T CELL COSTIMULATORY PATHWAYS – THE ROLE OF VITAMIN D$_3$

By

DAVID H. GARDNER

A thesis submitted to the University of Birmingham for the degree

of

DOCTOR OF PHILOSOPHY

School of Immunity and Infection
College of Medical and Dental Sciences
University of Birmingham
September 2015

UNIVERSITY OF BIRMINGHAM

University of Birmingham Research Archive

e-theses repository

Abstract

CD28-costimulatory signals interact with antigen-specific TCR signals to enhance T cell activation, proliferation and differentiation. The regulation of CD28-costimulation is controlled by CTLA-4 through its shared affinity for the CD28-ligands CD80 and CD86. CTLA-4-ig (abatacept) has emerged as an effective treatment for rheumatoid arthritis. The aims of this study were to consider the factors that influence CD28-costimulation requirements during *in vitro* T cell stimulation in order to identify strategies that may predict or improve clinical responses to abatacept-treatment.

The efficacy of abatacept during *in vitro* T cell stimulation inversely correlated with parameters that increased the strength of TCR-stimulation. The simultaneous inhibition of TCR- and CD28-signals by Cyclosporine A and abatacept respectively promoted the inhibition of T cell activation above the level seen by either agent alone. The active form of vitamin D3, $1,25(OH)_2D_3$, acted in a comparable manner to CsA to increase CD28-costimulation requirements by specifically inhibiting TCR-driven activation.

These findings suggest that clinical responses to abatacept treatment may be determined by the strength of TCR stimulus that underlies T cell activation. Furthermore, that vitamin D3 may represent a useful adjunct to enhance clinical responses to abatacept.

Acknowledgements

Contents

Chapter 3: Impact of relative APC numbers on T cell CD28-costimulation requirements............................ 66

Chapter 4: 1,25(OH)$_2$D$_3$ promotes the efficacy of CD28-costimulation blockade by abatacept 110

Chapter 5: Can *in vitro* T cell activation thresholds be utilized to predict disease outcome in early arthritis patients? 157

Chapter 6: General discussion and future work 182

Appendix 197

Publications 198

References 199

List of figures/tables

List of Abbreviations

1,25(OH)$_2$D$_3$: 1,25-Dihydroxyvitamin D$_3$

ACPA: Anti-Citrullinated Peptide Antibodies

ANOVA: Analysis of variance

Akt: Protein kinase B

AP-1: Activator Protein-1

AP-2: Adaptor Protein-2

APC: Antigen Presenting Cell

Bcl-xL: B cell lymphoma-extra large

BSA: Bovine Serum Albumin

BTLA: B and T Lymphocyte Attenuator

CD: Cluster of Differentiation

CHO: Chinese Hamster Ovary

CIA: Collagen Induced Arthritis

CsA: Cyclosporine A

CTLA-4: Cytotoxic T Lymphocyte Antigen-4

CTV: CellTrace™ Violet

DAG: Diacylglycerol

DC: Dendritic cell

EAE: Experimental Autoimmune Encephalomyelitis

EDTA: Ethylenediamine tetra-acetic acid

FoxP3: Forkhead box P3

GEF: Guanine-nuclear-Exchange Factor

GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor

ICAM: Intercellular Adhesion Molecule

ICOS: Inducible Costimulator

IFN: Interferon

IL: Interleukin

IP$_3$: Inositol 1,4,5-trisphosphate

ITAM: Immunoreceptor Tyrosine-based Activation Motif

iT$_{reg}$: Induced T$_{reg}$

KO: Knock-out

LAT: Linker for the Activation of T cells

LFA-1: Lymphocyte function-associated antigen-1

LPS: Lipopolysaccharide

MAP: Mitogen Activated Protein

MFI: Mean Fluorescence Intensity

MHC: Major Histocompatibility Complex

mTOR: Mammalian Target of Rapamycin

NFAT: Nuclear Factor of Activated T cells

NFκB: Nuclear Factor Kappa B

NOD: Non-obese diabetic

nT$_{reg}$: Natural T$_{reg}$

PAMP: Pathogen Associated Molecular Pattern

PBS: Phosphate Buffered Saline

PBMC: Peripheral Blood Mononuclear Cells

PD-1: Programmed cell death-1

PDK1: Phosphoinositide-dependent kinase 1

PI3K: Phosphatidylinositol 3-kinase

PIP$_2$: Phosphatidylinositol-4,5-bisphosphate

PKCθ: Protein kinase C theta

PLCγ1: Phospholipase Cγ1

PMA: Phorbol Myristate Acetate

pMHC: Peptide-MHC complex

PTPN22: Protein tyrosine phosphatase, non-receptor type 22

RA: Rheumatoid arthritis

RasGRP: RAS guanyl nucleotide releasing protein

SH2: Src Homology 2

SHP: Src-homology 2 domain-containing phosphatase

SLE: Systemic Lupus Erythematosus

SLP-76: SH2 domain containing leukocyte protein

SOS: Son of Sevenless

STAT: Signal Transducer and Activator of Transcription

TCR: T cell receptor

T$_{eff}$: T effector cell

T$_{fh}$: T follicular helper cell

T_h: T helper cell

TGF: Transforming Growth Factor

TNF: Tumour Necrosis Factor

T_{reg}: Regulatory T cell

VDR: Vitamin D Receptor

WASp: Wiskott-Aldrich syndrome Protein

WT: Wild-type

ZAP-70: Zeta Associated Protein Kinase-70

Chapter 1: Introduction

Following activation, CD4$^+$ effector T cell (T$_{eff}$) populations coordinate the activities of the adaptive immune system (denHaan et al., 2014) and interact with various components of the innate immune system (Strutt et al., 2011). As such, appropriate T cell activation represents a crucial step in maintaining protection during immune challenge. Accordingly, the requirement to regulate T cell activation signals is critical since inappropriate T cell activation leads to further activation of the wider immune system and its potentially destructive capacity. This is clearly demonstrated by a central role played by activated T cells in various autoimmune diseases (Xing and Hogquist, 2012). Therefore, the regulation of T cell activation is a crucial factor in maintaining immune tolerance. In this thesis I explore how the adjustment of T cell activation conditions influences immune function.

1.1. T cell subsets

T cells originate from hematopoietic progenitor cells that initially develop within the bone marrow. These T cell precursors subsequently traffic to the thymus where their development to a mature T cell is completed (Bhandoola et al., 2003). This transition includes the acquisition of a functional T cell receptor (TCR) following TCR gene rearrangement and subsequent positive selection (Vrisekoop et al., 2014) and CD4-CD8 lineage differentiation (Wang and Bosselut, 2009). Additionally, the process of negative selection shapes

the TCR repertoire to limit the egress of "self" reactive T cells from the thymus. Negative selection is influenced by the expression of an array of tissue specific antigens by epithelial cells within the thymic medulla that are expressed under the control of the transcription factor autoimmune regulator (AIRE) (Anderson and Su, 2011). Autoreactive T cells that display high affinity for antigen either undergo clonal deletion through the induction of apoptosis or are subjected to clonal diversion which entails their differentiation into regulatory T cells (T_{reg}) (Xing and Hogquist, 2012). It is those T cells that survive both positive and negative selection that constitute our peripheral T cell repertoire.

T_{reg} differentiation and function is dependent upon the forkhead box P3 (FoxP3) transcription factor (Benoist and Mathis, 2012). Mice deficient in FoxP3 display a lethal autoimmune phenotype (Fontenot et al., 2003). In humans, mutations within the *FOXP3* gene result in immune dysregulation polyendocrinopathy X-linked syndrome (IPEX) (Wildin et al., 2001; Bennett et al., 2001). IPEX is characterized by T_{reg} dysfunction and autoimmunity, clearly demonstrating the importance of T_{reg} in the regulation of the immune system (Ziegler, 2006). In the periphery, T_{reg} utilize an array of effector molecules to maintain tolerance. For example, T_{reg} express various molecules that suppress the activation and function of other lymphocytes such as Cytotoxic T Lymphocyte Antigen (CTLA)-4 (Wing et al., 2008) and Programmed Death (PD)-1 and its ligands PD-L1 and PD-L2 (Gotot et al., 2012; Park et al., 2015). Additionally, T_{reg} produce various anti-inflammatory cytokines including Transforming growth factor (TGF)-β, Interleukin (IL)-10 and IL-35 (Benoist

and Mathis, 2012). It has been suggested that T_{reg} mediate granzyme/perforin dependent cytotoxicity of activated T cells and antigen presenting cells (APC) subsets to regulate the magnitude of T cell responses and delivery of T cell activation signals (Grossman et al., 2004; Zhao et al., 2006; Salti et al., 2011). Finally, several lines of evidence suggest that T_{reg} suppress T cell activation indirectly by modulating the phenotype and stimulatory capacity of APCs (Shevach, 2009).

Mature T cells that enter the periphery can be classified in terms of their mutually exclusive expression of the TCR co-receptors CD4 and CD8 (Wang and Bosselut, 2009). CD8 specifically interacts with major histocompatibility (MHC)- class I molecules, which are expressed on the surface of all nucleated cells and present endogenous peptides that are derived from transcription. This is central to the function of CD8^{+} cytotoxic T cells in mediating immunity against intracellular pathogens (Zhang and Bevan, 2011). In contrast, CD4 specifically interacts with MHC-II that is expressed by professional APCs and presents exogenous peptides that are processed following internalization by endocytosis.

CD4^{+} T cells recirculate between the bloodstream and secondary lymphoid organs via the lymphatic system. Lymphoid tissue architecture provides a platform for T cells to interact and scan the surface of APCs for cognate antigen (Itano and Jenkins, 2003). Upon activation CD4^{+} fulfill a "helper" function by enhancing the activities of other components of the immune system. In 1989, Mosmann and Coffman outlined a model of CD4^{+} T cell

differentiation based upon differential cytokine expression. For example, specific production of IL-2 and Interferon (IFN)-γ by T helper (T_h) 1 cells and of IL-4 and IL-5 by T_h2 cells. This differential cytokine production was proposed to underlie differential helper functions with T_h1 cells primarily mediating cellular immunity via effects upon macrophages and $CD8^+$ T cells and T_h2 cells mediating humoral immunity in association with effects upon B cells and antibody class switching (Mosmann and Coffman, 1989). To some extent, this model has been supported by the identification that the transcription factors T-bet (Szabo et al., 2000) and GATA-3 (Zheng and Flavell, 1997) act as master-regulators of T_h1 and T_h2 lineage differentiation respectively and that there are specific conditions optimal for the polarization of T cells during stimulation to T_h1 or T_h2 outcomes (Constant and Bottomly, 1997).

Several observations suggest that a model of mutually exclusive T_h1 vs. T_h2 differentiation is overly simplistic. For instance, human T cells frequently display either shared characteristics of, or plasticity between, T_h1 and T_h2 lineage differentiation (Messi et al., 2003; Krawczyk et al., 2007; Hegazy et al., 2010; Peine et al., 2013; Tumes et al., 2013). Significantly, several additional T_h lineages have been defined which play important roles in T cell mediated immunity and regulation including induced T_{reg} (iT_{reg}), Th17, Th9 and T follicular helper (T_{fh}) cells. For example, naïve T cell stimulation in the presence of TGF-β promotes the generation of iT_{reg} that are characterized by FoxP3 expression (Chen et al., 2003; Fantini et al., 2004). These employ comparable suppressive mechanisms to "natural" T_{reg} (nT_{reg}) that are generated in the thymus. However, the activities of both T_{reg} populations are

required to effectively maintain tolerance at least in part because nT$_{reg}$ and iT$_{reg}$ display a non-overlapping TCR repertoire (Haribhai et al., 2011). Thus iT$_{reg}$, are induced by exposure to antigen in a non-inflammatory environment where TGF-β predominates and this serves as a mechanism of peripheral tolerance that reinforces centrally induced tolerance.

TGFβ signaling in the presence of other immunomodulatory cytokines also determines T cell differentiation towards the T$_h$9 or T$_h$17 lineage. T$_h$9 cells are generated in the presence of TGF-β and IL-4 (Veldhoen et al., 2008; Dardalhon et al., 2008) and express high IL-9 levels under the control of the control of the transcription factor PU.1 (Gerlach et al., 2014). These cells interact with other T$_h$ lineages to mediate inflammatory processes and autoimmunity in a variety of animal models (Kaplan et al., 2015). The development of the T$_h$17 cell subset, characterized by the production of IL-17, is promoted by TGF-β, however, this occurs on a background of proinflammatory cytokines such IL-6, IL-21 and IL-23 (Aggarwal et al., 2003; Bettelli et al., 2006; Korn et al., 2007; Zhou et al., 2007). These proinflammatory cytokines positively enforce T$_h$17 differentiation and negatively regulate iT$_{reg}$ expression by upregulating the transcription factor retinoic-acid receptor related orphan nuclear receptor (ROR)γt that acts as a master regulator of T$_h$17 differentiation and function and simultaneously inhibiting FoxP3 expression (Korn et al., 2008). T$_h$17 cells have been found to play a prominent role in chronic inflammation and autoimmune diseases. This is associated with the induction of a highly proinflammatory cytokine response following IL-17 signaling primarily among stromal and myeloid cells (Weaver

et al., 2007). Additionally T_h17 cells produce several additional proinflammatory cytokines including the related cytokine IL-17F, IL-6, TNFα, IL-21 and IL-22 that act to reinforce T_h17 differentiation and propagate inflammation (Damsker et al., 2010).

T_{fh} differentiation is controlled by the transcription factor Bcl6 (Johnston et al., 2009). Following activation, these cells migrate to B cell follicles in association with their characteristic expression of the chemokine receptor CXCR5 (Breitfeld et al., 2000). Here, T_{fh} cells utilize various surface and effector molecules including CD40L, Inducible T cell Costimulator (ICOS), IL-4 and IL-21 to promote B cell responses by initiating germinal center formation and directly interacting with B cells to facilitate affinity maturation (Crotty, 2014).

1.2. A two-signal model for T cell activation

Control over the initiation of $CD4^+$ T cell responses is associated with a requirement for multiple signals that contribute towards T cell activation thresholds. This, in effect, generates a checkpoint that must be passed before T cell activation can ensue. In 1970, Bretscher and Cohn proposed a "two-signal" model for antibody responses based upon differential responses towards antigen in the periphery (Bretscher and Cohn, 1970). This hypothesis was expanded upon by investigations into allograft responses by Lafferty and Woolnough who suggested that it was T cell activation that occurred as the result of an "inductive stimulus" in addition to antigen-stimulation (signal 1) (Lafferty and Woolnough, 1977). It was suggested that the absence of this

inductive stimulus promoted a state of "proliferative non-responsiveness to subsequent stimulation" (Quill and Schwartz, 1987). Several receptors have now been identified to display this costimulatory or accessory function by supporting antigen-stimulation via the T cell receptor (TCR) (Chen and Flies, 2013). Probably the predominant source of initial costimulation is via CD28, a T cell surface protein that is constitutively expressed by resting CD4^{+} T cells which interacts with ligands (CD80 and CD86) expressed by APCs (Sansom, 2000). Together, TCR- and CD28-driven signals determine the extent and efficiency of T cell activation and provide an opportunity for regulation of this process.

1.3. Signal one: TCR signaling

CD4^{+} TCR-signals are driven following the ligation of the TCR by cognate antigen presented in the context of major histocompatibility complex (MHC) II (pMHC). This recognition of pMHC complexes by the TCR is mediated by an extracellular polypeptide heterodimer comprising an association between a single TCRα and TCRβ chain (Wange and Samelson, 1996). Additionally, a small proportion of T cells express a TCR that comprises an association between a TCRγ chain and a TCRδ chain (Vantourout and Hayday, 2013). These γδ T cells do not require antigen processing and presentation by MHC for activation, however, in some cases they interact with non-classical MHC gamma delta (Luoma et al., 2014). Their function is not entirely understood but appears to contribute towards immunity at epithelial surfaces (Vantourout and Hayday, 2013). αβ TCRs are restricted to antigen binding in the context

of MHC possibly due to the coevolution of complementary structural motifs that mediate TCR-MHC interactions and which facilitate interactions between hypervariable regions of the TCR with antigen (Garcia et al., 2009). These hypervariable regions are generated by TCR gene rearrangement during T cell development and are expressed within complementarity determining regions (CDR) within the membrane distal variable domains of the TCRα and TCRβ chains. CDR3α and CDR3β play a particularly prominent role due to their placement over the MHC binding groove during TCR-MHC interactions (Wucherpfennig et al., 2010). The hypervariability within these CDRs is central to the function of T cells in recognizing and responding to a diverse group of epitopes.

1.3.1. Balancing the sensitivity and specificity of T cell responses

Even where the agonist peptide displays optimal affinity, interactions between the TCR and pMHC display an inherently low affinity and are short-lived (Valitutti et al., 1995). Nevertheless, TCR interactions with cognate peptide are extremely sensitive. For example, CD4^{+} T cells are activated when just 0.03% surface MHC-II present cognate antigen (Demotz et al., 1990). Furthermore, CD4^{+} T cells display transient calcium flux in response to a single cognate pMHC-II complex (Irvine et al., 2002). However, this level sensitivity could be predicted to be detrimental to the requirement of T cells to display a functional specificity for specific pMHC interactions that ultimately determines the specificity of T cell activation. The requirement for T cell responses that are both sensitive and specific is partly achieved through the activities of positive and negative thymic selection which produces a diverse

and functional TCR repertoire that displays high affinity interactions with "non-self" antigen and an inherently low affinity for "self" (Morris and Allen, 2012). Additionally, the serial triggering model for TCR activation predicts that the rapid off-rate of pMHC:TCR interactions balances this requirement for sensitive and specific T cell responses to antigen by rendering a requirement for serial triggering of TCRs (Valitutti and Lanzavecchia, 1997). As such, this model predicts that few cognate pMHC complexes can activate many TCRs in order to promote the sensitivity of TCR binding, similarly, that small reductions TCR affinities for pMHC potently inhibit this serial TCR triggering. Nonetheless, the problem of TCR triggering is still poorly understood and a number of models have been proposed (Davis and van der Merwe, 2006).

1.3.2. TCR signaling pathways

TCR triggering initiates a complex signaling cascade that is illustrated in **fig. 1.1**. In simplistic terms, productive TCR signaling can be suggested to occur due the combined activation of Nuclear Factor of Activated T cells (NFAT), Activator Protein (AP)-1 and Nuclear Factor (NF) κB transcription factors and their subsequent regulation of genes such as IL-2 that mediate T cell activation, proliferation and differentiation (Crabtree and Clipstone, 1994).

Signals are transmitted from the T cell surface upon antigen recognition by the TCRαβ heterodimer due to its association within the TCR/CD3 complex consisting of the CD3εδ, CD3εγ heterodimers and TCRζ homodimer (Wange and Samelson, 1996). This complex lacks intrinsic kinase activity, however, each CD3 chain comprises an immunoreceptor tyrosine-based activation

motif (ITAM), furthermore, each TCRζ chain comprises three ITAMs (Wange and Samelson, 1996). The Src family kinase member Lck, which physically associates with the TCR co-receptor and is brought into close proximity to the TCR/CD3 complex by antigen binding (Veillette et al., 1988), phosphorylates tyrosine residues within these ITAMs. Additionally, the Src kinase family member Fyn plays a role in the initiation of TCR signaling via its association with CD3 chains (Timson Gauen et al., 1992) but appears to have a more prominent role in thymocytes than peripheral T cells (Stein et al., 1992).

Phosphorylation of ITAMs within the CD3 complex results in the recruitment of zeta chain associated protein kinase (ZAP)-70 to the signaling complex via its Src Homology 2 (SH2) domains and its subsequent activation by both autophosphorylation and the activity of Lck (Iwashima et al., 1994). Upon activation, ZAP-70 phosphorylates multiple tyrosine residues on the adaptor protein linker for the activation of T cells (LAT) and SH2 domain containing leukocyte protein (SLP)-76 (Wang et al., 2010a).

LAT is localized at the cell membrane and its phosphorylation facilitates the recruitment of and interaction with SLP-76. These two scaffold proteins act as a platform for an elaborate signaling complex that mediates downstream TCR signals by recruiting various SH2 domain-containing proteins (Koretzky et al., 2006). For example, LAT promotes the activation of the Ras-ERK mitogen-activated protein (MAP)-kinase pathway. This occurs as the adaptor protein growth-factor-receptor-bound protein 2 (Grb2) binds to phosphorylated LAT; this subsequently recruits Son of Sevenless (SOS), which acts as a guanine-

nuclear-exchange factor (GEF) for the small GTPase Ras (Balagopalan et al., 2010). Additionally, phosphorylated SLP-76 recruits the adaptor protein Nck and the GEF Vav-1 that promotes subsequent Wiskott-Aldrich syndrome protein (WASp) recruitment and activation (Zeng et al., 2003). Activated WASp regulates T cell activation by effects upon the actin cytoskeleton which stabilize T cell-APC interactions (Sims et al., 2007). This regulation of the actin cytoskeleton during stimulation has emerged as a crucial aspect of T cell activation in that actin remodeling is necessary for the transduction of proximal TCR signaling events (e.g. Lck/ZAP-70 activation) to downstream TCR signaling (e.g. calcium flux and ERK phosphorylation) (Tan et al., 2014).

Another important step in the TCR signaling cascade is the recruitment and activation of phospholipase Cγ1 (PLCγ1) to the LAT-SLP-76 complex promotes the generation of the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacyglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2). By recruiting RAS guanyl nucleotide releasing protein (RasGRP) to the plasma membrane, DAG promotes the activation of the MAP kinase pathway (Huse, 2009), which in turn promotes the transcription of the AP-1 subunit *c-Fos* (Huang and Wange, 2004). DAG also positively regulates NFκB and AP-1 mediated transcription through the activation of protein kinase C-θ (PKCθ) (Arendt et al., 2002). Whilst DAG predominantly regulates the AP-1 transcription factor, IP_3 promotes NFAT nuclear translocation. This is mediated via an elevation in intracellular Ca^{2+} initially from intracellular stores and secondarily via a Ca^{2+} dependent activation of calcium release activated channels (CRAC) that mediate an extracellular Ca^{2+} flux (Hogan et al., 2003).

Elevated intracellular Ca^{2+} subsequently activates the calcium-binding messenger protein Calmodulin that activates the phosphatase calcineurin. In turn, calcineurin dephosphorylates NFAT that facilitates its nuclear translocation (Macián, 2005). NFAT and AP-1 are known to cooperate and synergistically enhance cytokines such as IL-2 and IL-4 that regulate T cell activation, differentiation and effector function (Macián et al., 2001).

1.3.3. The impact of TCR stimulus strength on the T cell response

Signaling pathways can be activated in a 'digital' or 'analog' manner. In this context, digital activation represents 'all-or-nothing' signaling events and is therefore 'switch-like' upon reaching a specific activation threshold. In contrast, analog signals are graded where the magnitude of the output is determined by the strength of the input (i.e. strength of TCR stimulation). Several TCR signaling modules display a digital activation pattern which facilitates the requirement for T cells to respond with specificity towards particular antigen (Germain, 2010). Accordingly, highly sensitive T cell responses have been observed in which T cells respond to a single specific pMHC complex with Tumour Necrosis Factor (TNF)-α and IL-2 production. Under these conditions, increasing pMHC numbers augmented the proportion of responding T cells but not the magnitude of cytokine production by activated T cells on a per cell basis (Huang et al., 2013). These observations are implying a digital response to TCR stimulation. In association with this, TCR-induced NFκB activation (Kingeter et al., 2010), NFAT translocation (Podtschaske et al., 2007) and ERK signaling (Altan-Bonnet and Germain, 2005) display a digital activation pattern in response to graded TCR stimuli.
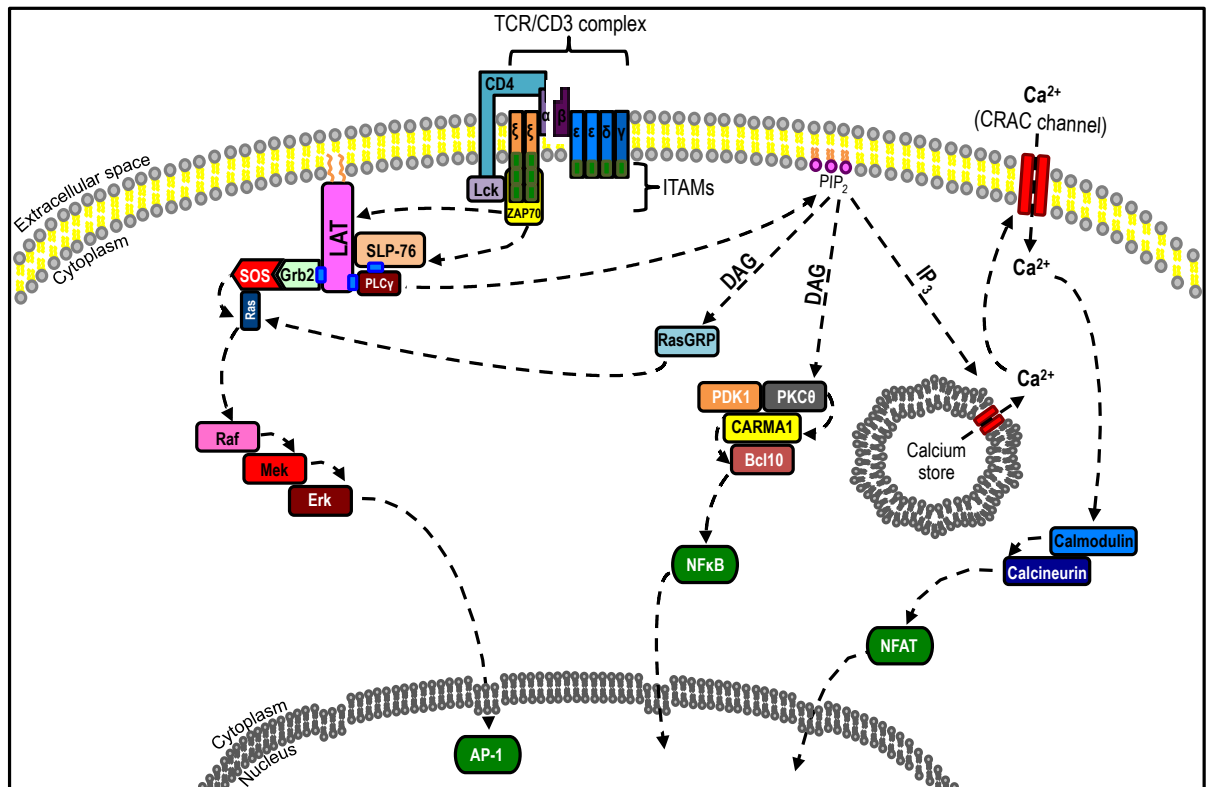
Fig. 1.1. TCR ligation initiates a complex signaling cascade. CD4^{+} T cells interact with pMHC complexes presented upon the surface of professional APCs. TCR ligation brings Lck via its association with CD4 into close proximity to ITAMs within the CD3 complex. Lck phosphorylates ITAMs which facilitates ZAP-70 association with the TCR/CD3 complex. ZAP-70 phosphorylates LAT and SLP-76 on multiple residues which subsequently act as scaffold proteins that facilitate downstream TCR signaling. These signals result in the activation of the transcription factors AP-1, NFκB and NFAT which regulate genes that guide T cell activation, proliferation and differentiation.

Digital activation of TCR pathways suggests that activation occurs when a certain signaling threshold is achieved such that activation only occurs in response to robust TCR stimulation. Nevertheless, productive CD8$^+$ T cell responses can occur following stimulation by very low affinity antigen although these responses are characterized by qualitative differences T cell phenotype and response kinetics such as an abbreviated proliferative response (Zehn et al., 2009). Similarly, CD4$^+$ T cells respond effectively to altered peptide ligands that mediate low affinity TCR interactions, however, altered antigen-affinity also has a qualitative impact upon the T cell response, in particular, high affinity ligands promote T$_h$1 type responses whilst low affinity ligands promote T$_h$2 responses (Pfeiffer et al., 1995; Nicholson et al., 1995; Tao et al., 1997b; Keck et al., 2014). Additionally, it has been suggested that TCR-signal strength determines the outcome of T cell proliferation with regards to T cell "fitness" e.g. T cells activated in response to high strength stimulation display enhanced survival, whilst T cells that receive weak stimulation undergo apoptosis and fail to proliferate in response to IL-2 and IL-7 when the activation stimulus is withdrawn (Gett et al., 2003).

On the surface, these observations are difficult to reconcile with an all-or-nothing digital TCR signaling response. One possibility is that these qualitative differences in T cell responses occurring in conjunction with different TCR signal strengths reflect different contributions of costimulatory signals towards activation. Additionally, it has been suggested that altered peptide ligands that display reduced TCR affinities alter TCR/CD3 phosphorylation with an associated inhibition of ZAP-70 activation (Sloan-

Lancaster et al., 1994; Madrenas et al., 1995). It has been suggested that incomplete ZAP-70 activation subsequently prevents SOS recruitment to phosphorylated LAT, preventing digital activation of the Ras-ERK pathway which becomes dependent upon less effective analog activation by RasGRP (Das et al., 2009). These upstream perturbations in the TCR signaling cascade lead to differences in transcription factor translocation to the nucleus. For example, differential AP-1 subunit composition including a prevalence of Jun-Jun homodimers, rather than Fos-Jun heterodimers that favor T_h2 differentiation, have been observed in response to reduced ERK activation by low affinity TCR-engagement (Jorritsma et al., 2003). Therefore, various components within the TCR signaling network display different activation thresholds such that decreased TCR stimulus strength leads to activation of elements, but not the entirety of the TCR-signaling cascade. Whilst this may be sufficient to mediate activation, it promotes qualitative differences in responding T cells.

1.4. Signal two: CD28-costimulation

As the predominant source of T cell costimulation, CD28-signaling promotes the generation of effector T cell populations through effects upon proliferation, survival and differentiation (Boomer and Green, 2010). CD28 is a 44kDa glycoprotein and comprises a membrane distal extracellular immunoglobulin variable-like domain (IgV) (Carreno and Collins, 2002). Interactions between CD28 and the structurally related B7 family members CD80 and CD86 are mediated via an MYPPPY motif within the FG loop of the IgV domain of CD28

(Evans et al., 2005). CD28 exists as a homodimer at the cell surface through a disulphide linkage between complimentary cysteine residues within the linker region that connects CD28 IgV and transmembrane domains (Schwartz et al., 2002) and the formation of a complimentary interface between corresponding IgV domains (Evans et al., 2005). Despite this dimerization, CD28 participates in monovalent interactions with CD80 and CD86 (Collins et al., 2002). These monovalent interactions are crucial to the regulation of CD28-signals because CD28 mutants that are capable of bivalent interactions are constitutively activated due to receptor clustering which promotes antigen-independent T cell activation (Dennehy et al., 2006).

Whilst CD28 is constitutively expressed by resting T cells its ligands CD80 and CD86 are only upregulated by APCs in response to inflammation (Banchereau and Steinman, 1998) providing a mechanism to maintain control over T cell activation. The inductive nature of these ligands by APCs in response to either pathogen-associated molecular patterns or danger signals derived from damaged cells is central to the infectious-nonself and danger models of immune activation respectively (Matzinger, 2002).

Upon interaction with CD80 and CD86, CD28 signaling has been strongly associated with the activity of phosphatidylinositol 3-kinase (PI3K) following its interaction with a YMNM motif with the CD28-cytoplasmic tail (Pagès et al., 1994; Prasad et al., 1994; Cai et al., 1995). The generation of phosphatidylinositol (3,4)-bisphosphate (PIP_2) and phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) by PI3-kinase promotes the recruitment of the

Plekstrin homology domain containing proteins Akt and phosphoinositide-dependent kinase 1 (PDK1) to the plasma membrane (Boomer and Green, 2010). PDK1-deficient T cells fail to produce IL-2 following stimulation because PDK1 mediates functional NFκB activation. This occurs as PDK1 participates in complex formation with PKCθ and CARMA1 which leads to sequential PKCθ and CARMA1 activation and ultimately to NFκB activation (Park et al., 2009). PDK1 also activates Akt by phosphorylation (Hemmings and Restuccia, 2012). Constitutive expression in T cells of active Akt negates the requirement for CD28-costimulation for functional T cell responses demonstrating a central role in the mediation of CD28-signals (Rathmell et al., 2003). Upon activation, Akt promotes the activity of the serine/threonine kinase mammalian target of rapamycin (mTOR) (Hemmings and Restuccia, 2012). By controlling various cellular and metabolic programs, mTOR directs the outcome of T cell stimulation by integrating cues received from activation signals and the microenvironment (Zeng and Chi, 2014).

CD28-signals are additionally mediated via a PYAP motif. This facilitates signaling by several mediators involved in TCR-signal transduction, for example, Vav-1 and Lck (Boomer and Green, 2010). Indeed, genomic changes following CD28-costimulation are mediated by the transcription factors NFAT, AP-1 and NFκB in a similar fashion to TCR-driven signals (Boomer and Green, 2010). As such, transcriptional profiles induced by TCR- and CD28-activation show striking similarity with the main distinction being quantitative differences in the extent of gene upregulation rather than the number of genes induced (Riley et al., 2002). Indeed, it has been suggested

that CD28-costimulation primarily serves to compensate for a relatively weak TCR stimulus and vice versa through cooperation towards a combined activation threshold (Acuto and Michel, 2003). This is supported by the apparent amplification by CD28-costimulation of TCR induced Vav-1 (Michel et al., 2000), Lck (Holdorf et al., 2002), PKCθ (Coudronniere et al., 2000), Ca^{2+} and PLCγ1 (Michel et al., 2001) signals. This signal integration occurs to the extent that CD28-costimulation reduces the number of TCRs that are required to achieve activation thresholds (Viola and Lanzavecchia, 1996).

1.5. Functions of CD28-costimulation

Despite the significant overlap between TCR and CD28 signaling pathways, CD28 has a clear impact upon T cell activation and differentiation due to the amplification of TCR driven signals (Acuto and Michel, 2003). This is most clearly demonstrated by CD28-KO mice which display compromised T cell responses (Shahinian et al., 1993; Green et al., 1994). As a result of these positive impacts, CD28-costimulation has been widely viewed as an essential requirement for T cell activation and a control point that can be targeted therapeutically (Linsley and Nadler, 2009).

1.5.1. Upregulation of IL-2 signaling

Strongly linked with each of the outcomes of CD28-costimulation is the ability to enhance T cell expression of and signaling by the T cell growth factor IL-2. For example, CD28-costimulation promotes IL-2 gene expression (Fraser et al., 1991) and promotes the stability of IL-2 mRNAs (Lindstein et al., 1989);

together, these factors combine towards elevated IL-2 production following CD28-signals. IL-2 subsequently functions in an autocrine manner to influence T cell proliferation and differentiation (Boyman and Sprent, 2012). Additionally, CD28-KO mice display reduced expression of the IL-2-receptor (IL-2R) α-chain (CD25) following stimulation (Shahinian et al., 1993). This can be partially attributed to the deficiency of IL-2 resulting from the absence of CD28-costimulation since IL-2 positively regulates CD25 expression via a feed-forward loop (John et al., 1996). However, CD28 also appears to act by an IL-2 independent mechanism to induce CD25 expression (Toyooka et al., 1996). Following expression, CD25 participates in the multimeric high-affinity IL-2R by associating with the IL-2Rβ subunit (CD122) and the common cytokine receptor-γ chain (CD132) (Boyman and Sprent, 2012). The dimeric IL-2Rβγ displays a low affinity for IL-2 (dissociation constant (K_d) = 10^{-9} M). Although this is sufficient for some responsiveness to IL-2 binding, CD25 expression significantly enhances the IL-2-R affinity for IL-2 (K_d = 10^{-11} M) (Taniguchi and Minami, 1993). Thus by enhancing CD25 expression, CD28 also enhances responsiveness to IL-2.

1.5.2. T cell proliferation

CD28-KO mice display significantly decreased activated T cell counts following TCR-stimulation (Shahinian et al., 1993; Green et al., 1994). The large alterations in cell counts arising from the presence of CD28-costimulation can be the result of relatively subtle kinetic alterations in parameters affecting T cell proliferation (Gett and Hodgkin, 2000). However, the specific nature of the impact of CD28 upon the cell cycle has been

controversial and appears to vary depending upon the stimulation system that is used. For example, Gett and Hodgkin used a monoclonal antibody (mAb) driven stimulation system to demonstrate reduced time to division following stimulation in the presence of CD28-signals but no significant effect upon subsequent division time (Gett and Hodgkin, 2000). In contrast, Bonnevier and Mueller used an *in vitro* antigen-driven system to show that CD28-costimulation controls T cell blastogenesis (occurring as T cells undergo the G_0-G_1 phase transition) as well as the subsequent division rate of T cell blasts (Bonnevier and Mueller, 2002). Consistent with these data, several studies have demonstrated a significant impact of CD28-costimulation upon cell cycle progression particularly at the G_1-S phase transition (Boonen et al., 1999; Appleman et al., 2000; 2002; Song et al., 2007). This regulation of late G_1 events by CD28 is mediated by both IL-2 dependent and IL-2 independent signals (Appleman et al., 2000).

1.5.3. T cell survival

CD28-signaling also enhances the magnitude of T cell responses by enhancing the survival of proliferating T cells, for example, by promoting increased expression of the anti-apoptotic factor Bcl-xL (Noel et al., 1996). Also, CD28-dependent downregulation of TCR induced CD95-L expression (Collette et al., 1997) delays or inhibits Activation Induced Cell Death, a mechanism whereby TCR induced activation can ultimately lead to resolution of a T cell response through the induction of apoptosis within the effector population (Kerstan and Hünig, 2004). Again, CD28-mediated IL-2 signaling also serves to enhance the survival of activated T cells by maintaining

appropriate cell cycle progression (Boise and Thompson, 1996).

1.5.4. Regulation of T cell metabolism

Upon stimulation, T cells undergo an active metabolic shift from oxidative phosphorylation towards glycolysis in order to meet the elevated energy demands required by increasing cell growth, macromolecule biosynthesis and proliferation (Jones and Thompson, 2007). CD28-costimulation facilitates this switch through a PI3K-Akt dependent enhancement of glucose uptake and glycolytic rate that meets the energy demands required for effective T cell responses (Frauwirth et al., 2002; Jacobs et al., 2008). Of central importance to the coordination of T cell metabolic pathways by CD28 is its regulation of mTOR (Zheng et al., 2009). It has been suggested that the association between T cell anergy and the absence of costimulation is associated with this regulation of mTOR by CD28. For example, mTOR inhibition by rapamycin induces T cell anergy even in the presence of CD28-signaling (Powell et al., 1999). Thus, CD28 may act via mTOR to control T cell immunity or tolerance.

1.5.5. T cell differentiation

It is becoming increasingly clear that outcomes following T cell stimulation including T cell differentiation are determined by the nature of activation signals (van Panhuys et al., 2014; Keck et al., 2014). In keeping with this concept, CD28-costimulation has been proposed to favor T_h2 differentiation whereas TCR-signals promote T_h1 differentiation (Lenschow et al., 1996; Tao et al., 1997a). Additionally, CD28 costimulation promotes the generation and

maintenance of T_{fh} cells (Platt et al., 2010; Linterman et al., 2014; Wang et al., 2015a). Similarly, the generation of nT_{reg} in the thymus (Tai et al., 2005) and iT_{reg} in the periphery (Guo et al., 2008) are CD28-dependent processes. Therefore, it is apparent that the availability of CD28-costimulation adjusts the nature and control of T cell responses through various effects upon T cell differentiation.

1.5.6. T_{reg} homeostasis

In addition to enhancing the generation of T_{reg} subsets, CD28 controls their maintenance and homeostasis within the periphery. For example, the development of autoimmune diabetes is promoted in CD80/CD86- or CD28-deficient non-obese diabetic mice (NOD) relative to wild-type (WT) controls and this is associated with the absence of $CD4^+CD25^+$ T_{reg} populations (Salomon et al., 2000). This loss of T_{reg} in the absence of CD28 was found to occur due to effects upon both the generation of T_{reg} in the thymus as well as their homeostasis in the periphery. Specifically, CD28-signals were found to be required for IL-2 production by conventional T cells that supported T_{reg} survival as well as maintaining T_{reg} CD25 expression and therefore IL-2 responsiveness (Tang et al., 2003). The requirement for a post-thymic cell – intrinsic CD28-signal for the maintenance of T_{reg} populations has been verified using inducible-CD28 deletion systems (Gogishvili et al., 2013; Zhang et al., 2013). Bone marrow chimera experiments also demonstrated that IL-2 derived from CD28-WT T cells could not rescue the survival of CD28-KO T_{reg} thereby demonstrating an indispensible requirement for this CD28 signaling in T_{reg} homeostasis (Gogishvili et al., 2013). Nevertheless, IL-2 also appears to

play an important role in T$_{reg}$ function and survival in the periphery because IL-2 deficiency in mice promotes a lethal autoimmune syndrome (Sadlack et al., 1995; Suzuki et al., 1995). Similarly, in humans, CD25 deficiency causes an IPEX-like syndrome that is characterized by immune dysregulation (Caudy et al., 2007). This occurs in conjunction with defects in T$_{reg}$ survival within the periphery but not induction within the thymus (D'Cruz and Klein, 2005; Fontenot et al., 2005). Furthermore, IL-2 signaling is required for T$_{reg}$ function (DeLaRosa et al., 2004; Furtado et al., 2002) at least partly by maintaining FoxP3 expression levels (Chen et al., 2011). However, T$_{reg}$ display an intrinsic defect in IL-2 production due to the suppression of the *Il2* gene by the T$_{reg}$ transcription factor Helios and FoxP3 (Popmihajlov and Smith, 2008; Baine et al., 2013) and therefore depend upon CD28-mediated IL-2 production by local conventional T cells. These observations suggest that CD28 and IL-2 signaling cooperate towards the maintenance of T$_{reg}$ homeostasis and function.

1.6. Regulation of CD28-costimulation

As the major source of T cell costimulation, the delivery of CD28-signaling is strictly regulated. This is largely achieved by limiting the availability of the CD28–ligands CD80/CD86 by both environmental factors and active regulatory mechanisms. Immature or resting APC subsets express low CD80/CD86 levels (Chung et al., 2003; Zheng et al., 2004; Mittelbrunn et al., 2009) although CD86 can display a more constitutive expression pattern amongst certain professional APC subsets (Azuma et al., 1993). During

maturation, APCs increase CD80/CD86 expression levels and the molecular components required to participate in productive interactions with T cells. This maturation can be driven by an overwhelmingly proinflammatory environment (Banchereau and Steinman, 1998) or due to direct contact with T cells via the CD40-CD154 pathway (Caux et al., 1994). Additionally, immune responses are associated with increased recruitment of APCs to inflamed lymph nodes (MartIn-Fontecha et al., 2003) which promotes T cell acquisition of both TCR and costimulatory signals.

Expression of the CD28-family member Cytotoxic T Lymphocyte Antigen-4 CTLA-4 by both FoxP3^{+} T_{reg} (Wing et al., 2008) and activated T cells (Manzotti et al., 2006; Wang et al., 2012) reflects the requirement to regulate the availability of CD28-signaling in order to suppress immune activation and control T cell activation. Like CD28, Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) also interacts with CD80 and CD86 but with higher affinity. Furthermore, the functional consequences of interaction differ – whilst interaction with CD28 results in activation, CD80/CD86 binding to CTLA-4 inhibits T cell responses (Sansom and Walker, 2006). The importance of this CTLA-4 mediated immune regulation is demonstrated by the lethal lymphoproliferative disorder that is associated with the absence of CTLA-4 in mice (Tivol et al., 1995; Waterhouse et al., 1995). The loss of immune regulation in CTLA-4-KO mice is driven by CD28 since the interruption of CD28-signaling prevents disease (Mandelbrot et al., 1999). In humans, heterozygous mutations in CTLA-4 that result in impaired expression levels or function are associated with severe immune dysregulation and autoimmunity

(Schubert et al., 2014; Kuehn et al., 2014). Similarly, various CTLA-4 polymorphisms have been associated with risk factors for autoimmune diseases, for example rheumatoid arthritis (RA) (Li et al., 2012b), type I diabetes (Chen et al., 2013), primary biliary cirrhosis (Li et al., 2012a) and systemic lupus erythematosus (SLE) (Liu and Zhang, 2013).

Temporal changes in the prevailing interactions between members of the CD28/CTLA-4:CD80/CD86 system occur throughout the course of T cell activation. This occurs due to differences in binding-affinities between receptor-ligand interactions along with differential patterns of expression and receptor dimerization. For example, in contrast to CD28, CTLA-4 is not expressed by resting T cells and is upregulated following activation such that peak expression levels are achieved 24-48 hours following stimulation (Linsley et al., 1992; Walunas et al., 1994). Therefore, in the early stages of T cell stimulation, interactions between CD28 and CD80/CD86 predominate to promote costimulatory signaling. Biophysical studies demonstrate that, relative to their CD28-binding affinity, CD80 and CD86 bind to CTLA-4 with approximately 8-fold and 20-fold increased affinities respectively (Collins et al., 2002). Therefore, CTLA-4 outcompetes CD28 for CD80/CD86 binding, and this represents an important parameter of the capacity for CTLA-4 to inhibit CD28-driven T cell activation that contributes towards the resolution of T cell responses (Walker and Sansom, 2011). Where CD28 and CTLA-4 are both expressed, a particular bias towards CTLA-4:CD80 interactions are observed. This occurs due to several factors, firstly, because CTLA-4 binds to CD80 with particularly high affinity (K_d = 0.2µM) while its affinity for CD86 is

lower (K_d = 2.6µM) (Collins et al., 2002). Furthermore both CTLA-4 and CD80 form homodimers and interact bivalently; as such, CTLA-4:CD80 interaction results in the formation of oligomeric lattices at the T cell-APC interface which is thought to promote the avidity of interactions (Stamper et al., 2001). These factors suggest that CTLA-4 expression biases the CD28:CD80/CD86 pathway in favor of relatively low-affinity CD28:CD86 interactions (K_d = 20µM) (Collins et al., 2002). It has been suggested that this may influence the phenotype of activated T cells due to qualitative differences between CD80 and CD86-driven signals (Manzotti et al., 2006). Furthermore, it may partially underlie the observation that CD86 plays a more dominant role in costimulatory signaling compared to CD80 in that CD86-KO mice are more severely immunocompromised than CD80-KO mice (Borriello et al., 1997).

Some disagreement has existed as to the exact manner by which CTLA-4 functions to control T cell responses (Walker and Sansom, 2011). For example, CTLA-4 has been widely proposed to function by a cell-intrinsic mechanism i.e. by a direct effect upon the CTLA-4 expressing cell. This is proposed to occur through the generation of negative signaling delivered via CTLA-4 following its ligation by CD80/CD86. This is mediated by the recruitment of the protein tyrosine phosphatases Src-homology 2 domain-containing phosphatase (SHP)-2 (Marengère et al., 1996; Lee et al., 1998) and protein phosphatase 2A (PP2A) (Chuang et al., 2000; Parry et al., 2005) by the CTLA-4 cytoplasmic domain. Functioning in this manner, CTLA-4 ligation among conventional T cells has been found to reduce T cell proliferation and IL-2 production following anti-CD3/anti-CD28 stimulation

(Krummel and Allison, 1996; Brunner et al., 1999). One mechanism by which this may occur is through a CTLA-4 dependent inhibition of proximal TCR signaling events (Lee et al., 1998; Guntermann and Alexander, 2002) and prevention of lipid raft formation within the T cell membrane that serves to facilitate TCR-signaling (Martin et al., 2001). Additionally, CTLA-4 is thought to interfere with the TCR-stop signal whereby T cells make stable contacts with APCs upon antigen-stimulation (Schneider et al., 2006) and enhance the induction of FoxP3 and a regulatory phenotype by activated T cells (Zheng et al., 2006; Barnes et al., 2013). Additionally, it has been suggested that signals delivered via T_{reg} associated CTLA-4 serve to maintain T_{reg} suppressive function (Kong et al., 2011). However it is unclear how negative signaling in conventional T cells translates to positive signaling for T_{reg} function; this represents one of several inconsistencies that have arisen between studies investigating the mechanisms and functional outcomes associated with CTLA-4 mediated signaling in T cells (Walker and Sansom, 2015).

Several lines of evidence suggest that CTLA-4 may function independent of the generation of negative signals. For example, CTLA-4 mutants that do not express a cytoplasmic domain maintain a regulatory capacity (Masteller et al., 2000). Furthermore, bone marrow chimeric mouse models have demonstrated that CTLA-4-deficient T cells do not mediate lethal lymphoproliferative disease in the presence of CTLA-4-replete T cells, this suggests that CTLA-4 regulates the activity of other T cells in a cell-extrinsic manner (Bachmann et al., 1999; Chikuma and Bluestone, 2007). An important mechanism by which this occurs is via a process of CD80/CD86 trans-

endocytosis in which CD80/CD86 are bound by CTLA-4 then transferred and subsequently degraded within the CTLA-4 expressing cell (Qureshi, et al., 2011). The intracellular trafficking of CTLA-4 supports this process. In contrast to CD28, which is expressed at the cell surface, CTLA-4 expression is predominantly associated with intracellular vesicles (Sansom and Walker, 2006). CTLA-4 cycles to the cell surface in response to elevated intracellular Ca^{2+} concentrations occurring due to TCR stimulation and localizes at the APC-T cell interface at sites of TCR engagement where interactions with CD80 and CD86 occur (Linsley et al., 1996). However, surface CTLA-4 is rapidly internalized and this is mediated via a clathrin-dependent mechanism that is mediated by the interaction between the clathrin adaptor protein (AP)-2 and the CTLA-4 cytoplasmic domain via a tyrosine-based YVKM motif (Schneider et al., 1999). This endocytic motif is evolutionarily conserved which suggests selective pressure upon CTLA-4 receptor recycling and therefore that CTLA-4 intracellular trafficking is central to its function (Kaur et al., 2013). Upon internalization, CTLA-4 recycles to the cell surface via an association with recycling endosomes; alternatively, it can be directed to lysosomes where CTLA-4 and interacting CD80 and CD86 molecules that have been removed from the APC surface are degraded (Qureshi et al., 2012). Therefore, the net result of CTLA-4 activity is the downregulation of CD80/CD86 from the APC surface that denies CD28-ligand interactions and inhibits CD28-dependent T cell activation (Qureshi et al., 2011; Hou et al., 2015).

1.7. CD28-independent costimulatory pathways

Although T cell responses in CD28-KO animals are inhibited, they are not entirely absent (Shahinian et al., 1993). This is demonstrated by the enhanced progression of autoimmune diabetes in CD28-KO NOD mice (Lenschow et al., 1996). Similarly, CD28-KO mice remain susceptible to the induction of experimental autoimmune encephalomyelitis (EAE) (Chitnis et al., 2001). Cytotoxic T cell responses also occur in response to allostimulation in the absence of CD28 in that CD28-KO mice are capable of normal skin allograft rejection (Kawai et al., 1996) and acute lethal graft-versus-host disease (Speiser et al., 1997). These responses are mediated, at least in part, by various CD28-independent costimulatory pathways.

1.7.1. TNF-receptor superfamily members

Several members of the TNF-receptor superfamily have been suggested to provide costimulatory support to TCR-stimulation (Croft, 2009). Such alternative costimulatory molecules include OX40 which interacts with OX40-ligand on APCs; inhibition of OX40 ligation suppresses T cell activation above the level that is seen by anti-CD80/anti-CD86 alone (Akiba et al., 1999). Similarly, OX40L blockade prevented the development of EAE in CD28-KO mice but had no impact on wild-type (WT) controls suggesting that OX40 signals can compensate for an absence of CD28-costimulation (Chitnis et al., 2001). This apparent costimulatory activity occurs relatively late during T cell activation since OX40 is only expressed by activated T cells (Weinberg et al., 1998) where maximal expression appears dependent upon CD28-signals (Akiba et al., 1999; Walker et al., 1999). Acting in this way, OX40 signals

appear to fine-tune T cell responses via effects upon T cell differentiation, for example, by biasing T cell activation towards a T_h2 type response by promoting IL-4 production by effector T cells (Ohshima et al., 1998).

Similar to OX40, in the presence of CD28, signals delivered by the TNF-superfamily members 4-1BB and CD27 modulate T cell effector function and the generation of memory T cells (Vinay and Kwon, 1998; Hendriks et al., 2000; Wen et al., 2002; Denoeud and Moser, 2011). However, 4-1BB ligation can also compensate for an absence of CD28 to support the generation of T cell activation (DeBenedette et al., 1997; Bukczynski et al., 2003). Similarly, CD27 signals enhance TCR-driven T cell proliferation (Kobata et al., 1994; Hintzen et al., 1995), though this occurs in an IL-2 independent manner suggesting that this does not represent a true costimulatory signal (Watts and DeBenedette, 1999). Signals delivered via CD27 and 4-1BB are particularly important in $CD8^+$ T cell responses (Shuford et al., 1997; Yamada et al., 2005) that are thought to be less dependent upon CD28-costimulation (Goldstein et al., 1998; Wang et al., 2000), at least in part due to the relative prevalence of $CD8^+CD28^-$ T cells (Borthwick et al., 2000).

1.7.2. CD28-superfamily members

The primary CD28-superfamily associated with the delivery of CD28-independent costimulatory signaling is ICOS. This molecule is structurally and functionally related to CD28 and binds to its ligand ICOS-L (B7-H2) via a FDPPPY motif that is related to the MYPPPY motif of CD28 and CTLA-4 (Chen and Flies, 2013). Whilst $CD4^+$ T cells constitutively express CD28,

ICOS is only expressed upon activation (Simpson et al., 2010); furthermore, CD28 signals appear necessary for maximal ICOS expression (McAdam et al., 2000). Transcriptional profiles induced by the CD28-superfamily member ICOS show striking similarity to that induced by CD28 (Riley et al., 2002). As such, ICOS ligation provides efficient costimulation of *in vitro* T cell activation (Hutloff et al., 1999; Yoshinaga et al., 1999). Similarly, ICOS substitutes for CD28-costimulation to drive T cell responses in CD28-KO mice (Suh et al., 2004) particularly when mice are crossed onto a background that confers constitutive ICOS expression (Linterman et al., 2009). *In vivo* models also demonstrate that ICOS signals not only cooperate with CD28 to promote T cell activation and proliferation but also are necessary for IL-4 production by activated T cells and T cell dependent B cell responses (Dong et al., 2001; Tafuri et al., 2001). As such, it has been suggested that ICOS signals are indispensable for humoral immune responses in CD28-KO mice (Suh et al., 2004). These functions underlie the importance of ICOS expression by T_{fh} cells (Simpson et al., 2010)

In contrast to ICOS, the CD28-superfamily member PD-1 promotes the inhibition of T cell responses following its ligation by PD-L1 (B7-H1) and PD-L2 (B7-DC) (Francisco et al., 2010). PD-1 deficient mice are susceptible to the development of autoimmunity (Nishimura et al., 1999; 2001; Wang et al., 2005; Zhang and Braun, 2014) but display a less aggressive phenotype than CTLA-4-KO mice (Riella et al., 2012b). Indeed, PD-1 functions differently to CTLA-4 (Parry et al., 2005) and it has been suggested that blockade of CTLA-4 and PD-1 leads to a synergistic enhancement of T cell responses in cancer

therapy (Intlekofer and Thompson, 2013). Underlying the tolerogenic function of PD-1 is an inhibition of T cell activation signaling (Sheppard et al., 2004; Yokosuka et al., 2012; Wei et al., 2013) and positive effects upon T_{reg} differentiation and function (Francisco et al., 2009; Chen et al., 2014).

Novel B7 and CD28 superfamily members have been described where the interaction partners and consequences of interaction are not fully understood (Greenwald et al., 2005). For example, B7-H3 is expressed by fibroblasts, dendritic cells, and monocytes (Chapoval et al., 2001; Tran et al., 2008). The consequence of its interaction with an as yet unidentified receptor on activated T cells is controversial; some studies identify a role in the costimulation of T cell responses (Chapoval et al., 2001; Luo et al., 2004) and others suggest a co-inhibitory function (Ling et al., 2003; Leitner et al., 2009). Additionally, B and T lymphocyte attenuator (BTLA) is expressed by activated T cells and participates in inhibitory interactions with an as yet unidentified counter receptor (Watanabe et al., 2003). Several novel interactions between known B7 and CD28 superfamily members have also been described. For example, the interaction between PD-L1 and CD80 has been proposed to inhibit T cell responses (Butte et al., 2007; Park et al., 2010) whilst ICOS-L is thought to deliver a costimulatory signal via CD28 (Yao et al., 2011). The relative importance of these pathways is unclear but is likely to confer an added dimension to the control of T cell responses. The various interactions between CD28 and B7-superfamily members are summarized in **fig. 1.2**.
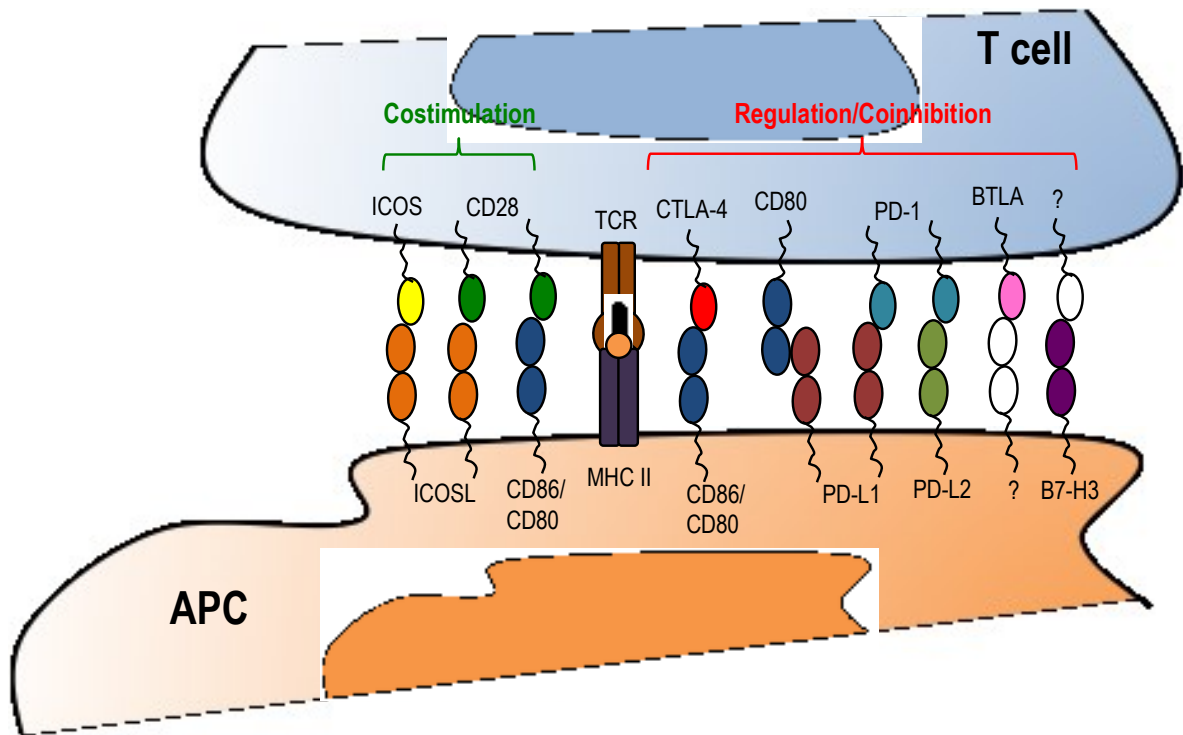
Fig. 1.2. Interactions between CD28- and B7- family members. CD28 is constitutively expressed by CD4^{+} T cells and interacts with the B7-family members CD80 and CD86. CD28 ligand binding results in signalling that positively impacts upon T cell activation and differentiation. These signals are regulated by CTLA-4 which also binds to CD80 and CD86 but with higher affinity. Additional costimulatory signals are delivered following the ligation of ICOS upon activated T cells by ICOS-L. Productive interactions between ICOS-L and CD28 has also been proposed. PD-1 participates in inhibitory signalling following ligation by PD-L1 and PD-L2. Additionally, PD-L1 has been found to interact with CD80 to inhibit T cell activation. BTLA on T cells and B7-H3 on APCs inhibit T cell activation in association with as yet unidentified interaction partners.

1.7.3 CD40-CD154

The CD40-CD154 signalling axis has been recognized as a crucial CD28 independent costimulatory pathway. CD154 is upregulated by activated T cells whilst CD40 is widely expressed by APC (Grewal and Flavell, 1996). Interactions between these molecules results in bidirectional signalling that induces change to the activation status and differentiation of both the T cell and the APC. For instance, CD154 engagement generates a costimulatory signal that facilitates TCR driven activation and proliferation (Wu et al., 1995; Munroe and Bishop, 2007). With regards to the APC, CD40 ligation induces an increase in the expression of costimulatory molecules (including CD80 and CD86) and MHC-II (Ma and Clark, 2009), whilst in macrophages, signals delivered via CD40 promote activation including the production of proinflammatory cytokines such as TNFα and IFNγ (Stout et al., 1996; Buhtoiarov et al., 2005). The absence of CD154 expression results in X-linked hyper IgM syndrome, which is characterized by a defect in germinal center formation and B cell isotype switching occurring as the result of reduced T_{fh} cell differentiation and function (Ma and Deenick, 2014).

Importantly, the CD40-CD154 pathway has been found to represent a prominent signaling axis in transplantation rejection. As such, blocking antibodies against both CD154 and CD40 have been found to promote allograft survival in both mice and humans (Kinnear et al., 2013). It is thought that this at least partly involves the loss of CD80 upregulation following CD40 signaling in APC subsets (Hancock et al., 1996). However, a synergistic relationship between CD154 and CD80/CD86 blockade has been reported

(Yamada et al., 2002), suggesting that the impact of CD154 blockade upon of CD80/CD86 expression does not exclusively account for the defective alloresponse and likely involves costimulation delivered via CD154. Taken together, these lines of evidence demonstrate the capacity for modification of multiple aspects of an adaptive immune response induced by CD40-CD154 signals.

1.7.4 Adhesion molecules

DC-T cell interactions mediated by adhesion molecules have been associated with direct costimulatory activities by means of increased T cell activation and proliferation including CD2-CD58 (Davis and van der Merwe, 1996), ICAM-3-DC SIGN (Martinez, 2005), CD6-CD166 (Hassan et al., 2004) and CD31 interactions (Elias et al., 1998). A widely cited interaction with a primary role in adhesion and a secondary role in costimulatory signaling is mediated by LFA-1-ICAM-1 interactions (Wang et al., 2009). In CD28-KO mice it has been suggested that ICAM-1 is crucial for T cell responses (Gaglia et al., 2000). However, it seems likely that this occurs primarily through the stabilization of T cell interactions that enhance the delivery of TCR signals (Bachmann et al., 1997).

1.7.5. Cytokines/Chemokines

Proinflammatory cytokines have been proposed to act as 'signal 3' during T cell stimulation by guiding T cell differentiation and effector function (Curtsinger and Mescher, 2010). However, it is recognized that these signals have a direct effect upon T cell activation and proliferation. IL-1 represents an

example of this and has been widely reported to act as a costimulatory signal, in particular for T_h2 cell proliferation (Lichtman et al., 1988; Huber et al., 1998). In this context it has been found that IL-1 acts in a comparable manner to CD28 by synergizing with TCR signals to promote NF-κB activation (McKean et al., 1995). Subsequent effects upon proliferation are at least partially associated with an upregulation of IL-2 signaling (Kalli et al., 1998). Similarly, T cell activation and cytokine production is elevated in response to TCR-stimulation in combination with various immunomodulatory cytokines, for example, IL-4 (Brown et al., 1988; Gett and Hodgkin, 2000) and IL-12 (Germann et al., 1993). Additionally, TNFα and IL-6 provide a costimulatory signal for CD8^{+} T cell activation driven by anti-CD3 stimulation (Sepulveda et al., 1999). Inhibition of IL-15 prevents CD28-independent CD8^{+} T cell driven transplant rejection (Ferrari-Lacraz et al., 2001). This cytokine stimulation occurs to the extent that T cell activation, in particular effector memory T cells, proliferate and acquire effector function in response to cytokine treatment, either IL-2 or IL-15 in combination with TNFα and IL-6, in the absence of overt TCR stimulation (Unutmaz et al., 1994; Sebbag et al., 1997; Brennan et al., 2002; 2008).

Another form of interaction that regulates T cell activation results from signaling by the chemokine receptors CCR5, CXCR12 and CCR7. These have been shown to provide costimulation and modulate T cell activation thresholds (Molon et al., 2005; Gollmer et al., 2009).

1.8. Therapeutic manipulation of the CD28 costimulatory pathway

Despite the fact that several costimulatory pathways can, to some extent, compensate for a relative absence CD28, it is clear that CD28 is the apical checkpoint for T cell costimulation. Importantly, CD28-signaling is thought to play a key role in the pathogenesis of T cell mediated autoimmunity. For example, CD28 signals are required for the optimal induction of collagen-induced arthritis (CIA) (Tada et al., 1999a), murine lupus (Tada et al., 1999b), EAE (Chang et al., 1999), autoimmune neuritis (Zhu et al., 2001) and graft-versus-host disease (Yu et al., 1998). Furthermore, the particularly strong association between CTLA-4 polymorphisms and autoimmunity suggests that a failure to regulate CD28-signaling leads to a breakdown in tolerance (Gough et al., 2005). It could be suggested that polymorphisms in CTLA-4 are more strongly associated with susceptibility to autoimmunity than CD28 itself (Ahmed et al., 2001; Djilali-Saiah et al., 2001; Wood et al., 2002; Ban et al., 2003). This highlights the importance of regulation of the CD28:CD80/CD86 axis such that under homeostatic conditions tolerance predominates and that during infection that immune responses can be promoted. Various strategies have been utilized, either to interfere with the availability of CD28-costimulation so as to skew this system in favor of immune tolerance, or, to promote CD28-signaling thereby enhancing immune responses in cancer therapy. These strategies are summarized in **fig. 1.4.**

1.8.1. Blocking anti-CD80/anti-CD86

Blocking antibodies to CD80 and CD86 were developed as a first strategy for blocking the CD28/CTLA-4/B7 pathway but have not been widely used in a clinical setting. Results from animal models in which they were tested are somewhat confusing as anti-CD86 inhibited autoimmune diabetes in non-obese diabetic (NOD) mice (Lenschow et al., 1995a), but enhanced disease in models of EAE (Racke et al., 1995; Miller et al., 1995). By contrast, anti-CD80 alone accelerated diabetes but reduced EAE pathology (Lenschow et al., 1995; Miller et al., 1995). In transplantation models, several studies identified that a combination of anti-CD80 and anti-CD86 were required for optimal suppression of T cell activation, but that anti-CD86 alone was more effective than anti-CD80 (Lenschow et al., 1995b; Pearson et al., 1997; Woodward et al., 1998). These results are reminiscent of CD80 and CD86 knock-out mice in which CD86 KO display the more profoundly immunocompromised phenotype (Borriello et al., 1997). Furthermore, they highlight the necessity to ensure adequate blockade of CD86 in immunosuppressive treatment. An additional limitation of these strategies is that the relative roles of CD80 and CD86 in CD28 and CTLA-4 biology remain somewhat unclear. It is possible that a better understanding of any differences may facilitate more subtle and targeted manipulation of T cell activation.
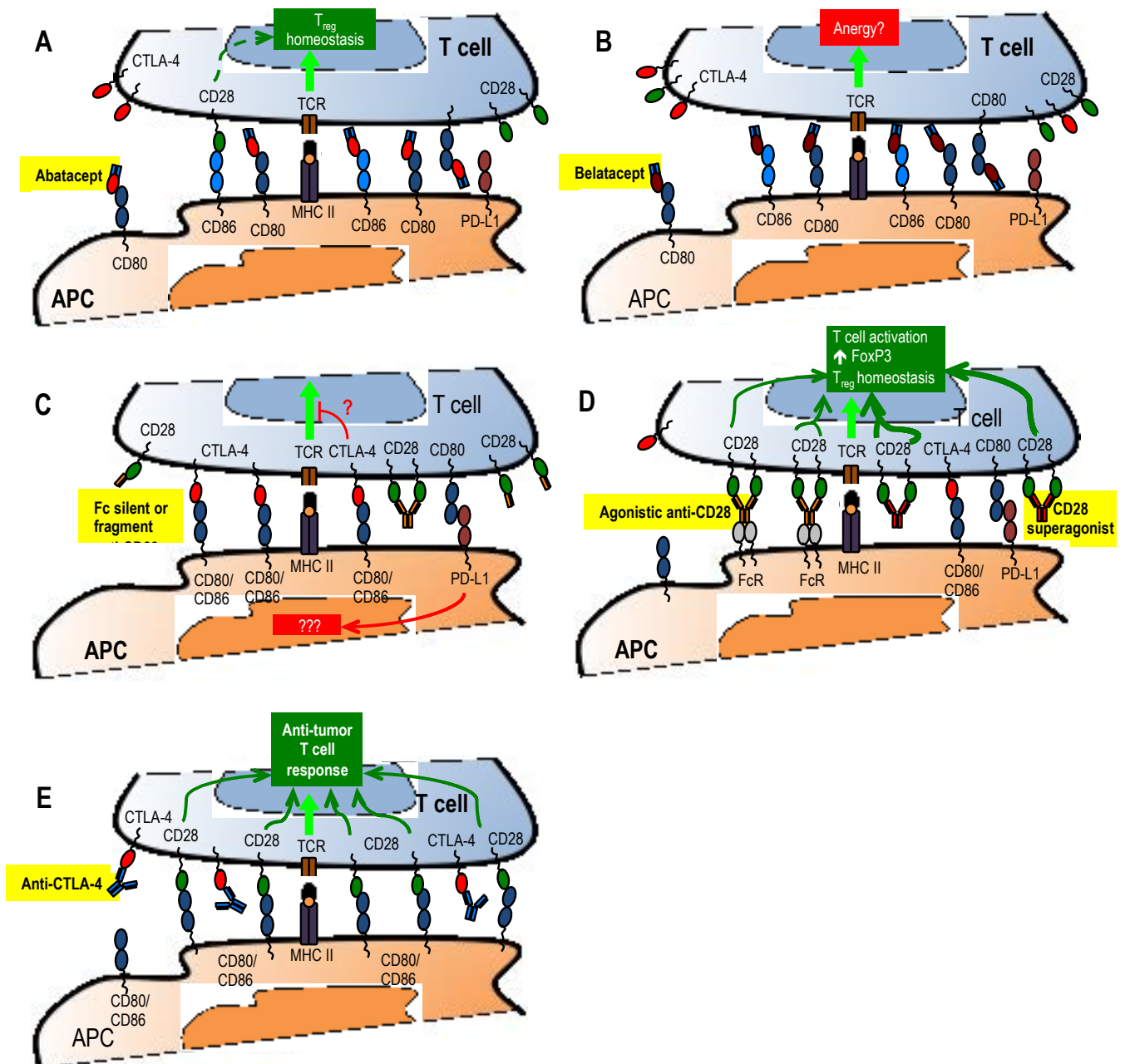
Fig. 1.3. Outcomes associated with manipulation of the CD28/CTLA-4 pathway. Several strategies are illustrated where the therapeutic agent is in yellow. **A**) Abatacept inhibits T cell activation by preventing CD28 interactions with CD80 (dark blue) and CD86 (light blue). A low relative affinity for CD86 may facilitate low level CD28-costimulation that is sufficient to maintain T_{reg} homeostasis. **B**) Belatacept displays an elevated affinity CD80 and CD86 which confers more potent blockade of CD28-costimulation. **C**) CD28 can be targeted directly with Fc-silent or anti-CD28 fragment antibodies to inhibit costimulatory signaling. This approach may allow other potentially beneficial interactions involving CD80 such as with CTLA-4 or PD-L1. The potential for unwanted T_{eff} stimulation can occur in this instance. **D**) Agonistic anti-CD28 can be utilized to inhibit T cell responses via positive effects upon T_{reg} generation and homeostasis. **E**) Anti-tumor responses can be elicited in association with CTLA-4 blockade. Here, the loss of CD28-regulation by CTLA-4 promotes T cell activation.

1.8.2. Soluble CTLA-4 analogues

CTLA-4 has been exploited as an inhibitor of CD28-costimulation mainly in the context of the fusion protein CTLA-4-ig. By competing with CD28 for ligand binding, CTLA-4-ig has been shown to inhibit T cell responses in models of EAE (Khoury et al., 1995), CIA (Webb et al., 1996), SLE (Finck et al., 1994) and autoimmune diabetes (Lenschow et al., 1995a). Furthermore, CTLA-4-ig promoted allograft survival in several transplantation models (Porier, et al. 2011).

A humanized version of CTLA-4-ig incorporating a modified IgG Fc domain (Abatacept; Bristol-Myers Squibb) is approved by the FDA and EMA for the treatment of moderate to severe RA in patients who have not responded to other disease-modifying anti-rheumatic drugs (DMARDs). Its efficacy in the treatment of RA has been verified in numerous placebo-controlled clinical trials in which it shows similar efficacy to other biological RA therapies such as TNFα antagonists (Buch et al., 2008).

Despite the utilization of abatacept in the treatment of RA, an apparent lack of efficacy of abatacept was found in non-human primate models of transplantation (Larsen et al., 2005). This was proposed to occur due to the inherently low affinity of native CTLA-4 for CD86 and led to the development a higher affinity CTLA-4-ig variant, LEA29Y (Belatacept; Bristol-Myers Squibb) which differs in two amino acid residues within the CTLA-4 binding region. This modification translates into around 4 and 2 times elevated avidities for

CD86 and CD80 respectively with an associated increased potency of CD28-blockade (Larsen et al., 2005). Belatacept has subsequently been approved for clinical use in immunosuppression regimens following renal transplantation as a result of favorable results from clinical trials. In the context of renal transplantation, alternatives to T cell antagonism with calcineurin-inhibitors such as cyclosporine A (CsA) are particularly desirable due to the long-term nephrotoxic side-effects of such drugs (Nankivell et al., 2003). In a phase III clinical trial evaluating the efficacy of belatacept in immunosuppression following renal transplantation (Vincenti et al., 2010), belatacept promoted equivalent patient/graft survival at a 12 month end point in comparison to CsA based treatment. Furthermore, compared to CsA, belatacept was associated with the preservation of renal function as indicated by mean glomerular filtration rate. However, belatacept treatment was associated with an increased prevalence of acute rejection when compared to CsA treated patients. This effect was not observed in the previous phase II trials (Vincenti et al., 2005) and remains to be confirmed. However, if such enhanced acute rejection is a real side-effect it is clearly of clinical significance and the mechanisms underlying it deserve considerable attention.

Despite a relatively clear rationale for the application of CTLA-4-ig in terms of its ability to outcompete CD28 for B7 ligand, the actual mechanisms underlying clinical responses are controversial. Despite results which demonstrated the induction of T cell anergy following CTLA-4-ig treatment *in vitro* (Tan et al., 1993), there is little evidence to suggest that either abatacept or belatacept promote long-term tolerance. For example, in a non-human

primate transplantation model, a treatment regimen including belatacept promoted allograft survival, however, withdrawal of belatacept therapy was followed by acute rejection (Lo et al., 2013). It is therefore possible that rather than completely attenuating T cell activation, which may be predicted based upon the perspective that costimulation is essential for T cell activation, that the clinical effects of abatacept could be attributed to qualitative differences in T cell differentiation. For example, it has been suggested that abatacept prevents T_{fh} differentiation that results in defective T cell dependent-B cell responses (Platt et al., 2010). Induction of a negative signal into the APC following B7 engagement of CTLA-4-ig has also been suggested as a mechanism of CTLA-4-ig action. This signal has been proposed to alter proinflammatory cytokine expression (Wenink et al., 2012), migration (Bonelli et al., 2012) or involve induction of indoleamine 2,3-dioxygenase (IDO) with subsequent immunomodulatory effects upon tryptophan metabolism (Grohmann et al., 2002). However, induction of IDO may be a product of the CTLA-4-ig variant used since abatacept inhibited T cell responses independent of IDO (Davis et al., 2008). Furthermore, treatment of APCs with either abatacept or belatacept *in vitro* failed to induce transcriptional changes in APCs (Carman et al., 2009).

1.8.3. Blocking anti-CTLA-4 antibody

The role of CTLA-4 as a negative regulator of CD28-signaling has led to the development of blocking CTLA-4 antibodies in cancer therapy with the aim of promoting T cell anti-tumour responses. The efficacy of this approach has been demonstrated in clinical trials evaluating the fully humanized anti-CTLA-

4 antibodies ipilimumab and tremelimumab in numerous tumour types (Grosso and Jure-Kunkel, 2013). Significantly, ipilimumab is now approved for the treatment of advanced or metastatic melanoma. This therapy induces an increase in the ratio of T_{eff} relative to T_{reg} and the basis of how this works demonstrates the mechanism of CTLA-4 mediated control of immunity. Whilst a decrease in absolute tumour-infiltrating T_{reg} counts has been reported in a murine melanoma model, this was found to occur in an FcR dependent manner by antibody-dependent cell-mediated cytotoxicity (ADCC) rather than interfering with any requirement by T_{reg} for CTLA-4 ligation (Simpson et al., 2013). Furthermore, in patients with either melanoma or metastatic renal cell cancer, ipilimumab therapy has been found to promote both CD4^{+} and CD8^{+} T cell activation rather than exerting effects on T_{reg} function (Maker et al., 2005). This is consistent with a model of CTLA-4 functioning in a cell extrinsic manner. For example, the absence of CTLA-4 mediated trans-endocytosis leads to elevated B7 expression by APCs that promotes the availability of CD28-signaling and thereby enhances T cell activation. Accordingly, melanoma patients who achieve clinical responses to therapy are more likely to experience immune-related adverse events (i.e. to break tolerance in association with anti-CTLA-4 therapy) (Attia et al., 2005). Whilst this is unsurprising when considering that CTLA-4 KO mice exhibit a lethal lymphoproliferative disorder (Tivol et al., 1995), it demonstrates the importance of CTLA-4 to the regulation of T cell activation thresholds. Furthermore, it shows that by modifying this threshold via CTLA-4 inhibition that T cell responses, albeit poorly controlled ones, can be generated.

1.8.4. CD28-specific antibodies

A drawback to B7 blockade is its potential to inhibit other important co-inhibitory and co-stimulatory interactions, an example being the interaction of CD80 with PD-L1 (Butte et al., 2007; Park et al., 2010). At present the precise role of these additional interactions is poorly understood but may nonetheless impact *in vivo* function. One way to bypass this is to directly target CD28 using antagonistic anti-CD28. However, where the aim is to suppress T cell activation, these antibodies need to be screened for agonist activity (Gardner et al., 2014). A degree of complexity exists in predicting the functional outcome of bivalent anti-CD28 in terms of agonistic vs. antagonistic properties, for example, the anti-CD28 mAb JJ319 behaves as an agonist during *in vitro* T cell stimulation and an antagonist using *in vivo* systems (Poirier et al., 2011). To avoid these difficulties, interest has turned towards the use of monovalent Fab fragments in costimulation blockade which are incapable of clustering CD28-molecules and consequential costimulatory signalling following cross-linking (Vanhove et al., 2003). For example, a monovalent anti-CD28 domain antibody inhibits *in vitro* T cell proliferation and cytokine production and T cell-dependent B cell responses in cynomolgous macaques (Suchard et al., 2013). Furthermore, in cynomolgous macaques, monovalent antagonistic anti-CD28 inhibits acute and chronic rejection of heart allografts especially when used in association with CsA (Poirier et al., 2010).

An alternative approach is to utilize the pro-regulatory function of CD28 using agonistic anti-CD28. A profound example of the agonistic capacity of anti-

CD28 is demonstrated by the induction of T cell activation and proliferation in the absence of overt TCR stimulation (Siefken et al., 1997; Tacke et al., 1997). Furthermore, these superagonists have been found to promote tolerance in several *in vivo* models via an amplification of T_{reg} (Beyersdorf et al., 2005a; Rodríguez-Palmero et al., 2006; Kitazawa et al., 2008; Beyersdorf et al., 2009; Wang et al., 2010b). Nevertheless, initial use of the CD28-superagonist TGN1412 in clinical trials was halted after treatment induced severe adverse events mediated by a cytokine-release syndrome (Hünig, 2012). This has been proposed to occur due to an unexpected CD28-driven hyper stimulation of effector memory T cells (Römer et al., 2011). Further studies have suggested that the use of TGN1412 at much lower concentrations than the initial clinical trial can result in the selective expansion of T_{reg} without influencing proinflammatory cytokine expression by conventional T cells (Tabares et al., 2014).

1.9. Rheumatoid arthritis: The role of T cells

RA is a chronic, inflammatory autoimmune disease that affects 0.5-1% of the population with a female to male ratio of 3:1 (Humphreys et al., 2013). The characteristic foci of the inflammatory process are the peripheral joints where the interactions between a mixed inflammatory infiltrate and an expanded stromal network mediate synovial hyperplasia and ultimately bone and cartilage destruction. Additional systemic pathology including cardiovascular and pulmonary involvement can also contribute towards adverse clinical

outcomes including an elevated mortality rate among RA patients (McInnes and Schett, 2011).

Genetic and environmental risk factors play an important and interacting role in the development and progression of RA (Yarwood et al., 2014). Studies of RA heritability in twin studies suggest that genetic risk factors accounts for just 60% of RA disease susceptibility (MacGregor et al., 2000). This is indicative of a strong environmental component to RA susceptibility. Nevertheless, RA is a heterogeneous disease where genetic risk factors cluster with specific environmental risk factors between patient sub-groups. The strongest genetic association with RA is provided by the MHC-II locus and is suggestive of a role played by $CD4^+$ T cells in RA susceptibility, perhaps by responding to antigens presented by MHC molecules.

Recent advances in treatment strategies, for example, the use of biological therapies, have improved prognosis for patients including the potential, in some patients, to induce disease remission and avoid the risk of the development of erosive disease (Klarenbeek et al., 2010) particularly when treatment is started early after symptom onset (van der Linden et al., 2010). A number of strategies that have been successfully utilized in the treatment of RA target (or at least have the potential to target) T cells, either directly or indirectly. A direct effect upon T cell activation and differentiation can be mediated by drugs such as CsA, abatacept, tocilizumab (an anti-IL-6 receptor mAb). Indirect effects include the inhibition of APC maturation (e.g. with TNFα

antagonists). The success would be predicted based upon the viewpoint that T cells play a central role in RA pathology (Cope et al., 2007)

1.9.1. The role of T cells in early RA

T cells are widely held to play an important role in RA pathogenesis. However, in contrast to other organ specific autoimmune diseases, the identification of a defined joint-specific autoantigen underlying the initiation of RA has been problematic (Cope et al., 2007). However, recent studies have suggested that RA may be associated with immunity towards neo-epitopes created by protein post-translational modifications including citrullination (Schellekens et al., 1998), carbamylation (Shi et al., 2011) or acetylation (Juarez et al., 2015). The best-characterized example is protein citrullination. Anti-citrullinated peptide antibodies (ACPA) can be detected prior to symptom onset (Nielen et al., 2004) and this facilitates the detection of ACPA as a diagnostic and prognostic indicator for RA (Zendman et al., 2006). A similar role for post-translational modifications of antigen is seen in other autoimmune diseases, for example, coeliac disease (which also has a strong HLA association) where the antigen (gluten) is well defined but deamidation catalyzed by transglutaminase 2 affects antigen presentation (Sollid and Jabri, 2011).

A role played by post-translational protein modifications such as citrullination in the development of the autoimmune response underlying RA has a genetic and environmental basis that is consistent with RA etiology. For example, citrullination promotes the stability of pMHC complexes and subsequent T cell priming in mice that are engineered to express the shared epitope (Hill et al.,

2003). The observation that the shared epitope could represent a risk factor for the initiation of immune responses towards citrullinated peptides has been supported by the observation that the shared epitope positively correlates with patients who display ACPA seropositivity (Huizinga et al., 2005). However, the shared epitope/ACPA paradigm also has a significant environmental basis. For instance, smoking represents an important risk factor for the development of RA (Stolt et al., 2003). By inducing peptide citrullination, previous smoking strongly correlates with the presence of ACPA, furthermore, this relationship was only observed in the presence of the shared epitope (Klareskog et al., 2006). Thus an interaction between a genetic risk factor (HLA-DR β1 shared epitope) and an environmental risk factor (smoking) interact to promote the development of ACPA-seropositive RA. This observation is consistent with RA initiation occurring at mucosal surfaces where the generation of citrullinated peptides occurs not only through exposure to tobacco smoke but also the mucosal microbiome (Catrina et al., 2014). Additionally, these studies demonstrate the concept of RA as a heterogeneous disease in that ACPA-seropositive RA is strongly associated with HLA-DR β1 alleles but ACPA-seronegative RA is not (Klareskog et al., 2009). The clustering of RA genetic risk factors and features of pathology within RA patient subgroups suggest that various biomarkers could be utilized to predict patient prognosis and optimise treatment strategies (Choy et al., 2013).

Several further genetic risk factors for the development of RA are associated with T cell activation and signaling. For example, PTPN22 encodes a phosphatase that negatively regulates Lck following TCR stimulation and an

allelic variant (620W) is associated with several autoimmune diseases including RA (Gregersen et al., 2006). It should be noted that the association between PTPN22 and RA does not automatically implicate T cells as the causative cell type underlying the development of RA since PTPN22 variants may function in other leukocytes e.g. neutrophils (Bayley et al., 2015). However, an association between mutations in the negative regulators of T cell activation signaling CTLA-4 and PD-1 and RA development have also been reported (Lin et al., 2004; Prokunina et al., 2004). Additionally, polymorphisms within the *IL2RA* and *IL2RB* genes that encode the IL-2R α and β chains respectively appear to correlate with RA (Cope et al., 2007) and could theoretically affect either T cell activation or T_{reg} homeostasis. Together, these studies provide genetic evidence that suggest that T cells play a role in the development and pathology of RA.

1.9.2. The role of T cells in established RA

T cells play a prominent role in the inflammatory processes occurring within affected joints in RA. Here T cells infiltrate the synovial membrane and interact with other lymphocytes. In some cases T cells coalesce with B cells to form tertiary lymphoid like structures that potentially facilitate B cell differentiation and high-affinity autoantibody production (Pitzalis et al., 2014). Additionally, T cells interact with and modify the behavior of resident stromal and myeloid cells. For example, T cells promote the release of proinflammatory mediators by monocytes, macrophages and fibroblasts during *in vitro* co-culture experiments (McInnes et al., 1997; Sebbag et al., 1997; Tran et al., 2007; van Hamburg et al., 2011). T_h17 cells appear to play a

particularly prominent role in mediating inflammation in RA. This may be predicted based upon the observation of impaired induction of CIA in IL-17 deficient mice (Nakae et al., 2003). Similarly, in humans, patients with early RA display increased T_h17 cell frequencies in the peripheral blood along with a selective enrichment of T_h17 cells in synovial fluid samples (Leipe et al., 2010). Additionally, in patients with established RA, disease activity positively correlates with T_h17 frequencies (Leipe et al., 2010). Although $FoxP3^+$ T_{reg} are present within the synovial lining of inflamed joints, these display a reduced suppressive capacity within an overwhelmingly inflammatory environment (Nie et al., 2013; Gao et al., 2015) and an increased tendency towards T_h17 plasticity (Wang et al., 2015b).

A key feature of the T cell response in rheumatoid arthritis is its non-resolving nature (Cope, 2008). However, this does not necessarily mean that the activity and contribution the T cells are consistent throughout the course of RA pathology. For example, cytokine analysis within synovial fluid samples shows a number of T cell derived cytokines including IL-2, IL-4 and IL-17 in samples derived from early RA patients but not from established RA patients (Raza et al., 2005). Indeed, T cells from established arthritis demonstrate an anergic phenotype that is characterized by hyporesponsiveness to activation signals and an absence of IL-2 production (Firestein et al., 1988; Allen et al., 1995). Nevertheless, synovial T cells also display resistance to apoptosis (Salmon et al., 1997; Raza et al., 2006) thus their proinflammatory interactions with resident cells and other lymphocytes persist. These observations appear to suggest a pattern where T cells actively initiate an autoimmune inflammatory

response in early RA but play a more passive role in established RA through, for example, interactions with resident stromal cells. It could therefore be suggested that where T cells are targeted in RA therapy, this would be most effective when treatment is administered early after symptom onset where T cells play a more active role in mediating inflammation. Indeed, the APIPPRA (Arthritis Prevention in the Pre-clinical Phase of Rheumatoid Arthritis with Abatacept) trial is currently evaluating the efficacy of abatacept therapy in individuals who are at high-risk for the development of RA prior to joint swelling with the aim of inhibiting T cell activation at a very early stage of pathology.

1.9.3. The role of abatacept in the treatment of RA

The inhibition of CD28-costimulation by abatacept has been utilized as a strategy in the treatment of RA. The safety and efficacy of this approach has been demonstrated by several phase II and phase III clinical trials (Moreland et al., 2002; Kremer et al., 2003; 2005; Genovese et al., 2005; Kremer et al., 2006a; Schiff et al., 2008). Briefly, the phase III ATTAIN (Abatacept Trial in Treatment of Anti-TNF Inadequate Responders) trial demonstrated that abatacept was effective for the treatment of patients with established RA (with a mean duration of disease of 12.2 years) who had not responded to TNFα inhibition, i.e. a difficult to treat population with longstanding disease (Genovese et al., 2005). Furthermore, a direct comparison of abatacept with a TNFα blocking agent (infliximab) in the phase III ATTEST (Abatacept or infliximab vs. placebo, a Trial for Tolerability, Efficacy and Safety in Treating rheumatoid arthritis) study demonstrated that the efficacy of abatacept was

equivalent to that of TNFα inhibition in a population of RA patients defined by their prior inadequate response to methotrexate (Schiff et al., 2008). These studies contributed to the utilization of abatacept in the treatment of RA patients who have failed several conventional and biological DMARDs and to the approval of this drug in the UK by the National Institute for Health and Care Excellence (NICE) (NICE, 2013). Whilst these trials have shown the therapeutic benefits of abatacept in **controlling** disease activity in established RA, the ADJUST (Abatacept study to Determine the effectiveness in preventing the development of rheumatoid arthritis in patients with Undifferentiated inflammatory arthritis and to evaluate Safety and Tolerability) trial has suggested that abatacept could be utilized to delay RA development in patients with early undifferentiated arthritis (Emery et al., 2010). This observation is consistent with the idea of T cells playing an active role in the initiation and early phases of RA and support the suggestion that abatacept may be particularly useful for the treatment of patients with early RA.

1.10. Project Aims

A consistent finding of studies evaluating the efficacy of abatacept for the treatment of RA is a degree of variability between patient responses to treatment. For example, in the ATTAIN patient cohort with active RA who did not respond well to anti-TNFα therapy, only 50% of abatacept-treated patients achieved an American College of Rheumatology (ACR)-20 response (denoting a 20% improvement in several disease activity indicators) (Genovese et al., 2005). Similarly, in the abatacept treatment arm of the

ATTEST study, only 67% patients achieved an ACR 20 response (Schiff et al., 2008).

These studies demonstrate that a proportion of patients achieve a negligible response to treatment and the factors underlying this are unclear. One possibility is that these patients may be refractory to all available treatment. Alternatively, and consistent with the idea of RA as a heterogeneous disease, this may represent a different RA sub-population in which T cells play a less prominent role. Finally, a key hypothesis in relation to this work is that under some conditions, CD28-costimulation may not always be essential to T cell activation and other stimulatory pathways may compensate for its absence. As such the initial the initial aim of this thesis is:

1. **To develop and utilise *in vitro* assays to identify factors and conditions that influence the relative contribution of CD28 toward T cell activation.** This will inform clinical decisions relating to improving responses and predicting outcomes of abatacept treatment.

One strategy to counteract the failure for abatacept to work in some RA this would be to combine abatacept with other treatments that target CD28-independent costimulatory pathways. An initial attempt to evaluate the feasibility of combining abatacept with anti-TNFα treatment did not enhance the efficacy of treatment and was associated with adverse events concerns including serious infections (Weinblatt et al., 2007), potentially due to the dual suppression of adaptive immunity and an important mediator of innate

immunity. Furthermore, this approach was hindered by a lack of clarity associated with the roles played by various CD28-independent costimulatory pathways.

2. **To use an understanding of conditions where CD28-independent activation occurs to investigate the potential utilization of abatacept in combination with other immunomodulatory agents in order to promote the outcome of therapy.** The potential for cooperation between abatacept and vitamin D3 towards the suppression of T cell activation will be investigated. Vitamin D3 supplementation has previously been found to promote the upregulation of CTLA-4 during T cell activation. This suggests the possibility for an overlap between abatacept and vitamin D3 mediated suppression of T cell activation. As such, the hypothesis underlying this area of work is that vitamin D3 supplementation will promote the efficacy of abatacept during *in vitro* T cell stimulation.

Chapter 2: Materials and Methods

2.1. Cell culture

Transfected Chinese hamster ovary (CHO) cells were cultured in DMEM (Life Technologies, Paisley, UK) supplemented with 10% (v/v) foetal bovine serum (FBS; Biosera Ltd, Uckfield, UK), 50U/mL penicillin and streptomycin (Life Technologies) and 200µM L-glutamine (Life Technologies) and incubated at 37°C in a humidified atmosphere of 5% CO_2. Cells were passaged upon confluence, typically every 48-72 hrs. CHO cell lines expressing CD80, CD86, FcRγII (CD32; FcR) or FcR/CD80 were generated by other laboratory members and as previously described (Qureshi et al., 2011).

2.2. PBMC isolation

PBMCs were isolated from leukocyte reduction system cones (National Blood Service, Birmingham, UK) by Ficoll-Paque (GE healthcare, Buckingham, UK) density gradient centrifugation. 25mL blood diluted 1:4 with PBS was layered onto 15mL Ficoll-Paque in 50mL Falcon tubes. Following 25 min centrifugation (1060g, 25 min) PBMCs were retrieved from the Ficoll-Plasma interface using sterile Pasteur pipettes. Subsequently, PBMCs were washed twice by centrifugation in PBS, once at 1060g for 10 min and once at 260g for 5 min. Cells were subsequently washed twice (490g, 5 min) in MACS buffer (2mM EDTA, 0.5% (w/v) BSA in PBS) followed by re-suspension at 100 x 10^6 PBMC/mL in MACS buffer.

2.3. CD4$^+$CD25$^-$ T cell purification

CD4$^+$CD25$^-$ T cells were purified using a custom EasySep™ CD4$^+$CD25$^-$ enrichment kit (StemCell Technologies, Meylan, France) according to manufacturer's instructions. Briefly, 50µL/mL PBMCs enrichment antibody cocktail was added and incubated for 5 min at room temperature. EasySep® Magnetic particles were then added at 100µL/mL PBMCs and incubated for 5 min at room temperature followed by purification using an EasySep® magnet according to manufacturer's instructions. Purified cells were washed twice by centrifugation in PBS (490xg, 5 min) and resuspended in RPMI medium. Cell purity was determined by immunofluorescence labeling and flow cytometric analysis and was frequently found to be ≥97% CD3$^+$CD4$^+$CD25$^-$.

2.4. Monocyte purification and culture

Monocytes were purified by negative selection using an EasySep™ monocyte enrichment kit (StemCell Technologies) according to manufacturer's instructions. Purified monocytes were washed by centrifugation twice in PBS (490xg, 5 min) and were resuspended at 1 x10^6 cells/well in 24 well plates. For monocyte-derived dendritic cell (DCs) differentiation, monocytes were cultured in RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 10% (v/v) foetal bovine serum (FBS; Biosera Ltd), 50U/mL penicillin and streptomycin and 200µM L-glutamine (subsequently referred to as RPMI/FBS) and in the presence of GM-CSF (800 U/mL; Berlex laboratories, Richmond,

CA, USA) and IL-4 (500 U/mL; Miltenyi Biotec, Bisley, UK) for 7 days, incubated at 37°C/5% CO_2. Where indicated, DCs were matured by exposure to 100ng/mL lipopolysaccharide (LPS) (Sigma) for 24hrs for analysis of surface markers.

2.5. CD4$^+$CD25$^-$ T cell stimulation

All T cell stimulations were performed in 96 well flat-bottom plates in RPMI-FBS. For DC-driven stimulations, 1×10^5 T cells per well were co-cultured with DCs at DC:T cell ratios of 1:5, 1:10, 1:20 or 1:40. Stimulation was generally carried out over a period of 5 days unless otherwise stated. Stimulations were treated with anti-CD3 (OKT3; 0.5µg/mL, unless otherwise stated). Alternatively, T cell stimulation was driven by toxic shock syndrome toxin (TSST)-1 at indicated concentrations (Toxin Technology, Sarasota, FL, USA). Analysis of TSST-1 driven activation was accompanied by identification of T cells expressing variable β2$^+$ (vβ2) TCRs by immunofluorescence labeling which display high affinity for TSST-1.

For CHO cell driven stimulations CHO-transfectants were glutaraldehyde fixed prior to stimulation. Briefly, 4×10^6 CHO cells were incubated in 1 mL 0.025% (w/v) glutaraldehyde for 3 minutes. Fixed cells were then washed once with RPMI/FBS then twice with PBS by centrifugation. Cells were subsequently resuspended in RPMI-FBS. For stimulation, 1×10^5 T cells were cultured in the presence of 0.5µg/mL anti-CD3 and fixed CHO transfectants at CHO:T cell ratio of 1:5 in RPMI-FBS incubated at 37°C for 5 days. Where indicated, CHO

FcR were used to mediate T cell activation in response to cross-linked anti-CD3 or anti-CD28 (clone 9.3) used at indicated concentrations, generally 0.5µg/mL.

Where indicated, T cells were also stimulated for 5 days with Dynabeads® Human T-activator CD3/CD28 beads (Life Technologies) according to manufacturer's instructions.

T cell stimulations were also variously treated with abatacept (20µg/mL; Bristol Myers Squibb), $1,25(OH)_2D_3$ (10nM; Sigma-Aldrich), IL-2 (200U/mL; Peprotech) or CsA (at indicated concentrations; Sigma-Aldrich). The concentration of $1,25(OH)_2D_3$ used were determined from previous experience (Jeffery et al., 2012) and similar to those used by others (Penna et al., 2007; Palmer et al., 2011). The vehicle for $1,25(OH)_2D_3$ was ethanol, which was diluted to a final concentration of 0.01% (v/v) during experiments; vehicle controls showed no effect upon experimental outcomes. PBS vehicle was used for all other reagents.

2.6. Flow cytometry

Antibodies used for immunofluorescence labeling are detailed in **table 2.1**. For analysis of cell surface proteins, cells were recovered following stimulation and washed once by centrifugation in PBS and incubated with relevant antibodies in PBS supplemented with 2% (v/v) goat serum (Sigma-Aldrich) for 30 min at 4°C. T cell counts per sample were determined relative

to AccuCheck counting beads (Invitrogen) in which the addition of 7µL beads represented 7000 counting beads. All data were acquired using a CyAn™ ADP Flow Cytometer (DakoCytomation, Ely, UK) and were analysed using FlowJo™ software (TreeStar, Ashland, OR, USA).

2.7. Intracellular FoxP3 and CTLA-4 staining

A FoxP3 staining buffer kit (eBioscience) was used for intracellular staining of FoxP3 and total CTLA-4 expression in accordance with manufacturer's instructions. Briefly, samples were added to 5ml polypropylene tubes and washed once by centrifugation in PBS (490g, 5 min). Cells were fixed by re-suspending in 500µg/mL FoxP3 Fixation/Permeabilization solution (eBioscience) and incubating at 4°C overnight. Cells were subsequently washed twice by centrifugation (490g, 5 min), once in PBS then with FoxP3 permeabilization Buffer (eBioscience). Cells were subsequently stained for FoxP3, CTLA-4 and CD25 expression for 45 min at 4°C then washed twice by centrifugation (490g, 5 min), again, once with FoxP3 Permeabilization Buffer (eBioscience) then once in PBS (490g, 5 min).

2.8. Phosflow staining

Staining for pSTAT5 was performed using a BD Phosflow™ buffer kit (BD Biosciences). Briefly, T cells were stimulated under indicated conditions, then washed by centrifugation in PBS (490g, 5 min). Cells were fixed by addition of an equal volume of BD Phosflow™ Fix buffer I (BD Biosciences) warmed to

37°C for 12 min. Cells were then washed by centrifugation in PBS-FBS (600g, 6 min). Cells were permeabilized by adding 1mL cold (-20°C) BD Phosflow™ Perm buffer III then incubated for 30 min at 4°C. Cells were then washed twice by centrifugation in 3mL PBS-FBS (600g, 6 min) and stained at room temperature with anti-pSTAT5 for 40 min followed by flow cytometric analysis.

2.9. Intracellular cytokine staining

For the detection of cytokine expression, T cells were stimulated for 5 days under indicated conditions and then restimulated for 4 hours with 50ng/mL PMA (Sigma-Aldrich) and 1µM ionomycin (Sigma-Aldrich) in the presence of 10µg/mL brefeldin A (Sigma-Aldrich). Cells were then washed by centrifugation (490g, 5 min), with PBS then fixed with 3% (w/v) paraformaldehyde in PBS for 12 min at room temperature. Subsequently cells were permeabilized with 0.1% (w/v) saponin in PBS and stained at room temperature for 30 min.

2.10. IL-2 ELISA

Supernatants from T cell stimulations were collected at indicated time points following stimulation and stored at -20°C until analysis. IL-2 levels were determined using a human IL-2 Ready-SET-Go!® ELISA kit (eBioscience) according to manufacturer's instructions.

Table 2.1. List of Antibodies used in immunofluorescence staining for flow cytometry analysis. (Abbreviations: Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Cyanine (Cy), Peridinin chlorophyll (PerCP), eFluor® 450 (e450)

Target	Clone	Isotype	Conjugate	Manufacturer	Volume per test (µL)
CD3	UCHT1	Mouse IgG1,κ	Allophycocyanin	BD Pharmingen	1.5
CD3	SK7	Mouse IgG1,κ	PerCP	BD Biosciences	1.5
CD3	SK7	Mouse IgG1,κ	PE-Cy7	BD Pharmingen	1.5
CD4	RPA-T4	Mouse IgG1,κ	FITC	BD Pharmingen	2
CD4	RPA-T4	Mouse IgG1,κ	PE	BD Pharmingen	2
CD4	RPA-T4	Mouse IgG1,κ	Allophycocyanin	BD Pharmingen	2
CD4	OKT4	Mouse IgG2b,κ	e450	eBioscience	2
CD14	MφP9	Mouse IgG2b,κ	PerCP	BD Biosciences	2
CD25	2A3	Mouse IgG1,κ	FITC	BD Biosciences	1.5
CD25	M-A251	Mouse IgG1,κ	PE	BD Pharmingen	3
CD25	M-A251	Mouse IgG1,κ	Allophycocyanin	BD Pharmingen	1.5
CD27	M-T271	Mouse IgG1,κ	FITC	BD Pharmingen	1.5
CD28	CD28.2	Mouse IgG1,κ	Allophycocyanin	BD Pharmingen	2
CD28	CD28.2	Mouse IgG1,κ	PE	BD Pharmingen	2
CD45RA	HI100	Mouse IgG2b,κ	e450	eBioscience	0.5
CD45RA	HI100	Mouse IgG2b,κ	PE	BD Pharmingen	1.5
CD45RO	UCHL1	Mouse IgG2a,κ	FITC	BD Pharmingen	2.5
CD62L	DREG-56	Mouse IgG1,κ	Allophycocyanin	BD Pharmingen	2
CD69	FN50	Mouse IgG1,κ	FITC	BD Pharmingen	2
CD71	M-A712	Mouse IgG2a,κ	Allophycocyanin	BD Pharmingen	2
CD80	L307.4	Mouse IgG1,κ	PE	BD Pharmingen	2
CD86	2331 (FUN-1)	Mouse IgG1,κ	FITC	BD Pharmingen	2
CD127	eBioRDR5	Mouse IgG1,κ	PE-Cy7	eBioscience	1.5
CTLA-4	BNI3	Mouse IgG2a,κ	PE	BD Pharmingen	1
FOXP3	PCH101	Rat IgG2a,κ	Allophycocyanin	eBioscience	1
IL-2	MQ1-17H12	Rat IgG2a,κ	Allophycocyanin	eBioscience	0.3
ICOS	DX29	Mouse IgG1,κ	PE	BD Pharmingen	3
IL-17A	eBio64CAP17	Mouse IgG1,κ	PE	eBioscience	0.5
IL-21	eBio3A3-N2	Mouse IgG1,κ	PE	eBioscience	1
IFNγ	B27	Mouse IgG1,κ	Allophycocyanin	BD Pharmingen	0.4
Ki67	B56	Mouse IgG1,κ	PerCP-Cy5.5	BD Pharmingen	1.5
OX40	ACT35	Mouse IgG1,κ	PE	BD Pharmingen	3
PD-1	MIH4	Mouse IgG1,κ	Allophycocyanin	BD Pharmingen	2
pSTAT5 (pY694)	Clone 47/Stat5(pY694)	Mouse IgG1,κ	PE	BD Phosflow	4
TCR vβ2	MPB2D5	Mouse IgG1,κ	PE	Beckman Coulter Immunotech	2
TNFα	MAb11	Mouse IgG1,κ	FITC	eBioscience	1

2.11. CD4$^+$ T cell proliferation assays

Prior to stimulation, CD4$^+$CD25$^-$ T cells were labeled with CellTrace™ Violet (CTV; Life Technologies). T cells were resuspended in 1mL PBS and CTV was added at a 1/1000 dilution for a final concentration of 5µM. Cells were incubated for 20 min at 37°C and protected from light. Unbound dye was quenched by addition of 20mL RPMI/FBS (10% v/v) for 10 min at 37°C. Cells were washed twice by centrifugation (490g, 5 min) in PBS prior to stimulation.

Following stimulation T cell proliferation was analysed by flow cytometry. Proliferation profiles were modeled using the Flowjo proliferation platform to determine various parameters defining T cell proliferation. Each of these statistics have advantages and disadvantages (Roederer, 2011). Division index represents the average number of divisions undergone by all cells in the original culture. In contrast, the proliferation index excludes undivided cells and is thus a measure of how far cells have divided. CD28-costimulation influences both the number of cells that enter the cell cycle and the number of divisions undergone. The division index accounts for both these parameters of T cell proliferation and was therefore frequently used to express T cell proliferation following stimulation.

2.12. Detection of T cell apoptosis

Following 5 days incubation, T cell apoptosis was determined by analysis of mitochondrial depolarization, a marker of early apoptosis (Zamzami et al., 1995). Cells were washed and incubated with 23ng/mL 3,3'

dihexyloxacarbocyanine iodide ($DiOC_6$) (Molecular Probes, Eugene, OR, USA) for 20 minutes at 37°C. Cells were then washed twice by centrifugation in PBS (490g, 5 min) and suspended in 400µL RPMI/FBS (2% v/v). Cells were analysed by flow cytometry and apoptotic cells were characterized as a $DiOC_6^{low}$ population that was verified by forward/side scatter profiles.

2.13. Stimulation of early arthritis patient PBMC samples

Heparinized peripheral blood samples were collected from patients within the Birmingham Early Arthritis cohort. Research ethics committee approval was obtained for the collection of this material and all patients gave written informed consent. Blood samples were diluted 1:2 with PBS supplemented with 2% (v/v) FBS (PBS-FBS). PBMCs were isolated by Ficoll-Paque (GE healthcare) density gradient centrifugation as previously described. Purified PBMCs were then washed by centrifugation in PBS-FBS, once at 1060g for 10 min, then once at 260g for 5 min, finally once at 490g, 5 min. PBMCs were then resuspended in RPMI-FBS.

PBMC populations were characterized by immunofluorescence labeling followed by flow cytometric analysis. Specifically PBMCs samples were considered in terms of monocyte:CD4 T cell ratios by $CD14^+$ and $CD3^+CD4^+$ expression respectively. The $CD4^+$ naïve:memory ratio was determined by differential expression of CD45RO positivity for memory T cells and CD45RA positivity for naïve T cells. Relative T_{reg} frequencies within the $CD3^+CD4^+$ population were determined by $CD25^{high}CD127^{low}$ expression.

PBMCs were stimulated in 96 well flat-bottom plates in a total volume of 200µL/well RPMI/FBS with $2x10^5$ PBMCs/well. PBMCs were stimulated with concentrations ranging from 0.5-0.00005µg/mL anti-CD3 for 4 days at 37°C either in the presence or absence of 20µg/mL abatacept.

Following stimulation, PBMCs were stained for surface marker expression. Ki67, a marker of proliferation, was primarily used to indicate T cell activation. This was stained for following cell fixation and permeabilization using a FoxP3 staining buffer kit (eBioscience) as described previously.

2.14. Statistics

Statistical analyses were performed using SPSS version 22 (SPSS, Inc., Chicago, IL, USA) or using Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Interactions between two factors, for example, $1,25(OH)_2D_3$ and abatacept, were analysed by repeated measures within-subjects two-factor analysis with Huynf-Felt correction. Normality was determined as part of this analysis by the distribution of residuals from the mean values in quantile-quantile plots as described (Ghasemi and Zahediasl, 2012). Where indicated, data were $\log_{10}$ transformed in order to reduce skewing of data that did not appear to be normally distributed. *P* values for the interaction effect and main effects for the individual factors are reported. Interaction reflects the impact of one factor upon the other (Slinker, 1998), for example, the extent to which the presence of $1,25(OH)_2D_3$ impacts upon the effect of abatacept. Where

significant interaction effects were identified, main effects for the individual factors are not reported and individual comparisons between experimental groups were made using two-tailed, paired t tests. P values <0.05 were considered significant.

Chapter 3: Impact of relative APC numbers on T cell CD28-costimulation requirements

3.1. Introduction

Several clinical trials have demonstrated abatacept to be effective in the treatment of RA, however, a significant proportion of patients display a limited clinical response to treatment (Buch et al., 2008). This inherent variability between patient responses to abatacept is reminiscent of clinical responses to other biological therapies used in the treatment of RA (Maneiro et al., 2013).

Differences between clinical responses to abatacept blockade may be attributed to various factors. RA is a heterogeneous disease and variable responses to treatment may reflect differences in underlying disease processes between patients that determine outcomes of treatment. For example, T cells may play a more dominant role in some subgroups. Additionally, the potency of abatacept blockade has been proposed to be suboptimal, especially in the context of non-human primate models of organ transplantation and this prompted the development of a CTLA-4-ig variant (belatacept) that binds to CD80/CD86 with higher affinity (Larsen et al., 2005). Alternatively, the timing of costimulation blockade may not overlap with a time frame in which CD28 is actively contributing towards T cell activation. Finally, CD28 may not always be crucial to T cell activation and other T cell stimulatory pathways may compensate for an absence of CD28-costimulation as seen in CD28-deficient mice. The identification of conditions under which CD28 requirements are limited may therefore identify strategies for

combination therapies or other approaches that promote patient responses to costimulation blockade.

In this chapter, the impact of abatacept blockade upon *in vitro* T cell stimulation was determined. The results show that the level of abatacept sensitivity is inversely correlated with the strength of TCR stimulation. Surprisingly, the DC:T cell ratio at which T cells are stimulated partly influences T cell activation thresholds such that TCR-stimulation is more effective in the presence of high relative DC numbers. As such, DC:T cell ratio influences CD28-costimulation requirements and therefore the efficacy of CD28-costimulation blockade.

3.2. Results

3.2.1. Abatacept inhibits T cell proliferation driven by both CD80 and CD86

Initially, to test the efficacy of T cell stimulation blockade by abatacept *in vitro*, CTV-labeled CD4$^+$CD25$^-$ human T cells were stimulated with soluble anti-CD3 and CHO cells expressing either CD80 or CD86 to provide CD28-costimulation. Treatment with abatacept at a concentration of 20μg/mL robustly inhibited T cell proliferation driven by either CD80 or CD86 (**fig. 3.1**). As predicted, abatacept had no impact upon T cell proliferation driven by anti-CD3/anti-CD28 coated beads due to the absence of CD28 ligands in this system (**fig. 3.1**). These experiments therefore established both the specificity and efficacy of abatacept blockade for future experiments.
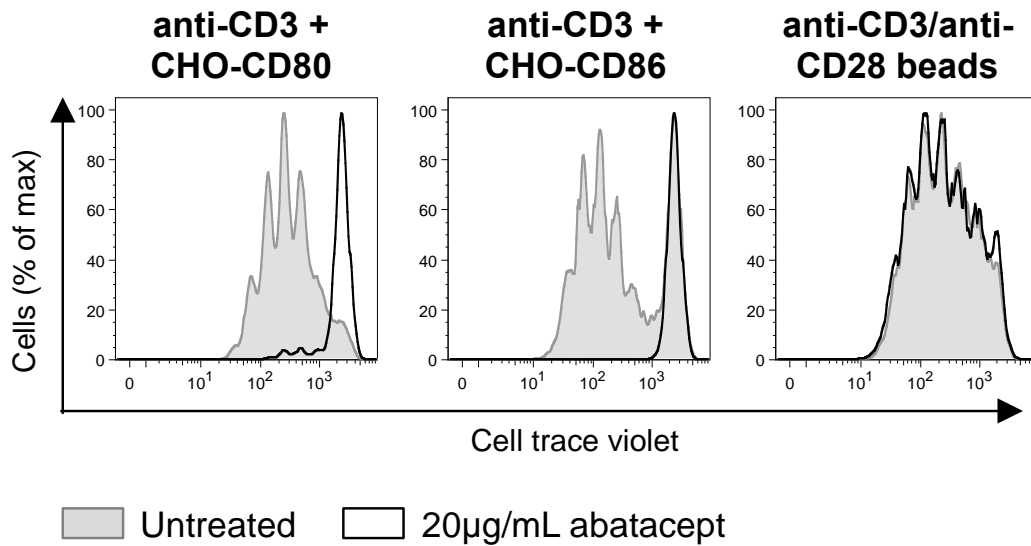
Fig. 3.1. Abatacept inhibits CD28-driven T cell proliferation. CTV labeled CD4$^+$CD25$^-$ T cells were incubated with 0.5µg/mL anti-CD3 and either CHO-CD80, CHO-CD86 or anti-CD3/anti-CD28 coated beads and with or without 20µg/mL abatacept for 5 days followed by flow cytometric analysis of CTV dilution. Representative flow cytometry data from one representative experiment of more than 5 performed.
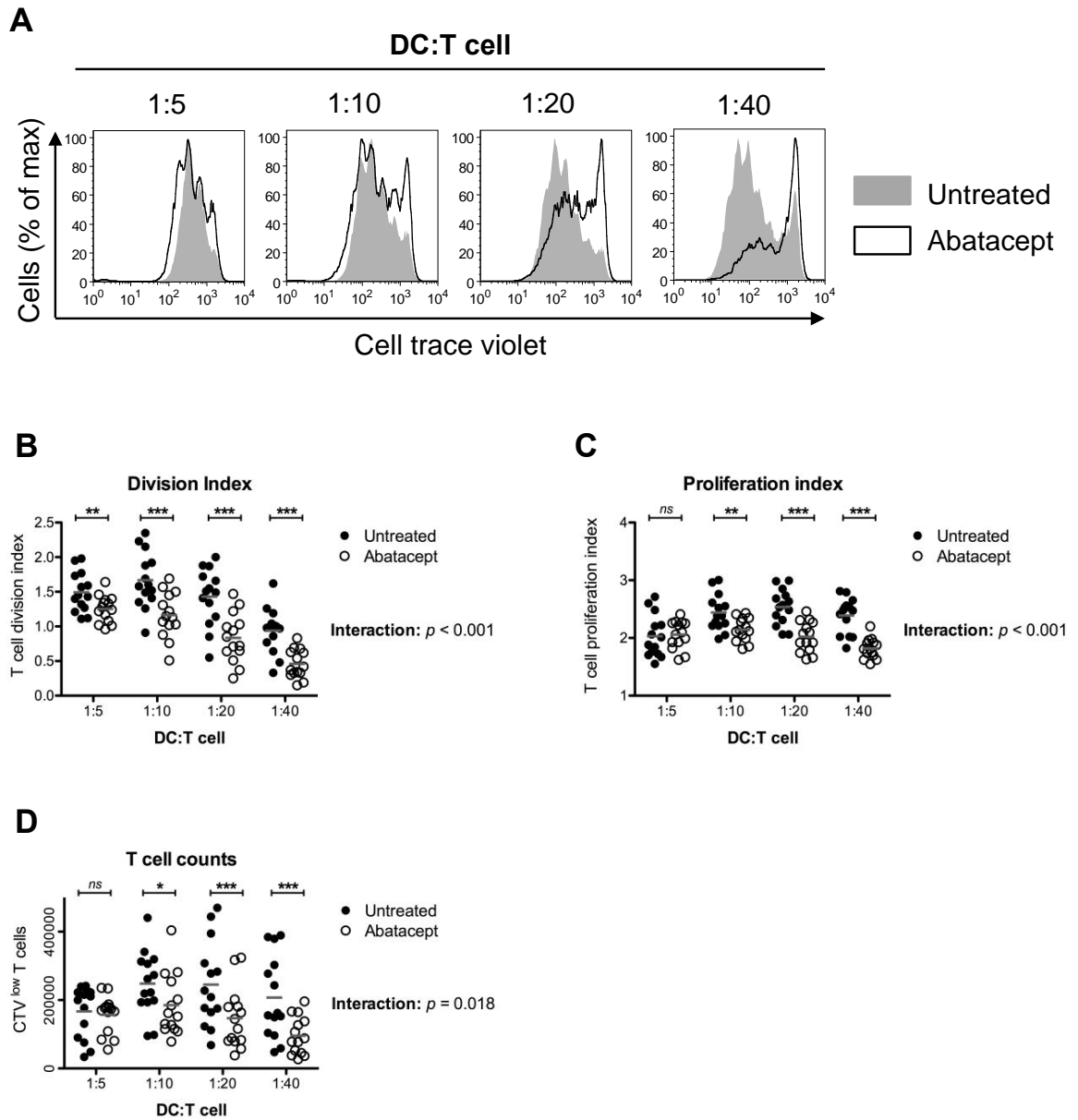
Fig. 3.2. Relative APC numbers determine T cell CD28-costimulation requirements. CTV labeled T cells were co-cultured with DCs at indicated DC:T cell ratios and stimulated with 0.5µg/mL anti-CD3 for 5 days -/+ (20µg/mL) abatacept. **A)** Representative flow cytometric data. CTV proliferation data from independent experiments ($n = 14$) was modeled and expressed as either division index (**B**) or proliferation index (**C**). **D)** CTV^{low} (i.e. divided) T cell counts were determined relative to a known quantity of beads added to each sample prior to flow cytometric analysis. p values are derived from two-way ANOVA. Further analyses was performed using paired, two-tailed t tests; ns not significant *p ≤0.05 **p ≤0.01, ***p ≤0.001.

3.2.2. High relative DC numbers inhibit suppression of T cell activation by abatacept

In order to further assess the impact of CD28-costimulation upon T cell activation T cells were again stimulated with 0.5μg/mL soluble anti-CD3 but this time with monocyte-derived dendritic cells (DCs) as a source of costimulation in the presence or absence of abatacept. Interestingly, very limited inhibition of T cell proliferation by abatacept was observed when DCs were used at high numbers relative to responder T cells (**fig. 3.2A**; 1 DC: 5 T cell). However, reducing this DC:T cell ratio (down to 1:40) progressively increased abatacept sensitivity both in terms of T cell proliferation (**fig. 3.2A/B**) and absolute T cell counts (**fig 3.2D**). This indicated that T cell activation driven by low relative DC numbers was more dependent upon CD28-costimulation.

In addition to effects upon abatacept-sensitivity, relative DC numbers had striking effects upon T cell proliferation. CTV plots were modeled and T cell proliferation was expressed as both division index and proliferation index. These statistics differ in that division index accounts the average number of divisions undergone by the overall T cell population whereas proliferation index represents the average number of divisions by divided T cells only and therefore remains uninfluenced by the proportion of T cells that enter the cell cycle. Unsurprisingly, the proportion of T cells that entered the cell cycle decreased as DC numbers were reduced suggesting that DC contact/costimulation became limiting at low numbers (**fig. 3.2A**). This heavily influenced the T cell division index, this significantly decreased as relative DC

numbers were reduced (**fig. 3.2B**). Interestingly, there was also an apparent inhibition in the extent of T cell division in response to high relative DC numbers. Although more T cells committed to proliferation in response to high DC:T cell ratios they tended to undergo fewer divisions. As such, there was a trend towards increased proliferation index as DC:T cell was reduced (**fig. 3.2C**). Together, these data therefore demonstrate that the DC:T cell ratio at which T cells are stimulated affects not only the suppression by abatacept but also the extent of the proliferative response following T cell stimulation. In general, the concept that high intensity T cell stimulation results in more proliferation commitment but lower numbers of divisions is a consistent finding throughout this thesis.

In order to rule out the obvious possibility that 20µg/mL abatacept was insufficient to saturate the increased amounts of CD80/CD86 expression when higher DC numbers were used, T cells were stimulated in the presence of either high (1 DC:5 T cell) or low (1 DC: 20 T cells) relative DC numbers with CD28-costimulation blockade by higher abatacept concentrations. However, increasing abatacept dose beyond 20µg/mL did not increase inhibition of T cell proliferative responses (**fig. 3.3**). Moreover, the dose response to abatacept was parallel between the two ratio's albeit set at different division levels. This further suggested that the difference in efficacy of abatacept treatment was not limited by the concentrations used.
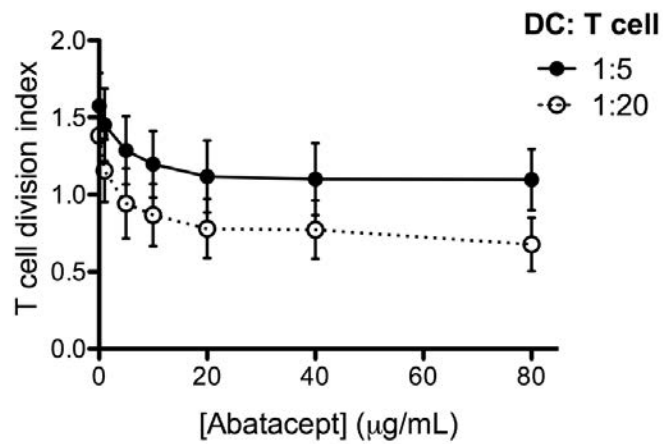
Fig. 3.3. Increased abatacept concentrations fail to inhibit abatacept-resistant T cell proliferation. CTV-labeled T cells were stimulated with 0.5µg/mL anti-CD3 at a DC:T cell ratio of 1:5 or 1:20 and were treated with indicated abatacept concentrations. T cell proliferation was determined by flow cytometric analysis. Mean division index values ±S.E. from independent experiments ($n = 4$).
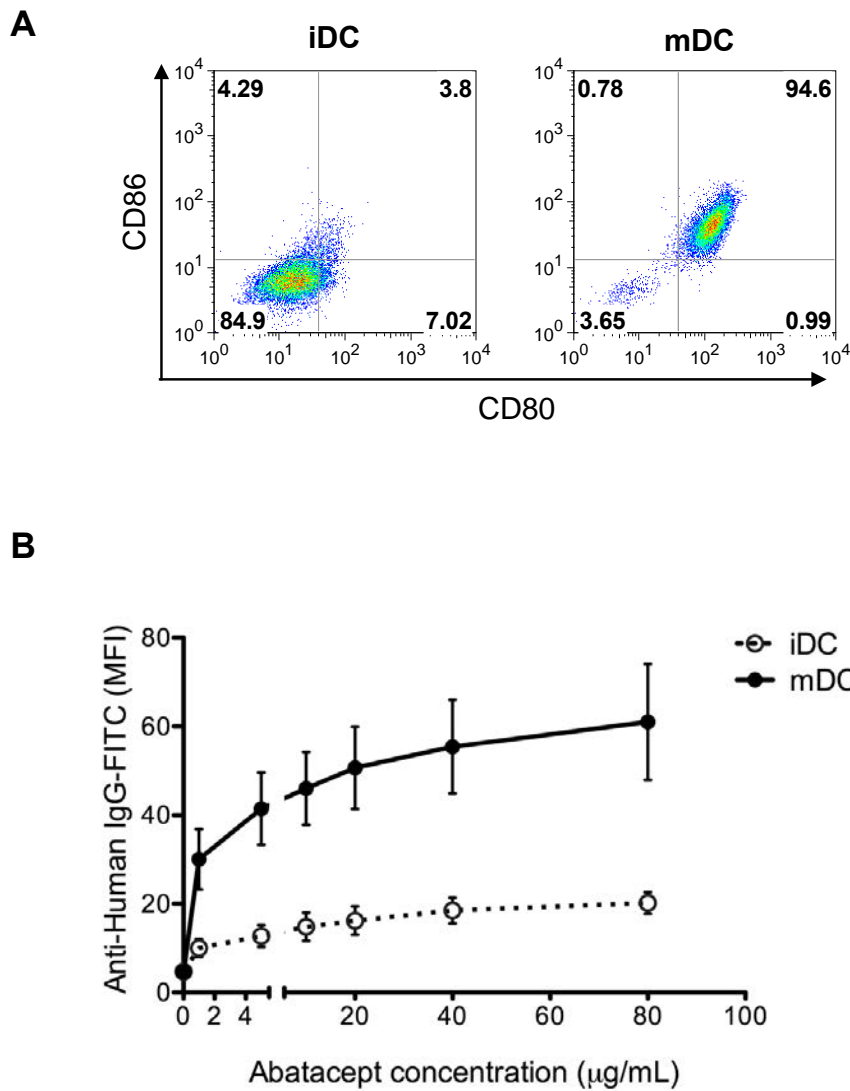
Fig. 3.4. Abatacept saturates DC CD80/CD86 expression levels. Immature DCs (iDC) were differentiated from monocytes by exposure to GM-CSF/IL-4 for 7 days. Mature DCs (mDC) were generated by further exposure to 100ng/mL LPS for 24 hours. (**A**) CD80 and CD86 expression levels were determined by immunofluorescence staining followed by flow cytometric analysis. (**B**) iDC and mDC were incubated with various abatacept concentrations, abatacept binding was subsequently detected by secondary staining with anti-IgG (Fc specific)-FITC. MFI values were determined by flow cytometric analysis. Mean values ±S.E. from independent experiments ($n = 4$).

In the above assays, T cells were incubated in the presence of immature DCs which express relatively low CD80/CD86 levels. However, these levels are upregulated during the course of T cell stimulation by direct contact with T cells and exposure to inflammatory cytokines (Banchereau and Steinman, 1998). This upregulation of CD80/CD86 could be mimicked by exposure to 100ng/mL LPS for 24 hours (**fig. 3.4A**). To check the saturation of CD80/CD86 levels by abatacept on both immature and LPS matured DCs, these cells were incubated with various abatacept concentrations. Abatacept binding was subsequently detected by staining for the abatacept-Fc domain with anti-IgG-FITC. These data showed increased anti-IgG-FITC MFI for mDCs compared to iDCs as expected. No substantial increase in anti-IgG-FITC MFI was observed when mDCs were exposed to abatacept concentrations greater than 20µg/mL (**fig. 3.4B**). Together, these data indicate that almost maximal saturation of CD80/CD86 occurs with abatacept at 20µg/mL. A concentration of 20µg/mL abatacept was therefore used in subsequent experiments which is broadly equivalent to steady state plasma concentrations achieved in RA patients treated with a standard dosing regimen (Ma et al., 2009).

Taken together, these data suggest that T cell proliferation occurring in the presence of abatacept was due to CD28-independent T cell activation. Importantly, the presence of increased relative DC numbers altered CD28-dependency despite saturating abatacept levels and no overt change in the intensity of the TCR signal provided at the level of an individual TCR. This is indicated by the illustration in **fig. 3.5**.

High DC:T cell ratio

Low DC:T cell ratio

= Unstimulated T cell   = Stimulated T cell   = DC   = anti-CD3   = TCR/CD3   = CD28   = CD80/CD86
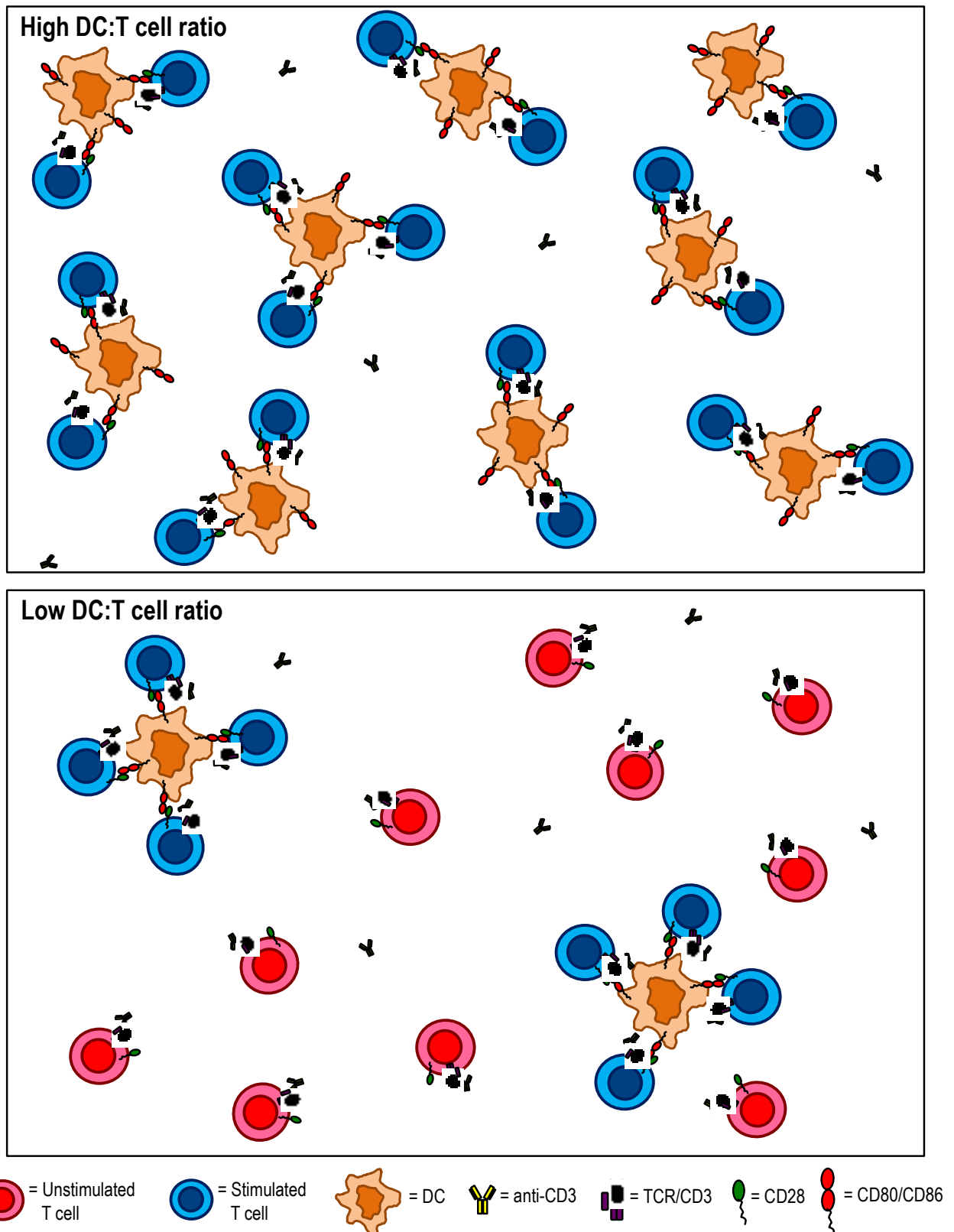
Fig. 3.5. Visual representation of *in vitro* T cell stimulation at high vs. low DC: T cell ratios. An equivalent number of T cells are stimulated with an equivalent anti-CD3 concentration but different relative DC numbers. This would be predicted to influence the proportion of T cells that undergo activation but not the quality of stimulation received on a per cell basis.
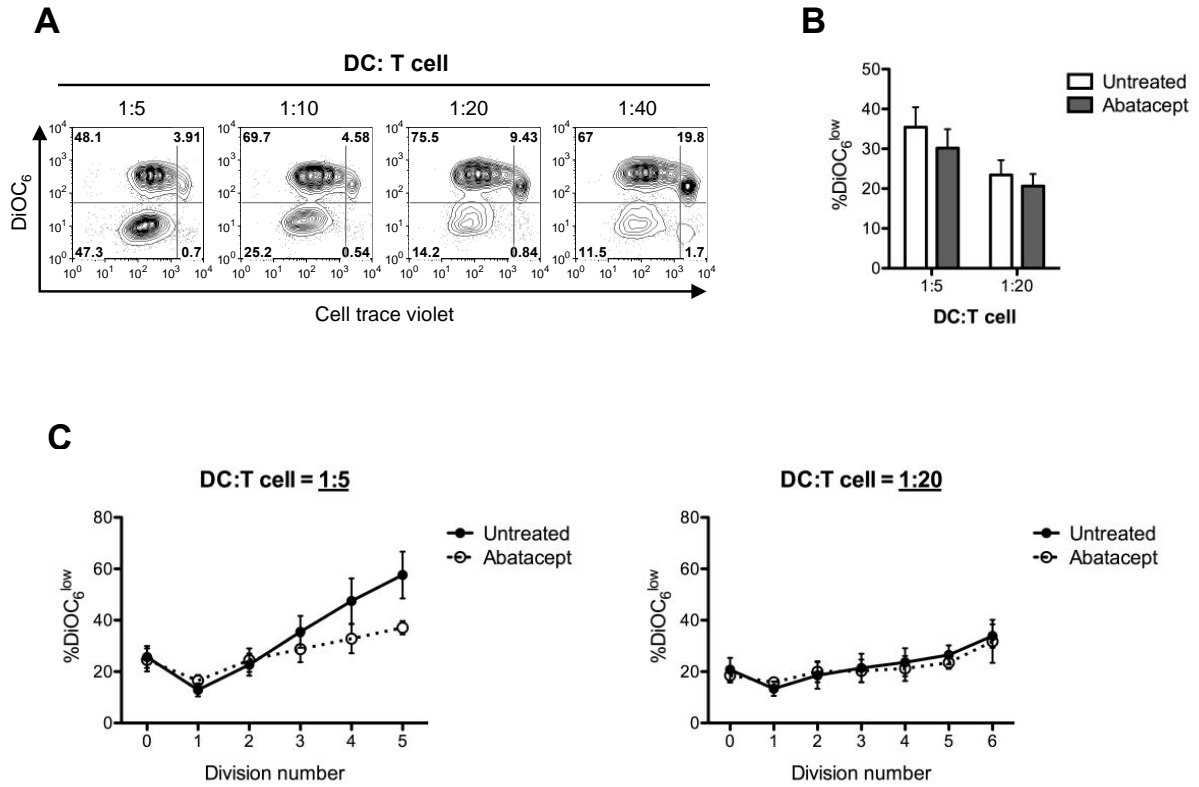
Fig. 3.6. T cell apoptosis is increased by stimulation at high DC:T cell ratios. CTV-labeled T cells were stimulated with 0.5µg/mL anti-CD3 at a DC:T cell ratio of either 1:5 or 1:20 and -/+ (20µg/mL) abatacept for 5 days. T cell apoptosis was determined by $DiOC_6$ labeling followed by flow cytometric analysis where $DiOC_6^{low}$ cells are apoptotic. **A**) Flow cytometry data from one representative experiment. **B**) Data from independent experiments (n = 3) showing the % $DiOC_6^{low}$ T cell when gating on CTV^{low} populations. Mean values ±S.E. **C**) The % $DiOC_6^{low}$ T cells expressed according to CTV division number. Data points represent mean values ±S.E.

3.2.3. High relative DC numbers promote T cell death

Previous work has demonstrated increased DO11.10 T cell apoptosis when stimulated by high relative OVA-pulsed DC numbers. (Höpken et al., 2005). This resulted in a similar proliferative arrest observed in **fig. 3.2A/C** when T cells were stimulated at high DC:T cell ratios (e.g. 1:5). Therefore, the impact of the DC:T cell ratio on T cell death during anti-CD3 driven T cell activation was investigated. Following 5 days stimulation, an increasing proportion of divided (CTV^{low}) but subsequently apoptotic ($DiOC_6^{low}$) T cells appeared as relative DC number increased (**fig. 3.6A**). This finding is indicative of elevated T cell apoptosis among proliferating T cells when stimulation was driven at high DC:T cell ratios reminiscent of activation induced death seen with high levels of TCR stimulation (Kishimoto and Sprent, 1999; Boissonnas and Combadiere, 2004). CD28-blockade by abatacept had a limited overall impact upon T cell death (**fig. 3.6B**). However by expressing T cell death according to the T cell division number, a trend was apparent whereby there was decreased death with the presence of abatacept treatment among T cells that had undergone ≥4 divisions but only at a high DC:T cell ratio (**fig. 3.6C**). However, CD28-costimulation is viewed as a pro-survival signal during T cell activation (Noel et al., 1996). The effect of abatacept on T cell survival/apoptosis is thus likely to be contextual and suggests that CD28 does not necessarily provide a survival signal under all stimulation conditions. Together, these data suggest that anti-CD3 driven T cell stimulation at elevated DC:T cell ratios promotes activation induced T cell death and at least partially underlies the reduced expansion of T cell populations under these conditions.
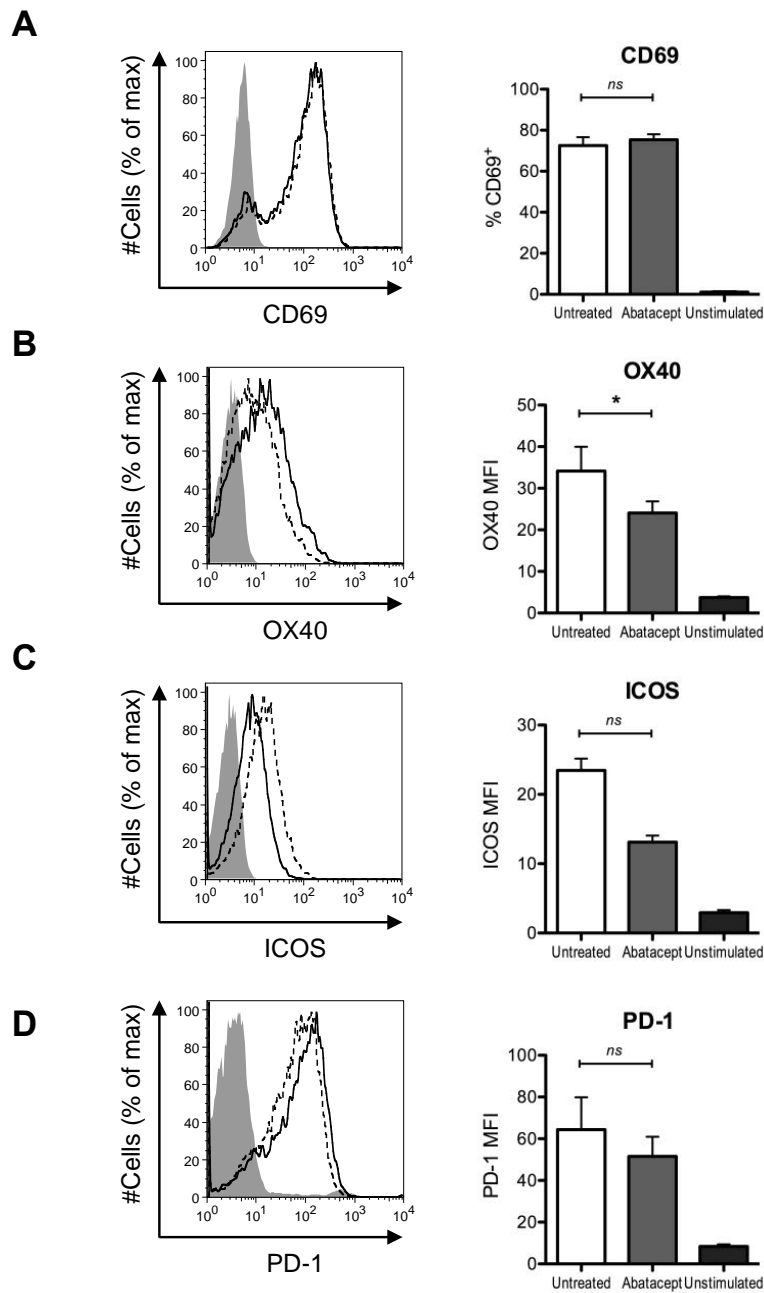
Fig. 3.7. Analysis of early T cell activation markers following CD28-costimulation blockade by abatacept. CD4$^+$CD25$^-$ T cells were stimulated with 0.5µg/mL anti-CD3 and DCs at a ratio of 1 DC:5 T cells for 2 days. The expression levels of the T cell activation markers, CD69 (**A**), OX40 (**B**), ICOS (**C**) and PD-1 (**D**) were assessed by immunofluorescence staining and flow cytometric analysis. Representative flow cytometry data and mean MFI values from independent experiments (n = 6 for **A**, **B** and **D**; n = 5 for **C**). *p ≤0.05, two-tailed Wilcoxon-matched pairs test.
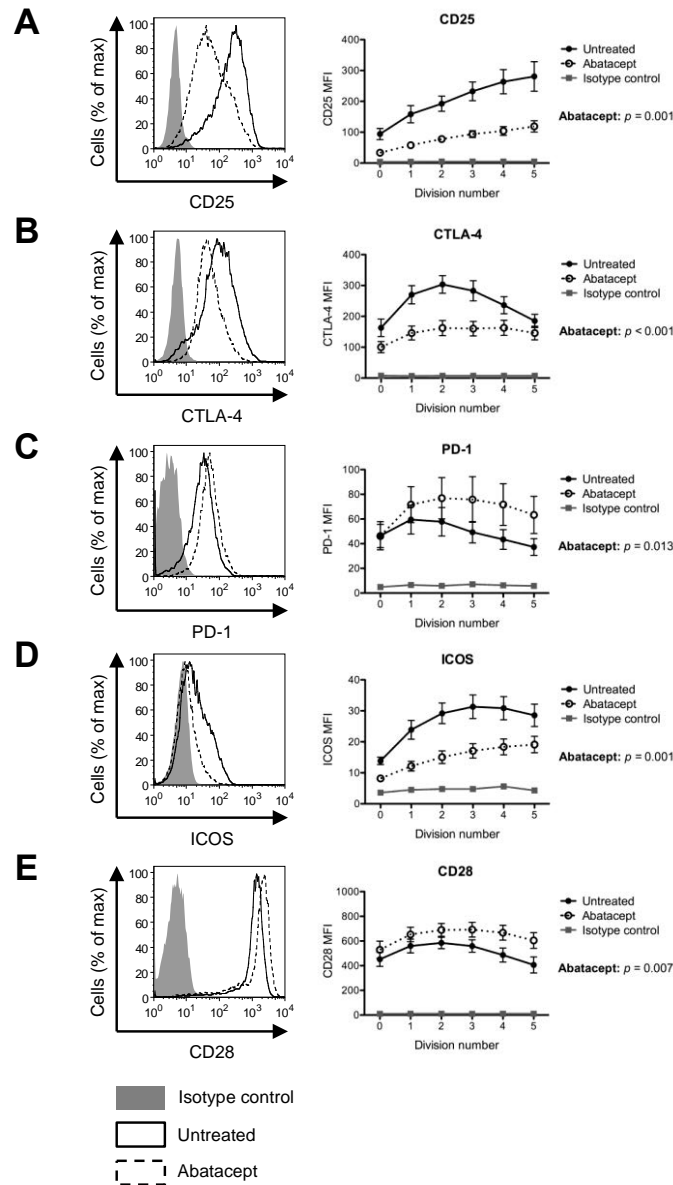
Fig. 3.8. High-dose anti-CD3 promotes abatacept-resistant proliferation of T cells with an altered phenotype. Activated T cells stimulated for 5 days with DCs (at a DC:T cell ratio of 1:5) and 0.5µg/mL anti-CD3 -/+ (20µg/mL) abatacept were assessed for CD25 (**A**), CTLA-4 (**B**), PD-1 (**C**), ICOS (**D**) and CD28 (**D**) expression by flow cytometric analysis. Representative data (gated only on CTV^{low} T cells) are shown as histograms and mean fluorescence intensity (MFI) values at each CTV division number from 8 independent experiments for which data points represent mean ±S.E values. *p* values are derived from two-way ANOVA but the main effect of abatacept only is represented.

3.2.4. Abatacept-blockade alters the expression of effector molecules on proliferating T cells

Although T cell proliferation remained largely unaffected by abatacept when stimulating with at high DC:T cell ratios, experiments were performed to determine whether the absence of costimulatory signaling via CD28 affected the phenotype of activated T cells. The expression of various T cell effector molecules was therefore assessed following T cell activation with 0.5µg/mL anti-CD3 and DCs either with or without abatacept treatment. Initially, the expression of activation markers following 2 days stimulation was determined, which is prior to T cell proliferation. Considering there was limited impact upon T cell proliferation at a DC:T cell ratio of 1:5 it was unsurprising that we found no impact of abatacept treatment upon CD69 expression (**fig. 3.7A**). Whilst we observed limited expression levels of the TNF-superfamily member OX40 at very early time points, this expression did appear to be inhibited by CD28-costimulation blockade (**fig. 3.7B**) which is consistent with previous studies (Walker et al., 1999). Despite previous reports suggesting an inhibition of the CD28-superfamily members ICOS and PD-1 by abatacept treatment (Platt et al., 2010), a non-significant impact upon the expression of these molecules was observed following costimulation blockade at this early (2 day) time point, although a trend towards inhibition was observed (**fig. 3.7C/D**).

The expression of T cell activation markers at a later stage of activation was also determined at 5 days following T cell proliferation. In contrast to the earlier time point, these experiments revealed that the expression of CD25 was markedly reduced on T cells activated in the presence of abatacept (**fig.**

3.8A). Markers of T cell regulation were also differentially affected by abatacept in that the expression of CTLA-4 was inhibited (**fig. 3.8B**) but PD-1 was slightly increased. Specifically, the absence of CD28-costimulation prevented the downregulation of PD-1 by T cells in higher division numbers (**fig. 3.8C**). At this time-point a significant inhibition of ICOS expression by abatacept was observed (**fig. 3.8D**). The levels of CD28 itself were also significantly higher among T cells activated in the presence of abatacept (**fig. 3.8E**). Therefore whilst blockade of costimulation at a DC:T cell ratio of 1:5 did not stop T cell proliferation, the phenotype of proliferating T cells (gated on dilution of CTV) was nonetheless affected by abatacept.

3.2.5. Anti-CD3 cross-linking promotes CD28-independent T cell activation

In order to investigate the nature of CD28-independent T cell activation in the context of anti-CD3, I compared T cells activated with various CHO cell transfectants (as artificial APCs) in the presence of a single dose of soluble anti-CD3 (0.5µg/mL anti-CD3) (**fig. 3.9A**). As a control, T cell proliferation driven by anti-CD3/anti-CD28 coated beads was not suppressed by abatacept due to the absence of CD80/CD86 in this system. In contrast, abatacept strongly inhibited T cell proliferation driven by CHO-CD80, even when present at concentrations as low as 0.5µg/mL (**fig. 3.9B**). Strikingly, abatacept failed to suppress T cell proliferation mediated by cells expressing an Fc receptor (CD32) and CD80 (CHO-FcR/CD80). This transfectant model was generated in order to evaluate the impact of anti-CD3 crosslinking upon signal strength. Thus, strong anti-CD3 signaling could drive CD28-independent T cell

proliferation, however, in order for this signal to reach the threshold for T cell activation there was a requirement for FcR mediated anti-CD3 cross-linking between T cell and APC. As such, the potency of anti-CD3 stimulation appeared to be the critical factor in determining T cell activation in the absence of CD28-costimulation.

In order to further explore the impact of anti-CD3 cross-linking upon CD28-signal requirements, $CD4^+CD25^-$ T cells were stimulated with glutaraldehyde-fixed CHO FcR/CD80 at varying CHO:T cell ratios (**fig. 3.10**). These experiments revealed a similar effect upon abatacept sensitivity to that seen with DC-driven activation in that abatacept blockade became more effective with decreasing CHO FcR/CD80:T cell ratios. Importantly, this observation demonstrated that elevated CD28-requirements at low relative APC numbers were not the result of a specific DC effect as the APC used in this context was a fixed CHO transfectant cell. Rather, there appeared to be a fundamentally different signaling experience received by T cells at different relative APC numbers despite no change in the amount of TCR agonist present. Specifically, there appeared to be enhanced efficacy of anti-CD3 stimulation in the presence of high relative APC numbers such that CD28 costimulation was not required for T cell proliferation. One plausible concept which could explain such observations is that commitment of T cells to cell division may involve more than a single APC interaction and that the numbers of DC-T cell interactions within a certain time-frame is important.
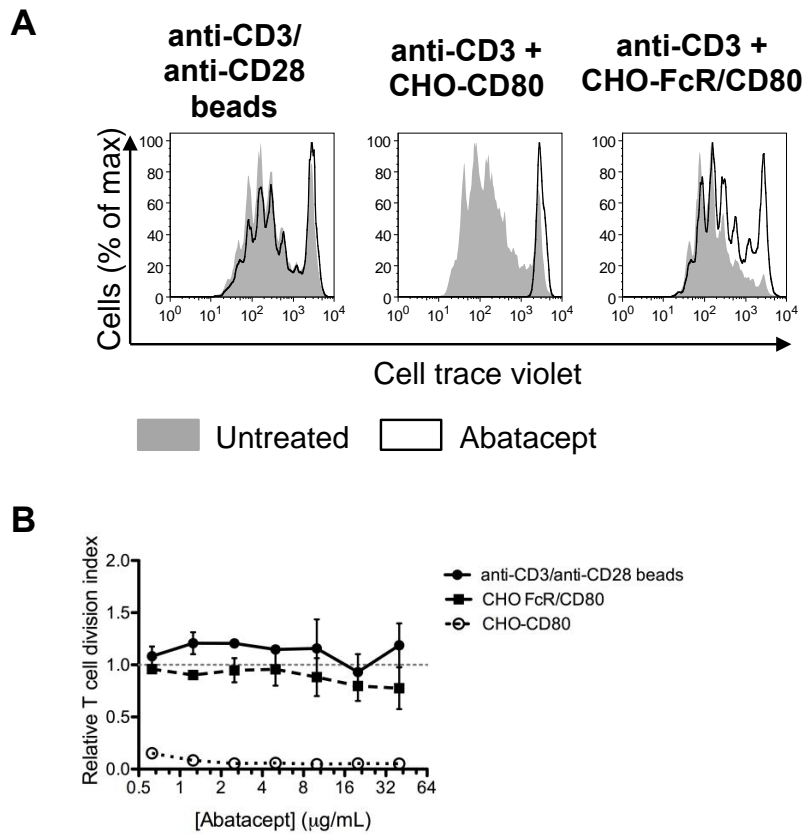
Fig. 3.9. Anti-CD3 cross-linking promotes abatacept-resistant T cell proliferation. CTV-labeled T cells were stimulated -/+ (20 μg/mL) abatacept with anti-CD3/anti-CD28 beads or 0.5μg/mL anti-CD3 in the presence of either CHO-CD80 or CHO-FcR/CD80 for 5 days. CTV dilution was determined by flow cytometric analysis. (**A**) Representative flow cytometry data. (**B**) Relative division index data normalized to 0μg/mL abatacept controls. Mean values ±S.E. from independent experiments (*n* = 3).

A

DC:T cell

1:5   1:10   1:20   1:40

Cells (% of max)

Cell trace violet

Untreated   Abatacept

B

CHO FcR/CD80

T cell division index

CHO:T cell

● Untreated
○ Abatacept
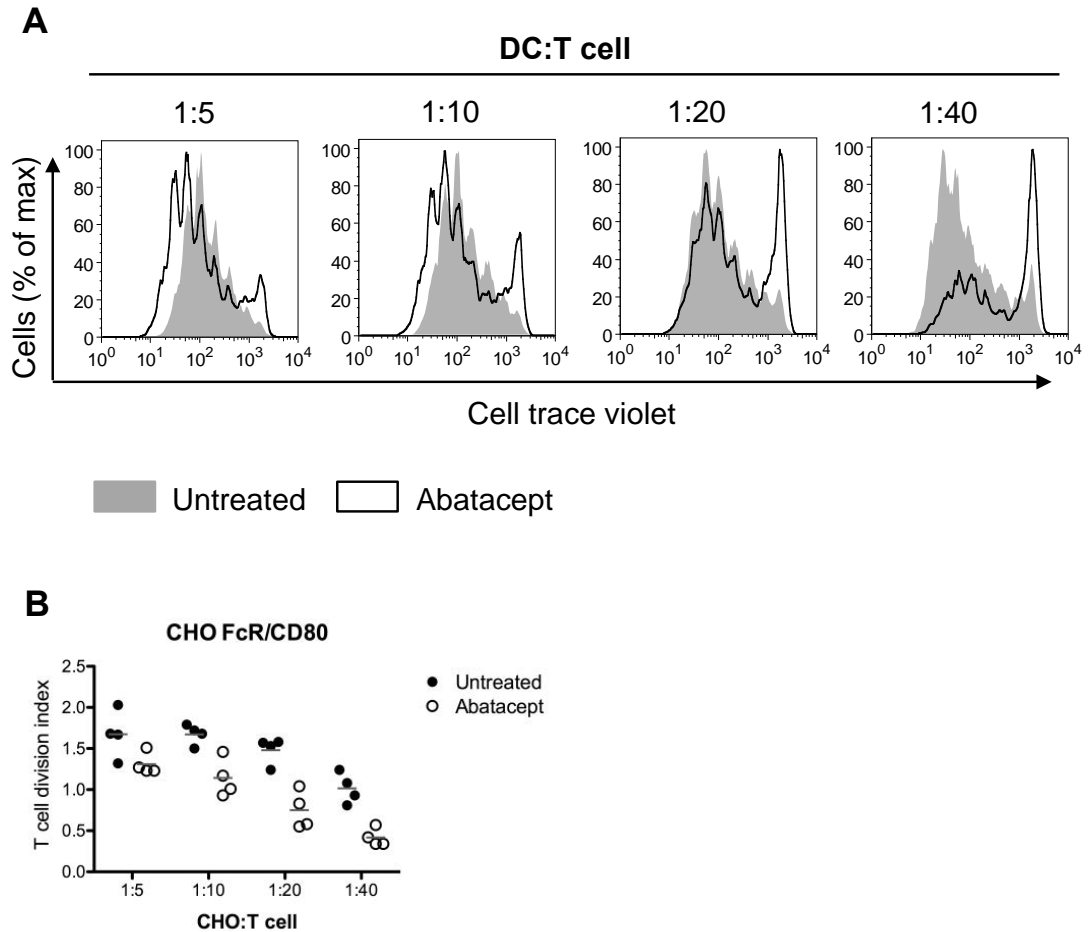
Fig. 3.10. **Relative APC numbers influence abatacept sensitivity when T cell activation is driven by fixed CHO cells**. CTV-labeled T cells were stimulated with 0.5µg/mL anti-CD3 in conjunction with CHO-FcR/CD80 for 5 days. **A**) Representative flow cytometry data showing CTV dilution. **B**) Division index data from independent experiments (n = 4). Horizontal bars represent mean values.

Since the nature of anti-CD3 stimulation affected abatacept-sensitivity in a CHO cell driven T cell activation system, I wanted to determine the impact of directly altering the potency of anti-CD3 stimulation in DC-driven T cell stimulation. T cells were therefore stimulated with a variety of anti-CD3 concentrations and in the presence of either high or low relative DC-numbers. Interestingly, these experiments showed that T cell activation could occur in response to anti-CD3 concentrations significantly lower than 500ng/mL (**fig. 3.11**). Furthermore, when stimulating at a DC:T cell ratio of 1:5, T cell proliferation at low anti-CD3 concentrations (≤0.5ng/mL) were abatacept sensitive, again indicating sufficient abatacept was available in the presence of high DC numbers to saturate CD80/CD86 expression levels (**fig. 3.11A/B**). In contrast, stimulation at higher anti-CD3 concentrations was abatacept-resistant. As such, we identified statistically significant interaction effects between anti-CD3 concentration and abatacept upon both T cell division index and actual T cell counts (**fig. 3.11B**). This confirmed that the effect of abatacept was dependent upon anti-CD3 concentration at this DC:T cell ratio. In contrast, T cell proliferation driven by low relative DC numbers (1DC:20 T cells) was abatacept sensitive regardless of anti-CD3 concentration (**fig. 3.11C**). Together, these data strongly suggest that the efficacy of CD28-blockade by abatacept is dependent upon the strength of TCR-stimulation, and that stimulation at high relative DC numbers in some way enhances the potency of anti-CD3 driven stimulation such that significant T cell proliferation can occur in the absence of CD28-costimulation.
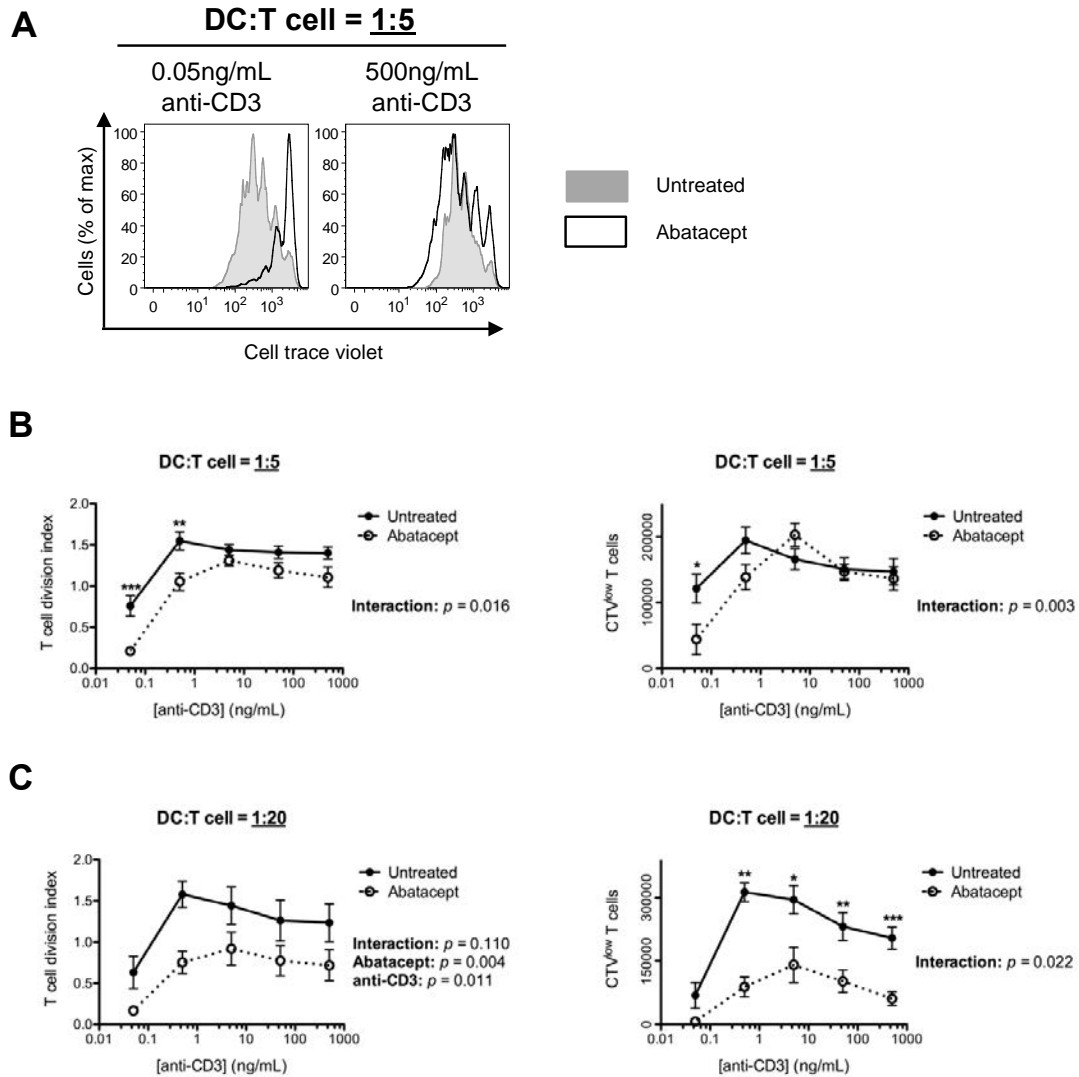
Fig. 3.11. **Efficacy of CD28-blockade by abatacept is dependent upon the strength of TCR-stimulation.** CTV-labeled T cells were co-cultured with DCs at a DC:T cell ratio of 1:5 or 1:20 and stimulated with indicated anti-CD3 concentrations for 5 days. **A**) Representative flow cytometry showing CTV dilution for T cells stimulated at DC:T cell ratio of 1:5. Division index values and CTV^{low} T cell counts are shown for T cell stimulations driven at a DC:T cell ratio of 1:5 (**B**) and 1:20 (**C**) from multiple independent experiments (n = 8 for 1 DC:5 T cells, n = 5 for 1 DC:20 T cells). Data points represent mean values ±S.E. p values are derived from two-way ANOVA. Further analyses was performed using paired, two-tailed t tests; *p ≤0.05 **p ≤0.01, ***p ≤0.001.
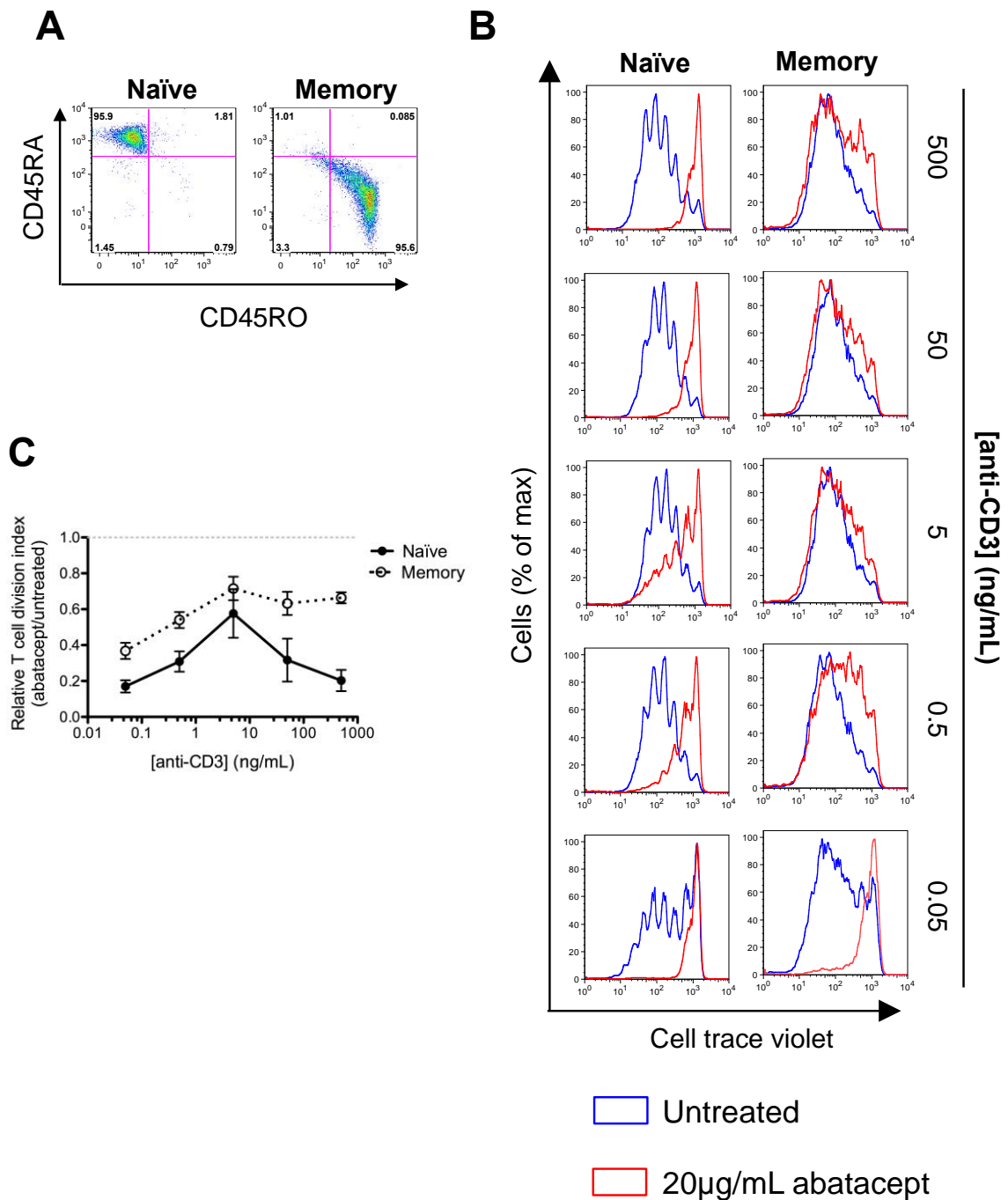
Fig. 3.12. **Naïve and Memory T cells have distinct CD28-costimulation thresholds.**
A) CD4$^+$CD25$^-$ were separated into naïve and memory populations based upon CD45RA$^+$ for naïve T cells and CD45RO$^+$ for memory T cells. **B**) Representative flow cytometry data showing CTV dilution following naïve and memory T cell stimulation with various anti-CD3 concentration and DCs at a ratio of 1 DC:5 T cells for 5 days. **C**) Relative division index values where abatacept treated samples are normalized to untreated controls ($n = 4$). Data points represent mean values ±S.E.
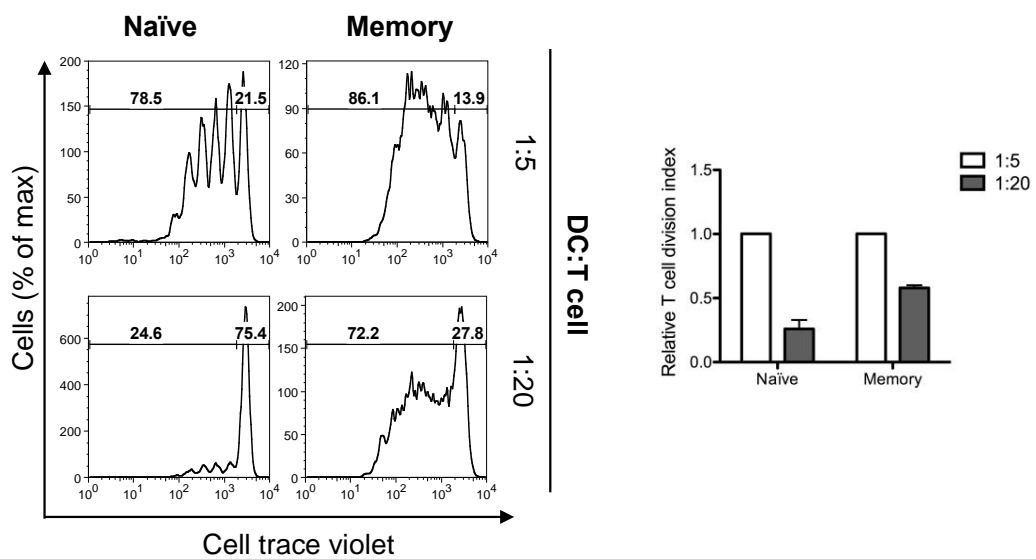
Fig. 3.13. **Naïve T cells display a tendency towards reduced proliferation when stimulated at low DC:T cell ratios.** CTV-labeled CD4$^+$CD45RA$^+$ naïve T cells and CD4$^+$CD45RO$^+$ memory T cells were stimulated with 0.5µg/mL anti-CD3 and DCs at a DC:T cell ratio of 1:5 or 1:20. Representative flow cytometry data and data from multiple experiments (n = 3) where division index values from stimulation at a ratio of 1 DC:20 T cells are normalized to 1DC:5 T cell stimulations. Data points represent mean values ±S.E.

3.2.6. Naïve and Memory T cells have distinct CD28-costimulation thresholds.

Evidence suggesting that abatacept-sensitivity during *in vitro* T cell stimulation was determined by the strength of TCR stimulus was obtained by stimulation of a CD4$^+$CD25$^-$ population where the relative proportions of naïve and memory T cells varied between donors. We therefore compared the impact of abatacept upon purified naïve and memory T cell populations separately, since naïve T cells are known to display different activation thresholds (Rogers et al., 2000). These populations were purified on the basis of differential CD45 isoform expression where naïve T cells are CD45RA$^+$CD45RO$^-$ and memory T cells are CD45RA$^-$CD45RO$^+$ (**fig. 3.12A**). Consistent with published data (Croft et al., 1994; London et al., 2000), we found that memory T cell proliferation was more abatacept-resistant than that of naive T cells when stimulation was driven by high anti-CD3 concentrations, however, memory T cell proliferation became abatacept sensitive at low anti-CD3 concentrations (**fig. 3.12B/C**). Interestingly, a pattern was observed whereby there was a relative increase in naïve T cell proliferation in the presence of abatacept at intermediate anti-CD3 concentrations (5ng/mL anti-CD3) relative to higher concentrations (**fig 3.12B/C**). The factors underlying this effect are unclear but these data demonstrate that the intensity of anti-CD3 stimulation affects the CD28-costimulation requirements of both naïve and memory T cells although the nature of this impact appears to differ.
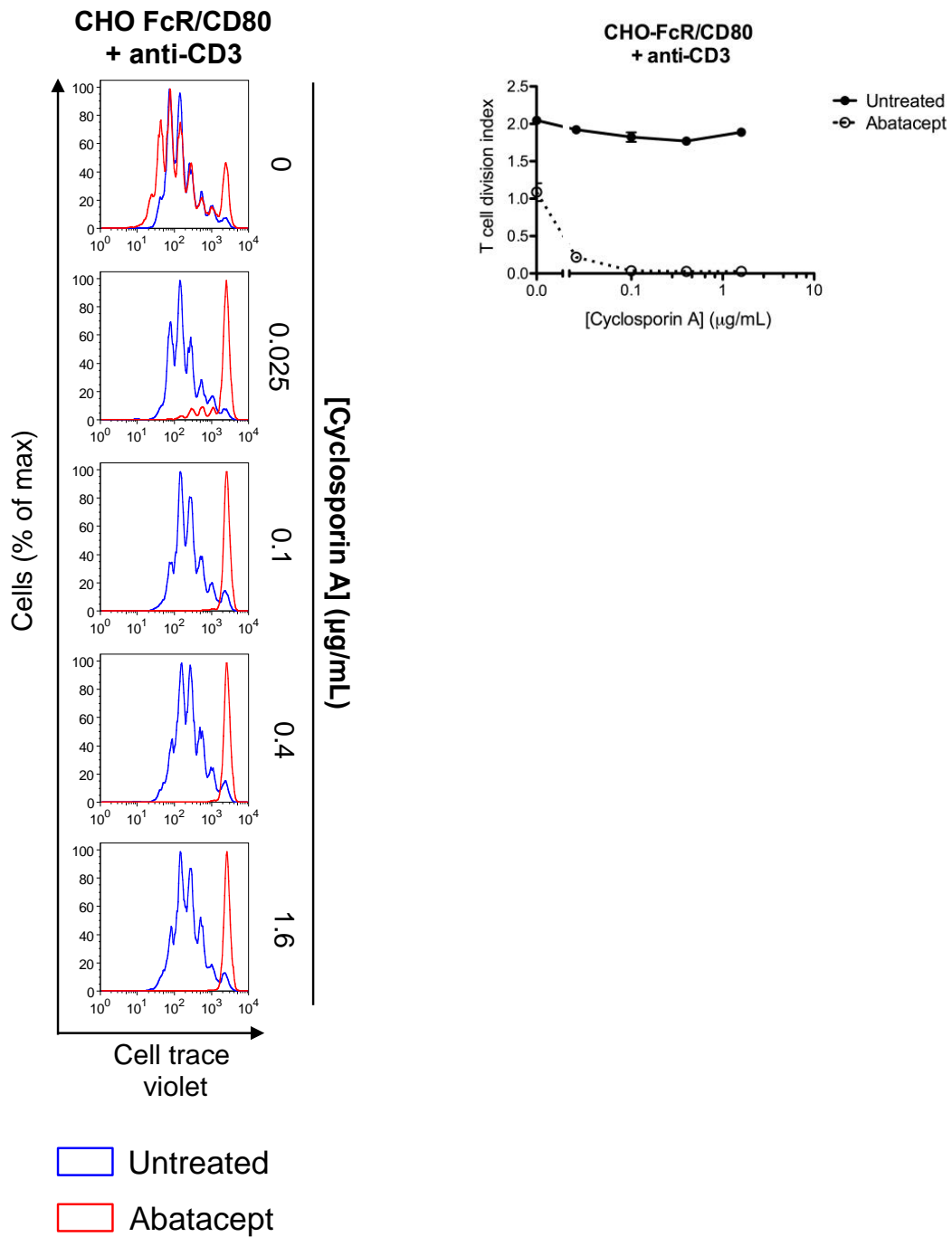
Fig. 3.14. Cyclosporin A in combination with abatacept inhibits CHO FcR/CD80 driven T cell stimulation. CTV-labeled T cells were stimulated with glutaraldehyde fixed 0.5µg/mL anti-CD3 with various CsA concentrations and 20µg/mL abatacept for 5 days. CTV dilution was determined by flow cytometric analysis. Representative flow cytometry data and mean division index data ±S.E. from multiple independent experiments ($n = 4$).

Having assessed the impact of varying the anti-CD3 concentration on abatacept sensitivity, we then determined the impact of varying DC numbers on naïve and memory T cell stimulation. These data suggest that the proliferative response of naïve T cells was inhibited by stimulation at lower DC:T cells (1 DC:20 T cells; **fig. 3.13**). Whilst the response of memory T cells was also inhibited this did not appear to occur to the same extent. Further experiments are required for appropriate statistical analysis of these trends. Nevertheless, these data appear to support the concept that naïve T cells have a higher activation threshold than memory T cells and that this can be achieved through the provision of CD28-costimulation, high anti-CD3 concentration or stimulation by high relative DC numbers.

3.2.7. Abatacept-resistant T cell proliferation is CsA-sensitive

The calcineurin inhibitor, CsA acts to inhibit NFAT translocation to the nucleus (Matsuda and Koyasu, 2000) and acting in this manner has been defined as a TCR-specific inhibitor of T cell activation (Geginat et al., 2000). In contrast, CD28-drives CsA-resistant T cell activation (June et al., 1987). T cells were therefore activated in the presence of CsA and abatacept in order to further explore the interplay between TCR and CD28 driven signals. To verify this approach we initially activated T cells using fixed CHO-FcR/CD80 cells. These data demonstrate that the presence of CsA, even at low concentrations (0.025µg mL), attenuates abatacept-resistant T cell proliferation under these conditions (**fig. 3.14**). These data suggest that targeting TCR-stimulation (e.g. using CsA) promotes the efficacy of CD28-costimulation blockade by abatacept.
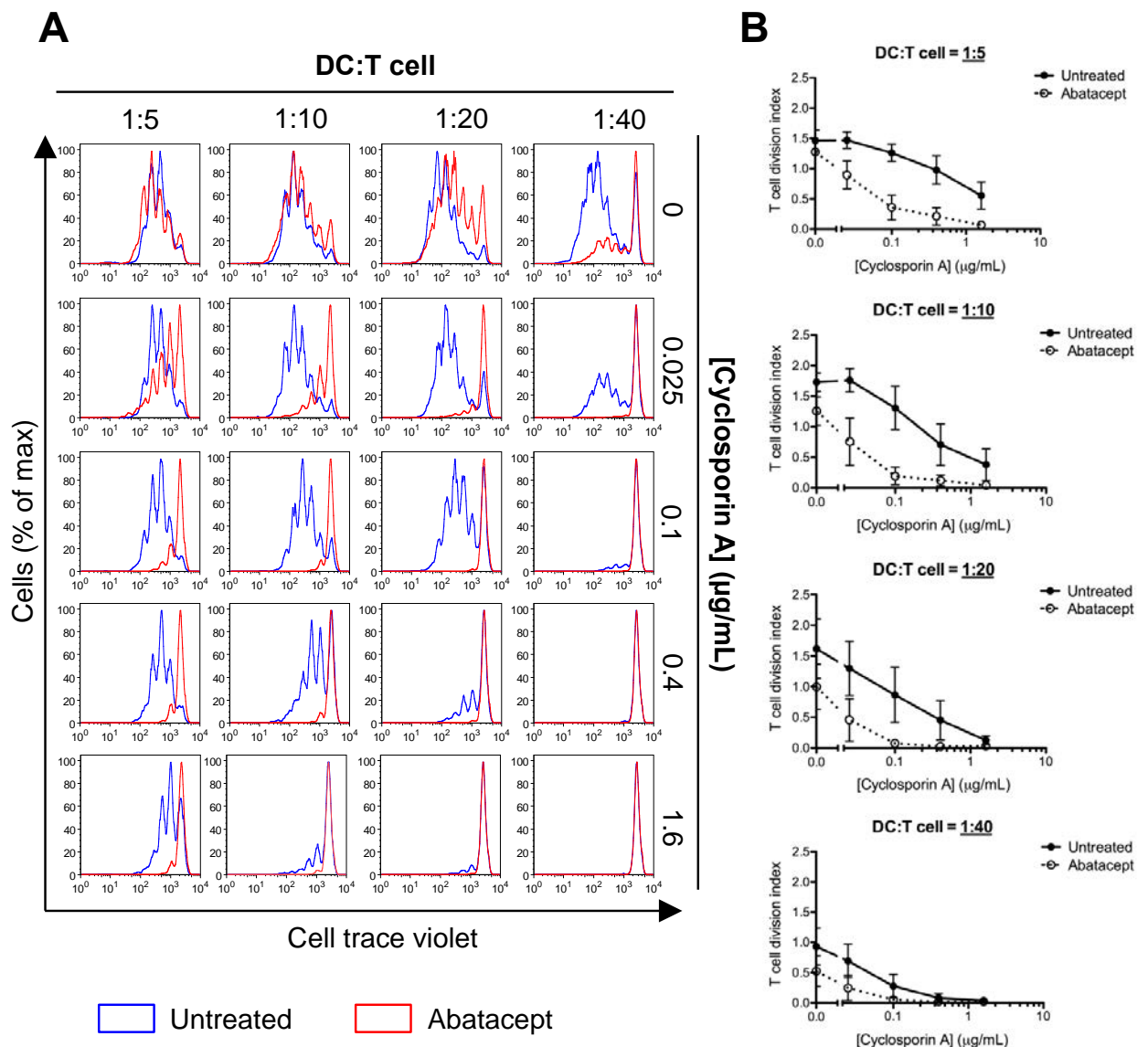
Fig. 3.15. Cyclosporin A and abatacept act in synergistic manner to inhibit T cell proliferation. CTV-labeled T cells were co-cultured with DCs at indicated DC:T cell ratios and stimulated with 0.5µg/mL anti-CD3 with various CsA concentrations and 20µg/mL abatacept where indicated for 5 days. CTV dilution was determined by flow cytometric analysis. **A**) Representative flow cytometry data. **B**) Mean division index data ±S.E. from multiple independent experiments (*n* = 3).

In order to explore the interaction between strength of TCR-stimulation and relative DC numbers, T cells were again stimulated in the presence of both CsA and abatacept using DCs as the APCs. As with CHO-FcR/CD80-driven stimulation, the combination of CsA and abatacept inhibited DC mediated T cell proliferation to a greater extent than with abatacept alone (e.g. compare red traces 0.025µg/mL CsA vs. 0µg/mL CsA **fig. 3.15A**). This effect was observed at all DC:T cell ratios tested. However, the CsA concentration required to entirely inhibit T cell proliferation was higher at elevated DC:T cell ratios (e.g. see blue traces 0.025µg/mL CsA; **fig. 3.15A**). This suggests that at high DC:T cell ratios, TCR signaling is more potent, more CsA-resistant and more CD28-independent. These data also demonstrate the extent to which the inhibition by CsA treatment alone was influenced by the DC:T cell ratio (e.g. compare solid lines between different DC:T cell ratios **fig. 3.15B**). For instance, when stimulated at a DC:T cell of 1:40, an inhibition of T cell proliferation occurred in response to low CsA concentrations alone (≥ 0.1 µg/mL CsA). In contrast, at a ratio of 1:5, T cell proliferation still occurred at the highest CsA concentrations tested (**fig. 3.15A/B**). It has been suggested that costimulatory signaling underlies this CsA-resistant T cell activation (Geginat et al., 2000). Therefore, CD28-dependent T cell proliferation also appeared to occur more effectively at high DC:T cell ratios.

A

B

DC:T cell

1:5   1:20   **Unstimulated**

vβ2 TCR

vBeta2 TCR MFI = 50   vBeta2 TCR MFI = 101   vBeta2 TCR MFI= 83.8

Cell trace violet

vβ2 TCR

□ Untreated
■ Abatacept

vβ2 TCR MFI

1:5   1:20

DC:T cell
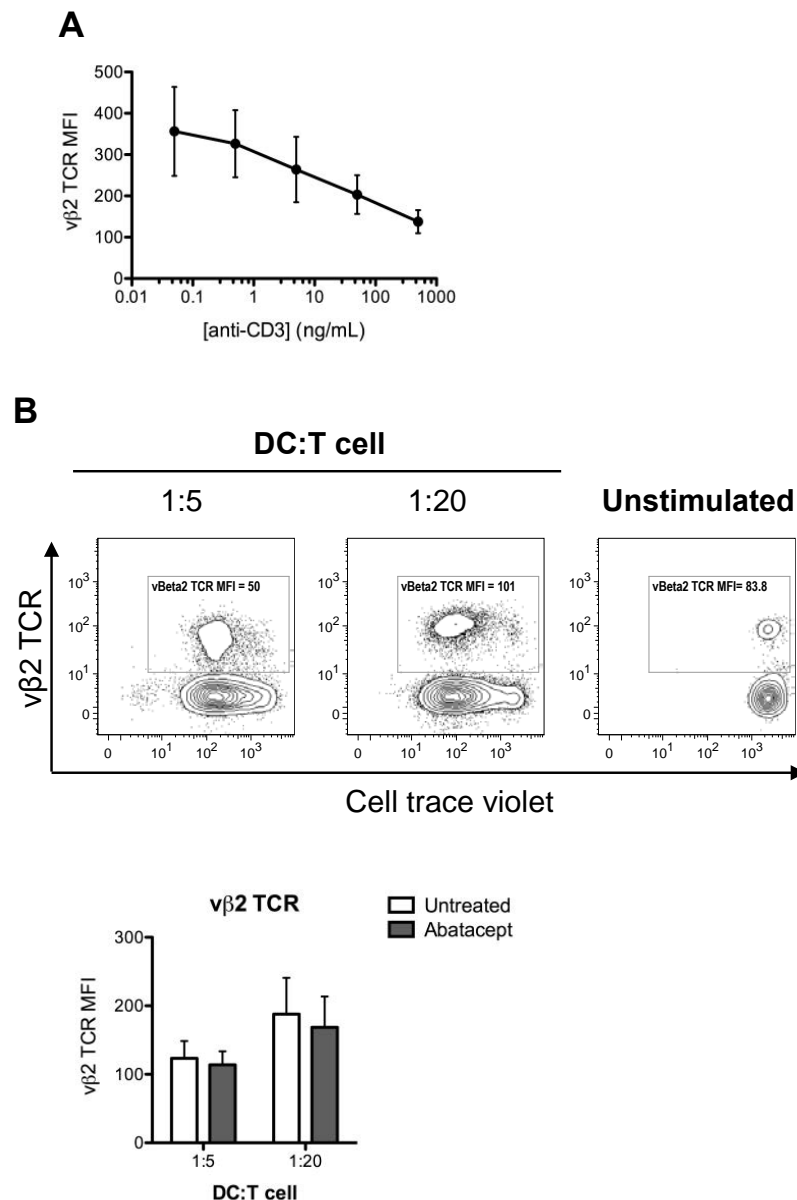
Fig. 3.16. Antigen-independent TCR downregulation tends to be increased when T cell stimulation is mediated by high relative DC numbers. CTV-labeled T cells were co-cultured with anti-CD3 and DCs. vβ2 TCR expression levels were determined by immunofluorescence staining followed by flow cytometric analysis. **A**) T cells were stimulated with indicated anti-CD3 concentrations at a DC:T cell ratio of 1:5 for 5 days. Data points represent mean values ±S.E. from independent experiments (n = 3). **B**) T cells were stimulated with 0.5µg/mL anti-CD3 at a DC:T cell ratio of 1:5 or 1:20, -/+ 20µg/mL abatacept for 5 days. Representative flow cytometry data and combined data showing vβ2 TCR MFI (gated on CTV^{low} T cells) from independent experiments (n = 5).

3.2.8. T cell stimulation at high DC:T cell ratios promotes TCR-downregulation

The extent of TCR downregulation following stimulation has been identified as a putative marker of the strength of TCR stimulus (Monjas et al., 2004). TCR-downregulation can occur as a result of endocytosis following TCR-ligation. Alternatively, protein tyrosine kinase dependent signals prompt downregulation of non-engaged TCRs (San José et al., 2000; Monjas et al., 2004). Since it seemed that T cell activation at high DC:T cell ratios altered the intensity of T cell activation signals I wanted to determine whether differences in TCR downregulation could be detected between different DC:T cell ratios. To determine the validity of this approach T cells were stimulated at a DC:T cell ratio of 1:5 but with different anti-CD3 concentrations to manipulate TCR signals and measured the surface expression of $v\beta2^+$ TCRs as a proxy for all TCRs. These data demonstrated that by increasing anti-CD3 concentration the surface expression of $v\beta2$ TCRs tended to be reduced, indicating that TCR downregulation maybe promoted by high intensity anti-CD3 stimulation (**fig. 3.16A**). Furthermore, downregulation of $v\beta2$ TCR surface expression levels following stimulation at low DC:T cell ratios (1DC:20 T cell) appeared to be less compared to a higher (1DC:5 T cell) ratio (**fig. 3.16B**), however, additional experiments are required for appropriate statistical analysis of this trend. These differences did not appear particularly robust which may be due to the lack of direct TCR-engagement during anti-CD3 driven stimulation. However, the trend did support the conclusion that stimulation at high DC:T cell ratios enhances the intensity of T cell activation signals.
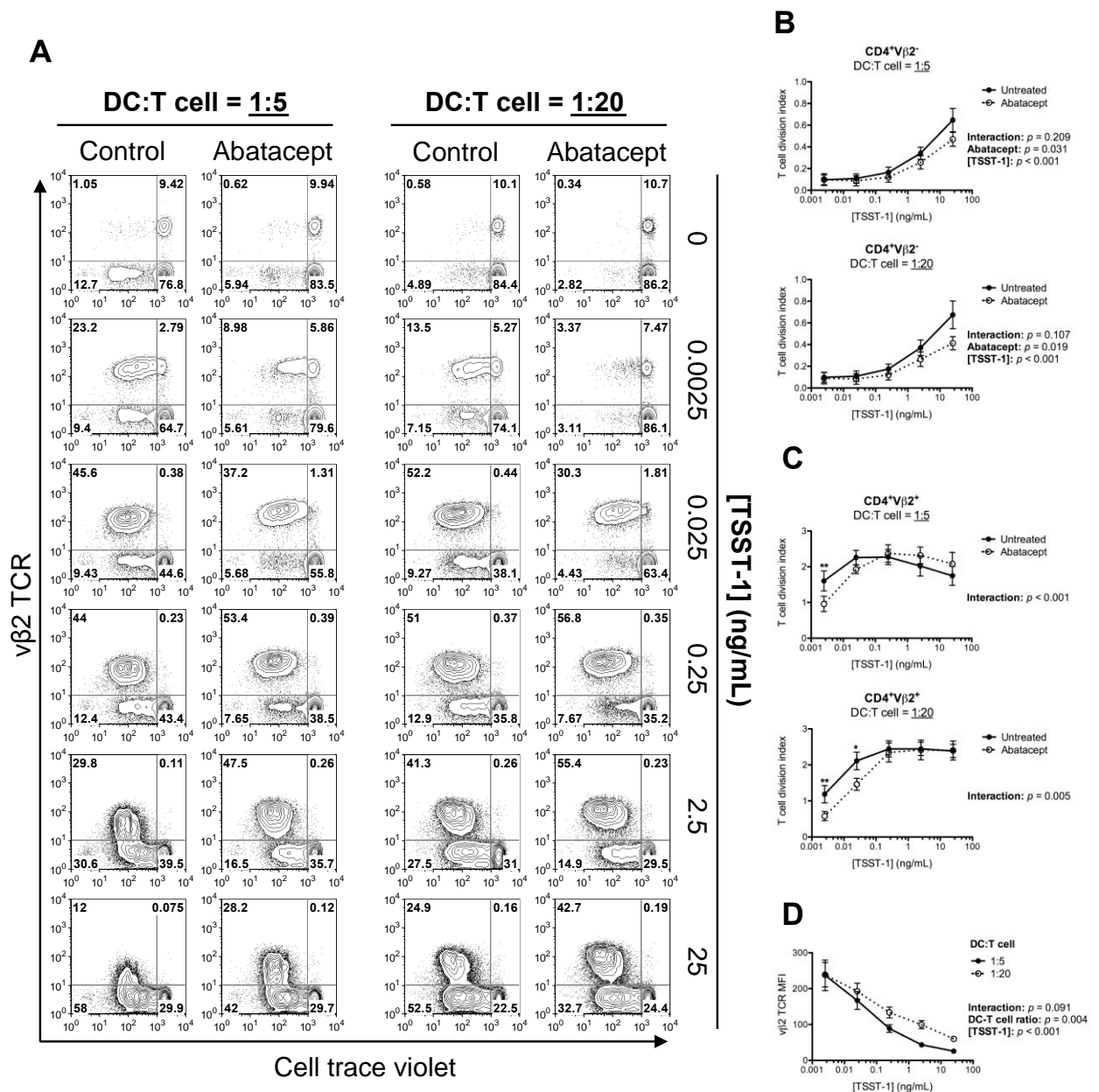
Fig. 3.17. Superantigen affinity, concentration and relative DC numbers determine T cell CD28-requirements. CTV-labeled T cells were stimulated with various TSST-1 concentrations and DCs -/+ (20µg/mL) abatacept for 5 days. vβ2 TCR expression levels were determined by immunofluorescence staining followed by flow cytometric analysis. **A**) Representative flow cytometry data. **B**) Mean division index values for CD4+vβ2- T cells. Data points represent mean values ±S.E. from independent experiments ($n = 7$). **C**) Mean division index values for CD4+vβ2+ T cells. Data points represent mean values ±S.E. from independent experiments ($n = 7$). **D**) vβ2 TCR expression levels were determined in T cells stimulated at a DC:T cell ratio of 1:5 compared to 1:20 and expressed as MFI. Data points represent mean values ±S.E. from independent experiments ($n = 7$). p values are derived from two-way ANOVA. Further analyses was performed using paired, two-tailed t tests; *ns* not significant *$p \leq 0.05$ **$p \leq 0.01$.

3.2.9. DC:T cell ratio influences T cell activation thresholds in superantigen-driven T cell activation

To further explore the interaction between TCR stimulus strength and CD28-costimulation requirements and to ensure that the impact of DC:T cell upon abatacept-sensitivity was not an artifact of anti-CD3 stimulation, T cells were stimulated with the staphylococcal superantigen TSST-1. TSST-1 facilitates simultaneous interaction between TCRs that contain the vβ2 domain and MHC-II molecules (Li et al., 1999). Therefore, this model allowed us to alter parameters that affect the intensity of TCR-stimulation in the context of different DC:T cell ratios using a system other than anti-CD3 crosslinking. Results from these experiments further supported the hypothesis that strong TCR stimulation overcomes CD28-requirements. Firstly, at higher TSST-1 concentrations, the induction of T cell activation by TSST-1 was less restricted to vβ2^{+} T cell responders since lower affinity vβ2^{-} T cells also proliferated (**fig. 3.17A/B**). However, whilst abatacept did not affect the proliferation of vβ2^{+} T cells at this high dose of TSST-1, proliferation of vβ2^{-} T cells was inhibited by abatacept and was therefore CD28-dependent (e.g. 2.5ng/mL TSST-1 **fig. 3.17A**). Thus, CD28-costimulation was required to support T cell proliferation in association with low affinity TCR-engagement but was not necessarily required by high affinity TCRs. Similar to observations with anti-CD3 stimulation, abatacept had no impact upon vβ2^{+} T cell proliferation in response to high TSST-1 concentrations (≥0.25ng/mL TSST-1), however, at low TSST-1 concentrations stimulations became abatacept-sensitive (**fig. 3.17A/C**). Therefore, CD28-costimulation also drives T cell proliferation when superantigen is at low concentration. Importantly, vβ2^{+} T cell proliferation

became abatacept sensitive at ten-fold higher TSST-1 concentration when stimulating with a DC:T cell ratio of 1:20 compared to a 1:5 ratio (**fig. 3.17C**). This suggests that high DC:T cell ratios can compensate for reduced antigen-load by enhancing the potency of TCR-driven stimulation. In support of this, greater vβ2 TCR downregulation was apparent when stimulating with high TSST-1 concentrations in 1:5 instead of 1:20 DC:T cell co-cultures (**fig. 3.17A/D**). Since TCR downregulation broadly correlates with strength of stimulation, these data provide further evidence that increasing the DC:T cell ratio promotes the delivery of T cell activation signals that facilitate CD28-independent T cell proliferation.

3.3. Discussion

As the predominant source of T cell costimulation, CD28-signaling enhances the generation of T cell effector populations via effects upon proliferation, survival and differentiation (Boomer and Green, 2010). Blocking this signal using abatacept is effective for the treatment of several T cell related diseases (Linsley and Nadler, 2009). However, responses to treatment are variable and suboptimal in a large proportion of patients. Therefore, the identification of factors that influence treatment responses along with the development of approaches to enhance responsiveness is an important clinical challenge.

The blockade of T cell activation by abatacept is dependent upon the extent to which CD28 contributes to T cell activation. However, the fact that T cell responses can occur in the absence of CD28-costimulation has been

demonstrated in CD28-knock out (KO) mouse models. In these models, *in vitro* T cell responses to concanavalin A, anti-CD3 and alloantigen were impaired but not entirely absent (Shahinian et al., 1993; Green et al., 1994). Interestingly, *in vivo* humoral responses were also highly defective (Shahinian et al., 1993) consistent with a role for CD28 in the development of T follicular helper cell differentiation (Platt et al., 2010). In contrast, T cell mediated immune responses to lymphocytic choriomeningitis virus which is widely regarded as a strong antigenic stimulus were normal (Shahinian et al., 1993). CD28-KO T cells are also capable of mediating both skin allograft rejection (Kawai et al., 1996), acute lethal graft-versus-host disease (Speiser et al., 1997) and diabetogenic responses in NOD mice (Lenschow et al., 1996). Thus T cell mediated immunity persists in a CD28-deficient environment.

T cell activation in CD28-knock out models has been found to be dependent upon the strength (Bachmann et al., 1996) and duration (Kündig et al., 1996) of TCR stimulation. This suggests that strong TCR-stimulation can compensate for a deficit of CD28-costimulation. Consistent with this concept, we have found that in the context of *in vitro* T cell activation, factors that increase the strength of TCR signaling can override T cell CD28-requirements and therefore promote abatacept-resistance. For example, we found that abatacept robustly inhibited T cell proliferation driven by soluble anti-CD3 and CHO-CD80 cells, in contrast, cross-linked anti-CD3 (at the same dose) mediated by CHO-FcR/CD80 promoted abatacept resistant T cell activation. Additionally, as a specific inhibitor of TCR signaling events (Geginat et al., 2000), we found that the combination of CsA and CD28-costimulation

blockade by abatacept synergize to potently suppress T cell activation, indicating that reducing TCR signaling increases abatacept responsiveness. Similarly, while responses to TSST-1 by high affinity T cells ($v\beta2^+$ TCR) were abatacept-resistant and therefore did not depend upon CD28 costimulation, proliferation became increasingly CD28-dependent at lower TSST-1 concentrations. Interestingly, we also observed T cell proliferation by cells expressing low affinity ($v\beta2^-$) TCRs, however, this was abatacept-sensitive and therefore CD28-dependent, even at high TSST-1 concentrations. Together these data make a clear case for the intensity of TCR signaling affecting abatacept sensitivity.

In keeping with previous observations, we have found that naïve T cells display greater CD28-requirements in comparison to memory T cells. These differences are associated with the reduced threshold for memory T cell activation that underlies the generation of rapid and robust secondary responses to antigen (Farber, 2009). Interestingly, distinct differences in TCR signal transduction between naïve and memory T cells have been observed wherein the magnitude of downstream TCR signals are enhanced in memory relative to naïve T cells (Kalland et al., 2011). These differences may reflect elevated expression levels or function of TCR-signaling mediators such as Zap-70 (Chandok et al., 2007), NFAT (Dienz et al., 2007), PLC-γ1 (vonEssen et al., 2010) or PKC-θ (vonEssen et al., 2013). Similarly, memory T cells express higher levels of adhesion molecules that promote the stability of interactions with APCs in memory compared to naïve T cell subsets (Sanders et al., 1988; Buckle and Hogg, 1990; Hamann et al., 1997). Several studies

have suggested differences between the impact of distinct CD45 isoforms that define naïve and memory subsets upon TCR signaling pathways (Novak et al., 1994; McKenney et al., 1995; Leitenberg et al., 1996). Additionally, more substantial lipid rafts with a greater content of TCRs and proximal signaling molecules within the plasma membrane of memory compared to naïve CD8$^+$ T cells has been suggested to enhance the magnitude of downstream TCR signaling upon antigen exposure (Kersh et al., 2003). Together these differences favor TCR-driven responses in memory T cells and would appear to promote CD28-independent responses that are not inhibited by abatacept. Nonetheless it is probably the case that memory T cells are still costimulation sensitive albeit over a different dose range of TCR stimulation compared with naïve T cells.

These results demonstrate that robust TCR stimulation can overcome a deficiency of CD28-costimulation and vice versa. Therefore factors that promote TCR driven responses such as increased antigen density or affinity or memory/naïve status promote CD28-independent T cell responses. Nonetheless, CD28-signaling does appear to impact T cell differentiation. I have shown that an absence of costimulation modifies the expression of markers of effector function by activated T cells including an inhibition of pro-regulatory outcomes. These data therefore support the concept that TCR and CD28 cooperate towards a combined activation threshold (Acuto and Michel, 2003). There is a clear biological basis for the idea that CD28 acts a quantitative support to TCR stimulation in that there is a significant degree of overlap between TCR and CD28 signaling mediators. A substantial function of

CD28 engagement appears to be the amplification of TCR signaling pathways (Michel et al., 2000; 2001; Holdorf et al., 2002) which may underlie the reduced TCR signaling threshold for T cell activation that occurs in the presence of costimulation (Viola and Lanzavecchia, 1996). Furthermore, the analysis of gene expression following CD28 engagement alone shows significant overlap to that induced by TCR-stimulated cells (Diehn et al., 2002; Riley et al., 2002; Wakamatsu et al., 2013). Nonetheless, the relative resistance of CD28-signaling to CsA as well as phenotypic changes supports the possibility that some CD28 signals are unique.

An interesting observation reported here is that the number of antigen presenting cells influences the strength of TCR signaling. Accordingly this affects the requirement for CD28-costimulation and ultimately abatacept sensitivity. Whilst it is easy to appreciate that the number of APCs may influence the actual number of T cells that become stimulated (see **fig. 3.5.**), it is less obvious why the intensity of TCR signals should change with APC number. In particular why do higher numbers of APC appear to enhance TCR pathway stimulation as seen by TCR downregulation, sensitivity to CsA and resistance to abatacept?

In simplistic terms, T cell activation occurs as the result of the integration of TCR and costimulatory signals following interactions with APCs. Generally, this has been thought to arise as the result of a productive interaction between a T cell and an APC presenting cognate antigen. Such interactions occur as T cells scan the surface of DCs for cognate antigen:MHC complexes

in an antigen-independent manner that utilizes interaction between ICAM-3 with DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) (Montoya et al., 2002). High affinity interactions between pMHC and TCR subsequently produce threshold signaling events and the generation of a TCR "stop signal" which is largely mediated by conformational changes in T cell LFA-1 (Dustin and Springer, 1989; Dustin et al., 1997). However, cognate interactions between T cells and APCs within a 3D collagen matrix were characterized by transient encounters that nevertheless support activation signaling (Gunzer et al., 2000). These results prompted a new paradigm for T cell priming in which activation thresholds can be achieved by the summation of multiple, sub threshold interactions rather than as a result of a single "all or nothing" signaling event (Friedl and Gunzer, 2001).

This concept of sequential interactions between APC and T cell during priming has been supported using *in vivo* mouse models in which T cell interactions with APCs are visualized within lymphoid tissue using multi-photon microscopy. These studies have led to the characterization of a multi-phase process (Mempel et al., 2004) whereby T cells encountering cognate antigen initially assume transient contacts with APCs for around 8 hours which resemble non-specific interactions with APCs. However, these interactions lead to CD69 upregulation and support the transition towards a phase (lasting ~12 hours) that is characterized by more stable APC:T cell interactions. The transition to these stable interactions is essential to the generation of effective CD4^{+} T cell responses (Celli et al., 2007). The dynamics associated with this progression from sequential to more stable

contacts between T cells and APCs are modified by various factors including APC maturation state (Hugues et al., 2004; Mittelbrunn et al., 2009) and the activity of T_{reg} (Tang et al., 2006; Tadokoro et al., 2006). Crucially, elevated antigen dose (Henrickson et al., 2008) or TCR-affinity (Skokos et al., 2007) decreases the duration of phase one and facilitates the transition to more stable APC:T cell interaction. This implies that the number of interactions required to meet activation thresholds are decreased if T cells can accumulate TCR signals more effectively from serial contacts.

Our results suggest that a serial encounter model for T cell activation can modify T cell CD28-requirements due to the interactive nature between TCR and costimulatory signaling. We find that *in vitro* T cell activation driven by soluble anti-CD3 and allogeneic T cells becomes increasingly CD28-dependent as DC:T cell ratios are reduced. This observation cannot be explained by a mechanism of T cell activation in which stimulatory signals are delivered by a single encounter between DC and T cell. Such a concept would only account for a change in the proportion of T cells that are activated in response to different relative DC numbers, i.e. more DC should support the priming of more T cells but not change the quality of the TCR signal. Instead, we observe increased potency of anti-CD3 stimulation in the presence of high relative DC numbers (thereby reducing abatacept efficacy) which strongly suggests that T cells integrate anti-CD3 driven activation signals as a result of multiple interactions with DCs. Importantly, the influence of the DC:T cell ratio upon CD28-requirements was not restricted to stimulation driven by anti-CD3 but was also apparent in superantigen-driven activation and seen using a

fixed transfectant model. Accordingly, we found that T cell responses to TSST-1 became abatacept sensitive at a 10 fold higher TSST-1 concentration when stimulating at a DC:T cell of 1:5 compared to 1:20. Furthermore, as a putative marker of T cell stimulus strength, we observed more significant TCR-downregulation associated with high- compared to low-relative DC numbers. Given that we observed the same effects using FcR/CD80 transfected cells, this effect of APC number influencing TCR potency is not restricted to DCs but is a general feature of the number of APCs.

The frequency of interactions between APC and T cell during activation may act to modify the signaling experience of T cells in at two least ways. Firstly, the accumulation of signals between successive APC interactions may directly influence signal intensity. The summation of TCR-signals from multiple DC interactions imposes a requirement for T cell "memory" of previous interactions. It has been proposed that this memory is analogous to the temporal summation of presynaptic potentials into the magnitude of postsynaptic potentials in neuronal cell activation (Rachmilewitz and Lanzavecchia, 2002). It has been found that a proportion of naïve T cells progress from G_0 to the G_1 phase of the cell cycle but remain undivided following short term, sub threshold periods of stimulation. These cells subsequently respond to a second sub-threshold period of stimulation whereas cells that remained in G_0 following the primary stimulation did not. Therefore, T cells that enter G_1 integrate multiple sub-threshold stimulations in order to reach the threshold to for proliferation (Munitic et al., 2005).

A mechanism has been identified whereby T cells may 'remember' signals from previous APC interactions through the temporal dynamics that are associated with NFAT translocation to the nucleus following TCR signaling (Marangoni et al., 2013) – whilst NFAT import occurs rapidly, export from the nucleus is much slower, such that sequential interactions can trigger NFAT translocation when a signal has not yet entirely resolved from a previous interaction. The result is an incremental rise in NFAT translocation between DC:T cell interactions which acts to propagate the outcome of TCR signaling. Similarly, transient TCR signals facilitate the upregulation and phosphorylation of the AP-1 component cFos, importantly, the active cFos levels remain elevated for at least 24 hours following this suboptimal TCR stimulation thereby reducing the activation threshold for subsequent TCR-signals (Clark et al., 2011).

In the context of results presented in this study, DC:T cell contacts are frequent in the presence of high relative DC numbers which could allow for an overlap between sub threshold signaling events that arise as a result of serial interactions. The summation of these events ultimately meets T cell activation thresholds, even in the absence of costimulation. In contrast, during T cell activation at low DC:T cell ratios, contacts between DC and T cell are rare to an extent that does not facilitate summation of TCR signaling within an appropriate time frame (Celli et al., 2005). As such, T cell activation becomes more dependent upon CD28-costimulation.

The reduction in CD28-costimulation requirements at high DC:T cell has

implications not only for the inhibition of T cell responses by abatacept but also for CTLA-4 mediated control of T cell activation in immune regulation by trans-endocytosis of CD80 and CD86 (Qureshi et al., 2011). In this way, CTLA-4 is essential to the activity of FoxP3$^+$ T$_{reg}$ which modify the availability of CD28-costimulation below a threshold level that is inhibitory to the initiation of autoimmune responses under tolerogenic conditions (Wing et al., 2008). Similarly, conventional T cells express CTLA-4 and regulate T cell activation in a cell-extrinsic manner (Corse and Allison, 2012; Wang et al., 2012), potentially via a feedback mechanism whereby T cells reduce the stimulatory capacity of APCs to promote the resolution of a response. Elevated relative APC numbers within a system can serve to inhibit CTLA-4 mediated suppression of T cell activation in at least two ways. Firstly, the corresponding increase in CD80/CD86 levels serves to increase the availability of CD28-costimulation to T cells within the system and therefore the amount of CTLA-4 mediated trans-endocytosis that is required to inhibit T cell activation (Hou et al., 2015). Secondly, as shown here, due to the facilitation of TCR stimulation in the presence of high relative DC numbers, T cell activation can occur independent of CD28 and is therefore outside the control of CTLA-4. Under such conditions, inhibition of T cell activation mediated by PD-1 may become more important to immune regulation as this has been found to target early TCR signaling events (Sheppard et al., 2004; Yokosuka et al., 2012; Wei et al., 2013).

An important consideration in relation to this work is the extent to which these observations are translated to T cell priming during *in vivo* stimulation. Indeed,

several factors add extra layers of complexity to the impact of DC:T cell ratios upon *in vivo* T cell priming within lymphoid tissue. For example, lymphoid architecture is maintained *in vivo* in such a way that maximizes cellular interactions. This occurs to the extent that a single DC may contact as many 5000 T cells per hour (Miller et al., 2004). As such the capacity for T cells to integrate signals from multiple DCs *in vivo* is extensive. Despite this, high affinity interactions between TCR and cognate antigen are rare within the lymph node. As such, many interactions between T cells and DCs may appear non-productive. Nevertheless, these low-affinity interactions have been found to be crucial to antigen responsiveness by generating 'basal activation' among T cells that facilitates subsequent responses to stimulation (Stefanová et al., 2002; Contento et al., 2010; Römer et al., 2011). These lines of evidence suggest that the frequency of DC:T cell interactions may modify T cell activation thresholds during both *in vivo* and *in vitro* stimulation.

These lines of evidence demonstrate that an important future direction is to test how these factors affect the extent to which DC:T cell ratios affect costimulation requirements using *in vivo* models. For example, a mouse model of DC depletion has been reported in which CD11c^{+} express the diphtheria toxin receptor and are deleted upon administration of diphtheria toxin (Hochweller et al., 2010). T cells derived from mice depleted of DCs are characterized by impaired TCR signaling and immune synapse formation upon exposure to cognate antigen *in vitro*. This model could be utilized with a mixed bone marrow chimera approach to delete different relative numbers of DCs in order to determine the impact of DC:T cell ratio upon costimulation

requirements. Similarly, Henrickson, *et al.* utilized an adoptive cell transfer approach in which different relative numbers of AG-pulsed DCs were transferred to recipient mice in order to test the impact of lymph node APC density upon parameters of T cell activation (Henrickson et al., 2008). An alternative approach to these experiments would be to change the number of Ag specific T cells relative to fixed DC numbers. In relation to this, bone marrow chimeras between Recombination-activating gene (RAG)-2 deficient and TCR transgenic mice have been generated which display variable frequency of Ag-specific T cells (Laouar and Crispe, 2000).

Overall, the results presented in this chapter demonstrate a mechanism whereby TCR stimulus strength is determined by the initial DC:T cell ratio at which T cells are activated such that in the presence of high DC numbers, T cell activation can occur independently of CD28-costimulation. This observation suggests that the relative availability of local APCs acts as environmental cue that changes the signaling experience delivered during T cell priming. This has clear implications for the use of abatacept in the treatment of T cell mediated diseases. Additionally, these observations are important to the interpretation of results of experiments in which T cells have been stimulated *in vitro.* For example, Davis, *et al.* demonstrated robust suppression by abatacept of allogeneic DC-driven T cell activation at a DC:T cell ratio of 1:50 (Davis et al., 2008). However, our results suggest that any such suppression is context specific and depends strongly upon the DC:T cell ratio at which T cell stimulation is induced.

Chapter 4: 1,25(OH)$_2$D$_3$ promotes the efficacy of CD28-costimulation blockade by abatacept

4.1. Introduction

The extent to which CD28-blockade by abatacept inhibits T cell activation is dependent upon the capacity for other stimulatory pathways to compensate for the absence of CD28-signals. However, studies of CD28-KO mice suggest that although T cell responses are impaired they are not entirely absent As such, a lack of efficacy associated with CD28-blockade in therapy, for example in the treatment of RA or in immunosuppression following renal transplantation, may be expected in some situations where T cell stimulation is driven by non-CD28 signals. Consistent with this, an improvement in the outcome of T cell responses following costimulation blockade has been identified by combining CTLA-4-ig with other immunomodulatory agent. For example, interruption of the CD40:CD154 pathway in association with CTLA-4-ig therapy results in the synergistic enhancement of allograft survival in both murine and non-human primate transplantation models (Gilson et al., 2009; Badell et al., 2012). Administration of alefacept, a CD2 specific fusion protein (leukocyte function-associated antigen (LFA)-3-ig) led to memory T cell depletion and promoted renal allograft survival in a non-human primates following abatacept treatment (Weaver et al., 2009). Likewise, belatacept in combination with the mTOR inhibitor sirolimus promoted allograft survival to a greater extent than with belatacept therapy alone (Lo et al., 2013). These

studies challenge the perspective of CD28 as a fundamental requirement for T cell activation. As such, evidence suggests that optimal responses to CD28-blockade require its combination with other immunomodulatory agents.

It has been argued that CD28-signaling acts as a quantitative support to TCR stimulation (Acuto and Michel, 2003), i.e. that the relative absence of TCR-signals can be compensated for by a predominantly CD28-driven response. In support of this, results from chapter 3 suggest that substantial T cell activation can occur in the presence of abatacept treatment using *in vitro* assays and that this activation is associated with the potency of TCR-stimulation. Furthermore, a combination of CsA (which essentially targets the productivity of TCR-signaling) and CTLA-4-ig led to potent suppression of T cell activation above the level of suppression seen by CsA or abatacept alone (Geginat et al., 2000).

Results in this chapter suggest that the active form of vitamin D3, $1,25(OH)_2D_3$, acts in a similar manner to CsA by acting directly upon T cells to inhibit TCR-driven stimulation in the absence of costimulation. Thus, by increasing reliance on CD28-costimulation, $1,25(OH)_2D_3$ renders T cell responses more abatacept-sensitive. These data suggest that vitamin D3 supplementation may be a simple approach to improve outcomes of CD28-blockade in treatment for patients with T cell mediated inflammatory diseases.
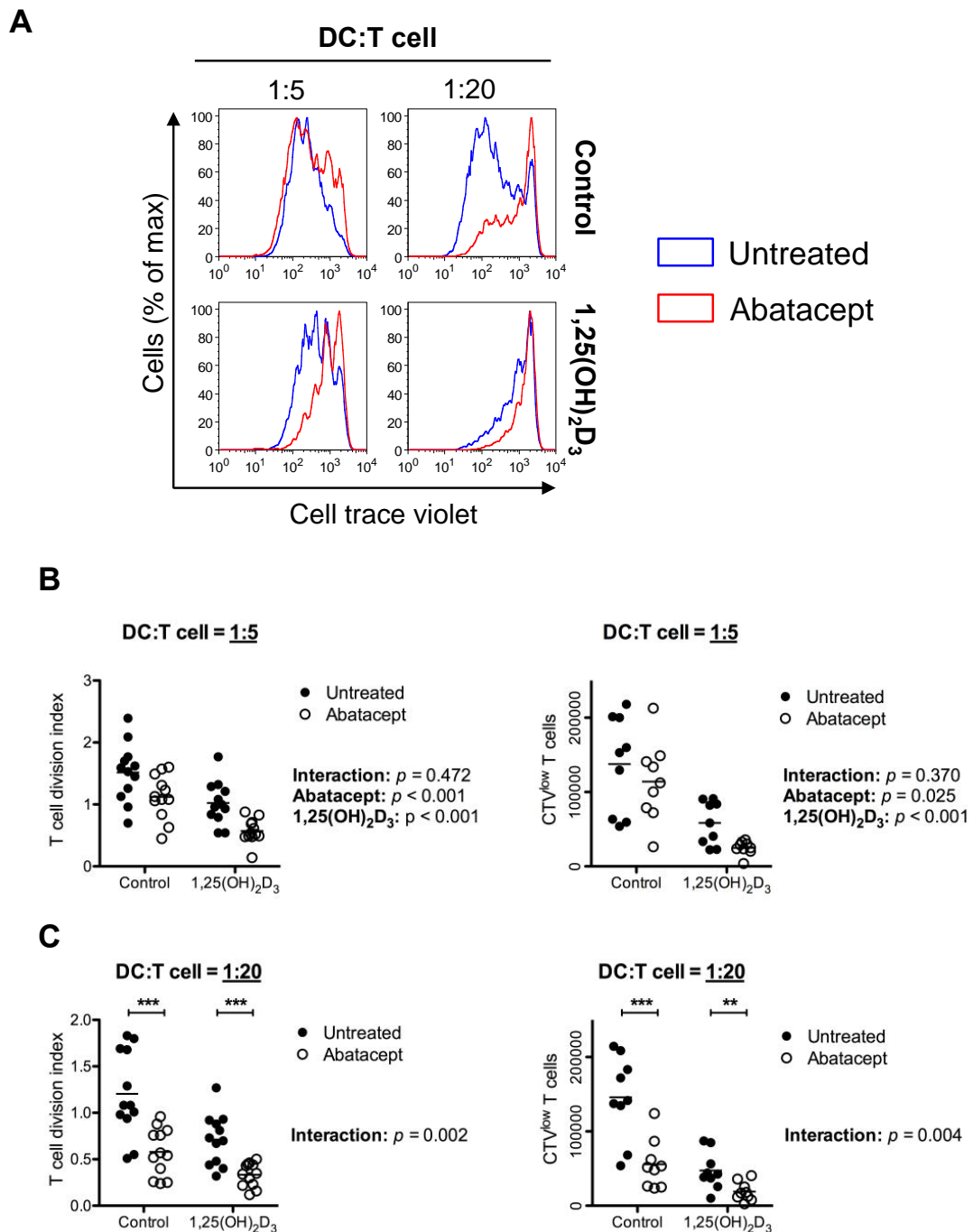
Fig. 4.1. 1,25(OH)$_2$D$_3$ increases the suppression of T cell proliferation by abatacept. CTV labeled CD4$^+$CD25$^-$ were incubated with 500ng/mL anti-CD3 and allogeneic DCs with or without 20µg/mL abatacept and 10nM 1,25(OH)$_2$D$_3$ for 5 days. **A**) Flow cytometric data showing CTV dilution from one representative experiment. Combined data from independent experiments expressed as either division index values or total numbers of proliferating (CTV^{low}) T cells for stimulations at a DC:T cell ratio of 1:5 (**B**) or 1:20 (**C**). Horizontal bars represent mean values. *p* values are derived from two-way ANOVA. Further analyses were performed using paired, two-tailed *t* tests where **$p \leq 0.01$, ***$p \leq 0.001$.

4.2. Results

4.2.1. 1,25(OH)$_2$D$_3$ enhances the efficacy of abatacept via a T cell intrinsic mechanism

Previous studies have identified effects of 1,25(OH)$_2$D$_3$, upon CD4$^+$ T cell activation and differentiation (Peelen et al., 2011). I determined how 1,25(OH)$_2$D$_3$ affected the inhibition of T cell responses by abatacept. Interestingly, the combination of 1,25(OH)$_2$D$_3$ and abatacept inhibited T cell proliferation to a greater extent than was observed with abatacept alone (**fig. 4.1A**). Two-way ANOVA analysis of T cell proliferation data from multiple donors where T cell activation was driven at a DC:T cell ratio of 1:5 data demonstrated a non-significant interaction effect between 1,25(OH)$_2$D$_3$ and abatacept; however, significant overall effects were observed for both agents (**fig. 4.1B**). These observations suggest that 1,25(OH)$_2$D$_3$ and abatacept combine to suppress T cell proliferation through an additive rather than a synergistic effect under these conditions. The combination of 1,25(OH)$_2$D$_3$ with abatacept almost entirely inhibited T cell proliferation where T cell proliferation was driven at a lower DC:T cell ratio (1DC:20 T cells; **fig 4.1A/C**). However, under these conditions, abatacept alone robustly inhibited T cell proliferation, which was considered to occur due to reduced potency of anti-CD3 signaling as suggested by data in chapter 3 of this thesis **fig 4.1C**. Together, these data suggested that 1,25(OH)$_2$D$_3$ supplementation enhances the efficacy of abatacept treatment and that the cooperation is more beneficial at higher DC:T cell ratios where the strength of TCR stimulation is higher.
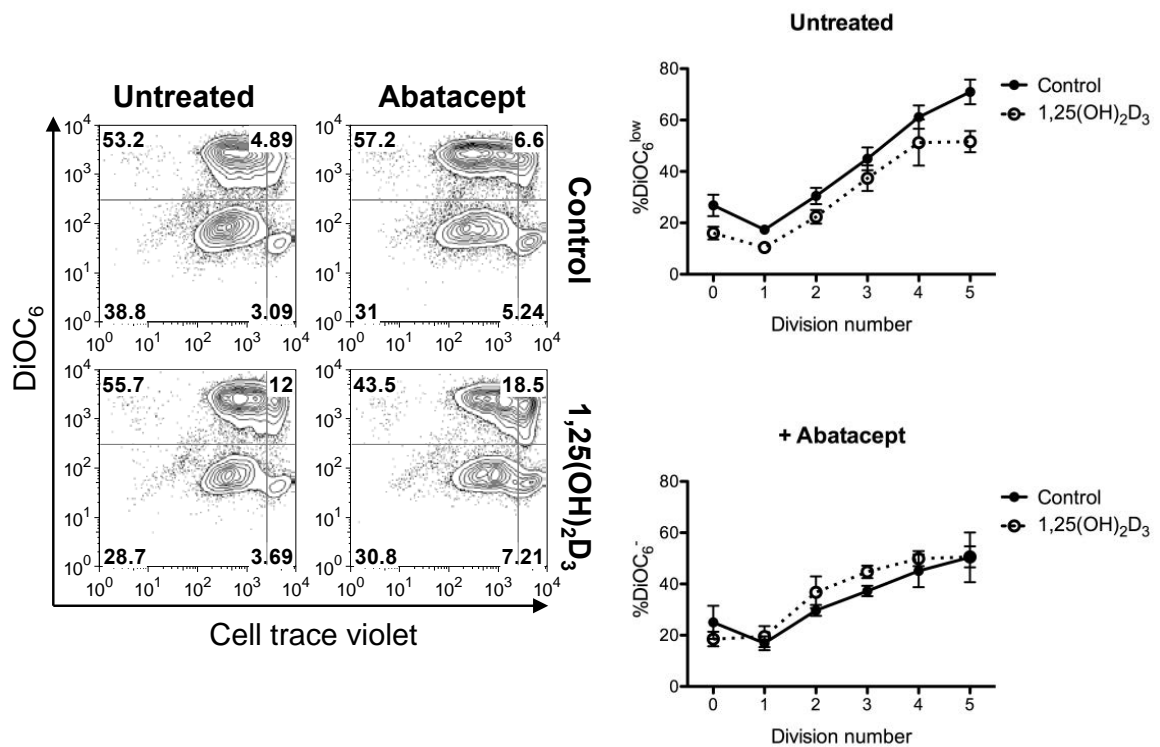
Fig. 4.2. Abatacept in conjunction with 1,25(OH)$_2$D$_3$ does not influence T cell apoptosis. CTV-labeled CD4$^+$ CD25$^-$ were activated with 0.5µg/mL anti-CD3 and DCs at a ratio of 1 DC:5 T cells in association with 20µg/mL abatacept and 10nM 1,25(OH)$_2$D$_3$ for 5 days. Activated T cells were stained with DiOC$_6$ and analysed by flow cytometry with DiOC$_6$^{low} cells representing apoptotic cells. Representative flow cytometry data and mean ±S.E values values from independent experiments (*n*=3).
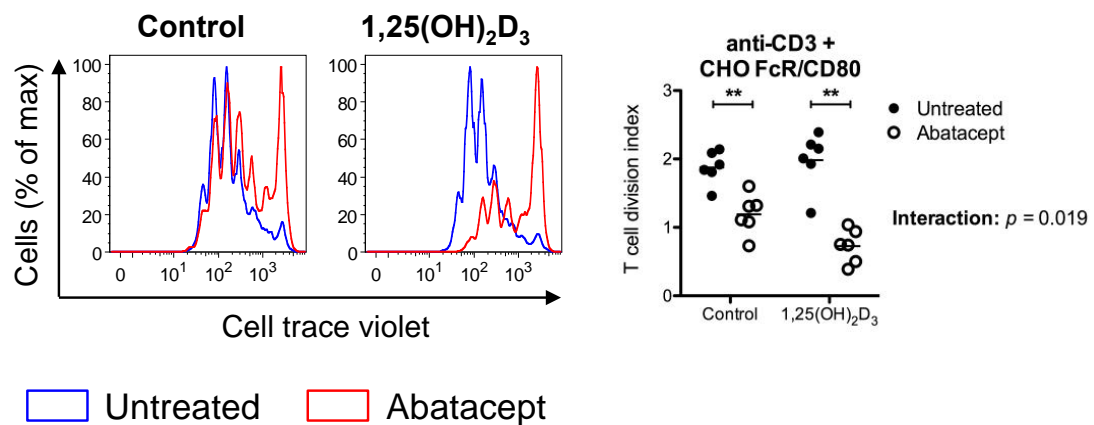
Fig. 4.3. 1,25(OH)$_2$D$_3$ increases the suppression of T cell proliferation by abatacept via a T cell intrinsic mechanism. CTV labeled CD4$^+$CD25$^-$ were incubated with 500ng/mL anti-CD3 and glutaraldehyde fixed CHO-FcR/CD80 with or without 20µg/mL abatacept and 10nM 1,25(OH)$_2$D$_3$ for 5 days. Flow cytometric data showing CTV dilution from one representative experiment. Combined data from independent experiments expressed as division index values. Horizontal bars represent mean values. *p* value for interaction between 1,25(OH)$_2$D$_3$ and abatacept was determined by two-way ANOVA. Further analyses were performed using paired, two-tailed *t* tests where **$p \leq 0.01$.
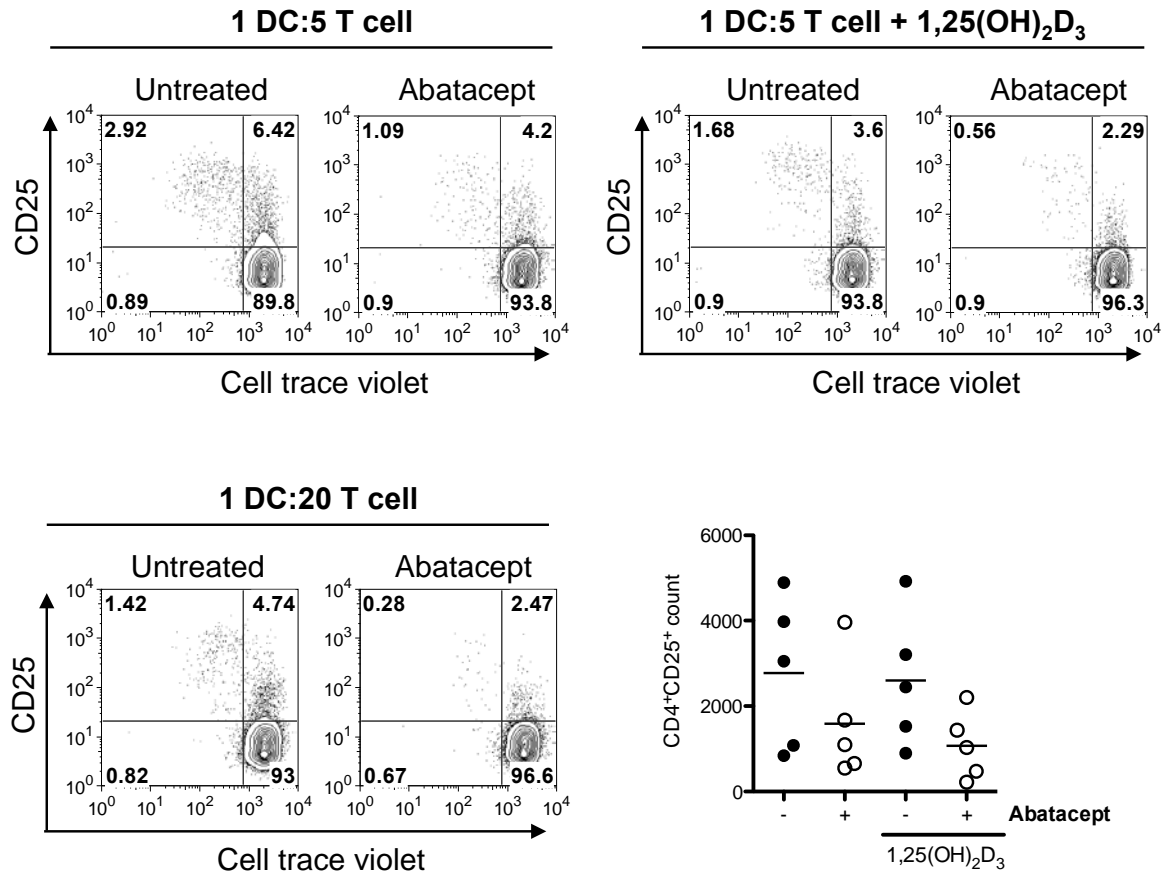
Fig. 4.4. T cell alloresponses are abatacept-sensitive. CTV labeled CD4$^+$CD25$^-$ were cultured in the presence of allogeneic DCs at a DC:T cell ratio of either 1:5 or 1:20 and with either 20µg/mL abatacept and 10nM 1,25(OH)$_2$D$_3$ or both for 5 days. CD25 expression was determined by immunofluorescence staining and flow cytometric analysis. T cell counts were determined relative to counting beads. Representative flow cytometry data showing CTV dilution relative to CD25 expression and data from multiple experiments showing CD4$^+$CD25$^+$ T cell counts. Horizontal bars represent mean values.

A

B
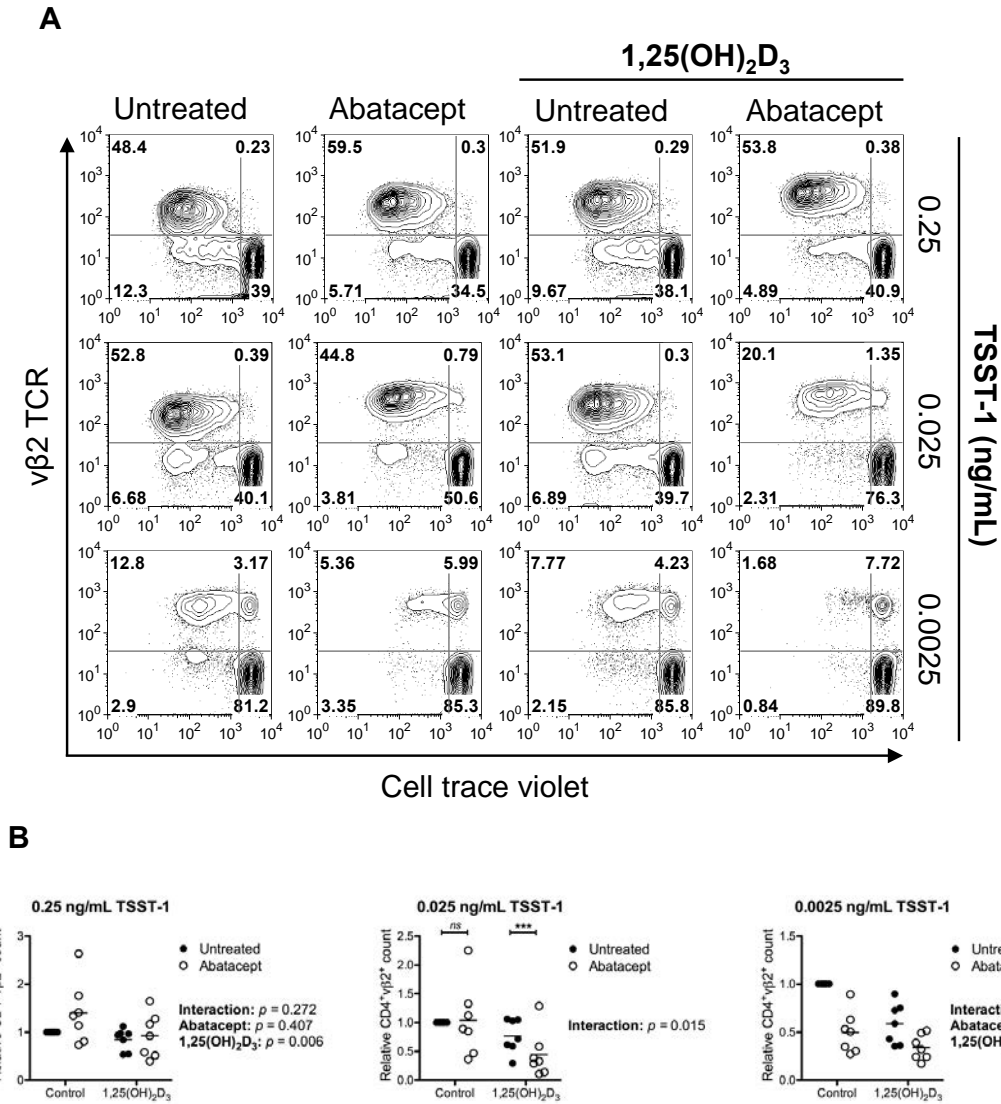
Fig. 4.5. 1,25(OH)$_2$D$_3$ promotes suppression of T cell proliferation by abatacept in superantigen stimulations. CTV labeled CD4$^+$CD25$^-$ were incubated with indicated TSST-1 concentrations and allogeneic DCs and with 20µg/mL abatacept, 10nM 1,25(OH)$_2$D$_3$ or both for 5 days. **A**) Flow cytometric data showing CTV dilution of vβ2$^+$ T cells from one representative experiment. **B**) CD4$^+$vβ2$^+$ T cell counts were determined relative to a known quantity of beads added to each sample prior to flow cytometric analysis. Horizontal bars represent mean values from independent experiments (n = 7). Data were log$_{10}$ transformed prior to statistical analysis. p values were determined by two-way ANOVA. Further analyses were performed using paired, two-tailed t tests where *p ≤0.05, **p ≤0.01.

These differences in the proliferative response resulting from the combination of $1,25(OH)_2D_3$ with abatacept did not appear to be associated with differences in the induction of T cell apoptosis since the combination of $1,25(OH)_2D_3$ with abatacept did not strongly influence the proportion of $DiOC_6^{low}$ staining cells following 5 days stimulation relative to abatacept-treatment alone (**fig. 4.2**).

T cells were stimulated with anti-CD3 in association with fixed CHO FcR/CD80 to determine whether $1,25(OH)_2D_3$ was acting upon the DC, T cell, or both. This system provided costimulation via a fixed artificial APC that could not be influenced by $1,25(OH)_2D_3$. Interestingly, $1,25(OH)_2D_3$ alone failed to inhibit T cell proliferation under these conditions of stimulation (**fig. 4.3**) in contrast to DC-mediated activation (**fig. 4.1**). Importantly, in the absence of $1,25(OH)_2D_3$-responsive APCs, $1,25(OH)_2D_3$ continued to enhance suppression of T cell proliferation by abatacept (**fig. 4.3**). These data therefore support a T cell intrinsic mechanism whereby $1,25(OH)_2D_3$ acts to enhance the control of T cell responses by abatacept in the absence of APCs. Furthermore, having previously found that this abatacept-resistant proliferation was associated with the strength of TCR-stimulation, it appeared that $1,25(OH)_2D_3$ specifically targeted TCR-driven T cell activation.

4.2.2. T cell alloresponses are inhibited by abatacept

The impact of the combination of abatacept with $1,25(OH)_2D_3$ upon T cell activation and proliferation was determined under a variety of activation conditions that were independent of anti-CD3 cross-linking. For instance

allogeneic DCs alone were used to determine the effect of $1,25(OH)_2D_3$ upon T cell alloresponses. These data suggested that when stimulating T cells with allogeneic DCs at a ratio of 1 DC:5 T cells that the response was minimal; frequently just 2-5% $CD4^+$ T cells were observed to be $CD25^{high}CTV^{low}$ following 5 days stimulation (**fig. 4.4A**). Unsurprisingly, reducing the number of allogeneic DCs limited the potency of this response (**fig. 4.4B**). Consistent with previous data this response was CD28-dependent as suggested by the inhibition of T cell proliferation by abatacept. Therefore, the combination of $1,25(OH)_2D_3$ with abatacept was not significantly beneficial under these conditions. Together, these data suggest that alloresponses do not represent a suitable system to analyze CD28-independent T cell responses.

4.2.3. $1,25(OH)_2D_3$ promotes suppression of superantigen-driven T cell activation by abatacept

To determine the impact of $1,25(OH)_2D_3$ in a system in which TCR-stimulus strength could be controlled, T cells were activated with TSST-1. Again, no significant advantage was observed when combining $1,25(OH)_2D_3$ with abatacept in terms of the activation of low affinity $v\beta2^-$ responder T cells since abatacept alone was inhibitory to T cell activation (**fig. 4.5A**). However, $1,25(OH)_2D_3$ and abatacept cooperatively inhibited $v\beta2^+$ T cell proliferation at lower TSST-1 concentrations. For example, significant interaction effect between $1,25(OH)_2D_3$ and abatacept was observed when stimulation was driven by 0.025ng/mL TSST-1 (**fig. 4.5B**). This indicates that suppression by abatacept was dependent upon the presence of $1,25(OH)_2D_3$. At an even lower TSST-1 concentration (0.0025ng/mL), no interaction between

1,25$(OH)_2D_3$ and abatacept was observed, however, both agents had overall independent effects and therefore combined in an additive manner to suppress vβ2$^+$ T cell proliferation (**fig. 4.5B**). In contrast, during stimulation with a higher TSST-1 concentration of 0.25ng/mL, the proliferation of high affinity vβ2$^+$ responder T cells was abatacept-resistant, even in the presence of 1,25$(OH)_2D_3$ (**fig. 4.5A/B**). These data demonstrate that strong TCR-stimulation overcomes the impact of 1,25$(OH)_2D_3$ upon abatacept-resistant T cell proliferation.

4.2.4. The combination of 1,25$(OH)_2D_3$ and abatacept suppress proinflammatory cytokine expression by activated T cells

Vitamin D3 has previously been previously shown to affect the expression of pro-inflammatory cytokines. The impact of 1,25$(OH)_2D_3$ and abatacept co-treatment on the expression of inflammatory cytokines by activated T cells was therefore investigated. These data revealed independent, additive effects of abatacept and 1,25$(OH)_2D_3$ in reducing the frequencies of proliferating T cells that were IFNγ$^+$ and TNFα$^+$ (**fig. 4.6A/D**). Thus, the combination of both agents favored the suppression of these inflammatory cytokines. Interestingly, a trend towards increased IL-17$^+$ T cells was observed when blocking CD28-costimulation, in keeping with previous observations (Bouguermouh et al., 2009; Riella et al., 2012a), however, this effect was lost in the presence of 1,25$(OH)_2D_3$ (**fig. 4.6B/D**). Finally, there was a striking inhibition of IL-21 by activated T cells in the presence of 1,25$(OH)_2D_3$ although no effect was observed following abatacept-treatment alone (**fig. 4.6C/D**). Together, these data suggest that the combination of 1,25$(OH)_2D_3$ supplementation enhances

the efficacy of abatacept treatment affecting both T cell proliferation and inflammatory cytokine production.

4.2.5. 1,25(OH)$_2$D$_3$ amplifies CD28-dependent FoxP3 and CTLA-4 expression

In order to explore the apparent interaction between 1,25(OH)$_2$D$_3$ and abatacept, their impact upon FoxP3 and CTLA-4 expression by activated T cells was explored. Interestingly, T cell stimulation in the presence of abatacept inhibited the induction of markers of T cell regulation, specifically, FoxP3, CTLA-4 and CD25 among proliferating T cells (**fig. 4.7A**). In contrast, 1,25(OH)$_2$D$_3$ supplementation promoted the induction of these proteins which is consistent with previous data (Jeffery et al., 2009). However, this was not observed when CD28-costimulation was blocked by abatacept (**fig. 4.7A**). As such, significant interaction effects between 1,25(OH)$_2$D$_3$ and abatacept were identified for CD25 and CTLA-4 MFI as well as the proportion of proliferating T cells that expressed FoxP3 (**fig. 4.7B**). This suggests that the effect of 1,25(OH)$_2$D$_3$ upon these proteins is dependent upon the presence of CD28-costimulation. Exogenous IL-2 supplementation partially restored the 1,25(OH)$_2$D$_3$ mediated upregulation of FoxP3 and CTLA-4 expression by proliferating T cells in the presence of abatacept (**fig. 4.7A**). The interaction effect between 1,25(OH)$_2$D$_3$ and CD28-blockade in the expression of CD25 and CTLA-4 was not observed in the presence of exogenous IL-2 which implicated the CD28-driven IL-2 production in the expression of these proteins (**fig. 4.7B**). However, the interaction effect between 1,25(OH)$_2$D$_3$ and abatacept was maintained for FoxP3 expression by proliferating T cells even

in the presence of IL-2 which suggests non-IL-2 mediated effects of CD28-signals upon FoxP3 induction (**fig. 4.7B**). Together, these data demonstrate a degree of CD28-dependence in the induction of a regulatory T cell phenotype that occurs in association with $1,25(OH)_2D_3$ treatment. Part of this interaction appears to involve the CD28-driven production of IL-2.

The interaction between $1,25(OH)_2D_3$ and CD28-costimulation in the induction of a regulatory phenotype of activated T cells may be attributed to the fact that $1,25(OH)_2D_3$ promoted CD28-expression by T cells (**fig. 4.8**). Therefore, $1,25(OH)_2D_3$ appeared to bias T cell activation in favor of CD28-driven activation that could facilitate elevated CD25, FoxP3 and CTLA-4 expression.

Experiments were also performed to determine the impact of $1,25(OH)_2D_3$ supplementation upon PD-1 expression which is another CD28-superfamily member that is involved in T cell regulation (Francisco et al., 2010). Interestingly, whilst $1,25(OH)_2D_3$ enhanced CTLA-4 expression, it inhibited that of PD-1. In contrast, T cell proliferation occurring in the presence of abatacept, which, was mediated by anti-CD3 stimulation, tended to result in slightly higher PD-1 levels (**fig. 4.9**). Therefore, the altered contribution of CD28 towards T cell activation appears to be associated with a rebalance between CTLA-4 and PD-1 mediated regulation such that CTLA-4 is increased in association with a more CD28-driven response.
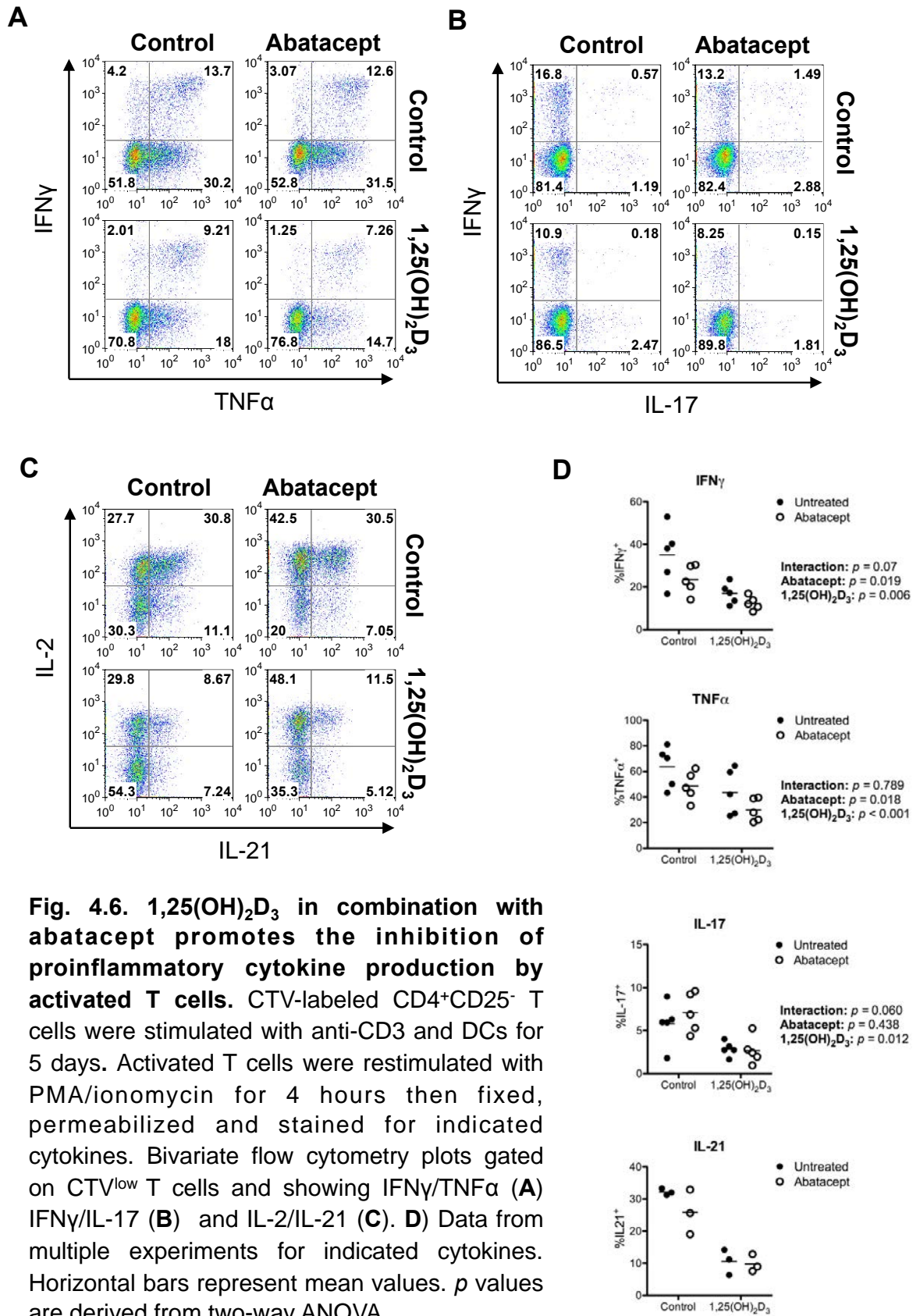
Fig. 4.6. 1,25(OH)₂D₃ in combination with abatacept promotes the inhibition of proinflammatory cytokine production by activated T cells. CTV-labeled CD4⁺CD25⁻ T cells were stimulated with anti-CD3 and DCs for 5 days. Activated T cells were restimulated with PMA/ionomycin for 4 hours then fixed, permeabilized and stained for indicated cytokines. Bivariate flow cytometry plots gated on CTV^{low} T cells and showing IFNγ/TNFα (**A**) IFNγ/IL-17 (**B**) and IL-2/IL-21 (**C**). **D**) Data from multiple experiments for indicated cytokines. Horizontal bars represent mean values. *p* values are derived from two-way ANOVA.
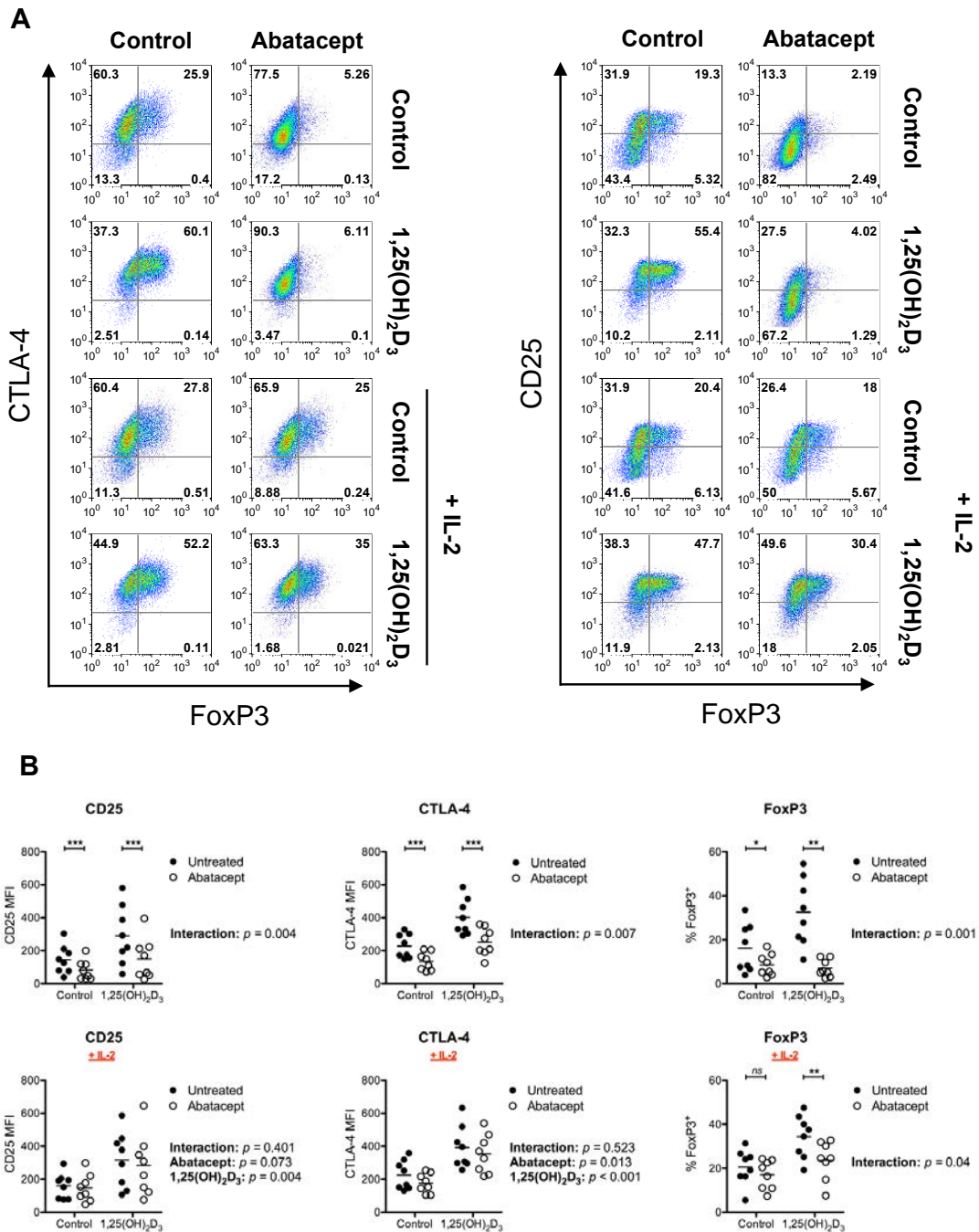
Fig. 4.7. Abatacept inhibits the induction of a regulatory T cell phenotype following 1,25(OH)₂D₃ supplementation. CD4⁺CD25⁻ were activated with 0.5µg/mL anti-CD3 and allogeneic DCs. Where indicated, stimulations were treated with 20µg/mL abatacept, 10nM 1,25(OH)₂D₃ or 200U/mL IL-2. **A**) Activated T cells were fixed, permeabilized and stained for CD25, FoxP3 and CTLA-4 by immunofluorescence staining. Representative flow cytometry data. **B**) Data from multiple experiments (*n* = 8) gated on CTVlow T cells showing CTLA-4/CD25 MFI values and %FoxP3⁺. *p* values are derived from two-way ANOVA, further analyses were performed using paired, two-tailed *t* tests where *ns* not significant, *p ≤0.05, **p ≤0.01, ***p ≤0.001.
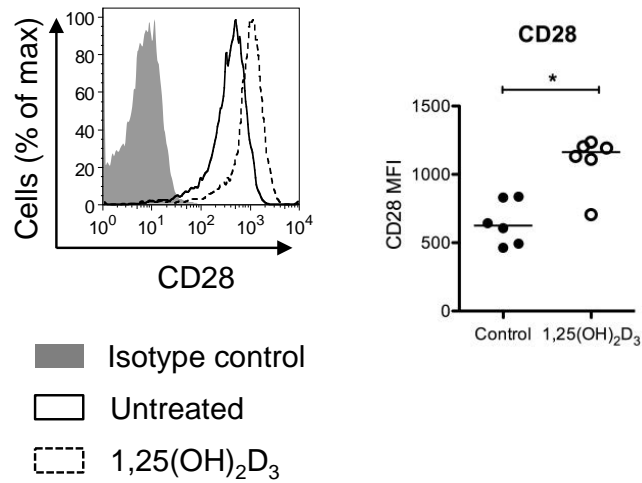
Fig. 4.8. 1,25(OH)₂D₃ promotes CD28 expression by activated T cells. CTV-labeled CD4⁺CD25⁻ were stimulated with 0.5µg/mL anti-CD3 and allogeneic DCs in the presence or absence of 10nM 1,25(OH)₂D₃ for 5 days. CD28 expression was determined by immunofluorescence labeling and flow cytometric analysis. Representative flow cytometry data and data from multiple experiments showing CD28 MFI values among CTVlow T cells. Horizontal bars represent median values. *ns* not significant **p* ≤0.05 paired, two-tailed Wilcoxon-matched pairs test.
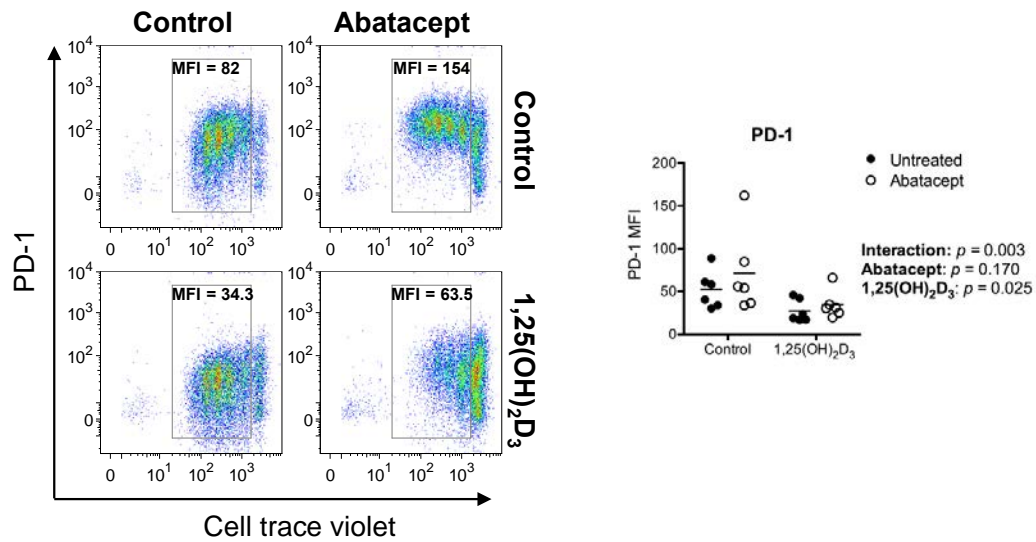
Fig. 4.9. 1,25(OH)$_2$D$_3$ inhibits PD-1 expression by activated T cells. CTV labeled CD4$^+$CD25$^-$ were stimulated with 0.5µg/mL anti-CD3 and allogeneic DCs and with either 20µg/mL abatacept and 10nM 1,25(OH)$_2$D$_3$ or both for 5 days. PD-1 expression was determined by immunofluorescence labeling and flow cytometric analysis. Representative flow cytometry data and data from multiple experiments showing PD-1 MFI values among CTV^{low} T cells. Horizontal bars represent mean values. *p* values are derived from two-way ANOVA.

4.2.6. T cell proliferation occurs in response to cross-linked anti-CD3 or anti-CD28 alone

Since T cell proliferation in the presence of abatacept occurred in the context of high intensity anti-CD3 signaling, but was rendered abatacept sensitive by $1,25(OH)_2D_3$, I explored the hypothesis that $1,25(OH)_2D_3$ targets the TCR/CD3 pathway thereby making T cell proliferation more CD28-dependent. To compare the TCR/CD3 and CD28 pathways directly, T cells were activated using anti-CD3 or anti-CD28 cross-linked by FcR transfected CHO cells. In this system, both pathways promoted T cell proliferation when stimulation was induced at antibody concentrations ≥0.05 µg/mL, with anti-CD28 potentially acting in a comparable manner to CD28-superagonists (**fig. 4.10**). A concentration of 0.5µg anti-CD3 or anti-CD28 was used in subsequent experiments as this concentration tended to induce the most comparable proliferation levels. It is important to note that at this concentration fewer T cells committed to cell division in response to anti-CD28 compared to anti-CD3, however, those T cells that entered the cell cycle underwent an equivalent number of divisions following 5 days stimulation.

A variety of experiments were performed in order to demonstrate that stimulation by cross-linked anti-CD3 and anti-CD28 engagement was fundamentally different. For example, the sensitivity of anti-CD3 and anti-CD28 stimulations to CsA was determined. As expected, given the reliance of TCR signaling on the calcineurin/NFAT pathway, CsA potently inhibited proliferation of anti-CD3-treated cells whilst anti-CD28 stimulated T cells were less affected by CsA (**fig. 4.11**). Analysis of T cell activation markers also

revealed differences between anti-CD3 and anti-CD28 stimulated T cells. For example, anti-CD28 promoted increased expression of ICOS and CD80 relative to anti-CD3 stimulated T cells (**fig. 4.12**). Similarly, OX40 and transferrin receptor (CD71) were more strongly upregulated following CD28-driven activation (**fig. 4.12**). Finally, no evidence of CD62L and CD27 downregulation was observed following anti-CD3 stimulation but this did occur in response to anti-CD28 driven activation (**fig. 4.12**). Together, these data are indicative of differences between anti-CD3 and anti-CD28 stimulation.

The impact of anti-CD3 or anti-CD28 driven activation upon markers of T cell regulation was determined. These experiments demonstrated a stronger bias towards a regulatory T cell phenotype with increased FoxP3 and CTLA-4 and CD25 expression levels when proliferation was induced by cross-linked anti-CD28 compared to anti-CD3 (**fig. 4.13**). CTLA-4 and CD25 expression levels could be increased to levels that were comparable with anti-CD28 driven activation through the combined activity of $1,25(OH)_2D_3$ and exogenous IL-2. Interestingly, $1,25(OH)_2D_3$ treatment only significantly increased FoxP3 expression when activation was driven by anti-CD28. In contrast, FoxP3 expression levels remained low following anti-CD3 stimulation regardless of $1,25(OH)_2D_3$ and IL-2 supplementation. In keeping with the effect of abatacept and $1,25(OH)_2D_3$ upon DC-driven T cell activation, the data suggest that 1,25(OH)2D3 works in association with CD28-costimulation to enhance FoxP3 expression by activated T cells. Importantly, these data demonstrate several points of distinction between T cell differentiation when stimulation is mediated by either cross-linked anti-CD3 or anti-CD28.
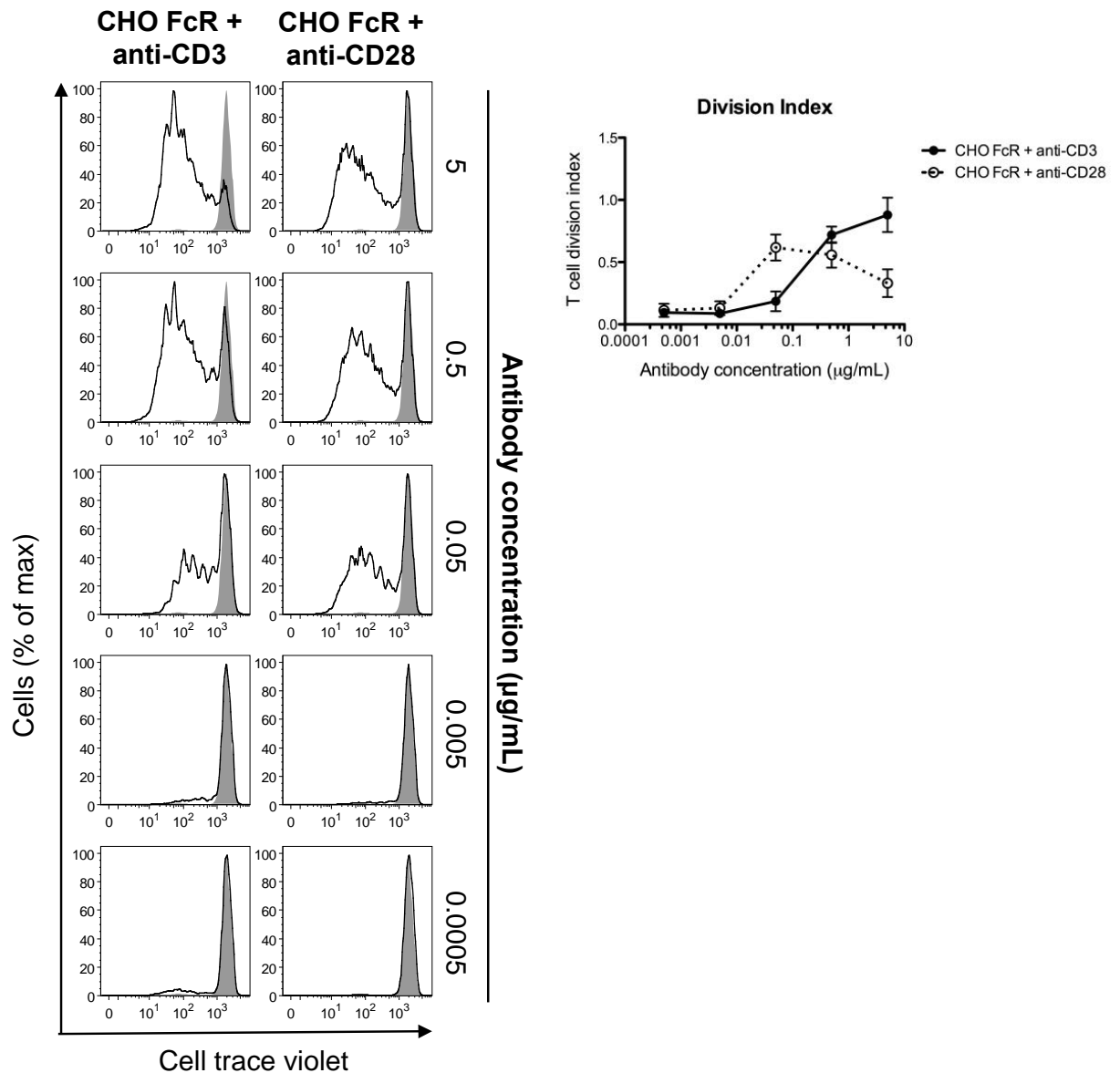
Fig. 4.10. T cell proliferation occurs in response to cross-linked anti-CD3 and anti-CD28 independently. CTV labeled T cells were stimulated with fixed CHO-FcR and either anti-CD3 or anti-CD28 for 5 days. Representative histograms showing CTV dilution, shaded histograms represent CHO-FcR alone. Combined data from 4 independent experiments are also summarized. Data points represent mean values ± S.E.
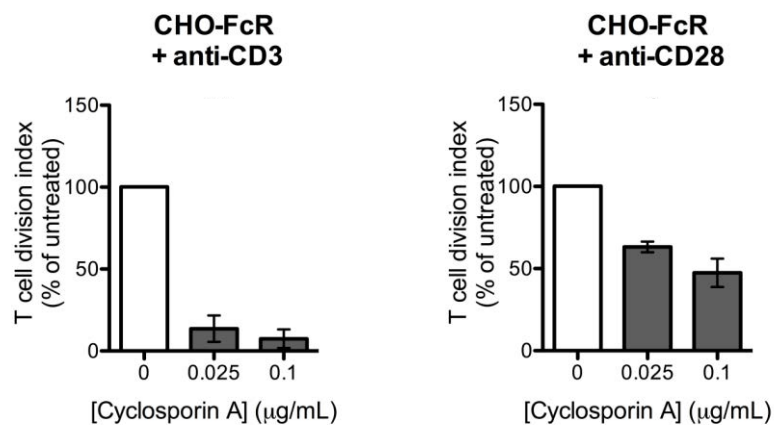
Fig. 4.11. The impact of CsA upon anti-CD3 and anti-CD28 driven T cell proliferation. T cells were stimulated with fixed CHO-FcR and either anti-CD3 or anti-CD28. Stimulations from were treated with indicated CsA concentrations. CTV dilution was analysed following 5 days stimulation. Bar charts show the division index values at each concentration plotted as a percentage of the division index for the untreated control from independent experiments (n = 3). Data points represent mean values ± S.E.
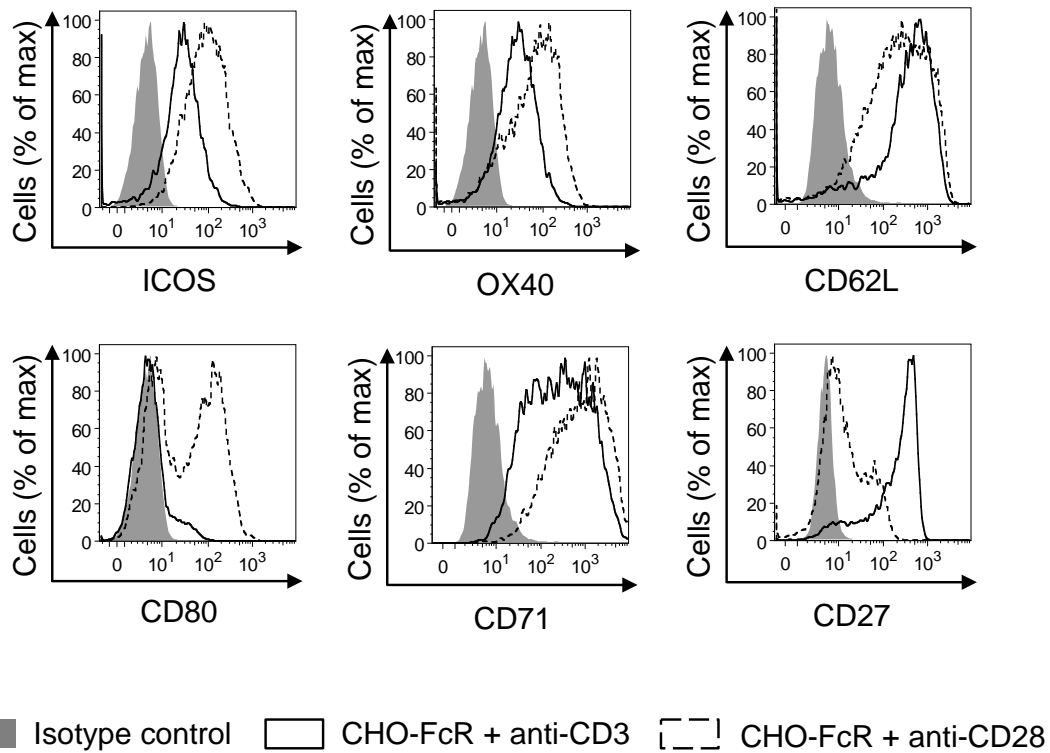
Fig. 4.12. T cell activation driven by anti-CD3 and anti-CD28 promotes distinct effector T cell phenotypes. T cells were stimulated with fixed CHO-FcR and either anti-CD3 or anti-CD28 for 5 days. Expression of indicated surface markers by dividing T cells was measured by immunofluorescence labeling and flow cytometric analysis. Representative histograms gated on CTV^{low} T cells from $n > 3$ experiments are shown.
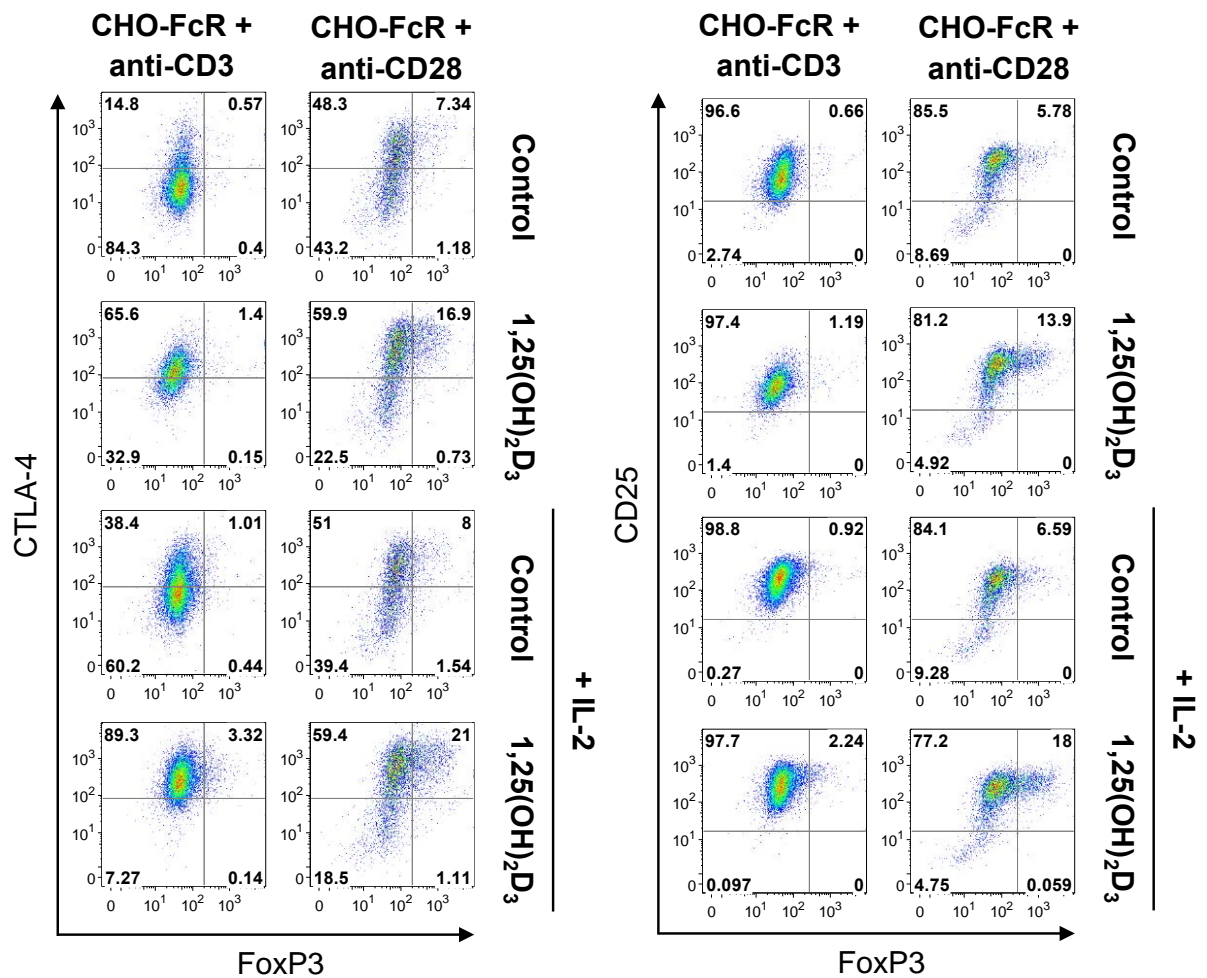
Fig. 4.13 T cell activation driven by anti-CD28 promotes a pro-regulatory phenotype. T cells stimulated with anti-CD3 or anti-CD28 were stained for CTLA-4, CD25 and FoxP3 and expression measured by flow cytometry. Flow cytometry data from one representative experiment of 5 independent experiments that were performed.

4.2.7. 1,25(OH)$_2$D$_3$ suppresses anti-CD3 but not anti-CD28 driven T cell activation

Having characterized a system in which CD3 and CD28-driven responses could be compared directly, the impact of 1,25(OH)$_2$D$_3$ on T cell responses driven by anti-CD3 or anti-CD28 pathways was assessed. These experiments demonstrated that 1,25(OH)$_2$D$_3$ markedly reduced the proliferation of T cells stimulated by anti-CD3, but did not affect T cells stimulated by anti-CD28 (**fig. 4.14**) suggesting that 1,25(OH)$_2$D$_3$ treatment predominantly targets T cells stimulated via the CD3 pathway. One of the primary endpoints of CD28-costimulation is an upregulation of IL-2 signaling (Boomer and Green, 2010). Interestingly, by supplementing exogenous IL-2 the inhibition of anti-CD3 driven activation by 1,25(OH)$_2$D$_3$ was no longer seen (**fig. 4.15**). Similar experiments were performed to determine how IL-2 supplementation affected the cooperation between 1,25(OH)$_2$D$_3$ and abatacept in DC-driven assays. These data suggested that IL-2 could substitute for the lack of CD28-signaling both in the presence or absence of 1,25(OH)$_2$D$_3$ treatment, (**fig. 4.16A**) although a modest overall effect of abatacept was still observed across multiple donors following IL-2 supplementation (**fig. 4.16B**).
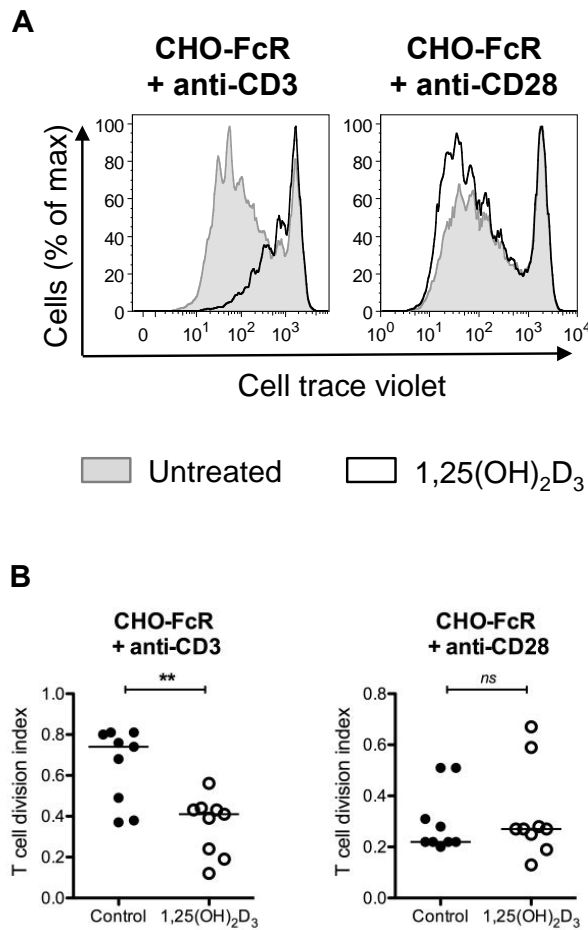
Fig. 4.14. 1,25(OH)$_2$D$_3$ suppresses TCR but not CD28 induced T cell proliferation. CD4$^+$CD25$^-$ CTV labeled T cells were stimulated with anti-CD3 or anti-CD28 in association with CHO-FcR with or without 10nM 1,25(OH)$_2$D$_3$ T cell proliferation was determined by flow cytometry. **A**) Representative CTV histograms showing the impact of 10nM 1,25(OH)$_2$D$_3$ on anti-CD3 and anti-CD28 driven T cell at 5 days. Data from multiple experiments are summarized in **B**. Horizontal bars indicate median values. Significance was tested by Wilcoxon matched pairs tests. *******p* ≤0.01
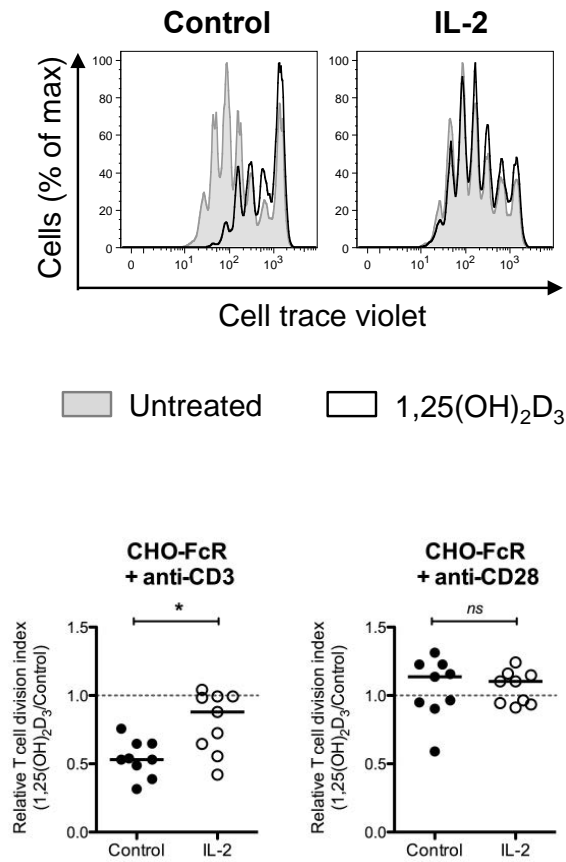
Fig. 4.15. Exogenous IL-2 prevents 1,25(OH)₂D₃ mediated suppression of anti-CD3 driven proliferation. CD4⁺CD25⁻ CTV labeled T cells were stimulated with anti-CD3 or anti-CD28 in association CHO-FcR for 5 days and were treated with 10nM 1,25(OH)₂D₃, 200U/mL IL-2 or both. CTV dilution was determined by flow cytometry. Representative CTV histograms showing the impact of 1,25(OH)₂D₃ and exogenous IL-2 upon T cell proliferation. Data from multiple experiments are also summarized where the effect of 1,25(OH)₂D₃ upon T cell division index is normalized to untreated controls. Horizontal bars indicate median values. Significance was tested by Wilcoxon matched pairs tests. *ns* not significant **p* ≤0.05
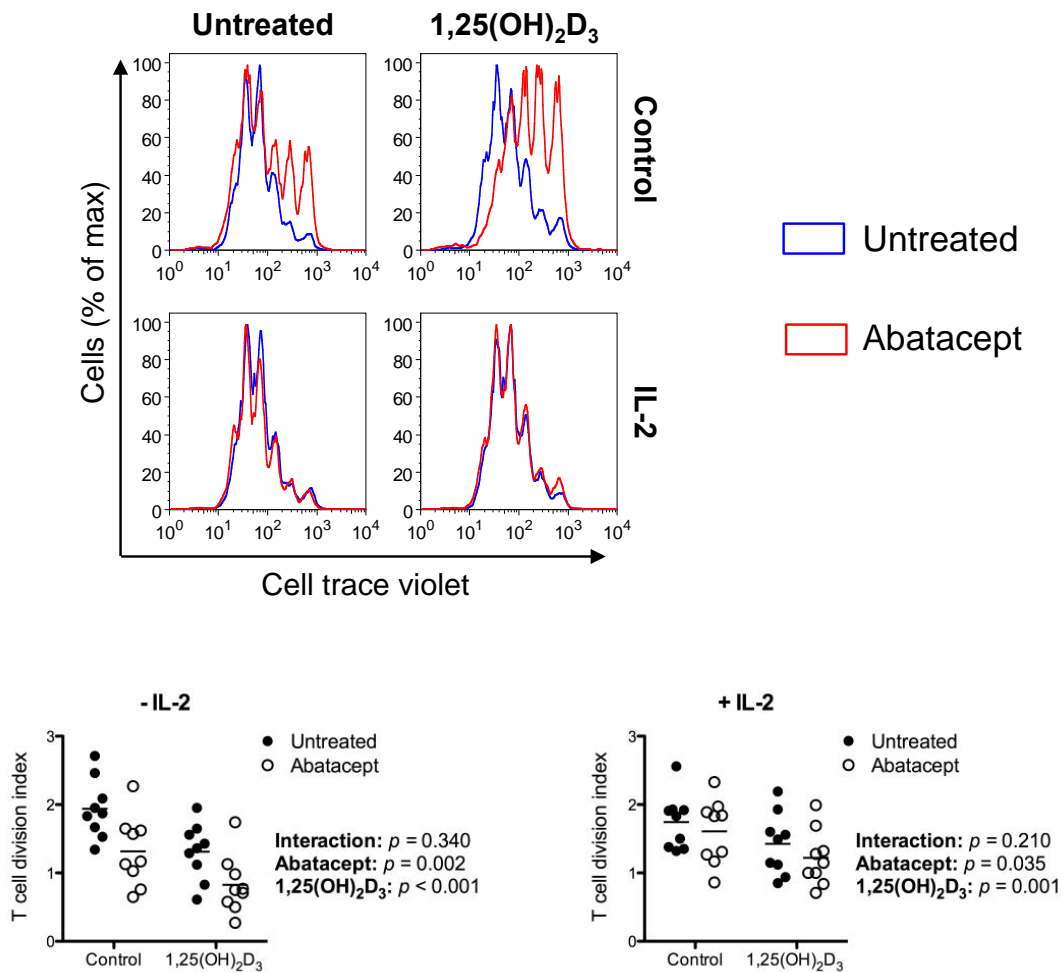
Fig. 4.16. Exogenous IL-2 prevents the inhibition of T cell proliferation mediated by the combination of 1,25(OH)$_2$D$_3$ and abatacept. CD4$^+$CD25$^-$ CTV labeled T cells were stimulated with anti-CD3 and DCs at a DC:T cell ratio of 1:5 and in the presence of 20μg/mL abatacept, 10nM 1,25(OH)$_2$D$_3$, or 200U/mL IL-2. CTV dilution was determined by flow cytometry. Representative CTV histograms. Data from multiple experiments expressed as T cell division index are also summarized. Horizontal bars represent mean values. *P* values are derived from two-way ANOVA

These experiments suggested that the $1,25(OH)_2D_3$ resistant T cell proliferation that was mediated by anti-CD28 driven stimulation may have been at least partially associated with increased IL-2 signaling occurring under these conditions. The analysis of IL-2 production following either anti-CD3 or anti-CD28-driven stimulation clearly demonstrated that anti-CD28 stimulation promoted high and sustained IL-2 production (**fig. 4.17A;** see red traces) that was more pronounced than with anti-CD3 driven activation). In contrast, anti-CD3 driven stimulation led to transient IL-2 production by T cells that could not be detected beyond 24-36h stimulation (**fig. 4.17A;** see blue traces). This limited IL-2 production following anti-CD3 stimulation was necessary for activation because anti-IL-2 treatment entirely inhibited both anti-CD3 and anti-CD28-driven activation (data not shown). Consistent with this, comparable levels of STAT5 phosphorylation (pSTAT5), which is a proximal component of the IL-2 signaling pathway (Lin and Leonard, 2000), could be detected following anti-CD3 and anti-CD28 driven T cell stimulation at 16h (**fig. 4.17B**). However, following 6 days stimulation, pSTAT5 could only be detected in anti-CD28 driven cultures (**fig. 4.17B**). This reflected sustained IL-2 production following strong CD28-costimulation. Importantly, $1,25(OH)_2D_3$ did not appear to inhibit T cell proliferation in the absence of CD28-costimulation via a direct impact upon IL-2 production since IL-2 production (**fig. 4.17A**) and signaling (**fig. 4.17B**) in the context of anti-CD3 stimulation did not appear to be affected by $1,25(OH)_2D_3$.
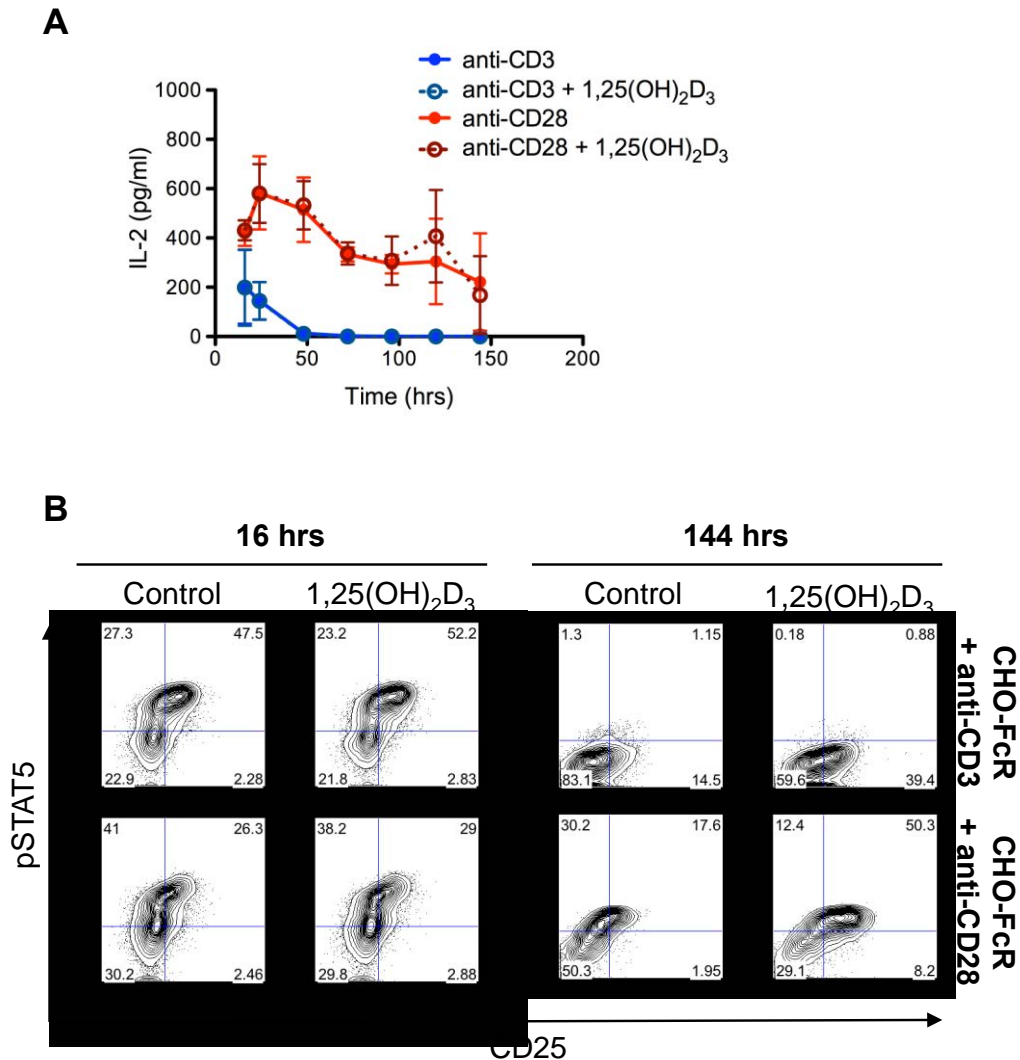
Fig. 4.17. 1,25(OH)$_2$D$_3$ does not alter IL-2 production by activated T cells following either anti-CD3 or anti-CD28 driven proliferation. CD4$^+$CD25$^-$ CTV labeled T cells were stimulated with anti-CD3 or anti-CD28 in association CHO-FcR in the presence of absence of 10nM 1,25(OH)$_2$D$_3$. Supernatants were recovered at indicated time points. **A**) IL-2 concentration was determined by ELISA. Data points represent mean values ± S.E. from 3 independent experiments. **B**) T cells were stained for pSTAT5 expression levels following either 16h or 144h. Representative flow cytometry data from 1 independent experiment of 3 performed.

4.2.8. 1,25(OH)$_2$D$_3$ does not interact with low dose CsA to modify abatacept-sensitivity

Results suggested that 1,25(OH)$_2$D$_3$ acted in a similar manner to CsA (see **fig. 3.14**) by targeting abatacept-resistant T cell proliferation that was driven by anti-CD3 stimulation. Experiments were performed to determine how CsA interacted with 1,25(OH)$_2$D$_3$ in the context of CD28-blockade particularly at CsA concentrations that did not entirely inhibit T cell proliferation in association with abatacept treatment. The presence of CsA at higher concentrations (>0.1µg/mL CsA) overwhelmingly inhibited T cell proliferation when combined with abatacept blockade in both CHO FcR/CD8 and DC-driven stimulations (**fig. 4.18**). However, some T cell proliferation occurred when abatacept was combined with lower CsA concentrations. At these CsA concentrations, the presence of CsA did not strikingly inhibit T cell proliferation above the level of combination of 1,25(OH)$_2$D$_3$ and abatacept alone (**fig. 4.18**). As such, inhibition of TCR-driven activation by 1,25(OH)$_2$D$_3$ did not seem to compensate for suboptimal TCR inhibition by CsA to promote abatacept sensitivity.
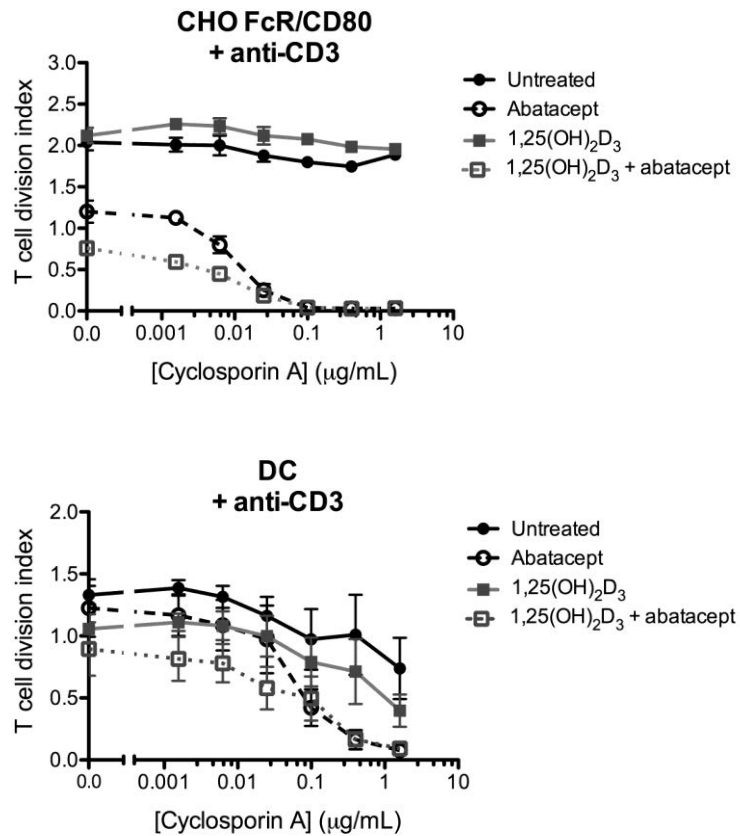
Fig. 4.18. 1,25(OH)$_2$D$_3$ does not interact with low dose CsA to modify abatacept-sensitivity. CD4$^+$CD25$^-$ CTV labeled T cells were stimulated with either anti-CD3 and CHO FcR/CD80 (**A**) or anti-CD3 and DCs (**B**) and in the presence of 20µg/mL abatacept, 10nM 1,25(OH)$_2$D$_3$, or indicated CsA concentrations. CTV dilution was determined by flow cytometry. Data from multiple experiments (*n* = 4) expressed as T cell division index are summarized. Data points represent mean values ± S.E.

4.2.9. 1,25(OH)$_2$D$_3$ acts to inhibit anti-CD3 driven proliferation rather than signal intensity

The inhibition of anti-CD3 driven T cell proliferation by 1,25(OH)$_2$D$_3$ could theoretically have arisen from either decreased signal intensity which subsequently reduced proliferation or alternatively via a direct impact upon the proliferative response by inhibiting cell cycle progression. Since the VDR is expressed at low levels by resting T cells and is induced following TCR-stimulation (Provvedini et al., 1983; vonEssen et al., 2010) it seemed more likely that the effect would be a later one that targeted the proliferative response. Various experiments were performed to test this hypothesis. By assessing T cell proliferation at different points in response to anti-CD3 driven activation it was apparent that early T cell proliferation remained largely unaffected following 1,25(OH)$_2$D$_3$ supplementation (**fig. 4.19;** day 3) but became more evident at later time points (**fig. 4.19**; day 5/6). Similar effects were observed in DC driven stimulations. For instance, a relatively constant division rate was observed under control conditions particularly when T cells were stimulated at a DC:T cell ratio of 1:20 (**fig. 4.20**). However, T cell expansion appeared to stall after 4 days stimulation following 1,25(OH)$_2$D$_3$ treatment both in the presence and absence of abatacept which seemed indicative of defective T cell proliferation (**fig. 4.20**). Interestingly, this stalled T cell proliferation was still apparent in conjunction with 1,25(OH)$_2$D$_3$ treatment alone suggesting that CD28-costimulation only partly overcomes the impact of 1,25(OH)$_2$D$_3$ upon proliferation under these conditions.
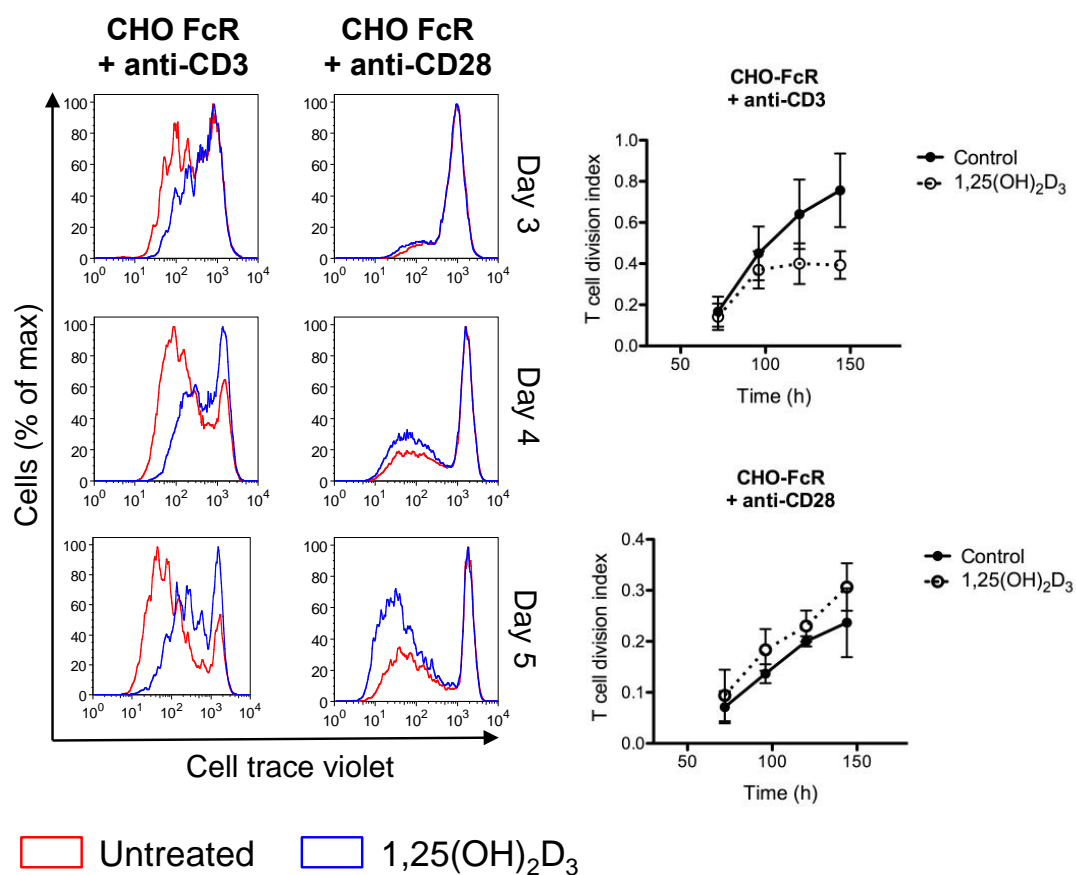
Fig. 4.19. Limited inhibition of early anti-CD3 driven T cell proliferation by 1,25(OH)$_2$D$_3$. CD4$^+$CD25$^-$ CTV labeled T cells were stimulated with anti-CD3 or anti-CD28 in association with CHO-FcR with or without 10nM 1,25(OH)$_2$D$_3$. T cell proliferation was determined at various time-points by flow cytometry. Representative CTV histograms and data from multiple experiments (*n* = 3). Data points represent mean values ± S.E.
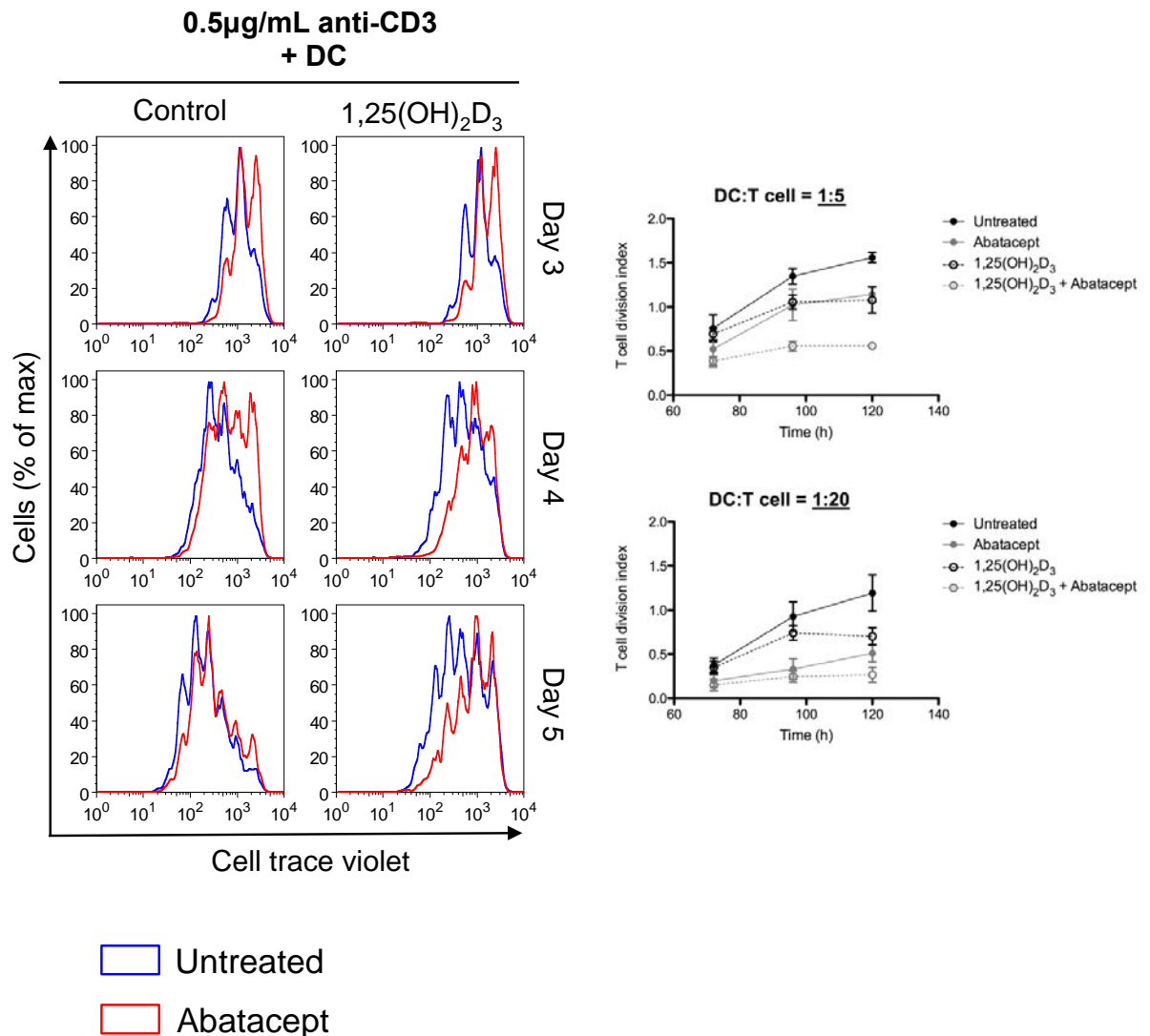
Fig. 4.20. Limited inhibition of T cell proliferation by 1,25(OH)$_2$D$_3$ and abatacept at early time-points CD4$^+$CD25$^-$ CTV labeled T cells were stimulated with anti-CD3 and DCs at a DC:T cell ratio of 1:5 or 1:20 with either 20µg/mL abatacept,10nM 1,25(OH)$_2$D$_3$ or both. T cell proliferation was determined at various time-points by flow cytometry. Representative CTV histograms showing T cell stimulation at a DC:T cell ratio of 1:5. Also, data from multiple experiments (n = 3). Data points represent mean values ± S.E.
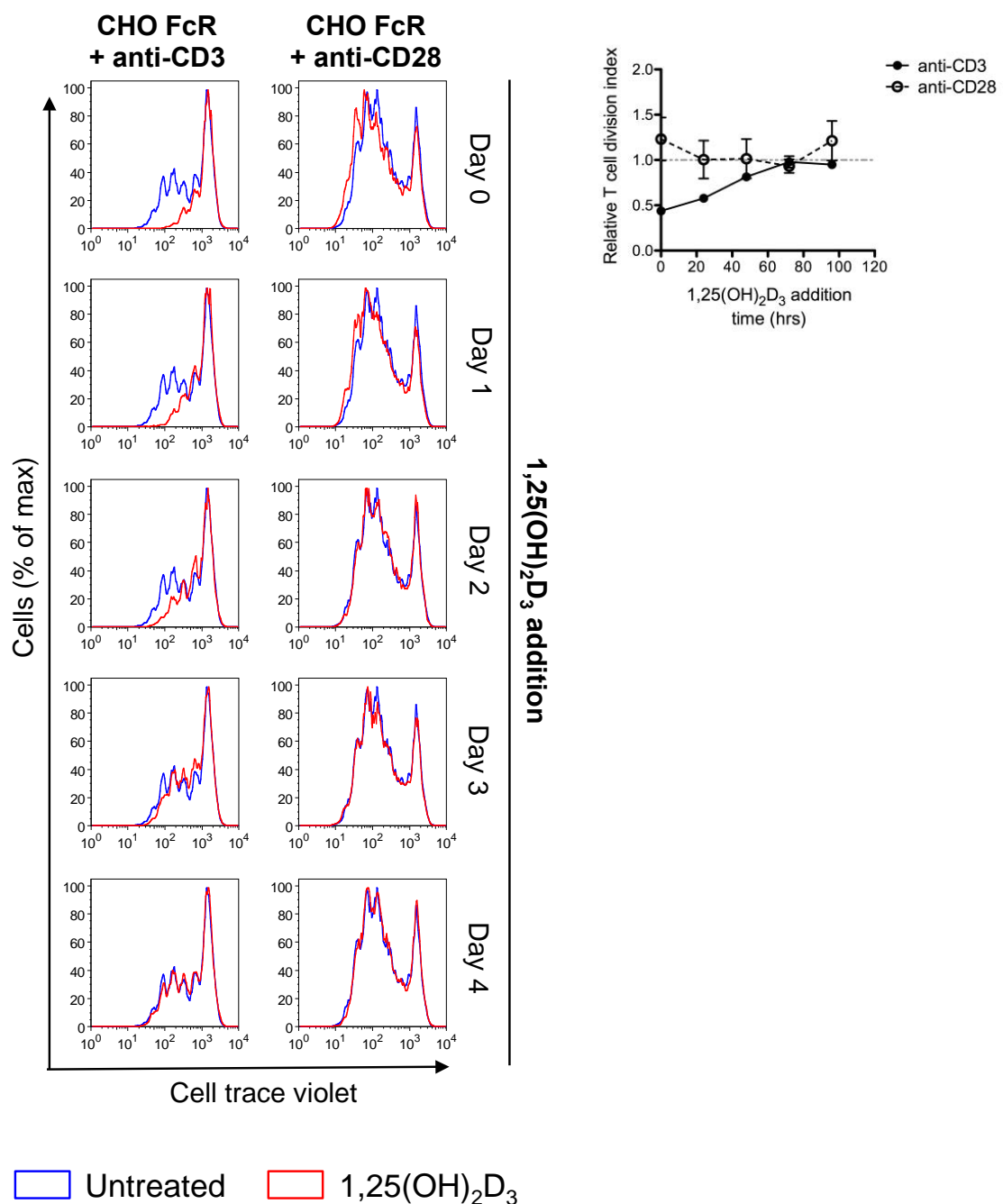
Fig. 4.21. The impact of delayed 1,25(OH)$_2$D$_3$ supplementation upon anti-CD3 driven T cell proliferation. CD4$^+$CD25$^-$ CTV labeled T cells were stimulated with anti-CD3 or anti-CD28 in association with CHO-FcR. 10nM 1,25(OH)$_2$D$_3$ was supplemented at indicated time-points where day 0 is prior to stimulation. T cell proliferation was determined by flow cytometry following 5 days stimulation. Representative CTV histograms and data from multiple experiments (*n* = 3). Data points represent mean values ± S.E.

144
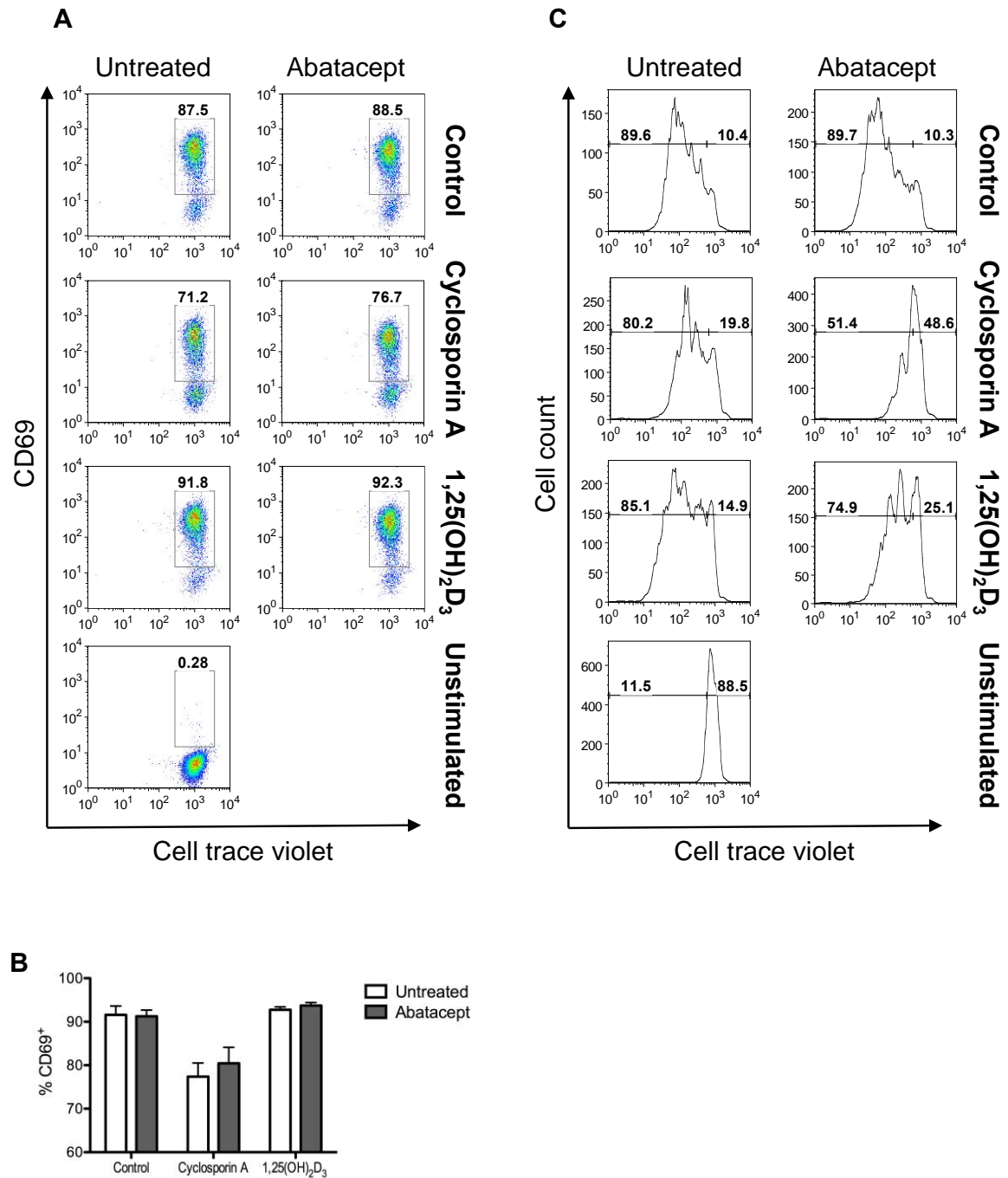
Fig. 4.22. 1,25(OH)$_2$D$_3$ supplementation fails to inhibit CD69 expression following 24 hrs. stimulation. CD4$^+$CD25$^-$ CTV labeled T cells were stimulated with anti-CD3 and DCs and treated with 10nM 1,25(OH)$_2$D$_3$ or 0.1µg/mL CsA either with or 20µg/mL abatacept. CD69 expression levels were determined by immunofluorescence staining and flow cytometric analysis. **A**) Representative flow cytometric data. **B**) Data from multiple experiments (n = 3) showing proportion of CD4$^+$ T cells staining positive for CD69. Data points represent mean values± S.E. **C**) T cell proliferation was determined by flow cytometry following 5 days stimulation. Representative CTV histograms.

These results appeared to suggest that $1,25(OH)_2D_3$ was acting to inhibit anti-CD3 driven T cell proliferation via a late effect rather than inhibiting the intensity of initial activation signal. However, by supplementing $1,25(OH)_2D_3$ at different time points post-anti-CD3 stimulation it was apparent that addition beyond 2 days did not appreciably inhibit T cell proliferation (**fig. 4.21**). Thus, $1,25(OH)_2D_3$ may target a relatively early activation event following anti-CD3 stimulation but this effect does not become apparent until T cell proliferation has occurred.

CD69 expression can be used as a tool to identify early T cell activation since peak expression is observed around 24 hours following TCR stimulation however mRNA is detected less than an hour following stimulation (Testi et al., 1994). To confirm that $1,25(OH)_2D_3$ was not affecting the strength of the initial TCR-signal the impact of $1,25(OH)_2D_3$ upon the early activation marker CD69 was determined. The data revealed no significant impact of $1,25(OH)_2D_3$ on CD69 expression following 24 hours stimulation (**fig. 4.22 A/B**); indeed, a trend was observed towards increased CD69 in the presence of $1,25(OH)_2D_3$ relative to control conditions across several experiments (**fig. 4.22B**). The impact of CsA upon CD69 was also utilized a control since this is known to directly target TCR signaling via the calcineurin inhibition and could therefore be predicted to inhibit early T cell activation events. However, CsA treatment was only associated with a modest decrease in CD69 expression (**fig. 4.22A/B**) even when used in combination with abatacept where a robust inhibition of T cell proliferation was observed following 5 days stimulation (**fig. 4.22C**). As such these data suggest that CD69 positivity does not necessarily

define a T cell that will go on to proliferation. However, the fact that $1,25(OH)_2D_3$ fails to inhibit CD69 expression suggests that it inhibits a TCR-driven activation event after the initial TCR signal.

4.3. Discussion

T cell activation thresholds are achieved following the integration of TCR and CD28-signals (Viola and Lanzavecchia, 1996). As such, results presented throughout this chapter demonstrate that strategies which selectively reduce the strength of the TCR stimulation act to promote CD28-costimulation requirements. For example, the calcineurin inhibitor, CsA, has been regarded a TCR-pathway selective inhibitor (Geginat et al., 2000), whilst CD28 costimulation promotes CsA insensitive T cell responses (June et al., 1987). When used at a low concentration, CsA, like abatacept, failed to inhibit T cell proliferation, however, the combination of a low CsA concentration with abatacept was highly effective at inhibiting activation. Other groups have also demonstrated synergy between CsA and either anti-CD80/CD86 blocking antibodies or CTLA-4-Ig for the inhibition of alloresponses in *in vitro* mixed lymphocyte reactions and organ transplantation models (Bolling et al., 1994; Van Gool et al., 1994; Perico et al., 1995; Ossevoort et al., 1999). Thus CsA, which is already used in the treatment of T cell mediated inflammatory diseases including RA (Cope et al., 2007), may serve to improve clinical responses to abatacept therapy. However, the adverse effects of calcineurin inhibition limit the viability of this option (van den Borne et al., 1999; Gremese and Ferraccioli, 2004; Liu et al., 2007). As such, the identification of

alternative TCR specific inhibitors is of interest. Here, a novel role for the active form of vitamin D3, $1,25(OH)_2D_3$, as an inhibitor of TCR but not CD28 driven T cell responses is reported. Importantly, like CsA, $1,25(OH)_2D_3$ was able to enhance the efficacy of abatacept in the blockade of anti-CD3 and DC driven T cell stimulations.

Data presented here suggest that, under conditions of sufficient TCR stimulation, T cell responses can occur in the absence of CD28-costimulation. Likewise, the availability of CD28-costimulation compensates for a weak TCR signal to promote T cell activation. For instance, T cell proliferation occurred in the absence of TCR stimulation in response to CD28-engagement alone mediated by cross-linked anti-CD28. This did not occur in response to soluble anti-CD28 alone suggesting that FcR-mediated cross-linking was crucial to the generation of threshold signaling, potentially relating to the size-dependent exclusion of CD45 from the T cell-CHO-FcR interface as predicted by the kinetic segregation model of T cell activation signaling (Davis and van der Merwe, 2006). As with other conventional agonistic anti-CD28 mAb, 9.3 binds to an epitope related to the CD28-MYPPPY ligand-binding motif (Peach et al., 1994). In contrast, superagonistic anti-CD28 binds to the membrane-proximal C"D loop of the CD28 immunoglobulin like domain (Lühder et al., 2003). However, our results using cross-linked 9.3 appear to be analogous to the utilization of CD28-superagonists as a mechanism to drive T cell activation by CD28-engagement in the absence of an obvious TCR-stimulus (Siefken et al., 1997; Tacke et al., 1997). In this manner, CD28-superagonists have been found to expand T_{reg} populations in various model systems

(Beyersdorf et al., 2005b) consistent with my observations of a pro-regulatory phenotype driven by CD28-costimulation. Despite these results, the use of the CD28 superagonist TGN1412 in clinical trials led to cytokine storm potentiated by widespread T cell activation particularly among effector memory T cell populations (Hünig, 2012). This example clearly demonstrates the importance of balancing both the pro-stimulatory and pro-regulatory functions of CD28 during the therapeutic modification of CD28-costimulation (Sansom and Walker, 2013). In this work, signaling via CD28 in isolation facilitated the comparison between TCR and CD28 driven signals in terms of their sensitivity to $1,25(OH)_2D_3$.

Vitamin D has been widely investigated in terms of its influence on T cell activation and effector function due to a strong correlation between vitamin D3 deficiency and autoimmunity (Holick, 2007). This is thought to arise due to diverse immunomodulatory properties of vitamin D, influencing both innate and adaptive immunity. Chromatin immunoprecipitation analysis in lymphoblastoid cell lines suggests 229 genes to be differentially affected by $1,25(OH)_2D_3$ treatment (Ramagopalan et al., 2010). Since both APCs and T cells express the VDR, effects of $1,25(OH)_2D_3$ on T cell responses may occur through either a direct effect on the T cell or an indirect effect via the APC (Peelen et al., 2011). By using an artificial fixed CHO system to stimulate T cells it was apparent that $1,25(OH)_2D_3$ can act via a T cell intrinsic mechanism to inhibit TCR-induced proliferation and promote abatacept sensitivity. An important consideration will be to verify these findings using *in vivo* models of autoimmunity or transplantation. In particular the potential to test the efficacy

of CD28-blockade using these models on a VDR deficient background would be valuable. Nevertheless, the widespread expression of the VDR complicates the interpretation of these experiments. As such, models in which VDR-deficient T cells can be transferred to wild-type recipients would be advantageous.

It has been speculated that the primary mechanisms by which vitamin D3 regulates T cell responses is via effects upon T cell proliferation and cytokine production (Cantorna and Waddell, 2014). In this study, various lines of evidence suggest that, in the absence of CD28-costimulation, $1,25(OH)_2D_3$ targets the proliferative response to T cell stimulation rather than the initial intensity of stimulation. For example, the inhibition of anti-CD3 T cell stimulation was more pronounced at later time points (i.e. following 5 days stimulation) whereas at earlier time points (i.e. day 3-4) inhibition was less prominent. In support of this, several reports suggests vitamin D3 mediated inhibition of cell cycle progression in various cell lineages, particularly at the G_1/S phase transition (Samuel and Sitrin, 2008). This may be at least partly associated with the capacity of vitamin D3 (or vitamin D3 analogs) to upregulate the cyclin-dependent kinase (CDK) inhibitors p21 and p27 that control entry into S phase (Liu et al., 1996; Kawa et al., 1997; Cozzolino et al., 2001). However, preliminary experiments have revealed no significant differences in the expression of either p21 or p27 following $1,25(OH)_2D_3$ supplementation (data not shown).

During T cell proliferation, the G_1/S phase transition is strongly associated with CD28-signaling (Appleman et al., 2000). However, in the absence of CD28-costimulation, it has been found that TCR-driven, Jak3 dependent cytokine signals can drive late G_1 events such as the upregulation of cyclin D3, cyclin E and cyclin A (Shi et al., 2009). An attractive hypothesis for the interaction between vitamin D3 and abatacept may be the inhibition of TCR-driven cytokine signaling by vitamin D3.

IL-2 is an example of a cytokine that signals via a Jak-3 dependent mechanism (Kirken et al., 1995). Furthermore, the T cell G_1/S phase transition is at least partially IL-2 dependent (Appleman et al., 2000). Although CD28 is most strongly associated with increased IL-2 signaling resulting both from an upregulation of IL-2 expression (Fraser et al., 1991) and mRNA stability (Lindstein et al., 1989), IL-2 expression is also upregulated, albeit to a lesser extent, following TCR stimulation alone (Umlauf et al., 1995; Shapiro et al., 1997). Several studies have suggested an inhibition of IL-2 production following vitamin D3 treatment (Tsoukas et al., 1984; Rigby et al., 1984; 1985; Thien et al., 2005) which has been thought to arise due to a direct inhibition of NFAT binding activated vitamin D receptor (VDR) at the IL-2 promoter (Alroy et al., 1995). Interestingly, NFAT appears to be important to the expression of IL-2 by TCR signaling alone (Shapiro et al., 1997) suggesting vitamin D could suppress TCR induced IL-2 whilst abatacept targets CD28 induced IL-2. However, although the absence of CD28 strongly inhibited IL-2 production, I have found no difference between IL-2 expression levels resulting from $1,25(OH)_2D_3$ exposure either in the presence or absence of CD28-

costimulation in contrast to these previous studies. Furthermore, $1,25(OH)_2D_3$ promotes CD25 expression and is therefore likely to facilitate IL-2 signaling. This suggests that vitamin D3 does not enhance abatacept efficacy by a direct effect upon IL-2 signaling. It is important however to note that IL-2 supplementation did overcome the defect in proliferation caused by the combination of vitamin D3 and abatacept, this suggests that high IL-2, either through supplementation or as a result of costimulation, antagonizes vitamin D3 mediated inhibition of T cell proliferation.

Another example of a cytokine that signals in a Jak-3 dependent manner is IL-21 (Leonard and Spolski, 2005). Interestingly, IL-21 can compensate for the absence of IL-2 as a growth factor (Attridge et al., 2012). Strikingly, $1,25(OH)_2D_3$ treatment results in an inhibition of IL-21 by activated T cells following $1,25(OH)_2D_3$ treatment which is consistent with other studies (Jeffery et al., 2009; Bruce et al., 2011; Jeffery et al., 2012). These results suggest that abatacept could act to inhibit CD28-driven IL-2 production rendering T cell proliferation dependent upon TCR driven IL-21 production. The inhibition of IL-21 production by vitamin D3 would therefore inhibit T cell proliferation under these conditions. The potential for suppression of IL-21 suggests some degree of overlap between vitamin D3 mediated effects upon T cell proliferation and proinflammatory cytokine expression. A useful future direction would be to determine the outcome of abatacept blockade in models of inflammation or transplantation upon an IL-21 deficient background to further explore the interaction between IL-21 and costimulation thresholds.

An important conclusion from the work presented in this chapter is that the impact of vitamin D3 upon T cell proliferation is dependent upon the context of the activation conditions. This is reflected by previous studies, for example, whilst vitamin D3 inhibits *in vitro* T cell activation driven by phytohemagglutanin (Rigby et al., 1984; Lemire et al., 1984; Tsoukas et al., 1984; Correale et al., 2009) this does not occur when T cells receive costimulation via anti-CD28 (Vanham et al., 1989). Similarly, no effect by vitamin D3 upon T cell proliferation is observed when T cells are stimulated with anti-CD3/anti-CD28 coated beads (Jeffery et al., 2009). I have shown that although $1,25(OH)_2D_3$ suppresses T cell proliferation driven by anti-CD3 and DCs, no impact is observed when stimulating with anti-CD3 and FcR/CD80 expressing CHO cells (which express significantly more CD80 than DCs) or by anti-CD28 stimulated T cells. These discrepancies appear to be associated with the relative contributions of TCR and CD28 to T cell activation. Therefore, just as strength of TCR-stimulation underlies abatacept-resistant T cell proliferation, robust CD28-costimulation may mediate vitamin D3-resistant T cell responses despite the inhibition of TCR signaling pathways by vitamin D3. This feature of vitamin D3 mediated inhibition of T cell responses may underlie an apparent lack of efficacy in some clinical trials analyzing the use of vitamin D3 supplementation for the treatment of the T cell mediated inflammatory diseases such as multiple sclerosis (Kampman et al., 2012).

In addition to influencing T cell activation, vitamin D has been widely reported to modify T cell effector function, for example, by inhibiting proinflammatory

cytokine production by T_h1, T_h9 and T_h17 cells (Reichel et al., 1987; Lemire et al., 1995; Jeffery et al., 2009; Colin et al., 2010; Palmer et al., 2011) and biasing differentiation towards T_h2 responses (Boonstra et al., 2001). Notably, vitamin D also promotes a regulatory phenotype characterized by increased expression of FoxP3 and CTLA-4 (Jeffery et al., 2009). It is possible that these effects are at least partly associated with redistribution in the relative contributions of TCR and CD28 signals towards T cell activation that results from vitamin D3 supplementation. This concept is consistent with the observation that T cell differentiation is influenced by the nature of activation signals (van Panhuys et al., 2014; Keck et al., 2014). For example, T_h1 (Tao et al., 1997a) and T_h17 (Bouguermouh et al., 2009) type responses are favored following TCR-driven activation which appears to be inhibited by vitamin D3. In contrast, CD28 signaling, favors T_h2 (Tao et al., 1997a) and T_{reg} differentiation (Gabryšová et al., 2011).

An interesting feature of the direct effect of vitamin D3 on T cells during stimulation is a reported VDR-dependent upregulation of PLC-γ1 by naïve T cells (vonEssen et al., 2010). This at least partially underlies an enhancement of TCR-driven stimulation by primed T cells relative to naïve counterparts. However, this would suggest that vitamin D3 has both positive and negative effects on a TCR-driven response that appears paradoxical. Nevertheless it could be suggested that any inhibition of T cell proliferation by vitamin D3 in the absence of CD28-costimulation forms a negative feedback mechanism to confer control over ongoing T cell responses (Kongsbak et al., 2013).

In my assays, vitamin D3 significantly increases CD28 expression by proliferating T cells. These data are supported by other studies suggesting that vitamin D3 can increase the expression levels of CD28 during *in vitro* stimulation of CD4$^+$ T cells derived from healthy controls or multiple sclerosis patients (Kickler et al., 2012). Additionally, vitamin D3 prevents the downregulation of CD28 by TNF-α among CD4$^+$ T cells in patients with primary sclerosing cholangitis (Liaskou et al., 2014). An upregulation of CD28 by vitamin D3 may facilitate T cell activation despite the corresponding inhibition of TCR-stimulation where CD80/CD86 are available. However, to some extent, this CD28-upregulation is paradoxical due to the increased expression of CTLA-4 that occurs in parallel and which serves to limit CD28-costimulation by depletion of CD80/CD86 via trans-endocytosis (Qureshi et al., 2011). The timing of these events may allow a window in which CD28 is elevated prior to CTLA-4 upregulation in order to facilitate costimulation initially but to control its availability in the long term.

Data presented here suggest that the induction of a pro-regulatory phenotype among activated T cells following 1,25(OH)$_2$D$_3$ could at least in part, be mediated by vitamin D3 tipping the T cell activation in favor of a CD28 driven response that is more pro regulatory in nature. T cell stimulation in the presence of 1,25(OH)$_2$D$_3$ promotes a regulatory phenotype characterized by increased expression of FoxP3 and CTLA-4; however this effect of vitamin D3 is blocked by abatacept. In support of this, Gabryšová, et al. found that the generation of induced T$_{reg}$ populations from naïve T cells *in vitro* was associated with the balance of this integrative threshold between TCR and

CD28 signaling (Gabryšová et al., 2011). Their findings suggest that reducing anti-CD3 stimulation in the presence of strong CD28-costimulation provided the most effective conditions for FoxP3 expression, which is consistent with the impact of vitamin D3 upon T cell activation signals. Again, this highlights the inherent tension between inhibiting effector responses and at the same time influencing regulatory outcomes (Sansom and Walker, 2013).

Any influence of costimulation-blockade upon T cell regulation may be offset by the fact that vitamin D3 and abatacept combine to more potently inhibit T cell proliferation. Thus greater inhibition of T cell proliferation as well as the reduction of inflammatory cytokines due to the presence of $1,25(OH)_2D_3$ favor its combination with abatacept as a potential treatment of inflammatory diseases.

Chapter 5: Can *in vitro* T cell activation thresholds be utilized to predict disease outcome in early arthritis patients?

5.1   Introduction

The TCR repertoire is shaped by T cell development within the thymus so that mature T cells in the periphery display weak autoreactivity. As such, autoimmune thresholds are set at a high level to prevent autoimmune T cell responses and perturbations in negative selection are associated with systemic autoimmunity (Palmer, 2003). Additionally, due to the integrative relationship between TCR and CD28 signals in achieving T cell activation thresholds, the control of CD28 signaling is essential to maintaining tolerance. Based upon this notion, it is unsurprising that a loss of CD28-regulation associated with CTLA-4 deficiency or altered function is associated with CD28-driven autoimmunity (Tivol et al., 1995; Waterhouse et al., 1995; Mandelbrot et al., 1999).

RA is an autoimmune disease where important genetic predisposing factors are MHCII and PTPN22 genotype (McInnes and Schett, 2011). By virtue of the MHCII variable affinity for antigen and the influence of PTPN22 on downstream TCR signal potency, both of these molecules may influence thymic selection and peripheral T cell activation thresholds. Several studies have identified reproducible intra-individual variations between various parameters of immune responses within standardized *in vitro* assays (Ye et al., 2014; Raj et al., 2014; Lee et al., 2014). The aims of this work presented

in this chapter are two-fold. Firstly, to determine whether differences between patterns of *in vitro* T cell activation within standardized *in vitro* assays could be identified between early arthritis patients who go on to develop RA relative to patients with transient joint inflammation. Secondly, to determine whether *in vitro* responsiveness to abatacept predicted subsequent clinical responsiveness.

5.2 Results

5.2.1. Ki67 as a marker of T cell activation

In order to assess variations in patterns of *in vitro* T cell activation between early arthritis patients of different eventual outcomes, a simple *in vitro* assay was developed in which T cells, derived from patients from the Birmingham Early Arthritis Cohort, were stimulated by various concentrations of anti-CD3, with or without 20µg/mL abatacept. The inclusion criteria for this cohort was a patient presenting with either [i], inflammatory arthritis as indicated by clinically apparent synovial swelling affecting at least one joint, or [ii], a history considered by the assessing Rheumatologist to be indicative of new onset inflammatory arthritis in the absence of overt joint swelling. Patients already treated with DMARDs or with symptoms indicative of degenerative disease or other causes of joint pain were not included within this study.

To circumvent difficulties associated with limited peripheral blood volumes, T cells were stimulated within whole PBMC samples. Previous experiments throughout this thesis have demonstrated T cell proliferation as indicated by CTV dilution to be a useful marker of T cell activation, however, CTV labeling

was not compatible with PBMC stimulation. As such, Ki67, which is upregulated as cells exit the G_0 cell cycle phases and is subsequently expressed throughout the cell cycle (Scholzen and Gerdes, 2000), was utilized as a marker of T cell proliferation. A typical gating strategy for analysis of Ki67 expression by T cells stimulated by anti-CD3 is shown in **fig. 5.1A**. This demonstrates Ki67 expression exclusively within the CD25$^+$ population when gating on CD3$^+$CD4$^+$ T cells. Time course analysis demonstrated Ki67 expression levels to be more pronounced following 4 days stimulation and that this decreased following 5-6 days proliferation (**fig. 5.1B**). This seemed consistent with the idea of stalled proliferation at later time points, as such, samples were analysed following 4 days stimulation for this study.

5.2.2. Differential in vitro TCR- and CD28-thresholds between individual early RA patients

As part of the optimization of an assay by which to compare T cell activation between early arthritis patients, qualitative differences in patterns of T cell proliferation were observed between patients in response to a range of anti-CD3 concentrations and abatacept treatment. For example, results from three patients (SWB EAC 0014, SW EAC 0069 and SWB EAC 0052) are shown in **fig. 5.2** and are representative of this inter-individual variability. For example, patient SW EAC 0014 displayed a fairly substantial Ki67$^+$ T cell population following stimulation by 0.05ng/mL anti-CD3 which is relatively unaffected by abatacept (**fig. 5.2A**) whilst SW EAC 0069 displayed abatacept sensitive proliferation under these conditions (**fig. 5.2B**). In contrast, patient SWB EAC 0052 showed limited T cell proliferation in response to stimulation by

0.05ng/mL anti-CD3 (**fig. 5.2C**). Together, these responses seem indicative of a degree of heterogeneity between *in vitro* T cell responses among early arthritis patients.

In order to assess how these parameters of *in vitro* T cell activation related to disease outcome, PBMC samples were stimulated with varying concentrations of anti-CD3 with or without 20µg/mL abatacept and T cell proliferation was assessed at 4 days by Ki67 labeling. The results from a small early arthritis patient cohort are summarized in **fig. 5.3A**. The clinical details for these patients are presented in **table 7.1** (Appendix). Unsurprisingly, these results demonstrate that the proportion of $CD3^+CD4^+$ T cells staining positive for Ki67 decreased as the anti-CD3 concentration driving proliferation was reduced. However, abatacept had a consistent but modest effect upon T cell proliferation at all anti-CD3 concentrations tested, this may indicate that CD28 plays a relatively limited role within this stimulation system. T cell proliferation in response to anti-CD3 stimulation was stratified relative to patient outcome determined at 12-month follow-up. These outcomes were categorized as RA, Persistent Non-RA, Resolving Non-RA or inflammatory arthralgia. A comparison of the clinical details between these sub-groups is summarized in **table 5.1**. However, categorization of *in vitro* T cell proliferation according to these outcomes was not associated with convincing differences between patient sub-groups (**fig. 5.3B/C**). It is possible that this poor resolution between patient sub-groups reflects limitations associated with either a small overall sample size or an inherent variability associated with PBMC-based T cell stimulation.
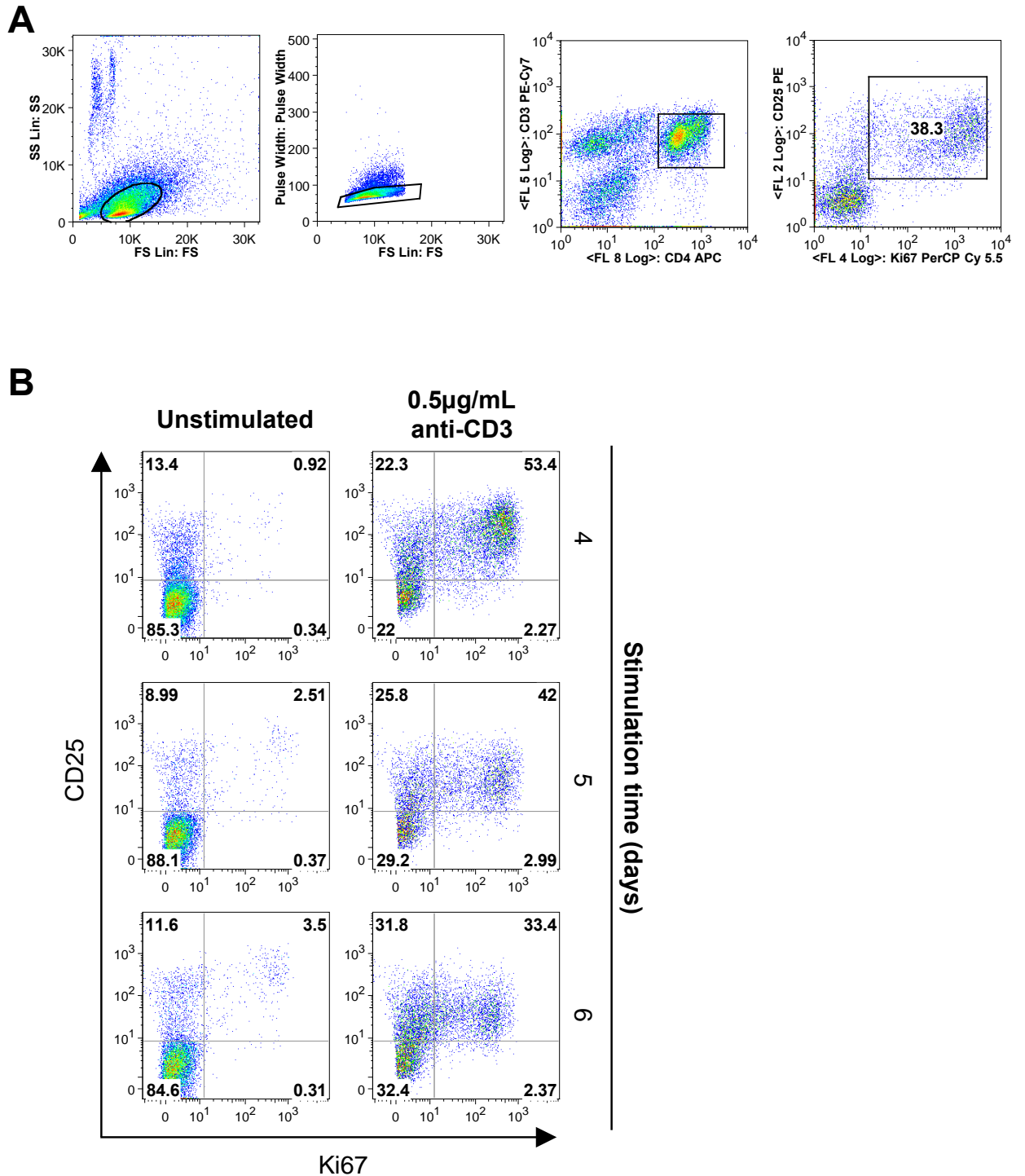
Fig. 5.1. Ki67 is expressed by CD3⁺CD4⁺CD25⁺ activated T cells. PBMC samples were generated by density gradient centrifugation. T cells were stimulated with 0.5μg/mL. **A**) Characteristic gating strategy for Ki67 analysis following anti-CD3 stimulation. Following initial discrimination based upon forward scatter (FS) vs. side scatter (SS) cells were gated on a low pulse width in order to exclude doublets. Ki67 expression was determined within the CD3⁺CD4⁺ population. **B**) Flow cytometry data for time course analysis showing Ki67 vs. CD25 expression (by CD3⁺CD4⁺ T cells) following stimulation for indicated time periods.
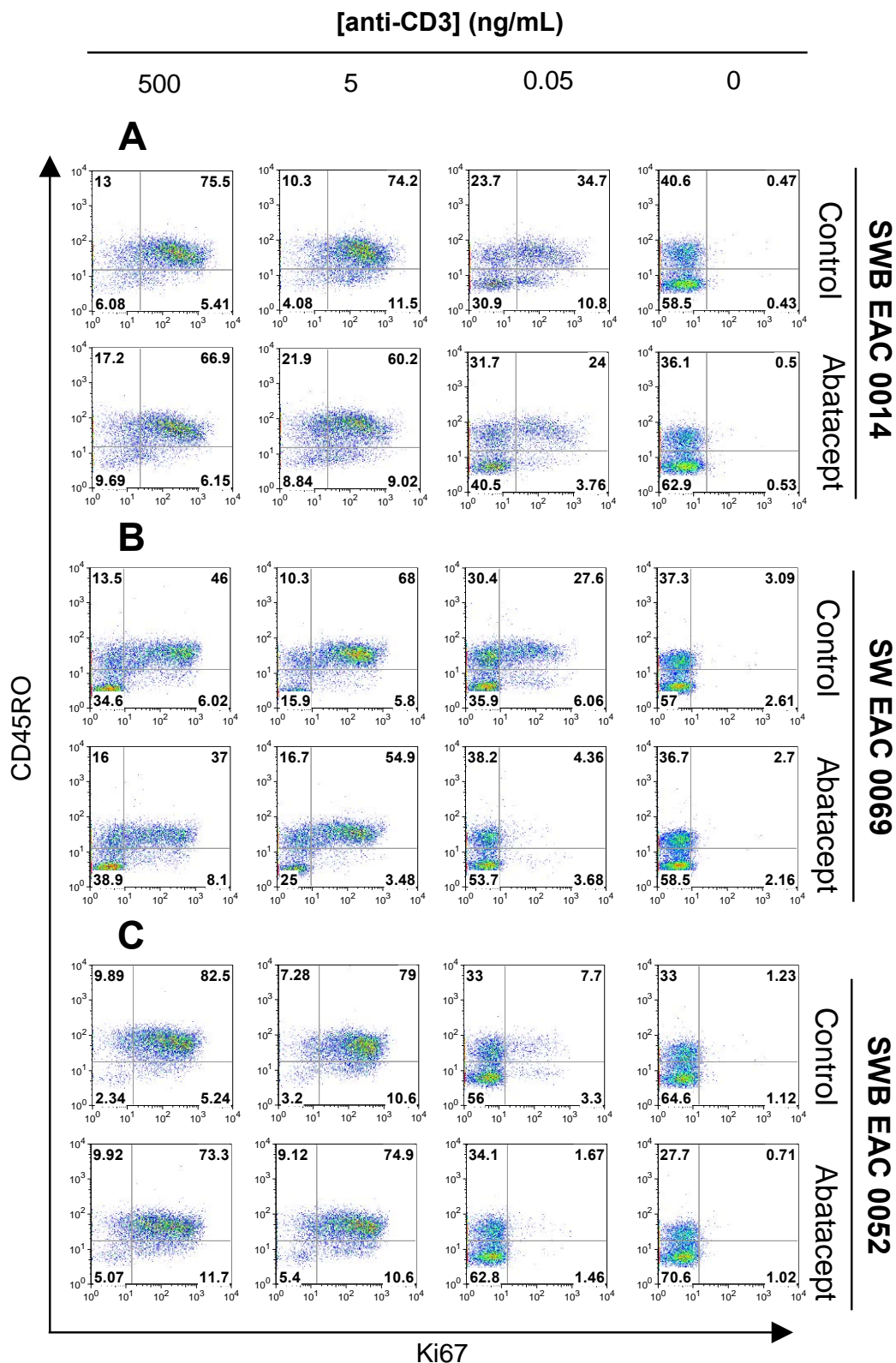
Fig. 5.2. Differential patterns of Ki67 staining following T cell stimulation by various anti-CD3 concentrations. PBMC samples were generated by density gradient centrifugation. T cells were stimulated with indicated anti-CD3 concentrations -/+ (20µg/mL) abatacept for 5 days. Representative flow cytometry showing data from 3 different early arthritis patients (**A**) SWB EAC 0014 (**B**) SWB EAC 0014 (**C**) SWB EAC 0052.
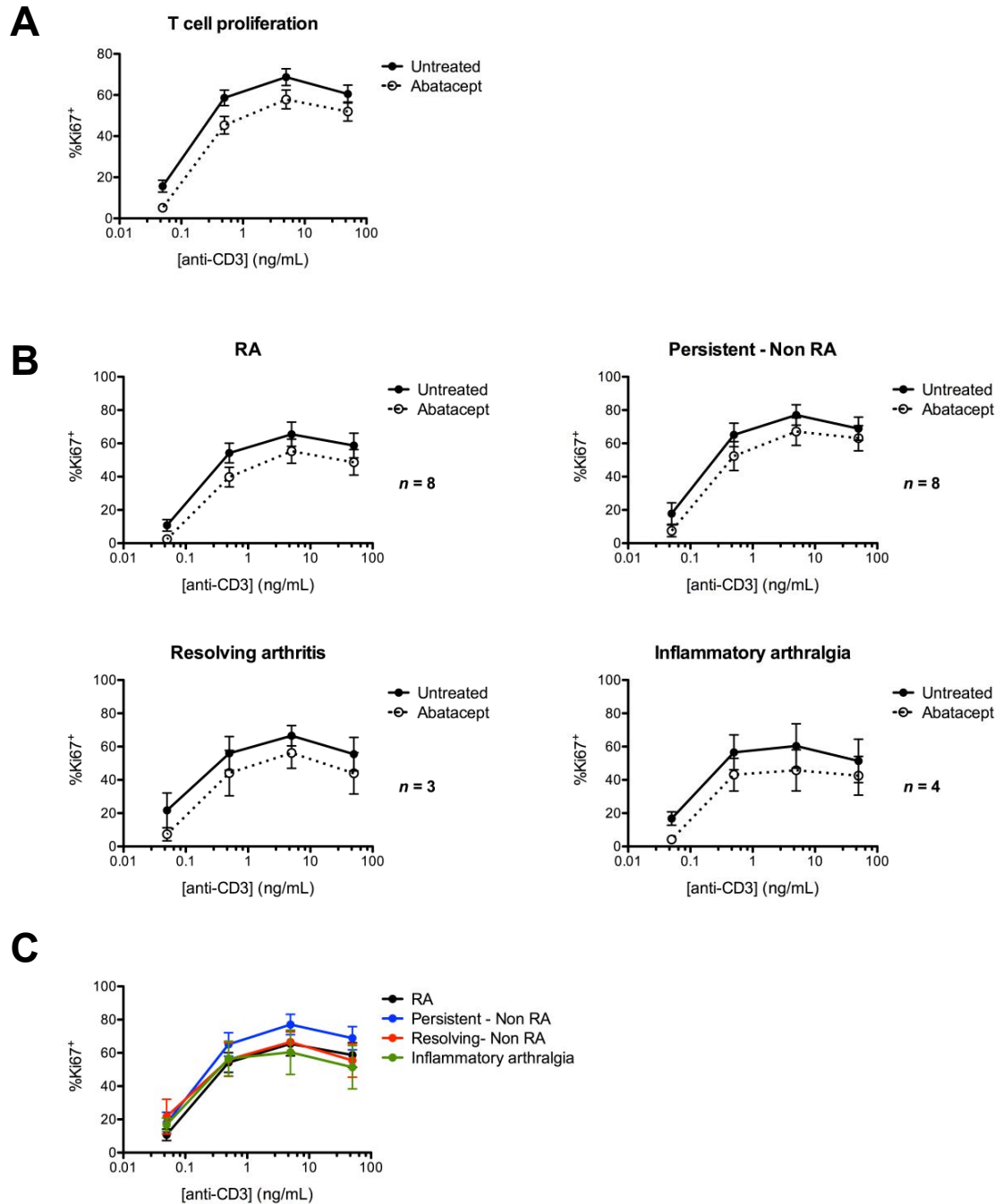
Fig. 5.3. Differential patterns of *in vitro* T cell proliferation in response to anti-CD3 among early arthritis patients do not correlate with outcome of disease. PBMC samples were isolated from whole blood derived early arthritis patients. 2 x 10⁵ PBMCs were stimulated with various anti-CD3 concentrations and -/+ (20μg/mL) abatacept. Following 4 days stimulation T cell proliferation was determined by Ki67 staining. **A**) The %Ki67⁺ T cells (gated on CD3⁺CD4⁺ cells) following 4 days stimulation from a cohort of 23 early arthritis patients. **B**) The %Ki67⁺ T cells from **A** are stratified by outcome of early arthritis as RA (n = 8), Persistent non-RA (n = 8), resolving arthritis (n = 3) or inflammatory arthritis (n = 4). **C**) A comparison of the %Ki67⁺ T cells under control conditions only. All data points represent mean values ±S.E.

Table 5.1. Clinical data for early arthritis patient sub-groups stratified according to clinical outcome (Abbreviations: 68-tender joint count (TJC-68), 66-swollen joint count (SJC-66), 28-tender joint count (TJC-28), 28-swollen joint count (SJC-28), C Reactive Protein (CRP), Erythrocyte sedimentation rate (ESR), Disease activity score 28 (DAS28), rheumatoid factor (RF), Cyclic Citrullinated Peptide Antibody (CCP)).

Classified as:	Patient	Gender	Age	Symptom duration (wks)	TJC 68	SJC 66	TJC 28	SJC 28	CRP (mg/L)	ESR (mm/hr)	DAS28 (ESR)	% RF positive *	% CCP positive ‡	25(OH)2D3 (ng/mL)	Patient global
RA	SW EAC 0084	Female	46.5 ± 15.8	77.6 ± 108.1	8.9 ± 7.4	4.1 ± 1.8	5.4 ± 5.3	3.5 ± 1.8	10.8 ± 13.9	24.3 ± 12.1	4.4 ± 1.0	57.1	75	39.3 ± 23.1	52.0 ± 21.1
	SW EAC 0085	Female													
	SW EAC 0087	Female													
	SW EAC 0089	Male													
	SW EAC 0093	Female													
	SW EAC 0104	Female													
	SW EAC 0105	Female													
	SW EAC 0111	Female													
Persistent non-RA	SW EAC 0090	Male	54.3 ± 15.1	52.3 ± 86.2	5.6 ± 2.6	3.4 ± 3.2	4.2 ± 2.2	2.6 ± 2.6	14.1 ± 9.1	24.3 ± 15.5	4.1 ± 1.1	25	0	42.7 ± 18.9	42.3 ± 25.2
	SW EAC 0106	Female													
	SW EAC 0107	Male													
	SW EAC 0113	Male													
	SW EAC 0120	Male													
	SW EAC 0127	Male													
	SW EAC 0128	Male													
	SW EAC 0129	Male													
Resolving arthritis	SW EAC 0035	Male	58.7 ± 17.2	37.3 ± 18.4	3.3 ± 4.2	2.3 ± 3.2	2.7 ± 4.6	2.3 ± 3.2	4.3 ± 3.5	15 ± 11.5	2.8 ± 2.1	50	0	82.8 ± 8.7	24.7 ± 14.6
	SW EAC 0116	Male													
	SW EAC 0119	Female													
Inflammatory arthralgia	SW EAC 0083	Female	54.3 ± 16.3	108.3 ± 61.0	4.5 ± 3.9	0 ± 0	4.0 ± 4.2	0 ± 0	9.5 ± 9.5	41.33 ± 28.18	3.9 ± 1.1	25	25	75.5 ± 53.0	50.8 ± 27.7
	SW EAC 0094	Female													
	SW EAC 0100	Female													
	SW EAC 0114	Female													

Values represent mean values ± standard deviation
• RF positivity was determined as a titer ≥20U/mL
‡ CCP positivity was defined as a titer ≥ 7U/mL

164

5.2.3. In vitro T cell activation thresholds as an indicator of clinical responses to abatacept

An additional aim for this study was to identify the capacity for patterns of *in vitro* T cell stimulation to predict likely patient responses to abatacept treatment. To address this, PBMC samples were obtained from patients who were about to commence abatacept treatment. For logistical reasons, samples were only obtained from 2 established RA patients whose clinical details are outlined in **table 5.2.** Responses to abatacept treatment were defined at 12 month follow up according to the European League against Rheumatism (EULAR) response criteria (van Gestel et al., 1999) whereby patient SW BSF 0042 showed a moderate response and patient SW BSF 0042 displayed a good response. Analysis of *in vitro* T cell proliferation to graded anti-CD3 stimulation and abatacept treatment in relation to these clinical responses were considered in **fig. 5.4A/B**. Significant conclusions cannot be drawn from this due to the limited sample size. However, the data do reveal an impact of abatacept upon T cell proliferation in these two patients. A larger sample size including patients showing a poor response to abatacept would be needed to address was in vitro abatacept responsiveness correlated with eventual clinical outcomes. An additional drawback associated with the method of T cell stimulation in the context of whole PBMC samples is the limited abatacept sensitivity across the anti-CD3 titration range.

Table 5.2. Clinical data for patients commencing abatacept treatment. (Abbreviations: 28-tender joint count (TJC-28), 28-swollen joint count (SJC-28), Disease activity score 28 (DAS28), C Reactive Protein (CRP), Erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), Cyclic Citrullinated Peptide Antibody (CCP).

Patient	Date	Final diagnosis	Gender	Age	Smoking status	TJC 28	SJC28	Patient Global	CRP (mg/L)	ESR (mm/hr)	DAS28 (ESR)	RF (U/mL)	CCP (U/mL)	25(OH)D3 (ng/mL)	EULAR response
SW BSF 0042	Abatacept begun: 07/04/2014	RA	Female	44	Current	4	3	73	2	32	5.06	positive (90.1)	positive (17)	31.03.2014 - 13.9	MODERATE RESPONSE
	12 month-follow up: 24/08/2015					1	0	80	1	28	4.02				
SW BSF 0050	Abatacept begun: 01/05/2014	RA	Male	75	Never	7	4	54	1	13	4.6	positive (77.9)	positive (>340.0)	13.06.2013 - 74.9	GOOD RESPONSE
	12 month-follow up: 12/05/2015					1	0	20	-	27	3.15				

166
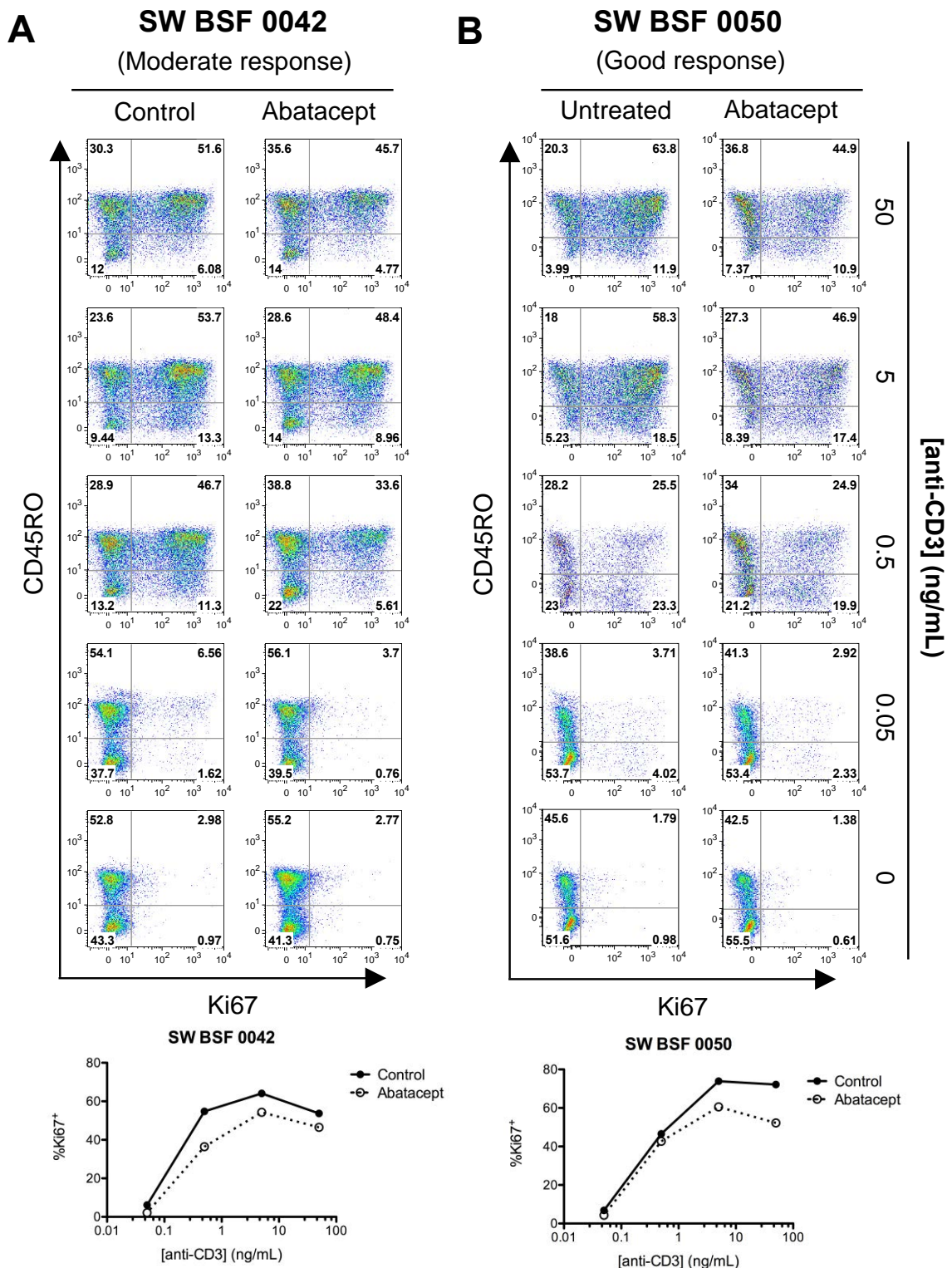
Fig. 5.4. *In vitro* T cell stimulation as a predictive test for likely responses to abatacept-treatment. PBMC samples were isolated from 2 patients, SW BSF 0042 with established arthritis who were commencing abatacept treatment. 2×10^5 PBMCs were stimulated with various anti-CD3 concentrations and -/+ (20μg/mL) abatacept. Following 4 days stimulation T cell proliferation was determined by Ki67 staining.

5.2.4. Monocyte:T cell ratios within PBMC samples determine the extent of T cell activation and abatacept sensitivity

Data presented in chapter 3 of this thesis demonstrates that relative APC numbers affect the quality of T cell stimulation such that abatacept resistant T cell proliferation is observed at high APC:T cell ratios. As such, it seemed that the proportion of monocytes within the PBMC sample relative to $CD3^+CD4^+$ T cells could impact upon parameters of T cell activation. Ki67 expression and abatacept sensitivity were therefore expressed relative to the $CD14^+:CD3^+CD4^+$ ratio determined by an *ex vivo* staining panel used to characterize the PBMC samples prior to stimulation. These data suggest that high $CD14^+$ monocytes relative to $CD3^+CD4^+$ T cells within the PBMC sample at the time of stimulation increases the proportion of $CD3^+CD4^+$ T cells that express Ki67 following 4 days stimulation when stimulation was driven by higher anti-CD3 concentrations between (**fig. 5.5A**; 50-0.5ng/mL anti-CD3). Additionally, the influence of the monocyte:T cell ratios at initial stimulation on the abatacept sensitivity of the T cell response was assessed. Abatacept sensitivity was determined as the proportion of $CD3^+CD4^+$ T cells expressing Ki67 at day 4 under abatacept treated conditions relative to untreated controls. These data demonstrated a statistically significant (although relatively weak) correlation whereby abatacept resistance increased in association with elevated monocyte:T cell ratios when stimulation was driven by higher anti-CD3 concentrations (**fig. 5.5B;** 50-0.5ng/mL anti CD3). This correlation was not seen when stimulation was driven by the lowest anti-CD3 concentration tested of 0.05ng/mL perhaps because any proliferation that occurs under these conditions is more dependent upon CD28-costimulation

as shown in **fig. 5.5B**. This supports the concept that CD28-independent proliferation occurs more effectively at higher APC:T cell ratios due to the summation of cross-linked anti-CD3 signaling between successive APC interactions. These monocyte:T cell ratios within the periphery did not however appear to significantly correlate with either disease outcome (**fig. 5.6A**) or disease activity (**fig. 5.6B**) as defined by DAS28 score. As such, in the context of these assays the monocyte:T cell ratio can be considered an extraneous factor that determines variability between patterns of T cell proliferation in response to anti-CD3 stimulation.

Patterns of T cell activation were also expressed relative to the proportion of CD45RO$^+$ memory T cells within the CD3$^+$CD4$^+$ population at initial stimulation. This revealed no overall correlation at any of the anti-CD3 concentrations tested with the proportion of T cells that expressed Ki67 following 4 days stimulation (**fig. 5.7A**). Similarly, the impact of abatacept upon Ki67 expression following 4 days stimulation did not strongly correlate with the proportion of CD4$^+$CD45RO$^+$ T cells within the original PBMC population was stimulation was mediated by higher anti-CD3 concentrations (**fig. 5.7B** 50-0.5ng/mL anti-CD3). When stimulating T cells with 0.05ng/mL anti-CD3, a statistically significant correlation was observed whereby an increased proportion of CD45RO$^+$ of memory T cells promoted increasingly abatacept-sensitive T cell proliferation (**fig. 5.7B**). This result was unexpected but may reflect the CD28-dependent proliferation of memory T cells at low anti-CD3 concentrations along with an absence of naïve T cell proliferation, even in the presence of abatacept, at low anti-CD3 concentrations. Another

potential source of variation in *in vitro* T cell responses under these conditions between patients was the relative abundance of T_{reg} within the $CD3^+CD4^+$ population. The proportion of $CD3^+CD4^+$ T cells that displayed a T_{reg} phenotype was determined by an *ex vivo* staining panel where $CD25^{high}CD127^{low}$ staining was considered to be indicative of the T_{reg} population (Liu et al., 2006). However, these experiments revealed no significant correlation between the proportion of $CD25^{high}CD127^{low}$ within the initial $CD3^+CD4^+$ population and either Ki67 expression (**fig. 5.8A**) or the impact of abatacept upon Ki67 expression (**fig. 5.8B**). This may be predicted based upon the observation that abatacept had a limited impact upon T cell proliferation under these conditions in association with the idea that CTLA-4 expression is essential to the activity of T_{reg} (Wing et al., 2008).

5.2.5. PBMC stimulation by anti-CD3 is associated with limited CD25 upregulation by activated T cells

Fig. 3A demonstrated a modest impact of abatacept upon T cell proliferation even at low anti-CD3 concentrations. In contrast, purified $CD4^+CD25^-$ T cells stimulated with allogeneic DCs and anti-CD3 demonstrated a consistent degree of proliferation at low anti-CD3 concentrations although this was CD28-dependent and therefore abatacept sensitive (see **fig. 3.11**). One possibility was that stimulation occurred in a CD28-independent manner in association with high monocyte:T cell ratios (across the 23 early arthritis patients that were tested in this study the mean monocyte:T cell ratio within PBMC samples was 1:3.2).
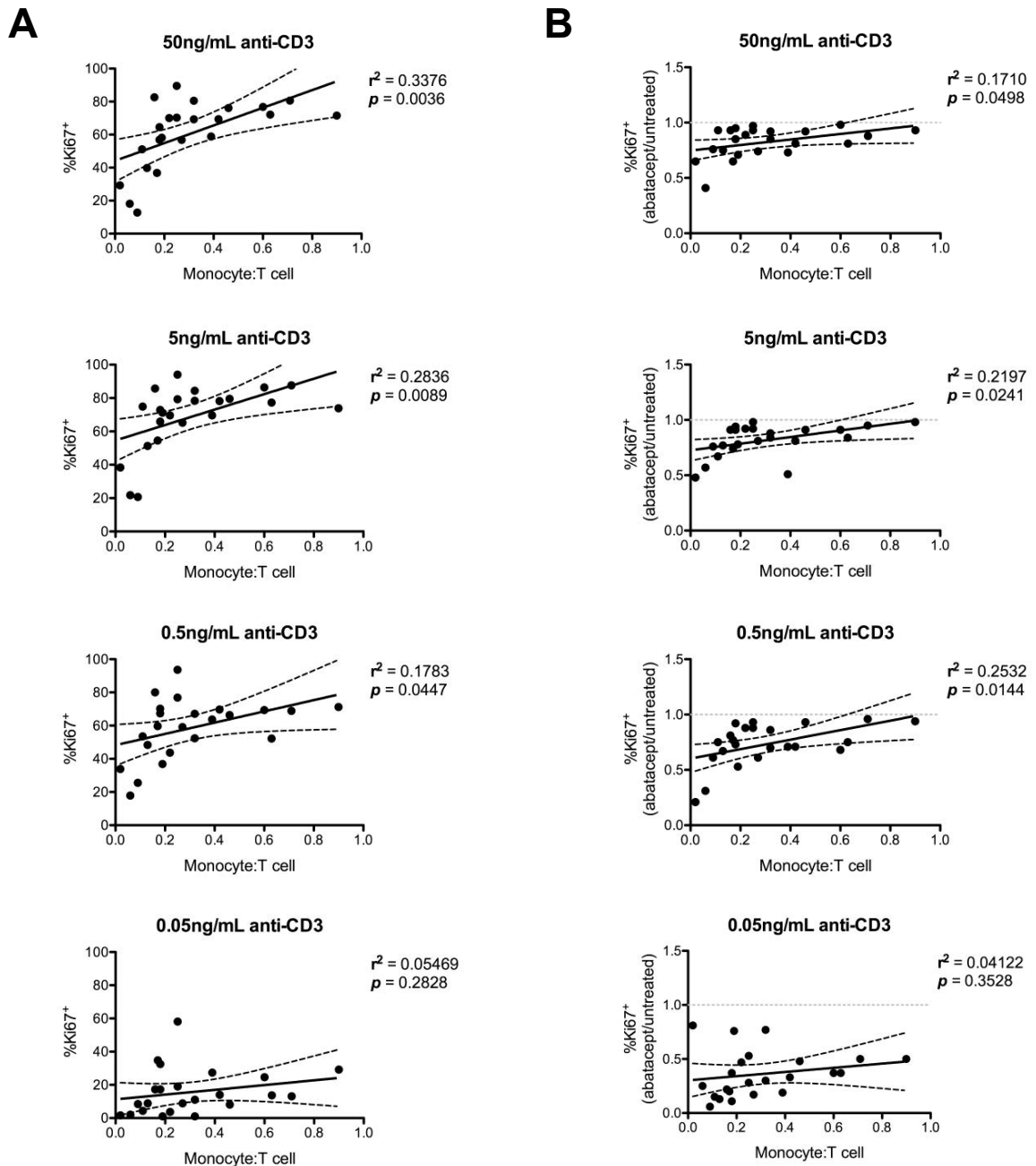
Fig. 5.5. CD14$^+$ Monocyte to CD3$^+$CD4$^+$ ratio within PBMC samples determines the extent of T cell proliferation and efficacy of abatacept blockade following anti-CD3 stimulation. PBMC samples were isolated from whole blood derived from 23 early arthritis patients. Monocyte:T cell ratios were determined *ex vivo* and expressed as decimals i.e. CD14$^+$ divided by CD3$^+$CD4$^+$. 2 x 10^5 cells were stimulated with various anti-CD3 concentrations and -/+ (20μg/mL) abatacept. Following 4 days stimulation T cell proliferation was determined by Ki67 staining. **A**) The relationship between *ex vivo* monocyte:T cell ratios and the % Ki67$^+$ T cells (gating on CD3$^+$CD4$^+$). **B**) The relationship between the outcome of abatacept-blockade and *ex vivo* monocyte:T cell ratios. The impact of abatacept upon T cell proliferation following was determined as the % Ki67$^+$ T cells following abatacept treatment normalized to untreated controls. *p* values are derived from linear regression analysis and dotted lines represent the 95% confidence band.

A

B

$r^2 = 0.00007$
$p = 0.9708$
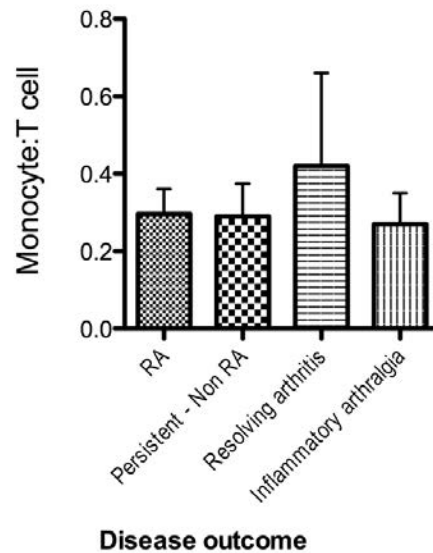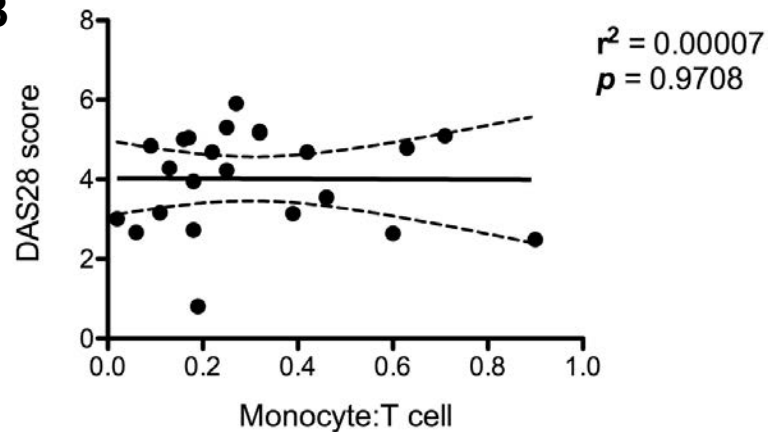
Fig. 5.6. Monocyte:T cell ratios within the peripheral blood of RA patients does not reflect disease outcome or activity. *Ex vivo* monocyte:T cell ratios were determined by flow cytometry and expressed relative to various disease outcomes (**A**) or disease activity as indicated by DAS28 scores (**B**). *p* value is derived from linear regression analysis and dotted lines represent the 95% confidence band.
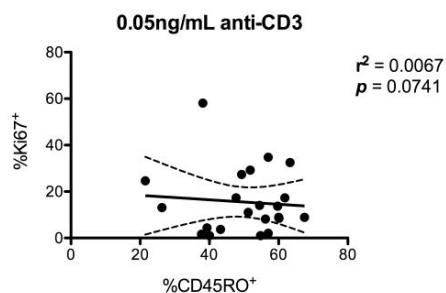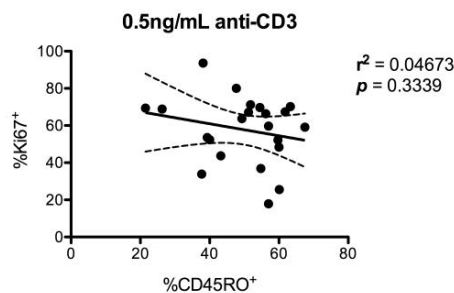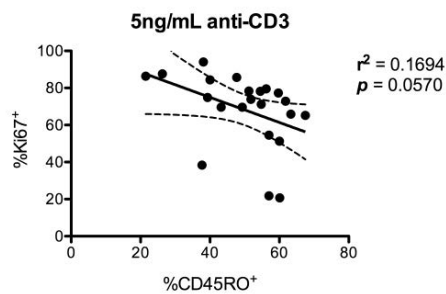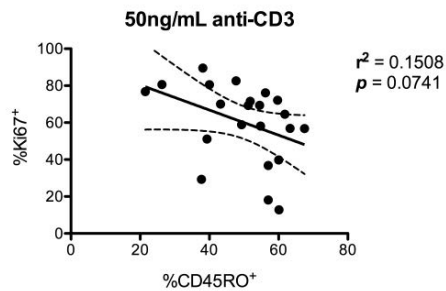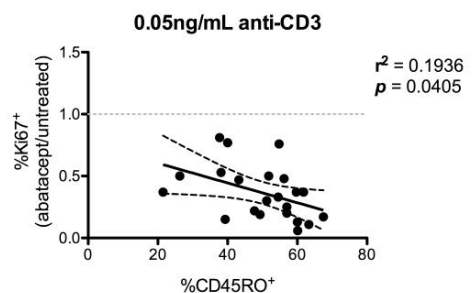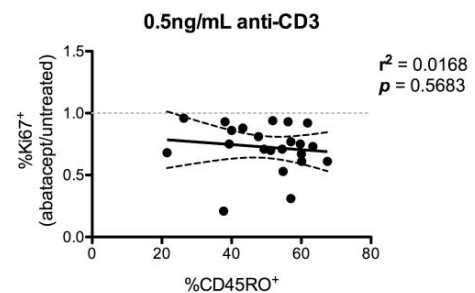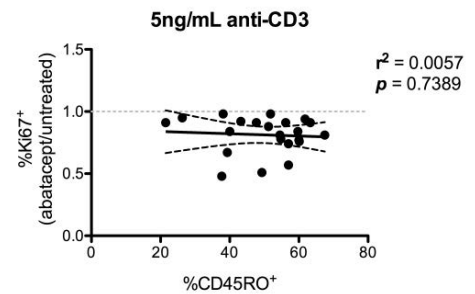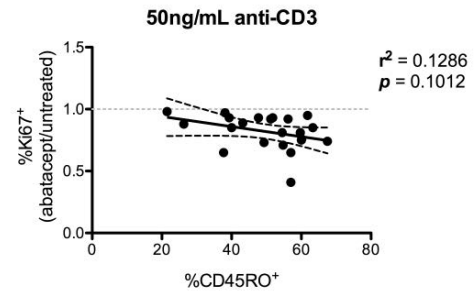
Fig. 5.7. The proportion of CD3⁺CD4⁺CD45RO⁺ memory T cells within PBMC samples does not strongly influence T cell proliferation or the efficacy of abatacept following anti-CD3 stimulation. PBMC samples were isolated from whole blood derived from 23 early arthritis patients. % CD45RO T cells (gating on CD3⁺CD4⁺ cells) were determined prior to stimulation. 2×10^5 cells were stimulated with various anti-CD3 concentrations and -/+ (20µg/mL) abatacept. Following 4 days stimulation T cell proliferation was determined by Ki67 staining. **A**) The relationship between % CD45RO⁺ T cells at stimulation and the % Ki67⁺ T cells following activation (gating on CD3⁺CD4⁺). **B**) The relationship between the outcome of abatacept-blockade and *ex vivo* CD45RO⁺ T cells. The impact of abatacept upon T cell proliferation following was determined as the % Ki67⁺ T cells following abatacept treatment normalized to untreated controls. *p* values are derived from linear regression analysis and dotted lines represent the 95% confidence band.
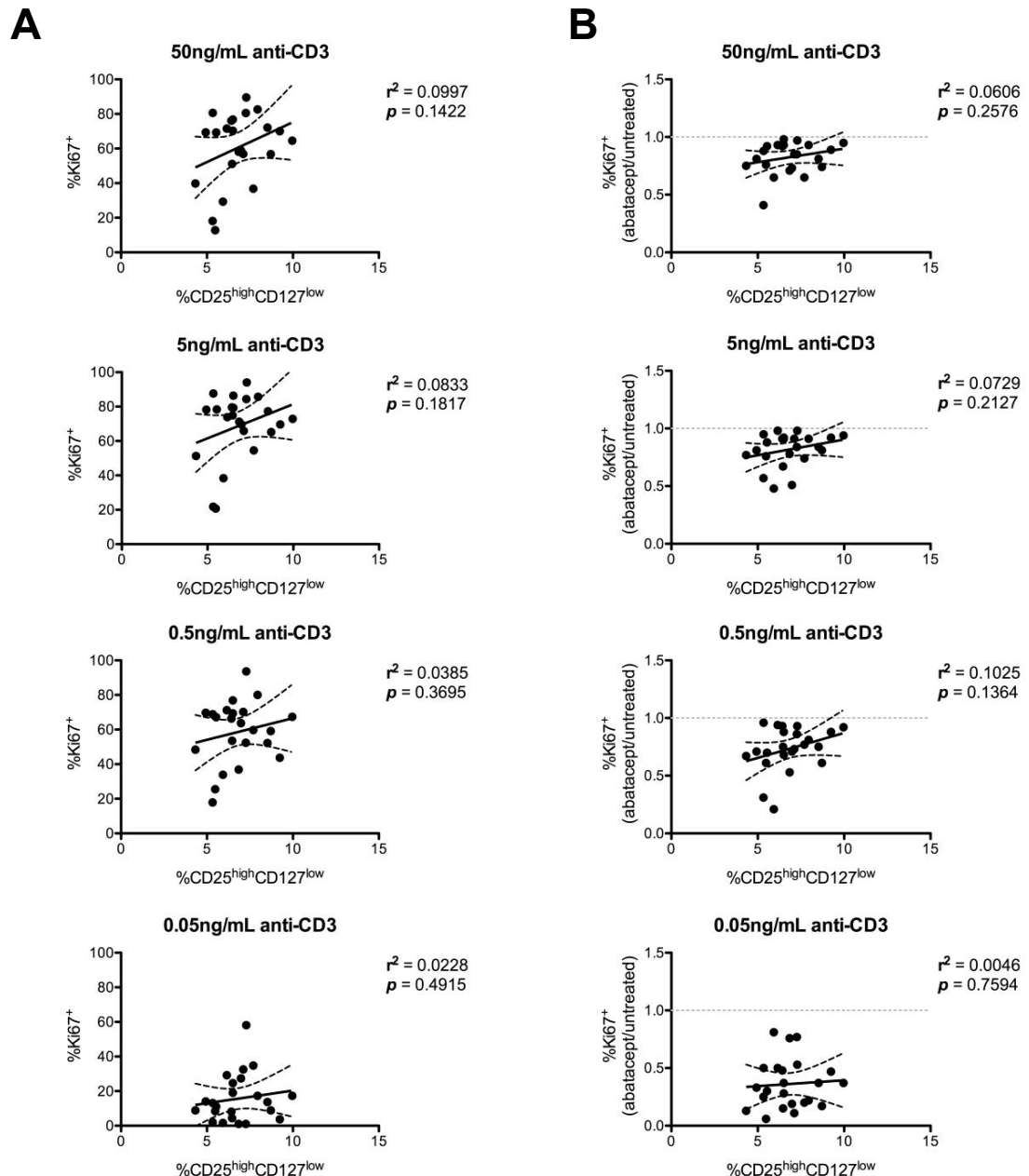
Fig. 5.8. The proportion of CD25^{high}CD127^{low} T cells within PBMC samples does not affect the extent of T cell proliferation or the efficacy of abatacept blockade following anti-CD3 stimulation. PBMC samples were isolated from whole blood derived from 23 early arthritis patients. The proportion of CD3$^+$CD4$^+$ T cells staining CD25^{high} CD127^{low} was determined *ex vivo.* 2 x 10^5 cells were stimulated with various anti-CD3 concentrations and -/+ (20µg/mL) abatacept. Following 4 days stimulation T cell proliferation was determined by Ki67 staining. **A**) The relationship between *the* % CD25^{high}CD127^{low} prior to stimulation and the % Ki67$^+$ T cells (gating on CD3$^+$CD4$^+$) following 4 days stimulation. **B**) The relationship between *the* % CD25^{high}CD127^{low} prior to stimulation and the outcome of abatacept-blockade. The impact of abatacept upon T cell proliferation following was determined as the % Ki67$^+$ T cells following abatacept treatment normalized to untreated controls. *p* values are derived from linear regression analysis and dotted lines represent the 95% confidence band.
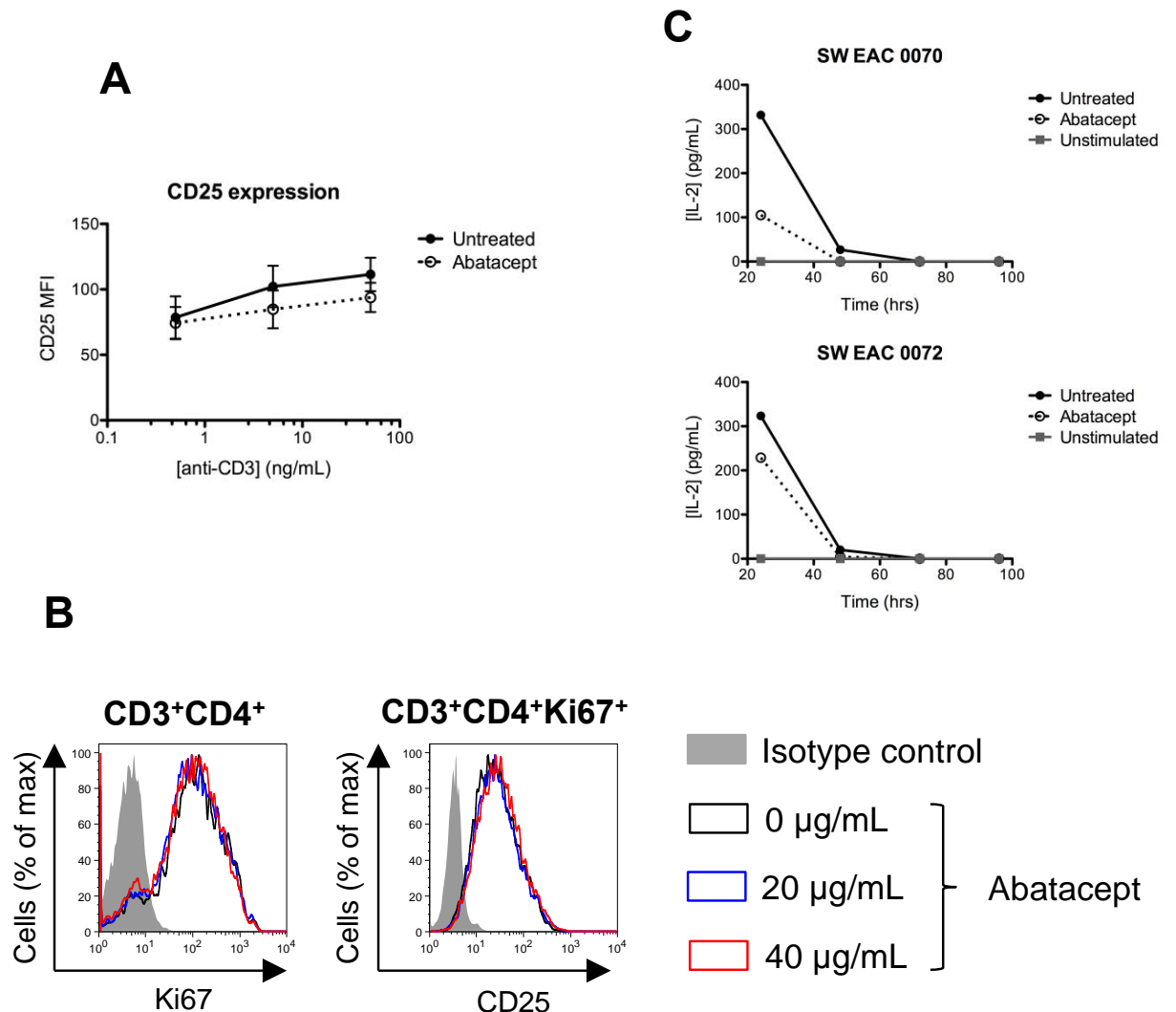
Fig. 5.9. CD28 blockade fails to significantly inhibit CD25 expression following anti-CD3 driven T cell proliferation in the context of whole PBMC samples. A) PBMC samples were isolated from whole blood derived from 23 early arthritis patients. 2×10^5 cells were stimulated with various anti-CD3 concentrations and -/+ (20µg/mL) abatacept. Following 4 days stimulation T cell proliferation was determined by Ki67 staining. **A**) CD25 MFI is shown for CD3+CD4+Ki67+ T cells. **B**) Flow cytometry data showing the impact of indicated abatacept concentrations upon the proportion of CD3+CD4+ T cells that express Ki67 and the expression of CD25 by CD3+CD4+Ki67+ T cells following stimulation with 500ng/mL anti-CD3. Data shows 1 representative experiment of 3 performed. **C**) IL-2 production was determined by ELISA from supernatants taken at indicated time points following PBMC stimulation by 500ng/mL anti-CD3 -/+ (20µg/mL) abatacept. Data points represent mean values of 3 technical replicates for 2 early arthritis patients (SW EAC 0070 and SW EAC 0072).

Additionally, it seemed plausible that T cell proliferation driven by anti-CD3 within the context of whole PBMC samples did not facilitate robust CD28-signaling, perhaps due to the provision of costimulation predominantly by monocytes. To explore this, the impact of abatacept upon CD25, which is a CD28-dependent activation marker (see **fig. 3.8**), was determined within the CD3$^+$CD4$^+$KI67$^+$ population (i.e. activated T cells). In contrast to DC driven assays, abatacept displayed a very modest effect upon CD25 expression levels (**fig. 5.9A**). Addition of a higher abatacept concentration of 40μg/mL abatacept did not affect either the proportion of CD3$^+$CD4$^+$ T cells that expressed Ki67 or CD25 expression by CD3$^+$CD4$^+$Ki67$^+$ T cells (**fig. 5.9B**), therefore the failure to inhibit CD25 was not due to incomplete CD80/CD86 blockade. IL-2 ELISAs were performed on two separate donors. These data demonstrated IL-2 production by T cells at early time-points (24-48h) following anti-CD3 stimulation (**fig. 5.9C**). Although abatacept was observed to suppress IL-2 (particularly for patient SW EAC 0070) following 24hrs stimulation, IL-2 production was transient and appeared more comparable to the pattern of IL-2 production induced by anti-CD3 than anti-CD28 driven T cell activation presented in **fig. 4.17**. Together, these observations may suggest that monocytes within PBMC samples do not support robust CD28-dependent T cell proliferation. As such, the upregulation of CD28-dependent activation markers e.g. CD25/IL-2 under control conditions is limited and therefore not strongly affected by abatacept. Therefore, T cell proliferation under these conditions is strongly dependent upon anti-CD3 stimulation. Therefore, T cell stimulation within the context of whole PBMC samples does

not appear to represent an optimal system by which to assess inter-individual differences between CD28-requirements and abatacept sensitivity.

5.3   Discussion

Considerable data now support the concept that early clinical intervention dramatically enhances outcomes in patients with RA, in particular, reducing the rate of long term joint damage (Raza and Filer, 2015). The optimal strategy for the effective management of patients with early arthritis would be to aggressively treat patients at a very early stage who are at risk of developing aggressive forms of the disease based upon prognostic biomarkers that indicate adverse disease outcomes. Current approaches focus on treating patients at the earliest stages of clinically apparent synovitis (for example, early undifferentiated arthritis and early RA) but ongoing trials are assessing the effects of therapy in patients at even earlier at risk stages (van Steenbergen et al., 2013), for example in the presence of RA related autoantibodies (Rantapää-Dahlqvist et al., 2003) but before joint swelling has actually occurred.

The factors that dictate progression from early-undifferentiated arthritis to persistent RA are not entirely clear. One possibility is that patients who are eventually going to develop RA display reduced T cell activation thresholds. This may in part be due to the presence of variants in genes of relevance to T cell activation and regulation, providing an inherent bias towards autoimmunity and chronic inflammatory responses. Consistent with this idea,

previous work has demonstrated that RA patients display more effective ERK activation upon stimulation relative to healthy controls which predisposes patients to effective TCR-driven T cell activation in response to suboptimal stimulation (Singh et al., 2009).

In this study, the aim was to utilize *in vitro* assays as a basis to assess inter-individual T cell thresholds between patient subgroups. Peripheral T cells were stimulated by various anti-CD3 concentrations and abatacept in the context of whole PBMC samples. However, the variability associated with PBMC samples created a potential problem in this context. In particular, the monocyte:T cell ratio strongly affected the outcome of stimulation. This is consistent with data presented in chapter 3 of this thesis whereby the APC:T cell ratio affects stimulus strength as T cells integrate signals from multiple APC interactions. A more suitable approach may be to purify distinct T cell populations from the periphery and stimulate under more standardized conditions such as with varying anti-CD3 and anti-CD28 concentrations although this has a significant drawback of requiring larger whole blood volumes from which to isolate T cells.

Another factor that complicates the management of patients with early arthritis is the heterogeneity within and between various forms of inflammatory arthritis, for example, the different responses to therapies seen in patients with early and established RA. In cancer, treatment strategies are frequently guided by an assessment of biomarkers predictive of treatment response

(Chan and Behrens, 2013). However, such biomarkers are generally less clearly defined within the context of RA (Miossec et al., 2011).

With regards to predicting the efficacy of abatacept treatment, one possibility would be to utilize *in vitro* T cell stimulation in a comparable manner to this study to determine abatacept sensitivity perhaps dictated by an innate bias towards TCR driven responses. However, T cell stimulation in the context of mixed PBMC samples results in relatively limited CD28-driven responses as indicated by a general lack of abatacept-mediated suppression. Within the context of PBMC samples the primary source of costimulation are monocytes although a small population of DCs does exist within the peripheral blood. $CD14^+$ monocytes did express CD86 in these experiments (data not shown). However, monocytes and T cells display different patterns of adhesion molecule expression (Brown et al., 1997). These differences may promote different T cell interaction dynamics with monocytes vs. DCs that could inhibit optimal CD28-costimulation. Together, the inherent variability along with the limited contribution of CD28 towards T cell proliferation in these PBMC-based assays makes observations with regards to abatacept efficacy difficult to interpret.

The optimal approach towards utilizing *in vitro* assays to predict patient responses would be to develop a more standardized assay with less inherent variability. One possibility would be to use transfected cell lines (i.e. CHO-CD80 or CD86 transfectants as utilized in chapters 3 and 4 of this thesis) to drive activation of purified T cell populations derived from patients. The main

drawback to this approach in this instance was the instability of CD80 or CD86 expression on an experiment-to-experiment basis. The generation of more stably transfected cell lines may therefore be a useful future generation in relation to this work. Another approach to this assay would be test CD28-costimulation requirements independently of abatacept-blockade. For example, the ability for purified T cell populations to respond to different relative concentrations of plate-bound anti-CD3 or anti-CD28 could be used as an indicator of the capacity for TCR-driven T cell activation independent of CD28-costimulation thereby implying the propensity for abatacept-resistant proliferation to occur.

Another approach to predicting patient responses to abatacept treatment would be to focus directly upon the T cells that are driving pathology within the synovium within early arthritis patients and to identify markers that are consistent with a CD28-dependent phenotype. The feasibility of this idea is demonstrated by the observation of a "costimulation signature" revealed by transcriptome analysis within T cells that is predictive of an aggressive course of disease in several forms of autoimmunity (McKinney et al., 2015). By determining the stimulation history of T cells in this way in the context of RA, it may provide an indicator as to how much CD28 is actively contributing to pathology and therefore be predictive of likely outcomes of abatacept-treatment. A similar approach has been proposed to predict treatment responses to other biological DMARDs. For example, high soluble ICAM-1 and low serum CXCL13 levels correlates with myeloid cell driven RA and robust responses to TNFα inhibition. In contrast, low soluble ICAM-1 and high

serum CXCL13 represents a more lymphoid pattern of RA and is associated with increased responses to tocilizumab (Dennis et al., 2014). The implementation of a range of these predictive tests for RA prognosis and treatment efficacy, especially in relation to early arthritis patients would represent a step towards more personalized and effective treatment.

Chapter 6: General discussion and future work

6.1 General discussion

T cell activation thresholds are achieved by the integration of signals initiated by TCR ligation and additional costimulatory signals that are predominantly delivered via the CD28:CD80/CD86 system (Viola and Lanzavecchia, 1996). This has been proposed to result in a system whereby strong TCR signaling overcomes a relative absence of costimulation and vice versa (Acuto and Michel, 2003). Nevertheless, CD28-costimulation blockade has been utilized as a strategy for the treatment of T cell mediated pathologies such as RA. However, the impact of TCR stimulation has not been widely considered in relation to this.

In this study, factors affecting the efficacy of CD28-costimulation blockade by abatacept during *in vitro* T cell stimulation have been investigated. Overall, the data suggest that the intensity of TCR-stimulation determines costimulation requirements. Indeed, the outcome of clinical trials investigating the efficacy of abatacept in the treatment of RA frequently suggest a substantial proportion of patients with either negligible or limited clinical responses (Moreland et al., 2002; Kremer et al., 2003; Genovese et al., 2005; Kremer et al., 2006b; Schiff et al., 2008). As such, it may be suggested that in some cases, that the efficacy of abatacept in the treatment of RA would be determined in part by the strength of the TCR stimulus that drives the pathological T cell response.

Several factors determine the potency of stimulation that is generated following TCR ligation. A combination of these factors may contribute towards the generation of TCR signals that are potent enough to mediate CD28-independent (and therefore abatacept-resistant) T cell proliferation. In addition to more intuitive parameters of TCR stimulus strength such as antigen affinity or concentration, evidence from this study suggest that relative APC numbers influence the strength of TCR stimulation. The result of this was that high DC numbers relative to responder T cells promoted abatacept-resistant T cell proliferation during *in vitro* T cell stimulation. This finding is in contrast to the idea that T cell priming occurs as a product of a single productive APC:T cell interaction and suggests that T cells integrate activation signals from multiple DC interactions. In many ways the translation of this observation to *in vivo* T cell stimulation is challenging. However, it is known that DC numbers are increased during chronic inflammation (Banchereau and Steinman, 1998), therefore, this is a factor that may contribute towards the outcome of abatacept treatment.

A more intuitive factor that determines TCR–stimulus strength is antigen affinity. It could be suggested that the majority of autoreactive TCRs within the periphery display relatively low affinity towards autoantigen due to the impact of negative selection upon the TCR repertoire. This would suggest a scenario whereby autoimmune T cell responses are largely CD28-dependent in association with suboptimal TCR stimulation. As such, autoimmune responses could conceivably be biased towards abatacept-sensitivity. However, it is thought that a substantial proportion of autoreactive T cells

escape negative selection and enter the periphery (Bouneaud et al., 2000). Furthermore, the greatest potential for involvement in autoimmune responses is among TCRs that are close to the affinity threshold for negative selection which represent those T cell clones that are most likely to escape deletion and enter the periphery (Koehli et al., 2014). These observations would suggest the potential for autoreactive T cells to arise within the periphery that display sufficient affinity for autoantigen that facilitates abatacept resistant T cell activation.

Understanding the affinity of TCR:antigen interactions that underlie T cell activation is more complex than was implied a historical dogma asserting that an individual TCR was strictly specific to a single antigen. For instance, the analysis of the memory T cell compartment of unexposed adults demonstrates the presence of virus specific T cells that have undergone the naïve to memory transition (Su et al., 2013). This arises due to TCR cross-reactivity between environmental and viral antigens. In relation to this, it is clear that specific T cell responses towards a specific peptide are significantly polyclonal in nature due to TCR binding degeneracy (Sewell, 2012). This cross-reactivity is essential for comprehensive immune coverage since the size of the TCR repertoire is significantly outnumbered by the amount of potential foreign antigens (Mason, 1998; Sewell, 2012). As such, TCR binding degeneracy exists to the extent that a single CD8$^+$ T cell clone responds to >1 million distinct peptides although many of these interactions are characterized by a relatively low affinity (Wooldridge et al., 2012). The structural homology between various peptides that interact with a cross-reactive TCR is variable

(Wilson et al., 2004). At one extreme, peptides can show no obvious homology whereby TCRs interact with structurally unrelated peptides in a promiscuous manner. In other cases there is considerable structural correspondence between peptides that and interact with a TCR due to molecular mimicry. Alternatively, structural similarity can be observed at specific residues within an epitope that form points of contact with the TCR. This was found to underlie low affinity interactions between autoreactive TCRs and microbial peptides which contribute towards pathogenesis within a humanized mouse model of multiple sclerosis (Harkiolaki et al., 2009).

TCR cross-reactivity creates a basis whereby many T cell clones respond to a specific antigen but that the TCR binding affinity differs between individual T cells that contribute to the response. This opens the possibility for differential contributions of TCR- and CD28-signals towards the activation of various T cell clones that contribute towards a particular response. Consistent with this idea, results in this thesis suggested that during *in vitro* T cell responses to TSST-1, that low affinity $v\beta2^-$ T cells respond in an abatacept-sensitive manner. In contrast, the proliferation of high affinity $v\beta2^+$ T cells was more resistant to abatacept. These observations suggest that in the context of autoimmunity that abatacept may serve to tune an immune response by inhibiting T cells that display weaker affinities for autoantigen whilst those that display higher affinities are more likely to be abatacept-resistant.

It could be suggested that those T cells that are activated by a predominantly TCR-driven response are those T cells that represent the most potent drivers

of pathology. For example, TCR-driven stimulation has been found to bias T cell differentiation towards T_h1 (Tao et al., 1997a) or T_h17 (Bouguermouh et al., 2009) differentiation. The activities of these proinflammatory T cell subsets are frequently associated with chronic inflammatory disorders and autoimmune disease (Damsker et al., 2010). In contrast, a predominant contribution of CD28-costimulation towards T cell priming has been thought to skew T cell differentiation of T_h2 responses (Tao et al., 1997a). Based upon this, it may be speculated that the identification of T_h2 driven responses may be more sensitive to abatacept blockade. In relation to this, a transient T_h2 polarized response is observed in early arthritis patients (Raza et al., 2005), this may highlight the necessity to utilize abatacept treatment at earlier stages of RA. Another hypothesis based upon these ideas would be that RA patient who are positive for autoantibodies, might demonstrate more substantial T_h2 responses and have the potential to respond more robustly to abatacept treatment. In support of this idea, it has been suggested that greater clinical responses to abatacept-treatment are observed in patients who display ACPA-seropositivity (Gottenberg et al., 2012; Pieper et al., 2013). However, this does not appear to be the case in terms of rheumatoid factor (RF)-seropositivity which predicts better responses for rituximab (anti-CD20) but not abatacept (Maneiro et al., 2013).

An additional factor that can influence TCR signal strength and potentially therefore the outcome of CD28-blockade is individual genetic polymorphisms associated with TCR signaling. Several studies have identified significant inter-individual variations between various parameters of immune responses

(Ye et al., 2014; Raj et al., 2014; Lee et al., 2014). This suggests the capacity for differences between T cell responses of individuals and therefore an innate propensity towards abatacept resistance or sensitivity. Interestingly, an increased responsiveness to TCR-stimulation has been shown among RA patients relative to healthy controls (Singh et al., 2009). This occurred in association with enhanced ERK activation among RA patients that acts to prevent negative regulation of Lck by the tyrosine phosphatase SHP-1 (Stefanová et al., 2002). It is possible that this elevated TCR-signaling may be associated with an innate tendency towards CD28-independent T cell activation in RA and therefore abatacept-resistance.

In addition to the impact of genetic polymorphisms upon TCR-driven activation in the periphery, it is also important to consider the impact of changes in TCR-signaling thresholds caused by genetic polymorphisms upon thymic T cell selection. For example, the tyrosine phosphatase PTPN22 negatively regulates signals delivered via the TCR (Wu et al., 2006). The 620W PTPN22 allelic variant has been implicated as a risk factor in several autoimmune diseases including RA (Gregersen et al., 2006). Given this association with autoimmunity it would be expected that the 620W allelic variant would be associated with a loss of function resulting in elevated TCR stimulation. However, it has been suggested that the allelic variant is associated with gain-of-function that promotes T cell hypo-responsiveness to TCR stimulation. As such, it has been postulated that the 620W PTPN22 variant may drive autoimmunity by altering T cells thresholds during central tolerance (Gregersen et al., 2006). Similarly, a mutation within the ZAP-70

gene, another TCR-signaling mediator, promotes autoimmune arthritis in mice occurring due to defects in negative selection leading to increased prevalence of autoreactive T cells within the periphery (Sakaguchi et al., 2003). These studies highlight significant complexity in understanding inter-individual differences between responses to TCR-stimulation due to the interaction between numerous genetic factors.

An additional factor that is involved in the generation of TCR signals is the nature of interactions between APCs and T cells. It is interesting to note that several adhesion molecules have been found to display costimulatory function. For example, it is suggested that costimulation is delivered via T cell surface LFA-1 (Dubey et al., 1995) and ICAM-1 (Chirathaworn et al., 2002). Similarly, ICAM-3 expressed at the T cell surface has been suggested to act as a costimulatory molecule (Hernandez-Caselles et al., 1993; Berney et al., 1999; Martinez, 2005). The generation of "outside-in" signaling pathways delivered via LFA-1 that lead to IL-2 production has been partially elucidated, this is suggestive of some degree of costimulatory function (Ni et al., 1999; Wang et al., 2009). However, in other cases adhesion molecules may simply act as accessory molecules that facilitate the acquisition or duration of TCR signals (Bachmann et al., 1997; Graf et al., 2007; Balkow et al., 2010). It is possible that the capacity for a costimulatory pathway to drive T cell activation in the absence of TCR-stimulation may serve as a point of differentiation between molecules that fulfill a true costimulatory vs. an accessory function. In relation to this, stimulation by cross-linked CD28 in the absence of TCR-

stimulation can promote T cell proliferation due to the generation of a strong (and likely supra-physiological) stimulus that is reminiscent of the activity of CD28 superagonists (Hünig, 2012).

Where a robust TCR signal is delivered, abatacept fails to completely suppress T cell activation and may in fact heighten inflammatory processes by favoring T_h17 differentiation (Bouguermouh et al., 2009; Riella et al., 2012a). It may therefore be concluded that abatacept treatment would be most effective for patients in whom pathology is associated with weak TCR-stimulation. To enhance clinical responses to abatacept in those in whom pathology is associated with a strong TCR-stimulus, the combination of abatacept with other immunomodulatory therapies that target TCR signaling may prove beneficial. For instance, in this study, an interaction between CsA and abatacept led to robust suppression of T cell proliferation. This finding has also been suggested by several other studies (Bolling et al., 1994; Van Gool et al., 1994; Perico et al., 1995; Ossevoort et al., 1999). Unsurprisingly, the combination of CsA with abatacept was less beneficial under stimulation conditions where CD28-requirements were lower. For example, abatacept and CsA alone robustly inhibited the proliferation of T cells when stimulation was mediated at a low DC:T cell ratio, therefore, the combination of both agents under these conditions was unnecessary for potent suppression. In contrast, at a high DC:T cell ratio, CsA inhibited CD28-independent T cell proliferation that was driven by an increased potency of anti-CD3 stimulation. Therefore, an ability to identify instances where strong TCR stimulation is present may allow the use of CsA to be appropriately targeted. This is an

important consideration because a significant drawback to the combination of CsA with abatacept for the treatment of RA is the inherent toxicity that is associated with calcineurin-inhibition (van den Borne et al., 1999; Gremese and Ferraccioli, 2004; Liu et al., 2007). It is possible that an increased efficacy of treatment associated with the combination of CsA in combination with abatacept may allow CsA treatment at reduced doses compared to with CsA monotherapy. Nevertheless, this does not however circumvent the need to understand the antigen specificity of T cells underlying RA. Additionally, the identification of novel strategies to inhibit TCR-driven activation could serve to enhance the efficacy of abatacept-treatment.

An important consideration in relation to the safety profile that is associated with abatacept treatment. A direct comparison of the efficacy of abatacept against infliximab therapy for RA in the ATTEST trial demonstrated a reduced incidence of serious infections associated with abatacept treatment relative to infliximab (Schiff et al., 2008). It is possible to conceive a scenario where high intensity TCR-driven T cell activation occurring in conjunction with infection drives protective T cell responses, even in the presence of CD28-blockade by abatacept. As such, the incidence of severe infections in association with simultaneous blockade of TCR and CD28 signals deserves further consideration.

The redistribution between TCR and CD28 signals that results from abatacept treatment and the altered T cell differentiation that occurs as a product of this also deserves further consideration, in particular, the apparent trade off

between effects upon T_{reg} populations and effector T cells. For example, it has been suggested that abatacept therapy in the treatment of RA leads to a decrease in regulatory T cell subsets (Pieper et al., 2013). Similarly, CTLA-4-ig has a deleterious effect upon $CD4^+CD25^+$ T_{reg} in mouse models (Salomon et al., 2000; Tang et al., 2003). Interestingly, in an MHC II-mismatched cardiac transplantation model, CTLA-4-ig promoted allograft rejection in a manner that was associated with a loss of nT_{reg} and subsequent increased T_{eff}: T_{reg} ratio. In contrast, the dominant impact of CTLA-4-ig was the suppression of T_{eff} responses in a fully allogeneic cardiac transplant model in which CTLA-4-ig significantly enhanced allograft survival (Riella et al., 2012a). Thus, the outcome of any negative impact upon T cell regulation following CD28-blockade depends upon the relative contribution of T_{reg} to disease processes.

Any impact upon T cell regulation represents a potential unwanted effect associated with CD28-blockade but would be predicted given the role of CD28 in T_{reg} biology. For example, the generation, function and maintenance of nT_{reg} in the periphery appear to be highly CD28-dependent processes (Gogishvili et al., 2013; Zhang et al., 2013). The impact of CD28-signals upon iT_{reg} is less clear. For example, strong CD28-costimulation is actually found to supress iT_{reg} induction *in vitro* (Semple et al., 2011). In other cases, it is reported that the generation of iT_{reg} is dependent upon CD28-signals (Guo et al., 2008), although this may depend upon the context of accompanying TCR stimulation intensity (Gabryšová et al., 2011). As such, optimal conditions for iT_{reg} induction may be tied to the overall strength of stimulation. The role for

CD28 in T_{reg} biology is likely to be associated with the upregulation of IL-2 signaling since IL-2 is essential for T_{reg} maintenance and function (Nelson, 2004; DeLaRosa et al., 2004; Maloy and Powrie, 2005). Therefore, IL-2 blockade in therapy is also associated with a defect in T cell regulation (Pilat and Wekerle, 2012). CsA is also known to induce and propagate autoimmunity under certain conditions (Prud'homme et al., 1991). Again, the immunological basis underlying this is thought to be associated with effects upon T_{reg} arising from the inhibition of IL-2 production (Mantel et al., 2006; Malek, 2008; Presser et al., 2009).

Several experiments in this study show a loss of markers of T cell regulation such as FoxP3 and CTLA-4 by proliferating T cells following abatacept treatment. Furthermore, abatacept inhibited the upregulation of FoxP3 and CTLA-4 occurring in the presence of $1,25(OH)_2D_3$ supplementation suggesting that $1,25(OH)_2D_3$ acts in association with CD28 to promote a regulatory phenotype. However, it could be predicted that any loss of regulation would be offset by the extent to which abatacept, or indeed, the combination of $1,25(OH)_2D_3$ and abatacept, supress T cell responses.

It is interesting that despite a proposed negative impact of CD28-costimulation blockade upon T cell regulation, reports do not suggest instances of RA severity increasing following abatacept-treatment. Nevertheless, the induction of other autoimmune diseases following abatacept treatment in RA has been reported (Buch et al., 2008). However, the factors underlying this have not been widely considered. It has been suggested that a detrimental impact upon

T_{reg} underlies higher acute rejection rates associated with belatacept-based immunosuppression compared to CsA (Riella and Sayegh, 2013). However, in opposition to this view, a study evaluating the impact of belatacept upon T_{reg} following renal transplantation actually found an increased prevalence of $CD3^{+}FoxP3^{+}$ relative $CD3^{+}FoxP3^{-}$ following belatacept treatment compared to CsA within graft biopsies during acute rejection belatacept therapy (Bluestone et al., 2008). This suggests that the trend towards acute rejection associated with belatacept therapy is not associated with deleterious effects upon T_{reg}. In the case of RA, it is possible that any deleterious impact upon T_{reg} is inconsequential due to the fact that T_{reg} in RA display defective function (Byng-Maddick and Ehrenstein, 2015).

Interestingly, belatacept dosing has been found to confer only 80% saturation of CD86 expression levels at the time of basal levels (Bluestone et al., 2008). Indeed, it may be speculated that the affinity of CTLA-4 has specifically evolved in order to allow CD28 access to CD86 even in the presence of CTLA-4 expressed constitutively by T_{reg}. Therefore, abatacept which confers less potent blockade of CD80 and CD86, may inhibit T cell responses whilst sparing T_{reg} and thereby represent a balanced blocker of costimulation (Gardner et al., 2014).

6.2 Future directions

A consistent theme throughout this work has been that strong TCR stimulation can overcome CD28-requirements thereby promoting abatacept-resistant T

cell proliferation. A useful future direction in relation to this would be to learn how to discriminate between predominantly TCR- vs. CD28-driven T cell responses. This may facilitate the identification of biomarkers upon which to predict responses to abatacept-treatment. In relation to this, T cell stimulation by cross-linked anti-CD3 or anti-CD28 independently generates T cell responses and is associated with distinct expression profiles of surface activation markers. An interesting future study may be to determine the expression of these markers by activated T cells isolated from sites of inflammation and to utilize these differences to determine the relative contribution made by TCR vs. CD28 towards activation. This information could be utilized in order to predict clinical responses to abatacept downstream.

Another future direction may be to classify patient clinical responses to abatacept according to genetic risk factors that determine the strength of the TCR signal, for example, to determine PTPN22 status in the context of the outcome of abatacept treatment. In relation to this, it may also be relevant to determine the impact of CTLA-4 polymorphisms upon abatacept treatment. This is because CTLA-4 mediated transendocytosis of CD80 and CD86 serves to inhibit CD28-ligation (Qureshi et al., 2011) and therefore acts in a manner that is comparable to the mechanism of action of abatacept. As such, a defect in CTLA-4 function may imply that disease is CD28-driven and therefore inherently abatacept-sensitive.

Data presented in this study support a novel function for the active form of vitamin D3, $1,25(OH)_2D_3$, as an inhibitor of TCR-driven T cell proliferation. Thus, in cases where abatacept-resistant T cell activation occurs due to strong TCR stimulus strength, vitamin D3 supplementation can enhance the efficacy of treatment in a similar manner to CsA. Identifying the outcomes of abatacept treatment retrospectively from previous clinical trials relative to vitamin D3 status (where this information is available) could further substantiate these data from *in vitro* assays.

Further studies are required to determine how the observations of a negative effect of CD28-blockade upon T cell regulation during *in vitro* studies and mouse models translate to clinical outcomes of abatacept and belatacept therapy. An interesting future direction may be to compare the impact of abatacept to belatacept upon T_{reg} populations.

6.3 Final summary

The efficacy of abatacept in the inhibition of *in vitro* T cell stimulation is strongly associated with the potency of TCR-stimulation. As such, T cell stimulation by low anti-CD3 or TSST-1 concentrations is abatacept sensitive. Results also suggested that relative DC numbers affected TCR-stimulus strength such that stimulation at high DC:T cell ratios facilitated CD28-independent T cell proliferation. This has implications for the use of abatacept in an inflammatory environment in which DC maturation and numbers in inflamed lymph nodes are increased. Together, these data suggest that

suboptimal RA patient responses to abatacept could be partly determined by various parameters that determine the strength of the TCR stimulus and highlight the importance of inhibiting TCR- and CD28-driven activation signals simultaneously. One way to achieve this is through combining abatacept with the calcineurin inhibitor CsA. Alternatively, data presented here suggests that the active vitamin D3 metabolite, $1,25(OH)_2D_3$ specifically inhibits TCR-driven activation in the absence of CD28-costimulation. In addition to this effect, $1,25(OH)_2D_3$ enhances CD28-expression and therefore may facilitate costimulation during the earlier phases of T cell stimulation. The result of these effects is the induction of a CD28-dependent T cell response that is characterised by the expression of a CD28-driven T cell phenotype including high CTLA-4 expression. By increasing CD28 requirements, vitamin D3 supplementation may serve to enhance clinical responses to abatacept in the treatment of T cell mediated inflammatory diseases.

Appendix

Table 7.1. Clinical data for early arthritis patients. (Abbreviations: 68-tender joint count (TJC-68), 66-swollen joint count (SJC-66), 28-tender joint count (TJC-28), 28-swollen joint count (SJC-28), C-Reactive Protein (CRP), Erythrocyte sedimentation rate (ESR), Disease activity score 28 (DAS28), rheumatoid factor (RF), Cyclic Citrullinated Peptide Antibody (CCP).

Sample number	Sample Date	Final diagnosis	Gender	Age	Symptom Duration (wks)	Smoking status	TJC 68	SJC 66	TJC 28	SJC28	CRP (mg/L)	ESR (mm/hr)	DAS28 (ESR)	RF (U/mL)	CCP (U/mL)	25(OH)D3 (ng/mL)	Patient Global
SW EAC 0083	17/03/14	Inflammatory Arthralgia	Female	54	114	Ever	3	0	1	0	8	no data	3.14	<11.0	1.3	39.6	80
SW EAC 0084	17/03/14	RA Persistant	Female	49	12	Never	0	4	0	4	1	8	2.67	92.2	114	29	47
SW EAC 0085	07/04/14	RA Persistant	Female	25	207	Never	16	4	6	4	24	30	5.21	<11.0	4.6	77	64
SW EAC 0087	07/04/14	RA Persistant	Female	27	11	Ever	10	5	7	5	6	8	4.28	33.2	8	no data	51
SW EAC 0089	28/04/14	RA Persistant	Male	69	14	Ever	17	7	14	5	8	22	5.17	121	290	21.9	20
SW EAC 0090	12/05/14	Persistent Psoriatic Arthritis	Male	61	38	Ever	10	9	6	6	22	32	5.1	<11.0	0.6	36.9	44
SW EAC 0093	09/06/14	RA Persistant	Female	62	290	Never	18	3	12	3	3	27	5.91	161	33	47.2	84
SW EAC 0094	16/06/14	Inflammatory Arthralgia	Female	39	21	Never	9	0	9	0	1	19	4.69	<11.0	1.3	149.1	68
SW EAC 0100	30/06/14	Inflammatory Arthralgia	Female	77	159	Ever	6	0	6	0	6	73	4.84	192	105	34.3	33
SW EAC 0104	14/07/14	RA Persistant	Female	39	25	Ever	4	2	2	2	2	21	3.95	<11.0	10	36	45
SW EAC 0105	14/07/14	RA Persistent	Female	45	25	Never	1	2	1	0	3	38	3.55	14.5	298	7.5	32
SW EAC 0035	21/07/14	Palindromic Rheumatism	Male	53	53	Current	0	0	0	0	1	2	0.81	no data	no data	no data	23
SW EAC 0106	21/07/14	Unclassified Inflammatory Arthritis Persistent	Female	45	11	Never	2	2	1	1	4	8	3.01	<11.0	0.7	63.5	51
SW EAC 0107	21/07/14	Unclassified Inflammatory Arthritis Persistent	Male	49	58	Current	6	2	6	2	2	5	3.16	<11.0	0.5	47	19
SW EAC 0111	22/09/14	RA Persistant	Female	56	37	Ever	5	6	1	5	40	40	4.79	no data	0.5	56.2	73
SW EAC 0113	06/10/14	Persistent Psoriatic Arthritis	Male	61	22	Never	6	6	5	5	14	16	4.69	<11.0	0.6	48	62
SW EAC 0114	06/10/14	Resolving inlammatory arthralgia	Female	47	139	Ever	0	0	0	0	23	32	2.73	<11.0	1	79.1	22
SW EAC 0116	27/10/14	Resolving Sarcoid Arthritis	Male	45	42	Never	2	1	0	1	4	19	2.49	105	1	88.9	11
SW EAC 0119	03/11/14	Resolving unclassified inflammatory arthritis	Female	78	17	Current	8	6	8	6	8	24	5.05	<11.0	<0.1	76.6	40
SW EAC 0120	03/11/14	Persistent Psoriatic Arthritis	Male	27	261	Ever	6	1	2	0	20	8	2.64	<11.0	0.5	70.1	28
SW EAC 0127	08/12/14	Persistent unclassified inflammatory arthritis	Male	63	4	Ever	2	1	2	1	26	52	5.01	38.4	1.3	10.5	84
SW EAC 0128	08/12/14	Persistent unclassified inflammatory arthritis	Male	78	5	Ever	6	0	6	0	19	33	4.23	710	1.2	32.2	4
SW EAC 0129	15/12/14	Persistent Psoriatic Arthritis	Male	50	19	Never	7	6	6	6	6	41	5.31	<11.0	no data	33	47

Publications

The following paper is derived from elements of this work:

Gardner, D.H., Jeffery, L.E., Soskic, B., Briggs, Z., Hou, T-Z., Raza, K. & Sansom, D.M. (2015) $1,25(OH)_2D_3$ promotes the efficacy of CD28-costimulation blockade by abatacept. *The Journal of Immunology.* 195: 2657-2665.

References

Acuto, O. and Michel, F. (2003) CD28-mediated co-stimulation: a quantitative support for TCR signalling. **Nature Reviews Immunology**, 3 (12): 939–951

Aggarwal, S., Ghilardi, N., Xie, M.-H., et al. (2003) Interleukin-23 Promotes a Distinct CD4 T Cell Activation State Characterized by the Production of Interleukin-17. **Journal of Biological Chemistry**, 278 (3): 1910–1914

Ahmed, S., Ihara, K., Kanemitsu, S., et al. (2001) Association of CTLA-4 but not CD28 gene polymorphisms with systemic lupus erythematosus in the Japanese population. **Rheumatology**, 40 (6): 662–667

Akiba, H., Oshima, H., Takeda, K., et al. (1999) CD28-independent costimulation of T cells by OX40 ligand and CD70 on activated B cells. **Journal of Immunology**, 162 (12): 7058–7066

Allen, M.E., Young, S.P., Michell, R.H., et al. (1995) Altered T lymphocyte signaling in rheumatoid arthritis. **Eur. J. Immunol.**, 25 (6): 1547–1554

Alroy, I., Towers, T.L. and Freedman, L.P. (1995) Transcriptional repression of the interleukin-2 gene by vitamin D3: direct inhibition of NFATp/AP-1 complex formation by a nuclear hormone receptor. **Molecular and Cellular Biology**, 15 (10): 5789–5799

Altan-Bonnet, G. and Germain, R.N. (2005) Modeling T cell Antigen Discrimination Based on Feedback Control of Digital ERK Responses. **PLoS Biology**, 3 (11): e356

Anderson, M.S. and Su, M.A. (2011) Aire and T cell development. **Current opinion in immunology**

Appleman, L.J., Berezovskaya, A., Grass, I., et al. (2000) CD28 costimulation mediates T cell expansion via IL-2-independent and IL-2-dependent regulation of cell cycle progression. **Journal of Immunology**, 164 (1): 144–151

Appleman, L.J., van Puijenbroek, A.A.F.L., Shu, K.M., et al. (2002) CD28 costimulation mediates down-regulation of p27kip1 and cell cycle progression by activation of the PI3K/PKB signaling pathway in primary human T cells. **Journal of Immunology**, 168 (6): 2729–2736

Arendt, C.W., Albrecht, B., Soos, T.J., et al. (2002) Protein kinase C-θ: signaling from the center of the T-cell synapse. **Current opinion in immunology**, 14 (3): 323–330

Attia, P., Phan, G.Q., Maker, A.V., et al. (2005) Autoimmunity correlates with tumor regression in patients with metastatic melanoma treated with anti-cytotoxic T-lymphocyte antigen-4. **Journal of Clinical Oncology**, 23 (25): 6043–6053

Attridge, K., Wang, C.J., Wardzinski, L., et al. (2012) IL-21 inhibits T cell IL-2 production and impairs Treg homeostasis. **Blood**, 119 (20): 4656–4664

Azuma, M., Ito, D., Yagita, H., et al. (1993) B70 antigen is a second ligand for CTLA-4 and CD28. **Nature**, 366 (6450): 76–79

Bachmann, M.F., Köhler, G., Ecabert, B., et al. (1999) Cutting Edge: Lymphoproliferative Disease in the Absence of CTLA-4 Is Not T Cell Autonomous. **Journal of Immunology**, 163 (3): 1128–1131

Bachmann, M.F., McKall-Faienza, K., Schmits, R., et al. (1997) Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. **Immunity**, 7 (4): 549–557

Bachmann, M.F., Sebzda, E., Kündig, T.M., et al. (1996) T cell responses are governed by avidity and co-stimulatory thresholds. **Eur. J. Immunol.**, 26 (9): 2017–2022

Badell, I.R., Russell, M.C., Cardona, K., et al. (2012) CTLA4Ig Prevents Alloantibody Formation Following Nonhuman Primate Islet Transplantation Using the CD40-Specific Antibody 3A8. **American Journal of Transplantation**, 12 (7): 1918–1923

Baine, I., Basu, S., Ames, R., et al. (2013) Helios induces epigenetic silencing of IL2 gene expression in regulatory T cells. **Journal of Immunology**, 190 (3): 1008–1016

Balagopalan, L., Coussens, N.P., Sherman, E., et al. (2010) The LAT story: A Tale of Cooperativity, Coordination, and Choreography. **Cold Spring Harbor Perspectives in Biology**, 2 (8): a005512

Balkow, S., Heinz, S., Schmidbauer, P., et al. (2010) LFA-1 activity state on dendritic cells regulates contact duration with T cells and promotes T-cell priming. **Blood**, 116 (11): 1885–1894

Ban, Y., Davies, T.F., Greenberg, D.A., et al. (2003) Analysis of the CTLA-4, CD28, and inducible costimulator (ICOS) genes in autoimmune thyroid disease. **Genes and immunity**, 4 (8): 586–593

Banchereau, J. and Steinman, R.M. (1998) Dendritic cells and the control of immunity. **Nature**, 392 (6673): 245–252

Barnes, M.J., Griseri, T., Johnson, A.M.F., et al. (2013) CTLA-4 promotes Foxp3 induction and regulatory T cell accumulation in the intestinal lamina propria. **Mucosal immunology**, 6 (2): 324–334

Bayley, R., Kite, K.A., McGettrick, H.M., et al. (2015) The autoimmune-associated genetic variant PTPN22 R620W enhances neutrophil activation and function in patients with rheumatoid arthritis and healthy individuals. **Annals of the Rheumatic Diseases**, 74 (8): 1588–1595

Bennett, C.L., Christie, J., Ramsdell, F., et al. (2001) The immune

dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. **Nature Genetics**, 27 (1): 20–21

Benoist, C. and Mathis, D. (2012) Treg cells, life history, and diversity. **Cold Spring Harbor Perspectives in Biology**, 4 (9): a007021

Berney, S.M., Schaan, T., Alexander, J.S., et al. (1999) ICAM-3 (CD50) cross-linking augments signaling in CD3-activated peripheral human T lymphocytes. **Journal of Leukocyte Biology**, 65 (6): 867–874

Bettelli, E., Carrier, Y., Gao, W., et al. (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. **Nature Immunology**, 441 (7090): 235–238

Beyersdorf, N., Ding, X., Hünig, T., et al. (2009) Superagonistic CD28 stimulation of allogeneic T cells protects from acute graft-versus-host disease. **Blood**, 114 (20): 4575–4582

Beyersdorf, N., Gaupp, S., Balbach, K., et al. (2005a) Selective targeting of regulatory T cells with CD28 superagonists allows effective therapy of experimental autoimmune encephalomyelitis. **The Journal of experimental medicine**, 202 (3): 445–455

Beyersdorf, N., Hanke, T., Kerkau, T., et al. (2005b) Superagonistic anti-CD28 antibodies: potent activators of regulatory T cells for the therapy of autoimmune diseases. **Ann. Rheum. Dis.**, 64(Suppl 4): iv91–95

Bhandoola, A., Sambandam, A., Allman, D., et al. (2003) Early T Lineage Progenitors: New Insights, but Old Questions Remain. **Journal of Immunology**, 171 (11): 5653–5658

Bluestone, J.A., Liu, W., Yabu, J.M., et al. (2008) The effect of costimulatory and interleukin 2 receptor blockade on regulatory T cells in renal transplantation. **American Journal of Transplantation**, 8 (10): 2086–2096

Boise, L.H. and Thompson, C.B. (1996) Hierarchical control of lymphocyte survival. **Science**, 274 (5284): 67–68

Boissonnas, A. and Combadiere, B. (2004) Interplay between cell division and cell death during TCR triggering. **Eur. J. Immunol.**, 34 (9): 2430–2438

Bolling, S.F., Lin, H., Wei, R.-Q., et al. (1994) The Effect of Combination Cyclosporine and CTLA4-Ig Therapy on Cardiac Allograft Survival. **Journal of Surgical Research**, 57 (1): 60–64

Bonelli, M., Ferner, E., Göschl, L., et al. (2012) Abatacept (CTLA-4Ig) treatment reduces the migratory capacity of monocytes in patients with rheumatoid arthritis (RA). **Arthritis & Rheumatism**, 65: 599–607

Bonnevier, J.L. and Mueller, D.L. (2002) Cutting edge: B7/CD28 interactions regulate cell cycle progression independent of the strength of TCR signaling. **Journal of Immunology**, 169 (12): 6659–6663

Boomer, J.S. and Green, J.M. (2010) An Enigmatic Tail of CD28 Signaling. **Cold Spring Harbor Perspectives in Biology**, 2 (8): a002436

Boonen, G.J., van Dijk, A.M., Verdonck, L.F., et al. (1999) CD28 induces cell cycle progression by IL-2-independent down-regulation of p27kip1 expression in human peripheral T lymphocytes. **Eur. J. Immunol.**, 29 (3): 789–798

Boonstra, A., Barrat, F.J., Crain, C., et al. (2001) 1α,25-dihydroxyvitamin D3 has a direct effect on naive CD4$^+$ T cells to enhance the development of Th2 cells. **Journal of Immunology**, 167 (9): 4974–4980

Borriello, F., Sethna, M.P., Boyd, S.D., et al. (1997) B7-1 and B7-2 Have Overlapping, Critical Roles in Immunoglobulin Class Switching and Germinal Center Formation. **Immunity**, 6 (3): 303–313

Borthwick, N.J., Lowdell, M., Salmon, M., et al. (2000) Loss of CD28 expression on CD8$^+$ T cells is induced by IL-2 receptor gamma chain signalling cytokines and type I IFN, and increases susceptibility to activation-induced apoptosis. **International Immunology**, 12 (7): 1005–1013

Bouguermouh, S., Fortin, G., Baba, N., et al. (2009) CD28 co-stimulation down regulates Th17 development. **PLoS ONE**, 4 (3): e5087

Bouneaud, C., Kourilsky, P. and Bousso, P. (2000) 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**, 13 (6): 829–840

Boyman, O. and Sprent, J. (2012) The role of interleukin-2 during homeostasis and activation of the immune system. **Nature Reviews Immunology**, 12 (3): 180–190

Breitfeld, D., Ohl, L., Kremmer, E., et al. (2000) Follicular B helper T cells Express CXC Chemokine Receptor 5, Localize to B Cell Follicles, and Support Immunoglobulin Production. **The Journal of experimental medicine**, 192 (11): 1545–1552

Brennan, F.M., Hayes, A.L., Ciesielski, C.J., et al. (2002) Evidence That Rheumatoid Arthritis Synovial T cells Are Similar to Cytokine-Activated T Cells: Involvement of Phosphatidylinositol 3-Kinase and Nuclear Factor κB Pathways in Tumor Necrosis Factor α Production in Rheumatoid Arthritis. **Arthritis & Rheumatism**, 46 (1): 31–41

Brennan, F.M., Smith, N.M.G., Owen, S., et al. (2008) Resting CD4$^+$ effector memory T cells are precursors of bystander-activated effectors: a surrogate model of rheumatoid arthritis synovial T-cell function. **Arthritis Research & Therapy**, 10: R36

Bretscher, P. and Cohn, M. (1970) A theory of self-nonself discrimination. **Science**, 169 (3950): 1042–1049

Brown, K.A., Bedford, P., Macey, M., et al. (1997) Human blood dendritic cells: binding to vascular endothelium and expression of adhesion molecules.

Clinical & Experimental Immunology, 107 (3): 601–607

Brown, M., Hu-Li, J. and Paul, W.E. (1988) IL-4/B cell stimulatory factor 1 stimulates T cell growth by an IL-2-independent mechanism. **Journal of Immunology**, 141 (2): 504–511

Bruce, D., Yu, S., Ooi, J.H., et al. (2011) Converging pathways lead to overproduction of IL-17 in the absence of vitamin D signaling. **International Immunology**, 23 (8): 519–528

Brunner, M.C., Chambers, C.A., Chan, F.K., et al. (1999) CTLA-4-Mediated Inhibition of Early Events of T Cell Proliferation. **Journal of Immunology**, 162 (10): 5813–5820

Buch, M.H., Vital, E.M. and Emery, P. (2008) Abatacept in the treatment of rheumatoid arthritis. **Arthritis Research & Therapy**, 10 (Suppl 1)

Buckle, A.M. and Hogg, N. (1990) Human memory T cells express intercellular adhesion molecule-1 which can be increased by interleukin 2 and interferon-γ. **Eur. J. Immunol.**, 20 (2): 337–341

Buhtoiarov, I.N., Lum, H., Berke, G., et al. (2005) CD40 ligation activates murine macrophages via an IFN-γ-dependent mechanism resulting in tumor cell destruction in vitro. **Journal of Immunology**, 174 (10): 6013–6022

Bukczynski, J., Wen, T. and Watts, T.H. (2003) Costimulation of human CD28(-) T cells by 4-1BB ligand. **Eur. J. Immunol.**, 33 (2): 446–454

Butte, M.J., Keir, M.E., Phamduy, T.B., et al. (2007) Programmed death-1 Ligand 1 Interacts Specifically with the B7-1 Costimulatory Molecule to Inhibit T cell Responses. **Immunity**, 27 (1): 111–122

Byng-Maddick, R. and Ehrenstein, M.R. (2015) The impact of biological therapy on regulatory T cells in rheumatoid arthritis. **Rheumatology**, 54 (5): 768–775

Cai, Y.C., Cefai, D., Schneider, H., et al. (1995) Selective CD28pYMNM Mutations Implicate Phosphatidylinositol 3-Kinase in CD86-CD28-Mediated Costimulation. **Immunity**, 3 (4): 417–426

Cantorna, M.T. and Waddell, A. (2014) The vitamin D receptor turns off chronically activated T cells. **Annals of the New York Academy of Sciences**, 1317: 70–75

Carman, J.A., Davis, P.M., Yang, W.-P., et al. (2009) Abatacept Does Not Induce Direct Gene Expression Changes in Antigen-Presenting Cells. **Journal of Clinical Immunology**, 29 (4): 479–489

Carreno, B.M. and Collins, M. (2002) The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. **Immunology**, 20: 29–53

Catrina, A.I., Deane, K.D. and Scher, J.U. (2014) Gene, environment, microbiome and mucosal immune tolerance in rheumatoid arthritis. **Rheumatology**, [Epub ahead of print]

Caudy, A.A., Reddy, S.T., Chatila, T., et al. (2007) CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like syndrome, and defective IL-10 expression from CD4 lymphocytes. **The Journal of allergy and clinical immunology**, 119 (2): 482–487

Caux, C., Massacrier, C., Vanbervliet, B., et al. (1994) Activation of human dendritic cells through CD40 cross-linking. **The Journal of experimental medicine**, 180 (4): 1263–1272

Celli, S., Garcia, Z. and Bousso, P. (2005) CD4 T cells integrate signals delivered during successive DC encounters in vivo. **The Journal of experimental medicine**, 202 (9): 1271–1278

Celli, S., Lemaitre, F. and Bousso, P. (2007) Real-Time Manipulation of T Cell-Dendritic Cell Interactions In Vivo Reveals the Importance of Prolonged Contacts for CD4$^+$ T Cell Activation. **Immunity**, 27 (4): 625–634

Chan, A.C. and Behrens, T.W. (2013) Personalizing medicine for autoimmune and inflammatory diseases. **Nature Immunology**, 14 (2): 106–109

Chandok, M.R., Okoye, F.I., Ndejembi, M.P., et al. (2007) A biochemical signature for rapid recall of memory CD4 T cells. **Journal of Immunology**, 179 (6): 3689–3698

Chang, T.T., Jabs, C., Sobel, R.A., et al. (1999) Studies in B7-deficient mice reveal a critical role for B7 costimulation in both induction and effector phases of experimental autoimmune encephalomyelitis. **The Journal of experimental medicine**, 190 (5): 733–740

Chapoval, A.I., Ni, J., Lau, J.S., et al. (2001) B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. **Nature Immunology**, 2 (3): 269–274

Chen, L. and Flies, D.B. (2013) Molecular mechanisms of T cell co-stimulation and co-inhibition. **Nature Reviews Immunology**, 13 (4): 227–242

Chen, Q., Kim, Y.C., Laurence, A., et al. (2011) IL-2 controls the stability of Foxp3 expression in TGF-beta-induced Foxp3+ T cells in vivo. **Journal of Immunology**, 186 (11): 6329–6337

Chen, W., Jin, W., Hardegen, N., et al. (2003) Conversion of Peripheral CD4$^+$CD25$^-$ Naive T cells to CD4$^+$CD25$^+$ Regulatory T Cells by TGF-β Induction of Transcription Factor Foxp3. **The Journal of experimental medicine**, 198 (12): 1875–1886

Chen, X., Fosco, D., Kline, D.E., et al. (2014) PD-1 regulates extrathymic regulatory T-cell differentiation. **Eur. J. Immunol.**, 44 (9): 2603–2616

Chen, Z., Fei, M., Da Fu, et al. (2013) Association between cytotoxic T lymphocyte antigen-4 polymorphism and type 1 diabetes: A meta-analysis. **Gene**, 516 (2): 263–270

Chikuma, S. and Bluestone, J.A. (2007) Expression of CTLA-4 and FOXP3 in *cis* protects from lethal lymphoproliferative disease. **Eur. J. Immunol.**, 37 (5): 1285–1289

Chirathaworn, C., Kohlmeier, J.E., Tibbetts, S.A., et al. (2002) Stimulation through intercellular adhesion molecule-1 provides a second signal for T cell activation. **Journal of Immunology**, 168 (11): 5530–5537

Chitnis, T., Najafian, N., Abdallah, K.A., et al. (2001) CD28-independent induction of experimental autoimmune encephalomyelitis. **Journal of Clinical Investigation**, 107 (5): 575–583

Choy, E.H., Kavanaugh, A.F. and Jones, S.A. (2013) The problem of choice: current biologic agents and future prospects in RA. **Nature Reviews Rheumatology**, 9 (3): 154–163

Chuang, E., Fisher, T.S., Morgan, R.W., et al. (2000) The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A. **Immunity**, 13 (3): 313–322

Chung, J.B., Wells, A.D., Adler, S., et al. (2003) Incomplete activation of CD4 T cells by antigen-presenting transitional immature B cells: implications for peripheral B and T cell responsiveness. **Journal of Immunology**, 171 (4): 1758–1767

Clark, C.E., Hasan, M. and Bousso, P. (2011) A Role for the Immediate Early Gene Product c-fos in Imprinting T Cells with Short-Term Memory for Signal Summation. **PLoS ONE**, 6 (4): e18916

Colin, E.M., Asmawidjaja, P.S., van Hamburg, J.P., et al. (2010) 1,25-Dihydroxyvitamin D3 modulates Th17 Polarization and Interleukin-22 Expression by Memory T cells From Patients With Early Rheumatoid Arthritis. **Arthritis & Rheumatism**, 62 (1): 132–142

Collette, Y., Razanajaona, D., Ghiotto, M., et al. (1997) CD28 can promote T cell survival through a phosphatidylinositol 3-kinase-independent mechanism. **Eur. J. Immunol.**, 27 (12): 3283–3289

Collins, A.V., Brodie, D.W., Gilbert, R.J.C., et al. (2002) The interaction properties of costimulatory molecules revisited. **Immunity**, 17 (2): 201–210

Constant, S.L. and Bottomly, K. (1997) Induction of Th1 and Th2 CD4^{+} T Cell Responses: The Alternative Approaches. **Immunology**, 15: 297–322

Contento, R.L., Campello, S., Trovato, A.E., et al. (2010) Adhesion shapes T cells for prompt and sustained T-cell receptor signalling. **The EMBO journal**, 29 (23): 4035–4047

Cope, A.P. (2008) T cells in rheumatoid arthritis. **Arthritis Research & Therapy**, 10 (Suppl 1): S1

Cope, A.P., Schulze-Koops, H. and Aringer, M. (2007) The central role of T cells in rheumatoid arthritis. **Clinical and Experimental Rheumatology**, 25 (Suppl. 46): S4–S11

Correale, J., Celica Ysrraelit, M. and Ines Gaitan, M. (2009) Immunomodulatory effects of Vitamin D in multiple sclerosis. **Brain**, 132: 1146–1160

Corse, E. and Allison, J.P. (2012) Cutting Edge: CTLA-4 on Effector T Cells Inhibits In *Trans*. **Journal of Immunology**, 189: 1123–1127

Coudronniere, N., Villalba, M., Englund, N., et al. (2000) NF-κB activation induced by T cell receptor/CD28 costimulation is mediated by protein kinase C-θ. **Proc. Natl. Acad. Sci. U.S.A.**, 97 (7): 3394–3399

Cozzolino, M., Lu, Y., Finch, J., et al. (2001) p21WAF1 and TGF-alpha mediate parathyroid growth arrest by vitamin D and high calcium. **Kidney International**, 60 (6): 2109–2117

Crabtree, G.R. and Clipstone, N.A. (1994) Signal transmission between the plasma membrane and nucleus of T lymphocytes. **Biochemistry**, 63: 1045–1083

Croft, M. (2009) The role of TNF superfamily members in T-cell function and diseases. **Nature Reviews Immunology**, 9 (4): 271–285

Croft, M., Bradley, L.M. and Swain, S.L. (1994) Naive Versus Memory CD4 T Cell Response to Antigen. **Journal of Immunology**, 152: 2575–2685

Crotty, S. (2014) T follicular helper cell differentiation, function, and roles in disease. **Immunity**, 41 (4): 529–542

Curtsinger, J.M. and Mescher, M.F. (2010) Inflammatory cytokines as a third signal for T cell activation. **Current opinion in immunology**, 22 (3): 333–340

D'Cruz, L.M. and Klein, L. (2005) Development and function of agonist-induced $CD25^+Foxp3^+$ regulatory T cells in the absence of interleukin 2 signaling. **Nature Immunology**, 6 (11): 1152–1159

Damsker, J.M., Hansen, A.M. and Caspi, R.R. (2010) Th1 and Th17 cells: adversaries and collaborators. **Annals of the New York Academy of Sciences**, 1183: 211–221

Dardalhon, V., Awasthi, A., Kwon, H., et al. (2008) IL-4 inhibits TGF-β-induced $Foxp3^+$ T cells and, together with TGF-β, generates $IL-9^+$ $IL-10^+$ $Foxp3^-$ effector T cells. **Nature Immunology**, 9 (12): 1347–1355

Das, J., Ho, M., Zikherman, J., et al. (2009) Digital Signaling and Hysteresis Characterize Ras Activation in Lymphoid Cells. **Cell**, 136 (2): 337–351

Davis, P.M., Nadler, S.G., Stetsko, D.K., et al. (2008) Abatacept modulates human dendritic cell-stimulated T-cell proliferation and effector function independent of IDO induction. **Clinical Immunology**, 126 (1): 38–47

Davis, S.J. and van der Merwe, P.A. (1996) The structure and ligand interactions of CD2: implications for T-cell function. **Immunology Today**, 17 (4): 177–187

Davis, S.J. and van der Merwe, P.A. (2006) The kinetic-segregation model: TCR triggering and beyond. **Nature Immunology**, 7 (8): 803–809

DeBenedette, M.A., Shahinian, A., Mak, T.W., et al. (1997) Costimulation of $CD28^-$ T Lymphocytes by 4-1BB Ligand. **Journal of Immunology**, 158 (2): 551–559

DeLaRosa, M., Rutz, S., Dorninger, H., et al. (2004) Interleukin- 2 is essential for $CD4^+$ $CD25^+$ regulatory T cell function. **Eur. J. Immunol.**, 34: 2480–2488

Demotz, S., Grey, H.M. and Sette, A. (1990) The Minimal Number of Class II MHC-Antigen Complexes Needed for T Cell Activation. **Science**, 249 (4972): 1028–1030

denHaan, J.M.M., Arens, R. and van Zelm, M.C. (2014) The activation of the adaptive immune system: cross-talk between antigen-presenting cells, T cells and B cells. **Immunology letters**, 162: 103–112

Dennehy, K.M., Elias, F., Zeder-Lutz, G., et al. (2006) Cutting Edge: Monovalency of CD28 Maintains the Antigen Dependence of T cell Costimulatory Responses. **Journal of Immunology**, 176 (10): 5725–5729

Dennis, G., Holweg, C.T.J., Kummerfeld, S.K., et al. (2014) Synovial phenotypes in rheumatoid arthritis correlate with response to biologic therapeutics. **Arthritis Research & Therapy**, 16 (2): R90

Denoeud, J. and Moser, M. (2011) Role of CD27/CD70 pathway of activation in immunity and tolerance. **Journal of Leukocyte Biology**, 89 (2): 195–203

Diehn, M., Alizadeh, A.A., Rando, O.J., et al. (2002) Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation. **Proc. Natl. Acad. Sci. U.S.A.**, 99 (18): 11796–11801

Dienz, O., Eaton, S.M., Krahl, T.J., et al. (2007) Accumulation of NFAT mediates IL-2 expression in memory, but not naïve, CD4+ T cells. **Proc. Natl. Acad. Sci. U.S.A.**, 104 (17): 7175–7180

Djilali-Saiah, I., Ouellette, P., Caillat-Zucman, S., et al. (2001) CTLA-4/CD28 Region Polymorphisms in Children From Families With Autoimmune Hepatitis. **Human Immunology**, 62 (12): 1356–1362

Dong, C., Juedes, A.E., Temann, U.A., et al. (2001) ICOS co-stimulatory receptor is essential for T-cell activation and function. **Nature**, 409 (6816): 97–101

Dubey, C., Croft, M. and Swain, S.L. (1995) Costimulatory requirements of naive CD4+ T cells. ICAM-1 or B7-1 can costimulate naive CD4 T cell activation but both are required for optimum response. **Journal of Immunology**, 155 (1): 45–57

Dustin, M.L. and Springer, T.A. (1989) T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. **Nature**, 341 (6243): 619–624

Dustin, M.L., Bromley, S.K., Kan, Z.Z., et al. (1997) Antigen receptor engagement delivers a stop signal to migrating T lymphocytes. **Proc Natl Acad Sci**, 94 (8): 3909–3913

Elias, C.G., Spellberg, J.P., Karan-Tamir, B., et al. (1998) Ligation of CD31/PECAM-1 modulates the function of lymphocytes, monocytes and neutrophils. **Eur. J. Immunol.**, 28 (6): 1948–1958

Emery, P., Durez, P., Dougados, M., et al. (2010) 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). **Annals of the Rheumatic Diseases**, 69 (3): 510–516

Evans, E.J., Esnouf, R.M., Manso-Sancho, R., et al. (2005) Crystal structure of a soluble CD28-Fab complex. **Nature Immunology**, 6 (3): 271–279

Fantini, M.C., Becker, C., Monteleone, G., et al. (2004) Cutting edge: TGF-β Induces a Regulatory Phenotype in CD4$^+$CD25$^-$ T Cells through Foxp3 Induction and Down-Regulation of Smad7. **Journal of Immunology**, 172 (9): 5149–5153

Farber, D.L. (2009) Biochemical signaling pathways for memory T cell recall. **Seminars in Immunology**, 21 (2): 84–91

Ferrari-Lacraz, S., Zheng, X.X., Kim, Y.S., et al. (2001) An antagonist IL-15/Fc protein prevents costimulation blockade-resistant rejection. **Journal of Immunology**, 167 (6): 3478–3485

Finck, B.K., Linsley, P.S. and Wofsy, D. (1994) Treatment of murine lupus with CTLA4Ig. **Science**, 265 (5176): 1225–1227

Firestein, G.S., Xu, W.D., Townsend, K., et al. (1988) Cytokines in chronic inflammatory arthritis. I. Failure to detect T cell lymphokines (interleukin 2 and interleukin 3) and presence of macrophage colony-stimulating factor (CSF-1) and a novel mast cell growth factor in rheumatoid synovitis. **The Journal of experimental medicine**, 168 (5): 1573–1586

Fontenot, J.D., Gavin, M.A. and Rudensky, A.Y. (2003) Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. **Nature Immunology**, 4 (4): 330–336

Fontenot, J.D., Rasmussen, J.P., Gavin, M.A., et al. (2005) A function for interleukin 2 in Foxp3-expressing regulatory T cells. **Nature Immunology**, 6

(11): 1142–1151

Francisco, L.M., Sage, P.T. and Sharpe, A.H. (2010) The PD-1 pathway in tolerance and autoimmunity. **Immunological Reviews**, 236 (1): 219–242

Francisco, L.M., Salinas, V.H., Brown, K.E., et al. (2009) PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. **Journal of Experimental Medicine**, 206 (13): 3015–3029

Fraser, J.D., Irving, B.A., Crabtree, G.R., et al. (1991) Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. **Science**, 251 (4991): 313–316

Frauwirth, K.A., Riley, J.L., Harris, M.H., et al. (2002) The CD28 signaling pathway regulates glucose metabolism. **Immunity**, 16 (6): 769–777

Friedl, P. and Gunzer, M. (2001) Interaction of T cells with APCs: the serial encounter model. **Trends in immunology**, 22 (4): 187–191

Furtado, G.C., de Lafaille, M.A.C., Kutchukhidze, N., et al. (2002) Interleukin 2 Signaling Is Required for CD4$^+$ Regulatory T Cell Function. **The Journal of experimental medicine**, 196 (6): 851–857

Gabryšová, L., Christensen, J.R., Wu, X., et al. (2011) Integrated T-cell receptor and costimulatory signals determine TGF-β-dependent differentiation and maintenance of Foxp3$^+$ regulatory T cells. **Eur. J. Immunol.**, 41 (5): 1242–1248

Gaglia, J.L., Greenfield, E.A., Mattoo, A., et al. (2000) Intercellular adhesion molecule 1 is critical for activation of CD28-deficient T cells. **Journal of Immunology**, 165 (11): 6091–6098

Gao, Y., Tang, J., Chen, W., et al. (2015) Inflammation negatively regulates FOXP3 and regulatory T-cell function via DBC1. **PNAS**, 112 (25): E3246–54

Garcia, K.C., Adams, J.J., Feng, D., et al. (2009) The molecular basis of TCR germline bias for MHC is surprisingly simple. **Nature Immunology**, 10 (2): 143–147

Gardner, D., Jeffery, L.E. and Sansom, D.M. (2014) Understanding the CD28/CTLA-4 (CD152) Pathway and Its Implications for Costimulatory Blockade. **American Journal of Transplantation**, 14: 1985–1991

Geginat, J., Clissi, B., Moro, M., et al. (2000) CD28 and LFA-1 contribute to cyclosporin A-resistant T cell growth by stabilizing the IL-2 mRNA through distinct signaling pathways. **Eur. J. Immunol.**, 30 (4): 1136–1144

Genovese, M.C., Becker, J.-C., Schiff, M., et al. (2005) Abatacept for Rheumatoid Arthritis Refractory to Tumor Necrosis Factor α Inhibition. **New England Journal of Medicine**, 353: 1114–1123

Gerlach, K., Hwang, Y., Nikolaev, A., et al. (2014) T$_H$9 cells that express the

transcription factor PU.1 drive T cell-mediated colitis via IL-9 receptor signaling in intestinal epithelial cells. **Nature Immunology**, 15 (7): 676–686

Germain, R.N. (2010) Computational analysis of T cell receptor signaling and ligand discrimination – Past, present, and future. **FEBS Letters**, 584: 4814–4822

Germann, T., Gately, M.K., Schoenhaut, D.S., et al. (1993) Interleukin-12/T cell stimulating factor, a cytokine with multiple effects on T helper type 1 (Th1) but not on Th2 cells. **Eur. J. Immunol.**, 23 (8): 1762–1770

Gett, A.V. and Hodgkin, P.D. (2000) A cellular calculus for signal integration by T cells. **Nature Immunology**, 1 (3): 239–244

Gett, A.V., Sallusto, F., Lanzavecchia, A., et al. (2003) T cell fitness determined by signal strength. **Nature Immunology**, 4 (4): 355–360

Ghasemi, A. and Zahediasl, S. (2012) Normality tests for statistical analysis: a guide for non-statisticians. **International journal of endocrinology and metabolism**, 10 (2): 486–489

Gilson, C.R., Milas, Z., Gangappa, S., et al. (2009) Anti-CD40 Monoclonal Antibody Synergizes with CTLA4-Ig in Promoting Long-Term Graft Survival in Murine Models of Transplantation. **Journal of Immunology**, 183: 1625–1635

Gogishvili, T., Lühder, F., Goebbels, S., et al. (2013) Cell-intrinsic and - extrinsic control of Treg-cell homeostasis and function revealed by induced CD28 deletion. **Eur. J. Immunol.**, 43 (1): 188–193

Goldstein, J.S., Chen, T., Brunswick, M., et al. (1998) Purified MHC class I and peptide complexes activate naive CD8+ T cells independently of the CD28/B7 and LFA-1/ICAM-1 costimulatory interactions. **Journal of Immunology**, 160 (7): 3180–3187

Gollmer, K., Asperti-Boursin, F., Tanaka, Y., et al. (2009) CCL21 mediates CD4^{+} T-cell costimulation via a DOCK2/Rac-dependent pathway. **Blood**, 114 (3): 580–588

Gotot, J., Gottschalk, C., Leopold, S., et al. (2012) Regulatory T cells use programmed death 1 ligands to directly suppress autoreactive B cells in vivo. **Proc. Natl. Acad. Sci. U.S.A.**, 109 (26): 10468–10473

Gottenberg, J.E., Ravaud, P., Cantagrel, A., et al. (2012) Positivity for anti-cyclic citrullinated peptide is associated with a better response to abatacept: data from the "Orencia and Rheumatoid Arthritis" registry. **Annals of the Rheumatic Diseases**, 71 (11): 1815–1819

Gough, S.C.L., Walker, L.S.K. and Sansom, D.M. (2005) CTLA4 gene polymorphism and autoimmunity. **Immunological Reviews**, 204: 102–115

Graf, B., Bushnell, T. and Miller, J. (2007) LFA-1-Mediated T Cell Costimulation through Increased Localization of TCR/Class II Complexes to

the Central Supramolecular Activation Cluster and Exclusion of CD45 from the Immunological Synapse. **Journal of Immunology**, 179 (3): 1616–1624

Green, J.M., Noel, P.J., Sperling, A.I., et al. (1994) Absence of B7-dependent responses in CD28-deficient mice. **Immunity**, 1 (6): 501–508

Greenwald, R.J., Freeman, G.J. and Sharpe, A.H. (2005) The B7 family revisited. **Immunology**, 23: 515–548

Gregersen, P.K., Lee, H.-S., Batliwalla, F., et al. (2006) PTPN22: Setting thresholds for autoimmunity. **Seminars in Immunology**, 18 (4): 214–223

Gremese, E. and Ferraccioli, G.F. (2004) Benefit/risk of cyclosporine in rheumatoid arthritis. **Clinical and Experimental Rheumatology**, 22 (5 Suppl 35): S101–7

Grewal, I.S. and Flavell, R.A. (1996) The Role of CD40 Ligand in Costimulation and T-Cell Activation. **Immunological Reviews**, 153: 85–106

Grohmann, U., Orabona, C., Fallarino, F., et al. (2002) CTLA-4-Ig regulates tryptophan catabolism in vivo. **Nature Immunology**, 3 (11): 1097–1101

Grossman, W.J., Verbsky, J.W., Barchet, W., et al. (2004) Human T regulatory cells can use the perforin pathway to cause autologous target cell death. **Immunity**, 21 (4): 589–601

Grosso, J.F. and Jure-Kunkel, M.N. (2013) CTLA-4 blockade in tumor models: an overview of preclinical and translational research. **Cancer immunity**, 13: 1–14

Guntermann, C. and Alexander, D.R. (2002) CTLA-4 Suppresses Proximal TCR Signaling in Resting Human CD4^{+} T Cells by Inhibiting ZAP-70 Tyr[319] Phosphorylation: A Potential Role for Tyrosine Phosphatases. **Journal of Immunology**, 168 (9): 4420–4429

Gunzer, M., Schäfer, A., Borgmann, S., et al. (2000) Antigen Presentation in Extracellular Matrix: Interactions of T cells with Dendritic Cells are Dynamic, Short Lived, and Sequential. **Immunity**, 13 (3): 323–332

Guo, F., Iclozan, C., Suh, W.-K., et al. (2008) CD28 controls differentiation of regulatory T cells from naive CD4 T cells. **Journal of Immunology**, 181 (4): 2285–2291

Hamann, D., Baars, P.A., Rep, M.H., et al. (1997) Phenotypic and functional separation of memory and effector human CD8+ T cells. **The Journal of experimental medicine**, 186 (9): 1407–1418

Hancock, W.W., Sayegh, M.H., Zheng, X.G., et al. (1996) Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection. **Proc. Natl. Acad. Sci. U.S.A.**, 93 (24): 13967–13972

Haribhai, D., Williams, J.B., Jia, S., et al. (2011) A Requisite Role for Induced Regulatory T Cells in Tolerance Based on Expanding Antigen Receptor Diversity. **Immunity**, 35 (1): 109–122

Harkiolaki, M., Holmes, S.L., Svendsen, P., et al. (2009) T Cell-Mediated Autoimmune Disease Due to Low-Affinity Crossreactivityto Common Microbial Peptides. **Immunity**, 30 (3): 348–357

Hassan, N.J., Barclay, A.N. and Brown, M.H. (2004) Frontline: Optimal T cell activation requires the engagement of CD6 and CD166. **Eur. J. Immunol.**, 34 (4): 930–940

Hegazy, A.N., Peine, M., Helmstetter, C., et al. (2010) Interferons Direct Th2 Cell Reprogramming to Generate a Stable GATA-3$^+$T-bet$^+$ Cell Subset with Combined Th2 and Th1 Cell Functions. **Immunity**, 32 (1): 116–128

Hemmings, B.A. and Restuccia, D.F. (2012) PI3K-PKB/Akt Pathway. **Cold Spring Harbor Perspectives in Biology**, 4 (9): a011189

Hendriks, J., Gravestein, L.A., Tesselaar, K., et al. (2000) CD27 is required for generation and long-term maintenance of T cell immunity. **Nature Immunology**, 1 (5): 433–440

Henrickson, S.E., Mempel, T.R., Mazo, I.B., et al. (2008) T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation. **Nature Immunology**, 9 (3): 282–291

Hernandez-Caselles, T., Rubio, G., Campanero, M.R., et al. (1993) ICAM-3, the third LFA-1 counterreceptor, is a co-stimulatory molecule for both resting and activated T lymphocytes. **Eur. J. Immunol.**, 23 (11): 2799–2806

Hill, J.A., Southwood, S., Sette, A., et al. (2003) Cutting Edge: The Conversion of Arginine to Citrulline Allows for a High-Affinity Peptide Interaction with the Rheumatoid Arthritis-Associated HLA-DRB1*0401 MHC Class II Molecule. **Journal of Immunology**, 171 (2): 538–541

Hintzen, R.Q., Lens, S.M., Lammers, K., et al. (1995) Engagement of CD27 with its ligand CD70 provides a second signal for T cell activation. **Journal of Immunology**, 154 (6): 2612–2623

Hochweller, K., Wabnitz, G.H., Samstag, Y., et al. (2010) Dendritic cells control T cell tonic signaling required for responsiveness to foreign antigen. **PNAS**, 107 (13): 5931–5936

Hogan, P.G., Chen, L., Nardone, J., et al. (2003) Transcriptional regulation by calcium, calcineurin, and NFAT. **Genes & Development**, 17 (18): 2205–2232

Holdorf, A.D., Lee, K.-H., Burack, W.R., et al. (2002) Regulation of Lck activity by CD4 and CD28 in the immunological synapse. **Nature Immunology**, 3 (3): 259–264

Holick, M.F. (2007) Vitamin D Deficiency. **New England Journal of**

Medicine, 357 (3): 266–281

Hou, T.Z., Qureshi, O.S., Wang, C.J., et al. (2015) A Transendocytosis Model of CTLA-4 Function Predicts Its Suppressive Behavior on Regulatory T Cells. **Journal of Immunology**, 194: 2148–2159

Höpken, U.E., Lehmann, I., Droese, J., et al. (2005) The ratio between dendritic cells and T cells determines the outcome of their encounter: proliferation versus deletion. **Eur. J. Immunol.**, 35 (10): 2851–2863

Huang, J., Brameshuber, M., Zeng, X., et al. (2013) A Single Peptide-Major Histocompatibility Complex Ligand Triggers Digital Cytokine Secretion in CD4$^+$ T Cells. **Immunity**, 39 (5): 846–857

Huang, Y. and Wange, R.L. (2004) T cell receptor signaling: beyond complex complexes. **Journal of Biological Chemistry**, 279 (28): 28827–28830

Huber, M., Beuscher, H.U., Rohwer, P., et al. (1998) Costimulation via TCR and IL-1 Receptor Reveals a Novel IL-1α-Mediated Autocrine Pathway of Th2 Cell Proliferation. **Journal of Immunology**, 160 (9): 4242–4247

Hugues, S., Fetler, L., Bonifaz, L., et al. (2004) Distinct T cell dynamics in lymph nodes during the induction of tolerance and immunity. **Nature Immunology**, 5: 1235–1242

Huizinga, T.W.J., Amos, C.I., van der Helm-van Mil, A.H.M., et al. (2005) Refining the Complex Rheumatoid Arthritis Phenotype Based on Specificity of the HLA-DRB1 Shared Epitope for Antibodies to Citrullinated Proteins. **Arthritis & Rheumatism**, 52 (11): 3433–3438

Humphreys, J.H., Verstappen, S.M.M., Hyrich, K.L., et al. (2013) The incidence of rheumatoid arthritis in the UK: comparisons using the 2010 ACR/EULAR classification criteria and the 1987 ACR classification criteria. Results from the Norfolk Arthritis Register. **Annals of the Rheumatic Diseases**, 72 (8): 1315–1320

Huse, M. (2009) The T-cell-receptor signaling network. **Journal of cell science**, 122 (9): 1269–1273

Hutloff, A., Dittrich, A.M., Beier, K.C., et al. (1999) ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. **Nature**, 397 (6716): 263–266

Hünig, T. (2012) The storm has cleared: lessons from the CD28 superagonist TGN1412 trial. **Nature Reviews Immunology**, 12 (5): 317–318

Intlekofer, A.M. and Thompson, C.B. (2013) At the bench: preclinical rationale for CTLA-4 and PD-1 blockade as cancer immunotherapy. **Journal of Leukocyte Biology**, 94 (1): 25–39

Irvine, D.J., Purbhoo, M.A., Krogsgaard, M., et al. (2002) Direct observation of ligand recognition by T cells. **Nature**, 419 (6909): 845–849

Itano, A.A. and Jenkins, M.K. (2003) Antigen presentation to naive CD4 T cells in the lymph node. **Nature Immunology**, 4 (8): 733–739

Iwashima, M., Irving, B.A., van Oers, N.S., et al. (1994) Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. **Science**, 263 (5150): 1136–1139

Jacobs, S.R., Herman, C.E., Maciver, N.J., et al. (2008) Glucose uptake is limiting in T cell activation and requires CD28-mediated Akt-dependent and independent pathways. **Journal of Immunology**, 180 (7): 4476–4486

Jeffery, L.E., Burke, F., Mura, M., et al. (2009) 1,25-Dihydroxyvitamin D3 and IL-2 Combine to Inhibit T Cell Production of Inflammatory Cytokines and Promote Development of Regulatory T Cells Expressing CTLA-4 and FoxP3. **Journal of Immunology**, 183: 5458–5467

Jeffery, L.E., Wood, A.M., Qureshi, O.S., et al. (2012) Availability of 25-hydroxyvitamin D(3) to APCs controls the balance between regulatory and inflammatory T cell responses. **Journal of Immunology**, 189 (11): 5155–5164

John, S., Robbins, C.M. and Leonard, W.J. (1996) An IL-2 response element in the human IL-2 receptor alpha chain promoter is a composite element that binds Stat5, Elf-1, HMG-I(Y) and a GATA family protein. **The EMBO journal**, 15 (20): 5627–5635

Johnston, R.J., Poholek, A.C., DiToro, D., et al. (2009) Bcl6 and Blimp-1 Are Reciprocal and Antagonistic Regulators of T Follicular Helper Cell Differentiation. **Science**, 325 (5943): 1006–1010

Jones, R.G. and Thompson, C.B. (2007) Revving the engine: signal transduction fuels T cell activation. **Immunity**, 27 (2): 173–178

Jorritsma, P.J., Brogdon, J.L. and Bottomly, K. (2003) Role of TCR-induced extracellular signal-regulated kinase activation in the regulation of early IL-4 expression in naive CD4+ T cells. **Journal of Immunology**, 170 (5): 2427–2434

Juarez, M., Bang, H., Hammar, F., et al. (2015) Identification of novel antiacetylated vimentin antibodies in patients with early inflammatory arthritis. **Annals of the Rheumatic Diseases**

June, C.H., Ledbetter, J.A., Gillespie, M.M., et al. (1987) T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. **Molecular and Cellular Biology**, 7 (12): 4472–4481

Kalland, M.E., Oberprieler, N.G., Vang, T., et al. (2011) T Cell-Signaling Network Analysis Reveals Distinct Differences between CD28 and CD2 Costimulation Responses in Various Subsets and in the MAPK Pathway between Resting and Activated Regulatory T Cells. **Journal of Immunology**, 187 (10): 5233–5245

Kalli, K., Huntoon, C., Bell, M., et al. (1998) **Mechanism responsible for T-cell antigen receptor-and CD28-or interleukin 1 (IL-1) receptor-initiated regulation of IL-2 gene expression by NF-κB**.

Kampman, M.T., Steffensen, L.H., Mellgren, S.I., et al. (2012) Effect of vitamin D3 supplementation on relapses, disease progression, and measures of function in persons with multiple sclerosis: exploratory outcomes from a double-blind randomised controlled trial. **Multiple Sclerosis Journal**, 18 (8): 1144–1151

Kaplan, M.H., Hufford, M.M. and Olson, M.R. (2015) The development and in vivo function of T helper 9 cells. **Nature Reviews Immunology**, 15 (5): 295–307

Kaur, S., Qureshi, O.S. and Sansom, D.M. (2013) Comparison of the Intracellular Trafficking Itinerary of CTLA-4 Orthologues. **PLoS ONE**, 8 (4): e60903

Kawa, S., Nikaido, T., Aoki, Y., et al. (1997) Vitamin D analogues up-regulate p21 and p27 during growth inhibition of pancreatic cancer cell lines. **British Journal of Cancer**, 76 (7): 884–889

Kawai, K., Shahinian, A., Mak, T.W., et al. (1996) Skin allograft rejection in CD28-deficient mice. **Transplantation**, 61 (3): 352–355

Keck, S., Schmaler, M., Ganter, S., et al. (2014) Antigen affinity and antigen dose exert distinct influences on CD4 T-cell differentiation. **PNAS**, 111 (41): 14852–14857

Kersh, E.N., Kaech, S.M., Onami, T.M., et al. (2003) TCR signal transduction in antigen-specific memory CD8 T cells. **Journal of Immunology**, 170 (11): 5455–5463

Kerstan, A. and Hünig, T. (2004) Cutting Edge: Distinct TCR- and CD28-Derived Signals Regulate CD95L, Bcl-xL, and the Survival of Primary T Cells. **Journal of Immunology**, 172 (3): 1341–1345

Khoury, S.J., Akalin, E., Chandraker, A., et al. (1995) CD28-B7 costimulatory blockade by CTLA4Ig prevents actively induced experimental autoimmune encephalomyelitis and inhibits Th1 but spares Th2 cytokines in the central nervous system. **Journal of Immunology**, 155 (10): 4521–4524

Kickler, K., Ni-Choileain, S., Williams, A., et al. (2012) Calcitriol modulates the CD46 pathway in T cells. **PLoS ONE**, 7 (10): e48486–e48486

Kingeter, L.M., Paul, S., Maynard, S.K., et al. (2010) Cutting Edge: TCR Ligation Triggers Digital Activation of NF- B. **Journal of Immunology**, 185 (8): 4520–4524

Kinnear, G., Jones, N.D. and Wood, K.J. (2013) Costimulation Blockade: Current Perspectives and Implications for Therapy. **Transplantation**, 95 (4): 527–535

Kirken, R.A., Rui, H., Malabarba, M.G., et al. (1995) Activation of JAK3, but not JAK1, is critical for IL-2-induced proliferation and STAT5 recruitment by a COOH-terminal region of the IL-2 receptor beta-chain. **Cytokine**, 7 (7): 689–700

Kishimoto, H. and Sprent, J. (1999) Strong TCR ligation without costimulation causes rapid onset of Fas-dependent apoptosis of naive murine CD4+ T cells. **Journal of Immunology**, 163 (4): 1817–1826

Kitazawa, Y., Fujino, M., Sakai, T., et al. (2008) Foxp3-expressing Regulatory T Cells Expanded With CD28 Superagonist Antibody Can Prevent Rat Cardiac Allograft Rejection. **The Journal of Heart and Lung Transplantation**, 27 (4): 362–371

Klarenbeek, N.B., Kerstens, P.J.S.M., Huizinga, T.W.J., et al. (2010) Recent advances in the management of rheumatoid arthritis. **BMJ**, 341: c6942

Klareskog, L., Catrina, A.I. and Paget, S. (2009) Rheumatoid arthritis. **Lancet (London, England)**, 373 (9664): 659–672

Klareskog, L., Stolt, P., Lundberg, K., et al. (2006) A New Model for an Etiology of Rheumatoid Arthritis: Smoking May Trigger HLA-DR (Shared Epitope)-Restricted Immune Reactions to Autoantigens Modified by Citrullination. **Arthritis & Rheumatism**, 54 (1): 38–46

Kobata, T., Agematsu, K., Kameoka, J., et al. (1994) CD27 is a signal-transducing molecule involved in CD45RA+ naive T cell costimulation. **Journal of Immunology**, 153 (12): 5422–5432

Koehli, S., Naeher, D., Galati-Fournier, V., et al. (2014) Optimal T-cell receptor affinity for inducing autoimmunity. **PNAS**, 111 (48): 17248–17253

Kong, K.-F., Yokosuka, T., Canonigo-Balancio, A.J., et al. (2011) A motif in the V3 domain of the kinase PKC-θ determines its localization in the immunological synapse and functions in T cells via association with CD28. **Nature**, 12 (11): 1105–1112

Kongsbak, M., Levring, T.B., Geisler, C., et al. (2013) The vitamin D receptor and T cell function. **Frontiers in Immunology**, 4: 1–10

Koretzky, G.A., Abtahian, F. and Silverman, M.A. (2006) SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. **Nature Reviews Immunology**, 6 (1): 67–78

Korn, T., Bettelli, E., Gao, W., et al. (2007) IL-21 initiates an alternative pathway to induce proinflammatory TH17 cells. **Nature**, 448 (7152): 484–487

Korn, T., Mitsdoerffer, M., Croxford, A.L., et al. (2008) IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3^{+} regulatory T cells. **PNAS**, 105 (47): 18460–18465

Krawczyk, C.M., Shen, H. and Pearce, E.J. (2007) Functional Plasticity in

Memory T Helper Cell Responses. **The Journal of Immunology**, 178 (7): 4080–4088

Kremer, J.M., Dougados, M., Emery, P., et al. (2005) Treatment of rheumatoid arthritis with the selective costimulation modulator abatacept: Twelve-month results of a phase iib, double-blind, randomized, placebo-controlled trial. **Arthritis & Rheumatism**, 52 (8): 2263–2271

Kremer, J.M., Genant, H.K., Moreland, L.W., et al. (2006a) Effects of abatacept in patients with methotrexate-resistant active rheumatoid arthritis: a randomized trial. **Annals of Internal Medicine**, 144 (12): 865–876

Kremer, J.M., Genant, H.K., Moreland, L.W., et al. (2006b) Effects of Abatacept in Patients with Methotrexate-Resistant Active Rheumatoid Arthritis. **Annals of Internal Medicine**, 144 (12): 865–876

Kremer, J.M., Westhovens, R., Leon, M., et al. (2003) Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. **New England Journal of Medicine**, 349 (20): 1907–1915

Krummel, M.F. and Allison, J.P. (1996) CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. **The Journal of experimental medicine**, 183 (6): 2533–2540

Kuehn, H.S., Ouyang, W., Lo, B., et al. (2014) Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. **Science**, 345 (6204): 1623–1627

Kündig, T.M., Shahinian, A., Kawai, K., et al. (1996) Duration of TCR Stimulation Determines Costimulatory Requirement of T Cells. **Immunity**, 5 (1): 41–52

Lafferty, K.J. and Woolnough, J. (1977) The origin and mechanism of the allograft reaction. **Immunological Reviews**, 35: 231–262

Laouar, Y. and Crispe, I.N. (2000) Functional flexibility in T cells: independent regulation of CD4$^+$ T cell proliferation and effector function in vivo. **Immunity**, 13 (3): 291–301

Larsen, C.P., Pearson, T.C., Adams, A.B., et al. (2005) Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. **American Journal of Transplantation**, 5 (3): 443–453

Lee, K.M., Chuang, E., Griffin, M., et al. (1998) Molecular basis of T cell inactivation by CTLA-4. **Science**, 282 (5397): 2263–2266

Lee, M.N., Ye, C., Villani, A.-C., et al. (2014) Common genetic variants modulate pathogen-sensing responses in human dendritic cells. **Science**, 343 (6175): 1246980

Leipe, J., Grunke, M., Dechant, C., et al. (2010) Role of Th17 cells in human

autoimmune arthritis. **Arthritis & Rheumatism**, 62 (10): 2876–2885

Leitenberg, D., Novak, T.J., Farber, D., et al. (1996) The extracellular domain of CD45 controls association with the CD4-T cell receptor complex and the response to antigen-specific stimulation. **The Journal of experimental medicine**, 183 (1): 249–259

Leitner, J., Klauser, C., Pickl, W.F., et al. (2009) B7-H3 is a potent inhibitor of human T-cell activation: No evidence for B7-H3 and TREML2 interaction. **Eur. J. Immunol.**, 39 (7): 1754–1764

Lemire, J.M., Adams, J.S., Sakai, R., et al. (1984) 1α,25-Dihydroxyvitamin D_3 Suppresses Proliferation and Immunoglobulin Production by Normal Human Peripheral Blood Mononuclear Cells. **Journal of Clinical Investigation**, 74: 657–661

Lemire, J.M., Archer, D.C., Beck, L., et al. (1995) Immunosuppressive actions of 1,25-Dihydroxyvitamin D_3: Preferential inhibition of Th_1 functions. **The Journal of nutrition**, 125 (6 Suppl): 1704S–1708S

Lenschow, D.J., Herold, K.C., Rhee, L., et al. (1996) CD28/B7 regulation of Th1 and Th2 subsets in the development of autoimmune diabetes. **Immunity**, 5 (3): 285–293

Lenschow, D.J., Ho, S.C., Sattar, H., et al. (1995a) 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**, 181 (3): 1145–1155

Lenschow, D.J., Zeng, Y., Hathcock, K.S., et al. (1995b) Inhibition of transplant rejection following treatment with anti-B7-2 and anti-B7-1 antibodies. **Transplantation**, 60 (10): 1171–1178

Leonard, W.J. and Spolski, R. (2005) Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. **Nature Reviews Immunology**, 5 (9): 688–698

Li, H., Llera, A., Malchiodi, E.L., et al. (1999) The Structural Basis of T cell activation by Superantigens. **Annual Review of Immunology**, 17: 435–466

Li, M., Zheng, H., Li, T., et al. (2012a) Cytotoxic T-lymphocyte associated antigen-4 gene polymorphisms and primary biliary cirrhosis: A systematic review. **Journal of Gastroenterology and Hepatology**, 27 (7): 1159–1166

Li, X., Zhang, C., Zhang, J., et al. (2012b) Polymorphisms in the CTLA-4 Gene and Rheumatoid Arthritis Susceptibility: A Meta-analysis. **Journal of Clinical Immunology**, 32 (3): 530–539

Liaskou, E., Jeffery, L.E., Trivedi, P.J., et al. (2014) Loss of CD28 Expression by Liver-infiltrating T cells Contributes to Pathogenesis of Primary Sclerosing Cholangitis. **Gastroenterology**, 147: 221–232

Lichtman, A.H., Chin, J., Schmidt, J.A., et al. (1988) Role of interleukin 1 in the activation of T lymphocytes. **Proc. Natl. Acad. Sci. U.S.A.**, 85 (24): 9699–9703

Lin, J.X. and Leonard, W.J. (2000) The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. **Oncogene**, 19 (21): 2566–2576

Lin, S.-C., Yen, J.-H., Tsai, J.-J., et al. (2004) Association of a Programmed Death 1 Gene Polymorphism With the Development of Rheumatoid Arthritis, but Not Systemic Lupus Erythematosus. **Arthritis & Rheumatism**, 50 (3): 770–775

Lindstein, T., June, C.H., Ledbetter, J.A., et al. (1989) Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. **Science**, 244 (4902): 339–343

Ling, V., Wu, P.W., Spaulding, V., et al. (2003) Duplication of primate and rodent B7-H3 immunoglobulin V- and C-like domains: divergent history of functional redundancy and exon loss. **Genomics**, 82 (3): 365–377

Linsley, P.S. and Nadler, S.G. (2009) The clinical utility of inhibiting CD28-mediated costimulation. **Immunological Reviews**, 229 (1): 307–321

Linsley, P.S., Bradshaw, J., Greene, J., et al. (1996) Intracellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement. **Immunity**, 4 (6): 535–543

Linsley, P.S., Greene, J.L., Tan, P., et al. (1992) Coexpression and Functional Cooperation of CTLA-4 and CD28 on Activated T Lymphocytes. **The Journal of experimental medicine**, 176 (6): 1595–1604

Linterman, M.A., Denton, A.E., Divekar, D.P., et al. (2014) CD28 expression is required after T cell priming for helper T cell responses and protective immunity to infection. **eLife**, 3: –

Linterman, M.A., Rigby, R.J., Wong, R., et al. (2009) Roquin differentiates the specialized functions of duplicated T cell costimulatory receptor genes *Cd28* and *Icos*. **Immunity**, 30 (2): 228–241

Liu, E.H., Siegel, R.M., Harlan, D.M., et al. (2007) T cell-directed therapies: lessons learned and future prospects. **Nature Immunology**, 8 (1): 25–30

Liu, J. and Zhang, H. (2013) -1722T/C polymorphism (rs733618) of CTLA-4 significantly associated with systemic lupus erythematosus (SLE): A comprehensive meta-analysis. **Human Immunology**, 74 (3): 341–347

Liu, M., Lee, M.H., Cohen, M., et al. (1996) Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. **Genes & Development**, 10 (2): 142–153

Liu, W., Putnam, A.L., Xu-Yu, Z., et al. (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells.

The Journal of experimental medicine, 203 (7): 1701–1711

Lo, D.J., Anderson, D.J., Weaver, T.A., et al. (2013) Belatacept and Sirolimus Prolong Nonhuman Primate Renal Allograft Survival Without a Requirement for Memory T Cell Depletion. **American Journal of Transplantation**, 13: 320–328

London, C.A., Lodge, M.P. and Abbas, A.K. (2000) Functional responses and costimulator dependence of memory CD4+ T cells. **Journal of Immunology**, 164 (1): 265–272

Luo, L., Chapoval, A.I., Flies, D.B., et al. (2004) B7-H3 Enhances Tumor Immunity in vivo by Costimulating Rapid Clonal Expansion of Antigen-Specific CD8$^+$ Cytolytic T Cells. **Journal of Immunology**, 173 (9): 5445–5450

Luoma, A.M., Castro, C.D. and Adams, E.J. (2014) γδ T cell surveillance via CD1 molecules. **Trends in immunology**, 35 (12): 613–621

Lühder, F., Huang, Y., Dennehy, K.M., et al. (2003) Topological Requirements and Signaling Properties of T Cell–activating, Anti-CD28 Antibody Superagonists. **The Journal of experimental medicine**, 197: 955–966

Ma, C.S. and Deenick, E.K. (2014) Human T follicular helper (Tfh) cells and disease. **Immunology and Cell Biology**

Ma, D.Y. and Clark, E.A. (2009) The role of CD40 and CD154/CD40L in dendritic cells. **Seminars in Immunology**, 21 (5): 265–272

Ma, Y., Lin, B.-R., Lin, B., et al. (2009) Pharmacokinetics of CTLA4Ig fusion protein in healthy volunteers and patients with rheumatoid arthritis. **Acta pharmacologica Sinica**, 30 (3): 364–371

MacGregor, A.J., Snieder, H., Rigby, A.S., et al. (2000) Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. **Arthritis & Rheumatism**, 43 (1): 30–37

Macián, F. (2005) NFAT proteins: key regulators of T-cell development and function. **Nature Reviews Immunology**, 5 (6): 472–484

Macián, F., López-Rodríguez, C. and Rao, A. (2001) Partners in transcription: NFAT and AP-1. **Oncogene**, 20 (19): 2476–2489

Madrenas, J., Wange, R.L., Wang, J.L., et al. (1995) ζ Phosphorylation Without ZAP-70 Activation Induced by TCR Antagonists or Partial Agonists. **Science**, 267 (5197): 515–518

Maker, A.V., Attia, P. and Rosenberg, S.A. (2005) Analysis of the cellular mechanism of antitumor responses and autoimmunity in patients treated with CTLA-4 blockade. **Journal of Immunology**, 175 (11): 7746–7754

Malek, T.R. (2008) The biology of interleukin-2. **Annual Review of Immunology**, 26: 453–479

Maloy, K.J. and Powrie, F. (2005) Fueling regulation: IL-2 keeps CD4$^+$ T$_{reg}$ cells fit. **Nature Immunology**, 6 (11): 1071–1072

Mandelbrot, D.A., McAdam, A.J. and Sharpe, A.H. (1999) B7-1 or B7-2 is required to produce the lymphoproliferative phenotype in mice lacking cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). **The Journal of experimental medicine**, 189 (2): 435–440

Maneiro, R.J., Salgado, E., Carmona, L., et al. (2013) Rheumatoid factor as predictor of response to abatacept, rituximab and tocilizumab in rheumatoid arthritis: Systematic review and meta-analysis. **Seminars in Arthritis and Rheumatism**, 43 (1): 9–17

Mantel, P.-Y., Ouaked, N., Rückert, B., et al. (2006) Molecular mechanisms underlying FOXP3 induction in human T cells. **Journal of Immunology**, 176: 3593–3602

Manzotti, C.N., Liu, M.K.P., Burke, F., et al. (2006) Integration of CD28 and CTLA-4 function results in differential responses of T cells to CD80 and CD86. **Eur. J. Immunol.**, 36 (6): 1413–1422

Marangoni, F., Murooka, T.T., Manzo, T., et al. (2013) The Transcription Factor NFAT Exhibits Signal Memory during Serial T Cell Interactions with Antigen-Presenting Cells. **Immunity**, 38: 237–249

Marengère, L.E., Waterhouse, P., Duncan, G.S., et al. (1996) Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4. **Science**, 272 (5265): 1170–1173

Martin, M., Schneider, H., Azouz, A., et al. (2001) Cytotoxic T lymphocyte Antigen 4 and CD28 Modulate Cell Surface Raft Expression in Their Regulation of T cell Function. **The Journal of experimental medicine**, 194 (11): 1675–1681

Martln-Fontecha, A., Sebastiani, S., Höpken, U.E., et al. (2003) Regulation of Dendritic Cell Migration to the Draining Lymph Node: Impact on T Lymphocyte Traffic and Priming. **The Journal of experimental medicine**, 198 (4): 615–621

Martinez, O. (2005) DC-SIGN, but not sDC-SIGN, can modulate IL-2 production from PMA- and anti-CD3-stimulated primary human CD4 T cells. **International Immunology**, 17 (6): 769–778

Mason, D. (1998) A very high level of crossreactivity is an essential feature of the T-cell receptor. **Immunology Today**, 19 (9): 395–404

Masteller, E.L., Chuang, E., Mullen, A.C., et al. (2000) Structural Analysis of CTLA-4 Function In Vivo. **Journal of Immunology**, 164 (10): 5319–5327

Matsuda, S. and Koyasu, S. (2000) Mechanisms of action of cyclosporine. **Immunopharmacology**, 47: 119–125

Matzinger, P. (2002) The Danger Model: A Renewed Sense of Self. **Science**, 296 (5566): 301–305

McAdam, A.J., Chang, T.T., Lumelsky, A.E., et al. (2000) Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4$^+$ T cells. **Journal of Immunology**, 165 (9): 5035–5040

McInnes, I.B. and Schett, G. (2011) The Pathogenesis of Rheumatoid Arthritis. **New England Journal of Medicine**, 365 (23): 2205–2219

McInnes, I.B., Leung, B.P., Sturrock, R.D., et al. (1997) Interleukin-15 Mediates T cell-Dependent Regulation of Tumor Necrosis Factor-α Production in Rheumatoid Arthritis. **Nature medicine**, 3 (2): 189–195

McKean, D.J., Bell, M., Huntoon, C., et al. (1995) IL-1 receptor and TCR signals synergize to activate NF-kB

-mediated gene transcription. **International Immunology**, 7 (1): 9–20

McKenney, D.W., Onodera, H., Gorman, L., et al. (1995) Distinct isoforms of the CD45 protein-tyrosine phosphatase differentially regulate interleukin 2 secretion and activation signal pathways involving Vav in T cells. **Journal of Biological Chemistry**, 270 (42): 24949–24954

McKinney, E.F., Lee, J.C., Jayne, D.R.W., et al. (2015) T-cell exhaustion, co-stimulation and clinical outcome in autoimmunity and infection. **Nature**, 523 (7562): 612–616

Mempel, T.R., Henrickson, S.E. and Andrian, von, U.H. (2004) T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. **Nature**, 427 (6970): 154–159

Messi, M., Giacchetto, I., Nagata, K., et al. (2003) Memory and flexibility of cytokine gene expression as separable properties of human T_H1 and T_H2 lymphocytes. **Nature Immunology**, 4 (1): 78–86

Michel, F., Attal-Bonnefoy, G., Mangino, G., et al. (2001) CD28 as a Molecular Amplifier Extending TCR Ligation and Signaling Capabilities. **Immunity**, 15 (6): 935–945

Michel, F., Mangino, G., Attal-Bonnefoy, G., et al. (2000) CD28 utilizes Vav-1 to enhance TCR-proximal signaling and NF-AT activation. **Journal of Immunology**, 165 (7): 3820–3829

Miller, M.J., Hejazi, A.S., Wei, S.H., et al. (2004) T cell repertoire scanning is promoted by dynamic dendritic cell behavior and random T cell motility in the lymph node. **Proc. Natl. Acad. Sci. U.S.A.**, 101 (4): 998–1003

Miller, S.D., Vanderlugt, C.L., Lenschow, D.J., et al. (1995) Blockade of CD28/B7-1 interaction prevents epitope spreading and clinical relapses of murine EAE. **Immunity**, 3 (6): 739–745

Miossec, P., Verweij, C.L., Klareskog, L., et al. (2011) Biomarkers and personalised medicine in rheumatoid arthritis: a proposal for interactions between academia, industry and regulatory bodies. **Annals of the Rheumatic Diseases**, 70 (10): 1713–1718

Mittelbrunn, M., del Hoyo, G.M., Lopez-Bravo, M., et al. (2009) Imaging of plasmacytoid dendritic cell interactions with T cells. **Blood**, 113 (1): 75–84

Molon, B., Gri, G., Bettella, M., et al. (2005) T cell costimulation by chemokine receptors. **Nature Immunology**, 6 (5): 465–471

Monjas, A., Alcover, A. and Alarcón, B. (2004) Engaged and bystander T cell receptors are down-modulated by different endocytotic pathways. **Journal of Biological Chemistry**, 279 (53): 55376–55384

Montoya, M.C., Sancho, D., Bonello, G., et al. (2002) Role of ICAM-3 in the initial interaction of T lymphocytes and APCs. **Nature Immunology**, 3 (2): 159–168

Moreland, L.W., Alten, R., Van den Bosch, F., et al. (2002) Costimulatory blockade in patients with rheumatoid arthritis: a pilot, dose-finding, double-blind, placebo-controlled clinical trial evaluating CTLA-4Ig and LEA29Y eighty-five days after the first infusion. **Arthritis & Rheumatism**, 46 (6): 1470–1479

Morris, G.P. and Allen, P.M. (2012) How the TCR balances sensitivity and specificity for the recognition of self and pathogens. **Nature Immunology**, 13 (2): 121–128

Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: Different Patterns of Lymphokine Secretion Lead to Different Functional Properties. **Immunology**, 7: 145–173

Munitic, I., Ryan, P.E. and Ashwell, J.D. (2005) T cells in G1 provide a memory-like response to secondary stimulation. **Journal of Immunology**, 174 (7): 4010–4018

Munroe, M.E. and Bishop, G.A. (2007) A Costimulatory Function for T Cell CD40. **The Journal of Immunology**, 178 (2): 671–682

Nakae, S., Nambu, A., Sudo, K., et al. (2003) Suppression of Immune Induction of Collagen-Induced Arthritis in IL-17-Deficient Mice. **The Journal of Immunology**, 171 (11): 6173–6177

Nankivell, B.J., Borrows, R.J., Fung, C.L.-S., et al. (2003) The natural history of chronic allograft nephropathy. **New England Journal of Medicine**, 349 (24): 2326–2333

Nelson, B.H. (2004) IL-2, Regulatory T Cells, and Tolerance. **Journal of Immunology**, 172 (7): 3983–3988

Ni, H.-T., Deeths, M.J., Li, W., et al. (1999) Signaling Pathways Activated by Leukocyte Function-Associated Ag-1-Dependent Costimulation. **Journal of**

Immunology, 162 (9): 5183–5189

NICE (2013) **Abatacept for treating rheumatoid arthritis after the failure of conventional disease-modifying anti-rheumatic drugs (rapid review of technology appraisal guidance 234)**. London: nice.org.uk

Nicholson, L.B., Greer, J.M., Sobel, R.A., et al. (1995) An Altered Peptide Ligand Mediates Immune Deviation and Prevents Autoimmune Encephalomyelitis. **Immunity**, 3 (4): 397–405

Nie, H., Zheng, Y., Li, R., et al. (2013) Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF-α in rheumatoid arthritis. **Nature medicine**, 19 (3): 322–328

Nielen, M.M.J., van Schaardenburg, D., Reesink, H.W., et al. (2004) Specific Autoantibodies Precede the Symptoms of Rheumatoid Arthritis: A Study of Serial Measurements in Blood Donors. **Arthritis & Rheumatism**, 50 (2): 380–386

Nishimura, H., Nose, M., Hiai, H., et al. (1999) Development of lupus-like autoimmune diseases by disruption of the *PD-1* gene encoding an ITIM motif-carrying immunoreceptor. **Immunity**, 11 (2): 141–151

Nishimura, H., Okazaki, T., Tanaka, Y., et al. (2001) Autoimmune Dilated Cardiomyopathy in PD-1 Receptor-Deficient Mice. **Science**, 291 (5502): 319–322

Noel, P.J., Boise, L.H., Green, J.M., et al. (1996) CD28 costimulation prevents cell death during primary T cell activation. **Journal of Immunology**, 157 (2): 636–642

Novak, T.J., Farber, D., Leitenberg, D., et al. (1994) Isoforms of the transmembrane tyrosine phosphatase CD45 differentially affect T cell recognition. **Immunity**, 1 (2): 109–119

Ohshima, Y., Yang, L.P., Uchiyama, T., et al. (1998) OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human CD4^{+} T cells into high IL-4-producing effectors. **Blood**, 92 (9): 3338–3345

Ossevoort, M.A., Lorré, K., Boon, L., et al. (1999) Prolonged skin graft survival by administration of anti-CD80 monoclonal antibody with cyclosporin A. **Journal of Immunotherapy**, 22 (5): 381–389

Pagès, F., Ragueneau, M., Rottapel, R., et al. (1994) Binding of phosphatidyl-inositol-3-OH kinase to CD28 is required for T-cell signalling. **Nature Immunology**, 369: 327–329

Palmer, E. (2003) Negative selection-Clearing out the bad apples from the T-cell repertoire. **Nature Reviews Immunology**, 3 (5): 383–391

Palmer, M.T., Lee, Y.K., Maynard, C.L., et al. (2011) Lineage-specific Effects

of 1,25-Dihydroxyvitamin D3 on the Development of Effector CD4 T Cells. **Journal of Biological Chemistry**, 286 (2): 997–1004

Park, H.J., Park, J.S., Jeong, Y.H., et al. (2015) PD-1 Upregulated on Regulatory T Cells during Chronic Virus Infection Enhances the Suppression of CD8^{+} T Cell Immune Response via the Interaction with PD-L1 Expressed on CD8^{+} T Cells. **The Journal of Immunology**, 194 (12): 5801–5811

Park, J.-J., Omiya, R., Matsumura, Y., et al. (2010) B7-H1/CD80 interaction is required for the induction and maintenance of peripheral T-cell tolerance. **Blood**, 116 (8): 1291–1298

Park, S.-G., Schulze-Luehrman, J., Hayden, M.S., et al. (2009) The kinase PDK1 integrates T cell antigen receptor and CD28 coreceptor signaling to induce NF-κB and activate T cells. **Nature Immunology**, 10 (2): 158–166

Parry, R.V., Chemnitz, J.M., Frauwirth, K.A., et al. (2005) CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. **Molecular and Cellular Biology**, 25 (21): 9543–9553

Peach, R.J., Bajorath, J., Brady, W., et al. (1994) Complimentarity Determining Region 1 (CDR1)- and CDR3-analagous Regions in CTLA4 and CD28 Determine the Binding to B7-1. **The Journal of experimental medicine**, 180: 2049–2058

Pearson, T.C., Alexander, D.Z., Corbascio, M., et al. (1997) Analysis of the B7 costimulatory pathway in allograft rejection. **Transplantation**, 63 (10): 1463–1469

Peelen, E., Knippenberg, S., Muris, A.-H., et al. (2011) Effects of vitamin D on the peripheral adaptive immune system: A review. **Autoimmunity Reviews**, 10 (12): 733–743

Peine, M., Rausch, S., Helmstetter, C., et al. (2013) Stable T-bet[+]GATA-3^{+} Th1/Th2 Hybrid Cells Arise In Vivo, Can Develop Directly from Naive Precursors, and Limit Immunopathologic Inflammation. **PLoS Biology**, 11 (8): e1001633–e1001633

Penna, G., Amuchastegui, S., Giarratana, N., et al. (2007) 1,25-Dihydroxyvitamin D3 Selectively Modulates Tolerogenic Properties in Myeloid but Not Plasmacytoid Dendritic Cells. **Journal of Immunology**, 178 (1): 145–153

Perico, N., Imberti, O., Bontempelli, M., et al. (1995) Toward novel antirejection strategies: in vivo immunosuppressive properties of CTLA4Ig. **Kidney International**, 47 (1): 241–246

Pfeiffer, C., Stein, J., Southwood, S., et al. (1995) Altered Peptide Ligands Can Control CD4 T Lymphocyte Differentiation In Vivo. **The Journal of experimental medicine**, 181 (4): 1569–1574

Pieper, J., Herrath, J., Raghavan, S., et al. (2013) CTLA4-Ig (abatacept)

therapy modulates T cell effector functions in autoantibody-positive rheumatoid arthritis patients. **BMC immunology**, 14: 34

Pilat, N. and Wekerle, T. (2012) Belatacept and Tregs: friends or foes? **Immunotherapy**, 4 (4): 351–354

Pitzalis, C., Jones, G.W., Bombardieri, M., et al. (2014) Ectopic lymphoid-like structures in infection, cancer and autoimmunity. **Nature Reviews Immunology**, 14 (7): 447–462

Platt, A.M., Gibson, V.B., Patakas, A., et al. (2010) Abatacept limits breach of self-tolerance in a murine model of arthritis via effects on the generation of T follicular helper cells. **Journal of Immunology**, 185 (3): 1558–1567

Podtschaske, M., Benary, U., Zwinger, S., et al. (2007) Digital NFATc2 Activation per Cell Transforms Graded T Cell Receptor Activation into an All-or-None IL-2 Expression Kanellopoulos, J. (ed.). **PLoS ONE**, 2 (9): e935

Poirier, N., Azimzadeh, A.M., Zhang, T., et al. (2010) Inducing CTLA-4-dependent Immune Regulation by Selective CD28 blockade Promotes Regulatory T cells in Organ Transplantation. **Science Translational Medicine**, 2 (17): 17ra10

Poirier, N., Blancho, G. and Vanhove, B. (2011) A more selective costimulatory blockade of the CD28-B7 pathway. **Transplant International**, 24 (1): 2–11

Popmihajlov, Z. and Smith, K.A. (2008) Negative feedback regulation of T cells via interleukin-2 and FOXP3 reciprocity. **PLoS ONE**, 3 (2): e1581

Powell, J.D., Lerner, C.G. and Schwartz, R.H. (1999) Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation. **Journal of Immunology**, 162 (5): 2775–2784

Prasad, K.V., Cai, Y.C., Raab, M., et al. (1994) T-cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr(P)-Met-Xaa-Met motif. **Proc. Natl. Acad. Sci. U.S.A.**, 91 (7): 2834–2838

Presser, D., Sester, U., Mohrbach, J., et al. (2009) Differential kinetics of effector and regulatory T cells in patients on calcineurin inhibitor-based drug regimens. **Kidney International**, 76 (5): 557–566

Prokunina, L., Padyukov, L., Bennet, A., et al. (2004) Association of the PD-1.3A Allele of the PDCD1 Gene in Patients With Rheumatoid Arthritis Negative for Rheumatoid Factor and the Shared Epitope. **Arthritis & Rheumatism**, 50 (6): 1770–1773

Provvedini, D.M., Tsoukas, C.D., Deftos, L.J., et al. (1983) 1,25-dihydroxyvitamin D3 receptors in human leukocytes. **Science**, 221 (4616): 1181–1183

Prud'homme, G.J., Parfrey, N.A. and Vanier, L.E. (1991) Cyclosporine-

induced autoimmunity and immune hyperreactivity. **Autoimmunity**, 9 (4): 345–356

Quill, H. and Schwartz, R.H. (1987) Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. **Journal of Immunology**, 138 (11): 3704–3712

Qureshi, O.S., Kaur, S., Hou, T.Z., et al. (2012) Constitutive Clathrin-mediated Endocytosis of CTLA-4 Persists during T Cell Activation. **Journal of Biological Chemistry**, 287 (12): 9429–9440

Qureshi, O.S., Zheng, Y., Nakamura, K., et al. (2011) Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. **Science**, 332 (6029): 600–603

Rachmilewitz, J. and Lanzavecchia, A. (2002) A temporal and spatial summation model for T-cell activation: signal integration and antigen decoding. **Trends in immunology**, 23: 592–595

Racke, M.K., Scott, D.E., Quigley, L., et al. (1995) Distinct Roles for B7-1 (Cd-80) and B7-2 (Cd-86) in the Initiation of Experimental Allergic Encephalomyelitis. **Journal of Clinical Investigation**, 96 (5): 2195–2203

Raj, T., Rothamel, K., Mostafavi, S., et al. (2014) Polarization of the effects of autoimmune and neurodegenerative risk alleles in leukocytes. **Science**, 344 (6183): 519–523

Ramagopalan, S.V., Heger, A., Berlanga, A.J., et al. (2010) A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. **Genome research**, 20 (10): 1352–1360

Rantapää-Dahlqvist, S., Ben A W de Jong, Berglin, E., et al. (2003) Antibodies Against Cyclic Citrullinated Peptide and IgA Rheumatoid Factor Predict the Development of Rheumatoid Arthritis. **Arthritis & Rheumatism**, 48 (10): 2741–2749

Rathmell, J.C., Elstrom, R.L., Cinalli, R.M., et al. (2003) Activated Akt promotes increased resting T cell size, CD28-independent T cell growth, and development of autoimmunity and lymphoma. **Eur. J. Immunol.**, 33 (8): 2223–2232

Raza, K. and Filer, A. (2015) The therapeutic window of opportunity in rheumatoid arthritis: does it ever close? **Annals of the Rheumatic Diseases**, 74 (5): 793–794

Raza, K., Falciani, F., Curnow, S.J., et al. (2005) Early rheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine profile of T cell and stromal cell origin. **Arthritis Research & Therapy**, 7 (4): R784–R795

Raza, K., Scheel-Toellner, D., Lee, C.-Y., et al. (2006) Synovial fluid leukocyte apoptosis is inhibited in patients with very early rheumatoid arthritis. **Arthritis**

Research & Therapy, 8 (4): R120–R120

Reichel, H., Koeffler, H.P., Tobler, A., et al. (1987) 1 α, 25-Dihydroxyvitamin D$_3$ inhibits Ƴ-interferon synthesis by normal human peripheral blood lymphocytes. **Proc Natl Acad Sci**, 84: 3385–3389

Riella, L.V. and Sayegh, M.H. (2013) T-cell co-stimulatory blockade in transplantation: two steps forward one step back! **Expert Opinion in Biological Therapy**, 13 (11): 1557–1568

Riella, L.V., Liu, T., Yang, J., et al. (2012a) Deleterious Effect of CTLA4-Ig on a Treg-Dependent Transplant Model. **American Journal of Transplantation**, 12 (4): 846–855

Riella, L.V., Paterson, A.M., Sharpe, A.H., et al. (2012b) Role of the PD-1 pathway in the immune response. **American Journal of Transplantation**, 12 (10): 2575–2587

Rigby, W.F., Noelle, R.J., Krause, K., et al. (1985) The effects of 1,25-dihydroxyvitamin D3 on human T lymphocyte activation and proliferation: a cell cycle analysis. **Journal of Immunology**, 135: 2279–2286

Rigby, W.F., Stacy, T. and Fanger, M.W. (1984) Inhibition of T lymphocyte mitogenesis by 1,25-dihydroxyvitamin D3 (calcitriol). **Journal of Clinical Investigation**, 74 (4): 1451–1455

Riley, J.L., Mao, M., Kobayashi, S., et al. (2002) Modulation of TCR-induced transcriptional profiles by ligation of CD28, ICOS, and CTLA-4 receptors. **Proc. Natl. Acad. Sci. U.S.A.**, 99 (18): 11790–11795

Rodríguez-Palmero, M., Franch, A., Castell, M., et al. (2006) Effective treatment of adjuvant arthritis with a stimulatory CD28-specific monoclonal antibody. **The Journal of Rheumatology**, 33 (1): 110–118

Roederer, M. (2011) Interpretation of cellular proliferation data: Avoid the panglossian. **Cytometry Part A**, 79A (2): 95–101

Rogers, P.R., Dubey, C. and Swain, S.L. (2000) Qualitative Changes Accompany Memory T Cell Generation: Faster, More Effective Responses at Lower Doses of Antigen. **Journal of Immunology**, 164: 2338–2346

Römer, P.S., Berr, S., Avota, E., et al. (2011) Preculture of PBMCs at high cell density increases sensitivity OF T-cell responses, revealing cytokine release by CD28 superagonist TGN1412. **Blood**, 118 (26): 6724–6726

Sadlack, B., Löhler, J., Schorle, H., et al. (1995) Generalized autoimmune disease in interleukin-2-deficient mice is triggered by an uncontrolled activation and proliferation of CD4+ T cells. **Eur. J. Immunol.**, 25 (11): 3053–3059

Sakaguchi, N., Takahashi, T., Hata, H., et al. (2003) Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis

in mice. **Nature**, 426 (6965): 454–460

Salmon, M., Scheel-Toellner, D., Huissoon, A.P., et al. (1997) Inhibition of T cell apoptosis in the rheumatoid synovium. **Journal of Clinical Investigation**, 99 (3): 439–446

Salomon, B., Lenschow, D.J., Rhee, L., et al. (2000) B7/CD28 Costimulation Is Essential for the Homeostasis of the CD4$^+$CD25$^+$ Immunoregulatory T Cells that Control Autoimmune Diabetes. **Immunity**, 12 (4): 431–440

Salti, S.M., Hammelev, E.M., Grewal, J.L., et al. (2011) Granzyme B Regulates Antiviral CD8$^+$ T Cell Responses. **Journal of Immunology**, 187 (12): 6301–6309

Samuel, S. and Sitrin, M.D. (2008) Vitamin D's role in cell proliferation and differentiation. **Nutrition reviews**, 66 (10 Suppl 2): S116–124

San José, E., Borroto, A., Niedergang, F., et al. (2000) Triggering the TCR complex causes the downregulation of nonengaged receptors by a signal transduction-dependent mechanism. **Immunity**, 12 (2): 161–170

Sanders, M.E., Makgoba, M.W., Sharrow, S.O., et al. (1988) Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-gamma production. **Journal of Immunology**, 140: 1401–1407

Sansom, D.M. (2000) CD28, CTLA-4 and their ligands: who does what and to whom? **Immunology**, 101 (2): 169–177

Sansom, D.M. and Walker, L.S.K. (2006) The role of CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) in regulatory T-cell biology. **Immunological Reviews**, 212: 131–148

Sansom, D.M. and Walker, L.S.K. (2013) CD28 costimulation: walking the immunological tightrope. **Eur. J. Immunol.**, 43 (1): 42–45

Schellekens, G.A., de Jong, B.A., van den Hoogen, F.H., et al. (1998) Citrulline is an Essential Constituent of Antigenic Determinants Recognized by Rheumatoid Arthritis-specific Autoantibodies. **Journal of Clinical Investigation**, 101 (1): 273–281

Schiff, M., Keiserman, M., Codding, C., et al. (2008) Efficacy and safety of abatacept or infliximab vs placebo in ATTEST: a phase III, multi-centre, randomised, double-blind, placebo-controlled study in patients with rheumatoid arthritis and an inadequate response to methotrexate. **Annals of the Rheumatic Diseases**, 67 (8): 1096–1103

Schneider, H., Downey, J., Smith, A., et al. (2006) Reversal of the TCR Stop Signal by CTLA-4. **Science**, 313 (5795): 1972–1975

Schneider, H., Martin, M., Agarraberes, F.A., et al. (1999) Cytolytic T

Lymphocyte-Associated Antigen-4 and the TCR ζ/CD3 Complex, But Not CD28, Interact with Clathrin Adaptor Complexes AP-1 and AP-2. **Journal of Immunology**, 163 (4): 1868–1879

Scholzen, T. and Gerdes, J. (2000) The Ki-67 Protein: From the Known and the Unknown. **Journal Of Cellular Physiology**, 182 (3): 311–322

Schubert, D., Bode, C., Kenefeck, R., et al. (2014) Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. **Nature medicine**, 20 (12): 1410–1418

Schwartz, J.-C.D., Zhang, X., Nathenson, S.G., et al. (2002) Structural mechanisms of costimulation. **Nature Immunology**, 3 (5): 427–434

Sebbag, M., Parry, S.L., Brennan, F.M., et al. (1997) Cytokine stimulation of T lymphocytes regulates their capacity to induce monocyte production of tumor necrosis factor-alpha, but not interleukin-10: possible relevance to pathophysiology of rheumatoid arthritis. **Eur. J. Immunol.**, 27 (3): 624–632

Semple, K., Nguyen, A., Yu, Y., et al. (2011) Strong CD28 costimulation suppresses induction of regulatory T cells from naive precursors through Lck signaling. **Blood**, 117 (11): 3096–3103

Sepulveda, H., Cerwenka, A., Morgan, T., et al. (1999) CD28, IL-2-independent costimulatory pathways for CD8 T lymphocyte activation. **Journal of Immunology**, 163 (3): 1133–1142

Sewell, A.K. (2012) Why must T cells be cross-reactive? **Nature Reviews Immunology**, 12 (9): 669–677

Shahinian, A., Pfeffer, K., Lee, K.P., et al. (1993) Differential T cell costimulatory requirements in CD28-deficient mice. **Science**, 261: 609–612

Shapiro, V.S., Truitt, K.E., Imboden, J.B., et al. (1997) CD28 mediates transcriptional upregulation of the interleukin-2 (IL-2) promoter through a composite element containing the CD28RE and NF-IL-2B AP-1 sites. **Molecular and Cellular Biology**, 17 (7): 4051–4058

Sheppard, K.-A., Fitz, L.J., Lee, J.M., et al. (2004) PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3ζ signalosome and downstream signaling to PKCθ. **FEBS Letters**, 574: 37–41

Shevach, E.M. (2009) Mechanisms of Foxp3^{+} T Regulatory Cell-Mediated Suppression. **Immunity**, 30 (5): 636–645

Shi, J., Knevel, R., Suwannalai, P., et al. (2011) Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. **Proc Natl Acad Sci**, 108 (42): 17372–17377

Shi, M., Lin, T.H., Appell, K.C., et al. (2009) Cell cycle progression following naive T cell activation is independent of Jak3/common gamma-chain cytokine signals. **Journal of Immunology**, 183 (7): 4493–4501

Shuford, W.W., Klussman, K., Tritchler, D.D., et al. (1997) 4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. **The Journal of experimental medicine**, 186 (1): 47–55

Siefken, R., Kurrle, R. and Schwinzer, R. (1997) CD28-Mediated Activation of Resting Human T Cells without Costimulation of the CD3/TCR Complex. **Cellular Immunology**, 176: 59–65

Simpson, T.R., Li, F., Montalvo-Ortiz, W., et al. (2013) Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. **Journal of Experimental Medicine**, 210 (9): 1695–1710

Simpson, T.R., Quezada, S.A. and Allison, J.P. (2010) Regulation of CD4 T cell activation and effector function by inducible costimulator (ICOS). **Current opinion in immunology**, 22 (3): 326–332

Sims, T.N., Soos, T.J., Xenias, H.S., et al. (2007) Opposing Effects of PKCθ and WASp on Symmetry Breaking and Relocation of the Immunological Synapse. **Cell**, 129 (4): 773–785

Singh, K., Deshpande, P., Pryshchep, S., et al. (2009) ERK-dependent T cell receptor threshold calibration in rheumatoid arthritis. **The Journal of Immunology**, 183 (12): 8258–8267

Skokos, D., Shakhar, G., Varma, R., et al. (2007) Peptide-MHC potency governs dynamic interactions between T cells and dendritic cells in lymph nodes. **Nature Immunology**, 8 (8): 835–844

Slinker, B.K. (1998) The Statistics of Synergism. **Journal of Molecular and Cellular Cardiology**, 30: 723–731

Sloan-Lancaster, J., Shaw, A.S., Rothbard, J.B., et al. (1994) Partial T Cell Signaling: Altered Phospho-ζ and Lack of Zap70 Recruitment in APL-Induced T Cell Anergy. **Cell**, 79 (5): 913–922

Sollid, L.M. and Jabri, B. (2011) Celiac disease and transglutaminase 2: a model for posttranslational modification of antigens and HLA association in the pathogenesis of autoimmune disorders. **Current opinion in immunology**

Song, J., Salek-Ardakani, S., So, T., et al. (2007) The kinases aurora B and mTOR regulate the G1-S cell cycle progression of T lymphocytes. **Nature Immunology**, 8 (1): 64–73

Speiser, D.E., Bachmann, M.F., Shahinian, A., et al. (1997) Acute graft-versus-host disease without costimulation via CD28. **Transplantation**, 63 (7): 1042–1044

Stamper, C.C., Zhang, Y., Tobin, J.F., et al. (2001) Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses. **Nature**, 410 (6828): 608–611

Stefanová, I., Dorfman, J.R. and Germain, R.N. (2002) Self-recognition promotes the foreign antigen sensitivity of naive T lymphocytes. **Nature**, 420 (6914): 429–434

Stein, P.L., Lee, H.M., Rich, S., et al. (1992) pp59^{fyn} Mutant Mice Display Differential Signaling in Thymocytes and Peripheral T Cells. **Cell**, 70 (5): 741–750

Stolt, P., Bengtsson, C., Nordmark, B., et al. (2003) Quantification of the influence of cigarette smoking on rheumatoid arthritis: results from a population based case-control study, using incident cases. **Annals of the Rheumatic Diseases**, 62 (9): 835–841

Stout, R.D., Suttles, J., Xu, J., et al. (1996) Impaired T cell-mediated macrophage activation in CD40 ligand-deficient mice. **Journal of Immunology**, 156 (1): 8–11

Strutt, T.M., McKinstry, K.K. and Swain, S.L. (2011) Control of innate immunity by memory CD4 T Cells. **Adv Exp Med Biol**, 780 (6): 57–68

Su, L.F., Kidd, B.A., Han, A., et al. (2013) Virus-specific CD4$^+$ memory-phenotype T cells are abundant in unexposed adults. **Immunity**, 38 (2): 373–383

Suchard, S.J., Davis, P.M., Kansal, S., et al. (2013) A Monovalent Anti-Human CD28 Domain Antibody Antagonist: Preclinical Efficacy and Safety. **Journal of Immunology**, 191 (9): 4599–4610

Suh, W.-K., Tafuri, A.A., Berg-Brown, N.N.N., et al. (2004) The inducible costimulator plays the major costimulatory role in humoral immune responses in the absence of CD28. **Journal of Immunology**, 172 (10): 5917–5923

Suzuki, H., Kündig, T.M., Furlonger, C., et al. (1995) Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta. **Science**, 268 (5216): 1472–1476

Szabo, S.J., Kim, S.T., Costa, G.L., et al. (2000) A Novel Transcription Factor, T-bet, Directs Th1 Lineage Commitment. **Cell**, 100 (6): 655–669

Tabares, P., Berr, S., Römer, P.S., et al. (2014) Human regulatory T cells are selectively activated by low-dose application of the CD28 superagonist TGN1412/TAB08. **Eur. J. Immunol.**, 44 (4): 1225–1236

Tacke, M., Hanke, G., Hanke, T., et al. (1997) CD28-mediated induction of proliferation in resting T cells in vitro and in vivo without engagement of the T cell receptor: evidence for functionally distinct forms of CD28. **Eur. J. Immunol.**, 27 (1): 239–247

Tada, Y., Nagasawa, K., Ho, A., et al. (1999a) CD28-Deficient Mice Are Highly Resistant to Collagen-Induced Arthritis. **Journal of Immunology**, 162 (1): 203–208

Tada, Y., Nagasawa, K., Ho, A., et al. (1999b) Role of the Costimulatory Molecule CD28 in the Development of Lupus in MRL/lpr Mice. **Journal of Immunology**, 163 (6): 3153–3159

Tadokoro, C.E., Shakhar, G., Shen, S., et al. (2006) Regulatory T cells inhibit stable contacts between CD4^{+} T cells and dendritic cells in vivo. **The Journal of experimental medicine**, 203: 505–511

Tafuri, A., Shahinian, A., Bladt, F., et al. (2001) ICOS is essential for effective T-helper-cell responses. **Nature**, 409 (6816): 105–109

Tai, X., Cowan, M., Feigenbaum, L., et al. (2005) CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. **Nature Immunology**, 6 (2): 152–162

Tan, P., Anasetti, C., Hansen, J.A., et al. (1993) Induction of alloantigen-specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. **The Journal of experimental medicine**, 177: 165–173

Tan, Y.X., Manz, B.N., Freedman, T.S., et al. (2014) Inhibition of the kinase Csk in thymocytes reveals a requirement for actin remodeling in the initiation of full TCR signaling. **Nature Immunology**, 15 (2): 186–194

Tang, Q., Adams, J.Y., Tooley, A.J., et al. (2006) Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. **Nature Immunology**, 7: 83–92

Tang, Q., Henriksen, K.J., Boden, E.K., et al. (2003) Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. **Journal of Immunology**, 171 (7): 3348–3352

Taniguchi, T. and Minami, Y. (1993) The IL-2/IL-2 receptor system: a current overview. **Cell**, 73 (1): 5–8

Tao, X., Constant, S., Jorritsma, P., et al. (1997a) Strength of TCR Signal Determines the Costimulatory Requirements for Th1 and Th2 CD4^{+} T Cell Differentiation. **Journal of Immunology**, 159: 5956–5963

Tao, X., Grant, C., Constant, S., et al. (1997b) Induction of IL-4-Producing CD4^{+} T cells by Antigenic Peptides Altered for TCR Binding. **Journal of Immunology**, 158 (9): 4237–4244

Testi, R., D'Ambrosio, D., De Maria, R., et al. (1994) The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. **Immunology Today**, 15 (10): 479–483

Thien, R., Baier, K., Pietschmann, P., et al. (2005) Interactions of 1 alpha,25-dihydroxyvitamin D3 with IL-12 and IL-4 on cytokine expression of human T lymphocytes. **The Journal of allergy and clinical immunology**, 116 (3): 683–689

Timson Gauen, L.K., Kong, A.N., Samelson, L.E., et al. (1992) p59fyn Tyrosine Kinase Associates with Multiple T-Cell Receptor Subunits through Its Unique Amino-Terminal Domain. **Molecular and Cellular Biology**, 12 (12): 5438–5446

Tivol, E.A., Borriello, F., Scweitzer, A.N., et al. (1995) Loss of CTLA-4 Leads to Massive Lymphoproliferation and Fatal Multiorgan Tissue Destruction, Revealing a Critical Negative Regulatory Role of CTLA-4. **Immunity**, 3 (5): 541–547

Toyooka, K., Maruo, S., Iwahori, T., et al. (1996) CD28 co-stimulatory signals induce IL-2 receptor expression on antigen-stimulated virgin T cells by an IL-2-independent mechanism. **International Immunology**, 8 (2): 159–169

Tran, C.N., Lundy, S.K., White, P.T., et al. (2007) Molecular Interactions between T Cells and Fibroblast-Like Synoviocytes: Role of Membrane Tumor Necrosis Factor-α on Cytokine-Activated T Cells. **The American Journal of Pathology**, 171 (5): 1588–1598

Tran, C.N., Thacker, S.G., Louie, D.M., et al. (2008) Interactions of T cells with fibroblast-like synoviocytes: role of the B7 family costimulatory ligand B7-H3. **Journal of Immunology**, 180 (5): 2989–2998

Tsoukas, C.D., Provvedini, D.M. and Manolagas, S.C. (1984) 1,25-dihydroxyvitamin D3: a novel immunoregulatory hormone. **Science**, 224 (4656): 1438–1440

Tumes, D.J., Onodera, A., Suzuki, A., et al. (2013) The Polycomb Protein Ezh2 Regulates Differentiation and Plasticity of CD4$^+$ T Helper Type 1 and Type 2 Cells. **Immunity**, 39 (5): 819–832

Umlauf, S.W., Beverly, B., Lantz, O., et al. (1995) Regulation of Interleukin 2 Gene Expression by CD28 Costimulation in Mouse T-Cell Clones: both Nuclear and Cytoplasmic RNAs Are Regulated with Complex Kinetics. **Molecular and Cellular Biology**, 15: 3197–3205

Unutmaz, D., Pileri, P. and Abrignani, S. (1994) Antigen-independent Activation of Naive and Memory Resting T Cells by a Cytokine Combination. **The Journal of experimental medicine**, 180 (3): 1159–1164

Valitutti, S. and Lanzavecchia, A. (1997) Serial triggering of TCRs: a basis for the sensitivity and specificity of antigen recognition. **Immunology Today**, 18 (6): 299–304

Valitutti, S., Müller, S., Cella, M., et al. (1995) Serial triggering of many T-cell receptors by a few peptide-MHC complexes. **Nature**, 375 (6527): 148–151

van den Borne, B., Landewe, R., The, H., et al. (1999) Cyclosporin A therapy in rheumatoid arthritis: only strict application of the guidelines for safe use can prevent irreversible renal function loss. **Rheumatology**, 38 (3): 254–259

van der Linden, M.P.M., le Cessie, S., Raza, K., et al. (2010) Long-Term

Impact of Delay in Assessment of Patients With Early Arthritis. **Arthritis & Rheumatism**, 62 (12): 3537–3546

van Gestel, A.M., Anderson, J.J., van Riel, P.L., et al. (1999) ACR and EULAR improvement criteria have comparable validity in rheumatoid arthritis trials. American College of Rheumatology European League of Associations for Rheumatology. **The Journal of Rheumatology**, 26 (3): 705–711

Van Gool, S.W., Ceuppens, J.L., Walter, H., et al. (1994) Synergy between cyclosporin A and a monoclonal antibody to B7 in blocking alloantigen-induced T-cell activation. **Blood**, 83 (1): 176–183

van Hamburg, J.P., Asmawidjaja, P.S., Davelaar, N., et al. (2011) Th17 Cells, but Not Th1 Cells, From Patients With Early Rheumatoid Arthritis Are Potent Inducers of Matrix Metalloproteinases and Proinflammatory Cytokines Upon Synovial Fibroblast Interaction, Including Autocrine Interleukin-17A Production. **Arthritis & Rheumatism**, 63 (1): 73–83

van Panhuys, N., Klauschen, F. and Germain, R.N. (2014) T-Cell-Receptor-Dependent Signal Intensity Dominantly Controls $CD4^+$ T Cell Polarization In Vivo. **Immunity**, 41 (1): 63–74

van Steenbergen, H.W., Huizinga, T.W.J. and van der Helm-van Mil, A.H.M. (2013) The Preclinical Phase of Rheumatoid Arthritis: What Is Acknowledged and What Needs to Be Assessed? **Arthritis & Rheumatism**, 65 (9): 2219–2232

Vanham, G., Ceuppens, J.L. and Bouillon, R. (1989) T Lymphocytes and Their CD4 Subset Are Direct Targets for the Inhibitory Effect of Calcitriol. **Cellular Immunology**, 124 (2): 320–333

Vanhove, B., Laflamme, G., Coulon, F., et al. (2003) Selective blockade of CD28 and not CTLA-4 with a single-chain Fv-α_1-antitrypsin fusion antibody. **Blood**, 102 (2): 564–570

Vantourout, P. and Hayday, A. (2013) Six-of-the-best: unique contributions of γδ T cells to immunology. **Nature Reviews Immunology**, 13 (2): 88–100

Veillette, A., Bookman, M.A., Horak, E.M., et al. (1988) The CD4 and CD8 T Cell Surface Antigens Are Associated with the Internal Membrane Tyrosine-Krotein kinase p56lck. **Cell**, 55 (2): 301–308

Veldhoen, M., Uyttenhove, C., van Snick, J., et al. (2008) Transforming growth factor-β "reprograms" the differentiation of T helper 2 cells and promotes an interleukin 9–producing subset. **Nature Immunology**, 9 (12): 1341–1346

Vinay, D.S. and Kwon, B.S. (1998) Role of 4-1BB in immune responses. **Seminars in Immunology**, 10 (6): 481–489

Vincenti, F., Charpentier, B., Vanrenterghem, Y., et al. (2010) A phase III study of belatacept-based immunosuppression regimens versus cyclosporine

in renal transplant recipients (BENEFIT study). **American Journal of Transplantation**, 10 (3): 535–546

Vincenti, F., Larsen, C., Durrbach, A., et al. (2005) Costimulation Blockade with Belatacept in Renal Transplantation. **New England Journal of Medicine**, 353 (8): 770–781

Viola, A. and Lanzavecchia, A. (1996) T cell activation determined by T cell receptor number and tunable thresholds. **Science**, 273 (5271): 104–106

vonEssen, M.R., Kongsbak, M., Levring, T.B., et al. (2013) PKCθ exists in an oxidized inactive form in naive human T cells. **Eur. J. Immunol.**, 43: 1659–1666

vonEssen, M.R., Kongsbak, M., Schjerling, P., et al. (2010) Vitamin D controls T cell antigen receptor signaling and activation of human T cells. **Nature Immunology**, 11 (4): 344–349

Vrisekoop, N., Monteiro, J.P., Mandl, J.N., et al. (2014) Revisiting thymic positive selection and the mature T cell repertoire for antigen. **Immunity**, 41 (2): 181–190

Wakamatsu, E., Mathis, D. and Benoist, C. (2013) Convergent and divergent effects of costimulatory molecules in conventional and regulatory CD4+ T cells. **Proc. Natl. Acad. Sci. U.S.A.**, 110 (3): 1023–1028

Walker, L.S., Gulbranson-Judge, A., Flynn, S., et al. (1999) Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers. **The Journal of experimental medicine**, 190 (8): 1115–1122

Walker, L.S.K. and Sansom, D.M. (2011) The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. **Nature Reviews Immunology**, 11 (12): 852–863

Walker, L.S.K. and Sansom, D.M. (2015) Confusing signals: Recent progress in CTLA-4 biology. **Trends in immunology**, 36 (2): 63–70

Walunas, T.L., Lenschow, D.J., Bakker, C.Y., et al. (1994) CTLA-4 can function as a negative regulator of T cell activation. **Immunity**, 1 (5): 405–413

Wang, B., Maile, R., Greenwood, R., et al. (2000) Naive CD8+ T cells do not require costimulation for proliferation and differentiation into cytotoxic effector cells. **Journal of Immunology**, 164 (3): 1216–1222

Wang, C.J., Heuts, F., Ovcinnikovs, V., et al. (2015a) CTLA-4 controls follicular helper T-cell differentiation by regulating the strength of CD28 engagement. **Proc. Natl. Acad. Sci. U.S.A.**, 112 (2): 524–529

Wang, C.J., Kenefeck, R., Wardzinski, L., et al. (2012) Cutting Edge: Cell-Extrinsic Immune Regulation by CTLA-4 Expressed on Conventional T Cells. **Journal of Immunology**, 189 (3): 1118–1122

Wang, H., Kadlecek, T.A., Au-Yeung, B.B., et al. (2010a) ZAP-70: an essential kinase in T-cell signaling. **Cold Spring Harbor Perspectives in Biology**, 2 (5): a002279

Wang, H., Wei, B., Bismuth, G., et al. (2009) SLP-76-ADAP adaptor module regulates LFA-1 mediated costimulation and T cell motility. **PNAS**, 106 (30): 12436–12441

Wang, J., Yoshida, T., Nakaki, F., et al. (2005) Establishment of NOD-Pdcd1-/- mice as an efficient animal model of type I diabetes. **Proc. Natl. Acad. Sci. U.S.A.**, 102 (33): 11823–11828

Wang, L. and Bosselut, R. (2009) CD4-CD8 Lineage Differentiation: Thpoking into the Nucleus. **Journal of Immunology**, 183 (5): 2903–2910

Wang, S., Liu, J., Wang, M., et al. (2010b) Treatment and Prevention of Experimental Autoimmune Myocarditis with CD28 Superagonists. **Cardiology**, 115 (2): 107–113

Wang, T., Sun, X., Zhao, J., et al. (2015b) Regulatory T cells in rheumatoid arthritis showed increased plasticity toward Th17 but retained suppressive function in peripheral blood. **Annals of the Rheumatic Diseases**, 74 (6): 1293–1301

Wange, R.L. and Samelson, L.E. (1996) Complex Complexes: Signaling at the TCR. **Immunity**, 5: 197–205

Watanabe, N., Gavrieli, M., Sedy, J.R., et al. (2003) BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. **Nature Immunology**, 4 (7): 670–679

Waterhouse, P., Penninger, J.M., Timms, E., et al. (1995) Lymphoproliferative disorders with Early Lethality in Mice Deficient in *Ctla-4*. **Science**, 270 (5238): 985–988

Watts, T.H. and DeBenedette, M.A. (1999) T cell co-stimulatory molecules other than CD28. **Current opinion in immunology**, 11: 286–293

Weaver, C.T., Hatton, R.D., Mangan, P.R., et al. (2007) IL-17 Family cytokines and the expanding Diversity of Effector T Cell Lineages. **Immunology**, 25: 821–852

Weaver, T.A., Charafeddine, A.H., Agarwal, A., et al. (2009) Alefacept promotes co-stimulation blockade based allograft survival in nonhuman primates. **Nature medicine**, 15 (7): 746–749

Webb, L.M., Walmsley, M.J. and Feldmann, M. (1996) Prevention and amelioration of collagen-induced arthritis by blockade of the CD28 co-stimulatory pathway: requirement for both B7-1 and B7-2. **Eur. J. Immunol.**, 26 (10): 2320–2328

Wei, F., Zhong, S., Ma, Z., et al. (2013) Strength of PD-1 signaling

differentially affects T-cell effector functions. **PNAS**, 110 (27): E2480–9

Weinberg, A.D., Vella, A.T. and Croft, M. (1998) OX-40: life beyond the effector T cell stage. **Seminars in Immunology**, 10 (6): 471–480

Weinblatt, M., Schiff, M., Goldman, A., et al. (2007) Selective costimulation modulation using abatacept in patients with active rheumatoid arthritis while receiving etanercept: a randomised clinical trial. **Ann. Rheum. Dis.**, 66: 228–234

Wen, T., Bukczynski, J. and Watts, T.H. (2002) 4-1BB ligand-mediated costimulation of human T cells induces CD4 and CD8 T cell expansion, cytokine production, and the development of cytolytic effector function. **Journal of Immunology**, 168 (10): 4897–4906

Wenink, M.H., Santegoets, K.C.M., Platt, A.M., et al. (2012) Abatacept modulates proinflammatory macrophage responses upon cytokine-activated T cell and Toll-like receptor ligand stimulation. **Annals of the Rheumatic Diseases**, 71 (1): 80–83

Wildin, R.S., Ramsdell, F., Peake, J., et al. (2001) X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. **Nature Genetics**, 27 (1): 18–20

Wilson, D.B., Wilson, D.H., Schroder, K., et al. (2004) Specificity and degeneracy of T cells. **Molecular Immunology**, 40: 1047–1055

Wing, K., Onishi, Y., Prieto-Martin, P., et al. (2008) CTLA-4 control over Foxp3$^+$ Regulatory T Cell Function. **Science**, 322 (5899): 271–275

Wood, J.P., Pani, M.A., Bieda, K., et al. (2002) A recently described polymorphism in the CD28 gene on chromosome 2q33 is not associated with susceptibility to type 1 diabetes. **European Journal of Immunogenetics**, 29 (4): 347–349

Woodward, J.E., Bayer, A.L., Chavin, K.D., et al. (1998) T-cell alterations in cardiac allograft recipients after B7 (CD80 and CD86) blockade. **Transplantation**, 66 (1): 14–20

Wooldridge, L., Ekeruche-Makinde, J., van den Berg, H.A., et al. (2012) A Single Autoimmune T Cell Receptor Recognizes More Than a Million Different Peptides. **Journal of Biological Chemistry**, 287 (2): 1168–1177

Wu, J., Katrekar, A., Honigberg, L.A., et al. (2006) Identification of substrates of human protein-tyrosine phosphatase PTPN22. **Journal of Biological Chemistry**, 281 (16): 11002–11010

Wu, Y., Xu, J., Shinde, S., et al. (1995) Rapid induction of a novel costimulatory activity on B cells by CD40 ligand. **Current Biology**, 5 (11): 1303–1311

Wucherpfennig, K.W., Gagnon, E., Call, M.J., et al. (2010) Structural biology

of the T-cell receptor: insights into receptor assembly, ligand recognition, and initiation of signaling. **Cold Spring Harbor Perspectives in Biology**, 2 (4): a005140

Xing, Y. and Hogquist, K.A. (2012) T-cell tolerance: central and peripheral. **Cold Spring Harbor Perspectives in Biology**, 4: a006957

Yamada, A., Salama, A.D. and Sayegh, M.H. (2002) The role of novel T cell costimulatory pathways in autoimmunity and transplantation. **Journal of the American Society of Nephrology**, 13 (2): 559–575

Yamada, A., Salama, A.D., Sho, M., et al. (2005) CD70 signaling is critical for CD28-independent CD8^{+} T cell-mediated alloimmune responses in vivo. **Journal of Immunology**, 174 (3): 1357–1364

Yao, S., Zhu, Y., Zhu, G., et al. (2011) B7-H2 is a Costimulatory Ligand for CD28 in Human. **Immunity**, 34 (5): 729–740

Yarwood, A., Huizinga, T.W.J. and Worthington, J. (2014) The genetics of rheumatoid arthritis: risk and protection in different stages of the evolution of RA. **Rheumatology**, [Epub ahead of print]

Ye, C.J., Feng, T., Kwon, H.-K., et al. (2014) Intersection of population variation and autoimmunity genetics in human T cell activation. **Science**, 345 (6202): 1254665–1254665

Yokosuka, T., Takamatsu, M., Kobayashi-Imanishi, W., et al. (2012) Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. **The Journal of experimental medicine**, 209: 1201–1217

Yoshinaga, S.K., Whoriskey, J.S., Khare, S.D., et al. (1999) T-cell co-stimulation through B7RP-1 and ICOS. **Nature**, 402 (6763): 827–832

Yu, X.Z., Martin, P.J. and Anasetti, C. (1998) Role of CD28 in acute graft-versus-host disease. **Blood**, 92 (8): 2963–2970

Zamzami, N., Marchetti, P., Castedo, M., et al. (1995) Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. **The Journal of experimental medicine**, 181 (5): 1661–1672

Zehn, D., Lee, S.Y. and Bevan, M.J. (2009) Complete but curtailed T-cell response to very low-affinity antigen. **Nature Immunology**, 458 (7235): 211–214

Zendman, A.J.W., van Venrooij, W.J. and Pruijn, G.J.M. (2006) Use and significance of anti-CCP autoantibodies in rheumatoid arthritis. **Rheumatology**, 45 (1): 20–25

Zeng, H. and Chi, H. (2014) mTOR signaling and transcriptional regulation in T lymphocytes. **Transcription**, 5 (2): e28263

Zeng, R., Cannon, J.L., Abraham, R.T., et al. (2003) SLP-76 Coordinates Nck-Dependent Wiskott-Aldrich Syndrome Protein Recruitment with Vav-1/Cdc42-Dependent Wiskott-Aldrich Syndrome Protein Activation at the T cell-APC Contact Site. **Journal of Immunology**, 171 (3): 1360–1368

Zhang, J. and Braun, M.Y. (2014) PD-1 deletion restores susceptibility to experimental autoimmune encephalomyelitis in miR-155-deficient mice. **International Immunology**, 26 (7): 407–415

Zhang, N. and Bevan, M.J. (2011) CD8$^+$ T cells: Foot Soldiers of the Immune System. **Immunity**, 35 (2): 161–168

Zhang, R., Huynh, A., Whitcher, G., et al. (2013) An obligate cell-intrinsic function for CD28 in Tregs. **The Journal of clinical investigation**, 123 (2): 580–593

Zhao, D.-M., Thornton, A.M., DiPaolo, R.J., et al. (2006) Activated CD4$^+$CD25$^+$ T cells selectively kill B lymphocytes. **Blood**, 107 (10): 3925–3932

Zheng, S.G., Wang, J.H., Stohl, W., et al. (2006) TGF-β Requires CTLA-4 Early after T Cell Activation to Induce FoxP3 and Generate Adaptive CD4$^+$CD25$^+$ Regulatory Cells. **Journal of Immunology**, 176 (6): 3321–3329

Zheng, W. and Flavell, R.A. (1997) The Transcription Factor GATA-3 Is Necessary and Sufficient for Th2 Cytokine Gene Expression in CD4 T Cells. **Cell**, 89 (4): 587–596

Zheng, Y., Delgoffe, G.M., Meyer, C.F., et al. (2009) Anergic T cells are metabolically anergic. **Journal of Immunology**, 183 (10): 6095–6101

Zheng, Y., Manzotti, C.N., Liu, M., et al. (2004) CD86 and CD80 differentially modulate the suppressive function of human regulatory T cells. **Journal of Immunology**, 172 (5): 2778–2784

Zhou, L., Ivanov, I.I., Spolski, R., et al. (2007) IL-6 programs T$_H$-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. **Nature Immunology**, 8 (9): 967–974

Zhu, Y., Ljunggren, H., Mix, E., et al. (2001) CD28-B7 costimulation: a critical role for initiation and development of experimental autoimmune neuritis in C57BL/6 mice. **Journal of Neuroimmunology**, 114 (1-2): 114–121

Ziegler, S.F. (2006) FOXP3: Of mice and men. **Ann. Rev. Immunol.**, 24: 209–226