Role of Interleukin 21 in CD4 T Cell Regulation

by

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ABSTRACT

IL-21 is crucial for anti-viral defense, the germinal center reaction and anti-tumour immunity. Conversely, it has been implicated in various autoimmune conditions, including type-1 diabetes. This study set out to explore how IL-21 influences CD4 T cell immune responses, with particular emphasis on its ability to counteract Treg-mediated suppression. These experiments revealed that IL-21 acted on conventional CD4 T cells to release them from suppression. This was associated with loss of Treg homeostasis, as IL-21 was able to inhibit IL-2 production and could substitute for IL-2 in conventional but not regulatory T cells.

Analysis of how CD4 T cell responses are controlled was broadened by investigation of the CTLA-4 pathway, a major regulator of T cell immunity. We showed that CTLA-4 could decrease the level of CD86 expression on APCs by trans-endocytosis in vivo, thereby limiting T cell CD28 signalling. In a further development, we showed that IL-21 could directly upregulate CD86 expression by B cells, illustrating the opposing functions of CTLA-4 and IL-21.

Finally, we explored how the nature of T cell activation influences cytokine production and pathogenicity. These experiments revealed that IL-21 production by CD4 T cells was strongly induced during responses driven by DCs, whilst stimulation with B cells promoted IFNγ expression. Moreover, T cells activated in the presence of DCs were profoundly diabetogenic in an adoptive transfer system, unlike those co-stimulated with B cells. These data provide new insight into the regulation of CD4 T cell responses and how levels of IL-21 produced in vivo could modulate the balance between tolerance and immunity.
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<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>AID</td>
<td>activation-induced cytidine deaminase</td>
</tr>
<tr>
<td>AIRE</td>
<td>autoimmune regulator</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>Bcl</td>
<td>B cell lymphoma</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>Blimp-1</td>
<td>B lymphocyte-induced maturation protein-1</td>
</tr>
<tr>
<td>CCR</td>
<td>CC chemokine receptor</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>cTEC</td>
<td>cortical thymic epithelial cell</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSR</td>
<td>class switch recombination</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T lymphocyte antigen-4</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DN</td>
<td>double negative</td>
</tr>
<tr>
<td>DP</td>
<td>double positive</td>
</tr>
<tr>
<td>EAA</td>
<td>essential amino acids</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EAU</td>
<td>experimental autoimmune uveitis</td>
</tr>
<tr>
<td>Floxed</td>
<td>flanked by LoxP</td>
</tr>
<tr>
<td>Foxp3</td>
<td>forkhead box p3</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
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1. INTRODUCTION

1.1. Overview of the immune system

The vertebrate immune system encompasses myriad exquisite mechanisms for host defence against pathogenic microorganisms. Broadly speaking, the immune response can be divided into two complementary arms based on relative specificity and temporal delay. Innate immunity involves rapid, but only broadly specific, reactivity to a given inoculum, whilst adaptive immunity provides delayed, but highly specific responses. In recent years, it has become increasingly clear that the overlap between these divisions is not insignificant, serving only to emphasise their highly integrated nature.

1.1.1. Innate immunity

The innate arm of the immune system represents various forms of first-line defence, but initially involves exposed sites such as skin or mucosal surfaces. These tissues not only form physical barriers to entry, but also secrete anti-microbial peptides, such as defensins and cathelicidins (Lehrer & Ganz 1999). Should these defences be overcome, various cell populations mount coordinated attacks to achieve pathogen clearance, including macrophages, mast cells, NK cells, dendritic cells and neutrophils. These populations have in common their expression of germline-encoded receptors, which recognize highly conserved pathogen-associated molecular patterns (PAMPs). It is these receptors that provide the innate immune system its rapidity and broad specificity, and crucially, that trigger phagocytosis (Janeway & Medzhitov 2002). An example, the Toll-like receptors (TLRs) form an expansive
range of pattern recognition receptors (PRRs), their principal ligands including both bacterial and viral components such as dsRNA (TLR3), LPS (TLR4), Flagellin (TLR5) and CpG (TLR9) (Beutler 2009).

Mannose, expressed by various pathogenic microbes, binds to another well-characterised PRR called mannose-binding lectin. This receptor is capable of activating the complement pathway, an enzymatic cascade that ultimately leads to cell lysis. An alternative pathway to complement activation is induced by the binding of C3b to various microbial cell wall or other surface components. Thus, complement represents a further critical mediator of innate immunity. Importantly, amongst other processes, it has considerable overlap with the adaptive immune system, which is utilised in situations where innate immunity is eluded or overwhelmed. Indeed, the classical complement pathway is reliant on antibody complexed with antigen for its activation, a process dependent on adaptive immunity (Walport 2001).

**1.1.2. Adaptive immunity**

One of the crucial switches between innate and adaptive immunity is at the point of endocytic uptake of pathogens by dendritic cells. Upon TLR ligation, bacteria are internalised into phagosomes, wherein antigens are selected for presentation at the cell membrane by major histocompatibility complex (MHC) class II. If a dendritic cell becomes infected by an intracellular pathogen, such as a virus, it may still trigger the adaptive immune system. In this scenario, cytosolic RIG-1 like receptors recognise viral PAMPs, and viral antigens are then presented at the cell membrane by MHC class I molecules. During this process, dendritic cells
mature and migrate to secondary lymphoid organs, such as lymph nodes, to present antigen (Iwasaki & Medzhitov 2010).

T lymphocytes, one of two primary components of adaptive immunity, recognise antigen presented in the manner outlined above. These cells bear highly specific receptors, known as T cell receptors (TCRs), each recognising a particular antigen. Unlike the germline encoded PRRs utilised by the innate immune system, TCRs are generated by specialised gene rearrangement mechanisms, a process termed V(D)J recombination. Due to the tens or even hundreds of different gene segments of each type (V, D or J) that may exist at a given TCR locus, their random recombination gives rise to a great number of possible TCR specificities (Gellert 2002). Upon antigen encounter, naïve T lymphocytes become activated, proliferate and migrate to sites of infection. A small number may differentiate into memory cells, an important process permitting rapid recall responses if they re-encounter their cognate antigen (Sallusto et al. 2004).

The second major cell type involved in adaptive immunity is the B lymphocyte. These cells recognise intact protein either presented by dendritic cells, or encountered in the extracellular space (Qi et al. 2006). As with T lymphocytes, these cells express high affinity receptors, in this case termed B cell receptors (BCRs). These are similarly generated by V(D)J recombination, but for the B cell this occurs at immunoglobulin loci (Gellert 2002). Upon antigen encounter, B cells receive T cell help and become either short-lived antibody-secreting plasma cells or form germinal centers, leading to the production of long-lived memory B cells. In a similar manner to T lymphocytes, antigen re-encounter of these cells promotes rapid recall responses, which involve proliferation and plasma cell differentiation.
The co-ordinated efforts of T and B cell subsets, initiated in the lymph nodes and spleen, thus provide for a highly effective mechanism of host protection against infection.

1.1.3. T lymphocytes

T lymphocytes originate from bone marrow resident multipotential haematopoietic stem cells (HSCs). These differentiate through a number of stages before becoming either common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs). The latter are known to express high levels of IL-7Rα, require the transcription factor Ikaros, and are precursors for both the B and T cell lineages. Later stages in T cell development require migration of progenitor populations to the thymus, via the blood (Bhandoola & Sambandam 2006).

Upon entering the thymic parenchyma, T cell progenitors migrate into the cortex where they express both CD4 and CD8, transferring from the double negative (DN), to the double positive stage (DP). These cells undergo further differentiation into single positive thymocytes (SP) and thus express either CD4 or CD8 exclusively. At this stage, their high-level expression of CCR7 attracts them into the thymic medulla, through the cortico-medullary junction. Here, SP thymocytes mature and express spingosine-1-phosphate receptor (S1P1), which permits thymic exit into the circulation (Takahama 2006).

As mature T lymphocytes, CD4+ and CD8+ T cells serve very different functions. CD8+ T cells are most important for defence against intracellular pathogens such as viruses and certain bacterial species. They are directly cytolytic, utilising the Fas/FasL and
perforin/granzyme pathways, amongst others, to clear infected cells (Wong & Pamer 2003). CD4+ T cells, on the other hand, are not generally considered to be directly cytotoxic; rather they produce a wide variety of cytokines and chemokines to recruit and enhance the phagocytic activity of macrophages and, critically, help B cells produce high affinity antibody (Zhu & Paul 2008).

To perform the roles outlined above, T lymphocytes must be presented antigen in such a way that it can ligate the TCR. This requires that the antigen be processed and then presented complexed to MHC molecules. CD8+ T cells require presentation by MHC class I (Grommé & Neefjes 2002), whilst CD4+ T cells require presentation by MHC class II (Villadangos 2001). Due to the fact that any cell could potentially fall victim to an intracellular pathogen, MHC class I is expressed ubiquitously (Rock 1996). On the other hand, MHC class II expression is restricted to professional antigen presenting cell (APC) populations, including B cells, macrophages and dendritic cells (Janeway Jr et al. 1987).

1.1.4. Co-stimulation

The TCR is a heterodimer composed of an α chain of 39-46kd and a β chain of 40-44kd (Allison et al. 1982), connected by a disulphide bond (Samelson 1985). These units form the highly variable region of a larger complex, also consisting of the invariable components ζ and CD3, which in itself has three subunits; γ, δ and ε. The α and β chains of the TCR are substituted by γ and δ chains in a subset of T cells termed γδ T cells (Klausner et al. 1990). Upon TCR ligation, signalling is mediated by CD3 and entails a sequence of phosphorylation events involving ZAP-70 (Huppa & Davis 2003). However, this signal alone is not sufficient
to drive the activation of naïve T lymphocytes. For this to occur co-stimulation is required, acting as an additional network of signals that additively promote T cell responses (Lenschow et al. 1996a). Collectively, interactions between co-stimulatory receptors and ligands, and between TCR and antigen/MHC complexes provide for the optimal initiation of T cell responses.

A number of different co-stimulatory pathways exist, including that of OX40 and its ligand (Gramaglia et al. 1998), PD1 and its ligands (Ishida et al. 1992), and ICOS and its ligand (Coyle & Gutierrez-Ramos 2001). However, co-stimulation is primarily regulated by the CD28 and CTLA-4 receptors, and their ligands CD80 and CD86 (Carreno & Collins 2002). Engagement of T cell-expressed CD28 by either CD80 or CD86 delivers a strong co-stimulatory signal (Jenkins et al. 1991). Conversely, engagement of CTLA-4, also expressed by T cells, by either of the same ligands serves as an inhibitory interaction (Thompson & Allison 1997). Both CD80 and CD86 are primarily APC restricted and are thought to be expressed at low levels by B cells, DCs and macrophages, which upregulate these ligands upon activation (Adam et al. 1998). Whilst CTLA-4 demonstrates far greater affinity for both CD80 and CD86, unlike CD28 it is not constitutively expressed by conventional T cells, also requiring activation for its expression. Balancing of the interactions between these receptors and their ligands permits T cell activation specifically within the confines of the required response (Sharpe & Freeman 2002). When such balancing is disrupted, the importance of co-stimulation for T cell activation becomes clear, as CTLA-4-/- mice develop a lethal lymphoproliferative syndrome (Waterhouse et al. 1995; Tivol et al. 1995) and CD28-/- mice demonstrate impaired T cell responses (Shahinian et al. 1993).
1.1.5. B lymphocytes

As with T cells, B lymphocytes originate from bone marrow resident CLPs. However, unlike T cells, they remain in the bone marrow to undergo maturation. Immediately after the CLP stage, developing B cells upregulate B220 and CD45R, but do not yet express CD19, which identifies all later B cell stages. These have a varied nomenclature but can be broken down into fractions (Fr.) from A through E. All stages after Fr. A (pre-Pro-B) are heavily reliant on V(D)J recombination. Upon maturation, B cells migrate from the bone marrow into the blood. In the periphery, B cells can be broadly divided into B1 B cells, which reside primarily in the peritoneal cavity, and B2 B cells, which are found in the spleen and lymph nodes (Hardy & Hayakawa 2001).

The primary function of B cells is the secretion of high affinity antibody during the adaptive phase of a given immune response. Upon BCR ligation, B cells process antigen and present it complexed with MHC class II to CD4+ T cells at the border of the B cell follicle and T cell zone. Here, B cells require accessory signals provided by T lymphocytes for their complete activation and further differentiation. Help is primarily provided by CD40 signalling and can also be promoted by IL-21 and ICOS-mediated IL-10 production (Vinuesa et al. 2005b). After receiving T cell help, B cells can either exit the follicle to differentiate into short-lived plasma cells, or migrate into B cell follicles to establish germinal centers. Here, B cells undergo somatic hypermutation, rapidly rearranging their antibody variable genes to produce antibody of increasing affinity for the antigen concerned. This process, termed affinity maturation, generates long-lived plasma cells, many of which migrate to the bone marrow and continue secreting antibody for many weeks. It is also the source of memory B cells, which rapidly differentiate into plasma cells if they re-encounter antigen (Allen et al. 2007).
Apart from the high antigen specificity provided by the variable regions of antibody, class switch recombination (CSR) replaces the Cµ gene with other heavy chain constant genes, giving rise to switching of Ig isotype from IgM to IgA, IgE or IgG. These isotypes offer a further level of variation in antibody structure, with each being functionally distinct. CSR occurs upon B cell activation and, like somatic hypermutation, is dependent on the enzyme activation-induced cytidine deaminase (AID) (Muramatsu et al. 2000).
1.2. Immunological tolerance and autoimmunity

The capacity to generate a huge variety of antigen specificities inherent to V(D)J recombination inevitably leads to the formation of autoreactive B and T lymphocytes. The immune system therefore employs robust mechanisms to abort these populations as they arise, in an effort to tolerise to self. Broadly speaking, tolerance is mediated within the thymus and bone marrow, termed central tolerance, or in the periphery, termed peripheral tolerance.

1.2.1. Central tolerance

Central tolerance consists of a number of checkpoints developing T cells pass through before fully maturing and migrating out of the thymus. The first exists at the DN stage (CD4-CD8-), wherein those cells that have failed to successfully rearrange their TCR β chain locus are not permitted to progress to the DP stage (CD4+CD8+). At this point, cortical thymic epithelial cells (cTEC) present MHC-peptide complexes to the remaining T cell cohort. This ensures that they recognise self-MHC, but also serves to guide SP T cell development, as those that interact successfully with MHC class I downregulate CD4, and with MHC class II downregulate CD8. T cells that fail to interact with a minimum level of affinity and avidity undergo apoptotic death by neglect, as they are deemed unresponsive and are essentially useless (Kyewski & Klein 2006). Cells that are permitted to progress migrate into the medulla, where medullary thymic epithelial cells (mTEC) present a vast array of self-antigens. The autoimmune regulator (AIRE) has been demonstrated to control the expression of the majority of these antigens, although other transcription factors are likely involved (Anderson 2002). Those cells that bind with sufficient affinity and avidity are deemed autoreactive and are either aborted, anergised or are driven toward the regulatory lineage. So
efficient is central tolerance that of the approximately $5 \times 10^7$ T cells generated daily in the thymus, only $1-2 \times 10^6$ are actually released (Kyewski & Klein 2006).

For B lymphocytes, central tolerance occurs in the bone marrow. As with T cells, the random nature of V(D)J recombination produces BCRs reactive to self. It has been estimated that unacceptably high degrees of autoreactivity are demonstrated by up to 50% of BCRs. To prevent B cells expressing these receptors escaping to the periphery, mechanisms exist to ensure that they become anergised or are deleted. When a BCR is encountered that is deemed autoreactive, a process termed receptor editing is utilised to further rearrange V gene segments. If this pathway is unsuccessful in generating BCRs with reduced reactivity to self, deletion occurs. It is thought that avidity plays a role in determining whether autoreactive B cells are anergised or deleted. Weaker signals are thought to promote the former, whilst stronger signals promote receptor editing. Receptors expressed by B cells that are very weakly ligated by self-antigen are permitted, allowing these cells to migrate to the periphery. This process is termed clonal ignorance (Shlomchik 2008).

1.2.2. Peripheral tolerance

Should autoreactive T cell clones evade negative selection in the thymus, a number of mechanisms exist to maintain tolerance in the periphery. Peripheral tolerisation of autoreactive T lymphocytes is particularly important, as not only are they inherently pathogenic, self-reactive B cells require their help to both differentiate into plasma cells and form germinal centers.
One relatively simple mechanism that maintains tolerance in the periphery is the physical separation of autoreactive T cells from self-antigen, such as that mediated by the blood brain barrier. Expression of CD62L and CCR7 by all naïve T cells promotes lymph node entry, thereby minimising their migration into non-lymphoid organs, where expression of tissue-restricted antigen (TRA) is high. Immune-privileged sites are particularly resistant to the entry of naïve T cells, as they express very low levels of integrin and selectin receptors, which are required for crossing of the endothelium (Mueller 2010). In addition, expression of cytokines such as TGFβ is high within immune-privileged tissues such as the eye, so that T cells that do enter find themselves in an immunosuppressive environment (Granstein et al. 1990). However, the majority of naïve T lymphocytes are not required to enter the tissues to encounter antigen, as dendritic cell migration facilitates its presentation in the lymph nodes. Tolerogenic dendritic cells are a specialised subset that acquire TRA in the absence of infection or inflammation. Antigen presentation by these cells induces anergy, or Fas-mediated cell death, of self-reactive T cells, ensuring that such cells can be effectively targeted in lymphoid organs. As the complete activation of T cells requires accessory signals, autoreactive TCR ligation in the absence of co-stimulation provides for a potent mechanism of anergy induction (Mueller 2010).

Foxp3+ regulatory T cells (discussed further in section 1.4) provide another critical mechanism for peripheral tolerance. As mentioned earlier, highly autoreactive T lymphocytes may be driven toward the natural Treg lineage during thymic maturation. These cells are then released into the periphery as potent regulators, capable of suppressing inappropriate T cell responses where necessary. Induction of Foxp3 expression in peripheral T cells (iTreg) provides for a further regulatory mechanism. This occurs in response to the localised secretion
of cytokines, such as TGFβ, or metabolites, such as retinoic acid, and is thought to be particularly important for oral tolerance (Sakaguchi et al. 2009). Thus ignorance, anergy, deletion and active regulation by natural and induced Treg contribute to the maintenance of peripheral tolerance.

1.2.3. Autoimmunity and type-1 diabetes

Despite the wide array of mechanisms employed by the immune system to prevent autoreactive T cells from persisting in or indeed ever reaching the periphery, a number inevitably do. Unchallenged, these cells are responsible for a range of autoimmune diseases that, dependent on their antigen specificity, can be systemic or localised to particular organs. Due to the targeting of nucleoproteins such as Ro and La, expressed in all nucleated cells, systemic lupus erythematosus (SLE) represents a systemic autoimmune disease (Casciola-Rosen et al. 1994). On the other hand, type-1 diabetes is an example of a tissue-specific autoimmune condition, as the antigens targeted here appear to be limited to the pancreatic islets, such as the insulin B chain. It is characterised by the T cell-mediated destruction of insulin-secreting pancreatic β cells, eventually resulting in total insulin deficiency. The ensuing loss of blood glucose homeostasis leads to an increased risk of kidney failure, heart disease and glaucoma, and has a significant risk of mortality due to diabetic ketoacidosis if left untreated (Atkinson & Maclaren 1994; Daneman 2006).

Although representing only 5-10% of all diabetes cases worldwide, type-1 diabetes incidence continues to rise. The factors influencing the risk of type-1 diabetes encompass both environmental and genetic, with the latter thought to determine both susceptibility and
resistance. In humans, the average prevalence risk in siblings is roughly 15 times greater than in the general population. A number of chromosomal regions have been heavily associated with type-1 diabetes risk, including those of the human leukocyte antigen (HLA), of which HLA Class II is a major susceptibility locus, and the insulin gene (Pociot & McDermott 2002).

As diabetes is typically only diagnosed upon sufficient damage to the pancreas such that loss of blood glucose homeostasis occurs, it is very difficult to determine the underlying processes that lead to its onset in humans. For this reason, the study of diabetes-prone mouse strains, particularly the non-obese diabetic (NOD), has become a critical tool in furthering our understanding of the disease. Using such models, we now appreciate some of the critical cell populations involved and the interactions that must take place between them. It has become abundantly clear that CD4 and CD8 T cells can contribute significantly to type-1 diabetes onset (Miller et al. 1988; Bendelac et al. 1987; Katz et al. 1993; Wong et al. 1996; Shizuru et al. 1988; Peterson & Haskins 1996). Dendritic cells are also known to be involved (Ludewig et al. 1998; Dahlén et al. 1998), as are B1 and B2 B cells (Serreze et al. 1996; Serreze et al. 1998; Bouaziz et al. 2007; Ryan et al. 2010). On the other hand, regulatory T cells are crucial for protection against type-1 diabetes (Sarween et al. 2004; You et al. 2007; Tang et al. 2004b; Lindley et al. 2005).

1.2.4. Studying type-1 diabetes using TCR transgenic mouse models

The NOD mouse model has traditionally been used to study type-1 diabetes. Whilst this strain provides a wealth of opportunities for in vivo study, not all mice on this background develop
the disease. Further, as the T cells in NOD animals express a wide range of TCR specificities, it is very difficult to study antigen specific responses using this model. However, NOD mice have been found to express the BDC2.5 TCR, which recognises a specific β-cell antigen. Adoptive transfer of T cell clones expressing the BDC2.5 TCR accelerated diabetes onset in young NOD animals (Haskins & McDuffie 1990). BDC2.5 TCR transgenic mice have since been generated, and activated T cells from these mice were only found in the pancreatic islets and lymph node (Högland et al. 1999).

To study antigen specific responses in type-1 diabetes, our lab has utilised the DO11xRIP-mOVA mouse model. These mice express a transgene encoding the DO11.10 TCR, which is reactive to chicken egg ovalbumin (OVA). They also express a transgene encoding OVA under the control of the rat insulin promoter (RIP), with consequent expression of OVA by insulin producing pancreatic β cells (Davey et al. 2002). Due to the thymic expression of OVA during negative selection, antigen specific natural regulatory T cells (nTreg) are also generated. However, although these delay disease, all mice eventually become diabetic (Clough et al. 2008).

Using an adoptive transfer model of type-1 diabetes in which DO11.10 TCR transgenic T cells were adoptively transferred into RIP-mOVA recipients, our lab has explored the parameters of the pathogenic response that are under Treg control (Sarween et al. 2004). One of the major advantages of the adoptive transfer model is that gene expression can be varied within the conventional and regulatory T cell populations independently. Indeed, we have recently demonstrated that antigen specific Treg lacking CTLA-4 expression failed to regulate diabetes, using this adoptive transfer system (Schmidt et al. 2009).
1.3. T helper cell differentiation pathways

As orchestrators of many of the critical mechanisms employed during the adaptive immune response, CD4+ T helper cells have been the subject of intense research for a number of decades. Upon antigen encounter, these cells are driven to differentiate in response to co-stimulatory signals and the local cytokine milieu. A number of differentiation pathways exist, with each resultant subset identified primarily by their expression of specific master transcription factors and cytokines. Moreover, specific subsets are known to participate in particular responses to infection and autoimmune diseases (Fig. 1.01).

1.3.1. T helper 1 (Th1) cells

Although the T helper cell nomenclature used today was first proposed by Tada and colleagues in 1978, it was Mosmann and Coffman that first defined T helper cell lineages based on their characteristic expression of specific cytokines (Tada et al. 1978; Mosmann et al. 2005). In the latter report, Th1 cells were identified by their production of IFNγ. It was later determined that IL-12 was critical for the induction of Th1 cells (Manetti et al. 1993; Hsieh et al. 2008), and that this was dependent on signal transducer and activator of transcription (Stat)3 and 4 (Jacobson et al. 1995; Szabo et al. 1995). Finally, it was discovered that this differentiation state was committed by expression of the transcription factor T-bet (Szabo et al. 2000).

Originally, Th1 cells were determined to be most important for cell-mediated immunity, but have also been implicated in a number of autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE), the mouse model commonly used to study multiple
Figure 1.01. T helper cell differentiation pathways. Originally, T helper cells were thought to polarise toward either the Th1 or Th2 lineages in response to IL-12 or IL-4 respectively. Later, it was observed that TGFβ promoted inducible Treg differentiation, and that addition of either IL-6 or IL-21 prompted Th17 lineage commitment. Most recently, the Tfh program has been defined, and is induced by IL-21 signalling. Adapted from (King et al. 2008).
sclerosis (Lafaille et al. 1997; Baron et al. 1993; van der Veen & Stohlman 1993). In type-1 diabetes, T cells that accelerated disease onset in NOD mice produced Th1 cytokines in response to islet in vitro (Bergman & Haskins 1994). Also in NOD mice, blockade of IFNγ was shown to be protective against diabetes onset (Debray-Sachs et al. 1991; Campbell et al. 1991). Further, NOD T cells are known to produce high levels of IFNγ in response glutamic acid decarboxylase (GAD), a known β cell antigen implicated in type-1 diabetes (Tisch et al. 1993; Kaufman et al. 1993). Recently, T-bet deficiency was shown to be completely protective against diabetes onset in NOD animals (Esensten et al. 2009).

1.3.2. T helper 2 (Th2) cells

In their initial cytokine based T helper cell classification, Mosmann and Coffman also described Th2 cells (Mosmann et al. 2005). These were later determined to express IL-4 and IL-13 in a lineage specific manner (Brown et al. 1989; McKenzie et al. 1993), and both are known to be important for Th2 polarisation (McKenzie et al. 1998; Kopf et al. 1993; Kühn et al. 1991; Swain et al. 1990). In this respect, IL-4 is known to act in a Stat6-dependent manner (Hou et al. 1994). In 1997, Zheng and Flavell determined that GATA-3 was the Th2 lineage master transcription factor, with minimal expression amongst differentiated Th1 clones (Zheng & Flavell 1997).

Th2 cells are associated with B cell help during humoral immunity, but have also been linked with allergy and autoimmunity. In the case of the former, it has been shown that Th2 cells activate eosinophils and mast cells, and promote the increased production of IgE, resulting in allergic inflammation and atopy (Romagnani 1994). Whilst type-1 diabetes has traditionally
been associated with Th1 cells, the Th2 lineage can influence the disease. Th2 cells have been shown to promote insulitis in NOD.scid mice (Pakala et al. 1997), but were tolerogenic when polarised by intranasal immunisation in NOD animals (Tian et al. 1996). Similarly, although EAE has classically been considered to be Th1 mediated, *in vitro* differentiated Th2 cells have also been shown to induce the disease in lymphopenic hosts (Lafaille et al. 1997).

1.3.3. T helper 17 (Th17) cells

The existence of Th17 cells was originally proposed in a report concerning the effects of IL-23 on the induction of EAE by IL-17 producing CD4+ T cells (Langrish et al. 2005). Later, the identification of the Th17 specific transcription factor RORγt confirmed that these were a distinct lineage (Ivanov et al. 2006). Whilst it was initially suggested that IL-23 was important for Th17 polarisation, this was not in fact the case. Instead, IL-6 and TGFβ were found to be required during Th17 induction (Veldhoen et al. 2006; Bettelli et al. 2006; Mangan et al. 2006), with IL-23 found to play a role in Th17 maintenance (Mangan et al. 2006). IL-21, alongside TGFβ, is also known to be capable of inducing Th17 cells (Yang et al. 2008; Korn et al. 2007), and is thought to sustain their production of IL-17 (Wei et al. 2007), although as these roles overlap with those of IL-6 it is not absolutely required (Sonderegger et al. 2008). Further, Stat3 signalling, which can be induced by either IL-6 or IL-21, is required for Th17 induction *in vitro* (Mathur et al. 2007) and *in vivo* (Harris et al. 2007). Recently, it has been shown that TGFβ is not absolutely required for Th17 differentiation, when substituted by IL-1β (Ghoreschi et al. 2010).
Th17 cells are known to be important during host defence against infection by fungi (Huang et al. 2004) and extracellular bacteria (Happel et al. 2005). However, they are most prominently associated with deleterious responses to self, having been implicated in a wide variety of autoimmune conditions. Originally, the Th17 lineage was associated with EAE (Langrish et al. 2005; Park et al. 2005; Bettelli et al. 2006) and arthritis (Lubberts et al. 2001; Lubberts et al. 2004; Bush et al. 2002), but roles have since been identified in type-1 diabetes (Emamaullee et al. 2009; Martin-Orozco et al. 2009) and SLE (Yang et al. 2009).

1.3.4. T follicular helper (Tfh) cells

The most recently defined CD4 T cell subset is that of T follicular helper cells, which were first proposed based on their expression of CXCR5 (Breitfeld et al. 2000; Schaeferl et al. 2000; Kim et al. 2001). However, the Tfh program was not wholly accepted until the discovery of the lineage specific transcription factor Bcl-6 (Nurieva et al. 2009; Yu et al. 2009; Johnston et al. 2009). Tfh cells are known to produce IL-21 (Bryant et al. 2007; Chtanova et al. 2004; Vinuesa et al. 2005a; Rasheed et al. 2006) and IL-4 (Reinhardt et al. 2009; King & Mohrs 2009), and IL-21 polarises naïve CD4 T cells toward Tfh differentiation (Vogelzang et al. 2008; Nurieva et al. 2008). A role for B cells has also been proposed in Tfh lineage commitment, as Tfh cells are absent in immunised B cell deficient mice (Haynes et al. 2007).

Tfh cells are known to localise to germinal centers through their expression of CXCR5, where they provide B cell help during antibody responses (Schaeferl et al. 2000), and are thus important during humoral immunity. They have also been implicated in a number of autoimmune diseases, including SLE (Hu et al. 2009; Vinuesa et al. 2005a) and collagen-
induced arthritis (Hu et al. 2009). A further role for Tfh cells in type-1 diabetes pathology has recently been suggested (Clough et al. 2008; Silva et al. 2011).

1.3.5. Other T helper cell subsets

In recent years, a number of novel T helper cell lineages have been proposed, but have yet to be widely accepted based on a lack of either cytokine or transcription factor specificity. Two prominent examples are the proposed Th22 and Th9 lineages.

IL-22 has been shown to be produced by Th17 cells and acted in synergy with IL-17 to promote the expression of antimicrobial peptides by primary keratinocytes (Liang et al. 2006). Soon after, it was confirmed that IL-23 was important in promoting the expression of IL-22 by Th17 populations (Kreymborg et al. 2007; Zheng et al. 2007). However, in humans, a population of IL-22 producing CD4+ T cells have been described that produce TNFα, but do not co-express IL-17, IFNγ or IL-4, and are involved in inflammatory skin disorders. These were suggested to be a distinct T helper cell lineage and were termed Th22 cells, following the nomenclature introduced with Th17 cells (Eyerich et al. 2009).

The Th9 lineage was originally proposed after two reports identified a population of CD4+ T cells that produced IL-9 but not IL-4, IFNγ or IL-17. As with Th17 cells, Th9 differentiation required TGFβ, in this case acting in concert with IL-4 to prevent Treg and Th2 differentiation (Dardalhon et al. 2008; Veldhoen et al. 2008). In humans, a similar population of cells has been described, also requiring TGFβ and IL-4 for their induction (Wong et al. 2010). Whilst Th9 cells also produce IL-10 they do not appear to be suppressive, but instead
have been implicated in the pathology of peripheral neuritis and colitis (Dardalhon et al. 2008).
1.4. Regulatory T cells

The concept of a T cell subpopulation with regulatory properties has existed for more than a quarter century, with early experiments demonstrating autoimmunity in lymphopenic mice receiving splenocytes depleted of Lyt-1$^{hi}$ (CD5+) cells (Sakaguchi et al. 1985). It later became clear that CD4+ regulatory T cells (Treg) could be isolated from naïve animals based on their expression of CD25 (Takahashi et al. 1998). However, it was not until more recently that regulatory T cells were defined as a distinct CD4 T cell lineage, with the discovery of the Treg specific master transcription factor Foxp3 (Fontenot et al. 2003; Fontenot et al. 2005a).

1.4.1. Regulatory T cell subsets

Broadly, regulatory T cells can be subdivided into those generated in the thymus during T cell maturation and those induced in the periphery from naïve conventional T cells. Those produced in the thymus, termed natural Treg (nTreg), constitute the majority of the peripheral Treg cohort. Natural Treg generation occurs in parallel with negative selection during CD4+ thymocyte development in the medulla, where TCR affinity for self-antigen is key to their selection. Prevailing dogma suggests that higher affinity is required than for conventional T cell selection, but not so high that deletion is induced (Jordan et al. 2001). Treg induced in this manner then migrate to the periphery, where they can be found shortly after birth (Kim et al. 2007).

Treg induction from naïve CD4+ conventional T cells is known to be dependent on TCR stimulation and provision of TGFβ (Chen et al. 2003), but can also be mediated by IL-2 (Laurence et al. 2007) or retinoic acid (Mucida et al. 2007). TGFβ-induced Treg (Th3 cells)
primarily produce TGFβ and have traditionally been associated with mucosal immunity, as they can be induced by gut resident dendritic cells during oral tolerisation protocols (Weiner 2001). Similarly, retinoic acid-induced Treg are also thought to play an important suppressive role in this setting (Mucida et al. 2007). Induced Treg (iTreg) have recently been shown to play a crucial role in mediating peripheral tolerance, by expanding the TCR repertoire of the total Treg pool (Haribhai et al. 2011). Expression of the Ikaros family member Helios is proposed to differentiate natural and induced Treg populations, its expression being restricted to the former (Thornton et al. 2010).

A further population of regulatory T cell has been proposed, which lacks Foxp3 expression. Termed Tr1 cells, this population is induced by IL-10, secretes IL-10 and TGFβ, and has been shown to be protective against EAE and colitis (Groux et al. 1997; Chen et al. 1994). Recent work has shown that IL-27 can also induce Tr1 cells, by inducing c-Maf, IL-21 and ICOS, which act co-ordinately to promote Tr1 differentiation (Pot et al. 2009). The lack of Foxp3 expression by Tr1 cells does not appear to impair their suppressive capacity (Vieira et al. 2004).

1.4.2. Factors necessary for Treg function and homeostasis

Regulatory T cells require a number of factors to maintain their function and homeostasis in the periphery. A role for IL-2 in Treg homeostasis was first suggested by reports that mice lacking IL-2 signalling developed spontaneous autoimmunity (Sadlack et al. 1995; Suzuki et al. 1995; Willerford et al. 1995). Neutralisation of IL-2 had a similar effect, and was attributed to decreased Treg numbers in the thymus and periphery (Setoguchi et al. 2005).
Roles for IL-2 include maintaining Treg survival (D'Cruz & Klein 2005) and promoting the expression of genes involved in cell metabolism and growth (Fontenot et al. 2005b). It has recently been demonstrated that Treg respond to IL-2 more rapidly than conventional T cells during an immune response (O'Gorman et al. 2009), and that low-dose IL-2 provision can both prevent and rescue disease in NOD mice by increasing Treg numbers (Tang et al. 2008; Grinberg-Bleyer et al. 2010a). IL-2 has also been shown to be important for Treg suppression in vitro (Thornton et al. 2004).

The co-stimulatory receptor CD28 is also known to be critical for Treg homeostasis. Diabetes pathology is exacerbated in CD28-/-NOD mice and whilst this was originally attributed to skewing of T helper cell subsets (Lenschow et al. 1996b), it subsequently became clear that these mice exhibited roughly fivefold fewer Treg in the periphery. Moreover, adoptive transfer of CD28-sufficient Treg into CD28-/-NOD mice delayed, and in some cases prevented, diabetes onset (Salomon et al. 2000).

Consistent with its role in opposing the CD28 pathway, CTLA-4 has recently been shown to tightly regulate the size of the peripheral Treg compartment. Analysis of CTLA-4-/- mice, which develop a fatal lymphoproliferative phenotype, surprisingly demonstrated a massively enlarged peripheral Treg population. This was accounted for not by altered thymic development or output, but by massively increased Treg proliferation in the periphery (Schmidt et al. 2009). In a different system utilising CTLA-4 conditional knockout mice, wherein CTLA-4 deletion is limited to the Foxp3+ Treg compartment, Treg thymic development and output were again determined to be normal. However, Treg numbers in
secondary lymphoid organs such as spleen and lymph node were hugely elevated (Wing et al. 2008).

Other factors are now emerging as also playing significant roles in maintaining Treg homeostasis and function. IFNγ, for example, has been suggested to play an important role in promoting CD25 expression and Treg proliferation, and IFNγ-deficiency promoted inflammation in a mouse model of autoimmune myocarditis (Afanasyeva et al. 2005). Similarly, TNFα has recently been suggested to promote Treg expansion and suppression, with this mechanism thought to be important during the suppression of disease in an adoptive transfer model of type-1 diabetes (Grinberg-Bleyer et al. 2010b).

1.4.3. Mechanisms of Treg-mediated suppression

It is increasingly clear that Treg have a wide arsenal of mechanisms at their disposal for the suppression of deleterious immune responses. These encompass effects on both APCs and conventional T cells, and include contact mediated pathways and the secretion of soluble factors (Fig. 1.02).

As TGFβ has traditionally been associated with immunosuppression, it became an early candidate as a mediator of Treg suppressive function. Indeed, it was shown that Treg produce TGFβ and that TGFβ blockade prevented suppression of conventional T cells in vitro (Nakamura et al. 2001). It was later demonstrated that whilst TGFβ-deficient Treg were capable of in vitro suppression, they were unable to prevent disease in an adoptive transfer model of colitis (Nakamura et al. 2004). Certainly, the role of TGFβ appears to demonstrate
Figure 1.02. Treg suppression is mediated by numerous mechanisms. Treg can secrete soluble suppressive factors, such as TGFβ or IL-35, or can induce apoptosis in target cell populations via the granzyme/perforin pathway. They can also deprive target cell populations of essential growth factors or catalyse the extracellular accumulation of immunosuppressive nucleotides. By targeting APCs, Treg can deprive T cells of the co-stimulatory ligand necessary for their activation and proliferation. Reproduced from (Vignali et al. 2008).
redundancy, as conventional T cells that were deficient in TGFβ signalling pathways were still suppressible by Treg in vitro (Piccirillo et al. 2002).

Similarly, IL-10 was initially proposed as a Treg suppressive cytokine due to its past association with suppression of immune responses. In support of this, regulatory T cells from IL-10−/− mice were unable to suppress colitis and whilst wildtype Treg were capable of suppression, this was abrogated upon provision of a blocking IL-10R antibody (Asseman et al. 1999). Further support came from a report that demonstrated IL-10 production by Treg, as measured by mRNA expression and protein secretion. This report also demonstrated that IL-10−/− Treg lacked suppressive function (Collison et al. 2009).

In recent years the Vignali laboratory have proposed IL-35 as an important immunosuppressive cytokine produced by regulatory T cells. IL-35 is a heterodimer composed of IL-27β and IL-12α (p35), the mRNAs for which are expressed constitutively by Treg but not by resting or activated conventional T cells. Treg incapable of producing either subunit due to genetic deletion demonstrated reduced suppressive activity in vitro and failed to reverse colitis in vivo (Collison et al. 2007). The group later demonstrated that Treg suppression across a permeable membrane was partially dependent on IL-35 (Collison et al. 2009).

Interestingly, whilst IL-9 has been identified as an effector cytokine for both Th17 cells and the proposed Th9 lineage, it is also thought to be produced by regulatory T cells (Lu et al. 2006). Provision of exogenous IL-9 has been demonstrated to enhance Treg suppression in vitro, whilst its blockade counteracts suppression. Furthermore, IL-9R−/− mice developed
more severe EAE than did wildtype animals in a murine model (Elyaman et al. 2009). Thus, the role of IL-9 in immune regulation remains controversial.

A further mechanism postulated to be utilised by Treg is the induction of conventional T cell apoptosis by their production of granzyme B. Its expression by Treg is upregulated upon activation, in a glucocorticoid-induced TNF-like receptor (GITR)-dependent manner. Regulatory T cells isolated from granzyme B-/- mice demonstrated decreased suppressive ability compared to wildtype Treg in vitro, and this correlated with their decreased ability to induce apoptosis in the conventional T cell population (Gondek et al. 2005).

IL-2 is critically important for Treg survival. Accordingly, Treg constitutively express high levels of the IL-2Rα chain (CD25). This has lead to the suggestion that Treg might act as an IL-2 sink, starving conventional T cells of an important growth factor (Thornton & Shevach 1998a). Support for this concept was provided by a chimeric suppression assay wherein murine Treg were used to suppress human conventional T cells. In this setting the IL-2Rα and β chains could be selectively blocked on Treg, and this effectively counteracted suppression (La Rosa et al. 2004). In addition, provision of IL-2 to in vitro assays prevented Treg suppression (Thornton & Shevach 1998a; La Rosa et al. 2004). It was later demonstrated that Treg could passively induce conventional T cell apoptosis by consuming IL-2, and that this could be overcome by providing exogenous IL-2 (Pandiyan et al. 2007).

Deprivation of growth and survival factors by Treg is not limited to IL-2 however, but also extends to essential amino acids (EAAs). In this case however, deprivation is mediated passively, by inducing the expression of enzymes that consume EAAs in dendritic cells in
in vitro and in skin grafts in a mouse model of graft vs. host disease. In response to reduced EAA availability, conventional T cells demonstrated reduced mammalian target of rapamycin (mTOR) signalling and failed to proliferate (Cobbold et al. 2009).

The adenosine A2A receptor is expressed by conventional T cells and mediates their inhibition. Treg are known to express the CD73/CD39 ectoenzymatic complex, which catalyses the production of adenosine from extracellular nucleotides, and it was therefore proposed that this might be an important suppressive mechanism. Accordingly, Treg isolated from CD39-deficient mice demonstrated impaired suppressive function in vitro and, unlike wildtype Treg, were incapable of suppressing graft rejection in a murine transplant model (Deaglio et al. 2007).

Regulatory T cells also constitutively express CTLA-4 and many reports have attempted to assess its requirement during suppression. In vitro suppression assays have yielded contradictory results, with antibody mediated CTLA-4 blockade appearing to counteract suppression (Takahashi et al. 2000; Tang et al. 2004a) whilst CTLA-4-deficient Treg maintain their ability to suppress in some cases (Tang et al. 2004a; Schmidt et al. 2009), but not others (Wing et al. 2008). The difference between CTLA-4 blockade and deficiency has been suggested to be due to a compensatory mechanism whereby CTLA-4/- Treg produce more TGFβ to maintain suppressive function (Tang et al. 2004a).

Whilst CTLA-4 is known to be important for Treg homeostasis, the fact that its deficiency leads to massively increased Treg numbers but a total lack of immune regulation suggests a critical role for CTLA-4 in mediating suppression in vivo (Schmidt et al. 2009; Wing et al.)
In agreement with this concept, CTLA-4-deficient Treg were completely incapable of preventing disease onset in an adoptive transfer model of type-1 diabetes (Schmidt et al. 2009), or of preventing the proliferation of adoptively transferred T cells in lymphopenic hosts (Sojka et al. 2009).

Exactly how CTLA-4 expression confers suppressive ability has been a contentious issue. It was originally proposed that CTLA-4 signalling might promote the expression of TGFβ, thus mediating suppression (Chen et al. 1998). However, others have shown that CTLA-4 ligation did not promote TGFβ expression by T cells and could mediate T cell inhibition independently of TGFβ (Sullivan et al. 2001). Further, TGFβ has been shown to be ineffective at blocking suppression mediated by CTLA-4-expressing Treg (Friedline et al. 2009). It has been suggested that CTLA-4 might mediate back signalling into dendritic cells through CD80 or CD86, promoting indoleamine 2,3-dioxygenase (IDO) expression and mediating suppression via apoptosis (Grohmann et al. 2002). It has also been reported that Treg can mediate the decreased expression of CD80 and CD86 by dendritic cells in murine and human studies, thereby inhibiting their ability to co-stimulate conventional T cells (Wing et al. 2008; Schmidt et al. 2009; Misra et al. 2004).
1.5. Interleukin 21

Cytokines are essential mediators of both innate and adaptive immunity. As mentioned previously, CD4 T cells in particular make use of cytokines to orchestrate other cell populations during the clearance of infection. Cytokine expression profiles define T helper cell lineages and are associated with responses to distinct infections. For example, Th1 cells that secrete IFN$\gamma$ have traditionally been linked with defence against intracellular pathogens. On the other hand, aberrant cytokine production is implicated in a wide array of autoimmune diseases. A recently identified member of the common $\gamma$-chain cytokine family, interleukin (IL)-21 is a perfect example, serving a critical role during host defence, but also during the onset and maintenance of autoimmunity.

1.5.1. Interleukin 21: basic biology and function

IL-21 and its receptor were first discovered in 2000 by Parrish-Novak and colleagues, the receptor also being identified by another group at this time who termed it the novel interleukin receptor (NILR) (Parrish-Novak et al. 2000; Ozaki et al. 2000). IL-21R has highest amino acid sequence homology to IL-2R$\beta$ and IL-4R$\alpha$, its gene lying physically adjacent to that of IL-4R$\alpha$ on chromosome 16 (Ozaki et al. 2000). At this time, IL-21R expression was determined to be lymphoid tissue restricted, being found in thymus, lymph node, spleen and peripheral blood (Parrish-Novak et al. 2000). It was later discovered that IL-21R signaled as a heterodimeric complex consisting of the IL-21R$\alpha$ chain and the common $\gamma$-chain (Asao et al. 2001; Habib et al. 2002), and that it was expressed by CD4 and CD8 T cells, NK cells and B cells (Jin et al. 2004). Its mRNA was also found to be expressed by myeloid DCs (Brandt et al. 2003a). IL-21R signalling is known to be mediated via Jak 1 and
Jak 3 (Habib et al. 2002), through Stat1 and Stat3, and to a lesser extent Stat5 (Zeng et al. 2007). Despite its receptor’s broad expression, IL-21 mRNA was only detected in activated CD4 T cells (Parrish-Novak et al. 2000), and its expression by this population is known to be induced by IL-6 (Suto et al. 2008). Production of IL-21 has been associated with the Th1 (Chtanova et al. 2004), Th2 (Wurster et al. 2002), Th17 (Korn et al. 2007; Wei et al. 2007; Nurieva et al. 2007) and Tfh (Rasheed et al. 2006; Vinuesa et al. 2005a; Chtanova et al. 2004) lineages.

The effects of IL-21 on particular cell populations are broad. It is known to promote CD8 T cell proliferation alone (Li et al. 2005) and in synergy with IL-7 or IL-15 (Zeng et al. 2005) and maintains high-level expression of CD28 (Li et al. 2005; Alves et al. 2005). IL-21 has also been shown to promote cytotoxic effector function in CD8 T cells (Casey & Mescher 2007), and to promote the expression of perforin in memory and effector subsets (White et al. 2007).

For CD4 T cells, IL-21 is known to synergise with TGFβ to promote Th17 differentiation and thus prevent Treg induction (Yang et al. 2008; Korn et al. 2007), and to maintain IL-17 production (Wei et al. 2007). It is also important in promoting Tfh differentiation (Vogelzang et al. 2008; Nurieva et al. 2008) and, by repressing eomesodermin expression, IL-21 downregulates IFNγ production by Th1 cells. It is thought to suppress Foxp3 expression by regulatory T cells (Li & Yee 2008) and augment the differentiation of the proposed Th9 lineage (Wong et al. 2010).
The effects of IL-21 on B cells are rather more complicated. In the context of BCR ligation, it promotes B cell activation and proliferation, whilst in the context of CD40 signalling it promotes both proliferation and apoptosis (Jin et al. 2004). For B cells stimulated with lipopolysaccharide (LPS) or CpG DNA, IL-21 inhibited proliferation and induced apoptosis in a Bim-dependent manner (Jin et al. 2004). IL-21-mediated apoptosis was also induced in resting B cells or those stimulated with IL-4, and correlated with the downregulation of the antiapoptotic factors Bcl-2 and Bcl-xL (Mehta et al. 2003). By its induction of Blimp-1, IL-21 promotes post-switch and plasma cell differentiation (Ettinger et al. 2005; Ozaki et al. 2004), and the effects of IL-6 on the same are mediated by IL-21 (Dienz et al. 2009). Further, IL-21 promotes Bcl-6 expression by B cells, which is critical for the maintenance of the germinal center response (Linterman et al. 2010; Zotos et al. 2010). In terms of antibody production, IL-21 is known to prevent IgE production by inhibiting Cε transcription (Suto et al. 2002), but promote the production of IgG1 (Ozaki et al. 2002).

There is a striking lack of data on the effects of IL-21 on dendritic cells, which may perhaps reflect the uncertainty surrounding whether this population expresses the IL-21Rα chain. IL-21 appears to inhibit dendritic cell maturation, as demonstrated by their decreased expression of MHC class II and greater antigen uptake. These dendritic cells had poor T cell-stimulatory capacity and did not become activated upon exposure to LPS (Brandt et al. 2003b; Brandt et al. 2003a). IL-21 is also thought to be important for the maintenance of CCR7 expression by dendritic cells, and thus in mediating their trafficking to lymphoid organs (Jin et al. 2009).

The report that originally described IL-21 and its receptor demonstrated a role in promoting NK cell expansion from bone marrow precursors (Parrish-Novak et al. 2000). It was further
shown that IL-21 promoted NK cell maturation from human cord blood precursors (Sivori et al. 2003). For activated NK cells, IL-21 enhanced their cytotoxic activity and expression of IFNγ, but could not prevent their apoptosis. Conversely, the effects of IL-21 on resting NK cells appeared to be inhibitory, as these cells could no longer proliferate in response to IL-15 (Kasaian et al. 2002).

1.5.2. Interleukin 21 in host defence

Due to its production by Tfh cells, IL-21 is known to play an important role during humoral immunity. IL-21R-deficiency in B cells leads to a diminished germinal center reaction and thus a reduced response to sheep red blood cells (Linterman et al. 2010). Similarly, germinal center persistence is prevented and plasma cell formation reduced in mice immunised with protein antigen in the absence of IL-21 signalling (Zotos et al. 2010). In IL-21R-/- mice infected with *Toxoplasma gondii*, serum levels of IgG1, IgG2a, IgG2b and IgG3 were all lower than in infected wildtype mice (Ozaki et al. 2002).

IL-21 is also thought to play a crucial role during viral infection. Initially, it was shown that high-level IL-21 mRNA transcription occurred in CD4+ T cells in mice challenged with herpes simplex virus type 2 or lymphocytic choriomeningitis virus (LCMV) (Holm et al. 2006). IL-21R-/- mice were shown to be able to resolve acute, but not chronic LCMV infection. This was attributed to defective long-term CD8 T cell functionality and maintenance, as demonstrated by their reduced numbers and expression of IFNγ compared with wildtype CD8 T cells (Fröhlich et al. 2009). Recently, the age-related ability to clear
hepatitis B virus (HBV) has been shown to correlate strongly with the expression of IL-21 mRNA in the liver in a mouse model of HBV infection (Publicover et al. 2011).

Due to its effects on both T cells and NK cells, that IL-21 is capable of mediating potent anti-tumour responses is unsurprising. In an early report, systemic production of IL-21 promoted NK cell cytolytic activity and strongly inhibited the growth of MCA205 fibrosarcoma and B16 melanoma (Wang et al. 2003). In a different system, wherein TS/A murine mammary adenocarcinoma cells were genetically altered to express IL-21 and subsequently injected into syngeneic mice, only small tumours developed that were rejected by 90% of recipients. Rejection was shown to be dependent on CD8 T cells and the expression of IFNγ (Di Carlo et al. 2004). In a model of established B16 melanoma, intraperitoneal administration of IL-21 induced tumour regression by enhancing naïve and memory CD8 T cell proliferation (Zeng et al. 2005).

1.5.3. Interleukin 21 in autoimmunity

Whilst numerous roles during defence against infection are apparent, a burgeoning literature exists confirming the associations of IL-21 with autoimmune disease. In the BXSB-Yaa mouse model of SLE for example, IL-21R-deficiency was completely protective. These mice developed none of the characteristic abnormalities associated with SLE, with a complete lack of renal disease and autoantibody production (Bubier et al. 2009).

In a mouse model of experimental autoimmune uveitis (EAU), IL-21R-/- animals were found to be more resistant to disease than wildtype mice. Further, adoptive transfer of IL-21R-/-
antigen specific lymphocytes induced EAU with a greatly reduced severity than did wildtype cells (Wang et al. 2011). Similarly, IL-21 deficiency has been shown to be protective against EAE onset (Nurieva et al. 2007), and administration of IL-21 before EAE induction promoted enhanced CNS infiltration and greater disease severity (Vollmer et al. 2005). However, another study that also utilised IL-21R-deficient mice demonstrated no protection whatsoever from EAE, with disease severity perhaps slightly enhanced compared to wildtype mice (Coquet et al. 2008).

There is also evidence for a role for IL-21 in the pathogenesis of type-1 diabetes. Three reports in recent years have demonstrated that IL-21R-deficiency completely protects NOD mice from type-1 diabetes onset (Datta & Sarvetnick 2008; Spolski et al. 2008; Sutherland et al. 2009). These mice exhibited little to no lymphocytic infiltration into the pancreatic islets and far reduced autoantibody titres compared with NOD mice on a wildtype background (Sutherland et al. 2009). A defect in effector T cells was proposed to be responsible for disease prevention, as adoptive transfer of IL-21R/-NOD splenocytes into NOD.scid recipients failed to induce diabetes onset (Sutherland et al. 2009). The report by Spolski and colleagues suggests that the T cell defect might be explained by a lack of IL-17 production by CD4 T cells in IL-21R-deficient NOD animals, although to the contrary Sutherland et al., observed increased IL-17 production in these mice (Sutherland et al. 2009; Spolski et al. 2008).

Previous work in our laboratory has shown that effector T cells in the pancreatic lymph nodes of DO11xRIP-mOVA diabetic animals express IL-21, and that T cells from these mice are resistant to Treg suppression (Clough et al. 2008). Importantly, we and others have shown
that IL-21 can effectively counteract suppression mediated by Treg *in vitro* (Clough et al. 2008; Peluso et al. 2007) and *in vivo* (Clough et al. 2008). This prompted the suggestion that CD4 T cell-derived IL-21 might promote diabetes onset by disrupting Treg function. However, quite how IL-21 was able to release conventional T cells from suppression remained unclear.

### 1.5.4. IL-21R-/- mice

IL-21R-deficient mice have been widely used to study both the *in vivo* and *in vitro* effects of IL-21 signalling on immune responses. IL-21R-/- mice appear phenotypically normal, with similar proportions of monocyte, lymphocyte and granulocyte populations as in wildtype animals. However, these mice have an approximately twofold increased level of circulating IgE antibody (Frohlich et al. 2006). We have obtained IL-21R-deficient mice on a BALB/c background from the Kopf laboratory at ETH Zurich. These mice were utilised extensively throughout this project.
1.6. Aims and Objectives

IL-21 is involved in various autoimmune conditions, though the precise mechanism by which it contributes to immune pathology in these diseases remains unclear. Numerous reports suggest a role for T cells, which is perhaps unsurprising considering the broad range of effects IL-21 has on these cells. Moreover, CD4 T cells are known to produce IL-21 at significant levels. IL-21 is known to counteract the Treg-mediated suppression of conventional CD4 T cells and this may therefore represent a route to autoimmunity, via the erosion of peripheral tolerance. A major aim for this project was thus to investigate in more detail the effects of IL-21 on Treg suppression. We wished to ascertain whether IL-21 acted universally to counteract suppression, or whether its effects were influenced by T cell activation conditions, for example by the particular cell population providing co-stimulation. We also wished to establish whether the counteraction of suppression by IL-21 reflected an ability to disable Treg suppression, render conventional T cells resistant to Treg effects or alternatively, was mediated by signalling to the APC population. Another objective was to define novel targets for IL-21 during T cell activation, and to further investigate the differentiation and function of IL-21-producing T helper cells.
2. MATERIALS AND METHODS

2.1. Mice

2.1.1. Mice

BALB/c and DO11.10 TCR transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). RIP-mOVA mice on a BALB/c background were provided by W. Heath (WEHI, Melbourne, Australia). CTLA-4-/- mice were a gift from A. Sharpe (Harvard University, Boston, USA). RAG2-/- mice were purchased from Taconic laboratories (Germantown, New York, USA) and were bred heterozygously. DO11.10 TCR transgenic mice were bred with RIP-mOVA mice to generate DO11xRIP-mOVA mice. DO11.10 TCR transgenic mice and RIP-mOVA mice were bred with RAG2-/- mice to generate RAG2-/- DO11.10 TCR transgenic mice and RAG2-/- RIP-mOVA mice. CTLA-4-/- mice were bred with RAG2-/- DO11.10 TCR transgenic mice to generate CTLA-4-/- RAG2-/- DO11.10 TCR transgenic mice. RAG2-/- DO11.10 TCR transgenic mice and CTLA-4-/- RAG2-/- DO11.10 TCR transgenic mice were bred with RAG2-/- RIP-mOVA mice to generate RAG2-/- DO11xRIP-mOVA mice and CTLA-4-/- RAG2-/- DO11xRIP-mOVA progeny. CD28-/- mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). CD28-/- mice were bred with RIP-mOVA mice to generate CD28-/mOVA+ mice. IL-21R-/- mice were a gift from M. Kopf (ETH, Zurich, Switzerland). IL-21R-/- mice were bred with DO11.10 TCR transgenic mice to generate IL-21R-/- DO11.10 TCR transgenic progeny. p110δ<sup>D910A</sup> mice were provided by K. Okkenhaug (Babraham Institute, Cambridge, UK). Mice were housed at
the University of Birmingham Biomedical Services Unit and were used in accordance with Home Office regulations.

2.1.2. Assessment of cultured T cell pathogenicity in vivo

2.5x10^4 wildtype or IL-21R−/- DO11.10 TCR transgenic MACS (Miltenyi Biotec) sorted CD4+CD25- cells were cultured with either 5x10^4 MACS sorted CD19+ B cells or 2.5x10^3 bone marrow-derived immature dendritic cells in 200µl C10 (see appendix) with 0.8µg/ml soluble anti-CD3 (BD Biosciences) for 3 days at 37°C, 5% CO₂. Cells were then harvested and MoFlo (DakoCytomation) sorted to isolate CD4+ cells. 1.8x10^6 sorted CD4+ cells were injected intravenously in 400µl PBS (Sigma) into lightly irradiated (3.5Gy) CD28−/-mOVA+ mice. Recipients were culled at day 21 and tissues harvested. Absolute cell counts were determined by acquiring all cells of the pancreas, pancreatic lymph node or inguinal lymph node by flow cytometry.

2.1.3. Blood glucose measurement

Blood glucose was measured using an Ascensia Elite XL blood glucose meter (Bayer, USA). Mice were considered overtly diabetic when producing blood glucose measurements of ≥250mg/dL.
2.1.4. Bone marrow transduction and generation of RAG2-/- CD86-GFP mice

GFP-tagged CD86 was generated by PCR to remove the stop codon. The fusion sequence was cloned into IRES-GFP deleted MIGR1 vector (gifted by W. Pear, University of Pennsylvania, Philadelphia, USA). The packaging cell line Plat-E was transfected with the retroviral vector using Lipofectamine 2000 (Invitrogen) to prepare retroviral supernatants. To prepare bone marrow cells, 150mg/kg 5-fluorouracil (Sigma) was given to adult RAG2-/- mice by intraperitoneal injection and total bone marrow cells were flushed from tibias and femurs. Bone marrow cells were cultured in IMDM (Invitrogen) supplemented with 20% FCS (Sigma), penicillin (Invitrogen), streptomycin (Invitrogen), L-glutamine (Invitrogen), sodium pyruvate (Invitrogen), nonessential amino acids (Invitrogen), 50ng/ml IL-6 (Peprotech), 10ng/ml IL-3 (Peprotech) and 50ng/ml stem cell factor (Invitrogen). After 48, 72, and 96 hours, cells were spin-infected with retrovirus by addition of 10ug/ml polybrene (Sigma) and centrifugation for 90 mins. Transduced bone marrow was injected intravenously into irradiated (450 Rad) RAG2-/- recipients. Thanks are due to Kyoko Nakamura for transduction and adoptive transfer of bone marrow.

2.1.5. Assessment of trans-endocytosis of CD86 by CTLA-4 in vivo

3 weeks following intravenous injection of transduced bone marrow, 1-5x10^6 MACS sorted CD4+CD25+ cells from DO11xRIP-mOVA mice or 1-5x10^6 MACS sorted CD4+ cells from DO11.10 TCR transgenic mice, RAG2-/- DO11xRIP-mOVA mice or CTLA-4-/- RAG2-/- DO11xRIP-mOVA mice were adoptively transferred by intravenous injection in 400µl PBS. After 24 hours, recipients were immunised by intraperitoneal injection with 100µg OVA/alum (Sigma) in 100µl PBS. After 7 days, mice received 100µg OVA peptide (Anaspec) in 400µl
PBS by intravenous injection, followed by 600µg of chloroquine (Sigma) in 100µl PBS by intraperitoneal injection 3 hours later. Spleens were harvested after a further 3 hours.
2.2. Flow cytometry and confocal laser microscopy

2.2.1. Cell isolation

Single cell suspensions were isolated from lymph nodes and spleens by mashing through a wire mesh in cold P2 (see appendix). Cells were isolated from pancreatic lymph nodes by tearing with forceps in P2. For spleens and peritoneal lavage fluid, red blood cells were lysed using 2ml lysis buffer (see appendix) for 3 mins at room temperature before dilution with 7ml P2 to stop the reaction. For the isolation of dendritic cells, spleens were cut into small fragments and digested with 0.5mg/ml collagenase/dispase (Roche) for 25 mins in 5ml C10 at 37°C, 5% CO₂ before mashing through a wire mesh and lysis of red blood cells. Lymphocytes were isolated from the pancreas by tearing in cold pancreas buffer (see appendix) before centrifugation. Cells were then resuspended in 2ml of warm pancreas digest solution (see appendix) and incubated at 37°C, 5% CO₂ for 10 mins. After passing through a 40µm cell strainer (BD Biosciences), cells were layered over lympholyte-M (Cedarlane Laboratories, Ontario, Canada) and centrifuged for 20 mins at 1000g, 4°C. Lymphocytes were then isolated by recovering cells at the interface and washing with P2.

2.2.2. Restimulation for intracellular cytokine staining

2x10⁶ cells were cultured in 1ml C10 in a 24 well flat bottom plate with 50ng/ml Phorbol Myristate Acetate (PMA) (Sigma) and 1.5µM Ionomycin (Sigma) at 37°C, 5% CO₂. After 1 hour, 10µg/ml Brefeldin A (Sigma) was added before culturing for a further 4 hours.
2.2.3. Staining for flow cytometry/confocal laser microscopy

Cell surface antigens were stained for 10 mins at 4°C in P2 containing 5% goat serum followed by washing with 500µl P2. For intracellular staining of CTLA-4, Foxp3, DO11.10 TCR and/or cytokines, cells were fixed and permeabilised with paraformaldehyde and saponin containing reagents according to the manufacturer’s instructions (eBioscience). Stains were added and cells incubated at 4°C for 30 mins.

2.2.4. IL-2 secretion assay

For measurement of secreted IL-2, cells were washed with 10ml MACS buffer (see appendix) before resuspension in 80µl cold C10 and addition of 20µl IL-2 catch reagent (Miltenyi Biotec). Cells were incubated on ice for 5 mins before dilution with 35ml warm C10 and incubation at 37°C, 5% CO₂ for 35 mins under slow continuous rotation. 15ml cold MACS buffer was then added before incubation on ice for 10 mins. Following centrifugation, cells were resuspended in 80µl cold MACS buffer and 20µl PE-conjugated IL-2 detection antibody (Miltenyi Biotec) and incubated for 10 mins on ice. Cells were then washed with 10ml cold MACS buffer.

2.2.5. Antibodies for flow cytometry/confocal laser microscopy

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Table 2.01. Antibodies for flow cytometry/confocal laser microscopy

2.2.6. Acquisition and analysis

For flow cytometry, all cells were acquired using either FACScalibur (BD Biosciences) or Dako CyAn (DakoCyTomation) flow cytometers using CellQuest Pro (BD Biosciences) or Summit (DakoCyTomation) respectively. Analysis was performed using FlowJo (TreeStar). For confocal laser microscopy, all cells were imaged using a Zeiss LSM 510 or a Zeiss LSM 780 inverted laser scanning confocal microscope with the help of Omar Qureshi. Cells were analysed in glass bottom culture dishes (MatTek) with a 100x oil immersion objective. Images were processed using ImageJ (NIH, Maryland, USA).
2.3. Cell sorting and differentiation

2.3.1. Magnetic cell sorting (MACS)

For positive selection of CD4+ T cells, CD19+ B cells or CD11c+ dendritic cells, single cell suspensions from spleen or lymph node were obtained as described previously and labelled with 10µl microbeads (Miltenyi Biotec) per 10^7 cells for 15 mins at 4°C. Cells were then washed and passed through an LS column (Miltenyi Biotec). Positively selected cells were retained on the column and eluted with MACS buffer to obtain the enriched fraction. For selection of CD4+CD25+ and CD4+CD25- T cells, single cell suspensions from lymph node were obtained as described previously and labelled with 15µl biotin-antibody cocktail (Miltenyi Biotec) per 10^7 cells for 10 mins at 4°C. 20µl of anti-biotin microbeads (Miltenyi Biotec) and 10µl CD25 PE (Miltenyi Biotec) were then added per 10^7 cells and cells incubated at 4°C for a further 15 mins. Cells were then washed and passed through an LD column (Miltenyi Biotec). CD4+ cells were collected from the run-off and were further labelled with 10µl anti-PE microbeads (Miltenyi Biotec) per 10^7 cells for 15 mins at 4°C. Cells were then washed and passed through an MS column (Miltenyi Biotec). CD4+CD25- cells were enriched in the run-off whilst CD4+CD25+ enriched cells were retained on the column and eluted with MACS buffer.

2.3.2. Fluorescence activated cell sorting (FACS)

Cultured cells were harvested, washed and stained in 600µl C2 (see appendix) for 10 mins at 4°C. Cells were then diluted in 10ml C10 before being passed through a 40µm cell strainer.
(BD Biosciences). Cells were then centrifuged and resuspended in 500µl C2 before sorting using a MoFlo high speed cell sorter (Beckman Coulter), operated by Roger Bird.

2.3.3. Generation of dendritic cells from murine bone marrow

Femurs were harvested and cleared of tissue before washing in 70% EtOH (Sigma) and then PBS. Marrow was flushed from both femurs with PBS before red cell lysis. Remaining cells were washed with PBS before culture of 2x10⁶ cells in 10ml C10 supplemented with 200U/ml of recombinant murine GM-CSF (Peprotech) at 37°C, 5% CO₂. At day 3, a further 10ml C10 supplemented with 200U/ml of recombinant murine GM-CSF was added to the culture. At days 6 and 8, 10ml cultured cells were collected, centrifuged and resuspended in 10ml C10 supplemented with 200U/ml of recombinant murine GM-CSF before replating. At day 10, cells were fed as described at days 6 and 8, although C10 was supplemented with 100U/ml of recombinant murine GM-CSF. For maturation of dendritic cells, C10 was further supplemented at day 10 with 1µg/ml of LPS (E. coli O26:B6, Sigma). Cells were then harvested at day 11.
2.4. In vitro cell culture

2.4.1. In vitro Suppression assays

Cells were cultured in duplicate in round-bottom 96 well plates in 200µl C10. MACS sorted wildtype or IL-21R-/- CD4+CD25- conventional T cells (in some experiments labelled with 1µM CFSE or derived from Thy1.1 or Thy1.2 donors) were added at 2.5x10^4 per well with MACS sorted wildtype, IL-21R-/- or CD28-/- CD4+CD25+ Treg (in some experiments derived from Thy1.1 or Thy1.2 donors) at ratios of 1:1, 0.5:1, 0.25:1 or 0:1 (Treg: Tconv). MACS sorted wildtype or IL-21R-/- CD19+ B cells, MACS sorted wildtype CD11c+ splenic dendritic cells, mature bone marrow-derived dendritic cells or wildtype or IL-21R-/- bone marrow-derived immature dendritic cells were added at ratios of 1:1, 2:1 or 4:1 (B cell: Tconv), 1:2, 1:5, 1:10, 1:20 or 1:40 (bone marrow-derived DC: Tconv) or 1:2 (splenic DC: Tconv). Cells were cultured alone or with 50, 100 or 200ng/ml murine IL-21 (Peprotech), 100ng/ml murine IFNγ (Peprotech), 10µg/ml anti-IFNγ (BD Biosciences), 20ng/ml TNFα (Peprotech), 5µg/ml anti-TNFα (Peprotech), 100µg/ml anti-CTLA-4 (4F10, provided by J. Bluestone, University of California at San Francisco, USA) or control hamster anti-mouse IgG. TCR ligation was provided by addition of 0.8µg/ml soluble α-CD3 (BD Biosciences). Cells were incubated at 37°C, 5% CO₂ for 72 hours. Plates were then harvested by washing with P2 and stained for flow cytometry as previously described. Cell counts were determined by acquiring all cultured cells.
2.4.2. Long-term cultures for assessment of proliferation, cytokines or IL-21Rα

Cells were cultured in triplicate in round-bottom 96 well plates in 200µl C10. MACS sorted wildtype or IL-21R-/- BALB/c or DO11.10 TCR transgenic CD4+CD25- conventional T cells (in some experiments derived from Thy1.1 or Thy1.2 donors) were added at 2.5x10^4 per well with MACS sorted wildtype CD4+CD25+ Treg (in some experiments derived from Thy1.1 or Thy1.2 donors) at ratios of 0.5:1 or 0:1 (Treg: Tconv). MACS sorted wildtype CD19+ B cells or wildtype bone marrow-derived immature dendritic cells were added at ratios of 2:1 (B cell: Tconv) or 1:10 (bone marrow-derived DC: Tconv). To assess the effect of IL-21-mediated CD86 upregulation on Tconv proliferation, B cells were pre-incubated for 15 hours alone or in the presence of 200ng/ml IL-21. Cells were cultured alone or with 200ng/ml murine IL-21 (Peprotech), 5µg/ml anti-IL-2 (BD Biosciences), 10µg/ml anti-CD80 (BioXcell), 10µg/ml anti-CD86 (BioXcell), 5µg/ml anti-TNFα (Peprotech) or control hamster anti-mouse IgG. TCR ligation was provided by addition of 0.8µg/ml soluble α-CD3 (BD Biosciences). Cells were incubated at 37°C, 5% CO₂ for 72 hours. For assessment of proliferation and IL-21Rα staining, wells were harvested at 24 hours, 48 hours and 72 hours. For cytokine stains, plates were harvested after 72 hours and restimulated as described previously. Cell counts were determined by acquiring all cultured cells by flow cytometry.

2.4.3. Short-term cultures for assessment of cell surface antigens

Cells were cultured in triplicate in round-bottom 96 well plates in 200µl C10. For phenotyping of conventional T cells after culture with B cells or for assessing the target of IL-21 for CD86 upregulation, MACS sorted wildtype or IL-21R-/- CD4+CD25- conventional T cells were added at 2.5x10^4 per well with MACS sorted wildtype or IL-21R-/- CD19+ B cells
at a ratio of 2:1 (B cell: Tconv). For assessing the role of p110δ in CD86 upregulation, 2x10⁵ MACS sorted wildtype or p110δ<sup>D910A</sup> CD19+ B cells were used. For assessing CD86 upregulation by APCs, CD86 expression over time, IL-21 dose dependency for CD86 upregulation and CD86 expression with LPS, 2x10⁵ splenocytes were used. Cells were cultured alone or with 25, 50, 100 or 200ng/ml murine IL-21 (Peprotech), 10ng/ml murine IL-4 (Peprotech) or 10µg/ml LPS (<i>E. coli</i> O26:B6, Sigma). Where provided, TCR ligation was provided by addition of 0.8µg/ml soluble α-CD3 (BD Biosciences). Cells were incubated at 37°C, 5% CO₂ for 15 hours. For assessment of CD86 upregulation over time, wells were harvested at 0.5, 2, 4, 6, 8 and 16 hours. Plates were washed with P2 and cells stained for flow cytometry as described previously.

### 2.4.4. Treg survival assays

Cells were cultured in triplicate in round-bottom 96 well plates in 200µl C10. MACS sorted wildtype CD4+CD25+ Treg were added at 2.5x10⁴ per well. Cells were cultured alone, with 20ng/ml murine IL-2 (Peprotech) or 200ng/ml murine IL-21 (Peprotech). Cells were incubated at 37°C, 5% CO₂ for 72 hours. Plates were then harvested by washing with P2 and stained for flow cytometry as previously described. Cell counts were determined by acquiring all cultured cells.

### 2.4.5. CHO transfectants

Human CTLA-4 cDNA was cloned into a CMV expression vector pCDNA3.1 and CTLA-4-expressing CHO cells generated by electroporation. CD86-GFP-expressing CHO cells were
generated as described previously using an IRES-GFP deleted MIGR1 vector (gifted by W. Pear, University of Pennsylvania, Philadelphia, USA) inserted by electroporation. Cells were grown in DMEM (Invitrogen) containing 10% FCS (Sigma). Cells expressing the plasmid were selected using 500µg/ml G418 and MoFlo cell sorting. Cells were cultured at 37°C, 5% CO₂ and were passaged by trypsinization. CHO cells expressing murine MHC Class II were provided by G. Freeman (Dana Faber Cancer Institute, Boston, USA) and transfected with mouse CD86-GFP by Amaxa nucleofection (Lonza). Thanks are due to Omar Qureshi for generating these CHO cell lines.

2.4.6. Assessment of trans-endocytosis of CD86 by CTLA-4 in vitro

Cells were cultured in flat-bottom 48 well plates in 1ml C10. For assessing trans-endocytosis by murine T cells, MACS sorted wildtype or CTLA-4-/- CD4+ T cells were added at 1x10⁵ per well with 1x10⁵ CHO cells expressing CD86-GFP. For assessing antigen-dependency of trans-endocytosis by murine T cells, MACS sorted DO11.10 TCR transgenic CD4+ T cells were added at 1x10⁵ per well with 1x10⁵ CHO cells expressing CD86-GFP and MHC class II. TCR ligation was provided by addition of 0.5µg/ml soluble α-CD3 (BD Biosciences) or 1µg/ml OVA peptide (Anaspec). Cells were incubated at 37°C, 5% CO₂ for 15 hours. Plates were then harvested and washed with P2 before cells were stained for confocal microscopy as described previously. For assessing the requirement for inhibition of lysosomal degradation, 1x10⁶ Far Red (Invitrogen) labelled CTLA-4-expressing CHO cells were cultured with 1x10⁶ CD86-GFP-expressing CHO cells in a 24 well flat bottom plate in 1ml C10 alone, or in the presence of 50µM chloroquine (Sigma). Cells were incubated at 37°C, 5% CO₂ for 15 hours.
Plates were then harvested and washed with P2 before cells were stained for confocal microscopy as described previously.
2.5. Immunohistology

2.5.1. Preparation of sections for immunohistochemistry
Pancreas’ were harvested and slowly frozen in O.C.T. compound (Tissue-Tek) above liquid nitrogen before storage at -80°C. Serial sections were cut at 6µm thickness using a cryostat at -20°C onto glass slides (Menzel-Glaser). After air-drying, sections were fixed in 100% acetone (Scientific and Chemical Supplies) at 4°C for 20 mins before drying at room temperature for a further 20 mins and storage at -20°C.

2.5.2. Protocol for immunohistochemical staining
Sections were re-hydrated in PBS before staining at room temperature with primary antibodies for 1 hour (Table 2). Sections were then washed with TRIS buffer at pH7.2 (Sigma). Peroxidase or biotin conjugated secondary antibodies (pre-adsorbed in 5% normal mouse serum) were then added for 45 mins at room temperature (Table 3). Biotinylated antibodies were detected with streptavidin AB complex-alkaline phosphate (DakoCytomation), followed by Naphthol AS-MX phosphate substrate (Sigma) and Fast Blue BB salt (Sigma) with Levamisole (Sigma). Peroxidase conjugated antibodies were detected with DAB solution (Sigma). Images were acquired using a Leica DM6000 B light microscope and processed using ImageJ (NIH, Maryland, USA).
2.5.3. Antibodies for immunohistochemical staining

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Table 2.02. Primary antibodies for immunohistochemical staining.

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Table 2.03. Secondary antibodies for immunohistochemical staining.
2.6. Molecular biology

2.6.1. RNA isolation and generation of cDNA by reverse transcription PCR

RNA was isolated from frozen cell pellets by addition of 800µl RNAbee (Biogenesis) before extraction with 80µl chloroform (Sigma) on ice for 5 mins. After centrifugation, RNA precipitation was performed with 400µl isopropanol (Sigma) at -20°C for 20 mins. RNA was then washed with 75% EtOH (Sigma) before heating to 70°C for 5 mins followed by transfer to ice. Reverse transcription PCR was then carried out using M-MLV reverse transcriptase (Invitrogen) and Oligo dT primers (Invitrogen).

2.6.2. qPCR protocol

Reactions were performed using 2x PCR mastermix (Applied Biosystems) and TaqMan gene expression assays (Applied Biosystems) (Table 4) in 96 well microAmp plates (Applied Biosystems) Expression was quantified using an Mx3000P cycler (Stratagene, Agilent Technologies) and normalized against β-actin expression. β-actin primers and probe were purchased from Eurogentec:

Forward: 5’ CGT GAA AAG ATG ACC CAG 3’
Reverse: 5’ TGG TAC GAC CAG AGG CAT 3’

PCR program:

1 cycle - 50°C, 2 mins
1 cycle - 95°C, 10 mins
40 cycles - 95°C, 15 secs followed by 60°C, 1 min
2.6.3. TaqMan gene expression assays

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Table 2.04. TaqMan gene expression assays.
2.7. Statistics

Statistical analysis was performed by unpaired T-test using GraphPad Prism (GraphPad Software). All analyses were two-tailed with a 95% confidence interval. *, \( p = <0.05 \), **, \( p = <0.01 \), ***, \( p = <0.001 \), ns = not significant.
3. COUNTERACTION OF TREG-MEDIATED SUPPRESSION BY INTERLEUKIN 21

3.1. Introduction

One of the earliest reported functions for interleukin 21 (IL-21), like other common \(\gamma\)-chain cytokines, was as a T cell growth factor in both man and mouse (Parrish-Novak et al. 2000). In the years since these experiments were performed, we and others have identified a critical role for IL-21 in counteracting the Treg-mediated suppression of murine (Clough et al. 2008) and human (Peluso et al. 2007) CD4+ conventional T cells. It could therefore be envisaged that these mechanisms would synergise to promote uninhibited T cell responses. Mechanistically, it remained unclear whether IL-21 was promoting a resistance to Treg suppression in conventional T cells, or a loss of function or homeostasis in the Treg population. Thus, we generated a multi-parameter, flow cytometry based suppression assay, utilising IL-21R deficient cell populations, to gain further insight into the conditions necessary for release from suppression to occur.
3.2. Results

3.2.1. IL-21 is expressed in DO11xRIP-mOVA mice

We have previously demonstrated that pancreatic lymph node CD4+CD25- T cells expressed IL-21 transcripts during the onset of type-1 diabetes in DO11xRIP-mOVA mice at 12 weeks of age. The same subset of cells taken from healthy DO11xRIP-mOVA mice however, at 6 weeks of age, expressed relatively little (Clough et al. 2008). What remained unclear was whether IL-21 mRNA expression continued throughout the course of the disease, or indeed whether IL-21 was significantly expressed in the pancreas, the major site of autoimmune pathology. To address these questions we first expanded our IL-21 mRNA expression dataset. CD4+CD25+ and CD4+CD25- T cells from DO11xRIP-mOVA pancreatic or inguinal lymph node were pooled and sorted at not only 6 and 12, but also 16 and 19 weeks of age, before quantitative real time PCR was undertaken to assess IL-21 mRNA expression throughout. This revealed a striking increase in IL-21 transcripts between six and 12 weeks of age, as previously reported by our group (Clough et al. 2008). IL-21 levels remained high at 16 weeks and were still raised, although not as markedly, at 19 weeks of age in pancreatic lymph node CD4+CD25- conventional T cells. Very little IL-21 mRNA was detected in CD4+CD25+ cells taken from this site, nor in peripheral T cells isolated from the inguinal lymph nodes (Fig. 3.01 A). This indicated that mRNA for IL-21 was being highly transcribed in conventional T cells during the onset and throughout the course of the disease. Further, this expression appeared to be limited to T cells in the lymph node (LN) draining the site of self-antigen (pancreatic LN) rather than in non-draining LN (inguinal LN).
Figure 3.01. IL-21 is expressed in DO11xRIP-mOVA mice. (A) CD4+CD25+ Treg and CD4+CD25- Tconv from the pancreatic or inguinal lymph nodes of DO11xRIP-mOVA mice (6 for each time point) were analysed by Taqman qPCR for the presence of IL-21 transcripts relative to an endogenous β-actin reference. (B) Single cell suspensions from the indicated tissues of DO11xRIP-mOVA mice (aged 8, 12 or 18 weeks) were restimulated and stained for surface CD4 and intracellular Foxp3 and IL-21 for analysis by flow cytometry. Graph shows IL-21 staining for gated CD4+Foxp3- cells. Bars represent means and SEM is shown for 3 mice per age group.
To assess whether IL-21 was being expressed in the pancreas during disease onset and beyond, we opted to undertake intracellular cytokine staining on pancreatic T cells. This was primarily due to the limited number of T cells it is possible to MoFlo sort from pancreatic tissue, a necessary step for analysis by quantitative real time PCR. Single cell suspensions of lymphocytes from DO11xRIP-mOVA mice, aged 8, 12 or 18 weeks, were therefore restimulated and stained for intracellular expression of IL-21 protein and analysed by flow cytometry. Cells were obtained from the pancreas, pancreatic lymph node, inguinal lymph nodes and spleen. As demonstrated in Fig. 3.01 B, the highest frequency of IL-21 producing CD4+Foxp3- conventional T cells was consistently found in the pancreas, equaled only by those of the spleen at 18 weeks of age. In all other tissues, IL-21 expression was far lower throughout the time course. The gradual increase in frequency of IL-21+ T cells in the spleen over time could possibly reflect a recirculating population of autoreactive conventional T cells. It is unclear why IL-21 protein and mRNA expression do not correlate in the pancreatic lymph node. Perhaps this reflects a delay in translation, manifesting as IL-21 protein expression in the pancreas after priming in the lymph node. What is clear is that a substantial fraction of pancreas-infiltrating conventional T cells express IL-21, even in mice as young as 8 weeks of age. Thus, there appears to be a large window within which IL-21 might exert its effects at the site of autoimmune attack.

3.2.2. Assessment of Treg-mediated suppression utilising a flow cytometry based in vitro assay

As mentioned earlier, we have previously demonstrated a role for IL-21 in counteracting the Treg-mediated suppression of CD4+ conventional T cells. However, these data were collected
using tritiated thymidine counts to assess proliferation. The major issue with this technique is that any proliferating cells within a given assay, be they conventional T cells, regulatory T cells or APCs, could incorporate thymidine. This confounded the determination of those cell populations that were being suppressed and indeed, those that were relieved from suppression in the case of the addition of IL-21. All that could accurately be observed was that universally, proliferation was inhibited. In an attempt to avoid this issue, others have utilised irradiated whole splenocytes to provide co-stimulation in their assays (Thornton & Shevach 1998b; Wing et al. 2008; Szymczak-Workman et al. 2009). However, whilst this precludes the proliferation of the APC population, it does not account for the potential of Treg to proliferate in vitro (Thornton et al. 2004; Zou et al. 2010). We therefore sought to optimise a flow cytometry based suppression assay that would enable the identification, thereby permitting the quantification and phenotyping, of each cell population.

In beginning to optimise a flow cytometry based assay, we first needed to confirm that we could sort highly pure populations of conventional and regulatory T cells, as well as B cells to provide co-stimulation, from BALB/c lymphoid tissues. We sorted CD19+ B cells from spleen and CD4+ conventional (CD25-) and regulatory (CD25+) T cells from lymph node, using Miltenyi MACS cell purification kits. As demonstrated in Fig. 3.02, we achieved greater than 98% purity for all cell populations when analysed by flow cytometry. To further assess the purity of our starting cell populations, we felt it important to determine whether our CD4+CD25+ T cells were, in fact, regulatory T cells. It has previously been demonstrated that the constitutive expression of CD25 by murine CD4+ T cells correlates with their expression of the Treg lineage specific transcription factor Foxp3 (Fontenot et al. 2003; Fontenot et al. 2005a). We therefore stained for the expression of both CD25 and Foxp3
Figure 3.02. Validation of MACS CD4+CD25+ and CD19+ cell sorting kits. Single cell suspensions from BALB/c lymph node were MACS sorted to isolate CD4+CD25+ Treg and CD4+CD25- Tconv. CD19+ B cells were positively selected from BALB/c spleen. Purified cells were surface stained for CD4, CD25 and CD19 for flow cytometric analysis. Representative dot plots show (A) Sorted lymph node CD4+CD25- Tconv and CD4+CD25+ Treg and (B) Sorted splenic CD19+ B cells. Data are representative of >10 experiments.
within the CD4+ compartment of BALB/c lymph node. We found that almost exactly the same frequency of CD25+ cells existed as did Foxp3+ cells amongst the CD4+ population. Further, analysis of co-staining revealed that almost all of the CD4+Foxp3+ population co-expressed CD25 (Fig. 3.03). On the basis of these data, we were satisfied that we could obtain highly pure populations of conventional and regulatory T cells, and B cells, for use in our assays. However, we also considered the possibility that once in co-culture, either population of T cell might alter their expression of Foxp3, confounding the accurate determination of conventional T cell number. To address this issue, we employed a congenic system, purifying Treg from Thy1.2+ and conventional T cells from Thy1.1+ BALB/c mice. This permitted the identification of these cell populations post-culture, by simply staining for Thy1.1 and Thy1.2 expression. In this manner, we were able to monitor any change in the expression of Foxp3 by either conventional or regulatory T cells.

To be able to assess Treg suppression, it was important that we design an assay that permitted the robust activation and proliferation of conventional T cells, without overcoming their susceptibility to suppression. It was thus relevant to consider both strength of TCR ligation and provision of co-stimulatory ligands. It has been observed that utilising plate-bound, rather than soluble, anti-CD3 in both murine (Thornton & Shevach 1998a) and human (Baecher-Allan & Viglietta 2002) suppression assays is less supportive of Treg suppression. Further, an agonistic anti-CD28 antibody was also shown to prevent Treg suppression in murine assays (Thornton et al. 2004; Thornton & Shevach 1998a). On the basis of these data, we set about first optimizing a robust conventional T cell proliferative response using a titration of both soluble anti-CD3 and B cells to provide co-stimulation. We observed marked increases in conventional T cell proliferation, after three days, with increasing B cell dose. Interestingly,
Figure 3.03. Expression of CD25 correlates with that of Foxp3 in BALB/c mice. Single cell suspensions from BALB/c lymph node were stained for surface CD4 and CD25, and intracellular Foxp3 for analysis by flow cytometry. Representative dot plots show CD25 and Foxp3 expression for gated CD4+ cells (upper panel) and Foxp3/CD25 co-staining for gated CD4+ cells (lower panel). Data are representative of >5 experiments.
there was little effect observed when increasing the concentration of soluble anti-CD3 (Fig. 3.04 A). We next sought to determine how these variables might alter the capacity for Treg-mediated suppression. Using the same titrations of B cell number and anti-CD3 concentration, we supplemented our assays with increasing numbers of Treg, up to a maximum ratio of 1 conventional to 1 regulatory T cell. Again, we found that B cell dose was important as increasing B cell numbers lead to a decrease in the susceptibility of conventional T cells to suppression (Fig. 3.04 B, C, D). For example, at a regulatory to conventional T cell ratio of 0.25:1, suppression was only observed at the lowest dose of B cells (Fig. 3.04 B). Results for the titration of anti-CD3 were more variable, but showed an overall trend toward inhibiting suppression at higher doses. This was particularly clear at a B cell to conventional T cell ratio of 2:1 (Fig. 3.04 C). Overall, we concluded that this ratio of B cell to conventional T cell, along with an anti-CD3 dose of 0.8µg/ml provided a balance between agreeable conventional T cell counts and clear susceptibility to Treg suppression.

3.2.3. IL-21 counteracts Treg-mediated immune suppression

Having optimised our suppression assay, it was next important to revisit the question of whether IL-21 could counteract Treg suppression. To achieve this, we set up our assay in the presence or absence of 200ng/ml IL-21, a dose used by us and others in previous in vitro studies (Ostiguy et al. 2007; Mehta et al. 2003; Clough et al. 2008). We observed a clear overcoming of suppression in cultures incubated with IL-21, in terms of both conventional T cell count (Fig. 3.05 A) and CFSE dilution (Fig. 3.05 B), even at a Treg to conventional T cell ratio of 1:1. In addition, because analysis by flow cytometry permits phenotyping of the conventional T cell population post-culture, we assessed their expression of the activation
Figure 3.04. B cell based in vitro suppression assay optimisation. 2.5x10^4 Tconv were cultured with B cells at B cell to Tconv ratios of 1:1, 2:1 and 4:1, with anti-CD3 at 0.5, 0.8 or 1µg/ml. Treg were present at the indicated Treg to Tconv ratios. After 3 days cells were stained for surface CD4 and CD19, and intracellular Foxp3 for analysis by flow cytometry. Graphs show absolute cell counts for CD4+ Tconv in the absence of Treg (A) and relative cell counts for CD4+Foxp3- Tconv in the presence of Treg at B cell to Tconv ratios of 1:1 (B), 2:1 (C) and 4:1 (D). In the latter, Tconv numbers are presented as a percentage, relative to the cell count where no Treg are present.
Figure 3.05. IL-21 counteracts the ability of Treg to inhibit Tconv proliferation. 2.5x10^4 CFSE-labelled Tconv were cultured with 5x10^4 B cells, with 0.8µg/ml anti-CD3 and the indicated ratios of Treg, alone or in the presence of 200ng/ml IL-21. After 3 days cells were stained for surface CD4, and CD19, and intracellular Foxp3 for analysis by flow cytometry. (A) Graph shows cell counts for CD4+Foxp3- Tconv. Counts are presented as a percentage, relative to the cell count where no Treg are present. Bars represent means and SEM is shown for 5 experiments. (B) Representative histograms show CFSE dilution for CD4+Foxp3- Tconv when cultured with Treg alone (upper panels), or in the presence of IL-21 (lower panels). Data are representative of >5 experiments. *, p = <0.05, **, p = <0.01, ***, p = <0.001.
markers CD69 and CD25. **Fig. 3.06 A and B** clearly demonstrate that as Treg numbers increased, the frequency of CD25+ and CD69+ conventional T cells respectively, declined. However, provision of IL-21 rescued the expression of both markers, even at higher doses of Treg, clearly demonstrating its counter-suppressive capacity in this respect.

That we could observe such a profound release from suppression by IL-21 at 200ng/ml lead us to question whether lower concentrations might reveal a dose dependency. We therefore repeated our suppression assay with a titration of IL-21 from 50 to 200ng/ml. **Fig. 3.07** demonstrates that IL-21 was increasingly effective at counteracting suppression as its dose was increased. This is particularly clear at a Treg to conventional T cell ratio of 1:1, wherein a significant increase in conventional T cell count was observed between all doses.

### 3.2.4. IL-21 does not counteract suppression in dendritic cell co-stimulated responses

To this point, we have only addressed the counter-suppressive function of IL-21 in respect of B cell-driven responses. In NOD mice, it has been established that both B cells and DCs are required for optimal antigen-specific T cell proliferation *in vivo* (Bouaziz et al. 2007). Based on this data, we felt it important to repeat our assays, with DCs providing co-stimulation, to assess whether IL-21 could also counteract suppression in this setting. Both splenic (George et al. 2003; Pasare & Medzhitov 2003) and bone marrow-derived (Kubo et al. 2004) DCs have been demonstrated to support Treg-mediated suppression *in vitro*, to a similar extent as B cells (George et al. 2003). We felt that, due to the number of DCs we would require, it would be preferable to utilise the latter in our assays. Using a protocol described by Lutz and colleagues (Lutz et al. 1999), we generated bone marrow-derived DCs from BALB/c mice.
Figure 3.06. IL-21 counteracts the ability of Treg to inhibit Tconv activation. 2.5x10^4 Tconv were cultured with 5x10^4 B cells, with 0.8µg/ml anti-CD3 and the indicated ratios of Treg, alone or in the presence of 200ng/ml IL-21. After 3 days cells were stained for surface CD4, CD25, CD69 and CD19, and intracellular Foxp3 for analysis by flow cytometry. Representative dot plots show CD25 (A) and CD69 (B) expression for gated CD4+Foxp3- Tconv when cultured with Treg alone (upper panels), or in the presence of IL-21 (lower panels). Data are representative of >5 experiments.
Figure 3.07. Release from Treg-mediated suppression is IL-21 dose-dependent. 2.5x10^4 Tconv were cultured with 5x10^4 B cells, with 0.8µg/ml anti-CD3 and the indicated ratios of Treg, alone or in the presence of the indicated concentrations of IL-21. After 3 days cells were stained for surface CD4 and CD19, and intracellular Foxp3. Graph shows cell counts for CD4+Foxp3- Tconv, which are presented as a percentage, relative to the cell count where no Treg are present. Bars represent means and SEM is shown for 3 experiments. *, p = <0.05, **, p = <0.01.
and assessed their expression of a number of DC markers. The cells generated by this protocol expressed CD11c, high levels of MHC class II and CD11b, and did not express B220, a profile consistent with that of classical DCs (Steinman et al. 1997) (Fig. 3.08).

In optimising a bone marrow-derived DC based assay, we assessed whether either immature DCs or mature DCs (having been cultured overnight with 1µg/ml LPS) were able to support Treg-mediated suppression. Mature DCs have previously been shown to be unsupportive of suppression (Kubo et al. 2004), data we were able to confirm across a range of mature DC to conventional T cell ratios. Whilst the number of mature DCs clearly influenced the extent to which conventional T cell proliferation was supported, Treg were unable to suppress this response, even at the highest ratio of Treg to conventional T cell (Fig. 3.09 A). Conversely, immature DCs were effective at supporting Treg suppression and this effect was only lost at the highest ratio of DC analysed (1:2) (Fig. 3.09 B). Fig. 3.09 C highlights the differential capacity of mature and immature DCs to support Treg-mediated suppression. Based on these data, we opted to use an immature DC to conventional T cell ratio of 1:10 going forward, as this appeared to provide a balance between supporting both proliferation and suppression.

With our immature DC based assay optimised, we proceeded to assess whether IL-21 was capable of counteracting suppression in this setting. As in our B cell-driven assays, 200ng/ml IL-21 was added and conventional T cell counts determined at day three. Strikingly, we observed that even at this dose, IL-21 was unable to prevent suppression, in terms of measuring either conventional T cell count (Fig. 3.10 A) or CFSE dilution (Fig. 3.10 B). Assaying CD69 expression by conventional T cells confirmed these data, demonstrating robust suppression of CD69 by Treg in either the absence or presence of IL-21 (Fig. 3.11). To
Figure 3.08. BALB/c Bone marrow-derived DCs express the phenotypic hallmarks of classical DCs. Single cell suspensions of bone marrow-derived DCs were surface stained for the expression of CD11c, MHC class II, B220 and CD11b. Representative dot plots show CD11c/MHC class II co-staining (upper panel) and B220 and CD11b staining for gated CD11c+MHC class II+ cells (lower panel). Data are representative of >5 experiments.
Figure 3.09. Bone marrow-derived DC based in vitro suppression assay optimisation. 2.5x10^4 Tconv were cultured with immature (i)DCs or LPS matured (m)DCs at DC to Tconv ratios as indicated, with anti-CD3 at 0.8µg/ml. Treg were present at the indicated ratios of Treg to Tconv. After 3 days cells were stained for surface CD4, and CD11c, and intracellular Foxp3. Bar graphs show cell counts for CD4+Foxp3- Tconv when cultured with mDCs (A) or iDCs (B). Line graph shows percentage suppression (relative to no Treg condition) when Tconv are cultured with iDCs or mDCs at a DC to Tconv ratio of 1:10 (C).
Figure 3.10. IL-21 does not counteract Treg-mediated suppression of Tconv proliferation in bone marrow-derived iDC based in vitro suppression assays. 2.5x10^5 Tconv were cultured with 2.5x10^3 iDCs, with anti-CD3 at 0.8µg/ml and the indicated ratios of Treg, alone or in the presence of 200ng/ml IL-21. After 3 days cells were stained for surface CD4 and CD11c, and intracellular Foxp3. (A) Graph shows cell counts for CD4+Foxp3-Tconv. Counts are presented as a percentage, relative to the cell count where no Treg are present. Bars represent means and SEM is shown for 3 experiments. (B) Representative histograms show CFSE dilution for CD4+Foxp3-Tconv when cultured with Treg alone (upper panels), or in the presence of IL-21 (lower panels). Data are representative of 3 experiments. ns = not significant.
Figure 3.11. IL-21 does not counteract Treg-mediated suppression of Tconv activation in iDC based in vitro suppression assays. 2.5x10^4 Tconv were cultured with 2.5x10^3 iDCs, with anti-CD3 at 0.8µg/ml and the indicated ratios of Treg, alone or in the presence of 200ng/ml IL-21. After 3 days cells were stained for surface CD4, CD69 and CD11c, and intracellular Foxp3. Representative dot plots show CD69 expression for CD4+Foxp3- Tconv when cultured with Treg alone (upper panels), or in the presence of IL-21 (lower panels). Data are representative of 3 experiments.
further corroborate these results, we repeated this assay using DCs freshly isolated from BALB/c spleen, at a DC to conventional T cell ratio of 1:10, as used in our bone marrow-derived DC assay and by others for splenic DC-driven responses (Pasare & Medzhitov 2003). Similarly, we found no release from suppression occurred with the addition of IL-21 (Fig. 3.12). Thus, in stark contrast to B cell-driven responses, abrogation of Treg-mediated suppression by IL-21 is incompatible with T cell responses driven by DCs.

3.2.5. Release from Treg suppression requires IL-21 signalling to conventional T cells

Due to the profound difference in responsiveness to IL-21 between DC and B cell co-stimulated assays, we hypothesised that IL-21 signalling to APCs might be the determining factor in overcoming suppression. Perhaps the simplest explanation would be that there was differential expression of the IL-21Rα chain between the two assays. IL-21Rα is expressed by lymphoid cells and activated NK cells (Parrish-Novak et al. 2000), and is expressed on naïve and more so on activated CD4+ T cells, CD8+ T cells and B cells. Very low levels of IL-21Rα were expressed on B220- cells in the bone marrow, implying that expression was absent from pre-DCs (Jin et al. 2004). However, (Brandt et al. 2003a) observed receptor expression on bone marrow-derived DCs irrespective of their activation state. In characterising CD4+ T cell subsets, it has been reported that IL-21Rα is also expressed on murine CD4+CD25+ Treg (Jang et al. 2009).

To allow us to assess the specificity of our antibody staining, and to permit future experiments to dissect the cell populations targeted by IL-21Rα signalling, we obtained mice deficient in the IL-21Rα subunit from Professor Manfred Kopf (Frohlich et al. 2006). To
Figure 3.12. IL-21 does not counteract Treg-mediated suppression in splenic DC based in vitro suppression assays. 2.5x10^4 Tconv were cultured with 2.5x10^3 splenic DCs, with anti-CD3 at 0.8µg/ml and the indicated ratios of Treg, alone or in the presence of 200ng/ml IL-21. After 3 days cells were stained for surface CD4 and CD11c, and intracellular Foxp3. Graph shows cell counts for CD4+Foxp3- Tconv. Counts are presented as a percentage, relative to the cell count where no Treg are present.
assess expression of IL-21Rα on the cell types used in our assays, we stained B cells, conventional and regulatory T cells, and bone marrow-derived DCs from both BALB/c and IL-21Rα deficient mice for IL-21Rα expression. In IL-21R-/- animals, we observed no positive staining on any cell population, demonstrating the specificity of this antibody. In BALB/c mice, IL-21Rα was expressed on the vast majority of B cells and conventional and regulatory T cells, although little to no expression was demonstrable for bone marrow-derived DCs (Fig. 3.13). We next assessed the expression of the receptor at the mRNA level by real-time PCR, using pure populations of each cell type. In this experiment, we found transcripts for IL-21Rα in all of the populations analysed (Fig. 3.14). This suggested that, in the case of bone marrow-derived DCs, either post-transcriptional regulation had prevented expression or trafficking of the protein, or that its expression was such that it was below the level of detection for flow cytometry. Thus, because IL-21Rα expression by DCs was either low or null compared to that of B cells, it appeared plausible that differential levels of IL-21 signalling to APCs might be responsible for determining whether release from suppression was supported. Based on this, we hypothesised that utilising IL-21R-/- B cells in our suppression assay might have a significant impact on the counter-suppressive effects of IL-21. However, before using cells from IL-21R-/- mice in a functional assay, we wished to check that these mice were phenotypically normal, containing a complete repertoire of immune cells, indicative of normal development and homeostasis. Previous studies suggested that IL-21Rα was expressed in both the thymus, at high levels (Ozaki et al. 2000; Parrish-Novak et al. 2000), and in bone marrow cells (Jin et al. 2004). Nonetheless, IL-21R-/- mice showed no apparent developmental abnormalities and proportions of lymphocytes, granulocytes and monocytes appeared normal (Frohlich et al. 2006). Our own phenotyping supported this data, with IL-21Rα deficient animals having normal frequencies of conventional and regulatory T
Figure 3.13. Expression of IL-21Rα by bone marrow-derived DCs, B cells, Tconv and Treg. BALB/c lymph node cells and bone marrow-derived DCs were stained for the expression of CD4, CD19, CD11c, MHC class II and IL-21Rα, and intracellular Foxp3. Representative histograms show IL-21Rα expression for the indicated cell populations from BALB/c (left panel) and IL-21R-/- (right panel) mice. Staining with an isotype control Ab is also shown. Data are representative of 3 experiments.
Figure 3.14. Expression of IL-21Rα by bone marrow-derived DCs, B cells, Tconv and Treg by qPCR. Bone marrow-derived DCs, B cells, Tconv and Treg were analysed by Taqman qPCR for the presence of IL-21Rα transcripts, relative to an endogenous β-actin reference.
cells (Fig. 3.15 A, B and C) and displaying parity, in the correlation between CD25 and Foxp3 in the Treg population, when compared with age matched BALB/c mice (Fig. 3.16). Frequencies of CD8+ T cells and B cells (Fig. 3.17 A, B and C) and classical DCs (Fig. 3.18 A and B) also appeared normal.

Based on the above data, we proceeded to substitute wildtype B cells with IL-21R-/- B cells in our suppression assay, to ascertain whether this prevented the effects of IL-21 on suppression. In addition, because wildtype conventional and regulatory T cells also expressed IL-21Rα, we included control conditions in which each of these populations was substituted with their IL-21R-/- counterparts. As demonstrated previously (Fig. 3.05 and 3.07), when all cell populations were IL-21R sufficient, IL-21 potently permitted conventional T cells to overcome suppression (Fig. 3.19, red bars). When the same assay was performed with all cell populations being IL-21R-/-, IL-21 was unable to overcome suppression, demonstrating a clear lack of redundancy for the IL-21Rα chain (Fig. 3.19, black bars). Conducting the assay with wildtype Treg and B cells, but with IL-21R-/- Tconv, produced a striking result, with a complete restoration in suppression of Tconv proliferation (Fig. 3.19, green bars). On the other hand, in conditions where either Treg or B cells were IL-21R-deficient, with all other populations being wildtype, IL-21 permitted release from suppression (Fig. 3.19, blue and orange bars). These data indicated that loss of IL-21 signalling in conventional T cells alone was sufficient to prevent IL-21 from counteracting Treg suppression. Analysis of CD69 expression provided a broadly similar picture. When all cell populations were IL-21R sufficient, IL-21 prevented Treg-mediated CD69 downregulation (Fig. 3.20, top row). Conversely, when all cells were IL-21R-/-, IL-21 did not counteract Treg-mediated CD69 downregulation (Fig. 3.20, bottom row). The greatest effect of IL-21Rα deficiency, in terms
Figure 3.15. Comparison of Tconv and Treg frequencies between BALB/c and IL-21R-/- mice. BALB/c or IL-21R-/- lymph node or splenic cells were stained for surface expression of CD4 and intracellular Foxp3. Representative dot plots (A) show cell frequencies and graphs show collated data for CD4+Foxp3- Tconv (B) and CD4+Foxp3+ Treg (C). Bars represent means and SEM is shown for 3 of each genotype. *ns* = not significant.
Figure 3.16. Correlation of CD25 with Foxp3 in T cells between BALB/c and IL-21R-/mice. BALB/c or IL-21R-/ lymph node or splenic cells were stained for surface expression of CD4 and CD25, and intracellular Foxp3. Representative dot plots show Foxp3/CD25 co-staining for gated CD4+ T cells. Data are representative of 3 mice per genotype.
Figure 3.17. Comparison of CD8 T cell and B cell frequencies between BALB/c and IL-21R-/− mice. BALB/c or IL-21R-/− lymph node or splenic cells were stained for surface expression of CD19 and CD8. Representative dot plots (A) show cell frequencies and graphs show collated data for CD19+ B cells (B) and CD8+ T cells (C). Bars represent means and SEM is shown for 3 of each genotype. ns = not significant.
Figure 3.18. Comparison of classical DC frequencies between BALB/c and IL-21R-/-
mice. BALB/c or IL-21R-/- splenic cells were stained for surface expression of CD3, CD19, CD11c and CD11b. Representative dot plots (A) show cell frequencies and graph shows collated data for CD11c+ DCs (B). Bars represent means and SEM is shown for 3 of each genotype. *ns* = not significant.
Figure 3.19. IL-21 signalling to Tconv counteracts the ability of Treg to inhibit their proliferation. 2.5x10^4 BALB/c or IL-21R^-/- Tconv were cultured with 5x10^4 BALB/c or IL-21R^-/- B cells, with 0.8μg/ml anti-CD3 and the indicated ratios of BALB/c or IL-21R^-/- Treg, in the presence of 200ng/ml IL-21. After 3 days cells were stained for surface CD4 and CD19, and intracellular Foxp3. Graph shows cell counts for wildtype (WT) or IL-21R^-/- CD4^+Foxp3^- Tconv when cultured with combinations of WT or IL-21R^-/- cell populations as indicated, in the presence of IL-21. Counts are presented as a percentage, relative to the cell count where no Treg are present. Data are representative of 4 experiments.
Figure 3.20. IL-21 signalling to Tconv counteracts the ability of Treg to inhibit their activation. 2.5x10⁶ BALB/c or IL-21R-/− Tconv were cultured with 5x10⁶ BALB/c or IL-21R-/− B cells, with 0.8µg/ml anti-CD3 and the indicated ratios of BALB/c or IL-21R-/− Treg, in the presence of 200ng/ml IL-21. After 3 days cells were stained for surface CD4 and CD19, and intracellular Foxp3. Representative dot plots show CD69 expression by WT or IL-21R-/− CD4+Foxp3- Tconv when cultured with combinations of WT or IL-21R-/− cell populations as indicated, in the presence of IL-21. Data are representative of 4 experiments.
of modulating CD69 expression, was again on conventional T cells (Fig. 3.20, fourth row). There was also a minor effect of IL-21Rα deficiency on B cells in some, but not all, assays as using these cells, CD69 expression was not completely restored in the presence of IL-21 (Fig. 3.20, second row).

The above result indicated that IL-21 signalling to Treg was not involved in counteracting suppression. However, data from a previous study has indicated that IL-21 might release human CD8+ T cells from Treg suppression by inhibiting Foxp3 expression in the CD4+ T cell population (Li & Yee 2008). We therefore wished to test whether IL-21 was downregulating Foxp3 expression by Treg in our suppression assay. As mentioned previously, use of the Thy1.1/Thy1.2 congenic system permits the starting Thy1.1+ Treg population to be tracked throughout a given culture, regardless of the maintenance of Foxp3 expression. We therefore initiated a standard assay, alone or in the presence of 200ng/ml IL-21, using a regulatory to conventional T cell ratio of 1:1. To directly assess the effects of IL-21 signalling to Treg, we used conventional T cells from IL-21R-/- mice. After three days in culture, Foxp3 expression was assessed for Thy1.1+ cells. We found that provision of IL-21 lead to a significant decrease in Foxp3 expression by Treg across multiple experiments (Fig. 3.21 A and B), although clearly it remained distinct from that of Thy1.2+ conventional T cells (Fig. 3.21 A). As a definitive way of excluding Treg as candidates for the target of IL-21 in counteracting suppression, we performed a suppression assay wherein only Treg could receive IL-21 signals. In this assay, both B cells and conventional T cells lacked the IL-21Rα chain. IL-21 was either absent from the assay or added at 200ng/ml. Under these conditions, IL-21 was completely incapable of counteracting suppression with, if anything, a small but significant enhancement in Treg suppression in the presence of IL-21 (Fig. 3.22). These data
Figure 3.21. IL-21 suppresses Foxp3 expression by Treg. 2.5x10⁴ IL-21R⁻/⁻ Thy1.2+ Tconv were cultured with 5x10⁴ BALB/c B cells, with 0.8µg/ml anti-CD3 and BALB/c Thy1.2+ Treg at a ratio of 1:1, alone or in the presence of 200ng/ml IL-21. After 3 days cells were stained for surface Thy1.1, Thy1.2 and CD19, and intracellular Foxp3. (A) Histogram shows representative Foxp3 expression for Thy1.1+ Treg cultured alone or in the presence of IL-21. Foxp3 expression amongst Thy1.2+ Tconv is presented for comparison. Foxp3 MFI is shown for Thy1.1+ Treg. (B) Graph shows collated Foxp3 MFI data for gated Thy1.1+ Treg cultured alone or in the presence of IL-21. Bars represent mean values across 7 experiments. *** p = <0.001.
Figure 3.22. IL-21 does not directly inhibit Treg suppressive function. 2.5x10^4 IL-21R/- Tconv were cultured with 5x10^4 IL-21R/- B cells, with 0.8µg/ml anti-CD3 and the indicated ratios of BALB/c Treg, alone or in the presence of 200ng/ml IL-21. After 3 days at 37°C cells were stained for surface CD4 and CD19, and intracellular Foxp3. Graph shows cell counts for CD4+Foxp3- Tconv. Counts are presented as a percentage, relative to the cell count where no Treg are present. Bars represent means and SEM is shown for 3 experiments. *, p = <0.05, **, p = <0.01, ns = not significant.
provided strong evidence that there was no role for IL-21 signalling to the Treg population in mediating release from Treg suppression.

That release from suppression required IL-21Rα expression by conventional T cells, but was also dependent on the APC population used to co-stimulate, appeared somewhat paradoxical. Initially, we considered that DCs might be inhibiting IL-21Rα expression by conventional T cells, thus rendering them incapable of responding to IL-21. As others have shown that IL-21Rα expression by CD4+ T cells is upregulated upon activation (Jin et al. 2004), it was possible that B cells permitted this outcome, whilst DCs did not. We therefore activated conventional T cells over three days, with either B cells or bone marrow-derived DCs, assaying conventional T cell IL-21Rα expression pre-culture and every day thereafter. We found that activation by either APC population induced upregulated IL-21Rα expression by conventional T cells, with a slightly greater increase observed in B cell-driven cultures (Fig. 3.23). In both assays, IL-21Rα expression was maintained at a higher level than that of naïve conventional T cells throughout. These experiments indicated that IL-21Rα expression was upregulated in both B cell and DC driven cultures. It seemed unlikely that the very slight increase seen in B cell assays could account for the difference in sensitivity to IL-21 observed in suppression assays.

In light of the above result, we next considered that DCs might be capable of supporting the counteraction of suppression by IL-21, but that direct IL-21 signalling to DCs might prevent this, by downregulating a receptor or ligand for example. As stated previously, whilst surface expression of the IL-21Rα chain was almost undetectable on DCs, they did express its mRNA. Further, others have shown responses to IL-21 by DCs (Brandt et al. 2003a) and have
Figure 3.23. Tconv IL-21R expression is maintained throughout 3 day cultures with B cells or bone marrow-derived DCs. 2.5x10⁴ Tconv were cultured with either 5x10⁴ B cells or 2.5x10³ iDCs, and 0.8μg/ml anti-CD3. At days 0, 1, 2 and 3 cells were stained for CD4, CD19, CD11c and IL-21Rα. Representative dot plots show IL-21Rα expression for gated CD4+ when cultured with B cells (left panel) or iDCs (right panel). Staining with an isotype control Ab is also shown. Data are representative of 2 experiments.
even demonstrated its uptake into these cells within 30 minutes of co-culture (Brandt et al. 2003b). To address the effects of IL-21 signalling directly to DCs, we generated DCs from both wildtype and IL-21R-deficient mouse bone marrow, and used them to drive suppression assays in the presence or absence of IL-21. As demonstrated in previous assays, IL-21 was unable to counteract Treg suppression where wildtype DCs were used as co-stimulators. The same was also true when IL-21R-/- DCs were used to drive conventional T cells, with Treg still able to suppress both their proliferation (Fig. 3.24 A) and CD69 expression (Fig. 3.24 B). If IL-21 signalling to DCs prevented release from suppression, neutralising this effect by using IL-21R-/- DCs should have enabled IL-21 to counteract suppression, at least to some extent. That this did not occur suggested that there was no role for IL-21 signalling to DCs in these assays.

It has previously been shown that activated CD4+ T cells are a major source of IL-21 (Parrish-Novak et al. 2000). Over the past decade, Th1 (Chtanova et al. 2004), Th2 (Wurster et al. 2002), Th17 (Korn et al. 2007; Wei et al. 2007; Nurieva et al. 2007) and Tfh (Rasheed et al. 2006; Vinuesa et al. 2005a; Chtanova et al. 2004) cells have all variously been identified as producers of IL-21 at either the mRNA or protein level. Critically, IL-6 is known to be important for the induction of IL-21 (Zhou et al. 2007; Suto et al. 2008), and DCs have been identified as a source of IL-6 (Kopf et al. 1998). We therefore considered whether DCs might be inducing IL-21 production by conventional T cells in our assays. In this scenario, endogenous IL-21 provision may have saturated conventional T cell expressed IL-21R, but its counter-suppressive effects might have been negated during the optimisation of the assay. If this were the case, utilising IL-21R-deficient conventional T cells in our DC-driven suppression assay might thus yield enhanced suppression. To test this, we conducted DC-
Figure 3.24. Comparison of WT and IL-21R/- iDCs in *in vitro* suppression assays. 2.5x10^4 Tconv were cultured with 2.5x10^3 BALB/c or IL-21R/- iDCs, with anti-CD3 at 0.8µg/ml and the indicated ratios of Treg, alone or in the presence of 200ng/ml IL-21. After 3 days cells were stained for surface CD4, CD69 and CD11c, and intracellular Foxp3. (A) Graph shows cell counts for CD4+Foxp3- Tconv when cultured with WT or IL-21R/- iDCs as indicated. Counts are presented as a percentage, relative to the cell count where no Treg are present. (B) Representative dot plots show CD69 expression for CD4+Foxp3- Tconv when cultured with WT iDCs (upper panels), or IL-21R/- iDCs (lower panels). Data are representative of 2 experiments.
based suppression assays wherein conventional T cells derived from either wildtype or IL-21R-/- mice. Results from this experiment revealed that IL-21R-/- conventional T cells were no better suppressed than wildtype conventional T cells (Fig. 3.25).

In collectively assessing these data, conventional T cells were clearly the major target for IL-21 during release from suppression. Paradoxically, the APC population was critical in determining whether release from suppression was supported, with DCs being unsupportive of the effects of IL-21. However, this did not appear to be mediated by DCs modulating IL-21Rα expression by conventional T cells, nor by direct IL-21 signalling to DCs. Further, DC-driven assays did not appear to be saturated with endogenously produced IL-21, which may have negated the effects of its exogenous provision. Thus, there appeared to be fundamental differences in whether IL-21 could counteract Treg suppression, determined by whether T cell activation was provided by B cells or DCs.

### 3.2.6. IL-21 inhibits the production of IL-2 by conventional T cells

In order to assess the mechanism by which IL-21 counteracted suppression, we analysed its capacity to regulate a panel of conventional T cell expressed surface antigens, in B cell-driven responses. In particular, we examined CD28 as IL-21 is known to positively regulate its expression on CD8+ T cells (Alves et al. 2005) and CD28 signalling can confer resistance to Treg suppression (Itoh et al. 1999). ICOS was also of interest as IL-21 has been shown to induce its expression in CD4+ T cells (Vogelzang et al. 2008). We cultured conventional T cells in combination with B cells alone, with anti-CD3, IL-21 or both for 15 hours before analysis by flow cytometry. As shown in Fig. 3.26, IL-21 induced very little change in any of
Figure 3.25. Comparison of WT and IL-21R-/- Tconv in iDC based in vitro suppression assays. 2.5x10^4 BALB/c or IL-21R-/- Tconv were cultured with 2.5x10^3 iDCs, with anti-CD3 at 0.8µg/ml and the indicated ratios of Treg, alone or in the presence of 200ng/ml IL-21. After 3 days cells were stained for surface CD4 and CD11c, and intracellular Foxp3 for analysis by flow cytometry. Graph shows cell counts for WT or IL-21R-/- CD4+Foxp3- Tconv as indicated. Counts are presented as a percentage, relative to the cell count where no Treg are present.
Figure 3.26. Tconv phenotyping after 15hr culture with IL-21. 2.5x10^4 Tconv were cultured with 5x10^4 B cells, with or without 0.8µg/ml anti-CD3 and with or without 200ng/ml IL-21. After a 15hr incubation cells were stained for surface CD4, CD28, 41BB, ICOS, PD-1, GITR, CD25, OX40, CD69, CD62L and CD103, and intracellular Foxp3. Histograms show staining for gated CD4+Foxp3- Tconv when cultured alone or in the presence of IL-21. Staining with an isotype control Ab is also shown.
the antigens we assayed. In anti-CD3 stimulated conditions, IL-21 very slightly downregulated 41BB, marginally upregulated OX40, CD25 and ICOS, and appeared to maintain higher levels of CD62L expression. Interestingly, no modulation of CD28 was observed in the presence of IL-21, in either the presence or absence of anti-CD3. Ultimately, it did not appear likely that any of the very minor changes in expression seen in this experiment could be responsible for the profound effects of IL-21 on Treg suppression.

We next considered that IL-21 signalling to conventional T cells might be altering the cytokine milieu produced within our assay, shifting the balance away from suppression and toward activation. We therefore examined the expression levels of a panel of cytokines, in the presence or absence of IL-21. IL-2 was initially identified as a growth factor for CD4+ T cells (Smith 1988) and its role as a critical homeostatic factor for regulatory T cells has recently emerged (reviewed in (Dooms & Abbas 2010)). Similar roles have also been identified for both the Th1 specific cytokine IFNγ (Feng et al. 2008; Afanasyeva et al. 2005) and TNFα (Grinberg-Bleyer et al. 2010b). Further, IL-10 is known to be a potent immunoregulatory cytokine with the capability of directly suppressing T cell responses (de Waal Malefyt et al. 1993), and its expression can be promoted by IL-21 (Spolski et al. 2009; Pot et al. 2009). IL-4, as a signature Th2 cytokine (Mosmann et al. 2005), was also assessed, as was the expression of IL-21 itself, as the production of IL-21 is known to be positively regulated in an autocrine manner (Suto et al. 2008). We found that, when cultured with B cells and anti-CD3, conventional T cells produced high levels of IL-2, IFNγ and TNFα (Fig. 3.27), but negligible levels of IL-4, IL-10 and IL-21 (Fig. 3.28). In agreement with a number of publications, IL-21 dramatically inhibited the production of IFNγ (Frohlich et al. 2006; Suto et al. 2006; Wurster et al. 2002), with strong inhibition of IL-2 and TNFα also observed (Fig. 3.27). Conversely,
Figure 3.27. IL-21 inhibits IL-2, IFNγ & TNFα production by Tconv. 2.5x10^4 Tconv were cultured with 5x10^4 B cells and 0.8µg/ml anti-CD3, alone or in the presence of 200ng/ml IL-21. After 3 days cells were restimulated and stained for surface CD4 and intracellular Foxp3, IL-2, IFNγ and TNFα. Representative contour plots show expression profiles and graphs show collated data for IL-2 (A) and (B), IFNγ (C) and (D), and TNFα (E) and (F) within gated CD4+Foxp3- Tconv. Bars represent means across 6 experiments. **, p = <0.01, ***, p = <0.001.
Figure 3.28. IL-21 does not alter the expression of IL-4, IL-10 and IL-21 by Tconv. 2.5x10^4 Tconv were cultured with 5x10^4 B cells and 0.8μg/ml anti-CD3, alone or in the presence of 200ng/ml IL-21. After 3 days cells were restimulated and stained for surface CD4 and intracellular Foxp3, IL-4, IL-10 and IL-21. Representative contour plots show expression profiles and graphs show collated data for IL-4 (A) and (B), IL-10 (C) and (D), and IL-21 (E) and (F) within gated CD4+Foxp3- Tconv. Bars represent means across 5-6 experiments as indicated. *ns* = not significant.
IL-21 did not alter the expression of either IL-4 or IL-21, nor did it induce IL-10 (Fig. 3.28). Thus, we considered the possibility that the effects of IL-21 on suppression could be mediated by its ability to inhibit the production of IFNγ, TNFα or IL-2.

We focused initially on IFNγ and TNFα, and examined whether altering their availability in our assays would influence Treg suppression. To this end, we supplemented our assays with these cytokines, or added blocking anti-IFNγ or anti-TNFα, and assessed conventional T cell counts after three days. We found that suppression was not enhanced upon provision of IFNγ, nor was it inhibited in conditions where IFNγ was limited (Fig. 3.29 A). A similar situation was seen when TNFα availability was manipulated; Treg suppression proceeded unaltered (Fig. 3.29 B). These data implied that the effects of IL-21 could not be mediated by inhibiting either IFNγ or TNFα production, as these cytokines had no significant effect on Treg suppression.

In addition to IFNγ and TNFα, our data had shown that IL-21 could also inhibit IL-2 production by conventional T cells (Fig. 3.27). Therefore, we next considered whether this property might allow IL-21 to alter Treg suppression. As mentioned previously, both conventional and regulatory T cells require IL-2. For the former, IL-2 is an important growth factor (Smith 1988), whereas it is a critical survival factor for Treg (D'Cruz & Klein 2005). With this in mind, we attempted to dissect the effects of IL-21 mediated IL-2 deprivation in our assay. In particular, we were interested as to whether IL-21 could substitute for IL-2. We found that, in B cell-driven responses, blockade of IL-2 abrogated conventional T cell proliferation. However, provision of IL-21 in this setting almost completely restored their proliferation, demonstrating the redundancy of IL-2 in this regard (Fig. 3.30). This
Figure 3.29. Altered availability of IFNγ or TNFα does not modulate Treg suppression. 2.5x10⁴ Tconv were cultured with 5x10⁴ B cells, with 0.8µg/ml anti-CD3 and the indicated ratios of Treg, alone or in the presence of 100ng/ml IFNγ, 10µg/ml anti-IFNγ, 20ng/ml TNFα or 5µg/ml anti-TNFα. After 3 days cells were stained for surface CD4 and CD19, and intracellular Foxp3 for analysis by flow cytometry. Graphs show cell counts for CD4+Foxp3-Tconv when cultured with IFNγ or anti-IFNγ (A), or TNFα or anti-TNFα (B). Counts are presented as a percentage, relative to the cell count where no Treg are present. Bars represent means and SEM is shown for 3 experiments. In some experiments isotype-matched control Abs were used to control for the blocking Abs indicated. ns = not significant.
Figure 3.30. IL-21 can substitute for IL-2 in supporting Tconv proliferation. 2.5x10^4 Tconv were cultured with 5x10^4 B cells and 0.8μg/ml anti-CD3, alone or in the presence of either 5μg/ml anti-IL-2, 200ng/ml IL-21 or both. After 3 days cells were stained for surface CD4 and CD19, and intracellular Foxp3. Graph shows cell counts for CD4+Foxp3- Tconv. Counts are presented as a percentage, relative to the cell count where no cytokine/blockade are present. Bars represent means and SEM is shown for 3 experiments. **, p = <0.01, ***, p = <0.001.
experiment demonstrated that, unlike for IFNγ or TNFα, we could not simply alter the availability of IL-2 and assess suppression, because interpretation of such an approach would be confounded by the influence of IL-2 on conventional T cell proliferation.

To further explore IL-21-mediated IL-2 downregulation, we assessed whether the secretion of IL-2 by conventional T cells was also inhibited in the presence of IL-21. This was important, as it determined whether Treg in our assays might actually be encountering reduced availability of IL-2 in the extracellular cytokine milieu. Culturing conventional T cells with B cells and anti-CD3, we found that provision of IL-21 decreased the level of secreted IL-2 to a similar extent as it decreased the level of intracellular protein \( \text{Fig. 3.31.} \). Moreover, it was important to determine whether inhibition of IL-2 production was mediated by IL-21 signalling to conventional T cells, as these were the primary target for IL-21 during release from suppression. We therefore determined IL-2 expression by IL-21Rα-deficient conventional T cells, when cultured with B cells and anti-CD3. In this scenario, we observed a complete restoration of IL-2 production in the presence of IL-21, identifying conventional T cells as the obligate target of IL-21 in both counteracting suppression and inhibiting IL-2 expression \( \text{Fig. 3.32.} \). These data would not necessarily have had any particular implications for suppression, as long as Treg were capable of producing IL-2 of their own. However, it has been demonstrated by a number of groups that this is not the case (Takahashi et al. 1998; Ono et al. 2007; Hori et al. 2003; Wolf et al. 2001; Papiernik et al. 1998). When cultured with conventional T cells, B cells and anti-CD3, we also found negligible production of IL-2 by Treg \( \text{Fig. 3.33.} \), confirming these previous findings, and that conventional T cells were the major IL-2 producing population in our assays. This suggested that the inhibition of their production of IL-2, by IL-21, would therefore represent a universal decrease in the amount of
Figure 3.31. IL-21 inhibits IL-2 secretion by Tconv. 2.5x10^4 Tconv were cultured with 5x10^4 B cells and 0.8µg/ml anti-CD3, alone or in the presence of 200ng/ml IL-21. After 3 days cells were restimulated and stained for CD4 and secreted IL-2. Contour plots show IL-2 staining for gated CD4+ Tconv. Data are representative of 2 experiments.
Figure 3.32. **IL-21 acts directly on Tconv to inhibit IL-2 production.** 2.5x10^4 IL-21R/- Tconv were cultured with 5x10^4 BALB/c B cells and 0.8µg/ml anti-CD3, alone or in the presence of 200ng/ml IL-21. After 3 days cells were restimulated and stained for surface CD4, and intracellular Foxp3 and IL-2. Representative contour plots show IL-2 staining for gated CD4+Foxp3- Tconv (A), and graph shows collated IL-2 expression data for these cells (B). Bars represent means and SEM is shown for 3 experiments. *ns* = not significant.
Figure 3.33. Comparison of IL-2 expression between Treg and Tconv. 2.5x10^4 Tconv were cultured with 5x10^5 B cells, 0.8µg/ml anti-CD3, in the presence of 1.25x10^4 Treg. After 3 days cells were restimulated and stained for surface CD4 and intracellular Foxp3 and IL-2 for analysis by flow cytometry. Representative contour plots show IL-2 staining for gated CD4+Foxp3- Tconv or CD4+Foxp3+ Treg (A), and graph shows collated IL-2 expression data (B). Bars represent means and SEM is shown for 5 experiments. ***, p = <0.001
available IL-2.

At this point, it was important to consider that Treg have been shown to suppress IL-2 mRNA (Sojka et al. 2005; Thornton & Shevach 1998a; Takahashi et al. 1998) and protein (Sojka et al. 2005; Takahashi et al. 1998) production by conventional T cells. In B cell-driven suppression assays, we found that at high doses of Treg, IL-2 production by conventional T cells was suppressed, in accordance with the above studies. However, at a regulatory to conventional T cell ratio of 0.5:1, Treg potently suppressed conventional T cell proliferation and IFNγ production (Fig. 3.34.), a known function of Treg (Sojka & Fowell 2011), but IL-2 expression was not inhibited. This led us to postulate that Treg were selectively maintaining IL-2 at high levels when suppression was incomplete. Further, this provided an ideal setting within which to investigate the effects of IL-21 on IL-2 production, in the context of Treg suppression. Using this setup, we found that IL-21 was still capable of suppressing IL-2 expression by conventional T cells (Fig. 3.35.).

Overall, IL-21 was found to inhibit the production of IL-2, IFNγ and TNFα by conventional T cells, but we did not find any role for IFNγ or TNFα in mediating Treg suppression. Due to the fundamental role played by IL-2 in Treg homeostasis, its modulation by IL-21 warranted further investigation. We found that IL-21 also inhibited IL-2 secretion, and could substitute for IL-2 in promoting T cell proliferation. Further, inhibition of IL-2 production was mediated by IL-21 signalling directly to conventional T cells. As Treg did not produce IL-2, they were dependent on its expression by conventional T cells. We showed that Treg preferentially permitted IL-2 production over that of IFNγ, and that in their presence, IL-21 maintained its ability to suppress IL-2, likely leading to a universal decrease in IL-2 availability.
Figure 3.34. Treg permit IL-2 expression by Tconv whilst suppressing their proliferation and production of IFNγ. 2.5x10^4 Tconv were cultured with 5x10^4 B cells and 0.8µg/ml anti-CD3, alone or in the presence of 1.25x10^4 Treg. After 3 days cells were restimulated and stained for surface CD4 and intracellular IL-2, IFNγ and Foxp3. Representative contour plots show expression profiles and graphs show collated data for IL-2 (A) and (B), and IFNγ (C) and (D), expression by CD4+Foxp3- Tconv. Bar graph shows cell counts for CD4+Foxp3- Tconv (E). Counts are presented as a percentage, relative to the cell count where no Treg are present. Bars represent means and SEM is shown for 5 experiments. **, p = <0.01, ***, p = <0.001, ns = not significant.
Figure 3.35. IL-21 suppresses IL-2 production by Tconv in the presence of Treg. 2.5x10^4 Tconv were cultured with 5x10^4 B cells, 0.8µg/ml anti-CD3 and 1.25x10^4 Treg, alone or in the presence of 200ng/ml IL-21. After 3 days cells were restimulated and stained for surface CD4, and intracellular Foxp3 and IL-2. Representative contour plots show IL-2 staining for gated CD4+Foxp3- Tconv (A), and graph shows collated IL-2 expression data (B). Bars represent means and SEM is shown for 6 experiments. **, p = <0.01.
3.2.7. IL-21 indirectly affects Treg homeostasis

We previously demonstrated that IL-21 could substitute for IL-2 in the conventional T cell population. As IL-2 is critical for Treg survival, we next wished to assess whether IL-21 could also substitute for IL-2 in this respect. We therefore conducted a Treg survival assay wherein Treg were cultured alone, or in the presence of either IL-2 or IL-21. In this experiment, we found that provision of IL-2 lead to a striking increase in Treg recovery, but that IL-21 was ineffective, leading to a similar cell recovery as that seen in the absence of cytokine (Fig. 3.36 A). Moreover, only where IL-2 was provided did the vast majority of recovered cells maintain their expression of Foxp3 and CD25 (Fig. 3.36 B). Thus, IL-21 was able to substitute for IL-2 in conventional T cell proliferation, but not in regulatory T cell survival. This potentially had major implications for our suppression assay, as it suggested that when IL-2 deprivation was induced by IL-21, this would selectively affect the Treg population. To address this possibility, we determined the absolute number of Treg in our suppression assays when cultured with or without IL-21. We found a significant decrease in the number of Treg recovered at day three when IL-21 was present, suggesting that IL-21 mediated IL-2 deprivation was negatively impacting on Treg homeostasis (Fig. 3.37 A). In addition, total IL-2 blockade lead to an even greater decrease in Treg recovery, providing further support for this concept (Fig. 3.37 A). Finally, repeating this experiment using IL-21Ra-deficient conventional T cells showed that Treg counts were no longer decreased in the presence of IL-21. This demonstrated that its effects on regulatory T cells were mediated indirectly via signalling to conventional T cells, the obligate targets for IL-21 during release from Treg suppression.

The above data lead us to conclude that IL-21 was inhibiting IL-2 production by conventional
Figure 3.36. IL-21 cannot substitute for IL-2 in maintaining Treg homeostasis. $2.5 \times 10^4$ Treg were cultured alone or in the presence of either 20ng/ml IL-2 or 200ng/ml IL-21. After 3 days cells were stained for surface CD4 and CD25, and intracellular Foxp3. Representative contour plots show Foxp3 and CD25 co-expression for gated CD4+ cells (A). Graph shows total cell recovery, presented as fold change in cell count relative to that where no cytokine is present (B). Bars represent means and SEM is shown for 3 experiments. **, $p = <0.01$. 
Figure 3.37. IL-21 indirectly affects Treg homeostasis. 2.5x10^4 BALB/c or IL-21R-/Tconv were cultured with 5x10^4 BALB/c B cells, with 0.8µg/ml anti-CD3 and 1.25x10^4 BALB/c Treg alone, or in the presence of either 200ng/ml IL-21 or 5µg/ml anti-IL-2. After 3 days cells were stained for surface CD19 and CD4, and intracellular Foxp3. Graph shows cell counts for CD4+Foxp3+ Treg when cultured with wildtype Tconv (A), or IL-21R-/ Tconv (B). Counts are presented as a percentage, relative to the cell count where no cytokine/blockade are present. Bars represent means and SEM is shown for 4 experiments. **, p = <0.01, ***, p = <0.001.
T cells, depriving Treg of a non-redundant survival factor, thereby relieving suppression. Recalling our earlier data, suppression assays driven by DCs were resistant to the effects of IL-21, with suppression proceeding uninhibited. Based on these results, we considered the possibility that the downregulation of IL-2 by IL-21 might not be supported in DC-driven responses. To test this, we assessed IL-2 expression by conventional T cells cultured with DCs and anti-CD3, in the presence or absence of IL-21. Fig. 3.38 clearly demonstrates that IL-21 was completely unable to suppress IL-2 production by conventional T cells in this setup. A differential ability to inhibit IL-2 production could therefore offer a potential explanation for the differences in sensitivity of Treg suppression to IL-21, between DC and B cell-driven assays.
Figure 3.38. IL-21 does not inhibit IL-2 production by Tconv in bone marrow-derived DC-driven responses. 2.5x10⁴ Tconv were cultured with 2.5x10³ iDCs and 0.8μg/ml anti-CD3, alone or in the presence of 200ng/ml IL-21. After 3 days cells were restimulated and stained for surface CD4 and CD11c, and intracellular Foxp3 and IL-2. Representative contour plots show IL-2 staining for gated CD4+Foxp3- Tconv (A), and graph shows collated IL-2 expression data (B). Bars represent means across 7 experiments. *ns* = not significant.
3.3. Discussion

Regulatory T cells are known to utilise an ever-expanding array of mechanisms to suppress conventional T cell responses. In contrast, the number of factors required to maintain their homeostasis and function appears more limited. Perhaps principal amongst these is IL-2, as demonstrated by the fatal autoimmune condition observed in mice with disrupted IL-2 signalling (Setoguchi et al. 2005; Willerford et al. 1995; Sadlack et al. 1995; Suzuki et al. 1995).

Foxp3 expression prevents the transcription of a number of key cytokine genes. Consequently, Treg do not produce IL-2 and are reliant on its secretion by conventional T cells (Bettelli et al. 2005), for whom IL-2 is an important growth factor. Treg therefore tightly control the production and availability of IL-2 to maintain dominant tolerance. They are known to limit IL-2 mRNA expression by conventional T cells, effectively controlling the amount of IL-2 secreted into the local milieu (Thornton & Shevach 1998a). In addition, their constitutive expression of the IL-2Rα chain ensures that they respond to IL-2 more rapidly than conventional T cells during an immune response (O’Gorman et al. 2009), and that they are well placed to compete for locally available IL-2. This not only ensures Treg survival, but is also thought to promote suppression, as conventional T cells become deprived of IL-2 signals (Pandiyan et al. 2007). Deprivation in this manner becomes self-reinforcing, as conventional T cells are then unable to upregulate IL-2Rα, and are thus incapable of competing for IL-2 (Busse et al. 2010). This form of regulation is critically important, as it is suggested that only a very small number of strongly activated conventional T cells express sufficient levels of IL-2Rα to compete effectively with Treg (Feinerman et al. 2010). As T cells are known to form synapses into which IL-2 is secreted (Sabatos et al. 2008), and Treg
require close proximity to conventional T cells to mediate IL-2 deprivation (Busse et al. 2010), it is logical to propose that Treg also form such synapses with conventional T cells to ensure the efficient consumption of IL-2 and thus the dominance of immune suppression.

In this chapter we have shown that IL-21 counteracts Treg-mediated suppression by signalling to conventional T cells, and that one of its major effects on this population is the inhibition of IL-2 production and secretion. Whilst it has been shown that IL-21 can suppress Foxp3 expression (Li & Yee 2008), this effect is minor when compared with other cytokines and was not responsible for the counteraction of suppression, as in assays where Treg alone expressed IL-21Rα, suppression was intact. Thus, the effects of IL-21 on Treg were not responsible for the counteraction of suppression.

In considering the possible effects of the downregulation of IL-2 by IL-21, it is plausible to suggest that both conventional and regulatory T cell populations would be impaired, as both require this cytokine for their growth and survival respectively. However, we show that IL-21 can almost completely substitute for IL-2 as a conventional T cell growth factor, thus achieving IL-2 independent proliferation. That there is redundancy in the cytokine requirement for T cell proliferation is unsurprising; the lymphoproliferative disease observed in mice with disrupted IL-2 signalling demonstrates as much. On the other hand, IL-21 was unable to substitute for IL-2 in maintaining Treg survival (D'Cruz & Klein 2005). This is in agreement with other reports demonstrating that IL-21 cannot substitute for IL-2 in promoting Treg proliferation (Comes et al. 2006; Li & Yee 2008). This selective support for the conventional T cell population is further compounded by the fact that these cells express high levels of IL-2Rα in the presence of IL-21. Thus, in the context of already decreased IL-2
availability, conventional T cells are fully equipped to compete with Treg for the IL-2 that remains. We suggest that these conditions heavily favour conventional T cell proliferation and result in impaired Treg homeostasis, as shown by their decreased cell counts where IL-21 is present.

To test whether alterations in IFNγ or TNFα availability were responsible for mediating the effects of IL-21, we were able to utilise blocking antibodies against these cytokines. However, such an approach was unsuitable for revealing the role of IL-2, since IL-2 blockade inhibited conventional T cell proliferation. Blockade of IL-2 in a standard suppression assay therefore confounds the assessment of suppression. In addition, provision of exogenous IL-2 only serves to interrupt its exquisite regulation by Treg, as outlined above, thereby preventing suppression (Thornton & Shevach 1998a). Thus, without the ability to specifically target IL-2 to Treg, it is difficult to increase IL-2 levels without affecting conventional T cells. However, in a chimeric suppression assay system using human Treg and murine conventional T cells, de la Rosa and colleagues were able to selectively block IL-2 signalling to Treg, and found that this prevented suppression (La Rosa et al. 2004). This setup is in many ways analogous to the situation we generate by provision of IL-21, in that conventional T cells are provided the growth factors they require, whilst Treg are specifically denied theirs.

Further mechanistic insight into the counteraction of suppression by IL-21 could be gained by determining the signalling pathways that mediate the inhibition of IL-2 production. IL-21 is known to signal primarily via Stat3, although it can also activate Stat1 and to a lesser extent Stat5 (Zeng et al. 2007). Interestingly, it has recently been shown that Stat3 activation counteracts the Treg-mediated suppression of human T cells, and that this is the signalling
pathway that mediates the counter-suppressive effects of IL-6 (Goodman et al. 2011). In addition, IL-6 is known to overcome suppression in murine studies (Pasare & Medzhitov 2003) and strongly induces the expression of IL-21 by murine CD4+ T cells (Suto et al. 2008). It would thus be interesting to investigate whether IL-6 mediates its effects indirectly by inducing IL-21, as it does in the induction of antibody production by B cells (Dienz et al. 2009). On the other hand, IL-27 is another example of a cytokine with the ability to inhibit IL-2 production, and this effect is mediated by Stat1 phosphorylation (Owaki et al. 2006). The inhibition of IL-2 production by IL-21 could therefore be mediated by signalling through either Stat1 or Stat3.

A major result demonstrated in this chapter was that only B cells supported the counter-suppressive effects of IL-21, whilst DCs did not. Intriguingly, DCs were also unsupportive of the inhibitory effect of IL-21 on IL-2 production, providing further support for the role of IL-2 inhibition in overcoming suppression. Initially, we considered whether IL-21 might be inhibiting DCs and that this might be responsible for their lack of support for the effects of IL-21 on conventional T cells. Others have reported negative regulation of DC maturation and expression of co-stimulatory ligands by IL-21 (Brandt et al. 2003a; Ansen et al. 2008). However, using IL-21Rα-deficient DCs in our suppression assays did not then permit release from suppression by IL-21, suggesting that inhibition of DCs by IL-21 was not masking the counteraction of suppression. We also considered that DCs might be downregulating IL-21Rα expression by conventional T cells. This would have accounted for the inability of IL-21 to suppress IL-2 expression by this population. However, we found that DCs did not negatively regulate IL-21Rα expression by conventional T cells, and in fact supported its upregulation during activation.
Over the past decade tolerogenic DCs have emerged as a regulatory APC population whose primary function is to prevent inappropriate T cell-mediated immunity. Tolerogenic DCs are known to play an important role during negative selection in the thymus, and maintain peripheral tolerance by anergising or deleting autoreactive T cells and promoting Treg induction and function. We therefore considered that the DCs used in our assays might be tolerogenic, as these cells are known to permit T cell proliferation before mediating their deletion (Steinman et al. 2003). This would potentially account for the inability of IL-21 to counteract suppression in both splenic and bone marrow-derived DC stimulations, as both populations are considered immature, and would thus be considered tolerogenic in vivo. Provision of LPS to bone marrow-derived DC cultures would negate their tolerogenicity by inducing maturation, and their ability to overcome suppression even in the absence of IL-21 is supportive of this. However, in chapter 6 we characterise the cytokine expression profiles of conventional T cells cultured with either B cells or DCs and find in both cases high-level production of inflammatory cytokines, such as TNFα. Moreover, DO11.10 TCR+ conventional T cells cultured with immature bone marrow-derived DCs are highly pathogenic in our adoptive transfer protocol for type-1 diabetes induction, suggesting that these cells are not being tolerised.

A further possibility is that DCs in these assays promote enhanced Treg function, permitting them to dominantly regulate IL-2 expression by conventional T cells. It has been shown that DC-expressed CD80 enhances Treg suppression (Zheng et al. 2004), and in chapter 4 we show that DCs constitutively express CD80, whilst B cells do not. Thus enhanced IL-2 regulation by Treg cannot be ruled out in DC assays.
As mentioned earlier, Treg mediate immune suppression using numerous mechanisms, and factors that inhibit a single suppressive pathway cannot account for the compensatory utilisation of others in order to maintain regulation. In support for this, it has been suggested that CTLA-4-deficient Treg can still suppress conventional T cell responses \textit{in vitro} through their production of TGFβ (Tang et al. 2004a). By inhibiting the production of IL-2, IL-21 targets an important Treg homeostatic factor. Thus, unlike with the inhibition of individual suppressive mechanisms, there is limited scope for compensation as there is a lack of redundancy for IL-2 in promoting Treg survival. IL-21 may therefore be significantly advantaged when compared with other cytokines with the potential to counteract Treg-mediated suppression.

In the future, it will be important to demonstrate that IL-21 can downregulate IL-2 production by conventional T cells, and to correlate this with the counteraction of suppression, \textit{in vivo}. In an attempt to achieve this, we will utilise an adoptive transfer system in which DO11.10 TCR+ conventional T cells are transferred into OVA immunised BALB/c or IL-21R-/ recipients, alone or in the presence of Treg. Upon provision of IL-21 by daily injection, we can then assess suppression based on conventional T cell proliferation, and IL-2 expression by intracellular cytokine staining. It would also be advantageous to show that Treg harvested from our suppression assays show signs of IL-2 deprivation. The Rudensky lab have previously described a panel of genes that are downregulated in Treg with disrupted IL-2 signalling, which could be utilised to this end (Fontenot et al. 2005b). As mentioned previously, it would be interesting to determine the mechanism by which IL-21 inhibits IL-2 production. Initially, we could simply assess whether IL-21 suppresses IL-2 mRNA
expression by conventional T cells, before potentially utilising cells from mice deficient in particular Stat proteins, such as Stat1 or Stat3.
4. MODULATION OF CD80 AND CD86 BY INTERLEUKIN 21

4.1. Introduction

In the previous chapter we noted that IL-21 has been shown to positively regulate the expression of CD28 on T cells (Alves et al. 2005). However, very little has been published concerning the regulation of the CD28 ligands CD80 and CD86 by IL-21. One study has demonstrated that CD80 and CD86 levels were slightly increased on human monocyte-derived DCs when generated in the presence of IL-21 (Williams et al. 2010). Conversely, CD80 was unaltered and CD86 slightly downregulated when mouse bone marrow-derived DCs were generated in the presence of IL-21 (Brandt et al. 2003a). On anti-CD40 stimulated B cells, IL-21 appeared to do very little to alter CD86 expression, whilst CD80 was not examined (Chen et al. 2007). Further, no kinetic or mechanistic data appear to exist concerning any of these results. As expression levels of CD80 and CD86, and how they are regulated, have important implications for the initiation of immune responses, we set out to address the role of IL-21 in modulating CD80 and CD86 expression on B cell subsets and DCs.
4.2. Results

4.2.1. IL-21 upregulates CD86 expression by B2 B cells

To assess the effects of IL-21 on CD80 and CD86 expression, we first sought to determine which of our APC populations expressed the IL-21Rα chain. In chapter 3, we demonstrated that whilst DCs appeared to express IL-21Rα mRNA, protein levels were below the level of detection (Fig. 3.13 & 3.14). Nonetheless, DCs have been reported to respond to IL-21 (Brandt et al. 2003a; Brandt et al. 2003b), suggesting some level of receptor expression at the cell surface. Whole splenic B cells were found to strongly express IL-21Rα (Fig. 3.13), however we had not at that point determined receptor expression by different B cell subsets. To this end we compared levels of IL-21Rα on splenic B cells with those on B cells residing in the peritoneal cavity. Splenic B cells are predominantly of the B2 subset, whilst B1 cells are the major B cell population found in the peritoneal cavity (Arnold et al. 1994). We therefore divided B cells harvested from these sites based on CD5 expression, with CD5- cells considered B2 B cells. It is important to note that peritoneal CD5- B cells may not represent a true B2 B cell population, and have been suggested to display an intermediate phenotype between the two subsets (Hastings et al. 2006). We found consistently high levels of IL-21Rα expression on both splenic and peritoneal lavage B2 B cells. Surprisingly, expression levels on B1 B cells were far lower, with the majority staining negatively (Fig. 4.01). Thus, B cell subsets differentially expressed IL-21Rα.

The above data suggested that B2 B cells expressed the highest levels of IL-21Rα, implying that they might be particularly suitable targets for the modulation of CD80 and CD86 by IL-21. Before addressing this possibility however, we determined resting levels of CD80 and
Figure 4.01. Expression of IL-21Rα by B1 and B2 B cells. Single cell suspensions from BALB/c spleen and peritoneal lavage fluid were stained for the expression of CD19, CD5 and IL-21Rα. Representative histograms show IL-21Rα and isotype control staining for CD19+CD5+ B1 and CD19+CD5- B2 B cells. Data are representative of 3 experiments.
CD86 on splenic DCs and B2 B cells, and peritoneal lavage B1 B cells. The generally accepted dogma is that resting APCs express low levels of both co-stimulatory ligands (Adam et al. 1998). However, resting DCs have been shown to express relatively high levels of both CD80 (Larsen et al. 1992) and CD86 (Azuma et al. 1993), as have B1 B cells (Ray et al. 2004). Our own analysis was broadly supportive of these findings. Almost all DCs expressed CD80, with a sizeable proportion expressing CD86 (Fig. 4.02). B2 B cells expressed very low levels of either ligand, whilst B1 B cells constitutively expressed both. To assess the effect of IL-21 on CD80 and CD86 expression, bulk splenocytes or peritoneal lavage cells were cultured in the presence or absence of IL-21 for 15 hours. We found that provision of IL-21 upregulated CD86 expression by B2 B cells, but had no significant effect on B1 B cells or DCs (Fig. 4.03). Conversely, IL-21 did not upregulate CD80 expression by B2 B cells, with levels on B1 B cells and DCs similarly unaffected (Fig. 4.04).

Overall, this analysis revealed that IL-21 was able to significantly upregulate CD86 expression by B2 B cells. Lack of responsiveness of B1 B cells to IL-21 might reflect their lower expression of IL-21Rα, or the fact that their expression levels of CD80 and CD86 was already high. Similarly, CD86 levels on DCs were not significantly modified by IL-21, and this may also be due to low level IL-21Rα expression. That no modulation of CD80 expression was observed in any of the populations assayed suggested that CD80 was not a target for IL-21, at least under these experimental conditions. We therefore studied the effects of IL-21 on CD86 expression by B2 B cells in greater detail.
Figure 4.02. Expression of CD80 and CD86 by DCs, B1 B cells & B2 B cells. Single cell suspensions from BALB/c spleen and peritoneal lavage fluid were stained for CD3, CD19, CD5, CD11c, MHC class II, CD80 and CD86. Representative contour plots show CD80, CD86 and isotype control staining for splenic CD3-CD19-CD11c+MHC class II+ DCs (A), splenic CD19+CD5- B2 B cells (B), and peritoneal lavage fluid CD19+CD5+ B1 B cells. Data are representative of 3 experiments.
Figure. 4.03. **Effect of IL-21 on CD86 expression by DCs and B cell subsets.** 2x10^5 BALB/c splenocytes or peritoneal lavage cells were cultured for 15hrs, alone or in the presence of 200ng/ml IL-21. Cells were then stained for surface CD19, CD5, CD11c, MHC class II, CD3 and CD86. Representative contour plots show CD86 staining and MFI, and graphs show collated data for gated splenic CD19+CD5- B2 B cells (A) and (B), peritoneal lavage CD19+CD5+ B1 B cells (C) and (D), and splenic CD11c+MHC class II+ DCs (E) and (F). Bars represent means and SEM is shown for 3 experiments. **, p = <0.01, ns = not significant.
**Figure. 4.04. Effect of IL-21 on CD80 expression by DCs and B cell subsets.** 2x10^5 BALB/c splenocytes or peritoneal lavage cells were cultured for 15hrs, alone or in the presence of 200ng/ml IL-21. Cells were then stained for surface CD19, CD5, CD11c, MHC class II, CD3 and CD80. Representative contour plots show CD80 staining and MFI, and graphs show collated data for gated splenic CD19+CD5- B2 B cells (A) and (B), peritoneal lavage CD19+CD5+ B1 B cells (C) and (D), and splenic CD11c+MHC class II+ DCs (E) and (F). Bars represent means and SEM is shown for 3 experiments. ns = not significant.
4.2.2. Characterisation of the effects of IL-21 on CD86 expression

It has previously been shown that IL-21 has a variety of effects on B cells. These include regulating their proliferation (Jin et al. 2004; Parrish-Novak et al. 2000), survival and apoptosis (Jin et al. 2004; Mehta et al. 2003), and differentiation and production of antibody (Zotos et al. 2010; Linterman et al. 2010; Vogelzang et al. 2008; Ettinger et al. 2005; Bryant et al. 2007; Ozaki et al. 2002). In vitro experiments have shown that IL-21 can exert these effects on B cells at doses as low as 30ng/ml (Jin et al. 2004). Because we initially observed the upregulation of CD86 by B cells at an IL-21 dose of 200ng/ml, we performed a titration to assess whether IL-21 could maintain its effects at lower concentrations. We found a dose-dependent induction of CD86 by IL-21, with effects seen at as low as 25ng/ml. CD86 MFI increased with each increase in IL-21 concentration, up to our maximum dose of 200ng/ml (Fig. 4.05).

It is well established that B cells upregulate CD86 expression upon activation (Adam et al. 1998). Kinetic analysis demonstrated that CD86 was induced by 6 hours post-stimulation and was maximally expressed by 24 hours (Lenschow et al. 1993). Because of the potent nature of the induction of CD86 by IL-21, we considered that its effects might be mediated rapidly, and thus set about establishing a time course. We cultured bulk splenocytes over the course of 30 minutes to 16 hours, in the presence or absence of IL-21, assessing CD86 expression at regular intervals. We observed significant increases in CD86 expression by B cells within four hours of exposure to IL-21, with maximal induction by only eight hours. Further, IL-21 maintained high levels of CD86 expression up to 15 hours after cultures were initiated (Fig. 4.06).
Figure 4.05. Upregulation of B cell-expressed CD86 is IL-21 dose-dependent. 2x10^5 BALB/c splenocytes were cultured for 15hrs, alone or in the presence of the indicated concentrations of IL-21. Cells were then stained for the expression of CD19 and CD86. Histograms show CD86 staining for gated CD19+ B cells (A) and graph shows CD86 MFI for these cells (B).
Figure 4.06. Upregulation of B cell-expressed CD86 by IL-21 occurs within 4 hours. 2x10^5 BALB/c splenocytes were cultured for the indicated time periods, alone or in the presence of 200ng/ml IL-21. Cells were then stained for surface CD19 and CD86. Histograms show CD86 staining for gated CD19+ B cells (A) and graph shows collated data (B). Dots represent means and SEM is shown for 3 experiments. *, p = <0.05, **, p = <0.01, ***, p = <0.001, ns = not significant.
In continuing to assess the potency of IL-21, we compared its ability to upregulate CD86 with that of lipopolysaccharide (LPS), a TLR4 ligand with established capacity for promoting the expression of co-stimulatory ligands by B cells (Razi-Wolf et al. 1992; Lenschow et al. 1993). To this end, we cultured bulk splenocytes alone, with 200ng/ml IL-21, or with a high dose of LPS (10µg/ml) for 15 hours. We found that whilst LPS broadly induced CD86 expression in the majority of B cells, IL-21 induced significantly greater expression on a per cell basis, as determined by mean fluorescence intensity (Fig. 4.07). Collectively, these data identify a novel function for IL-21 in inducing the expression of CD86 by B2 B cells. This effect was rapid, occurring in less than four hours, and highly potent, as judged by comparison with high doses of LPS. B cells appear to be highly sensitive to this mechanism, as upregulation of CD86 by IL-21 occurred from as low as 25ng/ml of IL-21, with increasing concentrations yielding greater CD86 expression.

4.2.3. Direct upregulation of CD86 by IL-21 is dependent on phosphoinositide 3-kinase p110δ and promotes T cell proliferation

Having established a role for IL-21 in promoting CD86 expression by B cells, we next wished to address the mechanism by which this function was mediated. It is well known that CD40 ligation during cognate interactions between B and T cells can induce the expression of both CD80 and CD86 (Ranheim & Kipps 1993; Roy et al. 1995). We therefore considered that in our previous experiments, IL-21 might have been activating T cells to upregulate CD40L, thereby driving CD86 expression indirectly. We therefore co-cultured highly pure populations of B cells and conventional T cells, from wildtype or IL-21R-/- mice, to assess which cell type was the target for IL-21. In cultures of wildtype B cells and IL-21R-/- conventional T
Figure 4.07. Effect of IL-21 and LPS on B cell CD86 expression. 2x10^5 BALB/c splenocytes were cultured for 15hrs, alone or in the presence of either 10µg/ml LPS or 200ng/ml IL-21. Cells were then stained for surface CD19 and CD86. Histograms show CD86 staining for gated CD19+ B cells (A) and graph shows collated data (B). Bars represent means and SEM is shown for 4 experiments. **, p < 0.01.
cells, no defect in the ability of IL-21 to upregulate CD86 was observed, when compared to cultures in which all populations derived from wildtype mice (Fig. 4.08). On the other hand, when B cells alone lacked IL-21Rα expression, we found a complete block on the effects of IL-21 on CD86 expression (Fig. 4.08). This clearly demonstrated that the upregulation of CD86 by IL-21 required direct signalling to B cells, with little to no role for indirect effects mediated by signalling to conventional T cells.

A critical role for phosphoinositide 3-kinase (PI3K) has been demonstrated in many aspects of B cell homeostasis and function (reviewed in (Okkenhaug & Vanhaesebroeck 2003)). It is notably a downstream target for CD40 signalling (Ren et al. 1994), a receptor known to upregulate CD86 expression. The p110δ subunit regulates B cell development and proliferation (Beer-Hammer et al. 2010), and also B cell receptor signalling (Okkenhaug et al. 2002; Bilancio et al. 2006). Due to the variety of functions for this subunit in B cells, we considered the possibility that it might also play a role in regulating CD86 expression. To assess this, we utilised p110δD910A mutant mice expressing a catalytically inactive mutant form of the p110δ subunit (Okkenhaug et al. 2002). B cells from wildtype or p110δD910A donors were cultured for 15 hours, alone or in the presence of 200ng/ml IL-21. Cultures were also setup in the presence of 10ng/ml IL-4, another common γ-chain signalling cytokine whose B cell-expressed IL-4R signalling is known to be regulated by p110δ (Bilancio et al. 2006). We found that IL-4 induced expression of CD86 in the majority of wildtype B cells. Whilst frequencies of the highest expressors were reduced in p110δD910A B cells, the majority remained positive for CD86 (Fig. 4.09 A). In striking contrast, an almost complete abrogation of CD86 induction by IL-21 was observed in p110δD910A B cells (Fig. 4.09 B). Thus, IL-21 and IL-4 induced CD86 expression by B cells via different signalling pathways, with the
Figure 4.08. Upregulation of CD86 by IL-21 requires direct IL-21R signalling to B cells. 2.5x10^4 WT or IL-21R-/- Tconv were cultured for 15hrs with 5x10^5 WT or IL-21R-/- B cells, alone or in the presence of 200ng/ml IL-21. Cells were then stained for surface CD19 and CD86. Contour plots show CD86 staining for gated CD19+ B cells.
Figure 4.09. Upregulation of B cell-expressed CD86 by IL-21 is phosphoinositide 3-kinase p110δ-dependent. 2x10^5 BALB/c or p110δ^{D910A} B cells were cultured for 15 hours, alone or in the presence of either 10ng/ml IL-4 or 200ng/ml IL-21. Cells were then stained for surface CD19 and CD86. Representative histograms show CD86 staining and MFI for gated WT or p110δ^{D910A} CD19+ B cells when cultured with IL-4 (A), or IL-21 (B). Data are representative of 2 experiments.
effects of IL-21 being entirely dependent on p110δ.

As the effects of IL-21 on CD86 expression were mediated through direct signalling to B cells, we wished to assess whether this might indirectly promote T cell responses. As mentioned earlier, resting B cells express very little CD86 (Adam et al. 1998), so it was not inconceivable that its robust induction by IL-21 might positively regulate T cell proliferation. To explore this possibility, we pre-incubated B cells for 15 hours, alone or with 200ng/ml IL-21, before co-culturing either population with conventional T cells and anti-CD3. Whilst no significant differences in cell count were observed at days one and two, a significantly greater number of conventional T cells were recovered after three days in culture with IL-21 pre-incubated B cells (Fig. 4.10). These data are consistent with the idea that CD86 upregulation by IL-21 can have functional consequences for T cell proliferation, although we cannot exclude the contribution of other IL-21-mediated effects in this assay.
Figure 4.10. Pre-incubation of B cells with IL-21 promotes Tconv proliferation. B cells were cultured for 15hrs, alone or in the presence of 200ng/ml IL-21. 2.5x10^4 Tconv were then cultured with 5x10^4 of either type of pre-incubated B cell and anti-CD3 at 0.8µg/ml. At the indicated time points cells were stained for surface CD4 and CD19. Graph shows absolute cell counts for CD4+ Tconv. Dots represent means and SEM is shown for 3 experiments. *, p = <0.05.
4.3. Discussion

Co-stimulatory signals critically regulate T cell responses during antigen presentation by APCs. Sufficiently high-level expression of ligands such as CD80 and CD86 ensures robust T cell activation and proliferation. Too little provision of these ligands induces T cell anergy or deletion, and forms an important peripheral tolerance mechanism mediated by tolerogenic DCs (Mueller 2010). During a given immune response, it has been suggested that CTLA-4 may inhibit immune responses simply by competing with CD28 for CD80 and CD86 interactions. This has been demonstrated by the protective effect of introducing a transgene encoding a CTLA-4 mutant lacking its cytoplasmic domain into CTLA-4-deficient mice (Masteller et al. 2000). Regulation of co-stimulatory ligand expression levels is therefore likely to be important in dictating the balance between CD28 and CTLA-4 engagement, and thus whether T cell activation is supported.

In this chapter we describe the promotion of CD86 expression by IL-21 and demonstrate that this effect is limited to B2 B cells. We found very little effect of IL-21 on CD86 expression by DCs or B1 B cells, which likely reflects their low level expression of the IL-21Rα chain. Brandt and colleagues have previously reported that bone marrow-derived DCs generated in the presence of IL-21 expressed lower levels of CD86 (Brandt et al. 2003a). It is therefore possible that myeloid precursors express higher levels of IL-21Rα than fully differentiated DCs, or that the effects of IL-21 in these cultures were mediated indirectly through other precursor populations.

Expression of CD86 by resting B cells is negligible but is rapidly induced in response to activation signals such as those mediated by CD40L (Adam et al. 1998). Early experiments
investigating the kinetics of CD86 upregulation determined that activated B cells begin to upregulate CD86 within 6 hours, and that it is maximally expressed within 24 hours (Lenschow et al. 1993). In this chapter we show that in the absence of activation signals IL-21 induces CD86 expression within only 4 hours. We further demonstrate that maximal induction is achieved within 8 hours and is maintained to at least 16 hours. It is therefore likely that promotion of CD86 expression by IL-21 will significantly enhance T cell proliferation in vivo in a given time period, as the response may be propagated more rapidly. In support of this, we show that enhanced provision of CD86 by pre-incubating B cells with IL-21 significantly enhances conventional T cell proliferation in a 3 day in vitro assay.

It has previously been shown that IL-21 promotes B cell proliferation through phosphoinositide-3 kinase (PI3K) (Zeng et al. 2007). Herein we demonstrate an unappreciated role for this pathway in IL-21-mediated B cell responses, as B cells expressing a catalytically inactive p110δ subunit were unable to upregulate CD86 in response to IL-21. IL-21 is known to promote CD4 and CD8 T cell survival in the absence of TCR ligation by activating the PI3K pathway (Ostiguy et al. 2007). However, as these experiments were conducted using whole murine lymph node cells, an intriguing possibility is that this effect is not mediated by direct signalling to T cells, but by the induction of CD86 expression by B cells, as CD28 signalling is known to promote T cell survival (Budd 2001). This might therefore explain the importance of PI3K in IL-21-mediated T cell survival.

T follicular helper (Tfh) cells have emerged in recent years as a distinct T helper cell population whose main function is to provide help for B cells during the germinal center reaction. A number of characteristics are associated with Tfh cells, including their expression
of CXCR5 and the transcription factor Bcl-6 (Crotty 2011). That Tfh cells also express IL-21 has been extensively documented (Bryant et al. 2007; Chtanova et al. 2004; Vinuesa et al. 2005a; Rasheed et al. 2006), and a role for this cytokine in their polarisation has since been described (Vogelzang et al. 2008; Nurieva et al. 2008). In a recent review article a number of potential Tfh differentiation checkpoints are proposed, one of which involves final lineage commitment not occurring until CD4 T cells arrive at the T-B interface (Crotty 2011). This would imply that direct interactions with B cells provide some of the necessary signals required for Tfh differentiation. One such signal B cells could potentially provide is CD86 co-stimulation, which is known to be important during germinal center formation. Indeed, it has recently been demonstrated that CD86 deficiency profoundly impairs the development of Tfh cells (Salek-Ardakani et al. 2011). It is therefore possible that the production of IL-21 by Tfh cells promotes further Tfh differentiation directly via positive feedback, but also indirectly by inducing high-level CD86 expression by B cells. CD86 expression has also been shown to be important for plasma cell generation and antibody production (Salek-Ardakani et al. 2011). It is therefore possible that defects in the germinal center response linked to disrupted IL-21 signalling to B cells (Linterman et al. 2010) may in part represent impaired T cell help due to reduced CD86 signalling. In considering these hypotheses, it is interesting that Tfh are also known to produce IL-4 (Reinhardt et al. 2009; King & Mohrs 2009), and that IL-4 can also induce CD86 expression by B cells, as demonstrated herein.

It is important to note that it is unlikely that the effects of IL-21 on CD86 expression would be limited solely to germinal center responses. IL-21 is not specifically expressed by Tfh cells; in fact its production has been linked with the majority of T helper cell lineages over the past decade. Th17 cells particularly have been shown to produce IL-21 (Korn et al. 2007; Wei
et al. 2007; Nurieva et al. 2007), and IL-21 is capable of inducing Th17 differentiation (Yang et al. 2008; Korn et al. 2007). As IL-17 is suggested to positively regulate a number of co-stimulatory molecules, including CD86 (Antonysamy et al. 1999), IL-21 may both directly and indirectly promote CD86 expression.

As proposed earlier, it will be important to test the significance of IL-21-mediated CD86 upregulation in an *in vivo* setting, which can be assessed using an adoptive transfer system in IL-21R-/- mice. Transfer of antigen loaded wildtype B cells and IL-21Rα-deficient DO11.10 TCR+ conventional T cells should then ensure an antigen specific T cell response wherein only transferred B cells can receive IL-21 signals upon its provision. As we have also recently acquired CD86-/- mice, extending these experiments to include B cells from these animals will provide further insight into the effects of IL-21-mediated CD86 induction on T cell responses.
5. TRANS-ENDOCYTOSIS OF CD86 BY CTLA-4

5.1. Introduction

In chapter 3 we studied the ability of IL-21 to counteract Treg-mediated suppression. However, the specific mechanisms responsible for suppression were not investigated. The subject of a great body of work, Treg have been found to regulate utilising a myriad of receptors, ligands and soluble factors (reviewed in (Tang & Bluestone 2008; Vignali et al. 2008)). CTLA-4 is one such example, playing a critical role in immune regulation, as demonstrated by CTLA-4-/- mice which develop a fatal lymphoproliferative syndrome (Tivol et al. 1995; Waterhouse et al. 1995). The capacity of CTLA-4 to confer suppressive function to Treg has been confirmed by numerous studies (Sojka et al. 2009; Takahashi et al. 2000; Friedline et al. 2009; Wing et al. 2008; Read et al. 2006; Schmidt et al. 2009). Our lab has previously shown that in an adoptive transfer model of autoimmune diabetes, Treg were unable to regulate disease if they lacked expression of CTLA-4 (Schmidt et al. 2009). Quite how CTLA-4 acts to control immune responses remains a contentious issue. Originally, a cell autonomous role for CTLA-4 was described, but this concept has been complicated by the above Treg data, which imply cell extrinsic functions (reviewed in (Walker & Sansom 2011; Wing et al. 2011)). We therefore set out to assess the role of Treg-expressed CTLA-4 in our suppression assays, and to further investigate the non-cell autonomous actions of CTLA-4.
5.2. Results

5.2.1. A role for CTLA-4 in *in vitro* suppression

The discovery that, unlike conventional T cells, Treg constitutively express CTLA-4 suggested the possibility of a role for this protein in Treg function (Takahashi et al. 2000; Read et al. 2000). Over a decade ago, it was demonstrated that blockade of CTLA-4 using anti-CTLA-4 antibody could prevent Treg suppression *in vitro* (Takahashi et al. 2000). However, this result remains controversial as others have shown no impairment of Treg suppression by this method (Thornton & Shevach 1998a; Oida et al. 2006). We therefore used anti-CTLA-4 antibody to assess whether Treg suppression was mediated by CTLA-4 in our own suppression assays. In these studies, blockade of CTLA-4 partially counteracted the ability of Treg to suppress conventional T cell proliferation, as measured by CFSE dilution (Fig. 5.01). This was particularly evident at a Treg to conventional T cell ratio of 1:1, where only 18.1% of cells remained in the undivided peak in the presence of αCTLA-4, compared with 38.5% in the presence of control antibody. Thus Treg suppression in our assays was, at least in part, dependent on CTLA-4.

5.2.2. Acquisition of CD86 by CTLA-4-expressing cells *in vitro*

The above data suggested that Treg were utilising CTLA-4 to suppress T cell proliferation in a cell extrinsic manner. Previously, it was shown that lymphocyte deficient recipient mice reconstituted with mixed CTLA-4-/- and CTLA-4+/+ bone marrow did not develop multi-organ inflammation, unlike recipients in which all cells lacked CTLA-4 (Bachmann et al. 1999; Homann et al. 2006). This suggested that wildtype CTLA-4-expressing cells could
Figure 5.01. CTLA-4 blockade counteracts the ability of Treg to inhibit Tconv proliferation. 2.5x10^4 CFSE-labelled Tconv were cultured with 5x10^4 B cells, 0.8µg/ml anti-CD3 and the indicated ratios of Treg, in the presence of 100µg/ml anti-CTLA-4 or control Ab. After 3 days cells were stained for surface CD4 and CD19. Histograms show CFSE dilution for CD4+CFSE+ Tconv when cultured with Treg alone (upper panels), or in the presence of anti-CTLA-4 (lower panels).
regulate those lacking CTLA-4 expression. Further, Friedline and colleagues (Friedline et al. 2009) have demonstrated that wildtype, but not CTLA-4-deficient, Treg could regulate CTLA-4-/- T cells in vivo. These studies provided considerable evidence that CTLA-4 could function in a non-cell autonomous fashion, but mechanistic data was lacking. Although TGFβ has been linked with CTLA-4 function, CTLA-4-/- cells, which were unable to regulate, did not produce less TGFβ (Bachmann et al. 1999), nor did TGFβ blockade prevent suppression by CTLA-4-sufficient Treg (Friedline et al. 2009). This latter report also found no apparent role for IL-10 in mediating wildtype Treg function, as IL-10R-/- CTLA-4-/- T cells remained effectively suppressed. A role for CTLA-4 in back signalling to APCs has been suggested, with signalling through CD80 and CD86 inducing indoleamine 2,3-dioxygenase (IDO)-dependent tryptophan catabolism and vulnerability to apoptosis (Grohmann et al. 2002). It has also been suggested that CTLA-4-mediates the downregulation of CD80 and CD86 by APCs (Wing et al. 2008; Schmidt et al. 2009; Onishi et al. 2008).

Because CTLA-4 is known to be highly endocytic, with surface CTLA-4 rapidly re-internalising in a clathrin-mediated process (Chuang et al. 1997), we became interested in whether the downregulation of CD80 and CD86 might reflect transfer of bound ligand into CTLA-4-expressing cells. To test this, in collaboration with the Sansom lab, we cultured CHO cells expressing CD86-GFP with Far Red-labelled CHO cells expressing human CTLA-4, analysing GFP transfer by flow cytometry. As CTLA-4 is known to undergo lysosomal degradation upon internalisation (Iida et al. 2000), we used the lysosomal inhibitor chloroquine to prevent the potential degradation of any internalized CD86-GFP. After only three hours, we noted a striking accumulation of CD86-GFP in chloroquine treated Far Red+
cells. This indicated that not only was CD86-GFP being transferred into CTLA-4-expressing cells, but also that it was being degraded in lysosomes once internalised (Fig. 5.02).

We next wished to test whether ligand transfer could be observed using cells naturally expressing CTLA-4, rather than CHO cells transfected with CTLA-4. For these experiments, we moved to a murine system as our lab maintains a colony of CTLA-4-/- mice. Accordingly, a comparison of wildtype and CTLA-4-/- animals revealed a complete lack of CTLA-4 expression amongst CD4+ lymphocytes (Fig. 5.03 A). We therefore assessed the ability of both wildtype and CTLA-4-/- T cells to acquire CD86-GFP from CHO cells in vitro. Because TCR ligation is known to induce CTLA-4 cycling (Finn et al. 1997), we added anti-CD3 to these cultures and determined CD86-GFP transfer after 15 hours. In these experiments, we observed considerable transfer of CD86-GFP into wildtype CD4+ T cells, but none whatsoever using CTLA-4-deficient cells under the same conditions (Fig. 5.03 B).

In the previous experiment we used anti-CD3 to enhance CTLA-4 expression. To further dissect the role of TCR signalling in trans-endocytosis we utilised a TCR transgenic system. DO11.10 TCR transgenic CD4+ cells were cultured with CD86-GFP-expressing CHO cells, alone or in the presence of OVA peptide, their cognate antigen. We found transfer of CD86-GFP into T cells only in conditions where OVA peptide was present (Fig. 5.04). Thus, transfer of CD86 into CD4+ cells occurred rapidly, was dependent on the expression of CTLA-4 and required TCR ligation. Further, acquisition was limited to CD25+ cells and once transferred, CD86-GFP appeared to be degraded.
Figure 5.02. Chloroquine is required to prevent lysosomal degradation of acquired CD86-GFP. 1x10⁶ CTLA-4-expressing Far Red-labelled CHO cells were cultured with 1x10⁶ CD86-GFP-expressing CHO cells for 3 hours, alone or in the presence of 50µM chloroquine before analysis by flow cytometry. Representative contour plots show acquired CD86-GFP for gated Far Red+ CTLA-4-expressing CHO cells. Data are representative of 3 experiments.
Figure 5.03. Murine CTLA-4-expressing CD4+CD25+ cells can effectively acquire CD86 in vitro. (A) Single cell suspensions from BALB/c or CTLA-4-/- lymph node were stained for surface CD4 expression and intracellular CTLA-4. Representative dot plots show CTLA-4 expression by gated lymphocytes. (B) 1x10^5 BALB/c or CTLA-4-/- T cells were cultured for 15hrs with 0.5µg/ml anti-CD3 and 1x10^5 murine CD86-GFP-expressing CHO cells. Cells were then stained for surface CD4 and CD25. Confocal microscopy images show distribution of CD86-GFP (green) in WT and CTLA-4-/- T cell cultures. Data are representative of 2 experiments.
Figure 5.04. Acquisition of CD86 by CD4+CD25+ cells is antigen-dependent. 1x10^5 DO11.10 TCR transgenic T cells were cultured with 1x10^5 CHO cells expressing murine MHC Class II and CD86-GFP for 15hrs, alone or with 1µg/ml OVA peptide. Cells were then stained for surface CD4 and CD25. Confocal microscopy images show distribution of CD86-GFP (green) in cultures absent of, or containing, OVA peptide. Data are representative of 2 experiments.
5.2.3. Acquisition of CD86 by CTLA-4-expressing cells in vivo

The above data suggested that CTLA-4 could effectively deplete CHO cells of CD86 by acquiring and degrading it. These results are consistent with the findings of others, that CTLA-4-expressing Treg were able to reduce expression levels of both CD80 and CD86 on DCs and B cells (Wing et al. 2008; Schmidt et al. 2009). To directly test whether CTLA-4 could acquire ligand from APCs in vivo, we generated an adoptive transfer system, described in detail in Fig. 5.05. Briefly, RAG-/- bone marrow was retrovirally transfected with CD86-GFP and adoptively transferred into irradiated RAG-/- hosts, generating CD86-GFP-expressing APCs. OVA specific T cells or Treg were then transferred, followed by immunisation and restimulation with OVA, to assess their acquisition of CD86-GFP. To mimic our original in vitro experiments as closely as possible, chloroquine was given to recipients during restimulation. In the first of a series of experiments, we assessed whether DO11.10 TCR transgenic CD4+ T cells could acquire CD86-GFP from reconstituted APCs, and whether this was influenced by TCR ligation. We observed acquisition of CD86-GFP by CD4+ T cells only in mice that had been restimulated with OVA peptide, and interestingly, only ever in cells expressing CD25 (Fig. 5.06 A, B). This confirmed that trans-endocytosis of CD86 could occur in vivo and was antigen-dependent.

In the above experiment, we could not determine whether cells that had acquired CD86-GFP were conventional or regulatory T cells. In this model system, CD4+ T cells were harvested from DO11.10 TCR transgenic mice lacking expression of the antigen for this receptor (OVA), before adoptive transfer. As natural Treg are generated in the thymus upon being presented self antigen by medullary thymic epithelial cells (Aschenbrenner et al. 2007), these mice have a diminished peripheral Treg compartment. On this basis, it was likely that the
Figure 5.05. Protocol for trans-endocytosis of CD86 by CTLA-4 in vivo. These experiments were undertaken collaboratively; Kyoko Nakamura performed retroviral infections and Omar Qureshi assisted with confocal microscopy.
Figure 5.06. Acquisition of CD86 by CD4+CD25+ cells is antigen-dependent in vivo. 1-5x10^6 DO11.10 TCR transgenic T cells were adoptively transferred into irradiated RAG-/- BALB/c recipients reconstituted with CD86-GFP-transfected RAG-/- BALB/c bone marrow, according to the protocol described in Fig. 5.05. Recipients were either restimulated with OVA peptide for 6hrs or untreated. Splenocytes were then stained for surface CD4 and CD25. (A) Confocal microscopy images show presence or absence of CD86-GFP (green) in splenocytes of OVA-restimulated or control recipients. (B) Graph shows the frequency of the indicated populations across multiple confocal fields for splenocytes from OVA-restimulated or control recipients. 100 cells were assessed for each condition. Data are representative of 2 experiments.
cells acquiring CD86-GFP in recipient animals were activated T cells. To precisely determine whether both regulatory and conventional T cells could endocytose CD86, we used CD4+ T cells from DO11.10 TCR transgenic mice or CD4+CD25+ T cells from DO11xRIP-mOVA mice that, due to the thymic expression of OVA, generate OVA specific peripheral Treg. These cells were transferred into the reconstituted recipients described earlier, followed by immunisation and restimulation with OVA. Upon harvest, we observed acquisition of CD86-GFP by CD4+CD25+ cells from both donor strains (Fig. 5.07 A). In assessing Foxp3 expression by CD4+CD25+ cells, as expected, we found that these were predominantly Foxp3- conventional T cells when donors were DO11.10 TCR transgenic mice, and Foxp3+ Treg when donors were DO11xRIP-mOVA mice (Fig. 5.07 B). These data clearly demonstrated that both regulatory and activated conventional T cells could acquire CD86-GFP from APCs in vivo.

To formally demonstrate the requirement for CTLA-4 in trans-endocytosis in vivo, we again utilised the CTLA-4-/- mouse strain. CTLA-4-/- mice bred to express the DO11 TCR still develop lymphoproliferative disease, since the T cells can utilise endogenous TCRα genes to generate autoreactive TCRs. Therefore, when crossed to a RAG-/- background they remain healthy. These mice were also crossed with RIP-mOVA mice to permit Treg generation. Thus, we transferred CD4+ T cells from CTLA-4+/+ or CTLA-4-/- RAG-/- DO11xRIP-mOVA mice according to our adoptive transfer protocol. Upon harvesting recipients, we found that transfer of CD86-GFP into CD4+CD25+ cells had occurred where donor T cells were CTLA-4-sufficient, but none whatsoever was observed using CTLA-4-/- T cells (Fig. 5.08). Furthermore, there was no evidence of acquisition of GFP when T cells were transferred into mice expressing control-GFP on their APCs (Fig. 5.09).
Figure 5.07. Both Tconv and Treg can acquire CD86 in vivo. 1-5x10^6 CD4+ cells from DO11 or CD4+CD25+ cells from DO11xRIP-mOVA donors were adoptively transferred into irradiated RAG-/- BALB/c recipients reconstituted with CD86-GFP-transfected RAG-/- BALB/c bone marrow, according to the protocol described in figure 5.05. Splenocytes were then stained for surface CD4 and CD25, and intracellular Foxp3. (A) Confocal microscopy images show distribution of CD86-GFP (green) in splenocytes of DO11 or DO11xRIP-mOVA T cell recipients. (B) Dot plots show Foxp3 staining for gated CD4+CD25+ splenic T cells from DO11 or DO11xRIP-mOVA T cell recipients. Data are representative of 2 experiments.
Figure 5.08. CTLA-4 expression is essential for acquisition of CD86 in vivo. 1-5x10^6 CD4+ T cells from CTLA-4+/+ or CTLA-4-/- RAG-/- DO11xRIP-mOVA donors were adoptively transferred into irradiated RAG-/- BALB/c recipients reconstituted with CD86-GFP-transfected BALB/c RAG-/- bone marrow, according to the protocol described in figure 5.05. Splenocytes were then stained for surface CD4 and CD25. (A) Confocal microscopy images show distribution of CD86-GFP (green) in splenocytes of CTLA-4+/+ or CTLA-4-/- T cell recipients. (B) Graph shows the frequency of the indicated populations across multiple confocal fields for splenocytes from recipients of CTLA-4+/+ or CTLA-4-/- T cells. 100 cells were assessed for each condition. Data are representative of 2 experiments.
Figure 5.09. CD86-GFP, but not control-GFP, is acquired by DO11.10 TCR transgenic T cells in vivo. 1×10⁶ DO11.10 TCR transgenic T cells were adoptively transferred into irradiated RAG-/- BALB/c recipients reconstituted with CD86-GFP or control GFP-transfected RAG-/- BALB/c bone marrow, according to the protocol described in Fig. 5.05. Splenocytes were then stained for surface CD4 and CD25. Confocal microscopy images show distribution of CD86-GFP (green) in splenocytes of CD86-GFP or control GFP-transfected recipients. Data are representative of 2 experiments.
In summary, we have demonstrated a novel mechanism by which CTLA-4-expressing cells might mediate suppression in a cell extrinsic manner. Trans-endocytosis by CTLA-4 occurs both in vitro and in vivo, and can be mediated by CTLA-4 expression by both regulatory and activated conventional T cells. It is dependent on TCR ligation and is specific for its ligand, CD86. This work, and confirmation that trans-endocytosis by CTLA-4 further extends to its second ligand, CD80, has recently been published (Qureshi et al. 2011).
5.3. Discussion

A role for CTLA-4 during in vitro Treg-mediated suppression has been the subject of a number of reports. Their often-conflicting results have made this a contentious subject, with a lack of clarity surrounding exactly how CTLA-4 functions serving only to complicate things further. Utilising blocking anti-CTLA-4 antibodies, a number of groups have reported diminished or completely abrogated suppression by Treg (Tang et al. 2004a; Takahashi et al. 2000), whilst others have found that suppression remains intact (Oida et al. 2006; Quezada et al. 2006). Unlike blocking antibodies, which may also target CTLA-4 expressing activated conventional T cells, the use of CTLA-4-deficient Treg in these assays specifically assesses the role of Treg-expressed CTLA-4. Approaching the question in this manner, a number of studies have found that Treg suppression is abrogated (Takahashi et al. 2000; Wing et al. 2008), whilst others have found that it is not (Tang et al. 2004a; Schmidt et al. 2009). The likely causes of the contradictory nature of these reports are the differences in culture conditions used to assess Treg suppression in vitro. These include the use of different populations of cells to provide co-stimulation and differences in the method and strength of TCR ligation. In the suppression assay system used throughout this work, we were able to demonstrate an effect of antibody-mediated CTLA-4 blockade on Treg suppression, although this was highly dependent on the ratio of B cells to conventional T cells and the dose of anti-CD3 used (our unpublished data).

Interestingly, a role for Treg-expressed CTLA-4 in in vivo suppression is more firmly established, and we have shown that CTLA-4-deficient Treg lacked suppressive function in an adoptive transfer model of type-1 diabetes (Schmidt et al. 2009). Likewise, others have shown that CTLA-4-deficient Treg were unable to suppress cytokine production and disease...
onset in an adoptive transfer model of colitis (Sojka et al. 2009). Further, absence of CTLA-4 expression specifically by the Treg population has been shown to induce lethal autoimmunity (Wing et al. 2008). In the latter study, mice expressing a floxed CTLA-4 gene were crossed with those expressing Cre under the control of the Foxp3 promoter, resulting in the absence of CTLA-4 expression from Foxp3+ cells. As with CTLA-4−/− mice, this caused fatal disease, although this was slightly delayed by comparison, demonstrating that CTLA-4-deficiency in Treg alone is sufficient to confer early lethality (Wing et al. 2008). CTLA-4-blockade has also been used to successfully block Treg suppression in vivo, in this case in an adoptive transfer model of colitis (Read et al. 2006).

The above data are important as they imply a cell extrinsic role for CTLA-4, a concept that is well demonstrated using chimeric bone marrow or T cell transfers wherein 50% of cells express CTLA-4 and 50% do not. By transferring wildtype and CTLA-4−/− bone marrow at a 1:1 ratio into irradiated RAG2−/− recipients, Bachmann and colleagues demonstrated that CTLA-4-expressing cells were capable of preventing the lethality observed in mice receiving only CTLA-4-deficient bone marrow (Bachmann et al. 1999). This data was later confirmed by Friedline and colleagues, who further showed that co-transfer of wildtype Treg and CTLA-4-deficient T cells at a 1:10 ratio into lymphopenic recipients did not lead to disease onset or lethality (Bachmann et al. 1999; Friedline et al. 2009; Homann et al. 2006). It has been suggested that cell intrinsic signalling to Treg might induce the secretion of suppressive cytokines, such as TGFβ. However, CTLA-4-deficient Treg produce similar levels of TGFβ to their wildtype counterparts (Bachmann et al. 1999), and TGFβ blockade does not prevent CTLA-4-expressing Treg from mediating suppression (Friedline et al. 2009). Thus, quite how CTLA-4 mediates its cell extrinsic effects has remained incompletely understood.
In this chapter and in a related publication (Qureshi et al. 2011), we have demonstrated a non-T cell autonomous mechanism whereby CTLA-4 can acquire CD86 in trans from antigen presenting cells. This process leaves APCs stripped of co-stimulatory ligands and unable to prime T cell activation and thus potentially explains how CTLA-4 can regulate T cell responses in a cell extrinsic manner. In support of this, a number of groups have observed reduced expression of co-stimulatory ligands by APCs when these cells have been in contact with those expressing CTLA-4 (Wing et al. 2008; Schmidt et al. 2009; Onishi et al. 2008). Of particular note, the capture and transfer of CD86 provides a functional explanation for the endocytic nature of CTLA-4, which rapidly re-internalises in a clathrin-dependent process (Chuang et al. 1997).

One particularly important point regarding the acquisition of CD86 by CTLA-4 is that the mechanism is intact for both conventional and regulatory T cells. Thus, the expression of functional CTLA-4 by conventional T cells does not necessarily imply cell intrinsic signalling. Rather, the expression of CTLA-4 by activated T cells may serve to limit the activation and proliferation of other T cells by reducing the availability of co-stimulatory ligands. This perhaps explains why Treg need constitute such a small proportion of the peripheral T cell pool, as activated T cells can themselves acquire regulatory properties.

During the course of the in vivo experiments described here, we determined that trans-endocytosis of CD86 by CTLA-4 was triggered by TCR ligation. Using DO11.10 TCR transgenic CD4+ T cells, we observed ligand transfer into CTLA-4-expressing cells only when recipient mice had been restimulated with OVA. For conventional T cells, this could simply be explained by the fact that they do not express CTLA-4 until they become activated.
Regulatory T cells on the other hand constitutively express CTLA-4, and the promotion of its cycling by TCR ligation (Finn et al. 1997) likely promotes CD86 acquisition. In addition, Treg are known to require TCR ligation for suppressive function (Thornton & Shevach 2000), which may in part be explained by enhanced cycling of CTLA-4.

The controversial nature of CTLA-4 function has lead to numerous proposed mechanisms for its cell extrinsic role. The endocytic process described in this chapter is a development of the ligand competition model, wherein CTLA-4 simply competes with CD28 for ligand interactions. A different model proposes that CTLA-4 is capable of back signalling through its ligands into APCs to promote IDO expression. This enzyme catabolises Tryptophan, the localised depletion of which impairs T cell proliferation (Fallarino et al. 2003; Grohmann et al. 2002). However, it seems unlikely that this mechanism is entirely responsible for the cell extrinsic function of CTLA-4, as IDO-deficient mice do not develop the fatal lymphoproliferative disease observed in CTLA-4/- mice (Walker & Sansom 2011).

In future experiments, it will be interesting to assess the role of factors known to promote Treg function in regulating trans-endocytosis of co-stimulatory ligands. Two such examples are IL-2 and CD28, which are both crucial for Treg function in vitro and in vivo. Our lab maintains CD28-deficient mice, and whilst Treg frequencies are reduced in these animals, they can be isolated for functional analysis. Indeed, that CD28/- Treg cannot suppress in in vitro suppression assays (Fig. 6.04) may be indicative of impaired CTLA-4 function. Using a similar adoptive transfer system as that described in this chapter, future experiments could utilise CD28-deficient DO11.10 TCR transgenic T cells to assess the role of CD28 in mediating trans-endocytosis in vivo.
6. APCs Program Distinct T Helper Cell Phenotypes

6.1. Introduction

One of the notable findings to arise from chapter 3 was the profound difference in the way suppression assays responded to IL-21 when proliferation was driven by either B cells or DCs. Suppression in the context of B cell-mediated co-stimulation was entirely overcome in the presence of IL-21 (Fig. 3.05 & 3.06). On the other hand, DC-driven assays appeared unresponsive to IL-21, with Treg suppression remaining intact (Fig. 3.10). This led us to consider that there might be fundamental differences in the way these cell populations were promoting T cell responses.

Whilst type-1 diabetes is defined as being T cell-mediated, B cells and DCs have been shown to play crucial roles in both the induction and maintenance of its pathology. Specifically, the antigen presenting functions of both have been highlighted as contributing factors in this disease (Wong et al. 2004; Ludewig et al. 1998). A greater understanding of precisely how each APC promotes T cell responses is thus beneficial. We therefore set out to assess how conventional T cell activation and proliferation was co-stimulated, and how their differentiation was modulated, between DC and B cell assays. Further, we examined how the nature of these stimulations altered the diabetogenic capacity of autoreactive T cells.
6.2. Results

6.2.1. B cells and DCs differentially co-stimulate T cell responses

Because conventional T cells in our previous experiments responded so differently to IL-21 between B cell and DC-driven assays, we wished to determine whether these APCs were providing distinct co-stimulatory signals. To achieve this, we cultured conventional T cells with either B cells or bone marrow-derived DCs, under conditions of antibody-mediated blockade of co-stimulatory factors. We then determined proliferation of conventional T cells after three days. CD80 or CD86 blockade was used as these are considered the primary co-stimulatory ligands during T cell responses (Adam et al. 1998). Blockade of TNFα was also utilised as TNFα has previously been shown to co-stimulate CD8+ T cells in the absence of CD28 signalling (Sepulveda et al. 1999). Further, in chapter 3 we showed significant TNFα production by conventional T cells in B cell-stimulated assays (Fig. 3.27). However, as clearly shown in Fig. 6.01 A, conventional T cell proliferation was almost entirely dependent on CD86 in B cell assays, with no significant role for either TNFα or CD80. In DC-driven responses, only TNFα blockade had a significant impact on proliferation, although this effect was not as profound as CD86 blockade in B cell stimulations (Fig. 6.01 B). From these data, we concluded that B cell and DC-driven responses were stimulated differently, with B cell co-stimulation dependent almost entirely on CD86, and that of DCs dependent to a large extent on TNFα.

We next wished to determine how these distinct stimulations altered conventional T cell activation. To this end, we cultured B cells or DCs with conventional T cells for three days and assayed expression of the activation markers CD69 and CD25, and of CD62L, which is
Figure 6.01. **B cell-mediated co-stimulation is dependent on CD86.** 2.5x10⁴ Tconv were cultured with either 5x10⁴ B cells or 2.5x10³ iDCs and 0.8µg/ml anti-CD3, alone or in the presence of either 10µg/ml anti-CD80, 10µg/ml anti-CD86 or 5µg/ml anti-TNFα. After 3 days cells were stained for surface CD4, CD11c and CD19, and intracellular Foxp3. Graphs show cell counts for CD4+Foxp3- Tconv when cultured with B cells (A) or iDCs (B). Counts are presented as a percentage, relative to the cell count where no blockade is present. Bars represent means and SEM is shown for 3 experiments. *, p = <0.05, **, p = <0.01, ns = not significant.
expressed by naïve T cells (Rosen 2004). We found comparably high expression of both CD25 and CD69 by conventional T cells cultured with either APC. However, whilst DCs promoted a significant downregulation in CD62L expression, B cell co-stimulated T cells maintained high-level expression, with almost all cells staining positive after three days (Fig. 6.02). Thus, whilst both APCs appeared to activate conventional T cells equivalently, CD62L expression was selectively maintained in B cell-driven responses.

6.2.2. B cells and DCs program distinct T helper cell cytokine expression profiles

As both B cell and DC stimulations appeared to readily promote conventional T cell activation, at least as judged by CD25 and CD69, we next assessed the expression of a panel of T cell cytokines. Conventional T cells were cultured with either B cells or DCs and their expression profiles analysed at day three. We assayed the Th1 and Th2 specific cytokines IFNγ and IL-4 respectively (reviewed in (Paul & Seder 1994)), and the Th17 specific cytokine IL-17 (Harrington et al. 2005; Park et al. 2005). As mentioned above, B cell assays resulted in high levels of TNFα production (Fig. 3.27), so it was included here for comparison. Finally, our interest in IL-21 led us to also examine its expression between APC stimulations. We found that conventional T cells cultured with B cells expressed high levels of IFNγ, but almost no IL-17 or IL-21. In stark contrast, DC-stimulated T cells expressed low levels of IFNγ by comparison, significantly higher levels of IL-17 and roughly half expressed high levels of IL-21. TNFα was expressed almost equivalently between assays, whilst little IL-4 was detected in either (Fig. 6.03).

In collectively assessing these data, B cell-driven responses were almost entirely dependent
Figure 6.02. CD62L expression by Tconv is maintained in the context of B cell-mediated co-stimulation. 2.5x10^4 Tconv were cultured with either 5x10^4 B cells or 2.5x10^3 iDCs, and 0.8µg/ml anti-CD3. After 3 days cells were stained for surface CD4, CD11c, CD19, CD25, CD69 and CD62L, and intracellular Foxp3. Representative dot plots show expression levels and graphs show collated data for CD25 (A) and (B), CD69 (C) and (D), and CD62L (E) and (F), for CD4+Foxp3- Tconv. Bars represent means and SEM is shown for 3 experiments. **, p = <0.01, ns = not significant.
Figure 6.03. B cells and DCs promote distinct Tconv cytokine expression profiles. 2.5x10^4 Tconv were cultured with either 5x10^4 B cells or 2.5x10^3 iDCs, and 0.8µg/ml anti-CD3. After 3 days cells were restimulated and stained for surface CD4 and intracellular IFNγ, IL-17, IL-21, TNFα and IL-4. Representative contour plots show expression levels and graphs show collated data for IFNγ (A) and (B), IL-17 (C) and (D), IL-21 (E) and (F), TNFα (G) and (H), and IL-4 (I) and (J), by CD4+ Tconv. Bars represent means across 5-9 experiments as indicated. *** p = <0.001, ns = not significant.
on CD86, involved the maintenance of CD62L expression during activation, and appeared to skew toward an IFNγ-producing Th1 phenotype. Conversely, DC stimulations were significantly dependent on TNFα and appeared to promote the expression of IL-21.

6.2.3. DC-stimulated conventional T cells are more diabetogenic than those driven by B cells

As mentioned previously, the APC functions of both B cells and DCs are known to be important contributing factors in type-1 diabetes pathogenesis (Wong et al. 2004; Ludewig et al. 1998). That their stimulation of conventional T cells should give rise to such different cytokine and CD62L expression profiles led us to question whether this might have implications for the diabetogenic capacity of autoreactive T cells. To assess this, we devised an adoptive transfer system in which cultured DO11.10 TCR transgenic donor T cells were transferred into recipient mice expressing OVA in the pancreas under the control of the RIP. This protocol was based on a similar method used by Camacho and colleagues (Camacho et al. 2001), but differed in that our recipients lacked CD28 expression. The advantages of using our system were two-fold. Firstly, CD28-/- mice had diminished frequencies of Foxp3+ Treg (Fig. 6.04 A & B), with those that remained lacking suppressive function, as determined by in vitro suppression assays (Fig. 6.04 C). This reduced the possibility that differences in diabetogenicity might have been caused by effects on Treg, such as those mediated by IL-21, produced exclusively by conventional T cells cultured with DCs. Secondly, lack of CD28 expression by host T cells reduced the likelihood of bystander activation, making them far less likely to participate in any pathogenic process mediated by adoptively transferred T cells. Thus, DO11.10 TCR transgenic T cells were cultured with anti-CD3 and either B cells or DCs
Figure 6.04. Reduced frequencies and impaired suppressive function of Treg from CD28-/− mice. Single cell suspensions from BALB/c or CD28-/− lymph node were stained for surface expression of CD4 and CD19, and intracellular Foxp3. Representative dot plots show CD4+Foxp3+ Treg frequencies within gated CD4+ lymphocytes (A), and graph shows collated data for these cells (B). 2.5×10⁴ WT Tconv were cultured with 5×10⁴ WT B cells, with 0.8μg/ml anti-CD3 and the indicated ratios of WT or CD28-/− Treg. After 3 days cells were stained for surface CD4 and CD19, and intracellular Foxp3. Graph shows cell counts for CD4+Foxp3− Tconv (C). Counts are presented as a percentage, relative to the cell count where no Treg are present. Bars represent means and SEM is shown for ≥3 experiments. *, p = <0.05, **, p = <0.01, ***, p = <0.001.
for three days, then CD4+ T cells were purified by MoFlo sorting and adoptively transferred into CD28-/-mOVA+ recipients. A small number of cultured cells were also stained for DO11.10 TCR, to ensure their expression profiles were comparable and would not be responsible for any differences in pathogenicity \textit{in vivo}. As demonstrated in Fig. 6.05, expression of this TCR was consistent between conventional T cells cultured with B cells or DCs.

To monitor differences in pathogenicity between B cell and DC co-stimulated conventional T cells, blood glucose measurements were taken from recipient mice at regular intervals, starting at day five. We found that recipients of DC-stimulated T cells rapidly lost the ability to control blood glucose homeostasis, with all becoming diabetic within 12 days. On the other hand, recipients of T cells from B cell cultures remained normoglycaemic at day 12, with significantly lower mean blood glucose levels at all subsequent time points examined (Fig. 6.06). We therefore considered that the difference in blood glucose levels might be due to increased entry of DC-stimulated T cells into the pancreas. To test this, recipients were sacrificed at day 21 and tissues taken for analysis. Initially, we quantified DO11.10 TCR+ conventional T cells in the pancreas, pancreatic lymph node and inguinal lymph nodes by flow cytometry. We found these cells accumulated preferentially in the pancreases of DC-stimulated T cell recipients, over other tissues. Conversely, far fewer DO11.10 TCR+ conventional T cells were found in the pancreases of B cell-stimulated T cell recipients, which instead accumulated in the pancreatic lymph node (Fig. 6.07 A). To confirm these findings, we undertook histological staining for insulin and CD4 in pancreas sections from day 21 recipients. These stains demonstrated many more CD4+ T cells infiltrating islets of DC-stimulated T cell recipients (Fig. 6.07 B).
Figure 6.05. Tconv cultured with B cells or DCs maintain equivalent DO11.10 TCR expression. 2.5x10^4 DO11.10 TCR transgenic Tconv were cultured with either 5x10^4 B cells or 2.5x10^3 iDCs, and 0.8µg/ml anti-CD3. After 3 days cells were stained for surface CD4, CD11c, CD19 and DO11.10 TCR. Representative dot plots show DO11.10 TCR expression by gated CD4+ Tconv.
Figure 6.06 Pathogenicity of DC-cultured versus B cell-cultured Tconv following adoptive transfer. 1.8x10^6 MoFlo sorted CD4+ DO11.10 TCR transgenic Tconv, having been cultured with B cells or DCs, and 0.8\mu g/ml anti-CD3 for 3 days, were adoptively transferred into lightly irradiated CD28-/-mOVA+ recipients via tail vein injection. Blood glucose was measured at the indicated time points. Graph shows collated blood glucose measurements. Dots represent means and SEM is shown for 7 mice per condition. Grey line indicates 250mg/dL threshold blood glucose for mice to be considered diabetic. *, p = <0.05, **, p = <0.01, ns = not significant.
Figure 6.07. Adoptively transferred DC-stimulated Tconv accumulate preferentially in the pancreas. 1.8x10^6 MoFlo sorted CD4+ DO11.10 TCR transgenic Tconv, having been cultured with B cells or DCs, and 0.8µg/ml anti-CD3 for 3 days, were adoptively transferred into lightly irradiated CD28-/-mOVA+ recipients via tail vein injection. At day 21, mice were sacrificed and tissues harvested. (A) Single cell suspensions from the indicated tissues were stained for surface CD4 and intracellular DO11.10 TCR and Foxp3. Graph shows absolute cell counts for CD4+DO11.10 TCR+Foxp3- cells. Bars represent means and SEM is shown for 4 mice per condition. (B) Pancreas sections were stained for CD4 and insulin for analysis by light microscopy (x20). *, p < 0.05. ns = not significant.
It has recently been shown that highly purified populations of IL-17 producing T cells can alter their cytokine expression profile after adoptive transfer (Bending et al. 2009). We therefore questioned whether the cytokine profiles of B cell or DC-stimulated T cells had changed during the course of our in vivo experiments. To test this, we harvested various tissues from recipient mice at day 21 and assessed the expression of IL-21, IFN\(\gamma\), TNF\(\alpha\) and IL-17 by DO11.10 TCR+ cells, as these had all variously been expressed pre-transfer. These analyses showed that T cells cultured with B cells before adoptive transfer broadly retained their Th1-like phenotype, expressing high levels of IFN\(\gamma\) and TNF\(\alpha\) in all tissues, but little IL-21. Curiously, IL-17, which had been expressed by less than 2% of B cell-cultured T cells pre-transfer, was detectable in roughly 12% of pancreas-infiltrating T cells. This increased expression was specific to the pancreas, as it was at least two-fold greater than in any other tissue (Fig. 6.08). For T cells cultured with DCs, cytokine expression changed greatly post-transfer. Where IL-21 had been expressed by over 50% of these cells pre-transfer, by day 21 this had decreased to less than 10%. This level of expression was now broadly in line with levels expressed by B cell-stimulated T cells in all tissues except the inguinal lymph node, where it was significantly higher at around 17% (Fig. 6.08 A). Strikingly, the majority of DC-stimulated T cells in the pancreas expressed high levels of IFN\(\gamma\), when less than 10% were IFN\(\gamma^+\) pre-transfer. Such was the increase that a significantly higher percentage expressed IFN\(\gamma\) than B cell-stimulated T cells in the same tissue. Moreover, this was the only tissue in which this difference occurred, with the majority of DC-stimulated T cells lacking IFN\(\gamma\) expression in all others (Fig. 6.08 B). TNF\(\alpha\) and IL-17 expression remained broadly unchanged, with relatively high and low levels of each cytokine respectively (Fig. 6.08 C & D).
Figure 6.08. Adoptively transferred DC-stimulated Tconv expressing IFN\(\gamma\) are enriched in the pancreas. 1.8x10\(^6\) MoFlo sorted CD4+ DO11.10 TCR transgenic Tconv, having been cultured with B cells or DCs, and 0.8µg/ml anti-CD3 for 3 days, were adoptively transferred into lightly irradiated CD28-/-mOVA+ recipients via tail vein injection. At day 21, mice were sacrificed and single cell suspensions from the indicated tissues restimulated and stained for surface CD4, and intracellular DO11.10 TCR, Foxp3, IFN\(\gamma\), IL-17, IL-21 and TNF\(\alpha\). Graphs show IL-21 (A), IFN\(\gamma\) (B), TNF\(\alpha\) (C) and IL-17 (D) expression for gated CD4+DO11.10 TCR+Foxp3- Tconv within the indicated tissues and pre-transfer. Bars represent means and SEM is shown for 4 (pancreas) or 6 (all other tissues) mice per condition. *, \(p = <0.05\), **, \(p = <0.01\), ***, \(p = <0.001\), ns = not significant.
A

% of CD4+DO11.10 TCR+ cells that are IL-21+

B

% of CD4+DO11.10 TCR+ cells that are IFNγ+
C

% of CD4+DO11.10 TCR+ cells that are TNFα+

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D

% of CD4+DO11.10 TCR+ cells that are IL-17+

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* p < 0.05
** p < 0.001
*** p < 0.0001
Overall, conventional T cells stimulated with DCs were more diabetogenic than those stimulated with B cells, causing diabetes onset within only 12 days of adoptive transfer in all recipients. DC-driven T cells accumulated preferentially in the pancreas, whereas those driven by B cells were most abundant in the pancreatic lymph node. In line with this finding, recipients of DC-stimulated T cells demonstrated enhanced pancreatic islet infiltration by CD4+ T cells. In assessing cytokine production, we noted that B cell-stimulated T cells broadly retained their pre-transfer phenotype of high IFNγ expression in all tissues. Moreover, whilst DC-driven T cells made very little before adoptive transfer, a strikingly high proportion expressed IFNγ in the pancreas by day 21 post-transfer, significantly more than B cell-stimulated T cells. Interestingly, the high expression of IL-21 observed pre-transfer by DC-driven T cells was notably absent by day 21 after adoptive transfer, with low levels expressed in all tissues.

6.2.4. Abrogated IL-21 signalling to DC-stimulated conventional T cells ameliorates diabetes

One of the most striking differences between B cell and DC-stimulated conventional T cells was in their production of IL-21. T cells stimulated with DCs in vitro not only produced far more IL-21, they were also significantly more pathogenic in our adoptive transfer model of type-1 diabetes. As IL-21 production appeared to decline post-transfer, we postulated that autocrine IL-21 signalling during culture, or shortly thereafter, might be responsible for the enhanced diabetogenicity of DC-driven T cells. This concept was supported by adoptive transfer experiments using IL-21Rα-deficient CD4+ T cells, which have indicated that ameliorated disease associated with loss of IL-21 signalling is due to a CD4+ T cell-intrinsic
defect (Spolski et al. 2008; Hanash et al. 2011; Oh et al. 2010). We therefore repeated our adoptive transfer experiments using B cell or DC-stimulated IL-21R-/- conventional T cells. We began by assessing how the phenotype of DC-activated T cells was influenced by autocrine IL-21 signalling. Wildtype T cells stimulated in this way had been found to downregulate CD62L (Fig. 6.02) and express high levels of IL-21, but low levels of IFNγ (Fig. 6.03). To test the role of autocrine IL-21 signalling in conferring this phenotype, we cultured wildtype or IL-21R-/- conventional T cells with B cells or DCs for three days in the presence of anti-CD3. We observed a significant increase in CD62L expression by DC-stimulated IL-21R-/- T cells compared to their wildtype counterparts (Fig. 6.09). We also observed a significant decrease in IL-21 production by IL-21R-/- T cells from DC cultures (Fig. 6.10). This is in agreement with previous work suggesting that IL-21 production by CD4+ T cells is regulated in an autocrine manner (Suto et al. 2008). In B cell cultures, we found an almost doubling in the expression of IFNγ by T cells in the context of IL-21Rα-deficiency (Fig. 6.11). This implied that very low-level production of IL-21 in B cell cultures was sufficient to partially inhibit IFNγ expression, in agreement with our previous data (Fig. 3.27), and that of others (Suto et al. 2008), concerning the regulation of IFNγ production by IL-21.

Upon transferring cultured IL-21R-/- conventional T cells into RIP-mOVA-expressing CD28-/- recipients, we observed a profound difference in blood glucose homeostasis, with DC-stimulated IL-21R-/- T cell recipients all measuring normoglycaemic by day 21. In contrast, and as demonstrated previously, wildtype T cells cultured with DCs caused diabetes in all recipients by day 12 (Fig. 6.12 A). For recipients of B cell-driven IL-21R-/- conventional T cells, no significant difference in diabetes onset was observed when compared with recipients
Figure 6.09. IL-21 inhibits CD62L expression by DC-stimulated Tconv. 2.5x10^4 WT or IL-21R-/- DO11.10 TCR transgenic Tconv were cultured with either 5x10^5 B cells or 2.5x10^3 immature DCs, and 0.8µg/ml anti-CD3. After 3 days cells were stained for surface CD4 and CD62L. Representative dot plots show CD62L staining for gated CD4+ Tconv (A) and graph shows collated data for these cells (B). Bars represent means and SEM is shown for 3 experiments. *, p = <0.05, ns = not significant.
Figure 6.10. Autocrine promotion of IL-21 expression in DC-stimulated Tconv. 2.5x10⁴ WT or IL-21R/- DO11.10 TCR transgenic Tconv were cultured with either 5x10⁴ B cells or 2.5x10³ immature DCs, and 0.8µg/ml anti-CD3. After 3 days cells were restimulated and stained for surface CD4 and intracellular IL-21. Representative contour plots show IL-21 staining for gated CD4+ Tconv (A) and graph shows collated data for these cells (B). Bars represent means and SEM is shown for 3 experiments. **, p = <0.01, ns = not significant.
Figure 6.11. IL-21 inhibits IFNγ expression by B cell-stimulated Tconv. 2.5x10^4 WT or IL-21R-/- DO11.10 TCR transgenic Tconv were cultured with either 5x10^4 B cells or 2.5x10^3 immature DCs, and 0.8µg/ml anti-CD3. After 3 days cells were restimulated and stained for surface CD4 and intracellular IFNγ. Representative contour plots show IFNγ staining for gated CD4+ Tconv (A) and graph shows collated data for these cells (B). Bars represent means and SEM is shown for 3 experiments. *, p = <0.05, ns = not significant.
Figure 6.12. Adoptively transferred DC-stimulated IL-21R-/- Tconv are less diabetogenic than wildtype Tconv. 1.8x10^6 MoFlo sorted CD4+ WT or IL-21R-/- DO11.10 TCR transgenic Tconv, having been cultured with B cells or DCs, and 0.8µg/ml anti-CD3 for 3 days, were adoptively transferred into lightly irradiated CD28-/-mOVA+ recipients via tail vein injection. Blood glucose was measured at the indicated time points. Graphs show collated blood glucose measurements for recipients of WT or IL-21R-/- Tconv stimulated with either DCs (A) or B cells (B). Dots represent means and SEM is shown for 3 mice per condition. Grey line indicates 250mg/dL threshold blood glucose for mice to be considered diabetic. *, p <0.05, **, p <0.01, ***, p <0.001, ns = not significant.
of wildtype B cell-stimulated T cells (Fig. 6.12 B), despite increased IFNγ expression pre-transfer (Fig. 6.11 B). We therefore concluded that autocrine IL-21 signalling to conventional T cells was required for the profound diabetogenicity characteristic of DC-stimulated T cells. Potential explanations for this included induced high-level IL-21 production or the downregulation of CD62L expression. On the other hand, lack of IL-21 signalling to B cell-stimulated conventional T cells did not alter their pathogenicity in vivo, even though they produced far higher levels of IFNγ pre-transfer.

In our previous adoptive transfer harvest data, we found that DC-stimulated wildtype T cells accumulated preferentially in the pancreas. Because IL-21R-/- T cells stimulated in this way did not cause diabetes, we wondered whether they were now unable to accumulate at this site. To assess this, we harvested recipients of DC or B cell-driven wildtype or IL-21Rα-deficient T cells at day 21 and quantified DO11.10 TCR+ conventional T cells in various tissues. To obtain accurate conventional T cell numbers, we gated based on the expression of CD4 and DO11.10 TCR, and lack of Foxp3 expression. However, this gating strategy revealed a notable induction of Foxp3 in the pancreas amongst IL-21R-/- T cell recipients from both B cell and DC cultures (Fig. 6.13). These data may reflect the fact that IL-21 has been shown to suppress Foxp3 expression (Li & Yee 2008) and induction in vitro (Nurieva et al. 2007) and in vivo (Buch er et al. 2009). Gating these Foxp3+ cells out, we found that DC-stimulated conventional T cells no longer accumulated in the pancreas, when compared with data for wildtype T cells from Fig. 6.07 (Fig. 6.14 A). Interestingly, IL-21R-/- conventional T cells cultured with B cells no longer accumulated in the pancreatic lymph node, with counts roughly equivalent in all tissues examined (Fig. 6.14 B).
Figure 6.13. Adoptively transferred *in vitro* stimulated IL-21R-/− Tconv upregulate Foxp3 in the pancreas. 1.8x10⁶ MoFlo sorted CD4+ WT or IL-21R-/− DO11.10 TCR transgenic Tconv, having been cultured with B cells or DCs, and 0.8µg/ml anti-CD3 for 3 days, were adoptively transferred into lightly irradiated CD28-/−mOVA+ recipients via tail vein injection. At day 21, mice were sacrificed and single cell suspensions from the indicated tissues stained for surface CD4 and intracellular DO11.10 TCR and Foxp3. Representative dot plots show Foxp3 staining for gated pancreatic WT or IL-21R-/− CD4+DO11.10 TCR+ cells. Data are representative of 3 mice per condition.
Figure 6.14. Adoptively transferred DC-stimulated IL-21R-/- Tconv do not accumulate in the pancreas. 1.8x10⁶ MoFlo sorted CD4+ WT or IL-21R-/- DO11.10 TCR transgenic Tconv, having been cultured with B cells or DCs, and 0.8µg/ml anti-CD3 for 3 days, were adoptively transferred into lightly irradiated CD28-/-mOVA+ recipients via tail vein injection. At day 21, mice were sacrificed and single cell suspensions from the indicated tissues stained for surface CD4 and intracellular DO11.10 TCR and Foxp3. Graphs show absolute cell counts for WT or IL-21R-/-CD4+DO11.10 TCR+Foxp3- cells, having been stimulated pre-transfer with either DCs (A) or B cells (B). Bars represent means and SEM is shown for 3 mice per condition. *, p < 0.05, ns = not significant.
In Fig. 6.08, we showed that the phenotype of DC-driven T cells changed dramatically after adoptive transfer. We therefore wished to test whether such changes in cytokine expression would also hold true for DC-stimulated IL-21R/- T cells. To this end, we harvested recipients of DC or B cell-stimulated IL-21Rα-deficient conventional T cells at day 21 and assessed the expression of IL-21 and IFNγ by DO11.10 TCR+ conventional T cells in various tissues. As shown in Fig. 6.15 A, DC-stimulated IL-21R/- T cells expressed broadly equivalent levels of IL-21 post-transfer as DC-stimulated wildtype T cells from Fig. 6.08 A. However, this did not represent diminished IL-21 expression in this case, as DC-driven IL-21R/- T cells made far less IL-21 before adoptive transfer than did wildtype T cells. In a similar fashion, IL-21R/- T cells cultured with B cells pre-transfer produced equivalent levels of IL-21 after adoptive transfer as wildtype T cells (Fig. 6.15 B). Interestingly, DC-stimulated IL-21Rα-deficient conventional T cells expressed equivalently high levels of IFNγ in the pancreas as wildtype T cells, but in all other tissues expression was significantly higher (Fig. 6.16 A). This may reflect the slight but not statistically significant increased expression of IFNγ by these cells pre-transfer. Curiously, IL-21R/- T cells cultured with B cells made significantly more IFNγ in the pancreas than wildtype cells, but not in any other tissue (Fig. 6.16 B).

These data demonstrated that loss of IL-21 signalling to conventional T cells stimulated with DCs was found to prevent their ability to induce diabetes upon adoptive transfer. The reason for the loss of pathogenicity in the absence of IL-21 signalling is not yet known, although clearly pancreas entry was impaired in this setting. Potential explanations include changes in CD62L expression and cytokine profile, or possibly the induction of Foxp3. Further experiments will be required to assess the influence of these changes over T cell pathogenicity. Regardless of IL-21Rα expression, IFNγ was consistently produced at high
Figure 6.15. Day 21 IL-21 expression for adoptively transferred in vitro co-stimulated WT and IL-21R-/- Tconv. 1.8x10⁶ MoFlo sorted CD4+ WT or IL-21R-/- DO11.10 TCR transgenic Tconv, having been cultured with B cells or DCs, and 0.8µg/ml anti-CD3 for 3 days, were adoptively transferred into lightly irradiated CD28-/-mOVA+ recipients via tail vein injection. At day 21, mice were sacrificed and single cell suspensions from the indicated tissues restimulated and stained for surface CD4 and intracellular DO11.10 TCR, Foxp3 and IL-21. Graphs show collated IL-21 expression for WT or IL-21R-/- CD4+DO11.10 TCR+Foxp3- Tconv, having been stimulated pre-transfer with either DCs (A) or B cells (B). Pre-transfer expression is shown for comparison. Bars represent means and SEM is shown for 3 mice per condition. ns = not significant.
Figure 6.16. Day 21 IFNγ expression for adoptively transferred in vitro co-stimulated WT and IL-21R-/- Tconv. 1.8x10^6 MoFlo sorted CD4+ WT or IL-21R-/- DO11.10 TCR transgenic Tconv, having been cultured with B cells or DCs, and 0.8µg/ml anti-CD3 for 3 days, were adoptively transferred into lightly irradiated CD28-/-mOVA+ recipients via tail vein injection. At day 21, mice were sacrificed and single cell suspensions from the indicated tissues restimulated and stained for surface CD4 and intracellular DO11.10 TCR, Foxp3 and IFNγ. Graphs show collated IFNγ expression for WT or IL-21R-/-CD4+DO11.10 TCR+Foxp3- Tconv, having been stimulated pre-transfer with either DCs (A) or B cells (B). Pre-transfer expression is shown for comparison. Bars represent means and SEM is shown for 3 mice per condition. *, p = <0.05, **, p = <0.01, ***, p = <0.001, ns = not significant.
levels in the pancreas, even in cases where very little of this cytokine was evident pre-
transfer. Intriguingly, despite increased expression of IFNγ in the pancreas, this was not
sufficient to confer a pathogenic phenotype. This was clearly demonstrated by B cell-
stimulated T cells, which consistently expressed high levels of IFNγ but displayed reduced to
no pathogenicity compared with DC-cultured T cells.
6.3. Discussion

Co-stimulation of T helper cell activation and proliferation is thought to be mediated primarily by the interactions of CD80 and CD86 with CD28. In this chapter we have shown that B cell-driven responses were almost entirely dependent on CD86, but not CD80. This may be due to the rapidity with which CD86 can be upregulated upon activation when compared with CD80, which is only maximally induced after 48-72 hours (Lenschow et al. 1993). Strikingly, we found no significant effect of blocking either CD80 or CD86 in DC-driven assays, but TNFα blockade significantly reduced proliferation. It has previously been reported that T cells can be co-stimulated in the absence of CD28 signals (Akiba et al. 1999) which, in agreement with our data, can be substituted by TNFα or IL-6 (Sepulveda et al. 1999). However, blockade of TNFα by no means completely abrogated conventional T cell proliferation, implying that DC-mediated co-stimulation was dependent on more than a single accessory interaction.

A striking observation presented in this chapter was that B cells and DCs promoted distinct T helper cell cytokine profiles. T cells co-stimulated with B cells adopted a Th1 phenotype characterised by high IFNγ expression, whilst those stimulated with DCs produced high levels of IL-21. The differences between these responses are likely due to the differential expression of cytokines by B cells and DCs. IL-6 is known to potently induce IL-21 expression by CD4+ T cells (Suto et al. 2008), and DCs have been reported to produce IL-6 (Kopf et al. 1998; Pasare & Medzhitov 2003). Indeed, IL-6 is partially responsible for the induction of IL-21 in DC-driven assays, as other members of our group have shown that IL-21 expression is reduced in the presence of an anti-IL-6 antibody (our unpublished data). It is also possible that the observed differential dependence on particular methods of co-stimulation determines the
distinct cytokine expression patterns shown here. In support of this, it has previously been shown that CD80 and CD86 differentially skew the balance between the Th1 and Th2 lineages in a mouse model of experimental autoimmune encephalomyelitis (EAE) (Kuchroo et al. 1995).

As mentioned previously, a number of stages are suggested to exist during the differentiation of Tfh cells. In a recently proposed model, initiation of the Tfh program was mediated by interactions with DCs, whilst lineage commitment was determined later during interactions with B cells (Crotty 2011). It is therefore possible that the induction of IL-21 by DC-expressed IL-6 is partially responsible for Tfh initiation. That DCs induce IL-6-mediated IL-21 production by naïve CD4 T cells would be particularly important for the germinal center (GC) response, as GC B cells have been reported to lack the ability to produce IL-6 (Burdin et al. 1996).

Perhaps the most significant result observed during this chapter was the differential pathogenicity of DO11.10 TCR transgenic conventional T cells after culture with either B cells or DCs. After culture with DCs, these T cells were highly diabetogenic upon adoptive transfer into CD28/-mOVA+ recipients, with hyperglycaemia observed in all recipients within 12 days. On the other hand, recipients of conventional T cells cultured with B cells had significantly lower blood glucose levels until at least day 21. Further investigation revealed significant islet infiltration only in recipients given DC-stimulated T cells, with these cells accumulating preferentially at this site over any other. In contrast, B cell-stimulated T cells accumulated in the pancreatic lymph node, with only small numbers observed in the pancreas.
Because of the differences in cytokine expression observed between B cell and DC cultures, we wondered whether these might be responsible for the observed differences in conventional T cell diabetogenicity. Traditionally, IFNγ has been associated with type-1 diabetes as IFNγ blockade was protective in NOD mice (Debray-Sachs et al. 1991; Campbell et al. 1991), as was T-bet deficiency, the master transcription factor for IFNγ-producing Th1 cells (Esensten et al. 2009). Interestingly, whilst conventional T cells stimulated with B cells expressed high levels of IFNγ both pre-transfer and in vivo, they did not mediate diabetes onset. However, whilst DC-driven T cells made very little IFNγ in vitro, they expressed even higher levels than B cell-stimulated T cells in the pancreas, and were highly pathogenic. As T cells co-stimulated with DCs expressed low levels of IL-21 in the pancreas, and because IL-21R/- T cells did not accumulate at this site, it is possible that IL-21 facilitates tissue entry of these cells, whilst IFNγ might then be critical for islet inflammation and destruction. A recent report by Sarra and colleagues provides considerable support for this hypothesis. Their data demonstrate that provision of IL-21 enhances skin infiltration of CD4+ T cells in a model of psoriasis, and that its neutralisation reduces inflammation. Crucially, they show that skin-infiltrating CD4+ T cells express high levels of IFNγ, and that in the same experiments conducted with IFNγ/- mice, IL-21 is still capable of mediating entry into the skin, but inflammation no longer occurs (Sarra et al. 2011).

We also considered whether the differential expression of CD62L between conventional T cells stimulated with B cells or DCs might account for the differences in pathogenicity observed. B cell-driven T cells expressed far higher levels of CD62L pre-transfer, and because CD62L mediates lymph node entry, we wondered whether this might account for their accumulation in the pancreatic lymph node. Indeed, others have shown that adoptively
transferred autoreactive CD4 T cells expressing low levels of CD62L demonstrated enhanced pathogenicity compared with their CD62L^{hi} counterparts (Amend et al. 2006). In agreement with this, adoptive transfer of DC-stimulated IL-21R^{-/-} conventional T cells, which expressed significantly higher levels of CD62L, did not lead to diabetes onset, and did not accumulate in the pancreas.

In considering the effects of IL-21 on the above processes, it is important to consider that data generated using IL-21R_{α}-deficient conventional T cells must be interpreted with the caveat that a significant proportion expressed Foxp3 by day 21 post-transfer, which may have suppressed the response. It is possible that this expression represents the expansion of a small population of contaminating Foxp3^{+} Treg, which could not be completely excluded during conventional T cell isolations before in vitro culture. It might also represent de novo Foxp3 induction, a phenomenon reported by others where IL-21 signalling to T cells is disrupted (Bucher et al. 2009). It is not possible to assess Foxp3 expression in T cells pre-transfer, as this process requires fixation and permeabilisation. One way to explore whether our data represents Foxp3 induction or expansion of Foxp3-expressing cells would be to utilise T cells from Foxp3-GFP mice, as this would allow us to transfer highly pure Foxp3^{-} populations.

In future experiments it will be important to further investigate the apparent difference in pathogenicity between conventional T cells stimulated with B cells versus DCs. As we observed increased Foxp3 expression using IL-21R^{-/-} T cells, we could alternatively try blocking IL-21 signalling using an IL-21R-Fc chimera, although this would not allow us to distinguish between the effects of IL-21 on donor cells and on recipient mice. We could also use blocking anti-CD62L antibody in B cell-driven T cell transfers to see if this gives rise to
equivalent pathogenicity to T cells stimulated with DCs. As DC-cultured T cells were present in far higher numbers in the pancreas, it is possible that they were more readily able to enter this site via the expression of relevant chemokine receptors or adhesion molecules. Assessing differential expression of these molecules between DC and B cell-stimulated T cells might therefore be informative. Chemokine receptors in particular would be of interest as IL-21 is thought to regulate CCR7 expression on DCs (Jin et al. 2009), and is produced at high levels in DC-driven responses.
7. General Discussion

In this thesis we have explored some of the fundamental mechanisms underlying CD4 T cell activation and regulation. These have significant implications for our understanding of both how immune responses to infectious agents are mounted, and the underlying causes of autoimmune disease. In previous chapters we have suggested that the induction of IL-21 by DCs, and the potent effects of this cytokine on CD86 expression by B cells, might be important during the development of T follicular helper (Tfh) cells. Bcl-6 is the master transcription factor for Tfh cells (Yu et al. 2009; Nurieva et al. 2009) and also critically regulates the germinal center B cell phenotype (Ranuncolo et al. 2007). Interestingly, it is known that Stat5, which is activated during IL-2 signalling, potently represses Bcl-6 expression in B cells (Walker et al. 2007; Scheeren et al. 2005). Further, it has recently been shown that the induction of Blimp-1 expression by IL-2-mediated Stat5 activation potently suppresses Bcl-6 expression, and thus Tfh induction (Johnston et al. 2012). The work presented in this thesis therefore suggests that IL-21 may further promote Bcl-6 expression indirectly, by inhibiting IL-2 production.

Over the past year, a number of groups have observed a distinct Treg population found in germinal centers (GCs), termed T follicular regulatory cells (Tfr) (Linterman et al. 2011; Chung et al. 2011; Wollenberg et al. 2011). These cells display a phenotype characteristic of Tfh cells, expressing PD-1, CXCR5 and Bcl-6, but also express Foxp3 and Blimp-1. They derive from thymic Treg, rather than naive CD4 T cells or Tfh cells, and are thought to be crucial for regulating germinal center reactions, exerting control over both Tfh and GC B cell numbers (Linterman et al. 2011). It is yet to be determined whether IL-2 is critical for the
homeostasis of this novel population, though the fact that they derive from Foxp3+ precursors strongly suggests that this is the case. If so, the inhibition of IL-2 expression by IL-21 may serve to regulate Tfr numbers, permitting appropriate germinal center reactions. Loss of this pathway in IL-21R−/− animals may therefore lead to an increase in Tfr numbers, which may contribute to the significantly diminished germinal center responses observed in these mice (Linterman et al. 2010).

IL-21 is known to play a crucial role in a number of autoimmune diseases. A particularly strong link has been implicated with autoimmune diabetes, as disrupted IL-21 signalling in NOD mice is protective (Spolski et al. 2008; Sutherland et al. 2009; Datta & Sarvetnick 2008). However, mechanistic data concerning the role played by IL-21 in this disease is lacking. Jin and colleagues have shown that IL-21 is essential for the expression of CCR7 by DCs. Thus, in its absence these cells do not traffic from the skin to present antigen in draining lymph nodes in a model of atopic dermatitis (Jin et al. 2009). Lack of antigen presentation by DCs might therefore account for the lack of islet infiltration seen in IL-21R−/− NOD mice. However, it is suggested that protection is mediated by a T cell defect in these animals, due to the reduced expression of IL-17 by CD4 T cells. Although IL-21 can promote Th17 differentiation, only Spolski and colleagues observed reduced IL-17 production in IL-21Rα-deficient NOD animals (Spolski et al. 2008), whilst others showed an increase in the production of this cytokine (Sutherland et al. 2009). A role for IL-17 in mediating the effects of IL-21 during autoimmune diabetes is therefore controversial, although some insight might be gained by assessing NOD mice treated with anti-IL-17 antibody, whom are partially protected (Emamaullee et al. 2009).
As IL-21R-/ NOD mice demonstrated an almost complete lack of islet infiltration, it is possible that, as suggested in chapter 6 and by Sarra and colleagues (Sarra et al. 2011), IL-21 is crucial for the entry of autoreactive T cells to sites where autoantigen is expressed. It may also be the case that IL-2 production is increased in these animals and that this leads to an increase in the number of Treg, preventing disease. Although Treg frequencies in the pancreatic lymph node appeared to be equivalent between wildtype and IL-21R-/ NOD mice, their numbers in the pancreas were not assessed (Spolski et al. 2008; Sutherland et al. 2009). Potential alterations in Treg homeostasis could certainly be important, as many studies have established a link between reduced Treg frequencies and autoimmune disease. For example, anti-CD25 treatment of DO11xRIP-mOVA mice to deplete Treg lead to diabetes onset 6 weeks earlier than in untreated animals (Clough et al. 2008).

Regulatory T cell homeostasis and function is critically dependent on conventional T cell-expressed cytokines. TNFα has been reported to boost Treg function (Grinberg-Bleyer et al. 2010b) and IL-2 is crucial for their survival and metabolic fitness (Fontenot et al. 2005b; D'Cruz & Klein 2005). This dependency stems from the inability of Treg to produce cytokines such as IL-2, due to their expression of the transcriptional repressor Foxp3. As a result, the size of the Treg pool can be directly linked to the number of IL-2 producing conventional T cells (Almeida et al. 2006). The demonstration that IL-21 can directly inhibit IL-2 expression by CD4 T cells, and that this has a marked impact on Treg homeostasis, suggests that a balance between these two cytokines may be crucial for the maintenance of immune tolerance.
The opposing roles of IL-2 and IL-21 are not limited to Treg homeostasis. It has previously been reported by Hinrichs and colleagues that IL-21 promotes CD8 T cell effector function, and can therefore enhance tumour regression in an adoptive transfer model (Hinrichs et al. 2008). In this study, by contrast, IL-2 potently inhibited CD8 T cell function, with subsequent inhibition of tumour regression. These cytokines were further linked, as IL-21 was able to counteract the effects of IL-2 (Hinrichs et al. 2008), suggesting that the balance between IL-2 and IL-21 may also be crucial in determining CD8 T cell responses. It has recently been suggested that relative levels of IL-2 and IL-21 mRNA expression in NOD mice may be important in determining protection against diabetes. The IL-2 and IL-21 genes are located within the NOD Idd3 locus, which is strongly associated with susceptibility to diabetes. In NOD mice, these genes are equivalently highly transcribed, but the instability of IL-2 mRNA is such that very little protein is actually produced. On the other hand, IL-21 mRNA is far more stable, leading to enhanced expression of IL-21 relative to IL-2. Importantly, this difference was not observed in diabetes resistant C57BL/6 mice (McGuire et al. 2009). In addition, reduced IL-2 production due to the loss of one Il2 allele lead to immune dysregulation and autoimmunity (Yamanouchi et al. 2007).

Collectively, these data suggest profoundly opposite effects for IL-2 and IL-21 on numerous cell populations, and that imbalances between these cytokines may predispose to autoimmunity. The effects of these cytokines on the Treg pool suggest that therapeutic strategies designed to boost Treg in autoimmune disease may be more effective when incorporating methods that inhibit IL-21 signalling. Conversely, in the case of cancer therapies, Treg depletion strategies that incorporate IL-21 provision may be particularly effective in promoting anti-tumour immunity. With respect to this latter point, it has recently
been shown that anti-CD25 treatment in combination with an IL-21 secreting cellular vaccine
cured 70% of recipients in a mouse tumour model, whereas Treg depletion alone had no effect
(Comes et al. 2006).
APPENDIX

C10
RPMI 1640 (GIBCO)
100U/ml penicillin (GIBCO)
100µg/ml streptomycin (GIBCO)
50µM 2-Mercaptoethanol (Sigma)
10% FCS (2% for C2) (Sigma)

P2
PBS (Sigma)
2% FCS (Sigma)

Lysis buffer
15mM TRIS hydrochloride
112mM ammonium chloride
pH 7.2

Pancreas buffer
PBS (Sigma)
5% FCS (sigma)
56mM glucose (Sigma)
2µg/ml aprotinin (Roche)
50µg/ml TLCK (Roche)
**Pancreas digest solution**

PBS (Sigma)

15% FCS (Sigma)

50µg/ml Liberase CI (Roche)

50µg/ml DNAse (Sigma)

**MACS buffer**

PBS (Sigma)

0.5% FCS (Sigma)

2mM EDTA (Sigma)


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