CD28 COSTIMULATION IN T CELLS:

REQUIREMENTS, OUTCOMES AND REGULATION

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ABSTRACT

The costimulatory receptor CD28 and its inhibitory counterpart CTLA-4 share the same ligands and comprise a crucial checkpoint in T cell activation. We previously described a novel mechanism of CTLA-4 function, whereby CTLA-4 removes its ligands from antigen presenting cells by trans-endocytosis. This reduces the availability of costimulatory ligands for CD28 engagement and thereby regulates T cell activation. The main aims of this project were to study the mechanism of trans-endocytosis and its use by other regulatory T cell receptors, and to begin to examine the functional implications of reducing the availability of costimulatory molecules for CD4 T cell responses.

Surprisingly, it was found that the YVKM motif required for CTLA-4 endocytosis is not essential for trans-endocytosis. Unexpectedly, it was revealed that PD-1 and OX40 can also internalise their ligands, although perhaps not via the same mechanism as CTLA-4 trans-endocytosis. It was also shown that altering the availability of CD28 ligands affects the extent of T cell proliferation, suggesting that CTLA-4 trans-endocytosis can finely tune the T cell response. Furthermore, it was observed that CD28 costimulation is not always required for T cell activation and proliferation, but CD28 engagement is required for the optimal upregulation of a number of effector proteins and for T_H2 cytokine production. Interestingly, T cells activated in the absence of CD28 signalling were not classically anergic. Strikingly, it was also found that memory T cells are dependent on CD28 costimulation.

These findings regarding the mechanism of CTLA-4 trans-endocytosis, CD4 T cell requirements for costimulation and the outcomes of CD28 blockade may hopefully lead to the design of more specific and effective therapies for autoimmunity, cancer and transplant rejection.

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ABBREVIATIONS AND ACRONYMS

ABC = antibody binding capacity
AKT = protein kinase B (PKB)
AP-1 = activator protein 1
AP-2 = adaptor protein 2
APC = antigen presenting cells
Bcl-xL = B cell lymphoma-extra large
BSA = bovine serum albumin
Cbl-b = Casitas B-lineage lymphoma
CCR7 = chemokine receptor 7
CD = cluster of differentiation
CHO = Chinese hamster ovary
CSK = C-terminal SRC kinase
CTLA-4 = cytotoxic T lymphocyte antigen-4
CTV = cell trace violet
CXCR5 = chemokine receptor 5
DC = dendritic cells
EDTA = ethylenediamine tetra-acetic acid
Egr = early growth response protein
FACS = fluorescence activated cell sorting
FoxP3 = forkhead box P3
Fyn = proto-oncogene tyrosine-protein kinase
GADS = GRB2-related adaptor protein
GFP = green fluorescent protein
GRAIL = gene related to anergy in lymphocytes
GRB2 = growth factor receptor bound protein 2
ICOS = inducible T cell costimulator
IFN = interferon
IL = interleukin
ICAM = intercellular adhesion molecule
IP_3 = inositol-1,4,5-triphosphate
ITAM = immunoreceptor tyrosine-based activation motif
Itch = itchy homologue E3 ubiquitin protein ligase
ITK = interleukin 2-inducible T cell kinase

iTreg = induced Treg

LAT = linker for activation of T cells

LBPA = lysobisphosphatidic acid

Lck = lymphocyte-specific protein tyrosine kinase

LFA = lymphocyte function-associated antigen

Lyp = lymphoid tyrosine phosphatase

MACS = magnetic-activated cell separation

MAPK = mitogen-activated protein kinase

MFI = mean fluorescence intensity

MHC = major histocompatibility complex

mTOR = mammalian target of rapamycin

NFAT = nuclear factor of activated T cells

NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells

PAG = phosphprotein associated with glycosphingolipid-enriched microdomains

PBMC = peripheral blood mononuclear cells

PBS = phosphate buffered saline

PCR = polymerase chain reaction

PD-1 = programmed cell death protein 1

PDK1 = phosphoinositide-dependent kinase 1

PI3K = phosphoinositide 3-kinase

PIP_2 = phosphatidylinositol-4,5-bisphosphate

PKCθ = protein kinase C-theta

PLCγ1 = phospholipase C-gamma 1

PMA = phorbol myristate acetate

PTPN22 = protein tyrosine phosphatase non-receptor type 22 (also known as Lyp)

Ras = rat sarcoma (GTPase)

RPMI = Roswell Park Memorial Institute

rtTA = reverse transactivator protein

RORγt = RAR-related orphan receptor gamma 2

SD = standard deviation

SEM = standard error of the mean

SLP76 = SH2 domain-containing leukocyte protein of 76kDa

T-bet = T-box transcription factor TBX21

Tet = tetracycline

TCR = T cell receptor

TGFβ = transforming growth factor-beta

T_H = T helper

TNFα = tumour necrosis factor-alpha

TRE = tetracycline response element

Treg = regulatory T cell

ZAP70 = zeta-chain-associated protein kinase-70

1 INTRODUCTION

1.1 Immune system

The immune system is comprised of many types of highly specialised cells that act together to defend the body against a wide range of pathogenic microorganisms and parasites. There are two main stages of an immune response (Chaplin, 2010). Initially, a rapid innate response is triggered when immune cells recognise common pathogen-associated molecular patterns such as generic bacterial proteins and viral DNA or RNA. This non-specific response involves several cell types including granulocytes, that release cytotoxic substances to kill microbes or infected cells, and phagocytes that ingest and destroy microbes. Phagocytes break down the proteins they ingest and present them as peptides on their cell surface. Recognition of specific foreign peptides (also called antigens) by other cells initiates the adaptive immune response which produces long-lived memory cells that will provide immunity against subsequent encounters with the same antigen.

The adaptive immune response involves two types of lymphocyte - T cells and B cells. T cells are divided into two main groups according to the co-receptor they express. CD8 T cells are largely responsible for the destruction of virally-infected cells while CD4 T cells produce cytokines that promote phagocytosis and provide help for B cell activation. Activated B cells secrete antigen-specific antibodies that either directly neutralise pathogens or label them as targets for destruction by other cells. The specificity of the adaptive immune response makes it more efficient and limits unwanted tissue damage. However, ironically, the random gene rearrangement necessary to produce the diversity of receptors required to recognise all possible pathogens can result in the generation of cells that recognise self peptides. Self-reactive lymphocytes can cause autoimmune disease where chronic inflammation leads to the destruction of host tissues (Davidson and Diamond, 2001; Ohashi, 2002). Therefore the immune system needs to be carefully regulated in order to allow effective responses against

infections while preventing autoimmunity. This project focuses on the regulation of CD4 T cells, in particular the characteristics and functions of the costimulatory molecules CD28 and CTLA-4 and related receptors. This chapter provides an overview of CD4 T cell biology then focuses on the current understanding of the roles of CD28 and CTLA-4 and their ligands followed by an outline of the main aims of this project.

1.2 T cell antigen recognition

Antigens must be presented in a certain way in order to be recognised by T cells, that is, they must be bound to a major histocompatibility complex (MHC) molecule. MHC molecules are cell surface glycoproteins, of which there are two classes (Bonilla and Oettgen, 2010). MHC class I molecules are expressed by all nucleated cell types. They present peptides that are derived from intracellular pathogens, especially viruses, and are recognised by the CD8 co-receptor. MHC class II molecules are only expressed by activated professional antigen presenting cells (APC) including B cells, dendritic cells (DC), monocytes and macrophages. MHC class II molecules display peptides derived from ingested pathogens to CD4 T cells. APC are activated by danger signals induced by bacterial proteins such as lipopolysaccharide (LPS) or by products of damaged cells (Matzinger, 2002). Activated APC not only present antigen to T cells but also provide additional stimulatory signals required for full T cell activation. APC and lymphocytes are brought into close contact in specialised lymphoid tissues and organs such as lymph nodes and the spleen in order for antigen surveillance and closer interactions to occur.

T cells recognise and bind antigen:MHC complexes via their T cell receptor (TCR) (**figure 1.1**). Unique TCR are created by genetic recombination of a number of possible variants for each TCR region. This occurs during T cell development in the thymus and results in the expression of a distinct TCR by every T cell. A positive selection process ensures that the TCR can recognise MHC molecules and then a process of negative selection deletes T cells

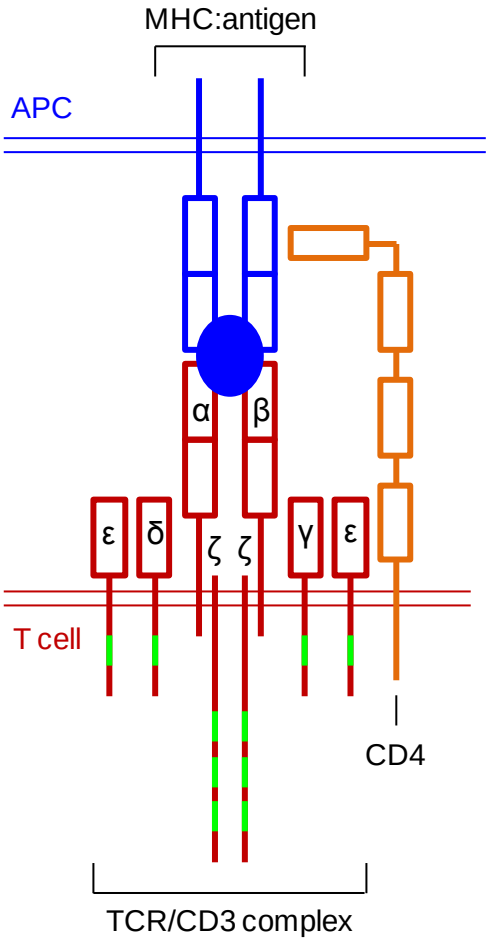


Figure 1.1 TCR structure and interaction with antigen:MHC. The TCR (red) is comprised of one α and one β chain and a CD3 complex which comprises two ζ chains, two ε chains, one δ chain and one γ chain. The α and β chains of the TCR bind their specific antigen (blue oval) while the co-receptor CD4 (orange) binds the MHC molecule (blue). This causes the phosphorylation of ITAMS (green) in the cytoplasmic tails of the CD3 chains.

whose TCR binds strongly to self peptides expressed by thymic medullary epithelial cells (Klein et al., 2009). Previously it was believed that a single TCR was specific for a single peptide antigen (Bonilla and Oettgen, 2010). However it is now appreciated that there are not enough T cells in the human body to recognise all the possible antigenic peptides if each TCR only recognises a single peptide sequence. In fact it has been found that a single TCR can recognise many different peptides in the context of a single MHC and this cross-reactivity allows less than 10^8 TCR to recognise a much greater number of peptides (Wooldridge et al., 2012; Sewell, 2012). Thymic selection should prevent the release of autoreactive cells into the periphery, however cells with low affinity for self antigens can escape and TCR cross-reactivity with foreign and self peptides may facilitate the development of autoimmune disease. Therefore further regulatory checkpoints are required in the periphery to avoid autoreactive T cell responses.

1.3 TCR signalling and immune synapse formation

The TCR of conventional T cells comprises of antigen-specific α and β chains and a CD3 complex (Wucherpfennig et al., 2010). TCR signalling occurs upon antigen recognition by the TCR and simultaneous binding of the co-receptor CD4 to MHC. The signalling cascade begins with the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) in the cytosolic domains of the CD3 chains by kinases Lck and Fyn, which are associated with CD4 and CD3 respectively. This initiates the recruitment, phosphorylation and activation of Zap70, which in turn activates the linker for activation of T cells (LAT). LAT recruits other kinases and molecules which mediate downstream signalling pathways leading to the activation of transcription factors such as NFAT that regulate genes controlling proliferation and differentiation (**figure 1.2**) (Brownlie and Zamoyska, 2013). TCR signalling via LAT also enhances cell adhesion by increasing the affinity of the integrin LFA-1 for its ligand ICAM-1. Additionally TCR signalling initiates actin polymerisation for the cytoskeletal remodelling required for signalling complex formation and immune synapse formation.

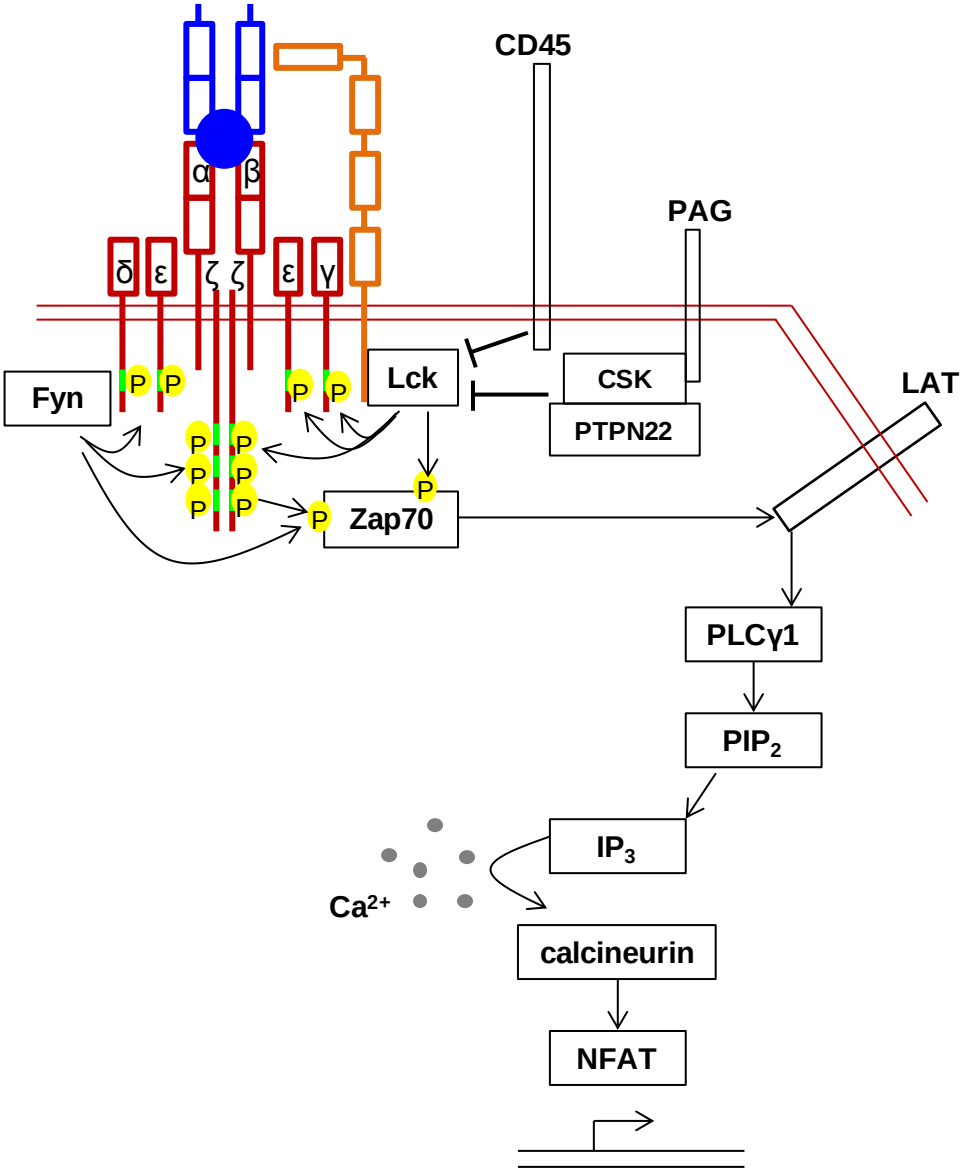


Figure 1.2 TCR signalling. Upon TCR (red) binding to antigen:MHC,(blue) the ITAMs (green) within the CD3 chains of the TCR complex are phosphorylated (yellow) by kinases Lck and Fyn, which are associated with CD4 (orange) and CD3 respectively. This allows Zap70 to be recruited and in turn phosphorylated and activated so it can recruit and phosphorylate the linker for activation of T cells (LAT). LAT recruits molecules such as SLP76, ITK, GADS, GRB2 and Vav to form a signalosome complex to mediate downstream signalling pathways (not shown). LAT also recruits PLCγ1 which hydrolyses PIP_2 to produce IP_3. IP_3 causes the release of calcium, which activates calcineurin, which activates the transcription factor NFAT. NFAT translocates to the nucleus and drives the transcription of genes associated with cell cycle progression and differentiation. TCR signalling is negatively regulated by phosphatases CD45 and PTPN22 which dephosphorylate the activating tyrosine residue of Lck to inactivate Lck. CSK also regulates Lck by phosphorylating an inhibitory tyrosine residue within Lck. CSK is recruited by PAG then binds to PTPN22.

An immune synapse forms at the area of contact between a T cell and APC. It has an organised structure with different molecules located in distinct regions (**figure 1.3**) (Monks et al., 1998; Huppa and Davis, 2003; Fooksman et al., 2010; Dustin et al., 2010; Rodriguez-Fernandez et al., 2010; Alarcon et al., 2011). Initially upon antigen binding TCR molecules form microclusters where TCR, Zap70 and LAT molecules are brought into close proximity to initiate proximal TCR signalling. Upon prolonged signalling TCR molecules translocate to a central supramolecular activation cluster (cSMAC) in the centre of the immune synapse. The cSMAC is surrounded by a region called the peripheral (p)SMAC which consists of a ring of adhesion molecules such as LFA-1. Around the pSMAC a distal (d)SMAC is formed comprising large proteins such as CD45. Generally smaller molecules are found at the centre of the immune synapse while large molecules are found around the edge, therefore it has been proposed that synapse formation is driven by molecule size (Burroughs and Wulfing, 2002). Conveniently, the immune synapse brings the TCR and Lck into close proximity and moves the inhibitory phosphatase CD45 away. Notably the type of immune synapse differs depending on the type of APC involved. The classic bull's eye pattern consisting of the three SMAC regions is characteristic of T cell-B cell interactions while T cell-DC contacts consist of multifocal synapses where TCR molecules cluster at multiple sites at the cell interface (Brossard et al., 2005). These multifocal immune synapses with DC are usually more transient, lasting only minutes as opposed to hours.

The function of the immune synapse has been controversial. Traditionally it was believed that synapse formation was necessary for prolonged TCR signalling and full T cell activation. However active Zap70 and Lck are often only detected in association with the TCR during the first 15-30 minutes following antigen encounter, before mature synapse formation (Lee et al., 2002). Indeed, tyrosine phosphorylated proteins are not found in the cSMAC of T cells activated with a strong agonist, although they are present in the cSMAC upon T cell stimulation with a weak agonist (Cemerski et al., 2008), suggesting that the immune synapse

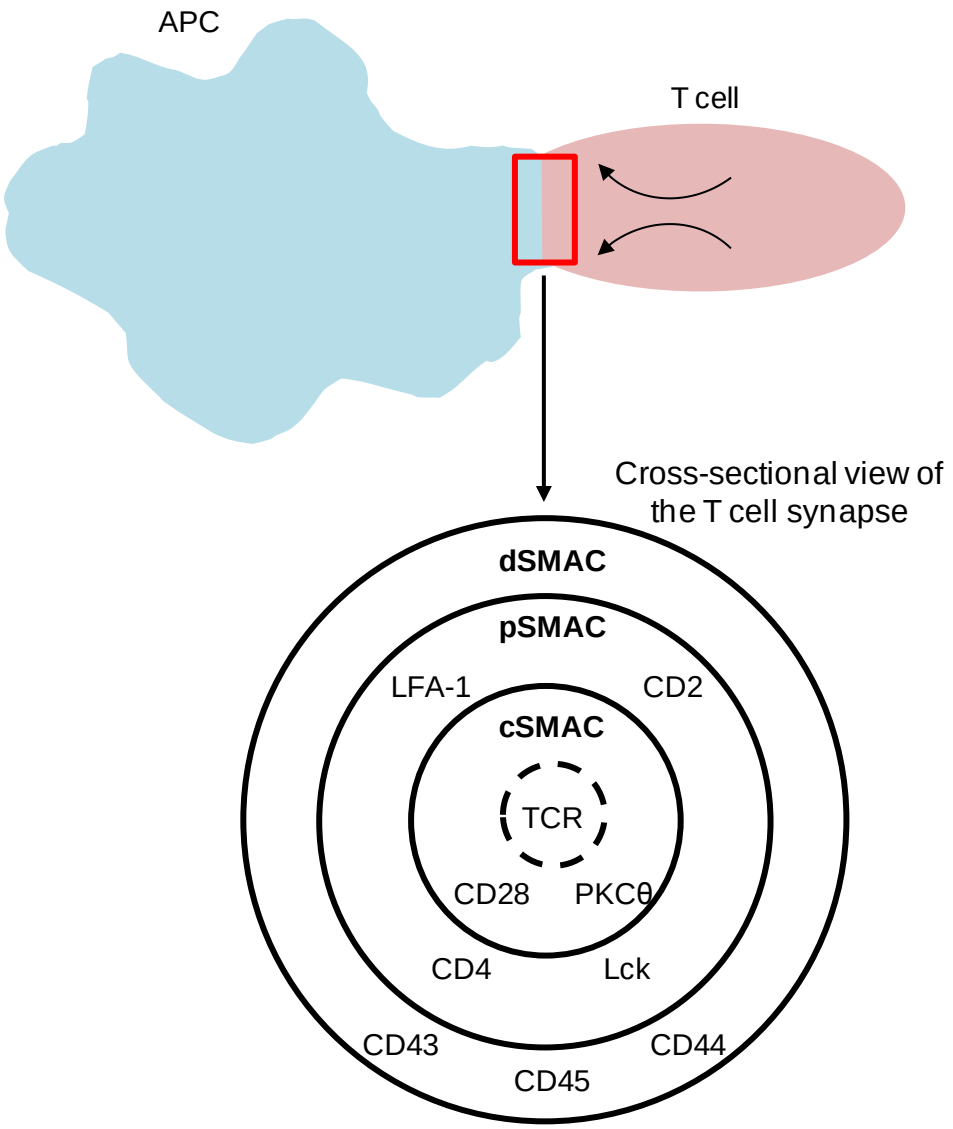


Figure 1.3 Immune synapse. Upon TCR signalling following recognition of specific peptide:MHC presented by an APC, cell surface receptors, filamentous actin and lipids polarise towards one end of the T cell (represented by arrows) to form an immune synapse at the area of contact between the T cell and APC (red box). A flat interface forms between the two cells where receptors and signalling molecules localise into distinct regions called supramolecular activation clusters (SMAC). TCR molecules translocate into the middle of the central (c)SMAC surrounded by CD28 and PKCθ. Around this the peripheral (p)SMAC forms comprising CD4, Lck and adhesion molecules LFA-1 and CD2. Large proteins such as CD43, CD44 and CD45 are excluded into the distal (d)SMAC.

may enhance or sustain TCR signalling in the context of weak antigen stimulation. More recent research has shown that within the cSMAC TCR molecules accumulate at the core while CD28 and PKCθ exist around the edge of the cSMAC, which is where signalling occurs (Tseng et al., 2008; Yokosuka et al., 2008). So the immune synapse is possibly not essential for T cell activation, but does help coordinate signalling molecules. Immune synapse formation is important for cell adhesion and promoting a long stable contact with an APC to allow the T cell to survey the range and amount of antigen presented by the APC (Dustin et al., 2010).

It is now appreciated that the immune synapse may actually play a role in TCR downregulation as the ubiquitin ligase Cbl-b and the multi-vesicular body marker LBPA are localised in the cSMAC with TCR molecules (Vardhana et al., 2010), indicative of active degradation and internalisation. Consistent with this, it has been shown that TCR molecules are continuously migrating from the dSMAC to the cSMAC, where they lose their association with other signalling molecules (Varma et al., 2006). It is also believed that the immune synapse may form to allow targeted cytokine release to and from both the T cell and APC to drive differentiation (see **section 1.4 CD4 T cell differentiation**). The IFNγ receptor has been found to localise preferentially to the immune synapse (Maldonado et al., 2004), suggesting that immune synapse formation could drive T_H1 differentiation. Additionally, the cell polarisation that occurs with immune synapse formation may promote asymmetric cell division, which can influence T cell differentiation and memory formation (Chang et al., 2007).

1.4 CD4 T cell differentiation

Upon T cell stimulation many transcriptional pathways are activated to drive the expression of proteins required for full activation, proliferation, effector functions and lineage commitment (Shipkova and Wieland, 2012). Early markers of T cell activation include the

interleukin (IL)-2 receptor alpha chain (CD25), CD69 and CD40-ligand (CD40L). CD25 and CD69 are involved in the initiation of T cell proliferation and CD40L is important for macrophage and B cell activation (Minami et al., 1993; Testi et al., 1989; van Kooten and Banchereau, 2000). Upregulation of the transferrin receptor CD71 is also important for the transport of iron into the cell to support the increase in metabolism required for T cell activation and expansion (Ponka and Lok, 1999). Numerous costimulatory molecules such as inducible costimulatory molecule (ICOS), cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed cell death protein-1 (PD-1), and OX40 are also upregulated, which have important regulatory and effector functions as described later (Bakdash et al., 2013).

In addition to the protein upregulation associated with T cell activation, many proteins are downregulated upon activation. T cells that have never encountered their specific antigen in the periphery are termed “naive” and they express naive T cell markers that make them easy to identify, which are downregulated on activated “effector” T cells. One way to distinguish naive and effector/memory T cells is based on which CD45 isoform they express. CD45 is a protein tyrosine phosphatase that plays a crucial role in T cell activation and different isoforms of its extracellular domain exist (Altin and Sloan, 1997). Naive T cells express CD45RA, which is the longest isoform, whereas effector T cells express CD45RO, which is the shortest (Clement, 1992). As well as the naive T cell marker CD45RA, homing receptors that are required by naive cells to enter secondary lymphoid tissues via high endothelial venules, such as CD62L and CCR7, are downregulated on effector cells (Picker et al., 1990; Sallusto et al., 1998). Another naive T cell marker is the costimulatory molecule CD27, which is expressed at low levels by naive T cells and is initially upregulated upon T cell activation, but its expression is eventually turned off in effector cells (de Jong et al., 1991; Hintzen et al., 1993).

CD4 T cells carry out many effector functions. For example they secrete intercellular signalling molecules called cytokines to help activate other immune cells. Therefore they are often referred to as T helper (T_H) cells. Activated T_H cells differentiate into various subtypes depending on their environment. T_H cell subtypes differ in their expression of transcription factors and each secrete of a particular range of cytokines (**figure 1.4**) (Zhu et al., 2010). The best characterised phenotypes are T_H1 and T_H2. IL-12 induces the development of T_H1 cells, which express the transcription factor T-bet and produce large amounts of IL-2 and IFN-γ. T_H1 cells promote cell-mediated immune responses via macrophage activation and CD8 T cell proliferation. On the other hand, IL-4 promotes differentiation towards the T_H2 phenotype where cells express the transcription factor GATA-3 and secrete mostly IL-4, IL-5 and IL-13, which are involved in antibody and allergic responses. Additional T_H cell subtypes have also been established including T_H17 cells which express the transcription factor RORγt and produce IL-17 and IL-22. T_H17 cells are usually involved in responses against bacterial infections by helping to recruit neutrophils and they are also associated with autoimmunity (Maddur et al., 2012). Another specialised subset of CD4 T cells are follicular T helper cells (T_{FH}), which express the transcription factor Bcl6 and are distinguished by their expression of CXCR5, PD-1, ICOS and IL-21 (Crotty, 2011). T_{FH} are required for the formation of germinal centres and subsequently the differentiation of B cells into antibody-producing plasma cells. It is also now believed that regulatory T cells (Treg) can be derived from peripheral CD4 T cells, which are referred to as induced Treg (iTreg) to distinguish them from natural Treg generated in the thymus. iTregs inhibit effector T cell expansion via both cell contact-mediated mechanisms and the secretion of anti-inflammatory cytokines such as TGF-β and IL-10 (Schmitt and Williams, 2013). Normally a single effector phenotype is predominant in a T cell response. However in order for T cells to terminally differentiate they must first receive all the necessary stimuli and become fully activated. TCR signalling alone is not usually sufficient for full T cell activation. A costimulatory signal is typically required in addition to

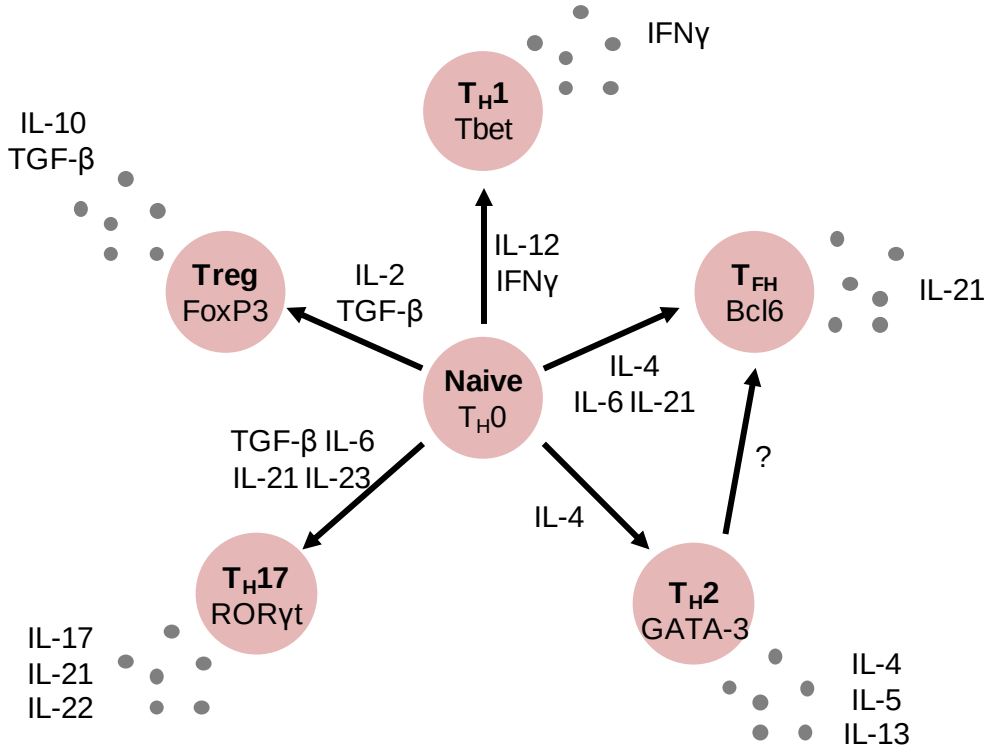


Figure 1.4 Naive T cells can differentiate into a variety of different lineages depending on the cytokine milieu. The cytokines that promote differentiation into each phenotype are indicated next to the arrows. The classification given to each subset is indicated in bold and the transcription factor which acts as the master regulator for each subset is also shown. The main cytokines produced by each cell type are shown.

antigen recognition in order to carefully control T cell activation. This is known as the two-signal model of T cell activation (**figure 1.5**).

1.5 Costimulatory pathways

The concept that a second signal is required for T cell activation was originally proposed by Lafferty and Woolnough (1977). This mechanism of positive regulation ensures that T cells are only activated in the presence of professional APC. This reduces the chance of a T cell being activated by self-peptides because costimulatory ligands are mainly expressed by APC activated by pathogen-induced danger signals and not by non-immune cells. There are several known costimulatory receptors that can stimulate naive T cells including CD28, CD2, LFA-1, CD81 and CD44. CD2 and its ligand LFA-3 are adhesion molecules responsible for promoting strong interactions between T cells and APC and they are also known to provide costimulation for T cell activation (Bierer et al., 1988; Green et al., 2000). Some reports suggest that CD2 enhances TCR signalling by promoting lipid raft aggregation to bring TCR molecules and signalling complexes into close proximity (Mestas and Hughes, 2001). Others suggest that CD2 can also directly induce activation signals (Kaizuka et al., 2009; Skanland et al., 2014). Another adhesion molecule LFA-1 and its ligand ICAM-1 can also provide a costimulatory signal to promote T cell activation (Van Seventer et al., 1990; Dubey et al., 1995). Alternatively, the tetraspanin molecule CD81 is also capable of costimulation (Sagi et al., 2012). There is no known ligand for CD81 but this receptor is believed to induce signal transduction through association with CD4 in tetraspanin-enriched microdomains (Yanez-Mo et al., 2009). Alternatively, CD44 is constitutively associated with the tyrosine kinases Lck and Fyn. Engagement of CD44 by hyaluronic acid promotes the recruitment of phosphokinases to amplify TCR signalling leading to T cell activation and proliferation (Foger et al., 2000). CD44 also rearranges the cytoskeleton to promote receptor clustering and enhance signal transduction (Foger et al., 2001). These receptors may enhance TCR

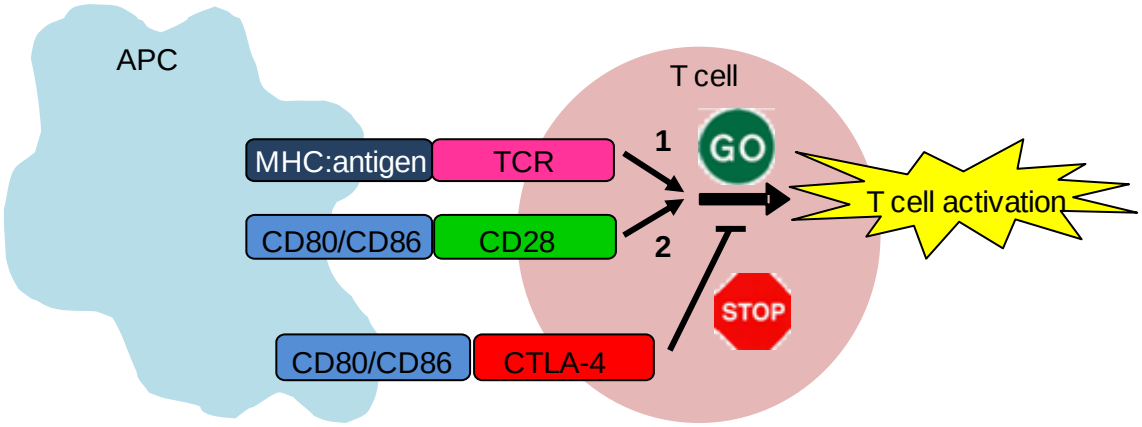


Figure 1.5 Two-signal model of T cell activation. Signal one is initiated when the TCR recognises is specific antigen presented by MHC. The costimulatory second signal is provided by CD28 when it engages CD80 or CD86 expressed by APC. When the combined strengths of signals one and two reach a critical threshold T cells become fully activated. CTLA-4 binds the same ligands as CD28 and inhibits T cell activation.

signalling to a lesser or greater extent, however CD28 is considered to be the primary and most potent costimulatory receptor (Wingren et al., 1995; Salomon and Bluestone, 2001).

1.6 CD28 signalling and function

CD28 is constitutively expressed by T cells and binds two ligands CD80 (B7-1) and CD86 (B7-2) which are expressed by APC. The importance of costimulatory CD28 signalling is demonstrated in CD28-deficient mice, which display poor T cell responses characterised by reduced T cell proliferation and IL-2 production, resulting in impaired germinal centre formation and reduced antibody production (Shahinian et al., 1993; Lucas et al., 1995; Ferguson et al., 1996). CD28 receptor signalling appears to augment TCR signalling and also activate additional signalling pathways (**figure 1.6**). CD28 costimulation decreases the threshold number of TCR that are required to be engaged for T cell activation (Viola and Lanzavecchia, 1996; Manz et al., 2011) and enhances TCR signalling capacity by increasing or sustaining the phosphorylation of substrates involved in early TCR signalling events such as ZAP70 (Tuosto and Acuto, 1998), Lck (Holdorf et al., 2002) and PLCγ1 (Michel et al., 2001). CD28 signalling activates several pathways, including that of phosphatidylinositol 3-kinase (PI3K) (Pages et al., 1994), which activates the protein kinases AKT and PDK1 (Parry et al., 1997; Park et al., 2009). CD28 is also responsible for the recruitment and activation of protein kinase C (PKCθ) and Carma1 (Yokosuka et al., 2008; Sanchez-Lockhart et al., 2008; Liang et al., 2013), which in concert with AKT and PDK1 activate the transcription factor nuclear factor-κB (NF-κB) (Park et al., 2009; Coudronniere et al., 2000). NF-κB controls the upregulation of genes required for cell cycle progression and survival (Khoshnan et al., 2000; Wan and DeGregori, 2003; Jones et al., 2002). CD28 signalling also leads to the activation of nuclear factor of activated T cells (NFAT) (Michel et al., 2000) and activator protein 1 (AP-1) (Rincon and Flavell, 1994), which are transcription factors that control cell proliferation, death and differentiation. CD28 signalling is integrated with signals from environmental cues, such as the cytokine milieu and the availability of nutrients, via the protein kinase mTOR, which

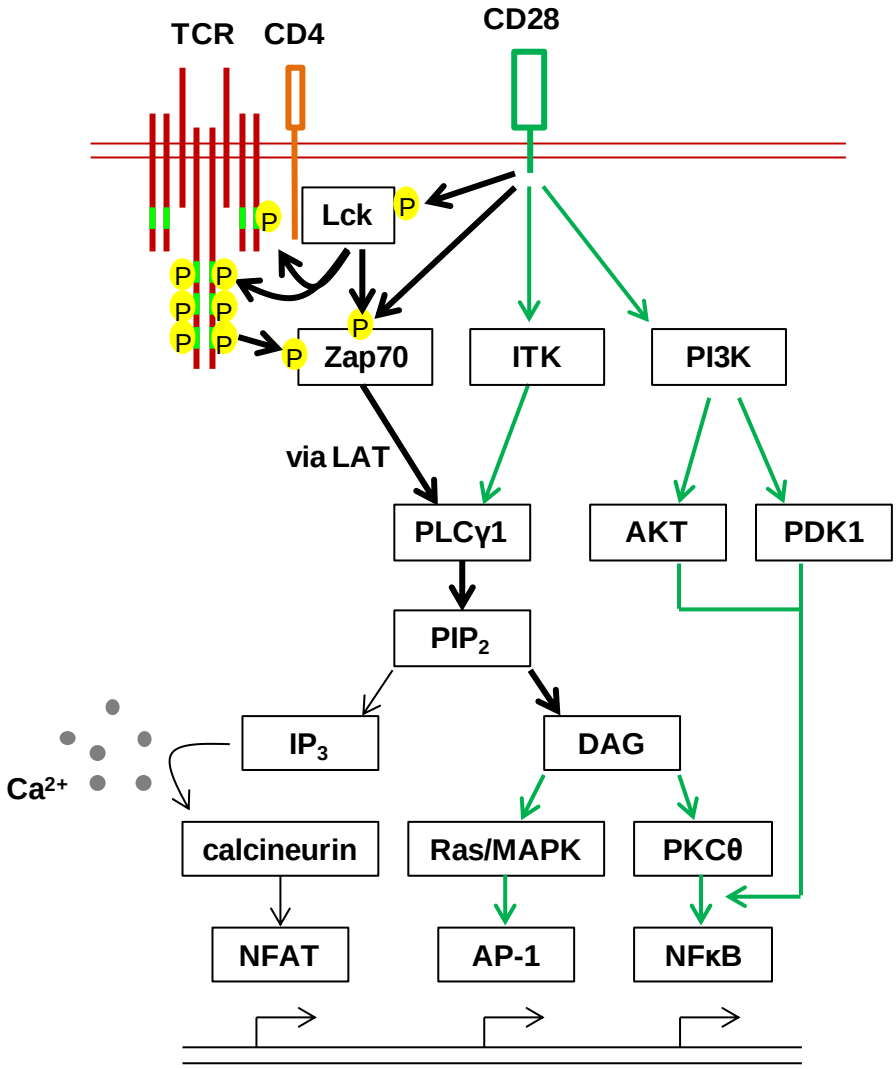


Figure 1.6 TCR and CD28 signalling pathways. This figure shows a simplified representation of some of the signalling pathways downstream of the TCR and CD28. The green arrows show CD28-dependent pathways and the bold black arrows show pathways amplified by CD28 activity. CD28 enhances TCR signalling by increasing Lck and Zap70 phosphorylation. CD28 ligation activates the PI3K pathway which activates AKT and PDK1. CD28 is also essential for PKCθ recruitment and activation which acts in concert with AKT and PDK1 to activate the transcription factor NF-κB which controls cell survival. CD28 is also required for Ras/MAPK signalling which activates AP-1. Without CD28 signalling and AP-1 activation, NFAT upregulates ubiquitin ligases which inhibit PKCθ and PLCγ1 leading to T cell anergy. Activation of both NFAT and AP-1 promotes T cell proliferation and differentiation.

prevents T cell anergy and enables full T cell activation, as described shortly (Zheng et al., 2009; Powell et al., 1999; Colombetti et al., 2006). Although the signalling pathways downstream of CD28 have been described in some detail, this is still a very active area of research and ongoing studies are continually adding to our understanding of these molecular pathways. Furthermore, the signalling motifs within the cytoplasmic tail of CD28 remain to be fully defined (Pagan et al., 2012; Ogawa et al., 2013; Boomer et al., 2014).

There are numerous functional outcomes of CD28 signalling. CD28 costimulation enhances T cell proliferation and cytokine production (Linsley et al., 1991a; Thompson et al., 1989). In particular, CD28 costimulation is required for optimal production of the growth factor IL-2 (Jenkins et al., 1991; Fraser et al., 1991). IL-2 acts in an autocrine and paracrine fashion to enhance T cell proliferation and differentiation (Smith, 1988; Brennan et al., 1997; Malek et al., 2001). Some evidence suggests that IL-2 does not directly control T cell division but instead promotes T cell survival (Ganusov et al., 2007). Notably CD28 can control cell cycle progression independently of IL-2 (Appleman et al., 2000; Lumsden et al., 2003). CD28 signalling also triggers the expression of a second wave of receptors, including ICOS, CTLA-4 and OX40 (McAdam et al., 2000; Finn et al., 1997; Walker et al., 1999). ICOS is another costimulatory molecule that enhances cytokine production by activated T cells (McAdam et al., 2000). In contrast CTLA-4 negatively regulates the T cell response and its function in relation to CD28 is the central theme of this project so will be described in detail shortly. OX40 enhances T cell expansion and survival and promotes the development of memory T cells (Croft et al., 2009). OX40 also upregulates the expression of CXCR5, which directs the migration of CD4 T cells to B cell follicles where they provide help for antibody responses (Walker et al., 2000).

Another important function of CD28 is to promote the upregulation of the metabolic machinery required for T cell activation, such as the translocation of the glucose transporter

GLUT1 to the cell surface (Frauwirth et al., 2002; Jacobs et al., 2008). CD28 costimulation also plays an important role in T cell survival, as it upregulates the expression of survival factors such as IL-2, upregulates the production of anti-apoptotic proteins such as Bcl-xL (Boise et al., 1995), and represses the expression of the pro-apoptotic transcription factor p73 (Wan and DeGregori, 2003). CD28 also prevents Fas-mediated apoptosis by reducing Fas-ligand expression, increasing the expression of c-FLICE inhibitory protein and disrupting the formation of the death-inducing signalling complex (Kirchhoff et al., 2000; Jones et al., 2002). CD28 may also be important in the formation of the immune synapse (Viola et al., 1999). Indeed, it has recently been suggested that CD28 costimulation is required for actin remodelling, which in turn is required for full T cell activation (Tan et al., 2013). Additionally, there is evidence that CD28 signalling may bias towards a T_H2 response (Rulifson et al., 1997; Lenschow et al., 1996; Smeets et al., 2012; Rudulier et al., 2014). By regulating all these factors, CD28 plays an important role in producing an optimal T cell response. Dissecting the CD28 motifs and signalling pathways responsible for each of these distinct functions is an active area of research (Miller et al., 2009).

1.7 T cell anergy

T cell anergy is a state of hyporesponsiveness induced following incomplete T cell activation due to a lack of costimulation or recognition of a low affinity antigen (Schwartz, 2003; Choi and Schwartz, 2007). Therefore anergy is a mechanism of peripheral tolerance to avoid responses against self antigens (Adler et al., 2000; Huang et al., 2003). Upon restimulation anergic T cells are unable to proliferate or produce IL-2 and have impaired effector function but they are not induced to die (Hargreaves et al., 1997). T cells can become anergic whether or not they divided following their initial stimulation (Wells et al., 2001) and anergic cells are unable to respond upon restimulation even if costimulation is provided (Lumsden et al., 2003; Mittrucker et al., 1996; Zheng et al., 2009). Clonal anergy is the best described form of anergy, which is caused by sustained calcium signalling downstream of TCR

stimulation. Calcium mobilisation activates calcineurin and the transcription factor NFAT. Calcineurin increases the transcription of E3 ubiquitin ligases GRAIL, Itch and Cbl-b, which degrade proteins required for T cell activation such as PKCθ and PLCγ1 (Heissmeyer et al., 2004). In the absence of CD28 costimulation NFAT activates an alternative transcriptional program to the one induced in concert with AP-1. CD28 signalling normally activates the Ras/MAPK pathway which activates AP-1 (Su et al., 1994; Janardhan et al., 2011). Without AP-1, NFAT induces anergy via another transcription factor Egr (Bandyopadhyay et al., 2007). NFAT and Egr2/3 upregulate GRAIL and Cbl-b and inhibit T cell activation (Soto-Nieves et al., 2009; Macian et al., 2002; Safford et al., 2005). Egr2 also upregulates diacylglycerol kinase which inhibits the Ras/MAPK/AP-1 pathway (Zheng et al., 2012), which may explain the maintenance of anergy despite the subsequent provision of costimulation (Fields et al., 1996). In addition, CD28 normally prevents anergy by inhibiting a number of regulatory proteins: PTEN, a negative regulator of PI3K signalling (Buckler et al., 2006); $p27^{kip1}$ which inhibits T cell proliferation and effector function (Rowell et al., 2005); and Ikaros which represses IL-2 gene expression (Thomas et al., 2007). Another type of anergy known as adaptive tolerance or *in vivo* anergy is also caused by insufficient costimulation. This involves a block in tyrosine kinase activation leading to the inhibition of calcium mobilisation and a lack of PLCγ1 activation (Schwartz, 2003). This causes stunted proliferation of naive T cells and prevents all effector function. However, defining an unresponsive state as either clonal anergy or adaptive tolerance is an over simplification. There seem to be several distinct forms of anergy caused by various molecular mechanisms that require further investigation in order to fully understand all the pathways leading to immune tolerance (Choi and Schwartz, 2007).

1.8 CD28, mTOR and anergy

The mammalian target of rapamycin (mTOR) pathway plays an important role in controlling the fate of T cells. mTOR is a serine/threonine kinase which integrates signals from

costimulatory receptors, hormones, cytokines and amino acids and promotes full T cell activation if sufficient signals are received (**figure 1.7**). In essence, mTOR assesses the availability of nutrients such as iron and glucose, which are required to fuel the high metabolic demands of T cell activation. Without the necessary energy resources or costimulatory signals, mTOR is not activated and T cell anergy is induced (Cobbold et al., 2009; Zheng et al., 2009). Numerous studies have demonstrated that mTOR activity is essential for anergy avoidance (Powell et al., 1999; Zheng et al., 2009; Zheng et al., 2007). In the presence of the necessary signals, activation of mTOR activates programs to prevent apoptosis, initiate ribosome biogenesis, induce RNA translation and promote cell cycle progression (Delgoffe and Powell, 2009). Therefore mTOR acts downstream of CD28 to prevent anergy induction. For example, mTOR is required for CD28-mediated upregulation of the transferrin receptor CD71 (Edinger and Thompson, 2002), which is necessary for full T cell activation (Zheng et al., 2007), and failure to upregulate CD71 is associated with anergy (Zheng et al., 2009). Other outcomes of CD28 signalling are also implemented via mTOR. For example mTOR coordinates CD28-mediated T_H2 differentiation (Yang et al., 2013). IL-2 receptor signalling also activates the AKT-mTOR pathway leading to the translation of otubain-1, which degrades GRAIL and allows T cell proliferation (Lin et al., 2009). Therefore CD28 activates mTOR both directly and indirectly via IL-2 signalling (Colombetti et al., 2006). Given its important role in mediating CD28 signalling, mTOR represents a crucial regulatory checkpoint in T cell activation.

1.9 Regulatory T cells

Autoreactive T cells are controlled not only by anergy induction due to insufficient CD28 or mTOR signalling, but also by specialised regulatory T cells (Tregs) which play a critical role in maintaining peripheral tolerance (Sakaguchi et al., 2008). Natural Tregs (nTregs) derived from the thymus are the best characterised Tregs. CD28 costimulation is required for their

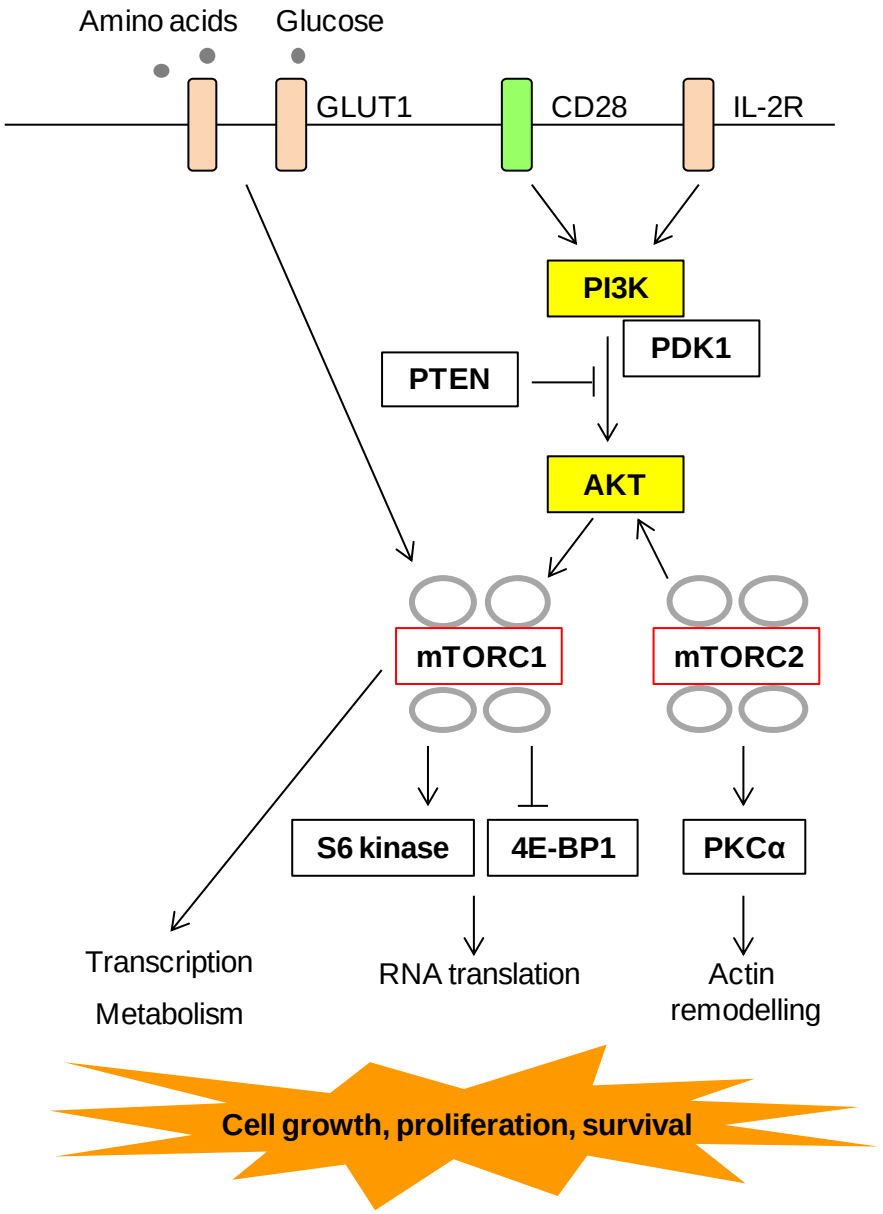


Figure 1.7 CD28-mTOR signalling. CD28 and IL-2 signalling activate the PI3K-AKT pathway, which leads to the activation of mTOR complex 1 (mTORC1). Stimuli from environmental factors such as nutrients also activate mTORC1. This leads to the phosphorylation of its substrates S6 kinase and 4E-BP1, which regulate various functions resulting in the promotion of T cell growth. The pathway leading to mTORC2 activation is less well understood but mTORC2 is known to enhance AKT phosphorylation and stimulate actin remodelling.

development, homeostasis and function (Salomon et al., 2000; Tai et al., 2005; Zhang et al., 2013; Liang et al., 2013) and they express the transcription factor FoxP3 which is required for Treg development (Hori et al., 2003; Fontenot et al., 2003b). Mutations in FoxP3 cause a loss of Treg function and consequently result in autoimmune disease, such as type 1 diabetes in *scurfy* mice and immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) in humans (Brunkow et al., 2001; Bennett et al., 2001). Tregs constitutively express the IL-2 receptor alpha chain CD25 and require IL-2 signalling to maintain homeostasis (Fontenot et al., 2005). Tregs use a variety of mechanisms to suppress effector T cell responses: aggregating around APC to out-compete naive or effector T cells for APC interaction; down-modulating costimulatory molecules on DC to prevent further T cell activation; secreting immunosuppressive cytokines IL-10, TGF-β and IL-35; and cell contact-dependent killing using granzymes or perforin (Shevach, 2009). Tregs constitutively express the inhibitory receptor CTLA-4 which plays a key role in Treg function (Read et al., 2000; Takahashi et al., 2000; Wing et al., 2008). The mechanism of action of CTLA-4 has been a controversial issue for many years and will be discussed presently.

Tregs can also be derived from peripheral naive CD4 T cells and are so-called induced Tregs (iTregs). iTregs can be divided into $FoxP3^+$ and $FoxP3^-$ subsets. $FoxP3^+$ iTreg are induced in the presence of TGF-β and to an extent have a similar phenotype and function to nTregs (Chen et al., 2003; Yadav et al., 2013). $FoxP3^-$ suppressive CD4 T cells, referred to as T regulatory type 1 (Tr1) cells or IL-10-secreting T cells, are derived from naive CD4 T cells rendered anergic due to chronic antigenic stimulation in the presence of IL-10 or due to a lack of CD28 costimulation (Groux et al., 1997; Buer et al., 1998; Lombardi et al., 1994; Koenen and Joosten, 2000; Boussiotis et al., 2000; Sundstedt et al., 2003; Levings et al., 2005; Gabrysova and Wraith, 2010). In addition, Tr1 cells have recently been shown to be induced by a defined subset of IL-10-producing tolerogenic DC (Gregori et al., 2010). It is not clear whether the IL-10-producing CD4 T cells described in these studies all belong to the

same population because some differences in phenotype were observed. However they are defined as Tr1 cells by their shared ability to suppress the activation of neighbouring CD4 T cells by the production of IL-10 (Roncarolo et al., 2006). IL-10 suppresses T cells by acting both directly on T cells by suppressing their IL-2 and TNF-α production leading to anergy, and indirectly by downregulating costimulatory molecule expression and pro-inflammatory cytokine production by APC (Groux et al., 1996; Ng et al., 2013). The discovery of these different types of Tregs demonstrates the diversity and importance of mechanisms of peripheral tolerance.

1.10 Negative regulation of T cells by CTLA-4

An important molecule involved in the negative regulation of T cells is the inhibitory receptor CTLA-4. A lack of CTLA-4 in mice results in fatal autoimmunity caused by uncontrolled self-reactive T cells (Tivol et al., 1995; Waterhouse et al., 1995). In humans CTLA-4 polymorphisms are associated with rheumatoid arthritis (Li et al., 2012), type I diabetes (Chen et al., 2013) and other autoimmune diseases (Ueda et al., 2003; Fernandez-Mestre et al., 2009). CTLA-4 is expressed by activated CD4 T cells and Tregs (Krummel and Allison, 1995; Brunet et al., 1987; Read et al., 2000; Takahashi et al., 2000) and binds the same ligands (CD80 and CD86) as CD28 (Linsley et al., 1991b). CTLA-4 is induced on activated T cells in a CD28-dependent manner and reaches optimal expression by day three (Alegre et al., 1996). CTLA-4 plays a role in the inhibition of T cell proliferation and IL-2 production (Walunas et al., 1996), and thereby contributes to the termination of an immune response. On the other hand, Tregs constitutively express CTLA-4 and are therefore able to prevent the initiation of aberrant responses. As the CD28/CTLA-4 pathway controls the initiation and termination of T cell responses, it is targeted therapeutically to reduce autoimmunity and transplant rejection and to encourage anti-tumour immunity. For example, a soluble fusion protein called abatacept consisting of the extracellular domain of CTLA-4 and an IgG Fc domain (CTLA-4-Ig), which binds to CD80/86 thereby blocking CD28 costimulation, is used

to treat autoimmune diseases such as rheumatoid arthritis (Linsley and Nadler, 2009; Podojil and Miller, 2013). Similarly a higher affinity CTLA-4-Ig molecule belatacept has more recently been approved for the treatment of kidney transplant patients (Wojciechowski and Vincenti, 2012).

1.11 Cell intrinsic models of CTLA-4 function

Several mechanisms of action have been proposed for CTLA-4, the simplest being that CTLA-4 inhibits T cell activation by out-competing CD28 for ligand binding and therefore preventing CD28-dependent T cell activation. This theory is supported by the fact that CD80 and CD86 bind to CTLA-4 with much greater avidity than to CD28 (Greene et al., 1996; van der Merwe et al., 1997; Collins et al., 2002; Jansson et al., 2005). CTLA-4 has also been shown to cluster in particular regions within the immunological synapse where it competes with CD28 and inhibits CD28-dependent recruitment of PKC-θ (Yokosuka et al., 2010). Another hypothesis is that CTLA-4 inhibits T cell activation by producing an intracellular negative signal that overrides activating signals. In support of this, it has been shown that PI3K associates with the cytoplasmic domain of CTLA-4 (Schneider et al., 1995; Bradshaw et al., 1997; Zhang and Allison, 1997). Phosphatases SHP-1, SHP-2 and PP2A have also been shown to bind CTLA-4 (Chuang et al., 2000; Marengere et al., 1996; Zhang and Allison, 1997; Teft et al., 2009). CTLA-4 may recruit these phosphatases to inhibit membrane-proximal TCR signalling (Lee et al., 1998; Guntermann and Alexander, 2002). However it is unlikely that CTLA-4 activates a downstream signalling pathway with inhibitory outcomes because PI3K is associated with stimulatory pathways and the significance of PP2A binding CTLA-4 remains controversial. Some reports suggest that this interaction facilitates CTLA-4-mediated inhibition of AKT (Parry et al., 2005), whereas others have found that PP2A represses CTLA-4 function (Baroja et al., 2002). In fact Teft et al. (2009) showed that the CTLA-4-PP2A interaction was not required for T cell inhibition but that CD28 expression was required for inhibition, indicating that CTLA-4 functions by directly suppressing CD28 function

as opposed to creating independent negative signals. In short, a downstream signalling pathway originating from CTLA-4 has not yet been elucidated. Furthermore a cell intrinsic inhibitory signal via CTLA-4 cannot easily explain the cell extrinsic suppressive function of Tregs.

The models of ligand competition and negative signalling are based on CTLA-4 functioning at the cell surface. However CTLA-4 is mainly intracellular and its cell surface expression is only transient and very low due to rapid endocytosis and continual intracellular trafficking (Leung et al., 1995; Alegre et al., 1996; Linsley et al., 1996; Mead et al., 2005; Qureshi et al., 2012). Upon T cell stimulation CTLA-4 cell surface expression is upregulated by trafficking of CTLA-4-containing vesicles to the plasma membrane (Linsley et al., 1996; Iida et al., 2000) via a chaperone called the TCR-interacting molecule (TRIM) (Valk et al., 2006). Although there is a substantial increase in exocytosis of CTLA-4 during T cell activation, there is actually only a small increase in the steady state levels of surface CTLA-4 (Mead et al., 2005). The majority of CTLA-4 expression is still intracellular (Iida et al., 2000), indicating that increased exocytosis is also accompanied by continued endocytosis (Linsley et al., 1996; Mead et al., 2005). CTLA-4 is internalised by clathrin-dependent endocytosis (Mead et al., 2005), which is mediated by a region in the cytoplasmic tail of CTLA-4 containing a YVKM motif that is recognised by the clathrin adaptor complex AP-2 (Bradshaw et al., 1997; Shiratori et al., 1997; Zhang and Allison, 1997). Once internalised, CTLA-4 is either rapidly degraded in lysosomes (Alegre et al., 1996; Egen and Allison, 2002; Iida et al., 2000; Schneider et al., 1999; Oki et al., 1999) or it accumulates in a perinuclear compartment where it is resistant to degradation, from which it may be recycled back to the plasma membrane (Linsley et al., 1996; Mead et al., 2005). The intracellular nature of CTLA-4 suggests that its primary function is not as a cell membrane signalling molecule. Indeed, Bradshaw et al. (1997) suggested that PI3K may be involved in regulating CTLA-4 trafficking not signalling.

1.12 Cell extrinsic models of CTLA-4 function

Studies using mixed chimeras have shown that CTLA-4$^+$ T cells can regulate the responses of CTLA-4-deficient T cells, which demonstrates that CTLA-4 functions extrinsically (Bachmann et al., 1999; Homann et al., 2006). More recently it has been confirmed that CTLA-4 functions in a cell extrinsic manner via both effector T cells and Tregs (Friedline et al., 2009; Wang et al., 2012; Corse and Allison, 2012). One proposed cell extrinsic mechanism of CTLA-4 is that CTLA-4 interaction with CD80/86 on APC upregulates indoleamine 2,3-dioxygenase (IDO) expression, causing increased tryptophan catabolism (Grohmann et al., 2002; Fallarino et al., 2003). The conversion of tryptophan, an amino acid essential for T cell proliferation, into pro-apoptotic metabolites that delete certain T cell subsets, leads to the inhibition of T cell responses (Fallarino et al., 2002). Alternatively, it was recently published by our laboratory that CTLA-4 can function extrinsically by removing CD80 and CD86 from APC by trans-endocytosis to reduce their capacity to provide costimulation to T cells (**figure 1.8**) (Qureshi et al., 2011). In short, CTLA-4 acts like a molecular hoover, ripping its ligands from APC in an endocytic process where the entire receptor-ligand complex is internalised so that fewer ligands are available to interact with CD28. Upon internalisation, ligand is degraded while CTLA-4 is recycled back to the cell surface to capture more ligand, which provides an explanation for the continual intracellular trafficking of CTLA-4. Trans-endocytosis if therefore consistent with the cell biology of CTLA-4 and explains the cell extrinsic function of CTLA-4.

1.13 Trans-endocytosis and trogocytosis in the immune system

In support of the model of trans-endocytosis as the mechanism of CTLA-4 function, it has been reported that Tregs can reduce the expression of CD80 and CD86 on DC in a contact-dependent manner (Cederbom et al., 2000; Vendetti et al., 2000; Grundstrom et al., 2003). More recently it was demonstrated that this requires CTLA-4 and impairs the ability of the DC

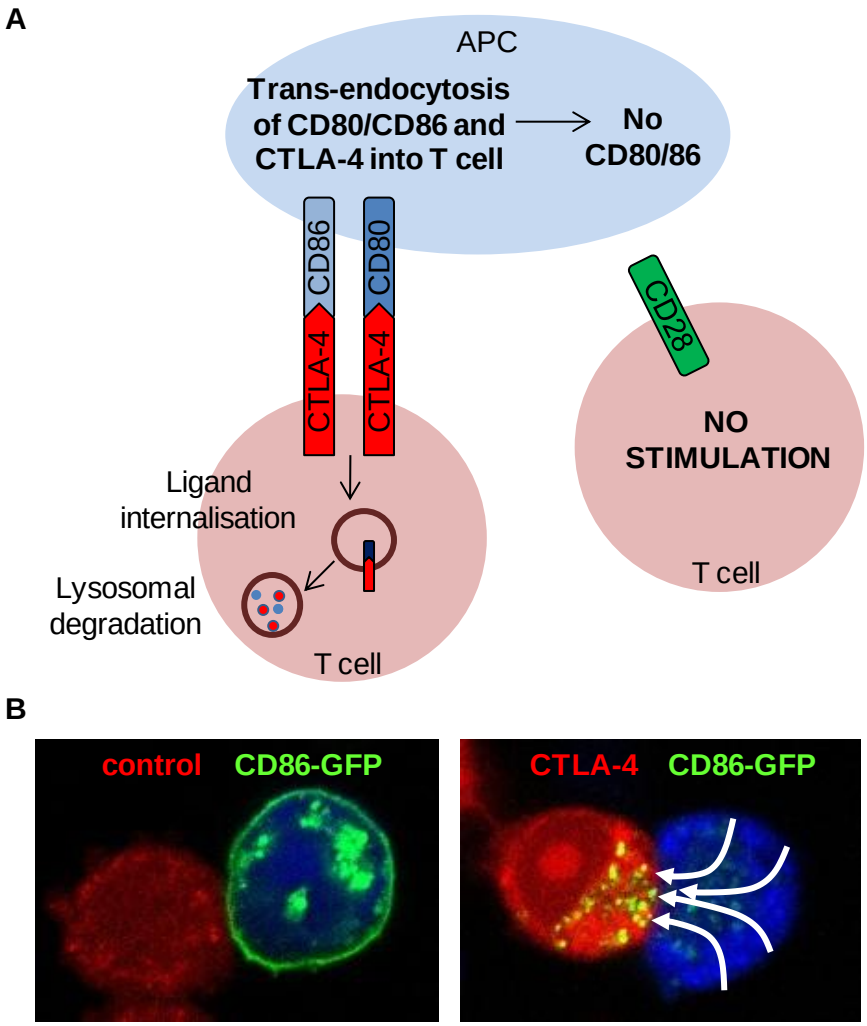


Figure 1.8 Trans-endocytosis. **(A)** Schematic representation of trans-endocytosis. CD80/86 expressed on an APC binds CTLA-4 expressed by an effector T cell or Treg. CTLA-4 and bound ligand are internalised via trans-endocytosis into endocytic vesicles. Internalised proteins are degraded in lysosomal compartments. As a result the APC has reduced CD80/86 expression so there are not enough ligands available to engage CD28. Therefore costimulation is not provided to other T cells, which prevents their activation. **(B)** Cells expressing CD86-GFP (blue) were incubated with untransfected control cells or cells expressing CTLA-4 (red) than analysed by confocal microscopy. Left: CD86-GFP was expressed on the cell surface. Right: CD86-GFP was internalised by CTLA-4-expressing cells.

to stimulate $CD4^+CD25^-$ T cell proliferation (Oderup et al., 2006; Wing et al., 2008; Onishi et al., 2008). Oderup et al. showed that proliferation was restored in the presence of CD28 antibody, confirming that the lack of response was due to insufficient CD28 costimulation. Consistent with this, their stimulation of T cells using latex beads coated with anti-CD3 and varying amounts of CD80/86 showed that reducing the availability of CD80/86 reduced the proliferative response. Furthermore, Treg-mediated suppression was more effective when fewer costimulatory ligands were available, which has also been demonstrated by others (Ermann et al., 2001; George et al., 2003). Altogether these findings demonstrate that reducing the availability of costimulatory ligands is functionally relevant. Additionally it has been shown that Treg cell contact-dependent suppression requires the formation of Treg aggregates on DC to allow sustained Treg-DC interaction (Onishi et al., 2008). This suggests that CTLA-4-mediated Treg function crucially involves prolonged contact with DC rather than effector T cells, which implies that the model of CTLA-4 removing costimulatory ligands from DC by trans-endocytosis is a viable and important regulatory mechanism.

A role for trans-endocytosis in the immune system is not a novel concept. For example, interactions between CD47 (expressed by T cells) and its counterpart SHPS-1 (expressed by DC) provide costimulation for T cell activation (Tomizawa et al., 2007). Trans-endocytosis of CD47 and SHPS-1 can occur in either direction and is believed to regulate their function (Kusakari et al., 2008). The trans-endocytosis of other endocytic proteins and their counterparts has also been described. For example the extracellular domain of the Notch receptor is removed upon interaction with its ligand and internalised by the ligand-expressing cell via trans-endocytosis (Parks et al., 2000; Nichols et al., 2007). This process is required for Notch receptor signalling, which is essential for regulating the transcription of proteins involved in many cellular processes during embryonic development. Also the sevenless transmembrane tyrosine kinase receptor, responsible for directing cell fate during Drosophila retinal development, internalises its ligand the bride of sevenless by trans-endocytosis

(Cagan et al., 1992). Similarly, bi-directional trans-endocytosis of ephrinB ligand:EphB receptor complexes has been defined as a mechanism to terminate cell adhesion and promote contact-mediated repulsion of neurons (Zimmer et al., 2003; Marston et al., 2003).

In general the transfer of proteins between immune cells is very common, but this is mainly via trogocytosis which is a very different process to trans-endocytosis. Trans-endocytosis involves the transfer and internalisation of a specific ligand upon encounter with its cognate receptor and the captured proteins are transferred from the donor cell directly into intracellular vesicles via endocytosis (Kusakari et al., 2008). On the other hand, trogocytosis involves the transfer of plasma membrane fragments between cells triggered by receptor-ligand interactions at intercellular contacts such as the immune synapse (Joly and Hudrisier, 2003; Davis, 2007). In trogocytosis proteins are transferred to the recipient cell surface via cell membrane exchange and only later internalised and degraded (Huang et al., 1999; Hwang et al., 2000; Williams et al., 2007). Many mechanisms for trogocytosis have been proposed including protein uprooting, transfer of enclosed membrane bodies or vesicles, intercellular membrane fusion creating membrane bridges, and membrane nanotubules (Davis, 2007; Ahmed and Xiang, 2011). There are many reports of protein transfer between APC and lymphocytes by trogocytosis, including the acquisition of peptide:MHC and costimulatory ligands from APC by T cells (Hwang et al., 2000; Hudrisier et al., 2007). For example independent reports have shown the transfer of CD80 to the cell membrane of CD4 T cells in a CD28-dependent manner where CD80 acquisition was increased upon TCR engagement and in correlation with the level of CD80 expression by the APC (Sabzevari et al., 2001; Tatari-Calderone et al., 2002). It remains controversial whether the transfer of MHC and costimulatory molecules from APC to T cells by trogocytosis has any functional significance (Ahmed and Xiang, 2011). Some reports suggest that acquisition of these proteins by T cells allows them to sustain their own activation (Zhou et al., 2005), while others indicate that T cells with acquired peptide:MHC and CD80 can extrinsically stimulate

naive T cells (Game et al., 2005). Alternatively, this process may terminate T cell responses (Tsang et al., 2003), or trogocytosis may simply have evolved as a means of intercellular communication or to share resources (Joly and Hudrisier, 2003). While the molecular mechanisms of both trogocytosis and trans-endocytosis remain to be fully elucidated, they are clearly distinct mechanisms of intercellular protein transfer which can easily be distinguished from one another.

1.14 CD28/CTLA-4 family proteins: ICOS and PD-1

Other members of the immunoglobulin superfamily that are related to CD28 and CTLA-4 include ICOS and PD-1 (**figure 1.9**). ICOS is not constitutively expressed by T cells but is upregulated upon T cell activation in response to TCR and CD28 signalling (McAdam et al., 2000). There is only one known ligand for ICOS (LICOS also known as B7-H2) and upon ligand interaction ICOS activates the PI3K-AKT pathway leading to the activation of transcription factors NFAT and c-Maf (Nurieva et al., 2007). ICOS is a costimulatory receptor whose function overlaps with CD28 to promote T cell differentiation and enhance effector cell function, including the production of cytokines, especially IL-4 (Hutloff et al., 1999; Dong et al., 2001a). ICOS costimulation is also important for germinal centre formation as it promotes the development of T_{FH} which regulate germinal centre B cell differentiation (Dong et al., 2001b; Akiba et al., 2005). Interestingly, LICOS has been shown to interact with CD28 and CTLA-4 and appears to provide effective costimulation via CD28 but is not as efficient as CD80 (Yao et al., 2011). The consequences of CTLA-4-LICOS interactions remain to be explored.

PD-1 on the other hand, like CTLA-4, plays an inhibitory role in T cell activation and is crucial for maintaining tolerance as shown by the development of autoimmunity in PD-1-deficient mice (Zhang et al., 2006; Nishimura et al., 1999). PD-1 is expressed at low levels on resting T cells and B cells and is upregulated further upon activation (Agata et al., 1996). PD-1

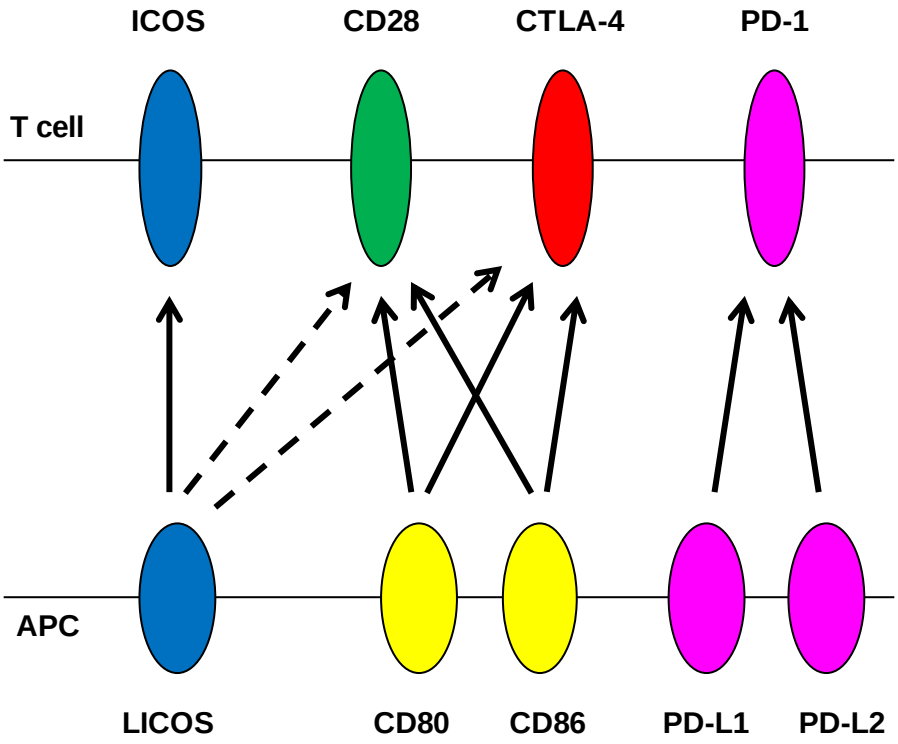


Figure 1.9 The CD28 family. The subgroup of receptors within the immunoglobulin superfamily that are closely related to CD28 are shown. ICOS and CD28 have stimulatory effects on T cell activation while CTLA-4 and PD-1 are inhibitory. Solid arrows represent known receptor-ligand interactions. Dashed arrows indicate putative interactions. CD80 and PD-L1 are also reported to interact with each other.

shares 23% homology with CTLA-4, but unlike CTLA-4 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) in its cytoplasmic tail for intracellular signalling (Shinohara et al., 1994; Okazaki et al., 2001). PD-1 engagement leads to tyrosine phosphorylation and activation of SHP-2 which inhibits membrane-proximal TCR signalling and blocks AKT and Ras signalling pathways (Latchman et al., 2001; Patsoukis et al., 2012). In the presence of low antigen concentrations and CD28 signalling, PD-1 inhibits T cell proliferation and cytokine production (Freeman et al., 2000; Latchman et al., 2001). In the presence of high antigen concentrations, PD-1 engagement cannot inhibit T cell proliferation but can still limit cytokine production (Latchman et al., 2001). PD-1 also promotes peripheral Treg development, maintains FoxP3 expression and enhances the suppressive activity of iTregs (Francisco et al., 2009). PD-1 binds two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC), which have different expression patterns (Freeman et al., 2000; Latchman et al., 2001). PD-L1 is expressed not only by APC, but also by T cells including Tregs, and in non-lymphoid tissues such as heart and lung. In contrast, PD-L2 expression is mainly limited to DC and monocytes. Incidentally, the expression of PD-1 ligands and also LICOS in both lymphoid and non-lymphoid tissues suggests that ICOS and PD-1 play an important role in peripheral tolerance (Swallow et al., 1999; Freeman et al., 2000). Although ICOS and PD-1 are known to be signalling receptors expressed at the cell surface like CD28 (Hutloff et al., 1999; Sheppard et al., 2004), it is worth investigating whether they are able to use trans-endocytosis as an alternative mechanism of action, especially PD-1 which plays a similar role to CTLA-4 in T cell regulation and tolerance.

1.15 Quantity of costimulatory ligands required for T cell stimulation

The model of CTLA-4 removing its ligands from APC by trans-endocytosis to prevent CD28 costimulation predicts that there is a minimum number of ligands required for effective costimulation and that changing CD80/86 expression levels has a measurable impact on the T cell response. It is uncertain whether costimulation works in an “all or nothing” manner,

where there is a threshold at which CD80/86 levels change from being insufficient to sufficient for T cell activation, or whether costimulation can be provided in a graded manner where increasing levels of CD80 or CD86 create a response curve where partial or slower T cell responses can occur. Measurement of T cell proliferation in response to a range of CD80 expression levels has shown there is a threshold CD80 expression level that must be reached before T cell proliferation occurs, which is dependent on antigen type and concentration (Murtaza et al., 1999; Chen et al., 2000). The reported responses seemed to be graded according to CD80 expression level, but this is still a relatively unexplored area of T cell activation and the requirements for CD86 remain unknown. Therefore one of the aims of this project was to use an inducible expression system to study the effect of altering the number of costimulatory ligands on CD4 T cell responses, in order to further understand the requirements for CD28 costimulation and the relevance of CTLA-4 trans-endocytosis.

1.16 CD80 and CD86: What is the difference?

It is currently unclear why there are two different ligands for CD28 and CTLA-4. CD80 and CD86 have surprisingly different structural and biochemical characteristics. For instance, CD80 and CD86 have only 26% amino acid identity (Freeman et al., 1993) and CD80 molecules form dimers, while CD86 is monomeric (Ikemizu et al., 2000). CD80 binds CD28 and CTLA-4 with higher affinity than CD86, although CD80 has a stronger bias towards CTLA-4 interactions rather than CD28, so relative to CTLA-4 binding, CD86 binds CD28 more effectively (Collins et al., 2002; Linsley et al., 1994). This suggests that CD80 could be the more potent costimulatory molecule, but that in the presence of CTLA-4, CD86 may in fact be the more dominant CD28 ligand (Pentcheva-Hoang et al., 2004; Jansson et al., 2005). The two ligands also have different expression patterns. CD86 is constitutively expressed by most APC and its expression is rapidly upregulated upon activation, unlike CD80, which is only expressed upon APC activation and is upregulated at a slower rate and

to much lower levels than CD86 (Lenschow et al., 1993). This pattern of expression indicates that CD86 may be more important in the initial stages of T cell activation.

It is unclear whether there is any functional difference between CD80 and CD86. Consistent with the hypothesis that CD86 is the more effective CD28 ligand, CD86 deficiency has been shown to reduce cytokine production more dramatically than CD80 deficiency (Schweitzer et al., 1997). It has also been demonstrated that CD86 blockade inhibits naive T cell proliferation and effector function more than blocking CD80 (Manickasingham et al., 1998). Similarly, CD86-deficient mice were shown to be more immuno-compromised than CD80-deficient mice (Borriello et al., 1997). In support of this, Mark et al. (2000) showed that costimulation via CD86 more potently induced an allergic T cell response than CD80. In addition, others have reported that disease severity was more significantly reduced in CD86-deficient non-obese diabetic (NOD) mice compared to CD80-deficient animals, suggesting that CD86 plays a more dominant role in disease onset and progression than CD80 (Girvin et al., 2000). Interestingly, despite having reduced tissue infiltration, CD80-deficient mice actually displayed increased T cell proliferation. This suggests that while CD80 does contribute to the migration of T cells to the site of inflammation, CD80 may be more important for negatively regulating T cell responses than stimulating them. However other studies report contradicting data suggesting that CD80 is the more important or potent CD28 ligand (Fleischer et al., 1996; Olsson et al., 1998; Fields et al., 1998).

There is also debate as to whether CD80 and CD86 can differentially direct T cell differentiation. Some studies have shown that blocking one ligand at a time can alternatively exacerbate or attenuate disease, which suggests that CD80 and/or CD86 can skew a T cell response towards a particular phenotype. For example, in the NOD mouse model, where disease is driven by autoreactive T_H1 cells, treatment with anti-CD86 blocking antibody prevented disease development, whereas anti-CD80 treatment accelerated disease

development, suggesting that CD86 generates a T_H1 response whereas CD80 may favour T_H2 differentiation (Lenschow et al., 1995). However the opposite was found using the mouse model of experimental allergic encephalomyelitis, which is also driven by autoreactive T_H1 cells. In this case, anti-CD80 blocking antibody increased IL-4 production and reduced disease incidence, while blocking CD86 increased IFN-γ production and increased disease severity (Kuchroo et al., 1995). This suggests that CD80 promotes T_H1 differentiation while CD86 favours T_H2 differentiation. Similarly, a recent study found that a lack of CD80 was associated with increased IL-4 production and ameliorated *Streptococcus*-induced arthritis, whereas a lack of CD86 exacerbated disease (Puliti et al., 2010). In another T_H1-driven disease model CD80 deficiency reduced disease while CD86 deficiency increased disease, again indicating that CD80 preferentially drives T_H1 responses while CD86 favours T_H2 differentiation (Odobasic et al., 2005). More recently the same group showed that in a mouse model of arthritis, CD80 costimulation reduced IL-4 production, while CD86 increased IL-17 production (Odobasic et al., 2008). Inhibition of CD86 was more effective at reducing disease severity than CD80 blockade, which could indicate that CD86 is the more potent CD28 ligand, or that the ability of CD86 to promote T_H17 responses makes it more pathogenic.

In contrast, there are also studies showing that CD80 and CD86 both stimulate T_H1 and T_H2 responses with no particular bias (Fields et al., 1998; Schweitzer et al., 1997). Other studies show no difference in the magnitude or phenotype of the T cell response following CD28 costimulation via CD80 compared to CD86, suggesting both ligands have identical roles and are therefore redundant (Levine et al., 1995; Lanier et al., 1995; Natesan et al., 1996; Greenwald et al., 1997; Lespagnard et al., 1998; Vasilevko et al., 2002; Bhatt et al., 2013). So it remains uncertain whether there are quantitative or qualitative differences between CD80 and CD86 costimulation. However the evolution of two different ligands for CD28 and CTLA-4 that vary considerably in their structure and relative avidities for their receptors strongly suggests they have non-redundant roles. Nevertheless CD80 and CD86 both bind

the same regions of CD28 and CTLA-4, which both contain the MYPPPY ligand-binding amino acid motif, suggesting that engagement by either ligand would have the same biological consequences for each receptor. Indeed, ligation of CD28 by both CD80 and CD86 leads to the recruitment of PI3K (Ghiotto-Ragueneau et al., 1996), suggesting they stimulate the same downstream transcriptional programs. However there are conflicting data regarding the relative potency of the two ligands in activating PI3K, so it remains unclear whether they produce quantitatively different signals (Ghiotto-Ragueneau et al., 1996; Slavik et al., 1999). The suggested differences in functional outcomes of CD80 and CD86 may be due to the different times at which they are expressed and the combination of other stimulatory molecules with which they are expressed. Also the affinities of CD80 and CD86 for CD28 are different so the duration of CD28 signalling may differ with each ligand, resulting in different response sizes. The disparity in results between studies is probably due to the use of different experimental methods and complex animal models. A simple model where CD80 and CD86 can be compared under identical conditions and at equal levels would allow a fair comparison. Another aim of this project was to use an inducible expression system to control the levels of CD80 and CD86 and compare their functional capabilities.

1.17 CD28-independent T cell responses

While it is clear that CD28 costimulation has many important functions, as shown by CD28 deficient mice (Shahinian et al., 1993; Lucas et al., 1995; Ferguson et al., 1996), animals lacking CD28 still survive and can reject tissue grafts and resist infection (Brown et al., 1996; Kawai et al., 1996; Honstettre et al., 2006; Hogan et al., 2001). This indicates that there are CD28-independent costimulatory pathways and raises questions about which CD28 functions are essential and which are redundant, and whether there are differences in the phenotype or long term fate of T cells stimulated with and without CD28 costimulation. While in some cases CD28 knockout mice cannot produce T cells responses (Khoruts et al., 1998; Kearney et al., 1995; Saha et al., 1996), it has been demonstrated in numerous

studies that some T cells in CD28-deficient mice can still respond, although proliferation rate and IL-2 production are often lower than normal (Shahinian et al., 1993; Lucas et al., 1995; Green et al., 1994; Ding and Shevach, 1996). Others have shown that T cells stimulated in the absence of CD28 costimulation can start proliferating as normal but often cease to proliferate after 2-4 days and do not survive long term (Sperling et al., 1996; Lumsden et al., 2003; Nurieva et al., 2006; Mittrucker et al., 1996). Several studies have shown that CD28-independent T cell activation requires a high affinity TCR antigen or a high dose of antigen (Lucas et al., 1995; Lumsden et al., 2003; Bachmann et al., 1996; Dubey et al., 1995). Cross-linking the TCR with immobilised anti-TCR/CD3 also elicits a normal proliferative T cell response in the absence of CD28 (Green et al., 1994; Cassell, 2001). However upon re-stimulation, the secondary T cell response is frequently reduced (Green et al., 1994) or cells are unresponsive (Mittrucker et al., 1996), suggesting that T cells may be primed in the absence of CD28 costimulation but that ultimately they are anergic and are unlikely to produce efficient effector or memory responses. Indeed, T cells stimulated in the absence of CD28 costimulation display impaired cytokine production (Thompson et al., 1989) and increased expression of E3 ubiquitin ligases, which are characteristic of T cell anergy (Nurieva et al., 2006).

However it is important to note that many of the studies showing CD28-independent responses *in vivo* only related to CD8 T cells (Kundig et al., 1996; Bachmann et al., 1996; Goldstein et al., 1998; Szot et al., 2001). Only about 50% of CD8 T cells express CD28, in contrast to nearly all CD4 T cells (Boucher et al., 1998), which indicates that these two subsets may use different costimulatory pathways. Also, most studies have been done in mice, which may have different costimulatory requirements to humans. Furthermore, most studies have only looked at early T cell proliferation and IL-2 production and not the effector phenotype or function or the long term fate of T cells stimulated in the absence of CD28 costimulation. Therefore another aim of this project was to study the outcome of CD28-

independent T cell stimulation in human cells. These studies were additionally intended to put the role of CTLA-4-ligand trans-endocytosis into context to understand its functional significance.

1.18 Memory T cells and their costimulatory requirements

Memory T cells, which develop following T cell activation and survive after the response has been resolved, are long-lived and provide lasting immunity against their specific antigen by responding rapidly to control and eliminate subsequent infections before symptoms manifest (Garcia et al., 1999). There are currently two defined subsets of memory T cells. Central memory T cells express CD62L and CCR7 to enter lymphoid tissues while effector memory T cells lack these molecules and survey non-lymphoid tissues (Sallusto et al., 1999). While it is clear that naive T cells require CD28 costimulation to produce an optimal response, the costimulatory requirements of memory T cells remain controversial. A number of early studies suggested that memory T cells do not require costimulation (Luqman and Bottomly, 1992; Croft et al., 1994; London et al., 2000). This concept was supported by evidence that memory T cells have higher affinity for antigen and require less antigen for activation (Sallusto et al., 1999; Savage et al., 1999; Busch and Pamer, 1999; Rogers et al., 2000; Kumar et al., 2011; Richer et al., 2013), which implies that less costimulation would be required. In addition, memory T cells are more resistant to suppression by Tregs (Afzali et al., 2011). However more recent reports have shown that both naive and memory T cells are dependent on CD28 costimulation (Fuse et al., 2011; Ndejembi et al., 2006; Sallusto et al., 1999; Garcia et al., 2004; Borowski et al., 2007; Garidou et al., 2009). The contradictory evidence probably reflects the fact that the costimulatory requirements of T cells, including memory cells, depend on the quality and quantity of antigen. For example, one study showed that a secondary response to attenuated *Salmonella* required CD28 engagement but a response against a cloned *Salmonella* antigen did not, suggesting that the type of antigen dictates the costimulation requirements (Garcia et al., 2004). In addition, the costimulatory

requirements of memory T cells may depend on their activation status and history. For example it has been found that memory T cells in vaccinated individuals required CD28 costimulation for a recall response, but that recently activated T cells did not require costimulation to respond (Yi-qun et al., 1996). Similarly it has been shown that central memory T cells require CD28 costimulation for effective proliferation and cytokine production while effector memory cells in the target tissue are CD28 independent (Fontenot et al., 2003a; Teijaro et al., 2009; Bottcher et al., 2013). Memory T cells may be activated without CD28 costimulation in some contexts because they are maintained in the G_1 phase of the cell cycle through CD27 and 4-1BB costimulation so they can respond efficiently to antigen without CD28 costimulation, unless they have been deprived of contact with DC, in which case they are more dependent on costimulation upon antigen encounter (Allam et al., 2009). Furthermore, the costimulatory requirements for a memory response may depend on the amount and duration of antigen exposure during the primary response in which the memory cells were generated (Floyd et al., 2011). Overall, the emerging importance of CD28 costimulation for memory T cell responses prompts further investigation into the relative costimulatory requirements of naive and memory CD4 T cells.

1.19 The requirements and outcomes of T cell activation depend on the type of antigen presenting cell

When investigating T cell activation and differentiation it is also important to consider the variation between different types of APC. DC, B cells, monocytes and macrophages differ in many of their characteristics, including costimulatory molecule expression, which may influence CD28-dependency and T cell fate. For example APC have different levels of MHC molecule expression: Monocytes and naive B cells express low levels of MHC; mature B cells, macrophages and immature DC express intermediate levels; and mature DC express high levels (Hart, 1997). This may affect how much antigen they can present, and therefore the strength of the stimulus they can provide and how many additional signals may be

required for T cell activation. Furthermore, the shape of the APC can affect its ability to make contact with and present antigen to T cells. For example monocytes and naive B cells are small and round with limited ruffles so they only make contact with T cells by chance. Activated B cells have a greater capacity than naive B cells to stimulate T cells, but DC have by far the best T cell priming ability due to their diverse shapes and dynamic filopodia and dendrites which they use for making contact with T cells (Gunzer et al., 2000; Gunzer et al., 2004). Once contact has been made, DC have an active tubulin-driven transport system to concentrate the presentation of MHC:peptide at the DC-T cell interface (Boes et al., 2002), which may partly explain why DC don't need more structured long-lasting immune synapses like B cells. Also, DC form stronger interactions with T cells than B cells or macrophages, which causes increased calcium mobilisation and more TCR and PKCθ molecules to translocate to the immune synapse, which results in greater T cell proliferation and cytokine production (Rothoeft et al., 2003; Lim et al., 2012). Different types of APC also express different repertoires and amounts of costimulatory molecules and chemokines, which determine what type of T cell they can attract, and also how that T cell may differentiate. Consistent with all these differences between APC, some studies have shown that while B cells can activate memory T cells, they have little or no capacity to prime naive T cells, whereas DC can stimulate both naive and memory T cells (Ronchese and Hausmann, 1993; Cassell and Schwartz, 1994). Therefore naive and memory T cells may largely be activated by different types of APC, which could explain the possible variation in their costimulatory requirements. Additionally, different APC present different types of antigen, which may determine the costimulatory requirements and T cell fate. For example DC can activate T cells with processed and presented antigen-immunoglobulin IgG complexes but macrophages and B cells cannot (de Jong et al., 2006). In terms of T cell differentiation, DC induce IL-4- and IFNγ-producing T cells whereas macrophages mostly induce IL-4-producing T cells (Rothoeft et al., 2003). Similarly B cells preferentially induce T cells to produce IL-4 (Macaulay et al., 1997). Alternatively B cells may cause naive T cells to differentiate into

Tregs upon prolonged contact (Reichardt et al., 2007). In summary, while CD28 costimulation appears to be important for T cell activation by both DC and B cells (Lim et al., 2012), a T cells requirement for CD28 may depend on the context of the APC.

1.20 Aims

Our laboratory established a novel mechanism for CTLA-4, whereby CTLA-4 captures its ligands CD80 and CD86 from APC by trans-endocytosis. One aim of this project was to enhance our understanding of this process and to investigate whether related receptors can also remove their ligands by trans-endocytosis:

1) **Study the use of trans-endocytosis by regulatory T cell receptors**
    - Develop a flow cytometry assay to study the trans-endocytosis of CTLA-4 and its ligands and to distinguish trans-endocytosis from trogocytosis
    - Determine whether CD80 and CD86 undergo trans-endocytosis equivalently
    - Investigate whether other proteins are able to remove their ligands by trans-endocytosis

The removal of costimulatory ligands from APC by CTLA-4 reduces their capacity to provide CD28 costimulation to T cells and therefore inhibits T cell activation. This generates the prediction that there is a minimum number of costimulatory ligands required for T cell activation. The second aim of this project was to test this prediction:

2) **Study the requirements for T cell costimulation**
    - Quantify the number of costimulatory ligand molecules required for T cell activation and determine whether changing the amount of costimulation has a graded or ungraded effect on the T cell response
    - Investigate the context in which costimulation is required and in what time frame costimulation must be provided
    - Compare CD80 and CD86 function quantitatively and qualitatively

Finally, CD28 costimulation is traditionally believed to be crucial for an effective T cell response. However CD28-independent T cell activation is reported to occur, although the functionality of these T cells remains to be fully analysed. The outcomes of restricting CD28 costimulation are of particular interest since the discovery that CTLA-4 removes the ligands it shares with CD28 from APC by trans-endocytosis. Therefore the third aim of this project was to explore the consequences of limiting CD28 costimulation:

3) Examine the outcomes of CD28 costimulation for CD4 T cell responses

- Study the fate of T cells activated independently of CD28
- Investigate whether T cells can avoid anergy without CD28 costimulation
- Compare the requirements of naive and memory T cells for costimulation

2 METHODS

2.1 Experimental plan

As outlined in **section 1.20** the first aim of this project was to study the use of trans-endocytosis by regulatory T cell receptors. In order to study trans-endocytosis, CHO cells were transfected with the human gene for the receptor of interest or with DNA encoding a ligand-GFP fusion protein (**sections 2.3-2.6** and **sections 2.8-2.10**). Trans-endocytosis could therefore be measured by ligand-GFP acquisition by receptor-expressing cells using flow cytometry of confocal microscopy (**sections 2.11-2.12**).

Another aim of this project was to quantify the number of costimulatory ligand molecules required for T cell activation. In order to determine the level of CD28 ligand required for T cell activation, a Tet-inducible expression system was used to express different amounts of CD80 or CD86 on CHO cells (**section 2.2** and **sections 2.4-2.8**). These cells were used with anti-CD3 to stimulate T cells. The T cell response to different levels of CD28 ligand was assessed by measuring T cell proliferation, protein expression or cytokine production (**sections 2.13-2.20**).

The third aim of this project was to examine the outcomes of CD28 costimulation by comparing the fates of T cells stimulated with and without CD28 costimulation. In order to activate T cells in the absence of CD28 costimulation, T cells required strong TCR signalling which was provided by cells expressing the Fcγ receptor II cross-linking anti-CD3 on the T cell surface.

2.2 Cloning of genes into Tet-inducible expression vector

pTRE3G plasmid DNA (Clontech) was transformed into Subcloning Efficiency DH5α Competent Cells (Invitrogen) according to the supplier's instructions. Transformed bacteria

were grown on agar plates containing 100µg/ml ampicillin (Sigma-Aldrich) (1.5% select agar (Sigma-Aldrich) in Circlegrow (MP Biomedicals)). To amplify and purify the plasmid DNA, a single bacterial colony was used to inoculate Circlegrow broth containing 100µg/ml ampicillin, which was shaken at 37°C overnight. Plasmid DNA was purified using the QIAprep spin miniprep kit (QIAGEN) according to the manufacturer's instructions.

Primers were designed to amplify human CD80 or CD86 coding sequences from plasmid DNA and to add flanking restriction sites to allow cloning into the pTRE3G vector. Primers were purchased from Eurofins MWG Operon.

Primers:

CD80 forward: CCC GCC GGA **GTC GAC** GAA GCC ATG GGC CAC ACA CGG AGG CAG

CD80 reverse: CCC GCG TAC **GGA TCC** ACA CTG TTA TAC AGG GCG TAC ACT TTC

CD86 forward: CCC GCC GGA **GTC GAC** TGC ACT ATG GGA CTG AGT AAC ATT CTC

CD86 reverse: CCC GCG TAC **GAT ATC** CTT TAA TTA AAA ACA TGT ATC ACT TTT

GTC GAC = Sal I restriction site

GGA TCC = BamHI restriction site

GAT ATC = EcoRV restriction site

ATG = start codon

TTA = stop codon

CD80 and CD86 genes (see **appendix** for sequences) were amplified from full length wild type DNA (CD80 cDNA was cloned previously in our laboratory and CD86 cDNA was a gift from L. Lanier (Azuma et al., 1993a)) by polymerase chain reaction (PCR) using 5ng/µl DNA, 2.5mM dNTPs (Fermentas), 5mM $MgCl_2$ (Bioline), 10µM forward primer, 10µM reverse primer and 2.5u *Pfu* polymerase (Fermentas). PCR was carried out according to the

following cycles (optimised by Claire Manzotti in our laboratory) using a Peltier thermal cycler PTC-225 (MJ Research):

95°C 5 minutes

95°C 1 minute
55°C 1 minute
72°C 1.5 minutes
} 25 cycles

72°C 10 minutes

PCR product size was verified by agarose gel electrophoresis where ~100ng DNA mixed with loading dye (Promega) was loaded per well alongside ~100ng DNA ladder (New England BioLabs) in a 1% agarose (Geneflow) gel containing SYBR safe DNA gel stain (Invitrogen) which was submerged in TAE buffer (Geneflow) and 100V applied for 70-80 minutes. PCR products were purified using a QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions. Total PCR product and 7.4µg pTRE3G DNA were digested with restriction enzymes SalI and BamHI or SalI and EcoRV (Promega) at 37°C for 3 hours. Digested DNA fragment sizes were verified by agarose gel electrophoresis (using 1.5% Seakem GTG agarose) then insert DNA was purified using a QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions, while plasmid DNA was gel extracted and purified using a QIAquick gel extraction kit. Genes of interest were inserted into pTRE3G using T4 DNA ligase (Fermentas) according to the manufacturer's instructions. 3µl of the ligation reaction was used to transform Subcloning Efficiency DH5α Competent Cells according to the manufacturer's instructions. Bacterial colonies grown on ampicillin (100µg/ml) agar plates were screened for plasmid DNA containing an insert of the correct size by direct colony PCR using primers specific for sequences flanking the vector cloning site followed by gel electrophoresis.

Vector-specific primers:

pTRE3G forward: CTC CCT ATC AGT GAT AGA GAA CGT

pTRE3G reverse: TAT TAC CGC CTT TGA GTG AGC TGA

Plasmid DNA from positive colonies was purified using the QIAprep spin miniprep kit (QIAGEN) according to the manufacturer's instructions. DNA was fully sequenced by Eurofins MWG Operon. A larger volume of more concentrated plasmid DNA (maxiprep) was prepared from the bacterial colony or culture grown for miniprep using a PureLink HiPure plasmid filter purification kit (Invitrogen) according to the manufacturer's instructions.

2.3 Generation of GFP fusion proteins

Genes of interest (human LICOS, LFA-3, PD-L1, PD-L2) (see **appendix** for sequences) were amplified by PCR (LICOS cDNA was a gift from S. Davis and PD-L1/PD-L2 cDNA were previously cloned by M. Liu in our laboratory) according to the method in **section 2.1** to add flanking restriction sites (BglII and SalI) and mutate the stop codon. Restriction sites were chosen that were in the cloning site of plasmid pEGFP-N3 (Invitrogen) and in frame with the start codon of the GFP coding sequence. Genes of interest were cloned into pEGFP-N3 using the same methods as those described in **section 2.1** for cloning CD80 and CD86 into pTRE3G except that DNA was amplified using TOP10 chemically competent *E. coli* (Invitrogen) which were selected using 50µg/ml kanamycin (Sigma-Aldrich).

Primers:

LICOS forward: CGC GCG **AGA TCT** CGC ACC ATG CGG CTG GGC AGT C

LICOS reverse: CGC GCG **GTC GAC** CTC CGG TCC AAC GTG GCC AGT G

PD-L1 forward: CGC GCG **AGA TCT** CGC AAG ATG AGG ATA TTT GCT G

PD-L1 reverse: CGC GCG **GTC GAC** GCT GGA TGA CGT CTC CTC CAA

PD-L2 forward: CGC GCG **AGA TCT** CAG AAC ATG ATC TTC CTC CTG CTA

PD-L2 reverse: CGC GCG **GTC GAC** ACA GGT TAA GAT AGC ACT GTT CAC

LFA-3 forward: CGC GCG **AGA TCT** CGA GCC ATG GTT GCT GGG AGC G

LFA-3 reverse: CGC GCG **GTC GAC** TCT TCT CAA TAA AAG AAC ATT CAT

AGA TCT = BglII restriction site

GTC GAC = SalI restriction site

pEGFP-N3 forward: GGC GGT AGG CGT GTA CGG TGG G

pEGFP-N3 reverse: GGA CAA GCT CGA CCT GCC GC

2.4 CHO cell culture

CHO cells were cultured in vented 75 cm^2 tissue culture flasks with Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (BioSera), 2mM L-glutamine (Sigma-Aldrich) and 1% penicillin and streptomycin (Invitrogen) in a humidified incubator at 37°C in 5% CO_2. When the cells reached ~90% confluency, the culture medium was removed and cells were washed with phosphate buffered saline (PBS) (prepared using tablets from Oxoid) then incubated at 37°C with 0.05% trypsin-EDTA (Invitrogen) for up to 5 minutes. Trypsin was quenched by adding 5 volumes of culture medium. ~10% of the cells were retained to continue cell culture and fresh culture medium was added to a total volume of 10 ml. Remaining cells were used for experimentation.

2.5 CHO cell protein expression analysis

~$0.2x10^6$ cells were re-suspended in ~100µl culture medium and incubated with the appropriate antibody (see **table 2.1**) at 4°C for 30 minutes. Cells were washed in PBS (all washes involve centrifugation at 490g for 5 minutes and removal of the supernatant unless stated otherwise) then re-suspended in ~1ml PBS and analysed by flow cytometry using a DakoCytomation $CyAn_{ADP}$ device (Beckman Coulter). Data was collected using Summit v4.3 software (Beckman Coulter) and analysed using FlowJo v8.5.3 (Treestar). Un-transfected

CHO cells were used as negative control samples, which were treated in the same way and incubated with the same antibodies as the test samples.

2.6 Cell transfection and selection

To make cell lines expressing FcR, CD2, PD-1 and GFP fusion proteins, ~2x10^6 CHO cells were transfected with 2-3µg of plasmid DNA using an Amaxa nucleofector device (Lonza Cologne AG) with Cell Line Nucleofector Kit T for CHO-K1 cells [ATCC] and program U-23 for high transfection efficiency according to the manufacturer's instructions. (Human CD2 and PD-1 DNA sequences had already been cloned into expression vector pcDNA3.1 (Invitrogen) in our laboratory by M. Liu and FcR cDNA was a gift from J. Allen and was cloned into expression vector pCR3.1 (Invitrogen) by D. Sansom). Transfected cells were selected for by antibody staining followed by either fluorescence activated cell sorting (FACS) using a BD FACSAria cell sorter, or incubation with sheep anti-mouse IgG-coated magnetic Dynabeads (Invitrogen) to positively select cells using a magnet. Stable cell lines were established by culturing cells with 250-500µg/ml geneticin (Invitrogen). Protein expression levels were kept as similar as possible for all receptor-expressing cell lines and all ligand-GFP-expressing cell lines. Mouse OX40L-GFP and mOX40 CHO cell lines were generated by Omar Qureshi. The AVKM CTLA-4 CHO cell line was created by Satdip Kaur as previously described (Qureshi et al., 2012). CD28, wild type CTLA-4, CD80-GFP and CD86-GFP CHO cell lines already existed in the laboratory, generated as previously described (Qureshi et al., 2011).

To make Tet-inducible cell lines, CHO cells already expressing a Tet-On regulatory plasmid (purchased from Clontech) were co-transfected by nucleofection (as above) with ~1.5µg pTRE3G containing CD80- or CD86-coding DNA and 0.25µg pcDNA3.1 (Invitrogen) containing a hygromycin resistance cassette. Cells were selected as above by FACS or magnetic cell sorting then stable cell lines were grown in DMEM containing 10% tetracycline-free fetal bovine serum (BioSera), 2mM L-glutamine, 1% penicillin and streptomycin,

1.2mg/ml hygromycin (Invitrogen) and 100µg/ml geneticin. Tet-On cells were incubated with 1µg/ml doxycycline (Sigma-Aldrich) for two days prior to sorting to induce expression of selectable protein. Stable but heterogeneous inducible cell lines were diluted and grown in 96 well plates to produce colonies of cells cloned from a single cell. Where only a single colony grew per well the cells were expanded and tested for their homogeneity in protein expression and their responsiveness to different doses of doxycycline. One clone of each cell line was chosen that expressed the protein of interest in a dose-dependent manner at a wide range of levels.

2.7 Tet-inducible system

Figure 2.1 shows how the Tet system regulates protein expression. A range of cell densities, doxycycline concentrations and treatment times were tested to establish a protocol for inducing optimum protein expression at a range of levels. The final working protocol that was used for inducing cells with which to stimulate T cells was as follows. ~$1.5x10^6$ cells in a 4ml volume of culture medium were added per $25cm^2$ culture flask. Serial dilution of doxycycline in culture medium produced 5x working concentrations of 5000, 1000, 500, 375 and 250ng/ml. Adding 1ml doxycycline dilution per flask gave final concentration of 1000, 200, 100, 75 or 50ng/ml doxycycline. Cells were incubated at 37°C for 42-48 hours. Cells were dissociated using trypsin and washed, then the number of protein molecules expressed per cell was determined as described in the following section.

2.8 Determining protein molecule number from antibody fluorescence intensity

Quantum Simply Cellular microspheres (Bangs Laboratories) with known numbers of antibody binding sites were used to calculate the antibody binding capacity (ABC) of cells and thus the number of protein molecules per cell. These tools were used to determine the number of costimulatory ligands induced on Tet-On cells and to accurately compare the cell surface expression levels of different receptors being tested for their ability to remove their

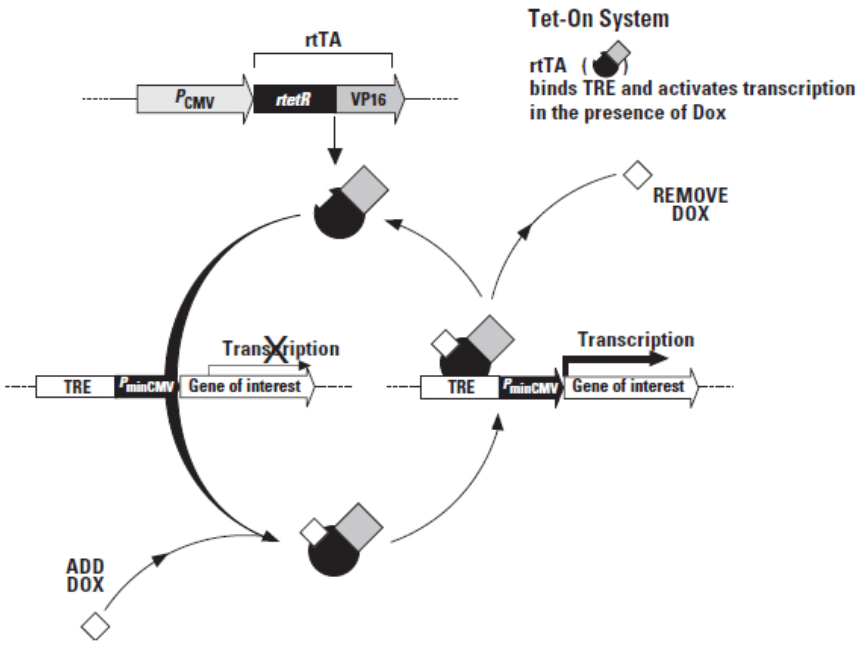


Figure 2.1 Tet-On system. The reverse transactivator protein (rtTA) is encoded by a regulatory plasmid. In the absence of doxycycline gene transcription for the response plasmid is switched off but when doxycycline binds to the rtTA it can bind the tetracycline response element (TRE) upstream of the gene of interest and drive transcription in a dose-dependent manner.

ligands by trans-endocytosis. One drop of each of four samples of microspheres with different ABC were added to 100µl culture medium in separate FACS tubes. 4µl antibody was added per sample except one sample of microspheres were left unstained to determine background fluorescence. Samples were incubated on ice for 30 minutes then washed in PBS. Cells were stained in the same way. All samples were analysed by flow cytometry on the same day on the same instrument using the same settings. The mean fluorescence intensity (MFI) measured for the microspheres was plotted against the ABC to produce a standard curve from which the number of surface protein molecules per cell could be derived using the measured MFI (**figure 4.4**).

2.9 Protein localisation by confocal microscopy

CHO cells were seeded in a chamber slide and incubated at 37°C for 6 hours to allow them to adhere to the bottom of the slide. Fluorescently-labelled antibody was added directly to the chambers and incubated at 37°C for 30 minutes before observation by confocal microscopy. Imaging was performed using a Zeiss LSM 780 inverted laser scanning confocal microscope and images were processed using ImageJ v1.44o (Wayne Rasband, NIH).

2.10 Endocytosis assay

$1x10^6$ CHO cells were labelled in a volume of 300µl of culture medium with 3µl fluorescently-labelled primary antibody (CTLA-4-PE, CD28-PE, PD-1-APC or CD2-APC) on ice for 30 minutes. Cells were washed in ice cold culture medium then re-suspended in 250µl fresh ice cold culture medium. 50µl of cell suspension was transferred to each of five FACS tubes on ice. Cells were incubated at 37°C for 0, 1, 5, 15 or 30 minutes then returned to 4°C. Cells were labelled with an appropriate anti-mouse secondary antibody on ice for 30 minutes. Each cell line was also stained with primary and secondary antibodies alone on ice. Cells were washed in ice cold culture medium and then re-suspended in ice cold PBS and kept at 4°C until analysis by flow cytometry. All flow cytometry was performed using a

DakoCytomation $CyAn_{ADP}$ device. Data was collected using Summit v4.3 software and analysed using FlowJo v8.5.3.

2.11 Trans-endocytosis assay by flow cytometry

CHO cells expressing ligand-GFP fusion proteins were incubated with 1µM Cell Trace Far Red DDAO-SE (Invitrogen) for 10 minutes at room temperature or 5µM Cell Trace Violet (CTV) (Invitrogen) for 20 minutes at 37°C then washed in culture medium. 100,000 unlabelled receptor-expressing cells were added per FACS tube then labelled ligand-GFP-expressing cells were added at the specified ratio. Where specified, cells were incubated with 10-40mM NH_4Cl (Sigma-Aldrich), 25nM bafilomycin A (Sigma-Aldrich) or 100µM chloroquine (Sigma-Aldrich). Cells were incubated in a volume of 400µl in a water bath at 37°C for 1-6 hours as specified then put on ice. Cells were vortexed, antibody was added to label ligand or GFP molecules present on the cell surface, then cells were aspirated up and down to separate them and incubated on ice for 30 minutes. Cells were washed and re-suspended in PBS then kept on ice while analysed by flow cytometry. Cells were aspirated up and down immediately before analysis to separate cells.

2.12 Trans-endocytosis assay by confocal microscopy

CHO cells expressing ligand-GFP fusion proteins were incubated with 5µM CTV for 20 minutes at 37°C while receptor-expressing CHO cells were incubated with 1µM Cell Trace Far Red for 10 minutes at room temperature. All cells were washed in culture medium then ~750,000 of each cell type were added per well in a glass bottom 24 well plate and incubated in the presence or absence of 10mM NH_4Cl at 37°C for 3-6 hours. Cells were observed by confocal microscopy. Imaging was performed using a Zeiss LSM 780 inverted laser scanning confocal microscope using a 100x oil immersion objective with excitation at 488nm, 543nm and 633nm. Constant laser powers and acquisition parameters were maintained throughout individual experiments. Data were analysed using ImageJ.

2.13 Isolation of T cells from whole blood

Blood from leukocyte cones purchased from the National Blood Service (Birmingham) was diluted five times with PBS and 25ml was layered onto 15ml Ficoll Paque PLUS (GE healthcare) then centrifuged at 1060g (2200 rpm) for 25 minutes without a brake. The buffy coat containing peripheral blood mononuclear cells (PBMCs) was recovered using a Pasteur pipette. PBMCs were re-suspended in 50ml PBS and centrifuged at 1060g for 10 minutes. PBMCs were washed again in 50ml PBS with centrifugation at 260g (1100 rpm) for 5 minutes. Two further washes, the first in 50ml PBS and the second in 50ml MACS buffer (2mM EDTA, 0.5% BSA in PBS), were performed with centrifugation at 490g (1500 rpm) for 5 minutes. PBMCs were re-suspended in MACS buffer at $100x10^6$ cells/ml and transferred to a 15ml Falcon tube. $CD4^+CD25^-$ T cells were negatively selected as follows: EasySep™ $CD4^+CD25^-$ T cell enrichment cocktail (StemCell Technologies) was added at 50µl/ml. After 10 minutes at room temperature, magnetic particles were added at 100µl/ml and left for 5 minutes. MACS buffer was added to a total volume of 8ml and the tube placed into an EasySep™ magnet for 5 minutes. $CD4^+CD25^-$ T cells were poured into a fresh tube by gently inverting the magnet, while magnetically labelled unwanted cells remained in the tube. The magnetic cell removal step was repeated for higher purity, then $CD4^+CD25^-$ T cells were washed once in PBS with centrifugation at 490 g for 5 minutes and re-suspended in PBS or RPMI 1640 culture medium containing 10% FBS, 2mM L-glutamine and 1% penicillin and streptomycin (herein referred to as RPMI). Cell purity was consistently >97% as determined by antibody labelling and flow cytometry. For some experiments (where specified) naïve and memory T cells were subsequently isolated from the $CD4^+CD25^-$ fraction by negative selection using EasySep™ naïve or memory $CD4^+$ T cell enrichment kits (StemCell Technologies) according to the manufacturer's instructions. For naive T cell enrichment unwanted cells are targeted for removal with Tetrameric Antibody Complexes recognizing CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCRγ/δ, glycophorin A and

dextran-coated magnetic particles. $CD45RO^+$ cells are targeted for removal with a biotinylated anti-CD45RO antibody and a bispecific Tetrameric Antibody Complex that recognizes biotin and dextran. For memory T cell enrichment unwanted cells are targeted for removal with Tetrameric Antibody Complexes recognizing CD8, CD14, CD16, CD19, CD20, CD36, CD45RA, CD56, CD123, TCRγ/δ, glycophorin A and dextran-coated magnetic particles.

2.14 T cell proliferation assay

All washes involved centrifugation at 490g for 5 minutes.

Purified T cells were re-suspended in PBS at ~$5x10^6$ cells/ml and labelled by incubating with 5μM CTV for 20 minutes at 37°C. Labelling was stopped by adding 20ml RPMI containing 10% FBS. After 5 minutes cells were washed once in PBS then re-suspended in RPMI at $2x10^6$ cells/ml. Cells were used immediately or left at 37°C overnight.

CHO cells were fixed by re-suspending 3-$4x10^6$ cells in 1ml 0.025% glutaraldehyde (Sigma-Aldrich) for 2-3 minutes, agitating gently. 10ml culture medium was added to neutralise the glutaraldehyde then cells were washed twice in PBS. CHO cells were re-suspended in RPMI and used immediately or stored at 4°C overnight.

T cell proliferation assays were carried out in round bottom 96 well culture plates. $0.1x10^6$ CTV-labelled T cells and $0.02x10^6$ CHO cells were added per well to give a CHO:T cell ratio of 1:5 (for all experiments in chapter five) or different numbers of CHO cells were added as specified. T cells were cultured in a 200μl volume of RPMI with 1μg/ml anti-CD3 (clone OKT) (unless otherwise specified) or 0.25-250ng/ml TSST-1 (Sigma-Aldrich) and 20μg/ml CTLA-4-Ig (Abatacept, Bristol-Myers Squibb) and/or 200U/ml IL-2 (Peprotech) where specified. Cultures containing only T cells and anti-CD3 were always set up to show there were no

contaminating APCs and to use as unstimulated controls. Cells were incubated at 37°C for 5 days. Cells were transferred to FACS tubes and washed in 1ml PBS. Cells were stained as necessary (refer to section 2.15) then washed and re-suspended in 400µl PBS. To determine the total number of T cells per culture, 7µl AccuCheck counting beads (Invitrogen) were added before analysis by flow cytometry.

2.15 T cell restimulation assay

$CD4^+CD25^-$ T cells were labelled with 2.5µM CFSE (Molecular Probes) for 10 minutes at room temperature then cells were washed in 10ml RPMI followed by a wash in PBS. Cells were re-suspended in RPMI for culture. ~$0.5x10^6$ CFSE-labelled $CD4^+CD25^-$ T cells and ~$0.1x10^6$ glutaraldehyde fixed CHO cells with 1µg/ml anti-CD3 were cultured in a 1ml volume per well in a 24 well plate at 37°C for 7-8 days. Cells cultured in the same conditions were combined and washed in PBS. Responding T cells were purified by FACS by sorting for all cells that had diluted CFSE fluorescence. Secondary stimulations were set up according to the T cell proliferation assay in the previous section.

2.16 T cell surface and total protein staining

Experiments were set up as for the T cell proliferation assay (section 2.13). Cells were incubated at 37°C for two or five days as indicated. Cells were transferred to FACS tubes and washed in 1ml PBS by centrifugation. For surface protein staining, cells were re-suspended in the residual volume after washing then 25µl of pre-prepared antibody cocktail diluted in PBS with 2% goat serum (Sigma-Aldrich) was added per tube (see **table 2.1** for final antibody volumes per sample). Cells were incubated at 4°C for 30 then washed in 1ml PBS. All stained cells were re-suspended in 400µl PBS and where appropriate 7µl counting beads were added. Samples were kept on ice until analysis by flow cytometry. As a negative control, stimulated T cells were incubated with isotype control antibodies for each

fluorochrome conjugate at 4°C for 30 minutes. Single antibody stains were also performed for each fluorochrome in order to compensate multi-colour samples.

For intracellular (total) protein staining of FoxP3, once cells had undergone surface staining and been washed, cells were re-suspended in 500µl fixation/permeabilisation buffer (eBioscience), which was made by mixing concentrate and diluent at a 1:3 ratio. Cells were incubated at 4°C for 1 hour then washed in 1ml PBS. Cells were washed again with 1ml 1x permeabilisation buffer (eBioscience) then re-suspended in the residual volume of permeabilisation buffer. 1µl FoxP3 antibody was added then cells were incubated at 4°C for 45 minutes. Cells were washed in 1ml 1x permeabilisation buffer then washed in 1ml PBS. Cells were re-suspended in PBS and analysed by flow cytometry.

For intracellular (total) staining of pSTAT5 and pS6, cells were re-suspended in 1ml PBS then 1ml 6% formaldehyde (Acros) was added. Cells were incubated at 37°C for 10 minutes, pelleted by centrifugation, re-suspended in the residual volume and put on ice. 1ml ice cold methanol (Fisher Scientific) was added and cells were incubated on ice for 10 minutes. Cells were washed with 2ml 0.5% BSA (Acros) in PBS then washed again in 1ml 0.5% BSA. Cells were left at room temperature for 10 minutes for the BSA to block non-specific protein interactions then 25µl pre-prepared antibody cocktail diluted in PBS with 2% goat serum was added and incubated at room temperature for 30 minutes. Cells were washed once in 0.5% BSA and again in PBS then re-suspended in PBS for analysis by flow cytometry.

2.17 Intracellular cytokine staining

After two days in culture T cells were either treated with 10µg/ml brefeldin A (Sigma-Aldrich) for 5-15 hours, or they were restimulated with 0.5µM phorbol myristate acetate (PMA) (Sigma-Aldrich) and 0.1mg/ml ionomycin (Sigma-Aldrich) for 5 hours and treated with 10µg/ml brefeldin A for the final 3.5 hours. Cells were transferred to FACS tubes and washed

in PBS. Cells were fixed in 3% paraformaldehyde (Sigma-Aldrich) at room temperature for 10 minutes then washed in PBS. Cells were permeabilised with 0.1% saponin (Acros) in PBS, pelleted by centrifugation, re-suspended in 75µl 2% goat serum in saponin and left at room temperature for 15 minutes to block non-specific protein interactions. 25µl pre-prepared antibody cocktail diluted in PBS with 2% goat serum was added and cells were incubated at room temperature for 40 minutes. Cells were washed once in saponin and twice in PBS before being re-suspended in PBS and analysed by flow cytometry.

2.18 Measuring transferrin uptake

Transferrin from human serum conjugated to Alexa Fluor 488 (Invitrogen) was diluted in RPMI to 1mg/ml then 1µl was added per well containing a 200µl volume to give a final concentration of 5µg/ml. Cells were incubated at 37°C for five days then washed and analysed by flow cytometry.

2.19 Measurement of cytokine production by multiplex bead immunoassay

T cell cultures were set up according to the T cell proliferation assay (**section 2.14**). Culture supernatants were collected on day five and stored at -20°C until analysis. A double dilution of half of each sample was made and every sample was processed in duplicate (one replicate half the concentration of the other). Cytokines were labelled using a human cytokine 25-plex antibody bead kit in a 96 well plate format (Invitrogen) according to the manufacturer's instructions. Cytokine concentration was measured by immunofluorescence detected using a Bio-Plex 200 (Bio-Rad) and calculated by Bio-Plex Manager 6.1 software.

2.20 Flow cytometry and data analysis

All flow cytometry was performed using a DakoCytomation $CyAn_{ADP}$ device. For all flow cytometry experiments at least 10,000 events for the cells of interest were collected or as many as possible. Data was collected using Summit v4.3 software and analysed using

FlowJo v8.5.3 software. The gating strategies used for flow cytometry analysis are shown in **figure 2.2**. For experiments using solely CHO cells, the live CHO cells were gated first according to forward and side scatter to exclude dead cells and then singlets were gated based upon pulse width to exclude aggregated cells before analysis the cells of interest, such as the GFP expression of donors and recipients in trans-endocytosis assays (**figure 2.2A**). Similarly for T cell studies the live T cells were gated first, followed by singlets, then CHO cells were excluded by gating out $APC^{high}APC\text{-}Cy7^{high}$ cells (glutaraldehyde fixed CHO cells are highly auto-fluorescent) (**figure 2.2B**). Then either the whole T cell population was analysed to determine proliferation or protein expression, or only responding T cells (T cells that had divided at least once) were analysed as specified. Responders were gated based on dilution of CTV fluorescence to exclude non-activated T cells and then just their protein expression was analysed. The percentage of T cells that committed to divide was calculated based on CTV dilution using the Proliferation platform in FlowJo. The proliferation index (average number of divisions made by cells that entered cell cycle) was also determined by the Proliferation platform in FlowJo. The absolute number of T cells was determined using AccuCheck counting beads which were identified by forward and side scatter. 7µl of beads (containing 7000 beads) were added per sample. Using the ratio of beads to T cells and the known total number of beads, the total number of T cells that were present in the whole sample was calculated as follows: Total number of T cells = number of T cells collected / number of beads collected x 7000.

2.21 Statistical analysis and data processing

GraphPad Prism 6 software was used to calculate all the statistics including the mean, standard deviation (SD) and standard error of the mean (SEM). The non-parametric Wilcoxon matched-pair signed rank test or the paired t test was used to determine statistically significant differences between conditions. To calculate the minimum number of costimulatory ligands required to initiate T cell proliferation or protein upregulation in chapter

4, the average number of ligand molecules was plotted as log(x) against the parameter of interest using a function in Prism to transform the x values. Only data values >0 were included. Then Prism software was used to fit a line with linear regression to the graph and the number of ligands required to initiate proliferation or protein expression was interpolated from where the line intercepted the x axis at y=0.

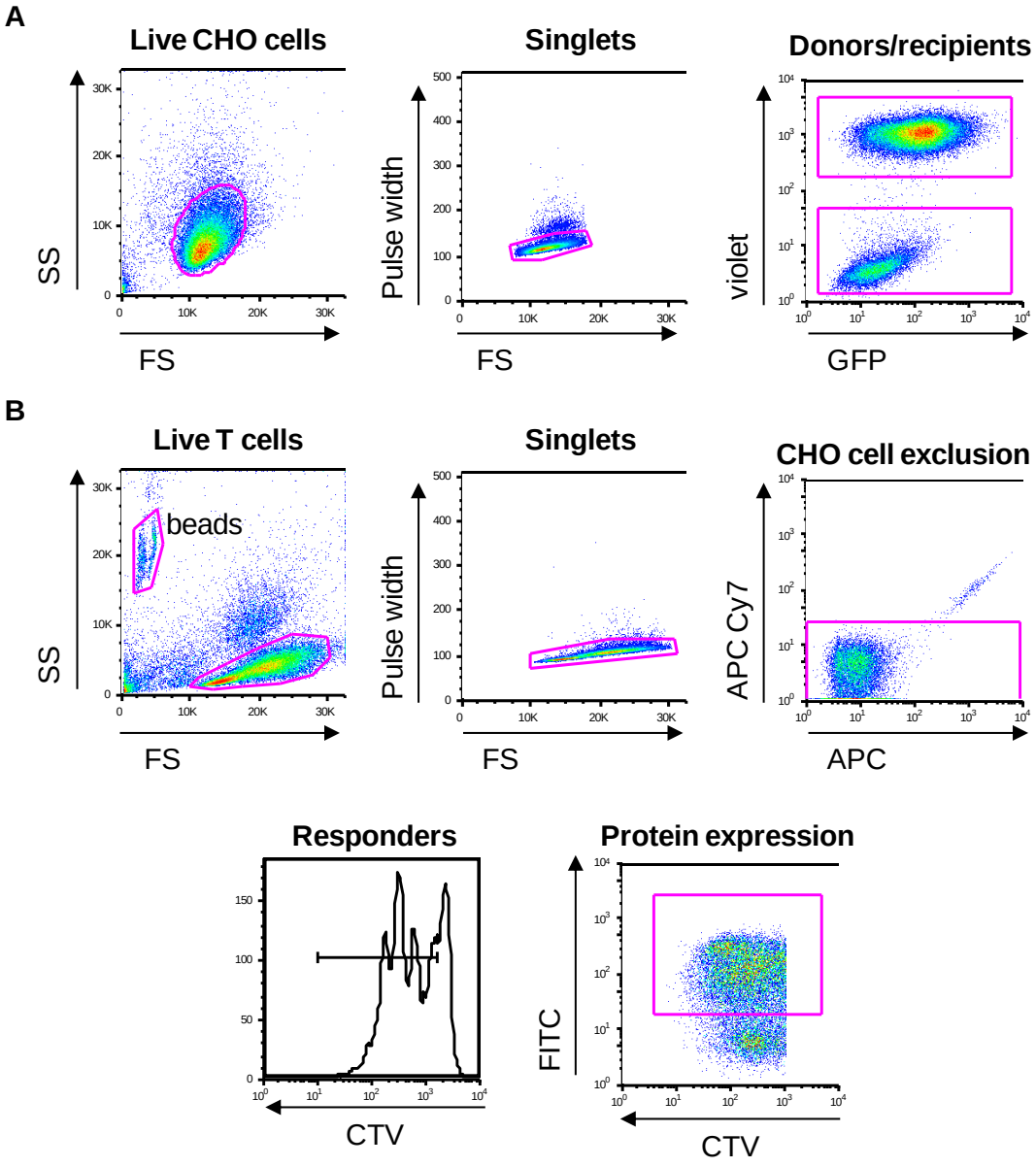


Figure 2.2 Gating strategies for flow cytometric analysis. Flow cytometry data was analysed using FlowJo software. **(A)** Live CHO cells were gated according to forward (FS) and side scatter (SS) to exclude dead cells. Singlets were gated based on pulse width to exclude cell aggregates. Live single cells were analysed as appropriate; for example donors and recipients were gated for trans-endocytosis assays. **(B)** Live T cells were gated according to FS and SS to exclude dead cells. Counting beads were also gated based on FS and SS. Singlets were gated based on pulse width to exclude cell aggregates. CHO cells were excluded by gating out $APC^{high}APC\text{-}Cy7^{high}$ cells. Responders (T cells that were activated and proliferating) were gated based on CTV dilution. Responders were analysed for protein expression, here $FITC^{+}$ cells are gated as an example.

Table 2.1 Antibodies
The manufacturer and volume used per sample are listed for all antibodies used, which are anti-human unless otherwise stated.

Antibody	Volume per sample	Manufacturer	Catalogue number
CCR7-PeCy7	2µl	BioLegend	353226
CD127-PeCy7	1µl	eBioscience	25-1278-42
CD2-APC	2µl	BD Pharmingen	560642
CD25-FITC	1.5µl	BD Pharmingen	345796
CD27-FITC	1.5µl	BD Pharmingen	555440
CD28-APC	2µl	BD Pharmingen	559770
CD28-PE	2µl	BD Pharmingen	555729
CD30-PE	2µl	eBioscience	12-0309-42
CD4-PE	2µl	BD Pharmingen	555347
CD4-APC	2µl	BD Pharmingen	555349
CD40L-PE (CD154)	2µl	BD Pharmingen	555700
CD45RA-PE	2µl	BD Pharmingen	555489
CD45RA-FITC	2µl	BD Pharmingen	555488
CD45RO-FITC	2µl	BD Pharmingen	555492
CD62L-APC	2µl	BD Pharmingen	559772
CD69-APC	2µl	BD Pharmingen	555533
CD71-APC	2µl	BD Pharmingen	551374
CD80-PE	2µl	BD Pharmingen	557227
CD80-FITC	2µl	BD Pharmingen	557226
CD86-PE	2µl	BD Pharmingen	555658
CD86-APC	2µl	BD Pharmingen	555660
CD86-FITC	2µl	BD Pharmingen	555657
CTLA-4-APC (CD152)	2µl	BD Pharmingen	555855
CTLA-4-PE (CD152)	1µl	BD Pharmingen	555853
FcR-APC (CD32)	2µl	BD Pharmingen	559769
HLA-DR-APC	2µl	BD Pharmingen	559866
IFNy-Alexa Fluor 488	1µl	eBioscience	53-7319-42
IgG-Alexa Fluor 555 (Donkey Anti-mouse)	1µl	Invitrogen	A31570
IgG-Alexa Fluor 647 (Chicken Anti-mouse)	1µl	Invitrogen	A21463
IL-2-APC	1µl	eBioscience	17-7029-82
IL-4-PeCy7	1µl	eBioscience	25-7049-82
IL-10-PE	5µl	BD Pharmingen	559337
IL-17-PE	0.5µl	eBioscience	12-7178-42
IL-21-Alexa Fluor 647	1.5µl	BD Pharmingen	560493
Foxp3-APC	1 µl	eBioscience	17-4776-42
GFP-APC	2µl	R&D systems	IC4240A
ICOS-PE (CD278)	3 µl	BD Pharmingen	557802
LFA-3-PE (CD58)	2µl	BD Pharmingen	340295
LICOS-PE (B7-H2)	2µl	BD Pharmingen	552502
OX40-PE (CD134)	2µl	BD Pharmingen	555838

Antibody	Volume per sample	Manufacturer	Catalogue number
OX40-APC (Anti-mouse)	2µl	eBioscience	17-1341-80
OX40L-PE (Anti-mouse)	2µl	eBioscience	12-5905-82
PD-1-APC (CD279)	2µl	BD Pharmingen	558694
PD-L1-PE (B7-H1)	2µl	BD Pharmingen	557924
PD-L2-APC (B7-DC)	2µl	BD Pharmingen	557926
Phospho-S6-Alexa Fluor 647	0.5µl	Cell Signaling Tech	4851S
TCR Vβ2-PE	2µl	Beckman Coulter	PN IM2213
Phospho-STAT5-Alexa Fluor 488	1µl	Cell Signaling Tech	3939S
TNFα-e450	1µl	eBioscience	48-7349-42

3 METHODS FOR STUDYING CTLA-4 TRANS-ENDOCYTOSIS AND ITS USE BY RELATED RECEPTORS

3.1 Characterisation of cell lines expressing coinhibitory or costimulatory receptors

Following the demonstration that CTLA-4 can remove its ligands from opposing cells by trans-endocytosis (Qureshi et al., 2011), the primary aim of the studies in this chapter was to determine whether related proteins also have this ability. Closely related members of the immunoglobulin superfamily include the costimulatory receptors CD28, ICOS and CD2, and another coinhibitory receptor PD-1. In order to study these receptors in a simple and convenient manner, stable CHO cell lines expressing the human form of these proteins were developed. Unfortunately ICOS protein could not be expressed no matter which DNA construct, expression vector, transfection method or cell line was used so the study of this protein was not ultimately pursued.

Once the cell lines were generated and stably expressed the protein of interest, basic characteristics of the proteins were assessed. Firstly the localisation of CTLA-4, CD28, CD2 and PD-1 was compared by confocal microscopy (**figure 3.1**). As expected CTLA-4 was located almost exclusively in intracellular vesicles, while CD28, CD2 and PD-1 were expressed predominantly at the plasma membrane. Next, protein internalisation from the cell surface was measured by flow cytometry (**figure 3.2**). More than 50% of CTLA-4 molecules were removed from the cell surface within five minutes, indicative of rapid endocytosis. In contrast CD28 and CD2 were internalised at a much slower rate and PD-1 was not internalised at all. Taken together these data show that CTLA-4 is an endocytic protein mainly located intracellularly, whereas CD28, CD2 and PD-1 are predominantly plasma membrane proteins.

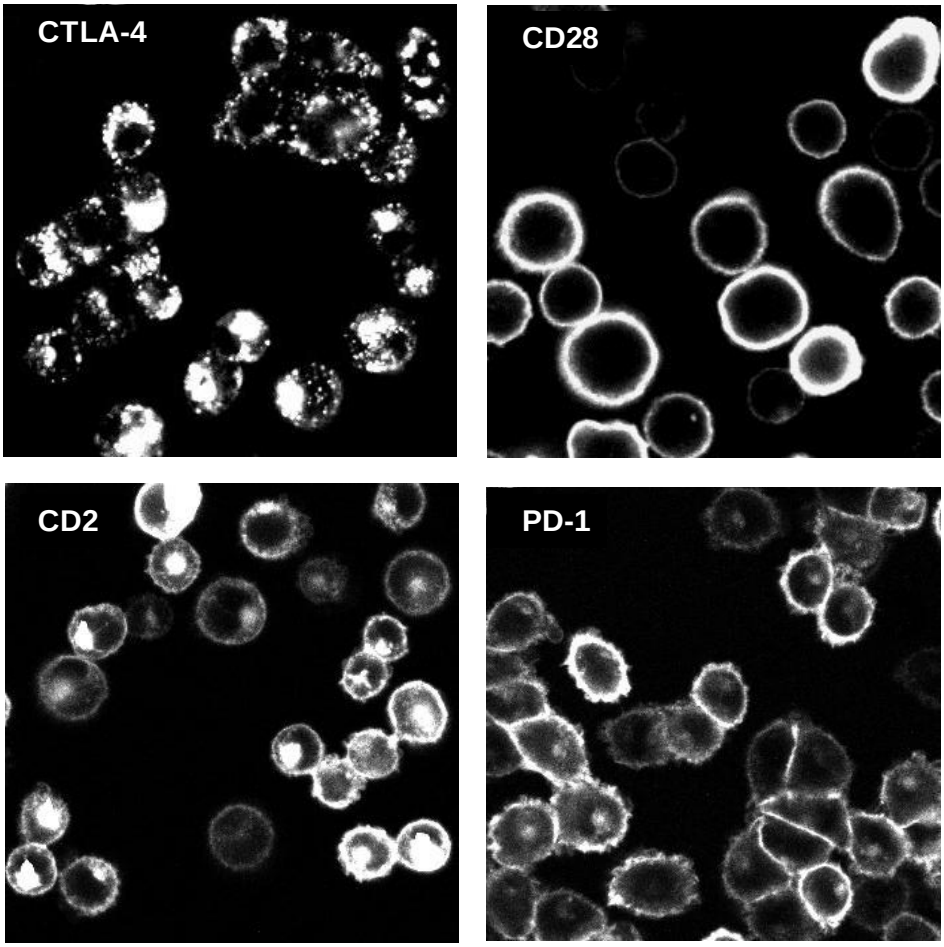


Figure 3.1 CTLA-4 is mainly located in intracellular vesicles whereas CD28, CD2 and PD-1 are located predominantly at the cell surface. Transfected CHO cells were stained using fluorescently-labelled antibodies at 37°C for 30 minutes then cells were observed by confocal microscopy. Images are representative of at least four micrographs.

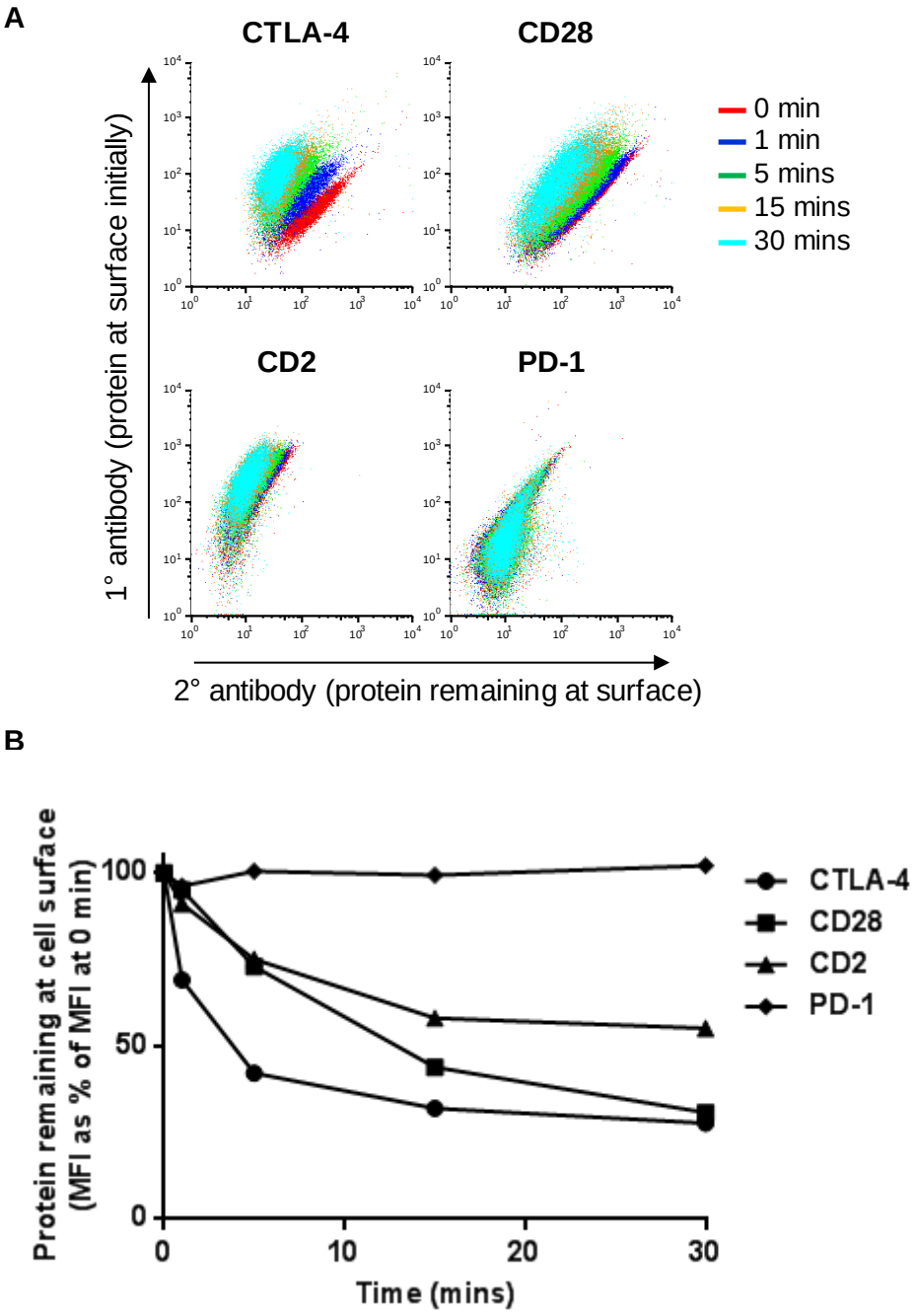


Figure 3.2 CTLA-4 is rapidly internalised from the cell surface whereas PD-1 is a stable membrane protein. Transfected CHO cells were incubated with fluorescently-labelled 1° antibody at 4°C to label protein at the cell surface. Cells were raised to 37°C for 0-30 minutes to allow protein internalisation. Cells were then incubated with fluorescently-labelled 2° antibody at 4°C to co-stain protein labelled with 1° antibody that remained at the cell surface. Cells were analysed by flow cytometry. **(A)** Plots show 1° antibody fluorescence against 2° antibody fluorescence. All time points for each protein are overlaid. A decrease in 2° antibody fluorescence (left shift) as time increases indicates a reduction in protein expression at the cell surface over time. **(B)** Graph shows the amount of protein remaining at the cell surface over time as measured by 2° antibody MFI shown as a percentage of the MFI at 0 minutes. Data are representative of two independent experiments.

Having established basic characteristics of the receptors of interest, the next objective was to determine whether they could remove their ligands by trans-endocytosis. In order to locate ligand easily, CHO cell lines expressing ligand-GFP fusion proteins were generated with the GFP tag located on the cytoplasmic domain. Although ICOS-expressing cells could not be produced LICOS-GFP cells were still generated to test the reported interactions of LICOS with CTLA-4 and CD28 (Yao et al., 2011). Before using these cells it was verified that GFP fluorescence and ligand expression were directly correlated so that localisation and measurement of GFP could be used to identify and quantify ligand protein.

3.2 Establishing a flow cytometry assay for analysing trans-endocytosis

An experimental method needed to be established that would allow the analysis of large numbers of cells simultaneously. Therefore flow cytometry was used to measure ligand-GFP acquisition by receptor-expressing cells (recipients). Ligand-GFP expressing cells (donors) were additionally labelled with a cell trace dye (far red or violet) to distinguish them from the recipients which may subsequently acquire GFP. Blank (un-transfected) cells were also incubated with donors as a negative control, which were used to set the gates for data analysis. **Figure 3.3** shows an example of CD80 trans-endocytosis by CTLA-4. This reveals the acquisition of CD80-GFP by over 50% of CTLA-4-expressing cells (middle panel) and a distinct population of donor cells with reduced CD80-GFP expression compared to the control. Additionally an inhibitor of lysosomal degradation, ammonium chloride (NH_4Cl), was used to prevent the degradation of internalised proteins. This increased the number of recipients that could be detected as having acquired CD80-GFP. These data illustrate the basic hallmarks of trans-endocytosis.

Trans-endocytosis is a continuous process because once CTLA-4-ligand complexes have been internalised either both proteins are targeted for degradation or CTLA-4 is recycled back to the plasma membrane to capture more ligand (Qureshi et al., 2011). Therefore an

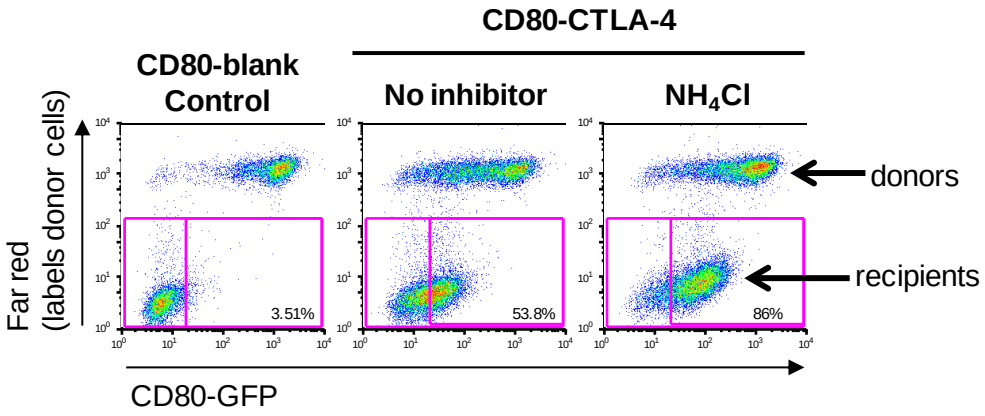


Figure 3.3 Trans-endocytosis of CTLA-4 and CD80 was measured by flow cytometry. Far red-labelled CHO CD80-GFP cells (donors) were incubated with blank or CTLA-4-expressing cells (recipients) at a 1:1 ratio +/- 10mM NH_4Cl at 37°C for 3 hours then analysed by flow cytometry. Plots show GFP fluorescence against far red fluorescence. Recipients are gated into GFP^- and GFP^+ populations and the percentage of GFP^+ recipients is shown.

increasing amount of ligand is captured over time. This is clearly demonstrated in **figure 3.4**, which shows the number of CTLA-4-expressing cells that acquired CD80 increased significantly between one and six hours. The amount of ligand acquired by each cell also increased as shown by the greater recipient GFP fluorescence over time. **Figure 3.4B** shows the total amount of CD80 removed from donor cells as the product of the percentage of recipients that acquired ligand and the average amount of ligand each cell acquired (GFP MFI). Again NH_4Cl increased the amount of CD80 detected on CTLA-4 recipients by preventing ligand degradation (not statistically significant). On the other hand, although CD80-GFP was transferred to CD28-expressing cells, the amount of ligand transfer did not increase significantly over time, suggesting that the acquisition of ligand was through a single event after which no further ligand was captured. Furthermore, NH_4Cl did not increase the amount of ligand detected on CD28-expressing cells. Taken together these results indicate that CD28 does not capture ligand by trans-endocytosis because CD80 was not accumulated over time or targeted to lysosomal pathways. These data demonstrate ways to distinguish between trans-endocytosis and trogocytosis.

In addition a further means to differentiate between trans-endocytosis and trogocytosis was tested. To determine whether CD80 acquired by CD28-expressing cells was still present on the recipient cell surface rather than internalised, ligand was detected by antibody staining at 4°C (**figure 3.5**). Indeed the majority of ligand acquired by CD28-expressing cells was detected at the cell surface, indicating that ligand was acquired by trogocytosis and not internalised by trans-endocytosis. In contrast very little CD80 remained at the cell surface of CTLA-4-expressing cells showing that ligand was readily internalised by trans-endocytosis. These data demonstrate that ligand transfer to CD28-expressing cells is due to trogocytosis and that trans-endocytosis is specific to CTLA-4. Using this method ligand acquisition by trogocytosis could be excluded from the analysis in order to exclusively measure ligand internalisation by trans-endocytosis. **Figure 3.6A** shows the gating strategy used to quantify

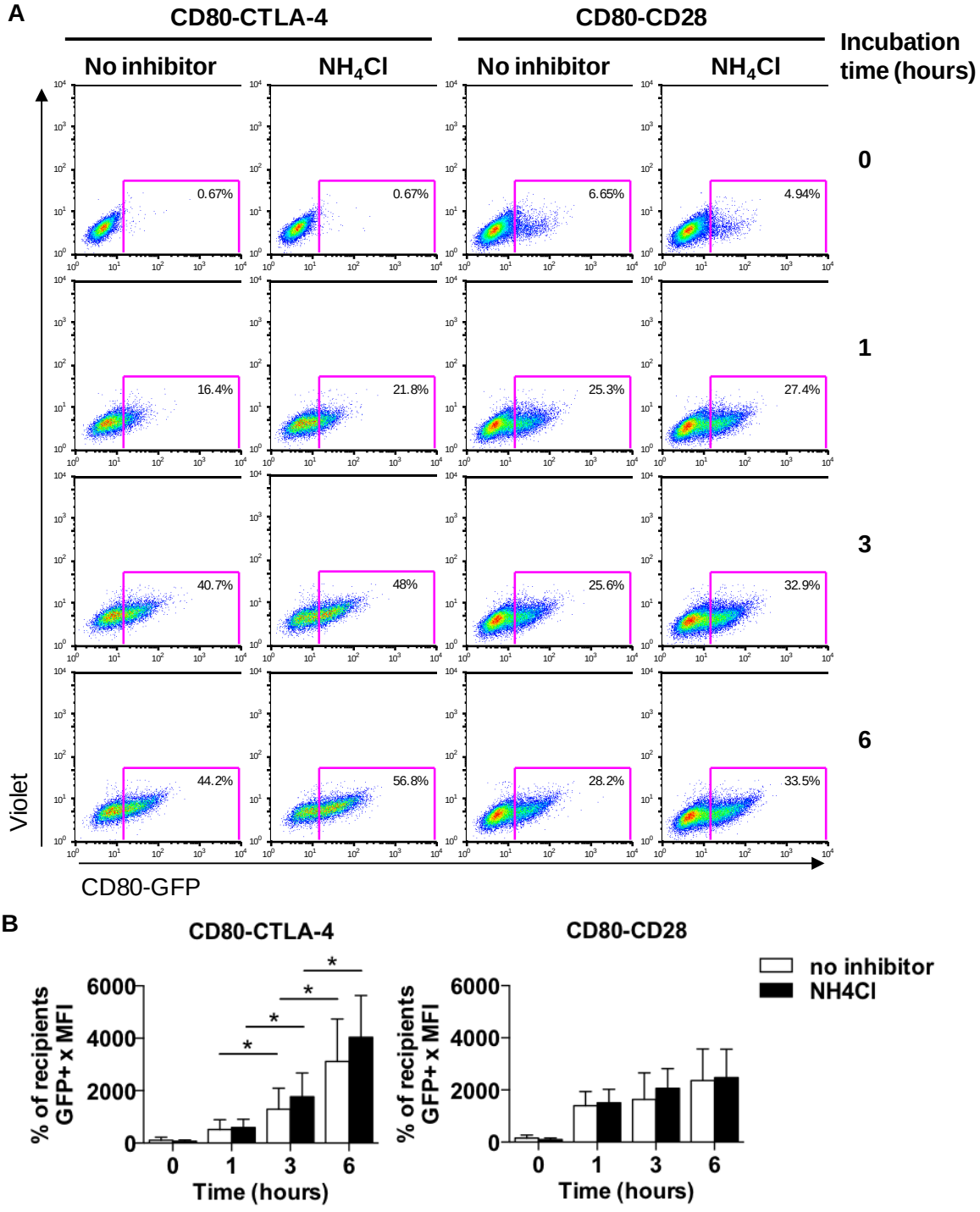


Figure 3.4 CTLA-4 captures ligand by trans-endocytosis which is time dependent and leads to ligand degradation. Violet-labelled CHO CD80-GFP cells were incubated with CTLA-4- or CD28-expressing cells at a 1:1 ratio +/- 10mM NH_4Cl at 37°C for 0-6 hours then analysed by flow cytometry. Data are representative of five independent experiments. **(A)** Plots show GFP fluorescence for recipient cells (violet negative) only. GFP^+ cells are gated and the percentage of GFP^+ recipients is shown. **(B)** Graphs show the mean (+SD) product of the percentage of recipients that acquired GFP and the GFP MFI of the GFP^+ recipients using data from five independent experiments. Statistically significant differences between data were determined using the paired t test *P<0.05.

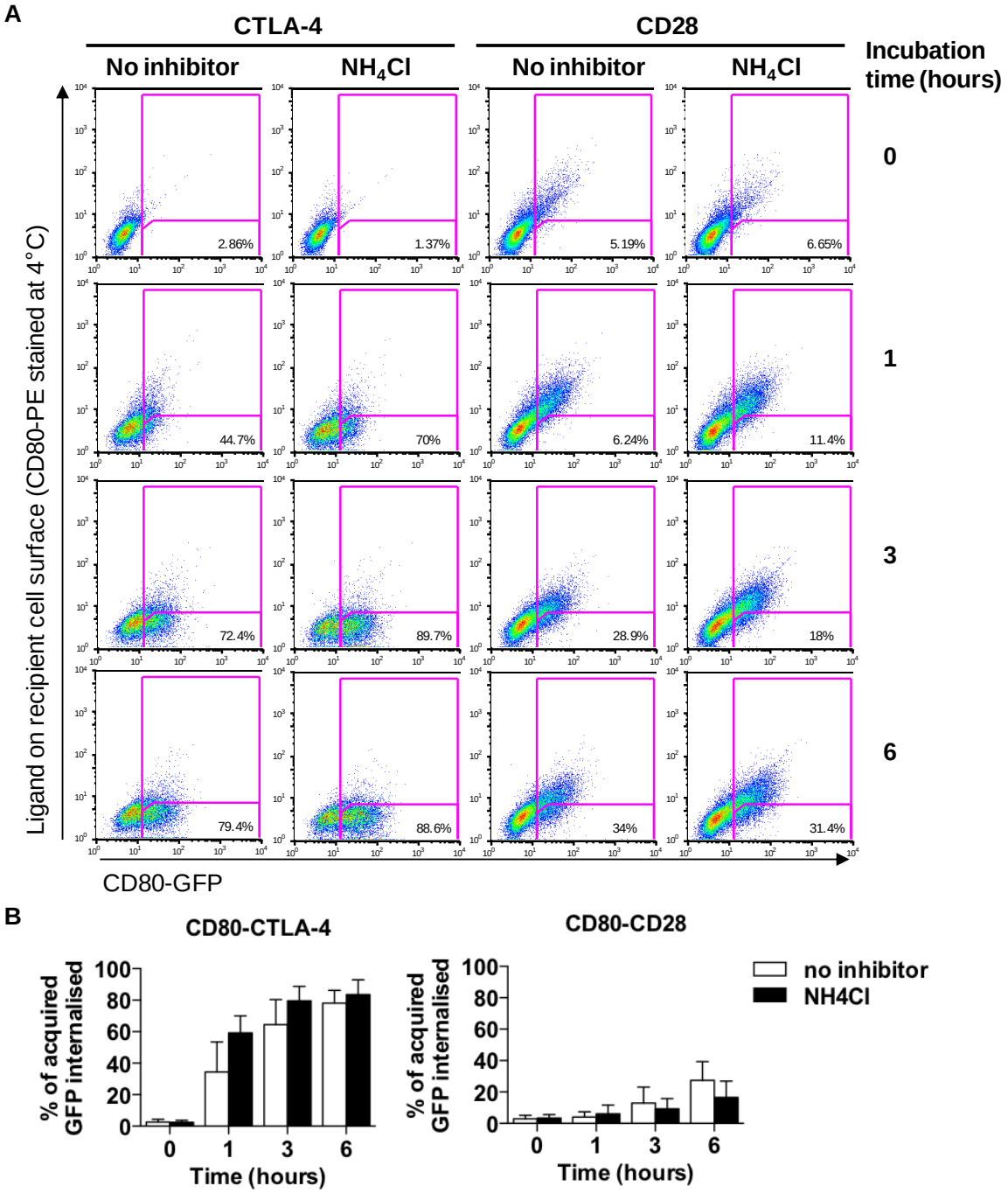


Figure 3.5 CTLA-4 internalises ligand via trans-endocytosis whereas CD28 only transfers ligand to the cell surface by trogocytosis. Violet-labelled CD80-GFP cells were incubated with CTLA-4- or CD28-expressing cells at a 1:1 ratio +/- 10mM NH_4Cl at 37°C for 0-6 hours then put on ice. Cells were disaggregated and labelled with CD80-PE antibody at 4°C for 30 minutes then analysed by flow cytometry. Data are representative of five independent experiments. **(A)** Plots show recipient cells only (gated on lack of violet fluorescence). Plots show CD80 labelled on the cell surface (PE fluorescence) against GFP fluorescence. GFP^+ cells are gated and divided into PE^+ and PE^- populations. The percentage of GFP^+ recipients with internalised ligand (not labelled on the cell surface) is shown. **(B)** Graphs show the mean (+SD) percentage of acquired GFP that was internalised using data from five independent experiments.

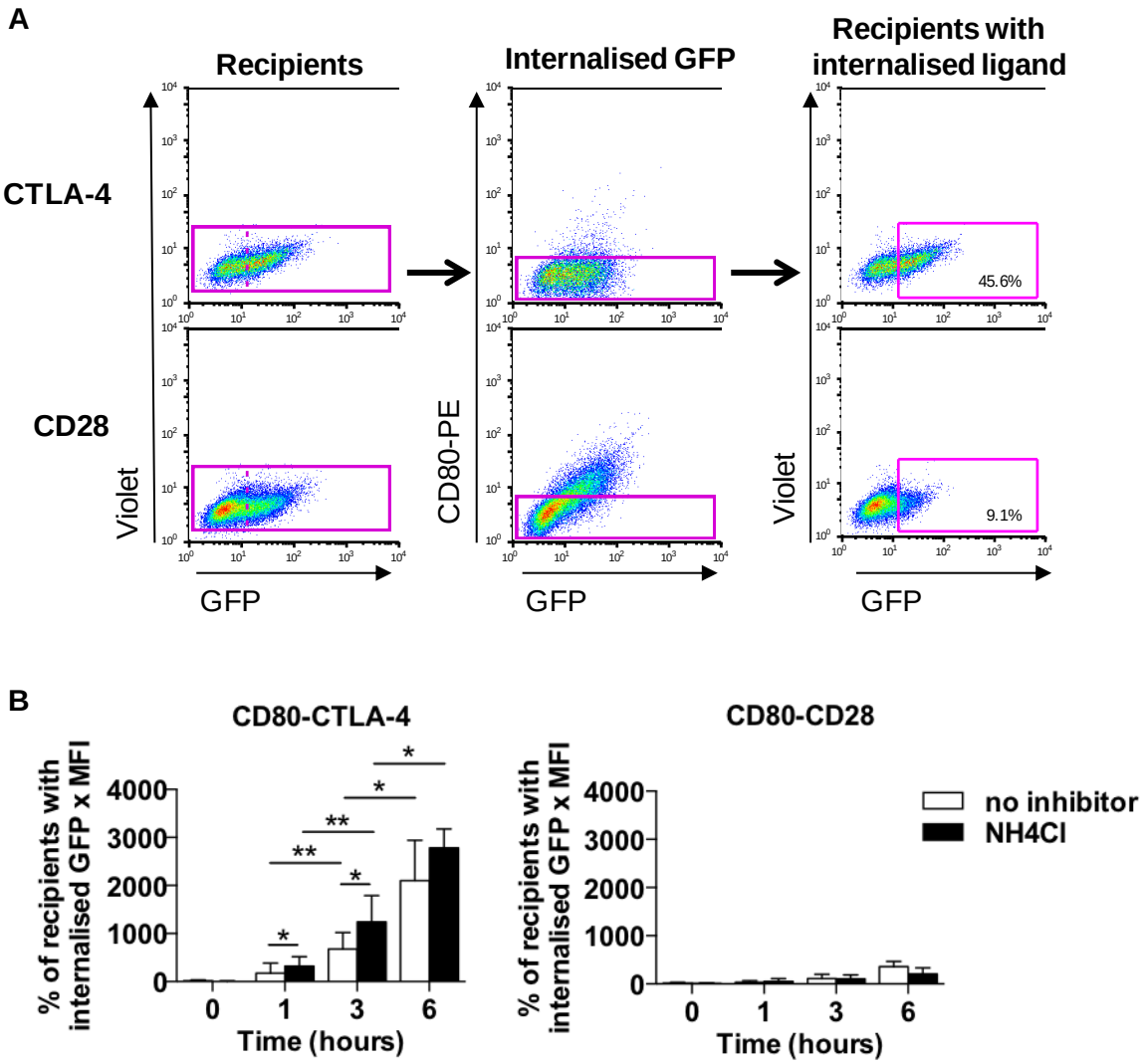


Figure 3.6 CTLA-4 removes ligand molecules by trans-endocytosis while CD28 does not. Violet-labelled CD80-GFP cells were incubated with CTLA-4- or CD28-expressing cells +/- 10mM NH_4Cl at 37°C for 0-6 hours then put on ice. Cells were disaggregated and labelled with CD80-PE antibody at 4°C for 30 minutes then analysed by flow cytometry. **(A)** Gating strategy for analysis of trans-endocytosis using 4h incubation with NH_4Cl as an example. Headings refer to the cells gated in pink. Each plot shows the gated cells from the previous plot. The percentage of recipients with internalised GFP is shown in the right hand panels (frequency of grandparent). The dashed line in the left hand panels divides GFP^- and GFP^+ cells for reference. **(B)** Graphs show the mean (+SD) product of the percentage of recipients that internalised GFP and the GFP MFI of the GFP^+ recipients using data from five independent experiments. Statistically significant differences between data were determined using the paired t test *P<0.05 **P<0.01.

trans-endocytosis. Recipients with ligand present on the cell surface were excluded (middle panels) so only ligand internalisation would be shown (right panels). The percentage shown in the GFP^+ gate is the frequency of the grandparent population, which represents the percentage of all recipients that internalised ligand by trans-endocytosis. **Figure 3.6B** shows the quantitation of trans-endocytosis. In contrast to the results shown in **figure 3.4**, which show global GFP acquisition, the capture of CD80 by CD28 was substantially lower than by CTLA-4 when only internalised ligand was taken into account. Taken together, the data presented here show that CTLA-4 has the ability to remove and internalise its ligands by trans-endocytosis but that CD28 does not do this. These experiments therefore established a robust method for measuring trans-endocytosis and distinguishing this process from trogocytosis.

3.3 CD80 is captured more efficiently by CTLA-4 trans-endocytosis than CD86

Once a protocol for studying trans-endocytosis by flow cytometry had been established the relative capture of the two ligands CD80 and CD86 by CTLA-4-mediated trans-endocytosis was compared. Donor cells were present in excess to allow the maximum proportion of recipients to capture ligand. It was found that more cells acquired CD80 than CD86 and more CD80 protein was internalised per cell than CD86 (**figures 3.7 and 3.9**) (although there were not enough replicates for this difference to reach statistical significance). This indicates that CD80 is removed more efficiently by trans-endocytosis than CD86. In the presence of excess donors the accumulation of ligand by recipients in the presence of NH_4Cl was very high. As expected, neither ligand was internalised to a great extent by CD28 (**figures 3.8 and 3.9**), indicating that CD28 does not perform trans-endocytosis.

In order to visualise trans-endocytosis and verify these results using a different approach, cells were observed by confocal microscopy (**figure 3.10**). The trans-endocytosis of CTLA-4 and CD86 was clearly visible. Numerous GFP-containing vesicles were present inside CTLA-

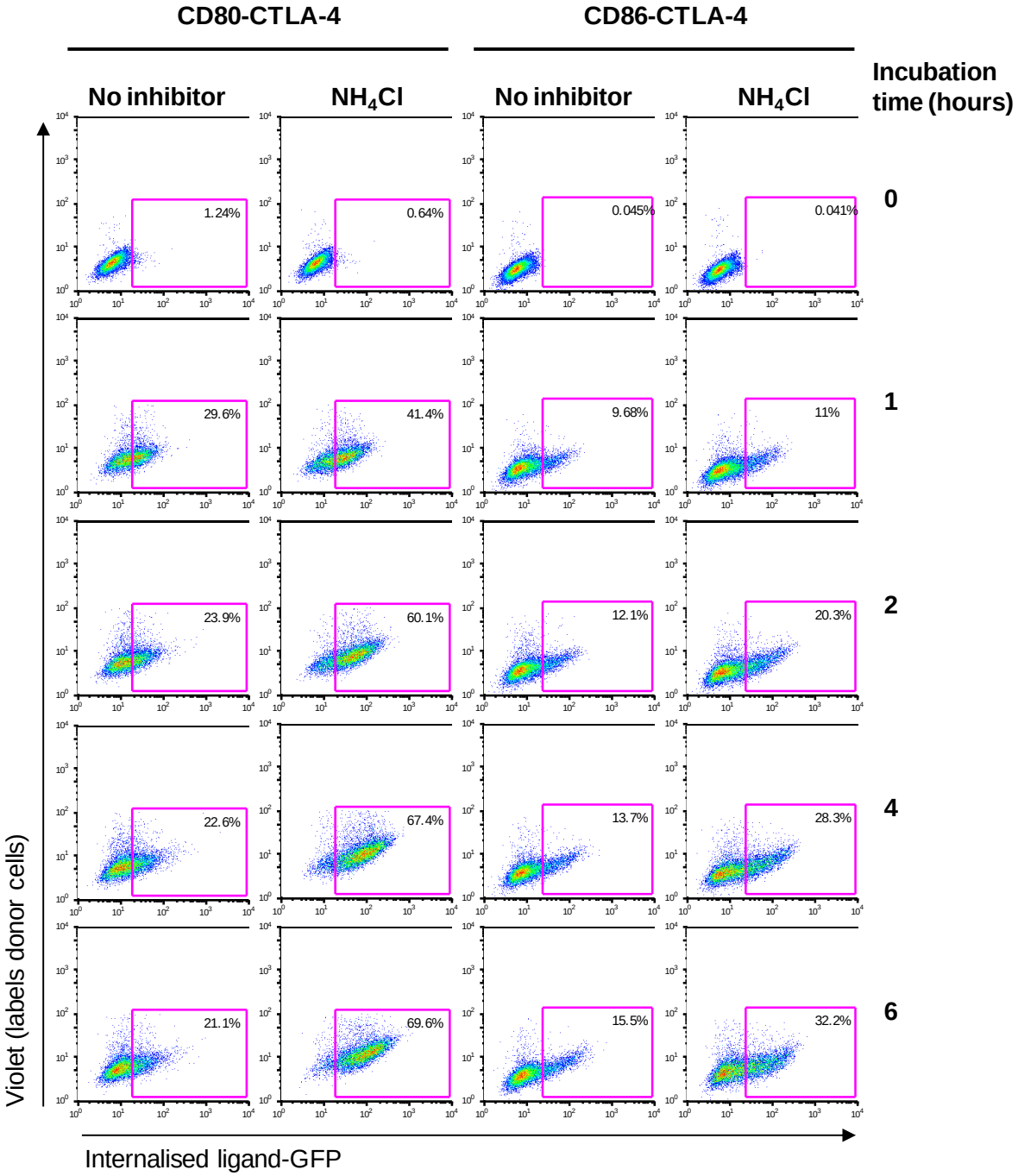


Figure 3.7 CD80 is captured more efficiently than CD86 via CTLA-4-mediated trans-endocytosis. Violet-labelled ligand-GFP cells were incubated with CTLA-4-expressing cells at a 4:1 ratio +/- 40mM NH_4Cl at 37°C for 0-6 hours then put on ice. Cells were disaggregated and labelled with PE-conjugated ligand-specific antibody at 4°C for 30 minutes then analysed by flow cytometry. Plots show recipients excluding those with ligand on their cell surface. GFP^+ cells are gated. The percentage of all recipients with internalised GFP is shown. Data are representative of three independent experiments.

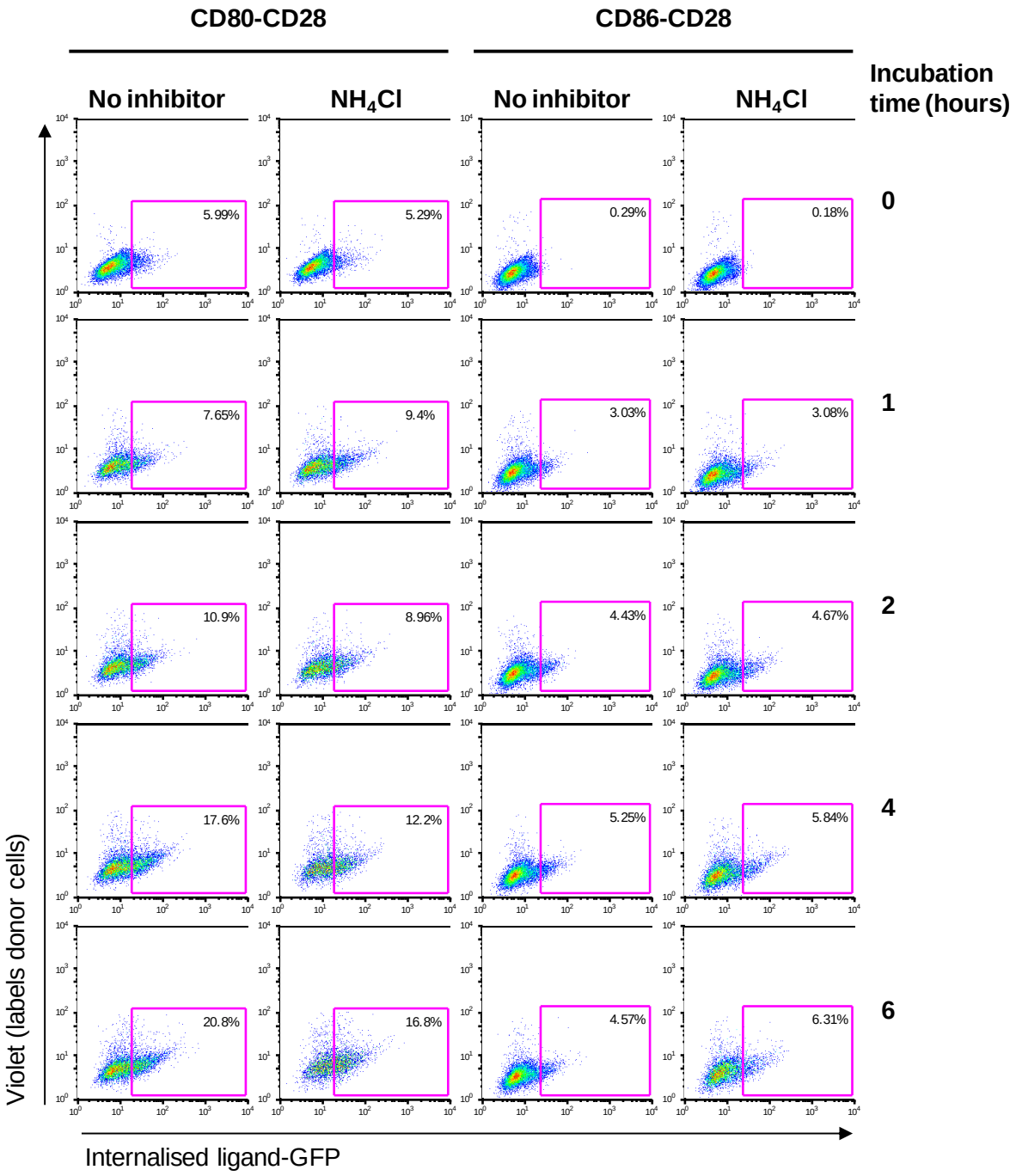


Figure 3.8 CD28 cannot capture either of its ligands by trans-endocytosis. Violet-labelled ligand-GFP cells were incubated with CD28-expressing cells at a 4:1 ratio +/- 40mM NH_4Cl at 37°C for 0-6 hours then put on ice. Cells were disaggregated and labelled with PE-conjugated ligand-specific antibody at 4°C for 30 minutes then analysed by flow cytometry. Plots show recipients excluding those with ligand on their cell surface. GFP^+ cells are gated. The percentage of all recipients with internalised GFP is shown. Data are representative of three independent experiments.

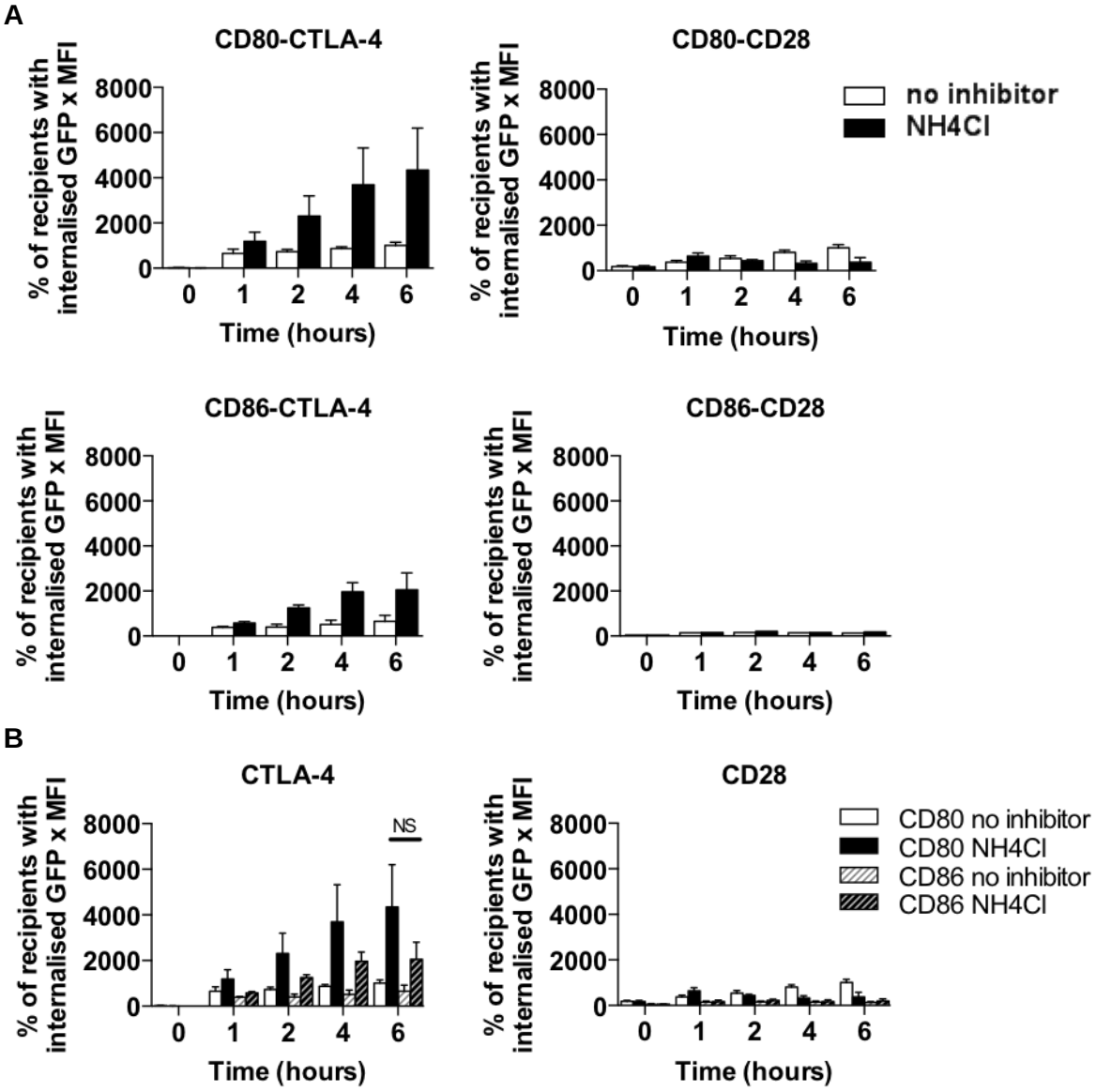


Figure 3.9 Comparing the trans-endocytosis of CD80 and CD86. Violet-labelled ligand-GFP cells were incubated with CTLA-4- or CD28-expressing cells at a 4:1 ratio +/- 40mM NH_4Cl at 37°C for 0-6 hours then put on ice. Cells were disaggregated and labelled with PE-conjugated ligand-specific antibody at 4°C for 30 minutes then analysed by flow cytometry. Graphs show the mean (+SEM) product of the percentage of recipients that internalised GFP and the GFP MFI of the GFP^+ recipients using data from three independent experiments. **(A)** Each receptor-ligand pair shown separately. **(B)** Graphs directly compare CD80 and CD86 internalisation for each receptor. There were not enough replicates for the data to reach statistical significance (NS) according to the t test.

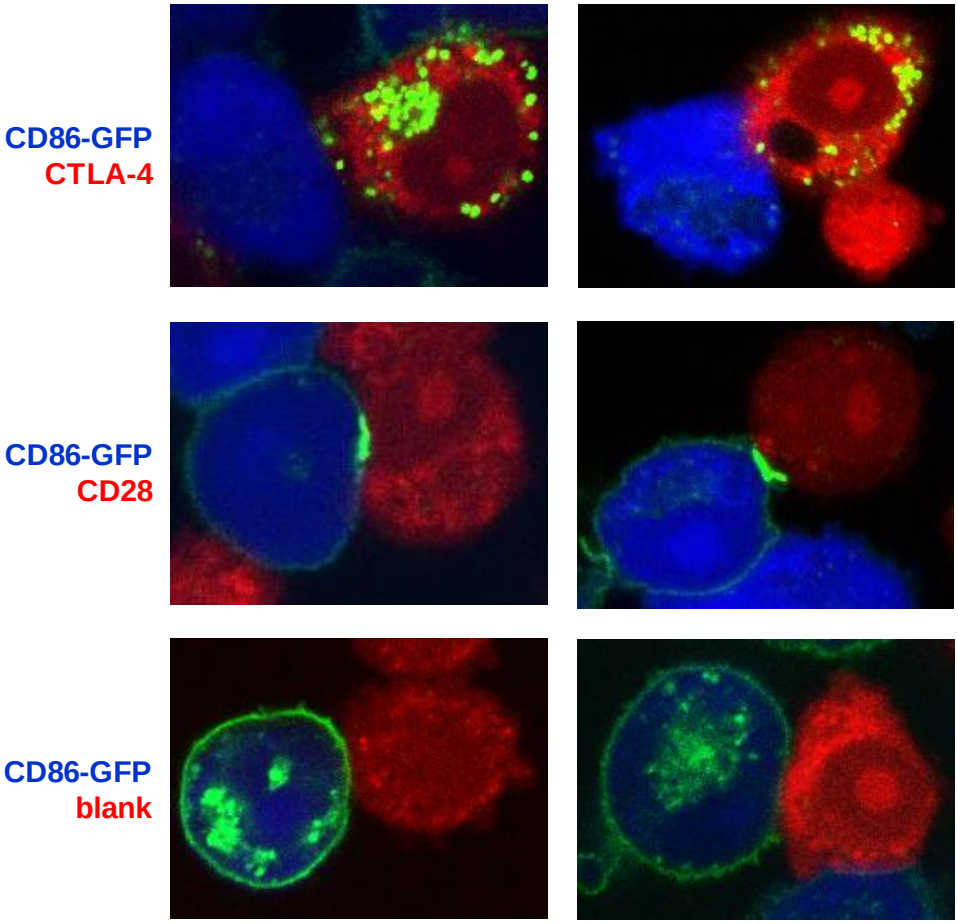


Figure 3.10 CTLA-4 ligands are targeted to intracellular vesicles during trans-endocytosis. Violet-labelled CD86-GFP cells were incubated with far red-labelled receptor-expressing cells or blank cells at a 1:1 ratio in the presence of 10mM NH_4Cl at 37°C for 3 hours then analysed by confocal microscopy. Images are representative of at least 150 cells observed over two independent experiments. Images of CD86-GFP cells with blank cells are representative of all ligand-GFP with blank cell controls which were included in all experiments but omitted from subsequent figures for simplicity.

4-expressing cells (red) and no CD86-GFP remained on the donor cells (violet). In contrast, no CD86-GFP was seen inside CD28-expressing cells and CD86-GFP was visible on the plasma membrane of donor cells. Receptor-ligand interactions were evident by the accumulation of CD86-GFP at cell-cell contacts. A comparison of the two CTLA-4 ligands in the absence of NH_4Cl showed a trend for more cells internalising CD80 than CD86 (**figure 3.11**) (although this difference was not statistically significant). In the presence of NH_4Cl the average number of cells that acquired CD80 and CD86 was similar (although the difference between the two ligands was statistically significant because the standard deviation was not high). Consistent with the general trend also shown by flow cytometry (**figures 3.7 and 3.9**) that trans-endocytosis of CD80 is more efficient than CD86, no CD80 remained on the plasma membrane of donor cells whereas a few donors still possessed CD86 (**figure 3.11A**). In contrast, most donors in the presence of CD28-expressing cells still expressed ligand, and ligand was rarely internalised by CD28-expressing cells, with the exception of CD80 in the presence of NH_4Cl. However the frequency of CD28-expressing cells that acquired CD80 was less than frequency of CTLA-4 recipients. Ligand transfer was receptor-specific because none was seen upon contact with un-transfected blank cells (**figure 3.10**).

3.4 A CTLA-4 variant that is expressed at the cell surface and not rapidly internalised can still capture ligand by trans-endocytosis

In order to investigate whether the constitutive endocytosis of CTLA-4 is necessary for trans-endocytosis, a CTLA-4 mutant which is expressed at the cell surface and undergoes minimal endocytosis was also studied. It is known that the YVKM motif in the cytoplasmic tail of CTLA-4 is the binding site for the clathrin adaptor complex AP-2 which is required for clathrin-mediated endocytosis (Zhang and Allison, 1997). The tyrosine residue in this motif was mutated to produce an AVKM CTLA-4 variant. Unlike wild type CTLA-4, this variant protein was expressed mainly at the cell surface and not internalised to a great extent (**figure 3.12**). Surprisingly however the AVKM CTLA-4 variant was able to capture CD80 and CD86

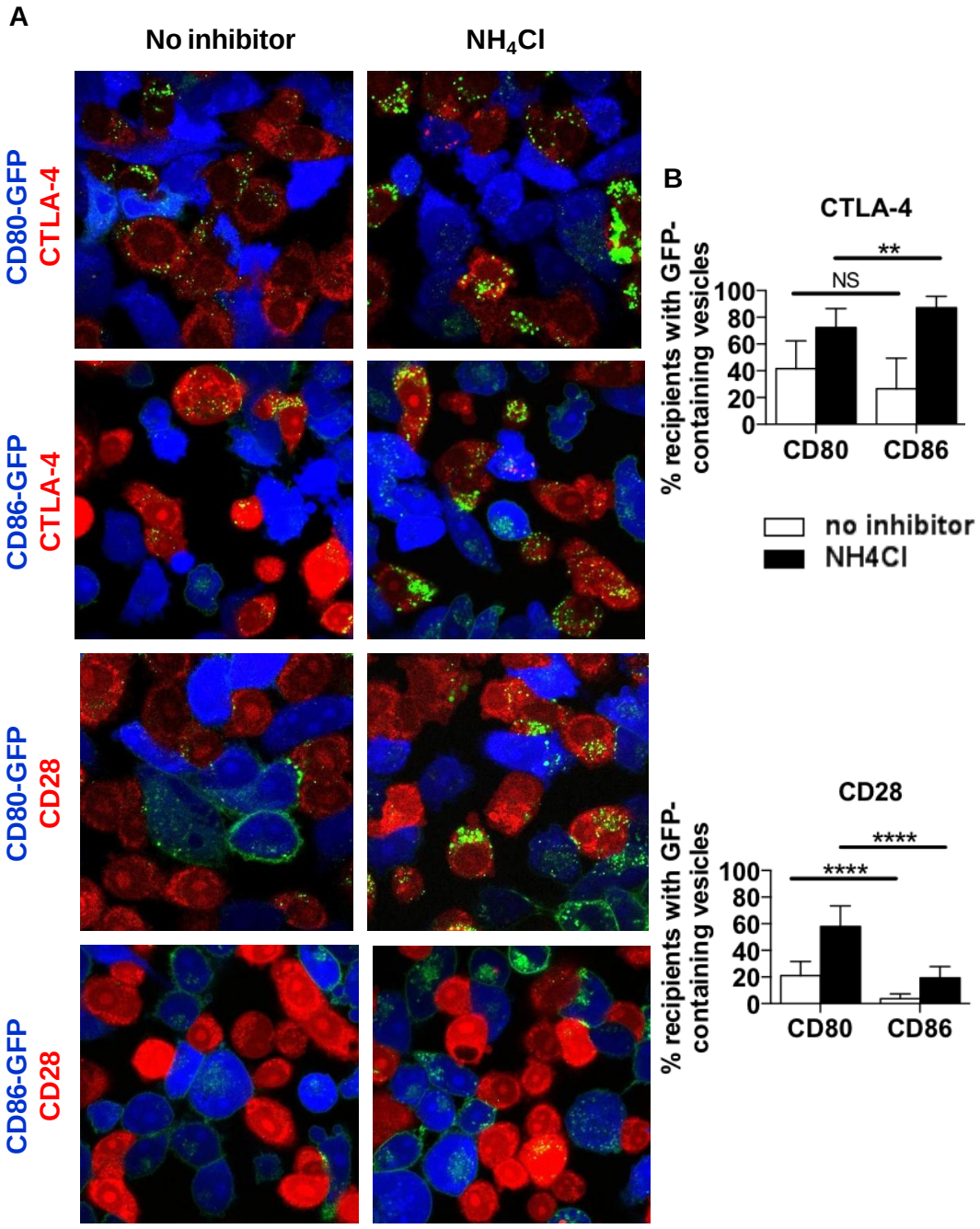


Figure 3.11 CD80 is more readily captured by trans-endocytosis than CD86. Violet-labelled ligand-GFP cells were incubated with far red-labelled receptor-expressing cells at a 1:1 ratio +/- 10mM NH_4Cl at 37°C for 6 hours then analysed by confocal microscopy. **(A)** Each image is representative of at least 16 micrographs collected over two independent experiments. **(B)** Graphs show the mean (+SD) percentage of recipients with ligand-GFP-containing vesicles within them. For each condition a total of 380-600 cells were analysed from two independent experiments. Statistically significant differences between data were determined using the t test **P<0.01 ****P<0.0001 NS=not significant.

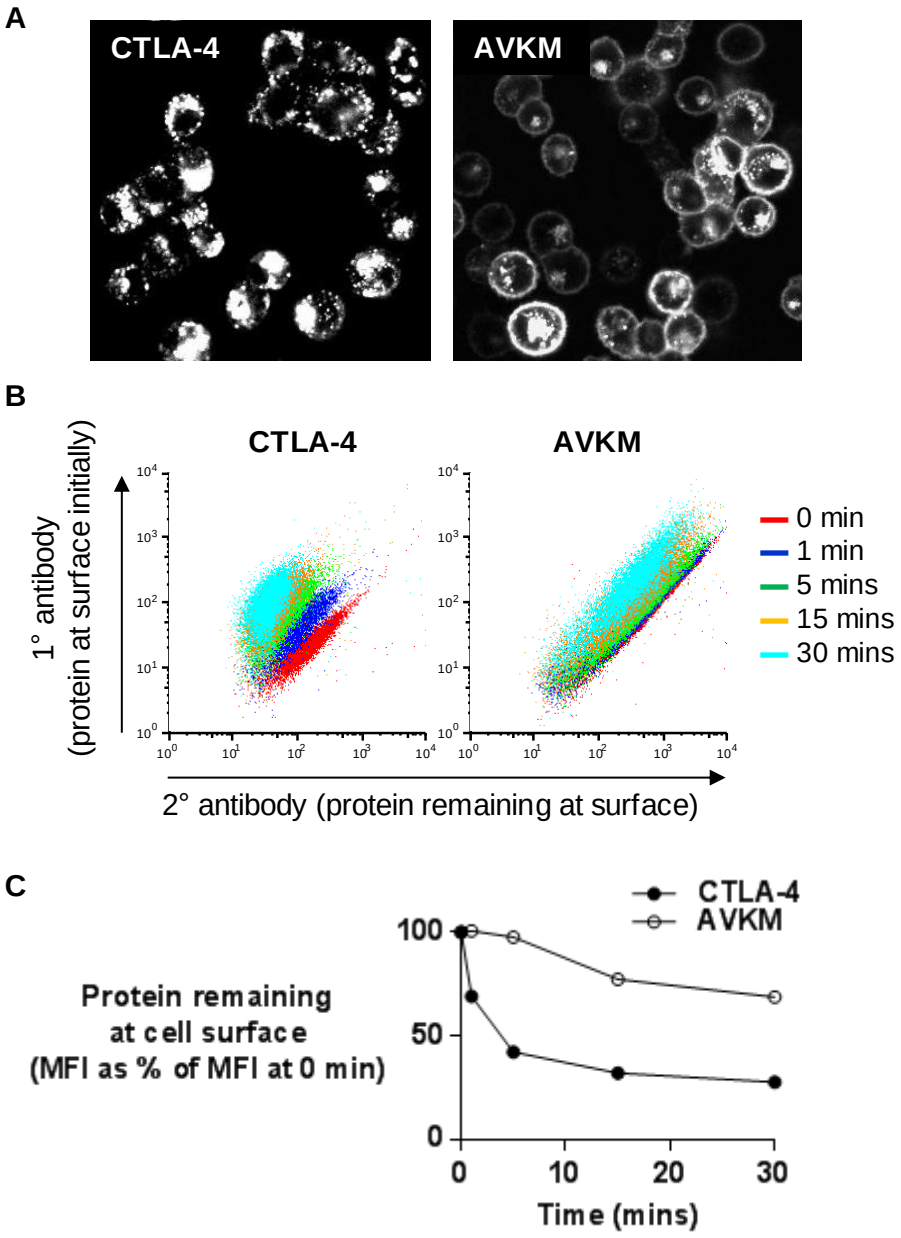


Figure 3.12 Mutation of the tyrosine in the YVKM motif in the cytoplasmic tail of CTLA-4 to AVKM results in a cell surface protein with limited endocytic activity. **(A)** Cells were incubated with fluorescently-labelled CTLA-4 antibody at 37°C then imaged by confocal microscopy. Images are representative of three micrographs. **(B-C)** Cells were incubated with fluorescently-labelled 1° antibody at 4°C to label protein at the cell surface. Cells were raised to 37°C for 0-30 minutes to allow protein internalisation. Cells were then incubated with fluorescently-labelled 2° antibody at 4°C to co-stain protein labelled with 1° antibody that remained at the cell surface. Cells were analysed by flow cytometry. Data are representative of two independent experiments. (*B*) Plots show 1° antibody fluorescence against 2° antibody fluorescence. All time points for each protein are overlaid. A decrease in 2° antibody fluorescence (left shift) as time increases indicates a reduction in protein expression at the cell surface over time. (*C*) Graph shows the amount of protein remaining at the cell surface over time as measured by 2° antibody MFI shown as a percentage of the MFI at 0 minutes.

by trans-endocytosis (**figures 3.13 and 3.14A**). Consistent with trans-endocytosis, the amount of ligand detected in recipients increased in the presence of NH_4Cl and the amount of ligand internalised increased over time. On average CD80 was captured more efficiently than CD86, as with wild type CTLA-4, although the difference between the two ligands was not as great (and did not reach statistical significance).

A comparison of the rate of trans-endocytosis by wild type and AVKM variant CTLA-4 revealed that the internalisation of CD86 was similar for both forms of CTLA-4 (**figure 3.14B**). Although the initial rate of internalisation of CD80 was similar for both CTLA-4 variants, the maximum amount of ligand acquisition was higher by wild type CTLA-4 (although this difference did not reach statistical significance). Both the frequency of cells that internalised ligand and most noticeably the amount of ligand acquired was considerably greater with wild type CTLA-4 compared to the AVKM variant (refer to **figure 3.7** and **figure 3.13**). This suggests that the YVKM motif increases the efficiency of trans-endocytosis, but that clathrin-mediated endocytosis is not essential, which indicates that alternative pathways can also direct trans-endocytosis.

3.5 CTLA-4 does not capture LICOS by trans-endocytosis

A recent report showed that LICOS (ICOS ligand) also interacts with CD28 and CTLA-4 (Yao et al., 2011), therefore the trans-endocytosis of LICOS was tested by flow cytometry. It was found that no LICOS protein was transferred by either receptor (**figure 3.15**). Furthermore, imaging by confocal microscopy indicated that CTLA-4 and CD28 did not interact effectively with LICOS because the ligand did not accumulate at cell-cell contacts (**figure 3.16**). In fact others have shown that LICOS only binds CD28 and CTLA-4 at 25°C and not at the physiological temperature of 37°C (Brodie et al., 2000). Ultimately these results suggest that LICOS is not an obvious natural ligand of CTLA-4 and demonstrate that trans-endocytosis only occurs upon very specific receptor-ligand binding.

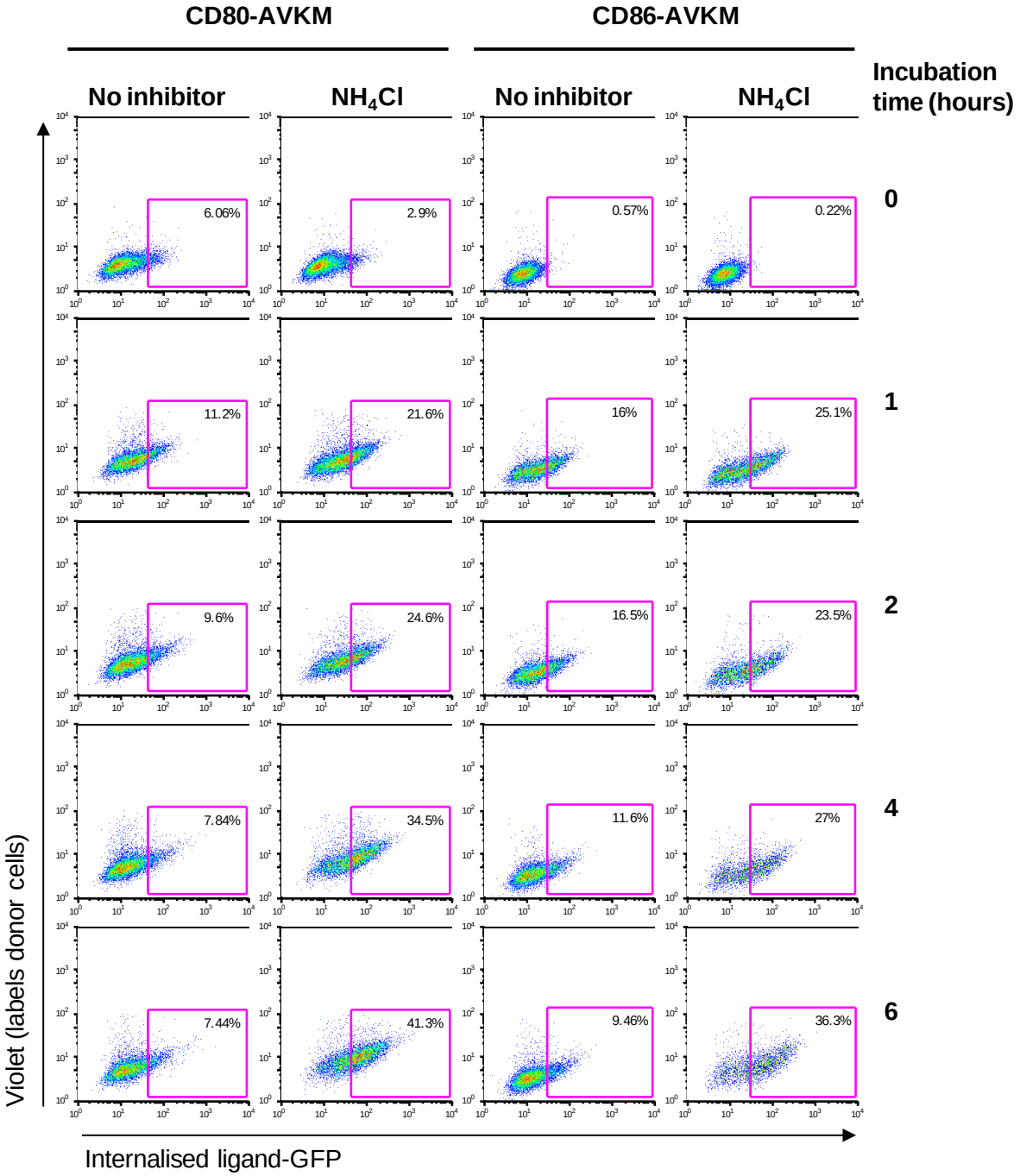


Figure 3.13 Clathrin-mediated endocytosis is not essential for trans-endocytosis. Violet-labelled ligand-GFP cells were incubated with AVKM-expressing cells at a 4:1 ratio +/- 40mM NH_4Cl at 37°C for 0-6 hours then put on ice. Cells were disaggregated and labelled with PE-conjugated ligand-specific antibody at 4°C for 30 minutes then analysed by flow cytometry. Plots show recipients excluding those with ligand on their cell surface. GFP^+ cells are gated. The percentage of all recipients with internalised GFP is shown. Data are representative of three independent experiments.

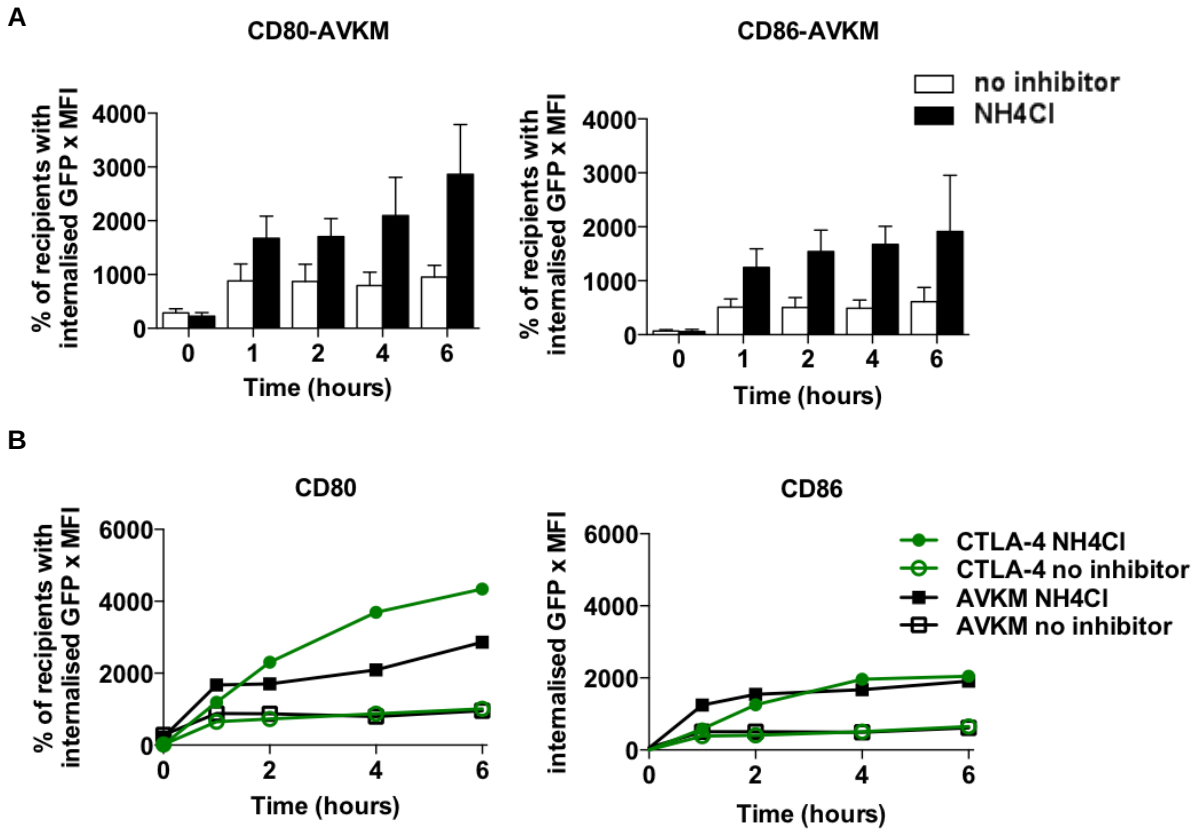


Figure 3.14 The CTLA-4 YVKM motif improves the efficiency of trans-endocytosis. Violet-labelled ligand-GFP cells were incubated with wild type or AVKM CTLA-4-expressing cells at a 4:1 ratio +/- 40mM NH_4Cl at 37°C for 0-6 hours then put on ice. Cells were disaggregated and labelled with PE-conjugated ligand-specific antibody at 4°C for 30 minutes then analysed by flow cytometry. **(A)** Graphs show the mean (+SEM) product of the percentage of AVKM recipients that internalised GFP and the GFP MFI of the GFP^+ recipients using data from three independent experiments. The differences between CD80 and CD86 did not reach statistical significance. **(B)** Graphs show the mean product of the percentage of recipients that internalised GFP and the GFP MFI of the GFP^+ recipients using data from three independent experiments comparing wild type and AVKM CTLA-4. The differences between CD80 and CD86 did not reach statistical significance.

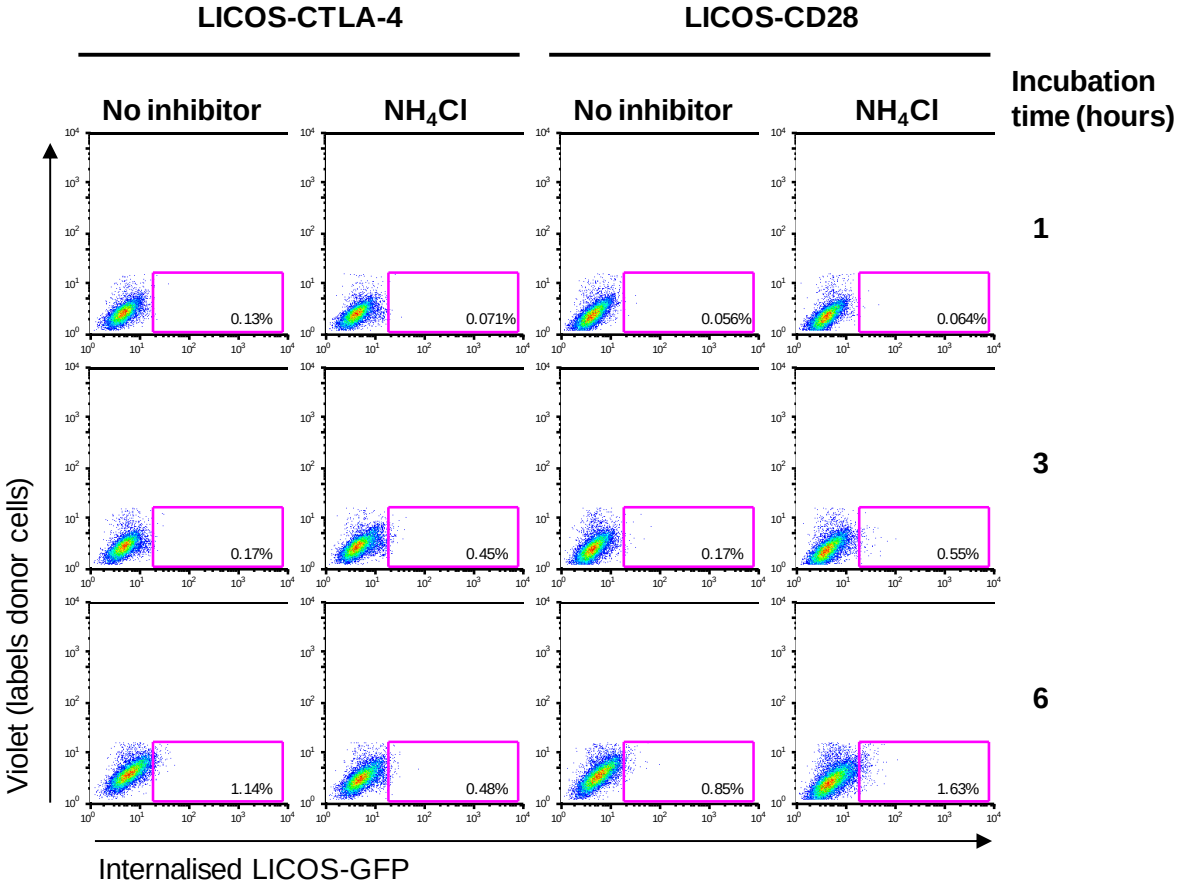


Figure 3.15 CTLA-4 does not internalise LICOS by trans-endocytosis. Violet-labelled LICOS-GFP cells were incubated with CD28- or CTLA-4-expressing cells at a 1:1 ratio +/- 10mM NH_4Cl at 37°C for 1, 3 or 6 hours then put on ice. Cells were disaggregated and labelled with PE-conjugated ligand-specific antibody at 4°C for 30 minutes then analysed by flow cytometry. Plots show recipients excluding those with ligand on their cell surface. GFP^+ cells are gated. The percentage of all recipients with internalised GFP is shown. Data are representative of three independent experiments.

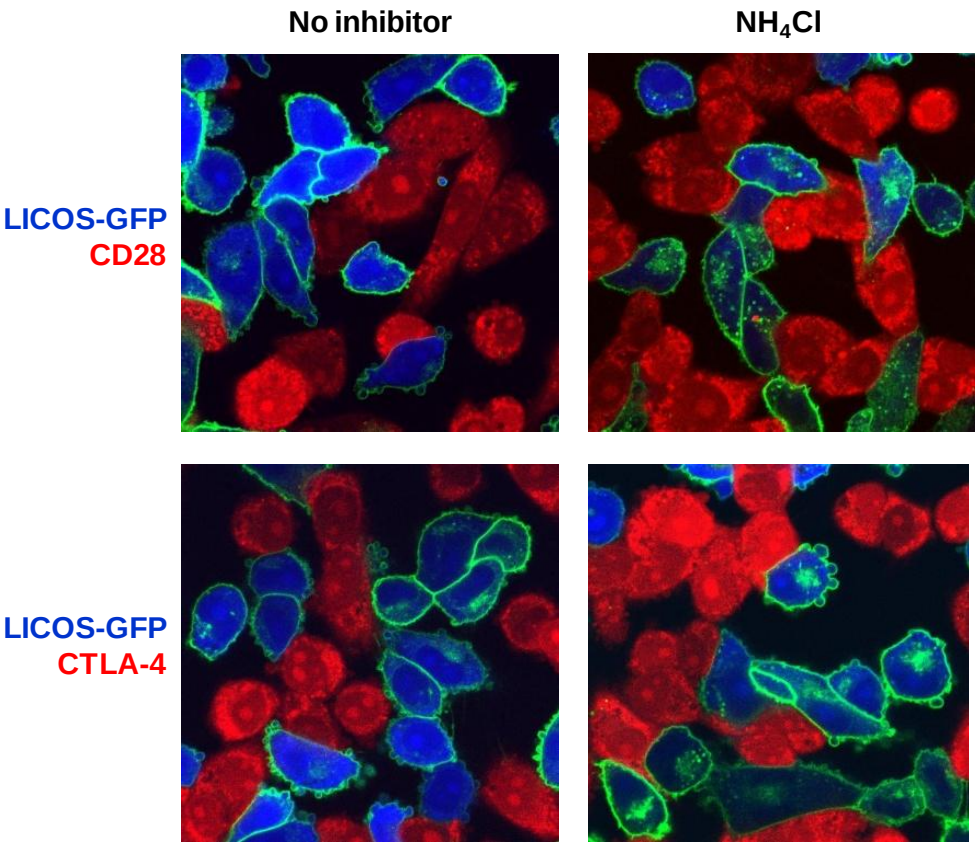


Figure 3.16 LICOS does not interact with CD28 or CTLA-4. Violet-labelled LICOS-GFP cells were incubated with far red-labelled receptor-expressing cells at a 1:1 ratio +/- 10mM NH_4Cl at 37°C for 6 hours then analysed by confocal microscopy. Each image is representative of at least 16 micrographs collected over two independent experiments.

3.6 Ammonium chloride, bafilomycin A and chloroquine are all effective inhibitors of lysosomal degradation useful for studying trans-endocytosis

Other inhibitors of lysosomal degradation were tested that may be useful for future studies with primary T cells *in vitro* and *in vivo*. NH_4Cl was used routinely due to its low cost, however bafilomycin A is more appropriate for use with primary T cells, and chloroquine could be used *in vivo* as it has been used therapeutically. Comparative studies showed that the percentage of CTLA-4 recipients that internalised CD80 and CD86 was similar in the presence of NH_4Cl, chloroquine and bafilomycin A (**figure 3.17**). Although the initial rate of trans-endocytosis and the maximum percentage of CTLA-4-expressing cells that internalised ligand was greater in the presence of bafilomycin A compared to other inhibitors, this may simply have been due to a more effective dose. In the future a range of inhibitor concentrations should be tested. Nonetheless, all three inhibitors prevented protein degradation to reveal comparable levels of trans-endocytosis, so they would all be suitable and useful for future studies.

3.7 CD2 is not capable of trans-endocytosis

Following the study of trans-endocytosis by CTLA-4, related receptors were then investigated. CD2 is another member of the immunoglobulin superfamily but unlike CD28, CTLA-4, ICOS and PD-1 it contains two immunoglobulin domains rather than only one. CD2 functions as both a costimulatory receptor and an adhesion molecule upon interaction with its ligand LFA-3. CD2-expressing cells were analysed by flow cytometry and confocal microscopy and despite being expressed at higher levels than CD28 or CTLA-4, the CD2 receptor did not capture its ligand LFA-3 by trans-endocytosis (**figure 3.18**). This was not due to a lack of receptor-ligand interactions because accumulation of LFA-3-GFP could be seen at points of contact with CD2-expressing cells. Finding instances where trans-endocytosis does not occur improves our understanding of the process because that means this mechanism is restricted to particular proteins and their specific ligands, and is not a

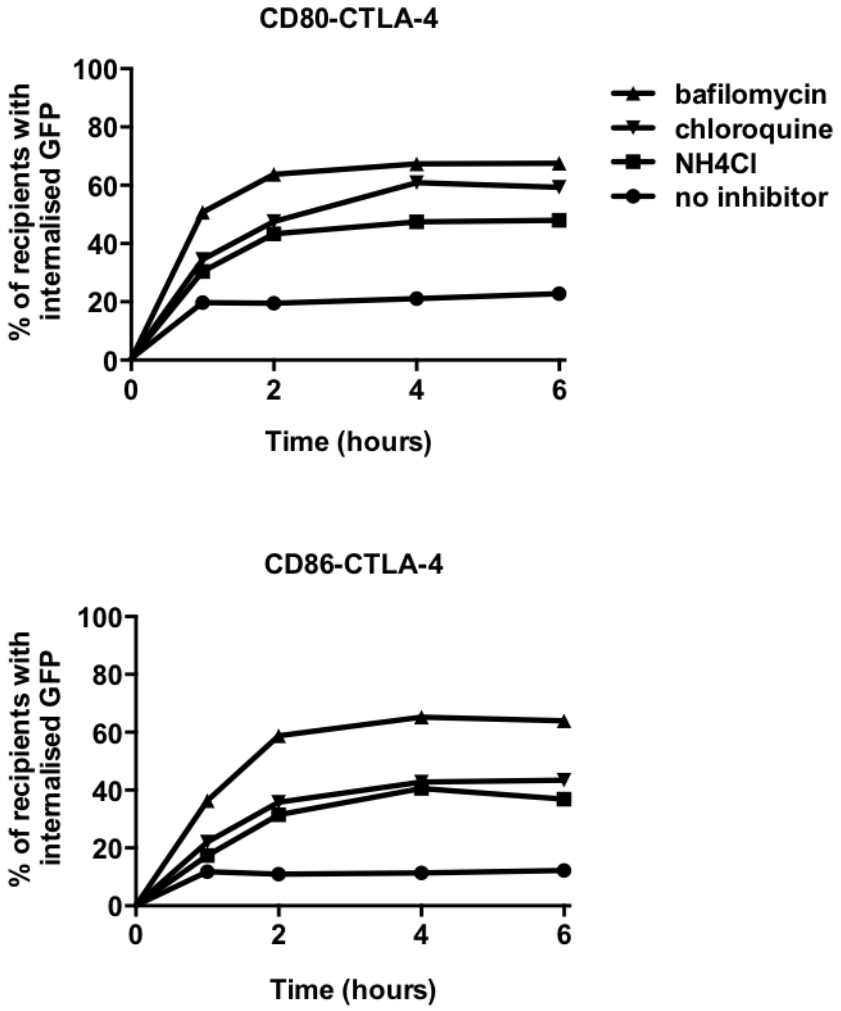


Figure 3.17 Using different inhibitors of lysosomal degradation to study trans-endocytosis. Violet-labelled ligand-GFP cells were incubated with CTLA-4-expressing cells at a 4:1 ratio at 37°C for 0-6 hours either untreated or in the presence of 40mM NH_4Cl or 25nM bafilomycin A or 100µM chloroquine. Cells were then put on ice, disaggregated and labelled with ligand-specific antibody at 4°C for 30 minutes before analysis by flow cytometry. Graphs show the mean percentage of total recipients that internalised ligand-GFP using data from three independent experiments.

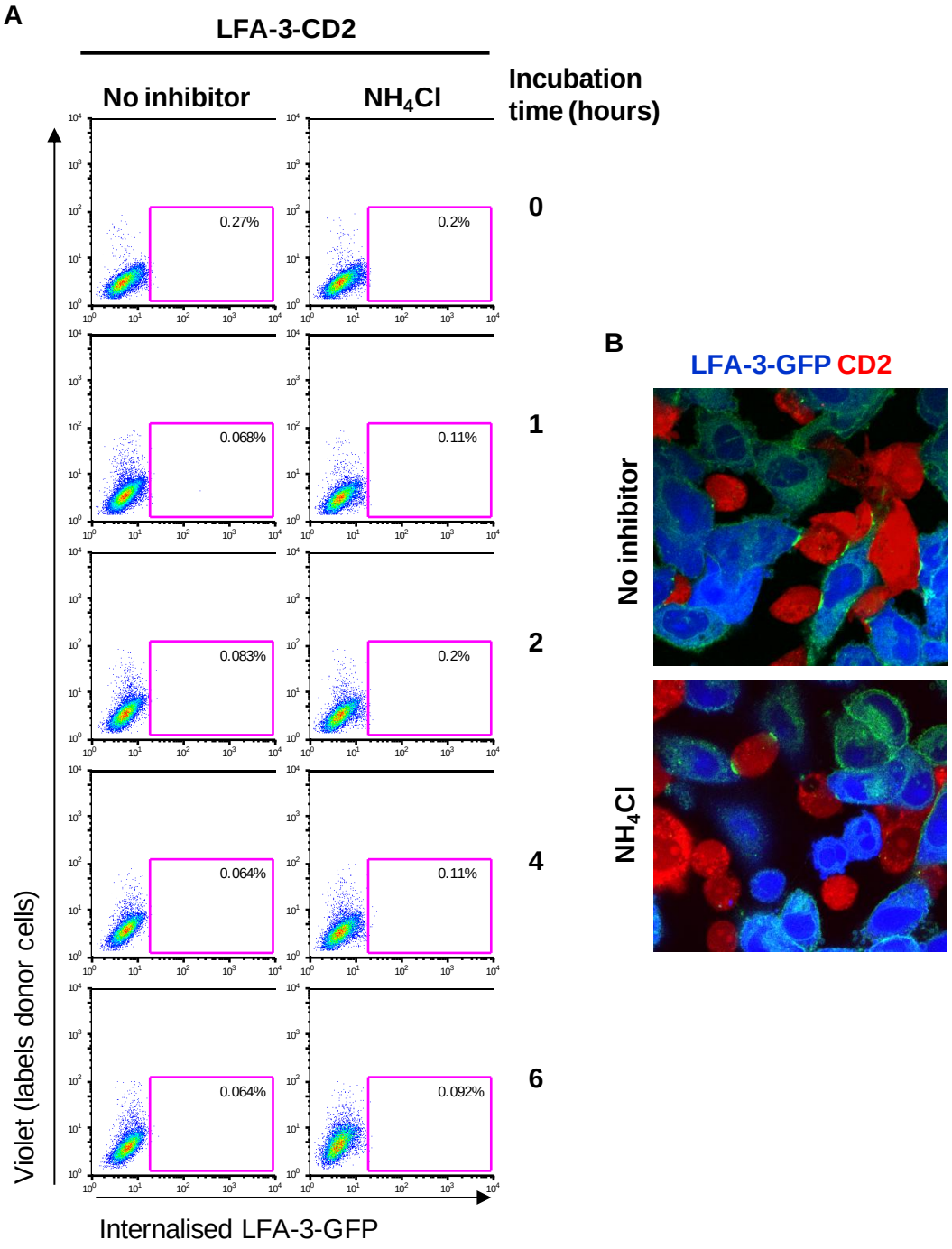


Figure 3.18 CD2 does not remove ligand by trans-endocytosis. **(A)** Violet-labelled LFA-3-GFP cells were incubated with CD2-expressing cells at a 4:1 ratio +/- 40mM NH_4Cl at 37°C for 0-6 hours then put on ice. Cells were disaggregated and labelled with LFA-3-PE antibody at 4°C for 30 minutes then analysed by flow cytometry. Plots show recipients excluding those with ligand on their cell surface. GFP^+ cells are gated. The percentage of all recipients with internalised GFP is shown. Data are representative of three independent experiments. **(B)** Violet-labelled LFA-3-GFP cells were incubated with far red-labelled CD2-expressing cells at a 1:1 ratio +/- 10mM NH_4Cl at 37°C for 6 hours then analysed by confocal microscopy. Each image is representative of at least 16 micrographs collected over two independent experiments.

seemingly generic phenomenon like trogocytosis. These results indicate that trans-endocytosis is not a common function of multiple receptors on T cells and that there are specific requirements for trans-endocytosis.

3.8 PD-1 internalises both its ligands

Next PD-1 was studied, which is another inhibitory receptor like CTLA-4. Interestingly it was found that PD-1 internalised both its ligands (**figure 3.19**), although the number of recipients that internalised GFP and the amount they acquired did not increase over time, suggesting that ligand acquisition by PD-1 utilises a different mechanism to CTLA-4. Yet NH_4Cl did increase the amount of internalised ligand detected, and in the absence of any lysosomal inhibitors the amount of internalised ligand decreased over time, indicating that PD-1 ligands were targeted to degradation pathways. Imaging by confocal microscopy confirmed that PD-L1 and PD-L2 were both located in intracellular vesicles in PD-1-expressing cells (**figure 3.20**). Whilst initially surprising considering that PD-1 is a plasma membrane protein with known signalling functions, these data show that PD-1 can also internalise its ligands. Although whether this occurs by the same mechanism as CTLA-4 trans-endocytosis or a different mechanism is unclear at present.

3.9 An unrelated costimulatory molecule OX40 also internalises its ligand

Since starting this project a colleague observed that the mouse ortholog of OX40 could also remove its ligand from opposing cells therefore I also assessed OX40 in my assays. Flow cytometric analysis of mOX40L acquisition by mOX40-expressing cells revealed that the majority of cells internalise ligand (**figure 3.21**). Like PD-1, more internalised ligand was detected in the presence of NH_4Cl and the amount of internalised ligand detected decreased over time in the absence of NH_4Cl, but the amount of ligand acquired did not increase over time. Imaging by confocal microscopy showed that a very high proportion of mOX40-expressing cells captured and internalised ligand (**figure 3.22**). These results suggest that

ligand internalisation may not be restricted to CTLA-4 and that in fact both coinhibitory and costimulatory receptors belonging to different protein families may have this ability. Overall these studies reveal that the removal of ligand from opposing cells by PD-1 and OX40 is a major feature of these receptors, although the functional significance and the mechanisms involved are uncertain and require further investigation.

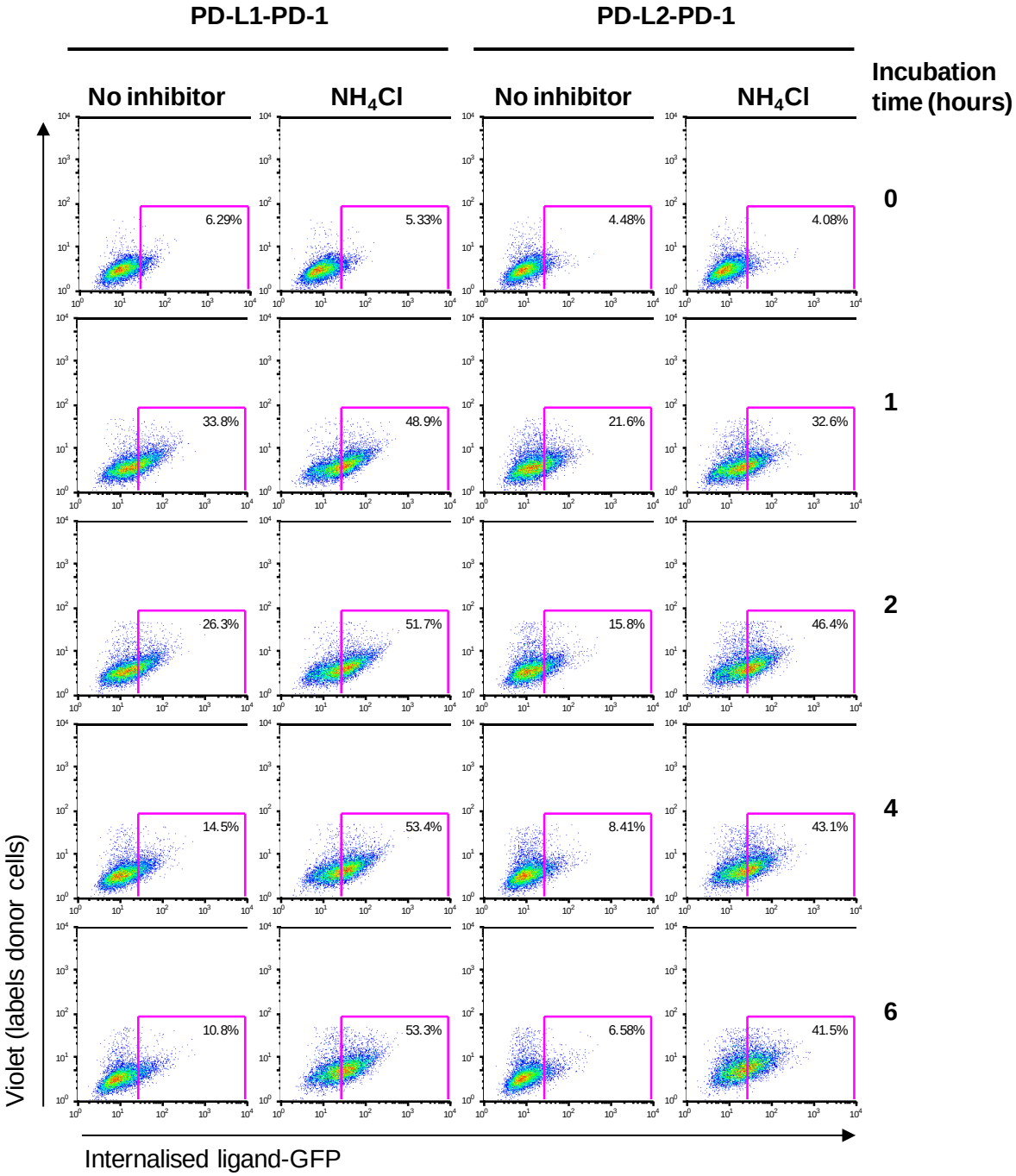


Figure 3.19 PD-1 internalises PD-L1 and PD-L2. Violet-labelled ligand-GFP cells were incubated with PD-1-expressing cells at a 4:1 ratio +/- 40mM NH_4Cl at 37°C for 0-6 hours then put on ice. Cells were disaggregated and labelled with fluorescently-labelled ligand-specific antibody at 4°C for 30 minutes then analysed by flow cytometry. Plots show recipients excluding those with ligand on their cell surface. The percentage of all recipients with internalised GFP is shown. Data are representative of three independent experiments.

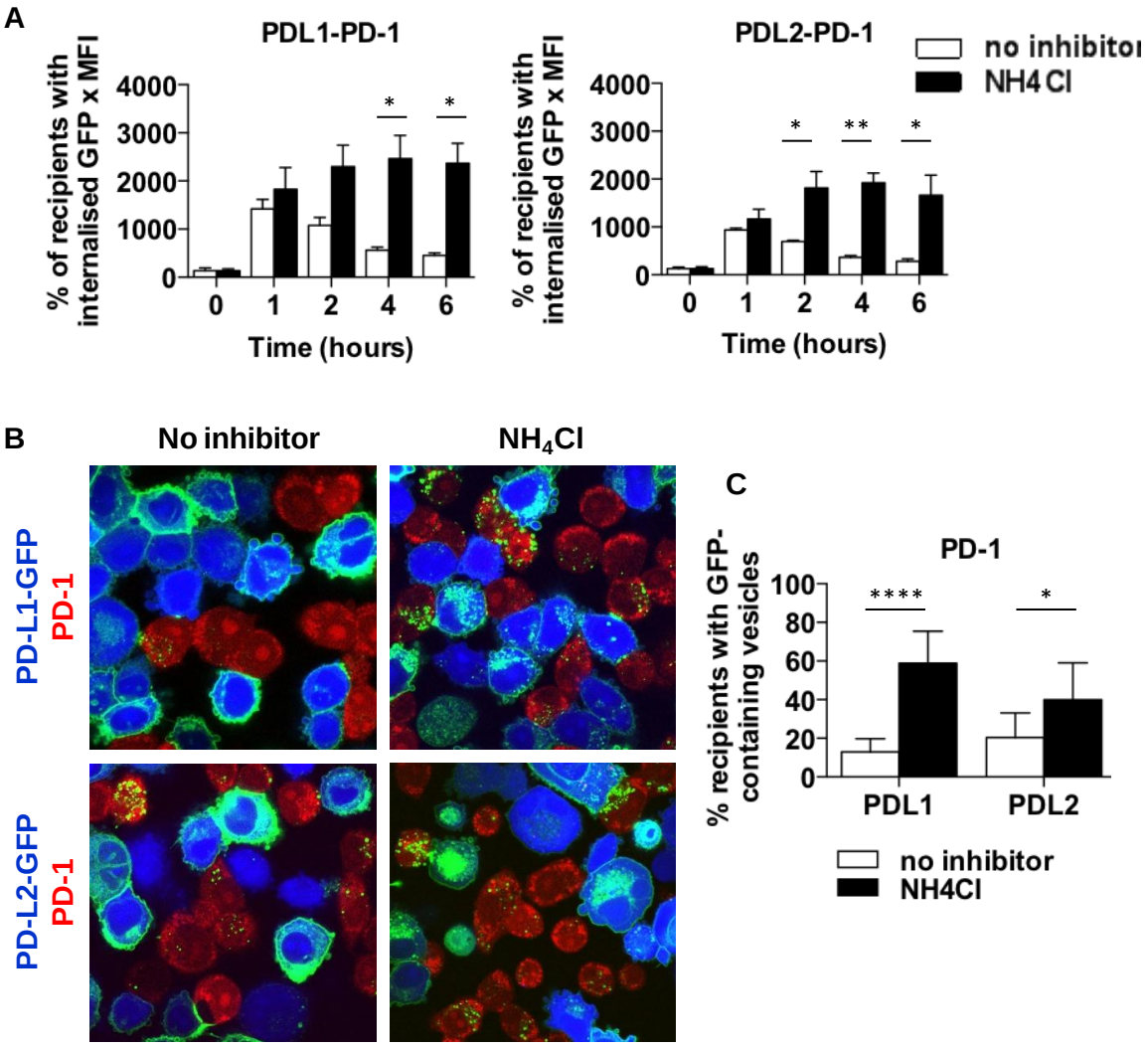


Figure 3.20 Internalised PD-1 ligands are present in intracellular vesicles and targeted for lysosomal degradation. **(A)** Violet-labelled ligand-GFP cells were incubated with PD-1-expressing cells at a 4:1 ratio +/- 40mM NH_4Cl at 37°C for 0-6 hours then put on ice. Cells were disaggregated and labelled with fluorescently-labelled ligand-specific antibody at 4°C for 30 minutes then analysed by flow cytometry. Graphs show the mean (+SEM) product of the percentage of recipients that internalised GFP and the GFP MFI of the GFP^+ recipients using data from three independent experiments. **(B-C)** Violet-labelled ligand-GFP cells were incubated with far red-labelled PD-1-expressing cells at a 1:1 ratio +/- 10mM NH_4Cl at 37°C for 6 hours then analysed by confocal microscopy. *(B)* Each image is representative of at least 24 micrographs collected over three independent experiments. *(C)* Graph shows the mean (+SEM) percentage of recipients with ligand-GFP-containing vesicles within them using data from three independent experiments. A total of 550-730 cells were analysed for each condition. Statistically significant differences between data were determined using the paired t test *P<0.05 **P<0.01 ****P<0.0001.

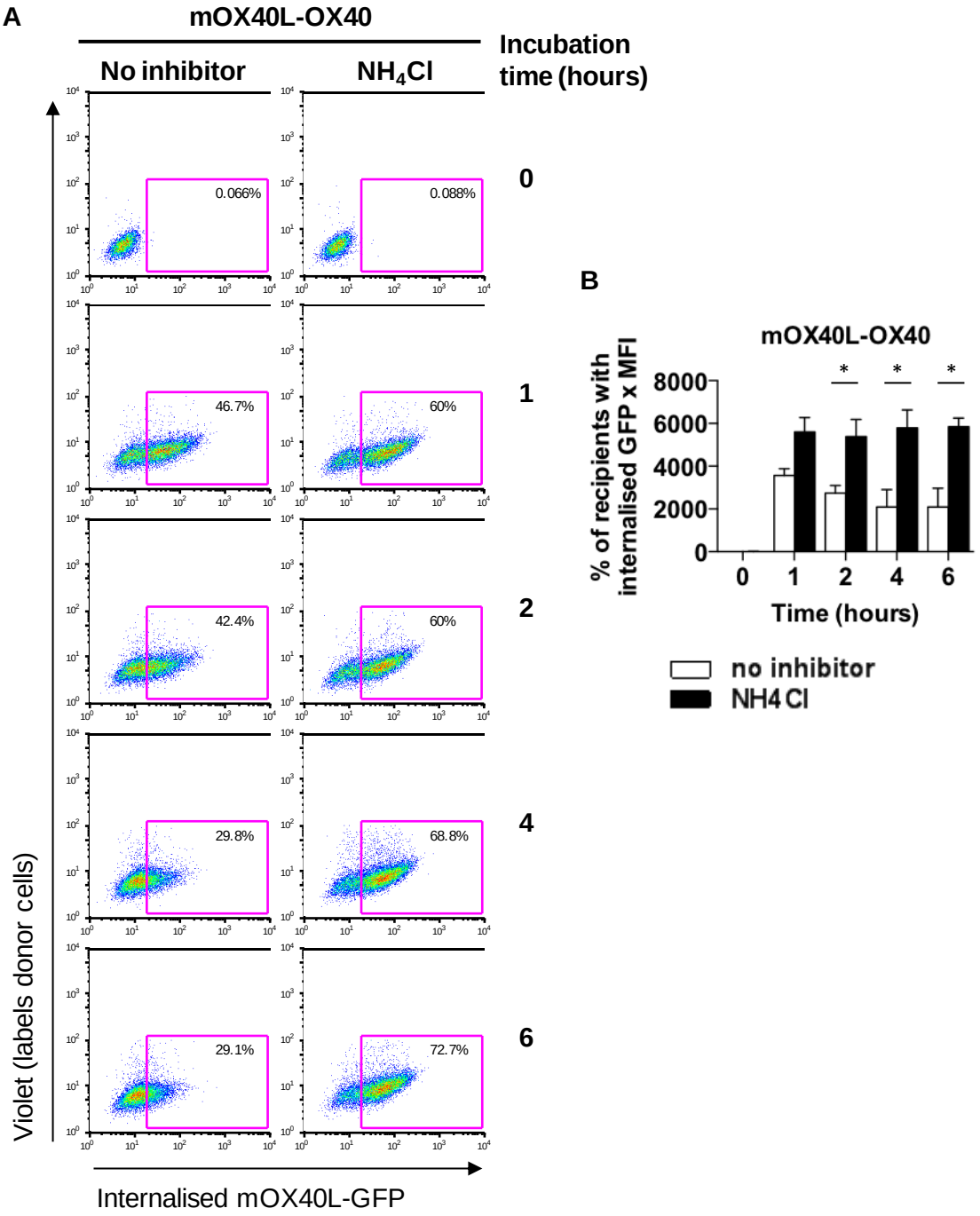


Figure 3.21 Mouse OX40 internalises its ligand. Violet-labelled mOX40L-GFP cells were incubated with mOX40-expressing cells at a 4:1 ratio +/- 40mM NH_4Cl at 37°C for 0-6 hours then put on ice. Cells were disaggregated and labelled with mOX40L-PE antibody at 4°C for 30 minutes then analysed by flow cytometry. **(A)** Plots show recipients excluding those with ligand on their cell surface. GFP^+ cells are gated. The percentage of all recipients with internalised GFP is shown. Data are representative of three independent experiments. **(B)** Graphs show the mean (+SEM) product of the percentage of recipients that internalised GFP and the GFP MFI of the GFP^+ recipients using data from three independent experiments. Statistically significant differences between data were determined using the paired t test *P<0.05.

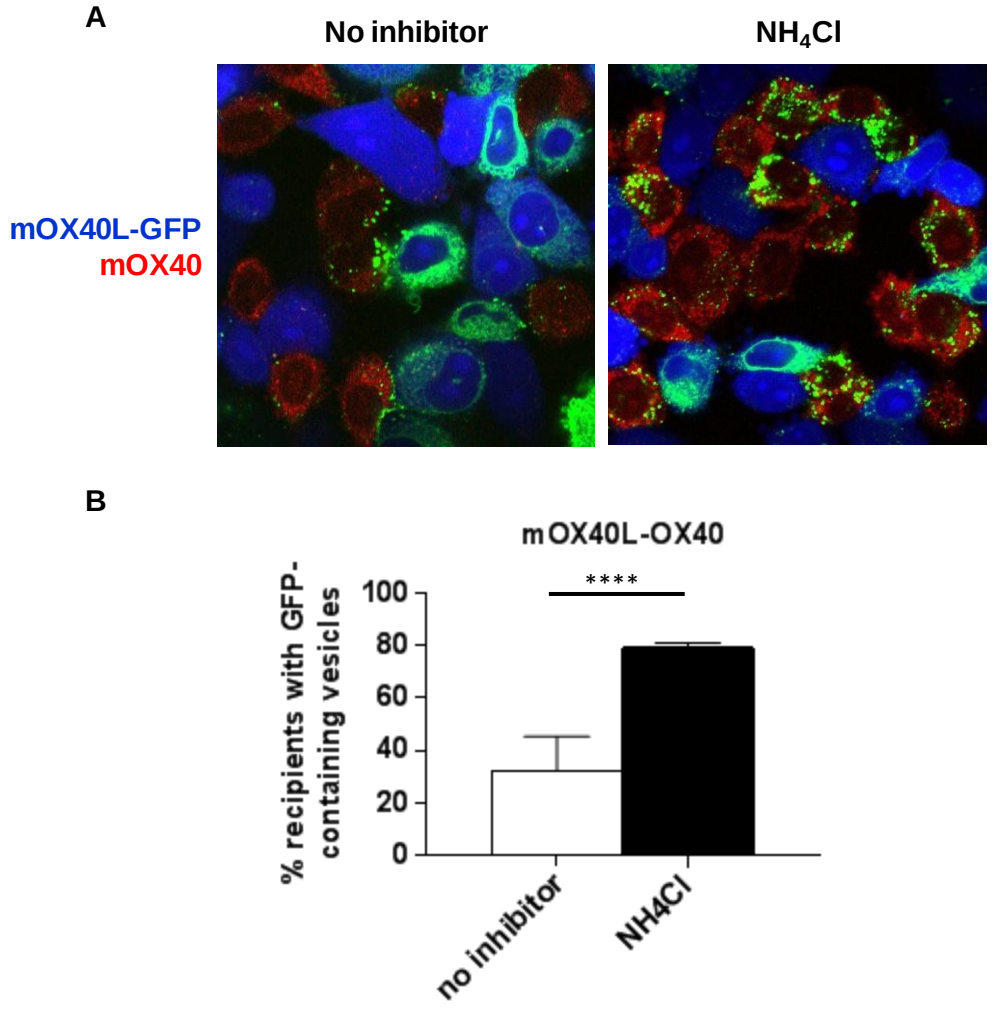


Figure 3.22 Mouse OX40 can remove its ligand by trans-endocytosis. Violet-labelled mOX40L-GFP cells were incubated with far red-labelled mOX40-expressing cells at a 1:1 ratio +/- 10mM NH_4Cl at 37°C for 6 hours then analysed by confocal microscopy. **(A)** Each image is representative of at least 24 micrographs taken over three independent experiments. **(B)** Graph shows the mean (+SD) percentage of recipients with ligand-GFP-containing vesicles within them using data from three independent experiments. A total of 525-545 cells were analysed for each condition. Statistically significant differences between data were determined using the t test ****P<0.0001.

3.10 Discussion

This project evolved from the discovery that CTLA-4 captures and internalises its ligands from APC by trans-endocytosis. During this process the entire ligand molecule is removed from the donor cell and the receptor-ligand complex is internalised via endocytic pathways and transported within intracellular vesicles before lysosomal degradation (Qureshi et al., 2011). The principle aims of the studies in this chapter were to establish a flow cytometry assay to measure trans-endocytosis, to continue to investigate the mechanism of trans-endocytosis via CTLA-4, and to explore the possible use of trans-endocytosis by other receptors. With regards to the first objective, it was demonstrated that trans-endocytosis could reproducibly be measured quickly and easily by analysing ligand-GFP acquisition by recipient cells using the high throughput method of flow cytometry. Trans-endocytosis was characterised by an increase in ligand acquisition over time and by the ability of lysosomal inhibitors to increase ligand detection. This reflects the cell biology of CTLA-4, which undergoes continual intracellular trafficking (Linsley et al., 1996; Mead et al., 2005; Qureshi et al., 2012). CTLA-4 is either recycled back to the plasma membrane to potentially engage other ligand molecules, or the receptor is targeted to lysosomal degradation along with its ligand. These features distinguish trans-endocytosis from trogocytosis, which is a process whereby plasma membrane fragments are transferred between cells (Joly and Hudrisier, 2003; Davis, 2007). During trogocytosis the initial transfer of proteins is to the cell surface only, then transferred proteins may subsequently be internalised and degraded. In contrast, trans-endocytosis involves direct protein internalisation. Therefore antibody labelling of ligands present on the cell surface was performed as an additional way to differentiate trans-endocytosis and trogocytosis. In addition, confocal microscopy was used for visual confirmation of ligand internalisation. Together, use of flow cytometry and confocal microscopy allowed the analysis of a large population of cells and visualisation of trans-endocytosis to produce both reproducible and reliable data.

3.10.1 The YVKM motif required for clathrin-mediated endocytosis is not essential for CTLA-4 trans-endocytosis

Understanding the molecular mechanism of trans-endocytosis may improve our understanding of T cell regulation and facilitate the identification of disease pathways and potential therapeutic targets. Initially it was predicted that the constitutive endocytosis of CTLA-4 and the process of trans-endocytosis use the same machinery and pathways. The YVKM motif in the cytoplasmic tail of CTLA-4 is the binding site of AP-2 and is therefore important for its clathrin-mediated endocytosis (Zhang and Allison, 1997). Indeed, a CTLA-4 variant with the tyrosine residue in this motif substituted for an alanine displayed limited endocytosis and was therefore highly expressed at the plasma membrane. Somewhat surprisingly the AVKM CTLA-4 variant was still able to capture ligand by trans-endocytosis, albeit less efficiently than wild type CTLA-4. This suggests that clathrin-mediated endocytosis is not essential for ligand trans-endocytosis and that other pathways can provide an alternative route for ligand internalisation. However it must be noted that the cell surface expression levels of wild type and AVKM variant CTLA-4 were very different and the amount of protein able to engage ligand is likely to affect the rate of trans-endocytosis. The amount of AVKM variant CTLA-4 at the plasma membrane was much greater than wild type CTLA-4, which may have increased the ability of cells expressing the variant to capture ligand. The data may therefore exaggerate the efficiency of AVKM variant CTLA-4 at trans-endocytosis compared to wild type CTLA-4, so inhibiting clathrin-mediated endocytosis may have a more severe impact on trans-endocytosis than the data suggest. This may also explain why the mutation appeared to have little effect on CD86 acquisition. Nonetheless other studies carried out in our laboratory, which controlled for differences in protein expression, were consistent with these findings that the YVKM motif is beneficial but not critical for ligand acquisition by CTLA-4. Interestingly, CTLA-4 with this tyrosine residue substitution is still relatively functional *in vivo* (Masteller et al., 2000). Furthermore, CTLA-4 lacking its entire cytoplasmic domain does not cause mice to develop lymphoproliferative disease to the same

extent as CTLA-4 knockout, suggesting that the suppressive function of CTLA-4 does not completely require elements within the cytoplasmic tail (Masteller et al., 2000; Takahashi et al., 2005). In fact a recent study has suggested that the extracellular domain of CTLA-4 is sufficient for Treg suppression of effector T cells (Tai et al., 2012). Although mice with tailless CTLA-4 do not develop fatal autoimmunity, they do have abnormal numbers of activated T cells which are skewed towards the T_H2 lineage making them vulnerable to particular infections (Masteller et al., 2000). Also, mutation of the tyrosine in the YVKM motif reduces the suppressive activity of Tregs (Stumpf et al., 2014). These studies suggest that CTLA-4 may be able to exert some function solely via its extracellular domain, but the cytoplasmic domain may be required for full regulatory function. Consistent with this, our laboratory has found that the trans-endocytosis of CD80 is not completely inhibited by deletion of the CTLA-4 cytoplasmic tail (Qureshi et al., manuscript in preparation). Overall the current data show that clathrin-mediated endocytosis of CTLA-4 is not essential for ligand trans-endocytosis and that the regions of CTLA-4 that control trans-endocytosis remain to be elucidated.

3.10.2 CD80 is captured more efficiently by trans-endocytosis than CD86

Another interesting finding was that CD80 appeared to be captured more efficiently than CD86. Unfortunately this result was not statistically significant despite the fact that the average amount of CD86 captured by CTLA-4 was half the average amount of CD80 internalised by trans-endocytosis as measured by flow cytometry. This was likely due to the low number of replicate experiments (n=3) and the wide standard deviation, which could be due to variation in ligand expression on different days, although every effort was made to maintain consistent and comparable ligand expression levels between cell lines and experiments. Similarly, the difference in CD80 and CD86 internalisation seen by confocal microscopy was not statistically significant as these results were the average of only two experiments. In one experiment CD80 trans-endocytosis was higher than CD86, but in the other it was lower. A repeat of these experiments would have been beneficial to determine

which of these results was reproducible. Overall this data and unpublished data of others in the lab (Omar Qureshi) support the conclusion that CD80 is captured by trans-endocytosis more efficiently than CD86. This may be due to their different structures and binding properties. CTLA-4 has much higher affinity for CD80 than CD86, and both CD80 and CTLA-4 form bivalent dimers, whereas CD86 is monomeric (Collins et al., 2002). Therefore CTLA-4 can bind twice as many CD80 molecules as it can CD86 molecules, so theoretically twice as much CD80 could be internalised via a given number of CTLA-4 molecules compared to CD86. It is also possible that CD80 and CD86 are internalised via different mechanisms. Indeed, mutation of both tyrosine residues and all lysine residues within the cytoplasmic tail of CTLA-4 prevents the internalisation of CD86 but CD80 trans-endocytosis cannot be completely inhibited, suggesting that alternative mechanisms may exist for the acquisition of CD80 (Qureshi et al., manuscript in preparation). Further investigation revealed that CD80-CTLA-4 interactions (but not CD86-CTLA-4) can cause the formation of tubular membrane protrusions that precede intracellular vesicle formation without the need for active cellular machinery. It is known that high density protein clustering can cause membrane deformation and tubule formation is required for vesicle formation (Stachowiak et al., 2010). Thus one possibility is that the bivalent nature of both CD80 and CTLA-4 dimers means that they can accumulate to form a lattice structure (Jansson et al., 2005), which might drive membrane invagination leading to trans-endocytosis. Ultimately it appears that the different structures and binding characteristics of CD80 and CD86 seem to determine how well they are captured by CTLA-4.

3.10.3 CD28 does not capture ligand by trans-endocytosis

Overall my data showed very effective capture of ligand by CTLA-4 but limited ligand internalisation via CD28. This was expected considering the contrasting cellular localisation and opposing functional outcomes of the two receptors. However, surprisingly, observations by confocal microscopy revealed a proportion of CD28-expressing cells with intracellular

vesicles containing CD80 in the presence of the lysosomal inhibitor NH_4Cl. Importantly, the percentage of CD28-expressing cells that acquired CD80 was still lower than the proportion of CTLA-4-expressing cells that did so. Taking all of the available data into account, it seems most likely that CD80 was internalised with CD28 following plasma membrane transfer by trogocytosis. It is known that CD80 can be transferred via CD28 to naive T cells by trogocytosis and that proteins transferred in this way are subsequently internalised for degradation (Hwang et al., 2000; Hudrisier et al., 2007). The amount of ligand acquired by CD28-expressing cells may have been more than expected via trogocytosis but CD28 is not a completely stable surface protein, it is subject to endocytosis as shown by my studies and others (Cefai et al., 1998; Badour et al., 2007). Therefore associated ligand internalisation following transfer by trogocytosis may be accelerated by CD28 endocytosis. It has even been suggested that iTregs can capture CD80/86 by trogocytosis independently of CD28 and CTLA-4 (Gu et al., 2012). It is plausible that the high expression of CD28 and CD80 in these cell lines enabled the exchange of a greater amount of protein than usual by trogocytosis, which then accumulated in lysosomal compartments due to the presence of NH_4Cl. Live imaging would be helpful in future studies in order to confirm this, as this would show whether ligand internalisation occurred actively during intercellular interactions (trans-endocytosis) or whether ligand was only transferred to the recipient cell surface as the cells parted and was only internalised later (trogocytosis). Additionally, it may be possible to distinguish between these processes more confidently by fixation of the donor cells. It has been shown that acquisition of HLA-DR1, CD80 and CD86 via trogocytosis is inhibited by fixation of the APC, suggesting this process requires membrane fluidity (Game et al., 2005). On the other hand trans-endocytosis is not inhibited even if the donor cells are fixed (my unpublished observations) therefore the simple method of fixing donor cells may provide a way to resolve these issues in the future.

3.10.4 PD-1-expressing cells internalise PD-L1 and PD-L2

Intriguingly the studies presented here have shown that PD-1 is able to internalise its ligands. In contrast to CTLA-4 trans-endocytosis, the number of PD-1-expressing cells that internalised ligand and the amount they acquired did not increase over time, suggesting that ligand acquisition by PD-1 may not occur by trans-endocytosis, but by a different mechanism to CTLA-4. Indeed the amount of ligand internalised by PD-1-expressing cells decreased over time in the absence of NH_4Cl, suggesting that the rate of ligand capture declines over time or that PD-1 ligands were degraded more rapidly than CTLA-4 ligands because different pathways are involved. Alternatively it is possible that PD-1 and its ligands were internalised by trans-endocytosis but ligand acquisition did not increase over time because PD-1 was not recycled back to plasma membrane. However it was not expected that PD-1 would be capable of trans-endocytosis because unlike CTLA-4, PD-1 is expressed predominantly at the cell surface and does not appear to be endocytic. Although as already discussed, receptor endocytosis may not be a prerequisite for trans-endocytosis. Also PD-1 has lower affinity for its ligands than CTLA-4 (Youngnak et al., 2003; Cheng et al., 2013) and PD-1 and its ligands are monomeric (Zhang et al., 2004; Lazar-Molnar et al., 2008). Therefore the interaction between PD-1 and its ligands was not expected to be strong enough to remove ligand molecules from opposing cells. Also, although CD80 and CD86 downregulation has been reported on DC in the presence of Tregs in many independent studies, no difference in PD-L1 or PD-L2 expression could be seen on iTreg-conditioned DC (DiPaolo et al., 2007). Instead PD-1 ligand may have been transferred by trogocytosis followed by internalisation. Indeed, transfer of PD-L1 from APC and tumour cells to CD8 T cells via trogocytosis has been reported (Gary et al., 2012). The acquired ligand enabled these T cells to trigger apoptosis in neighbouring PD-1-expressing cells, which the authors proposed could be a novel regulatory mechanism. Yet it is unclear by what mechanism ligand was transferred in my studies because although PD-1 ligands were not detected on the surface of recipient cells, suggesting ligand was not transferred by trogocytosis, there was no increase in ligand

acquisition over time which is characteristic of trans-endocytosis. Live imaging will therefore be required to explore the internalisation of PD-1 ligands further.

While the mechanism of ligand acquisition by PD-1-expressing cells remains to be defined, the functional significance of PD-1 ligand transfer is also unclear. Trans-endocytosis is consistent with the functional characteristics of CTLA-4, but there is not an obvious role for PD-1 trans-endocytosis. PD-1 and CTLA-4 are both negative regulators of T cell activation which inhibit T cell proliferation and cytokine production (Freeman et al., 2000; Latchman et al., 2001; Chemnitz et al., 2004; Walunas et al., 1996). This is highlighted by the development of autoimmunity in CTLA-4- and PD-1-deficient mice, although the phenotype is much more severe in CTLA-4 knockout mice which die within a few weeks after birth (Nishimura et al., 1999; Tivol et al., 1995; Waterhouse et al., 1995). It has been reported that both proteins suppress T cell responses by inhibiting glucose uptake and AKT phosphorylation (Parry et al., 2005). Therefore they do seem to have some similar functions but importantly they have different functional motifs. PD-1 contains a tyrosine-based ITSM motif which is a binding site for SHP-1 and SHP-2 and is required for PD-1-mediated inhibition of PI3K-AKT pathways (Chemnitz et al., 2004; Parry et al., 2005). While PD-1 appears to regulate signalling pathways, a signalling mechanism for CTLA-4 is less well defined. Consistent with this, PD-1 was found to suppress significantly more TCR/CD28-induced changes in gene transcription than CTLA-4, supporting a role for PD-1 in regulating intracellular events while CTLA-4 prevents the initiation of CD28 signalling by sequestering ligands (Parry et al., 2005). However a more recent study showed that neither CTLA-4 nor PD-1 influenced many TCR-induced transcriptional changes, suggesting that neither receptor has significant intracellular signalling functions and that they may use similar inhibitory mechanisms such as ligand competition or removal (Wakamatsu et al., 2013). Thus, whilst reports are inconsistent with each other, it is possible that CTLA-4 and PD-1 share some functional similarities.

Although our current understanding of the structure and function of PD-1 does not explain the internalisation of PD-1 and its ligands by trans-endocytosis, there are a few possible explanations for the internalisation of PD-1 ligands. Given its similarities with CTLA-4, the most logical reason for PD-1 to capture its ligands by trans-endocytosis would be if there were a second receptor for the two ligands PD-L1 and PD-L2 that is stimulatory. Thus PD-1 could inhibit T cell activation by ligand sequestration, in a similar fashion to the CD28/CTLA-4 system. Some early studies suggested that PD-L1 and PD-L2 can provide costimulation to T cells to enhance proliferation, although these studies used ligand-Ig fusion proteins which may have actually blocked PD-1 and prevented inhibition via this receptor rather than trigger stimulatory signals (Dong et al., 1999; Tseng et al., 2001; Tamura et al., 2001). Yet *in vivo* studies using DC from PD-L2 knockout mice showed they had reduced ability to stimulate T cell responses, again suggesting that PD-L2 can provide costimulation (Shin et al., 2003; Shin et al., 2005). Furthermore, this group showed that PD-L2-Ig stimulated PD-1-deficient T cells, supporting the concept that a second receptor exists for PD-L2 that has a stimulatory function. Consistent with this, PD-L2-expressing tumour cells triggered T cell responses leading to tumour rejection, including when only PD-1-deficient T cells were used (Liu et al., 2003). Moreover, PD-L1 and PD-L2 mutants unable to bind PD-1 were able to stimulate T cell proliferation (Wang et al., 2003). However numerous studies have been unable to replicate these findings (Pfistershammer et al., 2006; Brown et al., 2003). Overall it still seems unlikely that PD-1 removes its ligands as part of its inhibitory function.

Alternatively PD-1 may capture its ligands to negatively regulate its own function to put a brake on T cell inhibition and Treg development. In fact the trans-endocytosis of CD47 and SHPS-1 is believed to negatively regulate their function (Kusakari et al., 2008). The downregulation of PD-1 and its ligands may be necessary to eliminate persistent infections. For example, PD-1 is highly expressed on exhausted T cells in mice with chronic LCMV

infection but blockade of PD-L1 restored T cell function and reduced viral load (Barber et al., 2006). Thus it is possible that ligand internalisation by PD-1 evolved to control the inhibitory effects of this receptor in order to overcome T cell inhibition when necessary. Further investigation will be required to establish the functional importance of the discovery that PD-1 can internalise its ligands.

3.10.5 OX40-expressing cells acquire OX40L

The studies in this chapter also revealed that the mouse ortholog of OX40 can internalise its ligand. OX40 enhances T cell expansion and survival and promotes the development of memory T cells (Croft et al., 2009). OX40 also inhibits Treg suppression (Valzasina et al., 2005; Vu et al., 2007; Kitamura et al., 2009). Therefore OX40 ligand acquisition may serve to negatively regulate these stimulatory functions and terminate an immune response. Interestingly it has been suggested that reverse signalling through OX40L can enhance the activity of APC (Matsumura et al., 1999; Wang et al., 2004). The capture of OX40L may regulate these signalling events. Alternatively, trans-endocytosis could be a mechanism of OX40/OX40L downregulation which occurs as T cells differentiate into memory cells. Conversely, it has been suggested that OX40 promotes rather than inhibits Treg suppressive activity (Griseri et al., 2010). Sequestering OX40L by trans-endocytosis could be a mechanism by which this is possible. However others have found that OX40 merely increases Treg survival (Piconese et al., 2010). Intriguingly, a study has shown that OX40 can form a heterodimer with 4-1BB (another TNF receptor), and upon ligation they were coendocytosed and T cell proliferation was suppressed (Ma et al., 2005). Similarly, OX40 has been shown to inhibit T cell proliferation under certain priming conditions (Kim et al., 2005). T cell inhibition was associated with OX40L downregulation on DC and T cells and the authors speculated this function of OX40 served to halt T cell proliferation while additional DC signals were integrated in order to avoid unwanted immune responses. Therefore there are circumstances which could be explained by OX40 trans-endocytosis, however OX40 is a

plasma membrane protein not generally known to be endocytic. Also, costimulatory signalling pathways downstream of OX40 are well established (Song et al., 2004; Song et al., 2008), opposing the idea that OX40 may be inhibitory. Therefore speculation about the significance of ligand acquisition by PD-1 and OX40 should be avoided until further investigations in primary human T cells are carried out to support these findings. It remains to be verified whether activated T cells and Tregs expressing physiological levels of these receptors internalise ligand and live imaging will be required to determine whether this phenomenon is truly trans-endocytosis or due to another molecular mechanism.

3.10.6 Summary

In summary, it has been revealed that the YVKM motif of CTLA-4 is not essential for trans-endocytosis, suggesting that clathrin-mediated endocytosis plays a redundant role in this process. Therefore further studies are required in order to establish the detailed molecular mechanism of trans-endocytosis. In addition, the functional impact of trans-endocytosis in a physiological setting needs further investigation. Here only ligand acquisition was measured but what is functionally more important to consider is whether this causes a significant loss of ligand on the donor cells. A key question that remains to be answered is whether changes in ligand levels for short periods of time significantly alter T cell fate. In other words, do changes in ligand levels have a graded effect on T cell responses and for how long is ligand downregulation effective? The relative contribution of this mechanism to T cell regulation, the context in which it is most relevant, and the biological consequences of restricting CD28 costimulation remain to be fully explored. The subsequent chapters in this thesis aim to address some of these questions to help us understand the role of trans-endocytosis in T cell tolerance, which will hopefully reveal more effective ways of targeting the CD28/CTLA-4 pathway in the treatment of autoimmune diseases and cancer.

4 INVESTIGATING CD4 T CELL REQUIREMENTS FOR CD28 COSTIMULATION

4.1 CD28 costimulation is required to initiate and maintain a T cell response

The previous chapter demonstrated the ability of CTLA-4 to remove its ligands from other cells by trans-endocytosis, which limits the number of ligands available for CD28 costimulation. To determine the functional significance of this feature of CTLA-4, we need to better understand the costimulatory requirements of CD4 T cells. Therefore studies in this chapter were designed to test when T cells require costimulation and how much they need. Initial experiments were carried out using $CD4^+CD25^-$ T cells stimulated with anti-CD3 and CD28 costimulation provided in the form of CD80-expressing CHO cells either immediately (0h) or at various time points after TCR stimulation (**figures 4.1A-B**). Analysis of T cell proliferation at day five showed that delaying CD28 costimulation reduced the number of T cells that committed to divide and the response was significantly impaired if sufficient costimulation was not received within 24 hours following TCR stimulation. These results show that TCR stimulation and CD28 costimulation are required within a limited time of each other in order to initiate a T cell response. These data also indicate that restricting the availability of CD28 costimulation has a major impact on the size of the response. Next, in order to determine whether CD28 costimulation is required to maintain T cell proliferation, CD28 costimulation was inhibited 24 hours after T cell stimulation by adding CTLA-4-Ig to the culture (**figure 4.1C**). T cell proliferation was severely impaired, showing that not only is CD28 critical for initiating proliferation, it is also required to maintain proliferation. This suggests that regulation of CD28 costimulation after the response has started is still functionally significant. Together these data show that CD28 costimulation is required for both initiation and maintenance of CD4 T cell responses and therefore suggest that CD28 inhibition by CTLA-4 would be effective at either phase of the response.

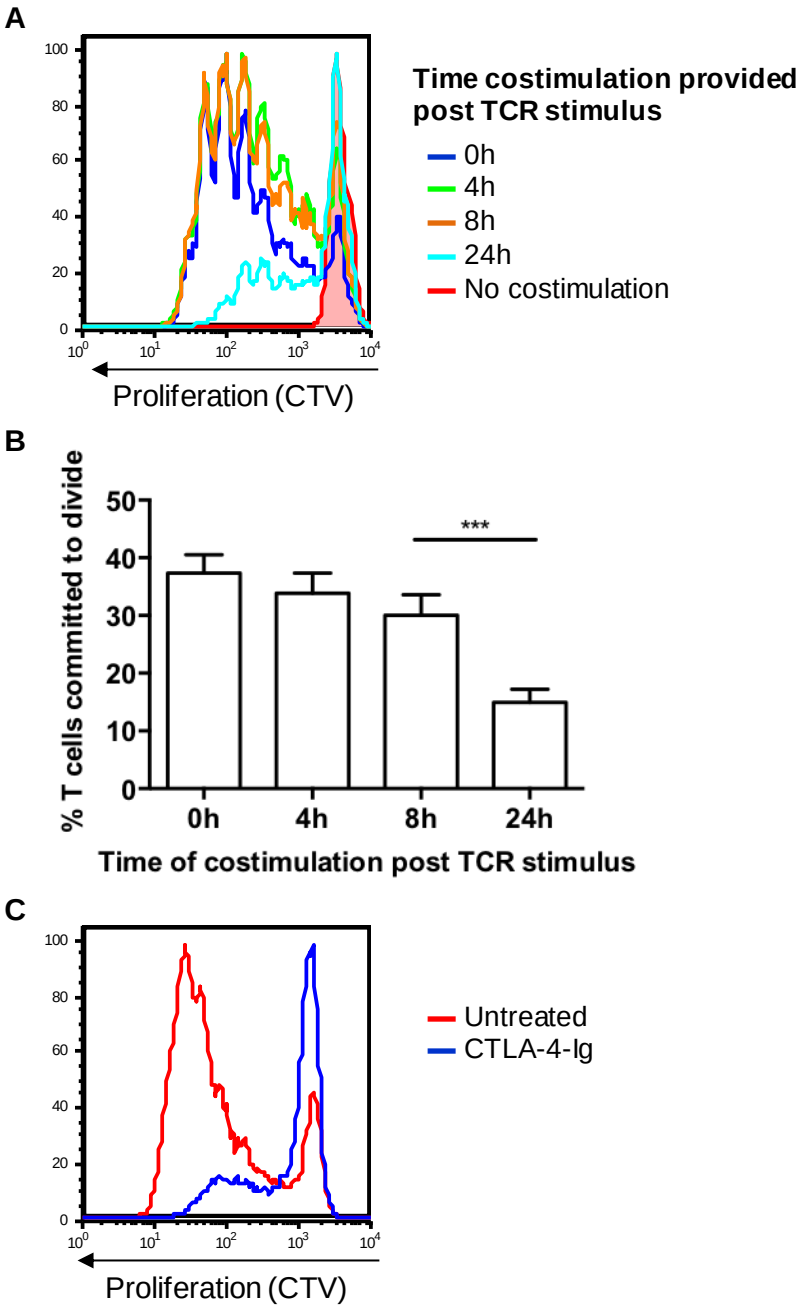


Figure 4.1 Restricting the availability of CD28 costimulation impairs the initiation and maintenance of T cell expansion. CTV-labelled $CD4^{+}CD25^{-}$ T cells were cultured with 1µg/ml anti-CD3. Fixed CD80-expressing CHO cells were added 0, 4, 8 or 24 hours after the anti-CD3 or no CD80 cells were added (no costimulation). Cells were incubated for five days then proliferation was analysed by flow cytometry. **(A)** Histograms show T cell proliferation (CTV fluorescence) for each condition overlaid. Data are representative of four independent experiments. **(B)** Graph shows the mean (+SEM) percentage of T cells that committed to divide for each condition using at least duplicate measurements from four independent experiments. Statistically significant differences between data were determined by the Wilcoxon test ***P<0.001. **(C)** CD80 cells and anti-CD3 were added to the T cell culture simultaneously and 18µg/ml CTLA-4-Ig or the equivalent volume of media (untreated) was added after 24 hours. Histogram shows T cell proliferation on day five.

4.2 Generating inducible cell lines and optimising their use

Our work showing that CTLA-4 removes its ligands from APC to reduce their capacity to provide CD28 costimulation to T cells generated the prediction that there is a minimum number of costimulatory ligands required for T cell activation. This prediction was tested by generating an inducible gene expression system that enabled the induction of CD80 or CD86 expression over a wide and continuous range of levels (refer to **sections 2.5-2.6** and **figure 2.1**). Briefly, human CD80 and CD86 were cloned into tetracycline-inducible response plasmids, which were transfected into CHO cells expressing the transactivator protein required for gene transcription. Cells were treated with doxycycline which is required for the transactivator protein to bind to the promoter region. Protein expression was induced in a dose dependent manner. These “Tet-On” cells were used to determine the number of ligands required for T cell activation.

Once the cell lines were stable, a protocol for using these cells to stimulate T cells was optimised. Firstly the time to reach maximal protein expression after doxycycline treatment was established. Protein expression level was maximal after 48 hours but started to decline after 72 hours therefore cells were used on the second day following doxycycline treatment (**figure 4.2A**). To verify that doxycycline did not directly affect T cell proliferation, cells constitutively expressing CD80 were cultured in the presence or absence of doxycycline for two days then used to stimulate T cells. This revealed that T cell proliferation was the same whether the T cells were cultured with untreated or doxycycline-treated cells (**figure 4.2B**), indicating that traces of doxycycline in the culture system did not affect T cell proliferation by acting on T cells directly. Finally, to verify that Tet-On CD80 inducible cells were able to stimulate T cell responses, they were treated with the highest recommended dose of doxycycline to induce the maximum level of CD80 expression, then they were fixed and cultured with T cells in the presence of anti-CD3. T cell proliferation was analysed at day five and showed that T cells were able to respond to costimulation using the Tet-On system

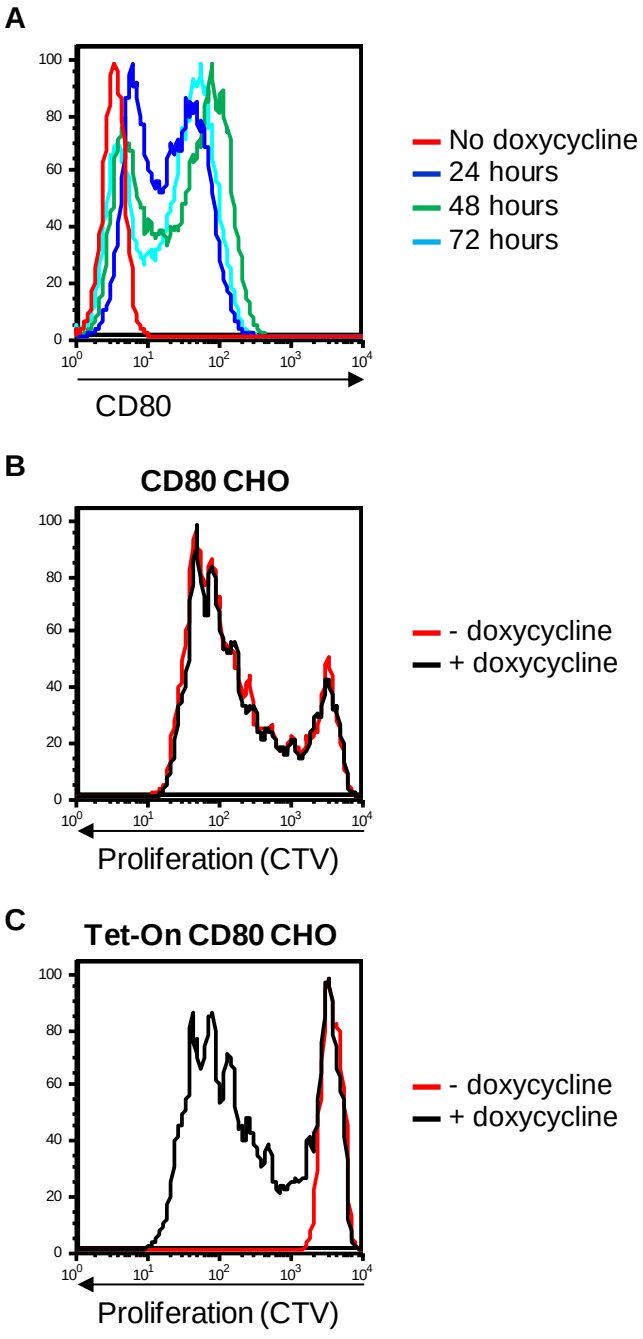


Figure 4.2 Optimising and validating the use of Tet-On cells to stimulate T cell responses. **(A)** Tet-On CD80 cells were incubated at 37°C for 24, 48 or 72 hours in the presence of 1000ng/ml doxycycline. Cells were labelled with CD80-PE antibody and analysed by flow cytometry. Overlaid histograms show CD80 expression at each time point. **(B)** CD80 CHO cells were incubated with or without 1000ng/ml doxycycline for 48 hours then fixed and incubated with CTV-labelled $CD4^+CD25^-$ T cells in the presence of 1µg/ml anti-CD3 for five days. T cells proliferation was analysed by flow cytometry. Histograms show T cell proliferation (CTV fluorescence) for each condition overlaid. **(C)** Same as *(B)* using Tet-On CD80 cells. All data are representative of at least two independent experiments.

(**figure 4.2C**). Next different concentrations of doxycycline were tested to find doses that induced a range of protein expression levels (**figure 4.3**). For CD80-expressing cells, at lower doses of doxycycline not all cells were induced to express CD80 so there were two distinct expression levels within the population. Unfortunately more homogenous expression could not be obtained even when multiple Tet-On CHO clones were screened. For CD86-expressing cells, a more homogenous population expressing similar levels of CD86 was observed. There was some background expression of CD86 but this level of protein could not facilitate a T cell response. The average number of ligand molecules per cell was determined by flow cytometry before every experiment using antibody-binding beads and fluorescent antibodies as described in **figure 4.4** and **section 2.7**. This therefore provided an experimental system with which to test the number of costimulatory ligands required for T cell activation.

4.3 CD80 is the more potent CD28 ligand

In order to determine whether there is a threshold number of costimulatory ligands required for a T cell response, human naive CD4 T cells were incubated with anti-CD3 and Tet-On cells induced to express different levels of CD80 or CD86. After five days proliferation was analysed by flow cytometry (**figure 4.5A**). This showed that the percentage of T cells that committed to divide increased as the number of costimulatory ligands per cell increased. The initial shallowness of the response curve (especially for CD86) revealed that there was a lack of response until a certain threshold number of costimulatory ligands was available then a proliferative response was initiated (**figure 4.5B** zoomed view). Plotting the number of costimulatory molecules on a logarithmic scale produced a straight line which allowed the interpolation of the number of costimulatory molecules required to initiate a proliferative response (**figures 4.5C-D**). More CD86 molecules were required per cell (>300,000) in order to elicit T cell proliferation compared to CD80 (~200,000), suggesting that CD80 is the more potent or efficient ligand. In addition, CD80 stimulated more T cells to commit to division than

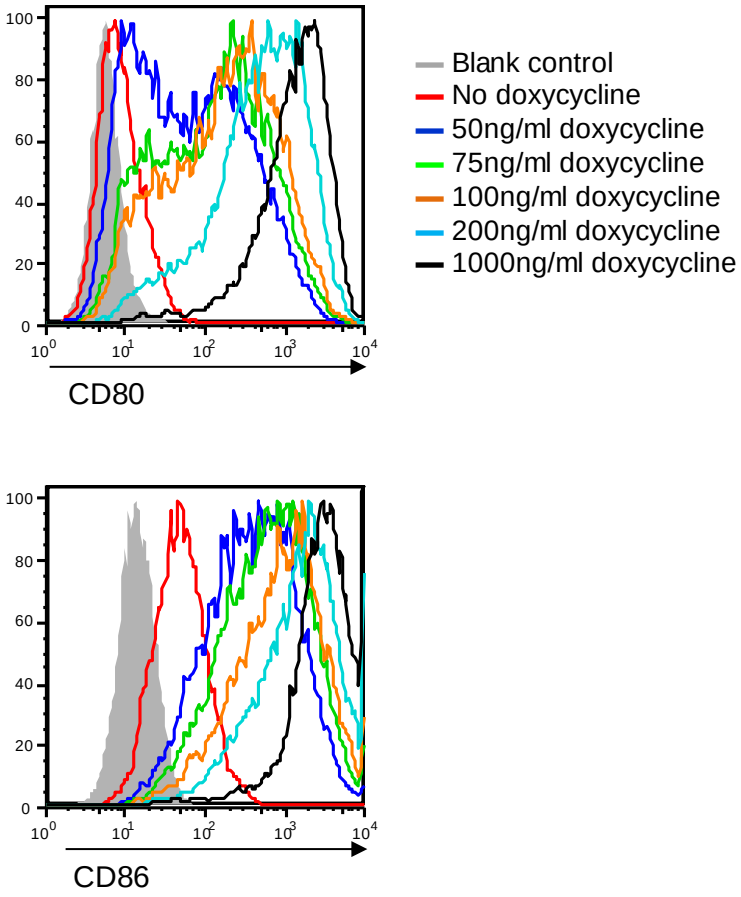
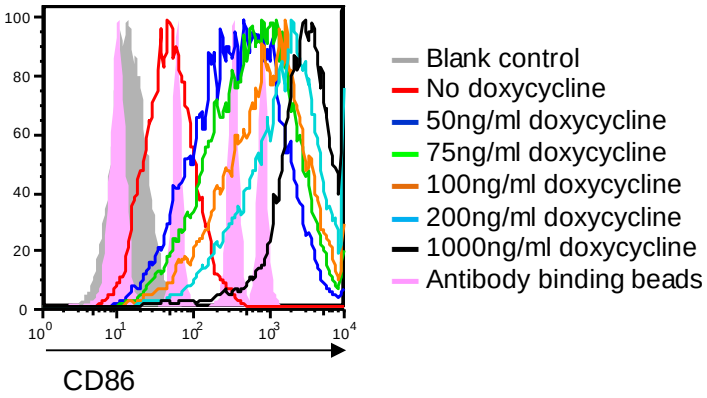


Figure 4.3 CD80 and CD86 expression was induced by doxycycline in a dose-dependent manner. Cells were incubated at 37°C for 42-48 hours in the presence of 1000, 200, 100, 75, 50 or 0 ng/ml doxycycline. Cells were incubated with fluorescently-labelled antibody and analysed by flow cytometry. Overlaid histograms show CD80-PE or CD86-PE fluorescence after treatment with each concentration of doxycycline. Blank (un-transfected) cells were used as a negative control.

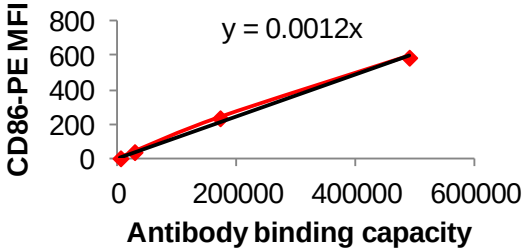
A



B

Antibody binding capacity of beads	CD86-PE MFI
4675	8.53
28353	44.40
172189	241.00
490505	593.00

C



D

	A	B	C	D	E
		CD86-PE MFI	MFI minus background	Average number of CD86 molecules per cell	Average number of CD86 molecules per cell minus background
2	unlabelled beads	1.05			
3	blank control	12			
4	no doxycycline	18	6	5000	4125
5	50ng/ml doxycycline	365	353	294167	293292
6	75ng/ml doxycycline	687	675	562500	561625
7	100ng/ml doxycycline	798	786	655000	654125
8	200ng/ml doxycycline	1413	1401	1167500	1166625
9	1000ng/ml doxycycline	2134	2122	1768333	1767458
			C9=B9-B3	D9=C9/0.0012	E9=D9-(B2/0.0012)

Figure 4.4 The number of ligand molecules per cell was calculated using antibody-binding beads. Tet-On CD86 cells were incubated at 37°C for 42-48 hours in the presence of 1000, 200, 100, 75, 50 or 0 ng/ml doxycycline. These cells, blank cells and antibody binding beads of four different antibody binding capacities were then incubated with CD86-PE antibody and analysed by flow cytometry. **(A)** Overlaid histograms show the CD86-PE fluorescence of each population of beads and cells. **(B)** Table shows the antibody binding capacity of the four types of beads and their CD86-PE MFI. **(C)** Values from the table *(B)* were used to create a standard curve and the equation of the line is shown. **(D)** Table shows how the number of ligands per cell was calculated using the MFI of the cells and the slope of the standard curve. The formulae for row 9 are shown in red as an example. Background fluorescence of the cells and beads was subtracted.

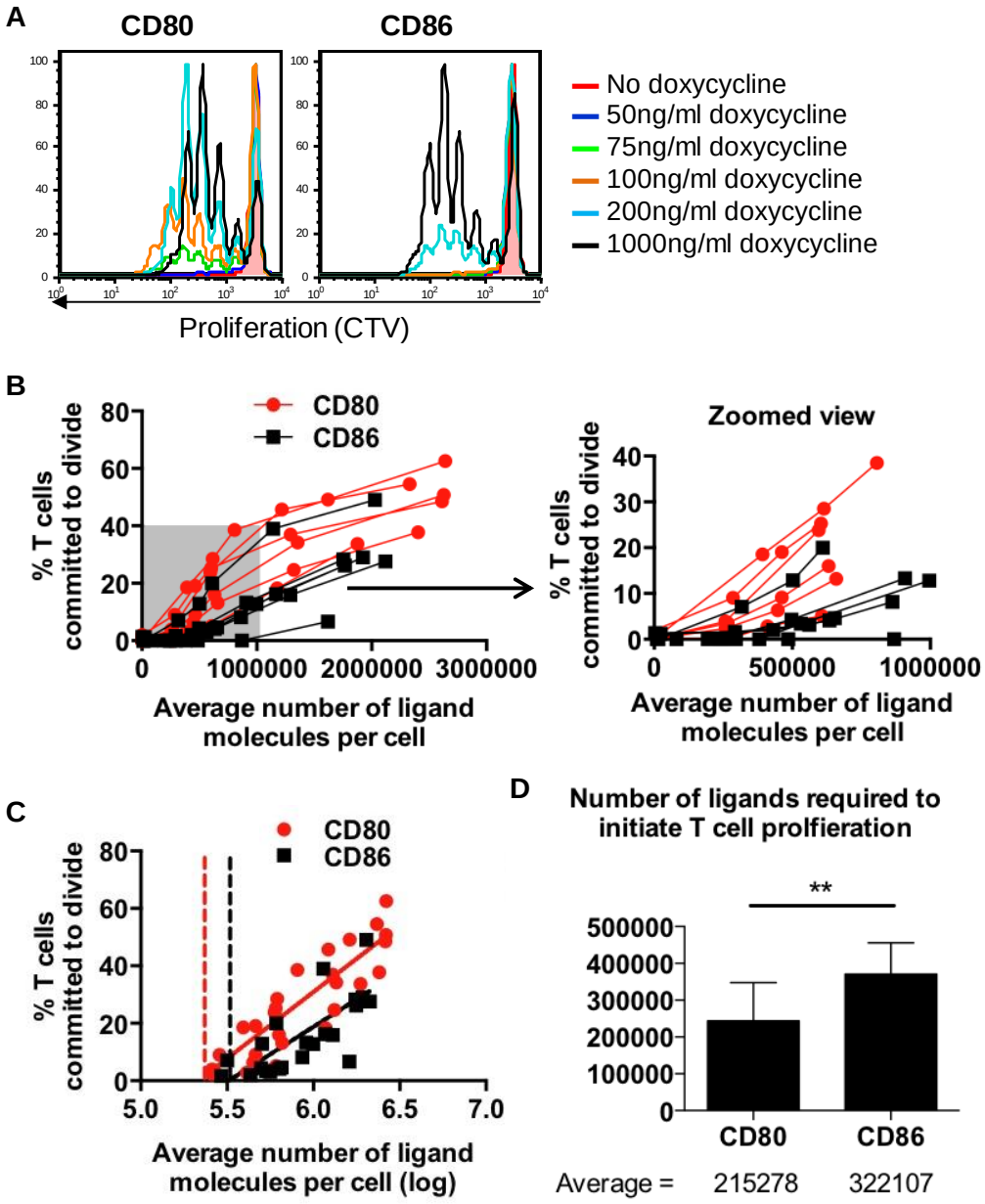


Figure 4.5 Calculating the threshold number of costimulatory ligands required to initiate T cell proliferation. Tet-On cells were incubated at 37°C for 42-48 hours in the presence of 1000, 200, 100, 75, 50 or 0 ng/ml doxycycline then washed and fixed with glutaraldehyde. Cells were incubated with CTV-labelled naive CD4 T cells at a ratio of 1:2 (CHO:T cells) with 1µg/ml anti-CD3 for five days then analysed by flow cytometry. **(A)** Overlaid histograms show T cell proliferation (CTV fluorescence). Data are representative of six independent experiments. **(B)** Graph (left) shows the percentage of T cells that committed to divide. Each data point is the average of triplicate samples. Each line shows one of six independent experiments. Graph (right) shows zoomed in view of grey area in left-hand graph. **(C)** Graph shows the same data as *(B)* with the number of costimulatory ligands per cell plotted as log(x). Where the straight line intercepts the x axis (shown by dashed line), this represents the number of ligands required to initiate T cell proliferation. **(D)** Graph shows the mean (+SD) number of ligand molecules required for T cell proliferation interpolated from graph *(C)*. The difference in the number of CD80 and CD86 molecules required was statistically significant according to the paired t test **P<0.01.

CD86 when they were expressed at equivalent levels above the threshold required for T cell activation. Taking one experiment as an example, when $1.8x10^6$ ligands were available per cell, CD86 stimulated ~26% of T cells to divide whereas CD80 stimulated ~40% of T cells to enter division (**figure 4.6A-B**). These studies therefore revealed that less CD80 is required than CD86 to initiate a T cell response and to increase the size of the response, in line with their relative affinities for CD28.

Interestingly it was found that the shape of the response profiles differed between CD80 and CD86. When CD80 was provided at increasing levels it was found that the proliferative T cell response was non-linear. At low levels of ligand expression a small increase in ligand number resulted in a large increase in T cell proliferation, but at higher levels of ligand availability the T cell response started to plateau, meaning that changes in ligand number had no further impact on T cell proliferation. On the other hand, where CD86 was the costimulatory ligand, the response produced a straight line meaning there was a direct linear relationship between ligand availability and T cell proliferation. This is demonstrated in **figure 4.6C**, which shows the difference in T cell proliferation was greater with CD86 compared to CD80 when the number of ligands available was reduced by half. One implication of this finding is that the removal of CD86 by trans-endocytosis may have a greater impact on the T cell response than the removal of CD80. This linear relationship between CD86 expression and T cell expansion may be important for Tregs to easily prevent the initiation of unwanted responses by removing ligand by trans-endocytosis, especially in cases of autoantigen recognition in a non-inflammatory environment when only CD86 is expressed.

In addition to proliferation, T cell upregulation of CD40L and CD25 was measured in response to varying numbers of costimulatory molecules (**figure 4.7**). In general the number of costimulatory ligands required to upregulate CD40L and CD25 was comparable to the number of costimulatory molecules required per cell to initiate T cell proliferation (**figures**

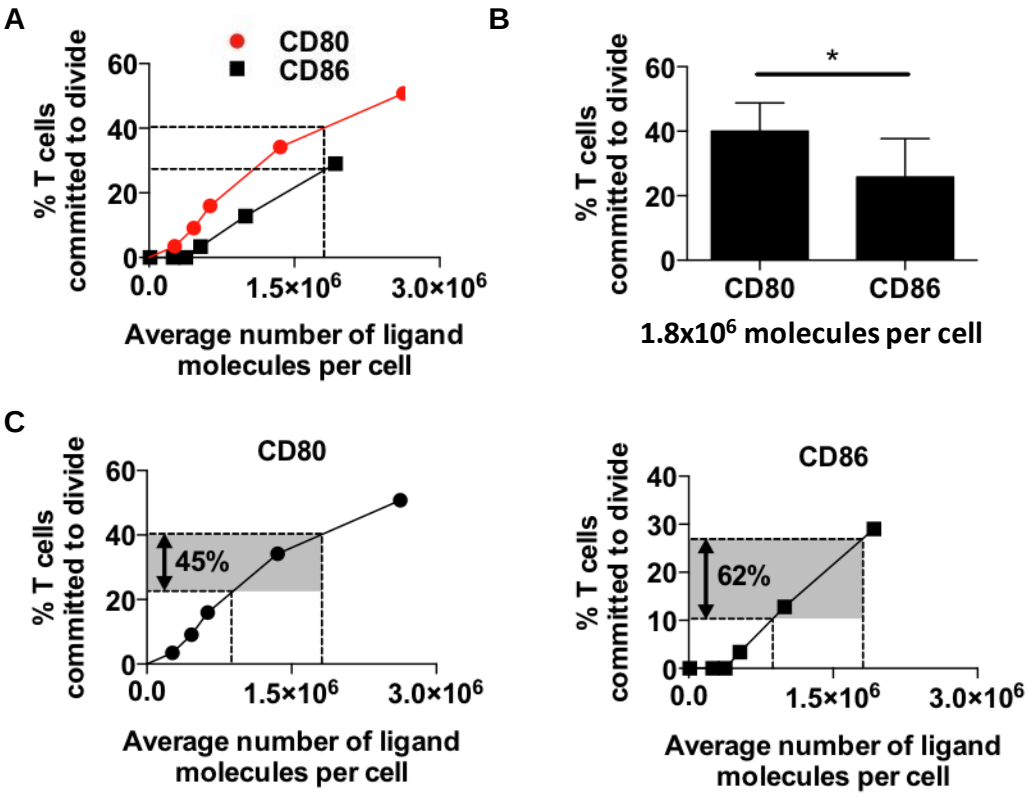
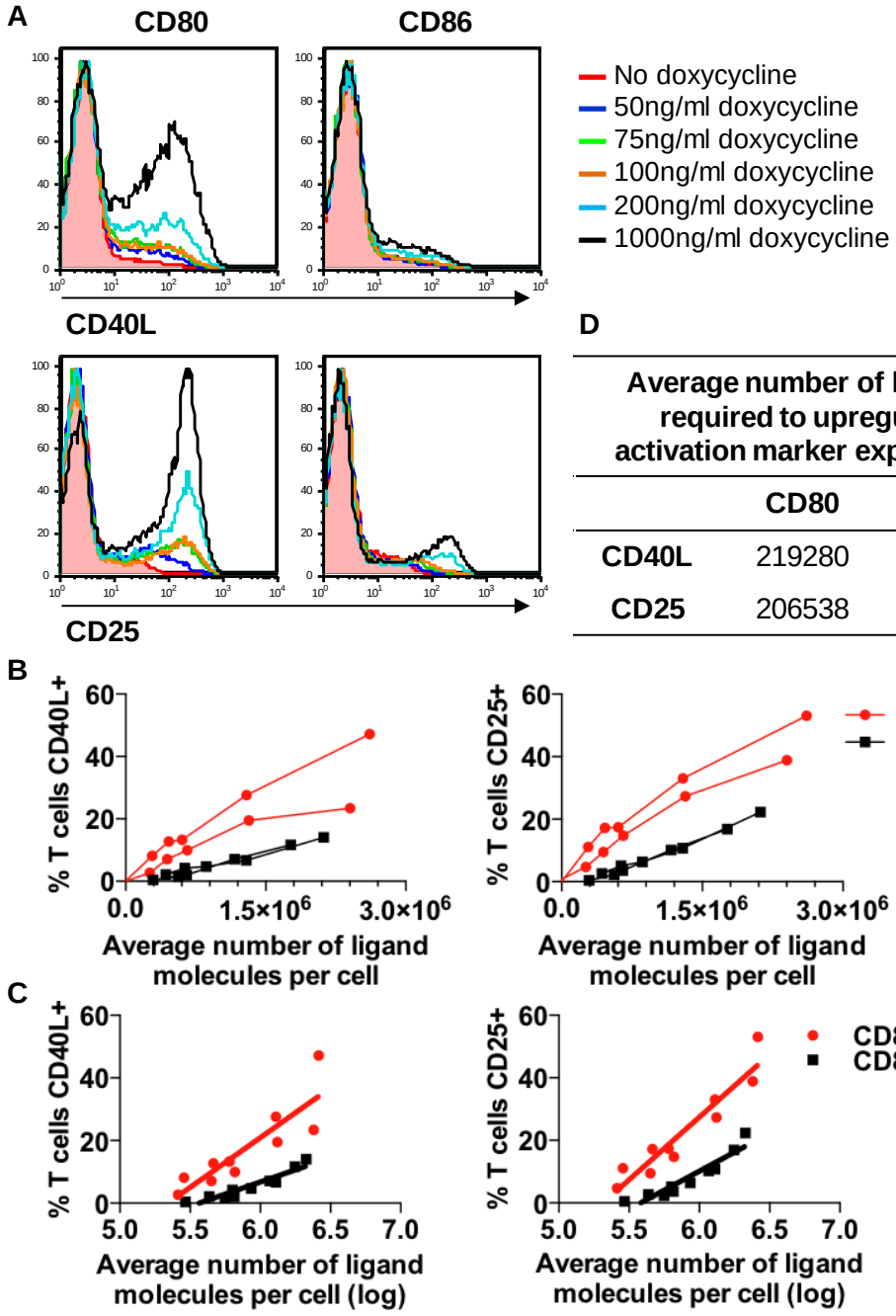


Figure 4.6 Analysing the effects of changing the number of CD80 molecules compared to CD86. Tet-On cells were incubated at 37°C for 42-48 hours in the presence of 1000, 200, 100, 75, 50 or 0 ng/ml doxycycline then washed and fixed with glutaraldehyde. Cells were incubated with CTV-labelled naive CD4 T cells at a ratio of 1:2 (CHO:T cells) with 1µg/ml anti-CD3 for five days then analysed by flow cytometry. **(A)** Graph shows the number of costimulatory ligand molecules per cell against T cell proliferation for one experiment representative of six. Dashed lines indicate the difference in efficiency between CD80 and CD86. **(B)** Graph shows the mean (+SD) percentage of T cells that committed to divide in the presence of CHO cells expressing an average of $1.8x10^6$ ligand molecules per cell. The difference between CD80 and CD86 was statistically significant according to the t test *P<0.05. **(C)** Graphs show the percentage of T cells that committed to divide. Data are representative of six independent experiments. Dashed lines indicate T cell proliferation in the presence of $0.9x10^6$ and $1.8x10^6$ ligand molecules. Grey area and annotation indicate the difference in T cell proliferation when ligand number is changed.



D

Average number of ligands required to upregulate activation marker expression		
	CD80	**CD86**
CD40L	219280	365595
CD25	206538	381066

Figure 4.7 CD80 is the more potent costimulatory ligand during T cell activation. Tet-On cells were incubated with doxycycline for 42-48 hours then washed and fixed with glutaraldehyde. Cells were incubated with naive CD4 T cells at a ratio of 1:2 (CHO:T cells) with 1µg/ml anti-CD3 for two days. T cells were labelled with antibodies then analysed by flow cytometry. **(A)** Overlaid histograms show CD40L or CD25 expression. Data are representative of two independent experiments. **(B)** Graphs show the percentage of T cells that upregulated CD40L or CD25. Data from two independent experiments are shown. **(C)** Graphs show the number of costimulatory ligands per cell plotted as log(x) against the percentage of cells expressing CD40L or CD25. Where the straight line intercepts the x axis this represents the number of ligands required to initiate protein upregulation. **(D)** Table shows the average number of ligand molecules required for T cell marker upregulation interpolated from graphs *(C)*.

4.5D and 4.7D). These data reinforce the view that CD80 is the more potent CD28 ligand because less CD80 molecules were required to upregulate CD40L and CD25 expression than CD86. Furthermore, CD80 stimulated more T cells to upregulate these activation markers than the same number of CD86 molecules. Together the analysis of T cell proliferation and activation marker upregulation showed that CD80 and CD86 produce quantitatively different responses.

4.4 TCR signal strength and costimulatory cell number determine the level of CD28 costimulation required

It has been reported that CD28 signalling reduces the number of TCR molecules that need to be engaged in order to trigger T cell activation (Viola and Lanzavecchia, 1996). To test whether increasing the TCR signal could likewise reduce the number of CD28 ligand molecules required for T cell activation, Tet-On cells induced to express different levels of CD80 or CD86 were used to stimulate T cells in the presence of a range of anti-CD3 concentrations. Indeed, increasing the anti-CD3 concentration decreased the number of costimulatory ligands required to initiate T cell proliferation and increased the maximum number of T cells that entered division (**figure 4.8**). This indicates that the strength of the TCR signal affects the amount of CD28 costimulation required and suggests that the two signals are additive or synergistic. For example, there was equivalent T cell proliferation in settings where there was a relatively high anti-CD3 concentration (4µg/ml) and ~$1.3x10^6$ CD80 molecules per cell, and where there was a lower anti-CD3 concentration (0.5µg/ml) but twice the number (~$2.6x10^6$) of CD80 molecules (**figure 4.8B**). This implies that TCR and CD28 signals can compensate for each other and therefore that the combination of both signals together controls T cell activation.

It was also investigated whether the total number of cells expressing costimulatory molecules could have an impact on T cell activation in addition to the density of ligands per cell. It was

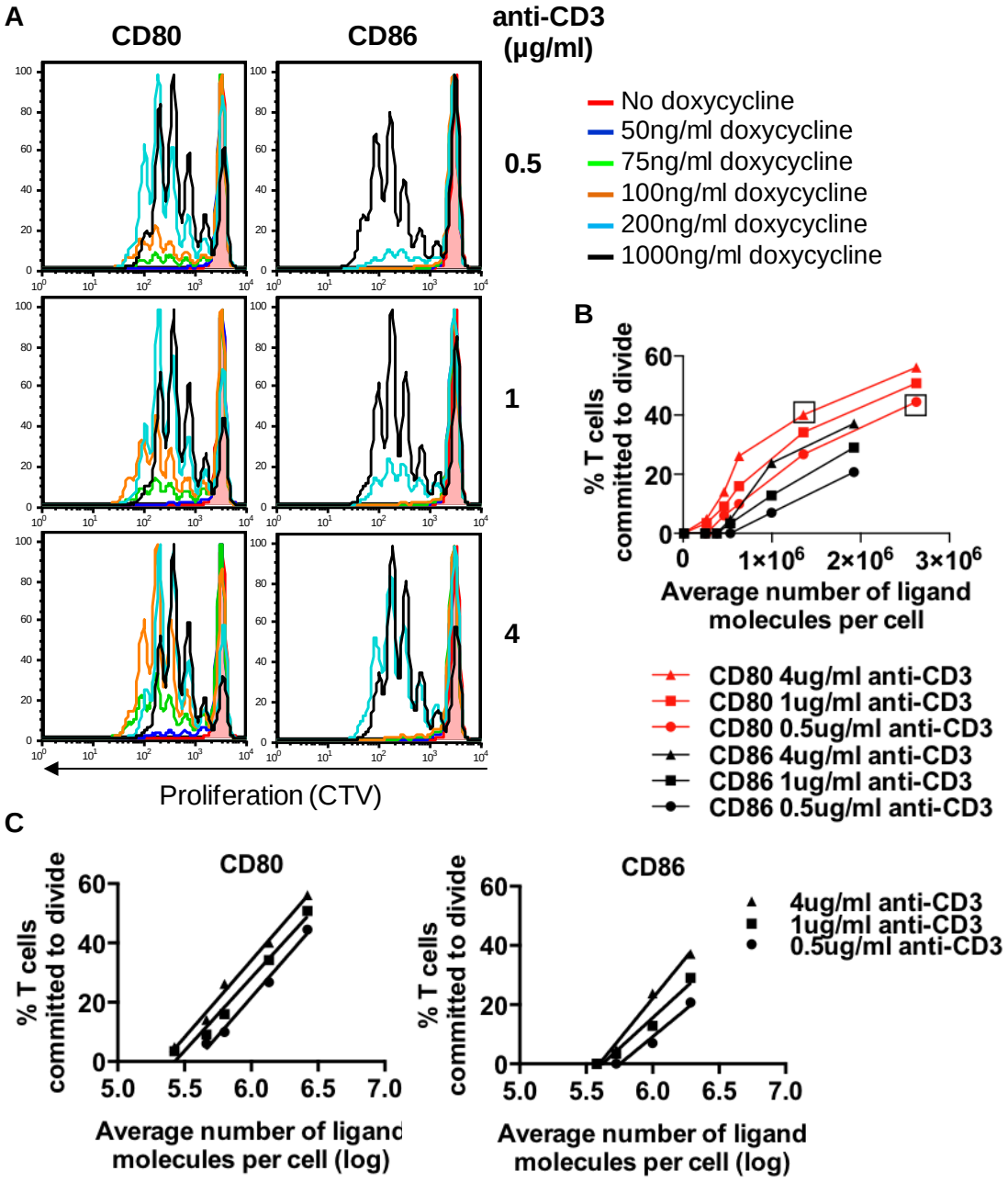
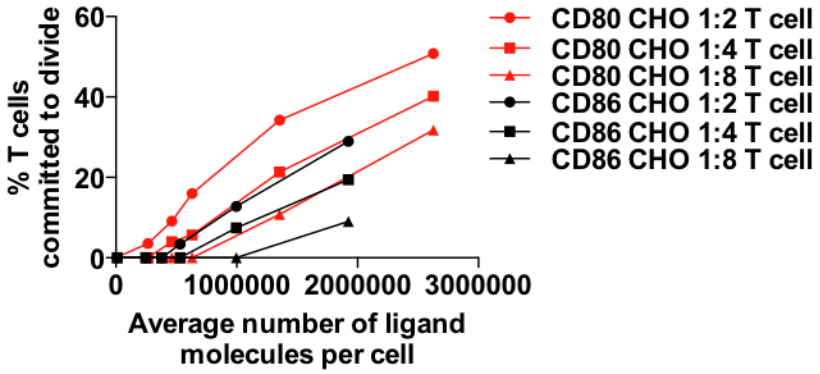


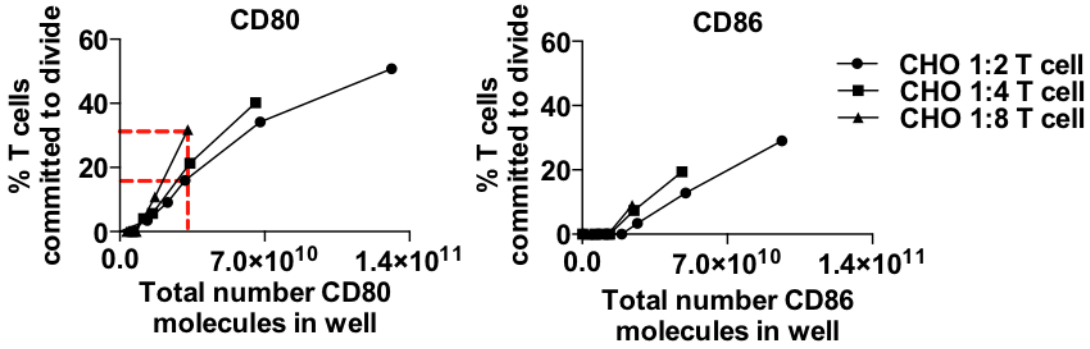
Figure 4.8 Increasing the TCR signal reduces the number of costimulatory ligands required for T cell proliferation. Tet-On cells were incubated at 37°C for 42-48 hours in the presence of 1000, 200, 100, 75, 50 or 0 ng/ml doxycycline then washed and fixed with glutaraldehyde. Cells were incubated with CTV-labelled naive CD4 T cells at a ratio of 1:2 (CHO:T cells) with 4, 1 or 0.5µg/ml anti-CD3 for five days then analysed by flow cytometry. Data are representative of three independent experiments. **(A)** Overlaid histograms show T cell proliferation (CTV fluorescence). **(B)** Graph shows the percentage of T cells that committed to divide. Each line shows T cell proliferation in response to a different concentration of anti-CD3 for each ligand. Boxed points indicate data referred to in the text. **(C)** Graphs show the number of costimulatory ligands per cell plotted as log(x) against T cell proliferation. Where the straight lines intercept the x axis, this represents the number of ligands required to initiate T cell proliferation.

found that reducing the number of cells expressing CD80 or CD86 reduced T cell proliferation and increased the number of costimulatory ligands required per cell to initiate a response (**figure 4.9A**). Therefore the number of costimulatory cells available affects the number of costimulatory molecules they are required to express. This suggests that T cells are sensitive to both the number of cells available to provide costimulation and the number of costimulatory ligands they possess. Based on this data it therefore seems possible that T cells can incorporate costimulatory signals from multiple APC. To explore this concept further, it was determined whether the total number of costimulatory ligands in the local environment was relevant to the T cell response. The total amount of ligand in the T cell culture was calculated by multiplying the average number of ligand molecules per cell by the number of cells in each well, and this value was plotted against T cell proliferation (**figure 4.9B**). Strikingly, the total amount of ligand required to initiate a response was the broadly similar whether there were a few cells expressing high levels of ligand or many cells expressing fewer ligand molecules, as shown by the response curves which largely overlapped at the start of the response even when different numbers of cells were used (**figure 4.9B-C**). This shows that even if there was only a low density of costimulatory ligands per cell, T cells can still become activated if there are enough costimulatory cells present. These data suggest that T cells may integrate signals through contact with a few cells expressing costimulatory ligands at high density or with many cells with lower ligand density. Intriguingly the maximum size of the response differed even if the same total amount of ligand was available. For example when ~$3.2x10^6$ CD80 molecules were available, ~32% of T cells entered division when there were few cells (CHO 1:8 T cell) expressing CD80 at a high density (**figure 4.9B**). In comparison only ~16% of T cells entered division when more cells were present (CHO 1:2 T cell) but expressing less CD80. This implies that ligand density has more impact on T cell proliferation than the number of ligand-expressing cells. In summary these data suggest that T cells may incorporate costimulatory signals from multiple

A



B



C

Total number of ligand molecules required to initiate T cell proliferation		
CHO:T cell ratio	**CD80**	**CD86**
1:2	$1.3x10^{10}$	$2.3x10^{10}$
1:4	$1.1x10^{10}$	$1.7x10^{10}$
1:8	$1.2x10^{10}$	-
Average	**$1.2x10^{10}$**	**$2x10^{10}$**

Figure 4.9 The number of cells providing costimulation is relevant to the T cell response. Tet-On cells were incubated at 37°C for 42-48 hours in the presence of 1000, 200, 100, 75, 50 or 0 ng/ml doxycycline then washed and fixed with glutaraldehyde. Cells were incubated with CTV-labelled naive CD4 T cells at a ratio of 1:2, 1:4 or 1:8 (CHO:T cells). The number of T cells was fixed at 100,000 per well and the number of CHO cells reduced accordingly. T cells were stimulated with 1µg/ml anti-CD3 for five days then analysed by flow cytometry. Data are representative of two independent experiments. **(A)** Graph shows the percentage of T cells that committed to divide. Each line shows the proliferation of T cells cultured with different numbers of CD80- or CD86-expressing cells. **(B)** Graphs show the percentage of T cells that committed to divide against the total number of costimulatory ligands in the well at each CHO:T cell ratio. Dashed lines indicate data points referred to in the text. **(C)** Table shows the total number of ligands required to initiate T cell proliferation interpolated from data in *(B)*.

APC to reach the threshold required for activation, although it appears to be more efficient for APC to express a higher density of costimulatory ligands.

4.5 CD80 and CD86 differentially control the T cell response

Although CD80 stimulated more T cells to commit to division, T cells that did respond divided further in the presence of CD86, meaning they had a greater proliferation index (**figure 4.10**). It is unclear whether this resulted from differences in the quality of CD28 signalling from CD80 and CD86 or from differences in the availability of space and nutrients due to the disparate numbers of expanding cells. To explore the possibility of qualitative differences between CD80 and CD86 engagement, the expression levels of surface proteins were measured on responding T cells at day five (**figure 4.11**). No differences were found in the expression levels of differentiation markers or costimulatory receptors between T cells receiving CD28 costimulation through CD80 engagement as opposed to CD86. This suggests that CD80 and CD86 do not produce qualitatively different signals. However it has previously been reported that CD80 and CD86 differentially control T cell lineage commitment (Odobasic et al., 2005; Puliti et al., 2010). Unfortunately reports on this topic are inconsistent, possibly due to variations in the ratio of CD80 and CD86 expressed and the type of antigen used. Therefore I took the simple approach of testing each ligand individually and used the inducible expression system to produce cells with similar levels of each ligand. To compare the two ligands, supernatants were collected from cultures where the sizes of the T cell responses were similar and cytokine concentration was measured correcting for any difference in CD80 and CD86 expression level. It was found that there were differences in cytokine production in response to CD80 versus CD86 (**figure 4.12**). Although not statistically significant, there was a trend for greater IL-2 production in response to CD80 compared to CD86, which is consistent with the concept that CD80 is the more potent ligand and could explain the greater number of T cells that committed to divide in other experiments. In addition, the T_H1-associated cytokine IFNγ was produced at higher levels in

response to CD80 while the T_H2-associated cytokine IL-13 was secreted more in response to CD86. These data support the possibility that the two ligands produce quantitatively or qualitatively different signals, specifically that CD86 preferentially directs or enhances a T_H2 response while CD80 produces a T_H1 response. Unfortunately not enough experiments were carried out to determine whether these results would reach statistical significance and no IL-4 or IL-5 production was detected in these assays. Overall, although further investigations are required, these data suggest that CD80 and CD86 may differentially regulate T cell phenotype.

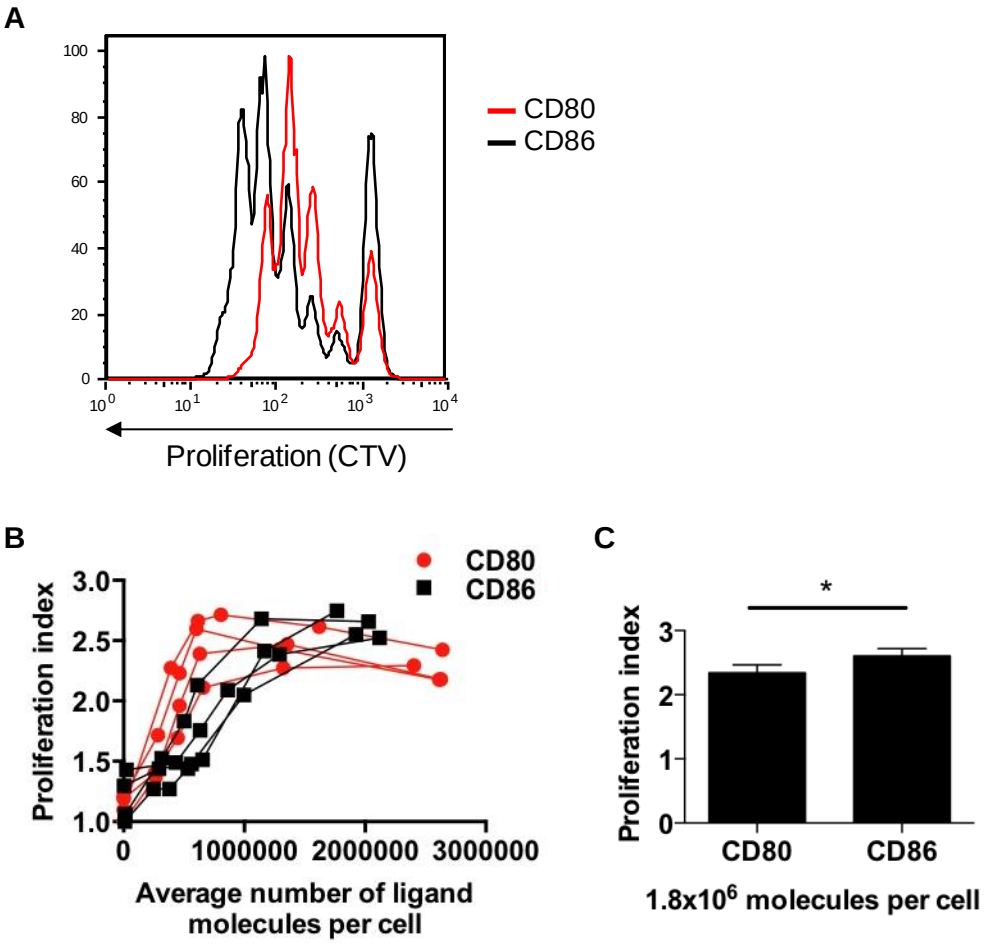


Figure 4.10 CD28 costimulation via CD86 causes T cells to divide further. Tet-On cells were incubated at 37°C for 42-48 hours in the presence of 1000, 200, 100, 75, 50 or 0 ng/ml doxycycline then washed and fixed with glutaraldehyde. Cells were incubated with CTV-labelled naive CD4 T cells at a ratio of 1:2 (CHO:T cells) with 1µg/ml anti-CD3 for five days then analysed by flow cytometry. **(A)** Histograms show T cell proliferation in the presence of an average of 2612785 CD80 molecules or 1767458 CD86 molecules. Data are representative of four independent experiments. **(B)** Graph shows the proliferation index (average number of divisions made by cells that entered cell cycle) of T cells cultured in the presence of cells expressing different numbers of costimulatory ligands. Each data point shows the average of triplicate samples. Each line shows one of four independent experiments. **(C)** Graph shows the mean (+SD) proliferation index of T cells stimulated in the presence of CHO cells expressing an average of $1.8x10^6$ ligand molecules per cell. The difference between CD80 and CD86 was statistically significant according to the t test *P<0.05.

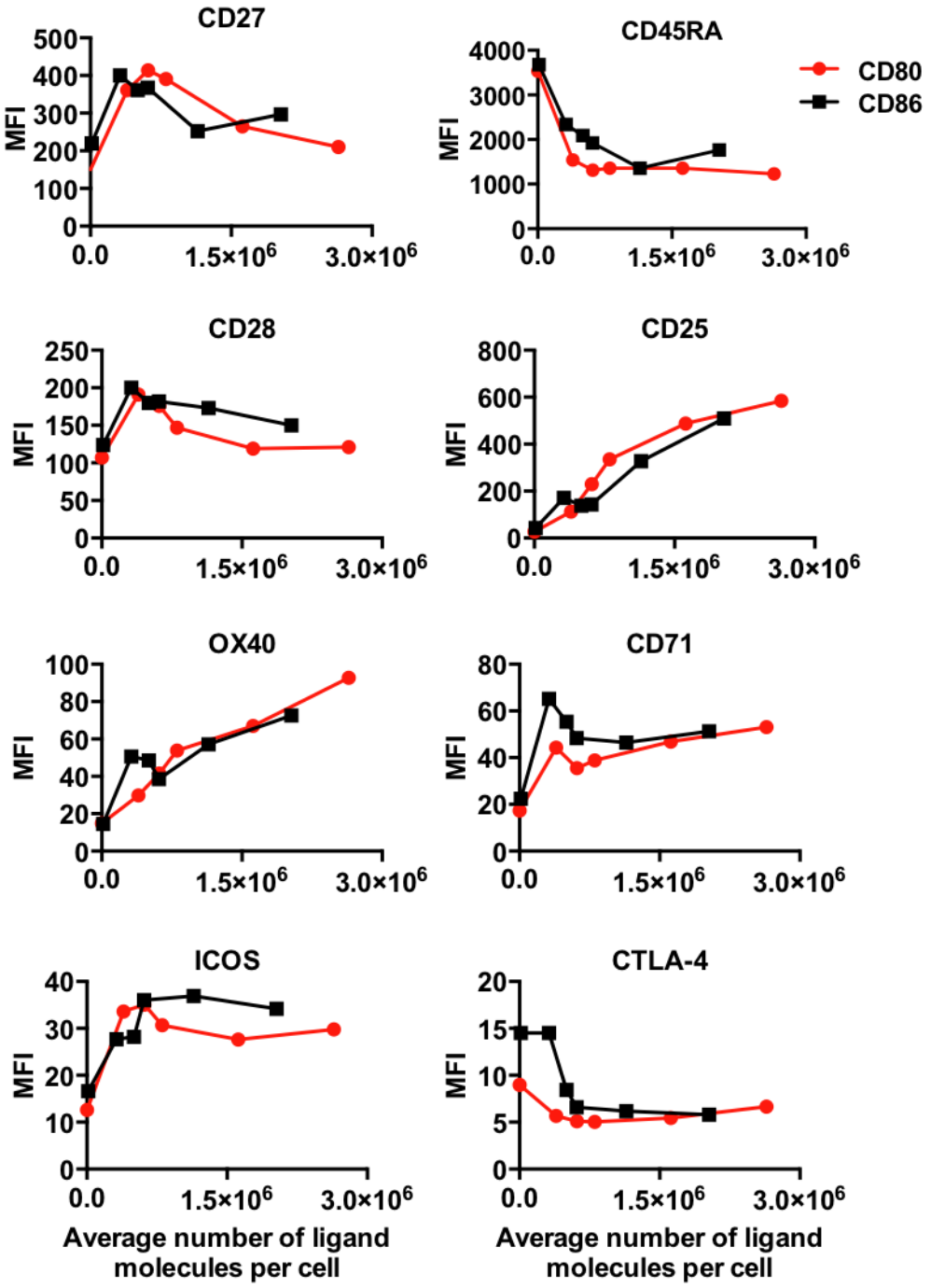


Figure 4.11 CD28 costimulation via CD80 and CD86 results in comparable protein expression profiles. Tet-On cells were incubated at 37°C for 42-48 hours in the presence of 1000, 200, 100, 75, 50 or 0 ng/ml doxycycline then washed and fixed with glutaraldehyde. Cells were incubated with CTV-labelled naive CD4 T cells at a ratio of 1:2 (CHO:T cells) with 1µg/ml anti-CD3 for five days. Cells were labelled for surface proteins then analysed by flow cytometry. Graphs show the MFI of surface markers expressed on proliferating T cells. Data are representative of three independent experiments.

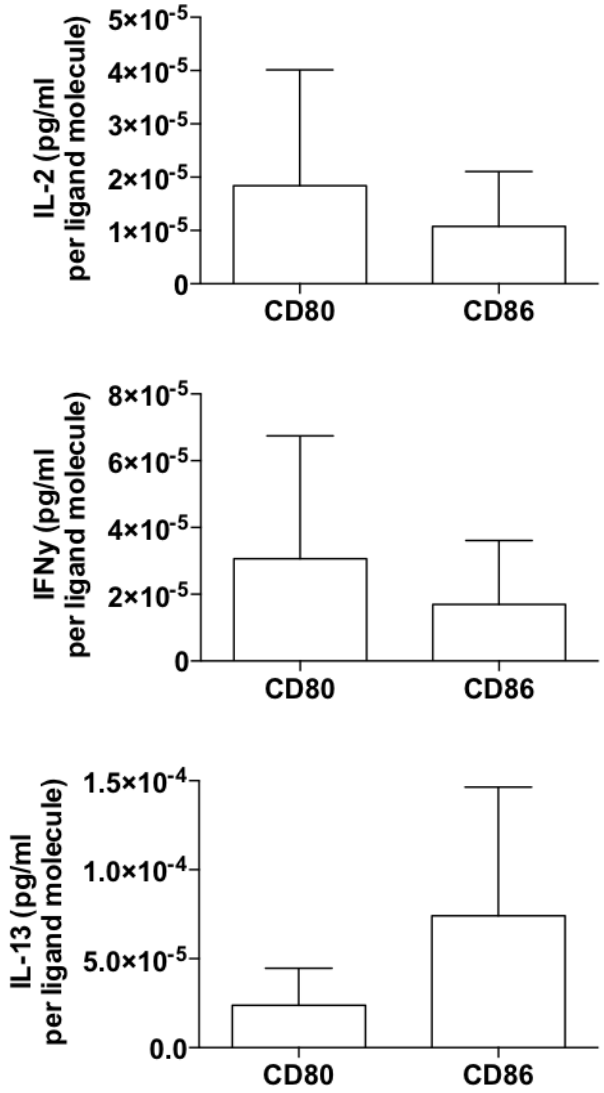


Figure 4.12 CD80 and CD86 may differentially direct T cell lineage commitment. Tet-On CD80 cells were incubated in the presence of 200ng/ml doxycycline and Tet-On CD86 cells with 1000ng/ml doxycycline, then cells were washed and fixed with glutaraldehyde. Cells were incubated with naive CD4 T cells at a ratio of 1:2 (CHO:T cells) with 1µg/ml anti-CD3 for five days. Culture supernatants were collected and cytokine concentration was analysed by multiplex bead immunoassay. Graphs show average (+SD) cytokine concentration divided by the average number of costimulatory ligands expressed per cell, determined from duplicate samples collected in three independent experiments. There were not enough replicate experiments for the differences between CD80 and CD86 to be statistically significant.

4.6 Discussion

4.6.1 Reducing the availability of costimulatory ligands incrementally reduces T cell proliferation

The main objective of the studies in this chapter was to determine the requirements of CD4 T cells for CD28 costimulation in order to better understand the implications of costimulatory ligand trans-endocytosis via CTLA-4. The discovery that CTLA-4 removes costimulatory ligands from APC to reduce their capacity to stimulate T cells generated the prediction that there is a threshold level of costimulation required for effective T cell activation. The studies presented here were designed to test this prediction. Using an inducible ligand expression system, the number of costimulatory molecules required to initiate T cell expansion was quantified and the wider impact of changing the availability of costimulatory ligands was investigated. As expected there was a threshold level of costimulation required to initiate a T cell response. The number of CD80 molecules required per cell to trigger T cell division was higher than previously reported (Chen et al., 2000) and higher than the levels normally expressed by APC (Jansson et al., 2005). This may have been due to the weak TCR stimulus used (soluble anti-CD3) and the lack of additional stimulatory signals normally provided by APC. Ultimately I did not attempt to express physiological levels of ligand, given that the cells were chemically fixed (making surface ligands immobile) and lacked adhesion molecules that normally promote T cell contact. Therefore the required number of ligand molecules per cell was likely to have been much greater than normal in this setting, in order to engage sufficient CD28 molecules within the area of intercellular contact. Nevertheless this experimental model could be used to observe the relative requirements for CD80 and CD86, and to examine the consequences of changing costimulatory ligand expression levels above the minimum threshold.

It was found that increasing the availability of costimulatory molecules increased the number of T cells that committed to division, showing that the T cell response is tunable rather than

"all or nothing". These results are supported by similar findings from other studies (Chen et al., 2000; Murtaza et al., 1999; Oderup et al., 2006). In addition, a study of the efficacy of CTLA-4-Ig showed that increasing the drug concentration gradually reduced T cell proliferation and at a certain concentration proliferation was completely inhibited, supporting the concept of a costimulation threshold and showing that partial inhibition or reducing costimulatory ligands is effective at altering T cell responses (Larsen et al., 2005). Also, simulations of synaptic accumulation of costimulatory molecules revealed that because the affinity of CD28 for its ligands is so low, small changes in ligand expression level may easily affect receptor-ligand complex formation and hence CD28 signalling (Jansson et al., 2005). In support of this, Tregs have been shown to downregulate CD80 and CD86 on DC in a CTLA-4-dependent manner which correlates with inhibition of effector T cell proliferation (Oderup et al., 2006; Onishi et al., 2008; Wing et al., 2008). It has also been shown that Treg-mediated downregulation of CD80/86 on DC can reduce IL-2 production and prevent effector T cell differentiation (Kastenmuller et al., 2011). Overall these findings indicate that even if trans-endocytosis via CTLA-4 cannot reduce ligand expression below an absolute threshold required to initiate T cell expansion, small reductions in ligand number may proportionally reduce the size of the T cell response. Therefore trans-endocytosis by CTLA-4 may be a valuable mechanism for fine-tuning a T cell response.

4.6.2 CD28 costimulation is required to initiate and maintain T cell proliferation

The importance of the timing of T cell regulation was also investigated. A recent study found that CD28 was required to sustain but not initiate T cell proliferation (Pagan et al., 2012). In contrast I found that CD28 costimulation was important for both the initiation and maintenance of T cell proliferation. In order to significantly impair T cell proliferation, costimulation had to be withheld for over eight hours. Therefore for CTLA-4-mediated trans-endocytosis of ligand to be an effective mechanism of T cell inhibition upon initial exposure to antigen, CTLA-4 would have to continually remove ligands for a sustained period of time at a

faster rate than they are upregulated by activated APC. Alternatively, CTLA-4 trans-endocytosis may only be relevant in non-inflammatory settings where CD80 and CD86 expression is low. This scenario is supported by studies showing that Treg-mediated suppression is more effective in settings where there are fewer costimulatory molecules (Ermann et al., 2001; George et al., 2003; Oderup et al., 2006). Therefore the removal of costimulatory ligands by trans-endocytosis can probably control autoimmune responses in the absence of infection, but will not have a detrimental impact on T cell responses to foreign pathogens.

Unfortunately autoimmunity still occurs in some cases, possibly because costimulatory molecules are highly expressed due to alternative stimuli. Indeed, autoimmunity is strongly associated with infection, which promotes APC activation. Activated APC express high levels of costimulatory molecules and create an inflammatory environment, which theoretically enables the activation of autoreactive T cells that otherwise would have been rendered anergic. For example type I diabetes has been linked to a patient history of respiratory infections and enterovirus infection (Beyerlein et al., 2013; Cubas-Duenas et al., 2013), and Epstein-Barr virus is a risk factor for rheumatoid arthritis (Croia et al., 2013). Alternatively, genetic polymorphisms affecting the CD28/CTLA-4 pathway are associated with autoimmunity (Ueda et al., 2003; Fernandez-Mestre et al., 2009; Li et al., 2012; Chen et al., 2013). Individuals that succumb to autoimmune disease may have insufficient or impaired CTLA-4, or may express high levels of costimulatory ligands that recover their expression rapidly. In summary, my data show that CD28 costimulation is required within a short time frame to initiate T cell proliferation. CTLA-4 may only be able to effectively control CD80 and CD86 expression and completely abrogate an unwanted response when the costimulatory ligand expression level is suboptimal.

Subsequent experiments showed that CD28 costimulation was also required to maintain proliferation because CD28 ligand blockade one day after T cell activation impaired T cell division. This implies that not only does the availability of CD28 costimulation determine the number of T cells that commit to divide but it also determines the maximum number of times those T cell will divide. Therefore costimulation blockade can control the size of the response even once it has started and contribute to the termination of the response. While CTLA-4 is constitutively expressed by Tregs, it is not expressed by naive T cells, therefore Tregs probably play the dominant role in preventing the instigation of unwanted responses. On the other hand, the upregulation of CTLA-4 on activated effector T cells following stimulation may be well timed to help terminate T cell proliferation in order to avoid a prolonged response that may lead to unwanted tissue damage. This concept is supported by a study showing that a lack of CTLA-4 on Tregs leads to inappropriate T cell activation and expansion, but that CTLA-4 expressed by effector T cells prevents these cells from infiltrating and damaging normal tissues (Jain et al., 2010). Furthermore, mice with CTLA-4-deficient Tregs but CTLA-4-sufficient conventional T cells survive longer than completely CTLA-4-deficient animals (Wing et al., 2008), consistent with a role for CTLA-4 on effector T cells in limiting responses. In addition, blocking CTLA-4 on both Tregs and effector T cells can synergistically enhance T cell responses (Peggs et al., 2009). Therefore CTLA-4 on both T cell subsets is functionally important and may function differently. One possibility is that CTLA-4 employs different mechanisms on each cell type (Wing et al., 2011). However recently, CTLA-4 has been shown to function extrinsically on effector T cells and Tregs alike (Wang et al., 2012; Corse and Allison, 2012). Therefore it may be the different times of CTLA-4 expression by these cells that dictates how they differentially mediate suppression and maintain tolerance. In summary, my data show that CD28 costimulation is required to maintain T cell proliferation, suggesting that ligand trans-endocytosis by CTLA-4 may be effective at all stages of the T cell response. Different T cells types may be responsible for regulating costimulation at different times.

4.6.3 Comparing the potency and function of CD80 and CD86

I also observed that in order to stimulate T cell activation and proliferation, a greater level of CD86 expression was required compared to CD80, which implies than CD80 is the more potent CD28 ligand. In addition, CD80 could activate a greater percentage of T cells and stimulate more T cells to enter division than CD86. Several *in vitro* studies agree that CD80 is the strongest ligand in terms of stimulating greater T cell proliferation and cytokine production (Fleischer et al., 1996; Fields et al., 1998; Olsson et al., 1998). This is not surprising considering the higher affinity CD28 has for CD80 and its higher valency (Collins et al., 2002), which may facilitate stronger CD28 signalling. In contrast *in vivo* studies suggest that CD86 is in fact the more dominant ligand. For example, one of the first studies of CD86-deficient mice showed they were unable to form germinal centres and their antibody production was diminished, whereas there was no marked effect in CD80-deficient mice (Borriello et al., 1997). In support of this, knocking out CD86 was more successful at reducing disease in a NOD mouse model than deleting CD80 (Girvin et al., 2000). Furthermore, a lack of CD86 alleviated allergic responses more significantly than loss of CD80 (Mark et al., 2000). One possibility is that CD86 may be the more stimulatory ligand *in vivo* because when both receptors and both ligands are present together, due to the varying affinities of all the possible interactions, CD86 engages more CD28 molecules than CTLA-4 while CD80 shows a bias towards CTLA-4, so in fact CD28 is more frequently engaged by CD86 rather than CD80 (Jansson et al., 2005; Pentcheva-Hoang et al., 2004; Collins et al., 2002). Another major factor in the differences seen between CD80 and CD86 knockout mice may be the higher expression of CD86 compared to CD80. CD86 is constitutively expressed by APC and its expression is highly upregulated upon activation, whereas CD80 is only expressed on mature APC and not as highly as CD86 (Lenschow et al., 1993). Indeed, a more recent study found that mucosal immune responses were more significantly inhibited in CD86-deficient mice compared to CD80-deficient, but it was observed that none of the

adjuvants stimulated very efficient CD80 upregulation, therefore CD86 may play a bigger costimulatory role simply because it is the main ligand expressed (Zhang et al., 2007). In summary, my experiments showed that CD80 is the more potent ligand because it stimulated more T cells to commit to divide, which in direct comparison with CD86 in a minimalist *in vitro* model, probably reflects their different affinities for CD28. However *in vivo* studies suggest that CD86 is the more dominant CD28 ligand, probably due to the complexities of different receptor-ligand affinities and ligand expression patterns. In the future, in order to replicate more physiological conditions, the inducible protein expression system could be used to express both ligands together at different ratios or at different times to explore the relative potencies of CD80 and CD86 further.

Another interesting observation was that although more CD86 was required to initiate a T cell response and less cells committed to divide, the responding cells divided further than with CD80 costimulation. This could suggest that the two ligands provide signals of different qualities. Alternatively, the greater number of expanding T cells stimulated with CD80 may have led to restricted space and nutrients and so inhibited the continuation of growth. Indeed it has been reported that a strong stimulus such as high antigen concentration can result in less proliferation (Murtaza et al., 1999; Cassell, 2001; Manzotti et al., 2006). In fact it has recently been shown that higher numbers of responding T cells inhibit expansion (Quiel et al., 2011). Not excluding this possibility, it is also feasible that T cell proliferation stimulated by CD80 was not maintained as long due to stronger inhibition by CTLA-4. When CTLA-4 is upregulated on activated T cells, perhaps CD80, which has a higher affinity for CTLA-4, is more rapidly captured by trans-endocytosis, which reduces CD28 costimulation and terminates the response. In contrast, although changing the level of CD86 appeared to have more impact on T cell expansion, CD86 is not as effectively captured by CTLA-4 (as shown in the previous chapter) so inhibition of CD86-mediated responses may be slower. We have previously reported similar results where more T cells committed to divide when costimulated

with CD80 compared to CD86 but they divided fewer times (Manzotti et al., 2006). The number of divisions with CD80 increased in the presence of anti-CTLA-4 blocking antibody, showing that although CD80 is the more potent costimulatory ligand, it is more effectively inhibited by CTLA-4, and therefore CD86 stimulates more efficient T cell proliferation. In summary, my studies have shown that CD80 stimulates more T cells to divide than CD86 but the responding T cells proliferate fewer times. Also, although T cell proliferation was more sensitive to changes in CD86 expression, CD80 was more efficiently captured by trans-endocytosis, therefore the outcomes of this regulatory mechanism are not straightforward. Understanding the CD28/CTLA-4 system is an ongoing challenge but continued research will hopefully allow us to manipulate it better in the treatment of disease.

Another unresolved debate is whether CD80 and CD86 produce qualitatively different signals via CD28. A large number of studies have found no functional difference between the two ligands (Fields et al., 1998; Schweitzer et al., 1997; Levine et al., 1995; Lanier et al., 1995; Natesan et al., 1996; Greenwald et al., 1997; Lespagnard et al., 1998; Vasilevko et al., 2002; Bhatt et al., 2013). However there is some evidence that CD86 may promote T_H2 responses while CD80 promotes T_H1 differentiation (Freeman et al., 1995; Kuchroo et al., 1995; Odobasic et al., 2005). Alternatively, CD86 may promote T_H2 responses while CD80 does not influence differentiation in a particular direction (Tao et al., 1997; Zhang et al., 2007). It is not clear how the two ligands could activate different signalling pathways because the binding sites for CD80 and CD86 on CD28 are highly overlapping, although they are not identical (Kariv et al., 1996). The MYPPPY motif is required for CD80 and CD86 binding but some other residues involved vary between the two ligands. The ligation of CD28 by both CD80 and CD86 leads to the recruitment of PI3K (Ghiotto-Ragueneau et al., 1996), suggesting they stimulate the same downstream pathways, yet a thorough investigation of the signalling events following CD80 versus CD86 ligation of CD28 is lacking. The data presented here seem to support the view that CD86 drives T_H2 differentiation while CD80

drives T_H1 development, because CD86 costimulation resulted in higher IL-13 production while CD80 costimulation caused greater IFNγ production. However the production of a wider range of cytokines needs to be analysed in order to fully define lineage commitment. I have only begun to address the question of why there are two CD28 ligands, but hopefully the ability to compare the two ligands at similar expression levels using the inducible expression system could soon allow us to resolve some of the issues discussed.

4.6.4 The sum of TCR and CD28 signalling combined controls T cell activation

Analysis of the costimulatory requirements of T cells stimulated with different doses of anti-CD3 revealed that TCR and CD28 stimulation seem to compensate for one another, suggesting that these two signals are additive or synergistic. Others have made similar observations that low doses of TCR antigen (or anti-CD3) with high CD28 ligand availability and vice versa can stimulate the same amount of T cell proliferation, showing that the combined sum of TCR and CD28 signalling events determines the T cell outcome (Murtaza et al., 1999; Chen et al., 2000). Over the years much work has addressed the question of whether the TCR and CD28 have shared or overlapping signalling pathways, or whether they activate distinct pathways. Many studies have shown that CD28 costimulation enhances TCR signalling by increasing or sustaining the phosphorylation of substrates involved in early TCR signalling events, such as ZAP70 (Tuosto and Acuto, 1998), Vav-1 (Salojin et al., 1999; Dennehy et al., 2007), Lck (Holdorf et al., 2002) and PLCγ (Michel et al., 2001). These studies indicate that TCR and CD28 signalling are integrated and that CD28 provides a quantitative signal to amplify or sustain the TCR signal for longer. Consistent with this, microarray studies have shown that CD28 predominantly amplifies the TCR-mediated transcriptional response, although CD28 does mediate some distinct transcriptional changes (Diehn et al., 2002; Riley et al., 2002). A more recent study made similar observations but in addition found that CD28 controls many more genes than TCR signalling alone by regulating alternative splicing of mRNA rather than gene transcription (Butte et al., 2012). CD28 may

mediate alternative splicing via the global regulator hnRNPLL, whose expression is CD28-dependent. Altogether there is considerable evidence that CD28 shares downstream activation pathways with the TCR and therefore enhances the TCR signal.

However there also appear to be many non-redundant roles of CD28. Early reports demonstrated that some outcomes of CD28 costimulation were resistant to calcineurin inhibitors, suggesting that CD28 activates signalling pathways distinct from calcium-dependent TCR signals (Ghosh et al., 1996; Geginat et al., 2000). More recently it has been shown that both the TCR and CD28 are required for optimal PKCθ function. PKCθ is initially recruited to TCR-CD28 microclusters within the immune synapse then CD28 and PKCθ co-localise in distinct regions separate from TCR (Yokosuka et al., 2008). The TCR and CD28 therefore work cooperatively to recruit PKCθ, but CD28 does not simply increase PKCθ activation, CD28 is essential for retaining PKCθ in the immune synapse and CD28-PKCθ interactions are critical for PKCθ-mediated activation of NFκB (Sanchez-Lockhart et al., 2008; Isakov and Altman, 2012). This indicates that some outcomes of T cell activation are dependent on CD28 and are not just augmented by costimulation, especially the upregulation of survival factors via NFκB (Kerstan and Hunig, 2004; Takeda et al., 2008; Tuosto, 2011). It is therefore becoming apparent that even though there are many shared signalling mediators downstream of the TCR and CD28, they still each produce unique signals and outcomes (Muscolini et al., 2013). One possibility is that TCR and CD28 signalling activate different subunits of the same transcription factor which each control different genes (Marinari et al., 2004). In summary, my data and that of others suggest that TCR and CD28 signalling are integrated so CD28 costimulation augments the T cell response to antigen and therefore an increase in either signal reduces the requirement for the other. Therefore T cells may commit to divide once a combined threshold of TCR and CD28 activation has been reached, but differences in the relative contributions of TCR and CD28 may lead to differences in survival or differentiation. For example it has been shown

that only weak TCR stimulation with strong CD28 stimulation is sufficient for a T_H2 response and that strong TCR with weak CD28 stimulation does not allow T_H2 differentiation (Tao et al., 1997). These findings are supported by a more recent report showing that CD3 stimulation skews towards a T_H1 response while CD28 stimulation skews towards T_H2 differentiation (Smeets et al., 2012). This question regarding the contributions of CD28 to T cell differentiation and effector function will be explored in the next chapter.

4.6.5 T cells seem to integrate signals from multiple APC

My data also pointed towards the possibility that T cells can integrate costimulatory signals from multiple APC, as increasing the number of costimulatory cells increased the percentage of T cells that committed to divide. Although this may simply have been because more T cells were likely to make a successful contact with a costimulatory cell when more were available. Indeed, even if relatively high levels of costimulatory molecules were expressed per cell, if there was a low CHO:T cell ratio, there was no T cell proliferation. However, low levels of costimulatory molecules per cell were sufficient to initiate T cell proliferation if there were enough costimulatory cells present. This suggested that T cells may be able to assimilate costimulatory signals from multiple cells. If each T cell is only required to interact with one costimulatory cell, then the initiation of T cell proliferation would always require the same threshold number of costimulatory molecules per cell. However there was not a fixed number of costimulatory molecules required per cell for T cell activation. If more costimulatory cells were present they could express less costimulatory ligands, implying that T cells do not require a certain number of CD28 receptors to be engaged at once for T cell activation, which supports the possibility that costimulatory signals can be accumulated through interactions with multiple cells.

The simplistic view that T cell activation occurs as the result of one productive interaction between a T cell and an APC has been challenged in recent years. The highly organised

immune synapse that forms between a T cell and APC was initially believed to sustain TCR signalling and it was thought that this was necessary for full T cell activation (Monks et al., 1998). However this is now known not to be the case and the immune synapse may in fact be involved in downregulating the TCR (Alarcon et al., 2011). Interestingly it has been found that “tonic” TCR signalling through frequent weak interactions with self-peptides maintains T cells in a partially activated state that is necessary in order for them to be effectively activated upon encountering foreign antigen (Stefanova et al., 2002; Hochweller et al., 2010). This suggests that interactions with multiple cells contribute to T cell activation. Maybe costimulatory pathways are also “primed” in this way or can contribute to tonic TCR signalling (Garbi and Kreutzberg, 2012). Others have shown that T cell priming consists of three separate phases: 1) T cells experience multiple short-lived interactions with APC, progressively reduce their motility and upregulate activation markers; 2) T cells form prolonged stable interactions with APC and begin to secrete cytokines; 3) T cells resume migration and continue to make brief contacts with APC (Mempel et al., 2004). Importantly the initial transient T cell-APC interactions observed by Mempel and colleagues were productive, as shown by the upregulation of CD69, which suggests that short serial receptor engagements contribute to T cell activation. Another group also described distinct stages of T cell activation where CD69 was upregulated during initial transient T cell-APC interactions (Miller et al., 2004). Consistent with this, others have shown that repeated transient T cell-APC interactions are sufficient to induce calcium signalling and the upregulation of activation markers (Gunzer et al., 2000). Additionally, another study found that repeated interruption of T cell signalling then reforming of the immune synapse did not prevent T cell activation, and what’s more the total signalling time correlated with the level of cytokine production, suggesting that intermittent signalling can be summated (Faroudi et al., 2003). Together these data suggest that T cells may integrate signals from serial interactions with multiple APC (Bousso, 2008).

Similar to my findings regarding costimulatory molecules, it has been found that T cell activation is dependent on the total amount of antigen present, which indicates that T cells can sense both antigen density per cell and the number of APC (Henrickson et al., 2008). When DC presented antigen at high density, T cells formed stable contacts with them relatively quickly and became activated, but at low antigen density T cells made short successive contacts for a longer period of time before they eventually formed a stable contact and were fully activated. This provides further evidence that T cells can integrate TCR signals from multiple APC and my data suggest that costimulatory signals may also be summated in a similar way. A recent study has suggested that T cells may be able to integrate signals from serial interactions by retaining the transcription factor NFAT in the nucleus where it remains transcriptionally active between transient interactions with APC (Marangoni et al., 2013). In summary, there is increasing evidence that T cells can integrate signals from serial interactions with different APC. My data support this concept and suggest that this includes the accumulation of CD28 signals. This model of T cell activation may explain how autoreactive T cells can become activated by low levels of antigen and costimulatory molecules.

4.6.6 Summary

To conclude, the studies in this chapter have shown that small changes in the number of costimulatory ligands have a noticeable effect on T cell proliferation, suggesting that trans-endocytosis via CTLA-4 may be able to fine tune a T cell response. However CTLA-4-ligand trans-endocytosis may only be able to completely prevent T cell activation in non-inflammatory conditions, such as in cases of auto-reactivity. These studies also showed there were different costimulation requirements depending on the strength of the TCR stimulus. The TCR signal from an autoantigen will be weak, so more CD28 costimulation will be required. Therefore again the data suggest that limiting the availability of costimulatory molecules would have more impact on an autoimmune response. The finding that the

combined levels of TCR and CD28 stimulation control T cell proliferation supports the model that these two signals are integrated. Finally, T cells appear to be able to integrate signals from different APC, which may allow them to respond to lower levels of antigen and costimulation in some settings.

5 STUDIES ON THE OUTCOMES OF LIMITING CD28 COSTIMULATION

5.1 CD28 costimulation is not always required for T cell activation and proliferation

Previous research has suggested that CD4 T cells have an absolute requirement for CD28 costimulation and that anergy results from a lack of CD28 engagement. However CD28 independent T cell responses do occur in CD28 knockout mice and in certain conditions, such as in the presence of strong TCR signalling. In order to investigate the outcomes of restricting the provision of CD28 costimulation, a simple experimental approach was used where CD28 ligands were either present or absent. Three independent but related experimental conditions were tested: 1) CHO cells expressing the Fcγ receptor II (FcR) were used to cross-link anti-CD3; 2) CHO cells co-expressing FcR and CD80 provided both cross-linked TCR and CD28 costimulation; 3) soluble anti-CD3 was used with costimulation provided by CD80 CHO cells. The availability of CD80 was therefore restricted by using CHO cells lacking CD80 expression or by the addition of the blocking antibody CTLA-4-Ig to the T cell culture. Both of these methods for preventing CD28 costimulation were routinely used in parallel to verify observations: FcR CD80 CHO cells were used with or without CTLA-4-Ig to eliminate effects caused by variation in protein expression between different cells lines, whilst FcR CHO cells were also used to avoid the possibility that CTLA-4-Ig may not effectively block all CD80 molecules. The above systems were used to analyse the proliferation of T cells stimulated in the presence or absence of CD28 costimulation (**figure 5.1**). When CD80 CHO cells lacking FcR were cultured with T cells in the presence of soluble anti-CD3 to provide a weak TCR stimulus, up to 50% of the T cells entered division. However T cell proliferation was completely abrogated when CTLA-4-Ig was added to block CD28 costimulation. In contrast, a substantial proportion of T cells committed to divide in both the presence and absence of CD28 costimulation when FcR-expressing CHO cells were used, demonstrating that the level of TCR signalling dictates the requirements for CD28 costimulation. Although T cell proliferation was robust without CD28 costimulation in the

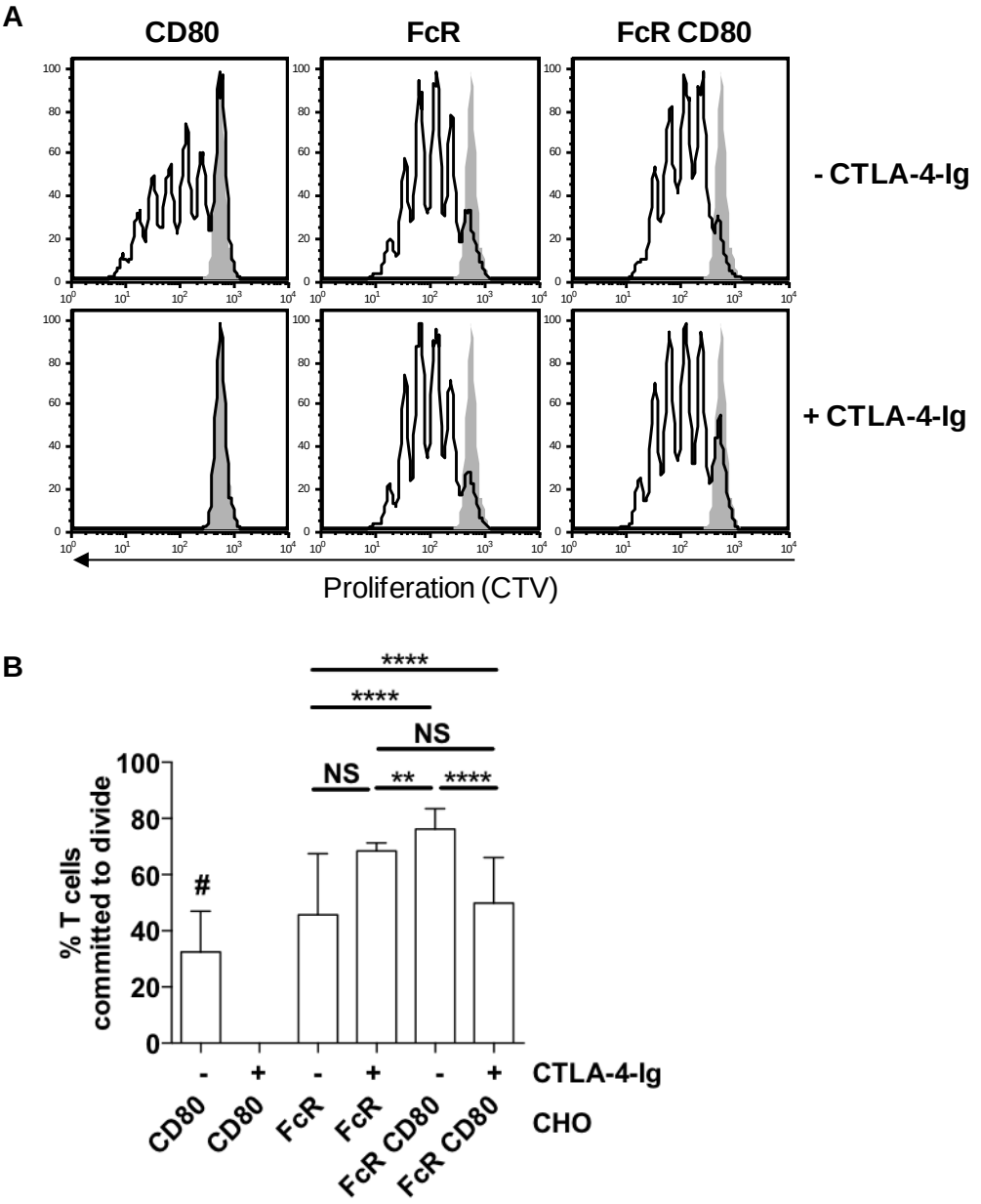


Figure 5.1 CD28 costimulation is not required for T cell proliferation. CTV-labelled $CD4^{+}CD25^{-}$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing CD80, FcR or FcR CD80 +/-20µg/ml CTLA-4-Ig for five days then proliferation was analysed by flow cytometry. **(A)** Histograms show dilution of cell trace violet as cells proliferate. Grey peaks represent unstimulated T cells. Data are representative of 21 independent experiments except FcR +CTLA-4-Ig where n=3. **(B)** Graph shows the mean percentage (+SD) of T cells that committed to divide using data from up to 21 independent experiments except FcR +CTLA-4-Ig where n=3. Statistically significant differences between data were determined by the Wilcoxon test **P<0.01 ****P<0.0001 NS=not significant.
T cell proliferation in the presence of CD80 CHO was significantly different to that in the presence of FcR –CTLA-4-Ig and FcR CD80+/-CTLA-4-Ig (P<0.0001) but NS different to FcR +CTLA-4-Ig (not shown).

context of strong TCR signalling, CD28 dependency was nonetheless variable between individuals. This implies that costimulatory requirements may vary between individuals however these results showed that CD28 costimulation was not always required to initiate a proliferative T cell response.

It has been reported that activated T cells can also express CD28 ligands (Sansom and Hall, 1993; Wyss-Coray et al., 1993; Azuma et al., 1993b; Hirokawa et al., 1995; Podojil and Miller, 2009; Radziewicz et al., 2010; Rudulier et al., 2014), therefore T cells were analysed for CD80 and CD86 expression to see whether T cells themselves can provide a source of CD28 costimulation in these studies. Interestingly, it was observed that CD80 and CD86 were expressed on T cells in a CD28 dependent manner (**figure 5.2**). In order to eliminate the possibility that CD80 or CD86 expression on T cells was providing CD28 costimulation in "costimulation deficient" conditions, CTLA-4-Ig was added with FcR CHO cells. T cell proliferation was unaffected (**figure 5.1A**), suggesting that no effective CD28 costimulation was provided by T cell expression of CD28 ligands.

T cell responses were also measured by the upregulation of early activation markers (**figure 5.3**). Similarly to T cell proliferation, CD25 and CD69 upregulation were CD28-independent. T cells upregulated CD25 and CD69 to similar levels when cultured with FcR CHO cells regardless of whether CD28 costimulation was available. On the other hand, optimal CD40L expression required both strong TCR and CD28 signalling. This demonstrates that although CD28 signalling is not required for T cell activation and proliferation, CD28 costimulation is important for other functions.

5.2 CD28 costimulation is required for T cells to acquire an effector phenotype

Having found that T cell activation and proliferation were largely CD28-independent, it was tested whether CD28 is required for the differentiation of T cells. It was found that although

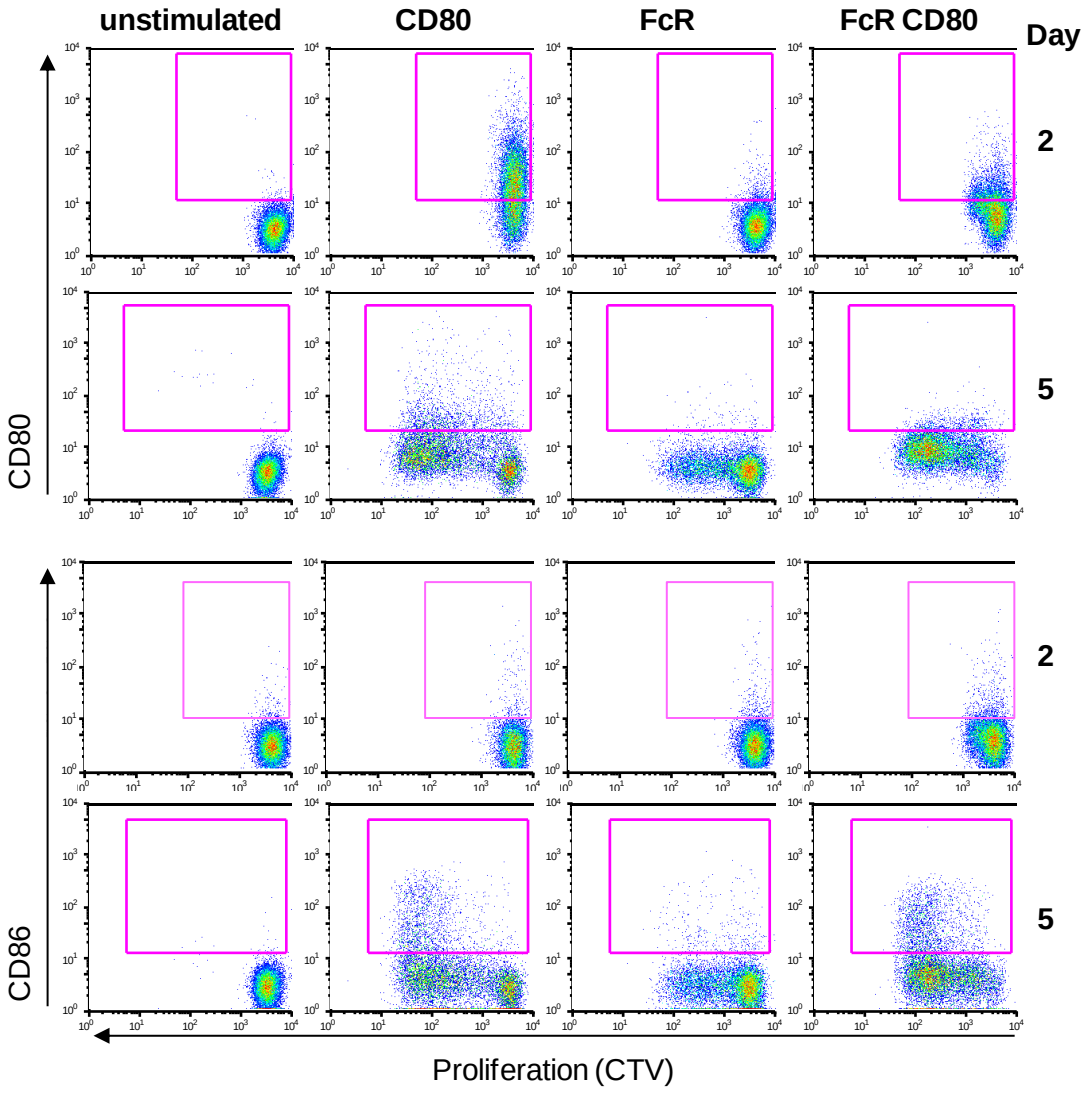


Figure 5.2 CD80 and CD86 are expressed on T cells activated in the presence of CD28 costimulation. CTV-labelled $CD4^{+}CD25^{-}$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing CD80, FcR or FcR CD80 +/-20µg/ml CTLA-4-Ig for two or five days then stained for surface protein expression and analysed by flow cytometry. Plots show CTV fluorescence against CD80 or CD86 expression. $CD80/86^{+}$ cells are gated. Data are representative of five independent experiments.

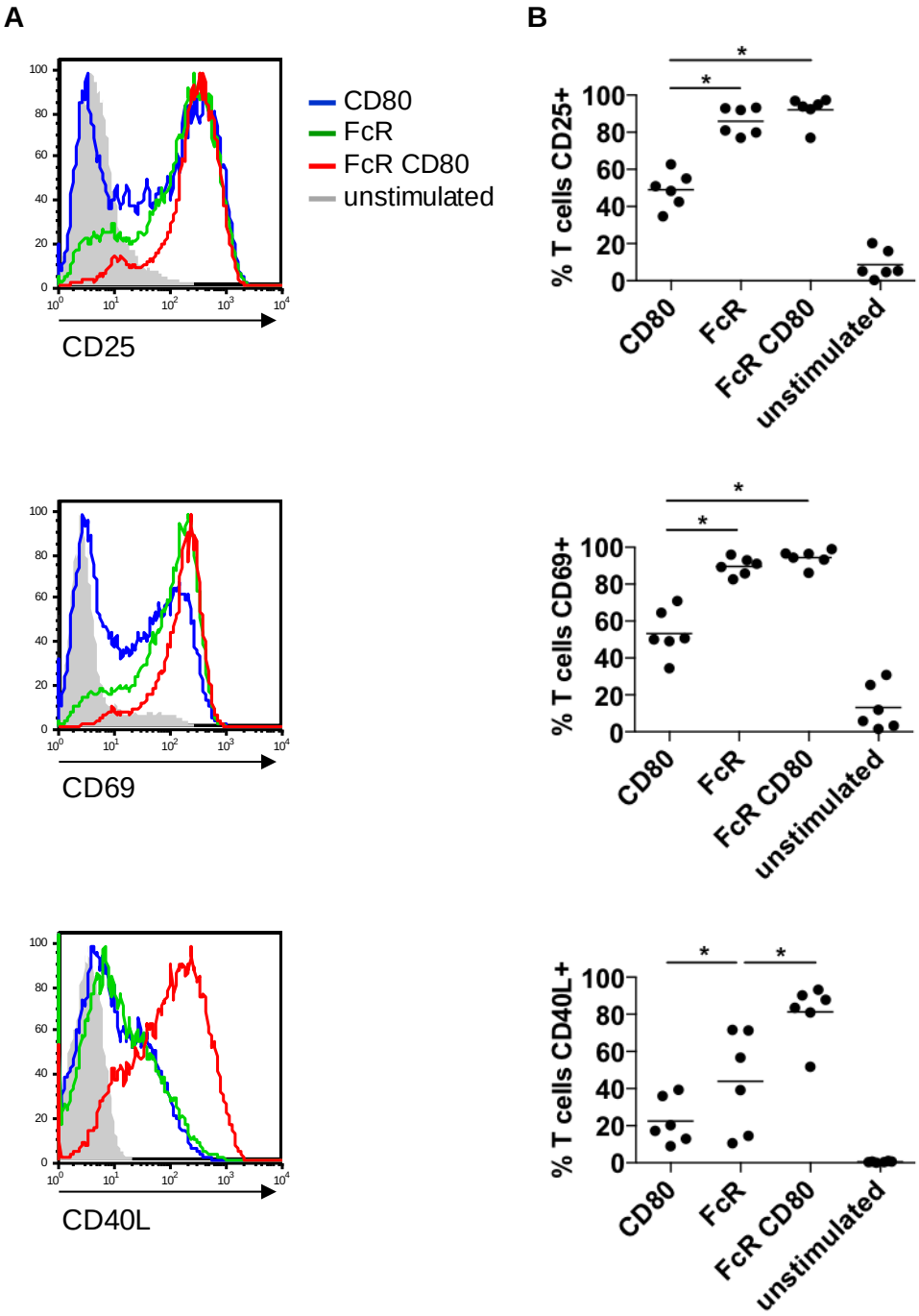


Figure 5.3 Upregulation of early activation markers CD25 and CD69 is CD28 independent but maximal CD40L expression requires CD28 costimulation. $CD4^+CD25^-$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing CD80, FcR or FcR CD80 for two days then surface proteins were stained with fluorescently-labelled antibodies and analysed by flow cytometry. **(A)** Histograms show CD25, CD69 and CD40L expression for each condition overlaid. Data are representative of six independent experiments. **(B)** Graphs show the percentage of T cells expressing each activation marker. Data from six independent experiments are shown. Horizontal bars represent the mean of six experiments. Statistically significant differences between data were determined by the Wilcoxon test *P<0.05.

all responding cells upregulated CD45RO independently of CD28 costimulation, CD45RA expression was less effectively downregulated in the absence of CD28 costimulation (**figure 5.4**). Other naive T cell markers including CD27, CCR7 and CD62L also failed to be downregulated without CD28 costimulation (**figure 5.5**), suggesting that CD28 costimulation is required to promote the phenotypic changes associated with effector T cells.

Next the expression of important effector proteins on responding T cells was analysed, such as the upregulation of costimulatory receptors ICOS and OX40 (**figure 5.6 and 5.7**). The upregulation of these effector proteins was largely CD28-dependent, and CD28 costimulation maintained their expression at higher levels throughout cell division. Similarly, the expression of CTLA-4 at the cell surface required CD28 costimulation (**figure 5.8**). This expression was likely due to both *de novo* synthesis and increased protein trafficking because the total amount of CTLA-4 and the amount in the cycling pool were also CD28 dependent (**figure 5.8B-C**). Together these data show that CD28 costimulation is important for the upregulation of secondary costimulatory receptors and the expression of the regulatory receptor CTLA-4.

Expression of the transferrin receptor CD71 was also analysed as a measure of the capacity for iron uptake and therefore the metabolic state of the T cells. It was found that the level of CD71 expression was significantly impaired in the absence of CD28 costimulation (**figure 5.9**). This restricted the amount of transferrin that was obtained by T cells cultured in the absence of CD28. This was not surprising because CD71 expression is documented to be controlled by CD28 signalling via mTOR (Edinger and Thompson, 2002). CD28 signalling and the downstream activation of mTOR are also reported to be required for the upregulation of other nutrient transporters such as GLUT1 (Frauwirth et al., 2002; Jacobs et al., 2008). A loss of CD28-mediated mTOR activation and the consequential impact on iron and glucose uptake may explain the impaired ability of T cells to upregulate the expression of other proteins such as those described above. To assess the contribution of the mTOR pathway in

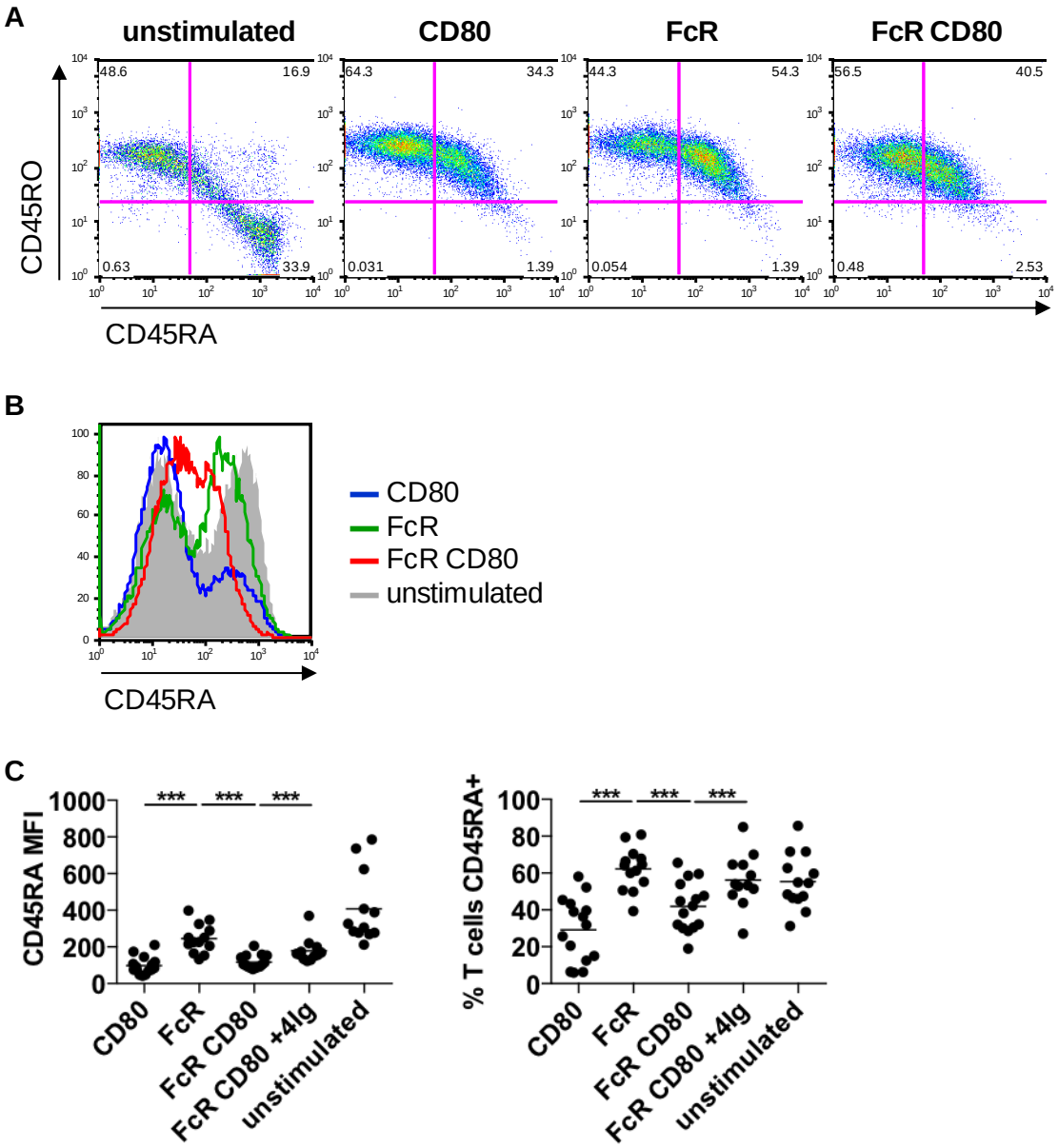


Figure 5.4 T cells stimulated in the absence of CD28 costimulation upregulate CD45RO but do not downregulate CD45RA. CTV-labelled $CD4^{+}CD25^{-}$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing CD80, FcR or FcR CD80 +/- 20µg/ml CTLA-4-Ig for five days then stained for CD45 expression and analysed by flow cytometry. **(A)** Plots show CD45RA expression against CD45RO expression of responder T cells only or all unstimulated T cells. The percentage of cells in each quadrant is shown. Data are representative of seven independent experiments. **(B)** Overlaid histograms show CD45RA expression for each condition. Data are representative of 11 independent experiments. **(C)** Graphs show the CD45RA MFI of responding T cells only or all unstimulated T cells (left) and the percentage of responder or unstimulated T cells expressing CD45RA (right). Data from 11 independent experiments are shown. Horizontal bars represent the mean of 11 experiments. Statistically significant differences between data were determined by the Wilcoxon test ***P<0.001.

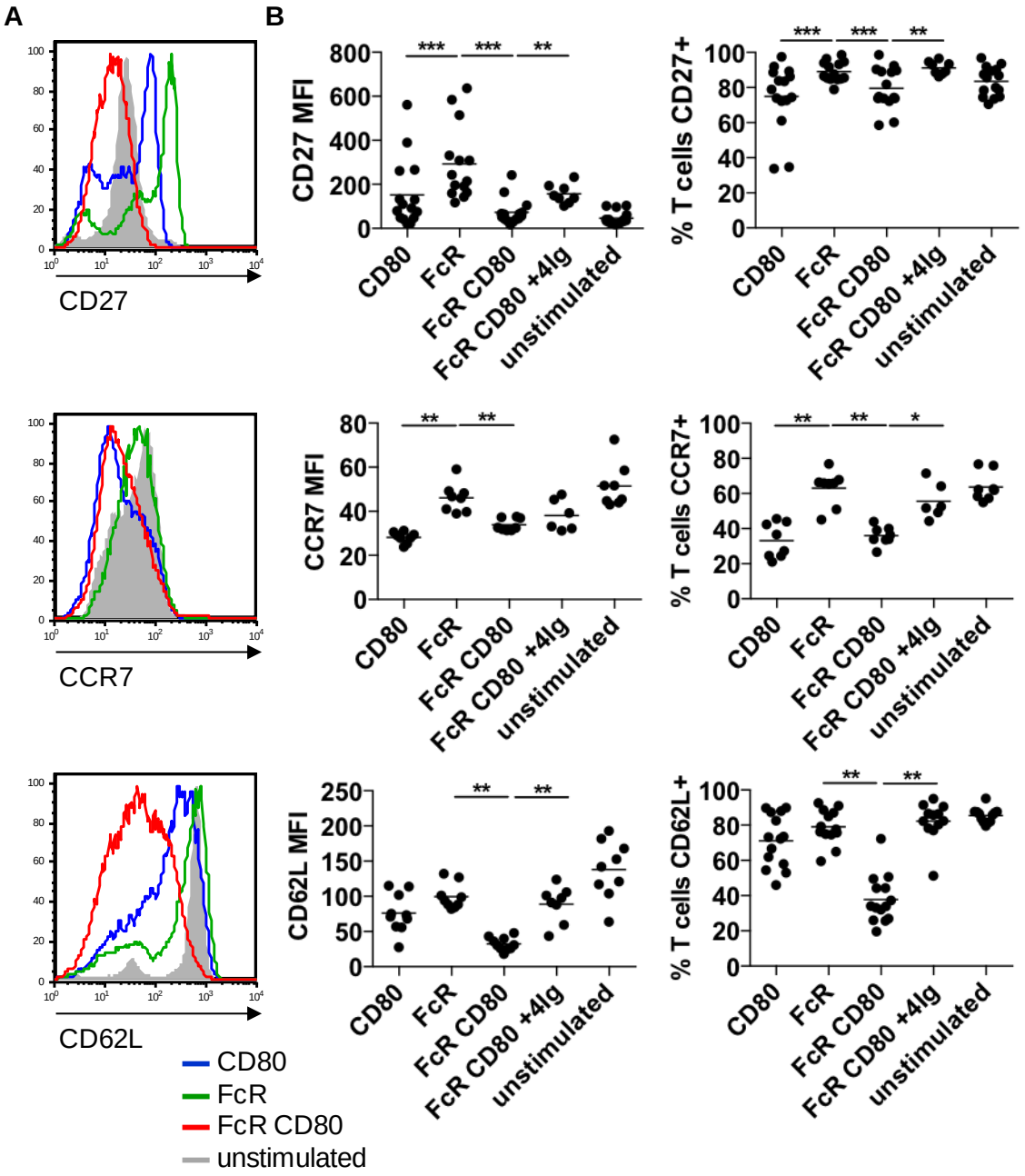


Figure 5.5 T cells stimulated in the absence of CD28 costimulation do not downregulate naive T cell markers. CTV-labelled $CD4^{+}CD25^{-}$ T cells were stimulated for five days then stained for surface protein expression and analysed by flow cytometry. **(A)** Overlaid histograms show CD27, CCR7 and CD62L expression for each condition. Data are representative of 13, 4 and 12 independent experiments respectively. **(B)** Graphs show the MFI of responding T cells only or all unstimulated T cells (left) and the percentage of responder or unstimulated T cells expressing the protein (right). Data from 4-13 independent experiments are shown. Horizontal bars represent the mean of all experiments. Statistically significant differences between data were determined by the Wilcoxon test *P<0.05; **P<0.01; ***P<0.001.

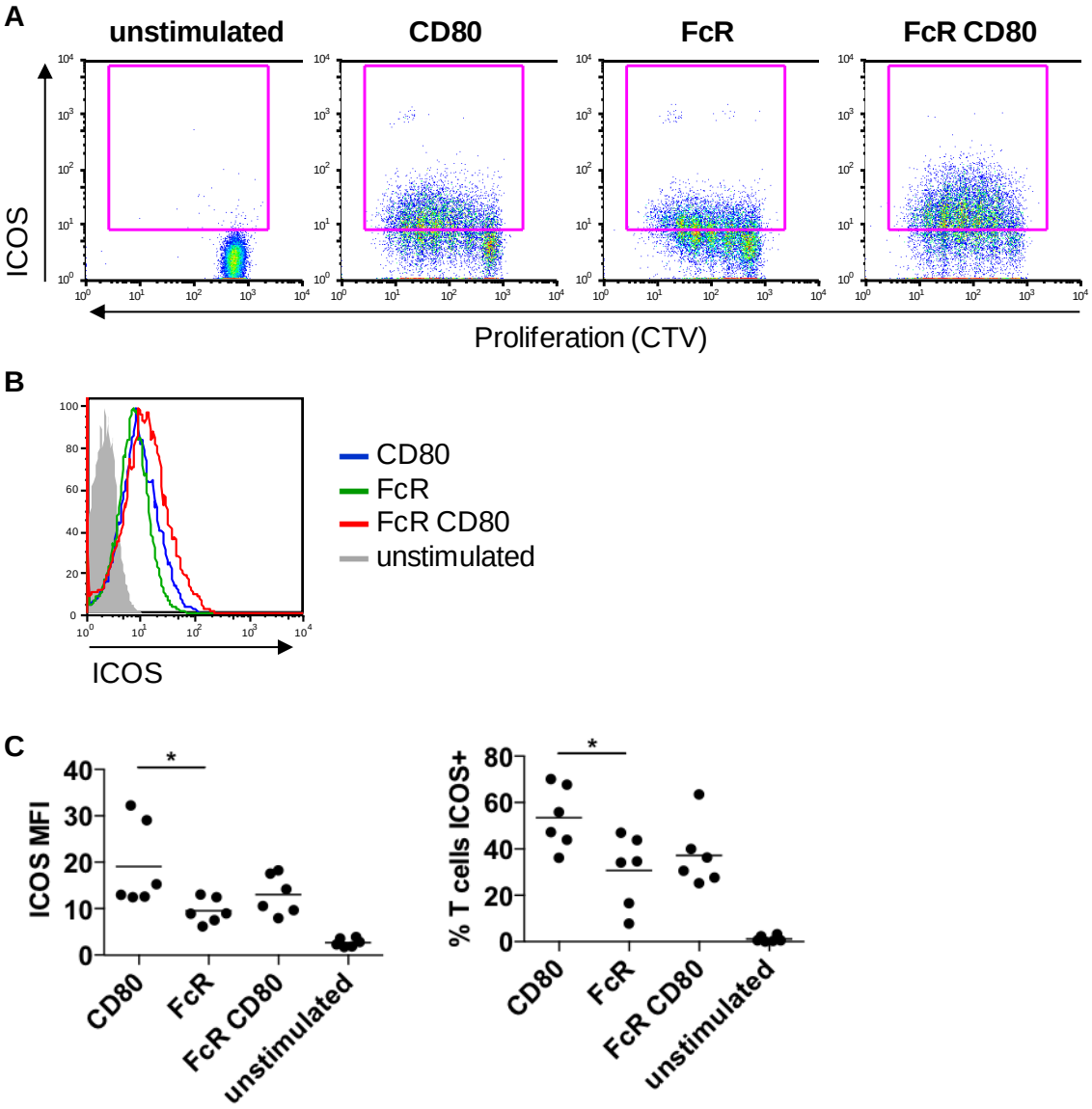


Figure 5.6 ICOS upregulation is impaired in the absence of CD28 costimulation. CTV-labelled $CD4^{+}CD25^{-}$ T cells were stimulated for five days then stained for ICOS expression and analysed by flow cytometry. **(A)** Plots show CTV fluorescence against ICOS expression. $ICOS^{+}$ cells are gated. Data are representative of six independent experiments. **(B)** Overlaid histograms show ICOS expression for each condition. Data are representative of six independent experiments. **(C)** Graphs show the ICOS MFI of responding T cells only or all unstimulated T cells (left) and the percentage of responder or unstimulated T cells expressing ICOS (right). Data from six independent experiments are shown. Horizontal bars represent the mean of all experiments. Statistically significant differences between data were determined by the Wilcoxon test *P<0.05.

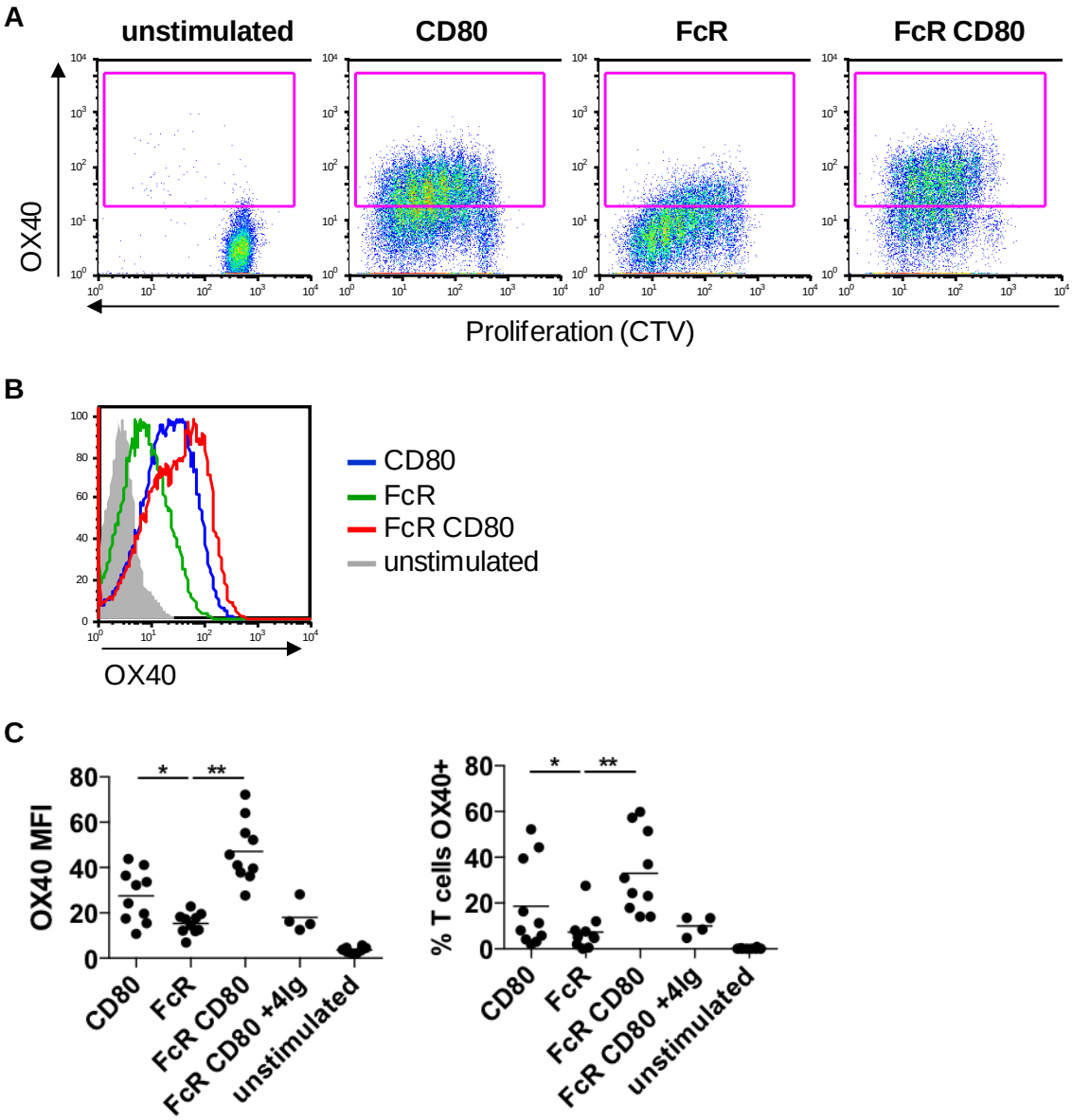


Figure 5.7 OX40 expression is CD28 dependent. CTV-labelled $CD4^{+}CD25^{-}$ T cells were stimulated for five days then stained for OX40 expression and analysed by flow cytometry. **(A)** Plots show CTV fluorescence against OX40 expression. $OX40^{+}$ cells are gated. Data are representative of nine independent experiments. **(B)** Overlaid histograms show OX40 expression for each condition. Data are representative of nine independent experiments. **(C)** Graphs show the OX40 MFI of responding T cells only or all unstimulated T cells (left) and the percentage of responder or unstimulated T cells expressing OX40 (right). Data from nine independent experiments are shown. Horizontal bars represent the mean of all experiments. Statistically significant differences between data were determined by the Wilcoxon test *P<0.05; **P<0.01.

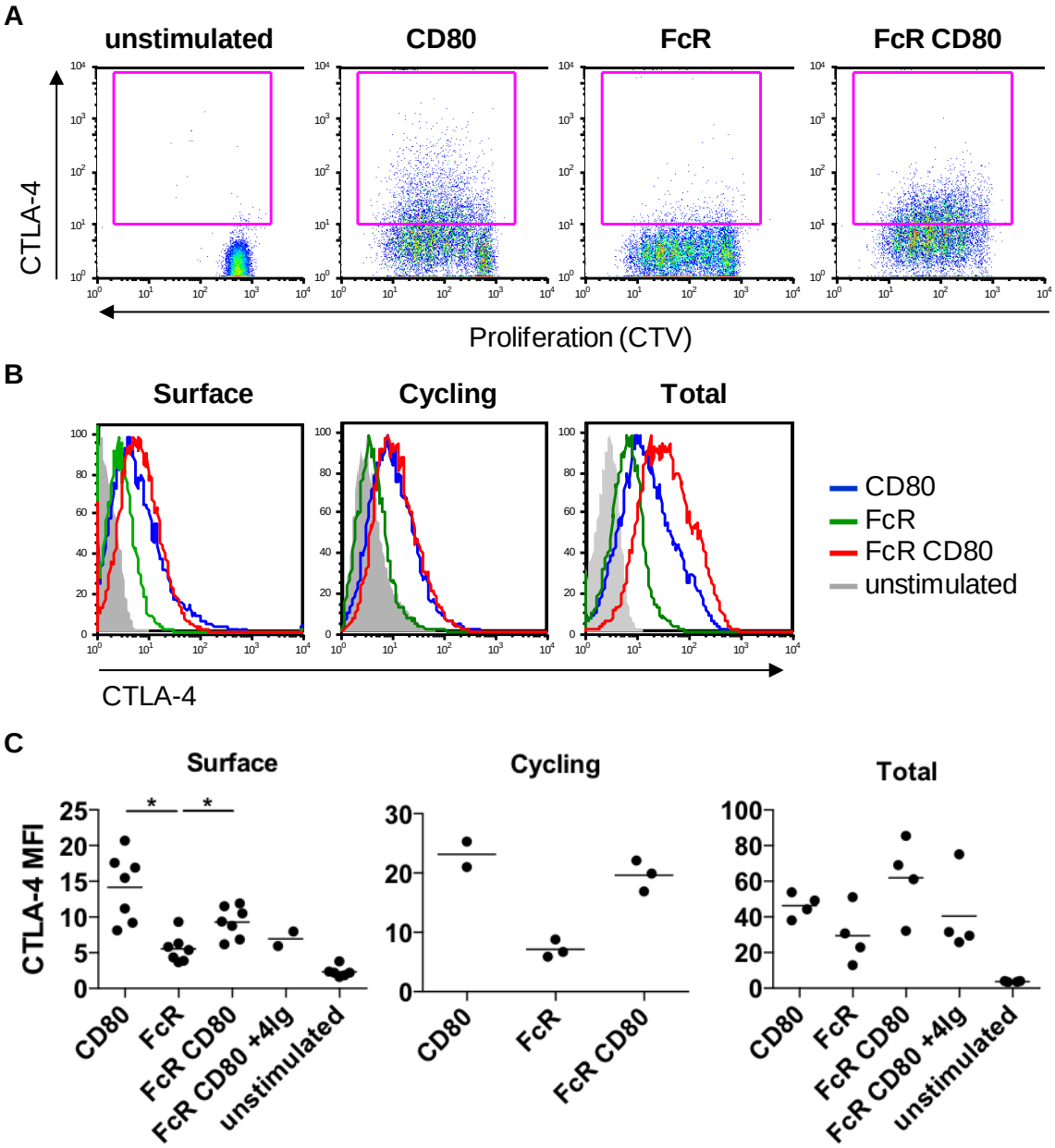


Figure 5.8 CTLA-4 expression is CD28 dependent. CTV-labelled $CD4^{+}CD25^{-}$ T cells were stimulated for five days. Cells were stained for CTLA-4 expression (surface at 4°C; cycling at 37°C; cell fixation and permeabilisation for total stain) and analysed by flow cytometry. **(A)** Plots show CTV fluorescence against CTLA-4 expression at the cell surface. $CTLA\text{-}4^{+}$ cells are gated. Data are representative of seven independent experiments. **(B)** Overlaid histograms show surface, cycling and total CTLA-4 expression. Data are representative of seven, three and four independent experiments respectively. **(C)** Graphs show the CTLA-4 MFI of responding T cells only or all unstimulated T cells. Data points for each condition are from independent experiments. Horizontal bars represent the mean of all experiments. Statistically significant differences between data were determined by the Wilcoxon test *P<0.05.

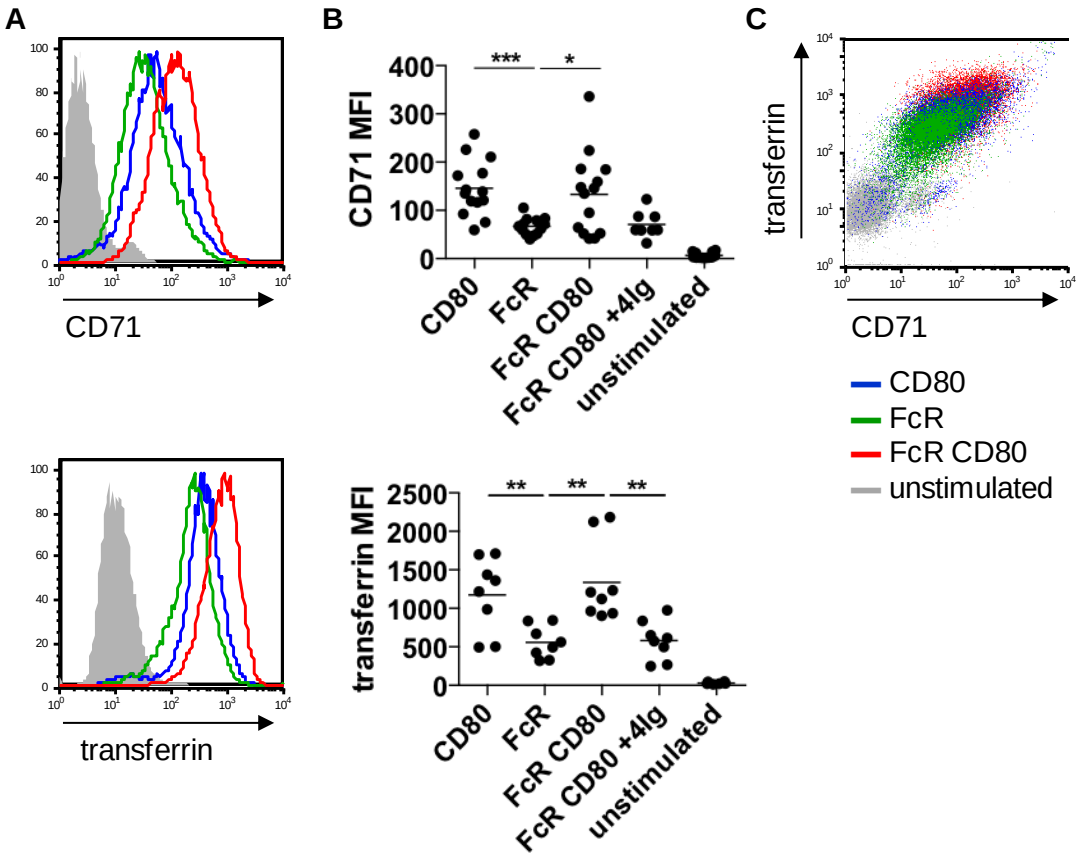


Figure 5.9 The level of CD71 expression and consequently the amount of transferrin uptake are limited in the absence of CD28 costimulation. $CD4^{+}CD25^{-}$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing CD80, FcR or FcR CD80 +/-20µg/ml CTLA-4-Ig plus 5µg/ml fluorochrome-conjugated transferrin for five days then stained for CD71 expression and analysed by flow cytometry. **(A)** Overlaid histograms show CD71 and transferrin fluorescence for each condition. Data are representative of 13 and 7 independent experiments respectively. **(B)** Graphs show the CD71 and transferrin MFI of responding T cells only or all unstimulated T cells. Data from 7-13 independent experiments are shown. Horizontal bars represent the mean of all experiments. Statistically significant differences between data were determined by the Wilcoxon test *P<0.05; **P<0.01; ***P<0.001. **(C)** Plot shows CD71 expression against transferrin fluorescence for each condition overlaid. Data are representative of seven independent experiments.

CD28 function, ribosomal S6 kinase phosphorylation was analysed as a measure of mTORC1 activity. It was found that S6 kinase phosphorylation only occurred to a large extent in the presence of both a strong TCR signal and CD28 costimulation (FcR CD80 CHO) (**figure 5.10**). S6 kinase phosphorylation was only detected in a few T cells in response to a weak TCR signal despite CD28 signalling (CD80 CHO), suggesting that CD28 costimulation is not sufficient for mTORC1 activity and that both strong TCR and CD28 stimulation are required for effective phosphorylation of S6 kinase. These data support findings that mTOR is a downstream mediator of CD28 signalling, but also suggest that CD28 can signal independently of mTOR because even when the TCR signal was not strong enough to activate mTOR (CD80 CHO), CD28-dependent changes were still observed (**figures 5.4-5.9**).

A common feature of many receptors is their ability to regulate their own expression. Analysis of CD28 expression by activated T cells revealed that CD28 expression was regulated differently depending on the TCR and costimulatory signals provided (**figure 5.11**). CD28 was completely downregulated by day two in the presence of soluble anti-CD3 and CD80 CHO cells, but expression was almost restored to resting levels by day five. These findings are consistent with the model that CD28 is transiently downregulated via negative feedback mechanisms upon CD28 receptor engagement (Eck et al., 1997). However in the presence of FcR-expressing CHO cells, CD28 expression on T cells was upregulated. The level of CD28 upregulation was significantly higher in the absence of CD28 ligation, and unlike in the presence of CD28 costimulation, had not declined towards normal levels by day five. This implies that a strong TCR signal upregulates CD28 expression, which perhaps overrides any self regulation by CD28. The particularly high CD28 expression in the absence of costimulation may be a compensatory mechanism employed by T cells to try to acquire the costimulatory signals required for proliferation and differentiation. Overall these data show that CD28 expression reflects the strength of TCR and costimulatory signals received

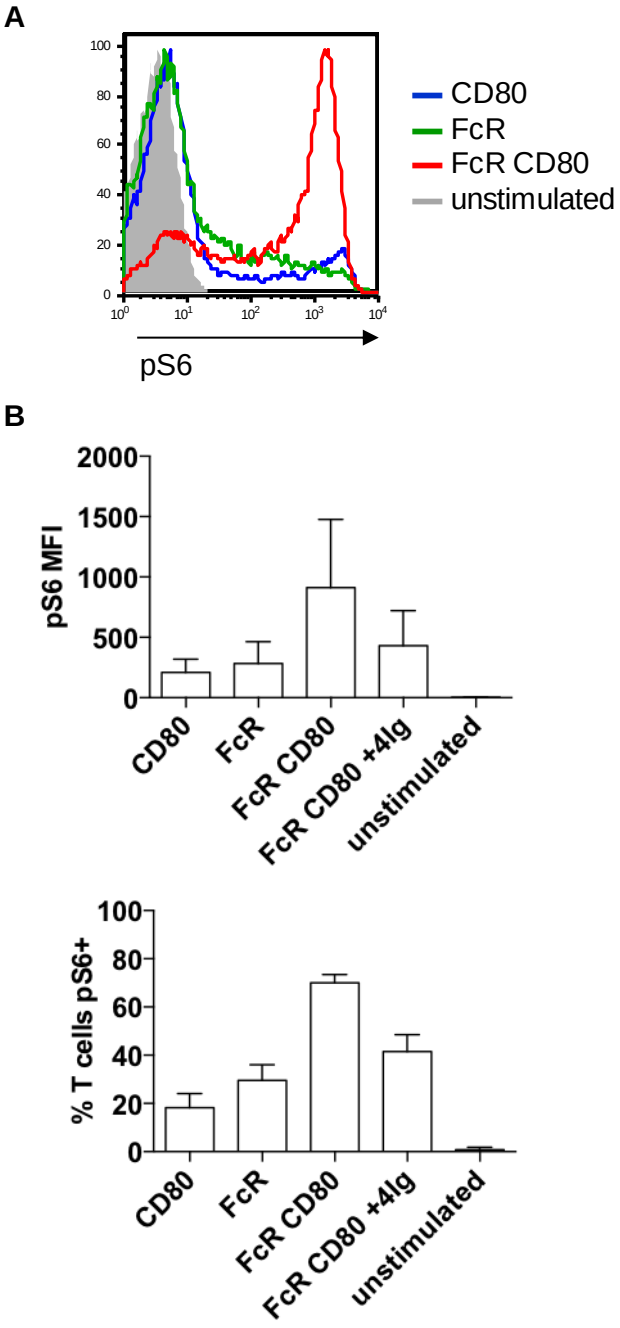


Figure 5.10 CD28 costimulation is required for activation of the mTOR pathway. $CD4^+CD25^-$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing CD80, FcR or FcR CD80 +/-20µg/ml CTLA-4-Ig for two days. Cells were fixed and permeabilised then stained for pS6 expression and analysed by flow cytometry. **(A)** Overlaid histograms show pS6 expression. Data are representative of three independent experiments. **(B)** Graphs show the mean (+SD) pS6 MFI and the mean (+SD) percentage of T cells expressing pS6 using data from three independent experiments. There were not enough replicate data for differences to reach statistical significance.

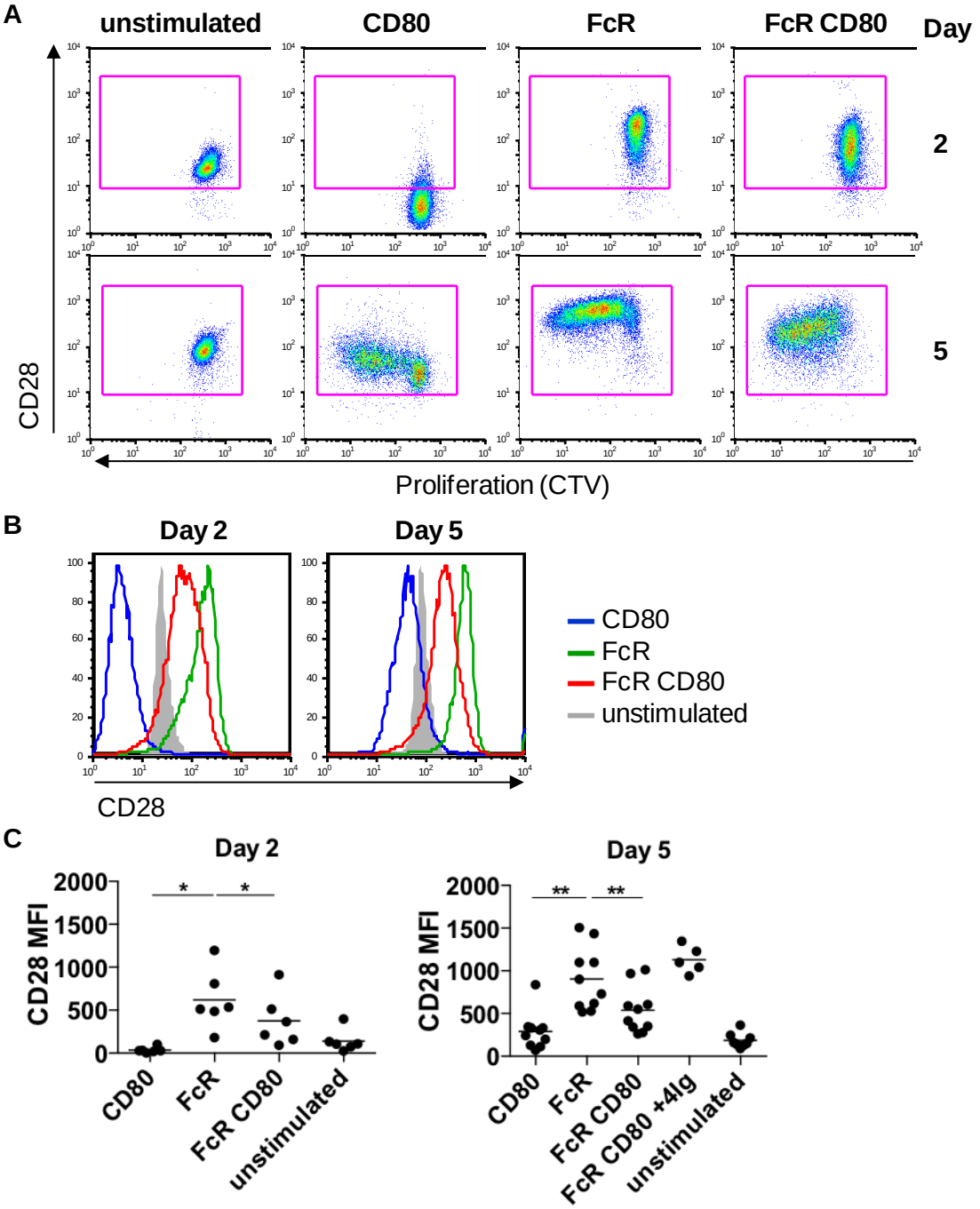


Figure 5.11 CD28 expression is regulated by TCR and CD28 signalling. CTV-labelled $CD4^{+}CD25^{-}$ T cells were stimulated for two or five days then stained for CD28 expression and analysed by flow cytometry. **(A)** Plots show CTV fluorescence against CD28 expression. $CD28^{+}$ cells are gated. Data are representative of at least six independent experiments. **(B)** Overlaid histograms show CD28 expression for each condition. Data are representative of at least six independent experiments. **(C)** Graphs show the CD28 MFI of responding T cells only or all unstimulated T cells. Data from six (day 2) and nine (day 5) independent experiments are shown. Horizontal bars represent the mean of all experiments. Statistically significant differences between data were determined by the Wilcoxon test *P<0.05; **P<0.01.

by the T cell and raises the possibility of using CD28 expression as a marker of whether T cells received CD28 costimulation.

In order to test the outcomes of limiting CD28 costimulation with a more physiological stimulus, naive CD4 T cells were stimulated with TSST antigen presented by the MHC molecule HLA-DR4 expressed by CHO cells. These CHO cells co-expressed CD80 to provide CD28 costimulation which was blocked as required by CTLA-4-Ig. Consistent with my previous data, T cell proliferation did not require CD28 costimulation, however CD28, CD71 and OX40 expression were CD28-dependent (**figure 5.12**). The downregulation of naive T cell markers also required CD28 costimulation (**figure 5.13**). Therefore using two different TCR stimuli I observed that CD28 costimulation was not essential for T cell proliferation but is crucial for optimal expression of effector molecules by activated T cells.

5.3 TCR signalling and CD28 signalling are not redundant

In the previous chapter it was demonstrated that the strength of the TCR signal determines the amount of CD28 stimulation required to initiate T cell proliferation. This suggests that the combined number of TCR and CD28 receptors that are engaged controls T cell division. **Figures 5.12 and 5.13** show that restricting the availability of CD28 costimulation limits the upregulation of effector molecules and instead T cells retain a more naive phenotype. To investigate whether increasing the TCR signal when CD28 costimulation is restricted can compensate for the lack of CD28 signalling and rescue the protein expression, the antigen concentration was increased ten-fold. The percentage of T cells that proliferated in response to 25ng/ml TSST with costimulation was similar to that which responded to a higher antigen dose of 250ng/ml TSST but in the absence of CD28 costimulation (**figure 5.14**). This again provides evidence that a stronger TCR signal reduces the requirement for CD28 costimulation. When the phenotypes of the responding T cells in these two conditions were compared, although the extent of proliferation was the same, naive T cell markers were not

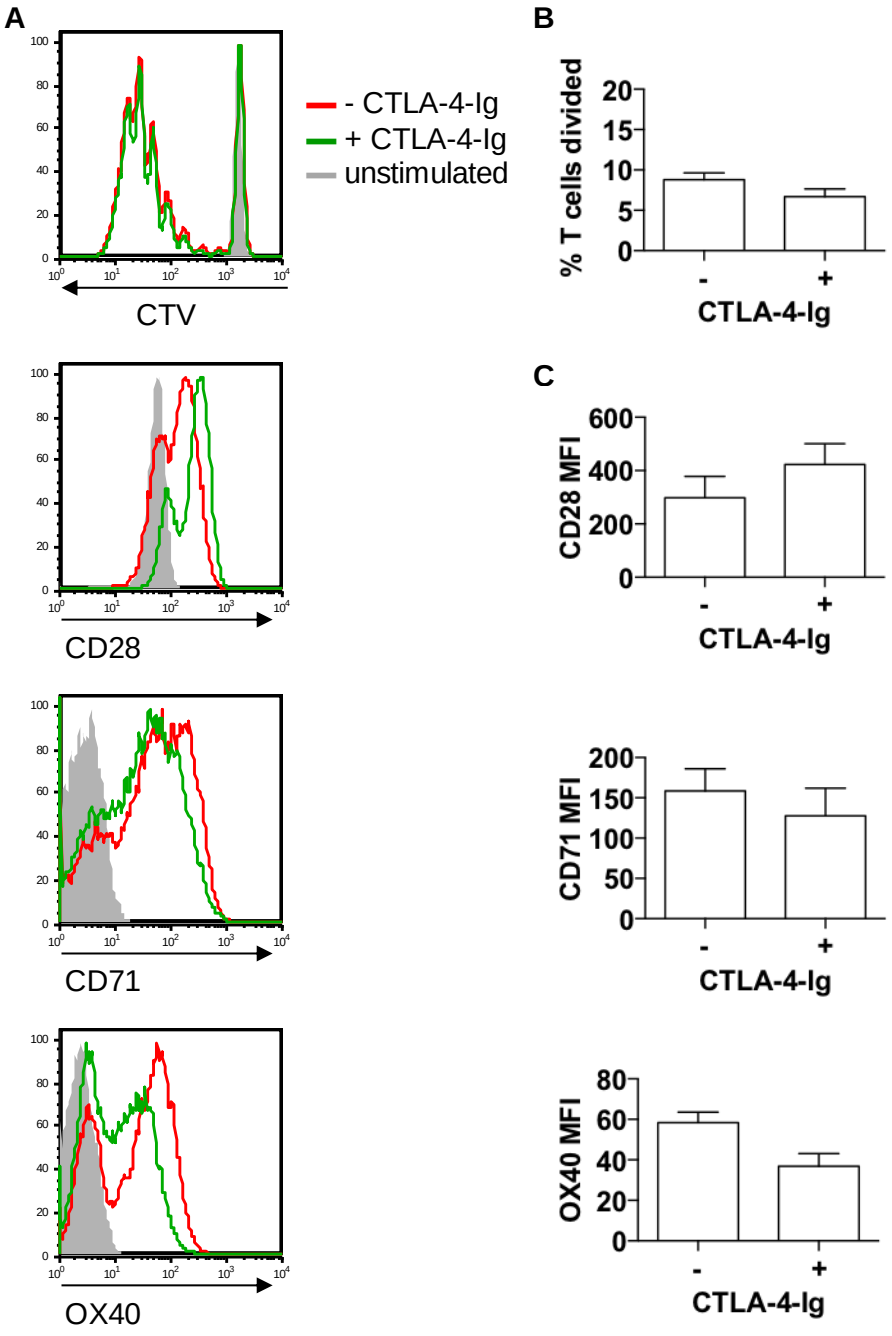


Figure 5.12 Protein expression is still largely CD28-dependent when T cells are stimulated with antigen. CTV-labelled $CD4^{+}CD25^{-}$ naive T cells were incubated at 37°C with 25ng/ml TSST and fixed CHO cells expressing HLA-DR4 CD80 +/-20µg/ml CTLA-4-Ig for five days then stained for surface protein expression and analysed by flow cytometry. **(A)** Overlaid histograms show T cell proliferation (CTV fluorescence) or surface protein expression for each condition. Data are representative of three independent experiments. **(B)** Graph shows the mean (+SD) percentage of T cells that committed to divide using data from three independent experiments. **(C)** Graphs show the mean (+SEM) MFI of responding T cells only using data from three independent experiments. There were not enough replicate data for differences to reach statistical significance.

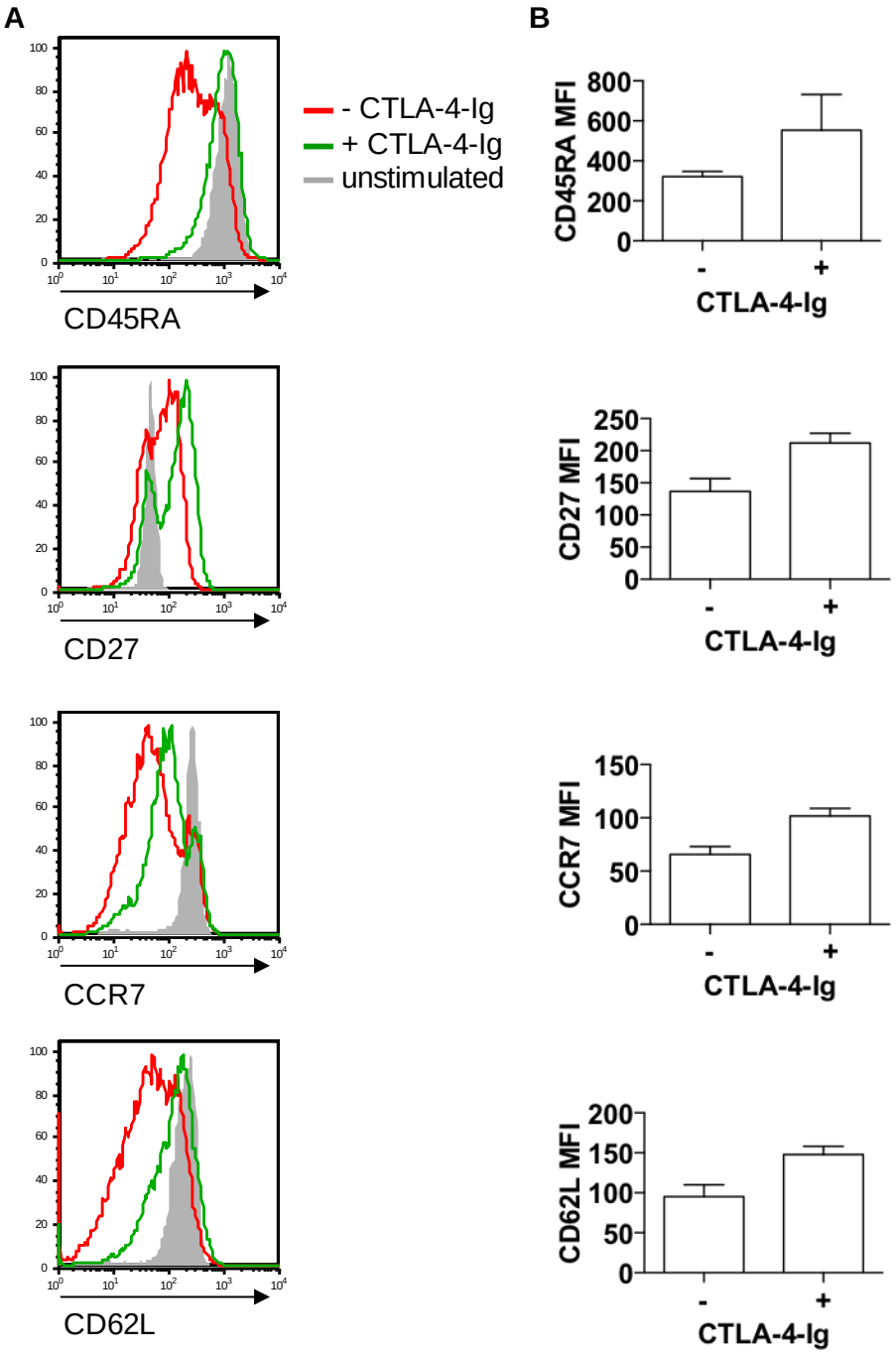


Figure 5.13 Antigen-stimulated T cells do not efficiently downregulate naive T cell markers in the absence of CD28 costimulation. CTV-labelled $CD4^{+}CD25^{-}$ naive T cells were incubated at 37°C with 25ng/ml TSST and fixed CHO cells expressing HLA-DR4 CD80 +/-20µg/ml CTLA-4-Ig for five days then stained for surface protein expression and analysed by flow cytometry. **(A)** Overlaid histograms show protein expression for each condition. Data are representative of three independent experiments. **(B)** Graphs show the mean (+SEM) MFI of responding T cells using data from three independent experiments. There were not enough replicate data for differences to reach statistical significance.

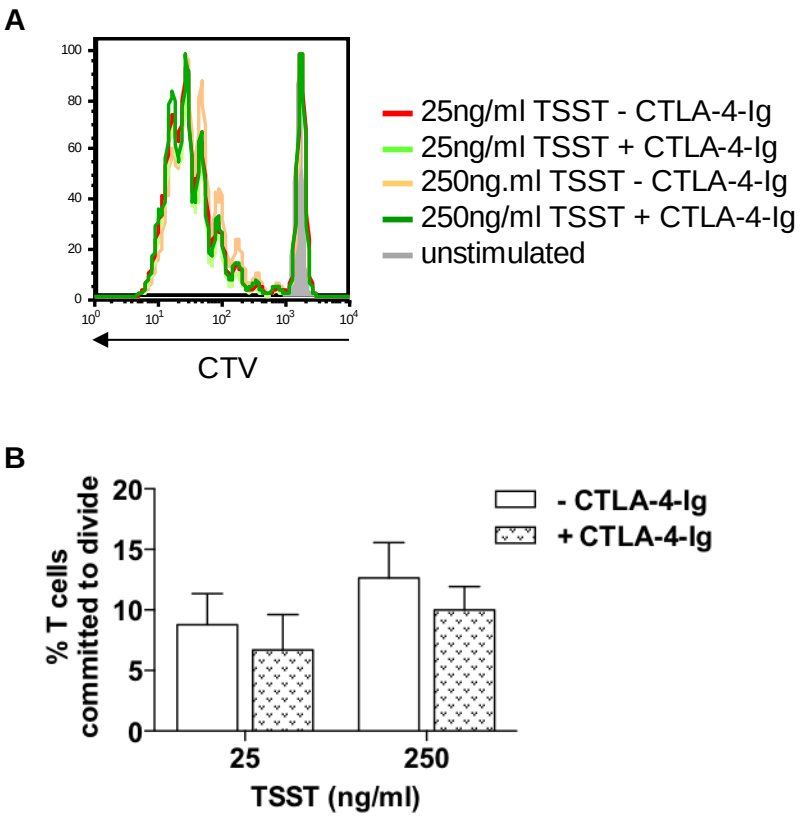


Figure 5.14 The combined strength of TCR and CD28 stimulation controls T cell proliferation. CTV-labelled $CD4^+CD25^-$ naive T cells were stimulated with fixed CHO cells expressing HLA-DR4 CD80 and 25-250ng/ml TSST +/- 20µg/ml CTLA-4-Ig. Cells were incubated at 37°C for five days then analysed by flow cytometry. **(A)** Overlaid histograms show T cell proliferation (CTV fluorescence). Data are representative of three independent experiments. **(B)** Graph shows the mean (+SD) percentage of T cells that committed to divide using triplicate measurements from three independent experiments. There were not enough replicate data for differences to reach statistical significance.

downregulated in the absence of CD28 costimulation and additionally OX40 and CD71 expression were impaired without CD28 signalling (**figure 5.15**). These findings are consistent with the concept that CD28 costimulation is not absolutely required for T cell proliferation but it is crucial for T cells to adopt an effector phenotype, because increased TCR stimulation could not compensate for the lack of CD28 to implement the changes in protein expression associated with full T cell activation. This indicates that CD28 does not simply enhance TCR signalling to promote effector molecule expression, it controls protein expression independently of the TCR. To explore the relative contributions of TCR and CD28 signalling further, it was tested whether these signals are additive or synergistic by stimulating T cells with anti-CD3, anti-CD28 or both antibodies together. It was found that simultaneous ligation of both receptors caused more T cells to commit to divide than the sum of those that responded to either signal alone (**figure 5.16**). This suggests that the two signals are synergistic rather than simply additive, which again supports the concept that the TCR and CD28 activate separate as well as shared pathways.

5.4 CD28 costimulation influences T cell differentiation

Having studied the role of CD28 costimulation in regulating the expression of effector molecules by activated T cells, the requirement for CD28 costimulation in T cell lineage commitment was investigated. Firstly the development of induced Tregs was assessed. It was found that FoxP3 was only significantly induced in response to a strong TCR signal in combination with CD28 costimulation (**figure 5.17**). These cells were identified as induced Tregs by their $FoxP3^{+}CD25^{+}CD127^{-}CTLA\text{-}4^{+}$ phenotype. This was consistent with published reports that the magnitude of both TCR and CD28 signals finely control iTreg development (Gabrysova et al., 2011), however the stability and function of these FoxP3-expressing cells was not investigated further. Next the importance of CD28 in the generation of specialised effector phenotypes was studied by analysing intracellular cytokine profiles. As expected the production of IL-2 was strongly enhanced by CD28 costimulation (**figure 5.18**). The

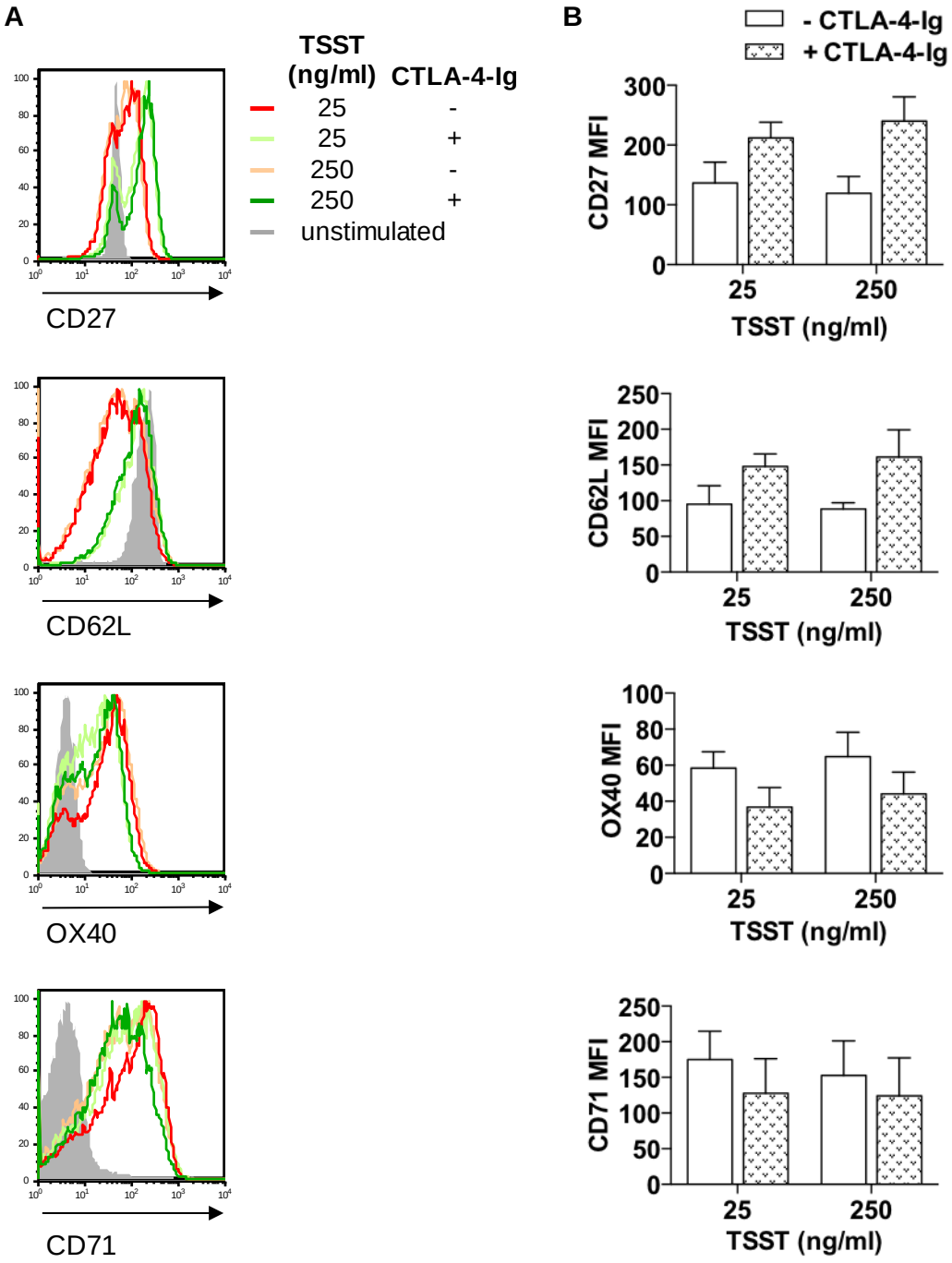


Figure 5.15 Increasing TCR stimulation cannot compensate for lack of CD28 costimulation to rescue the upregulation of effector proteins. $CD4^{+}CD25^{-}$ naive T cells were stimulated with fixed CHO cells expressing HLA-DR4 CD80 and 25-250ng/ml TSST +/- 20µg/ml CTLA-4-Ig. Cells were incubated at 37°C for five days then stained for surface protein expression and analysed by flow cytometry. **(A)** Overlaid histograms show surface protein expression. Data are representative of three independent experiments. **(B)** Graphs show the mean (+SD) MFI of responding T cells only using data from three independent experiments. There were not enough replicate data for differences to reach statistical significance.

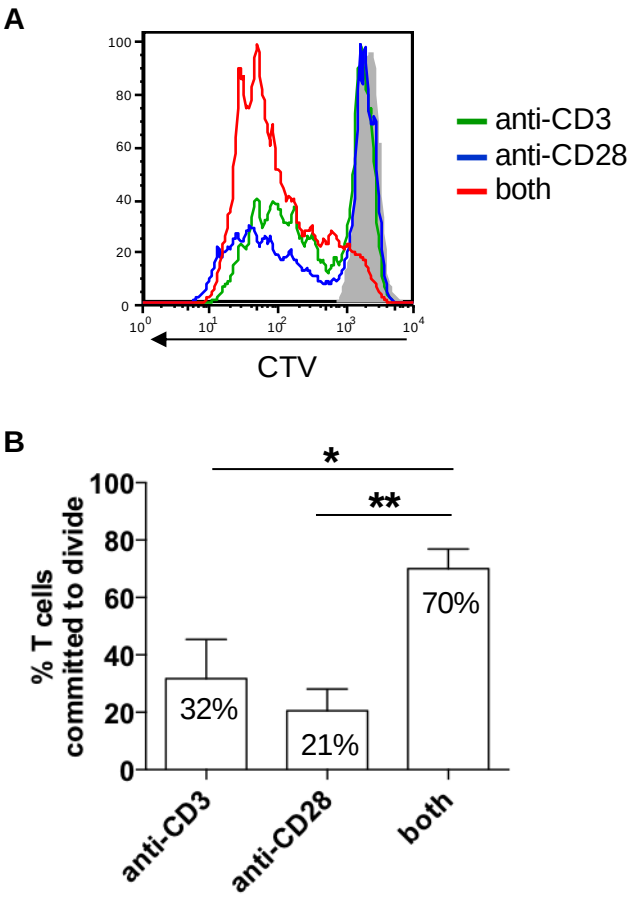


Figure 5.16 TCR and CD28 signalling are synergistic. CTV-labelled $CD4^+CD25^-$ T cells were stimulated with fixed FcR CHO cells in the presence of 1µg/ml anti-CD3, 1µg/ml anti-CD28 or both antibodies for five days then analysed by flow cytometry. **(A)** Overlaid histograms show T cell proliferation (CTV fluorescence) for each condition. Data are representative of three independent experiments. **(B)** Graph shows the mean (+SEM) percentage of T cells that committed to divide using data from three independent experiments. Differences between data were statistically significant according to the paired t test *P<0.05 **P<0.01.

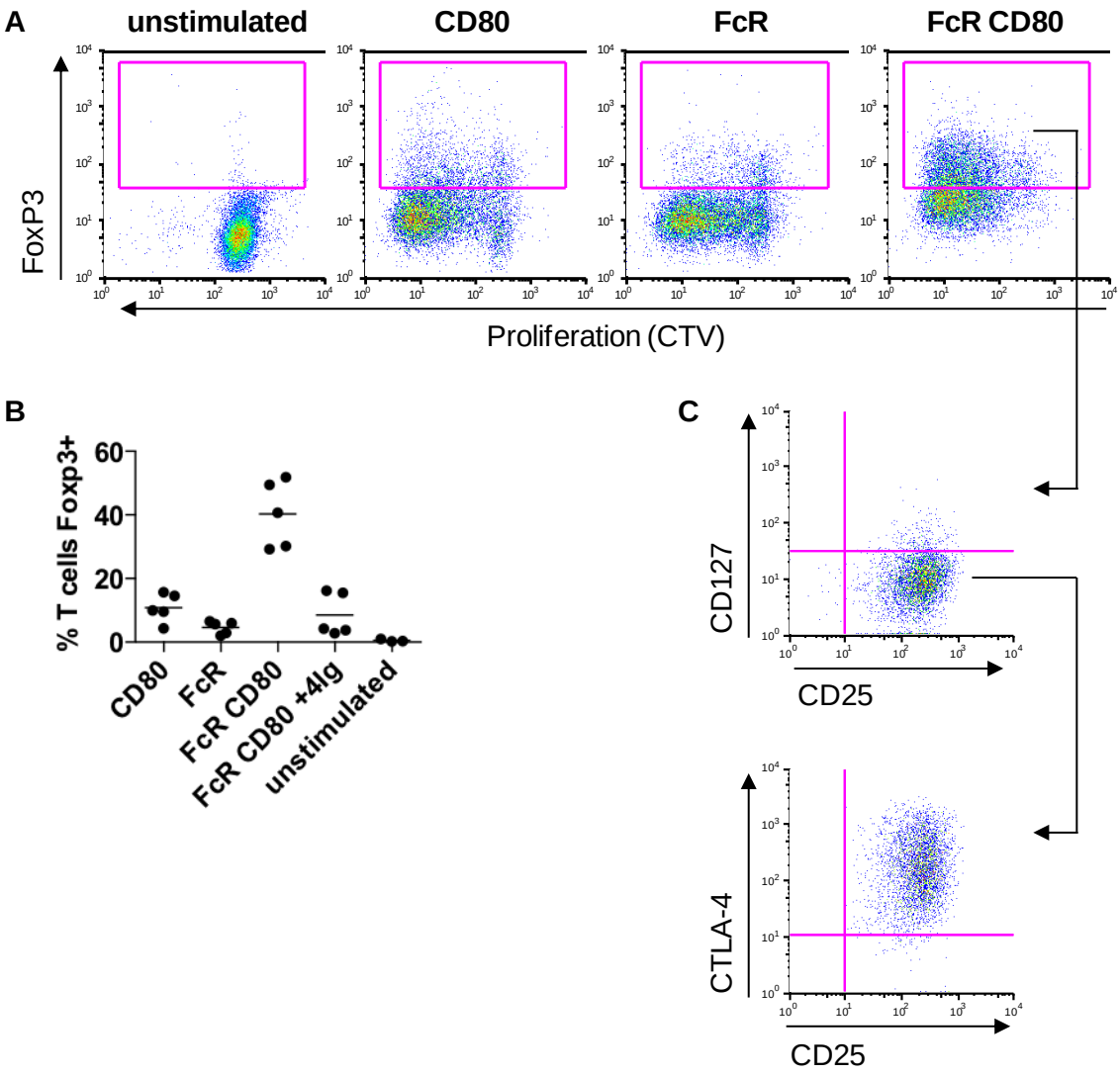


Figure 5.17 Induced Treg development requires CD28 costimulation. CTV-labelled $CD4^{+}CD25^{-}$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing CD80, FcR or FcR CD80 +/-20µg/ml CTLA-4-Ig for five days. Cells were stained for cell surface CD25 and CD127 expression. Then cells were fixed and permeabilised and stained for FoxP3 and CTLA-4 expression before analysis by flow cytometry. **(A)** Plots show CTV fluorescence against FoxP3 expression. $FoxP3^{+}$ cells are gated. Data are representative of four independent experiments. **(B)** Graph shows the percentage of responder or unstimulated T cells expressing FoxP3. Data from four independent experiments are shown. Horizontal bars represent the mean of all experiments. **(C)** Plots show other phenotypic markers expressed by $FoxP3^{+}$ cells generated after stimulation with FcR CD80 CHO cells.

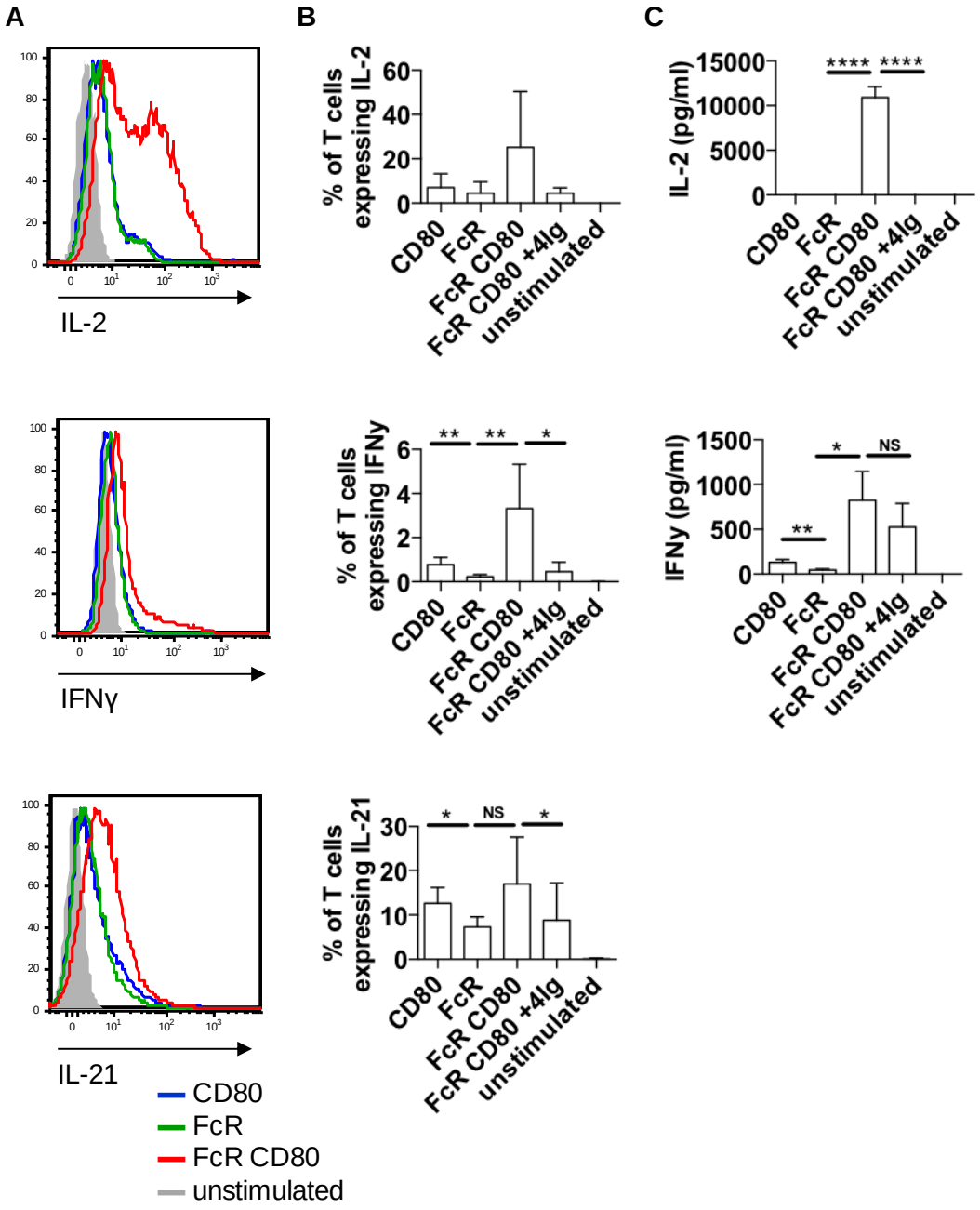


Figure 5.18 CD28 costimulation enhances IL-2, IFNγ and IL-21 production. $CD4^{+}CD25^{-}$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing CD80, FcR or FcR CD80 +/-20µg/ml CTLA-4-Ig for two or five days. **(A)** On day two cells were treated with 10µg/ml brefeldin A only for 5-15 hours then fixed, permeabilised and stained with fluorescently-labelled antibodies. Cells were analysed by flow cytometry. Overlaid histograms show intracellular cytokine staining for each condition. Data are representative of four independent experiments. **(B)** Graphs show the mean (+SD) percentage of T cells expressing the cytokine using data from four independent experiments. **(C)** On day five culture supernatants were collected and secreted cytokine concentration was measured by multiplex bead immunoassay. Graphs show the mean (+SD) cytokine concentration measured from duplicates in five independent experiments. Statistically significant differences between data were determined by the paired t test *P<0.05 **P<0.01 ****P<0.0001 NS=not significant.

requirement of CD28 costimulation for robust IL-2 production and the established role of IL-2 in T cell proliferation and differentiation may explain the incomplete differentiation of T cells lacking CD28 costimulation. The role of IL-2 in CD28-mediated outcomes will be discussed shortly. Meanwhile, production of the T_H1 cytokine IFNγ was also strongly TCR and CD28 dependent, and similarly the expression of IL-21 was limited without CD28 costimulation (**figure 5.18**). In order to see rare cell populations, T cells were restimulated with PMA and ionomycin. This approach revealed that IL-17 production was CD28-dependent (**figure 5.19**). Interestingly, the frequency of IL-10-expressing cells was increased in the absence of CD28 costimulation (**figure 5.19**). Taken together these data suggest that CD28 costimulation promotes the generation of multiple inflammatory T cell subtypes and normally suppresses the production of anti-inflammatory responses. Finally the production of T_H2 cytokines was analysed and most strikingly it was found that the secretion of IL-4, IL-5 and IL-13 was distinctly CD28 dependent in that very little of these cytokines were produced by TCR ligation alone (**figure 5.20**), consistent with reports that CD28 signalling promotes T_H2 differentiation. Overall these data show that both strong TCR and CD28 signalling are required for optimal cytokine production. In addition, the absence of CD28 costimulation favours the development of IL-10 producing T cells and even with weak TCR stimulation CD28 promotes the production of T_H2-associated cytokines.

5.5 Lack of CD28 costimulation does not lead to anergy

It has been widely reported that CD28 costimulation prevents the induction of anergy (Schwartz, 2003; Buckler et al., 2006; Zheng et al., 2009). Therefore using my system it was tested whether T cells primarily stimulated in the absence of CD28 costimulation could then proliferate after a second stimulation in the presence of costimulation or whether they become anergic. Surprisingly it was found that T cells expanded initially in the absence of CD28 costimulation could still produce a second proliferative response if CD28 costimulation was provided (**figure 5.21**). T cell expansion was comparable to that of T cells that received

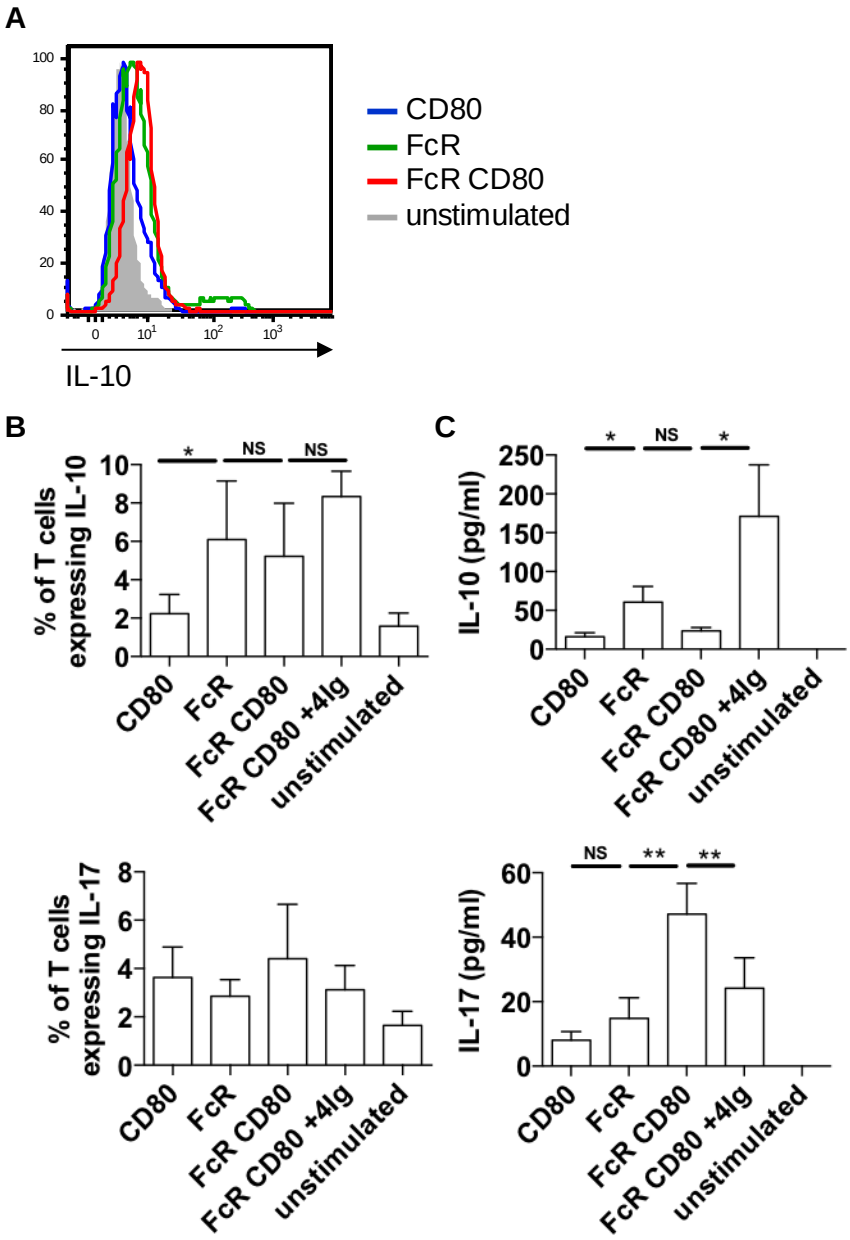


Figure 5.19 T cells stimulated in the absence of CD28 costimulation secrete more IL-10 but IL-17 production is impaired. $CD4^{+}CD25^{-}$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing CD80, FcR or FcR CD80 +/-20µg/ml CTLA-4-Ig for two or five days. **(A)** On day two cells were stimulated with 0.5µM PMA and 0.1mg/ml ionomycin then treated with 10µg/ml brefeldin A for 3.5 hours. Cells were fixed, permeabilised and stained with IL-10 or IL-17 antibody then analysed by flow cytometry. Overlaid histograms show intracellular cytokine staining for each condition. Data are representative of four independent experiments. **(B)** Graphs show the mean (+SD) percentage of T cells expressing the cytokine using data from four independent experiments. **(C)** On day five culture supernatants were collected and secreted cytokine concentration was measured by multiplex bead immunoassay. Graphs show the mean (+SD) cytokine concentration measured from duplicates in five independent experiments. Statistically significant differences between data were determined by the paired t test *P<0.05 **P<0.01 NS=not significant.

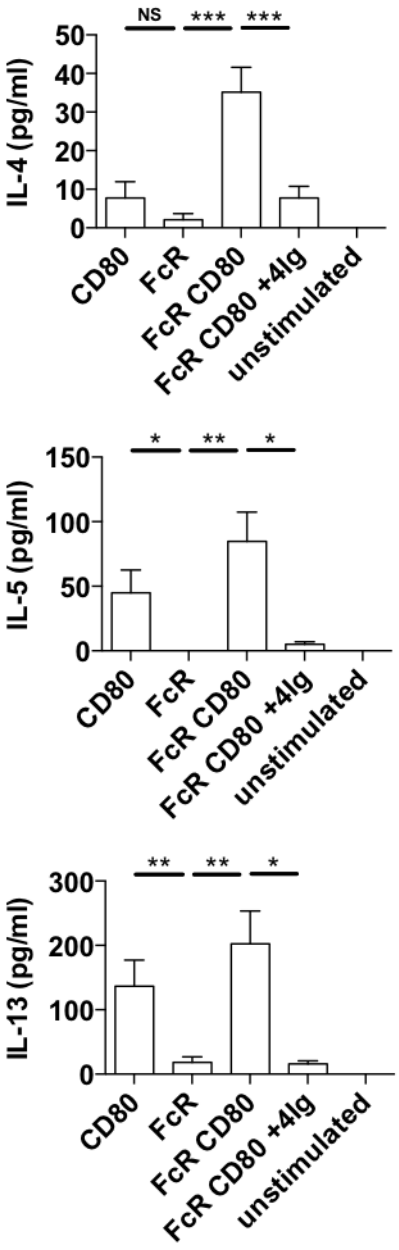


Figure 5.20 CD28 costimulation strongly increases the production of T_H2 cytokines. $CD4^+CD25^-$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing CD80, FcR or FcR CD80 +/-20µg/ml CTLA-4-Ig for five days. Culture supernatants were collected and secreted cytokine concentration was measured by multiplex bead immunoassay. Graphs show the mean (+SD) cytokine concentration measured from duplicates in five independent experiments. Statistically significant differences between data were determined by the paired t test *P<0.05 **P<0.01 ***P<0.001 NS=not significant.

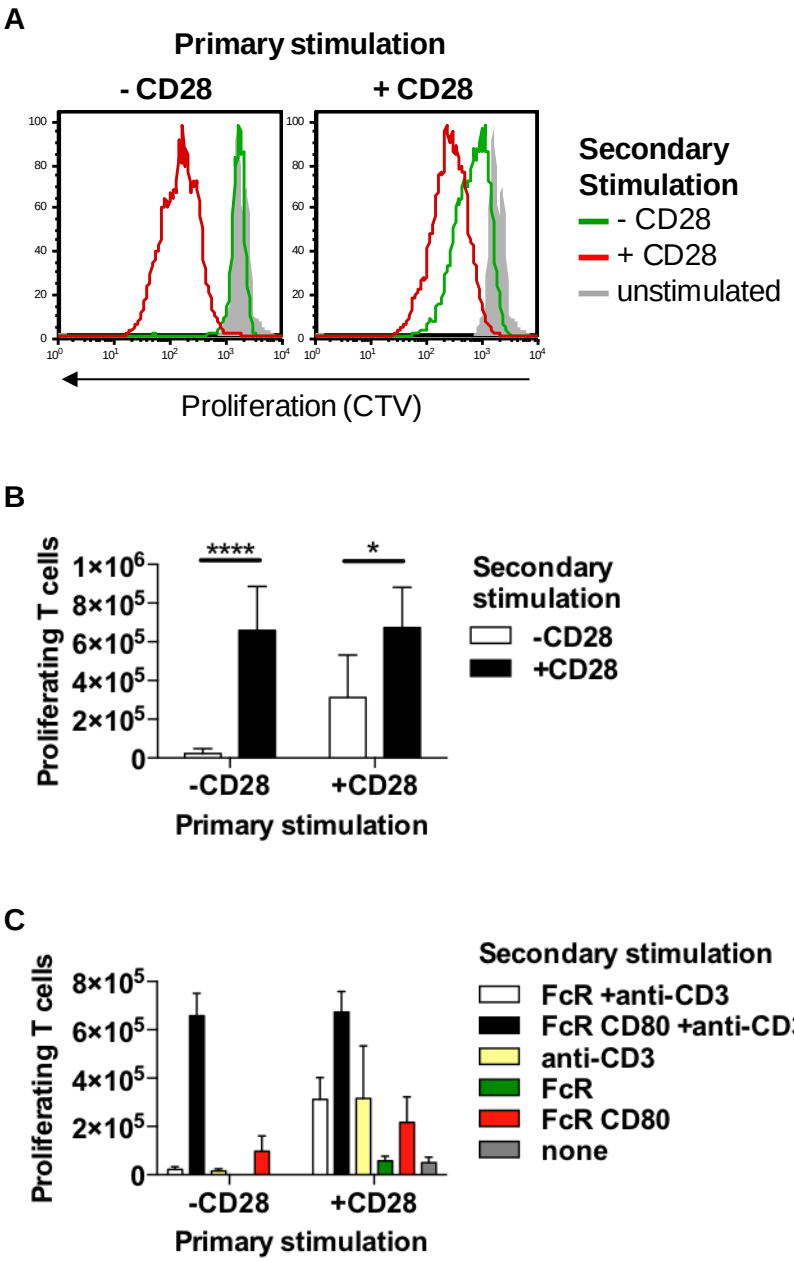


Figure 5.21 T cells stimulated in the absence of CD28 costimulation do not become anergic. $CD4^{+}CD25^{-}$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing FcR (-CD28) or FcR CD80 (+CD28) for 7-8 days. T cells were CTV-labelled and restimulated by incubation with FcR or FcR CD80 CHO +/-1µg/ml anti-CD3, with anti-CD3 alone or none of these for five days then analysed by flow cytometry. **(A)** Overlaid histograms show the proliferation (CTV fluorescence) of T cells restimulated with FcR or FcR CD80 CHO and 1µg/ml anti-CD3. Data are representative of seven independent experiments. **(B)** Graph shows the mean (+SD) number of proliferating T cells in the same conditions as *(A)* using data from six independent experiments. Statistically significant differences between data were determined by the t test *P<0.05 ****P<0.0001. **(C)** Graph shows the mean (+SEM) number of proliferating T cells in all conditions.

CD28 costimulation during both stimulations. Also in the presence of CD28 costimulation during the second stimulation there was high CD25, ICOS and CD71 expression on T cells during their secondary response whether or not they received CD28 costimulation in the primary stimulation (**figure 5.22**) and these cells showed further signs of maturation by down-regulating CD27 and CD62L (**figure 5.23**). Taken together these data indicate that lack of CD28 costimulation did not condemn T cells to unresponsiveness and that they can subsequently proliferate and differentiate as well as T cells that were primed in the context of CD28 signalling. Interestingly even T cells that were stimulated twice without CD28 costimulation, although they did not produce a second proliferative response, were not induced to die (**figure 5.24**).

It has been shown that IL-2 can rescue anergy (Schwartz, 2003; Bishop et al., 2009) therefore it is possible that CD28 signals during the second stimulation reversed anergy by increasing IL-2 production. Therefore it was tested whether the addition of IL-2 during the second stimulation after T cells were primed in the absence of CD28 costimulation could also promote a secondary response. Indeed it was found that IL-2 stimulated a secondary proliferative response of an equivalent magnitude to CD28 signalling when T cells primed in the absence of CD28 costimulation were restimulated (**figure 5.25A-B**). This suggests that CD28 signalling promotes a secondary response or reverses anergy through enhancing IL-2 production. However unlike CD28 signalling, IL-2 signalling during the second stimulation was not sufficient for optimal ICOS upregulation or downregulation of CD27 and CD62L (**figure 5.25C-D** and **figure 5.26**). This indicates that outcomes of CD28 signalling independent of IL-2 are required for full T cell activation and function. Furthermore, because IL-2 was not sufficient for the full maturation of T cells primed without CD28 costimulation and others have shown that T cell anergy cannot be reversed by subsequent provision of CD28 costimulation (Lumsden et al., 2003; Mittrucker et al., 1996; Zheng et al., 2009), it seems that the T cells primed without CD28 costimulation were not anergic. In summary,

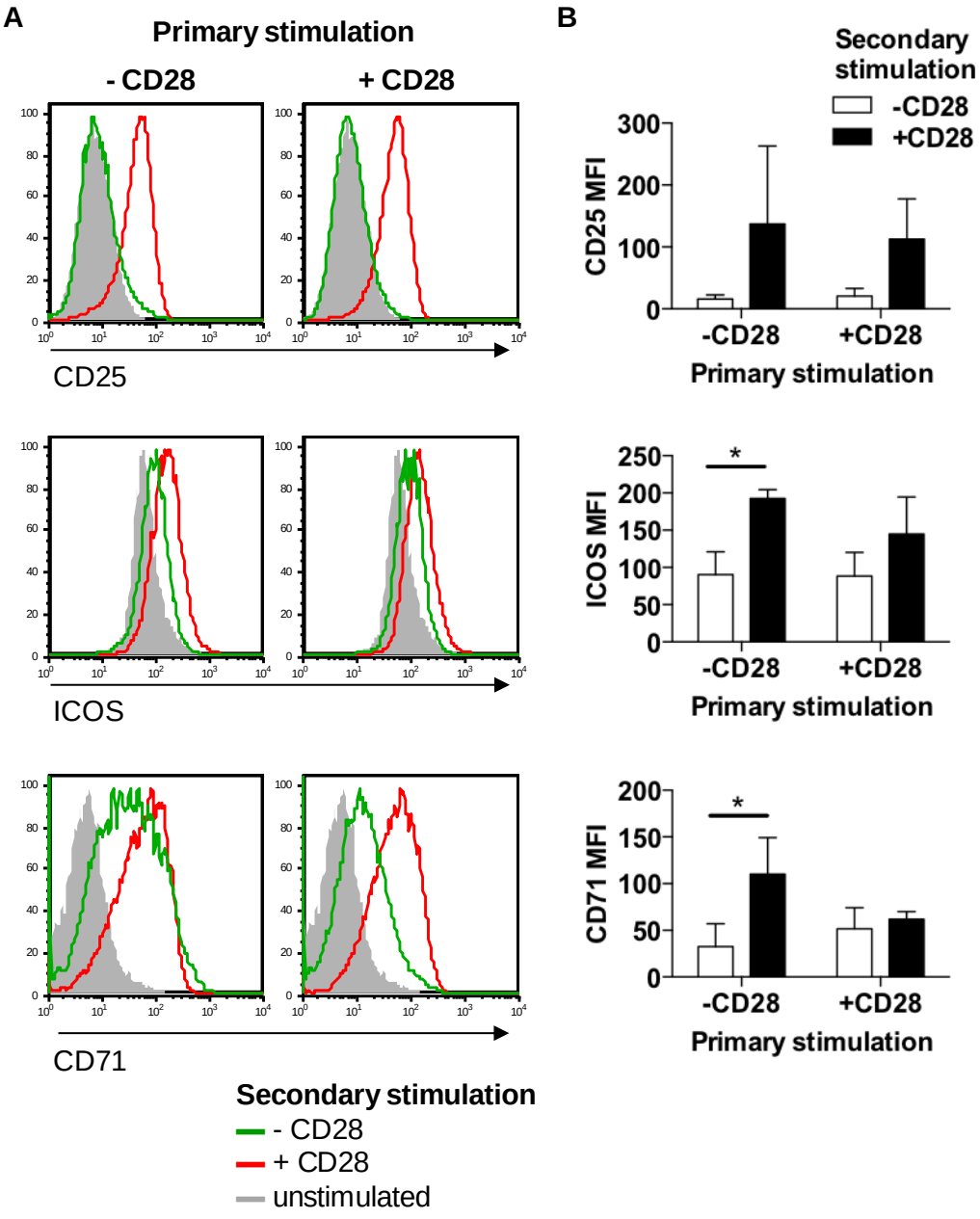


Figure 5.22 Restimulated T cells that did not receive CD28 costimulation in their primary stimulation upregulate effector T cell receptors. $CD4^{+}CD25^{-}$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing FcR (-CD28) or FcR CD80 (+CD28) for 7-8 days. T cells were restimulated by incubation with FcR or FcR CD80 CHO and 1µg/ml anti-CD3 for five days. Surface proteins were stained using fluorescently-labelled antibodies then cells were analysed by flow cytometry. **(A)** Overlaid histograms show T cell protein expression (MFI). Data are representative of at least two independent experiments. **(B)** Graphs show the mean (+SD) MFI using data from 2-4 independent experiments. For ICOS high background fluorescence was subtracted. Statistically significant differences between data were determined by the t test *P<0.05.

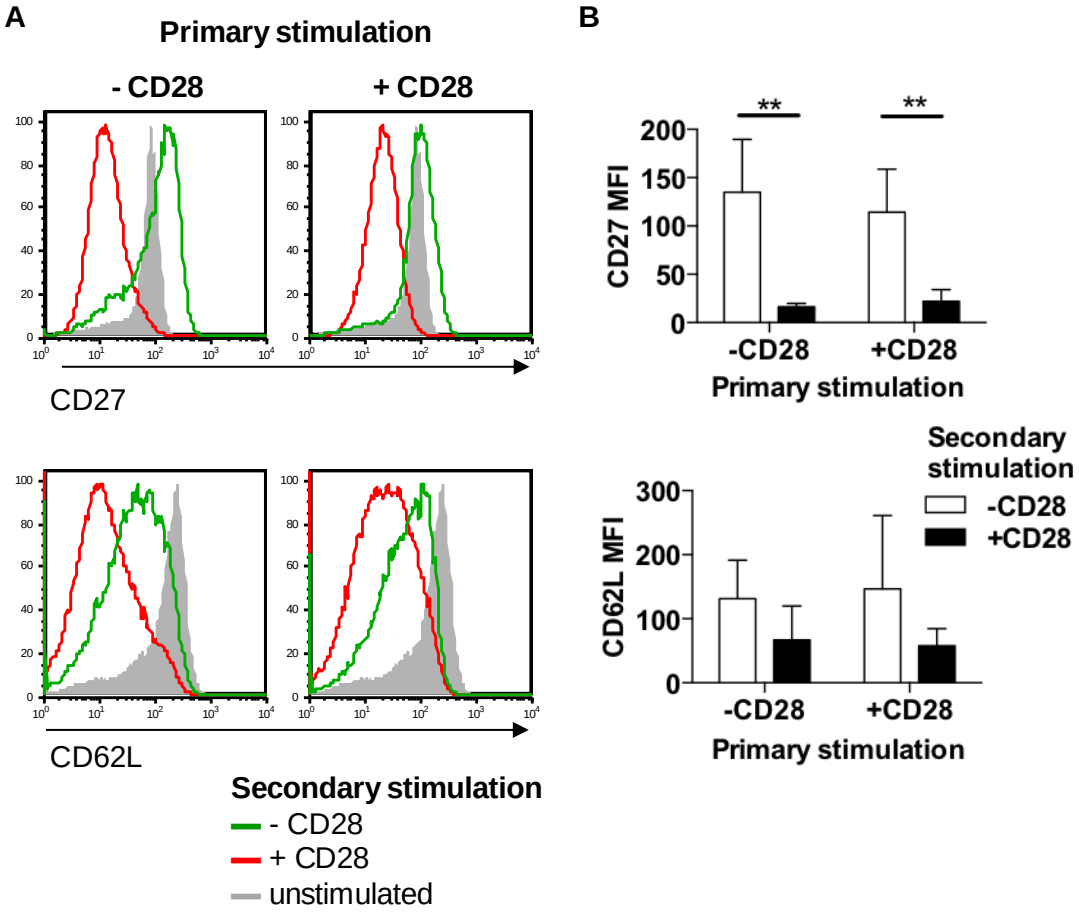


Figure 5.23 Restimulated T cells that did not receive CD28 costimulation in their primary stimulation downregulate naive T cell markers. $CD4^{+}CD25^{-}$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing FcR (-CD28) or FcR CD80 (+CD28) for 7-8 days. T cells were restimulated by incubation with FcR or FcR CD80 CHO and 1µg/ml anti-CD3 for five days. Surface proteins were stained using fluorescently-labelled antibodies then cells were analysed by flow cytometry. **(A)** Overlaid histograms show T cell protein expression (MFI). Data are representative of five independent experiments. **(B)** Graphs show the mean (+SD) MFI using data from five independent experiments. Statistically significant differences between data were determined by the t test **P<0.01.

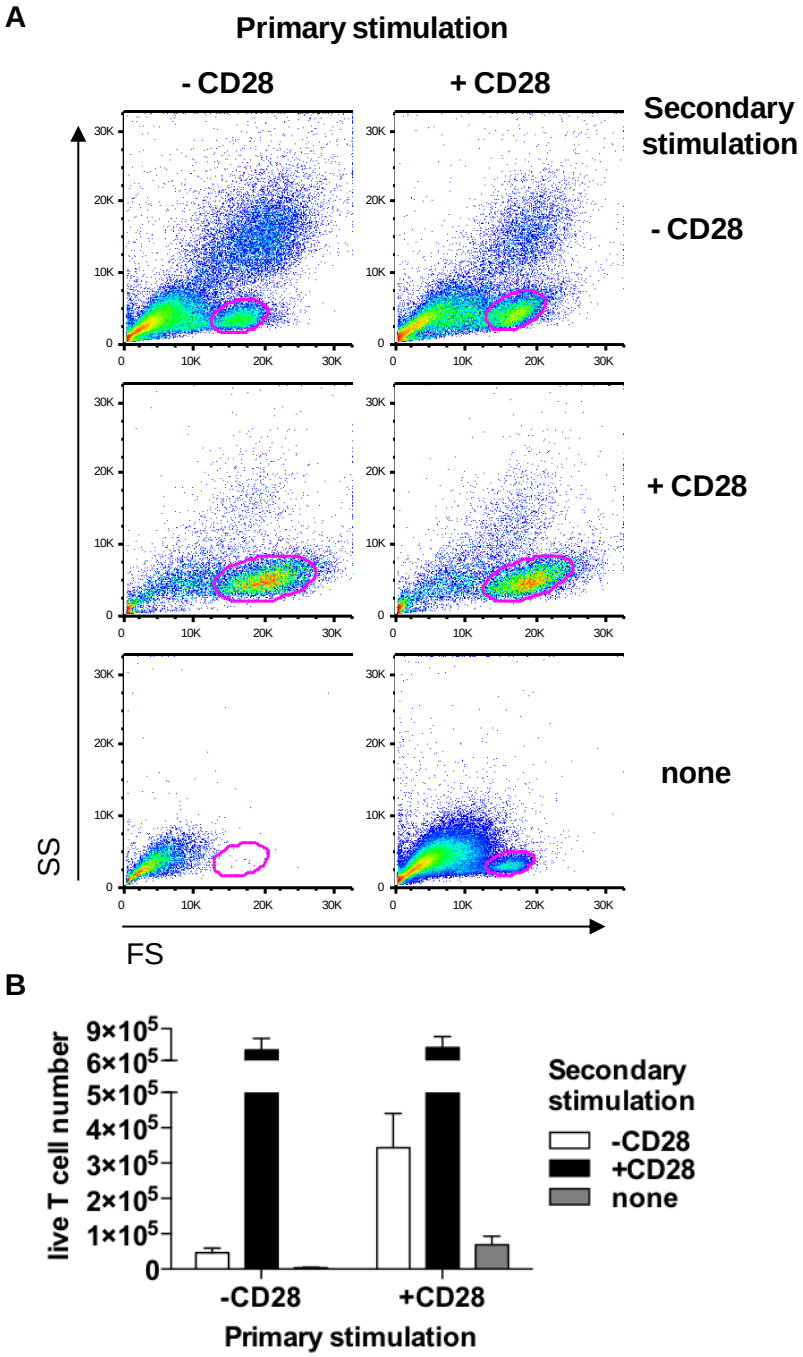


Figure 5.24 T cells that are stimulated twice using CD3 antibody without CD28 costimulation are not induced to die. $CD4^+CD25^-$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing FcR (-CD28) or FcR CD80 (+CD28) for 7-8 days. $1x10^5$ T cells were restimulated by incubation with FcR or FcR CD80 CHO and 1µg/ml anti-CD3 for five days then analysed by flow cytometry. **(A)** Plots show all cells in the culture according to their forward (FS) and side (SS) scatter. Live T cells are gated. Data are representative of five independent experiments. **(B)** Graph shows the mean (+SEM) number of live T cells using data from five independent experiments.

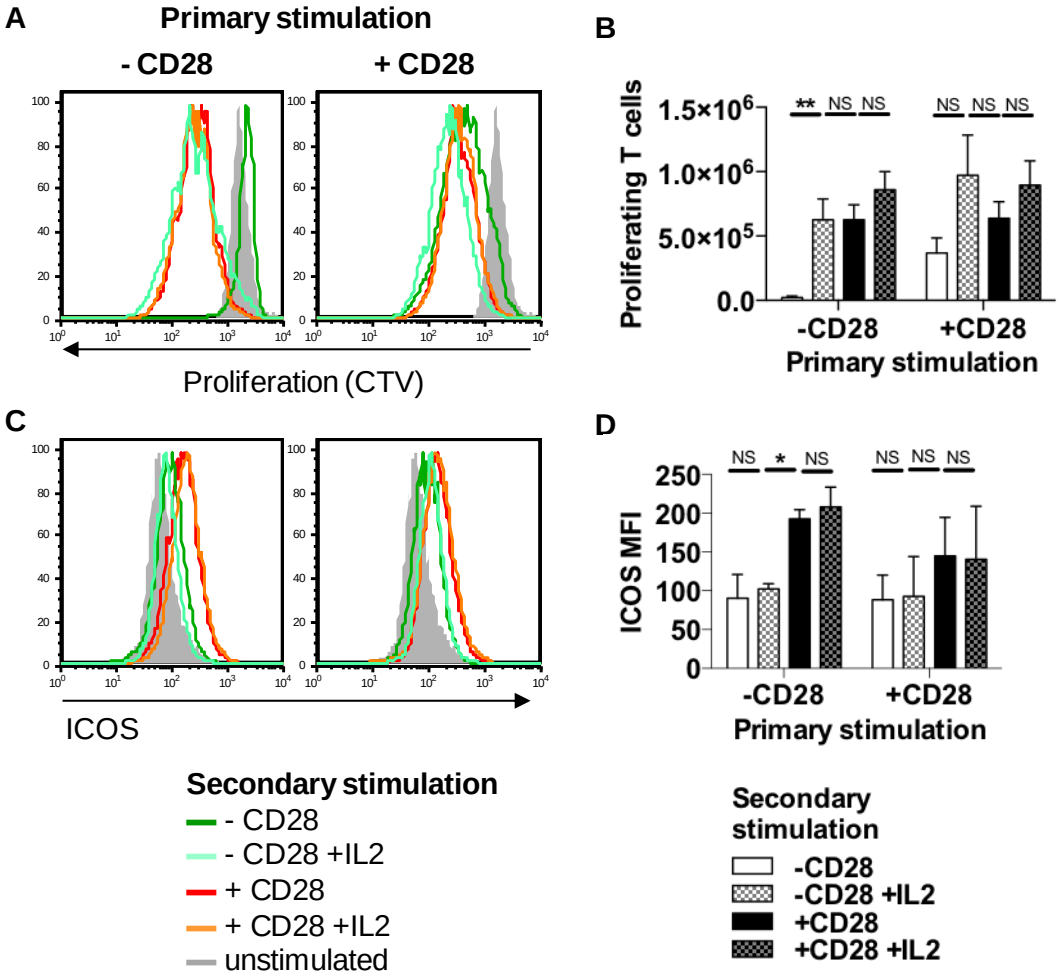


Figure 5.25 IL-2 can rescue the proliferation of T cells restimulated in the absence of CD28 costimulation but not ICOS upregulation. $CD4^+CD25^-$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing FcR (-CD28) or FcR CD80 (+CD28) for 7-8 days. T cells were CTV-labelled and restimulated by incubation with FcR or FcR CD80 CHO +/- 1µg/ml anti-CD3 +/- 200U/ml IL-2 for five days then analysed by flow cytometry. **(A)** Overlaid histograms show the proliferation (CTV fluorescence) of T cells restimulated with FcR or FcR CD80 CHO and 1µg/ml anti-CD3 +/- IL-2. Data are representative of four independent experiments. **(B)** Graph shows the mean (+SEM) number of proliferating T cells in the same conditions as *(A)* using data from four independent experiments. **(C)** Overlaid histograms show ICOS expression (MFI). Data are representative of two independent experiments. **(D)** Graph shows the mean (+SD) ICOS MFI using data from two independent experiments. Statistically significant differences between data were determined by the t test *P<0.05 **P<0.01 NS=not significant.

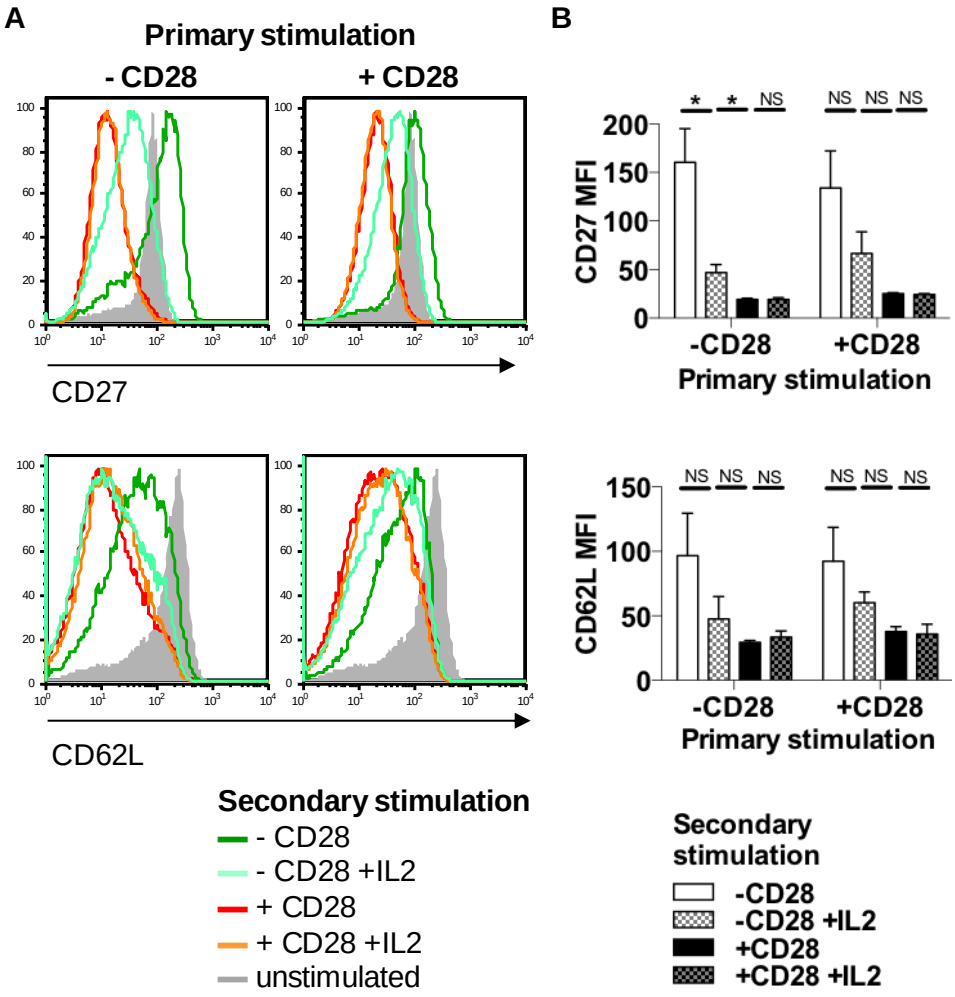


Figure 5.26 IL-2 only partially rescues the downregulation of naive T cell markers by T cells restimulated in the absence of CD28 costimulation. $CD4^{+}CD25^{-}$ T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing FcR (-CD28) or FcR CD80 (+CD28) for 7-8 days. T cells were restimulated by incubation with FcR or FcR CD80 CHO and 1µg/ml anti-CD3 +/- 200U/ml IL-2 for five days. Surface proteins were stained using fluorescently-labelled antibodies then cells were analysed by flow cytometry. **(A)** Overlaid histograms show T cell protein expression (MFI). Data are representative of two independent experiments. **(B)** Graphs show the mean (+SD) MFI using data from two independent experiments. Statistically significant differences between data were determined by the t test *P<0.05 NS=not significant.

contrary to expectation, these results indicate that in the context of strong TCR signalling, a lack of CD28 costimulation does not induce anergy or apoptosis in this system.

5.6 IL-2 dependent and IL-2 independent functions of CD28

It is well documented that IL-2 production is strongly upregulated by CD28 as shown in **figure 5.18**. Accordingly, signalling events downstream of IL-2 receptor stimulation such as STAT5 phosphorylation were influenced by CD28 stimulation (**figure 5.27A**). IL-2 promotes T cell growth and differentiation through a variety of mechanisms and is responsible for many of the outcomes of CD28 costimulation. However in the previous section it was shown that IL-2 could not promote ICOS upregulation and T cell maturation to the same extent as CD28 signalling during a secondary response, regardless of whether or not CD28 costimulation was provided during priming (**figures 5.25-5.26**), suggesting that IL-2 does not mediate all functions downstream of CD28. To investigate this further, the roles of CD28 and IL-2 in the priming of naive T cells were studied. In order to distinguish between CD28 costimulation and IL-2 signalling, exogenous IL-2 was added to T cell cultures where CD28 costimulation was unavailable to determine which effects of costimulation blockade were rescued. Interestingly, it was found that reduced T cell proliferation caused by lack of CD28 costimulation was almost completely restored by the addition of IL-2 (**figure 5.27B-C**), showing that proliferation is mainly driven by IL-2. Despite its effects on proliferation, IL-2 did not promote CD45RA downregulation (**figure 5.27D-E**) and did not restore CD71 or OX40 expression (**figure 5.28A-B**) in the absence of CD28 costimulation. This indicates that the role of CD28 costimulation in these aspects of T cell differentiation is IL-2-independent. Although it is possible that IL-2 may have been unable to rescue the T cell response because expression of the IL-2 receptor alpha chain CD25 was lower without CD28 costimulation (**figure 5.28C**). It was somewhat surprising that CD71 was not upregulated in response to IL-2 despite this signalling pathway being an alternative to CD28 upstream of mTOR, although the data already discussed did indirectly suggest that mTOR is not necessarily always

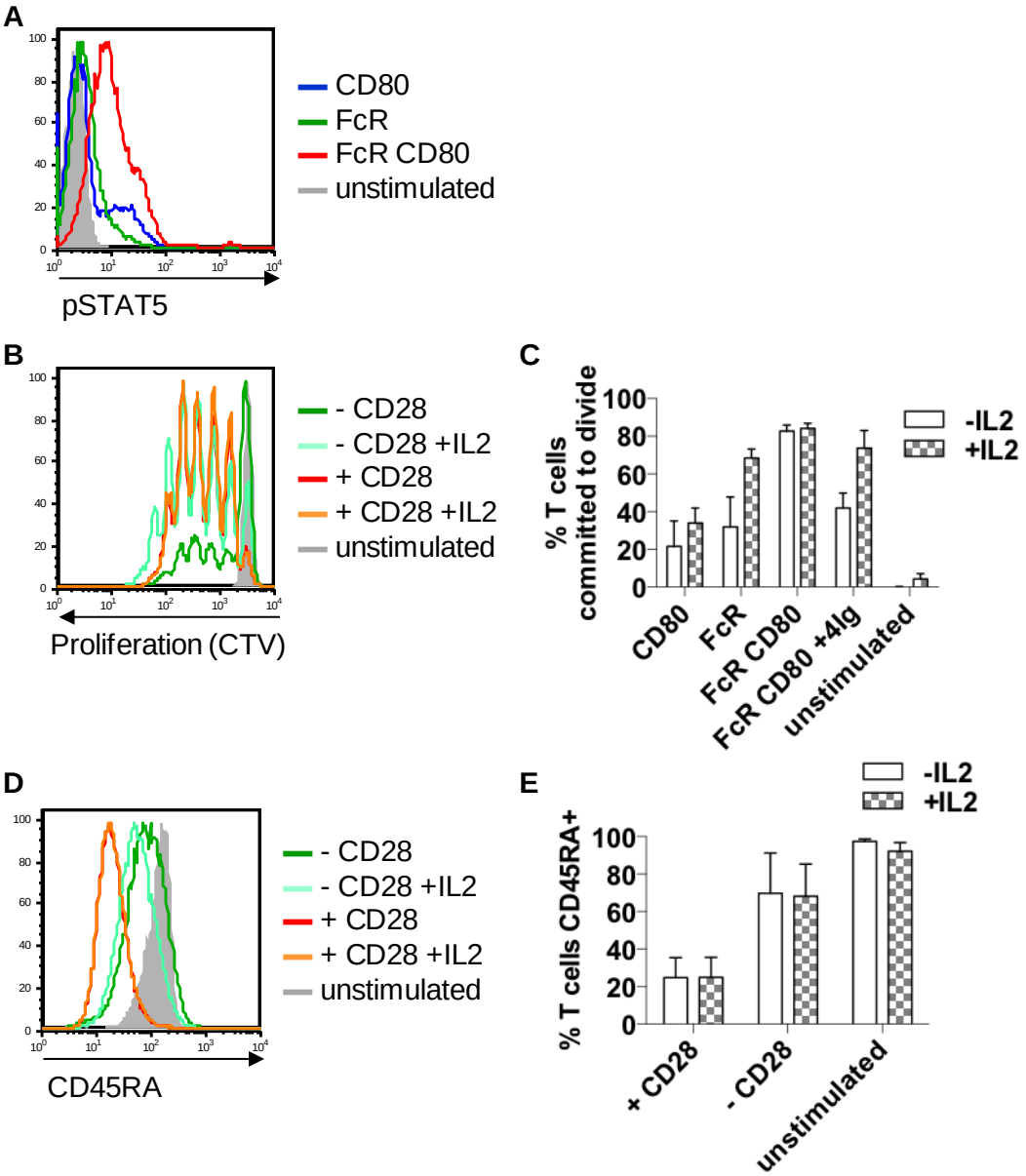


Figure 5.27 IL-2 can compensate for lack of CD28 costimulation to promote naive T cell proliferation but not CD45RA downregulation. **(A)** $CD4^+CD25^-$ naive T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing CD80, FcR or FcR CD80 for two days. Cells were fixed and permeabilised then stained for pSTAT5 expression and analysed by flow cytometry. Overlaid histograms show pSTAT5 expression. Data are representative of three independent experiments. **(B-E)** CTV-labelled $CD4^+CD25^-$ naive T cells were stimulated with 1µg/ml anti-CD3 and fixed CHO cells expressing CD80, FcR or FcR CD80 +/-20µg/ml CTLA-4-Ig +/-200U/ml IL-2 for five days then stained for CD45RA expression and analysed by flow cytometry. Data are representative of three independent experiments. *(B)* Overlaid histograms show T cell proliferation (CTV fluorescence) when cultured with FcR CHO (-CD28) or FcR CD80 CHO (+CD28) +/-IL-2. *(C)* Graph shows the mean (+SEM) percentage of T cells that committed to divide using data from three independent experiments. *(D)* Overlaid histograms show CD45RA expression. *(E)* Graph shows the mean (+SEM) percentage of responding T cells or unstimulated T cells expressing CD45RA using data from three independent experiments. There were not enough replicate data for differences to reach statistical significance.

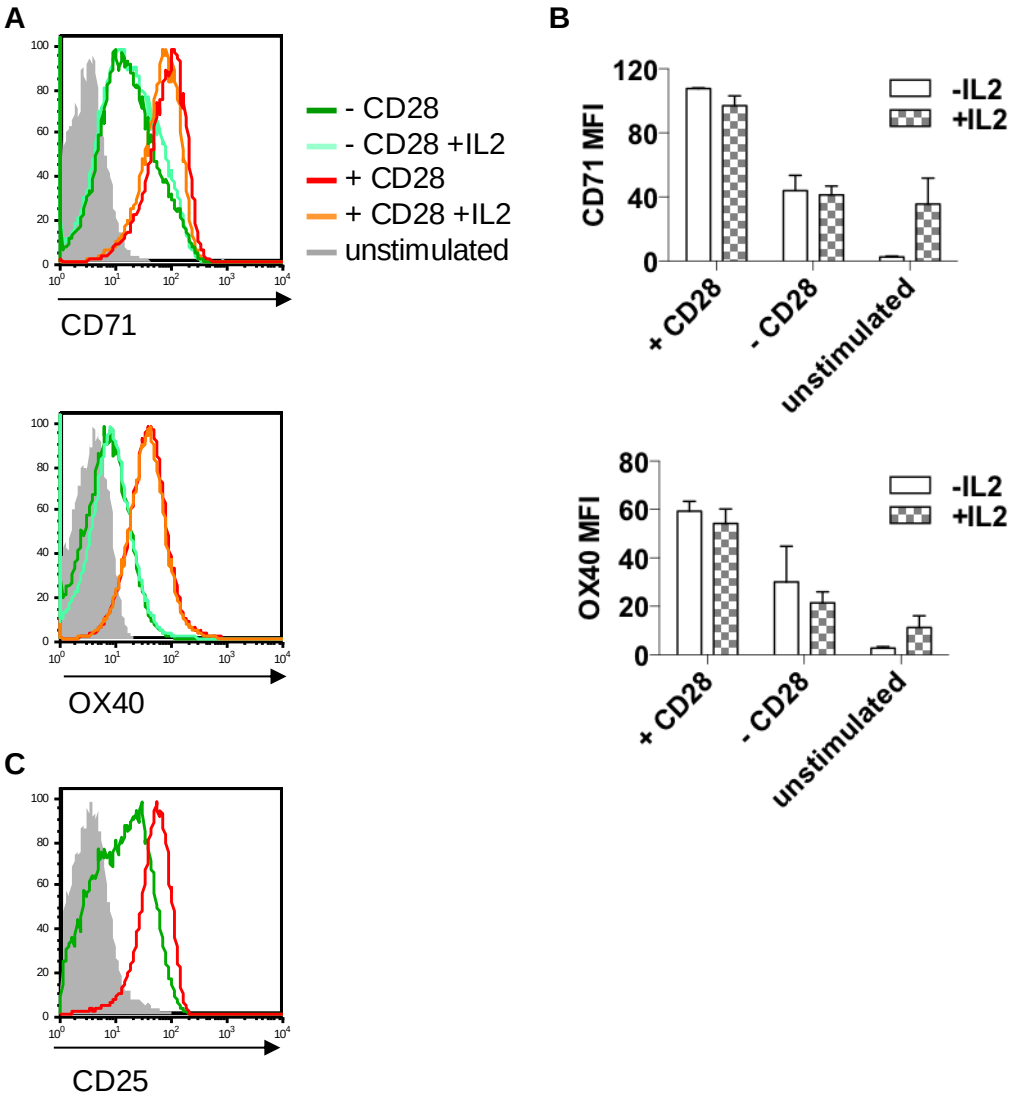


Figure 5.28 IL-2 cannot rescue CD71 or OX40 upregulation by naive T cells in the absence of CD28 costimulation. $CD4^{+}CD25^{-}$ naive T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing FcR or FcR CD80 +/-200U/ml IL-2 for five days then stained for surface protein expression and analysed by flow cytometry. Data are representative of three independent experiments. **(A)** Overlaid histograms show surface protein expression by T cells cultured with FcR CHO (-CD28) or FcR CD80 CHO (+CD28) +/- IL-2. **(B)** Graphs show the mean (+SEM) MFI of responding T cells only or all unstimulated T cells using data from three independent experiments. There were not enough replicate data for differences to reach statistical significance. **(C)** Overlaid histogram shows surface CD25 expression of T cells cultured with or without CD28 costimulation.

needed for CD71 upregulation. Overall these data support the view that CD28 costimulation has both IL-2-dependent and IL-2-independent outcomes.

5.7 Memory T cells are more CD28 dependent than naive T cells

Having investigated the effects of restricting CD28 costimulation upon a mixed resting T cell population and naive T cell priming, the relative costimulatory requirements of naive and memory T cells were examined (**figure 5.29**). It was observed that upon receiving strong TCR stimulation and CD28 costimulation (FcR CD80 CHO), a similarly high proportion of naive and memory T cells entered division (**figure 5.29B**). However when TCR signalling was weaker (CD80 CHO) both responses were smaller, but nearly double the percentage of memory T cells committed to divide in comparison to naive T cells, suggesting that memory cells are more sensitive to TCR activation. However when CD28 costimulation was not provided, there was less memory T cell division than naive. In fact despite a stronger TCR stimulus (FcR CHO compared to CD80 CHO), memory T cell division was reduced in the absence of CD28 costimulation. This suggests that memory T cells are more dependent on CD28 costimulation. This is demonstrated in **figure 5.29C**, which shows that the percentage decrease in T cell proliferation when CD28 costimulation was inhibited was greater for memory cells. In support of these findings, when T cells were stimulated with TSST antigen and HLA-DR4 CD80 CHO cells, memory T cell proliferation was again inhibited more dramatically than naive T cell proliferation by lack of CD28 costimulation (**figure 5.30**). The data clearly show that proportionally memory T cell proliferation is reduced the most when CD28 costimulation is inhibited. Altogether these data indicate that memory T cells are more sensitive to a lack of CD28 costimulation but more sensitive to weak TCR stimuli. The costimulatory requirements of memory T cells for effector molecule expression was also examined. It was found that memory T cells, like naive T cells, require CD28 costimulation for optimal upregulation of CD40L, ICOS and OX40 (**figure 5.31**). These data indicate that not only do memory T cells require CD28 costimulation for proliferation they also require

CD28 signals for full effector function. Overall these data suggest that memory T cells are not always CD28-independent.

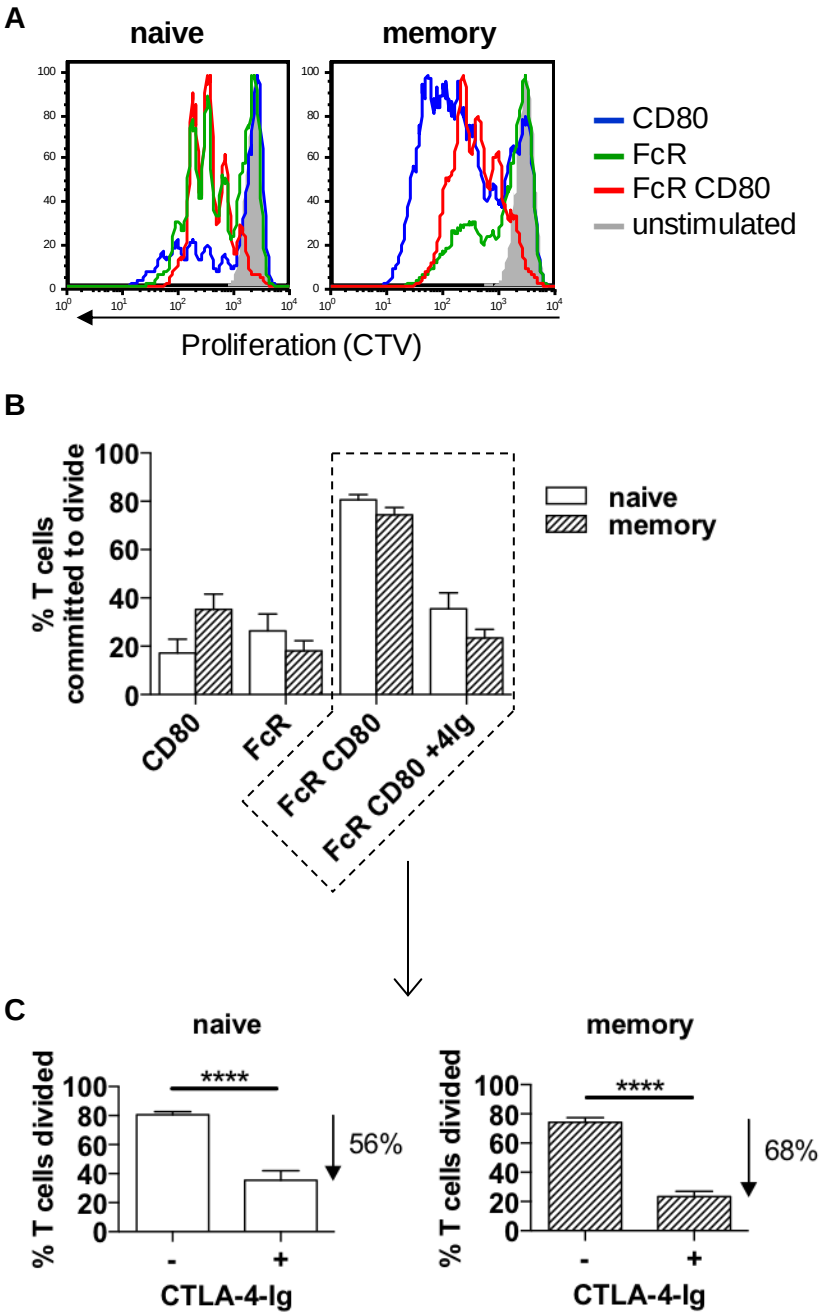


Figure 5.29 Memory T cells are CD28 dependent. CTV-labelled $CD4^{+}CD25^{-}$ naive or memory T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing CD80, FcR or FcR CD80 +/-20µg/ml CTLA-4-Ig for five days then analysed by flow cytometry. **(A)** Overlaid histograms show T cell proliferation (CTV fluorescence). Data are representative of nine independent experiments. **(B)** Graph shows the mean (+SEM) percentage of T cells that committed to divide using replicate data from nine independent experiments. None of the differences between naive and memory responses were statistically significant. **(C)** Graphs show the mean (+SEM) percentage of T cells that committed to divide when cultured with FcR CD80 CHO (data outlined in *(B)*). The percentage decrease in proliferation caused by the addition of CTLA-4-Ig is shown. Differences between data were statistically significant according to the t test ****P<0.0001.

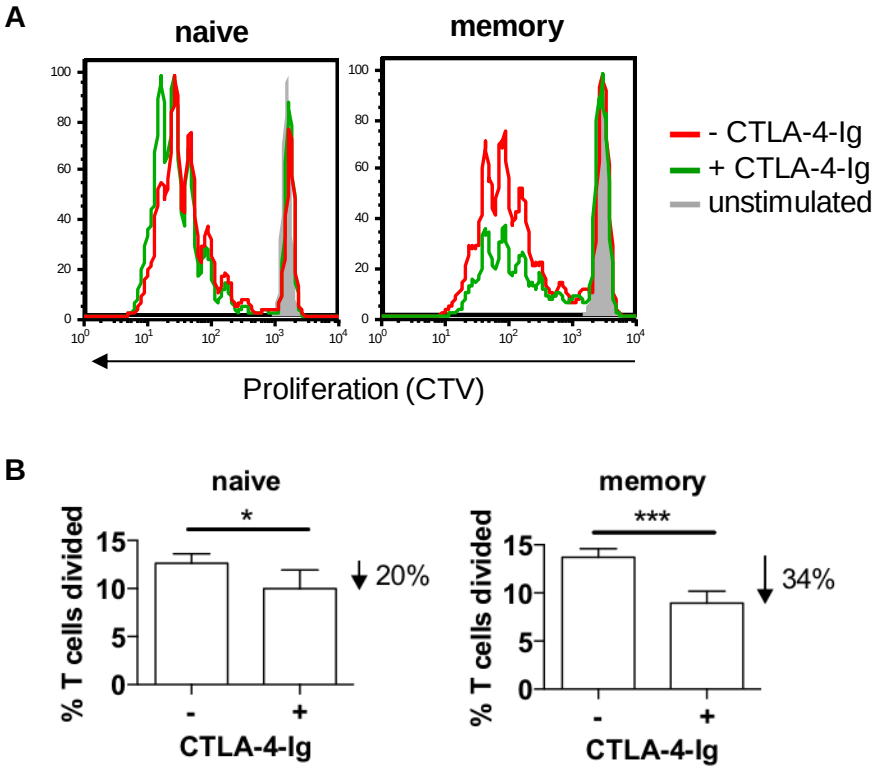


Figure 5.30 Antigen-stimulated memory T cells are more CD28 dependent than naive T cells. CTV-labelled $CD4^{+}CD25^{-}$ naive or memory T cells were incubated at 37°C with 250ng/ml TSST and fixed CHO cells expressing HLA-DR4 CD80 +/-20µg/ml CTLA-4-Ig for five days then analysed by flow cytometry. **(A)** Overlaid histograms show T cell proliferation (CTV fluorescence). Data are representative of three independent experiments. **(B)** Graphs show the mean (+SEM) percentage of T cells that committed to divide using triplicate measurements from three independent experiments. The percentage decrease in proliferation caused by the addition of CTLA-4-Ig is shown. Differences between data were statistically significant according to the t test *P<0.05 ***P<0.001.

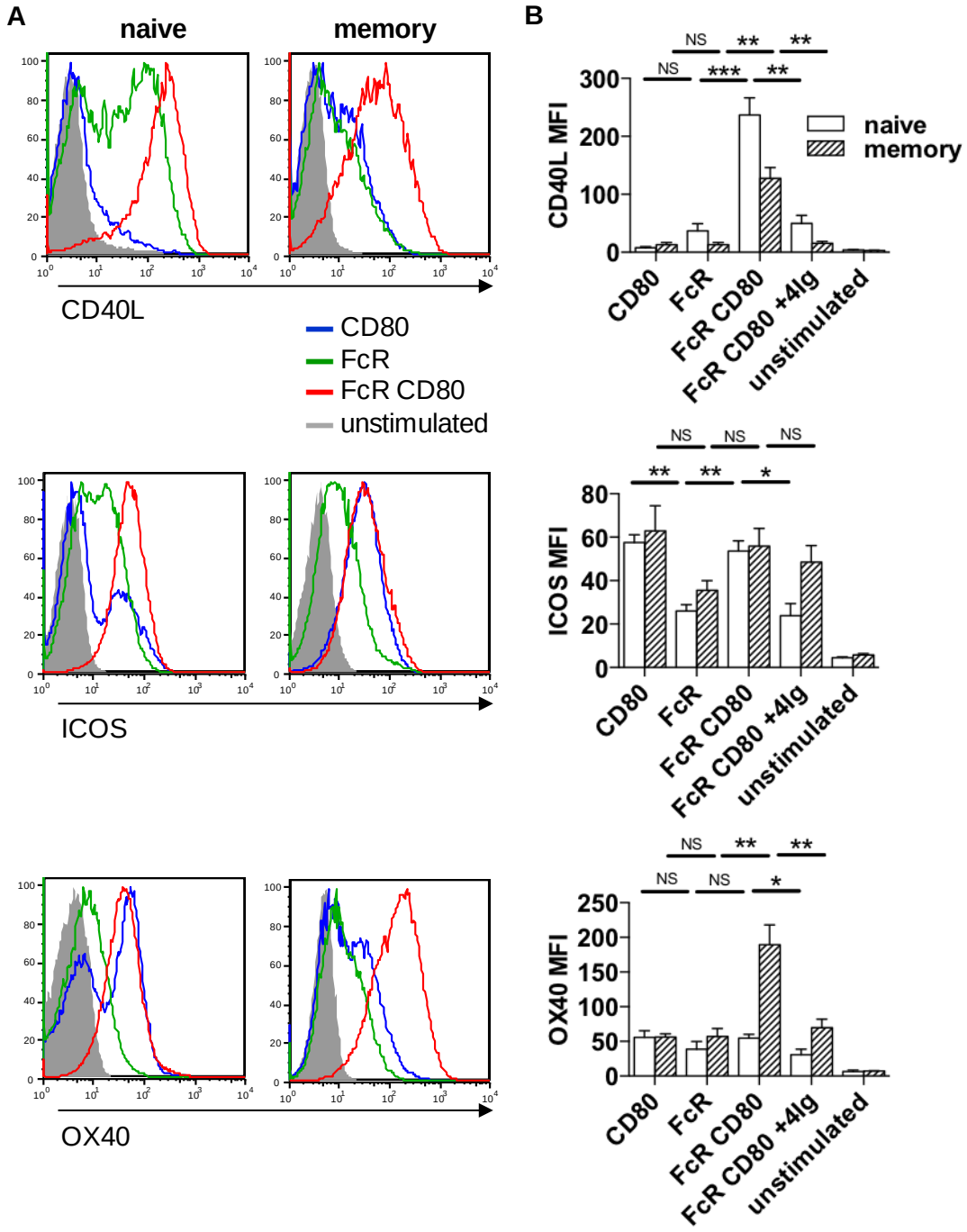


Figure 5.31 Memory T cells require CD28 costimulation for effector molecule expression. $CD4^{+}CD25^{-}$ naive or memory T cells were incubated at 37°C with 1µg/ml anti-CD3 and fixed CHO cells expressing CD80, FcR or FcR CD80 +/-20µg/ml CTLA-4-Ig for two or five days then stained for surface protein expression and analysed by flow cytometry. **(A)** Overlaid histograms show CD40L expression (day 2) and ICOS and OX40 MFI (day 5). Data are representative of at least three independent experiments. **(B)** Graphs show the mean (+SEM) MFI of all T cells (CD40L) or responding T cells only (ICOS and OX40) using data from 3-6 independent experiments. Differences between data were statistically significant according to the t test *P<0.05 **P<0.001 ***P<0.001 NS=not significant.

5.8 Discussion

The aim of this chapter was to study the outcomes of restricting the availability of CD28 costimulation to CD4 T cells in order to better understand the potential functional impact of ligand trans-endocytosis by CTLA-4. The two-signal model states that T cell activation requires both a TCR signal and a costimulatory signal via CD28, and yet CD28-deficient mice can still produce T cell responses (Brown et al., 1996; Kawai et al., 1996; Honstettre et al., 2006; Hogan et al., 2001). The studies presented here revealed that CD28 is not always essential for T cell activation or proliferation, but the phenotype of T cells stimulated in the absence of CD28 costimulation is dramatically altered. My data suggest that CD28-independent responses are predominantly driven by high intensity TCR signals and occur mainly in naive T cells. During CD28-independent responses, naive T cell markers remained highly expressed while the upregulation of proteins required for optimal function such as CD40L, ICOS, CD71 and OX40 was limited. Cytokine production was also reduced in the absence of CD28 costimulation, except IL-10 production, which was enhanced. Indeed, TCR stimulation without adequate costimulation has been shown to generate IL-10-producing T cells which suppress the activation of other T cells (Groux et al., 1997; Buer et al., 1998; Lombardi et al., 1994; Koenen and Joosten, 2000; Boussiotis et al., 2000; Sundstedt et al., 2003; Levings et al., 2005; Gabrysova and Wraith, 2010). Altogether these results suggest that blocking CD28 may not completely inhibit T cell activation but the response may be suboptimal or skewed towards a particular phenotype. There have been similar findings in mouse models where a lack of CD28 costimulation impairs T cell differentiation and cytokine production but T cell expansion is not affected and the T cell response is still effective. For example, CD28-deficient *scurfy* (FoxP3 deficient) mice still develop autoimmune disease (Singh et al., 2007; Eberlein et al., 2012) and CD80/86-deficient mice can still clear a viral infection (Singh et al., 2007; Eberlein et al., 2012). This suggests that T cells stimulated in the absence of CD28 costimulation have an abnormal phenotype but still produce an effective response in the absence of Tregs or in the presence of strong antigen. My studies

were consistent with these reports. I stimulated T cells by cross-linking CD3 in the absence of nTregs and found that a lack of CD28 costimulation did not prevent T cell activation or expansion, and protein expression and cytokine production were not completely abrogated. The production of some cytokines, in particular those associated with T_H2 responses, seemed more CD28 dependent than others, suggesting that T cell differentiation may be skewed. Overall it is apparent that Treg function and the strength of TCR signalling influence the fate of T cells stimulated in CD28-deficient conditions.

5.8.1 Investigating the role of CD28 in FoxP3 expression

To further explore T cell differentiation in the absence of CD28 costimulation, I studied the generation of FoxP3-expressing cells. I found that both sufficient TCR stimulation and CD28 costimulation were required for FoxP3 expression. It was initially surprising that FoxP3-expressing cells were generated in response to the stronger TCR stimulus (1µg/ml anti-CD3 cross-linked via FcR CD80 CHO) but not in response to weaker TCR stimulation (soluble anti-CD3 with CD80 CHO), because it has been shown that suboptimal TCR signals are required for iTreg differentiation while strong TCR signals inhibit iTreg development (Turner et al., 2009; Gottschalk et al., 2010; Gabrysova et al., 2011; Long et al., 2011; Molinero et al., 2011; Qiao et al., 2013). Although what these reports described as a strong TCR stimulus was in fact a very high dose of antigen. Instead, using 1µg/ml of plate bound anti-CD3 was found not to be too strong a stimulus for Treg induction, which is more consistent with my observations (Gabrysova et al., 2011; Molinero et al., 2011). The role of CD28 in iTreg development is controversial. Some studies have shown that CD28 is required for Treg induction (Liang et al., 2005; Guo et al., 2008), although possibly only due to the increased production of IL-2 (Davidson et al., 2007). In contrast, others have shown that a lack of CD28 engagement or weak CD28 stimulation is optimal for Treg induction (Benson et al., 2007; Semple et al., 2011; Ma et al., 2012; Etemire et al., 2013). This may be because strong PI3K-AKT-mTOR signalling inhibits FoxP3 upregulation and iTreg development (Haxhinasto

et al., 2008; Sauer et al., 2008; Turner et al., 2009; Tomasoni et al., 2011; Etemire et al., 2013). Indeed, mTOR-deficient T cells all differentiate into Tregs (Delgoffe et al., 2009). As the TCR and CD28 are both upstream of the PI3K-AKT-mTOR pathway, it is not surprising that a recent report demonstrated that the combined strength of TCR and CD28 signalling determined the extent of FoxP3 induction (Gabrysova et al., 2011). Indeed, the inhibition of FoxP3 expression upon strong stimulation in their study was mediated in part by the PI3K-AKT-mTOR pathway. Consistent with my findings, this report showed that relatively weak TCR (1µg/ml cross-linked anti-CD3) and strong CD28 stimulation was optimal for FoxP3 upregulation, while weak stimulation of both receptors resulted in low FoxP3 expression. This suggests that CD28 blockade may impair Treg induction. As already discussed, my results indicate that a lack of CD28 costimulation diminishes but doesn't completely abrogate the production of effector cytokines, including the T_H17-associated cytokines IL-17 and IL-21, which are strongly implicated in the pathogenesis of autoimmune diseases such as rheumatoid arthritis (Azizi et al., 2013). Unfortunately if a lack of CD28 costimulation also prevents $FoxP3^+$ Treg induction, the T cells that do effectively differentiate into T helper cells may not be suppressed as they should be in the absence of costimulation. Recent research has shown that the differentiation of naive T cells into T_H17 and Treg lineages is mutually exclusive and that the relative activity of these two populations may be instrumental in the development of autoimmunity versus tolerance (Afzali et al., 2007; Afzali et al., 2010). Overall, my data indicate that the generation of iTregs requires CD28 costimulation, while the production of effector cytokines is reduced but not inhibited by CD28 blockade. This indicates that therapies targeting the CD28 pathway should be used with caution because CD28 controls both effector and regulatory fates.

5.8.2 The requirement for CD28 costimulation varies between individuals

Although T cell proliferation was always initiated to some extent in the absence of CD28 costimulation, the percentage of T cells that committed to divide varied quite widely between

donors. This suggests that costimulatory requirements differ between individuals, which may contribute to differences in susceptibility to autoimmune disease. For example, less dependence on CD28 costimulation may allow T cell activation in the absence of infection and make T cell inhibition via CTLA-4 less effective. In fact, altered TCR signalling thresholds have been observed in autoimmunity, which may reduce the requirement for CD28 costimulation and so reduce the impact of blocking this pathway (Singh et al., 2009; Sakaguchi et al., 2012). Also there is often an expanded population of $CD4^{+}CD28^{null}$ T cells in autoimmune disease, which may contribute to disease pathology (Thewissen et al., 2007; Pieper et al., 2013). Furthermore, differences in costimulatory requirements may partly explain the variation between patients in the efficacy of CTLA-4-Ig in treating rheumatoid arthritis (Genovese, 2005; Schiff et al., 2008). Indeed a recent study of T cells from rheumatoid arthritis patients showed there was a general trend for upregulation of inflammatory cytokines when CTLA-4 was blocked and downregulation of cytokine production when CD28 was blocked, however in some patients the opposite was observed or the response was unaffected by costimulation blockade (Kormendy et al., 2013). These apparent differences in CD28 dependency may be due to genetic factors and may partly explain the differences in the incidence of autoimmune diseases between populations (Tobon et al., 2010). Indeed, the genetic background of B7-deficient mice determines whether or not CD28 costimulation is required for the induction of autoimmunity (Jabs et al., 2002). While CD28 independence may or may not be a means by which tolerance can be broken, CTLA-4-Ig is nonetheless an effective treatment for many patients. My data support the possibility that some T cells are CD28-dependent while others are CD28-independent, which may account for the variability in the efficacy of CTLA-4-Ig. According to my studies, measuring the expression of surface proteins including CD28, CD40L, OX40 and CD71 on T cells from patients with autoimmune disease may be a simple way to determine whether the T cells received CD28 costimulation. Analysis of these biomarkers could provide valuable information about the disease mechanism or patient suitability for CTLA-4-Ig treatment.

5.8.3 TCR and CD28 signalling are synergistic

In the previous chapter it was shown that increasing the TCR stimulus reduced the amount of costimulation required to initiate T cell proliferation. This suggests that the combined strength of TCR and CD28 signalling controls T cell division. Using the FcR CHO model to cross-link anti-CD3 provides a strong enough signal so that CD28 costimulation is not required at all to initiate T cell proliferation. This supports evidence that TCR and CD28 signalling pathways are integrated and therefore TCR and CD28 signalling combined control T cell activation (Tuosto and Acuto, 1998; Salojin et al., 1999; Dennehy et al., 2007; Holdorf et al., 2002; Michel et al., 2001; Diehn et al., 2002; Riley et al., 2002), suggesting that if there is sufficient stimulation of one of these receptors the other is not required. However using the FcR CHO model I found that despite robust proliferation in the absence of CD28 costimulation, the T cells retained naive characteristics and failed to optimally upregulate effector proteins such as OX40 and CD71. Therefore I tested whether increasing the TCR signal could compensate for this and found that strong TCR signalling did not reduce the need for CD28 engagement to promote these phenotypic changes upon T cell activation. This indicates that CD28 does not solely amplify TCR signals but it also has unique functions mediated by distinct signalling pathways. My data show that CD28 is specifically required for OX40 and CD71 expression, which are vital for full T cell activation and expansion, survival and anergy avoidance, B cell help and the development of memory T cells (Ponka and Lok, 1999; Walker et al., 2000; Zheng et al., 2007; Zheng et al., 2009; Croft et al., 2009). Others have also shown that CD28 signalling is necessary for T_H2 differentiation, which cannot be rescued by strong TCR signalling (Tao et al., 1997; Manickasingham et al., 1998; Smeets et al., 2012). My data also demonstrated the strict requirement for CD28 costimulation for T_H2 cytokine production. Furthermore, stimulation of T cells with both anti-CD3 and anti-CD28 together resulted in greater proliferation than stimulation with either antibody alone, suggesting that the two signals are synergistic. Although it is possible one receptor activates downstream signalling

molecules more effectively than the other, these data support the model that TCR and CD28 have both overlapping and distinct signalling pathways.

5.8.4 mTOR and IL-2 mediate some but not all functions of CD28 costimulation

In recent years the serine/threonine kinase mTOR has come to be known as a major regulator of the T cell response that integrates signals from a variety of stimuli (Delgoffe and Powell, 2009). CD28-mediated activation of the PI3K/AKT pathway leads to the activation of mTOR, which then promotes many outcomes of CD28 signalling such as the upregulation of CD71 and T cell differentiation (Edinger and Thompson, 2002; Tomasoni et al., 2011). CD28 also indirectly activates mTOR by enhancing IL-2 production, as IL-2 receptor signalling also activates the PI3K/AKT pathway upstream of mTOR (Colombetti et al., 2006; Lin et al., 2009). My data show that strong TCR and CD28 signalling were both required for phosphorylation of the mTOR substrate S6 kinase and that weaker TCR signalling and CD28 costimulation was not sufficient. Yet CD71 was highly upregulated in response to CD28 signalling no matter what the TCR signal strength, suggesting that CD28 also functions independently of pS6. Indeed, PI3K signalling can activate both mTOR-dependent and mTOR-independent pathways (Gamper and Powell, 2012). However it is important to note that S6 kinase phosphorylation only measures mTORC1 activity and not mTORC2, and it has been suggested that phosphorylation of the S6 kinase residues threonine 421/serine 424 is more representative of mTOR activity in T cells than phosphorylation at serine 235/236 (Zheng et al., 2007). Ongoing studies of downstream effectors of CD28 signalling, in particular mTOR, may provide further therapeutic targets for treating autoimmunity and manipulating the immune system in organ transplantation and cancer.

It has long been known that IL-2 production is greatly enhanced following CD28 engagement and that IL-2 promotes T cell growth and survival. To investigate the extent to which IL-2 is responsible for the downstream effects of CD28 costimulation, exogenous IL-2 was provided

in the absence of CD28 engagement. It was found that IL-2 rescued naive T cell proliferation, showing that IL-2 can promote T cell proliferation in the absence of CD28 signals. Indeed others have also shown that IL-2 can restore T cell proliferation in the absence of CD28 costimulation if there is sufficient antigen (Lumsden et al., 2003). Although IL-2 is known to be a potent stimulator of T cell division, there are clearly IL-2-independent mechanisms of cell cycle progression (Appleman et al., 2000; Colombetti et al., 2006; Lupino et al., 2010). In contrast to its positive effects on T cell proliferation, IL-2 could not rescue CD45RA downregulation or the expression of OX40 and CD71, which indicates again that there are IL-2-independent functions of CD28. Notably, IL-2 could not rescue CD28 deficiency despite being an upstream activator of mTOR, again showing that mTOR does not mediate all CD28 functions. Understanding the complex signalling pathways separating and integrating CD28, IL-2 and mTOR signalling may allow us to finely control T cell activation to avoid unwanted responses without inhibiting responses to infection.

5.8.5 A lack of CD28 costimulation is not sufficient to induce T cell anergy

Interestingly it was found that T cells primed in the absence of CD28 costimulation could be restimulated to produce a robust proliferative response and upregulate effector molecules if CD28 costimulation was subsequently provided. This suggests that a lack of CD28 does not necessarily render T cells unresponsive to restimulation as long as costimulation is provided with the second exposure to antigen. It was also observed that T cells which received costimulation during their primary stimulation could subsequently divide once or twice in the presence of TCR stimulation or CD28 costimulation alone. However these cells no longer expressed CD25, ICOS and CD71, so this limited cell division was probably a continuation of the primary response and should not be considered a CD28-independent secondary response. Despite having shown that resting T cells could be activated and proliferate in the absence of CD28, I expected the expanded cells to be unresponsive to subsequent stimulation. Previous studies have shown that T cells can go through an effector phase

before tolerance induction (Huang et al., 2003) but ultimately a lack of CD28 costimulation is believed to result in anergy so the T cells cannot be restimulated (Lumsden et al., 2003; Schwartz, 2003). However some reports have shown that T cells primed without CD28 costimulation can be restimulated with costimulation (Lane et al., 1996), although unlike my findings, most found that proliferation and cytokine production were reduced during the second response (Schweitzer and Sharpe, 1998; Fuse et al., 2008). In fact Schweitzer and Sharpe found that there was a complete lack of IL-4 production, suggesting that T cells primed without CD28 costimulation can be restimulated to proliferate but they cannot differentiate into T_H2 effector cells. Unfortunately cytokine production was not measured in my studies so the effector function of the restimulated T cells primed without CD28 costimulation remains unclear. However T cells primed without CD28 costimulation did upregulate CD25, ICOS and OX40 to normal levels upon second stimulation with CD28, suggesting that the cells were not completely non-functional. This suggests that tolerance may not be successfully induced by lack of CD28 costimulation upon priming, which indicates how autoimmunity can develop if CD28 is subsequently engaged. This may be possible in an inflammatory environment and explains the strong association of autoimmunity with infection (Beyerlein et al., 2013; Cubas-Duenas et al., 2013; Croia et al., 2013).

In addition, I found that T cells stimulated twice in the absence of CD28 costimulation did not enter cell division but importantly they were not deleted. This was surprising because a lack of CD28 costimulation is reported to lead to cell death (Lumsden et al., 2003) as CD28 signalling normally inhibits apoptosis and promotes cell survival (Boise et al., 1995; Wan and DeGregori, 2003; Kirchhoff et al., 2000; Jones et al., 2002). It is possible that although the T cells stimulated twice without CD28 costimulation were not deleted they may have become anergic. It has been shown that autoreactive T cells chronically stimulated *in vivo* become anergic but are not deleted (Steinert et al., 2012). In the future it would be interesting to include further rounds of restimulation and to analyse T cell differentiation. It has been

reported that prolonged or repeated TCR stimulation causes unresponsiveness and the development of IL-10-producing cells (Groux et al., 1997; Buer et al., 1998; Lombardi et al., 1994; Koenen and Joosten, 2000; Boussiotis et al., 2000; Sundstedt et al., 2003; Levings et al., 2005; Gabrysova and Wraith, 2010). Overall these data show that CD28 signals are not necessarily required to prevent anergy or cell death but they are needed to produce a secondary or memory T cell response. This implies that CD28 is involved in chronic autoimmune disease and therefore this pathway remains a promising therapeutic target.

5.8.6 Memory T cells also require CD28 costimulation

Having demonstrated that CD28 costimulation was required to restimulate T cells primed *in vitro*, I wanted to investigate the costimulatory requirements of memory T cells further. Interestingly, my data show that memory CD4 T cells require CD28 costimulation for an optimal proliferative response. Furthermore, it was found that memory T cells were more dependent on CD28 costimulation than naive cells for cell division. In addition memory T cells required CD28 signalling for the upregulation of CD40L, ICOS and OX40, suggesting that memory T cell function is CD28-dependent. These findings support recent studies in mice, that contrary to initial *in vitro* studies, have shown that memory T cells do require CD28 costimulation (Ndejembi et al., 2006; Garidou et al., 2009; Fuse et al., 2011; Ndlovu et al., 2014). In addition, other research has shown that central memory T cells require CD28 costimulation for effective proliferation and cytokine production while effector memory cells in the target tissue are CD28 independent, which may explain some of the discrepancy between studies (Fontenot et al., 2003a; Teijaro et al., 2009; Bottcher et al., 2013). Consistent with my data, Teijaro et al. showed that the effect of costimulation blockade on antibody production was greater during a memory response compared to naive. In fact it was recently shown that in the absence of CD28 costimulation there was greater phosphorylation of TCR-proximal signalling molecules in naive T cells compared to memory cells, suggesting that memory T cells may be more dependent on CD28 to amplify TCR signals (Kalland et al.,

2011). Although these signalling events were still stronger in naive T cells than memory cells in the event of CD28 engagement, therefore these differences may depend on more than CD28. It has been reported that memory T cells have a higher RNA content than naive cells, supposedly to allow rapid proliferation and translation of effector molecules without the need to wait for the activation of transcription (Allam et al., 2009; Giardino Torchia et al., 2013). However this does not necessarily make memory T cells CD28 independent because it has recently been suggested that CD28 is less important for gene transcription and more important for alternative splicing of RNA (Butte et al., 2012), so perhaps memory T cells still require CD28 after all. Also, it has been shown that memory T cells like naive cells need CD28 costimulation to upregulate Bcl-xL for survival and to downregulate Bcl-2 to allow cell cycle progression (Boesteanu and Katsikis, 2009).

The conflicting publications regarding the costimulatory requirements of memory T cells may be due to differences in the antigen type, dose or exposure time, which are all factors shown to alter the costimulatory requirements of memory T cells (Garcia et al., 2004; Floyd et al., 2011). Studies may also vary depending on how the T cell response was measured because it has been postulated that there are two TCR signalling pathways in memory T cells that have different functional outcomes and differential requirements for CD28. These consist of an early pathway to cytokine production that does not require CD28 costimulation and a late pathway to proliferation and IL-2 production which does require CD28 (Farber, 2009). In summary, the data presented here show that memory T cells are dependent on CD28 costimulation. Memory T cells were tested using a simple experimental model here and in the presence of additional costimulatory molecules memory T cells may be less dependent on CD28, therefore further studies using DC to test memory T cell requirements for CD28 costimulation will be very interesting. Nonetheless my findings are consistent with other recent research in this field. CD28 dependency of memory T cells should make it harder for autoimmunity to develop. In the future it would be interesting to compare the memory T cell

response of patients with autoimmunity against their healthy relatives to investigate whether autoreactive memory T cells have altered costimulatory requirements.

5.8.7 Summary

Overall these studies showed that inhibition of CD28 signalling did not prevent T cell activation and proliferation but T cell differentiation was affected. In particular, the generation of T_H2 helper cells and iTregs was impaired, while production of the anti-inflammatory cytokine IL-10 was increased. Together with reduced production of inflammatory cytokines and limited expression of stimulatory receptors and nutrient transporters, these findings suggest that inhibition of CD28 costimulation by CTLA-4 may not be sufficient to avoid T cell activation and proliferation but overall the effector response will be dampened. To add further complexity to this finely balanced system, costimulatory requirements appear to vary between individuals and T cells, which may partly explain the development of autoimmunity and the variable efficacy of CTLA-4-Ig treatment. These studies showed that the expression of certain T cell proteins is dependent on CD28 signalling, therefore analysis of these markers may allow us to predict which patients would respond to CD28 blockade. It was also found that CD28 signals were not required to prevent anergy or cell death but they were needed to produce a secondary T cell response. Furthermore, it was found that memory T cells were very sensitive to a lack of CD28 costimulation and were in fact more CD28-dependent that naive T cells in the conditions tested. This suggests that CD28 plays a role in chronic autoimmune disease and is therefore a suitable target for treating established autoimmunity.

6 FINAL DISCUSSION

6.1 Background

The immune system has evolved to protect us against a diverse range of pathogens. TCR diversity is achieved through TCR gene rearrangement, although unfortunately this also results in the generation of autoreactive T cells that can cause autoimmune disease. To restrict the release of autoreactive T cells from the thymus, cells expressing receptors with strong affinity for self antigens are deleted. However some autoreactive T cells still escape into the periphery. Understanding how T cells are activated and regulated may help us develop novel therapies to enhance or dampen the immune system to treat cancer or autoimmune disease. CD28 costimulation is a key checkpoint that positively regulates T cell activation. CTLA-4 is the counter-receptor for CD28 which negatively regulates T cell activation. The CD28-CTLA-4 axis is therefore an attractive target for manipulating T cell immunity. Fully understanding the functional mechanisms of these receptors is necessary in order to design specific and effective therapeutics.

CD28 has long been known to be critical for T cells responses (Shahinian et al., 1993; Lucas et al., 1995; Ferguson et al., 1996) and yet CD28-deficient mice can still have effective T cell immunity (Brown et al., 1996; Kawai et al., 1996; Honstettre et al., 2006; Hogan et al., 2001). The exact role of CD28 costimulation is therefore not entirely clear and it remains to be fully defined in what context CD28 signalling is required and when it is redundant. It is also unclear what outcomes of T cell activation are strictly CD28-dependent. In addition, the functional mechanism of CTLA-4 has been a matter of debate for many years. Some studies have presented CTLA-4 as providing a cell intrinsic negative signal (Schneider et al., 1995; Bradshaw et al., 1997; Zhang and Allison, 1997), while in vivo models have shown CTLA-4 to function extrinsically (Bachmann et al., 1999; Homann et al., 2006; Friedline et al., 2009; Wang et al., 2012; Corse and Allison, 2012). CTLA-4 has also been shown to play a major

role in Treg suppressive function (Read et al., 2000; Takahashi et al., 2000; Wing et al., 2008), which implicates a cell extrinsic mechanism of action. Consistent with the cell extrinsic model of CTLA-4 function and its cell biology, our laboratory has described a novel mechanism whereby CTLA-4 removes its ligands from opposing cells by trans-endocytosis (Qureshi et al., 2011). In light of this, the primary aim of this project was to study the molecular mechanism of CTLA-4 trans-endocytosis further and to begin to examine the functional implications of reducing the availability of costimulatory molecules.

6.2 Clathrin-mediated endocytosis is not essential for CTLA-4 trans-endocytosis

In order to study trans-endocytosis two complementary methods were used: high throughput flow cytometry and confocal microscopy for visualisation. Using these techniques to measure ligand acquisition by CTLA-4-expressing cells, it was found that the YVKM motif in the cytoplasmic tail of CTLA-4 required for clathrin-mediated endocytosis was not essential for CTLA-4 trans-endocytosis. This was surprising because I expected trans-endocytosis to utilise the same machinery as that used for the constitutive endocytosis of CTLA-4. The molecular pathways involved in trans-endocytosis therefore remain to be elucidated. The trans-endocytosis of CD80 and CD86 was also compared and a trend was found suggesting that CD80 is captured more efficiently than CD86. Given the structural differences between these two ligands there is a possibility that they are captured via different mechanisms. In order to fully establish the molecular mechanisms and kinetics of trans-endocytosis further studies are required.

6.3 Altering the availability of CD28 ligands finely controls T cell proliferation

The functional implications of removing costimulatory ligands by CTLA-4 trans-endocytosis were also investigated. It was found that CD28 ligand availability had a graded effect on the number of T cells that committed to divide, suggesting that CTLA-4 trans-endocytosis can fine-tune the size of the T cell response. Furthermore, CD28 costimulation was important for

both initiating and maintaining T cell proliferation, suggesting that negative regulation via CTLA-4 is effective at all stages of the T cell response. Interestingly, a lack of CD28 costimulation did not completely inhibit T cell proliferation unless costimulation was blocked for a sustained period, suggesting that CTLA-4 trans-endocytosis may be most effective in the context of low costimulatory molecule expression or non-inflammatory conditions. My studies also showed that the level of costimulatory ligands required for T cell activation depended on the strength of the TCR stimulus. More costimulatory molecules were needed to initiate T cell proliferation in the presence of low TCR stimulation. Given that autoreactive TCR have lower antigen affinity, the data suggest that limiting the availability of costimulatory molecules would have more impact on an autoimmune response.

6.4 T cells that do not receive CD28 costimulation can escape anergy and death

Additional studies were performed to examine the fate of T cells activated in absence of CD28 costimulation. It was found that CD28-independent responses required a strong TCR stimulus and mainly occurred in naive T cells not memory T cells. Additionally it was found that CD28 costimulation was required for a fully effective response because T cells that did not receive CD28 costimulation did not optimally upregulate effector molecules or produce T_H2 cytokines. Yet surprisingly, T cells primed in the absence of CD28 costimulation did not become anergic because they could be restimulated upon second exposure to antigen if CD28 was subsequently provided. These cells appeared to be fully activated as they upregulated CD25, ICOS and CD71, although cytokine production was not measured. Nevertheless, the data imply that autoreactive T cells could be primed in non-inflammatory conditions and persist, and later become reactivated during an infection when activated APC can provide sufficient costimulation. Furthermore, it was found that T cells stimulated twice in the absence of CD28 costimulation were not deleted, again demonstrating that a lack of CD28 costimulation does not always ensure tolerance.

6.5 Memory T cells require CD28 costimulation

Crucially, CD28 was required for a secondary response *in vitro*, and consistent with this *ex vivo* memory T cells also required CD28 costimulation for proliferation and effector molecule expression. These findings were particularly interesting because they contradict the traditional view that memory T cells are less dependent on costimulation, and support emerging evidence that memory T cells do in fact require CD28 signals. My data even suggest that memory T cells are more dependent on CD28 than naive T cells. Importantly this suggests that CD28 is involved in chronic autoimmune disease which is driven by memory T cells, and CD28 is therefore an attractive and relevant therapeutic target.

6.6 Therapies that target more specific proteins or cell populations could improve drug efficacy and safety

Drugs used to systemically block CD28 or CTLA-4 can have unwanted side effects. For example the CTLA-4 blocking antibody Ipilimumab has shown great success in treating melanoma and other malignant cancers (O'Day et al., 2007; Hodi et al., 2010), however CTLA-4 blockade to enhance tumour immunity often causes unwanted autoimmunity and inflammation (Della Vittoria Scarpati et al., 2014). On the other hand, CD28 blockade to treat autoimmunity and prevent transplant rejection may inhibit memory responses making patients vulnerable to infections they were vaccinated against or previously exposed to (Linsley and Nadler, 2009). Understanding these costimulatory pathways in more detail may allow us to target them more specifically. Interestingly, a recent study found that ITK-mediated CD28 signals are not required for T cell activation or proliferation but are required for autoreactive T cells to enter non-lymphoid tissues (Jain et al., 2013), so potentially this pathway could be targeted to treat autoimmunity without systemic immunosuppression. Alternatively, specific T cells could be targeted using a new technology consisting of soluble monoclonal TCR molecules fused to an immune-modulating component (Liddy et al., 2012),

for example an autoantigen-specific TCR linked to CTLA-4 could be used to bind to the target tissue and inhibit localised T cells (Sewell, 2012).

6.7 Costimulatory requirements vary between individuals: Biomarkers to stratify patients and tailored treatments may improve disease outcome

Another issue to take into consideration when targeting CD28/CTLA-4 is patient variability. My studies have shown that a lack of CD28 costimulation has a variable effect on T cell proliferation between individuals. Consistent with this, the efficacy of abatacept has been shown to vary considerably between patients (Genovese, 2005; Schiff et al., 2008; Kormendy et al., 2013), suggesting that patients may inherently have different costimulatory requirements, or perhaps that disease can occur without CD28. Notably, my data and that of others (Rulifson et al., 1997; Lenschow et al., 1996; Smeets et al., 2012) have shown that CD28 mainly promotes T_H2 responses, which may partly explain why CD28 blockade is not always effective at treating a T_H1 and T_H17 driven disease such as rheumatoid arthritis (Boissier et al., 2012). Alternatively, differences in costimulatory requirements may contribute to differences in susceptibility to autoimmunity, in that less dependence on CD28 costimulation may allow T cell activation in non-inflammatory conditions and make CTLA-4-Ig less effective. Also, altered TCR signalling thresholds have been observed in autoimmunity (Singh et al., 2009; Sakaguchi et al., 2012), which may reduce the requirement for CD28 costimulation and so reduce the impact of blocking this pathway. It would be interesting to test T cells from the same donor on repeated occasions to investigate whether every individual has different but consistent costimulatory requirements and to subsequently study how those requirements relate to genotype. It has been shown that the genetic background of mice determines whether they require costimulation for the development of autoimmunity (Jabs et al., 2002). In humans, genetic polymorphisms in numerous genes, including CD28, CTLA-4, ICOS, HLA-DR4, PTPN22 and ZAP70 have been associated with autoimmunity (Raychaudhuri et al., 2009; Kim et al., 2010; Li et al., 2012; Chen et al., 2013; Zouidi et al.,

2014). It is likely that the unique combination of genetic variants of these and other genes determines the T cell dependency on CD28 and how well T cells can be regulated by CTLA-4, all contributing to give each individual a unique likelihood of autoimmunity. Interestingly, there are distinct differences in CD28, CTLA-4 and ICOS haplotypes between separate populations, which may contribute to observed differences in the incidence of autoimmune diseases between populations (Butty et al., 2007). My studies have shown that expression of surface proteins including CD28, CD40L, OX40 and CD71 is dependent on CD28 costimulation. Therefore measuring the expression of these proteins on T cells from patients with autoimmune disease may be a simple way to determine whether the T cells received CD28 costimulation. Analysis of these biomarkers could therefore provide valuable information about the disease mechanism or patient suitability for CTLA-4-Ig treatment.

6.8 Future perspectives

Intriguingly, my studies found that PD-1 can internalise its ligands, although the kinetics of ligand acquisition differed from CTLA-4 trans-endocytosis. There is not known to be a stimulatory receptor for PD-L1 and PD-L2 which would explain the purpose for removing these ligands as part of the inhibitory function of PD-1, although some studies have indicated PD-L1 and PD-L2 may have stimulatory effects (Dong et al., 1999; Tseng et al., 2001; Tamura et al., 2001; Wang et al., 2003; Shin et al., 2003; Shin et al., 2005). Alternatively ligand internalisation may be part of a negative feedback mechanism by which PD-1 regulates its own function. Further studies are required to confirm whether PD-1 ligand internalisation occurs by trogocytosis, trans-endocytosis or by another mechanism, and also to begin to investigate the functional role of this phenomenon. Similarly, OX40 ligand acquisition was also observed during my studies, which I speculate could be a mechanism of negative regulation to inhibit T cell expansion. Further work is required to verify whether human OX40 can also capture ligand and subsequently to explore the role of this mechanism in T cell biology.

Another question that remains to be answered is why there are two different CD28 ligands. This question could be addressed using the inducible expression system I have developed, by expressing the two ligands together at different ratios or at different times in order to compare their functionality, including most importantly their individual and combined effects on cytokine production. Additionally, the importance of CD28 costimulation may depend on the expression of other costimulatory molecules and on other characteristics of the APC. The experiments presented here used a very simple model to begin to quantify the level of CD28 costimulation required in a controlled setting without unknown influential factors, but in the future it would be interesting to use real APC of different types and knockdown costimulatory ligands with siRNA to study the costimulatory requirements of T cells in a more physiological context.

6.9 Final summary

Understanding the molecular mechanism of trans-endocytosis may reveal novel ways of targeting the CD28-CTLA-4 system in order to manipulate T cell responses in the context of autoimmunity, anti-tumour immunity and transplant rejection. Similarly, a more thorough understanding of CD28 function may allow the design of better targeted drugs and more appropriate tests for drug efficacy. This project has highlighted that while CD28 costimulation is not always required for T cell activation, controlling the number of costimulatory ligands can fine tune the proliferative T cell response. However CD28 dependency does vary between individuals. In addition, I have shown that CD28 is required for the upregulation of several important effector molecules and for T_H2 cytokine production. Importantly, contrary to early publications, I also found that a lack of CD28 costimulation does not necessarily cause anergy or T cell deletion. Most significantly my studies suggest that memory T cells are dependent on CD28 costimulation, which has important implications for immune therapies.

7 APPENDIX

Human CD80 cDNA sequence (CCDS2989.1 published at www.ncbi.nlm.nih/gov) cloned into vector pTRE3G and sequence verified after cloned into expression vector

atgggccacacacggaggcagggaacatcaccatccaagtgtccatacctcaatttctttcagctctt
ggtgctggctggtctttctcacttctgttcaggtgttatccacgtgaccaaggaagtgaaagaagtgg
caacgctgtcctgtggtcacaatgtttctgttgaagagctggcacaaactcgcatctactggcaaaag
gagaagaaaatggtgctgactatgatgtctggggacatgaatatatggcccgagtacaagaaccggac
catctttgatatcactaataacctctccattgtgatcctggctctgcgcccatctgacgagggcacat
acgagtgtgttgttctgaagtatgaaaaagacgctttcaagcgggaacacctggctgaagtgacgtta
tcagtcaaagctgacttccctacacctagtatatctgactttgaaattccaacttctaatattagaag
gataatttgctcaacctctggaggttttccagagcctcacctctcctggttggaaaatggagaagaat
taaatgccatcaacacaacagtttcccaagatcctgaaactgagctctatgctgttagcagcaaactg
gatttcaatatgacaaccaaccacagcttcatgtgtctcatcaagtatggacatttaagagtgaatca
gaccttcaactggaatacaaccaagcaagagcattttcctgataacctgctcccatcctgggccatta
ccttaatctcagtaaatggaatttttgtgatatgctgcctgacctactgctttgccccaagatgcaga
gagagaaggaggaatgagagattgagaagggaaagtgtacgccctgtataa

Human CD86 isoform 1 cDNA sequence (CCDS3009.1 published at www.ncbi.nlm.nih/gov) cloned into vector pTRE3G and sequence verified after cloned into expression vector

atggatccccagtgcactatgggactgagtaacattctctttgtgatggccttcctgctctctggtgc
tgctcctctgaagattcaagcttatttcaatgagactgcagacctgccatgccaatttgcaaactctc
aaaaccaaagcctgagtgagctagtagtattttggcaggaccaggaaaacttggttctgaatgaggta
tacttaggcaaagagaaatttgacagtgttcattccaagtatatgggccgcacaagttttgattcgga
cagttggaccctgagacttcacaatcttcagatcaaggacaagggcttgtatcaatgtatcatccatc
acaaaaagcccacaggaatgattcgcatccaccagatgaattctgaactgtcagtgcttgctaacttc
agtcaacctgaaatagtaccaatttctaatataacagaaaatgtgtacataaatttgacctgctcatc
tatacacggttacccagaacctaagaagatgagtgttttgctaagaaccaagaattcaactatcgagt
atgatggtgttatgcagaaatctcaagataatgtcacagaactgtacgacgtttccatcagcttgtct
gtttcattccctgatgttacgagcaatatgaccatcttctgtattctggaaactgacaagacgcggct
tttatcttcacctttctctatagagcttgaggaccctcagcctcccccagaccacattccttggatta
cagctgtacttccaacagttattatatgtgtgatggttttctgtctaattctatggaaatggaagaag
aagaagcggcctcgcaactcttataaatgtggaaccaacacaatggagagggaagagagtgaacagac
caagaaaagagaaaaaatccatatacctgaaagatctgatgaagcccagcgtgtttttaaaagttcga
agacatcttcatgcgacaaaagtgatacatgtttttaa

Human PD-L1 isoform A cDNA sequence (CCDS6464.1 published at www.ncbi.nlm.nih/gov) cloned into vector pEGFP-N3 and sequence verified after cloned into expression vector (Note: primer was designed to mutate stop codon during cloning to add GFP tag to protein)

ATGAGGATATTTGCTGTCTTTATATTCATGACCTACTGGCATTTGCTGAACGCATTTACTGTCACGGT
TCCCAAGGACCTATATGTGGTAGAGTATGGTAGCAATATGACAATTGAATGCAAATTCCCAGTAGAAA
AACAATTAGACCTGGCTGCACTAATTGTCTATTGGGAAATGGAGGATAAGAACATTATTCAATTTGTG
CATGGAGAGGAAGACCTGAAGGTTCAGCATAGTAGCTACAGACAGAGGGCCCGGCTGTTGAAGGACCA
GCTCTCCCTGGGAAATGCTGCACTTCAGATCACAGATGTGAAATTGCAGGATGCAGGGGTGTACCGCT
GCATGATCAGCTATGGTGGTGCCGACTACAAGCGAATTACTGTGAAAGTCAATGCCCCATACAACAAA
ATCAACCAAAGAATTTTGGTTGTGGATCCAGTCACCTCTGAACATGAACTGACATGTCAGGCTGAGGG
CTACCCCAAGGCCGAAGTCATCTGGACAAGCAGTGACCATCAAGTCCTGAGTGGTAAGACCACCACCA
CCAATTCCAAGAGAGAGGAGAAGCTTTTCAATGTGACCAGCACACTGAGAATCAACACAACAACTAAT

GAGATTTTCTACTGCACTTTTAGGAGATTAGATCCTGAGGAAAACCATACAGCTGAATTGGTCATCCC
AGAACTACCTCTGGCACATCCTCCAAATGAAAGGACTCACTTGGTAATTCTGGGAGCCATCTTATTAT
GCCTTGGTGTAGCACTGACATTCATCTTCCGTTTAAGAAAAGGGAGAATGATGGATGTGAAAAAATGT
GGCATCCAAGATACAAACTCAAAGAAGCAAAGTGATACACATTTGGAGGAGACGTAA

Human PD-L2 cDNA sequence (CCDS6465.1 published at www.ncbi.nlm.nih/gov) cloned into vector pEGFP-N3 and sequence verified after cloned into expression vector (Note: primer was designed to mutate stop codon during cloning to add GFP tag to protein)

atgatcttcctcctgctaatgttgagcctggaattgcagcttcaccagatagcagctttattcacagt
gacagtccctaaggaactgtacataatagagcatggcagcaatgtgaccctggaatgcaactttgaca
ctggaagtcatgtgaaccttggagcaataacagccagtttgcaaaaggtggaaaatgatacatcccca
caccgtgaaagagccactttgctggaggagcagctgcccctagggaaggcctcgttccacatacctca
agtccaagtgagggacgaaggacagtaccaatgcataatcatctatggggtcgcctgggactacaagt
acctgactctgaaagtcaaagcttcctacaggaaaataaacactcacatcctaaaggttccagaaaca
gatgaggtagagctcacctgccaggctacaggttatcctctggcagaagtatcctggccaaacgtcag
cgttcctgccaacaccagccactccaggacccctgaaggcctctaccaggtcaccagtgttctgcgcc
taaagccacccccctggcagaaacttcagctgtgtgttctggaatactcacgtgagggaacttactttg
gccagcattgaccttcaaagtcagatggaacccaggacccatccaacttggctgcttcacattttcat
ccccttctgcatcattgctttcattttcatagccacagtgatagccctaagaaaacaactctgtcaaa
agctgtattcttcaaaagacacaacaaaaagacctgtcaccacaacaaagagggaagtgaacagtgct
atctga

Human LFA-3 variant 3 (GPI-linked isoform) cDNA sequence (NCBI Reference Sequence NR_026665.1 published by Wallich et al. (1998)) cloned into vector pEGFP-N3 and sequence verified after cloned into expression vector (Note: primer was designed to mutate stop codon during cloning to add GFP tag to protein)

ATGGTTGCTGGGAGCGACGCGGGGCGGGCCCTGGGGGTCCTCAGCGTGGTCTGCCTGCTGCACTGCTT
TGGTTTCATCAGCTGTTTTTCCCAACAAATATATGGTGTTGTGTATGGGAATGTAACTTTCCATGTAC
CAAGCAATGTGCCTTTAAAAGAGGTCCTATGGAAAAAACAAAAGGATAAAGTTGCAGAACTGGAAAAT
TCTGAATTCAGAGCTTTCTCATCTTTTAAAAATAGGGTTTATTTAGACACTGTGTCAGGTAGCCTCAC
TATCTACAACTTAACATCATCAGATGAAGATGAGTATGAAATGGAATCGCCAAATATTACTGATACCA
TGAAGTTCTTTCTTTATGTGCTTGAGTCTCTTCCATCTCCCACACTAACTTGTGCATTGACTAATGGA
AGCATTGAAGTCCAATGCATGATACCAGAGCATTACAACAGCCATCGAGGACTTATAATGTACTCATG
GGATTGTCCTATGGAGCAATGTAAACGTAACTCAACCAGTATATATTTTAAGATGGAAAATGATCTTC
CACAAAAAATACAGTGTACTCTTAGCAATCCATTATTTAATACAACATCATCAATCATTTTGACAACC
TGTATCCCAAGCAGCGGTCATTCAAGACACAGATATGCACTTATACCCATACCATTAGCAGTAATTAC
AACATGTATTGTGCTGTATATGAATGTTCTTTAATTGAGAAGACAATTTCTTCATTTTTAGGTATTCT
GAAATGTGACAGAAAACCAGACAGAACCAACTCCAATTGA

Human LICOS isoform A cDNA sequence (CCDS42952.1 published at www.ncbi.nlm.nih/gov) cloned into vector pEGFP-N3 and sequence verified after cloned into expression vector (Note: primer was designed to mutate stop codon during cloning to add GFP tag to protein)

ATGCGGCTGGGCAGTCCTGGACTGCTCTTCCTGCTCTTCAGCAGCCTTCGAGCTGATACTCAGGAGAA
GGAAGTCAGAGCGATGGTAGGCAGCGACGTGGAGCTCAGCTGCGCTTGCCCTGAAGGAAGCCGTTTTG
ATTTAAATGATGTTTACGTATATTGGCAAACCAGTGAGTCGAAAACCGTGGTGACCTACCACATCCCA
CAGAACAGCTCCTTGGAAAACGTGGACAGCCGCTACCGGAACCGAGCCCTGATGTCACCGGCCGGCAT
GCTGCGGGGCGACTTCTCCCTGCGCTTGTTCAACGTCACCCCCCAGGACGAGCAGAAGTTTCACTGCC
TGGTGTTGAGCCAATCCCTGGGATTCCAGGAGGTTTTGAGCGTTGAGGTTACACTGCATGTGGCAGCA
AACTTCAGCGTGCCCGTCGTCAGCGCCCCCCACAGCCCCTCCCAGGATGAGCTCACCTTCACGTGTAC

ATCCATAAACGGCTACCCCAGGCCCAACGTGTACTGGATCAATAAGACGGACAACAGCCTGCTGGACC
AGGCTCTGCAGAATGACACCGTCTTCTTGAACATGCGGGGCTTGTATGACGTGGTCAGCGTGCTGAGG
ATCGCACGGACCCCCAGCGTGAACATTGGCTGCTGCATAGAGAACGTGCTTCTGCAGCAGAACCTGAC
TGTCGGCAGCCAGACAGGAAATGACATCGGAGAGAGAGACAAGATCACAGAGAATCCAGTCAGTACCG
GCGAGAAAAACGCGGCCACGTGGAGCATCCTGGCTGTCCTGTGCCTGCTTGTGGTCGTGGCGGTGGCC
ATAGGCTGGGTGTGCAGGGACCGATGCCTCCAACACAGCTATGCAGGTGCCTGGGCTGTGAGTCCGGA
GACAGAGCTCACTGGCCACGTTTGA

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