Role of the CTLA-4 Receptor in Regulatory T Cell Development, Homeostasis and Function

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Abstract

Autoimmunity can occur when self-reactive lymphocytes of the adaptive immune system are activated upon encounter with antigen. This can lead to the development of debilitating and potentially life-threatening autoimmune diseases such as type-1 diabetes, rheumatoid arthritis and multiple sclerosis. Regulatory T cells (Tregs) are a subset of CD4+ T cells that express the lineage-specific transcription factor *Foxp3* and exert dominant peripheral tolerance to maintain immune homeostasis. It is therefore important to fully understand the underlying mechanisms of Treg development, homeostasis and function due to the positive and negative effects that therapeutic manipulation could have on this essential T lymphocyte population.

Many effector molecules have been proposed to have a central role in regulatory T cell function, and it is now clear that Tregs are equipped with multiple mechanisms by which to exert suppressive function. It has been reported that the cytotoxic T lymphocyte antigen-4 (CTLA-4) receptor is constitutively expressed by regulatory T cells and a role for this molecule in Treg suppression has been suggested.

This investigation revealed a role for CTLA-4 in maintaining homeostasis of the peripheral regulatory T cell compartment. In addition, using a transgenic mouse model that permitted the development of antigen-specific *Ctla-4*-deficient Tregs, a role for the CTLA-4 receptor in Treg suppressive function was identified. The data obtained suggest that the CTLA-4 receptor may function on regulatory T cells by modulating CD80/CD86 co-stimulatory molecule expression by antigen-presenting cells, and hence their capacity to activate conventional T cells to generate effector T cells and instigate an effective immune response.

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List of Abbreviations

ADP……..adenosine diphosphate
AIRE…….autoimmune regulator
AMP……...……....adenosine monophosphate
APC……..antigen-presenting cell
ATP……..adenosine triphosphate
BCR……...B cell receptor
BM……...bone marrow
BrdU……5-bromo2'-deoxy-uridine
CD……..cluster of differentiation
cDNA……complimentary deoxyribonucleic acid
CFSE……....carboxyfluorescein succinimidyl ester
CHO……..chinese hamster ovary
CNS……..central nervous system
Cre…….Cre recombinase
cTEC……..cortical thymic epithelial cell
CTL……cytotoxic T lymphocyte
CTLA-4……..cytotoxic T lymphocyte antigen-4
CXCR……..CXC chemokine receptor
DC……dendritic cell
DNA……...deoxyribonucleic acid
EAE……..experimental autoimmune encephalomyelitis
Ebi…….Epstein-Barr virus-induced gene
Fig.……...figure
Floxed……flanked by LoxP
FOXP3……...forkhead box P3
GALT……..gut-associated lymphoid tissue
GATA3……GATA binding protein 3
GC……germinal centre
GFP……...green fluorescent protein
GITR……..glucocorticoid-induced tumor necrosis factor receptor

GM-CSF……..granulocyte macrophage colony-stimulating factor
IBD……..inflammatory bowel disease
ICOS…….inducible T cell co-stimulator
IDO……indoleamine 2,3-dioxygenase
IFN-γ……interferon-gamma
Ig……immunoglobulin
IL……interleukin
IPEX……..immunodysregulation polyendocrinopathy enteropathy X-linked
L……..ligand
LFA-1……lymphocyte function-associated antigen-1
LN……..lymph node
LPS…….lipopolysaccharide
MFI……..median fluorescent intensity
MHC……...major histocompatibility complex
mRNA……...messenger ribonucleic acid
MS…….multiple sclerosis
mTEC……medullary thymic epithelial cell
NK……natural killer
NOD……non-obese diabetic
Nrp-1…….neuropilin-1
OPG…….osteoprotegerin
OVA…….ovalbumin
PAMP……..pathogen-associated molecular pattern
PBS……..phosphate buffered saline
PCR……polymerase chain reaction
PD……...programmed death
PMA…….phorbol myristate acetate
PRR……..pattern-recognition receptor
qPCR……..quantitative polymerase chain reaction
R……...receptor
r…….recombinant
RA…….rheumatoid arthritis

Rag2……...recombination activating gene 2
RANK…….receptor activator of nuclear factor κ B
RIP……rat insulin promoter
RNA……ribonucleic acid
RORγt…….retinoic acid-related orphan nuclear hormone receptor-gamma t
RUNX…….runt-related transcription factor
SCID……...severe combined immunodeficiency
sCTLA-4……soluble cytotoxic T lymphocyte antigen-4
SLE……systemic lupus erythematosis
STAT5…….signal transducer and activator of transcription 5
T1D…….type-1 diabetes mellitus
T-bet……...T-box transcription factor 21
Tconv…….conventional T cell
TCR……...T cell receptor
Teff……effector T cell
TFH……T follicular helper
TGF-β……transforming growth factor-beta
T_H1…….type 1 helper T cell
T_H2…….type 2 helper T cell
T_H3…….type 3 helper T cell
T_H17…….helper T cell that produces IL-17
TLR……toll-like receptor
TNF-α……...tumor necrosis factor-alpha
Tr1……...type 1 regulatory T cell
TRANCE……..tumor necrosis factor-related activation-induced cytokine
Treg……regulatory T cell
WASp…….. …….Wiskott-Aldrich syndrome protein
WT……wild type

1.0 Introduction

1.1 Immunity

All organisms are at risk of infection and disease caused by invasion of unwanted, foreign pathogens. Therefore, various mechanisms of immunity have evolved to provide protection. The vertebrate immune system is a highly effective biological defence mechanism; potentially pathogenic microorganisms are continually encountered on a daily basis, but illness is a relatively rare occurrence. Immunity in vertebrates consists of complex, highly regulated interactions between many types of tissues, cells and proteins, which are divided into two parts: the innate and the adaptive immune responses.

1.1.1 The Innate Immune System.

Innate immunity is a basic, first line of defence against infection. Innate defence mechanisms actively exist within an organism prior to encounter with pathogens, and therefore act quickly and efficiently to eliminate invading microorganisms and prevent infection. The physical and chemical mechanisms of the mammalian innate immune system are evident throughout the evolution of multicellular organisms. The epithelial surfaces of the skin and internal mucosa provide a physical barrier to infection. Additionally, pathogen invasion is inhibited by the secretion of anti-microbial enzymes and peptides in, for example, saliva and tears [1]. If pathogens do manage to overcome the physical epithelial barrier and enter the organism this can trigger the activation of an enzymatic cascade known as the complement system [2]. Effector functions of complement include the direct lysis of bacteria- or virus-infected cells and promotion of pathogen clearance by the phagocytic cells of the innate immune system.

In addition to physical and chemical elements, there is also a cellular component of innate immunity. Cells of the innate immune system are leucocytes of myeloid origin; this includes macrophages, neutrophils and dendritic cells (DCs) which ingest and destroy invading pathogens by phagocytosis. Innate immune cells recognise conserved structures which are common to many pathogens, but not found on host cells [3]. These pathogen-associated molecular patterns (PAMPs) are identified by pattern-recognition receptors (PRRs) on the surface of innate leucocytes [4], for example, toll-like receptors (TLRs) [5]. Innate immunity

depends on invariant, germ-line encoded receptors which trigger an almost immediate attack on pathogens. However, infectious microorganisms are not always successfully eliminated by the innate immune system because pathogens evolve to escape detection by the genome-specified pattern-recognition receptors. When innate immunity is bypassed, evaded or overwhelmed, there is also the adaptive immune response to control infection.

1.1.2 The Adaptive Immune System.

Adaptive immunity has evolved to overcome the constraints of innate immunity in that a specific immune response is generated to a particular pathogen and results in immunological memory to provide enhanced protection against re-infection. The adaptive immune system consists of leucocytes of lymphoid origin, namely T lymphocytes and B lymphocytes. The importance of adaptive immunity is illustrated by the severe immuno-compromised phenotypes associated with defects in the adaptive immune system [6].

The clonal selection theory, first hypothesised by Macfarlane Burnet in the 1950s, describes the basic principle of the adaptive immune response [7]. Each lymphocyte has cell-surface antigen-receptors of a single specificity, and upon encounter with its specific antigen the particular lymphocyte is activated to proliferate. This generates many progeny cells, all with the same cell-surface antigen-receptors specific for a particular pathogen, which differentiate into either effector lymphocytes to eliminate the infection or memory lymphocytes that are primed to produce a rapid response if the particular pathogen is encountered again. In order to generate a population of individual, specific lymphocytes capable of recognising diverse pathogens, the cell-surface antigen-receptors are not germ-line encoded but formed by the random recombination of variable receptor gene segments [8].

The adaptive immune system is initiated by the innate immune system upon infection by a pathogen. Peripheral lymphoid organs such as the spleen and lymph nodes (LNs) are highly organised structures specialised to promote the interaction of innate immune cells with lymphocytes to activate the adaptive immune response. Dendritic cells are long-lived, specialised phagocytic cells present in most tissues where they provide surveillance against infecting pathogens. In addition to pattern-recognition receptor-mediated phagocytosis of pathogens, dendritic cells continually sample the local extracellular environment by the

receptor-independent process of macropinocytosis. Uptake of a pathogenic agent activates tissue-resident dendritic cells to migrate to nearby draining lymph nodes, mature into a specialised antigen-presenting cell (APC) and interact with re-circulating T lymphocytes, thereby initiating an adaptive immune response [9].

1.1.3 T Lymphocytes.

All lymphocytes originate from haematopoietic stem cell progenitors in the bone marrow. T cell precursors then migrate to the thymus where they undergo maturation into naïve T lymphocytes [10], which are then exported to re-circulate in the peripheral blood and lymphatic system. Two broad classes of T lymphocytes develop in the thymus from a common CD4+CD8+ precursor; CD4+ helper T cells which activate other cells of the immune system, and CD8+ cytotoxic T cells that destroy virally infected cells.

T cell receptors (TCRs) recognise antigens as peptide fragments displayed on the surface of antigen-presenting cells, such as dendritic cells. Antigen-presenting cells process antigens which are then delivered to the cell surface and presented by specialised glycoproteins called major histocompatibility complex (MHC) molecules that bind the CD4 and CD8 co-receptors on T cells. CD8+ T cells are MHC class I restricted, which is expressed on all nucleated cells and displays endogenously derived peptides from intracellular pathogens infecting the cell. CD4+ T cells are MHC class II restricted which displays exogenously derived peptides from extracellular pathogens [11]. However, the binding of an antigen-specific T cell receptor and CD4 or CD8 co-receptor to a peptide:MHC complex is not sufficient for naïve T cell activation. A second, independent co-stimulatory signal is also required. The antigen-presenting cell displaying the peptide:MHC complex simultaneously expresses the ligands, CD80 and CD86, that interact with the co-stimulatory receptor CD28 on the T cell surface to deliver an activation signal alongside the antigen-specific signal via the T cell receptor [12].

Upon full T cell activation following T cell receptor stimulation by antigen and CD28-mediated co-stimulation, naïve T cells differentiate into effector T cells (Teff). Effector T cells can be classified into functional groups that detect antigen from different types of pathogen and therefore eliminate it accordingly. Activated CD8+ T cells

differentiate into cytotoxic T lymphocytes (CTLs) and have the ability to kill infected target cells. Apoptosis of an infected target cell is induced by one of two main mechanisms [13]. Cytotoxic T lymphocytes can release cytotoxins; perforin disrupts the target cell membrane allowing entry of granzyme. Alternatively, CD8+ T cells can express the FAS ligand which interacts with the death receptor FAS on the target cell. To ensure that cytotoxic T lymphocytes only target infected cells, exogenously derived peptide antigens are excluded from the MHC class I pathway [14]. CD4+ helper T lymphocytes produce cytokines to recruit and activate other immune cells. T_H1 CD4+ T cells express the transcription factor T-bet and secrete interferon-gamma (IFN-γ) to activate macrophages as part of an inflammatory response [15], whereas production of interleukin-4 (IL-4), IL-5 and IL-13 by GATA3-expressing T_H2 CD4+ T cells initiates a B cell-mediated humoral response [16]. More recently, an additional CD4+ helper T cell subset has been identified to be involved in orchestrating the inflammatory response. T_H17 cells express the transcription factor RORγt and produce the cytokines IL-17, IL-21 and IL-22 [17, 18]. The T_H17 immune response is thought to protect against fungal infections and Gram-negative bacteria through the recruitment and activation of neutrophils [19].

It takes several days for clonal expansion and differentiation into effector T cells, therefore an important element of the adaptive immune response is the generation of immunological memory. During the differentiation of effector T lymphocytes, CD4+ and CD8+ memory T cells are also generated. Memory T cells are long-lived and ensure the rapid instigation of a secondary, antigen-specific adaptive immune response upon subsequent infection with the same pathogen [20].

1.1.4 B Lymphocytes.

In addition to the cellular T lymphocyte response, adaptive immunity consists of the humoral immune response in which antibody-producing B cells protect the extracellular spaces from pathogenic infection. B lymphocytes, like T cells, originate from haematopoietic stem cell progenitors in the bone marrow. However, unlike T cells, B cells remain in the bone marrow to mature. Antigen recognition activates B cells to differentiate into plasma cells that secrete antibody; the effector molecule of humoral immunity. Similar to the T lymphocyte response,

memory B cells are also generated to provide long-term immunity to re-infection with the same pathogen [21].

The B cell receptor (BCR) is a membrane-bound antibody (immunoglobulin) that upon antigen-recognition transmits an activation signal into the B cell to initiate plasma cell differentiation. Like T lymphocytes, B cells require more than simply antigen alone for full activation to differentiate into antibody-secreting plasma cells; CD4+ helper T cells provide the requisite accessory signals [22]. Therefore, the B cell receptor also delivers bound antigen into the B cell for degradation and processing, and MHC class II molecules then present the peptides on the B cell surface to CD4+ helper T cells. An example accessory signal provided by helper T cells is CD40 ligand (CD40L), that upon interaction with CD40 on B cells drives resting B cells into cell cycle and increases expression of CD80 and CD86 co-stimulatory molecules which enhance the mutual T-B lymphocyte interaction by sustaining T cell growth and differentiation [23].

Upon encounter with antigen in the presence of helper T cells, naïve B cells differentiate into antibody-producing plasma cells at sites within lymphoid tissues known as germinal centres (GCs) [24, 25]. Germinal centres are areas of intense proliferation where B cells undergo somatic hypermutation of the antigen recognising, variable portion of antibody to create immunoglobulin diversity and improve antibody affinity for antigen. Antibodies are versatile as effector molecules; the effector function of antibodies is determined by the Fc tail region, and therefore different isotypes of antibody are produced to confer functional diversity to the humoral response. Different cytokines preferentially induce immunoglobulin class switching in the germinal centre reaction to produce different isotypes of antibody such as IgG and IgE. Secreted antibody functions in three main ways to mediate the humoral response to infection [26]. Firstly, antibodies can neutralise a pathogen or toxin by blocking adherence to host cells and consequent infection. Secondly, recognition of antigen by antibody creates an immunoglobulin coat which opsonises the bound pathogen or toxin for identification and destruction by phagocytic cells of the immune system. Lastly, antibodies bound to pathogens can also activate the complement system that results in the formation of a membrane attack complex which disrupts the microbial cell membrane, thereby destroying the pathogen.

1.2 Autoimmunity

Highly specific, adaptive immune responses towards such a variety of pathogenic antigens are possible due to the random generation of many diverse lymphocyte receptors. However, in order to ensure that the immune receptor repertoire is large enough to recognise a significant amount of the almost infinite number of potentially pathogenic antigens, some self-reactive lymphocytes are predictably generated. Autoimmunity can occur when an immune response is mounted against self-tissue antigens as opposed to invading, foreign pathogens. Pathologically, autoimmune disease results from this immune-mediated attack of self-tissues. Consequently, there are immunological tolerance mechanisms that actively prevent autoimmunity but still allow the effective control of infection [27].

1.2.1 Immunological Tolerance.

The random generation of many diverse lymphocyte receptors to provide effective immunity against the almost infinite number of pathogens that could be encountered inevitably generates some that recognise self-antigens. Immunological tolerance is the active failure to mount an immune response to a particular antigen, and therefore protects against autoimmunity.

Self-reactive T and B lymphocytes can be generated; therefore both T cells and B cells can be made immunologically tolerant. During B lymphocyte development, self-reactive B cell receptors are deleted by undergoing negative selection upon recognition of self-molecules present in the bone marrow [28]. Peripheral tolerance mechanisms that control any self-reactive B cells which escaped clonal deletion are not that well understood. Upon encounter with self-antigen, peripheral B cells can be induced to undergo apoptosis [29, 30] or become anergic [27]. B cell activation and differentiation into antibody-producing plasma cells requires T cell help, consequently T cell tolerance is much more rigorous.

Similar to B cell tolerance, T cell tolerance involves both central tolerance mechanisms that occur within the thymus during T cell development, and peripheral tolerance mechanisms to control any self-reactive T cells that circumvent central tolerance and are exported to the periphery where self-antigen could be encountered (*Fig. 1.01*). During thymic development,

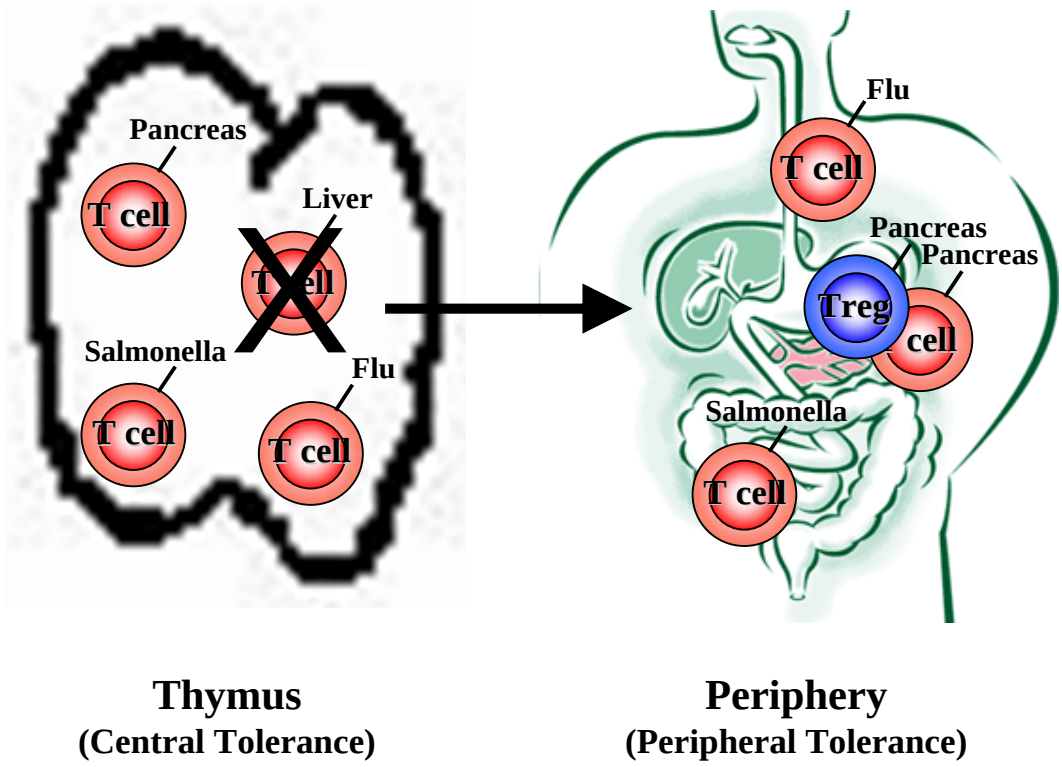


Figure 1.01. A diagram to illustrate immunological T cell tolerance. In the thymus (left) T lymphocytes develop bearing T cell receptors that recognise an almost infinite repertoire of different antigens. Central tolerance mechanisms exist to delete highly self-reactive T cells by negative selection. Mature thymocytes are exported to the periphery to provide immunity against invading pathogens (right). Peripheral tolerance mechanisms such as regulatory T cells control the unwanted activation of any self-reactive T cells, that escaped negative selection in the thymus, upon encounter with their self-antigen.

T lymphocytes undergo both positive selection to ensure self-MHC is recognised and negative selection to delete T cells that recognise self-antigens [31].

Positive selection occurs in the thymic cortex and is mediated by cortical thymic epithelial cells (cTECs). CD4/CD8 double positive thymocytes are positively selected on their ability to recognise self-MHC molecules. Developing thymocytes that fail to bind self-MHC molecules on the thymic stroma within the cortex are induced to undergo programmed cell death. The MHC gene is highly polymorphic which increases the range of antigens that can be presented to T cells. However, this MHC allelic diversity means that T lymphocytes are MHC-restricted in their antigen recognition. Thus, positive selection identifies T lymphocytes that are capable of responding to infected host cells. During positive selection, CD4/CD8 double positive thymocytes are also coordinated to express the CD4 or the CD8 co-receptor according to recognition of MHC class II or MHC class I molecules, respectively.

Following positive selection in the thymic cortex, T lymphocytes with strongly self-reactive receptors are deleted in the medulla by negative selection. The autoimmune regulator AIRE (a transcription factor) promotes the expression of self-antigens by medullary thymic epithelial cells (mTECs) [32]. This promiscuous gene expression of tissue-specific antigens within the thymus identifies any highly self-reactive T cells that have been generated and those that pose the greatest threat are induced to undergo apoptosis. There is also now evidence for intercellular self-antigen transfer between thymic epithelial cells and thymic dendritic cells within the medulla microenvironment which would increase the efficacy of negative selection by broadening the cellular basis of self-antigen presentation for tolerance induction [33].

There is a fine balance between the elimination of highly self-reactive T lymphocytes and the development of a diverse T cell receptor repertoire to provide efficient protection against pathogenic infection. Consequently, some self-reactive T cells evade central tolerance and are exported to the periphery. Thus, peripheral tolerance mechanisms exist to control potentially self-reactive T cells and the development of autoimmunity. One mechanism of peripheral tolerance is the generation of immunoprivileged sites where anatomical barriers

prevent lymphocytes encountering antigen and the cytokine environment inhibits the development of an immune response [34]. These are generally tissues that upon mal-function could hinder the survival of an organism, such as the central nervous system (CNS), eye and placenta. Also, T cells can be made unresponsive upon antigen encounter by the induction of anergy. T cell stimulation with antigen via the T cell receptor in the absence of CD28 co-stimulation has been reported to induce an anergic state of unresponsiveness [35-38]. Finally, peripheral tolerance is actively maintained by a subset of T lymphocytes called regulatory T cells (Tregs) [39]. This specialised population of CD4+ T cells is thought to maintain immune homeostasis by suppressing the inappropriate activation of T cells, such as any self-reactive T cells that encounter antigen to thereby prevent autoimmunity and the development of autoimmune disease. In addition to these natural mechanisms of peripheral tolerance, immunological tolerance can be induced or acquired in the periphery [40]. For example, in oral tolerance, continually encountered, non-self food proteins induce the immune system to adapt to external antigens that pose no threat, and failure to acquire tolerance can lead to the development of inflammatory bowel disease (IBD). Clinically, the induction of tolerance is of key importance in tissue transplantation.

1.2.2 Type-1 Diabetes.

Dysregulated immune homeostasis can result upon loss of immunological tolerance and lead to the development of autoimmunity. There are many different types of autoimmune diseases mediated by both self-reactive T and B lymphocytes. Autoimmune diseases are broadly classified as systemic when the self-antigens are ubiquitous, for example systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), or organ-specific when disease is limited to auto-antigens of a particular organ, such as type 1 diabetes (T1D) and multiple sclerosis (MS) [41].

Type-1 diabetes is one of three main forms of diabetes. It is a T cell-mediated autoimmune disease in which insulin-producing beta-cells of the islets of langerhans in the pancreas are destroyed by the immune system. Upon islet cell destruction, the decrease in production of insulin by the pancreas results in a loss of control over blood glucose homeostasis and consequently hyperglycemia. Long-term, chronic disease can result in limb amputation due to gangrene, renal failure, blindness, coma and stroke [42].

Susceptibility to type-1 diabetes is thought to be genetically inherited [43]. However, environmental stimuli such as viral infection or stress seem to trigger disease onset [44]. Particularly in the developed western world the incidence of type-1 diabetes is on the increase [45]. Currently there is no true, complete cure for type-1 diabetes. Kidney-pancreas organ transplants have proved to be successful in making patients insulin-independent [46]. This treatment does however require long-term immunosuppressive drug therapy. Thus, at present treatment of type-1 diabetes requires that patients control the disease with regular insulin injections to maintain glucose homeostasis.

The pathological mechanisms that lead to the development of type-1 diabetes are not fully understood; although there is strong evidence that disease is associated with loss of immunological self-tolerance. Due to their function of mediating immunological peripheral tolerance, defects in regulatory T cell number or function may be critically involved in the development of type-1 diabetes and many other autoimmune diseases. Early studies reported reduced numbers of regulatory T cells in type-1 diabetic patients [47]. However, subsequent studies have found no difference in the frequency of CD4+ CD25+ Tregs between type-1 diabetic subjects and normal controls [48-50], even when FOXP3 was used as a more specific lineage marker for regulatory T cells [42]. One of these studies found that even though type-1 diabetic patients had normal numbers of peripheral regulatory T cells, these Tregs were defective in their functional ability to suppress effector T cell proliferation during *in vitro* co-culture [48]. On the other hand, isolation of regulatory T cells as only the CD4+ CD25hi population did not identify a difference in *in vitro* Treg suppressive capacity between type-1 diabetic patients and normal controls [49]. Type-1 diabetes is difficult to study in humans as clinical diagnosis of loss of blood glucose control by insulin occurs after manifestation of widespread pancreatic islet destruction. Therefore animal models of type-1 diabetes have been valuable tools for studying the onset and progression of the disease.

1.2.3 Transgenic, Adoptive Transfer Mouse Model of Autoimmune Diabetes.

The non-obese diabetic (NOD) mouse which spontaneously develops diabetes similar to the human disease is frequently used to study type-1 diabetes and the role of regulatory T cells in autoimmunity [51]. However, the Tregs present in these model animals have to be analyzed

as whole heterogeneous populations because their antigen-receptor repertoire recognizes a huge variety of self-antigens encountered in the thymus during development. Our laboratory has developed a transgenic mouse model to facilitate the study of regulatory T cells with a single antigen specificity [52, 53] (*Fig. 1.02*).

DO11.10 Rag2-/- transgenic mice contain CD4+ T lymphocytes that possess the *DO11.10* transgenic T cell receptor, which can be specifically identified using a fluorescently labeled monoclonal antibody (KJ-126). The *DO11.10* transgenic TCR recognizes the chicken egg glycoprotein ovalbumin (OVA). In RIP-*mOVA* transgenic mice expression of the membrane-bound form of OVA is under the control of the rat insulin promoter (RIP), therefore these mice specifically express the OVA protein on the β cells of the pancreas. Consequently, adoptive transfer of *DO11.10 Rag2*-/- CD4+ T cells into RIP-*mOVA* mice, which are also *Rag2*-/- and therefore T and B lymphocyte deficient, causes pancreatic islet destruction and development of overt diabetes, which can be monitored by blood glucose readings. Additionally, antigen-specific (KJ-126+) CD4+ CD25+ regulatory T cells can be obtained from *DO11.10 Rag2*-/- x RIP-*mOVA Rag2*-/- mice because the rat insulin promoter also drives *mOVA* expression in the thymus, therefore in these double positive mice a regulatory T cell population develops with a single *DO11.10* TCR specificity. Co-transfer of antigen-specific (KJ-126+) CD4+ CD25+ regulatory T cells with *DO11.10 Rag2*-/- CD4+ T cells into RIP-*mOVA Rag2*-/- mice suppresses the development of diabetes. This transgenic, adoptive transfer mouse model of autoimmune diabetes is therefore a very useful tool for studying antigen-specific Tregs *in-vivo* [52] as 100% of these mice spontaneously develop diabetes.

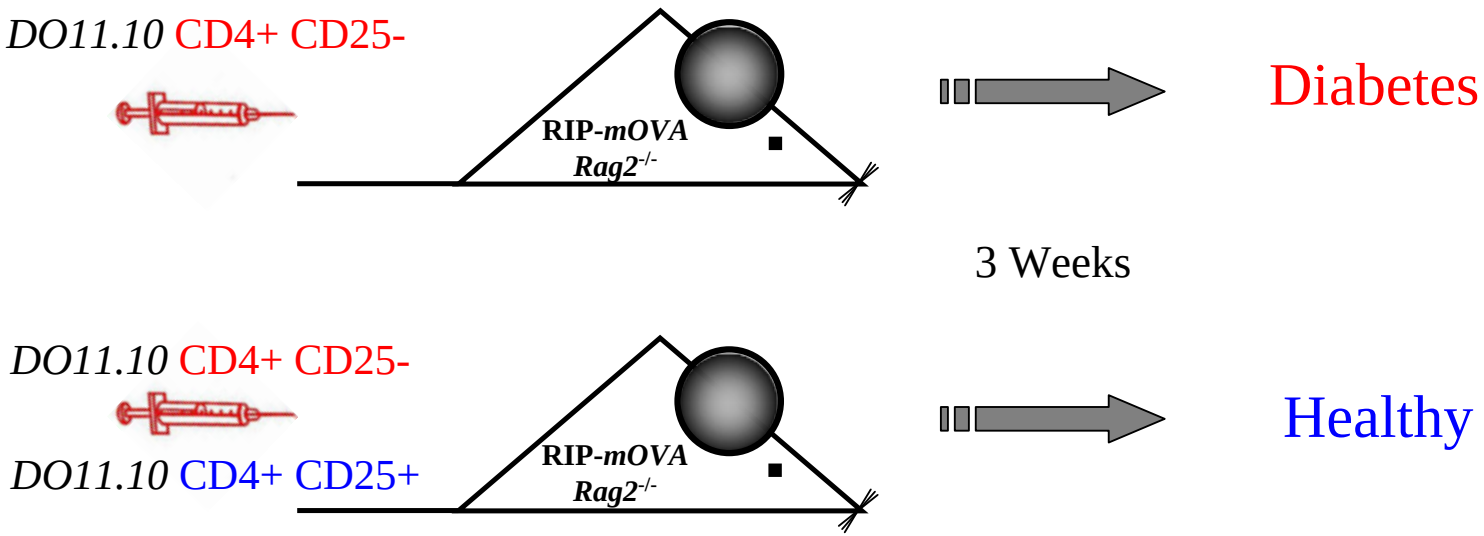


Figure 1.02. Transgenic, adoptive transfer mouse model of autoimmune diabetes. Adoptive transfer of TCR-transgenic (*DO11.10*) CD4+ CD25- T cells, that specifically recognise the model antigen ovalbumin, into RIP-*mOVA Rag2*-/- host mice, which express the model antigen ovalbumin under the control of the rat insulin promoter, causes pancreatic islet destruction and development of overt diabetes in around 3 weeks. Co-transfer of TCR-transgenic (*DO11.10*) CD4+ CD25+ Tregs suppresses the development of diabetes.

1.3 Regulatory T Cells

Regulatory T cells are a subset of CD4+ T cells specialised to exert dominant immunological tolerance in the periphery, by suppressing the activation of effector T cells, to maintain homeostasis. The concept of specialised suppressor T cells was an active area of cellular immunology research in the 1970s. However, with the molecular biology revolution of the 1980s; CD4+ helper T cells and CD8+ cytotoxic T cells were cloned, but no suppressor T cell subset could be identified [54]. This led to widespread questioning of the very existence of a specialised suppressor T cell subset. Almost 15 years ago, there came a renaissance in the suppressor T cell field when Sakaguchi and colleagues demonstrated the existence of naturally occurring, thymus-derived CD4+ T cells that expressed the interleukin-2 (IL-2) receptor α-chain (CD25+), and which protected from autoimmunity to maintain peripheral immunological tolerance [39]. This CD4+ CD25+ T cell subset was subsequently found to inhibit effector T cell responses both *in vitro* and *in vivo* [55-57], and are now termed regulatory T cells.

Regulatory T cells were firmly established as a distinct T cell subset with the identification of the lineage-specific forkhead box P3 transcription factor, FOXP3 [58-60]. *Foxp3* encodes a member of the forkhead / winged-helix family of transcription factors, also known as *Scurfin*. Genetic mutations of *Foxp3* in both mice and humans leads to the development of severe, fatal autoimmune disease; scurfy and immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), respectively [61, 62]. Since the identification of *Foxp3* as a regulatory T cell-lineage marker, it has been established that FOXP3 has an essential role in maintaining the transcriptional and function programs of Tregs (reviewed in [63]). More recently, the molecular mechanisms that maintain *Foxp3* expression in Tregs have begun to be elucidated; for instance a complex of the transcription factors RUNX and CBFbeta has been implicated [64].

CD4+ FOXP3+ regulatory T cells critically control unwanted effector T cell responses, such as those towards self-antigens during autoimmune diseases and those towards non-harmful foreign antigens during allergic reactions. Therefore, it is important to fully understand the development, homeostasis and function of the regulatory T cell subset due to the therapeutic potential to treat autoimmunity and allergy [65]. Modulation of the regulatory T cell

population also has therapeutic implications in the promotion of tolerance following transplantation [66]. Additionally, the CD4+ FOXP3+ regulatory T cell population is one of the main barriers to overcome in cancer therapy as Tregs function to suppress anti-tumor immune responses. Murine studies have shown that Treg depletion leads to enhanced tumor immunity [67-70], and CD4+ FOXP3+ Tregs have been found to be increased in the peripheral blood and, in some instances, tumor infiltrating lymphocytes of human cancer patients [71].

1.3.1 Development of Natural Regulatory T cells in the Thymus.

Natural CD4+ FOXP3+ regulatory T cells constitute 5-10% of the peripheral CD4+ T cell population. It was realised that natural Tregs develop in the thymus and migrate to the periphery as studies in mice found that neonatal thymectomy resulted in lethal autoimmunity and a lack of peripheral regulatory T cells [72]. It is still not completely understood how CD4+ FOXP3+ regulatory T cells develop in the thymus. It was initially proposed that differentiation into the FOXP3+ Treg lineage is determined according to TCR-signal strength during thymic development. As discussed previously, during thymic development all T lymphocytes that receive a weak TCR-signal are positively selected to survive and develop, but a very strong TCR-signal induces apoptosis and the self-reactive T cells undergo negative selection. An intermediate TCR-signal was hypothesised to up-regulate *Foxp3* expression and commit the developing T cell to the Treg lineage (reviewed in [73]).

Studies using TCR-transgenic mice support the requirement of intra-thymic self-antigen encounter for FOXP3+ regulatory T cell differentiation as Tregs do not develop in mice that express a single, transgenic TCR unless the cognate antigen for the transgenic TCR is expressed in the thymus [74, 75]. Analysis of conventional and regulatory T cell receptor genes originally identified minimal overlap between the two T cell populations with regulatory, but not conventional, T cells expressing self-antigen specific TCRs [76]. In contrast, more recent analyses of conventional and regulatory T cell receptor sequences, by the same laboratory, suggest that Tregs express a polyclonal TCR repertoire which does not preferentially recognise self-antigens, but is comparable to the polyclonal TCR repertoire of conventional T cells (Tconv) [77].

As discussed above, positive selection of developing thymocytes occurs in the cortical region of the thymus, and then AIRE-induced, promiscuous self-antigen expression in the thymic medulla mediates negative selection of self-reactive T lymphocytes. Some studies suggest that FOXP3+ regulatory T cells arise at the CD4 single-positive stage, alongside negative selection, in the thymic medulla [78, 79]. Consistent with this, FOXP3+ cells can only be found in the medullary region of *Foxp3*-GFP reporter mice thymi [80]. On the other hand, it has also been found that differentiation into the FOXP3+ Treg lineage may be initiated in cortical CD4/CD8 double-positive thymocytes earlier in thymic development [81-84]. In addition to induction of Treg differentiation by cortical thymic epithelial cells, medullary thymic epithelial cells or thymic dendritic cells, it has recently been proposed that developmental control of immature thymocyte differentiation into the regulatory T cell lineage is T cell-intrinsic and does not require a dedicated antigen-presenting cell [85].

In addition to antigen-encounter, CD28-mediated co-stimulation has been found to be required for CD4+ FOXP3+ regulatory T cell thymic development. Analysis of *CD28*-deficient mice and anti-CD80/anti-CD86 antibody treated mice identified a significant decrease in the percentage and number of CD4 single-positive CD25+ thymocytes [86]. A role for the CD28 co-stimulatory receptor homologue, CTLA-4, in FOXP3+ Treg development has not been specifically addressed. However, no overall defects in thymic selection and thymocyte development have been identified in *Ctla-4*-deficient mice [87, 88].

Regulatory T cells constitutively express the IL-2 receptor α-chain, CD25, and there is substantial evidence that IL-2 signalling plays a role in Treg development. *IL-2*-knockout and *IL-2Rα/β*-knockout mice have a substantially reduced CD4+ CD25+ Treg population and exhibit similar severe and fatal autoimmunity to *Foxp3*-deficient mice [89-93]. Adoptive transfer experiments or generation of mixed bone marrow chimeras to correct the deficient Treg population inhibited the lethal autoimmunity [94-96]. Furthermore, thymus-specific expression of IL-2Rβ in *IL-2Rβ*-/- mice restored the production of functional CD4+ CD25+ Tregs and prevented fatal autoimmunity [93, 97]. The IL-2 cytokine is a T cell growth factor, and it has been proposed that IL-2 signalling functions in regulatory T cell thymic development to drive expansion of this population following initial production of thymic CD4+ CD25+ Tregs [98].

Transforming growth factor-beta (TGF-β) is another cytokine that has been extensively studied in determining the molecular mechanisms involved in thymic Treg differentiation as mice deficient in TGF-β also have a similar severe autoimmunity phenotype to *Foxp3*-deficient and *IL-2*-deficient mice [99, 100]. Initial analysis of *TGF-β*-knockout and *TGF-βRII*-knockout mice found an unaltered, or if anything augmented, thymic FOXP3+ population [101-104]. However, it has recently been proposed that TGF-β signalling plays a formerly unrecognised role in the thymic development of CD4+ CD25+ FOXP3+ regulatory T cells that was previously masked by the increased IL-2 present in *TGF-βRI*-knockout mice [105].

1.3.2 Adaptive Regulatory T Cells in the Periphery.

In addition to natural regulatory T cells that develop in the thymus and are exported to the periphery to exert dominant immunological tolerance, *Foxp3* expression can be induced in peripheral CD4+ FOXP3- conventional T cells to generate adaptive Tregs (also called T_H3 cells). It is thought that adaptive regulatory T cells have a functional role in controlling allergic inflammation and maintaining mucosal tolerance to continuously encountered, non-harmful environmental and food antigens (reviewed in [106]). Induction of *Foxp3* expression *in vivo* by foreign antigen has been demonstrated by a number of different methods [107-110], and more recently the adaptive Tregs generated have been shown to functionally exert suppressive activity [111-113].

The requirements and molecular mechanisms of *Foxp3* induction to generate adaptive Tregs are still not completely understood. *In vitro*, TGF-β has been found to induce *Foxp3* expression [114]. Consistent with this, neutralisation of TGF-β *in vivo* has been found to impair oral tolerance and inhibit the differentiation of antigen-specific adaptive Tregs [110, 115]. Specifically in the gut-associated lymphoid tissue (GALT), retinoic acid present in this microenvironment has been suggested to play an important role in the TGF-β induction of *Foxp3* expression and generation of adaptive Tregs [116-118]. However, more recent evidence has found that retinoic acid may facilitate TGF-β induction of FOXP3+ Tregs by inhibiting the generation of effector T_H17 cells [119]. Recently, the Runt-related transcription factors RUNX1 and RUNX3 have been demonstrated to bind to the *Foxp3* promoter as the

molecular link between TGF-β receptor signalling and induction of *Foxp3* expression in the generation of adaptive Tregs [120].

Similar to the differentiation of natural FOXP3+ Tregs in the thymus, the co-stimulatory receptor CD28 has been found to be required for the *in vitro* TGF-β-mediated induction of *Foxp3* expression [121]. It was established that the requirement for CD28 in adaptive Treg generation was mediated by IL-2, which is in line with experiments using *IL-2*-deficient T cells and IL-2 neutralisation that demonstrated a requirement for IL-2 in TGF-β-mediated *in vitro Foxp3* induction [122]. In contrast to natural Treg generation, it has been suggested that the homologue of the CD28 receptor, CTLA-4, has an essential role in the TGF-β-mediated *in vitro* induction of *Foxp3* [123]. Another inhibitory signalling pathway has also been proposed to regulate the generation of adaptive Tregs as programmed death 1 ligand (PD-L1) was found to be required for the TGF-β-mediated *in vitro* induction of *Foxp3* expression [124]. On the other hand, an additional co-stimulatory pathway mediated by OX40 has been identified as an inhibitor of the induction of *Foxp3* expression [125], data suggest that this is due to the induction of memory T cells *in vivo* [126].

In addition to the induction of *Foxp3* by TGF-β to generate the adaptive regulatory T cells discussed above (T_H3 cells), IL-10 has been found to induce CD4+ T cells to become type 1 regulatory T cells (Tr1 cells). Tr1 cells do not express *Foxp3* [127], but produce high levels of the inhibitory cytokines IL-10 and TGF-β to mediate suppression [128]. Tr1 cells have been shown to play a critical role in the prevention of inflammatory bowel disease and regulation of allergic responses [129].

1.3.3 Peripheral Homeostasis of Regulatory T Cells.

There have been major advances in knowledge and understanding of CD4+ FOXP3+ natural and adaptive Treg generation in the thymus and periphery, respectively. However, relatively little is known about the mechanisms that control the size and stability of the peripheral Treg population. To date similar factors to those involved in the development of regulatory T cells have been implicated in peripheral Treg homeostasis, such as CD28 co-stimulation and the cytokines IL-2 and TGF-β (reviewed in [130]).

As discussed above, CD28-mediated signaling has been implicated in the thymic development of regulatory T cells. CD28 signals are also required for the peripheral homeostasis of Tregs [131] as the reduced thymic Treg numbers observed in *CD28*-deficient mice are not restored in the periphery. Peripheral CD4+ CD25+ Tregs are also decreased upon anti-CD80/anti-CD86 blocking antibody treatment. It is unclear from these experiments whether the requirement for CD28 directly controls Treg survival and proliferation, or indirectly regulates production of the T cell growth factor IL-2 from conventional T cells. Although, an investigation found that upon adoptive transfer into *CD28*-/- mice, wild type Treg survival was impaired [86].

Consistent with the potential role of the cytokine IL-2 in Treg homeostasis implicated by studies on the role of the CD28 receptor, *IL-2*-knockout and *IL-2Rα/β*-knockout mice have a decreased CD4+ CD25+ Treg population and develop severe autoimmunity [89-93]. Furthermore, a role for IL-2 in the peripheral maintenance of regulatory T cells is suggested by a competitive setting model in which in the presence of wild type FOXP3+ Tregs, *IL-2Rα*-/- FOXP3+ Tregs do not survive [80]. Similarly, TGF-β has been proposed to function in peripheral Treg homeostasis because both *TGF-βI*-/- and *TGF-βRII*-/- mice have been found to have reduced numbers of peripheral FOXP3+ Tregs [101, 103, 104].

More recently, other molecules have been proposed to function in the maintenance of peripheral regulatory T cell homeostasis. The Wiskott-Aldrich syndrome protein (WASp) is essential for optimal T cell activation and WAS patients are immunodeficient. However, WAS patients are also susceptible to systemic autoimmunity, and even though thymic Tregs develop normally in *WASp*-/- mice the peripheral CD4+ FOXP3+ Treg population is reduced [132]. Mice deficient for the cellular adhesion molecule LFA-1 also have a significantly reduced peripheral Treg population. Although, in this study, the decreased number of peripheral Tregs was attributed to a reduced capacity of *LFA-1*-/- conventional T cells to be induced to become adaptive Tregs [133].

1.3.4 Mechanisms of Regulatory T Cell Function.

Since the identification of the CD4+ CD25+ FOXP3+ regulatory T cell subset there has been numerous studies investigating the mechanism(s) of Treg suppression due to the huge

therapeutic potential in the modulation of Treg function to improve treatment for autoimmunity, allergy and cancer. From both *in vitro* and *in vivo* studies, it has become clear that regulatory T cells do not rely on just one mechanism of suppression, but rather there are multiple mechanisms by which Tregs can exert suppressive function (*Fig. 1.03*) (reviewed in [134-137]).

There is debate over the cellular target of Treg-mediated suppression. *In vitro* suppression assays of effector T cell responses can be performed in the absence of antigen-presenting cells using bead-coupled, plate-bound or soluble antibodies to provide the necessary TCR and CD28 activation stimulus. This suggests that regulatory T cells target responder, effector T cells directly to achieve suppression. Due to the constitutive expression of the IL-2 receptor α-chain (CD25) by regulatory T cells, it has been suggested that Tregs may compete with FOXP3- conventional T cells for the T cell growth factor IL-2, thereby inhibiting Tconv proliferation and inducing apoptosis due to cytokine deprivation [138]. In line with this hypothesis, IL-2 has been found to be essential for Treg homeostasis *in vivo* [139] and Tregs do not produce IL-2 as FOXP3 suppresses IL-2 transcription [140], therefore regulatory T cells could act as a sink for the IL-2 cytokine. However, CD25 is the low-affinity receptor for IL-2 and expression of the high-affinity IL-2 receptor complex has not been quantified on FOXP3+ regulatory T cells compared to activated, effector FOXP3- conventional T cells (reviewed in [136]).

It has also been hypothesised that other cytokines could mediate Treg suppressive function. CD4+ FOXP3+ regulatory T cells produce inhibitory cytokines such as IL-10, IL-35 and TGF-β. *In vivo* adoptive transfer studies using Tregs from *IL-10*-/- mice provided evidence of a role for IL-10 in regulatory T cell suppressive function as *IL-10*-deficient Tregs failed to prevent inflammatory bowel disease [141] or protect against experimental autoimmune encephalomyelitis (EAE) [142]. IL-35 is a newly proposed inhibitory cytokine and is a member of the IL-12 heterodimeric cytokine family, composed of Epstein-Barr virus-induced gene 3 (*Ebi3*) and *IL-12a* (*p35*). *In vitro Ebi3*-/- and *IL-12a*-/- Tregs were found to have reduced suppressive activity over responder effector T cells, and neither *Ebi3*-/- nor *IL-12a*-/- Tregs were able to protect against inflammatory bowel disease *in vivo* [143]. However, it has been reported that human CD4+ CD25+ FOXP3+ regulatory T cells do not constitutively

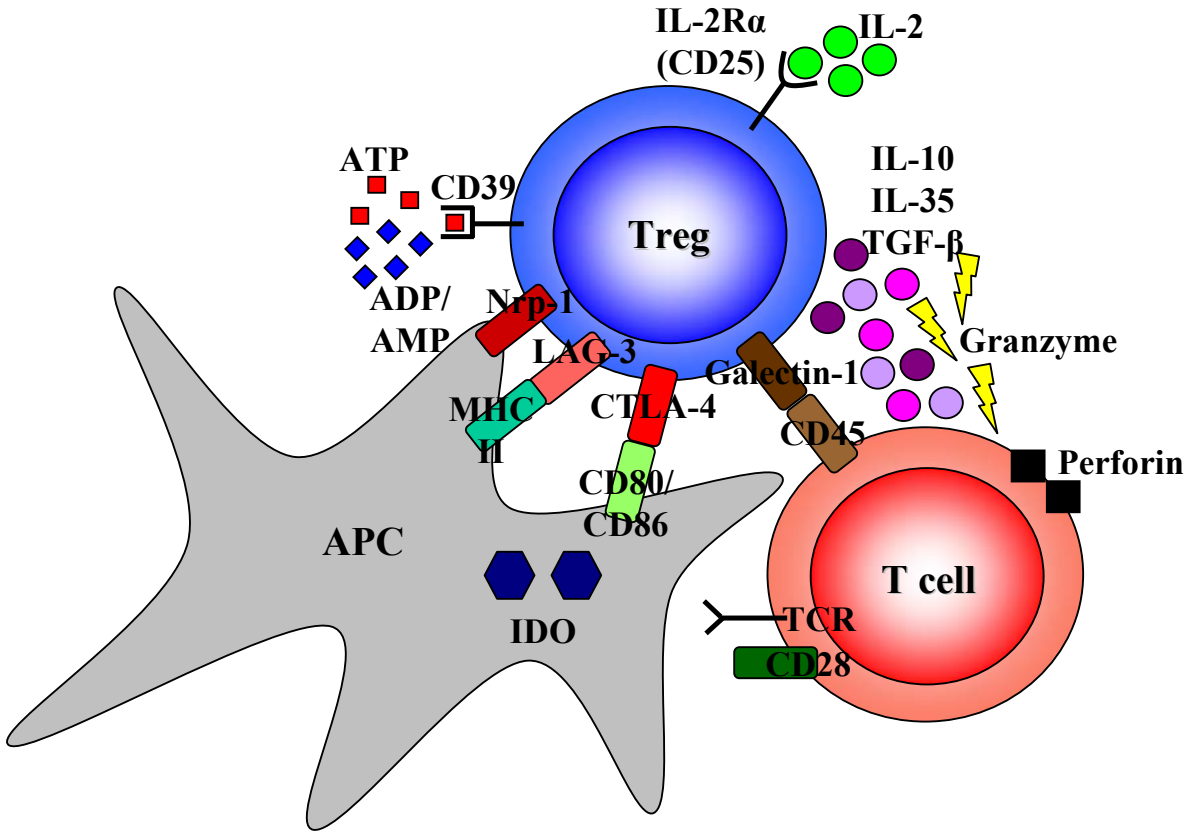


Figure 1.03. A diagram to illustrate the proposed mechanisms of regulatory T cell function. Decreased APC antigen-presentation and co-stimulation by CD39 hydrolysis of ATP to ADP or AMP, Nrp-1-mediated long Treg:APC interaction, LAG-3 ligation of MHC II, CTLA-4 binding of CD80/CD86. Induction of IDO. Induction of T cell apoptosis by Galectin-1 binding of CD45, granzyme and perforin-mediated cytolysis. Treg production of inhibitory cytokines (IL-10, IL-35, TGF-β). T cell cytokine deprivation by CD25-mediated IL-2 consumption.

express IL-35 [144]. As discussed previously, mice deficient in TGF-β suffer from a fatal, autoimmune diseased phenotype and TGF-β has been found to induce *Foxp3* expression. However, there is controversy over the role of TGF-β as an effector molecule of regulatory T cell suppressive function. Initially, *in vitro* Treg suppression was shown to be reversed by anti-TGF-β antibody [145]. However, later studies found that anti-TGF-β or soluble TGF-βRII did not abrogate Treg suppressive function [146]. *In vivo*, abrogation of wild-type Treg protection against inflammatory bowel disease with anti-TGF-β neutralizing antibodies suggests TGF-β is involved in Treg control of intestinal homeostasis [147]. On the other hand, anti-TGF-β was also found to abrogate the function of *TGF-β1*-/- Tregs which suggests that TGF-β is required to control intestinal inflammation, but can be produced by another, non-regulatory T cell source [102].

In addition to the secretion of inhibitory cytokines by FOXP+ regulatory T cells to induce cell cycle arrest in FOXP3- effector T cells, it has been proposed that Tregs can express surface molecules such as Galectins which induce cell cycle arrest, apoptosis and inhibit pro-inflammatory cytokine production by effector T cells upon interaction with many responder cell surface glycoproteins such as CD45. *In vitro*, both mouse and human Treg suppressive function was reduced upon blockade of galectin-1, and suppression by regulatory T cells isolated from *galectin-1*-deficient mice was also impaired [148]. Another potential mechanism by which it has been hypothesised that Tregs could induce apoptosis and directly target responder, effector T cells to achieve suppression is granzyme-mediated cytolysis. For instance, activated human CD4+ CD25+ FOXP3+ regulatory T cells have been shown to express granzyme A and induce the apoptosis of CD4+ and CD8+ responder T cells in a perforin-dependent manner [149].

However, as well as directly affecting the effector T cells, regulatory T cells could also control an immune response by targeting the antigen-presenting cells. As explained previously, antigen-presenting cells such as dendritic cells present peptides complexed to MHC molecules, along with co-stimulatory molecules, to activate T cells via TCR ligation and CD28 co-stimulation. Two-photon laser-scanning microscopy studies demonstrated in lymph nodes *in situ* that direct interactions occur between Tregs and antigen-presenting cells, and this was associated with decreased antigen-presenting cell capacity to stimulate effector

T cells [150]. A number of mechanisms have been proposed by which effector molecules on CD4+ FOXP3+ regulatory T cells modulate the capacity of antigen-presenting cells to prime FOXP3- conventional T cells.

It has been suggested that pro-longed interaction of regulatory T cells with antigen-presenting cells can prevent maturation of the dendritic cells; this restricts conventional T cell access to peptide:MHC complexes and limits their activation. Neuropilin-1 (Nrp-1) is a receptor that is preferentially expressed by regulatory T cells and recently been shown to promote long interactions between Tregs and immature dendritic cells, and blockade of which abrogates Treg-mediated suppression *in vitro* [151]. Another cell surface receptor expressed by regulatory T cells that has been proposed to maintain dendritic cells in an immature state and limit their capacity to present antigen to and co-stimulate T cells is LAG-3. LAG-3 binds to MHC class II molecules and induces an inhibitory signal which prevents dendritic cell maturation [152]. It has also been hypothesised that regulatory T cell expression of CD39 functions to limit the co-stimulatory capacity of antigen-presenting cells. Adenosine triphosphate (ATP) can up-regulate CD86 expression by dendritic cells during inflammation in response to cell damage. However, Tregs express the ectoenzyme CD39 which modulates extracellular ATP by hydrolysis to adenosine diphosphate (ADP) or adenosine monophosphate (AMP) [153].

Constitutive expression of CTLA-4 by CD4+ CD25+ regulatory T cells [154, 155] and the severe lymphoproliferative disease observed in *Ctla-4* knockout mice [156, 157] suggest that the CTLA-4 receptor is a good candidate effector molecule for Treg function. CTLA-4 binds the same ligands on antigen-presenting cells as the CD28 co-stimulatory receptor (CD80 and CD86) with a 20-100 fold higher affinity [158]. It has therefore been hypothesised that the binding of CTLA-4 on Tregs to CD80 and CD86 on antigen-presenting cells could also prevent the priming and co-stimulation required to activate conventional T cells by ligand competition. It has also been proposed that this interaction of CTLA-4 on Tregs with its ligands on antigen-presenting cells prevents dendritic cell maturation, or even down-regulates CD80 and CD86 expression [159, 160]. In addition it has been suggested that upon binding of CTLA-4 on Tregs to its ligands on antigen-presenting cells, CD80 and CD86 can back-signal into the dendritic cell to stimulate production of the enzyme

indoleamine 2,3-dioxygenase (IDO) [161, 162]. IDO catabolises an essential amino acid for T cell proliferation, tryptophan, therefore it has been proposed that induction of active IDO negatively affects T cell survival [163].

Another receptor that is constitutively expressed by regulatory T cells compared to being up-regulated by conventional T cells upon activation is the co-stimulatory and survival glucocorticoid-induced tumor necrosis factor receptor, GITR. It has been proposed that GITR functions to negatively regulate Treg-mediated suppression [164, 165]. However, *GITR*-deficiency does not alter immune homeostasis [166] and it seems more likely that antibody-mediated modulation of GITR targets and enhances effector T cell responses rather than abrogate regulatory T cell function [167, 168].

Very recently, a role for microRNAs (specifically miRNA 155 [169]) in the maintenance and function of regulatory T cells has been revealed [170, 171]. However, the detailed molecular mechanisms are not yet known.

1.4 The Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) Receptor

The *Ctla-4* gene was originally identified during the screening of mouse cytotoxic T cell-derived cDNA libraries for T cell specific genes [172], hence the name cytotoxic T lymphocyte associated antigen. The *Ctla-4* gene is homologous to the *CD28* co-stimulatory receptor gene; there is a high degree of nucleotide sequence similarity and the genes map to a similar chromosomal location [173]. The CTLA-4 receptor (also known as CD152) is a type-1 transmembrane glycoprotein member of the immunoglobulin superfamily and exists as a disulphide-linked homodimer [174]. In addition to homology with CD28, CTLA-4 was identified as a second receptor for the CD28 co-stimulatory receptor ligands (CD80 and CD86) [175-178]. The cell surface expression levels of CTLA-4 on resting T cells are very low. It is difficult to measure CTLA-4 expression as this receptor largely exists intracellularly, and upon cell activation is translocated to the cell surface. However, cell surface expression levels of CTLA-4 on activated T cells are limited due to rapid clathrin-mediated endocytosis of the CTLA-4 receptor [179]. A soluble form of CTLA-4 (sCTLA-4) has also been described which is a splice variant that lacks the membrane-tethering region of the CTLA-4 receptor [180].

1.4.1 The CD28 Co-Stimulatory Receptor and CD80/CD86 Ligands.

The CD28 co-stimulatory receptor is a 44kDa, disulphide-linked homodimeric member of the immunoglobulin superfamily constitutively expressed on the surface of both CD4+ and CD8+ T cells. As discussed previously, CD28 receptor ligation, by the binding of the ligands CD80 and CD86, along with TCR-stimulation, by antigen presented on MHC, results in the full activation of naïve T cells [181]. A number of different T cell responses occur following CD28-costimulated activation such as proliferation, enhanced cell survival by the up-regulation of the anti-apoptotic protein Bcl-$_{xL}$, and increased cytokine (IL-2, TNF-α, IFN-γ and GM-CSF) production via mRNA stabilisation [182]. *CD28* gene ablated mice have a much reduced frequency of peripheral T cells which could be attributed to defective survival and proliferation upon the loss of CD28-mediated co-stimulation. Interestingly, it has been reported that *CD28*-deficient mice can still generate T cell responses to viruses and allografts [183, 184], which is indicative of a CD28-independent element to T cell responses [185]. However, CD28-mediated co-stimulation is essential for the activation of T cells that provide B cell help for germinal centre and antibody class switching responses as *CD28*-deficient

mice lack germinal centres [186] due to an inability to optimally induce the chemokine receptor CXCR5, which directs T cell migration into B follicles [187].

The CTLA-4 receptor binds the same ligands as the CD28 co-stimulatory receptor, namely CD80 and CD86. Like the CD28 and CTLA-4 receptors, CD80 and CD86 are members of the immunoglobulin superfamily. CD80 and CD86 are cell surface glycoproteins expressed by antigen-presenting cells and the level of CD80 and CD86 expression seems to depend on the activation state of the cells (reviewed in [188]). Both CD80 and CD86 bind the same receptors and it was initially thought that these ligands were functionally equivalent [189, 190]. Both the CD28 and CTLA-4 receptors preferentially bind to CD80 over CD86, but the CTLA-4 receptor has a 20-100 fold higher affinity for both ligands compared to the CD28 co-stimulatory receptor [158]. However, the functional roles of CD80 and CD86 are still not fully understood. CD80 and CD86 are only about 25% identical in sequence (reviewed in [191]) and structural data suggest that CD80 exists as a dimer [192], whereas this has not been shown for CD86. Additionally, studies using knockout mice revealed that *CD86*-deficient mice were more severely immunocompromised than *CD80*-deficient mice, which suggests differential stimulatory and inhibitory functions for the two ligands [190]. However, when investigating the functional differences between CD80 and CD86 *in* vivo, blocking antibody studies have yielded conflicting results [193-195]. More recently, studies using Chinese hamster ovary (CHO) cell transfectants that express either CD80 or CD86 have revealed that CD80 is a more potent co-stimulator than CD86 [196].

1.4.2 CTLA-4 Receptor Function.

It is now regarded that the CTLA-4 receptor functions opposite the CD28 co-stimulatory receptor to negatively regulate T cell responses. Early studies implicated the CTLA-4 receptor in the augmentation of CD28-mediated co-stimulation as anti-CTLA-4 monoclonal antibodies were found to enhance the proliferation of anti-CD3/anti-CD28 activated T cells [197, 198]. However, it was soon realised that the stimulatory effects observed upon blockade of CTLA-4 could be attributed to the disruption of T cell negative control as the CTLA-4 receptor was found to inhibit antigen-driven, CD28 co-stimulated proliferation and IL-2 production by CD4+ T cells [199-202].

Negative regulation of T cell responses is clearly illustrated by *Ctla-4* gene ablated mice which develop a lethal lymphoproliferative disease and die by 3 to 4 weeks of age [156, 157]. *Ctla-4*-/- mice exhibit a massive expansion of polyclonal T cells leading to lymphocytic infiltration and tissue destruction of multiple organs, such as the heart, liver and pancreas. It has been demonstrated that the lymphoproliferative phenotype of *Ctla-4*-/- mice is mediated by CD4+ T cells as CD4+ T cell depletion abrogated disease [203]. Additionally, CD28 co-stimulation is required for this CD4+ T cell-mediated lymphoproliferative syndrome as CTLA-4-Ig treatment of *Ctla-4*-deficient mice prevents the fatal disease [204] and *Ctla-4*-/- mice that are also deficient in *CD80* and *CD86* do not develop the lymphoproliferative disorder [190, 205]. Similarly, this lethal disease is abrogated in mice deficient in both *Ctla-4* and *CD28* [206].

Using anti-CTLA-4 blocking antibodies and a murine model of experimental autoimmune encephalomyelitis (EAE), a role for the CTLA-4 receptor in mediating the inhibition of an ongoing immune response and regulating autoimmunity has been suggested [207]. This functional role of CTLA-4 in regulating the development of EAE was later attributed to control over the frequency of the primed CD4+ T cell population [208]. It has been proposed that the CTLA-4-mediated restriction of proliferating CD4+ T cell clonal expansion is cell-autonomous and regulated by the induction of CTLA-4 expression upon T cell activation [209]. A role for the CTLA-4 receptor in the maintenance of T cell homeostasis has also been shown using TCR-transgenic *Ctla-4*-/- mice in which the lethal lymphoproliferative disease associated with *Ctla-4*-deficiency was delayed and the antigen-specific CD4+ T cell population was activated and expanded [210].

Studying CTLA-4 is severely hampered by the fatal lymphoproliferative syndrome of *Ctla-4*-/- mice and research into the CTLA-4 receptor is therefore often carried out using blocking antibodies and *in vitro* tissue culture systems. Alternatively, *in-vivo,* disease in *Ctla-4*-/- mice is delayed or prevented by CTLA-4-Ig injection, anti-CD80/86 blocking antibody treatment or breeding onto a *CD80/86*-/- background. However, these methods also interfere with CD28 signalling, so results cannot be attributed directly to the loss of CTLA-4. To overcome the lethal lymphoproliferative syndrome that develops in *Ctla-4*-/- animals it is also possible to breed *Ctla-4*-deficient mice onto a TCR-transgenic background, directing

TCR specificity to a non-self antigen. To preclude rearrangements of second T cell receptors that might recognise self-antigens these animals can be maintained on a *Rag2*-deficient background, and accordingly TCR-transgenic *Ctla-4*-/- *Rag2*-/- mice do not develop lymphoproliferative disease and their peripheral T cells bear a naïve phenotype [210, 211]. This model system has proved useful in dissecting the role of CTLA-4 in non-regulatory T cells [52, 210, 211], although it cannot be used to elucidate the role of CTLA-4 in FOXP3+ Tregs since TCR-transgenic *Rag2*-/- animals do not develop Tregs due to the absence of cognate antigen expression in the thymus [212].

1.4.3 Mechanisms of CTLA-4 Receptor Function.

The underlying mechanism(s) of CTLA-4 receptor function are still not fully understood. Both cell-intrinsic and cell-extrinsic models of CTLA-4 receptor function have been proposed. Early studies with antibodies to CTLA-4 found that CTLA-4 ligation inhibited T cell proliferation and IL-2 production [199-202]. Following on from these results, it has been proposed that upon ligand-binding, the CTLA-4 receptor transduces a negative signal within the T cell that inhibits TCR / CD28 signalling and T cell activation. It has been suggested that the transduction of TCR and CD28 activating signals by protein kinases is regulated by the CTLA-4-mediated recruitment of phosphatases [213, 214]. More recently, another mechanism by which it has been hypothesised that the CTLA-4 receptor negatively affects TCR signalling is the inhibition of microcluster formation in T cells [215] because following TCR ligation, signalling molecules such as the protein kinase ZAP70 form clusters to transduce an activating signal to induce T cell proliferation and effector cytokine expression. In addition, two other cell-intrinsic models for CTLA-4 receptor function have been proposed that suggest the T cell response is negatively regulated by the disruption of immunological synapse formation. Firstly, it has been reported that CTLA-4 could block lipid-raft formation in response to TCR ligation, thereby disturbing the recruitment of adhesion and signalling molecules required to form a stable immunological synapse for T cell activation [216-218]. Secondly, it has been hypothesised that the CTLA-4 receptor is involved in reversing the TCR-induced ‘stop’ signal which is normally required to reduce T cell motility and allow the formation of a stable interaction with an antigen-presenting cell (reviewed in [219]).

Cell-intrinsic, negative signalling models of CTLA-4 receptor function are not consistent with the ability of CTLA-4-expressing cells to exert dominant tolerance over CTLA-4-deficient cells in mixed bone marrow chimeras [220, 221]. Furthermore, using mixed bone marrow chimeras, it has been shown that CTLA-4 is not involved in the down-regulation of specific T cell responses in a T cell-autonomous fashion because following viral infection, activated and expanded antigen-specific CTLA-4-deficient T cells contracted indistinguishably from antigen-specific CTLA-4-sufficient T cells [222]. Cell-extrinsic models of CTLA-4 receptor function in the down-regulation of T cell responses have also been proposed. As discussed above, not only does the CTLA-4 receptor share its ligands with the CD28 co-stimulatory receptor, but both CD80 and CD86 have preferential affinity for CTLA-4 compared with CD28. This has formed the basis of the ligand competition model for CTLA-4-mediated inhibition of T cell activation in which preferential binding of CTLA-4 to CD80 and CD86 would deprive T cells of CD28 co-stimulatory signals. Consistent with this hypothesis, transgenic expression of a truncated CTLA-4 receptor that lacks the cytoplasmic domain needed for signal transduction, ameliorated the severe lymphoproliferative disease seen in *Ctla-4* knockout mice [223]. Upon ligand binding, it has also been suggested that the CTLA-4 receptor could function in a cell-extrinsic manner by 'back-signalling' via CD80 and CD86 into antigen-presenting cells. CD80/CD86 engagement of CTLA-4 has been reported to up-regulate expression of the enzyme indoleamine 2,3-dioxygenase (IDO) in dendritic cells [224, 225]. IDO degrades the essential T cell proliferation metabolite, tryptophan and has therefore been proposed as an immunosuppressive mechanism [163]. However, *Ido* knockout mice do not exhibit the same severe lymphoproliferative syndrome phenotype as *Ctla-4*-deficient mice [226].

1.4.4 CTLA-4 and Regulatory T Cells.

It was first suggested that the CTLA-4 receptor may have a role in regulatory T cell function when it was found that CD4+ CD25+ Tregs constitutively express CTLA-4 [155]. The severe lymphoproliferative syndrome exhibited by *Ctla-4*-deficient mice is also consistent with a role for the CTLA-4 receptor in immune regulation and is similar to the *Foxp3*-/- scurfy mouse phenotype [60]. Moreover, a role for CTLA-4 in Treg suppressive function would be in line with the observation that CTLA-4 and FOXP3 need to be expressed by the same cell to provide optimal protection from the lethal lymphoproliferative diseases associated with

deficiency of both pathways [227]. In addition polymorphisms in the *Ctla-4* gene have been associated with several autoimmune diseases such as type 1 diabetes, thyroiditis, systemic lupus erythematosus and rheumatoid arthritis (reviewed in [228, 229]). However, functional studies into the role of CTLA-4 in Treg-mediated suppression have yielded contradictory results.

In-vitro analysis has found that that *Ctla-4*-deficient Tregs retain suppressive function [230]. Although, to circumvent the lymphoproliferative and activated cellular phenotype associated with *Ctla-4*-/- mice, this study carried out experiments with CD25+ CD4 single positive thymocytes rather than peripheral Tregs. It has also been demonstrated that *Ctla-4*-deficient regulatory T cells exert suppressive function *in vitro* because they develop a compensatory suppressive mechanism involving the production of TGF-β [231]. However, in this investigation *Ctla-4*-/- mice were treated with anti-CD80 and anti-CD86 monoclonal antibodies or expressed the *Ctla-4-Ig* transgene in order to delay the overt lymphoproliferative syndrome. Therefore, these regulatory T cells that were analysed for suppressive function developed in the absence of CD28 signalling, and it has been discussed above that a requirement for the CD28 co-stimulatory receptor in Treg development and homeostasis has been identified.

In vivo the role of the CTLA-4 receptor in regulatory T cell suppression of colitis has been investigated [232]. *Ctla-4*-/- Tregs were found to prevent colitis *in vivo*, however these Tregs were isolated from *Ctla-4*-/- mice that were also *CD80*- and *CD86*-deficient. Although, to circumvent this issue *Ctla-4*-deficient Tregs were isolated from mixed bone marrow chimeras using a congenic marker and these Tregs were similarly found to prevent the development of colitis *in vivo*. In contrast, in this same study, anti-CTLA-4 blocking antibody treatment abrogated Treg mediated suppression of colitis *in vivo*, even when appropriate measures were taken to ensure the monoclonal antibody only targeted the Treg population and did not simultaneously affect the conventional T cell population. The NOD mouse model of autoimmune diabetes has also been used to assess the role of the CTLA-4 receptor in regulatory T cell suppressive function [233]. Blocking anti-CTLA-4 antibody treatment was found to accelerate progression of diabetes in NOD mice. Control adoptive transfer experiments using NOD-SCID mice found that the anti-CTLA-4 monoclonal antibody

preferentially targeted the Treg population as antibody treatment following adoptive transfer of effector cells alone did not accelerate diabetes progression, whereas antibody treatment abrogated the suppressive function of co-transferred Tregs.

Overall, the role of the CTLA-4 receptor in regulatory T cell suppressive function is still not clear. Also, it is not known if, like its homologue CD28, the CTLA-4 receptor has a functional role in the generation of regulatory T cells. Although, it has been reported that CD25+ cells isolated from *Ctla-4*-deficient mice express *Foxp3* mRNA [231]. Additionally, if the CTLA-4 receptor has a role on regulatory T cells it is not known whether this is mechanistically Treg-intrinsic to modulate TCR signalling or Treg-extrinsic to modulate antigen-presenting cells. Many of the studies into the mechanism of CTLA-4 receptor function that have been discussed above in 1.4.2 investigated activated (CTLA-4-expressing) CD4+ FOXP3- conventional T cells or the CD4+ T cell population as a whole and did not specifically analyse the CD4+ FOXP3+ regulatory T cell population. It is clear that many of the proposed mechanisms for CTLA-4 receptor inhibition of T cell activation could also be applicable to regulatory T cell suppressive function. For instance, CD4+ CD25+ regulatory T cells have been shown to down-regulate CD80 and CD86 on dendritic cells [159]. Also, CD4+ CD25+ Tregs have been reported to modulate tryptophan catabolism via the induction of IDO in dendritic cells [161]. Therefore, there are still many unanswered questions regarding the CTLA-4 receptor biology of regulatory T cells.

1.5 Aims and Objectives

CD4+ FOXP3+ regulatory T cells are a key lymphocyte population in the maintenance of immunological tolerance. It is therefore important to understand the molecular pathways that control regulatory T cell homeostasis and function. This investigation focused in particular on the role of the CTLA-4 receptor in regulatory T cell biology. The main objectives of this project were to understand the contribution of the CTLA-4 receptor to Treg selection in the thymus, peripheral Treg homeostasis (cellular proliferation and FOXP3 induction) and Treg suppressive function. Overall, this project aimed to provide new insight into the role of CTLA-4 in the regulation of T cell immune responses, in particular those that could result in autoimmunity.

2.0 MATERIALS AND METHODS

2.1 Mice

2.1.1 Mice. BALB/c, *DO11.10* TCR transgenic and *CD28*-/- mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Rat insulin promoter (RIP)-*mOVA* mice on a BALB/c background expressing membrane OVA under the control of the rat insulin promoter (from line 296-1B) were a gift from W. Heath (Walter and Eliza Hall Institute, Melbourne, Australia). *Rag2*-/- mice were purchased from Taconic Laboratories (Germantown, NY). *Ctla-4*-/- mice were provided by Arlene Sharpe (Harvard, Boston, MA). *Ctla-4*-/- mice were bred heterozygously. *DO11.10* mice and RIP-*mOVA* mice were bred to a *Rag2*-/- background to generate *DO11.10 Rag2*-/- and RIP-*mOVA Rag2*-/- mice. *Ctla-4*-/- and *CD28*-/- mice were backcrossed with *DO11.10 Rag2*-/- mice to generate *DO11.10 Rag2*-/- *Ctla-4*-/- and *DO11.10 Rag2*-/- *CD28*-/ mice. *DO11.10 Rag2*-/- and *DO11.10 Rag2*-/- *Ctla-4*-/- were crossed with RIP-*mOVA Rag*2-/- to generate *DO11.10* x RIP-*mOVA Rag2*-/- and *DO11.10* x RIP-*mOVA Rag2*-/- *Ctla-4*-/- progeny. *Foxp3*+/- females were kindly provided by A. Rudensky and used to obtain *Foxp3*-/- mice. *OX40*-/- mice were provided by M. Bachmann. *CD30*-/- mice were provided by H. Takimoto. *OX40*-/- C*D30*-/- mice were generated by breeding the two single knockout strains and backcrossing heterozygote offspring. *Rank*-/- mice on the C57BL/6 background were provided by J. Penninger. C57BL/6 mice were used from the Birmingham Biomedical Services Unit stock. Mice were housed at the Birmingham Biomedical Services Unit and were used according to Home Office regulations.

2.1.2 Adoptive T Cell Transfers. For assessment of *Ctla-4*-/- Treg pathogenicity: 1-2 x 10^6 *Ctla-4*-/- lymphocytes or high speed cell sorted (Mo-Flo, DakoCytomation) CD4+ CD25- conventional T cells and CD4+ CD25hi regulatory T cells from lymph nodes of BALB/c wild type and *Ctla-4*-/- mice were adoptively transferred into *Rag2*-/- mice. These were injected intravenously in 400µl of phosphate buffered saline (PBS) (Sigma). For assessment of wild type Treg control of *Ctla-4*-/- disease: *Ctla-4*-/- lymphocytes alone or plus 0.6 x 10^6 MACS separated CD4+ CD25- conventional T cells or CD4+ CD25+ regulatory T cells from lymph nodes of BALB/c wild type mice were adoptively transferred into *Rag2*-/- mice. These were injected intravenously in 400µl of PBS. For adoptive transfer of *Ctla-4*-/- lymphocytes into

BALB/c or *CD28*-/- mice: 50 x 10^6 *Ctla-4*-/- lymphocytes were injected intravenously in 400µl of PBS into BALB/c and *CD28*-/- mice. For adoptive transfer model of autoimmune diabetes: 0.2 x 10^6 high speed cell sorted CD4+ CD25- from lymph nodes of *DO11.10* x RIP-*mOVA Rag2*-/- were injected intravenously in 400µl of PBS into RIP-*mOVA Rag2*-/- mice alone or plus 0.2 x 10^6 high speed cell sorted CD4+ CD25+ from lymph nodes of *DO11.10* x RIP-*mOVA Rag2*-/- or *DO11.10* x RIP-*mOVA Rag2*-/- *Ctla-4*-/- mice. Weight loss is expressed as a percentage of maximal weight, and LN counts refer to pooled axillary, inguinal, brachial and mesenteric lymph nodes.

2.1.3 Bone Marrow Chimeras. 8 x 10^6 THY1 depleted bone marrow (MACS depletion) from BALB/c or *Ctla-4-/-* mice was injected intravenously in 400µl of PBS into *Rag2*-/- mice (irradiated with 5Gy) either alone or as a BALB/c : *Ctla-4-/-* 50:50 mix.

2.1.4 *In Vivo* Anti-CTLA-4 / Anti-ICOSL Blocking Antibody Treatment. Six to eight week old BALB/c mice were administered with 500µg of blocking anti-CTLA-4 monoclonal antibody (4F10, gift from J. Bluestone, University of California, San Francisco) or 200µg of anti-ICOSL blocking antibody (HK5.3, ebioscience) or control hamster IgG by intra-peritoneal injection twice weekly. 4F10 was grown in Miniperm bioreactors (Grenier Bio-One) using IgG-depleted FCS and purified by passing over staphylococcal protein A (GE Healthcare).

2.1.5 *In Vivo* OVA Treatment. Six to eight week old *DO11.10 Rag2*-/-, *DO11.10 Rag2*-/- *Ctla-4*-/- and *DO11.10 Rag2*-/- *CD28*-/- mice were injected (intra-peritoneal) with 50µg Imject Ovalbumin (Thermo Scientific) in 100ul PBS or equivalent volume of PBS as a control. Treatment was administered on day 0, 1, 2, 5 and 6 and tissue was harvested on day7.

2.1.6 *In Vivo* BrdU Treatment. Two week old BALB/c mice were given 1mg 5-bromo2'-deoxy-uridine (BrdU) (BD Pharmingen) in 100µl by intra-peritoneal injection every twenty-four hours for three days. Mice were harvested twenty-four hours following last injection.

2.1.7 Diabetes Monitoring. Blood glucose was measured using Ascensia Elite XL blood glucose meter (Bayer, USA). Overt diabetes was classed as having a blood glucose ≥250mg/dl.

2.2 Immunohistology

2.2.1 Section Preparation. Spleens were flash frozen in liquid nitrogen and stored at -80°C until use. Tissue was sectioned by cryostat at -20°C. Serial sections 5μm in thickness were cut onto glass slides (Hendley-Essex). Sections were air dried for 30 minutes, fixed in 100% cold acetone (Scientific and Chemical supplies) at 4°C for 20 minutes and dried at room temperature for 10 minutes. Slides were stored in sealed polyethylene bags at -20°C until use.

2.2.2 Immunochemistry. Slides were re-hydrated in PBS before use. Sections were then stained with primary antibodies directed against murine epitopes for one hour at room temperature (Table 1). Sections were washed in TRIS (pH7.2) (Sigma) and biotin- or HRP-conjugated secondary antibodies pre-adsorbed with normal mouse serum added for 45minutes at room temperature (Table 2). HRP-conjugated antibodies were finally detected with Diaminobenzidine (DAB) solution (Sigma). Biotinylated antibodies were detected with streptavidinABComplex-alkaline phosphate (DakoCytomation), followed by Naphthol AS-MX Phosphate–substrate and Fast Blue BB salt with Levamisole (all Sigma). Images were taken with a Nikon eclipse E400 light microscope and Nikon coolpix 950 digital camera and red, green, blue levels adjusted in Adobe Photoshop version 8.0. Images processed for presentation with ImageJ (Rasband W.S., ImageJ, U.S. National Insitute of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/ 1997-2009)

2.2.3 Immunofluorescence. As for immunochemistry, slides were re-hydrated in PBS before use. Sections were then blocked with 10% goat serum (Sigma) or if goat primary antibodies were used sections were blocked with 10% donkey serum (Sigma). Sections were then stained with primary antibodies directed against murine epitopes for one hour at room temperature (Table 1). Sections were washed in PBS (Sigma) and secondary antibodies pre-adsorbed with normal mouse serum added for 45minutes at room temperature (Table 2). For three-step amplifications, tertiary antibodies were added for 40minutes at room temperature (Table 2). Slides were mounted with Vectorshield (Vector Laboratories) and images were obtained by confocal microscopy (Zeiss LSM 510). Images processed with ImageJ (Rasband W.S., ImageJ, U.S. National Insitute of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/ 1997-2009)

2.2.4 H & E Staining. Tissue was placed in 10% formalin (Sigma, 4% formaldehyde w/v) at 4°C for 5 days. Processing of tissue, cutting of sections and hematoxylin and eosion staining was kindly carried out by the pathology department.

2.2.5 Antibodies.

Specificity	Clone	Host	Conjugate	Supplier	Dilution
CD11c	N418	Hamster	Purified	Purified from Supernatant	1/10
CD4	GK1.5	Rat	Purified	BD	1/1000
CD4		Rat	AF-647	Conjugated by D. Withers	1/100
CD86	GL1	Rat	FITC	BD	1/100
FOXP3	FJK-16s	Rat	Biotinylated	ebioscience	1/50
IgD		Sheep	Purified	The Binding Site, Birmingham, UK	1/200
Ki67		Rabbit	Purified	J. Gerdes, Borstel, Germany	1/500
PNA			Biotinylated	Vector Labs	1/150

Table 1. Primary antibodies used in immunohistology.

Specificity	Host	Conjugate	Supplier	Amount / Dilution
biotin		Streptavidin AF555	Invitrogen	1/1000
FITC	Rabbit	Purified	Dako	1/500
Hamster	Goat	Cy5	Jackson Labs	1/60
Rabbit	Donkey	AF488	Invitrogen	1/100
Rat	Rabbit	Biotinylated	Dako	1/600
Sheep / Goat	Donkey	Peroxidase	The Binding Site, Birmingham, UK	1/100

Table 2. Secondary antibodies used in immunohistology.

2.3 Flow Cytometry

2.3.1 Cell Isolation. Single cell suspensions were isolated from peripheral lymph nodes (LNs), spleens and thymi by mashing through a wire mesh with cold PBS containing 2% FCS (Sigma). For spleens and thymi this was followed by red blood cell removal by incubation for ten minutes at 37°C in 3-7ml of lysis buffer (see appendix), cells were washed thoroughly in ice-cold PBS containing 2% FCS to stop the reaction. In some instances spleens were digested with 0.5mg/ml collagenase / dispase (Roche), 0.25mg/ml Liberase (Roche) and 0.1mg/ml DNase (Sigma) in complete medium (C10) (see appendix) for 45minutes at 37°C prior to mashing though a wire mesh and red blood cell lysis. To isolate non-thymocytes thymi were teased apart and digested with 0.5mg/ml collagenase / dispase (Roche) in C10 for 45minutes at 37°C on a shaker. A second digest with 1X trypsin (Sigma) in 0.02% EDTA (Sigma) was carried out at 37°C for ten minutes. Samples were filtered by passing through a 40μm cell strainer (BD Biosciences). Lymphocytes from the pancreas were obtained by tearing the pancreas in cold PBS containing 5% FCS, 56mM glucose (Sigma), 2μg/ml aproptinin (Roche) and 50μg/ml TLCK (Roche). Following centrifugation, samples were resuspended in 2ml pre-warmed PBS containing 15% FCS and Liberase CI (Roche). Samples were passed through a 40μm cell strainer (BD) and lymphocytes were recovered by centrifugation at 1000 x g, 4°C for 20 minutes over lympholyte-M (Cedarlane Laboratories, Ontario, Canada) and then washed in PBS containing 2% FCS. Cells from the draining and non-draining pancreatic lymph nodes (PanLNs) were obtained by tearing open the tissue in PBS containing 2% FCS.

2.3.2 Staining Protocol. Cells were surface stained for fifteen minutes at 4°C with 5% goat serum and washed with 1ml PBS containing 2% FCS. For intracellular CTLA-4, Ki67, FOXP3 and cytokine staining, cells were fixed and permeabilised according to the manufacturer's instructions (eBioscience) with reagents containing paraformaldehyde and saponin and then intracellular antibodies were added. For intracellular cytokine staining 2×10^6 cells per well (24 well plate) were cultured with 50ng/ml Phorbol Myristate Acetate (PMA) (Sigma) and 1.5μM Ionomycin (Sigma) for 4-6hours. After one hour 1μl/ml of Golgi Stop (BD Pharmingen) was added per well. For intracellular BrdU staining, cells were fixed and permeabilised according to the manufacturer's instructions (BectonDickinson) with reagents containing paraformaldehyde and saponin. 100μl of 300μg/ml DNase (BD

Pharmingen BrdU Kit) was added and cells incubated for one hour at 37°C in a water bath and then intracellular antibodies were added. For Annexin V staining, cells were resuspended in 100ul of cold binding buffer (see appendix) and incubated with annexin V antibody for 15 minutes at room temperature before another 500ul of cold binding buffer was added before analysis. All cell samples were analysed by flow cytometry (FACScalibur™ or DakoCytomation™ and FlowJo™ software) (BectonDickinson).

2.3.3 Antibodies.

Specificity	Clone	Conjugate	Supplier	Dilution
ANNEXIN V		FITC	BD 556419	5µl
B220	RA3-6B2	PE	BD 553090	1/100
Bcl-2		FITC	BD 51-15024X	18µl
Biotin		Streptavidin APC	BD 554067	1/100
Biotin		Streptavidin PE	BD 554061	1/100
CD11c	N418	FITC	ebioscience 11-0114-82	1/50
CD11c	N418	PE	ebioscience 12-0114-82	1/50
CD19	1D3	APC	BD 550992	1/200
CD19	1D3	FITC	BD 553785	1/200
CD25	PC61	APC	BD 557192	1/100
CD25	PC61.5	PE-Cy7	ebioscience 25-0251-82	1/50
CD25	PC61	PE	BD 553866	1/100
CD27	LG.7F9	APC	ebioscience 17-0271-82	1/50
CD28	37.51	Biotin	BD 553296	1/10
CD3e	145-2C11	PerCP	BD 553067	1/75
CD3e	145-2C11	PE-Cy5.5	ebioscience 35-0031-82	1/75
CD4	RM4-5	PerCP	BD 553052	1/50
CD4	GK1.5	FITC	BD 553729	1/100
CD40	1C10	Biotin	ebioscience 13-0401-85	1µl
CD45.2	104	FITC	ebioscience 11-0454-82	1/200
CD62L	MEL-14	APC	BD 553152	1/50
CD62L	MEL-14	PE-Cy7	ebioscience 25-0621-82	1/50

CD69	H1.2F3	FITC	BD 553236	1/50
CD69	H1.2F3	PE	BD 553237	1/50
CD8	53-6.7	FITC	BD 553031	1/100
CD80	16-10A1	FITC	BD 553768	1/50
CD80	16-10A1	PE	BD 553769	1/50
CD86	GL1	FITC	BD553691	1/50
CD86	GL1	PE	BD 553692	1/50
CD8a	53-7.6	APC	ebioscience 17-0081-82	1/100
CTLA-4	UC10-4F10-11	PE	BD 553720	1µl
CXCR5	2G8	Biotin	BD 551960	2.5µl
EpCam	G8.8	AF-647	Hybridoma gift from Moon Kim	1/700
FAS		PE		1/75
FOXP3	FJK.16S	APC	ebioscience 17-5773-82	2µl
FOXP3	FJK.16S	Pacific Blue	ebioscience 57-5773-80	2µl
FOXP3	FJK-16S	PE	ebioscience 12-5773-82	1.5µl
FOXP3	FJK-16S	FITC	ebioscience 11-5773-82	1µl
I-Ad	AMS-32.1	Biotin	BD 553546	1/200
ICOS	7E.17G9	Biotin	ebioscience 13-9942-81	1/50
ICOS	7E.17G9	PE	ebioscience 12-9942-82	1/50
ICOSL	HK5.3	PE	ebioscience 12-5985-81	1/50
IFN-γ	XMG1.2	APC	BD 554413	1µl
IFN-γ	XMG1.2	PE	BD 554412	1µl
IFN-γ	XMG1.2	AF-488	BD 557724	1µl
IL-17	TC11-18H10	PE	BD 559502	1µl
IL-17	TC11-18H10	FITC	BD 560221	1µl
IL-2	JES6-5H4	FITC	BD 554427	1µl
IL-2	JES6-5H4	PE	BD 554428	1µl
IL-4	11B11	APC	BD 554436	1µl
KJ	DO11.10	PE	CALTAG mm7504	1/50
PD-1	J43	PE	ebioscience 12-9985-82	1/50

THY1.1	H1551	APC	ebioscience 17-0900-82	1/400
THY1.2	53-2.1	APC	ebioscience 17-0902-83	1/400
TRANCE	IK2215	Biotin	ebioscience 13-5952-82	1µl

Table 3. Antibodies used in flow cytometry.

2.4 Cell Separation / Sorting

2.4.1 Magnetic Cell Separation (MACS). Single cell suspensions were obtained from spleen or lymph nodes as described. For positive selections cells were labelled with 10ul microbeads (Miltenyi Biotec) per 10^7cells and passed through an LS column. The magnetically labelled cells were retained in the column, and eluted to give the desired fraction. For negative selections cells were labelled with 15µl of biotin-conjugated antibody cocktail (Miltenyi Biotec) per 10^7 cells, and passed through an LD column. The magnetically labelled cells were retained in the column and the flow-through collected as the desired fraction.

2.4.2 Fluorescent Activated Cell Sorting (FACS). Single cell suspensions were isolated from lymph nodes as described. Cells were surface stained in 600µl C10, for 10 minutes on ice before being passed through a 40µm cell strainer (BD Biosciences). Desired cell populations were purified by high speed cell sorting (Beckman Coulter High Speed Cell Sorter). Thank you to Roger Bird for carrying out the cell sorting.

2.5 *In Vitro* Cell Culture

2.5.1 Overnight Cultures.

For CD28-depedency assays 1 x 10^5 wild type BALB/c or *CD28*-/- MACS separated CD4+ lymphocytes were cultured overnight in a 96-well, round-bottomed plate plus or minus 500ng/ml anti-CD3 (BD Pharmingen) and 1µg/ml anti-CD28 (BD Pharmingen) at 37°C, 5% CO_2. The next day wells were harvested by washing with PBS and stained with CD4, ICOS, CD69, TRANCE and intracellular FOXP3, CTLA-4 for analysis by flow cytometry as described above.

For *in vitro* overnight culture with lipopolysaccharide (LPS) 1 x 10^5 wild type BALB/c splenocytes were cultured overnight in a 96-well, round-bottomed plate with 10µg/ml LPS (Sigma) at 37°C, 5% CO_2. The next day wells were harvested by washing with PBS and stained with CD3, CD19, CD80 and CD86 for analysis by flow cytometry as described above.

For analysis of ICOS expression in a '*Ctla-4*-/- environment' 3 x 10^5 lymphocytes from 18 day old BALB/c or *Ctla-4*-/- mice were cultured in a 96-well, round-bottomed plate at 37°C, 5% CO_2 for two days. Supernatants were removed and added to 2 x 105 wild type BALB/c lymphocytes which were cultured overnight in a 96-well, round-bottomed plate at 37°, 5% CO_2. The next day wells were harvested by washing with PBS and stained with CD4, ICOS, CD69 and intracellular FOXP3 for analysis by flow cytometry as described above.

2.5.2 CTLA-4 Cycling.

Wild type BALB/c lymphocytes were surface stained with CD4 and CD25 for analysis by flow cytometry as described above. 1 x 10^6 lymphocytes were cultured in a 96-well, round bottomed plate plus or minus 50ng/ml Phorbol Myristate Acetate (PMA) (Sigma) and 1.5µM Ionomycin (Sigma) with 1µl anti-CTLA-4-PE antibody at 37°C, 5% CO_2 for various lengths of time. For harvest wells were washed with PBS and stained intracellular with CD4 and FOXP3 for analysis by flow cytometry as described above.

2.5.3 TGF-β Induction.

A 48-well, flat-bottomed plate was pre-coated with 10µg/ml anti-CD3 (BD Pharmingen) in PBS and stored at -4°C overnight. The anti-CD3 was removed to leave plate-bound anti-CD3 and 2.5 x 10^5 lymphocytes from *DO11.10 Rag2*-/- mice that were either wild type, *Ctla-4*-/- or *CD28*-/- were cultured in the pre-coated 48-well plate with 1.25 x 10^6 irradiated BALB/c

splenocytes (20Gy) plus or minus 3ng/ml TGF-β (Peprotech) and 100 I. U. recombinant IL-2 (Peprotech) at 37°C, 5% CO_2 for 3 days. For harvest wells were washed with PBS and stained with CD4, KJ-126, CD25 and intracellular FOXP3 for analysis by flow cytometry as described above.

2.5.4 Suppression Assays. Cells were cultured in triplicate in round-bottomed 96 well plates in complete medium (see appendix). CD4+ CD27+ CD25- conventional T cells were added at 2.5 x 10^4 per well. In co-culture experiments, purified CD4+ CD27hi CD25hi Tregs were added at a ratio of 1:1, 0.5:1 or 0.25:1 (Tregs:Tcells). $CD19^+$ purified APCs were added at a ratio of 1:6 (Tcells:APCs). Cells were stimulated with 1µg/ml of soluble anti-CD3 (BD Pharmingen). After 72 hours cells were harvested and stained with CD4, THY1.1 and intracellular FOXP3 for analysis by flow cytometry as described above.

2.5.5 Anergy Assay.

Congenically marked THY1.1+ MACS separated CD4+ CD25- conventional T cells were co-cultured with wild type BALB/c or *Ctla-4*-/- CD4+ CD25hi CD27hi Tregs at a 1:1 ratio as described for the suppression assays above. On day 3, THY1.1+ conventional T cells were re-isolated by MACS positive selection (THY1.1-PE antibody 1/300, 10µl anti-PE MACS beads) and 2.5 x 10^4 cells re-stimulated in 96-well, round-bottomed plates with 1 x 10^5 irradiated BALB/s splenocytes (20Gy), 1µg/ml anti-CD3 (BD Pharmingen) and 100 I.U. recombinant IL-2 (Peprotech) at 37°C, 5% CO_2 for 3 days, 1µCi tritiated thymdine (Amersham, GE Healthcare) was added for the last 6 hours of culture before harvest.

2.5.6 T Cell / Treg and B Cell / DC Co-cultures.

4 x 10^6 MACS separated BALB/c splenic CD19+ or CD11c+ cells were co-cultured with 8 x 10^4 MACS separated BALB/c CD4+ CD25- T cells or CD4+ CD25+ Tregs or both, plus or minus 50µg/ml anti-CTLA-4 blocking antibody (4F10, gift from J. Bluestone, University of California, San Francisco) at 37°C, 5% CO_2. Cells were harvested and stained with CD3, CD19, CD11c, I-Ad and CD86 for analysis by flow cytometry as described above.

2.6 Molecular Biology

2.6.1 RNA Isolation.

Frozen cell pellets were thawed and the RNA was isolated from the cells by RNAzol (Biogenesis Ltd) homogenisation (800µl at room temperature), chloroform extraction (80µl for 5minutes on ice) and isopropanol precipitation (15minutes at -20°C).

2.6.2 cDNA Preparation.

Immediately following RNA extraction, samples were heated to 70°C for 5 minutes before transferring to ice. Reverse transcription PCR was then performed by using M-MLV avian reverse transcriptase and oligo(dT) primers (Invitrogen, Paisley, UK).

2.6.3 qPCR. Primers and probes of pre-made TaqMan Gene Expression Assays purchased from Applied Biosystems (see table 4) and used in accordance with manufacturers' instructions. Primer and probe titrations were determined and reactions made with PCR 2x mastermix (ABI) in 96 well microAmp plates (ABI). Gene expression was quantified using a Mx3000 PCR machine (Stratagene) and normalised to β-actin levels. β-actin primer and probe (Eurogentec): F: 5' CGT GAA AAG ATG ACC CAG 3' (0.07µm). R: 5' TGG TAC GAC CAG AGG CAT 3' (0.05µm). 1 cycle 50°C for 2minutes, followed by 1 cycle 95°C for 10minutes, followed by 40 cycles 95°C for 15seconds and 60°C for 1minute.

Quantitative SYBR Green PCR with RANK primers was conducted with SensiMix No-Ref SYBR Green Kit (Quantace) and used in accordance with the manufacturer's instructions and normalised to β-actin levels. RANK primers: F: 5' GCT GGC TAC CAC TGG AAC TC 3' (0.2µm). R: 5' GTG CAG TTG GTC CAA GGT TT 3' (0.2µm). 1 cycle 95°C for 10minutes, followed by 39 cycles 95°C for 15seconds, 62°C for 20seconds and 72°C for 5 seconds. Then melting curve of 30seconds up to 73°C, then next 37 cycles 5seconds increasing 1°C each time. Thank you to Sonia Parnell for carrying out the SYBR green qPCR.

2.6.4 TaqMan Gene Expression Assays.

Gene Name	TaqMan Assay ID
TGF-β	Mm03024053_m1
IL-17a	Mm00439619_m1
IL-21	Mm00517640_m1

Table 4. Applied Biosystems TaqMan assays.

2.7 Statistics

Unpaired T-tests were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA). All tests were two-tailed with 95% confidence intervals. $p \leq 0.05$ for statistical significance.

3.0 Role of CTLA-4 in Regulatory T Cell Development and Homeostasis

3.1 Introduction

CTLA-4 was first thought to be a co-stimulatory receptor because early studies using anti-CTLA-4 monoclonal antibodies interpreted enhanced proliferation of anti-CD3/anti-CD28 activated T cells as evidence for augmented CD28-mediated co-stimulation [197, 198]. It is now realised that the stimulatory effects observed upon blockade or loss of CTLA-4 can be attributed to the disruption of T cell negative control [199, 200]. The two receptors CD28 and CTLA-4 are now regarded to have functionally opposite roles in T cell responses.

Like the CD28 co-stimulatory receptor, CTLA-4 is predominantly found to be expressed by T lymphocytes. It is now known that CTLA-4 is constitutively expressed by the CD4+ CD25+ regulatory T cell subset [154, 155]. However, the role of CTLA-4 in Treg development and homeostasis is unclear. CTLA-4 binds to the same ligands (CD80 and CD86) as the CD28 co-stimulatory receptor. CD28 signalling has been shown to control both the thymic development and peripheral homeostasis of Tregs [86, 131]. On the other hand, it has previously been shown that thymic selection and thymocyte development is grossly normal in *Ctla-4*-deficient mice [87, 88]. However, these studies did not specifically assess the development of FOXP3+ Tregs in *Ctla-4* knockout mice as they were carried out prior to the identification of the transcription factor *Foxp3* as a specific marker for Tregs [60].

Due to the fatal lymphoproliferative disease that *Ctla-4* knockout mice succumb to around three weeks of age [156, 157], frequently, CD28 signalling is simultaneously abrogated by CD80/86 blockade or deficiency when studying *Ctla-4*-deficient mice [231, 232]. As discussed above, CD28 co-stimulation has been found to be required for the thymic generation and peripheral maintenance of regulatory T cells [86], therefore the results of these studies cannot be solely attributed to a lack of CTLA-4. Consequently, to investigate the role of CTLA-4 in regulatory T cell development and homeostasis, *Ctla-4*-deficient mice with unaltered CD28 signalling were analysed using FOXP3 monoclonal antibodies.

3.2 Results

3.2.1 Cellular expression of the CD28 and CTLA-4 receptors, and their ligands CD80 and CD86.

In order to understand a potential role for CTLA-4 on regulatory T cells, it was important to study the CTLA-4 receptor in the context of its ligands, CD80 and CD86, and also its homologue; the CD28 co-stimulatory receptor. An early objective of the project was therefore to analyse the cellular distribution of CTLA-4, CD28 and their ligands (CD80 and CD86). Since Tregs were to be identified by expression of the FOXP3 transcription factor, the specificity of the FOXP3 monoclonal antibody was first validated by staining wild type C57BL/6 mice and *Foxp3*-/- lymphocytes for analysis by flow cytometry. CD28 and CTLA-4 antibodies were similarly assessed using *Ctla-4*-/- and *CD28*-/- lymphocytes along with appropriate wild type BALB/c controls. It can be seen in *Fig. 3.01* that the FOXP3 (*A*), CD28 (*B*) and CTLA-4 (*D*) antibodies all specifically stained wild type, control lymphocytes as opposed to lymphocytes from the respective knockout mice. Also, the streptavidin secondary conjugate used to detect CD28 showed negligible non-specific binding (*C*). As well as validating the specificity of the monoclonal antibodies, this data confirmed gene ablation in the *Foxp3*, *CD28* and *Ctla-4* knockout mice.

It is known that T cells constitutively express the CD28 co-stimulatory receptor (reviewed in [188]). Flow cytometry was used to investigate the distribution of CD28 expression among the various T cell subtypes. It can be clearly seen in *Fig. 3.02* that CD4+ FOXP3- conventional T cell, CD4+ FOXP3+ regulatory T cell (*A*) and CD8+ T cell (*B*) populations all constitutively expressed a similar amount of the CD28 co-stimulatory receptor. The level of CD28 expression on CD8+ T cells was slightly higher than CD4+ T cells as indicated by the median fluorescent intensity of CD28-specific staining by flow cytometry. Within the CD4+ T cell population the median fluorescent intensity of CD28-specific staining on FOXP3+ cells was slightly lower than on FOXP3- cells.

CD28 is constitutively expressed by T cells, whereas CTLA-4 is not highly expressed by resting conventional T cells. Rather, upon activation *de novo* transcription of CTLA-4 is induced [185] and still, cell surface expression of the receptor is kept low by endocytotic

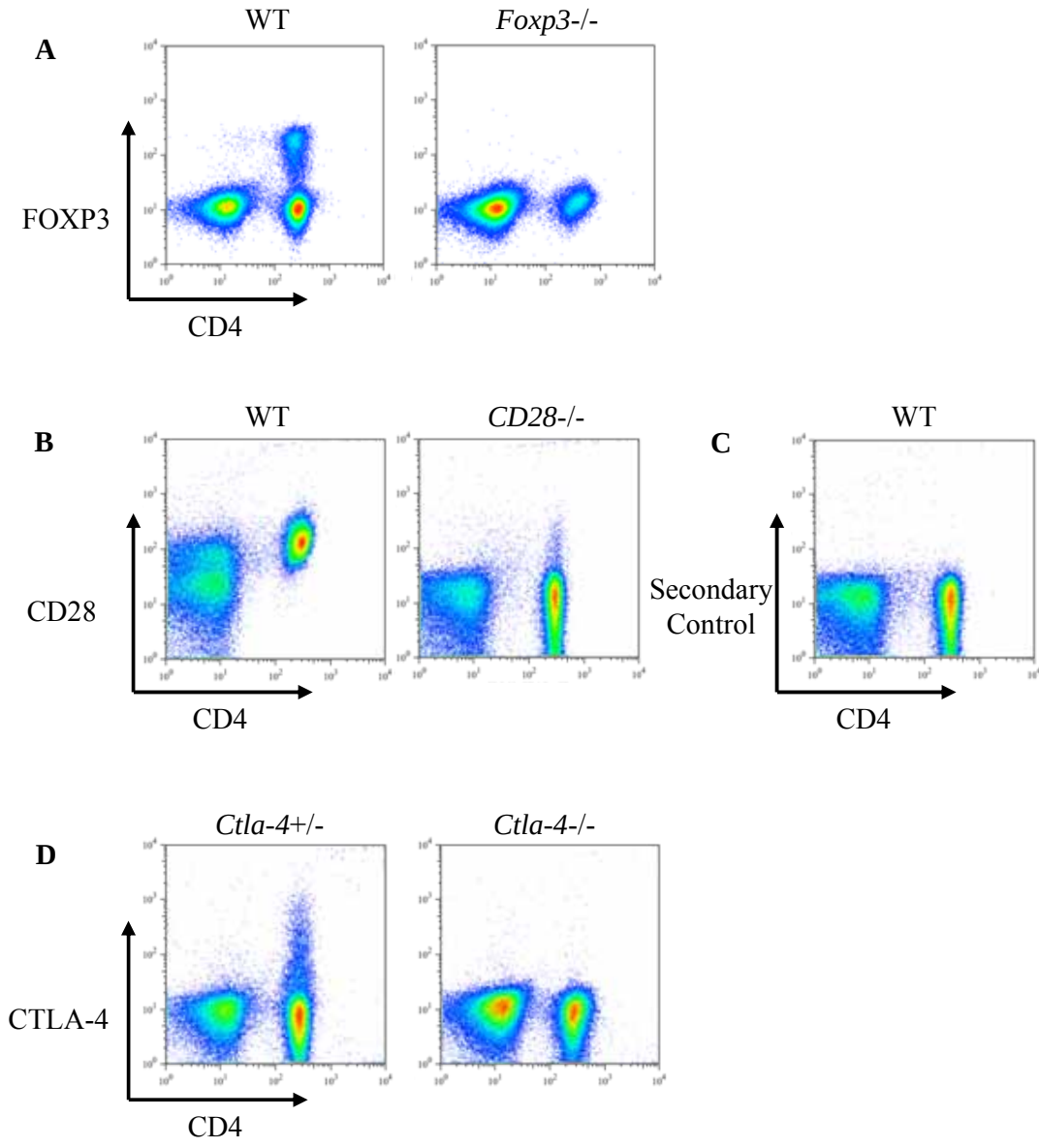


Figure 3.01. Validation of FOXP3, CD28 and CTLA-4 monoclonal antibodies by flow cytometry. Single cell suspensions from peripheral lymph nodes were surface stained with CD4, CD28 and intracellularly with CTLA-4 and FOXP3 for analysis by flow cytometry. Representative FACS plots of (A) three week old wild type C57BL/6 mice (left) and *Foxp3*-/- mice (right), (B) adult wild type BALB/c mice (left) and *CD28*-/- mice (right), (C) adult wild type BALB/c mice for secondary alone and (D) 15-18 day *Ctla-4*+/- mice (left) and *Ctla-4*-/- mice (right). All FACS plots show the live lymphocyte gate. Data representative of ≥5 mice.

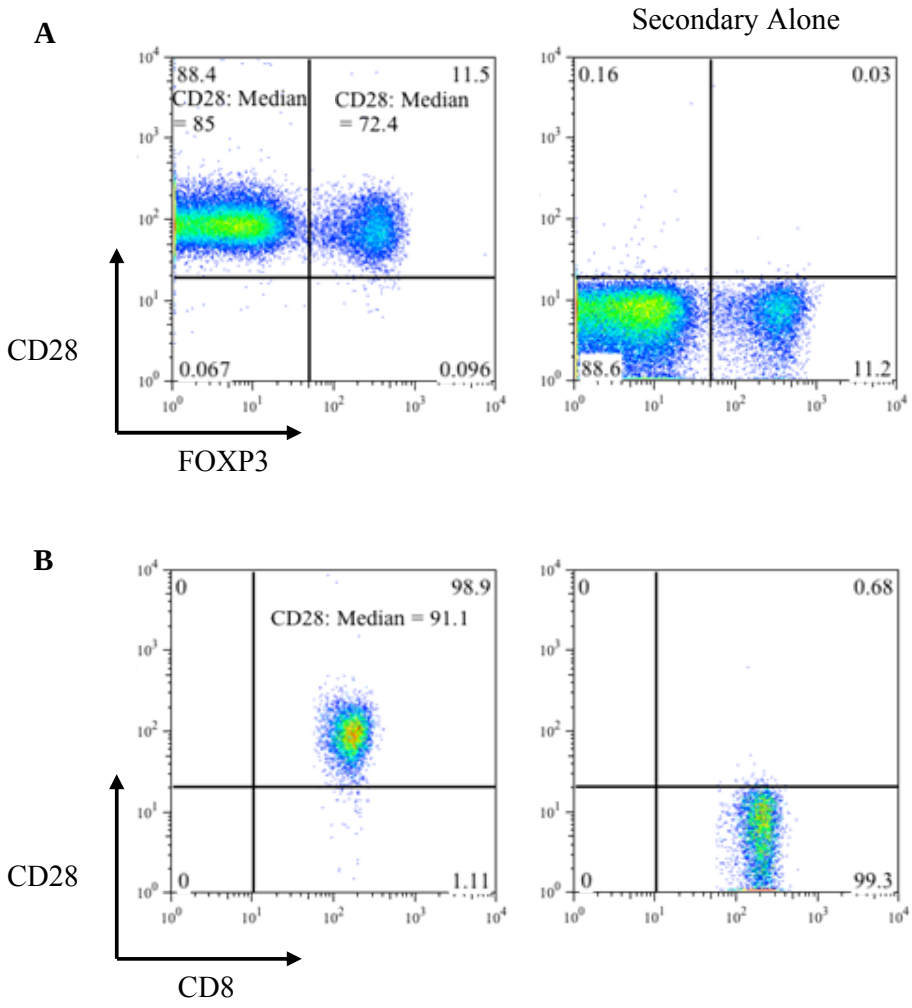


Figure 3.02. Cellular distribution of CD28 co-stimulatory receptor expression. Single cell suspensions from peripheral lymph nodes were surface stained with CD4, CD8, CD28 and intracellularly with FOXP3 for analysis by flow cytometry. Representative FACS plots for (A) CD4+ gated lymphocytes of adult wild type BALB/c mice (left) and for secondary alone (right) and (B) CD8+ gated lymphocytes of adult wild type BALB/c mice (left) and for secondary alone (right). Data representative of 3 mice. Median fluorescence intensity also shown.

mechanisms [234]. It is known that CD4+ CD25+ Tregs constitutively express the CTLA-4 receptor [154, 155]. However, these studies were carried out prior to the identification of the transcription factor *Foxp3* as a specific marker for Tregs, and the development of FOXP3-specific monoclonal antibodies. Therefore, Tregs were previously identified as the CD4+ CD25+ T cell population, which could include some activated (CD25+) conventional T cells (CD4+ FOXP3-). Consequently, the surface and intracellular expression of CTLA-4 by Tconv and Treg populations identified as CD4+ FOXP3- and CD4+ FOXP3+, respectively, was analysed by flow cytometry. Additionally, CTLA-4 expression by other lymphocyte populations (CD8+ T cells and B cells) was assessed by flow cytometry to investigate the cellular distribution of the CTLA-4 receptor. The FACS plots in *Fig. 3.03* show that neither FOXP3- nor FOXP3+ CD4+ T cells expressed CTLA-4 at the cell surface. On the other hand, intracellular CTLA-4 was detected in some CD4+ FOXP3- Tconv and the majority of CD4+ FOXP3+ Tregs (*A*). Only a small minority of CD8+ T cells expressed CTLA-4 (*C*). It has been suggested that CTLA-4 may be expressed by B cells [235-237]. However, CTLA-4 was not detected in CD19+ B cells (*Fig 3.03 D*).

As mentioned above, the CTLA-4 receptor is not highly expressed by resting conventional T cells. The up regulation of CTLA-4 by Tconv upon activation is thought to be CD28-dependent [202, 234], whereas the mechanistic control of CTLA-4 expression in Tregs is not known. It has previously been reported that CD28 co-stimulation was required to induce CTLA-4 expression in CD4/CD8 double positive thymocytes as part of Treg thymic development [238]. Using lymphocytes isolated from *CD28*-deficient mice and agonistic antibodies to CD3 and CD28, the CD28-dependency of intracellular CTLA-4 expression by CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs was investigated by *in vitro* overnight culture. As predicted there were fewer CD4+ lymphocytes in *CD28*-/- mice, however both CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs expressed intracellular CTLA-4 like lymphocytes from wild type mice (*Fig. 3.04 A*). *Fig. 3.04 B* shows that fewer *CD28*-deficient CD4+ FOXP3- conventional T cells expressed intracellular CTLA-4 compared to wild type (left graph). Intracellular CTLA-4 expression in Tconv increased in response to TCR-stimulation by anti-CD3 in both wild type and *CD28*-deficient lymphocytes. In wild type Tconv, CD28 co-stimulation with anti-CD28 in conjunction with TCR-stimulation with

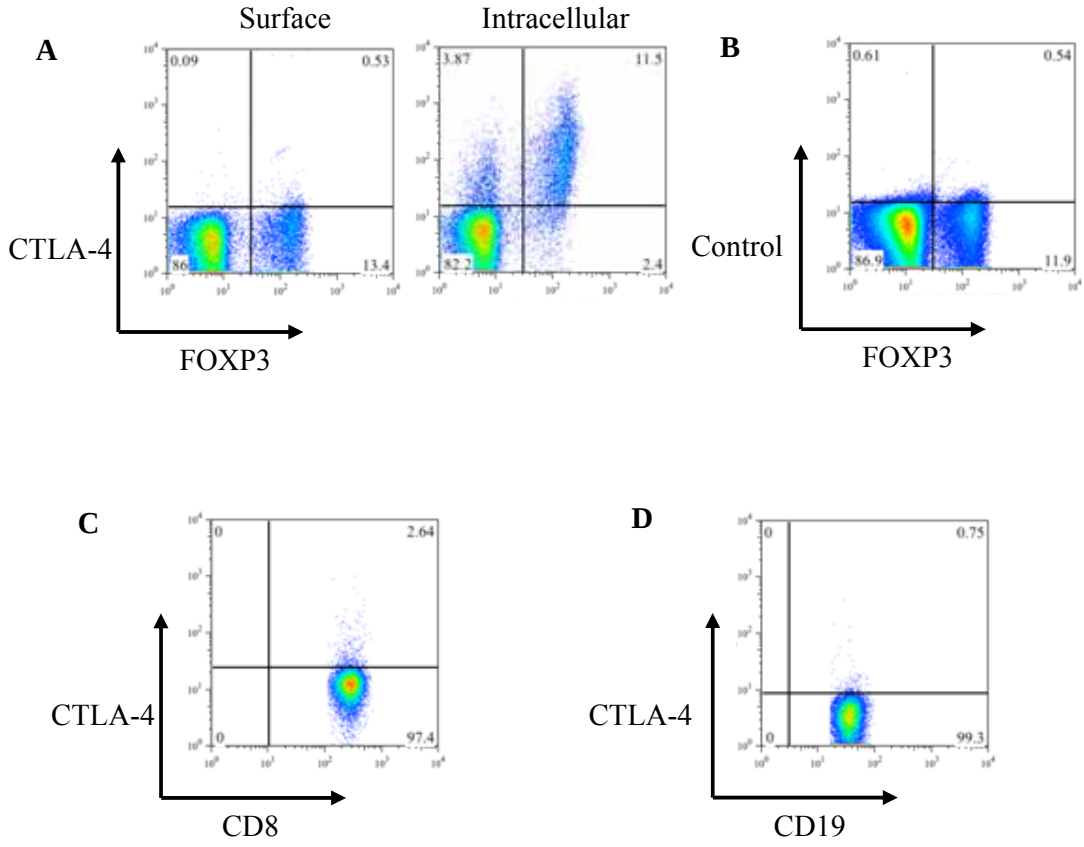


Figure 3.03. Cellular distribution of CTLA-4 receptor expression. Single cell suspensions from peripheral lymph nodes were surface stained with CD4, CD8, CD19, CTLA-4 and intracellularly with FOXP3, CTLA-4 and Control antibody for analysis by flow cytometry. Representative FACS plots for (A) CD4+ gated lymphocytes of adult wild type BALB/c mice for surface CTLA-4 (left) and intracellular CTLA-4 (right), (B) CD4+ gated lymphocytes of adult wild type BALB/c mice for intracellular control antibody, (C) CD8+ gated lymphocytes of adult wild type BALB/c mice for intracellular CTLA-4 and (D) CD19+ gated lymphocytes of adult wild type BALB/c mice for intracellular CTLA-4. Data representative of ≥3 mice.

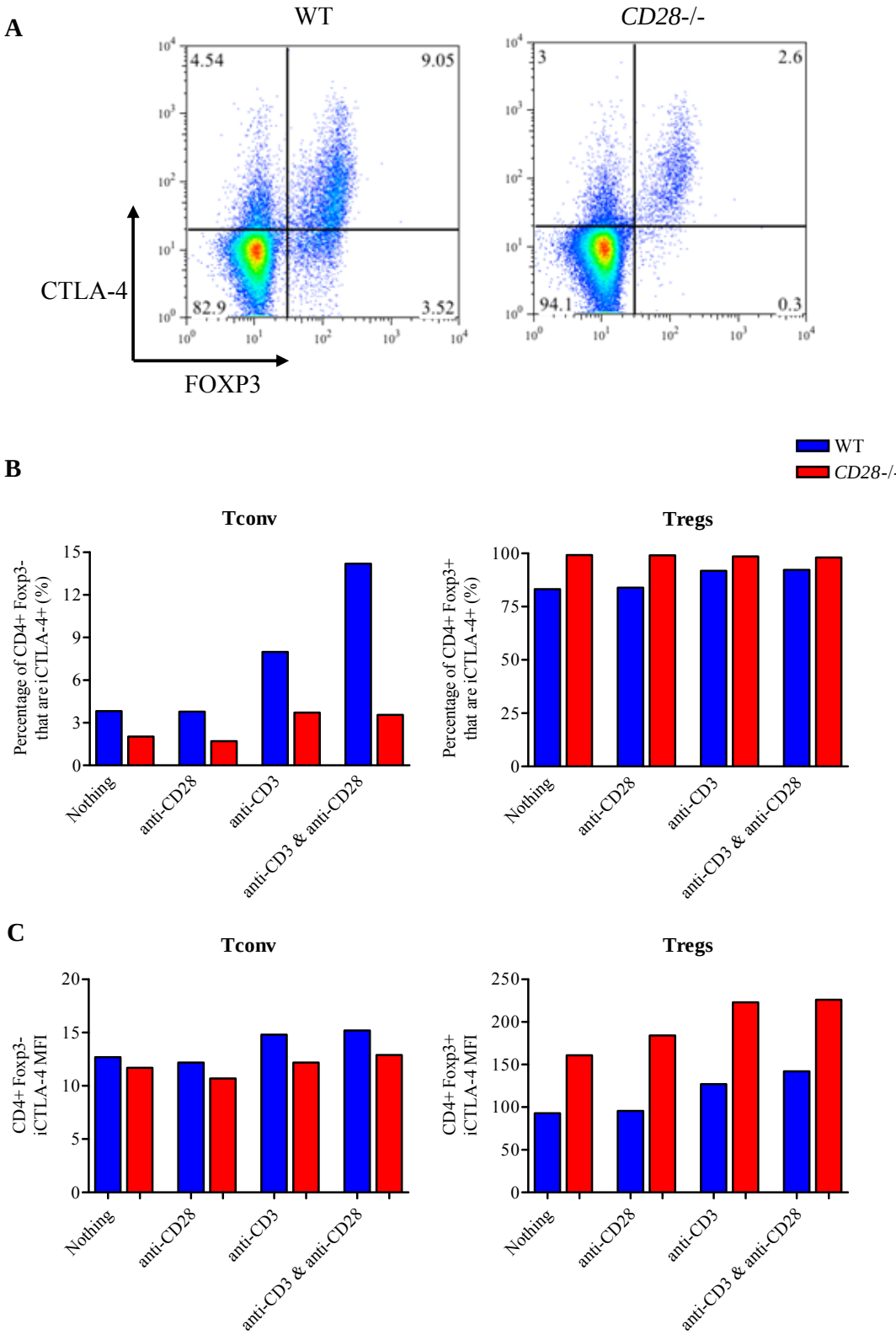

A
WT
CD28-/-
4.54
9.05
82.9
3.52
3
2.6
94.1
0.3
CTLA-4
FOXP3
B
WT
CD28-/-
Tconv
Tregs
Percentage of CD4+ Foxp3- that are iCTLA-4+ (%)
Percentage of CD4+ Foxp3+ that are iCTLA-4+ (%)
Nothing
anti-CD28
anti-CD3
anti-CD3 & anti-CD28
C
Tconv
Tregs
CD4+ Foxp3- iCTLA-4 MFI
CD4+ Foxp3+ iCTLA-4 MFI

Figure 3.04. CD28-dependency of intracellular CTLA-4 expression by CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs. (A) Single cell suspensions from peripheral lymph nodes were surface stained with CD4 and intracellularly with CTLA-4 and FOXP3 for analysis by flow cytometry. Representative FACS plots for CD4+ gated lymphocytes of adult wild type BALB/c mice for iCTLA-4 (left) and adult *CD28*-/- mice for iCTLA-4 (right). 2 X 10^5 cells adult BALB/c wild type (blue) or *CD28*-/- (red) single cell suspensions from peripheral lymph nodes were cultured overnight plus or minus 500ng/ml anti-CD3 and 1µg/ml anti-CD28. Cells were then surface stained with CD4 and intracellularly with CTLA-4 and FOXP3 for analysis by flow cytometry. (B) percentage iCTLA-4+ of gated CD4+ FOXP3- Tconv (left) and gated CD4+ FOXP3+ Tregs (right). (C) iCTLA-4 median fluorescence intensity of gated CD4+ FOXP3- Tconv (left) and gated CD4+ FOXP3+ Tregs (right). Representative graphs from three independent experiments are shown.

anti-CD3 further increased intracellular CTLA-4 expression, although CD28 co-stimulation alone had no affect. As predicted, the anti-CD28 antibody had no synergistic affect with anti-CD3 on *CD28*-/- lymphocytes. Collectively, these data suggest that a combination of TCR and CD28 signalling drive the up regulation of CTLA-4 in conventional T cells.

Parallel analyses were carried out on the CD4+ FOXP3+ regulatory T cell subset. Intracellular CTLA-4 expression by *CD28*-deficient CD4+ FOXP3+ Tregs (*Fig. 3.04 B and C*) was increased in both proportion and intensity compared to wild type. Similar to conventional T cells, Treg intracellular CTLA-4 expression increased upon TCR-stimulation with anti-CD3. However, CD28 co-stimulation had no synergistic affect with TCR-stimulation on intracellular CTLA-4 expression by CD4+ FOXP3+ Tregs. Overall, the data suggest that up regulation of intracellular CTLA-4 expression by CD4+ FOXP3- Tconv is CD28-dependent and CD28-independent for CD4+ FOXP3+ Tregs.

Any effect of CTLA-4 on the homeostasis of regulatory T cells would presumably depend upon encounter with one of its natural ligands; CD80 or CD86. The CD28 / CTLA-4 ligands, CD80 and CD86, are expressed by dendritic cell and B cell antigen-presenting cells (APCs) to provide T cell co-stimulation when antigen is presented to T cells via MHC molecules on APCs [239]. To examine the distribution of CD80 and CD86 expression by APCs flow cytometry was used to analyse wild type splenocytes. *Fig. 3.05 A* shows that CD3- CD11c+ MHC II+ dendritic cells constitutively expressed both CD80 and CD86. On the other hand, only a relatively small proportion of CD19+ B cells expressed CD80 and CD86 (*Fig. 3.05 B*). Nevertheless, B cells were capable of up regulating CD86 expression following activation by overnight culture with LPS (*Fig. 3.05 C*).

3.2.2 CTLA-4 receptor endocytosis.

As discussed above, it is known that CTLA-4 is poorly expressed at the cell surface (see *Fig. 3.03*). Upon T cell activation, CTLA-4 is thought to be translocated to the cell surface, but cell surface expression levels are limited due to rapid clathrin-mediated endocytosis of the CTLA-4 receptor [179]. Phorbol myristate acetate (PMA) T cell activation has been shown to increase CTLA-4 trafficking to the plasma membrane via a phospholipase D-dependent mechanism [240]. To test the capacity of CTLA-4 to undergo membrane trafficking in

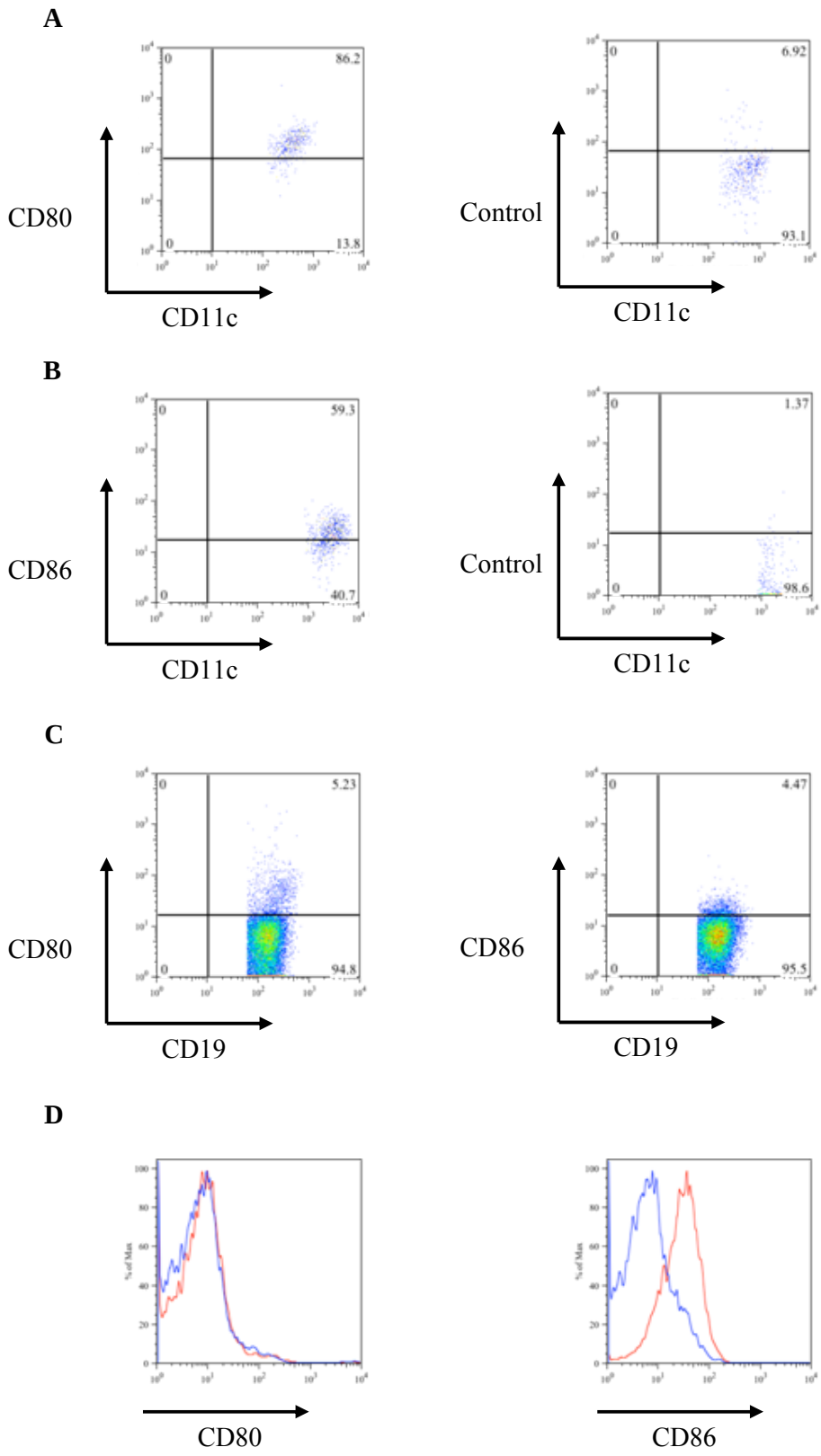

A
CD80
CD11c
86.2
13.8
Control
CD11c
6.92
93.1
B
CD86
CD11c
59.3
40.7
Control
CD11c
1.37
98.6
C
CD80
CD19
5.23
94.8
CD86
CD19
4.47
95.5
D
CD80
CD86

Figure 3.05. Cellular distribution of CD80/CD86 ligand expression. Splenocytes were surface stained with CD3, CD11c, CD19, CD80, CD86 and I-Ad for analysis by flow cytometry. (A) CD3- I-Ad+ CD11c+ gated lymphocytes of adult wild type BALB/c mice for CD80 (left) and control antibody (right). (B) CD3- I-Ad+ CD11c+ gated lymphocytes of adult wild type BALB/c mice for CD86 (left) and control antibody (right). (C) CD19+ gated lymphocytes of adult wild type BALB/c mice for CD80 (left) and CD86 (right). (D) 1 X 10^5 wild type BALB/c splenocytes were cultured overnight plus or minus 10µg/ml LPS. Cells were then surface stained with CD19, CD80 and CD86 for analysis by flow cytometry. Left histogram shows CD80 of gated CD19+ cultured plus (red) and minus (blue) LPS, and right histogram shows CD86 of gated CD19+ cultured plus (red) and minus (blue) LPS.

CD4+ FOXP3- conventional T cells and CD4+ FOXP3+ regulatory T cells, *in vitro* culture with PMA, ionomycin and extracellularly provided, fluorescently conjugated anti-CTLA-4 antibody was used. To investigate CTLA-4 receptor cycling in CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs whole lymphocytes were cultured plus or minus PMA and ionomycin with exogenous, fluorescently conjugated anti-CTLA-4 antibody. Over time, any CTLA-4 that trafficked to the cell surface would be fluorescently labelled by the exogenous anti-CTLA-4 and quantified upon analysis by flow cytometry.

The data in *Fig. 3.06* shows that over time the CTLA-4 receptor cycled in response to PMA and ionomycin as the proportion of cells positive for CTLA-4 increased from the minimal surface level of CTLA-4 expression towards the level of constitutively expressed, intracellular CTLA-4 upon fresh *ex vivo* staining. In the time-frame studied, Treg CTLA-4 expression was always greater than Tconv CTLA-4 expression. The CTLA-4 receptor cycling kinetics of Tconv and Tregs was similar; after 2hrs incubation with PMA and ionomycin there was a shift in the proportion of cells that had bound the fluorescently-conjugated anti-CTLA-4 antibody. For both Tconv and Tregs, the level of bound fluorescently-conjugated anti-CTLA-4 antibody after culture in medium alone did not increase above the minimal surface level of CTLA-4 expression. The data obtained suggest that the CTLA-4 receptor cycles in Tregs in a similar manner to Tconv, but the intracellular store of CTLA-4 in Tregs is constitutively larger than in Tconv.

3.2.3 CTLA-4 normally functions to restrict the proliferation of Tregs in the periphery.

To examine the role of CTLA-4 in Treg homeostasis, first, BALB/c mice genetically deficient for the *Ctla-4* gene were analysed. Mice in which the *Ctla-4* gene has been ablated succumb to a fatal lymphoproliferative syndrome by around 3 weeks of age [156, 157]. *Fig. 3.07 A* illustrates how homozygous *Ctla-4*-deficient mice were smaller than heterozygous littermate controls and exhibited lymphadenopathy and splenomegaly. The graph of Fig. *3.07 B* shows how in addition to looking larger by eye, the spleen from a *Ctla-4* knockout mouse was also of greater mass. The absolute cell counts of pooled peripheral lymph nodes and spleen of a *Ctla-4*-deficient mouse was significantly increased (*Fig. 3.07 C*).

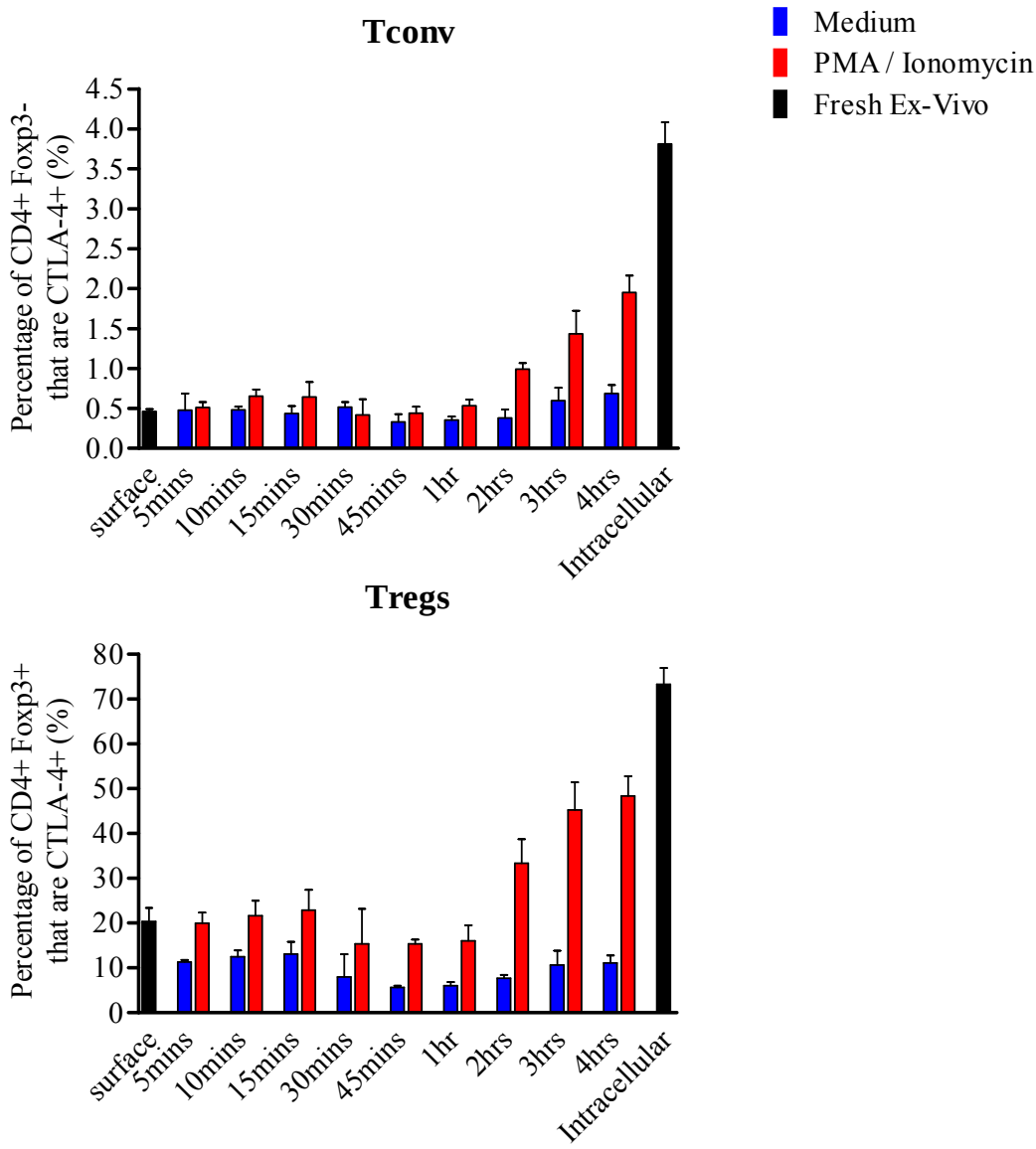
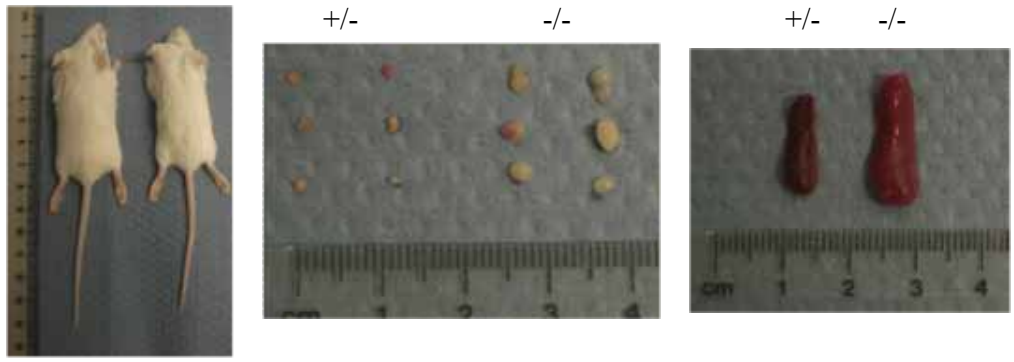
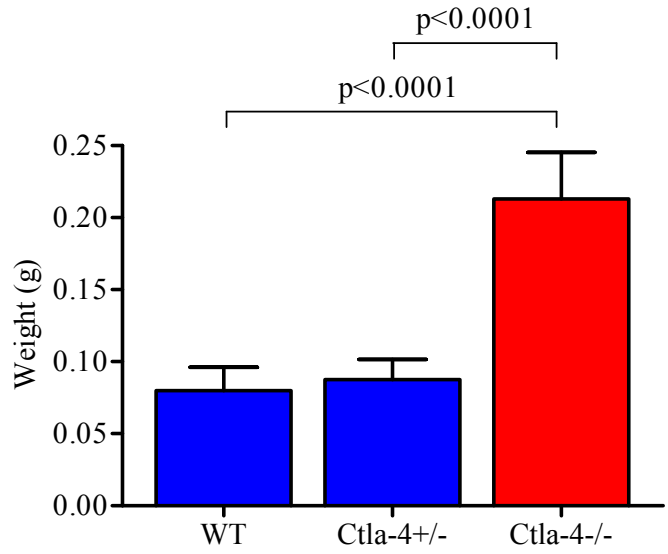


Figure 3.06. CTLA-4 receptor cycling in CD4+ FOXP3- conventional T cells and CD4+ FOXP3+ Tregs. Wild type adult BALB/c single cell suspensions from peripheral lymph nodes were stained with CD4. 1 X 10^6 lymphocytes were cultured for time stated plus or minus 50ng/ml PMA and 1.5µM Ionomycin and 2µg/ml CTLA-4-PE. Cells were then stained intracellularly with FOXP3 for analysis by flow cytometry. For fresh ex-vivo wild type adult BALB/c single cell suspensions from peripheral lymph nodes were surface stained with CD4, CTLA-4 and intracellularly CTLA-4 and FOXP3. Top graph shows CD4+ FOXP3- gated Tconv and bottom graph shows CD4+ FOXP3+ gated Tregs. Bars represent mean and standard deviation is shown for three independent experiments. Note the different scales on the two graphs.

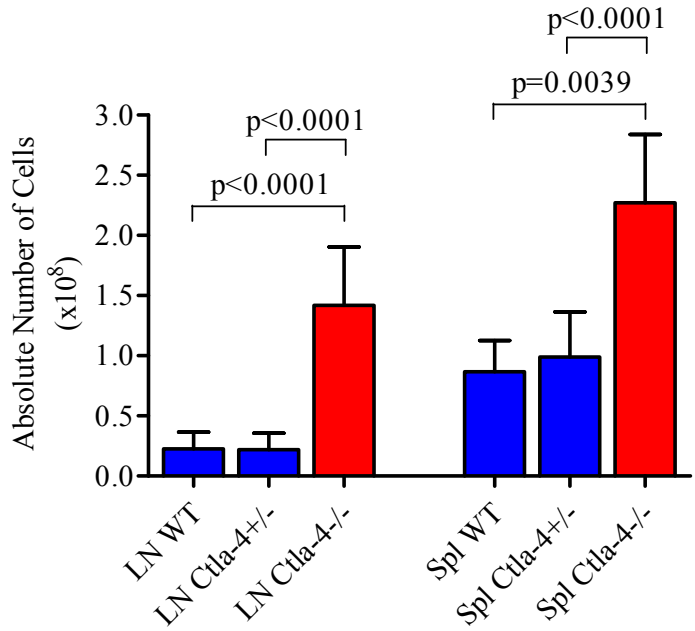
A


+/-
-/-
+/-
-/-
+/-
-/-

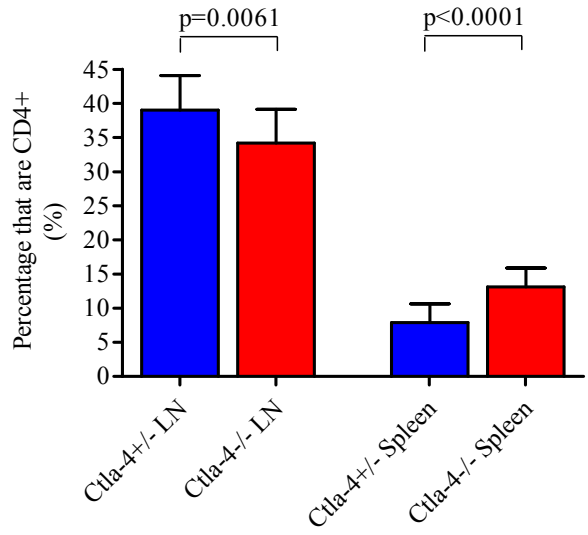
B

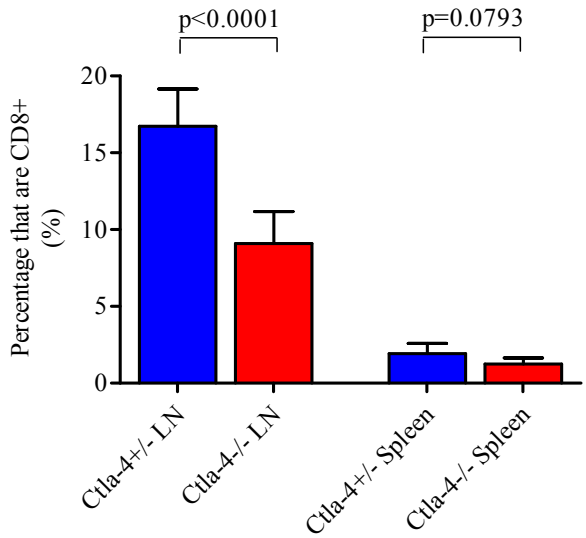

p<0.0001
p<0.0001
Weight (g)
0.25
0.20
0.15
0.10
0.05
0.00
WT
Ctla-4+/-
Ctla-4-/-

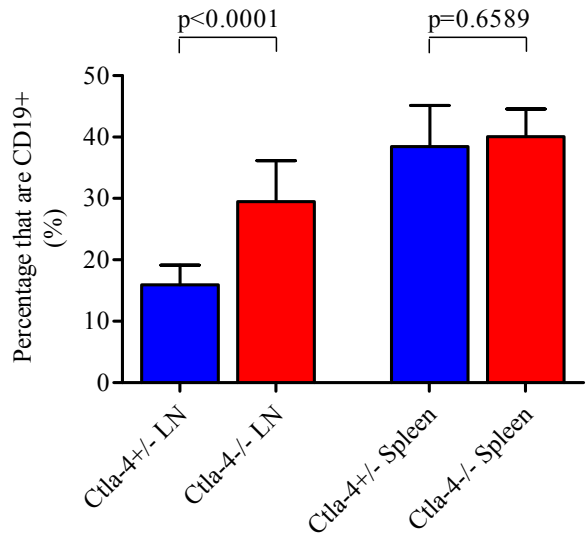
C


p<0.0001
p=0.0039
p<0.0001
p<0.0001
Absolute Number of Cells (x10^8)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
LN WT
LN Ctla-4+/-
LN Ctla-4-/-
Spl WT
Spl Ctla-4+/-
Spl Ctla-4-/-

D


p=0.0061
p<0.0001
Percentage that are CD4+ (%)
45
40
35
30
25
20
15
10
5
0
Ctla-4+/- LN
Ctla-4-/- LN
Ctla-4+/- Spleen
Ctla-4-/- Spleen


p<0.0001
p=0.0793
Percentage that are CD8+ (%)
20
15
10
5
0
Ctla-4+/- LN
Ctla-4-/- LN
Ctla-4+/- Spleen
Ctla-4-/- Spleen


p<0.0001
p=0.6589
Percentage that are CD19+ (%)
50
40
30
20
10
0
Ctla-4+/- LN
Ctla-4-/- LN
Ctla-4+/- Spleen
Ctla-4-/- Spleen

E

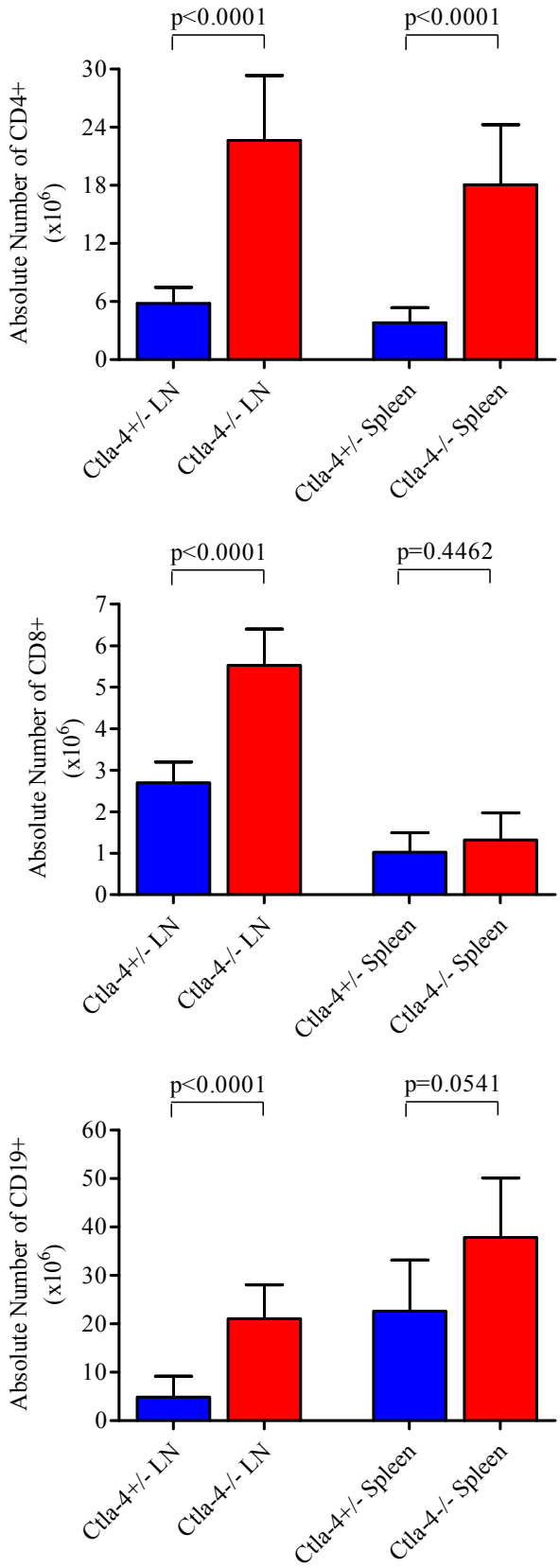
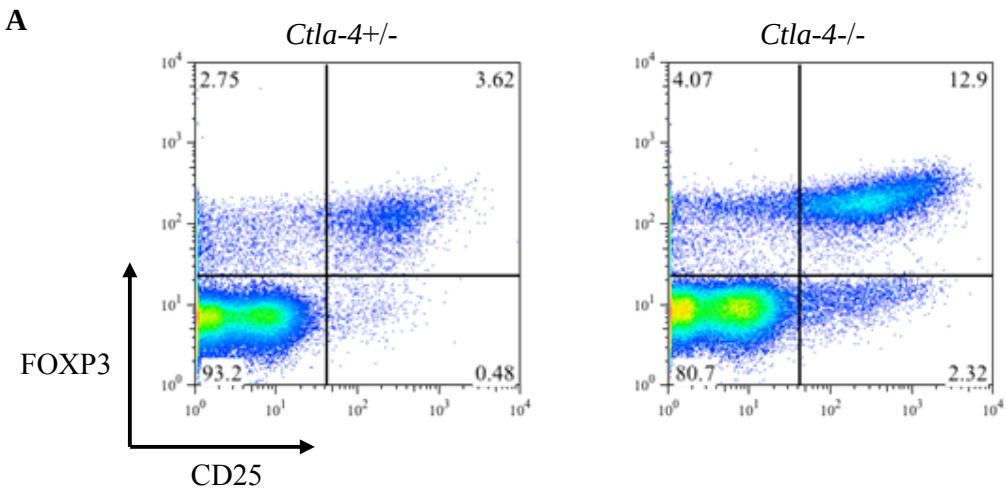
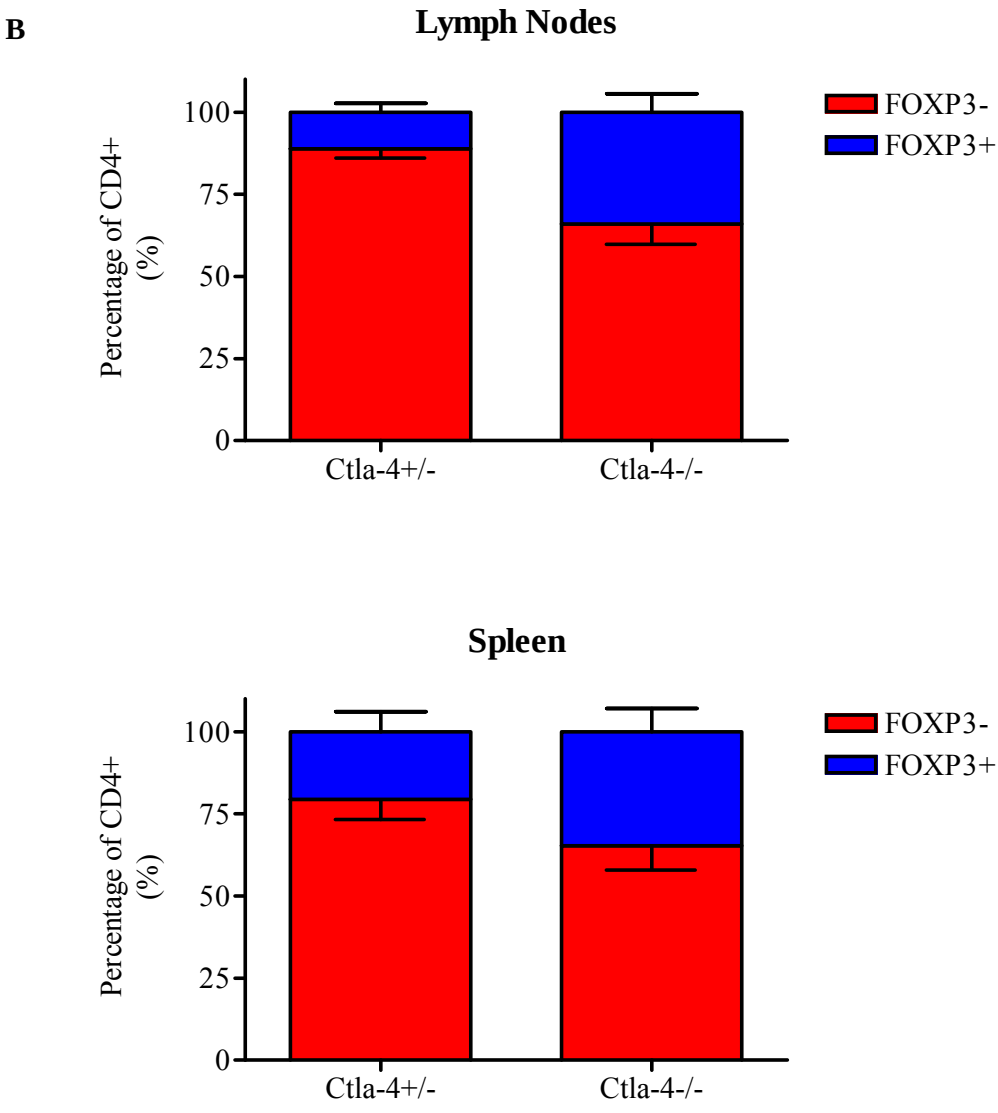

p<0.0001
p<0.0001
Absolute Number of CD4+ (x10^6)
30
24
18
12
6
0
Ctla-4+/- LN
Ctla-4-/- LN
Ctla-4+/- Spleen
Ctla-4-/- Spleen
p<0.0001
p=0.4462
Absolute Number of CD8+ (x10^6)
7
6
5
4
3
2
1
0
Ctla-4+/- LN
Ctla-4-/- LN
Ctla-4+/- Spleen
Ctla-4-/- Spleen
p<0.0001
p=0.0541
Absolute Number of CD19+ (x10^6)
60
50
40
30
20
10
0
Ctla-4+/- LN
Ctla-4-/- LN
Ctla-4+/- Spleen
Ctla-4-/- Spleen

Figure 3.07. *Ctla-4*-/- mouse phenotype. (A) Day 18 *Ctla-4*+/- and *Ctla-4*-/- mice (left). Centre shows brachial (top), axillary (middle) and inguinal (bottom) lymph nodes dissected from day 18 *Ctla-4*+/- and *Ctla-4*-/- mice. Day 18 *Ctla-4*+/- and *Ctla-4*-/- spleens (right). (B) Graph shows weight of 15-18 day old BALB/c, *Ctla-4*+/- and *Ctla-4*-/- spleens. (C) Graph shows absolute cell number for all lymph nodes and spleen from 15-18 day old BALB/c, *Ctla-4*+/- and *Ctla-4*-/- mice. (D) Graphs show percentage of CD4+ (top), CD8+ (middle) and CD19+ (bottom) in lymph nodes and spleen of 15-18 day old *Ctla-4*+/- and *Ctla-4*-/- mice. (E) Graphs show absolute number of CD4+ (top), CD8+ (middle) and CD19+ (bottom) in lymph nodes and spleen of 15-18 day old *Ctla-4*+/- and *Ctla-4*-/- mice. Bars represent mean values and standard deviation is shown for >5 mice. p values calculated by two-tailed, unpaired T-test for the means.

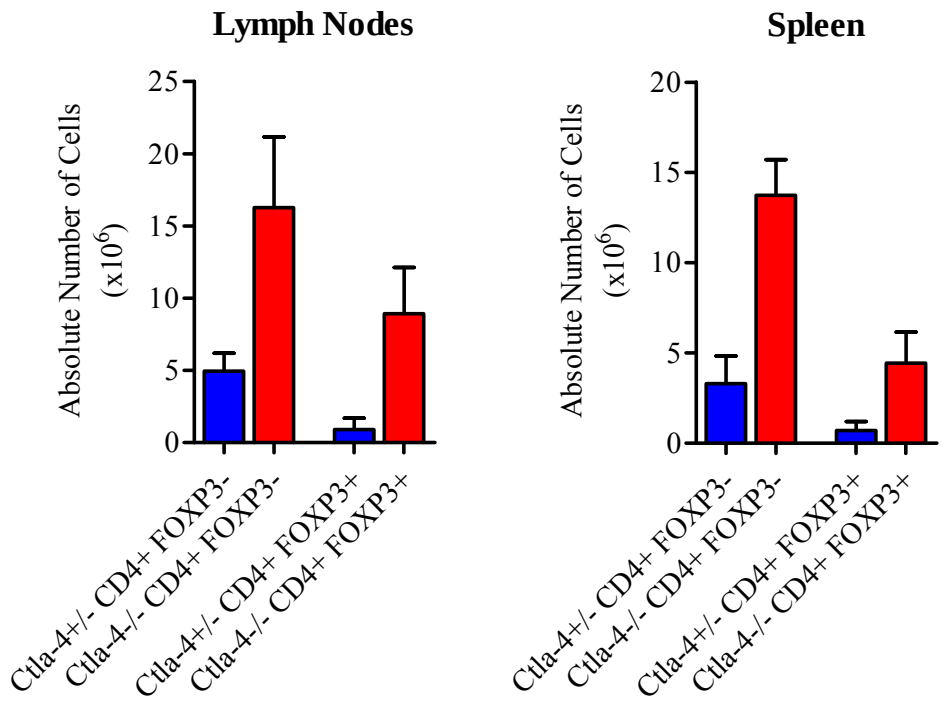
Phenotyping of *Ctla-4*-deficient mice was carried out by flow cytometry to investigate the cellular composition of the lymphocytes within the lymph nodes and spleen. Since no overt difference was observed between wild type and *Ctla-4* heterozygous mice, *Ctla-4*+/- littermate controls were used as a comparison for the *Ctla-4* knockout mice. Due to the large increase in lymph node and spleen size of *Ctla-4*-deficient mice, it can be seen in *Fig. 3.07 E* that the absolute numbers of CD4+ T cells, CD8+ T cells and CD19+ B cells was increased in the lymph nodes and spleen of *Ctla-4*-/- mice compared to littermate controls. However, the T cell and B cell percentage compositions of lymph nodes and spleen varied (*Fig. 3.07 D*). The percentage of CD4+ T cells was slightly decreased in the lymph nodes and increased in the spleen of *Ctla-4* knockout mice compared to littermate controls. These differences were small, but statistically significant. Whereas, the percentage of CD8+ T cells were decreased in *Ctla-4*-/- lymph nodes, but unaltered in the spleen. With the decreased CD4 and CD8 T cell proportion in the *Ctla-4*-/- lymph nodes there was a concurrent increase in CD19+ B cells. The B cell proportions in the *Ctla-4*-/- spleen however remained similar to that of the littermate controls.

The original phenotyping studies of *Ctla-4*-deficient mice when they were generated in the mid-1990s identified an augmented CD25+ T cell population [156, 157]. Due to alterations in other T cell activation markers such as increased CD69 and decreased CD62L, at the time, the increase in CD25+ T cells was attributed to an increase in activated T cells. These studies were carried out prior to the resurgence of CD4+ CD25+ Tregs and the identification of the transcription factor *Foxp3* as a specific marker for this T cell population. To test whether the augmented CD4+ CD25+ population in *Ctla-4*-deficient mice represented activated conventional T cells or regulatory T cells, FOXP3 expression was analysed in lymphocytes from *Ctla-4* knockout mice by flow cytometry. The FACS plots of *Fig. 3.08 A* show that in *Ctla-4*-/- mice there was an increase in the percentage of CD25+ FOXP3+ lymphocytes [241]. The phenotyping data of the *Ctla-4*-/- mice in *Fig. 3.07 D* suggested that there was not much change in the proportion of CD4+ T cells as a whole. The graphs of *Fig. 3.08 B* show the proportion of CD4+ T cells that were FOXP3- or FOXP3+, and it can be seen that there was a shift within the CD4+ T cell population in which the FOXP3+ lymphocytes were augmented in the absence of *Ctla-4*. Again, due to the lymphadenopathy and splenomegaly the absolute


A
Ctla-4+/-
Ctla-4-/-
2.75
3.62
93.2
0.48
4.07
12.9
80.7
2.32
FOXP3
CD25


B
Lymph Nodes
Percentage of CD4+ (%)
100
75
50
25
0
Ctla-4+/-
Ctla-4-/-
FOXP3-
FOXP3+
Spleen
Percentage of CD4+ (%)
100
75
50
25
0
Ctla-4+/-
Ctla-4-/-
FOXP3-
FOXP3+

C


Lymph Nodes
Spleen
Absolute Number of Cells (x10^6)
25
20
15
10
5
0
Ctla-4+/- CD4+ FOXP3-
Ctla-4-/- CD4+ FOXP3-
Ctla-4+/- CD4+ FOXP3+
Ctla-4-/- CD4+ FOXP3+

D

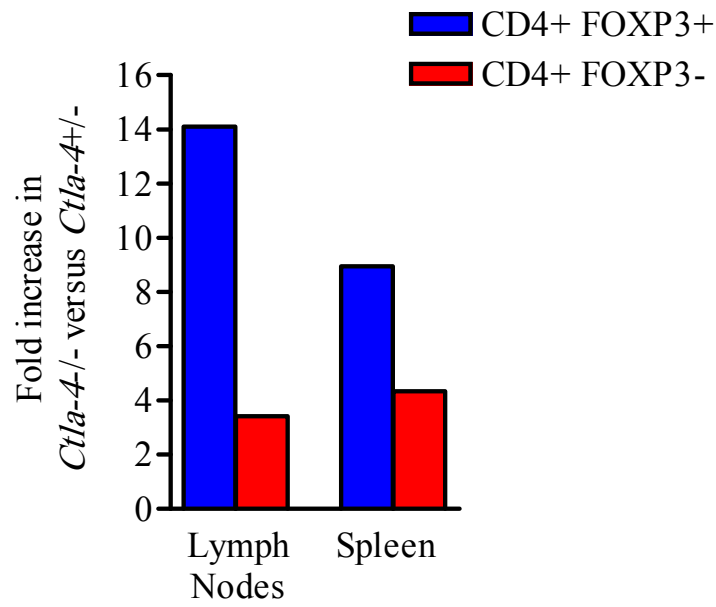

CD4+ FOXP3+
CD4+ FOXP3-
Fold increase in Ctla-4-/- versus Ctla-4+/-
16
14
12
10
8
6
4
2
0
Lymph Nodes
Spleen

Figure 3.08. *Ctla-4*-deficient mice have increased CD4+ FOXP3+ cells. Day 15-18 *Ctla-4*+/- and *Ctla-4*-/- single cell suspensions from peripheral lymph nodes were surface stained with CD4, CD25 and intracellularly with FOXP3 for analysis by flow cytometry. (A) CD25 and FOXP3 expression by day 15-18 *Ctla-4*+/- (left) and *Ctla-4*-/- (right) lymphocytes. (B) Graphs show percentage of CD4+ that are FOXP3- (red) or FOXP3+ (blue) in the lymph nodes (top) and spleen (bottom) of *Ctla-4*+/- and *Ctla-4*-/- mice. (C) Graphs show absolute number of CD4+ FOXP3- and CD4+ FOXP3+ in the lymph nodes (left) and spleen (right) of *Ctla-4*+/- (blue) and *Ctla-4*-/- (red) mice. (D) Fold increase in the absolute number of CD4+ FOXP3+ Tregs (blue) and CD4+ FOXP3- Tconv (red) in the lymph nodes and spleen of *Ctla-4*-/- versus *Ctla-4*+/- mice. Bars represent mean and standard deviation is shown for ≥5 mice.

numbers of both CD4+ FOXP3- and CD4+ FOXP3+ lymphocytes was increased in *Ctla-4*-deficient mice (*Fig. 3.08 C*). One possible explanation for the augmentation of CD4+ FOXP3+ lymphocytes in the periphery of *Ctla-4*-deficient mice is that thymocyte selection could be altered in the absence in *Ctla-4*, such that a greater proportion of CD4+ thymocytes are induced to express *Foxp3* and are then exported to the periphery. FOXP3 expression by *Ctla-4*-/- thymocytes was therefore analysed by flow cytometry. *Fig. 3.09 A* shows the gating strategy used to identify the four main thymocyte populations based on CD4 and CD8 expression. The proportions of CD4 single positive, CD8 single positive, CD4 / CD8 double negative and CD4 / CD8 double positive thymocytes were unaltered in the thymi of mice lacking *Ctla-4*. Within the CD4 single positive thymocyte gate, the percentage of FOXP3+ CD4+ thymocytes was similar in *Ctla-4*-deficient thymi compared to littermate controls (*Fig. 3.09 B*). The graph of *Fig. 3.09 C* represents combined data for four different *Ctla-4*-/- mice and four different *Ctla-4*+/- littermate controls, and shows that the percentage of CD4 single positive FOXP3+ thymocytes was unchanged. The data of *Fig. 3.09* was obtained using a piece of thymus as opposed to a whole thymus because due to the lymphadenopathy in *Ctla-4*-/- mice it was difficult to obtain single cell thymocyte suspensions from whole thymi that were not contaminated by parathymic lymph nodes (as previously reported by others [88]). Therefore, flow cytometry data was only used when no CD19+ B cell contamination was observed (illustrated in *Fig. 3.09 D*). Together the data suggest that the size of the CD4+ FOXP3+ regulatory T cell population selected intra-thymically is not modulated by the CTLA-4 pathway.

To compliment the above studies into a possible role of CTLA-4 in thymic selection, the distribution of intracellular CTLA-4 expression among the four main thymocyte subsets, based on CD4 and CD8 expression, was analysed by flow cytometry. *Fig. 3.10 A* shows that like for peripheral T cells, CD4 single positive thymocytes expressed intracellular CTLA-4 and this mainly corresponded to the FOXP3+ subset. CD8 single positive, CD4 / CD8 double positive and CD4 / CD8 double negative thymocyte populations expressed negligible amounts of intracellular CTLA-4.

As a comparison of CTLA-4 deficiency, mice lacking the homologous co-stimulatory receptor CD28 were also examined. It is known that CD28 is required for the thymic

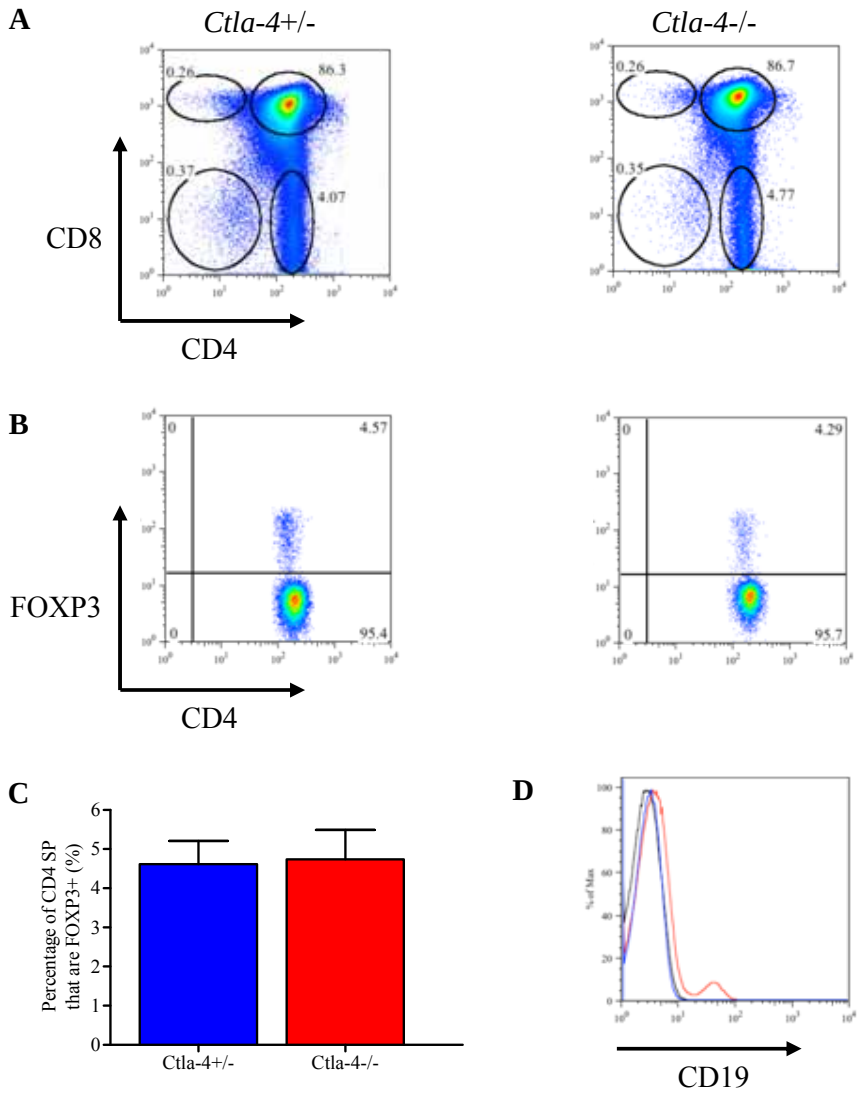


Figure 3.09. Normal proportion of CD4+ FOXP3+ Tregs in the thymus of *Ctla-4-/-* mice. Single cell suspensions from 15-18 day old *Ctla-4*+/- or *Ctla-4*-/- thymi were surface stained with CD4, CD8, CD19 and intracellularly with FOXP3 for analysis by flow cytometry. (A) Representative FACS plots showing the proportions of CD8 single positive, CD4 / CD8 double positive, CD4 / CD8 double negative and CD4 single positive thymocytes for *Ctla-4*+/- (left) and *Ctla-4*-/- (right). (B) Representative FACS plots for FOXP3 expression by CD4 SP-gated *Ctla-4*+/- (left) and *Ctla-4*-/- (right) thymocytes. (C) A graph to show the mean percent of CD4 SP-gated thymocytes that are FOXP3+ with standard deviations. (D) Histogram to illustrate the presence of CD19+ cells in the whole thymus of *Ctla-4*-/- (red) compared to *Ctla-4*+/- (black) and a carefully dissected piece of *Ctla-4*-/- thymus (blue) to avoid contamination with parathymic lymph node cells. Data represent 4 mice.

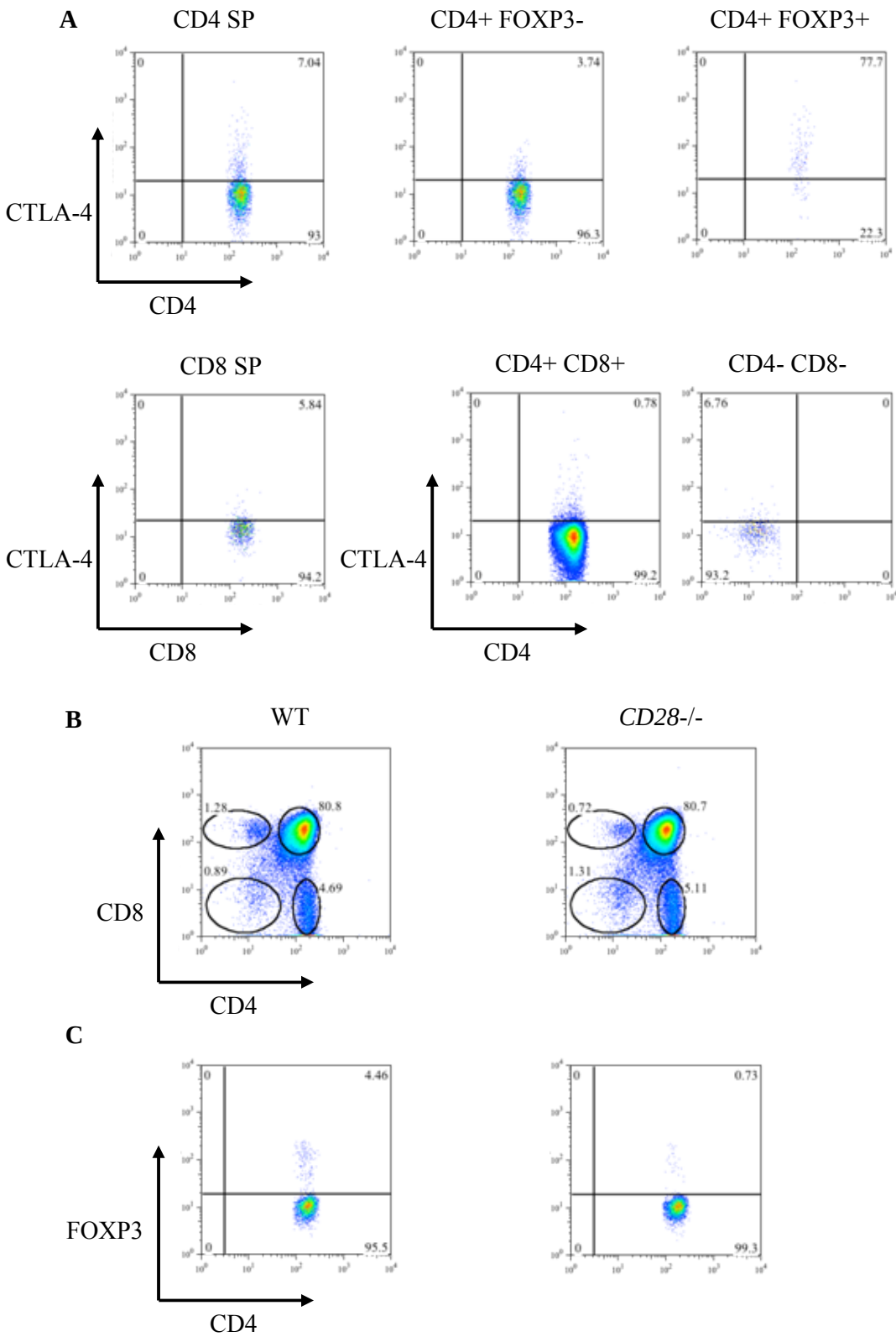

A
CD4 SP
CD4+ FOXP3-
CD4+ FOXP3+
7.04
93
3.74
96.3
77.7
22.3
CTLA-4
CD4
CD8 SP
CD4+ CD8+
CD4- CD8-
5.84
94.2
0.78
99.2
6.76
93.2
CTLA-4
CD8
CTLA-4
CD4
B
WT
CD28-/-
1.28
80.8
0.89
4.69
0.72
80.7
1.31
5.11
CD8
CD4
C
4.46
95.5
0.73
99.3
FOXP3
CD4

Figure 3.10. Distribution of CTLA-4 in wild type thymus and analysis of CD4+ FOXP3+ Tregs in *CD28*-/- thymus. Single cell suspensions from adult wild type BALB/c or *CD28*-/- thymi were surface stained with CD4, CD8 and intracellularly with CTLA-4 and FOXP3. Representative FACS plots showing (A) Intracellular CTLA-4 expression by the various CD4 and CD8 thymocyte subsets of adult wild type BALB/c thymus. (B) the proportions of CD8 single positive, CD4 / CD8 double positive, CD4 / CD8 double negative and CD4 single positive thymocytes for wild type BALB/c (left) and *CD28*-/- (right), (C) FOXP3 expression by CD4 SP-gated wild type BALB/c (left) and *CD28*-/- (right) thymocytes. Data represent ≥3 mice.

selection of CD4+ FOXP3+ Tregs [86, 131, 238]. Thymi from *CD28*-deficient mice were analysed for FOXP3 expression by flow cytometry as done previously for *Ctla-4*-deficient thymi. *Fig. 3.10 B* illustrates that similar to *Ctla-4*-deficient thymi, *CD28*-deficient thymi had unaltered proportions of CD4 single positive, CD8 single positive, CD4 / CD8 double negative and CD4 / CD8 double positive thymocytes to wild type thymi. The proportion of CD4 single positive thymocytes that was FOXP3+ on the other hand was significantly reduced in thymi lacking *CD28* (*Fig. 3.10 C*). Thus, the published observation that CD28 signalling controls FOXP3+ Treg selection in the thymus was repeatable, but a role for CTLA-4 in this process was not found.

The molecules, CD80 and CD86, are common ligands to both the CTLA-4 and CD28 receptors. Within the thymus it is hypothesised that medullary thymic epithelial cells (mTECs) present antigen to developing thymocytes as part of negative selection and FOXP3+ Treg differentiation [79, 242]. Therefore, the expression of the ligands, CD80 and CD86, was analysed on non-thymocytes by flow cytometry. Thymi were teased apart to remove the majority of thymocytes and digested with collagenase, dispase and trypsin so that EpCam+ (pan-epithelial cell marker) non-thymocytes (CD45-) could be identified. Using an isotype control to set the flow cytometry analysis gates *Fig. 3.11* shows that CD80 and CD86 expression by EpCam+ non-thymocytes was similar in wild type, *CD28*-deficient and *Ctla-4*-deficient thymi.

No thymic defect in Treg development was observed in the absence of *Ctla-4*, therefore the peripheral homeostasis of *Ctla-4*-deficient Tregs was investigated. Previous phenotyping analysis of *Ctla-4* knockout mice by others observed overt T cell activation as determined by increased CD69 and decreased CD62L expression [156, 157]. To assess the activation status of conventional T cells and regulatory T cells in *Ctla-4*-deficient mice, FOXP3 was used to discriminate the peripheral T cells. *Fig. 3.12* shows that both CD4+ FOXP3- Tconv (*A*) and CD4+ FOXP3+ Tregs (*B*) up-regulated CD69 and down-regulated CD62L in the periphery of *Ctla-4* knockout mice compared to littermate controls. Increased CD69 and decreased CD62L are consistent with a highly activated phenotype. Additionally, expression of the secondary co-stimulatory molecule ICOS was analysed because it is now known that ICOS expression increases upon T cell activation [243, 244]. In both CD4+ FOXP- Tconv

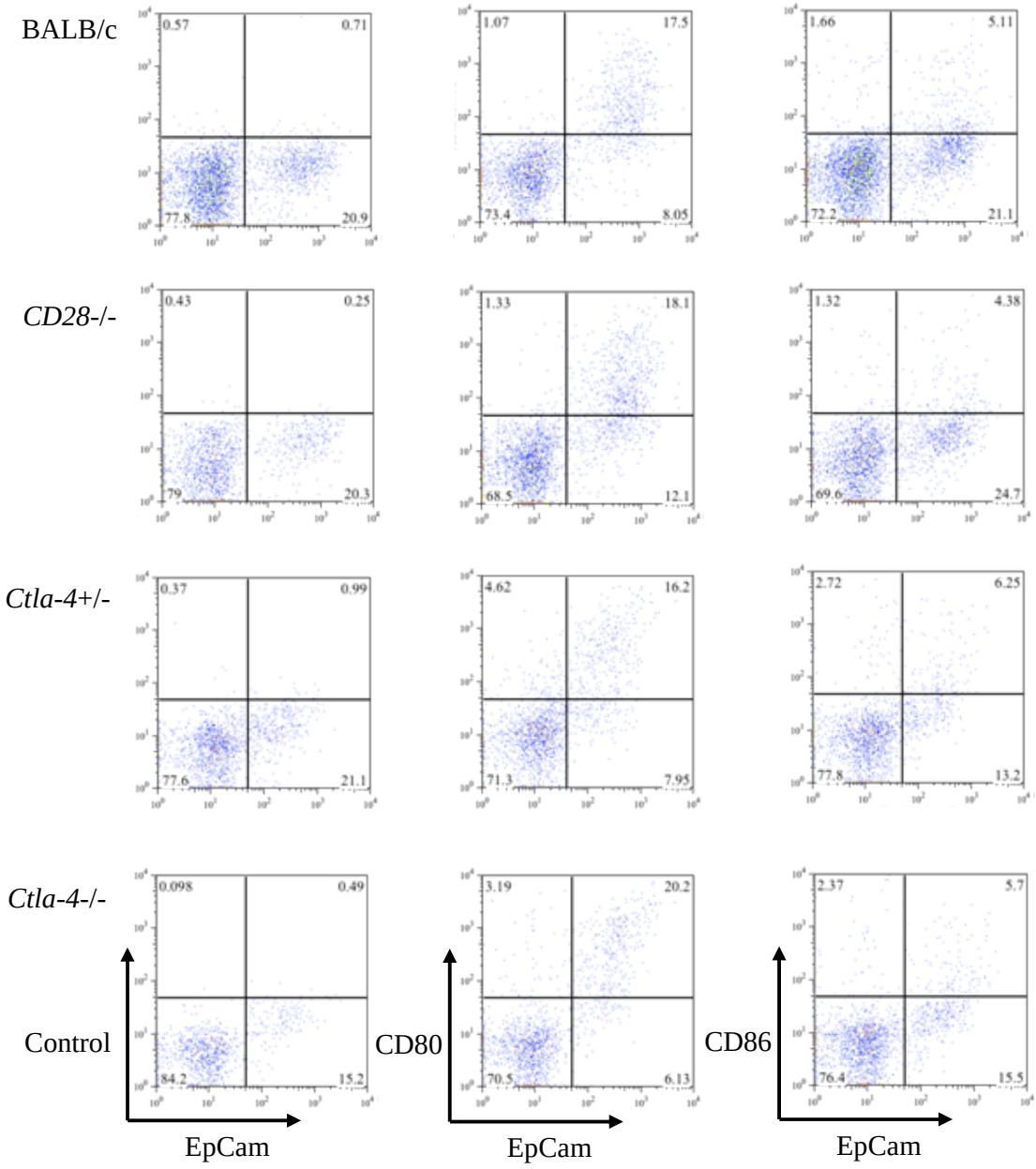
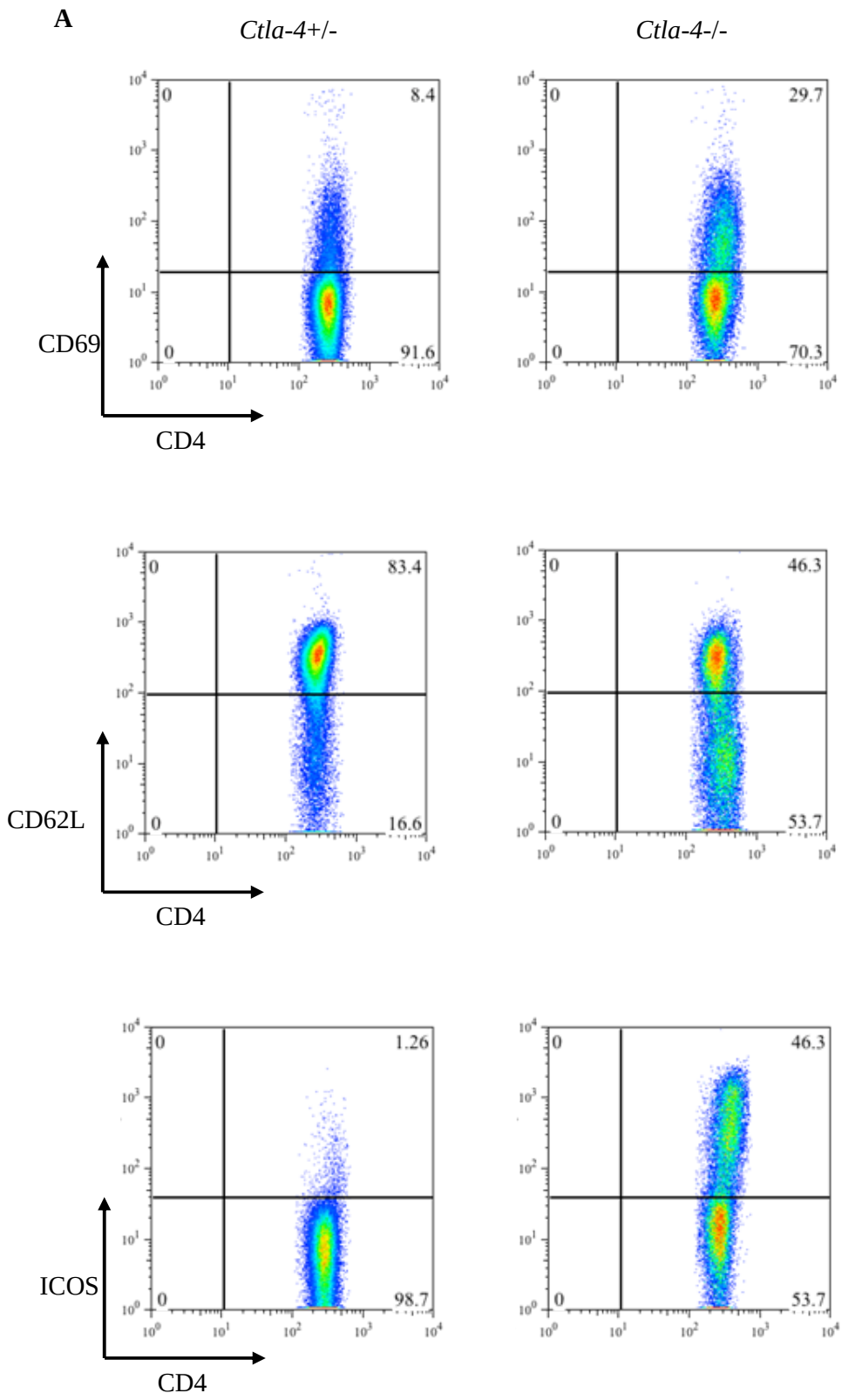


Figure 3.11. CD80 and CD86 expression by CD45- EpCam+ non-thymocytes. Adult wild type BALB/c, *CD28*-/-, 15-18 day old *Ctla-4*+/- and *Ctla-4*-/- thymi were teased to remove thymocytes, collagenase / dispase and trypsin digested and surface stained with CD45, EpCam, CD80, CD86 or control antibody for analysis by flow cytometry. Representative FACS plots to show CD80 (middle) and CD86 (right) expression by EpCam+ cells. All FACS plots show CD45- gate. Data representative of ≥3 mice.


A
Ctla-4+/-
Ctla-4-/-
CD69
CD4
8.4
91.6
29.7
70.3
CD62L
CD4
83.4
16.6
46.3
53.7
ICOS
CD4
1.26
98.7
46.3
53.7

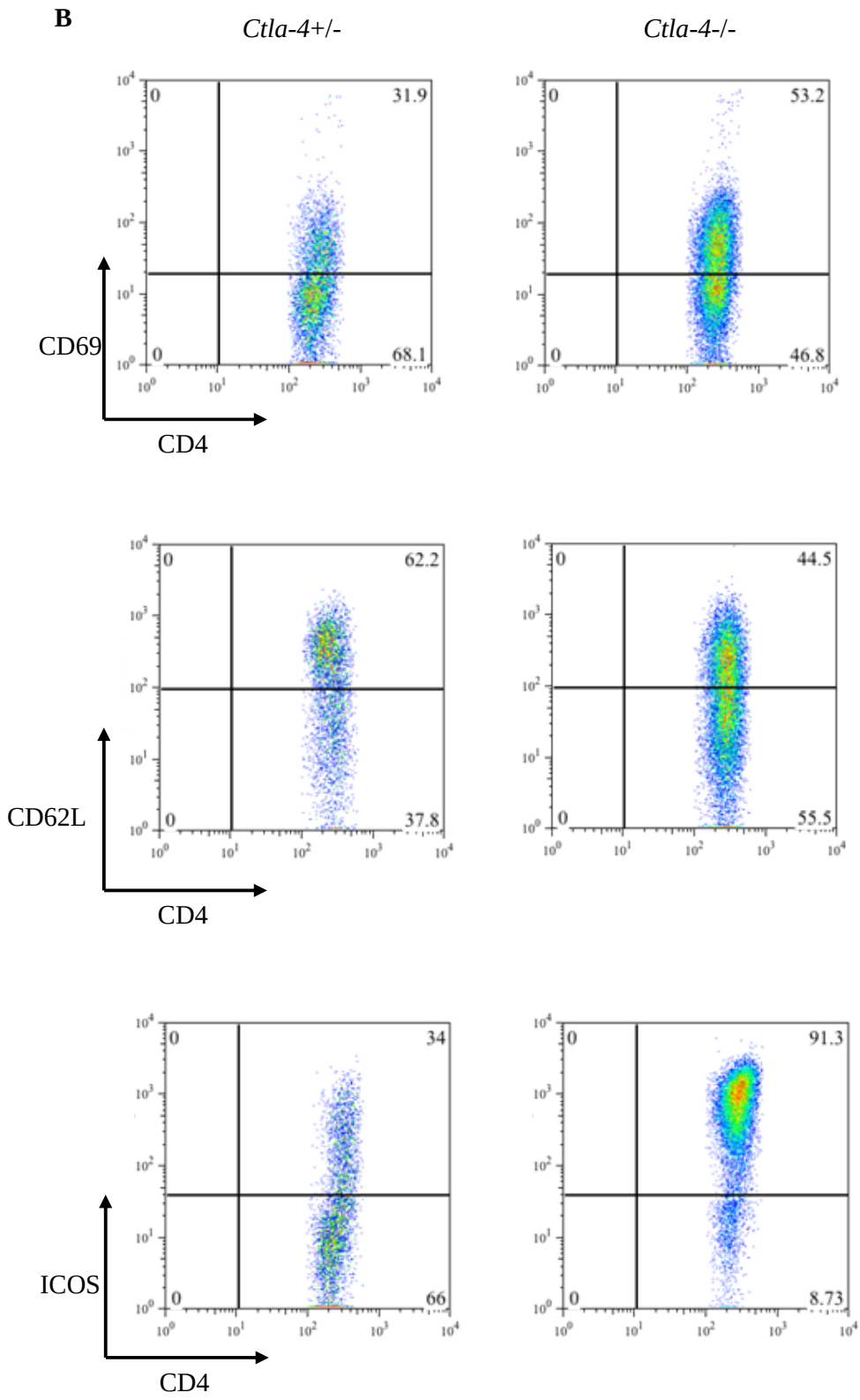

B
Ctla-4+/-
Ctla-4-/-
CD69
CD4
0
31.9
0
68.1
0
53.2
0
46.8
CD62L
CD4
0
62.2
0
37.8
0
44.5
0
55.5
ICOS
CD4
0
34
0
66
0
91.3
0
8.73

Figure 3.12. Activation marker expression by CD4+ FOXP3- conventional T cells and CD4+ FOXP3+ Tregs in *Ctla-4*-/- mice. Single cell suspensions from peripheral lymph nodes were surface stained with CD4, CD69, CD62L, ICOS and intracellularly with FOXP3 for analysis by flow cytometry. (A) Representative FACS plots of CD69 (top), CD62L (middle) and ICOS (bottom) for CD4+ FOXP3- gated lymphocytes of day 15-18 *Ctla-4*+/- (left) and day 15-18 *Ctla-4*-/- (right). (B) Representative FACS plots of CD69 (top), CD62L (middle) and ICOS (bottom) for CD4+ FOXP3+ gated lymphocytes of day 15-18 *Ctla-4*+/- (left) and day 15-18 *Ctla-4*-/- (right). Data representative of four mice.

(*Fig. 3.12 A*) and CD4+ FOXP+ Tregs (*Fig. 3.12 B*) there was a substantial increase in ICOS expression. Given the activated Treg phenotype observed above, enhanced proliferation could possibly explain the augmented FOXP3+ population in the periphery of *Ctla-4*-deficient mice. The proliferation of peripheral FOXP3+ cells in mice lacking *Ctla-4* was analysed using the nuclear protein expressed by proliferating cells; Ki67. Spleen sections from *Ctla-4* knockout and littermate controls were stained with CD4, FOXP3 and Ki67 for analysis by confocal microscopy. The confocal images in *Fig. 3.13 A* clearly show that many more FOXP3+ cells (red) in the *Ctla-4*-/- spleen were also Ki67+ (green) and undergoing proliferation (appearing yellow) compared to in the control littermate spleen. The immunoflourescence data obtained was quantified by scoring the images from the spleens of a number of different mice for the percentage of FOXP3+ cells that were also Ki67+ (yellow) (*Fig. 3.13 B*). There was a statistically significant increase in the percentage of proliferating FOXP3+ cells in *Ctla-4*-deficient spleens compared to littermate control spleens. Similar data was obtained by flow cytometry analysis of lymphocytes isolated from the lymph nodes of *Ctla-4* knockout mice compared to littermate controls. Intracellular staining for FOXP3 and Ki67 showed that there was a statistically significant increase in the proportion of proliferating (Ki67+) CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs in the absence of *Ctla-4* (*Fig. 3.14*).

The nuclear protein Ki67 that is present during all active phases of the cell cycle and absent in resting cells is becoming a more widely used cellular marker for determining the growth fraction of a given cell population [245]. Ki67 was the proliferation marker of choice as it is difficult to detect a more classical method such as 5-bromo-2-deoxyuridine (BrdU) incorporation along with FOXP3. It is also not easy to administer BrdU to *Ctla-4*-/- mice due to the early, fatal disease associated with ablation of the *Ctla-4* gene. The use of Ki67 as a marker of proliferation was validated by comparison with BrdU in CD4+ T cells of wild type BALB/c mice *in vivo* by flow cytometry. The representative FACS plots of *Fig. 3.15 A* and graph in *Fig. 3.15 B* show that Ki67 expression measured a similar proportion of proliferating CD4+ T cells to the detection of BrdU incorporation.

In *Fig. 3.14* it can be seen that *Ctla-4*+/- littermate control CD4+ FOXP3+ Tregs proliferate more than CD4+ FOXP3- Tconv. Some may view this Treg proliferation in naïve mice as

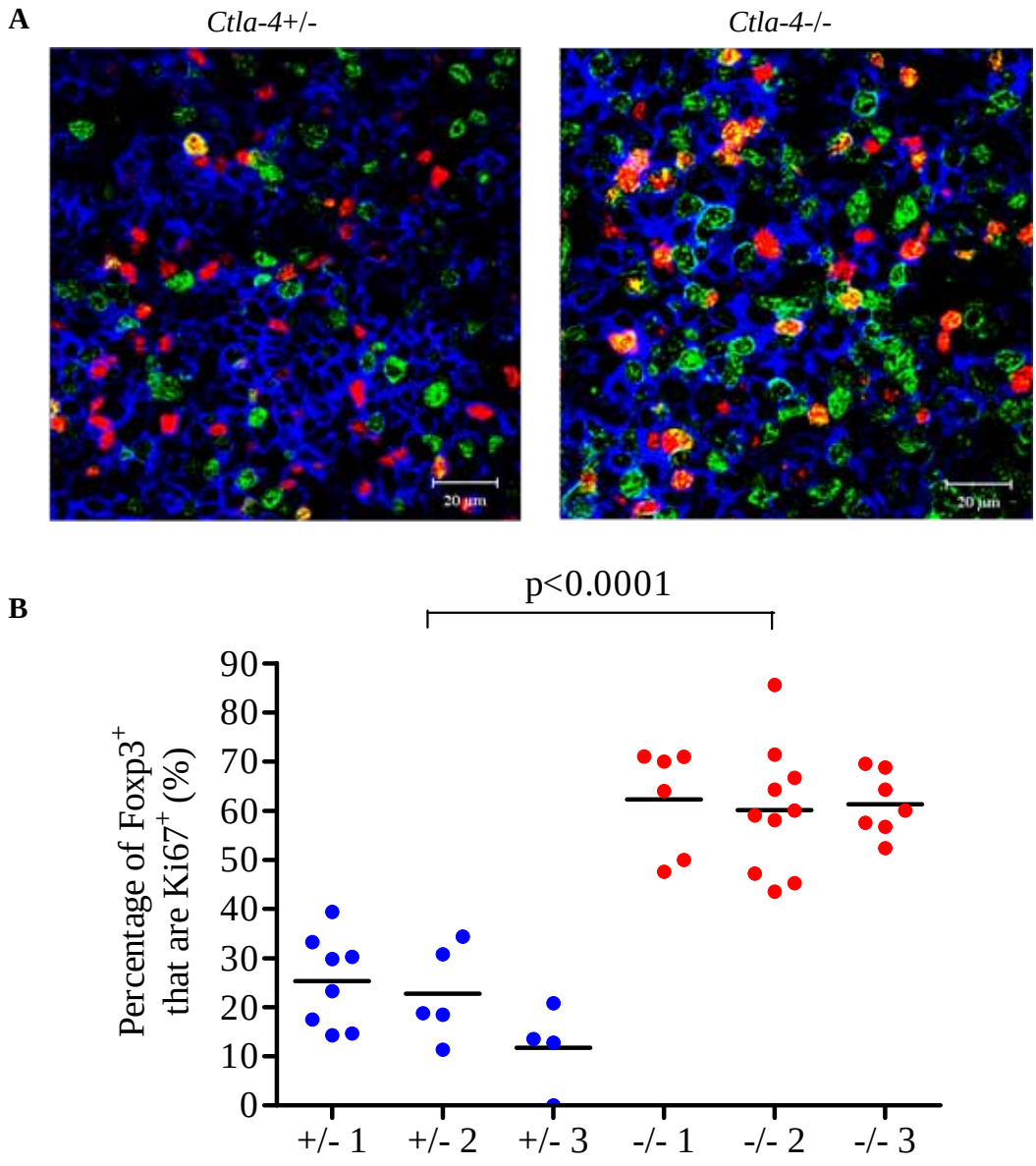


Figure 3.13. CD4+ FOXP3+ Tregs in the *Ctla-4*-/- spleen show increased proliferation. (A) Acetone fixed, 6µm spleen sections from 15-18 day old littermate *Ctla-4*+/- controls (left) or *Ctla-4*-/- (right) were stained with rat anti-CD4-647 (blue), rat anti-FOXP3-biotin, streptavidin-555 (red), rabbit anti-Ki67 and donkey anti-rabbit-488 (green) for analysis by confocal microscopy (x63). Proliferating FOXP3+ Ki67+ Tregs appear yellow. (B) Graph shows scoring of stained *Ctla-4*+/- (blue) and *Ctla-4*-/- (red) spleen sections for the percentage of FOXP3+ that are Ki67+. Each symbol represents a different field of view and each column a different mouse. >five fields were scored per mouse. Lines represent mean value. p value calculated by two-tailed, unpaired T-test for the means.

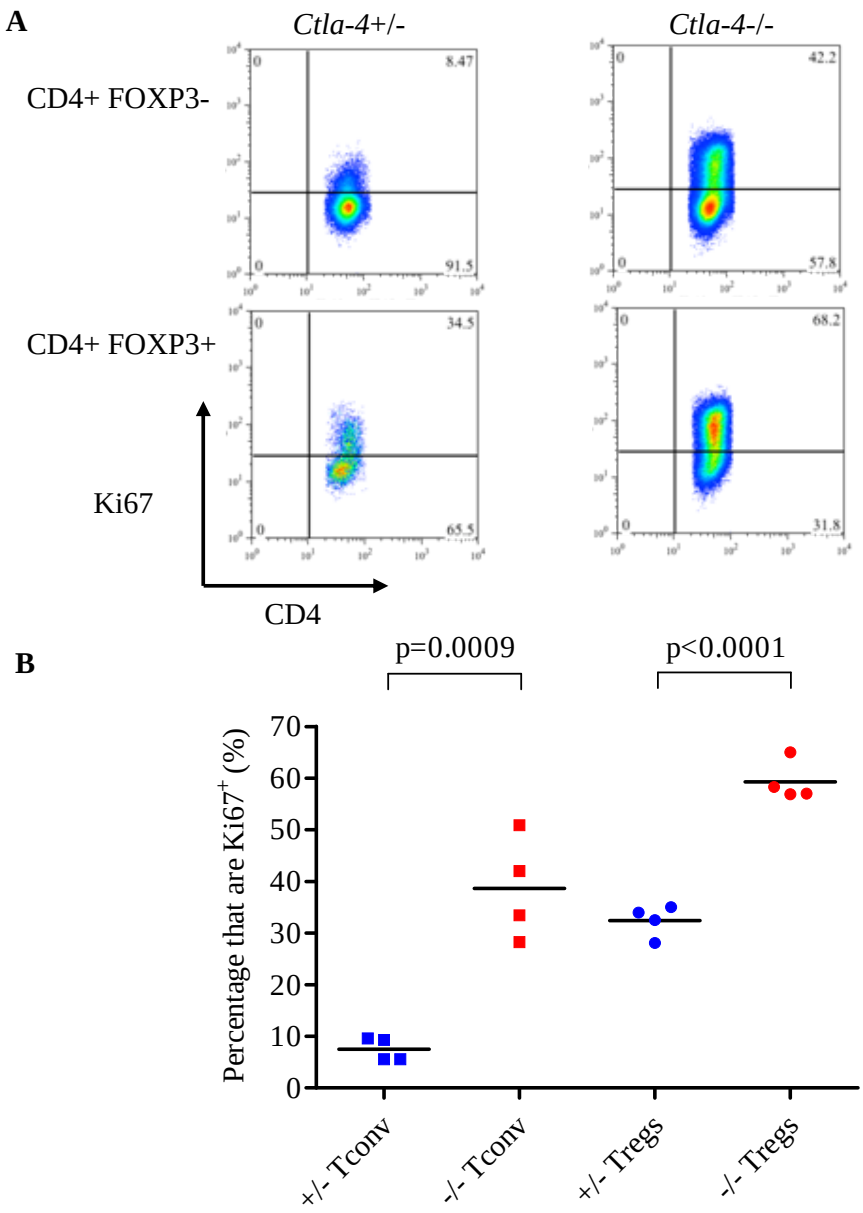


Figure 3.14. CD4+ FOXP3- conventional T cell and CD4+ FOXP3+ Treg proliferation in the peripheral lymph nodes of *Ctla-4*-/- mice. Single cell suspensions from peripheral lymph nodes were surface stained with CD4 and intracellularly with FOXP3 and Ki67 for analysis by flow cytometry. (A) Representative FACS plots of Ki67 expression by CD4+ FOXP3- gated lymphocytes (top) and CD4+ FOXP3+ gated lymphocytes (bottom) from 15-18 day old *Ctla-4*+/- (left) and *Ctla-4*-/- (right). (B) Graph shows percentage of CD4+ FOXP3- (squares) and CD4+ FOXP3+ (circles) that expressed Ki67 for four *Ctla-4*+/- (blue) and four *Ctla-4*-/- (red) mice. Lines indicate mean values. p values calculated by two-tailed, unpaired T-test for the means. Experiments carried out in collaboration with C. Wang.

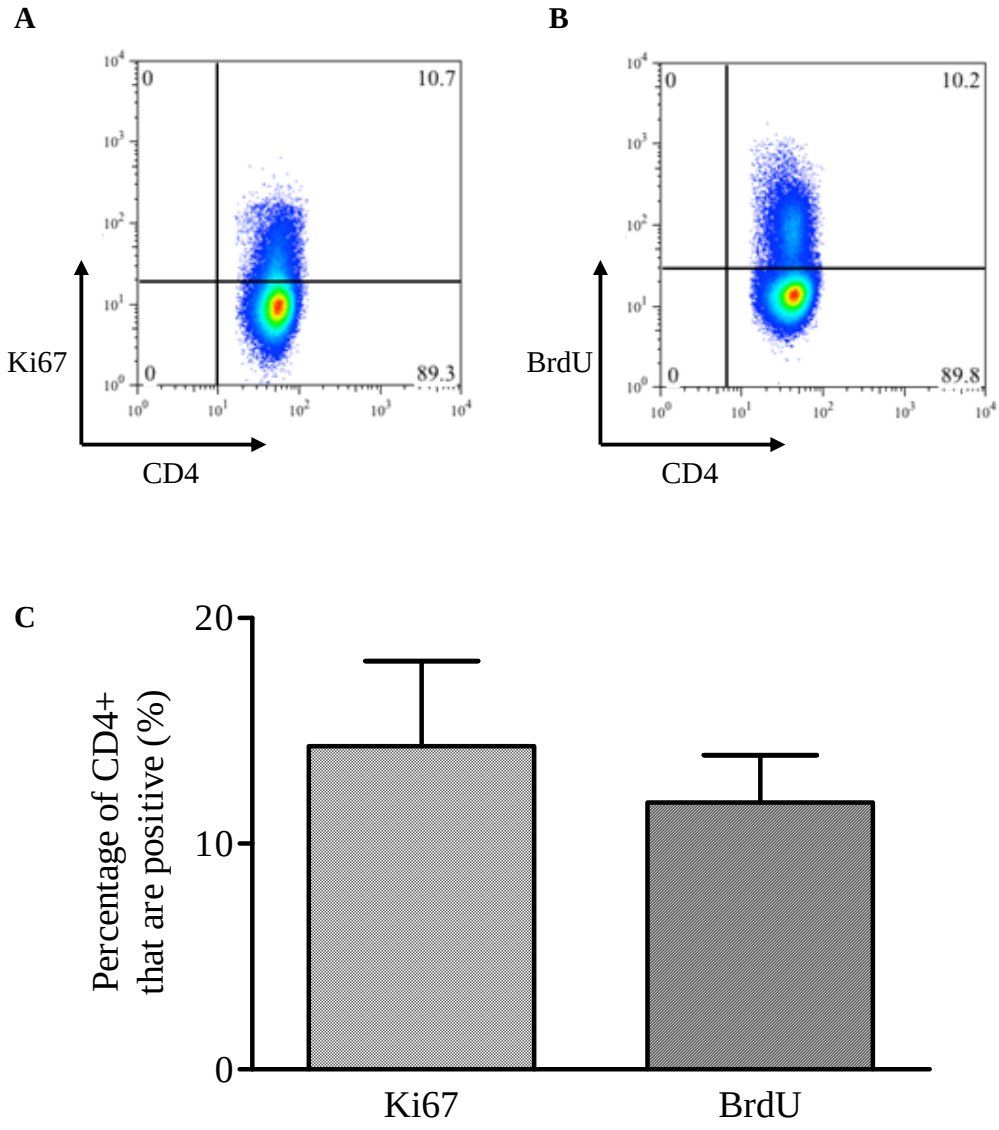


Figure 3.15. Validation of Ki67 as a marker of proliferation by comparison with BrdU. Two week old wild type BALB/c mice received 1mg BrdU by intra-peritoneal injection every twenty-four hours for three days. Twenty-four hours following last injection mice were harvested and single cell suspensions from peripheral lymph nodes were surface stained with CD4 and intracellularly with Ki67 or BrdU for analysis by flow cytometry. Representative FACS plots of Ki67 (A) and BrdU (B) expression by CD4+ gated lymphocytes. (C) Graph shows data for four mice. Bars represent means and standard deviation is shown.

rather high considering that *in vitro* Tregs are anergic [57]. However, in unmanipulated mice the CD4+ CD25+ Treg population has been shown to exhibit a surprising capacity for proliferation that has been suggested to reflect their continuous encounter with self antigens [246]. Additionally, due to the young age at which *Ctla-4*-/- mice have to be sacrificed there may also be lymphopenic affects seen in the age-matched *Ctla-4*+/- littermate controls. To investigate the proliferation of naïve conventional T cells and regulatory T cells, BALB/c mice were assessed during maturation from a neonate (1 week old) into an adult (>6 weeks old). It can be seen in *Fig. 3.16* that CD4+ FOXP3+ Treg proliferation, as determined by Ki67 expression, was generally greater than that of CD4+ FOXP3- Tconv proliferation. The graph of *Fig. 3.16* demonstrates that both Treg and Tconv proliferation was greater in young mice (1 week old) and decreased as the mice matured into adults (>6 weeks old).

3.2.4 Tregs deficient in *Ctla-4* undergo less cell death.

An augmented FOXP3+ population in the absence of *Ctla-4* could also be a consequence of decreased cell death. Therefore, the proportion of FOXP3+ cells undergoing cell death was analysed in the *Ctla-4* knockout mouse. Cells undergoing apoptosis translocate phosphatidylserine from the inner leaflet of the plasma membrane lipid bilayer to the outer layer [247] where, in the presence of calcium, the ANNEXIN V protein can then detect and bind this phospholipid-like molecule [248]. Unfortunately, analysis of ANNEXIN V binding by flow cytometry cannot be carried out with intracellular staining for FOXP3. Tregs in the mouse can be identified with the surface markers CD4 and CD25 as the CD4+ CD25+ population correlates very well with the CD4+ FOXP3+ population. However, in the *Ctla-4*-deficient mouse CD4+ FOXP3- Tconv exhibit an activated phenotype (*Fig. 3.12 A*) with increased expression of CD25 [156, 157]. *Fig. 3.17 A* illustrates that the CD4+ CD25+ population in the *Ctla-4*-/- mouse consists of only 85% FOXP3+ lymphocytes. Consequently, additional cell surface markers had to be indentified that could be used to represent a pure FOXP3+ Treg population. In humans where the regulatory CD4+ FOXP3+ population is confined to just the CD4+ CD25hi cells, CD127 is frequently used as an additional cell surface marker to isolate a pure population of CD4+ CD25hi CD127lo FOXP3+ Tregs [249, 250]. It has also been shown in an inflammatory environment (inflamed synovia) where Tconv have increased CD25 expression, that CD27 is a useful marker to distinguish Tregs from effector T cells. CD27 is constitutively expressed by T cells and upon activation Tregs

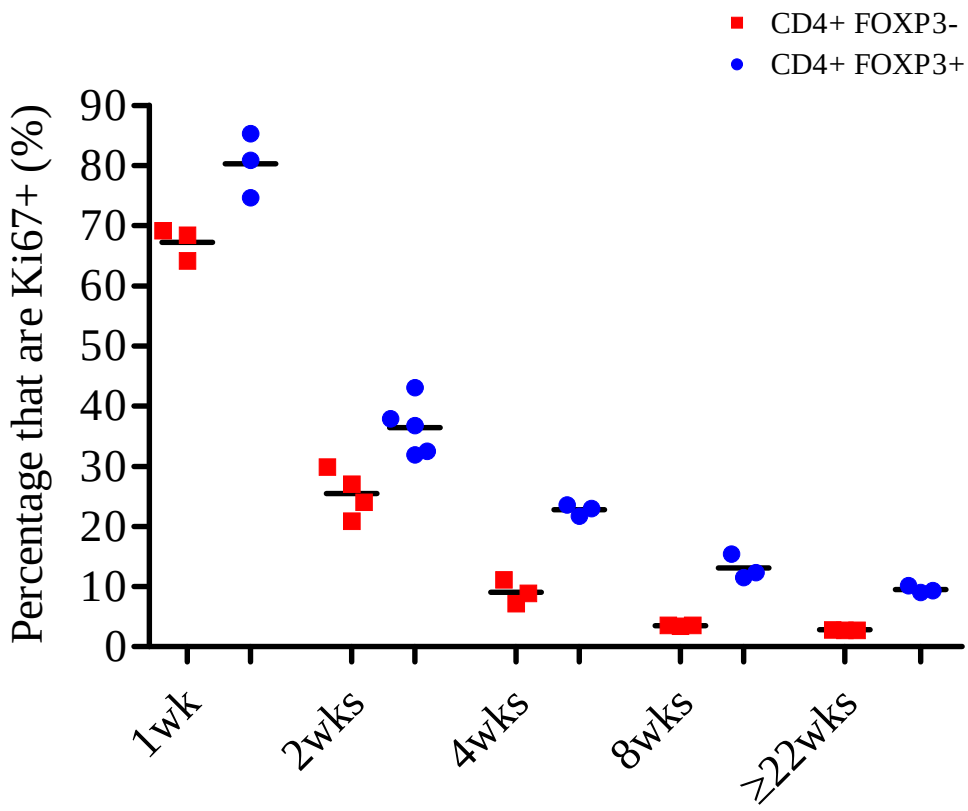


Figure 3.16. Timecourse of CD4+ FOXP3- conventional T cell and CD4+ FOXP3+ Treg proliferation. Single cell suspensions from wild type BALB/c peripheral lymph nodes were surface stained with CD4 and intracellularly with Ki67 and FOXP3 for analysis by flow cytometry. Graph shows percentage of gated population that express Ki67 for one week, two weeks, four weeks, eight weeks or ≥twenty-two week old mice. CD4+ FOXP3- gated lymphocytes represented by red squares and CD4+ FOXP3+ gated lymphocytes represented by blue circles. Each point represents a different mouse and lines indicate means.

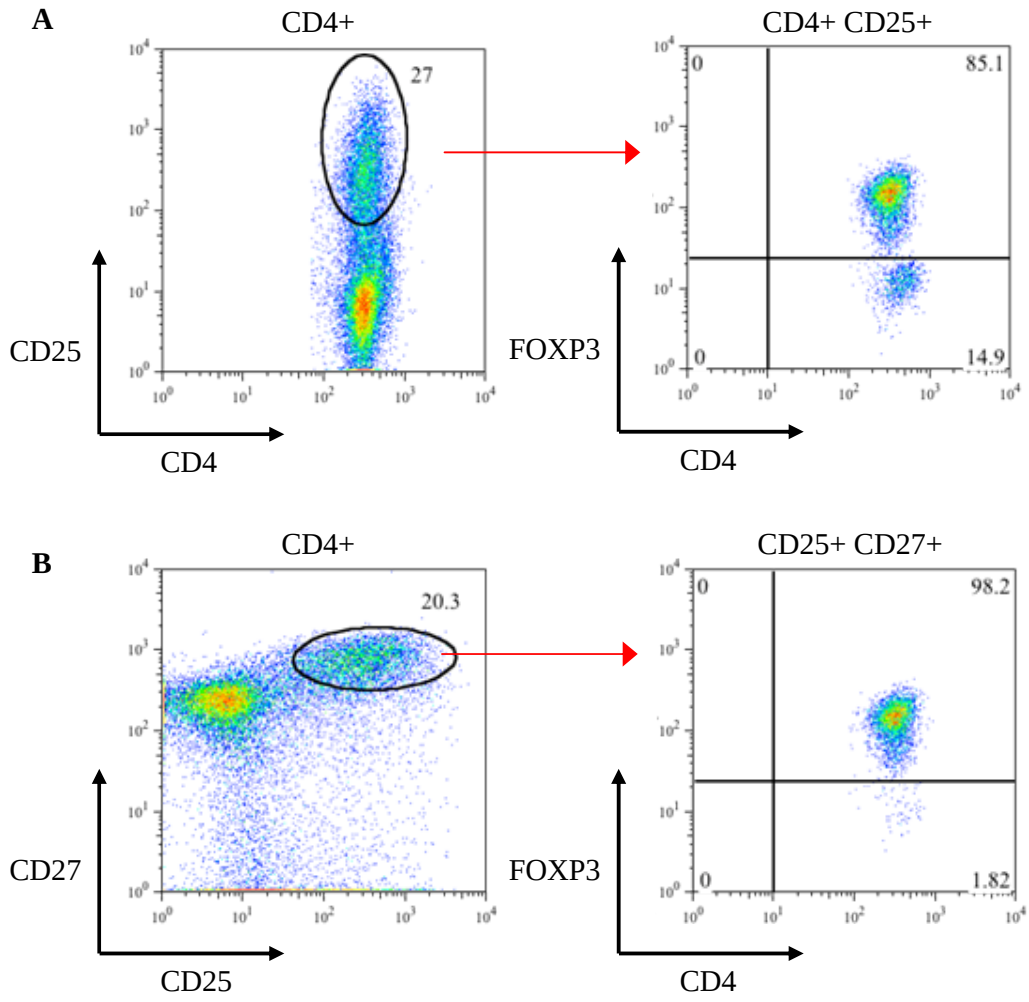


Figure 3.17. Discrimination of Tregs from activated conventional T cells using CD25 and CD27 cell surface markers. Single cell suspensions from 15-18 day old *Ctla-4*-/- peripheral lymph nodes were surface stained with CD4, CD25, CD27 and intracellularly with FOXP3 for analysis by flow cytometry. Representative FACS plots show (A) FOXP3 expression (right) of CD4+ CD25+ gated lymphocytes indicated on the left and (B) FOXP3 expression (right) of CD4+ CD25+ CD27hi gated lymphocytes indicated on the left. Experiments carried out in collaboration with C. Wang.

maintain expression of CD27, whereas Tconv down regulate CD27 [251]. It was therefore investigated whether CD27 could be used in conjunction with CD4 and CD25 as cell surface markers to isolate a pure CD4+ FOXP3+ population from the activated environment of *Ctla-4*-deficient mice. *Fig. 3.17 B* shows that when just the CD25+ CD27hi CD4+ T cell population was isolated it had a FOXP3+ purity of 98%. In future when intracellular FOXP3 cannot be used to identify Tregs, a reliable population could be identified as CD4+ CD25+ CD27hi.

To analyse the proportion of FOXP3+ Tregs undergoing cell death (apoptotic and necrotic), ANNEXIN V staining was carried out on peripheral lymphocytes along with the surface markers CD4, CD25 and CD27 for analysis by flow cytometry. The representative FACS plots and graph in *Fig. 3.18* show that there was slightly less cell death occurring within the Tconv population in *Ctla-4*-/- mice compared to wild type as indicated by reduced ANNEXIN V staining. Whereas there was a statistically significant reduction in ANNEXIN V staining and therefore cell death occurring within the Treg population in *Ctla-4*-/- mice compared to wild type controls. In addition to increased proliferation of peripheral Tregs in *Ctla-4*-deficient mice, decreased occurrence of cell death could also be contributing to the augmented FOXP3+ population observed.

To investigate the mechanism behind the observed reduction in regulatory T cell death in the *Ctla-4* knockout mice, FAS expression was analysed by flow cytometry. FAS (CD95) is a death receptor up-regulated by activated T cells and the ligation of which, by FAS ligand, induces apoptosis [252-254]. Mechanistically, the triggering of signalling pathways can be regulated by the control of cell surface receptor expression. The representative histograms and graph in *Fig. 3.19* show that FAS expression by CD4+ FOXP3- Tconv was similar in lymphocytes from *Ctla-4*-deficient mice and littermate controls. A reduction in the intensity of FAS expression by CD4+ FOXP3+ Tregs from *Ctla-4* knockout mice was observed compared to lymphocytes from littermate controls. Reduced FAS receptor expression by Tregs either due to the cell-intrinsic loss of CTLA-4 or the environment of the *Ctla-4*-deficient mouse, could potentially account for the observed decrease in proportion of Tregs undergoing cell death in *Ctla-4*-/- mice.

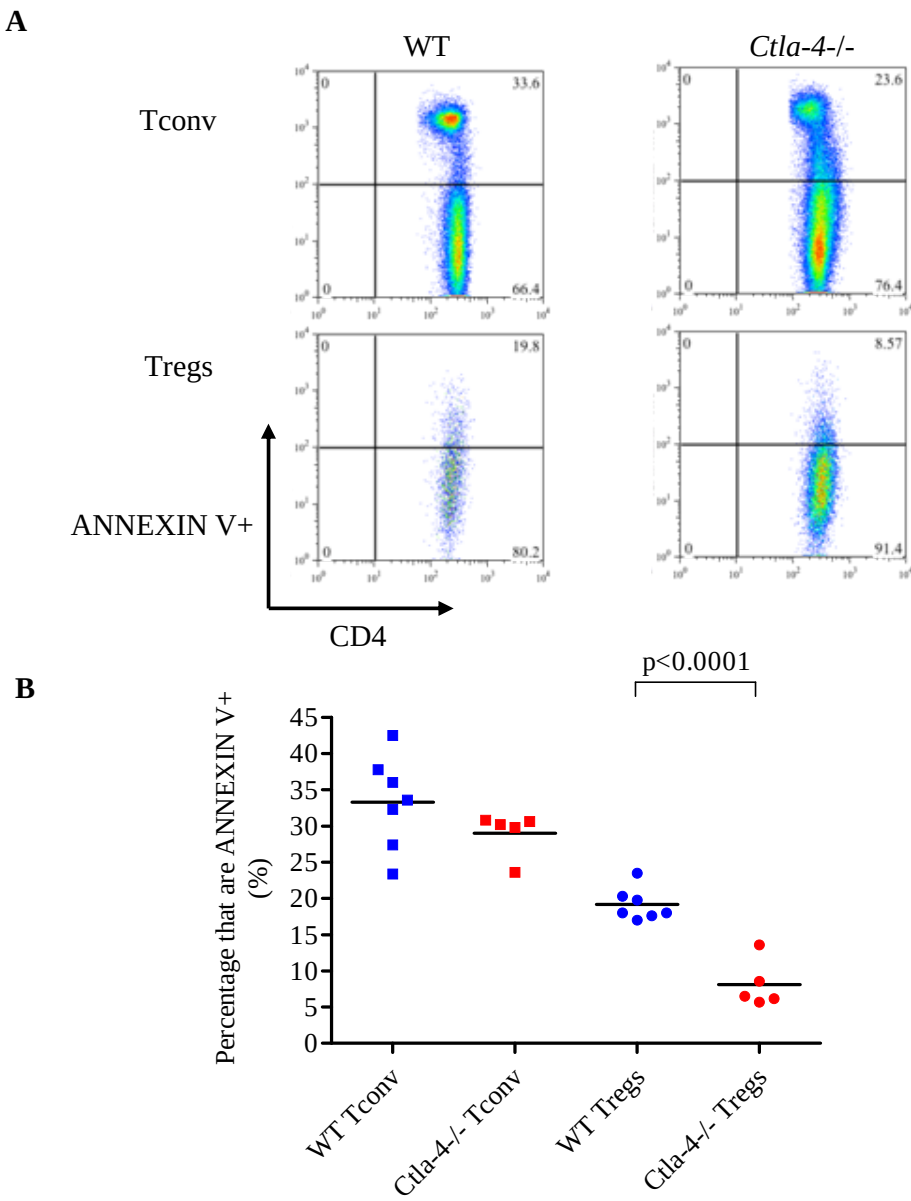


Figure 3.18. Cell Death analysis of *Ctla-4*-deficient CD4+ CD27low Tconv and CD4+ CD25+ CD27hi Tregs using ANNEXIN V. Single cell suspensions from 15-18 day old wild type BALB/c (left) and *Ctla-4*-/- (right) peripheral lymph nodes were surface stained with CD4, CD25, CD27 and ANNEXIN V for analysis by flow cytometry. (A) Representative FACS plots show ANNEXIN V expression by CD4+ CD27low gated Tconv (top) and CD4+ CD25+ CD27hi gated Tregs (bottom). (B) Graph shows data of CD4+ CD27low Tconv (squares) and CD4+ CD25+ CD27hi Tregs (circles) for >five wild type BALB/c (blue) and *Ctla-4*-/- (red) mice. Lines indicate mean values. p value calculated by two-tailed, unpaired T-test for the means.

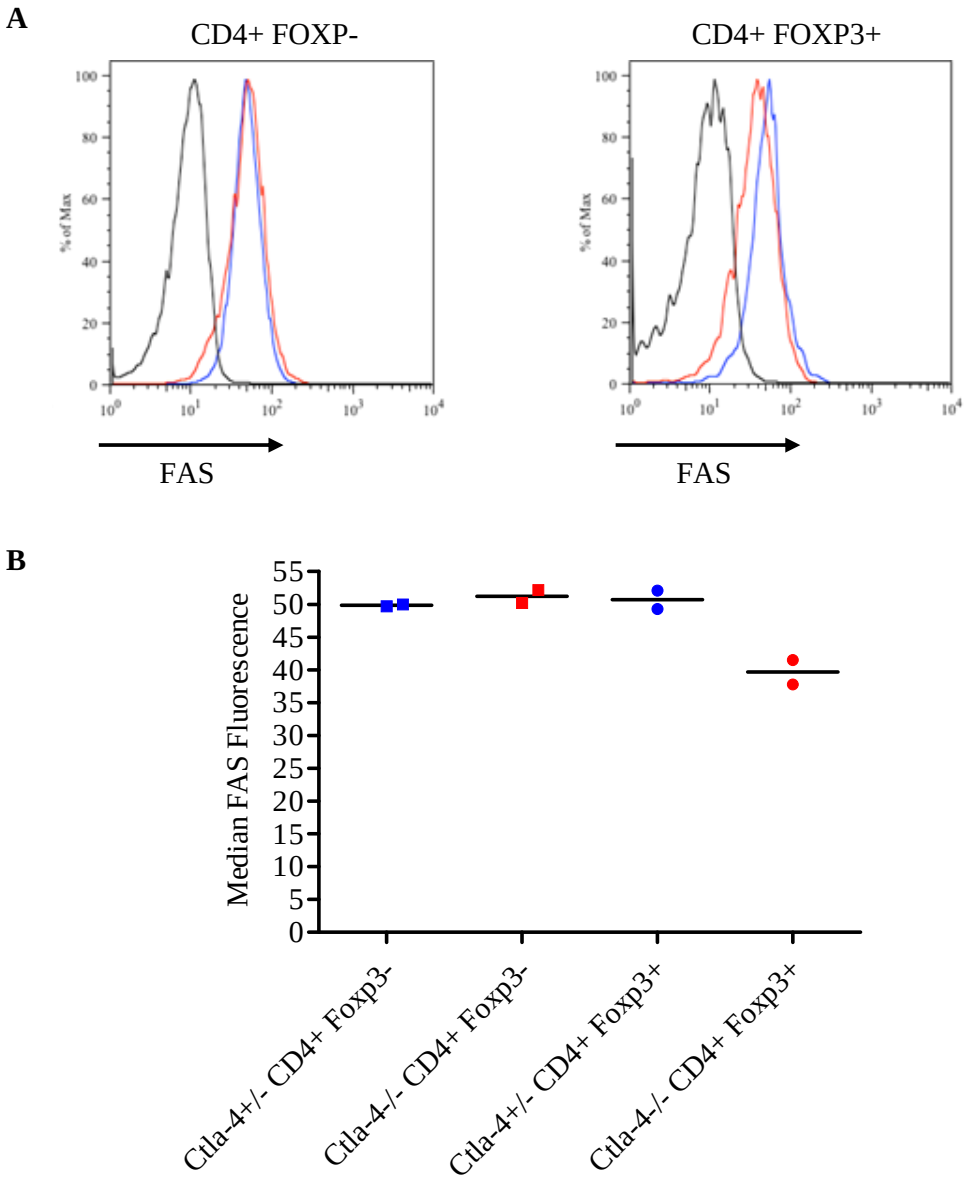


Figure 3.19. FAS expression by *Ctla-4*-/- CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs. Single cell suspensions from 15-18 day old *Ctla-4*+/- (blue) and *Ctla-4*-/- (red) peripheral lymph nodes were surface stained with CD4, FAS and intracellularly with FOXP3 for analysis by flow cytometry. (A) Representative histograms show FAS expression by CD4+ FOXP3- gated Tconv (left) and CD4+ FOXP3+ gated Tregs (right). Isotype control in black. (B) Graph shows data for FAS median fluorescence intensity of CD4+ FOXP3- (squares) and CD4+ FOXP3+ (circles) for two *Ctla-4*+/- (blue) and *Ctla-4*-/- (red) mice. Lines indicate mean values.

The amount of Tconv and Treg cell death measured in wild type mice in *Fig. 3.18* could be considered to be relatively high. These wild type mice were only 2-3 weeks old because they were age-matched to the 15-18 day old *Ctla-4*-/- mice. Tconv and Treg cell death was consequently assessed in the naïve BALB/c mouse as it matures from a neonate (2 weeks old) into an adult (>6 weeks old) by ANNEXIN V staining and flow cytometry. It can be seen in *Fig. 3.20* that CD4+ CD27low conventional T cell death, as determined by ANNEXIN V binding, did not vary over the timecourse studied, if anything ANNEXIN V binding to Tconv slightly increased as the BALB/c mice aged. CD4+ CD25+ CD27hi regulatory T cell death, as determined by ANNEXIN V binding, on the other hand decreased from 2 weeks to 4 weeks and then remained fairly constant.

3.2.5 Induction of the Treg-specific transcription factor FOXP3.

Natural CD4+ FOXP3+ Tregs are produced in the thymus and exported to the periphery where functionally they are main players in the maintenance of peripheral tolerance. More recently it has been established that expression of the transcription factor FOXP3 can be induced in CD4+ FOXP3- Tconv in the periphery to generate adaptive Tregs (reviewed [106]). To explore whether the augmented FOXP3+ population observed in the periphery of *Ctla-4*-/- mice could reflect increased induction of FOXP3 in peripheral T cells, experiments to test the role of CTLA-4 in FOXP3 induction were carried out.

In vitro, culture of CD4+ CD25- Tconv with TCR-stimulation and CD28 co-stimulation in the presence of TGF-β induces FOXP3 expression [114]. However, it is difficult to determine whether this is true FOXP3 induction or outgrowth of any contaminating FOXP3+ cells present in the isolated CD4+ CD25- population before culture. This technical problem can be overcome using lymphocytes from TCR-transgenic mice. T cells within *DO11.10* mice bear a transgenic T cell receptor specific for the model antigen OVA (KJ-126+). However, due to random TCR-rearrangement during T cell development some KJ-126-negative T cells can develop. The model antigen OVA is not promiscuously expressed within the thymus, therefore natural CD4+ FOXP3+ Tregs specific for OVA (KJ-126+) do not develop in the thymus and cannot be found in the periphery. Although, it is possible that KJ-126-negative T cells which develop due to random TCR-rearrangement could encounter their antigen within the thymus and be induced to become natural CD4+ FOXP3+ Tregs. Consequently, a

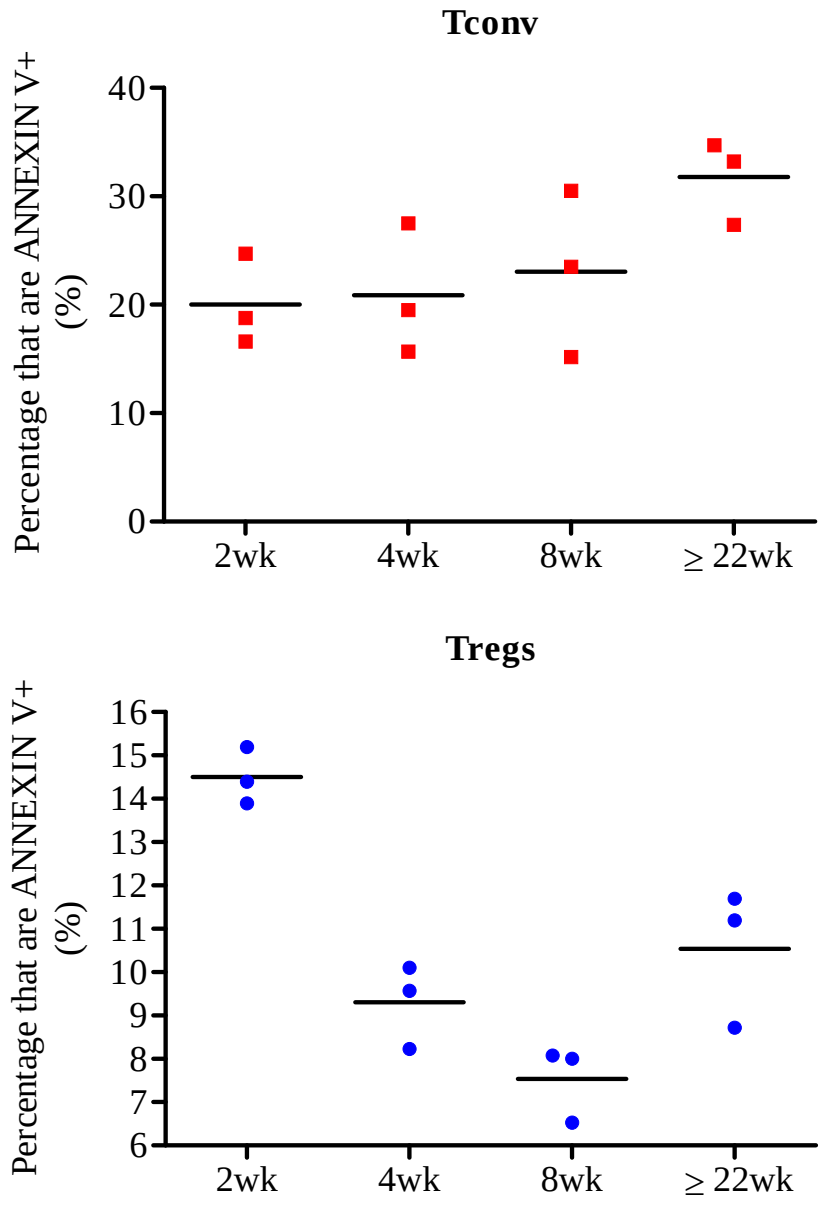


Figure 3.20. Timecourse of CD4+ CD27low Tconv and CD4+ CD25+ CD27hi Treg cell death by ANNEXIN V. Single cell suspensions from wild type BALB/c peripheral lymph nodes were surface stained with CD4, CD25, CD27 and ANNEXIN V for analysis by flow cytometry. Graphs show percentage of gated population that express ANNEXIN V. Data for three two weeks, four weeks, eight weeks or ≥twenty-two week old mice. CD4+ CD27low gated lymphocytes represented by red squares (top) and CD4+ CD25+ CD27hi gated lymphocytes represented by blue circles (bottom). Lines indicate means.

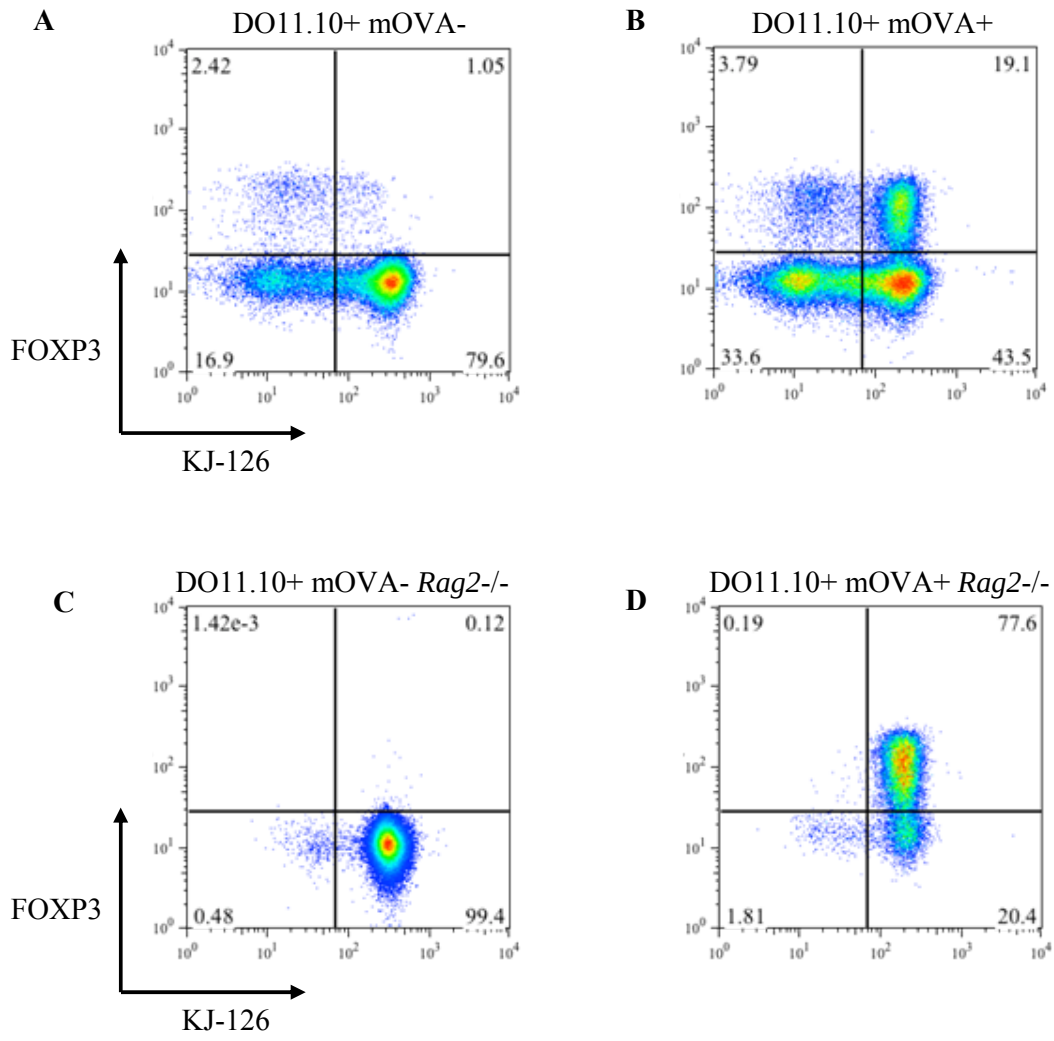


Figure 3.21. Analysis of antigen-specific CD4+ FOXP3+ Tregs in TCR-transgenic mice. Single cell suspensions from peripheral lymph nodes were surface stained with CD4, KJ-126 and intracellularly with FOXP for analysis by flow cytometry. Representative FACS plots of antigen-specific (KJ-126+) CD4+ FOXP3+ Tregs in (A) DO11.10+ mOVA-, (B) DO11.10+ mOVA+ mice, (C) DO11.10+ mOVA- *Rag2*-/- and (D) DO11.10+ mOVA+ *Rag2*-/- mice. All FACS plots CD4+ gated lymphocytes.

minority of CD4+ KJ-126- FOXP3+ lymphocytes are exported from the thymus and can be found in the periphery (*Fig. 3.21 A*). By maintaining TCR-transgenic mice on a *Rag2*-/- background (deficiency in the recombination activating gene), random TCR-rearrangements are prevented and no T cells encounter their antigen in the thymus which prevents thymic selection of FOXP3+ Tregs. Therefore, all T cells in the periphery are antigen-specific (KJ-126+) but FOXP3- (*Fig. 3.21 C*). In contrast, in *DO11.10* TCR-transgenic mice expression of the model antigen OVA under the control of the rat insulin promoter (RIP) means OVA is expressed in the thymus and natural CD4+ FOXP3+ Tregs specific for OVA (KJ-126+) develop and can be found in the periphery (*Fig. 3.21 B*). Maintenance of these double transgenic mice on a *Rag2*-/- background means that only OVA antigen-specific (KJ-126+) CD4+ FOXP3- conventional T cells and CD4+ FOXP3+ regulatory T cells develop (*Fig. 3.21 D*).

Lymphocytes from DO11.10+ mOVA- *Rag2*-/- mice that were either wild type, *Ctla-4*-/- or *CD28*-/- were used to investigate the role of CTLA-4 and CD28 in the induction of FOXP3 *in vitro*. *Fig. 3.22 A* shows a representative FACS plot of the FOXP3 expression induced in CD4+ KJ-126+ wild type DO11.10+ mOVA- *Rag2*-/- lymphocytes cultured for 3 days in the presence of TGF-β (44%). Addition of recombinant IL-2 augmented FOXP3 induction (58.2%). Negligible FOXP3 expression was seen following culture without TGF-β, plus or minus rIL-2. The graph of *Fig. 3.22 B* shows a similar percentage of CD4+ KJ-126+ cells were induced to express FOXP3 by TGF-β in both wild type and *Ctla-4*-deficient mice. FOXP3 induction by TGF-β was lower in CD4+ KJ-126+ lymphocytes that lacked *CD28*. Addition of rIL-2 slightly augmented the mean percentage of FOXP3 induced in wild type CD4+ KJ-126+ but had no affect on the TGF-β induction of FOXP3 by lymphocytes lacking *Ctla-4*. The percentage of FOXP3 expression induced in lymphocytes deficient in *CD28* was substantially increased upon addition of rIL-2. Analysis of the absolute numbers (*Fig. 3.22 C*) of FOXP3+ CD4+ KJ-126+ CD25+ induced indicated that in the absence of *CD28* there was decreased FOXP3 induction compared to wild type, whereas the absence of *Ctla-4* had no affect. Addition of rIL-2 slightly increased the absolute number of CD4+ KJ-126+ CD25+ FOXP3+ of wild type lymphocytes and *CD28*-/- lymphocytes relative to TGF-β induction in the absence of rIL-2. However, rIL-2 decreased the absolute number of CD4+ KJ-126+ CD25+ FOXP3+ induced by TGF-β compared to in the absence of rIL-2

A

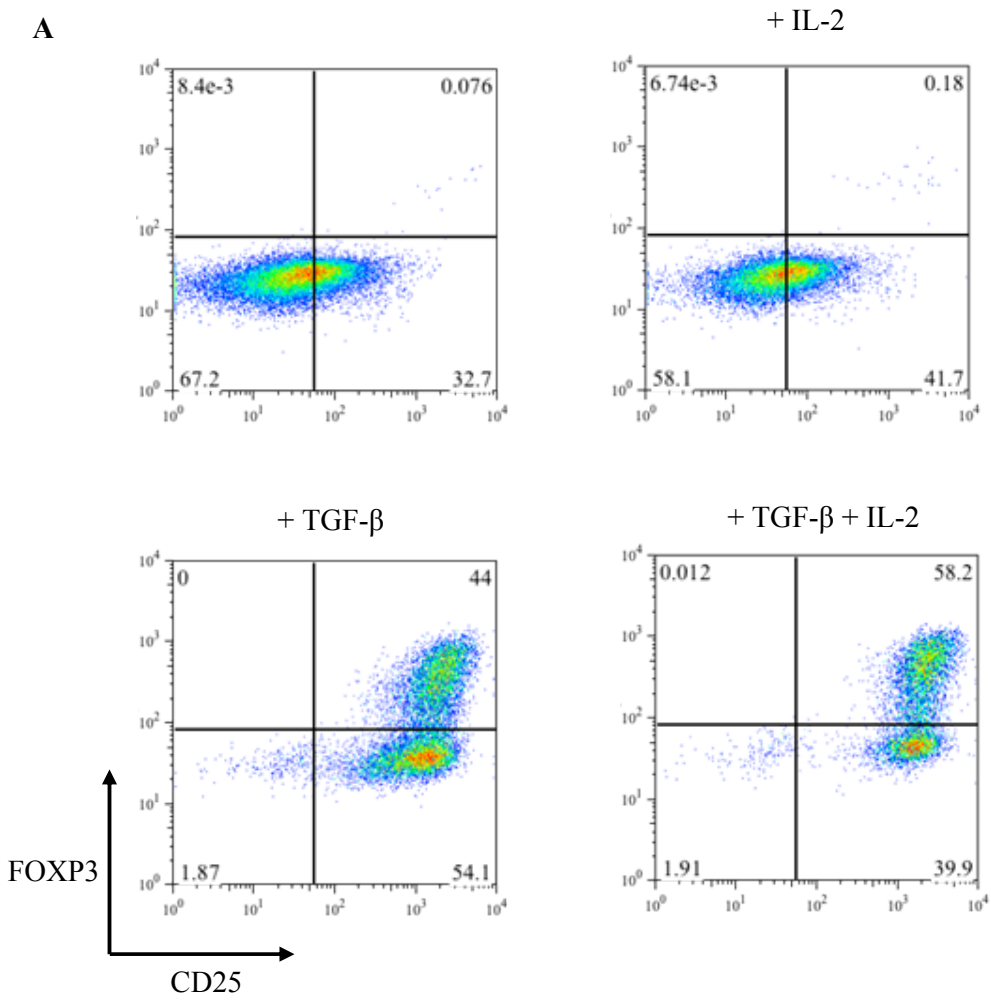
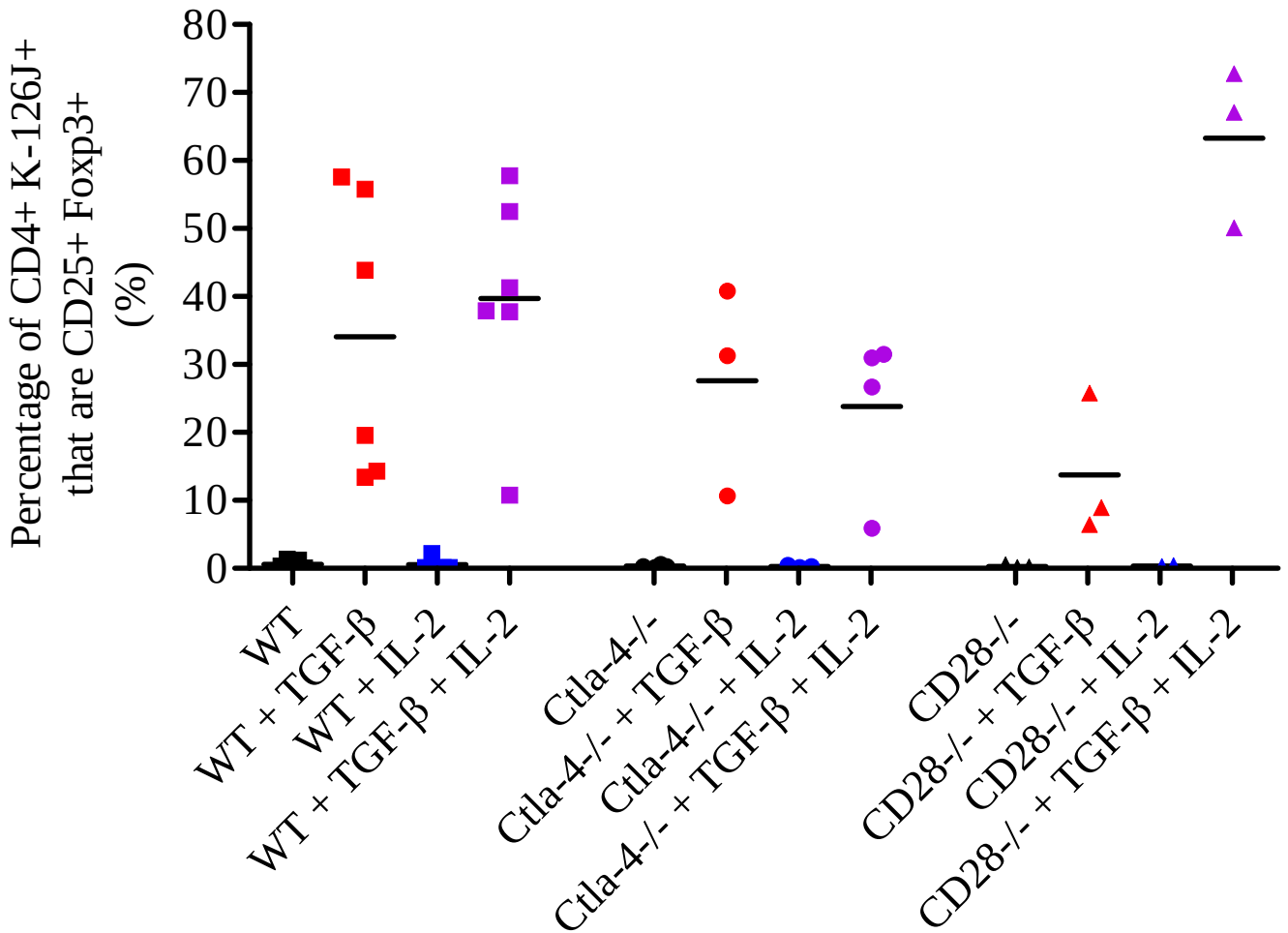
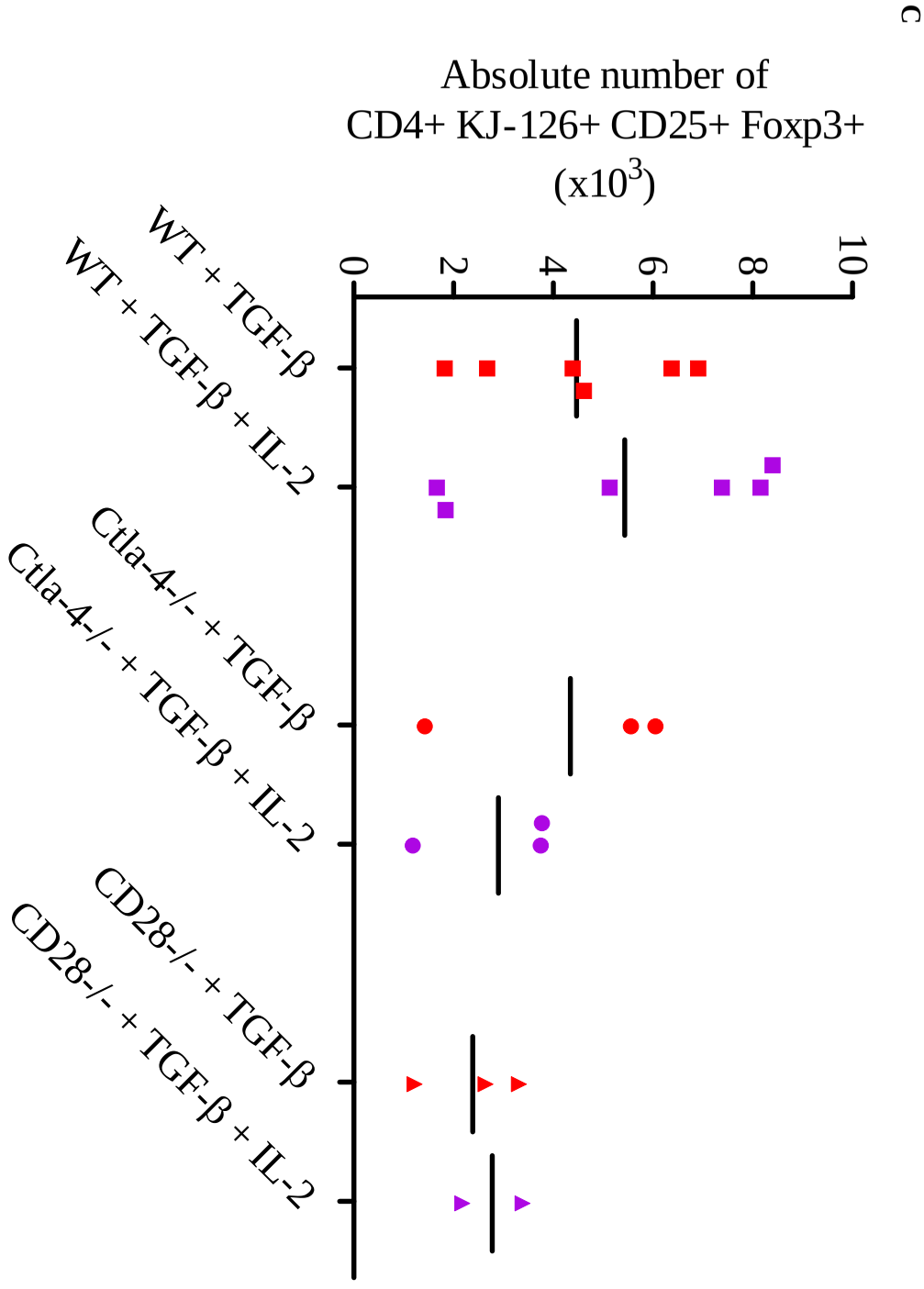

+ IL-2
8.4e-3
0.076
67.2
32.7
6.74e-3
0.18
58.1
41.7
+ TGF-β
0
44
1.87
54.1
+ TGF-β + IL-2
0.012
58.2
1.91
39.9
FOXP3
CD25

Figure 3.22. Induction of CD4+ FOXP3+ cells *in vitro* using TGF-β. Single cell suspensions of peripheral lymph nodes were cultured with 1μg/ml plate-bound anti-CD3, plus or minus 3ng/ml TGFβ and 100 I.U. recombinant IL-2. On day three, cells were harvested and surface stained with CD4, KJ, CD25 and intracellularly with FOXP3 for analysis by flow cytometry. (A) Representative FACS plots showing CD25 and FOXP3 expression of CD4+ KJ+ gated lymphocytes from DO11.10+ mOVA- *Rag2*-/- mice cultured (B) Graph shows data of the percentage of CD4+ KJ+ that are CD25+ FOXP3+ following culture with anti-CD3 alone (black), plus rIL-2 (blue), plus TGFβ (red) or plus TGFβ and rIL-2 (purple) for ≥three wild type DO11.10+ mOVA- *Rag2*-/- (squares), *Ctla-4*-/- DO11.10+ mOVA- *Rag2*-/- (circles) or *CD28*-/- DO11.10+ mOVA- *Rag2*-/- mice (triangles). (C) Graph shows data of the absolute number of CD4+ KJ+ CD25+ FOXP3+ following culture plus TGFβ (red) or TGFβ and rIL-2 (purple) for ≥three wild type DO11.10+ mOVA- *Rag2*-/- (squares), *Ctla-4*-/- DO11.10+ mOVA- *Rag2*-/- (circles) or *CD28*-/- DO11.10+ mOVA- *Rag2*-/- mice (triangles). Lines indicate mean values.

B


Percentage of CD4+ K-126J+ that are CD25+ Foxp3+ (%)
80
70
60
50
40
30
20
10
0
WT
WT + TGF-β
WT + IL-2
WT + TGF-β + IL-2
Ctla-4-/-
Ctla-4-/- + TGF-β
Ctla-4-/- + IL-2
Ctla-4-/- + TGF-β + IL-2
CD28-/-
CD28-/- + TGF-β
CD28-/- + IL-2
CD28-/- + TGF-β + IL-2

c


Absolute number of
CD4+ KJ-126+ CD25+ Foxp3+
(x10^3)
0
2
4
6
8
10
WT + TGF-β
WT + TGF-β + IL-2
Ctla-4-/- + TGF-β
Ctla-4-/- + TGF-β + IL-2
CD28-/- + TGF-β
CD28-/- + TGF-β + IL-2

for lymphocytes deficient in *Ctla-4*. Overall, the results obtained suggest that CTLA-4 is not required for FOXP3 induction by TGF-β, but neither does CTLA-4 normally function to restrict the TGF-β induction of FOXP3. The data are consistent with a role of CD28 in the TGF-β induction of FOXP3 expression, which could be overcome by addition of exogenous rIL-2.

Having examined the induction of FOXP3 *in vitro*, the next aim was to develop a system to examine this phenomenon *in vivo*. It has been found that *in vivo* sub immunogenic, continuous exposure to antigen induces FOXP3 expression [107]. A protocol was established in which *DO11.10*+ *mOVA*- *Rag2*-/- mice were immunised with 50μg OVA by intra-peritoneal injection on day 1, day 2, day 3, day 6 and day 7. On day 8 mice were harvested and expression of FOXP3 by peripheral CD4+ T cells was analysed by flow cytometry. *Fig. 3.23 A* shows that OVA immunisation induced a population of CD4+ FOXP3+ T cells that was not present in mice that received control PBS immunisations. As with the previous *in vitro* TGF-β FOXP3 induction experiments, *DO11.10*+ *mOVA Rag2*-/- mice that were wild type, *Ctla-4*-/- or *CD28*-/- were used to assess the role of CTLA-4 and CD28 in *in vivo* antigen-mediated FOXP3 induction. The graphs in *Fig. 3.22 B* suggest that CD28 was absolutely required for *in vivo* FOXP3 induction as the percentage and absolute number of CD4+ FOXP3+ cells obtained was negligible. In the absence of *Ctla-4* an augmented population of CD4+ FOXP3+ cells was induced *in vivo* in terms of both percentage composition and absolute number compared to that in wild type mice. The data therefore suggest that CTLA-4 normally functions to restrict the induction of peripheral CD4+ FOXP3+ lymphocytes *in vivo* in response to antigen.

To further explore the role of CD28 in FOXP3 expression, *in vitro* assays were carried out using lymphocytes isolated from *CD28*-deficient mice and agonistic antibodies to CD3 and CD28. *Fig. 3.24* shows that in both wild type and *CD28*-deficient CD4+ T cells the intensity of FOXP3 expression was increased upon TCR-stimulation with anti-CD3. As predicted, anti-CD28 did not affect FOXP3 expression by *CD28*-/- CD4+ T cells. Similarly, wild type CD4+ T cell FOXP3 expression was unaltered by anti-CD28-mediated co-stimulation. Overall, this data suggests that increased intensity of FOXP3 expression following TCR-mediated CD4+ T cell activation is not CD28-dependent.

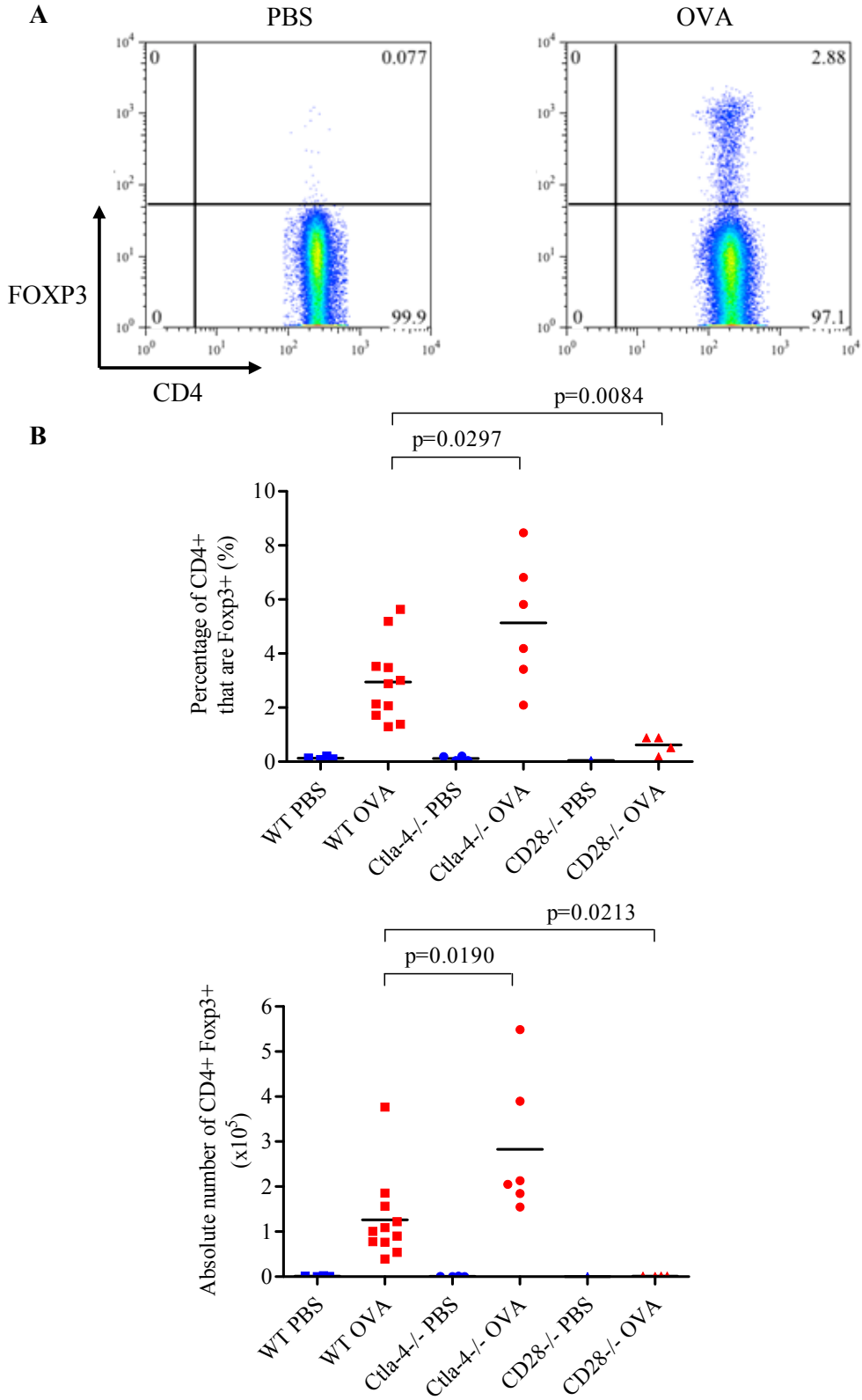
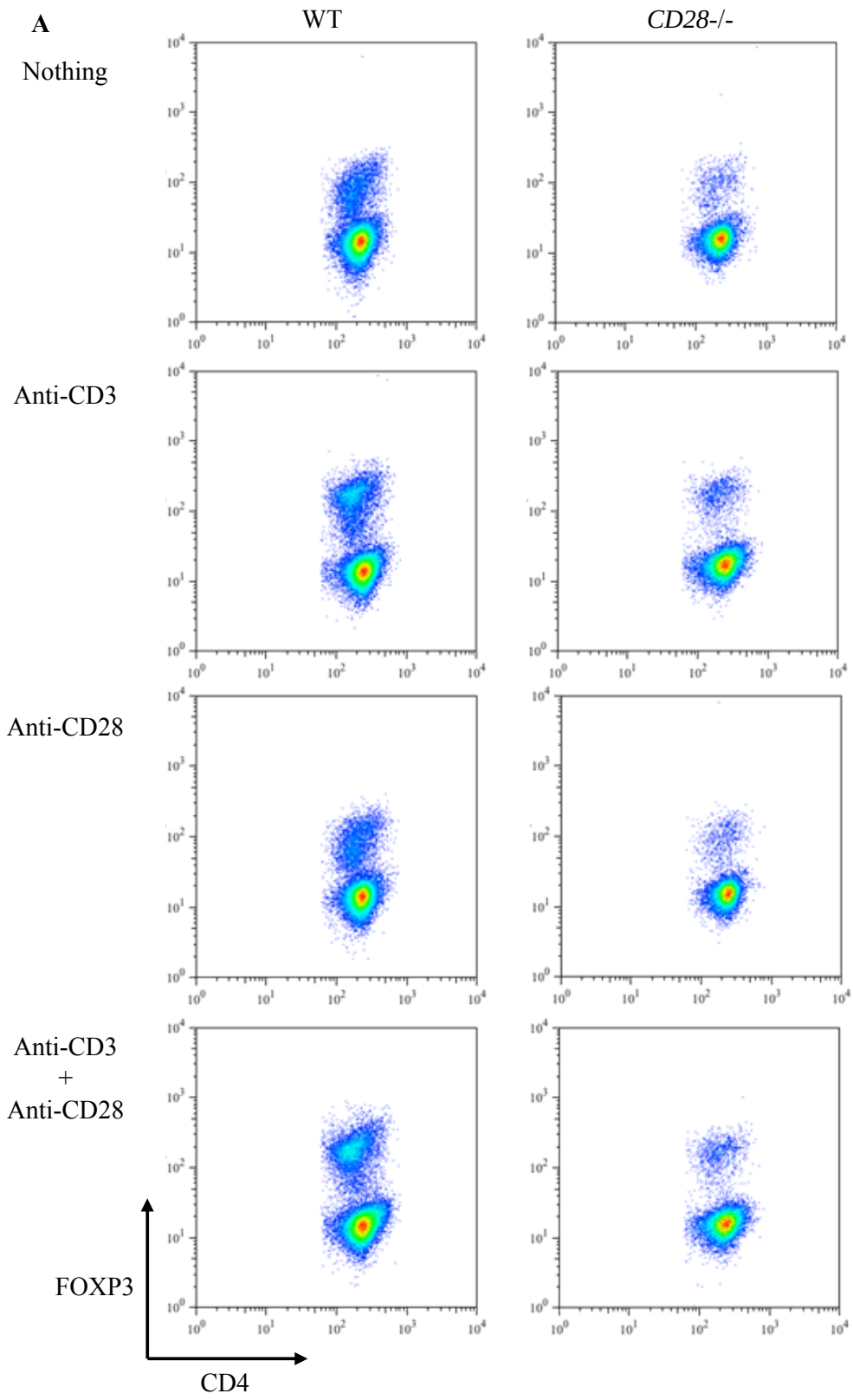

A
PBS
OVA
0
0.077
99.9
2.88
97.1
FOXP3
CD4
B
p=0.0084
p=0.0297
Percentage of CD4+ that are Foxp3+ (%)
WT PBS
WT OVA
Ctla-4-/- PBS
Ctla-4-/- OVA
CD28-/- PBS
CD28-/- OVA
p=0.0213
p=0.0190
Absolute number of CD4+ Foxp3+ (x10^5)

Figure 3.23. *In vivo* antigen-driven induction of CD4+ FOXP3+ cells. Wild type DO11.10+ mOVA- *Rag2*-/- (squares), *Ctla-4*-/- DO11.10+ mOVA- *Rag2*-/- (circles) or *CD28*-/- DO11.10+ mOVA- *Rag2*-/- mice (triangles) received 50µg OVA or PBS alone by intra-peritoneal injection on day 1, day 2, day 3, day 6 and day 7. Mice were harvested on day 8. Single cell suspensions of peripheral nodes were surface stained with CD4 and intracellularly with FOXP3 for analysis by flow cytometry. (A) Representative FACS plots showing FOXP3 expression of CD4+ gated lymphocytes from DO11.10+ mOVA- *Rag2*-/- mice that received PBS (left) or OVA (right). Graphs show data of (B) the percentage of CD4+ that are FOXP3+ (C) the absolute number of CD4+ FOXP3+ following PBS (blue) or OVA (red) treatment for ≥three wild type DO11.10+ mOVA- *Rag2*-/- (squares), *Ctla-4*-/- DO11.10+ mOVA- *Rag2*-/- (circles) or *CD28*-/- DO11.10+ mOVA- *Rag2*-/- mice (triangles). Lines indicate mean values. p values calculated by two-tailed, unpaired T-test for the means.


A
WT
CD28-/-
Nothing
Anti-CD3
Anti-CD28
Anti-CD3
+
Anti-CD28
FOXP3
CD4

B

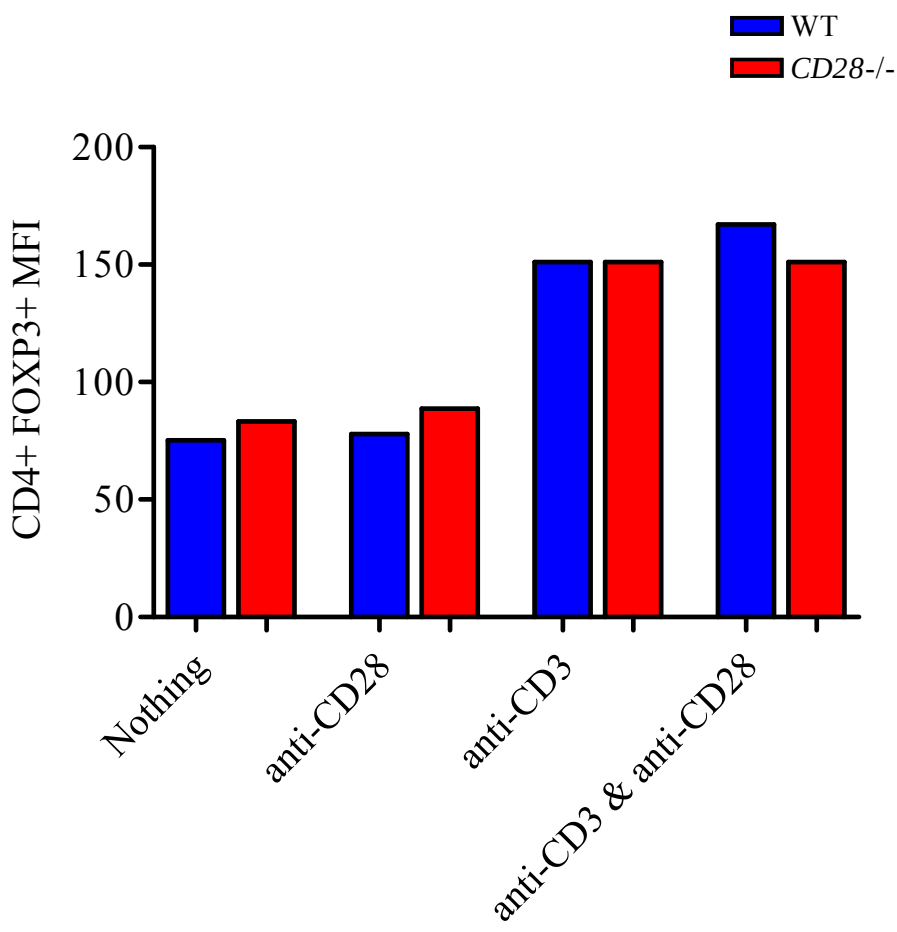

WT
CD28-/-
CD4+ FOXP3+ MFI
200
150
100
50
0
Nothing
anti-CD28
anti-CD3
anti-CD3 & anti-CD28

Figure 3.24. CD28-dependency of intracellular FOXP3 expression by CD4+ FOXP3+ Tregs. Adult BALB/c wild type or *CD28*-/- single cell suspensions from peripheral lymph nodes were cultured overnight plus or minus 500ng/ml anti-CD3 and 1μg/ml anti-CD28. Three wells were pooled and surface stained with CD4 and intracellularly with FOXP3 for analysis by flow cytometry. (A) Representative FACS plots of FOXP3 expression by gated CD4+ lymphocytes from wild type BALB/c (left) and *CD28*-/- (right) mice cultured plus or minus anti-CD3 and anti-CD28. (B) Graph shows FOXP3 median fluorescence intensity of gated CD4+ FOXP3+ Tregs. Representative graph from three independent experiments is shown.

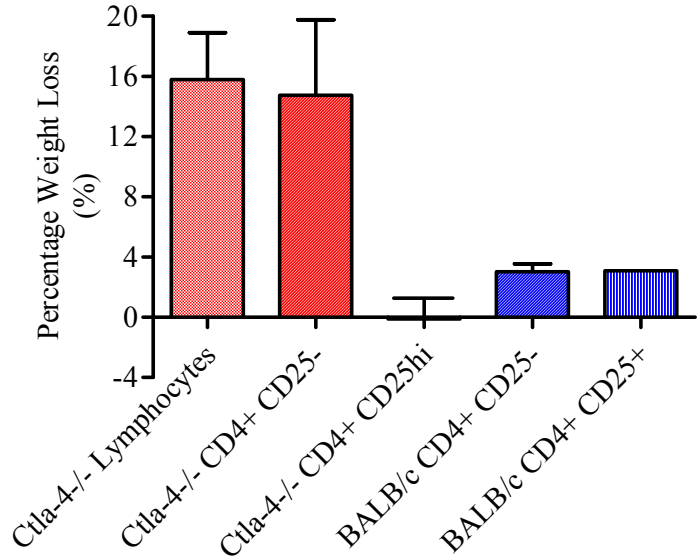
3.2.6 *Ctla-4*-deficiency and Treg pathogenicity.

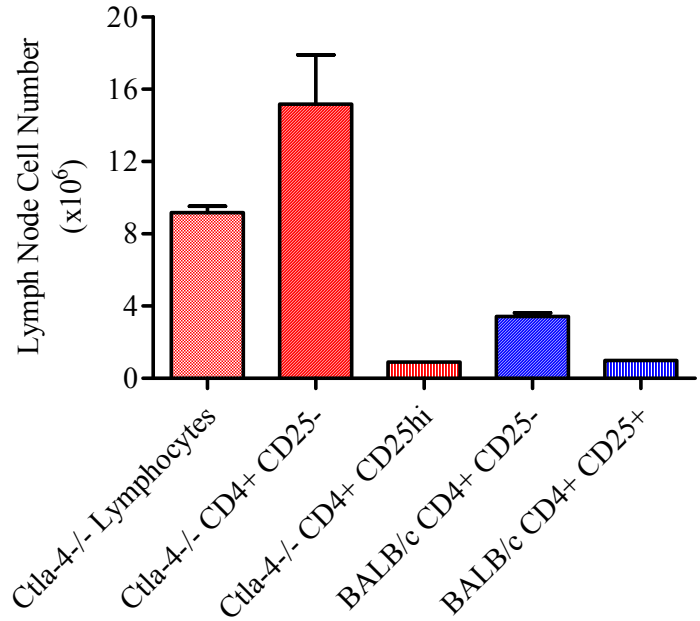
Mice in which the *Ctla-4* gene has been ablated die at around 3 weeks of age from a fatal lymphoproliferative syndrome with multi-organ lymphocytic infiltration [156, 157]. Yet, as described previously the CD4+ FOXP3+ population which constitutes the regulatory T cells was found to be augmented. One potential solution to this paradox is that CD4+ FOXP3+ Tregs that lack CTLA-4 have a pathogenic functional role as opposed to being regulatory.

To assess the inherent pathogenicity of the CD4+ FOXP3+ population in *Ctla-4*-deficient mice *in vivo* adoptive T cell transfers into *Rag2*-/- mice were carried out. Adoptive transfer of *Ctla-4*-/- lymphocytes or mo-flow sorted *Ctla-4*-/- CD4+ CD25- transferred disease into *Rag2*-/- recipient mice in 3-4 weeks. This was evident by severe weight loss and lymphadenopathy, as shown by increased absolute lymph node cell counts (*Fig. 3.25 A*). In addition to weight loss, organ infiltration was studied by hematoxylin and eosin (H & E) histology staining of heart tissue to assess the pathogenicity of *Ctla-4*-deficient Tregs. Others have shown that *Ctla-4*-/- mice with fatal lymphoproliferative syndrome have significant lymphocytic infiltration of many organs such as heart, lung and liver [157]. It was found that similar lymphocytic infiltration could be identified in the heart tissue of *Rag2*-/- recipient mice of *Ctla-4*-/- lymphocytes and *Ctla-4*-/- CD4+ CD25- Tconv (*Fig. 3.25 B*). In comparison for *Rag2*-/- mice that received *Ctla-4*-/- CD4+ CD25hi Tregs, weight loss and lymphadenopathy was negligible. Likewise, transfer of wild type CD4+ CD25- and CD4+ CD25+ caused no weight loss or lymphadenopathy (*Fig. 3.25 A*). H & E staining of the heart tissue from recipients of *Ctla-4*-/- CD4+ CD25hi Tregs showed no lymphocytic infiltration (*Fig. 3.25 B*) and was indistinguishable from heart tissue from mice that had received wild type CD4+ CD25- Tconv or CD4+ CD25+ Tregs (*Fig. 3.25 C*). These data suggest that *Ctla-4*-deficient Tregs are not themselves pathogenic.

Assessing the pathogenicity of *Ctla-4*-deficient Tregs by adoptive transfer into *Rag2*-/- mice does not account for interaction of Tregs with other lymphocytes such as B cells and Tconv via, for example, the provision of co-stimulatory ligands or the secretion of cytokines. For instance, regulatory T cells normally lack CD40L [52] which activated pathogenic effector T cells up regulate to co-stimulate antigen-presenting cells, such as B cells, though CD40 (reviewed [255]). One way in which loss of *Ctla-4* could potentially render Tregs pathogenic

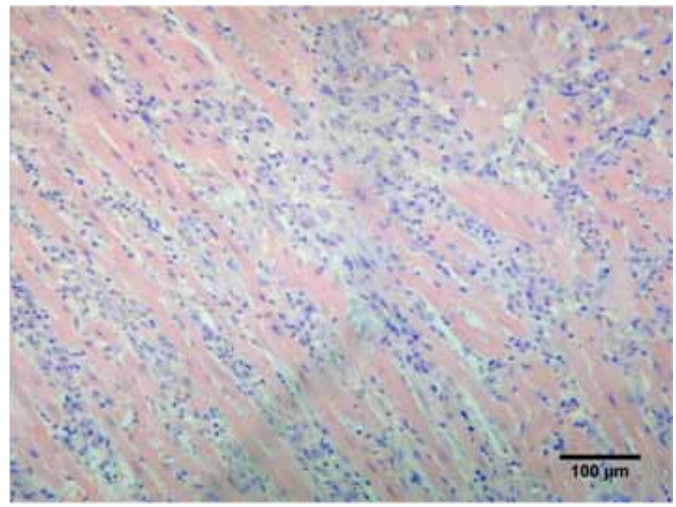
A


Percentage Weight Loss (%)
20
16
12
8
4
0
-4
Ctla-4-/- Lymphocytes
Ctla-4-/- CD4+ CD25-
Ctla-4-/- CD4+ CD25hi
BALB/c CD4+ CD25-
BALB/c CD4+ CD25+

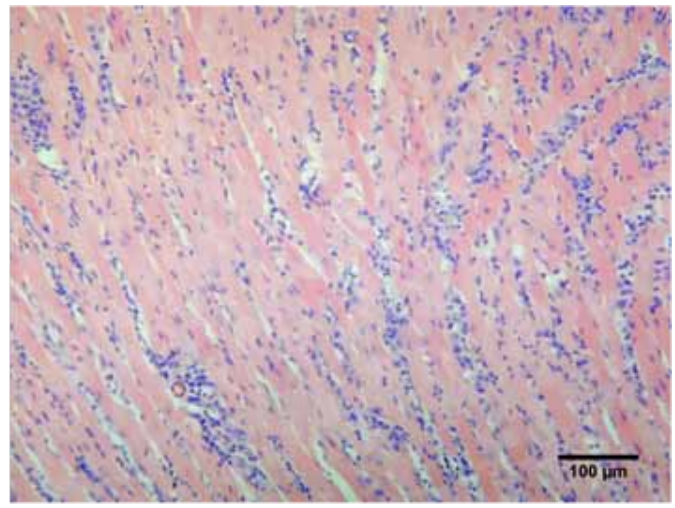

Lymph Node Cell Number (x10^6)
20
16
12
8
4
0
Ctla-4-/- Lymphocytes
Ctla-4-/- CD4+ CD25-
Ctla-4-/- CD4+ CD25hi
BALB/c CD4+ CD25-
BALB/c CD4+ CD25+

B

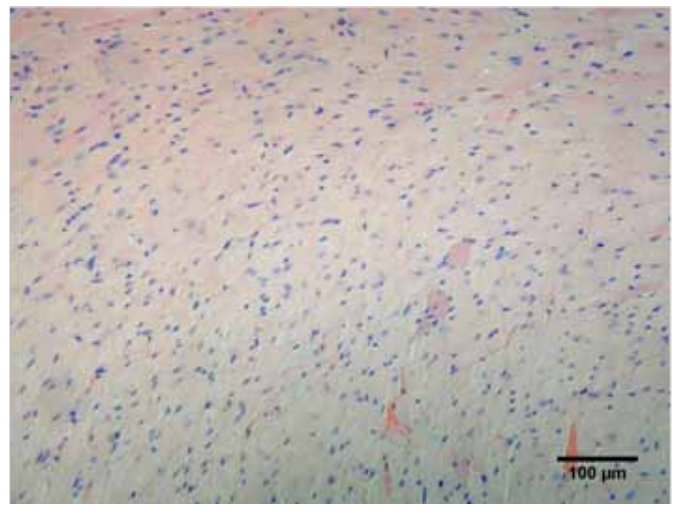
Ctla-4-/-
lymphocytes



Ctla-4-/-
CD4+ CD25-

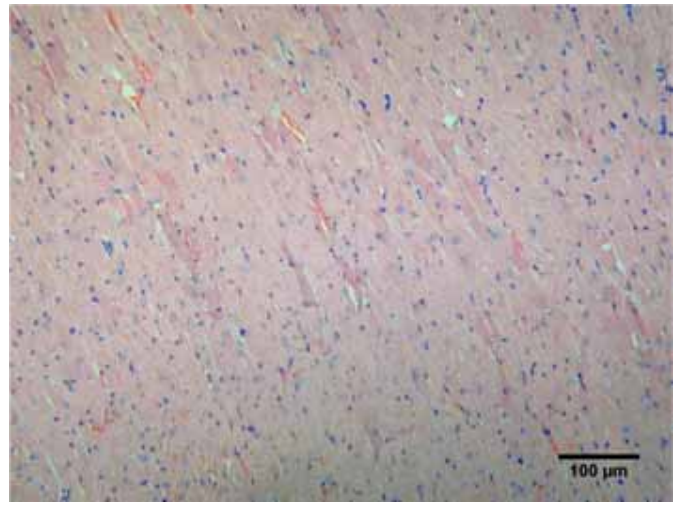


Ctla-4-/-
CD4+ CD25hi



C

BALB/c
CD4+ CD25-



BALB/c
CD4+ CD25+

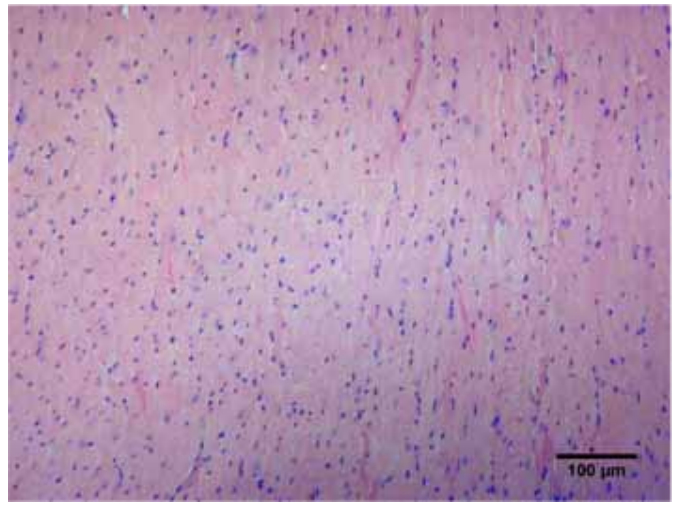


Figure 3.25. *Ctla-4*-deficient Tregs are not inherently pathogenic. 1-2 x 10^6 lymphocytes or mo-flow sorted CD4+ CD25- or CD4+ CD25hi lymph node cells from 15-18 day old *Ctla-4*-/- mice were intra-venially injected into *Rag2*-/- recipients. Mice were harvested 3-4 weeks following transfer. (A) Graphs show percentage weight loss (top) and lymph node cellularity (bottom) of recipient mice. Bars indicate mean values and standard deviations are shown for three-five mice. (B) H&E staining of fomaldehyde fixed heart tissue from recipients of *Ctla-4*-/- lymphocytes (top), *Ctla-4*-/- CD4+ CD25- (middle) or *Ctla-4*-/- CD4+ CD25hi (bottom). (C) H&E staining of fomaldehyde fixed heart tissue from recipients of BALB/c CD4+ CD25- (top) or BALB/c CD4+ CD25+ (bottom).

is by permitting the expression of CD40L. Consequently, CD40L expression by *Ctla-4*-deficient Tregs was analysed by flow cytometry. It can be seen in *Fig. 3.26* that wild type CD4+ FOXP3- Tconv expressed some CD40L and this was increased in Tconv from *Ctla-4*-/- mice. *Ctla-4*-deficient CD4+ FOXP3+ Tregs however did not express CD40L, similar to wild type Tregs.

It is also possible that *Ctla-4* could normally function to restrict the capacity of Tregs to make pro-inflammatory cytokines. Therefore, cytokines produced by Tregs isolated from *Ctla-4* knockout mice were investigated by quantitative polymerase chain reaction (qPCR) and intracellular cytokine staining analysis by flow cytometry. As described previously, the cell surface marker CD27 was successfully used along with CD4 and CD25 to identify CD4+ FOXP3+ Tregs from the activated environment of the *Ctla-4* knockout mouse when intracellular FOXP3 could not be used. mRNA and cDNA was obtained from mo-flow sorted CD4+ CD25- Tconv and CD4+ CD25+ CD27hi Tregs from *Ctla-4*-/- and age-matched wild type BALB/c lymphocytes. By qPCR, expression of mRNA transcripts for TGF-β, IL-17 and IL-21 by wild type and *Ctla-4*-/- Tconv and Tregs were determined (*Fig. 3.27*). There was not much change in expression of TGF-β by either Tconv or Tregs from *Ctla-4*-deficient mice compared to those T cell populations from wild type mice. On the other hand Tconv and particularly Tregs from *Ctla-4*-/- mice showed increased IL-17 expression compared to wild type, whereas the only population to show any IL-21 expression were the Tconv lacking *Ctla-4*.

To extend the qPCR studies above, cytokine expression was detected in FOXP3- and FOXP3+ CD4+ T cell populations at the protein level by intracellular cytokine staining. *Fig. 3.28* shows representative FACS plots for the detection of IL-2 (*A*), IFN-γ (*B*), TGF-β (*C*), IL-10 (*D*) and IL-17 (*E*) expressed by CD4+ FOXP3- Tconv and CD4+ FOXP3+ Treg populations in *Ctla-4*-/- mice and littermate controls. Flow cytometry analysis of Tconv and Treg cytokine expression was carried out for a number of different mice. The pro-inflammatory cytokines IL-2 (*F*) and IFN-γ (*G*) showed significantly increased expression by *Ctla-4*-/- Tconv, whereas neither *Ctla-4*-/- or *Ctla-4*+/- control Tregs showed any IL-2 production (*F*). There was, however, a statistically significant increase in IFN-γ

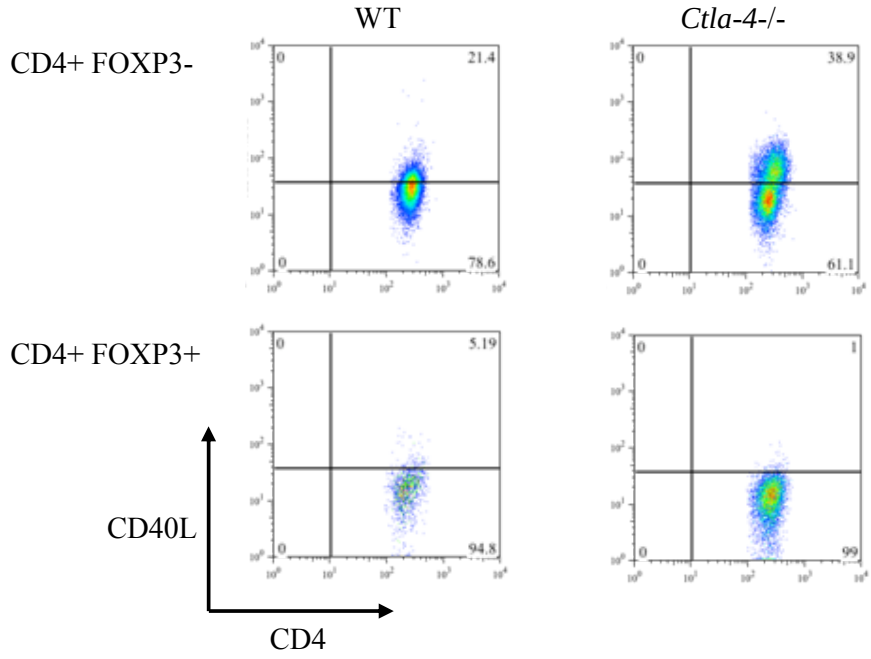


Figure 3.26. CD40 ligand expression by *Ctla-4*-deficient Tregs. 15-18 day old wild type BALB/c (left) or *Ctla-4*-/- (right) single cell suspensions from peripheral lymph nodes were surface stained with CD4 and intracellularly with CD40L and FOXP3 for analysis by flow cytometry. Representative FACS plots of iCD40Lexpression by CD4+ FOXP3- gated lymphocytes (top) and CD4+ FOXP3+ gated lymphocytes (bottom).

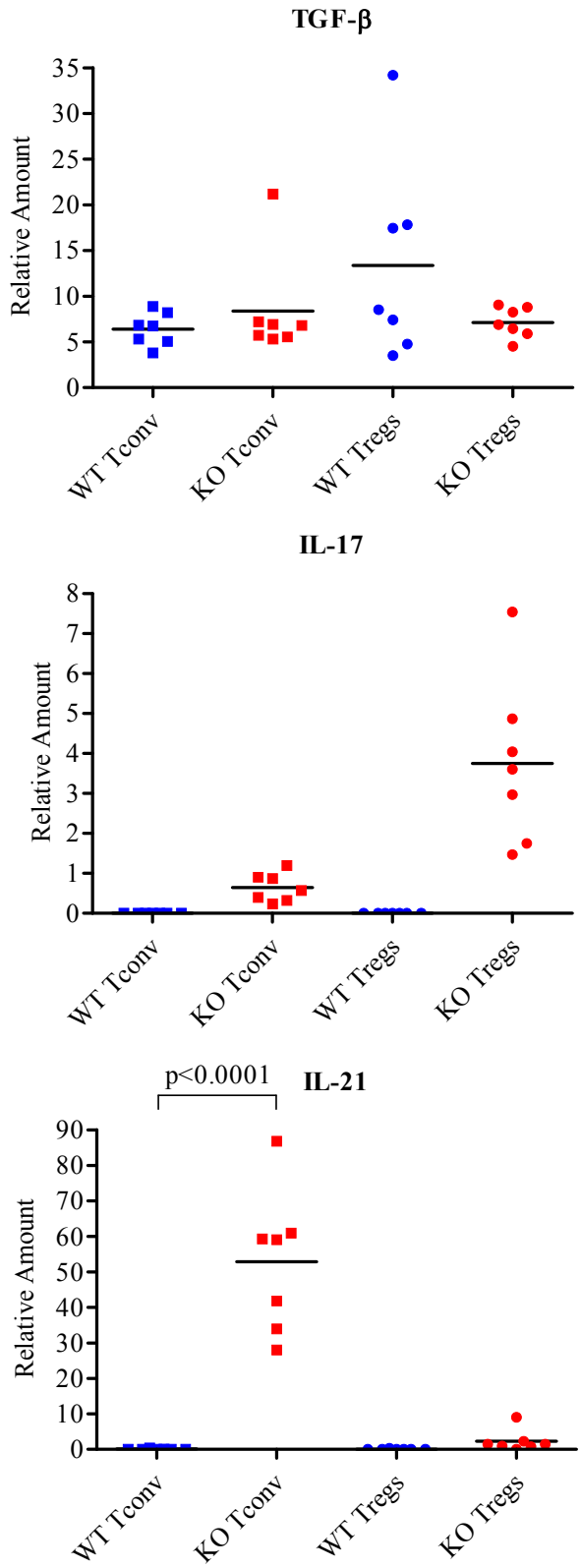
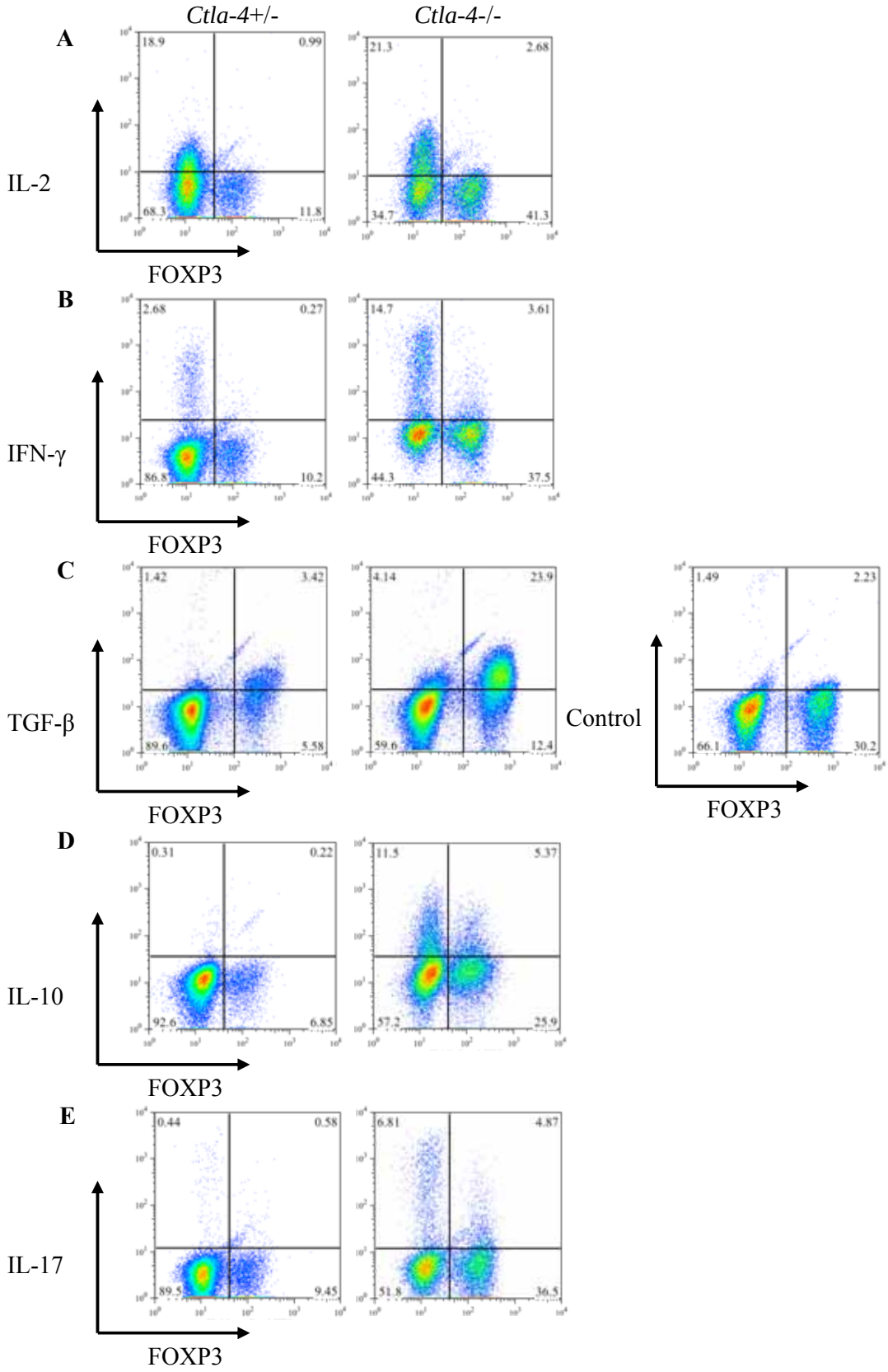
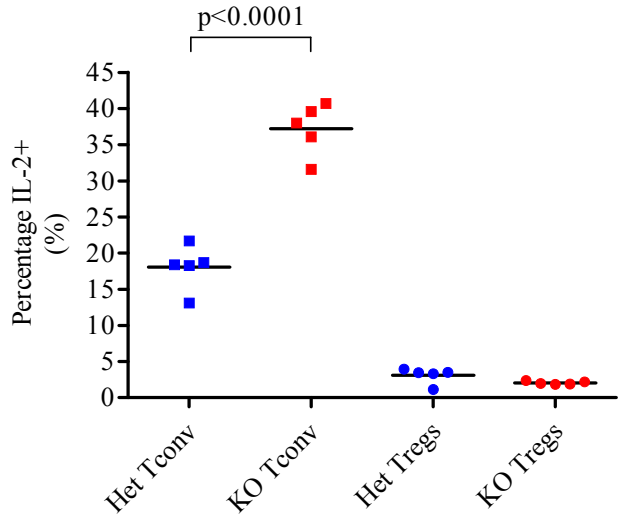

TGF-β
Relative Amount
35
30
25
20
15
10
5
0
WT Tconv
KO Tconv
WT Tregs
KO Tregs
IL-17
Relative Amount
8
7
6
5
4
3
2
1
0
WT Tconv
KO Tconv
WT Tregs
KO Tregs
p<0.0001
IL-21
Relative Amount
90
80
70
60
50
40
30
20
10
0
WT Tconv
KO Tconv
WT Tregs
KO Tregs

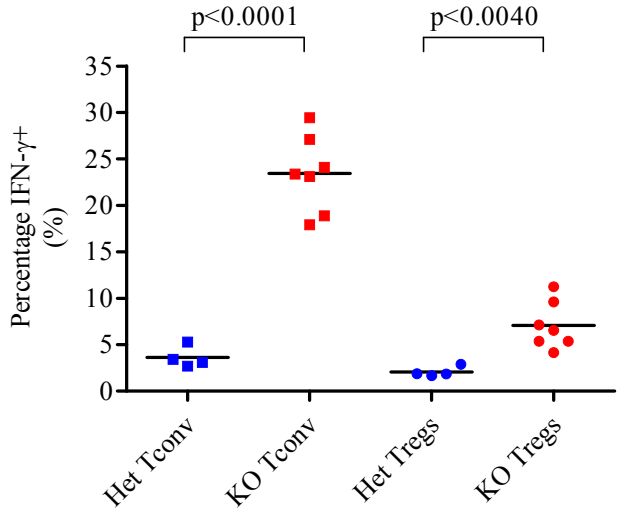
Figure 3.27. Real time qPCR for cytokine expression by *Ctla-4*-/- CD4+ CD25- Tconv and CD4+ CD25+ CD27hi Tregs. 15-18 day old wild type BALB/c or *Ctla-4*-/- single cell suspensions of peripheral lymph nodes were surface stained with CD4, CD25 and CD27 for mo-flow cell sorting. Tconv were isolated as CD4+ CD25- and Tregs were isolated as CD4+ CD25+ CD27hi. mRNA and cDNA was obtained from snap-frozen cell pellets and real time TaqMan qPCR carried out for the presence of TGF-β, IL-17 and IL-21 relative to β-actin. Graphs show data for CD4+ CD25- Tconv (squares) and CD4+ CD25+ CD27hi Treg (circles) cDNA samples obtained from ≥six different wild type (blue) or *Ctla-4*-/- (red) mice. Relative amount was calculated as the amount of target normalised to an endogenous β-actin reference. p values calculated by two-tailed, unpaired T-test for the means.


Ctla-4+/-
Ctla-4-/-
A
IL-2
18.9
0.99
68.3
11.8
21.3
2.68
34.7
41.3
FOXP3
B
IFN-γ
2.68
0.27
86.8
10.2
14.7
3.61
44.3
37.5
FOXP3
C
TGF-β
1.42
3.42
89.6
5.58
4.14
23.9
59.6
12.4
FOXP3
Control
1.49
2.23
66.1
30.2
FOXP3
D
IL-10
0.31
0.22
92.6
6.85
11.5
5.37
57.2
25.9
FOXP3
E
IL-17
0.44
0.58
89.5
9.45
6.81
4.87
51.8
36.5
FOXP3

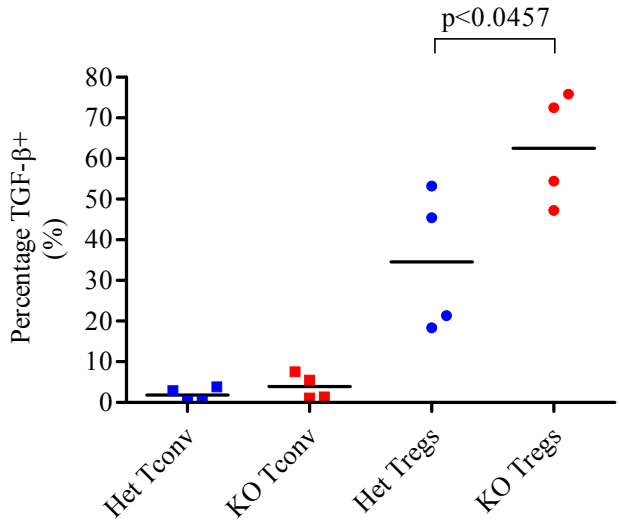
F


p<0.0001
Percentage IL-2+ (%)
45
40
35
30
25
20
15
10
5
0
Het Tconv
KO Tconv
Het Tregs
KO Tregs

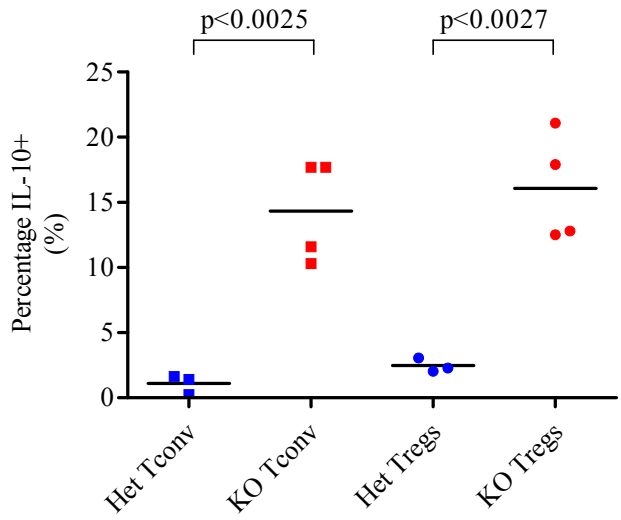
G


p<0.0001
p<0.0040
Percentage IFN-γ+ (%)
35
30
25
20
15
10
5
0
Het Tconv
KO Tconv
Het Tregs
KO Tregs

H


p<0.0457
Percentage TGF-β+ (%)
80
70
60
50
40
30
20
10
0
Het Tconv
KO Tconv
Het Tregs
KO Tregs

I


p<0.0025
p<0.0027
Percentage IL-10+ (%)
25
20
15
10
5
0
Het Tconv
KO Tconv
Het Tregs
KO Tregs

J

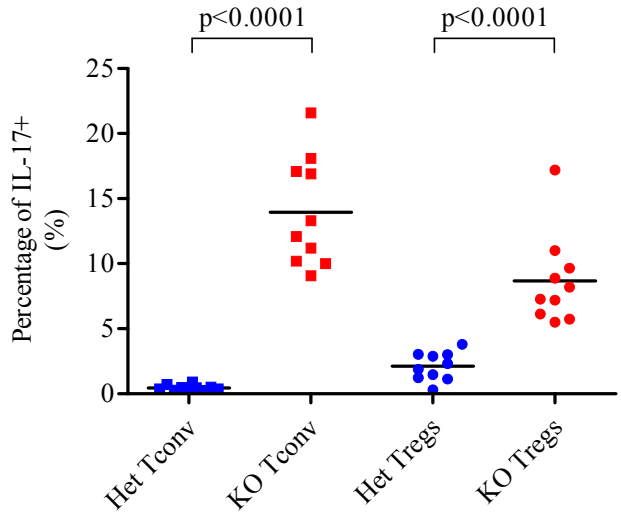

p<0.0001
p<0.0001
Percentage of IL-17+ (%)
25
20
15
10
5
0
Het Tconv
KO Tconv
Het Tregs
KO Tregs

Figure 3.28. Intracellular cytokine staining of *Ctla-4*-/- CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs. 15-18 day old *Ctla-4*+/- or *Ctla-4*-/- single cell suspensions of peripheral lymph nodes were cultured for four hours with 50ng/ml PMA and 1.5μM ionomycin, and 1μl/ml BD GolgiStop for the last three hours, at 37°C, 5% CO_2. Cells were then surface stained with CD4, and intracellularly with CD4, FOXP3, IL-2, IFN-γ, IL-17 for analysis by flow cytometry. For detection of TGF-β single cell suspensions were cultured with 2 x 10^6 anti-CD3/anti-CD28 Dynabeads per ml overnight at 37°C, 5% CO_2 prior to surface staining with CD4, TGF-β and intracellular staining with FOXP3 for analysis by flow cytometry. For detection of IL-10 single cell suspensions were cultured with 2 x 10^6 anti-CD3/anti-CD28 Dynabeads per ml overnight at 37°C, 5% CO_2 prior to staining with CD4, IL-10 and FOXP3 using a MACS secretion assay kit. Representative FACS plots for CD4+ gated *Ctla-4*+/- (left) or *Ctla-4*-/- (right) IL-2 (A), IFN-γ (B), TGF-β (C), IL-10 (D) and IL-17 (E) expression. Graphs show data for percentages of IL-2 (F), IFN-γ (G), TGF-β (H), IL-10 (I) and IL-17 (J) expression by CD4+ FOXP3- gated Tconv (squares) and CD4+ FOXP3+ gated Tregs (circles) from *Ctla-4*+/- (blue) or *Ctla-4*-/- (red) mice. Lines indicate mean values. p values calculated by two-tailed, unpaired T-test for the means.

production by Tregs from *Ctla-4*-deficient mice (*G*). A greater proportion of Tregs expressed TGF-β than Tconv, which was augmented with the lack of *Ctla-4* (*H*). Both IL-10 (*I*) and IL-17 (*J*) production by *Ctla-4*+/- control Tconv and Tregs was negligible, and both Tconv and Tregs from *Ctla-4*-/- mice produced statistically significant increased amounts of these cytokines.

To qualify the CD4+ CD25- Tconv and CD4+ CD25+ CD27hi Treg populations isolated by mo-flow sorting for qPCR analysis of mRNA expression it was decided to investigate the expression of mRNA transcripts for the T_H1 and T_H2 lineage transcription factors T-bet [15] and GATA3 [16], respectively. *Fig. 3.29* shows that mRNA transcripts for both T-bet and GATA3 were expressed by both the isolated Tconv and Treg populations. Tconv and Tregs from *Ctla-4*-/- mice showed increased expression of T-bet and GATA3 mRNA compared to the respective T cell populations from age-matched wild type control mice.

3.2.7 Role of CTLA-4 in T cell help for B cells.

By both qPCR and flow cytometry, increased IL-21 production was observed by Tconv from *Ctla-4*-deficient mice. IL-21 is a cytokine associated with T follicular helper (TFH) cells which are present within B cell follicles and involved in the initiation of the germinal centre reaction as part of the humoral immune response [256]. Early studies of *Ctla-4* knockout mice identified substantially increased serum IgE levels [156] which is consistent with the immunoglobulin class switching that occurs during the germinal centre B cell response [257]. The presence of germinal centres within the spleen of *Ctla-4*-deficient mice was therefore investigated by immunohistochemistry. In the age-matched wild type control spleen of *Fig. 3.30 A* CD4+ (blue) T zones and IgD+ (brown) B zones could be identified. In the *Ctla-4* knockout spleen, similar CD4+ (blue) T zones and IgD+ (brown) B zones could be determined, but also within the B zones an IgD negative area could be identified (*Fig. 3.30 B*). It is known that germinal centre B cells loose expression of IgD [258-261] and the antigen peanut agglutinin (PNA) is specifically expressed by germinal centre B cells [262]. Using serial sections, the IgD negative areas in the *Ctla-4*-/- spleen were found to be PNA+ (*Fig. 3.30 B*). No positive staining for PNA was identified in the age-matched wild type control spleen (*Fig. 3.30 A*). In conclusion, compared to an age-matched wild type control spleen, spontaneous germinal centres could be identified in spleens from

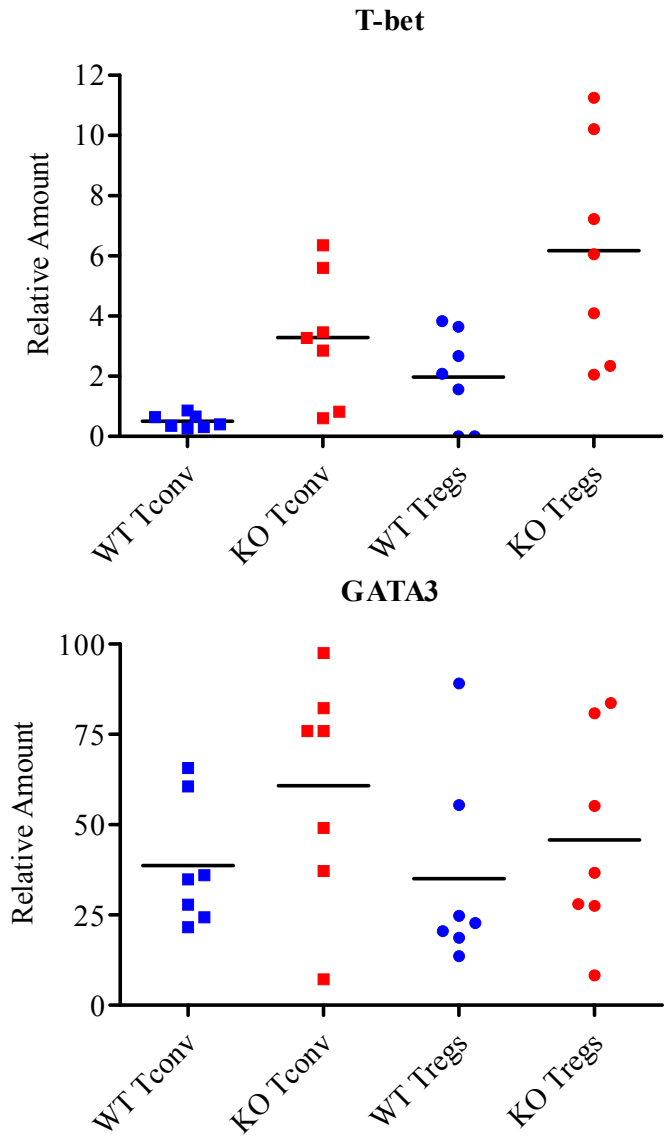
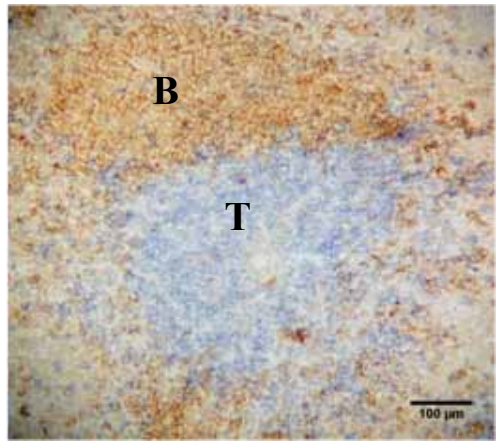


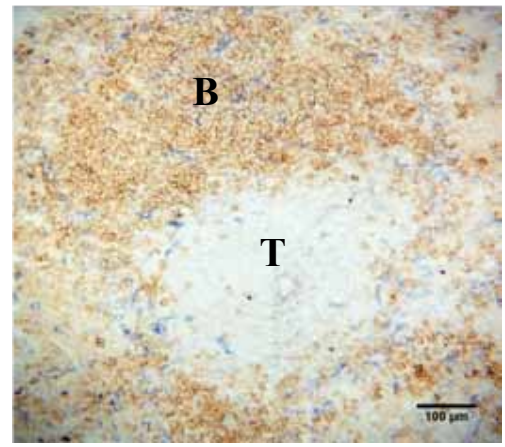
Figure 3.29. Real time qPCR for T_H1 and T_H2 transcription factor expression by mo-flow sorted CD4+ CD25- Tconv and CD4+ CD25+ CD27hi Tregs. 15-18 day old wild type BALB/c or *Ctla-4*-/- single cell suspensions of peripheral lymph nodes were surface stained with CD4, CD25 and CD27 for mo-flow cell sorting. Tconv were isolated as CD4+ CD25- and Tregs were isolated as CD4+ CD25+ CD27hi. mRNA and cDNA was obtained from snap-frozen cell pellets and real time TaqMan qPCR carried out for the presence of T-bet (top) and GATA3 (bottom) relative to β-actin. Graphs show data for CD4+ CD25- Tconv (squares) and CD4+ CD25+ CD27hi Treg (circles) cDNA samples obtained from ≥six different wild type (blue) or *Ctla-4*-/- (red) mice. Relative amount was calculated as the amount of target normalised to an endogenous β-actin reference.

A

WT
CD4/IgD

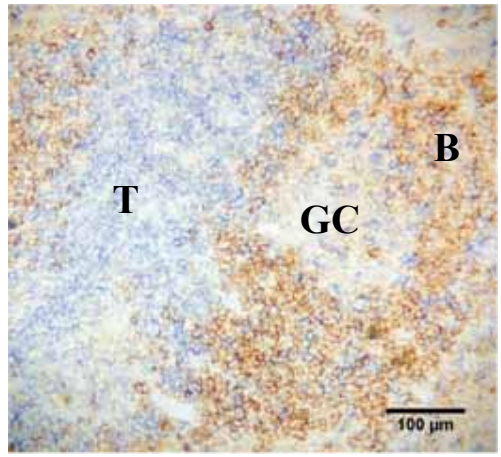


WT
PNA/IgD



B

Ctla-4-/-
CD4/IgD



Ctla-4-/-
PNA/IgD

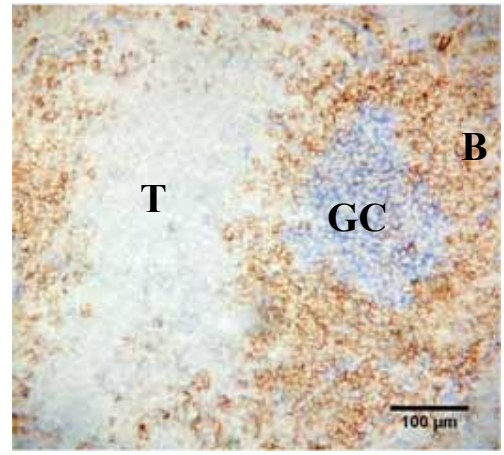


Figure 3.30. Germinal centre histology of *Ctla-4-/-* spleens. Acetone fixed, 6µm spleen sections from 15-18 day old wild type BALB/c (A) or *Ctla-4-/-* (B) were stained with IgD (brown) and CD4 (blue) (left) or PNA (blue) (right) for analysis by light microscopy (x10). T – T zone (CD4+), B – B zone (IgD+), GC – germinal centre (PNA+).

Ctla-4-deficient mice. To gain further insight into the development of spontaneous germinal centres in *Ctla-4*-deficient mice and the role of CTLA-4, the spleens of BALB/c mice following a timecourse of anti-CTLA-4 blocking antibody (4F10) treatment were analysed by immunohistochemistry for IgD, CD4 and PNA. In *Fig. 3.31 B* IgD negative areas within the B zones were identified at day 3 which were not present at day 1. These IgD negative areas within the B zones were increased in size and number at day 14 (*Fig. 3.31 C*). Analysis of serial sections showed the IgD negative areas were PNA+. The mean size of the PNA+ germinal centres in a number of mouse spleens throughout the timecourse of anti-CTLA-4 blocking antibody treatment was scored under the microscope (*Fig. 3.31 D*). Over time, anti-CTLA-4 blocking antibody treatment increased the mean germinal centre size towards that observed in *Ctla-4*-deficient mice.

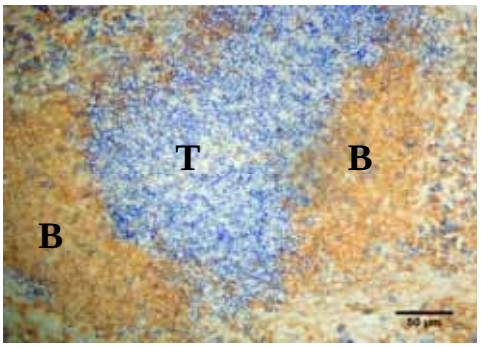
As shown in *Fig. 3.03 D*, B cells do not express CTLA-4. Therefore, the spontaneous germinal centres observed in *Ctla-4*-deficient mice could be due to dysregulated TFH cell homeostasis. It has been shown that Tregs can migrate to follicles and suppress germinal centre TFH cells [263], it could be that in the absence of CTLA-4 there is a loss of TFH cell regulation by Tregs and concurrent spontaneous germinal centre formation. The distribution of FOXP3+ cells within the BALB/c and *Ctla-4* knockout spleen was analysed by immunohistochemistry. In *Fig. 3.32 A* FOXP3+ cells (blue) could be identified within the IgD+ (brown) B zone follicle. Similarly, within the B zone follicle and germinal centre of *Ctla-4*-deficient spleens, FOXP3+ cells (blue) could be found (*Fig. 3.32 B*). If there is dysregulation of TFH cells by Tregs in *Ctla-4*-deficient mice, the data suggest this was not due to altered Treg trafficking and localisation.

To directly assess TFH cell homeostasis within the *Ctla-4*-deficient mouse, flow cytometry was used to quantify TFH cells. Many markers associated with the TFH cell phenotype are also expressed by Tregs such as ICOS and OX40, therefore the TFH cell population has historically been challenging to identify. PD-1 and CXCR5 currently seem the best markers used to distinguish TFH cells from other CD4+ T cell subtypes by flow cytometry [264]. Therefore CD4, PD-1 and CXCR5 cell surface expression was combined with FOXP3-intracellular expression to analyse the TFH cell population within the peripheral lymphocytes of the *Ctla-4* knockout mouse. The representative FACS plots (*Fig. 3.33 A*) and graph in

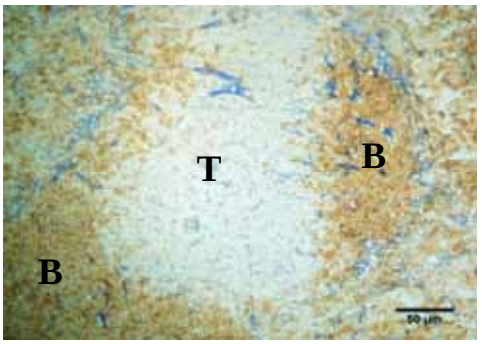
Fig. 3.33 C indicated that the CD4+ FOXP3- CXCR5+ PD-1+ TFH cell population was statistically significantly augmented in the absence of CTLA-4. As well as visual identification of germinal centres within *Ctla-4* knockout spleens and identification of an augmented TFH cell population within the peripheral lymphocytes of *Ctla-4*-deficient mice, in the absence of CTLA-4, CD19+ splenocytes showed increased FAS expression by flow cytometry (*Fig. 3.33 D*). The FAS receptor is known to be highly expressed by germinal centre B cells [265, 266].

A

Day 1
CD4/IgD

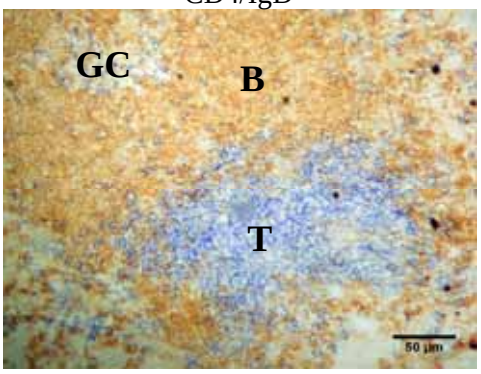


Day 1
PNA/IgD

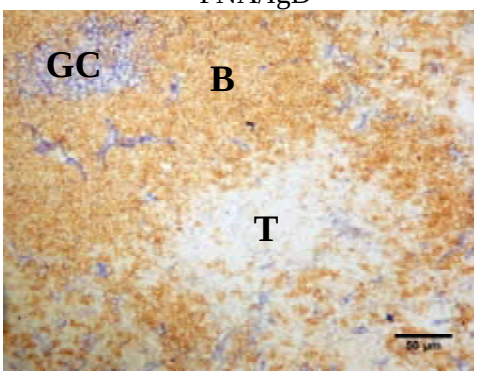


B

Day 3
CD4/IgD

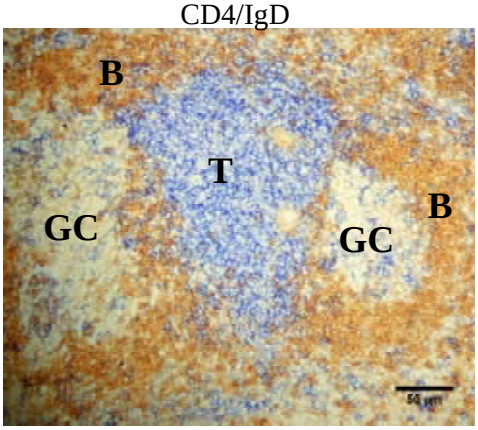


Day 3
PNA/IgD

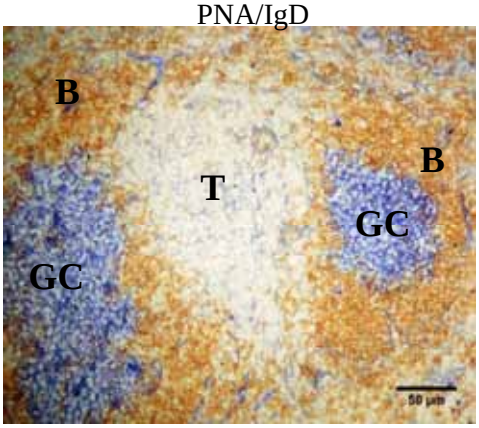


C

Day 14
CD4/IgD



Day 14
PNA/IgD



D

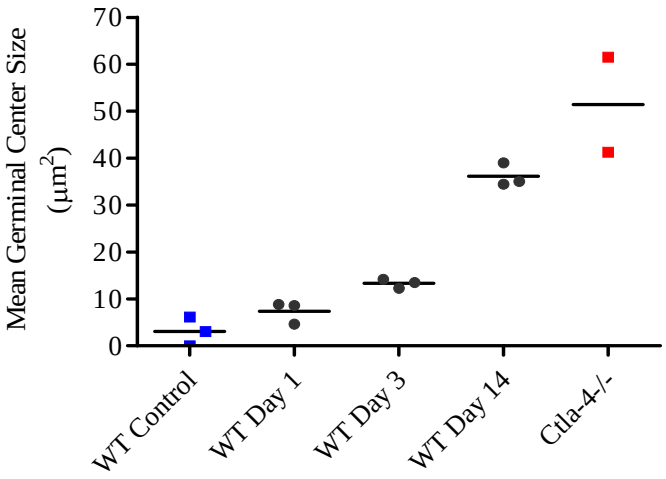

Mean Germinal Center Size
(μm2)
70
60
50
40
30
20
10
0
WT Control
WT Day 1
WT Day 3
WT Day 14
Ctla-4-/-

Figure 3.31. Analysis of germinal centre development in BALB/c spleen following an anti-CTLA-4 blocking antibody timecourse. Acetone fixed, 6µm spleen sections from 15-18 day old wild type BALB/c following treatment with 500µg anti-CTLA-4 blocking antibody (4F10) twice weekly by intra-peritoneal injection were stained with IgD (brown) and CD4 (blue) (left) or PNA (blue) (right) for analysis by light microscopy (x20). Representative pictures of mice harvested on day 1 (A), day 3 (B) and day 14 (C) of treatment. T – T zone (CD4+), B – B zone (IgD+), GC – germinal centre (PNA+). (D) Germinal centres were sized in μm^2 using a graticule and determining the area of PNA+ staining within IgD+ B cell follicles. Graph shows data from three wild type BALB/c mice (blue squares), wild type BALB/c mice day 1 after anti-CTLA-4 blocking antibody treatment, mice day 3, mice day 14 (grey circles) and *Ctla-4*-/- mice (red squares).

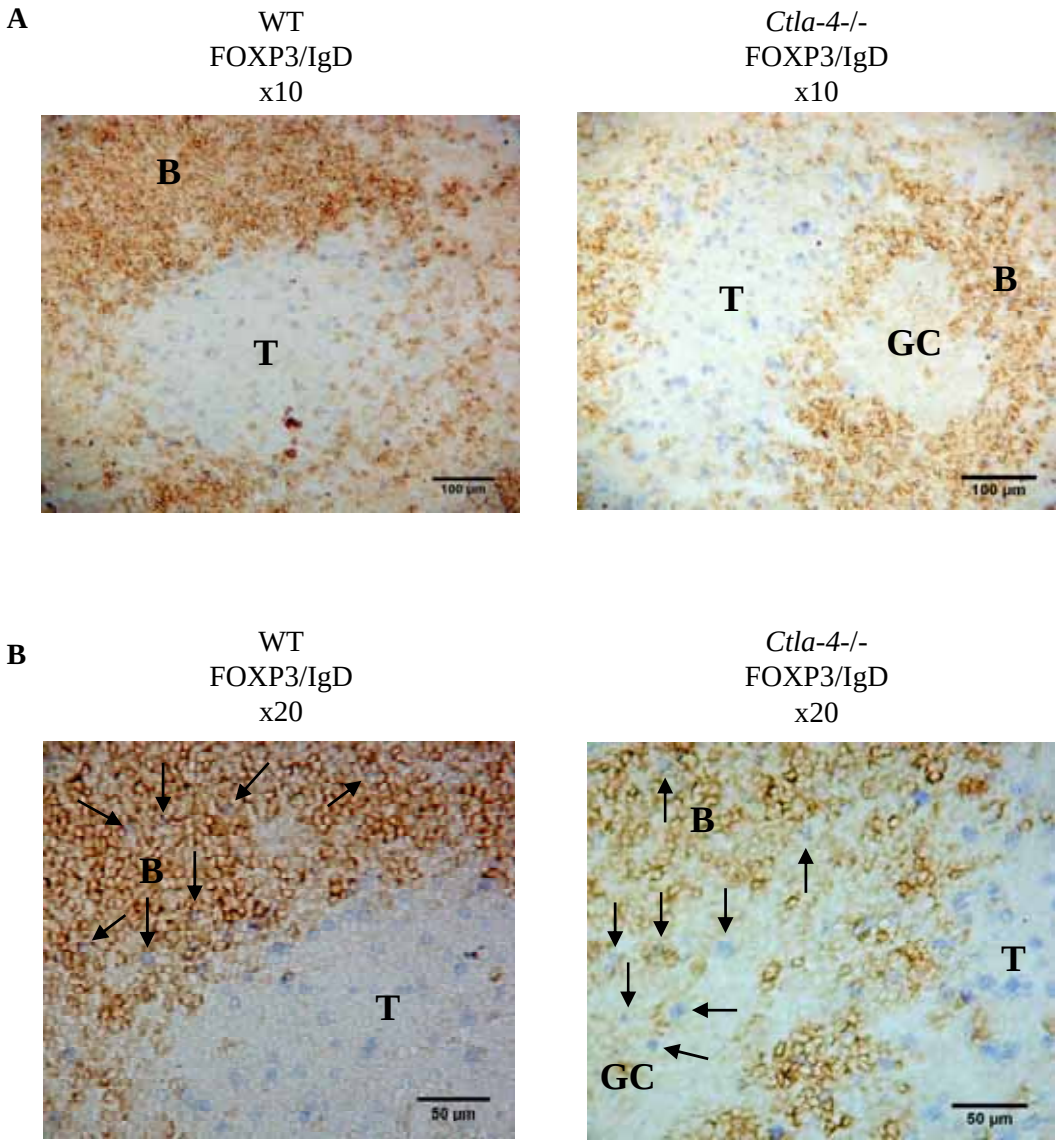
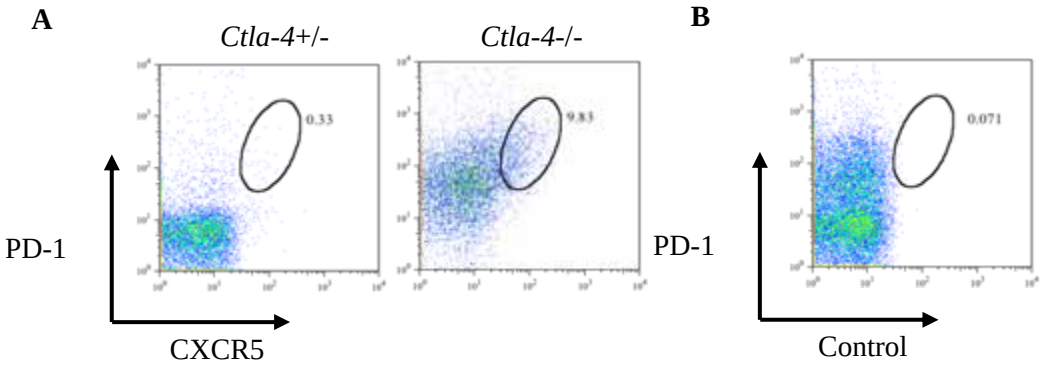
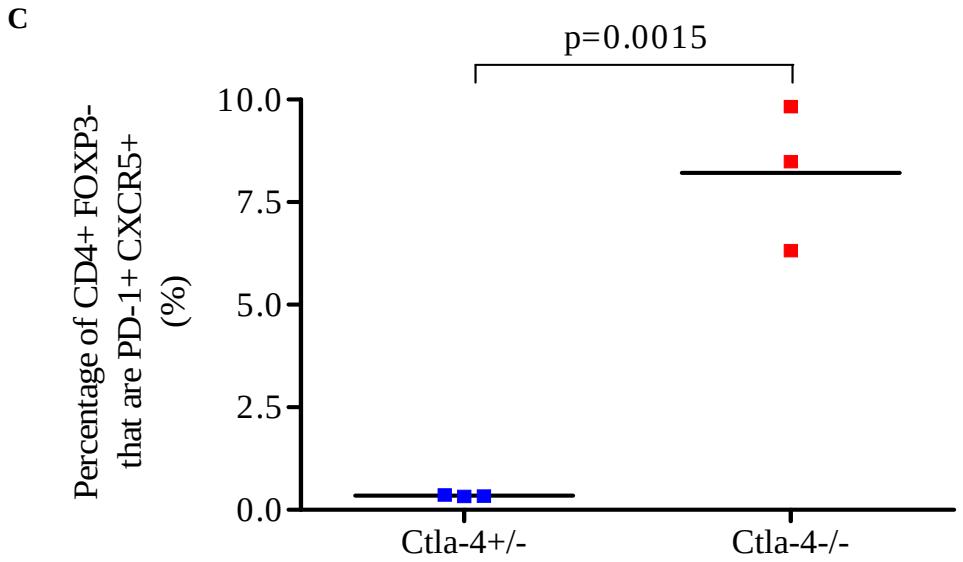


Figure 3.32. Localisation of FOXP3+ cells in BALB/c and *Ctla-4*-/- spleen. Acetone fixed, 6µm spleen sections from 15-18 day old wild type BALB/c (left) or *Ctla-4*-/- (right) were stained with IgD (brown) and FOXP3 (blue) for analysis by light microscopy (x10 - A and x20 - B). Arrows indicate FOXP3+ cells in B zone / germinal centre. T – T zone (CD4+), B – B zone (IgD+), GC – germinal centre (PNA+).


A
Ctla-4+/-
Ctla-4-/-
0.33
9.83
PD-1
CXCR5
B
0.071
PD-1
Control


C
p=0.0015
Percentage of CD4+ FOXP3- that are PD-1+ CXCR5+ (%)
10.0
7.5
5.0
2.5
0.0
Ctla-4+/-
Ctla-4-/-

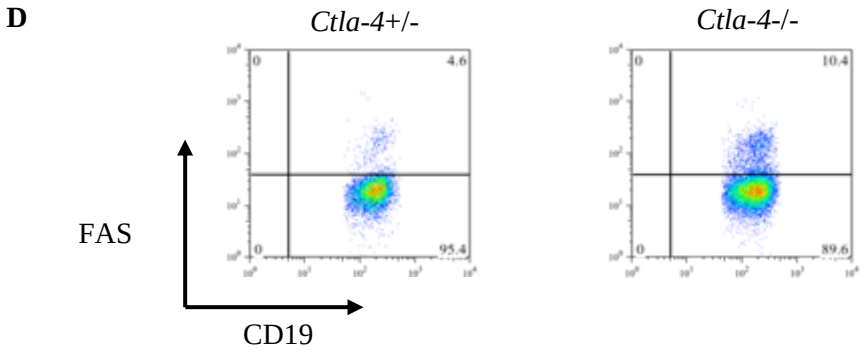

D
Ctla-4+/-
Ctla-4-/-
4.6
95.4
10.4
89.6
FAS
CD19

Figure 3.33. Analysis of T follicular helper cells in *Ctla-4*-/- mice. Single cell suspensions from 15-18 day old *Ctla-4*+/- and *Ctla-4*-/- splenocytes were surface stained with CD4, PD-1, CXCR5 and intracellularly with FOXP3 for analysis by flow cytometry. (A) Representative FACS plots show CXCR5 and PD-1 expression of CD4+ FOXP3- gated lymphocytes in *Ctla-4*+/- (left) and *Ctla-4*-/- (right) mice. (B) Representative FACS plot shows control antibody staining of CD4+ FOXP3- gated lymphocytes. (C) Graph shows data of the percentage of CD4+ FOXP3- that are CXCR5+ PD-1+ for three *Ctla-4*+/- (blue) and *Ctla-4*-/- (red) mice. Lines indicates mean value. (D) Single cell suspensions from 15-18 day old *Ctla-4*+/- (left) and *Ctla-4*-/- (right) splenocytes were surface stained with CD19 and FAS for analysis by flow cytometry. Representative FACS plots show FAS expression of CD19+ gated lymphocytes. p value calculated by two-tailed, unpaired T-test for the means.

3.3 Discussion

Analysis of *Ctla-4*-gene ablated mice in this present study identified an augmented FOXP3+ population. This is in agreement with an investigation which found a 30-40% increase in the frequency of CD4+ CD25+ regulatory T cells in the peripheral blood of healthy subjects homozygous for a single nucleotide *Ctla-4* polymorphism [267]. This finding is also consistent with recent data from a study in which anti-CTLA blocking antibody was used as a cancer immunotherapy in combination with GM-CSF to induce effector T cells to mediate tumour regression. However, it was found that rather than decrease the number of Tregs or impair their regulatory function, CTLA-4 blockade actually increased the number of CD4+ FOXP3+ Tregs at a lower dose than that required to expand the activated effector CD4+ T cells [268]. Kavanagh *et al*. suggested that CTLA-4 may inhibit Treg proliferation similar to its role on effector T cells. This is corroborated by the data obtained in this current investigation which found that CTLA-4 signalling normally functions to control the size of the peripheral CD4+ FOXP3+ compartment by modulating proliferation. Additionally, it has recently been reported by two other groups that CTLA-4 functions to negatively regulate Treg homeostasis and limit their frequency *in vivo* [269, 270].

Regulatory T cells have previously been shown to proliferate in response to self-antigen *in vivo* [52]. Therefore, it could be that upon self-antigen encounter in the periphery after exiting the thymus, CTLA-4 normally functions to restrict the magnitude of the CD4+ FOXP3+ proliferative response. Also, at the young age that *Ctla-4*-/- mice have to be studied, there may be lymphopenia-induced, homeostatic Treg proliferation. Consistent with this, the studies presented here of naïve BALB/c mice upon maturation from a neonate (1 week old) into an adult (>6 weeks old) found by expression of the cell cycle marker Ki67 that CD4+ FOXP3+ Treg proliferation decreased as the mice matured into adults. Additionally, high levels of the cytokine IL-2 have been found to overcome Treg anergy *in vitro* and stimulate their proliferation [57]. Increased IL-2 production by CD4+ FOXP3- conventional T cells was detected in *Ctla-4*-/- mice. It could therefore be the higher levels of IL-2 present in the environmental milieu that causes the increased proliferation of CD4+ FOXP3+ cells in *Ctla-4*-/- mice.

An altered cytokine milieu could also account for the decreased proportion of conventional and regulatory T cells found to be undergoing cell death in *Ctla-4*-deficient mice compared to age-matched BALB/c controls. The cytokine milieu of the *Ctla-4*-/- environment could promote cell growth and division and protect from cell death. For example, T cell growth factors such as IL-2, IL-7 and IL-15 that share a common-gamma signalling chain in their cell surface receptors induce viability of activated T lymphocytes, whereas mutations in the common-gamma chain are associated with the X-linked severe combined immunodeficiency (SCID) phenotype [271, 272] that appears to be due to apoptosis of developing T lymphocytes lacking the necessary viability inducing signals [273].

In addition to alterations in the proliferation and/or cell death of peripheral CD4+ FOXP3+ Tregs, the augmented FOXP3+ population observed in the *Ctla-4*-/- mouse could be the result of enhanced FOXP3 induction in CD4+ FOXP3- T cells. Others have previously identified an essential role for CTLA-4 in the TGF-β induction of *Foxp3* and suggested that a lack of peripheral, adaptive Tregs in *Ctla-4*-/- mice may contribute to the fatal lymphoproliferative syndrome of *Ctla-4*-deficient mice [123]. In the present study a role for CTLA-4 in the TGF-β induction of FOXP3 *in vitro* was not found as using TCR-transgenic mice a similar proportion of CD4+ CD25- T cells from *Ctla-4*-/- mice were induced to express FOXP3 as CD4+ CD25- T cells from wild type mice. The differences observed may be because in this present study TCR-transgenic *Rag2*-/- mice were used to ensure that the starting population of CD4+ CD25- T cells was naïve and truly FOXP3-. Also, in this current investigation the sensitive technique of flow cytometry was used to detect FOXP3 expression at the protein level as opposed to detection of *Foxp3* mRNA by PCR.

Additionally, the complimentary *in vivo* FOXP3 induction experiments carried out found no requirement for CTLA-4 in FOXP3 induction. If anything, the data suggests that CTLA-4 may normally function to restrict the induction of peripheral CD4+ FOXP3+ lymphocytes *in vivo* in response to antigen. The augmented FOXP3 induction observed in *Ctla-4*-/- mice *in vivo* (*Fig. 3.23*) could be the result of enhanced proliferation of induced FOXP3+ T cells upon the loss of CTLA-4 rather than increased induction of FOXP3. It would be interesting in future to investigate the involvement of proliferation in the augmented FOXP3 induction observed in *Ctla-4*-/- mice *in vivo* by the adoptive transfer of CFSE-labelled T cells prior to

antigen immunisation to induce FOXP3 expression. Upon harvest of wild type and *Ctla-4*-/- mice, the proliferation of the induced FOXP3+ T cell population could be assessed by CFSE dilution.

The role of the CD28 co-stimulatory receptor in FOXP3 induction was also investigated. Both TGF-β *in vitro* and antigen *in vivo* studies found a requirement for CD28 in FOXP3 induction. These studies using TCR-transgenic *Rag2*-/- mice to ensure a truly naïve FOXP3- starting population of T cells corroborate previous findings by others [121]. Although FOXP3 was not induced in *CD28*-deficient CD4+ CD25- T cells, addition of exogenous IL-2 to the *in vitro* TGF-β experiments induced FOXP3 which suggests the requirement of CD28 in FOXP3 induction is mediated by IL-2. The requirement of the CD28 receptor in FOXP3 induction indicates a role for the co-stimulatory ligands CD80 and CD86. The requirement of CD80 and CD86 in FOXP3 induction could be investigated in future by antibody-mediated blockade of each of these ligands during *in vitro* TGF-β induction or antigen induction *in vivo*. It would also be interesting to use antigen-presenting cells from *CD80*/*CD86*-deficient mice for *in-vitro* TGF-β induction, or generate TCR-transgenic *Rag2*-/- mice lacking CD80/86 for *in vivo* FOXP3 induction experiments.

The *in vitro* and *in vivo* experiments carried out induced FOXP3 expression in peripheral CD4+ CD25- (FOXP3-) T cells; however it was not determined whether the cells generated were fully functional regulatory T cells. It is difficult to isolate FOXP3 induced T cells as each protocol activates the conventional T cells to express CD25, therefore CD25 cannot be used as a cell surface marker to purify FOXP3+ T cells from activated FOXP3- T cells. Ideally, TCR-transgenic *Rag2*-/- FOXP3-GFP mice would be used to induce FOXP3+ T cells which could then be isolated on the basis of GFP expression and used in suppression assays to assess the functional activity of the FOXP3 induced T cells. It is believed that natural and induced / adaptive FOXP3+ regulatory T cells may differ in suppressive function due to the stability of *Foxp3* expression as determined by the methylation state of CpG motifs within the Treg-specific demethylated region (TSDR) (reviewed in [106]). Recently, it has been reported that instability of *Foxp3* expression can lead to the generation of pathogenic memory T cells *in vivo* and the development of autoimmune disease [274]. An *in vivo* microenvironment where it is believed induced / adaptive FOXP3+ Tregs may have a

functional role is the gut where immune tolerance to foreign food allergens and a non-inflammatory environment needs to be maintained. Within the gut-associated lymphoid tissue (GALT) a functionally specialised, mucosal dendritic cell population has been found to be important in the TGF-β induction of FOXP3+ Tregs via a retinoic acid-dependent mechanism [116-118]. CTLA-4 has been found to play an essential role in the function of regulatory cells that control intestinal inflammation [154]. Therefore, it would be interesting to assess the role of CTLA-4 in FOXP3 induction *in vivo* using TCR-transgenic *Rag2*-/- mice in a more physiological setting such as oral tolerance by oral gavage administration of antigen.

This investigation found that there was unaltered thymic selection of natural CD4+ FOXP3+ Tregs in *Ctla-4*-deficient mice. This suggests that the augmented peripheral CD4+ FOXP3+ Treg population identified in *Ctla-4*-/- mice is not due to increased thymic selection of Tregs and their export to the periphery. If CTLA-4 plays a functional role in the thymic development of Tregs, the data obtained here suggests that it is not in terms of restricting the proportion of CD4+ FOXP3+ Tregs that are selected. It would be interesting to analyse recent thymic emigrants in *Ctla-4*-/- mice because the augmented peripheral CD4+ FOXP3+ population observed could be due to altered frequency of thymocyte export. Recent thymic emigrants have been studied by others using intra-thymic injection of fluorescein, however none of the surface proteins investigated uniquely distinguished recent thymic emigrants from peripheral T cells or mature thymocytes [275]. Alternatively, T cell receptor excision circles can be used to identify recent thymic emigrants within the peripheral T cell pool [276]. The results of this current investigation differ to a recent report that found enhanced thymic selection of FOXP3+ regulatory T cells in *Ctla-4*-deficient mice protected from experimental autoimmune encephalomyelitis (EAE) [277]. However, in these studies Verhagen *et al*. used TCR-transgenic mice in which it is not truly understood how the thymic generation of conventional and regulatory T cell occurs.

The augmented FOXP3+ population observed in *Ctla-4* knockout mice raises a paradox as these mice suffer a fatal lymphoproliferative disease; however the regulatory T cell population is increased. It is possible that CD4+ FOXP3+ regulatory T cells lacking CTLA-4 have a pathogenic functional role as opposed to being regulatory. Assessment of the ability of

CD4+ CD25hi Tregs from *Ctla-4* knockout mice to transfer disease by adoptive transfer into *Rag2*-/- recipients did not identify an inherent pathogenic function within the FOXP3+ population. However, this experimental setup did not allow for the interaction of Tregs with other lymphocytes such as conventional T cells and B cells, for example via the secretion of cytokines.

Analysis of the cytokines produced by CD4+ FOXP3+ regulatory T cells in *Ctla-4*-/- mice identified an altered cytokine profile compared to CD4+ FOXP3+ Tregs in *Ctla-4*+/- littermate controls. This could modulate the homeostatic balance towards pathogenic effector T cells. A small, but reproducible increase in production of the T_H1 pro-inflammatory cytokine IFN-γ by *Ctla-4*-/- CD4+ FOXP3+ Tregs was observed. Thus, rather than having a regulatory function, CD4+ FOXP3+ Tregs in *Ctla-4*-/- mice may contribute to the initiation and amplification of immune activation. Additionally, increased production of TGF-β by CD4+ FOXP3+ Tregs in *Ctla-4*-/- mice was found. As discussed above, T cells activated with TGF-β can be induced to become FOXP3+ regulatory T cell [114]. Therefore, the increased TGF-β observed could contribute to the augmented FOXP3+ population in *Ctla-4*-/- mice. However, TGF-β has been found to be able to promote both regulatory and effector cell differentiation pathways depending on the other cytokines present in the local environment. Activation of T cells with TGF-β along with IL-6 or IL-21 results in the generation of T_H17 cells [278, 279]. T_H17 cells are a relatively newly classified CD4+ T cell subset distinct from T_H1, T_H2 and Tregs that produce IL-17 and express the lineage transcription factor RORγt [17, 18, 280]. T_H17 cells are believed to play a pro-inflammatory role in some autoimmune diseases as certain mouse models such as experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis are mediated mostly by T_H17 cells [281, 282]. An increase in IL-17 production by CD4+ FOXP3+ regulatory T cells was observed in *Ctla-4* knockout mice. Studies in mice initially found that TGF-β-induced CD4+ FOXP3+ Tregs were resistant to T_H17 conversion by IL-6 [283]. However, more recently human Tregs have been found to differentiate into IL-17-producing cells with the addition of other cytokines such as IL-1β or IL-21 rather than IL-6 [284, 285].

Expression of the T_H1 and T_H2 lineage transcription factors T-bet and GATA3 was detected in both wild type BALB/c and *Ctla-4*-/- CD4+ CD25- Tconv and CD4+ CD25+ CD27hi Treg

populations. This could be due to contamination of the mo-flow sorted cell preparations from which mRNA was extracted and analysed by qPCR. In future it would therefore be interesting to analyse expression of multiple transcription factors such as T-bet, GATA3 and RORγt in wild type and *Ctla-4*-/- CD4+ FOXP3- conventional T cells and CD4+ FOXP3+ regulatory T cells by flow cytometry. Recently, evidence has started to accumulate that suggests differentiation of CD4+ T cells into effector lineages is more plastic than previously appreciated (reviewed in [286]). For example, FOXP3+ Tregs have been shown to up regulate the T_H1-specifying transcription factor T-bet during type 1 inflammation [287].

Analysis of cytokines produced by CD4+ T cells in *Ctla-4* knockout mice found an increase in IL-21 production. IL-21 is the most recently described member of the common gamma chain signalling family of cytokines, which also includes IL-2, IL-4, IL-7, IL-9 and IL-15. A broad range of actions have been implicated for IL-21 from the expansion of CD8+ T cells, apoptosis of NK cells to the differentiation of B cells [288]. In recent years, a fundamental role for IL-21 in the generation of T follicular helper (TFH) cells has been established [289, 290]. Consistent with the substantially increased serum IgE levels identified in early studies of *Ctla-4* knockout mice [156], which indicates immunoglobulin class switching has occurred during the germinal centre B cell response [257], and increased IL-21 production by CD4+ FOXP3- Tconv measured in this study, spontaneous germinal centres were identified in the spleens of *Ctla-4*-/- mice. Few mouse strains have been described that develop spontaneous germinal centres, those that do typically develop an autoimmune, lupus-like disease or Type 1 diabetes [46]. Studies using *sanroque* mice, which exhibit a lupus-like phenotype, have identified excessive TFH cell numbers and aberrant germinal centre formation as a pathway to systemic autoimmunity [291]. In line with this, increased numbers of CD4+ FOXP3- CXCR5+ PD-1+ TFH cells were found in *Ctla-4*-/- mice. A role for CTLA-4 in the regulation of T cell help for germinal centre B cells has previously been suggested by others [292]. Thus, the fatal lymphoproliferative syndrome of *Ctla-4*-deficient mice is likely to be pathologically manifested by an aberrant B cell response and autoantibody production.

The augmented CD4+ FOXP3- CXCR5+ PD-1+ population and spontaneous germinal centres found in *Ctla-4*-/- mice suggests a role for CTLA-4 in the regulation of T cell help for

germinal centre B cells as discussed above. However, it is not clear whether TFH cells express CTLA-4 themselves, which is required to maintain their homeostasis, or if in the absence of CTLA-4 there is a loss of TFH cell regulation by Tregs. In this study it was found that in the absence of CTLA-4, FOXP3+ cells still traffic to and localise within the T zones and B zones in a similar manner to in wild type mice. It would be interesting in future to analyse CTLA-4 expression by CD4+ FOXP3- CXCR5+ PD-1+ TFH cells. A mouse model that may be suitable for this would be the *DO11.10* x RIP-*mOVA* transgenic mouse model of autoimmune diabetes as our laboratory has previously found elevated levels of IL-21 [53] and identified spontaneous germinal centres (C. Wang, personal communication).

4.0 Role of CTLA-4 in the Suppressive Function of Regulatory T Cells

4.1 Introduction

Regulatory T cells actively control effector T cell populations to maintain peripheral tolerance. It is now considered that loss of Treg function is a major factor in the development of autoimmune disease. Due to the constitutive expression of the CTLA-4 receptor by Tregs [154, 155] and the fatal lymphoproliferative syndrome observed in *Ctla-4*-deficient mice [156, 157], a role for CTLA-4 as an effector molecule in Treg function has been suggested. Furthermore, polymorphisms in the *Ctla-4* gene are linked with several autoimmune diseases such as type 1 diabetes, thyroiditis, systemic lupus erythematosus and rheumatoid arthritis (reviewed in [228, 229]).

A role for CTLA-4 as an effector molecule in Treg suppressive function would be consistent with the ability of *Ctla-4*-expressing cells to exert dominant tolerance over *Ctla-4*-deficient cells in mixed bone marrow chimeras [220]. Early studies with anti-CTLA-4 blocking antibodies abrogated disease protection by Tregs [154, 155], although in these experiments effector T cells could have also been affected by the CTLA-4 blockade treatment. Additionally, antibody-mediated CTLA-4 modulation studies have yielded conflicting results where in some instances Treg suppression is abrogated [155, 231, 232], but in other investigations Tregs retain suppressive function [56, 293]. It has even been reported that different batches of monoclonal antibody preparations sometimes yielded opposite results [294]. In studies where the role of CTLA-4 was specifically assessed in the Treg population, generation of *Ctla-4*-deficient Tregs relied on the simultaneous abrogation of CD28 signalling by CD80/86 blockade or deficiency [231, 232]. It is not clear whether or not Tregs that develop in the absence of CD28 are functionally equivalent to wild type Tregs, therefore the results of these studies cannot be solely attributed to a lack of CTLA-4.

To analyse the suppressive function of *Ctla-4*-deficient Tregs in a setting not complicated by lymphoproliferation or CD28 deficiency, a TCR-transgenic system was used to generate

antigen-specific Tregs lacking CTLA-4. The functional activity of *Ctla-4*-deficient Tregs was assessed *in vivo* using an adoptive transfer mouse model of autoimmune diabetes.

4.2 Results

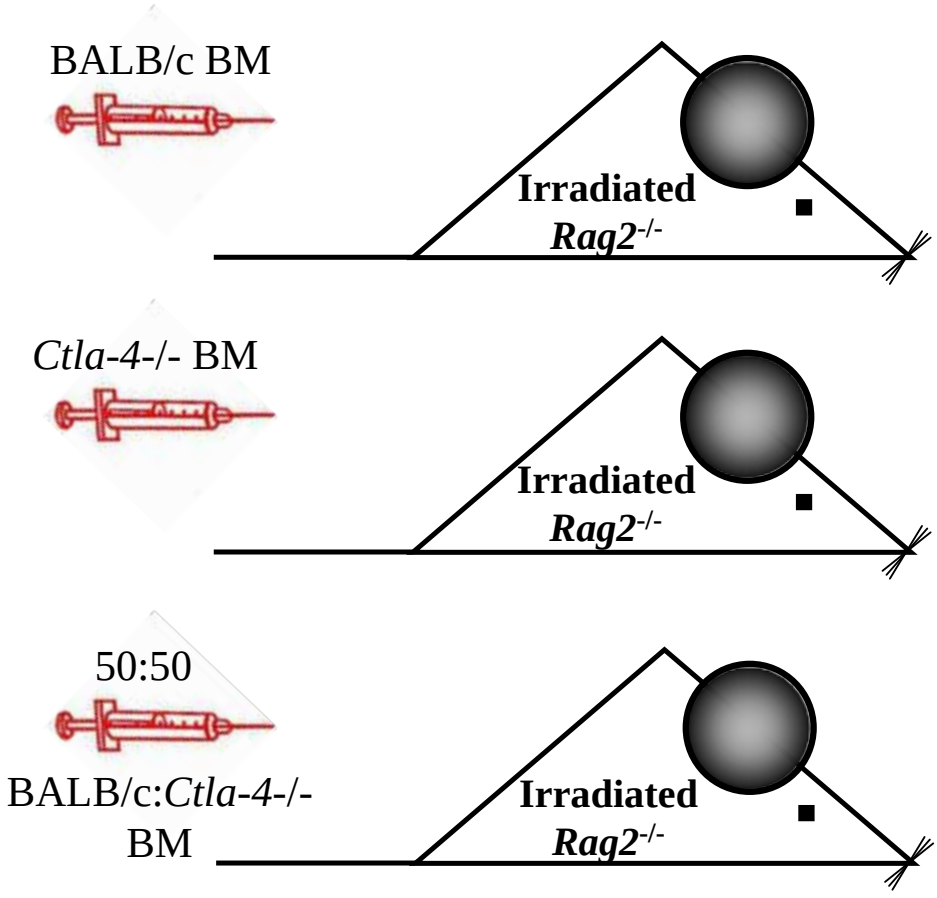
4.2.1 *In vivo* evidence for a role of the CTLA-4 receptor in Treg suppressive function.

Due to the fatal lymphoproliferative disease in mice where the *Ctla-4* gene has been ablated [156, 157] and the finding that CTLA-4 is constitutively expressed by regulatory T cells [154, 155], a role for CTLA-4 in Treg suppressive function has been hypothesised for some years. Over a decade ago, Bachmann *et al*. carried out elegant bone marrow chimera studies in which *Rag2*-/- mice reconstituted with a mixture of wild type C57BL/6 bone marrow and *Ctla-4*-/- bone marrow remained healthy and did not develop disease like *Rag2*-/- recipients of *Ctla-4*-/- bone marrow alone. These experiments showed that the lethal lymphoproliferative syndrome of *Ctla-4*-deficient mice was not T cell autonomous and could be prevented by factors produced by normal T cells [220].

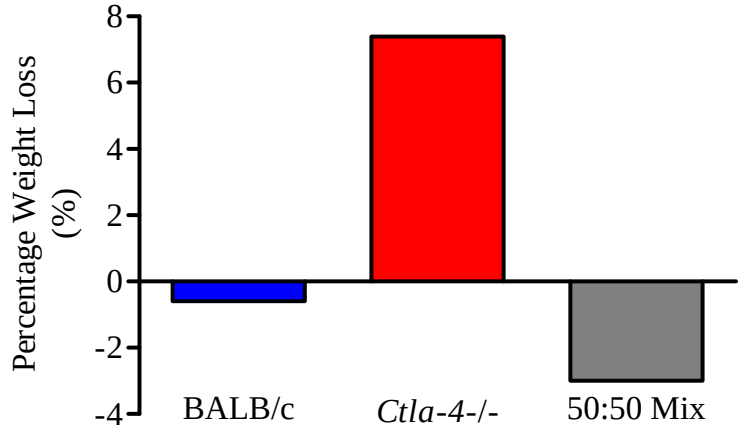
To assess the cell-extrinsic role of CTLA-4 for ourselves using our *Ctla-4*-/- mice on the BALB/c genetic background, similar bone marrow chimera experiments to those performed by Bachmann *et al*. were carried out. As illustrated in *Fig. 4.01 A*, T cell-depleted (THY1 negative) bone marrow from wild type BALB/c, *Ctla-4*-/- or a 50:50 mix was adoptively transferred into irradiated *Rag2*-deficient recipient mice. Five weeks following transfer, recipient mice of *Ctla-4*-deficient bone marrow had lost a substantial percentage of body weight, whereas mice that had received wild type BALB/c bone marrow or a 50:50 mix of wild type and *Ctla-4*-/- bone marrow had not lost weight and remained healthy (*Fig. 4.01 B*). In fact mixed bone marrow chimeras showed no signs of disease (such as weight loss of lymphoproliferation) at any time point examined.

When the recipient mice were sacrificed five weeks after transfer, the reconstitution of the peripheral lymphocyte compartment was analysed by flow cytometry using congenic markers. It was possible to distinguish lymphocytes derived from wild type BALB/c or *Ctla-4*-/- bone marrow on the basis of their expression of variants of the THY1 protein (THY1.1 and THY1.2, respectively). The CD4+ lymphocytes of recipient mice of wild type BALB/c bone marrow alone were THY1.1+, and THY1.1- in recipient mice of *Ctla-4*-/- bone marrow alone. The CD4+ lymphocytes of mice that received a 50:50 mix of bone marrow showed a 40:60

A


BALB/c BM
Irradiated
Rag2-/-
Ctla-4-/- BM
Irradiated
Rag2-/-
50:50
BALB/c:Ctla-4-/-
BM
Irradiated
Rag2-/-

B


Percentage Weight Loss (%)
8
6
4
2
0
-2
-4
BALB/c
Ctla-4-/-
50:50 Mix

C

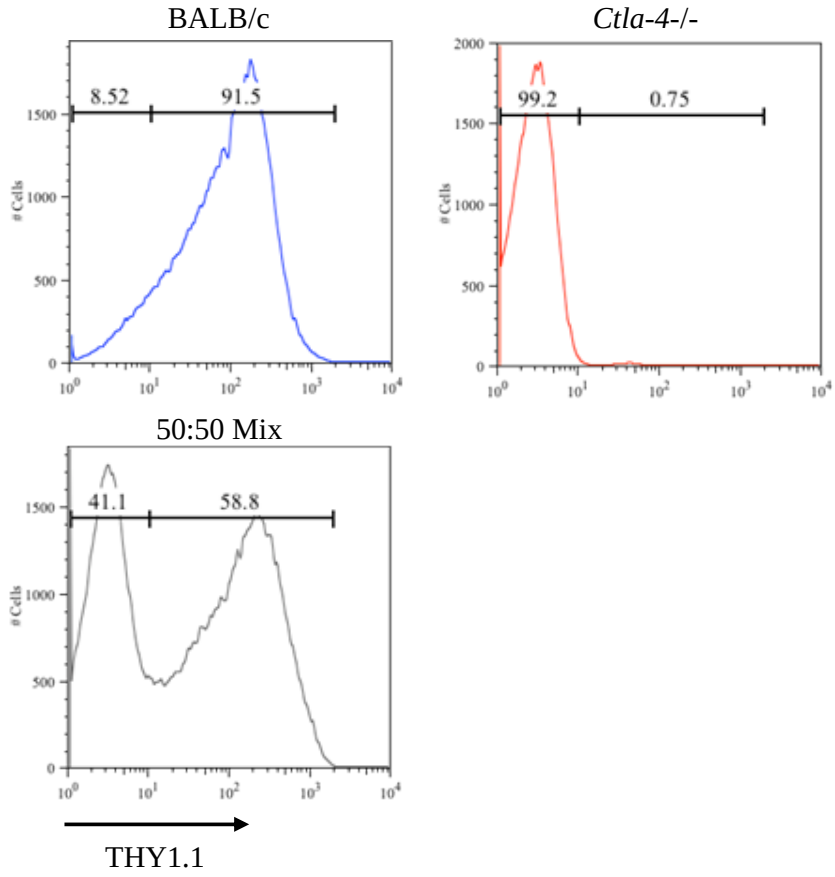

BALB/c
8.52
91.5
Cells
1500
1000
500
0
10^0
10^1
10^2
10^3
10^4
Ctla-4-/-
99.2
0.75
2000
50:50 Mix
41.1
58.8
THY1.1

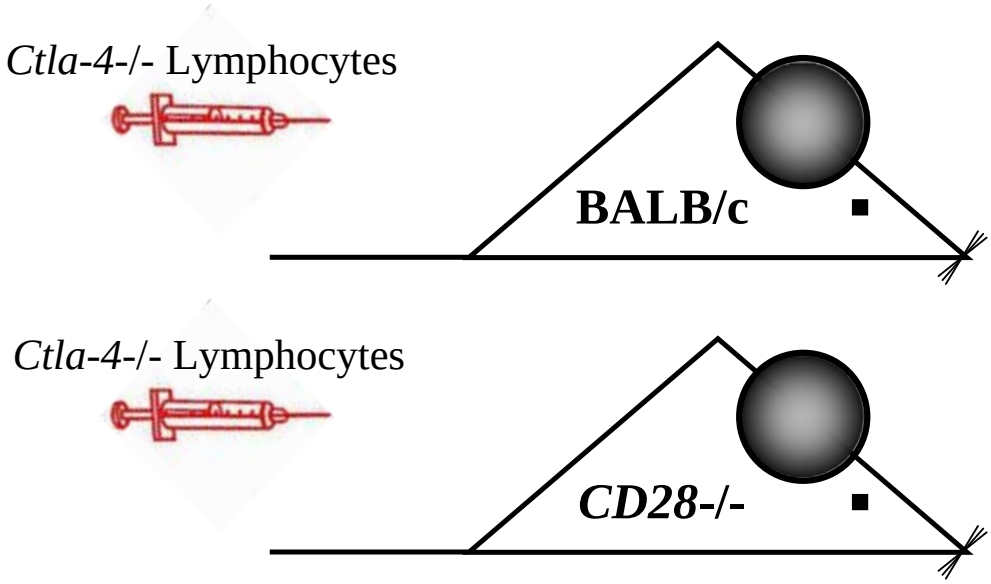
Figure 4.01. BALB/c : *Ctla-4*-/- Mixed Bone Marrow Chimeras. (A) THY1 depleted bone marrow from BALB/c or *Ctla-4*-/- mice was adoptively transferred into *Rag2*-/- mice (8 x 10^6 cells) either alone or as a 50:50 mix. Mice were harvested 5 weeks following transfer. (B) Graph shows the percentage weight loss of recipients mice for a representative experiment. (C) Histograms illustrate recipient peripheral lymph node composition of THY1.1+ BALB/c and THY1.1- *Ctla-4*-/- CD4+ gated lymphocytes. Data shown is representative of three independent adoptive transfer experiments. Experiments carried out in collaboration with C. Wang.

THY1.1-:THY1.1+ composition (*Fig. 4.01 C*). The THY1.1-:THY1.1+ ratio within the mixed bone marrow chimeras consistently ranged from 35:65 to 58:42.

The experiments carried out above, like those by Bachmann *et al*. suggest that wild type cells are capable of controlling the fatal lymphoproliferative disease associated with the loss of *Ctla-4* by gene ablation. This could be due to the presence of functional Tregs that express CTLA-4 in the wild type lymphocyte compartment that develops. To examine this in more detail and assess Treg control of *Ctla-4*-deficient lymphoproliferative disease, lymphocytes from *Ctla-4*-/- mice were adoptively transferred into wild type BALB/c mice which have a full complement of functional Tregs, or *CD28* knockout mice which have a deficient peripheral Treg population [131, 238, 295] (illustrated in *Fig. 4.02 A*). Four weeks following adoptive transfer, *CD28* knockout recipients of *Ctla-4*-deficient lymphocytes had lost greater than 15% of their body weight, whereas wild type BALB/c recipients of *Ctla-4*-/- lymphocytes and control BALB/c and *CD28*-/- mice remained healthy with no weight loss (*Fig. 4.02 B*). In conclusion, when *Ctla-4*-/- lymphocytes were transferred into BALB/c mice with a full complement of functional Tregs the recipient mice remained healthy, whereas *CD28*-/- recipients with a compromised peripheral Treg population succumbed to a severe wasting disease.

The adoptive transfer experiments carried out above imply that wild type CD4+ FOXP3+ Tregs can control the fatal lymphoproliferative syndrome of *Ctla-4* knockout mice. To directly test the capacity of Tregs to control disease induced by *Ctla-4*-deficiency, further *in vivo* adoptive transfer experiments were carried out. As illustrated in *Fig. 4.03* A, CD4+ CD25- lymphocytes from *Ctla-4*-deficient mice were adoptively transferred into *Rag2*-deficient mice either alone or with the co-transfer of wild type BALB/c CD4+ CD25- conventional T cells or wild type CD4+ CD25+ regulatory T cells. Two to three weeks following transfer, *Rag2*-/- recipients of *Ctla-4*-/- lymphocytes alone or plus wild type CD4+ CD25- Tconv had lost greater than 15% body weight (*Fig. 4.03 B*). Importantly, *Rag2*-/- recipient mice of *Ctla-4*-/- lymphocytes plus wild type CD4+ CD25+ Tregs remained healthy with no weight loss.

A


Ctla-4-/- Lymphocytes
BALB/c
Ctla-4-/- Lymphocytes
CD28-/-

B

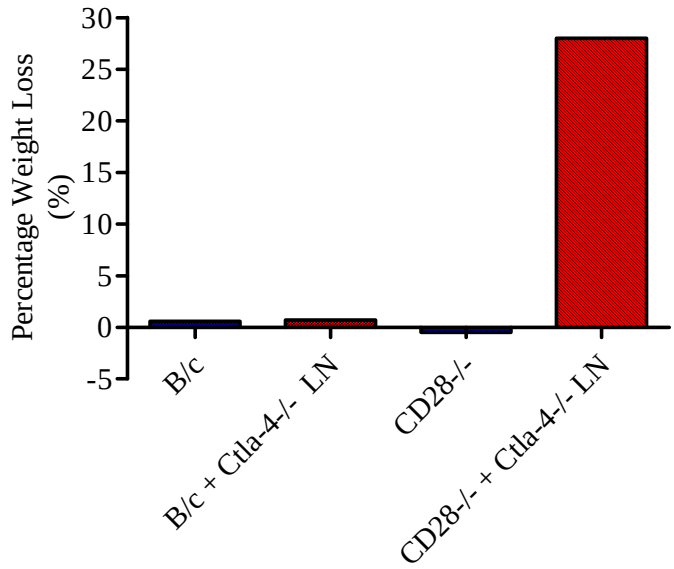
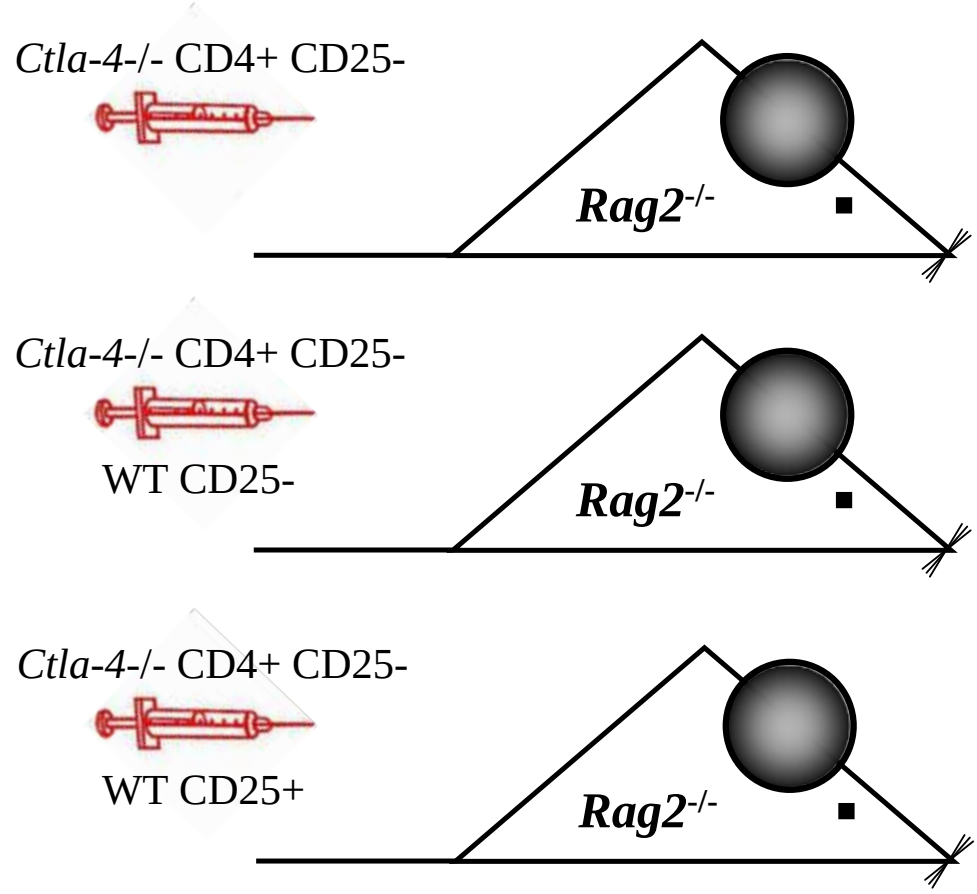
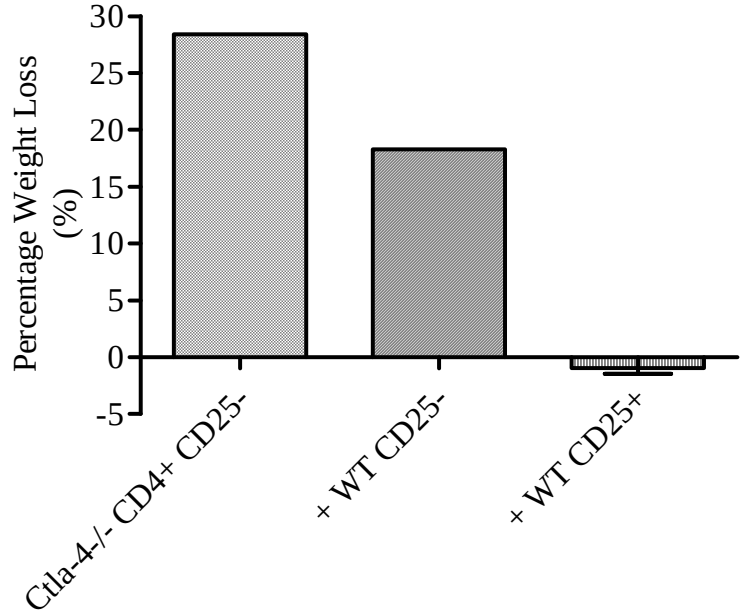

Percentage Weight Loss (%)
30
25
20
15
10
5
0
-5
B/c
B/c + Ctla-4-/- LN
CD28-/-
CD28-/- + Ctla-4-/- LN

Figure 4.02. Adoptive transfer of *Ctla-4*-/- lymphocytes causes disease in Treg-deficient *CD28*-/- mice. (A) Single cell suspensions from peripheral lymph nodes of *Ctla-4*-/- mice were adoptively transferred into BALB/c or *CD28*-/- mice (50 x 10^6 cells). Mice were harvested 4 weeks following transfer. (B) Graph shows the percentage weight loss of recipients mice for a representative experiment. Data shown is representative of two independent adoptive transfer experiments. Experiments carried out in collaboration with C. Wang.

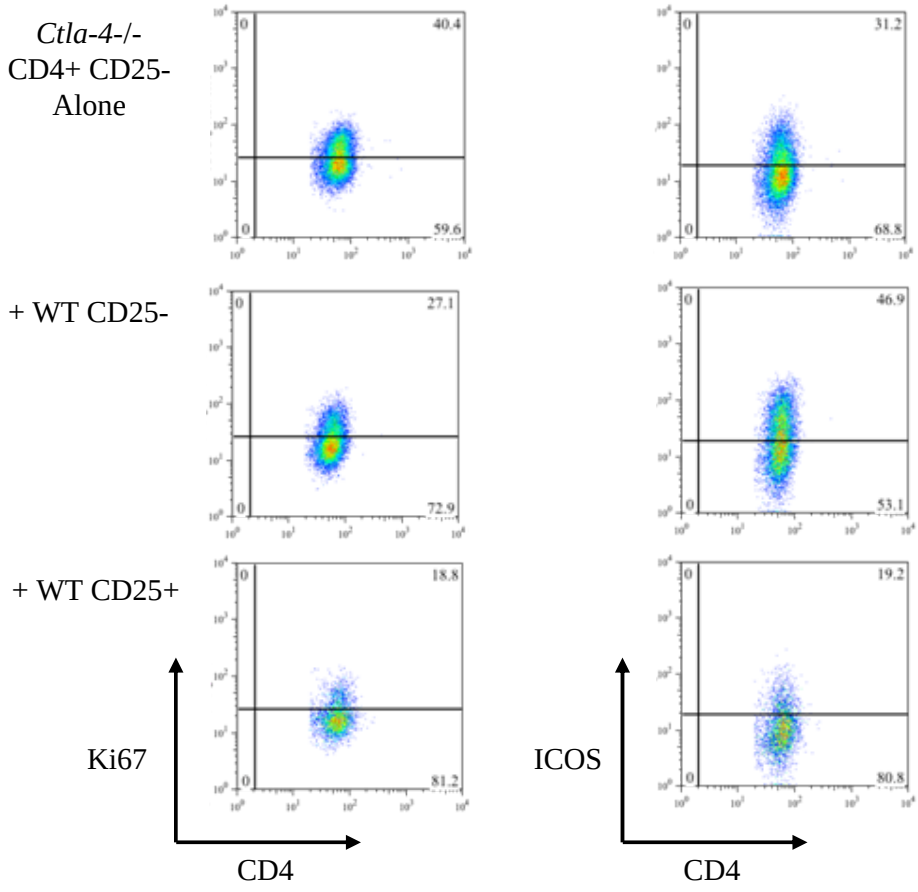
A


Ctla-4-/- CD4+ CD25-
Rag2-/-
Ctla-4-/- CD4+ CD25-
WT CD25-
Rag2-/-
Ctla-4-/- CD4+ CD25-
WT CD25+
Rag2-/-

B


Percentage Weight Loss (%)
30
25
20
15
10
5
0
-5
Ctla-4-/- CD4+ CD25-
+ WT CD25-
+ WT CD25+

C


Ctla-4-/-
CD4+ CD25-
Alone
+ WT CD25-
+ WT CD25+
40.4
59.6
31.2
68.8
27.1
72.9
46.9
53.1
18.8
81.2
19.2
80.8
Ki67
CD4
ICOS
CD4

D

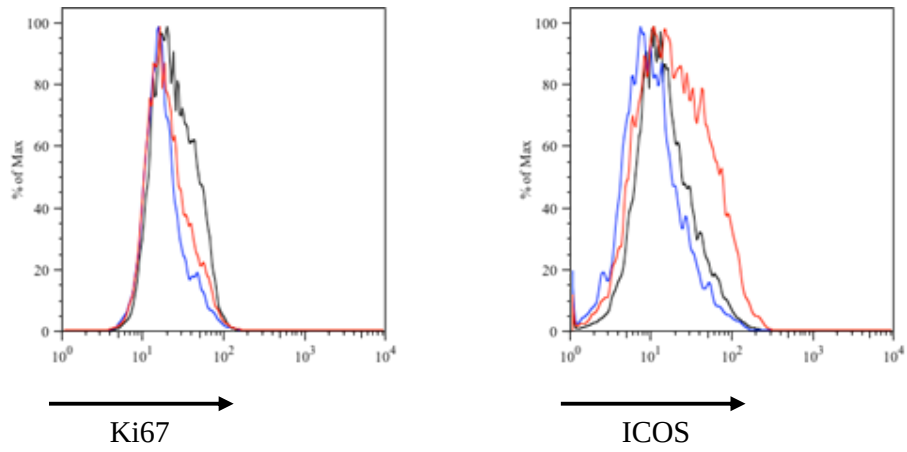

% of Max
Ki67
% of Max
ICOS

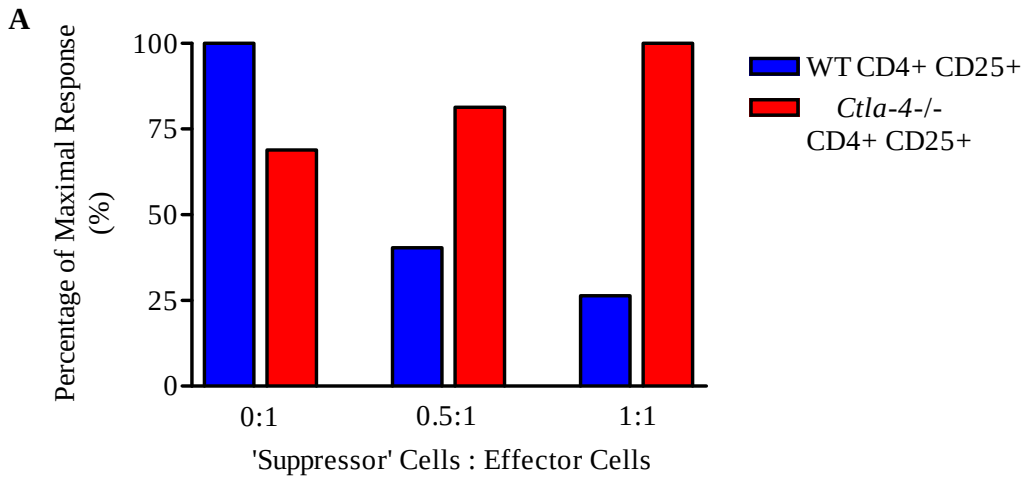
Figure 4.03. Wild type BALB/c CD4+ CD25+ Tregs protect *Rag2*-/- from disease following adoptive transfer of *Ctla-4*-/- lymphocytes. (A) MACS separated CD4+ CD25- from peripheral lymph nodes of *Ctla-4*-/- mice were adoptively transferred into *Rag2*-/- mice (0.6 x 10^6 cells) alone or plus 0.6 x 10^6 BALB/c MACS separated CD4+ CD25- Tconv or CD4+ CD25+ Tregs. Mice were harvested 2-3 weeks following transfer. (B) Graph shows the percentage weight loss of recipients mice for a representative experiment. (C) Single cell suspensions from peripheral lymph nodes of recipient mice were surface stained with CD4, ICOS and intracellular with FOXP3 and Ki67 for analysis by flow cytometry. Representative FACS plots are shown for Ki67 (left) and ICOS (right) expression by CD4+ FOXP3- gated lymphocytes for recipients of *Ctla-4*-/- CD4+ CD25- alone (top), plus BALB/c CD4+ CD25- Tconv (middle) or plus BALB/c CD4+ CD25+ Tregs (bottom). (D) Histograms of Ki67 (left) and ICOS (right) expression by CD4+ FOXP3- gated lymphocytes for recipients of *Ctla-4*-/- CD4+ CD25- alone (black), plus BALB/c CD4+ CD25- Tconv (red) or plus BALB/c CD4+ CD25+ Tregs (blue). Data shown is representative of three independent adoptive transfer experiments.

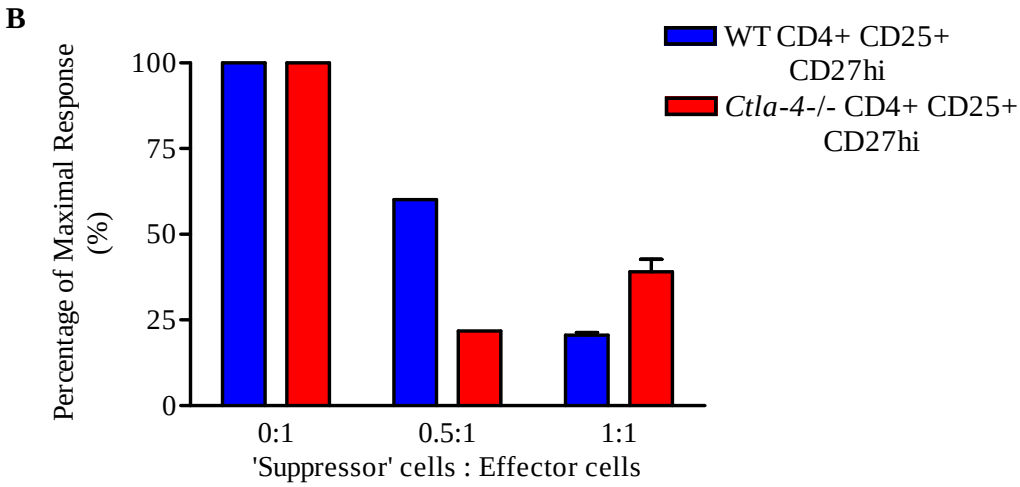
When the recipient mice were sacrificed the phenotype of congenically marked (THY1.1-) *Ctla-4*-deficient CD4+ FOXP3- Tconv was analysed by flow cytometry. Differences were only small, however, the CD4+ FOXP3- Tconv in *Rag2*-/- recipient mice of *Ctla-4*-deficient lymphocytes alone or co-transferred with wild type CD4+ CD25- Tconv expressed higher levels of the cell cycle marker Ki67 and secondary co-stimulatory molecule ICOS compared to the *Ctla-4*-deficient CD4+ FOXP3- Tconv in *Rag2*-/- recipients with co-transferred wild type CD4+ CD25+ Tregs (*Fig. 4.03 C and D*). Overall, the fatal lymphoproliferative disease of *Ctla-4*-deficient mice was adoptively transferred by CD4+ CD25- lymphocytes to *Rag2*-/- hosts. Co-transfer of wild type CD4+ CD25+ Tregs prevented disease transfer, whereas wild type CD4+ CD25- Tconv did not provide protection.

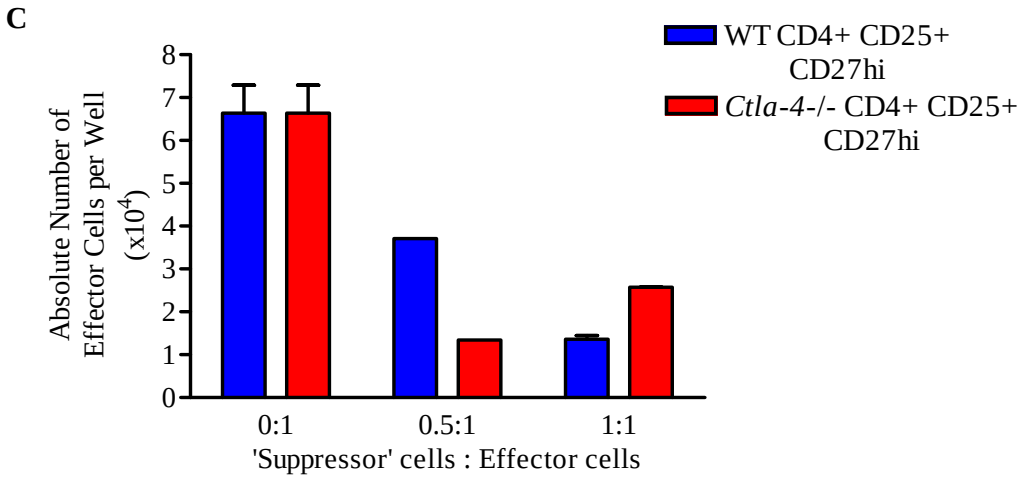
4.2.2 *Ctla-4*-deficient Tregs exert suppressive function *in vitro*.

The *in vivo* bone marrow chimera and adoptive transfer data in 4.2.1 suggests that Tregs in *Ctla-4*-deficient mice could be functionally defective, leading to the severe lymphoproliferative syndrome observed. The function of regulatory T cells is frequently assessed *in vitro* by their ability to suppress the proliferation and / or cytokine production of conventional T cells. To test the suppressive capacity of Tregs from *Ctla-4*-deficient mice, suppression of conventional T cell growth *in vitro* was analysed.

It was decided that a conventional tritiated thymidine-based *in vitro* suppression assay could not be used due to the spontaneous proliferation of *Ctla-4*-deficient Tregs observed in chapter 3. Instead a flow cytometry-based assay was developed in which congenically marked wild type CD4+ CD25- effector T cells (THY1.1+) were co-cultured with congenically marked wild type or *Ctla-4*-/- Tregs (THY1.2+) and flow cytometry was used to then quantify the absolute number of effector T cells after three days incubation. When Tregs were isolated from *Ctla-4* knockout mice using the cell surface markers CD4 and CD25 (CD4+ CD25+ lymphocytes), no suppression of effector T cell growth was observed (*Fig. 4.04 A*). However, as discussed in chapter 3, the use of CD27 as an additional cell surface marker to CD25 allows better discrimination of FOXP3+ cells in *Ctla-4*-/- mice. When Tregs were isolated from *Ctla-4* knockout mice using the additional cell surface marker CD27 (CD4+ CD25+ CD27hi lymphocytes), *Ctla-4*-deficient Tregs suppressed effector T cell


A
Percentage of Maximal Response (%)
100
75
50
25
0
0:1
0.5:1
1:1
'Suppressor' Cells : Effector Cells
WT CD4+ CD25+
Ctla-4-/- CD4+ CD25+


B
Percentage of Maximal Response (%)
100
75
50
25
0
0:1
0.5:1
1:1
'Suppressor' cells : Effector cells
WT CD4+ CD25+ CD27hi
Ctla-4-/- CD4+ CD25+ CD27hi


C
Absolute Number of Effector Cells per Well (x10^4)
8
7
6
5
4
3
2
1
0
0:1
0.5:1
1:1
'Suppressor' cells : Effector cells
WT CD4+ CD25+ CD27hi
Ctla-4-/- CD4+ CD25+ CD27hi

D

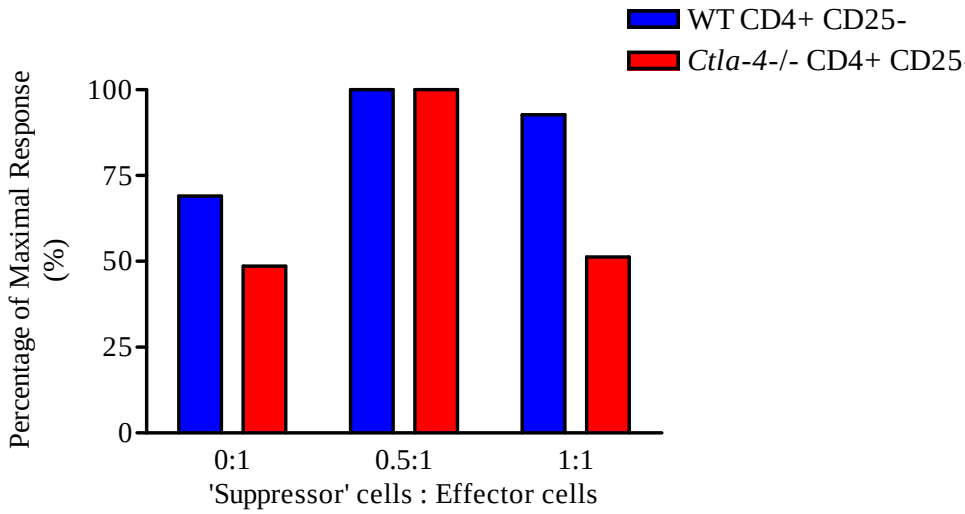

WT CD4+ CD25-
Ctla-4-/- CD4+ CD25-
Percentage of Maximal Response (%)
100
75
50
25
0
0:1
0.5:1
1:1
'Suppressor' cells : Effector cells

Figure 4.04. ***In vitro*** **analysis of** ***Ctla-4*****-deficient Treg suppressive function.** Single cell suspensions from BALB/c and *Ctla-4*-/- peripheral lymph nodes were mo flow sorted to obtain CD4+ CD25- Tconv and CD4+ CD25+ or CD4+ CD25+ CD27hi Tregs. 2.5 x 10^4 BALB/c Tconv were co-cultured in 96 well, U-bottomed plates with 15 x 10^4 MACS separated CD19+ from BALB/c spleen, 1µg/ml anti-CD3 and BALB/c or *Ctla-4*-/- Tregs at the indicated ratio for 3 days at 37°C, 5% CO_2. Cells were then surface stained with CD4, THY1.1 and intracellular for FOXP3 for analysis by flow cytometry. (A) Graph shows percentage of maximum THY1.1+ CD4+ FOXP3- effector T cell number following culture with indicated ratios of BALB/c or *Ctla-4*-/- CD4+ CD25+. (B) Graph shows percentage of maximum THY1.1+ CD4+ FOXP3- effector T cell number following culture with indicated ratios of BALB/c or *Ctla-4*-/- CD4+ CD25+ CD27hi Tregs. (C) Graph shows absolute number of THY1.1+ CD4+ FOXP3- effector T cells following culture with indicated ratios of BALB/c or *Ctla-4*-/- CD4+ CD25+ CD27hi Tregs. (D) Graph shows percentage of maximum THY1.1+ CD4+ FOXP3- effector T cell number following culture with indicated ratios of BALB/c or *Ctla-4*-/- CD4+ CD25- Tconv. Data shown is representative of four independent *in vitro* suppression assay experiments.

growth to a similar extent as wild type Tregs (*Fig. 4.04 B and C*). The difference in Treg suppressive function observed when CD27 was additionally used to isolate Tregs is likely to reflect the presence of activated (CD25+) CD4+ FOXP3- conventional T cells when Tregs were isolated solely using CD25.

As a control for the possibility that increasing the number of cells per well with the addition of Tregs suppresses effector T cell growth, CD4+ CD25- conventional T cells were added to the assay as mock 'suppressor' cells. *Fig. 4.04 D* shows that no suppression of effector T cell growth was observed in the presence of wild type or *Ctla-4* knockout CD4+ CD25- Tconv. Overall, Tregs from *Ctla-4* knockout mice exert suppressive function *in vitro* when isolated as a pure population free from contaminating, activated conventional T cells [241].

The *in vitro* analyses above suggest that Tregs lacking CTLA-4 can still function to suppress effector T cells. If Tregs in *Ctla-4*-deficient mice are functional, another possible explanation for the severe lymphoproliferative syndrome in CTLA-4 knockout mice is that the conventional T cells are resistant to Treg-mediated suppression. This hypothesis was tested by carrying out an *in vitro* suppression assays using CD4+ CD25- conventional T cells from *Ctla-4*-/- mice as effector cells (THY1.2+) and wild type BALB/c CD4+ CD25+ Tregs (THY1.1+). It was found that CD4+ CD25- conventional T cells from *Ctla-4*-deficient mice were susceptible to suppression by wild type CD4+ CD25+ Tregs *in vitro* (*Fig. 4.05*). This corroborates the *in vivo* data obtained in 4.2.1 where wild type Tregs protected *Rag2*-/- recipients from disease upon adoptive transfer of *Ctla-4*-/- lymphocytes. Overall, the *in vitro* suppression assays carried out suggest that Tregs from *Ctla-4*-deficient mice have suppressive function and conventional T cells from *Ctla-4*-/- mice are capable of being suppressed.

There is debate over whether regulatory T cells directly suppress effector T cells, or indirectly control them via modulation of antigen-presenting cells. Therefore, another potential explanation for the severe lymphoproliferative disease observed in *Ctla-4*-deficient mice is that the antigen-presenting cells in these mice are unable to support Treg-mediated suppression. To investigate Treg-mediated suppression of Tconv in the presence of antigen presenting cells from *Ctla-4* knockout mice, *in vitro* suppression assays were carried out. In

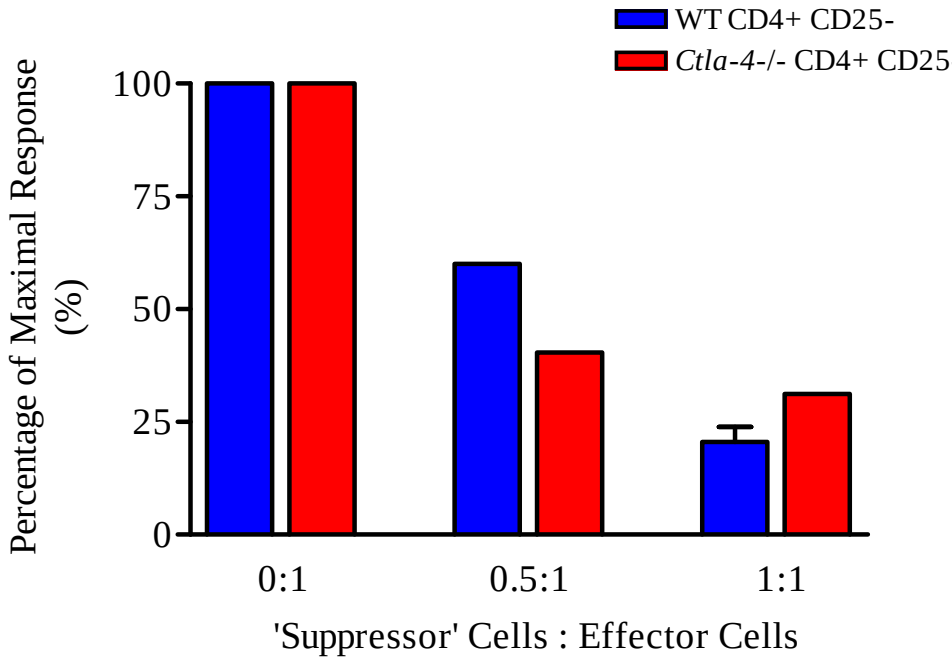


Figure 4.05. *In vitro* suppression of *Ctla-4*-deficient CD4+ CD25- Tconv. Single cell suspensions from BALB/c and *Ctla-4*-/- peripheral lymph nodes were MACS separated into CD4+ CD25- Tconv and CD4+ CD25+ Tregs. 2.5 x 10^4 BALB/c or *Ctla-4*-/- CD4+ CD25- Tconv were co-cultured in 96 well, U-bottomed plates with 15 x 10^4 MACS separated CD19+ from BALB/c spleen, 1µg/ml anti-CD3 and BALB/c CD4+ CD25+ Tregs at the indicated ratio for 3 days at 37°C, 5% CO_2. Cells were then surface stained with CD4, THY1.1, THY1.2 and intracellular for FOXP3 for analysis by flow cytometry. Graph shows percentage of maximum THY1.1+ or THY1.2+ CD4+ FOXP3- effector T cell number following culture with indicated ratio of BALB/c Tregs. Data shown is representative of two independent *in vitro* suppression assay experiments.

the presence of CD19+ B cells isolated from *Ctla-4*-/- mice, both wild type BALB/c and *Ctla-4*-deficient Tregs suppressed conventional T cell growth to the same extent (*Fig. 4.06*). In conclusion the data suggest that CD19+ B cells from *Ctla-4* knockout mice support *in vitro* Treg-mediated suppression of conventional T cells.

4.2.3 Effect of *Ctla-4*-deficiency on the generation and function of antigen-specific regulatory T cells.

It was found in 4.2.2 above that *Ctla-4*-deficient Tregs exert suppressive function *in vitro*. *In vitro* suppression assays are a useful tool for determining Treg suppressive activity, however; where possible, further insight can be obtained using *in vivo* mouse models to assess the suppressive function of Tregs. Our laboratory has developed an adoptive transfer mouse model of autoimmune diabetes. CD4+ antigen-specific (KJ-126+) CD25- conventional T cells from *DO11.10* TCR-transgenic *Rag2*-/- mice are adoptively transferred into *Rag2*-/- mice that express the membrane form of OVA (*DO11.10* antigen) under the control of the rat insulin promoter, and hence in the pancreatic islets (RIP-*mOVA*). This causes pancreatic islet destruction and development of overt diabetes with 100% penetrance. Co-transfer of antigen-specific (KJ-126+) CD4+ CD25+ regulatory T cells suppresses the induction of diabetes [53, 296]. As discussed in 3.2.5, in *DO11.10 Rag2*-/- mice all peripheral T cells are antigen-specific (KJ-126+) and FOXP3-negative. If *DO11.10 Rag2*-/- mice are crossed with RIP-*mOVA Rag2*-/- mice, antigen-specific (KJ-126+) CD4+ FOXP3+ Tregs are also generated in the thymus and exported to the periphery due to promiscuously expressed OVA antigen under the control of the autoimmune regulator AIRE in medullary thymic epithelial cells [32, 52, 242, 297] (*Fig. 3.21*). Therefore, antigen-specific Tregs can be generated by crossing *DO11.10 Rag2*-/- mice with RIP-*mOVA Rag2*-/- mice. By also introducing mice deficient in *Ctla-4* to the breeding colonies, antigen-specific *Ctla-4*-/- Tregs can be obtained.

To test whether *Ctla-4*-deficiency altered the frequency of antigen-specific FOXP3+ Tregs generated in the thymus, flow cytometry was used to analyse thymocyte FOXP3 expression. *Fig. 4.07 A* shows that no CD4+ KJ-126+ FOXP3+ Tregs were present in the thymus or periphery of wild type or *Ctla-4*-/- *DO11.10 Rag2*-/- mice. In *DO11.10* RIP-*mOVA Rag2*-/- mice CD4+ KJ-126+ FOXP3+ Tregs were present in the thymus and periphery. Introduction

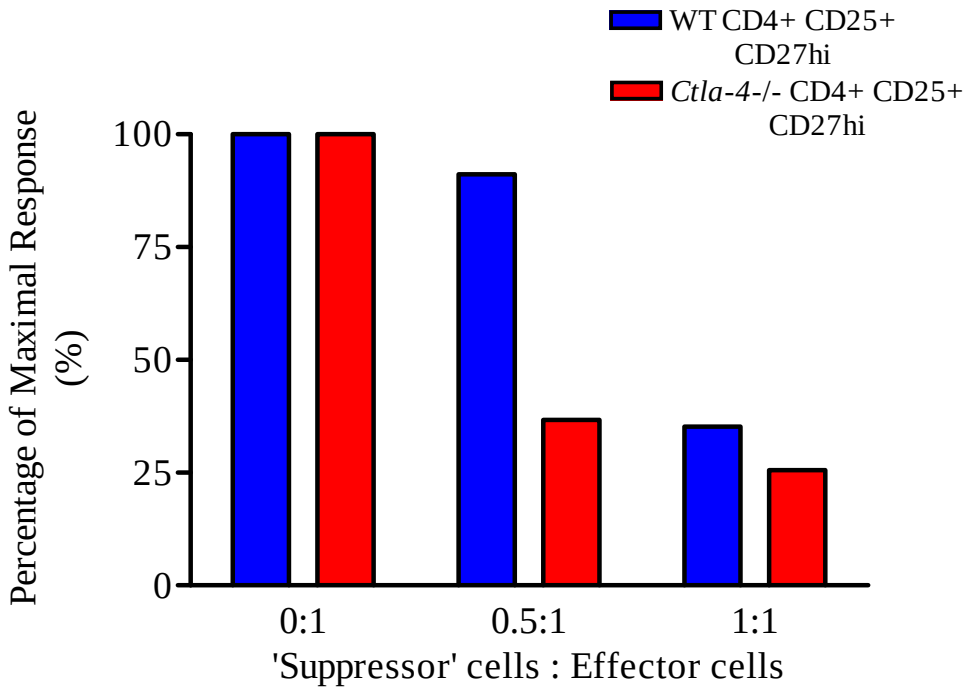
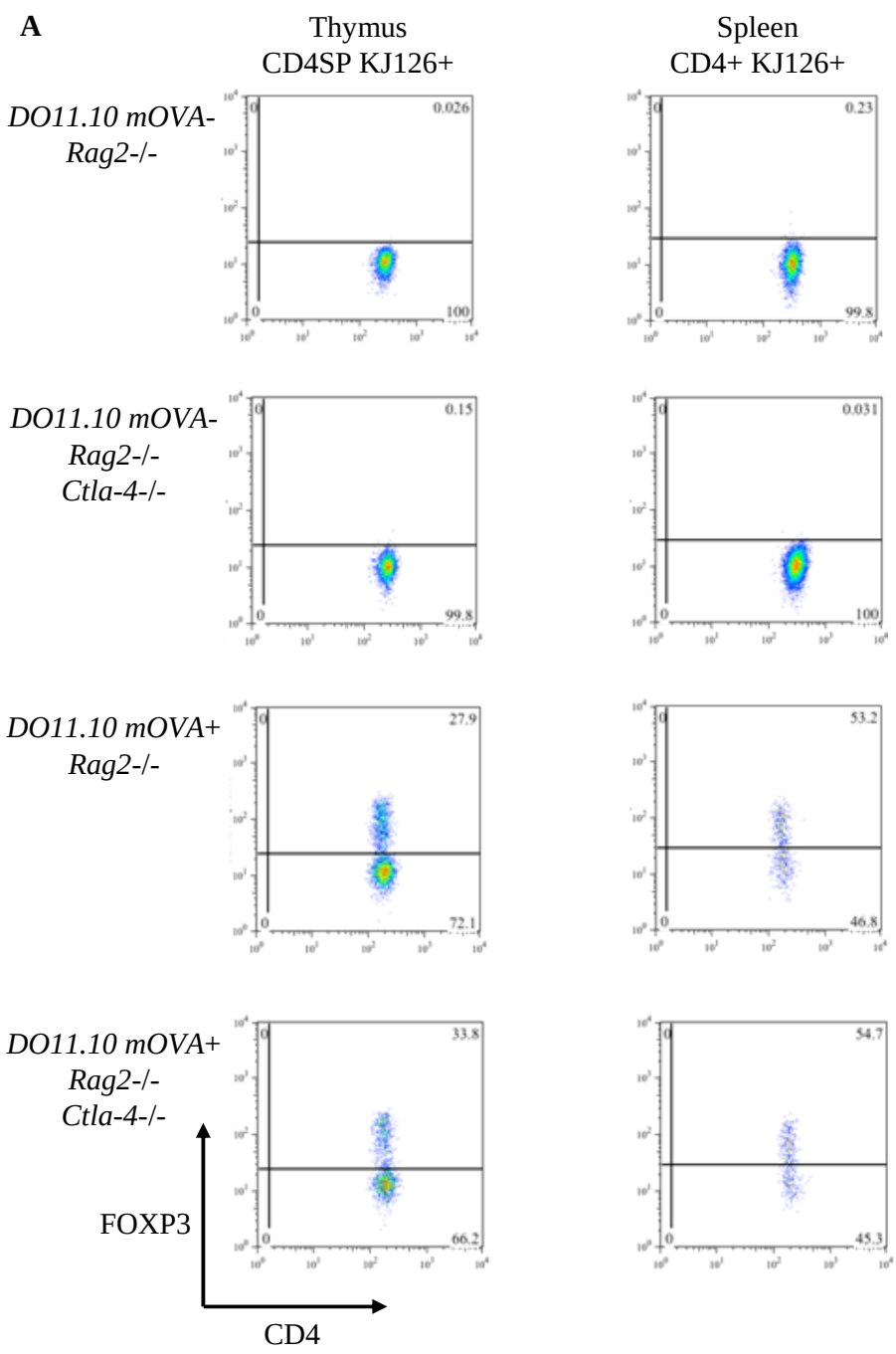


Figure 4.06. CD19+ B cells from *Ctla-4*-/- mice support *in vitro* suppression. Single cell suspensions from BALB/c and *Ctla-4*-/- peripheral lymph nodes were mo-flow sorted into CD4+ CD25- Tconv and CD4+ CD25+ CD27hi Tregs. 2.5 x 10^4 BALB/c Tconv were co-cultured in 96 well, U-bottomed plates with 15 x 10^4 MACS separated CD19+ from *Ctla-4*-/- spleen, 1µg/ml anti-CD3 and BALB/c or *Ctla-4*-/- Tregs at the indicated ratios for 3 days at 37°C, 5% CO_2. Cells were then surface stained with CD4, THY1.1 and intracellular for FOXP3 for analysis by flow cytometry. Graph shows percentage of maximum THY1.1+ or THY1.2+ CD4+ FOXP3- effector T cell number following culture with indicated ratio of BALB/c or *Ctla-4*-/- Tregs. Data shown is representative of two independent *in vitro* suppression assay experiments.


A
Thymus
CD4SP KJ126+
Spleen
CD4+ KJ126+
DO11.10 mOVA-
Rag2-/-
0.026
100
0.23
99.8
DO11.10 mOVA-
Rag2-/-
Ctla-4-/-
0.15
99.8
0.031
100
DO11.10 mOVA+
Rag2-/-
27.9
72.1
53.2
46.8
DO11.10 mOVA+
Rag2-/-
Ctla-4-/-
33.8
66.2
54.7
45.3
FOXP3
CD4

B

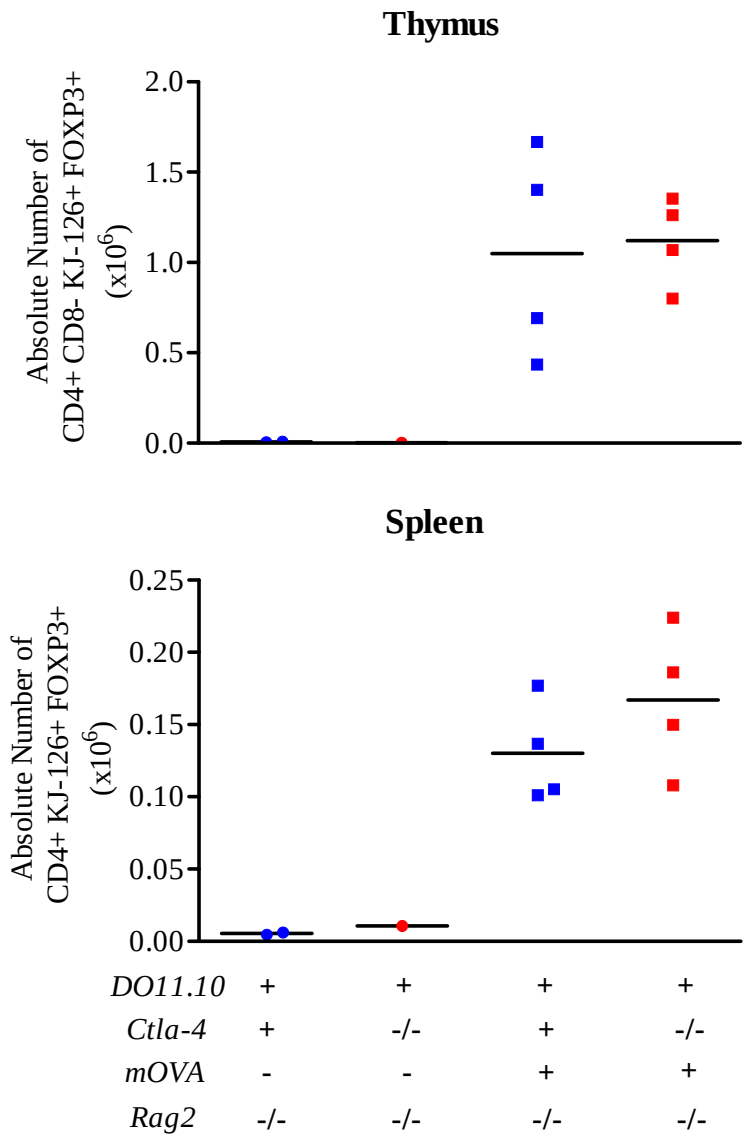
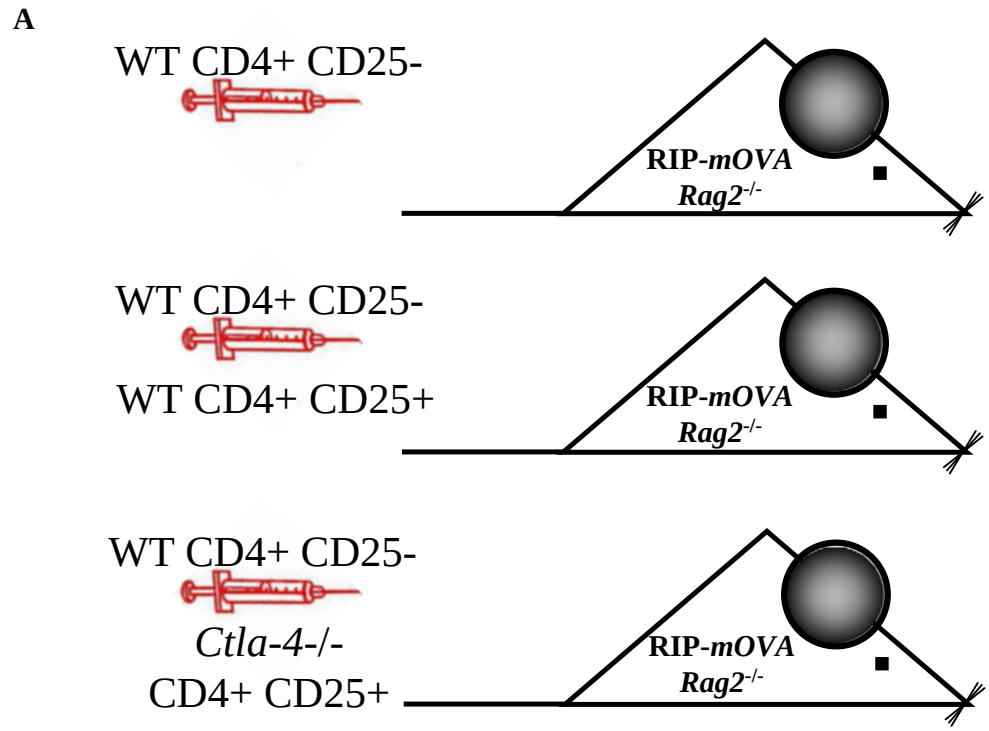

Thymus
Absolute Number of CD4+ CD8- KJ-126+ FOXP3+ (x10^6)
2.0
1.5
1.0
0.5
0.0
Spleen
Absolute Number of CD4+ KJ-126+ FOXP3+ (x10^6)
0.25
0.20
0.15
0.10
0.05
0.00
DO11.10 + + + +
Ctla-4 + -/- + -/-
mOVA - - + +
Rag2 -/- -/- -/- -/-

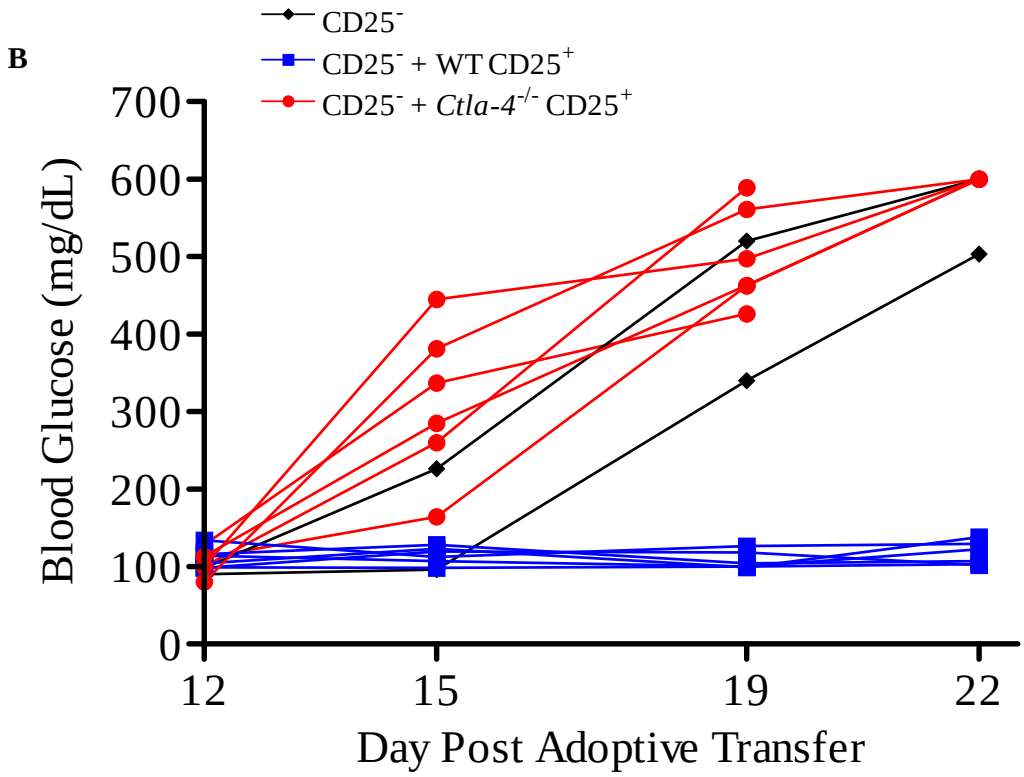
Figure 4.07. CD4+ FOXP3+ Tregs in TCR-Tg mice. Single cell suspensions from thymus and spleen were surface stained with CD4, KJ-126 and intracellular with FOXP3 for analysis by flow cytometry. (A) Representative FACS plots of antigen-specific (KJ-126+) CD4SP FOXP3+ Tregs in thymus (left) and CD4+ FOXP3+ Tregs in spleen for wild type and *Ctla-4-/- DO11.10 mOVA*- and *DO11.10 mOVA*+ mice. All FACS plots CD4+ KJ-126+ gated lymphocytes. (B) Graphs show absolute number of CD4+ CD8- KJ-126+ FOXP3+ (thymus) or CD4+ KJ-126+ FOXP3+ (spleen) in 6 week old mice of the indicated genotype. Experiments carried out in collaboration with C. Wang.

of *Ctla-4*-deficiency did not alter the absolute number of CD4+ KJ-126+ FOXP3+ Tregs generated in the thymus or then present in the periphery (*Fig. 4.07 B*).

Introducing the *Ctla-4*-deficiency to the TCR-transgenic mice did not alter the generation of antigen-specific regulatory T cells. Therefore, the adoptive transfer mouse model of diabetes described above was used to investigate the suppressive function of antigen-specific *Ctla-4*-deficient Tregs generated in a setting not complicated by lymphoproliferation. Wild type antigen-specific (KJ-126+) CD4+ CD25- conventional T cells were adoptively transferred into RIP-*mOVA Rag2*-/- mice either alone or co-transferred with wild type or *Ctla-4*-/- antigen-specific (KJ-126+) CD4+ CD25+ regulatory T cells (illustrated in *Fig. 4.08 A*). Diabetes progression was monitored by regularly obtaining blood glucose readings. The graph of *Fig. 4.08 B* shows that adoptive transfer of CD4+ CD25- Tconv alone (black lines) led to high blood glucose readings greater than 250mg/dL by 22 days post transfer. Consistent blood glucose readings of greater than 250mg/dL are indicative of overt diabetes. Co-transfer of wild type CD4+ CD25+ Tregs maintained low, normal blood glucose readings around 100-150mg/dL and suppressed the development of diabetes (blue lines of graph in *Fig. 4.08 B*). On the other hand, co-transfer of *Ctla-4*-deficient CD4+ CD25+ Tregs (red lines of graph in *Fig. 4.08 B*) did not maintain low blood glucose readings, and the blood glucose increased to give readings of greater than 250mg/dL by 22 days post transfer in a similar manner to the adoptive transfer of CD4+ CD25- Tconv alone. Overall, *Ctla-4*-deficient Tregs did not control the induction of diabetes in an adoptive transfer mouse model of diabetes as measured by blood glucose readings [241].

One possible explanation for the difference in blood glucose readings observed upon co-transfer of wild type or *Ctla-4*-/- Tregs is that the *Ctla-4*-deficient Tregs may not have survived as well as wild type Tregs after transfer or trafficked appropriately to the antigen-expressing tissue (pancreas) and draining lymph node. To investigate this hypothesis, when the mice were sacrificed, the presence of CD4+ FOXP3+ Tregs in the pancreas and draining pancreatic lymph node was analysed by flow cytometry. In mice that received *Ctla-4*-/- Tregs, CD4+ FOXP3+ Tregs could be detected in the pancreas and pancreatic lymph node as in mice that received wild type Tregs (*Fig. 4.08 C*). In conclusion, no difference was


A
WT CD4+ CD25-
RIP-mOVA
Rag2-/-
WT CD4+ CD25-
WT CD4+ CD25+
RIP-mOVA
Rag2-/-
WT CD4+ CD25-
Ctla-4-/-
CD4+ CD25+
RIP-mOVA
Rag2-/-


B
CD25-
CD25- + WT CD25+
CD25- + Ctla-4-/- CD25+
Blood Glucose (mg/dL)
700
600
500
400
300
200
100
0
12
15
19
22
Day Post Adoptive Transfer

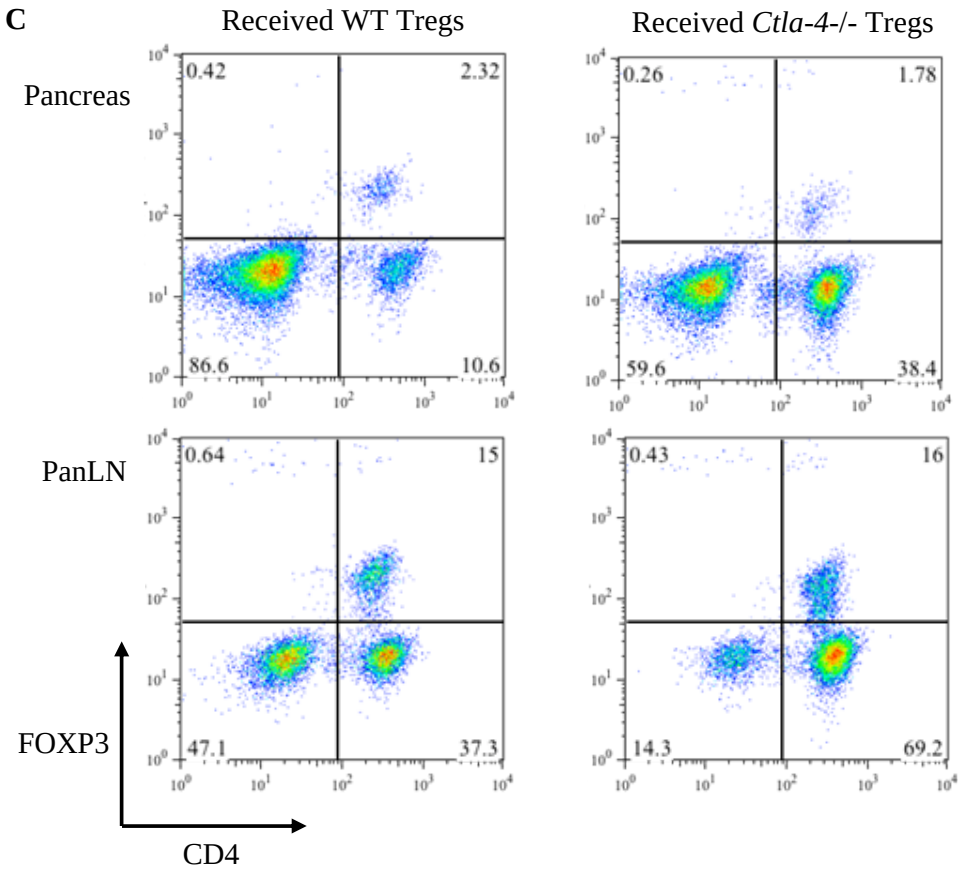

C
Received WT Tregs
Received Ctla-4-/- Tregs
Pancreas
PanLN
FOXP3
CD4
0.42
2.32
86.6
10.6
0.26
1.78
59.6
38.4
0.64
15
47.1
37.3
0.43
16
14.3
69.2

Figure 4.08. Antigen-specific Tregs deficient in *Ctla-4* lack regulatory function *in vivo*. (A) Single cell suspensions from peripheral lymph nodes of wild type and *Ctla-4*-/- *DO11.10 mOVA*+ *Rag2*-/- mice were mo-flow sorted into CD4+ CD25- Tconv and CD4+ CD25+ Tregs. WT CD4+ CD25- were adoptively transferred into RIP-*mOVA Rag2*-/- mice (0.2 x 10^6 cells) alone or plus 0.2 x 10^6 WT or *Ctla-4*-/- CD4+ CD25+. (B) Blood glucose was monitored throughout. Mice were harvested 3 weeks following transfer when recipients of WT CD4+ CD25- alone or plus *Ctla-4*-/- CD4+ CD25+ gave blood gluocose readings of 600mg/dL according to home office regulations. (C) Single cell suspension from pancreas (top) and pancreatic lymph node (bottom) of recipient mice were surface stained with CD4 and intracellular with FOXP3 for analysis by flow cytometry. Representative FACS plots are shown for recipients of WT (left) and *Ctla-4*-/- (right) CD4+ CD25+ Tregs. Experiments carried out in collaboration with C. Wang.

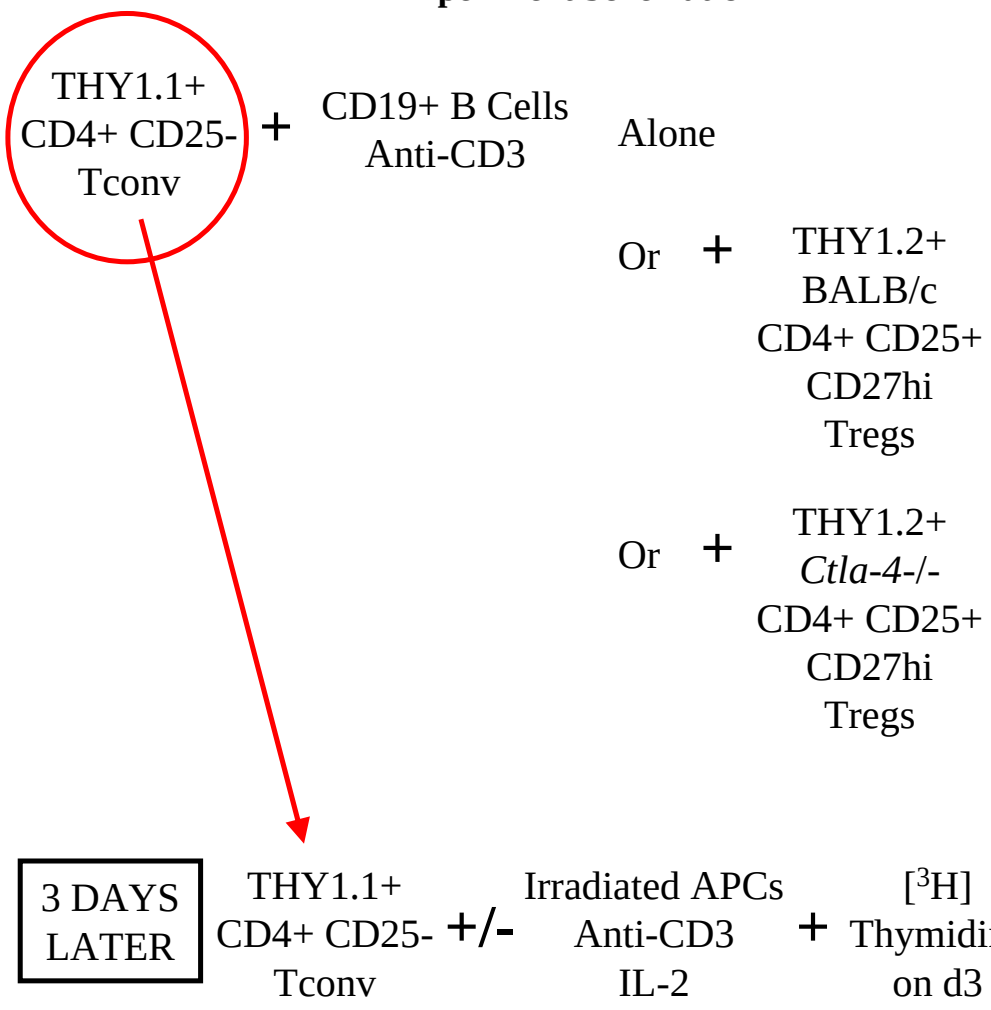
observed in the survival and trafficking to the antigen-expressing tissue or draining lymph node by wild type and *Ctla-4*-/- Tregs following adoptive transfer [241].

4.2.4 Induction of anergy in CD4+ CD25- conventional T cells following co-culture with *Ctla-4*-deficient Tregs.

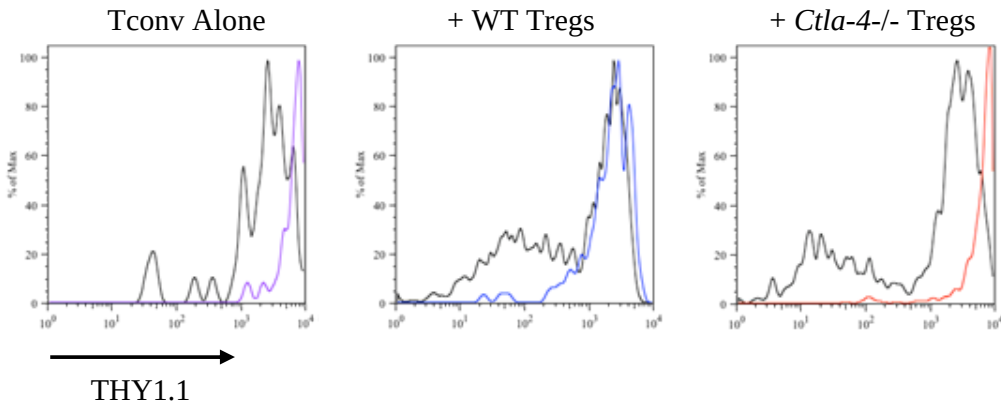
The *in vivo* data obtained in 4.2.3 suggests that CTLA-4 is required by regulatory T cells in the suppression of pancreatic islet autoimmunity. Although, in 4.2.2 Ctla-*4*-deficient Tregs were found to exert suppressive function *in vitro*. This raises the possibility that there may be redundancy in the manner in which regulatory T cells suppress conventional T cells. Maybe *Ctla-4*-/- Tregs use a different mechanism to compensate for the lack of CTLA-4. Many mechanisms have been proposed for how the CTLA-4 receptor functions in conventional T cells. The CTLA-4 receptor binds the same ligands CD80 and CD86 as the CD28 co-stimulatory receptor. Many hypotheses for the effector function of CTLA-4 on Tregs involve the prevention of ligand binding to CD28 thereby interrupting CD28-mediated co-stimulation. This could act as a mechanism of conventional T cell regulation as upon encounter with antigen via the TCR a second co-stimulatory signal through the CD28 receptor is thought to be required for full T cell activation [36, 298, 299]. Furthermore, T cell stimulation via TCR binding of antigen in the absence of the CD28 co-stimulatory signal has been reported to induce an anergic state of unresponsiveness in the conventional T cell [35-38].

To assess the hypothesis that the CTLA-4 receptor functions on Tregs by preventing CD28-mediated co-stimulation of conventional T cells thereby inducing a state of anergy; congenically marked (THY1.1+) CD4+ CD25- Tconv were co-cultured with wild type or *Ctla-4*-deficient Tregs in the presence of TCR-stimulation provided by soluble anti-CD3 and CD80/CD86-expressing CD19+ B cells, then re-isolated using the congenic marker and proliferation measured by tritiated thymidine incorporation in response to re-stimulation with irradiated splenocytes and soluble anti-CD3 plus or minus recombinant IL-2 (*Fig. 4.09 A*). With reference to *Fig. 4.09 C*, re-stimulation of Tconv, previously cultured without any Tregs, with anti-CD3 and antigen presenting cells induced proliferation (top graph, second bar from left) which was augmented with the addition of IL-2. Whereas, re-stimulation of Tconv, previously cultured with wild type Tregs, with anti-CD3 and antigen presenting cells did not

A **Experiment Schematic**



B



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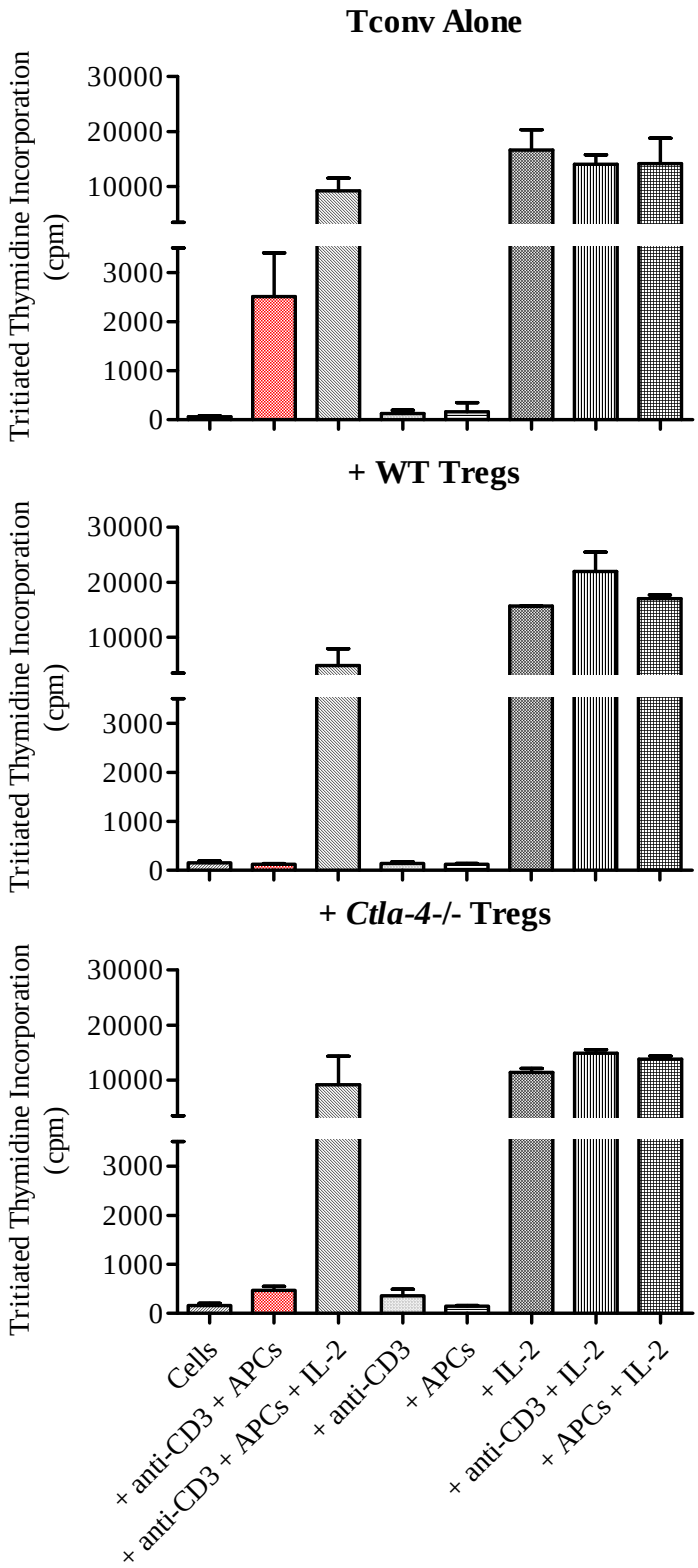
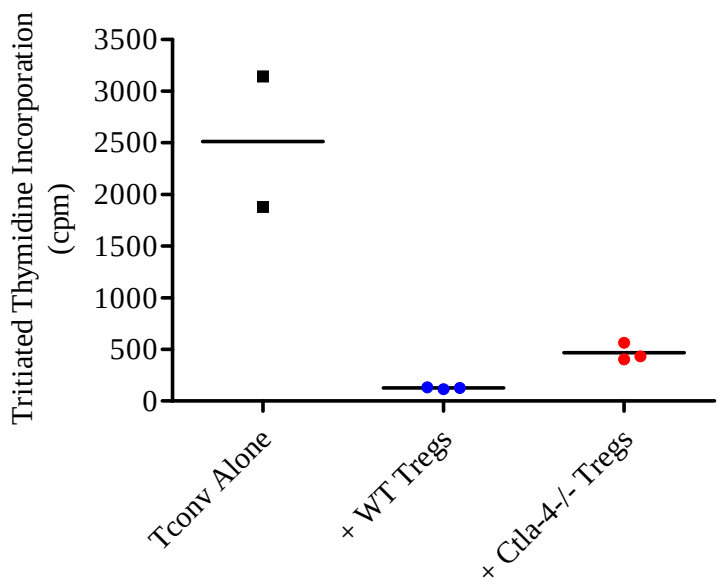

Tconv Alone
+ WT Tregs
+ *Ctla-4*-/- Tregs
Tritiated Thymidine Incorporation (cpm)
30000
20000
10000
3000
2000
1000
0
Cells
+ anti-CD3 + APCs
+ anti-CD3 + APCs + IL-2
+ anti-CD3
+ APCs
+ IL-2
+ anti-CD3 + IL-2
+ APCs + IL-2

D


Tritiated Thymidine Incorporation (cpm)
3500
3000
2500
2000
1500
1000
500
0
Tconv Alone
+ WT Tregs
+ Ctla-4-/- Tregs

E

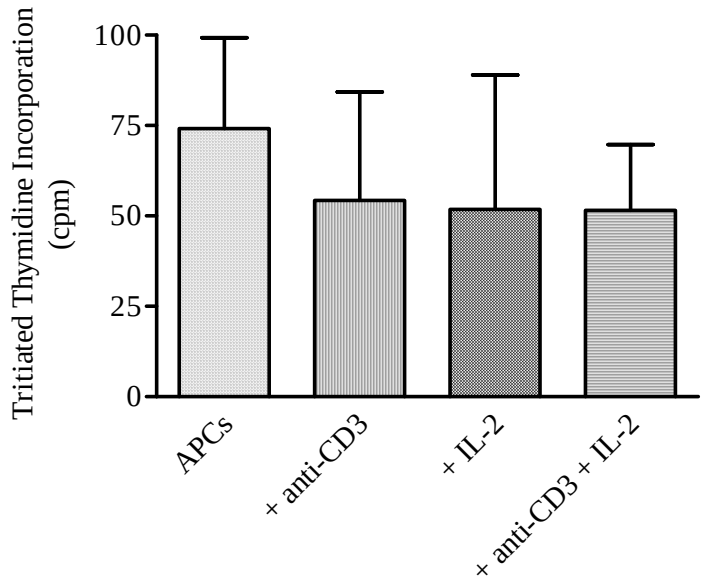

Tritiated Thymidine Incorporation (cpm)
100
75
50
25
0
APCs
+ anti-CD3
+ IL-2
+ anti-CD3 + IL-2

Figure 4.09. CD4+ CD25- Tconv co-cultured with *Ctla-4*-/- CD4+ CD25+ CD27hi Tregs are anergic upon re-stimulation. Single cell suspensions from THY1.1+ BALB/c peripheral lymph nodes were MACS separated to obtain CD4+ CD25- Tconv. Single cell suspensions from THY1.2+ BALB/c and THY1.2+ *Ctla-4*-/- peripheral lymph nodes were mo-flow sorted to obtain CD4+ CD25+ CD27hi Tregs. (A) 2.5 x 10^4 THY1.1+ BALB/c Tconv were co-cultured in 96 well, U-bottomed plates with 15 x 10^4 MACS separated CD19+ from BALB/c spleen, 1µg/ml anti-CD3 alone or plus THY1.2+ BALB/c or THY1.2+ *Ctla-4*-/- Tregs at a 1:1 ratio for 3 days. THY1.1+ CD4+ CD25- Tconv were then re-isolated by MACS separation and 2.5 x10^4 cells cultured in 96 well, U-bottomed plates with 1 x 10^5 irradiated BALB/c splenocytes plus or minus 1µg/ml anti-CD3 and 100I.U. rIL-2 for 3 days. Tritiated thymidine was added for the last 6hours of culture. (B) Co-cultured cells were surface stained with CD4 and THY1.1 for analysis by flow cytometry. Histograms show CD4+ THY1.1 purity before (black line) and after (coloured line) MACS re-isolation. (C) Graphs show tritiated thymidine incorporation of THY1.1+ CD4+ CD25- Tconv that were previously cultured alone (top), with WT Tregs (middle) or with *Ctla-4*-/- Tregs (bottom). (D) Graph to show tritiated thymidine incorporation of THY1.1+ CD4+ CD25- Tconv re-stimulated with irradiated splenocytes and anti-CD3. (E) Graph shows tritiated thymidine incorporation by irradiated splenocytes (APCs) plus or minus anti-CD3 and IL-2. Data shown is representative of two independent *in vitro* assay experiments.

induce proliferation (middle graph, second bar from left). This putative anergic state was overcome by the addition of IL-2 as seen by the proliferation induced. Similarly, re-stimulation of Tconv, previously cultured with *Ctla-4*-/- Tregs, with anti-CD3 and antigen-presenting cells did not induce proliferation (bottom graph, second bar from left) and IL-2 overcame the anergy by induction of proliferation (summarised in *Fig. 4.09 D*). Culture of the re-isolated Tconv alone or re-stimulation with anti-CD3 or antigen-presenting cells alone did not induce proliferation in any of the Tconv populations. This suggests that reasonably pure Tconv populations were re-isolated after the initial co-culture as also indicated by flow cytometric analysis for the THY1.1 congenic marker following Tconv re-isolation (*Fig. 4.09 B*). Under all re-stimulation conditions, addition of exogenous IL-2 induced a similar amount of proliferation in all the Tconv populations. Additionally, *Fig. 4.09 E* shows that the contribution of the irradiated splenocytes to the tritiated thymidine cell counts was minimal plus or minus anti-CD3 and IL-2.

4.2.5 Modulation of CD80/CD86 expression on antigen-presenting cells by CTLA-4 on Tregs.

The *in vivo* suppression of diabetes experiment in 4.2.3 revealed a clear role of CTLA-4 on regulatory T cells for suppression, at least in this disease setting. Even if there is redundancy in the Treg mode of action *in vitro*, the requirement of CTLA-4 by Tregs for suppression of pancreatic islet autoimmunity *in vivo* suggests that CTLA-4 nonetheless represents a key effector molecule in Treg suppressive function. The main mechanism that has been proposed as a cell-extrinsic effector function of CTLA-4 on regulatory T cells is the induction of indoleamine 2,3-dioxygenase (IDO) in dendritic cells (reviewed in [226]). However, not everyone agrees because functional evidence of the IDO pathway remains to be identified [300]. Colleagues within our institution (Dr. O. Qureshi and Dr. D. Sansom) have found evidence that CTLA-4 can remove its ligands from antigen-presenting cells in a trogocytosis-like manner. This could represent a mechanism of suppression by rendering APCs deficient in the co-stimulatory ligands needed to drive full T cell activation.

To investigate trogocytosis as a potential mechanism for CTLA-4 function, modulation of ligand (CD80 and CD86) expression on dendritic cell and B cell antigen-presenting cells by CTLA-4 on Tregs was explored. To assess modulation of dendritic cell CD80/CD86

expression by CTLA-4 on Tregs, CD11c+ dendritic cells were co-cultured with Tconv and/or Tregs plus or minus anti-CTLA-4 blocking antibody. CD86 expression by the CD11c+ dendritic cells was then analysed by flow cytometry. The representative histograms and graphs in *Fig. 4.10* show that the presence of Tregs in the co-culture down regulated CD86 expression by CD11c+ dendritic cells, and addition of anti-CTLA-4 blocking antibody abrogated this decrease in CD11c+ dendritic cell CD86 expression. By flow cytometry, expression of MHC class II by the CD11c+ dendritic cells was unaltered in all co-culture conditions. In conclusion, Tregs specifically down regulated CD86 expression by CD11c+ dendritic cells in a CTLA-4-dependent manner.

To investigate modulation of CD80/CD86 expression on antigen-presenting cells by CTLA-4 on Tregs *in vivo*, confocal microscopy was used to analyse CD86 expression by CD11c+ dendritic cells in the spleen of *Ctla-4* knockout mice. *Fig. 4.11 A* clearly shows that the intensity of CD86 expression in the spleen of *Ctla-4*-/- mice was higher than that in *Ctla-4*+/- littermate control spleens. From a number of fields of view of three *Ctla-4*-/- spleens and three *Ctla-4*+/- littermate control spleens, the median fluorescence intensity of CD86 expression by CD11c+ cells was quantified. It was found that the level of CD86 expression by CD11c+ cells in the spleens of *Ctla-4*-deficient mice was higher than that in *Ctla-4*+/- littermate controls spleens (*Fig. 4.11 B*).

To further analyse CD80/CD86 expression by antigen-presenting cells in *Ctla-4*-deficient mice flow cytometry was used. The representative histograms show that on both CD19+ B cells and CD3- CD11c+ MHC II+ dendritic cells the intensity of CD80 and CD86 expression was increased in *Ctla-4*-/- mice compared to *Ctla-4*+/- littermate controls (*Fig. 4.12 A*). Quantification of CD80 and CD86 median fluorescent intensity on CD19+ B cells (*Fig. 4.12 B*) and CD3- CD11c+ MHC II+ dendritic cells (*Fig. 4.12 C*) in a number of different mice found expression of both ligands was significantly increased in *Ctla-4*-/- mice compared to *Ctla-4*+/- littermate controls.

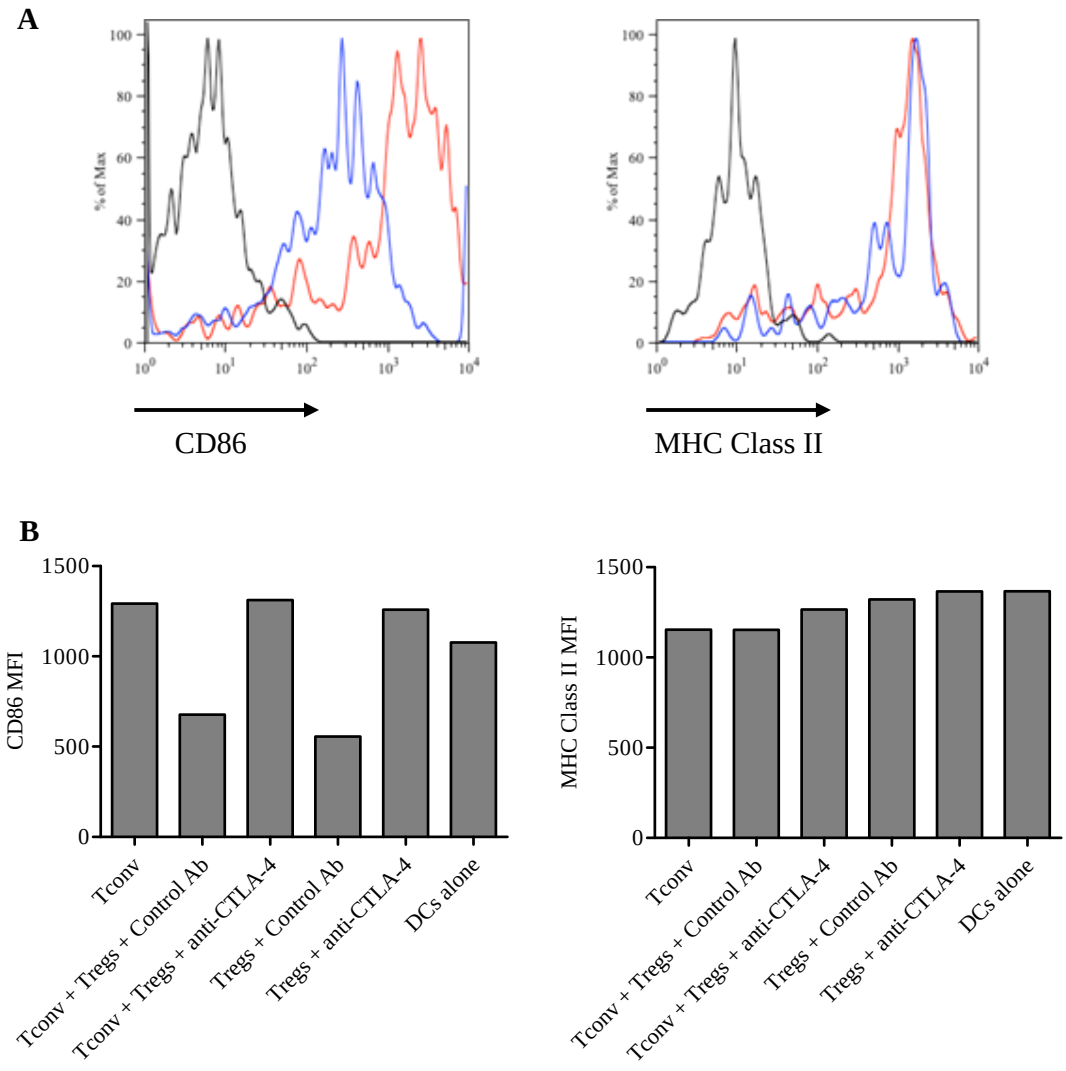
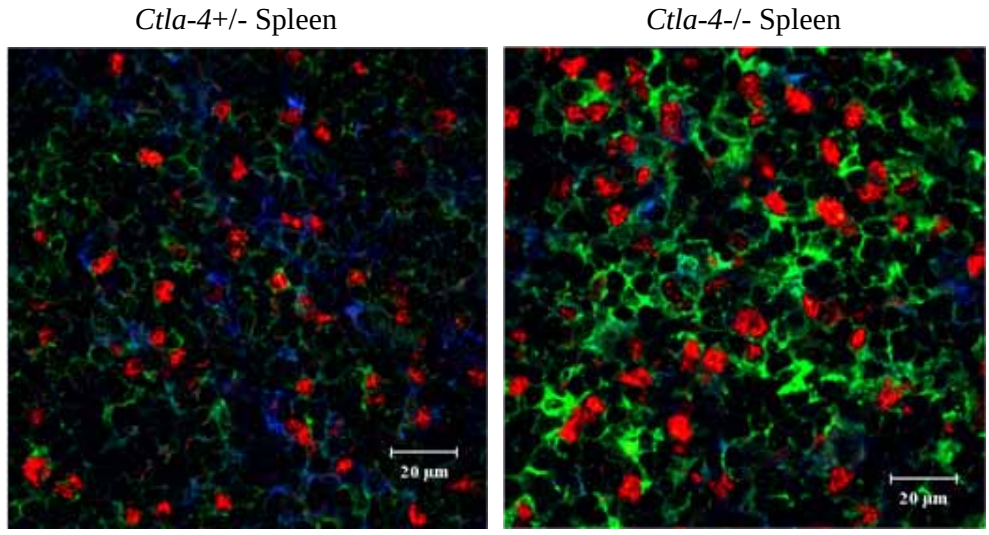


Figure 4.10. CTLA-4-dependent down-regulation of CD11c+ DC CD86 by Tregs. 4 x 10^4 MACS separated BALB/c splenic CD11c+ were cultured for 48hours with 8 x 104 CD4+ CD25- Tconv, CD4+ CD25+ Tregs or both plus or minus 50µg/ml anti-CTLA-4 blocking antibody (4F10). Cells were surface stained with CD11c, CD86 and MHC Class II for analysis by flow cytometry. (A) Representative histograms of CD86 (left) and MHC II (right) expression by CD11c+ gated cells. Black line = isotype control, Blue line = Tconv plus Tregs and red line = Tconv alone. (B) Graphs of CD86 (left) and MHC II (right) median fluorescence intensity on CD11c+ gated cells. Data shown is representative of three independent *in vitro* assay experiments.

A


Ctla-4+/- Spleen
Ctla-4-/- Spleen
20 µm
20 µm

B

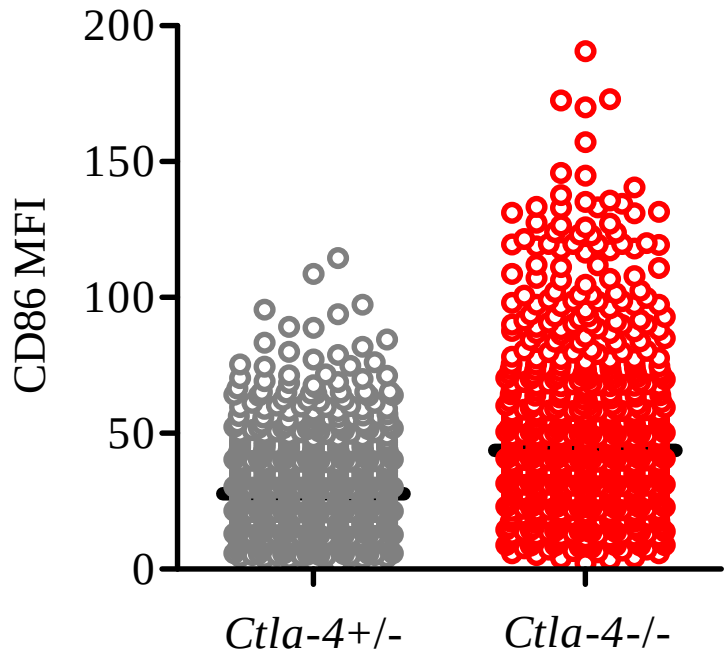
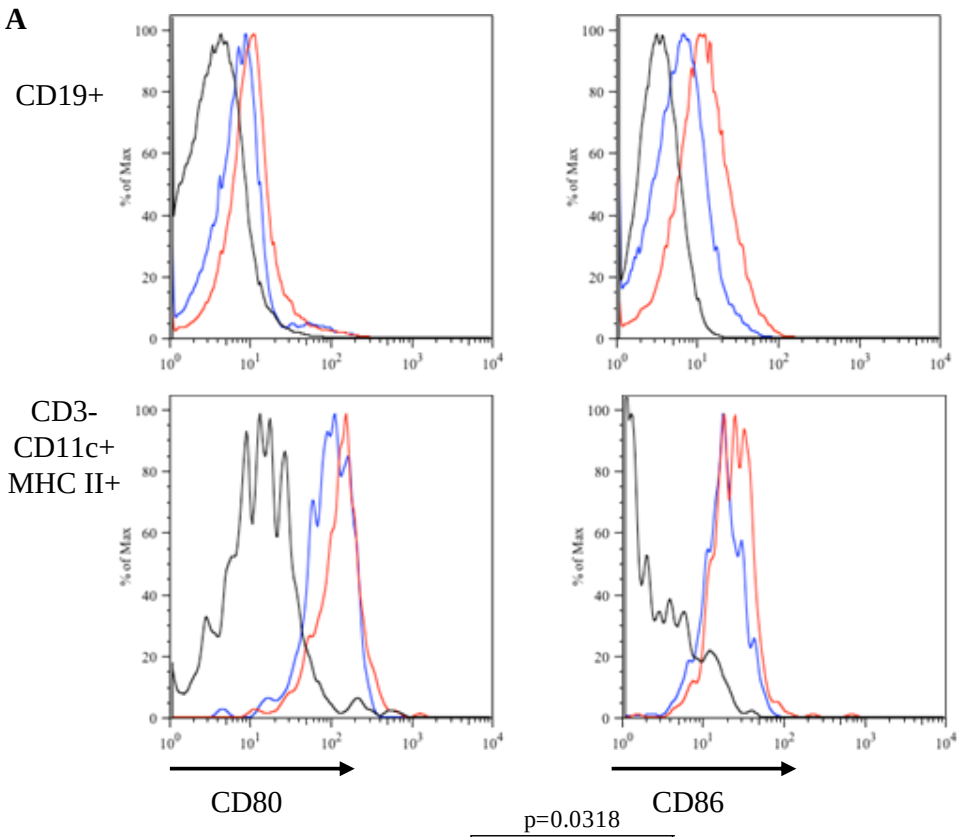
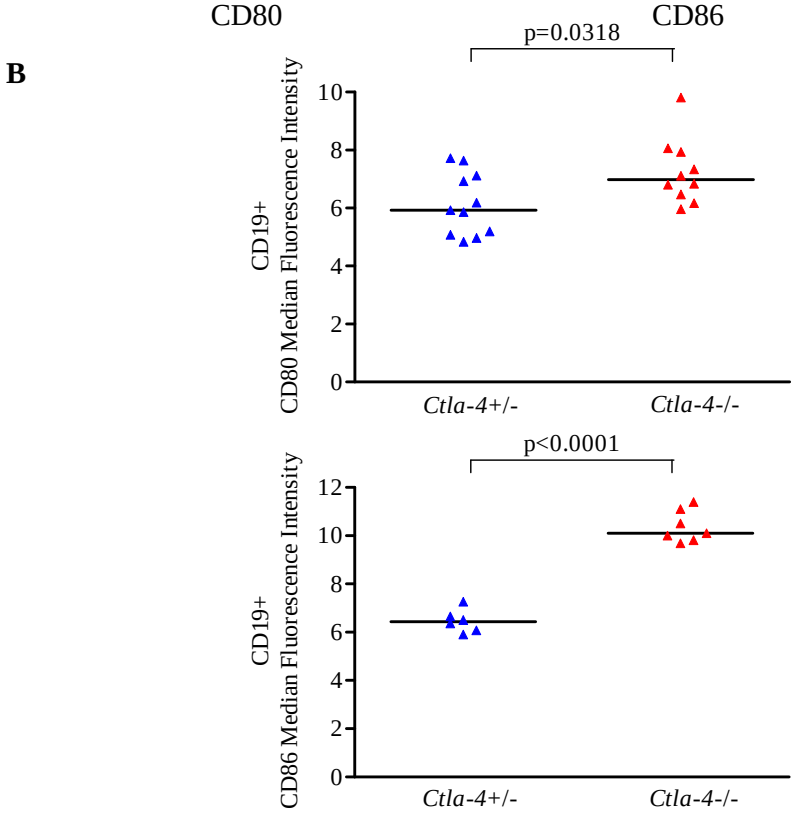

CD86 MFI
200
150
100
50
0
Ctla-4+/-
Ctla-4-/-

Figure 4.11. Comparison of CD86 expression by CD11c+ DCs in *Ctla-4*+/- and *Ctla-4*-/- spleen. Acetone fixed, 6µm spleen sections from 15-18 day old littermate *Ctla-4*+/- controls or *Ctla-4*-/- were stained with FOXP3 (red), CD11c (blue) and CD86 (green) for analysis by confocal microscopy (x63). (A) Representative images of *Ctla-4*+/- (left) and *Ctla-4*-/- (right) spleen. (B) Graph shows CD86 median fluorescence intensity of CD11c+ cells in *Ctla-4*+/- and *Ctla-4*-/- spleen. Each circle represents a different CD11c+ cell and lines indicate median. Data shown is representative of six fields of view from three tissue sections of three *Ctla-4*+/- spleens and three *Ctla-4*-/- spleens.


A
CD19+
CD3-
CD11c+
MHC II+
% of Max
CD80
CD86


B
p=0.0318
CD19+
CD80 Median Fluorescence Intensity
Ctla-4+/-
Ctla-4-/-
p<0.0001
CD19+
CD86 Median Fluorescence Intensity
Ctla-4+/-
Ctla-4-/-

C

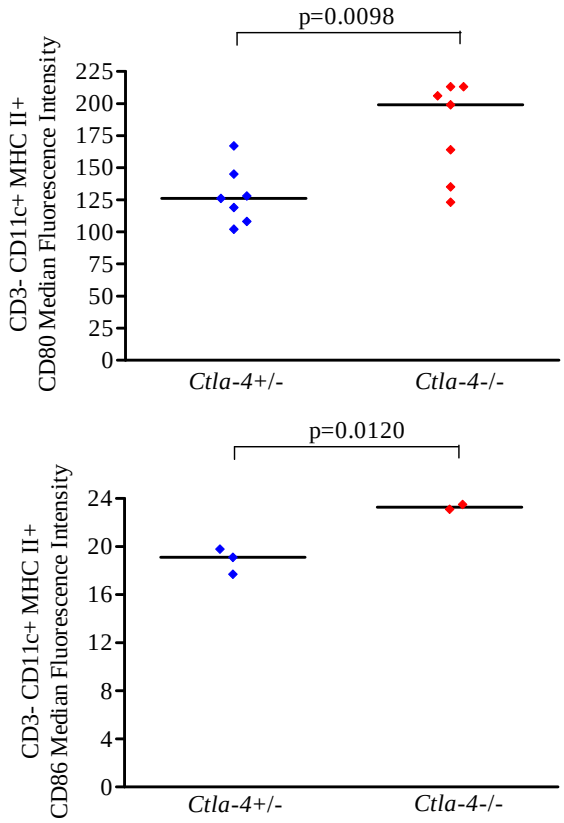

p=0.0098
CD3- CD11c+ MHC II+
CD80 Median Fluorescence Intensity
225
200
175
150
125
100
75
50
25
0
Ctla-4+/-
Ctla-4-/-
p=0.0120
CD3- CD11c+ MHC II+
CD86 Median Fluorescence Intensity
24
20
16
12
8
4
0
Ctla-4+/-
Ctla-4-/-

Figure 4.12. Analysis of CD80 and CD86 expression by CD19+ B Cells and CD3- CD11c+ MHC II+ DCs in *Ctla-4*+/- and *Ctla-4*-/- spleen. Single cell suspensions from *Ctla-4*+/- and *Ctla-4*-/- spleens were surface stained with CD3, CD19, CD11c, MHC Class II, CD80 and CD86 for analysis by flow cytometry. (A) Representative histograms show CD80 (left) and CD86 (right) expression by CD19+ B cells (top) and CD3- CD11c+ MHC II+ DCs (bottom) from *Ctla-4*+/- (blue) and *Ctla-4*-/- (red) mice. Isotype controls shown in black. Graphs show pooled data for CD80 and CD86 expression by CD19+ B cells (B) and CD3- CD11c+ MHC II+ DCs (C). Lines represent mean value. p values calculated by two-tailed, unpaired T-test for the means.

4.3 Discussion

Using *Ctla-4*-/- mice on the BALB/c genetic background, mixed bone marrow chimera experiments confirmed the cell-extrinsic protective role of CTLA-4 previously demonstrated by Bachmann *et al*. using *Ctla-4*-/- mice on the C57BL/6 genetic background [220]. Others have also previously found that radiation chimeras reconstituted with a mixture of *Ctla-4*+/+ and *Ctla-4*-/- bone marrow retained a normal phenotype when circumventing the experimental limitations associated with the fatal lymphoproliferative disease of *Ctla-4* knockout mice in order to study long term T cell immunity in the absence of CTLA-4 [221]. In this present study, two different *in vivo* adoptive transfer approaches found that CTLA-4-expressing, wild type CD4+ FOXP3+ regulatory T cells could control the lethal lymphoproliferative disease associated with *Ctla-4*-deficiency. These findings extend previous work carried out by Tivol and Gorski in which the adoptive transfer of *Ctla-4*-deficient splenocytes into *Rag2*-deficient mice resulted in fatal inflammation and tissue destruction similar to that seen in *Ctla-4*-deficient mice, whereas the co-transfer of an equivalent number of splenocytes from wild type animals allowed the recipient mice to survive indefinitely [301].

Overall, this suggests that the role of CTLA-4 in the maintenance of peripheral tolerance is more complex than the provision of an inhibitory signal into self-reactive CD4+ FOXP3- conventional T cells. Rather, the evidence indicates that CTLA-4 functions on CD4+ FOXP3+ regulatory T cells in the maintenance of peripheral tolerance. This is consistent with the recent demonstration by Friedline *et al*. using mixed stem cell chimeras and T cell transfer systems, that CTLA-4 was essential for Treg dominant control of a large pool of self-reactive T cells *in vivo* [302]. A role for CTLA-4 in Treg suppressive function would be consistent with the observation that CTLA-4 and FOXP3 need to be expressed on the same cell to provide optimal protection from the lethal lymphoproliferative disease associated with deficiency of either pathway [227].

It was found that Tregs from *Ctla-4* knockout mice exert suppressive function *in vitro* when isolated as a pure population free from contaminating, activated conventional T cells. Alternatively, within the *Ctla-4*-deficient mice, the self-reactive CD4+ FOXP3- conventional T cells could be resistant to suppression. Our laboratory has previously found that in a mouse

model of spontaneous autoimmune diabetes, over time the ability of Tregs to control autoimmunity is overcome by the acquisition of resistance to suppression within the auto-reactive, conventional T cell population [53]. Similar findings have been reported by others using non-obese diabetic (NOD) mice [303, 304]. Our laboratory found that resistance to Treg-mediated suppression was associated with the induction of IL-21 [53], furthermore, in chapter 3 of this present study, an increase in IL-21 mRNA was observed in conventional T cells from *Ctla-4*-deficient mice compared to wild type controls. However, it was also found that CD4+ CD25- conventional T cells from *Ctla-4*-deficient mice were susceptible to suppression by wild type CD4+ CD25+ Tregs *in vitro* that is consistent with the *in vivo* data obtained in which wild type Tregs protected *Rag2*-/- recipients from disease upon co-transfer with *Ctla-4*-/- lymphocytes.

The function of antigen-specific, *Ctla-4*-deficient regulatory T cells was assessed *in vivo* using an adoptive transfer mouse model of autoimmune diabetes. The generation of TCR-transgenic regulatory T cells lacking CTLA-4 allowed the role of this pathway in Treg suppressive function to be tested for the first time in a setting uncomplicated by lymphoproliferation or interrupted CD28-signalling. *Ctla-4*-deficient Tregs did not control the induction of diabetes in this adoptive transfer mouse model of diabetes as measured by blood glucose readings. It is likely that the requirement for CTLA-4 in Treg function varies depending on the disease setting because others have previously shown that *Ctla-4*-deficient Tregs were capable of preventing colitis induced by the transfer of CD4+ CD45RBhi cells into *Rag*-deficient recipients [232]. Interestingly, suppression of colitis by *Ctla-4*-/- Tregs, but not wild type Tregs, in this setting was highly dependent on IL-10. This suggests that pathological responses that can be controlled by IL-10 may not require expression of CTLA-4 by regulatory T cells. Consistent with this hypothesis, it has been shown that mice lacking IL-10 specifically in Tregs develop colitis but do not exhibit lymphoproliferation or systemic autoimmunity [305]. This implicates IL-10 as an important effector molecule for Treg function within the colon, but suggests that other effector mechanisms such as CTLA-4 could be essential for Treg function at other tissue sites. Consistent with this, knockdown of CTLA-4 by RNA interference triggers focused autoimmunity of the pancreas [306].

Consistent with a role for CTLA-4 in the negative regulation of the CD4+ T cell compartment, indicated by the lethal lymphoproliferative disease of *Ctla-4*-deficient mice, a requirement for CTLA-4 in Treg suppressive function *in vivo* was identified using an adoptive transfer mouse model of autoimmune diabetes. In line with this, Wing *et al*. recently generated *Ctla-4* conditional knockout mice in which *Ctla-4* was specifically deleted in CD4+ FOXP3+ T cells by crossing mice expressing Cre under the control of the *Foxp3* promoter with mice expressing a floxed *Ctla-4* gene. These *Ctla-4* conditional knockout mice succumbed to a similar fatal lymphoproliferative syndrome as that seen in *Ctla-4*-deficient mice, furthermore Treg-specific *Ctla-4* deficiency was found to impair the suppressive function of Tregs *in vitro* and *in vivo* [307]. Although it has been hypothesised for some years that CTLA-4 has a role in regulatory T cell suppressive function, the mechanism(s) of action are not well defined and remain a subject of debate.

There is also debate over whether Tregs function to suppress by direct contact with effector T cells, or via interaction with antigen-presenting cells. Two-photon laser-scanning microscopy studies have shown *in situ* that Tregs directly interact with antigen-presenting cells rather than effector T cells in order to control T cell priming by dendritic cells [150]. This could be mediated by the constitutively expressed CTLA-4 in Tregs as its ligands CD80 and CD86 are expressed by antigen-presenting cells. However, the control of Treg aggregation around dendritic cells has been found to critically depend on LFA-1 rather than CTLA-4 [308]. On the other hand, preferential affinity of CD80 and CD86 ligand binding of CTLA-4 compared with CD28 [158] has led to a ligand competition model of CTLA-4 function on Tregs. Tregs with constitutive expression of CTLA-4 could preferentially bind CD80 and CD86 ligands on antigen-presenting cells, thereby depriving effector T cells of CD28 co-stimulatory signals [185]. Furthermore, direct contact between Tregs and APCs has been shown to induce the down-modulation of co-stimulatory CD80 and CD86 molecules [159] which was then later found to be CTLA-4-dependent [160]. It has also been proposed that CTLA-4 on Tregs can alter APC function by inducing the production of the enzyme indoleamine 2,3-dioxygenase (IDO) through CD80/CD86 back-signalling [161, 162]. It has been suggested that as the enzyme IDO breaks down tryptophan, which is an essential amino acid for T cell proliferation, induction of active IDO would be dominantly immunosuppressive [163]. In addition to these Treg-extrinsic mechanisms of CTLA-4

function, a Treg-intrinsic role of CTLA-4 has recently been reported. Similar to the indicated role of CTLA-4 ligation dampening signalling pathways downstream of the TCR in conventional T cells [309], CTLA-4 was found to play an analogous role in altering Treg signalling responses [310].

Ligand competition and down-modulation of co-stimulatory molecules as hypotheses for the effector function of CTLA-4 on Tregs both involve the prevention of CD80/CD86 ligand binding to CD28 thereby interrupting CD28-mediated co-stimulation. This could act as a mechanism of conventional T cell regulation as upon encounter with antigen via the TCR a second co-stimulatory signal through the CD28 receptor is thought to be required for full T cell activation [36, 298, 299]. Furthermore, T cell stimulation via TCR binding of antigen in the absence of the CD28 co-stimulatory signal has been reported to induce an anergic state of unresponsiveness in the conventional T cell [35-38]. In this present study, *in vitro* assays found that effector T cells were anergic upon re-stimulation following co-culture with both wild type Tregs and *Ctla-4*-deficient Tregs. This experimental setup suggests that induction of an anergic state of unresponsiveness in conventional T cells following co-culture with regulatory T cells is not dependent upon CTLA-4 expression by the Treg population. Anergy is assessed as the inability to proliferate upon re-stimulation with TCR-stimulation and CD28 co-stimulation which is overcome with the addition of IL-2. This is difficult to analyse specifically as proliferation of the same T cells cannot be measured before and after addition of IL-2, therefore the IL-2 could be keeping cells alive rather than overcoming anergy in response to TCR-stimulation and CD28 co-stimulation. It has been reported by others that the induction of T cell anergy *in vivo* requires engagement of CTLA-4 on effector T cells to induce an abortive response [311].

Down-modulation of CD80/CD86 co-stimulatory molecules on APCs as an effector mechanism of Tregs was first hypothesised nearly ten years ago. Cederbom *et al.* demonstrated that in the presence of stimuli that would normally increase the expression of co-stimulatory molecules by dendritic cells, CD4+CD25+ T cells down-regulated the expression of CD80 and CD86 on dendritic cells *in vitro* [159]. This was later shown to be dependent upon CTLA-4 and furthermore the extent of down-modulation was functionally significant because Treg-cell conditioned dendritic cells induced poor T-cell proliferation

responses [160]. More recently, using conditional knockout mice, Wing *et al*. found that Treg-specific CTLA-4 deficiency impaired Treg-mediated down-regulation of CD80 and CD86 expression on dendritic cells [307]. As mentioned previously, colleagues within our institution (Dr. O. Qureshi and Dr. D. Sansom) have found evidence that CTLA-4 can remove its ligands from antigen-presenting cells in a trogocytosis-like manner. This could represent a mechanism of co-stimulatory molecule down-regulation by CTLA-4-expressing regulatory T cells.

Modulation of ligand (CD80 and CD86) expression on dendritic cell and B cell APCs by CTLA-4 on Tregs was explored in this investigation. In this study it was similarly found that following *in vitro* co-culture, Tregs specifically down regulated CD86 expression by CD11c+ dendritic cells and this was dependent upon CTLA-4. Consistent with this, confocal microscopy and flow cytometry analysis identified augmented CD80 and CD86 expression by CD11c+ dendritic cells and CD19+ B cells in *Ctla-4*-deficient mice. However, due to the lethal lymphoproliferative disease of *Ctla-4*-deficient mice the cellular environment is pro-inflammatory, therefore the increased expression of CD80 and CD86 by APCs observed could be due to cellular activation. In future it would be interesting to assess CD80 and CD86 expression by APCs in the adoptive transfer model of diabetes following co-transfer of wild type or *Ctla-4*-deficient Tregs. Removal of CD86 by CTLA-4 could then be assessed *in vivo* if RIP-*mOVA Rag2*-/- recipient mice were used that also expressed GFP-tagged CD86.

5.0 Role of Additional Accessory Pathways in Regulatory T Cell Homeostasis

5.1 Introduction

The data obtained in chapter 3 (role of CTLA-4 in regulatory T cell development and homeostasis) suggested that CTLA-4 plays a role in the homeostasis of peripheral regulatory T cells. However, it was unclear from the data obtained using *Ctla-4* knockout mice, with a severe lymphoproliferative syndrome, whether the altered Treg homeostasis observed was a direct cell-intrinsic result of the loss of CTLA-4 in the regulatory T cells, or in response to cell-extrinsic changes in the environment within the diseased mice.

To compliment the *Ctla-4* knockout mouse studies in chapter 3 and further dissect the mechanism by which CTLA-4 controls Treg homeostasis, anti-CTLA-4 blocking antibody *in vivo* experiments were carried out with *Ctla-4*-sufficient mice. The involvement of factors which are known to modulate regulatory T cell homeostasis, such as IL-2 signalling [57, 80], CD28 co-stimulation [131, 238, 295] and encounter with antigen [52] was investigated.

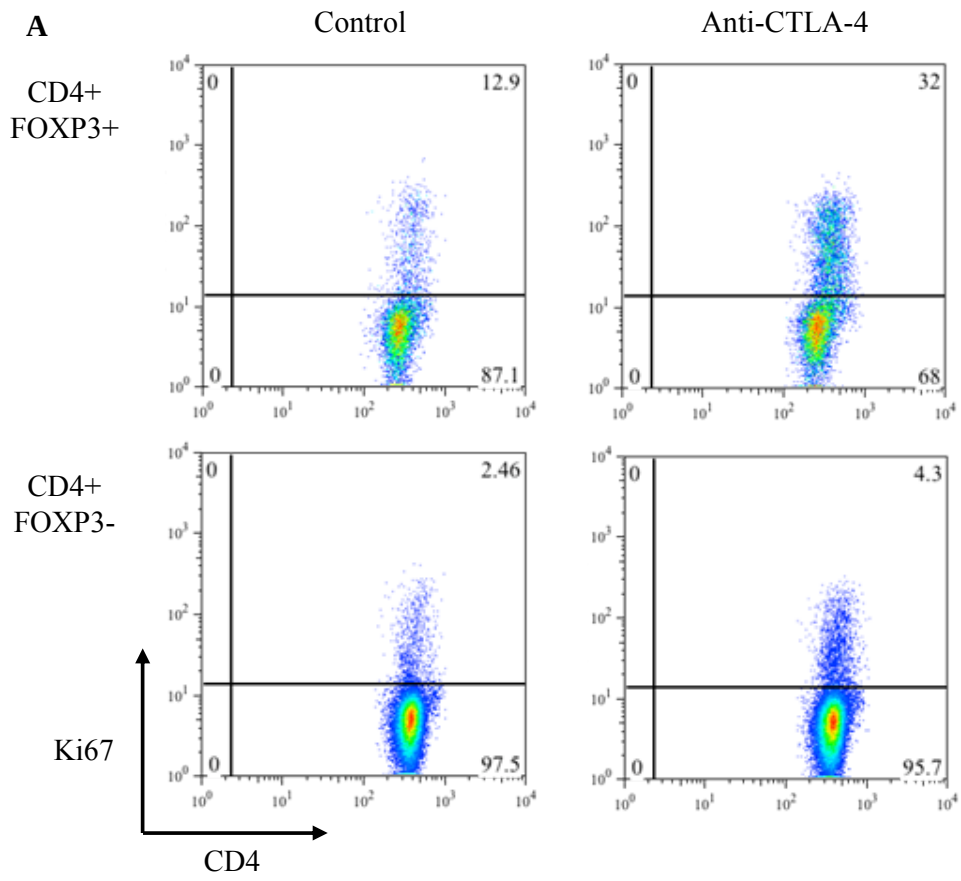
Additionally, accessory pathways such as those mediated by other CD28-superfamily members and tumor necrosis factor (TNF) receptors have been suggested to be functionally involved in the development and homeostasis of regulatory T cells [125, 312-316]. The involvement of the CD28-superfamily, secondary co-stimulatory molecule ICOS and TNF receptors OX40, CD30 and RANK, in the context of the role of the CTLA-4 pathway in Treg homeostasis, was studied both *in vitro* and *in vivo*.

5.2 Results

5.2.1 Mechanistic analysis of regulatory T cell proliferation resulting from a lack of CTLA-4.

In chapter 3, analysis of CD4+ FOXP3+ regulatory T cells in *Ctla-4*-deficient mice identified a potential role for the CTLA-4 receptor in the peripheral homeostasis of Tregs. Prior to the revival in the concept and existence of regulatory T cells, the CTLA-4 receptor had been found to function opposite the CD28 co-stimulatory receptor to negatively control T cell activation and proliferation [199, 200]. As it is now known CD4+ FOXP3+ regulatory T cells constitutively express CTLA-4 whereas CD4+ FOXP3- conventional T cells up regulate CTLA-4 only upon activation, the augmented Treg proliferation observed in *Ctla-4* knockout mice could be due to the cell-intrinsic loss of CTLA-4 in regulatory T cells. However, it is also possible that the lymphoproliferative environment within diseased *Ctla-4*-deficient mice alters peripheral CD4+ FOXP3+ Treg homeostasis in *Ctla-4* gene ablated mice. For example, high levels of cytokines such as IL-2 could be present which could potentially alter regulatory T cell proliferation.

To assess the loss of CTLA-4 signalling on CD4+ FOXP3+ Treg homeostasis in a healthy mouse, wild type BALB/c mice were treated with anti-CTLA-4 blocking antibody. On day eight, following twice weekly treatment with anti-CTLA-4 blocking antibody, CD4+ FOXP3+ Treg proliferation was assessed by flow cytometry analysis of Ki67 expression. Increased Ki67 expression by CD4+ FOXP3+ regulatory T cells was observed in mice treated with anti-CTLA-4 blocking antibody compared to controls. Expression of Ki67 by CD4+ FOXP3- conventional T cells was also increased, but proportionally not by as much as Ki67 expression by CD4+ FOXP3+ Tregs (*Fig. 5.01 A*). To explore whether it was possible to distinguish which T cell subset (CD4+ FOXP3- conventional T cells or CD4+ FOXP3+ regulatory T cells) responded to anti-CTLA-4 treatment, a time course analysis was undertaken. Three days following initiation of anti-CTLA-4 antibody-mediated blockade, CD4+ FOXP3+ Tregs showed a statistically significant increase in Ki67 expression ($p=0.0019$), whereas Ki67 expression by CD4+ FOXP3- Tconv at this time point was not significantly different from that in control treated mice ($p=0.3393$) (*Fig. 5.01 B*). The CD4+ FOXP3- Tconv population did show increased Ki67 expression following anti-CTLA


A
Control
Anti-CTLA-4
CD4+
FOXP3+
CD4+
FOXP3-
Ki67
CD4
12.9
87.1
32
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2.46
97.5
4.3
95.7

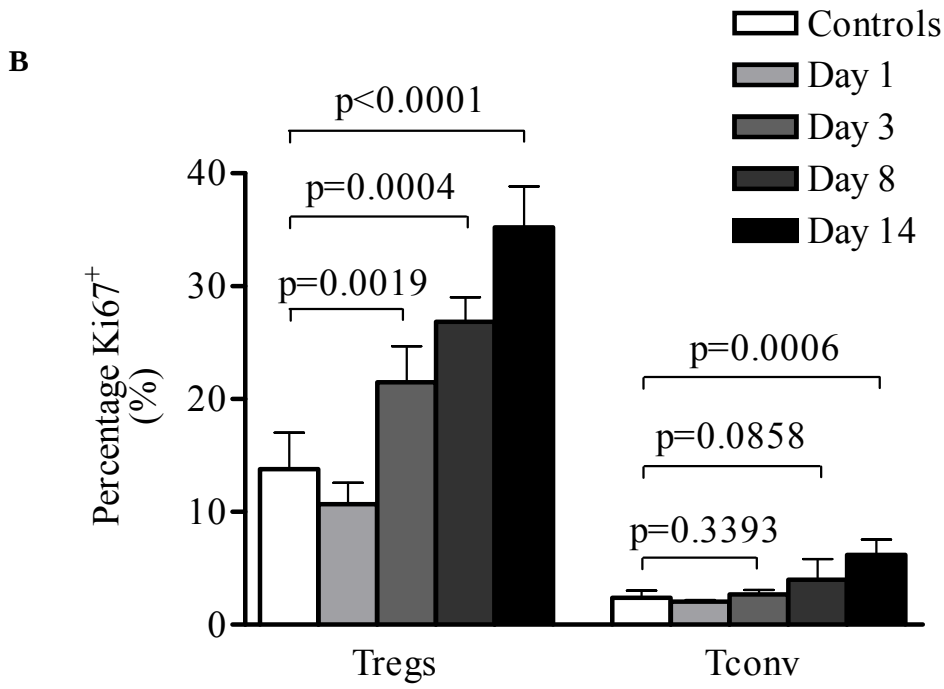

B
Controls
Day 1
Day 3
Day 8
Day 14
p<0.0001
p=0.0004
p=0.0019
p=0.0006
p=0.0858
p=0.3393
Percentage Ki67+ (%)
40
30
20
10
0
Tregs
Tconv

Figure 5.01. Antibody-mediated CTLA-4 blockade augments CD4+ FOXP3+ Treg proliferation *in vivo*. BALB/c mice received 500μg of anti-CTLA-4 blocking antibody (4F10) or control twice weekly by intra-peritoneal injection. Single cell suspensions from peripheral lymph nodes were surface stained with CD4 and intracellular with FOXP3 and Ki67 for analysis by flow cytometry. (A) Representative FACS plots of Ki67 expression by CD4+ FOXP3+ Tregs (top) and CD4+ FOXP- Tconv (bottom) from mice harvested on day 8. (B) Graph shows data for ≥3 mice harvested at each time point. Bars represent mean value and standard deviation is shown. p value calculated by two-tailed, unpaired T-test for the means.

blocking antibody treatment; however this manifested later than for the CD4+ FOXP3+ Treg population. The graph of *Fig. 5.01 B* shows that by day eight there was a trend toward slightly increased Ki67 expression by CD4+ FOXP3- cells in mice that had received anti-CTLA-4 blocking antibody, but this trend was not significant until day fourteen ($p=0.0006$).

Although the proliferation of conventional T cells was not altered at early time points by anti-CTLA-4 blocking antibody treatment, it remained possible that treatment induced a non-proliferative change in these cells that indirectly altered regulatory T cell homeostasis. In this regard it is known that upon activation, CD4+ FOXP3- conventional T cells produce the T cell growth factor cytokine IL-2 [317]. IL-2 has been found to overcome Treg anergy *in vitro* [57] and IL-2 signalling has since been identified as a critical requirement for Treg homeostasis *in vivo* [80]. Therefore, in both the *Ctla-4* gene ablated mice and wild type BALB/c mice treated with anti-CTLA-4 blocking antibody, the augmented proliferation of peripheral CD4+ FOXP3+ Tregs observed could be due to enhanced IL-2 production by CD4+ FOXP3- conventional T cells. On day three of the anti-CTLA-4 blocking antibody time course when CD4+ FOXP3+ Tregs, but not CD4+ FOXP3- Tconv, Ki67 expression was significantly increased, production of IL-2 by CD4+ non-Tregs was measured by intracellular cytokine staining and analysis by flow cytometry. IL-2 production by CD4+ non-Tregs was detected (*Fig. 5.02 A*) and this was unaltered following antibody-mediated CTLA-4 blockade (*Fig. 5.02 A and B*). The FACS plots of *Fig. 5.02 A* show that with and without anti-CTLA-4 blocking antibody treatment, CD4+ CD25+ CD27hi Tregs produced negligible IL-2. Overall, this data shows that antibody-mediated CTLA-4 blockade did not detectably affect IL-2 production by conventional T cells at day three of a time course of treatment, and therefore suggests that the augmented CD4+ FOXP3+ Treg proliferation observed was not dependent on enhanced IL-2 production by conventional T cells.

IL-2 is required to overcome the profound unresponsiveness regulatory T cells exhibit to TCR engagement *in vitro* [57], however it has previously been shown that Tregs proliferate in response to self-antigen *in vivo* [52]. It was therefore hypothesised in chapter 3 that upon self-antigen encounter in the periphery, after exiting the thymus, CTLA-4 could potentially function to restrict the magnitude of the CD4+ FOXP3+ proliferative response. To further the

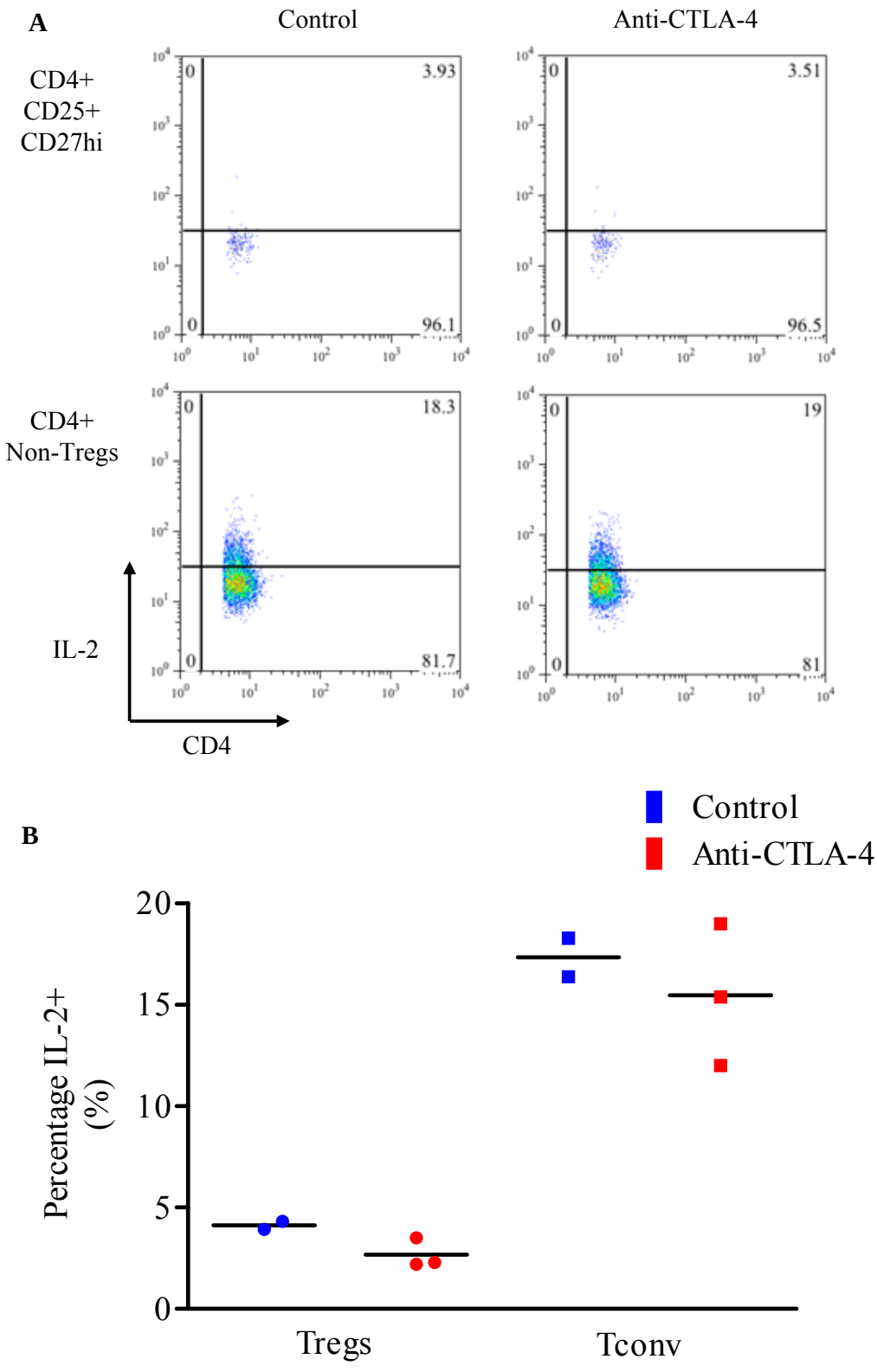

A
Control
Anti-CTLA-4
CD4+
CD25+
CD27hi
3.93
96.1
3.51
96.5
CD4+
Non-Tregs
18.3
81.7
19
81
IL-2
CD4
B
Control
Anti-CTLA-4
Percentage IL-2+
(%)
20
15
10
5
0
Tregs
Tconv

Figure 5.02. Antibody-mediated CTLA-4 blockade does not affect IL-2 production by conventional T cells at day 3. BALB/c mice received 500μg of anti-CTLA-4 blocking antibody (4F10) or control twice weekly by intra-peritoneal injection. Single cell suspensions from peripheral lymph nodes were cultured for four hours with PMA and ionomycin. Cells were then surface stained with CD4, CD25, CD27 and intracellular with IL-2 for analysis by flow cytometry. (A) Representative FACS plots of IL-2 production by CD4+ CD25+ CD27hi Tregs (top) and CD4+ non-Treg Tconv (bottom) from mice harvested on day 3. (B) Graph shows data for a number of different mice harvested on day 3. Lines represent mean value.

studies on the role of CTLA-4 in regulatory T cell homeostasis, Ki67 expression was investigated in transgenic mice (*DO11.10 RIP-mOVA Rag2-/-*) to analyse proliferation of a single clone of Tregs in response to antigen *in vivo*, in the presence or absence of CTLA-4. As mentioned previously, mice with germ line deletion of *Ctla-4* succumb to a severe lymphoproliferative syndrome. Therefore, TCR-transgenic mice (*DO11.10 RIP-mOVA Rag2-/-*) were used in chapter 4 to study the functional activity of Tregs lacking the CTLA-4 receptor in a setting uncomplicated by lymphoproliferation, but with an intact CD28 pathway. A key feature of this transgenic mouse model used is that antigen availability is restricted anatomically to the pancreas by the rat insulin promoter (RIP), such that T cells at most peripheral sites do not encounter antigen. This differs from the situation in the non-transgenic *Ctla-4*-/- mice that exhibit global polyclonal T cell activation in the periphery. Accordingly, CD4+ FOXP3+ regulatory T cells in the peripheral, inguinal lymph nodes of *DO11.10 RIP-mOVA Rag2*-/- mice remained largely undivided, even in the absence of CTLA-4, as indicated by expression of the cell cycle marker Ki67 (*Fig. 5.03*). To examine Tregs that had been exposed to antigen, lymphocytes were isolated from digested pancreata. Very few T cells could be recovered from the pancreata of these transgenic mice. However, CD4+ FOXP3+ Tregs isolated from the pancreas exhibited greater expression of Ki67 in mice that lacked CTLA-4. This data supports the idea that CTLA-4 signalling controls regulatory T cell proliferation in response to encounter with self-antigen.

Each mouse system used in this present study (*Ctla-4*-/-, TCR-transgenic and anti-CTLA-4 blocking antibody) to investigate Tregs lacking the CTLA-4 receptor has an intact CD28 co-stimulatory pathway. The CD28 receptor is a co-stimulatory molecule required for T cell activation and proliferation [182]. It has previously been established by others that CD28 co-stimulation supports the survival and promotes the self-renewal of peripheral regulatory T cells [86, 131]. Additionally, as discussed above, prior to the revival in the concept and existence of regulatory T cells, the CTLA-4 receptor had been found to function opposite the CD28 co-stimulatory receptor to negatively control T cell activation and proliferation [199, 200]. A key mechanism of action proposed for CTLA-4 is the binding and sequestering of its ligands, which are also common to the co-stimulatory receptor CD28, namely CD80 and CD86. In this model CTLA-4 essentially serves as a competitive inhibitor of the CD28 pathway. However, it has also been proposed that following ligation CTLA-4 can transmit a

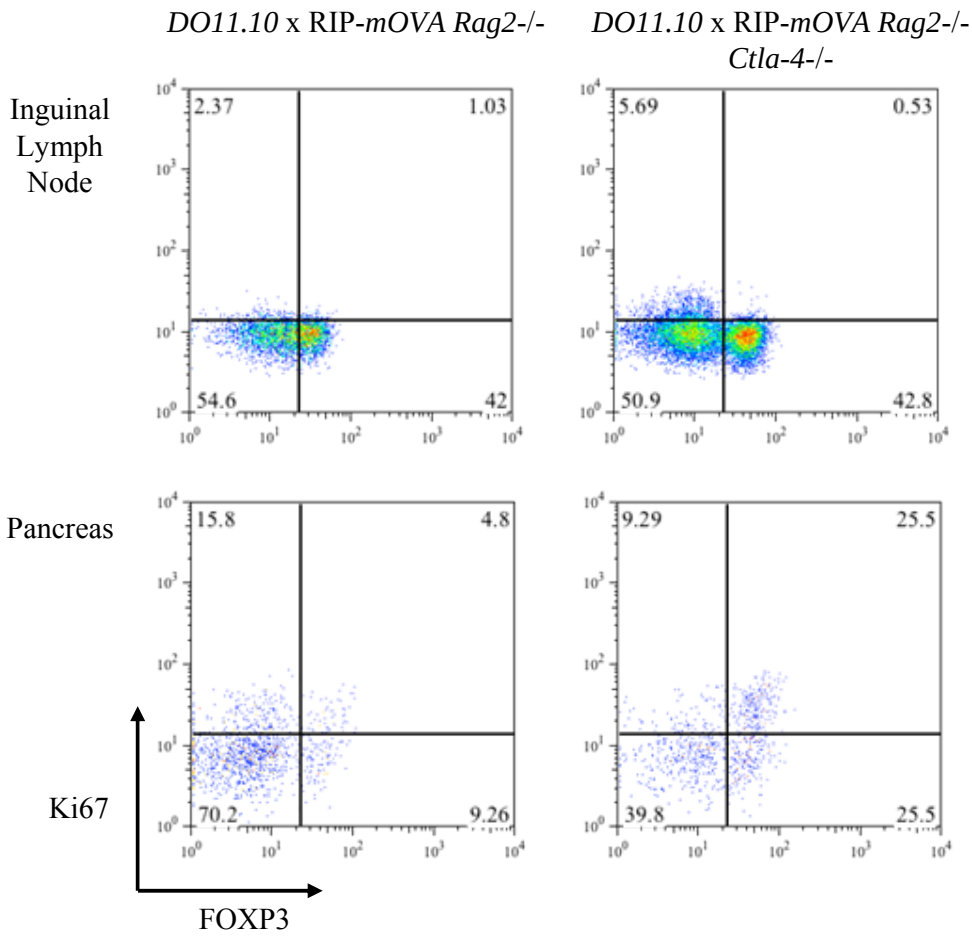
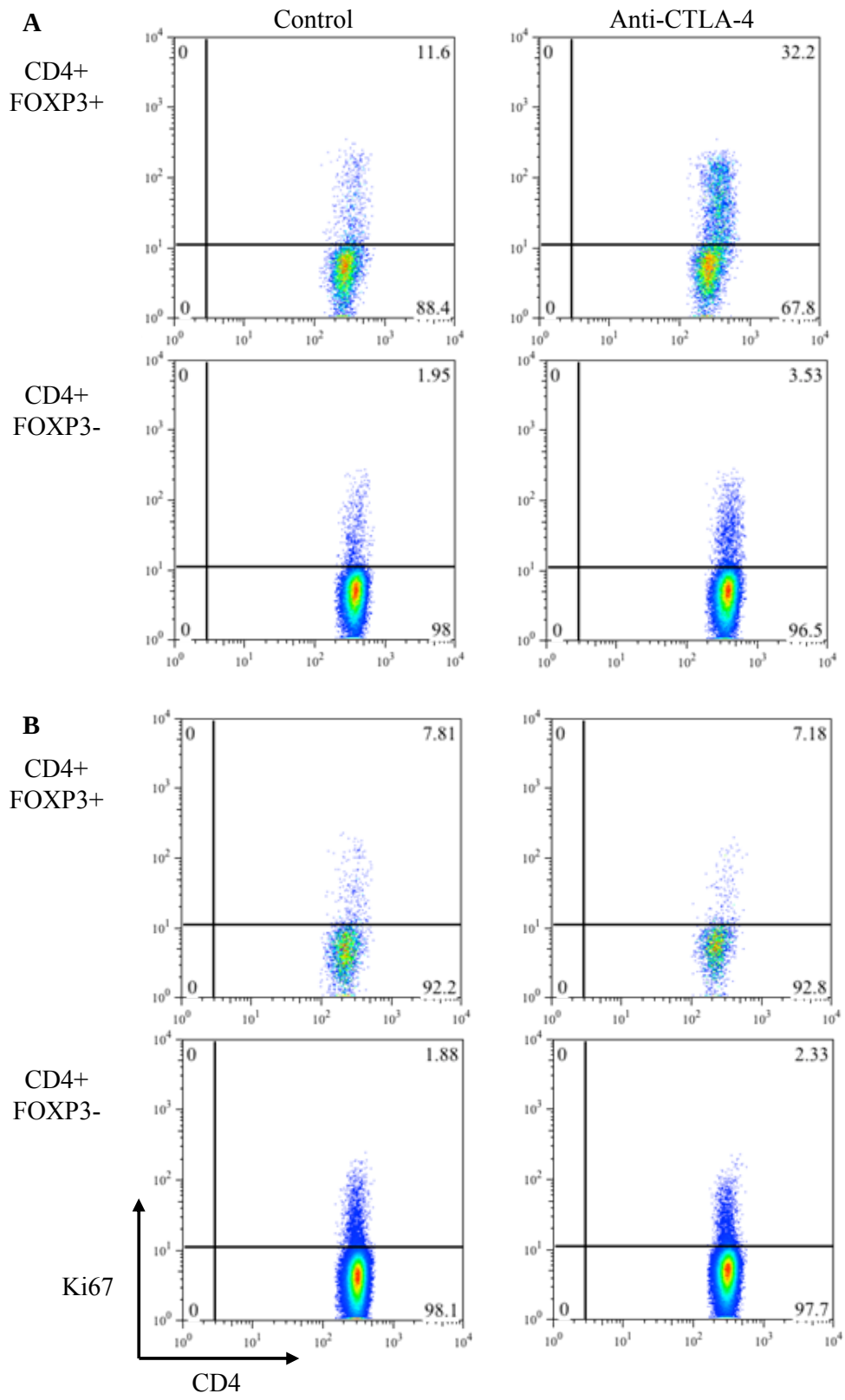


Figure 5.03. Antigen-specific CD4+ FOXP3+ Tregs deficient in CTLA-4 show augmented proliferation at the site of antigen expression. Wild type (left) and *Ctla-4*-/- (right) *DO11.10* x RIP-*mOVA Rag2*-/- single cell suspensions from non-draining inguinal lymph nodes (top) and pooled, digested pancreata (≥2) were surface stained with CD4 and intracellular with FOXP3 and Ki67 for analysis by flow cytometry. Representative FACS plots of Ki67 expression with respect to FOXP3 expression by CD4+ lymphocytes. Data is representative of two independent experiments.

negative signal [213, 318, 319]. In addition, in a small number of instances it has been suggested that CTLA-4 can function in a ligand-independent manner [43, 320]. In these latter two scenarios, CD28 would not need to be present for the effects of CTLA-4 blockade to manifest. However, CD28 would need to be present if CTLA-4 was working by limiting access of CD28 to its ligands. In an attempt to distinguish between these possibilities *CD28*-/- mice were used to ask whether the ability of anti-CTLA-4 blocking antibody treatment to augment Treg proliferation depended on CD28. *Fig. 5.04 A* shows the increased Ki67 expression observed previously (*Fig. 5.01*) by both CD4+ FOXP3- conventional T cells and CD4+ FOXP3+ regulatory T cells in wild type BALB/c mice on day eight of anti-CTLA-4 blocking antibody treatment. However, on day eight of anti-CTLA-4 blocking antibody treatment of *CD28* knockout mice, both CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs showed unaltered Ki67 expression (*Fig. 5.04 B*). The data obtained of Ki67 expression by CD4+ FOXP3+ Tregs (*Fig. 5.04 C*) and CD4+ FOXP3- Tconv (*Fig. 5.04 D*) for a number of control and anti-CTLA-4 antibody treated wild type BALB/c and *CD28*-/- mice is also shown graphically. These data suggest that the augmented CD4+ FOXP3+ Treg proliferation upon antibody-mediated CTLA-4 blockade is CD28-dependent.

5.2.2 Role of the secondary co-stimulatory molecule ICOS in CTLA-4 modulation of regulatory T cell homeostasis.

It was found in 5.2.1 above that the augmented CD4+ FOXP3+ Treg proliferation upon antibody-mediated CTLA-4 blockade was CD28-dependent, and CD28 co-stimulation has been previously implicated in the maintenance of peripheral Treg homeostasis [86, 131]. However, analysis of Ki67 expression by flow cytometry in *Fig. 5.04* identified some CD4+ FOXP3+ Tregs in the periphery of *CD28*-/- mice that expressed Ki67 and were therefore undergoing cell cycling (7.81% in *CD28*-/- versus 11.6% in wild type, in the experiment shown). Therefore, there may be some other molecule(s) involved in peripheral CD4+ FOXP3+ regulatory T cell proliferation and homeostasis. A good candidate molecule is the inducible T cell co-stimulator (ICOS). The secondary co-stimulatory molecule ICOS is expressed upon T cell upon activation and has been found to enhance T cell responses such as proliferation and IL-10 production [243, 321] as indicated by the defects in T cell activation and effector function in *Icos* knockout mice [322]. ICOS is also expressed by a higher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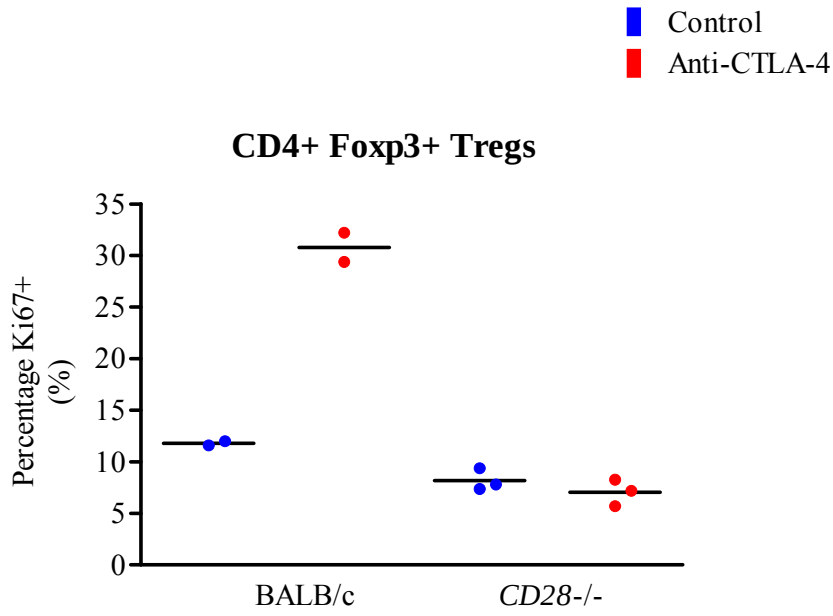
Control
Anti-CTLA-4
CD4+
FOXP3+
11.6
88.4
32.2
67.8
CD4+
FOXP3-
1.95
98
3.53
96.5
B
CD4+
FOXP3+
7.81
92.2
7.18
92.8
CD4+
FOXP3-
1.88
98.1
2.33
97.7
Ki67
CD4

C


Control
Anti-CTLA-4
CD4+ Foxp3+ Tregs
Percentage Ki67+ (%)
35
30
25
20
15
10
5
0
BALB/c
CD28-/-

D

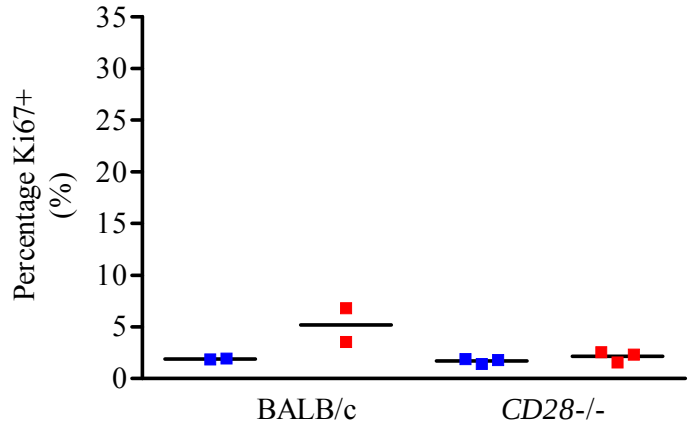

CD4+ Foxp3-Tconv
Percentage Ki67+ (%)
35
30
25
20
15
10
5
0
BALB/c
CD28-/-

Figure 5.04. Augmented CD4+ FOXP3+ Treg proliferation upon antibody-mediated CTLA-4 blockade is CD28-dependent. BALB/c and *CD28*-/- mice received 500μg of anti-CTLA-4 blocking antibody (4F10) or control twice weekly by intra-peritoneal injection. Single cell suspensions from peripheral lymph nodes were surface stained with CD4 and intracellular with FOXP3 and Ki67 for analysis by flow cytometry. Representative FACS plots of Ki67 expression by CD4+ FOXP3+ Tregs (top) and CD4+ FOXP- Tconv (bottom) from BALB/c (A) and *CD28*-/- (B) mice harvested on day 8. Graphs show data for ≥2 BALB/c (C) and *CD28*-/- (D) mice. Lines represent mean value. Experiments carried out in collaboration with C. Wang.

proportion of CD4+ FOXP3+ regulatory T cells than CD4+ FOXP3- conventional T cells in the steady state, and has been implicated in Treg homeostasis [313].

In chapter 3 it was found that expression of ICOS by both CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs was substantially increased in *Ctla-4* knockout mice (*Fig. 3.12*). This further highlights the potential involvement of the ICOS pathway in the augmented regulatory T cell proliferation upon the loss of CTLA-4 signalling. First, ICOS expression by conventional T cells and regulatory T cells was analysed following antibody-mediated CTLA-4 blockade in wild type BALB/c mice. On day eight of anti-CTLA-4 blocking antibody treatment, ICOS expression by CD4+ FOXP3+ Tregs was substantially increased, and there was a slight increase in the expression of ICOS by CD4+ FOXP3- Tconv (*Fig. 5.05*). This data shows that ICOS expression on CD4+ FOXP3+ regulatory T cells is augmented upon antibody-mediated blockade of the CTLA-4 pathway *in vivo*.

In 15-18 day old *Ctla-4* knockout mice a substantial increase in ICOS expression by conventional T cells and regulatory T cells was observed in chapter 3. However, as discussed above, after only a relatively short time (day 8) following antibody-mediated blockade of the CTLA-4 pathway, increased expression of ICOS by Tregs was observed. Therefore, CD4+ FOXP3+ regulatory T cells in one week old *Ctla-4*-deficient mice were analysed. As seen previously for the 15-18 day old *Ctla-4*-/- mice, the percentage of CD4+ T cells that expressed FOXP3 was increased in the lymph nodes and spleen of one week old *Ctla-4*-/- mice (*Fig. 5.06 A*). Interestingly, in one week old *Ctla-4*-/- mice, Ki67 expression by CD4+ FOXP3+ Tregs was increased, but in contrast to the data obtained for 15-18 day old *Ctla-4*-/- mice, Ki67 expression by CD4+ FOXP3- Tconv in one week old *Ctla-4*-/- mice was similar to that in *Ctla-4*+/- littermate controls (*Fig. 5.06 B*). ICOS expression by CD4+ FOXP3+ Tregs in one week old *Ctla-4*-/- mice was substantially increased, whereas the percentage of CD4+ FOXP3- Tconv that expressed ICOS was only slightly increased compared to *Ctla-4*+/- littermate controls (*Fig. 5.06 C*). Overall that data suggest that increased proliferation and expression of ICOS by CD4+ FOXP3+ regulatory T cells upon loss of the CTLA-4 pathway occurs before the increased proliferation and activation of CD4+ FOXP3- conventional T cells.

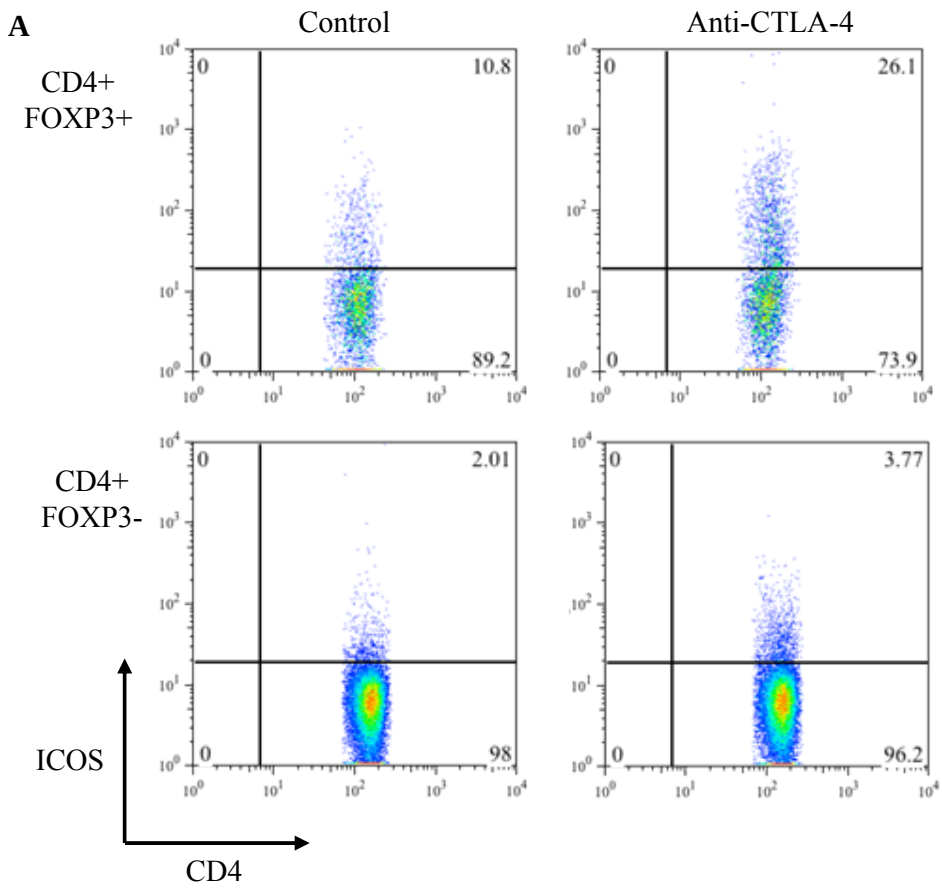
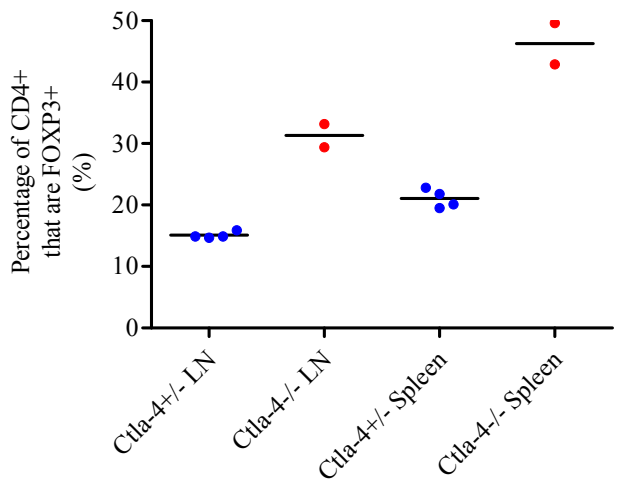
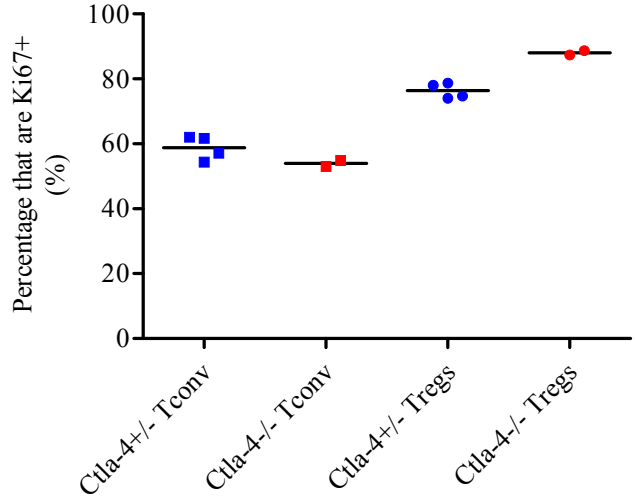


Figure 5.05. ICOS expression on CD4+ FOXP3+ Tregs is augmented upon CTLA-4 blockade *in vivo*. BALB/c mice received 500µg of anti-CTLA-4 blocking antibody (4F10) or control twice weekly by intra-peritoneal injection. Single cell suspensions from peripheral lymph nodes were surface stained with CD4, ICOS and intracellular with FOXP3 for analysis by flow cytometry. Representative FACS plots of ICOS expression by CD4+ FOXP3+ Tregs (top) and CD4+ FOXP- Tconv (bottom) from mice harvested on day 8.

A


Percentage of CD4+ that are FOXP3+ (%)
50
40
30
20
10
0
Ctla-4+/- LN
Ctla-4-/- LN
Ctla-4+/- Spleen
Ctla-4-/- Spleen

B


Percentage that are Ki67+ (%)
100
80
60
40
20
0
Ctla-4+/- Tconv
Ctla-4-/- Tconv
Ctla-4+/- Tregs
Ctla-4-/- Tregs

C

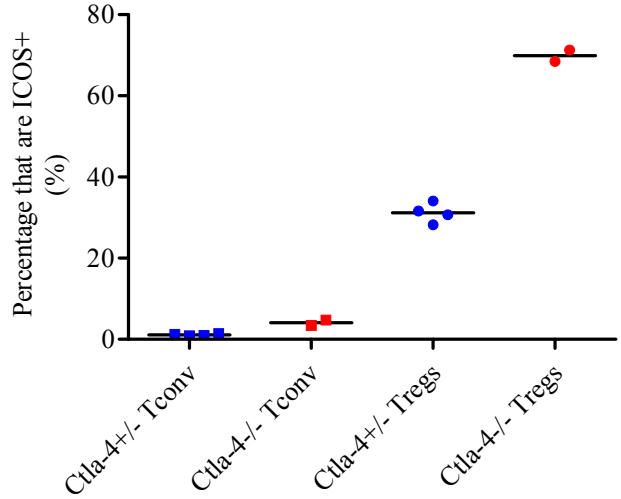

Percentage that are ICOS+ (%)
80
60
40
20
0
Ctla-4+/- Tconv
Ctla-4-/- Tconv
Ctla-4+/- Tregs
Ctla-4-/- Tregs

Figure 5.06. Analysis of CD4+ FOXP3+ Tregs in one week old *Ctla-4*-deficient mice. Day 6 *Ctla-4*+/- and *Ctla-4*-/- single cell suspensions from peripheral lymph nodes were surface stained with CD4, ICOS and intracellular with FOXP3 and Ki67 for analysis by flow cytometry. (A) Graph shows the percentage of CD4+ T cells that express FOXP3 in *Ctla-4*+/- littermate controls (blue) and *Ctla-4*-/- (red). (B) Graph shows the percentage of CD4+ FOXP3- Tconv (squares) and CD4+ FOXP3+ Tregs (circles) that express Ki67 in *Ctla-4*+/- littermate controls (blue) and *Ctla-4*-/- (red). (C) Graph shows the percentage of CD4+ FOXP3- Tconv (squares) and CD4+ FOXP3+ Tregs (circles) that express ICOS in *Ctla-4*+/- littermate controls (blue) and *Ctla-4*-/- (red). Lines represent mean value.

Along with altered receptor expression, the activity of a signalling pathway is also modulated by expression of the ligand(s). To obtain an indication of the potential activity of the ICOS signalling pathway in *Ctla-4*-deficient mice, ICOS ligand (ICOSL) expression by CD19+ B cells was analysed by flow cytometry. In *Fig. 5.07 A and B* it can be seen that the median fluorescent intensity (MFI) of ICOSL expression on the surface of CD19+ B cells was slightly decreased in *Ctla-4*-/- mice compared to age-matched BALB/c controls. In some instances, upon receptor-ligand interaction there is a concurrent down regulation of ligand expression as a means of modulating the activity of signalling via the receptor. Therefore, intracellular expression of ICOSL by CD19+ B cells was also analysed by flow cytometry. It was found that the MFI of intracellular ICOSL expression by CD19+ B cells in *Ctla-4*-/- mice was slightly increased compared to age-matched BALB/c controls (*Fig. 5.07*). However, these shifts in the intensity of ICOSL expression are only very small and more data is required to determine if these represent significant changes.

Upon T cell activation, the up regulation of ICOS has been reported to be CD28-dependent [244]. However, the mechanistic control of ICOS expression in Tregs is not known. Using lymphocytes isolated from *CD28*-deficient mice and agonistic antibodies to CD3 and CD28, the CD28-dependency of ICOS expression by CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs was investigated by *in vitro* overnight culture. *Fig. 5.08* shows that fewer *CD28*-deficient CD4+ FOXP3- conventional T cells (Tconv) expressed ICOS compared to wild type. ICOS expression in Tconv increased in response to TCR-stimulation by anti-CD3 in wild type and slightly in *CD28*-deficient lymphocytes. In wild type Tconv, CD28 co-stimulation with anti-CD28 in conjunction with TCR-stimulation with anti-CD3 further increased ICOS expression, although CD28 co-stimulation alone had no affect. As predicted, the anti-CD28 antibody had no affect on *CD28*-/- lymphocytes. Collectively, these data suggest that a combination of TCR and CD28 signalling drive the up regulation of ICOS in conventional T cells.

Parallel analyses were carried out on the CD4+ FOXP3+ regulatory T cell subset. ICOS expression by *CD28*-deficient CD4+ FOXP3+ Tregs (*Fig. 5.08*) was similar to wild type. Similar to conventional T cells, Treg ICOS expression increased upon TCR-stimulation with anti-CD3, and CD28 co-stimulation further increased ICOS expression. Again, the anti-CD28

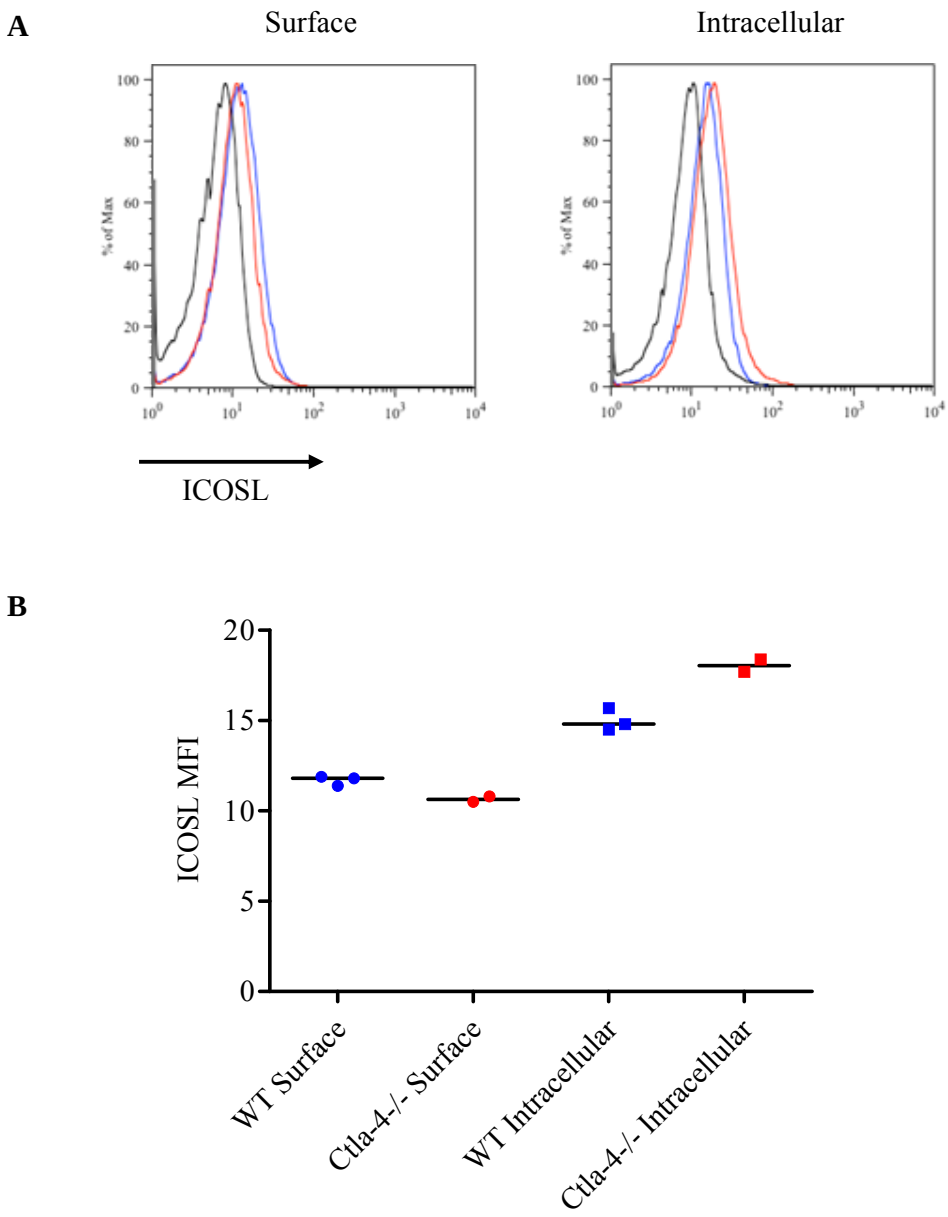
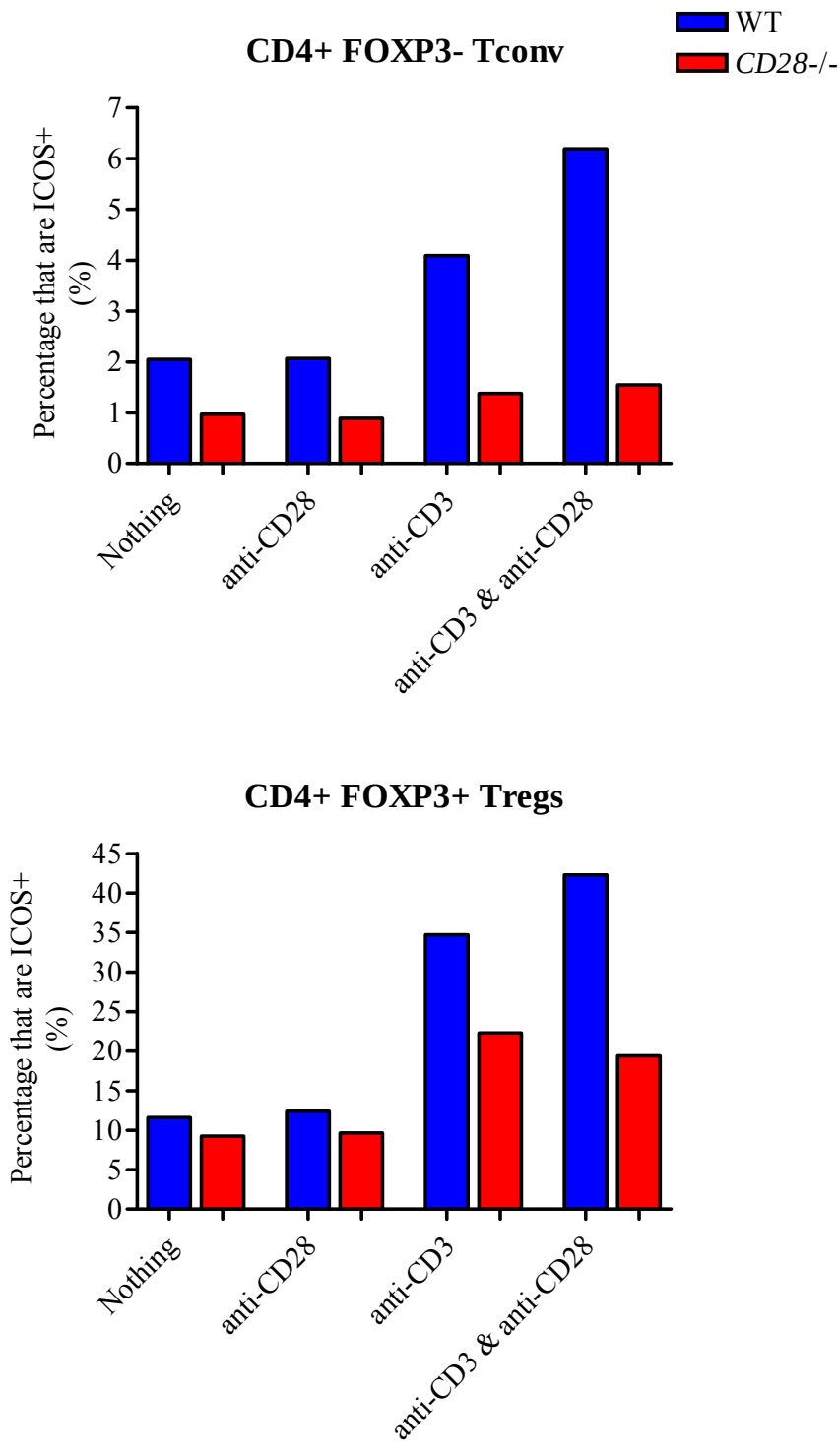


Figure 5.07. Analysis of ICOSL expression by CD19+ B cells in *Ctla-4*-deficient mice. Day 15-18 BALB/c and *Ctla-4*-/- single cell suspensions from peripheral lymph nodes were surface stained with CD19, ICOSL and intracellular with ICOSL for analysis by flow cytometry. (A) Representative histograms for surface (left) and intracellular (right) expression of ICOSL by CD19+ gated lymphocytes of day 15-18 BALB/c mice (blue) and *Ctla-4*-/- mice (red). Isotype controls in black. (B) Graph shows data for surface (circles) and intracellular (squares) ICOSL median fluorescent intensity (MFI) on CD19+ B cells for a number of BALBC/c (blue) and *Ctla-4*-/- (red) mice. Lines represent median value.

A


CD4+ FOXP3- Tconv
WT
CD28-/-
Percentage that are ICOS+ (%)
0
1
2
3
4
5
6
7
Nothing
anti-CD28
anti-CD3
anti-CD3 & anti-CD28
CD4+ FOXP3+ Tregs
Percentage that are ICOS+ (%)
0
5
10
15
20
25
30
35
40
45
Nothing
anti-CD28
anti-CD3
anti-CD3 & anti-CD28

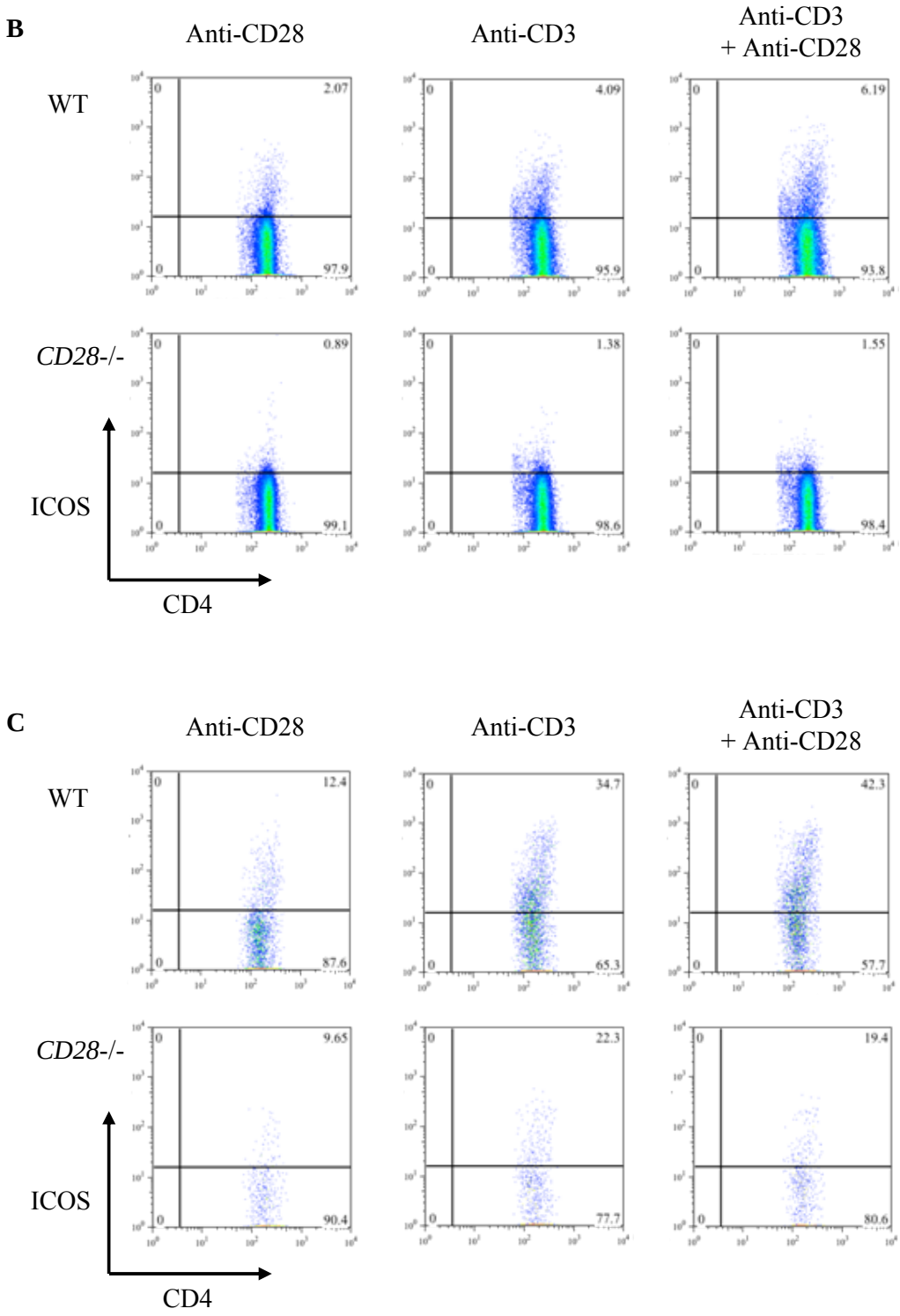

B
Anti-CD28
Anti-CD3
Anti-CD3
+ Anti-CD28
WT
CD28-/-
ICOS
CD4
2.07
97.9
4.09
95.9
6.19
93.8
0.89
99.1
1.38
98.6
1.55
98.4
C
Anti-CD28
Anti-CD3
Anti-CD3
+ Anti-CD28
WT
CD28-/-
ICOS
CD4
12.4
87.6
34.7
65.3
42.3
57.7
9.65
90.4
22.3
77.7
19.4
80.6

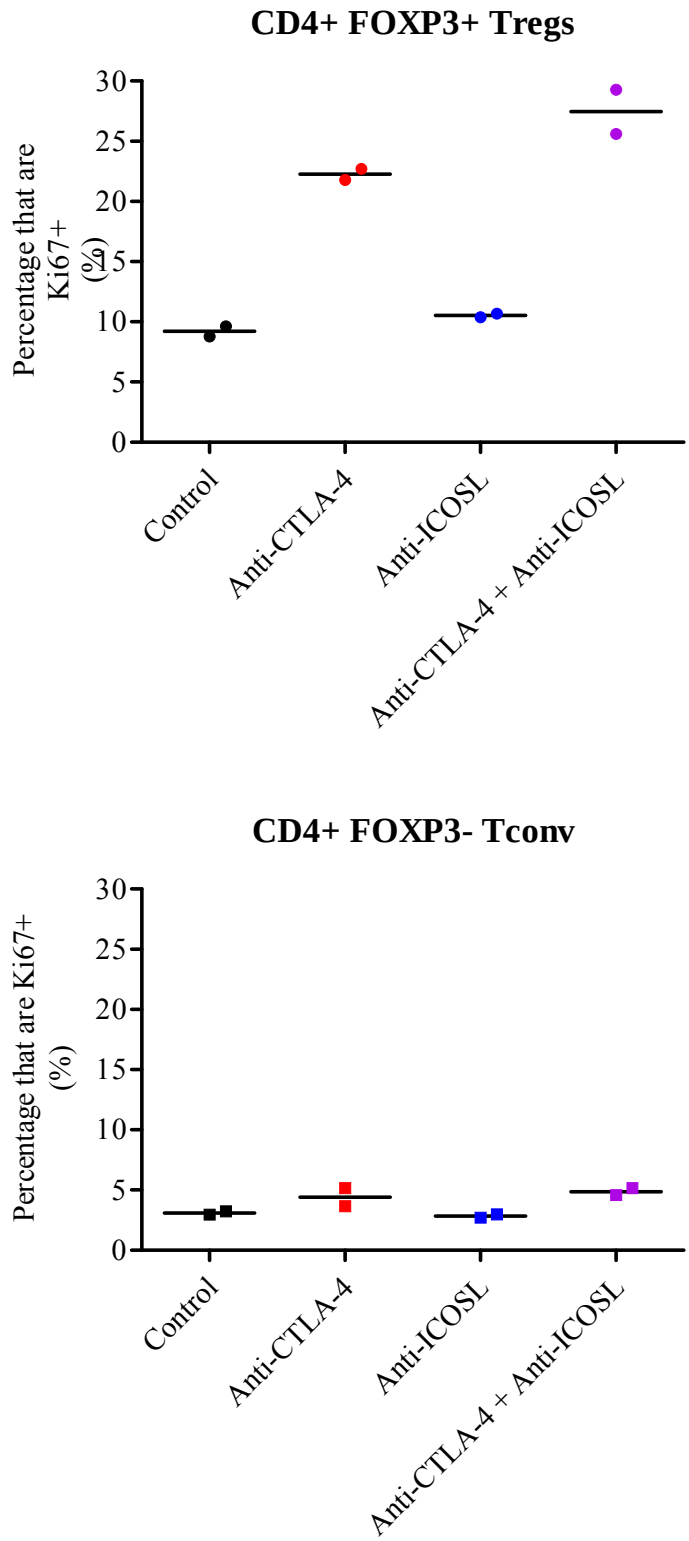
Figure 5.08. CD28-dependency of ICOS expression by CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs. Adult BALB/c wild type or *CD28*-/- single cell suspensions from peripheral lymph nodes were cultured overnight plus or minus 500ng/ml anti-CD3 and 1μg/ml anti-CD28. Three wells were pooled and surface stained with CD4, ICOS and intracellular with FOXP3 for analysis by flow cytometry. (A) Graphs show ICOS expression by gated CD4+ FOXP3- Tconv (top) and CD4+ FOXP3+ Tregs (bottom). Representative graph from three independent experiments is shown. Representative FACS plots of ICOS expression by CD4+ FOXP3- Tconv (B) and CD4+ FOXP3+ Tregs (C) following culture with anti-CD3 and/or anti-CD28 are also shown.

antibody had no affect on *CD28*-/- lymphocytes. Overall, the data suggest that up regulation of ICOS expression by both CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs is augmented by CD28 signalling.

From the ICOS and ICOSL expression patterns observed upon loss of the CTLA-4 pathway in this study, and the generally accepted role of ICOS as a secondary co-stimulatory molecule, it could be hypothesised that the ICOS signalling pathway may be involved in the augmented Treg proliferation observed in *Ctla-4*-/- mice and anti-CTLA-4 blocking antibody treated mice. To investigate the role of ICOSL-ICOS signalling in the augmented Treg proliferation observed upon loss of CTLA-4 signalling, *in vivo* experiments were carried out in which wild type BALB/c mice were treated with anti-CTLA-4 blocking antibody in the presence or absence of anti-ICOSL blocking antibody. The graphs of *Fig. 5.09* show increased Ki67 (*A*) and ICOS (*B*) expression by CD4+ FOXP3+ Tregs (top) and CD4+ FOXP3- Tconv (bottom) day five after anti-CTLA-4 blocking antibody treatment, as seen previously. Treatment with anti-ICOSL blocking antibody alone had no affect on Ki67 or ICOS expression by either CD4+ FOXP3- Tconv or CD4+ FOXP3+ Tregs. Combined blockade of both the CTLA-4 and ICOS pathways by treatment with anti-CTLA-4 and anti-ICOSL blocking antibodies did not abrogate the increase in Ki67 expression by both CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs caused by anti-CTLA-4 blocking antibody treatment alone. Overall, the data suggest that the ICOSL-ICOS pathway is not responsible for the augmented Treg proliferation observed upon loss of the CTLA-4 pathway following antibody-mediated blockade.

The increased ICOS expression observed on CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs in *Ctla-4*-deficient mice and following anti-CTLA-4 blocking antibody treatment of wild type BALB/c mice could be a cell-intrinsic result of the loss of CTLA-4 signalling, or it could be due to factors in the local environment. In an attempt to distinguish between these possibilities *in vitro* experiments were carried out in which wild type BALB/c lymphocytes were cultured in control medium or supernatants obtained from the *in vitro* culture of wild type BALB/c or *Ctla-4*-/- lymphocytes. ICOS expression on CD4+ FOXP3- Tconv was similar following culture in control medium, wild type-derived supernatant or *Ctla-4*-/--derived supernatant. On the other hand, CD4+ FOXP3+ Treg expression of ICOS

A


CD4+ FOXP3+ Tregs
Percentage that are Ki67+ (%)
30
25
20
15
10
5
0
Control
Anti-CTLA-4
Anti-ICOSL
Anti-CTLA-4 + Anti-ICOSL
CD4+ FOXP3- Tconv
Percentage that are Ki67+ (%)
30
25
20
15
10
5
0
Control
Anti-CTLA-4
Anti-ICOSL
Anti-CTLA-4 + Anti-ICOSL

B

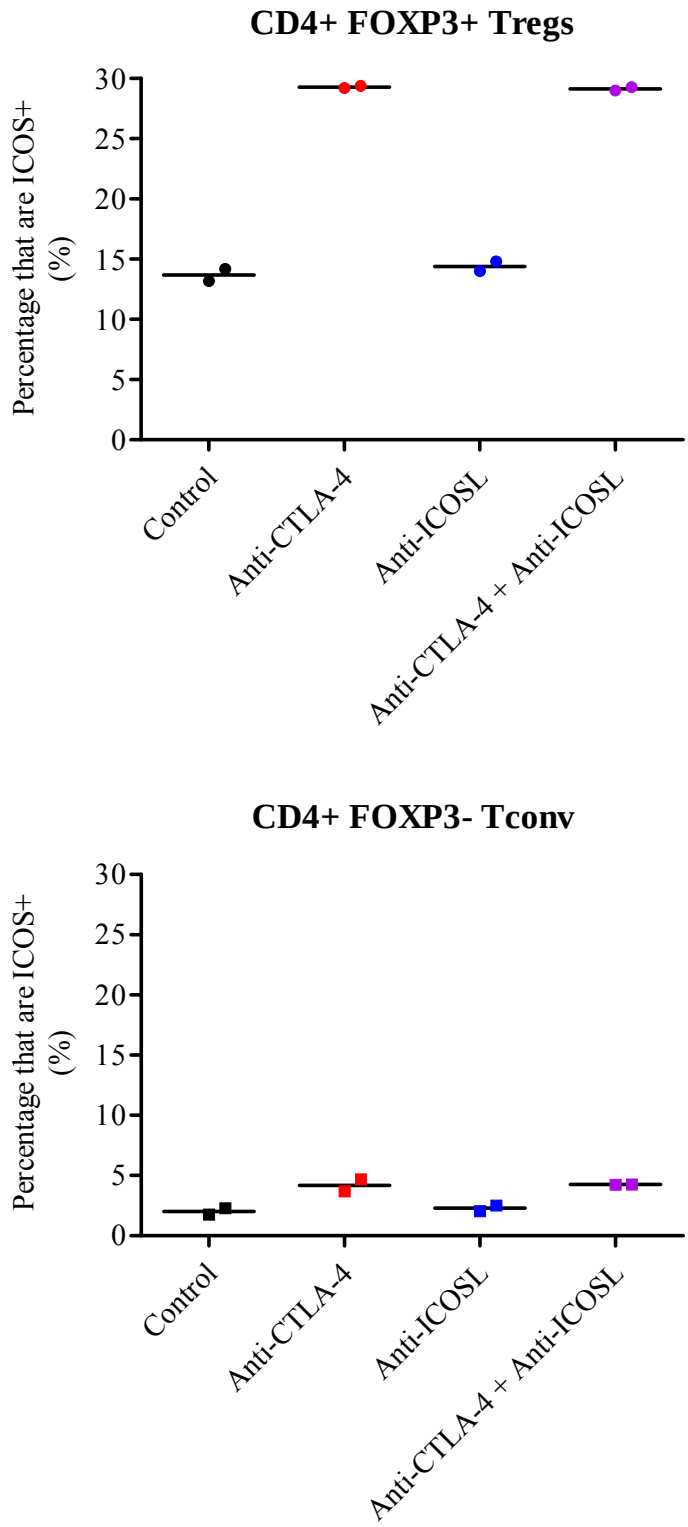

CD4+ FOXP3+ Tregs
Percentage that are ICOS+ (%)
30
25
20
15
10
5
0
Control
Anti-CTLA-4
Anti-ICOSL
Anti-CTLA-4 + Anti-ICOSL
CD4+ FOXP3- Tconv
Percentage that are ICOS+ (%)
30
25
20
15
10
5
0
Control
Anti-CTLA-4
Anti-ICOSL
Anti-CTLA-4 + Anti-ICOSL

Figure 5.09. *In vivo* analysis of the ICOS-dependency of augmented CD4+ FOXP3+ Treg proliferation upon antibody-mediated CTLA-4-blockade. BALB/c mice received 500μg of anti-CTLA-4 blocking antibody (4F10) plus or minus 200μg of anti-ICOSL blocking antibody (HK5.3) or control twice weekly by intra-peritoneal injection. Mice were harvested on day 5. Single cell suspensions from peripheral lymph nodes were surface stained with CD4, ICOS and intracellular with FOXP3 and Ki67 for analysis by flow cytometry. Graphs show Ki67 (A) and ICOS (B) expression by CD4+ FOXP3+ Tregs (top) and CD4+ FOXP3- Tconv (bottom). Lines represent mean value.

was increased following culture in *Ctla-4*-/--derived supernatant compared to control medium or wild type-derived supernatant (*Fig. 5.10*). Overall, the *in vitro* data suggest that lymphocytes in *Ctla-4*-deficient mice produce some factor(s) that can up regulate ICOS expression by CD4+ FOXP3+ Tregs.

5.2.3 Role of the TNF receptors OX40 and CD30 in CTLA-4 modulation of regulatory T cell homeostasis.

In addition to ICOS, the tumor necrosis factor (TNF) receptor OX40 is also known as a secondary co-stimulatory molecule expressed by activated T cells [323]. It has been found that OX40 is involved in the T cell-dependent humoral response [324], CD4 T cell memory [325] and, along with another TNF receptor expressed by activated T cells CD30, T cell survival (reviewed in [326, 327]). Additionally, OX40 is expressed by regulatory T cells and it has been proposed that OX40 is involved in Treg induction and function [125, 314]. Therefore, following the studies on ICOS above, the TNF receptors OX40 and CD30 were other good candidate molecules to be involved in the modulation of regulatory T cell homeostasis. First, CD4+ FOXP3+ regulatory T cells were analysed in *OX40*-/- *CD30*-/- mice to assess the role of these two TNF receptors in Treg development and homeostasis. The graph of *Fig. 5.11 A* indicates that in the peripheral lymph nodes of mice lacking *OX40* and *CD30* there was no difference in the percentage of CD4+ T cells that were FOXP3+ compared to C57BL/6 controls. However, the data show that a slightly increased proportion of CD4+ T cells expressed FOXP3 in the thymi of *OX40*-/- *CD30*-/- compared to in wild type control thymi. To examine whether the OX40 and CD30 pathways influence the proliferation of mature Tregs in the periphery, Ki67 expression was analysed by flow cytometry. The FACS plots of *Fig. 5.11 B* show that there was not really any change in Ki67 expression by peripheral CD4+ FOXP3- conventional T cells or CD4+ FOXP3+ regulatory T cells lacking *OX40* or both *OX40* and *CD30* compared to wild type C57BL/6 controls.

To test whether deficiency in the OX40 or CD30 pathways altered the augmented Treg proliferation upon antibody-mediated blockade of the CTLA-4 pathway, mice lacking *OX40* (*OX40*-/-) or both *OX40* and *CD30* (*OX40*-/- *CD30*-/-) were treated with anti-CTLA-4 blocking antibody. On day eight after treatment with anti-CTLA-4 blocking antibody, Ki67 expression by both Tconv and Tregs in wild type C57BL/6 mice was increased, as seen

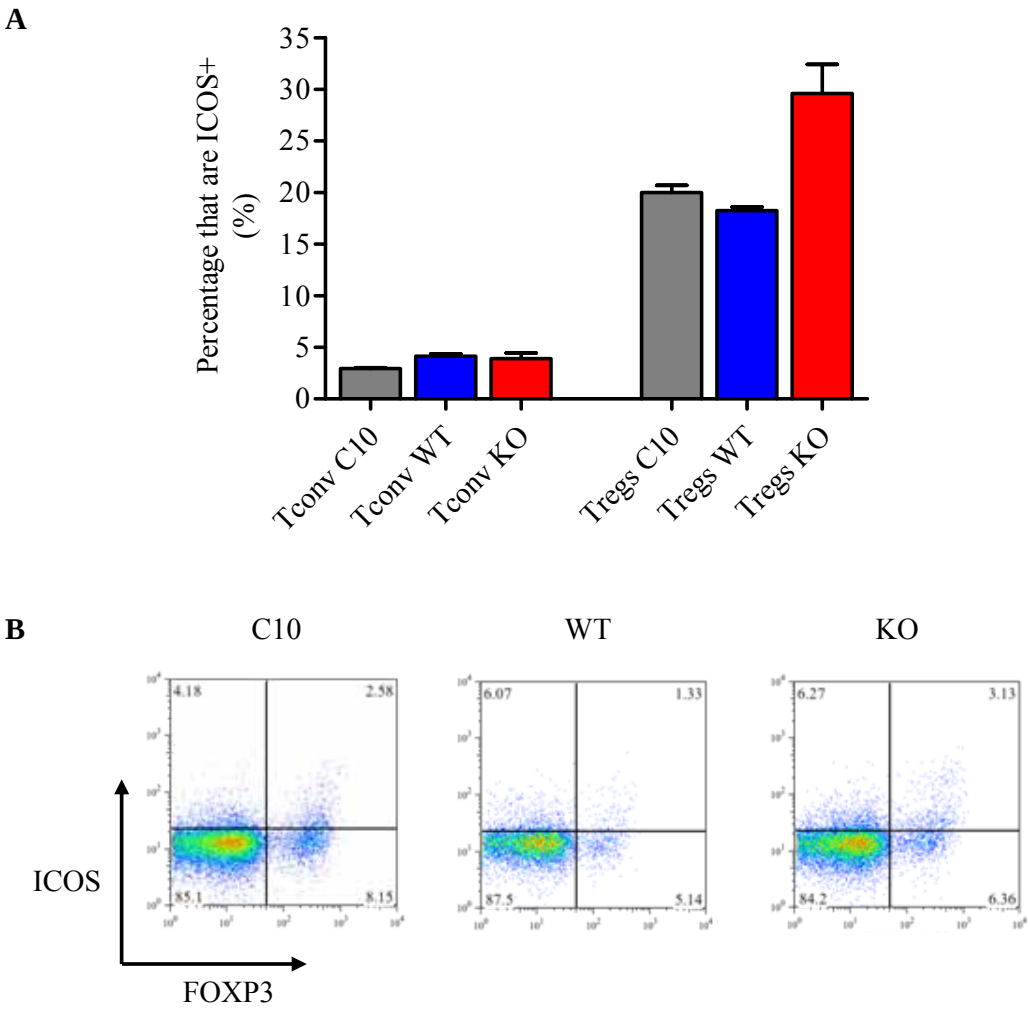
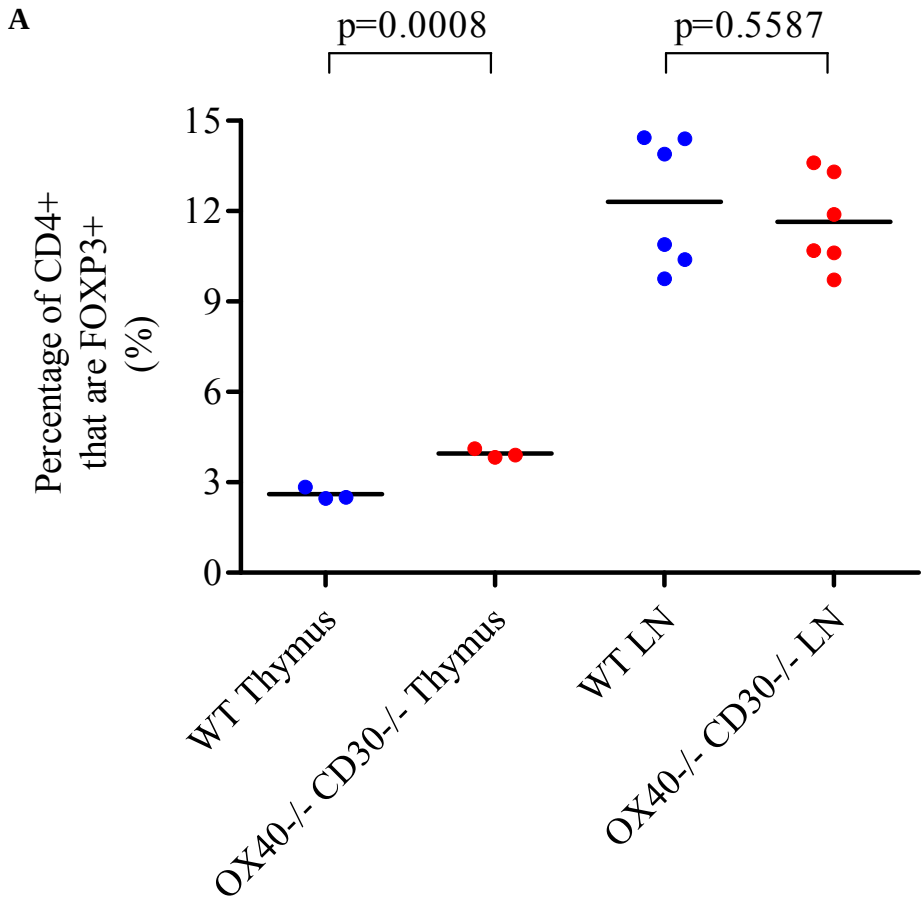


Figure 5.10. *In vitro* analysis of CD4+ FOXP3- Tconv and CD4+ FOXP3+ Treg ICOS expression in a '*Ctla-4*-/- environment'. 2 x 10^5 wild type BALB/c lymphocytes were cultured in control medium (C10), wild type supernatant (WT) or *Ctla-4*-/- supernatant (KO) overnight. The next day cells were analysed by flow cytometry for ICOS expression by CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs. Supernatants were obtained from culture of 3 x 10^5 wild type BALB/c or *Ctla-4*-/- lymphocytes for two days. (A) Graph to show ICOS expression by CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs following culture in control medium (C10), wild type supernatant (WT) or *Ctla-4*-/- supernatant (KO). Bars on graphs represent mean and standard deviation is shown. (B) Representative FACS plots of FOXP3 and ICOS expression by CD4+ gated lymphocytes following culture in control medium (C10), wild type supernatant (WT) or *Ctla-4*-/- supernatant (KO).


A
p=0.0008
p=0.5587
Percentage of CD4+ that are FOXP3+ (%)
15
12
9
6
3
0
WT Thymus
OX40-/- CD30-/- Thymus
WT LN
OX40-/- CD30-/- LN

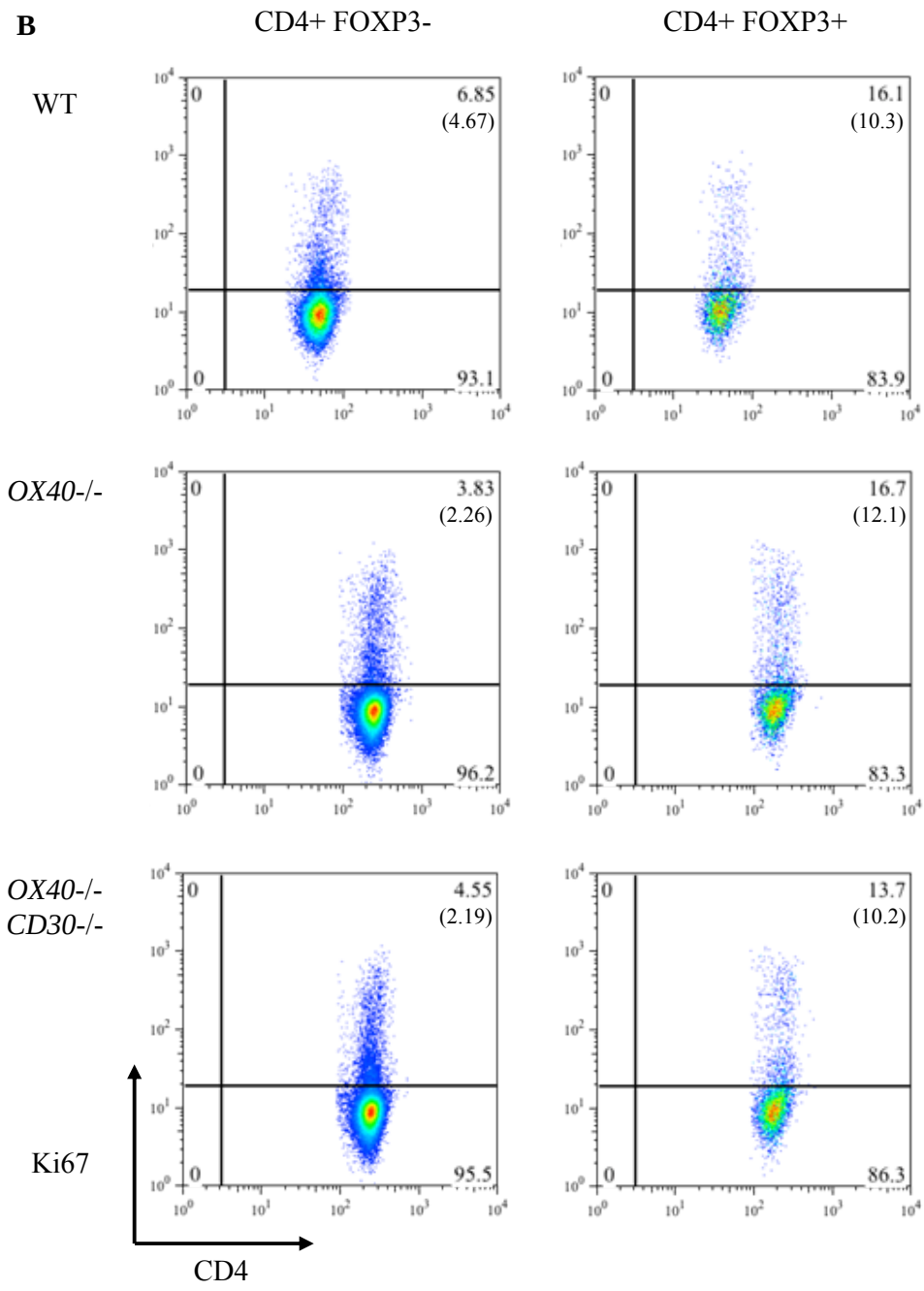

B
CD4+ FOXP3-
CD4+ FOXP3+
WT
OX40-/-
OX40-/-
CD30-/-
Ki67
CD4
6.85
(4.67)
93.1
16.1
(10.3)
83.9
3.83
(2.26)
96.2
16.7
(12.1)
83.3
4.55
(2.19)
95.5
13.7
(10.2)
86.3

Figure 5.11. Analysis of CD4+ FOXP3+ Tregs in mice lacking OX40 and CD30. Single cell suspensions from thymi and peripheral lymph nodes of wild type C57BL/6 (WT), *OX40*-/- and *OX40*-/- x *CD30*-/- mice were surface stained with CD4 and intracellular with FOXP3 and Ki67 for analysis by flow cytometry. (A) Graph shows the percentage of CD4+ that were FOXP3+ in the thymi and peripheral lymph nodes of wild type C57BL/6 or *OX40*-/- x *CD30*-/- mice. (B) Representative FACS plots of Ki67 expression by CD4+ FOXP3- Tconv (left) and CD4+ FOXP3+ Tregs (right) from the lymph nodes of wild type C57BL/6 (top), *OX40*-/- (middle) and *OX40*-/- x *CD30*-/- (bottom) mice. Data are representative of 2 mice. p values calculated by two-tailed, unpaired T-test for the means.

previously with BALB/c mice. Anti-CTLA-4 blocking antibody treatment also increased Ki67 expression by Tconv and Tregs in *OX40*-/- and *OX40*-/- *CD30*-/- mice (*Fig. 5.12*). Overall, the data suggest that regulatory T cell proliferation upon the loss of the CTLA-4 pathway by anti-CTLA-4 blocking antibody treatment is not OX40/CD30-dependent.

5.2.4 Role of the TNF receptor RANK in regulatory T cell development and homeostasis.
Another TNF receptor that is of interest with respect to regulatory T cell development and homeostasis is receptor activator of NF-κB (RANK). RANK was originally identified to be expressed by dendritic cells [328] and interaction with RANKL on activated T cells established a role for the RANKL-RANK pathway in DC function and survival [329]. Additionally, the RANKL-RANK pathway was found to be involved in bone physiology following the identification of RANK expression on chondrocytes and osteoclasts [330]. RANK is also expressed by endothelial cells, specifically medullary thymic epithelial cells (mTECs), and a role for RANK in positive selection-mediated thymic medulla formation has been proposed [315, 331]. mTECs, that promiscuously express self-antigen under the control of the autoimmune regulator AIRE, have been proposed to functionally determine whether thymocytes develop into FOXP3+ regulatory T cells [79]. This consequently infers a role for the RANKL-RANK pathway in regulatory T cell development.

In addition to dendritic cells, osteoclasts and mTECs, RANK has also been reported to be expressed by activated T cells [328, 329]. It is technically difficult to study the cellular distribution of RANK expression as good monoclonal antibodies are not available. Due to the activated cellular phenotype in *Ctla-4*-deficient mice with severe lymphoproliferative disease, it was decided to investigate *Rank* expression by SYBR green qPCR using sorted cell populations from these mice. Some *Rank* expression was detected in both wild type and *Ctla-4*-/- whole lymphocyte and purified CD4+ cell preparations (*Fig. 5.13 A*). The relative amount of *Rank* expression detected in wild type and *Ctla-4*-/- lymphocytes and CD4+ was two logs lower than that expressed by the foetal thymic organ culture cells used as a positive control. *Rank* expression was also detected in wild type and *Ctla-4*-/- purified CD4+ CD25- conventional T cell and CD4+ CD25+ CD27hi regulatory T cell preparations (*Fig. 5.13 B*). With the purified Tconv and Treg populations a three log lower relative amount of *Rank* was detected compared to in the foetal thymic organ culture cells used as a positive control.

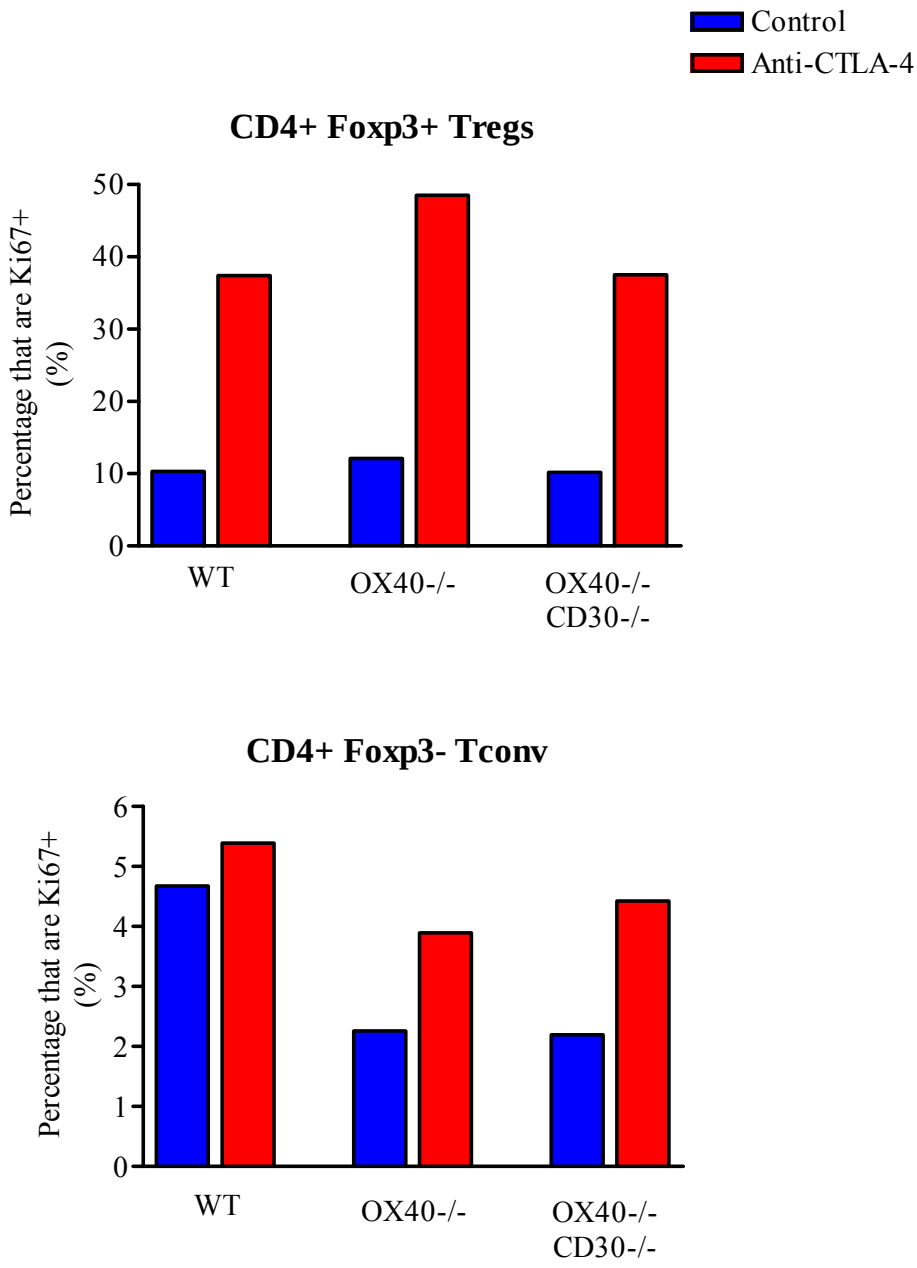


Figure 5.12. Augmented CD4+ FOXP3+ Treg proliferation upon antibody-mediated CTLA-4 blockade is not OX40 / CD30-dependent. C57BL/6, *OX40*-/- and *OX40*-/- x *CD30*-/- mice received 500µg of anti-CTLA-4 blocking antibody (4F10) or control twice weekly by intra-peritoneal injection. Single cell suspensions from peripheral lymph nodes were surface stained with CD4 and intracellular with FOXP3 and Ki67 for analysis by flow cytometry. Graphs show Ki67 expression by CD4+ FOXP+ Tregs (A) and CD4+ FOXP3- Tconv (B) harvested on day 8.

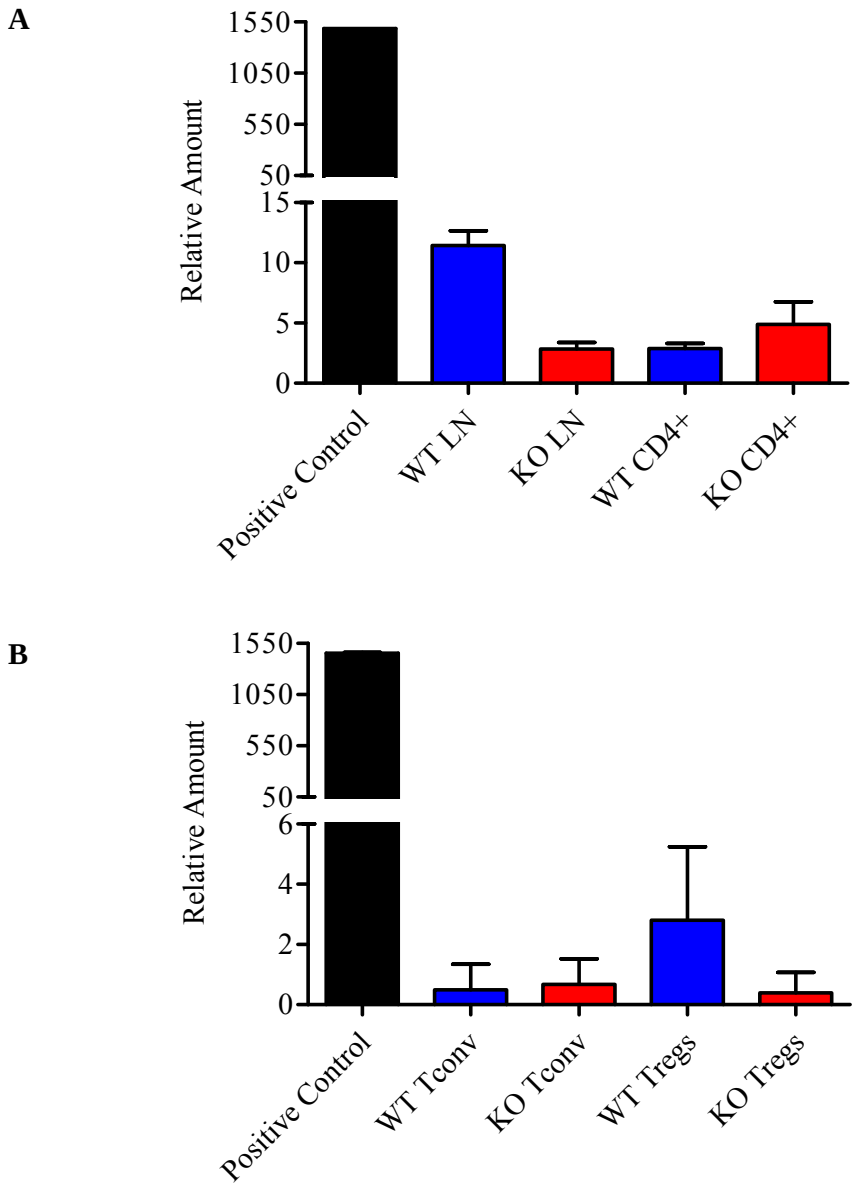


Figure 5.13. Real time qPCR for RANK expression. 15-18 day old wild type BALB/c or *Ctla-4*-/- single cell suspensions of pooled lymph nodes were surface stained with CD4, CD25 and CD27 for mo-flow cell sorting. CD4+ T cells were isolated and Tconv were isolated as CD4+ CD25- and Tregs were isolated as CD4+ CD25+ CD27hi. mRNA and cDNA was obtained from snap-frozen cell pellets and real time SYBR Green qPCR carried out for the presence of RANK relative to β-actin. Graphs show pooled data for triplicates of cDNA samples obtained from three different wild type (blue) or *Ctla-4*-/- (red) mice. Relative amount was calculated as the amount of target normalised to an endogenous β-actin reference. Experiments carried out in collaboration with S. Parnell.

In contrast to RANK, good RANK ligand (or TRANCE) monoclonal antibodies are available. To compliment the RANK expression studies above, the cellular distribution of TRANCE (RANKL) expression was analysed by flow cytometry in *Ctla-4*-deficient mice and littermate controls. In *Ctla-4*-/- mice a substantially increased proportion of CD4+ T cells expressed TRANCE (*Fig. 5.14 B*). Therefore, TRANCE expression by CD4+ FOXP3- conventional T cells and CD4+ FOXP3+ regulatory T cells within the CD4+ T cell population was investigated. Some CD4+ FOXP3- Tconv expressed TRANCE in *Ctla-4*+/- littermate controls; strikingly, this was greatly increased in *Ctla-4*-/- mice (*Fig. 5.14 C*). No difference was observed in the TRANCE expression by CD4+ FOXP3+ regulatory T cells in *Ctla-4*-/- mice compared to *Ctla-4*+/- littermate controls (*Fig. 5.14 D*).

The increased TRANCE expression observed above on CD4+ T cells from *Ctla-4*-/- mice could be due to the cell-intrinsic loss of CTLA-4, or it could be a result of the lymphoproliferative environment within the diseased *Ctla-4*-deficient mice. To examine the effect of CTLA-4 blockade in mice not experiencing lymphoproliferation, the cellular distribution of TRANCE (RANKL) expression was analysed by flow cytometry in wild type BALB/c mice treated with anti-CTLA-4 blocking antibody. *Fig. 5.15* shows that in control treated mice similar TRANCE expression was detected on both CD4+ FOXP3- conventional T cells and CD4+ FOXP3+ regulatory T cells, as previously found in *Ctla-4*+/- littermate controls. On day eight after antibody-mediated CTLA-4 blockade, TRANCE expression only slightly increased on the FOXP3- conventional CD4+ T cell population (*Fig. 5.15 C*). The TRANCE expression after anti-CTLA-4 blocking antibody treatment was lower than that previously observed in *Ctla-4*-deficient mice (*Fig. 5.14*). As seen in *Ctla-4* knockout mice, there was not really any change in TRANCE expression on CD4+ FOXP3+ Tregs following anti-CTLA-4 blocking antibody treatment (*Fig. 5.15 D*).

The data obtained above suggest that TRANCE expression by CD4+ FOXP3- T cells is increased upon loss of CTLA-4 signalling. Since CTLA-4 functions to antagonise CD28 signalling, the CD28-dependency of TRANCE expression by CD4+ FOXP3- conventional T cells and CD4+ FOXP3+ regulatory T cells was investigated using lymphocytes isolated from *CD28*-deficient mice and agonistic antibodies to CD3 and CD28 by *in vitro* overnight culture. *Fig. 5.16 A* shows that fewer *CD28*-deficient CD4+ FOXP3- conventional T cells

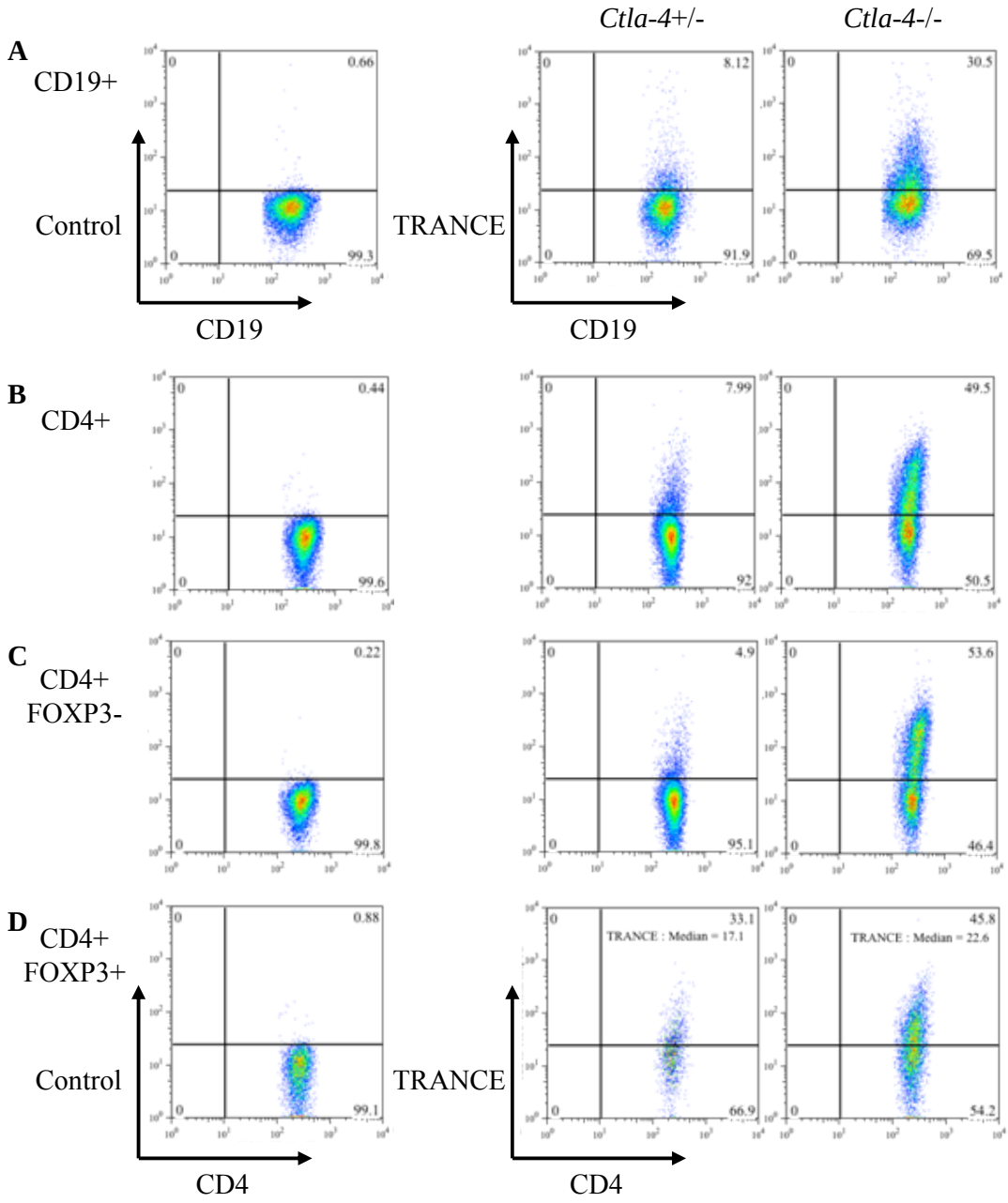


Figure 5.14. Cellular distribution of TRANCE (RANKL) expression in *Ctla-4*-deficient mice. Single cell suspensions from peripheral lymph nodes were surface stained with CD4, CD19, TRANCE and intracellular with FOXP3 for analysis by flow cytometry. Representative FACS plots for CD19+ (A), CD4+ (B), CD4+ FOXP3- (C) and CD4+ FOXP3+ (D) gated lymphocytes of 15-18 day old *Ctla-4*+/- and *Ctla-4*-/- mice (right) and for secondary antibody alone (left). Data representative of 4 mice.

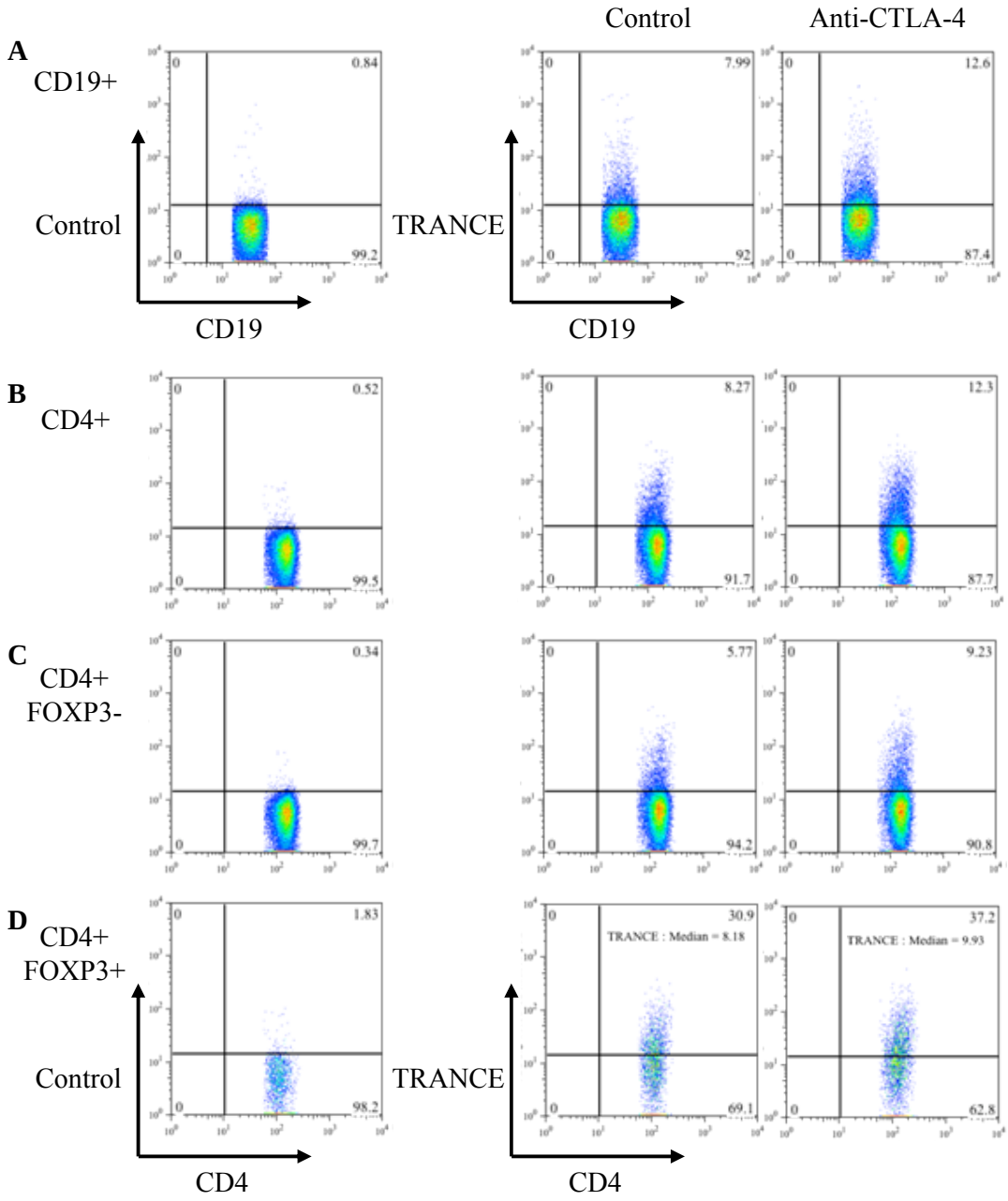
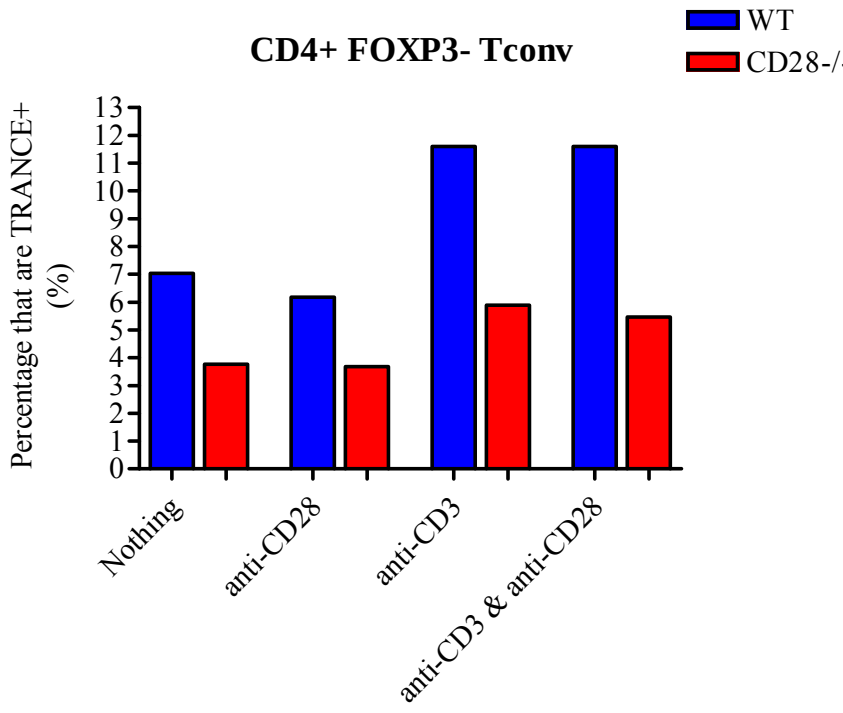


Figure 5.15. TRANCE (RANKL) expression upon antibody-mediated CTLA-4 blockade *in vivo*. BALB/c mice received 500µg of anti-CTLA-4 blocking antibody (4F10) or control twice weekly by intra-peritoneal injection. Single cell suspensions from peripheral lymph nodes were surface stained with CD4, CD19, TRANCE and intracellular with FOXP3 for analysis by flow cytometry. Representative FACS plots of TRANCE expression by CD19+ B cells (A), CD4+ T cells (B), CD4+ FOXP3- Tconv (C) and CD4+ FOXP3+ Tregs (D) from mice harvested on day 8.

A


CD4+ FOXP3- Tconv
WT
CD28-/-
Percentage that are TRANCE+ (%)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Nothing
anti-CD28
anti-CD3
anti-CD3 & anti-CD28

B

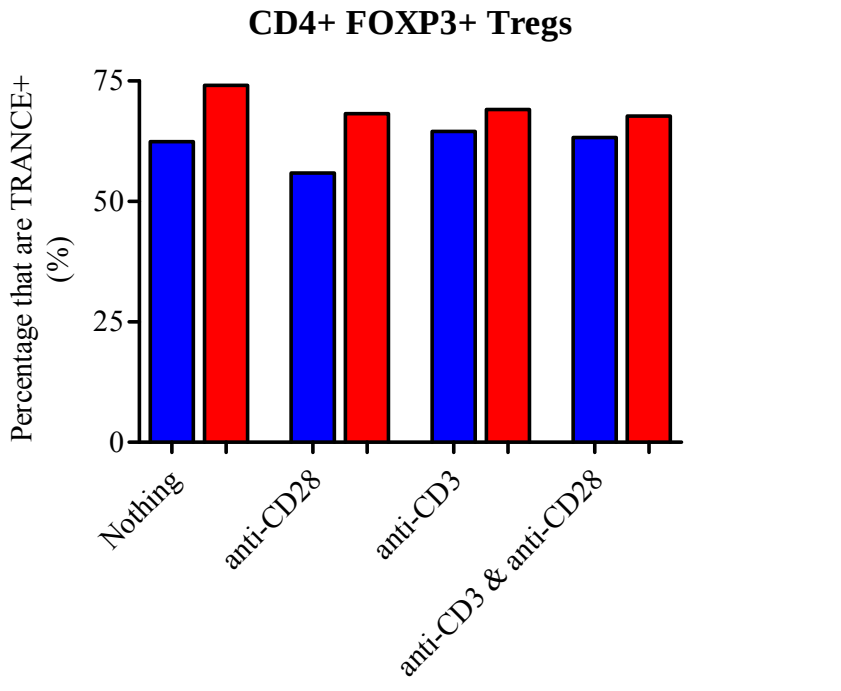
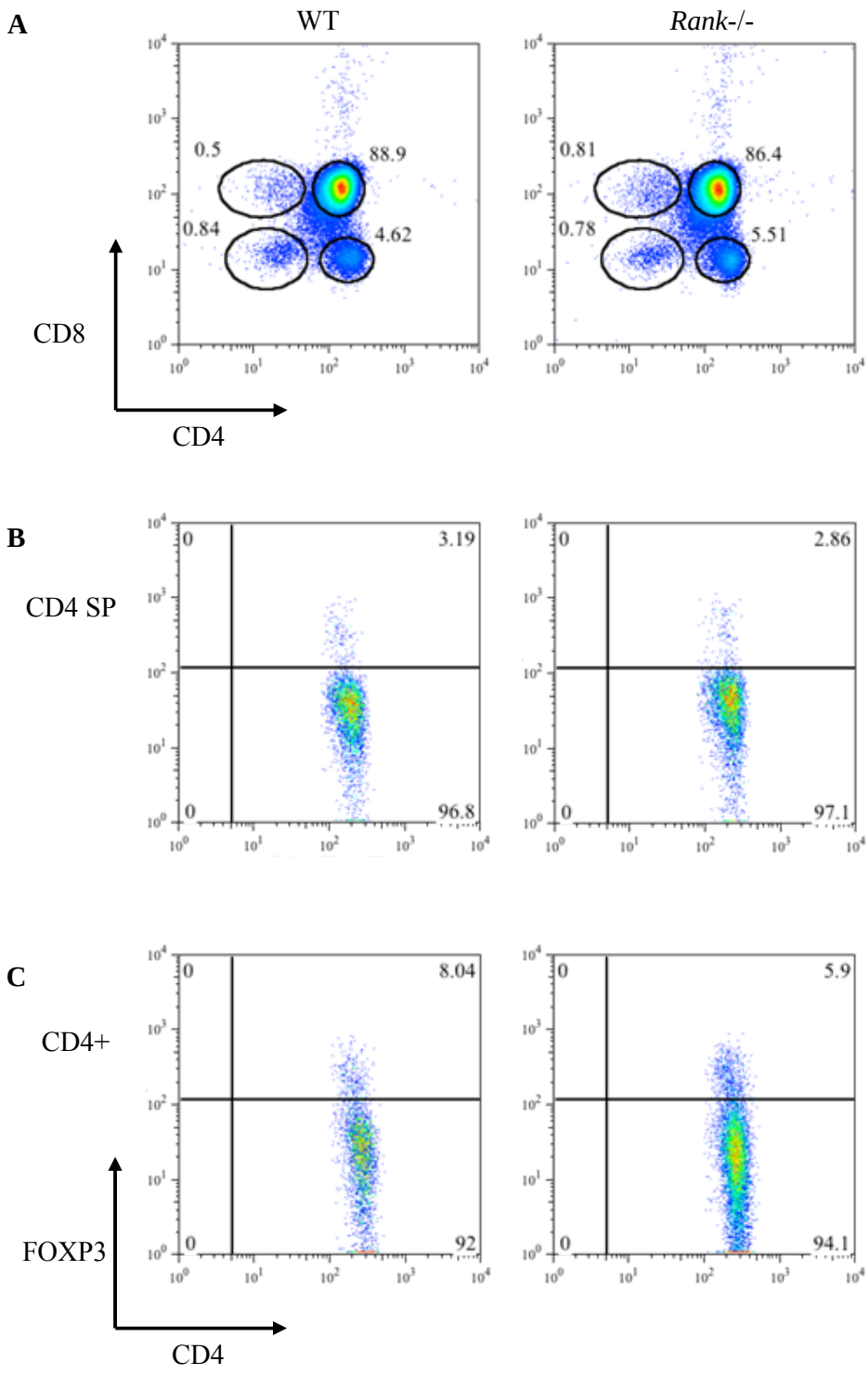

CD4+ FOXP3+ Tregs
Percentage that are TRANCE+ (%)
0
25
50
75
Nothing
anti-CD28
anti-CD3
anti-CD3 & anti-CD28

Figure 5.16. CD28-dependency of TRANCE (RANKL) expression by CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs. Adult BALB/c wild type or *CD28*-/- single cell suspensions from peripheral lymph nodes were cultured overnight plus or minus 500ng/ml anti-CD3 and 1µg/ml anti-CD28. Three wells were pooled and surface stained with CD4, TRANCE and intracellular with FOXP3 for analysis by flow cytometry. Graphs show TRANCE expression by gated CD4+ FOXP3- Tconv (top) and CD4+ FOXP3+ Tregs (bottom). Representative graph from three independent experiments is shown.

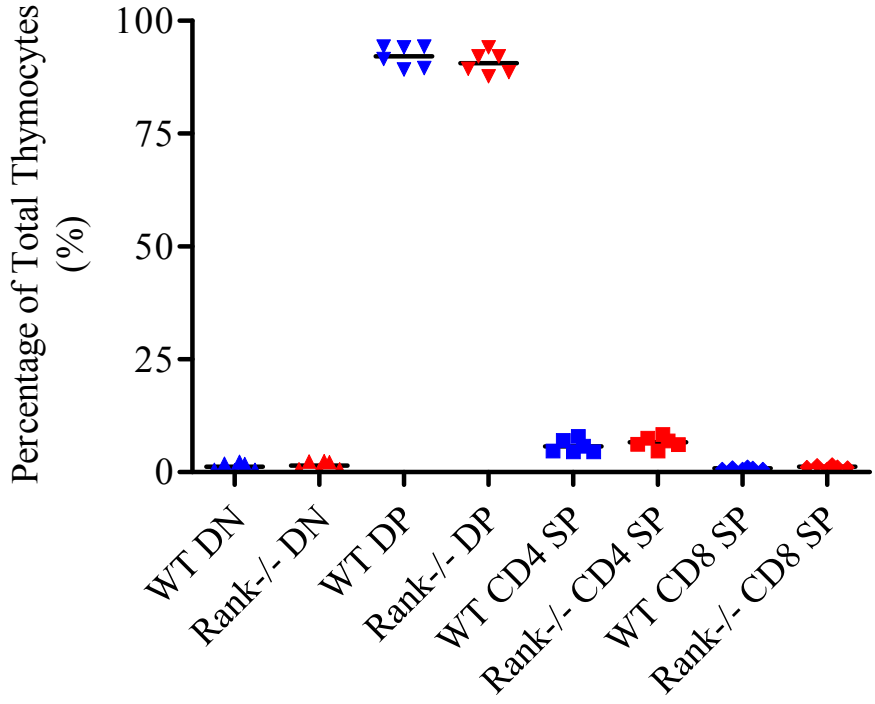
(Tconv) expressed TRANCE compared to wild type. TRANCE expression in Tconv increased in response to TCR-stimulation by anti-CD3 in wild type and *CD28*-deficient lymphocytes. In wild type Tconv, TRANCE expression following CD28 co-stimulation with anti-CD28 in conjunction with TCR-stimulation with anti-CD3 was similar to TCR-stimulation with anti-CD3 alone. As predicted, the anti-CD28 antibody had no affect on *CD28*-/- lymphocytes. Collectively, these data suggest that the up regulation of TRANCE in conventional T cells is CD28-independent.

Parallel analyses were carried out on the CD4+ FOXP3+ regulatory T cell subset. TRANCE expression by *CD28*-deficient CD4+ FOXP3+ Tregs (*Fig. 5.16 B*) was increased compared to wild type. In wild type and *CD28*-/- Tregs TCR-stimulation or CD28 co-stimulation did not alter TRANCE expression. Overall, the data suggest that TRANCE expression by CD4+ FOXP3+ regulatory T cells is not augmented by anti-CD3 or anti-CD28 treatment.

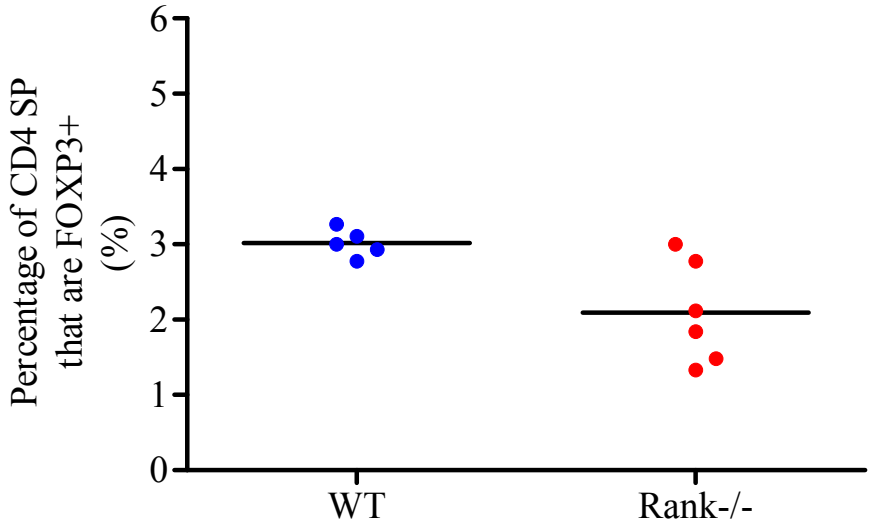
To investigate the role of the TNF receptor RANK in regulatory T cell development and homeostasis *in vivo, Rank* knockout mice were analysed by flow cytometry. No difference in the proportions of the CD4/CD8 T cell populations within the thymus were observed in *Rank*-deficient mice compared to C57BL/6 age-matched controls (*Fig. 5.17 A and D*). However, the proportion of CD4 single positive thymocytes that expressed FOXP3 was slightly decreased (*Fig. 5.17 B and E*). This suggests that the thymic development of CD4+ FOXP3+ regulatory T cells is negatively affected in *Rank*-deficient mice, but CD4+ FOXP3+ Tregs are not absent altogether. *Rank* knockout mice lack lymph nodes, therefore splenocytes were analysed to assess the peripheral homeostasis of CD4+ FOXP3+ Tregs. *Fig. 5.17 C and F* show that in the periphery the proportion of CD4+ T cells that expressed FOXP3 was decreased in *Rank*-/- mice compared to age-matched wild type controls. Even with the decreased proportion of CD4+ FOXP3+ Tregs in *Rank*-/- mice, the absolute number of peripheral CD4+ FOXP3+ Tregs was increased compared to C57BL/6 age-matched controls (*Fig. 5.17 G*). This could be due to the splenomegaly in *Rank*-deficient mice as shown by the greater spleen mass (*Fig. 5.17 H*) and increased overall number of splenocytes (*Fig. 5.17 I*) in the *Rank*-/- spleen compared to the spleen of a C57BL/6 age-matched controls.


A
WT
Rank-/-
0.5
88.9
0.84
4.62
0.81
86.4
0.78
5.51
CD8
CD4
B
CD4 SP
0
3.19
0
96.8
0
2.86
0
97.1
C
CD4+
0
8.04
0
92
0
5.9
0
94.1
FOXP3
CD4

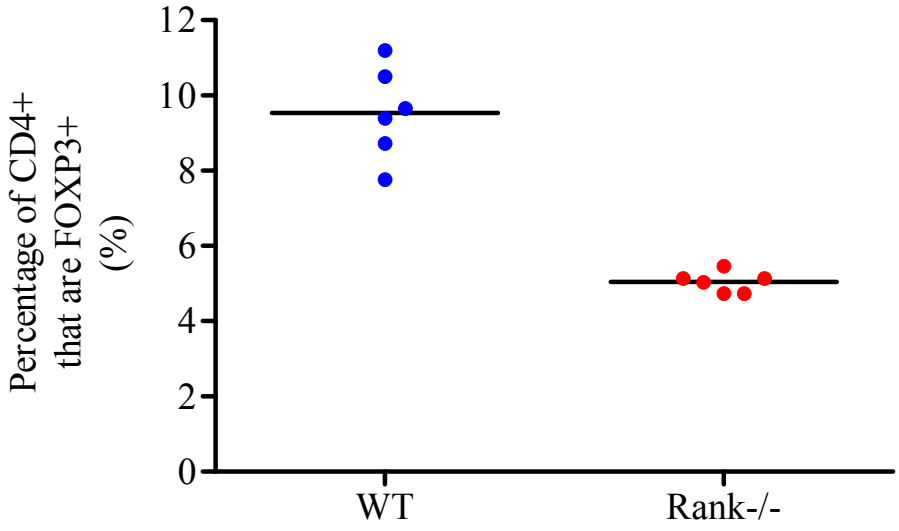
D


Percentage of Total Thymocytes (%)
100
75
50
25
0
WT DN
Rank-/- DN
WT DP
Rank-/- DP
WT CD4 SP
Rank-/- CD4 SP
WT CD8 SP
Rank-/- CD8 SP

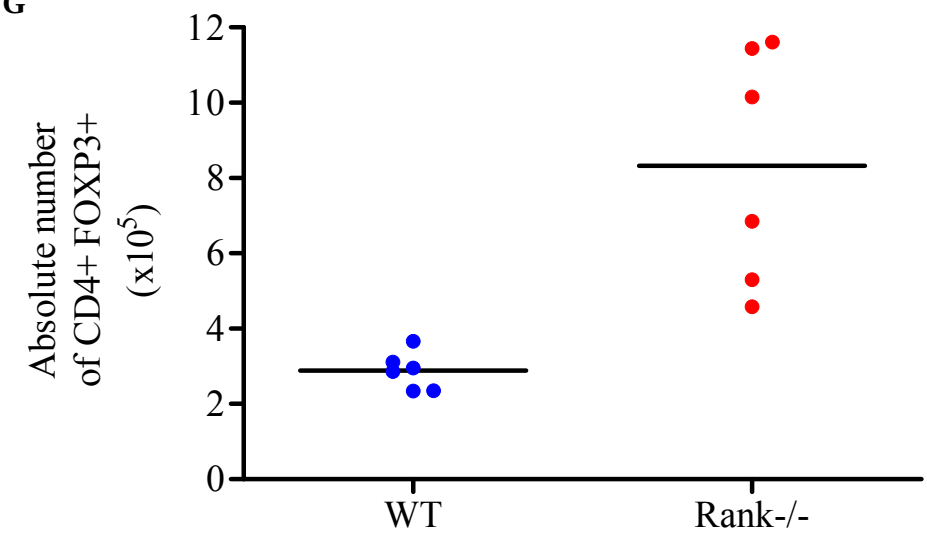
E


Percentage of CD4 SP that are FOXP3+ (%)
6
5
4
3
2
1
0
WT
Rank-/-

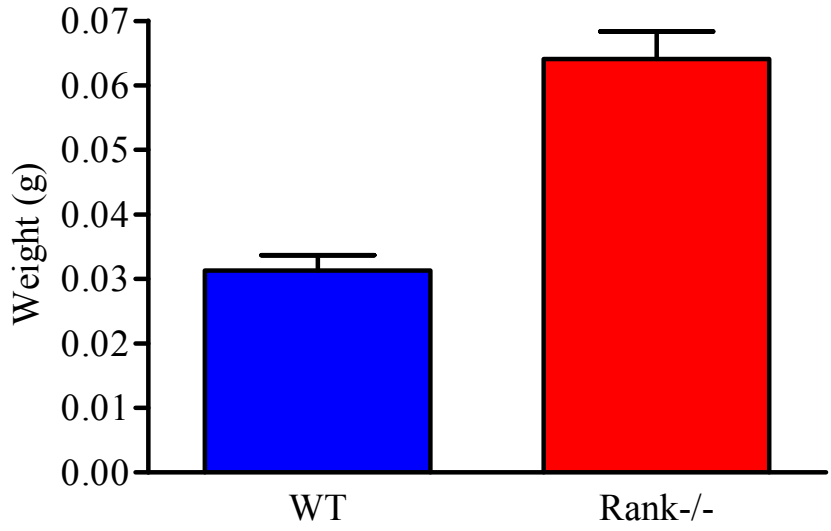
F


Percentage of CD4+ that are FOXP3+ (%)
12
10
8
6
4
2
0
WT
Rank-/-

G


Absolute number of CD4+ FOXP3+ (x10^5)
12
10
8
6
4
2
0
WT
Rank-/-

H


0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Weight (g)
WT
Rank-/-

I

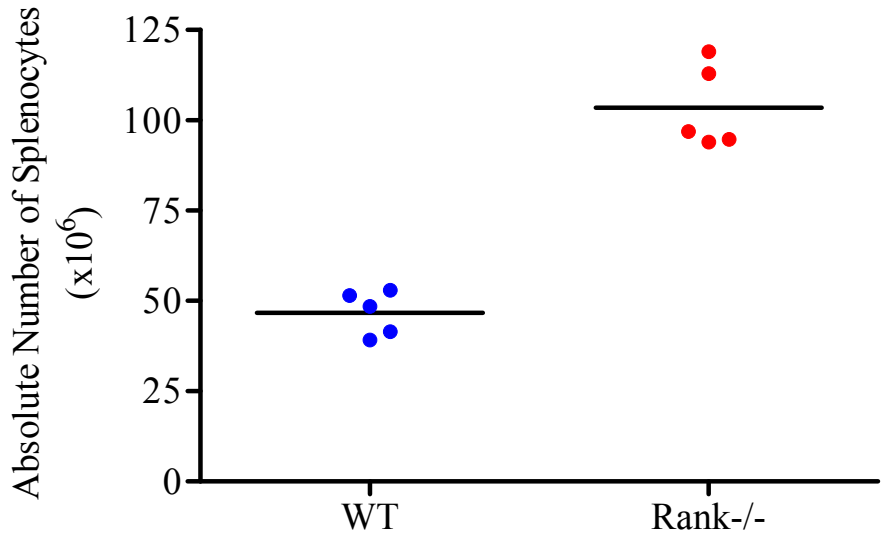
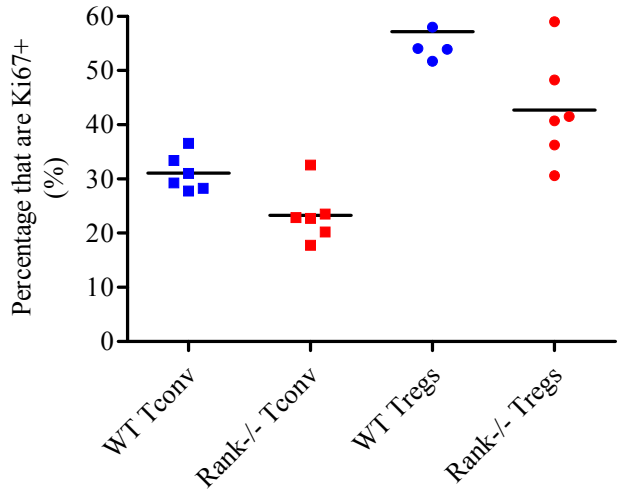

125
100
75
50
25
0
Absolute Number of Splenocytes
(x10^6)
WT
Rank-/-

Figure 5.17. CD4+ FOXP3+ cells are present in the thymus and peripheral spleen of *Rank*-/- mice. Single cell suspensions from the thymi and spleens of 15 day old *Rank*-/- mice and age-matched wild type C57BL/6 control mice were analysed by flow cytometry. Representative FACS plots for (A) CD4 and CD8 expression by thymocytes, (B) FOXP3 expression by CD4 single positive thymocytes and (C) FOXP3 expression by CD4+ gated splenocytes. (D) Graph shows CD4/CD8 percentage composition of the thymus for a number of mice. (E) Graph to show FOXP3 expression by CD4 single positive thymocytes. (F) Graph shows FOXP3 expression by CD4+ gated splenocytes. (G) Graph to show the absolute number of CD4+ FOXP3+ splenocytes. (H) Graph shows weight of 15 day old C57BL/6 and *Rank*-/- spleens. (I) Graph to show absolute cell counts of 15 day old C57BL/6 and *Rank*-/- spleens. Each point on the graphs represents a different mouse and lines indicate mean values.

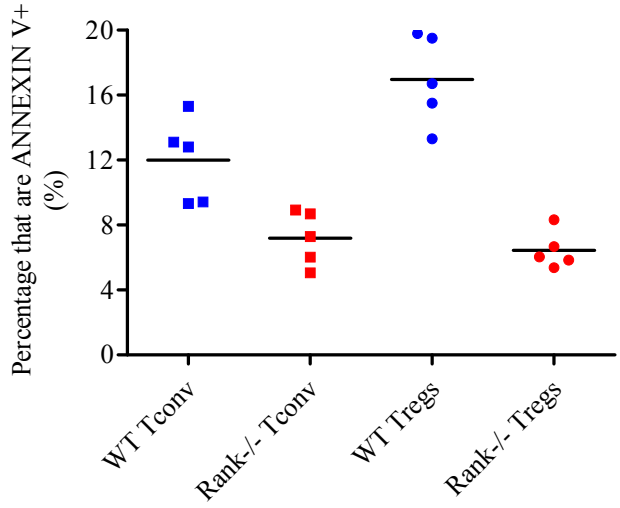
The data above suggest that CD4+ FOXP3+ Treg homeostasis is negatively affected in *Rank*-deficient mice. This might be accounted for by the reduced selection of CD4+ FOXP3+ Tregs in the thymi of *Rank* knockout mice or altered Treg proliferation and/or survival in the periphery of *Rank*-/- mice. To further explore peripheral CD4+ FOXP3+ regulatory T cell homeostasis in *Rank*-deficient mice, CD4+ T cells in *Rank*-/- splenocytes were phenotyped by flow cytometry. The proportion of CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs undergoing proliferation, as indicated by expression of the cell cycle marker Ki67, was decreased in *Rank*-deficient mice compared to age-matched wild type controls (*Fig. 5.18 A*). Additionally, expression of the cell death marker ANNEXIN V was also decreased in both *Rank*-/- conventional T cells and regulatory T cells (*Fig. 5.18 B*). Consistent with the reduced T cell death in *Rank*-deficient mice, Tconv and Tregs expressed increased amounts of the anti-apoptotic Bcl-2 protein compared to their wild type counterparts (*Fig. 5.18 C*).

In this study it has previously been discussed how the co-stimulatory receptor CD28 has a major role in regulatory T cell homeostasis. Similarly, the CTLA-4 receptor which shares the common ligands CD80 and CD86 with CD28 has been found to function in the control of peripheral Treg homeostasis. As discussed above, the peripheral homeostasis of Tregs was found to be altered in *Rank*-/- mice. Therefore, the expression of the CD28 and CTLA-4 receptors was analysed in *Rank*-deficient mice. In CD4+ FOXP3- conventional T cells and CD4+ FOXP3+ regulatory T cells from *Rank* knockout mice expression of both CD28 and CTLA-4 was similar to that in C57BL/6 age-matched controls (*Fig. 5.18 D and E*). To further examine the peripheral homeostasis of T cells in *Rank*-deficient mice flow cytometry was used to phenotype the activation status of T cells by investigating CD69, CD62L and ICOS expression. CD69 expression by CD4+ FOXP3+ Tregs in *Rank*-/- mice was increased compared to age-matched wild type controls (Fig. 5.18 F). However, no change in the proportion of Tconv or Tregs that down regulated CD62L was observed in *Rank* knockout mice (*Fig. 5.18 G*). Similarly, ICOS expression was unchanged, or if anything slightly decreased on CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs from *Rank*-deficient mice (*Fig. 5.18 H*). Also, expression of RANK ligand (TRANCE) by conventional T cells and regulatory T cells was increased in *Rank*-/- mice compared to C57BL/6 age-matched controls (*Fig. 5.18 I*). Overall, the CD4+ T cells in the periphery of *Rank*-deficient mice did not exhibit a phenotype indicative of an activated T cell.

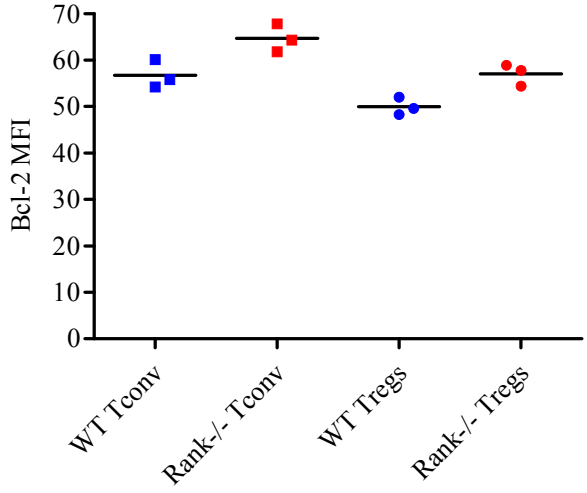
A


Percentage that are Ki67+ (%)
60
50
40
30
20
10
0
WT Tconv
Rank-/- Tconv
WT Tregs
Rank-/- Tregs

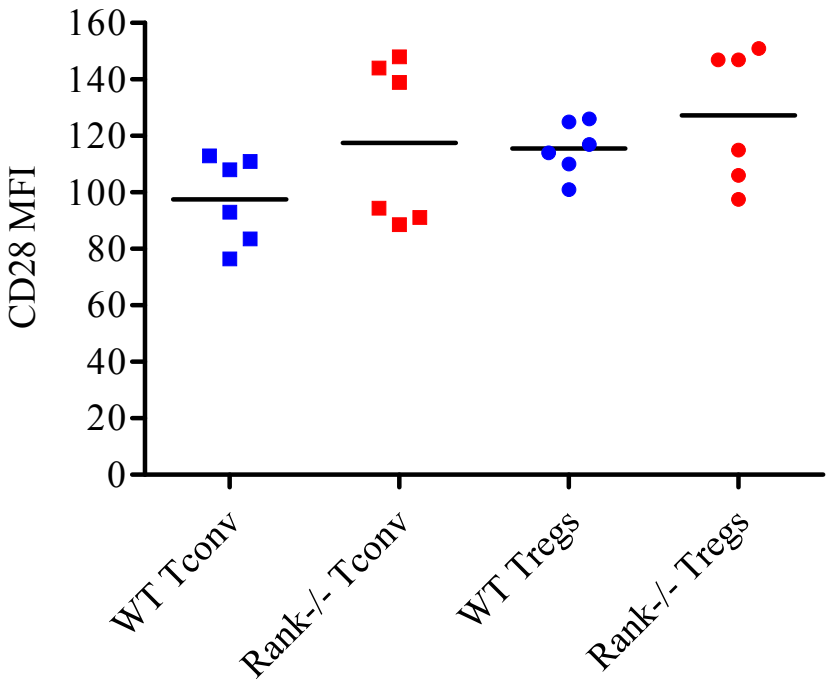
B


Percentage that are ANNEXIN V+ (%)
20
16
12
8
4
0
WT Tconv
Rank-/- Tconv
WT Tregs
Rank-/- Tregs

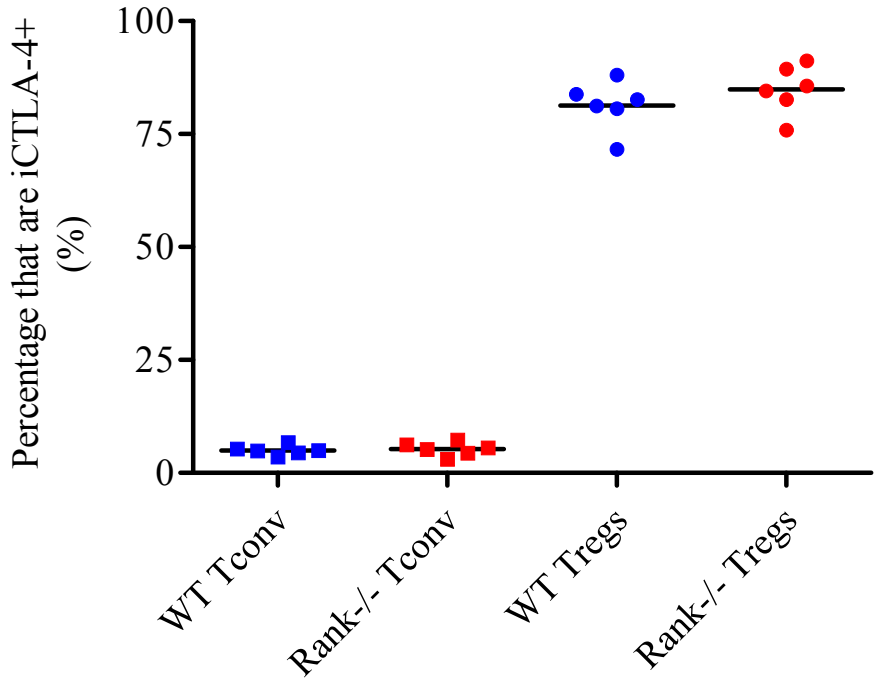
C


Bcl-2 MFI
70
60
50
40
30
20
10
0
WT Tconv
Rank-/- Tconv
WT Tregs
Rank-/- Tregs

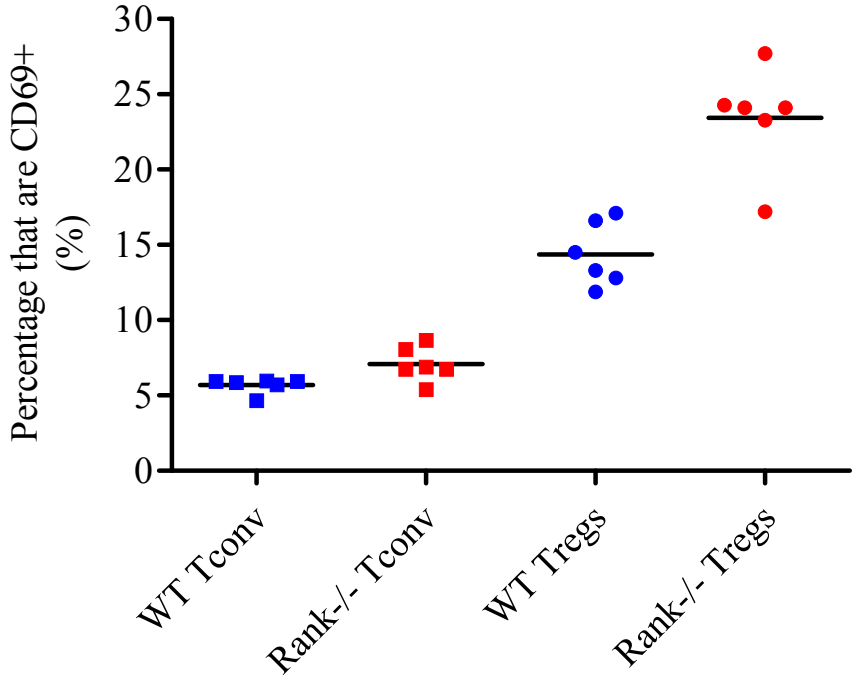
D


160
140
120
100
80
60
40
20
0
CD28 MFI
WT Tconv
Rank-/- Tconv
WT Tregs
Rank-/- Tregs

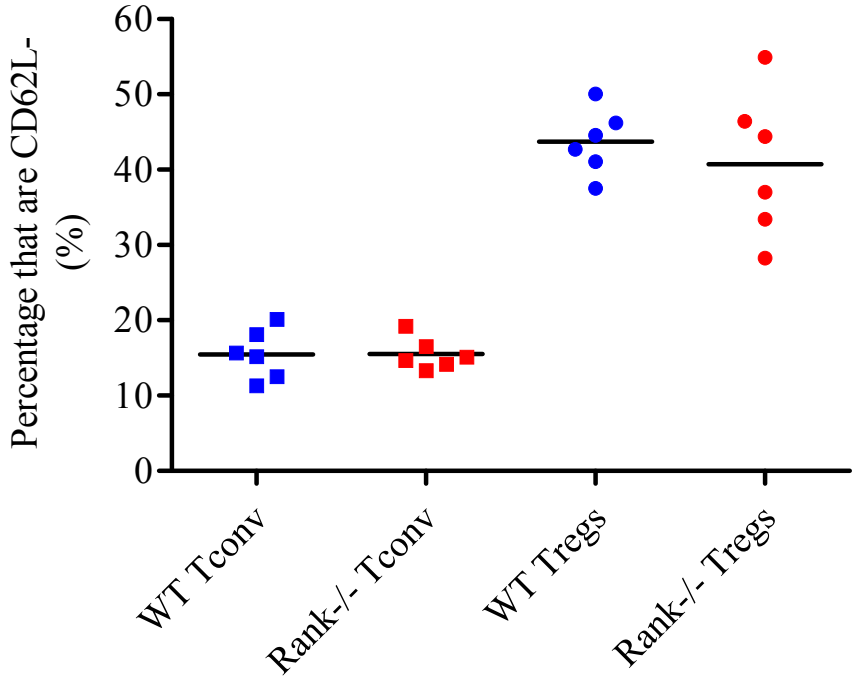
E


100
75
50
25
0
Percentage that are iCTLA-4+ (%)
WT Tconv
Rank-/- Tconv
WT Tregs
Rank-/- Tregs

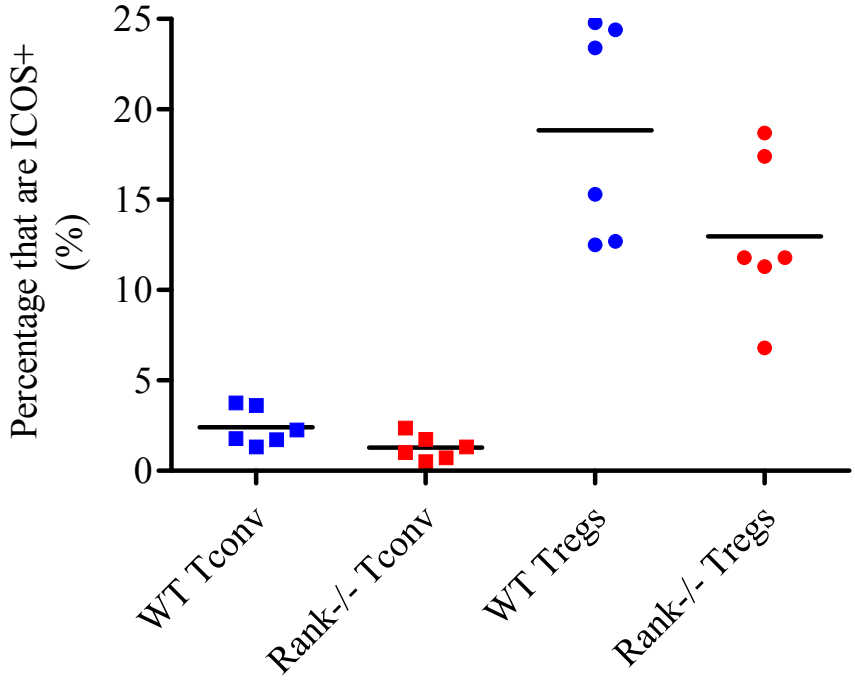
F


Percentage that are CD69+ (%)
30
25
20
15
10
5
0
WT Tconv
Rank-/- Tconv
WT Tregs
Rank-/- Tregs

G


Percentage that are CD62L- (%)
60
50
40
30
20
10
0
WT Tconv
Rank-/- Tconv
WT Tregs
Rank-/- Tregs

H


Percentage that are ICOS+ (%)
25
20
15
10
5
0
WT Tconv
Rank-/- Tconv
WT Tregs
Rank-/- Tregs

I

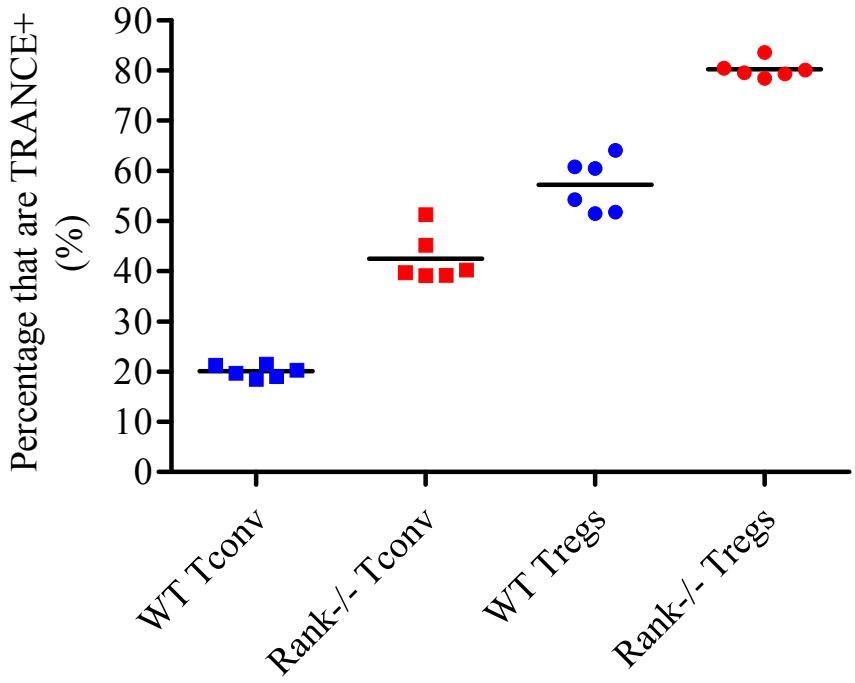

Percentage that are TRANCE+ (%)
90
80
70
60
50
40
30
20
10
0
WT Tconv
Rank-/- Tconv
WT Tregs
Rank-/- Tregs

Figure 5.18. CD4+ FOXP3- Tconv and CD4+ FOXP3+ Treg phenotype in *Rank*-/- mice. Day 15 C57BL/6 and *Rank*-/- splenocytes were analysed by flow cytometry for Ki67 (A), ANNEXIN V (B), Bcl-2 (C), CD28 (D), CTLA-4 (E), CD69 (F), CD62L (G), ICOS (H) and TRANCE (I) expression by CD4+ FOXP3- Tconv and CD4+ FOXP3+ Tregs. Each point on the graphs represents a different mouse and lines indicate mean values.

Having identified a population of CD4+ FOXP3+ cells in the periphery of *Rank*-deficient mice it is of interest to investigate the suppressive function of these regulatory T cells. CD4+ CD25+ Tregs were sorted from wild type and *Rank*-/- splenocytes and assayed for suppressive function in an *in vitro* assay. It was found that CD4+ CD25+ Tregs from *Rank*-/- mice suppressed the growth of CD4+ CD25- effector T cells to the same extent as wild type CD4+ CD25+ Tregs (*Fig. 5.19*). In summary, CD4+ FOXP3+ regulatory T cells develop in the thymus of *Rank*-deficient mice and are exported to the periphery. These peripheral CD4+ CD25+ Tregs in *Rank* knockout mice exert suppressive function *in vitro*.

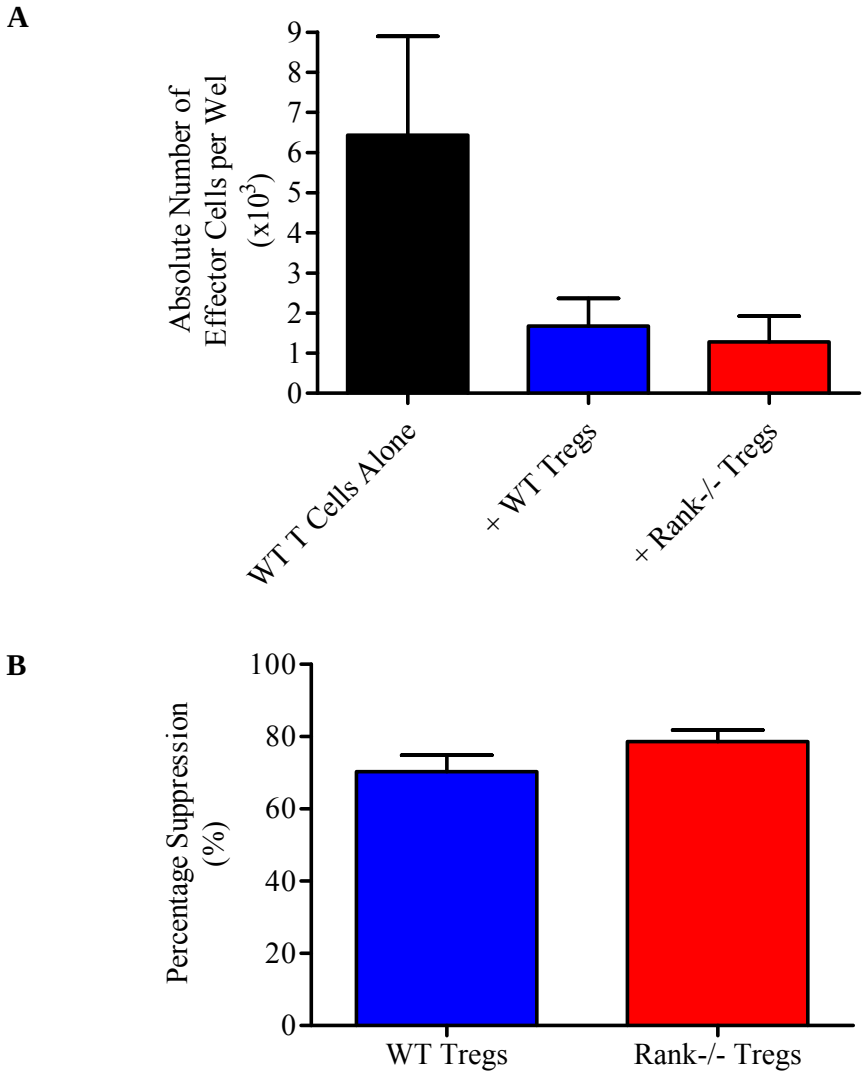


Figure 5.19. CD4+ CD25+ Tregs from *Rank*-/- mice suppress effector T cell proliferation *in vitro*. Single cell suspensions from C57BL/6 and *Rank*-/- spleens were MACS separated to obtain CD4+ CD25- Tconv and CD4+ CD25+ Tregs. 2.5 x 10^4 C57BL/6 Tconv were co-cultured with 15 x 10^4 MACS separated CD19+ from C57BL/6 spleen, 1µg/ml anti-CD3 and C57BL/6 or *Rank*-/- Tregs at a 1:1 ratio for 3 days. Cells were then surface stained with CD4, CD45.1 and intracellular for FOXP3 for analysis by flow cytometry. (A) Graph shows absolute number of CD45.1+ CD4+ FOXP3- effector T cells following culture with C57BL/6 or *Rank*-/- Tregs. (B) Graph shows the percentage suppression as determined using the absolute number of effector T cells when cultured with and without Tregs. Bars represent means and standard deviations are shown. Data shown is representative of two independent *in vitro* suppression assay experiments.

5.3 Discussion

A potential role for the CTLA-4 receptor in the peripheral homeostasis of Tregs was previously identified in chapter 3 of this study. However, it was unclear from the data obtained using *Ctla-4* knockout mice, with a severe lymphoproliferative syndrome, whether the altered Treg homeostasis observed was a direct cell-intrinsic result of the loss of CTLA-4 in the regulatory T cells, or in response to cell-extrinsic changes in the environment within the diseased mice. Antibody-mediated CTLA-4 blockade studies in healthy adult mice triggered an increase in CD4+ FOXP3+ regulatory T cell proliferation. CD4+ FOXP3+ Tregs would be expected to be the initial cellular target of anti-CTLA-4 blocking antibody since they constitutively express CTLA-4 [154, 155], whereas CTLA-4 is activation induced in CD4+ FOXP3- conventional T cells. The delayed kinetics of the CD4+ FOXP3- conventional T cell proliferative response to anti-CTLA-4 blocking antibody could be because CTLA-4 is up regulated only upon activation in CD4+ FOXP3- Tconv. Alternatively, the data obtained in chapter 4 of this study identified a role for CTLA-4 in Treg function. Thus, anti-CTLA-4 blocking antibody treatment may alter the function of the targeted Treg population which could lead to the expansion of uncontrolled effector T cells. It would be interesting to continue anti-CTLA-4 blocking antibody treatment for a longer period of time to see if upon long term blockade of the CTLA-4 pathway, healthy adult mice succumb to a similar lymphoproliferative disease as gene-ablated mice.

Although the proliferation of conventional T cells was not altered at early time points by anti-CTLA-4 blocking antibody treatment, a non-proliferative change in these cells could be induced that indirectly affects regulatory T cell homeostasis. For instance, it is known that upon activation, CD4+ FOXP3- conventional T cells produce the T cell growth factor cytokine IL-2 [317]. IL-2 has been found to overcome Treg anergy *in vitro* [57] and IL-2 signalling has since been identified as a critical requirement for Treg homeostasis *in vivo* [80]. Furthermore, augmented production of IL-2 by CD4+ FOXP3- conventional T cells in *Ctla-4* knockout mice was observed in chapter 3 (*Fig. 3.26 F*). However, the short-term anti-CTLA-4 blockade experiments in normal mice did not identify any alterations in IL-2 production by CD4+ non-Tregs; rather, they suggest that the steady-state Treg turnover seen *in vivo* [246] is subject to continuous inhibition via CTLA-4. It is possible that the

dysregulated Treg homeostasis is exacerbated in the setting of *Ctla-4*-deficiency given the global lymphocyte activation and production of cytokines that further affect Treg homeostasis.

STAT5 activation has been identified as crucial for signal transduction downstream of the IL-2 receptor and therefore maintenance of regulatory T cells [332, 333]. Consequently, it is possible that other common gamma chain cytokines, such as IL-7 and IL-15, which also activate STAT5, may additionally contribute to the maintenance of Treg homeostasis. The number of FOXP3+ Tregs is not altered by IL-7 or IL-15 deficiency, however disrupted IL-2 receptor signalling plus the loss of IL-7 or IL-15-induced signalling results in a greater decrease in Tregs compared to absence of IL-2-induced signalling alone (reviewed in [334]). It would therefore be interesting to analyse IL-7 and IL-15 production in *Ctla-4*-deficient mice and in wild type BALB/c mice following antibody-mediated CTLA-4 blockade. Although, it has been reported that other common gamma chain cytokines can promote the survival of Tregs, but only IL-2 induces their proliferation and clonal expansion [335].

The CD28 co-stimulatory receptor is a second signal required for T cell activation and proliferation following TCR stimulation by antigen [182]. It has also been established that CD28 co-stimulation supports the survival and promotes the self-renewal of peripheral regulatory T cells [86, 131]. Data was obtained in this present study which supports the idea that CTLA-4 signalling controls regulatory T cell proliferation in response to encounter with self-antigen. Furthermore, the augmented CD4+ FOXP3+ Treg proliferation upon antibody-mediated CTLA-4 blockade was found to be CD28-dependent. This is as would be predicted from the maintenance of *Ctla-4*-deficient mice on a *CD80/CD86*-/- background or anti-CD80/86 blocking antibody treatment to overcome the lethal lymphoproliferative syndrome that develops in *Ctla-4* gene-ablated mice and hinders their study.

The secondary co-stimulatory molecule ICOS was not found to be involved in the augmented Treg proliferation observed upon loss of the CTLA-4 pathway following antibody-mediated blockade. Although, others have previously shown that ICOS critically controls the pool size of FOXP3+ regulatory T cells, as well as effector-memory T cells, in the steady-state and in antigen-specific immune reactions [313]. However, in this study by Burmeister *et al*. analysis

of the CD4+ T cell population as a whole found that ICOS did not influence cell cycle progression, but rather promoted cell survival. Consequently, the decreased occurrence of peripheral Treg cell death observed in *Ctla-4*-deficient mice (chapter 3.4) could be the result of the substantially increased ICOS expression by CD4+ FOXP3+ Tregs in *Ctla-4*-/- mice.

Additionally, the role of the TNF receptor RANK in regulatory T cell development and homeostasis was investigated. A role for the RANKL-RANK pathway in regulatory T cell development has been inferred because RANK has been proposed to be required for positive selection-mediated thymic medulla formation [315, 331], and it has been suggested that medullary thymic epithelial cells (mTECs), that promiscuously express self-antigen under the control of the autoimmune regulator AIRE, functionally determine whether thymocytes develop into FOXP3+ regulatory T cells [79]. The data obtained in this study found that RANK was not absolutely required for regulatory T cell development, as CD4+ FOXP3+ T cells were identified in the thymus and periphery of *Rank*-deficient mice. This could be because the thymic medulla may not necessarily be the exclusive site of central tolerance induction. Liston *et al*. have shown that cortical thymic epithelial cells (cTECs) are sufficient for supporting FOXP3+ Treg lineage differentiation [84]. Furthermore, it has recently been reported by Wirnsberger, Mair and Klein that FOXP3+ regulatory T cell development does not require a dedicated antigen-presenting cell, but rather there is T cell-intrinsic control of thymocyte Treg lineage differentiation [85].

In the present study the peripheral Tregs that developed in *Rank*-deficient mice were found to exert suppressive function *in vitro*. As highlighted in this investigation, it is still not clear whether or not CD4+ FOXP3+ Tregs express RANK and therefore whether it is directly or indirectly required for Treg function. In future, the RANK signal detected by the SYBR green qPCR could be verified by analysis of cDNA obtained from *Rank*-/- cell preparations. Recently, Totsuka *et al*. found that the RANK-RANKL pathway was not required for CD4+ CD25+ Tregs to suppress the proliferation of CD4+ responder cell *in vitro*, but was critically involved in Treg-mediated suppression of colitis *in vivo* [316]. It was hypothesised that RANK may indirectly modulate Tregs via the interaction of RANKL (TRANCE) on Tregs with RANK-expressing dendritic cells. Consistent with this, in this present study TRANCE expression by CD4+ T cells was augmented upon the loss of the CTLA-4 pathway.

This could possibly induce dendritic cell production of T cell growth and differentiation factors such as IL-15 [329] which has surprisingly been shown to induce dose-dependent Treg proliferation in a similar manner to IL-2 [336]. In line with this, Loser *et al*. reported that in the skin, epidermal TRANCE modulated regulatory T cell homeostasis via the activation of dendritic cells, although the molecular mechanisms underlying this control of Treg numbers was not determined [337]. Similarly, it has recently been reported that dendritic cells feedback to control regulatory T cell homeostasis *in vivo* as it was found loss of dendritic cells lead to a loss of Tregs and vice versa [338]. RANK is also expressed by chondrocytes and osteroclasts and been found to be involved in bone physiology [330]. These studies by Kong *et al*. used *Ctla-4*-deficient mice as a source of spontaneously activated T cells that expressed TRANCE, and found that all *Ctla-4*-/- mice displayed severe osteoporosis compared with littermate controls. This suggests that the augmented TRANCE expression by CD4+ T cells in *Ctla-4*-deficient mice is significant enough to have a physiological effect via RANK.

In future, it would be interesting to compliment the *Rank*-/- studies with analysis of *Trance*-deficient mice. Also, it would be useful to generate *Rank*-/- foetal liver chimeras due to the multiple bone and lymphoid developmental defects in *Rank*-deficient mice which lead to a very short life-expectancy. Additionally, the affect of positively and negatively modulating the RANK-RANKL pathway on regulatory T cell homeostasis could be investigated *in vitro* and *in vivo* with agonistic and antagonistic reagents, for example osteoprotegerin (OPG) is a soluble decoy receptor for TRANCE.

6.0 General Discussion

In the periphery, regulatory T cells function to maintain immune homeostasis by suppressing the activation of any self-reactive T cells that avoided thymic central tolerance. It is now well established that loss of peripheral tolerance leads to autoimmunity. With the relatively recent revival in the concept of regulatory T cells, there has been renewed excitement in the possible therapeutic applications of Tregs for autoimmune diseases. However, clinical implementation has proved challenging. For treatment of autoimmune diseases and maintenance of tolerance following transplantation, the main aim is to boost regulatory T cell numbers and function. However, this therapeutic augmentation of tolerance does not want to be at the expense of protective immunity to infection. Additionally, when developing treatments to suppress effector T cells, a simultaneous negative effect on the regulatory T cell population is not desirable. On the other hand, regulatory T cell maintained tolerance is one of the main barriers to overcome in cancer therapy.

The CTLA-4 receptor has long been thought to be important in the maintenance of peripheral tolerance since the observation that gene ablation of *Ctla-4* in mice leads to the development of a lethal lymphoproliferation syndrome [156, 157]. The data obtained in this investigation extend knowledge and understanding of the *Ctla-4* knockout mouse phenotype to include dysregulated Treg homeostasis and function. This is in line with the constitutive expression of the CTLA-4 receptor by regulatory T cells and the hypothesis of a role for CTLA-4 in Treg suppressive function. Overall, it was found that defects in the CTLA-4 receptor may uncouple Treg number and function. This could potentially account for the enhanced regulatory T cell numbers sometimes observed in autoimmune diseases [249, 339] as polymorphisms in *Ctla-4* are associated with autoimmunity [228, 229]. CTLA-4 blockade has recently been shown to increase regulatory T cell numbers in a mouse melanoma model [340] and in prostate cancer patients [268]. These findings could be viewed as discouraging since they suggest regulation is boosted alongside anti-tumor immunity following CTLA-4 blockade. However, in this present study it was found that Treg suppression *in vivo* was compromised in the absence of CTLA-4. This raises the possibility that Tregs expanded by CTLA-4 blockade may show impaired regulation. Thus, CTLA-4 blockade may enhance anti-tumor responses both by increasing effector T cell activation and simultaneously

decreasing Treg function. This was recently assessed in a study using a transgenic mouse model of melanoma that expressed human CTLA-4 to evaluate the independent contributions of CTLA-4 blockade on the conventional and regulatory T cell compartments during cancer immunotherapy [341]. It was found that CTLA-4 blockade on conventional T cells, but not regulatory T cells enhanced anti-tumor responses, whereas CTLA-4 blockade of both T cell populations had synergistic anti-tumor activity. It is possible that enhanced proliferation of regulatory T cells impairs their suppressive function. However, it has previously been demonstrated by others that congenically marked, antigen-specific Tregs tracked *in vivo* exhibit efficient suppression despite extensive proliferation [342].

If in the future Tregs were to be used in the clinic as an immunotherapy for autoimmune diseases, the *ex-vivo* expansion of Tregs would be required to obtain sufficient numbers as natural Tregs constitute only 5-10% of peripheral blood lymphocytes. Investigations such as this present study into the role of various pathways in Tregs homeostasis are critical in the development of a robust *ex-vivo* Treg expansion protocol. For example, the data obtained here suggest that blockade of the CTLA-4 pathway could potentially be used to expand regulatory T cells. However, it was also found that loss of the CTLA-4 pathway impaired regulatory T cell suppressive function in an adoptive transfer mouse model of autoimmune diabetes. Therefore, further work is required to determine whether short-term blockade of the CTLA-4 pathway affects the function of regulatory T cells in the long-term, which could depend on the mechanism by which CTLA-4 functions in Treg suppressive activity.

To obtain sufficient numbers of regulatory T cells to use as immunotherapy, expression of FOXP3 could also be induced in CD4+ FOXP3- conventional T cells. As discussed previously in this study, various *in vitro* experiments and *in vivo* mouse models have clearly demonstrated that CD4+ FOXP3- conventional T cells can be effectively induced to express FOXP3. However, little is known about the physiological occurrence of FOXP3+ regulatory T cell induction in humans. Nadkarni *et al*. have found that anti-TNF-α antibody (infliximab) therapy gives rise to a newly generated CD4+ CD25hi FOXP3+ Treg population in rheumatoid arthritis patients responding to treatment. The data obtained was consistent with the TGF-β-dependent differentiation of CD62L- CD4+ CD25hi FOXP3+ regulatory T cells with potent suppressor activity from CD4+ CD25- T cells [343]. The data obtained in this

present study found that the CTLA-4 receptor may function to restrict the induction of FOXP3 expression in CD4+ FOXP3- conventional T cells. Modulation of CTLA-4 could therefore possibly be used in future to therapeutically induce FOXP3+ regulatory T cells. However, this current investigation found using an *in vivo* mouse model that upon loss of the CTLA-4 receptor, regulatory T cell suppressive function was impaired. Relatively little is understood about the underlying mechanisms of induced Treg suppression. TGF-β-induced FOXP3+ regulatory T cells have previously been shown to exert suppressive function *in vivo* [344, 345]. The data is consistent with antigen-specific Tregs being more potent suppressors than polyclonal Tregs [346, 347], and it has also been demonstrated that TGF-β-induced Tregs can function by inhibiting the stimulatory capacity of dendritic cells [113].

In order to make significant progress in the therapeutic modulation of regulatory T cells it is therefore important to fully understand the cellular and molecular mechanisms underlying Treg homeostasis and function. A major therapeutic goal in the modulation of immune responses is manipulation of the CTLA-4 pathway. However, the initial rationale for this approach (blockade of effector T cell inhibition [348]) has been complicated by the emergence of regulatory T cells which constitutively express the CTLA-4 receptor. The design of strategies to target the CTLA-4 pathway needs to take into account potential effects on both the effector and regulatory T cell populations. Some pathways have been found to possibly differentially regulate regulatory T cells and conventional T cells, but no single pathway has been found to regulate either Tregs or Tconv exclusively. For example, superagonistic anti-CD28 antibodies were identified as a promising novel treatment for autoimmune disease due to the preferential expansion and activation of regulatory T cells over conventional T cells (reviewed in [349]). However, the phase one human clinical trial resulted in a life-threatening cytokine release syndrome [350] as regulatory T cells did not rapidly control the conventional T cell response to the CD28 superagonist stimulation [351]. Comprehensive understanding of regulatory T cell homeostasis and function is particularly important with respect to the plasticity between T cell subsets which has recently become evident, for example, the reciprocal relationship between adaptive Tregs and pro-inflammatory T_H17 cells (reviewed in [352, 353]). For instance, it has recently been demonstrated that FOXP3+ regulatory T cells can be induced to express the pro-inflammatory cytokine IL-17 [285, 354, 355]. Similarly, under the inflammatory conditions of the

Ctla-4-deficient mouse phenotype, it was found in this present study that CD4+ FOXP3+ regulatory T cells were capable of producing IL-17.

Intriguingly, in this present study it was found that *Ctla-4*-deficient Tregs were capable of suppressing effector T cells *in vitro*, but were unable to control the development of autoimmune diabetes *in vivo*. Thus, the capacity to regulate immune responses *in vivo* is not always accurately predicted from *in vitro* suppression assays. This has previously been documented for IL-10 and TGF-β [141, 147]. The discrepancy between *in vitro* and *in vivo* suppression assays may reflect the fact that regulatory T cells are clearly equipped with multiple modes of action [134-137, 356], any of which may be sufficient for suppression in a controlled *in vitro* environment. Consistent with this, it has previously been shown that the ability of *Ctla-4*-/- Tregs to suppress *in vitro* is partially dependent on TGF-β [231], suggesting that this pathway can compensate for the lack of CTLA-4. In chapter 3 of this study it was found that CD4+ FOXP3+ Tregs from *Ctla-4*-deficient mice produced increased TGF-β compared to littermate controls. In contrast, the mechanistic requirements for *in vivo* suppression may vary depending on the nature of the immune response and the tissue site. *In vivo* the trafficking of regulatory T cells to the appropriate target site to mediate suppression can be affected by many things, for example chemokine gradients that direct cellular movement or the crossing of the blood vessel endothelium. Furthermore, it is difficult to manipulate and analyse *in vivo* the process of suppression, such as the active number of regulatory T cells, effector T cells and antigen-presenting cells. In addition there is still the continued debate over where regulatory T cells exert suppressive function *in vivo*; the target tissue or draining lymph node. Finally, over the last decade *in vitro* suppression assays have been widely used to investigate the suppressive function of regulatory T cells, however, no standard, uniform method has been employed with different groups developing and utilising diverse assays.

Overall, the data obtained in this investigation shed new light on why defects in the CTLA-4 receptor could be linked to the onset of autoimmunity and how therapeutic modulation of CTLA-4 could affect the regulatory T cell population.

Appendix

Binding Buffer:

0.1M HEPES, pH 7.4

1.4M NaCl

25mM $CaCl_2$

Complete Medium (C10):

RPMI

100units/ml Penicillin (GIBCO)

100µg/ml Streptomycin (GIBCO)

50µM 2-Mecaptoethanol (2-Me)

10% FCS

Lysis Buffer:

15mM Tris Hydrochloride

112mM Ammonium Chloride

pH 7.2

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