An Investigation of the Impact of CD4⁺CD25⁺ Regulatory T Cells on Immune Responses Induced In Vivo.

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A Thesis Submitted to Cardiff University in Candidature for the Degree of Doctor of Philosophy

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August 2007
Declaration

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed .......................................... (Candidate) Date 10/12/07

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This thesis is being submitted in partial fulfilment of the requirements for the degree of PhD.

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This thesis is the result of my own independent work/investigation, except where otherwise stated. Other sources are acknowledged by explicit references.

Signed .......................................... (Candidate) Date 10/12/07

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“Anybody who has been seriously engaged in scientific work of any kind realises that over the entrance to the gates of the temple of science are written the words: 'Ye must have faith.'”

Max Planck

Dedication

To Chris, who put up with me during my PhD, and still wanted to marry me.
Acknowledgements

Good questions and well explored answers are the basis for any thesis and there is fine art to both. Luckily I have had many people to share ideas and formulate theories with over the past 3 years, and it gives me great pleasure to have the opportunity to thank them now.

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Abstract

CD4⁺CD25⁺ regulatory T cells (Treg) are known to inhibit T cell responses; however their site of action and whether they suppress other immune responses has not been well characterised. Using a model of tumour rejection involving a melanoma cell line expressing Fas ligand (B16FasL) the effect of Treg on these responses has been studied. NK cells and macrophages were found to play a critical role in rejection of B16FasL. Depletion of Treg enhanced tumour rejection, whilst adoptive transfer of Treg inhibited tumour rejection, indicating that Treg suppress innate immune responses. Experiments performed to identify the Treg target indicated that Treg inhibit the cytolytic activity of NK cells. Furthermore, depletion of Treg enhanced the inflammatory infiltrate within 24 hours, which consisted of elevated numbers of neutrophils, indicating that Treg not only inhibit innate immune responses, but also act rapidly.

Another aim of this project was to identify whether Treg act in lymphoid organs or at the tumour site. T cells from transgenic mice, in which lymph node homing receptor CD62L expression is maintained upon activation (LΔP), were predicted not to enter inflamed tissue. However, experiments showed that T cells from LΔP mice could enter inflamed lungs following influenza infection. Characterisation of memory T cell responses indicated that LΔP mice exhibit delayed viral clearance, despite comparable cell numbers, cytolytic activity, and levels of CD107a and IFNγ. Control transgenic mice in which CD62L can be shed, showed no defect in viral clearance indicating that inability to shed CD62L in LΔP mice did not affect T cell migration but compromised anti-viral immunity. Although not suitable to study location of Treg action, this data indicated that CD62L shedding is important for memory T cell responses.
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- Neutrophils are Able to Lyse B16FasL In Vitro
- Histological Differences Between Isotype Control and Anti-CD25+ Depleting Antibody Treated Mice
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</thead>
<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>AICD</td>
<td>activation induced cell death</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>B16FasL</td>
<td>B16-F10 transfected with Fas Ligand</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine monophosphate</td>
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<tr>
<td>CFSE</td>
<td>carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
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<td>CLA</td>
<td>cutaneous lymphocyte antigen</td>
</tr>
<tr>
<td>CTL</td>
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</tr>
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</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
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<td>DN</td>
<td>double negative</td>
</tr>
<tr>
<td>DP</td>
<td>double positive</td>
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<tr>
<td>DTH</td>
<td>delayed type hypersensitivity</td>
</tr>
<tr>
<td>DTR</td>
<td>diphtheria toxin receptor</td>
</tr>
<tr>
<td>E-NTPDase</td>
<td>ectonucleoside triphosphate diphosphohydrolases</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain protein</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas Ligand</td>
</tr>
<tr>
<td>Flu</td>
<td>influenza virus</td>
</tr>
<tr>
<td>(E)GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GITR</td>
<td>glucocorticoid-induced TNFR-related protein</td>
</tr>
<tr>
<td>GITRL</td>
<td>GITR ligand</td>
</tr>
<tr>
<td>GVHD</td>
<td>graft-vs.-host disease</td>
</tr>
<tr>
<td>HA</td>
<td>haemagglutinin</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>haematoxylin and eosin</td>
</tr>
<tr>
<td>HEV</td>
<td>high endothelial venules</td>
</tr>
<tr>
<td>IDO</td>
<td>indoleamine 2,3-dioxygenase</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>Interleukin-8 receptor</td>
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<td>IFNγ</td>
<td>Interferon gamma</td>
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<td>IEL</td>
<td>Intraepithelial lymphocytes</td>
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<td>i.n.</td>
<td>intranasal</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
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<td>LdLN</td>
<td>lung draining lymph node</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LFA-1</td>
<td>lymphocyte function-associated antigen 1</td>
</tr>
<tr>
<td>MAdCAM-1</td>
<td>mucosal addressin cell adhesion molecule-1</td>
</tr>
<tr>
<td>MFI</td>
<td>geometric mean fluorescence intensity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<td>-------------</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inhibitory protein</td>
</tr>
<tr>
<td>mLN</td>
<td>mesenteric lymph nodes</td>
</tr>
<tr>
<td>MLV</td>
<td>multi-lamella vesicles</td>
</tr>
<tr>
<td>NA</td>
<td>neuraminidase</td>
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<tr>
<td>NF-AT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NOD</td>
<td>non-obese diabetes</td>
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<tr>
<td>NP68</td>
<td>peptide from flu nucleoprotein (NP&lt;sub&gt;366-374&lt;/sub&gt;)</td>
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<td>pathogen associated molecular patterns</td>
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<td>peripheral blood mononuclear cells</td>
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<td>pLN</td>
<td>peripheral lymph nodes</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear</td>
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Chapter 1 - Introduction

The immune system has evolved to protect the host from a wide range of pathogens and it comprises an innate and adaptive immune arm. Cells of the innate immune system respond immediately to signals induced upon injury. Innate immune cells express germline encoded receptors capable of recognising pathogen associated molecular patterns (PAMPs), which upon ligation by microbial products activate cells and induce cytokine/chemokine secretion, triggering effector cells to degranulate, and attack invaders (reviewed in (Pasare and Medzhitov 2005; Bianchi 2007; Trinchieri and Sher 2007). Although innate immune responses are effective first lines of defence in limiting pathogen dissemination, an aggressive pathogen may not be eliminated and the innate immune response may cause extensive damage to the host through continual release of toxic mediators.

The adaptive immune system has evolved to facilitate pathogen clearance and includes development of memory responses capable of more rapid clearance upon a second encounter with pathogen. Although initiation of adaptive immune responses (principally T and B cells) takes longer, the clearance of invading pathogens is directed in a highly antigen specific manner. Unlike innate immune cells, adaptive immune cells use DNA recombination to generate both the T cell receptor (TCR) and the B cell receptor (BCR), from a selection of germline encoded genes, during cell development (reviewed in (Goldrath and Bevan 1999; Nemazee 2000). It is estimated that $10^{18}$ different TCR can be generated in this way, each with the potential to recognise pathogen-derived antigens; however receptors recognising host antigens could also be generated during this process (Janeway et al. 2001) pg 138).
Excessive immune responses to pathogens, inappropriate responses to environmental antigens and auto-immune responses can be extremely detrimental to the host and the immune system has evolved mechanisms to regulate these responses. In particular, the regulation of T cell responses has been extensively studied. The development of T cells in the thymus is an intricate process during which cells are selected for the ability of their TCR to bind with low affinity the major histocompatibility complex (MHC) in association with peptides derived from host proteins (self-peptides) (Janeway 1994; Fink and Bevan 1995; Starr et al. 2003). This process is required in order that T cells can recognise MHC in the periphery, however there is the potential to produce self-reactive T cells. The majority of T cells binding MHC:self-peptide with high affinity are deleted in the thymus (Kappler et al. 1987; Ramsdell and Fowlkes 1990; Nossal 1994) and can also be induced to undergo apoptosis/become anergic in the periphery upon contact with self-antigen in the absence of costimulation (Rocha and von Boehmer 1991). Although these mechanisms limit the number of self-reactive T cells in the periphery, they are imperfect and sub-populations of T cells that are capable of actively suppressing T cell responses have been identified (Gershon and Kondo 1970; Sakaguchi et al. 1995).

1.1. Immunosuppressive T Cells

Evidence for a population of T cells involved in immunosuppression was initially gathered during the 1970s. Gershon et al reported that a population of thymocytes was involved in maintaining peripheral tolerance, and this population was termed suppressor T cells (Ts) (Gershon and Kondo 1970; Gershon and Kondo 1971; Gershon et al. 1972; Gershon et al. 1974; Gershon 1975). It was discovered that removal of the thymus from mice at day 3 of life induced widespread autoimmunity later in life, whereas day 7 thymectomy did not. Furthermore, thymus transplant or injection of T cells from adult mice before day 14 of life prevented autoimmune disease, suggesting that a population of
T cells with the ability to suppress autoimmune T cells, emigrated out of the thymus after
day 3 but before day 7 (Kojima and Prehn 1981; Sakaguchi et al. 1985). Many other
studies over the following years characterised these cells further, clearly indicating that
they prevented autoimmunity (Cohen and Wekerle 1973; Penhale et al. 1976; Cooke et
al. 1978; Muraoka and Miller 1980) and suppressed responses in an antigen specific
manner (Tada and Takemori 1974; Weinberger et al. 1979; Smith and Howard 1980;
Green et al. 1983). It was originally proposed that Ts cells mediated their biological
functions via soluble, antigen-specific factors containing a region of the MHC (termed I-J),
however, despite numerous attempts, molecular studies failed to identify a
Corresponding I-J gene within the MHC. Unfortunately, few researchers pursued Ts cells
and the field was abandoned.

It wasn't until the mid 1990s that the field of immunosuppressive T cells was
rediscovered. With more sophisticated experimental tools, the initial experiments with
mice thymectomised on day 3 of life were repeated. These mice developed widespread
autoimmune disease that could be rescued by adoptive transfer of CD4+ T cells from
normal adult mice. A significant advance was the identification of the population
containing the T cell suppressive capacity as CD4+ T cells coexpressing the IL-2 receptor
alpha chain, CD25 (Sakaguchi et al. 1995; Suri-Payer et al. 1996). Adoptive transfer of
CD4+CD25- T cells (Asano et al. 1996) or CD25+ T cell-depleted mature thymocytes
(Itoh et al. 1999; Seddon and Mason 2000) into syngeneic T cell deficient mice caused
similar autoimmune disease which could be inhibited by cotransfer of CD4+CD25+ T
cells. Further in vitro analysis revealed this population was capable of suppressing clonal
(Suri-Payer et al. 1998) and polyclonal CD4+ T cell responses and suggested suppression
was mediated in a contact-dependent fashion, requiring activation through the TCR in
order to become suppressive (Thornton and Shevach 1998; Thornton and Shevach 2000). Since these cells are present in normal naïve mice and exit the thymus functionally mature, they are frequently referred to as naturally occurring CD4⁺CD25⁺ regulatory T cells (Treg).

1.2. Naturally Occurring CD4⁺CD25⁺ Regulatory T Cells

1.2.1. Phenotype and Function

CD4⁺CD25⁺ T cells comprise 5-10% of both human and mouse peripheral CD4⁺ T cells. Although in naïve mice the vast majority of this population shows suppressive capacity, in humans (and in mice undergoing an active immune response) this is less clear cut, as activated conventional T cells also express CD25. Various studies have identified those cells expressing the highest levels of CD25 as Treg in humans, which represent 1-3% of peripheral CD4⁺ T cells, although differentiating between high and low expression is still subjective (Baecher-Allan et al. 2001).

Besides CD25, a range of other molecules have been detected on the surface of Treg, particularly CD62L (L-selectin), CD103 (αE integrin subunit), GITR (glucocorticoid-induced TNFR-related protein) and CTLA-4 (cytotoxic T lymphocyte associated antigen-4) and high levels of CD45RB. These markers aided more specific characterisation of Treg and their function. Using combinations of these markers investigators reported that not only do Treg inhibit autoimmune disease caused by CD4⁺CD25⁻ T cells (Groux et al. 1997; Read et al. 2000), they also inhibit responses in murine models of allergy, and parasite infection (Belkaid et al. 2002), indicating that their activity may not be confined to suppression of self-reactive T cells.
Numerous reports indicated that suppression *in vitro* is contact dependent and could not be blocked by anti-IL-10/TGFβ blocking antibodies. However, in many *in vivo* experimental models there is a requirement for Treg to produce IL-10 (Belkaid *et al.* 2002) or TGFβ, although some reports have suggested that the source of TGFβ is not Treg (Fahlen *et al.* 2005; Kullberg *et al.* 2005). The importance of these cytokines is discussed later. *In vitro* Treg do not proliferate in response to TCR stimulation (are anergic) unless supplied with high concentrations of IL-2 (Thornton and Shevach 1998), although TCR stimulation is reported to be required for suppression of both CD4+ and CD8+ T cell responses (Piccirillo and Shevach 2001). However, reports also indicate that Treg are not anergic *in vivo*, and that IL-2 is not involved (Walker *et al.* 2003a). Resolving the differences between *in vitro* and *in vivo* work has proved difficult without a unique marker for Treg.

### 1.2.2. FOXP3

More recently, the transcription factor FOXP3 (also known as Scurfin) has been shown to be involved in the function of Treg. Studies of the scurfy mouse strain, which develop similar disorders to day 3 thymectomised mice (Godfrey *et al.* 1991; Blair *et al.* 1994; Clark *et al.* 1999), identified a mutation in the X-linked recessive gene *Foxp3*, encoding a forkhead-winged-helix transcription factor (Brunkow *et al.* 2001). In humans with X-linked recessive disease, IPEX, who suffer from multi-organ autoimmune diseases, allergy and IBD, mutations were also identified in the *Foxp3* gene (Wildin *et al.* 2001; Wildin *et al.* 2002). Initially it was hypothesised that FOXP3 was a repressor of transcription which regulated T cell activation and therefore limited immunopathology (Schubert *et al.* 2001). However it was not until 2003 that a link between FOXP3 and Treg was confirmed.
Expression of *Foxp3* mRNA was identified in mouse CD4+CD25+CD8- thymocytes and peripheral CD4+ T cells predominantly, though not exclusively, in the CD25+ population (Hori *et al.* 2003). Forced expression of *Foxp3* in T cells using retroviral vectors and IL-2, upregulated CTLA-4, GITR and CD103 and resulted in a population that failed to proliferate and produce cytokines in response to TCR stimulation. Importantly, these cells were capable of suppressing CD4+CD25+ T cell proliferation, in a contact-dependant manner and required TCR stimulation in order to do so. Furthermore, these cells were able to inhibit IBD when cotransferred with disease causing CD4+CD25+CD45RBhi T cells into SCID mice. These findings were supported by two reports published shortly after, in which retrovirally transduced CD4+CD25+ T cells and T cells from *Foxp3* transgenic mice were utilised respectively (Fontenot *et al.* 2003; Khattri *et al.* 2003). Further studies using a reporter allele of *Foxp3* indicated that only cells that expressed FOXP3 had regulatory activity, and that conventional T cells did not upregulate FOXP3 upon activation (Fontenot *et al.* 2005b).

The discovery of FOXP3 as a Treg marker enabled more accurate identification of Treg. It was confirmed that the majority of Treg in humans are CD25hi by RT PCR and there were two isoforms of FOXP3 identified (Allan *et al.* 2005). The recent availability of an anti-FOXP3 antibody has aided characterisation of other cell surface markers on Treg; however, the nuclear localisation of FOXP3 precludes staining of live cells, due to the requirement for permeabilisation, and therefore direct functional analysis.

Initially, human studies reported that FOXP3 was found only in CD4+CD25hi T cells that exert suppressive activity *in vitro*. Subsequent reports of FOXP3 expression in activated conventional T cells indicated that FOXP3 expression in humans is not confined to Treg, although expression generally correlated with anergy and regulatory activity (Walker *et
al. 2003b; Allan et al. 2005; Morgan et al. 2005). Prolonged TCR stimulation in the absence of costimulation, also leads to FOXP3 expression and acquisition of suppressor function (Walker et al. 2003b).

A recent study aimed to clarify the field by comparing stimulated CD4\(^+\)CD25\(^-\) T cells to CD4\(^+\)CD25\(^+\) T cells from 9 donors (Wang et al. 2007). In this study, CD4\(^+\)CD25\(^-\) T cells from all donors upregulated FOXP3 upon TCR stimulation, and were hyporesponsive to further stimulation, however only a third of these exhibited suppressive activity in vitro. The suppressive capacity correlated with the stability of FOXP3 expression, with FOXP3 maintained at high levels in the suppressive cells and rapidly lost in those cells unable to suppress. The emerging hypothesis is that transient FOXP3 expression attenuates effector function whilst sustained expression confers regulatory activity in human T cells.

This hypothesis is supported by initial reports describing FOXP3 as a factor that regulated T cell activation by inhibiting NF-AT mediated gene transcription (Schubert et al. 2001). NF-AT (nuclear factor of activated T cells), AP-1 (activator protein-1) and NF-\(\kappa\)B are induced upon TCR stimulation and lead to T cell activation and IL-2 gene transcription. The Foxp3 promoter region contains binding sites for both NF-AT and AP-1 (Mantel et al. 2006) and others have shown that FOXP3 directly interacts with NF-AT and NF-\(\kappa\)B, blocking their ability to induce IL-2, IL-4 and IFN\(\gamma\) gene transcription (Bettelli et al. 2005). The NF-AT-FOXP3 complex has also been shown to bind DNA and be required for Treg suppressive capacity and upregulation of the Treg associated markers CTLA-4 and CD25 (Wu et al. 2006).
The NF-AT family includes four closely related members; NF-ATc1, NF-ATc2, NF-ATc3 and NF-ATc4, of which NF-ATc2 and NF-ATc3 appear to be dispensable for Treg generation (Bopp et al. 2005). The results of a study on NF-ATc2/c3 double deficient mice showed that although mice displayed a phenotype similar to FOXP3 deficient mice, there was normal development of FOXP3^+ CD4^+ CD25^+ T cells. The phenotype was found to be due to conventional T cells being unresponsive to Treg mediated suppression. Since these mice display elevated levels of NF-ATc1 (Ranger et al. 1998a; Ranger et al. 1998b) it is unclear whether this family member compensates for the loss of the others or NF-ATc2 and NF-ATc3 are not required for the generation of Treg.

1.2.3. Other Types of Regulatory T Cell

The discovery that FOXP3 expression generates an immunosuppressive phenotype has facilitated the identification of other types of regulatory cells. Increasing evidence indicates that regulatory T cells can be induced from CD4^+ CD25^- T cells in the periphery, either upon stimulation amidst an immunosuppressive environment or by repeated antigen stimulation (Groux et al. 1997; Walker et al. 2003b). Immature Dendritic Cells (DC: 'professional' APC), thought to induce anergy or death in T cells recognising antigens expressed on their surface, have also been implicated in regulatory T cell induction (Jonuleit et al. 2000; Mahnke et al. 2003).

Stimulation of the TCR in the presence of TGFβ is also known to induce regulatory T cell phenotype in conventional CD4^+ T cells (Chen et al. 2003a), which requires CD28 and CTLA-4 signalling (Liu et al. 2006b; Zheng et al. 2006). TGFβ also induces a population of CD8^+ regulatory T cells although these cells are less well characterised (Rifa'i et al. 2004; Xystrakis et al. 2004; Zheng et al. 2004). The expression of FOXP3, however, is not always required to confer the ability to suppress immune responses as a
number of investigators have reported a population of FOXP3-negative T cells, secreting IL-10 that can inhibit immune responses \textit{in vitro} and \textit{in vivo} (Levings \textit{et al.} 2001; Vieira \textit{et al.} 2004).

\textbf{1.3. Development in the Thymus}

Conventional T cells (T\textsubscript{conv}) develop in the thymus from CD4\textsuperscript{+}CD8\textsuperscript{-} (double negative - DN) precursors. These precursors have generated a functional TCR\textbeta chain, paired with a surrogate TCR\textalpha chain, by the CD4\textsuperscript{+}CD8\textsuperscript{+} (double positive - DP) stage. Subsequent generation of a functional TCR\textalpha chain allows the precursor to be positively, then negatively selected based on interactions with MHC molecules in the thymus. DP T cells become apoptotic in the absence of stimulation through the newly formed TCR.

Recognition of MHC/self-peptide in the thymic cortex rescues the T cell and commits the cell to either the CD4\textsuperscript{+} or CD8\textsuperscript{+} T cell lineage. However the affinity of the TCR interaction with MHC/self-peptide must be low as T cells bearing a high affinity TCR are subsequently deleted by a process referred to as negative selection.

Similar to conventional T cells, Treg development requires MHC class II/self-peptide expression on thymic APC, in particular on thymic epithelial cells from the cortex (Bensinger \textit{et al.} 2001; Jordan \textit{et al.} 2001; Apostolou \textit{et al.} 2002). Studies of developing thymocytes in TCR transgenic mice, with different affinities for influenza virus haemagglutinin (HA), also expressing another transgene encoding HA, have indicated that CD4\textsuperscript{+}CD25\textsuperscript{+} thymocytes require a TCR with a higher affinity for self-peptide in order to develop into Treg, and that low affinity TCR do not induce Treg development (Jordan \textit{et al.} 2001; Apostolou \textit{et al.} 2002). This conclusion was corroborated in other TCR transgenic models such as the D011.10 model (Kawahata \textit{et al.} 2002). Investigators
have also reported an increase of Treg precursors in the presence of specific antigen at the DP stage of development, where these cells acquire the Treg markers CD25 and CTLA-4 (Fontenot et al. 2005b; Cabarrocas et al. 2006). Another report indicated that DP thymocytes from MHC class II deficient mice express FOXP3 and suppress severe colitis (Krajina et al. 2004). Together these reports suggest that the commitment to the Treg lineage is made early in T cell development, at the DP stage, possibly upon high affinity interaction of their TCR with MHC expressing self peptides (Figure 1.1A).

The latter stages of Treg development are more controversial. Given the high affinity of the Treg precursor TCR, it is unclear how these cells proceed through negative selection. An early report suggested that Treg precursors were more resistant to negative selection (Papiernik et al. 1998), however recent reports have indicated that Treg precursors expressing a TCR for a neo-self antigen are deleted at the single positive (SP) stage of development, since there is a reduction in proportion of Treg when compared to the DP stage (Caton et al. 2004; Cabarrocas et al. 2006). This suggests that only Treg with TCR affinities above that required for Treg induction and below that which causes deletion would persist. However, the existence of clonotypic Treg and Tconv cells in transgenic mice suggests that this hypothesis is oversimplified and suggests other signals are also involved in Treg lineage commitment (Figure 1.1B and C).
Figure 1.1. Schematic Representations of Possible Associations Between TCR Affinity and Treg Selection

Hypothesis (A) suggests high TCR affinity alone instructs Treg development. However the overlap of repertoire of Tconv and Treg suggests other factors are involved as in (B) and (C). Hypothesis (B) suggests this overlap may be due to the strength of TCR signal, possibly related to the concentration of TCR on the surface. Hypothesis (C) suggests that Treg may develop separately to Tconv and require different signals to differentiate.
1.3.1. The Role of FOXP3 in Treg Development

Since forced FOXP3 expression is sufficient to convert Tconv cells into a Treg phenotype, it has been suggested that FOXP3 is required for the development of Treg in the thymus (Fontenot et al. 2003; Hori et al. 2003), indeed FOXP3 expression is detected on DP thymocytes in the thymic medulla (Fontenot et al. 2005b; Cabarrocas et al. 2006). However whether or not FOXP3 expression commits developing T cells to the Treg lineage or is a result of such a commitment remains unclear.

In a bid to address this question, two groups of investigators tracked Treg development in mice where FOXP3 transcription could be studied in the absence of functional protein (Gavin et al. 2007; Lin et al. 2007). These studies inserted DNA encoding EGFP/GFP and a stop codon into the Foxp3 locus. Since Foxp3 is X-linked, male mice carrying the gene, referred to here as Foxp3gfp, developed autoimmune disease, whereas heterozygous females (Foxp3gfp/+ ) did not. X chromosome inactivation in these females lead to half the Treg bearing wild-type Foxp3, therefore studies of Treg development in Foxp3gfp/+ females allowed Foxp3gfp Treg analysis in healthy mice. Although Foxp3gfp Treg lacked the ability to suppress T cell responses; they expressed high levels of CD25 and CTLA-4, proliferated poorly and produced only minimal IL-2 suggesting that FOXP3 expression is not required for these characteristics.

Both studies reported a similar proportion of Foxp3gfp Treg in the thymus when compared to wild-type Treg at the DP stage of development, suggesting that FOXP3 expression is not required for CD25 and CTLA-4 upregulation, seen in the early stages of Treg development. However the proportion of Treg precursors at the SP stage was reduced in Foxp3gfp Treg suggesting that FOXP3 enhances Treg precursor survival upon maturation.
The expression of FOXP3 stabilises CD25 and CTLA-4 expression, confers suppressive activity, and functional FOXP3 is required to maintain Foxp3 transcription. This is supported by a report described earlier, where only stable expression of FOXP3 correlates with suppressive capacity in activated human conventional T cells (Wang et al. 2007). Together these reports support the hypothesis that positive selection of T cells with high affinity induces FOXP3 expression, which in turn allows some Treg precursors to escape deletion. The stability of FOXP3 expression might then determine the ultimate commitment to either the Treg or Tconv lineage, although how this might be controlled is unclear. The availability of antigen or level of TCR expression are factors that might affect signalling.

A similar, though less pronounced phenotype is evident in dicer-deficient Treg (Cobb et al. 2006). Deficiency in Dicer, the RNAse III enzyme that generates functional miRNAs (micro RNAs which post-transcriptionally regulate gene expression), results in lower precursor Treg numbers at the SP stage, a lower number of Treg in the periphery, and ultimately immune pathology. This defect was cell autonomous as generation of dicer-deficient Treg in a wild-type thymus did not rescue the phenotype. Dicer-deficiency also precluded induction of FOXP3 expression in CD4+CD25+ T cells by TGFβ, which may suggest that miRNAs are important for FOXP3 induction during naturally occurring Treg development in the thymus.

Depletion of Treg by targeting CD25 has been the most specific way of removing Treg from a healthy immune system for more than a decade (Onizuka et al. 1999). Although not a unique marker of Treg, depletion of CD25+ cells relieves immune suppression in a number of experimental models and in the majority of cases this does not cause severe autoimmune disease (Onizuka et al. 1999; Long et al. 2003; Benghiat et al. 2005;
Loughry et al. 2005). Although day 3 thymectomy does cause autoimmune disease it is less severe than in scurfy mice, which are deficient in FOXP3 and develop rapid, fatal autoimmune disease. Due to identification of other non-CD25 expressing regulatory T cells, it was hypothesised that these remaining regulatory cells were sufficient to control rampant autoimmune disease (Groux et al. 1997; Walker et al. 2003b). However the possible role of FOXP3 in the development of self-specific Treg led to the hypothesis that in the absence of FOXP3 those cells with high TCR affinity which would have become Treg are added to the autoreactive T cell pool, contributing to autoimmune disease. Another possibility is that Treg are required early in life to aid homeostatic expansion of Tconv and prevent autoimmunity at this stage.

A recent paper aimed to distinguish between these alternatives by generating mice in which depletion of FOXP3+ cells in vivo could be achieved using diphtheria toxin (Kim et al. 2007). Investigators inserted a construct encoding GFP fused to human diphtheria toxin receptor (DTR) into the untranslated region of Foxp3. Treatment of mice with diphtheria toxin targeted FOXP3 expressing cells resulting in specific depletion of Treg after 7 days treatment, with 97% depletion by day 2. Normal numbers of Treg recovered within 10-15 days once treatment was removed. The report indicated that Foxp3DTR mice, when treated with diphtheria toxin from birth, developed similar autoimmune disease to scurfy (Foxp3−/−) mice, suggesting that self-reactive T cells that failed to commit to the Treg lineage are not the major cause of autoimmunity in these mice. This conclusion is supported by another study using a similar model (Lahl et al. 2007). In adult mice, depletion of FOXP3+ cells resulted in an even more rapid induction of autoimmunity, also suggesting that autoimmunity is not simply due to a defect in homeostatic regulation that occurs early in life (Figure 1.2).
Figure 1.2. Does FOXP3 Deficiency Result in Increased Numbers of Autoreactive Tconv

(A) Represents the T cell repertoire in wild-type mice. (B) Represents the repertoire in mice depleted of Treg using anti-CD25 antibody, post Treg development. In FOXP3 deficient mice, do T cells that fail to commit to the Treg lineage and therefore contribute to autoimmunity as in (C), or do they fail to survive as in (B).
1.3.2. IL-2 and IL-2R

IL-2 has often been implicated in Treg development and function as mice deficient in either IL-2, IL-2Rα (CD25) or IL-2Rβ (CD122) develop wide ranging autoimmunity similar to FOXP3 deficient mice (Malek and Bayer 2004), and neutralising anti-IL-2 antibody has a similar effect (Setoguchi et al. 2005). Recent studies using Foxp3 reporter mice have indicated that IL-2 and IL-2Rα deficient mice do generate FOXP3+ thymocytes although in substantially reduced numbers (Fontenot et al. 2005a). These Treg were equally able to suppress T cell responses when compared to wild-type Treg in vitro, suggesting that IL-2 signalling is not required for Treg function. The few Treg that are generated in these mice are attributed to other cytokines signalling through the IL-2 receptor common γ-chain (Fontenot et al. 2005a), as mice deficient for this receptor chain have a complete lack of Treg, suggesting that FOXP3 expression may require some signalling through this receptor.

Since Tconv cells found in IL-2Rα deficient mice display an activated phenotype and therefore could produce cytokines able to signal through this receptor, the authors aimed to reduce inflammatory environment by generating mixed bone marrow chimeric mice in which no autoimmunity was evident (Fontenot et al. 2005a). In these experiments, T cell-depleted CD45.1+ (wild-type) and CD45.1- bone marrow (consisting of IL-2Rα+/+ IL-2Rα-/- cells mixed at a ratio of 1:1) was used to reconstitute lethally irradiated wild-type (CD45.1+) mice. In mice receiving a mixture of IL-2Rα+/+ and wild-type bone marrow, IL-2Rα+/+ Treg were equally able to compete with wild-type Treg, however in mice receiving IL-2Rα-/+ and wild-type bone marrow, IL-2Rα-/+ Treg only represented a small proportion of SP thymocytes and an even smaller proportion of lymph node cells,
indicating that in the absence of an inflammatory environment IL-2Rα⁻ Treg are impaired in their survival. Together this data suggests that although IL-2 signalling is not required for FOXP3 expression and Treg function, signalling through its receptors is important for homeostasis. Interestingly, a recent report indicated that a single amino acid deletion in the leucine zipper region of FOXP3 prevented oligomerisation disrupting the association with the IL-2 promoter and limiting repression of \textit{IL-2} transcription (Li \textit{et al.} 2007).

### 1.4. Activation

Early \textit{in vitro} experiments indicated that Treg activation required stimulation through the TCR; however a polyclonal population of naïve Treg can inhibit antigen-specific effector cells within 7 days of adoptive transfer. These observations lead to the hypothesis that Treg are specific for self antigen, which is supported by the data gathered on Treg development in the thymus.

Another report has indicated that Treg cells can share TCR specificity with Tconv (Hsieh \textit{et al.} 2006). This study utilised mice bearing a non-variable TCRβ chain thereby limiting TCR repertoires enough to study variability by sequencing. Comparisons between TCRα chains on Treg vs. Tconv indicated that there was some overlap. The study also reported that T cells transduced with Treg TCRs proliferated more than those transduced with Tconv TCRs upon adoptive transfer into normal mice, suggesting that Treg TCRs, as a population are more self-reactive. The TCR repertoire of activated T cells isolated from FOXP3 deficient mice, which suffer from severe autoimmune disease, is similar to that of Treg, again suggesting that at least a proportion of Treg are self-specific.
If Treg are activated by self-antigen it is possible that they are consistently activated *in vivo*. Supporting this hypothesis, one study isolated a population of CD4⁺CD25⁺CD134(OX-40)⁺ Treg from naïve rats that could inhibit responses *ex vivo* without additional stimulation (Nolte-'t Hoen *et al.* 2004). This population expressed activated T cell markers which suggested that they were recently activated *in vivo*. The authors also reported a CD134⁺ Treg population which expressed naïve cell markers and required stimulation through the TCR to become effective, which may suggest that these cells do not recognise self antigen, or they require additional stimulation.

However, the majority of reports indicate that exerting their suppressive effects *ex vivo* requires restimulation. An alternative hypothesis is that TCR stimulation by self antigens alone may not be sufficient for Treg activation and other signals may contribute. One paper reported that Treg require either IL-2 or IL-4, presumably from the targeted effector cells, as well as TCR stimulation in order to become suppressive (Thornton *et al.* 2004). However, the authors also reported that Treg suppression could be induced by IL-2 in the absence of TCR stimulation. It is possible that these Treg received this TCR stimulation *in vivo*, similar to those isolated by Nolte-'t Hoen *et al.*, but required an additional stimulatory signal, however it could suggest that Treg could be activated by a purely inflammatory environment.

In support of these hypotheses, Toll-like receptor (TLR) stimulation can also increase Treg proliferation and suppressive capacity (Caramalho *et al.* 2003). TLRs are germline encoded receptors that recognise PAMPs and certain endogenous molecules released by cells during inflammation. They are expressed on a wide range of cells and usually result in stimulation of immune responses. Treg have been shown to express TLR-4, -5, -7 and -
8, which principally bind LPS, flagellin, single stranded RNA and DNA. Furthermore TLR-4 stimulation with LPS could induce Treg proliferation in the presence of IL-2 and in the absence of APC. LPS stimulation also enhances the suppressive function of Treg in vitro, with LPS activated Treg being equally capable of limiting intestinal inflammation as freshly isolated Treg in vivo.

However, TLR-ligation on Tconv enhances TCR stimulation and TLR ligation on DC stimulates maturation and secretion of IL-6, which renders Tconv resistant to Treg suppression (Pasare and Medzhitov 2003). This apparent conflict is readily resolved with two observations. Firstly, the concentration of LPS (TLR-4 ligand) required to activate Treg is 3x higher than that required to activate DC (Banchereau and Steinman 1998), thus ensuring that a microbial infection can be controlled whilst endotoxic shock is limited by activation of Treg in higher amounts of LPS. Secondly, activation through TLR-2 induces proliferation of Treg with transiently impaired suppressive capacity (Sutmuller et al. 2006), suggesting that during the initial priming and effector phase of an immune response effector cells can proceed unheeded, with Treg controlling the contraction of the immune response.

Other reports focusing on activation by TCR stimulation have shown that Treg bearing identical TCR to their naïve Tconv counterparts require lower doses of cognate antigen in order to be activated (Takahashi et al. 1998). Indeed suppression is lost at higher doses (Stephens et al. 2005) suggesting that Treg suppression occurs when antigenic stimulation is low, preventing autoimmunity in weakly self-reactive Tconv.
Although the majority of reports have suggested that Treg bear self-reactive TCR, reports of Treg which inhibit immune responses to pathogens, such as *Leishmania*-specific regulatory T cells (Suffia *et al.* 2006), suggest that Treg can also be foreign antigen specific. Although it is possible that these regulatory T cells could be induced in the periphery, by adoptive transfer of Ly5.1⁺ Treg from naïve mice, the authors showed that the *Leishmania*-specific regulatory T cells in infected mice were derived from the naturally occurring Treg population.

1.5. Mechanisms of Suppression

The mechanisms by which Treg exert their suppression remain a subject of debate. Many studies have shown that suppression is contact dependent *in vitro*, as suppression was not evident in wells where cells have been separated by membranes. However, a number of studies have also indicated that cytokines such as IL-10 and TGFβ are involved in mediating suppression, particularly *in vivo*. In this section the molecules thought to be involved and their possible mode of action are discussed.

1.5.1. Cytotoxic T Lymphocyte Antigen 4

Spontaneous diabetes is exacerbated in CD80/CD86 deficient and CD28 deficient NOD mice, which was found to be due to a low number of Treg (Salomon *et al.* 2000). These molecules are usually associated with T cell costimulation and deficiency was postulated to prevent diabetes. The observation that Treg constitutively expressed the other CD80/CD86 ligand, Cytotoxic T Lymphocyte Antigen 4 (CTLA-4 or CD152), with its deficiency or blockade in normal mice leading to lymphoproliferative and organ-specific autoimmune disease similar to that in FOXP3 deficient mice, suggested a role for CTLA-
4 in the mechanism of Treg mediated suppression (Tivol et al. 1995; Waterhouse et al. 1995).

However, Treg isolated from CTLA-4 deficient mice appear to function normally in
vitro, inhibiting T cell proliferation, suggesting that CTLA-4 would not be the only
mechanism of suppression (Tang et al. 2004). Furthermore the suppressive effect
appeared to be mediated by IL-10. This result is supported by a recent study into the
effects of B7/CTLA-4 deficient Treg (Read et al. 2006). As well as similar
immunosuppressive function in vitro, CTLA-4 deficient Treg were also able to inhibit
colitis induced by wild-type Tconv. Interestingly, the mechanism employed by CTLA-4
deficient Treg differs from that employed by wild-type Treg as anti-CTLA-4 treatment,
which blocks wild-type Treg activity, could not block the suppressive effect of CTLA-4
deficient Treg, indicating that another mechanism may be employed. This experiment
also suggested that the anti-CTLA-4 antibody is not acting as costimulation on the
responding T cells as there is no enhanced proliferation. The possible involvement of
CTLA-4 in Treg development complicates the interpretation of these experiments as Treg
from CTLA-4 deficient mice may not develop in the same way as their wild-type
counterparts and therefore may develop alternate suppressive mechanisms. The
generation of conditional knock-out mice will prove invaluable in resolving these issues.

The ligands for CTLA-4 are mainly expressed on APC, however T cells can also
upregulate CD80, and to a lesser extent CD86, suggesting that CTLA-4 may be acting
directly upon the responding T cells (Paust et al. 2004), although this remains to be
shown. The result of CTLA-4-CD80/CD86 interactions between Treg and APC also
remains unclear. Binding studies have indicated that CTLA-4 has a higher affinity, and
double the valency, for its ligands than CD28, leading to the hypothesis that CTLA-4 on Treg competes with CD28 on Tconv for CD80/CD86 binding sites on APC and thereby reduces costimulation for Tconv (Ikemizu et al. 2000; Collins et al. 2002). Similarly this interaction could induce downregulation of CD80/CD86 on the APC and therefore reduce their capacity to activate T cells resulting in a less pronounced immune response (Cederbom et al. 2000; Oderup et al. 2006).

Another hypothesis is that CTLA-4, upon ligation with CD80/CD86 on APC, provides a costimulatory signal to Treg, activating them to suppress. However, mice lacking the cytoplasmic region or tyrosine residues of CTLA-4 do not develop the lymphoproliferative disease seen in CTLA-4 deficient animals suggesting that signalling back through CTLA-4 to Treg is not necessary for suppression (Nakaseko et al. 1999; Baroja et al. 2000). It has also been reported that CD80/CD86 signalling induces IDO (indoleamine 2,3-dioxygenase) production in DC, which is known to have immunosuppressive effects (Fallarino et al. 2006).
Figure 1.3. Possible Ways in Which CTLA-4 Could be Involved Treg Suppressive Mechanisms

(A) CTLA-4 could compete with CD28 for ligands CD80/CD86. (B) CTLA-4 signalling could downregulate CD80/CD86. Both of these would limit costimulation signals received by Tconv. (C) CTLA-4 could induce a Treg stimulatory signal from the APC. (D) CTLA-4 could induce IDO production by APC which has immunosuppressive capacity.
1.5.2. Glucocorticoid-Induced TNFR-Related Protein

As with CD25, glucocorticoid-induced TNFR-related protein (GITR) is expressed at high levels on the majority of Treg and can be upregulated in other T cells upon activation (McHugh et al. 2002; Shimizu et al. 2002). GITR ligation with an agonist anti-GITR antibody enhances T cell responses in vitro and leads to eradication of tumour in tumour bearing mice (Ko et al. 2005), suggesting a role for GITR in the mechanism of Treg suppression. Indeed anti-GITR antibodies can abrogate Treg anergy in the presence of IL-2 without TCR stimulation (McHugh et al. 2002; Shimizu et al. 2002). However, use of GITR deficient T cells indicated that anti-GITR antibodies affected responder T cells to become resistant to Treg suppression rather than sending an inhibitory signal to Treg (Ronchetti et al. 2004; Stephens et al. 2004).

Since GITR ligand (GITRL) is expressed on a range of APC (Stephens et al. 2004), it is possible that high expression of GITR on Treg may sequester ligand away from the activated T cells. Supporting this hypothesis is the observation that GITR is upregulated upon TCR stimulation and GITRL is upregulated upon APC maturation, providing a means to overcome Treg blocking. However it is also possible that GITR signalling acts upon both Treg and Tconv in a similar way, augmenting proliferation and enhancing function of each cell type with the outcome dependent on a fine balance of precursor number and activation state (Shevach and Stephens 2006).

1.5.3. Transforming Growth Factor β

Transforming Growth Factor β (TGFβ) is a secreted and cell-associated polypeptide with immunoregulatory properties. It can be secreted by a range of cell types including B cells, macrophages and monocytes, and can regulate the function of an equally diverse range of
cells. The evidence implicating TGFβ in the mechanism of Treg suppression is rapidly accumulating. TGFβ has been shown to be important in the control of intestinal inflammation and diabetes (Read et al. 2000; Belghith et al. 2003) and TGFβ signalling has been shown to disrupt proliferation, cytokine production (Espevik et al. 1990) and cytotoxic function in activated T cells (Smyth et al. 1991). Its receptor is only upregulated on T cells upon activation suggesting that T cells only become receptive to suppression after activation (Chen and Wahl 2003). TGFβ has also been shown to induce FOXP3 expression in naïve Tconv responding to activation by DC (Chen et al. 2003a; Kim and Leonard 2007).

However, in many in vitro suppression assays anti-TGFβ does not inhibit the suppression of T cells by Treg. This is surprising considering 80% of Treg express membrane bound TGFβ once activated (Piccirillo et al. 2002) and CTLA-4 ligation enhances TGFβ production (Chen et al. 1998b; Gomes et al. 2000). Furthermore, numerous studies have implicated membrane bound TGFβ, both active and latent forms in direct cell contact dependent suppression of T cell responses (Nakamura et al. 2001; Chen et al. 2003a; Nakamura et al. 2004; Peng et al. 2004; Lim et al. 2005), suggesting that TGFβ is involved in the Treg suppressive mechanism.

Two recent reports have highlighted further corroboration between CTLA-4 and TGFβ signalling, which supports earlier reports that blocking of both TGFβ and CTLA-4 is required to prevent suppression (Annunziato et al. 2002). Zheng et al. reported that TGFβ could not induce regulatory T cell differentiation in CTLA-4 deficient mice, and blockade of CTLA-4 also prevented differentiation. Furthermore TGFβ induced upregulation of CD80, a CTLA-4 ligand, on responding T cells (Zheng et al. 2006).
These results were supported by another study which reported that CTLA-4 ligation resulted in accumulation of membrane bound TGFβ at the point of cell contact (Oida et al. 2006).

In contrast, reports utilising TGBβ deficient mice have reported that Treg from these mice develop normally in the thymus (Marie et al. 2005; Li et al. 2006a; Marie et al. 2006), suppress T cell responses in vitro (Piccirillo et al. 2002), and inhibit intestinal inflammation in vivo (Zorn et al. 2006). However, they are reduced in numbers in the periphery suggesting that TGFβ plays a role in Treg homeostasis (Huber et al. 2004; Marie et al. 2005). One report indicated that Treg deficient in the TGFβ receptor develop and function normally, however responder TGFβ receptor deficient T cells are able to escape regulation and induce intestinal inflammation (Fahlen et al. 2005). It is also possible that in TGBβ deficient mice, Treg utilise other mechanisms of suppression or induce TGFβ production by other cell types, however other studies will have to be carried out in order to resolve these issues.

1.5.4. Interleukin-10

Like TGFβ, Interleukin-10 (IL-10) is an immunoregulatory cytokine first recognised for its ability to inhibit the effector function of T cells, macrophages and monocytes, and has been shown to have diverse effects on a wide range of other cell types. Despite papers indicating that Treg suppression cannot be inhibited in vitro by anti-IL-10 blocking antibodies, production of IL-10 by Treg is required in order to inhibit immune responses in vivo. Intestinal inflammation, widely shown to be inhibited by adoptive transfer of Treg, could not be inhibited by IL-10 deficient Treg (Annacker et al. 2001). Blocking antibodies can also relieve suppression in this model, and in graft rejection models IL-10
receptor blocking antibody accelerates rejection (Kingsley et al. 2002). Similarly in models of parasitic infection, neutralisation of IL-10 results in pathogen clearance, which in Schistosomiasis can result in immune pathology (Hesse et al. 2004) and in Leishmania infection can cause ablation of a memory response (Belkaid et al. 2001), suggesting that IL-10 mediated curtailment of these immune responses is advantageous for the host.

Two studies have recently suggested that the location of IL-10 secretion is also important, with local secretion of IL-10 required to inhibit EAE (McGeachy et al. 2005) and intestinal inflammation (Uhlig et al. 2006). IL-10 has been shown to induce expression of inhibitory receptors on DC, such as B7-H4 (Kryczek et al. 2006), which is thought to prevent adequate activation of T cells. A recent paper has also indicated that the direct effect of IL-10 on effector T cells is mediated by src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1), an intracellular protein tyrosine phosphatase, involved in negatively regulating T-cell activation. In this report IL-10 activated SHP-1 can suppress T cell costimulation by dephosphorylating the CD28 and ICOS receptors within minutes of ligation, therefore preventing binding of PI3 kinase, essential for T cell activation signalling (Taylor et al. 2007).

1.5.5. ATP, AMP and Adenosine

It has long been known that adenosine triphosphate (ATP) released upon cell damage can induce proinflammatory responses in cells expressing purinergic P2 receptors (Khakh and North 2006). These receptors are expressed on immune and epithelial cells, with ligation on monocytes leading to release of IL-1, and ligation on DC leading to chemotaxis and maturation (Ferrari et al. 1997; Idzko et al. 2002; Khakh and North 2006). Extracellular levels of ATP can be controlled by ectonucleoside triphosphate diphosphohydrolases (E-NTPDase), which degrades it to adenosine monophosphate (AMP), thereby removing the
proinflammatory stimulus. One such E-NTPDase, CD39, is expressed on the surface of immune cells and has recently been shown to be involved in Treg inhibition of T cell responses (Borsellino et al. 2007; Deaglio et al. 2007). In addition to the conversion of ATP to AMP, another ectonucleotidase expressed on Treg, CD73, can dephosphorylate AMP to produce adenosine, which has also been reported to inhibit T cell proliferation (Huang et al. 1997; Armstrong et al. 2001).

Both studies indicated that CD39 was expressed on the majority of FOXP3+ Treg and that CD73 was also expressed, although on a smaller proportion (Borsellino et al. 2007; Deaglio et al. 2007). Expression of CD39 correlated with FOXP3 expression and transfection of Tconv with FOXP3 upregulated CD39, suggesting that CD39 plays a role in Treg function (Borsellino et al. 2007). The authors also show that activated Treg, although exhibiting similar levels of CD39 to non-activated Treg, are capable of consuming 40x the amount of ATP. Consumption of ATP prevented ATP mediated DC maturation and inhibited T cell proliferation, suggesting that Treg could inhibit initiation and potentiation of T cell responses using this mechanism. In addition ATP consumption resulted in the ability of Treg to survive administration of toxic levels of ATP suggesting that Treg could persist at sites of cell death.
Figure 1.4. Mechanism of Immune Suppression Mediated by CD39 and CD73

CD39 on the surface of Treg could deplete the local environment of proinflammatory ATP released by dying cells. P2 receptor ligation by ATP can induce DC chemotaxis and maturation as well as IL-1 secretion by monocytes and macrophages. By converting ATP to AMP this also provides the substrate for CD73. Conversion of AMP to adenosine could also suppress T cell responses via ligation of the A2A receptor.
Another study indicated that CD73 played an important role in Treg function, however the expression of CD39 is also required to generate its substrate (Deaglio et al. 2007). This study utilised mice lacking the receptor for the immunosuppressive adenosine (A2A receptor) to demonstrate that in the absence of adenosine signalling T cell responses could proceed, although suppression was not complete. This was most evident 6 days after stimulation in vitro as A2A receptor expression is upregulated at this time point, suggesting that effector T cells become receptive to suppression at the peak of the immune response. Furthermore, Treg from CD39 deficient mice are unable to inhibit skin graft rejection, indicating that this mechanism is also relevant in vivo.

1.5.6. Cyclic Adenosine Monophosphate

Cyclic adenosine monophosphate (cAMP), like adenosine, is known to be a potent inhibitor of T cell growth, differentiation and proliferation, by inducing the transcriptional repressor ICER (Bodor et al. 2007). ICER is then thought to compete with CREB for binding to the IL-2 promoter attenuating IL-2 production. High levels of cAMP have been detected in Treg, which is elevated upon activation suggesting that the suppressive activity may be regulated by cAMP (Bopp et al. 2007). In addition, Tconv cells incubated with Treg showed an increase in intracellular levels of cAMP, which was reproduced by administration of forskolin (cAMP-elevating agent), resulting in a reduction in proliferative capacity. Blocking of cAMP neutralised the inhibitory effect of Treg on IL-2 production and proliferation by responding T cells which displayed enhanced ICER expression.

Furthermore, the authors showed that cAMP could be transferred from Treg to target Tconv directly through gap junctions (Bopp et al. 2007). To demonstrate this, it was first shown that dye transfer could occur between activated Treg and responding Tconv in
Following this, the authors demonstrated that fluorescently labelled cAMP could be passed from Treg to Tconv, a process that could be blocked by peptides which inhibit gap junction formation. Together these results indicate that cAMP may mediate the contact dependent suppression widely reported.

1.6. Regulation of Other Types of Cell

Although initially characterised as being able to inhibit CD4+ T cell responses, there is increasing evidence to indicate that Treg suppress the activity of other immune cells. In vitro studies report that Treg can inhibit the activation and/or proliferation of both CD4+ and CD8+ T cells (Thornton and Shevach 1998; Piccirillo and Shevach 2001), and this is supported in vivo (Dubois et al. 2003). Treg can also suppress B cell proliferation, antibody production, and class switching in vitro (Nakamura et al. 2004; Lim et al. 2005), and autoantibody responses in vivo (Fields et al. 2005); although distinction between direct inhibition of B cells and inhibition of T cell help for B cells, and other cells, is difficult in vivo.

Interestingly, Treg have also been reported to inhibit DC. DC are the main activators of naïve T cells and are often referred to as ‘professional’ antigen presenting cells. As described earlier, Treg have been reported to downregulate co-stimulatory molecules CD80 and CD86 on DC, which was later found to be CTLA-4 dependent (Cederbom et al. 2000; Oderup et al. 2006). Another group had similar findings and reported that treatment of DC with anti-TGFβ resulted in a 10% increase in stimulatory capacity (Misra et al. 2004). It is therefore likely that one mechanism by which Treg suppress immune responses is by suppressing the activating capacity of DC. However, it is not the only mechanism as Treg suppression of CD4 T cell proliferation can occur in the absence of DC in vitro (Thornton and Shevach 1998). Although Serra et. al. found no role for
TGFβ in Treg suppression of DC maturation, ligation of the costimulatory molecule, CD40 or treatment with CpG DNA could render DC resistant to Treg mediated suppression. Since Tconv cells upregulate CD40 ligand upon activation, this suggests that Treg may help maintain DC in a non-stimulatory state until the initiation of a strong activation signal (Serra et al. 2003).

Treg do not only inhibit the capacity of DC to stimulate immune responses but they also inhibit the capacity of human monocytes and macrophages to stimulate T cell proliferation (Taams et al. 2005). Monocytes cultured with Treg expressed decreased levels of CD86, CD80, CD40, MHC class II, and proinflammatory cytokines when compared with monocytes cultured with conventional T cells. In another study, human monocyte survival induced by LPS treatment was inhibited by Treg (Venet et al. 2006). The report indicated that survival was inhibited by a proapoptotic mechanism involving the Fas/FasL pathway as Treg inhibition of monocyte survival was blocked by anti-FasL antibodies and reproduced by addition of recombinant soluble FasL. Furthermore Treg displayed elevated levels of FasL when compared to their Tconv counterparts.

These reports were interesting as they suggested that Treg suppression was not confined to the adaptive immune system. They supported the findings of an earlier study exploring the impact of Treg on the innate immune pathology seen in RAG deficient (RAG−/−) mice infected with Helicobacter hepaticus (Maloy et al. 2003). These mice lack the RAG recombinase gene required to generate both the TCR and the BCR and therefore lack T cells and B cells. Upon infection with H. hepaticus, which does not usually cause disease in immune competent mice, RAG−/− mice develop T cell-independent intestinal inflammation, comprising polymorphonuclear and mononuclear cells. This inflammation
could be inhibited by adoptive transfer of Treg, reducing the number of neutrophils, monocytes/macrophages, DC and NK cells in the spleen, lamina propria and mesenteric lymph node. Since both anti-IL-10 and anti-TGFβ blocking antibodies relieved suppression and IL-10-deficient Treg could not inhibit inflammation, it was suggested that these cytokines play a major role in suppressing the innate immune system.

In parallel, an observation made in our own laboratory suggested that Treg inhibit innate responses to tumour rejection. In these experiments RAG-/- mice were injected with CD4+CD25+ T cells, or control CD4+CD25- cells, both from naïve mice, and subsequently inoculated with B16 tumour cells. Although all mice grew tumours, tumours grew more rapidly in mice receiving Treg compared to mice receiving the control cell population, suggesting that there is a degree of tumour control exerted by the innate immune system which is suppressed by adoptive transfer of Treg. This led to the hypothesis that not only do Treg inhibit the adaptive arm of the immune system, but also inhibit innate immunity. One of the aims of the work described here was to explore this hypothesis and identify target cells.
1.7. Do Treg inhibit innate immune responses?

1.7.1 B16FasL Tumour Model

In order to explore the hypothesis that Treg inhibit the innate immune responses, a model of tumour rejection involving a melanoma cell line expressing Fas ligand (B16FasL) was used. Fas ligand (FasL/CD95) is a type I integral membrane protein of the tumour necrosis factor receptor family, which was initially reported to induce apoptotic death in Fas bearing cells (Suda et al. 1993). FasL induced cell death is involved in many aspects of T cell regulation including thymocyte deletion (Yonehara et al. 1994), T cell mediated cytotoxicity (Ju et al. 1994) and activated T cell deletion, either induced upon inappropriate activation (Alderson et al. 1995) or during termination of an immune response (Daniel and Krammer 1994).

To induce apoptosis, a FasL trimer is thought to bind three Fas molecules, which then oligomerise (reviewed in (Wajant et al. 2003), and depicted in Figure 1.3). This induces assembly of a signalling complex called DISC, which includes the Fas-associated death domain protein (FADD) and caspase 8. Depending on cell type, caspase 8 can either directly activate other caspases to induce apoptosis or can initiate an amplification loop. Fas signalling can also induce necrotic death, requiring FADD and a serine/threonine kinase RIP, in the absence of caspase 8.
Figure 1.5. Fas Signalling
Diagrammatic representation of known signalling pathways induced by Fas ligation adapted from Wajant et al. 2003. Solid lines indicate known associations, whilst dotted lines indicate pathways yet to be dissected. This is not exclusive and many of the down stream signalling molecules interact between pathways. Signals through TNF and IL-1 receptors are also known to modulate Fas signalling.
FasL expression, although mainly restricted to activated CD4+ and CD8+ T cells, has also been detected on tissue from so called ‘immune privileged’ sites (Siegel et al. 2000). The eye (Griffith et al. 1995) and the testis (Bellgrau et al. 1995) are examples of these tissues where a prolific immune response would be extremely detrimental to the host. It was therefore hypothesised that FasL expression in these tissues limits ensuing immune responses by inducing cell death in the infiltrating, activated T cells. The observation that a number of tumours isolated from patients, of both haematopoietic and non-haematopoietic origin (Hahne et al. 1996; Saas et al. 1997; Shiraki et al. 1997), expressed FasL suggested that FasL provided the tumour a means to escape destruction by invading T cells.

This assumption was extended and it was thought that graft rejection could also be prevented by gene transfer of FasL. A number of investigators began to assess the role of FasL in tumour immune escape/graft rejection by generating cells expressing FasL. A few reported T cell killing (O’Connell et al. 1996; Arai et al. 1997a; Cefai et al. 2001) or increased tumour growth (Bellgrau et al. 1995; Lau et al. 1996), however contrary to the hypothesis, others reported an increase in tumour/graft rejection (Allison et al. 1997; Arai et al. 1997b; Seino et al. 1997; Chen et al. 1998a).

Seino et al. studied tumour rejection by three different tumour cell lines from lymphoma (L5178Y), hepatoma (MH134) and melanoma (B16) forced to express FasL. When injected subcutaneously into syngeneic animals, non-transfected cells form tumours whereas FasL transfected tumours failed to form palpable tumours. This rejection was inhibited by anti-FasL neutralising antibodies and a bystander killing effect on non-transfected tumour cells was also reported. Tumour rejection was observed in T cell-
deficient \textit{nu/nu} mice and histological examination of the sites of injection 2 days later showed massive cellular infiltrates mainly consisting of neutrophils. TUNEL staining revealed that the majority of FasL transfected tumour cells were undergoing apoptosis at this time. Treatment of mice with agents to deplete various cell types also revealed a role for neutrophils in tumour rejection but not for NK cells or macrophages. Work with bone marrow chimera\textsuperscript{s} indicated that Fas on haematopoietic cells was important for rejection. Lastly, intraperitoneal injection of FasL expressing cells recruited immune cells, 80-90\% of which were neutrophils, capable of tumour lysis \textit{ex-vivo}. In combination these results suggested neutrophils were important for FasL-expressing tumour cell rejection (Seino \textit{et al.} 1997).

Arai \textit{et. al.} used adenoviral vectors to introduce FasL into pre-established Fas\textsuperscript{-} tumours and found that tumours began to regress within 24 hours, with no tumour detectable 2 days later. Histological analysis at 24 hours also revealed massive cell death by TUNEL staining. Upon injection of a stable transfectant of the cell line CT26 into \textit{nu/nu}, SCID and SCID-beige, which lack T, B and NK cells, all mice were able to reject tumour challenge, which is supported by the previous report indicating that rejection is T cell independent. Histological analysis identified polymorphonuclear (PMN) and mononuclear infiltrates, with the presence of neutrophils confirmed by antibody staining with anti-Gr-1 antibody (Arai \textit{et al.} 1997b).

One report suggests that the difference observed between these studies and the hypothesised outcome is due to the level of FasL expression (Chen \textit{et al.} 2003b). High levels of FasL expression allow the tumour to grow more quickly and yet with much larger neutrophil infiltrate, however only FasL\textsuperscript{low} tumour cells grew more quickly upon
neutrophil depletion. *In vitro* neutrophils lysed FasL\textsuperscript{low} cells more easily and when incubated with FasL\textsuperscript{high} cells neutrophils were impaired in their activation markers, suggesting that high FasL expression inactivates neutrophils. However another report found no difference in tumour growth upon differential FasL expression (Igney *et al.* 2003).

Chen *et. al.* attempted to resolve the opposing reports by studying FasL expressing cell lines injected into the immune privileged site, the eye. In contrast to subcutaneous injection, cells injected into the anterior chamber of the eye produced tumours, suggesting that it was the microenvironment that dictated the outcome. Human PMN preferentially lysed FasL-expressing cells *in vitro*, as did murine PMN, and depletion of other cell types within the preparation did not inhibit tumour lysis. Tumour lysis could be inhibited by addition of FasL-Fc Fusion protein and was reduced when PMN from Fas deficient, *lpr* mice were used. Furthermore, fluid from the eye could inhibit this lysis, with the responsible factor identified as TGFβ. The effect of TGFβ was suggested to be on the neutrophils since addition of TGFβ had no effect on FasL dependent killing of Jurkats by the tumour cells. In addition, doubly transfected FasL\textsuperscript{+}TGFβ\textsuperscript{+} tumour cells grew out when injected subcutaneously with few infiltrating neutrophils (Chen *et al.* 1998a).

Like other members of the TNF receptor family, FasL can be cleaved from the surface by matrix metalloproteinases and it was initially reported that soluble FasL had chemotactic activity for neutrophils (Seino *et al.* 1998; Ottonello *et al.* 1999); however this has been widely disputed (Behrens *et al.* 2001). Others have concluded that FasL can induce
apoptosis in the first infiltrating neutrophils which then act as to amplify the response by recruiting increasing neutrophil numbers (Shimizu et al. 2001).

However, an elegant study using cell lines transfected with different FasL constructs clearly indicated that membrane bound (i.e. non-cleavable) FasL was a more potent inducer of inflammation in vivo than wild-type FasL, and that soluble FasL alone was unable to induce inflammation (Hohlbaum et al. 2000). Injection of these cells into Fas deficient mice did not induce inflammation and other experiments indicated that a functional Fas death domain was required suggesting that Fas signalling on recipient cells was essential to induce inflammation. Interestingly, co-injection of naïve peritoneal cells from wild-type mice prevented tumour growth in Fas/FasL deficient mice by recruiting Fas deficient neutrophils, indicating that the proinflammatory event is Fas dependent, however neutrophil extravasation is not.

Following this report the authors utilised a membrane-bound cell-free form of FasL (vesicles) to assess the contribution of peritoneal cells to tumour attack (Hohlbaum et al. 2001). Injection of vesicles led to a rapid (within 4 hours) and transient induction of IL-1β, MIP-2, MIP-1α and MIP-1β RNA. MCP-1 RNA persisted longer and was detectable after 18 hours. The cytokine induction was associated with a rapid increase in neutrophil number and a rapid decrease in the number of cells expressing high levels of CD11b and F4/80, two macrophage markers, most likely due to FasL induced cell death. Indeed F4/80⁺ cells became annexin V positive within 15 minutes of vesicle injection and this could be replicated in vitro. Further analysis revealed that MIP-2 expression correlated with disappearance of the CD11b⁺ population, and in vitro the CD11b⁺ population was the only one to respond to FasL by producing cytokines and undergoing apoptosis.
Finally they coinjected purified CD11b$^{\text{high}}$ macrophages from naïve wild-type mice with vesicles and showed it restored neutrophil recruitment in Fas/FasL deficient mice.

Interestingly, tumour cells transfected with IL-8 (Lee et al. 2000a), MIP-1α (Nakashima et al. 1996), MIP-1β (Miyata et al. 2001) or MCP-1 (Bottazzi et al. 1992) also induced tumour regression and an inflammatory influx in vivo. This suggests that once initiated by any one of these factors, the inflammatory response is capable of tumour clearance.

Although macrophages were essential in order to induce a neutrophil infiltrate it remains unclear whether there is a requirement for apoptosis, cytokine/chemokine production or both. It has long been known that IL-1β plays a role in neutrophil recruitment, however it appears that its affect may be indirect. IL-1$^{-/-}$ mice have been reported to be defective in neutrophil recruitment to the peritoneum (Sayers et al. 1988) and Fas ligation has been reported to induce processing and secretion of IL-1β in cells harvested from the peritoneum following challenge with a FasL expressing cell line (Miwa et al. 1998). Although cells also underwent apoptosis, the process of cytokine secretion did not appear to be a consequence of cell degeneration as no IL-1β secretion was observed in non-stimulated cells in vitro. Stimulation of IL-1β secretion is caspase 1 independent as cells from ICE deficient mice were capable of IL-1β secretion. In agreement with the report described above, IL-1$^{-/-}$ mice presented with reduced neutrophil recruitment upon intraperitoneal injection with FasL expressing tumour cells, however both murine and human neutrophils show no chemotactic activity towards IL-1 in vitro (Georgilis et al. 1987), suggesting that another factor may bridge IL-1 production and neutrophil recruitment.
In contrast, MIP-2 and its human functional homologue IL-8 are recognised neutrophil chemoattractants and activate oxidative burst and release of lysosomal enzymes. Not only is MIP-2 found in the peritoneum upon Fas ligation but, human colonic epithelial cells have been reported to release IL-8 upon Fas ligation which was dependent on gene transcription (Abreu-Martin et al. 1995). CXCR2 is expressed on neutrophils and is the receptor for both MIP-2 and KC, another neutrophil chemoattractant. CXCR2 deficient mice were utilised to address the role of these cytokines in the rejection of FasL expressing cells (Shimizu et al. 2005). In this model, only macrophages were recruited to the peritoneum following intraperitoneal injection of FasL expressing cells. Since there was no defect in production of the other chemoattractants studied, it suggested a primary role of CXCR2 ligands in the recruitment of neutrophils. In agreement with the studies described by Hohlbaum et al. discussed earlier only wild-type macrophages co-injected with FasL expressing cells could induce neutrophil recruitment in Fas deficiency mice (Hohlbaum et al. 2000). All CXCR2 deficient mice survived FasL+ tumour challenge similar to wild type mice suggesting that neutrophils, although recruited in a large number, are not required for tumour rejection. This conclusion was supported by another study on mice with neutrophils deficient in cytotoxicity molecules p47phox or iNOS (Igney et al. 2005). In these mice, and in mice depleted of neutrophils using anti-Gr-1 depleting antibody, there was no difference in tumour growth when compared to control mice.

Within the laboratory, the melanoma cell line B16-F10 has also been transfected with FasL (B16FasL), which when inoculated subcutaneously, leads to 50% of C57BL/6 mice being capable of tumour rejection (Simon et al. 2002). This rejection was dependant on Fas binding as mutation of this region resulted in 0% tumour rejection. The truncation of
the cytoplasmic region had no affect on tumour rejection indicating that reverse signalling through FasL is not responsible for tumour rejection. Interestingly, there was a slight impairment of tumour rejection in MIP-1α deficient mice, which although it is primarily thought to recruit macrophages, has also been reported to act as a neutrophil chemoattractant (Lee et al. 2000a).

In summary, FasL expressing tumour cell lines have been extensively studied and the immune responses induced are well characterised, which is depicted in Figure 1.3. Although there is still more to be learned about the mechanism of tumour rejection, studies agree that innate immune cells are primarily responsible and can be sufficient for rejection. Overall, these reports indicate that B16FasL induces an inflammatory response and may therefore represent an ideal model in which to study the effects of Treg on innate immune responses.
Figure 1.6. Tumours Expressing FasL Induce Inflammation
This diagram shows the way in which FasL expressing tumours are thought to be rejected. FasL interacts with Fas on macrophages, and FADD signalling is required for apoptosis and concurrent chemokine release. MIP-2 and IL-1 have been shown to attract neutrophils which may participate in tumour clearance. The role of other cytokines released by macrophages, and the cells they attract, is unknown.
1.8. Location of Treg Suppression

Although extensive studies have been carried out to elucidate the mechanism of Treg suppression both in vitro and in vivo, whether Treg suppress immune responses in the secondary lymphoid organs or at the site of challenge, remains to be elucidated. The location of Treg suppression will dictate the cells that could be targets for immune suppression as all mechanisms of suppression previously described are short-ranged. If Treg are only active in the lymphoid tissue they are likely to encounter naïve and central memory T cells along with B cells, as these cells are able to circulate through lymph nodes. Furthermore, accumulating evidence suggests that the ability of DC to activate T cells is inhibited by Treg (Cederbom et al. 2000; Serra et al. 2003; Misra et al. 2004; Veldhoen et al. 2006) implying that Treg inhibit the priming of an immune response, which is most likely to occur in a lymph node. Intravital two-photon laser scanning microscopy has also indicated a role for Treg in destabilising contacts between DC and Tconv in lymph nodes (Tadokoro et al. 2006; Tang et al. 2006). However, if the activity of Treg is not restricted to lymphoid tissue, it is possible that they can inhibit the effector phase of an immune response, potentially inhibiting the action of both effector T cells and cells of the innate immune system to limit immunopathology.

Although migration of Tconv cells has been studied extensively, the migration, and therefore the location, of Treg is less well studied. Circulating T cells have various adhesion molecules and chemokine receptors which are thought to direct migration, some of which have been shown to be expressed on Treg. Movement into tissues occurs in three stages; tethering of cells on endothelium, chemokine activated adhesion and transmigration through endothelium, with each stage controlled by homing markers. Although there is a wide range of these markers identified, below is a summary of the best characterised and their role in T cell migration.
1.8.1. T Cell Homing to Lymphoid Tissue

Upon thymus exit, naïve Tconv express CD62L and CCR7. These molecules aid circulation through the lymphoid tissue facilitating encounter with antigen. CD62L (L-selectin) mediates rolling of lymphocytes in high endothelial venules (HEV) of peripheral and mucosal lymph nodes (Bradley et al. 1994) via binding of peripheral node addressin (PNAd) or mucosal addressin cell adhesion molecule-1 (MAdCAM-1) respectively and T cells from CD62L deficient mice have impaired entry into lymph nodes (Arbones et al. 1994) indicating that CD62L is required for lymph node entry.

Firm adhesion of T cells to the HEV requires upregulation of lymphocyte function-associated antigen 1 (LFA-1) which is stimulated by signalling through CCR7 by the chemokine CCL21 expressed by the endothelial cells. Within the lymph node, CCL21 and CCL19 attract CCR7+ T cells to the T cell area. Upon activation by DC expressing specific antigen, T cells downregulate CD62L and CCR7 and are programmed to express chemokine receptors and adhesion molecules that allow them to enter peripheral tissue.

Memory T cells however are mixed in phenotype, with central memory T cells (T_{CM}) expressing both CD62L and CCR7 and effector memory T cells (T_{EM}) expressing neither (Sallusto et al. 1999). T_{EM} require no additional stimulation in order to become effective and are thought to reside in peripheral tissues as the first line of defence, whereas T_{CM} require restimulation in order to exert their effector mechanisms and are thought to recirculate through lymphoid tissue. The expression of CD62L and CCR7 on the surface of T_{CM} supports this conclusion.
1.8.2. T cell Homing to Non-lymphoid Tissue

Once activated/reactivated in lymphoid tissue T cells need to migrate out and into tissue. Interestingly, effector T cells generated in different lymphoid organs display distinct tissue tropism (Campbell and Butcher 2002) as cells activated in mesenteric lymph nodes (mLN - draining the gut) express high levels of $\alpha_4\beta_7$ (Stagg et al. 2002; Mora et al. 2003) whereas skin draining lymph nodes (skinLN) stimulate upregulation of other molecules (Calzascia et al. 2005).

Several reports have suggested that DC in the mucosal draining lymph nodes induce T cells to express mucosal homing receptors, in particular $\alpha_4\beta_7$ and CCR9 (Stagg et al. 2002; Mora et al. 2003). The ligands for these receptors, MAdCAM-1 and CCL25 respectively, are found almost exclusively in the HEV of mLN, Peyer’s patches (PP) and postcapillary venules in the lamina propria. Furthermore CCL25 is produced by gut epithelia, suggesting that these interactions very precisely dictate migration of activated T cells into organs. Other receptors such as CCR6 and CCR10 (and their ligands CCL20 and CCL28) have also been implicated in migration to the small intestine (Kunkel et al. 2003). Intraepithelial lymphocytes (IEL) are another population of mucosal homing T cells, however the majority express a different integrin, $\alpha_E$ (CD103), associated with $\beta_7$ which is thought to regulate their homing as CD103 deficient mice are severely depleted of IEL (Schon et al. 1999).

Skin homing T cells express ligands for P and E-selectin as well as chemokine receptor CCR4 (ligands are CCL17 and CCL22) and/or CCR10 (ligand is CCL27). Cutaneous lymphocyte antigen (CLA) has been identified on T cells in the skin where it interacts with E-selectin expressed on endothelial cells, which is upregulated during inflammation.
(Kunkel and Butcher 2002). VCAM-1 is also upregulated on inflamed endothelium and its ligand, \(\alpha_4\beta_1\), is expressed on skin homing T cells in contrast to \(\alpha_4\beta_7\) which is downregulated (Mora and von Andrian 2006). CCL17 and CCL27 are also expressed in skin, although not exclusively, under non-inflammatory conditions by endothelial cells and keratinocytes respectively. They are also expressed by activated monocytes, macrophages and B cells, and mature DC, suggesting that they attract CCR4\(^+\) T cells to sites of ensuing immune responses. Although each receptor/ligand pair does not attract cells solely to the skin, reports indicate that the majority of cells identified in skin express more than one receptor, consolidating their commitment to normal and inflamed skin.

### 1.8.3. Treg Homing Markers

A wide range of homing markers have been reported to be expressed on Treg cells although few investigators completely agree. Originally identified as expressing high levels of CCR7 and CD62L (Itoh et al. 1999; Lepault and Gagnerault 2000), there has been a plethora of reports indicating that Treg express a range of different homing receptors. These differences may be due to methods of Treg isolation. Until recently, Treg isolation relied upon expression of CD25, which is also a marker of activated T cells, and therefore the chemokine receptor and adhesion molecule profile could have been contaminated with activated cells. In an attempt to exclude activated cells from these earlier studies, analysis was usually carried out on populations expressing cell markers such as CD45RO/A in humans (Iellem et al. 2001). Although this facilitated the study of Treg at the time, the discovery of FOXP3 as a more specific marker of Treg prevented the accidental exclusion of other Treg and allowed the study of Treg subpopulations. Another factor that may affect the study of homing markers is the tissue from which the Treg were isolated. Classically murine Treg were isolated from spleen and lymph nodes, and human Treg from peripheral blood, therefore conclusions drawn
from these experiments should not be applied generally to the Treg population as a whole just those found in that location.

Human Treg from peripheral blood expressed CCR4, which although has been implicated in inflamed skin homing, its ligands (CCL17 and CCL22) are also produced by activated APC. In combination with the observation that these cells also express CCR8, whose ligand, CCL1, is also expressed by professional APC, this suggested that these receptors regulate migration to activated lymphoid organs (Iellem et al. 2001). However the same group later reported these cells expressed high levels of CLA and low levels of α4β7 and CCR9, suggesting they were skewed toward skin and not gut homing (Iellem et al. 2003).

In reality the receptor expression was assessed on a population basis and all receptors tested were expressed by a proportion of Treg (Iellem et al. 2003; Clark and Kupper 2007). Murine studies of cardiac transplant indicated that recruitment of Treg was dependent on CCR4 as tolerance to allografts could not be induced in CCR4 deficient mice (Lee et al. 2005), which may suggest migration to lymphoid organs is required, however, CLA⁺CCR4⁺ Treg can be isolated from normal human skin (Hirahara et al. 2006; Clark and Kupper 2007).

CCR5, the receptor for the inflammatory cytokine CCL4, has also been detected on large proportions of Treg which are attracted by CCL4 (Bystry et al. 2001). In vivo, Treg deficient in CCR5 have been shown to be less effective at preventing graft-vs.-host disease (GVHD) (Wysocki et al. 2005) and favoured pathogen persistence in the *Leishmania major* model of infection due to inefficient Treg migration to the site of infection in the skin (Yurchenko et al. 2006).
Although first identified as a homing receptor for IEL in the gut, high levels of CD103 are expressed on IEL from other mucosal surfaces which aids binding to E-cadherin. Inflammatory skin disease in CD103 deficient mice suggested immune dysfunction, which could be transferred to wild-type mice by adoptive transfer of T cells (Schon et al. 2000). Expression was detected on a population of Treg which had enhanced regulatory activity when compared to their CD103⁺ counterparts (Lehmann et al. 2002). Later studies indicated that CD103⁻ Treg could not control antigen-induced arthritis (Huehn et al. 2004) or Leishmania major infection (Suffia et al. 2005), however, the latter study found no increase in suppressive capacity of CD103⁺ Treg but detected enhanced numbers of Treg in skin of wild-type mice, indicating that CD103 expression enables Treg migration into the skin. In contrast, a study of murine colitis found no role for CD103 on the surface of Treg but wild-type Treg could not control disease in CD103 deficient recipients (Annacker et al. 2005).

A role for E/P-selectin ligands in controlling Treg entry into inflamed tissues was also indicated by studies of FucTVII deficient mice, which lack an enzyme necessary to generate selectin ligands in T cells. Treg from these mice were unable to enter inflamed sites and suppress delayed type hypersensitivity (DTH) (Siegmund et al. 2005). Treg expressing CD103 have been shown to express low levels of CD62L and CCR7, whereas CD103⁻ Treg express high levels of these markers (Huehn et al. 2004; Siegmund et al. 2005). Interestingly, the latter cannot inhibit DTH or antigen-induced arthritis whereas Szanya et. al. have reported that CD4⁺CD25⁺CD62L⁻CCR7⁺ T cells and not those negative for CD62L were able to inhibit diabetes (Szanya et al. 2002). Two other reports support this observation as only the CD62L⁺ population of Treg could protect against GVHD and bone marrow graft rejection (Taylor et al. 2004; Ermann et al. 2005).
The conclusion drawn by the majority of the studies where CD62L expression is required to inhibit detrimental immune responses is that Treg require entry into lymph nodes in order to exert suppression, suggesting that the possible mechanism of action is inhibition of the initiation of an immune response. Whilst one study has shown that CD62L deficient Treg behave like CD62L deficient Tconv, in that they are unable to enter lymph nodes effectively (Venturi et al. 2007), these reports do not exclude the possibility that CD62L$^+$ Treg have a higher proliferative capacity, as reported by some (Fu et al. 2004; Ochando et al. 2005), and therefore can produce a greater number effective progenitors which are then able to inhibit immune responses.

Similar to Tconv, recent reports have indicated that organ selective homing in Treg can be programmed by DC in the respective lymph node. In one study CFSE labelled OVA-specific Treg were adoptively transferred into wild-type hosts which were later challenged with OVA. Cells recovered from the mLN predominantly expressed $\alpha_4\beta_7$ and CCR9, whereas those from peripheral lymph nodes (pLN) were $\alpha_4\beta_7$ negative and expressed E/P selectin ligands (Siewert et al. 2007). Another study successfully induced ear homing Treg by prior incubation with Langerhans cells (Schwarz et al. 2007). A study of human Treg also drew parallels between Treg and Tconv as unlike most other studies it aimed to identify subpopulations of Treg expressing cohorts of homing molecules (Lim et al. 2006). This study identified a subpopulation of CD45RA$^+$CD62L$^+$CCR7$^+$ naïve-like Treg as well as CD45RO$^+$ memory-like populations which predominantly expressed tissue homing receptors. A proportion of CD45RO$^+$ cells also expressed CD62L and CCR7 which suggests that the memory Treg pool may contain both central and effector memory-like cells.
In summary, studies show that Treg express homing markers that would direct migration into both lymphoid and non-lymphoid tissue, although whether these markers are expressed on the same cell remains to be determined. Furthermore, Treg have also been isolated from both lymphoid tissue and peripheral tissue in steady state and inflammatory situations. However, investigations into the ability of Treg expressing different markers to inhibit immune responses in vivo have reported conflicting results, leaving the question of whether Treg inhibit initiation or the effector phase of an ensuing immune response unanswered. These possibilities are not mutually exclusive, and may involve different Treg subpopulations. One aim of this thesis is to identify the location of Treg action in vivo and therefore help address these questions.

1.8.4. CD62L Transgenic Mice

Previous work has indicated that only Treg expressing high levels of CD62L are able to inhibit various immune responses in vivo (Szanya et al. 2002; Fu et al. 2004; Taylor et al. 2004; Ermann et al. 2005), which has been attributed to preferential migration to lymph nodes over peripheral tissue. Although Treg may inhibit the initiation of the immune response in the lymph node, reports have not excluded the possibility that these Treg give rise to progeny that can migrate into tissue and inhibit the effector phase of the immune response. In order to address this hypothesis, a model in which Treg might be retained within lymph nodes, was characterised.

CD62L mediates rolling of lymphocytes in high endothelial venules of pLN and TCR engagement, or cross-linking with anti-CD62L antibody or ligand, causes proteolytic shedding of its ectodomain (Ley et al. 1995). Shedding has been shown to correlate with loss of lymph node entry (Hamann et al. 2000), as has complete deficiency (Arbones et
al. 1994), and studies of T cells isolated from inflammatory sites show low levels of CD62L expression (Mobley and Dailey 1992; Hou and Doherty 1993; Rigby and Dailey 2000). In combination, these results suggest that maintenance of CD62L expression on T cells could retain T cells in the lymph nodes and prevent access to inflamed tissue.

If maintenance of high levels of CD62L expression prevents T cell infiltration of peripheral tissues, Treg from mice that cannot downregulate CD62L could be used to address the hypothesis that Treg must migrate into tissues in order to exert their suppressive effects. In vitro studies using anti-CD3/CD28 antibodies have shown that CD62L undergoes complex changes in expression after TCR engagement (Chao et al. 1997). Accelerated shedding during the first 4 hours leads to rapid downregulation. CD62L is then re-expressed 24-48 hours later due to increased gene transcription, followed by late downregulation after 3-5 days due to accelerated shedding and decreased gene transcription (Figure 1.7) (Chao et al. 1997). Therefore in order to prevent CD62L downregulation, gene transcription must be maintained and proteolytic shedding prevented.
Figure 1.7. Schematic Representation of CD62L Levels on T Cells Following TCR Engagement
Following TCR engagement CD62L is lost from the cell surface by shedding within the first hour and remains low for up to 4 hours. Increased gene transcription and cessation of shedding results in an increase in CD62L expression with 2-3 fold increase in expression when compared to naïve T cells. CD62L expression then drops over the following 5 days.
Two strains of mice expressing either wild-type (WT) or a shedding-resistant form (LΔP) of CD62L driven by the hcd2 promoter were generated by the laboratory of Dr Ann Ager (Figure 1.8 duplicated from (Galkina et al. 2003). Numerous studies have shown that protein expression driven using the hcd2 promoter is directed to T cells and is maintained upon activation (Zhumabekov et al. 1995; Bromley et al. 2005), therefore wild-type CD62L expressed under this promoter does not undergo transcriptional downregulation post T cell activation (WT mice). The additional substitution of the proteolysis sensitive membrane proximal region of CD62L with that of the proteolysis insensitive CD62P (P-selectin), generated a shedding resistant form of CD62L (LΔP mice). CD62L transgenic mice were backcrossed to B6 CD62L−/− to eliminate endogenous CD62L and transgenic lines expressing levels of CD62L comparable with those on T cells in B6 mice were chosen. Therefore LΔP mice potentially present a model in which to address the hypothesis that maintained CD62L expression stops Treg access to peripheral tissue subsequently preventing exertion of their suppressive effects.

The inability of T cells to downregulate CD62L did not affect cellularity or subset composition of pLN, mLN, PP or spleen in LΔP mice, and shedding was resisted in vitro upon PMA or cognate peptide (CD8) stimulation, unlike WT CD62L. Lymphocyte rolling under flow conditions and the ability of naïve cells to home to pLN were also not affected, although LΔP T cells accumulated within the HEV suggesting that CD62L shedding may inhibit movement across HEV into pLN. In vivo stimulation with cognate peptide reduced the ability of WT CD8+ cells to enter LN when compared to LΔP CD8+ cells, however activated LΔP CD8+ cells were inferior to naive LΔP CD8+ cells in their ability to migrate into lymph nodes, suggesting other factors also play a role.
Directed expression to T cells using hcd2 cassette

Backcross on to C57BL/6 CD62L−/− background

Figure 1.8. Construction of CD62L Mutants
Schematic representation of the Membrane Proximal Region (MPR) of mouse CD62L in relation to the whole molecule (SCR, short consensus repeat). Location of a primary cleavage site in wild-type CD62L (WT) is shown together with amino acid sequence of corresponding LAP mutant, containing the MPR of CD62P which is naturally shorter and lacks the cleavage site. This figure is duplicated from Galkina et al 2003.
As T cell migration in the context of an active immune response had not yet been studied in these mice, it was first important to establish whether or not maintained CD62L expression prevented T cell access of inflamed tissue. Since insufficient information is known about Treg migration, the migration of conventional T cells under inflammatory conditions in WT and LAP mice was studied in the context of an influenza infection.

1.8.5. Influenza Virus Model

The influenza model has been used extensively to study T cell responses, because the site of infection and therefore the target organ for T cells is known. Influenza virus is an enveloped RNA virus of which three strains exist (A, B and C) each characterized by the antigenic properties of internal components. Influenza A viruses can be further categorized into subtypes on the basis of two surface antigens present in the lipid bilayer: haemagglutinin (HA), responsible for binding to host cells, and neuraminidase (NA), which facilitates release of viral progeny from infected cells. These antigens are known to be the main targets of the immune system.

Influenza virus infection is restricted to the epithelial cells and monocytes/macrophages of the respiratory tract, and infection can cause tissue destruction directly during the lytic phase of the virus life cycle or indirectly by induction of interferon α/β production (Garcia-Sastre et al. 1998). The innate response is not sufficient to control the virus and the adaptive immune system is enlisted. CD8+ cytotoxic T cells can directly kill infected cells aided by CD4+ helper T cells (Th), which also aid production of neutralising antibodies by B cells.

Typically the virus is controlled within 7-10 days, with CD8+ T cells activated and proliferating within lymph nodes 3-4 days after infection, subsequently migrating to the
lung around days 5-7 (Tripp et al. 1995a; Topham et al. 1997; Lawrence and Braciale 2004). CD8+ T cells exert their effector functions in the lung, producing antiviral cytokines and lysing target cells by a mechanism involving perforin and/or Fas (Topham et al. 1997). The importance of virus-specific CD8+ T cells is demonstrated by observations in mice which lack CD8+ T cells. A study on β2-microglobulin- and therefore MHC class I- deficient mice indicated that viral clearance was delayed in the absence of CD8+ T cells (Eichelberger et al. 1991), and in the case of a more pathogenic strain, viral infection was more prolific, resulting in increased morbidity (Bender et al. 1992).

B cells are also important for viral clearance. B cells are responsible for the generation of neutralizing antibodies to external viral coat proteins (Gerhard et al. 1997). B cell deficient, μMT mice can clear less pathogenic forms of virus with slightly delayed kinetics (Topham et al. 1996), however more pathogenic strains result in increased morbidity (Mozdzanowska et al. 1997).

CD4+ T cells are also recruited to the lung in large numbers peaking at day 6-7 (Baumgarth and Kelso 1996a; Baumgarth and Kelso 1996b; Roman et al. 2002). Although the role of CD4+ T cells appears secondary to CD8+ T cells and B cells, they provide cytokines important for CD8+ effector and memory cell generation along with facilitating antibody production. Upon infection of CD4 deficient, MHC class II deficient mice with influenza, influenza-specific CD8+ T cells had limited clonal expansion but maintained equivalent cytotoxic ability at the peak of infection, resulting in a slight delay in viral clearance (Tripp et al. 1995b; Ribardy et al. 2000). In contrast, numerous studies have indicated that CD4 deficient mice do not exhibit impaired viral clearance, even with
more pathogenic strains (Allan et al. 1990) (Mozdzanowska et al. 2000). However, the support provided by CD4\(^+\) T cells is more evident in the absence of other arms of the immune system, as \(\mu\)MT were even more susceptible to mortality, where recruitment of CD8\(^+\) T cells to the lung was reduced, suggesting that CD8\(^+\) T cells required CD4\(^+\) T cell help (Mozdzanowska et al. 2000; Riberdy et al. 2000).

IFN\(\gamma\) production by both CD8\(^+\) and CD4\(^+\) T cells is also elevated during influenza infection (Roman et al. 2002). IFN\(\gamma\) can induce macrophage activation, upregulate the expression of MHC class I and II molecules and activate NK cells (Welsh et al. 1991), possibly contributing to the innate clearance of virus, however, there have been mixed reports concerning the relevance of IFN\(\gamma\) in mediating viral clearance. Experiments carried out in vitro have reported no requirement for IFN\(\gamma\) with reports suggesting that in the absence of IFN\(\gamma\) in vitro CD4\(^+\) T cells acquire direct lytic ability (Graham et al. 1993). In contrast, in vivo studies have indicated that IFN\(\gamma\) from CD4\(^+\) T cells enhances survival and IFN\(\gamma\) deficient CD8\(^+\) T cells caused greater immune pathology in the lungs (Wiley et al. 2001). A recent study has also demonstrated that IFN\(\gamma\) signalling, acting on T cells themselves, regulates trafficking CD8\(^+\) T cells from the lymph node to the lung (Turner et al. 2007).

In summary, both CD4\(^+\) and CD8\(^+\) T cells respond to influenza infection with known kinetics and migrate into the site of infection and inflammation, the lung, where they exert their effector functions. This well characterised model provides a way in which to characterise the migration of T cells within WT and LAP mice, with a view to utilising these mice to study the location of action of Treg.
1.9. **Aims and Objectives**

CD4+CD25+ regulatory T cells are a subset of T cells that have been shown to suppress T cell responses both *in vitro* and *in vivo*. Recent evidence has suggested that Treg may also inhibit other non-T cell responses. One aim of this thesis is to assess whether the suppressive effect of Treg is confined to T cells or whether the cells are capable of inhibiting innate immune responses. To achieve this aim a model of tumour rejection involving a melanoma cell line expressing Fas ligand (B16FasL) was utilised. B16FasL induces an inflammatory response and rejection is thought to involve innate immune cells, particularly neutrophils and macrophages.

The first objective of this study was to elucidate the nature of the innate immune response to B16FasL following *in vivo* challenge of mice with the tumour cells. These experiments are described in Chapters 3 and 4. The second objective was to study the effect of Treg upon these responses. These experiments are also described in Chapters 3 and 4.

The ability of Treg to inhibit immune responses, T cell or otherwise, is dependent on the ability of Treg to locate and act upon target cells. The mechanisms of Treg suppression described so far require cell contact or are dependent on short range immunosuppressive cytokines indicating that Treg are most likely to exert their suppressive effect locally. The location of Treg action will determine the cells with which Treg are in close contact, highlighting the possible cellular targets. Furthermore, identification of the location of Treg action may also indicate the stage at which Treg are effective. For instance, if confined to the lymph nodes Treg may only inhibit initiation and priming of an immune response and not the effector phase. Another aim of this thesis is to identify the location of Treg action *in vivo*.
To achieve this aim CD62L transgenic mice were utilised. CD62L is a lymphoid homing marker, downregulation of which is thought to allow access to peripheral tissues. It was hypothesised that T cells from LΔP mice, which maintain CD62L expression post activation in vitro, would fail to migrate into the periphery during an inflammatory response. Therefore LΔP mice potentially present a model in which to address the hypothesis that maintained CD62L expression stops Treg access to peripheral tissue subsequently preventing exertion of their suppressive effects.

Therefore, the third objective of this study was to analyse the suitability of this mouse strain for studies of Treg activity in vivo. To fulfil this objective, the in vivo migration of T cells was studied using a model of influenza infection, where the migration of wild-type T cells is already well described. These experiments are described in Chapters 5.

In summary, the work presented in this thesis describes investigations into the effect of Treg on cells involved in the innate immune rejection of B16FasL. Subsequently, this study explored the use of CD62L transgenic mice as a model to investigate the location of Treg action.
Chapter 2 - Materials and Methods

2.1. Mice

C57BL/6 (B6) and C57BL/6 Rag 1 deficient mice (RAG<sup>+</sup>) purchased from Harlan (Oxford, UK) or bred at Biomedical Services (Cardiff, UK) were maintained at Biomedical Services (Cardiff, UK). WT and LAP mice were generated as previously described (Galkina et al. 2003), bred at NIMR (London, UK), and maintained at Biomedical Services (Cardiff, UK). Mice were housed in Filter-top cages throughout experimental procedures unless infected with virus when animals were housed in Scantainers. All experiments were performed in compliance with Home Office regulations.

2.2. Cell Culture

2.2.1. Tumour Cell Lines

B16F10 (B16) and B16F10 transfected with Fas Ligand (B16FasL) were generated as previously described (Simon et al. 2002) and were maintained in R10 which consists of RPMI 1640 medium (Gibco - Invitrogen, Carlsbad, USA) supplemented with 10% foetal calf serum (FCS) (Gibco-Invitrogen, Carlsbad, USA), penicillin-streptomycin, L-glutamine, non-essential amino-acids (Life Technologies- Invitrogen, Carlsbad, USA) and 50μM of 2β-mercaptoethanol (βMe) (Sigma-Aldrich, St Louis, USA). In the case of B16FasL, G418 was added to the media at a final concentration of 1.5mg/ml in order to maintain expression of FasL. Tumour cells were either injected subcutaneously (10<sup>5</sup> in 100μl of PBS) or intraperitoneally (2x10<sup>6</sup> in 100μl of PBS). Tumour growth was
monitored in mice weekly by parting the hairs and using callipers to measure length (l) and width (w) of tumour. Tumour volume was calculated according to the following equation: \( \frac{2\pi lw}{6} \).

2.2.2. Other Cell Lines

Yac-1 (TIB-160; American Type Culture Collection, Teddington UK), RMA, and RMA-S cells were also maintained in R10. TK cells were maintained in DMEM medium (Gibco-Invitrogen, Carlsbad, USA) supplemented with 10% foetal calf serum (FCS), penicillin-streptomycin, L-glutamine, and non-essential amino-acids (D10).

2.2.3. Hybridomas and In Vivo Depletion

Hybridomas secreting CD25- (PC61, rat IgG1 (Lowenthal et al. 1985)), E. coli β-galactosidase- (GL113, rat IgG1, isotype control), NK1.1- (PK136, mouse IgG2a (Koo and Peppard 1984)), Gr-1- (RB6-8C5, rat IgG2b (Seino et al. 1998)) and TGFB- (1D11, mouse IgG1 (Dasch et al. 1989)) specific monoclonal antibodies (mAbs) and their efficiency to deplete their respective cell subset have been described previously (Table 2.1). Hybridomas were grown using the CELLine CL1000 system (INTEGRA Biosciences Chur, Switzerland) with R10 used in the cell compartment and R0 (R10 without FCS) used in the media compartment and cell compartment supernatant collected for monoclonal antibody (mAb) purification every 7 days.

Administration frequency and quantity of depleting antibody used was dependant on target cell turnover. For short-term experiments (<3 days), 0.5mg of each antibody was injected i.p. 1 day prior to tumour inoculation. For tumour rejection experiments (>60 days), 0.5mg of PC61, GL113 and/or PK136, were administered i.p. 1 and 3 days prior to tumour inoculation. Due to the turnover of Neutrophils, 300µg of RB6-8C5 was
administered every second day from 1 day prior to tumour inoculation. Macrophage depletion was achieved by injection of 1mg of Carrageenan resuspended in PBS i.p. 1 and 3 days prior to tumour inoculation and 3 days later.

Table 2.1. Agents Used in Depletion Experiments
Details of antibodies and other agents used to deplete indicated cell types *in vivo* are summarised below.

<table>
<thead>
<tr>
<th>Depleting Agent</th>
<th>Species and Isotype</th>
<th>Target</th>
<th>Used to deplete</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC61</td>
<td>Rat IgG1</td>
<td>CD25</td>
<td>Regulatory T cells,</td>
</tr>
<tr>
<td>GL113</td>
<td>Rat IgG1</td>
<td><em>E. coli</em> β-galactosidase</td>
<td>None – isotype control</td>
</tr>
<tr>
<td>PK136</td>
<td>Mouse IgG2a</td>
<td>NK1.1</td>
<td>NK Cells</td>
</tr>
<tr>
<td>RB6-8C5</td>
<td>Rat IgG2b</td>
<td>Gr-1 (Ly6G)</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>Carrageenan</td>
<td></td>
<td>Phagocytes</td>
<td>Macrophages</td>
</tr>
</tbody>
</table>
2.3. **Cell Isolation**

2.3.1. Mouse CD4⁺CD25⁺ and CD4⁺CD25⁻ cells

CD4⁺CD25⁺ T cells were purified by negative selection using Dynabeads and subsequent positive selection using MACS beads. Spleen and lymph node cell suspensions prepared from naïve C57BL/6 mice were resuspended at 10⁸ cells/ml in HBSS (Gibco- Invitrogen, Carlsbad, USA) and mixed at a 2:1 ratio (volume/volume) with an antibody cocktail containing 10 µg/ml rat anti-B220, -Mac-1, -CD8, -MHC class II and -NK1.1 antibodies in HBSS/0.1% BSA in order to enrich CD4⁺ cells. After a 20-minute incubation on ice the cells were washed twice in HBSS and Dynabead-conjugated sheep anti-rat IgG antibodies (Dynal - Invitrogen, Carlsbad, USA) were added at a ratio of 1 Dynabead per spleen cell. After a further 20 minute incubation at 4°C, Dynabead-bound cells were magnetically separated according to the manufacturer’s instructions. Dynabead bound cells were discarded and a quarter of the original number of Dynabeads were added to the cells and a second round of negative selection was performed as above. Cells that were not Dynabead bound (enriched for CD4⁺ cells) were subsequently incubated with anti-CD25 antibodies conjugated to R-Phycoerythrin (PE) (Miltenyi Biotec, Bergisch Gladbach, Germany) and purified using microbeads conjugated to anti-PE antibodies according to the manufacturers instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Alternatively CD4⁺CD25⁺ and CD4⁺CD25⁻ were purified using the MACS purification kit according to manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were then either injected intravenously (i.v.) into mice or used *in vitro*. A purity of greater than 90% CD4⁺CD25⁺ cells was obtained in all experiments (See Appendix Figure A.3).
2.3.2. Mouse Neutrophils (PMN)

Bone marrow was collected from naïve mice and PMN isolated by density centrifugation. Ends of bones were removed and marrow flushed out using a 27 gauge needle and syringe filled with RPMI 1640 medium. Cells were collected by centrifugation 350 x g for 5min, resuspended and red blood cells lysed by addition of 3ml 0.2% NaCl followed immediately by 3ml 1.6% NaCl. Cells were washed immediately in RPMI 1640 medium and cells resuspended at 2ml/mouse. A Percoll (Amersham Biosciences-GE Healthcare Little Chalfont, UK) gradient was prepared as follows: Stock Percoll solution was prepared by adding 9 volumes of Percoll to 1 volume of 10x PBS. This was then used to generate solutions of 72%, 64% and 52% Percoll diluted in PBS. 2.5ml of each was then layered into a 15ml tube using a 21G needle and 5ml syringe. 2ml of the bone marrow preparation was then layered on top and the gradient centrifuged at 1500 x g for 30min at room temperature with no brake. The lower ring of cells constituted mainly mature neutrophils and was collected with minimal Percoll using a Pasteur pipette. Cells were then washed twice in >10ml R10 and used immediately. Purity of PMN was assessed by Giemsa staining of methanol fixed cells on positively charged slides and was greater than 90%.

2.3.3. Mouse Dendritic Cells

Dendritic Cells were prepared from bone marrow collected from limbs of B6 mice aged between 6 and 8 weeks. Ends of bones were removed and marrow flushed out using a 27 gauge needle and syringe filled with RPMI 1640 medium. Cells were collected by centrifugation 350 x g for 5min, resuspended and red blood cells lysed by addition of 1ml RBC lysis buffer (Biolegend, San Diego USA) for 5min at room temperature. Cells were washed immediately in RPMI 1640 medium and passed through a cell strainer. The total cells from 1 mouse were then resuspended in 50ml of R10 supplemented with 200U/ml
recombinant mouse Granulocyte Macrophage-Colony Stimulating Factor (rmGM-CSF, Peprotech, Rocky Hill, USA) and plated at 1ml per well of a 24 multi-well (MW) plate. On day 3 of culture, non adherent cells were gently removed and fresh R10 added. DC were immature and ready for experimentation from Day 4. For DC maturation positive control 2ug/ml of LPS (Sigma-Aldrich, St Louis, USA) was added to wells.

2.3.4. Mouse Peritoneal Lavage Cells

Mice were injected i.p. with 2x10^6 B16F10 or B16FasL. The peritoneal lavage cells were collected by injecting 6ml PBS with 2mM EDTA and 0.5% BSA into the peritoneum of sacrificed mice at the indicated times after tumour inoculation. 6ml of fluid was recovered in every case. Cells were counted and used for flow cytometry, cytospin and/or Chromium release assay.

2.3.5. Human Neutrophils (PMN)

Polymorphonuclear Cells (PMN) from blood of healthy donors were collected in heparin and separated by density centrifugation. 6ml of 6% w/v Dextran 70 (Fisher Scientific UK, Loughborough, UK) resuspended in 0.9% NaCl was gently mixed with 24ml of fresh blood and RBC allowed to sediment at room temperature for 45-60min. The plasma and white blood cells (WBC) were layered on top of 20ml lymphoprep (Axis-Shield, Oslo, Norway) and centrifuged at 700 x g for 25min at room temperature with no brake. After centrifugation, all liquid was aspirated and the neutrophils and remaining RBC in the pellet resuspended in 1ml H_2O for 20-30sec to hypotonically lyse RBC. Cells were washed twice with R10 and used immediately. Purity of PMN was assessed by Giemsa staining of methanol fixed cells on positively charged slides and was greater than 95%.
2.3.6. Human CD4⁺CD25⁺ and CD4⁺CD25⁻ cells

Peripheral Blood Mononuclear Cells (PBMC) from blood of healthy donors were collected in heparin and separated by density centrifugation. Fresh Blood was layered on top of an equal volume of lymphoprep and centrifuged at 700 x g for 25min at room temperature with no brake. After centrifugation, PBMC were located at the interphase between the plasma and lymphoprep and were gently removed using a Pasteur pipette. Cells were washed twice in R10 and counted. CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were then purified using the MACS purification kit according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were then immediately used \textit{in vitro}. A purity of greater than 90% for CD4⁺CD25⁺ cells was obtained in all experiments.

2.4. Antibodies

2.4.1. Purification of Endotoxin Free Monoclonal Antibodies for \textit{In-Vivo} Use

All glassware and other materials were treated with 0.5M NaOH for 20 min to remove all traces of endotoxin and sterile, tissue culture grade plastics were used throughout. Buffers were also made up with highly purified AnalR water (BDH-VWR International, West Chester, USA). Cell debris was removed from hybridoma supernatant by centrifugation at 6000 x g for 30min and the protein in the supernatant precipitated by addition of an equal volume of saturated ammonium sulphate (660g in 1L of AnalR water) for 16 hours at 4°C. The protein was collected by centrifugation at 6000 x g for 30min and resuspended in minimal AnalR water and the process repeated. The protein solution was then dialysed against 3 x 2L PBS with intervals of greater than 6 hours. The resulting protein solution was then run on a denaturing protein gel (SDS-PAGE (Laemmli 1970)) to evaluate the purity.
2.4.2. Endotoxin Free, Azide Free, Antibodies for In Vitro Stimulation

Endotoxin free, azide free mAbs raised against CD28 (37.58) and CD3 (50A2) were purchased from Leinco Technologies Inc. St. Louis, USA.

2.5. Cell Identification

2.5.1. Fluorescent Staining

Anti -CD8α-FITC, -IFNγ-FITC, -CD11b-FITC, -CD62L-FITC, -CD25-PE, -βTCR-PE, -CD4-Alexa Fluor 610, -IFNγ-Alexa Fluor 610, -CD8α-Alexa Fluor 647, -CD4-Alexa Fluor 647, -F4/80-APC and biotinylated anti-MHC Class II antibodies were purchased from Caltag Laboratories, Burlingame, USA, as were rat IgG1 and IgG2a isotype control antibodies. Anti -CD86-FITC, -CD80-FITC, -CD107a-FITC, -NK1.1-FITC (PK136), -NK1.1-PE (PK136), -NK1.1-purified (3A4), -CD8α-PerCpCy5.5, -Gr-1- PerCpCy5.5, -CD11c-APC, -CD16/CD32 antibodies and Streptavidin PerCpCy5.5 were purchased from BD Pharmingen, Franklin Lakes, USA, as were rat IgG2b, mouse IgG2a and hamster IgG isotype control antibodies. PE conjugated MHC class I tetramers were produced within the laboratory. Samples were also stained using an anti-FOXP3-PE staining kit (Ebioscience, San Diego, USA).

Extracellular Staining

All staining and washes were carried out in FACS buffer (PBS + 2% FCS + 2mM EDTA) and on ice to minimise antibody internalisation. Single cell suspensions were obtained, and non-specific binding of Fc limited by incubation with 0.25μg of anti-CD16/CD32 (Fc blocking antibody 2.4G2) per 10^6 cells in 25μl, for 20 min. Cells were then washed twice. If required, 0.1μg of MHC class I tetramer in 25μl was added to cells 15 min prior to
addition of staining antibodies. Cells were then incubated with 0.25µg of antibody per $10^6$ cells in 25µl of FACS buffer for 20min. In cases where directly conjugated antibodies were not used, immunoglobulin-specific secondary antibodies were added after 2 washes. Cells were then fixed in FACS fix (FACS buffer +2% Formalin) and analysed by flow cytometry (FACS Calibur, BD, Franklin Lakes, USA). Cell Quest Pro and Flowjo were used to analyse the resulting data.

**Intracellular Staining**

Any cell surface staining required was performed as described above. Intracellular staining was then performed using Cytofix/Cytoperm Kit (BD Pharmingen, Franklin Lakes, USA) according to the manufacturer's instructions. Cells were resuspended in FACS fix and analysed by flow cytometry (FACS Calibur). Cell Quest Pro and Flowjo were used to analyse the resulting data.

**2.5.2. Cytospin of Lavaged Cells**

Cytofunnels were assembled as described in the manufacturer instructions. 240µl of lavage fluid was added to the Cytofunnel assembly, consisting of funnel, absorbent card, glass slide and metal clamp. The Cytofunnel was then placed in the Cytospin for 10 minutes at 1000 r.p.m. Slides were then air dried and stained using a Wright–Giemsa stain, rinsed in deionised water and allowed to air dry.
2.6. **Histology**

2.6.1. **Paraffin Embedded Sections**

Tissue collected for histology was fixed in Zinc fixative (0.1M Tris HCl [pH 7.4] with 0.05% Ca acetate, 0.5% Zn acetate and 0.5% Zn chloride) (Beckstead 1994) for 24-72hrs at 4°C. Tissue was then embedded in paraffin wax and 5μm sections cut and placed on positively charged glass slides. Sections were then either stained with haematoxylin and eosin (H&E) or subjected to immunohistochemistry.

2.6.2. **Cellular Mass Size Determination**

Sections of injected skin were taken every 300μm and stained with H&E. The diameter (D) and width (W) of infiltrate on each section was measured on a calibrated microscope and an estimate made of the total tumour volume based on Figure 2.1.

2.6.3. **Immunohistochemistry**

**Macrophage/Monocyte marker – F4/80**

Sections were dehydrated over 30min by washing in xylene followed by ethanol and finishing in water. Sections were equilibrated in Tris-buffered saline (TBS) for 30min prior to blocking with TBS supplemented with 1% bovine serum albumin (BSA) and 2% rat serum (Blocking buffer) for 30min. Blocking buffer was removed and primary antibody, rat anti-mouse F4/80 (Abcam, Cambridge, UK) diluted 1:500 in blocking buffer, added overnight at 4°C in a humidity chamber. Slides were rinsed with TBS and washed in TBS/1%BSA for 10min followed by incubation with the secondary antibody, Alkaline Phosphatase (AP) conjugated goat anti-rat (Abcam, Cambridge, UK) diluted 1:100 in TBS/1%BSA, for 1hr at room temperature. Slides were rinsed with TBS and washed in TBS/1%BSA for 10min followed by incubation with the tertiary antibody, AP
The volume of the shape below is: \( \frac{1}{3} \pi (r_1 + r_2 + r_1 \cdot r_2) \)

The circle at either end represents the serial sections through the skin with the distance between the sections (h) being 300\(\mu\)m. However the area of cell mass on each section is elliptical in shape with a diameter of (D) and the width (W). To calculate \( r \), it was assumed that area of an ellipse with a diameter of D and a width of W is equal to that of a circle with a radius of \( r \):

Area of an ellipse = \( \pi D W \) and Area of a circle = \( \pi r^2 \) so \( \pi D W = \pi r^2 \)

Therefore \( r = \sqrt{\frac{\pi D W}{\pi}} = \sqrt{D W} \)

Therefore the volume of the shape is:

\[
\frac{300\pi(\sqrt{D W} + \sqrt{D W} \cdot \sqrt{D W})}{3} = 100\pi(\sqrt{D W} + \sqrt{D W} + \sqrt{D W} \cdot \sqrt{D W})
\]

Assuming that the cellular mass ends at the midpoint between the last section it is seen on and the next, the total volume would resemble the diagram below and therefore the sum of each shape results in the total volume of the cellular mass.

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**Figure 2.1. Estimating the Total Volume of the Cellular Mass**

Using the assumption and calculations shown, an estimate of the total volume of cellular mass can be calculated from the diameter (D) and the width (W) of cellular mass on each section.
conjugated mouse anti-AP (Sigma-Aldrich, St Louis, USA) diluted 1:200 in TBS/1%BSA, for 30min at room temperature. Slides were rinsed with TBS and washed in TBS/1%BSA for 10min. Sections were then developed using Sigma-Fast Fast-Red (Sigma-Aldrich, St Louis, USA) according to manufacturer’s instructions and counterstained with haematoxylin.

**Neutrophil marker – IL-8R**

Sections were dehydrated over 30min by washing in xylene followed by ethanol and finishing in water. Slides were then microwaved for 3 x 5min in 10mM Citrate Acid pH 6 and allowed to cool naturally. Sections were equilibrated in PBS for 30min prior to blocking of peroxidase activity with 1% H₂O₂ for 5mins. Non-specific antibody binding was blocked by incubation with PBS supplemented with 1% bovine serum albumin (BSA) and 2% rabbit serum (Blocking buffer) for 30min. Blocking buffer was removed and primary antibody, rabbit anti-mouse IL-8RB (K-19) (Santa Cruz Biotechnology, Santa Cruz, USA) diluted 1:200 in blocking buffer, added overnight at 4°C in a humidity chamber. Slides were rinsed with PBS and washed in PBS/1%BSA for 10min, followed by incubation with the secondary antibody; biotinylated swine anti-rabbit (Dako, Glostrup, Denmark) diluted 1:300 in PBS/1%BSA, for 1hr at room temperature. Slides were rinsed with PBS and washed in PBS/1%BSA for 10min followed by incubation with the tertiary antibody, horseradish peroxidase (HRP) conjugated Extravidin (Sigma-Aldrich, St Louis, USA) diluted 1:50 in PBS/1%BSA, for 30min at room temperature. Slides were rinsed with PBS and washed in PBS/1%BSA for 10min. Sections were then developed using a DAB substrate kit (VectorLabs, Burlingame, USA) according to manufacturer’s instructions and counterstained with haematoxylin.
2.7. Influenza Virus

2.7.1. Preparation of Influenza Virus Stocks

Recombinant influenza A virus strain E61-13-H17 (H17;H3N2), amplified in embryonated chicken eggs, was generated by Dr Rosa Gonzalves, and Dr John Skehel from National Institute for Medical Research London. The virus was titrated from allantoic fluid by performing a haemagglutination assay.

2.7.2. Infection

Mice were infected intra-nasally (i.n.) with 20 haemagglutination units (HAU) of Influenza virus H17 in 20μl of PBS. 8 days post-infection, perfused lungs, lung draining lymph nodes (LDLN), blood and spleen were harvested for immunostaining and/or functional assays.

2.8. Vaccinia Virus

Recombinant Vaccinia Virus (rVV) expressing a MHC class I-restricted peptide epitope (NP68) derived from the influenza nucleoprotein (rVVNPp) has previously been described by (Townsend et al. 1988) The control rVV expressed a MHC class I-restricted peptide epitope from melanoma antigen Trp2 (rVVTrp2) (Overwijk et al. 1998).

2.8.1. Preparation of Recombinant Vaccinia Virus Stocks

Expansion of rVV was carried out in TK⁻ cells. An 80% confluent monolayer of TK⁻ cells was infected with $10^7$ plaque forming units (pfu) in 5ml of VDM (D10 supplemented with 0.05% bovine serum albumin (BSA)). Cells were incubated for 2 hours at 37°C then an additional 15ml of D10 was added for 48 hours. Cells were subsequently harvested.
and subjected to three freeze thaw cycles. Cell debris was removed by centrifugation and Vaccinia titres in the supernatant determined by plaque assay.

2.8.2. Infection

Mice were injected intraperitoneally (i.p.) with 50μl of rVV at 10^8 pfu/ml. At days 1, 3, 5, and 8 post-infection, perfused lungs, lung draining lymph nodes (LDLN), ovaries, ovary draining lymph nodes (ODLN), blood and spleen were harvested for viral titres, immunostaining and/or functional assays.

2.8.3. Vaccinia Virus Titres

Stock rVV and infected tissue titres were determined by plaque assay on a monolayer of TK^- cells. Tissue was homogenized and debris removed before serial dilution in D10. TK^- cells in a 24 MW plate were infected with 200μl of the dilutions for 2 hours at 37°C. 1ml of D10 was then added to each well for 24 hours before staining with Giemsa. Pfu per sample was calculated after enumeration of plaques in wells with between 30 and 300 plaques.

2.9. Determination of Functional Capacity

2.9.1. Chromium Release Assay

The chromium (^{51}Cr) release assay assesses the ability of effectors to lyse particular targets and is used in this thesis to measure the activity of NK cells, neutrophils and CD8^+ T cells.
Peritoneal Lavage Killing Assay

Mice were injected i.p. with 2x10^6 B16F10 or B16FasL. The peritoneal lavage was collected using PBS with 2mM EDTA and 0.5% BSA at the indicated times after tumour inoculation. Cells were counted and resuspended in RPMI at 5x10^6/ml. 3 fold dilutions of cell suspensions were made in duplicate in a 96 MW plate. 10^4 51Cr labelled B16 or B16FasL cells were added to wells for 4-5hr at 37°C. For minimal and maximal lysis, cells were incubated with medium or 5% Triton X100 respectively. Lavage activity was measured by 51Cr release with the formula; % lysis = [(sample - min)/(max - min)] x100.

Neutrophil Killing Assay

PMN from healthy human donors or mouse bone marrow were collected and resuspended in RPMI. Double dilutions of PMN were made in duplicate in a 96 MW plate at indicated ratios. 10^4 51Cr labelled B16 or B16FasL cells were added to wells for 4-5hr/18-20hr at 37°C. For minimal and maximal lysis, cells were incubated with medium or 5% Triton X100 respectively. Lavage lytic activity was measured by 51Cr release with the formula; % lysis = [(sample - min)/(max - min)] x100.

CTL Killing Assay

4x10^6 spleen cells were stimulated in vitro with 1x10^6 NP68 (ASNENMDAM - Research Genetics- Invitrogen, Carlsbad, USA) peptide loaded (10^-5M), irradiated splenocytes in a 24 MW plate. 10U/ml of rIL-2 was added at day 2. On day 5, cells from 4 wells were collected washed and resuspended in 600μl RPMI. 3 fold dilutions of cell suspensions were made in duplicate in a 96·MW plate. 10^4 51Cr labelled NP68/control peptide loaded B16 cells were added to wells for 4hr at 37°C. For minimal and maximal lysis, cells were
incubated with medium or 5% Triton X100 respectively. CTL activity was measured by 
$^{51}$Cr release with the formula; \( \% \text{ lysis} = \frac{(\text{sample} - \text{min})/(\text{max} - \text{min})}{100} \).

2.9.2. IFNγ and CD107a Staining

Intracellular Staining

Tissues from mice were mechanically dissociated and cells incubated for 4hr at 37°C in 
the presence of 3μM monensin (Sigma-Aldrich, St Louis, USA). During this time cells 
were stimulated with either; 1μg/ml ionomycin and 20ng/ml PMA (Sigma-Aldrich, St 
Louis, USA), or NP68/irrelevant peptide. For intracellular CD107a staining anti-CD107a 
was also added during stimulation at 1:500. Surface and intracellular stains were then 
carried out as described above.

Surface Staining

Highly activated cells from infected mice could be stained directly ex-vivo with anti-
IFNγ and anti-CD107a as described for extracellular staining

2.9.3. Statistical Analysis

All statistical analyses were performed using GraphPad Prism 3.0. Where there was no 
statistically significant difference the data is marked ns. For \( p \) values less than 0.05 data is 
marked with *, if less than 0.01 it is marked with **, and differences with a \( p \) value of 
less than 0.001 are marked with ***.
Chapter 3 – Innate Immune Responses to B16FasL are Inhibited by CD4⁺CD25⁺ Regulatory T Cells

3.1. Introduction

CD4⁺CD25⁺ regulatory T cells (Treg) are a subset of T cells that have been shown to suppress T cell responses both in vitro and in vivo, as described in detail in the Introduction. One aim of this thesis is to assess whether the suppressive effect of Treg is confined to T cells or whether the cells are capable of inhibiting other non T cell responses. This Chapter describes experiments performed to assess the ability of Treg to inhibit innate immune responses.

The hypothesis that Treg inhibit innate immune responses was first formulated on the basis of an observation made in the laboratory using the tumour cell line, B16. In these experiments, RAG⁻/⁻ mice were injected with CD4⁺CD25⁺ T cells or control CD4⁺CD25⁻ cells, both from naïve mice, followed by inoculation of B16 tumour cells. Although all mice grew tumours, tumours grew more rapidly in mice receiving Treg compared to mice receiving the control cell population, suggesting that there is a degree of tumour control exerted by the innate immune system which is suppressed by adoptive transfer of Treg.

In a model of tumour rejection involving a melanoma cell line expressing Fas ligand (B16FasL), in which 50% of B6 mice inoculated subcutaneously are capable of tumour rejection (Simon et al. 2002), rejection is thought to be largely T cell independent and accompanied by an inflammatory infiltrate at the site of tumour inoculation (Seino et al. 1997; Chen et al. 1998a). A number of FasL expressing tumour cell lines have been shown to be more susceptible to rejection in vivo than their parental cell lines, and studies
of intraperitoneally injected FasL<sup>+</sup> tumours indicate a major role for the innate immune response, particularly neutrophils and macrophages, in the rejection process (Hohlbaum et al. 2001; Chen et al. 2003b). Recent studies show that in this system membrane-bound FasL is a potent mediator of inflammation (Hohlbaum et al. 2000) and neutrophil apoptosis, mediated via FasL expression, is crucial for the induction of inflammation (Shimizu et al. 2001). Overall, these data indicate that B16FasL induces an inflammatory response and may therefore represent an ideal model in which to study the effects of Treg on innate immune responses.

This Chapter first describes experiments that characterise the innate immune response to the melanoma cell line B16FasL, and then investigates the hypothesis that Treg inhibit this response. Subsequent investigations will focus on the effect of Treg on individual populations of innate immune cells.
3.2. Results

3.2.1. The Innate Immune Response is Sufficient for B16FasL Rejection

To address whether Treg impinge on innate immune responses, it was essential to characterise the immune response to B16FasL. Numerous studies have shown that enhanced rejection of FasL expressing tumour cell lines coincides with the induction of a pronounced inflammatory response (Seino et al. 1997; Chen et al. 1998a; Hohlbaum et al. 2001; Chen et al. 2003b). To determine whether this innate immune response is sufficient for B16FasL rejection, these cells were injected s.c. into both B6 and RAG\(^{-/-}\) mice which lack T and B cells. As described previously (Simon et al. 2002), approximately 50% of B6 mice were able to reject the tumour challenge. Interestingly RAG\(^{-/-}\) mice were equally able to reject B16FasL (Figure 3.1), indicating that the innate immune response can result in tumour rejection in the absence of B cells and T cells.

3.2.2. NK cells and Macrophages are Important for B16FasL Rejection

Having determined that the innate immune response is sufficient for B16FasL rejection, experiments were undertaken to examine which cells were responsible for this tumour rejection by depleting neutrophils, macrophages and NK cells in vivo prior to tumour inoculation. Neutrophil and NK cell depletion with anti-Gr-1 (RB6-8C5) and anti-NK1.1 (PK136) depleting antibodies has previously been described (Koo and Peppard 1984; Seino et al. 1997), as has depletion of macrophages with carrageenan (Seino et al. 1997). To check effective depletion in this model, mice
Figure 3.1. RAG\(^{-/-}\) and B6 Mice are Equally Able to Reject B16FasL Tumour Challenge

B6 and RAG\(^{-/-}\) mice were injected s.c. with \(10^5\) B16FasL and tumour growth monitored over 100 days. Bars indicate mean percentage rejection±SEM of 3 experiments, with the number of tumour free mice/total number of mice injected indicated at day 100.
treated with each depleting agent were monitored for the presence of these cell types (Figure 3.2A&B). Neutrophils were identified as SSC\textsuperscript{hi}CD11b\textsuperscript{+}Gr-1\textsuperscript{hi}F4/80\textsuperscript{+}, macrophages as SSC\textsuperscript{int}CD11b\textsuperscript{+}Gr-1\textsuperscript{lo-int}F4/80\textsuperscript{+} (see Appendix Figure A.1) and NK cells as SSC\textsuperscript{lo}NK1.1\textsuperscript{+}. Depletion of neutrophils was reflected by the total number of cells recovered from the lavage, which was reduced by approximately 30% in B16FasL treated mice (from 4.68\times10^6 \pm 1.49\times10^6 to 1.58\times10^6 \pm 0.25\times10^6). Depletion of NK cells was confirmed by staining with a different monoclonal antibody also thought to recognise NK1.1 (3A4). Depletion of each cell type was achieved for 10 days using the regime described in Materials and Methods. Figure 3.3A shows that depletion of macrophages and NK cells in B6 mice inhibits rejection of B16FasL, whereas depletion of neutrophils had no effect on rejection. In RAG\textsuperscript{-/-} mice, neutrophils and NK cells were important for B16FasL rejection (Figure 3.3B). Depletion of macrophages in RAG\textsuperscript{-/-} mice reduced the number of tumour free mice but not to a statistically significant level.

3.2.3. Peritoneal Challenge Model

The data described above indicate that innate immune cells are sufficient for B16FasL rejection. However, functional analysis of cells from the site of subcutaneous tumour challenge is hampered because isolation of sufficient viable cells is difficult. It is for this reason that an in vivo peritoneal tumour cell challenge model was established.

3.2.4. Neutrophils, NK cells and Macrophages are Recruited upon Intraperitoneal Injection of B16FasL

To identify the cells recruited to the tumour site, B16FasL was injected into the peritoneum of B6 mice and immune cells recruited to the site collected by peritoneal lavage 18 hours later. The lavage was then evaluated by flow cytometry for its cellular
Figure 3.2. Identification and Depletion of Neutrophils, NK cells and Macrophages

The presence or absence of neutrophils, NK cells and macrophages can be determined by flow cytometry. (A) Cells were identified by forward (FSC) and side (SSC) scatter, and by antibody binding to CD11b, NK1.1, Gr-1 and F4/80: SSC$^{hi}$CD11b$^+$Gr-1$^{hi}$F4/80$^+$ (neutrophils); SSC$^{lo}$NK1.1$^+$ (NK cells); SSC$^{int}$CD11b$^+$Gr-1$^{lo-int}$F4/80$^{hi}$ (resident macrophages) SSC$^{int}$CD11b$^+$Gr-1$^{int}$F4/80$^{int}$ (recruited macrophages). (B) Mice were depleted of neutrophils, NK cells and macrophages by treatment with RB6-8C5, PK136 and carrageenan respectively and the presence of cells determined by FACS analysis. FACS plots are representative of 10 mice per group.
Figure 3.3. Depletion of Neutrophils, NK cells and Macrophages

B6 and RAG\(^{-/-}\) mice were depleted of neutrophils, NK cells and macrophages by treatment with RB6-8C5, PK136 and carrageenan respectively. Mice were challenged s.c. 1 day later with \(10^5\) B16FasL and tumour growth monitored over 60 days. Bars indicate mean percentage rejection ± SEM of 3 experiments, with the number of tumour free mice/total number indicated. Statistical significance was evaluated by Fisher’s Exact test (* \(p<0.05\)).
B6 mice were either challenged i.p. with 2 x 10^6 B16FasL [closed symbols] or an equal volume of PBS [open symbols]. 18 hours later, the peritoneum was lavaged and collected cells analysed by flow cytometry. Each symbol represents the percent of live cells in one mouse, with the mean indicated by the line. Statistical significance was determined by Mann-Whitney test (* p<0.05).

Figure 3.4. Neutrophils, NK cells and Macrophages are Recruited Following Intraperitoneal Injection of B16FasL

B6 mice were either challenged i.p. with 2 x 10^6 B16FasL [closed symbols] or an equal volume of PBS [open symbols]. 18 hours later, the peritoneum was lavaged and collected cells analysed by flow cytometry. Each symbol represents the percent of live cells in one mouse, with the mean indicated by the line. Statistical significance was determined by Mann-Whitney test (* p<0.05).
composition. Neutrophils, NK cells and macrophages were recruited to the peritoneum following injection of B16FasL (Figure 3.4).

**3.2.5. NK Cells Isolated from the Peritoneum of B16FasL Challenged Mice Can Lyse Tumour Cells**

In order to assess the anti-tumour activity of lavage cells, cells from the peritoneum of B16FasL challenged mice were used as effectors against chromium labelled tumour cells. Interestingly, lavage cells were able to lyse B16 (Figure 3.5A) and B16FasL *ex vivo*. The action of both neutrophils and NK cells can be cytolytic so it was hypothesised that these cell types may be responsible for the lytic activity of lavage cells. To investigate this, B6 mice were depleted of neutrophils and NK cells *in vivo* using RB6-8C5 and PK136 respectively (Figure 3.5B). *In vivo* depletion of NK cells but not neutrophils reduced the *ex-vivo* lysis of the tumour by the lavaged cells. In order to limit the possibility that depleting antibodies alter lytic activity, depletion of neutrophils and NK cells from the peritoneal lavage was also carried out *ex vivo* (Figure 3.5C). Similarly, depletion of NK cells but not neutrophils *ex vivo* completely abolished tumour lysis, also indicating that other cells do not contribute to tumour lysis in these experiments.

**3.2.6. B16FasL Express NK Cell Activating Ligands**

Since NK cells can receive stimulatory signals through the NKG2D receptor and are known to recognise the absence of MHC class I, B16 and B16FasL were tested for expression of the NKG2D ligand Rae-1 and levels of MHC class I. Both B16 and B16FasL were positive for the murine NKG2D ligand Rae-1 (Figure 3.6). The tumour cells also expressed low levels of MHC class I when compared to spleen cells (Figure 3.6). The data shown here and above indicate that NK cells are recruited to the peritoneum upon B16FasL injection where they are capable of direct tumour lysis.
Figure 3.5. NK Cells in the Lavage of B16FasL Challenged Mice are Capable of Tumour Lysis Ex-Vivo

Mice were challenged i.p. with $2 \times 10^6$ B16FasL. 18 hours later, the peritoneum was lavaged and collected cells used as effectors against chromium labelled B16 (A). (B) 1 day prior to B16FasL challenge mice were depleted in vivo of neutrophils (RB6-8C5) or NK cells (PK136) or remained undepleted. (C) Lavage cells were depleted ex vivo of neutrophils, NK cells or both using magnetic beads, prior to incubation with chromium labelled B16.
Figure 3.5 (cont.). NK Cells in the Lavage of B16FasL challenged mice are capable of Tumour Lysis Ex-Vivo
Mice were challenged i.p. with 2x10⁶ B16FasL. 18 hours later, the peritoneum was lavaged and collected cells used as effectors against chromium labelled B16. (A) Each symbol represents percent tumour lysis for each mouse with the mean indicated by the bar. (B) 1 day prior to B16FasL challenge mice were depleted in vivo of neutrophils (RB6-8C5) or NK cells (PK136) or remained undepleted. Graph shows mean percent tumour lysis ± SEM of 5 mice per group. (C) Lavage cells were depleted ex vivo of neutrophils, NK cells or both using magnetic beads, prior to incubation with chromium labelled B16. Graph shows mean percent tumour lysis ± SEM of 5 mice per group and are representative of 3 independent experiments.
Figure 3.6. B16 and B16FasL Express NKG2D Ligand, Rae-1 and Low MHC Class I

B16 and B16FasL were immunostained with anti-Rae-1, anti-MHC class I [open histograms] or appropriate isotype control antibody [filled histograms], and evaluated by flow cytometry. Similarly stained naive spleen cells are included for reference.
3.2.7. Treg Inhibit Innate Immune Rejection of B16FasL

The experiments described above indicate that the innate immune system is sufficient for rejection of B16FasL. Furthermore, NK cells recruited by B16FasL have been shown to have direct cytotoxic activity. It was now possible to test the hypothesis that Treg inhibit innate immunity. This hypothesis was investigated by depleting Treg in B6 mice prior to B16FasL challenge. Depletion of Treg is accomplished via administration of anti-CD25 depleting antibody, PC61. Administration of 1mg of PC61 results in an approximately 4-fold reduction in the number of CD4+CD25+ splenocytes and an approximately 2-fold reduction in CD4+FOXP3+ splenocytes for around three weeks after injection (Figure 3.7A&B and Appendix Figure A.2). Mice treated with PC61 or isotype control antibody GL113 were then challenged s.c. with B16FasL and tumour growth monitored over 60 days (Figure 3.7C). As expected 50% of GL113 treated mice rejected B16FasL challenge which was enhanced to 100% rejection upon PC61 treatment. Since the data shown previously indicate that B16FasL is rejected by innate immune responses, this data suggests that removal of Treg promotes rejection of the tumour cells by enhancing this response.

This conclusion is supported by further experiments injecting RAG-/- mice with CD4+CD25+ or CD4+CD25- cells purified from naïve B6 mice, or PBS alone, and one day later inoculating them with B16FasL (Figure 3.8). Tumour growth was monitored weekly for at least 100 days. Approximately 50% of the mice inoculated with CD4+CD25- cells or PBS rejected the B16FasL inoculum. However, no rejection was observed in the mice receiving CD4+CD25+ cells indicating that Treg can inhibit the innate immune system. Collectively these data indicate that CD4+CD25+ Treg inhibit innate immune responses that are capable of tumour rejection in B6 and RAG-/- mice.
Figure 3.7. CD25⁺ Cell Depletion Enhances Rejection of B16FasL in B6 mice

Mice were treated with either isotype control antibody (GL113) or anti-CD25 depleting antibody (PC61) and the presence of CD4⁺CD25⁺ and CD4⁺FOXP3⁺ cells evaluated by FACS. Representative FACS plots are given in (A) and the number of cells for each mouse is shown in (B). Statistical significance was evaluated by Mann-Whitney test (** p<0.01, *** p<0.001). Mice were challenged s.c. 1 day later with 10⁵ B16FasL and tumour growth monitored over 100 days (C). Bars indicate mean percentage rejection ± SEM of 3 experiments, with the number of tumour free mice/total number indicated. Statistical significance was evaluated by Fisher's Exact test (*** p<0.001).
Figure 3.8. Adoptive Transfer of CD4+CD25+ Cells Inhibits Rejection of B16FasL in RAG'' Mice

RAG'' mice were injected i.v with either 10^6 purified CD4+CD25+ cells [solid line], 10^6 purified CD4+CD25' cells [dotted line] or an equal volume of PBS [dashed line]. 1 day later, mice were injected s.c. with 10^5 B16FasL and tumour growth monitored over 100 days (A). The numbers of mice in each group are indicated in parentheses. The data is representative of 2 independent experiments summarised in (B). Bars indicate mean percentage rejection ± SEM, with the number of tumour free mice/total number injected indicated. Statistical significance was evaluated by Fisher's Exact test (* p=0.05).
3.2.8. Treg Inhibit NK Dependent Ex Vivo Tumour Lysis by Lavage Cells

Since NK cells were the only cell type shown to be important for tumour rejection in both B6 and RAG⁻/⁻ mice, it was hypothesised that Treg cells inhibit the activity of B16FasL-induced NK cells. The intraperitoneal tumour challenge model showed that NK cells were responsible for tumour lysis and therefore provided a system to study the effect of Treg on NK cell function. To determine whether Treg inhibit NK cell mediated tumour lysis, purified CD4⁺CD25⁺ cells or CD4⁺CD25⁻ cells were injected concomitantly with B16FasL tumour cells into the peritoneum of RAG⁻/⁻ mice. 18 hours later the lavage cells were harvested and used as effectors against chromium labelled B16 and B16FasL. Figure 3.9 shows that the percentage tumour lysis was reduced in mice receiving CD4⁺CD25⁺ cells compared to those receiving CD4⁺CD25⁻ cells. In agreement with this data are the results from B6 mice treated with either GL113 or PC61 with/without PK136 (Figure 3.10). Treatment with PC61 alone enhanced tumour lysis; however in the absence of NK cells, PC61 treatment could not enhance tumour lysis. Together these experiments indicate that the increase in tumour lysis is due to NK cell activity and not activity of another cell type and therefore show that Treg inhibit the activity of NK cells.

3.2.9. Treg Reduce the Percentage of NK cells in the Lavage

Altered NK activity could be the result of altered activity on a per cell basis, altered recruitment or a product of the two. To address this, the proportion of NK cells in the lavage was monitored by flow cytometry. In B6 mice, adoptive transfer of purified CD4⁺CD25⁺ cells reduced the percentage of NK cells when compared to CD4⁺CD25⁻ cells (Figure 3.11). The same experiment in RAG⁻/⁻ mice yielded similar results.
RAG" mice were injected i.p. with 10^6 purified CD4^+CD25^+ cells or CD4^+CD25^- cells. At the same time mice were injected i.p. with 2x10^6 B16FasL. 18 hours later, the peritoneum was lavaged and collected cells used as effectors against chromium labelled B16 and B16FasL. Graph shows mean percent tumour lysis ± SEM of 5 mice per group. Statistical significance was evaluated by Mann-Whitney test (* p<0.05, ** p<0.01).
Figure 3.10. CD25+ Cell Depletion Enhances NK Cell Dependent Tumour Lysis by Lavaged Cells from B16FasL Challenged B6 Mice

B6 mice were treated with either isotype control antibody (GL113) or anti-CD25 depleting antibody (PC61) with/without anti-NK1.1 depleting antibody (PK136) 1 day prior to injection i.p. with 2x10^6 B16FasL. 18 hours later, the peritoneum was lavaged and collected cells analysed by flow cytometry for the presence of NK cells. Symbols represent the percent tumour lysis for each mouse at a ratio of 50 lavage cells : 1 tumour cell. Bars represent the mean tumour lysis ± SEM. Data shown is representative of 3 independent experiments. Statistical significance was evaluated by unpaired t-test (* p<0.05).
Figure 3.11. Adoptive Transfer of CD4+CD25+ Cells Reduces the Percentage of NK Cells in the Lavage of B16FasL Challenged B6 and RAG-/- Mice

B6 and RAG-/- mice were injected i.p. with 10^6 purified CD4+CD25+ cells [open bars] or 10^6 purified CD4+CD25- cells [shaded bars]. At the same time mice were injected i.p. with 2x10^6 B16FasL. 18 hours later, the peritoneum was lavaged and collected cells analysed by flow cytometry for the presence of NK cells. Bars represent the mean percent of lavaged cells that are NK cells (SSC<sub>lo</sub> NK1.1<sup>+</sup>) ± SEM from 5 B6 and 2 RAG-/- mice per group. Statistical significance was evaluated by Mann-Whitney test (** p < 0.01).
however could not be analysed for statistical significance due to the low number of mice used. Furthermore, treatment of B6 mice with PC61 tended to increase the percentage of NK cells in the lavage however it was not statistically significant (Figure 3.12). This data goes some way to support the hypothesis that Treg inhibit NK cell recruitment to the site of B16FasL challenge but further experiments are required to corroborate this observation.

3.2.10. Treg Directly Inhibit NK Dependent *Ex Vivo* Tumour Lysis by Lavage Cells

Experiments were done in collaboration with Dr Simon at Oxford University, to determine whether Treg inhibit NK cell activity on a per cell basis. CD4⁺CD25⁺ cells or CD4⁺CD25⁻ cells purified from naïve B6 mice were either left un-stimulated or stimulated with anti-CD3 antibodies and irradiated antigen presenting cells. These cells were then added to lavage cells obtained from B6 mice injected intraperitoneally with B16FasL at a ratio of 1:1 (Figure 3.13). Un-stimulated cells did not affect tumour lysis (data not shown). Stimulated CD4⁺CD25⁻ cells neither lysed tumour cells directly nor affected tumour lysis when mixed with lavaged cells. Only stimulated CD4⁺CD25⁺ cells were able to inhibit tumour lysis and these cells also inhibited proliferation of CD4⁺CD25⁻ cells in a conventional Treg suppression assay (72.4 ± 4.0 % inhibition at a ratio of 1:1 - Appendix Figure A.3).
Figure 3.12. The Effect of CD25+ Cell Depletion on the Percentage of NK Cells in the Lavage of B16FasL Challenged B6 Mice

B6 mice were treated with either isotype control antibody (GL113) or anti-CD25 depleting antibody (PC61) 1 day prior to injection i.p. with 2x10^6 B16FasL. 18 hours later, the peritoneum was lavaged and collected cells analysed by flow cytometry for the presence of NK cells. Symbols represent the percent of lavaged cells that are NK cells (SSCl NK1.1⁺) in one mouse with bars indicating the mean. Data are a summary of 2 independent experiments.
Figure 3.13. *In Vitro* Activated CD4^+^CD25^+^ Cells Inhibit Tumour Lysis by Lavaged Cells from B16FasL Challenged B6 Mice

CD4^+^CD25^+^ cells or CD4^+^CD25^−^ cells purified from naive B6 mice were stimulated with anti-CD3 and irradiated antigen presenting cells. These cells were then added to lavage cells obtained from B6 mice injected i.p. with 2x10^6^ B16FasL 18 hours previously, at a ratio of 1:1. These cells also inhibited proliferation of CD4^+^CD25^−^ cells in a conventional Treg suppression assay (data not shown). Bars represent the mean percentage tumour lysis ± SD of triplicate wells. Statistical significance was evaluated by Mann-Whitney test (*p<0.05).
3.3. Discussion

As described in the Introduction, the aims of the experiments described in this Chapter were; 1) to characterise the immune response to B16FasL; 2) to assess the ability of Treg to inhibit innate immune responses; and 3) to investigate the effect of Treg, if any, on individual innate immune cells. This was done by utilising the melanoma cell line B16 engineered to express FasL, as FasL⁺ tumour cell lines have been reported to induce an innate immune response.

3.3.1. Characterising the Immune Response to B16FasL

The observation that RAG⁻/⁻ mice are equally able to reject s.c. B16FasL tumour challenge when compared to immunocompetent B6 mice demonstrates that innate immune responses are sufficient for B16FasL rejection. In order to characterise this response further two models were used. Injection of B16FasL subcutaneously allowed the identification of cells involved in tumour rejection. The model of intraperitoneal injection of B16FasL was developed in order to allow functional analyses of cells involved in this rejection process.

Upon i.p. injection of B16FasL, NK cells, neutrophils and macrophages were recruited to the peritoneum and the proportion of resident macrophages were concomitantly reduced as indicated by other studies (Hohlbaum et al. 2001). Macrophages may promote tumour rejection directly by production of inflammatory cytokines and/or by recruitment of other cells (Hohlbaum et al. 2001). Fas⁺ macrophages have been shown to secrete chemokines which act as attractants for both NK cells and neutrophils, upon engagement with FasL (Hohlbaum et al. 2001; Shimizu et al. 2005). To assess the role of macrophages in B16FasL rejection, mice were treated with carrageenan in order to deplete macrophages.
prior to s.c. injection of B16FasL. While carrageenan treatment reduced the percentage of mice able to reject B16FasL tumour challenge, agreeing with the current literature that macrophages are important for rejection, the interpretation of results is complicated by the fact that treatment with carrageenan also induced a neutrophil infiltrate (seen in figure 3.2B). Attempts were made to deplete macrophages using multi-lamella vesicles (MLV) containing Clodronate, which is thought to be a cleaner depletion method (Rooijen and Sanders 1994); however effective depletion was unsuccessful. Therefore, a role for macrophages could not be definitively characterised in these experiments.

Cells lavaged from the peritoneum following i.p. injection of B16FasL showed ex vivo cytolytic activity towards tumour cells. This was dependent on NK cells as depletion of NK cells, in vivo or in vitro, abolished lavage cytolytic activity. This result is not surprising considering that both B16 and B16FasL express Rae-1, and low MHC class I. Rae-1 is a ligand for the NK cell activating receptor NKG2D (Yokoyama et al. 2004) which along with the low levels of MHC class I, are factors known to enhance recognition by NK cells. Moreover, depletion of NK cells prior to s.c. injection of B16FasL reduced the percentage of mice able to reject tumour challenge in both B6 and RAG$^-$ mice, indicating that NK cells are critical for B16FasL rejection.

The role of neutrophils is less clear cut as depletion in B6 mice did not affect rejection whereas depletion in RAG$^-$ mice completely abolished rejection. Other studies have shown neutrophils recruited upon i.p. injection of FasL expressing cells were responsible for tumour lysis ex vivo (Seino et al. 1997; Chen et al. 1998a). In contrast to these studies, depletion of neutrophils prior to B16FasL challenge had no effect on ex vivo tumour cell lysis. Removal of neutrophils from the lavage also had no effect on ex vivo
tumour cell lysis. These results are in agreement with those in which tumour growth in mice deficient in neutrophil cytotoxicity was the same as that in wild-type mice when challenged with FasL expressing tumours (Igney et al. 2005).

Overall:

- Innate immune cells are sufficient for B16FasL rejection.
- Macrophages are recruited by B16FasL, however their precise role has yet to be defined;
- Neutrophils although recruited by B16FasL, do not contribute to ex vivo tumour lysis and are not essential for B16FasL rejection in B6 mice;
- NK cells are essential for s.c. B16FasL rejection and are capable of tumour lysis ex vivo suggesting that they are the main effector cell for B16FasL rejection.

3.3.2. Treg Inhibit Innate Immune Responses

Having established that B16FasL is rejected by innate immune cells, the next step was to assess the ability of Treg to inhibit this rejection. In order to address this, B6 mice were treated with PC61 prior to B16FasL inoculation. PC61 is a monoclonal antibody specific for CD25 and has been widely used to deplete naturally occurring Treg in naïve mice which constitutively express CD25 (Onizuka et al. 1999). Currently there are no unique cell surface markers identified for Treg that would allow specific targeted depletion in vivo. Although the majority of CD4⁺CD25⁺ cells were depleted by PC61 treatment, only approximately half of CD4⁺FOXP3⁺ cells were depleted. Despite this, PC61 treatment enhanced B16FasL rejection from 50% to 100%. Since B16FasL has been shown to be rejected by innate immune cells, this suggests that Treg may suppress innate immune responses. Furthermore, adoptive transfer of purified CD4⁺CD25⁺ cells, which have been
shown to contain the Treg population (Sakaguchi et al. 1995) prevented B16FasL rejection in 100% of RAG^{−/−} mice indicating that Treg do indeed inhibit the innate immune system.

### 3.3.3. Treg Inhibit NK Cell Activity

The ability of Treg to suppress tumour lysis by lavaged cells was then assessed. In RAG^{−/−} mice adoptive transfer of CD4^{+}CD25^{+} cells inhibited lysis and in B6 mice PC61 treatment enhanced lysis. This tumour lysis was shown to be dependent on NK cells and, in the absence of NK cells, PC61 treatment could not enhance lysis. This demonstrates that depletion of Treg did not enhance the activity of another cell type, which then acts in addition to existing NK cell activity and therefore indicates that Treg inhibit NK cell activity.

Treg inhibition of NK cell activity manifests in two ways. Data presented in this chapter demonstrates the ability of Treg, activated by anti-CD3 antibody and irradiated APC, to inhibit the NK cell dependent cytotoxic activity of lavage cells. During the course of this investigation similar findings have been published by other investigators (Trzonkowski et al. 2004; Ghiringhelli et al. 2005; Smyth et al. 2006). Trzonkowski et al. showed that coincubation of human NK cells with purified CD4^{+}CD25^{+} cells inhibited IFNγ and perforin production and resulted in a reduction of NK cytotoxic activity. The mechanism remained elusive but enhanced generation of NK-Treg conjugates in vitro was reported along with increased IL-10 production.

A study of GIST (gastrointestinal stromal tumour–bearing) patients treated with Gleevec STI571 in order to enhance anti-tumour NK activity, showed that Treg numbers were
enhanced in patients which did not display NK cell induction (Ghiringhelli et al. 2005). Treg were shown to inhibit NK cell cytotoxicity and IFNγ secretion in a non fixation sensitive manner, later identified as membrane bound TGFβ. Neutralising anti-TGFβ antibody could prevent inhibition and soluble TGFβ mimicked the effect of Treg by altering expression of inhibitory receptor NKG2D. In parallel, experiments carried out in the murine system produced similar findings. Adoptive transfer of Treg inhibited NK target cell lysis by splenocytes and reduced the expression of NKG2D, yet adoptive transfer of TGFβ−/− Treg did not. Adoptive transfer of Treg also resulted in more lung metastases in nu/nu mice challenged with the melanoma B16 transfected with NKG2D ligand Rae. In addition, they reported that scurfy (Foxp3 deficient) mice had enhanced NK target cell lysis by splenocytes and Treg depletion with PC61 enhanced splenic NK proliferation.

Similar findings were presented using various tumour cell lines expressing Rae-1 isoforms in the murine system (Smyth et al. 2006). However, in contrast to the study by Ghiringhelli et al. and in agreement with our findings, only activated Treg were able to inhibit NK cell cytotoxicity. Since Ghiringhelli et al. did not attempt this experiment in their murine system these differences may be due to differences between human and mouse Treg. Neutralising anti-TGFβ antibody also restored cytotoxicity, unlike anti-IL10 antibody, and this was shown to be contact dependent. Another disparity was that no enhanced NK cell cytotoxicity or proliferation was detected in PC61 treated mice. The effect of Treg on NK cells was also demonstrated in vivo by adoptive transfer of activated Treg into RAG−/− mice prior to i.v. tumour challenge, resulting in increased lung metastases. No increase in lung metastases was observed upon Treg transfer in the absence of NK cells.
In accordance with the observation that Treg inhibits NKG2D-mediated NK cell tumour lysis, both B16F10 and B16FasL used in these experiments express the NK2GD ligand, Rae-1. Both of latter studies demonstrate that suppression by the Treg is dependent on TGFβ. However, in the B16FasL model, TGFβ does not completely account for the suppressive effect of Treg on NK cells, since neutralisation of TGFβ in vivo using the TGFβ-specific neutralising antibody, 1D11, did not increase tumour lysis within the peritoneal lavage (data not shown). However, it was observed that treatment with 1D11 increased NK cell recruitment into peritoneum following injection of tumour cells. The increase, although not statistically significant, was consistent thus raising the possibility that production of TGFβ by Treg also impedes recruitment of NK cells.

Indeed, the suppressive effect of Treg on NK cytotoxicity may be magnified in vivo by Treg inhibition of NK cell migration to the site of tumour challenge. Adoptive transfer of CD4+CD25+ cells into RAG2−/− mice prior to B16FasL challenge impaired the recruitment of NK cells to the peritoneum and the converse was seen in B6 mice treated with PC61 however the difference was not significant. Studies of NK migration following virus infection support a role for MIP-1α, a chemokine whose production is driven by Type I interferons (IFNs) (Salazar-Mather et al. 2002; Yokoyama et al. 2004). Hohlbaum et. al showed MIP-1α production by cells isolated from the peritoneum post challenge with FasL expressing cells and another study later confirmed that macrophages were the source (Hohlbaum et al. 2001; Shimizu et al. 2005). Therefore the stimulation of MIP-1α secretion by Fas ligation on resident macrophages may recruit NK cells and ultimately lead to tumour rejection. Indeed, this laboratory has previously shown that the ability to reject B16FasL is impaired in MIP-1α deficient mice (Simon et al. 2002; Jones et al. 2003), a finding that may be attributable to defective NK cell migration to the tumour
site. The mechanism by which Treg inhibit NK cell migration may not be a direct effect on NK cells but an inhibition of cytokine production by macrophages. Further studies are required in order to elucidate this mechanism.

In summary, this Chapter demonstrates that Treg can inhibit innate immune rejection of B16FasL. In agreement with recently published studies it has also been shown that Treg can directly inhibit NK cell cytotoxicity of tumour cells. However, this chapter has also identified a possible role for Treg in the regulation of NK cell migration to sites of immune challenge. It still remains unclear whether this is a direct effect on NK cells or an indirect effect through the suppression of chemoattractant production by other cell types and is a matter for further investigation.
Chapter 4 – Neutrophil Recruitment by B16FasL is Inhibited by CD4⁺CD25⁺ Regulatory T Cells

4.1. Introduction

In the previous chapter, a tumour cell line expressing Fas ligand (B16FasL) was shown to be rejected by the innate immune system, and was used to address the hypothesis that Treg suppress innate immune responses. Indeed, in vivo depletion of Treg in wild-type B6 mice enhanced tumour rejection whilst adoptive transfer of Treg into RAG⁻/⁻ mice inhibited tumour rejection indicating that Treg do suppress innate immune responses.

An article published during the course of this study supports these findings. The study of Helicobacter hepaticus infected RAG⁻/⁻ mice showed that adoptive transfer of CD4⁺CD25⁺CD45RB⁻ cells (Treg) from naïve B6 mice inhibited T cell-independent intestinal inflammation via IL-10 and TGFβ (Maloy et al. 2003). In this model of chronic inflammation, numbers of neutrophils, monocytes/macrophages, NK cells and dendritic cells (DC) were lower in spleens of mice receiving Treg, indicating a role for Treg in the control of chronic (systemic) inflammation.

The inhibition of multiple cell types involved in chronic inflammation by Treg would be advantageous to the host, preventing excessive immunopathology and possibly death. However, unlike the model studied by Maloy et al., the B16FasL tumour model is one of acute inflammation. Since acute inflammation can be detected within hours, the ability of Treg to suppress B16FasL rejection would suggest that Treg might also act rapidly to suppress the innate immune system.
Although the data presented in the previous chapter indicate that Treg inhibit NK cells, in order to address the hypothesis that Treg suppress acute inflammatory responses, the effect of Treg on other innate immune cells must be studied. It has been shown in the previous chapter that B16FasL tumour challenge induces the recruitment of neutrophils and macrophages and that Treg depletion enhances B16FasL rejection. This Chapter describes experiments designed to address the hypothesis that Treg also inhibit neutrophil and macrophage responses, with a view to exploring further the theory that Treg are rapidly active.
4.2. Results

4.2.1. Treg Depletion Enhances B16FasL Rejection in NK Depleted Mice

To characterise more fully the impact of Treg on the innate immune system, experiments were performed to determine whether cell types other than NK cells, were required for tumour rejection after treatment of mice with CD25-specific depleting antibodies. To explore the hypothesis that cells of the innate immune system, other than NK cells, may be inhibited by Treg in our model of B16FasL rejection, mice were treated with both PK136 and PC61, in order to deplete NK cells and CD25+ cells respectively, followed by s.c. challenge with B16FasL (Figure 4.1A and B). Figure 4.1A is a duplication of Figure 3.3A for reference. Interestingly, PC61 treatment could still enhance rejection in those mice depleted of NK cells suggesting that Treg could be inhibiting the action of other cell types involved in B16FasL rejection.

4.2.2. Treg Depletion Cannot Enhance B16FasL Rejection in Neutrophil Depleted Mice

Despite the finding in the previous chapter that neutrophils are not essential for B16FasL rejection in B6 mice, they are recruited to the peritoneum following i.p. injection of B16FasL in agreement with many other studies (Seino et al. 1997; Chen et al. 1998a; Hohlbaum et al. 2001). It is possible therefore that although not essential, they contribute to tumour rejection and that this anti-tumoural activity is inhibited by Treg. In order to test this hypothesis, mice were treated with both PC61 and RB6-8C5, to deplete neutrophils, followed by s.c. challenge with B16FasL (Figure 4.2A and B). Figure 4.2A
Figure 4.1. CD25+ Cell Depletion Enhances Tumour Rejection in the Absence of NK Cells

Mice were treated with either (A) Isotype control antibody (GL113) or (B) anti-CD25 depleting antibody (PC61), with/without anti-NK1.1 depleting antibody (PK136). Mice were injected s.c. 1 day later with $10^5$ B16FasL and tumour growth monitored over 100 days. Bars indicate mean percentage rejection ± SEM of 3 experiments, with the number of tumour free mice/total number indicated. Statistical significance was evaluated by Fisher’s Exact test (* p<0.05).
Figure 4.2. CD25⁺ Cell Depletion Cannot Enhance Tumour Rejection in the Absence of Neutrophils

Mice were treated with either (A) Isotype control antibody (GL113) or (B) anti-CD25 depleting antibody (PC61), with/without anti-NK1.1 (PK136) or anti-Gr-1 (RB6-8C5) depleting antibodies. Mice were injected s.c. 1 day later with 10⁵ B16FasL and tumour growth monitored over 100 days. Bars indicate mean percentage rejection ± SEM of 3 experiments, with the number of tumour free mice/total number indicated. Statistical significance was evaluated by Fisher’s Exact test (** p<0.01).
is a duplication of Figure 3.3A for reference. No enhancement of rejection was observed in neutrophil depleted mice upon PC61 treatment indicating that neutrophils are important for the increased rejection seen in PC61 treated mice, suggesting that their activity is normally inhibited by Treg.

4.2.3. Neutrophil Recruitment to the Peritoneum Following B16FasL Injection is Not Affected by Treg Depletion

The ability of Treg to inhibit neutrophils could manifest as reduced neutrophil numbers at the site of challenge or reduced activity on a per cell basis. In order to characterise the effect of Treg on neutrophil numbers, cells recruited to the peritoneum at different time points by B16FasL challenge were analysed for their neutrophil content. In the previous chapter, the peritoneal model of tumour inoculation was used in order to recover recruited cells for analysis and at 18 hours post tumour inoculation, there was no difference in the number of neutrophils recovered from the lavage of PC61 and GL113 (isotype control antibody) treated mice. The lifespan of neutrophils is known to be short, with the most generous estimates being 24 hours for infiltrating neutrophils, and just 10 hours for circulating neutrophils (Yamashiro et al. 2001). For this reason it was hypothesised that an increase in neutrophil number upon Treg depletion might be observed at different time points given that neutrophil infiltration is detectable after just 1 hour. In order to address this, GL113 and PC61 treated mice were injected i.p. with B16FasL and the cells from peritoneal cavity collected by lavage 1, 3, 6, 18, 24, and 36 hours later. The number of recruited neutrophils for each mouse was assessed by flow cytometry as $SSC^{hi}CD11b^+Gr-1^{hi}F4/80^-$ as described in Chapter 3 (Figure 4.3). No difference in neutrophil recruitment was observed at any time point, post i.p. injection of B16FasL, between groups of mice, suggesting that Treg do not enhance neutrophil recruitment to the peritoneum.
Figure 4.3. Neutrophil Numbers in Lavage Fluid are Not Affected by CD25⁺ Cell Depletion

Mice were treated with either isotype control antibody (GL113 – solid line) or anti-CD25 depleting antibody (PC61 – dotted line) and injected i.p 1 day later with 2×10⁶ B16FasL. At the indicated time points, mice were sacrificed and the peritoneal cavity lavaged. Cells from the lavage were counted and immunostained for CD11b, Gr-1 and F4/80. Presence of neutrophils (SSC⁻⁴CD11b⁺Gr-1hiF4/80⁻) in the lavage fluid was analysed by flow cytometry as in Chapter 3. Mice were analysed individually and data shown are the mean ± SEM of >5 mice per group.
4.2.4. Treg Depletion Results in an Increased Number of Nuclear Segments in Neutrophils Isolated from the Peritoneum Following B16FasL injection

Although no differences in neutrophil numbers were observed in the above experiment, cytospins generated from 18 hour lavaged cells showed phenotypic differences in neutrophils isolated from GL113, compared to PC61, treated mice. Cytospins were generated from 240µl of lavage fluid and enabled visualisation of recruited cells. Cell counts confirmed that there was no difference in the number of recruited neutrophils (data not shown), however it was noted that neutrophils from PC61 treated mice had an increased number of nuclear segments. Examples of segmented nuclei are given in Figure 4.4A, where segments are joined by thin strands of chromatin. Figure 4.4B and 4.4C are examples of neutrophils isolated from GL113 and PC61 treated mice respectively. Upon enumeration, it was evident that the proportion of neutrophils with a higher number of segments was increased in PC61 treated mice (Figure 4.4D) which resulted in an increase in the average number of segments per neutrophil (Figure 4.4E).

4.2.5. Treg Depletion Results in an Increase in Cellular Mass at the Site of B16FasL Injection

Whilst the peritoneal model is useful, it was hypothesized that neutrophil recruitment and access to tumour may be different in the skin. Injection of fluid subcutaneously (with or without tumour cells) causes visible distortion and possibly cellular stress due to the inability to diffuse quickly, the effect of which is less severe upon intraperitoneal injection, and this process could more potently initiate neutrophil infiltration. In addition, s.c. injection restricts tumour cells to a discrete area of the skin unlike in the peritoneal model where cells have a much greater volume in which to settle. There are also distinct
Figure 4.4. Increased Number of Nuclear Segments in Neutrophils from CD25⁺ Cell Depleted Mice

Mice were treated with either isotype control (GL113) or anti-CD25 depleting antibody (PC61) and injected i.p 1 day later with 2x10⁶ B16FasL. 18 hours later, mice were sacrificed and the peritoneal cavity lavaged. Cytospins generated of lavaged cells were stained and number of segments in 100 neutrophils assessed. (A) Examples of segmented neutrophils. (B) and (C) Representative photographs of cytospins generated from GL113 (B) and PC61 (C) treated mice. (D) Indicates proportion of neutrophils with given number of segments. Mice were analysed individually and data shown are the mean ± SEM of 4 mice per group. (E) Indicates the average number of segments in neutrophils isolated from 4 mice per group, with bars indicating the mean per group. Statistical significance was evaluated by Mann Whitney test (*p=0.0286).
populations of cells found in the skin which could significantly contribute to neutrophil activation and recruitment, such as keratinocytes which are a rich source of IL-1. Migration of neutrophils towards tumour cells could also be more restricted in skin due to being surrounded by extracellular matrix, unlike in the peritoneum. For these reasons it was important to study the neutrophil infiltration in the skin.

Isolation of cells from the precise site of s.c. inoculation is difficult at such early time points due to size and number of cells, however, histological analysis of the site of inoculation allowed the study of the cellular infiltrate at the non-palpable B16FasL inoculation site. B6 mice treated with GL113 or PC61 were injected s.c. with $10^5$ B16FasL and 4, 24, 96, and 240 hours after tumour injection mice were sacrificed and injected skin removed for histology. Tissue was embedded in paraffin and 5μm sections cut at 300μm intervals throughout the skin. Sections were then H&E stained to locate the midsection of the tumour infiltrate (Figure 4.5). It is clear from all sections that there is a large amount of cell death, as indicated by the lack of cellular cohesion and presence of fragmented nuclei. Fragmented nuclei often resemble the polymorphonuclear phenotype of neutrophils and it is therefore difficult to distinguish different cell types within these areas by histology. There is however a visible increase in size of the cellular mass at 24 hours in the PC61 treated group compared with the GL113 treated group (Figure 4.4C and D). Analysis of the total volume of the cellular mass, calculated as described in Chapter 2 (Figure 2.1), in each of >4 mice per group supported this observation (Figure 4.6). There is a rapid increase in size of cellular mass between 4 and 24 hours which tends to be greater in the PC61 treated mice, although not quite significantly different. It is unlikely that this increase is due to tumour cell division and, due to the early time point, is most likely to consist of tumour cells and an inflammatory infiltrate. Between 24
Figure 4.5. Cellular Mass at the Site of B16FasL Injection is Larger in CD25⁺ Cell Depleted Mice
Mice were treated with either isotype control antibody (GL113 – A-G) or anti-CD25 depleting antibody (PC61 – H-N) were injected s.c. 1 day later with 10⁵ B16FasL. At the indicated time points, mice were sacrificed and skin surrounding the injection site collected for histology. H&E stained 5µm paraffin sections were generated throughout the skin. Sections shown are representative of >4 mice per group. (continued on next page)
Figure 4.6. Total Volume of Cellular Mass at the Site of B16FasL Injection is Larger in CD25⁺ Cell Depleted Mice

Mice were treated with either isotype control antibody (GL113 – solid line) or anti-CD25 depleting antibody (PC61 – dotted line) were injected s.c. 1 day later with 10⁵ B16FasL. At the indicated time points, mice were sacrificed and skin surrounding the injection site collect for histology. H&E stained 5μm paraffin sections were generated every 300μm throughout the skin. An estimation of the volume of cellular mass was based on the area of cellular mass on each section and the distance between them, as described in Figure 2.1. Data shown are mean ± SEM of >4 mice per group. Statistical significance was evaluated by Mann Whitney test.
and 96 hours there is a switch as control mice have larger cellular masses, coinciding with an increased number of live tumour cells.

4.2.6. Treg Depletion Results in Enhanced Neutrophil Recruitment to the Site of B16FasL Injection

In order to ascertain the precise constituents of the cellular mass, and therefore address the hypothesis that Treg inhibit neutrophil recruitment, immunohistochemistry was performed. IL-8 and its mouse counterpart, MIP-2, attract neutrophils to the site of inflammation and the IL-8 receptor (IL-8R) is expressed on the majority of mature neutrophils. For this reason, an antibody to the murine IL-8R was used, in conjunction with haematoxylin nuclear staining, to identify neutrophils in the cellular mass (Figure 4.7). Visualisation of anti-IL8R antibody with horseradish peroxidase and DAB substrate produced a dark brown product. Due to the large amount of cell debris, which non-specifically binds antibodies, identification of cells within areas of cell death was obscured thus hampering colorimetric analysis. However in other areas neutrophils were identified as polymorphonuclear cells which express IL-8R (dark brown staining). For this reason the midsections of cellular mass for each mouse were blindly ranked according to the extent of neutrophil infiltrate. The slides were then grouped into those with similar infiltrates which resulted in scores out of 5 (Figure 4.8A). Since a difference in the neutrophil infiltrate between groups was more obvious at 24 hours, the neutrophils were counted on these slides. The results show an increased number of neutrophils in PC61 treated mice at 24 hours post injection. The results reflect the data on increased cellular mass in PC61 treated mice, with a significant increase in neutrophil infiltrate evident at 24 hours. In combination these results suggest that Treg inhibit neutrophil recruitment in the skin.
Figure 4.7. Neutrophil Recruitment to the Site of B16FasL Injection is Greater in CD25⁺ Cell Depleted Mice at 24 Hours

(A) Mice were treated with either isotype control antibody (GL113) or anti-CD25 depleting antibody (PC61) were injected s.c. 1 day later with 10⁵ B16FasL. At 24 hours mice were sacrificed and skin surrounding the injection site collected for histology. IL-8R stained 5μm paraffin sections were generated to identify neutrophils. Areas of neutrophil infiltration are enclosed by lines. 2 sections from each group are shown and are representative of 5 mice per group. All photographs are taken at x5 magnification. (B) Examples of neutrophils (indicated by yellow arrows) which stain dark brown with multilobed nuclei visible at x 40 magnification.
Figure 4.8. Neutrophil Recruitment to the Site of B16FasL Injection is Greater in CD25\(^+\) Cell Depleted Mice at 24 Hours

Mice were treated with either isotype control antibody (GL113 - closed symbols) or anti-CD25 depleting antibody (PC61 - open symbols) were injected s.c. 1 day later with \(10^5\) B16FasL. At the indicated time points, mice were sacrificed and skin surrounding the injection site collected for histology. 5\(\mu\)m paraffin sections were generated throughout the skin and midsections from each mouse stained with anti-IL-8R antibody to identify neutrophils. (A) Sections were blindly ordered according to the amount of neutrophil infiltrate. Those showing similar infiltrate were grouped together and groups were assigned a score from those in group 0 having no neutrophils to those in group 5 having the greatest number of neutrophils. (B) The numbers of neutrophils, assessed as polymorphonuclear cells with IL-8R expression, are given for sections taken at 24 hours post injection. Each symbol represents the score for one mouse. Statistical significance was evaluated by Mann Whitney test (* \(p<0.05\)).
4.2.7. Neutrophils are Able to Lyse B16FasL \textit{In Vitro}

Although present at the site of B16FasL injection, the capacity of neutrophils to contribute to tumour rejection remains unclear. The experiments in the previous chapter suggest that neutrophils recruited to the peritoneum are unable to lyse tumour cells \textit{ex-vivo} (Figure 3.5). However due to the short life span of activated neutrophils, it is possible that their lytic activity wanes once recruited and activated in the peritoneum. In order to address the hypothesis that neutrophils can lyse tumour cells, mature neutrophils isolated from mouse bone marrow were used as effectors against chromium labelled B16 and B16FasL (Figure 4.9A). The results show that neutrophils can lyse tumour cells \textit{in vitro} and that they show enhanced lytic activity against B16FasL. Furthermore, neutrophils isolated from the peripheral blood of healthy volunteers also lysed B16FasL, but not B16, \textit{in vitro} (Figure 4.9B).

4.2.8. Histological Differences Between Isotype Control and Anti-CD25 Depleting Antibody Treated Mice

During analysis of neutrophil numbers, it was noticed that there were two forms of cellular mass displaying different histological characteristics (Figure 4.8). In one type, cells are confined to a single layer of the skin, in a rounded symmetrical shape, surrounded by normal tissue (Figure 4.10A). In the other type, inflammatory cells are spread throughout the layers of the skin, forming irregular shaped cellular masses (Figure 4.10B). Strikingly, upon assessment of sections for these characteristics, none of the sections from PC61 treated mice, and only half of the GL113 treated mice, displayed the 'confined' phenotype (Figure 4.10C). This is particularly interesting when compared to the percentage of mice that reject these tumours; 50% in GL113 treated mice and 100% in PC61 treated mice. Furthermore, there is a positive correlation between confined
Figure 4.9. Isolated Neutrophils Lyse B16FasL In Vitro
Neutrophils isolated from the bone marrow of mice (A) or blood of healthy volunteers (B) were used directly as effectors against Chromium labelled B16 or B16FasL at a ratio of 40:1. Bars indicate mean ± SD of duplicates and graphs are representative of 2 experiments.
Figure 4.10. Confinement of Cellular Mass

Mice were treated with either isotype control antibody (GL113) or anti-CD25 depleting (PC61) antibody were injected s.c. 1 day later with $10^5$ B16FasL. At 4, 24, 96 and 240 hours post injection, mice were sacrificed and skin surrounding the injection site collected for histology. H&E stained 5µm paraffin sections were generated throughout the skin. Sections shown are representative confined (A) and non-confined (B) cellular masses. (C) The percentage of mice displaying the confined phenotype of cellular mass. (D) Live tumour cells show irregular nuclear staining indicated by yellow arrows. (E) Non-confined and confined cellular masses were studied for the presence of live tumour cells. Statistical significance was evaluated by Fisher’s exact test (*** p<0.001).
cellular masses and those containing live tumour cells (Figure 4.10D and E). Together this data suggests a link between the confined phenotype and tumour outgrowth.

4.2.9. Macrophage Recruitment to the Peritoneum Following B16FasL injection is Not Affected by Treg Depletion

Based on studies of intraperitoneally injected tumour, others have implicated macrophages in the chain of events resulting in rejection of FasL+ tumours. It is thought that FasL induction of apoptosis in Fas+ macrophages (Hohlbaum et al. 2000; Hohlbaum et al. 2001), results in release of neutrophil chemotactic factors. In order to assess their involvement in rejection and a possible difference in Treg depleted mice, GL113 and PC61 treated mice were injected i.p. with B16FasL and the cells from peritoneal cavity collected by lavage. The number of resident and recruited macrophages was then assessed by flow cytometry. As described in Chapter 3 resident macrophages in the lavage fluid were identified as SSCintCD11b+Gr-1intF4/80hi (Figure 4.11A) and recruited macrophages as SSCintCD11b+Gr-1intF4/80int (Figure 4.11B). In support of a previous study (Hohlbaum et al. 2001), a drop in macrophage number was observed up to 6 hours post B16FasL injection, however no difference in macrophage recruitment was observed at any time point between GL113 and PC61 treated mice, suggesting that Treg do not enhance macrophage recruitment to the peritoneum.
Figure 4.11. Macrophage Numbers in Lavage Fluid are Not Affected by CD25+ Cell Depletion

Mice were treated with either isotype control antibody (GL113 – solid line) or anti-CD25 depleting antibody (PC61 – dotted line) and injected i.p 1 day later with 2x10^6 B16FasL. At the indicated time points, mice were sacrificed and the peritoneal cavity lavaged. Cells from the lavage were counted and immunostained for CD11b, Gr-1 and F4/80. Presence of (A) resident (SSC^intCD11b^Gr-1^intF4/80^int) and (B) recruited (SSC^intCD11b^Gr-1^intF4/80^int) macrophages in the lavage fluid was analysed by flow cytometry as in Chapter 3. Mice were analysed individually and data shown are the mean ± SEM of >5 mice per group.
4.2.10. The Cellular Mass Does Not Include Macrophages

As described earlier for neutrophils, the events in the peritoneum may be different to that in the skin. In addition, the peritoneum has a large, distinct resident population of macrophages which is likely to be different to that found in the skin. Again, histological analysis of the site of inoculation provides the ideal way to study macrophages at the B16FasL inoculation site. For this reason, serial sections of those obtained for IL-8R staining were immunostained with anti-F4/80 antibody, a macrophage / monocyte marker, in conjunction with haematoxylin nuclear staining, to identify macrophages in the cellular mass. Visualisation of anti-F4/80 antibody with Alkaline Phosphatase and Fast-Red substrate produced a red product. Surprisingly, there were no F4/80+ cells in the cellular mass (Figure 4.12 A-D) and only a few in the surrounding tissue; for comparison, a section from a mouse injected with the parental cell line B16 showed a much more evident macrophage infiltrate (Figure 4.12E). Furthermore there was no difference in macrophage number detected between groups of mice. It is therefore possible that peritoneal events reflect those in the skin in that macrophages numbers are depleted at very early time point which simultaneously prevents detection of any difference in macrophages between GL113 and PC61 treated mice using this method.
Figure 4.12. Absence of Macrophages in B16FasL Injected Skin
Mice were treated with either isotype control antibody (GL113 – A&C) or anti-CD25 depleting antibody (PC61 – B&D) and injected s.c. 1 day later with $10^5$ B16FasL. At the indicated time points, mice were sacrificed and skin surrounding the injection site collected for histology. 5µm paraffin sections were generated at 300µm throughout the skin. Midsections of the cellular infiltrate were then immunostained with anti-F4/80 antibody and visualised with Alkaline Phosphatase and Fast-Red to give a red substrate. (E) Unlike B16FasL, mice injected with B16 exhibit a macrophage infiltrate. All photographs are taken at x20 magnification.
4.3. Discussion

One aim of this thesis was to address the hypothesis that Treg suppress innate immune activity. Having shown in the previous chapter that Treg inhibit NK cell activity it was important to assess the effect of Treg on other cells of the innate immune response, in particular neutrophils and macrophages. This was done by utilising the melanoma cell line B16FasL which has been shown to induce neutrophil and macrophage infiltration.

4.3.1. Neutrophils

The ability of Treg-depleted mice to reject B16FasL in the absence of NK cells indicated that other cell types were involved in enhancing rejection. In addition, Treg depletion in the absence of neutrophils failed to enhance rejection, indicating that neutrophils are at least partly responsible for the enhanced tumour rejection observed in the absence of Treg. Together these results suggest that neutrophils are inhibited by Treg.

Treg inhibition of neutrophils could be a result of suppressed neutrophil recruitment and/or suppressed activity. Data presented in this chapter shows that although the recruitment of neutrophils to the peritoneum following i.p. injection of B16FasL was not inhibited by Treg, the ability of neutrophils to infiltrate the subcutaneous site of tumour challenge was enhanced by Treg depletion. These results reflect those from studies of Helicobacter hepaticus infected RAG\(^{+/−}\) mice where it was found that adoptive transfer of Treg reduced neutrophil numbers in the spleen and lamina propria (Maloy et al. 2003), although the model is that of chronic inflammation. Since intestinal inflammation induced by H. hepaticus could not be inhibited by Treg isolated from IL-10\(^{+/−}\) mice and was blocked by in vivo administration of anti-IL-10R and anti-TGF\(\beta\) antibodies, it is likely that these soluble factors are involved in the inhibition of neutrophil recruitment.
In the experiments described here, neutrophils isolated from mice and humans were able to lyse B16FasL indicating that neutrophils are capable of tumour lysis; however, target cell lysis could not be inhibited in vitro by addition of anti-CD3/CD28 activated Treg (data not shown). In contrast, a recent study of human neutrophils reporting that LPS-activated CD4+CD25+ Treg suppressed PMA-induced reactive oxygen intermediate (ROI) production and CD62L expression in neutrophils, whilst increasing CD11b expression, all of which are markers of neutrophil activation (Lewkowicz et al. 2006). Furthermore, LPS-activated CD4+CD25+ Treg suppressed LPS-induced neutrophil production of IL-8, TNFα and IL-6 and enhanced neutrophil survival, all of which could be blocked by addition of anti-IL-10, anti-TGFβ and anti-TLR4 antibodies. Interestingly, anti-CD3/CD28 activated Treg could also suppress cytokine production and neutrophil survival; however neither could be inhibited by anti-IL-10 or anti-TGFβ antibody addition.

Both of these studies highlight a role for IL-10 and TGFβ in inhibition of neutrophils by Treg. Whether Treg act through these cytokines or through interactions with immune and non-immune cells, to inhibit neutrophil recruitment in response to B16FasL challenge, has not yet been addressed. It is possible that Treg inhibit the activity of keratinocytes, fibroblasts and other cells resident in the skin, known to be able to secrete inflammatory mediators such as IL-1, TNFα and IL-8. Treg could also act to downregulate expression of chemokine/cytokine receptors and adhesion molecules on neutrophils and/or the corresponding ligands on the target endothelium in the skin, such as E-Selectin, therefore limiting the ability of neutrophils to transmigrate into tissues. Interestingly, IL-10 is known to inhibit production of MIP-1α, IL-8/MIP-2 and KC/GROα by macrophages and neutrophils (Moore et al. 2001). IL-8 (Abreu-Martin et al. 1995) and MIP-1α (Lee et al.
2000b) are known to recruit neutrophils and therefore IL-10 could inhibit neutrophil attraction to sites of inflammation. TGFβ also inhibits TNFα induced IL-8 expression by endothelial cells (MacDonald et al. 1999), whilst downregulating E-Selectin and VCAM-1, known to be important for neutrophil transmigration, and thereby inhibit entry of tissue by neutrophils (Smith et al. 1996; Park et al. 2000). One study showed that TGFβ inhibited lysis of FasL expressing tumour cells by neutrophils (Chen et al. 1998a).

Although these cytokines have been implicated in the modulation of neutrophil activity, the sources of these cytokines are vast, ranging from leukocytes to macrophages to keratinocytes, complicating direct association with Treg (Moore et al. 2001; Li et al. 2006b). Treatment of mice with anti-IL-10 and anti-TGFβ antibodies prior to tumour challenge will go some way to addressing the hypothesis that the mechanism of Treg suppression is mediated through these cytokines. However, these experiments will not determine the source of the cytokine, as Treg could induce IL-10 and/or TGFβ expression by other cells. Treg isolated from mice deficient in IL-10 and/or TGFβ could be utilised to differentiate between these two possibilities. In summary, there is an abundance of ways in which Treg could inhibit neutrophil recruitment but further careful experimentation is needed.

The increase in neutrophil nuclear segmentation upon Treg depletion is an interesting observation. The segmentation of nuclei in human neutrophils from peripheral blood has long been used as a diagnostic tool. A lower number of segments indicates a 'younger' neutrophil with the ratio of bands (single segmented immature neutrophils) to total neutrophil number often given as a result. A 'shift to the left' or an increase in bands is indicative of severe infection, where there is enhanced neutrophil exit from the bone
marrow before complete maturation (Davis et al. 2006). A ‘shift to the right’ or an increase in average number of segments, is rarer. Hypersegmentation is strongly associated with anaemia, in particular Megaloblastic anaemia caused by Vitamin B12 and/or Folic Acid deficiency (Gulley et al. 1990), and is evident in other non-infectious inflammatory conditions such as in rats with pulmonary inflammation caused by graphite dust (Anderson et al. 1989). Others have reported hypersegmentation in neutrophils from irradiated mice (Gagnon et al. 2003) and patients with Chemotherapy-induced acral erythema, an inflammatory skin condition (Tsuruta et al. 2000). Since the model used in this thesis is also one of an inflammatory reaction in the absence of infection, it is interesting that Treg depletion enhances the proportion of highly segmented neutrophils.

Historically hypersegmentation is associated with older neutrophils and prolonged survival. Although the literature on the activity of hypersegmented neutrophils is limited, this phenotype is also seen in patients and rats during the first few hours after treatment with G-CSF (Ulrich et al. 1988). Recombinant G-CSF is used therapeutically to relieve neutropenia, as it has been shown to stimulate granulocytopenesis. In addition, G-CSF can promote survival, phagocytic and cytolytic capacity, along with production of ROI in mature neutrophils (Brach et al. 1992; Spiekermann et al. 1997). A recent report also indicated that in vitro treatment of human neutrophils with G-CSF, delayed neutrophil apoptosis, prevented deterioration of chemotaxis towards C5a and IL-8 usually associated with neutrophil aging, and maintained expression of chemokine receptors CXCR1 and CXCR2 (Wolach et al. 2007). These reports suggest that hypersegmentation is indeed associated with prolonged neutrophil survival. Since Treg depletion enhanced the proportion of highly segmented neutrophils, this may suggest that Treg inhibit neutrophil survival. This hypothesis is supported by a recent study by Lewkowicz et. al., in which
activated Treg enhanced human neutrophil apoptosis. This hypothesis could also explain
the enhanced neutrophil numbers observed during B16FasL challenge, as neutrophils
persist at the site of injection (Lewkowicz et al. 2006).

In order to further investigate the impact of Treg on neutrophil responses, particularly the
effect on recruitment versus survival, there are a number of approaches that could be
taken. Since neutrophil recruitment is at least in part dependant on soluble mediators, it
would be important to quantify these chemoattractants at the site of tumour challenge.
This could be carried out by real-time PCR of mediators important for neutrophil
recruitment, such as MIP-2 and KC. Although real-time PCR quantifies the amount of
specific mRNA present in sample at one time, a kinetic analysis may also be important to
determine at what point levels may be increased. Measurement of other inflammatory
mediators, such as IL-1β, in these kinetic experiments would also help address which, if
any, may initiate an increase in the others. This method could also be used to assay the
quantity of IL-10 and TGFβ.

Another set of experiments to address the impact of Treg on neutrophil survival could be
carried out in vitro. Monitoring of neutrophil survival in the presence or absence of
(activated) Treg, by Annexin V/Propidium Iodide staining or by exclusion of 7AAD,
would help address the hypothesis that Treg directly effect neutrophil survival. However,
this experiment does not exclude the probability that the microenvironment in the skin is
important in this process. Neutrophil maturation and activation could also be studied in
this way, for example by monitoring ROI production, cell surface marker expression or
cytokine expression.
4.3.2. Macrophages

A recent study of monocytes from human peripheral blood indicated that Treg inhibit the capacity of these cells to act as antigen presenting cells (Taams et al. 2005). Unlike CD4+CD25-T cells, coculture of CD4+CD25+ Treg with monocytes did not induce T cell proliferation or TNFα, IFNγ, or IL-10 cytokine production although the T cells showed similar activation markers. Studies of costimulatory molecules expressed by these monocytes revealed that CD4+CD25+ Treg did not induce upregulation of CD40, CD80 and MHC class II and reduced the levels of CD86. Coculture of CD4+CD25+ Treg with both CD4+CD25-T cells and monocytes inhibited the proinflammatory effects seen in cultures without CD4+CD25+ Treg. Repurified monocytes from cocultures with CD4+CD25+ Treg were unable to stimulate T cell proliferation unlike those from cultures without T cells and those with CD4+CD25-T cells. Furthermore, LPS induced production of TNFα and IL-6 was inhibited by preculture of monocytes with CD4+CD25+ Treg. Although these data indicate that Treg do inhibit the ability of blood derived monocytes to act as antigen presenting cells; whether or not this is the case for tissue macrophages remains to be delineated.

Using the techniques described here, identification of an inhibitory effect of Treg on macrophages proved elusive. There was no effect on numbers of either resident or recruited macrophages upon depletion of Treg, however this does not rule out the possibility that macrophages are inhibited by Treg. Unfortunately histological analysis of macrophage recruitment was also unfruitful, most likely due to the reported macrophage depleting affect of Fas ligation (Hohlbaum et al. 2001), as injection of the parental cell line B16 induced a substantial macrophage infiltrate. This complicating factor makes monitoring macrophage recruitment difficult, however a way of bypassing this is to
utilise Fas$^{br}$ mice. These mice have a mutation in Fas which is non-functional and prevents Fas mediated cell death. Histological analysis of B16FasL injected Fas$^{br}$ mice treated with PC61 might help resolve this issue. It is particularly important to resolve this issue as macrophages have been shown to secrete chemokines which attract both NK cells and neutrophils (Hohlbaum et al. 2001; Shimizu et al. 2005) suggesting that inhibition of their recruitment might be an indirect effect of Treg inhibition of macrophage chemoattractant production.

4.3.3. Are Treg Rapidly Active?

The two histological phenotypes seen in tumour sections are interesting observations, particularly as the confined phenotype is associated with the presence of live tumour cells. In contrast to the confined phenotype, the skin surrounding the tumour cells in non-confined tumours is heavily infiltrated with inflammatory cells and the percentage of mice able to reject B16FasL tumour challenge seems to correspond with the percentage of tumours which are non-confined. Moreover, 50% of GL113 mice bear the non-confined phenotype, compared to 100% of PC61 treated mice. This could suggest that Treg depletion enhances a pre-existing immune response, as opposed to uncovering another arm of the immune system. If accurate, this would support the hypothesis that Treg act rapidly to suppress an ensuing immune response as this difference is seen from 4 hours post injection. Unfortunately, using this model it is not possible to otherwise identify those GL113 treated mice that will eventually go on to reject tumour challenge at this early time point, and therefore draw definitive conclusions.

The hypothesis that Treg either act rapidly or constitutively, goes against the current literature on conventional naïve T cell activation. Naïve T cell activation principally occurs in lymph nodes upon contact with mature DC, which present antigen in the
context of costimulatory molecules. This process first requires DC to collect antigen and migrate to the lymph node and is slow in comparison to an innate immune response. It is therefore unlikely that Treg able to suppress acute inflammatory responses are activated in this way upon B16FasL challenge.

This assumes that Treg are equivalent to naïve T cells, which is not unreasonable based on the observation that Treg in mice express similar markers to naïve T cells (CD62L and CCR7) (Itoh et al. 1999; Lepault and Gagnerault 2000), however it is also possible that rapidly acting Treg are akin to memory T cells, in that they do not require the same stringent costimulatory signals as naïve T cells in order to become activated upon challenge. Central memory T cells (T_{CM}) also express CD62L and CCR7 but are more slowly activated than their effector memory (T_{EM}) counterparts expressing low levels of these markers.

Numerous studies have identified populations of regulatory T cells expressing memory T cell markers. Some early reports in mice identified a population of regulatory T cells expressing low levels the naïve T cell marker CD45RB (Read et al. 2000), and in humans, the majority of regulatory T cells express the memory marker CD45RO, lack the naïve T cell marker CD45RA and have low levels of CD45RB (Taams et al. 2001; Taams et al. 2002). In fact, only recent studies have identified a naïve regulatory T cell population in adult humans (Valmori et al. 2005; Seddiki et al. 2006). However another recent report indicated that these cells have an increased turnover, like memory T cells, but are present in elderly individuals suggesting that they may not be wholly derived from the thymus (Vukmanovic-Stejic et al. 2006).
If memory Treg are similar to memory T cells, this suggests that they have previously been activated by antigen and are activated again upon B16FasL injection. Since B16FasL is a tumour cell line derived from B6 mice, it is likely that the antigen recognised by the Treg is a self-antigen. Several reports have suggested the TCR repertoire of Treg contains self-reactive TCR (Cabarrocas et al. 2006), substantiating the possibility that Treg are initially activated by self-peptides and exist as memory cells either being constantly activated by self-peptide on non-antigen presenting cells or are activated upon tissue destruction. Furthermore, studies have identified memory-like populations of Treg, with one reporting the ability of these cells to inhibit conventional T cell proliferation ex vivo without additional TCR stimulation (Nolte-'t Hoen et al. 2004).

The ability of Treg to suppress an innate immune response in a discrete site so rapidly would also suggest that Treg patrol the periphery. If Treg are indeed similar to memory T cells, it is possible that they share the ability to enter non-lymphoid tissue. Since the ability of cells to enter peripheral tissues is regulated by chemokine receptor and adhesion molecule expression, the reports of several studies showing expression of CLA, a ligand for E-Selectin expressed in normal and inflamed skin, on human peripheral blood Treg indicate that Treg are capable of skin infiltration (Rao et al. 2002). Furthermore, there are an increasing number of reports indicating that Treg can be found in peripheral tissue in steady state, in particular they have been isolated from skin in both mice (Suffia et al. 2005) and humans (Clark and Kupper 2007) suggesting that Treg do indeed patrol the peripheral tissue prior to immune challenge. In combination these reports support the hypothesis that Treg are present in the periphery prior to tumour challenge.
In summary, the results shown in this chapter indicate that the recruitment of neutrophils is inhibited by Treg. Migration and infiltration of tissues by inflammatory cells is regulated by various chemoattractants and adhesion molecules on both neutrophils and on the endothelium which are produced / upregulated in response to injury or infection. It is therefore possible that Treg may not only directly inhibit innate immune cell activity but also suppress mobilisation of an innate immune response via inhibition of chemoattractant secretion and/or adhesion molecule upregulation by the tissue under attack. If this is the case it would provide a mechanism by which Treg can inhibit innate immune cell recruitment and suppress inflammation in general.

The advantage of inhibiting acute inflammatory immune responses may not be immediately obvious. Aside from the ability to prevent excessive immunopathology, one role could be in regulating the ability of wounds to heal, as inflammation has been shown to significantly affect wound closure and scar tissue formation (Eming et al. 2007). Although cells of the innate immune system are potent anti-microbial effectors and are important for the clearance of microbes in open wounds, in tissue injury which does not compromise the skin barrier, suppression of inflammation could limit further tissue damage by granule release. In a non-sterile wound it is conceivable that the presence of microbial molecules or inflammatory mediators could override the effect of Treg by rendering cells refractive to suppression.

Overall, the data presented in this Chapter add to the current literature which is slowly altering the way in which innate and adaptive immune system are viewed. Previously, they were thought of as separate entities with cell interactions primarily being confined within one system. Increasingly, reports indicate that the complexity of cell interactions has been largely underestimated. The possibility that Treg inhibit innate immune
responses in general, would add to the intricacy; however the mechanism and extent of suppression requires further detailed investigation.
Chapter 5 – Investigation of the Location of CD4⁺CD25⁺ Regulatory T Cell Action

5.1. Introduction

The ability of Treg to inhibit conventional T cell responses is now widely accepted. Extensive studies have been carried out in order to elucidate the mechanism of suppression both in vitro and in vivo, yet few have focused on the location of action of Treg. One objective of this thesis was to identify the location of Treg action in vivo.

Whether Treg suppress immune responses in the lymph nodes or at the site of challenge, remains to be elucidated. The implications are that Treg may be able to suppress different cell types depending on their location or be effective at different stages of an immune response. For instance, if Treg are only active in the lymph node they are likely to encounter naïve and central memory T cells along with B cells, as these cells are able to circulate through lymph nodes. Furthermore, accumulating evidence suggests that the ability of DC to activate T cells is inhibited by Treg (Cederbom et al. 2000; Serra et al. 2003; Misra et al. 2004; Veldhoen et al. 2006), implying that Treg inhibit the priming of an immune response. If the activity of Treg is not restricted to lymphoid tissue, it is possible that they are inhibiting the effector phase of an immune response, potentially inhibiting the action of both effector T cells and cells of the innate immune system to limit immunopathology.

Identification of the location of Treg action may also help analyse the mechanism of Treg suppression. In vivo studies of the mechanisms Treg use to mediate suppression have highlighted roles for soluble factors such as TGFβ and IL-10, and yet in vitro studies
consistently show a requirement for cell-cell contact. The identification of membrane bound TGFβ on the surface of Treg (Nakamura et al. 2001; Kim et al. 2007) may go some way to resolving this conflict but it doesn’t explain the requirement in some models for IL-10. The requirement for cell contact suggests that the anatomical location and migration of Treg \textit{in vivo} is critical for their suppression of immune responses. Furthermore, a cell contact dependent mechanism might confer upon Treg the ability to actively select a target cell. However the use of soluble mediators to exert suppression would allow Treg to target a larger number of cells, not necessarily restricted to the organ of Treg location. Another explanation is that Treg can induce a cell contact independent, suppressive phenotype in other cells via a cell contact dependent mechanism (Dieckmann et al. 2002; Jonuleit et al. 2002). Either way the mechanisms by which Treg exert their suppressive activity might be highlighted upon studying their location of action.

Although migration of conventional T cells has been studied extensively, the migration, and therefore the location, of Treg is less well studied. Circulating T cells have various adhesion molecules and chemokine receptors which are thought to direct migration; one such adhesion molecule is CD62L, which is expressed on the majority of Treg and naive T cells (Sallusto et al. 1999; Wherry et al. 2003; Bouneaud et al. 2005). Rolling of lymphocytes in high endothelial venules of peripheral lymph nodes is mediated by CD62L, the ectodomain of which can be proteolytically shed following T cell activation. Loss of CD62L prevents T cell access of LN (Hamann et al. 2000) and T cells isolated from inflammatory sites express low levels of CD62L (Mobley and Dailey 1992; Hou and Doherty 1993; Rigby and Dailey 2000). These observations suggest CD62L downregulation may be required for LN exit and/or entry into inflamed tissue.
If CD62L downregulation on T cells is required for infiltration of peripheral tissues, Treg from mice that cannot downregulate CD62L could be used to address the hypothesis that Treg must migrate into tissues in order to exert their suppressive effects. *In vitro* studies of CD62L expression following TCR engagement indicate that both shedding and gene transcription are involved in regulating surface levels of CD62L (Chao *et al.* 1997), as described in Chapter 1. Therefore in order to prevent CD62L downregulation, gene transcription must be maintained and proteolytic shedding prevented.

The laboratory of Dr Ann Ager generated two strains of mice expressing either wild-type or a shedding-resistant form of CD62L driven by the *hcd2* promoter (Figure 5.1 duplicated from Galkina *et al.* 2003). Protein expression driven using the *hcd2* promoter has been shown to be directed to T cells, and is maintained upon activation (Zhumabekov *et al.* 1995; Bromley *et al.* 2005), therefore wild-type CD62L expressed under this promoter should not undergo transcriptional downregulation post T cell activation (WT mice). Substitution of the proteolysis sensitive membrane proximal region of CD62L with that of the proteolysis insensitive CD62P, generated a shedding resistant form of CD62L (LΔP mice). Therefore LΔP mice, in which proteolytic shedding is prevented and gene transcription maintained, could potentially be used to address the hypothesis that prevention of CD62L downregulation on Treg inhibits their access to peripheral tissue subsequently preventing exertion of their suppressive effects.
Lectin domain

EFG domain

Membrane proximal region

Cleavage site

WT CQ ETNR SFSKIKEGDYN PLF
LAP CQ AGTL T IQEA* * * * * * PLF

• Directed expression to T cells using hcd2 cassette
• Backcross on to C57BL/6 CD62L−/− background

Figure 5.1. Construction of CD62L Mutants
Schematic representation of the Membrane Proximal Region (MPR) of mouse CD62L in relation to the whole molecule (SCR, short consensus repeat). Location of a primary cleavage site in wild-type CD62L (WT) is shown together with amino acid sequence of corresponding LAP mutant, containing the MPR of CD62P which is naturally shorter and lacks the cleavage site. This figure is duplicated from Galkina et al 2003.
T cell migration during an active immune response had not yet been studied in these mice, therefore it was first important to validate the hypothesis that maintained CD62L expression prevented T cell access of inflamed tissue. Since little is known about Treg migration, the migration of conventional T cells in WT and LAP mice was studied during influenza infection. The influenza model has been used extensively to study T cell responses, because the site of infection and therefore the target organ for T cells is known. This Chapter describes experiments carried out in order to characterise T cell migration in WT and LAP mice, with a view to utilising these mice to study the location of action of Treg.
5.2. Results

5.2.1. CD4⁺ and CD8⁺ T cells From LΔP Mice can Infiltrate Influenza Infected Lungs

In order to characterise the migratory pathways of CD62L transgenic T cells, a robust model of influenza A virus (flu) infection was used to validate the hypothesis that failure to shed CD62L impairs migration of T cells to sites of viral infection. Infiltration of T cells into the lung following intranasal (i.n.) infection with flu was studied at the peak of the immune response (Kedzierska et al. 2006). B6, WT and LΔP mice were sacrificed 8 days post infection, lungs were collected and numbers of infiltrating T cells assessed by flow cytometry. Due to the highly vascularised nature of the lung, it was hypothesised that the few T cells harvested from lungs of LΔP mice were located within the blood vessels and not within the lung tissue. In order to address this hypothesis, lungs were perfused with PBS:EDTA prior to tissue harvest. As expected, lungs harvested from B6 mice infected with flu showed marked infiltration with both CD4⁺ and CD8⁺ T cells (Figure 5.2). Surprisingly, there was no difference in T cells infiltrating the lung in WT and LΔP mice when compared to each other and to control B6 mice. Similar results were gathered from the lung draining lymph node (LdLN) and spleen. Repeated experiments confirmed this result suggesting that maintained CD62L expression on T cells does not impair their ability to enter inflamed tissues.
Figure 5.2. B6, WT and LΔP T Cells Are Equally Able to Enter the Tissue of Flu-Infected Lungs
B6, WT and LΔP mice were infected i.n. with 20 HAU of influenza virus. Eight days after infection, lungs were perfused and cells isolated from the lungs, lung draining lymph nodes (LdLN) and spleens were stained with antibodies to CD4 and CD8, and then evaluated by FACS. The total numbers of CD4⁺ or CD8⁺ cells in lungs, LdLN and spleens of B6 (squares), WT (triangles) and LΔP (circles) mice are shown. Each symbol represents an individual mouse and solid lines represent the means within each group. Data are representative of two individual experiments. Statistical significance was evaluated using a Mann Whitney test (* p<0.05).
5.2.2. CD8* but not CD4* T cells From LAP Mice Maintain CD62L Expression

In order to verify that expression of CD62L was indeed maintained on T cells in infected mice, particularly in LAP mice, cells harvested from organs were also immunostained with anti-CD62L antibody. As expected, a large proportion of CD4+ and CD8+ cells in the spleen of B6 mice were CD62Llo with WT T cells showing downregulation on a proportion of cells due to accelerated shedding (Figure 5.3). Despite studies indicating that CD62L on LAP T cells is not shed upon TCR stimulation in vitro (Galkina et al. 2003), a significant proportion of CD4+ cells in flu-infected LAP mice were CD62Llo in lungs and spleen (Figure 5.4). However, CD62L expression remained high on the majority of CD4+ cells isolated from the LdLN. In contrast the majority of CD8+ T cells remained CD62Lhi in lungs, LdLN and spleen (Figure 5.5). This indicates differential regulation of CD62L expression in CD4+ and CD8+ T cells, as discussed later in more detail.

Unfortunately the ability of CD4+ cells from LAP mice to downregulate CD62L expression and enter inflamed tissue excludes the possibility of utilising these mice to identify the location of CD4+CD25+ Treg action. However, the observation that CD8+ T cells from LAP mice, which do maintain high CD62L expression, also migrate into infected lungs contradicts the current dogma that T cells need to downregulate CD62L in order to enter inflamed tissue and warrants further investigation.
Figure 5.3. CD4⁺ T Cells from LAP Mice Can Downregulate CD62L in Contrast to CD8⁺ T Cells

B6, WT and LAP mice were infected i.n. with 20 HAU of influenza virus. Eight days after infection, cells isolated from the spleen were stained with antibodies to CD4, CD8 and CD62L, and then evaluated by FACS. Representative FACS plots of CD62L versus either CD4 or CD8 staining on splenocytes from B6, WT and LAP mice (5 mice per group) are shown.
Figure 5.4. CD4⁺ T cells from LAP Mice Show Similar Levels of CD62L Expression in Lungs and Spleen When Compared to B6 and WT Mice

B6, WT and LAP mice were infected i.n. with 20 HAU of influenza virus. Eight days after infection, cells isolated from the spleen were stained with antibodies to CD4 and CD62L, and then evaluated by FACS. The percentage of CD4⁺ cells in lungs, LdLN and spleens of B6 (squares), WT (triangles) and LAP (circles) mice expressing CD62L at high levels are shown. Each symbol represents an individual mouse and solid lines represent the means within each group. Data are representative of two individual experiments with a minimum of 4 mice per group. Statistical significance was evaluated using a Mann Whitney test (** p<0.01).
Figure 5.5. CD8+ T Cells from LΔP Mice Maintain CD62L Expression in Lungs, LdLN and Spleen When Compared to B6 and WT Mice

B6, WT and LΔP mice were infected i.n. with 20 HAU of influenza virus. Eight days after infection, cells isolated from the spleen were stained with antibodies to CD8 and CD62L, and then evaluated by FACS. The percentage of CD8+ cells in lungs, LdLN and spleens of B6 (squares), WT (triangles) and LΔP (circles) mice expressing CD62L at high levels are shown. Each symbol represents an individual mouse and solid lines represent the means within each group. Data are representative of two individual experiments with a minimum of 4 mice per group. Statistical significance was evaluated using a Mann Whitney test (* p<0.05, ** p<0.01).
5.2.3. Investigation of CD8$^+$ T cells in LAP Mice

In an experiment to determine whether the cells in the lungs of influenza-infected mice were antigen-specific effector cells, it was important to distinguish between non-activated and activated CD8$^+$ T cells, as normal lymphocyte trafficking had been reported in naïve WT and LAP mice (Galkina et al. 2003). The anti-influenza A response has been well characterised and CD8$^+$ T cells specific for an immunodominant epitope from influenza nucleoprotein can be detected using fluorescently labelled soluble H2-D$^b$ tetramers containing the NP$_{366-374}$ (NP68) peptide. This permitted monitoring of flu-specific CD8$^+$ effector cell generation during infection and the presence of flu-specific memory CD8$^+$ T cells once the infection has been cleared. Therefore, in order to determine the effect of maintained CD62L expression on activated CD8$^+$ T cell infiltration of infected tissues, B6, WT and LAP mice were infected i.n. with influenza A virus. 8 days post infection, lungs were perfused and, together with lung draining lymph nodes (LdLN) and spleen, numbers of CD8$^+$, tetramer$^+$ (tet$^+$) T cells infiltrating each organ assessed by flow cytometry (Figure 5.6, and Appendix Figure A.4). Control staining with NP68 tetramer and irrelevant tetramer (gp33), on both B6 and F5 (transgenic for a TCR recognising NP68) mice is shown in Appendix Figure A.7, along with representative FACS plot of NP68 Tetramer staining of organs from infected mice.

In agreement with the data on total CD8$^+$ T cells, the number of CD8$^+$/tet$^+$ T cells was similar in the lungs and spleen of B6, WT and LAP mice, however there was an increase in the number of CD8$^+$/tet$^+$ T cells in the LdLN of LAP mice when compared to B6 mice. The percentage of CD8$^+$/tet$^+$ cells expressing CD62L at high levels was also assessed in these experiments. Figure 5.7 shows downregulation of CD62L on B6 CD8$^+$/tet$^+$ cells in
Figure 5.6. Maintained CD62L Expression Does Not Prevent Flu-Specific CD8+ Cells From Entering Lung Tissue

B6, WT and LAP mice were infected i.n. with 20 HAU of influenza virus. Eight days after infection, lungs were perfused and cells isolated from the lungs, draining lymph nodes (LdLN) and spleens were stained with antibodies to CD8 and with NP68-tetramers and evaluated by FACS. The total numbers of CD8+ tet+ cells in lungs, LdLN and spleens of B6 (squares), WT (triangles) and LAP (circles) mice are shown. Each symbol represents an individual mouse and data are a summary of two independent experiments using groups of at least 5 mice. Solid lines represent the means within each group. Statistical significance was evaluated using a Mann Whitney test (* p<0.05).
Figure 5.7. CD62L is Maintained at a High Level in CD62L Transgenic Mice

B6, WT and ΔP mice were infected i.n. with 20 HAU of influenza virus. Eight days after infection, lungs were perfused and cells isolated from the lungs, draining lymph nodes (LdLN) and spleens were stained with antibodies to CD8, CD62L and with NP68-tetramers and evaluated by FACS. The percentages of CD8+ tet+ cells that are CD62Lhi in lungs, LdLN and spleens of B6 (squares), WT (triangles) and ΔP (circles) mice are shown. Each symbol represents an individual mouse and solid lines represent the means within each group. Data are representative of two independent experiments with a minimum of 4 mice per group. Representative FACS plots of CD62L versus tetramer staining on CD8+ splenocytes in B6 mice are shown. Staining in a naïve B6 spleen is given for comparison.
lung, as would be expected, however the majority of WT cells retain CD62L on their surface and almost none of the LΔP cells downregulate CD62L. These findings were reflected in the LdLN and spleen. In combination these data confirm the finding above that although antigen activated CD8+ T cells maintain CD62L at high levels, they are able to enter inflamed tissue.

5.2.4. CD8+ T cells from B6, WT and LΔP mice are Capable of Target Cell Lysis and IFNγ Secretion

CD62L functions primarily as a LN homing adhesion molecule, and downregulation of CD62L expression has been shown to correlate with immediate effector function in CD8+ T cells (Oehen and Brduscha-Riem 1998). As suggested previously the purpose of CD62L downregulation could be to allow entry into inflamed tissues, however signalling via CD62L has also been shown to costimulate TCR proliferation (Nishijima et al. 2005); it is therefore possible that the level of CD62L expression regulates the proliferation and/or differentiation of activated T cells. By analogy with other cell surface molecules that undergo ectodomain proteolysis, such as CD44 and Notch (Gao and Pimplikar 2001; Okamoto et al. 2001), shedding may regulate cleavage of the CD62L cytoplasmic tail, resulting in effector function. In order to assess the functional capability of virally induced T cells, the ability of T cells harvested from flu-infected mice to lyse targets labelled with the immunodominant flu epitope, NP68, was assessed. Splenocytes isolated from flu-infected mice were stimulated with NP68 peptide-pulsed, irradiated splenocytes from naïve mice. Five days later, stimulated cells were collected and used as effectors in a chromium release assay against NP68- or irrelevant peptide- pulsed targets. Figure 5.8 shows that cytotoxic T lymphocytes (CTL) are generated in all mice and specific lysis is greater for NP68-pulsed targets in all groups.
Figure 5.8. CD62L Transgenic Mice are Capable of Cytolysis
B6, WT and LΔP mice were infected i.n. with 20 HAU of influenza virus. Eight days after infection, cells isolated from the spleens were stimulated with NP68-pulsed, irradiated APC. 5 days later cells were used as effectors in a chromium release assay against NP68- (dark bars) or irrelevant peptide- (light bars) labelled targets. The mean percentage specific lysis ± SEM for 5 mice per group is shown at a culture dilution of 1:3 targets.
IFNγ production by CD8+ T cells has also been implicated in viral clearance (Belz et al. 2001; Wiley et al. 2001) and therefore the ability of CD8+ T cells from flu-infected mice to produce IFNγ upon restimulation with PMA/ionomycin in the presence of monensin was assessed (Figure 5.9). There was no statistically significant difference in the numbers of IFNγ secreting CD8+ cells between B6, WT and LAP in lungs and spleen suggesting that there is no impairment in CD8+ T cell effector function.

5.2.5. CD8+tet+ cells Show Enhanced Levels of CD107a and IFNγ on their Surface Following Direct Ex Vivo Antibody Staining

Both of the above methods require in vitro stimulation of cells prior to analysis and it was hypothesised that this may overcome suboptimally activated effector function in the cells analysed. Furthermore, stimulation of cells isolated from flu-infected mice also resulted in a downregulation of the TCR such that resolution of a tetramer positive population was not possible, preventing distinction between flu-specific and non-specific responses. For these reasons the possibility of direct ex vivo staining of cells with CD107a and IFNγ was explored.

Cell surface expression of CD107a (LAMP-1) has been shown to correlate with degranulation and release of cytolytic molecules and has been used to assess the lytic capacity of cells (Betts et al. 2003). Usually the antibody to CD107a is incubated in conjunction with peptide stimulation and monensin for 4 hours in order to enhance staining. However it was found that when stained directly ex vivo, tet+ populations could be resolved and CD107a levels were elevated on tet+ cells when compared to tet- cells in infected mice. Similar results were found for IFNγ, allowing analysis of the functional...
Figure 5.9. CD62L Transgenic Mice are Equally Capable of IFNγ Production
B6, WT and LΔP mice were infected i.n. with 20 HAU of influenza virus. Eight days after infection, cells isolated from the lungs and spleens were stimulated PMA and ionomycin for 4 hours at 37°C in the presence of monensin. Cells were then stained with antibodies to CD8 and IFNγ and evaluated by FACS. The total numbers of CD8⁺ cells producing IFNγ in lungs, LdLN and spleens of B6 (squares), WT (triangles) and LΔP (circles) mice are shown. Each symbol represents an individual mouse and data are a summary of two independent experiments using groups of at least 5 mice. Solid lines represent the means within each group.
capacity of CD8⁺tet⁺ cells without the bias of *in vitro* stimulation. Therefore, in order to assess the functional capacity of flu-specific CD8⁺ T cells in B6, WT and LΔP mice, mice were infected i.n. with flu and sacrificed eight days later. Lungs were perfused and cells isolated from lungs and spleens were stained with antibodies to CD8, CD107a, IFNγ and tetramer.

In agreement with the results of the chromium release assay, the geometric mean fluorescence intensity (MFI) of CD107a was elevated on flu-specific CD8⁺tet⁺ cells when compared to CD8⁺tet⁻ cells (Figure 5.10 and Appendix Figure A.8). Furthermore, the levels of CD107a expression were similar in B6, WT and LΔP mice, implying that there is no defect in the capacity of CD8⁺tet⁺ T cells to lyse target cells. Similarly, IFNγ levels were higher on CD8⁺tet⁺ cells when compared to CD8⁺tet⁻ cells in all mice (Figure 5.11 and Appendix Figure A.8). Although B6 mice showed a slightly higher level of IFNγ expression in the lung when compared to CD62L transgenic mice, there was no difference in IFNγ expression between WT and LΔP mice. In combination with the results on stimulated cells, this data indicates that flu-specific CD8⁺ T cells from WT and LΔP mice express, at the cell surface, key cytotoxic molecules associated with two different effector functions and are recruited to infected tissues in normal numbers, suggesting that they are equally capable of viral clearance.

5.2.6. CD8⁺tet⁺ Memory Cell Numbers are Similar in B6, WT and LΔP mice

Having established that flu-specific CD8⁺ T cells which maintain CD62L expression can migrate to sites of inflammation and appear to function normally, the logical progression
Figure 5.10. CD62L Transgenic Mice Show Comparable Levels of CD107a to B6 Ex Vivo

B6, WT and LΔP mice were infected i.n. with 20 HAU of influenza virus. Eight days after infection, lungs were perfused and cells isolated from the lungs and spleens were stained directly ex vivo with antibodies to CD8, CD107a and with NP68-tetramers and evaluated by FACS. The geometric mean fluorescence intensities (MFI) of CD107a on CD8⁺tet⁺ and CD8⁺tet⁻ cells for B6 (squares), WT (triangles) and LΔP (circles) mice are shown, along with representative histograms. Each symbol represents an individual mouse and data are a summary of two independent experiments using groups of at least 5 mice. Solid lines represent the means within each group.
Figure 5.11. CD62L Transgenic Mice are Show Comparable Levels of IFNγ to B6 Ex Vivo

B6, WT and LAP mice were infected i.n. with 20 HAU of influenza virus. Eight days after infection, lungs were perfused and cells isolated from the lungs and spleens were stained directly ex vivo with antibodies to CD8, IFNγ and with NP68-tetramers and evaluated by FACS. The geometric mean fluorescence intensities (MFI) of IFNγ on CD8\(^{+}\)tet\(^{+}\) and CD8\(^{+}\)tet\(^{-}\) cells for B6 (squares), WT (triangles) and LAP (circles) mice are shown. Each symbol represents an individual mouse and data are a summary of two independent experiments using groups of at least 5 mice. Solid lines represent the means within each group.
was to study the memory response. Two populations of memory T cells have been identified in the literature; those that are CD62L\(^{−}\)CCR7\(^{−}\)CD45RA\(^{−}\) and display immediate effector function, termed effector memory T cells (Tem); and those that are CD62L\(^{+}\)CCR7\(^{+}\)CD45RA\(^{+}\) which are able to enter LN but need restimulation in order to display effector function and therefore termed central memory T cells (Tcm). Tem have been shown to distribute throughout peripheral non-lymphoid organs, a process thought to be dependent on CD62L and CCR7 downregulation and Tcm are thought to circulate through lymphoid organs. One hypothesis is that CD62L downregulation is required for memory cell differentiation.

In order to address this hypothesis, B6, WT and LΔP mice were infected i.n. with flu and numbers of flu-specific T cells assessed more than 100 days after infection. Cells isolated from perfused lungs, LdLN, spleens as well as non-involved ovaries and ovary draining lymph nodes (OdLN) were stained with antibodies to CD8, CD62L and with tetramers (Figure 5.12 and Appendix Figure A.5). The numbers of CD8\(^{+}\)tet\(^{+}\) cells isolated from both the lung and the ovary were similar for all mice suggesting that there was no impairment of Tem cell generation in CD62L transgenic mice. Furthermore, the numbers of CD8\(^{+}\)tet\(^{+}\) cells isolated from the spleen and lymph nodes of these mice were similar to each other and B6 mice, suggesting that Tcm generation was not impaired. In fact, there was a trend for increased numbers of memory T cells in lymph nodes of LΔP mice. Although the low numbers of cells isolated from each tissue prevented definitive analysis of CD62L levels, it was clear that LΔP CD8\(^{+}\)tet\(^{+}\) cells maintain CD62L and that there are varying levels in WT and B6 mice (Figure 5.13). These results suggest that downregulation of CD62L is not required for the generation and distribution of flu-specific memory CD8\(^{+}\) T cells.
Figure 5.12. Maintained Expression of CD62L Does Not Affect the Distribution or Numbers of Flu-Specific Memory CD8+ T Cells

B6, WT and LΔP mice were infected i.n. with 20 HAU of influenza virus. >100 days after infection, lungs were perfused and cells isolated from the lungs, lung draining lymph nodes (LdLN), ovaries, ovary draining lymph nodes (OdLN) and spleens were stained with antibodies to CD8 and with NP68-tetramers and evaluated by FACS. The total numbers of CD8+ tet+ cells in lungs, LdLN, ovaries, OdLN and spleens of B6 (squares), WT (triangles) and LΔP (circles) mice are shown. Each symbol represents an individual mouse and data are representative of two independent experiments using groups of at least 5 mice. Solid lines represent the means within each group. Statistical significance was evaluated using a Mann Whitney test (* p<0.05).
Figure 5.13. CD62L Expression is Maintained on Flu-Specific Memory CD8+ T Cells in LΔP Mice

B6, WT and LΔP mice were infected i.n. with 20 HAU of influenza virus. >100 days after infection, cells isolated from the ovaries, ovary draining lymph nodes (OdLN) and spleens were stained with antibodies to CD8, CD62L and with NP68-tetramers and evaluated by FACS. Representative FACS plots of CD62L versus Tetramer staining on CD8+ cells in ovaries, OdLN and spleens of B6, WT and LΔP mice are shown. Staining in a naïve B6 spleen is given for comparison.
5.2.7. Infection with Recombinant Vaccinia Virus

Whether or not the memory CD8+ T cells generated are capable of enhancing viral clearance during a secondary infection can be addressed using a model of recombinant vaccinia virus (rVV) infection. To avoid the potential complications of studying recruitment to the primary site of challenge, the ability of flu-immune mice to clear recombinant vaccinia virus expressing the flu NP68 epitope (rVVNP) (Townsend et al. 1988) was assessed. After systemic infection by i.p injection, vaccinia virus replicates in the ovaries and clearance can be measured by plaque assay of the ovary (Jones et al. 2003). In flu-immune B6 mice, i.e. those infected with flu more than 100 days previously, the flu-specific memory CD8+ T cells are able to clear rVVNP faster than naïve mice (Figure 5.14). Therefore any defect in the CD8+ T cell memory response is reflected by a higher viral titre at day 5 post rVV infection.

5.2.8. Failure to Shed CD62L on Memory CD8+ T Cells Results in Increased Viral Titres

To address the hypothesis that maintained CD62L expression affects the ability of memory T cells to clear a secondary infection, flu-immune B6, WT and ΔP mice were injected i.p. with either rVVNP or a control rVV expressing an irrelevant melanocyte antigen from melanoma (rVVTlp2) (Overwijk et al. 1998). Mice were sacrificed 5 days later and viral titres in ovaries evaluated (Figure 5.15). As expected, viral titres in flu-immune B6 mice receiving rVVNP were much lower than in those receiving rVVTlp2 (effectively a naïve vaccinia-specific response) and WT mice were equally capable of viral clearance, reflecting clearance by NP68 specific CD8+ T cells. In stark contrast, viral titres in ΔP mice receiving rVVNP were significantly higher, although clearance of rVVTlp2 was similar in all mice.
Clearance of rV VNPP

Figure 5.14. Diagrammatic Representation of rV VNPP Clearance in Naïve and Flu-Immune

The diagram indicates the pattern of expected viral titres for naive (red line) and flu-immune (blue line) B6 mice given recombinant vaccinia virus expressing NP68 (rV VNPP).
Flu-immune B6, WT and LAP mice were generated by i.n. infection with 20 HAU of influenza virus and >100 days later, mice were challenged with recombinant Vaccinia Virus expressing a MHC class I-restricted peptide epitope derived from the influenza nucleoprotein (rVWNPP) or an irrelevant antigen (rVVTrp2). Mice were sacrificed 5 days later and ovaries collected. Ovaries were homogenized and viral titres determined by plaque assay. Symbols represent the number of plaque forming units (pfu) per ovary in individual B6 (squares), WT (triangles) and LAP (circles) mice challenged with rVWNPP (closed symbols) or rVVTrp2 (open symbols) from two independent experiments using groups of 5 mice. Statistical significance was evaluated using Mann Whitney test (* p<0.05, *** p<0.001).
5.2.9. Distribution of Memory CD8\(^{+}\)tet\(^{+}\) cells is Similar in B6, WT and L\(\Delta\)P mice Challenged with rVWNPP

The defect in the memory CD8\(^{+}\) T cell response leading to higher viral titres could be due to a defect in the resident T\(_{\text{EM}}\) and/or recruited T\(_{\text{CM}}\). Previously, it was shown that similar numbers of memory T cells were generated in mice prior to the second infection. To determine whether the reduced clearance of virus in L\(\Delta\)P mice was due to lack of recruitment of NP68-specific CD8\(^{+}\) T cells during infection, ovaries, OdLN and spleens were harvested and numbers of CD8\(^{+}\)tet\(^{+}\) compared with those in WT and B6 mice (Figure 5.16 and Appendix Figure A.6). As found during primary flu infection, altered CD62L expression did not impair the ability of CD8\(^{+}\)tet\(^{+}\) cells to enter the site of inflammation, in this case the ovary. In fact, there was a trend towards enhanced numbers of CD8\(^{+}\)tet\(^{+}\) in all organs in L\(\Delta\)P mice. Continued analysis of CD62L expression confirmed it was maintained at high levels on CD8\(^{+}\)tet\(^{+}\) cells in L\(\Delta\)P mice challenged with rVWNPP (Figure 5.17).

5.2.10. CD8\(^{+}\)tet\(^{+}\) T cells from B6, WT and L\(\Delta\)P mice Challenged with rVWNPP are Capable of Target Cell Lysis and IFN\(\gamma\) Secretion

Since the distribution of memory CD8\(^{+}\)tet\(^{+}\) T cells to ovaries and their recruitment during ongoing infection were not altered in L\(\Delta\)P mice, it was hypothesised that there was a defect in the cytotoxic capacity of these cells. In order to address this hypothesis, the ability of splenocytes harvested from flu-infected mice to lyse targets labelled with NP68 peptide or an irrelevant peptide was assessed by chromium release assay (Figure 5.18). Although B6 mice had consistently higher cytolysis, WT and L\(\Delta\)P mice had comparable
Figure 5.16. Elevated Numbers of CD8\textsuperscript{+}tet\textsuperscript{+} Cells are Detected in Organs of L\Delta P Mice When Compared to B6 and WT Mice

Flu-immune B6, WT and L\Delta P mice were generated by i.n. infection with 20 HAU of influenza virus and \textgreater100 days later, mice were challenged with recombinant Vaccinia Virus expressing a MHC class I-restricted peptide epitope derived from the influenza nucleoprotein (rVWNPP). Mice were sacrificed 5 days later and ovaries, ovary draining lymph nodes (OdLN) and spleen collected and stained with antibodies to CD8 and with NP68-tetramers and evaluated by FACS. The total numbers of CD8\textsuperscript{+}tet\textsuperscript{+} cells in organs of rVWNPP challenged mice is given for B6 (squares), WT (triangles) and L\Delta P (circles) mice. Data are a summary of 2 independent experiments using a minimum of 5 mice per group. Statistical significance was evaluated using a Mann Whitney test (*** p<0.001).
Figure 5.17. CD62L Expression is Maintained on Flu-Specific Memory CD8+ T Cells in LAP Mice Upon Recombinant Vaccinia Virus Infection
Flu-immune B6, WT and LAP mice were generated by i.n. infection with 20 HAU of influenza virus and >100 days later, mice were challenged with recombinant Vaccinia Virus expressing a MHC class I-restricted peptide epitope derived from the influenza nucleoprotein (rVVPNPP). Mice were sacrificed 5 days later and ovaries, ovary draining lymph nodes (OdLN) and spleen collected and stained with antibodies to CD8, CD62L and with NP68-tetramers and evaluated by FACS. Representative FACS plots of CD62L versus tetramer staining on CD8+ cells isolated from organs in B6, WT and LAP mice (5 per group) are shown.
Figure 5.18. CD8$^+$ tet$^+$ Cells from B6, WT and LΔP Mice Show Similar Capacity to Lyse Target Cells

Flu-immune B6, WT and LΔP mice were generated by i.n. infection with 20 HAU of influenza virus and >100 days later, mice were challenged with recombinant Vaccinia Virus expressing a MHC class I-restricted peptide epitope derived from the influenza nucleoprotein (rVWNPP). Mice were sacrificed 5 days later and spleens were harvested for Chromium release assay. Cells isolated from the spleens were stimulated with NP68-pulsed, irradiated APC. 5 days later cells were used as effectors in a chromium release assay against NP68 peptide- (closed symbols) or irrelevant peptide- (open symbols) labelled targets. The percent specific lysis at 3 different culture dilutions is given for 1 representative mouse out of a group of 5 for B6 (squares), WT (triangles) and LΔP (circles) mice.
levels of cytolysis suggesting that the defect in viral clearance in LAP mice is not due to a
defect in the ability of memory CD8\(^+\) T cells to lyse virally infected targets.

Direct \textit{ex vivo} staining with anti-CD107a and anti-IFN\(\gamma\) antibodies was performed in flu-
immune mice challenged with rVWNPP or left unchallenged (Figure 5.19 and 5.20). In
agreement with the data from the chromium release assay, there was no difference in the
levels of CD107a on CD8\(^+\)tet\(^+\) splenocytes isolated from B6, WT or LAP mice. However,
there was a significant increase in the levels of CD107a on CD8\(^+\)tet\(^+\) cells isolated from
the ovaries and OdLN of LAP mice when compared to those from B6 and WT mice. A
similar result is seen when comparing levels of IFN\(\gamma\) on LAP and WT cells (Figure 5.20).
In combination with the observation that higher numbers of CD8\(^+\)tet\(^+\) cells can be seen in
LAP mice, this data supports the hypothesis that the NP68-specific response is greater in
these mice due to increased viral titres at this time point.
Figure 5.19. CD8⁺ tet⁺ Cells From B6, WT and LΔP Mice Have Levels of Cell Surface CD107a Expression

Flu-immune B6, WT and LΔP mice were generated by i.n. infection with 20 HAU of influenza virus and >100 days later, mice were challenged with recombinant Vaccinia Virus expressing a MHC class I-restricted peptide epitope derived from the influenza nucleoprotein (rVWNPP). Mice were sacrificed 5 days later and ovaries, ovary draining lymph nodes (OdLN) and spleen were harvested for immunostaining. Cells were stained directly ex vivo with NP68-tetramer, anti-CD8 and anti-CD107a antibody and evaluated by FACS. The geometric mean fluorescence intensity (MFI) of CD107a on CD8⁺ tet⁺ populations is given for unchallenged (open symbols) and rVWNPP challenged (closed symbols) mice. Data are representative of 2 independent experiments with a minimum of 5 mice per group. Each symbol represents a single mouse and the solid lines indicate means within each group. Statistical significance was evaluated using a Mann Whitney test (* p<0.05).
Figure 5.20. CD8$^+$ tet$^+$ Cells from B6, WT and LΔP Mice Show Similar Capacity to Produce IFNγ

Flu immune B6, WT and LΔP mice were generated by i.n. infection with 20 HAU of influenza virus and >100 days later, mice were challenged with recombinant Vaccinia Virus expressing a MHC class I-restricted peptide epitope derived from the influenza nucleoprotein (rVNP). Mice were sacrificed 5 days later and ovaries, ovary draining lymph nodes (OdLN) and spleen were harvested for immunostaining. Cells were stained directly ex vivo with NP68-tetramer, anti-CD8 and anti-IFNγ antibody and evaluated by FACS. The geometric mean fluorescence intensity (MFI) of IFNγ on CD8$^+$ tet$^+$ populations is given for unchallenged (open symbols) and rVNP challenged (closed symbols) mice. Data are representative of 2 independent experiments with a minimum of 5 mice per group. Each symbol represents a single mouse and the solid lines indicate means within each group. Statistical significance was evaluated using a Mann Whitney test (* p<0.05).
5.3. Discussion

The aim of this Chapter was to explore whether CD62L transgenic mice could be used to identify the location of Treg action. The hypothesis on which the study was to be based, that T cells unable to downregulate CD62L could not enter inflamed tissue, had to be tested initially. The findings of this initial study altered the course of the investigation as two important observations were made. CD62L expression could be downregulated on CD4$^+$ T cells in LΔP mice and therefore Treg with maintained CD62L expression could not be generated. However, maintenance of CD62L on CD8$^+$ T cells did not prevent access of inflamed tissue. The second observation proved interesting as it contradicted the current dogma and therefore warranted further investigation.

5.3.1. Characterising T cell migration CD62L Transgenic Mice

Maintained expression of CD62L was hypothesised to prevent T cell access of inflamed tissue therefore T cell migration into flu infected lungs was monitored. The slightly lower number of CD4$^+$ and CD8$^+$ T cells isolated from the lungs of LΔP mice when compared to B6 and WT mice initially suggested a slight impairment of T cell migration to inflamed tissue. However, repeated experiments where lungs were perfused prior to tissue harvest, yielded similar numbers of cells in both lung and LdLN, with reduced numbers of CD4$^+$ in the spleen of WT and LΔP mice when compared to B6 mice. Analysis of CD62L levels in these mice revealed that CD62L expression on CD4$^+$ T cells could be downregulated in lungs and spleen of infected LΔP mice. In contrast CD8$^+$ T cells from LΔP mice were unable to downregulate CD62L expression.

Although interesting, the mechanism of CD62L downregulation on these cells was not pursued in this thesis. Due to the similar FACS profiles of CD4$^+$ cells isolated from WT
and LA\textasciitilde P mice, the possibility that viral infection can induce an alternative shedding of CD62L from the surface of LA\textasciitilde P T cells must be explored, although this would oppose in \textit{vitro} studies indicating that LA\textasciitilde P T cells resist basal and PMA-induced shedding (Galkina \textit{et al.} 2003). Furthermore, CD8\textsuperscript{+} T cells isolated from LA\textasciitilde P mice crossed with F5/RAG\textsuperscript{+/-} mice, in which all lymphocytes express a MHC class I restricted TCR for flu nucleoprotein (NP68), also resisted cognate peptide induced shedding. An alternative hypothesis is that expression of CD62L is transcriptionally downregulated in these CD4\textsuperscript{+} cells, although numerous studies have utilised the human CD2 promoter to maintain expression of proteins upon T cell activation without loss of expression (Zhumabekov \textit{et al.} 1995; Bromley \textit{et al.} 2005).

\textbf{5.3.2. Location of Treg Action}

Unfortunately, the ability of CD4\textsuperscript{+} cells from LA\textasciitilde P mice to downregulate CD62L expression and enter inflamed tissue prevented utilisation of these mice to identify whether or not Treg excluded from inflamed tissue were impaired in their suppressive activity. This model was to be used to identify the location of Treg action \textit{in vivo}, however during the course of this project a plethora of other studies on Treg migration were published. While the majority of these studies focused on identification of chemokine receptor expression and chemotaxis by Treg isolated from peripheral blood of humans (Iellem \textit{et al.} 2003; Hirahara \textit{et al.} 2006; Lim \textit{et al.} 2006), or lymphoid organs of mice (Venturi \textit{et al.} 2007), thereby implying possible migration patterns, a few studied cells harvested from non-lymphoid organs (Siegmund \textit{et al.} 2005; Yurchenko \textit{et al.} 2006).
The wide ranging experimental methods may explain the lack of a consensus in reports, however there is some agreement on the markers of migration in humans and mice. In summary, the main migration markers shown to be expressed on Treg are CCR7, CD62L, CCR4, CLA, CCR5, and CD103 as discussed in the introduction. CCR7 is a receptor for lymphoid chemokines CCL19 and CCL21 and CD62L is an adhesion molecule known to be required for entry into lymph nodes. CCR4 is a receptor for CCL17 and CCL22 which are produced by macrophages, activated T cells and mature DC. CLA is a ligand for E-Selectin which is expressed in normal skin and inflamed endothelium. CCR5 is the receptor for CCL4, an inflammatory chemokine and CD103 is the αΕ subunit in a range of integrins which bind E-cadherin on epithelia. Each study has highlighted that the majority of Treg isolated by various techniques, expressed both lymphoid homing and non-lymphoid homing markers.

A recent study of murine Treg isolated from different locations aimed to identify cohorts of markers on Treg that may be associated with localisation (Sather et al. 2007). Treg isolated from normal skin express CD103 and CCR4 supporting earlier studies linking these markers to skin tropic Treg (Suffia et al. 2005; Hirahara et al. 2006). In addition, Treg in peripheral LN stimulated by antigen administered subcutaneously, downregulated CCR7 and upregulated CD103, CCR4 and E-selectin ligands and were found 5 days later in the skin. Furthermore this accumulation was impaired in the absence of CCR4, and CCR4 deficient Treg were unable to prevent cutaneous inflammation caused by wild-type CD4+ T cells.

Dissection of Treg subtypes may yet reveal individual Treg populations with specific tissue homing capacities, however given the current information it is possible that Treg
are promiscuous and retain the ability to circulate through lymph nodes whilst being able to enter inflamed tissue. This would allow the Treg to be present both at the priming of an immune response and at the effector stage and therefore bestows dual potential to limit excessive immune activation. Furthermore, the identification of skin homing receptors on Treg in peripheral blood and the isolation of Treg from normal skin in humans (Clark and Kupper 2007) and mice (Sather et al. 2007) suggests that Treg may patrol the peripheral tissue prior to immune challenge, conferring the ability to interact with rapid, innate immune and memory responses.

5.3.3. A New Focus – CD8⁺ T cells

Current belief is that the ability of T cells to enter inflamed tissue is permitted via CD62L downregulation based on the observation that T cells isolated from sites of inflammation are CD62L⁺. However, CD8⁺ T cells from LΔP mice maintain high CD62L expression and yet are able to migrate into infected lungs upon flu infection, indicating that CD62L downregulation is not required for entry into inflamed tissue. Therefore despite the lack of usefulness for studying Treg, further investigations were carried out in order to fully characterise flu-specific CD8⁺ T cells in these mice.

Since maintained CD62L expression does not prevent access of T cells to inflamed tissue, and cells isolated from sites of infection express low levels of CD62L, it was hypothesised that CD62L shedding and transcriptional downregulation might be involved in the generation or function of effector T cells. Furthermore, it was hypothesised that this may also impact upon memory cell generation, distribution and function. It was first important to assess the ability of flu-specific T cells to enter inflamed lung as these cells would be activated by stimulation through the TCR which would usually induce shedding and later transcriptional downregulation of CD62L.
The influenza model has been widely used to assess T cell responses to infection and tetramers to the MHC class I restricted immunodominant peptide have been employed to identify flu-specific CD8⁺ T cells (Townsend and Skehel 1984). Studies of CD8⁺tet⁺ cells supported those on total CD8⁺ T cells in that there was no significant difference in number of cells recruited to the lung or isolated from the spleen between B6, WT or LAP mice. There was a trend towards slightly enhanced numbers of CD8⁺tet⁺ cells isolated from the LdLN. The lack of a difference between mice was reflected in the ability of CD8⁺ cells to lyse NP68 labelled targets and produce IFNγ. This result was reproduced upon direct ex vivo staining of CD8⁺tet⁺ cells with IFNγ and CD107a, a marker of lytic capacity. In combination these results indicate downregulation of CD62L is not a requirement for effector cell generation or migration to inflamed tissue. Furthermore, maintenance of CD62L expression does not impair the functional capacity of antigen specific CD8⁺ T cells.

5.3.4. Memory Cell Generation

The similar number of CD8⁺tet⁺ cells harvested from B6, WT and LAP mice 100 days post flu infection, suggested that maintained CD62L expression did not affect the distribution of flu-specific memory cells at the site of primary infection. Furthermore the number of CD8⁺tet⁺ cells harvested from distant, non-involved sites was also similar, in agreement with reports for other virus-specific memory cells (Masopust et al. 2001; Masopust et al. 2004). Interestingly, there is a trend towards increased numbers of CD8⁺tet⁺ cells harvested from LdLN in LAP mice, which is reproduced, although not significantly, in the OdLN. This may simply reflect the consequences of the primary
response, as elevated numbers were seen in LdLN 8 days post-infection, probably due to the slight increase in retention of cells crossing the HEV as reported previously (Galkina et al. 2003). Although the low cell number harvested from organs precluded definitive analysis of CD62L expression on flu-specific memory CD8+ T cells, it is clear from the FACS plots that expression on cells isolated from B6 and WT mice is varied whereas expression on LΔP T cells remains high. Together these results indicate downregulation of CD62L is not required for the generation and distribution of flu-specific memory T cells to non-lymphoid tissue, regardless of site of activation.

5.3.5. Failure to Shed CD62L Compromises Anti-Viral Immunity

Although it has been shown that the ability to generate memory T cells is similar amongst B6, WT and LΔP mice, the function of these memory T cells also had to be tested, and therefore the ability of mice to clear a secondary infection with rVV expressing the immunodominant flu peptide, NP68, was assessed. This model avoided complicating factors associated with infection in the same organ as, after systemic infection, vaccinia virus replicates to high titres in ovaries and clearance of rVV expressing CTL epitopes from the ovaries has been used as a measure of CD8+ T cell memory in a number of other studies (Karupiah et al. 1990; Jones et al. 2003).

Since the only difference between WT and LΔP mice is their ability to shed CD62L from their T cells, the inability of LΔP mice to clear rVVNPP when compared to both B6 and WT mice, indicates a significant role for CD62L shedding in the control of infection. However, some NP68-specific clearance was evident as titres in rVVNPP challenged mice were lower than rVVTrp2 challenged mice. In addition this defect was not due to
global impairment of the response to vaccinia virus as there was no difference in the ability to control rVVTrp2 infection between groups.

This impairment of viral clearance by memory CD8$^+$ T cells was not simply due to an inability of memory cells to migrate to the site of infection as there were in fact increased numbers of CD8$^+$tet$^+$ cells found in both the ovary and the spleen. These increased numbers may reflect the on going struggle to limit infection as viral titres are still high at this time point. Nor was the impairment due to a defect in function as determined by \textit{in vitro} analysis of lytic activity in stimulated cells. Further analysis of the levels of IFN$\gamma$ and CD107a on the surface of CD8$^+$tet$^+$ cells confirmed these results and indicated that cells isolated from LAP mice were not only functionally capable but were expressing higher levels of these markers than their WT and B6 counterparts. In summary, although memory CD8$^+$ T cells in LAP mice are equally capable of both target lysis and IFN$\gamma$ production and are recruited to infected tissues in normal numbers, they show defective viral clearance.

Clearance of virus by memory T cells is likely to depend on both memory T cells resident in tissue (T$_{EM}$) and those recruited upon infection (T$_{CM}$), the contributions of which have not yet been clearly defined. Given the current definition of T$_{EM}$ and T$_{CM}$, it is hypothesised that initial infection is limited by T$_{EM}$ and that T$_{CM}$, once activated in the lymphoid tissue, would migrate into the ovary to further control infection. This is highly probable as only a small increase in the number of CD8$^+$tet$^+$ cells is seen in the ovary up until day 3 of vaccinia infection, possibly due to T$_{EM}$ proliferation, followed by a rapid influx between day 3 and day 5 (data not shown). Since there were no differences in cell number or viral titre seen between groups of mice at these early time points, this suggests there is no defect in early responses to infection (data not shown).
If the primary target is the ovarian stroma, the efficiency of viral clearance will depend on the ability of T cells, most likely TCM, to enter/migrate through the ovarian tissue towards their target. The ability of cells to enter inflamed tissue is dictated by altered expression of inflammatory chemokines and/or adhesion molecules within the organ and is controlled by the infected stromal cells. One hypothesis is that in the absence of CD62L shedding, flu-specific cells in LAP mice are not optimally activated to express the correct adhesion and chemokine receptors required to gain access to their target infected cells within the ovary. Although viral titres were higher in LAP mice at day 5, clearance was not completely absent as by day 8 the titres in LAP mice were comparable to those in WT and B6 mice (data not shown). It is possible that, as virus replication proceeds relatively unchecked in LAP mice, the accompanying tissue destruction allows antigen-specific T cell entry, which then interact with and kill target cells more readily. However, the elevated numbers of CD8+ tet+ cells seen at day 5 may simply compensate for a delay in the preceding immune response.

Several mechanisms could be responsible for an early delay in viral clearance. CD62L signalling via ligands expressed on endothelial and stromal cells in the draining LN or the target organ could activate integrin- or chemokine-mediated adhesion and migration (Hwang et al. 1996; Giblin et al. 1997; Ding et al. 2003). Sustained CD62L signalling could therefore enhance adhesion such that the kinetics of T cell entry, migration within, and exit from these organs may be slowed, resulting in a slight delay in target cell attack. The absence of significant levels of soluble CD62L in LAP mice, which could compete for signalling with cell surface CD62L and limit adhesion or directly stimulate ligand expressing stromal cells, may also alter the kinetics of memory T cell migration through tissues. As alluded to previously, CD62L shedding could direct the subsequent genetic
programming of memory T cells required for optimal target cell interaction. Although there is little evidence to support this hypothesis in T cells, it has been reported that neutrophils expressing a shedding resistant mutant of CD62L are defective in chemokine directed migration (Venturi et al. 2003). To explore these differing hypotheses, it will be important to identify anatomical location and kinetics of CD62L shedding, and to determine whether ligands for CD62L are induced in virally infected ovaries. Further in depth studies of the kinetics of memory cell migration would also prove invaluable.

In summary, it is clear from the data presented here that the existing dogma that CD8⁺ T cells require CD62L downregulation in order to enter inflamed tissue needs to be revised. Furthermore, it has been shown that maintained CD62L does not affect CD8⁺ effector and memory cell generation and distribution to non-lymphoid organs, which is supported by the finding that T_CM of the phenotype CD62L⁺CCR7⁺, can be detected in non-lymphoid organs (Bouneaud et al. 2005; Unsoeld and Pircher 2005). Finally these results demonstrate a critical role for CD62L shedding in the control of viral infection by CD8⁺ memory T cells. Future studies are needed to determine whether this is truly a defect in cell migration or a defect in target cell recognition.
Chapter 6 - Final Discussion

6.1. Treg Inhibit Innate Immune Responses

The immune system has evolved to protect the host against invading pathogens; however it also has the potential to damage the host. In order to prevent inappropriate or excessive immune responses the immune system is intricately regulated. One source of immune regulation is naturally occurring CD4⁺CD25⁺ regulatory T cells, which have been shown to suppress T cell activation, proliferation and effector mechanisms in vitro and in vivo (Sakaguchi et al. 1995; Dubois et al. 2003), however T cells are not the sole cause of immunopathology. Therefore, whether or not Treg suppression is confined to T cells was an area that warranted exploration.

Reports indicating that Treg could also inhibit B cells (Nakamura et al. 2004; Fields et al. 2005; Lim et al. 2005) and DC (Cederbom et al. 2000; Misra et al. 2004; Oderup et al. 2006) suggested that suppression was not confined to T cells, however prior to the commencement of this study, only one report had addressed the possibility that Treg inhibit innate immune responses. In this study, Treg could inhibit chronic intestinal inflammation, reducing numbers of inflammatory cells in lymphoid tissue, indicating that Treg inhibit chronic inflammation (Maloy et al. 2003).

The work presented in this thesis demonstrates that Treg can inhibit the rejection of B16FasL, which has been shown to be rejected by innate immune responses. In contrast to Maloy et. al. the model of B16FasL rejection is one of acute inflammation in which Treg depletion can enhance responses within hours. Dissection of the cells involved indicated that activated Treg could directly inhibit the lytic ability of NK cells and may
restrict migration of NK cells to the site of tumour rejection. Furthermore, the data presented here implicates Treg in the inhibition of neutrophil recruitment to the site of tumour rejection.

6.1.1. Possible Modes of Action

The possible ways in which Treg could achieve this suppression are extremely varied and include direct and indirect mechanisms. TGFβ from Treg has been implicated in the suppression of NK cell activity (Ghiringhelli et al. 2005; Smyth et al. 2006), and along with IL-10 has a role in the control of T cell independent intestinal inflammation (Maloy et al. 2003) and neutrophil activity (Lewkowicz et al. 2006). These cytokines are well known for their immunosuppressive properties on various cell types and therefore represent strong candidates for direct suppression of innate immune responses in the B16FasL model. However the sources of these cytokines are varied complicating interpretation of experiments using anti-cytokine depleting antibodies in vivo. Although it has been demonstrated that TGFβ and IL-10 are involved in Treg mediated suppression of innate immune cells in vitro, particularly membrane bound TGFβ, Treg could induce production of these cytokine by other cell types in vivo via another mechanism. Further experiments utilising anti-TGFB and anti-IL-10 depleting antibodies in combination with Treg from cytokine deficient mice would help elucidate the role these cytokines play in Treg mediated immunosuppression.

Chemokine and cytokine production by local cells plays a critical role in the initiation and maintenance of an inflammatory response and therefore could be the target for Treg suppression. Inflammatory cells such as neutrophils and NK cells are recruited and activated by MIP-2(IL-8), IL-1β, MIP-1α and others which can be produced by local
macrophages, keratinocytes and epithelial cells. Another hypothesis is that Treg may influence neutrophil and NK cell activity indirectly by suppression of cytokine production. Identification of chemokines/cytokines produced in the skin following B16FasL challenge, in the presence and absence of Treg, would allow validation of this hypothesis and may provide insights into the target populations.

In support of this hypothesis, the recently described roles for CD39 and CD73 on the surface of Treg in converting proinflammatory ATP into immunosuppressive adenosine provides another possible mechanism by which Treg can suppress innate immune responses (Borsellino et al. 2007; Deaglio et al. 2007). ATP can be released upon cell death and ligation of its P2 receptor on immune cells can lead to IL-1β release in macrophages and activation of DC (Khakh and North 2006). Since the importance of macrophages for the initiation of inflammation following challenge with FasL expressing cell lines has been clearly demonstrated (Hohlbaum et al. 2000; Hohlbaum et al. 2001), this is also a strong candidate for the mechanism for Treg mediated suppression. The hypothesis that Treg deplete ATP and therefore inhibit macrophage production of IL-1β, leading to a reduction in neutrophil recruitment could be addressed using the B16FasL model in P2 receptor deficient mice or by utilising Treg deficient in CD39 and or CD73. The A2A receptor for adenosine has also been identified on the surface of NK cells (Lokshin et al. 2006) and neutrophils (Ernens et al. 2006) suggesting that the product of ATP degradation could also influence NK cells and neutrophils directly.

6.1.2. Implications

The novel observation that Treg can inhibit acute innate immune responses has a range of implications. Firstly, it adds to the growing body of literature indicating that Treg can suppress a multitude of immune cells, possibly acting to generally dampen down immune
responses. It also alters the way in which the immune system as a whole is viewed. Currently it is accepted that there are two arms of the immune system, innate and adaptive, within which cell interactions are confined. Combined with other evidence of 'cross-talk', the interaction between Treg from the adaptive immune system and cells of the innate immune system suggests that this view is oversimplified and perhaps inappropriate. The immune system is highly complex with each activated cell adding to the multitude of activation signals, explaining the requirement for strict regulation. The observation that Treg inhibit a plethora of immune cells may suggest that the mechanism by which they suppress is an evolutionarily old one that can globally suppress cell activity, leading to suppression of not only immune cells but non-immune cells too. However it may also suggest that Treg employ a multitude of effector mechanisms which may affect target cells more specifically.

Another question that remains unanswered is whether these Treg represent only a subpopulation of naturally occurring Treg. The ability of Treg to suppress the innate immune response to B16FasL, which is rapid by nature, suggests that Treg must also be exerting their effect within hours. This suggests that Treg must either be activated rapidly or be constantly active. The question then arises as how they are activated. The published evidence on Treg activation suggests that these cells are activated to suppress T cell responses through their TCR. The observation made in this thesis that only Treg from naïve mice activated through their TCR could inhibit tumour lysis by NK cells supports this hypothesis, suggesting that Treg that inhibit innate immune cells are also activated through their TCR. The recognition of self peptides by TCR on Treg has also been reported which suggests that Treg could be activated constantly by self antigen in the periphery and/or activated upon presentation of self peptides during an ensuing immune
response. In the first instance you might expect effector memory like Treg able to rapidly respond without costimulation and in the second there could be a mix of different phenotypes depending on the immune challenges already experienced by the host.

However the research into activation of Treg has focused on those able to inhibit T cell responses and therefore may not represent the way in which Treg are activated to suppress innate immune responses. Other innate receptors such as the TLRs may be responsible for activating Treg to inhibit innate immune cells. A variety of TLRs have been identified on the surface of Treg (Caramalho et al. 2003) and a number of recent reports have indicated that TLR ligation on Treg leads to suppression of T cells responses. Two reports indicated that TLR2 ligation with Pam$_2$Cys resulted in increased Treg proliferation, with temporary abrogation of suppression (Liu et al. 2006a; Sutmuller et al. 2006), which has also been reported upon TLR9 ligation with CpG oligodeoxynucleotide (Chiffoleau et al. 2007). Although TLRs were first described to bind pathogen associated molecules, it has been shown that they also bind host ‘stress’ associated molecules such as heat shock proteins (Ohashi et al. 2000; Asea et al. 2002; Vabulas et al. 2002). Indeed, ligation of TLR2 with heat shock protein, HSP60, on Treg, in conjunction with TCR stimulation, has been shown to augment inhibition of T cell responses (Zanin-Zhorov et al. 2006).

These reports suggest that Treg could also be activated though their TLR to suppress innate immune responses. In support of this hypothesis, it has been reported that TLR4 stimulation of human Treg with ultra pure LPS can induce inhibition of neutrophil activity (Lewkowicz et al. 2006). In contrast to other studies on suppression of T cell responses by TLR activated Treg, this report found no requirement for TCR stimulation.
and no temporary abrogation of suppressive activity, suggesting that the mechanism of suppression of innate immune responses is different to that of T cell responses. Further analysis of the effects of different TLR ligands will determine whether TLR ligation in general can stimulate Treg or whether specific TLRs/ligands stimulate suppression, whilst others stimulate Treg proliferation and temporary abrogation of suppression. Experiments stimulating Treg with TLR ligands in vitro and utilising TLR deficient Treg in vivo would address this hypothesis.

Although TCR stimulation is a likely way of activating Treg to suppress innate immune responses, this relies on the ability of the Treg TCR to recognise antigen, and therefore activation could be limited. Should TLR ligation stimulate Treg to suppress in the absence of TCR stimulation, this would provide a mechanism by which a greater number of Treg could be activated rapidly. It is also possible that other molecules and cytokines can bypass TCR stimulation, a hypothesis supported by the observation that Treg can be activated to suppress T cell responses by the addition of IL-2 (Thornton et al. 2004), although IL-2 is unlikely to be produced very early in the B16FasL rejection model.

The role of DC in the stimulation of Treg has also been explored. Although initial reports indicated that suppression of T cell responses by Treg in vitro was APC independent (Thornton and Shevach 1998), other reports indicate that DC can induce regulatory T cell proliferation and differentiation from naïve T cells (Jonuleit et al. 2000; Mahnke et al. 2003), and Treg can inhibit DC function (Cederbom et al. 2000; Oderup et al. 2006). A recent report has indicated that other cells can contribute to this interaction. Keratinocytes engineered to express high levels of RANKL, which is naturally upregulated upon ultraviolet light exposure and during certain types of inflammation, interact with RANK
on DC in the skin to enhance their ability to induce regulatory T cell expansion in the skin and draining lymph nodes (Loser et al. 2006). Induction of Treg expansion in this study requires TNFα production (Loser et al. 2006), a finding supported by the observation that regulatory T cells express higher levels of the TNF receptor 2 and TNFα enhances suppressive activity (Chen et al. 2007). Although it is not clear what proportion of cells are induced from Tconv in the study by Loser et al., it is interesting to note that inflammatory responses may enhance Treg activity, and in combination with the findings of this thesis, suggest that this could be a negative feedback loop to limit extensive inflammation.

However the role of TNFα and other inflammatory mediators in the regulation of Treg responses has also been implicated in the inhibition of Treg activity. In numerous studies of Rheumatoid Arthritis (RA) in humans, it has been shown that TNFα impairs both naturally occurring, and adaptive regulatory T cell function (Ehrenstein et al. 2004; van Amelsfort et al. 2007), and results in increased FOXP3 expression (Valencia et al. 2006). Other cytokines such as IL-7 and IL-15 have also been shown to limit suppressor function of regulatory T cells (Ruprecht et al. 2005). This dichotomy is similar to that involving TLR stimulation, where slight variations in ligand-receptor signalling or microenvironment could result in essentially opposite outcomes. Experiments to assess if suppression is temporarily abrogated in Treg treated with inflammatory cytokines would go some way to help resolve these differences.

In the B16FasL model it is still unclear how Treg may be activated to suppress innate immune responses. Each of the mechanisms described above are plausible in this model. Since the parental cell line B16 is a tumour cell line derived from B6 mice, it will contain
self-antigens and also tumour antigens, suggesting that Treg could be activated through their TCR upon recognition of host proteins or tumour associated host proteins. Equally, FasL expression results in large amounts of cell death in the local area, either directly or indirectly, which could lead to the release of ‘stress’ associated molecules which could ligate TLRs and may provide ligands for self-specific TCR. The cytokine milieu could also be a contributing factor, with inflammatory cytokines acting in concert to potently activate Treg.

Another hypothesis is that FasL costimulation may activate Treg directly. The Fas-FasL interaction has long been implicated in the homeostatic contraction of T cell responses, with expression of FasL being upregulated on activated T cells resulting in ‘autocrine suicide’ (Dhein et al. 1995). It has been reported that Treg express increased levels of Fas (Taams et al. 2001) and are more susceptible to FasL induced cell death in the absence of TCR stimulation when compared to Tconv (Fritzsching et al. 2005). However, upon TCR activation, Treg are less susceptible to cell death than their Tconv counterparts (Fritzsching et al. 2005). This suggests that Fas/FasL signalling differs between the two cell types and is possibly altered by other exogenous signals. It is therefore possible that Fas signalling could costimulate TCR signalling in surviving Treg, which would contribute towards the termination of immune responses. Indeed, a number of studies have reported that Fas signalling can costimulate suboptimal TCR stimulation in Tconv via caspase activation (Alam et al. 1999; Kennedy et al. 1999). Studying of the effect of Fas stimulation on the suppressive effect of Treg, using cells deficient in the apoptosis inducing pathway, would help address this hypothesis.
It is also possible that commensal bacteria, drawn into the skin upon injection of B16FasL, could activate Treg. This could be through TCR recognition of foreign antigens or via recognition by innate receptors such as TLRs, indeed LPS has been shown to activate Treg to suppress neutrophil activity (Lewkowicz et al. 2006). The reason why bacteria might activate Treg may not be immediately obvious, until you consider that the skin, like the gut, is covered with commensal, non-pathogenic bacteria and is constantly exposed to exogenous antigen. Responses to commensal bacteria on the skin must be tightly regulated in order to prevent unnecessary damage, which could result in compromising this physical barrier to pathogens. Although the mechanism is still debated, it is widely agreed that Tconv can become tolerant to commensal bacteria in the gut (and possibly the skin), either by inactivation/deletion due to lack of costimulation, or by active suppression (Iweala and Nagler 2006).

However innate immune receptors recognise molecules shared by both pathogenic and non-pathogenic micro-organisms, suggesting that regulation of innate immune responses must be by other mechanisms. Active suppression of both innate and adaptive immune responses by Treg in the skin and gut is therefore an attractive hypothesis. It is also possible that the ability to suppress innate immune responses is confined to those Treg within these organs. Recent studies have reported that APC isolated from the skin, gut and draining LN activate Treg to express skin and gut homing receptors respectively (Schwarz et al. 2007; Siewert et al. 2007). It is therefore possible that these APC also program these Treg to respond differently to micro-organisms. The study of Treg isolated from different anatomical locations may help address this hypothesis.
The location of Treg action would also impact upon their effectiveness in vivo. If located solely within the lymphoid tissue prior to immune challenge, Treg have the opportunity to interact with a wide range of immune cells with the potential to suppress both the initiation and maintenance of an immune response. The close contact of large numbers of immune cells in this situation would provide the ideal environment in which Treg could suppress responses by a contact-dependant mechanism as suggested by a number of studies in vitro (Thornton and Shevach 1998; Thornton and Shevach 2000; Nakamura et al. 2001).

However, it has been reported that although Treg are activated via their TCR in an antigen specific manner their suppression is antigen non-specific (Thornton and Shevach 2000), suggesting that Treg may suppress multiple responses indiscriminately. Potentially, this could prevent activation of cells in the lymph node required to generate an adequate immune response and lead to poor immune responses. In contrast, if Treg action occurs in the periphery, their action could be localised without affecting the ability of the host to mount responses to other simultaneous challenges. In this situation, it appears more likely that the mechanism(s) by which Treg exert their suppressive effects are not cell contact dependent since immune cells would be dispersed throughout the tissue.

In the B16FasL model, depletion of Treg altered the inflammatory influx within 24 hours, which might suggest that Treg are acting locally and not within lymphoid organs. The majority of inflammatory cells at this time point would have been recruited from the blood and therefore Treg in the blood or skin would be the likely effectors of suppression. Indeed, Treg have been isolated from normal skin (Suffia et al. 2005; Hirahara et al.
2006) supporting this hypothesis. Limiting Treg to lymphoid organs would help address this hypothesis. Unfortunately the CD62L transgenic mice used in this thesis were not appropriate to study the location of Treg action, however experiments on other mice where Treg entry into peripheral tissue is prevented could prove interesting.

6.1.3. Therapy

The manipulation of Treg has long been thought to be the key to eradicating cancer and resolving autoimmune disease. In addition, attenuating the inflammatory response would also be advantageous in numerous clinical settings. Delicate tissues such as the lung are extremely susceptible to damage by infiltrating innate immune cells (Chatterjee et al. 2007), and attenuation of this response can limit damage whilst other treatments could stem the cause of the inflammation. Damage caused to organs in this manner can often prove fatal so rapid reduction of inflammation would save lives.

Altering the suppression of the innate arm of the immune system may also help break the cycle in cases of chronic inflammation, where cytokines released by recruited inflammatory cells recruit more cells. Many of these disorders are perpetuated by T cell responses and therefore Treg provide an attractive way to limit both. The effect of Treg on innate immune cells may also have knock on effects on the initiation of an adaptive immune response and therefore altering responses early on may prevent initiation of an inappropriate adaptive response, or conversely could be used as an adjuvant to enhance to potency of a vaccination.
6.2. Shedding of CD62L is Important for Viral Clearance

Initially CD62L transgenic mice were hypothesised to be useful in the study of the location of Treg action. CD62L has been shown to mediate rolling of lymphocytes in HEV of pLN (Sallusto et al. 1999; Wherry et al. 2003; Bouneaud et al. 2005) and shedding of its ectodomain correlated with loss of LN entry (Hamann et al. 2000). In combination with studies on T cells isolated from inflammatory sites which reported low CD62L expression, this suggested that maintained CD62L expression may retain T cells in pLN. Had this been the case, mice unable to downregulate CD62L could have been utilised to study the location of Treg action.

However work carried out in this thesis to characterise WT and LΔP mice, in which T cells express wildtype and shedding resistant CD62L on a CD62L deficient background respectively, indicated that downregulation of CD62L was not required for CD8+ T cell entry into the inflamed tissue of the lung during influenza infection. This observation went against the current dogma and warranted further investigation into the effects of maintained CD62L expression, if any, on CD8+ T cell responses. Primary responses to flu appeared to be unaffected, showing comparable numbers and in vitro effector function of flu-specific CD8+ T cells in B6, WT and LΔP mice. Generation and distribution of flu-specific memory CD8+ T cells are also not affected as numbers of cells within various organs 100 days after flu infection were also comparable. Although displaying similar cytolytic function in vitro, corresponding with cell surface levels of CD107a and IFNγ, upon challenge with rVV expressing a flu epitope, flu-immune LΔP mice were impaired in their viral clearance when compared to both WT and B6 mice. Since the only
difference between WT and LΔP mice is the ability to shed CD62L, this indicated that failure to shed CD62L compromises anti-viral immunity.

6.2.1. Possible Modes of Action

The impaired anti-viral memory response in LΔP mice could be the result of a number of factors. In combination with the observation that viral titres are eventually controlled by LΔP mice (data not shown), a report indicating that T cells from these mice are retained around the HEV, may suggest a slight delay in the kinetics of an LΔP T cell response. Equally, retention in the HEV could lead to incorrect localisation of T cells within the LN altering the ability to interact with DC and mount an anti-viral response. Indeed, a study of CCR7 deficient CD8⁺ T cells indicated that localisation of T cells within the lymph node resulted in impaired viral clearance yet normal effector function in vitro (Junt et al. 2004). CD62L signalling via ligands expressed on endothelial and stromal cells in the draining LN or the target organ could also activate integrin- or chemokine-mediated adhesion and migration (Hwang et al. 1996; Giblin et al. 1997; Ding et al. 2003). Therefore sustained CD62L signalling could enhance adhesion such that the kinetics of T cell entry, migration within, and exit from these organs may be slowed, resulting in a slight delay in target cell attack. In order to address this, a detailed study of the relationship between viral titres and T cell infiltrate should be carried out. Adoptive transfer of CFSE labelled memory T cells transgenic for both CD62L and the TCR for NP68 (F5 mice), would provide information on the expansion of these cells during a response and the kinetics of migration of these cells to the ovary could also be tracked using this method.
Although flu-specific LΔP CD8⁺ T cells showed no impairment of cytolytic activity \textit{in vitro}, the ability of these cells to lyse target cells \textit{in vivo} has not been addressed in this thesis. Small differences in the ability of T cells to recognise/interact with target cells, may be overcome \textit{in vitro}, whereas \textit{in vivo} CTL assays may highlight a deficiency in anti-viral activity. These assays should be carried out during both the primary and memory response to ensure that the defect, if any, is not in generation of the memory response. A study of the adhesion molecules and cytokine receptors expressed by these cells would also provide useful information.

It is possible that the lack of circulating soluble CD62L is the cause of impaired anti-viral immunity. It is possible that soluble CD62L could compete for ligands and limit cell adhesion, which may manifest in shedding deficient mice as increased cell adhesion and retention in and around vessels. Another possibility is that soluble CD62L, or its internalisation, could signal directly to stromal tissue, altering its responses to invading T cells, possibly facilitating their entry by upregulating adhesion molecules. Since soluble CD62L can be detected in the serum during an ensuing immune response, it could act as a systemic signal, mobilising the immune system. Although T cells are the only source of soluble CD62L in WT mice it could be enough to amplify immune responses. An in depth analysis of soluble CD62L levels in WT and LΔP mice would determine if this is a valid hypothesis.

\textbf{6.2.2. CD62L and memory T cells}

The experiments using CD62L transgenic mice have not only put into question the view that CD62L downregulation is required for entry into inflamed tissue, but has demonstrated an important role for CD62L shedding on memory CD8⁺ T cells in viral clearance. The kinetics of CD62L shedding, its re-expression and subsequent
transcriptional downregulation on activated T cells suggests that CD62L may have a role during the initial stages of a T cell response in the lymph node and could be an integral part of differentiation of memory T cells.

Recent studies have suggested that memory cell populations are derived from responding T cells before day 3 in the primary response, opposing the theory that memory T cells are derived from effector T cells towards the end of the immune response (Kedzierska et al. 2007). It has been reported that a proportion of responding T cells, that were CD62L$^{\text{hi}}$ and maintained TCR diversity, persisted in the long-term, when compared to rapidly proliferating CD62L$^{\text{lo}}$ T cells, of which the majority were eliminated during the contraction phase (Kedzierska et al. 2006). This suggested that CD62L may be important during memory cell differentiation. However a later report adoptively transferred T cells, derived from influenza infected mice, into naïve recipients and reported that cells transferred from the draining lymph nodes and not the spleen resulted in a superior memory response, irrespective of CD62L expression (Kedzierska et al. 2007). This suggested that location in a lymph node rather than CD62L expression was important for identifying the most potent memory T cells. However, since CD62L is also involved in LN homing these two factors may be inextricably linked. Clearly, the role of CD62L in the generation of memory T cell responses requires further investigation.

Once generated, two forms of memory T cell are thought to exist, those that circulate through peripheral tissues, do not express CD62L and do not require restimulation to exert their effector functions (T$_{EM}$), and those that express CD62L, circulate through lymphoid tissue and require restimulation (T$_{CM}$). The expression of CD62L on T$_{CM}$ may simply allow LN access and therefore access to activation signals from DC, however it is
possible that CD62L and its shedding is involved in \( T_{CM} \) activation or function. It is not yet known whether shedding of CD62L from the surface of memory T cells post activation differs from that on naïve T cells, therefore further studies on the kinetics and consequences of CD62L shedding on \( T_{CM} \) may further distinguish between \( T_{CM} \) and \( T_{EM} \).

In summary, although little is known about the consequences of CD62L shedding in T cell responses, it is clear that it plays a significant role in viral clearance that is not simply due to a inability of T cells to migrate to the site of infection. Since the models used here are those of active infection a subtle defect or delay in the immune response can tip the balance in favour of viral replication.

6.2.3. Relevance to Disease

CD62L shedding clearly impacts on viral clearance, with higher viral loads in mice where shedding is prevented on memory T cells. Although not intended to be a study of human disease, this work suggests that CD62L shedding may impact upon susceptibility to viral infections, and possibly other infections. If viral titres peak much higher in individuals with defects in CD62L shedding this may turn a usually sub-clinical infection clinical, or increase severity of disease. Since the effect appears to be restricted to memory T cells, this suggests that where the majority may become immune to further infection, those with defects in CD62L shedding may be susceptible to further infection with the same or similar agents. This may also impact upon vaccination strategies. However, it is important to bear in mind the balance between activation and regulation during immune responses and this is only one model. In models where activation stimulus is stronger, the defect may be overcome, therefore studies using other infection models would address this hypothesis.
6.3. Conclusion

In one part of this thesis I have shown that Treg are able to inhibit acute innate immune responses as well as their well defined ability to inhibit T cells responses. Initially I demonstrated that tumour rejection involving B16FasL was mediated by innate immune cells and that this rejection was inhibited by Treg. Although postulated to be dependent on neutrophils, this study demonstrated that the cytolytic activity of cells recruited upon B16FasL was dependent on NK cells, thought to be activated by low levels of MHC Class I and NKG2D ligands expressed by the tumour. This cytolytic activity could be inhibited by adoptively transferred Treg in vivo and by activated Treg ex vivo. Furthermore, Treg depletion enhanced the number of neutrophils present at the site of B16FasL challenge and resulted in nuclear hypersegmentation of neutrophils, which is thought to be linked to enhanced survival (Wolach et al. 2007). However many questions still remain concerning the mechanisms of Treg activation and suppression in this model, in particular it is important to address whether or not these Treg represent a subpopulation of Treg or whether all Treg are capable of inhibiting innate immune responses. The results of these studies may lay the foundations of future studies on the manipulation of Treg to treat a variety of inflammatory diseases.

In the second part of this thesis, I have demonstrated that maintenance of CD62L expression on CD8\(^+\) T cells does not prevent entry into inflamed tissue and that it does not appear to affect effector T cell or memory T cell generation and distribution. However a crucial finding was that failure to shed CD62L on memory T cells resulted in a defect in viral clearance, which could not be attributed to a defect in migration of cells to inflamed tissue, cytolytic activity or IFN\(\gamma\) production. Identifying the mechanism involved in this impairment will lead to greater understanding of memory T cell
responses, which in turn may lead to insights into the generation of immunity to infectious agents.
Figure A.1. Characterising Resident and Recruited Macrophages.
Mice were challenged i.p. with 2x10^6 B16FasL or remained unchallenged. 2 hours and 24 hours later the peritoneum was lavaged and collected cells stained with anti-CD11b, -F4/80 and -Gr-1 antibodies, then evaluated by FACS. Macrophages present prior to B16FasL challenge were identified as CD11b^lo-intF4/80^hi, with higher FSC/SSC, were termed resident macrophages. Those emerging over time and identified as CD11b^hi-F4/80^{int-low}, with lower FSC/SSC, were recorded as recruited macrophages.
Figure A.2. Administration of PC61 depletes CD25+ cells resulting in reduced numbers of CD4+CD25+FOXP3+ cells but no change in CD4+CD25-FOXP3+ cell numbers.

(A) Mice were treated with either isotype control antibody (GL113) or anti-CD25 depleting antibody (PC61) and splenocytes stained with anti-CD4, -CD25, -FOXP3 antibodies then evaluated by FACS. Representative FACS plots, gated on CD4+ cells, are given in (A) and the number of CD4+CD25+FOXP3+ cells for each mouse is shown in (B). Statistical significance was evaluated by Mann-Whitney test. A representative FACS plot of splenocytes isolated from naïve mice stained with anti-CD4 and FOXP3 isotype control antibody is given in (C).
Figure A.3. CD4<sup>+</sup>CD25<sup>+</sup> Treg are purified from the spleen using magnetic cell sorting and exhibit suppressive function in vitro.

(A) Splenocytes were magnetically depleted of non-CD4<sup>+</sup> cells. Positive selection of CD25<sup>+</sup> cells from the CD4<sup>+</sup> enriched cells resulted in two populations, CD4<sup>+</sup>CD25<sup>−</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cells. Cells were stained with antibodies to CD4 and CD25 and evaluated by FACS. 2x10<sup>5</sup>CD4<sup>+</sup>CD25<sup>−</sup> were stimulated with 1x10<sup>5</sup> irradiated CD4<sup>+</sup> splenocytes (APC) and 1μg/ml anti-CD3 antibody for 3 days, with varying numbers of CD4<sup>+</sup>CD25<sup>+</sup> cells at the ratios shown. Proliferation was then assessed by radioactive thymidine incorporation 3 days later (B).
Figure A.4. Maintained CD62L Expression Does Not Prevent Flu-Specific CD8$^+$ Cells From Entering Lung Tissue

B6, WT and LΔP mice were infected i.n. with 20 HAU of influenza virus. Eight days after infection, lungs were perfused and cells isolated from the lungs, draining lymph nodes (LdLN) and spleens were stained with antibodies to CD8 and with NP68-tetramers and evaluated by FACS. The percentage of CD8$^+$ cells that are tet$^+$ in lungs, LdLN and spleens of B6 (squares), WT (triangles) and LΔP (circles) mice are shown. Each symbol represents an individual mouse and data are a summary of two independent experiments using groups of at least 5 mice. Solid lines represent the means within each group.
Figure A.5. Maintained Expression of CD62L Does Not Affect the Distribution or Numbers of Flu-Specific Memory CD8+ T Cells

B6, WT and LΔP mice were infected i.n. with 20 HAU of influenza virus. >100 days after infection, lungs were perfused and cells isolated from the lungs, lung draining lymph nodes (LdLN), ovaries, ovary draining lymph nodes (OdLN) and spleens were stained with antibodies to CD8 and with NP68-tetramers and evaluated by FACS. The percentage of CD8+ cells that are tet+ in lungs, LdLN, ovaries, OdLN and spleens of B6 (squares), WT (triangles) and LΔP (circles) mice are shown. Each symbol represents an individual mouse and data are representative of two independent experiments using groups of at least 5 mice. Solid lines represent the means within each group.
Figure A.6. Elevated Numbers of CD8\(^{+}\)tet\(^{+}\) Cells are Detected in Organs of L\(\Delta\)P Mice When Compared to B6 and WT Mice

Flu-immune B6, WT and L\(\Delta\)P mice were generated by i.n. infection with 20 HAU of influenza virus and >100 days later, mice were challenged with recombinant Vaccinia Virus expressing a MHC class I-restricted peptide epitope derived from the influenza nucleoprotein (rVWNPP). Mice were sacrificed 5 days later and ovaries, ovary draining lymph nodes (OdLN) and spleen collected and stained with antibodies to CD8 and with NP68-tetramers and evaluated by FACS. The percentage of CD8\(^{+}\) cells that are tet\(^{+}\) in organs of rVWNPP challenged mice is given for B6 (squares), WT (triangles) and L\(\Delta\)P (circles) mice. Data are a summary of 2 independent experiments using a minimum of 5 mice per group.
Figure A.7. Tetramer Staining.

(A) Splenocytes from naïve F5 (bearing a TCR specific for NP68), B6 or influenza infected B6 mice were stained with antibodies to CD8, with either NP68-tetramers, or irrelevant tetramers, gp33-tetramers, then evaluated by FACS. Representative FACS plots of CD8 versus Tetramer staining are given in (A). (B) B6, WT and LAP mice were infected i.n. with 20 HAU of influenza virus. Eight days after infection, lungs were perfused and cells isolated from the lungs and spleens were stained with antibodies to CD8 and with NP68-tetramers, and then evaluated by FACS as in Figure 5.6. Representative FACS plots of CD8 versus Tetramer staining are given in (B).
Figure A.8. CD62L Transgenic Mice Show Comparable Levels of CD107a to B6 Ex Vivo

B6, WT and LAP mice were infected i.n. with 20 HAU of influenza virus. Eight days after infection, lungs were perfused and cells isolated from the lungs and spleens were stained directly *ex vivo* with antibodies to CD8, CD107a, IFNγ and with NP68-tetramers and evaluated by FACS. (A) Representative FACS plots of isotype control staining for anti-IFNγ and -CD107a antibodies on CD8+ cells from lungs is given alongside staining with specific antibodies. (B) Representative FACS plots of CD107a versus IFNγ on CD8+tet+ or CD8+tet- cells from the lungs or spleen.
References


