CONTROL OF T CELL INTERLEUKIN 21 PRODUCTION IN A MOUSE MODEL OF TYPE-1 DIABETES

by

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ABSTRACT

IL-21 is a potent immune modulator crucial for the generation of protective anti-viral and humoral responses. Nonetheless, the detrimental effects of IL-21 are well documented in a variety of autoimmune diseases including type-1 diabetes. Although elevated IL-21 mRNA expression has been reported in mouse models of diabetes, it remained unclear which cells are responsible for its production and whether this cytokine was expressed by cells that infiltrate the pancreas. We addressed these questions by evaluating IL-21 production in the DO11xRIP-mOVA mouse model of type-1 diabetes. Our findings demonstrated that conventional CD4 T cells are the main source of IL-21 protein and T cell expression of this cytokine was markedly enriched within the pancreas, suggesting a potential role at the site of the autoimmune attack.

Since both dendritic cells and B cells are abundant within the pancreatic lesion, we explored the capacity of these cells to contribute to T cell IL-21 production. Our investigation revealed that bone marrow-derived dendritic cells are constitutively able to support T cell IL-21 production, whereas B cell stimulated cultures required additional stimuli such as the provision of exogenous IL-6. Interestingly, our study identified a novel CD25+ innate lymphoid cell population in the pancreas, which appears to be a counterpart of the innate lymphoid cell populations recently described in the gut and lungs. Pancreas-derived CD4-CD25+ innate lymphoid cells could promote T cell IL-21 production *in vitro*, raising the possibility that this population contributes to T cell IL-21 production within the autoimmune lesion.

Collectively, these data suggest that IL-21 production is a characteristic feature of pancreas-specific CD4 T cell responses. A better understanding of how this cytokine is elicited and how it contributes to autoimmune pathology is likely to be valuable for future therapeutic interventions.

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LIST OF ABBREVIATIONS

AID……..activation-induced cytidine deaminase

AIRE…….autoimmune regulator

APC…….……antigen presenting cells

BCR…….……..B cell receptor

CCR……...C-C chemokine receptor

CD…….....cluster of differentiation

CLP……...……..common lymphoid progenitors

CLR……...…….. C-type lectin receptors

CMP…….....common myeloid progenitors

CNS……..central nervous system

CSR……...class-switch recombination

cTEC……..…..cortical thymic epithelial cells

CTL……...cytotoxic T lymphocytes

CTLA-4…….…… cytotoxic T lymphocyte antigen 4

CXCR…….. ……..CXC chemokine receptors

DC……..dendritic cells

DN…….……..double negative

DP…….double positive

EAE…….....……..….experimental autoimmune encephalomyelitis

Foxp3…….....forkhead box P3

gp……..glycoprotein

HSC…….haematopoietic stem cells

ICAM……intercellular adhesion molecule

iDC…….....immature dendritic cells

IFNγ……...interferon γ

Ig……...immunoglobulins

IL……...…..interleukin

ILC…….innate lymphoid cells

IPEX……..……immunodysregulation, polyendocrinopathy, enteropathy, X-linked

Jak…….…...…janus kinase

LCMV…… lymphocytic choriomeningitis virus

LIF……..leukemia inhibitory factor

LN……..lymph node

LPS……...lipopolysaccharides

LTi…….....lymphoid tissue-inducer

MAPK……....mitogen-activated protein kinases

MBL……..……..mannose-binding lectin

mDC…….....mature dendritic cells

MHC……...major histocompatibility complex

mRNA……...……messenger ribonucleic acid

MS…..……..…….multiple sclerosis

mTEC……..……medullary thymic epithelial cells

NCR……....natural cytotoxicity triggering receptor

NK……..……. natural killer

NOD……..……. non-obese diabetic

OSM……oncostatin M

OVA…….ovalbumin

PAMP……...…….pathogen-associated molecular patterns

PD-1…….programmed death 1

PI3K……..phosphatidylinositol 3-kinase

PMA…….phorbol myristate acetate

PRR……...…….pattern recognition receptors

R…….…….receptor

RA…….rheumatoid arthritis

RAG……..……...recombination-activating gene

RIG-I……..….retinoic acid– inducible gene I

SHM…….……somatic hypermutation

SLE……..….systemic lupus erythematosus

SP……..…….single positive

Stat…… signal transducer and activator of transcription

Tconv……...……conventional T cells

TCR……..T cell receptor

Tfh……...T follicular helper

TGFβ……..transforming growth factor β

Th……T helper

TLR……. toll-like receptors

TNFα…….……....tumour necrosis factor α

Treg…….…….regulatory T cells

T1D……...……type-1 diabetes

V(D)J……..variable, diversity, joining

WT……..wildtype

1. INTRODUCTION

1.1. The immune response

The emergence of multicellular organisms (metazoans), providing a host environment for pathogenic microorganisms, resulted in the development of defense mechanisms to counteract this invasion. Despite continuous exposure to infectious agents, two separate arms of the immune system, namely innate and adaptive, mediate protection in vertebrates. Innate immune mechanisms serve as a first line of defense, facilitating immediate responses to a broad range of pathogens. Once the innate component is overwhelmed or subverted, the adaptive arm orchestrates highly specific responses that generate immunological memory for future protection. It has recently become apparent that the previously underappreciated interplay between these two complex systems is vital for protection against infectious agents.

1.1.1. Innate immunity

Prior to full engagement of the innate arm of the immune system, pathogens encounter a physical barrier consisting of host epithelium and internal mucosa that serve as a first level of defense. These tissues produce anti-microbial peptides, chiefly represented by defensins and cathelicidins, which provide an additional mechanism of protection at entry sites (Zanetti et al. 1995; Ganz 2003). Pathogens that manage to evade these physical and chemical components are targeted by various innate immune cells, which identify unique structures called pathogen-associated molecular patterns (PAMP). This ancient recognition of non-self is mediated by germline-encoded pattern recognition receptors (PRR) that are expressed by macrophages, neutrophils, dendritic cells (DC), mast cells, eosinophils and natural killer (NK) cells

(Janeway & Medzhitov 2002). One of the best described PRR families are the Toll-like receptors (TLR), which sense the presence of numerous bacterial and viral components by interacting with many ligands, for example: lipoprotein (TLR2), dsRNA (TLR3), LPS (TLR4), flagellin (TLR5), diacyl lipoprotein (TLR6) and CpG-DNA (TLR9) (Beutler 2009; Takeuchi & Akira 2010). In addition to TLR, C-type lectin receptors (CLR) are another class of transmembrane PRR, whereas the retinoic acid– inducible gene I (RIG-I)–like receptors (RLR) and the nucleotide-binding oligomerisation domain (NOD)-like receptors (NLR) represent their cytoplasmic counterparts (Takeuchi & Akira 2010; Iwasaki & Medzhitov 2010).

The complement system is another major defense mechanism of the innate immune arm against microbial agents. Binding of the C1q protein directly to the pathogen surface, C-reactive protein or antigen-antibody complexes activates the classical pathway of the complement cascade. Other pathways of complement mediated killing are triggered by the binding of the C3b protein to the pathogen surface (alternative pathway) or by the interaction between the mannose-binding lectin (MBL) receptor with various carbohydrates expressed by pathogens on their surface (lectin pathway) (Ricklin et al. 2010). Most of the infectious agents that are recognised by PRR or are marked by complement components are ultimately engulfed and digested by phagocytic cells such as neutrophils and macrophages. The failure of innate defenses to completely remove pathogenic microbes leads to the induction of adaptive mechanisms, which orchestrate further responses.

At the interface of the innate and adaptive arm of the immune system sit phagocytic cells such as DC and NK cells, which induce effector functions mediated by the adaptive compartment

(Hoebe et al. 2004). For example, bacterial products recognised by TLR on the surface of DC are taken up and trafficked into phagosomes to be later processed and presented to T cells by major histocompatability complex (MHC) class II molecules. While DC use the above mechanism for the detection of extracellular pathogens, they can also mediate the activation of the adaptive immune response when infected by intracellular pathogens such as viruses. In this instance, the recognition of viral PAMP through cytosolic RLR leads to presentation of viral proteins on MHC class I molecules (Iwasaki & Medzhitov 2010). Importantly, whether activated by extracellular or intracellular pathogens, DC provide additional signals that modulate T cell responses.

1.1.2. Adaptive immunity

The generation of a highly diverse receptor repertoire specific for a broad range of pathogen antigens is a principal feature of the adaptive immune system. Unlike germline-encoded PRR, receptors of the adaptive immune system are a result of genetic recombination that occurs during their formation. The main cellular components of the adaptive immune arm consist of T and B lymphocytes. It is their receptors that recognise pathogen-derived antigens and deliver activation signals to these immunocompetent cells (Litman et al. 2010). A unique feature of the adaptive immune system is also the ability to generate immunological memory. Both B and T cells facilitate rapid secondary responses triggered by the recognition of previously encountered cognate antigens.

The genetic rearrangement that results in assembly of the adaptive immune receptors, namely immunoglobulins (Ig) and T cell receptors (TCR), occurs in B and T lymphocytes and is termed V(D)J recombination. This process involves the rearrangement of variable (V),

diversity (D) and joining (J) gene segments, and is initiated by recombination-activating gene 1 (RAG1) and 2 (RAG2) proteins in B and T lymphocyte precursors (Gellert 2002). V(D)J recombination results in the generation of a hugely diverse immunoglobulin and TCR repertoire, which covers a broad spectrum of foreign peptides, ensuring robust immune responses.

Initially, immunoglobulins serve as a high affinity membrane-bound receptor (BCR) on naïve B cells. At the intermediate stage antigen-experienced B cells form close interactions with T helper cells and differentiate into short-lived plasma cells or initiate germinal centre formation. During the germinal centre reaction B cells bearing high specificity receptors are selected and differentiated into long-lived memory B cells. Subsequent encounter with cognate antigen leads to rapid expansion of memory B cells and their differentiation into immunoglobulin-secreting plasma cells. (McHeyzer-Williams & McHeyzer-Williams 2005). Likewise, naïve T cells bearing TCR highly specific for cognate antigen undergo activation and clonal expansion. As a result, a small fraction of T cells enter the memory pool, which confers rapid recall responses upon antigen re-challenge (Sallusto et al. 2004).

1.1.3. T lymphocytes

T lymphocytes are derived from haematopoietic stem cells (HSC) in a multistep process that involves migration from the bone marrow to the thymus and ultimately into the periphery. HSC with self-renewing potential that reside in the bone marrow differentiate into common lymphoid progenitors (CLP) or common myeloid progenitors (CMP). It is CLP that are developmentally lymphoid lineage restricted and give rise to B and T lymphocytes. These lineage negative precursors express high levels of FLT3 and IL-7Rα, but low levels of SCA-1

and KIT expression (Bhandoola & Sambandam 2006). In the next stage, T cell precursors exit the bone marrow and colonise the thymic parenchyma to undergo further development and selection. At this point, these cells are double negative (DN) for CD4 and CD8 expression. DN thymocytes then migrate into the cortex, where they differentiate into CD4+CD8+ double positive (DP) thymocytes. Successful TCRβ gene rearrangement mediated by pre-TCR signals is a prerequisite for progression from the double negative (DN, CD4-CD8-) to the double positive (DP, CD4+CD8+) stage (Boehmer et al. 2003). At the next checkpoint, termed positive selection, thymocytes migrate through the thymic cortex wherein they encounter cortical thymic epithelial cells (cTEC) displaying peptide-MHC complexes. Cells bearing TCR that form stable interactions with self-MHC molecules are selected for further differentiation, whereas the remaining CD4+CD8+ compartment undergoes death by neglect. Consequently, self-MHC restricted DP thymocytes become single positive (SP) CD4 or CD8 cells depending on whether they bind to MHC class II or MHC class I respectively (Kyewski & Klein 2006). As this part of differentiation also enriches for thymocytes that recognise self-antigen, further checkpoints are required to prevent autoreactive T cells entering the periphery. Thus, central tolerance mechanisms encompass the subsequent CCR7-driven migration of self-MHC restricted thymocytes to the thymic medulla, and the process of negative selection that occurs in this compartment. After tolerisation mechanisms eliminate SP thymocytes bearing self-reactive TCR, the remaining fraction exits the thymus into the periphery, where they constitute the naïve T cell pool (Takahama 2006).

Unlike with humoral immunity, the activation of the cellular component of the adaptive immune system requires antigenic peptides to be presented in the form of peptide-MHC

complexes to T cells. This process is MHC restricted, which means that peptides displayed by MHC class I molecules are recognised by T cells bearing the co-receptor CD8, whereas peptides displayed by MHC class II molecules are recognised by T cells bearing the co-receptor CD4 (Rudolph et al. 2006). MHC class I is typically expressed on the surface of nucleated cells, which allows infected cells to present antigens derived from intracellular pathogens to CD8 T cells. MHC class II is constitutively expressed on the surface of professional antigen presenting cells (APC) and displays antigens derived from extracellular pathogens to CD4 T cells (Van Laethem et al. 2012; Neefjes et al. 2011).

The initial signal required for T cell activation is triggered by ligation of the TCR by a peptide-MHC complex. TCR exist in two distinct heterodimers assembled from either α and β or γ and δ chains, in both cases joined by a disulfide bond (Rudolph et al. 2006). The majority of T cells found in the thymus and lymph nodes express αβ TCR, whereas the small fraction of T cells that bear γδ TCR reside in the skin and intestinal epithelium (Ciofani & Zúñiga-Pflücker 2010). Upon ligand interaction, αβ TCR form a complex with the ζζ homodimer and CD3, which consists of εδ and εγ heterodimers. This conformational change leads to a phosphorylation of CD3 subunits and ζ chains that ultimately activates ZAP-70. The subsequent signalling cascade activates genes responsible for the regulation of T cell function (Brownlie & Zamoyska 2013).

Although TCR ligation is a prerequisite for T cell activation, other co-stimulatory signals are also required. Following TCR engagement, the CD28 pathway provides the major co-stimulatory signal that enhances T cell responses (Jenkins et al. 1991). CD28 signalling is triggered upon its interaction with CD80 or CD86. Both ligands are expressed at low levels on

the surface of APC such as DC, B cells and monocytes. Upon pathogenic encounter, APC become activated and enhance their expression of CD80 and CD86 ligands (Lenschow et al. 1996). The absence of CD28 signals in T cells that received TCR ligation results in incomplete activation and T cell anergy (Jenkins & Schwartz 1987). This is part of the tolerance mechanisms, which ensures that T cells become unresponsive to autoantigens that they might encounter in the periphery (Schwartz 2003).

The engagement of both TCR and CD28 results in signal transduction mediated by the phosphoinositide 3- kinase (PI3K) family of proteins. As a part of the intracellular signalling machinery, PI3K plays a key role in T cell development, function and differentiation (haug 2013). For example, the p110δ catalytic subunit of class IA PI3K, the expression of which is restricted to leucocytes, is associated with signalling events during T cell activation. Murine T cells ($p110\delta^{D910A}$) with a catalytically inactive form of p110δ are characterised by reduced immune responses due to attenuated TCR and CD28 signals (Okkenhaug et al. 2002). Furthermore, $p110\gamma^{D910A}$ T cells have a decreased ability to proliferate and produce cytokines in antigen-specific responses with co-stimulation provided by APC (Okkenhaug et al. 2006).

Although co-stimulation is predominantly dependent on CD28 signals, T cell express a variety of other molecules that regulate their activation and function. A further T cell co-stimulatory pathway is mediated by ICOS, a CD28 superfamily member, which is rapidly upregulated on naïve T cells upon TCR ligation (Hutloff et al. 1999). This co-stimulatory molecule not only regulates T cell function through the promotion of IL-4, IL-5 and IL-13 but also provides signals during B and T cell interactions in germinal centres (Coyle et al. 2000; Choi et al. 2011). In addition, the CD154 molecule expressed on activated T cells has the

capacity to modulate adaptive immune responses mediated by B cells. The ligation of CD40 on B cells, which is the receptor for CD154, delivers signals that strongly promote humoral responses mediated by these cells. Furthermore, CD40/CD154 interactions enhance antigen presentation and the expression of a variety of accessory molecules and soluble mediators by APC, which in turn potentiate T cell responses (reviewed in (Quezada et al. 2004)). Other significant co-stimulatory molecules involved in the modulation of T cell responses include the CD28 superfamily member PD-1 (Freeman et al. 2000) and the TNFR family member OX40 (Kopf et al. 1999). The negative regulation of T cell responses is primarily mediated by CTLA-4 engagement by CD80 and CD86, ligands that are shared with CD28. Higher affinity for CD80 and CD86 allows CTLA-4, which is rapidly induced in activated T cells, to effectively compete with CD28 for their binding (Peach et al. 1994). The potency of this negative regulation becomes apparent in CTLA-4-/- mice, who die prematurely due to uncontrolled lymphoproliferation (Waterhouse et al. 1995; Tivol et al. 1995). Therefore, the opposing functions of CD28 and CTLA-4 play a decisive role in the modulation and fine-tuning of T cell responses (Sharpe & Freeman 2002).

Once antigen is presented in the context of MHC and co-stimulation signals are provided, naïve T cells become fully activated and undergo clonal expansion. Mature T cells then exit lymphoid organs and migrate to sites of infection to perform various effector functions. MHC class I restricted CD8 T cells mediate immune responses targeted towards intracellular pathogens. Infected cells can by destroyed by CD8 T cells either via induction of programmed cell death mediated by the Fas/FasL pathway, or via cell lysis mediated by localised perforin and granzyme release (Ju et al. 1995; Berke 1995). Due to their potent cytolytic properties, CD8 T cells are also termed cytotoxic T lymphocytes (CTL). Unlike CD8 T cells, CD4 T

cells orchestrate immune responses by providing help to other immune cells. Following activation, CD4 T cells release a variety of soluble mediators such as cytokines and chemokines, which modulate inflammatory, allergic, parasitic, humoral, cytolytic and regulatory responses (Swain et al. 2012).

1.1.4. B lymphocytes

B lymphocytes arise from the CLP during the differentiation process that occurs in the bone marrow. The early stages of B cell development are divided into fraction A, B, C and D, which are defined by the expression of specific surface markers. B cell precursors from fraction A to D are characterised by the expression of B220, but absence of immunoglobulins on the cell surface. Progression from fraction A to D is accompanied by V(D)J rearrangement and relies on various micro-environmental factors such as CXCL12, FLT3L, IL-7, SCF and RANKL. Immature B cells are derived from fraction D, and are positive for B220, CD19 and IgM, but negative for IgD. These cells migrate to secondary lymphoid organs such as the spleen, where they upregulate their expression of IgD to give rise to the mature B cell phenotype. Upon activation mature B cells differentiate into immunoglobulin-producing plasma cells that mediate humoral responses (Nagasawa 2006).

The generation of highly specific humoral responses occurs in a T cell-dependent process, which results in the development of affinity-matured long-lived plasma cells and memory B cells. It requires MHC class II bound antigen to be presented by activated B cells to antigen specific T cells (Allen et al. 2007). Upon provision of T cell help, selected B cells enter germinal centres where they undergo somatic hypermutation (SHM). This immunoglobulin gene diversification mechanism is initiated by activation-induced cytidine deaminase (AID)

and results in generation of higher affinity immunoglobulins due to point mutations occurring in the antibody variable regions (Odegard & Schatz 2006). Further AID-dependent immunoglobulin diversification is achieved by a mechanism termed class-switch recombination (CSR) in which the Cμ immunoglobulin heavy chain constant region is exchanged for Cγ, Cα or Cε alternatives. CSR permits generation of functionally distinct IgG, IgA or IgE antibody classes with the same antigen specificity (Xu et al. 2012). Ultimately, immunoglobulin-secreting plasma cells colonise the bone marrow, where BAFF and APRIL signals support their survival (Goodnow et al. 2010).

1.2. Immunological tolerance and dysregulation

The production of a highly diverse lymphocyte receptor repertoire, which is mediated by V(D)J gene rearrangement, carries an inherent risk of generating self-reactive B and T cells capable of inflicting autoimmune tissue damage. In order to prevent self-recognition, central tolerance mechanisms exist in the bone marrow and thymus to remove self-reactive B and T lymphocytes at the developmental stage. Additionally, protection against inappropriate immune responses in the periphery is provided by various tolerising strategies collectively termed peripheral tolerance.

1.2.1. Central tolerance

Central tolerance is a rigorous multistep selection mechanism that occurs during T cell development in the thymus. As a result, only around 5% of thymocytes are able to progress through this stringent process to finally give rise to the naïve T cell pool in the periphery. It is now known that negative selection in the thymic medulla can be orchestrated by both medullary thymic epithelial cells (mTEC) and thymic DC (Klein et al. 2009). The promiscuous expression of tissue-restricted antigens by mTEC is mostly regulated by a transcription factor termed the autoimmune regulator (AIRE), although the expression of proteins such as C-reactive protein (CRP) and glutamate decarboxylase 67 (GAD67) is thought to be AIRE-independent (Hogquist et al. 2005). It has been reported that the presentation of tissue-restricted antigens by thymic DC is the result of antigen transfer from mTEC (Koble & Kyewski 2009). Ultimately, cells bearing TCR that strongly interact with self-antigens presented by either mTEC or thymic DC are deleted. In this way, negative selection prevents the entry of auto-reactive T cell clones into the periphery. In addition, it

also allows a small proportion of self-reactive CD4+ thymocytes to differentiate into Foxp3+ Treg, ensuring that tolerance is maintained beyond the confines of the thymus (Lee et al. 2011).

Unlike T cells that undergo selection in the thymus, B cells are subject to central tolerance mechanisms during their development in the bone marrow. Although V(D)J recombination generates a very high proportion of BCR that recognise self-antigens, signals delivered by such BCR result in a developmental block and further gene rearrangement occurs. This process, termed receptor editing continues until the recombination of the immunoglobulin light (IgL) chain gene assembles a BCR that is unable to interact with self-antigens. Cells that fail to successfully rearrange the BCR undergo clonal deletion in the bone marrow (Nemazee 2006). The above tolerance mechanisms therefore prevent auto-reactive B cells from developmental progression and entry to the periphery.

1.2.2. Peripheral tolerance

Although central tolerance mechanisms are in place in order to deplete self-reactive lymphocytes in the bone marrow and thymus, the drive to widen the receptor repertoire to recognise a broad range of pathogenic antigens permits a fraction of self-reactive lymphocytes to be exported to the periphery. As previously mentioned, various control mechanisms exist in the periphery to keep auto-reactive B and T lymphocytes at bay.

One such control mechanism is that encompassed by the concept of immune privilege. Certain sites such as the brain, placenta and eye are protected from T cell entry, thereby

preventing unwanted tissue damage that might be mediated by T cell clones specific for antigens expressed in these immunoprivileged organs. For example, molecules such as CD200, TRAIL, and FasL are thought to control the entry of T cells into neurological tissues. The interaction between the latter molecule and Fas receptor expressed on T cells can trigger programmed cell death of these cells (Forrester et al. 2008).

Self-reactive T cells can also exist in the periphery in a state of ignorance. In this scenario, tissue-specific antigens fail to activate auto-reactive T cells as they are sufficiently scarce to prevent effective antigen presentation (Kurts et al. 1998). Alternatively, tolerisation of self-reactive T cells in the periphery can be accomplished via Fas/FasL-mediated apoptosis, or by co-stimulatory pathway-mediated anergy. Another form of peripheral control is phenotypic skewing, in which activated self-reactive T cells fail to inflict tissue damage due to deviations in their cytokine and chemokine receptor expression patterns (Walker & Abbas 2002). Likewise, B cells that recognise self-antigens in the periphery are thought to be subjected to control mechanisms such as deletion and anergy (Goodnow et al. 1988; Russell et al. 1991).

Auto-reactive T cells that evade thymic selection might also be tolerised in a T cell-extrinsic manner in the periphery. One set of cells important for this process are forkhead box P3 (Foxp3) positive regulatory T cells (Treg), which constitutively express CD25 and CTLA-4. Naturally occurring Treg develop in thymus and enter into the periphery where they suppress unwanted immune responses via numerous inhibitory mechanisms (Seddon & Mason 2000; Roncarolo & Battaglia 2007). Early evidence from CD25 depletion experiments demonstrated that these cells are critical for maintaining peripheral tolerance (Sakaguchi et al. 1995).

1.2.3. Normal and dysregulated immune responses

The generation of a protective immune response requires cooperation of both the humoral (B cell-mediated) and cellular (T cell-mediated) arms of the adaptive immune system. Antigen recognition mechanisms initiate the immune response that removes intracellular pathogens in a T cell-dependent manner, whereas both B and T cells orchestrate the clearance of extracellular pathogens. Despite stringent selection processes in the bone marrow and thymus, and multiple control mechanisms in the periphery, self-reactive B and T cells are able to instigate tissue damage in a number of autoimmune disorders. Although autoimmunity is often divided into B cell or T cell driven pathologies, both cell types are often present at the site of immune attack. Furthermore, lymphocyte-mediated damage might affect multiple sites (systemic) or a single organ (tissue-specific).

B lymphocytes play a key role in a number of autoimmune diseases, with one of their major contributions being the production of autoantibodies. For example, systemic lupus erythematosus (SLE) is chracterised by the generation of high titers of autoantibodies specific for nuclear antigens. These autoantibodies are involved in the formation of immune complexes, which are deposited in multiple sites such as the skin, kidneys, lungs and vascular walls (Shlomchik et al. 1994). B cells also contribute to the inflammatory responses observed in rheumatoid arthritis (RA). Although this autoimmune disease is strongly associated with T cell activation, autoantibodies such as rheumatoid factor and anti-citrulline are present in the majority of RA patients (Steiner & Smolen 2002). The inflammation of the joint, which is a hallmark of RA, is partially attributed to the accumulation of immune complexes, which subsequently activate the complement system and attract neutrophils to the site (Yanaba et al. 2008).

A number of pathologies in the central nervous system (CNS) are typically associated with T cell-mediated autoimmunity. Despite the immunoprivileged status of the CNS, multiple sclerosis (MS) is a well-documented autoimmune disease of the neurological tissues. In this pathology, auto-reactive T cells specific for myelin antigens orchestrate destruction of the myelin sheath around nerves, which ultimately results in the loss of neurological function (Goverman 2009). Evidence from the experimental autoimmune encephalomyelitis (EAE) mouse model of MS revealed that CD4 T cells are the main culprits responsible for the autoimmune damage. Myelin-specific CD4 T cell were traditionally associated with IFNγ production, however more recent studies underlined the importance of IL-17-producing CD4 T cells in the demyelination process (O'Connor et al. 2008). Furthermore, in the EAE model the latter population promoted formation of the ectopic lymphoid follicles, structures typically observed in autoimmune disorders such as MS, Sjögren's syndrome, rheumatoid arthritis and myasthenia gravis (Peters et al. 2011).

A critical component of normal immune responses is the Treg-mediated control of self-reactive T cells. The failure to suppress inappropriate immune responses by Treg might be the result of their insufficient numbers or functional defects, but resistance to suppression developed by effector T cells may also be an important factor (Buckner 2010). The requirement for adequate Treg numbers in order to control autoimmunity is clearly demonstrated in patients with the immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), in which the Treg pool is absent or malfunctions due to mutations in the transcription factor Foxp3 (Wildin et al. 2002). The defective expression of cytokines and surface molecules by which Treg mediate suppression has also been implied as a key factor in

preventing the suppression of autoimmune responses (Vignali et al. 2008). Finally, several cytokines such as IL-2, IL-7, IL-15 and IL-21 permit the resistance of effector T cells to Treg-mediated suppression (Walker 2009).

1.3. CD4 T cell differentiation pathways

CD4 T cells play a critical part in the immune response to invading pathogens. Following antigen encounter, naïve T cells rapidly proliferate and differentiate into distinct effector T cell subsets. Over the years, it has become clear that the type of pathogenic challenge and the local microenvironment influence the outcome of CD4 T cell differentiation. Recently, it has become apparent that differentiated T cell subsets display significant plasticity, particularly in terms of cytokine production. Inappropriate CD4 T cell differentiation results in defective effector function, which is the feature of several pathologies. Commitment towards particular CD4 differentiation pathways is ultimately determined by the induction of specific master transcription factors responsible for lineage stability (**Fig. 1.01**).

1.3.1. T helper 1 cells (Th1)

Seminal work by Mosmann and Coffman classified T helper cells based on their signature cytokine expression profiles. It is now widely accepted that Th1 cells are characterised by their production of IFNγ (Mosmann & Coffman 1989), and that their differentiation is initiated by IL-12 signals (Manetti et al. 1993; Rogge et al. 1997). Expression of the IFNγ gene is controlled by the transcription factor T-bet, which stabilises commitment towards the Th1 lineage (Szabo et al. 2000). Th1 cells are typically associated with antiviral and antimicrobial responses and cell-mediated immunity (Paul & Seder 1994). For example, expression of IFNγ by this subset promotes macrophage-mediated microbial killing (Suzuki et al. 1988). However, inappropriate activation of the Th1 programme has been associated with numerous autoimmune disorders. Early studies have demonstrated that Th1 clones were shown to be highly efficient at inducing mouse EAE, whereas Th2 clones were not (Khoruts

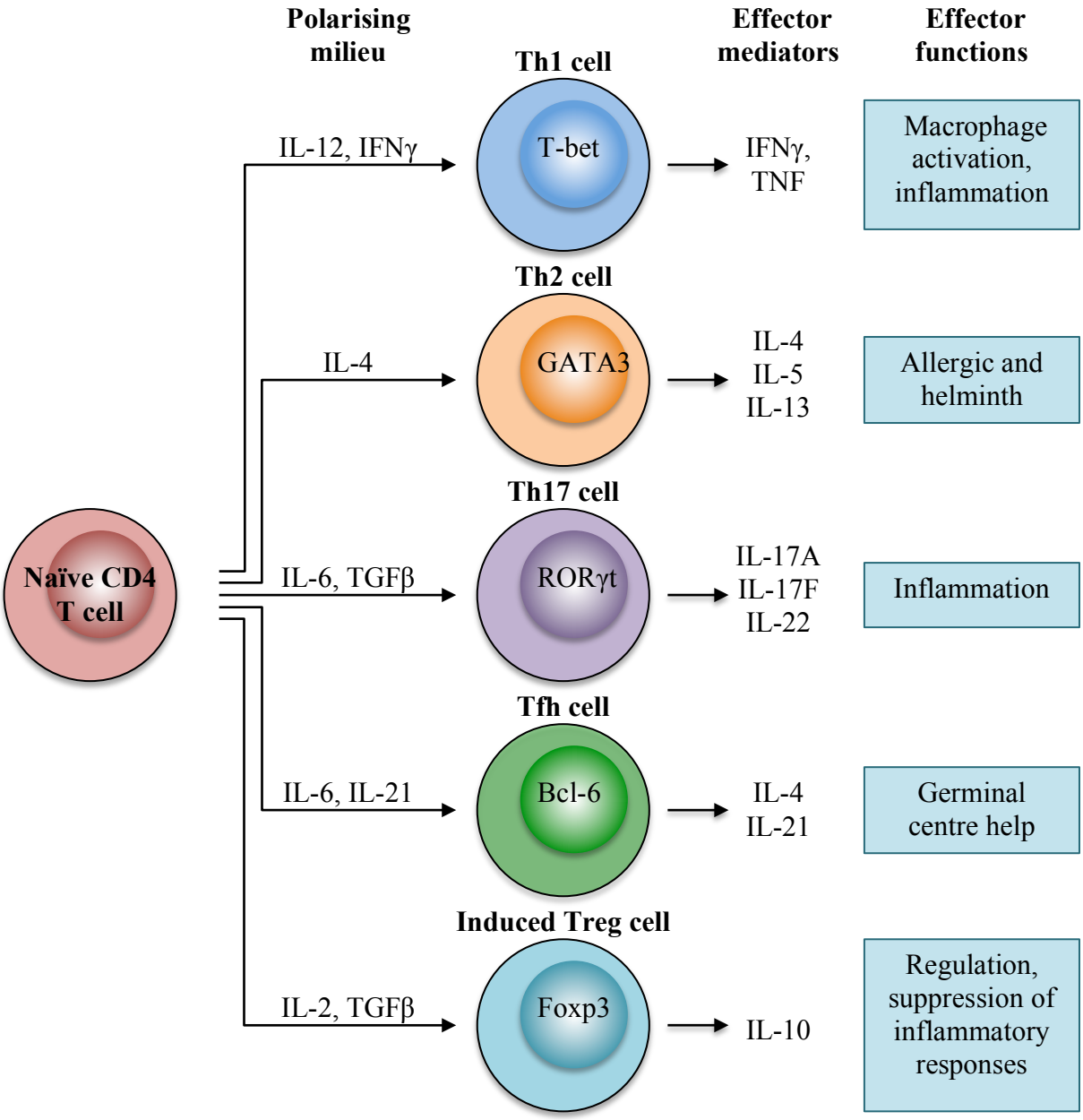


Figure 1.01. Differentiation pathways of activated CD4 T cells. Initially, Mosmann and Coffman defined the Th1 and Th2 differentiation pathways, induced by IL-12 and IL-4, respectively (Mosmann & Coffman 1989). Over the years, additional lineages including Th17, Tfh and induced Treg have been identified. Th17 lineage commitment is strongly associated with IL-6 and TGFβ signalling, whilst TGFβ signalling alone drives induced Treg differentiation. IL-21 and IL-6 are key inducers of the Tfh differentiation programme. Adapted from (Swain et al. 2012).

et al. 1995). Conversely, the administration of anti-IFNγ antibody in the non-obese diabetic (NOD) mouse model of type-1 diabetes reduced severity of disease (Debray-Sachs et al. 1991; Campbell et al. 1991).

1.3.2. T helper 2 cells (Th2)

Th2 cells were also originally described by Mosmann and Coffman, and are defined by their production of IL-4, IL-5 and IL-13 (Mosmann & Coffman 1989). Th2 differentiation is initiated by IL-4, which activates the master transcription factor for this lineage, GATA-3. Once activated, GATA-3 controls the expression of the IL-4, IL-5 and IL-13 genes (Zheng & Flavell 1997; Kopf et al. 1993). A requirement for IL-13 for Th2 differentiation has also been reported (McKenzie et al. 1998). Th2 cells play a crucial role in fighting extracellular parasites by promoting humoral responses mediated by B cells. The Th2 effector cytokine IL-4 is a key driver of B cell commitment towards IgE class switching, whereas IL-5 promotes recruitment of eosinophils (Geha et al. 2003; Coffman et al. 1989). However, exaggerated Th2 responses in allergy and asthma are also associated with increased production of IgE and recruitment of effector cells such eosinophils, basophils and mast cells. Ultimately, the cross-linking of mast cell-bound IgE by allergen leads to the degranulation and secretion of potent chemical mediators such as histamine and serotonin, which promote inflammation. (Sutton & Gould 1993).

1.3.3. T helper 17 cells (Th17)

Studies by Langrish and colleagues identified a distinct T cell population that drives autoimmunity in EAE mice by the production of IL-17 (Langrish et al. 2005). Initially, IL-23

was thought to be the main inducer of IL-17 production, later work however identified IL-6 and TGFβ as the key cytokines that initiate the Th17 programme (Veldhoen et al. 2006; Bettelli et al. 2006). Alternatively, Th17 differentiation can be promoted by IL-21 in concert with TGFβ, with the first cytokine acting in a self-potentiating manner (Korn et al. 2007; Nurieva et al. 2007). In the absence of the TGFβ signalling, the synergistic effect of IL-6, IL-23 and IL-1β has been shown to induce the Th17 programme (Ghoreschi et al. 2010). The activation of the Th17 differentiation pathway is controlled by the master transcription factor RORγt, which originally was thought to be induced by the TGFβ and IL-6 signalling (Ivanov et al. 2006).

Apart from IL-17, the Th17 cell subset is also associated with the secretion of IL-21 and IL-22 (Zheng et al. 2006; Liang et al. 2006). A protective effect of Th17 cells has been observed in infections mediated by gram-negative bacteria and fungi (Ye et al. 2001; Huang et al. 2004). The function of this population is also associated with host defence against *Bacteroides fragilis*, *Borrelia burgdoferi* and *Mycobacterium tuberculosis* (Infante-Duarte et al. 2000; Chung et al. 2003; LeibundGut-Landmann et al. 2007). However, there is also a strong link between Th17 cells and various autoimmune disorders. The deleterious effects of IL-17 signalling have been observed in animal models of arthritis (Bush et al. 2002; Nakae et al. 2003). Further observations confirmed that neutralisation of IL-17 in EAE mice resulted in improved clinical outcome (Hofstetter et al. 2005). An enrichment in the Th17 population was also found in SLE patients, and in the MRL/lpr mouse model of this disease (Yang et al. 2009).

1.3.4. T follicular helper cells (Tfh)

Tfh cells were initially identified by their expression of the chemokine receptor CXCR5, which allows them to enter B cell follicles and promote antibody-mediated responses (Breitfeld et al. 2000; Schaerli et al. 2000; Kim et al. 2001). These CD4+CXCR5+ T cells were further defined in mouse and human studies by their surface expression of ICOS and PD1, their expression of the transcription factor Bcl-6, and by their production of IL-21 (Chtanova et al. 2004; Rasheed et al. 2006; Vinuesa et al. 2005). Later observations confirmed that Tfh commitment is regulated by the induction of Bcl-6 (Nurieva et al. 2009; Johnston et al. 2009; Yu et al. 2009). Tfh cells provide crucial help to B cells to promote their differentiation into germinal centre B cells and plasma cells, thus supporting desired antibody-mediated immune responses. Conversely, the dysregulation of Tfh-mediated immunity has been linked with various autoimmune diseases. An increased proportion of circulating Tfh cells has been observed in SLE and rheumatoid arthritis patients (Simpson et al. 2010; J. Ma et al. 2012; Liu et al. 2012), and studies in mice also show an association between dysfunctional Tfh cell and lupus (Vinuesa et al. 2005; Linterman et al. 2009).

1.3.5. Regulatory T cells (Treg)

As previously mentioned, regulatory T cells are a critical component of peripheral tolerance mechanisms. These CD4+ suppressor T cells can be further divided into natural (n)Treg , which are exported from thymus, or induced (i)Treg, which differentiate from naïve T cells in the periphery. nTreg are thought to be selected in the thymus based on the high-affinity interaction between TCR and self-peptide in the medulla (Jordan et al. 2001). Initially, CD4+ Treg were identified by their constitutive surface CD25 expression, while Foxp3 was

subsequently proposed to be the Treg-specific transcription factor (Sakaguchi et al. 1995; Hori et al. 2003; Fontenot et al. 2003). Stable expression of Foxp3 in natural Treg, which is achieved by demethylation of its promoter, supports lineage commitment (Janson et al. 2008). The importance of Treg in maintaining peripheral tolerance becomes strikingly apparent in mice lacking Foxp3 expression. The absence of this master regulator results in severe lymphoproliferative disease and premature death (Brunkow et al. 2001).

The suppressive nature of Treg is thought to be associated with the expression of inhibitory cytokines such as IL-10, TGFβ and IL-35 (Asseman et al. 1999; Nakamura et al. 2001; Kearley 2005; Collison et al. 2007). In addition, constitutive expression of the inhibitory receptor CTLA-4 plays a key role in Treg-mediated suppression (Read et al. 2000). It is thought that CTLA-4 competes with its homolog molecule CD28 for binding of CD80 and CD86, therefore modulating co-stimulatory signals provided by APC although multiple other mechanisms have been proposed (Sansom & Walker 2006). The recent observation that CTLA-4 is capable of removing CD80 and CD80 ligands via trans-endocytosis, provided a novel explanation for the cell-extrinisic inhibitory function of this receptor (Qureshi et al. 2011).

The induction of Foxp3 expression in CD4+CD25- T cells mediated by provision of TCR and TGFβ signals gives rise to induced Treg (Chen et al. 2003). These cells phenotypically and functionally resemble natural Treg, but their Foxp3 expression can be transient. Several groups have demonstrated *de novo* induction of Treg in gut-associated lymphoid tissue. This conversion was facilitated by CD103+ DC co-stimulation, and depended on the synergistic effect of retinoic acid and TGFβ (Mucida et al. 2007; Coombes et al. 2007; Sun et al. 2007).

In addition to Foxp3-expressing Treg, various other regulatory subsets that lack the expression of this transcription factor have been identified. Th3 cells exert regulatory function through their secretion of TGFβ, a cytokine that is also required for their induction. These cells can be found in the gut-associated lymphoid tissue, where they modulate responses to oral antigens (Weiner 2001). Provision of IL-10 was shown to be important in the generation of a further regulatory T cell population, termed Tr1 cells. These cells are characterised by their production of IL-10, and have been shown to mediate the suppression of antigen-specific T cell responses in a mouse model of colitis (Groux et al. 1997). The suppressive capacity of Tr1 cells has been demonstrated to be independent of Foxp3 expression (Vieira et al. 2004).

1.3.6. Other proposed T helper cell subsets

Recent advances in understanding T cell differentiation processes led to the identification of two additional T cell lineages, namely Th9 and Th22, although their master transcription factors that are yet to be defined.

Two independent laboratories reported that IL-4 together with TGFβ supported the development of Th9 cells, which are characterised by their secretion of IL-9 and IL-10. Despite high levels of IL-10 production, Th9 cells were negative for Foxp3 expression and lacked suppressive capability (Dardalhon et al. 2008; Veldhoen et al. 2008). Indeed, the pro-inflammatory nature of Th9 cells was confirmed in Rag-1 deficient mice, where the adoptive transfer of this population promoted colitis and neuritis. Further support came from the study of EAE mice, wherein the adoptive transfer of Th9 cells supported disease development (Jäger et al. 2009).

The investigation of the signals that drive Th17 differentiation resulted in the observation that the IL-23-induced production of IL-22 mediated the development of skin inflammation in B6 mice treated with IL-23 (Y. Zheng et al. 2006). However, studies in humans suggested the IL-22-producing T cell population was distinct from Th17 cells, due to the low levels of the RORγt expression and the lack of IL-17 production (Duhen et al. 2009; Trifari et al. 2009). The expression of skin-homing receptors by these cells provides for their ability to modulate immune responses in the skin. Due to the limited concurrent expression of other cytokines with IL-22 in humans, it has been proposed that these cells should be termed Th22.

1.4. Interleukin 21

The generation of a protective immune response requires the collaboration of multiple cell types. Cytokines represent messengers of the immune system that provide essential communication between various cell populations. They deliver signals that tightly control the proliferation, differentiation and survival of the immune cells. Interleukin 21 (IL-21) is the latest addition to the common γ-chain cytokine family. This pleiotropic cytokine has been shown to be instrumental in the generation of protective immunity, although its deleterious effects are well documented and have been associated with a number of autoimmune diseases.

1.4.1. Interleukin 21 and its receptor

It has been more than a decade since Parrish-Novak *et al.* used cloning techniques to discover the IL-21 receptor (IL-21R) and subsequently its functional ligand IL-21 (Parrish-Novak et al. 2000). A parallel investigation by Ozaki and colleagues demonstrated that structurally IL-21R most closely resembled IL-2Rβ and IL-4Rα (Ozaki et al. 2000). Functional signalling through IL-21R requires assembly of a heterodimeric complex consisting of the IL-21Rα chain and the common γ-chain (Asao et al. 2001; Habib et al. 2002). Utilisation of the latter subunit makes IL-21 the most recent member of the common γ-chain cytokine family, which also includes IL-2, IL-4, IL-7, IL-9 and IL-15. IL-21 signalling is mediated by the recruitment of Jak1 and Jak3, which in turn activate Stat1 and Stat3 (Habib et al. 2002; Zeng et al. 2007). Furthermore, selective inhibition of the MAPK and PI3K pathways demonstrated that IL-21 also utilises these pathways for its signal transduction (Zeng et al. 2007). The initial investigation by Parrish Novak and colleagues revealed that the distribution of IL-21R was primarily lymphoid tissue restricted (Parrish-Novak et al. 2000). Later work established that a

variety of lymphoid cells including T cells, B cells, NK cells and DC expressed IL-21R (Jin et al. 2004; Brandt 2003; Leonard & Spolski 2005). In contrast to the extensive distribution of its receptor, IL-21 was originally found to be predominantly expressed by fully activated human CD4 T cells (Parrish-Novak et al. 2000). Since then, numerous CD4 T cell subsets have been associated with IL-21 production. Th1 cells were shown to express IL-21 mRNA, and IL-21 signals enhanced Th1 lineage commitment through the upregulation of T-bet and IFNγ (Chtanova et al. 2004; Strengell et al. 2002; Strengell et al. 2003). The expression of IL-21 mRNA has also been observed in the Th2 lineage (Wurster et al. 2002). Furthermore, IL-21 signals strongly supported the Th2 differentiation programme *in vivo* (Pesce et al. 2006; Frohlich et al. 2007). The Th17 lineage has been extensively associated with IL-21 production (Nurieva et al. 2007; Wei et al. 2007). In addition, IL-21 in concert with TGFβ was capable of replacing IL-6 signals in the promotion of Th17 differentiation (Korn et al. 2007). Impaired Th17 differentiation has also been reported in the absence of IL-21, which acts in a self-potentiating manner to maintain Th17 lineage commitment (Nurieva et al. 2007; Wei et al. 2007). A recent investigation using IL-21 reporter mice confirmed previous reports demonstrating a clear link between Tfh cells and IL-21 production (Lüthje et al. 2012; Chtanova et al. 2004; Vinuesa et al. 2005). In addition to CD4 T cells, activated NKT cells from the thymus, liver and spleen have shown to secret substantial amounts of IL-21 (Coquet et al. 2007).

Although the signals that promote IL-21 production are less well established, IL-6 has been suggested to be the main driver of IL-21 expression in T cells (Suto et al. 2008). This cytokine signals through a membrane-bound IL-6Rα (CD126) chain and a signal-transducing subunit glycoprotein 130 (gp130) (Lissilaa et al. 2010). The gp130 subunit is also commonly

used for signal transduction by other IL-6 family cytokines, such as IL-11, IL-27, leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotrophin-like cytokine (CLC) (Garbers et al. 2012). One study has indicated that IL-27 can induce IL-21 mRNA *in vitro* (Pot et al. 2009). Other investigations have demonstrated that Tfh maintenance and survival depends on IL-27-induced IL-21 production by these cells (Batten et al. 2010). In addition, human studies showed that IL-12 promoted the generation of IL-21-producing Tfh cells (Schmitt et al. 2009; Ma et al. 2009). IL-21 has been suggested to promote its own production by an autocrine feedback loop (Suto et al. 2008).

1.4.2. IL-21 in normal immune responses

IL-21 has been strongly associated with the generation of effective immune responses during chronic viral infections. Unlike in the acute phase of lymphocytic choriomeningitis virus (LCMV) infection, IL-21 signalling was essential for longer term antiviral CD8 T cell responses in the later stages of the infection. Furthermore, IL-21 acted directly on CD8 T cells, providing essential survival signals and maintaining their effector function (Elsaesser et al. 2009; Yi et al. 2009; Frohlich et al. 2009). Importantly, the exhaustion of CD8 cells due to defective IL-21 signalling might provide clues for our further understanding of immune responses in the presence of persistent antigenic burden, such as in the case of cancer.

The expression of IL-21 by Tfh cells is a prominent characteristic of effective humoral responses. Early experiments suggested that in the response to *Toxoplasma gondii* inoculation, IL-21R-/- mice had enhanced IgE production, whereas IgG1, IgG2a, IgG2b, and IgG3 titers were decreased. In humans, IL-21 promoted antibody class switching towards the IgG1 and IgG3 isotypes in anti-CD40 activated CD19+ B cells (Pène et al. 2004). IL-21 signalling to B

cells is known to be required for optimal affinity maturation, sustained Bcl-6 expression by germinal centre B cells and normal IgG1 production (Linterman et al. 2010; Zotos et al. 2010). In addition, IL-21 also regulates the germinal centre reaction by supporting Tfh differentiation (Nurieva et al. 2008; Vogelzang et al. 2008; Eto et al. 2011).

Numerous studies have demonstrated that IL-21 mediates anti-tumour responses by enhancing T cell and NK cell function. Syngeneic mice inoculated with murine colon carcinoma Colon 26 cells that constitutively expressed the IL-21 gene generated responses that lead to tumour destruction. This anti-tumour protection was characterised by increased lytic activity and IFNγ production by spleen cells from inoculated animals (Ugai et al. 2003). Likewise, B16F1 and MethA cells transduced to express IL-21 were rejected in *in vivo* studies of melanoma and fibrosarcoma tumor models, respectively. In these experiments, NK cells and CD8 T cells were thought to mediate anti-tumour responses through perforin secretion. Furthermore, pre-immunisation with irradiated B16F1-IL-21 cells provided a further protective effect in the great majority of mice that were re-challenged with B16F1 cells (Ma et al. 2003).

1.4.3. IL-21 in autoimmunity

Although IL-21 supports the generation of protective immunity, increasing evidence suggests that this cytokine appears to play a key role in the development of autoimmune disease. For example, it is thought that IL-21R signalling plays a major role in SLE. In sanroque mice, which develop a lupus-like pathology, gene expression analysis showed that CD4+ cells had increased expression levels of the IL-21 gene (Vinuesa et al. 2005). Moreover, this model is chracterised by elevated numbers of Tfh cells and high titers of autoantibody. Bubier and colleagues investigated the role of IL-21 in the development of SLE in BXSB-Yaa mice. The

levels of transcript for *il21* expressed in splenic T cells increased thoughout the progression of the disease in BXSB-Yaa compared with aged matched WT animals. The loss of IL-21 signalling in IL-21R-/- BXSB-Yaa mice prevented hypergammablobulinemia and autoantibody production. Furthermore, renal disease typical of SLE was absent in the kidneys of these mice (Bubier et al. 2009). In humans, enrichment in IL-21-producing CD4 and CD8 T cells was detected in the peripheral blood of SLE patients (Dolff et al. 2011).

IL-21 has also been implicated in the pathogenesis of multiple sclerosis. In the EAE model, injection of IL-21 before disease induction resulted in greater CNS infiltration and clinical score. Moreover, auto-reactive T cells isolated from these mice were more pathogenic in an adoptive transfer model of the same disease. These detrimental effects of IL-21 treatment were thought to be mediated by enhanced NK cell activity and IFNγ production (Vollmer et al. 2005). In addition, the impaired generation of Th17 cells in IL-21 deficient mice prevented EAE (Nurieva et al. 2007). The investigation of MS lesions in humans demonstrated that CD4 T cells and neurons expressed IL-21 during both the acute and chronic phases of the disease. This study also found that CD4 T cells, CD8 T cells, CD19 B cells and neurons expressed IL-21R, which indicates that IL-21 might act directly on any or all of these populations in MS (Tzartos et al. 2011). In contrast, Coquet and colleagues have demonstrated that IL-21 signalling is not required for the induction of EAE or for Th17 differentiation in IL-21-/- and IL-21R-/- mice (Coquet et al. 2008).

Evidence also suggests that IL-21 plays a role in rheumatoid arthritis. In a mouse model of collagen-induced arthritis, the blockade of IL-21 resulted in reduced disease severity (Young et al. 2007). The same group have demonstrated that Lewis rats immunised with Freund's

complete adjuvant showed clinical score improvement after administration of the IL-21R/Fc fusion protein. In humans, CD4 and CD8 T cells, B cells and NK cells isolated from peripheral blood and synovial fluid of rheumatoid arthritis patients demonstrated increased IL-21R expression. Furthermore, T cells from RA patients produced greater amounts of TNFα and IFNγ in response to IL-21 stimulation (Li et al. 2006).

The detrimental effect of the IL-21 signalling has been also implied in inflammatory bowel disease. Studies of the dextran sodium sulfate (DSS) and trinitrobenzenesulfonic acid (TNBS) mouse models of colitis found elevated levels of IL-21 mRNA and protein. The defective Th17 differentiation in IL-21-deficent mice had a protective effect, thus confirming prominent role of the IL-21 signalling in both models of colitis (Fina et al. 2008).

1.5. Type-1 diabetes

Type-1 diabetes (T1D) is a chronic autoimmune disorder triggered by the selective destruction of insulin-producing β-cells located in the pancreatic islets of Langerhans. The subsequent loss of β-cell mass results in insufficient production of insulin, the hormone that promotes glucose uptake by the liver, muscle and fat tissue (Aronoff et al. 2004). The failure to regulate carbohydrate metabolism may result in the development of retinopathy, renal disease or can even be fatal. Currently, the only available treatment involves the life-long administration of exogenous insulin. Future interventions will have to re-establish local tolerance mechanisms to permit the re-expansion and stabilisation of the β-cell compartment (Herold et al. 2013). It is therefore important to understand the role of the cellular mechanisms that lead to immune dysregulation in T1D.

1.5.1. Type-1 diabetes: background

The onset of T1D typically occurs in childhood with peak incidence at between 6 and 15 years of age. It is estimated that in the western world the prevalence of T1D can reach up to 40 in 100000 individuals (Patterson et al. 2009). Disease susceptibility appears to be regulated by a combination of genetic and environmental factors. Epidemiological studies demonstrated that the incidence of T1D was increased 15-fold in siblings compared with in the general population (Harjutsalo et al. 2005). The main T1D susceptibility genes are located within the human leukocyte antigen (HLA) region, with the *HLA-DR* and *HLA-DQ* genes being of particular significance (Concannon et al. 2009). In recent years, the genetic basis of T1D was also linked to polymorphisms within non-HLA genes such as insulin (*INS*), cytotoxic T lymphocyte protein 4 (*CTLA4*) and IL-2Rα (*IL2RA*) (Barrett et al. 2009). Studies in

homozygotic twins have demonstrated that approximately 65% of initially asymptomatic individuals will become diabetic as their siblings by the age of 60 (Redondo et al. 2008). The disease-free status of the remaining homozygotic twins suggests a significant involvement of environmental elements in this pathology.

It has been postulated that molecular mimicry between viral and β-cell antigens might potentially contribute to the development of autoimmune diabetes. Although some viral infections have been shown to have a protective effect, pathogens such as coxsackie virus B4, rubella virus, cytomegalovirus and rotavirus might play an inductive role in the development of T1D (Atkinson et al. 1994; Ou et al. 2000; Pak et al. 1990; Honeyman et al. 2010). Furthermore, evidence suggests that the introduction of foreign proteins derived from dairy and cereal products during infancy might be responsible for the increase in diabetes incidence in children of T1D parents (Knip et al. 2010; Ziegler et al. 2003; Norris et al. 2003).

The initial detection of autoantibodies in the serum of T1D patients suggested an autoimmune basis for this disease (Bottazzo et al. 1974). The most common autoantibodies are specific for numerous pancreas-derived molecules such as insulin, proinsulin, glutamate decarboxylase 65 (GAD65), protein tyrosine phosphatase and zinc transporter 8 (Wenzlau et al. 2007). Autoantibody status is a strong diagnostic and predictive marker of autoimmune diabetes. The presence of at least one autoantibody in the serum has been detected in more than 90% of patients with T1D. Studies suggest that about three quarters of direct relatives of T1D individuals that tested positively for autoantibodies will develop the disease in the near future (Krischer 2003; Orban et al. 2009; Herold et al. 2013). Although the production of

autoantibodies is a prominent clinical manifestation of autoimmune diabetes, there is little evidence to suggest that they induce the disease.

1.5.2. Observations from animal models of type-1 diabetes

Studying the cellular mechanisms that mediate β-cell death in humans is problematic, as most of the destruction occurs during the clinically silent phase. Over the years, a number of animal models emerged including the non-obese diabetic (NOD) mouse, the biobreeding (BB) rat and transgenic strains that provided valuable insight into the pathogenesis of T1D. Based on the collective evidence of these studies, it has been postulated that two 'checkpoints' exist during the initiation of autoimmune diabetes. Firstly, the recognition of β-cell antigens is a prerequisite to induce tissue damage. This might be associated with genetic factors or the release of self-antigens during waves of β-cell death (Mathis et al. 2001). The presence of a self-reactive repertoire however is insufficient as autoreactive cells are found in healthy individuals. The second checkpoint is associated with the loss of control over the self-reactive repertoire and progressive β-cell destruction. Factors that might facilitate the progression from active insulitis to overt diabetes include the release of pro-inflammatory mediators, reduced inhibitory signalling and defects in Treg survival and function (André et al. 1996).

The NOD mouse is one of the most studied models of T1D. These animals develop spontaneous diabetes with a female incidence bias between 10 and 30 weeks of age. Initially, a variety of mononuclear cells enter the NOD pancreas at around four weeks and engulf the β-cell islets leading to peri-insulitis. Histological evidence has demonstrated that the pancreatic infiltrate is predominantly made-up of CD4 T cells, however CD8 T cells, DC, B cells, NK cells and macrophages are also present. Ultimately, these mononuclear cells invade the islets

at around 10 weeks causing insulitis and mediating β-cell destruction (reviewed in (Anderson & Bluestone 2005). Seminal work by Shizuru and colleagues demonstrated that T cells are the primary mediators of pathogenic responses in the NOD model, although other cell types also contribute to the disease. The depletion of CD4 T cells in these mice reversed the mononuclear cell infiltration in the pancreas and prevented diabetes (Shizuru et al. 1988). Similarly, the treatment of diabetic NOD mice with anti-CD3 antibody resulted in the regression of the disease, thus confirming the important role played by T cells in orchestrating β-cell destruction (Chatenoud et al. 1994). Furthermore, adoptive transfer experiments demonstrated that the most effective protocol for disease induction required co-injection of CD4 and CD8 T cells into irradiated NOD recipients (Miller et al. 1988; Hutchings & Cooke 1990; Yagi et al. 1992; Phillips et al. 2009).

Over the years numerous studies have shed light on the autoantigens targeted by self-reactive CD4 and CD8 T cells in diabetes, including insulin, GAD, heat shock protein 60 (Hsp60) and insulinoma-associated protein 2 (IA-2) (reviewed in (Lieberman & DiLorenzo 2003)). Lejon and Fathman have demonstrated that the majority of autoreactive T cells are characterised by high expression of CD4 in the NOD pancreas. Furthermore, only a small number of $CD4^{hi}$ T cells isolated from this organ was required to transfer disease in the NOD-SCID system (Lejon & Fathman 1999). Once the pancreas-reactive T cells evade thymic selection and enter to the circulation, number of tolerising mechanisms exists in the periphery to prevent the pathogenic immune responses. It is therefore not surprising that another T cell subset, namely Treg cells, plays a role in the pathogenesis of T1D in NOD system. Despite CD28 being a major co-stimulatory receptor in T cells, CD28 deficiency in the NOD model results in a profound reduction in the Treg pool and accelerated disease (Salomon et al. 2000). IL-2 also

appears to be important for the regulation of Treg responses in NOD animals, as increased Treg numbers observed after low dose IL-2 administration were associated with the reversal of diabetes in NOD mice (Grinberg-Bleyer et al. 2010). In terms of the T cell involvement in the β-cell death in NOD mice, CD8 T cells have been shown to mediate killing through the Fas/FasL pathway (Amrani et al. 1999; Kreuwel et al. 1999).

Although T cells are primarily responsible for the autoimmune pathology observed in the NOD system, B cell involvement is clearly manifested by the high titers of autoantibody found in these animals. Despite this, evidence argues against a critical pathogenic role for circulating autoantibody, but suggests that effects on antigen presentation and modulating T cell migration may be important (Wong et al. 2004; Ryan et al. 2010). Two groups have demonstrated that the reversal of diabetes can be observed in NOD mice treated with B cell depleting antibodies (Hu et al. 2007; Fiorina et al. 2008). In both cases, an expansion of the Treg compartment within the CD4 T cell pool was observed, suggesting enhanced regulation was responsible for the protective effect in these mice.

The data concerning the involvement of innate immune cells in β-cell death identifies both pro-inflammatory and protective responses. The evidence suggests that DC facilitate presentation of islet-specific antigens during waves of β-cell death associated with tissue remodeling (Turley et al. 2003). Moreover, DC from NOD mice expressed TNFα and had an increased ability to produce IL-12 and prime T cell-mediated responses (Dahlén et al. 1998; Poligone et al. 2002). In contrast, the increased recruitment of DC in GM-CSF-treated NOD mice had a protective effect (Kared et al. 2005). Macrophages are another innate population that have been shown to play a pathogenic role in the development of T1D. The evidence

suggests that blockade of adhesion molecules associated with macrophage entry to the pancreas can prevent diabetes in the NOD model (Hutchings et al. 1990). It has been demonstrated that macrophages from NOD mice readily produce IL-12, which in turn regulates cytotoxic CD8 T cell responses in the pancreas (Alleva et al. 2000; Jun et al. 1999).

Alternative transgenic models of T1D have provided valuable advances in our understanding of the autoimmune pathology observed in this disease. Our laboratory studies immune responses against islet tissue using the DO11xRIP-mOVA mouse model. In this double transgenic model, the DO11.10 TCR, which is specific for residues 323-339 of chicken egg ovalbumin (OVA), is expressed in mice that also express OVA under the control of the rat insulin promoter. Subsequently, DO11 T cells recognise presented OVA peptide and mediate an immune attack restricted to β-cells in the pancreas. These mice develop diabetes between 10 and 12 weeks of age without any gender bias. Histological analyses demonstrate that DO11xRIP-mOVA mice develop insulitis from approximately three weeks of age. At this stage B cell entry into the pancreas precedes T cell arrival, however the steady influx of the latter population is observed from then onwards (Ryan et al. 2010). Other immune cells that infiltrate the DO11xRIP-mOVA pancreas also include DC and macrophages. CD4+Foxp3- T cells isolated from the pancreas of these animals are known to produce substantial levels of IFNγ and TNFα, pro-inflammatory mediators associated with T1D. Disease progression in DO11xRIP-mOVA mice was also correlated with a decreased proportion of Foxp3+ Treg cells in the pancreas-draining lymph node (Clough et al. 2008). The negative regulator CTLA-4, polymorphisms in which are associated with T1D, is critical for controlling β-cells specific T cell responses in an adoptive transfer system based on the DO11xRIP-mOVA

model. In this system, DO11 Treg cells deficient in CTLA-4 failed to control OVA-specific T cell responses and permitted the development of diabetes (Schmidt et al. 2009).

The B cell compartment appears to play an important pathological role in the DO11xRIP-mOVA diabetes model. These cells appear to modulate the effector function of DO11 T cells and mediate their entry to the pancreas. In this system, B cell deficiency is known to delay disease onset, as evidenced by crossing DO11xRIP-mOVA mice onto the Jh-/- background (Wesley et al. 2010).. Furthermore, B1 cell transfer into DO11xRIP-mOVA/RAG2-/- mice induced the expression of the adhesion molecule VCAM-1, which in turn facilitated T cell entry into the pancreas and promoted insulitis (Ryan et al. 2010). Similarly, Wesley and colleagues demonstrated that the transfer of CD8 T cells into DO11xRIP-mOVA/RAG2-/- mice resulted in the influx of pathogenic DO11 CD4 T cells into the pancreas, leading to insulitis (Wesley et al. 2010) These data suggest that both B1 cells and CD8 T cells can play the role of gatekeeper, with both populations facilitating pancreas infiltration by CD4 T cells during the initiating stages of the disease.

1.5.3. IL-21 in type-1 diabetes

Several studies have demonstrated the importance of IL-21 for the development of diabetes in NOD mice. Genetic analysis has shown that the *il21* gene is located at the *idd3* locus, a diabetes-associated region in the NOD mouse model (Wicker et al. 1994). King *et al.* have demonstrated an increased level of IL-21 and IL-21R expression in the T cell compartment of NOD mice (King et al. 2004). Back-crossing IL-21R deficient mice onto the NOD background almost completely abrogated development of diabetes in one study and gave

complete protection in other (Spolski et al. 2008; Sutherland et al. 2009). Sutherland and colleagues have also reported that increased levels of mRNA for IL-21 measured in the pancreas correlated with the progression of diabetes. Evidence from a double transgenic model in which β-cells express both IL-21 and OVA suggests that this cytokine induces the cytotoxic activity of transferred OVA-specific CD8 T cells, which in consequence promotes diabetes (Sutherland et al. 2013). We demonstrated that IL-21 mRNA was overexpressed in the pancreatic LN of diabetic DO11xRIP-mOVA mice. The overexpression of IL-21 was later narrowed to CD4+CD25- T cells. Furthermore, the provision of exogenous IL-21 has been shown to counteract Treg-mediated suppression in both *in vivo* and *in vitro* systems (Clough et al. 2008). Later, our group demonstrated that one mechanism by which IL-21 is able to counteract Treg suppression is mediated by the inhibition of IL-2 production by CD4+CD25- T cells (Attridge et al. 2012). Collective evidence from mouse models of T1D suggests that IL-21 signalling plays a prominent role in modulating the immune response associated with the development of autoimmune diabetes.

1.6. Innate lymphoid cells

In recent years, numerous groups have identified novel innate lymphoid cell populations that mediate protective immune responses, but may also contribute to the development of autoimmunity and allergy. These cells in many ways resemble existing innate lymphoid populations such as NK cells and lymphoid tissue-inducer (LTi) cells. Therefore, the consortium of leaders in the field proposed a new system of nomenclature that encompasses newly discovered cells and the existing immune players. They have agreed that these novel immune populations should be termed innate lymphoid cells (ILC), and that their nomenclature should be based on their expression of particular cytokines and transcription factors. Using this system, ILC have been subdivided into three distinct populations (**Fig. 1.02**) (Spits et al. 2013).

1.6.1. Innate lymphoid cells: biology and function

Unique characteristics including the absence of myeloid and DC markers, the lack of RAG gene expression, and lymphoid appearance are common features of the known ILC populations (Spits & Cupedo 2012). ILC are related to NK cells and LTi cells in that their development is dependent on the common γ-chain and the transcriptional repressor inhibitor of DNA binding 2 (ID2) (Yokota et al. 1999; Boos et al. 2007; Spits et al. 2013). Intriguingly, different ILC populations have been associated with distinct cytokine expression profiles highly reminiscent of those associated with T helper cell subsets.

Group 1 ILC are defined by IFNγ production and consist of NK cells and the newly discovered ILC1 population. NK cells were firstly described as effector lymphocytes of innate

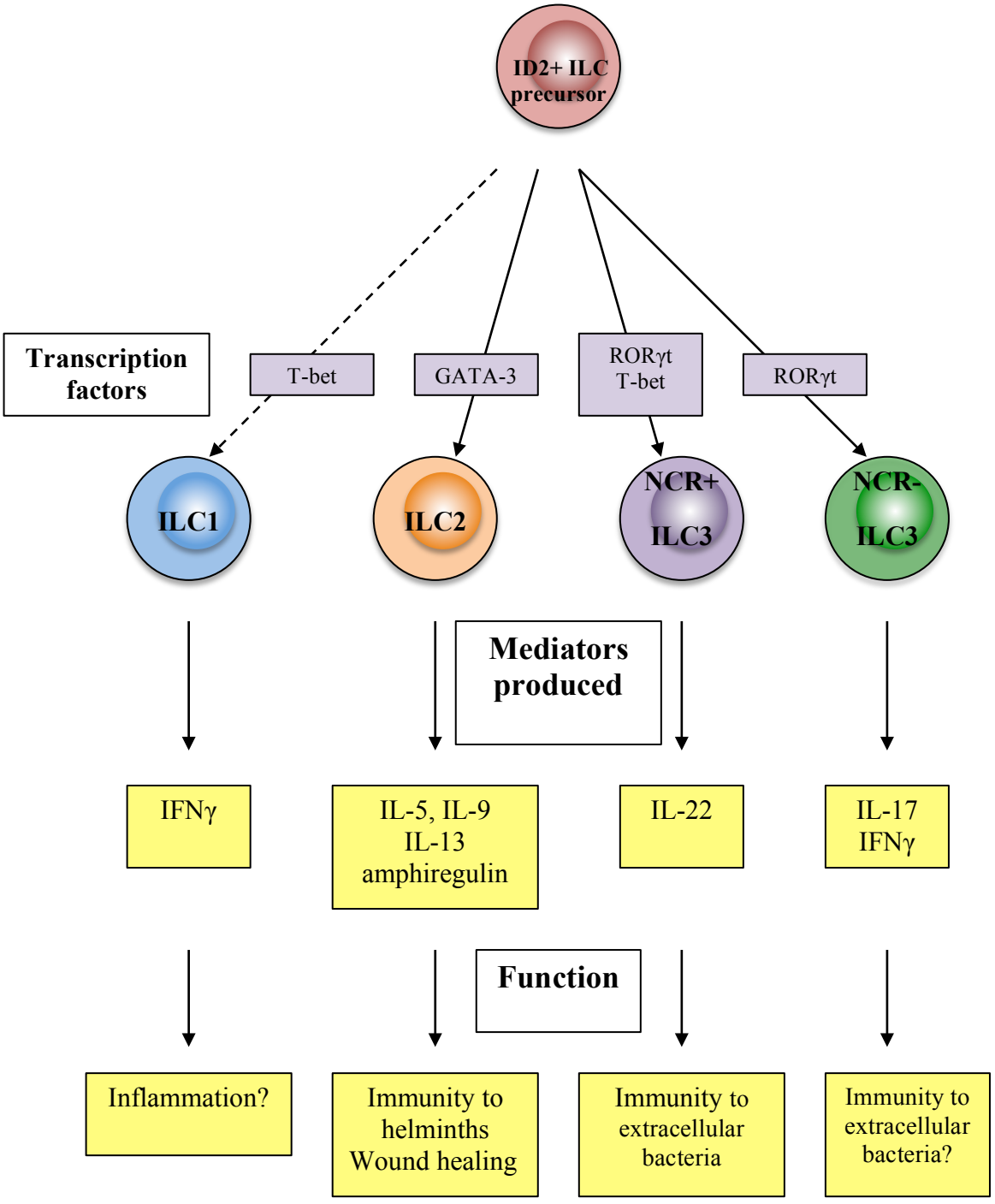


Figure 1.02. Proposed classification of novel innate lymphoid cell (ILC) populations. Recently, a consensus view to classify innate lymphoid cells based on their cytokine signature and function has been agreed. ILC1 secrete IFNγ and may mediate inflammation. ILC2 release cytokines that modulate protective responses to helminths and support wound healing. Protection against extracellular bacteria is mediated by ILC3, which produce IL-17, IL-22 and IFNγ. Adapted from (Spits et al. 2013; Walker et al. 2013).

origin that mediate strong anti-tumour responses (Kiessling et al. 1975; Herberman et al. 1975). NK cells mediate their effector functions through their production of the pro-inflammatory cytokines IFNγ and TNFα, and through their release of cytolytic perforins and granzymes (Vivier et al. 2011). Although categorisation of the IFNγ-producing ILC1 population as a separate group 1 ILC subset is still debatable, these cells have been shown to arise from RORγt positive ILC in mouse and man (Vonarbourg et al. 2010; Bernink et al. 2013). ILC1 development involves the downregulation of RORγt expression in response to IL-12, with the subsequent induction of the transcription factor T-bet and production of IFNγ. IFNγ-producing ILC1 have been associated with the development of colitis in RAG2-/- mice treated with anti-CD40 antibody (Vonarbourg et al. 2010). In addition, the ILC1 subset was enriched in the intestine of Crohn's disease patients, suggesting the potential involvement of this population in gut mucosa homeostasis (Bernink et al. 2013).

Group 2 ILC (ILC2) are characterised by production of type 2 cytokines. ILC2 were firstly identified in RAG2-/- mice based on their expression of IL-5 and IL-13, which was stimulated by IL-25 administration (Fort et al. 2001; Hurst et al. 2002). Later, numerous groups demonstrated that this population was also responsive to IL-33 stimulation and resided in the mesenteric lymph node, mesenteric fat-associated lymphoid clusters (FALC), spleen, liver, intestines and airways (Moro et al. 2010; Neill et al. 2010; Price et al. 2010; Barlow et al. 2012). ILC2 development is mediated by the activation of the transcription factors RORα and GATA3 (Halim et al. 2012; Wong et al. 2012; Hoyler et al. 2012; Mjösberg et al. 2012). In addition to IL-5 and IL-13, ILC2 have been shown to express IL-4, IL-6 and IL-9 (Neill & McKenzie 2011; Wilhelm et al. 2011). Due to their abundant expression of type 2 cytokines,

ILC2 play a key role in generating protective immune responses against helminths such as *Nippostrongylus brasiliensis* (Moro et al. 2010; Neill et al. 2010; Fallon 2006). Aside from the gut, this population is also present at mucosal sites in the human and murine lungs. In mice challenged with influenza virus, ILC2 mediated epithelial tissue repair in the lung by releasing the epidermal growth factor family member amphiregulin (Monticelli et al. 2011). In contrast, inappropriate activation of the ILC2 population has been associated with allergic responses in the airways (Chang et al. 2011; Bartemes et al. 2012).

Finally, group 3 ILC consist of LTi cells and the novel ILC3 subset, and are defined by their ability to secrete IL-17A and IL-22 (Spits et al. 2013). LTi cells are involved in the development of lymphoid tissue during embryogenesis (Mebius et al. 1997). The generation of this subset requires the transcription factor RORγt (Eberl et al. 2003). LTi cells are thought to produce high levels of IL-17A and IL-22 in response to IL-23 stimulation (Takatori et al. 2009). Apart from their instrumental role in lymphoid organogenesis, LTi cells have been shown to maintain memory CD4 T cell survival and mediate protective responses in the intestine of *Citrobacter rodentium* infected mice (Withers et al. 2012; Sonnenberg et al. 2011). The newly proposed natural cytotoxicity triggering receptor positive ILC3 (NCR+ ILC3) subset require RORγt for their differentiation, express the NK cell activating receptor NKp46 and secrete high levels of IL-22, but are phenotypically and functionally distinct from LTi and NK cells (Satoh-Takayama et al. 2008; Luci et al. 2008; Sanos et al. 2011). Due to the high level expression of IL-22, NCR+ ILC3 are thought to mediate protective responses in the intestinal mucosa to pathogens such as *Citrobacter rodentium* (Zheng et al. 2008; Lee et al. 2011). In contrast, a further subset of group 3 ILC exists that do not express NCR (NCR- ILC3). These cells are defined by the absence of NKp46 expression and produce IL-22, IFNγ

and IL-17A (Spits et al. 2013). It has been demonstrated that NCR- ILC3 orchestrate pathogenic responses in the *Helicobacter hepaticus*-induced mouse model of colitis (Buonocore et al. 2010).

Overall ILC cells constitute populations of emerging interest whose contribution to protective and aberrant immune responses is yet to be fully defined. Their ability to secrete large quantities of cytokines suggests they will have widespread functions in modulating adaptive and innate immune responses.

1.7. Aims and objectives

The aim of this project was to explore the production of the cytokine interleukin 21 (IL-21) in a mouse model of T1D, focusing in particular on how production of this cytokine varies between different tissue sites and throughout the course of the disease. Specifically we aimed to investigate whether islet-reactive T cells that infiltrated the pancreas were producing IL-21, and how IL-21 production at this site compared with that of T cells elsewhere. Furthermore we wished to establish whether IL-21 levels increased as mice progressed from a normoglycaemic state to overt diabetes.

We further aimed to investigate the signals that control IL-21 production by T cells, and specifically, how different types of antigen presenting cell, co-stimulatory signals or cytokines might influence T cell IL-21 expression *in vitro* and *in vivo*. Thus, the overall objectives were to evaluate IL-21 production in T1D pathology and to explore the signals that promote its expression.

2. MATERIALS AND METHODS

2.1. Mice

2.1.1. Mice

BALB/c, CD28-/- and DO11.10 TCR transgenic mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). RIP-mOVA mice on a BALB/c background were a kind gift from W. Heath (WEHI, Melbourne, Australia). IL-21R-/- mice were provided by M. Kopf (ETH, Zurich, Switzerland). CTLA-4-/- mice were obtained from A. Sharpe (Harvard University, Boston, USA). RAG2-/- and Jh-/- mice were purchased from Taconic laboratories (Germantown, New York, USA). p110δ^{D910A} mice were a gift from K. Okkenhaug (Babraham Institute, Cambridge, UK). DO11.10 TCR transgenic mice were crossed with RIP-mOVA mice to generate DO11xRIP-mOVA mice. CD28-/- mice were initially bred with RIP-mOVA and DO11.10 TCR mice to generate CD28-/-mOVA+ and CD28-/-DO11.10 TCR mice. CD28-/-mOVA+ and CD28-/-DO11.10 TCR mice were crossed to generate DO11xRIP-mOVAxCD28-/- progeny. Jh-/- mice were initially bred with RIP-mOVA and DO11.10 TCR mice to generate Jh-/-mOVA+ and Jh-/-DO11.10 TCR mice. Jh-/-mOVA+ and Jh-/-DO11.10 TCR mice were crossed to generate DO11xRIP-mOVAxJh-/- progeny. p110δ^{D910A} mice were initially bred with RIP-mOVA and DO11.10 TCR mice to generate p110δ^{D910A} mOVA+ and p110δ^{D910A} DO11.10 TCR mice. p110δ^{D910A}mOVA+ and p110δ^{D910A}DO11.10 TCR mice were crossed to generate DO11xRIP-mOVAxp110δ^{D910A} progeny. Mice were housed at the Biomedical Services Unit (University of Birmingham) and were used in accordance with Home Office regulations.

2.1.2. *In vivo* antigen-specific responses to alum-precipitated OVA

$1x10^6$ DO11.10 TCR transgenic MACS (Miltenyi Biotec) sorted CD4+ cells were CellTrace Violet (Invitrogen) labeled according to manufacturer instructions and injected intravenously in 400µl PBS (Sigma) into wildtype or Jh-/- mice. After 24 hours, recipients were immunised by intraperitoneal injection with 200 µg alum-precipitated OVA (Sigma) in 100µl PBS. Mice were sacrificed and spleens harvested at day 5, 11 or 14.

2.1.3. *In vivo* antigen-specific responses to peptide-loaded DC immunisation

$3x10^6$ DO11.10 TCR transgenic MACS (Miltenyi Biotec) sorted CD4+ cells were CellTrace Violet (Invitrogen) labeled according to the manufacturer's instructions and injected intravenously in 400µl PBS (Sigma) into wildtype mice. After 24 hours, recipients were immunised with $1.5x10^5$ bone marrow-derived dendritic cells loaded overnight with 1 µg/ml OVA peptide (Sigma) by intravenous injection in 400µl PBS (Sigma). Mice were sacrificed and spleens harvested at day 2, 3 or 6.

2.1.4. Blood glucose measurement

Blood glucose was monitored using an Ascensia Elite XL blood glucose meter (Bayer, USA). Mice were considered diabetic following two consecutive blood glucose readings of over 250mg/dL.

2.2. Flow cytometry

2.2.1. Cell isolation

Single cell suspensions were isolated from peripheral lymph nodes and spleens by mashing through a wire mesh in ice-cold P2 (see appendix). For spleens, red blood cells were lysed using 2ml lysis buffer (see appendix) for 3 minutes at room temperature before dilution with 8ml of cold P2 to stop the reaction. Cells were obtained from pancreatic lymph nodes by teasing apart tissue with forceps in cold P2. Lymphocytes were recovered from the pancreas by tearing in cold pancreas buffer (see appendix) before centrifugation. Cells were then digested in 2ml of pre-warmed pancreas digest solution (see appendix) for 10 minutes at 37°C on a shaker. The reaction was stopped with 8ml of cold C10 (see appendix) and cells were filtered through a 40μm cell strainer (BD Biosciences). Cells were then resuspended in pre-warmed C10, layered over lympholyte-M (Cedarlane Laboratories, Ontario, Canada) and centrifuged for 20 mins at 1000g, 4°C. Lymphocytes were then isolated by collecting cells at the interface and washing with P2.

2.2.2. Restimulation for intracellular cytokine staining

$2x10^6$ cells per well (24 well flat bottom plate) were cultured in 1ml C10 in the presence of 50ng/ml Phorbol Myristate Acetate (PMA) (Sigma) and 1.5μM Ionomycin (Sigma) at 37°C, 5% CO_2. After 1 hour, 10μg/ml Brefeldin A (Sigma) was added and samples were incubated for a further 4 hours.

2.2.3. Staining for flow cytometry

Cells were surface stained for 10 minutes at 4°C in P2 containing 5% goat serum followed by washing with 1ml P2. Prior to intracellular staining for Foxp3 and cytokines, cells were fixed and permeabilised with paraformaldehyde and saponin containing reagents according to the manufacturer's instructions (eBioscience). Intracellular antibodies were added and samples were incubated at 4°C for 30 minutess. All cell samples were acquired using a FACScalibur (BD Biosciences) or Dako CyAn (DakoCytomation) flow cytometer, followed by analysis using FlowJo (TreeStar) software.

2.2.3. Antibodies

Specificity	Clone	Conjugate	Supplier	Dilution
B220	RA3-6B2	PerCP	BD Biosciences	1/200
Biotin		Streptavidin FITC	BD Biosciences	1/200
CCR9	eBioCW-1.2	PE	eBioscience	1/100
CD3ε	17A2	APC eFluor 780	eBioscience	1/50
CD4	RM4-5	PerCP	BD Biosciences	1/50
CD4	RM4-5	APC	BD Biosciences	1/50
CD11b	M1/70	eFluor 450	eBioscience	1/100
CD11b	M1/70	FITC	eBioscience	1/50
CD11c	N418	PE	eBioscience	1/50
CD19	eBio 1D3	eFluor 450	eBioscience	1/200
CD19	MB19-1	FITC	eBioscience	1/100
CD25	PC61	APC	BD Biosciences	1/100

Specificity	Clone	Conjugate	Supplier	Dilution
CD25	PC61.5	PE-Cy7	eBioscience	1/100
CD43	S7	FITC	BD Biosciences	1/100
CD49b	DX5	Biotin	BD Biosciences	1/100
CD49d	R1-2	PE	BD Biosciences	1/100
CD62L	MEL-14	APC	BD Biosciences	1/50
CD80	16-10A1	PE	BD Biosciences	1/50
CD86	GL1	FITC	eBioscience	1/50
CD122	TM-Beta 1	PE	BD Biosciences	1/50
CD127	A7R34	FITC	eBioscience	1/100
c-Kit	2B8	APC	eBioscience	1/300
DO11.10 TCR	KJ1-26	APC	eBioscience	1/100 or 1µl
Foxp3	FJK-16s	APC	eBioscience	2µl
Foxp3	FJK-16s	FITC	eBioscience	1µl
I-Ad	AMS-32.1	FITC	BD Biosciences	1/300
I-A/I-E	M5/114.15.2	APC	eBioscience	1/200
ICAM	YN4/1.7.4.	PE	eBioscience	1/300
ICOS	7E.17G9	PE	eBioscience	1/50
IFNγ	XMG1.2	PE-Cy7	eBioscience	1µl
IL-2	JES6-5H4	Alexa Fluor 488	eBioscience	1µl
IL-5	TRFK5	APC	BD Biosciences	1µl
IL-13	eBio13A	PE	eBioscience	1µl
IL-17	TC11-18H10	Alexa Fluor 488	BD Biosciences	1µl

Specificity	Clone	Conjugate	Supplier	Dilution
IL-21	mhalx21	PE	eBioscience	1µl
Sca-1	D7	APC	BioLegend	1/200
ST2	245707	APC	R&D Systems	5µl
Thy1.2	53-2.1	APC	eBioscience	1/200
TNFα	MP6-XT22	eFluor 450	eBioscience	1µl

Table 2.01. Antibodies for flow cytometry

2.3. Cell sorting

2.3.1. Magnetic-activated cell sorting (MACS)

CD4+ T cells and CD19+ B cells were positively selected from single cell suspensions obtained from lymph node and spleen, respectively. Prior to separation cells were labelled with 10µl of CD4 or CD19 microbeads (Miltenyi Biotec) per 10^7 cells for 15 mins at 4°C. After washing, cells were passed through an LS column (Miltenyi Biotec) and the retained magnetically labelled fraction was then eluted with MACS buffer. CD4+CD25- T cells were sorted from single cell suspensions obtained from lymph node. Prior to separation cells were labelled with 15µl biotin-antibody cocktail (Miltenyi Biotec) per 10^7 cells for 10 mins at 4°C followed by 20µl of anti-biotin microbeads (Miltenyi Biotec) and 10µl CD25 PE (Miltenyi Biotec) per 10^7 cells for a further 15 mins at 4°C. After washing, cells were passed through an LD column (Miltenyi Biotec). The run-off containing CD4+ cells was collected and labelled with 10µl anti-PE microbeads (Miltenyi Biotec) per 10^7 cells for 15 mins at 4°C. After a wash cells were passed through an MS column (Miltenyi Biotec). The run-off contained the desired CD4+CD25- cell fraction and was collected.

2.3.2. Fluorescence activated cell sorting (FACS)

For fluorescence activated cell sorting cells were recovered from the pancreas as described previously and stained in 1ml C2 (see appendix) for 10 mins at 4°C. Cells were then washed in 10ml C2 before being passed through a 40µm cell strainer (BD Biosciences). The cell fraction of interest was sorted using a MoFlo high-speed cell sorter (Beckman Coulter), operated by Roger Bird and Shankar Suresh.

2.4. *In vitro* cell culture

2.4.1. Differentiation of dendritic cells from murine bone marrow

Harvested mouse femurs were cleared of tissue and washed in 70% ethanol (Sigma) followed by PBS. Marrow was recovered from femurs by flushing with PBS. After red cell lysis and washing with PBS, $2x10^6$ cells were cultured in 10ml C10 supplemented with 200U/ml of recombinant murine GM-CSF (Peprotech) at 37°C, 5% CO_2. An additional 10ml C10 containing 200U/ml of recombinant murine GM-CSF was added to the culture at day 3. At days 6 and 8, 10ml of the cell culture was harvested, centrifuged and resuspended in 10ml of fresh C10 containing 200U/ml of recombinant murine GM-CSF. At day 10, cultures were replenished as described at days 6 and 8, but with 100U/ml of recombinant murine GM-CSF. To mature dendritic cells, culture medium was further supplemented with 1μg/ml of LPS (E. coli O26:B6, Sigma) at day 10. Mature and immature dendritic cells were harvested at day 11.

2.4.2. *In vitro* cultures for assessment of T cell responses

Cells were cultured in 8 replicates in round-bottom 96 well plates in 200μl C10. MACS sorted BALB/c, IL-21R-/-, CD28-/-, p110δ ^{D910A}, or DO11.10 TCR transgenic CD4+CD25- conventional T cells were added at $2.5x10^4$ cells per well with MACS sorted BALB/c CD19+ B cells or BALB/c bone marrow-derived immature or mature dendritic at ratios of 3:1 and 6:1 (B cell: Tconv) or 1:10 and 1:5 (bone marrow-derived DC: Tconv). TCR ligation was provided by the addition of 0.8μg/ml soluble α-CD3 (BD Biosciences) or 1 μg/ml OVA peptide (Sigma). Where indicated, CD4+CD25- conventional T cells were activated with anti-CD3/anti-CD28 beads (Gibco) at a ratio of 1:1. For assessment of T cell cytokine responses,

cells were cultured in C10 alone or with 100 ng/ml murine IL-6 (Peprotech), 25 ng/ml murine IL-11 (R&D Systems), 5 ng/ml murine IL-12 (Peprotech), 50ng/ml murine IL-23 (eBioscience), 50 ng/ml murine IL-27 (R&D Systems), 10 µg/ml anti-IL-6 (R&D Systems), 10 µg/ml anti-gp130 (R&D Systems), 10 µg/ml anti-IL-27 (R&D Systems), 5 µg/ml anti-OSM (R&D Systems), or 10 µg/ml anti-LIF (R&D Systems). Cells were incubated at 37°C, 5% CO_2 and harvested for restimulation after 72 hours.

2.4.3. *In vitro* cultures of pancreas-sorted CD4-CD25+ cells and their effect on T cell responses

CD4-CD25+ cells were isolated and sorted from the pancreas' of RAG2-/- mice as previously described. Pancreatic CD4-CD25+ cells were then cultured in round-bottom 96 well plates in 200µl C10 containing 10 ng/ml murine IL-7 (R&D Systems) and 10 ng/ml murine IL-33 (Enzo Life Sciences) at 37°C, 5% CO_2 for 5 days. For assessment of the effect of cultured CD4-CD25+ on T cell responses, cells were cultured in in round-bottom 96 well plates in 200µl C10. After 5 days of pre-culturing, $5x10^3$ CD4-CD25+ cells were added per well with $2.5x10^4$ MACS sorted BALB/c CD4+CD25- conventional T cells. CD4+CD25- conventional T cells were cultured in C10 alone or with 10 ng/ml murine IL-7 (R&D Systems) and 10 ng/ml murine IL-33 (Enzo Life Sciences). CD4-CD25+ cells together with CD4+CD25- conventional T cells were cultured in C10 with 10 ng/ml murine IL-7 (R&D Systems) and 10 ng/ml murine IL-33 (Enzo Life Sciences) with or without 10 µg/ml anti-IL-6 (R&D Systems). CD4+CD25- conventional T cells were activated with anti-CD3/anti-CD28 beads (Gibco) at a ratio of 1:1. Cells were incubated at 37°C, 5% CO_2 and harvested for restimulation after 72 hours.

2.5. Immunohistology

2.5.1. Section preparation

Harvested pancreases were gently frozen in O.C.T. compound (Tissue-Tek) over liquid nitrogen and subsequently stored at -80°C until use. Serial six-micrometer pancreatic sections were cut at -20°C using a cryostat and mounted onto glass slides (Menzel-Glaser). Air-dried sections were later fixed in 100% acetone (Scientific and Chemical supplies) at 4°C for 20 minutes. After fixation, sections were dried at room temperature for 20 minutes and stored at -20°C until use.

2.5.2. Immunohistochemistry

Prior to staining, slides were brought to room temperature and tissues re-hydrated in PBS. Sections were stained with primary antibodies specific for murine antigens at room temperature for 1 hour (Table 2.02) and then washed with TRIS buffer at pH7.2 (Sigma). Alkaline phosphatase or biotin-conjugated secondary antibodies (pre-incubated with 10% normal mouse serum) were added to the sections for 45 minutes at room temperature (Table 2.03). Slides were then washed with TRIS buffer at pH7.2 and biotin-conjugated tertiary antibodies (pre-incubated with 10% normal mouse serum) were added for 30 minutes at room temperature where appropriate (Table 2.04). Biotinylated antibodies were detected with avidin Vectastein ABC complex- peroxidase (Vector Laboratories), followed by the addition of DAB solution (Sigma). Alkaline phosphatase-conjugated antibodies were detected with Naphthol AS-MX phosphate substrate (Sigma) and Fast Blue BB salt (Sigma) with Levamisole (Sigma).

2.5.3. Antibodies used for immunohistochemistry

Specificity	Clone	Host	Conjugate	Supplier	Dilution
CD25	7D4	Rat	Biotin	BD Biosciences	1/100
Insulin	H-86	Rabbit	Purified	Santa Cruz Biotechnology	1/50

Table 2.02. Primary antibodies used for immunohistochemistry.

Specificity	Host	Conjugate	Supplier	Dilution
Biotin	Sheep	Purified	Bethyl Laboratories	1/800
Rabbit	Swine	Alkaline phosphatase	Dako Cytomation	1/100

Table 2.03. Secondary antibodies used for immunohistochemistry.

Specificity	Host	Conjugate	Supplier	Dilution
Sheep	Donkey	Biotin	The Binding Site	1/100

Table 2.04. Tertiary antibody used for immunohistochemistry.

2.6. Molecular biology

2.6.1. RNA isolation and cDNA synthesis by reverse transcription PCR

Frozen cell pellets were homogenised with 800µl RNA-Bee (Biogenesis) followed by extraction with 80µl chloroform (Sigma) on ice for 5 minutes. Subsequently, RNA was obtained by precipitation with 400µl isopropanol (Sigma) at -20°C for 20 minutes and washed with 75% EtOH (Sigma). RNA was then heated to 70°C for 5 minutes and transferred on ice to chill. cDNA was obtained by reverse transcription PCR using M-MLV reverse transcriptase (Invitrogen) and Oligo dT primers (Invitrogen).

2.6.2. qPCR protocol

Reactions were carried out using 2x PCR Mastermix (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) (Table 2.05) in 96 or 384 well microAmp plates (Applied Biosystems). Gene targets were quantified using an Mx3000P cycler (Stratagene, Agilent Technologies) (96 well plates) or a 7900HT Real-Time PCR System (Applied Biosystems) (384 well plates) and normalised to β-actin expression. β-actin primers and probe (Eurogentec):

Forward: 5’ CGT GAA AAG ATG ACC CAG 3’

Reverse: 5’ TGG TAC GAC CAG AGG CAT 3’

PCR program:

Step 1: 50°C, 2 minutes

Step 2: 95°C, 10 minutes

Step 3 (40 cycles): 95°C, 15 seconds followed by 60°C, 1 minute

2.6.3. TaqMan Gene Expression Assays

Gene	TaqMan Assay ID
Csf2	Mm01290062_m1
IL-5	Mm00439646_m1
IL-6	Mm00446190_m1
IL-9	Mm00434305_m1
IL-10	Mm00439616_m1
IL-12p35	Mm00434165_m1
IL-13	Mm00434204_m1
IL-23p19	Mm00518984_m1
IL-27p28	Mm00461164_m1

Table 2.05. Gene targets in TaqMan Gene Expression Assays

2.7. Statistics

Data were analysed using GraphPad Prism (GraphPad Software) by unpaired or paired two-tailed T-test with a 95% confidence interval. *, p = <0.05, **, p = <0.01, ***, p = <0.001, ns = not significant.

3. ANALYSIS OF INTERLEUKIN 21 PRODUCTION IN MOUSE MODELS OF AUTOIMMUNITY

3.1. Introduction

Seminal work by Parrish-Novak and colleagues has identified an addition to the common γ-chain cytokine family, namely interleukin 21 (IL-21) (Parrish-Novak et al. 2000). This class I cytokine receptor ligand was first reported as a lymphocyte growth factor, with subsequent studies demonstrating that IL-21 may play an important role in type-1 diabetes (T1D) (Spolski et al. 2008; Sutherland et al. 2009) and other autoimmune diseases (Vinuesa et al. 2005; Kang et al. 2011). Our previous data indicated that IL-21 mRNA was upregulated in the DO11xRIP-mOVA mouse model of T1D (Clough et al. 2008) and that IL-21 could potentially contribute to immune dysregulation by counteracting Treg mediated suppression (Attridge et al. 2012). However, analysis of IL-21 at the protein level in mouse models of autoimmunity was lacking. In order to investigate this, a multi-colour flow cytometry approach was used to evaluate IL-21 protein at the single cell level in the DO11xRIP-mOVA mice. To complement our analysis of a tissue-specific model of autoimmunity, analysis of a systemic lymphoproliferative disease was also undertaken. For this purpose mice lacking the T cell regulatory protein, CTLA-4, were used. CTLA-4-/- mice are known to develop a lethal lymphoproliferative disease resulting in multi-organ infiltration and death within a few weeks of birth (Tivol et al. 1995; Waterhouse et al. 1995). The aim of this part of the project was therefore to assess whether IL-21-producing cells could be identified by flow cytometry in either the DO11xRIP-OVA or the CTLA4-/- models of immune dysregulation.

3.2. Results

3.2.1. Optimisation of IL-21 staining in DO11xRIP-mOVA mice

One of the major focuses in our group is to understand T cell responses in a dysregulated immune system. As part of this aim, our laboratory has developed the DO11xRIP-mOVA double transgenic mouse model of T1D. This model enables us to investigate the progression of an autoimmune response in which a self-tissue (the pancreatic islets that transgenically express OVA) is targeted and destroyed as a result of activation of OVA-specific T cells (DO11.10 TCR T cells). To better understand the progression of T1D in DO11xRIP-mOVA mice, blood glucose measurements were taken from three to 23 weeks of age. To that end, tail bleeds were performed and blood glucose levels were monitored using a glucometer. Mice are typically considered to be diabetic following two consecutive glucose readings of over 250mg/dl. The majority of DO11xRIP-mOVA mice younger than 10 weeks remained normoglycaemic, with blood glucose levels around 100-200mg/dl (**Fig. 3.01**). Progression to overt disease typically began between 10 and 12 weeks of age, with several readings being recorded that exceeded 250mg/dl. Blood glucose levels increased steadily to reach maximum measurable levels (600mg/dl) by approximately 18 weeks. Thus, the DO11xRIP-mOVA mouse model of T1D enables us to investigate T cells responses in a system where 100% of mice develop diabetes.

We have previously reported that the induction of IL-21 mRNA was associated with progression to overt diabetes in DO11xRIP-mOVA mice (Clough et al. 2008). Our initial data demonstrated that IL-21 mRNA was overexpressed in CD4+CD25- T cells isolated from the

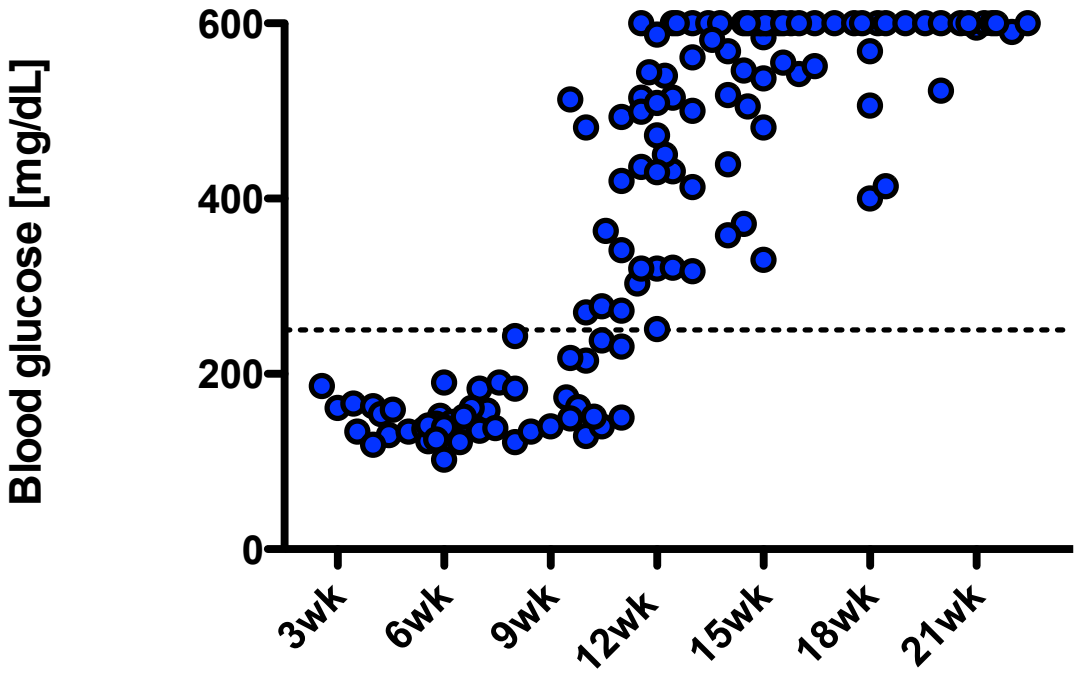


Figure 3.01. Blood glucose readings in DO11xRIP-mOVA mice (in collaboration with L.Clough). Graph shows blood glucose measurements in DO11xRIP-mOVA mice tested at different weeks of age. Dashed line indicates blood glucose level above which mice are considered diabetic.

pancreatic lymph node (LN) of 12-week-old (diabetic) mice compared to six-week-old (normoglycaemic) animals. However, little was known about the kinetics of IL-21 mRNA throughout the course of the disease. To address this, CD4+CD25- and CD4+CD25+ T cells were purified from the pancreatic LN of six, 11, 16 and 19 week-old mice and quantitative real time PCR analysis was carried out to measure IL-21 mRNA expression levels. The data generated showed that IL-21 levels peaked at 11 weeks, remained high at 16 weeks and tailed off at the later time point. IL-21 transcripts were predominantly detected in CD4+CD25- T cells, with very little being expressed in CD4+CD25+ T cells (**Fig. 3.02**). Thus our analysis revealed that the major source of IL-21 transcripts in the pancreas-draining LN was the CD4+CD25- conventional T cell population and that levels were upregulated between six and 11 weeks of age and thereafter remained high for at least another eight weeks.

At the time this project was initiated, there were no reports demonstrating the expression of IL-21 at the protein level. Given that IL-21 transcripts were present in the pancreatic LN of diabetic DO11xRIP-mOVA mice and this cytokine is able to counteract Treg mediated suppression in mouse and man (Peluso et al. 2007; Clough et al. 2008; Attridge et al. 2012), we sought to optimise intracellular cytokine staining to evaluate IL-21 expression in our mouse model of T1D. In order to establish reliable IL-21 staining, single cell suspensions from DO11xRIP-mOVA spleen were restimulated with PMA and ionomycin. Brefeldin A and GolgiStop were tested to select the most efficient protein transport inhibitor. Finally, the anti-IL-21 antibody clone, concentration and incubation time were assessed to optimise the staining protocol (data not shown). The result of this work is that we were able to develop a protocol that permitted us to detect IL-21 at the protein level (**Fig. 3.03**). Our previous work demonstrated that IL-21 mRNA was expressed by CD4+CD25- T cells, so we included

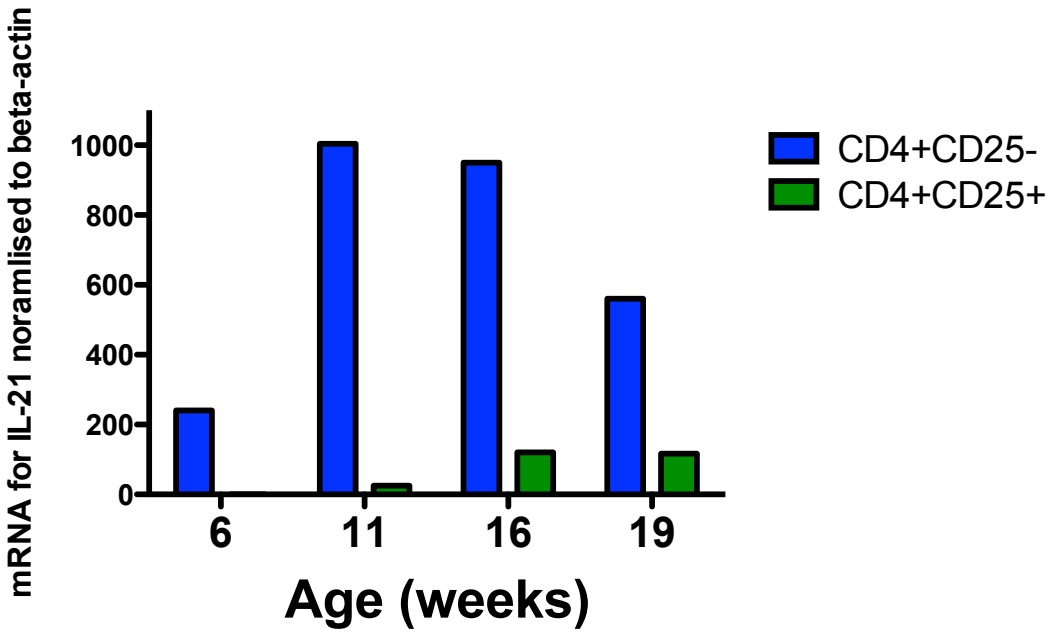


Figure 3.02. The expression of IL-21 mRNA in DO11xRIP-mOVA mice (in collaboration with K. Attridge). CD4+CD25- and CD4+CD25+ T cells were isolated from pancreatic lymph nodes of DO11xRIP-mOVA mice (n=6). The expression of IL-21 mRNA was evaluated by Taqman qPCR. IL-21 transcripts were normalised to endogenous beta-actin levels.

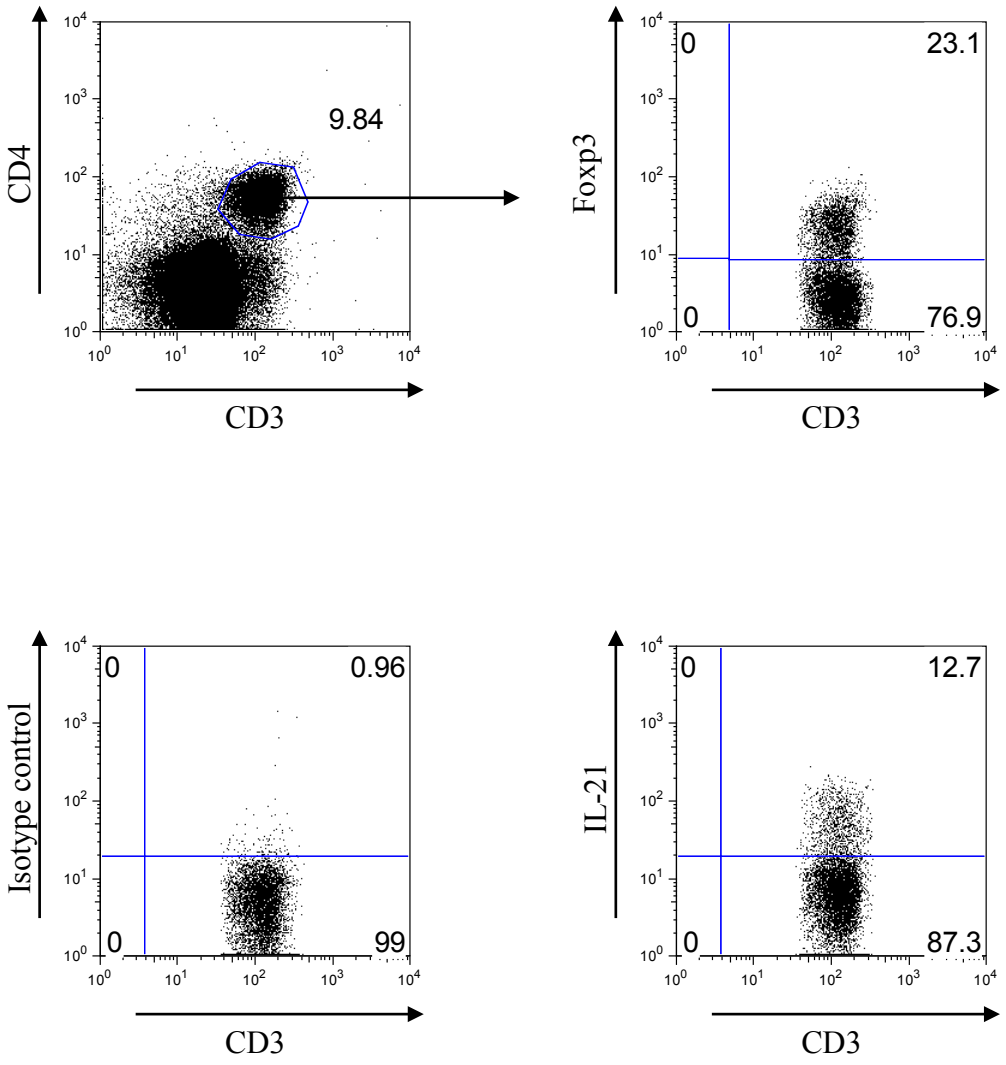


Figure 3.03. The expression of IL-21 in splenocytes from DO11xRIP-mOVA mice (18-week-old). Single cell suspensions from DO11xRIP-mOVA spleen were restimulated and stained for surface CD3 and CD4, and intracellular Foxp3 and IL-21. Dot plots show the gating strategy applied to identify CD4+ Foxp3- T cells by flow cytometry (top panel) and the expression of IL-21 in CD4+Foxp3- Tconv cells (bottom panel).

antibodies against the Treg lineage specific transcription factor Foxp3 to help us specifically identify Foxp3- conventional T cells. The data in **Fig. 3.03** illustrate the gating strategy used for CD3+CD4+ T cells and for the CD4+Foxp3- conventional T cell subset (top panel). The bottom panel shows the positive staining threshold set using an isotype control antibody and IL-21 expression in CD4+Foxp3- conventional T cells. This flow cytometry assay provided us with a useful tool to further evaluate the role of IL-21 in the DO11xRIP-mOVA mouse model of T1D.

3.2.2. IL-21 is markedly enriched in DO11xRIP-mOVA and CTLA-4-/- mice

Having optimised an intracellular cytokine stain, it was possible to complement our mRNA data by evaluating IL-21 expression at the protein level in DO11xRIP-mOVA mice. To achieve this, we isolated cells from the pancreatic LN (draining LN), inguinal LN (non-draining LN), spleen and pancreas (the organ of immune attack). Single cell suspensions from these tissues were restimulated and stained for intracellular IL-21 (**Fig. 3.04**). Surprisingly, although IL-21-producing T cells could be readily identified in the pancreatic LN, their proportion was only slightly higher than that seen in lymph nodes that did not drain the site of self-antigen expression (inguinal LN). Strikingly however the analysis revealed that IL-21 was highly produced by pancreas-infiltrating CD4+Foxp3- conventional T cells. A moderate frequency of IL-21-producing CD4+Foxp3- conventional T cells was detected in the spleen. Consistent with mRNA data, we observed that CD4+Foxp3+ regulatory T cells expressed negligible levels of IL-21 across all tissues. The above data suggests that T cell IL-21 production was enhanced at the site of the autoimmune attack and to a lesser extent in the spleen. While representative flow cytometry plots are shown in **Fig. 3.04**, collated data from several donor animals is presented in **Fig. 3.05**. These data show that there was a statistically

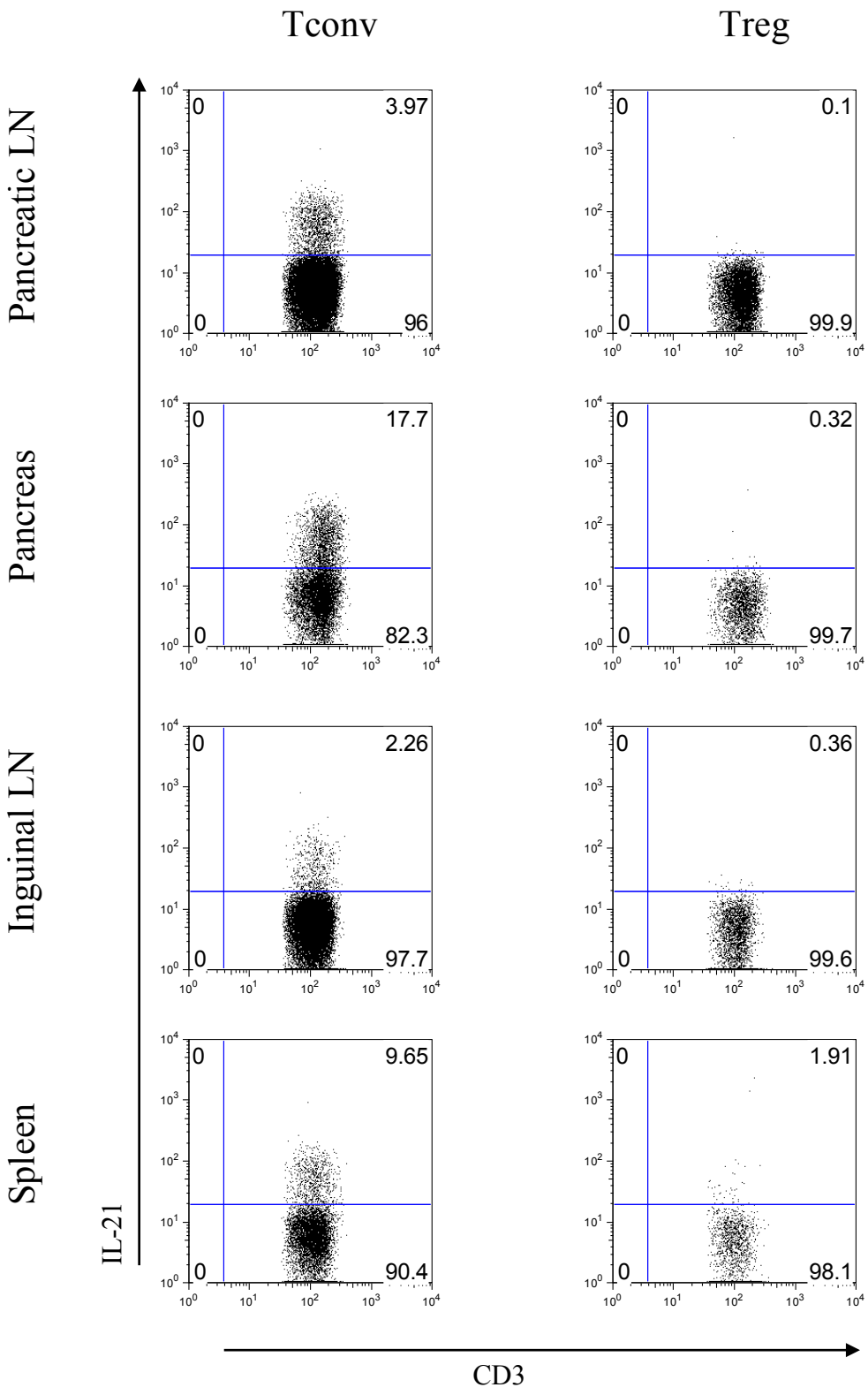


Figure 3.04. The expression of IL-21 in different tissues of DO11xRIP-mOVA mice (12-week-old). Single cell suspensions from pancreatic lymph node, pancreas, inguinal lymph node and spleen of DO11xRIP-mOVA mice were restimulated and stained for surface CD3 and CD4, and intracellular Foxp3 and IL-21. Representative dot plots show IL-21 staining in CD4+Foxp3- Tconv and CD4+CD25+ Treg cells.

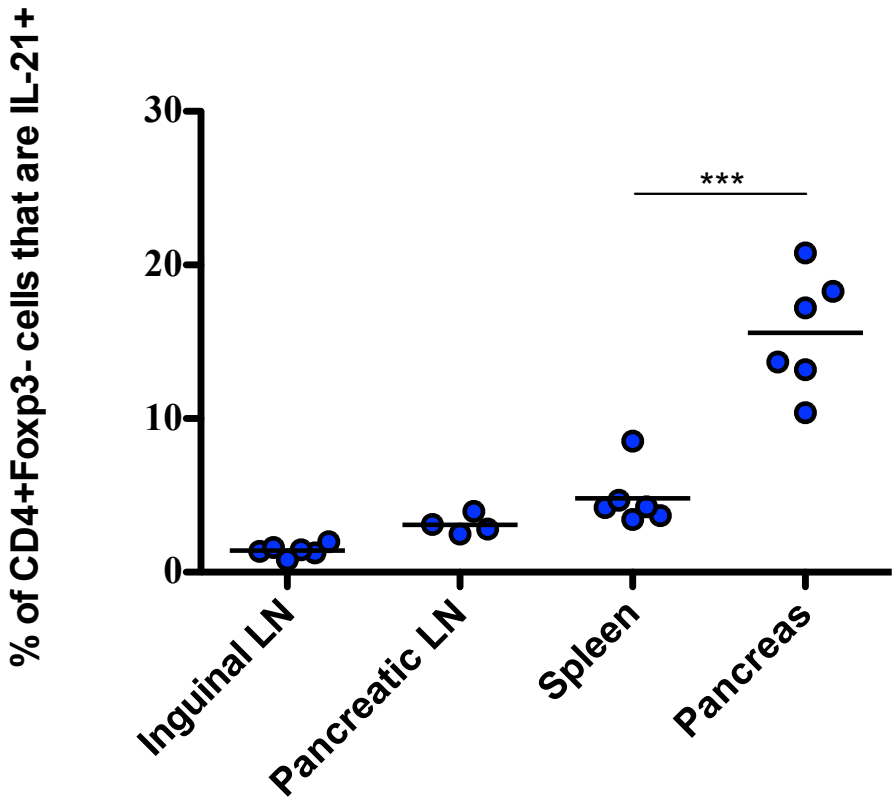


Figure 3.05. IL-21 producing Tconv are markedly enriched in the pancreases of DO11×RIP-mOVA mice. Single cell suspensions from indicated tissues of DO11xRIP-mOVA mice were restimulated and stained for surface CD4 and intracellular Foxp3 and IL-21. Graph shows collated data for IL-21 in CD4+Foxp3- T conv. Bars represent means across 6 experiments for the indicated tissue. ***, p=<0.001.

significant difference in IL-21 expression between cells isolated from the pancreas and spleen. Unlike at the site of immune attack, a substantially lower frequency of IL-21-producing CD4+Foxp3- conventional T cells was found in draining (pancreatic) and non-draining (inguinal) LN. This indicates that IL-21 expression was markedly enriched in the pancreas of DO11xRIP-mOVA mice, suggesting that this cytokine might potentially modulate immune responses *in situ* during the course of diabetes.

The above data demonstrated that IL-21 was substantially produced in the pancreas of normoglycaemic DO11xRIP-mOVA mice. However, this initial study only included a limited numbers of healthy animals, and little was known about how IL-21 levels varied throughout the course of disease progression. We wished to know whether IL-21 was present in the pancreas and other tissues at even earlier ages and how its expression varied over time. To address this, we evaluated IL-21 expression in the inguinal LN, pancreatic LN, pancreas and spleen from four to 18-week-old DO11xRIP-mOVA mice. **Fig. 3.06** clearly demonstrates that IL-21 expression was consistently enhanced in the pancreas across all ages. In contrast, a lower frequency of IL-21+ CD4+Foxp3- conventional T cells was observed in the inguinal LN and pancreatic LN. Moderate IL-21 levels were detected in the spleen throughout the progression of the disease. Interestingly, the lowest average frequency of IL-21-producing conventional T cells in the pancreas was found in 18-week-old mice. Although average proportions of IL-21-expressing CD4+Foxp3- T cells varied in the spleen throughout the time course, a slight upward trend was observed between 12 and 18 weeks. Overall, our time course studies revealed that pancreas-infiltrating conventional T cells expressed substantial levels of IL-21 throughout disease progression. Furthermore, we were able to detect IL-21-expressing diabetogenic T cells from four weeks of age onwards. Thus the autoreactive Tconv

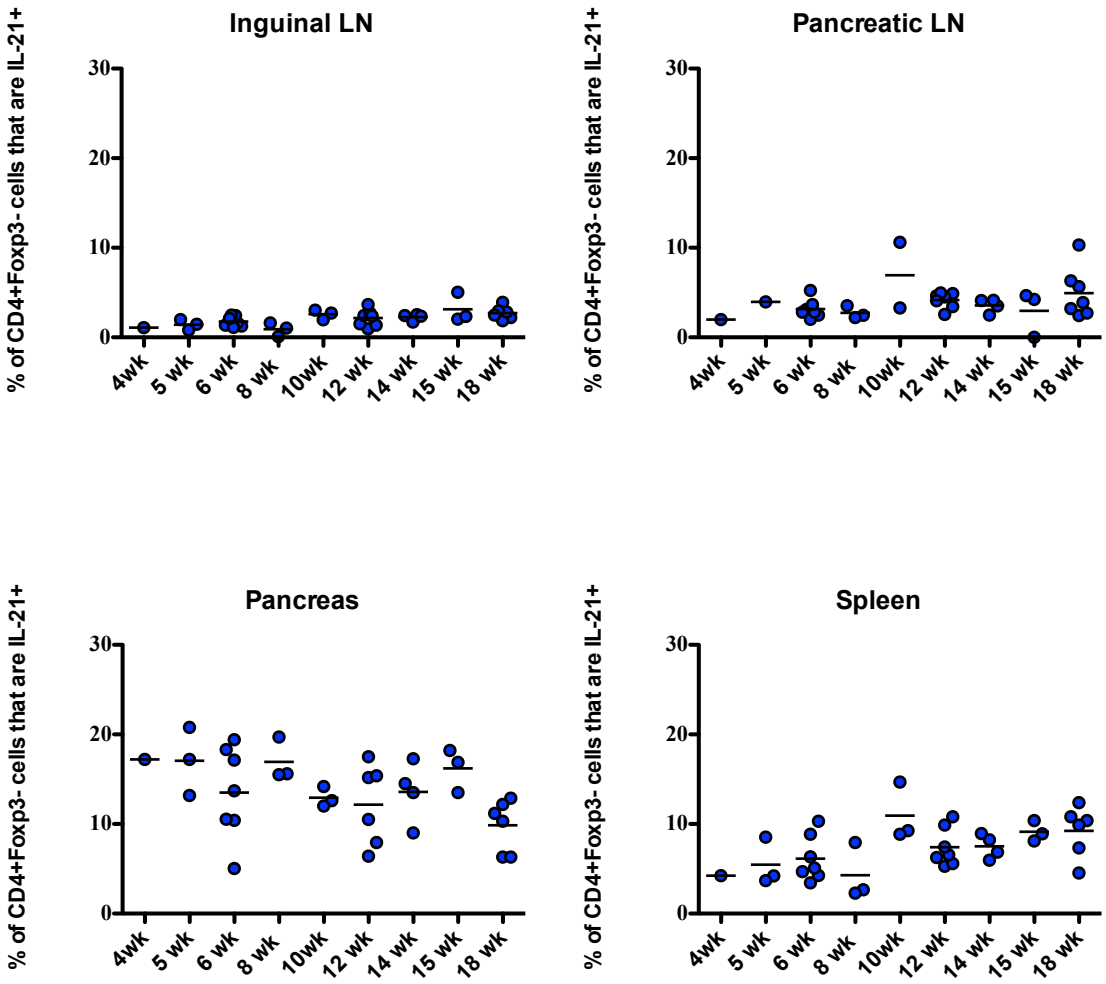


Figure 3.06. Time course analysis of IL-21 expression in DO11 x RIP-mOVA mice. Single cell suspensions from indicated tissues of DO11xRIP-mOVA mice were restimulated and stained for surface CD4 and intracellular Foxp3 and IL-21. Graphs show collated data for IL-21 expression in CD4+Foxp3- T conv. Bars represent means across indicated ages (weeks).

entering the pancreas at the very earliest stages of the autoimmune attack have the potential to elicit the cytokine IL-21. It should be noted that DO11 single transgenic mice and RIP-mOVA single transgenic mice were also assessed for IL-21 expression with levels being <3% in all tissues examined. Several studies have demonstrated that IFNγ and TNFα might be important in driving disease in T1D. Growing evidence suggests that IFNγ plays a major role in β-cell death either directly (Seewaldt et al. 2000) or indirectly via other mechanisms such as the induction of TNFα or IL-1β to name a few (reviewed in (Lehuen et al. 2010)). Additionally, TNFα has been shown to accelerate diabetes at an early stage of the disease (Christen et al. 2001; Lee et al. 2005). IL-2 is thought to be essential for Treg homeostasis, thus it might modulate immune regulation at the site of the immune attack. We therefore decided to evaluate the expression of IFNγ, TNFα and IL-2 by the IL-21-producing conventional T cells in the pancreas. To do this, we isolated cells from the pancreas of DO11xRIP-mOVA mice, and assessed their expression of intracellular IL-21, IFNγ, TNFα and IL-2. Pancreas-infiltrating conventional T cells highly expressed TNFα and IFNγ, and showed some expression of IL-2, although the stain was less well defined (**Fig. 3.07 A**). Of the IL-21-producing T cells, over half co-expressed IFNγ. The picture was even more striking for TNFα, with the vast majority of IL-21-producing cells also expressing this cytokine. **Fig. 3.07 B** illustrates that almost all IL-21+ conventional T cells co-expressed TNFα, while more than half of them also produced IFNγ. In contrast, most IL-2-producing CD4+Foxp3- T cells did not co-express IL-21 (although it is harder to be certain about this given the quality of the IL-2 staining). Thus, we found that the majority of IL-21+ conventional T cells could also co-express IFNγ or TNFα. As the presence of these two cytokines was previously linked with β-cell death, IL-21+TNF+ and IL-21+IFNγ+ conventional T cells might have a key role in driving the development of diabetes.

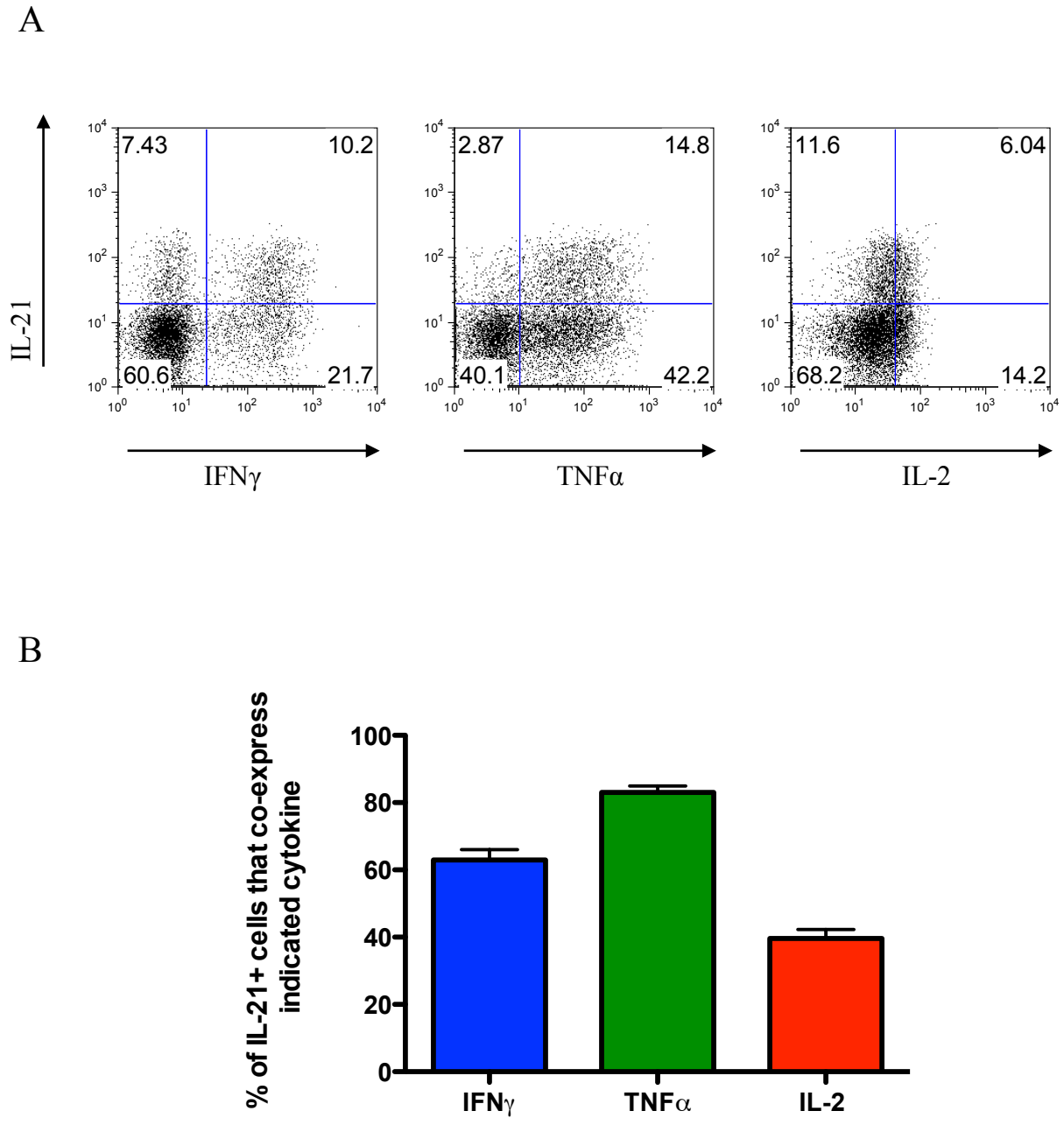


Figure 3.07. Co-expression of IL-21 with other cytokines in pancreas-infiltrating T cells isolated from DO11xRIP-mOVA mice. Single cell suspensions from DO11xRIP-mOVA pancreas were restimulated and stained for surface CD4 and intracellular Foxp3, IL-21, IFNγ, TNFα and IL-2. (**A**) Representative dot plots show IL-21 co-staining with IFNγ, TNFα and IL-2 in the pancreas infiltrating CD4+Foxp3- T conv. (**B**) Graph shows collated data for IFNγ, TNFα and IL-2 in IL-21+ Tconv. Bars represent means and SEM across 3 experiments for the indicated cytokine.

Upon activation, CD4+ naïve T cells can carry out their effector function by differentiating into distinct CD4+ subsets characterised by a unique cytokine imprint. A dogmatic division between Th1 and Th2 lineages associated with IFNγ and IL-4 production, respectively was revised upon the emergence of the pathogenic Th17 subset, characterised by IL-17 expression (Langrish et al. 2005). Since then several studies have demonstrated that IL-21 is expressed by the Th17 lineage and acts in an autocrine manner to generate this potentially pathological subset (Nurieva et al. 2007; Korn et al. 2007; Wei et al. 2007). We therefore wished to investigate the possible contribution of the Th17 subset to tissue injury in the DO11xRIP-mOVA mouse model of T1D. To assess this, we isolated T cells from the pancreases of eight, 11 and 14-week-old DO11xRIP-mOVA mice, and assessed their expression of intracellular IL-21 and IL-17. To check for diabetes status, tail bleeds were performed and blood glucose levels measured. As demonstrated in **Fig. 3.08 A**, the expression of IL-17, a signature cytokine of Th17 subset, remained very low through the time course. Furthermore, it appeared that the great majority of IL-21-producing T cells did not co-express IL-17. **Fig. 3.08 B** indicates that the 8-week-old mouse was determined to be normoglycaemic, whereas the 11 and 14-week-old animals had elevated glucose levels, thus allowing us to probe IL-17 expression in pre-diabetic, early diabetic and late-diabetic animals. Overall, unlike IFNγ, TNFα, IL-2 and IL-21, we found very low levels of IL-17 in the pancreas of DO11xRIP-mOVA mice regardless of their diabetes status. It therefore seems plausible to suggest that the Th17 population has a limited impact on tissue injury in our diabetes model.

In addition to examining IL-21 expression in a model of tissue-specific autoimmunity, we also wished to explore the potential expression of this cytokine in the context of systemic

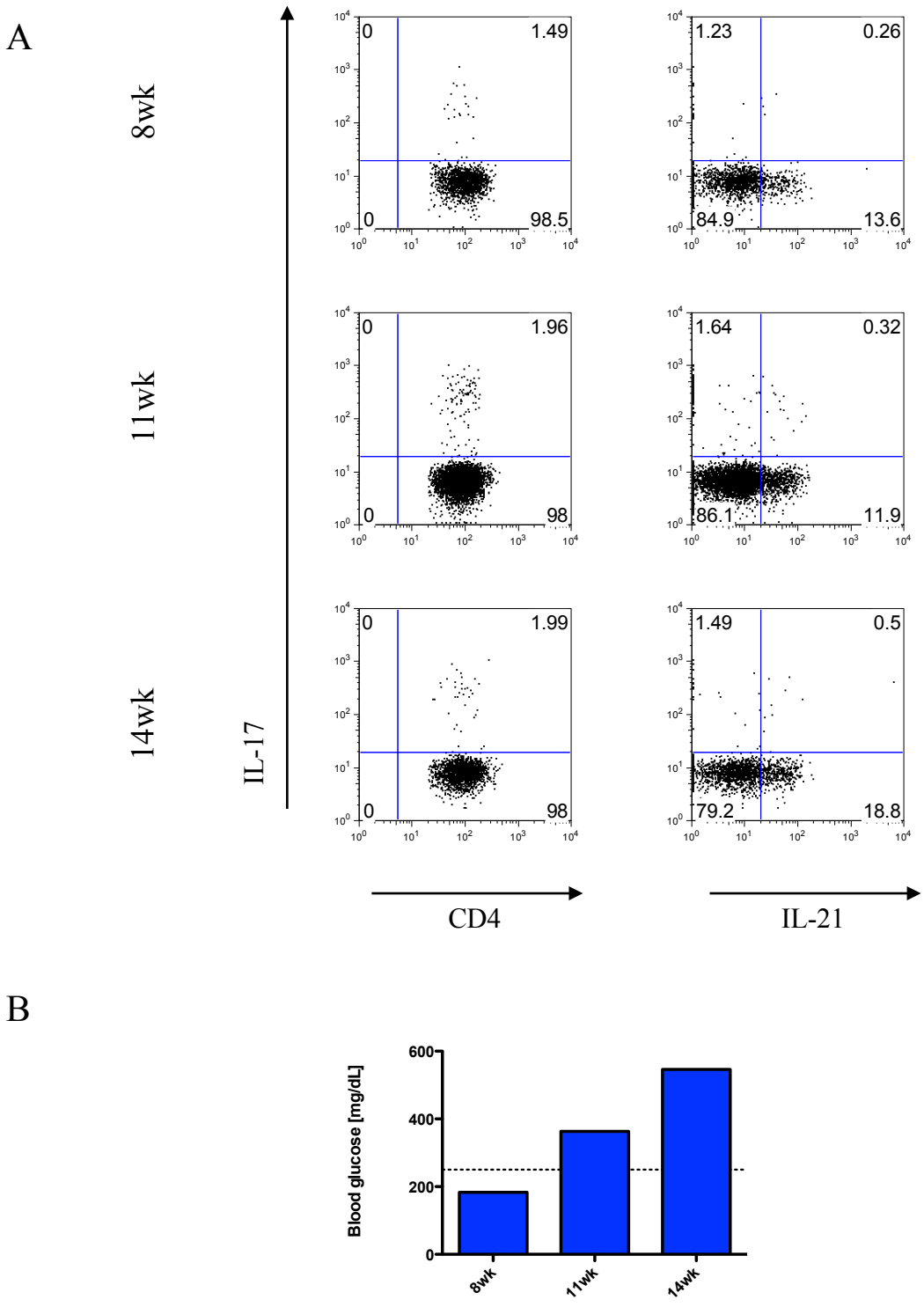


Figure 3.08. Expression of IL-17 by T cells infiltrating the pancreas in DO11xRIP-mOVA mice. Single cell suspensions from pancreases of DO11xRIP-mOVA mice were restimulated and stained for surface CD4 and intracellular Foxp3, IL-21 and IL-17. (**A**) Representative dot plots show IL-17 staining in CD4+Foxp3- Tconv and co-expression of IL-21. (**B**) Graph shows blood glucose measurements for DO11xRIP-mOVA mice used in this experiment. Dashed line indicates blood glucose level at which mice are considered diabetic.

autoimmune conditions. To this end we have also examined CTLA-4-/- mice. CTLA-4 is constitutively expressed on CD4+Foxp3+ regulatory T cells and activated CD4+Foxp3- conventional T cells. We and others have reported that Treg lacking CTLA-4 lose the ability to control T cell responses *in vivo* (Wing et al. 2008; Schmidt et al. 2009). Earlier evidence demonstrated that mice lacking this negative regulator developed a fatal multi-organ lymphoprolipherative disease (Tivol et al. 1995; Waterhouse et al. 1995). In this chapter, we revealed that IL-21 was markedly enriched in the target organ of tissue-specific autoimmunity. However, it was unclear whether IL-21 could be found in a systemic autoimmune disorder. To address this, Tconv (CD4+CD25-) and Treg (CD4+CD25+CD27hi) from peripheral lymph nodes of 15 to 18-day-old CTLA-4-/- or BALB/c mice were MoFlo sorted and IL-21 expression was evaluated by quantitative real time PCR. The inclusion of CD27 as a sorting marker was to avoid the isolation of activated T cells that downregulate CD27 (Tang & Bluestone 2006); this is necessary in CTLA-4-/- mice due to the large number of activated T cells in the periphery. **Fig. 3.09** illustrates that IL-21 mRNA expression levels were significantly higher in conventional T cells from CTLA-4-/- mice than their WT counterparts. Moreover, negligible levels of IL-21 transcripts were found in both CTLA-4-/- and WT regulatory T cells, which is in line with our mRNA analysis of DO11xRIP-mOVA mice. These data suggest that IL-21 mRNA is expressed in conventional T cells and that this cytokine might potentially contribute to the pathology in mice lacking CTLA-4. We therefore wished to evaluate IL-21 protein expression in CTLA4-/- animals. To this end, isolated cells from peripheral LN and spleen of CTLA-4-/- and control BALB/c animals were assessed for IL-21 expression by flow cytometry. As seen in **Fig. 3.10 A**, IL-21-producing CD4+Foxp3- conventional T cells could clearly be detected in the spleen of CTLA-4-/- mice. In contrast, CD4+Foxp3+ regulatory T cells expressed negligible amounts of IL-21 (data not shown),

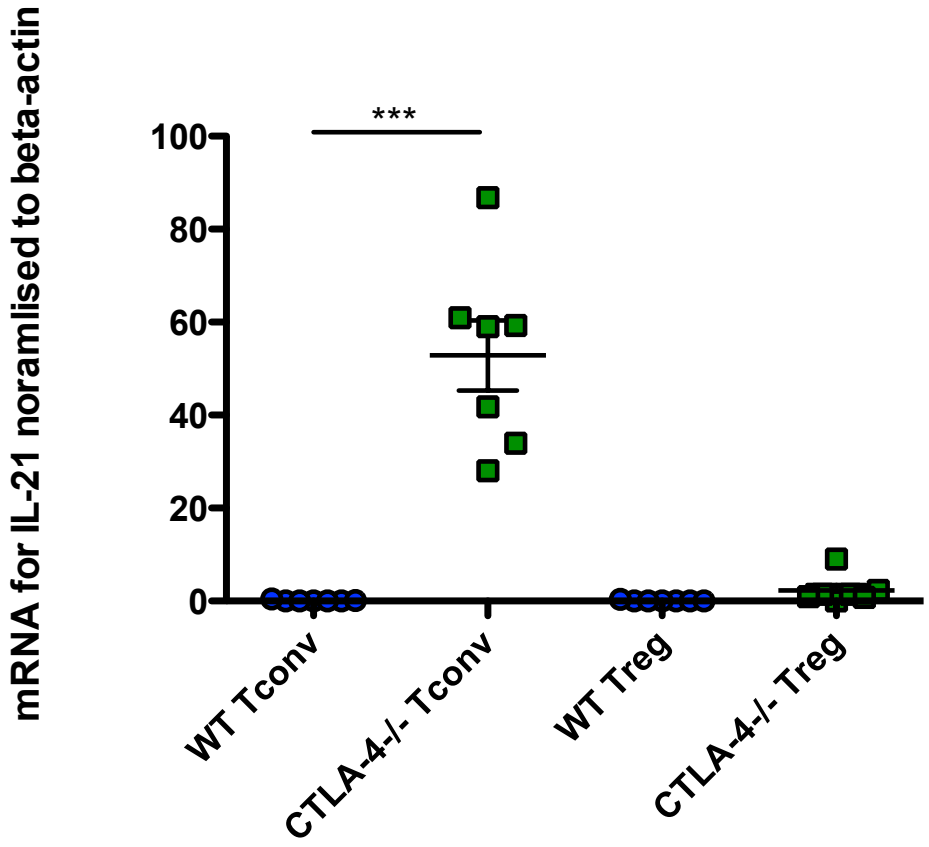


Figure 3.09. The expression of IL-21 mRNA in CTLA-4-/- mice (in collaboration with E.Schmidt). CD4+CD25- Tconv and CD4+CD25+CD27hi Treg cells were isolated from peripheral lymph nodes of 15 to18-days-old CTLA-4-/- or BALB/c mice. The expression of IL-21 mRNA was evaluated by Taqman qPCR. IL-21 transcripts were normalised to endogenous beta-actin levels. Bars represent means and SEM across 7 mice for the indicated cell subset. ***, p=<0.001

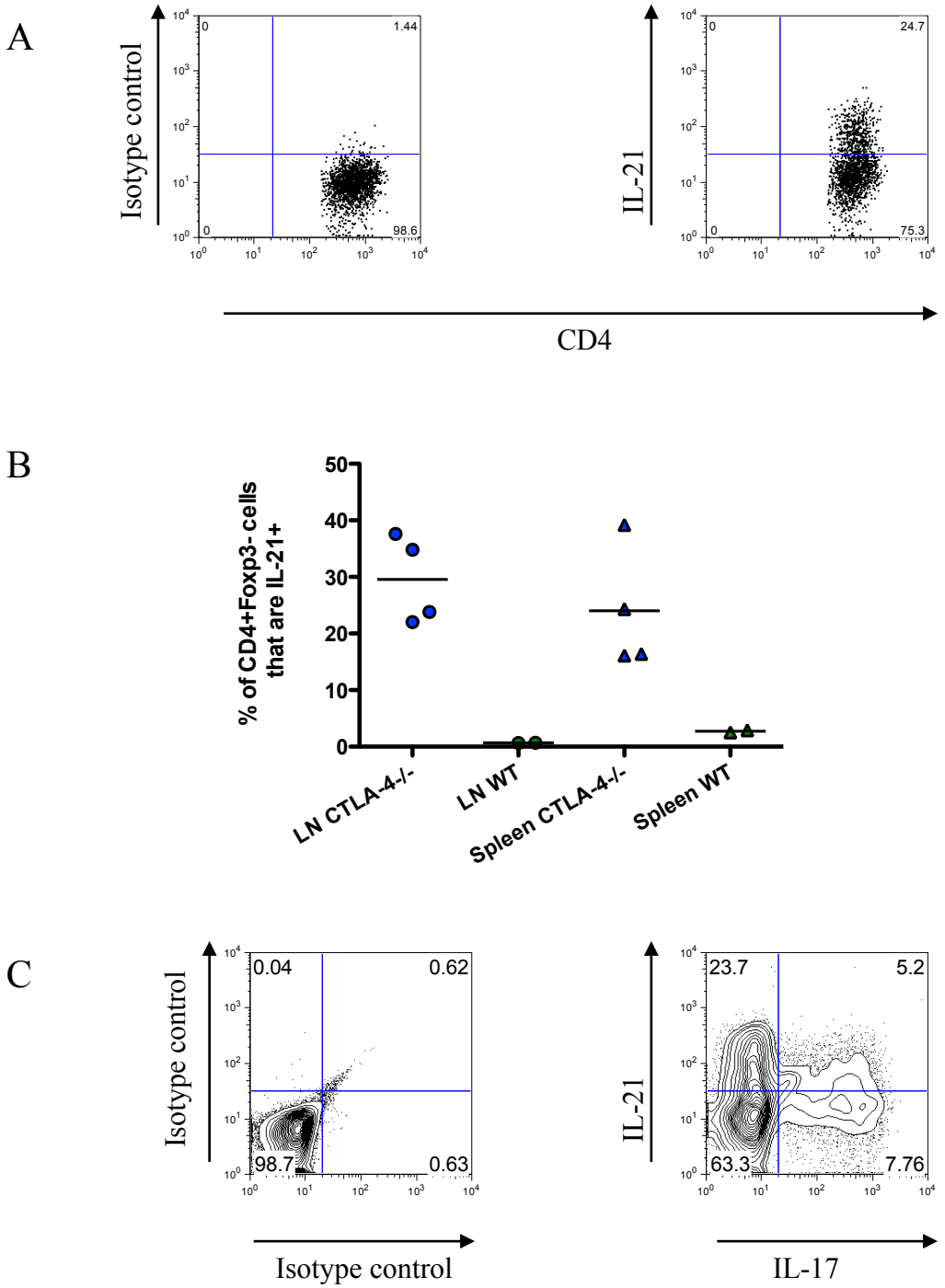


Figure 3.10. IL-21 is upregulated in CTLA-4-/- mice. Single cell suspensions from lymph nodes and spleen of CTLA-4-/- and BALB/c mice were restimulated and stained for surface CD4 and intracellular Foxp3, IL-21 and IL-17. (**A**) Representative dot plot shows IL-21 staining in CD4+Foxp3- Tconv isolated from CTLA-4-/- spleen. (**B**) Graph shows collated data with means for IL-21 expression in CD4+Foxp3- T conv isolated from the indicated tissues of CTLA-4-/- and BALB/c (WT) mice. (**C**) Representative contour plots show co-staining of IL-21 and IL-17 in CD4+Foxp3- Tconv isolated from the CTLA-4-/- spleen.

mirroring our findings in the DO11xRIP-mOVA model. With regard to IL-21 distribution, we found similar levels of IL-21-producing conventional T cells in peripheral LN and spleen (**Fig 3.10 B**). BALB/c animals (designated WT for wildtype) showed very little IL-21 expression in either conventional or regulatory T cells. In the DO11xRIP-mOVA model we showed that the IL-21 producing T cells did not co-produce IL-17 (**Fig. 3.08**). To assess whether this was also the case in a systemic model of autoimmunity we co-stained IL-21 and IL-17 in CTLA-4-/- mice. Intriguingly, we found that a substantial fraction of conventional T cells expressed IL-17, far higher than what we had observed in the DO11xRIP-mOVA mice (**Fig. 3.10 C**). Furthermore, a fraction of the IL-17+ conventional T cells also co-expressed IL-21. However, importantly when we assessed the IL-21-producing cells, only a very small minority of these co-expressed IL-17. This result suggests that the IL-21-producing cells seen in both the DO11xRIP-mOVA model of tissue-specific autoimmunity and in the CTLA4-/- model of systemic autoimmunity are not Th17 cells but rather represent a distinct population.

Overall, we demonstrated that IL-21 was found at the protein level in the DO11xRIP-mOVA pancreas and CTLA-4-/- peripheral lymph nodes and spleen. As IL-21 can counteract Treg-mediated suppression, it can be envisaged that IL-21-producing conventional T cells might potentially influence the local cytokine environment in the DO11xRIP-mOVA pancreas, promoting immune dysregulation and possibly contributing to the tissue damage. Furthermore, IL-21 might support maintenance of potentially pathogenic Th17 cells in the peripheral lymph nodes and spleen of CTLA-4-/- mice. Undoubtedly, IL-21 has the ability to modulate T cell responses in the organ-specific and systemic autoimmunity.

3.3. Discussion

Increasing evidence suggests that the IL-21 pathway is associated with the development of numerous autoimmune disorders (reviewed in (Søndergaard & Skak 2009)). The importance of this cytokine in the development of autoimmune diabetes has been previously demonstrated by three independent groups (Spolski et al. 2008; Sutherland et al. 2009; Datta & Sarvetnick 2008). Their studies examined the role of IL-21 signalling in the non-obese diabetic (NOD) mouse model of T1D. The authors showed that backcrossing IL-21R-/- mice onto the NOD background prevented lymphocytic pancreas infiltration and the development of diabetes. The most striking finding in this chapter was the overexpression of IL-21 protein in both the DO11xRIP-mOVA model of diabetes and in a systemic model of autoimmunity, the CTLA-4 deficient mouse.

Having previously detected IL-21 transcripts in pancreatic LN Tconv, our initial investigation aimed to evaluate the expression of IL-21 across different tissues in the DO11xRIP-mOVA mouse model of T1D (Clough et al. 2008). The present study revealed that IL-21 protein was significantly enriched in CD4+Foxp3- Tconv isolated from the pancreas of DO11xRIP-mOVA mice (**Fig. 3.05**). Given the fact that IL-21 has been shown to counteract Treg mediated suppression in mouse and man, this cytokine might potentially contribute to the immune dysregulation at the site of autoimmune attack (Clough et al. 2008; Peluso et al. 2007; Attridge et al. 2012). Although CD4+Foxp3- Tconv produced small amounts of IL-21 in the inguinal LN (non-draining), pancreatic LN (self antigen-draining) and spleen, this expression was substantially lower than in the pancreas. Further analysis aimed to evaluate the expression of IL-21 throughout the progression of the disease. Interestingly, we found that IL-21 was highly produced by T cells infiltrating pancreas of DO11xRIP-mOVA mice from

an early time point (four weeks of age) and this expression was maintained through to overt diabetes (**Fig. 3.06**). Hence, IL-21 signals might potentially modulate immune responses at any time point immediately following entry of lymphocytes into the pancreas. Furthermore, it appears that the expression of IL-21 in the spleen increased over the studied period. This suggests a rise in recirculating IL-21-producing Tconv over time, which might be explained by the declining availability of auto-antigen as β-cell pool is depleted.

Further phenotypic analysis of IL-21-producing Tconv in the pancreas revealed that the majority co-expressed the pro-inflammatory cytokines IFNγ and TNFα, both of which are associated with β-cell destruction (**Fig. 3.07**). In the healthy state, naïve T cells travel through lymphoid tissues and blood, however their access to the pancreas is restricted. This changes once the T cells encounter β-cell-derived antigens and become activated. In T1D, these cells gain the ability to access the pancreas and carry out their effector function mediated by Fas/FasL interactions and production of cytokines associated with inflammation such as IFNγ and TNFα (reviewed in (Mathis et al. 2001)). Early experiments implied that the ectopic expression of IFNγ restricted to insulin-producing β-cells was associated with lymphocytic infiltration and development of diabetes (Sarvetnick et al. 1988; Sarvetnick et al. 1990). Furthermore, IFNγ is believed to promote production of other pro-inflammatory mediators including TNFα and IL-1β (reviewed in (Lehuen et al. 2010). A more complex picture emerged from studies that examined the role of TNFα in the development of T1D. Yang and colleagues reported that the administration of recombinant TNFα at an early age accelerated the disease in NOD mice, whereas blockade had a protective effect (Yang et al. 1994). The same study also revealed the strikingly opposite result of TNFα treatment in older NOD mice, which showed delayed onset of diabetes. Later, the Flavell group investigated this

phenomenon utilising two different transgenic mouse models where TNFα was expressed under the rat insulin promoter either in the neonatal or adult NOD mice. Their findings were in line with Yang's work and confirmed that TNFα expression in the neonatal NOD mice promoted the development of diabetes, whilst it prevented the disease in adult animals (Grewal et al. 1996; Green et al. 1998).

We found that to some extent IL-21-producing conventional T cells also co-expressed IL-2 **(Fig. 3.07)**. Originally IL-2 was associated with T cell growth, however this perception has recently changed and this cytokine is now believed to be a key factor for Treg survival (reviewed in (Dooms & Abbas 2010)). Interestingly, Grinberg-Bleyer and colleagues demonstrated that treatment with low dose IL-2 reversed disease in the diabetogenic NOD mouse model (Grinberg-Bleyer et al. 2010). This protective effect was achieved by enhancing Treg survival and function that consequently downmodulated conventional T cell responses in these mice. We and others demonstrated that IL-21 releases conventional T cells from Treg suppression (Peluso et al. 2007; Clough et al. 2008). Our recent work suggests that IL-21 acts directly on conventional T cells to decrease their IL-2 production, thus compromising the function of the Treg compartment (Attridge et al. 2012). Therefore, it is plausible that IL-21 might potentially modulate IL-2 expression by conventional T cells in our model, which might have a detrimental effect on Treg function in the pancreas.

Our studies also looked at the expression of IL-17, a hallmark cytokine associated with the potentially pro-inflammatory Th17 subset. Numerous reports indicated that IL-21 is an important autocrine factor that regulates the generation of this T cell lineage (Nurieva et al. 2007; Korn et al. 2007; Wei et al. 2007). More recently, a number of reports suggested the

involvement of the Th17 subset in the development of autoimmune diabetes. BDC2.5 T cells differentiated into Th17 cells *in vitro* were shown to cause diabetes when transferred into NOD/SCID recipients (Martin-Orozco et al. 2009; Bending et al. 2009). However closer analysis revealed that these IL-17-producing T cells switched to expressing IFNγ after adoptive transfer. Disease could be blocked by inhibiting the IFNγ pathway, but not by targeting IL-17 (Martin-Orozco et al. 2009). One study suggested the blockade of IL-17 or IL-25 (IL-17E) in 10-week-old NOD mice prevented the development of diabetes (Emamaullee et al. 2009), however a separate report suggested a protective role for IL-17 in adoptive transfer models of diabetes (Han et al. 2010). Our data suggests that the pancreas-infiltrating conventional T cells expressed very low levels of IL-17 and this could be observed for normoglycaemic as well as hyperglycaemic animals (**Fig. 3.08**). It therefore seems plausible to suggest that the Th17 population has a limited impact on tissue injury in our diabetes model.

Our studies also looked at the potential expression of IL-21 in CTLA-4-/- mice, which unlike the DO11xRIP-mOVA model, is a systemic mouse model of autoimmunity. The absence of this negative regulator of the immune system leads to uncontrolled lymphoproliferation, which in consequence causes multi-organ infiltration and premature death after three weeks from birth (Tivol et al. 1995; Waterhouse et al. 1995). Interestingly, our preliminary mRNA data suggested that IL-21 transcripts were highly expressed in the peripheral LN and spleen of CTLA-4-/- mice (**Fig. 3.09**). Subsequently, we confirmed that conventional CD4+Foxp3- T cells expressed high amounts of IL-21 protein in these lymphoid organs (**Fig. 3.10 B**). The absence of CTLA-4 in T cells has been shown to support the generation of the Th2 response marked by enhanced expression of IL-4 and reduced production of IFNγ and IL-2 (Khattri et

al. 1999; Bour-Jordan et al. 2003). In light of these reports and our data, it is intriguing that the lack of CTLA-4 might potentially enhance IL-21, thereby inhibiting IL-2 production. This shift in the IL-21/IL-2 balance might possibly influence Treg mediated suppression and have serious implications in breaking immunological tolerance (Attridge et al. 2012). The high levels of IL-21 in the CTLA-4-/- mice also raised the question of whether its production comes from the Th17 subset. Our data however demonstrate that in this systemic model of autoimmunity a great majority of IL-21-producing conventional T cells did not express IL-17 (**Fig. 3.10 C**). This implies that the main source of IL-21 was not the Th17 lineage, but a different cell subset.

Future studies should investigate the effect of IL-21 blockade on disease progression in DO11xRIP-mOVA mice. It will be interesting to evaluate how this treatment would affect IFNγ and TNFα production in the pancreas, where IL-21 is mostly expressed. Furthermore, blocking IL-21/IL-21R interactions might possibly boost Treg function and survival through the increase of IL-2 production. Taking into consideration findings from the TNFα transgenic NOD studies, it will be also important to block IL-21 at different time points, as this might influence the outcome of the treatment. Alternatively, the role of IL-21 in T1D could also be examined by generation of DO11xRIP-mOVAxIL-21R-/- mice.

4. ANALYSIS OF PATHWAYS THAT PROMOTE T CELL IL-21 PRODUCTION.

4.1. Introduction

Data presented in the previous chapter indicated that IL-21 was highly expressed by pancreas-infiltrating T cells in DO11xRIP-mOVA mice, and in multiple organs of CTLA-4-/ mice. Although IL-21 production is currently mainly associated with the Th17 and Tfh lineages, others have shown that this cytokine can be also be produced by T cells differentiated into the Th1 and Th2 subsets (Wurster et al. 2002; Eto et al. 2011; Korn et al. 2007). Moreover, this cytokine is known to act in an autocrine manner to support the generation and maintenance of both the Th17 and Tfh subsets (Nurieva et al. 2007; Nurieva et al. 2008). However, a limited number of studies have focused on investigating the pathways that are involved in the promotion of IL-21 production. Suto and colleagues identified IL-6 as a key cytokine that drives IL-21 expression *in vitro* (Suto et al. 2008). In this chapter, we sought to carry out a comprehensive analysis of pathways that might be involved in the development of IL-21-producing T cells. Furthermore, our studies particularly aimed to generate a better understanding of how different antigen presenting cells (APC) affect the production of IL-21.

4.2. Results

4.2.1. Dendritic cells but not B cells can promote IL-21 production

Studies of Th17 differentiation indicated that not only was IL-6 essential for the development of this T cell subset but also that it induced their expression of IL-21 mRNA (Nurieva et al. 2007; Korn et al. 2007; Wei et al. 2007). Furthermore, the analysis performed by Suto and colleagues confirmed that IL-6 also promoted IL-21 expression at the protein level (Suto et al. 2008). Our studies aimed to evaluate IL-21 production at the single cell level in the context of co-stimulation provided by different APC populations. To this end, we decided to set up an *in vitro* assay with conventional T cells and APC derived from BALB/c mice. To exclude the possibility that the presence of CD4+CD25+ Treg was inhibiting differentiation to an IL-21-producing phenotype, the conventional T cells were depleted of CD25+ cells. We assessed the purity of MACS sorted CD4+CD25- conventional T cells and as indicated in **Fig. 4.01** purities were routinely greater than 98%.

Initially, we wished to verify the ability of IL-6 to induce IL-21 production by activated T cells, as reported by Suto and colleagues (Suto et al. 2008). To address this, we cultured sorted CD4+CD25- conventional T cells with anti-CD3/anti-CD28 beads at a 1:1 ratio, to provide TCR ligation and co-stimulation, in the presence or absence of 100ng/ml IL-6. After three days cells were restimulated and the expression of IL-21 and IFNγ evaluated by flow cytometric analysis. **Fig. 4.02 A** clearly demonstrates that T cell activation alone was not sufficient to promote IL-21 expression at substantial levels. In contrast, we observed a high frequency of IL-21-expressing T cells when exogenous IL-6 was provided. A large population

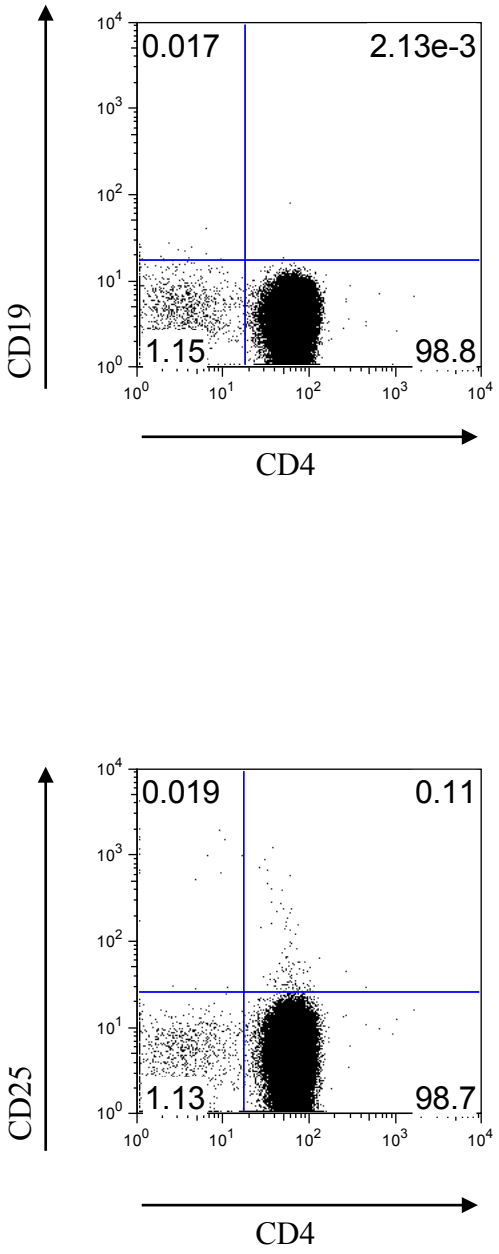


Figure 4.01. Validation of MACS CD4+CD25- isolation kits. MACS sorting was used to purify CD4+CD25- Tconv from BALB/c lymph node. Purified cells were surface stained for CD4, CD19 and CD25 and analysed by flow cytometry. Representative dot plots show purities for sorted CD4+CD25- Tconv.

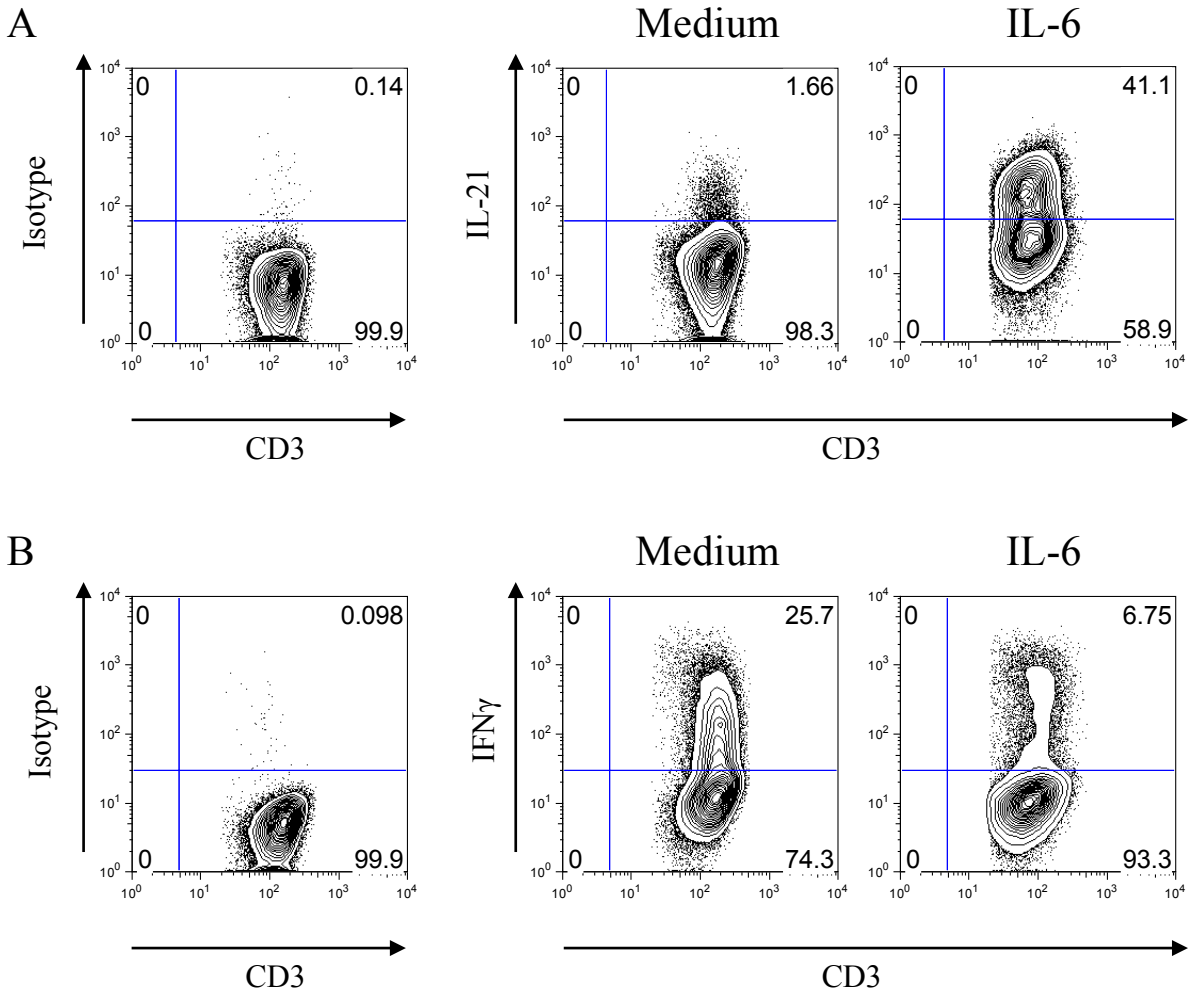


Figure 4.02. Expression profiles for IL-21 and IFNγ in anti-CD3/anti-CD28 bead-driven assays. $2.5x10^4$ Tconv were cultured with $2.5x10^4$ anti-CD3/anti-CD28 beads, alone or in the presence of 100ng/ml IL-6. After three days cells were restimulated and stained for surface CD3 and intracellular IL-21 and IFNγ. Representative contour plots show expression profiles for IL-21 (**A**) and IFNγ (**B**).

of IFNγ-producing T cells was detected in the absence of IL-6, which was diminished when this cytokine was added to the assay (**Fig. 4.02 B**). Repeated experiments (**Fig. 4.03)** confirmed that TCR ligation and CD28 co-stimulation were insufficient to drive a substantial IL-21 production and that the proportion of IL-21-expressing T cells was significantly higher when the exogenous IL-6 was provided. Thus we were able to repeat the findings of Suto and colleagues (Suto et al. 2008), giving us confidence in this result.

Antigen presentation is required for T cell priming, clonal expansion and migration to sites where they carry out their effector functions. It is believed that in T1D this process is initiated in the pancreas-draining LN by APC such as DC and B cells. Elegant studies by Gagnerault *et al.* demonstrated that the removal of the pancreatic LN from young NOD mice almost completely abrogated the development of diabetes (Gagnerault et al. 2002). Numerous reports showed that the antigen-presenting dendritic cells and B cells might potentially determine disease outcome in mouse models of T1D (Ludewig et al. 1998; Turley et al. 2003; Serreze et al. 1996; Greeley et al. 2001). Our own data suggests that dendritic cells and B cells are also present in the pancreatic LN and pancreas of DO11xRIP-mOVA mice. Furthermore, the depletion of B cells in this model prevented the development of diabetes (Ryan et al. 2010). In light of these findings we wished to establish whether signals provided by dendritic cells and B cells might modulate IL-21 production by T cells. In addition to providing TCR signals, such APC also contribute additional co-stimulatory and accessory signals that differ depending on APC type.

We therefore sought to obtain highly purified B cell and DC populations that could be used to initiate T cell responses *in vitro*. To this end, we purified CD19+ B cells from BALB/c spleen.

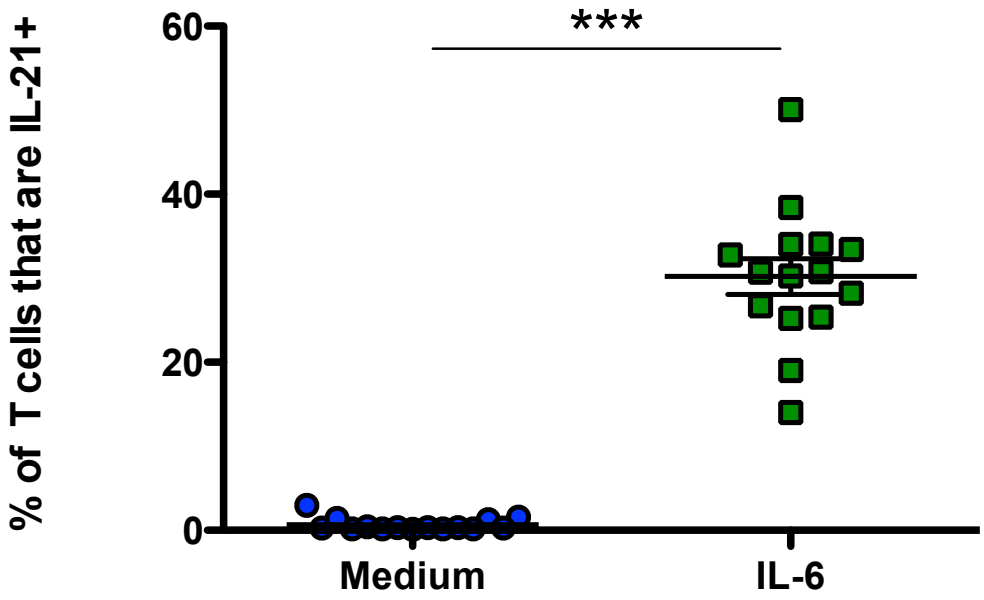


Figure 4.03. Exogenous IL-6 promotes IL-21 production in anti-CD3/anti-CD28 bead-driven assays. $2.5x10^4$ Tconv were cultured with $2.5x10^4$ anti-CD3/anti-CD28 beads, alone or in the presence of 100ng/ml IL-6. After three days cells were restimulated and stained for surface CD3 and intracellular IL-21. Graph shows collated data for IL-21 expression within gated CD3+ Tconv. Bars represent means across 15 experiments. ***, p=<0.001.

Due to the fact that our assay required high numbers of DC, we decided to differentiate bone marrow cells from BALB/c mice into the DC lineage following the method developed by Lutz *et al.* (Lutz et al. 1999). In some cases, the bone marrow-derived DC were matured over night with Lipopolysaccharides (LPS) to test whether their maturation status affects IL-21 production. CD19+ B cells, immature DC (iDC) and mature DC (mDC) were phenotyped for their expression of key markers. As shown in **Fig. 4.04** B cells expressed high levels of CD19 and MHC class II whilst lacking CD4, CD80 and CD86. We found that bone marrow-derived iDC and mDC expressed CD11c and CD11b which was in line with the classical DC phenotype outlined by Steinman and colleagues (Steinman et al. 1997). Moreover, we found high expression levels of MHC class II, CD80 and CD86, which were all further increased after over night incubation with LPS. In addition, our bone marrow-derived DC express the most recently identified marker of the classical DC phenotype, named zDC (Meredith et al. 2012), which is absent in other CD11c+ cell such as monocytes and plasmacytoid DC.

Initially, we were interested in determining whether signals provided by B cells are required for IL-21 production. To this end, CD19+ B cells were cultured with CD4+CD25- T cells at 3:1 and 6:1 ratios, with TCR ligation provided by soluble anti-CD3 antibody. After three days the expression of IL-21 and IFNγ was assessed by flow cytometry. The data in **Fig. 4.05** clearly show that very few IL-21-expressing cells were found for both ratios. In contrast, the great majority of T cells expressed IFNγ. This indicates that B cells failed to provide signals that promote IL-21 production, but were effective at inducing IFNγ expression. We previously showed that in the anti-CD3/anti-CD28 bead-driven assay, exogenous IL-6 acted directly on T cells to promote IL-21 expression. To test whether this was the same when T cell responses were driven by B cells, we assessed the effect of including exogenous IL-6.

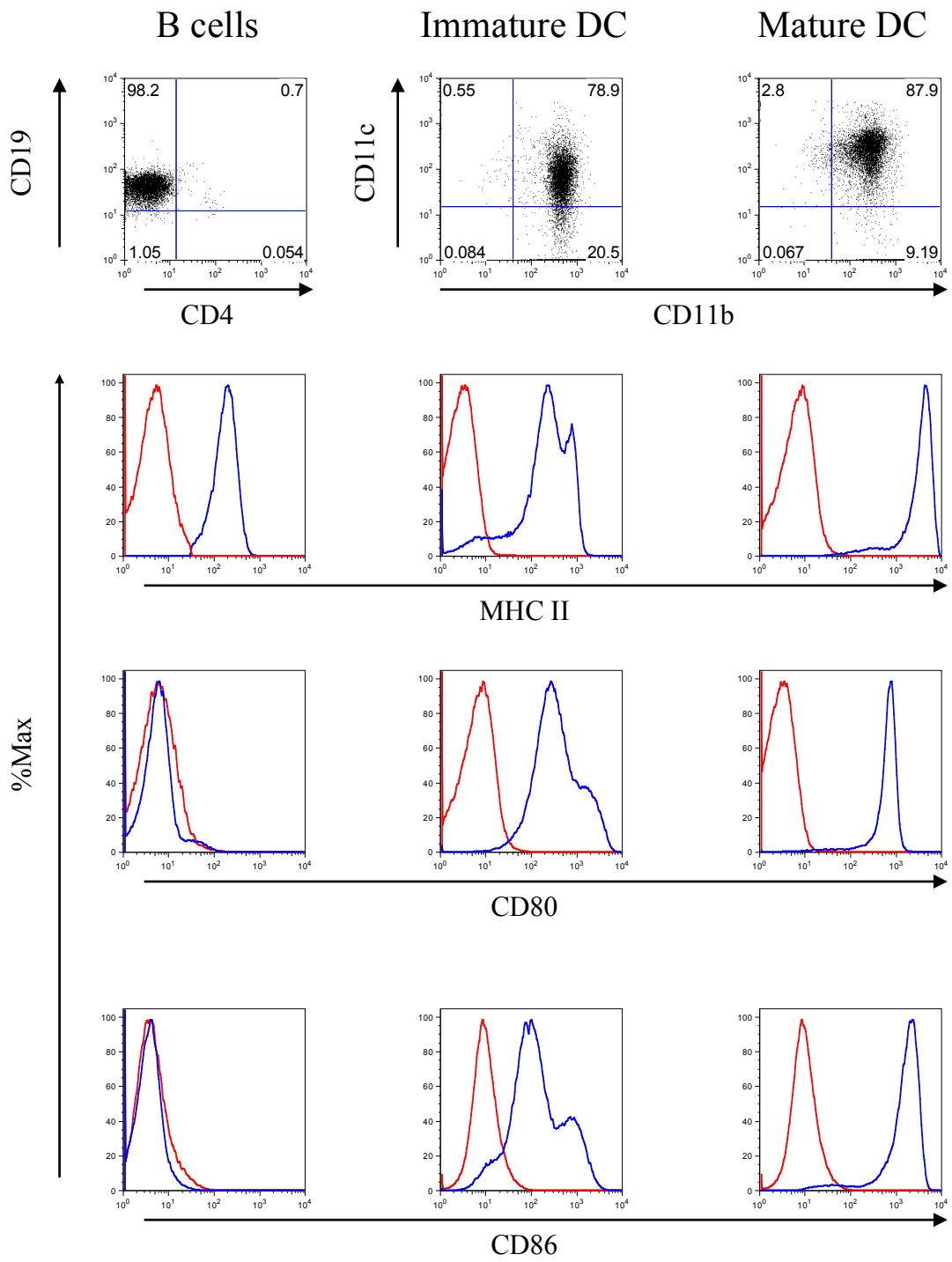


Figure 4.04. Phenotyping of purified B cells and bone marrow-derived DC. Single cells suspensions of MACS purified CD19+ cells were surface stained for CD4, CD19, MHC class II, CD80 and CD86. Bone marrow-derived immature DC or LPS-matured bone marrow-derived DC were surface stained for CD11b, CD11c, MHC class II, CD80 and CD86. Representative dot plots show CD4/CD19 co-staining and CD11b /CD11c co-staining for MACS purified CD19+ cells and bone marrow-derived DC, respectively (top row). Representative histograms show MHC class II, CD80 and CD86 staining in CD19+ B cells and bone marrow-derived DC.

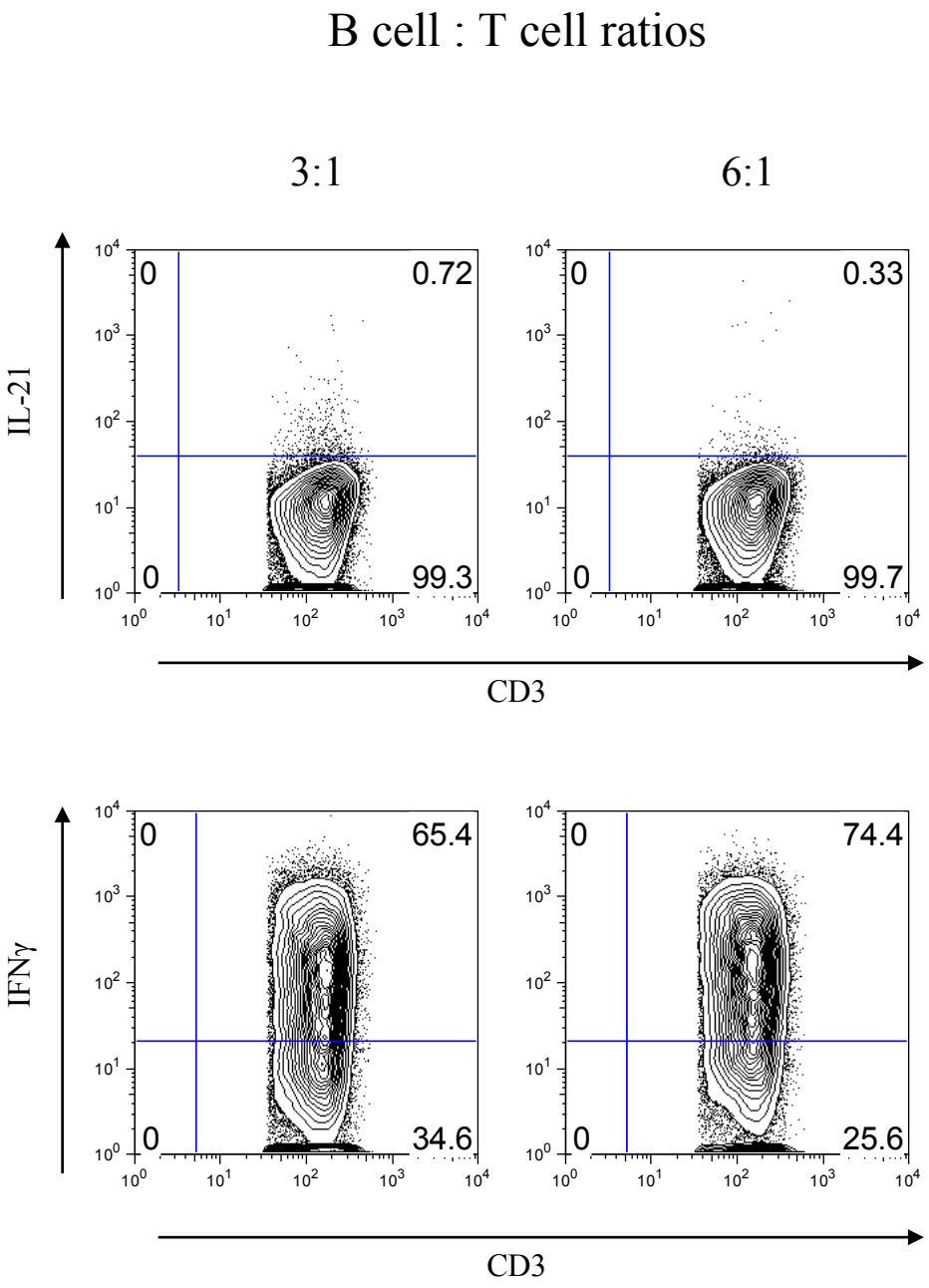


Figure 4.05. Expression profiles for IL-21 and IFNγ in B cell-driven assays. $2.5x10^4$ Tconv were cultured with $7.5x10^4$ or $15x10^4$ B cells and 0.8μg/ml anti-CD3. After three days cells were restimulated and stained for surface CD3 and intracellular IL-21 and IFNγ. Representative contour plots show expression profiles for IL-21 and IFNγ within gated Tconv.

CD19+ B cells and CD4+CD25- T cells were cultured at a 3:1 ratio, with TCR ligation provided by anti-CD3 antibody, in the presence or absence of 100ng/ml IL-6. **Fig. 4.06 A** illustrates that a high frequency of IL-21-expressing T cells was found in the cultures supplemented with IL-6. Extending our data set confirmed this finding, with a mean increase in IL-21 expression from 2% to 16% (**Fig. 4.06 B**). The increase in IL-21 in the presence of IL-6 was associated with a decrease in IFNγ production (**Fig. 4.07**) consistent with our previous observations in bead-driven assays. Overall, B cell-driven assays appeared to be similar to anti-CD3/anti-CD28 bead stimulations in respect of their modulation of IL-21 and IFNγ by exogenous IL-6. The above experiments illustrated that neither T cell activation nor B cell-derived signals were sufficient to induce IL-21 production.

We therefore sought to assess whether DC can influence IL-21 expression. To address this, immature or mature DC were cultured with CD4+CD25- T cells at 1:10 and 1:5 ratios, and TCR ligation was provided by anti-CD3 antibody. After three days we evaluated IL-21 and IFNγ expression by flow cytometric analysis. Intriguingly, we found that the substantial proportion of T cells produced IL-21. This was observed for the cultures driven by immature DC (**Fig. 4.08 A**) as well as mature DC (**Fig. 4.08 B**). To some extent, both DC populations supported the production of IFNγ although levels of this cytokine were considerably lower than the levels of IL-21. These findings indicate that in contrast to B cells, immature and mature DC appear to support the production of IL-21 by T cells, whereas an opposite pattern is observed for IFNγ expression.

Whilst both immature and mature DC appeared to promote IL-21 expression, we wished to test whether the provision of exogenous IL-6 could further enhance IL-21 expression in these

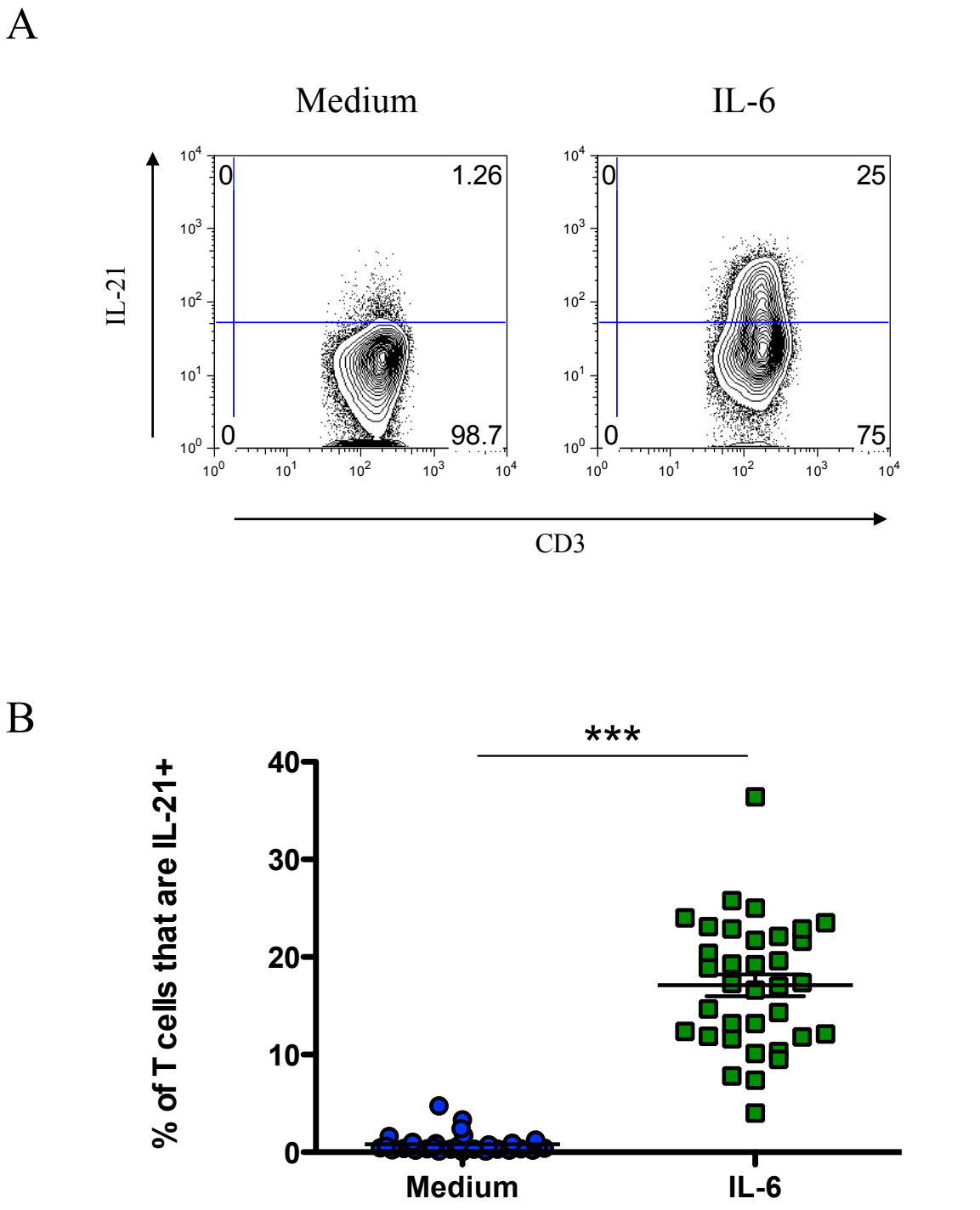


Figure 4.06. Exogenous IL-6 promotes IL-21 production in B cell-driven assays. $2.5x10^4$ Tconv were cultured with $7.5x10^4$ B cells and 0.8µg/ml anti-CD3, alone or in the presence of 100ng/ml IL-6. After three days cells were restimulated and stained for surface CD3 and intracellular IL-21. Representative contour plots show expression profiles for IL-21 (**A**). Graph shows collated data for IL-21 expression within gated Tconv (**B**). Bars represent means across 35 experiments. ***, p=<0.001.

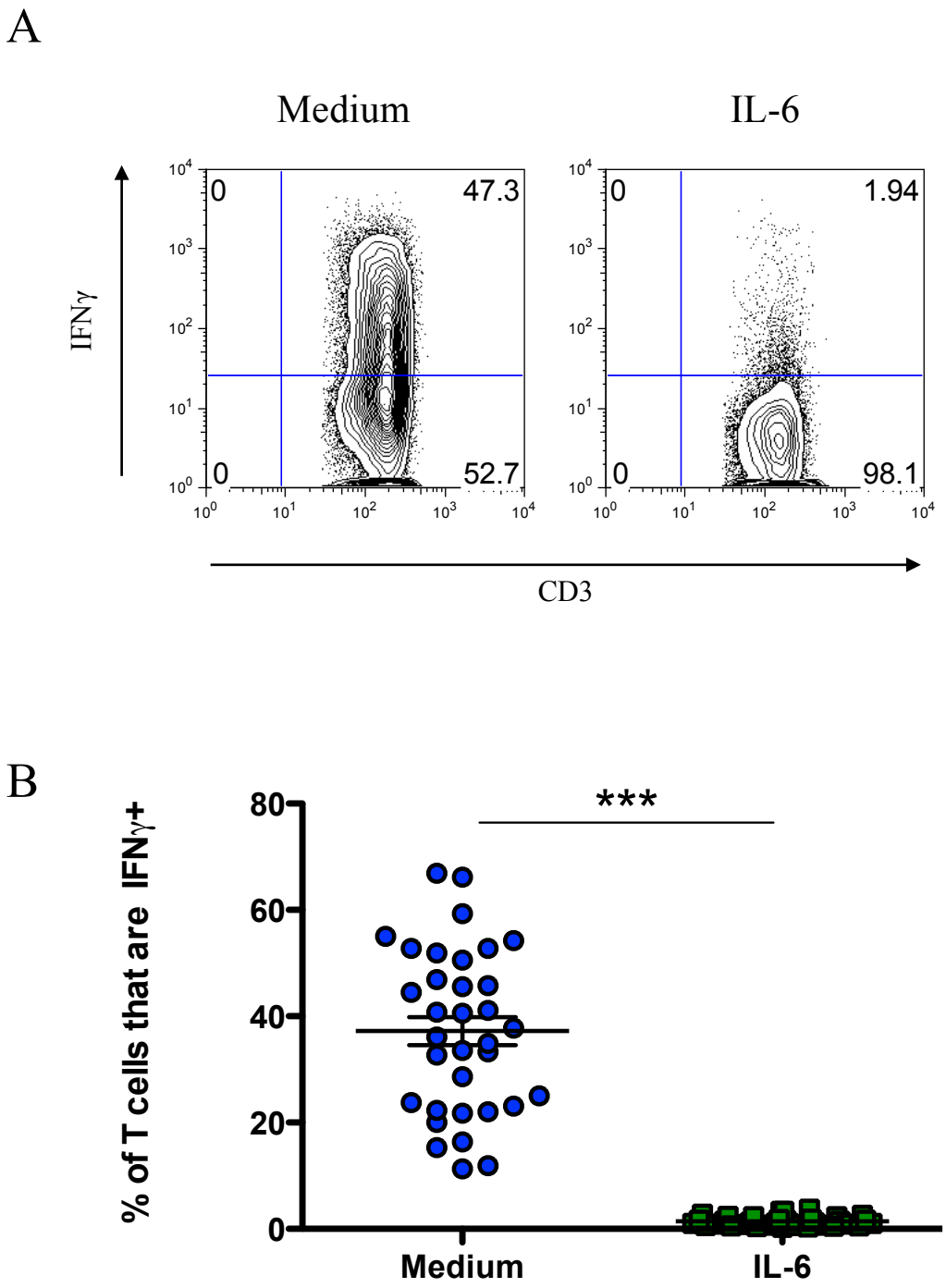


Figure 4.07. Provision of IL-6 in B cell-driven assays inhibits IFNγ production. $2.5x10^4$ Tconv were cultured with $7.5x10^4$ B cells and 0.8μg/ml anti-CD3, alone or in the presence of 100ng/ml IL-6. After three days cells were restimulated and stained for surface CD3 and intracellular IFNγ. Representative contour plots show expression profiles for IFNγ (**A**). Graph shows collated data for IFNγ expression within gated Tconv (**B**). Bars represent means across 34 experiments. ***, p=<0.001.

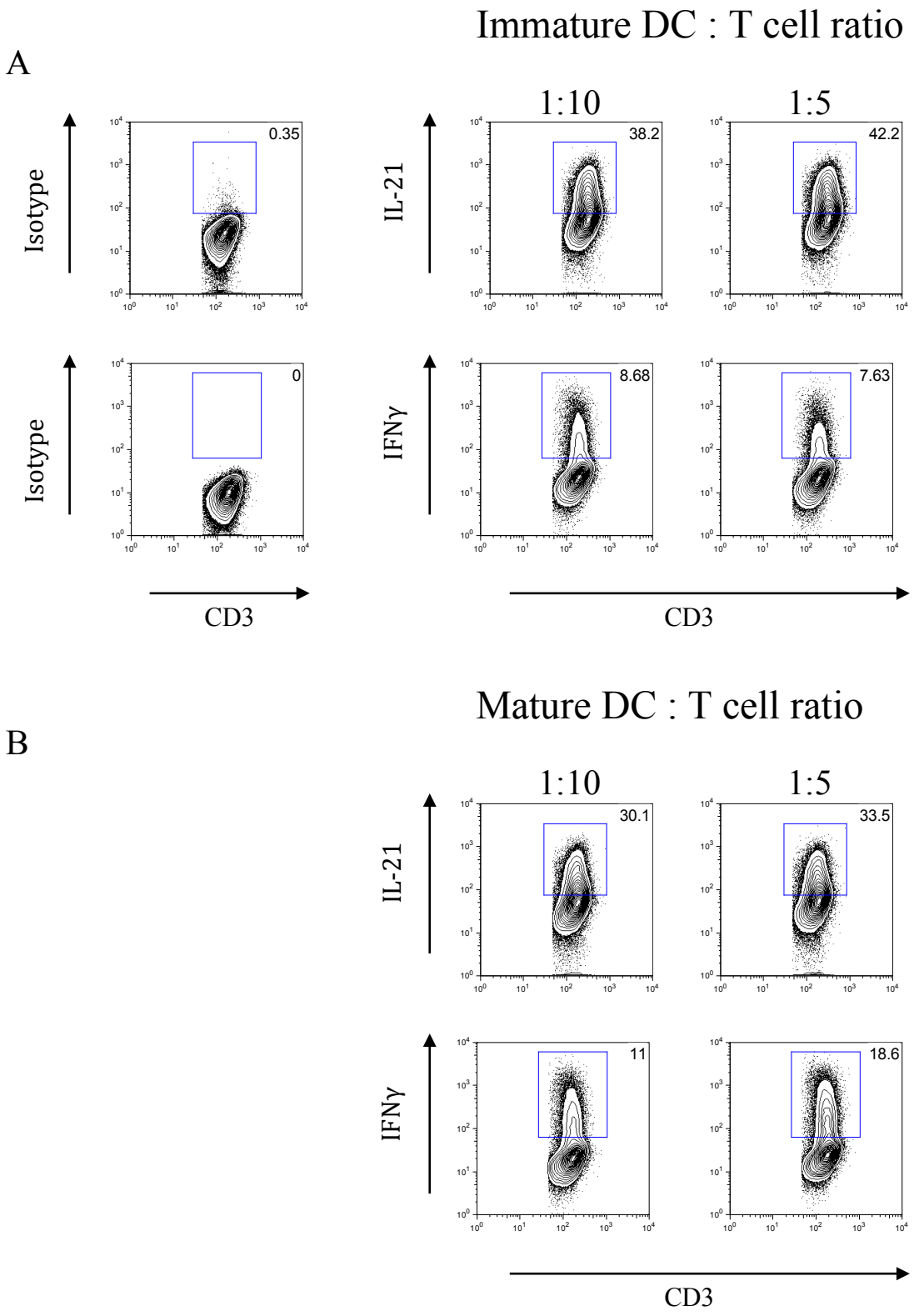


Figure 4.08. Expression profiles for IL-21 and IFNγ in immature and mature DC-driven assays. $2.5x10^4$ Tconv were cultured with $2.5x10^3$ or $5x10^3$ immature or mature DC and 0.8μg/ml anti-CD3. After three days cells were restimulated and stained for surface CD3 and intracellular IL-21 and IFNγ. Representative contour plots show expression profiles for IL-21 and IFNγ within gated CD3+ Tconv in immature (**A**) and mature (**B**) DC-driven assays.

cultures. To this end, we carried out our co-culture assay as described above, in the presence or absence of 100ng/ml IL-6. **Fig. 4.09** demonstrates that the provision of IL-6 in the immature DC-driven assay resulted in a small but significant increase in the proportion of IL-21-expressing T cells and this was observed for both the 1:5 and 1:10 ratios. Similarly, it appeared that IL-6 could also slightly enhance T cell IL-21 expression in the mature DC-driven assay, although this only reached significance at the 1:10 ratio. Thus our study suggests that IL-21 production can increase further in the immature DC-driven assay with the addition of IL-6, and to a lesser extent in mature DC-driven responses.

Until this point, our analysis of IL-21 expression had been restricted to a single timepoint after three days of stimulation. We therefore felt that it was important to evaluate the kinetics of IL-21 protein expression in these assays to get a better appreciation for when this differentiation programme was being switched on. To address this, immature DC or B cells were cultured with CD4+CD25- T cells at 1:10 and 3:1 ratios, respectively. TCR ligation was provided by anti-CD3 antibody in the presence or absence of IL-6. After one, two and three days we examined IL-21 expression by flow cytometry. These experiments revealed that in assays where co-stimulation was provided by immature DC, IL-21 production peaked at day two and was enhanced at every time point in the presence of IL-6 (**Fig 4.10 A**). Interestingly, we observed that in the B cell-driven assay the highest frequency of IL-21-producing T cells was detected after one day and that this proportion diminished throughout the remainder of the time course (**Fig 4.10 B**). These data indicated that IL-21 could be found early on when signals are provided by B cells and IL-6, whereas more time is required for IL-21 production in DC-driven responses, regardless of exogenously provided IL-6.

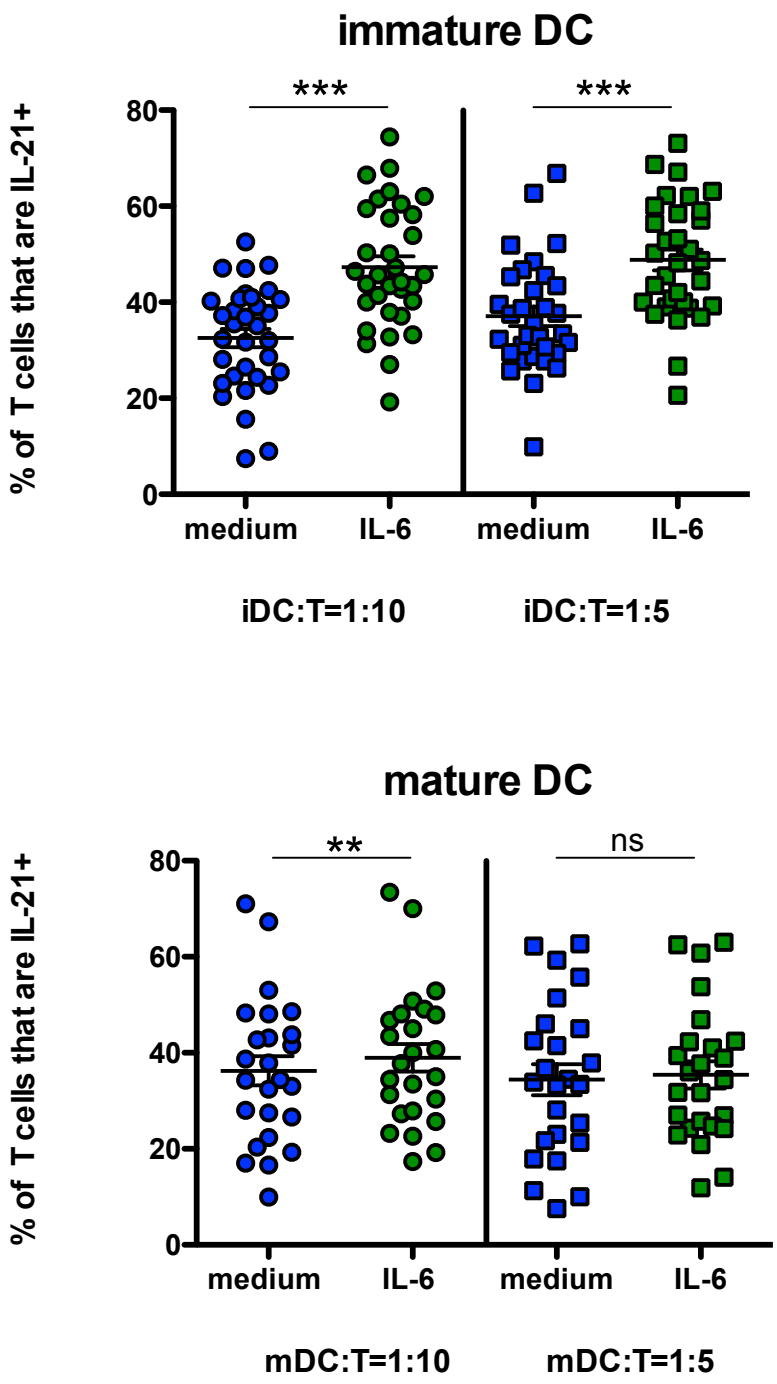


Figure 4.09. The effect of exogenous IL-6 on IL-21 production in immature and mature DC-driven assays. $2.5x10^4$ Tconv were cultured with $2.5x10^3$ or $5x10^3$ immature or mature DC and 0.8µg/ml anti-CD3, alone or in the presence of 100ng/ml IL-6. After three days cells were restimulated and stained for surface CD3 and intracellular IL-21. Graph shows collated data for IL-21 expression within gated Tconv. Bars represent means across 33 experiments. ***, p=<0.001, **, p=<0.01, ns = not significant.

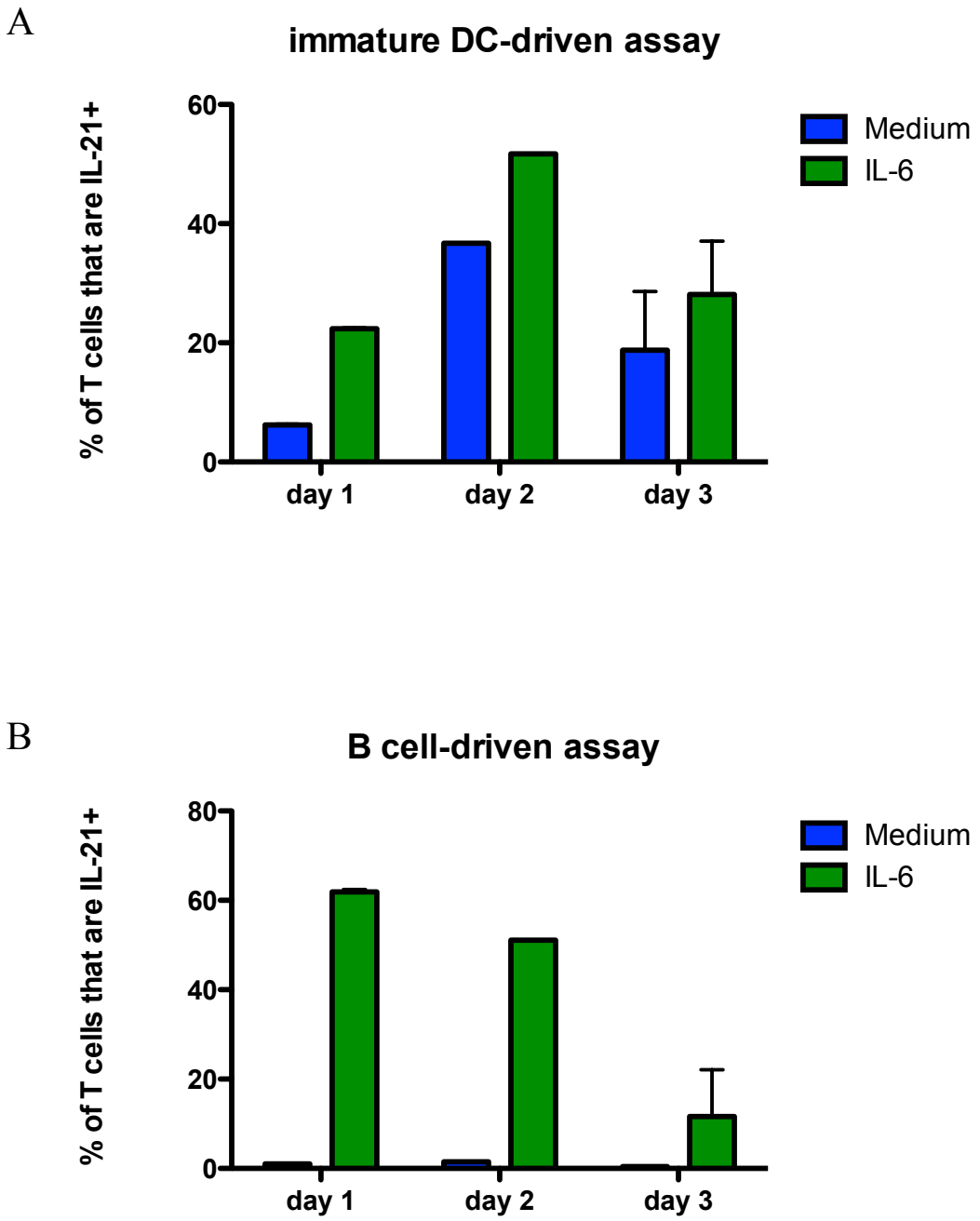


Figure 4.10. Timecourse for IL-21 production in immature DC and B cell-driven assays. $2.5x10^4$ Tconv were cultured with $2.5x10^3$ immature DC or $7.5x10^4$ B cells and 0.8μg/ml anti-CD3, alone or in the presence of 100ng/ml IL-6. After one, two and three days cells were restimulated and stained for surface CD3 and intracellular IL-21. Graph shows collated data for IL-21 expression within gated Tconv in the immature DC-driven assay (**A**) and B cell-driven assay (**B**). Bars represent means across 2 experiments.

Since we had shown that IL-6 was a potent inducer of T cell IL-21, and DC are known to produce IL-6 (Kopf et al. 1998; Pasare & Medzhitov 2003), we therefore sought to establish whether IL-21 induction in the DC-driven assays was dependent on provision of IL-6. Prior to testing the effect of anti-IL-6 antibody on IL-21 expression in DC-driven responses, we wished to validate this IL-6 blocking reagent. We therefore set up the anti-CD3/anti-CD28-driven assay alone, in the presence of anti-IL-6 antibody, exogenous IL-6 or both. As expected addition of IL-6 induced high levels of IL-21 expression, which was counteracted upon provision of IL-6 blocking antibody (**Fig. 4.11**). This assay confirmed the high quality of this reagent and allowed us to proceed with the blockade of IL-6 in the DC-driven assays. To test the dependence of IL-21 production on endogenous IL-6, we cultured immature and mature DC with CD4+CD25- T cells at 1:10 and 1:5 ratios with TCR ligation provided by anti-CD3 antibody, in the presence or absence of 10μg/ml anti-IL-6 antibody. **Fig. 4.12 A** illustrates that IL-21 expression was significantly reduced in immature DC-driven responses at both ratios in the presence of anti-IL-6 antibody. Similarly, we found that IL-6 blockade had a significant effect on IL-21 levels in mature DC-driven responses (**Fig. 4.12 B**). These data suggest that the promotion of IL-21 expression in DC-driven assays can be partially explained by endogenous IL-6 production. However it was notable that IL-6 blockade did not completely ablate IL-21 production.

In summary, the above studies aimed to evaluate the role of different APC populations in the promotion of T cell IL-21 production. We demonstrated that unlike B cells, DC were constitutively capable of driving IL-21 expression *in vitro*. Moreover, it appeared that this effect was dependent, at least in part on endogenous IL-6.

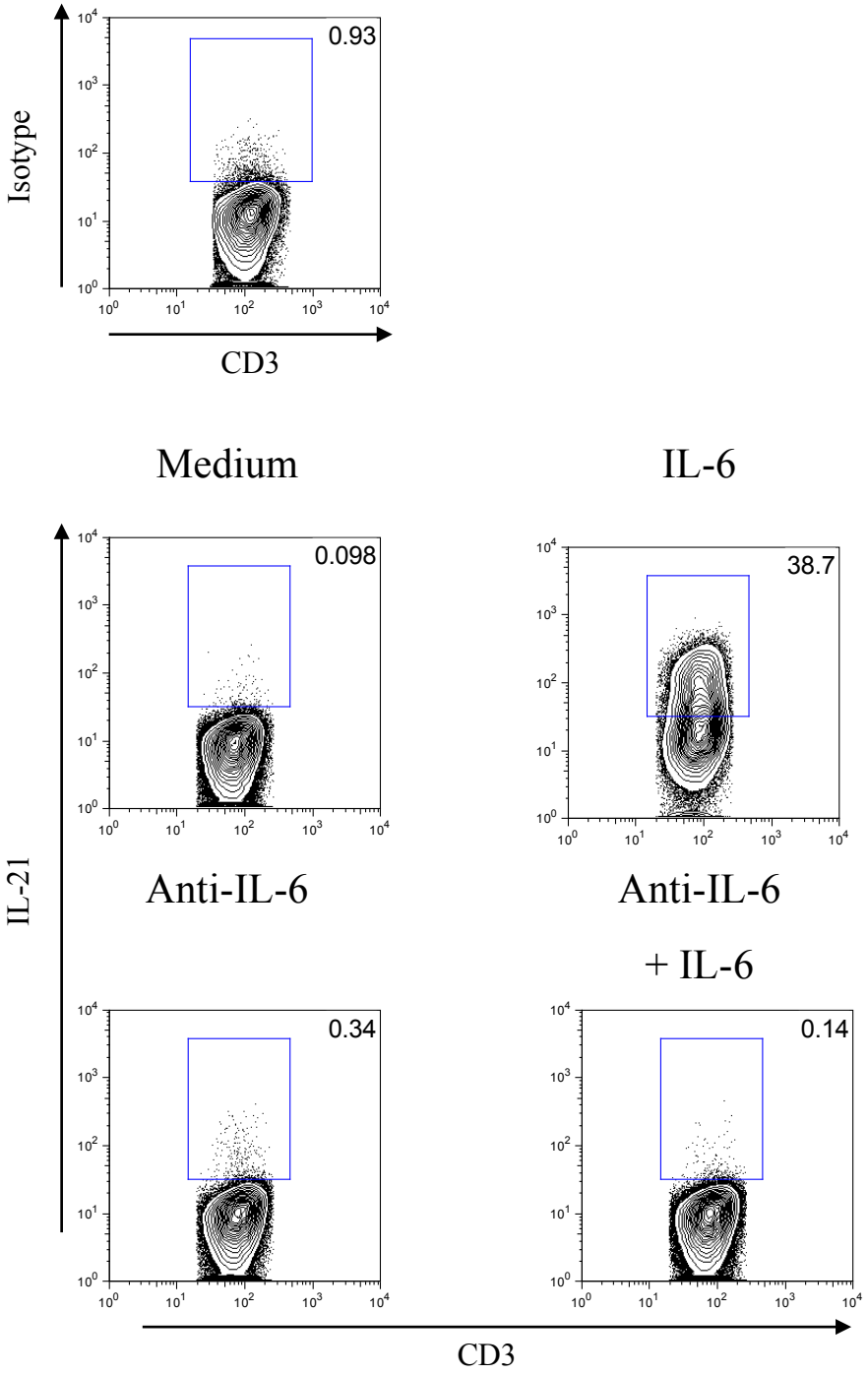
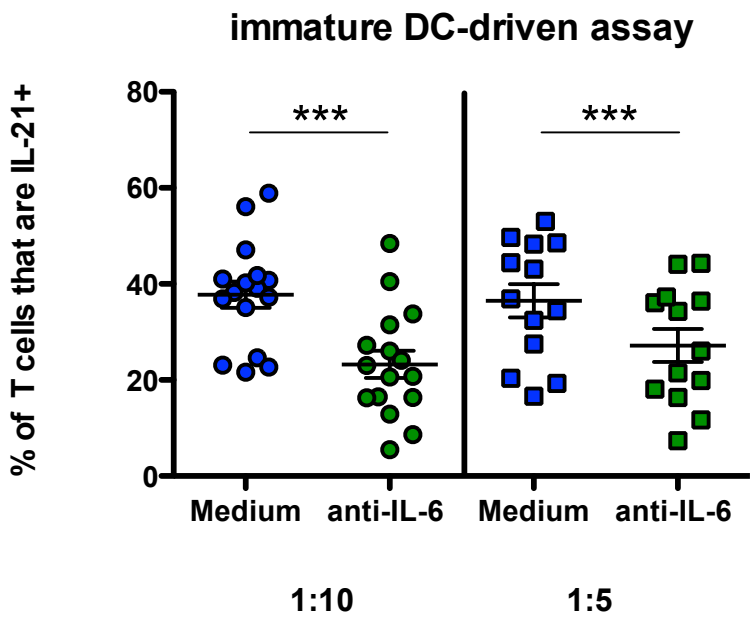


Figure 4.11. Validation of the anti-IL-6 blocking antibody. $2.5x10^4$ Tconv were cultured with $2.5x10^4$ anti-CD3/anti-CD28 beads in the presence of 10µg/ml anti-IL-6, 100ng/ml IL-6 or both. After three days cells were restimulated and stained for surface CD3 and intracellular IL-21. Representative contour plots show expression profiles for IL-21 within gated Tconv.

A



B

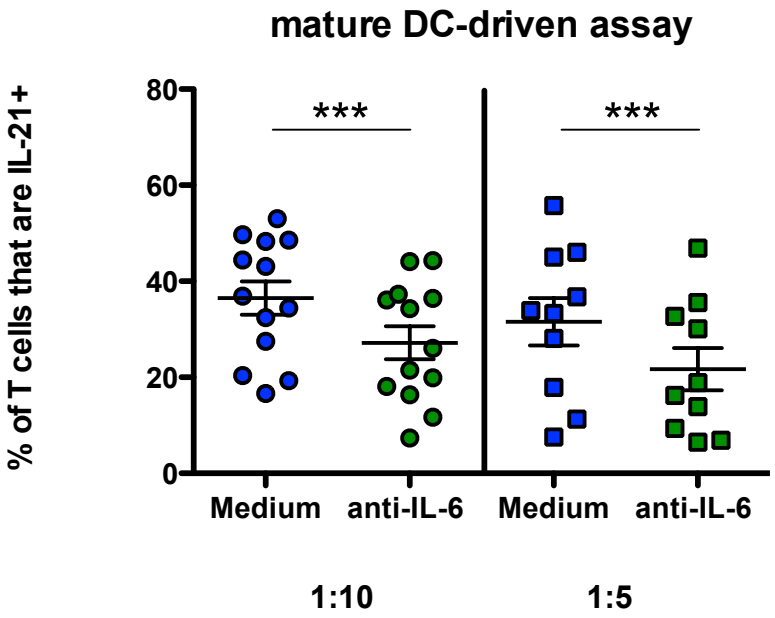


Figure 4.12. Promotion of IL-21 in DC-driven assays is partially dependent on endogenous IL-6. $2.5x10^4$ Tconv were cultured with $2.5x10^3$ or $5x10^3$ immature (**A**) or mature (**B**) DC and 0.8µg/ml anti-CD3, alone or in the presence of 10µg/ml anti-IL-6. After three days cells were restimulated and stained for surface CD3 and intracellular IL-21. Graph shows collated data for IL-21 expression within gated Tconv. Bars represent means across 17 experiments. ***, p=<0.001.

4.2.2. The role of the gp130 pathway in the promotion of IL-21 production

We previously demonstrated that DC can significantly enhance T cell IL-21 production, and that this was due at least in part to production of IL-6. However we noted that some IL-21 was still produced in the presence of anti-IL-6 antibodies, and it remained unclear what other signals might account for this residual IL-21. We therefore sought to investigate other potential inducing factors that could explain the remaining IL-21 production. As IL-6 was a major promoter of IL-21 expression, we initially focused our studies on investigating the pathway utilised by this cytokine. IL-6 signalling requires the formation of the IL-6–IL-6Rα complex, which is involved in the phosphorylation of the trans-membrane gp130 β-receptor subunit. This suggests that gp130 signal transduction might play a role in the promotion of IL-21 production. Other members of the IL-6-type cytokine family have such as IL-11, IL-27, leukemia inhibitory factor (LIF) and oncostatin M (OSM) been shown to signal through the gp130 pathway (reviewed in (Garbers et al. 2012)). Therefore, it was plausible that these molecules might potentially control IL-21 expression.

Firstly, we felt it was important to assess the effect of gp130 signalling on IL-21 expression. To this end, immature DC were cultured with CD4+CD25- T cells with anti-CD3 in the presence of anti-IL-6, anti-gp130, or both. **Fig. 4.13** illustrates that that anti-gp130 was more effective than anti-IL-6 at inhibiting T cell IL-21 production. These data indicate that other ligands that signal through gp130 may contribute to the promotion of IL-21 expression in the DC-driven assay.

It has been reported that IL-27 signalling induced the expression of IL-21R and IL-21 by activated T cells *in vitro* (Pot et al. 2009). We therefore sought to assess whether production of IL-27 is involved in the ability of DC to promote T cell IL-21 production. To this end, we

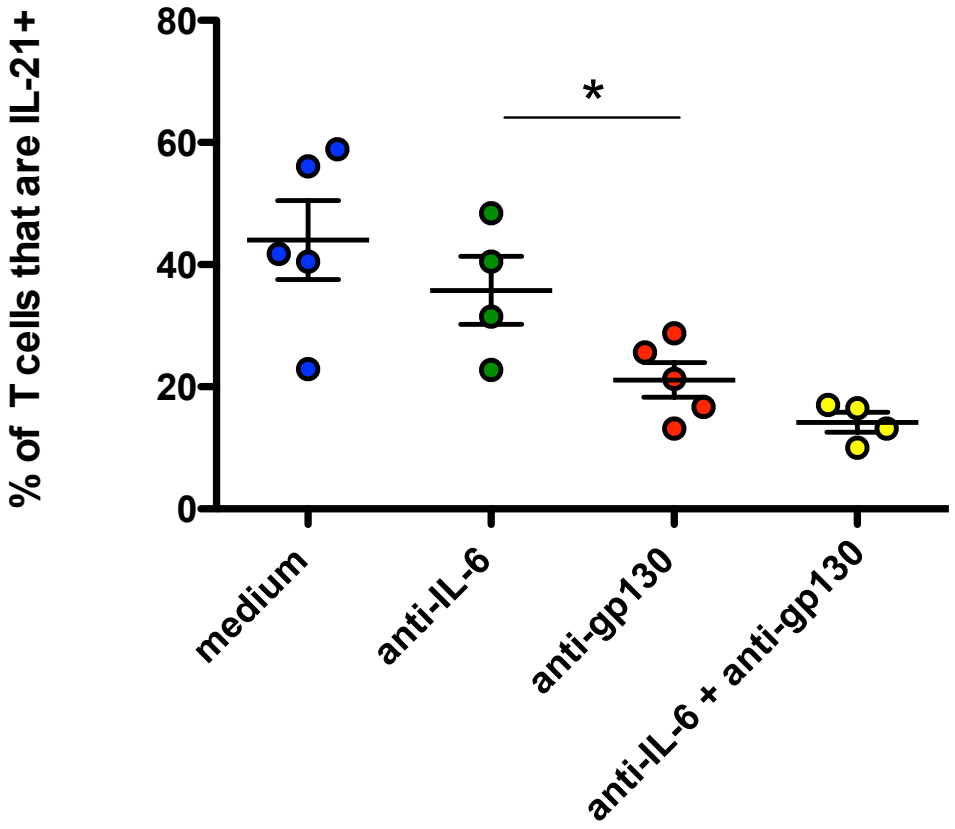


Figure 4.13. Role of the gp130 pathway in IL-21 production in DC-driven assays. $2.5x10^4$ Tconv were cultured with $2.5x10^3$ immature DC and 0.8µg/ml anti-CD3, alone or in the presence of 10µg/ml anti-IL-6, 10µg/ml anti-gp130 or both. After three days cells were restimulated and stained for surface CD3 and intracellular IL-21. Graph shows collated data for IL-21 expression within gated Tconv. Bars represent means and SEM across five experiments. *, p=<0.05.

set up immature and mature DC-driven assays alone or in the presence of anti-IL-27 antibodies. We also included antibodies to LIF and OSM since these cytokines also signal through gp130. As illustrated in **Fig 4.14**, the proportion of IL-21-expressing T cells appeared to be similar after the addition of anti-IL-27, anti-LIF and anti-OSM into these cultures. This indicated that blocking these cytokines had a very limited effect on the expression of IL-21 in DC-driven responses. To further explore the roles of these cytokines, we wished to test whether their exogenous provision would increase IL-21 production. We therefore assessed IL-21 production in cultures driven by B cells and anti-CD3 or anti-CD3/anti-CD28 beads alone, or in the presence of either IL-11 or IL-27 (we were unable to obtain OSM). The data in **Fig. 4.15** demonstrate that the production of IL-21 was unaltered after provision of IL-11 and IL-27. These results suggest a limited role for IL-11 and IL-27 in the induction of IL-21 expression in our assays.

Overall, the above studies demonstrated a clear role for the gp130 pathway in the modulation of IL-21 production, but with the exception of IL-6, none of the cytokines that we tested appeared to be involved.

4.2.3. The role of other cytokines in the modulation of IL-21 production

Studies in humans suggested that IL-12, the cytokine typically associated with Th1 differentiation, was able to induce IL-21 expression by activated T cells *in vitro* (Ma et al. 2009; Schmitt et al. 2009). The same reports provided evidence that IL-23, which is related to IL-12 by their shared utilisation of the IL-12p40 subunit, similarly promoted IL-21 production in humans. We therefore sought to test the ability of these cytokines to drive IL-21 expression in mouse T cells. Initial quantitative PCR analysis indicated that mRNA for IL-23 and IL-12,

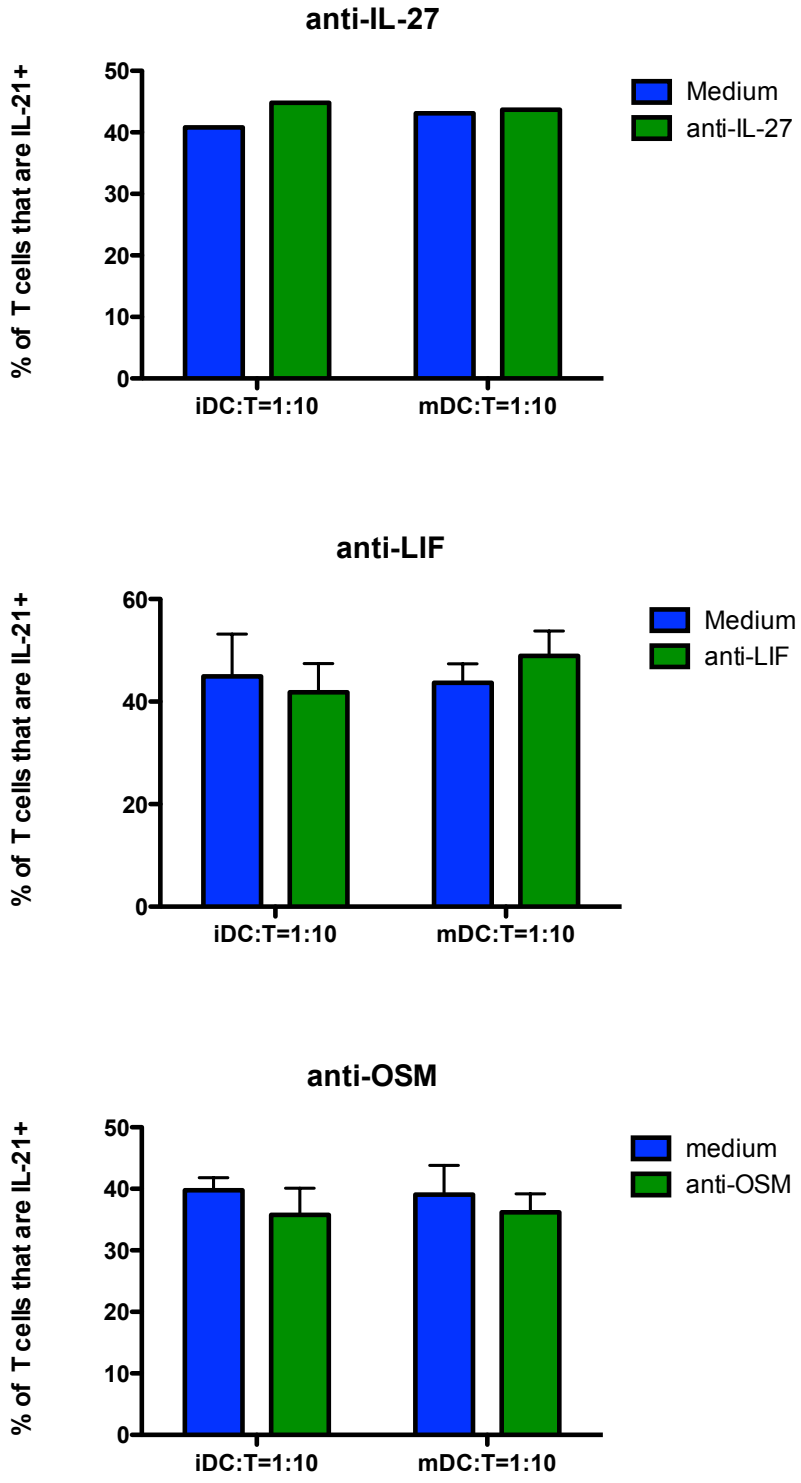


Figure 4.14. The effect of IL-27, LIF and OSM blockade on IL-21 production in DC-driven assays. $2.5x10^4$ Tconv were cultured with $2.5x10^3$ immature or mature DC and 0.8μg/ml anti-CD3, alone or in the presence of 10μg/ml anti-IL-27, 10μg/ml anti-LIF or 5μg/ml anti-OSM. After three days cells were restimulated and stained for surface CD3 and intracellular IL-21. Graphs show collated data for IL-21 within gated Tconv. Bars represent means and SEM across 3 experiments.

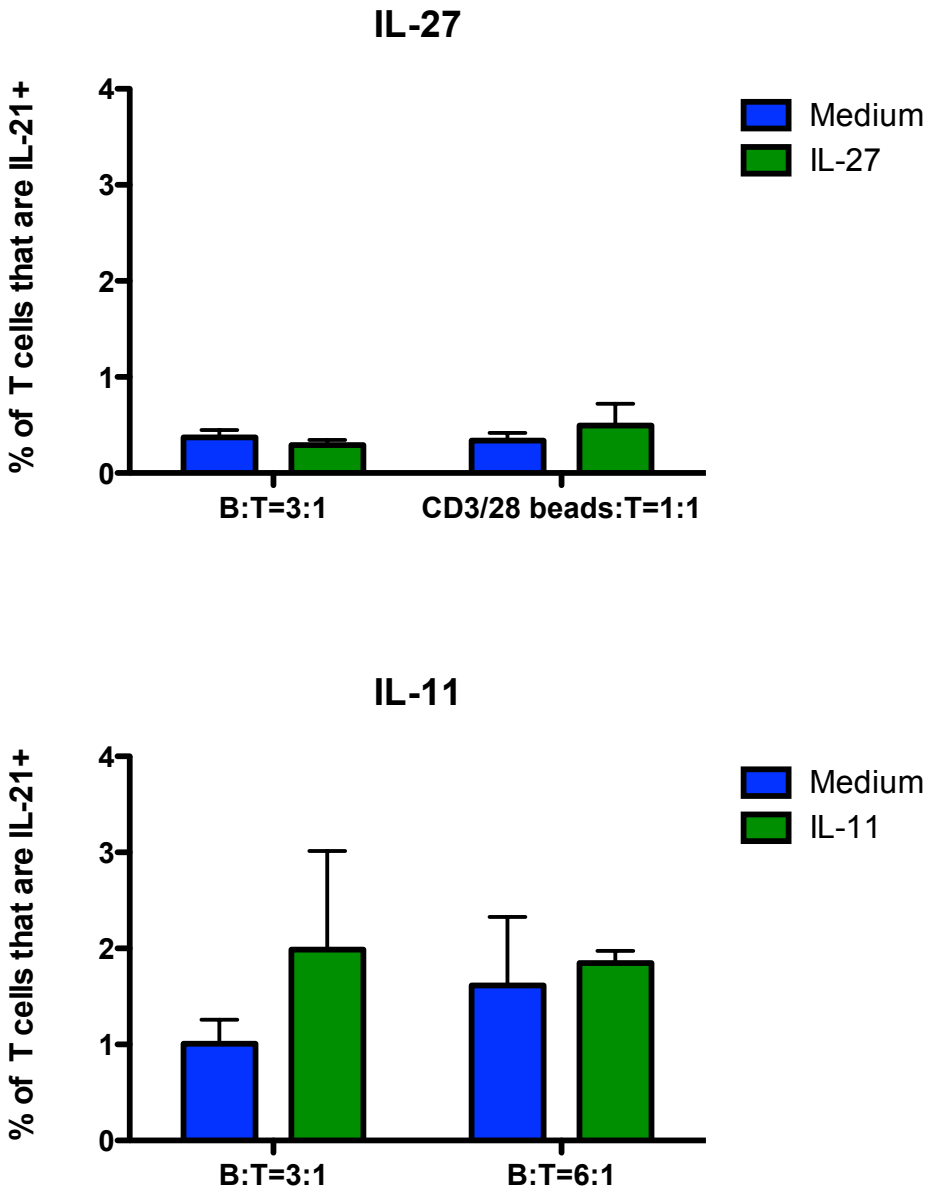


Figure 4.15. The effect of IL-27 and IL-11 on T cell IL-21 production. $2.5x10^4$ Tconv were cultured with $7.5x10^4$ or $15x10^4$ B cells and 0.8µg/ml anti-CD3, or 2.5x104 anti-CD3/anti-CD28 beads, alone or in the presence of 25ng/ml IL-11 or 50ng/ml IL-27. After three days cells were restimulated and stained for surface CD3 and intracellular IL-21. Graph shows collated data for IL-21 expression within gated Tconv. Bars represent means and SEM across 4 experiments.

in addition to IL-6, was produced by our bone-marrow derived DC populations and that B cells appeared to be a notable source of IL-12 (**Fig. 4.16**). As shown in **Fig. 4.17 A,** in contrast to what had been observed in human cells, IL-12 did not alter IL-21 expression in our murine cell cultures. As expected, upon the addition of IL-12 we observed an increase of IFNγ-expressing T cells, which demonstrated that this cytokine was biologically active and could influence T cell differentiation in our cultures. **Fig. 4.17 B** demonstrates that after the addition of exogenous IL-23 into B cell-driven assays, the production of IL-21 remained broadly unchanged. These data indicate that although IL-12 and IL-23 are believed to be important for the induction of IL-21 in humans, we were unable to confirm this in murine T cell differentiation studies.

It has been previously demonstrated that IL-21 supported generation of potentially pathogenic Th17 subset, and this was dependent on a positive feedback loop (Nurieva et al. 2007; Korn et al. 2007; Wei et al. 2007). Subsequently, Suto and colleagues demonstrated that T cells lacking IL-21R had an impaired ability to produce IL-21 (Suto et al. 2008). As the published work involved T cells activated using anti-CD3 and anti-CD28 antibodies, we wished to assess the role of autocrine IL-21 signalling in IL-21 expression in assays where DC or B cells were initiating the T cell response. To address this, we isolated CD4+CD25- T cells from peripheral lymph nodes of BALB/c or IL-21R-/- mice and evaluated IL-21 production in immature DC, mature DC, B cell and anti-CD3/anti-CD28 bead-driven responses alone or in the presence of exogenous IL-6. **Fig. 4.18** illustrates that the signals provided by DC were less effective at supporting IL-21 expression in IL-21R-/- T cells, and this was observed for both immature and mature DC stimulations at both ratios tested (top graph). The provision of IL-6 in the DC-driven assay enhanced the average proportion of IL-21-producing T cells when

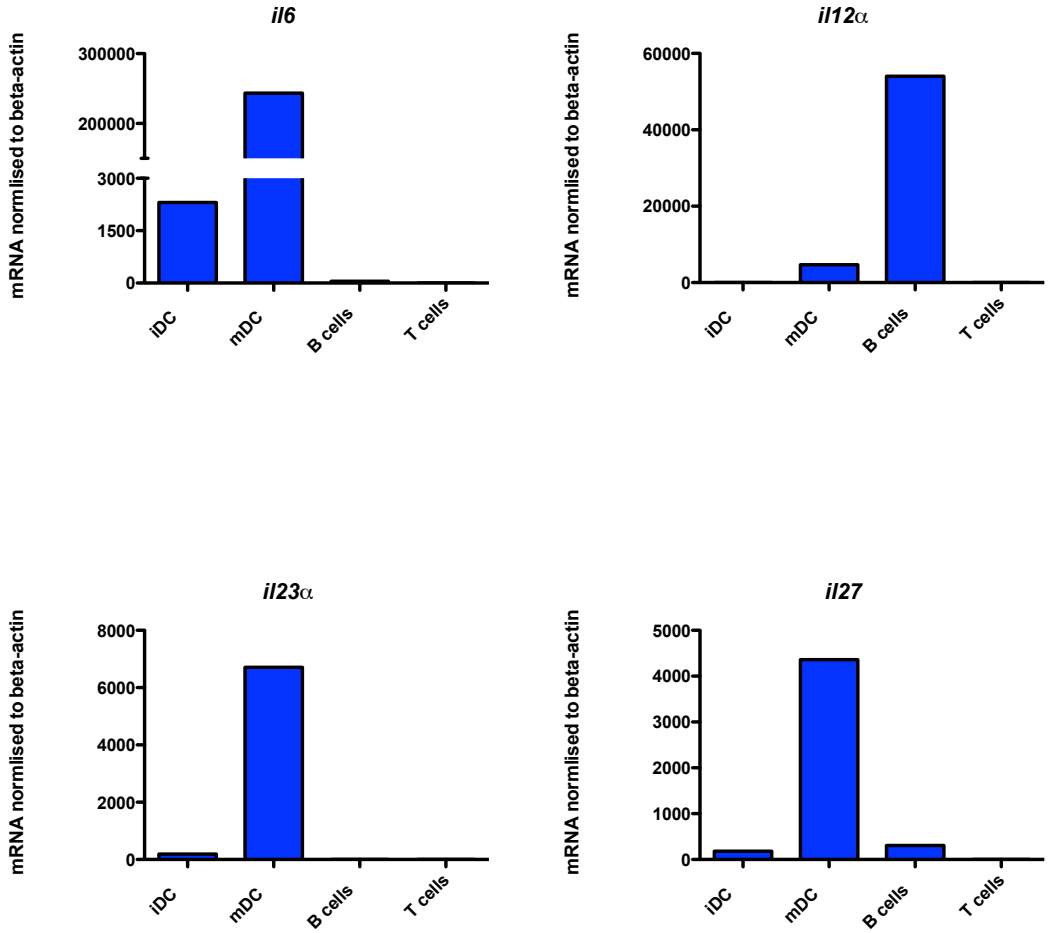


Figure 4.16. The expression of mRNA for IL-6, IL-12α, IL-23α, IL-27 in immature and mature DC, B cells and T cells. The expression of IL-6, IL-12α, IL-23α and IL-27 mRNA in immature and mature DC, B cells and Tconv was evaluated by Taqman qPCR. Transcripts were normalised against endogenous beta-actin levels.

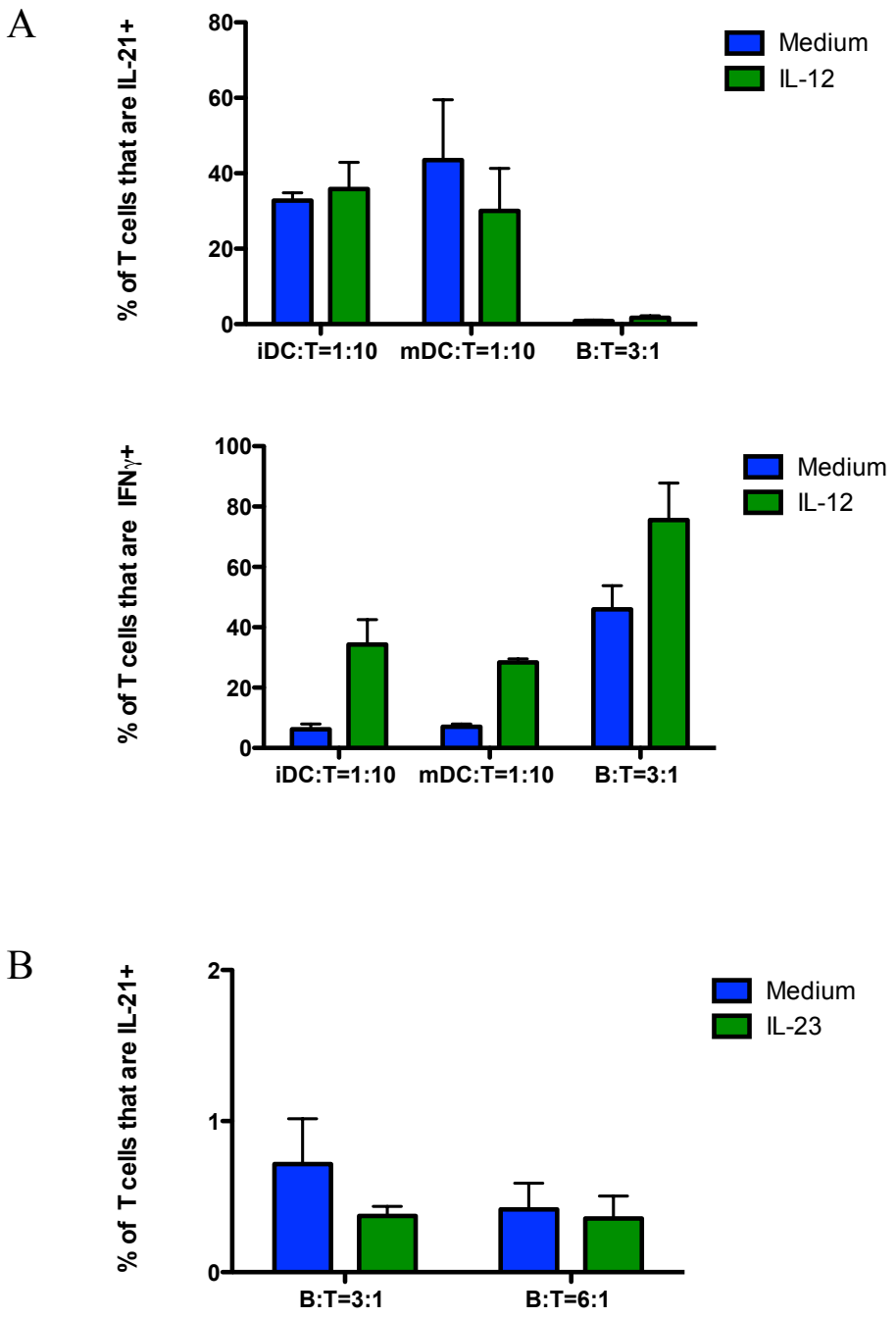


Figure 4.17. The effect of IL-12 and IL-23 on T cell IL-21 production. $2.5x10^4$ Tconv were cultured with immature or mature DC ($2.5x10^3$) or B cells ($7.5x10^4$ or $15x10^4$) and 0.8μg/ml anti-CD3, alone or in the presence of 5ng/ml IL-12 (**A**) or 50ng/ml IL-23 (**B**). After three days cells were restimulated and stained for surface CD3 and intracellular IL-21 and IFNγ. Graphs show collated data for IL-21 and IFNγ within gated Tconv. Bars represent means and SEM across >4 experiments.

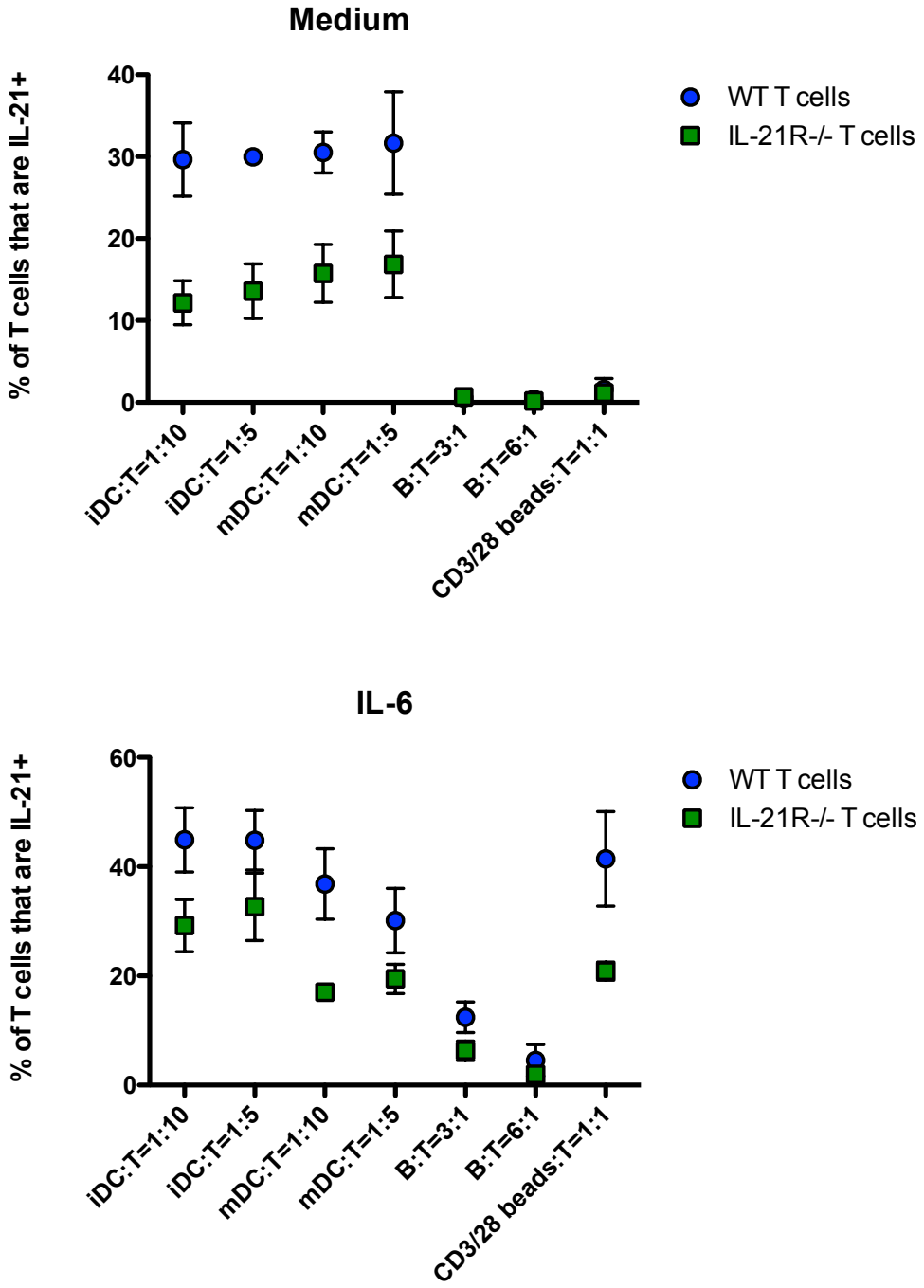


Figure 4.18. Role of autocrine IL-21 signalling in T cell IL-21 expression. $2.5x10^4$ Tconv from BALB/c or IL-21R-/- mice were cultured with immature or mature DC ($2.5x10^3$ or $5x10^3$), or B cells ($7.5x10^4$ or $15x10^4$), with 0.8μg/ml anti-CD3, alone or in the presence of 100ng/ml IL-6. Alternatively, $2.5x10^4$ Tconv from BALB/c or IL-21R-/- mouse were cultured with $2.5x10^4$ anti-CD3/anti-CD28 beads, alone or in the presence of 100ng/ml IL-6. After three days cells were restimulated and stained for surface CD3 and intracellular IL-21. Graphs show collated data for IL-21 expression within gated Tconv. Points and bars represent means and SEM respectively across 4 experiments.

these cells lacked IL-21R, however this increase was still smaller than that observed for WT T cells. Thus autocrine IL-21 signalling played a role in IL-21 production in assays driven by DC. In B cell driven assays, as expected the production of IL-21 required exogenous IL-6 provision, but T cells lacking IL-21R showed a less marked induction of IL-21. Similarly, the ability of T cells to receive IL-21 signals augmented IL-21 production from anti-CD3/anti-CD28 activated T cells response to IL-6. Together these data demonstrated that although IL-21 signals were not essential for IL-21 production in the DC stimulated cultures, this cytokine acts in an autocrine manner to further potentiate its expression.

4.2.4. The role of the CD28 and p110δ PI3K pathway in the modulation of IL-21 production

It is widely accepted that CD28 ligation, in addition to TCR engagement, is required for optimal T cell activation, differentiation and survival. However little is known about whether the CD28 pathway is essential for T cells to express IL-21. To address this question, we cultured immature or mature DC with CD4+CD25- T cells from peripheral lymph nodes of WT or CD28-/- mice with anti-CD3 and evaluated IL-21 production by T cells. We found that the average frequency of IL-21-expressing T cells was similar across different ratios and DC subsets in the WT and CD28-/- T cells (**Fig. 4.19 A**). Thus, this implies that IL-21 production in DC-driven assays is largely independent of CD28 co-stimulation.

A number of reports have suggested that the p110δ catalytic subunit of phosphatidylinositol 3-kinase (PI3K), an enzyme that is activated by TCR and CD28 ligation, is particularly important for the modulation of immune responses (Okkenhaug et al. 2002). It has also been

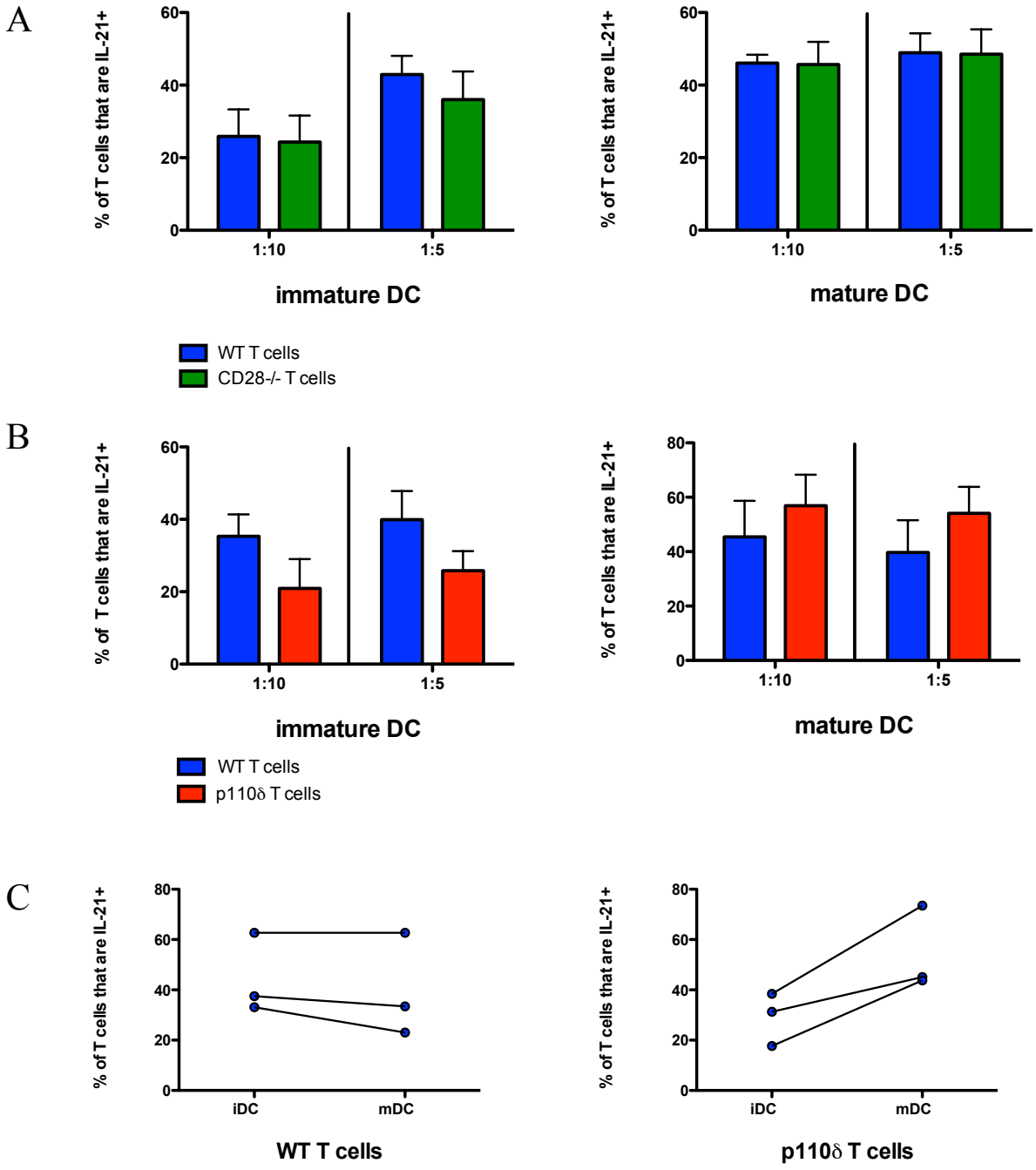


Figure 4.19. The ability of CD28 co-stimulation and the PI3K pathway to modulate IL-21 production. $2.5x10^4$ Tconv from BALB/c, CD28-/- or p110δ ^{D910A} mice were cultured with $2.5x10^3$ or $5x10^3$ immature or mature DC and 0.8μg/ml anti-CD3. After three days cells were restimulated and stained for surface CD3 and intracellular IL-21. Graphs show collated data for IL-21 expression within gated Tconv for WT vs CD28-/- (**A**) and WT vs p110δ ^{D910A} (**B**). Graphs show data sets (immature vs mature DC stimulation) for IL-21 within gated Tconv from WT and p110δ ^{D910A} mouse (**C**). Bars represent means and SEM across >3 experiments.

reported that T cells from p110δ^{D910A} mice (mutant with catalytically inactive form of p110δ) had reduced ability to produce IL-21 *in vitro* (Rolf et al. 2010). The latter finding was based on analysis of T cells activated with anti-CD3 and anti-CD28 in the presence of IL-6. In order to test the importance of the p110δ subunit for the promotion of IL-21 expression in the context of activation by DC, immature or mature DC were cultured with CD4+CD25- T cells from peripheral lymph nodes of WT or p110δ^{D910A} mice and anti-CD3. Intracellular IL-21 production was then assessed after three days. We observed that the average frequency of IL-21-expressing T cells tended to be slightly lower in p110δ^{D910A} mice in the immature DC-driven assay (**Fig. 4.19 B).** In contrast, when activated in the presence of mature DC, the average IL-21 expression tended to be slightly higher in p110δ^{D910A} mutant T cells. Thus, maturation of DC appeared to enhance IL-21 production by T cells from p110δ^{D910A} mice but not from WT animals (**Fig 4.19 C**). These observations are preliminary and await replication, but suggest that the signals provided by immature and mature DC could differentially modulate IL-21 expression in the p110δ^{D910A} T cells.

To further verify that CD28 was not required for T cell IL-21 production, we also performed *in vivo* analysis using CD28-deficient mice. Since we had shown that a major site for T cell IL-21 production *in vivo* was the infiltrated pancreas in DO11xRIP-mOVA mice, we generated the DO11xRIP-mOVAxCD28-/- model in which T cells are deficient in CD28. Initially, we wanted to assess whether CD28 deficiency is important for the development of diabetes. To address this, we took blood glucose readings from six, 12 and 18-week-old DO11xRIP-mOVAxCD28-/- mice. **Fig 4.20** illustrates that unlike CD28-sufficient animals, CD28-deficient DO11xRIP-mOVA mice rarely develop disease. Although most of these mice remained healthy, we were nevertheless able to isolate infiltrating cells from the pancreas.

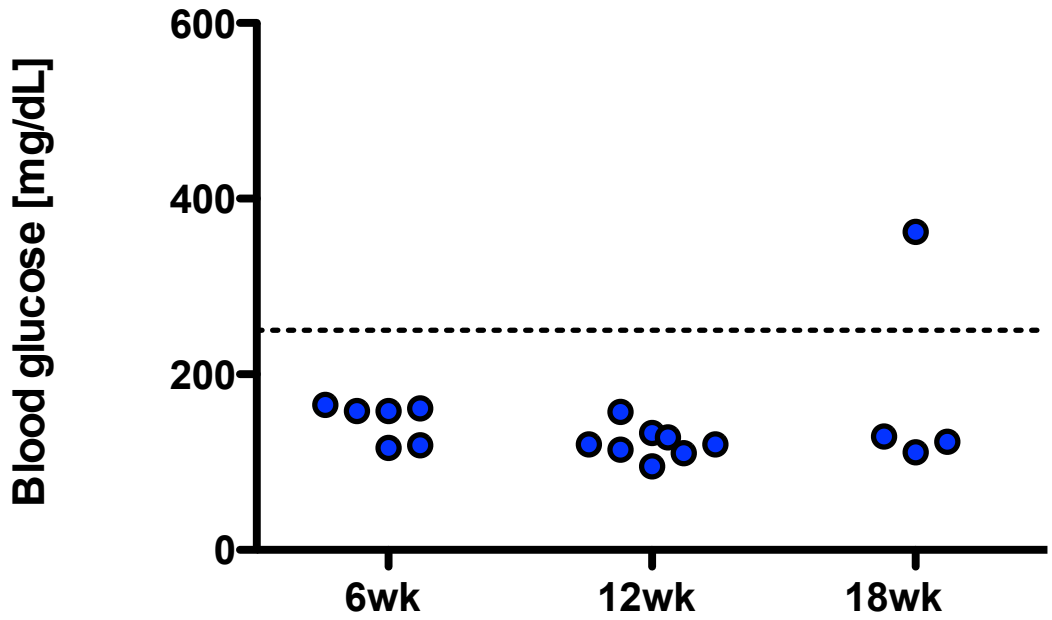


Figure 4.20. Blood glucose readings in DO11xRIP-mOVAxCD28-/- mice. Graph shows blood glucose measurements in DO11xRIP-mOVAxCD28-/- mice tested at different weeks of age. Dashed line indicates blood glucose level above which mice are considered diabetic. Note: the 18-week-old animal with raised blood glucose levels is included because this mouse was used for flow cytometric analysis in the next figure. However this was the only 18-week-old animal of >10 assessed to have raised blood glucose levels.

Our analysis revealed that the pancreas-infiltrating T cells in these animals were capable of measurable IL-21 production, but that the proportion of IL-21-producing cells was lower than in CD28-sufficient animals. This difference was even more marked when T cells from lymph node and spleen were assessed (**Fig. 4.21)**. This implies that unlike in the *in vitro* assay, the CD28 pathway might be important in driving IL-21 production *in vivo*.

In addition, we wished to further investigate the importance of the p110δ subunit of PI3K for the expression of IL-21 *in vivo*. We therefore took a similar approach and generated DO11xRIP-mOVAxp110δ^{D910A} model in which cells have inactive form of p110δ. Initially, it was important to get an appreciation for the role that p110δ deficiency might play in the development of diabetes. To address this, we took blood glucose readings from six to 18-week-old DO11xRIP-mOVAxp110δ^{D910A} mice. We found that in the absence of p110δ signalling, these mice remained normoglycaemic at every time point (**Fig. 4.22**). Our primary goal however was to evaluate the contribution of this pathway for the induction of IL-21 expression. We therefore isolated single cell suspensions from inguinal LN, pancreatic LN, pancreas and spleen of six, 12 and 18-week-old DO11xRIP-mOVAxp110δ^{D910A} mice and evaluated intracellular IL-21 by flow cytometry. We found that the proportion of IL-21+ conventional T cells was decreased in the inguinal LN, pancreatic LN and spleen of DO11xRIP-mOVAxp110δ $^{(D910A)}$ mice in comparison to age matched DO11xRIP-mOVA animals (**Fig. 4.23**). However, we observed that in the pancreas of p110δ deficient mice, IL-21 production was initially reduced at six weeks, but then recovered and matched the level found in DO11xRIP-mOVA animals at later time points. These data suggest that the contribution of the p110δ pathway to IL-21 production might vary between peripheral lymphoid tissues and the antigen-expressing pancreas. Thus overall our findings indicate that

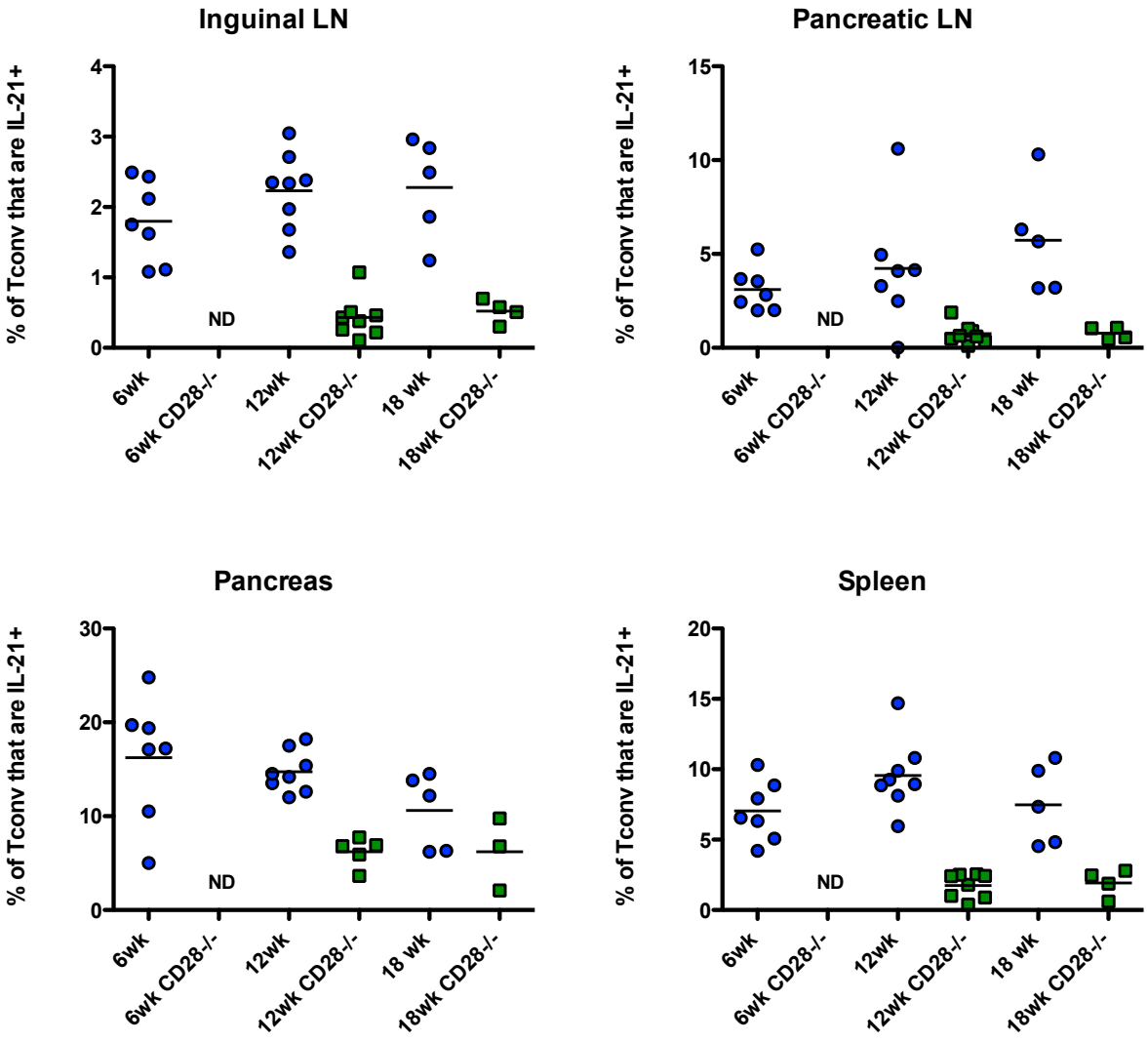


Figure 4.21. The ability of the CD28 pathway to modulate IL-21 production *in vivo*. Single cell suspensions from inguinal lymph node, pancreatic lymph node, pancreas and spleen of DO11xRIP-mOVA or DO11xRIP-mOVAxCD28-/- (CD28-/-) mice were restimulated and stained for surface CD4 and intracellular Foxp3 and IL-21. Graphs show collated data for IL-21 expression in CD4+Foxp3- Tconv from DO11xRIP-mOVA and DO11xRIP-mOVAxCD28-/- mice in the indicated tissues. Bars represent means across indicated mice and ages (weeks). ND = not determined.

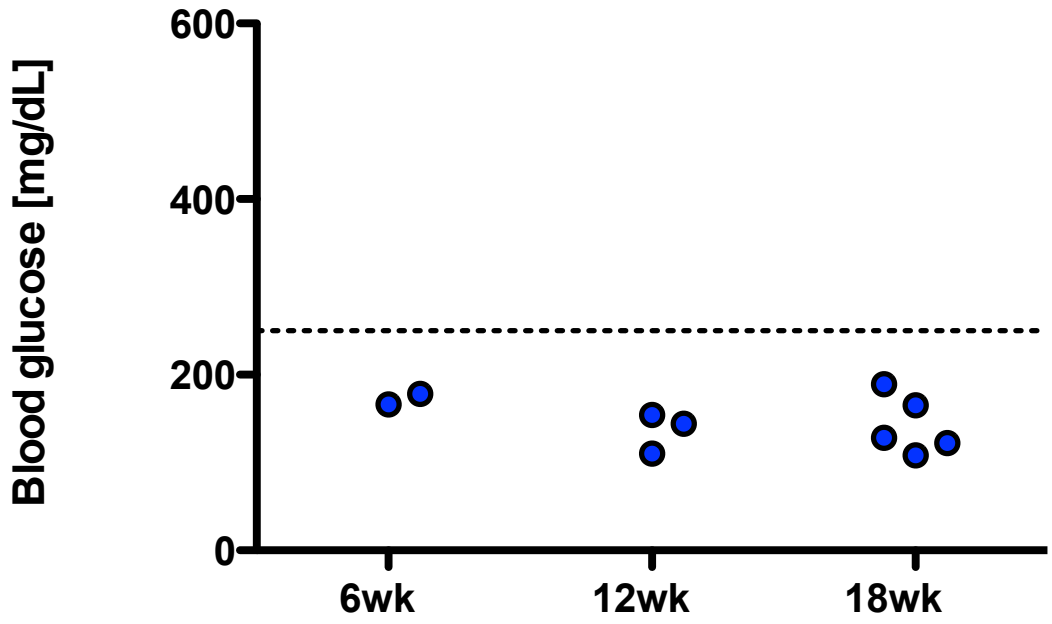


Figure 4.22. Blood glucose readings in DO11xRIP-mOVA x p110δ $^{(D910A)}$ mice. Graph shows blood glucose measurements in DO11xRIP-mOVA x p110δ $^{(D910A)}$ mice tested at different weeks of age. Dashed line indicates blood glucose level above which mice are considered diabetic.

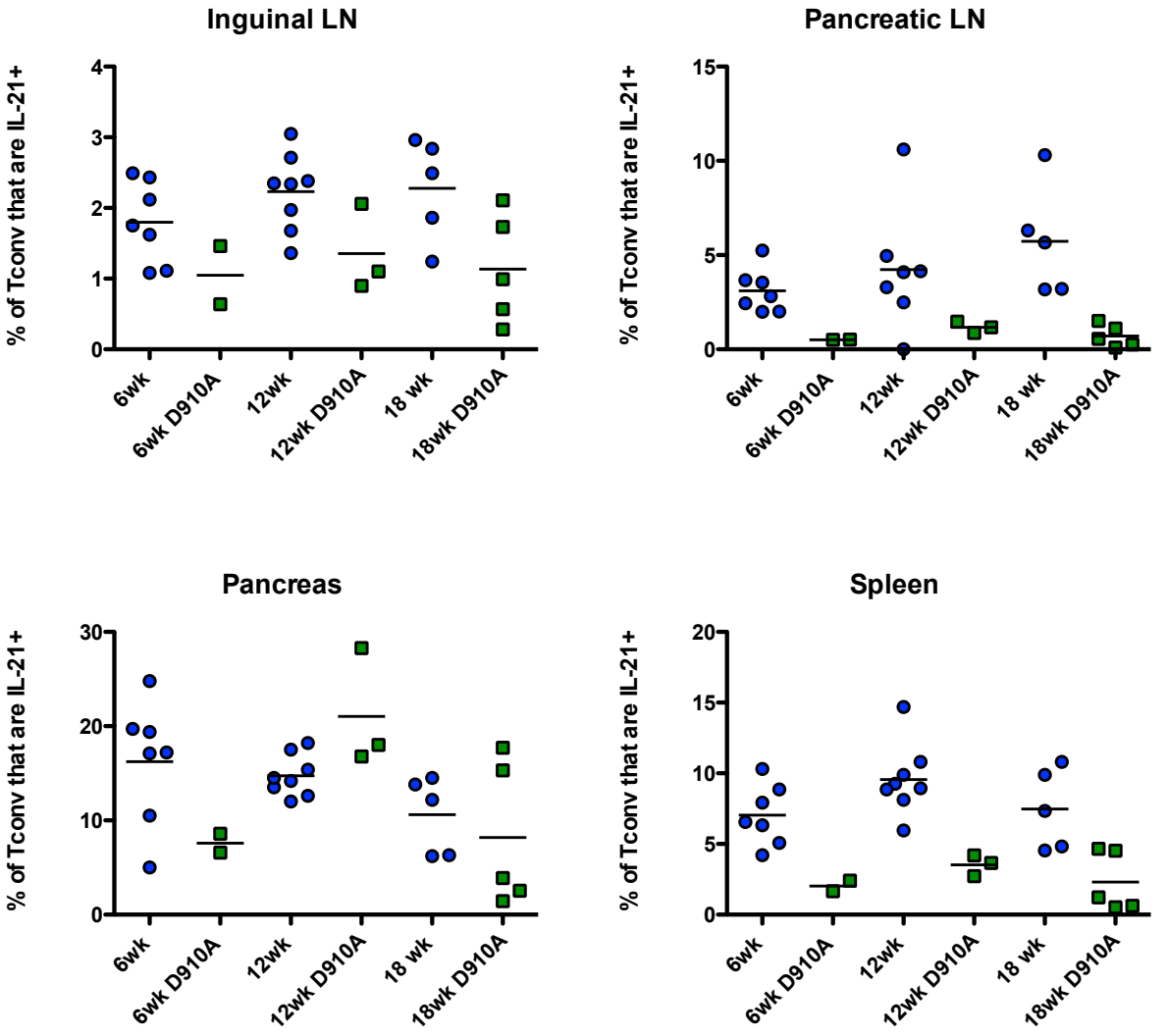


Figure 4.23. The ability of the PI3K pathway to modulate IL-21 production *in vivo*. Single cell suspensions from inguinal lymph node, pancreatic lymph node, pancreas and spleen of DO11xRIP-mOVA or DO11xRIP-mOVAx p110δ ^{D910A} (D910A) mice were restimulated and stained for surface CD4 and intracellular Foxp3 and IL-21. Graphs show collated data for IL-21 in CD4+Foxp3- Tconv from DO11xRIP-mOVA or DO11xRIP-mOVAx p110δ ^{D910A} mice in the indicated tissues. Bars represent means across indicated mice and ages (weeks).

neither CD28 nor the p110δ PI3K pathway are essential for T cell production of IL-21 *in vivo*, but that these signals may have a subtle influence on how much of this cytokine is produced at particular sites and times.

4.3. Discussion

The substantial presence of IL-21 protein in the DO11xRIP-mOVA pancreas, and throughout the lymphatic organs of CTLA-4-/- mice, provided the evidence that this cytokine might play a key role in the development of autoimmunity. Thus establishing the signals required for induction of IL-21 will be important for understanding the causes of the immune dysregulation. Previous studies, which showed a role for IL-21 in the generation of Th17 cells, also revealed that IL-6 could induce the expression of IL-21 mRNA (Nurieva et al. 2007). Later work by Suto and colleagues demonstrated that IL-6 signals strongly promote IL-21 protein expression by T cells (Suto et al. 2008). Initially, we confirmed this finding by investigating T cell responses in anti-CD3/anti-CD28 bead-driven cultures (**Fig. 4.03**). Given the fact that B cells and DC are present in the pancreatic infiltrate of DO11xRIP-mOVA mice, we also wished to examine how these different APC populations might modulate the production of IL-21 *in vitro*.

Initially, we wished to understand how the B cells modify T cell cytokine profiles. In our studies, B cell signals showed to skew the response towards the Th1 differentiation programme, with a high proportion of T cells expressing IFNγ (**Fig. 4.05**). Interestingly, the strong IFNγ responses observed in B cell-driven assays were inhibited after addition of exogenous IL-6 (**Fig. 4.07 B**). This finding agrees with the work of Korn and colleagues, which demonstrated that *in vitro* activated T cells substantially increased their expression of IFNγ in the absence of IL-6 signals (Korn et al. 2007). However little was known whether B cells acting as APC could provide the co-stimulation and other accessory signals that promote IL-21 production. Our data suggests that very little IL-21 was made in B cell-driven assays (**Fig. 4.06 B**). Therefore, in the context of APC signals, stimulation by B cells was

insufficient to induce IL-21 production *in vitro*. It is clear that under certain conditions B cells do have the capacity to produce IL-6. *In vitro* studies have shown that activated B cells can produce large amounts of IL-6 (Harris et al. 2000). Furthermore, IL-6-producing B cells were found in an *in vivo* mouse model of multiple sclerosis, and their presence strongly contributed to pathogenic responses (Barr et al. 2012). Therefore, it is possible that under the above circumstances B cells might be able to support T cell IL-21 production.

Having examined B cell-driven responses, we subsequently wished to examine the effect of DC signals on IL-21 production *in vitro*. The bone marrow-derived DC used in our studies expressed classical DC markers such as CD11c, CD11b, MHC class II, CD80, CD86 (**Fig. 4.04**) and also the recently identified transcription factor zDC (Steinman et al. 1997; Meredith et al. 2012; Satpathy et al. 2012). Unlike in B cell-driven assays, the signals provided by DC substantially induced IL-21 production (**Fig. 4.08**). This was observed in T cell responses driven by both immature and mature DC at two different APC to T cell ratios. The provision of exogenous IL-6 further enhanced IL-21 expression in immature DC-driven responses, but had a limited effect in mature DC-driven assays, particularly at higher APC to T cell ratios (**Fig. 4.09**). This study therefore revealed fundamental differences between DC and B cells in their ability to drive the IL-21 programme *in vitro*.

An investigation of the kinetics of IL-21 expression suggested that this cytokine was readily made one day after stimulation with B cells and IL-6, and its production then decreased over the remainder of the time course (**Fig. 4.10 A**). In the case of the DC-driven assay, the most robust IL-21 expression was seen at day two and three post stimulation (**Fig. 4.10 B**). The addition of exogenous IL-6 in these assays seemed to have a less pronounced effect on IL-21

production than in the B cell-driven responses. This might imply that perhaps DC provided other accessory signals that modulated IL-21 expression from day one onwards.

The fact that DC were capable of supporting IL-21 production raised the question of whether IL-6 was present in these assays. Two separate studies have demonstrated the ability of DC to express IL-6, therefore it was plausible that this cytokine was responsible for the observed induction of IL-21 (Kopf et al. 1998; Pasare & Medzhitov 2003). Having validated a blocking anti-IL-6, we investigated DC-driven responses in the presence of it (**Fig. 4.11**). Our data showed that IL-21 production was significantly reduced in the immature and mature DC-driven assays in the presence of this antibody (**Fig. 4.12**). This implies that the production of IL-21 observed in these responses was partially induced by IL-6. However since IL-6 blockade did not completely ameliorate IL-21 production, there was scope to investigate the involvement of other ligands that use similar signalling pathway to IL-6.

As the gp130 β-receptor trans-membrane subunit is engaged in signal transduction for the IL-6-type cytokine family, it was important to establish whether blocking this pathway would modulate IL-21 production in the immature DC-driven assays. We observed a trend towards greater inhibition of IL-21 in the presence of anti-gp130 antibodies than with IL-6 blockade alone (**Fig. 4.13**). This suggested the possibility that immature DC might use other members of the IL-6-type cytokine family that signal through gp130 to promote IL-21 production.

An additional candidate cytokine that signals through gp130 and has been associated with IL-21 modulation is IL-27. Pot and colleagues have previously demonstrated that IL-27 promoted IL-21 mRNA and protein expression in anti-CD3/anti-CD28 stimulated murine T

cell cultures (Pot et al. 2009). Similar *in vitro* studies using human cells revealed that IL-27 was a strong IL-21 inducer in CD4+CD45RA+CXCR5- T cells isolated from tonsil (Batten et al. 2010). IL-27 therefore represented a strong candidate that DC could potentially utilise to induce IL-21 in a gp130-dependent manner. However supplementation (**Fig. 4.15**) or blockade (**Fig. 4.14**) of IL-27 did not support this idea. In fact addition of IL-27 increased IFNγ production whereas blockade of IL-27 reduced IFNγ (data not shown). Since typically we found that IL-21 and IFNγ are reciprocally regulated (see **Fig. 4.07**), this result added weight to the lack of IL-21 induction by IL-27. We therefore investigated other gp130 binding ligands such as OSM and LIF to see whether they modulated IL-21 production in iDC-driven cultures. However, IL-21 expression was not altered in the presence of antagonistic anti-OSM or anti-LIF antibodies (**Fig. 4.14**). Likewise, the provision of exogenous IL-11 and IL-27 in both B cell and anti-CD3/anti-CD28 bead-driven assays had very little effect on IL-21 production (**Fig. 4.15**). Thus, although we lack robust internal controls for some of these reagents, the suggestion from this part of the study is that unlike IL-6, other gp130 binding cytokines failed to markedly modulate IL-21 *in vitro*.

Having assessed the contribution of the gp130 pathway, we wished to investigate other possible signals that might induce IL-21 production. Evidence from human studies indicated that both IL-12 and IL-23 promote the IL-21 programme in activated CD4+ T cells *in vitro* (Ma et al. 2009; Schmitt et al. 2009). Our PCR data revealed that DC used in our *in vitro* cultures expressed mRNA for IL-6, IL-23 and IL-27, whereas IL-12 transcripts were mainly found in B cells (**Fig. 4.16**). We therefore investigated whether exogenous IL-12 might regulate IL-21 expression when co-stimulation is provided by different APC populations. Unlike in humans, IL-12 appeared to lack the ability to support IL-21 production in T cell

cultures driven by either DC subset, or B cells (**Fig. 4.17 A**). In contrast, exogenous IL-12 substantially increased IFNγ production in these assays. While IL-23 was shown to drive IL-21 production in human studies, the addition of this cytokine in B cell-driven assays had little effect on murine IL-21 (**Fig. 4.17 B**). Therefore, these experiments illustrated that both IL-12 and IL-23 appear to have very little involvement in the promotion of IL-21 expression in mice.

Numerous Th17 differentiation studies revealed that the expression of IL-21 is regulated in an autocrine fashion (Nurieva et al. 2007; Korn et al. 2007; Wei et al. 2007). In addition, Suto and colleagues have also observed an autocrine effect whilst investigating the pathways that generate IL-21-producing T cells (Suto et al. 2008). We therefore wanted to assess the importance of a potential IL-21 feedback loop in our assays to explore whether IL-21 might be positively regulating its own production. Following stimulation with either immature or mature DC, IL-21 production was markedly decreased in T cells lacking the IL-21Rα subunit (**Fig. 4.18**). Interestingly, exogenous IL-6 was able to recover some IL-21 expression, suggesting that it could substitute for IL-21 in some settings. This effect was mostly observed for T cell responses driven by immature DC, and the frequency of IL-21-producting IL-21R-/- T cells was still lower than for their wildtype counterparts. In addition, the IL-21R-/- T cells had a reduced capacity to express IL-21 in cultures driven by B cells or anti-CD3/anti-CD28 beads, in the presence of IL-6. Overall, these experiments revealed the strong effect of an IL-21 feedback loop on the potentiation of IL-21 production by T cells.

We also examined the involvement of the CD28 and PI3K pathways on the modulation of IL-21 expression. The generation of successful immune responses is initiated by the engagement of TCR and CD28 co-stimulation. It is broadly accepted that CD28 ligation is associated with

the induction of IL-2 production (reviewed in (Lenschow et al. 1996)), and upregulation of the co-stimulatory molecule ICOS (McAdam et al. 2000). These events have been shown to be important for the survival and proliferation of activated T cells. However, little was known about whether the CD28 pathway is involved in the modulation of IL-21 responses. We initially examined this in the DC-driven assays and found that these responses were largely independent of CD28 signals (**Fig. 4.19 A**). Suh and colleagues showed that ICOS engagement enhanced humoral responses in the absence of CD28 signals *in vivo* (Suh et al. 2004). It is therefore plausible that ICOS or other accessory molecules, together with IL-6 present in DC-driven responses, may contribute to the promotion of IL-21 in CD28-/- T cells. We also assessed the role of CD28 in regulating IL-21 production *in vivo* by comparing T cells from DO11xRIP-mOVA mice and their CD28-/- counterparts. In contrast to the *in vitro* studies, CD28 signals played an important part in supporting IL-21 and accessory molecules appeared unable to compensate for the decreased production of this cytokine at most sites *in vivo* (**Fig. 4.21**). It is worth noting that in the pancreas, even CD28-/- T cells were capable of making some IL-21 suggesting possible compensatory pathways exclusive to this location.

It has been demonstrated that the p110δ catalytic subunit of PI3K plays an important role in T cell activation (Okkenhaug et al. 2002). More recent studies by Rolf and colleagues reported that CD4+ T cells with a catalytically inactive form of p110δ had a reduced ability to produce IL-21 (Rolf et al. 2010). We therefore wished to establish whether the p110δ subunit of PI3K could modulate IL-21 expression in assays driven by DC. Interestingly, our data suggests that the average IL-21 expression in $p110\delta^{D910A}$ mutant T cells was slightly increased in mature DC-driven responses (**Fig. 4.19 B**). In contrast, immature DC signals appeared to dampen IL-21 production in $p110\delta^{D910A}$ T cells. This investigation revealed that the modulation of IL-21

responses by p110δ might depend on the maturation status of the DC population (**Fig. 4.19 C**). It seems that accessory signals provided by mature DC play a substantial role in promoting IL-21 production by T cells with a defective p110δ subunit. Okkenhaug and colleagues reported CD28 signals were particularly important for T cell activation in $p110\delta^{D910A}$ mice (Okkenhaug et al. 2002). We have also generated DO11xRIP-mOVAx$p110\delta^{D910A}$ to assess the role of the p110δ subunit on IL-21 production in antigen-specific responses. Our data suggest that the p110δ pathway is not essential for the induction of IL-21 in these mice although we observed subtle differences in the proportion of IL-21-expressing T cells in different tissue sites when we compared p110δ mice with their age-matched counterparts (**Fig. 4.23**). Taken together, these data demonstrate that the p110δ subunit of PI3K is not absolutely required for the promotion of IL-21 production.

In this chapter we predominantly focused on investigating the signals that promote IL-21 in APC-driven responses. We found that B cell stimulation induced IFNγ production, whereas DC mainly supported IL-21 programme. It would be valuable to isolate these DC or B cell stimulated DO11 T cells and assess their pathogenicity in antigen-specific responses *in vivo*. It would be of particular importance to establish whether these cells maintain or change their initial cytokine signature. In the future it will be also important to generate more data in p110δ T cell assays and investigate the possible difference in IL-21 production between the immature and mature DC-driven responses. This disparity could be potentially due to higher expression of CD80 and CD86 ligands on mature DC, therefore it would be interesting to evaluate IL-21 production in the presence of an escalating dose of anti-CD80 and/or anti-CD86 antibodies.

5. ANALYSIS OF IL-21 PRODUCTION IN ANTIGEN-SPECIFIC RESPONSES

5.1. Introduction

In the previous chapter we investigated the signals that promote T cell IL-21 production. These experiments were mainly performed *in vitro* and T cell activation was driven by anti-CD3 antibodies. We therefore wished to extend this analysis to examine IL-21 production in response to antigen-specific T cell stimulation. Furthermore, it was not clear to what extent the *in vitro* stimulation assays mimicked the propensity of T cells to differentiate into IL-21-producers *in vivo*. We therefore wished to examine T cells responding to an antigen-specific stimulation *in vivo* to assess the magnitude and kinetics of IL-21 production. To gain insight into the relative roles of dendritic cells and B cells in promoting IL-21 production we performed experiments in which antigen-pulsed dendritic cells were used, and we also performed immunisation experiments comparing T cell IL-21 production in wildtype and B-cell deficient mice.

5.2. Results

5.2.1. IL-21 is produced in antigen-specific responses *in vitro* and *in vivo*

To test the propensity of T cells to make IL-21 during an antigen-specific response we took advantage of DO11 T cells, which express a TCR specific for ovalbumin. CD4+CD25- T cells from DO11 mice were cultured with immature DC or B cells, with TCR ligation provided by OVA peptide or anti-CD3 antibody. **Fig. 5.01 A** illustrates that a substantial proportion of T cells expressed IL-21, but not IFNγ, in response to stimulation provided by OVA peptide and DC. In contrast, high levels of IFNγ were found in the B cell-driven assay with OVA peptide stimulation. Thus, antigen-specific activation yielded similar responses to those seen previously with anti-CD3 stimulation. **Fig. 5.01 B** shows a side-by side comparison between antigen-specific and anti-CD3 stimulation; in both cases IL-21 is highly expressed in the DC-driven assay and IFNγ is predominantly made in B cell-driven responses.

Having demonstrated that IL-21 can be expressed in antigen-specific T cell responses *in vitro*, we next wanted to test whether IL-21 could be induced in an *in vivo* setting. To this end, we adoptively transferred CD4+ DO11 T cells into BALB/c mice and immunised recipients with alum-precipitated OVA (OVA/alum) the following day. After five, 11 and 14 days, single cell suspensions from spleens were assessed for the expression of CD3, DO11.10 TCR, IL-2, IL-21, IFNγ and TNFα by flow cytometry. **Fig. 5.02** demonstrates a clear expansion of the DO11 T cell population five days following OVA/alum challenge, compared with non-immunised recipients. Gating on these CD3+DO11.10 TCR+ T cells enabled us to evaluate the cytokine profiles produced by the antigen-specific response. As shown in **Fig. 5.03 A** approximately

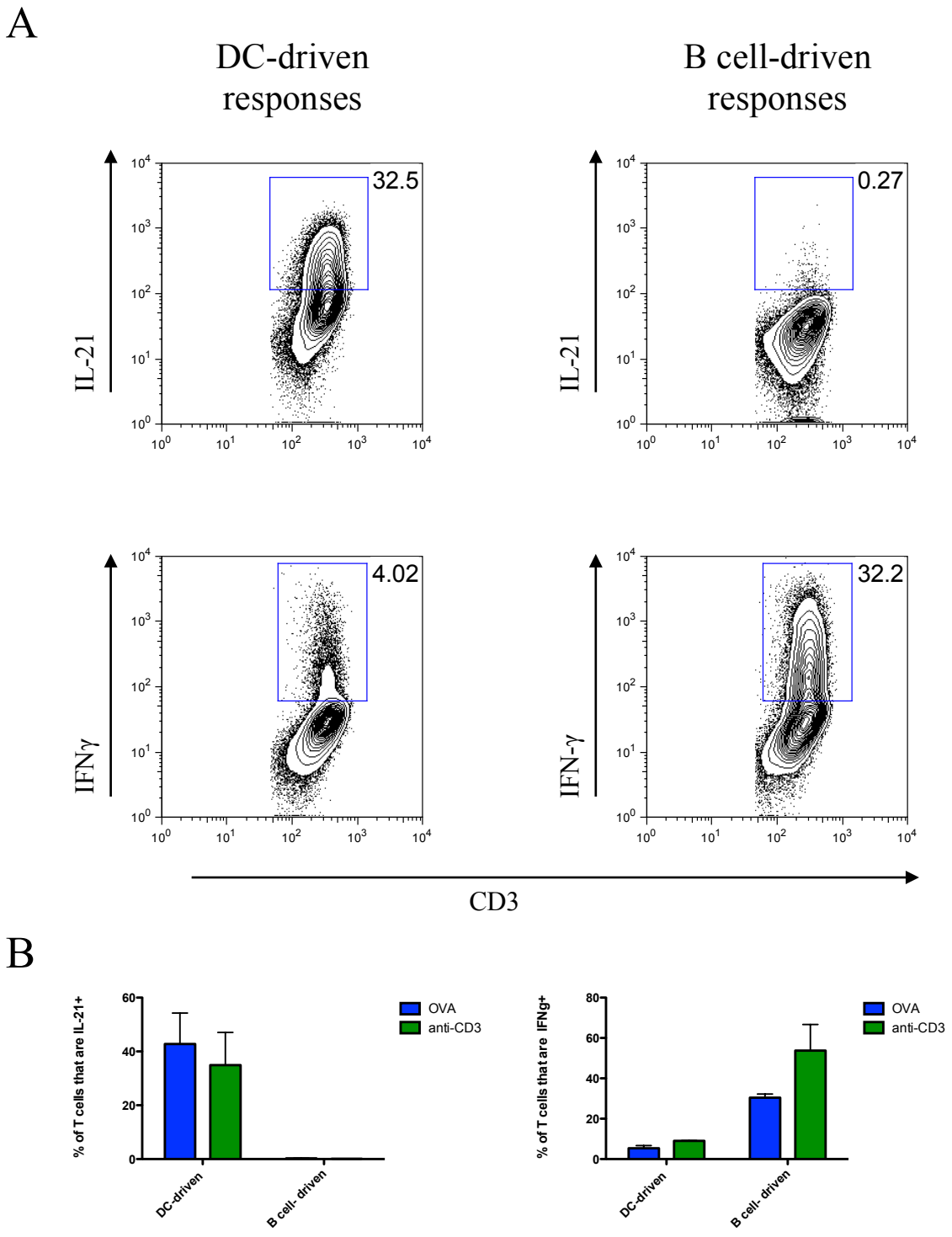


Figure 5.01. The production of IL-21 and IFNγ in antigen-specific responses *in vitro*. $2.5x10^4$ Tconv from DO11 mice were cultured with $2.5x10^3$ immature DC or $7.5x10^4$ B cells and 1μg/ml OVA peptide. After three days cells were restimulated and stained for surface CD3 and intracellular IL-21 and IFNγ. Representative contour plots show expression profiles for IL-21 and IFNγ within gated Tconv in the OVA peptide-driven assay (**A**). Graphs show collated data for IL-21 and IFNγ expression in cultures where TCR signal was provided by OVA peptide or anti-CD3 (**B**). Bars represent means and SEM across 2 experiments.

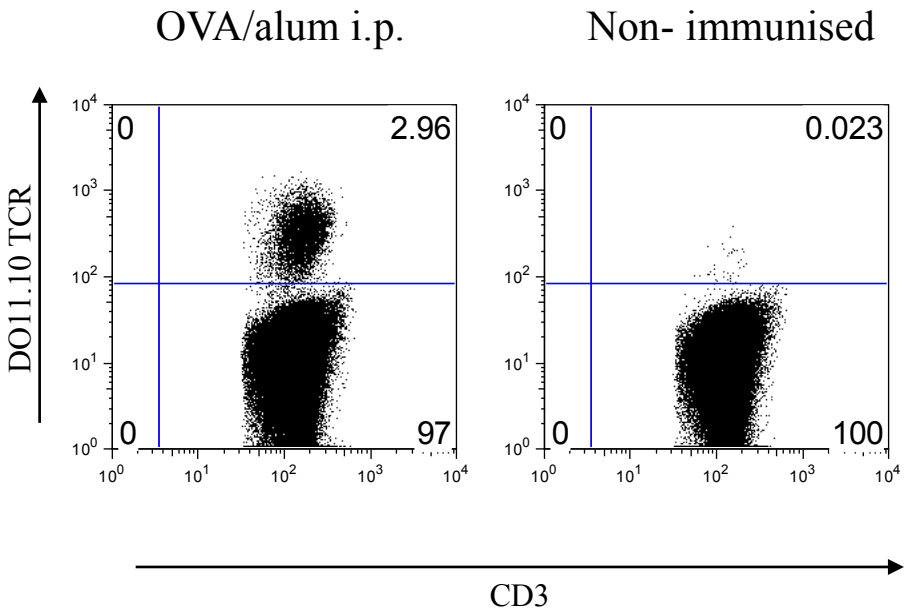


Figure 5.02. The CD4 T cell antigen-specific response to OVA/alum immunisation. $1x10^6$ CD4+ T cells from DO11 lymph node were transferred i.v. into BALB/c recipients. The following day recipients were immunised with 200µg OVA/alum i.p. five days post immunisation single cell suspensions from spleen were stained for surface CD3, CD4 and DO11.10 TCR. Representative dot plots show DO11.10 TCR staining within gated CD3+CD4+ T cells.

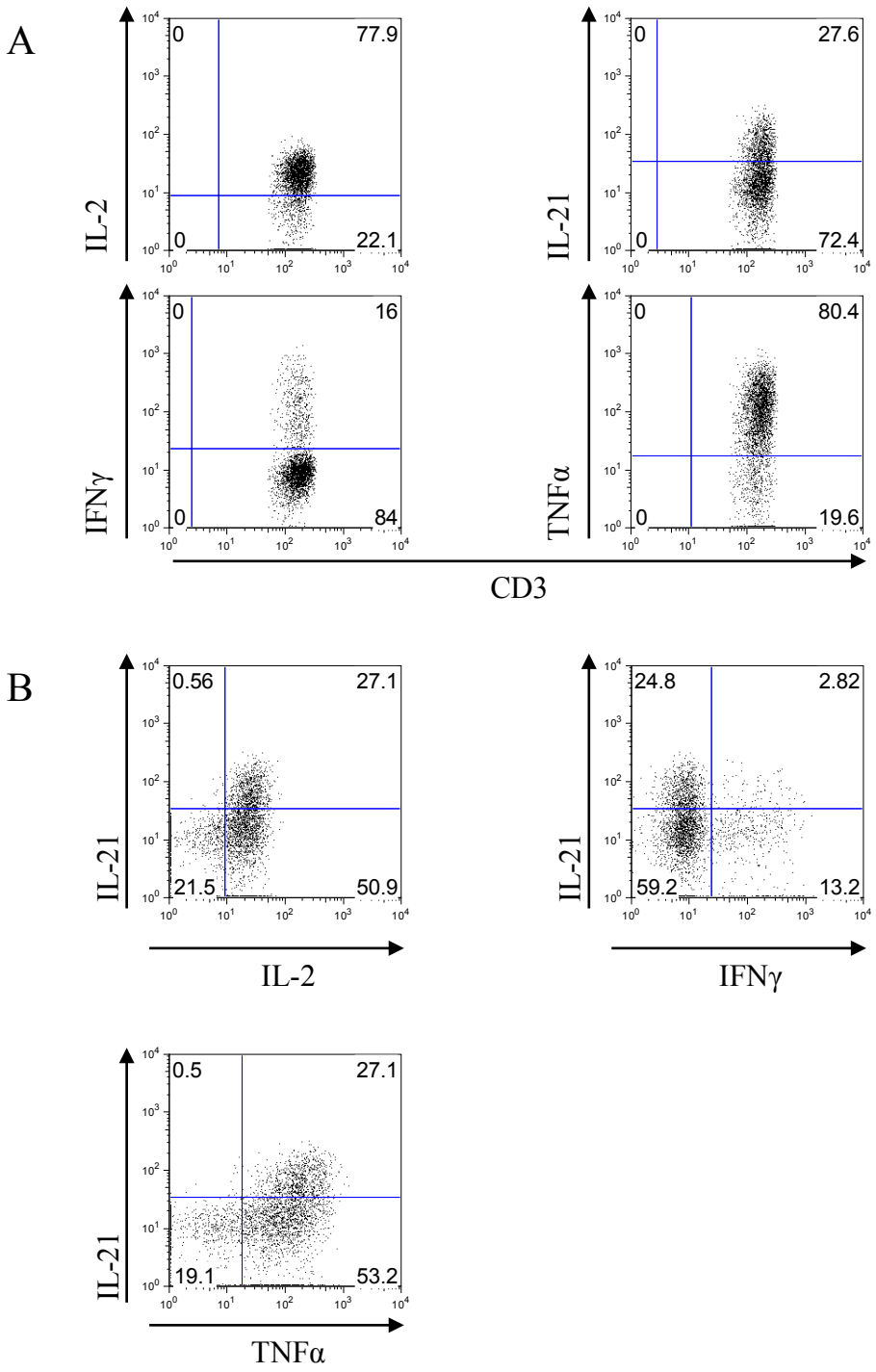


Figure 5.03. IL-21 can be detected during antigen–specific responses *in vivo*. 1x10^6 CD4+ T cells from DO11 lymph node were transferred i.v. into BALB/c recipients. The following day recipients were immunised with 200µg OVA/alum i.p. five days post immunisation single cell suspensions from spleen were restimulated and stained for surface CD3 and DO11.10 TCR, and intracellular IL-21, IL-2, IFNγ and TNFα. Representative dot plots show cytokine staining within gated CD3+ DO11.10 TCR+ T cells (**A**). Representative dot plots show IL-21 co-staining with IFNγ, TNFα and IL-2 within gated CD3+ DO11.10 TCR+ T cells (**B**).

one third of CD4+ DO11 T cells expressed IL-21. In addition, we found that the great majority of DO11 T cells produced IL-2 and TNFα, while much lower levels of IFNγ were detected. To get a better appreciation for the phenotype of the IL-21-producing DO11 T cells, we evaluated their co-expression of IL-21, IL-2, IFNγ and TNFα. It appeared that nearly all of the IL-21-expressing DO11 T cells co-stained for IL-2 and TNFα (**Fig 5.03 B**). In contrast, only approximately 10% of IL-21-producing DO11 T cells co-expressed IFNγ. Overall, these data indicate that IL-21 can be produced in antigen-specific T cell responses *in vivo*. These IL-21+ T cells also expressed the pro-inflammatory cytokine TNFα and the common γ-chain family cytokine IL-2.

As our preliminary research looked at day five post OVA/alum immunisation, we wished to further characterise these T cell responses at later time points. We therefore used the protocol described above and evaluated the expression of IL-21, IFNγ and IL-2 at five, 11 and 14 days post immunisation. **Fig. 5.04** illustrates that approximately 30% of T cells expressed IL-21 at day five and over the analysed time frame this production slightly increased. These data suggest that even two weeks after immunisation, IL-21 production by T cells remained high. The average frequency of IFNγ-expressing T cells increased from around 5% at day five to approximately 10% after two weeks. This production was however substantially lower than that observed for IL-21. It appears that the great majority of T cells were IL-2 positive (around 80%) five days following immunisation and this expression was slightly reduced over later time points. Overall, these results demonstrate that IL-21 is made during T cell responses driven by OVA/alum and that its expression stayed high throughout the investigated time course.

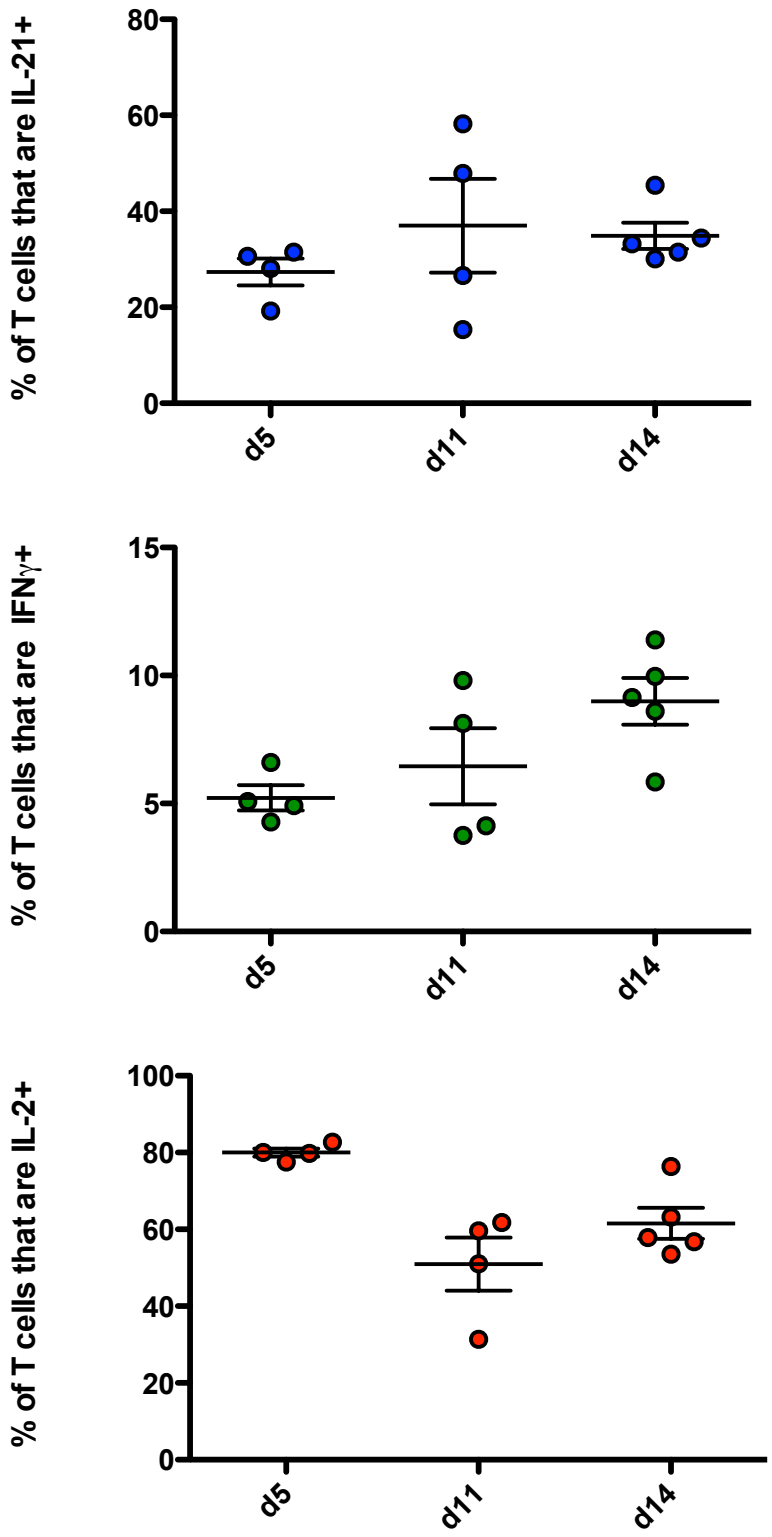


Figure 5.04. The kinetics of IL-21, IFNγ and IL-2 production in OVA/alum immunised mice. $1x10^6$ CD4+ T cells from DO11 lymph node were transferred i.v. into BALB/c recipients. The following day recipients were immunised with 200µg OVA/alum i.p. five, 11 and 14 days post immunisation single cell suspensions from spleen were restimulated and stained for surface CD3 and DO11.10 TCR and intracellular IL-21, IFNγ and IL-2. Graphs show collated data for IL-21, IFNγ and IL-2 expression within gated CD3+ DO11.10 TCR+ T cells. Bars represent means and SEM.

5.2.2. The importance of signals provided by B cells and DC for the promotion of IL-21 *in vivo*

In Chapter 4 we demonstrated that immature and mature DC provided signals that permitted T cell IL-21 production. It was therefore important to test whether these cells also supported IL-21 expression in an *in vivo* setting. To address this, we opted for an adoptive transfer model in which OVA peptide pulsed-DC drive CD4+ DO11 T cell responses in BALB/c hosts. Cell trace violet labeled CD4+ T cells from DO11 peripheral lymph nodes were thus transferred i.v. into BALB/c recipients. The following day the same mice were injected i.v. with either unpulsed DC or OVA peptide loaded DC. Single cell suspensions from spleen were stained for surface CD4, DO11.10 TCR and CD62L at day six post DC transfer. **Fig. 5.05 A** illustrates that a substantial CD4+ DO11.10 TCR+ population was found only in mice injected with peptide-loaded DC. Furthermore, our data demonstrates that the transferred CD4+ DO11 T cells expanded *in vivo* (**Fig. 5.05 B**) and this proliferation was characterised by the downregulation of CD62L (**Fig. 5.05 C**). This experiment indicates that the peptide-loaded DC were efficient at antigen presentation and effectively drove T cell responses *in vivo*.

We next wished to investigate further the DC-driven responses *in vivo* and phenotype the adoptively transferred CD4+ DO11 T cells for their expression of cytokines. To this end, we transferred fluorescently labeled CD4+ DO11 T cells with OVA peptide pulsed DC as described above and after six days assessed cytokine production by staining for intracellular IL-21, IFNγ and IL-2. The gating strategy for identifying CD4+ DO11.10 TCR+ T cells and their proliferation is shown in the top panel of **Fig 5.06**. We found that OVA peptide loaded DC supported the generation of IL-21-expressing DO11 T cells (approximately 15%). These responses were also characterised by substantial IFNγ production and very high IL-2

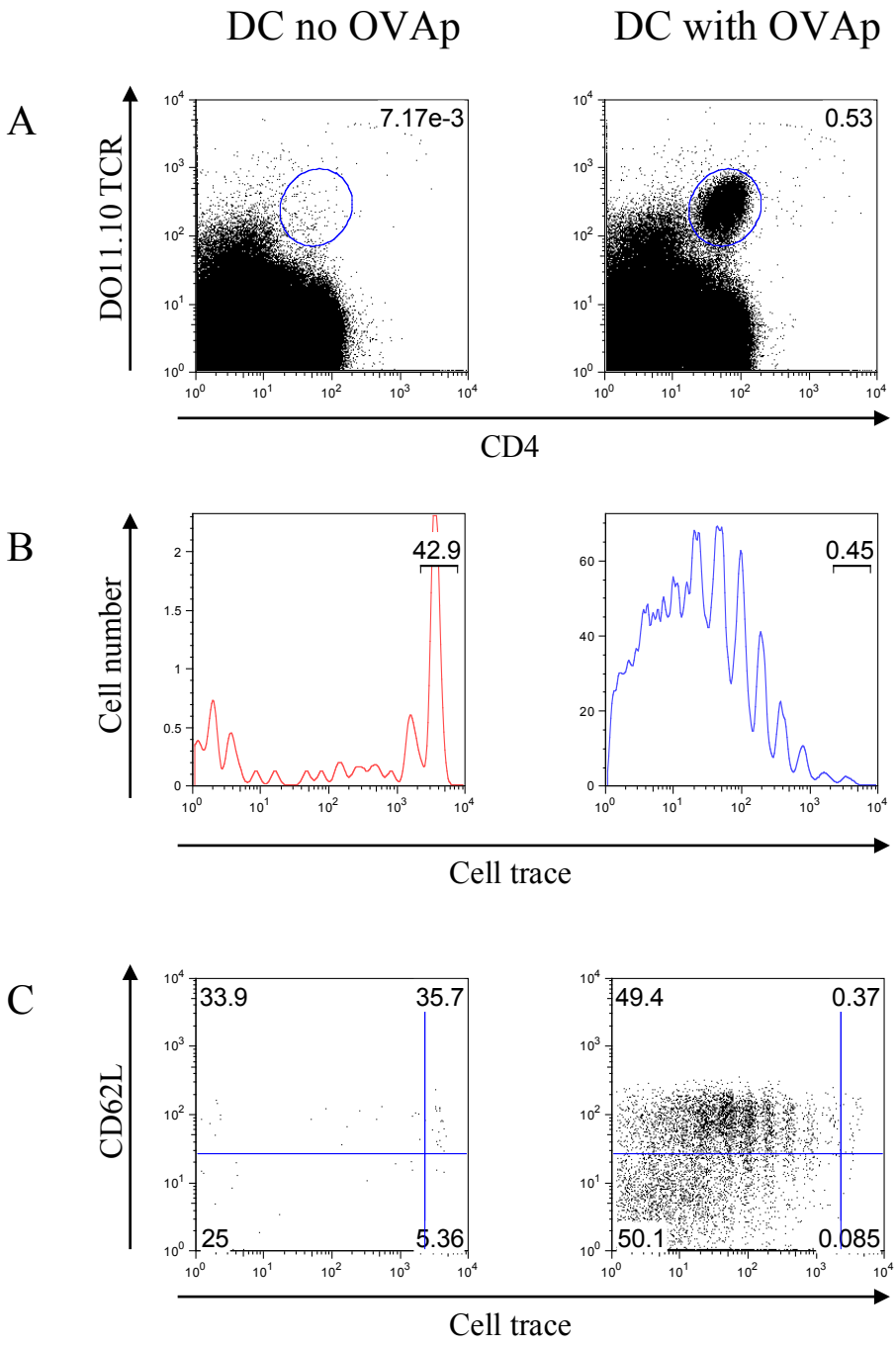


Figure 5.05. The ability of OVA loaded BM-DC to drive antigen-specific responses *in vivo*. $3x10^6$ fluorescently labeled CD4+ T cells from DO11 lymph node were transferred i.v. into BALB/c recipients. The following day $1.5x10^5$ BM-DC or BM-DC loaded overnight with 1µg/ml OVA peptide were transferred i.v. six days post immunisation single cell suspensions from spleen were stained for surface CD4 and DO11.10 TCR, and CD62L. Representative dot plots show DO11.10 TCR and CD4 staining within the lymphocyte gate (**A**). Representative histograms show cell trace violet dilutions within gated CD4+ DO11.10 TCR+ T cells (**B**). Representative dot plots show cell trace violet dilutions and CD62L staining within gated CD4+ DO11.10 TCR+ T cells (**C**).

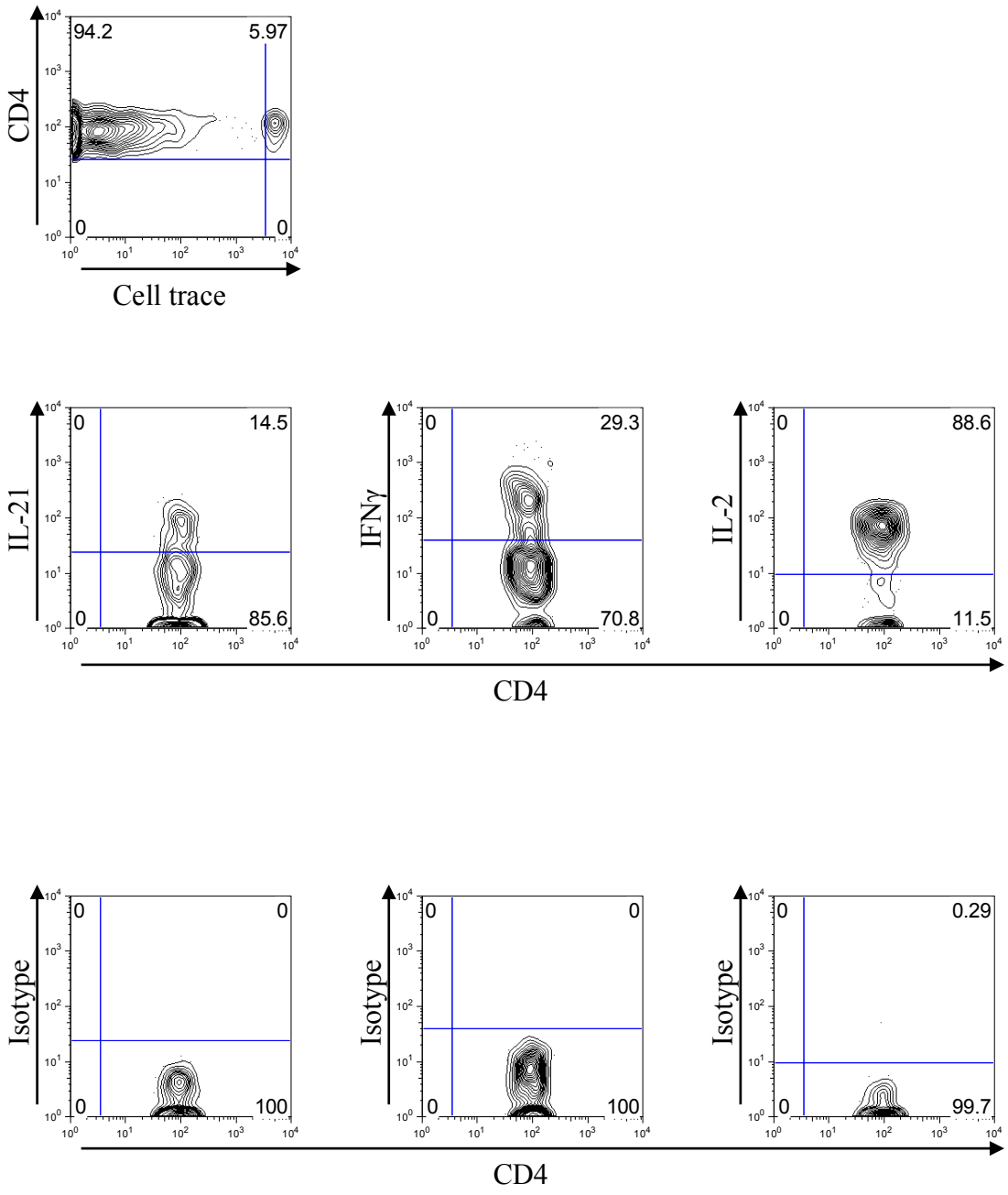


Figure 5.06. The ability of OVA peptide loaded BM-DC to promote cytokine production *in vivo*. $3x10^6$ CD4+ fluorescently labeled T cells from DO11 lymph node were transferred i.v. into BALB/c recipients. Following day $1.5x10^5$ BM-DC loaded overnight with 1μg/ml OVA peptide were transferred i.v. into recipients. After six days post immunisation single cell suspensions from spleen were stained for surface CD4 and DO11.10 TCR, and intracellular IL-2, IL-21 and IFNγ. Representative contour plots show cell trace violet dilutions within gated CD4+ DO11.10 TCR+ T cells (top panel). Representative contour plots show IL-2, IL-21 and IFNγ staining within gated CD4+DO11.10 TCR+ T cells (middle panel) and isotype controls (bottom panel).

expression (middle panel). We therefore demonstrated that bone marrow-derived DC are able to promote IL-21 production in both an *in vitro* culture system and an *in vivo* immunisation model.

Our *in vitro* data suggested that DC-activated T cells started to produce IL-21 after one day of culture and this production was substantially increased at days two and three. We therefore sought to assess the expression of IL-21, IFNγ and IL-2 at an earlier stage of the immunising DC-T cell interactions (days two and three). As demonstrated in **Fig. 5.07** the average expression of IL-21 was low at day two and slightly increased at day three to reach its highest level at day six (approximately 15% CD4+ DO11 T cells were IL-21+). Similarly, a low average frequency of IFNγ-expressing T cells was observed for the first two time points of the time course, which then substantially increased to around 30% by day six. With regard to IL-2 production, the majority of DO11 T cells expressed this cytokine from day three onwards (around 60% IL-2-expressing T cells at day three and 80% at day six). Thus, T cells responding to peptide pulsed DC *in vivo* expressed IL-2 as early as day three but did not appreciably produce IL-21 or IFNγ until day six.

Having evaluated the importance of bone marrow-derived DC in the promotion of IL-21, we next sought to investigate the role B cells play in the production of IL-21 *in vivo*. To address this, we adoptively transferred CD4+ DO11 T cells into either wildtype or B cell deficient (Jh-/-) mice and immunised recipients with OVA/alum the following day. After five, 11, and 14 days single cell suspensions from spleen were assessed for their expression of surface CD3, DO11.10 TCR and intracellular IL-21, IFNγ and IL-2 by flow cytometry. As demonstrated in **Fig. 5.08**, we found that a substantial proportion of DO11 T cells expressed IL-21 in the Jh-/-

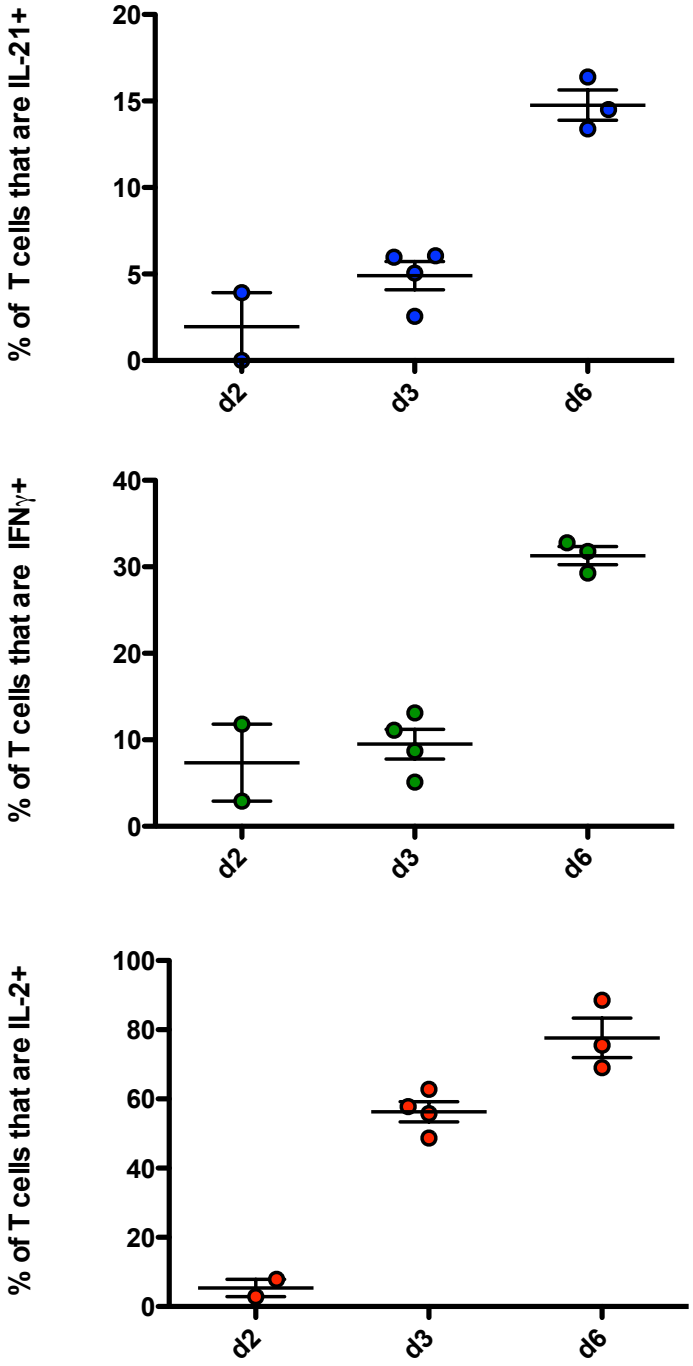


Figure 5.07. The kinetics of IL-21, IFNγ and IL-2 production in OVA peptide loaded BM-DC responses. IL-21, $3x10^6$ CD4+ fluorescently labeled T cells from DO11 lymph node were transferred i.v. into BALB/c recipients. Following day $1.5x10^5$ BM-DC loaded overnight with 1μg/ml OVA peptide were transferred i.v. two, three and six days post immunisation single cell suspensions from spleen were stained for surface CD4 and DO11.10 TCR, and intracellular IL-2, IL-21 and IFNγ. Graphs show collated data for IFNγ and IL-2 expression within gated CD4+ DO11.10 TCR+ T cells. Bars represent means and SEM.

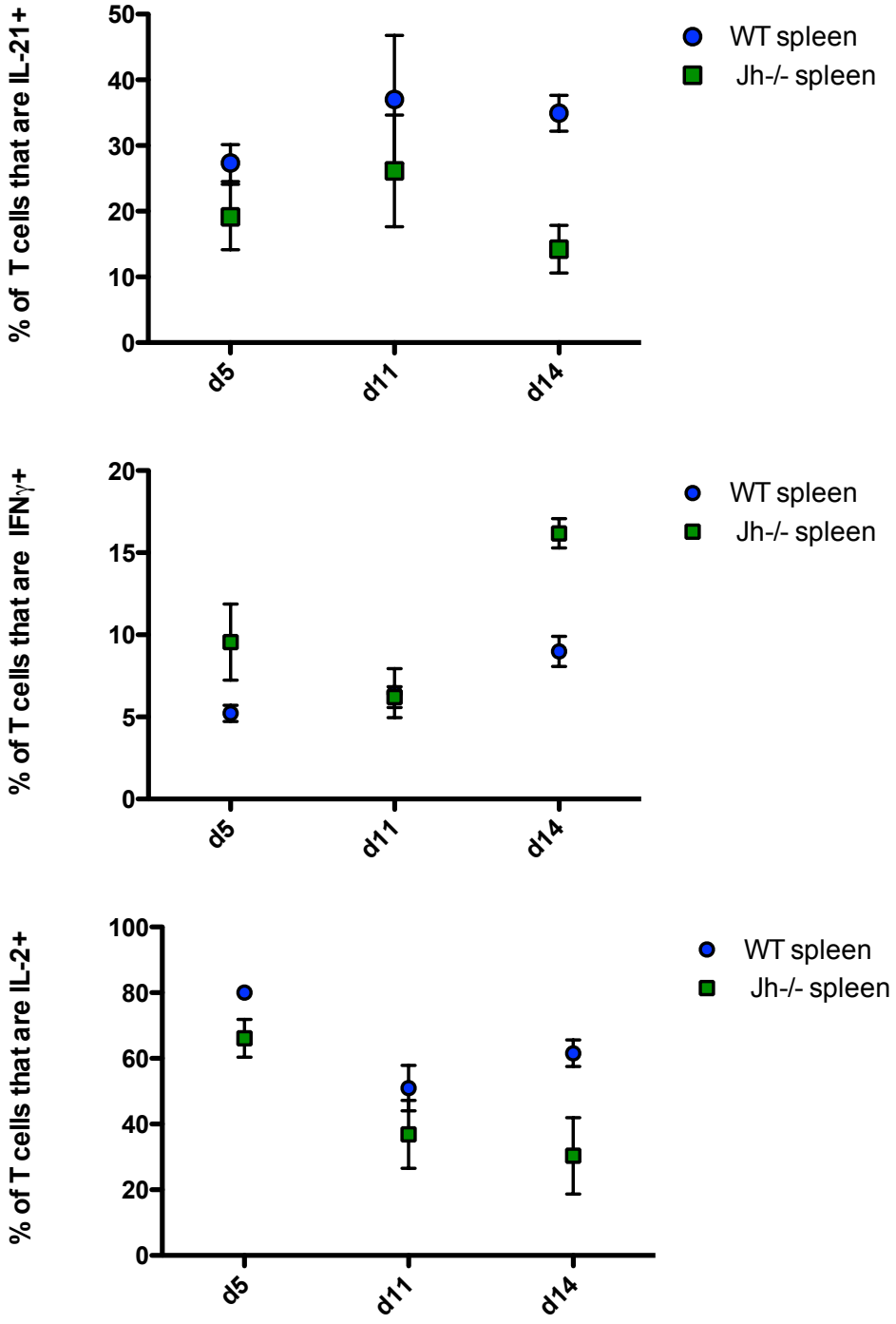


Figure 5.08. The ability of B cells to modulate IL-21 production *in vivo*. $1x10^6$ CD4+ T cells from DO11 lymph node were transferred i.v. into BALB/c or Jh-/- recipients. Following day recipients were immunised with 200µg OVA/alum i.p. six, 11 and 14 days post immunisation single cell suspensions from spleen were restimulated and stained for surface CD3 and DO11.10 TCR, and intracellular IL-21, IFNγ and IL-2. Graphs show collated data for IL-21, IFNγ and IL-2 expression within gated CD3+ DO11.10 TCR+ T cells. Points and bars represent means and SEM across four experiments.

spleen, thus it appears that B cells are not essential for the induction of IL-21 in this *in vivo* model. Although IL-21 levels were comparable with those observed in the WT host at day five and 11, it appeared that the average proportion of IL-21-expressing T cells was reduced from approximately 30% in WT to 15% in Jh-/- mice at day 14. These data clearly demonstrate that B cells might potentially play a limited role in the early response to immunisation, but their presence is important for the maintenance of IL-21 production at later stages. Furthermore, this investigation also shows that the average expression of IFNγ in Jh-/- mice at least matched that of WT hosts at days five and 11, and was greatly increased at the end of the time course. This indicates that the late reduction in IL-21 in B cell-deficient animals was specific rather than reflecting a generalised decrease in the levels of all cytokines. IL-2 was initially expressed by the majority of T cells (day five) and this proportion substantially decreased in the B cell deficient mice by day 14 (from approximately 60% IL-2-expressing T cells in WT to 30% in Jh-/- mice). Overall, these data demonstrate that B cells potentially modulated cytokine production by T cells during the later phase of the immune response, but had limited impact during the initial phase.

To gain further insight into the importance of B cell signals for the promotion of IL-21 *in vivo*, we decided to utilise the DO11xRIP-mOVA mouse model and examine the impact of rendering it deficient in B cells. Initially, we assessed the impact of B cell deficiency on the development of diabetes in DO11xRIP-mOVAxJh-/- mice. **Fig. 5.09** clearly demonstrates that DO11xRIP-mOVAxJh-/- mice still develop disease. It appears that the absence of B cells might potentially delay disease progression to overt diabetes, as blood glucose readings remained under 250mg/dl for 12-week-old animals. We next wished to assess IL-21 production in DO11xRIP-mOVAxJh-/- mice across different tissues and ages. Therefore,

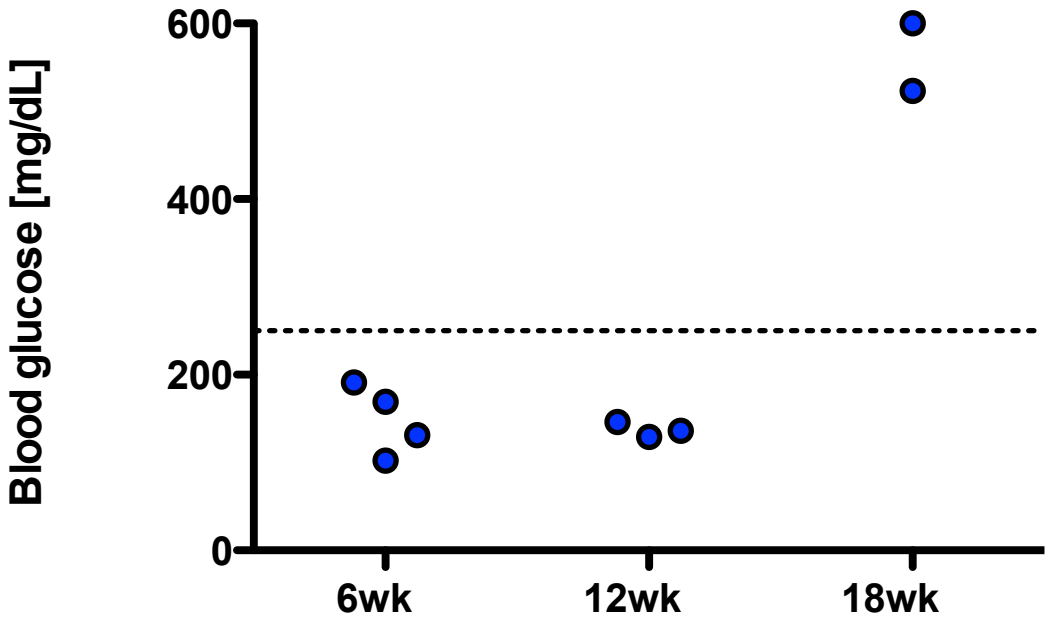


Figure 5.09. Blood glucose readings in DO11xRIP-mOVAxJh-/- mice. Graph shows blood glucose measurements in DO11xRIP-mOVAxJh-/- mice tested at different weeks of age. Dashed line indicates blood glucose level above which mice are considered diabetic.

single cell suspensions from the inguinal LN, pancreatic LN, pancreas and spleen of six, 12 and 18-week-old Jh-/- DP mice were evaluated for the expression of IL-21. **Fig. 5.10** illustrates that a very low proportion of IL-21+ conventional T cells was found in the inguinal LN, pancreatic LN and spleen of Jh-/- DP mice. The highest level of IL-21 production was observed in pancreas-infiltrating conventional T cells. Most importantly, this investigation revealed that IL-21 was substantially reduced across all tissues and ages in B cell deficient DO11xRIP-mOVA mice. This suggests that B cells might potentially support the generation or maintenance of IL-21-producing conventional T cells in the DO11xRIP-mOVA mouse model.

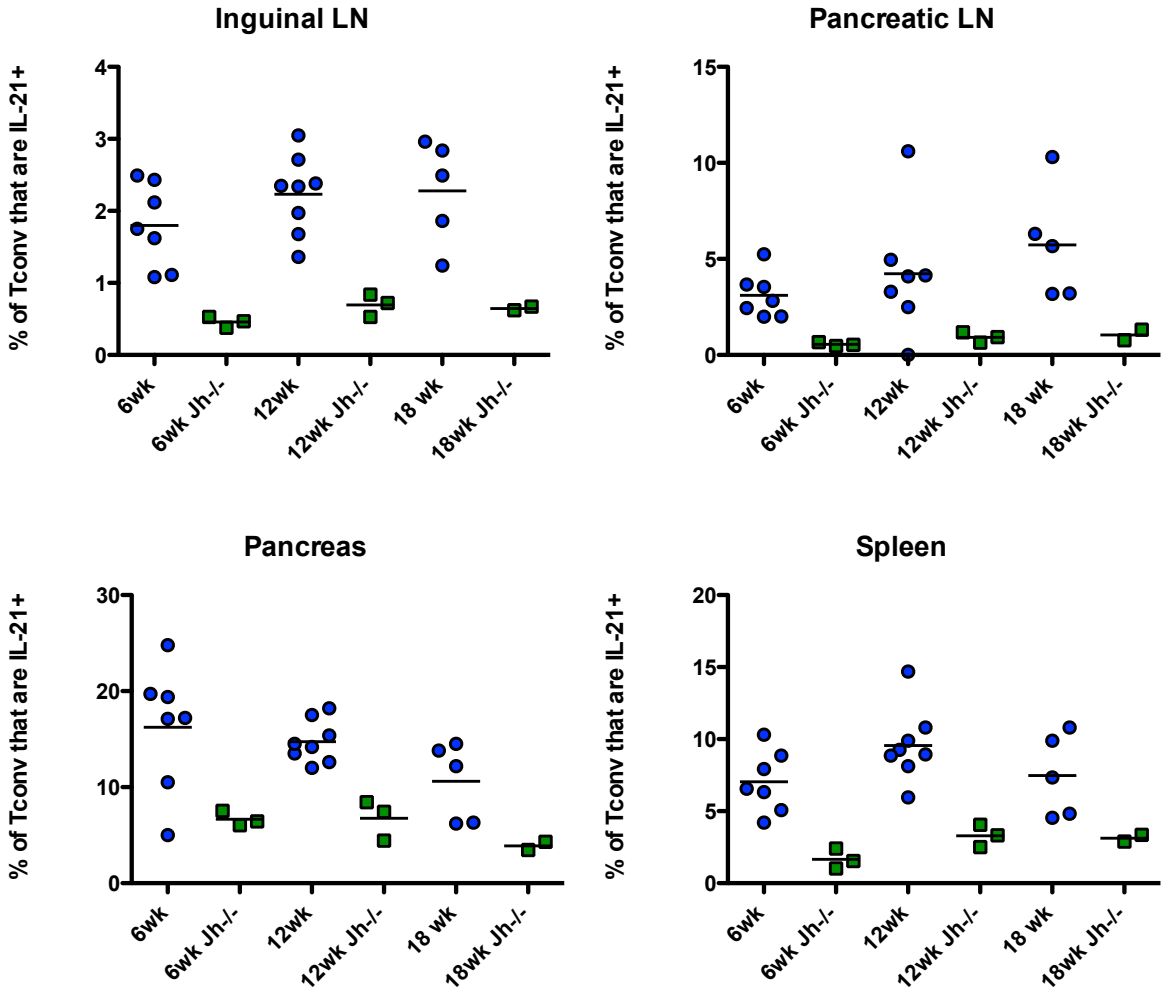


Figure 5.10. The ability of B cells to modulate IL-21 production in DO11xRIP-mOVA mice. Single cell suspensions from inguinal lymph node, pancreatic lymph node, pancreas and spleen of DO11xRIP-mOVA or DO11xRIP-mOVAxJh-/- (Jh-/-) mice were restimulated and stained for surface CD4 and intracellular Foxp3 and IL-21. Graphs show collated data for IL-21 expression in CD4+Foxp3- Tconv from DO11xRIP-mOVA or DO11xRIP-mOVAxJh-/- in the indicated tissues. Bars represent means across the indicated mice and ages (weeks).

5.3. Discussion

In this chapter we examined the involvement of DC and B cells in IL-21 production *in vivo*. Initially, it was important to establish whether peptide-specific T cell responses *in vitro* mimicked those observed for anti-CD3 antibody stimulation. Our data suggests that both methods of TCR activation yielded broadly similar patterns of cytokine expression. IL-21 was predominantly expressed in DC-driven assays, whereas B cell co-stimulation skewed T cell responses towards IFNγ production (**Fig. 5.01 A**). Having established cytokine profiles in the peptide-specific system, we consequently wished to examine IL-21 responses *in vivo*. To achieve this we transferred CD4+ DO11 T cells into BALB/c recipients and immunised them with OVA/alum. Initially, we investigated T cell expansion and cytokine production five days post immunisation. CD4+ DO11 T cells proliferated in response to OVA/alum and expressed substantial levels of IL-21 together with IL-2, IFNγ and TNFα (**Fig. 5.03**).

Interestingly, IL-21 was readily made as early as five days after peptide immunisation. Consistent with these data, Batten *et al.* reported the overexpression of IL-21 mRNA four days after immunisation with OVA emulsified in complete Freund's adjuvant (Batten et al. 2010). Therefore these two studies support the notion that IL-21 might potentially be involved in the early phase of an immune response. To gain further insight into the kinetics and magnitude of IL-21 expression, the timescale of these *in vivo* studies was extended and IL-21 production was examined at day 11 and 14 post immunisation. At the same time we also monitored the expression of IFNγ and IL-2 to get a more detailed picture of the cytokine expression profile in the OVA/alum system. We observed that CD4+ DO11 T cells maintained IL-21 expression throughout this two-week period (**Fig. 5.04**). The response to OVA peptide was characterised by a gradual increase in IFNγ production, while IL-2

remained high at all time points. A consistent level of IL-21 expression in the OVA/alum immunisation model might contribute to prolonging T cell responses *in vivo*, as this cytokine has been associated with T cell growth and survival (Parrish-Novak et al. 2000). It was somewhat surprising that a low proportion of DO11 T cells expressed pro-inflammatory IFNγ. This might potentially be explained by the presence of endogenous IL-6, that not only was a main inducer of IL-21 programme in our *in vitro* studies, but also inhibited strong IFNγ responses in the B cell-driven assays. It is also possible that IL-21 was inhibiting the production of IFNγ in this system, as we have observed this effect in previous studies (Attridge et al. 2012).

We have previously demonstrated that bone marrow-derived DC provided signals that promoted IL-21 production *in vitro*. The induction of IL-21 in these assays was mostly dependent on endogenous IL-6, which was confirmed by using anti-IL-6 antibodies and also by analysing mRNA expression from DC. Previous reports also indicated DC as a possible source of IL-6 (Kopf et al. 1998; Pasare & Medzhitov 2003). Consequently, we wished to test the propensity of T cells to produce IL-21 in response to peptide-loaded DC *in vivo*. This was addressed using OVA peptide-loaded bone marrow-derived DC and CD4+ DO11 T cells, which were both transferred into BALB/c recipients. Firstly, we demonstrated that DO11 T cells only expanded in response to peptide-loaded DC, but not DC alone. This was evident from the cell trace dilution profiles and the downregulation of the adhesion molecule CD62L, which is known to be downregulated upon T cell activation (**Fig. 5.05**). We demonstrated that a substantial proportion of T cells stimulated with peptide-loaded DC *in vivo* expressed IL-21 after six days (**Fig. 5.06**). The magnitude of IL-21 response was comparable with that observed in the OVA/alum system at day five. This indicates that bone marrow-derived DC

were able to induce IL-21 in an antigen-specific fashion *in vivo*. Furthermore, DC stimulation supported the generation of IFNγ- and IL-2- producing T cells six days after immunisation. To compare the peptide-pulsed DC responses with our *in vitro* findings, we also assessed IL-21 expression at days two and three. Our data showed that very little IL-21 was made at these early timepoints and that considerable levels of this cytokine were produced after six days (**Fig. 5.07**). Furthermore, it appeared that the peptide-loaded DC stimulation drove strong IL-2 responses, which occurred from day three onwards. We also found that the production of IFNγ by T cells was somewhat attenuated at these two early timepoints. Generally, it appeared that T cells started to produce considerable amounts of cytokine three days after peptide-loaded DC immunisation. This delay might potentially be explained by the time that is required for DC and T cells to migrate successfully to the lymphoid tissue before priming could occur. In summary, this investigation revealed that DC supported the generation of IL-21-producing T cells in an antigen-specific response *in vivo*.

Having evaluated IL-21 expression in the OVA/alum system, we wished to use this model to further elucidate the role of B cells in the induction of this cytokine. This was achieved by immunising B cell deficient versus wildtype recipient mice with OVA/alum and evaluating T cell cytokine expression over time. Our investigation demonstrated that the initial magnitude of IL-21 expression by CD4+ DO11 T cells at day five and 11 was comparable between B cell deficient and wildtype mice (**Fig. 5.08**). Intriguingly, we noticed a substantial reduction in IL-21 production in mice lacking a B cell compartment at day 14. It is therefore conceivable that B cell – T cell interactions might have a limited influence on the promotion of IL-21 initially after immunisation, whilst becoming more important at later stages. Recent evidence from IL-21 reporter mouse studies suggests that this cytokine, in addition to the

transcription factor Bcl-6 and chemokine receptor CXCR5, is strongly associated with the Tfh subset (Lüthje et al. 2012). Thus, the level of IL-21 production could potentially be modulated by the ability of T cell to differentiate into Tfh. Choi *et al.* demonstrated that late stage B cell - T cell interactions were particularly important for commitment towards the Tfh linage, and that preceding ICOS signals provided by DC were required for the induction of Bcl-6 and CXCR5 (Choi et al 2011). Thus, the lack of B cell signals that are required for Tfh lineage commitment might potentially explain the reduction in IL-21 expression observed in the late phase post immunisation.

To further dissect the role of B cell signals in the promotion of IL-21, we also took advantage of the DO11xRIP-mOVAxJh-/- mouse strain that lack a B cell compartment. This model allowed us to evaluate IL-21 expression in the setting of a prolonged immune response. Our data showed that in the absence of B cell signals, IL-21 production by Tconv was considerably reduced throughout all tissues and ages studied (**Fig. 5.10**). These findings suggest that B cell – T cell interactions play an important role in IL-21 production and maintenance during the acute and chronic phase of the immune response, but confirm that they are not absolutely required for IL-21 production.

Future work should address the question of whether early expression of IL-21 after OVA/alum immunisation was contributing to T cell proliferation. This could be tested in mice transferred with DO11 T cells and immunised with OVA/alum in the presence of anti-IL-21 antibodies or using IL-21R-/- DO11 T cells. The ability of DO11 T cells to proliferate could be established by Ki-67 expression, cell trace dilution profiles and absolute cell numbers. In terms of IL-21 responses induced by OVA-loaded DC, it would be interesting to perform the

IL-6 blockade *in vivo* to test whether this cytokine drove IL-21 production in this model. IL-21R-/- T cells could also be used to see whether IL-21 feedback is responsible for limiting IFNγ expression. Based on the evidence that Tfh cell are associated with high IL-21 production, further experiments should determine whether IL-21-producing T cells express CXCR5, PD-1 and Bcl-6. This analysis would establish whether the IL-21 production observed after OVA/alum immunisation is derived from Tfh cells.

6. CHARACTERISATION OF A NOVEL CELL SUBSET IN THE PANCREAS

6.1. Introduction

The research efforts of our group primarily focus on understanding the contribution of different pancreas-infiltrating cell populations to the development of diabetes. During attempts to dissect the role of B and T lymphocytes in this disease, we have found a population of lineage negative cells in the pancreas of DO11xRIP-mOVA mice. These cells were identified as CD4-CD25+ and appear to be exclusively located in the pancreas. Recently, numerous groups have reported the emergence of a new class of cells involved in type 2 immune responses (reviewed in (Neill & McKenzie 2011)). These lineage negative innate immune cells are characterised by their expression of CD25 and are associated with the modulation of immune responses to helminth infections and asthma through the production of IL-4, IL-5, IL-9 and IL-13. We therefore sought to further characterise the phenotype of pancreatic CD4-CD25+ cells and evaluate their influence over T cell responses.

6.2. Results

6.2.1. Characterisation of CD4-CD25+ cells in the pancreas

To better understand the contribution of different T cell subsets to the development of T1D, we have previously assessed the distribution of conventional T cells and Treg in DO11xRIP-mOVA mice. One of the approaches taken involved staining for surface CD25 as a key marker of the Treg lineage and, to a limited extent, of activated conventional CD4 T cells. This analysis revealed that CD25 was not only expressed by CD4 T cells in the lymph node and pancreas, but also by a CD4- population in the latter organ (**Fig. 6.01 A**). To phenotype the CD4-CD25+ population, we stained single cell suspensions from the pancreases of DO11xRIP-mOVA mice for CD3, CD4, CD25, Dx5, CD122, CD19, B220 and CD11c. As illustrated in **Fig. 6.01 B** CD4-CD25+ cells were negative for the natural killer (NK) cell marker Dx5 and low for CD122. They did not express CD19 or B220, which are associated with the B cell lineage. Furthermore, we also found very little expression of CD11c or CD3, markers for DC and T cells respectively. These data suggest that CD4-CD25+ cells did not represents members of the T cell, B cell, NK cell or DC lineages.

Various groups have identified lineage negative populations that are responsible for modulating type 2 immune responses (Moro et al. 2010; Price et al. 2010; Neill et al. 2010; Saenz et al. 2010). These new innate immune cells also expressed certain T cell-associated markers. To extend our phenotyping of pancreas-derived CD4-CD25+ cells, single cell suspensions from the DO11xRIP-mOVA pancreas were assessed for the expression of CD127, ST2, ICOS, ICAM, MHC II, CD11b, CD49d, cKit, Sca-1, CD43 and CCR9 by flow

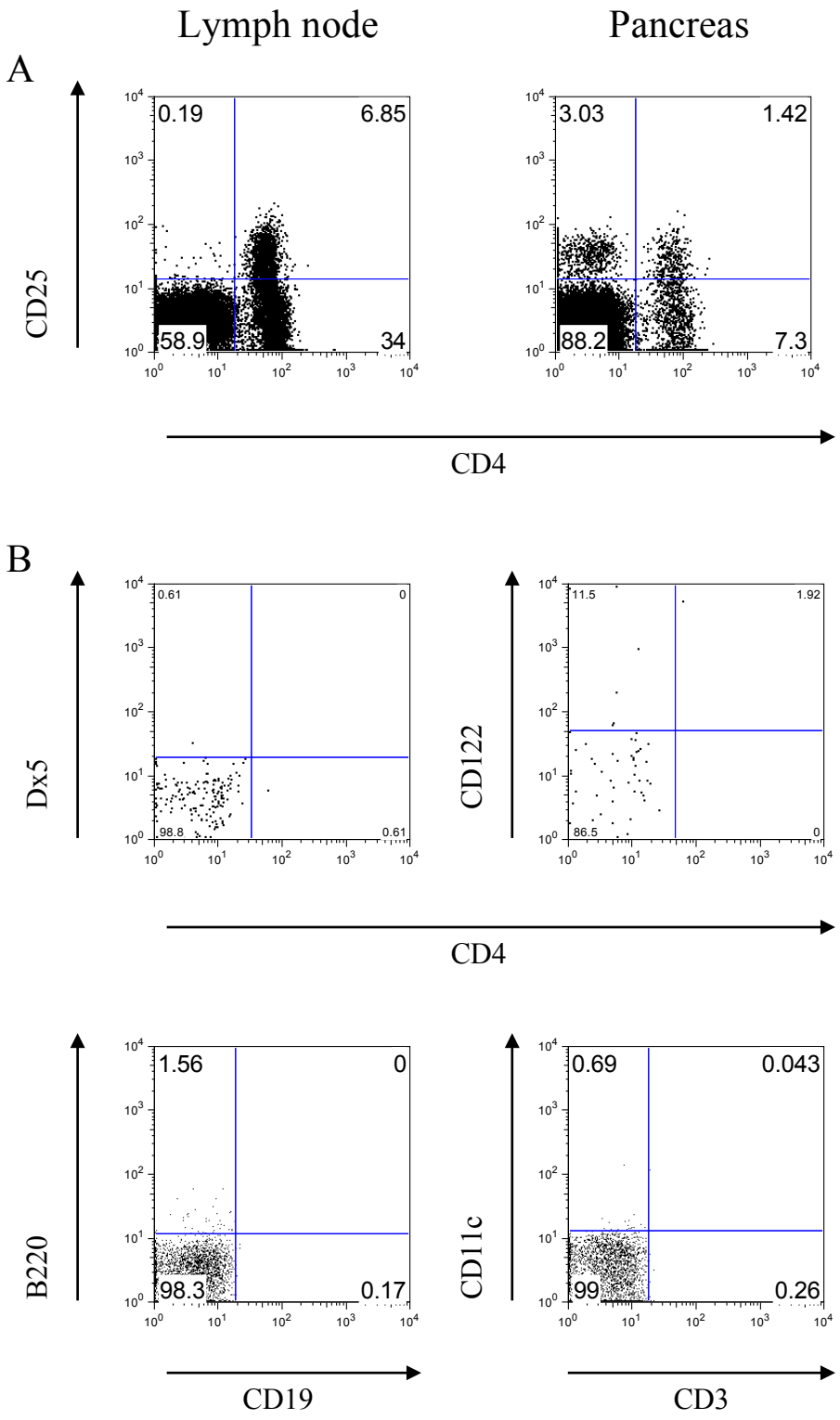


Figure 6.01. Identification of the unknown cell population in the DO11xRIP-mOVA pancreas. Single cell suspensions from inguinal lymph node and pancreas of DO11xRIP-mOVA mice were stained for surface CD3, CD4, CD25, Dx5, CD122, B220, CD19 and CD11c. Representative dot plots show CD4 and CD25 staining in the inguinal lymph node and pancreas (**A**). Representative dot plots show CD3, CD4, Dx5, CD122, B220, CD19 and CD11c staining within gated CD4-CD25+ cells in the pancreas (**B**).

cytometry. **Fig. 6.02** demonstrates that CD4-CD25+ cells expressed numerous T cell markers including CD127, ST2, ICOS, ICAM, CD49d, CD43 and CCR9. We also found expression of cKit and Sca-1 molecules, which are mostly associated with haematopoetic progenitor cells. Although CD4-CD25+ cells expressed MHC class II, they were negative for CD11b and other APC markers (described in **Fig. 6.01 B**). This analysis suggests that CD4-CD25+ cells share the expression of various markers with recently identified innate immune cells such as natural helper cells (NHC), nuocytes, innate helper 2 (Ih2) cells and multi-potent progenitor (MPP) type 2 cells (reviewed in (Neill & McKenzie 2011)).

It has been demonstrated that these novel lineage negative populations were able to modulate type 2 immune responses through their production of IL-4, IL-5, IL-6 and IL-13. We therefore wished to assess the expression of these type 2 cytokines in our pancreatic CD4-CD25+ cells. We decided to use the RAG2-/- strain to exclude any T or B lymphocyte contamination in the pancreas and simplify the gating strategy for CD4-CD25+ cells. Single cell suspensions from RAG2-/- pancreases were therefore stained for surface CD25 and intracellular IL-5 and IL-13. **Fig. 6.03** illustrates that approximately 10% of CD4-CD25+ cells expressed IL-5 and IL-13. Furthermore, we found that these type 2 cytokines were co-expressed by CD4-CD25+ cells.

In order to evaluate CD4-CD25+ cells for the expression of various cytokines by quantitative real time PCR and assess their effects on our functional *in vitro* assays, it was necessary to establish a cell sorting strategy for this population. It has been reported that the novel innate immune cell population expresses CD90, and we therefore decided to incorporate this marker

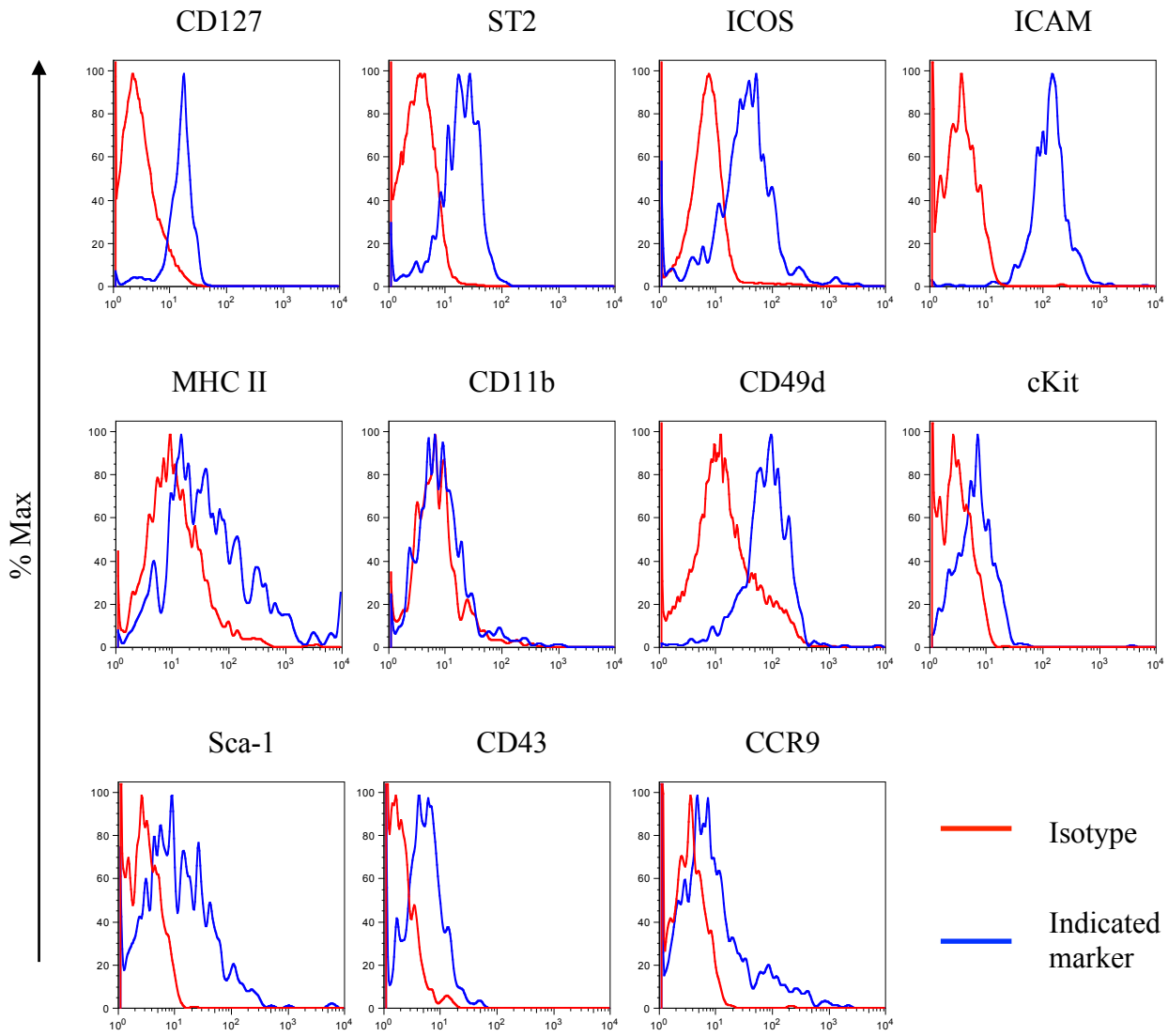


Figure 6.02. Phenotyping of CD4-CD25+ cells in DO11xRIP-mOVA pancreas. Single cell suspensions from DO11xRIP-mOVA pancreas were stained for surface CD4, CD25, CD127, ST2, ICOS, ICAM, MHC II, CD11b, CD49d, cKit, Sca-1, CD43 and CCR9. Representative histograms show expression levels for the indicated markers versus isotype control stains within gated CD4-CD25+ cells.

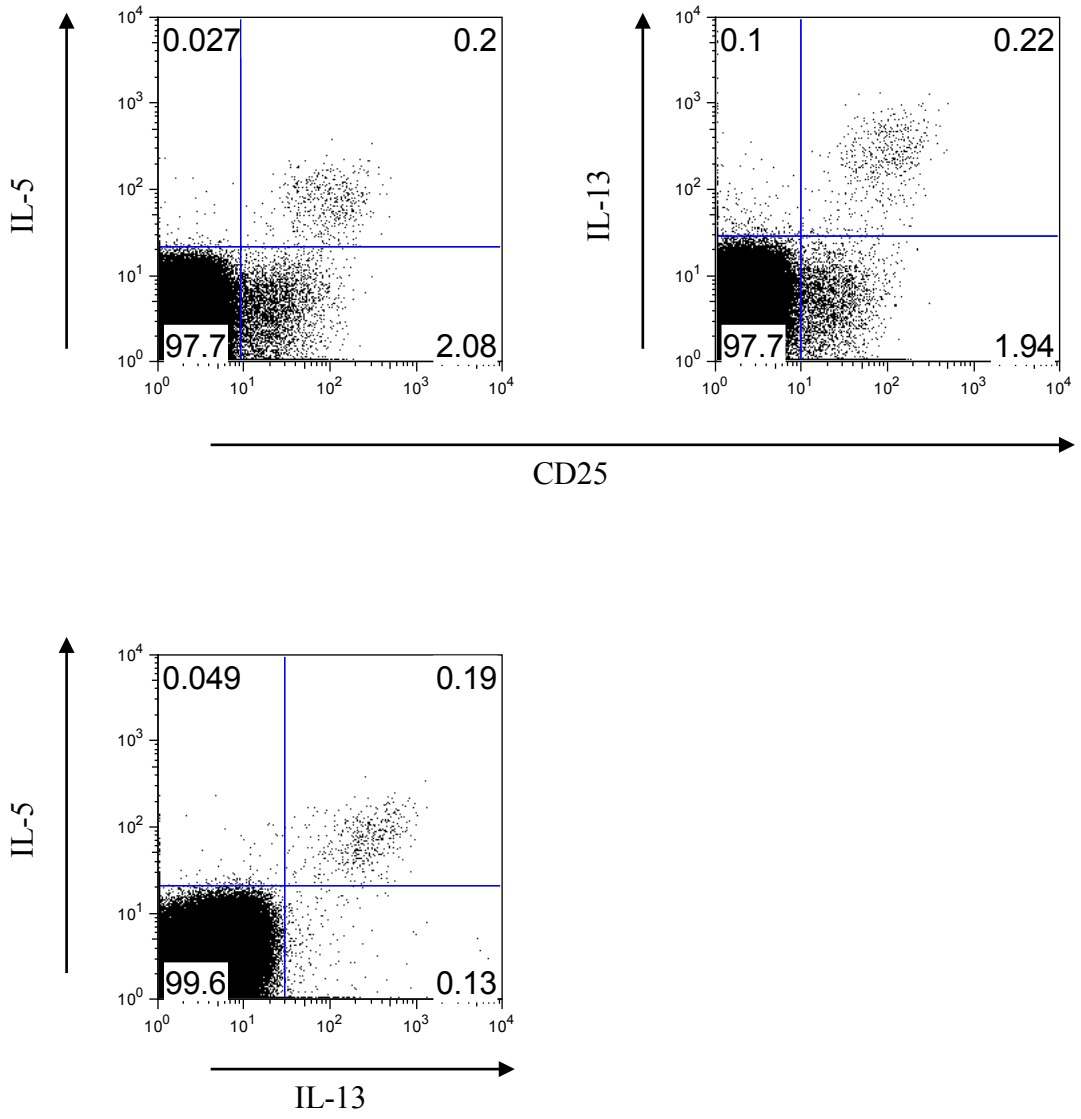


Figure 6.03. Expression of IL-5 and IL-13 in CD4-CD25+ cells. Single cell suspensions from the pancreas of RAG2-/- mice were restimulated and stained for surface CD25 and intracellular IL-5 and IL-13. Representative dot plots show CD25, IL-5 and IL-13 staining in the pancreas.

into our sorting strategy. Thus, single cell suspensions from the RAG2-/- pancreas were analysed for the expression of CD3, CD4, CD25 and CD90.2 by flow cytometry. As demonstrated in **Fig 6.04**, cells isolated from the pancreas were negative for CD3 and CD4, but expressed CD25 (around 2% were CD25+). We observed that a high proportion of CD25+ cells also expressed CD90.2. Thus, we were confident that CD90.2 together with CD25 were good markers for the identification of CD4-CD25+ cells in the pancreas.

Having established a sorting strategy we were now able to evaluate the expression of various cytokines by quantitative real time PCR analysis. To this end, we sorted CD4-CD25+ cells from RAG2-/- pancreases by gating on the CD25+CD90.2+ population and isolated RNA. The expression levels of GM-CSF, IL-5, IL-6, IL-9, IL-10 and IL-13 were assessed by quantitative real time PCR. **Fig 6.05** illustrates that the expression of GM-CSF, IL-6 and IL-5 transcripts were upregulated in these sorts. We were unable to detect mRNA for IL-9, IL-10 and IL-13. This analysis demonstrates that the pancreas-derived CD4-CD25+ cells have the potential to produce GM-CSF, IL-5 and IL-6. The lack of transcript expression for IL-9, IL-10 and IL-13 could either reflect a lack of expression or could reflect a technical failure since we were not able to robustly quality control all of these reagents. The fact that we could detect IL-13 protein in restimulated CD4-CD25+ cells, but not IL-13 mRNA in freshly isolated CD4-CD25+ could therefore either reflect a requirement for cellular stimulation or technical limitations in our quantitative real time PCR assay.

Phenotypic analysis of CD4-CD25+ cells revealed similarities with the newly described type 2 innate immune cells that were mostly distributed throughout tissues associated with gut immunity. Although our flow cytometric analysis was carried out on cells isolated from the

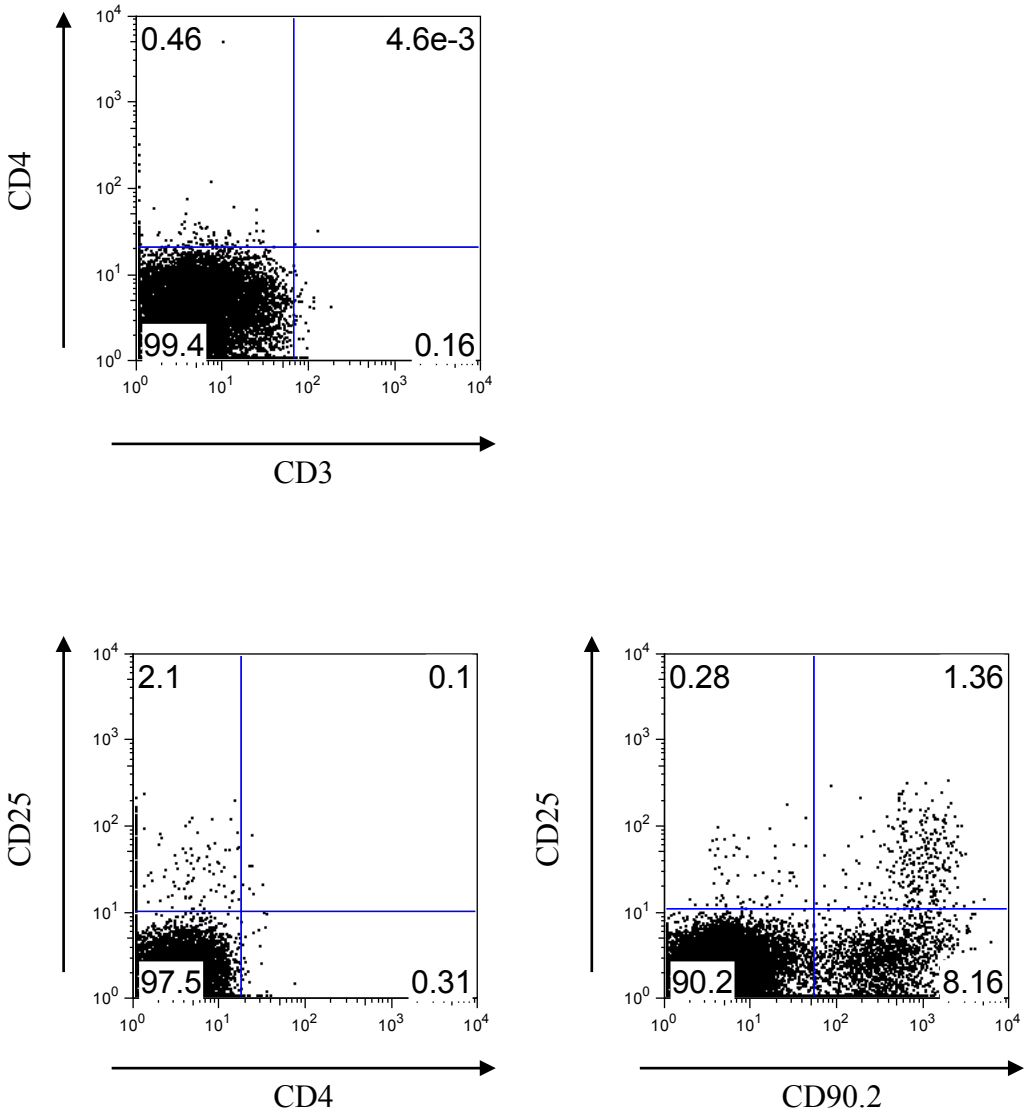


Figure 6.04. Sorting strategy for the isolation of CD4-CD25+ cells from the pancreas of RAG2-/- mice. Single cell suspensions from the pancreas of RAG2-/- mice were stained for surface CD3, CD4, CD25 and CD90.2. Representative dot plots show CD3, CD4, CD25 and CD90.2 staining in the pancreas.

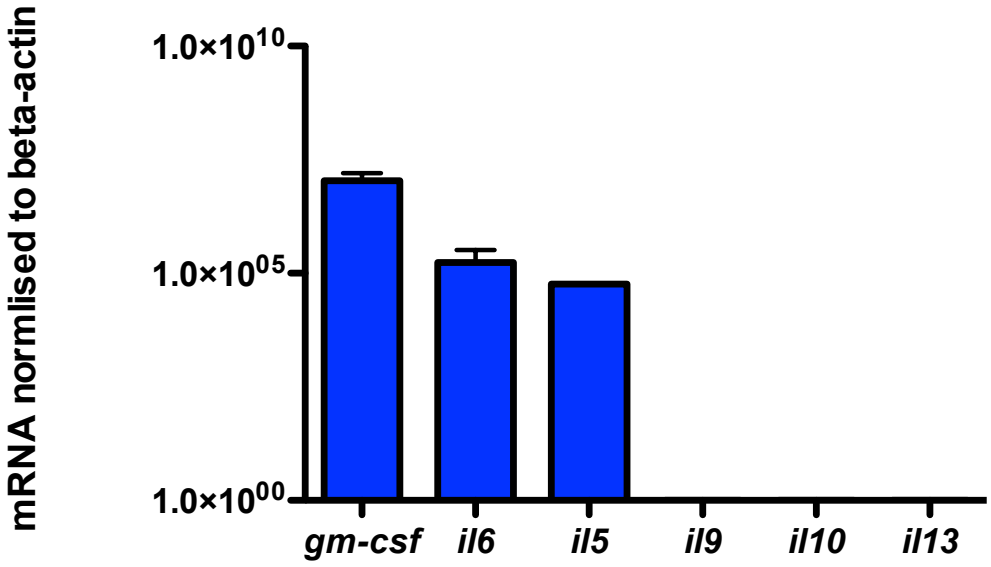


Figure 6.05. CD4-CD25+ cells express mRNA for GM-CSF, IL-5 and IL-6. The expression of GM-CSF, IL-5, IL-6, IL-9, IL-10 and IL-13 in MoFlo sorted CD25+CD90.2+ cells was evaluated by Taqman qPCR. Transcripts were normalised against endogenous beta-actin levels. Graph shows means and SEM across three sorts.

pancreas, this approach did not allow us to identify the location where CD4-CD25+ cells resided within the pancreas. To address this, frozen pancreas sections from RAG2-/- mice were stained for CD25 and insulin to establish the location of CD4-CD25+ cells in relation to the pancreatic islets. Since the CD4-CD25+ population is relatively small, few cells with this phenotype were found on any given pancreas section. However, as shown in **Fig 6.06**, preliminary data suggested that the majority of CD25+ cells (brown) resided at the border of pancreatic islets consisting of insulin-producing β-cells (blue). Thus, it is possible that the CD4-CD25+ cells might modulate the cytokine environment in the vicinity of the pancreatic islets.

6.2.2. CD4-CD25+ cells promote IL-21 production *in vitro*

Previous reports by Moro and Neill indicated that the newly identified innate immune cell population are responsive to IL-7 and IL-33, and these cytokines affect their survival, proliferation and cytokine production (Moro et al. 2010; Neill et al. 2010). We therefore wished to test whether IL-7 and IL-33 signals are important for the survival and proliferation of CD4-CD25+ cells. To this end, we sorted CD4-CD25+ cells from RAG2-/- pancreases (gating on CD25+CD90.2+ cells as previously described) and cultured them in the presence of IL-7 and IL-33 as described by Neill (Neill et al. 2010). After five days cells were counted and the expression of CD25, CD127 and ST2 was assessed by flow cytometric analysis. **Fig.6.07 A** illustrates that cultured CD4-CD25+ cells maintained their expression of CD25 and ST2 but expressed slightly lower levels of CD127. We also found that absolute cell numbers slightly increased after five days of incubation with IL-7 and IL-33 (**Fig. 6.07 B**). This experiment demonstrated that CD4-CD25+ cells expanded *in vitro* in response to IL-7 and IL-33 signals and maintained their expression of the indicated markers.

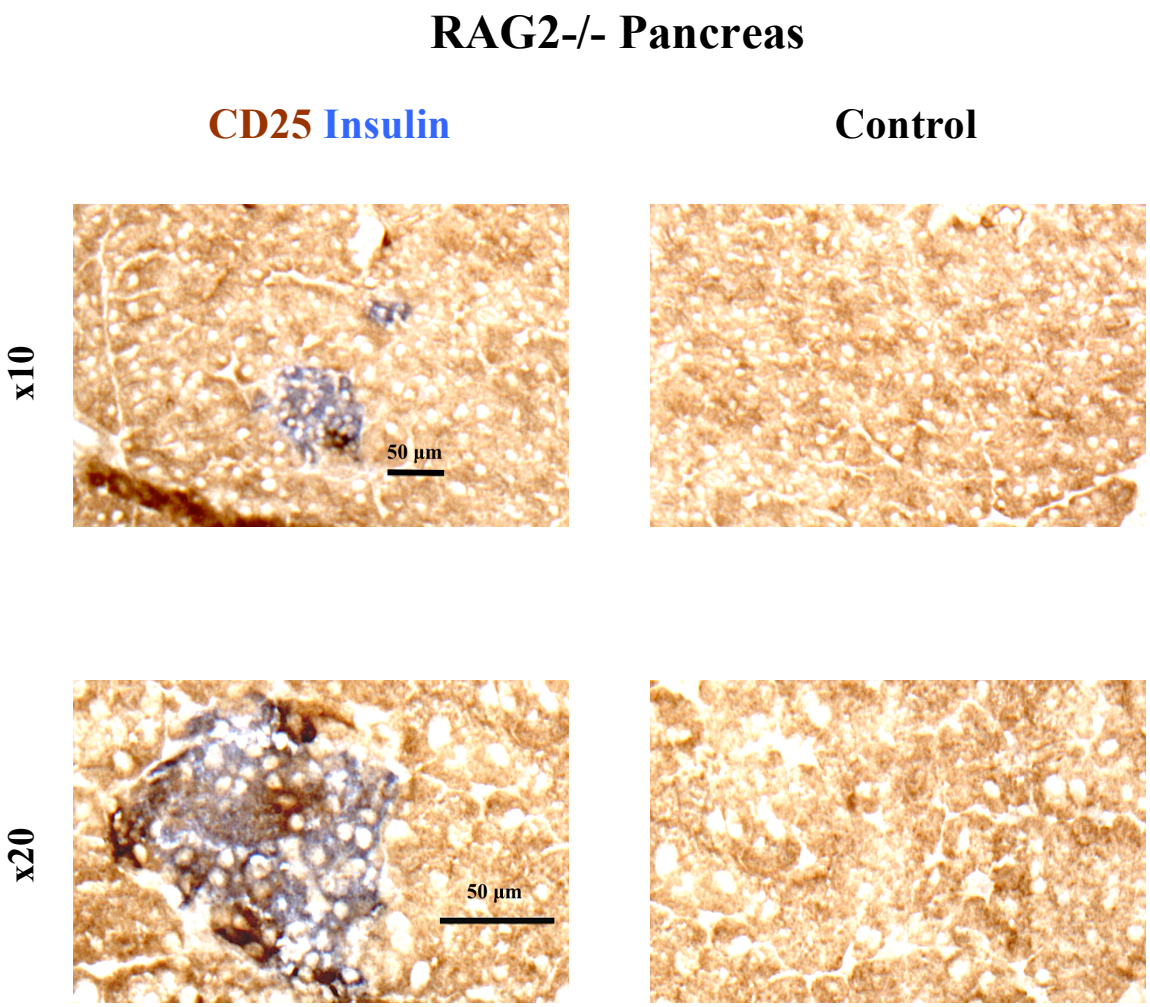


Figure 6.06. Localisation of CD4-CD25+ cells in the RAG2-/- pancreas. Frozen pancreas sections from RAG2-/- mouse were stained for CD25 (brown) and insulin (blue) for analysis by light microscopy.

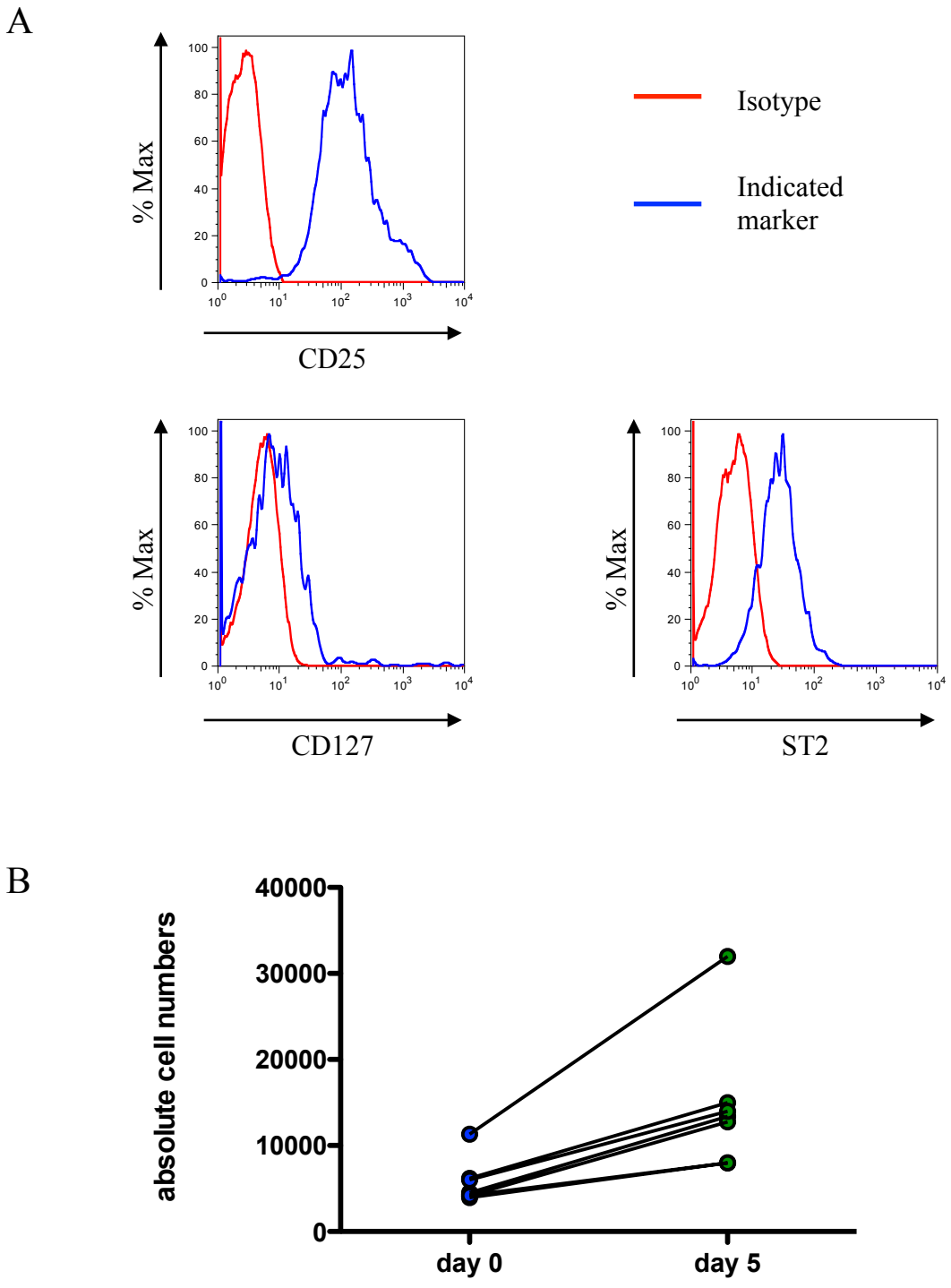


Figure 6.07. Characterisation of CD4-CD25+ cells after culture with IL-7 and IL-33. MoFlo sorted CD25+CD90.2+ cells were cultured for five days with 10ng/ml IL-7 and 10ng/ml IL-33. After five days cells were stained for surface CD25, CD127 and ST2. Representative histograms show CD25, CD127 and ST2 staining in the cultured cells (**A**). Graph shows absolute cell numbers at the start and after five days of culture (**B**).

We have reported in this chapter that CD4-CD25+ cells expressed IL-6 mRNA. Given our previous demonstration that IL-6 can promote IL-21 production, we were interested to explore whether the presence of CD4-CD25+ cells could modulate T cell cytokine production. To address this, we cultured *in vitro* expanded pancreatic CD4-CD25+ cells with CD4+CD25- T cells and anti-CD3/anti-CD28 beads, alone or with IL-7 and IL-33 to ensure survival of the CD4-CD25+ cells. After three days the expression of IL-21 and IFNγ was assessed by flow cytometry. As shown in **Fig 6.08**, the presence of CD4-CD25+ cells promoted IL-21 production by conventional T cells. This was independent of IL-7 and IL-33 as these cytokines failed to induce IL-21. This evidence suggests that CD4-CD25+ cells can provide the signals necessary for the promotion of IL-21. Our *in vitro* analysis of the pathways that modulate IL-21 production revealed that IL-6 was the main inducing factor for its expression. Consequently, we wished to establish whether the ability of CD4-CD25+ cells to promote IL-21 production by T cells was IL-6 dependent. To address this, CD4-CD25+ cells from the pancreas were cultured with CD4+CD25- conventional T cells in the presence or absence of anti-IL-6 antibody. These assays were driven by anti-CD3/anti-CD28 beads and exogenous IL-6 was used as a positive control. **Fig 6.09 A** illustrates that the great proportion of conventional T cells expressed IL-21 when CD4-CD25+ cells were present in the culture, while the addition of the anti-IL-6 antibody inhibited this expression. This suggests that the induction of IL-21 could likely be explained by the presence of endogenously produced IL-6. As demonstrated in the extended data set (**Fig. 6.09 B**), the average frequency of IL-21+ conventional T cells was dramatically reduced after the provision of anti-IL-6 antibody. Interestingly, IL-7 and IL-33 signals promoted the expression of IFNγ, which was substantially decreased when CD4-CD25+ cells were present in the culture. Overall, it

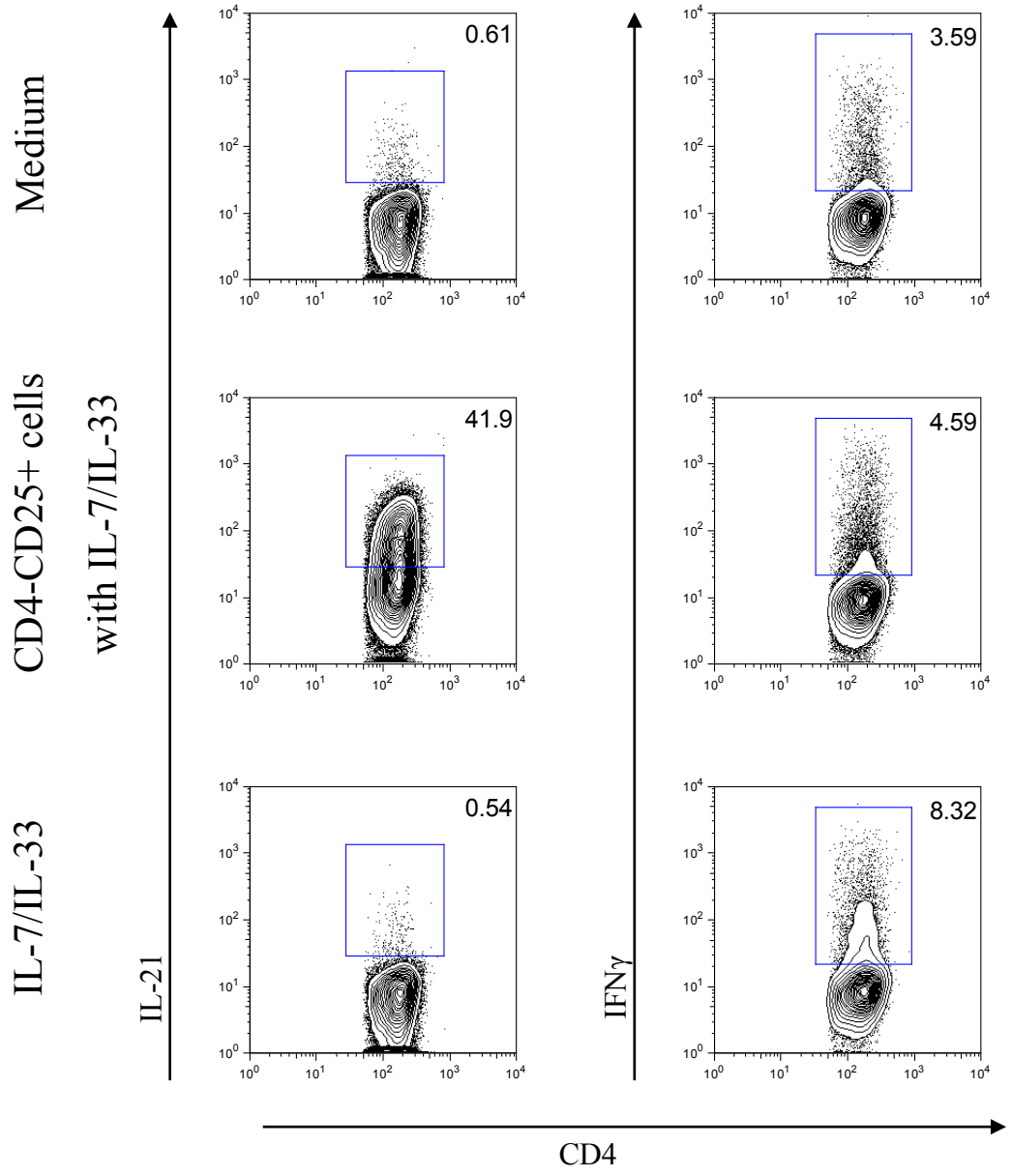


Figure 6.08. T cell cytokine production in the presence or absence of CD4-CD25+ cells. $2.5x10^4$ Tconv were cultured with $2.5x10^4$ anti-CD3/anti-CD28 beads, alone or with $5x10^3$ CD4-CD25+ cells (previously cultured for five days with 10ng/ml IL-7 and 10ng/ml IL-33). Where indicated the co-cultures were supplemented with 10ng/ml IL-7 and 10ng/ml IL-33. After three days cells were restimulated and stained for surface CD4 and intracellular IL-21 and IFNγ. Representative contour plots show expression profiles for IL-21 and IFNγ within gated CD4+ cells.

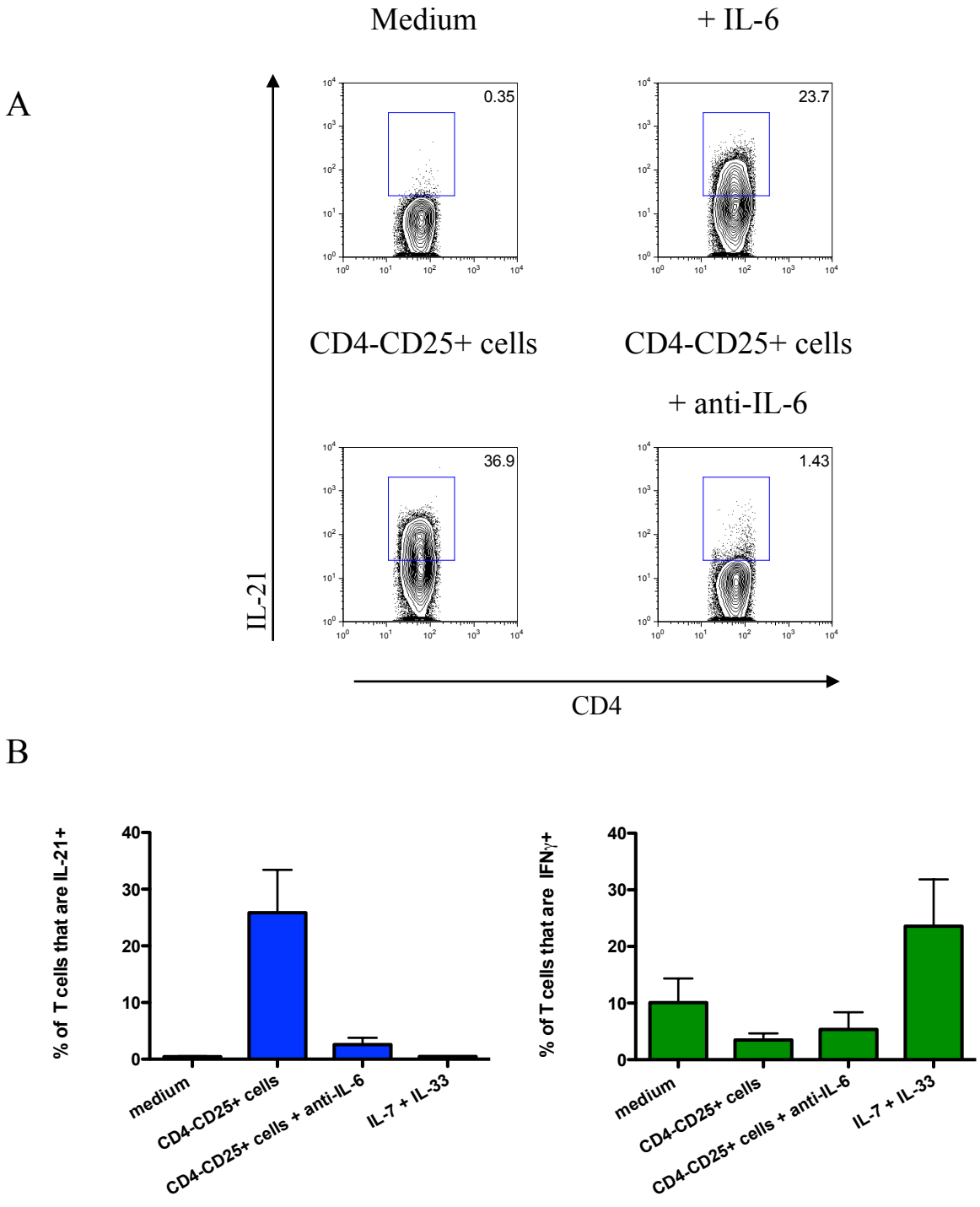


Figure 6.09. The effect of IL-6 blockade on Tconv cytokine production in co-cultures with CD4-CD25+ cells. $2.5x10^4$ Tconv were cultured with $2.5x10^4$ anti-CD3/anti-CD28 beads, alone or with $5x10^3$ CD4-CD25+ cells (previously cultured for five days with 10ng/ml IL-7 and 10ng/ml IL-33) or 100ng/ml IL-6. The co-cultures with CD4-CD25+ cells were supplemented with 10ng/ml IL-7 and 10ng/ml IL-33, alone or together with 10μg/ml anti-IL-6. After three days cells were restimulated and stained for surface CD4 and intracellular IL-21 and IFNγ. Representative contour plots show expression profiles for IL-21 (**A**). Graphs show collated data for IL-21 and IFNγ expression within gated Tconv (**B**). Bars represent means across four experiments.

appears that endogenous IL-6 was present in the cultures with CD4-CD25+ cells and that this cytokine promoted the production of IL-21.

6.3. Discussion

Numerous reports have demonstrated that the entry of a mixed population of leukocytes to the pancreas is associated with β-cell death and the onset of diabetes (reviewed in (Lehuen et al. 2010)). Our work suggests that that B cells, Tconv and Treg are found in the pancreatic infiltrate of the DO11xRIP-mOVA mice and therefore have the potential to modulate the disease process (Clough et al. 2008; Ryan et al. 2010). Whilst investigating the role of CD4+CD25+ Treg in the pancreas, we have identified a cell population that expresses high levels of CD25 but does not belong to the CD4 lineage (**Fig. 6.01 A**). To gain a better appreciation of the phenotype of these CD4-CD25+ cells, we further investigated their expression of lineage markers. Our initial analysis revealed that CD4-CD25+ cells were devoid of the T cell marker CD3 and the NK cell marker Dx5 (**Fig. 6.01 B**). This unknown cell subset was further negative for CD19, B220 and CD11c, and was therefore not associated with the B cell or DC lineages, respectively (**Fig. 6.01 C**).

In recent years numerous groups have reported the existence of a lineage negative population of cells that have recently been named innate lymphoid cells (ILC) (Spits et al. 2013). Based on their cytokine production, ILC were further divided into 3 subclasses: ILC1 (IFNγ-expressing), ILC2 (IL-5-, IL-9- and IL-13-expressing) and ILC3 (IL-22-expressing) (Walker et al. 2013). Intriguingly, Moro and colleagues have reported a population of cells residing in mesenteric lymphoid clusters that were lineage negative and expressed the surface markers cKit, Sca-1, CD25, CD127, ST2 and ICOS (Moro et al. 2010). These cells, identified as ILC2, controlled helminth infection though their high production of IL-13, and were also capable of releasing IL-5 and IL-6 *in vitro*. Similarly, Neill *et al.* have identified lineage-negative cells that produce large amounts of IL-13 and expand *in vivo* in response to exogenous IL-25 and

IL-33 (Neill et al. 2010). Their phenotype was defined by the presence of the surface molecules ICOS, Thy1.2, CCR9, CD-127, ST2 and IL-17BR. These cells were found across a variety of different tissues including the mesenteric lymph node, intestines, spleen, bone marrow and blood, and modulated responses to helminth infection through the production of additional type 2 cytokines (IL-4 and IL-5). Subsequently, more evidence has emerged suggesting that this ILC subset is strongly associated with type 2 immunity in response to IL-25 and IL-33 (Saenz et al. 2010; Price et al. 2010).

On the basis of the above data, we wished to phenotype the lineage-negative cells in the pancreas of DO11xRIP-mOVA mice for a panel of cell surface markers. Our analysis revealed that the CD4-CD25+ cells expressed high levels of CD127 and ST2, the receptors for IL-7 and IL-33, respectively (**Fig. 6.02**). These cells were also characterised by their expression of CCR9, ICAM and CD49d. These molecules are known to mediate recruitment and entry into tissues, which might potentially explain why CD4-CD25+ cells were found in the pancreas. Furthermore, we found these cells demonstrated expression of cKit and Sca-1, which are key markers of the ILC2 population (Moro et al. 2010). Although our pancreatic CD4-CD25+ cells were devoid of molecules specific for APC, they expressed moderate levels of MHC class II. It therefore appears that the lineage-negative CD25+ cells we identified in the pancreas shared a number of surface markers associated with the ILC2 population.

What remained unclear from the above experiments was whether the CD4-CD25+ cells found in the pancreas were able to produce type 2 cytokines, an integral characteristic of type 2 ILC. Solid evidence supports the notion that ILC2 mediate type 2 responses through high-level production of IL-5 and IL-13 (reviewed in (Walker et al. 2013)). To establish whether the

cells found in the pancreas produced type 2 cytokines, we opted to use the RAG2-/- strain, which are depleted of adaptive immune cells, including B and T cells. These experiments confirmed that CD4-CD25+ cells, like ILC, were of innate origin. Furthermore, a substantial fraction of lineage negative cells in the pancreas produced IL-5 and IL-13 (**Fig. 6.03**). Our data therefore suggests that in addition to previously reported sites (gut and airways), a linage-negative population might potentially modulate type 2 responses in the pancreas.

We next wished to develop a sorting strategy with the aim of isolating CD4-CD25+ cells from the pancreas. Introducing the ILC-specific CD90.2 marker allowed us to purify the CD25+CD90.2+ cells from the RAG2-/- pancreas (**Fig. 6.04**). We initially performed PCR analysis on these sorted cells to check for their expression of mRNA for other ILC-derived soluble factors. This investigation revealed that the pancreas-sorted cells expressed transcripts for GM-CSF (**Fig. 6.05**). The same cytokine was found in the supernatant of cultured cKit+Sca-1+ ILC isolated from mesenteric lymphoid clusters (Moro et al. 2010). In addition, sorted cells also expressed mRNA for IL-6, another soluble factor associated with the ILC subset (Neill et al. 2010; Moro et al. 2010). Our investigation implies that CD4-CD25+ cells isolated from the pancreas share their cytokine repertoire with group 2 ILC.

It was important at this stage to establish the location of CD4-CD25+ cells within the pancreas. To achieve this we again utilised the RAG2-/- strain, and stained for insulin and CD25 in frozen pancreas sections. Histologic analysis suggested that CD25+ cells were found within the insulin-producing islets, where they might potentially modulate immune responses in settings of autoimmune diabetes (**Fig. 6.06**).

We have previously demonstrated that IL-6 strongly induced IL-21, and the latter cytokine was enriched in the DO11xRIP-mOVA pancreas. Given that CD4-CD25+ cells were a source of IL-6 mRNA, it was conceivable that these cells might modulate the production of T cell-produced IL-21 within pancreatic islets. To address this, we sorted CD4-CD25+ cells from the RAG2-/- pancreas and expanded this population *in vitro*. Studies by Neill *et al.* demonstrated that not only did ILC2 cells express CD127 and ST2, but they also expanded preferentially in the presence of IL-7 and IL-33 *in vitro* (Neill et al. 2010). We found that the CD4-CD25+ cells proliferated moderately in response to an IL-7/IL-33 cytokine cocktail, and maintained their expression of CD25, CD127 and ST2 (**Fig. 6.07**). We next wished to assess whether these cells could modulate T cell responses *in vitro*. Our data are still fairly preliminary at this stage, but suggested that the presence of CD4-CD25+ cells in co-cultures promoted IL-21 production by T cells (**Fig. 6.08**). This observation could not be attributed to exogenous IL-7 and IL-33, as addition of this cytokine cocktail alone failed to induce IL-21 expression. Given the fact that CD4-CD25+ cells expressed mRNA for IL-6, our next aim was to establish whether this cytokine was responsible for the induction of IL-21. We found that IL-21 expression was strongly inhibited in cultures of T cells with CD4-CD25+ cells when anti-IL-6 antibody was added (**Fig. 6.09**). This suggested that IL-21 production by T cells was most likely induced by IL-6 produced by CD4-CD25+ cells. Thus, our investigation found that a novel population of innate lymphoid cells was capable of modulating immune responses in the pancreas through the release of cytokines. This cell subset might be of particular significance for the dysregulated immune system within the pancreatic islets in type-1 diabetes.

In the short term, it will be essential to assess the expression of the transcription factors RORα and GATA-3 in the CD4-CD25+ cells isolated from the pancreas, as these have been shown to be critical for the development of group 2 ILC. It will also be important to investigate the *in vivo* function of CD4-CD25+ cells. Initially, it will be interesting to transfer sorted CD4-CD25+CD90.2 cells into CD90.1 recipients, to assess the homing ability of these cells to the pancreas and lymphoid tissues. Furthermore, it will be interesting to test the ability of CD4-CD25+ cells to modulate immune responses in T1D. This can be achieved by utilising CD90.1+ DO11 T cells transferred into CD90.2+ RIP-mOVA/RAG2-/- recipients after depletion of host CD4-CD25+ cells using anti-CD90.2 antibody. In this experiment, disease progression would be monitored by taking blood glucose readings, and T cell function would be evaluated by intracellular cytokine staining.

7. GENERAL DISCUSSION

The main function of an appropriate immune response is to protect the host from pathogens whilst maintaining self-tolerance. Occasionally, the fine-tuning of the response might be lost, such that the ability to distinguish self from non-self is compromised. This leads to autoimmunity and inflammation involving a wide repertoire of immune cells. The major aim of this thesis was to investigate the early events that are associated with the organ-specific autoimmunity observed in the DO11xRIP-mOVA mouse model of T1D.

We and others have demonstrated that IL-21 is an important soluble mediator involved in the development of autoimmune diabetes (Spolski et al. 2008; Sutherland et al. 2009; Clough et al. 2008). Furthermore, the studies carried out by our group have revealed the strong immunomodulatory properties of this cytokine. We have previously shown that IL-21 is capable of releasing Tconv from Treg mediated suppression (Peluso et al. 2007; Clough et al. 2008). Our lab further demonstrated that IL-21 signalling to Tconv was associated with reduced IL-2 production by this population, thereby removing cytokine support for the Treg population (Attridge et al. 2012). While the decreased availability of IL-2 had a negative impact on Treg homeostasis, it had little effect on Tconv responses as these were successfully maintained by IL-21. Thus, T cell IL-21 production in the pancreas of DO11xRIP-mOVA mice, which we observed in this study, might play an important role in permitting autoreactive T cells to escape from the natural regulatory mechanisms that normally keep them in check.

In this thesis, we also identified IL-6 and IL-21 feedback as major pathways that support T cell IL-21 production in responses initiated by antigen presenting cells such as dendritic cells and B cells. Evidence suggests that IL-6 and IL-21 are strongly associated with numerous autoimmune disorders in which the disruption of IL-21 signalling often prevents disease. In rheumatoid arthritis, blockade of the IL-21 pathway improved disease symptoms in two distinct animal models of the disease (Young et al. 2007). Disease severity scores in the collagen-induced arthritis model were diminished after DBA/1 mice were treated with a murine IL-21 receptor Fc fusion protein (IL-21R.Fc). Similarly, the blockade of IL-21 signalling with IL-21R.Fc reversed adjuvant-induced arthritis in Lewis rats. Early studies in DBA/1 mice and other animal models of rheumatoid arthritis have established that IL-6 was excessively produced at sites of inflammation (Takai et al. 1989; Sugita et al. 1993). In light of our data it is possible that IL-6 may be responsible for the promotion of IL-21 responses in animal models of RA. Furthermore, Campbell *et. al* have identified IL-6 as one of the cytokines that might contribute to the development of T1D (Campbell et al. 1991). Increased levels of IL-6 have been detected in the pancreatic islet cultures isolated from diabetic NOD/Wehi mice, in which disease was induced by cyclophosphamide. This finding suggests that IL-6 may be readily available in the pancreas, and might therefore promote the IL-21 signals that have been associated with autoimmune diabetes in the NOD model (Spolski et al. 2008; Sutherland et al. 2009). The excessive production of IL-6 was also detected in sera from MRL/lpr lupus prone mice, and this increase was age-related (Tang et al. 1991). More recently, Herber and colleagues demonstrated that the blockade of IL-21 in MRL-Fas^{lpr} mice ameliorated disease symptoms (Herber et al. 2007). In another lupus model, the loss of IL-21R signalling was shown to protect BXSB-Yaa mice from disease (Bubier et al. 2009). These animals also demonstrated impaired IL-21 production by CD4+ICOS+ T cells in the

spleen, consistent with the autocrine feedback control of IL-21 production described herein. Collectively, these studies suggest that induction of IL-21 by IL-6 may be a factor in the pathogenesis of several autoimmune disorders, and that IL-6 might therefore be an upstream target for therapeutic intervention in diseases where IL-21 has a known pathogenic role.

Initial studies by the Kishimoto group identified IL-6 as a key modulator of B cell differentiation and antibody production (Muraguchi et al. 1988). The work in this thesis has established that this cytokine is also a major driver for IL-21 production in the context of APC stimulation. In recent years, IL-21-expressing Tfh cells have been shown to play a critical role in the development of germinal centres, providing a specialised environment for B – T cell interactions that lead to the generation of memory B cells and plasma cells (reviewed in (Vinuesa et al. 2009)). Interestingly IL-6 has been identified as a key signal for promoting Tfh differentiation in the context of chronic viral infection (Harker et al. 2011). Harker and colleagues identified two separate peaks of IL-6 production in the response to lymphocytic choriomeningitis virus (LCMV) challenge, and showed that the late wave of IL-6 improved Tfh responses and was critical for viral control. Given our own findings, perhaps the enhanced antibody responses observed during LCMV infection were partially dependent on the IL-6-induced release of IL-21 by Tfh cells. This report also suggested that follicular DC were the main source of IL-6, which is intriguing as the DC used in our studies promoted IL-21 production in part via IL-6.

Eto and colleagues have demonstrated that the synergistic effects of both IL-6 and IL-21 were required for optimal Tfh development, however the generation of germinal centre B cells is predominantly controlled by IL-21 (Eto et al. 2011). Interestingly, the studies in this thesis

have shown a requirement for the combined effect of IL-6 and IL-21 signalling for optimal T cell IL-21 production, which was impaired in IL-21R-/- T cells.

Our group has found a population of Tfh cells in the pancreatic LN of DO11xRIP-mOVA mice (our unpublished data). Although staining for Tfh markers such as CXCR5, PD-1 and IL-21 together was technically problematic, it seems plausible that some of the IL-21-producing T cells in the pancreas-draining LN might represent the Tfh subset. Elegant studies using IL-21 reporter mice showed that, after immunisation, a great majority of IL-21-producing T cells expressed lineage markers associated with Tfh cells (Lüthje et al. 2012).

In the last chapter of this thesis, we described a lineage-negative cell subset that resided in the pancreas and expressed type 2 cytokines. These CD4-CD25+ cells shared many characteristics with the newly identified group 2 ILC. It is somewhat intriguing that although T1D is strongly associated with type 1 responses, we found a population of cells located at the site of autoimmune destruction that are capable of releasing IL-5 and IL-13. To date several groups have shown the importance of ILC2 in gut pathology, however little was known about whether these cells were able to modulate T cell responses (reviewed in (Walker et al. 2013)). Our studies suggest that the pancreas-resident CD4-CD25+ cells can induce IL-21 production by T cells, most likely by providing IL-6. This implies that the lineage-negative population in the pancreas might influence emerging autoimmune responses by altering T cell cytokine production. In the future it will be interesting to perform *in vivo* studies to determine the role of the CD4-CD25+ cells in modulating the progression of T1D.

In summary, the pleiotropic effects of IL-21 have been associated with numerous autoimmune disorders including T1D. Understanding which signals trigger IL-21 production, and which cell types are responsible for providing those signals, will ultimately inform strategies to manipulate this axis therapeutically in the setting of autoimmunity.

APPENDIX

P2

Phosphate buffered saline (PBS) (Sigma)

2% foetal calf serum (FCS) (Sigma)

C10

RPMI 1640 (GIBCO)

100U/ml penicillin (GIBCO)

100µg/ml streptomycin (GIBCO)

50µM 2-mecaptoethanol (Sigma)

10% FCS (Sigma)

MACS Buffer

PBS (Sigma)

0.5% FCS (Sigma)

2mM EDTA (Sigma)

Lysis Buffer

15mM TRIS hydrochloride

112mM ammonium chloride

pH 7.2

Pancreas Buffer

PBS (Sigma)

5% FCS (Sigma)

50mM 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 deoxyribonuclease (Sigma)

LIST OF REFERENCES

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