Understanding Control of T cell Responses by CTLA-4

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Abstract

Cytotoxic T Lymphocyte Associate Antigen 4 (CTLA-4) is an important negative regulator of T cell activation. The protein is expressed in activated T cells and can also be found in regulatory T cells. The mechanism of action of this protein remains controversial; it has typically been associated with a cell intrinsic negative signal however, there is increasing evidence that CTLA-4 may act as an effector molecule. Surprisingly, we find that blocking CTLA-4 in a model of T cell activation driven by ligand-expressing transfectants has no effect on either proliferation or cytokine production, suggesting that CTLA-4 does not inhibit in this setting. In contrast, blocking CTLA-4 in a dendritic cell based assay enhances proliferation and cytokine production, only when the amount of co-stimulation is limiting. In these experiments CTLA-4 function correlates with decreased expression of B7 ligands on dendritic cells consistent with the removal of ligands by CTLA-4. Furthermore, the addition of CTLA-4 transfected Jurkat cells acts to suppress T cell responses consistent with a role for CTLA-4 as an effector of suppression. Overall our data do not support a role for CTLA-4 in delivering a ligand-dependent cell-intrinsic negative signal and instead suggest a role for CTLA-4 as an effector molecule which inhibits co-stimulation by APC.

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Introduction

1.1 The Immune Response

The immune system has evolved to protect the host from the array of pathogens encountered over its lifetime. The system requires tight regulation to ensure that the correct level of response is achieved. Too hesitant a response can result in the organism succumbing to infection; conversely, too vigorous a response can result in reactivity to self-antigens, which may result in autoimmunity. There are two broad types of immunity: innate and adaptive. The innate response provides the first line of defence against invading pathogens comprising a broad range of mechanisms designed to generate a rapid response against infection. Such mechanisms include physical barriers like the skin which stop antigens getting into the body, proteins such as the complement system which mediate the killing of pathogens, innate immune cells such as neutrophils and biologically active enzymes that cause the destruction of infected cells [1].

Unlike innate immunity, the adaptive response is antigen specific generating highly adapted responses to specific antigens, which result in memory. Two types of adaptive cells exist, T cells and B cells. Whilst B cells are responsible for antibody production, T cells recognise antigens expressed in a cellular context. T cell antigens are presented on the surface of cells by molecules known as major histocompatibility complexes (MHC). There are two classes of MHC; I and II and they differ in the type of cells that they present antigens on. MHC I molecules present short peptides of both self and non-self antigen on the surface on most cell types in the body. Conversely, MHC II molecules are used by antigen presenting cells (APCs) and they display peptides that have been processed within the cell after ingestion by the APC. The different classes of MHC molecules activate different types of

immune cells. For example, MHC I cells trigger a response from $CD8^+$ T-cells, which are involved in the cytotoxic killing of cells infected by intracellular pathogens. On the other hand, MHC II molecules cause $CD4^+$ T-cells to activate. The $CD4^+$ T-cells are known as helper cells and they are involved in the regulation of both cell mediated and humoral immunity [2]. Historically, there were two major types of helper T-cells known as Th1 and Th2 cells [3], however these subsets have since been expanded to include other cell types such as Th17 cells. Different helper T-cell subsets produce different cytokines upon activation with differing effector functions. For example, Th1 cells produce IL-2 and interferon γ (IFNγ), which stimulate macrophage antigen presentation and the proliferation of $CD8^+$ T-cells hence up-regulating the response against intracellular pathogens [3]. Th2 cells, on the other hand, produce IL-4, 5, 6 and 10, which drive B cells to produce antibodies [3]. In contrast, Th17 cells have been shown to produce IL-17 and play a role in the defence against a range of extracellular pathogens by producing a very strong inflammatory response. Indeed, it has been suggested that these cells are required to clear infection where the Th1 or Th2 response has been inefficient [4].

An important feature of the adaptive immune response is the generation of immune memory. This allows the immune system to act promptly upon detection of previously encountered antigens without causing serious disease. Memory T-cells are thought to differentiate into either central memory cells that circulate in secondary lymphoid organs, or effector memory cells that patrol peripheral tissues [5]. Memory cells survive after antigen clearance; there is some evidence to suggest that development of memory cells requires strong antigen driven signals during the primary response [6]. Subsequent survival is thought not to be dependent on MHC engagement [7, 8], instead it appears that IL-7 and IL-

15 engagement are required for maintenance of the CD4 memory population [9-11]. Despite their potential for survival, the numbers of CD4 memory cells are thought to decline over time with the population having a half life of around 10 years [12], however, it has been shown that numbers can be maintained with persistent viral antigen stimulation[13].

1.2 T-cell development and the T-cell Receptor

T-cells are produced in bone marrow but migrate to the thymus where they undergo the maturation process (**figure 1.2.1**). A number of critical processes take place in the thymus, including the rearrangement of genes encoding the T cell receptor and critically, the selection of T cells expressing TCRs of appropriate and "tolerable" specificities. The T-cell progenitors enter the thymus at the cortico-medullary junction [14] and become double negative 1 cells (DN1) which lack expression of both CD4 and CD8. The double negative cells pass through a further 3 stages as DN cells as they migrate from the cortico-medullary junction to the subcapsular zone. It is during this phase of development that the genes encoding the T-cell receptor (TCR) undergo rearrangement in a process known as V(D)J recombination. This allows the formation of a broad repertoire of antigen specific receptors [3] enabling the T-cell to recognise and hence mount responses against the pathogens it encounters. There are 2types of T-cell receptor (TCR); the most commonα:β receptor and the rarer γ:δ receptor [15]. In each case the extracellular part of the receptor is formed by both an α chain and β chain or a γ and δ chain which are covalently linked together by disulphide bonds [15]. In the cell the TCR forms an association with the CD3 complex; it is this complex that is required for signalling after TCR engagement [16]. After formation of the TCR, the DN cells develop into cells that are both $CD4^+$ and $CD8^+$; these cells are termed double positive cells (DP).

In the cortex, the DP cells are exposed to MHC and antigens. If the T-cells respond too strongly to these self antigens or if they don't respond at all, the cells undergo a process of deletion known as negative selection which helps remove self reactive T-cells [17]. Those cells that respond moderately are positively selected. These cells become single CD4 or CD8 cells and migrate to the medulla [18]. The medulla contains medullary thymic epithelial cells (mTEC), which express a diverse range of tissue specific antigens [19, 20]. The single positive cells are exposed to these antigens and are thought to undergo a further process of negative selection [17]. The mature single positive thymocytes are then exported from the thymus enabling them to enter the circulation. Ultimately, these processes result in a peripheral T cell repertoire, which is both capable of responding to a vast array of foreign antigens whilst at the same time limited in its capacity to respond to self antigens under normal conditions.

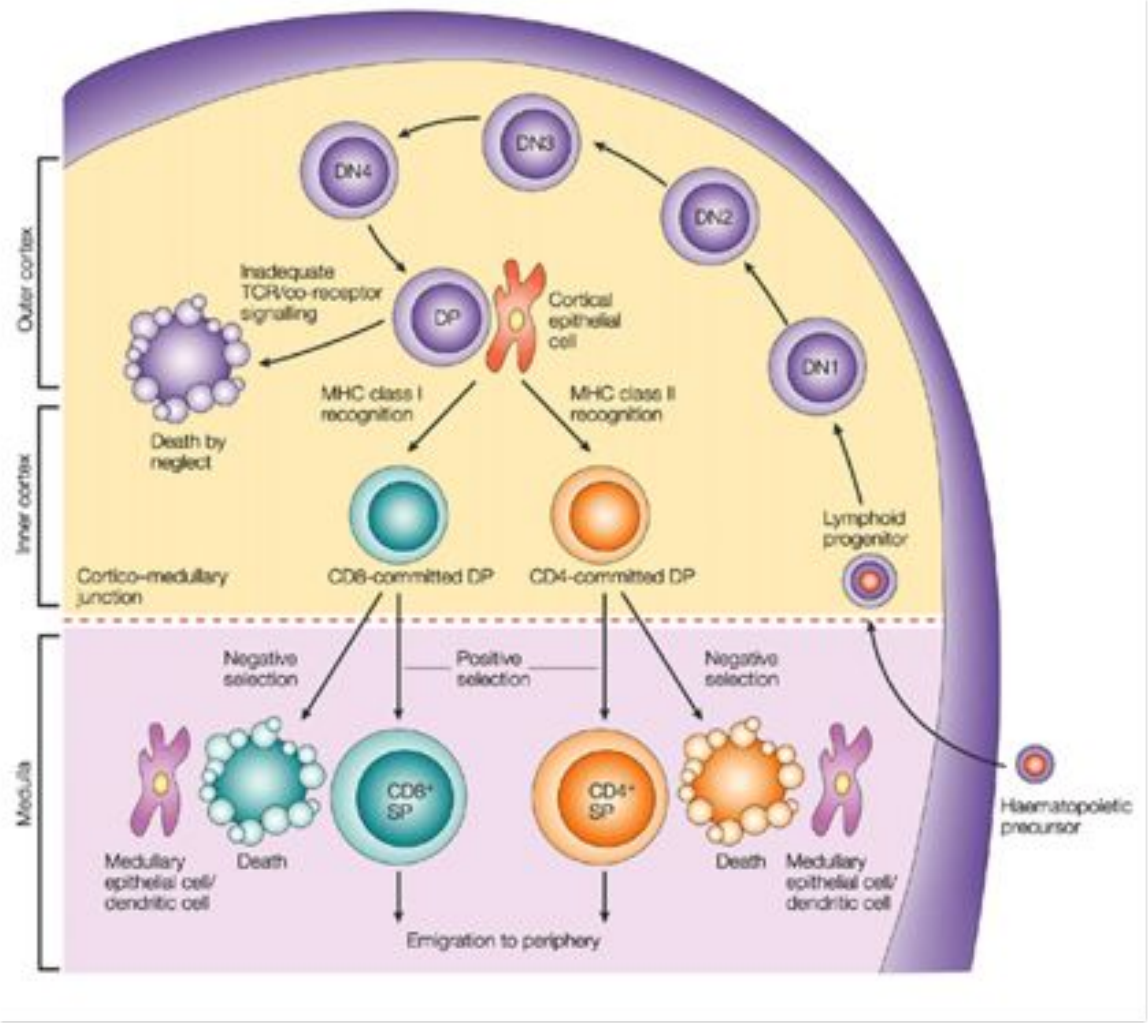

Figure 1.2.1: T cell development in the thymus. Lymphoid progenitor cells enter the thymus at the cortex and progress through 4 stages as DN cells. Rearrangement of the genes encoding the T cell receptor occurs at this stage. The progenitors then become DP cells and are exposed to self antigens. Those cells that respond to strongly are negatively selected and die by apoptosis. Those that respond moderately, are positively selected and migrate to the medulla where they undergo further positive and negative selection. The single positive cells that survive this process are exported from the thymus and enter periphery. Diagram adapted from Germain, 2002.

1.3 T-cell Activation

The effective response of T cells to a foreign antigen first requires the activation and expansion of relatively rare antigen-specific T cells. T-cell activation begins with antigen recognition by the T-cell receptor in the context of MHC molecules on the APC. The alpha/beta T-cell receptor itself is unable to signal and associates with CD3 complex which is responsible for delivering T-cell receptor signalling.

Upon antigen recognition the T-cell and the APC form an interaction known as an immunological synapse (IS) [21], figure 1.3.1. The structure of the IS allows organisation of the T-cell signalling equipment to aid activation. The IS can be organised into three distinct structural regions known as supramolecular activation clusters (SMAC) [22]. The central SMAC (cSMAC) contains the TCR and co-stimulatory molecules [22], whilst the peripheral SMAC (pSMAC) contains adhesion molecules such as LFA-1 [22, 23] and the distal SMAC (dSMAC) contains proteins such as CD45 [24]. Upon T-cell interaction with DCs, multifocal synapses have been shown to form [25, 26]. It is thought that TCR engages with signalling molecules in the dSMAC before migrating through the pSMAC, towards the cSMAC [27-30] making the synapse a structure that enables organisation of the T-cells signalling equipment to aid activation.

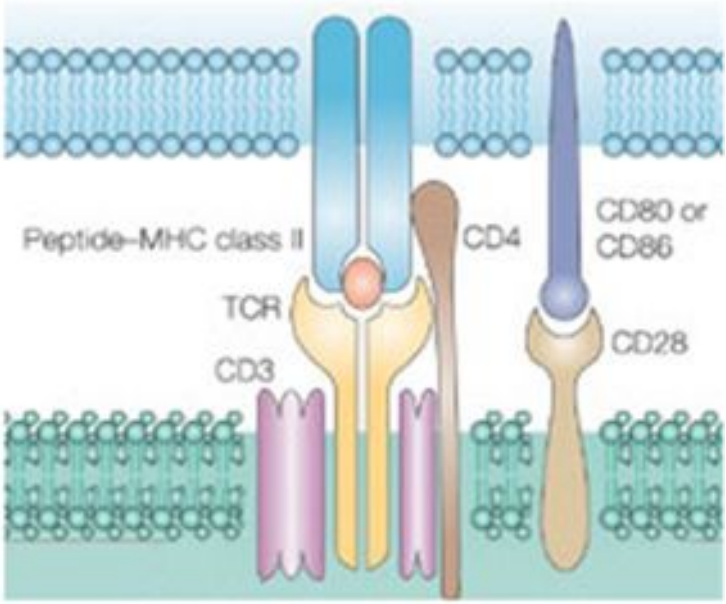

Figure 1.3.1: The Immunological Synapse (IS). The IS is a broad term for the interaction of TcR with the APC. The T cell receptor binds to the antigens presented on MHC class II molecules on the surface of the APC (blue) with the aid of the co-receptor, CD4. Signalling is then mediated by CD3. For full activation of T cells the co-stimulatory molecule CD28 binds to one of two ligands expressed on APC, namely CD80 and CD86. These interactions mediate cell proliferation, survival and cytokine production. Diagram adapted from Friedl *et al*, 2005.

A number of molecules are associated with T-cell receptor signalling. One of the first stages of signalling involves the phosphorylation of the immunoreceptor tyrosine based activation motif (ITAM) of the CD3 and TCR ζ chain by lck and fyn [31-33]. Most evidence points to lck being the major molecule responsible for ITAM phosphorylation, specifically due to the findings that lck knockout cells have significantly reduced ITAM phosphorylation and that this can be recovered by inducing expression of lck [34-37]. ITAM phosphorylation results in recruitment of ZAP-70 to the TCR ζ chain, which enables lck mediated activation of ZAP-70 [38, 39].

Activation of ZAP-70 leads to recruitment of two adapter proteins; linker for the activation of T-cells (LAT) and src homology 2 (SH2) domain containing leukocyte phosphorylation of 76kDa (SLP-76) [40, 41]. LAT binds to the PLCγ1 subunit of phosphoinositide 3-kinase (PI3K) which results in the breakdown of the molecule into two components, diaglycerol (DAG) and tris phosphate (IP3). DAG results in the activation of PKC and IP3 initiates the release of Ca^{2+}. In addition to this, LAT has been shown to activate the Ras-MAPK pathway by binding to Grb2, which leads to recruitment of son of sevenless (Sos) [42]. Finally, LAT can also activate NFAT function through recruitment of SLP-76, which is mediated by LAT binding to Gads [43-45]. Activation of these pathways results in differentiation of effector T-cells and drives their proliferation.

1.4 Co-stimulation

Despite its critical role in generating antigen specificity, TCR/CD3 signalling alone appears to be insufficient and can result in the T-cell becoming anergic or being destroyed [3]. Therefore, in order to generate a highly effective response additional co-stimulatory signal are required.

1.4.1 CD28

One of the most important co-stimulatory molecules identified to date is CD28. The importance of this protein in T-cell activation is most notably shown by the fact that CD28 $^{-/-}$ mice fail to mount sufficient immune responses when challenged with antigen [46]. These mice suffer from decreased IL-2 production, highly impaired ability for IgG class switching and limited T-cell function. This highlights the importance of CD28 in T-cell – B-cell collaboration and its essential role in the induction of an effective, high affinity, class switched antibody response [46]. CD28 binds to one of 2 ligands, CD80 or CD86, found on the surface of APCs where ligation of CD28 induces signalling pathways within the T-cell that result in full T-cell activation, survival, proliferation and cytokine production. Co-stimulation through CD28 is important for cytokine production and it has been shown that signalling via CD28 has been shown to be required for the induction of IL-2 [47, 48]. Further studies have suggested that the CD28 signalling pathway plays a critical role increasing enhancer activity of the IL-2 gene, as well as stabilising of the cytokine's mRNA [49, 50]. In addition to its role in inducing cytokine production, CD28 has been shown to enhance cell survival. Studies have shown that CD28 can up-regulate Bcl-xL and that this is decrease the susceptibility of T-cells to apoptosis [51-56].

The cytoplasmic tail of CD28 contains a YMNM motif that is known to mediate binding of the signalling molecules of PI3K and Grb2 upon phosphorylation following TCR activation [57-60]. Engagement of PI3K results in the production of the phosphatidylinositol (3,4)-bisphosphate (PIP2) and phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) lipids [61] which enable the recruitment proteins containing a pleckstrin homology (PH) domain such as phosphoinositide-dependent protein kinase 1 (PDK1) [62, 63]. Recruitment of PDK1 results in the phosphorylation and subsequent activation of protein kinase B (PKB) [64, 65]. Activation of PKB enables the phosphorylation of BAD, which releases Bcl-2 and Bcl-XL enabling them to exert their anti-apoptotic functions hence promoting T-cell survival [66]. CD28 can also signal by the recruitment of Grb2 to the asparagine residue of the YMNM motif [67, 68]. Grb2 is then thought to recruit Sos and vav [67, 69, 70], which results in the downstream activation of JNK [71]. Whilst the exact role of CD28 signals compared to the role of TCR signals is not well understood, it is clear that combined signals from both partners are required to generate an effective T cell response.

1.4.2 ICOS

ICOS (**I**nducible **COS**timulator) is a co-stimulatory molecule whose expression is up regulated upon T-cell activation [72]. Signalling by this molecule is initiated upon binding its ligand, ICOS-L, which can be found on B-cells, DCs and macrophages [73, 74]. Similar to CD28, ICOS signals through the recruitment of PI3K to its SH2 domain, which results in the downstream activation of JNK, p38 and ERK [75-77]. Further, ICOS has been shown to signal through the Akt pathway leading to the suggestion that the protein is required for cell survival [75, 77-79].

ICOS signalling is also thought to be important for the production of a number of cytokines, including IL-4, IL-10, INF-γ, TNF-α and IL-5 [72, 74, 76, 80{Dong, 2001 #591, 81, 82], this pathway is therefore thought to be required for optimal Th1 and Th2 cell responses and more recently T follicular helper responses. On top of this, there is evidence to suggest that the protein may have a role to play in survival and maintenance of Regulatory T-cells (Tregs) [81, 83-85].

1.4.3 Programmed Death 1 (PD-1)

PD-1 is a negative regulator of immune responses that is up regulated on both T and B-cells upon their activation [86-90]. The protein has two ligands; PD-L1 and PD-L2 [88, 91-93]. PD-L1 can be found on DCs, macrophages, T-cells and B-cells [88, 94, 95], whereas PD-L2 expression is limited to DCs and macrophages [88, 92, 93, 96, 97].

The tail of PD-1 contains an ITIM motif immediately followed by an immunoreceptor tyrosine-based switch motif (ITSM) motif [98] and it is this region that is thought to be important for PD-1 function [99]. It is thought that PD-1 could function through the recruitment of SHP-1 and SHP-2 to the ITSM resulting in decreased Akt function, possibly as a result of inhibition of PI3K activity [93, 99-102]. Other evidence points to the possibility that PD-1 is thought to function through deactivation of the AP-1 transcription factor [103-105].

1.5 Immunological Tolerance

Immunological tolerance is the term used to describe the fact that the immune system does not generally mount a response against self-antigens. There are two categories of immune tolerance, peripheral and central and both play an important role in protecting against the development of autoimmunity.

1.5.1 Central Tolerance

Central tolerance occurs during T lymphocyte development in the thymus. As stated previously, during development T-cells undergo two stages of selection. Firstly, positive selection, where only cells that react to antigens expressed on the thymic cortical epithelium survive. The second stage is negative selection where cells that react too strongly to self-antigens expressed on DCs and other epithelial cells in the medulla are removed. This second is stage is essential for limiting the number of self-reactive T-cells that enter the periphery thus reducing the occurrences of autoimmune disease. This is evidenced by the finding that inhibition of the negative selection step by the removal of the capacity for DCs and medullary epithelial cells to present antigens to the T-cells increases the incidence of pathogenic, self-reactive T- cells entering the periphery [106, 107].

A key feature of negative selection is that self-antigens from various tissues around the body can be expressed in the thymus, particularly in the medullary epithelial cells [19]. The transcription factor Autoimmune Regulator (Aire) was found to be expressed in the medulla and is thought to be responsible for tissue specific antigens being expressed within the thymus and is thus important in preventing autoimmunity [108, 109]. Consistent with this is the finding that autoimmune polyendocrinopathy candidiasis ectodermal dystrophy

(APECED) was associated with a defect in Aire [110]. Subsequent studies using Aire knockout mice showed that these animals developed autoimmunity and that this was specifically associated the absence of Aire [111, 112] adding further weight to the importance of the protein in negative selection. Exactly how Aire regulates gene expression remains open to question although there is some evidence to suggest that Aire binding directly to target DNA sequences in order to drive expression of self antigens [113].

Negative selection of T-cells requires the presentation of self-antigens either on mTECs or DCs [114, 115]. The mTEC cells are able to present antigens to T-cells directly [116-118], however, these cells have been shown to inefficiently process and present antigens using the classical MHC II pathway [117]. Instead, it is thought that mature mTECs attach antigen to MHC-II by a process known as macroautophagy where portions of the cell cytoplasm are enclosed in a membranous structure and are targeted towards lysosomes where the contents are broken down into smaller fragments [119]. These fragments can then be loaded onto MHC-II molecules and presented to T-cells. This process of MHC-II loading has been shown to be important for central tolerance as blocking of this mechanism inhibits positive selection and leads to the development of autoimmunity [120].

Self-antigens are also presented by DCs in the thymus. There is evidence to suggest that the thymic DC population are made up of cells that are derived in thymus as well as cells that migrate there from the periphery [121-123]. The migrating DCs are thought to bring peripheral antigens with them to assist in the negative selection of self reactive T-cells [124, 125]. More generally, there is evidence to suggest that DCs are able to acquire self-antigens from mTEC cells [118, 126-128] although the mechanism by which transfer occurs remains elusive. The fact that there is a naturally high turnover of mTEC cells and that the DCs are able to present antigens from intracellular compartments has led to the suggestion that the

DCs acquire antigen through the uptake of apoptotic cells [118, 129, 130]. Alternatively there is also evidence to suggest that the DCs may acquire antigens through removal of proteins from the surface of the mTECs [127, 128, 131, 132].

1.5.2 Peripheral Tolerance

Despite there being several mechanisms in place to delete overtly self reactive T-cells, it is clear that this process is incomplete [133-138]. Indeed it seems implausible that T cells reactive to all self-antigens could be deleted whilst retaining a sufficiently broad repertoire. For this reason, it is necessary to have other mechanisms in place that will control self reactive T-cells in the periphery. A number of mechanisms exist to control the expansion of self-reactive T-cells in the periphery including clonal deletion, anergy, regulatory T-cells and tolerogenic DCs.

1.5.2.1 Regulatory T-cells (Tregs) and peripheral tolerance

Another critical control point for self-reactive T cells is the generation of Tregs. These cells are generally identified as a subset of T-cells that constitutively express CD25 [139]. Depletion of Treg cells in mice results in development of autoimmune disorders, highlighting a requirement for these cells in tolerance [140, 141]. Additionally, it has been shown that stimulation of peripheral blood samples delpletied of Treg cells revealed the presence of responder T-cell populations that had reactivity to a number of self-antigens. Subsequent re-introduction of Tregs to these cultures was sufficient for suppression of these self-reactive cells [142-145]. Further, it is possible to induce tissue specific autoimmune

conditions by injecting self antigens into mice [146]. In experiments where Tregs were depleted prior to introduction of self-antigen, it was found that this exacerbated the resulting autoimmunity [147, 148]. Taken together, these data highlight the importance of Tregs in the maintenance of peripheral tolerance.

1.5.2.2 Clonal Deletion in the Periphery

It has been shown that clonal deletion of self-reactive T-cells s not restricted to the thymus and that this process can also occur in the periphery. T-cell survival in the periphery is dependent on the recognition of MHC molecules in the absence of antigen. This is evidenced by the finding that naïve T-cells transferred into MHC deficient mice have significantly shortened lifespans [149]. One of the pathways thought to be important in tolerance is the Fas/FasL pathway which causes cell to undergo apoptosis in a process known as activation induced cell death [150]. Both TCR signalling and Il-2 are required for the induction of FasL expression on T-cell [150]. The importance of this pathway of cell death in maintaining tolerance is indicated from findings that Fas or FasL knockout mice develop autoimmune disorders [151-153].

Other studies have indicated a role for passive cell death in controlling tolerance. Passive cell death occurs in the absence of growth factors that are required for T-cell survival and proliferation [154]. This is indicated by the finding that mice deficient in Bim, a pro-apoptotic factor, are resistant to cell death under conditions where cytokines are absent [155]. Further, Bim knockout mice developed autoimmunity, consistent with a role for this pathway in tolerance [155]. This pathway is thought to be important for the removal of T-cells after clearance of infection [156, 157]

1.5.2.3 Anergy

One suggested mechanism for preventing the activation of self reactive T-cells is known as anergy [158]. When T-cells encounter an antigen that results in signalling through the T-cell receptor without subsequent co-stimulation, they enter a state of hypo responsiveness [159]. The requirement for co-stimulation appears to relate to the need for IL-2 production in order to drive the T-cell through the cell cycle [160, 161]. Once these cells become anergic, it is thought that they are able to suppress other effector cells using cytokines such as IL-10 and TGF-β [162-165].

Anergised cells have been shown to have a distinct gene expression profile [166-168]. Specifically, it has been shown that anergic cells have up-regulated expression of ubiquitin E3 ligases, diaglycerol kinase α (DKGα), caspase 3 and Ikaros [168-170]. The genetic profile induced during anergy thought to be driven by nuclear factor of activated T-cells (NFAT) signalling as inhibition of this pathway by cyclosporine A inhibited expression of the genes associated with anergy [168]. This altered genetic profile of T-cells is thought to suppress TCR signalling, therefore suppressing T-cell proliferation [171-173].

1.5.2.4 Tolerogenic Dendritic Cells

DCs are thought to play a critical role in peripheral tolerance as removal of DCs in mice results in the activation of T-cells leading to fatal autoimmunity [174]. Initial reports suggested that immature DCs are capable of inducing tolerance [175-178] whereas mature DCs stimulate immunogenic responses [179-182], an effect thought to arise as a result of the

immature DCs delivering TCR stimulation with inadequate co-stimulatory signals [183]. Subsequent studies have shown that mature DCs can also have a regulatory phenotype. Specifically, it has been shown that DCs matured with tumour necrosis factor-α (TNF-α) had a regulatory phenotype and protected mice from the development of experimental autoimmune encephalomyelitis (EAE) [184]. Additionally, mature DCs have been shown to be capable of presenting self-antigens in draining lymph nodes, a process thought to be important for peripheral tolerance [185, 186]. Tolerogenic DCs are thought to function by up-regulating the inhibitory receptors PD-1 and CTLA-4 on T-cells as well as through the induction of Tregs [187, 188].

1.6 Regulatory T-cells

These cells are generally identified as a subset of T-cells that constitutively express CD25 [139]. Furthermore, these cells can be distinguished from activated T-cells by expression of the transcription factor forkhead box protein 3 (Foxp3). In humans however, while Treg do express FoxP3, a subset of activated T-cells have also been shown to be capable of up-regulating this transcription factor [189]. However, this expression of FoxP3 in activated T-cells appears to be transient and occurs at lower levels than is found in Treg [190]. Nonethless, in both humans and mice FoxP3 is absolutely required for the development and maintenance of Treg [191, 192] and accordingly, scurfy mice, which have a mutation if the FoxP3 gene develop lymphoproliferative and autoimmune disease [193]. In humans, a similar mutation in the FoxP3 gene results a condition known as immune dysregulation, polyendocrinopathy, enterophathy x-linked (IPEX) which is characterised by autoimmune endocrinopathy, type I diabetes and thyroiditis [194]. This profound autoimmune phenotype is testimony to the fact that our T cell repertoire is in fact highly self-reactive and that control of these cells is critical to prevent autoimmunity.

1.6.1 Treg Development

The majority of Regulatory T-cells develop in the thymus, known as natural Tregs, however, there is also a subset of Tregs that are induced in the periphery. Natural Treg cell development is thought to begin with induction of FoxP3 expression in the medulla of the thymus [195]. There is evidence to suggest that TCRs recognising self antigen with a medium avidity [116, 196-201] and subsequent co-stimulation is required to induce the development of Treg [202, 203]. Under these conditions, TCR signalling results in activation

of the NF-κB pathway, which is in turn responsible for the induction of FoxP3 expression [204-206]. The cytokines present in the thymus have also been observed to play a role, in particular, IL-2 has been shown to be crucial, further, Treg generation via IL-2 is thought to be mediated via JAK 1/3 and signal transducer and activator of transcription 5 (STAT 5) signalling [207-210]. Once a cell becomes a Treg, it leaves the thymus where it suppresses activation of effector T-cells against self-antigens and is thus a major player in mediating peripheral tolerance.

As well as natural Treg, it is also possible for effector T-cells to be converted into Tregs in the periphery; these cells are known as induced Tregs (iTreg). Induction of Treg occurs through a combination of TCR, IL2 and TGF-β signalling [211-213], although the exact mechanism and balance of signals required for iTreg induction remains elusive.

There are two subsets of iTregs: type 1 regulatory cells (Tr1) and Th3 cells. The Tr1 cells are a subset of regulatory cell that secrete high levels of IL-10 and TGF-β but that lack FoxP3 expression [162]. These Tr1 cells are thought to arise in the presence of IL-27 secreted from tolerogenic DCs [214-217]. Secretion of IL-27 is thought to activate the transcription factor Maf and the arylhydrocarbon receptor (Ahr) [218, 219].

The second subset of induced Tregs are the Th3 cells and these are thought to arise from a combination of IL-2 and TGF-β signalling in the periphery [211-213]. The signalling pathways induced by these molecules are thought to target transcription factors, such as STAT5, NFAT and CREB/ATF, to the FoxP3 promoter as well as inducing Smad signalling to stimulate the expression of FoxP3 [206, 220-222]. There is evidence to suggest that T-cells

lose the capacity for becoming Tregs 2-3 days post stimulation and that memory T-cells are unable to be converted to iTreg cells [212]. Additionally, it appears that low doses of high affinity ligands were necessary for generation of Tregs [223, 224]. Taken together, this data implies that a very specific set of environmental conditions are required for the generation of Th3 cells.

1.6.2 Mechanisms of Treg Mediated Suppression

There is a growing body of evidence to suggest that Tregs use various means of suppressing the action of effector T-cells depending on specific microevironmental conditions. Some of the major mechanisms are suggested below.

1.6.2.1 Cytotoxic T Lymphocyte Antigen 4 (CTLA-4)

CTLA-4 is an important negative regulator of T-cell activation with a role to play in the suppression of immune responses by Tregs [225]. This is indicated by the finding that Tregs express constiutively high levels of CTLA-4 in both humans and mice [226-229]. Further, blocking CTLA-4 results in mice results in the development of autoimmunity, whilst CTLA-$4^{-/-}$ mice die at an early each form severe immunoproliferative disorders [227, 229, 230]. CTLA-4 is thought function in Tregs by a number of mechanisms, including modulation of co-stimulatory ligands on DCs [230]. A further possible mechanism is through the induction of Indoleamine 2,3-dioxygenase (IDO) expression in the DC, which results in depletion of tryptophan and subsequent inhibition of responder T-cell proliferation [231].

1.6.2.2 IL-2

IL-2 is a cytokine that induces cell proliferation and survival [232, 233]. Mice that lack IL-2 receptor or the ability to produce Il-2 suffer lympoprolfierative disease [234-236] suggesting a role for this cytokine in prevention of autoimmunity. Indeed, it has been shown that Tregs can inhibit IL-2 production [237, 238] and that the lack of IL-2 results in apoptosis of responder T-cells [239]. The down regulation of IL-2 by Treg is thought to occur indirectly, possibly as a result of Treg mediated modulation of APC resulting in reduced co-stimulation and hence impaired IL-2 production [230]. An alternative suggestion is that since Tregs require IL-2 for their survival [240, 241], their presence results in localised depletion of IL-2 leading to apoptosis of the effector T-cells.

1.6.2.3 Transforming Growth Factor-β (TGF-β)

TGF-β is a member of the TGF-β superfamily with three subsets (TGF-β 1,2 and 3) being found in mammalian tissues [242]. TGF-β is thought to inhibit the proliferation of cells and drives cellular differentiation; it has also been identified as an important inducer of Treg cells [243, 244]. Tregs have been shown to produce high levels of both soluble and membrane bound TGF-β and mice whose cells are unable to produce the molecule succumb to autoimmune disorders suggesting a role for the molecule in immunosuppression [245-249]. TGF-β is thought to down-regulate IL-2 production by T-cells thus inhibiting effector cell expansion; this is supported by the finding that proliferation can be partially restored through the addition of exogenous IL-2 [250, 251]. In addition to modulation of IL-2, TGF-β is also thought to control proliferation via various cell cycle regulators such as c-myc, cdk4, Cyclin D2 and E [252-255].

The effects of TGF-β are thought not to be universal as the molecule seems to be able to inhibit naive T-cells more effectively than it can activated T-cells [256]. In fact, in activated T-cells, it has been shown that TGF-β drives T-cell proliferation and protects these cells from apoptosis suggesting that TGF-β's function is dependent on specific environmental factors [257, 258]. Regulatory T-cells may therefore be able to utilise TGF-β to prevent the initial activation of autoreactive T-cells in the periphery.

1.6.2.4 IL-10

IL-10 is thought to be particularly important for maintaining intestinal tolerance as IL-10 knockout mice develop intestinal inflammation, a condition that is overcome through the addition of Treg or IL-10 [259]. Further, there is evidence to suggest that IL-10 is specifically required for Treg mediated control of intestinal inflammation [260]. The cytokine is thought to impair the secretion of pro-inflammatory molecules such as TNF-α, IL-6 and GM-CSF form monocytes and macrophages, whilst simultaneously promoting the secretion of anti-inflammatory molecules like IL-1 and IL-12 [261-264]. Additionally, IL-10 downregulates surface expression of MHC-II, CD86 and the adhesion molecule CD54 thus impairing T-cell responses [265-267]. As well as modulating APCs, IL-10 can exert its effect directly on T-cells by inhibiting the production of IL-2, IFN-γ, IL-4 and IL-5 [268, 269]. The major effects of IL-10 are thought to be mediated through the activation of STAT 3 following engagement of the IL-10 receptor [270-272].

1.6.2.5 Other factors mediating Treg suppression

There has been some evidence to suggest that Tregs can mediate suppression in a contact dependent manner [229, 238], one possible mechanism for this could be through the action of granzyme, which results in the targeted apoptosis of responder cells [273, 274]. This method of control is supported by the finding that granzyme deficient Tregs have reduced capacity for suppression [274]. In addition to granzyme, both Galectin 1 and 10 have been implicated in Treg function [275, 276]. These molecules are members of the β-galactosides and could play a role in cell cycle arrest and inhibition of pro-inflammatory cytokines.

Another possible mechanism is through the action of IL-35, a relatively new cytokine that has been shown to be capable of suppressing effector T-cells. Certainly, it has been shown that Tregs lacking functional IL-35 are unable to inhibit effector cell proliferation [277]. The cytokine is made of and IL-12α and EBI_3 chain giving the molecule structural similarity to IL-12 and IL-27 [277], however, the specifics of the biology of how the cytokine exerts its regulatory function remain largely unknown.

1.7 Cytotoxic T Lymphocyte Associated Antigen-4 (CTLA-4)

CTLA-4 (also known as CD152) is an important negative regulator of T-cell activation. This is most strikingly illustrated by the fact that CTLA-4 knockout mice die at a very early age (3-4 weeks) from severe lymphoproliferative disorders [278, 279]. Further to this, it has been shown that blocking CTLA-4 with F(ab)s causes increased T-cell activation [280]. This negative regulatory role for CTLA-4 has been shown to require TCR stimulation and co-stimulation via CD28 [281, 282] although its exact mechanisms of action remain controversial.

CTLA-4 is a type I transmembrane glycoprotein consisting of an Ig-V like extracellular domain and a short 36 amino acid (aa) cytoplasmic domain [283]. It exists as a homodimeric molecule, the dimer is produced by the formation of disulphide linkages between the cysteine molecules at position 122 [284]. Further to this, glycosylation of the asparginine residues at positions 78 and 110 act to cement dimerisation [285, 286]. The extracellular portion contains a MYPPPY sequence that is required for the binding of the two CTLA-4 ligands CD80 and CD86 (also known as B7-1 and B7-2 respectively; [287]). This same binding site is conserved in the co-stimulatory molecule CD28. For the most part CTLA-4 expression is initiated upon activation of T-cells at which point, CTLA-4 is re-localised towards the immunological synapse where it associates with lipid rafts [288]. However, a small proportion of cells ubiquitously express the molecule. These cells are known as regulatory T-cells and are required to inhibit the activation of other immune cells. Only a small proportion of CTLA-4 is expressed on the cell surface at any given time, the majority of molecules are found in intracellular vesicles [289, 290]. The pool of CTLA-4 expressed internally is exported to the surface before being returned to the endocytic vesicles. This

trafficking has been shown to involve a region of the tail known as the Y_{201}VKM motif. This region allows an association with the AP50 subunit of the clathrin adaptor molecule AP-2 [291, 292], a molecule that mediates CTLA-4 internalisation. This process is thought to be controlled by phosphorylation since mutation of this Y_{201} residue prevents the AP-2 interaction and results in increased surface levels of CTLA-4 [293, 294].

1.7.1 **Biochemical Interactions**

Although CD28 and CTLA-4 bind to the same ligands, they do so with very different kinetics. CTLA-4 is capable of binding to both CD80 and CD86 with greater avidity and affinity. CTLA-4 binds to CD86 with a dissociation constant (Kd) of 2.6μM, and to CD80 with a Kd of 0.2μM [295]. Binding for these ligands with CD28 is considerably lower with Kds of 4μM and 20μM for CD80 and CD86 respectively [295]. In part, some of the differences in the ligand interactions may arise from the fact that CD80 exists as a dimer, whereas CD86 is a monomer [295, 296]. A dimeric CD80 has been shown to be capable binding two CTLA-4 or CD28 molecules at one time [297] which is thought to lead to the formation of lattice style arrangements on the surface of the T-cell [295]. In contrast, CD86 can only bind molecule can only bind one molecule of CD28 or CTLA-4 at a time [296, 298].

The fact that, comparatively speaking, CD86 binds CD28 more effectively than CD80 does led to the suggestion that CD86 is the more potent co-stimulatory ligand of the two [298]. Conversely, CTLA-4's enhanced binding to CD80 points to the possibility of this being the major inhibitory receptor [298]. Certainly, there have been several studies demonstrating that blocking CD86 results in impaired immune responses, whilst blocking CD80 has an enhancing effect [54, 55]. Furthermore, nonobese diabetic (NOD) mice treated with blocking CD80 antibodies developed disease more rapidly than controls, whereas those treated with blocking CD86 were protected [299]. An additional difference between the molecules is that CD28 has a monovalent binding profile, whereas CTLA-4 is bivalent [298]. This is thought to enable CTLA-4 to from more stable interactions with its ligands, compared to CD28 [298]. Exactly what role these binding differences play in these receptors function remains elusive at this time.

1.7.2 Mechanisms of action

The mechanisms by which CTLA-4 acts to suppress T-cell responses remain controversial and multiple mechanisms of CTLA-4 mediated inhibition have been suggested; these include direct negative signalling by the cytoplasmic domain of the molecule, sequestration of the ligands CD80 and CD86 away from CD28 [300] and the control of proliferation by regulatory T-cells. Further to this, there is evidence to suggest that CTLA-4 binding to its ligands causes back signalling into the APC resulting in tryptophan catabolism. These mechanisms are discussed in more detail below.

1.7.2.1 Sequestration of Ligands

The finding that CTLA-4 and the co-stimulatory molecule CD28 bind to the same ligands on DCs led to the suggestion that this had functional significance. Although they bind to the same ligands, it has been shown that CTLA-4 does so with greater avidity and affinity than CD28 [300-302]. It was thought that CTLA-4 could therefore sequester the ligands away from CD28 hence restricting the positive signal normally initiated by this molecule [283], such a model is only functional if the B7 ligands are limiting. This model is supported by the fact that after T-cell activation, CTLA-4 expression is induced and the molecules transported to the immunological synapse (IS) [303]. However, this mechanism may not be sufficient for down regulation of T-cell responses since it has been shown that transgenic mice expressing "tailless" CTLA-4 still suffer from lymphoproliferative disorders [304].

1.7.2.2 Signalling

The second suggested mechanism of CTLA-4 action is that it negatively signals via its cytoplasmic tail (**Figure 1.4.1**). The exact signalling methods employed by CTLA-4 remain unclear although it has been shown that CTLA-4 acts to reduce production of IL-2 and its receptor, as well as causing cell cycle arrest [280, 305]. It has been shown that the Y_{201}VKM motif of CTLA-4's tail can interact with the SH2 domain-containing tyrosine phophatase-2 (SHP-2) [291, 306]. This interaction occurs when the Y_{201} becomes phosphorylated and is

thought to inhibit T-cell responses by dephosphorylating TCR ζ and linker for activation of T-cells (LAT, [306-309]) hence down-regulating positive signalling pathways. However this mechanism remains controversial as it has been shown that mutating the Y_{201} and hence inhibiting phosphorylation does not diminish suppression [310].

CTLA-4 has also been shown to interact with PI3K however the importance of PI3K:CTLA-4 interactions in down regulating the immune responses remains contentious since inhibition of PI3K has little effect on CTLA-4 function [16]. PI3K is also recruited by CD28 [311], when PI3K is bound to CD28, Akt becomes activated [283]. This gave rise to the hypothesis that CTLA-4 binding to PI3K prevents its interaction with CD28, down-regulating signalling involved in driving T-cell activation [283]. More recently, it has been reported that CTLA-4 may regulate anergy via PI3K signalling [312].

It has been also been reported that CTLA-4 may be able to signal by binding to the serine/threonine phosphatase PP2A [313, 314]. This molecule is thought to interact with the tail of CTLA-4 preventing CTLA-4's inhibitory function [313]. Upon T-cell activation PP2A becomes phosphorylated causing it to dissociate from CTLA-4 freeing this molecule to inhibit T-cell inactivation [283]. This model is supported by the finding that mutation of the PP2A binding site increased CTLA-4's inhibitory capacity [313]. Overall, exactly how PP2A regulates CTLA-4 activity remains unknown [283]. In general, whilst much has been written about the inhibitory signals emanating from CTLA-4, there is still considerable uncertainty in the field as to the exact nature and indeed the functional importance of such signals.

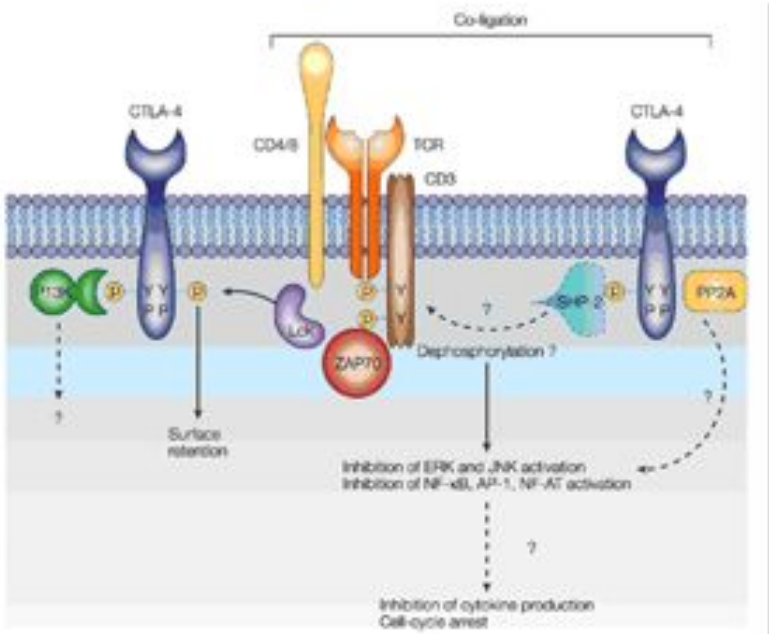

Figure 1.4.2.1: CTLA-4 Signalling Pathways. CLTA-4 is thought to associate with PI3K, a molecule that also interacts with CD28 activating signalling pathways. CTLA-4:PI3K interactions have unknown function as yet however it may sequester the molecule away from CD28. CTLA-4 is also thought to associate with SHP-2. Phosphorylation of Y201VKM results in the recruitment of SHP2. This is thought to cause the dephosphorylation of TCRζ and LAT causing a down regulation of positive signals from the TcR. CTLA-4 has also been shown to interact with PP2A. In inactive T-cells PP2A is associated with CTLA-4. Upon T-cell activation PP2A becomes phosphorylated causing it to detach from CTLA-4. This then frees CTLA-4 to negatively regulate proliferation of the T-cells. Diagram adapted from Friedl *et al*, 2005.

1.7.2.3 Cell Extrinsic Mechanisms of CTLA-4 Function

A number of recent studies have suggested that CTLA-4 is able to function by extrinsically regulating the proliferation of responder cells. One of the first studies demonstrating this was the finding that reconstituting RAG-2 deficient mice with a mixture of wild type and CTLA-$4^{-/-}$ bone marrow rescued these animals from disease [315]. It was subsequently shown that virus specific responses were equivalent in CTLA-4 knockout T-cells when compared to wild type cells in bone marrow chimeras [316]. Other data have suggested that expression of CTLA-4 within a population of T-cells is enough to confer the ability of these cells to act as suppressors [317]. Taken together, these data would suggest that the presence of CTLA-4 expressing cells is enough to regulate the proliferation of non-expressing cells. There are a number of other studies that show extrinsic function [230, 318, 319] and recent studies by the Walker and Allison labs has established that even conventional T cells can utilise CTLA-4 cell-extrinsically to regulate immune responses in vivo [320, 321].

1.7.2.4 CTLA-4 and Treg

One possible way that CTLA-4 could regulate proliferation of a responder population is through the action of regulatory T-cells (Treg). Tregs are a subset of cells that constitutively express CTLA-4.

The role of CTLA-4 on Treg was initially controversial as it was reported that CTLA-4 knockout mice contain FoxP3 expressing cells and that these cells are able to suppress immune responses [322]. However, increasing evidence for a role of CTLA-4 in Treg suppression arises from the finding that blocking CTLA-4 in Tregs with antibodies inhibits suppression [227, 237, 322-326]. In addition, a number of studies using CTLA-4 knockout regulatory T-cells have now shown a clear requirement for CTLA-4 for Treg function [230, 318, 319, 327]. Although the exact role played in Treg suppression is still unknown, a recent study using CTLA-4 conditional knockout T-cells showed CTLA-4 dependent modulation of the dendritic cell [230]. Also, recent work from our own laboratory showed that CTLA-4 was able to remove CD80 and CD86 from the surface of cells [328] suggesting a possible explanation of how CTLA-4 could exert its inhibitory function.

1.7.2.5 Indoleamine 2,3-dioxygenase (IDO)

Another proposed extrinsic mechanism is that CTLA-4 may be able to back signal through CD80 and CD86 after ligand binding. The CTLA-4:B7 interaction has been shown to increase the concentration of IDO, an enzyme involved in the breakdown of tryptophan, present in the APC [231]. Tryptophan is essential for T-cell proliferation [329] therefore increased IDO expression would reduce the capacity of T-cells to proliferate. This idea is supported in pregnancy when inhibition of IDO leads to abortion of allogeneic embryos [330]. The gene encoding IDO is thought to be induced by IFN-γ [331] indicating a possible role for IDO during inflammation. However, the importance of IDO to CTLA-4 responses remains subject to debate. Certainly, CTLA-4 Ig has been found up-regulate expression of IDO in a specific subset of DCs known as plasmacytoid DCs [332], suggesting that this mechanism may be functional under a specific set of conditions. However, signalling via IDO is unlikely to fully explain CTLA-4 function due to the fact that IDO knockout mice remain healthy [333].

1.8 Immune Mediated Disease

Although the immune system is designed to protect the body from invading pathogens whilst maintaining tolerance to self-antigens, sometimes the mechanisms in place to control the immune system are overcome. In situations like this, the immune system can cause disease. Two of the major immune mediated diseases types are autoimmunity and allergies.

1.8.1 Autoimmunity

Tight regulation of the immune system exists in order to ensure that a robust response is mounted against foreign antigens that would otherwise cause disease, whilst simultaneously ensuring that self-antigens are tolerated. However, some autoreactive cells escape this regulation and cause autoimmune disease. The onset of autoimmune diseases are thought to be triggered by a combination of genetic and environmental factors. It is thought that the most likely environmental factor that triggers autoimmunity is infection.

One theory of how infection causes autoimmune disease is through a process known as molecular mimicry. This is a process whereby viral or bacterial proteins that have structural similarities to self-antigens trigger immune responses that persist after clearance of the infection [334]. Indeed, there is mounting evidence that TCRs are able to cross react to peptide sequences with similar charge distributions and structures [335-337]. Further, a number of animal models that develop autoimmune disease from molecular mimicry exist, examples include herpes simplex virus infection leading to blindness, streptococcus

pyogenes antigens are thought to give rise to rheumatoid myocarditis and infection with H-2Kb restricted antigens resulting in autoimmune diabetes [338-343]. A second theory of how infection causes autoimmune disease is through bystander activation of autoreacive cells. It is thought that during the course of an inflammatory response, APCs pick up and present self-antigens that are released from localised tissue destruction during infection, and this results in activation of self-reactive T-cells [344]. Despite this, the role of infectious agents in the development of autoimmune disease remains difficult to prove conclusively and continues to be subject to further investigation.

Many autoimmune diseases are thought to have a genetic susceptibility coupled with environmental triggers. Twin studies looking at coeliac disease (CD) have shown strong genetic links with a concordance rate of up to 83% [345, 346]. However, this contrasts with much lower rates of concordance in other diseases such as RA. In general it is thought that multiple genetic loci are responsible for the genetic component of autoimmunity, however, the genes expressing MHC on chromosome 6p21 are thought to be a major factor in development and disease. Further studies have also identified possible associations with IL-2 and IL-21 genes [347-349]. There are, however, many other autoimmune diseases, such as rheumatoid arthritis (RA), that appear to have less of a genetic link [350-353]. Despite this, numerous genes have been implicated in RA suggesting that genetics is a factor [354-360]. Taken together, this data suggest that numerous factors can contribute to the onset of autoimmune disease.

1.8.2 CTLA-4 and Immune mediated disease

Regulatory T-cells have been shown to have a pivotal role in the maintenance of immune tolerance in the periphery and therefore in the prevention of autoimmune disease. With CTLA-4 suggested to be critical for Treg function, it is not surprising that defects in the gene expressing CTLA-4 are implicated in a number of autoimmune conditions including Graves' disease [361], Type 1 Diabetes [362, 363] and rheumatoid arthritis [364, 365].

Three polymorphisms have been identified within the locus encoded the CTLA-4 gene. It has been shown that there can be a dinucleotide repeat in exon 3 [366]. There has also been shown to be a G49A insertion in exon 1, which leads to the inclusion of alanine instead of threonine at this location [363]. There is a general lack of clarity pertaining to how this mutation affects the function of CTLA-4. Some studies have suggested that this mutation is reduces CTLA-4 expression due to inefficient mRNA leading to increased T-cell proliferation in these individuals [367-369]. Subsequent *in vitro* studies of this polymorphism have suggested that it leads to decreased surface expression of the protein due to abnormal glycosylation patterns [370]. In other studies, CTLA-4 appears to bind more strongly to its co-stimulatory molecules resulting in improved down regulation of immune responses [371]. A third reported polymorphism is a C-318T substitution in the promoter region [372] which has been shown to increase expression of the protein [373] possibly due to the fact that this mutation introduces a lymphoid enhancer factor-1 site to the promoter [367]. There is evidence to suggest that the increased expression of CTLA-4 plays a role in suppressing autoimmunity [374, 375].

1.9 CTLA-4 as a therapeutic target

CTLA-4's role in controlling peripheral tolerance to has led to it being a target for autoimmune therapy. Abatacept is an immunoglobulin molecule fused to the extra-cellular domain of CTLA-4 and is thought to function by inhibiting T-cell activation [376]. Abatacept is licensed for use in the treatment of rheumatoid arthritis (RA) where TNF-α therapy has failed [377], and in the EU, must be used as a combination therapy with methotrexate [378]. More recently, Abatacept has also been approved for the treatment of juvenile arthritis [379]. The treatment has been shown to successfully reduce the symptoms of RA in a number of patient groups [378]. Overall, therapy with abatapcept resulted in very few side effects when used as a treatment, common adverse reactions when they did occur included headache, respiratory infection and nausea [378].

It has been suggested that the immune system plays a role in inhibiting tumour progression although certain aspects of this idea have remained controversial [380]. In particular the theory of immunosurveillance has been subjected to much criticism over the years. This model suggested that transformed cells present tumour-associated antigens (TAAs) and that these molecules alert the immune system to the presence of the tumour [380]. Evidence for involvement of the immune system in tumour management arose from the observation that some tumours contain infiltrating lymphocytes [380]. For example, patients suffering from melanoma had a better prognosis if lymphocyte infiltration was detected [381]. This was subsequently found to be true of a number of other tumour types including ovarian [382], breast [383] and colorectal cancers [384, 385]. However, tumours that contained regulatory T-cells (Tregs) actually worsened the prognosis of that patient [386]. This led to the targeting of CTLA-4 in cancer immunotherapy.

There are currently two human anti-CTLA-4 monoclonal antibodies in clinical trials; these are ipilimumab (Bristol-Myers Squibb) and tremelimumab (Pfizer). These studies initially involved patients with metastatic melanoma [387] however, they have been extended to include patients with ovarian, breast, prostate and renal cancers [387, 388]. Both treatments have been used in clinical trials alone as well as in combination with other therapies [389]. Such trials showed evidence that administration of anti-CTLA-4 antibodies resulted in some anti-tumour responses, however, one of the negative sides of this type of therapy is the common occurrence of immune related adverse events (irAEs). These irAEs include dermatitis, colitis, uveitis, hepatitis and hypophystitis [387]. The presence irAEs have been shown to indicate to a certain extent that the anit-tumour treatments are having some effect [387] however, better treatments would cause the same anti-tumour effects without resulting in adverse reactions in the patients.

1.10 Project Aims

The main overall aim of this project was to look at the various proposed mechanisms of CTLA-4 action to try and elucidate exactly how this protein exerts its effects as a negative regulator of T-cell activation. One of the most commonly sated mechanisms of action for CTLA-4 is through the delivery of a cell intrinsic negative signal. Our hypothesis was that CTLA-4 did not function in this manner due to the lack of evidence demonstrating a negative signal when stimulating T-cells with natural ligand. Instead, the major evidence for a negative signal came from antibody cross -inking experiments. We therefore wished to design experiments to test the role of CTLA-4 when engaged by its natural ligands.

An additional aim was to study the role of CTLA-4 in Tregs, part of the project was therefore to isolate a highly pure population of Tregs from peripheral blood for use in functional sutides. In doing so, it was hoped that the function of CTLA-4 in Treg could be compared to that of convention T-cells to elucidate possible differences in the function of the protein in these cell types.

A further aim was to determine whether any CTLA-4 expressing cells could suppress the proliferation of a responder population. Based on the finding that CTLA-$4^{-/-}$ mice can be rescued from disease by the addition of wild type bone marrow [315] it was hypothesised that the presence of any CTLA-4 expressing cells in culture would be capable of regulating the proliferation of a responder population.

Since robust tools are an absolute requirement for studying CTLA-4 function, another aim of this project was to carry out a broad characterisation of anti-CTLA-4 antibodies for use in functional studies. Specifically, it was vital to ensure that the antibodies being were capable of blocking the interactions between CTLA-4 and its ligands as this would form the basis of the key experimental approach.

Chapter 2:

Materials and Methods

2.1 Antibodies

Details of the antibodies used in this study can be found in table 1.

Antibody Name	Target	Species Raised in	Isotype	Obtained From
BNI3	CTLA-4	Mouse	IgG2a	Pharmingen
26B	CTLA-4	Mouse	IgG2	B. Carreno (Wyeth Research)
11D4	CTLA-4	Mouse	IgG1	R. Peach (Bristol Myers Squibb)
11G1	CTLA-4	Mouse	IgG1	J. Allison (MSKCC)
10A8	CTLA-4	Mouse	IgG1	R. Peach (Bristol Myers Squibb)
Tremelimumab	CTLA-4	Human	IgG2	Pfizer

Table 1: Antibody Details. Name of the antibody, what molecule it reacts against, the species it was raised in and where the antibodies came from are all indicated in the table.

2.2 Flow Cytometry Antibodies

Target	Fluorophore	Clone	Species Raised in	Company
CD3	PerCP	SK7	Mouse IgG1	BD
CD4	FITC	RPA-T4	Mouse IgG1	BD
CD11c	APC	B-Ly6	Mouse IgG1	BD
CD25	PE/APC	M-A251	Mouse IgG1	BD
CD49d	FITC	MZ18-24A9	Mouse IgG2b	Miltenyi Biotec
CD80	PE	L307.4	Mouse IgG1	BD
CD86	PE	2331 (FUN-1)	Mouse IgG1	BD
CD127	FITC	eBioRDR5	Mouse IgG1	eBioscience
CD127	PE	HIL-7R-M21	Mouse IgG1	BD
CTLA-4	PE/APC/PE-Cy5	BNI3	Mouse IgG2a	BD
FoxP3	PE/APC	PCH101	Rat IgG2a	eBioscience
IL-2	PE	MQ1-17H12	Rat IgG2a	eBioscience
IL-17	PE	ebio64CAP17	Mouse IgG1	eBioscience
IL-21	PE	ebio3A3-N2	Mouse IgG1	eBioscience
IFNγ	eFlour 450	4S.B3	Mouse IgG1	eBioscience

Table 2: Details of Antibodies Used in Flow Cytometry Analysis. Details include target protein, fluorescent labels, clone and isotype of antibody, species the antibody was raised in and the company the antibody was obtained from. All antibodies react with the human protein.

2.3 Western Blotting Antibodies

Target	Clone	Dilution	Species Raised in	Company
Actin	AC-40	2500	Mouse	Sigma
CD86	C-19	1000	Goat	SantaCruz
CTLA-4	C-19	2500	Goat	SantaCruz
anti-mouse HRP	P0260	2500	Rabbit	Dako
anti-goat HRP	P0449	2500	Rabbit	Dako

Table 3: Details of Antibodies Used in Western Blot Analysis. Details include target protein, clone of antibody, species the antibody was raised in and the company the antibody was obtained from.

2.4 Maintenance of Cell Lines

All cells were grown in a 5% carbon dioxide environment at 37°C. CHO cell lines were maintained in Dulbecco's Modified Eagles Medium (DMEM, Gibco) supplemented with 10% foetal calf serum (FCS), 1% penicillin and streptomycin and 1% L-glutamine. Cell lines were expanded by removing media and washing cells with 5ml phosphate buffer saline (PBS, pH 7.2), 2ml of Trypsin EDTA was added to the cells which were then incubated until all of the cells had detached from the flask. The trypsin was neutralised with 8ml of media and an appropriate volume of this cell suspension was transferred to a new flask. The flask was then replenished with 20ml of fresh media.

T-cells and jurkat cell lines were maintained in RPMI (Gibco) supplemented with 10% FCS, 1% penicillin and streptomycin and 1% L-glutamine. Jurkat cells were maintained by removing cells from the flask and replenishing with fresh media.

2.5 Generation of CHO Cell Transfectants

CHO cell lines expressing CD80, CD86 or CTLA-4 were generated previously in the lab by electroporation of human cDNAs cloned into a CMV expression vector. Cells that were positive for the expression of the plasmid were selected using G418 (500μg/ml) treatment and were sorted by flow cytometry.

2.6 Generation of CTLA-4 expressing jurkat cells

The jurkat cell lines were generated by Y.Zheng at the university of Birmingham. To do this, CTLA4 was cloned into the MP71 retrovirus vector. Pheonix-A packaging cells were transfected with the vector using FUGENE6 transfection reagent (Roche Molecular Biochemical). Superanatants containing retrovirus were collected after 48 hours. Non-tissue culture treated 6-well plates were coated with RetroNectin (TaKaRa) at 30 mg/ml and blocked by 2% BSA. 1ml of the harvested retrovirus containing supernatants were added to the plates and centrifuged at 1500 rpm at room temperature for 15 minutes before the addition of 2x106 jurkat cells. Plates were centrifuged for a further 60minutes at 2000 rpm at 30°C. After 24 hours, the media was replaced with fresh RPMI and after 72 hours cells were sorted by staining with CTLA-4 PE (BD) on the MoFlow cell sorter.

2.7 Freezing of Cell Lines

Cells were split as described above. Then, cells were washed once in PBS and resuspended in DMEM containing 1% L-Glut, 1% Pen Strep, 10% FBS and 10% DMSO and were transferred to cryovials. Cryovials were incubated overnight at -80°C before being transferred to liquid nitrogen.

2.8 Conjugation of Anti-CTLA-4 Antibody to HRP

Tremelimumab was directly conjugated to horseradish peroxidise (HRP) for the enzyme linked immunosorbent assay (ELISA). Direct conjugation of these proteins was carried out using lighting link HRP conjugation kit (Innova Biosciences) as per manufacturer's instructions.

2.9 Enzyme-Linked ImmunoSorbent Assay (ELISA)

Plates were coated using 50µl of coating buffer (0.5M Sodium Carbonate, 0.5M Sodium Hydrogen Carbonate) containing CTLA-4 Ig (R&D Systems) at a concentration of 0.5µg/ml; plates were then incubated overnight at 4°C. After coating each plate was washed four times in PBS containing 0.1% Tween (PBS-T) and was blocked in a 10% bovine serum albumin (BSA) solution for 1 hour at 37°C. Each anti-CTLA-4 antibody (listed in table 1) was titrated from a top dose of either 10µg/ml or 1µg/ml in a series of 10 fold dilutions. The blocking solution was removed and 100µl of each titration was added to the wells, the plates were incubated at 37C for 1 hour. Plates were washed four times in PBS-T before the addition of 100µl of a goat anti mouse HRP secondary antibody (Dako), diluted 1:1000, plates were incubated for one hour at 37°C. The wells were washed four times in PBS-T. For detection, 100µl of TMB substrate (Biofx) was added to each well. After five minutes the reaction was stopped by the addition of STOP solution (Biofx) and the plates were read at 450nm.

2.10 Surface Plasmon Resonance

Binding affinities of anti-CTLA-4 antibodies was determined using the Biacore 3000 system (Biacore AB). To do this, CTLA-4 Ig was fixed to the surface of a CD5 chip by means of the amine coupling method (Biacore, GE Healthcare). All antibody samples were titrated from 100nM in a two-fold dilution series in HBS-EP Buffer (Biacore, GE Healthcare). To block non-specific binding, an anti-human IgG antibody was passed over the surface of the chip for 10 minutes. Then, the dilutions of antibodies were passed over the chip at a rate of 10µl per minute for 30 minutes, the flow was then stopped and the off rates were measured for a further 30 minutes. Between each antibody dilution, any bound material still on the surface was removed by passing a solution of 100mM glycine at pH 2.2 over the surface of the chip. The KD values were calculated by plotting the response units on a graph and carrying out a non-linear regression.

2.11 Antibody binding to CTLA-4 expressing CHO cells

A surface stain was carried out where cells were washed in PBS before adding the appropriate dilution of each antibody. Samples were incubated on ice for 30 minutes before being washed with PBS. An anti-mouse 488 (Invitrogen) or anti-human IgG FITC (Sigma) antibody was added and samples incubated for a further 30 minutes. Cells were washed once in PBS and remained on ice until flow cytrometry was conducted.

2.12 Ability of Antibodies to Block CTLA-4 ligand binding

Blocking experiments were carried out by incubating 0.1µg/ml of CTLA-4 Ig with various dilutions of each antibody on ice for 30 minutes. Then, 150,000 CHO-CD80 or CHO-CD86 cells were added and samples were incubated on ice for a further 30 minutes. The cells were washed once in PBS and centrifuged at 1800rpm for 5 min. To stain for the presence of the CTLA-4 Ig a 1:1000 dilution of FITC conjugated Fc specific anti-human IgG (Sigma) was incubated with the cells for 30 minutes on ice. Samples were washed and resuspended in PBS before carrying out flow cytometry. Alternatively, in the case of tremelimumab, the CTLA-4 Ig was directly conjugated to an Alexa Fluor 488 dye using the Zenon Human IgG labelling kit (Molecular Probes) as per manufacturer's instructions.

2.13 Isolation of PBMC

Buffy coats were obtained from the national blood service in Birmingham (NBS). The blood was diluted 1:1 in PBS before layering 25ml onto 15ml of Ficol-Paque (GE Healthcare) and centrifuged at 2200rpm for 25 minutes without brake. The serum layer was removed and the peripheral blood mononuclear cell (PBMC) layer containing lymphocytes was transferred to a fresh tube, which was filled with MACS buffer (PBS, EDTA and 0.5% BSA) and centrifuged at 1500rpm for 10 minutes. The pellet was resuspended and washed in MACS buffer a further 3 times before being diluted to a concentration of $100x10^6$ cells per ml.

2.14 Isolation of T-cells

T-cells were isolated from PBMC using 100µl of $CD4^+$ T-cell negative selection cocktail (EasySep, Stemcell) was added for every ml of cells, this was incubated at room temperature for 15 minutes. Then, 60µl of magnetic colloid beads was added per ml of suspension before again being incubated at room temperature for 15 minutes. Samples were made up to 9ml with MACS buffer and the tube was put in the EasySep magnet for 5 minutes. The supernatant containing $CD4^+$ cells were poured into a fresh tube. This process was carried out a further twice, cells were

counted and resuspended $100x10^6$ cells/ml. Some of these cells were kept for phenotype analysis, the remainder were subjected to a CD25 selection step. To do this, 100µl of CD25 selection cocktail (Miltenyi) per 1ml of cells was added. This was incubated on ice for 30 minutes before being washed and passed through the column once. This time, $CD25^-$ cells pass through the column and were collected.

2.15 Isolation of Monocytes

A similar process was used for the isolation of monocytes. This time, 100µl of human monocyte enrichment cocktail (EasySep, Stemcell) per ml of cells was added and incubated on ice for 30 minutes. Then, 60µl of magnetic beads were added per ml of cells and samples were incubated on ice for a further 30 minutes. Samples were made up to a volume of 9ml with MACS buffer before being added to the Easysep magnet. Samples were incubated for 5 minutes and unbound cells were poured into a fresh tube. This step was repeated a further twice, cells were counted and resuspended at $2x10^6$/ml in fully supplemented RPMI.

2.16 Isolation of Regulatory T-cells

Tregs were isolated from PBMCs using the EasySep $CD4^{+}CD127^{low}$ T-cell enrichment kit (Stemcell) as per manufacturer's instructions. To purify these cells further, the $CD4^{+}CD127^{low}$ cells were resuspended at $100x10^{6}$cells/ml. Then 7.5µl of CD49d FITC antibody was added per $100x10^{6}$ cells. Samples were incubated on ice for 30 minutes, washed once in MACS buffer before adding 20µl of anit-FITC beads (Miltenyi) per $100x10^{6}$ cells, samples were incubated on ice for a further 30 minutes. Samples were washed once in MACS buffer before being passed through two columns. The collected cells were counted and resuspended at $2x10^{6}$/ml in RPMI.

2.17 Differentiation of Dendritic Cells

Monocytes were placed into a 12 well plate at a density of $2x10^{6}$ cells per well and 500U/ml IL-4 (Peprotech) and 800U/ml GM-CSF (Peprotech) were added. Cells were cultured for 2 days before addition of 1ml of media containing IL-4 and GM-CSF; cells were incubated for a further 5 days. Half of the DCs were matured by adding 1µg/ml LPS (Sigma) 24 hours before the cells were used in an assay.

2.18 Gluteraldehyde Fixing of CHO Cells

Cells were washed once in PBS and the pellets were resuspended in 0.025% gluteraldehyde (Sigma) and incubated at room temperature for two minutes with vigorous agitation. Cells were washed three times in fully supplemented RPMI before finally being resuspended at $2x10^6$ cells per ml.

2.19 T-cell Proliferation Assays

An appropriate number of $CD25^-$ T-cells were washed in PBS before being incubated for 10 minutes in 2.5µm carboxyfluorescein succinimidyl ester (CFSE) with periodic gentle agitation. Cells were washed three times in fully supplemented RPMI before being resuspended in RPMI at a concentration of $2x10^6$ cells per ml. The appropriate numbers of live dendritic cells or fixed CHO cells were added to wells containing the labelled T-cells. All DC based experiments were carried out using live DCs, whereas all CHO experiments made use of fixed cells. The cultures were then stimulated with the indicated concentration of the OKT3 clone of the CD3 antibody and were allowed to grow for 5 days. Each sample was then analysed by flow cytometry. CTLA-4 blocking antibodies were added at a concentration of 40µg/ml, CTLA-4 Ig and blocking CD28, CD80 and CD86 antibodies were all used at 10µg/ml

and the IDO inhibitor 1-methyl tryptophan was added at a concentration of 500µM. For the jurkat suppression assays, jurkats were added at a ratio of 1 for every 3 T-cells.

2.20 Re-stimulation experiments

$CD25^-$ T-cells were stimulated with CD3/CD28 dynabeads for five days. At the point, the dynabeads were removed from samples using a magnet and the cells were transferred to a fresh tube. Fresh fully supplemented RPMI media was added and the cells incubated overnight at 37°C. The cells were then washed three times in RPMI and resuspended at $2x10^6$cells/ml.

2.21 Staining of Cells for Flow Cytometry

Surface stains were carried out by incubating cells with the indicated antibodies on ice for 30 minutes before washing three times in PBS. Cells were kept on ice for analysis or were fixed with 3% PFA prior to flow cytometry analysis. The CTLA-4 recycling stains were carried out by incubating the antibody on cells at 37°C. After staining, cells were washed three times in PBS. Intracellular staining was carried out by fixing cells in 3% PFA for ten minutes. Cells were washed once in PBS and once in PBS-Saponin (5% Saponin) to permeabilise the cells. Antibodies were diluted in PBS-Saponin and were incubated on samples for 30 minutes before washing once in PBS-saponin and once in PBS.

2.22 Cytokine Staining of T-cells

$CD4^+CD25^-$ T-cells were stimulated for 5 days in the presence of CD3 and co-stimulation as described above. On day five cells were re-stimulated with 50ng/ml PMA, 1µM ionomycin and 5µg/ml brefeldin A (Sigma) for four hours. Cells were washed once in PBS and were then fixed for 10 minutes in 3% PFA. Samples were washed once in PBS-Saponin before being incubated with an anti-IL-2 antibody. Alongside this, cells were also stained for the presence of a CTLA-4 antibody at room temperature for 30 minutes. Samples were washed once more in PBS-Saponin and were analysed by flow cytometry.

2.23 FoxP3 Staining of T-cells

FoxP3 stains were carried out using the staining kit from eBioscience as per protocol. Briefly, T-cells were washed three times in PBS and 0.5ml of Fix/Perm buffer was added. The cells were incubated overnight at 4°C before being washed once in permeablisation buffer. FoxP3 antibody was added and samples were incubated for one hour at 4°C before being washed once more in permeabilisation buffer followed by a further two washes in PBS.

2.24 Isolation of Protein

Samples were washed twice in cold PBS before adding 1mlµl of RIPA buffer (ThermoScientific) per $5x10^6$ cells. Samples were incubated on ice for 15 minutes and were centrifuged at 15,000rpm for 30 minutes. Supernatants were transferred to fresh tubes and stored at -20°C.

2.25 Protein Quantification by Bradford Assay

The protein in each sample was quantified using Bradford reagent (Biorad), which was diluted 1:5 in distilled water. Bovine serum albumin (BSA) was used to create a standard curve. One millilitre of Bradford reagent was added to each sample tube and the OD was read at 595nm. The OD value of each of the standards was plotted on a graph and this was used to calculate the amount of antibody in each sample.

2.26 Western Blot Analysis

A total quantity of 10µg of protein was diluted in protein loading buffer (50mM Tris-HCl pH 6.8, 2% SDS, 10% Glycerol, 1% β-Mercaptoethanol, 12.5mM EDTA and 0.02% Bromophenol Blue). Samples were incubated at 95°C for five minutes to denature the protein. Samples were loaded onto a 10% SDS-PAGE Gel composed of a layer of stacking gel (125mM Tris-HCl pH6.8, 0.1% SDS) and a layer of resolving gel (375mM Tris-HCl pH8.8 and 0.1% SDS) along with 10µl of ladder. The gel was filled with a running buffer (25mM Tris pH 8.3, 250mM Glycine, 0.1% SDS with the pH adjusted to 8.3) and samples were resolved at 100 Volts until the dye front reached the bottom of the gel. The stacking gel was removed and samples were transferred onto nitrocellulose membrane (Amersham Hybond-P membrane, GE Lifesciences) that had been pre-soaked in methanol for 5 seconds before being soaked in transfer buffer (25mM Tris, 192mM Glycine and 10% Methanol) for at least five minutes. Samples were transferred at 10 volts for 90 minutes and the membrane was blocked overnight in 10% blocking buffer (PBS-Tween with milk). One ten minute followed by three five minute washes were carried out using wash buffer (PBS-Tween) before addition of the primary antibody. The primary antibody was incubated on the sample for one hour, samples were washed again as described above and the secondary antibody was incubated on the membrane for a further hour. The membrane was washed again and then incubated with ECL (Amersham

ECL Prime, GE Healthcare) for one minute and membrane was developed for the appropriate time.

2.27 Statistical Analysis

All statistics were performed in Prism version 5.0. Details of specific tests are described in figure legends.

Chapter 3:

Characterisation of anti-CTLA-4 antibodies

3.1 Introduction

Antibodies targeting CTLA-4 are a useful tool in studying the function. In the past many groups have used this means of studying the role of CTLA-4, but with varying results. Some groups found that blocking protein function with anti-CTLA-4 resulted in inhibition of CTLA-4 mediated suppression [1-7] whereas; other groups observed that the use of antibodies had no effect on CTLA-4 function [8-10]. Such discrepancies may be explained by incomplete assessment of the capabilities of the antibodies used in each study. It was therefore important to understand what the antibodies were capable of. Our laboratory had a panel of anti-CTLA-4 antibodies about which very little was known other than claimed specificity for the protein. Moreover in the situation where antibodies to CTLA-4 do not reveal functional effects it is important to be confident of the characterisation of the antibody. We therefore conducted a full characterisation of these antibodies in order to facilitate interpretation of the functional outcomes in future experiments.

3.2 Results

The first stage in the characterisation process was to confirm that each of the antibodies were able to bind specifically to CTLA-4. To do this, an ELISA was developed whereby a plate was coated with CTLA-4 Ig. Increasing concentrations of each antibody were then incubated on the Ig, the plates were washed and presence of bound antibody was detected using an HRP conjugated secondary antibody. The exception to this is tremelimumab, which, as a result of the fact that both it and the CTLA-4 Ig are humanised, was directly conjugated to HRP. The presence of HRP was detected by TMB substrate, the reaction was stopped and plates read at 450nm. The optical densities (OD) were then plotted on the graphs as shown in **figure 3.2.1**.

Importantly, it can be seen that all of the antibodies were able to bind to CTLA-4 and that they interacted with the extracellular domain of the protein. Four of the antibodies, BNI3, 10A8, 26B Whole and 26B ScFv reached maximal binding at around 0.01µg/ml, 11D4 at 0.1µg/ml and both tremelimumab and 11D4 $F(ab)_2$ did not reach their maximum until 1µg/ml.

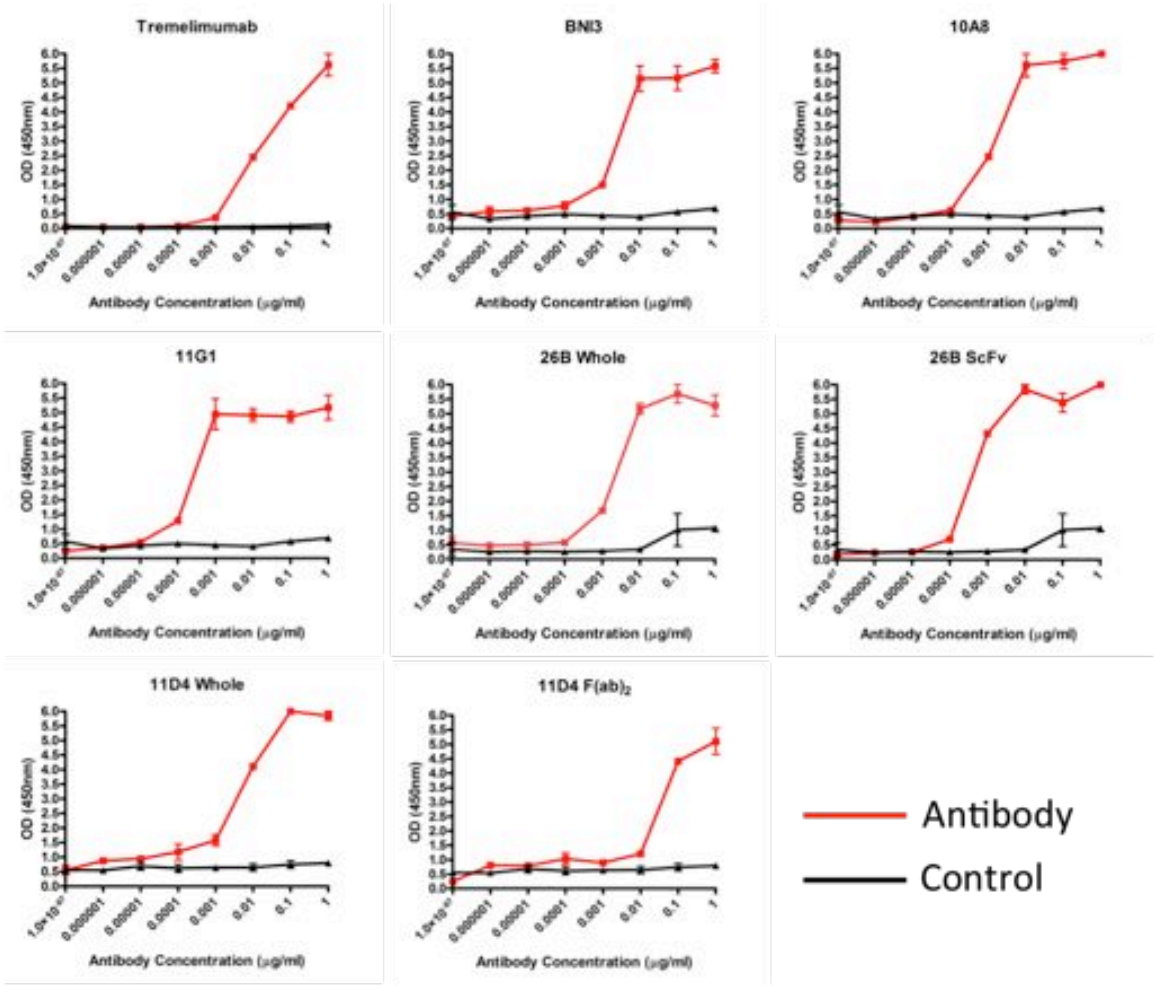

Figure 3.2.1: Anti-CTLA-4 antibodies bind to the extracellular domain of CTLA-4 Ig in an ELISA. ELISA plates were coated with 0.5μg/ml of CTLA-4 Ig, before the addition of increasing concentrations of each antibody. The presence of bound anti-CTLA-4 antibodies was detected by adding a secondary anti-mouse HRP antibody. Tremelimumab was directly conjugated to an HRP. TMB substrate was added to react with the HRP, the reaction was stopped and plates read at 450nm. Optical density reading were plotted on graphs as shown. The data is representative of at least three independent experiments.

These variations in peak binding values may reflect differing affinities of each antibody for CTLA-4; it was therefore decided to obtain data on affinities and avidities by surface Plasmon resonance. For this, a chip was coated with CTLA-4 Ig before increasing concentrations of each antibody were passed over the surface of the chip for thirty minutes to measure the on rate. The flow was stopped and the off rate measured for a further thirty minutes, the data for these are shown in **part A of figures 3.2.2 to 3.2.9**. To obtain data as to the affinity of antibody binding, calculated as the KD value, the maximal response unit from each dilution was plotted as a graph as shown in **part B of figures 3.2.2 to 3.2.9**. Interestingly, although tremelimumab appears to have one of the poorest binding curves of the antibodies in the ELISA, according to the BIAcore data, this antibody is the highest affinity with a KD value of just 1.051nM. It is also the antibody that appears to have the steepest off rate curve suggesting that although it binds very strongly, it does also fall off quickly. This may explain why the antibody appeared to have a lower binding capacity for CTLA-4 in the ELISA.

The 10A8 and BNI3 antibodies had a largely similar affinity with KD values at 6.891 and 6.908nM respectively, the next best binder was 11G1 with a KD of 13.89. It is interesting to note that the 26B Whole antibody had a KD value that is comparable to the ScFv version of the antibody. However, it appeared that once bound, the whole antibody was able to form a more stable complex than the Fv as evidenced by the rate at which the antibodies detached from CTLA-4 Ig upon stopping the flow. Both 11D4 antibodies had very poor binding curves and, surprisingly, it seems that

the whole version of the antibody was a worse binder than its equivalent $F(ab)_2$ fragment.

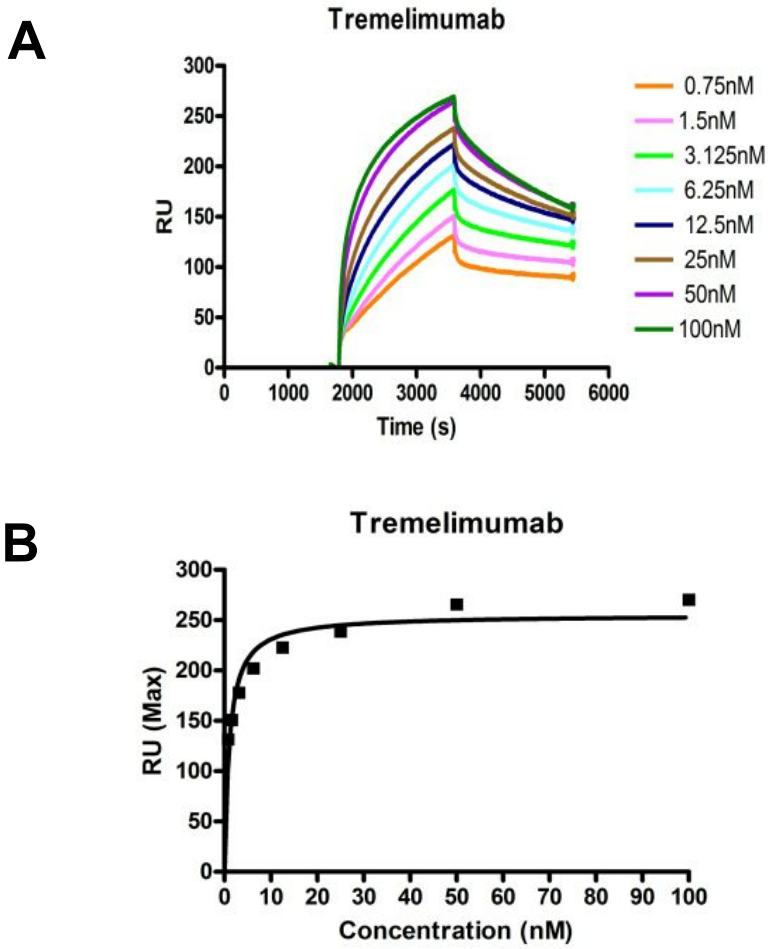

Figure 3.2.2: Kinetics of Tremelimumab Binding to CTLA-4 Ig. Binding kinetics were determined using surface plasmon resonance. CTLA-4 Ig was bound to a CM5 chip. Increasing concentrations of Tremelimumab were then passed over the surface of the chip and the amount of binding detected. After 30 minutes, the flow was stopped and the off rate measured. **A.** Binding plot of tremelimumab for CTLA-4 Ig at the various concentrations. **B.** The maximum response unit for each concentration was plotted in a graph and a non-linear regression one site binding model was performed in prism to calculate the KD value.

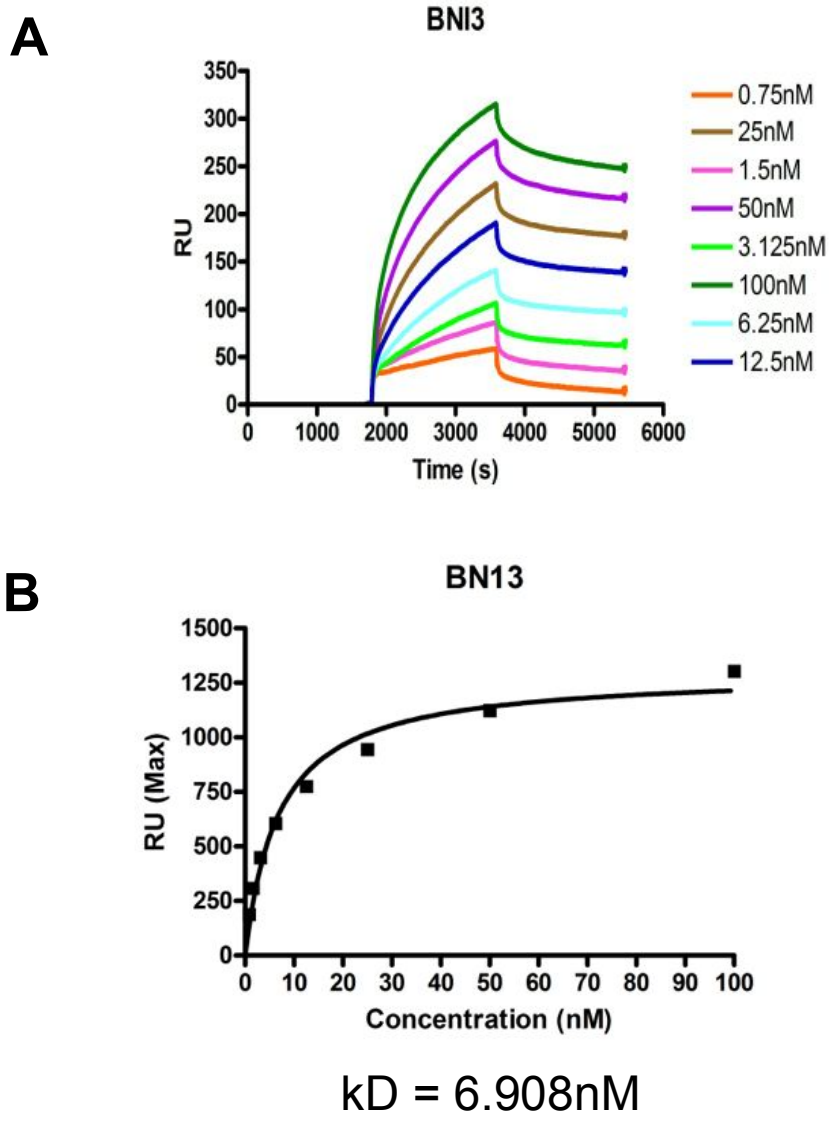

Figure 3.2.3: Kinetics of BNI3 Binding to CTLA-4 Ig. Binding kinetics were determined using surface plasmon resonance. CTLA-4 Ig was bound to a CM5 chip. Increasing concentrations of BNI3 were then passed over the surface of the chip and the amount of binding detected. **A.** Binding plot of BNI3 for CTLA-4 Ig at the various concentrations. **B.** The maximum response unit for each concentration was plotted in a graph and a non-linear regression one site binding model was performed in prism to calculate the KD value.

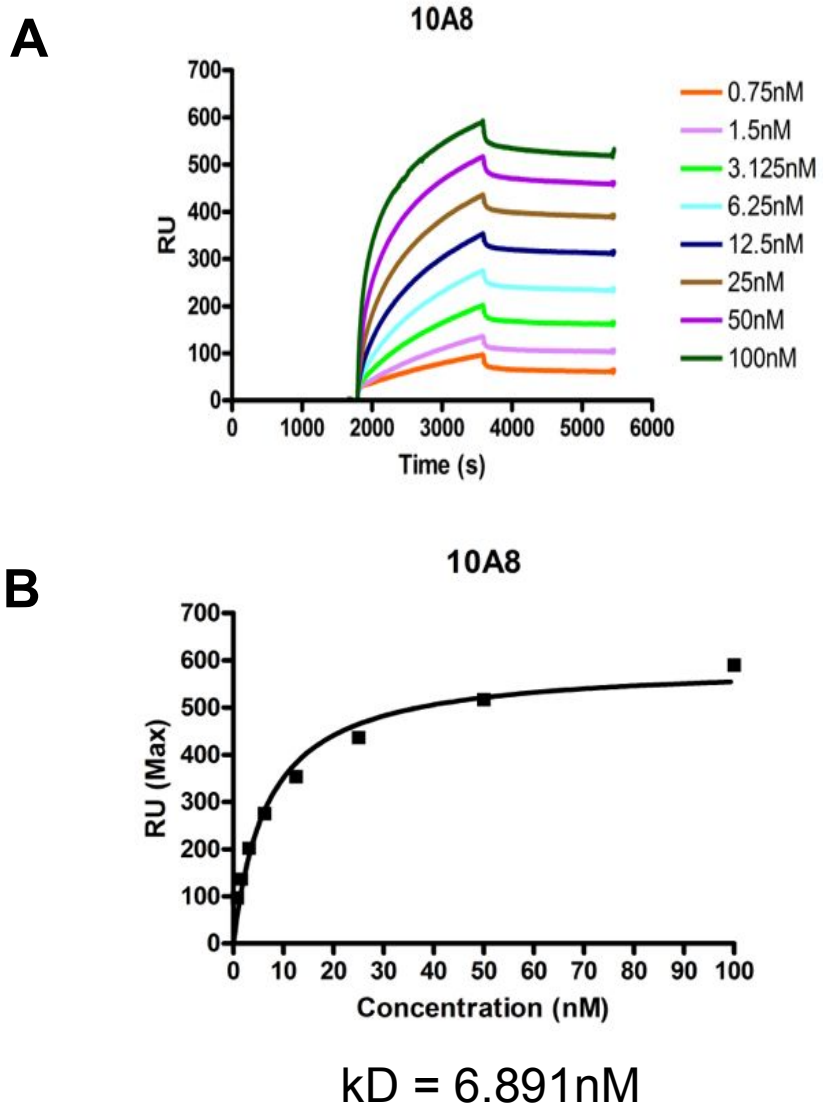

Figure 3.2.4: Kinetics of 10A8 Binding to CTLA-4 Ig. Binding kinetics were determined using surface plasmon resonance. CTLA-4 Ig was bound to a CM5 chip. Increasing concentrations of 10A8 were then passed over the surface of the chip and the amount of binding detected. **A.** Binding plot of 10A8 for CTLA-4 Ig at the various concentrations. **B.** The maximum response unit for each concentration was plotted in a graph and a non-linear regression one site binding model was performed in prism to calculate the KD value.

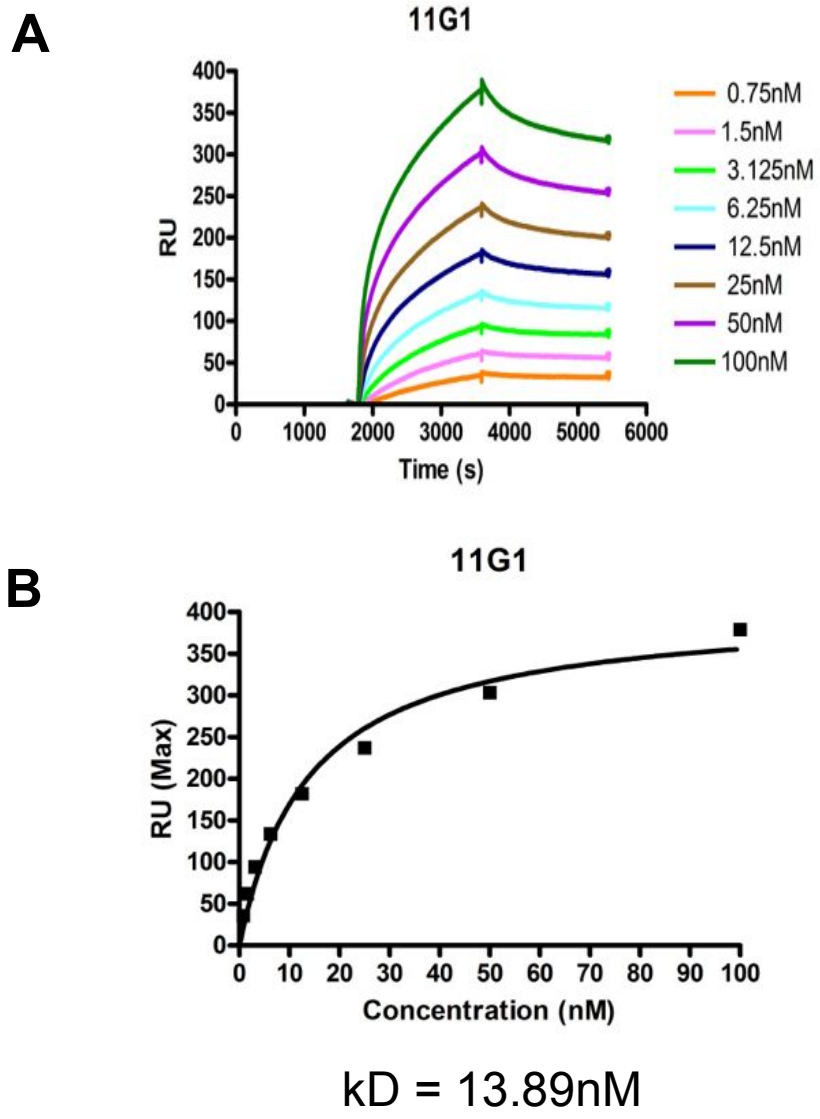

Figure 3.2.5: Kinetics of 11G1 Binding to CTLA-4 Ig. Binding kinetics were determined using surface plasmon resonance. CTLA-4 Ig was bound to a CM5 chip. Increasing concentrations of 11G1 were then passed over the surface of the chip and the amount of binding detected. **A.** Binding plot of 11G1 for CTLA-4 Ig at the various concentrations. **B.** The maximum response unit for each concentration was plotted in a graph and a non-linear regression one site binding model was performed in prism to calculate the KD value.

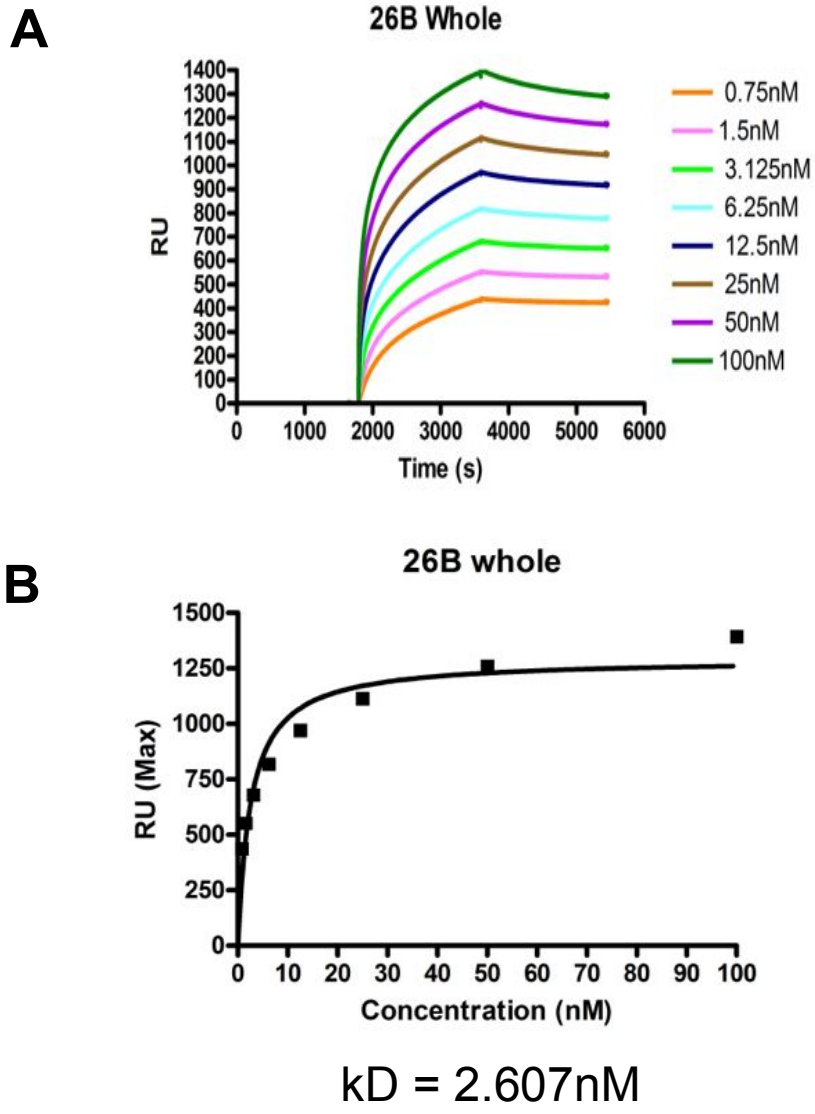

Figure 3.2.6: Kinetics of 26B Whole Binding to CTLA-4 Ig. Binding kinetics were determined using surface plasmon resonance. CTLA-4 Ig was bound to a CM5 chip. Increasing concentrations of 26B Whole were then passed over the surface of the chip and the amount of binding detected. **A.** Binding plot of 26B Whole for CTLA-4 Ig at the various concentrations. **B.** The maximum response unit for each concentration was plotted in a graph and a non-linear regression one site binding model was performed in prism to calculate the KD value.

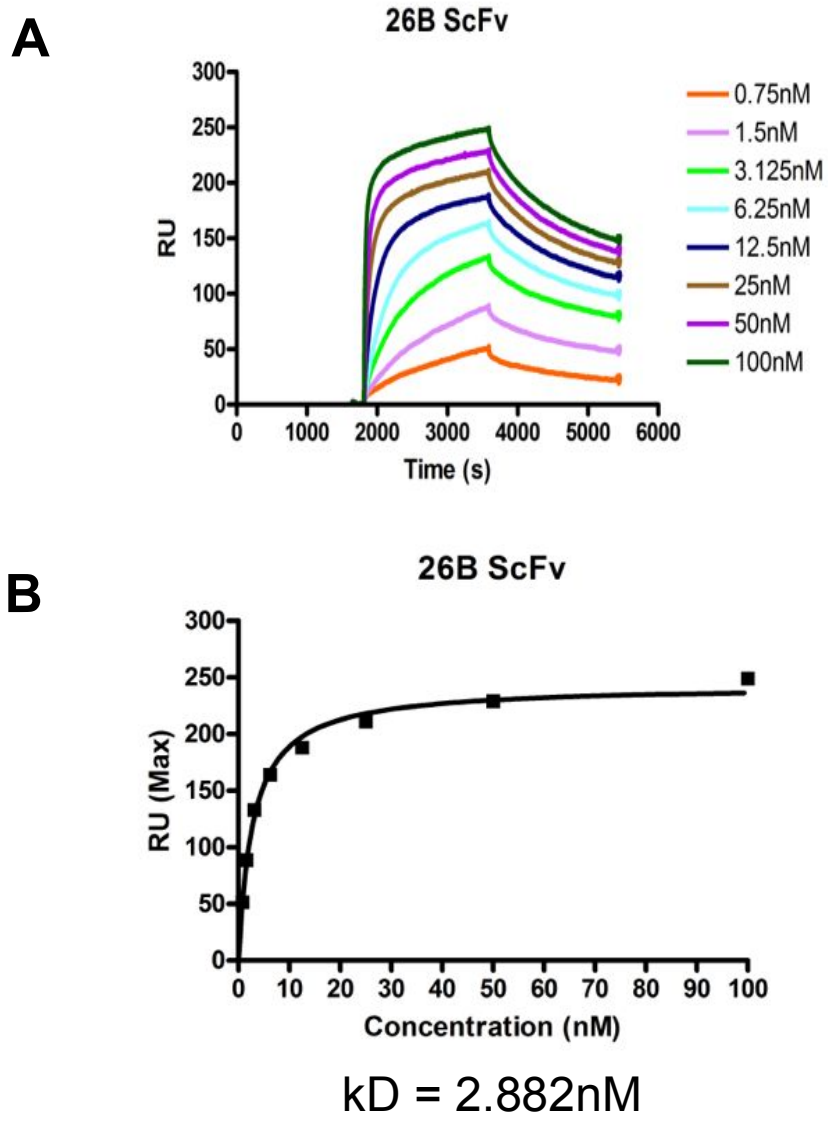

Figure 3.2.7: Kinetics of 26B ScFv Binding to CTLA-4 Ig. Binding kinetics were determined using surface plasmon resonance. CTLA-4 Ig was bound to a CM5 chip. Increasing concentrations of 26B ScFv were then passed over the surface of the chip and the amount of binding detected. **A.** Binding plot of 26B ScFv for CTLA-4 Ig at the various concentrations. **B.** The maximum response unit for each concentration was plotted in a graph and a non-linear regression one site binding model was performed in prism to calculate the KD value.

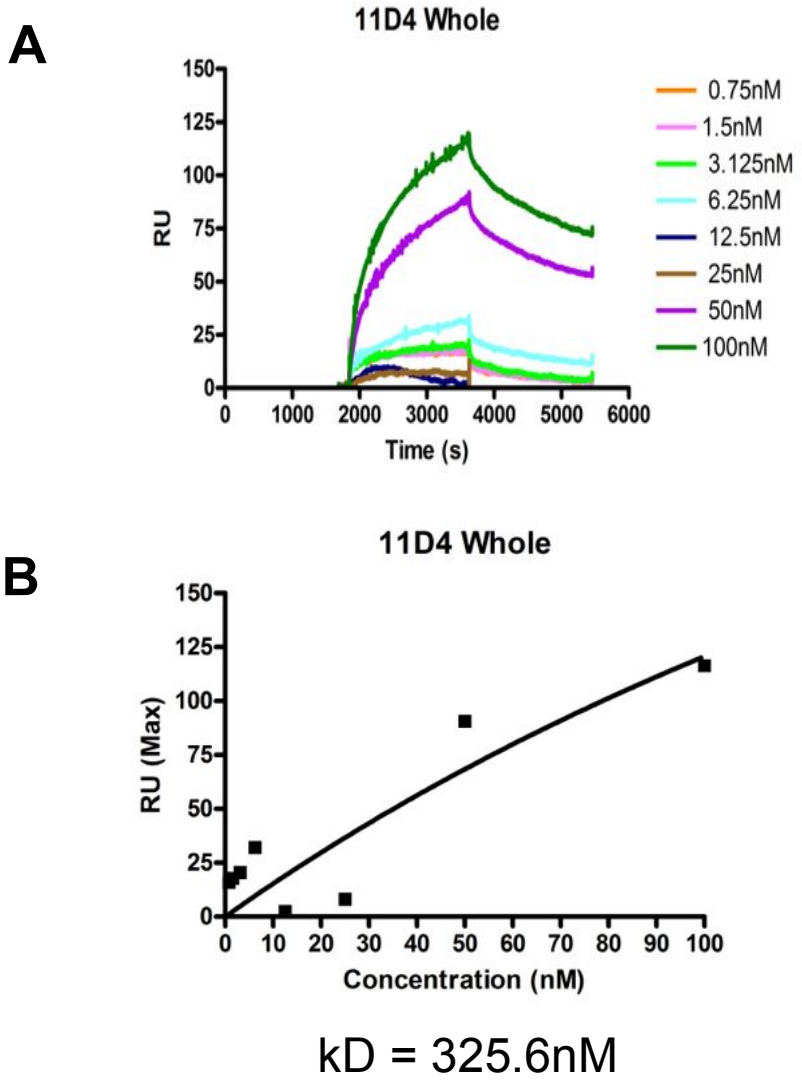

Figure 3.2.8: Kinetics of 11D4 Whole Binding to CTLA-4 Ig. Binding kinetics were determined using surface plasmon resonance. CTLA-4 Ig was bound to a CM5 chip. Increasing concentrations of 11D4 Whole were then passed over the surface of the chip and the amount of binding detected. **A.** Binding plot of 11D4 Whole for CTLA-4 Ig at the various concentrations. **B.** The maximum response unit for each concentration was plotted in a graph and a non-linear regression one site binding model was performed in prism to calculate the KD value.

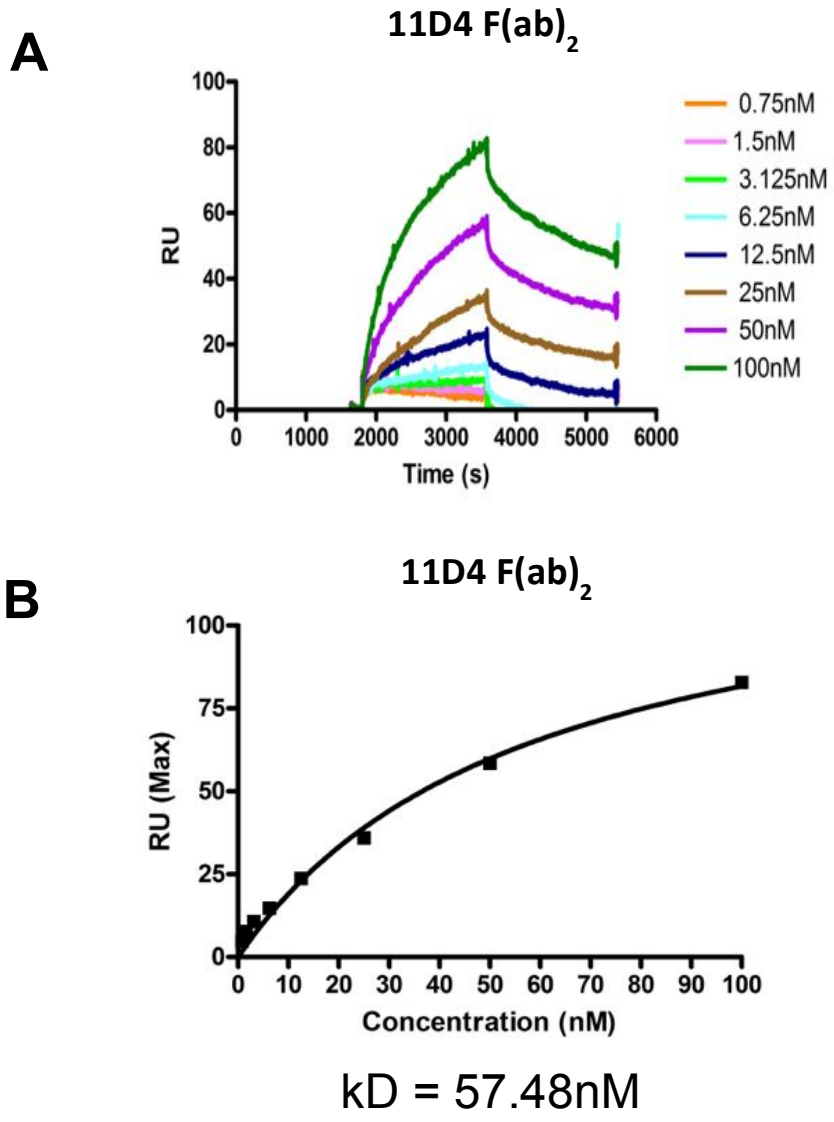

Figure 3.2.9: Kinetics of 11D4 F(ab)$_2$ Binding to CTLA-4 Ig. Binding kinetics were determined using surface plasmon resonance. CTLA-4 Ig was bound to a CM5 chip. Increasing concentrations of 11D4 F(ab)$_2$ were then passed over the surface of the chip and the amount of binding detected. **A.** Binding plot of 11D4 F(ab)$_2$ for CTLA-4 Ig at the various concentrations. **B.** The maximum response unit for each concentration was plotted in a graph and a non-linear regression one site binding model was performed in prism to calculate the KD value.

Having studied the binding of the antibodies to CTLA-4 Ig, it was decided to check that they were able to bind to full the full-length native protein. To do this, CHO cells expressing CTLA-4 were used. Initially, expression of CTLA-4 was confirmed by incubating the cells at 37°C for 30 minutes with a PE labelled CTLA-4 antibody. **Figure 3.2.10** shows that the cells expressed very high levels of CTLA-4.

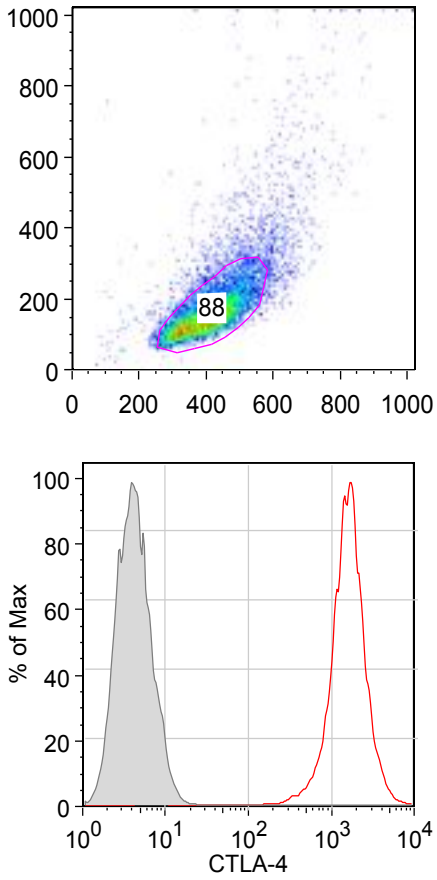

Figure 3.2.10 Phenotype of CHO-CTLA-4 Cells. CHO cells expressing CTLA-4 were labeled with CTLA-4 PE at 37°C for 30 minutes before being analysed by flow cytometry. The data presented is representative of the cell line.

To check that each of the antibodies could specifically recognise the full-length protein, CTLA-4 CHO cells were incubated with each antibody at 4°C for 30 minutes. The antibodies were then stained with an anti-human or anti-mouse FITC antibody and presence of bound antibody was determined using flow cytometry. **Figure 3.2.11** shows the results of these experiments. This figure shows that all of the antibodies could indeed recognise full length CTLA-4. Interestingly, although both 11D4 molecules had very poor BIAcore results, the fluorescent profiles of these antibodies upon binding full length CTLA-4 was the highest suggesting that a large amount of these antibodies could bind to the protein. Both 10A8 and 26B whole also seemed to have bright peaks of binding. The remaining antibodies had very uniform binding patterns.

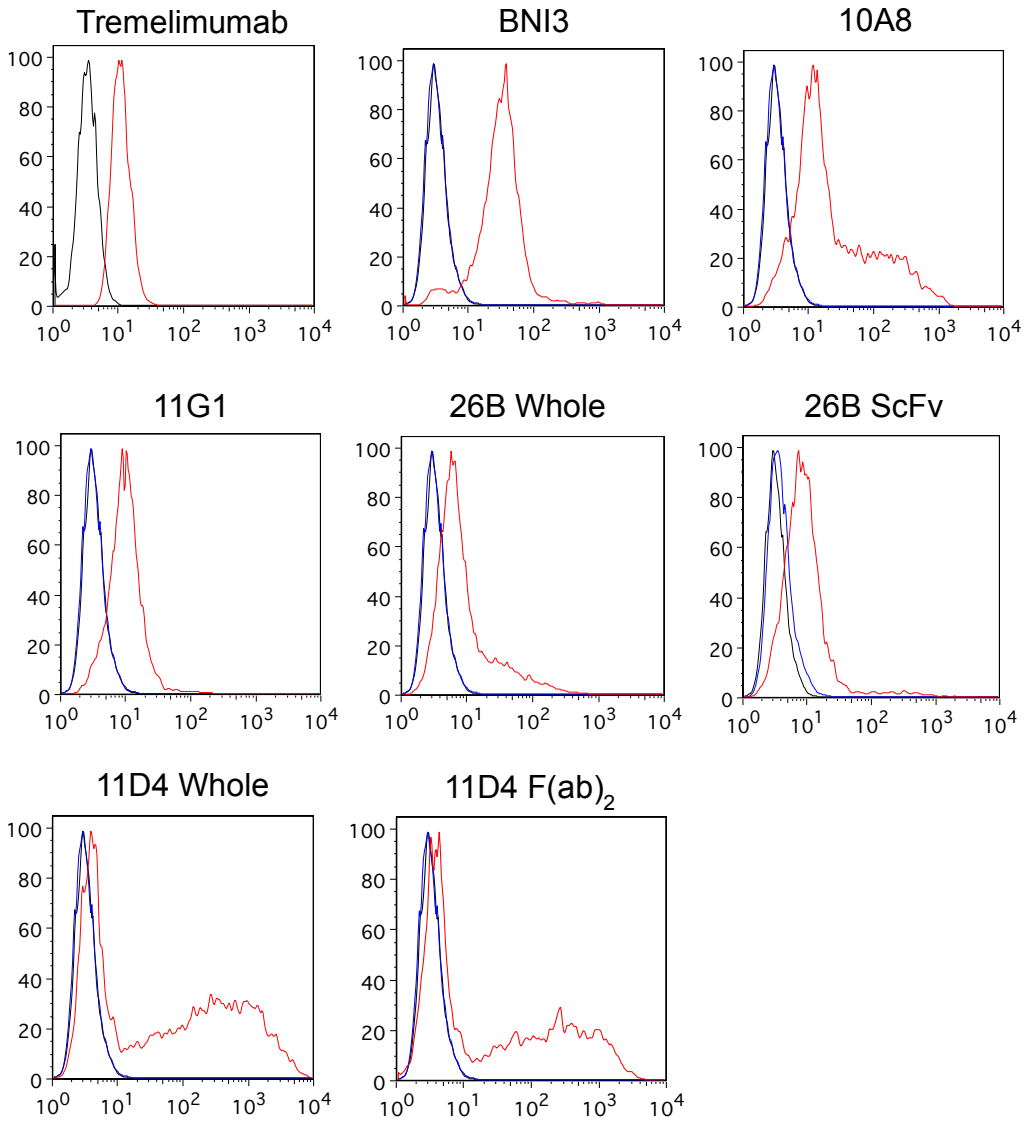

Figure 3.2.11: Anti-CTLA-4 antibodies bind to full length CTLA-4 expressed in CHO cells. The antibodies were incubated with CHO cells expressing CTLA-4 on ice for 30 minutes to stain the surface protein. Cells were then labelled with an anti-mouse FITC or anti-human FITC (for tremelimumab only) on ice for a further 30 minutes. The staining was then analysed by flow cytometry.

Having determined the binding characteristics of the antibodies, it was decided to test if the antibodies were able to disrupt CTLA-4 binding to its ligands. In order to carry out the blocking experiments successfully a concentration of CTLA-4 Ig that provided sub-maximal staining was desired to ensure that the antibodies and ligands could compete for CTLA-4 Ig binding. To find out this dose, CTLA-4 Ig was titrated from 20µg/ml to 0.0001µg/ml, each sample was subjected to flow cytometry analysis and the mean fluorescence was plotted on a graph (**figure 3.2.12**). From these data it was determined that using the CTLA-4 Ig at a fixed dose of 0.1µg/ml provided sub maximal staining for both CHO cells that stably express either CD80 or CD86 and this was the concentration of CTLA-4 Ig used for all subsequent blocking experiments.

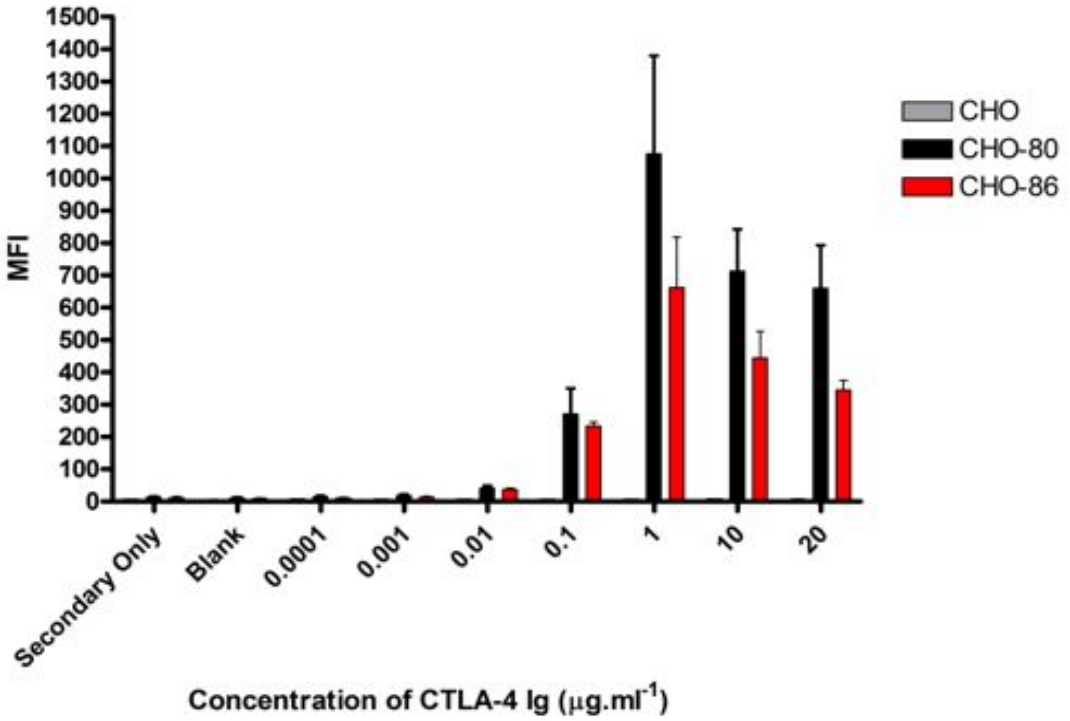

Figure 3.2.12: Titration of CTLA-4 Ig to achieve sub maximal staining of CHO cell tranfectants. A sub-maximal dose of CTLA-4 Ig for use in blocking experiments was determined by incubating 150,000 CHO, CHO-CD80 or CHO-CD86 cells for 30 minutes with various concentrations of CTLA-4 Ig. The cells were stained with an anti-human Fc specific antibody which was directly conjugated to a FITC fluorophore. Each sample was then analysed by flow cytometry and the mean flueoresence plotted on a graph. The data presented is representative of three independent experiments.

To address if the antibodies were blockers, an assay was developed where various dilutions of the antibody was incubated with CTLA-4 Ig that had been pre-labelled with a PE fluorophore. To this, CHO cells expressing either CD80 or CD86 were added. The detection of bound Ig was carried out by flow cytometry and the results are shown in **figure 3.2.13 and 3.2.14**. These data show that 10A8 and 11G1 are all capable of blocking CTLA-4 binding to both CD80 and CD86 and do so at a concentration of 1µg/ml. Interestingly, although the ELISA data suggested that tremelimumab seemed to have around 100 fold lower binding capacity than most of the other antibodies, this antibody turned out to be a very good blocking antibody and could also completely ablate ligand binding at 1µg/ml. Importantly, not all antibodies that recognise CTLA-4 make good blockers, for example, although 26B was able to recognise CTLA-4 Ig in the ELISA and was able to completely block CTLA-4 interactions with CD86, it was only able to partially inhibit binding to CD80. Further to this, 11D4 was also a very poor blocking antibody as it could only inhibit binding by around 50% for both ligands. These data highlight the importance of carrying out a thorough characterisation of antibodies for use in functional studies. Since tremelimumab appeared to be one of the best blocking antibodies, this was the antibody chosen for use in functional experiments.

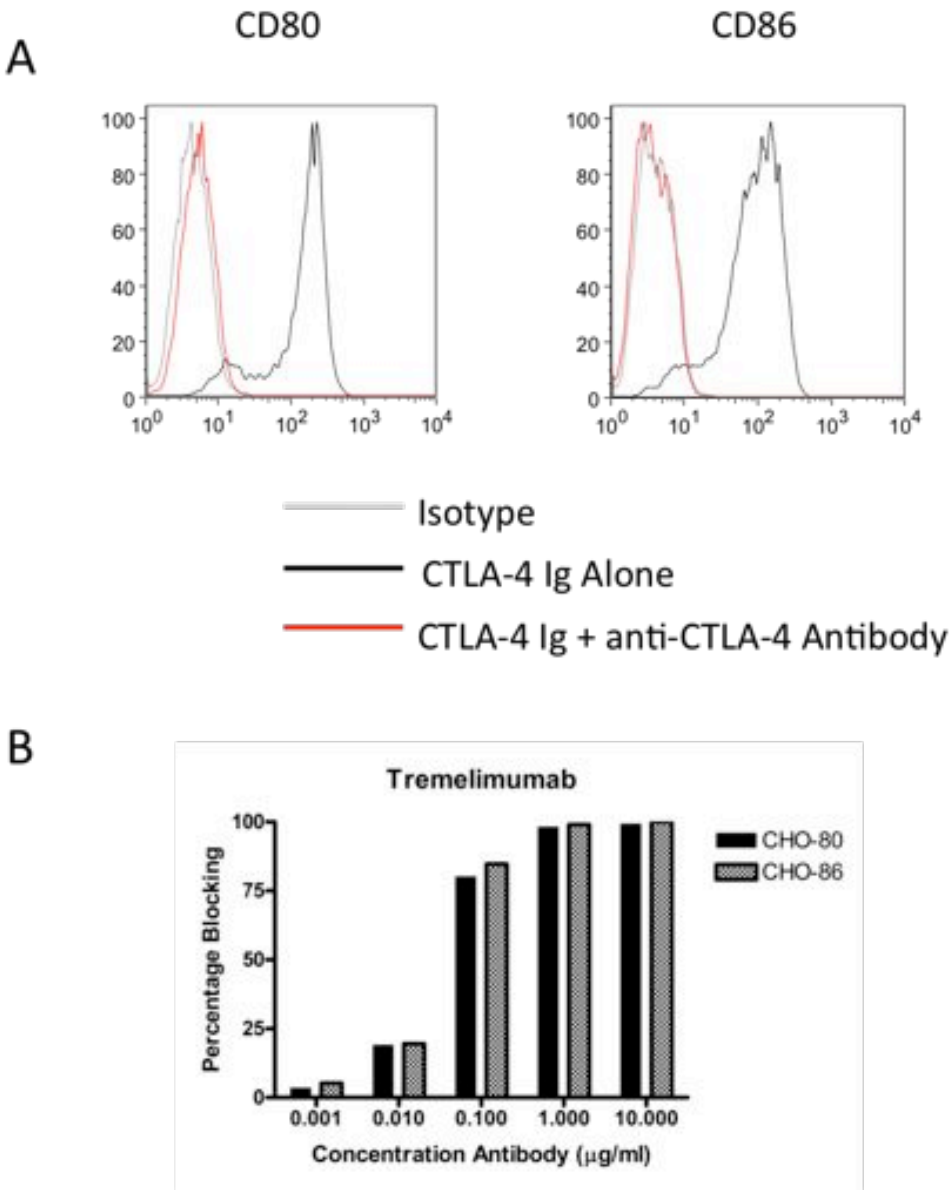

Figure 3.2.13: Tremelimumab blocks CTLA-4 binding to ligands expressed on CHO cells. CHO Cells expressing either CD80 or CD86 were incubated with 0.1µg/ml of CTLA-4 Ig, which had been directly conjugated to a FITC fluorophore, in the presence of increasing concentrations of anti-CTLA-4. **A.** Flow cytometry plots showing intensity of CTLA-4 Ig binding to ligand. **B.** Percentage blocking is calculated based on the MFI of CTLA-4 Ig staining in the presence of decreasing concentrations of anti-CTLA-4. The data presented are representative of at least three independent experiments.

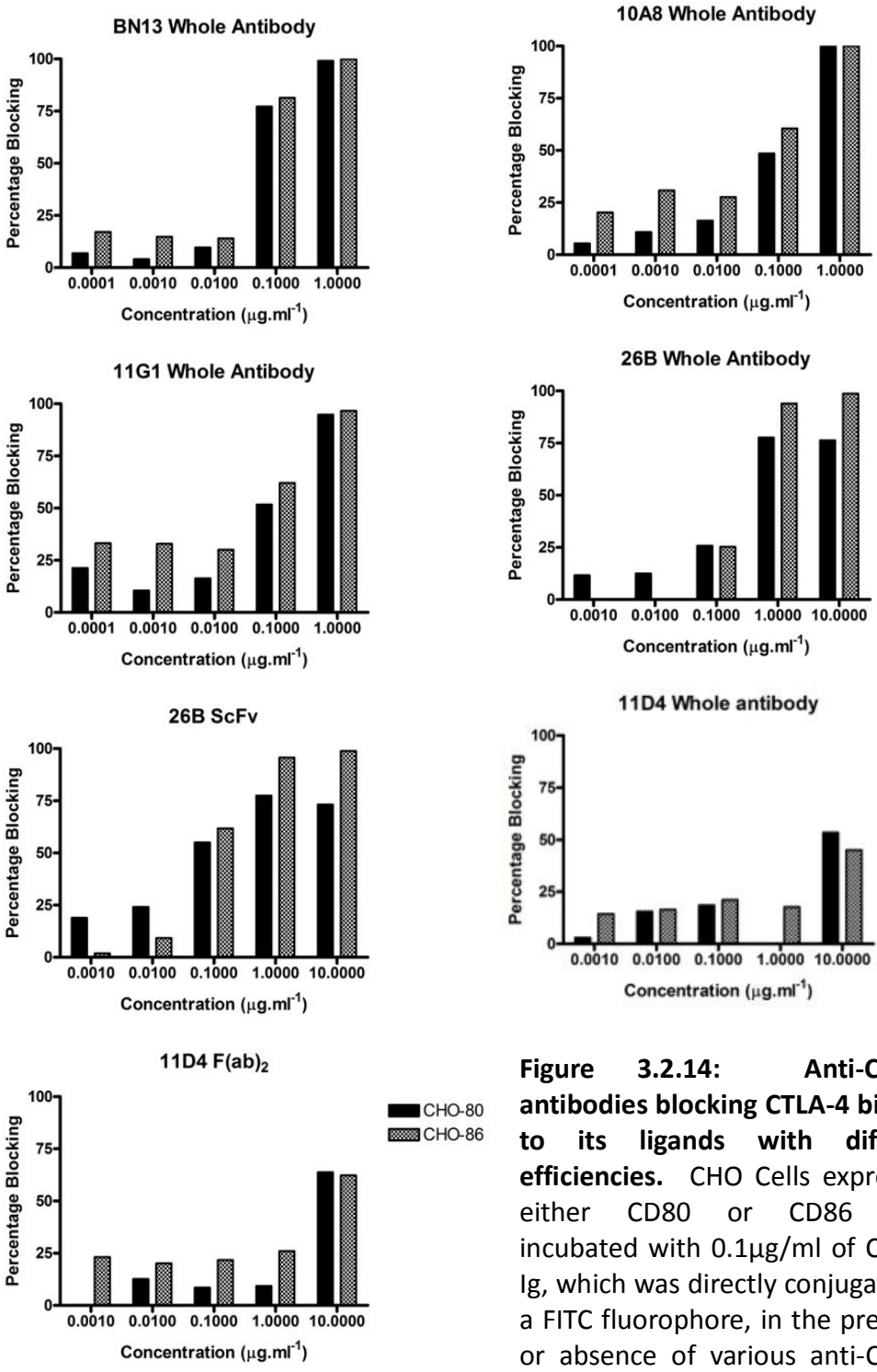

Figure 3.2.14: Anti-CTLA-4 antibodies blocking CTLA-4 binding to its ligands with differing efficiencies. CHO Cells expressing either CD80 or CD86 were incubated with 0.1μg/ml of CTLA-4 Ig, which was directly conjugated to a FITC fluorophore, in the presence or absence of various anti-CTLA-4 antibodies at the indicated concentrations. Percentage blocking are calculated based on the MFI of CTLA-4 Ig staining in the presence of decreasing concentrations of anti-CTLA-4.

To determine if blocking efficiency could be predicted using the KD, it was decided to plot the KD values against the percentage blocking obtained using 0.1μg/ml of each antibody (**figure 3.2.15**). A non-linear regression was fitted to the graph and the R^2 value calculated. This data shows that the blocking of both CD80 and CD86 followed a similar trend at this concentration of antibody. Further, it appears that the blocking potential of each antibody cannot be readily predicted by the KD value associated with the antibody.

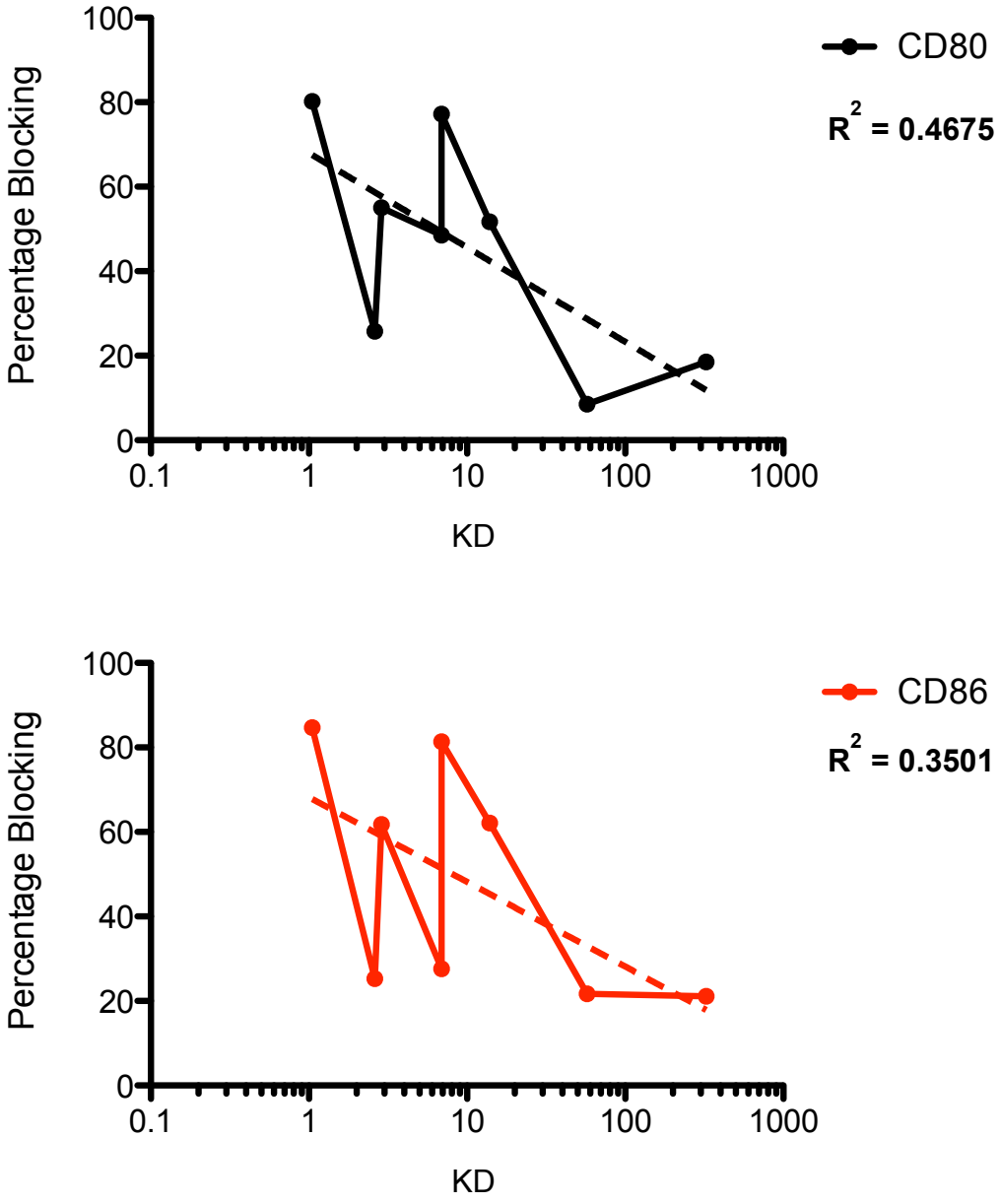

Figure 3.2.15: Does KD Value Predict Blocking Capability of Antibody: The KD values for each of the antibodies derived from the BIAcore experiments were plotted against the percentage blocking achieved using a concentration of anti-CTLA-4 antibody of 0.1µg/ml. The graphs were fitted with a non-linear regression and the R^2 value calculated.

3.3 Discussion

Antibodies against CTLA-4 provide both a useful tool in studying the importance of ligand binding to T-cell activation as well as presenting a potentially powerful target in the treatment of tumours. With this in mind it was decided to try to characterise a panel of antibodies that were available within the laboratory.

Very little was known about any of these antibodies, other than claimed specificity, so to begin the characterisation it was important for determine that the antibodies were able to recognise CTLA-4. The ELISA data confirmed that they all had the ability to bind the extra-cellular domain of the protein but that 11D4 required one hundred fold more antibody to achieve what was perceived as maximal binding. Further to this, tremelimumab appeared to reach its highest level of absorbance at 10μg/ml, thus requiring one thousand fold more antibody than the others. The fact that even at this concentration it cannot be guaranteed that tremelimumab has managed to saturate binding suggests that this is a fairly low affinity antibody.

However, an alternative explanation for the apparent poor binding of Tremelimumb in the ELISA experiments may be that there is saturation of the signal on the plate reader and that we are simply observing that the other antibodies saturate this signal earlier. A further difficulty with the ELISA data is the fact that

Tremelimumab had to be directly conjugated to HRP, whereas all of the other antibodies were detected by probing with a secondary HRP antibody. This makes direct comparison of the antibodies problematic. Whilst the ELISA was initially intended for use as a system to reveal the binding the characteristics of the antibodies for CTLA-4, these issues meant an alternative means of verification was required. The BIAcore analysis enabled a more sensitive approach to determine binding characteristics of the antibodies. Further as a result of the fact that binding could be detected in the absence of a secondary reagent, it meant that direct comparisons of the antibodies could be carried out.

In contrast to what was implied by the ELISA data, it was actually found that Tremelimumab had the lowest kD value when analysed by BIAcore. The antibody appeared to have a very fast on rate and, once the flow stopped, a large amount of the antibody fell off, before stabilisation of the curve. This binding profile may go some way to explaining the seemingly poor binding characteristics in the ELISA experiments.

As could be observed from the comparison of blocking capability with KDs derived from BIAcore, it was interesting to note that that not all of the antibodies had such good blocking potential as would be expected based on the binding profiles. For example, BNI3 and 10A8 had very similar KD values with 6.908nM and 6.891nM respectively, but their blocking potential at a concentration of 0.1μg/ml

was around 75% for BNI3 and 65% for 10A8. A possible reason for the variance in blocking capability of antibodies with similar KD values could be a result of the epitope that the antibodies recognise. It may be that the epitope recognised by BNI3 is more conducive to blocking CTLA-4:ligand interactions than that of 10A8.

It would have been useful to carry out some direct competition experiments between the antibodies to confirm the affinity data obtained from the BIAcore. However, this was not performed as there were insufficient quantities of some of the antibodies to carry out a comprehensive competition study. A further issue with competition studies is that the epitopes for most of the antibodies were unknown, which would possibly have made interpretation of any competition data more difficult.

It is interesting to note that some antibodies had varying blocking potentials depending on which ligand they were attempting to disrupt CTLA-4 binding to. One example of this is 26B. While this antibody seems able to completely block CD86 binding it can only inhibit CD80 binding by up to 75%. The antibody having a higher affinity for CTLA-4 than CD86 but a lower affinity for CTLA-4 than CD80 presumably causes this effect. The selective nature of this antibody makes it potentially useful in studying the importance of the individual ligands in CTLA-4 mediated suppression. The poor blocking status of 26B was perhaps not entirely surprising given its apparent binding site. The 26B antibody is one of the only antibodies tested where

the binding site has been determined (**Figure 3..3.1**). Mutational analysis carried out determined that 26B ScFv bound to the M11 epitope ^{65}SICT^{68} [11], a site located away from the ligand binding MYPPY motif. It could be that binding to this site causes a conformational change in the structure of the ligand-binding site and that this prevents ligand engagement.

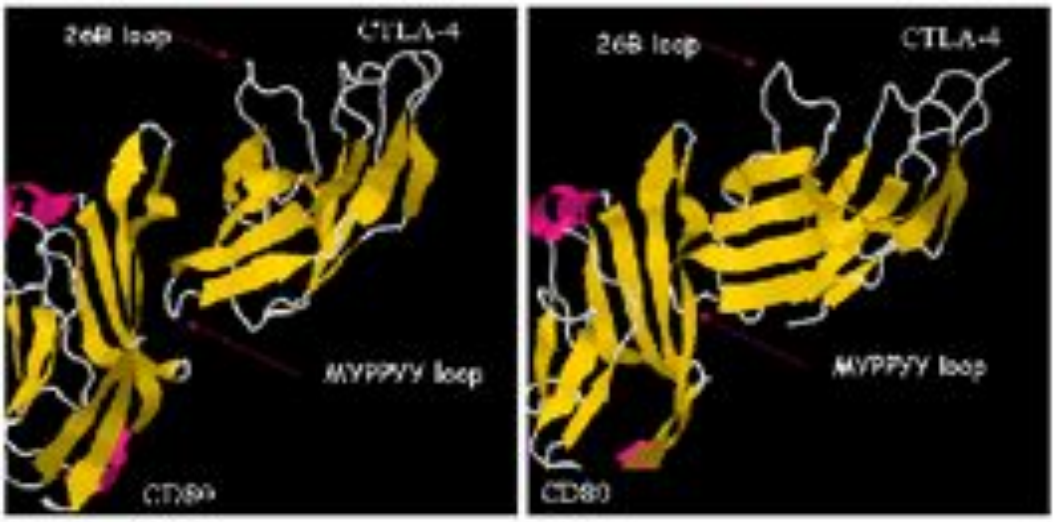

Figure 3.3.1: Crystal structure of CTLA-4 ligand binding domain. The left hand image shows the ligand binding portion of CTLA-4. The MYPPYY loop which allows binding to the CTLA-4 ligands is indicated. The diagram also indicates the region where mutational studies have placed 26B binding. This site is located away from the ligand binding site. The right hand image is the same except it has been rotated by 30°. Image adapted from the Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank; file ID 1I8L.pdb.

Some interesting observations could be made when looking at the ability of the antibodies to bind CTLA-4 expressed in CHO cell lines. From the surface stains for both 11D4 antibodies and 10A8, it appeared that only a proportion of the cells were being stained and that those cells that did become stained had very high fluorescent profiles. A simple explanation for these data would be that the antibody is aggregating and this is causing the bright fluorescence. Alternatively, it could be that these antibodies are only able to bind to a specific subset of CTLA-4 molecules. It is known that increased glycosylation of CTLA-4 is required for surface expression of the protein [12] as well as for the stabilisation of the CTLA-4 homodimer [13, 14]. It has also been shown that phosphorylation of the tail of CTLA-4 is required for inhibition of its interaction with clathrin adapter molecule AP-2 [15, 16] and hence increased surface expression. It may be that some of these modifications interrupt antibody binding to the protein resulting in the strange staining patterns observed

Overall this chapter highlighted the importance of fully characterising antibodies before using them in functional studies. Just because an antibody is able to bind to the extra-cellular region of CTLA-4, does not necessarily mean that it will be a good blocking antibody. Upon full assessment of the antibodies, it was decided to use tremelimumab in studying the function of CTLA-4, since it was one of the most potent blocking antibodies.

Chapter 4:

Assessing CTLA-4 Function

4.1 Introduction

The mechanism by which CTLA-4 functions to control T-cell responses has been subject of debate. Numerous modes of action have been proposed for CTLA-4 since it was first discovered, despite this, exactly how this protein modulates T-cell responses remains unknown. The broad aim of this project was to determine the context of CTLA-4 function by testing the predictions of various models that have been suggested. One of the most widely embraced mechanisms of action for CTLA-4 is that it delivers a negative signal into the T-cell upon ligand engagement. This model was first suggested after the discovery that cross-linking CTLA-4 with antibodies results in reduced IL-2 production and proliferation [1]. Subsequently, it has been shown that CTLA-$4^{-/-}$ mice expressing a form of CTLA-4 lacking the intracellular domain fails to entirely rescue the fatal phenotype of these mice [2]. This suggests that it is important for the protein to interact with the inside of the T-cell in order to function completely. A number of laboratories have shown evidence that CTLA-4 can interact with a number of signalling proteins including PI3K and SHP-2 suggesting CTLA-4 may function by signalling into the T-cell.

Having characterised a series of antibodies in the previous chapter, it was decided to use these as tools to study the function of CTLA-4 with a focus on determining if the protein is able to deliver a negative signal when stimulated with natural ligands. Accordingly, blocking the interaction between CTLA-4 and its ligands should reveal settings in which CTLA-4 is active by enhancing T-cell responses.

4.2 Results

4.2.1 Activating T-cells with CHO cells expressing CTLA-4 ligands

The theory about the negative signal has arisen from experiments using antibodies to cross-link CTLA-4 and observing a subsequent down regulation in proliferation and IL-2 production. However, to our knowledge, this mechanism has not been shown to occur upon engagement with CD80 and CD86 ligands. To determine whether CTLA-4 was inhibitory in a ligand driven model of T-cell activation, CHO cells expressing either CD80 or CD86 on their surface were used to provide co-stimulation. Our laboratory previously created stable CHO cells lines that express high levels of the B7 molecules (**figure 4.2.1**). To carry out these functional experiments, responder $CD25^-$ T-cells were isolated from peripheral blood mononuclear cells (PBMCs), the initial step in this process involved negative selection of $CD4^+$ T-cells which, as is shown in **figure 4.2.2**, resulted in a purity of around 87% of $CD3^+CD4^+$ T-cells. These cells were then subjected to a further negative selection step to isolate $CD25^-$ T-cells, which yielded a purity of greater than 90% $CD25^-$ T-cells. Purities of cells were assessed before every experiment and the data presented in figure 4.2.2 are representative of a typical yield of purified cells.

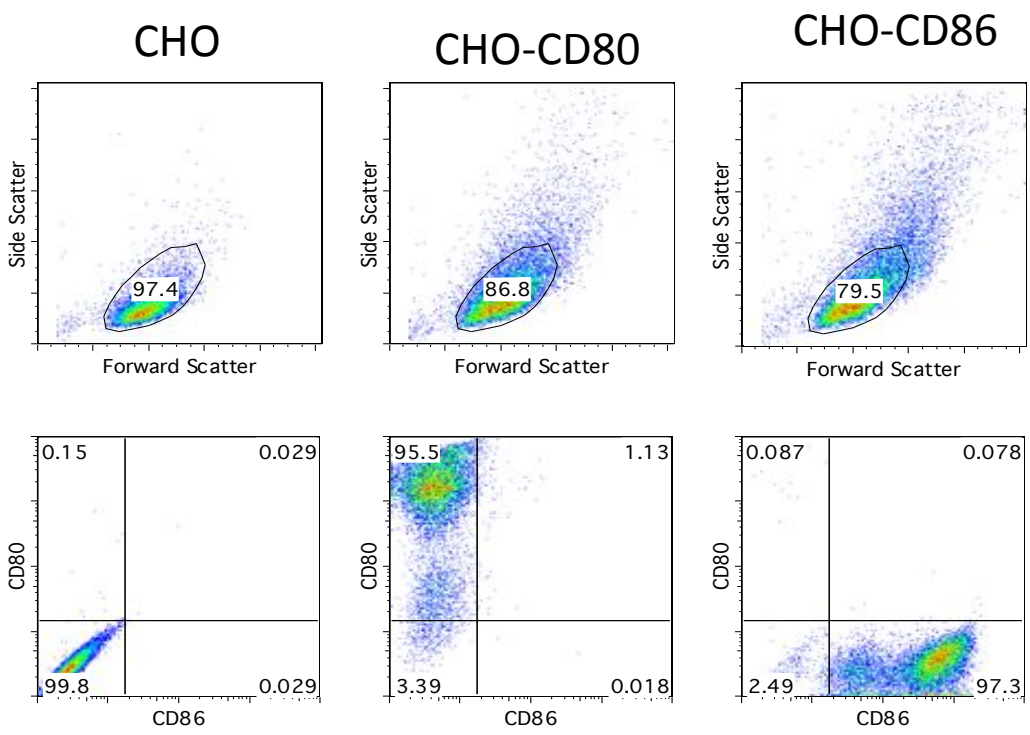

Figure 4.2.1: Phenotype of CHO cell transfectants. CHO cells, either untransfected or transfected with CD80 or CD86, were stained with CD86 FITC and CD80 PE on ice for 30 minutes before being analysed by flow cytometery Gates were drawn using the CD80 and CD86 staining on an untransfected CHO cell.

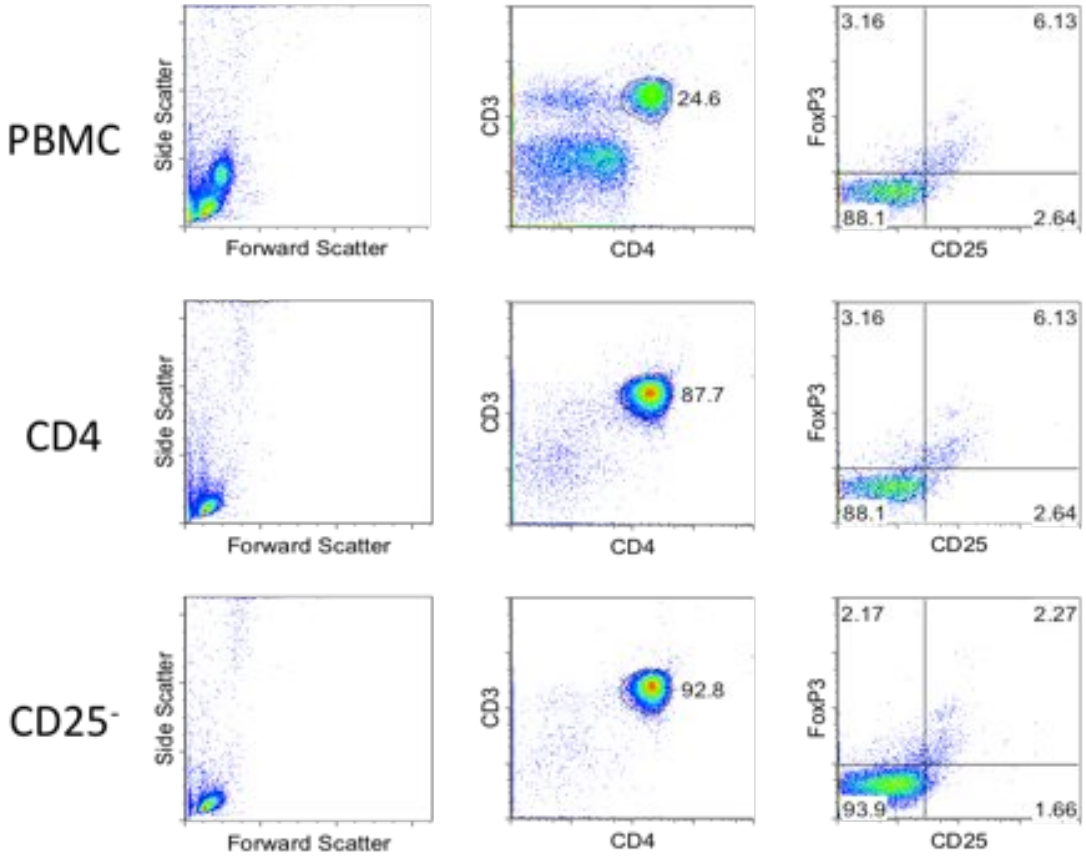

Figure 4.2.2 : Purity of T-cells isolated from PBMCs. T cells were isolated from PBMCs using a two step process. Step 1 involved the selection of $CD4^{+}$ T-cells, whilst step 2 saw the selection of $CD25^{-}$ T-cells. At each stage of the isolation process, samples of the T cell cultures were surface stained for CD3, CD4 and CD25 before being fixed, permeabilised and stained for FoxP3. Purities of T-cells were determined before each experiment. The data presented is representative of a typical yield.

It was first important to assess whether or not the CHO cells would provide adequate co-stimulation. To do this, $CD25^-$ T-cells were labelled with CFSE before being stimulated with CD3 in the presence of untransfected, CD80 or CD86 expressing CHO cells. On days 2, 3, 4 and 5 after stimulation the T-cells were stained for their CD25 expression and both this and the proliferation was measured by flow cytometry (**figure 4.2.3**). The T-cells stimulated with both CHO-CD80 and CHO-CD86 cells have begun to up-regulate expression of CD25 by day 2. By day 5, the majority of these cells are expressing this protein. Despite this up-regulation of CD25 by day 2, those cells stimulated with CHO-CD86 cells don't show signs of proliferation until day 3. The CHO-CD80 cells appear to stimulate the T-cells at a slower rate as these T-cells begin to proliferate by day 4, however, the purpose of the experiment was not to test the relative effects of each ligand and differences in expression may account for these effects. Nonetheless, in the case of both cell types, the majority of T-cells are proliferating by day 5. Importantly, it appears that those T-cells stimulated in the presence of untransfected CHOs neither proliferate nor up-regulate CD25, highlighting that this model requires the presence of the co-stimulatory ligands and that CD3 is not enough to drive activation. Taken together, these data indicate that the CHO cells do provide a suitable model for co-stimulation for which to study CTLA-4 function.

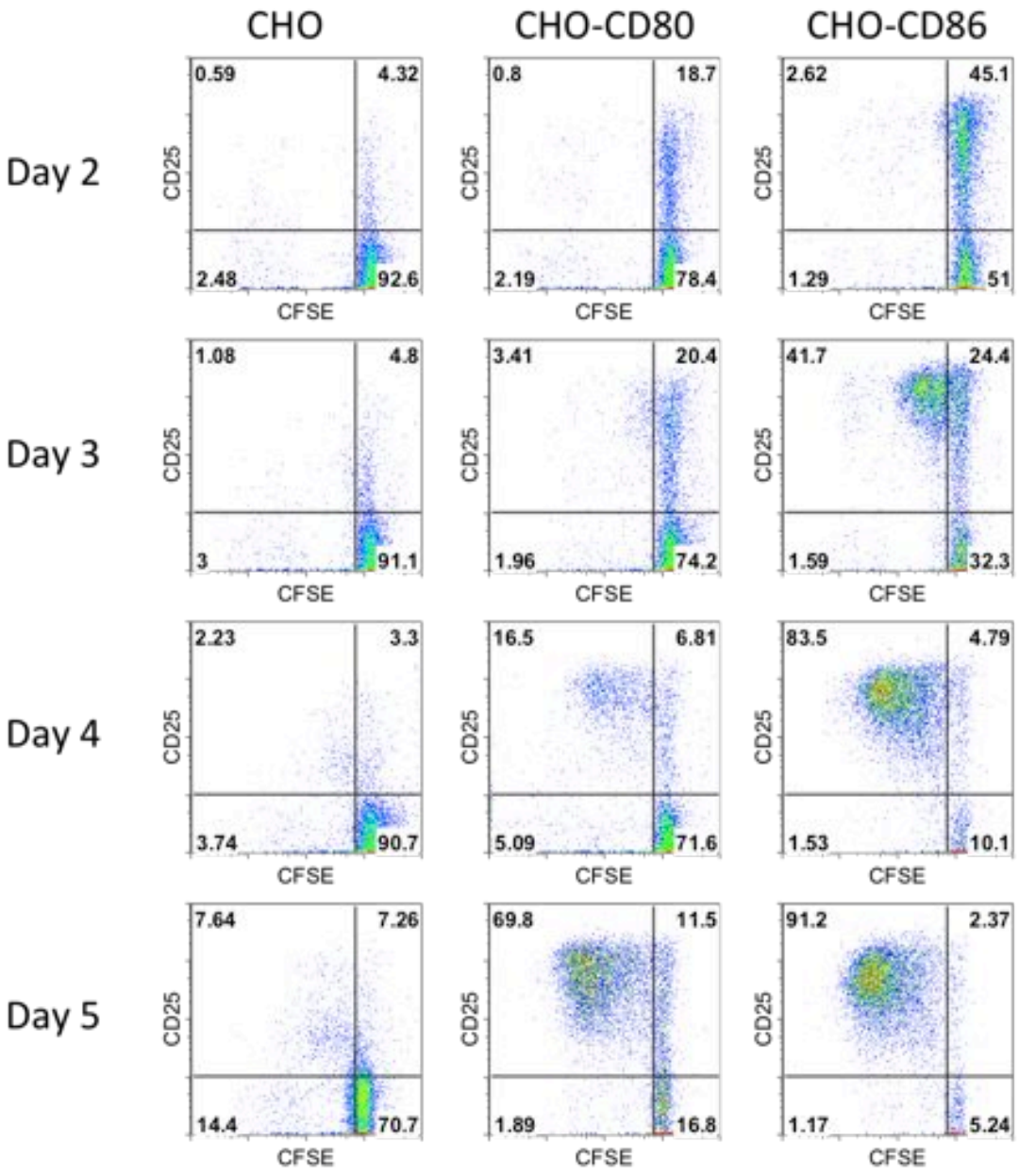

Figure 4.2.3: CHO cells expressing CD80 or CD86 can provide T-cell co-stimulation. CFSE labelled CD25$^-$ T-cells were stimulated with 10µg/ml of CD3 in the presence of gluteraldehyde fixed CHO, CHO-CD80 or CHO-CD86 expressing cells at a ratio of one CHO cell to three T-cells. At day 2, 3, 4 and 5 the cells were stained for the presence of surface CD25 by incubating the on ice for 30 minutes. Both CD25 and the amount of proliferation in the cultures was analysed by flow cytometry. The data presented is representative of five independent experiments.

To determine if CTLA-4 is able to deliver a negative signal in this setting, $CD25^-$ T-cells were labelled with CFSE and stimulated with CHO, CHO-CD80 or CHO-CD86 cells together with CD3. This was done in the presence or absence of the blocking antibody tremelimumab. After five days of stimulation the cells were analysed by flow cytometry and the data are shown in **figure 4.2.4**. Again we observed that the T-cell proliferation was co-stimulation dependent as there was very little proliferation when the untransfected cells are present compared to when the CD80 and CD86 cells provide the co-stimulation. Surprisingly, we observed that blocking CTLA-4 in this assay has no effect on T-cell proliferation, a finding not obviously consistent with this molecule delivering a cell intrinsic negative signal. To ensure that the T-cells were expressing CTLA-4 at this time point the T-cells were fixed, permeabilised and stained for the protein at day five (**figure 4.2.5**). From this, we see that all of the proliferating cells are expressing CTLA-4. The fact that blocking CTLA-4 is having no effect is therefore not a result of an absence of CTLA-4 expression. Thus in this model we observe ligand dependent T-cell stimulation and up-regulation of CTLA-4 yet despite both ligand and receptor being expressed no obvious inhibitory function of CTLA-4 is revealed.

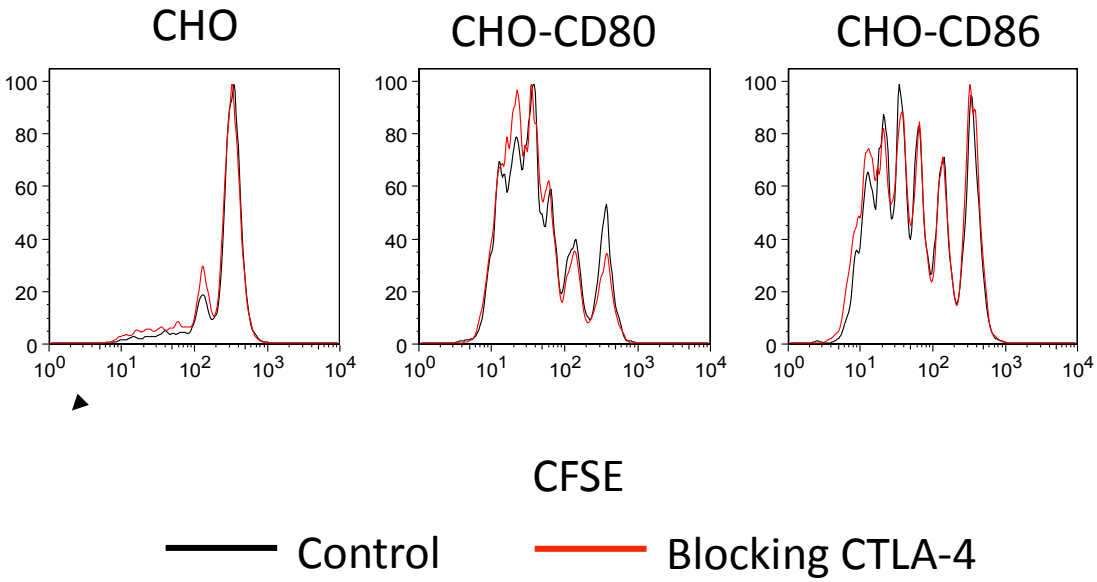

Figure 4.2.4: Blocking CTLA-4 has no effect on the proliferation of T-cells co-stimulated with CHO cell transfectants. CFSE labelled $CD25^-$ T-cells were stimulated with 10µg/ml of CD3 and gluteraldehyde fixed untransfected CHO, CHO-CD80 or CHO-CD86 expressing cells at a ratio of one CHO cell to three T-cells. The cultures were incubated in the presence or absence of a blocking CTLA-4 antibody (Tremelimumab, 50µg/ml). After five days the level of proliferation was determined by flow cytometry. The data presented is representative of five independent experiments.

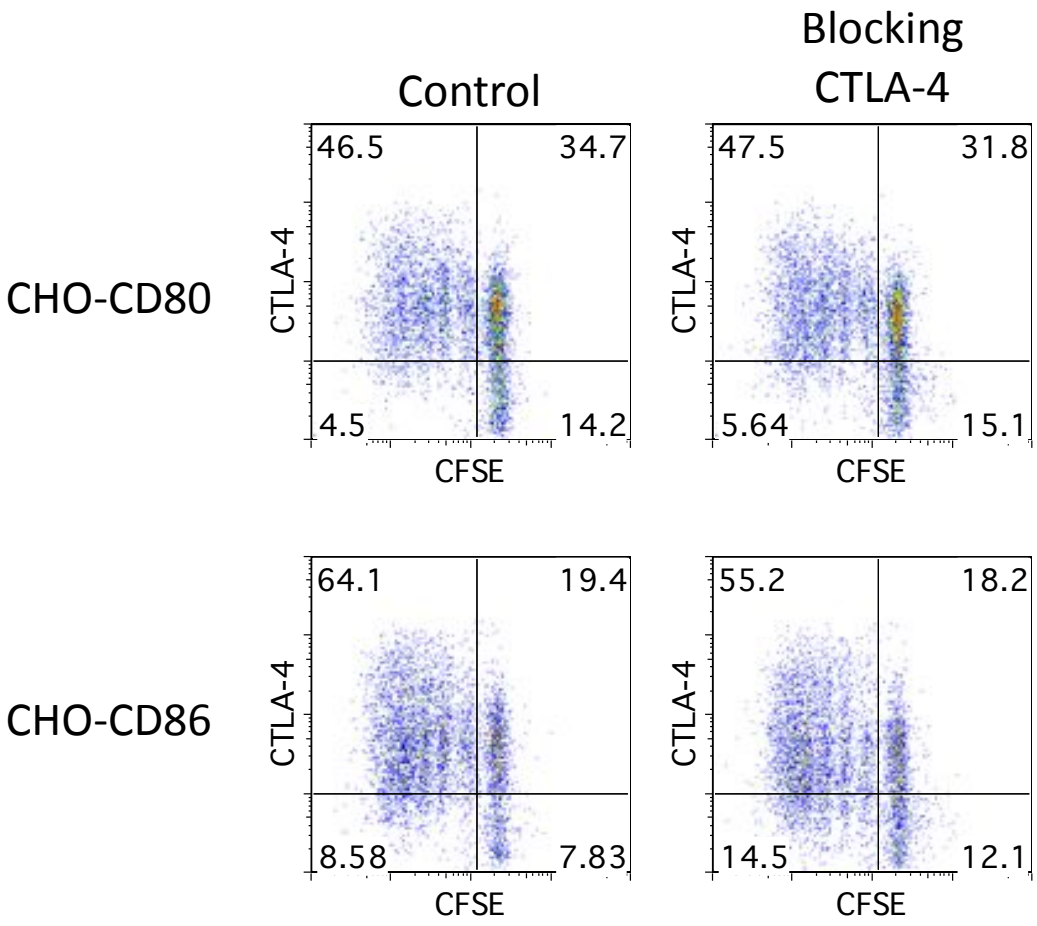

Figure 4.2.5: CD25$^-$ T-Cells co-stimulated with CHO cell transfectants express CTLA-4. CFSE labelled CD25$^-$ T-cells were stimulated with CD3 (10µg/ml) in the presence of gluteraldehyde fixed untransfected CHO, CHO-CD80 or CHO-CD86 expressing cells, at a ratio of one CHO cell to three T-cells. The cultures were incubated in the presence or absence tremelimumab (50µg/ml) to block CTLA-4. After five days the cells were fixed in 3% PFA and permeabilised in a 5% PBS-Saponin solution and stained for total CTLA-4 expression before being analysed by flow cytometry. The data presented are representative of five independent experiments.

The lack of antibody effects could possibly be explained by the fact that a very high numbers of CHO cells were used to provide co-stimulation. It was therefore decided to assess if a negative signal could be revealed by reducing numbers of CHO cells used to stimulate T-cells thereby weakening the co-stimulation via CD28. As can be seen from **figure 4.2.6**, titrating out the number of CHO cells, only revealed in effect in the cultures stimulated with CHO-86 cells at a 1:500 ratio, no other samples revealed any evidence of a negative signal.

It has been previously shown by other laboratories that cross-linking antibodies against CTLA-4 results in reduced IL-2 production. It was therefore decided to see if blocking CTLA-4 using non cross-linked antibody model would affect IL-2 expression in our CHO system. To do this, $CD25^-$ T-cells were stimulated as described. After 5 days of stimulation the samples were re-stimulated with PMA, Ionomycin and Brefeldin A for four hours to induce cytokine production, the cells were then fixed permeabilised and stained for the presence of IL-2, data is shown in **figure 4.2.7**. These data show that both CD80 and CD86 can stimulate IL-2 production in T-cells however it does appear that stimulating with CD86 results in more IL-2 producing cells than stimulating with CD80. Importantly however, Instead of enhancing IL-2 production, we observed that blocking CTLA-4 results in a slight decrease in overall IL-2 expression, a finding that is counter to previous observations. To investigate if CTLA-4 can influence other cytokine production, the

expression of IFNγ, IL-17 and IL-21 were also studied (**figure 4.2.8**). Again, both CHO-CD80 and CHO-CD86 cells stimulate the production of IFNγ, IL-17 and IL-21 to varying degrees. Thus in this model of T-cell stimulation we consistently found that blocking CTLA-4 had little effect on cytokine production.

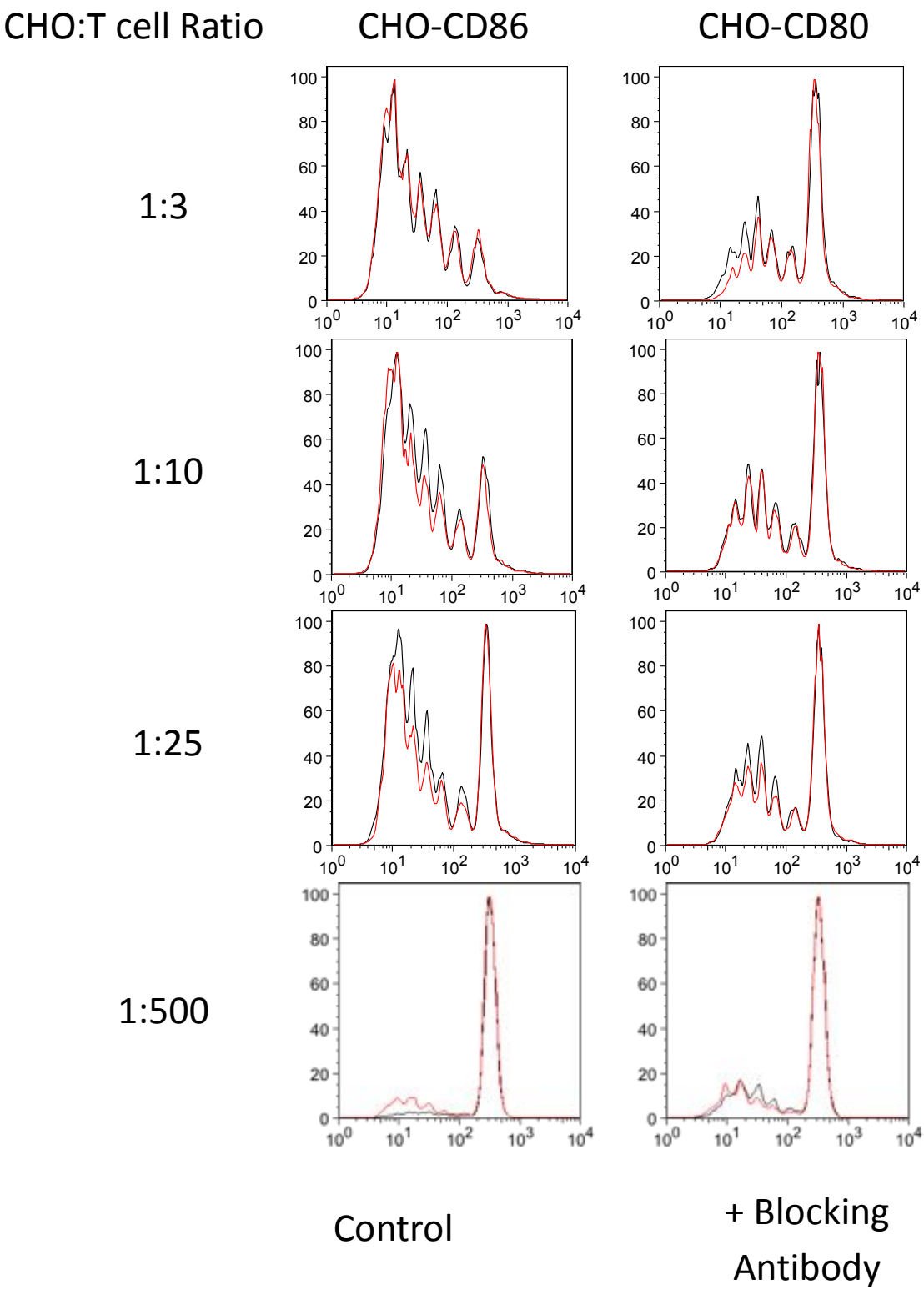

Figure 4.2.6: Titrating the number of co-stimulatory CHO cells does not reveal a negative signal. CFSE labelled $CD25^-$ T-cells were stimulated with CD3 (10µg/ml) and reducing numbers of gluteraldehyde fixed CHO cell transfectants in the presence or absence of the blocking CTLA-4 antibody Tremelimumab (50µg/ml). Proliferation was determined by flow cytometery after five days. The data presented is representative of five independent experiments.

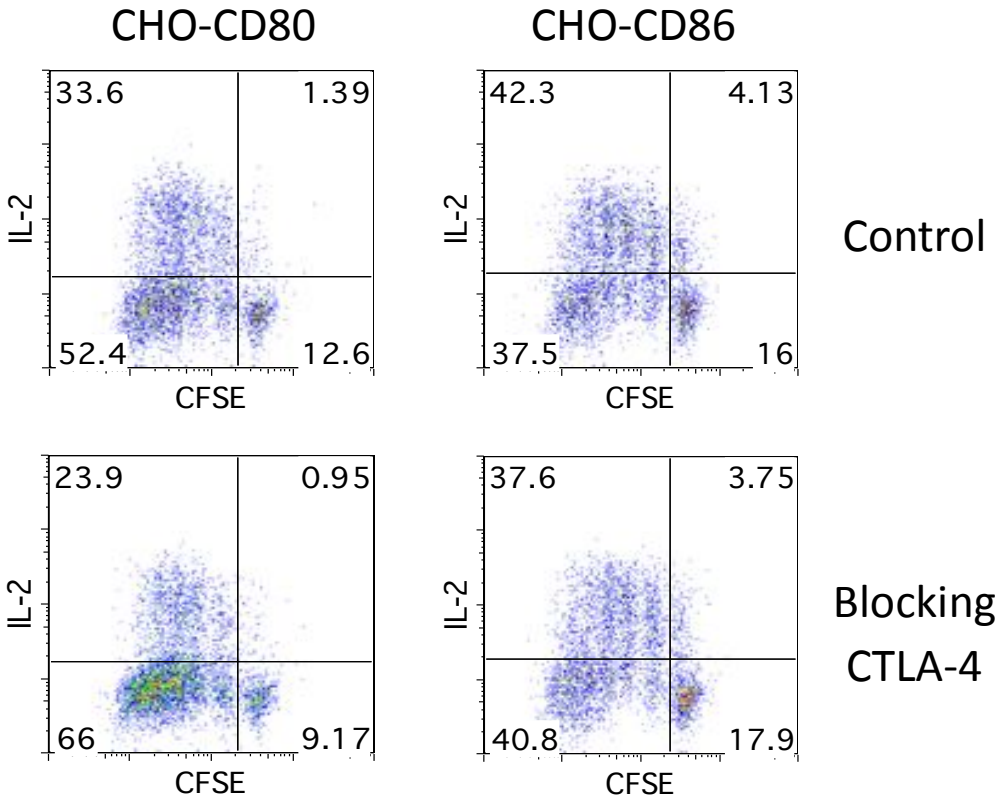

Figure 4.2.7: Blocking CTLA-4 does not enhance IL-2 production in a CHO cell transfectant driven assay of T-cell proliferation. CFSE labelled $CD25^-$ T-cells were stimulated with CD3 (10µg/ml) in the presence of gluteraldehyde fixed CHO cells expressing either CD80 or CD86 at a ratio of one CHO cell for every three T-cells. After 5 days, cultures were re-stimulated with PMA (50ng/ml), Ionomycin (1µM) and Brefeldin A (5µg/ml) for four hours, the cultures were then fixed with PFA (3%) and permeabilised in a 5% PBS-Saponin solution before being stained for IL-2 production. The data presented is representative of five independent experiments.

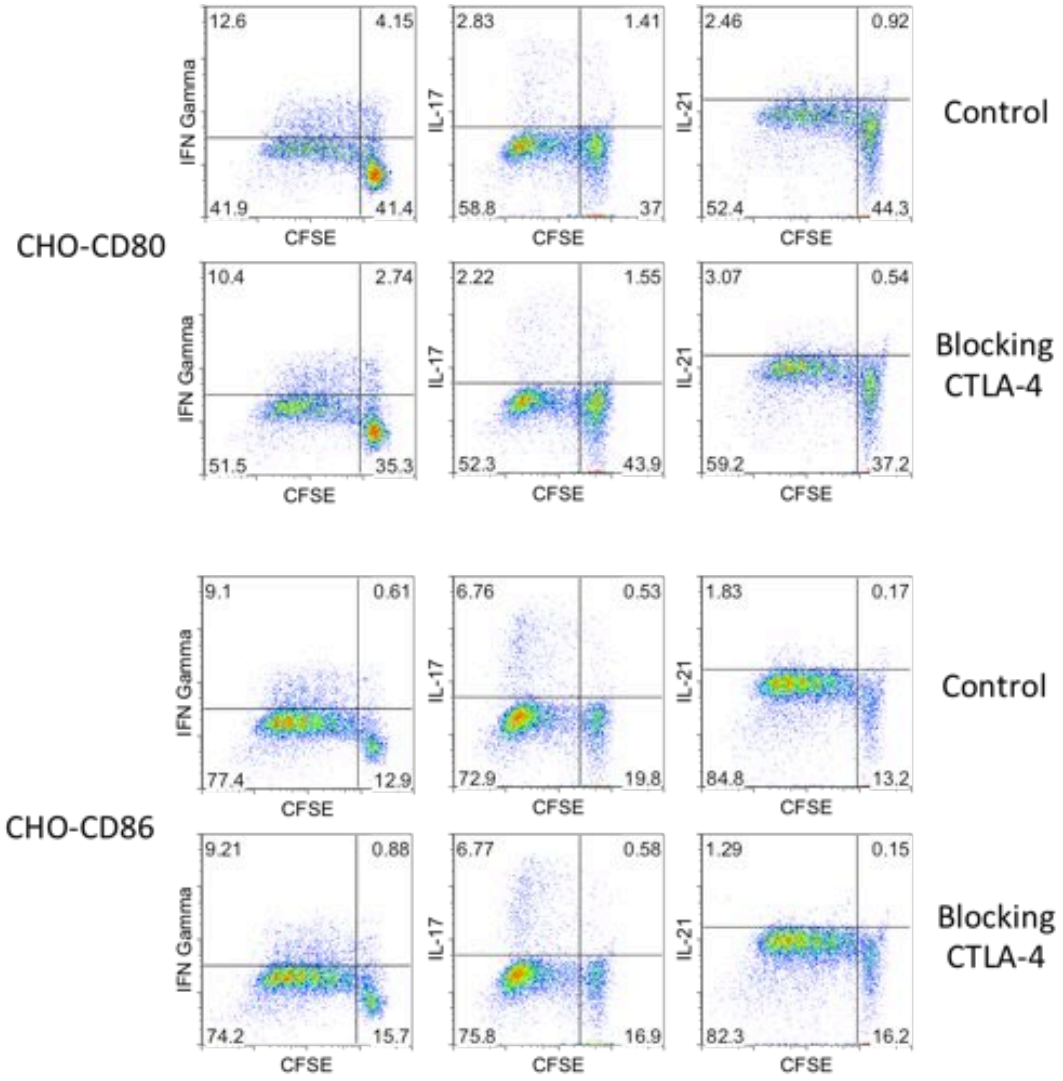

Figure 4.2.8: Blocking CTLA-4 has differential effects on the production of cytokines. CFSE labelled $CD25^-$ T-cells were stimulated with CD3 (10µg/ml) in the presence of gluteraldehyde fixed CHO cells expressing either CD80 or CD86 at a ratio of three T-cells to every one CHO cell. After 5 days, cultures were re-stimulated with PMA (50ng/ml), Ionomycin (1µM) and Brefeldin A (5µg/ml) for four hours before being stained for IL-17, IL-21 or IFNγ production. The data presented is representative of five independent experiments.

Since CTLA-4 does not become up-regulated until after the T-cell is activated, it could be argued that the lack of antibody affects may be a result of the fact that the target protein is not there at the beginning of the experiment, it was therefore decided to see if blocking CTLA-4 would have any effect on T-cell blasts. For this, $CD25^-$ T-cells were stimulated with CD3/28 dynabeads for five days. The beads were removed, the cells rested overnight before being CFSE labelled and re-stimulated with CD3 in the presence of CHO, CHO-80 and CHO-86 cells. The responder population were allowed to divide for a further five days before being analysed by flow cytometry. The data shown in **figure 4.2.9** represents the MFI of each sample such that the further the cells have divided, the lower the MFI. Again from this it can be seen that CHO cells trigger very little proliferation of the blasts but the CHO-CD80 and CHO-CD86 cells allow the blasts to proliferate indicating that the blasts require co-stimulation to divide. Again, we find that blocking CTLA-4 had no effect on the amount of proliferation the T-cells undergo. This is despite the fact that at the point of re-stimulation, all of the cells are expressing CTLA-4. It was next decided to see if any other antibodies could modulate T-cell proliferation in this assay. For this, CFSE labelled $CD25^-$ T-cells were stimulated with CD3 and the various types of CHO cells in the presence of anti-CTLA-4, CTLA-4 Ig and a blocking CD28 antibody (**figure 4.2.10**). Again, we find that blocking with anti-CTLA-4 has no effect on proliferation; however, if we add CTLA-4 Ig or CD28 antibodies we find that T-cell proliferation is completely ablated highlighting that blocking this co-stimulatory pathway does result in functional effects. Taken together, all of these data are inconsistent with the concept that CTLA-4 delivers a cell intrinsic negative signal.

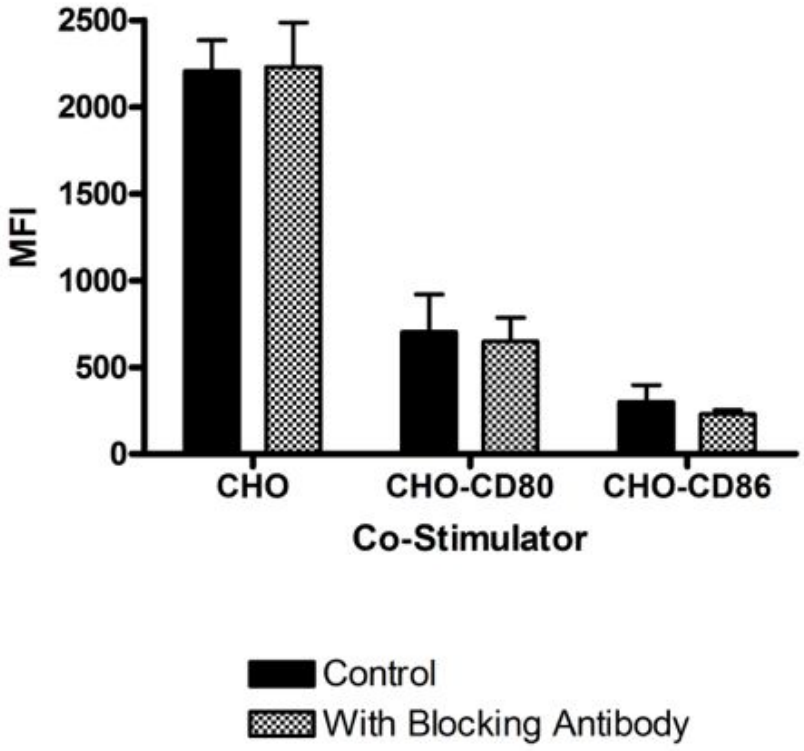

Figure 4.2.9: Blocking CTLA-4 upon re-stimulation of T-cell blasts with CHO cell transfectants does not affect T-cell proliferation. $CD25^-$ T-cells were stimulated with CD3/28 dynabeads for five days. The beads were removed and the cells were rested overnight in RPMI media. The cells were then labelled with CFSE and were stimulated for a further five days with gluteraldehyde fixed CHO cell transfectants in the presence or absence of a blocking CTLA-4 antibody (Tremelimumab; 50µg/ml). Proliferation was determined by flow cytometry, the mean fluorescence intensity values calculated and plotted in the graph as shown. Statistical analysis showed no significant difference between controls and test samples when using a Kruskal-Wallis test with a Dunn's post test. The data presented is representative of three independent experiments.

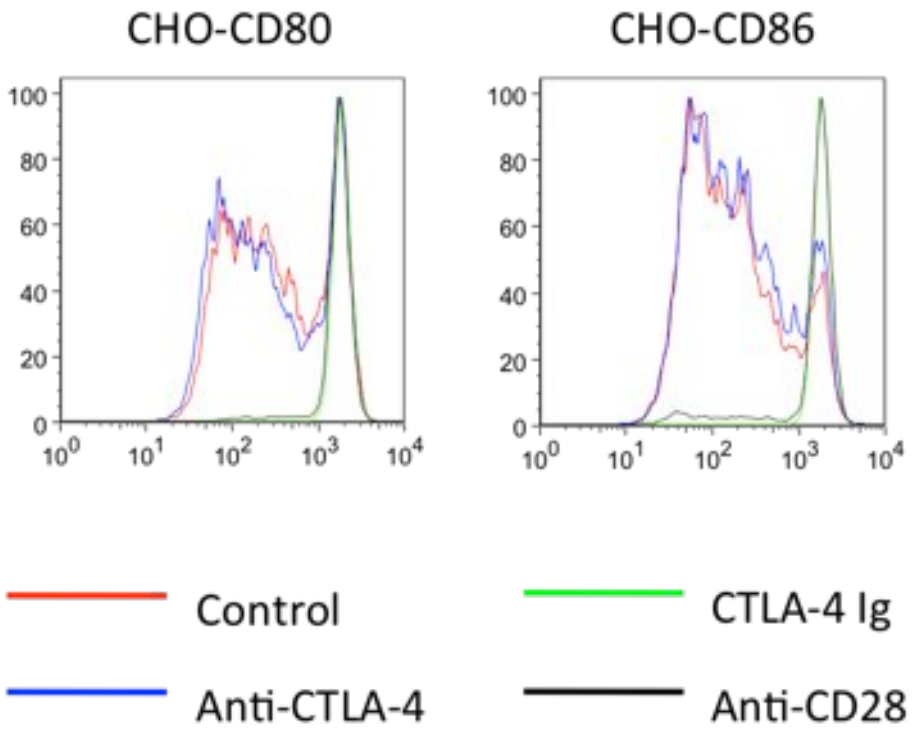

Figure 4.2.10: Anti-CD28 and CTLA-4 Ig are able to modulate T-cell proliferation where anti-CTLA-4 cannot. CFSE labelled $CD25^-$ T-cells were stimulated with CD3 (10µg/ml) in the presence of gluteraldehyde fixed CHO cells expressing either CD80 or CD86 at a ratio of one CHO cell to 10 T-cells (Control). Anti-CTLA-4 (Tremelimumab; 50µg/ml), CTLA-4 Ig (10µg/ml) or anti-CD28 (10µg/ml) were added to the cultures and the amount of proliferation was determined by flow cytomtery after 5 days. The data presented is representative of three independent experiments.

4.2.2 Does Blocking CTLA-4 Affect Proliferation When Using Dendritic Cells as APC?

The next stage in the process was to move away from using CHO transfectants and determine the effect of blocking CTLA-4 in a slightly more physiological setting, for this, dendritic cells were used to provide co-stimulation. The dendritic cells were derived from monocytes that were cultured with IL-4 and GM-CSF. After 6 days of culture, some of the DCs were matured by adding LPS. On day 7 the DCs were phenotyped by staining for CD80 and CD86, the data is shown in **figure 4.2.11**. The DCs were initially gated on CD11c expression, it is shown in this figure that upon maturation, the DCs up-regulate their expression of both CD80 and CD86. To ensure the DCs were able to provide effective T-cells co-stimulation, $CD25^-$ responder cells were labelled with CFSE and stimulated for five days in the presence of CD3 and either immature or mature DCs (**figure 4.2.12 – black line**). This indicates that the DCs can stimulate T-cells to divide. It appears that this division is co-stimulation dependent since the overall amount of proliferation can be limited by reducing the number of DCs in the assay. To confirm that the proliferation is dependent on B7 co-stimulation, it was decided to block CD80 and CD86 in these assays (**figure 4.2.12 – red line**). By blocking B7 ligands it is clear that T-cell proliferation is almost completely ablated indicating that this model of T-cell activation is ligand dependent.

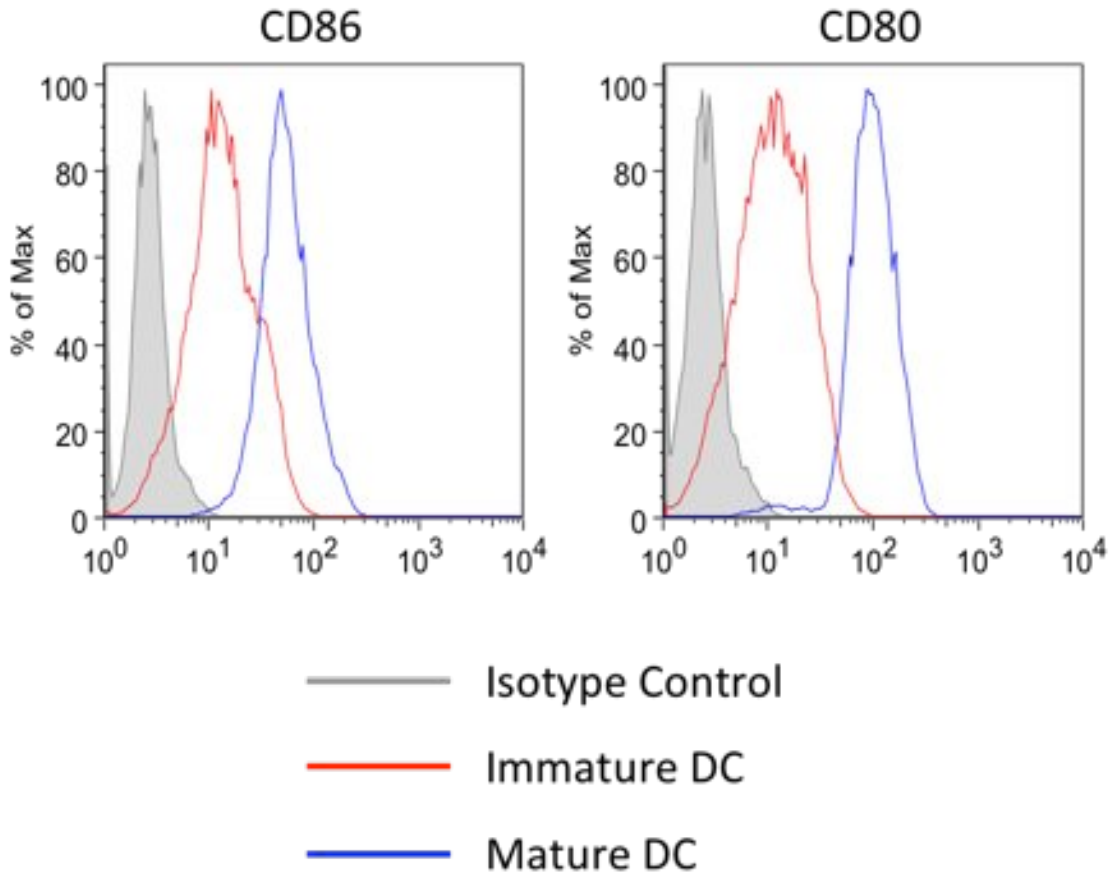

Figure 4.2.11: Phenotype of dendritic cells. Monocytes were cultured for 7 days in the presence of IL-4 (500U/ml) and (GM-CSF 800U/ml). On day 6 cells were either left untreated or matured overnight with 1µg/ml of LPS. Cell were stained on ice for 30 minutes with CD80 and CD86, DCs were gated on CD11c positive cells. Phenotypes were tested before every experiment and the data presented is representative of a typical culture.

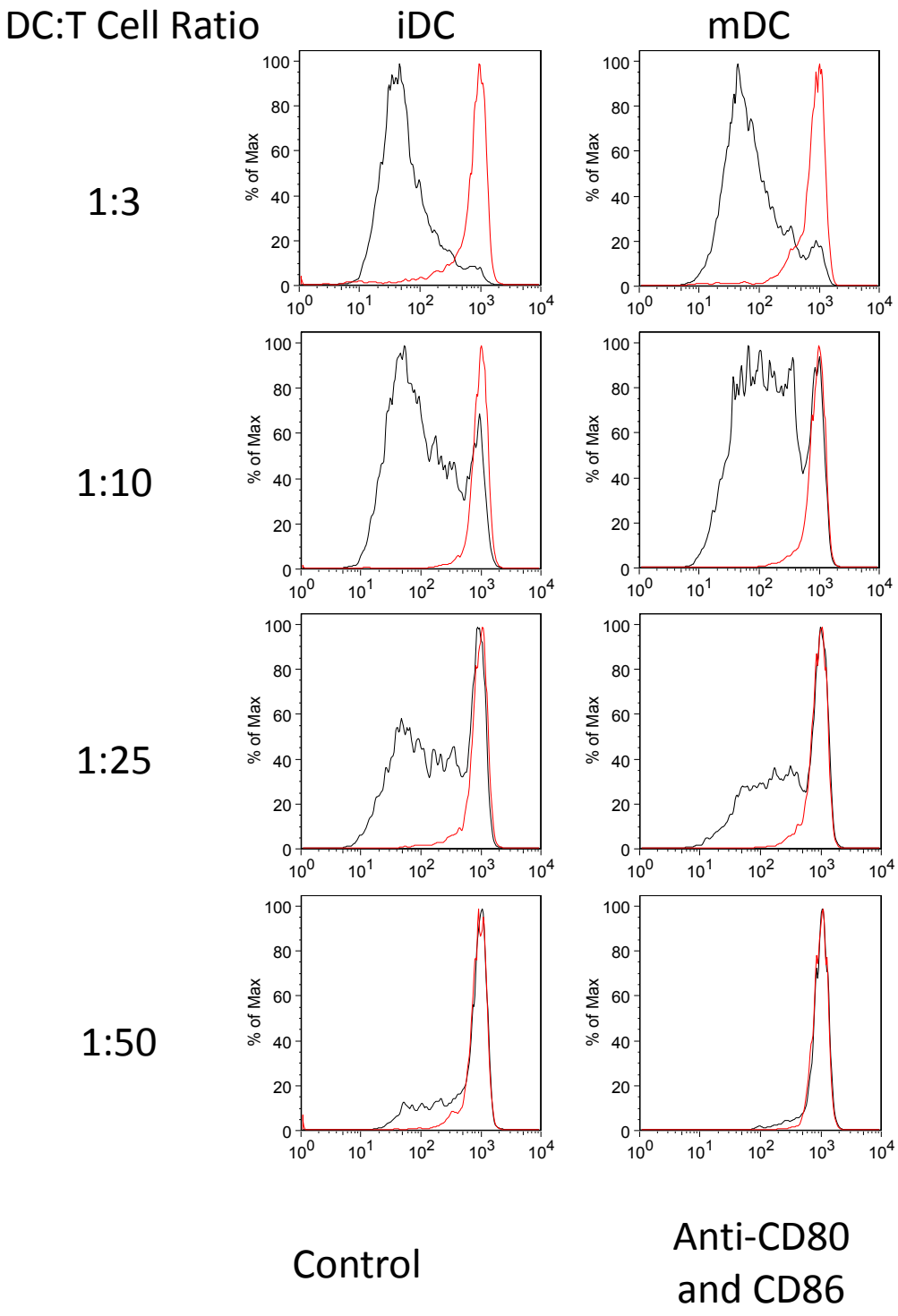

Figure 4.2.12: T-Cell responses are dependent on co-stimulation. $CD25^-$ T-cells were isolated and stimulated with 10µg/ml of CD3 in the presence of mature or immature DCs. Cultures were left untreated or were incubated for five days with blocking antibodies to CD80 and CD86 at a concentration of 10µg/ml each. T-cell proliferation was determine by flow cytometry

To assess the affect of blocking CTLA-4 in a DC based assay, T-cells were labelled with CFSE and were stimulated with CD3 and reducing numbers of DCs. As can be seen from **figure 4.2.13**, when high numbers of DCs are present, blocking CTLA-4 has little effect on T-cell proliferation. Interestingly, however, as the number of DCs is reduced, blocking CTLA-4 dramatically enhances proliferation. Again, the proliferating cells are all expressing CTLA-4 (**figure 4.2.14**). To find out if blocking CTLA-4 in the DC assay affects IL-2 production, the T-cells were analysed for their IL-2 expression. **Figure 4.2.15** shows that there is very little difference between blocked and control samples when high numbers of DCs are used, where there are fewer DCs however, there is a large increase in IL-2 production in the blocked samples. These data suggest that CTLA-4 may be more effective where it can compete effectively with CD28 for B7 engagement.

Again, it was decided to look at the affect blocking CTLA-4 had on IFNγ, IL-17 and IL-21: **figure 4.2.16.** At the 1/3 DC:T-cell ratio, blocking CTLA-4 seems to cause an overall decrease in both IFNγ and IL-17 expression whereas there is apparently no effect on IL-21. At the lower ratio of 1/25, it seems that blocking CTLA-4 results in a modest increase in both IFNγ and IL-17 but it appears that the treatment results in a decrease in IL-21 production. CTLA-4 therefore seems to have a more profound effect on IL-2 production than other cytokines.

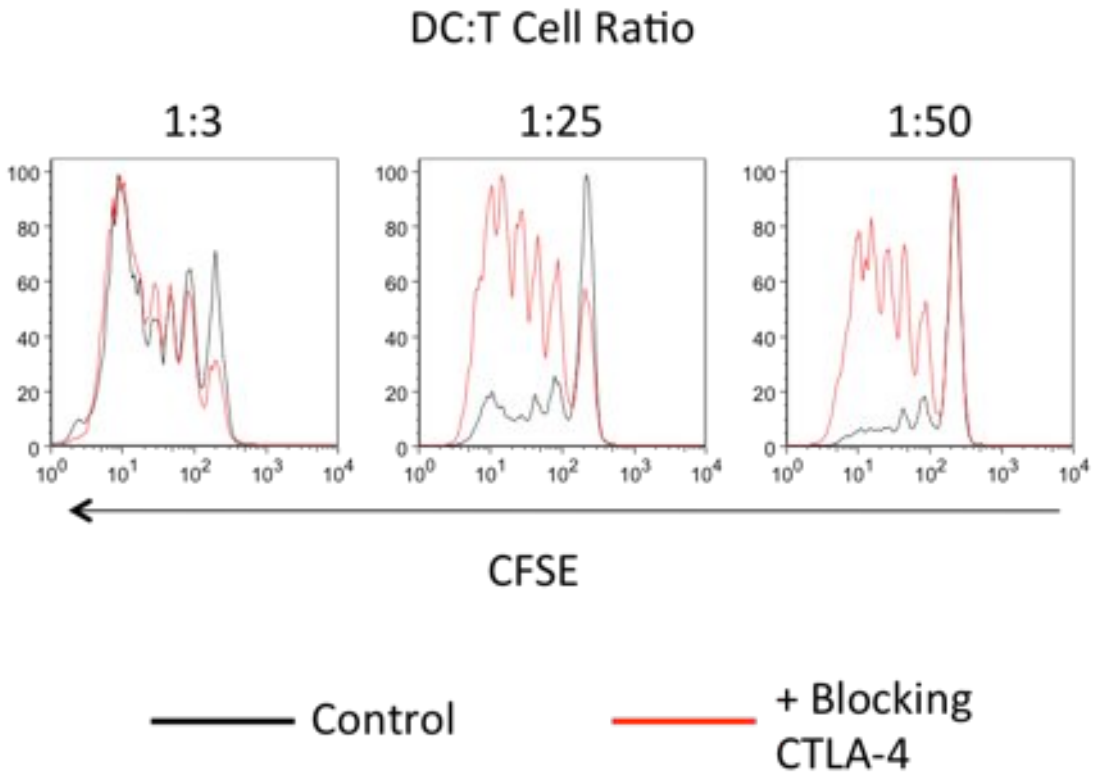

Figure 4.2.13: Blocking CTLA-4 in a DC based assay enhances T-cell proliferation when co-stimulation is limiting. CFSE labelled $CD25^-$ T-cells were stimulated with 10µg/ml of CD3 and the indicated ratio of immature monocyte-derived dendritic cells in the presence or absence of the blocking CTLA-4 antibody (Tremelimumab; 50µg/ml). After 5 days, proliferation of T-cells was analysed by flow cytometry. The data presented is representative of at least five independent experiments.

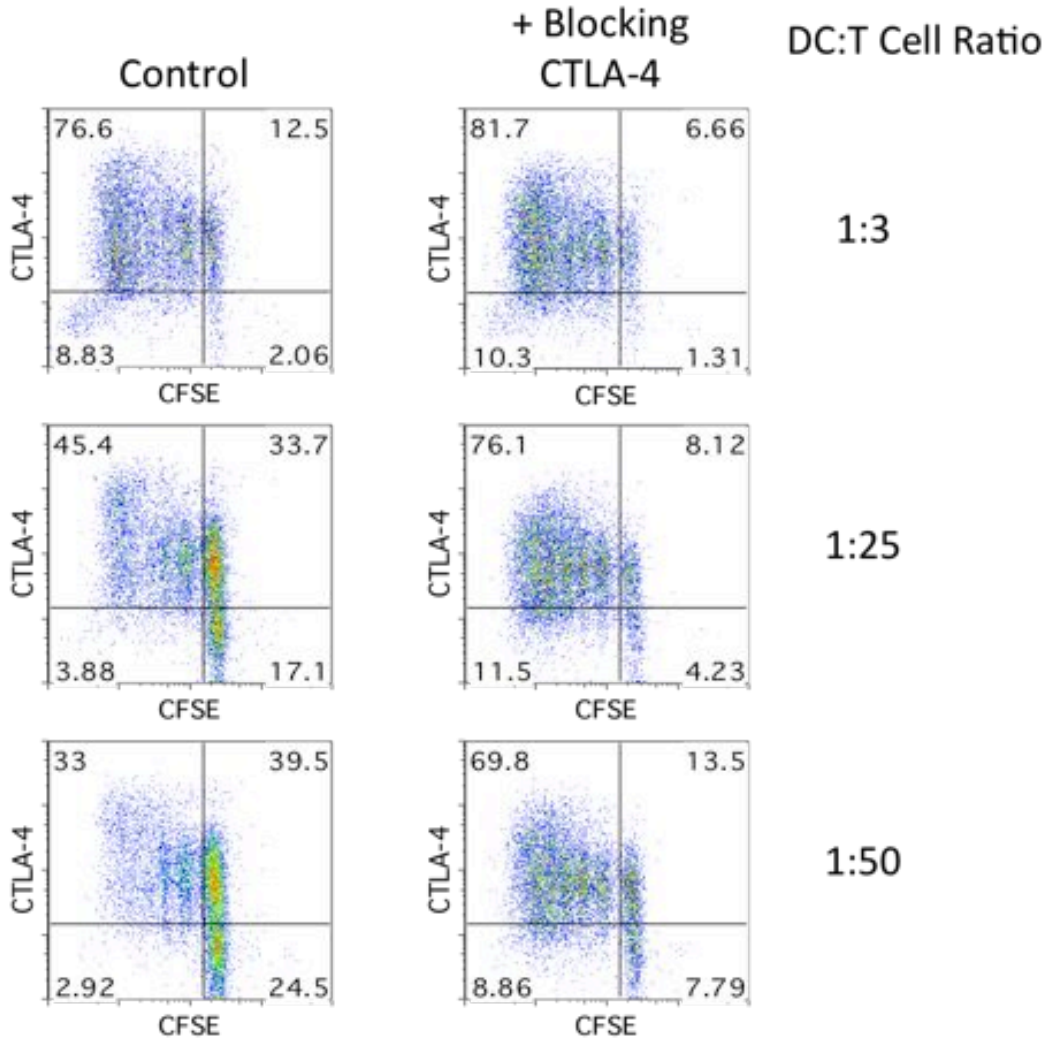

Figure 4.2.14: Proliferating cells express CTLA-4. CFSE labelled CD25$^-$ T-cells were stimulated with CD3 (10µg/ml) and immature DCs in the presence or absence of the blocking CTLA-4 antibody (Tremelimumab; 50µg/ml). After 5 days, cultures were fixed (3% PFA) and permeabilised (5% PBS-Saponin) to stain for the presence of total CTLA-4, cultures were analysed by flow cytometry. The data presented is representative of at least five independent experiments.

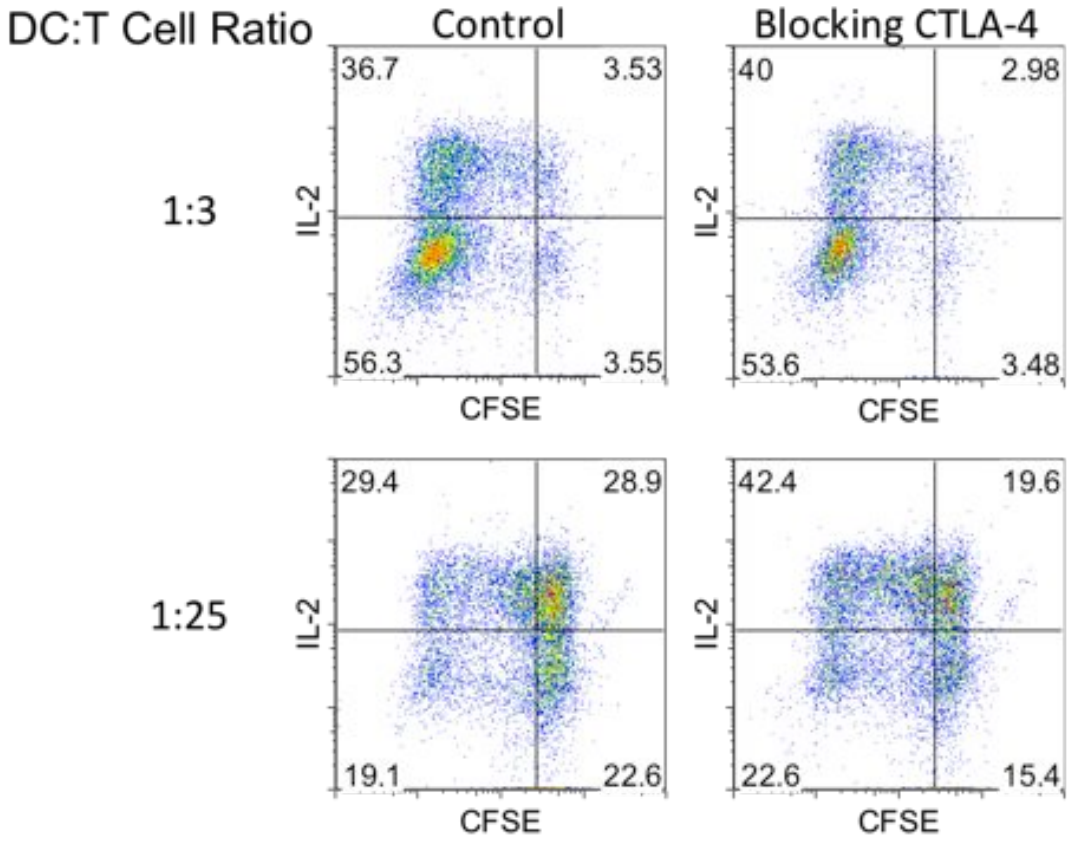

Figure 4.2.15: Blocking CTLA-4 enhances IL-2 production where the number of DCs used to provide co-stimulation is limited. CFSE labelled CD25- T-cells were stimulated with CD3 (10µg/ml) and the indicated ratios of immature DCs in the presence of Tremelimumab (50µg/ml). After 5 days, cultures were re-stimulated with PMA (50ng/ml), Ionomycin (1µM) and Brefeldin A (5µg/ml) for four hours before being fixed, permeabilised and stained for IL-2 production. Cultures were analysed by flow cytometery. The data presented is representative of five independent experiments.

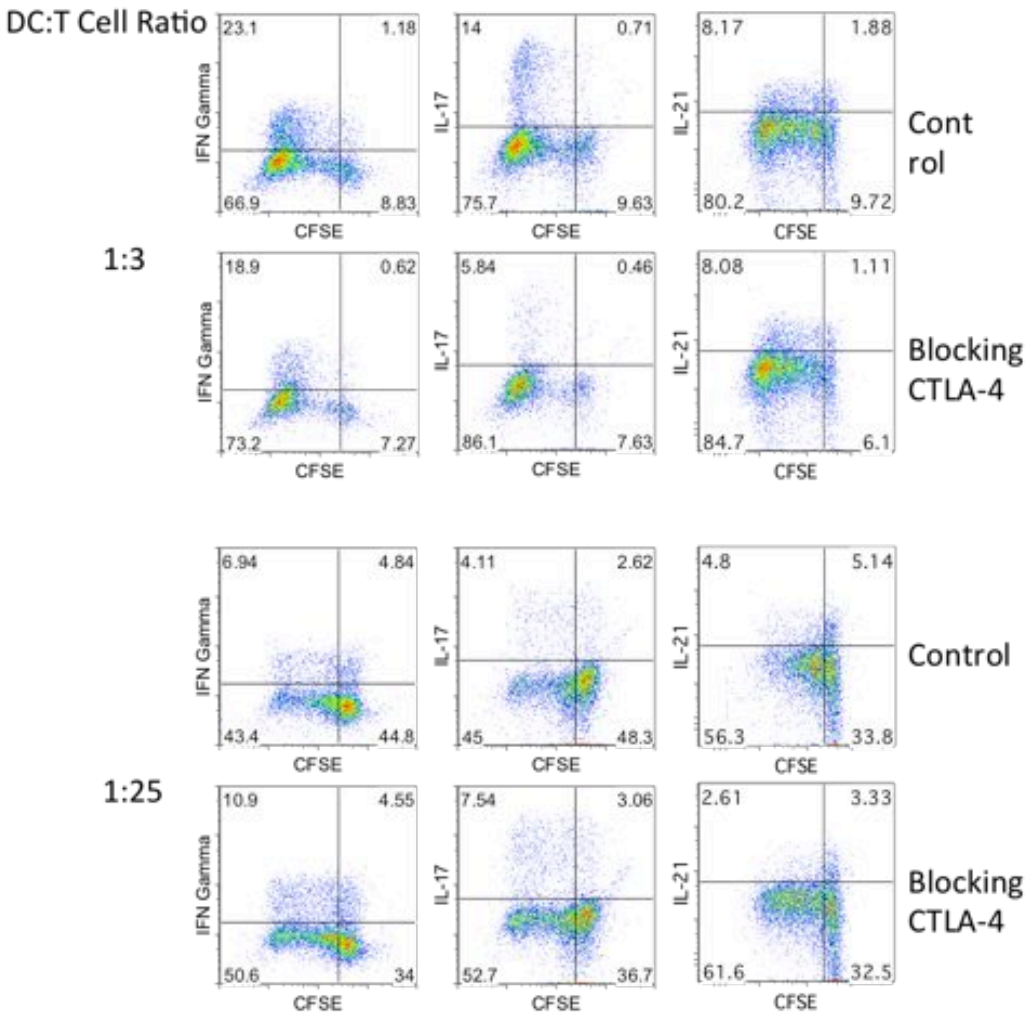

Figure 4.2.16: Blocking CTLA-4 has differential effects on T-cell cytokine production. CFSE labelled $CD25^-$ T-cells were stimulated with CD3 (10µg/ml) in the presence of the indicated ratios of immature DCs and Tremelimumab at 50µg/ml. After 5 days, cultures were re-stimulated with PMA (50ng/ml), Ionomycin (1µM) and Brefeldin A (5µg/ml) for four hours before being fixed, permeabilised and stained for IL-17, IL-21 or IFNγ production. Cells were analysed by FACS, the data presented is representative of three independent experiments.

To study the effect of blocking CTLA-4 upon re-stimulation, $CD25^-$ T-cells were stimulated with CD3/28 beads for five days, the beads removed and the T-cells rested over night before being labelled with CFSE and re-stimulated with CD3 and increasing numbers of DCs. After five days of re-stimulation, the amount of proliferation was determined by flow cytometry the data is shown in **figure 4.2.17**. As with the experiments carried out with CHO cells, these data are expressed as MFI such that the more cell division that occurs, the lower the MFI. These results mirror the primary response data in that reducing the number of DCs present, reduces the amount of proliferation that the T-cells undergo. Importantly, blocking CTLA-4 is again only effective where fewer DCs are present further arguing against the delivery of a negative signal.

Since T-cell proliferation appears to be entirely dependent on B7 co-stimulation and CTLA-4 appears to be more effective under conditions where it can out compete CD28 for ligand, it was decided to assess if the addition of a CD28 agonist would overcome the effects of blocking CTLA-4. To do this, CFSE labelled CD25- T-cells were stimulated with CD3 and DCs as described previously. This was done in the presence of anti-CTLA-4, dynabeads coupled to an agonistic CD28 antibody or a combination of both (**figure 4.2.18**) Again we see that blocking with CTLA-4 (red line) alone only affects the proliferation of T-cells when there are fewer DCs present. As expected, addition of the CD28 agonist enhances proliferation at this ratio of DCs (green line). Surprisingly, however, addition of both anti-CTLA-4 and the CD28 agonists together appear to have an additive effect.

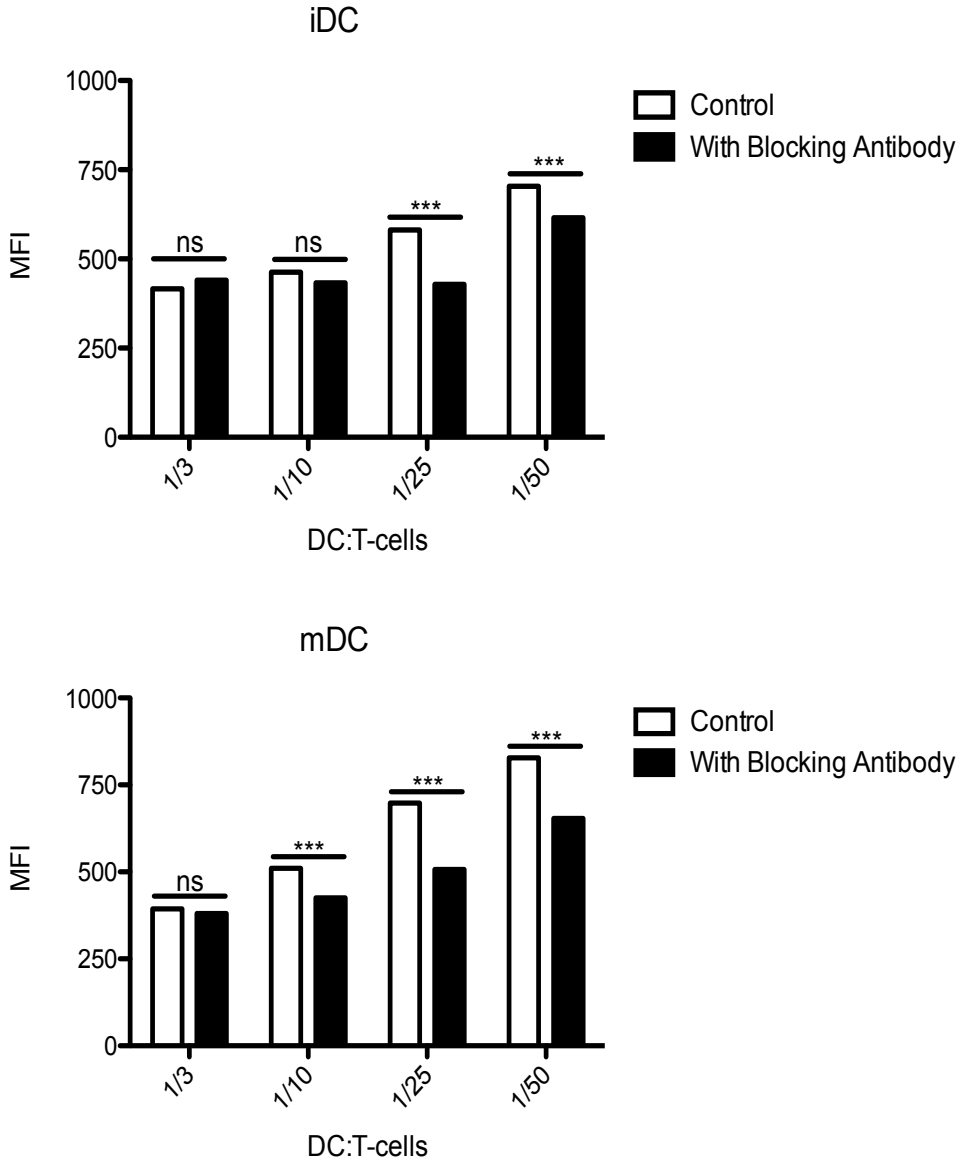

Figure 4.2.17: Blocking CTLA-4 enhances the proliferation of T-cell blasts co-stimulated with DCs. CFSE labelled CD25$^-$ T-cells were stimulated with CD3/28 dynabeads for five days. The beads were removed and the cells were rested overnight in RPMI media. The cells were then labelled with CFSE (2.5µM) and were stimulated for a further five days with CD3 (10µg/ml), DCs at the indicated ratios in the presence or absence of the blocking CTLA-4 antibody (Tremelimumab; 50µg/ml). Proliferation was determined by flow cytometry, the mean fluouresence intensity values calculated and plotted in the graph as shown. The data presented is representative of three independent experiments. Statistical analysis was carried out using a Kruskal-Wallis test with a Dunn's post test. ns = not significant, *** $p<0.001$.

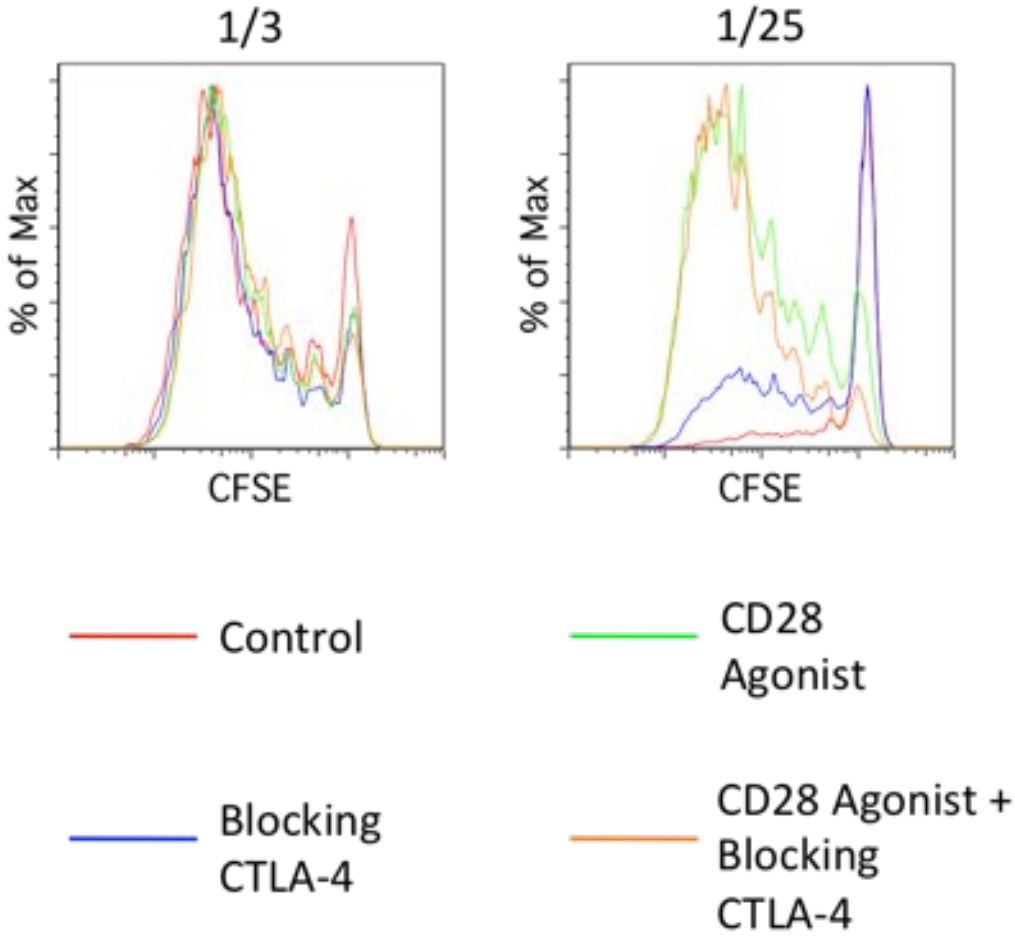

Figure 4.2.18: Does addition of a CD28 agonist overcome the effect of blocking CTLA-4. CFSE labelled $CD25^-$ T-cells were stimulated with CD3 (10µg/ml) in the presence of gluteraldehyde fixed CHO cells expressing either CD80 or CD86 at the indicated ratios. Anti-CTLA-4 (Tremelimumab; 50µg/ml), CD28 agonist (clone 9.3; 10µg/ml) or a combination of both were added to the cultures and the amount of proliferation was determined by flow cytometry after 5 days. The data presented is representative of three independent experiments.

4.3 Discussion

The theory that CTLA-4 delivers an inhibitory signal into the T-cell has been widely embraced however, all of the supporting data has come from experiments using antibodies against CTLA-4 [1, 3]. However, there are numerous problems associated with the use of cross-linked antibodies, it was therefore decided to look for evidence of a negative signal using natural CTLA-4 ligands. To do this, a model system using CHO cells that express either CD80 or CD86 on their surface was employed. The use of CHO cells to provide co-stimulation offers a useful model to test for a negative signal since the cells express relatively large amounts of ligands this reduces the likelihood of competition affecting the results. Further to this, the CHO cells are unable to produce signals that would enhance T-cell proliferation or indeed receive signals, therefore presenting a clean system to study the effect of blocking CTLA-4 on T-cell activation. The data presented suggest that CTLA-4 does not inhibit T-cell proliferation by delivering a ligand dependent inhibitory signal as there is no effect of blocking CTLA-4 on proliferation responses. Only when the CHO cells are titrated to very low levels do we see any effect of blocking CTLA-4. In the samples stimulated with CHO-86 cells where there was only one CHO cell to every 500 T-cells, we do start to see enhance proliferation of the T-cells. However, the fact that the number of co-stimulatory cells had to be titrated so low before an effect of blocking CTLA-4 was revealed further argues against the delivery of a negative signal. Instead, it is likely that blocking CTLA-4 at very low levels of co-stimulation is inhibiting the competition of this protein and CD28 for ligand engagement.

It may be suggested that the lack of effect of blocking CTLA-4 in CHO cell assays is because the antibody does not allow functional effects. However, the since the same antibody seems able to cause considerable enhancement of proliferation in DC based assays suggests otherwise. Moreover the antibodies used have been extensively characterised in chapter 3 ruling out trivial criticisms of non-blocking or low affinity. The data presented here does not rule out the possibility that CTLA-4 can signal without the need for engagement of B7. It has been shown in mice that a ligand independent splice variant of CTLA-4 (liCTLA-4) can be expressed and is associated with a number of autoimmune diseases such as type I diabetes [4]. This liCTLA-4 was subsequently shown to be able to inhibit T-cell proliferation and IFNγ production to the same extent as full length CTLA-4 [5]. However, recent studies by Wicker *et al* have shown that Li-CTLA-4 is unable to rescue the fatal CTLA-4 deficient phenotype, suggesting the role of this isoform is at best limited [2]. Further to this, it has been shown that full length CTLA-4 could inhibit proliferation and IL-2 production in the absence of ligand [6] suggesting that a negative signal could be ligand independent.

To test the effect of blocking CTLA-4 in a more physiological setting, it was decided to use dendritic cells as antigen presenting cells. Both mature and immature dendritic cells were used. It was perhaps surprising to note that immature dendritic cells provided just as effective co-stimulation as mature cells. It would be expected that mature DCs would be capable of driving greater proliferation in the

responder population due to the fact that they express higher levels of both CD80 and CD86 on their surface (as shown in figure 4.2.11). The equivalent levels of co-stimulation may be explained by the fact that the addition of the T-cells to the cultures results in the DCs maturing and hence driving further proliferation. Activated T-cells express CD40 Ligand (CD40L) [7] which binds to CD40 expressed on DCs. It has been shown that CD40-CD40L interactions can result in DC maturation categorised by enhanced CD80 and CD86 expression [8] and may account for the equivalent levels of proliferation observed. In the experiments carried out using DCs, it is interesting to note that whilst the antibody has very little effect on proliferation or IL-2 production when there are many DCs present, there are striking effects when there are fewer DCs. This further supports the concept that CTLA-4 does not deliver a ligand dependent T-cell intrinsic negative signal as it would be expected that if a negative signal was delivered, having more ligand present might produce a stronger signal. One of the most basic models for CTLA-4 function is that it is able to out-compete with CD28 for B7 ligand, binding due to its higher avidity and affinity for the protein thus reducing the activation signal that would otherwise be delivered into the cell [9-12]. The fact that CTLA-4 is more effective in a setting where the amount of ligand is limiting suggests the possibility that CTLA-4 could function by some form of competition with CD28. Although it is probable that this mechanism will have some influence on T-cell responses, if this mechanism was a major factor it would be expected that pronounced effects of blocking CTLA-4 would be observed upon reducing the number of transfected CHO cells used as co-stimulators and not just when the number of DCs were reduced.

An important feature of CTLA-4 biology is that the protein is only expressed in resting T-cells once these cells become activated. CTLA-4 can be detected on activated T-cells as early as 24-48 hours post stimulation [1, 13-15]. It could therefore be argued that the absence of CTLA-4 effects in the CHO cell experiments may be a result of the delayed expression of the protein on the surface of the T-cells. It has certainly been shown that primary responses in CTLA-$4^{-/-}$ CD8 T-cells are comparable to those of CTLA-$4^{+/+}$ cells, whereas, re-stimulated CTLA-$4^{-/-}$ CD8 T-cells are hyper-responsive when compared to the WT-cells [16]. There are however, numerous experiments in chimeric settings that show that CTLA-4 deficient T-cells are normal in the presence of other CTLA-4 expressing cells. This strongly suggests that a cell intrinsic role for CTLA-4 function is unlikely to be important *in vivo* [17-20]. The fact that blocking CTLA-4 was able to enhance T-cells responses when DCs were used to stimulate in both primary and re-stimulated responses argues that CTLA-4 is effective in both settings. It therefore follows that the lack of effects when using CHO cells to re-stimulate is further evidence against CTLA-4 acting as an intrinsic negative signal.

When CTLA-4 was initially associated with a negative signal, this conclusion was reached as a result of experiments that showed cross-linking CTLA-4 with antibody resulted in decreased IL-2 production [1]. The data presented here show that blocking CTLA-4 also resulted in increased IL-2 production, which is consistent with these early studies into CTLA-4 function. This enhancement of IL-2 production

is more pronounced when fewer DCs are present which reflects the proliferation data presented and implies that CTLA-4 is more effective when the APC is limiting.

It was decided to assess the effect of blocking CTLA-4 on the production of other pro-inflammatory cytokines. The data presented here show an example of the results from such experiments; however, the data was highly variable making it difficult to draw concrete conclusions from these experiments. The inconsistency in the data could be a result of donor variability and may suggest that there are factors other than CTLA-4 that are required for regulating the production of these cytokines.

Taken together, the data presented here are inconsistent with the idea that CTLA-4 is able to deliver a ligand-dependent, cell intrinsic negative signal. Instead, it appears that the protein functions by some means of competition with CD28 by virtue of the fact that blocking CTLA-4 is more effective when the numbers of APCs are limited.

Chapter 5:

CTLA-4 Acts as an Extrinsic Regulator of T-cell Activation

5.1 Introduction

In the previous chapter we found no evidence that CTLA-4 was able to deliver a ligand dependent negative signal. Blocking CTLA-4 did enhance the proliferation of T-cells in a DC based assay but only when the numbers of DCs were limiting. One interpretation of these data is that CTLA-4 could act extrinsically to regulate the DC and that this regulation is most effective when there are lower amounts of ligand available. The aim of this chapter was to test further the settings where CTLA-4 can effectively regulate the proliferation of responder cells and to try to assess the mechanisms that may account for this function.

5.2 Results

5.2.1 Can CTLA-4 expressing Cells Regulate the Proliferation of a Responder Population?

The existence regulatory T-cells, a subset of T-cell that constitutively expresses CTLA-4, has long been known. These cells are thought to regulate responder populations by various mechanisms. It was decided to assess the function of CTLA-4 in regulatory T-cell responses. Our laboratory previously isolated regulatory T-cells from PBMCs initially by CD4 negative selection followed by CD25 positive selection. **Figure 5.2.1.1** shows yields of each cell type at the difference stages of purification. This shows that only around 30% of the $CD25^+$ T-cells are Tregs based on them expressing both CD25 and FoxP3. In order to attempt to understand how CTLA-4 works in Tregs, it was important to try to improve the yield of these cells. It has been recently shown that selecting both CD127 low, CD49d low yields a pure population of "untouched" Tregs [1]. It was therefore attempted to improve the yield of Treg by first negatively selecting $CD4^+CD127^{low}$ cells using the "stemcell" isolation kit. **Figure 5.2.1.2** shows that this kit resulted in good purity of $CD3^+CD4^+$ cells and that about 99% of these cells expressed almost no CD127. This method of isolation alone resulted in only around 16% of cells expressing both CD25 and FoxP3. At this stage of selection, around 45% of the cells expressed CD49d. To isolate the CD49d negative cells, the $CD4^+CD127^{low}$ cells were incubated with a CD49d FITC labelled antibody before being labelled with anti-FITC magnetic beads. The labelled cells were then isolated from the samples using a magnet. This second

round of selection resulted in almost a 60% purity of Tregs based on CD25 and FoxP3 expression, double the purity of the previous method of Treg isolation.

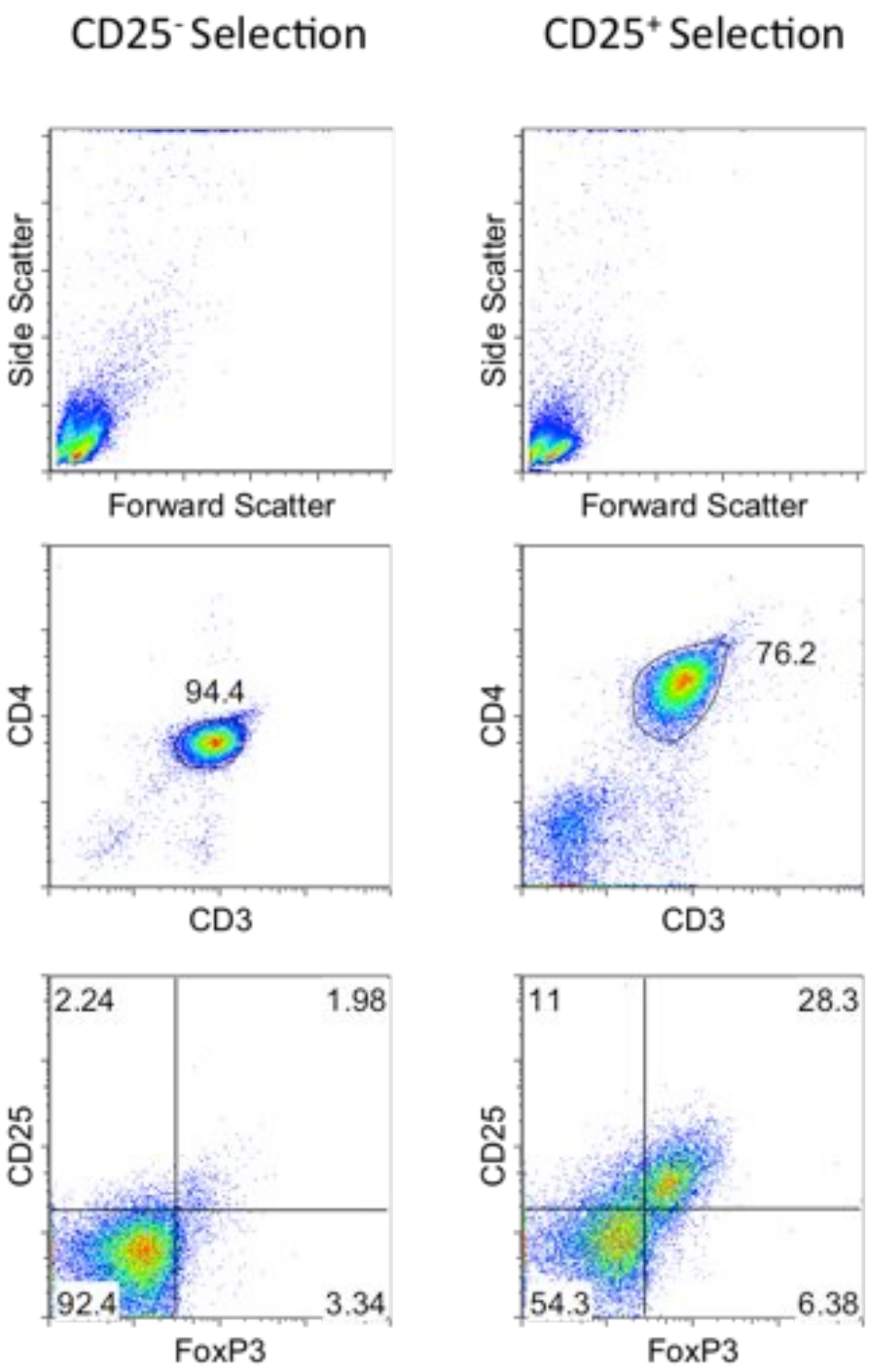

Figure 5.2.1.1: Purity of Tregs isolated by CD25 positive selection. $CD4^+$ T-cells were isolated from PBMCs before being incubated with CD25 selection beads. The cells were then passed through a column. Negative cells were collected before detaching the positive cells from the column. The isolated cells were stained for surface CD25, fixed, permeabilised and stained for FoxP3 and protein expression was determined by flow cytometry. Purities were determined before every experiment and the data presented is representative of a typical yield.

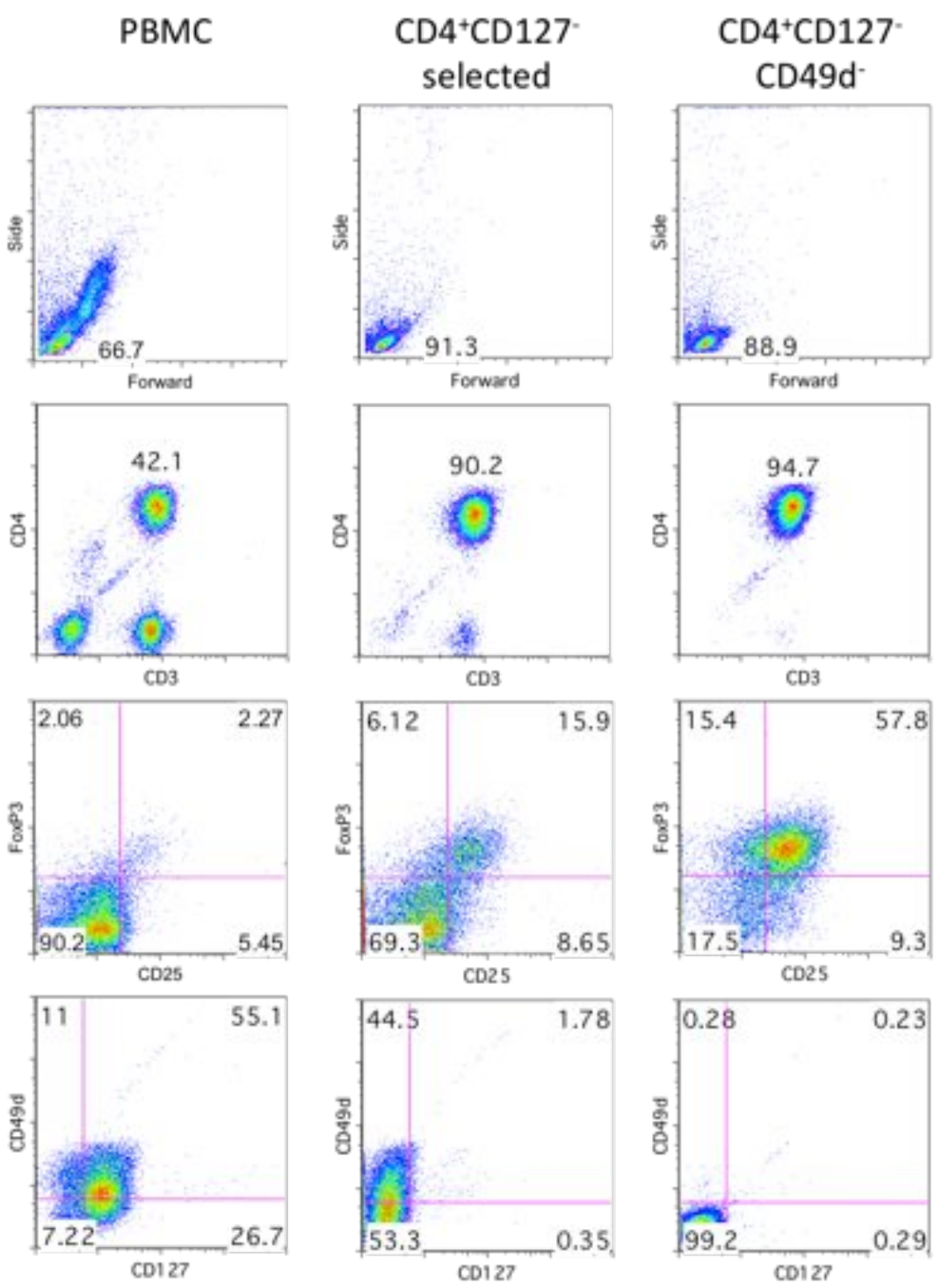

Figure 5.2.1.2: Negative selection of regulatory T-cells by a combination of CD49d and CD127 negative selection improves the yield of Tregs. PBMCs were treated with $CD4^{+}CD127^{-}$ negative selection cocktail, positive cells were removed from the sample by magnet. These cells were either stained for purity or subjected to further negative selection by CD49d. All purified cells were stained on the surface for CD4 and CD49d before being fixed, permeabilised and stained for FoxP3. Expression levels of protein were determined by flow cytometery. The data presented is representative of three independent experiments.

To assess the role of CTLA-4 on Treg function, CFSE labelled $CD25^-$ T-cells were stimulated with CD3 and DCs. Samples were treated with anti-CTLA-4, Tregs or a combination of both (**Figure 5.2.1.3**). Surprisingly, instead of suppressing responder cell proliferation, it appeared that addition of the Tregs actually enhanced T-cell proliferation. Further, it was found that blocking CTLA-4 in the absence of Tregs enhanced proliferation, but had no effect on T-cell proliferation when Tregs were present.

These results may reflect the fact that the isolated Tregs were still not pure enough for effective suppression and that contaminating activated T-cells contributed to proliferation. To address this issue, it was also decided to use jurkats as a suppressor population as wild type (WT) jurkats don't express CTLA-4. Our laboratory was able to transduce some of these cells so that they became CTLA-4 positive using a retroviral approach. Expression of CTLA-4 was confirmed by labelling the cells with a CTLA-4 antibody at 37°C. **Figure 5.2.1.4** shows that the WT jurkats were negative for CTLA-4 expression whereas the CTLA-4 jurkats expressed the protein. Further, since this labelling was carried out a 37°C, it appears that the CTLA-4 in these cells is recycling effectively between the membrane and the cytoplasm.

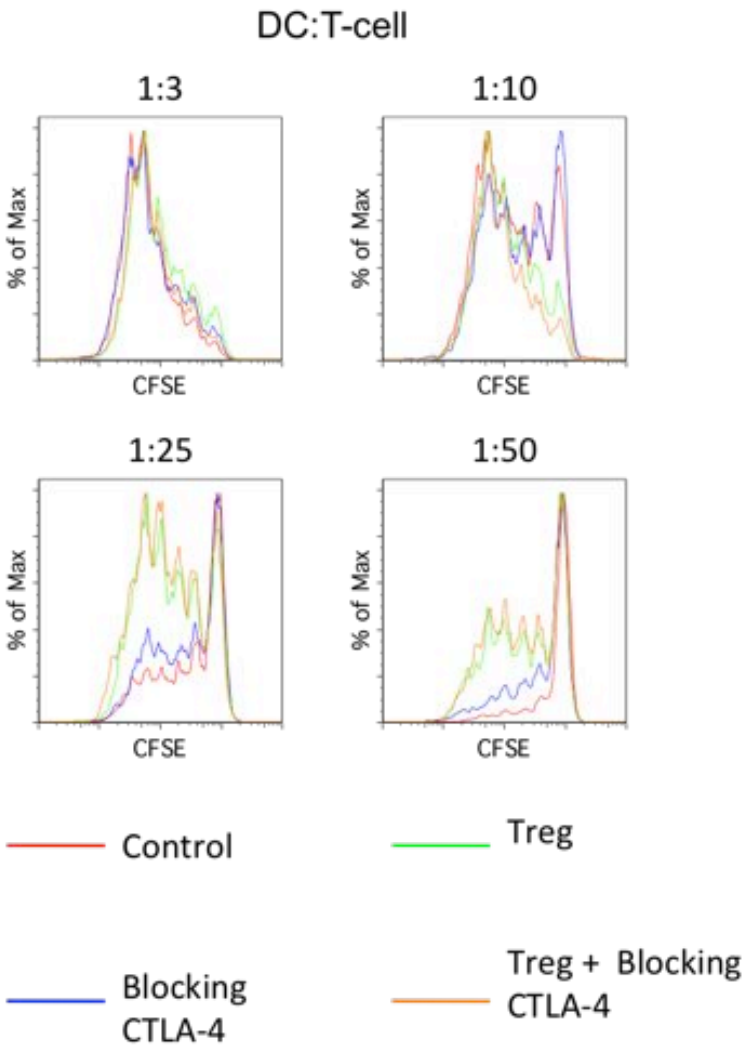

Figure 5.2.1.3: Addition of Treg cells enhances proliferation of responder CD25$^-$ T-cells. Responder CD25$^-$ T-cells were labelled with CFSE and were stimulated with CD3 (10µg/ml) and immature DCs at the ratios indicated. Tregs (1:3), blocking CTLA-4 antibody (Tremelimumab; 50µg/ml) or a combination of both were added. After 5 days of culture, the proliferation of the responder population was determined by flow cytometry. The data presented is representative of two independent experiments.

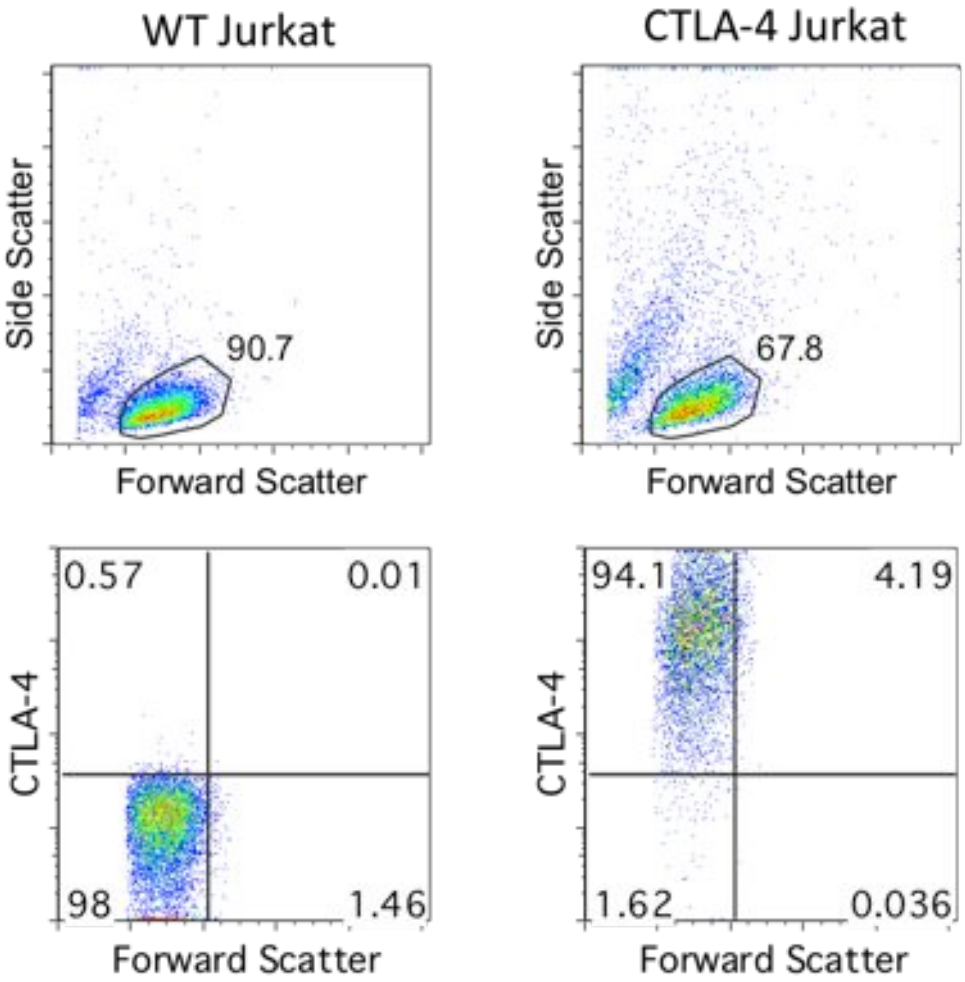

Figure 5.2.1.4: Jurkat cell lines express CTLA-4. Jurkat cells were incubated with a PE labelled anti-CTLA-4 antibody (BNI3) at 37°C for thirty minutes to stain the recycling pool of the protein. Protein expression was then determined by flow cytometry.

To find out if CTLA-4 expressing cells were able to suppress the proliferation of responder cells, CFSE labelled T-cells were stimulated with CD3 and DCs in the presence of either WT or CTLA-4 jurkats. As can be seen from **figure 5.2.1.5** when there were high numbers of DCs present, CTLA-4^+ jurkat cells had little effect on proliferation when compared to cells cultured in the presence of WT jurkats. However, as the numbers of DCs were reduced, it can be seen that CTLA-4 jurkats were able to suppress the proliferation of the CFSE-labelled responder T-cell population. These data suggest that CTLA-4 expressing cells are capable of suppressing the proliferation of surrounding cells, consistent with CTLA-4 acting as an external regulator of T-cell function. Furthermore the ability of jurkat cells to suppress is dependent on the number of APCs present suggesting that CTLA-4^+ jurkat cells are affecting the APC rather than the responder T-cells.

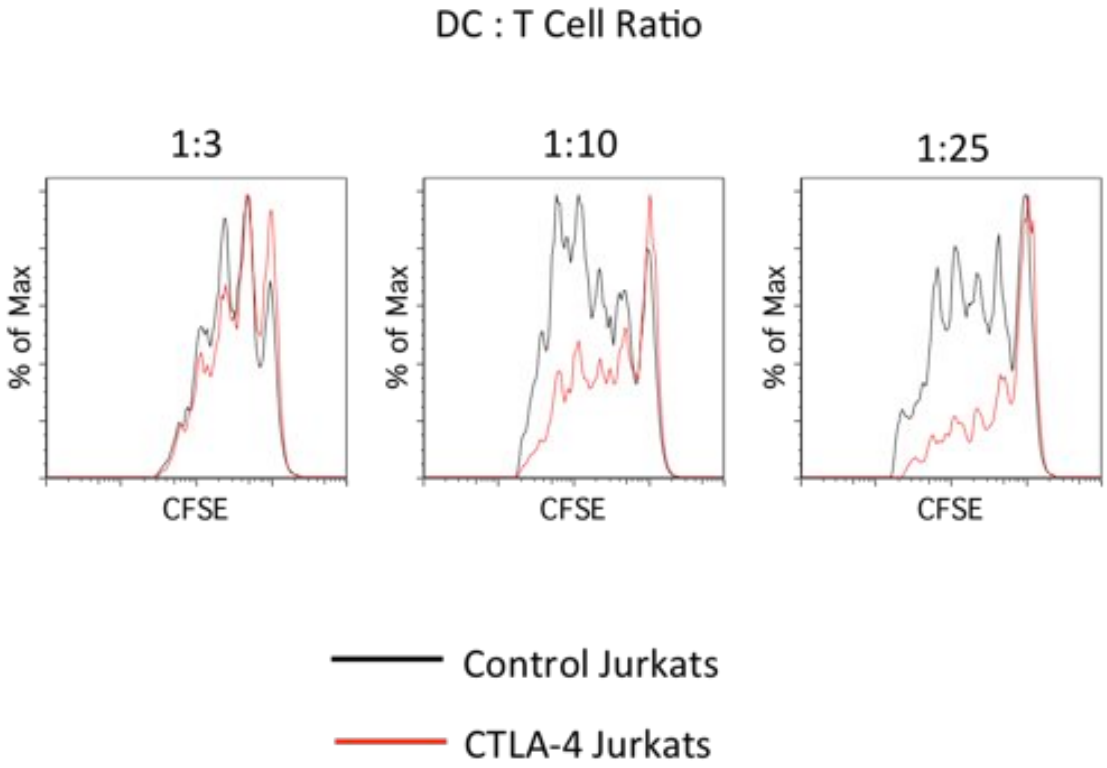

Figure 5.2.1.5: CTLA-4 expressing jurkats can suppress responder cell proliferation. Responder $CD25^-$ T-cells were labelled with CFSE and stimulated with CD3 (10µg/ml) and DCs at the indicated ratios. This was done in the presence of WT or CTLA-4 expressing jurkat cells at a ratio of one jurkat to three T-cells. The amount of proliferation of the responder population was determined after 5 days by flow cytometry. The data presented are representative of three independent experiments.

5.2.2 Determining CTLA-4 Effector Function

The data presented so far are consistent with CTLA-4 acting as an external effector molecule capable of affecting APCs. To address which mechanisms could account for the observations made, it was decided to look at models where CTLA-4 acts extrinsically to influence the DC. The first mechanism to be looked at was the role of IDO in these assays. It has been suggested that CTLA-4 is able to back signal into the dendritic cell upon ligand binding, this back signalling results in up-regulation of the enzyme IDO. IDO is required for the breakdown of tryptophan, an essential amino acid required for T-cell proliferation, thereby reducing the capacity for T-cells to divide [2, 3]. To test if IDO accounts for the enhanced proliferation and IL-2 production seen in dendritic cell assays upon blocking CTLA-4 it was decided to use 1-methyl tryptophan (1-MT), a widely used inhibitor of IDO function. Addition of 1-MT to our samples results in increased T-cell proliferation modestly only when using both mature and immature DCs at very high numbers (5:1 ratio, **figure 5.2.2.1**). However, as the numbers of DCs in the assay were reduced the effect of 1-MT is lost. In fact, at very low numbers of DCs (1:50 ratio) it seems that the 1-MT became toxic to the cells. The fact that the effects of CTLA-4 blocking antibodies are only seen at low DC numbers and the 1-MT effects are only seen at high DC numbers suggests that IDO does not account for the increase proliferation of our DC experiments above. To confirm this it was decided to carry out a blocking experiment at the 5:1 DC to T-cell ratio. As can be seen from **figure 5.2.2.2**, blocking CTLA-4 at this DC

ratio had no effect on proliferation. This argues against the fact that CTLA-4 is exerting its function via IDO in these assays.

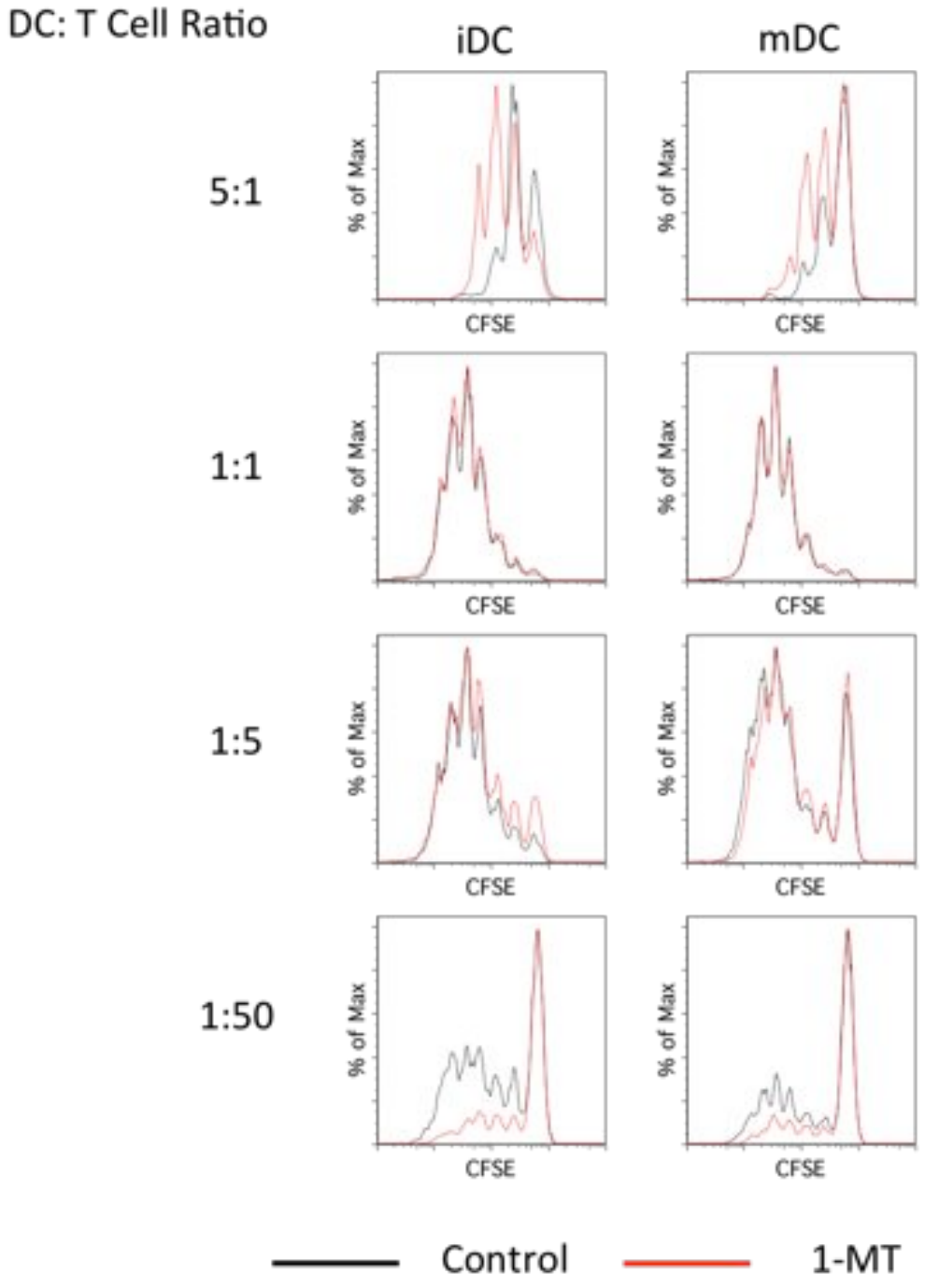

Figure 5.2.2.1: Enhanced proliferation after CTLA-4 blockade is not a result of IDO. CFSE labelled $CD25^-$ T-cells were stimulated with CD3 (10µg/ml) in the presence of either immature or mature DCs at the indicated ratos. Cultures were left untreated (control) or treated with the IDO inhibitor 1-methyl tryptophan (500µM). Proliferation of responder cells was determined by flow cytometry after 5 days. The data presented are representative of three independent experiments.

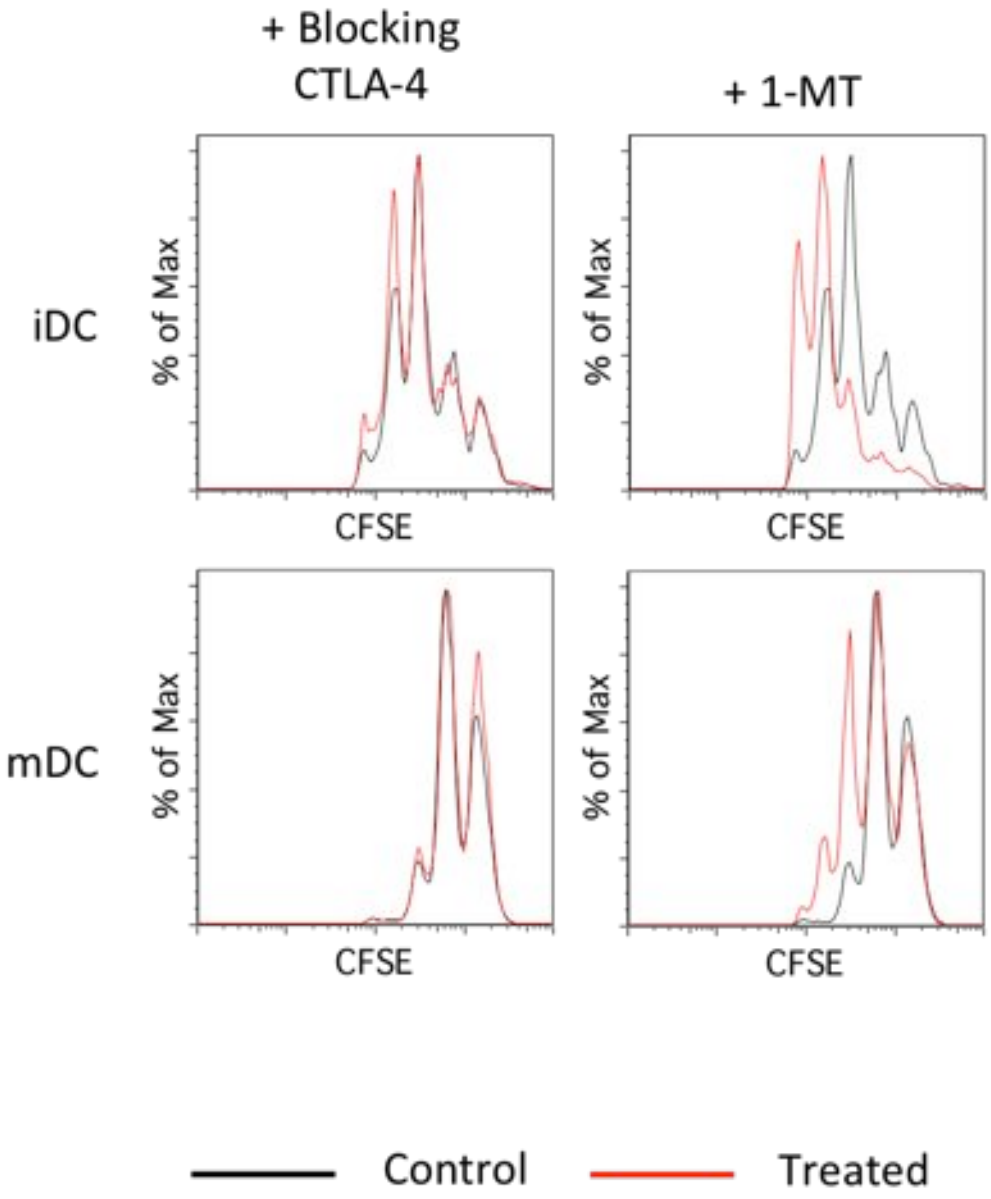

Figure 5.2.2.2: Effect of blocking CTLA-4 does not correlate with the effect of 1-MT inhibition. CFSE labelled CD25$^-$ T-cells were incubated with CD3 (10µg/ml) in the presence of mature or immature DCs at a ratio of 5 DCs for every T cell. Cells were left untreated or were treated with anti-CTLA-4 (Tremelimumab; 50µg/ml) or 1-MT (500µM). Cultures were analysed by flow cytometry after 5 days. The data presented is representative of three independent experiments.

During the course of my thesis work, data from our laboratory began to indicate that one role for CTLA-4 is to acquire CD86 from DCs [4]. To test if this could account for the observations made in DC assays and in the suppression of responder cells by CTLA-4 expressing jurkat cells it was decided to test if CTLA-4^{+} jurkat cells could indeed acquire CD86. To do this, CHO cells expressing CD86 GFP were incubated with either WT or CTLA-4 jurkats for six hours. To allow easy separation of the different cell types, the CHO cells were first labelled with a far red dye (Figure. The amount of GFP present in the jurkat was then determined by flow cytometry. **Figure 5.2.2.3** shows that the WT jurkats express very little GFP whereas those jurkats expressing CTLA-4 appear to have acquired a considerable amount of GFP. These data suggest that CD86 acquisition can occur in a CTLA-4 dependent manner in jurkat cells and could therefore act to suppress T-cell co-stimulation.

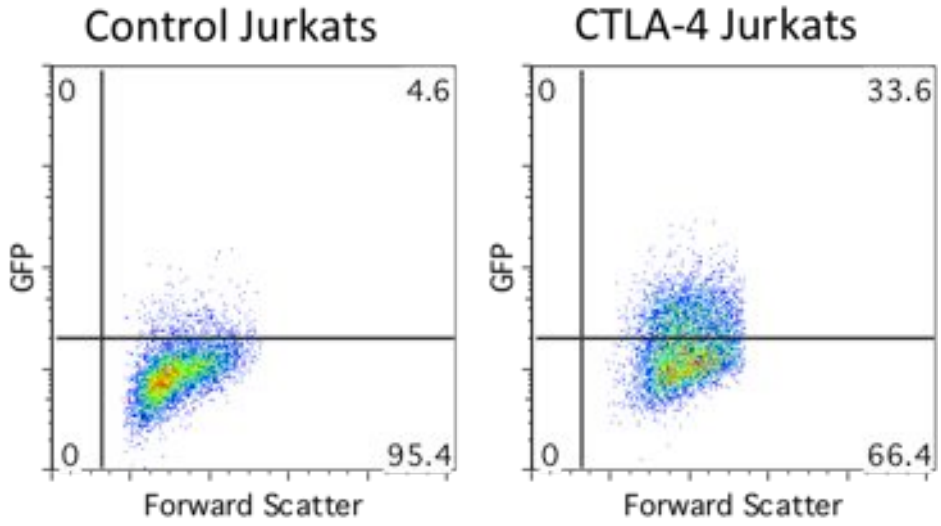

Figure 5.2.2.3: Jurkats can acquire CD86 GFP from CHO cells. WT or CTLA-4 jurkats were incubated with CHO cells expressing GFP tagged CD86 for 6 hours at 37°C. Presence of GFP tagged protein in the jukat cells was determined by flow cytometry. The data presented is representative of four independent experiments.

The next step was to determine if CD80 or CD86 acquisition by CTLA-4 could account for the functional effects observed in the DC assay. To do this, CFSE labelled $CD25^-$ T-cells were stimulated with CD3 and reducing numbers of DCs in the presence or absence of anti-CTLA-4. After five days, the cultures were stained for CD11c, CD80 and CD86 to assess the levels of these proteins on the DC. These markers and the T-cell proliferation were then analysed by flow cytometry. As can be seen from **figure 5.2.2.4**, the DCs were expressing both CD80 and CD86 and that the overall levels of B7 ligands increased when the DCs are incubated with T-cells and that the increase is less marked when DC numbers are reduced. Importantly, where high numbers of DCs were present (1/3) blocking CTLA-4 had little effect on the down regulation of B7 ligands. Whilst in contrast we observed that blocking CTLA-4 results in a marked increase in B7 expression on the DC. These data highlight that modulation of the DCs by CTLA-4 could account for the enhance proliferation observed when CTLA-4 is blocked.

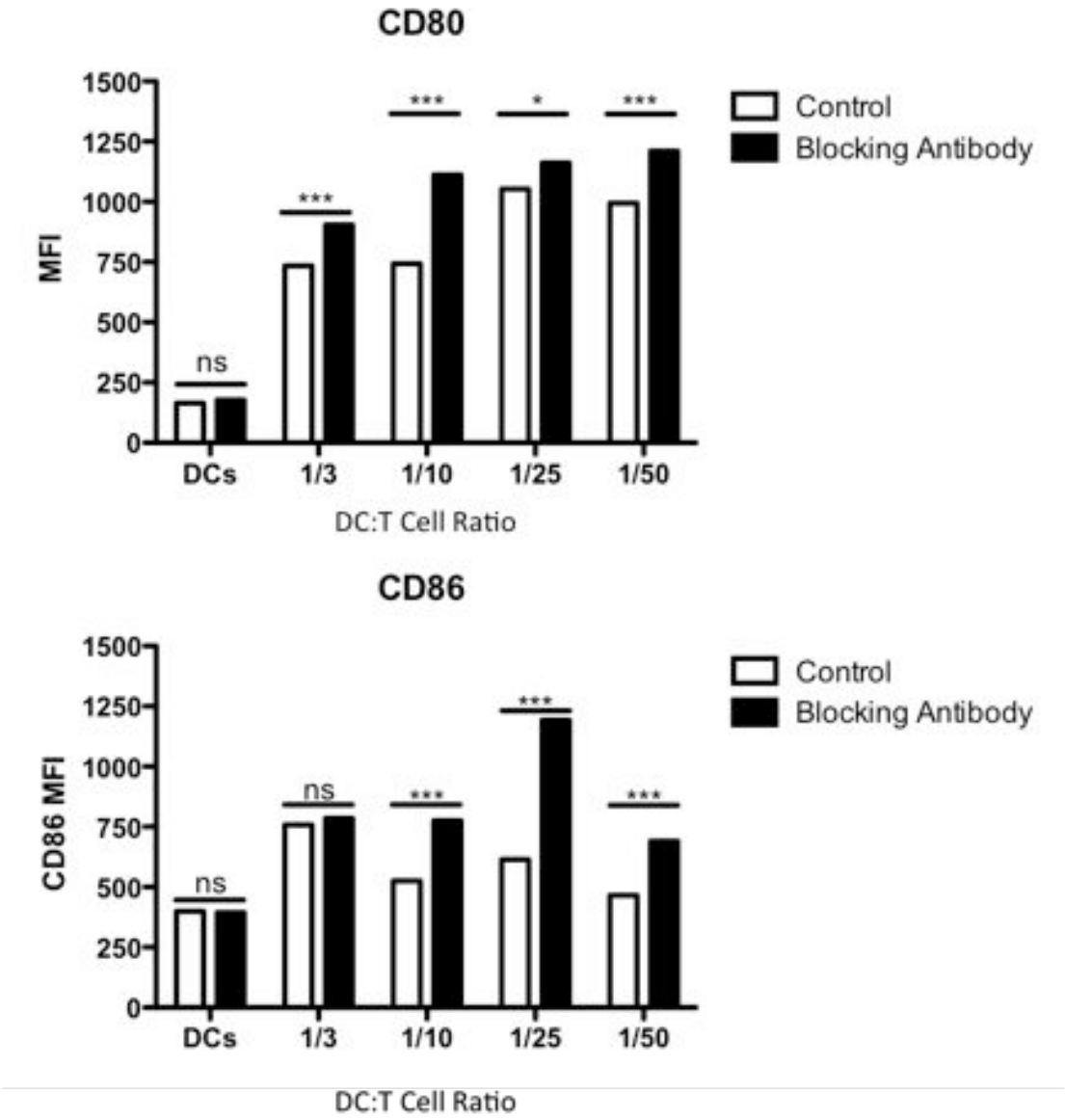

Figure 5.2.2.4: Blocking CTLA-4 results in the down regulation of CD80 and CD86 on DCs. CFSE labelled $CD25^-$ T-cells were incubated with CD3 (10µg/ml) and DCs at the indicated ratios in the presence or absence of blocking antibody (Tremelimumab; 50µg/ml) for five days. At day five, the cultures were stained for surface CD80 and CD86 and protein levels analysed by flow cytometry. The statistical analysis was performed using a Two-way Annova followed by a Bonferroni post test. ns = not significant, * $p<0.05$, *** $p<0.001$. The data presented is representative of five independent experiments.

Since it appears that CTLA-4 is able to acquire CD80 and CD86 from the APC, it was decided to assess if CD86 is degraded upon entry into the T-cell. To do this, CHO-CD86 GFP cells were incubated for six hours with either WT or CTLA-4 jurkat cells. This was done in the presence or absence of NH_4Cl, a known inhibitor of lysosomal degradation. **Figure 5.2.2.5** shows again the when the CHO cells were incubated with the WT jurkats that there was no acquisition of the GFP signal, further, incubating these cells with NH_4Cl had no effect. However, when the CHO-CD86 cells are incubated with CTLA-4 jurkats, around a third of the cells acquired GFP. Interestingly, when this sample is incubated with NH_4Cl, the overall number of CTLA-4 jurkat cells that have GFP increases two fold to around 60%. These data suggest that CD86 is degraded in lysosomes after it enters the CTLA-4 expressing cell. To confirm these data, it was decided to carry out a similar experiment but this time assessing overall protein concentration within cells by western blot analysis. This was done by mixing untransfected CHO cells with CHO-CD86 cells or mixing CHO-CTLA-4 cells with CHO-CD86 cells, both of which were done in the presence or absence of NH_4Cl (**Figure 5.2.2.6**). After six hours of incubation the all cells were collected, protein was extracted and the overall amount of CD86 in the samples was determined by western blot. As can be seen, in the CHO + CHO-CD86 sample, the amount of protein in the cells treated with NH_4Cl is increased relative to the untreated cells. Where CHO-CD86 cells are incubated with CHO-CTLA-4 cells it can be seen that there is an overall decrease in the amount of CD86 in these samples when compared to controls, this decrease in protein is recoverable with the addition of NH_4Cl. These data are consistent with the idea that CTLA-4 expressing cells can acquire CD86 and that the CD86 is degraded in lysosomes.

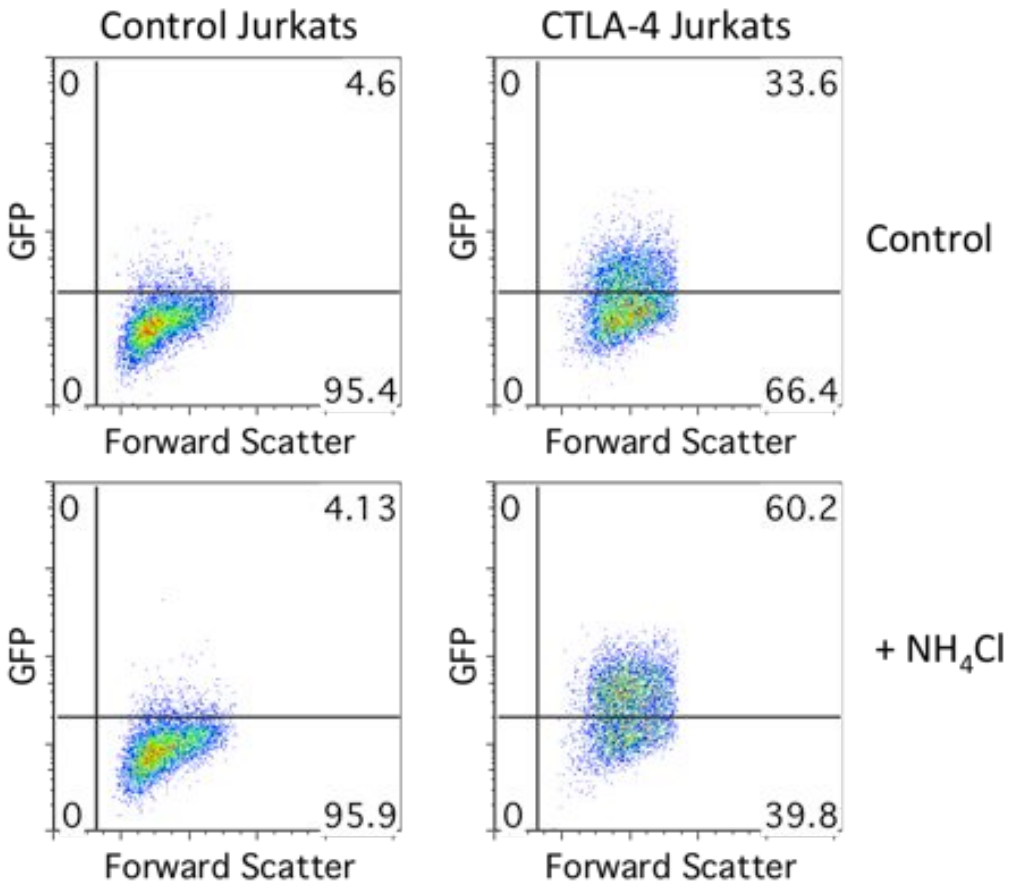

Figure 5.2.2.5: Acquired CD86 is degraded in the jurkat. WT or CTLA-4 jurkats were incubated with CHO cells expressing GFP tagged CD86 in the presence or absence of NH_4Cl (CONCENTRATION) for 6 hours at 37°C. Presence of GFP tagged protein in the jurkat cells was determined by flow cytometry.

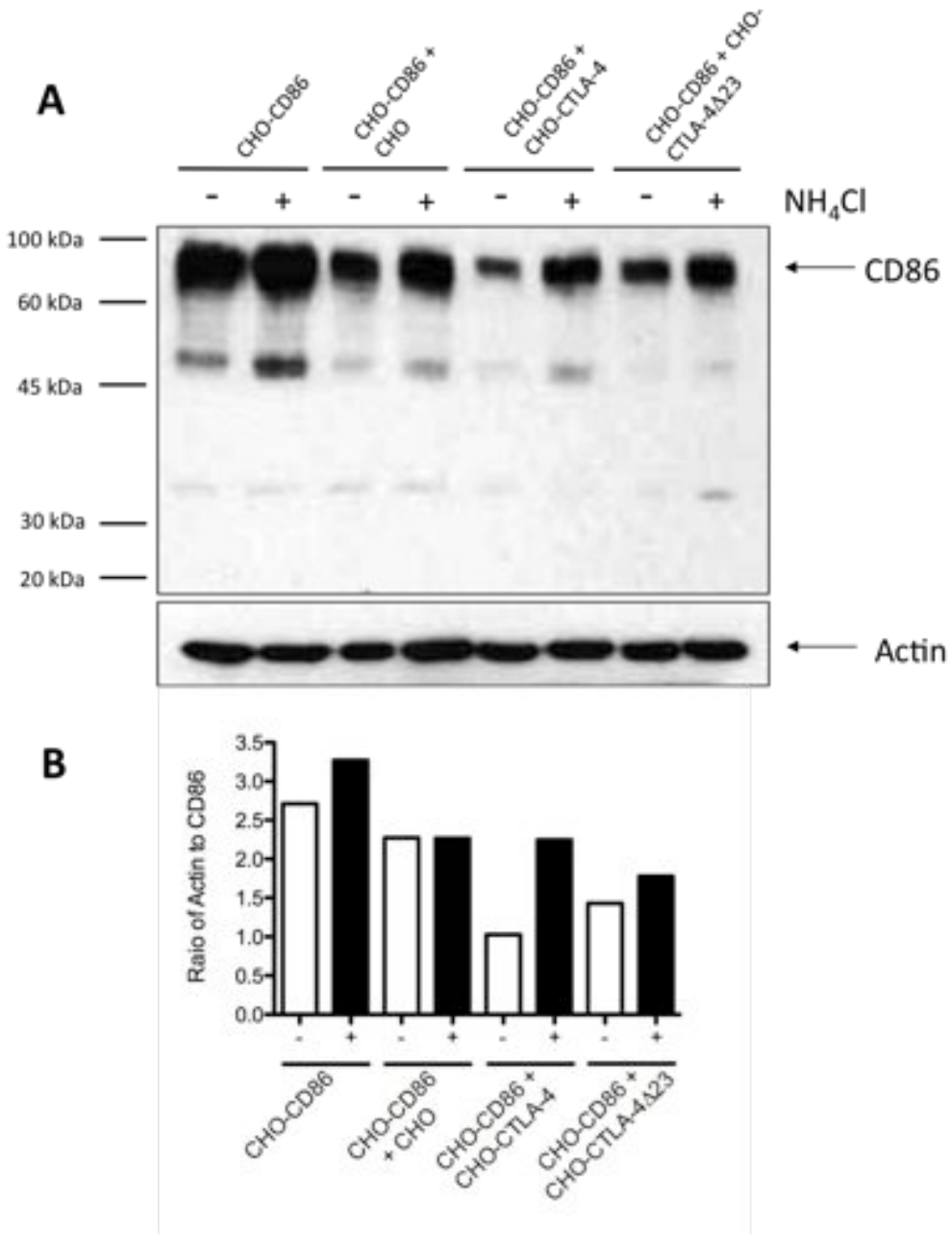

Figure 5.2.2.6: CD86 is degraded after acquisition by CTLA-4. CHO-CD86 cells were incubated for 6 hours alone, with CHO, CHO-CTLA-4 or CHO-CTLA-4Δ23 cells in the presence or absence of NH_4Cl (CONCENTRATION). **A**. The protein was extracted , separated by SDS-PAGE gel electrophoresis before being transferred to a membrane. The membrane was then blocked and probed with a primary antibody against CD86 (C-19; 1:1,000) or Actin (AC-40; 1:2,500) and a secondary HRP antibody (1:2,500). **B**. The intensity of bands was determined by plotting each sample as OD against distance and calculating the area under the curve. The ratio of sample to actin was then calculated and plotted in the graph as shown.

As a further control, CHO-CD86 cells were incubated with CHO-CTLA-4Δ23 cells (**Figure 5.2.2.6**). The CTLA-4Δ23 is a mutant form of CTLA-4 that is missing the final 23 amino acids of the tail. This mutant has increased surface expression relative to WT CTLA-4 and has been shown to be less effective at acquiring CD86 from neighbouring cells (OS Qureshi, unpublished observations). We found that the overall levels of CD86 in these cells is equivalent to the control well (CHO + CHO-CD86) indicating that the CD86 has to be internalised into the CTLA-4 expressing cell in order for it to be degraded. To find out if CTLA-4 is also degraded upon acquisition of CD86, CHO-CTLA-4 cells were incubated with either untransfected CHO's or CHO-CD86 cells. After six hours, the protein was extracted and western blot analysis was carried out to look at CTLA-4 (**Figure 5.2.2.7**). From these data it appears that CTLA-4 is also specifically degraded after acquiring CD86. Interestingly, however, it seems that CTLA-4 may be less effectively degraded when incubated with CD80 expressing cells. Whilst preliminary these data provide interesting insights into the possible differential fate of CTLA-4 when bound by its two natural ligands.

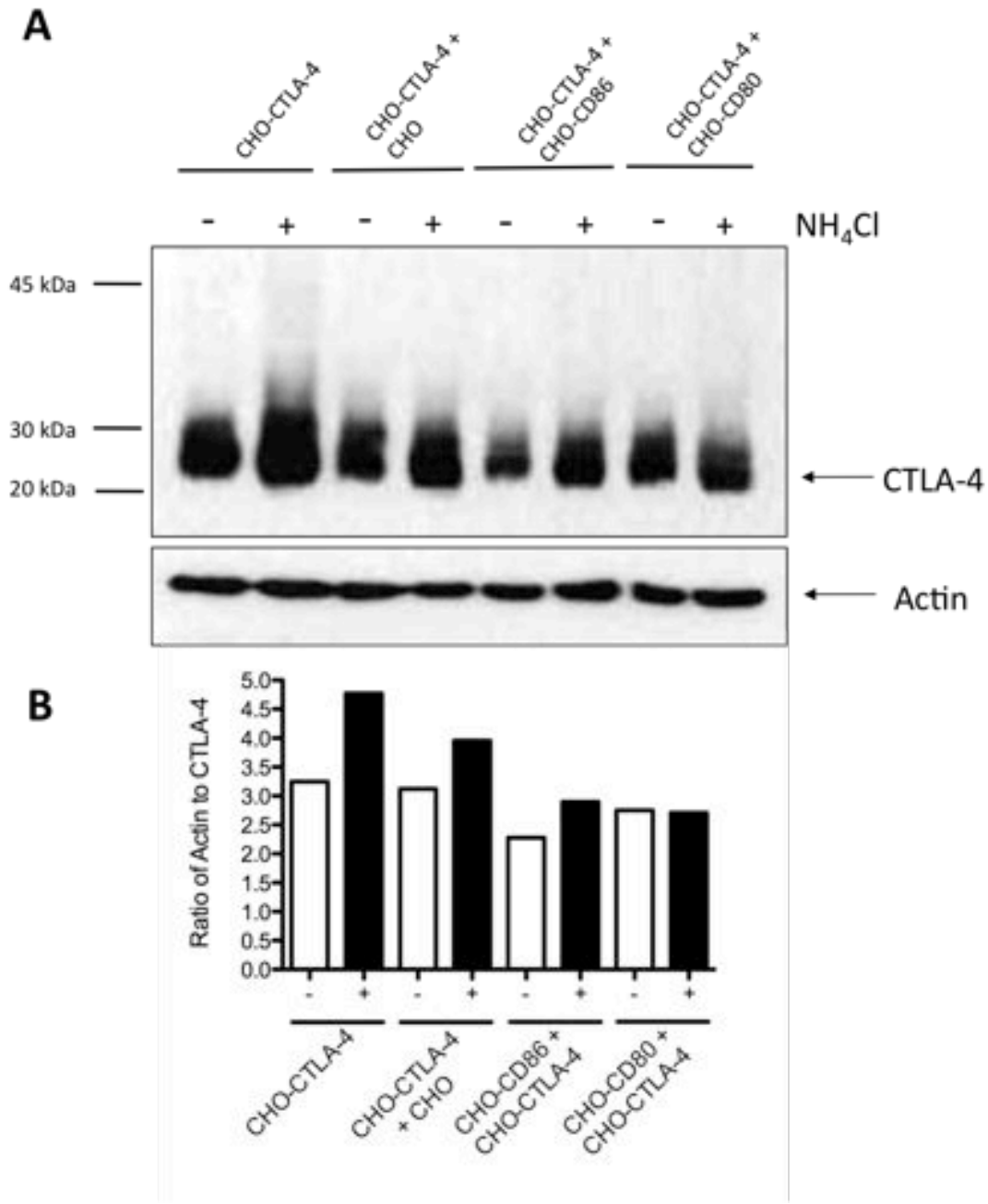

Figure 5.2.2.7: CTLA-4 is also degraded after acquiring CD86. CHO-CTLA-4 cells were incubated for 6 hours alone, with CHO, CHO-CD86 or CHO-CD80 cells in the presence or absence of NH_4Cl (CONCENTRATION). **A.** The protein was extracted , separated by SDS-PAGE gel electrophoresis before being transferred to a membrane. The membrane was then blocked and probed with a primary antibody against CTLA-4 (C-19; 1:2,500) or Actin (AC-40; 1:2,500) and a secondary HRP antibody (1:2,500). **B**. The intensity of bands was determined by plotting each sample as OD against distance and calculating the area under the curve. The ratio of sample to actin was then calculated and plotted in the graph as shown.

5.3 Discussion

Regulatory T-cells are a distinctive subset of T-cells that are thought to play an important role in immune tolerance, with data suggesting that these cells are defective in patients with various autoimmune diseases [5-7]. It would be expected that addition of Treg cells to cultures would result in the suppression of responder cell proliferation. It was therefore surprising to find that addition of these cells, in fact, enhanced the proliferation of the responder population. The most likely reason for this is down to the fact that the suppressor cell population constituted only 60% $CD25^{+}FoxP3^{+}$ cells. The contamination of the suppressor population with a large number of effector cells may have resulted in the production of additional pro-inflammatory cytokines and that are driving the responder to undergo further proliferation. More recent data from the laboratory have now shown that with pure Treg, CTLA-4 based suppression can be seen as predicted (DMS personal comments).

Isolation of a pure population of regulatory T-cells from PBMCs proved to be a difficult task, probably as a result of the fact that these cells exist in very small number within peripheral blood. To therefore study if CTLA-4 expressing cells were able to regulate the expression of a responder population, it was decided to use jurkat cells that had been transduced to express CTLA-4. This provided a useful model for studying the ability of CTLA-4 to control the proliferation of surrounding cells since WT jurkats could be used as a control. These experiments showed that

the presence of CTLA-4 expressing cells resulted in the inhibition of a responder population. The idea that CTLA-4 can regulate its external environment is not new. Indeed, it has been shown that both CD4 and CD8 T-cell responses to viral proteins are unaffected by the absence of cell-intrinsic expression of CTLA-4 and instead that these responses can be controlled by the presence of other CTLA-4 expressing cells [8]. Further, it has previously shown that CTLA-4 knockout mice reconstituted with WT bone marrow are rescued from disease [9] suggesting that the presence of CTLA-4 expressing cells can control the proliferation of other non-expressing cells. However, these data go further and show that suppression by CTLA-4 does not require a specialised Treg cell and that it can be carried out by any CTLA-4 expressing cell. This strongly suggests that CTLA-4 is itself sufficient to mediate suppression without the need for additional specialised machinery.

It is interesting to note that the use of a CTLA-4 expressing "regulatory" population of cells was most effective when the number of DCs in cultures were limited. These data reflect the concept presented in the previous chapter where blocking CTLA-4 with antibodies was most effective when there were fewer DCs in the culture and that CD80 and CD86 down regulation is most marked in cultures stimulated with fewer DCs. These data are consistent with the idea that CTLA-4 could be out competing CD28 for ligand engagement in a cell extrinsic manner. By depleting ligand levels on APC CTLA-4 can diminish CD28 co-stimulation. It was also interesting to find that CD86 appeared to be more readily depleted than CD80. It would perhaps be expected that CD80 would be the first ligand removed considering

that the CTLA-4:CD80 interaction is stronger than the CTLA-4:CD86 interaction. This finding may be in part due to the fact that CTLA-4 has been shown to form lattice structures with CD80 [10] which may anchor both proteins at the cell surface. In contrast, CD86 is thought not to form dimers [11] which may make this protein more susceptible to removal from the surface of the APC. Taken together, these data further suggest that CTLA-4 is acting as competition for CD28 resulting in reduced positive signalling.

One of the possible explanations for the lack of functional effects using CHO cells but not DCs may be down to the dendritic cells being signalled via CTLA-4 ligands to produce factors that inhibit T-cell proliferation. One of the most obvious candidates for this was via the production of IDO as previously reported. However, it was found that inhibiting IDO only affected proliferation in T-cells when there were very high numbers of DCs. Such high numbers of DCs may be required in order to produce enough IDO to deplete the tryptophan in the serum rich environment that the experiments were carried out in. It could be expected that the 1-MT is toxic to the cells and certainly it appears that when there are very few DCs in the culture, that treating with 1-MT results in a vast decrease in proliferation compared to untreated cells. However, where there are more DCs, the levels of proliferation between treated and untreated cells are largely equivalent suggesting that toxicity of the 1-MT is not a major factor in this experiment. It is thought that IDO can exist as both an inactive and active form and that activation of IDO occurs via IFNγ [12, 13]. It may therefore be the case that in those cultures with fewer DCs, fewer T-cells

become activated lowering the total amount of IFNγ in these cultures. It is interesting to note that in our experience, all mature DCs express IDO following stimulation, yet can effectively stimulate T-cell responses. Accordingly this suggests that there must be some specific context to IDO regulation. The fact that blocking CTLA-4 at such high numbers of DCs had no effect on proliferation suggests that IDO does not account for the functional effects seen in our assays. The relationship between CTLA-4 and IDO still remains enigmatic with little reproduction of the original literature. Whilst the situation remains unclear our data suggest that there are clear functions of CTLA-4 that do not relate to IDO.

The data presented here and elsewhere [4] show that one function of CTLA-4 is to acquire ligands from APC. We assessed the fate of the acquired ligands upon entry to the T-cell, it was found that specifically blocking degradation results in increased presence of CD86 GFP within CTLA-4 cells. This implies that the protein is indeed degraded upon acquisition. Interestingly, it appears that both CTLA-4 and CD86 are degraded once acquisition occurs. These findings may provide an explanation as to why CTLA-4 is recycled between the cytoplasm and the cell surface and why the protein to be targeted to lysosomes for degradation [14-17]. However, this data is still very preliminary and requires further work to fully corroborate the findings.

Overall, the data presented in this chapter are consistent with the idea that CTLA-4 is able to regulate the proliferation of surrounding cells by modulating APCs. Indeed, previous data has shown that regulatory T-cells are able to modulate the expression of the B7 lignads on DCs [18]. Further, our laboratory has recently published data showing that CTLA-4 can specifically down-regulate the expression of CD80 and CD86 [4].

Chapter 6:

Final Discussion

6.1 Final Discussion

There is little doubt that CTLA-4 acts as a negative regulator of T-cell activation, however, the mechanism, or indeed mechanisms, employed by the protein have been subject to much debate. The aim of this project was therefore to use well validated reagents to try and test the various suggested models of CTLA-4 mediated suppression in order to establish how this protein exerts its inhibitory function.

The aim of chapter three was to characterise a range of anti-CTLA-4 antibodies that our laboratory had in order to assess their suitability for tools in studying the role of CTLA-4. Although it appeared that all of the antibodies tested were able to bind to the extra-cellular domain of their target protein, it was interesting to note that this did not necessarily predict that they would be capable of blocking CTLA-4 interactions. These data highlight the importance of carrying out a comprehensive study on the antibodies prior to carrying out functional experiments using them. The importance of a full characterisation was particularly evident in experiments where no functional effects were observed upon blocking CTLA-4 in T-cells stimulated with B7 expressing CHO cells. If the blocking antibodies hadn't been fully tested then it could be concluded that CTLA-4 wasn't functioning in this setting. However, the fact that it was known that the antibodies were capable of blocking, together with the subsequent data using DCs as co-stimulators where large

functional effects were revealed, suggests that CTLA-4 just does not function in this assay despite its high levels of expression.

The reason for a lack of functional effects when blocking CTLA-4 in the CHO cell assays, even when the numbers of CHO cells present were very low is itself an interesting result. It supports the possibility that CTLA-4, despite popular belief, is unable to deliver a negative signal into the T-cells. If it were able to deliver a negative signal, it would be expected that having more ligand available would result in a stronger negative signal. It cannot be said that the lack of a negative signal is a result of the process of fixation as these same ligands are capable of driving T-cell proliferation in the first place and the fact that this proliferation is dependent on B7:CD28 interactions suggest that, despite fixation, the ligands are still functional. It is therefore reasonable to conclude that if a negative signal were important in restricting T-cell proliferation that this signal would also be deliverable in this setting and therefore observable in the CHO experiments.

The fact that there was no effect of blocking proliferation in a CHO cell assay but were sizeable effects under other conditions using DCs as APC, points to the possibility that CTLA-4 may be capable of acting on the DC. It could be that the process of fixation inhibits the removal of ligands from the surface of the CHO cell, which is why no effects of blocking CTLA-4 could be observed. In order to address this issue, CHO cells were irradiated or treated with mitomycin C before being used

to provide co-stimulation for the responder T-cell population. It was found, however, that the T-cells did not undergo proliferation when mitomycin C treated CHO cells were used. The reason for this is currently under investigation. One possible explanation could be due to the fact that CHO cells do not express adhesion molecules on their surface. One of the major adhesion molecules is LFA-1, which has been shown to be required for IS formation [1]. LFA-1 is thought to aid the formation of the pSMACs and hence assisting with TCR signalling [2]. It could be that the process of fixation holds the activation molecules in place, allowing optimal TCR signalling. In contrast, the live cells may be too fluid which, in the absence of adhesion molecules, results in inadequate activation signals resulting in impaired T-cell responses.

It was then decided to assess if the removal of ligands from DCs could be inhibited by fixing the APCSs prior to their use as co-stimulators. Again, however, it was found that fixed DCs were unable to provide co-stimulation for the T-cells. It would be interesting in future to find a method of blocking the removal of ligand on the DC and testing if blocking CTLA-4 still had a functional outcome. Despite these limitations the simple negative signal model is not supported by the above data, suggesting either very specific requirements for this function or that intrinsic inhibition is not a major mechanism.

The data presented in this thesis provide a little more clarity as to how CTLA-4 is able to modulate T-cell responses, a summary of which is shown in **figure 6.1**. It would appear that CTLA-4 and CD28 work to maintain the fine balance required between producing a sufficiently robust immune response to overcome infection, whilst maintaining tolerance to self antigens and thus preventing autoimmunity. There are several key features of the biology of both CD28 and CTLA-4 that seem to enable this fine balance to be met. Firstly, is the timing of protein expression. It has been found that CD28 expressed on all resting T-cells [3] and is therefore immediately available for engagement when an antigen is encountered. Conversely, it is only once the TCR engages with antigen together with co-stimulatory signals from CD28 engagement that high levels of CTLA-4 expression are induced [4]. Further, it has been demonstrated that CTLA-4 has a very high turnover rate with a half-life of roughly 2-4 hours [5]. The timing differences of the expression patterns would allow T-cells to initiate a response on encountering an infectious agent without the response being drowned out by CTLA-4 suppression.

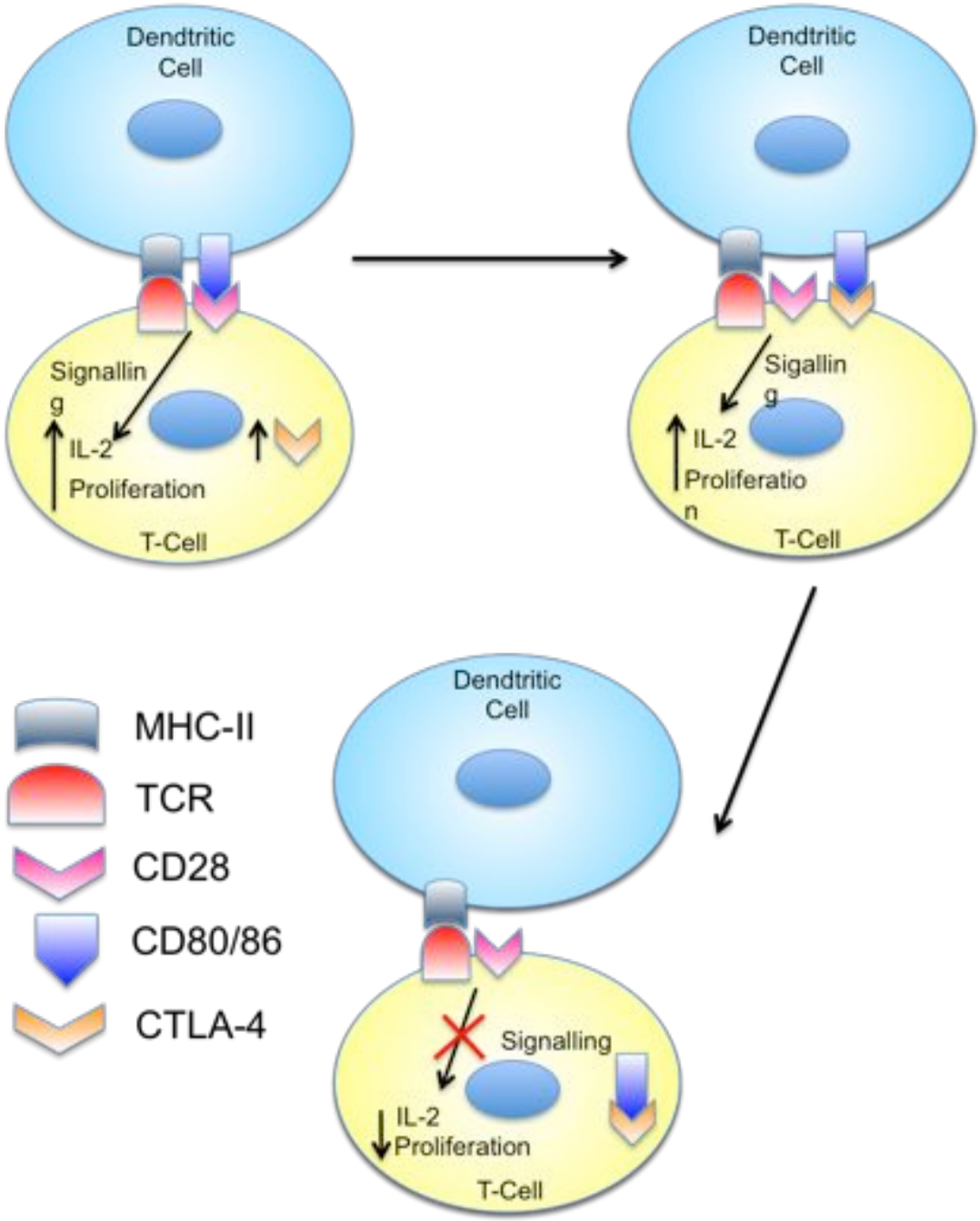

Figure 6.1: Summary Diagram of CTLA-4 Mediated Suppression. T-cells are activated after stimulation of the TCR together with CD28 mediated co-stimulation. Activation of the T-cells results in induced IL-2 production, proliferation and induction of CTLA-4 expression. CTLA-4 is then transported to the surface where it out competes CD28 for binding to CD80 and CD86. Upon engagement of CTLA-4 to its ligands, both molecules are internalised into the T-cell resulting in reduced levels of ligand on the surface of the DCs.

The second critical feature of these proteins biology is location of expression. CD28 is found at relatively high levels localised at the surface of the T-cell where it is readily available to engage with its ligands thus driving the early T-cell response to infection unimpeded. Conversely, CTLA-4 is found largely in intracellular vesicles with only a very small amount of the protein being found at the surface at any given time [5, 6]. Once the T-cell is activated, CTLA-4 is trafficked to the immunological synapse [7] before being re-internalised back into the T-cell. This limited surface expression would be expected to reduce the opportunity for CTLA-4 to engage with its ligands preventing T-cell responses from being prematurely suppressed.

A third important difference in the biology of CD28 and CTLA-4 that contributes to function is that CTLA-4 is able to bind to its ligands with greater avidity and affinity than CD28 thus enabling CTLA-4 to out-compete CD28 for ligand binding [8, 9]. This feature allows CTLA-4 to act as an efficient antagonist to a protein that is expressed earlier and more abundantly on the surface of the T-cell. The data presented in chapter 5 showing that CTLA-4 is able to down regulate the expression of CD80 and CD86 on the APC fits well with a competition model for suppression by CTLA-4. Firstly, the T-cell is activated by engagement of the TCR and co-stimulation by CD28. This results in expression of CTLA-4 which is transported to the IS where it competes with CD28 for ligand binding. Upon engagement, CTLA-4 and bound ligand are re-internalised and targeted to the lysosome for degradation [10] thus reducing the availability of B7 on the APC and hence the capacity for CD28 to provide further co-stimulation. It is interesting to note that in preliminary data

studying the fate of CTLA-4 after the removal of ligand suggests that the protein is only degraded after engaging with CD86 but not with CD80. It does appear that the CTLA-4 is capable of removing both ligands so these data could therefore imply that once internalised, the protein may be processed differently in lysosomes. Alternatively, it has been shown that CTLA-4 and CD80 can form lattice like structures on the surface of the T-cell [11]. The formation of such lattices may make it more difficult for CD80 to be removed from the surface of the APC and, consistent with this idea, the data presented in figure 5.2.3.4 does suggest that CD86 is more readily down-regulated than CD80.

The data showing that CTLA-4 expressing jurkat T-cells were able to suppress a responder population suggests that CTLA-4 may work in the same way in both helper and regulatory T-cells. It has been shown that Tregs constitutively express CTLA-4 [12], which may go some way to explaining their highly suppressive nature. Having CTLA-4 available from the first engagement may prevent or limit the delivery of the initial co-stimulatory signal from CD28 thus increasing the threshold for T-cell activation. There have also been some data suggesting that CTLA-4 may be able to reduce the contact time between the T-cell and the APC [13] and that lower levels of ligand are detected in the presence of CTLA-4 positive cells [14, 15], both of which may be a result of CTLA-4 depleting B7 ligands from the DC. The idea of CTLA-4 removing ligands from the APC provides a mechanism for CTLA-4 that would be functional in both settings.

The transfer of proteins between cells has been observed in a number of situations and appears to be particularly prevalent between cells of the immune system [16-19]. The exact molecular mechanisms driving the inter-cellular trafficking of proteins remain largely unknown. Two potential mechanisms of protein transfer have been identified; these are trogocytosis and nanotube formation [20]. Nanotubes are long membrane projections that connect T-cells enabling the transfer of information [21]. There is evidence to suggest that specific proteins can be directed to nanotubes [20] raising the possibility that this could play a role in the transfer of ligands between the DC and the T-cell. An alternative to nanotubes is trogocytosis, which is an active process of protein transfer that requires cell-cell contact. It would therefore be expected that upon transport to the immune synapse, that CTLA-4 could be in a position to induce trogocytosis to occur. Further, as a process, trogocytosis is thought to occur rapidly [22, 23], which could enable CTLA-4 to modulate T-cell responses quickly and effectively. Both nanotube formation and trogocytosis require further study to determine how they enable protein transfer. Additional work is also necessary to establish the mechanisms that CTLA-4 uses to remove ligands from the DCs.

Further work would begin by trying to determine if the “ripping” of CD86 from the surface of T-cells is a major mechanism for CTLA-4 function. It would be interesting to try to reveal CTLA-4 function using the CHO cell transfectants as stimulatory cells by finding a model where non-fixed CHO cells were able to drive T-cell proliferation. This may become

possible by including factors such as adhesion molecules in the CHO cell thereby enabling the T cell to engage more tightly with the CHO cell.

In parallel to this, it would be useful to develop a DC driven system where it is possible to inhibit the transfer of co-stimulatory molecules. This could take the form of a fixed DC that is capable of driving proliferation. To do this, it may be necessary to over express co-stimulatory molecules on the surface to overcome the fact that fixed DCs not able to drive T-cell proliferation. Alternatively, it may be possible to develop a DC that has its CD86 anchored to the membrane possibly by tethering the B7 ligands to integral membrane proteins. If the use of the modified DCs resulted in the loss of CTLA-4 effects when APCs are titrated, it would go some way towards showing that the transfer of co-stimulatory molecules is an important mechanism of CTLA-4 function.

Another avenue for investigation would be to assess the role of CTLA-4 on Treg cells. The jurkat experiments presented in this thesis suggest that any CTLA-4 expressing cell is capable of suppression. It would be useful to try to isolate a pure population of Treg cells and to assess their ability to suppress responder T-cell function when compared to non-regulatory cells. It may be that regulatory T-cells are more effective suppressors and that anti-CTLA-4 effects may be observed at an earlier time point when compared to non-regulatory cells. However, this could be driven by other means of suppression.

It would also be interesting to conduct further investigation of the preliminary western blot data suggesting that CD86 is degraded upon acquisition by CTLA-4. From the preliminary data it appears that CD86 is degraded where CTLA-4 is not, this could either be

because the proteins are becoming detached inside the T-cell or that new CTLA-4 is generated more rapidly than CD86. It is known that CTLA-4 is trafficked towards lysosomes so it could be that once in here, the pH of the lysosome causes CTLA-4 to detach from CD86 enabling the CTLA-4 to be redirected to the surface of the T-cell whilst the CD86 is degraded.

It was also interesting to note from the preliminary data that levels of CD86 declined where CD80 didn't. Further investigation would try to elucidate if this was important for maintaining the immune response active during the course of an infection. It would be interesting to make a single CD80 molecule that was incapable of dimerization to determine if this made it possible for CTLA-4 to remove this protein also.

Overall, there still remain a number of unknowns surrounding exactly how CTLA-4 modulates T-cell activation. Understanding how this protein works could have important therapeutic implications. There are a number of autoimmune diseases that are associated with defective CTLA-4 [24-28], further; it appears that blocking CTLA-4 function could improve the prognosis in certain cancer patients [29, 30]. Utilising the negative regulatory function of CTLA-4 has already proven to have some success in the treatment of RA [31]. It appears that one of the biggest issues with the use of blocking CTLA-4 antibodies as a treatment is a lack of specificity. Blocking CTLA-4 can result in the inhibition of all T-cell responses, which can increase susceptibility to infection and also result in a breakdown of peripheral tolerance causing the development of autoimmune conditions. Adverse reactions as a result of indiscriminate blocking of CTLA-4 on all expressing cells appear to correlate with

the effectiveness of the treatment against cancer [30]. If all CTLA-4 expressing cells are capable of suppressing then it is not surprising that blocking the proteins function results in a breakdown of tolerance and inducement of autoimmune conditions. It may be more appropriate to try to target the blocking antibody therapies to antigen specific T-cells in order to reduce the incidence of adverse reactions to the treatment.

Understanding processes up or downstream of CTLA-4 function may also be important in developing therapeutics. It may be possible to isolate areas of the pathway that will allow specific targeting towards CTLA-4.

Chapter 7:

References

1. Chaplin, D.D., *Overview of the immune response.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S3-23.
2. Chaplin, D.D., *1. Overview of the human immune response.* J Allergy Clin Immunol, 2006. **117**(2 Suppl Mini-Primer): p. S430-5.
3. Parkin, J. and B. Cohen, *An overview of the immune system.* Lancet, 2001. **357**(9270): p. 1777-89.
4. Bettelli, E., et al., *Induction and effector functions of T(H)17 cells.* Nature, 2008. **453**(7198): p. 1051-7.
5. Zielinski, C.E., et al., *Dissecting the human immunologic memory for pathogens.* Immunol Rev, 2011. **240**(1): p. 40-51.
6. Williams, M.A., E.V. Ravkov, and M.J. Bevan, *Rapid culling of the CD4+ T cell repertoire in the transition from effector to memory.* Immunity, 2008. **28**(4): p. 533-45.
7. Polic, B., et al., *How alpha beta T cells deal with induced TCR alpha ablation.* Proc Natl Acad Sci U S A, 2001. **98**(15): p. 8744-9.
8. Swain, S.L., H. Hu, and G. Huston, *Class II-independent generation of CD4 memory T cells from effectors.* Science, 1999. **286**(5443): p. 1381-3.
9. Kondrack, R.M., et al., *Interleukin 7 regulates the survival and generation of memory CD4 cells.* J Exp Med, 2003. **198**(12): p. 1797-806.
10. Lenz, D.C., et al., *IL-7 regulates basal homeostatic proliferation of antiviral CD4+T cell memory.* Proc Natl Acad Sci U S A, 2004. **101**(25): p. 9357-62.
11. Purton, J.F., et al., *Antiviral CD4+ memory T cells are IL-15 dependent.* J Exp Med, 2007. **204**(4): p. 951-61.
12. Hammarlund, E., et al., *Duration of antiviral immunity after smallpox vaccination.* Nat Med, 2003. **9**(9): p. 1131-7.
13. Lin, E., et al., *Heterogeneity among viral antigen-specific CD4+ T cells and their de novo recruitment during persistent polyomavirus infection.* J Immunol, 2010. **185**(3): p. 1692-700.
14. Lind, E.F., et al., *Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development.* J Exp Med, 2001. **194**(2): p. 127-34.
15. Kuhns, M.S., M.M. Davis, and K.C. Garcia, *Deconstructing the form and function of the TCR/CD3 complex.* Immunity, 2006. **24**(2): p. 133-9.
16. Alegre, M.L., K.A. Frauwirth, and C.B. Thompson, *T-cell regulation by CD28 and CTLA-4.* Nat Rev Immunol, 2001. **1**(3): p. 220-8.
17. Takahama, Y., *Journey through the thymus: stromal guides for T-cell development and selection.* Nat Rev Immunol, 2006. **6**(2): p. 127-35.
18. Witt, C.M., et al., *Directed migration of positively selected thymocytes visualized in real time.* PLoS Biol, 2005. **3**(6): p. e160.
19. Derbinski, J., et al., *Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self.* Nat Immunol, 2001. **2**(11): p. 1032-9.
20. Gotter, J., et al., *Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters.* J Exp Med, 2004. **199**(2): p. 155-66.
21. Reichardt, P., B. Dornbach, and M. Gunzer, *The molecular makeup and function of regulatory and effector synapses.* Immunological reviews, 2007. **218**: p. 165-77.
22. Monks, C.R., et al., *Three-dimensional segregation of supramolecular activation clusters in T cells.* Nature, 1998. **395**(6697): p. 82-6.
23. Lee, K.H., et al., *T cell receptor signaling precedes immunological synapse formation.* Science, 2002. **295**(5559): p. 1539-42.

24. Freiberg, B.A., et al., *Staging and resetting T cell activation in SMACs.* Nature immunology, 2002. **3**(10): p. 911-7.
25. Brossard, C., et al., *Multifocal structure of the T cell - dendritic cell synapse.* European journal of immunology, 2005. **35**(6): p. 1741-53.
26. Tseng, S.Y., et al., *T cell-dendritic cell immunological synapses contain TCR-dependent CD28-CD80 clusters that recruit protein kinase C theta.* Journal of immunology, 2008. **181**(7): p. 4852-63.
27. Campi, G., R. Varma, and M.L. Dustin, *Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling.* J Exp Med, 2005. **202**(8): p. 1031-6.
28. Yokosuka, T., et al., *Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76.* Nat Immunol, 2005. **6**(12): p. 1253-62.
29. Kaizuka, Y., et al., *Mechanisms for segregating T cell receptor and adhesion molecules during immunological synapse formation in Jurkat T cells.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(51): p. 20296-301.
30. Varma, R., et al., *T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster.* Immunity, 2006. **25**(1): p. 117-27.
31. Irving, B.A. and A. Weiss, *The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways.* Cell, 1991. **64**(5): p. 891-901.
32. Romeo, C., M. Amiot, and B. Seed, *Sequence requirements for induction of cytolysis by the T cell antigen/Fc receptor zeta chain.* Cell, 1992. **68**(5): p. 889-97.
33. Wegener, A.M., et al., *The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules.* Cell, 1992. **68**(1): p. 83-95.
34. al-Ramadi, B.K., et al., *Deficient expression of p56(lck) in Th2 cells leads to partial TCR signaling and a dysregulation in lymphokine mRNA levels.* J Immunol, 1996. **157**(11): p. 4751-61.
35. Gupta, S., et al., *The T-cell antigen receptor utilizes Lck, Raf-1, and MEK-1 for activating mitogen-activated protein kinase. Evidence for the existence of a second protein kinase C-dependent pathway in an Lck-negative Jurkat cell mutant.* J Biol Chem, 1994. **269**(25): p. 17349-57.
36. Straus, D.B. and A. Weiss, *Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor.* Cell, 1992. **70**(4): p. 585-93.
37. Williams, S., et al., *Reconstitution of T cell antigen receptor-induced Erk2 kinase activation in Lck-negative JCaM1 cells by Syk.* Eur J Biochem, 1997. **245**(1): p. 84-90.
38. Chan, A.C., et al., *Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function.* EMBO J, 1995. **14**(11): p. 2499-508.
39. Wange, R.L., et al., *Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70.* J Biol Chem, 1995. **270**(32): p. 18730-3.
40. Bubeck Wardenburg, J., et al., *Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function.* The Journal of biological chemistry, 1996. **271**(33): p. 19641-4.
41. Zhang, W., et al., *LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation.* Cell, 1998. **92**(1): p. 83-92.
42. Finco, T.S., et al., *LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway.* Immunity, 1998. **9**(5): p. 617-26.

43. Asada, H., et al., *Grf40, A novel Grb2 family member, is involved in T cell signaling through interaction with SLP-76 and LAT.* The Journal of experimental medicine, 1999. **189**(9): p. 1383-90.
44. Bourette, R.P., et al., *Mona, a novel hematopoietic-specific adaptor interacting with the macrophage colony-stimulating factor receptor, is implicated in monocyte/macrophage development.* The EMBO journal, 1998. **17**(24): p. 7273-81.
45. Liu, S.K. and C.J. McGlade, *Gads is a novel SH2 and SH3 domain-containing adaptor protein that binds to tyrosine-phosphorylated Shc.* Oncogene, 1998. **17**(24): p. 3073-82.
46. Shahinian, A., et al., *Differential T cell costimulatory requirements in CD28-deficient mice.* Science, 1993. **261**(5121): p. 609-12.
47. Jenkins, M.K., et al., *CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells.* Journal of immunology, 1991. **147**(8): p. 2461-6.
48. Norton, S.D., et al., *The CD28 ligand, B7, enhances IL-2 production by providing a costimulatory signal to T cells.* Journal of immunology, 1992. **149**(5): p. 1556-61.
49. Fraser, J.D., et al., *Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28.* Science, 1991. **251**(4991): p. 313-6.
50. Lindstein, T., et al., *Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway.* Science, 1989. **244**(4902): p. 339-43.
51. Sperling, A.I., et al., *CD28/B7 interactions deliver a unique signal to naive T cells that regulates cell survival but not early proliferation.* Journal of immunology, 1996. **157**(9): p. 3909-17.
52. Boise, L.H., et al., *CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL.* Immunity, 1995. **3**(1): p. 87-98.
53. Howland, K.C., et al., *The roles of CD28 and CD40 ligand in T cell activation and tolerance.* Journal of immunology, 2000. **164**(9): p. 4465-70.
54. Judge, T.A., et al., *The role of CD80, CD86, and CTLA4 in alloimmune responses and the induction of long-term allograft survival.* Journal of immunology, 1999. **162**(4): p. 1947-51.
55. Kearney, E.R., et al., *Antigen-dependent clonal expansion of a trace population of antigen-specific CD4+ T cells in vivo is dependent on CD28 costimulation and inhibited by CTLA-4.* Journal of immunology, 1995. **155**(3): p. 1032-6.
56. Vella, A.T., et al., *CD28 engagement and proinflammatory cytokines contribute to T cell expansion and long-term survival in vivo.* Journal of immunology, 1997. **158**(10): p. 4714-20.
57. August, A. and B. Dupont, *CD28 of T lymphocytes associates with phosphatidylinositol 3-kinase.* International immunology, 1994. **6**(5): p. 769-74.
58. Pages, F., et al., *Binding of phosphatidylinositol-3-OH kinase to CD28 is required for T-cell signalling.* Nature, 1994. **369**(6478): p. 327-9.
59. Prasad, K.V., et al., *T-cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr(P)-Met-Xaa-Met motif.* Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(7): p. 2834-8.
60. Truitt, K.E., C.M. Hicks, and J.B. Imboden, *Stimulation of CD28 triggers an association between CD28 and phosphatidylinositol 3-kinase in Jurkat T cells.* The Journal of experimental medicine, 1994. **179**(3): p. 1071-6.
61. Sasaki, T., et al., *Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration.* Science, 2000. **287**(5455): p. 1040-6.
62. Costello, P.S., M. Gallagher, and D.A. Cantrell, *Sustained and dynamic inositol lipid metabolism inside and outside the immunological synapse.* Nature immunology, 2002. **3**(11): p. 1082-9.

63. Harriague, J. and G. Bismuth, *Imaging antigen-induced PI3K activation in T cells.* Nature immunology, 2002. **3**(11): p. 1090-6.
64. Alessi, D.R., et al., *Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha.* Current biology : CB, 1997. **7**(4): p. 261-9.
65. Walker, K.S., et al., *Activation of protein kinase B beta and gamma isoforms by insulin in vivo and by 3-phosphoinositide-dependent protein kinase-1 in vitro: comparison with protein kinase B alpha.* The Biochemical journal, 1998. **331 (Pt 1)**: p. 299-308.
66. Yang, E., et al., *Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death.* Cell, 1995. **80**(2): p. 285-91.
67. Schneider, H., et al., *T cell antigen CD28 binds to the GRB-2/SOS complex, regulators of p21ras.* European journal of immunology, 1995. **25**(4): p. 1044-50.
68. Songyang, Z., et al., *SH2 domains recognize specific phosphopeptide sequences.* Cell, 1993. **72**(5): p. 767-78.
69. Ramos-Morales, F., et al., *The proline-rich region of Vav binds to Grb2 and Grb3-3.* Oncogene, 1995. **11**(8): p. 1665-9.
70. Ye, Z.S. and D. Baltimore, *Binding of Vav to Grb2 through dimerization of Src homology 3 domains.* Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(26): p. 12629-33.
71. Su, B., et al., *JNK is involved in signal integration during costimulation of T lymphocytes.* Cell, 1994. **77**(5): p. 727-36.
72. McAdam, A.J., et al., *Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells.* Journal of immunology, 2000. **165**(9): p. 5035-40.
73. Swallow, M.M., J.J. Wallin, and W.C. Sha, *B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFalpha.* Immunity, 1999. **11**(4): p. 423-32.
74. Yoshinaga, S.K., et al., *T-cell co-stimulation through B7RP-1 and ICOS.* Nature, 1999. **402**(6763): p. 827-32.
75. Arimura, Y., et al., *A co-stimulatory molecule on activated T cells, H4/ICOS, delivers specific signals in T(h) cells and regulates their responses.* International immunology, 2002. **14**(6): p. 555-66.
76. Feito, M.J., et al., *Mechanisms of H4/ICOS costimulation: effects on proximal TCR signals and MAP kinase pathways.* European journal of immunology, 2003. **33**(1): p. 204-14.
77. Fos, C., et al., *ICOS ligation recruits the p50alpha PI3K regulatory subunit to the immunological synapse.* Journal of immunology, 2008. **181**(3): p. 1969-77.
78. Fruman, D.A., *Phosphoinositide 3-kinase and its targets in B-cell and T-cell signaling.* Current opinion in immunology, 2004. **16**(3): p. 314-20.
79. Parry, R.V., et al., *CD28 and inducible costimulatory protein Src homology 2 binding domains show distinct regulation of phosphatidylinositol 3-kinase, Bcl-xL, and IL-2 expression in primary human CD4 T lymphocytes.* Journal of immunology, 2003. **171**(1): p. 166-74.
80. Gigoux, M., et al., *Inducible costimulator promotes helper T-cell differentiation through phosphoinositide 3-kinase.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(48): p. 20371-6.
81. Kopf, M., et al., *Inducible costimulator protein (ICOS) controls T helper cell subset polarization after virus and parasite infection.* The Journal of experimental medicine, 2000. **192**(1): p. 53-61.
82. Dong, C., et al., *ICOS co-stimulatory receptor is essential for T-cell activation and function.* Nature, 2001. **409**(6816): p. 97-101.

83. Burmeister, Y., et al., *ICOS controls the pool size of effector-memory and regulatory T cells.* Journal of immunology, 2008. **180**(2): p. 774-82.
84. Guo, F., et al., *CD28 controls differentiation of regulatory T cells from naive CD4 T cells.* Journal of immunology, 2008. **181**(4): p. 2285-91.
85. Ito, T., et al., *Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery.* Immunity, 2008. **28**(6): p. 870-80.
86. Agata, Y., et al., *Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes.* International immunology, 1996. **8**(5): p. 765-72.
87. Chemnitz, J.M., et al., *SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation.* Journal of immunology, 2004. **173**(2): p. 945-54.
88. Freeman, G.J., et al., *Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation.* The Journal of experimental medicine, 2000. **192**(7): p. 1027-34.
89. Nishimura, H., et al., *Developmentally regulated expression of the PD-1 protein on the surface of double-negative (CD4-CD8-) thymocytes.* International immunology, 1996. **8**(5): p. 773-80.
90. Vibhakar, R., et al., *Activation-induced expression of human programmed death-1 gene in T-lymphocytes.* Experimental cell research, 1997. **232**(1): p. 25-8.
91. Dong, H., et al., *B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion.* Nature medicine, 1999. **5**(12): p. 1365-9.
92. Ishida, M., et al., *Differential expression of PD-L1 and PD-L2, ligands for an inhibitory receptor PD-1, in the cells of lymphohematopoietic tissues.* Immunology letters, 2002. **84**(1): p. 57-62.
93. Latchman, Y., et al., *PD-L2 is a second ligand for PD-1 and inhibits T cell activation.* Nature immunology, 2001. **2**(3): p. 261-8.
94. Brown, J.A., et al., *Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production.* Journal of immunology, 2003. **170**(3): p. 1257-66.
95. Yamazaki, T., et al., *Expression of programmed death 1 ligands by murine T cells and APC.* Journal of immunology, 2002. **169**(10): p. 5538-45.
96. Ansari, M.J., et al., *The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice.* The Journal of experimental medicine, 2003. **198**(1): p. 63-9.
97. Loke, P. and J.P. Allison, *PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(9): p. 5336-41.
98. Riley, J.L., *PD-1 signaling in primary T cells.* Immunological reviews, 2009. **229**(1): p. 114-25.
99. Okazaki, T., et al., *PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(24): p. 13866-71.
100. Sathish, J.G., et al., *Constitutive association of SHP-1 with leukocyte-associated Ig-like receptor-1 in human T cells.* Journal of immunology, 2001. **166**(3): p. 1763-70.
101. Blair, P.J., et al., *CTLA-4 ligation delivers a unique signal to resting human CD4 T cells that inhibits interleukin-2 secretion but allows Bcl-X(L) induction.* Journal of immunology, 1998. **160**(1): p. 12-5.
102. Parry, R.V., et al., *CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms.* Molecular and cellular biology, 2005. **25**(21): p. 9543-53.

103. Quigley, M., et al., *Transcriptional analysis of HIV-specific CD8+ T cells shows that PD-1 inhibits T cell function by upregulating BATF.* Nature medicine, 2010. **16**(10): p. 1147-51.
104. Schraml, B.U., et al., *The AP-1 transcription factor Batf controls T(H)17 differentiation.* Nature, 2009. **460**(7253): p. 405-9.
105. Williams, K.L., et al., *Characterization of murine BATF: a negative regulator of activator protein-1 activity in the thymus.* European journal of immunology, 2001. **31**(5): p. 1620-7.
106. Laufer, T.M., et al., *Unopposed positive selection and autoreactivity in mice expressing class II MHC only on thymic cortex.* Nature, 1996. **383**(6595): p. 81-5.
107. Laufer, T.M., L. Fan, and L.H. Glimcher, *Self-reactive T cells selected on thymic cortical epithelium are polyclonal and are pathogenic in vivo.* J Immunol, 1999. **162**(9): p. 5078-84.
108. Heino, M., et al., *Autoimmune regulator is expressed in the cells regulating immune tolerance in thymus medulla.* Biochem Biophys Res Commun, 1999. **257**(3): p. 821-5.
109. Zuklys, S., et al., *Normal thymic architecture and negative selection are associated with Aire expression, the gene defective in the autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED).* J Immunol, 2000. **165**(4): p. 1976-83.
110. *An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains.* Nat Genet, 1997. **17**(4): p. 399-403.
111. Anderson, M.S., et al., *Projection of an immunological self shadow within the thymus by the aire protein.* Science, 2002. **298**(5597): p. 1395-401.
112. Ramsey, C., et al., *Aire deficient mice develop multiple features of APECED phenotype and show altered immune response.* Hum Mol Genet, 2002. **11**(4): p. 397-409.
113. Ruan, Q.G., et al., *The autoimmune regulator directly controls the expression of genes critical for thymic epithelial function.* J Immunol, 2007. **178**(11): p. 7173-80.
114. Gallegos, A.M. and M.J. Bevan, *Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation.* J Exp Med, 2004. **200**(8): p. 1039-49.
115. Mazda, O., et al., *Requirement of dendritic cells and B cells in the clonal deletion of Mls-reactive T cells in the thymus.* J Exp Med, 1991. **173**(3): p. 539-47.
116. Aschenbrenner, K., et al., *Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells.* Nat Immunol, 2007. **8**(4): p. 351-8.
117. Klein, L., B. Roettinger, and B. Kyewski, *Sampling of complementing self-antigen pools by thymic stromal cells maximizes the scope of central T cell tolerance.* Eur J Immunol, 2001. **31**(8): p. 2476-86.
118. Koble, C. and B. Kyewski, *The thymic medulla: a unique microenvironment for intercellular self-antigen transfer.* J Exp Med, 2009. **206**(7): p. 1505-13.
119. Mizushima, N. and D.J. Klionsky, *Protein turnover via autophagy: implications for metabolism.* Annu Rev Nutr, 2007. **27**: p. 19-40.
120. Nedjic, J., et al., *Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance.* Nature, 2008. **455**(7211): p. 396-400.
121. Donskoy, E. and I. Goldschneider, *Two developmentally distinct populations of dendritic cells inhabit the adult mouse thymus: demonstration by differential importation of hematogenous precursors under steady state conditions.* J Immunol, 2003. **170**(7): p. 3514-21.
122. Li, J., et al., *Thymus-homing peripheral dendritic cells constitute two of the three major subsets of dendritic cells in the steady-state thymus.* J Exp Med, 2009. **206**(3): p. 607-22.
123. Proietto, A.I., M.H. Lahoud, and L. Wu, *Distinct functional capacities of mouse thymic and splenic dendritic cell populations.* Immunol Cell Biol, 2008. **86**(8): p. 700-8.

124. Bonasio, R., et al., *Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus.* Nat Immunol, 2006. **7**(10): p. 1092-100.
125. Proietto, A.I., et al., *Dendritic cells in the thymus contribute to T-regulatory cell induction.* Proc Natl Acad Sci U S A, 2008. **105**(50): p. 19869-74.
126. Albert, M.L., B. Sauter, and N. Bhardwaj, *Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs.* Nature, 1998. **392**(6671): p. 86-9.
127. Barlow, A.K., X. He, and C. Janeway, Jr., *Exogenously provided peptides of a self-antigen can be processed into forms that are recognized by self-T cells.* J Exp Med, 1998. **187**(9): p. 1403-15.
128. Humblet, C., A. Rudensky, and B. Kyewski, *Presentation and intercellular transfer of self antigen within the thymic microenvironment: expression of the E alpha peptide-I-Ab complex by isolated thymic stromal cells.* Int Immunol, 1994. **6**(12): p. 1949-58.
129. Gabler, J., J. Arnold, and B. Kyewski, *Promiscuous gene expression and the developmental dynamics of medullary thymic epithelial cells.* Eur J Immunol, 2007. **37**(12): p. 3363-72.
130. Gray, D., et al., *Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire.* J Exp Med, 2007. **204**(11): p. 2521-8.
131. Harshyne, L.A., et al., *Dendritic cells acquire antigens from live cells for cross-presentation to CTL.* J Immunol, 2001. **166**(6): p. 3717-23.
132. Harshyne, L.A., et al., *A role for class A scavenger receptor in dendritic cell nibbling from live cells.* J Immunol, 2003. **170**(5): p. 2302-9.
133. Bouneaud, C., P. Kourilsky, and P. Bousso, *Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion.* Immunity, 2000. **13**(6): p. 829-40.
134. Fowell, D. and D. Mason, *Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the CD4+ T cell subset that inhibits this autoimmune potential.* J Exp Med, 1993. **177**(3): p. 627-36.
135. Liblau, R., et al., *T cell response to myelin basic protein epitopes in multiple sclerosis patients and healthy subjects.* Eur J Immunol, 1991. **21**(6): p. 1391-5.
136. Schluesener, H.J. and H. Wekerle, *Autoaggressive T lymphocyte lines recognizing the encephalitogenic region of myelin basic protein: in vitro selection from unprimed rat T lymphocyte populations.* J Immunol, 1985. **135**(5): p. 3128-33.
137. Sun, J.B., et al., *Autoreactive T and B cells responding to myelin proteolipid protein in multiple sclerosis and controls.* Eur J Immunol, 1991. **21**(6): p. 1461-8.
138. Wucherpfennig, K.W., et al., *Clonal expansion and persistence of human T cells specific for an immunodominant myelin basic protein peptide.* J Immunol, 1994. **152**(11): p. 5581-92.
139. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.* J Immunol, 1995. **155**(3): p. 1151-64.
140. Sakaguchi, S., *Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self.* Nature immunology, 2005. **6**(4): p. 345-52.
141. Singh, B., et al., *Control of intestinal inflammation by regulatory T cells.* Immunological reviews, 2001. **182**: p. 190-200.
142. Danke, N.A., et al., *Autoreactive T cells in healthy individuals.* Journal of immunology, 2004. **172**(10): p. 5967-72.
143. Gnjatic, S., et al., *NY-ESO-1 DNA vaccine induces T-cell responses that are suppressed by regulatory T cells.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2009. **15**(6): p. 2130-9.

144. Taams, L.S., et al., *Antigen-specific T cell suppression by human CD4+CD25+ regulatory T cells.* European journal of immunology, 2002. **32**(6): p. 1621-30.
145. Wing, K., et al., *CD4 T cell activation by myelin oligodendrocyte glycoprotein is suppressed by adult but not cord blood CD25+ T cells.* European journal of immunology, 2003. **33**(3): p. 579-87.
146. Baxter, A.G., *The origin and application of experimental autoimmune encephalomyelitis.* Nature reviews. Immunology, 2007. **7**(11): p. 904-12.
147. Kohm, A.P., et al., *Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis.* Journal of immunology, 2002. **169**(9): p. 4712-6.
148. Morris, G.P., et al., *H2A- and H2E-derived CD4+CD25+ regulatory T cells: a potential role in reciprocal inhibition by class II genes in autoimmune thyroiditis.* Journal of immunology, 2005. **174**(5): p. 3111-6.
149. Marrack, P., et al., *Homeostasis of alpha beta TCR+ T cells.* Nature immunology, 2000. **1**(2): p. 107-11.
150. Xing, Y. and K.A. Hogquist, *T-cell tolerance: central and peripheral.* Cold Spring Harbor perspectives in biology, 2012. **4**(6).
151. Fisher, G.H., et al., *Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome.* Cell, 1995. **81**(6): p. 935-46.
152. Nagata, S. and T. Suda, *Fas and Fas ligand: lpr and gld mutations.* Immunology today, 1995. **16**(1): p. 39-43.
153. Rieux-Laucat, F., et al., *Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity.* Science, 1995. **268**(5215): p. 1347-9.
154. Wells, A.D., et al., *The role of peripheral T-cell deletion in transplantation tolerance.* Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 2001. **356**(1409): p. 617-23.
155. Bouillet, P., et al., *Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity.* Science, 1999. **286**(5445): p. 1735-8.
156. Hildeman, D.A., et al., *Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim.* Immunity, 2002. **16**(6): p. 759-67.
157. Pellegrini, M., et al., *Shutdown of an acute T cell immune response to viral infection is mediated by the proapoptotic Bcl-2 homology 3-only protein Bim.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(24): p. 14175-80.
158. Lombardi, G., et al., *Anergic T cells as suppressor cells in vitro.* Science, 1994. **264**(5165): p. 1587-9.
159. Schwartz, R.H., *T cell anergy.* Annu Rev Immunol, 2003. **21**: p. 305-34.
160. Beverly, B., et al., *Reversal of in vitro T cell clonal anergy by IL-2 stimulation.* Int Immunol, 1992. **4**(6): p. 661-71.
161. Jenkins, M.K., *The role of cell division in the induction of clonal anergy.* Immunol Today, 1992. **13**(2): p. 69-73.
162. Groux, H., et al., *A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis.* Nature, 1997. **389**(6652): p. 737-42.
163. Barrat, F.J., et al., *In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines.* J Exp Med, 2002. **195**(5): p. 603-16.
164. Cottrez, F., et al., *T regulatory cells 1 inhibit a Th2-specific response in vivo.* J Immunol, 2000. **165**(9): p. 4848-53.

165. Faria, A.M. and H.L. Weiner, *Oral tolerance: mechanisms and therapeutic applications.* Adv Immunol, 1999. **73**: p. 153-264.
166. Knoechel, B., et al., *Functional and molecular comparison of anergic and regulatory T lymphocytes.* Journal of immunology, 2006. **176**(11): p. 6473-83.
167. Lechner, O., et al., *Fingerprints of anergic T cells.* Current biology : CB, 2001. **11**(8): p. 587-95.
168. Macian, F., C. Garcia-Rodriguez, and A. Rao, *Gene expression elicited by NFAT in the presence or absence of cooperative recruitment of Fos and Jun.* The EMBO journal, 2000. **19**(17): p. 4783-95.
169. Bandyopadhyay, S., N. Soto-Nieves, and F. Macian, *Transcriptional regulation of T cell tolerance.* Seminars in immunology, 2007. **19**(3): p. 180-7.
170. Heissmeyer, V., et al., *Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins.* Nature immunology, 2004. **5**(3): p. 255-65.
171. DeSilva, D.R., et al., *Anergic T cells are defective in both jun NH2-terminal kinase and mitogen-activated protein kinase signaling pathways.* The Journal of experimental medicine, 1996. **183**(5): p. 2017-23.
172. Fields, P.E., T.F. Gajewski, and F.W. Fitch, *Blocked Ras activation in anergic CD4+ T cells.* Science, 1996. **271**(5253): p. 1276-8.
173. Li, W., et al., *Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4+ T cells.* Science, 1996. **271**(5253): p. 1272-6.
174. Ohnmacht, C., et al., *Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity.* The Journal of experimental medicine, 2009. **206**(3): p. 549-59.
175. Bonifaz, L., et al., *Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance.* The Journal of experimental medicine, 2002. **196**(12): p. 1627-38.
176. Hawiger, D., et al., *Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo.* The Journal of experimental medicine, 2001. **194**(6): p. 769-79.
177. Liu, K., et al., *Immune tolerance after delivery of dying cells to dendritic cells in situ.* The Journal of experimental medicine, 2002. **196**(8): p. 1091-7.
178. Dhodapkar, M.V., et al., *Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells.* The Journal of experimental medicine, 2001. **193**(2): p. 233-8.
179. Inaba, K., et al., *Immunologic properties of purified epidermal Langerhans cells. Distinct requirements for stimulation of unprimed and sensitized T lymphocytes.* The Journal of experimental medicine, 1986. **164**(2): p. 605-13.
180. O'Doherty, U., et al., *Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium.* The Journal of experimental medicine, 1993. **178**(3): p. 1067-76.
181. Schuler, G. and R.M. Steinman, *Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro.* The Journal of experimental medicine, 1985. **161**(3): p. 526-46.
182. Witmer-Pack, M.D., et al., *Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells.* The Journal of experimental medicine, 1987. **166**(5): p. 1484-98.
183. Gallucci, S., M. Lolkema, and P. Matzinger, *Natural adjuvants: endogenous activators of dendritic cells.* Nature medicine, 1999. **5**(11): p. 1249-55.

184. Menges, M., et al., *Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity.* The Journal of experimental medicine, 2002. **195**(1): p. 15-21.
185. Huang, F.P., et al., *A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes.* The Journal of experimental medicine, 2000. **191**(3): p. 435-44.
186. Scheinecker, C., et al., *Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node.* The Journal of experimental medicine, 2002. **196**(8): p. 1079-90.
187. Probst, H.C., et al., *Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4.* Nature immunology, 2005. **6**(3): p. 280-6.
188. Schildknecht, A., et al., *FoxP3+ regulatory T cells essentially contribute to peripheral CD8+ T-cell tolerance induced by steady-state dendritic cells.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(1): p. 199-203.
189. !!! INVALID CITATION !!!
190. Gavin, M.A., et al., *Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development.* Proc Natl Acad Sci U S A, 2006. **103**(17): p. 6659-64.
191. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.* Nat Immunol, 2003. **4**(4): p. 330-6.
192. Hori, S., T. Nomura, and S. Sakaguchi, *Control of regulatory T cell development by the transcription factor Foxp3.* Science, 2003. **299**(5609): p. 1057-61.
193. Brunkow, M.E., et al., *Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse.* Nat Genet, 2001. **27**(1): p. 68-73.
194. Chatila, T.A., et al., *JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome.* J Clin Invest, 2000. **106**(12): p. R75-81.
195. Lee, H.M. and C.S. Hsieh, *Rare development of Foxp3+ thymocytes in the CD4+CD8+ subset.* J Immunol, 2009. **183**(4): p. 2261-6.
196. Bautista, J.L., et al., *Intraclonal competition limits the fate determination of regulatory T cells in the thymus.* Nat Immunol, 2009. **10**(6): p. 610-7.
197. Jordan, M.S., et al., *Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide.* Nat Immunol, 2001. **2**(4): p. 301-6.
198. Kawahata, K., et al., *Generation of CD4(+)CD25(+) regulatory T cells from autoreactive T cells simultaneously with their negative selection in the thymus and from nonautoreactive T cells by endogenous TCR expression.* J Immunol, 2002. **168**(9): p. 4399-405.
199. Lerman, M.A., et al., *CD4+ CD25+ regulatory T cell repertoire formation in response to varying expression of a neo-self-antigen.* J Immunol, 2004. **173**(1): p. 236-44.
200. Leung, M.W., S. Shen, and J.J. Lafaille, *TCR-dependent differentiation of thymic Foxp3+ cells is limited to small clonal sizes.* J Exp Med, 2009. **206**(10): p. 2121-30.
201. Picca, C.C., et al., *Thymocyte deletion can bias Treg formation toward low-abundance self-peptide.* Eur J Immunol, 2009. **39**(12): p. 3301-6.
202. Lio, C.W., et al., *CD28 facilitates the generation of Foxp3(-) cytokine responsive regulatory T cell precursors.* J Immunol, 2010. **184**(11): p. 6007-13.
203. Tai, X., et al., *CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2.* Nat Immunol, 2005. **6**(2): p. 152-62.

204. Long, M., et al., *Nuclear factor-kappaB modulates regulatory T cell development by directly regulating expression of Foxp3 transcription factor.* Immunity, 2009. **31**(6): p. 921-31.
205. Ruan, Q., et al., *Development of Foxp3(+) regulatory t cells is driven by the c-Rel enhanceosome.* Immunity, 2009. **31**(6): p. 932-40.
206. Zheng, Y., et al., *Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate.* Nature, 2010. **463**(7282): p. 808-12.
207. Malek, T.R., et al., *IL-2 family of cytokines in T regulatory cell development and homeostasis.* J Clin Immunol, 2008. **28**(6): p. 635-9.
208. Burchill, M.A., et al., *Linked T cell receptor and cytokine signaling govern the development of the regulatory T cell repertoire.* Immunity, 2008. **28**(1): p. 112-21.
209. Lio, C.W. and C.S. Hsieh, *A two-step process for thymic regulatory T cell development.* Immunity, 2008. **28**(1): p. 100-11.
210. Lu, L.F. and A. Rudensky, *Molecular orchestration of differentiation and function of regulatory T cells.* Genes & development, 2009. **23**(11): p. 1270-82.
211. Davidson, T.S., et al., *Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells.* J Immunol, 2007. **178**(7): p. 4022-6.
212. Selvaraj, R.K. and T.L. Geiger, *A kinetic and dynamic analysis of Foxp3 induced in T cells by TGF-beta.* J Immunol, 2007. **179**(2): p. 11 p following 1390.
213. Zheng, S.G., et al., *Generation ex vivo of TGF-beta-producing regulatory T cells from CD4+CD25- precursors.* J Immunol, 2002. **169**(8): p. 4183-9.
214. Awasthi, A., et al., *A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells.* Nature immunology, 2007. **8**(12): p. 1380-9.
215. Fitzgerald, D.C., et al., *Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells.* Nature immunology, 2007. **8**(12): p. 1372-9.
216. Ochando, J.C., et al., *Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts.* Nature immunology, 2006. **7**(6): p. 652-62.
217. Stumhofer, J.S., et al., *Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10.* Nature immunology, 2007. **8**(12): p. 1363-71.
218. Apetoh, L., et al., *The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27.* Nature immunology, 2010. **11**(9): p. 854-61.
219. Pot, C., et al., *Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells.* Journal of immunology, 2009. **183**(2): p. 797-801.
220. Kim, H.P. and W.J. Leonard, *CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation.* The Journal of experimental medicine, 2007. **204**(7): p. 1543-51.
221. Maruyama, T., et al., *Control of the differentiation of regulatory T cells and T(H)17 cells by the DNA-binding inhibitor Id3.* Nature immunology, 2011. **12**(1): p. 86-95.
222. Tone, Y., et al., *Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer.* Nature immunology, 2008. **9**(2): p. 194-202.
223. Gottschalk, R.A., E. Corse, and J.P. Allison, *TCR ligand density and affinity determine peripheral induction of Foxp3 in vivo.* The Journal of experimental medicine, 2010. **207**(8): p. 1701-11.
224. Oliveira, V.G., et al., *Sub-optimal CD4+ T-cell activation triggers autonomous TGF-beta-dependent conversion to Foxp3+ regulatory T cells.* European journal of immunology, 2011. **41**(5): p. 1249-55.

225. Wing, K. and S. Sakaguchi, *Regulatory T cells exert checks and balances on self tolerance and autoimmunity.* Nature immunology, 2010. **11**(1): p. 7-13.
226. Miyara, M., et al., *Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor.* Immunity, 2009. **30**(6): p. 899-911.
227. Read, S., V. Malmstrom, and F. Powrie, *Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation.* J Exp Med, 2000. **192**(2): p. 295-302.
228. Salomon, B., et al., *B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes.* Immunity, 2000. **12**(4): p. 431-40.
229. Takahashi, T., et al., *Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4.* J Exp Med, 2000. **192**(2): p. 303-10.
230. Wing, K., et al., *CTLA-4 control over Foxp3+ regulatory T cell function.* Science, 2008. **322**(5899): p. 271-5.
231. Grohmann, U., et al., *CTLA-4-Ig regulates tryptophan catabolism in vivo.* Nat Immunol, 2002. **3**(11): p. 1097-101.
232. Lenardo, M., et al., *Mature T lymphocyte apoptosis--immune regulation in a dynamic and unpredictable antigenic environment.* Annual review of immunology, 1999. **17**: p. 221-53.
233. Miyazaki, T., et al., *Three distinct IL-2 signaling pathways mediated by bcl-2, c-myc, and lck cooperate in hematopoietic cell proliferation.* Cell, 1995. **81**(2): p. 223-31.
234. Schorle, H., et al., *Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting.* Nature, 1991. **352**(6336): p. 621-4.
235. Suzuki, H., et al., *Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta.* Science, 1995. **268**(5216): p. 1472-6.
236. Willerford, D.M., et al., *Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment.* Immunity, 1995. **3**(4): p. 521-30.
237. Takahashi, T., et al., *Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state.* Int Immunol, 1998. **10**(12): p. 1969-80.
238. Thornton, A.M. and E.M. Shevach, *CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production.* J Exp Med, 1998. **188**(2): p. 287-96.
239. Pandiyan, P., et al., *CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells.* Nat Immunol, 2007. **8**(12): p. 1353-62.
240. Furtado, G.C., et al., *Interleukin 2 signaling is required for CD4(+) regulatory T cell function.* J Exp Med, 2002. **196**(6): p. 851-7.
241. Setoguchi, R., et al., *Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization.* J Exp Med, 2005. **201**(5): p. 723-35.
242. Li, M.O., et al., *Transforming growth factor-beta regulation of immune responses.* Annu Rev Immunol, 2006. **24**: p. 99-146.
243. Choi, M.E., et al., *Rat mesangial cell hypertrophy in response to transforming growth factor-beta 1.* Kidney Int, 1993. **44**(5): p. 948-58.
244. Marie, J.C., et al., *TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells.* J Exp Med, 2005. **201**(7): p. 1061-7.

245. Kulkarni, A.B., et al., *Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death.* Proc Natl Acad Sci U S A, 1993. **90**(2): p. 770-4.
246. Levings, M.K., et al., *Human CD25+CD4+ T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct from type 1 T regulatory cells.* J Exp Med, 2002. **196**(10): p. 1335-46.
247. Nakamura, K., et al., *TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice.* J Immunol, 2004. **172**(2): p. 834-42.
248. Nakamura, K., A. Kitani, and W. Strober, *Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta.* J Exp Med, 2001. **194**(5): p. 629-44.
249. Shull, M.M., et al., *Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease.* Nature, 1992. **359**(6397): p. 693-9.
250. Brabletz, T., et al., *Transforming growth factor beta and cyclosporin A inhibit the inducible activity of the interleukin-2 gene in T cells through a noncanonical octamer-binding site.* Mol Cell Biol, 1993. **13**(2): p. 1155-62.
251. Kehrl, J.H., et al., *Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth.* J Exp Med, 1986. **163**(5): p. 1037-50.
252. Genestier, L., et al., *Transforming growth factor beta1 inhibits Fas ligand expression and subsequent activation-induced cell death in T cells via downregulation of c-Myc.* J Exp Med, 1999. **189**(2): p. 231-9.
253. Nelson, B.H., et al., *Uncoupling of promitogenic and antiapoptotic functions of IL-2 by Smad-dependent TGF-beta signaling.* J Immunol, 2003. **170**(11): p. 5563-70.
254. Ruegemer, J.J., et al., *Regulatory effects of transforming growth factor-beta on IL-2- and IL-4-dependent T cell-cycle progression.* J Immunol, 1990. **144**(5): p. 1767-76.
255. Wolfraim, L.A., et al., *p21Cip1 and p27Kip1 act in synergy to alter the sensitivity of naive T cells to TGF-beta-mediated G1 arrest through modulation of IL-2 responsiveness.* J Immunol, 2004. **173**(5): p. 3093-102.
256. Cottrez, F. and H. Groux, *Regulation of TGF-beta response during T cell activation is modulated by IL-10.* J Immunol, 2001. **167**(2): p. 773-8.
257. Gunnlaugsdottir, B., S.M. Maggadottir, and B.R. Ludviksson, *Anti-CD28-induced co-stimulation and TCR avidity regulates the differential effect of TGF-beta1 on CD4+ and CD8+ naive human T-cells.* Int Immunol, 2005. **17**(1): p. 35-44.
258. Sung, J.L., J.T. Lin, and J.D. Gorham, *CD28 co-stimulation regulates the effect of transforming growth factor-beta1 on the proliferation of naive CD4+ T cells.* Int Immunopharmacol, 2003. **3**(2): p. 233-45.
259. Kuhn, R., et al., *Interleukin-10-deficient mice develop chronic enterocolitis.* Cell, 1993. **75**(2): p. 263-74.
260. Murai, M., et al., *Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis.* Nat Immunol, 2009. **10**(11): p. 1178-84.
261. de Waal Malefyt, R., et al., *Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes.* J Exp Med, 1991. **174**(5): p. 1209-20.
262. Fiorentino, D.F., et al., *IL-10 inhibits cytokine production by activated macrophages.* J Immunol, 1991. **147**(11): p. 3815-22.

263. Jenkins, J.K., M. Malyak, and W.P. Arend, *The effects of interleukin-10 on interleukin-1 receptor antagonist and interleukin-1 beta production in human monocytes and neutrophils.* Lymphokine Cytokine Res, 1994. **13**(1): p. 47-54.
264. D'Andrea, A., et al., *Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells.* J Exp Med, 1993. **178**(3): p. 1041-8.
265. Creery, W.D., et al., *Differential modulation of B7-1 and B7-2 isoform expression on human monocytes by cytokines which influence the development of T helper cell phenotype.* Eur J Immunol, 1996. **26**(6): p. 1273-7.
266. de Waal Malefyt, R., et al., *Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression.* J Exp Med, 1991. **174**(4): p. 915-24.
267. Willems, F., et al., *Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes.* Eur J Immunol, 1994. **24**(4): p. 1007-9.
268. Del Prete, G., et al., *Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production.* J Immunol, 1993. **150**(2): p. 353-60.
269. Groux, H., et al., *Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells.* J Exp Med, 1996. **184**(1): p. 19-29.
270. El Kasmi, K.C., et al., *General nature of the STAT3-activated anti-inflammatory response.* J Immunol, 2006. **177**(11): p. 7880-8.
271. Takeda, K., et al., *Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils.* Immunity, 1999. **10**(1): p. 39-49.
272. Williams, L., et al., *Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages.* J Immunol, 2004. **172**(1): p. 567-76.
273. Cao, X., et al., *Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance.* Immunity, 2007. **27**(4): p. 635-46.
274. Gondek, D.C., et al., *Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism.* Journal of immunology, 2005. **174**(4): p. 1783-6.
275. Garin, M.I., et al., *Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells.* Blood, 2007. **109**(5): p. 2058-65.
276. Kubach, J., et al., *Human CD4+CD25+ regulatory T cells: proteome analysis identifies galectin-10 as a novel marker essential for their anergy and suppressive function.* Blood, 2007. **110**(5): p. 1550-8.
277. Collison, L.W., et al., *The inhibitory cytokine IL-35 contributes to regulatory T-cell function.* Nature, 2007. **450**(7169): p. 566-9.
278. Tivol, E.A., et al., *Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4.* Immunity, 1995. **3**(5): p. 541-7.
279. Waterhouse, P., et al., *Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4.* Science, 1995. **270**(5238): p. 985-8.
280. Walunas, T.L., et al., *CTLA-4 can function as a negative regulator of T cell activation.* Immunity, 1994. **1**(5): p. 405-13.
281. Linsley, P.S., et al., *CTLA-4 is a second receptor for the B cell activation antigen B7.* J Exp Med, 1991. **174**(3): p. 561-9.

282. Waterhouse, P., et al., *Normal thymic selection, normal viability and decreased lymphoproliferation in T cell receptor-transgenic CTLA-4-deficient mice.* Eur J Immunol, 1997. **27**(8): p. 1887-92.
283. Teft, W.A., M.G. Kirchhof, and J. Madrenas, *A molecular perspective of CTLA-4 function.* Annu Rev Immunol, 2006. **24**: p. 65-97.
284. Linsley, P.S., et al., *Binding stoichiometry of the cytotoxic T lymphocyte-associated molecule-4 (CTLA-4). A disulfide-linked homodimer binds two CD86 molecules.* J Biol Chem, 1995. **270**(25): p. 15417-24.
285. Darlington, P.J., et al., *Hierarchical regulation of CTLA-4 dimer-based lattice formation and its biological relevance for T cell inactivation.* J Immunol, 2005. **175**(2): p. 996-1004.
286. Chun, T., H.J. Choi, and Y.H. Chung, *Two different forms of human CTLA-4 proteins following peripheral T cell activation.* Immunology letters, 2004. **91**(2-3): p. 213-20.
287. Balzano, C., et al., *CTLA-4 and CD28: similar proteins, neighbouring genes.* Int J Cancer Suppl, 1992. **7**: p. 28-32.
288. Darlington, P.J., et al., *Surface cytotoxic T lymphocyte-associated antigen 4 partitions within lipid rafts and relocates to the immunological synapse under conditions of inhibition of T cell activation.* J Exp Med, 2002. **195**(10): p. 1337-47.
289. Alegre, M.L., et al., *Regulation of surface and intracellular expression of CTLA4 on mouse T cells.* J Immunol, 1996. **157**(11): p. 4762-70.
290. Wang, X.B., et al., *Regulation of surface and intracellular expression of CTLA-4 on human peripheral T cells.* Scand J Immunol, 2001. **54**(5): p. 453-8.
291. Zhang, Y. and J.P. Allison, *Interaction of CTLA-4 with AP50, a clathrin-coated pit adaptor protein.* Proc Natl Acad Sci U S A, 1997. **94**(17): p. 9273-8.
292. Chuang, E., et al., *Interaction of CTLA-4 with the clathrin-associated protein AP50 results in ligand-independent endocytosis that limits cell surface expression.* J Immunol, 1997. **159**(1): p. 144-51.
293. Bradshaw, J.D., et al., *Interaction of the cytoplasmic tail of CTLA-4 (CD152) with a clathrin-associated protein is negatively regulated by tyrosine phosphorylation.* Biochemistry, 1997. **36**(50): p. 15975-82.
294. Shiratori, T., et al., *Tyrosine phosphorylation controls internalization of CTLA-4 by regulating its interaction with clathrin-associated adaptor complex AP-2.* Immunity, 1997. **6**(5): p. 583-9.
295. Ikemizu, S., et al., *Structure and dimerization of a soluble form of B7-1.* Immunity, 2000. **12**(1): p. 51-60.
296. Zhang, X., et al., *Crystal structure of the receptor-binding domain of human B7-2: insights into organization and signaling.* Proc Natl Acad Sci U S A, 2003. **100**(5): p. 2586-91.
297. Linsley, P.S., et al., *Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors.* Immunity, 1994. **1**(9): p. 793-801.
298. Collins, A.V., et al., *The interaction properties of costimulatory molecules revisited.* Immunity, 2002. **17**(2): p. 201-10.
299. Lenschow, D.J., et al., *Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse.* The Journal of experimental medicine, 1995. **181**(3): p. 1145-55.
300. Carreno, B.M., et al., *CTLA-4 (CD152) can inhibit T cell activation by two different mechanisms depending on its level of cell surface expression.* J Immunol, 2000. **165**(3): p. 1352-6.
301. Greene, J.L., et al., *Covalent dimerization of CD28/CTLA-4 and oligomerization of CD80/CD86 regulate T cell costimulatory interactions.* J Biol Chem, 1996. **271**(43): p. 26762-71.

302. Leung, H.T., et al., *Cytotoxic T lymphocyte-associated molecule-4, a high-avidity receptor for CD80 and CD86, contains an intracellular localization motif in its cytoplasmic tail.* J Biol Chem, 1995. **270**(42): p. 25107-14.
303. Linsley, P.S., et al., *Intracellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement.* Immunity, 1996. **4**(6): p. 535-43.
304. Masteller, E.L., et al., *Structural analysis of CTLA-4 function in vivo.* J Immunol, 2000. **164**(10): p. 5319-27.
305. Krummel, M.F. and J.P. Allison, *CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation.* J Exp Med, 1995. **182**(2): p. 459-65.
306. Marengere, L.E., et al., *Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4.* Science, 1996. **272**(5265): p. 1170-3.
307. Chuang, E., et al., *Regulation of cytotoxic T lymphocyte-associated molecule-4 by Src kinases.* J Immunol, 1999. **162**(3): p. 1270-7.
308. Miyatake, S., et al., *Src family tyrosine kinases associate with and phosphorylate CTLA-4 (CD152).* Biochem Biophys Res Commun, 1998. **249**(2): p. 444-8.
309. Yi, L.A., S. Hajialiasgar, and E. Chuang, *Tyrosine-mediated inhibitory signals contribute to CTLA-4 function in vivo.* Int Immunol, 2004. **16**(4): p. 539-47.
310. Baroja, M.L., et al., *The inhibitory function of CTLA-4 does not require its tyrosine phosphorylation.* J Immunol, 2000. **164**(1): p. 49-55.
311. Schneider, H., et al., *CTLA-4 binding to the lipid kinase phosphatidylinositol 3-kinase in T cells.* J Exp Med, 1995. **181**(1): p. 351-5.
312. Schneider, H., et al., *CTLA-4 activation of phosphatidylinositol 3-kinase (PI 3-K) and protein kinase B (PKB/AKT) sustains T-cell anergy without cell death.* PLoS One, 2008. **3**(12): p. e3842.
313. Baroja, M.L., et al., *Inhibition of CTLA-4 function by the regulatory subunit of serine/threonine phosphatase 2A.* J Immunol, 2002. **168**(10): p. 5070-8.
314. Chuang, E., et al., *The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A.* Immunity, 2000. **13**(3): p. 313-22.
315. Bachmann, M.F., et al., *Cutting edge: lymphoproliferative disease in the absence of CTLA-4 is not T cell autonomous.* J Immunol, 1999. **163**(3): p. 1128-31.
316. Homann, D., et al., *Lack of intrinsic CTLA-4 expression has minimal effect on regulation of antiviral T-cell immunity.* Journal of virology, 2006. **80**(1): p. 270-80.
317. Zheng, Y., et al., *Acquisition of suppressive function by activated human CD4+ CD25- T cells is associated with the expression of CTLA-4 not FoxP3.* J Immunol, 2008. **181**(3): p. 1683-91.
318. Friedline, R.H., et al., *CD4+ regulatory T cells require CTLA-4 for the maintenance of systemic tolerance.* J Exp Med, 2009. **206**(2): p. 421-34.
319. Schmidt, E.M., et al., *Ctla-4 controls regulatory T cell peripheral homeostasis and is required for suppression of pancreatic islet autoimmunity.* J Immunol, 2009. **182**(1): p. 274-82.
320. Corse, E. and J.P. Allison, *Cutting edge: ctla-4 on effector T cells inhibits in trans.* Journal of immunology, 2012. **189**(3): p. 1123-7.
321. Wang, C.J., et al., *Cutting edge: cell-extrinsic immune regulation by ctla-4 expressed on conventional T cells.* Journal of immunology, 2012. **189**(3): p. 1118-22.
322. Tang, Q., et al., *Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function.* Eur J Immunol, 2004. **34**(11): p. 2996-3005.
323. Kingsley, C.I., et al., *CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses.* J Immunol, 2002. **168**(3): p. 1080-6.
324. Loser, K., et al., *An important role of CD80/CD86-CTLA-4 signaling during photocarcinogenesis in mice.* J Immunol, 2005. **174**(9): p. 5298-305.

325. Manzotti, C.N., et al., *Inhibition of human T cell proliferation by CTLA-4 utilizes CD80 and requires CD25+ regulatory T cells.* Eur J Immunol, 2002. **32**(10): p. 2888-96.
326. Zheng, Y., et al., *CD86 and CD80 differentially modulate the suppressive function of human regulatory T cells.* J Immunol, 2004. **172**(5): p. 2778-84.
327. Kolar, P., et al., *CTLA-4 (CD152) controls homeostasis and suppressive capacity of regulatory T cells in mice.* Arthritis Rheum, 2009. **60**(1): p. 123-32.
328. Qureshi, O.S., et al., *Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4.* Science, 2011. **332**(6029): p. 600-3.
329. Munn, D.H., et al., *Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase.* Science, 2002. **297**(5588): p. 1867-70.
330. Mellor, A.L., et al., *Prevention of T cell-driven complement activation and inflammation by tryptophan catabolism during pregnancy.* Nat Immunol, 2001. **2**(1): p. 64-8.
331. Jung, I.D., et al., *Differential regulation of indoleamine 2,3-dioxygenase by lipopolysaccharide and interferon gamma in murine bone marrow derived dendritic cells.* FEBS Lett, 2007. **581**(7): p. 1449-56.
332. Mellor, A.L., et al., *Cutting edge: induced indoleamine 2,3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion.* J Immunol, 2003. **171**(4): p. 1652-5.
333. Baban, B., et al., *Indoleamine 2,3-dioxygenase expression is restricted to fetal trophoblast giant cells during murine gestation and is maternal genome specific.* J Reprod Immunol, 2004. **61**(2): p. 67-77.
334. Fujinami, R.S., et al., *Molecular mimicry in virus infection: crossreaction of measles virus phosphoprotein or of herpes simplex virus protein with human intermediate filaments.* Proceedings of the National Academy of Sciences of the United States of America, 1983. **80**(8): p. 2346-50.
335. Gregersen, J.W., et al., *Functional epistasis on a common MHC haplotype associated with multiple sclerosis.* Nature, 2006. **443**(7111): p. 574-7.
336. Lang, H.L., et al., *A functional and structural basis for TCR cross-reactivity in multiple sclerosis.* Nature immunology, 2002. **3**(10): p. 940-3.
337. Wucherpfennig, K.W. and J.L. Strominger, *Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein.* Cell, 1995. **80**(5): p. 695-705.
338. Agabi, Y.A., et al., *Seroprevalence of herpes simplex virus type-2 among patients attending the Sexually Transmitted Infections Clinic in Jos, Nigeria.* Journal of infection in developing countries, 2010. **4**(9): p. 572-5.
339. Christen, U., et al., *A viral epitope that mimics a self antigen can accelerate but not initiate autoimmune diabetes.* The Journal of clinical investigation, 2004. **114**(9): p. 1290-8.
340. Cunningham, M.W., et al., *Cytotoxic and viral neutralizing antibodies crossreact with streptococcal M protein, enteroviruses, and human cardiac myosin.* Proceedings of the National Academy of Sciences of the United States of America, 1992. **89**(4): p. 1320-4.
341. Deshpande, S.P., et al., *Herpes simplex virus-induced keratitis: evaluation of the role of molecular mimicry in lesion pathogenesis.* Journal of virology, 2001. **75**(7): p. 3077-88.
342. Honeyman, M.C., et al., *Evidence for molecular mimicry between human T cell epitopes in rotavirus and pancreatic islet autoantigens.* Journal of immunology, 2010. **184**(4): p. 2204-10.
343. Zhao, Z.S., et al., *Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection.* Science, 1998. **279**(5355): p. 1344-7.

344. Zipris, D., et al., *TLR activation synergizes with Kilham rat virus infection to induce diabetes in BBDR rats.* Journal of immunology, 2005. **174**(1): p. 131-42.
345. Greco, L., et al., *The first large population based twin study of coeliac disease.* Gut, 2002. **50**(5): p. 624-8.
346. Hervonen, K., et al., *Concordance of dermatitis herpetiformis and celiac disease in monozygous twins.* J Invest Dermatol, 2000. **115**(6): p. 990-3.
347. Heap, G.A. and D.A. van Heel, *Genetics and pathogenesis of coeliac disease.* Seminars in immunology, 2009. **21**(6): p. 346-54.
348. Plaza-Izurieta, L., et al., *Revisiting genome wide association studies (GWAS) in coeliac disease: replication study in Spanish population and expression analysis of candidate genes.* J Med Genet, 2011. **48**(7): p. 493-6.
349. van Heel, D.A., et al., *A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21.* Nat Genet, 2007. **39**(7): p. 827-9.
350. Aho, K., et al., *Occurrence of rheumatoid arthritis in a nationwide series of twins.* J Rheumatol, 1986. **13**(5): p. 899-902.
351. Jarvinen, P. and K. Aho, *Twin studies in rheumatic diseases.* Semin Arthritis Rheum, 1994. **24**(1): p. 19-28.
352. Silman, A.J., et al., *Twin concordance rates for rheumatoid arthritis: results from a nationwide study.* Br J Rheumatol, 1993. **32**(10): p. 903-7.
353. Svendsen, A.J., et al., *Relative importance of genetic effects in rheumatoid arthritis: historical cohort study of Danish nationwide twin population.* BMJ, 2002. **324**(7332): p. 264-6.
354. Eleftherohorinou, H., et al., *Pathway-driven gene stability selection of two rheumatoid arthritis GWAS identifies and validates new susceptibility genes in receptor mediated signalling pathways.* Hum Mol Genet, 2011. **20**(17): p. 3494-506.
355. Imboden, J.B., *The immunopathogenesis of rheumatoid arthritis.* Annu Rev Pathol, 2009. **4**: p. 417-34.
356. Plenge, R.M., et al., *Two independent alleles at 6q23 associated with risk of rheumatoid arthritis.* Nat Genet, 2007. **39**(12): p. 1477-82.
357. Plenge, R.M., et al., *TRAF1-C5 as a risk locus for rheumatoid arthritis--a genomewide study.* N Engl J Med, 2007. **357**(12): p. 1199-209.
358. Raychaudhuri, S., et al., *Common variants at CD40 and other loci confer risk of rheumatoid arthritis.* Nat Genet, 2008. **40**(10): p. 1216-23.
359. Remmers, E.F., et al., *STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus.* N Engl J Med, 2007. **357**(10): p. 977-86.
360. Stahl, E.A., et al., *Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci.* Nat Genet, 2010. **42**(6): p. 508-14.
361. Yanagawa, T., et al., *CTLA-4 gene polymorphism associated with Graves' disease in a Caucasian population.* J Clin Endocrinol Metab, 1995. **80**(1): p. 41-5.
362. Marron, M.P., et al., *Insulin-dependent diabetes mellitus (IDDM) is associated with CTLA4 polymorphisms in multiple ethnic groups.* Hum Mol Genet, 1997. **6**(8): p. 1275-82.
363. Nistico, L., et al., *The CTLA-4 gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes. Belgian Diabetes Registry.* Hum Mol Genet, 1996. **5**(7): p. 1075-80.
364. Lee, C.S., et al., *Association of CTLA4 gene A-G polymorphism with rheumatoid arthritis in Chinese.* Clin Rheumatol, 2003. **22**(3): p. 221-4.
365. Vaidya, B., et al., *An association between the CTLA4 exon 1 polymorphism and early rheumatoid arthritis with autoimmune endocrinopathies.* Rheumatology (Oxford), 2002. **41**(2): p. 180-3.

366. Polymeropoulos, M.H., et al., *Dinucleotide repeat polymorphism at the human CTLA4 gene.* Nucleic Acids Res, 1991. **19**(14): p. 4018.
367. Chistiakov, D.A., et al., *Genetic analysis and functional evaluation of the C/T(-318) and A/G(-1661) polymorphisms of the CTLA-4 gene in patients affected with Graves' disease.* Clin Immunol, 2006. **118**(2-3): p. 233-42.
368. Donnadieu, E., et al., *Allergy-associated polymorphisms of the Fc epsilon RI beta subunit do not impact its two amplification functions.* J Immunol, 2000. **165**(7): p. 3917-22.
369. Maurer, M., et al., *A polymorphism in the human cytotoxic T-lymphocyte antigen 4 (CTLA4) gene (exon 1 +49) alters T-cell activation.* Immunogenetics, 2002. **54**(1): p. 1-8.
370. Anjos, S., et al., *A common autoimmunity predisposing signal peptide variant of the cytotoxic T-lymphocyte antigen 4 results in inefficient glycosylation of the susceptibility allele.* J Biol Chem, 2002. **277**(48): p. 46478-86.
371. Sun, T., et al., *Functional genetic variations in cytotoxic T-lymphocyte antigen 4 and susceptibility to multiple types of cancer.* Cancer Res, 2008. **68**(17): p. 7025-34.
372. Deichmann, K., et al., *An Mse I RFLP in the human CTLA4 promotor.* Biochem Biophys Res Commun, 1996. **225**(3): p. 817-8.
373. Wang, X.B., et al., *A CTLA-4 gene polymorphism at position -318 in the promoter region affects the expression of protein.* Genes Immun, 2002. **3**(4): p. 233-4.
374. Anjos, S.M., M.C. Tessier, and C. Polychronakos, *Association of the cytotoxic T lymphocyte-associated antigen 4 gene with type 1 diabetes: evidence for independent effects of two polymorphisms on the same haplotype block.* J Clin Endocrinol Metab, 2004. **89**(12): p. 6257-65.
375. Ligers, A., et al., *CTLA-4 gene expression is influenced by promoter and exon 1 polymorphisms.* Genes Immun, 2001. **2**(3): p. 145-52.
376. Emery, P., et al., *Impact of T-cell costimulation modulation in patients with undifferentiated inflammatory arthritis or very early rheumatoid arthritis: a clinical and imaging study of abatacept (the ADJUST trial).* Ann Rheum Dis, 2010. **69**(3): p. 510-6.
377. Tak, P.P. and J.R. Kalden, *Advances in rheumatology: new targeted therapeutics.* Arthritis Res Ther, 2011. **13 Suppl 1**: p. S5.
378. Khraishi, M., A. Russell, and W.P. Olszynski, *Safety profile of abatacept in rheumatoid arthritis: a review.* Clin Ther, 2010. **32**(11): p. 1855-70.
379. Goldzweig, O. and P.J. Hashkes, *Abatacept in the treatment of polyarticular JIA: development, clinical utility, and place in therapy.* Drug Des Devel Ther, 2011. **5**: p. 61-70.
380. Prestwich, R.J., et al., *The immune system--is it relevant to cancer development, progression and treatment?* Clin Oncol (R Coll Radiol), 2008. **20**(2): p. 101-12.
381. Taylor, R.C., et al., *Tumor-infiltrating lymphocytes predict sentinel lymph node positivity in patients with cutaneous melanoma.* J Clin Oncol, 2007. **25**(7): p. 869-75.
382. Zhang, L., et al., *Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer.* N Engl J Med, 2003. **348**(3): p. 203-13.
383. Yoshimoto, M., G. Sakamoto, and Y. Ohashi, *Time dependency of the influence of prognostic factors on relapse in breast cancer.* Cancer, 1993. **72**(10): p. 2993-3001.
384. Baier, P.K., et al., *Analysis of the T cell receptor variability of tumor-infiltrating lymphocytes in colorectal carcinomas.* Tumour Biol, 1998. **19**(3): p. 205-12.
385. Galon, J., et al., *Type, density, and location of immune cells within human colorectal tumors predict clinical outcome.* Science, 2006. **313**(5795): p. 1960-4.

386. Wolf, D., et al., *The expression of the regulatory T cell-specific forkhead box transcription factor FoxP3 is associated with poor prognosis in ovarian cancer.* Clin Cancer Res, 2005. **11**(23): p. 8326-31.
387. O'Day, S.J., O. Hamid, and W.J. Urba, *Targeting cytotoxic T-lymphocyte antigen-4 (CTLA-4): a novel strategy for the treatment of melanoma and other malignancies.* Cancer, 2007. **110**(12): p. 2614-27.
388. Callahan, M.K., J.D. Wolchok, and J.P. Allison, *Anti-CTLA-4 antibody therapy: immune monitoring during clinical development of a novel immunotherapy.* Semin Oncol, 2010. **37**(5): p. 473-84.
389. Weber, J., *Review: anti-CTLA-4 antibody ipilimumab: case studies of clinical response and immune-related adverse events.* Oncologist, 2007. **12**(7): p. 864-72.