 is important in maintaining tolerance. Such a role conceptually fits with the expression of PD-L1, which is the only member of the immunoglobulin super-family to be expressed on non-haematopoietic cells. PD-1:PD-L1 has consequently been shown to play a vital role in tissue tolerance (Guleria et al., 2007; Keir et al., 2006), with PD-L1 expression often functioning as a barrier inhibiting T cell function within tissues. In the studies presented in this thesis, anti-
PD-1 failed to overcome tolerance already induced in OT-II cells, suggesting no role for PD-1 in the maintenance of peptide induced T cell tolerance. However, deletion was the primary mechanism of tolerance in this TCR-transgenic model of peptide tolerance. In a heterogeneous population of T cells, administration of anti-PD-1 following peptide tolerance induction could well affect the responsiveness of lower affinity peptide-reactive T cells which remain following peptide treatment.

The role of PD-1 mediated signals in T cell tolerance has primarily been investigated in TCR transgenic models. The demonstration that PD-1 signalling has the greatest influence on T cell responsiveness following low levels of TCR stimulus, (Freeman et al., 2000), makes it important to establish the effect of anti-PD-1 administration on a heterogeneous population of T cells, bearing TCR of different affinities. Studies in the PD-1−/− mouse would suggest that autoimmunity would develop (Nishimura et al., 1999; Nishimura et al., 2001). However, it would be important to determine why particular autoimmune phenotypes develop in certain strains of mice. Is this due to the expansion of T cells bearing low affinity TCRs for organ-specific peptides, and/or due to loss of tolerance specifically within tissues as PD-1:PD-L1 can no longer dampen T cell function?

Importantly, the role of PD-1 within the T cell compartment still poses many questions. If anti-PD-1 continues to perform well in clinical trails as an anti-cancer agent, these questions will need resolving. The use of anti-PD-1 for therapy over a prolonged period of time would seem ill-advised. Yet shorter periods of anti-PD-1 therapy could well prove highly effective in generating immunity to cancers. Essentially, choices will have to be made weighing the potential loss of immunological self-tolerance against the imminent threat posed by cancer.

**RANKL in tolerance and immunity**

Data presented in chapter 4 appeared to suggest a dichotomy in the effect of administration of anti-RANKL; with administration seen to enhance both tolerance and immunity. The mechanism behind this differential functioning remains unclear, but could reflect of one of two situations. If anti-RANKL is simply a blocking
antibody then it must be concluded that the blockade of RANKL during immunity prevents an inhibitory signal, whereas blockade of RANKL during tolerance prevents an activating signal. What could generate such a dichotomy of function? RANKL mediated signals could play different roles at different time-points during the activation of a T cell, i.e. temporal variations in RANKL function. Alternatively RANKL could mediate different signals depending on the context in which it is ligated, thereby having different effects on tolerance and immunity. However, no other costimulatory molecule mediates such a dual role in T cell functioning.

Here it is suggested that the anti-RANKL antibody used in the experiments described in chapter 4 mediates a signal to the T cell upon RANKL binding. Similar to other TNFR family members, RANKL has been shown to have the potential to reverse-signal upon RANK ligation (Chen et al., 2001; Wong et al., 1997b). Here it is suggested that these signals reinforce signal 1, and hence provide a mechanism by which anti-RANKL treatment could enhance both tolerance and immunity. Such a signal would in essence be in opposition to PD-1 mediated signals which effectively functions to dampen TCR mediated signalling, and prevents activation by weak TCR ligands. RANKL:RANK could function in opposition to these signals, and function to potentiate TCR mediated signals. *In vivo* this would have two functions, firstly enhancing T cell responses to weak TCR ligands and thereby aiding in the initiation of an immune response to a weak stimulus. Secondly, outside an inflammatory context, RANKL mediated signals could aid in the establishment of tolerance to peripherally expressed self-peptides. The mechanism by which ligation of RANKL might achieve this requires further investigation, because this anti-RANKL antibody could be used to enhance Ag-specific therapies for tolerance induction. Moreover, it is important to elucidate the function of this new costimulatory molecule and outline the reverse-signalling initiated within the T cell.

**PD-1 and RANKL in Peptide-induced tolerance**

It is clear that peptide-induced tolerance is an active process; following peptide treatment T cells transiently expand before being deleted (Kearney et al., 1994; Liblau et al., 1996). This initial T cell priming occurs despite limited expression of
costimulatory molecules by DC (Hochweller and Anderton, 2005). Thus the dialogue between the T cell and DC during the induction of tolerance is limited but sufficient to induce T cell expansion. The rapid up-regulation of RANKL during peptide induced T cell tolerance, but not immunity, suggests this molecule plays an important role in environments of limited costimulation. Here, it is suggested that ligation of RANKL mediates reverse-signalling to the T cell which positively feeds back on the TCR-p:MHC interaction and enhances it. PD-1 appears to play no role in the early signalling events during the induction of peptide tolerance (Figure 7.1). However, in T cells with a low affinity for peptide, PD-1 signalling may well completely prevent T cell activation and therefore the induction of both tolerance and immunity in these cells (Freeman et al., 2000; Keir et al., 2007). It is important to consider that both RANKL:RANK and PD-1:PD-L1 mediate two-way signals, to both the DC and the T cell. Therefore the roles of RANK and PD-L1 in peptide tolerance should be determined, especially the role of PD-L1 considering its expression on both T cells and DC and importantly the demonstration that it mediates a negative costimulatory signal (Butte et al., 2007). Forward- and reverse-signalling upon receptor-ligand binding highlights the complexity of costimulatory signals. A network of signals is formed which finely controls the activation of T cells, these consist of both positive and negative costimulatory signals. It would be of interest to determine whether such a network was at all quantifiable, whether receipt of a specific number of positive signals allows full T cell activation. Therefore it would be possible to determine a threshold for costimulation, above which immunity is induced and below which tolerance is induced.
Figure 7.1. Anti-RANKL and anti-PD-1 in tolerance and immunity.

a. During the induction of tolerance PD-1 mediates a negative signal to the T cell whereas RANKL mediates a positive signal to the T cell. Administration of anti-RANKL enhances the positive signal mediated by RANKL leading to better tolerance. Administration of anti-PD-1 disrupts PD-1 signals, removing the negative signal; however this has no effect on tolerance induction.

b. During the induction of immunity PD-1 mediates a negative signal to the T cell whereas RANKL mediates a positive signal to the T cell. Administration of anti-RANKL enhances the positive signal mediated by RANKL leading to greater clonal expansion and enhanced T cell immunity. Administration of anti-PD-1 disrupts PD-1 signals, removing the negative signal also resulting in greater clonal expansion and enhanced T cell immunity.
7.2 Signal 1

Studies have used APL in therapeutic tolerance models (Nicholson et al., 1995), some have even been translated into the clinic; though they have not met with success (Bielekova et al., 2000; Kappos et al., 2000). In the studies presented in this thesis the mechanism by which an APL of the Ac1-9 peptide of MBP induces tolerance was examined (Chapters 5 and 6). This peptide, 4Tyr, has a high affinity for MHC, and is a good tolerogen; inducing long lasting tolerance to the Ac1-9 peptide in both TCR transgenic mice (Burkhart et al., 1999) and wild-type mice (Metzler and Wraith, 1999). It was necessary to determine the mechanism by which this peptide induces tolerance following systemic administration, and in doing so ascertain its suitability for translation to the clinic. APL with altered affinities for the MHC could potentially be ideal therapeutics. Unlike APL which have altered TCR contacts, APL with altered MHC contacts should have the same effect on all peptide-reactive T cells; therefore not generating the problems with T cell fine-specificity seen when using APL with altered TCR contacts (Anderton et al., 1999; Anderton et al., 1998).

The data presented in this thesis have shown that 4Tyr induces a profound degree of tolerance to Ac1-9, but that this is achieved through a novel non-deletional mechanism. Peptide induced T cell tolerance has been frequently associated with deletion of peptide-reactive T cells (Kearney et al., 1994; Liblau et al., 1996; Pape et al., 1998). 4Tyr did not induce tolerance by such a mechanism, instead peptide-reactive T cells were seen to persist and retain the ability to make IL-2. This novel mechanism reflected the ability of 4Tyr to persist in a biologically relevant form, bound to the MHC, for up to 14 days after its administration; leading to chronic stimulation of the peptide-reactive T cells. A common outcome following repeated stimulation of T cells with Ag is the increased production of IL-10 (Broere et al., 2008; Burkhart et al., 1999; Kohyama et al., 2004). Production of IL-10 has also been reported upon repeated stimulation of Th1 cells (O'Garra and Vieira, 2007). Repeated stimulation of the TCR with lower doses of Ag has been associated with expression of foxp3 in Ag-reactive T cells (Apostolou and von Boehmer, 2004; Kretschmer et al., 2005). This has led to the proposition that repeated exposure of a
T cell to low levels of Ag induces foxp3 in Ag-reactive T cells, but repeated exposure to higher levels induces IL-10 production from Ag-reactive T cells. Tolerance induced by 4Tyr does not fall into either category because, although to induce tolerance a high dose of peptide is administered, the concentration of 4Tyr would decline over time, leaving by day 14 a very low density of 4Tyr:MHC complexes on the surface of DC. This was demonstrated by data showing that when Tg4 T cells were transferred 4 days or 7 days after 4Tyr treatment, the density of 4Tyr:MHC was such that deletional tolerance was induced. Moreover, when Tg4 cells were transferred 14 days after 4Tyr administration, the density of 4Tyr:MHC complexes was insufficient to induce tolerance. Such data generate a better understanding of peptide induced tolerance and show that the T cell tolerance induced is not only dependent on the time over which peptide-reactive T cells are stimulated (chronic versus limited stimulation) but also the density of p:MHC complexes on the DC. This allows for a quantitative model of peptide induced T cell tolerance to be proposed (Figure 7.2). The dose of peptide, and therefore the density of p:MHC complexes, which leads to deletion versus persistence of tolerant T cells could be determined. Continuing studies should examine how the density of peptide can influence tolerance to self-peptides in the steady-state. If tolerance to self-peptides can be mediated by different mechanisms (deletion versus foxp3 versus IL-10), how different peptides drive this and specifically which peptides drive the different mechanisms and the biochemical processes which control this would be important to establish. Thereby determining the optimal p:MHC kinetics for the various mechanisms of tolerance induction.

Data presented here show that 4Tyr:MHC complexes are displayed on CD11c+CD4+ DC during tolerance induction. This is probably due to the location of these DC within the spleen. However it remains possible that CD11c+CD4+ DC could be specifically programmed for tolerance induction. Other subsets of DC have been shown to be responsible for specific tasks during Ag presentation; 33D1+ DC preferentially process peptides for presentation on MHCII, DEC205+ DC preferentially process peptides for presentation on MHCI, and CD103+ DC from the gut, and CD8+CD205+ splenic DC have been shown to specialise in the induction of
foxp3 (Coombes and Powrie, 2008; Dudziak et al., 2007; Yamazaki et al., 2008). Thus, although a quantitative model of peptide tolerance induction has been suggested this does not exclude qualitative effects; mediated, for example, by the subset of DC on which the peptide is presented.

The persistence of tolerant Tg4 cells following 4Tyr treatment allowed the phenotype of these cells to be examined. In line with other studies, (Chiodetti et al., 2006; Dubois et al., 1998; Srinivasan and Frauwirth, 2007; Tanchot et al., 1998), data presented here show that persisting tolerant Tg4 cells cannot mediate calcium signalling following TCR ligation. As this defect was only manifest in the absence of excess calcium, these data highlight an interesting, and previously un-described, mechanism of tolerance; T cell responsiveness could be altered by changes in cell membrane potential. Differences in the expression of ion channels used to establish membrane potential has been noted when comparing naïve and memory T cells (Chandy et al., 2004). Therefore, altered expression of ion channels, and/or inhibitors of these channels could well be employed as a mechanism by which tolerance is maintained. The data presented here reiterate those shown by other studies, that tolerance induction alters only a few signalling pathways in T cells and does not globally alter all the signalling capabilities of a T cell.

Does the use of APL with high affinity for MHC have an application to the clinical setting? Firstly it would be very difficult to create disease-relevant peptides which have an extremely high affinity for MHC in an out-bred human population. Such a therapy may only be suitable for patients with specific HLA-haplotypes. Perhaps a more important consideration is the data showing that 4Tyr is maintained in a biologically relevant form for 14 days after administration. During this 14 days tolerance is induced in a mouse housed in a clean animal unit, but what if people were administered such a persisting peptide? Previous data from this laboratory has shown that administration of LPS, up to three days after 4Tyr administration can overcome tolerance induction and lead to T cell priming (Konkel and Anderton, unpublished observations). Such data would advise against the use of peptides with
high affinity for MHC in the clinic, as infection, with concomitant TLR-stimulation would result in induction of T cell immunity against the administered peptide.

Figure 7.2. Quantitative Model: manifestations of tolerance following induction by administration of peptide.
Data presented in this thesis and that from other studies suggests different manifestations of tolerance in T cells following peptide treatment are due to the following mechanisms; a, Repeated encounter of a T cell with DC presenting decreasing cognate p:MHC complexes over time results in the generation of tolerant T cells which persist and have the capacity to only make IL-2. b, Minimal repeated encounter of a T cell with DC presenting cognate p:MHC results in deletion of peptide-reactive T cells. c, Repeated encounter of a T cell with DC consistently presenting high levels of cognate p:MHC complexes over time results in the generation of IL-10 producing Tr1 cells. d, Repeated encounter of a T cell with DC consistently presenting low levels of cognate p:MHC complexes over time results in the generation foxp3+ T cells.
8. References


Chen, A.I., McAdam, A.J., Buhlmann, J.E., Scott, S., Lupher, M.L., Jr., Greenfield, E.A., Baum, P.R., Fanslow, W.C., Calderhead, D.M., Freeman, G.J., Sharpe,


regulatory T cell function within the context of experimental autoimmune encephalomyelitis. Vet Immunol Immunopathol 87, 357-372.


Ouyang, W., Ranganath, S.H., Weindel, K., Bhattacharya, D., Murphy, T.L., Sha, W.C., Murphy, K.M., 1998. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity 9, 745-755.


258


Rogers, P.R., Song, J., Gramaglia, I., Killeen, N., Croft, M., 2001. OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. Immunity 15, 445-455.


Smith, K.M., McAskill, F., Garside, P., 2002. Orally tolerized T cells are only able to enter B cell follicles following challenge with antigen in adjuvant, but they remain unable to provide B cell help. J Immunol 168, 4318-4325.


cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. Nature 408, 600-605.


Figure A.1. Anti-PD-1 enhances in vitro responses of CD8+ T cells to a greater extent than CD4+ T cells.
Splenocytes from C57BL/6 mice were stained with CFSE and cultured on plates coated with 8µg/ml anti-CD3. 50µg/ml of anti-PD-1 (black bar) or isotype control (white bar) was added to each culture well. 72 hours later CFSE dilution was determined by flow cytometry. The percent of a, CD4+ T cells or b, CD8+ T cells in each generation was calculated manually as described in materials and methods. Asterisk represent significant differences; *p<0.05, **p<0.0074, ***p<0.0001, as determined by unpaired T test. c-d, Tables showing the percentages of divided or undivided cells and the fold difference (division of one value by the other) or Δ (subtraction of one value from the other) between anti-PD-1 and isotype treated groups.
Error bars represent SEM and data is from 1 experiment with 3-4 wells per culture condition.
Figure A.2  Anti-RANKL enhances tolerance induced by 500µg pOVA.
Pooled LN responses from experiment described in Figure 4.2. Bar chart showing a, OT-II cells in the LN as a percentage of all CD4+ T cells and b, total number of OT-II cells in the LN. Ex vivo c-d, IL-2 and e-f, IFNγ production from LN cell cultures to 100µM pOVA. Figures c and d are the same graph; d has a smaller scale and does not include the PBS treated group. Figures e and f are the same graph; f has a smaller scale and does not include the PBS treated group.
Error bars represent SEM and data is representative of 4 experiments with 3-4 mice per group.
### a.

<table>
<thead>
<tr>
<th>Expression on anti-RANKL treated compared to isotype control treated T cells</th>
<th>Unstimulated cultures</th>
<th>Stimulated cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCRβ</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CD69</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CD62L</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CD44</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CD25</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CD28</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>OX40</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>PD-1</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>RANKL</td>
<td>n.d.</td>
<td>decreased</td>
</tr>
<tr>
<td>PD-L1</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Foxp3</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

### b.

<table>
<thead>
<tr>
<th>Expression on anti-RANKL treated compared to isotype control treated DC</th>
<th>Unstimulated cultures</th>
<th>Stimulated cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHCII</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CD80</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CD86</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>PD-L1</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>PD-L2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

**Figure A.3. The effect of anti-RANKL on the molecular expression of cultured splenocytes.**

Splenocytes from OT-II mice were cultures with 10μM of pOVA plus 10μg/ml of anti-RANKL or isotype control. The expression of various molecules was determined after 48, 72 and 96 hours of culture by flow cytometry. In separate experiments splenocytes from C57BL/6 mice were cultured with 2μg/ml anti-CD3 plus 10μg/ml of anti-RANKL or isotype control. The expression of various molecules was determined after 48, 72 and 96 hours of culture by flow cytometry. Tables show overall differences in staining between anti-RANKL treated and isotype treated cultures on a, CD4+ T cells and b, CD11c+ DC from both pOVA and anti-CD3 stimulated cultures; n.d. represents no difference. Data is representative of 2 experiments in which cultures were stimulated with pOVA and 2 experiments in which cultures were stimulated with anti-CD3.
Figure A.4. Anti-RANKL ablates LPS primed T cell immunity.

1-2x10^6 Ly5.1+OT-II cells were transferred to C57BL/6 mice 1 day prior to treatment with 500μg pOVA+30μg LPS i.v. At the same time as peptide treatment mice also received 250μg of anti-RANKL or isotype control i.p. Mice were sacrificed 7 days later. a, OT-II cells as a percentage of all CD4+ cells in the spleen. Ex vivo b, IL-2 production and c, proliferation from splenocytes to increasing concentrations of pOVA.

Error bars represent SEM and significant difference was determined by unpaired T test. Data is from 1 experiment with 2 mice per group.
Figure A.5. DC depletion after 3 doses of DTx i.p.
B10.PLxDOG mice were treated with 4Tyr or PBS i.v. and DC depleted by administration of 3 doses of ~22ng DTx/g of body weight given i.p. (as in Figure 5.14). Two days after the last DTx dose mice were sacrificed and spleens sampled, the populations of DC in the spleen were determined by flow cytometry. **a**, Depletion of pDC populations in the spleen. **b**, Depletion of conventional DC in the spleen. Plots are representative of 2 experiments with 2 mice per group.
Figure A.6. Tyrosine phosphorylation of stimulated tolerant and effector Tg4 cells.
2x10^6 Tg4 cells were transferred to B10.PL mice 1 day prior to treatment with 4Tyr or 4Tyr+LPS i.v. Mice were sacrificed 4 days later and splenocytes sorted by FACS into CD4+Ly5.1- cells (Host) and CD4+Ly5.1+ cells (Tg4). Equal numbers of sorted Tg4 or host cells were stimulated (+) by cross-linking CD3 or left unstimulated (-), for 5 minutes before cell lysis. Phospho-tyrosine levels were detected by Western blot. Similar loading was assured by re-probing the blot for β-actin. Blot is from 1 of 2 consistent experiments.