# The role of co-stimulatory receptors in the regulation of regulatory T cell migration.

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# Statement of Originality

I, Madhav Kishore, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

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Details of collaboration and publications:

Some sections in 'T cell metabolism and immune regulation' of the introduction are adapted from my co-authored review with Dr. David Coe and Prof. Federica Marelli-Berg. These are published in the manuscript: Coe DJ, Kishore M and Marelli-Berg F (2014) **Metabolic regulation of regulatory T cell development and function**. Front. Immunol. 5:590. doi: 10.3389/fimmu.2014.00590

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In this section, for most part, the in vivo murine procedures were carried out by postdoctoral researcher Dr Hongmei Fu and I assisted her in sample processing and data acquisition for these experiments. Time lapse microscopy experiments described were the results of collaboration with PhD student Elvira Infante and Prof. Anne J. Ridley at Kings College London (Figure 29). Here, the provision of cellular materials and data analysis was undertaken by me. Intravital microscopy and data analysis of intra-vital experiments described were performed by PhD student Beatrice Gittens at the William Harvey Research institute (Figure 24). Immunofluorescent staining and imaging experiments were conducted jointly with Dr. Guosu Wang and Dr.Hongmei Fu.

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Here the in vivo experiment in Figure 68 was carried out by Dr. Liang Ma.

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# Abstract

Once an immune response is initiated, a combination of several mechanisms coordinates and directs the homing of T cells to their target tissue. Co-stimulatory receptors expressed on the surface of T lymphocytes are known to actively regulate T cell motility and thus are crucial for regulating T cell migration. The B7 co-stimulatory receptor family members CD28 and CTLA-4 are well known to positively and negatively regulate effector T cell motility respectively. However, their effect on motility and subsequently migration has not been specifically studied in regulatory T cells (Tregs).

Tregs are a CD4+ T cell subset fundamental for maintaining immune homeostasis and their role in controlling autoimmunity has been well established through a variety of experimental animal models. Tregs differ from conventional T cells in their expression of CTLA-4. While conventional T cells express CTLA-4 only after activation, Tregs are known to express this negative co-stimulatory receptor constitutively. Additionally, Tregs display a distinct metabolic phenotype to that of conventional T cells. This thesis examines the impact of both co-stimulatory receptors on Treg migration while taking in account the consequences of the concomitant delivery of signals from both. The data in this study suggests that glycolysis, rather than lipid oxidation induced by CD28 promotes Treg migration through glycolytic enzymes including glucokinase, whose expression in T cells was previously unknown. In contrast, CTLA-4 inhibits CD28 induced Treg migration via inhibition of these glycolytic enzymes. The therapeutic implications of these results in the context of disease and transplantation are discussed.

CD31, an IgG like molecule, is expressed on a number of leukocytes including lymphocytes. In T cells, CD31 inhibits activation by recruiting phosphatases through its cytoplasmic ITIM domains and has thus been described as a co-inhibitory receptor. The role of this co-receptor in the regulation of T cell migration is investigated in the last part of this thesis. Our observations suggest that CD31 selectively signals in activated T cells to regulate their migration in response to inflammatory chemokines, revealing an additional role of this receptor in regulating T cell-mediated inflammation.

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# Abbreviations

| | |
|---|---|
| 1MT | 1-methyltryptophan |
| 2-DG | 2-Deoxy-D-glucose |
| 2-ME | 2-mercaptoethanol |
| 6-NBDG | 6-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-Deoxyglucose |
| Ag | Antigen |
| AICD | Activation-induced cell death |
| AMPK | AMP-activated protein kinase |
| APCs | Antigen presenting cells |
| bmDCs | Bone marrow-derived Dendritic Cells |
| BTLA | B and T lymphocyte attenuator |
| CIITA | Class II transactivator |
| CASK | Calmodulin associated serine/threonine kinase |
| CBL-B | Casitas-B-lineage lymphoma protein B |
| CFSE | Carboxyfluorescein succinimidyl ester |
| CIA | Collagen-induced arthritis |
| CLA | Cutaneous lymphocyte antigen |
| CLIP | Class II-associated invariant chain peptide |
| CNS | Central nervous system |
| CPT1a | Carnitine palmitoyltransferase 1a |
| cSMAC | Central supramolecular activation cluster |
| CTACK | Cutaneous T cell activating chemokine |
| CTLA-4 | Cytotoxic T lymphocyte associated antigen 4 |
| DCs | Dendritic cells |
| DDAO-SE | Dodecyldimethylamine oxide-succinimidyl ester |
| dLN | Draining lymph node |
| DMEM | Dulbecco's modified eagles medium |
| DOCK2 | Dedicator of cytokinesis 2 |
| EAE | Experimental autoimmune encephalomyelitis |
| ECAR | Extracellular acidification rate |
| ECs | Endothelial cells |
| EDTA | Ethylenediaminetetraacetic acid |
| ER | Endoplasmic reticulum |
| ERG | Ether- à –go-go related gene |
| ERKs | Extracellular-signal-regulated kinases |
| ERRα | Estrogen related receptor-alpha |
| ETX | Etomoxir |
| FAO | Fatty acid oxidation |
| Foxp3 | Forkhead box P3 |
| GAD | Glutamic acid decarboxylase |
| GAGs | Glycosoaminoglycans |
| GALT | Gut-associated lymphoid tissue |
| GCK | Glucokinase |
| GCs | Germinal centres |
| GEFs | Guanine nucleotide exchange factors |

| | |
|---|---|
| GlyCAM-1 | Glycosaminoglycan cell adhesion molecule 1 |
| GPCRs | G protein-coupled receptors |
| GTPase | Guanosine triphosphatase |
| HEVs | High endothelial venules |
| HMGCR | 3-hydroxy-3-methylglutaryl-CoA reductase |
| HVEM | Herpesvirus entry mediator |
| ICAM-1 | Intercellular adhesion molecule 1 |
| ICAM-2 | Intercellular adhesion molecule 2 |
| ICOS | Inducible T-cell costimulator |
| IDO | Indoleamine 2,3-dioxygenase |
| IFN-γ | Interferon gamma |
| Ig-CAMs | Ig superfamily |
| IgSE | Immunoglobulin superfamily |
| IL-2 | Interleukin-2 |
| IRF-3 | Interferon regulatory factor 3 |
| ITAMs | Immunoreceptor tyrosine-based activation motifs |
| ITIMs | Immunoreceptor tyrosine-based Inhibitory motifs |
| ITK | Interleukin-2 (IL-2)–inducible Tec kinase |
| $iT_{REG}$ | Inducible regulatory T cells |
| Jak | Janus Tyrosine Kinase |
| JAMs | Junctional adhesion molecules |
| JNK | Jun N-terminal kinase |
| LAT | Linker for activation |
| LBRC | Lateral border recycling compartment |
| LDH | Lactate dehydrogenase |
| LFA-1 | Lymphocyte function associated antigen 1 |
| LKB1 | Liver kinase B1 |
| Mac-1 | Macrophage-1 antigen |
| MAdCAM-1 | Mucosal addressin cell adhesion molecule 1 |
| MDC | Macrophage-derived chemokine |
| MET | Metformin |
| MHC | Major histocompatability complex |
| MLNs | Mesenteric lymph nodes |
| MLR | Mixed lymphocyte reaction |
| MS | Multiple sclerosis |
| mTOR | Mammalian target of rapamycin |
| mTORC1 | Mammalian target of rapamycin complex 1 |
| mTORC2 | Mammalian target of rapamycin complex 2 |
| ndLN | Non draining lymph node |
| NF-kB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NK cells | Natural killer cells |
| $nT_{REG}$ | Naturally ocurring regulatory T cells |
| OCR | Oxygen consumption rate |
| OXPHOS | Oxidative phosphorylation |
| PBMCs | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered solution |
| PCNA | Proliferating nuclear cell antigen |
| PD-1 | Programmed cell death protein 1 |

| | |
|---|---|
| PDH | Pyurvate dehydrogenase |
| PDHK | PDH Kinase |
| PDK1 | Phosphoinositide-dependent kinase 1 |
| PDZ | Postsynaptic density-95/ discs large/zona occludens-1 |
| PECAM-1 | Platelet endothelial cell adhesion molecule 1 |
| PI | Phosphoinositol |
| PI(3,4)P2 | Phosphoinositol 3,4-bisphosphate |
| PI(3,4,5)P3 | Phosphoinositol 3,4,5-triphosphate |
| PI(4)P | Phosphoinositol 4-bisphosphate |
| PI(4,5)P2 | Phosphoinositol 4,5-bisphosphate |
| PI3K | Phosphoinositide 3-kinase |
| PKB | Protein kinase B |
| PKC | Protein kinase C |
| PKCθ | Protein kinase C θ |
| PLC | Phospholipase C |
| pLN | Peripheral lymph node |
| PMA | Phorbol myristate acetate |
| PNAd | Peripheral lymph node addressin |
| PP2A | Protein phosphatase 2A |
| PSGL-1 | P-Selectin ligand, P-selectin glycoprotein ligand 1 |
| PTEN | Phosphatase and tensin homologue deleted on chromosome 10 |
| PTPases | Protein tyrosine phosphatases |
| Rac1 | Ras-related C3 botulinum toxin substrate 1 |
| RAG | Recombination-activating gene |
| Rap1 | Ras-proximate-1 or Ras-related protein 1 |
| Rapa | Rapamycin |
| RAPL | Ras association domain-containing protein 5 |
| RHEB | Ras Homolog Enriched In Brain |
| RIAM | Rap1 effector Rap1–GTP-interacting adapter molecule |
| S1PR | Sphingosine 1 phosphate receptor |
| SDF-1 | Stromal cell-derived factor 1 |
| SFK | Src family of tyrosine kinases |
| SHIP | SH2 domain-containing inositol 5'-phosphatase |
| SHP-1 | Src-homology 2 domain (SH2)-containing phosphatse-1 |
| SHP-2 | Src-homology 2 domain (SH2)-containing phosphatse-2 |
| SLP76 | SH2−domain−containing leukocyte protein of 76 kDa |
| SPL | Spleen |
| SRC | Spare respiratory capacity |
| SREPB-2 | Sterol-regulatory element binding protein-2 |
| STAT | Signal transducer and activator of transcription |
| TAP-1 | Transporter for antigen presentation 1 |
| TARC | Thymus and activation regulated chemokine |
| TCA | Tricarboxylic acid |
| $T_{CM}$ | T central memory cells |
| TCR | T cell receptor |
| TECK | Thymus expressed chemokine |
| $T_{EFF}$ | T effector cells |
| TEM | Trans-endothelial migration |

| | |
|---|---|
| $T_{EM}$ | T effector memory cells |
| TGF-β | Transforming growth factor beta |
| Tiam1 | T cell lymphoma invasion and metastasis 1 |
| TNFα | Tumour necrosis factor alpha |
| $T_{REG}$ | T regulatory cells |
| TSC1 | Tuberous sclerosis complex 1 |
| VCAM | Vascular cell adhesion molecule |
| VLA-4 | Very late induced antigen 4 |
| WASp | Wiskott-Aldrich syndrome protein |
| WT | Wildtype |
| ZAP-70 | Zeta-chain-associated protein kinase 70 |

# Chapter 1 Introduction

## 1.1 T cell migration: an overview

After their development and exit from the thymus, naïve T cells constantly recirculate through blood and lymphoid tissues where they recognise cognate antigen displayed by antigen presenting cells and undergo activation. Once activated, T cells differentiate into memory T cells and effector T cells (von Andrian and Mackay 2000). The effector T cells primarily localise to sites of inflammation while differentiated memory cells re-circulate between the peripheral and lymphoid tissues so as to prepare for secondary responses (Chalasani et al. 2002; Masopust et al. 2001; Weninger et al. 2001).

These migratory events rely upon the T cells exiting the bloodstream and lymphatic system through vascular endothelium by means of tightly regulated molecular interactions summarised in the multistep paradigm of lymphocyte migration (Springer 1994). Specifically, the lymphocytes must first tether and roll on the vascular endothelium mediated through the action of selectins and integrins. Chemokines bound to the luminal side of the vascular endothelium of postcapillary venules increase integrin affinity causing a firm arrest of the lymphocytes on the endothelium. Finally, lymphocytes cross the endothelial barrier through a complex process involving multiple molecular interactions known as trans-endothelial migration (TEM) or diapedesis. A similar stepwise process is also observed in the extravasation of other leukocytes such as neutrophils and monocytes.

## 1.1.1 Multistep paradigm of lymphocyte migration

### 1.1.1.1 Rolling

In the first step, lymphocytes traversing through the vascular endothelial lumen constantly roll across the endothelial walls by means of low affinity interactions of adhesion molecules. This slows the lymphocytes allowing interactions with the endothelial cells (ECs)(Garrood et al. 2006). Most important of these adhesion molecules are a group of specialised transmembrane glycoproteins known as selectins. Three selectins identified in humans and mice are L-selectin, P-selectin and E-selectin.

L-selectin (also known as CD62L) is constitutively expressed my most circulating leukocytes and is downregulated in lymphocytes following activation. Expressed on the tips of microvilli, this selectin plays a crucial role in the rolling and tethering of T cells at sites of inflammation (Bruehl et al. 1996; Stein et al. 1999). Loss of L-selectin as seen in L-selectin knockout mice, results in impaired rolling of lymphocytes in inflamed postcapillary venules and subsequently reduced infiltration through endothelium in these mice (Arbones et al. 1994; Tedder et al. 1995). L-selectin is also necessary for T cells to re-circulate to the secondary lymphoid organs (Bradley et al. 1994). Conversely, T cells from mice expressing reduced levels of L-selectin display increased rolling velocity in vivo (Galkina et al. 2007). L-selectin also binds to the P-Selectin ligand, P-selectin glycoprotein ligand 1 (PSGL-1) (Tu et al. 1996). PSGL-1 expressed by circulating leukocytes can bind the L-selectin of a previously tethered leukocyte, forming a positive feedback whereby an adherent leukocyte recruits more leukocytes exponentially (Walcheck et al. 1996). Ligands for L-selectin can be found in both inflammatory and lymphoid tissues. These are primarily glycosylated proteins which include glycosaminoglycan cell adhesion molecule 1 (GlyCAM-1), mucosal addressin cell adhesion molecule 1 (MAdCAM-1), CD34, Sgp200, podocalyxin and endomucin (S. D. Rosen 2004).

P-selectin (also known as CD62P) is expressed primarily on platelets and ECs of inflamed tissues. It is circulated to the plasma membrane from intracellular Weibel-Palade bodies in ECs (or α-granules in platelets) through stimulation by histamine and thrombin (Patel et al. 2002) within minutes thus mediating the early recruitment of platelets to tissue injury sites. The major ligand for P-selectin, PSGL-1 is expressed by circulating myeloid cells and T cells (Ley and Kansas 2004).

E-selectin (also known as CD62E) is also expressed on inflamed endothelium. Unlike P-selectin, E-selectin is regulated by increased transcription in response to inflammatory stimuli (Bevilacqua et

al. 1987). In humans, the major ligand for E-selectin is the cutaneous lymphocyte antigen (CLA), expressed by a majority of T cells at sites of chronic cutaneous inflammation (Berg et al. 1991). In mice, E-selectin binding of PSGL-1mediates Th1 lymphocyte migration (Hirata et al. 2000).

While selectins are the best characterised mediators of lymphocyte rolling, other molecules are also know to carry out this function. CD44, a transmembrane glycoprotein expressed in an activated form on subgroups of activated lymphocytes (reviewed inPonta et al. 2003) can mediate selectin independent rolling on inflamed tissue (DeGrendele et al. 1997). Its ligand hyaluronic acid is a polysaccharide component of the extracellular matrix, expression of which can be upregulated by pro inflammatory signals (Mohamadzadeh et al. 1998). Interactions of α4 integrins and β2 integrins expressed by lymphocytes with their endothelial ligands can also play a role in rolling (Alon et al. 1995; U. Jung et al. 1998; Kadono et al. 2002).

#### 1.1.1.2 Activation and firm arrest

While rolling allows for weak contact between the leukocytes and endothelial surface, if no further stimuli are provided to the leukocytes, they detach and return back to circulation. However, in the presence of luminally expressed activating molecules, they progress to firm adhesion (reviewed in Garrood et al. 2006). Thus, the next step in leukocyte extravasation relies on providing activating stimuli to the leukocytes by a group of small polypeptides known as chemokines.

Chemokines or chemoattractant cytokines have molecular weights between 8-12 kDA and can be grouped into four groups based on the number and position of the 4 conserved cysteine residues near the N-terminus (reviewed in Griffith et al. 2014; B. Moser et al. 2004). Chemokines with adjacent cysteines are CC chemokines, and are the most abundant. Chemokines with an amino acid between the two cysteines CXC and with only one conserved cysteine XC along with chemokines containing three inter cysteine amino acids $CX_3C$ form the other groups. Receptors for these chemokines expressed by leukocytes are 7 transmembrane G protein coupled receptors (GPCRs) which may bind to one chemokine specifically or bind different chemokines exhibiting considerable overlap. Binding of chemokines to their receptors results in enhanced leukocyte adhesiveness, allowing for the next migration step –firm adhesion - to proceed. Chemokines can also be classified into inflammatory or homeostatic based on the functions they carry out (reviewed in Ebert et al. 2005; Griffith et al. 2014). Inflammatory chemokines may be produced by

a number of cell types in response to inflammation to allow recruitment of leukocytes to inflammatory sites (redundant, non-tissue-specific). On the other hand, homeostatic chemokines control cellular traffic in the secondary lymphoid organs and contribute to the immune surveillance of the peripheral tissues (tissue-specific, non-redundant)(B. Moser et al. 2004). They also control cellular migration in the bone marrow.

Apically expressed chemokines, which allow attachment and signalling, need not necessarily be produced by the endothelial cell expressing it. Chemokines secreted elsewhere can be uptaken by transcytosis and anchored to the endothelium by glycosoaminoglycans (GAGs), synthesised by the endothelium (Middleton et al. 2002). The variety of chemokines in different tissues and the expression of multiple chemokine receptors by subgroups of leukocytes have been known to be responsible for the bulk of the homing pattern seen in the recruitment of leukocytes (reviewed in Griffith et al. 2014). Within the tissue extravasation process, chemokines also guide the lymphocytes through the layers underlying the endothelium.

An extensive list of chemokine receptors, cellular expression type and associated function are described in the Table 1 below adapted from (Griffith et al. 2014).

| Receptor | Immune cell expression | Key immune function |
|---|---|---|
| **G protein–coupled receptors** | | |
| CXCR1 | Neutrophil > monocyte, NK, mast cell, basophil, $CD8^+$ $T_{EFF}$ | Neutrophil trafficking |
| CXCR2 | Neutrophil > monocyte, NK, mast cell, basophil, $CD8^+$ T | B cell lymphopoiesis, neutrophil egress from bone marrow, neutrophil trafficking |
| CXCR3 | Th1, $CD8^+$ $T_{CM}$ and $T_{EM}$, NK, NKT, pDC, B cell, Treg, Tfh | Th1-type adaptive immunity |
| CXCR4 | Most (if not all) leukocytes | Hematopoiesis, organogenesis, bone marrow homing |
| CXCR5 | B cell, Tfh, Tfr, $CD8^+$ $T_{EM}$ | B and T cell trafficking in lymphoid tissue to B cell zone/follicles |
| CXCR6 | Th1, Th17, γδ T, ILC, NKT, NK, plasma cell | ILC function, adaptive immunity |
| CCR1 | Monocyte, macrophage, neutrophil, Th1, basophil, DC | Innate immunity, adaptive immunity |
| CCR2 | Monocyte, macrophage, Th1, iDC, basophil, NK | Monocyte trafficking, Th1-type adaptive immunity |
| CCR3 | Eosinophil > basophil, mast cell | Th2-type adaptive immunity, eosinophil distribution and trafficking |
| CCR4 | Th2, skin- and lung-homing T, Treg > Th17, $CD8^+$ T, monocyte, B cell, iDC | Homing of T cells to skin and lung, Th2-type immune response |
| CCR5 | Monocyte, macrophage, Th1, NK, Treg, $CD8^+$ T, DC, neutrophil | Type 1 adaptive immunity |
| CCR6 | Th17 > iDC, γδ T, NKT, NK, Treg, Tfh | iDC trafficking; GALT development, Th17 adaptive immune responses |
| CCR7 | naive T, $T_{CM}$, $T_{RCM}$, mDC, B cell | mDC, B cell, and T cell trafficking in lymphoid tissue to T cell zone, egress of DC and T cells from tissue |
| CCR8 | Th2, Treg, skin $T_{RM}$, γδ T, monocyte, macrophage | Immune surveillance in skin, type 2 adaptive immunity, thymopoiesis |
| CCR9 | Gut-homing T, thymocytes, B, DC, pDC | Homing of T cells to gut, GALT development and function, thymopoiesis |
| CCR10 | Skin-homing T cell, IgA-plasmablasts | Humoral immunity at mucosal sites, immune surveillance in skin |
| XCR1 | Cross-presenting $CD8^+$ DC, thymic DC | Antigen cross-presentation by $CD8^+$ DCs |
| CX3CR1 | Resident monocyte, macrophage, microglia, Th1, $CD8^+$ $T_{EM}$, NK, γδ T cell, DC | Patrolling monocytes in innate immunity, microglial cell and NK cell migration, type 1 adaptive immunity |
| **Atypical (nonsignaling) receptors** | | |
| ACKR1 (DARC; Duffy) | RBC, LEC | Chemokine transcytosis, chemokine scavenging |
| ACKR2 (D6) | LEC, DC, B cell | Chemokine scavenging |
| ACKR3 (CXCR7) | Stromal cells, B cell | Shaping chemokine gradients for CXCR4 |
| ACKR4 (CCRL1; CCX-CKR) | Thymic epithelium | Chemokine scavenging |

**Table 1 Chemokine receptors that are involved in cellular recruitment**

*This table adapted from (Griffith et al. 2014) gives an up to date list of chemokine receptors that have been discovered to regulate cellular trafficking including their cellular expression and associated function.*

**Chemokine receptor Structure and Activation**

All Chemokine receptors are 7 trans-membrane receptors with seven helical membrane spanning regions that are connected by extra-membranous loops (Allen et al. 2007). These N terminus and three extracellular loops are exposed to outer surface of the cell. While the C terminus and three intracellular loops face the cytoplasm. The signalling initiated through these receptors upon ligand binding is due, at least in part, to the presence of motifs within these cytoplasm facing loops. The DRY motif (Asp-Arg-Tyr) in the second intracellular loop is one such motif which is missing in the scavenger chemokine receptors (Rovati et al. 2007).These 'scavenging' or atypical chemokine receptors do not signal and are important for shaping chemokine gradients and dampening inflammation by scavenging chemokines in a GTPase independent manner. The N terminus is important for ligand binding.

Chemokine receptors are GPCRs where the (αβγ) heterotrimeric G proteins bind to the intracellular loops (Cabrera-Vera et al. 2003; Pierce et al. 2002). The Gα subunit interacts directly with GPCR intracellular loops and with the Gβ subunit, which in turn binds with the Gγ subunit. The Gα protein subunit contains the GTPase domain involved in the hydrolysis of GTP. In the inactive state, the Gα subunit remains bound to GDP. Upon ligand binding, a conformational change activates the heterotrimeric G protein inside the cell, causing dislocation of GDP from the Gα subunit and replacement by GTP. The Gα-GTP then dissociates from the receptor and Gβγ heterodimers, and both complexes activate downstream effector signalling molecules. Over-stimulation through continued stimuli can lead to receptor desensitisation and internalisation by agonist-dependent phosphorylation of the C terminus of the GPCR. This receptor phosphorylation subsequently promotes binding of proteins known as arrestins, which block further interaction with G proteins and initiates receptor internalisation through clathrin coated pits and lipid rafts/caveolae (Lefkowitz and Whalen 2004; Neel et al. 2005).

Upon chemokine binding, the N terminus of the receptor binds the N terminus of the chemokine in an extended fashion, orienting the chemokine into the helical bundle. Additionally, the other extracellular loops also bind to the globular body of the chemokine orienting the N terminus of the chemokine into the transmembrane helices.

### 1.1.1.3 Firm Adhesion

Once the chemokines provide activating signals via their receptors expressed on the lymphocytes, conformational changes brought about in the integrins allows for rapid increase in affinity leading to firm adhesion to the endothelial surface (Explained in detail later). These integrins are glycosylated protein heterodimers composed of a large alpha chain that pairs non-covalently with a smaller beta chain (reviewed in Danen and Sonnenberg 2003; van der Flier and Sonnenberg 2001). Integrins bind to components of the extracellular matrix or their ligands on the endothelial surface resulting in this firm adhesion. In T cells, two integrins are particularly important for extravasation. These are the very late induced antigen 4 (VLA-4, $\alpha_4\beta_1$ ) and Lymphocyte function associated antigen 1 (LFA 1).

LFA-1 binds to its two ligands (ICAM-1) and (ICAM-2) expressed on ECs. These ligands are Ig-like type 1 transmembrane glycoproteins with short cytoplasmic tails, single transmembrane regions and larger extracellular domains. Both ligands through their interactions with LFA-1 play important roles in the migration of T cells. While expression of ICAM-1 can be significantly increased following inflammatory stimuli such as IL-1 and TNFα (Gahmberg et al. 1997), ICAM-2 remains constitutively expressed on vascular and lymphatic endothelium with indifference to inflammatory stimuli (de Fougerolles et al. 1991; Geijtenbeek et al. 2000). Thus, ICAM-1 is important for the migration of T cells to the inflamed non lymphoid tissue. Antibody mediated blockade of ICAM-1 and not ICAM-2 was shown to reduce migration of T cells to inflamed skin *in vivo* (J. C. Lehmann et al. 2003). In the case of trafficking to lymph nodes, there is significant redundancy between these two ICAM molecules as antibody mediated blockade of either had little effect on migration to peripheral lymph nodes, while antibody mediated blockade of both dramatically reduced homing of T cells to the lymph nodes (J. C. Lehmann et al. 2003).

Like ICAM-1, the expression of vascular cell adhesion molecule (VCAM-1), the ligand for VLA-4 is greatly induced on endothelium upon stimulation through inflammatory mediators (Henninger et al. 1997). VCAM-1 has also been reported to support rolling, mediated through VLA-4, leading to firm adhesion without the need of chemokine stimulation (Alon et al. 1995). In addition, VLA-4 also binds another integrin $\alpha_4\beta_7$ expressed by subgroups of T cells (Carter and Wicks 2001).

#### 1.1.1.4 Integrin Activation

The arrest of tethered or rolling leukocytes on the target endothelium is almost entirely mediated by members of the integrin superfamily and their endothelial immunoglobulin superfamily (IgSF) ligands (reviewed in Ley et al. 2007; A. D. Luster et al. 2005). Interestingly, the majority of leukocytes maintain these integrins in largely inactive states (exceptions include T and B cell blasts, and some myeloid cells that constitutively express high or intermediate integrin affinity) and require their modulation for high-affinity and flow shear-resistant adhesiveness towards their specific endothelial ligands (Laudanna and Alon 2006). Previous studies conducted in both *in vitro* and *in vivo* have revealed that this increased integrin adhesiveness and microclustering occurs within a fraction of seconds following contact with endothelial ligands (Grabovsky et al. 2000; Shamri et al. 2005). As mentioned above, chemokine or chemoattractant binding to their G protein-coupled receptors (GPCRs) on the leukocyte surface initiates the high integrin avidity to ligand within sub-seconds at restricted leukocyte-endothelial contact points (Laudanna 2005). It is unclear whether this 'inside out' signalling via chemokines is locally transmitted at the endothelial contact zone or if the rolling leukocyte accumulates successive chemokine signals to surpass an activation threshold.

From a structural viewpoint, inside out signalling induces conformational changes in the integrins from their inactive bent clasped heterodimeric structure (reviewed in Ley et al. 2007) towards their unclasped heterodimeric form with extended ectodomains exhibiting intermediate to high affinity towards their ligands (Arnaout et al. 2005). In light of the short time frame involved in this process, integrins may exist in preformed associations with different cytoskeletal adaptors and membrane proteins such as tetraspanins, CD47, CD98 and CD44. However, chemokine-induced inside out signalling only partially accounts for the conformational changes that ensure firm arrest. Recent data suggests that bidirectional integrin signals induce the necessary rearrangements of the α and β subunits of the integrin cytoplasmic tails as well as changes in the extracellular domain that follow binding of extracellular ligands (reviewed in Abram and Lowell 2009). This associated 'outside in' signalling involves early coupling of tyrosine kinases, particularly those of the Src and Syk families. Rapid activation of these enzymes has been reported in lymphocytes, myeloid cells and platelets following $\beta_1$, $\beta_2$ and $\beta_3$ integrin engagement (Arias-Salgado et al. 2003). Deficiency of Syk kinase was described to block $\beta_1$, $\beta_2$ and $\beta_3$ integrin signalling events in neutrophils and macrophages (Mocsai et al. 2002). In Jurkat T cells, the

deficiency of the Zap-70 kinase (member of the Syk family of tyrosine kinases) has been shown to abrogate signalling events from the $\beta_1$ integrin (Epler et al. 2000).

A key protein that interacts with the α chain of integrins during the inside out signalling is the guanosine triphosphatase (GTPase) Rap1 (reviewed in Kinashi 2005). Expression of dominant negative Rap1 has been reported to inhibit TCR-mediated LFA-1 activation in Jurkat T cells (K. Katagiri et al. 2000), while overexpression of the Rap1-specific GAP, SPA1, inhibits rapid ICAM-dependent adhesion of primary T cells to vascular endothelium (Shimonaka et al. 2003). Rap1 acting through Ras association domain-containing protein 5 or RAPL has also been reported to alter integrin clustering (Koko Katagiri et al. 2003). A second mechanism that contributes to integrin activation mediated by Rap1 is through the facilitation of talin binding to the intracellular domain of the β-integrin subunit. The talin protein is a major integrin activating adaptor in leukocytes and platelets (Critchley 2009; S. Tadokoro et al. 2003). This talin binding is mediated by the Rap1 effector Rap1–GTP-interacting adapter molecule (RIAM) (Watanabe et al. 2008). The binding of talin to the β integrin subunit also results in the opening of the extracellular integrin headpiece to promote high-affinity-ligand binding. Conditional knockdown of talin in platelets of mice showed poor activation of integrins and increased resistance to arterial thrombosis mediated by platelet $\beta_1$ and $\beta_3$ integrins (Nieswandt et al. 2007; Petrich et al. 2007). Another family of proteins called Kindlins can also interact with integrin $\beta_1$ and $\beta_3$. Kindlin-1 and 2 are ubiquitously expressed whereas Kindlin-3 is restricted to hematopoietic cells (reviewed in Abram and Lowell 2009). Kindlin-3-deficient mice die due to severe bleeding as a result of platelet dysfunction resulting from defective inside out signalling via $\beta_3$ integrins (M. Moser et al. 2008), while, Kindlin-2 deficient embryonic stem cells in mice exhibit decreased adhesion on a variety of ligands (Montanez et al. 2008).

Thus 'inside out' and 'outside in' signalling induces both conformational changes to the integrin structure increasing affinity and also causes enhanced integrin clustering (increased avidity).This occurs at the apical surface of the endothelium leading to firm adhesion of the lymphocytes to the ECs.

#### 1.1.1.5 Trans-endothelial migration (diapedesis)

Once a lymphocyte has committed to trans-endothelial migration (TEM), the firmly-adhered cell changes shape and begins to display two distinct edges on the endothelium. These are the leading edge and the trailing edge (uropod) of the cell (del Pozo et al. 1995). This polarised state of the cell is the result of changes in the distribution of actin filaments initiated through signalling pathways resulting from the interaction of the lymphocyte with the endothelium. The leading edge is characterised by a high density of chemokine receptors and integrins highlighting its role in chemotaxis while the trailing edge or uropod is important for contact between polarised lymphocytes through the action of selectins (reviewed in Sanchez-Madrid and del Pozo 1999). In addition, cholesterol-rich structures known as lipid rafts that facilitate signalling are thought to be fundamental in establishing cell polarity (reviewed in Simons and Toomre 2000; Villalba et al. 2001), as cholesterol depletion abolished the formation of both the leading edge and the uropod in the study by (Gomez-Mouton et al. 2001).

Although migration through the endothelial cell itself (transcellular route)(Carman and Springer 2008; Feng et al. 1998) has been described, the majority of the lymphocyte migration occurs through the junctions between adjacent ECs (paracellular route). These junctions are formed through the interaction of dedicated adhesion molecules including junctional adhesion molecules (JAMs), platelet endothelial cell adhesion molecule (PECAM-1)/CD31, CD99, vascular endothelial (VE) cadherin, occludins and claudins (reviewed in W. A. Muller 2003). CD31 through homophillic interactions with CD31 expressed on T cells plays a crucial role in TEM (Described in detail below). Similar to CD31, CD99 also plays a role in TEM through homophillic interactions between endothelial cell junctions and T cells.

**CD31**

Platelet endothelial cell adhesion molecule 1 or PECAM-1 (CD31), a 130kD type I transmembrane cell adhesion molecules from the Ig superfamily (Ig-CAMs), is a key adhesion molecule involved in leukocyte trans-endothelial migration (P. J. Newman et al. 1990). This molecule is expressed on a number of hematopoietic and immune cells including platelets, neutrophils, monocytes, megakaryocytes, natural killer cells, T cell, B cells and on ECs where it is expressed on the borders of adjacent cells (reviewed in Jackson 2003; P. J. Newman 1997).

Structurally, CD31 is composed of 6 extracellular domains, a transmembrane portion, and a cytoplasmic tail. The 6 extracellular Ig-like domains are differentially glycosylated involving N-linked and O-linked glycosylation sites (Xie and Muller 1993). CD31 can interact through homophilic (or homotypic) fashion via its $1^{st}$ domain (reviewed in Newman and Newman 2003; Privratsky et al. 2010). In addition, a number of putative heterotypic ligands for CD31 have also been identified. These include the ADP-ribose cyclase (CD38) (via domain 2) (Deaglio et al. 1998), the neutrophil expressed CD177 (domain 6) (Sachs et al. 2007) and the integrin αvβ3 (domains 1 and 2) (Piali et al. 1995). Within the cytoplasmic tail, CD31 contains residues that serve for palmitoylation, phosphorylation and docking of cytosolic signalling molecules (Newman and Newman 2003). Importantly, CD31 bears two Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) that encompass $Tyr_{663}$ and $Tyr_{686}$ which when phosphorylated have been observed to recruit Src homology 2 (SH2) domain-containing proteins. SH2 domain containing proteins that have been previously identified to be recruited include SHP-2 (Newman and Newman 2003), SHP-1 (Henshall et al. 2001; Hua et al. 1998) Henshall et al. 2001), SH2 domain-containing inositol 5'-phosphatase (SHIP) (Pumphrey et al. 1999), phospholipase Cγ1 (PLCγ1) (Henshall et al. 2001) and members of the Src family of tyrosine kinases (SFK) (Lu et al. 1997; Masuda et al. 1997; Osawa et al. 1997) . Another residue in the cytoplasmic domain subjected to post-translational modification is $Cys_{595}$, which, when palmitoylated, can target CD31 to membrane microdomains enabling it to act as a regulator of cell signalling and apoptosis (Sardjono et al. 2006). An overview of the structure of CD31 is depicted in the Figure 1 below adapted from (Privratsky et al. 2010).

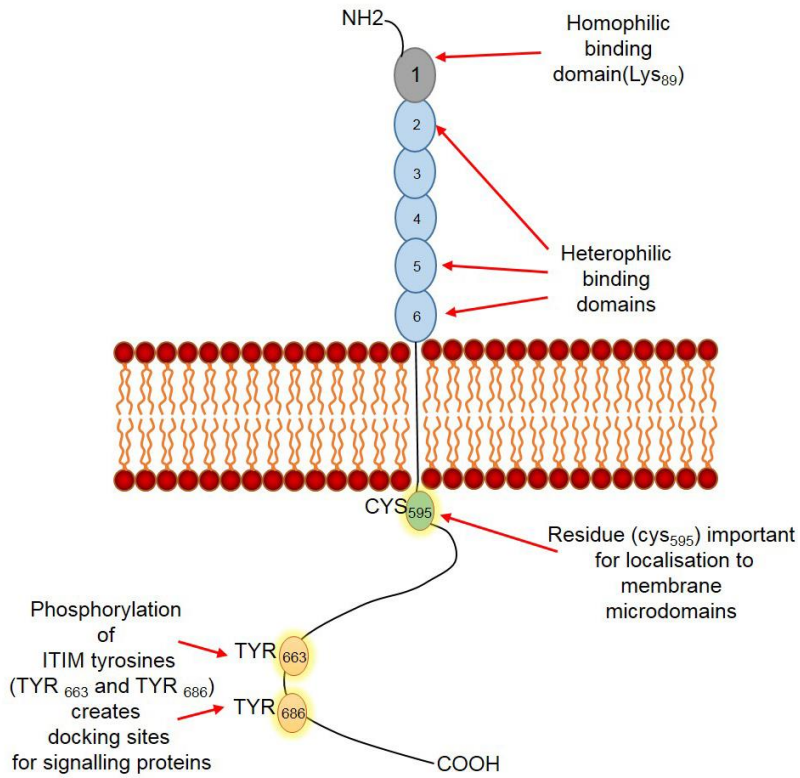



**Figure 1 Structure of CD31**

*The diagram indicates CD31 cytoplasmic signalling domains, including the ITIMS that recruit the SH2 domain containing phosphatases when phosphorylated, as well as the extracellulular domains that are important for binding ligands. Figure adapted from (Privratsky et al. 2010).*

#### The role of PECAM-1 (CD31) in TEM

Historically, CD31 remains a multifaceted molecule implicated in both pro-inflammatory and anti-inflammatory roles. Its pro-inflammatory aspect results from its ability to facilitate leukocyte trans-endothelial migration. The first studies to investigate this functional aspect utilised CD31 specific antibodies to report blocking of leukocyte transmigration across endothelial monolayers *in vitro* (W. A. Muller et al. 1993) and leukocyte accumulation at inflammatory sites *in vivo* (Vaporciyan et al. 1993). Following these initial reports, several other studies have established pro-inflammatory roles for CD31 primarily through its ability to support leukocyte emigration from vasculature to inflammatory sites. These studies are summarised in the Table 2 adapted from (Privratsky et al. 2010). Evidence implicating CD31 in leukocyte transmigration through

venular walls was first directly visualised using the technique of intravital microscopy in the studies by (Wakelin et al. 1996)and (Thompson et al. 2001) indicating inhibition of leukocyte TEM following treatment with CD31 blocking antibodies. Importantly, besides demonstrating that CD31 mediated leukocyte migration through ECs, CD31 can also mediate leukocyte migration through the perivascular basement membrane (Thompson et al. 2001).

Mechanistically, CD31 is known to facilitate TEM through its effects on both ECs and leukocytes during early and late stages of the leukocyte migration cascade.

**Leukocyte CD31 in TEM**

CD31 engagement on the leukocytes can initiate signalling events resulting in the activation of integrins. For example, $\beta_1$- and $\beta_2$-integrins in T cell subsets (Tanaka et al. 1992), $\beta_1$-integrins in macrophages (Vernon-Wilson et al. 2006; Vernon-Wilson et al. 2007), $\beta_2$-integrins in natural killer (NK) cells (Berman et al. 1996) and the $\alpha_M\beta_2$ (Mac-1, CD11b/CD18) integrin in monocytes and neutrophils (Berman and Muller 1995) have all been described to become activated downstream, following CD31 stimulation on these cell types. In neutrophils, homophilic CD31 engagements were reported to trigger upregulation of integrin $\alpha_6\beta_1$ – a receptor for laminin, a major component of the basal lamina (Dangerfield et al. 2002). Thus one of the main functions of leukocyte CD31 is to activate integrins following homophilic engagement resulting in efficient emigration through the endothelium and the sub-endothelial matrix. An exact mechanism through which CD31 engagement achieves integrin activation remains poorly understood. However, there is evidence to suggest the involvement of the Ras family GTPase, Rap1 in this process. Rap1 initiates cytoskeletal rearrangement and its activation has been observed in Jurkat T cells that were exposed to antibody mediated CD31 ligation (Reedquist et al. 2000). In addition, CD31 molecules exist on the cell surface in dynamic equilibrium between primers, dimers and oligomers, and this oligomerised state has been described as best suited for CD31 to serve as a positive regulator of integrin activation (T. Zhao and Newman 2001). There is also evidence that CD31 on leukocytes can modulate cell membrane potential which can enhance integrin mediated adhesion. For example, in phagocytes, CD31 inhibited membrane repolarization mediated by the ether- à –go-go related gene (ERG) ,a voltage gated potassium channel which enhances $\beta_1$-integrin-mediated firm adhesion (Vernon-Wilson et al. 2007).

**Endothelial CD31 in TEM**

On the other hand endothelial CD31 through its homophilic or heterophilic interactions with leukocytes can also facilitate leukocyte TEM. Besides simply facilitating adhesion, a series of elegant studies have identified CD31 as a main constituent of the recycling compartment on ECs, termed the lateral border recycling compartment (LBRC). The LBRC is a surface-connected membrane network located at the borders between adjacent ECs (Mamdouh et al. 2003).This network contains CD31, CD99, and junctional adhesion molecule (JAM)-A, and is recycled and targeted to the region of the cell where paracellular or transcellular migration is occurring (Mamdouh et al. 2003; Mamdouh et al. 2009). The study by (Mamdouh et al. 2003) indicated that CD31 interactions between EC and leukocytes are important for triggering targeted CD31 and LBRC-recycling, which are required for leukocyte transmigration. Later studies by the same group showed that both CD31 dependent or independent leukocyte TEM was dependent on LBRC recycling mediated by endothelial microtubules and kinesin family molecular motors (Mamdouh et al. 2008). Interestingly, the $Y_{663}$ but not the $Y_{686}$ ITIM-mediated recruitment of SHP-2 was essential for these functions. Indeed, blockade of CD31 via antibody on monocytes prevented endothelial CD31 targeting to the zone around the monocyte, thus, preventing TEM with little consequence to monocyte-endothelial cell adhesion (Dasgupta et al. 2009). It is unclear whether CD31 mediated downstream signalling in the ECs is important for leukocyte TEM. The study by (O'Brien et al. 2003) reported that leukocyte transmigration was independent of endothelial CD31 signalling using ITIM-mediated recruitment of SHP-2. Nonetheless, recruitment of CD31 to the LBRC and LBRC recycling via the cytoplasmic tail remain crucial events for leukocyte TEM.

**Junctional adhesion molecules (JAMs)**

JAMs are expressed on both ECs and T cells. As with CD31 and CD99, they are capable of homophilic interactions as well. The JAMs also act as integrin ligands binding both LFA-1 and VLA-4 on T cells (Ostermann et al. 2002). JAM A is concentrated at endothelial borders. While it normally engages in homophilic adhesion, under inflammatory conditions it can bind LFA-1 on T cells and is thus important in TEM (W. A. Muller 2003; Ostermann et al. 2002). Antibody mediated blockade of JAM A on human ECs was shown to reduce TEM *in vitro* (Ostermann et al. 2002).In *in vivo* studies, antibody-mediated blockade of JAM A resulted in reduced trans-endothelial

migration *(Woodfin et al. 2009).* JAM C can engage in homophilic adhesion or heterophilic adhesion with JAM B or CD11b/CD18 (Mac-1) (Chavakis et al. 2004; Johnson-Leger et al. 2002). JAM C is also known to interact with the integrin Mac-1 expressed on monocytes and neutrophils and thus, plays a role in their TEM (Chavakis et al. 2004). Human JAM-C is also expressed by platelets, monocytes, natural killer cells, dendritic cells, B cells and a subset of T cells (reviewed in Weber et al. 2007). However, JAM C has not been detected on mouse leukocytes.

While the exact mechanism through which JAM-A promotes leukocyte extravasation remains to be identified, it is likely to involve the association of this molecule with key mediators of cytoskeletal remodelling (reviewed in Weber et al. 2007). Localised at the intercellular contacts of ECs and epithelial cells, JAMs participate in the assembly and maintenance of junctions, signalling to cytoskeleton-associated proteins and recruiting cell polarity proteins to the junctions via their PDZ-binding-domain motif. JAM-A is apically positioned at tight junctions and interacts with Rap-1, calmodulin associated serine/threonine kinase (CASK), an atypical protein kinase C (PKC) complex and PSD-95/Disc Large/zona occludens protein-1 (ZO-1; PDZ)-containing proteins, all of which can act as scaffolds for larger complexes (Bazzoni et al. 2000; Ebnet et al. 2000). Blocking JAM-A has shown to reduce neutrophil or monocyte extravasation in inflammatory meningitis, peritonitis, skin or cremaster microcirculation, ischemia-reperfusion of heart and liver (reviewed in Weber et al. 2007).

**CD99**

CD99 is a heavily O-glycosylated 32-kD type I transmembrane protein that is present on thymocytes, T cells and many other hematopoietic cell types. It is also concentrated at the intercellular borders of confluent human ECs. Homophilic interactions between leukocyte and endothelial CD99 are known to play a crucial role in transmigration (W. A. Muller 2003). However, CD99 appears to have a role in distal stages of diapedesis, after the point at which CD31 has played its critical role. In the study by (Schenkel et al. 2002), leukocytes treated with antibody-mediated CD31 blockade were still inhibited from treatment with anti-CD99 after the anti-CD31 blockade had been removed. Contrariwise, leukocytes treated with anti-CD99 could no longer be inhibited from transmigration by anti-CD31 treatment after the anti-CD99 block had been removed. In this study, antibody-mediated blockade of CD99 arrested monocytes at appoint where they were partially through the junction. Similar observations were also reported in neutrophils by (Lou et al. 2007). Blocking of CD31 and CD99 has an additive effect on the

inhibition of migration, suggesting that both molecules likely regulate distinct aspects of diapedesis (Schenkel et al. 2002).

While CD31 and CD99 do not play a role in the step preceding transmigration (firm adhesion), VCAM-1, ICAM-1 and JAMs are thought to have a roles in both tight adhesion and transmigration stages. Clustering of VCAM-1 and ICAM-1 on the ECs has been observed as the leukocyte approaches the ECs border (Barreiro et al. 2002; Carman and Springer 2004). Once adherent, ICAM-1 is enriched under the leukocyte as it migrates and continues to surround it as it transmigrates. The actin cytoskeleton is involved in this process; specifically, the Src-dependent phosphorylation of the actin-binding molecule cortactin is required for ICAM-1 clustering (Yang et al. 2006). Furthermore, ICAM-1 clustering further induces phosphorylation of cortactin inducing even more clustering (Durieu-Trautmann et al. 1994). Similarly, VCAM-1 clustering and enrichment under leukocytes has also been reported (Barreiro et al. 2002).

The intracellular signalling following adhesion allows for the opening of the endothelial barriers and also results in the breakage and formation of the leukocyte-endothelial bonds. This further induces retraction of the uropod and protrusion of the leading edge. The leading edge protrusion towards and through the junctional barriers is mediated through means of its polarised chemokine receptors. Within the leukocytes, dramatic reorganization of membrane domains and the cytoskeleton enables a polarised morphology with an actin-rich lamellipodium at the front and a uropod at the rear (reviewed in Sanchez-Madrid and Serrador 2009). The formation of these structures are controlled by members of the Rho GTPase family of proteins, including RhoA, Rac1/2 and Cdc42 (reviewed in Heasman and Ridley 2010). Recently, a study has established that Rho A-depleted cells lack both lamellipodia and uropods, and instead have narrow protrusions extending from a rounded cell body (Heasman et al. 2010).

Thus multiple processes are integrated to achieve effective migration across an endothelial barrier. It has been suggested that considerable overlap between the different steps of the multi-step model of leukocyte extravasation also exists and that there is substantial cooperation between adhesion molecules at all stages (W. A. Muller 2003). After crossing the endothelial barrier, the lymphocyte must cross the perivascular basement membrane. The mechanisms involved in this process remain incompletely defined, however, studies have identified that this is achieved through the interaction of integrins on lymphocytes with proteins on the basement membrane known as laminins (Hohenester and Yurchenco 2013; Kenne et al. 2006).

## 1.1.2 Homing of T cells

The selectivity associated with the recruitment of specific subgroups of T cells to distinct locations within the body suggest that T cells do not migrate in a random fashion but are guided to their destinations through the actions of key molecules. While naïve T cells constantly recirculate from blood back to the lymphoid organs, upon activation they acquire specific receptors termed 'homing receptors' enabling them access to the non-lymphoid tissue (reviewed in Mora and von Andrian 2006). On the other hand, vascular endothelium selectively displays tissue specific ligands for these homing receptors known as vascular addressins, further increasing the specificity of T cell recruitment. Chemokine receptor ligands expressed selectively by endothelium in certain tissues adds an additional another layer of specificity to the recruitment.

### 1.1.2.1 Homing of naïve T cells to peripheral lymphoid organs (PLNs)

Naïve T cells traffic into the peripheral lymphoid organs through specialised vessels known as high endothelial venules (HEV) (Mackay et al. 1990; Vestweber 2003; Weninger et al. 2003). Located with the T cell zones of the lymph nodes, they are lined by specialised ECs that display addressins. The peripheral lymph node addressin (PNAd) is highly expressed on the HEVs of the PLNs and mesenteric lymph node of the gut and binds the L-selectin displayed by naïve T cells (Streeter et al. 1988). In humans PNAd is a complex made up of podocalyxin and endomucin and GLyCAM-1 thus mediating binding through selectins. These HEVs also display the chemokine ligands CCL19 and CCL21 which bind the chemokine receptor CCR7 abundantly expressed on naïve T cells and subsets of memory T cells (Baekkevold et al. 2001). Thus the naïve T cells utilise the aforementioned migration steps to enter the PLNs. Briefly, the interaction of L-selectin with the addressin on the HEVs induce tethering and rolling (reviewed in Ebert et al. 2005). The chemokine receptor CCR7 signalling through its ligands displayed on the HEVs induces the inside out signalling causing the high affinity LFA-1 binding to ICAM-1 displayed on the endothelium, resulting in arrest of the cell. The T cell then exits through the endothelial junctions and enters the T cell zone where chemokines are present.

Another chemokine receptor, described to contribute to lymph node homing is the CXCR4 receptor which binds stromal cell-derived factor 1 (SDF-1, CXCL12) (Campbell et al. 2003). In the absence of CCR7 ligation by CCL19/21, some cells were still observed to be able to migrate to the lymph nodes and this was abolished in CXCR4 deficient mice in the study by (Okada et al. 2002).

An additional pathway involved in the entry as well as the exit of T cells to the lymph nodes is the sphingosine 1 phosphate (S1P) receptor signalling pathway (Chi and Flavell 2005; Rosen and Goetzl 2005). Sphingosine 1 phosphate is a phospholipid expressed at low concentrations in the lymphoid tissue and at high concentrations in the lymph and blood itself. This concentration gradient is maintained through the action of the enzyme S1P lyase expressed ubiquitously in the tissues (Schwab et al. 2005). The phospholipid binds to its receptor S1PR causing internalisation thereby reducing its expression. As a naïve T cells circulate in the blood, the high concentrations of S1P reduce their receptor expression and prevent entry into the tissues (reviewed in Vera Reitsema 2014). Once the naïve T cell enters the lymph node, the low concentration induces S1P receptor (S1PR) upregulation. However, the receptor upregulation can take several hours allowing enough time for antigen presentation to occur, following which the activated effector cells are subjected back to an increasing S1P concentration gradient allowing for the exit of the cell via the efferent lymphatics. Blockade of S1PR using the drug FTY720 prevents T cell egress from the lymph nodes thus acting as an immunosuppressive agent (reviewed in Cyster 2005; Matloubian et al. 2004). In addition, it has been reported that naïve T cells from S1PR-deficient mice adoptively transferred into wildtype (WT) mice were able to populate the lymph nodes but were unable to exit (Matloubian et al. 2004).

#### 1.1.2.2 Homing of T cells to peripheral non lymphoid and inflamed tissues

Upon antigen activation, naïve T cells proliferate and differentiate into short lived effector T cells ($T_{EFF}$) and into a small fraction of long lived memory T cells. The memory T cells can be further grouped into central memory ($T_{CM}$) and effector memory T cells ($T_{EM}$) (reviwed in Sallusto et al. 2004; Sallusto and Lanzavecchia 2009). The former express L-selectin, CCR7 and thus, are able to traffic in a manner similar to that of naïve T cells. However, they also acquire the necessary homing receptors enabling access to sites of chronic inflammation (Ley and Kansas 2004). $T_{CM}$ cells do not have immediate effector functions but are responsible for secondary recall responses thus providing antigenic memory (Manjunath et al. 2001). The $T_{EM}$ cells lack expression of L-selectin and CCR7 and instead acquire tissue specific integrins thus becoming able to traffic back to the extra-lymphoid sites where an immediate effector response is required (Sallusto et al. 1999; von Andrian and Mackay 2000). $T_{EM}$ cells do not traffic to resting lymph nodes and are mainly found in the blood, spleen and peripheral tissues and have immediate effector functions (Masopust et al. 2001). $T_{EFF}$ cells represent the major fraction of differentiated T cells that undergo apoptosis soon

after the primary response (contraction phase) (Sprent and Webb 1995). Thus $T_{EFF}$ and $T_{EM}$ form the 'effector arm' of the T cell responses. A diagram summarising the migratory properties of naïve,$T_{CM}$, $T_{EFF}$ and $T_{EM}$ is given in Figure 2below adapted from (Ward and Marelli-Berg 2009).

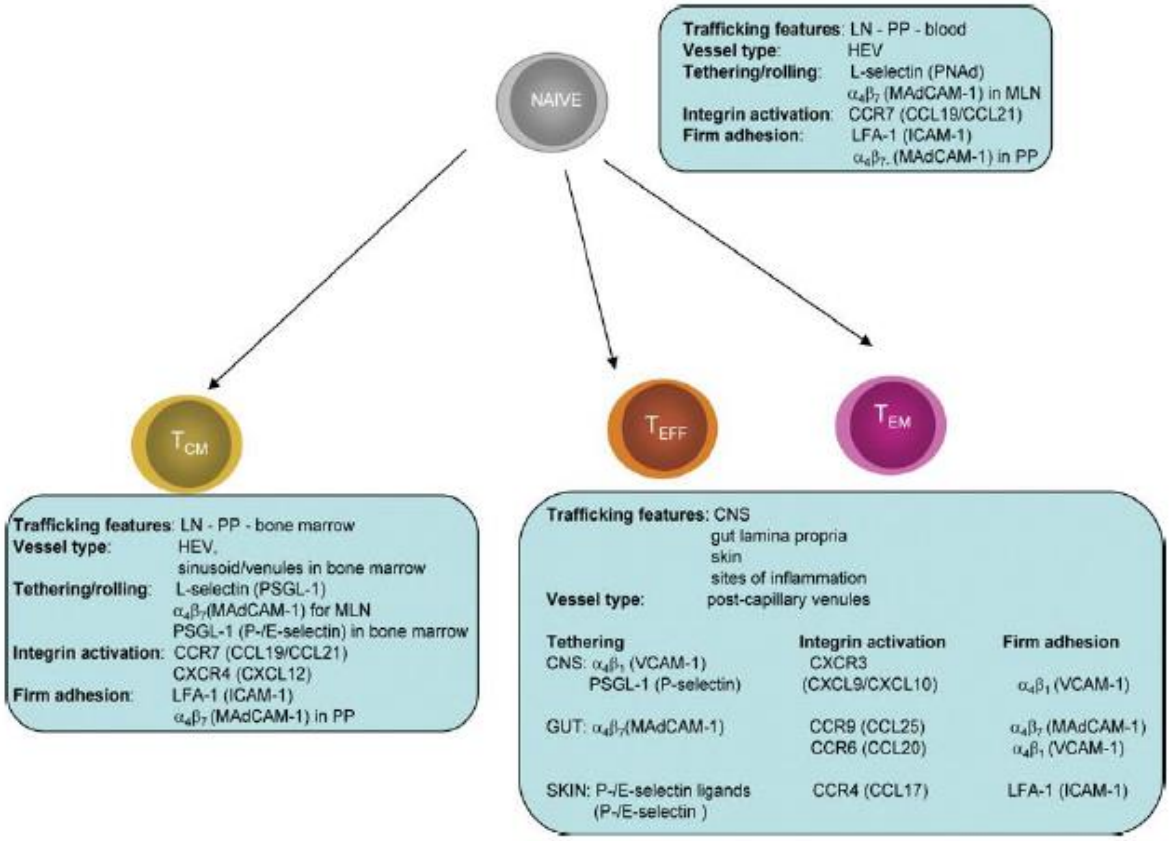



**Figure 2 Molecular interactions required for homing of different subsets of T cells to their respective sites.**

*Naïve T cells predominately traffic to the secondary lymphoid tissues such as the peripheral lymph nodes (PLNs), PP and MLNs in the gut. Memory T cells predominately travel to the non-lymphoid tissue such as the skin, the lamina propria of the gut, and the central nervous system. The figure is adapted from (Ward and Marelli-Berg 2009).*

As the T cells become activated, they begin to express the ligands for E-selectin and P-selectin. They also upregulate the expression of LFA-1, VLA-4 and specific chemokine receptors enabling them access to the inflamed sites where an effector response is to begin. On the other end, the endothelium at the site of infection, in response to inflammatory signals such as cytokines

upregulate E-selectin, P-selectin and ligands for integrins expressed on activated T cells such as ICAM-1 and VCAM-1 (reviewed in W. A. Muller 2003). Inflammatory chemokines produced from the endothelium, tissue specific macrophages and other cells induce motility and transmigration of the activated T cells to enter the site of infection.

The chemokine receptor CXCR3 is expressed on activated T cells plays a key role in the effector responses (Bonecchi et al. 1998). CXCR3 binds to CXCL10 expressed by ECs, monocytes and fibroblasts in response to interferon gamma (IFN-γ) produced in inflammation(A. D. Luster and Ravetch 1987b). Several other chemokine receptors have been implicated in selectively recruit subsets of effector/memory T cells to different inflamed tissues (See Table 1).

Within the secondary lymphoid tissues, differentiating $T_{EFF}$ cells can acquire specific homing receptors and chemokine receptors thus enabling specific subsets, best suited to respond to certain tissues, to selectively migrate to those tissues. Clear examples of this are observed in the migration of effector T cells to the gut and skin. Effector T cells migrating to the skin express the E-selectin ligand cutaneous lymphocyte antigen (CLA) (Berg et al. 1991). They also express chemokine receptors CCR4 and CCR10 which bind chemokines CCL17 (also known as thymus and activation regulated chemokine TARC )(J. J. Campbell et al. 1999; Reiss et al. 2001) and CCL27 (the cutaneous T cell activating chemokine CTACK) expressed in inflamed skin (Morales et al. 1999; Reiss et al. 2001). Effector T cells that migrate to the gut are known to express integrin $\alpha_4\beta_7$ which binds MAdCAM-1 expressed on gut endothelium (Berlin et al. 1993; Lefrancois et al. 1999).These cells also express chemokine receptor CCR9 which binds CCL25 (also known as thymus expressed chemokine TECK) (Stenstad et al. 2006), a chemokine expressed in the inflamed bowel. Indeed, antibody blockade of either CCR9 or TECK has been described to significantly reduce T cell migration to the small intestine (Hosoe et al. 2004; Rivera-Nieves et al. 2006).

### 1.1.2.3 Mechanisms of T cell homing induction

As mentioned above, activation of naïve T cells by antigen presentation can differentiate T cells to specific subsets capable of selective recruitment to tissues. This implies that signals delivered to the naïve T cells during antigen presentation may also induce homing properties. Thus, it is reasonable to suggest that the lymphoid microenvironment, including the resident APCs such as the DCs influence the gain of selective homing properties for tissues.

T cells activated in the cutaneous associated lymphoid tissue express high levels of CLA while those activated in intestinal lymphoid tissue acquire integrin $\alpha_4\beta_7$ expression. Indeed, dendritic cells from the GALT such as those found in the mesenteric lymph nodes and Peyers patches induce high expression of $\alpha_4\beta_7$ and CCR9 on T cell while reducing CCR4 *in vitro* (Johansson-Lindbom et al. 2003; Mora et al. 2003; Mora et al. 2005). Additionally, these T cells migrated more towards the chemokine CCL25 and had reduced expression of L-selectin (Johansson-Lindbom et al. 2003; Mora et al. 2003). The mechanism involved was later described to occur through the action of the Vitamin A metabolite retinoic acid on a subset of dendritic cells found primarily in the MLNs and Peyers patches (Iwata et al. 2004). Interestingly, Vitamin A deficient mice have reduced $\alpha_4\beta_7$ expressing T cells as well as depletion of T cells from the lamina propria of the gut (Iwata et al. 2004; Mora et al. 2005).

Similarly, T cells stimulated with DCs from the peripheral lymph nodes exhibited higher levels of fucosyltransferase-VII (FucT-VII), the enzyme required for post translation modification of PSGL-1, as well as high levels of P and E selectins in the study by (Mora et al. 2005). In addition, they displayed high levels of CCR4 and migrated more efficiently to the skin when adoptively transferred. Another mechanism postulated to explain this phenomenon was through the action of the vitamin D3 metabolite, $1,25(OH)_2D_3$. DCs isolated from the skin draining lymph nodes are demonstrated to convert vitamin D3 to $1,25(OH)_2D_3$ which would then act on T cells imprinting a skin homing phenotype (Sigmundsdottir et al. 2007).

It is important to bear in mind that T cell homing phenotypes are reversible. For example, activated T cells from the Peyer's patches re-stimulated with peripheral lymph node DCs are able to express PSGL-1 (Mora et al. 2005). Similarly peripheral lymph node T cells re-stimulated with Peyer's patches DCs can also acquire $\alpha_4\beta_7$ expression.

## 1.1.3 Regulation of T cell Migration

### 1.1.3.1 Molecular mediators involved in the regulation of T cell motility

**Role of Rho family GTPases in T cell migration**

In naïve T cells, the Rac GEF (guanine-nucleotide exchange factor) DOCK (dedicator of cytokinesis) 2 initiates the early wave of chemokine induced F actin formation. (Fukui et al. 2001), have previously demonstrated that DOCK-2 deficient T lymphocytes exhibit reduced chemokine induced F-actin formation, cell polarity and *in vitro* migration. Furthermore, DOCK2 deficient T cells were observed to have reduced interstitial motility within the lymphoid tissue (César Nombela-Arrieta et al. 2007). DOCK2 is also required for the chemokine-induced T cell adhesion under shear stress mediated by the integrin α4β1 (García-Bernal et al. 2006).

Another Rac GEF Tiam1 (T-cell lymphoma invasion and metastasis 1) has been demonstrated to be involved during chemokine-induced T-cell migration and associates with members of the Par (partitioning defective) polarity complex that include Par3 and PKC (protein kinase C)-ζ. The complex segregates at the leading edge of the cells and is important in establishing the anterior posterior axis (Gérard et al. 2007; Ludford-Menting et al. 2005).

Vav, another Rac GEF is also known to be involved in activation of integrins and adherence of T cells. Overexpression of Vav mutants, as demonstrated by (Vicente-Manzanares et al. 2005) diminished lymphocyte polarisation, actin polymerisation and migration responses to the chemokine CXCL12. However, opposing these results, Vav-1 deficient cells exhibited normal migration in the study performed by (Vicente-Manzanares et al. 2005).This could reflect the compensation of other Vav isoforms.

Rho-dependent signalling is also thought to be a key component of T cell migration and adhesion in response to several chemokines in mature T-cells and thymocytes (Cronshaw et al. 2004; Z. Li et al. 2005). In thymocytes, Rho-A activation was necessary for integrin activation induced by Rap1 and Rac (Vielkind et al. 2005). In addition, Rho-A controls the LFA-1 high-affinity state triggering by chemokines along with the lateral mobility induced by chemokines (Giagulli et al. 2004).

### 1.1.3.2 The role of PI3 Kinase in T cell migration

A large body of evidence has implicated Phosphoinositide 3-kinase (PI3K) signalling as a major mediator of cell motility and migration of leukocytes. In T cells, PI3K-dependent signals are induced by chemokine receptor, TCR and co-stimulatory receptor activation known to contribute to several aspects of T lymphocyte differentiation and homing as well as reorganization of actin and other components of the general migratory machinery (reviewed in Ward and Marelli-Berg 2009).

The class IA PI3 kinases are made up of a regulatory subunit and an associated catalytic subunit (reviewed in Ward 2006). Five regulatory subunits, encoded by three genes are known to exist in the class I PI3 kinases. The PI3K3r1 gene encodes p85α, p55α and p50α, the PI3Kr2 encodes p85β and the PI3Kr3 encodes p55γ regulatory subunits respectively. These subunits can bind to three catalytic isoforms p110α, p110β and p110δ and recruit the complex to the plasma membrane upon receptor ligation and phosphorylation. In T cells p85α and p50α are most abundant. A single class 1B catalytic isoform, p110γ can pair with either a p84 or p101 regulatory subunit and can be activated downstream of G protein coupled receptors (GPCRs). The class I PI3 kinases phosphorylate their substrates phosphoinositol (PI), PI(4)P (phosphoinositol 4-bisphosphate) and PI(4,5)P2 (phosphoinositol 4,5-bisphosphate) and PI(3,4)P2 (phosphoinositol 3,4-bisphosphate) into the resulting product PI(3,4,5)P3 (phosphoinositol 3,4,5-triphosphate). The resulting PI(3,4,5)P3 phospholipids bind to signalling proteins bearing a lipid binding domain such as PH (pleckstrin homology) and Phox homology domains. In resting T cells, the levels of PI(3,4,5)P are low but readily increase following activation (reviewed in Crabbe et al. 2007; Deane and Fruman 2004; Vanhaesebroeck et al. 2005). Phosphatases PTEN (phosphatase and tensin homologue deleted on chromosome 10) and SHIP (SH2 containing inositol phosphatase) dephosphorylate these PI(3,4,5)P3 back to PI(3,4)P2 thereby acting as negative regulators of PI3 kinase activity (S. J. Harris et al. 2008). Upon stimulation, pleckstrin homology domain–containing proteins such as Akt, phosphoinositide-dependent kinase 1 (PDK1), and phospholipase C (PLC) recognise and bind to newly generated and enriched PIP3, resulting in its transient membrane relocalization and consequent activation (Xue and Hemmings 2013).

Using genetic and pharmacological means, the contribution of individual classes of PI3 kinase to T cell migration has been extensively studied (Ward and Marelli-Berg 2009). These investigations have revealed class I PI3K isoforms P110γ and P110δ to be the dominant isoforms involved in T cell migration. In *in vitro* studies, p110γ deficient CD4 and CD8 T cell migration to chemokines

CXCL12, CCL19 and CCL21 is decreased significantly as compared to T cells from WT mice (Reif et al. 2004). However, in other studies using experimental systems that more accurately represent the physiological conditions of migrating cells, PI3Kγ inhibitors have had little effect on T cell migration (Martin et al. 2008). This may be a result occurring through off target activity of the PI3K selective inhibitors. Using multiphoton microscopy, studies have observed pan PI3K inhibitors to have no effect on the migration of T cells within the T cell zones of the secondary lymphoid organs (Asperti-Boursin et al. 2007; Worbs et al. 2007). It is important to note that in this regard several other PI3K independent signalling mechanisms could regulate migration. Rac guanine nucleotide exchange factors (GEFs) including DOCK2, Tiam1 and Vav have been implicated in T cell adhesion and migratory events (Ward and Marelli-Berg 2009) as described earlier. With respect to migration of activated T lymphocytes, the P110δ isoform is believed to be the dominant isoform coupled to the TCR and CD28. These aspects are further examined in the subsequent sections.

Besides these functions, PI3K-Akt signalling has an impact on the regulation of nutrient uptake and metabolism via downstream engagement of the mammalian target of rapamycin (mTOR) kinase complex. This complex co-ordinates nutrient sensing pathways to that of metabolism. Details regarding the PI3K-akt-mTOR axis are discussed later in the section 1.3.4.1.

### 1.1.3.3 Regulation of Migration by the T cell Receptor

While the acquisition of homing receptors and chemokine receptors confers a level of specificity to migrating T cells, additional mechanisms must exist to maximise the efficiency of specific memory T cell recruitment to antigen-rich large tissues. For example, the human gut can be as long as 6 metres in adult humans. A gut homing T cell would need additional methods through which it can find and localise to specific antigen-rich sites within the gut. One mechanism believed to provide this additional specificity is through the action of antigen presentation by the endothelium and its subsequent recognition by the TCRs of T cells interacting with the endothelium as a consequence of inflammation.

**T cell recruitment by antigen-presenting endothelium**

In humans, ECs express both Class I and Class II Major Histocompatibility Complex molecules. In mice, the ECs display only Class I molecules but can rapidly upregulate class II MHC molecules in response to stimulation via IFN-γ (Choi et al. 2004; Haraldsen et al. 1998; Savage et al. 1995). The extent of expression is described to vary amongst different tissues with non-lymphoid vascular ECs having the highest expression (reviewed in Choi et al. 2004). Along with the expression of MHC molecules, ECs are also known to be able to present antigen to T cells (Bagai et al. 2005; Greening et al. 2003; Haraldsen et al. 1998; Murray et al. 1994). Furthermore, a study in mice concluded that vascular ECs could cross present antigen to CD8 T cells – a property believed to be restricted to dendritic cells - and this process was mediated by transporter for antigen presentation (TAP1)- and Proteasome-dependent pathways (Bagai et al. 2005).

For T cells to become activated, two key signals are required for a full activation response. The first is mediated by TCR engagement by the cognate antigenic peptide displayed on the MHC molecule. The second signal occurs via co-stimulatory molecules such as CD80 (B7-1) and CD86 (B7-2) which can engage CD28 receptors on the T cells. The absence of a secondary signal can lead to state of unresponsiveness known as anergy (Schwartz 1996). Besides CD28, other molecules such as inducible co-stimulatory molecules such as ICOS and OX40 which bind to their ligands are also known to provide co-stimulatory signals for T cells (reviewed in Choi et al. 2004). In humans, ECs lack expression of both CD80 and CD86 while in mice, ECs display low levels of CD80 (Marelli-Berg et al. 2001b; Marelli-Berg and Jarmin 2004a). Co-stimulatory molecules such as the ICOS-L

and PD-1 are expressed constitutively on human ECs and are believed to prevent anergy induction upon antigen presentation by the endothelium (reviewed in Rudd and Schneider 2003).

As ECs are capable of displaying antigen to T cells, it raises the question as to whether such antigen presentation could result in proliferation and further activation. It is therefore postulated that the functional consequence of antigen presentation differs in light of the context it occurs in. Dendritic cells presenting antigen to T cells induce a TCR activated 'stop signal' which causes loss of migratory ability and polarisation to the APC resulting in proliferation (Dustin et al. 1997). In contrast endothelial cell antigen presentation to T cells does not result in proliferation in the absence of cytokines (Lodge and Haisch 1993; Pardi and Bender 1991). Interestingly, EC antigen presentation was observed to increase migration (Marelli-Berg and Jarmin 2004a) while presentation by epithelial cells resulted in anergy and loss of migratory ability (Marelli-Berg et al. 2001b). Loading of ECs with cognate antigen was observed to reduce migration of cross presented CD8 T cells *in vitro* suggesting that avidity of the signal may be important on determining the functional consequence of antigen presentation (Marelli-Berg et al. 2001a).

Thus antigen recognition by ECs can directly enhance T cell transendothelial migration without inducing anergy leading to the hypothesis that antigens are presented by the endothelium of infiltrating vessels. Several *in vivo* studies have given evidence in support of this hypothesis. In an experimental autoimmune encephalomyelitis (EAE) model, expression of MHC Class II molecules by the vascular endothelium in the central nervous system (CNS) was demonstrated to be crucial for T cell infiltration to the CNS (Sobel et al. 1984).

In another *in vivo* model of autoimmune diabetes in non-obese diabetic (NOD) mice, insulin specific CD8 T cell recruitment was abrogated in IFN-γ deficient NOD mice due to impaired autoantigen presentation by the MHC Class I and Class II molecules (Savinov et al. 2001). This effect could be reversed by systemic application of IFN-γ. In a separate study, islet specific homing by insulin specific CD8 T cells was impaired due to deficient insulin secretion, highlighting the cross presentation of local antigens by the endothelium (Savinov et al. 2003).

A mouse model incorporating male minor histocompatability antigen HY- specific CD4 T cells showed selective recruitment of these CD4 T cells in the inflamed peritoneum of male but not in that of female mice (Marelli-Berg et al. 2004b). Visualisation of the vascular bed of the peritoneum showed cognate recognition of antigen by the endothelium resulted in no difference in rolling or adhesion but a significant increase in diapedesis.

In the human system, antigen presentation by ECs was revealed to increase the motility of antigen specific memory CD4 T cells through endothelial monolayers *in vitro* (Marelli-Berg et al. 1999b). In addition, CD8 T cell clones were shown to migrate more efficiently through murine endothelial monolayers treated with murine IFN-γ than through endothelial monolayers that were not treated (Marelli-Berg et al. 2004b). In a separate study, IFN-γ treated glutamic acid decarboxylase (GAD) pulsed ECs were identified to increase the migration of GAD specific T cells *in vitro* (Greening et al. 2003). Here, antibody blockade of MHC inhibited the migration.

Collectively, these studies indicate that antigen-presenting endothelium facilitates the recruitment of antigen specific memory T cells to antigen-rich inflammatory sites.

**IFN-γ signalling and MHC upregulation**

IFN-γ is a type II interferon secreted by a variety of lymphoid cells that include CD4+ T cells, CD8+ T cells, NK cells and B cells. Also, APCs such as monocytes/macrophages and DCs are known to secrete IFN- γ locally, which is crucial for early host defences against pathogens. Deficiency in IFN-γ signalling has been correlated to reduced natural resistance against bacterial, viral and parasitic infections in mice. IFN- γ production is controlled by cytokines such as IL-12 and IL-18. In contrast, IFN- γ is negatively regulated by cytokines such as IL-4, IL-10, TGF-β, and by glucocorticoids.

The functional receptor for IFN- γ is a heterodimer composed of two ligand binding α chains (IFNGR1) and two signal transducing β chains (IFNGR2) (Bach et al. 1997).The IFN- γ receptor chains lack any intrinsic kinase/phosphatase activity and thus, associates with signalling machinery for signal transduction (Schroder et al. 2004). Binding of the IFN- γ to the IFN- γ receptor α chains enables receptor activation. The intracellular domains of the IFN- γ receptor bear binding motifs for the Janus tyrosine kinases (Jak)1/2 and the latent cytosolic factor, signal transducer and activator of transcription (STAT)1. The membrane proximal Jak binding motif LPKS in humans is located at residues 266-269, while, the STAT1 binding YDKPH motif are positioned at residues 440-444 (Schroder et al. 2004). In humans, the receptor-ligand internalisation is mediated through the isoleucine-leucine sequence at residues 270 and 271.

The Jak-STAT signalling pathway involves receptor recruitment and activation of members of both Janus family of kinases and the STAT family of transcriptional activators that control transcription of a variety of previously identified target genes. Upon ligand binding, Jak2

undergoes auto-phosphorylation and activation which then trans-phosphorylates Jak1 (Briscoe et al. 1996). This activated Jak1 then phosphorylates the tyrosine residue at the YDKPH domain enabling recruitment of latent STAT1 via its SH2 domain (Greenlund et al. 1995; Heim et al. 1995). The receptor recruited STAT1 pair (one on each α chain) becomes phosphorylated which in turn induces disassociation of the STAT1 homodimer and translocation to the nucleus. The SH2 domains that are necessary for dimerization also enable the STAT1 pair to bind to the promoter regions of IFN-γ activation site elements to initiate/suppress transcription of IFN-γ-regulated genes. Consequently, STAT1 deficient mice exhibit increased susceptibility to microbial and viral infections (Durbin et al. 1996; Meraz et al. 1996).

In contrast, negative regulation of the IFN-γ signalling is mediated through means of ligand-receptor internalisation. This ligand-receptor complex enters the endosomal pathway, where the complex disassociates (Farrar and Schreiber 1993; Schreiber and Farrar 1993). Eventually, the uncoupled and dephosphorylated receptor is recycled to the cell surface while the ligand is degraded. Additionally, specific feedback inhibitors such as SOCS-1 inhibit IFN-γ signalling by binding to the Jak1/2 and interfering with the tyrosine kinase activity (Alexander et al. 1999). Receptor and Jak dephosphorylation can also be mediated via PTPs such as SHP-1 and SHP-2 (Haque et al. 1997; You et al. 1999).

IFN-γ signalling mediated upregulation multiple functions in the Class I MHC antigen presentation pathway to increase the quantity and diversity of peptides presented on the cell surface (In context of Class I MHC) (Schroder et al. 2004). Stimulation via IFN-γ induces a replacement in the proteasome protein subunits which increases the quantity, quality and repertoire of peptides for Class I MHC loading. The proteasome enzymatic subunits are β1, β2, and β5 of unstimulated cells are replaced with the LMP-2 MECL-1 and LMP-7 subunits upon IFN-γ signalling (Schroder et al. 2004). These enzymatic subunits serve to increase levels and diversity of epitopes presented for CD8 T cell recognition in the context of Class I MHC. Furthermore, IFN-γ signalling induces PA28 activator, which increases the peptide generation by associating with the proteasome and altering proteasome proteolytic cleavage (Groettrup et al. 1996). Additionally, IFN-γ signalling induces TAP-1 protein that transports peptides from the cytosol to the endoplasmic reticulum (ER) lumen (York and Rock 1996). The TAP protein also transiently associates with the Class I MHC to aid in efficient peptide loading (Grandea et al. 1995; York and Rock 1996). IFN-γ signalling also upregulates other molecules such as the tapasin and GP96 chaperones which aid in the efficient peptide: Class I MHC complex (Anonymous 1994; Suto and Srivastava 1995).

The IFN-γ signalling mediated upregulation of peptide: Class II MHC molecules occurs by promoting expression of several molecules including the invariant (Ii) chain, cathepsins B, H and L (Lafuse et al. 1995; Lah et al. 1995) and lysosomal proteases involved in the production of antigenic peptides for Class II MHC loading. The transmembrane Ii chain chaperone aids in trafficking of Class II MHC molecules from ER to the MHC II endosomal compartment (MIIC)(Cresswell 1994; Wolf and Ploegh 1995). Furthermore, An Ii-derived peptide, class II-associated invariant chain peptide (CLIP) binds to the Class II MHC peptide-binding groove preventing inappropriate binding (Cresswell 1994; Wolf and Ploegh 1995). Regulators of peptide accessibility, such as DMA and DMB, remove CLIP from peptide binding grooves thus, enabling the groove to be accessible for peptide loading (Kern et al. 1995). Moreover, IFN-γ signalling upregulates Class II transactivator (CIITA) which acts as a switch for rapid upregulation of class II MHC molecule constituents such as the Ii chain and DM molecules (Boss 1997).

**Molecular mediators of antigen-dependent regulation of T cell motility**

Besides Class I PI3 Kinases, previous studies have identified a number of key molecular mediators that regulate migration in response to TCR signalling. These include the GEF Vav1 and the Rho GTPase Rac (Ward and Marelli-Berg 2009). Vav1−/− T cells display severe migratory defects *in vitro* and have impaired recruitment to antigen-rich tissue resulting from inability to engage sustained TCR signalling (David et al. 2009; Garcia-Bernal et al. 2005). T cells deficient in the Rac isoform Rac 2 also exhibit defects in TCR-induced signalling and proliferation as well as reduced chemotaxis to chemokines (Faroudi et al. 2010). On the other hand, increased Rac1 activity following TCR engagement leads to the loss of stable migratory polarity while T cells expressing constitutively active Rac1 produce multiple lamellae, lack a distinguishable uropod and exhibit impaired migration *in vitro (Cernuda-Morollón et al. 2010)*. Thus, Rac1 signalling is necessary for regulating T cell migration by inhibiting the migratory polarity formation. This process is necessary for the TCR induced 'stop signals' that facilitate interaction with antigen presenting cells and retention at inflammatory sites.

### 1.1.3.4 Regulation of Migration via co-stimulatory receptors

**An overview of CD28 co-stimulation**

As previously mentioned, naïve T cells require two signals for their activation. This two signal model of T cell activation, first derived from the work proposed by (Bretscher and Cohn 1970), hypothesises that the first signal is provided through the engagement of a specific antigen in the context of an MHC molecule by the TCR on the T cell. The second signal, a nonspecific one, is provided by the engagement of certain group of receptors by a number of distinct molecular interactions that occur between a T cell and APC. The best characterised of these receptors is the CD28 co-stimulatory receptor which binds to its ligands B7-1 and B7-2 on the APC (reviewed in Rudd and Schneider 2003). Activation of naïve T cells without CD28 signals leads to state of long lasting hyporesponsive state known as T cell clonal anergy, or cell death (Schwartz 1996). In humans, CD28 is expressed constitutively on all naïve and activated CD4 T cells and approximately 50% of CD8 T cells (Topp et al. 2003). Individuals of older age or with chronic infections are seen to have higher number of CD28 –ve T cells, suggesting loss of CD28 correlates with immune senescence (Posnett et al. 1999). Although, a distinct subset of immunosuppressive CD8+ T cells that are CD28 -ve have been identified in mice (Ben-David et al. 2007), CD28 is expressed on all T cells and is not down regulated with age or chronic disease (reviewed in Strioga et al. 2011).

CD28 has been described as a positive co-stimulator of immune responses due to its effect on the activation of T cells. Co-ligation of CD28 with the T cell receptor reduces the threshold for activation by reducing the number of TCR stimulations necessary for activation and by decreasing the duration of TCR engagement (reviewed in Alegre et al. 2001a; Brunner-Weinzierl et al. 2004; Salomon and Bluestone 2001). In addition, CD28 ligation was observed to favour survival rather than apoptosis following TCR stimulation by the upregulation of pro-survival anti-apoptotic protein BCL-XL (Parry et al. 2003). Among other functions, co-ligation of CD28 is known to upregulate the secretion of cytokines, most importantly IL-2, a pro survival cytokine (Kane et al. 2001). This was found to be mediated by both increased transcription of the IL-2 gene as well as increased stabilization of IL-2 mRNA (Parry et al. 2003). CD28 also enhances glucose metabolism by increasing expression of the glucose transporter Glut1 (Jacobs et al. 2008). Thus, several different pathways are integrated by CD28 receptor signalling to result in increased T cell activation and survival thus strengthening its identity as a positive co-stimulatory molecule.

The CD28 signalling pathway is only a part in a complex network of structurally related signalling pathways. Other members of the B7 family of co-stimulatory receptors include cytotoxic T lymphocyte associated antigen 4 (CTLA-4/CD152), the inducible co stimulatory receptor (ICOS), programmed death receptor 1 (PD-1) and B and T lymphocyte attenuator (BTLA) (reviewed in Greenwald et al. 2005; Sharpe and Freeman 2002).These bind to their ligands B7-1/B7-2, ICOSL (B7h), PDL-1/PDL-2 and HVEM respectively, expressed on antigen presenting cells. While CD28 is constitutively expressed, the expression of the other CD28 family members by T cells is rapidly and transiently upregulated after activation.

**CD28 and Migration**

The CD28 co-stimulatory receptor is well known to induce signalling pathways that mediate adhesion and cytoskeleton rearrangements. These signalling pathways are crucial in mediating T cell motility and thus, CD28 has emerged as a positive regulator of T cell migration.

Crosslinking of CD28 on human CD4 T cells induced a rapid upregulation of integrin mediated adhesion to fibronectin, ICAM-1 and VCAM-1 which was described to be dependent on the activity of the Protein kinase C (PKC) (Shimizu et al. 1992). Furthermore, CD28 was described to rapidly increase LFA-1-ICAM-1 interactions between T cells and B7-1 transfected melanoma cells, a process shown to be dependent on PKC activation (Turcovski-Corrales et al. 1995). More evidence suggesting a role in adhesion was provided by another study showing increased β1 integrin-mediated adhesion of a myelomonocytic cell line following CD28 activation (Zell et al. 1996). CD28 mediated tyrosine phosphorylation of the adaptor molecule Casitas-B-lineage lymphoma protein (CBL)-B at its cytoplasmic Src homology 2 (SH2) domain resulting in recruitment and activation of PI3-kinase was observed to be the driving molecular mechanism of this phenomenon (Krawczyk et al. 2005; Zell et al. 1998). Interestingly, this pathway was noted to be distinct from the direct association of PI3K with the SH2 binding domain motif (Wooten et al. 1999). CD28 also plays an important role in cytoskeletal rearrangements in T cells. CD28 triggering induced upregulation of F actin through the activity of the Ras homologue GTpases Rac1 and Cdc42 (Kaga et al. 1998; Salazar-Fontana et al. 2003). In a separate study, Cdc42 activation following CD28 engagement was described to occur through the action of Src kinases independently of ZAP 70 (Zeta-chain-associated protein kinase 70) activation through TCR engagement (Salazar-Fontana et al. 2003). In the study by (Michel et al. 2000), CD28 was

observed to activate Vav1 independently of the TCR. In addition, CD28 engagement induced actin polymerisation through interaction with the Wiskott-Aldrich syndrome protein (WASp) via PI3-Kinase (Badour et al. 2007). WASp protein is also known to couple TCR signals with cytoskeletal rearrangements (Watanabe et al. 2013).

*In vivo* experimental models of autoimmune diseases were crucial for establishing a role of CD28 in the regulation of T cell migration. Using these models, loss of CD28 signalling was shown to lead to inefficient localisation of T cells following priming. In a model of EAE, CD28 deficient mice showed significantly delayed type hypersensitivity and reduced disease severity while CD28 deficient T cells continued to exhibit vigorous responses to myelin derived antigens ex vivo (Girvin et al. 2000). Interestingly, in the absence of CD28 ligands the adoptive transfer of encephalitogenic T cells localised to meningeal but not to parenchymal sites of the CNS (Chang et al. 1999). In a separate study utilizing an EAE model in female NOD mice, antibody blockade of the CD28 ligands CD80 and CD86 abrogated tissue infiltration, inflammation and demyelination (Chang et al. 1999; Girvin et al. 2000).

CD28 can also affect the migration of primed T cells to the germinal centers (GC) of secondary lymphoid organs in order to sustain B cell responses. In one study, CD28 dependant upregulation of OX40 was observed to be crucial for the upregulation of the chemokine receptor CXCR5 (necessary for migration to B cell zones) (Walker 1999). In addition, CD28-deficient mice failed to develop GCs in the Peyer's patches, spleen, or other peripheral lymph nodes.

Direct evidence implicating CD28 in the regulation of primed T cell migration to antigenic tissue was provided by a study utilizing transgenic mice bearing a point mutation at the PI3K binding motif (YMNF) of the cytoplasmic tail of the CD28 receptor ($CD28^{Y170F}$) (Mirenda et al. 2007). T cells from these $CD28^{Y170F}$ mice bear a CD28 receptor unable to recruit class I PI3Kinases but still maintain normal clonal expansion and IL-2 production (Okkenhaug et al. 2001). In the study by (Mirenda et al. 2007), OT II (ovalbumin specific, MHC class II restricted TCR transgenic) and OT-II/$CD28^{Y170F}$ double transgenic mice were generated and their T cells were described to proliferate equally following immunization with ovalbumin. However, only OT II T cells were able to localise to ovalbumin-expressing peritoneal cavity and tissue suggesting that PI3K recruitment via the YMNF motif was crucial for recruitment of T cells to antigenic sites.

It has also been observed that dominant CD28 signalling can override the regulation of memory T cell trafficking by the TCR and homing receptors and lead to unregulated T cell trafficking to non-lymphoid tissue (Mirenda et al. 2007). Optimal antibody activation of CD28 in HY-specific H2-Db-

restricted CD8+ T resulted in the unregulated infiltration of various organs such as the gut, liver, kidney spleen and heart in syngeneic female mice. This effect occurred specifically in memory rather than naïve T cells and the effect was abrogated in memory $CD28^{Y170F}$ HY-specific CD8+ T cells (Mirenda et al. 2007).

Conversely, this enhanced homing of T cells to multiple organs involved a vast number of memory T cells and not naïve T cells and may explain the effects seen in patients treated with a humanised CD28 super-agonist (TGN1412) in the TeGenero Trial at Northwick Park. Here, healthy human volunteers, after receiving this CD28 super-agonist, were reported to suffer from multiple organ failure and a 'cytokine storm' which is believed to have occurred due to uncontrolled infiltration of memory T cells into non-lymphoid tissue causing damage to the tissue through the release of pro-inflammatory cytokines TNFα, IL-2, IL-10 and IFN−γ (Suntharalingam et al. 2006). Given that in humans 50% of T cells are of a memory phenotype compared to that of laboratory mice which have a relatively large naïve T cell repertoire, it is plausible that the superagonist may have triggered unregulated non lymphoid tissue infiltration by memory T cells that would not be seen in mice kept in sterile conditions (Eastwood et al. 2010) (reviewed in Marelli-Berg et al. 2007).

In this context, it is important to note that it is highly likely that co-stimulation mediated regulation of migration results from interaction with antigen presenting cells rather than ECs that are also know to display antigens in response to inflammatory stimuli (reviewed in Marelli-Berg et al. 2007). Evidence supporting this includes the observation that human ECs do not express B7 co-receptors while, murine ECs only express low levels of B7-1 (Marelli-Berg et al. 1999a; Marelli-Berg et al. 2000). Furthermore murine ECs do not upregulate B7-1/2 expression in response to cytokine mediated stimulation (Marelli-Berg et al. 2000). Similarly, immature dendritic cells also express very low levels of B7 molecules.

**CTLA-4: an overview.**

Cytotoxic T-Lymphocyte Antigen 4(CTLA-4), also known as CD152, is a negative regulator of the immune responses. CTLA-4 bears significant structural similarities and shares approximately 30% homology with CD28. It also shares binding specificity to the CD28 ligands CD80 and CD86 and actively competes for its ligands (Walker and Sansom 2011). However, the binding affinity of CTLA-4 to its ligands is 20-50 fold higher and exhibits different kinetics *in vitro.* The regulation of the negative signals provided by CTLA-4 is believed to be a consequence of its tightly regulated and timely expression.

While CD28 is constitutively expressed by T cells, surface expression of CTLA-4 on T cells, excluding regulatory T cells, only occurs after activation (Brunet et al. 1987; Freeman et al. 1992; Linsley et al. 1992). In resting T cells, little CTLA-4 is expressed on the surface with the majority mainly located within intracellular vesicles (Alegre et al. 1996a; Linsley et al. 1996). This occurs by virtue of its interaction with the u2 subunit of the clathrin adaptor protein complex AP2 (Chuang et al. 1997; Y. Zhang and Allison 1997). TCR engagement rapidly induces trafficking of CTLA-4 from the intracellular vesicles to the cell surface with maximal expression reported to occur between 48-72 hours post-activation (M. L. Alegre et al. 1996b; Linsley et al. 1996). However, CTLA-4 endocytosis processes are still seen to occur even at the stage of maximal surface expression. Thus, the expression of CTLA-4 by activated T cells is transient, as this molecule is rapidly internalised by endocytosis.

The contribution of CTLA-4 signal to the regulation of T cell migration was first reported by studies in CTLA-4 knockout mice. These mice rapidly developed lymphoproliferative disease with multiorgan lymphocytic infiltration, tissue destruction, severe myocarditis, pancreatitis, and died by 3-4 weeks of age. (Tivol et al. 1995; Waterhouse et al. 1995)

**CTLA-4 function**

Unlike CD28, much less is known about how CTLA-4 functions as a negative modulator of immune responses. Several models have been proposed to explain its mode of action (reviewed in Rudd 2008; Walker and Sansom 2011). These models can be further divided into those in which CTLA-4 ligation affects the T cell that expresses it (cell-intrinsic models) and those in which CTLA-4 carries out its effects through other cells (cell extrinsic models)

**Cell intrinsic mechanisms**

CTLA-4 has a similar ligand specificity as CD28 for its ligands CD80 and CD86, however CTLA-4 binds with a 50 -100 fold greater avidity to them. Thus, one model hypothesises that the CTLA-4 functions by simply outcompeting CD28 for its ligands. Evidence in favour of this hypothesis was given in the study by (Masteller et al. 2000). In their study, a truncated variant of the CTLA-4 gene lacking its cytoplasmic domain, necessary for signalling, was described as 'sufficient' in retarding the disease onset in CTLA-4-/- mice (Masteller et al. 2000). High expression of this gene variant in CTLA-4-/- deficient mice did result in lymphodenopathy but little tissue infiltration. As such, the mice survived to adulthood and were able to reproduce. This suggested that the binding ectodomain of CTLA-4 could compete with the CD28 molecule thereby preventing positive co-stimulation and subsequently proliferation of the T cells. It also suggested that the cytoplasmic domains were necessary for preventing the massive tissue infiltration observed in CTLA-4 deficient mice. However, these observations were complicated by the fact that CD28 signalling was required for the onset of the disease in CTLA-4-/- mice (Tivol 1997). Conversely, blockade of CD28 by means of an antibody or through expression of an inhibitory variant of the CD28 gene could prevent the disease in CTLA-4-/- mice (Tai et al. 2007). Furthermore, since conventional T cells only express CTLA-4 after activation, it is unlikely that this mechanism would restrain the priming of naïve cells (Walker and Sansom 2011).

Another mechanism through which CTLA-4 functions in T cells was described by imaging studies analysing the formation of lipid rafts that act as platforms for signalling in T cells. While CD28 signalling was shown to promote lipid raft formation following TCR stimulation, CTLA-4 had an opposite effect (Chikuma et al. 2003; Martin et al. 2001; Rudd et al. 2002). Thus this model hypothesises that CTLA-4 functions by simply decreasing the formation of these lipid rafts in naïve T cells which hold key components required for TCR signalling. However, this model suffers a caveat in that the CTLA-4 effect on secondary responses are much less pronounced than that observed in naïve T cells even though the total CTLA-4 in naïve T cells is likely to be much lower.

A similar model showed that CTLA-4 ligation inhibits the formation of microclusters (Schneider et al. 2007). Following TCR ligation, TCR molecules arrange themselves into packed structures at the point of engagement. These 'microclusters' assemble the necessary signalling proteins and tyrosine kinases within seconds of TCR ligation (Yokosuka 2005). These include the kinase ζ-chain-associated protein kinase of 70 kDa (ZAP70) and adaptors, such as the Linker for activation (LAT) and SH2-domain-containing leukocyte protein of 76 kDa (SLP76) (Bunnell 2006;

Yokosuka 2005). In (Schneider et al. 2007)'s study, co-ligation of TCR and CTLA-4 blocked the formation of ZAP70 containing microclusters in T cells. The resulting impairment of microcluster formation was thought to dampen the TCR signals necessary for T cell proliferation. Another study by (Yokosuka 2010), gave a spatiotemporal basis for CTLA-4 mediated negative signalling at the central supramolecular activation cluster (cSMAC) of the immunological synapse. In this study, CTLA-4 directly competed with CD28 for its ligands at the cSMAC, ultimately resulting in the exclusion of CD28 from the cSMAC. This study provides further evidence for competition between CD28 and CTLA-4. In addition, their observations indicated that CTLA-4 inhibited the translocation of Protein kinase C θ (PKCθ) and a scaffolding protein CARMA1 necessary for CD28 mediated positive co-stimulation at the cSMAC, thus, providing further negative regulation.

Another model hypothesises that CTLA-4 exerts is negative regulation by engagement of negative signalling molecules that regulate TCR signalling. CTLA4 is thought to bind the protein tyrosine phosphatases (PTPases) SHP2 (sRC homology 2 (SH2)‐domain‐containing PTPase 2) (K. M. Lee 1998) , PP2A (protein phosphatase 2A) (Chuang 2000) and SYP (also called PTPN11) (Marengere 1996).These phosphatases are known to inhibit T cell activation by dephosphorylating key signalling molecules downstream of the TCR. Evidence in favour of this was given in the studies by (Martin et al. 2001; Schneider 2002). Here, antibody-induced co-ligation of CTLA-4 with the TCR reduced phosphorylation of CD3ζ and the linker for activation of T cells (LAT) along with reduction in phosphorylation levels of the extracellular-signal-regulated kinases (ERKs). While LAT proteins bind other activating proteins downstream, ERKs are required for the production of IL-2. Indeed, CTLA-4 deficient cells show reduced ERK activity. However, this model too has some downfalls. Firstly, CTLA-4 does not bear binding sites for the SHP2 molecules suggesting an indirect connection (H. Schneider and Rudd 2000). In addition, SHP2 has also been described to be a positive regulator of ERKs (Frearson and Alexander 1998). Secondly, while PP2A clearly inhibits cell growth by acting on Protein Kinase B (PKB/ Akt), a kinase with multiple functions within the cell, there is low level of CTLA-4 binding to PP2A relative to its great abundance in the cells and several reports have also identified CD28 to bind PP2A (Chambers and Allison 1999). The relationship between PP2A and these co-stimulatory molecules remains to be investigated. Nonetheless, it is plausible that phosphatases such as PP2A and SHP2 may be utilised by CTLA-4 to provide negative regulation to the T cells. In a separate study, ligation of CTLA-4 led to the upregulation of E3 ligase Casitas B‐lineage lymphoma B (CBL‐B), an E3 ligase of the ubiquitylation pathway that has been linked to autoimmunity (Bachmaier 2000; Chiang 2000). CBL-B deficient mice develop autoimmunity but fail however to develop the lethal disease exhibited in CTLA-4 deficient mice (Bachmaier 2000).

Finally, CTLA-4 has been proposed to function by inducing the reversal of the TCR induced stop signal (reviewed inRudd 2008). TCR triggering initiates a reduction or slowing of T cell motility. This 'stop signal' was observed to be necessary to form stable contacts with APCs, thus, allowing for the formation of the stable immunological synapses between the T cells and APCs (Dustin et al. 1997). (Schneider 2006) demonstrated that triggering of CTLA-4 reverses this stop signal both *in vivo* and *in vitro*. Furthermore, CTLA-4 deficient cells were described to have reduced motility and more stable contacts when treated with peptide pulsed antigen presenting cells *in vitro*. This model also suggests that the increase in motility following CTLA-4 triggering could be explained through increased activation and clustering of LFA1 via means of recruitment of the GTPase RAP1 by CTLA-4 (Dillon et al. 2005). Although high affinity LFA-1 interaction with its ligand inter cellular adhesion molecule (ICAM-1) is required for cell immobilization, it is also required for increased cell motility. Indeed, CTLA-4 deficient T cells showed impaired adhesion to ICAM-1 *in vitro (Schneider et al. 2005)*. Thus, this model hypothesises that CTLA-4 functions by reversing TCR mediated stop signals which reduce the dwell times of T cell with APCs ultimately raising the threshold for activation.

**Cell extrinsic mechanisms**

Initial studies investigating the role of the B7 pathways in T cells showed that CTLA-4 blinding to CD80 and CD86, expressed by antigen presenting cells greatly reduced the activation of the T cells (Krummel and Allison 1995).

One mechanism believed to explain the extrinsic function of CTLA-4 is through the activation of the enzyme Indoleamine 2,3-dioxygenase (IDO). The interaction of CTLA-4Ig to CD80 and CD86 expressed on antigen presenting dendritic cells stimulates them to express IDO (Fallarino 2003; Grohmann 2002). IDO catabolises the amino acid tryptophan into kynurenine, a tryptophan catabolite toxic to T cells neighbouring the dendritic cells thereby suppressing immune activation and preventing further activation of effector T cells (Munn 1999). Evidence to support this was given in the study where an IDO inhibitor 1- methyltryptophan (1MT) prevented the CTLA-4Ig induced survival of pancreatic islet allografts (U. Grohmann et al. 2002). However, this function of CTLA-4 still is another controversial topic. This is due to the fact that IDO-deficient mice lack the phenotype seen in CTLA-4 deficient mice. Additionally, 1MT was also observed to modify DC function independently of IDO (Agaugue et al. 2006).

Preliminary studies observing CTLA-4 expressing T cell interaction with APCs showed that CTLA-4 engagement with CD80/86 on the APC resulted in the downregulation of these molecules without any effect on other co stimulatory molecules ligands such as CD40 or even MHC Class II (Oderup et al. 2006; Schildknecht 2010). Interestingly this effect was not inhibited through the action of the IDO inhibitor 1MT (Oderup et al. 2006).

Recent studies have indicated that CTLA-4 can physically remove its natural ligands on APCs following binding thereby reducing positive co-stimulation by CD28 (Soskic et al. 2014; Walker and Sansom 2015). Elegant Imaging studies revealed that following binding to its natural ligands, these are removed from the APC and CTLA-4:B7 complexes undergo rapid endocytosis and are degraded within lysosomes. (Hou et al. 2015a; Qureshi et al. 2011) In essence, this model represents an extension of the direct competition model between CD28 and CTLA-4, whereby CTLA-4 not only competes with CD28 for its ligands but also reduces their availability to CD28.

One of the simplest models believed to account for CTLA-4 function hypothesises that ligation of CTLA-4 expressed on regulatory T cells switches on their suppressive ability. In the study by (Barnes et al. 2013), the B7 ligands CD80 and CD86 were capable of inducing Foxp3+ Treg populations *in vitro* and in and in an *in vivo* T cell transfer model of colitis. Additionally, the authors observed enhanced expression of Foxp3, a key nuclear transcription factor for regulatory T cell function, following the B7 ligand treatment. Regulatory T cells (Tregs) express CTLA-4 constitutively and are able to suppress the activation of conventional T cells (described in 1.2.2). The role of CTLA-4 in Treg cells will be discussed in section 1.2.2.1.

**CTLA-4 and migration**

Whether the CTLA-4 co stimulatory molecule functions to mediate pro or inhibitory migratory signals remains as yet controversial. It is likely that CTLA-4 may regulate migration of T cells in conjunction with signals from the TCR or even through means of inhibiting pro migratory signals induced by CD28 stimulation.

As mentioned previously, CTLA-4 triggering induces clustering of LFA-1 integrin *in vitro* through the activation of the small GTPase Rap1 (Dillon et al. 2005; Katagiri 2000). In addition, CTLA-4 ligation was noted to enhance motility of T cells on ICAM-1 coated plates and thus is believed to share similar adhesion and motility regulating signalling pathways and effects as CD28 (Schneider et al. 2005).

However, *in vivo* studies have showed that CTLA-4 ligation induces signals that are antagonistic to CD28 induced pro-migratory signals at least during T cell migration to non-lymphoid tissue (reviewed in Marelli-Berg et al. 2007). CTLA-4 is thought to negate the CD28 signals that positively regulate T cell extravasation through endothelial barriers at the site of antigen localisation. This was clearly demonstrated in the studies showing that intra-peritoneally injected, CTLA-4 ligated, CD8+ HY-specific restricted C6 T cells clones were prevented from entering the peritoneal membrane (Mirenda et al. 2007). Interestingly, this negative regulation of endothelial extravasation of T cells by CTLA-4 occurred in a strictly “cognate recognition of antigen”-dependant i.e in conjunction with antigen-receptor triggering.

Finally, it was proposed that CTLA-4 may provide its inhibitory effects on migration of T cells by reversing CD3/CD28-mediated phosphorylation via the selective recruitment of phosphatases such as SHP-1 and SHP-2. The cytoplasmic domain of CTLA-4 has been reported to interact with the tyrosine phosphatase SHP-2 and the serine/threonine phosphatase PP2A.

In one *in vivo* study, migration of Th1 differentiated CD4+ T cells to sites of inflammation was increased via enhanced chemotaxis towards chemokines ligands of CCR5 and CCR7 when CTLA-4 signals were given together with CD28 and TCR signals (Knieke et al. 2009). In the same study, CTLA-4 signals appeared to increase activation of the PI3K-Akt pathway induced by the CCR5 ligand CCL4.

In summary, the overall effect of CTLA-4 signals on PI3Kinase activation and T cell motility remains controversial.

**The interplay between CD28 and CTLA-4 signals in the regulation of T cell migration**

Both CD28 and CTLA-4 are known to impact signalling downstream of the TCR. However, a thorough analysis of the competition between signals from the two opposing co-receptors as well as the selective use of signalling pathways that affect migration are yet to be defined. Recently, a study by (Jain et al. 2013), demonstrated that CD28 and the interleukin-2 (IL-2)–inducible Tec kinase (ITK) regulate the trafficking of autoreactive T cells. PI3K signalling enables ITK recruitment to both the T cell receptor (TCR) and CD28 upon stimulation. Phosphorylation of ITK activates phospholipase C-γ1, leading to calcium (Ca2+) mobilization and actin polarization to the site of TCR stimulation. In addition, ITK has also been observed to become activated through $\beta_1$ integrins and is involved in Cdc42- and Rac-mediated chemokine-induced migration (Takesono et al. 2004). In this study, concurrent ablation of CD28 and ITK enabled self-reactive Ctla4−/− T cells to accumulate in secondary lymphoid organs without blocking spontaneous T cell activation. Additionally, the authors (Jain et al. 2013) demonstrated that the Itk−/−Ctla4−/− mice remain healthy, mount antiviral immune responses and exhibit a long lifespan, even though excessive spontaneous T cell activation and proliferation in lymphoid organs is observed.

Similarly, other signalling pathways that are engaged by these co-stimulatory receptors, and display common points of interaction, may account for their roles in regulation of migration of T cells. Yet, these pathways remain to be investigated.

**CD31 as a negative co-stimulator of T cells**

Previously we have described CD31 as a crucial mediator of TEM facilitating recruitment of leukocytes to inflammatory sites. As mentioned before, CD31 is a multifaceted Ig-like molecule that has been described to have both pro-inflammatory and anti-inflammatory functions. While the pro-inflammatory role largely accounts from its ability to facilitate TEM of leukocytes towards inflamed tissues, its anti-inflammatory roles are thought to occur via two mechanisms.

Firstly, CD31 is important for the maintenance of the vascular barrier integrity (Privratsky et al. 2011). This function of CD31 was first described by (Ferrero et al. 1995) who showed that treatment with a blocking antibody specific for CD31 increased the permeability of endothelial monolayers *in vitro*. In another study, CD31 deficient mice exhibited increased inflammatory cell infiltration into the brain parenchyma in a model of EAE, and this phenotype was correlated with enhanced barrier function of CD31- expressing endothelial monolayers (Graesser et al. 2002).

Secondly, and more importantly for this study, several reports have highlighted CD31 as a crucial immuno-regulatory receptor acting to suppress leukocyte activation and cytokine secretion. A summary of all these reports, adapted from (Privratsky et al. 2010) are highlighted in the Table 2 given below. We shall, however, describe the immunomodulatory role of CD31 in detail in the next section.

| Inflammatory model | Results | Mouse strain | References |
|---|---|---|---|
| In vitro B lymphocyte activation assays | B cells expressing PECAM-1 have lower proliferation rates in response to BCR cross-linking and decreased antibody production in response to T-independent antigens | C57BL/6J | (Wilkinson et al. 2002) |
| Spontaneous immune complex-mediated glomerulonephritis | Expression of PECAM-1 helps to prevent the spontaneous development of autoantibodies and immune complex-mediated glomerulonephritis | C57BL/6J | (Wilkinson et al. 2002) |
| In vitro T lymphocyte activation assays | Cross-linking of antibody bound PECAM-1 attenuates calcium mobilization following CD3 cross-linking | N/A | (Newton-Nash and Newman 1999) |
| IgE-dependent systemic and local anaphylaxis | Expression of PECAM-1 is correlated with lower serum histamine concentrations and decreased tissue swelling and mast cell degranulation following exposure to allergic stimuli | C57BL/6J | (Wong et al. 2002) |
| Collagen-induced arthritis | PECAM-$1^{+/+}$ mice have delayed onset of arthritis with decreased pro-inflammatory cytokine production and leukocyte infiltration into joints compared to PECAM-$1^{-/-}$ mice | C57BL/6J | (Tada et al. 2003;Wong et al. 2005) |
| Experimental autoimmune encephalitis (EAE) | Expression of PECAM-1 delays the onset of EAE, decreases parenchymal inflammatory cell infiltration and promotes vascular integrity | C57BL/6J | (Graesser et al. 2002) |
| LPS-induced endotoxic shock | Expression of PECAM-1 is protective in LPS-induced endotoxemia and is correlated with decreased production of pro-inflammatory cytokines and increased vascular integrity | C57BL/6J | (Carrithers et al. 2005;Maas et al. 2005) |
| In vitro cytokine production by macrophages | Ligation and cross-linking of PECAM-1 by CD38-Fc fusion protein inhibits pro-inflammatory cytokine production in LPS-stimulated macrophages | N/A | (Rui et al. 2007) |
| Atherogenic-diet-induced steatohepatitis | Expression of PECAM-1 delays onset and lessens severity of atherogenic diet-induced steatohepatitis by decreasing pro-inflammatory cytokine production and leukocyte infiltration | C57BL/6J | (Goel et al. 2007) |
| Atherosclerosis in LDL receptor$^{-/-}$ mice | Expression of PECAM-1 is correlated with decreased atherosclerotic lesion area in the total aorta with preferential protection in the aortic sinus, descending aorta, and the branching arteries of the aortic arch | C57BL/6J | (Goel et al. 2008) |

## Table 2 Anti-inflammatory functions of CD31

*This table lists various studies where CD31 expression has had an anti-inflammatory effect either delaying or reducing disease severity in disease models. It also indicates studies where CD31 crosslinking or ligation has shown to incite anti-inflammatory functions. This table is adapted from (Privratsky et al. 2010)*

#### Signalling Function of CD31

CD31 medites its intracellular signalling via the two ITIM motifs in the cytoplasmic tail (Newman and Newman 2003). Upon homophilic or heterohpilic engagement of CD31 or through the action on other stimuli (TCR triggering), the tyrosine residues at postions 663 and 686 within these ITIM domains become phosphorylated. However, CD31 is generally not found to be tyrosine phosphorylated in cells maintained in a resting state. Since CD31 does not bear an intrinsic kinase activity, the exact kinases involved in this process have been the subject of intense investigation.The Src family of kinases is thought to be the dominant kinase family involved in this process (Cao et al. 1998; Cicmil et al. 2000). However, studies have identified Src-independent tyrosine phosphorylation of CD31 via the Csk and Syk family of protein kinases (Cao et al. 1998; Sagawa et al. 1997). Once phosphorylated, these ITIM motifs allow for the recruitment of Src homology 2 (SH2) domain-containing signaling proteins which can then initiate signalling pathways further downstream.

CD31 inhibitory receptor signalling extends to several cell types such as mast cells, macrophages and dendritic cells and lymphocytes. In a study by (Wong et al. 2002), CD31 signals suppressed mast cell activation, which prevented systemic and local IGE-dependent anaphylactic reactions when animals were challenged with allergic stimuli. In macrophages, ligation of CD31 by the CD38-Fc fusion protein was identified to negatively regulate TLR-4 signalling likely through ITIM/SHP-2 interactions (Rui et al. 2007). Recently, in DCs, activating the CD31/SH2 domain-containing tyrosine phosphatase-signalling pathway with an agonist was described by (Clement et al. 2014) to reduce DC maturation. This rendered them more tolerogenic towards recall antigens in both *in vitro* and *in vivo*.

**CD31-mediated inhibition of Leukocyte activation**

CD31-mediated inhibition of T cell activation was first identified in the study by (Newton-Nash and Newman 1999) where CD31 was described to attenuate TCR-induced mobilization of calcium from intracellular stores. Recently, a study by (Ma et al. 2010) provided direct evidence that CD31 regulates T cell activation *in vivo* through partial inhibition of proximal TCR signalling, specifically Zap-70 phosphorylation. In B cells, the reduction of calcium mobilization following CD31 crosslinking was shown to be mediated by recruitment of SHP-2 phosphatase via the cytoplasmic ITIM domains of CD31 (D. K. Newman et al. 2001). Together, these studies indicate that CD31 can inhibit activation of T and B cells by recruiting (SH2) domain-containing tyrosine protein phosphatases that dephosphorylate key mediators in the proximal TCR/BCR signalling thereby increasing the overall threshold for activation. Figure 3 adapted from (Marelli-Berg et al. 2013) depicts an overview of CD31-mediated inhibition of T cell activation. In support of this notion, CD31 deficient mice exhibit aberrant proliferation and activation of B cells, which correlates with the development of autoimmune disease in older mice (Wilkinson et al. 2002).

Even though the studies mentioned above suggests that the CD31 negatively inhibits T cell activation, CD31-deficient mice do not display autoimmune symptoms under steady state conditions and instead, exhibit a very mild phenotype with a normal number of T cells (G. S. Duncan et al. 1999a). However, under conditions of inflammatory stress, lack of CD31 affects the extent of T-cell mediated inflammation in mice. Given below is a table adapted from (Marelli-Berg et al. 2013) that describes various models of disease that have been studied in CD31-deficient mice and the effect of CD31 deficiency on the outcome of the disease.

The absence of any autoimmune symptoms in CD31 deficient mice during steady state conditions (i.e. absence of any inflammation/immune response) may be a consequence of an additional role of CD31 in maintaining cell survival in leukocytes. In (Ma et al. 2010)'s study, CD31-deficient mice exhibited accelerated and pronounced allograft and tumour rejection. However, their study also revealed that, following the enhanced expansion, CD31 deficient T cells displayed increased activation-induced cell death (AICD). This result was credited to CD31 signalling-mediated activation of the extracellular-signal-regulated kinase (Erk), which associates with pro-survival pathways independently of TCR signalling. In a separate study by (Ross et al. 2011), using a model of salmonella infection, CD31-deficient mice displayed delayed clearance of pathogens, in spite of increased T cell activation. The authors claimed that increased proliferation and activation by the CD31-deficient CD4 T cells did not increase pathogen clearance as reduced survival following proliferation resulted in slower accumulation of activated cells, which are essential for pathogen removal. Thus CD31 signalling not only increases the threshold for T cell activation, but, also increases T cell survival.

| Disease model | Effect of CD31 deficiency | Reference |
|---|---|---|
| Induction of EAE with MOG peptide in C56BL/6 mice | Enhanced leukocyte extravasation and accelerated onset of EAE | (Graesser et al., 2002) |
| CIA in DBA/1 mice | Enhanced arthritis | (Tada et al., 2003; Wong et al., 2005) |
| Exposure to the bacterial endotoxin LPS | Septic shock | (Maas et al., 2005) |
| Laser-induced and $FeCl_3$ endothelial injury | Accelerated vascular occlusion (thrombosis) | (Falati et al., 2006) |
| Diet-induced non-alcoholic steatohepatitis | Progressive liver disease | (Goel et al., 2007) |
| LDLR KO (hypercholesterolemic) mice | Accelerated atherosclerosis | (Goel et al., 2008) |
| ApoE-deficient (hypercholesterolemic) mice | Inhibited atherosclerosis | (Harry et al., 2008) |
| Bone marrow hematopoietic cell engraftment | Hypersensitivity to macrophage CSF and receptor activator of NF-κB ligand; osteoclastic bone loss | (Wu et al., 2009) |
| Lipopolysaccharide (LPS)-induced endotoxemia | Cytokine storm and acute respiratory distress syndrome due to accumulation of cytokine-producing leukocytes at sites of inflammation | (Privratsky et al., 2010). |
| Tumor and skin allograft | Enhanced tumor and skin allograft rejection | (Ma et al., 2010) |
| Systemic *Salmonella* infection | Delayed pathogen clearance despite increased T-cell activation due to enhanced T-cell susceptibility to AICD | (Ross et al., 2011) |
| Generation of endothelial cell barrier *in vitro* | Loss of endothelial barrier integrity | (Privratsky et al., 2011) |

**Table 3 CD31-Deficiency in disease models**

*The table given above describes various studies that indicate the effect of CD31 deficiency in a disease models and is adapted from (Marelli-Berg et al. 2013)*

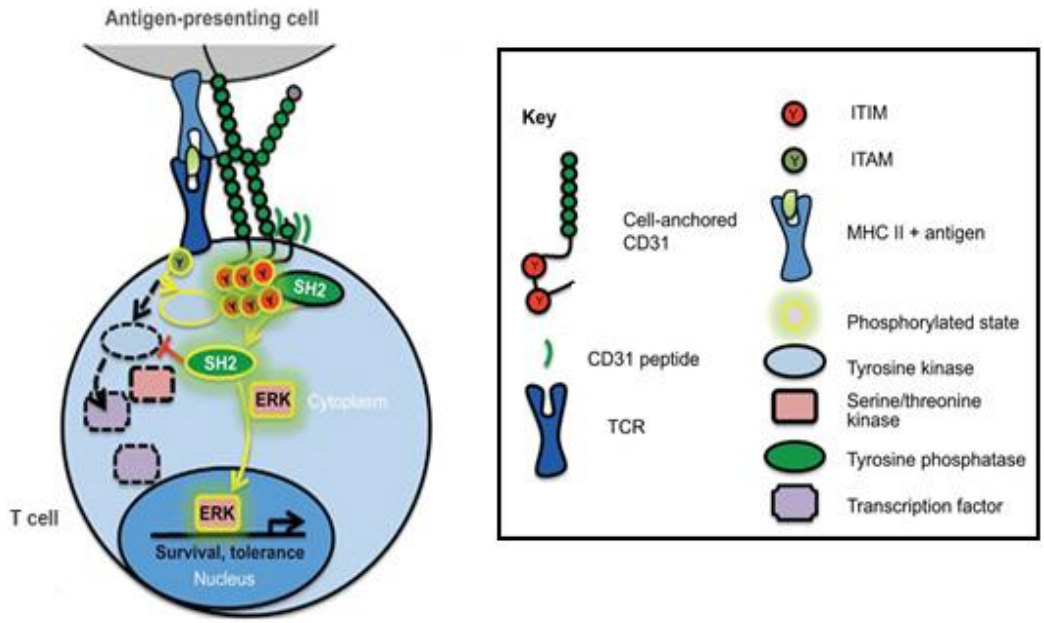



**Figure 3 CD31-mediated inhibition of T cell activation and induction of Erk**

*This figure indicates how CD31 increases the threshold for T cell activation. The recruitment of (SH2) domain-containing tyrosine protein phosphatases that dephosphorylate key proximal TCR kinases inhibits activation and regulates proliferation. CD31 signalling also induces Erk phosphorylation that protects against TCR induced AICD. Figure adapted from(Marelli-Berg et al. 2013).*

**CD31-mediated inhibition of cytokine production**

Although incompletely understood, several reports have highlighted the role of CD31 in regulating cytokine production. Studies by (Maas et al. 2005) and (Carrithers et al. 2005) showed that CD31 suppressed the production of pro-inflammatory cytokines following exposure to endotoxin. Another study by (Tada et al. 2003), reported enhanced production of the pro-inflammatory cytokine IFN-γ by the CD31 deficient lymphocytes in response to collagen in a CIA model. In another report, CD31 suppressed cytokine production in a model of non-alcoholic steatohepatitis (Goel et al. 2007). In DCs, loss of CD31 from the surface following maturation has been associated with their increased production of inflammatory cytokines (Clement et al. 2014). The exact mechanism through which CD31 regulates cytokine production remains elusive. However, (Rui et al. 2007) have reported the involvement of the inhibition of c-Jun N-terminal kinase (JNK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and interferon regulatory factor 3 (IRF-3) signalling pathways by CD31 signalling in macrophages.

**CD31 and T cell migration**

In recent years, studies have tried to elucidate the role of CD31 in leukocyte recruitment. *In vitro* studies that used antibody-mediated blockade of CD31 were the first to show the importance of CD31 in TEM of T cells. Studies by (Manes et al. 2010; Manes and Pober 2011) revealed that anti–CD31 antibody blocking abrogated TCR-dependent migration of human effector memory CD4 T cells induced by APCs.

Previous *in vivo* studies were unable to determine the impact of CD31 signalling selectively on T cell migration as they did not distinguish the effect of the CD31-deficiency in T cells and the endothelium separately (reviewed in Marelli-Berg et al. 2013). Recently, conclusive evidence to suggest a role for CD31 in T cell trafficking was provided by the study by (Ma et al. 2012). In their study, CD31-deficiency resulted in impaired trafficking of naïve T cells to the secondary lymphoid tissue. Here, CD31-/- naïve T cells adoptively transferred into CD31-expressing (CD31+/+) WT mice displayed reduced migration into the secondary lymphoid tissues as compared to CD31-expressing naïve T cells. However, CD31-deficient mice display normal colonization of secondary lymphoid structure. Thus, it is likely that this effect is masked through compensatory mechanisms from other molecules involved in TEM (reviewed in Nourshargh and Marelli-Berg 2005).

In addition, this study demonstrated that CD31-deficiency impaired constitutive trafficking of effector T cells and inflammation-induced extravasation into antigen-rich tissue sites. Here, effector memory HY (male antigen)-specific T cells, from female CD31-deficient mice, were adoptively transferred into male mice. The authors demonstrate that, under non-inflammatory conditions, CD31-deficient HY-specific T cells show significantly impaired ability to access non lymphoid, non-antigenic tissue of the CD31 competent male mice when compared against WT HY-specific T cells. Interestingly, when the HY-specific T cells from WT female mice were adoptively transferred to CD31-deficient male recipients, similar results were also observed by the authors. This suggests that CD31 interactions between T cell and endothelium are essential for the constant re-circulation of effector memory T cells into non-lymphoid tissue. When the experiments were repeated under inflammatory conditions, induced by IFN-γ injection to the peritoneum, HY-specific T cells from CD31-deficient female mice were demonstrated to have impaired migration to antigen rich peritoneum of WT male mice. However, when HY-specific T cells from WT female mice were adoptively transferred into CD31-deficient or WT male recipients treated with IFN-γ, recruitment of HY-specific T cells from CD31 deficient to the peritoneum was significantly increased. Thus, antigen-specific memory T cell extravasation under inflammatory

conditions was enhanced by the selective lack of CD31 on the endothelium. This is in line with earlier observations that indicate enhanced disease severity in experimental models of autoimmune disease in CD31-deficient mice (reviewed in Marelli-Berg et al. 2013; Privratsky et al. 2010).

## 1.2 Regulatory T cells

A hallmark feature of the immune system is the ability to effectively recognise and mount an immune response against a foreign pathogen or antigen while maintaining a state of acceptance to antigens that are expressed natively. This state of acceptance, also described as 'tolerance' is clearly observed in transplantation settings where the immune system of the recipient fails to initiate immune responses against antigens present in the syngeneic donor grafts while readily rejecting mis-matched donor grafts following a strong immune reaction to its allo-antigens. Under steady state conditions, failure of the immune system to distinguish between self and non-self can be linked to the development and onset of progressing autoimmune diseases. Originally thought to be only dependent on negative selection in the thymus (central tolerance, tolerance is now recognised as a dynamic aspect of the mature immune system (peripheral tolerance), where many auto/self-reactive T cells (T cells expressing TCRs for self-antigens) are present in a normal T cell repertoire (Yan and Mamula 2002). These self-reactive T cells are kept at bay through the action of a number of mechanisms.

Regulatory T cells are crucial in the maintenance of peripheral tolerance. Historically, regulatory T cells were identified as T suppressor cells due to their ability to suppress the activation of other T cells and subsequently the immune response (reviewed in Sakaguchi et al. 2007). The ability of these cells to maintain tolerance is best exemplified by the severe systemic autoimmunity and lymphoproliferative disease observed in FoxP3-deficient (Scurfy) mice and in humans carrying mutations in the FOXP3 gene, the expression of which is crucial for Treg function. Besides preventing autoimmunity, Tregs play a key role in regulating immunity against infections of viral, bacterial and parasitic origin (reviewed in Q. Tang and Bluestone 2008b). Thus these cells retain a dual role in not just regulating protective anti-tumour and anti-pathogen immunity, but also in preventing inflammatory disease by restraining aberrant responses to innocuous antigens.

Regulatory T cells are a small subset of CD4+ T cells that exist within the body as a heterogeneous population exhibiting different surface receptors depending on their localisation and functional state. Regulatory T cells can be broadly classified into two categories: naturally occurring regulatory T cells (nTregs) which develop in the thymus and inducible regulatory T cells (iTregs) that develop in the periphery (reviewed in Curotto de Lafaille and Lafaille ; Sakaguchi et al. 2008; Sakaguchi et al. 2010). nTregs mature from thymocytes expressing T cell receptors with weak affinity to self-antigens that escape the process of thymic selection. Inducible Tregs develop from

conventional naïve CD4+ T cells following stimulation under tolerogenic conditions. These conditions may include, but are not limited to, the action of TGF- β and IL-10. While nTregs and iTregs share expression of a number of markers and surface receptors, the stability of the FOXP3 gene expression was observed to be quite distinct between the two populations. In a mouse model of transplantation, a comparison of the efficacy in preventing rejection in transplants showed nTregs to perform substantially better. iTregs generated *in vitro* lost Foxp3 expression, their ability to maintain tolerance and regained some of their effector function facilitating graft rejection (Tonkin et al. 2008). Following this, a gene microarray analysis of the genome of nTregs and iTregs revealed differences in the methylated and unmethylated states of key genes associated with the stable expression of the FOXP3 gene (Waight et al. 2015).

## 1.2.1 Treg generation and differentiation

### 1.2.1.1 TCR in Treg development

Tregs develop in the thymus from the pool of double positive (DP) cells and TCR engagement with self-peptide-MHC Class II complex and CD28-B7 interactions are crucial for this process (Maloy and Powrie 2001). Mice with transgenic TCRs for foreign or non-thymically expressed proteins such as chicken ovalbumin or myelin basic protein bred onto RAG deficient backgrounds bear very few or no Tregs. While on a RAG sufficient background, TCR specific for self-peptides allow for the generation of Tregs (Apostolou et al. 2002; Itoh et al. 1999; Olivares-Villagomez et al. 2000). Interestingly, mice expressing cognate antigen in TCR-transgenic mice develop a higher proportion and number of Tregs (Jordan et al. 2001; H.-M. Lee et al. 2012). Thus, TCR recognition of self-peptides is essential for the thymic generation of nTregs.

Tregs develop in the thymus with a TCR repertoire that overlaps with that of autoreactive T cells in Foxp3-deficient mice. It is widely believed that naturally occurring Tregs develop with TCRs with avidity to self-peptide MHC II which is intermediate between positive selection (weak avidity) and negative selection (strong avidity) (Hsieh et al. 2012).Evidence in support of this hypothesis was provided by a study where retroviral expression of high frequency of TCRα from genes from Tregs and not from Teffs into a TCR transgenic RAG deficient background conferred the ability to rapidly expand upon transfer into a lymphopenic host (Lathrop et al. 2008).

### 1.2.1.2 CD28 and Treg development

CD28 co-stimulation is also required for the development of thymic Tregs. CD28-/- and B7-1 / 2 deficient mice show marked reduction in Treg numbers (Salomon et al. 2000). During thymic selection, CD28 provides co-stimulation for TCR-mediated negative selection of self-reactive double positive thymocytes. CD28 signalling not only induces IL-2 production, but also delivers a cell-intrinsic signal required for TCR-mediated Foxp3 expression (X. Tai et al. 2005b). CD28-induced Lck signals and the downstream activation of NF-κB transcription factor family member c-Rel have also been linked to Treg differentiation (Tai et al. 2005a; Vang et al. 2010). During the stages of thymic development, CD28 signals operate independently of TCR signals by sustaining

the development of Treg precursors and preventing their negative selection (Lio and Hsieh 2008a; Lio et al. 2010).

In the periphery, CD28 signals are crucial for Treg homeostasis (Bour-Jordan and Bluestone 2009). *In vivo* studies indicate CD28 signals as necessary for the survival and self-renewal of adoptively transferred Treg cells (Tang et al. 2003). Evidence to support this was observed when blockade of CD28 with CTLA-4Ig exacerbated autoimmune diabetes in NOD mice associated with a loss in Tregs (Salomon et al. 2000). In addition, B7-1- and B7-2-deficient mice on a NOD background also developed exacerbated autoimmune diabetes associated with reduced peripheral Tregs (Salomon et al. 2000). Further studies investigated the role of CD28 signalling in Tregs by selective genetic ablation of CD28 in the Foxp3+ CD4+ subset (Zhang et al. 2013). These mice displayed autoimmunity characterised by skin inflammation and decreased thymic Tregs. The authors observed reduced proliferation and survival of CD28-deficient Tregs that were located in the periphery. Thus, CD28 plays key roles in both Treg thymic generation as well as in Treg post-maturational proliferation and survival.

Similar to nTregs, induction of Tregs from conventional CD4+CD25- T cells also requires CD28 co-stimulation as was indicated by the study by (Guo et al. 2008). As in mice, effective proliferation of human Tregs requires CD28 co-stimulation that cannot be substituted by IL-2 (Hombach et al. 2007).

#### 1.2.1.3 CTLA-4 in Treg development

As discussed before, CTLA-4 expression on Tregs, unlike in Teffs, is constitutive. Furthermore, Treg activation also induces upregulation of CTLA-4 expression with rapid translocation to the cell surface (Mead et al. 2005). Like many aspects of CTLA-4 activity, its role in thymic selection of nTregs also remains controversial. As mentioned previously, CTLA-4 deficient mice develop lymphoproliferative disease and die within weeks due to multi-organ infiltration and tissue destruction mediated by self-reactive T cells (Chambers et al. 1997; Tivol et al. 1995; Waterhouse et al. 1995). Studies carried out to investigate the molecular mechanisms involved revealed that CTLA-4 along with other signals play a definitive role during thymic selection by setting the threshold for the selection of TCRs, the absence of which allows the selection of TCRs of low affinity (Verhagen et al. 2013). Here, the authors demonstrated that the CTLA-4 expressed within the corticomedullary region of the thymus acts during negative selection by tuning the TCR

repertoire of the self-reactive conventional T cells and those of Tregs. Using a TCR transgenic mouse model specific for the myelin basic protein peptide, loss of CTLA-4 was observed to result in the selection of endogenous TCRa chains of altered peptides thus widening the diversity of the Tconv cells and skewing the repertoire of developing thymocytes towards that of regulatory T cells. Thus, CTLA-4 not only affects the thymic generation of FoxP3+ Treg cells but also, Tconv cells.

In another independent study, no differences in thymic output of Tregs were reported between WT and CTLA-4-deficient mice within 15-17 days after birth (J. Verhagen et al. 2009). In this study, an OVA peptide specific, TCR transgenic mouse model revealed increased frequencies of Tregs in the periphery due to enhanced proliferation of CTLA-4-deficient Tregs responding to self-antigens. Furthermore, antibody-mediated blockade of CTLA-4 was described to augment Treg proliferation (Kavanagh et al. 2008; A. L. Tang et al. 2008). These findings also explain why CTLA-4 deficient mice have higher frequency of Foxp3-expressing Tregs even though CTLA-4 functions by counteracting CD28 signals, crucial for thymic generation of Tregs.

#### 1.2.1.4 IL-2 and Treg development

In addition to the expression of the nuclear transcription factor Foxp3, Tregs are also known to express high levels of the high affinity IL-2 receptor CD25 (Sakaguchi et al. 1995). In mice, CD25 along with Foxp3 expression is used to identify Treg populations. IL-2 is crucial for the generation of Treg populations in both the thymus and the periphery (Bayer et al. 2007; Cheng et al. 2013). Thus, it is not surprising that IL-2 deficient mice develop lethal multi-organ autoimmunity and lymphoproliferation which can be prevented by the adoptive transfer of WT T cells or the reconstitution of the CD25+ T cell fraction (Malek et al. 2002). During positive selection in the thymus, TCR signalling induces the upregulation of CD25. This upregulation is maintained by IL-2 signalling to generate the CD4+CD25+ cells containing the Treg precursors (Lio and Hsieh 2008b). Following CD25 signalling, STAT5-dependent signalling leads to induction of Foxp3 expression and Treg maturation (Burchill et al. 2007; Yao et al. 2007). Within the periphery and in TGFβ-induced Tregs, transcription of Foxp3 and subsequent Treg development is dependent on IL-2 signalling (Zheng et al. 2007a). However, mice lacking CD25 or IL-2 have detectable Tregs, although with low Foxp3 expression, suggesting that the role of IL-2 in Treg development may be partially redundant

and other mechanisms (cytokines like IL-4 and IL-7) may compensate for IL-2 signalling during Treg development (Yates et al. 2007).

IL-2 signalling is critically involved in the maintenance of *in vivo* Treg homeostasis. Genetic defects in IL-2 signalling or antibody mediated neutralization of IL-2 have revealed substantial reduction of Tregs in the periphery, where Tregs lacking CD25 are quickly outcompeted by WT Tregs (Bayer et al. 2007; Fontenot et al. 2005). In humans, genetic variations in the IL-2 and CD25 genes have been correlated with susceptibility to autoimmune diseases such as Type 1 Diabetes, multiple sclerosis and celiac disease owing to the reduction in the numbers of circulating Tregs (Cavanillas et al. 2010; Dendrou and Wicker 2008; van Heel et al. 2007).

### 1.2.2 Mechanisms of Treg-mediated Suppression

To exert their regulatory function, Tregs are known to modulate the activity of a variety of components of both the innate and adaptive immune system. Following their discovery, several studies identified a plethora of mechanisms through which Tregs suppress and ultimately regulate immunity against foreign pathogens and maintain tolerance to self-antigens. A series of reports highlighted that the type of mechanisms through which Tregs mediated suppression was largely dependent on the nature of the immune response, the causing agent, the immunological makeup of the host and the site where suppression occurs (Corthay 2009; Sakaguchi et al. 2009; Schmidt et al. 2012). Nonetheless, evidence is now accumulating to indicate the role of Tregs in every adaptive immune response, either physiological or pathological. In this section, these different mechanisms are briefly described.

With the initial experiments performed *in vitro*, cell-cell contact between Treg and responding T cells was observed to be necessary. Indeed, Treg cells failed to suppress activation of effector T cells when separated from the Teffs by a semi-permeable membrane *in vitro*. Studies by (Cao et al. 2007; Gondek et al. 2005) described *in vitro* killing of responding effector CD4 and CD8 T cells through the release of cytolytic proteins granzyme B and Perforin by Tregs, following direct cell-cell contact. However, imaging studies did not detect stable contact formation between Treg-Teffs *in vivo*, a prerequisite for direct cell contact mediated killing.

Another mechanism believed to play a role in Treg mediated suppression was through removal of the pro-survival cytokine IL-2 from the vicinity of the activated CD4 and CD8 effector cells via the high affinity CD25 receptor expressed abundantly on Tregs (Busse et al. 2010). However, this 'IL-2 sink' theory was plagued by the fact that *in vivo* activation of Teffs can remain effective in the absence or little presence of IL-2 depending upon the type of *in vivo* model used. Other mechanisms through which Tregs modify the microenvironment of activating/activated Teffs include the activities of the ecto-enzymes CD39, CD73 and that of galectins expressed by Tregs (Deaglio et al. 2007; Garin et al. 2007; Kubach et al. 2007). CD39 and CD73 catabolise the production of immunosuppressive adenosine while Galectin 1 and Galectin 10 are functionally important for human Tregs to be effective at suppression. Additionally, the upregulation of intracellular cyclic AMP by Tregs was also shown to inhibit T cell proliferation and IL-2 formation (Bopp et al. 2007). While these mechanisms add additional suppressive power to Tregs, they alone are insufficient as loss or removal of any one of these mechanisms does not replicate the

autoimmune and lymphoproliferative phenotype occuring in the absence of Tregs. This suggests that the key mechanisms crucial for Treg function lie elsewhere.

Tregs are also known to downplay immune responses by directly interfering with the ability of antigen presenting cells (APCs) to activate effector T cells. Tregs outcompete Teffs in forming aggregates around APCs, thereby physically restricting access of antigen presentation to Teffs (Onishi et al. 2008; Schneider 2006).

In several *in vivo* models, Tregs mediate suppression though the action of secreted immunoregulatory cytokines. Tregs are known to produce high amounts of membrane-bound and soluble immunoregulatory cytokine TGF-β (Nakamura et al. 2001). TGF-β1 deficient mice develop T cell mediated autoimmunity shortly after birth and mice with T cell-specific TGF-β1 deficiency show enhanced Th1 and Th2 responses and immunopathology including colitis (Li et al. 2006; Li et al. 2007; van Ginkel et al. 1999).

IL-10, another immunoregulatory cytokine secreted by Tregs, has been identified to exert immunosuppressive effects on several cell types (Moore et al. 2001). Interestingly, blocking IL-10 or using Tregs deficient in IL-10 abrogates the protective effect of Tregs on T cell in transfer-induced colitis model of inflammation (Asseman et al. 1999). Interestingly, IL-10 mediated immunosuppression by Tregs seems to control memory/antigen-experienced T cells during prevention or cure of colitis rather than the naïve cells (Asseman et al. 2003; Uhlig et al. 2006). IL-10 or IL-10 receptor deficient mice do not develop autoimmunity, but are susceptible to colitis in the presence of triggering flora (Kuhn et al. 1993; Spencer et al. 1998). In one study, Tregs were observed to need IL-10 (and not TGF-β) to control IFN-γ production by T cells in the inflamed skin (Sojka and Fowell 2011). Recent studies have further confirmed that IL-10 receptor signalling is required in Tregs as well as in Th17 cells in order to suppress colonic Th17 responses (Chaudhry et al. 2011).

The cytokine IL-35 is yet another Treg-secreted immunosuppressive cytokine that directly inhibits conventional T cell proliferation (Collison et al. 2007). In a model of inflammatory bowel disease, Tregs deficient in IL-35 show reduced suppressive ability (Collison et al. 2007). However, unlike in mice, human Tregs do no produce IL-35 (Bardel et al. 2008). Nonetheless, treatment of naïve T cells with IL-35 in humans and mice results in the generation of a so-called $iT_R35$ regulatory population that mediates suppression via IL-35 without requiring IL-10, TGF-β, or Foxp3 (Collison et al. 2010).

#### 1.2.2.1 CTLA-4 in Treg function

Several mechanisms of suppression may be employed by Tregs to achieve immune regulation depending upon a number of factors including the location of the immune response. However, it has been hypothesised that one central core mechanism of suppression, under the control of Foxp3, must be shared by every Treg cell in any location in both human and mice (Tang and Bluestone 2008a). CTLA-4 dependent suppression is a likely candidate for such a core mechanism. Unlike conventional T cells, regulatory T cells constitutively express CTLA-4. As mentioned previously, CTLA-4 contributes to maintaining tolerance towards self-antigens through a variety of molecular mechanisms including modification of APCs and the production of IDO. Thus, Tregs are believed to be endowed with the ability to suppress the activity of conventional T cells using CTLA-4 in a similar manner to that used by other conventional T cells that express CTLA-4.

Indeed, CTLA-4 deficient Tregs are believed to have impaired suppressive ability *in vivo*. In one study, antigen specific Tregs deficient in CTLA-4 were unable to prevent disease in a mouse model of autoimmune diabetes (Schmidt et al. 2009). Additionally, Treg-specific deletion of CTLA-4 in mice using a Foxp3-Cre system precipitated in autoimmune disease similar to that in CTLA-4 deficient mice, although the life span of these mice was significantly extended (Chikuma and Bluestone 2007; Wing et al. 2008). The less severe phenotype of these mice compared to that of CTLA-4 deficient mice is likely due to CTLA-4 expressed by conventional T cells. As described before, CTLA-4 expressed by conventional T cells following activation plays a key role in preventing autoimmunity and maintaining immune homeostasis. In another study, CTLA-4-deficient Tregs were unable to control the expansion of CD4 target cells within a lymphopenic environment and were unable to inhibit the cytokine production associated with homeostatic expansion (Sojka et al. 2009).

Foxp3 together with other transcription factors are required to upregulate the expression of CTLA-4 by binding to the promoter region of the CTLA-4 gene, indicating that Foxp3 may sustain the high expression of CTLA-4 in Tregs (Marson et al. 2007; Y. Wu et al. 2006).

Studies have also highlighted the ability of Treg-expressed CTLA-4 to modulate the activity of APCs. Indeed, Tregs that lack CTLA-4 were unable to inhibit DC upregulation of CD80 and CD86 as compared to WT Tregs (Hou et al. 2015b). Similarly, ligation of CD80 and CD86 by CTLA-4 expressed by Tregs induced the activation of the IDO pathway (Grohmann 2002) leading to the production of immunosuppressive kynurenin or the activation of transcription factor foxo3, which

mediates inhibition of cytokine production by DCs Transcription factor foxo3 controls the magnitude of T cell immune responses by modulating the function of dendritic cells (Dejean et al. 2009).

#### 1.2.2.2 CD31 and regulatory T cells

So far, very few studies have addressed the role of the Ig-like CD31 molecule specifically on the regulatory T cell subset. Given the involvement of this molecule in T cell activation, cytokine production and migration, CD31 may play a crucial role in maintaining tolerance through its action on regulatory T cells. The recent study by (Ma et al. 2012) investigated this aspect and observed CD31-deficient CD4+CD25+ FOXP3+ Tregs to display defective regulatory functions *in vivo*. Similar findings were also observed *in vivo* by the study by (J. Haas et al. 2007), who reported reduction in CD31-expressing Tregs in patients with multiple sclerosis (MS). They also reported a reduction in this Treg pool within the peripheral blood with aging. However the molecular mechanism underlying this effect is at present unclear. The functional consequences of homophilic engagement of CD31 between T cells and Tregs has not been investigated. As CD31 can modulate ZAP-70 activity (Ma et al. 2010) and TCR-triggering is necessary for Treg function, one can speculate that the modulation of Treg-proximal TCR signaling (ZAP-70) through engagement of CD31 by APCs is required for their optimal suppressive activity. This aspect of Treg cell biology remains to be investigated.

### 1.2.3 Tregs in Transplantation

Tregs have been described to be crucially involved in the induction and maintenance of tolerance post-transplant, as well as the prevention of graft rejection in animal models (Reviewed inWalsh et al. 2004). Similarly, evidence suggests that the levels of Foxp3 and intra-graft Treg frequency can be correlated to the clinical graft acceptance, survival and function of the transplanted grafts (Chauhan et al. 2009; Zuber et al. 2013). Indeed, strategies have been devised to promote tolerance of transplanted graft through selective generation of Treg within grafts by pharmacological means. Notably, the immunosuppressive drug rapamycin (sirolimus) inhibits signalling by its eponymous target, the mammalian target of rapamycin (mTOR) which integrates cellular metabolism and proliferation pathways and has been observed to enhance generation of Tregs while suppressing T effector cell proliferation following transplantation (Saunders et al. 2001).

Adoptive transfer of Tregs has emerged as an attractive therapeutic alternative to conventional immunosuppressive strategies that are involved in transplantation to ensure graft survival and prevention of graft rejection (Nagahama et al. 2007). These strategies have an advantage in reducing the risks of uncontrolled infection and tumour growth associated with conventional immunosuppressive strategies which rely solely on pharmacological agents to prevent graft rejection.

Overview of rejection and transplantation tolerance

Once an organ/tissue is grafted onto a recipient, the T cells of the recipient respond to antigens that are displayed by the graft in a similar fashion as to any foreign protein. Rejection of graft tissue occurs as a consequence of polymorphisms in histocompatability genes, primarily those located within the MHC. The T cells from the recipient respond to foreign (allogeneic) MHC. As a result a large population of activated effector cells, primarily T cells and macrophages are generated which mediate graft destruction.

There are two key pathways through which alloresponsive T cells can recognise antigens present in the transplanted tissue (Reviewed in Afzali et al. 2007). In the direct pathway, the responding T cells recognise intact MHC molecules on the surface of donor derived APCs, whereas in the indirect pathway, recipient APCs process donor derived allo MHC molecules into peptides which are then presented to T cells by self- MHC molecules. It is widely believed that direct pathway of recognition contributes to the immediate or acute phase of rejection mediated when graft

resident donor APCs migrate to the surrounding lymphoid tissue where they stimulate alloresponsive T cells (Benichou 1999). As the donor derived APCs are relatively short lived, the indirect pathway of allorecognition predominates as the alloresponse progresses and thus forms the chronic phase of rejection. In addition to these two pathways, a semidirect pathway of allorecognition has been observed in which host/recipient APCs acquire intact MHC molecules from donor APCs from the graft via means of cell-cell contact and are thus able to recognise self-antigens via the direct recognition pathway (Herrera et al. 2004). Thus the semidirect pathway enables a single host APC to present allopeptides via self-MHC to CD4+ T helper cells as well as stimulate cytotoxic CD8+ T cells via a donor acquired Class I MHC molecule.

Experimental methods of induction of transplantational tolerance can be divided into two categories. Central tolerance refers to the process by which bone marrow transplants are preceded to organ transplants in an attempt to induce generation of hematopoietic chimerism (Sprent and Kishimoto 2001). As such, donor reactive T cells which develop are deleted in the thymus during thymocyte maturation in a manner similar to self-reactive T cells. Peripheral tolerance strategies rely on blocking/modulating T cell activation or growth factor receptor pathways in mature T cells through pharmacological agents or antibodies (Mueller 2010). Conversely, Tregs were observed to be crucial for maintaining peripheral tolerance within transplanted grafts by inhibiting responses of both resident T cells as well as that of new thymic emigrants.

#### 1.2.3.1 Antigen specificity of Tregs in transplantation

Previous studies investigating solid organ transplantation in murine models have shown antigen specificity of Tregs to be advantageous at preventing rejection in solid organ transplants. However in models of GVHD, the advantage of antigen specificity over polyclonal specificity is only moderate (Pidala and Anasetti 2010). Like conventional T effector cells, Tregs recognise alloantigens through both direct and indirect recognition pathways. Interestingly, Tregs with direct antigen specificity were observed to be very effective at preventing acute rejection following transplantation while Tregs with indirect allospecificity are crucial in preventing chronic rejection later after transplantation (Tsang et al. 2008). The expansion of Tregs with direct specificity for human transplant therapies has been easier to achieve using allogeneic monocyte-derived dendritic cells, peripheral blood mononuclear cells (PBMCs), or allogeneic B cells as APCs

(W. Tu et al. 2008). These Tregs have been reported to be suppressive at very low Treg-Teff ratios and in human skin transplants are more potent over polyclonal Tregs at preventing rejection (Graca et al. 2002). Expanding Tregs with indirect allospecificity has been proven more difficult to achieve. The first protocols developed involved peptide-pulsing of APCs with alloantigens which is an inefficient process (only 1% of the available MHC molecules are occupied by the peptide) (Jiang et al. 2003; Tsang et al. 2006). Newer protocols using beads coated with recombinant peptide along with MHCII and anti CD28 mAB and tetramer based cell sorting of antigen specific Tregs have been used for better Treg cell-based therapies to combat chronic rejection (Masteller et al. 2005).

Transplantation studies have reported that the continuous presence of antigen is a requirement for maintenance of transplantation tolerance (Chen et al. 1996; Onodera et al. 1998). This observation, along with the fact that indirect allorecognition pathways proceeds once the acute phase of rejection has died down, suggests that the indirect pathway of allorecognition is the dominant pathway utilised by Tregs. Studies carried out by (Yamada et al. 2001) using mice lacking class II MHC (indirect recognition only) and recipients that lack MHC class II molecules in the periphery but possess CD4 T cells as a result of transgenic expression of class II MHC on the thymic epithelium (direct recognition only) indicated indirect recognition of alloantigens to be crucial for the ability to tolerize over direct recognition pathways. Tregs can thus achieve tolerance of a grafted tissue through means of recognizing a small subset of graft derived antigens which can then block further responses to other graft expressed antigens in a method termed “linked suppression”(See section 1.2.2).

# 1.3 T cell metabolism and immune regulation

Although signalling through receptors such as the TCR, the co-stimulatory receptors, cytokine receptors and others affect the proliferation and differentiation of T cells, nutrient availability and environmental cues also play a key role in supporting these events. Like all other cells, T cells also coordinate their differentiation and proliferation with their metabolic demands and availability of essential nutrients. Glucose, amino acids, and lipids are the primary metabolic fuels for all lymphocytes. However, different T cell subsets are now being identified which exhibit unique metabolic patterns adapted to their function. For example, In the study by (Peter et al. 2010), the authors indicated that T cells switch between highly proliferative (activated T cell and developing thymocyte) and quiescent (memory, naïve and anergic T cells) states characterised by distinct metabolic pathways in each. Furthermore, Teff cells upon activation switch from primarily mitochondria-dependent oxidative phosphorylation (OXPHOS) ( abundant in resting T cells) to aerobic glycolysis, a phenomenon termed the "Warburg effect" (Warburg 1956). This may be because glycolysis can fuel the demand for rapid generation of fundamental building blocks of the proliferating cell, such as amino acids, lipids, complex carbohydrates, and ribonucleotides that the relatively efficient OXPHOS cannot provide (Caro-Maldonado et al. 2012).

## 1.3.1 T cell activation and metabolism

Resting naïve or memory T cells utilise oxidation of glucose, lipids and glutamine as their primary source of energy. When unstimulated, resting T cells do not grow or proliferate but still require energy for cell survival. Thus, these cells maintain a clear demand for energy, with little need for biosynthesis. Consistent with these metabolic requirements, resting T cells exhibit a balanced use of glucose, amino acids and lipids that undergo oxidation in the mitochondria for maximal ATP generation (Bental and Deutsch 1993). In naïve T cells, the homeostatic survival signals from the TCR and cytokine interleukin-7 receptor (IL-7R) that maintain survival also maintain this basal metabolism (Rathmell et al. 2001). Naïve T cells that are deprived of these signals begin to degrade cell surface glucose transporter Glut1 and undergo cellular atrophy that leads to reduced rates of glucose metabolism and induction of pro-apoptotic Bcl-2 family proteins (Coloff et al. 2011; Rathmell et al. 2000; Rathmell et al. 2001; Wofford et al. 2008). Consistent with this hypothesis, the study by (Jacobs et al. 2010) demonstrated that the conditional deletion of the IL-

7 receptor in mature T cells resulted in their poor survival *in vitro* and *in vivo* which was associated with reduced basal rates of glycolysis and shortened lifespan.

Upon activation through TCR and CD28 co-stimulation, T cells rapidly activate metabolic programs that closely resemble that of many cancer cells, with high rates of glucose uptake and aerobic glycolysis (Michalek and Rathmell 2010; Warburg 1956). In addition, oxidation of the amino acid glutamine (glutaminolysis) increases to maintain tricarboxylic acid (TCA) cycle flux and provide a source of ATP and biosynthetic precursors (Newsholme et al. 1985). In contrast, oxidation of lipids decreases while lipid synthesis increases to facilitate the building of new membranes.

Following TCR and CD28-induced co-stimulation, the PI3K-Akt-mTOR pathway facilitates the trafficking of the glucose transporter Glut1 to the cell surface to enhance glucose uptake (Barthel et al. 1999). Furthermore, PI3K-Akt-mTOR signals induce the activity of glycolytic enzymes as well as the nuclear transcription factors sterol-regulatory element binding proteins (SREPBs) to stimulate lipid synthesis (Krycer et al. 2010). Previously, (Luu et al. 2012) have shown that Akt and mTOR kinases acutely upregulate SREPB-2, a nuclear transcription factor that controls cholesterol production also necessary for building membranes. Additionally, the nuclear hormone receptor estrogen related receptor-alpha (ERRα) increases following T cell activation (Michalek et al. 2011b) and can induce the expression of TCA and electron transport genes, essential for efficient mitochondrial metabolism.

While glucose and glutamine metabolism increase following T cell activation, lipid metabolism switches from oxidation to synthesis. This occurs mainly through signalling and substrate-mediated inhibition mechanisms. The PI3-Akt pathway was presented by (Deberardinis et al. 2006) to downregulate the activity of the enzyme Carnitine palmitoyltransferase Ia (CPT1a) which leads to inhibition of lipid oxidation. The CPT1a enzyme is responsible for the formation of acyl carnitines enabling the transport of long chain fatty acids into the mitochondria for oxidation thus controlling FAO. Furthermore, production of the lipid biosynthesis precursor malonyl-CoA from TCA derived citrate further inhibits CPT1a and fatty acid oxidation (McGarry et al. 1977).

While aerobic glycolysis serves as the primary source of energy following T cell activation, mitochondrial OXPHOS continues and can increase (reviewed in Waickman and Powell 2012). However, rather than lipid oxidation, the mitochochondrial oxidation of glucose and glutamine serves as the primary source of oxidative energy. Previously, (O'Rourke and Rider 1989) showed that 10% of the glucose in activated lymphocytes was driven to the mitochondria and entirely oxidized, which could support approximately 65% of the ATP generated by the cell. (Roos and

Loos 1973) also showed that 15% of the total ATP generation came from aerobic glycolysis, while the rest from OXPHOS.

Additionally, the oxidation of glutamine also contributes as an energy source. Following entry into the TCA cycle as α-ketoglutarate, glutamine-derived carbons can exit the mitochondria as malate or citrate. These can then be used for lipid synthesis or to generate lactate and produce NADPH by malic enzyme and replenish cytosolic $NAD^+$ by lactate dehydrogenase (Caro-Maldonado et al. 2012).

Once an immune response begins to subside, the vast majority of Teffs die off while a few develop into long lasting memory T cells. This transition from stimulated to long-lived quiescent T cells involves metabolic reprogramming, as cells no longer require biosynthesis and instead require efficient supply of ATP. Thus memory T cells switch from glycolysis to oxidative metabolism. In support of this hypothesis, inhibiting glycolysis and stimulating lipid oxidation at the peak of an immune response has been shown to enhance memory T cell development (Sukumar et al. 2013). In CD8 memory T cells, mitochondrial lipid oxidation dominates. Additionally, unlike in naïve T cells, CD8 memory T cells display much higher levels of lipid oxidation and vastly higher mitochondrial spare respiratory capacity (SRC) (van der Windt et al. 2012). The SRC represents the extent to which cells can increase oxygen consumption beyond the basal rate of respiration, with adequate nutrients, when placed under stress. Thus, the SRC does not measure the direct rates of OXPHOS but reflects the cell's ability to respond and rapidly increase respiration and, therefore, ATP production. The SRC is mediated through increase in the electron transport as well as by an increase in the overall density of mitochondria per cell. More recently, the study by (van der Windt et al. 2012) have demonstrated that the source of the memory T cell oxidative metabolism and SRC is largely mediated through lipid oxidation; a process regulated in part through the expression of CPT1a. Indeed, their study indicated that modulation of CPT1a influences both SRC and memory generation.

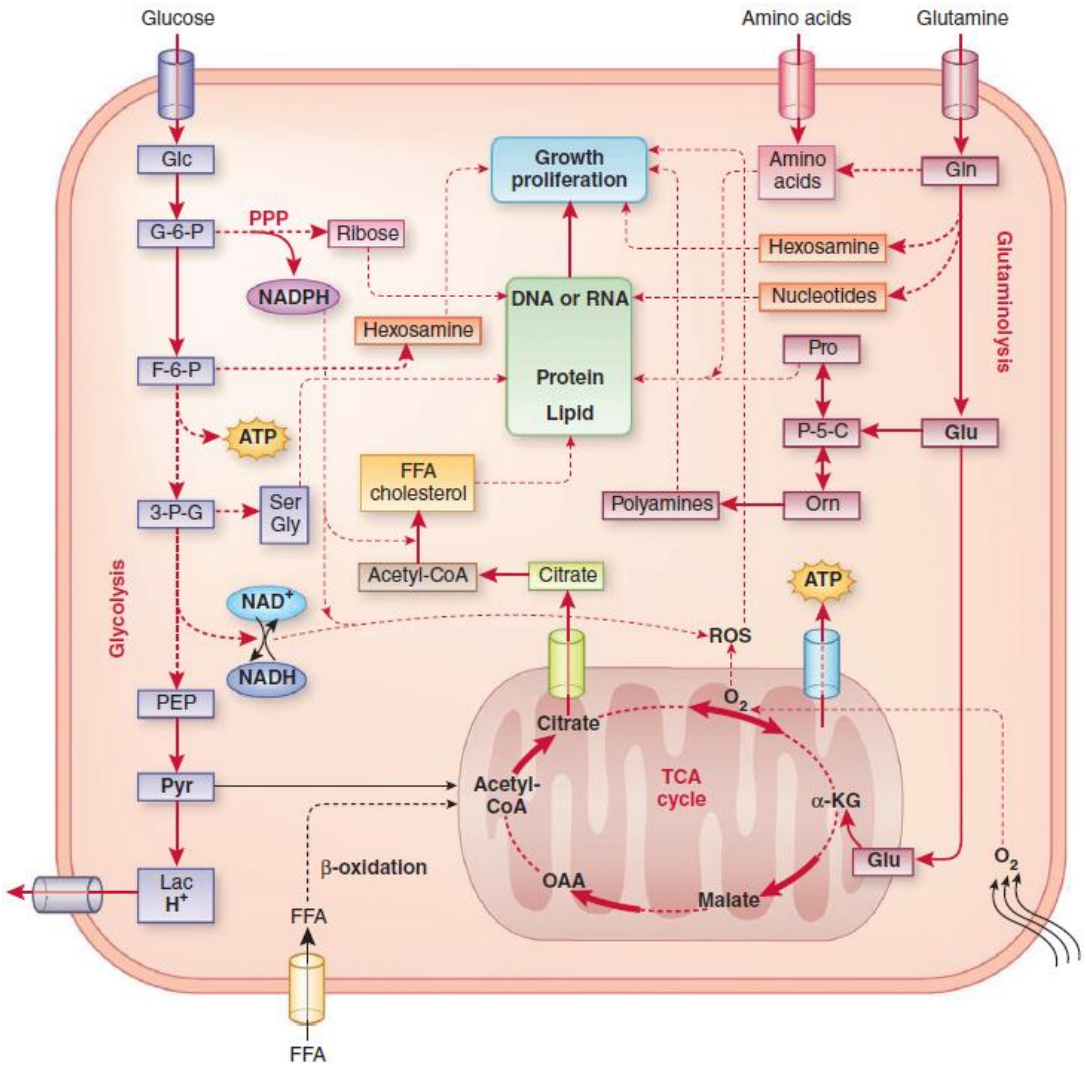



**Figure 4 T cell metabolic reprogramming**

*In naïve and memory T cells, mitochondrial oxidation of glucose and fatty acids through the TCA cycle supplies the energy for basic cellular functions. After activation, β-oxidation of fatty acids decreases and other metabolic processes (indicated in red), including glycolysis and glutaminolysis, increase. The glucose catabolic pathways shifts more towards production of NADPH and 5-carbon ribose (via the pentose phosphate pathway, (PPP)) at glucose -6-phosphate (G-6-P) and detours toward lactate production (aerobic glycolysis) at pyruvate. Meanwhile, glutaminolysis provides the α-ketoglutarate (α-KG), which enters the mitochondrial TCA cylcle, provides energy and a carbon resource for lipids. This metabolic reprogramming is optimised for cell growth and proliferation by providing carbon and ATP. Once differentiated, long lived memory CD8 T cells rely on the β-oxidation of fatty acids as a source of energy. F-6-P, fructose-6-phosphate; 3-P-G, glycerate-3-phosphate; Pyr, pyruvate; Lac, lactate; FFA, free fatty acids; OAA, oxaloacetate; PEP, phosphoenolpyruvate; P-5-C, 1-pyrroline-5-carboxylate; Glu, glutamate; Orn, ornithine.* ***Figure adapted from (Wang and green, 2012).***

## 1.3.2 Metabolic Pathways in Tregs

Functionally distinct T cell subsets exhibit diverse energetic and biosynthetic pathways for their energy requirements. Several reports have highlighted the existence of different metabolic 'signatures' in Treg and Teff cells that affect their differentiation and function. These are briefly described below.

### 1.3.2.1 Lipid oxidation

One of the key metabolic features that distinguish Tregs from Teffs is their dependency on lipid oxidation as a primary energy source. Unlike activated Teffs, Tregs display high fatty acid oxidation (FAO) rates. The study by (Michalek et al. 2011c) demonstrated that the addition of the drug Etomoxir, known to block fatty acid oxidation, led to the reduction in the number of Tregs generated under Treg differentiating conditions *in vitro* (Michalek et al. 2011c). Etomoxir is an inhibitor of CPT1, a rate-limiting enzyme in FAO located on the outer surfaces of the inner and outer mitochondrial membranes. Thus, the authors conclude that FAO is necessary for Treg differentiation.

A recent study by (H. Zeng et al. 2013c) has indicated that Tregs require lipid and cholesterol metabolism, with the mevalonate pathway particularly important for coordinating Treg cell proliferation and upregulation of the co-stimulatory receptors CTLA-4 and ICOS to establish Treg functional competency. In this study, Tregs treated with statins, pharmacological inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR, the rate-limiting enzyme of the mevalonate pathway), impaired the suppressive activity of Tregs in *in vitro* suppression assays. This functional defect could be corrected upon the addition of mevalonate, the immediate downstream product of HMGCR. The authors conclude that upregulated lipid biosynthesis via the mevalonate pathway promotes the expression of effector molecules and the immunosuppressive activity of Tregs. Figure 5 below depicts the mevalonate pathway in T cells and statin-mediated inhibition of this pathway adapted from (Zeiser et al. 2009).

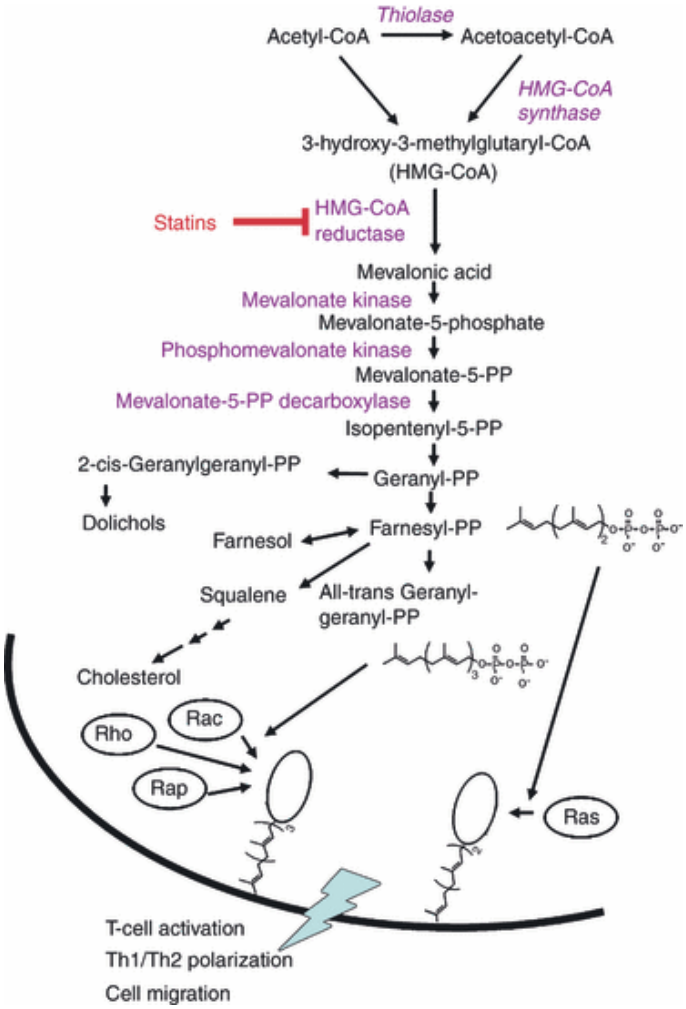



**Figure 5 Statin inhibition of the mevalonate pathway in T cells**

*Statins such as Simvastatin and Atorvastatin block the activity of HMG-CoA reductase, the rate limiting enzyme of this pathway which causes reduction in farnesylated and geranylated downstream proteins. In addition, downstream prenylation of GTPases such as Rho-B, Rac1 and Ras , necessary for binding to cell membranes is disengaged. This is thought to inhibit activation and regulate Th1/Th2 polarisation. Figure adapted from (Zeiser et al. 2009).*

### 1.3.2.2 AMP-activated protein kinase (AMPK) pathway

The AMP-activated protein kinase (AMPK) is a heterotrimeric kinase consisting of a catalytic α subunit, a regulatory β subunit, and an AMP-binding γ subunit. AMPK acts as an intracellular energy sensor and is activated in response to low energy levels indicated by fluctuations in AMP: ATP ratio. AMP binding to the γ subunit results in allosteric activation of this enzyme (W. H. Lee and Kim 2010). In addition, AMPK can also be activated in an AMP-independent manner mediated by phosphorylation of threonine-172 on the α subunit by upstream kinases. Following activation, AMPK restores ATP homeostasis through the upregulation of several genes involved in processes such as glucose oxidation and, more importantly, lipid oxidation. Among these is CPT-1, crucial for mitochondrial oxidative lipid metabolism (Kompare and Rizzo 2008). Additionally, AMPK also preserves CPT-1 activity by inhibiting acetyl-CoA carboxylase which is itself a suppressor of CPT-1, thus further promoting fatty acid oxidation (W. H. Lee and Kim 2010).

(Michalek et al. 2011c) have demonstrated that both nTregs and iTregs display high levels of AMPK activity, and pharmacological activation of AMPK by metformin, a drug used to treat diabetes mellitus increased Treg generation *in vitro*. In addition, activation of AMPK via metformin increased the Treg population in the CD4+ T cell compartment in an *in vivo* murine model of asthma (Michalek et al. 2011a). Here, the authors described that mice sensitised by aerosol to ovalbumin in the presence of metformin, and challenged 21 days later exhibited an increase in the frequency and number of Foxp3+ Tregs in the draining lymph nodes as compared to mice immunized in the absence of metformin. However, no change in airway responsiveness was noted even though there were fewer lymphocytes recovered in the bronchial alveolar lavage in the metformin treated animals.

In models of EAE, T cells from mice afflicted with EAE show reduced levels of AMPK protein and genetic deletion of AMPK in mice renders them more susceptible to EAE. In this study, $AMPK^{-/-}$ mice were observed to have higher clinical scores, CNS cellular infiltration, and *ex vivo* levels of IFN-γ production than their WT counterparts (Nath et al. 2009). Moreover, in the study by (MacIver et al. 2011), knocking out liver kinase B1 (LKB1), an upstream activator of AMPK resulted in similar observations such as increased IFN-γ and IL-17 pro-inflammatory cytokine production by the LKB1-/- T cells. Taken together, these studies suggest AMPK activity to be a crucial metabolic pathway of Tregs. An overview of the AMPK pathway is depicted in the Figure 8 of Section 1.3.3.2.

### 1.3.2.3 Glucose oxidation

Recent studies have suggested that Tregs do not maintain an obligatory reliance on a particular fuel source such as lipids, but rather on the mitochondrial oxidative metabolism of either lipids or glucose. Pyurvate dehydrogenase (PDH) is an enzyme that catalyses the conversion of cytosolic pyruvate into mitochondrial acetyl-CoA for oxidative metabolism and thus, serves as a key bifurcation point between T cell glycolytic and oxidative metabolism. PDH is inhibited by the PDH Kinase (PDHK), which suppresses pyruvate oxidation and instead promotes its conversion into lactate via lactate dehydrogenase (LDH). In a study by (Ostroukhova et al. 2012), inhibition of PDHK1 via Dichloroacetate (DCA, inhibitor of PDHK1) led to upregulation of IL-10 production and induction of Foxp3. In this study, intraperitoneal DCA addition before intranasal ragweed exposure was shown to reduce lactate production and airway inflammation associated along with an increased number of Foxp3+ Treg cells in a mouse model of asthma. More recently, a study by (Gerriets et al. 2015) has confirmed that Tregs have reduced levels of PDHK1 activity and, furthermore, that inhibition or knockdown of PDHK1 increases Treg generation. Inhibition of PDHK1 in their study modulated immunity with decreased Th17 and increased Treg populations resulting in protection of animals against disease in a murine EAE model. An additional observation in their study was that inhibition of glycolysis via 2-DG reduced Teff proliferation while increasing Tregs proliferation *in vitro*. However, inhibition of the electron transport chain (thus, mitochondrial oxidation) by rotenone sharply reduced Treg proliferation. Collectively these reports highlight the importance of mitochondrial oxidation (of both glucose and lipids) in Treg development and function.

### 1.3.2.4 Similarity to CD8 memory T cells

The above studies indicate that Tregs display metabolic phenotypes that are similar to that of long-lived memory CD8 T cells. Like Tregs, CD8 memory T cells display higher AMPK activity and FAO (Araki and Ahmed 2013). In a study by (Pearce et al. 2009), treatment with metformin induced the generation of CD8 memory T cells *in vivo* upon *Listeria monocytogenes* infection. Recently, (Gerriets et al. 2015) have demonstrated that Tregs also have high SRC similar to what is observed in CD8 memory T cells. Therefore, it is likely that mitochondrial metabolism and oxidation of both glucose and lipids may serve as an efficient means to maintain long term survival of Tregs in a manner similar to what is observed in CD8 memory T cells.

### 1.3.3 The mTOR Pathway in T Cells

The mammalian target of rapamycin (mTOR) pathway is a key nutrient sensing pathway that integrates fundamental cell processes such as proliferation, differentiation and cellular function to nutritional environmental status. mTOR is a 290kD serine/threonine protein kinase belonging to the PI3K-related kinase family that integrates signals from both internal and external stimuli to pathways that control aspects of cell growth and metabolism (Schieke et al. 2006).These include the response to hypoxia, and the biogenesis and oxidative capacity of mitochondria. mTOR also regulates critical processes such as cytoskeletal organization, transcription and protein synthesis (Dowling et al. 2010; Vivanco and Sawyers 2002). In addition, mTOR is also known to integrate signals from growth factors such as insulin and insulin like growth factors to the cellular metabolism (Vivanco and Sawyers 2002; Zoncu et al. 2011).

mTOR derives its name from its naturally occurring inhibitor rapamycin, a powerful immunosuppressive compound referred to as Sirolimus (Douros and Suffness 1981). Rapamycin was first discovered in the soil bacterium *Streptomyces hygroscopicus* from the Easter islands. mTOR was the name given to the catalytic subunit formed by the two complexes mTORC1 and mTORC2. The two distinct complexes are known to phosphorylate different substrates and regulate distinct cellular functions. Rapamycin-sensitive mTORC1 forms the fundamental nutrient sensing complex that is activated by Akt kinase downstream of PI3K signalling (via the TCR, co-stimulatory receptors, and cytokines) whereas the rapamycin-insensitive mTORC2 controls spatial aspects of cell growth through activation of cytoskeletal component (Cybulski and Hall 2009). mTORC1, composed of the serine/threonine kinase mTOR itself, the scaffolding protein RAPTOR, the positive accessory proteins FKBP12, Deptor, and mLST8, and a regulatory subunit PRAS40 is a target of Akt downstream of PI3K signalling (Laplante and Sabatini 2009). Rapamycin, as an immunosuppressive drug, binds to FKB12 and disrupts the formation and function of the TORC1 complex (Sabatini et al. 1994). Conversely, TORC1 activation drives protein synthesis via phosphorylation of downstram kinase S6K1, which phosphorylates the ribosomal protein S6 and initiates the translation of messenger RNA. The mTORC2 complex is composed of mTOR, the accessory proteins mLST8, RICTOR, deptor, Protor1/2 and mSIN1 (Sarbassov et al. 2004). mTORC2 activates the kinase Akt by phosphorylating serine at position 473 of the Akt kinase (Hresko and Mueckler 2005; Sarbassov et al. 2005) thereby activating the PI3K-Akt pathway. Thus, Akt lies both upstream and downstream of mTOR. mTORC2 is known to regulate cell polarisation and responses to chemotactic signals via G protein-coupled activation of RAS (Charest et al. 2010).

Interestingly, TORC2 is thought to be negatively regulated by TORC1 activity via SAPK interacting protein 1 (Sin1, regulatory component of TORC2) phosphorylation (Liu et al. 2013). Thus, rapamycin can indirectly activate mTORC2 in the short term, however, long term inhibition of mTORC1 (over days) by rapamycin may also inhibit mTORC2 activation (Sarbassov et al. 2006).

Figure 6 below depicts an overview of mTOR signalling pathways in mammalian cells and is adapted from (Yang and Guan, 2007).

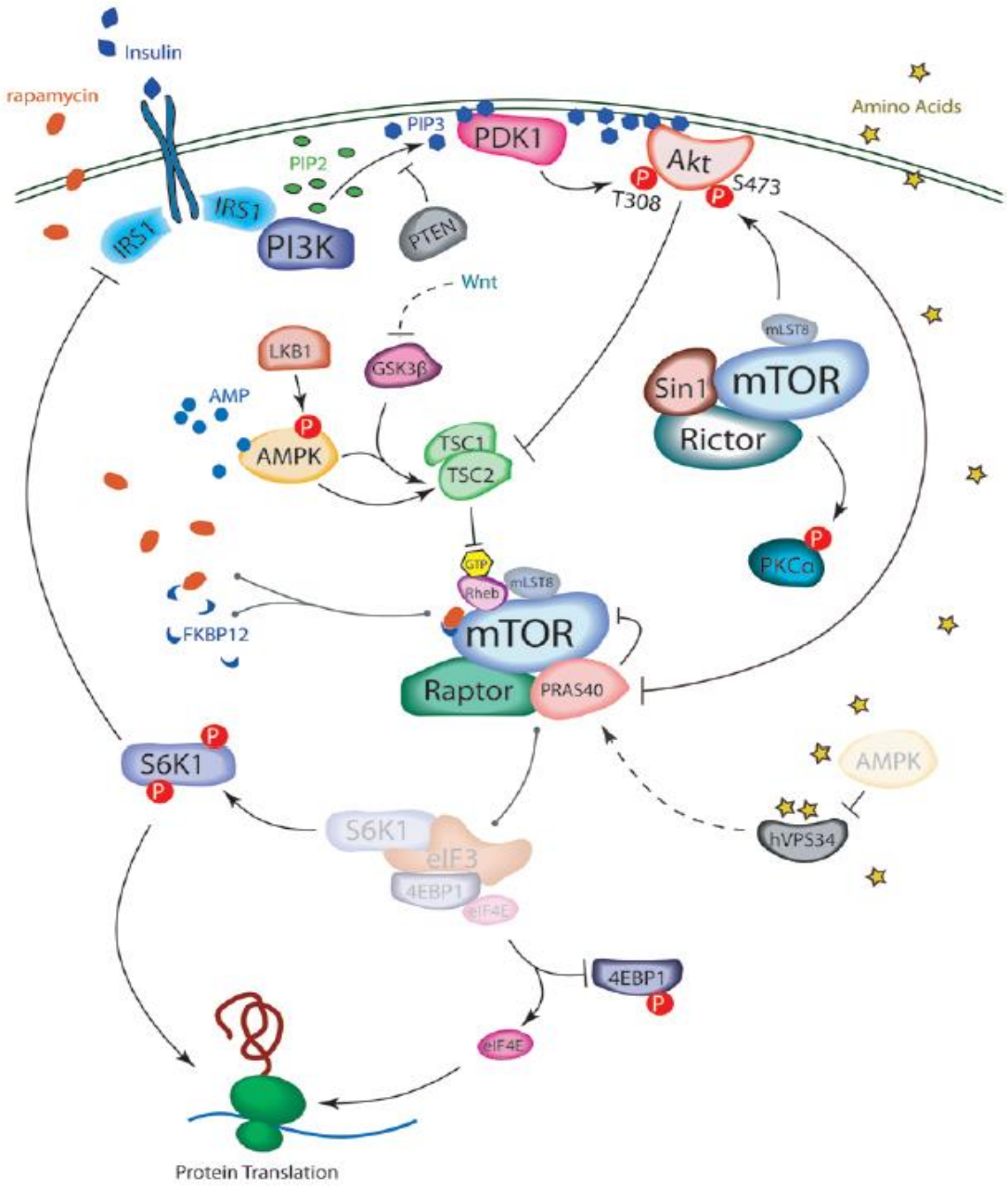



**Figure 6 mTOR signalling network in mammalian cells**

*mTORC1 is the rapamycin sensitive complex of mTOR formed of mTOR,Raptor,mLST8, FKBP12, and PRAS40. TSC1/2-Rheb is the major regulator of mTORC1. Signals via growth factors/PI3K-Akt are led downstream via TSC1/2-Rheb to activate mTORC1, thus, enabling phosphorylation of downstream S6K1 and 4EBP1.The mTORC2 complex, formed of mTOR, Rictor, Sin1, and mLST8 phosphorylates Akt at Serine position 473. Arrows represent activation, bars represent inhibition, and dots represent binding. Figure adapted from (Yang and Guan 2007).*

### 1.3.3.1 mTOR regulates peripheral T cell homeostasis, activation and differentiation

As mentioned previously, different subsets of T cells have been shown to use distinct bioenergetics and metabolic pathways to meet their energy requirements. Given its central role in coupling upstream signals to that of metabolic inputs, mTOR activity is described in various studies as a regulator of T cell homeostasis, activation and differentiation. During the transition from naïve to activated, to memory, T cells coordinate an intricate network of epigenetic, transcriptional, and metabolic programs, many of which are directly influenced by mTOR activation (Chi 2012; Yang and Chi 2012; H. Zeng and Chi 2013).

In naïve CD4 and CD8 T cells, which require low TCR and IL-7 receptor signalling to maintain T cell homeostasis (Sprent and Surh 2011), low levels of mTOR signalling appear to be required to maintain T cell quiescence. In support of this, T cells deficient in tuberous sclerosis complex 1 (TSC1, an inhibitor of RHEB and thus, an inhibitor of mTORC1) display excessive mTORC1 signalling with aberrant cell cycling, reduced homeostatic proliferation in response to IL-7 and hyper-responsiveness to TCR induced apoptosis (O'Brien et al. 2011; Wu et al. 2011; Zhang et al. 2012).

Once stimulated, T cells that undergo activation require mTORC1 signalling to drive entry into the cell cycle from quiescence. In line with this, mTORC1, RHEB and Raptor deficient T cells display defects in antigen-induced proliferation (Delgoffe et al. 2009; Yang et al. 2013). Furthermore, rapamycin treatment or loss of raptor in naive, but not in proliferating T cells, blocks clonal expansion and promotes T cell anergy (Yang et al. 2013; Zheng et al. 2007b). Interestingly, raptor-deficient T cells also display decreased glycolysis, OXPHOS, and/or lipogenesis (Wang et al. 2011; Yang et al. 2013). Collectively, these studies indicate that mTOR signalling is crucial for T cell activation and proliferation.

Th1, Th2, and Th17 cells express high surface levels of the glucose transporter Glut1 and are highly glycolytic. Conversely, in the absence of mTOR, polarisation towards the CD4 T helper subsets Th1, Th2 and Th17 are all impaired. Thus, these T cells require the induction of mTOR activity for their differentiation. In Th1 T cells, mTORC1 activity is thought to be crucial for differentiation (Delgoffe et al. 2011; Lee et al. 2010). In Th2 cells, both mTORC1 and mTORC2 activities are necessary for differentiation (Lee et al. 2010; Yang et al. 2013). The relevance of mTORC2 in Th1 cell differentiation is still controversial. In Th17 T cells, genetic deletion of Hypoxia inducible factor 1 alpha (HIF1α), a transcription factor functionally regulated by mTORC1, impairs transcriptional activation of RORγt and therefore, Th17 differentiation (Dang et al. 2011; Shi et al. 2011).

Figure 7 below gives an overview of how mTOR signalling controls peripheral T cell fate decisions as adapted from (Chapman and Chi 2014).

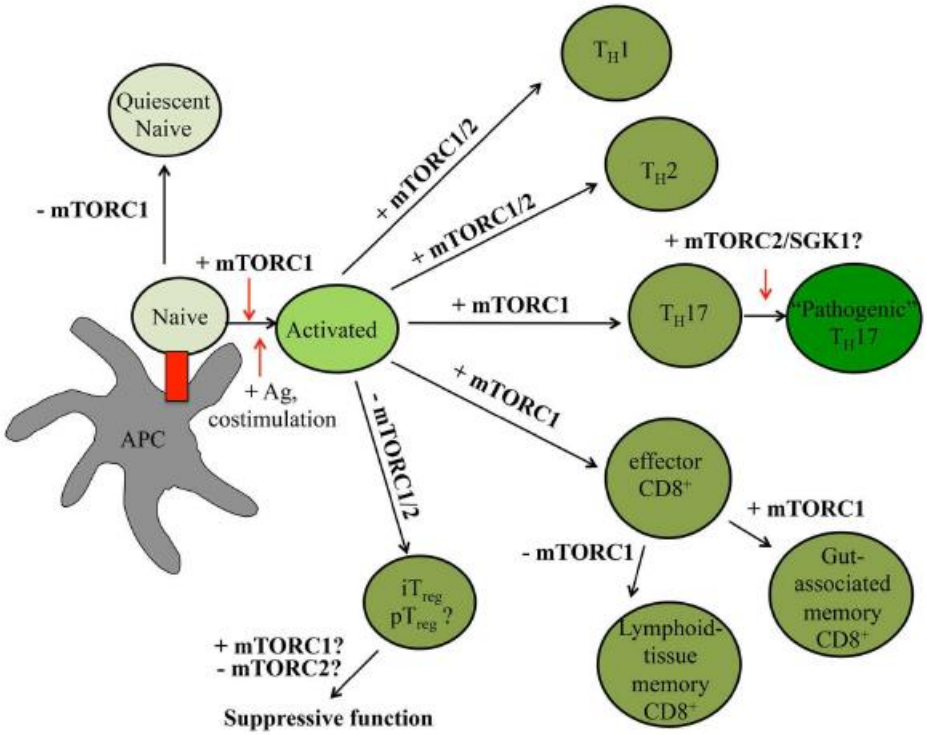



**Figure 7 mTOR regulates peripheral T cell fate decisions**

*In the peripheral tissues, low levels of mTORC1 signalling controls T cell quiescence. Following TCR and co-stimulatory signals, T cells undergo clonal expansion. Under the influence of different cytokines, T cell differentiate into different CD4+ Teffs lineages. CD8 T cells differentiate into either effector or memory T cells. The roles of mTORC1 and mTORC2 in various T cell states are indicated in the figure, with (+) signs representing positive roles and (–) signs indicating negative roles. Question marks (?) indicate pathways warranting further study or under investigation. Figure adapted from (Chapman and Chi 2014).*

In contrast, CD8 memory T cells, which have high lipid oxidation rates, mTOR inhibition was shown to be a crucial event for their differentiation. In a study by (Shrestha et al. 2014), TSC1-deficient CD8 T cells display high mTORC1 activity and impaired CD8 memory differentiation

following IL-15 stimulation. Similar to CD8 memory T cells, Tregs also display high lipid oxidation rates. Furthermore, inhibition of mTORC1 activation has been shown to be required for differentiation of inducible Tregs (iTregs) and is described in detail in the next section.

#### 1.3.3.2 mTOR Inhibition and Treg Differentiation

Tregs exhibit a very distinct metabolic signature which features low expression of the glucose transporter Glut1 and high lipid oxidation rates *in vitro* (Michalek et al. 2011c). These distinct metabolic characteristics can be directly linked to the varying mTOR activity in conventional and regulatory T cells. Conventional CD4 and CD8 T cells, upon activation, meet the enhanced energy requirement using the mTOR pathway to upregulte aerobic glycolysis. Selective inhibition of mTOR by rapamycin prevents the generation of Teff responses and promotes the generation of Tregs (Battaglia et al. 2005; Powell et al. 1999; Vanasek et al. 2001; Zheng et al. 2007b). Knocking out mTOR specifically in T cells was revealed to lead to the impaired development of $T_{eff}$ responses while favouring $T_{reg}$ differentiation (Zeng et al. 2013a). CD4+ T cells lacking both mTORC1 and mTORC2 complexes fail to differentiate into any CD4 Teff lineage (Th1, Th2, or Th17), and preferentially develop a Treg cell phenotype (Delgoffe et al. 2009; Delgoffe et al. 2011). In a study by (Shi et al. 2011), blocking glycolysis promoted iTreg cell generation through the inhibition of the transcription factor HIF1α. As described before, HIF1α is functionally regulated by mTORC1.

In Tregs, AMP kinase activity and preferential lipid oxidation (described above) are a primary source of energy production. APMK activation is also known to inhibit mTORC1 activation. AMPK phosphorylates and activates TSC2, a GTPase-activating protein that forms a complex with TSC1 which subsequently inactivates RHEB. This results in loss of activation of mTORC1 (Hardie et al. 2006). Thus, AMPK activity in Tregs, which promotes mitochondrial oxidative metabolism indicates reduced mTORC1. In the study by (Michalek et al. 2011c) (described before) inhibition of mitochondrial lipid uptake and oxidation pathways by Etomoxir abrogated the generation of Treg without altering Teff differentiation. Furthermore, in this study, Tregs expressed lower levels of the glucose transporter Glut1 as compared to Teff, and transgenic CD4+ T cells overexpressing Glut1 develop fewer Treg. Overall, these studies indicate that inhibition of mTOR and subsequently glycolysis, while increasing FAO is crucial in the development and differentiation of iTregs. An overview of AMPK signalling in Tregs is provided in Figure 8 below adapted from (Fleming et al. 2011).

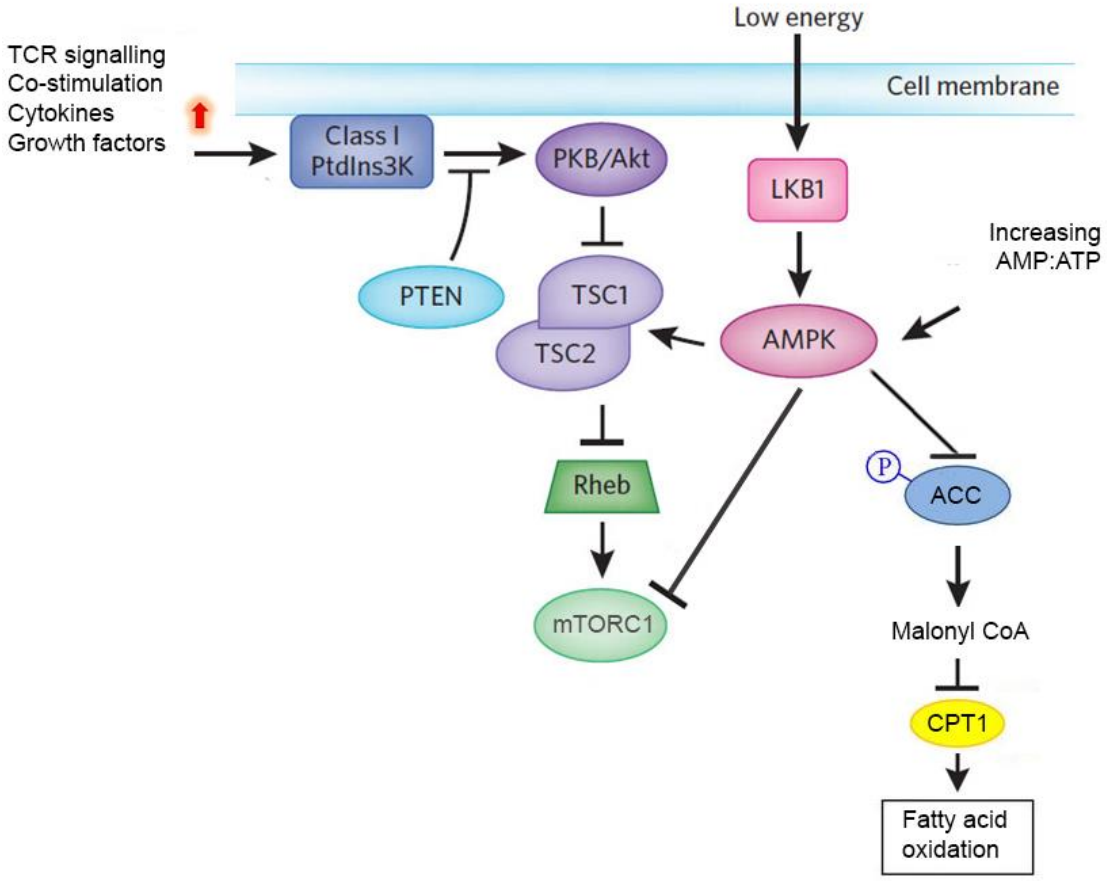



**Figure 8 AMPK-mTORC1 signalling in Tregs**

*AMPK undergoes activation due to increasing AMP: ATP ratios and upstream activation of LKB1. Once activated, APMK inhibits mTORC1 activity either directly or through activation of the TSC1/2 complex. The TSC1/2 complex is a negative regulator of mTORC1. Additionally, AMPK blocks acetyl-CoA carboxylase (ACC), to inhibit suppression of CPT1 activation and thus increases fatty acid oxidation. Figure adapted from (Fleming et al. 2011).*

### 1.3.3.3 mTOR and Treg Function

From the above mentioned discoveries, it could be reasonably assumed that inhibiting mTOR can increase Treg differentiation. However, recent findings by (H. Zeng et al. 2013b) indicate a crucial

role of the mTORC1 complex to the suppressive activity of Tregs . In this study, mTORC1 activity and Glut1 expression were higher in Tregs than naive T cells under steady state conditions. Selective genetic deletion of Raptor in the CD4+ Foxp3+ T cell subset led to the early onset of a fatal autoimmune disease in mice (H. Zeng et al. 2013b). Interestingly, this disease was described to be similar to that witnessed in Scurfy mice that bear a loss-of-function mutation in the Foxp3 transcription factor, indicating impaired Treg function.

Mechanistically, (Zeng et al. 2013a) established that the mTORC1 pathway in Tregs was necessary to initiate the upregulation of lipid metabolism so as to enable surface CTLA-4 and ICOS for Treg-mediated suppression. In addition, mTORC1 is also known to induce mitochondrial oxidative metabolism (Cunningham et al. 2007). Taken together, these results indicate that mTORC1 activity is essential for Treg function.

#### 1.3.3.4 Oscillation of mTOR activity in Tregs.

From the above mention studies it is clear that Tregs require mTORC1 for their functional fitness. However, inhibition of mTORC1 is also necessary for Treg differentiation and proliferation. This indicates the presence of a dynamic regulation of mTORC1 activity in Tregs. To explain this, the report by (Procaccini et al. 2010) described an 'oscillatory model' of mTOR activation in Tregs. Here, mTOR activity in Tregs oscillates between low and high activation states. The intermittent reduction in mTOR signalling followed by its enhanced activation by means of TCR triggering and IL-2 stimulation was shown to be necessary for Treg proliferation. However, inhibition of mTOR signalling necessary for Treg division was short-lived and prolonged incubation with rapamycin (>1Hr) ablated Treg proliferation. This study identified the adipocyte hormone leptin as a key signal that regulates mTOR activity *in vivo*, promoting Treg proliferation. In addition, Treg-produced leptin was identified to contribute to mTOR activation in an autocrine manner.

## 1.3.4 T cell metabolism and migration

### 1.3.4.1 PI3K-Akt-mTOR axis and T cell migration

Like in all other motile cells, the metabolic status that is maintained in T cells through both extracellular and intracellular stimuli, has a profound effect on their migration. One important and evolutionally conserved function of the PI3K-Akt-mTOR pathway in T cells is to regulate cell metabolism and protein synthesis (Jones and Thompson 2007). These processes are crucial to T cell migration and are therefore likely to be closely intertwined with molecular pathways involved in T cell migration. For example, regulation of T cell migration via chemokine receptors was previously shown to occur partly through the action of the catalytic subunit p110γ of the PI3K. As mentioned previously, p110γ-deficient CD4 and CD8 T cells show compromised migration to chemokines and fail to recruit inflammatory sites. T cell migration is a highly energy dependant process and it is plausible that chemokine induced signalling may affect T cell migration through regulation of metabolism. Some tentative evidence to suggest this hypothesis is provided by a study linking chemokine receptor signalling to cell growth and metabolism in transformed T cells and developing thymocytes (Janas et al. 2010; Lo et al. 2010). However a direct relationship between chemokine receptor signalling and T cell metabolism remains to be established.

In addition to the effect on chemokine receptors, the indirect action of T cell metabolism on the expression of adhesion receptors may also regulate trafficking of T cells. Accordingly, PI3Kδ signalling through Akt and the Foxo transcription factors not only regulates the expression of CCR7 and $S1P_1$, but also that of CD62L integrin. Transcriptional control of CD62L by p110δ was observed to be mediated by mTOR through regulation of the transcription factor Krüppel-like Factor 2 (KLF2) (Sinclair et al. 2008). KLF2 is a key modulator of homing receptor expression which binds to the promoter of CD62L and is necessary and sufficient for CD62L expression (Bai et al. 2007; Carlson et al. 2006; Sebzda et al. 2008). In CD8 T cells, disruption of the PI3Kδ/Akt signalling prevents the down-regulation of CD62L, CCR7, and S1P1, and thus, these T cells retain a lymph node trafficking pattern rather than migrating to non-lymphoid inflammed tissues (Finlay and Cantrell 2010; Andrew N. Macintyre et al. 2011b). These observations are in line with those that show aberrant memory T cell trafficking or PI3Kδ inhibited memory T cell trafficking to peripheral sites (Jarmin et al. 2008). Similar results were seen with memory T cells from mice bearing a disruptive point mutation in the p110δ ($p110\delta^{D910A}$) catalytic subunit of PI3K rendering it inactive (Jarmin et al. 2008).

Conversely, it is also worth noting that factors that may affect T cell homing and thus the peripheral tissue destination of T cells will also affect T cell metabolism, albeit indirectly, by determining the cytokine environment to which they are exposed to. A study of the differentiation of CD8 T cells through the related cytokines IL2 and IL15 illustrates the differential regulation of T cell metabolism by distinct cytokine environments. IL2 induced Akt-mTOR promotes elevated glucose metabolism and glycolysis while IL15 does not maintain this metabolic state and T cells responding to IL15 have reduced nutrient uptake and glycolysis (Cornish et al. 2006; A. N. Macintyre et al. 2011a).

In summary, while several studies have provided indirect evidence linking the PIP3 induced Akt-mTOR activation pathways to the T cell metabolism, the energy requirement for T cell migration has only recently been investigated (R. Haas et al. 2015). However, the metabolic pathways required by Tregs have not been studied. This is particularly interesting given the different metabolic setup of conventional T cells and Tregs.

# Chapter 2 Hypothesis and Aims

In conventional T cells, CD28 and CTLA-4 receptors are known to engage signalling pathways that regulate adhesion and motility. Therefore, we hypothesised that these molecules would also regulate motility in the Treg subset which is known to express these co-receptors. In addition, both CD28 and CTLA-4 molecules are both constitutively expressed in Tregs and as such are likely to have a distinct impact on their migration compared to the effect exerted on conventional Teffs where CTLA-4 is only expressed transiently after activation.

Furthermore, the novel T cell co-inhibitory receptor molecule CD31 is known to recruit phosphatases, which are capable of dephosphorylating key signalling effector proteins that regulate T cell migration. Thus, we predict that CD31 would regulate the migration of T cells.

The specific aims of this study include:

- To establish a role for TCR antigen recognition in the migration of Tregs
- To assess the role of CD28 and CTLA-4 in Treg migration *in vitro* and *in vivo* and to determine if their impact on Treg migration occurs independently of TCR signalling.
- To establish the effect of concomitant signalling of both CD28 and CTLA-4 signal on the overall T cell motility and thus Treg migration *in vitro* and *in vivo*
- To assess the impact of CD31 signalling on T cell migration to chemokines *in vitro* and *in vivo*

# Chapter 3 Materials and Methods

# *3.1* Mice

All mice used in the experiments of this study were 7-11 weeks. C57BL/6, BALB/c and CBA/Ca mice were purchased from Charles River (UK) and IFN-γR KO mice were purchased from The Jackson Laboratory (USA). The Foxp3-GFP (Foxp3-IRES-EGFP) knock in mice on the C57BL/6 background were kindly provided by Dr B Malissen (Centre d'Immunologie de Marseille-Luminy, Marseille, France). Marilyn female mice, bearing a transgenic TCR specific for the male minor transplantation antigen HY peptide epitope NAGFNSNRANSSRSS and restricted by H2-Ab, have been previously described (Lantz et al. 2000). These mice were bred on a recombination activating gene 2 deficient (RAG2−/−) background. The $CD28^{Y170F}$ transgenic mice on the C57BL/6 background were a kind gift provided by Dr K Okkenhaug. Excised secondary lymphoid organs from 4 week old CTLA-4KO mice (of H-2u haplotype) were provided by Prof D Wraith (Bristol). The CD31-/- mice were present in house and details regarding derivation are given in the study by (Gordon S. Duncan et al. 1999b)

All in vivo experiments were conducted with strict adherence to the Home office guidelines (PPL 70/7443) following approval by the Queen Mary University of London Ethics committee.

## 3.1.1 Mouse genotyping

Mouse genotyping was performed by PCR. Briefly, genomic DNA was extracted from tail or ear clippings through NAOH-HCl method. 0.5cm clippings were treated first with 100µl alkaline lysis buffer *(*25 mM NaOH, 0.2 mM EDTA in $H_2O$) at 95°C for 30 minutes followed by 100ul of neutralization buffer (40 mM Tris.HCl in $H_20$). PCR reactions were carried out using the thermal cycler (Applied Biosystems). PCR end products were analysed on a 0.8-1% agarose gel.

Primers for $CD28^{Y170F}$

| | |
|---|---|
| CD28-L150 | 5'- GTAGATAGCAACGAGGTCAGC-3' |
| CD28-R778 | 5'- CAGAGCAGTGATGATGAGCA-3' |
| Actin-F | 5'- GGTGTCATGGTAGGTATGGGT-3' |
| Actin-R | 5'- CGCACAATCTCACGTTCAG-3' |

Primers for CD28Ex2

CD28-int2 5'- CCTGAGTCCTGATCTGTCAGACT-3'
CD28-ex2 5'- CTGCTTGTGGTAGATAGCAACGA-3'
CD28 HSV-TKv2 5'- ATTCGCCAATGACAAGACGCTGG-3'

Primers for P110δ$^{D910A}$

D150: 5'- GGG GTC TGA ATA CAG AGA GAA T -3'
D153: 5'- CCA TTT ATG GCT ATT CTG TTC -3'

Primers for IFN-γR KO

oIMR0587: 5'- CCC ATT TAG ATC CTA CAT ACG AAA CAT ACG G -3' (wild type)
oIMR0588: 5'- TTT CTG TCA TCA TGG AAA GGA GGG ATA CAG -3' (wild type)
oIMR6916: 5'- CTT GGG TGG AGA GGC TAT TC -3' (mutant forward)
oIMR6917: 5'- AGG TGA GAT GAC AGG AGA TC -3' (mutant reverse)

# 3.2 Cells

## 3.2.1 Endothelial Cells

### 3.2.1.1 Isolation of primary microvascular endothelial cells

Murine lung microvascular endothelial cells were isolated as previously described (Marelli-Berg et al. 2000). Mouse lungs were diced into 2-3mm$^3$ blocks, washed in phosphate buffered saline (PBS; Sigma-Aldrich, Cat# D8537) and digested in a solution containing 0.5mg/ml Type IV Collagenase (Sigma, Cat# C5138) for 30 minutes in a humidified incubator maintained at 37°C. A 70um cell strainer (Fisher scientific, Cat# 22363548) was then used to remove undigested tissue while the digested tissue was collected and centrifuged at low rpm (<250g). The supernatant was aspirated and further digested in trypsin/ ethylenediaminetetraacetic acid (EDTA) (Gibco, Cat# E7889) at 37 °C for 5 minutes to create a uniform cell suspension. The cell suspension was then washed with PBS at a high centrifuge speed (>300g) and resuspended in DMEM (Gibco, Cat# 11966-025) containing 10% Foetal bovine serum (Seralab, Cat# A210009). The cells were then seeded in 2% gelatinated (Sigma-Aldrich, Cat# 1393) 25 cm2 culture flasks (Helena biosciences, Cat# 90026) for 24 hours. After 24 hours, non-adherent cells were removed by washing with warm PBS and complete EC medium (mentioned below) was added to the culture.

### 3.2.1.2 Culture of ECs

EC medium consisted of Dulbecco's Modified Eagle media (DMEM, Gibco, Cat# 41966-052) supplemented with 2 mM glutamine (Gibco, Cat# 250-30), 50 IU/mL penicillin (Gibco, Cat# 15140-122), 50 µg/mL streptomycin (Gibco, Cat# 15140-122), 50 µM 2-Mercaptoethanol (2-ME) (Gibco, Cat# 31350-010), 1mM sodium pyruvate (Gibco, Cat# 11360-039), 20mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) (Gibco, Cat# 15630-056), 1% non-essential amino acids (Gibco, Cat# 11140-050), 20% FCS and 150µg/ml EC growth supplement (Sigma-Aldrich, Cat# E0760). Medium was replaced every 48 hours. When confluent, cells were detached with trypsin/EDTA (Gibco, Cat# T4049) and passaged.
For functional assays, ECs were used between passages 2 and 8 and treated with 600 U/mL murine IFN-γ (PeproTech, Cat# 315-05) for 48 to 72 hours prior to use in experiments. This led to up-regulation of MHC class I, induction of MHC class II and several adhesion molecules (Class II upregulation shown in Figure 9 below)

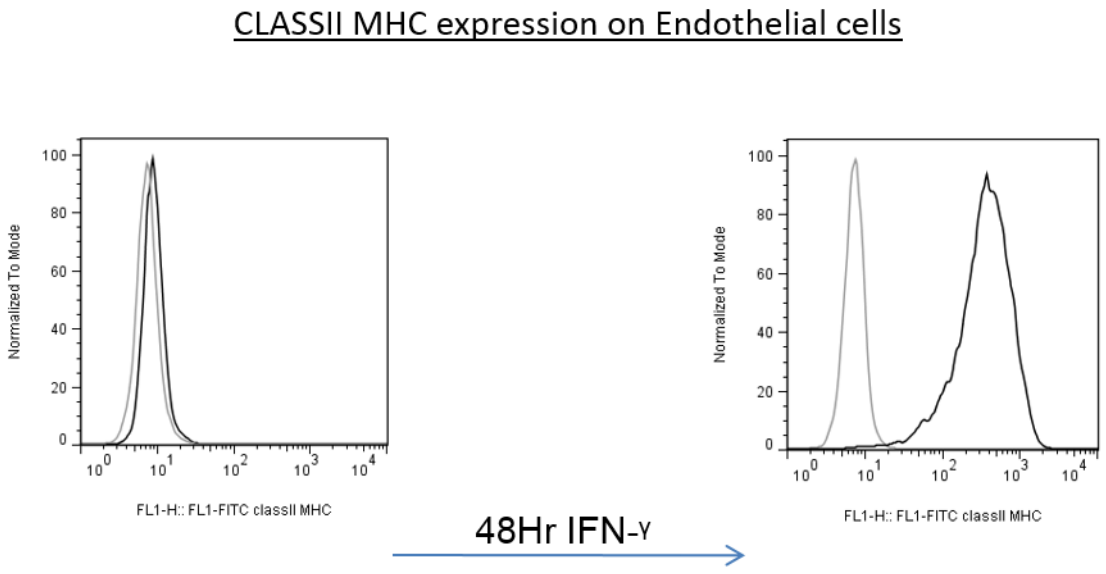



**Figure 9 Class II MHC expression on endothelial cells**

*This histograms in this figure display the surface expression of class II MHC molecules by murine microvascular endothelial cells* observed by flow cytometry*. Treatment with* 600U/ml *IFN-γ for a period between 48-72 hours induces class II MHC upregulation. Dark peaks indicate*

*expression via flurochrome labelled anti-Class II antibody while grey peaks are representative of isotype.*

## 3.2.2 Bone marrow-derived Dendritic Cells (bmDCs)

### 3.2.2.1 Isolation of bone marrow-derived Dendritic Cells (bmDCs)

Bone marrow-derived DCs were obtained from WT BALB/c (H2-d) mice. Femurs and tibias from 7–to-10-week-old female mice were removed and BM cells were flushed out with PBS using a 27 gauge needle (Becton Dickinson, Cat# 302200). Red blood cells were lysed from the cell suspension with lysis buffer (Sigma-Aldrich, Cat# R7757). BM cells ($5 \times 10^6$) were seeded per well in a 6 well plate (Helena bioscience, Cat# 92006) in DC medium as described in section below.

### 3.2.2.2 Culture of Dendritic cells

Bone marrow-derived dendritic cells were cultured in RPMI 1640 medium (Gibco, Cat# 21875-034) supplemented with 10% FCS, 2mM glutamine, 50 IU/mL penicillin, 50 µg/mL streptomycin, 50 µM 2-ME and 2% murine granulocyte-macrophage colony stimulating factor (GM-CSF) obtained from the supernatant of the GMCSF hybridoma (gift from Dr. Jian-Guo Chai, Imperial College, London, UK). Cells were cultured at 37°C in the presence of 5% CO2. On days 3 and 5, fresh culture medium was added to the plates.
For Treg-DC co-cultures, immature bmDCs were collected and used on day 6 of culture. For functional assays, immature DCs were matured overnight with 100ng/ml lipopolysaccharide (LPS) (Invivogen, Cat# tlrl-3pelps) and were used between 7-to-10 days post-isolation.

## 3.2.3 T cells

### 3.2.3.1 Isolation of T cells

Primary T cells were isolated from the lymph nodes and spleen of the mice. Mice were dissected and the lymph nodes and spleen removed. A uniform cell suspension was prepared by mashing the lymph nodes and spleens through a 70um cell strainer. Cells were then used directly or further subjected to immunomagnetic separation to obtain specific T cell subsets.

### 3.2.3.2 Culture of HY-specific T cells

Effector memory CD4+ and CD8+ T cells specific for the male-specific minor transplantation antigen HY peptide Dby epitope and restricted by H2-Ab and for the Uty epitope restricted by H2-Db, respectively, were obtained from WT female mice by 2 fortnightly intraperitoneal (i.p.) immunization of female mice with male splenocytes, and further expansion of isolated T lymphocytes with male splenocytes *in vitro*, as previously described (Jarmin et al. 2008; Marelli-Berg et al. 2004a). T cell populations were composed by approximately 20% CD8+ and 75% CD4+ T cells. The T cells were cultured at 37°C in the presence of 5% CO2 using T cell medium consisting of RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 50 IU/mL penicillin, 50 µg/mL streptomycin, 10mM HEPES and 50 µM 2-ME. Additionally, 20U/ml of IL-2 (Roche, Cat# 10799068001) was supplemented to the medium.
For use in functional assays, viable T cells were selected between days 6-8 by density gradient centrifugation with Ficoll-Paque (GE Healthcare, Cat# 17-1440-02).

### 3.2.3.3 Culture of H2-d allospecific T effector cells

T effector cells were isolated using negative immunomagnetic selection kits for CD4+CD25- T cells (Invitrogen Dynal, Cat#11463D) from a mixture of lymphocytes cell suspension obtained from dissected lymph nodes and spleens of WT C57BL/6 (H2-b haplotype) mice. Purified CD4+CD25-T cells were then co-cultured with irradiated (60Gy) splenocytes ($50x10^6$ splenocytes per $5x10^6$ lymphocytes) from BALB/c (H2-d haplotype) to selectively expand H2-

d alloantigen specific cross reactive T cells. T cells were cultured in complete T cell medium with 20U/ml of IL-2.

#### 3.2.3.4 Culture of H2-d allospecific Tregs

CD4+CD25+ Treg cells were isolated from spleen and LNs using the Dynabeads® FlowComp™ Mouse CD4+CD25+ Treg Cells Kit (Invitrogen Dynal, Cat#11463D). To expand H2d-allospecific CD4+CD25+ Tregs, cells isolated from C57BL/6 (H2-b) mice were stimulated weekly with either irradiated or mytomycin C (Sigma-Aldrich, Cat# M4287) inactivated immature BALB/c-derived (H2-d) DCs at a ratio of 5:1 (Treg:DC). The co-cultures were maintained in complete T cell medium supplemented with 10U/ml IL-2. Cells were harvested and seeded at an optimal density of $1.5 \times 10^6$ Treg cells per well of a 24-well tissue culture (Helena bioscience, Cat# 92024) plate each week. The percentage of CD4+Foxp3+ cells after two weeks of culture was greater than 95%. For extremely high purity of Treg cells (>99%), CD4+CD25+Foxp3+ cells were obtained from Foxp3-GFP reporter mice through FACS.
For use in functional assays, Tregs were removed from co-cultures between days 6-8.

## 3.3 Antibody-mediated T cell activation

Activated T cells were obtained by polyclonal stimulation of LN cells with plate-bound anti-CD3 (1 µg/ml, eBiosciences, Cat# 16-0032-85) and plate-bound anti-CD28 (5 µg/ml, eBiosciences, Cat# 16-0281-86) in in complete T cell medium supplemented with 20 U/ml recombinant IL-2 (Roche, West Sussex, UK) for 7 days at 37°C. Antibody coating of tissue culture plates was performed by incubating antibodies in 200µl of Tris buffer (pH 8.5) at 37°C for a period of 1 hour.

## 3.4 Lymphocyte trans-endothelial migration assay

Primary microvascular endothelial cells treated with IFN-γ for 48–72 h were seeded ($3 \times 10^4$) and cultured on 2% gelatin-coated Transwell tissue culture well inserts (diameter, 6.5 mm) containing 3-µm pore size (Costar, Cat# CLS3472-48EA) polycarbonate membranes in EC medium for 16 h to from a monolayer. T cells ($5 \times 10^5$) resuspended in migration medium (RPMI 1640 supplemented with 2% fetal bovine serum) were added to each insert and left to migrate through the monolayer; the well volume was also replaced with fresh migration media. The number of migrated T cells was determined by hemocytometric counting of the cells present in the well media at different time points over a 24-h period. Figure 10 below depicts a diagrammatic overview of this assay.

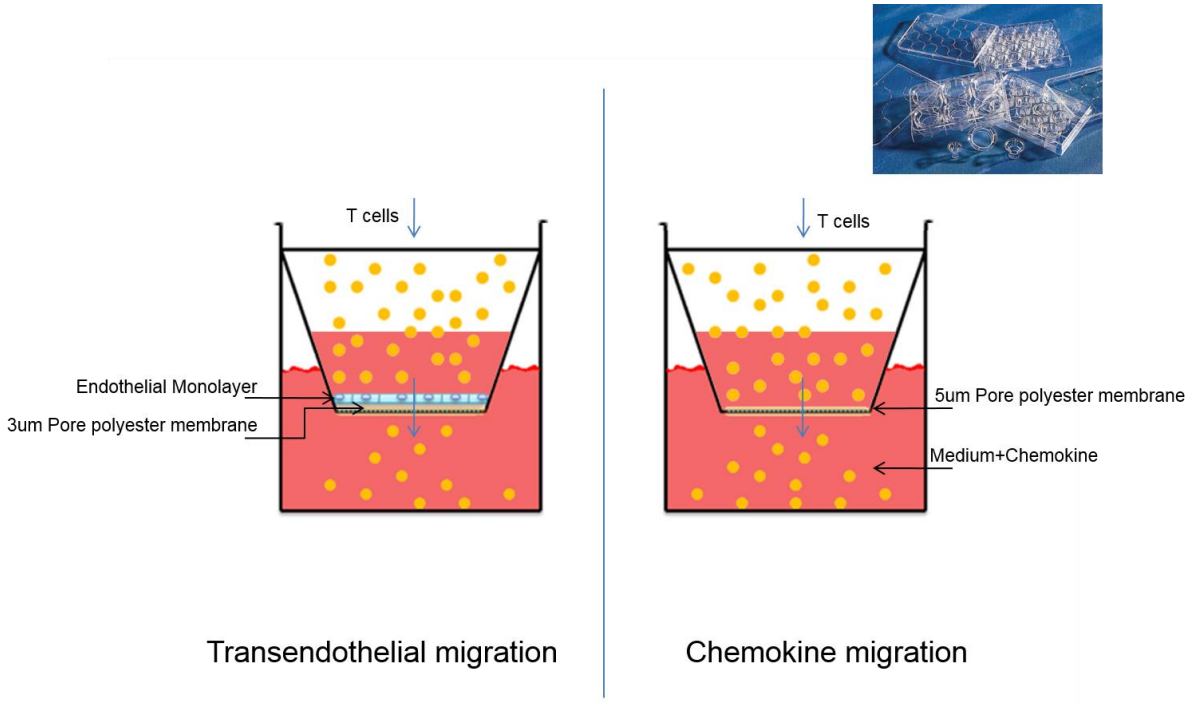



**Figure 10 Illustrative overview of *in vitro* migration assays**

## 3.5 Lymphocyte chemokinesis assay

Approx. 5–10 × $10^5$ T cells were seeded onto Transwell™ tissue culture well inserts (diameter, 6.5 mm) with 5-µm pore size (Costar, Cat# CLS3421-48EA) polycarbonate membranes and chemokine containing migration media was placed in the bottom of the well. The number of migrated cells was determined by hemocytometric counting of the cells present in the well media at indicated time points. Figure 1 above also depicts a diagrammatic overview of this assay. The table below indicates the chemokines and the concentrations that were used in this
study.

| Chemokine | Concentration used | Supplier | Catalogue no. |
|---|---|---|---|
| MIP-3β (CCL19) | 200ng/ml | Peprotech | 250-27B |
| Murine Exodus-2 (CCL21) | 200ng/ml | Peprotech | 250-13 |
| Murine IP-10 (CXCL10) | 300ng/ml | Peprotech | 250-16 |
| Murine SDF-1α (CXCL12) | 300ng/ml | Peprotech | 250-20A |

**Table 4 Chemokine and chemokine concentrations in chemokinesis assays**

## 3.6 Mixed lymphocyte reaction assay

Treg suppressive ability was measured using mixed lymphocyte reaction (MLR) or mixed lymphocyte culture (MLC) assays. Responder T cells isolated from LNs/Spleen were activated by plate-bound anti-CD3 anti-CD28 antibody or DCs in triplicates in a 96 well plate following which Tregs were added to the wells at varying ratios in T cell medium. The co-culture plates were incubated for 48 hours at 37°C after which the cells were pulsed with 1µCi/well 3H thymidine and incubated overnight at 37°C in the presence of 5% CO2. The reaction was halted by freezing the plates at -20°C; plates were later thawed and harvested using Tomtec harvester 96 (Receptor Technologies, Adderbury UK) and onto filter paper (Perkin Elmer). Cell proliferation was measured by radioactivity incorporation and read by using Wallac Microbeta counter for Windows (Perkin Elmer)

## 3.7 Induction of CD28 and CTLA-4 signalling

Induction of CD28 and CTLA-4 signalling was performed as has been described previously in the study by (Schneider et al. 2005) and by (Wells et al. 2001).To induce co-stimulatory signals via CD28 and CTLA-4 co-receptors for functional assay, cells were incubated with antibodies targeting the functional domains of the co-receptors. To induce CD28 signalling, T cells were treated with a mixture of hamster anti-mouse CD28 (5µg/5x$10^6$ cells) (clone: 37.52, Bio-Rad, Cat# MCA1363) and goat anti-hamster immunoglobulin (Ig) (2.5µg/5x$10^6$ cells) (Bio-Rad, Cat# STAR104) in migration medium for 45 minutes at 37°C in the presence of 5% CO2. Similarly, CTLA-4 signalling was achieved by incubating T cells with a mixture of hamster anti-mouse CTLA-4 (5µg/5x$10^6$ cells) (clone: UC10-4F10-11, Becton Dickinson, Cat# 553718), and goat anti-hamster immunoglobulin (Ig) (2.5µg/5x$10^6$ cells) (Bio-Rad, Cat# STAR104). A hamster IgG isotype control was used to observe any non-specific effects of the antibody stimulation (Bio-Rad, Cat# MCA2356). T cells were washed in PBS prior to use in the experiments.

## 3.8 Fluorescent labelling of viable T cells

For labelling T cells with fluorescent probes, T cells were washed with PBS, counted and resuspended in PBS at a final concentration of $10^7$/ml. If necessary, dead cells were removed using density gradient centrifugation with Ficoll-Paque prior to re-suspension. Labelling of T cells with PKH26 (Sigma-Aldrich, Cat# PKH26GL-1KT), a cell linker dye for cell membranes was performed using manufacture instructions. PKH26 labelling added at a final concentration of 5µM, and the cells were incubated at room temperature for 5 minutes. The reaction was inactivated by adding an equal volume of FBS to the cell suspension and the cells were washed in PBS containing 10% FBS for 10 minutes. Labelling of T cells with succinimidyl ester dyes CFSE (Invitrogen, Cat# C1157) or DDAO-SE (Invitrogen, Cat# C34553) was performed by incubating the T cells in PBS containing final concentration of 3.3µm CFSE or 1.3µm DDAO-SE for 10-15 minutes at room temperature. The reaction was terminated by adding equal volume FBS and the cells were then washed with PBS containing 10% FBS for 10 minutes.

## 3.9 Peritoneal model of T cell recruitment

To observe *in vivo* recruitment of T cells we used the previously described model of T cell recruitment (Mirenda et al. 2007).Either PKH26 or CFSE or DDAO-SE labelled T cells ($10^7$) were injected intra-venously (i.v.) into mice that have previously received IFN-γ (600U; administered intra-peritoneal injection (i.p.) 48 to 72 hours before. This IFN-γ treatment induces local upregulation of MHC molecules by the endothelium enabling T cell recruitment. Labelled T cells recovered via peritoneal lavage were analysed 16 hours later using flow cytometric analysis. In addition, the draining mesenteric lymph node and other secondary lymphoid tissue such as the spleen were analysed using flow cytometry to observe recruitment to those tissues.

# 3.10 Imaging and microscopy

## 3.10.1 Time lapse microscopy

Primary ECs were seeded onto wells of a 24-well tissue culture plate for 24hours to obtain confluent monolayers. A total of $5 \times 10^5$ T cells were seeded onto confluent endothelial monolayers and their migration was recorded using an optimized Nikon Eclipse TE2000 microscope, equipped with a × 20 objective and Metamorph software (Molecular Devices LLC). Images were taken every minute over a 60-min period and the migration speed (μm min−1) of the cells was determined using ImageJ analysis software (National Institute of Health). About 30–40 cells were tracked in every experimental condition.

## 3.10.2 Widefield deconvolution fluorescence microscopy

Tissues samples were excised, embedded in Optimal Cutting Temperature compound (OCT; Thermo Fisher Scientific, Cat# 12678646), snap-frozen and stored until analysis. Frozen tissue sections were laid onto Polysine coated microscope slides (VWR International, Cat# 47100), air dried and then fixed with ice cold acetone (Sigma, Cat# 534064) for 10 min. Tissue sections were washed in PBS, blocked with serum for 3 hours and stained using mentioned primary antibodies at 4 °C for 24 h. Excess antibody was washed away with PBS and tissues were stained with indicated secondary antibodies along with DAPI (4',6-diamidino-2-phenylindole) (Invitrogen/LifeTechnologies, Cat# D1306) for 30 min at room temperature. Slides were washed, mounted in ProLong Gold Antifade Reagent (Invitrogen/Life Technologies, Cat# P36930) and visualized using a Zeiss Z1 fluorescence microscope (Carl Zeiss, UK) equipped with an AxioCam MRm Cooled monochrome digital camera and an Apotome 2 Imaging unit. Images were acquired using a Plan Apochromat 20x/0.8 NA objective and Axiovision software version 4.8 (Carl Zeiss, UK).

### 3.10.3 Confocal microscopy

Cells were allowed to adhere onto poly-l-lysine coated coverslips and fixed in 4% paraformaldehyde (Thermo Fisher Scientific, Cat# 28906) for 5-10 minutes at room temperature. Where mentioned, permeabilization was carried out using 0.2% Triton X-100 (Sigma-Aldrich, Cat# X100-500ML) in PBS for 5 minutes. Cells were then washed in PBS, blocked in blocking buffer (PBS containing 0.1% Fish skin gelatin (Sigma-Aldrich, Cat# G7765) and 1% serum of the species giving rise to secondary antibodies) for 3-6 hours and then stained with appropriate primary antibodies at 4°C for 16 hours in the dark. Following staining, cells were washed again and stained with corresponding secondary antibodies and DAPI for 30 minutes at room temperature. After multiple PBS washes coverslips were mounted onto slides using ProLong Gold antifade and then examined using a Leica SP5 confocal microscope equipped with a 63×1.4 NA objective. Confocal images and z-stacks were acquired and analysed by Leica LAS software. Repositioning of scale bars and image layouts were prepared using Adobe Photoshop (Adobe Systems). All images in a group were treated equally.

### 3.10.4 Intravital microscopy

T cell–EC interactions were visualized by intravital microscopy of the cremasteric muscle vasculature of male mice pre-treated with intrascrotal injection of 1,200 U IFN-γ for 72 h. Mice were then anesthetized by i,p. administration of ketamine (150 mg kg−1) (Ketalar; Parke-Davis, Cambridge, UK) and 7.5 mg kg−1 xylazine (Rompun; Bayer, Newbury, UK) and placed on a custom-built, heated (37 °C) microscope stage. The jugular vein was cannulated and the cremaster muscle was exteriorized through an incision in the scrotum. One testis was gently drawn out to allow the cremaster muscle to be opened and pinned outflat over the optical window within the microscope stage. The tissue was kept warm and moist throughout each experiment by superfusion with 37 °C bicarbonate buffered saline. A total of $2.5 \times 10^7$ CFSE (20 µm) labelled T cells were infused into the cannulated jugular vein and the cremaster muscle post capillary venules were imaged 1 h later using an Olympus BX61W1 microscope with an Olympus UMPlanFL × 20/0.50 W water immersion objective (Carl Zeiss). The set-up was connected to an Olympus BXUCB lamp, Uniblitz VCMD1 shutter driver and DG4-700 shutter instrument. The set-up used a Hamamatsu C9300 digital camera

with a Videoscope VS4-1845 image intensifier attached. All videos were captured and analysis carried out using Slidebook 5.0 software (Intelligent Imaging TTL).

Rolling cells were defined as fluorescent T cells moving slower than the flowing erythrocytes, and rolling flux was quantified as the number of rolling cells moving past a fixed point on the venular wall per 5 min. Firmly adherent leukocytes were defined as fluorescent T cells that remained stationary for at least 30 s within a 450 um vessel segment. Transmigrated cells were defined as those cells in the extravascular tissue of the 340–450 µm field of view, either side of the vessel segments quantified at least two sections of 2–5 vessels were studied in each animal.

# 3.11 Assays of T cell metabolism

## 3.11.1 *In vitro* 6NBDG uptake assay

Freshly isolated T cells or cultured T cells were washed in PBS and resuspended in glucose free T cell medium (Gibco, Cat# 11879-020) containing various mentioned signalling antibodies and incubated for 45 minutes at 37°C with 5% CO2. A final concentration of 400µM 6-NBDG (Life Technologies, Cat# N23106) in glucose free T cell medium was then added to the cells and the cells were further incubated for an additional 10-15 minutes. Finally, the cells were washed twice with warm PBS and resuspended in flow cytometry buffer and placed on ice. Immediate analysis was performed using flow cytometry to observe fluorescence uptake by the T cells.

## 3.11.2 *In vivo* 6NBDG uptake assay

To measure glucose uptake activity of T cells *in vivo*, PKH26 labelled T cells ($3x10^6$) were injected intra-peritoneally (I.P) into mice. A second I.P injection of 6NBDG (400µM in Sterile water) was given to the mice immediately afterwards. After a 1 hour period, the mice were sacrificed and the mesenteric (draining) lymph nodes and spleen collected for analysis by

flow cytometry. Widefield microscopy of the peritoneal membranes was performed to observe influx of labelled T cells into the peritoneal membrane. PKH26+ T cells infiltrating the membranes were further analysed for 6NBDG uptake (green fluorescence) using image analysis software ImageJ. A cell count of labelled cells in 10 (10x) field views images was performed manually to determine differences in T cell infiltration.

### 3.11.3 Measurement of ECAR and OCR

Real time bioenergetics analysis of extracellular acidification rates (ECAR) and oxygen consumption rates (OCR) of T cells subjected to antibody stimulation was performed using the XF analyser (Seahorse biosciences). T cells were cultured in serum free, unbuffered XF assay medium (Seahorse biosciences, Cat# 102365-100) for 1 hour following which they were seeded ($6X10^5$/well) into the seahorse XF24 cell plates for analysis. Perturbation profiling of the use of metabolic pathways by T cells was achieved by the addition of oligomycin (1 µM), Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 1µM), Antimycin A (1µM), rotenone (1 µM), D-glucose (10mM), 2-Deoxy-D-glucose (2DG, 50mM; all from Sigma-Aldrich). Experiments with the Seahorse system were done with the following assay conditions: 2 min mixture; 2 minutes wait; and 4–5 min measurement. Metabolic parameters were then calculated.

## 3.12 Flow Cytometry

### 3.12.1 Cytometer and analysis software

Flow cytometric analysis of T cells was performed using either a 3 laser equipped FACSCalibur (Becton Dickinson, Oxford, UK) connected to a Mac computer (OS 9) with Cellquest as the acquisition software or on a 4 laser equipped LSR II Fortessa machine (Becton Dickinson) using FACSDiva as acquisition software.
Acquired samples were analysed using Flowjo (Treestar, USA) by gating on live cells on the forward and side scatter plot.

## 3.12.2 Surface staining

For surface staining, cells were resuspended ($10^7$/ml) and stained with flurochrome conjugated antibodies in 100µl of Flow cytometry buffer made of PBS containing 0.1% sodium azide (Sigma-Aldrich, Cat# S2002-25G) and 1% FBS at 4°C for 30 minutes. CCR7 antibody (4B12) staining was performed at 37°C for 30 mins. Optimal antibody concentrations for staining were calculated based on manufacturer instructions. Following staining, cells were washed and resuspended with flow cytometry buffer and analysed immediately. Alternatively, for delayed analysis, cells were fixed in fixation buffer (flow cytometry buffer containing 1% Formaldehyde (Sigma-Aldrich, Cat# 15,812-7)) for 30 minutes at 4°C , washed and stored in flow cytometry buffer at 4°C. The table below indicates the fluorescent antibodies that were used in this study

| **Antibody** | **Clone** | **Conjugate** | **Isotype** | **Supplier** | **Catalogue no.** |
|---|---|---|---|---|---|
| Anti-CD3 | 17A2 | PE/Cy7 | Rat IgG2b, κ | Biolegend | 100220 |
| Anti-CD4 | GK1.5, RM4-5 | PE, APC, efluor450 | Rat IgG2a, κ | eBioscience | 12-0042-82 |
| Anti-CD8 | 53-6.7 | PE, APC, FITC | Rat IgG2a, κ | eBioscience | 11-0081-82 |
| Anti-CD11a (LFA-1) | M17/4 | PE | Rat IgG2a, κ | eBioscience | 11-0111-81 |
| Anti-CD11c | N418 | Alexa Fluor® 647 | Armenian Hamster IgG | Biolegend | 117312 |
| Anti-CD14 | Sa2-8 | PE | Rat IgG2a, κ | eBioscience | 12-0141-82 |
| Anti-CD25 (IL-2 receptor) | PC61.5 | PE, PE/Cy7 | Rat IgG1, λ | eBioscience | 12-0251-81 |
| Anti-CD28 | 37.51 | PE | Syrian Hamster IgG | eBioscience | 12-0281-82 |
| Anti-CD29 (VLA-4) | HMβ1-1 | APC/Cy7 | Armenian Hamster IgG | Biolegend | 102226 |
| Anti-CD31 (PECAM-1) | 390, MEC 13.3 | PE | Rat IgG2a, κ | eBioscience | 12-0311-81 |
| Anti-CD40 | 3/23 | PE | Rat IgG2a, κ | Biolegend | 124607 |
| Anti-CD44 | IM7 | PE | Rat IgG2a, κ | eBioscience | 12-0441-81 |
| Anti-CD49d (Integrin alpha 4) | 9C10 (MFR4.B) | PE | Rat IgG2a, κ | Biolegend | 103705 |

| | | | | | |
|---|---|---|---|---|---|
| Anti-CD54 (ICAM-1) | YN1/1.7.4 | PE | Rat IgG2b, κ | Biolegend | 116105 |
| Anti-CD62L (L-Selectin) | MEL-14 | PE, PE/Cy7 | Rat IgG2a, κ | eBioscience | 12-0621-81 |
| Anti-CD69 | H1.2F3 | PE, PE/Cy7 | Armenian Hamster IgG | eBioscience | 12-0691-81 |
| Anti-CD80 (B7-1) | 3H5 | PE | Mouse (BALB/c) IgG1, κ | Becton Dickinson | 555014 |
| Anti-CD86 (B7-2) | GL1 | PE | Rat IgG2a, κ | eBioscience | 12-0862-81 |
| Anti-CD102 (ICAM-2) | 3C4(mIC2/4) | FITC | Rat IgG2a, κ | Becton Dickinson | 553326 |
| Anti-CD103 (Integrin alpha E) | 2E7 | PE | Armenian Hamster IgG | eBioscience | 14-1031-81 |
| Anti-CD106 (VCAM-1) | 429 | FITC | Rat IgG2a, κ | eBioscience | 11-1061-81 |
| Anti-CD119 (IFNγ Receptor1) | 2E2 | PE | Armenian Hamster IgG | eBioscience | 12-1191-82 |
| Anti-CD152 (CTLA-4) | UC10-4B9 | PE, PE-Cy7, APC | Armenian Hamster IgG | Biolegend | 106306 |
| Anti-Mouse CD183 (CXCR3) | CXCR3-173 | PE | Armenian Hamster IgG | eBioscience | 12-1831-82 |
| Anti CD184 (CXCR4) | 2B11 | PE | Rat IgG2b, κ | eBioscience | 12-9991-81 |
| Anti CD194 (CCR4) | 2G12 | PE | Armenian Hamster IgG | Biolegend | 131203 |
| Anti CD195 (CCR5) | HM-CCR5 (7A4) | PE | Armenian Hamster IgG | eBioscience | 12-1951-81 |
| Anti CD197 (CCR7) | 4B12 | PE, PE-Cy7, APC | Rat IgG2a, κ | eBioscience | 12-1971-82 |
| Anti I-A/I-E (Class II MHC) | M5/114.15.2 | PE | Rat IgG2b, κ | Biolegend | 107602 |
| Anti-Foxp3 | FJK-16s | Alexa Fluor® 647, Alexa Fluor® 488, APC | Rat IgG2a, κ | eBioscience | 53-5773-82 |
| Anti-phospho-Akt (Ser473) | - | Purified | Rabbit IgG | Cell Signalling | 9271 |
| Anti-PCNA | PC10 | PE | Mouse IgG2a, κ | Biolegend | 307908 |
| Anti-Vβ6 | RR4-7 | PE | Rat IgG2b, κ | eBioscience | 17-5795-82 |

**Table 5 Fluorescent conjugated antibodies used for flow cytometry**

### 3.12.3 Intracellular staining

For intracellular Foxp3 staining, eBioscience Anti-Mouse/Rat Foxp3 Staining Set APC (clone FJK-16S) kit was used. Cells were resuspended ($10^7$/ml) and stained with surface antigens as mentioned above and then fixed/permeabilized for 30 minutes at 4°C using Fixation/Permeabilization working solution made from mixing 1 part of the fixation/permebilization concentrate (eBioscience, Cat# 00-5123) to 3 parts of the fixation/permebilization diluent (eBioscience, Cat# 00-5223 ). The cells were then washed twice in 1X permeabilization buffer (eBioscience, Cat# 00-8333) and stained with fluorochrome conjugated-Foxp3 antibody in 1X permeabilization buffer for 30 minutes at 4°C. A final wash with 1X permeabilization buffer was performed and the cells were then centrifuged and resuspended in 200ul of flow cytometry buffer.

For intracellular staining of phosphorylated antigens such as Akt, after surface staining, cells were fixed in ice cold 90% methanol (VWR International, Cat# 20847.307) for 10 minutes at –20°C. Cells were then washed twice in PBS. Intracellular staining was carried out after initially blocking permeabilized cells in buffer (0.5% BSA/PBS + Na3VO4) at room temperature for 30 min and incubation with a dilution of 1:100 of Phospho-Akt (Ser473 rabbit anti-mouse antibody )(Cell Signalling Technology, Cat# 9271) for 30 min at room temperature. Cells were then washed and stained with secondary APC-F(ab)2 fragment donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch, Cat# 711-116-152) at 1:100 for 30 min at room temperature. After a final wash, cells were resuspended and then analyzed using flow cytometry.

## 3.13 Western Blotting

Total cell lysates were obtained in 3M NaCl, 1M TRIS-base (pH 7.5), 1.0% Triton X-100, phosphatase inhibitor cocktail 2 (Sigma-Aldrich, Cat# P5726) and protease inhibitor (Sigma-Aldrich, Cat# P8340). 20 mg of cell lysate was subjected to SDS-PAGE, under reducing conditions. We used NuPAGE® 4-12% Bis-Tris Gel (LifeTechnologies, Cat# WG1401BOX) with the XCellSureLock™ Mini-Cell (Life Technologies, Cat# EI0001), that require 800 ml of NuPAGE® MES SDS Running Buffer (20X) (LifeTechnologies, Cat# NP0002). After electrophoresis, proteins were

transferred into a nitrocellulose filter membrane (Protan; Schleicher & Schuell BioScience) using a XCell II™ Blot Module (LifeTechnologies, Cat# EI0002) in, NuPAGE® transfer buffer (20X) (LifeTechnologies, Cat# NP0006), with NuPAGE® Antioxidant (LifeTechnologies , Cat# NP0005) and 10% methanol. Membranes were placed in 5% nonfat milk in PBS plus 0.05% Tween 20 (PBST) (Cell signalling Technology, Cat# 9809) at 25°C for 1 hours to block the nonspecific binding sites. Filters were incubated overnight with specific antibodies, before being washed three times in PBST and then incubated with a peroxidase-conjugated secondary antibody (GE Healthcare). After washing with PBST, peroxidase activity was detected with the Lumi-light western blotting substrate (Roche, Cat#12015200001) or with the Supersignal West Femto (Thermo Scientific, Cat#34095). All antibodies were used at 1:1000 in PBST 5% BSA. The filters were also probed with an Actin and ERK 1/2 antibody (Santa Cruz Biotechnology Inc.), 1:1000 in PBST 5% non-fat milk, to normalize the amount of loaded protein. All filters were quantified by densitometric analysis of the bands, using the program ImageJ software.

## 3.14 Statistical Analyses

An unpaired student's t-Test test was used do assess significance of all experiments presented. All reported P values are 2-sided. P values of less than 0.05 were considered significant.

Experimental data sets from the seahorse analyser were analysed using a one-way ANOVA using Bonferroni correction or Kruskal-Wallis with Dunn's post-test to take into account of multiple comparisons. Results with $p < 0.05$ were considered statistically significant. Where indicated 'n' represent the number of replicates in *in vitro* experiments or number of animals in *in vivo* experiments. N signifies the number of experiments.

# Chapter 4 Results

# 4.1 Part 1: Self-recognition of endothelium enables Treg trafficking

## 4.1.1 Introduction

CD4+ CD25+ Foxp3+ Tregs constitute a small fraction of the total T cell repertoire. Despite this numerical disadvantage, these Tregs form a crucial component of the immune system that maintains tolerance within the peripheral tissues. Tregs can exert their immunomodulatory roles in both lymphoid and non-lymphoid tissues. During the initial stages of an immune response, effective migration and localisation of Tregs within the lymph nodes is vital for the regulation of T cell clonal expansion and differentiation (Ding et al. 2012). However, once an effector immune response becomes established, regulation of local inflammation in the peripheral target tissues becomes crucial (Nguyen et al. 2007; Rudensky and Campbell 2006).

The importance of Treg co-localisation with Teffs to the periphery has been highlighted in several studies of autoimmunity. For example, in Foxp3 deficient scurfy mice, widespread autoimmunity can be circumvented by the adoptive transfer of Foxp3+ Tregs. Interestingly, restoration of Treg compartment with a-1, 3-fucosyltransferase VII-deficient Tregs that are incapable of migrating to skin results in the prevention of autoimmune disease in all tissues but the skin (Dudda et al. 2008). Similarly, loss of CCR4 expression in Tregs disables their recruitment to the skin and lung resulting in skin and lung inflammatory diseases (Sather et al. 2007).

During an effector immune response in the periphery, the establishment of an optimal Treg:Teff ratio is essential for immune regulation (Tang et al. 2012). Thus rapid recruitment of Tregs relative to Teffs is required to control tissue damage emphasizing the need for efficient recruitment (Fan et al. 2010). Given that Tregs exhibit reduced proliferative potential (comparatively to $T_{naive}$ and $T_{cm}$) and have extremely low proportions in the peripheral blood, this suggests the existence of mechanisms that favour their convergence and localisation to the inflammatory sites.

Similar to Teffs, following activation, Tregs also acquire distinct chemokine receptors and adhesion molecules enabling their recruitment and retention to sites where regulation is required (Bach 2003; Luster 2002; Wood and Sakaguchi 2003). For example, activated Tregs express $\alpha_E$ (CD103) $\beta_{7,}$ the ligand for E cadherin, which enables their efficient localisation into inflamed tissue to suppress the effector phase of the immune response (Huehn et al. 2004; Lehmann et al. 2002). There is evidence that suggest the existence of Treg subsets which preferentially localise to distinct inflammatory environments. For instance, CXCR3 expressing Tregs are capable of migrating and regulating Th1 cell induced inflammatory responses within the liver, and CNS in EAE (Muller et al. 2007). CCR6+ Tregs accumulate in Th17 cell induced inflammatory sites that also include the CNS in EAE models (Yamazaki et al. 2008). However, the redundancy in the molecular interactions mediating Treg recruitment to several distinct tissue sites suggest that further mechanisms must be in place which enhance Treg recirculation during inflammation.

Furthermore, it is unclear how Tregs are able to effectively and rapidly access, as well as accumulate to, non-lymphoid tissue relative to Teffs. Given that Treg mediated suppression needs to be coordinated with a sufficient delay to allow the effector response to ensue, but prior to the occurrence of tissue damage, mechanisms must exist to ensure these timely events.

As we have previously mentioned, the endothelial cells lining microvascular vessels are capable of capturing, processing and displaying antigens on MHC molecules in response to inflammatory stimuli (Marelli-Berg and Jarmin 2004b). This allows T cells bearing specific TCRs for those antigens to be recruited, enabling antigen-specific T cell recruitment into tissues. There is evidence that Tregs also traffic in response to antigen recognition. Recently, a study has reported that Tregs recruit and compartmentalize in a site-specific manner in the lymph nodes over time, an effect that may be regulated by antigen recognition as indicated by the enrichment of recently activated CD69-expressing Tregs in the lymph nodes after adoptive transfer (Lieberman et al. 2012). In addition, mice expressing an OX40-cre allele that is prominently expressed within Tregs cells, and a conditional null allele for the gene encoding p56Lck (thus, impaired TCR signalling) exhibit preferential redistribution of Tregs to lymph nodes rather than to non-lymphoid tissue associated with abnormal Treg cell homeostasis (Kim et al. 2009).

Assuming the widely accepted theory suggesting that Tregs recognize self-derived peptides, it is possible that self-peptide presentation by the endothelium may drive the rapid and efficient recruitment of Tregs to inflamed tissue thus reaching the functional numbers necessary for regulation. Furthermore, the exact mechanism through which an optimal Treg:Teff ratio becomes established thus preventing tissue destruction remains to be identified.

### 4.1.2 Aims

Given that Tregs express TCRs with affinities for self-derived antigens and that TCR-driven antigen recognition is crucial for recruitment of antigen specific T cells to the periphery, we hypothesize that recognition of self-derived peptides displayed by the endothelium may be an essential component for the efficient recruitment of Tregs towards non-lymphoid inflammatory tissue. Furthermore, Class II MHC upregulation and peptide display by the endothelium in response to localised inflammatory stimuli, such as IFN- γ, could serve as a strategic element for Treg recruitment. To test this hypothesis we designed the following experimental strategies.

1. Adopt and optimise existing methods for *in vitro* expansion of Tregs of known antigen specificity in numbers sufficient for *in vivo* trafficking studies.
2. Assess the role of cognate recognition of self-derived peptides displayed by the endothelium in Treg recruitment.
3. Assess the role of IFN- γ -induced Class II MHC upregulation in Treg recruitment.
4. Elucidate the mechanism through which optimal Treg:Teff ratios necessary for regulation are established in peripheral immune responses.

## 4.1.3 Results

### 4.1.3.1 Expansion of Foxp3 expressing regulatory T cells.

Tregs constitute a minute fraction of the total T cell repertoire. In normal wild type mice approximately 4-12% of their CD4+ T cell compartment constitutes Foxp3+ cells (L. Zhao et al. 2007), while in humans this ranges from 4-9% (Baecher-Allan et al. 2001). Although this highlights the remarkable efficiency with which the Tregs regulate the immune responses, it poses challenges for the study of the migratory and homing characteristics of these cells *in vivo*. Therefore, we optimized an existing Treg culture protocol that enabled us to expand allospecific Treg populations *in vitro*.

Briefly, purified regulatory T cells were first isolated from the lymph nodes and spleens of C57BL/6 mice using commercially available magnetic cell sorting kits. These kits utilize negative selection of CD4+ cells followed by positive selection of CD25+ cells. CD25 a receptor for IL-2, a pro survival and proliferation cytokine, is expressed constitutively on regulatory T cells in mice. However, conventional naïve T cells are also known to upregulate the CD25 receptor following activation. Therefore in order to obtain pure regulatory T cell populations we utilised the Foxp3-GFP reporter mice (C57BL/6 background). Foxp3 is a nuclear transcription factor crucial for Treg function and serves as a key marker for Treg identification in mice. From these mice we were able to sort GFP positive Foxp3 expressing CD4 T cells using flow cytometry. Once isolated, the CD4+CD25+ Foxp3+ Treg populations were stimulated with immature bone marrow derived dendritic cells in the presence of IL-2. While splenic antigen presenting cells have been shown to induce low Treg proliferation, immature bone marrow derived dendritic cells are excellent promoters of Treg proliferation (Marguti et al. 2009; Zhou et al. 2013). Using a ratio of 1:5 (DC:Treg), the Tregs from a H2-B (C57BL/6) background were re-stimulated every 7 days using bone marrow derived dendritic cells (bmDCs) from H2-D (BALB/) mice. The proliferation observed was comparable to that of Teff populations during the first 3 days of co-culture. In this manner, only those Tregs which recognised allo-antigens receive proliferative signals thus skewing the TCR reactivity of this polyclonal population towards allo-antigens overtime. These expanded Tregs were seen to inhibit the proliferation of allogeneic responder T cells in an *in vitro* mixed lymphocytic reaction (Figure 11).

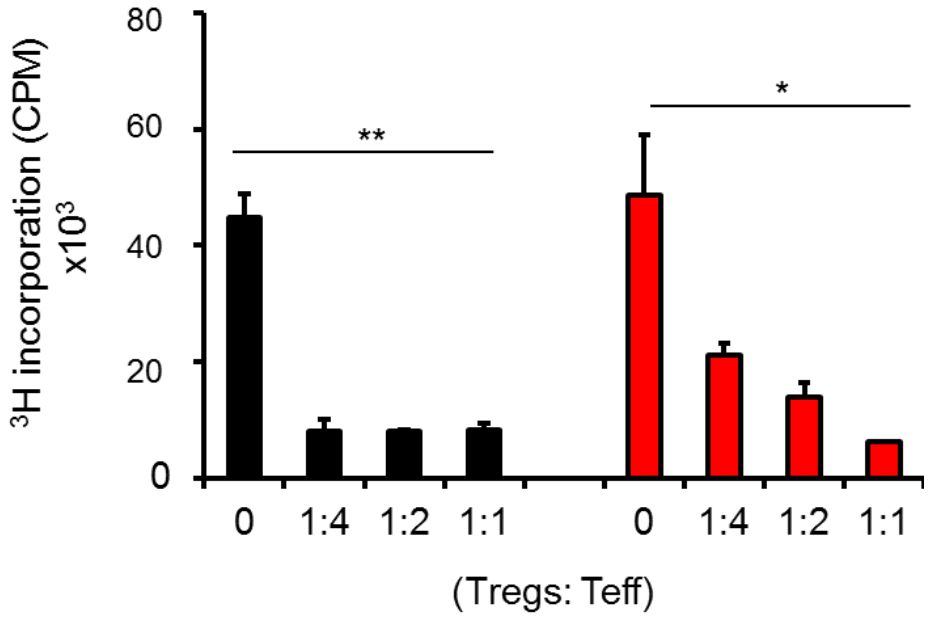



**Figure 11 Allospecific Tregs (H2-B) expanded by *in vitro* culture with BALB/c-derived, (H2-D) immature DCs and IL-2 can suppress allogeneic T cell proliferation.**

*This panel shows increasing numbers of allospecific Tregs added to co-cultures of C57BL/6 conventional naïve T cells ($10^5$) stimulated with H2-D (BALB/c) -derived DCs ($10^3$, black bars), or CD3/CD28 activating antibodies (red bars). T-cell proliferation was measured as $^3$HTdR incorporation in triplicate cultures (N=5). Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. *P<0.05, **P<0.01.*

Flow cytometric analysis showed that the Tregs upregulated expression of surface markers indicative of memory, such as chemokine receptor CCR4 and alpha E integrin chain (CD103) (Figure 12). The loss of L-Selectin (CD62L) over extended periods of *in vitro* culture was also observed, consistent with observations from other groups.

Using this technique, we were successful in the generation of allospecific Treg and Teff populations from wild type and Foxp3-GFP reporter mice.

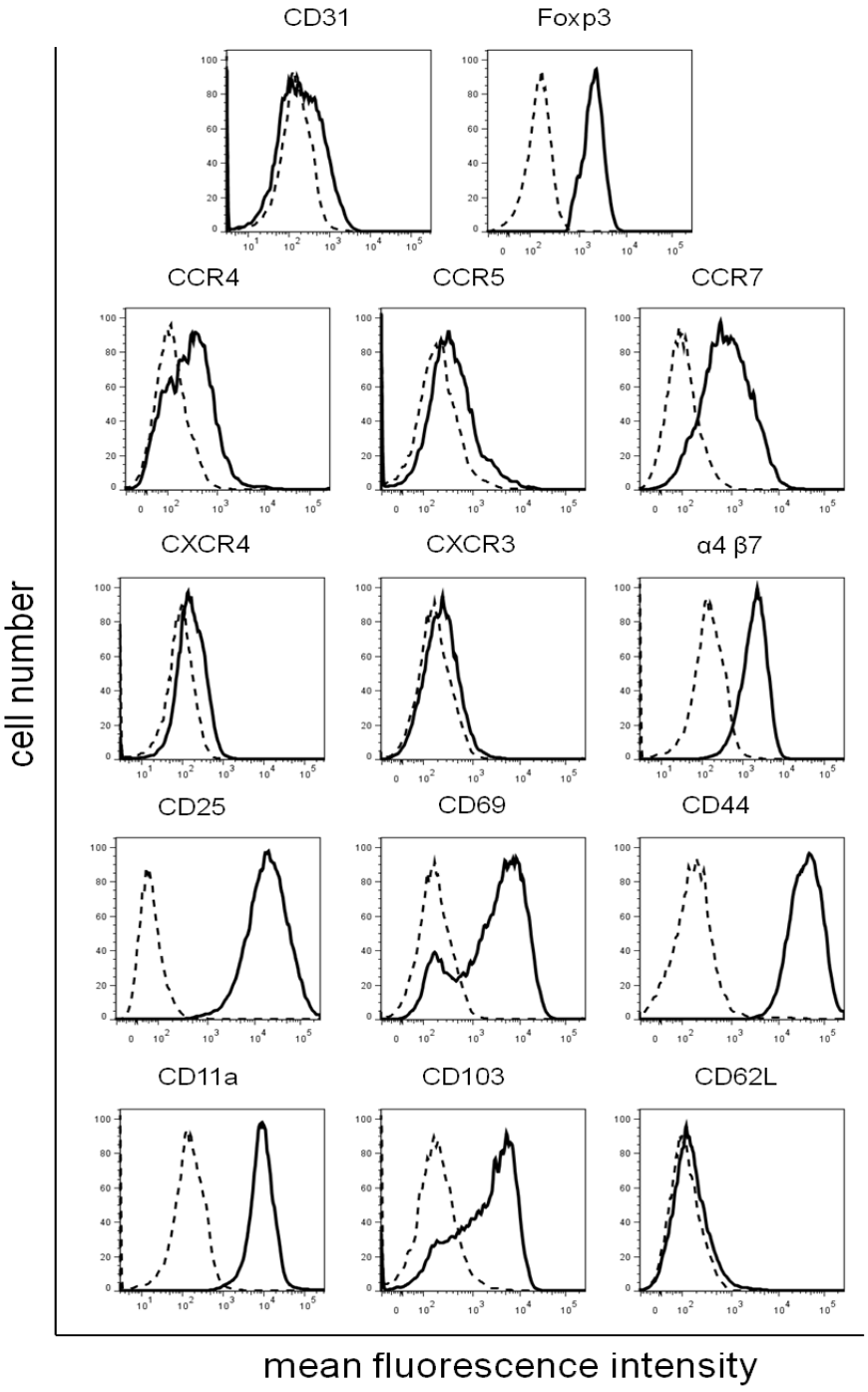



**Figure 12 Allospecific Tregs (H2-D) expanded by *in vitro* culture with BALB/c-derived, (H2-D) immature DCs and IL-2 express surface markers indicative of memory**

*Panel **a**. indicates surface staining of chemokine receptor and adhesion molecules on the in vitro expanded allospecific Tregs as analysed through flow cytometry. The histograms are representative of 3 experiments (N=3). The dotted lines in the histograms represent staining using antibody isotype controls.*

### 4.1.3.2 Self-Recognition is required for Treg Trafficking

It has been well established that T cells selectively recruit to inflamed non-lymphoid tissue through recognition of specific antigens that are displayed by the endothelial cells lining the nearby vessels (Marelli-Berg et al. 1999b) (Marelli-Berg et al. 2004b). Local inflammatory stimuli induce these endothelial cells to capture process and display antigens on MHC molecules. Given that regulatory T cells have antigen specificity towards self-derived peptides, we hypothesised that Tregs would respond to self-antigen presentation by the endothelium, which would lead to efficient Tregs migration *in vivo*. In order to assess Tregs recruitment *in vivo*, we utilized an established murine model of Treg recruitment to antigen-enriched peritoneum. In this model, a low dose of IFN- γ given via the intra-peritoneal (i.p.) route induces local upregulation of MHC molecules. We first compared the recruitment of Tregs from the circulation into the peritoneum of syngeneic (C57BL/6, H2-B) and irrelevant (CBA/Ca, H2-K) mice. CD4+ T cells isolated from Foxp3-GFP reporter mice ($10^7$/mouse) were pooled (to ensure equal numbers of Treg populations in the cell preparation) and injected intravenously (i.v.) in IFN-γ-treated or untreated recipients. Significantly larger number of Tregs (GFP+) migrated to the peritoneal cavity of syngeneic recipients previously exposed to IFN-γ, compared with those detected in the lavage of IFN-γ-treated irrelevant (CBA/Ca, H2-K) mice and non-treated syngeneic recipients after 16 hours (Figure 13).

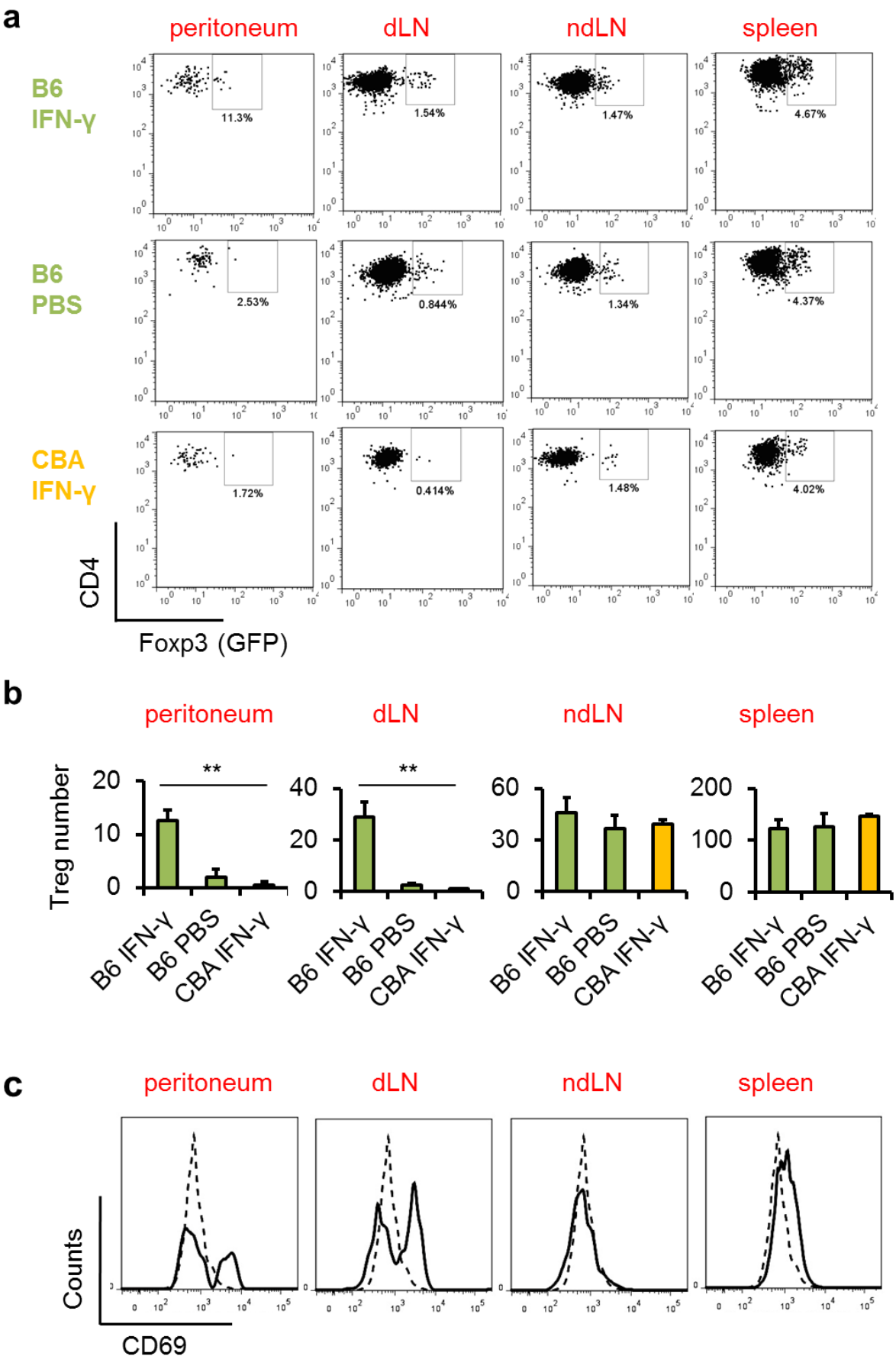

a
peritoneum
dLN
ndLN
spleen
B6
IFN-γ
11.3%
1.54%
1.47%
4.67%
B6
PBS
2.53%
0.844%
1.34%
4.37%
CBA
IFN-γ
1.72%
0.414%
1.48%
4.02%
CD4
Foxp3 (GFP)
b
peritoneum
dLN
ndLN
spleen
Treg number
**
**
B6 IFN-γ
B6 PBS
CBA IFN-γ
c
peritoneum
dLN
ndLN
spleen
Counts
CD69

**Figure 13 Antigen recognition facilitates Treg trafficking.**

*Total CD4+ T cells from Foxp3-GFP reporter mice ($10^7$/mouse) were injected intravenously into syngeneic H2-B (C57BL/6) or irrelevant H2-K (CBA/Ca) mice that had received an intraperitoneal injection of 600 U IFN-γ 72 h earlier. Some C57BL/6 recipient received saline solution alone. The presence of GFP+ Tregs in the peritoneal lavage, dLN and spleen was analysed by flow cytometry 16 h later. Tregs were identified by gating on the CD4+GFP+ population. Representative dot plots are shown in panel **a**. The mean number of Treg cells (in the total CD4+ population) of Treg cells detected in the peritoneal cavity and lymphoid organs are shown in panel **b**. Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. (n=3, N=4) **P<0.01. (c) GFP+ Tregs retrieved in the same sites from IFN-γ-treated syngeneic C57BL/6 recipients were stained with anti-CD69-PE/Cy7 (clone H1.2F3). CD69 expression was analysed by flow cytometry, shown in panel **c**. The dotted line shows non-specific staining with an Isotype-matched control antibody. The histograms are representative of three cell preparations.*

Interestingly, 50% of Tregs recruited to the peritoneal cavity and draining lymph nodes exhibited high expression of CD69 indicative of recent T cell receptor engagement in this population. In addition, Tregs were preferentially recruited to the draining lymph nodes (dLNs, mesenteric) of IFN-γ-treated syngeneic, but not irrelevant mice, suggesting that antigen presentation also affects their localization to secondary lymphoid tissue.

To further confirm that antigen recognition drives Treg recruitment we tested the possibility that, within a polyclonal Treg population, Tregs are selectively recruited based on their TCR specificity. To this aim, we enriched GFP-tagged Tregs cross-reactive for H2-D alloantigens by *in vitro* expansion using immature BALB/c derived bone marrow derived dendritic cells and IL-2 as described earlier. Next, we carried out *in vitro* based lymphocyte migration assays to observe transendothelial migration of these *in vitro* expanded Tregs through IFN- γ treated endothelial cell monolayers of allogeneic (BALB/c, H2-D), syngeneic (C57BL/6, H2-B) and irrelevant (CBA/Ca, H2-K) origin. Interestingly, migration of H2-D allospecific Tregs through allogeneic endothelium was greater than through endothelium of syngeneic and irrelevant origin (Figure 14).

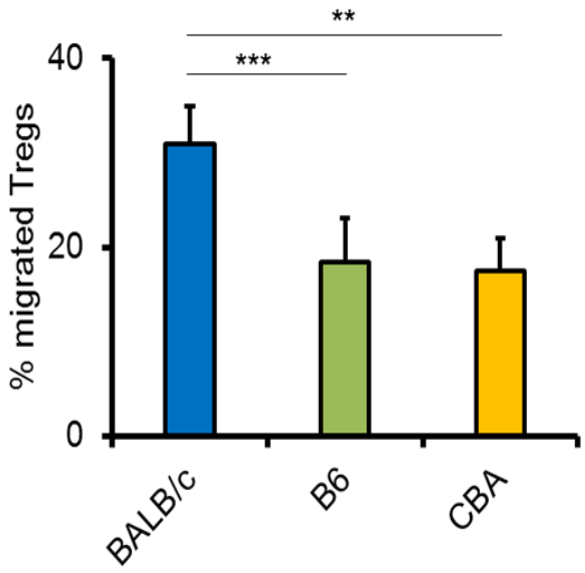



**Figure 14 Allospecific Tregs migrate more efficiently through allogeneic endothelium *in vitro*.**

*H2-D-allospecific Tregs expanded via in vitro culture were then placed over 48hr IFN-γ-treated syngeneic H2-B (C57BL/6), allogeneic H2-D (BALB/c) or irrelevant H2-K (CBA/Ca) endothelial cell monolayers in a trans-endothelial transwell migration assay. Following 24Hr, the number of migrated cells in the bottom of the transwell was evaluated by hemocytometric counting. Results were expressed as average percentage of migrated cells Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. (n=3, N=4) *P<0.05*

Following these *in vitro* experiments, we observed trafficking of these expanded cross-reactive Tregs into the peritoneum of allogeneic (BALB/c, H2-D), syngeneic (C57BL/6, H2-B) and irrelevant (CBA/Ca, H2-K) recipient mice using the peritoneal model of T cell recruitment described above. Similar to the *in vitro* experiments, the migration of these Tregs was significantly higher to the peritoneal cavity and draining mesenteric lymph nodes in IFN- γ treated alloantigen-expressing (BALB/c, H2-K) recipients (Figure 15).

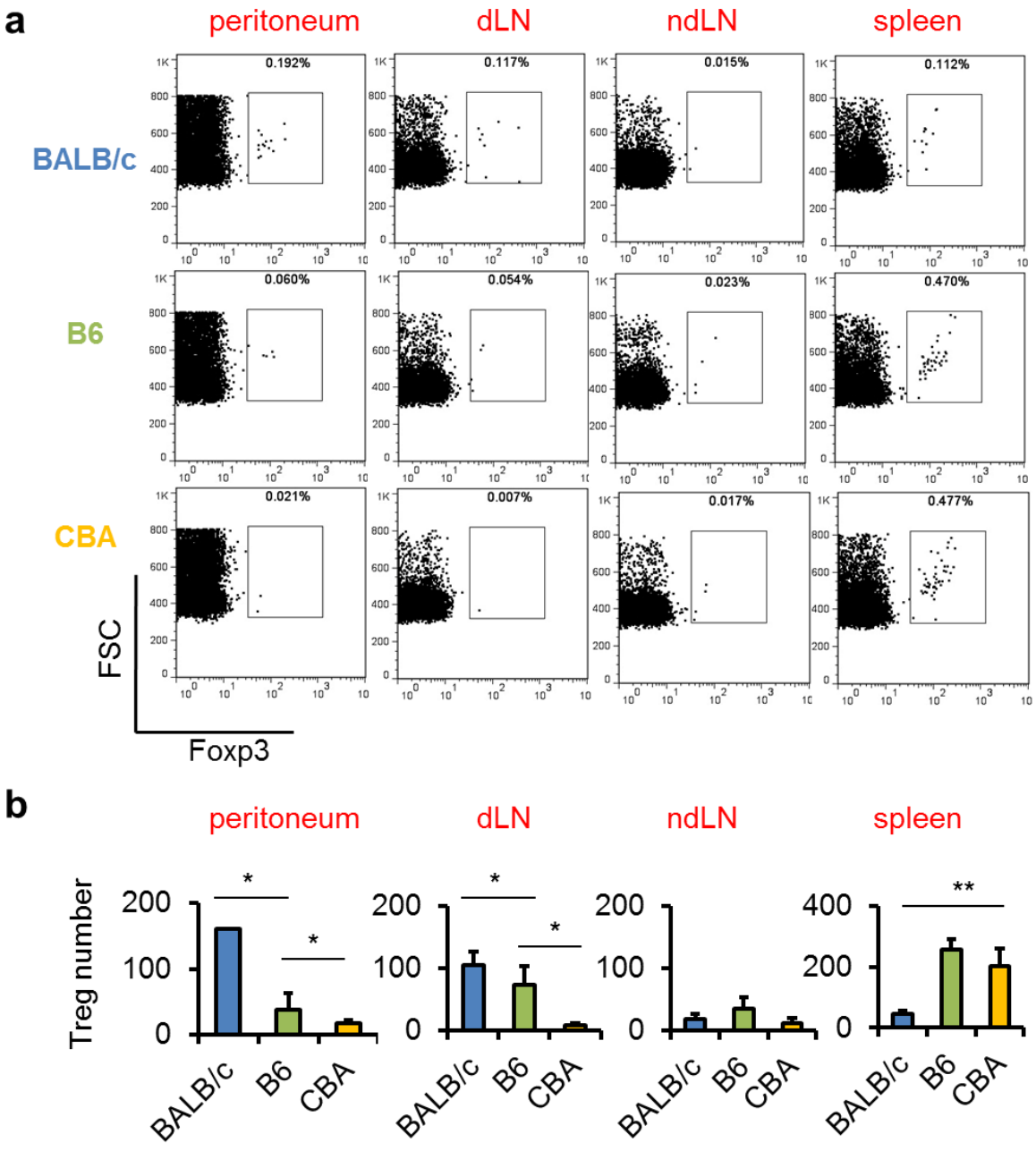



**Figure 15 Allospecific Tregs migrate more efficiently to the peritoneal cavity of allogeneic mice.**

*CFSE-labelled H2-D-allospecific Treg cells were injected intravenously ($10^7$/mouse) into allogeneic H2-D (BALB/c), syngeneic H2-B (C57BL/6) or irrelevant H2-K (CBA/Ca) mice that had received an intraperitoneal injection of 600 U IFN-γ 72 h earlier. The presence of GFP+ Tregs in lavage, mesenteric dLNs, non-dLNs and spleen was analysed by flow cytometry 16 h later. Representative dot plots are shown in panel **a**. The mean number of Treg cells (in the total CD4+ population) detected in the peritoneal cavity and lymphoid organs is shown in panel **b**. Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. (n=3, N=4) *P<0.05, **P<0.01.*

Having established that antigen recognition plays a role in Treg migration to inflamed non-lymphoid tissue, we investigated whether recognition of endogenous peptides (putative 'self-antigens') in the context of self-MHC Class II molecules is instrumental in efficient Treg localisation to target tissue. To address this, the GFP –Foxp3 reporter mice were crossed with a TCR-transgenic mouse strain bearing TCR specificity for the HY-derived peptide in the context of H2-Ab (Marilyn mice). The male minor histocompatibility antigen HY encoded by the Y chromosome, is expressed by all male cells. Subsequently, HY-specific T cells are removed in the thymus by negative selection in males but are present in females. In the F1 Marilyn GFP-Foxp3 mice, the mean percentage of CD4+ Vβ6+ Marilyn T cells as a proportion of total lymphocytes was 12.2% (±2.5%). In the same mice, the mean percentage of naturally occurring Tregs (GFP+) as a proportion of Marilyn T cells was 3.5% (±1.4%). CD4+ T cells purified from the female F1 Marilyn GFP-Foxp3 mice were purified and pooled and injected into IFN- γ treated male or female syngeneic (C57BL/6) recipients. These recipient mice were genetically identical to the donor mice with the exception of the HY antigen, which is only expressed in male animals. Foxp3+ Vβ6+ T cells were seen to be higher in the peritoneal cavity and dLNs of IFN-γ treated male mice than in that of female mice treated with IFN-γ (Figure 16). These data suggest that recognition of self-peptides in the context of self-MHC Class II molecules can drive the recruitment of Tregs to non-lymphoid tissue.

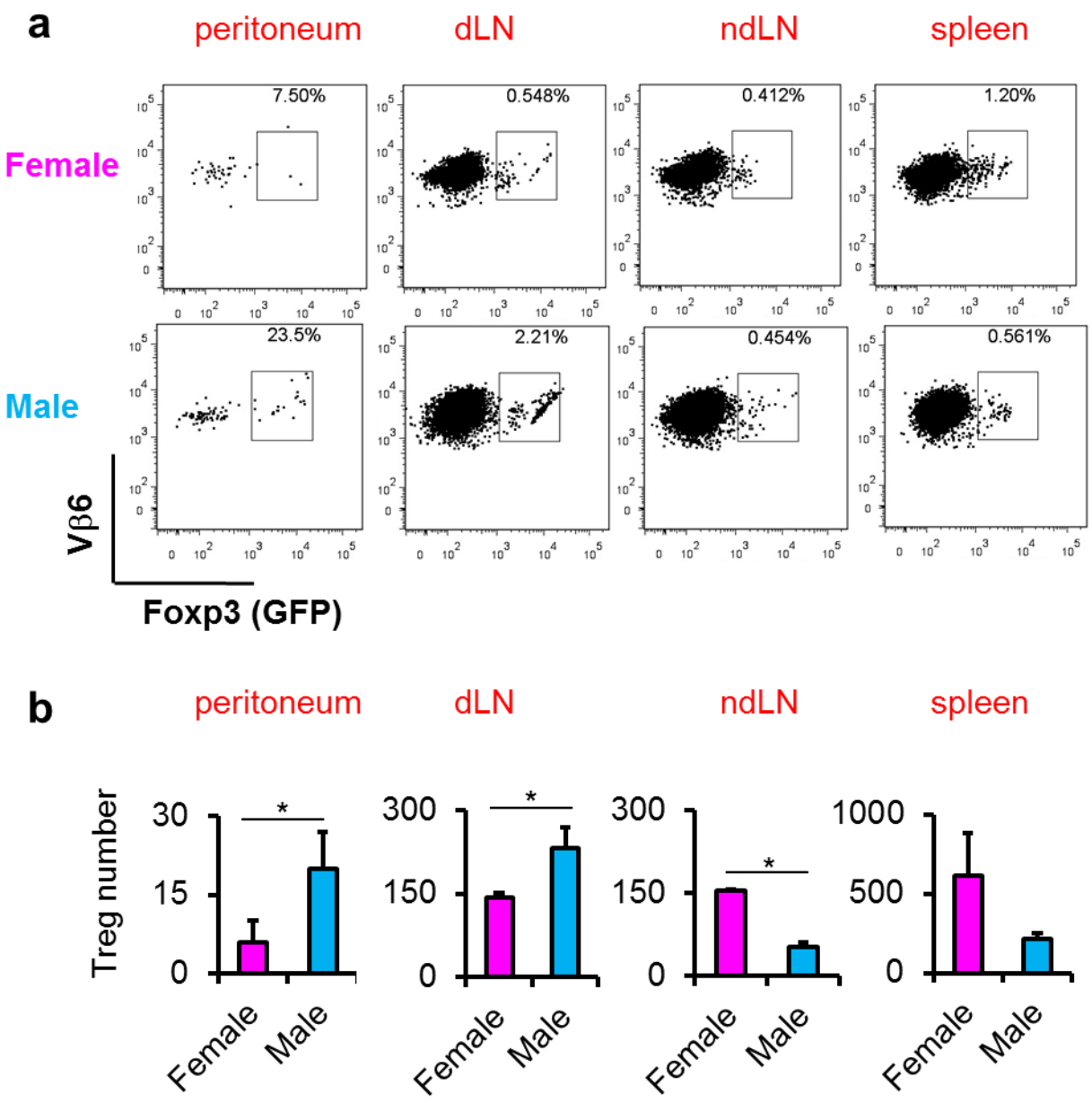



**Figure 16 Self-recognition drives Treg recruitment**

*Total CD4+ T cells from GFP-Foxp3 Marilyn F1 mice ($10^7$/mouse) were injected intravenously into syngeneic male or female C57BL/6 mice that had received an intraperitoneal injection of 600 U IFN-γ 72 h earlier. An anti-Vβ6 antibody was used to identify Marilyn T cells. The presence of GFP+ Vβ6+ Tregs in lavage, dLN and spleen was analysed by flow cytometry 16 h later. Tregs were identified by gating on the CD4+ T-cell population. Representative dot plots are shown in panel a. The mean number of Treg cells (in the total CD4+ population) of Treg cells detected in the peritoneal cavity and lymphoid organs is shown in panel b. Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. (n=3, N=2) *P<0.05.*

#### 4.1.3.3 IFN-γ induction of MHC Class II expression by the endothelium is required for Treg recruitment.

For T cells to efficiently localise to antigen specific sites within the non-lymphoid tissue, endothelial cells lining the microvasculature react to inflammatory stimuli by presenting processed antigens bound to Class II MHC molecules that are displayed for specific T cell recruitment (Marelli-Berg and Jarmin 2004b). To further evaluate the requirement for MHC Class II expression by the endothelium in the recruitment of Tregs, we performed *in vitro* trans-endothelial migration assays to observe the migration of Tregs from Foxp3-GFP reporter through IFN-γ pre-treated endothelium, where Class II MHC was blocked using an antibody. Treg migration through activated endothelium treated with the Class II MHC blocking antibody prior to the migration assay was diminished compared to the activated endothelium treated with an isotype control antibody (Figure 17).

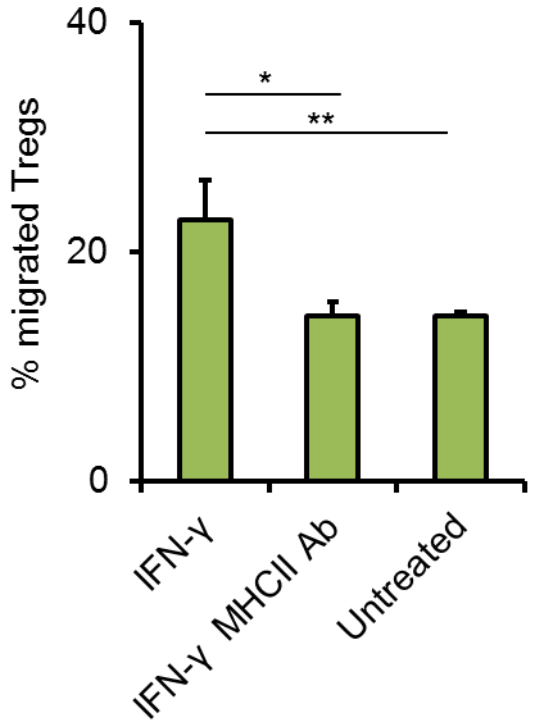



**Figure 17 Antibody mediated blockade of class II MHC inhibits Treg trans-endothelial migration *in vitro*.**

*H2-D-allospecific Tregs expanded via in vitro culture were placed over 48hr IFN-γ -treated syngeneic H2-B (C57BL/6) endothelial cell monolayers in a trans-endothelial transwell-based migration assay. In parallel, H2-D-allospecific Tregs migrated through syngeneic H2-B (C57BL/6) endothelial cell monolayers previously treated with 500 µg anti-MHC class II antibody (clone M5/114, BioXCell). Following 24Hr, the number of migrated cells in the bottom of the transwell was evaluated by hemocytometric counting. Results were expressed as average percentage of migrated cells. Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. (n=3, N=4) *P<0.05, **P<0.01.*

Similarly, to observe this effect in *vivo*, we carried out experiments where freshly isolated Tregs from Foxp3-GFP reporter mice were injected into IFN-γ pre-treated (i.p.) mice that had either been given an intra-peritoneal injection of a Class II blocking antibody or its corresponding isotype control. As expected, the recruitment of GFP+ Tregs into the peritoneum and draining lymph nodes of mice that had received the Class II blocking antibody was significantly reduced as compared to those that had received the isotype control (Figure 18).

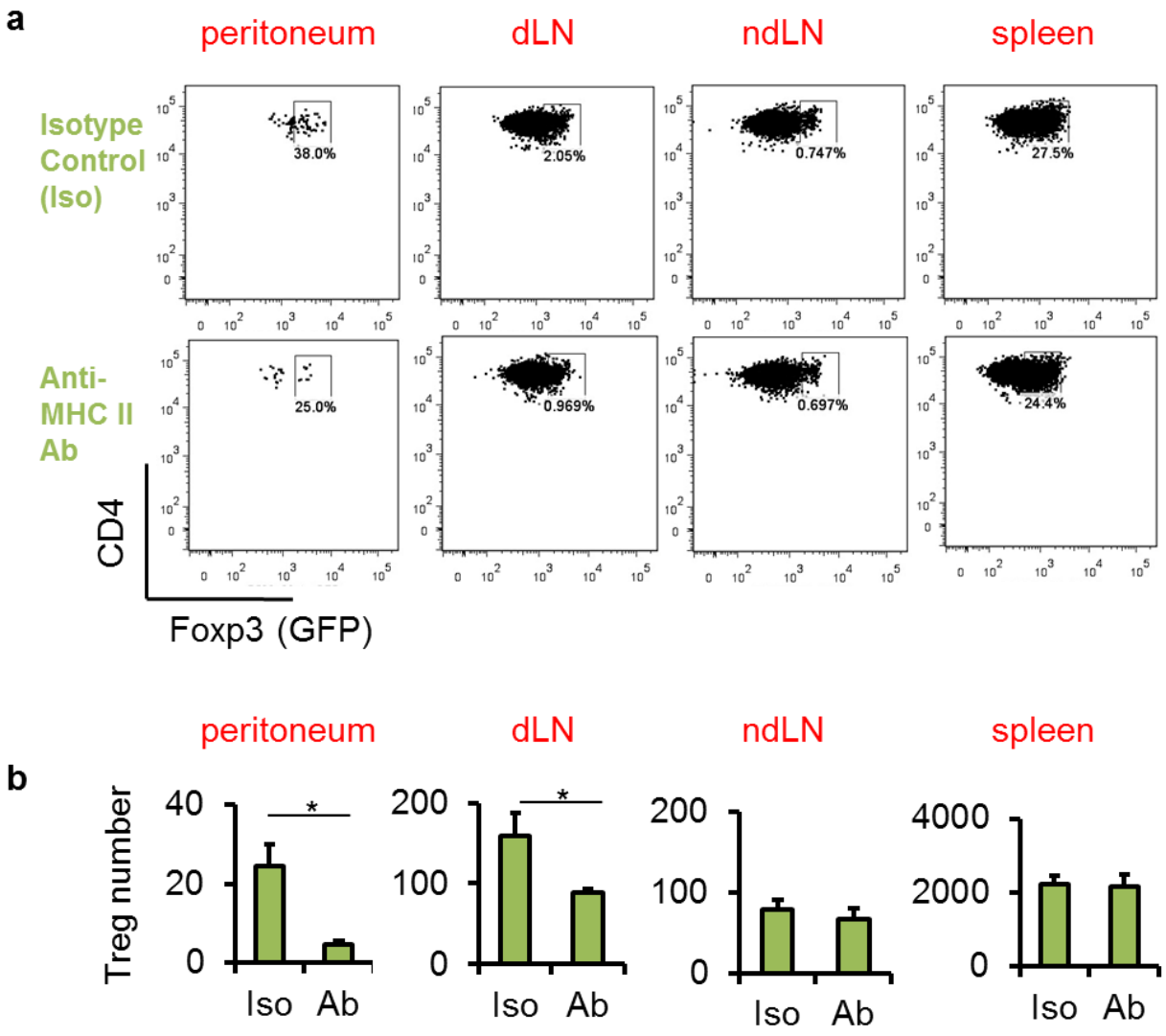



**Figure 18 Treg recruitment requires MHC class II molecule expression by target tissue**

*Total CD4+ T cells isolated from Foxp3-GFP reporter mice were injected intravenously into syngeneic C57BL/6 mice ($10^7$/mouse) that had received an intraperitoneal injection of 600 U IFN-γ 72 h earlier. Prior to Treg adoptive transfer, recipient mice received either 500 µg anti-MHC class II antibody (clone M5/114, BioXCell) or isotype control antibody. The presence of GFP+ Tregs in the peritoneal lavage, dLN, ndLN and spleen was analysed by flow cytometry 16 h later. Tregs were identified by gating on the CD4+GFP+ population. Representative dot plots are shown in panel **a**. The mean number of Treg cells (in the total CD4+ population) detected in the peritoneal cavity and lymphoid organs is shown in panel **b**. Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. (n=3, N=3) *P<0.05.*

In addition to inducing MHC Class II molecule expression by the endothelial cells, IFN-γ can also induce upregulation of adhesion markers such as VCAM-1 and ICAM-1 which can also affect the recruitment of T cells (Figure 19)(Paleolog et al. 1992).

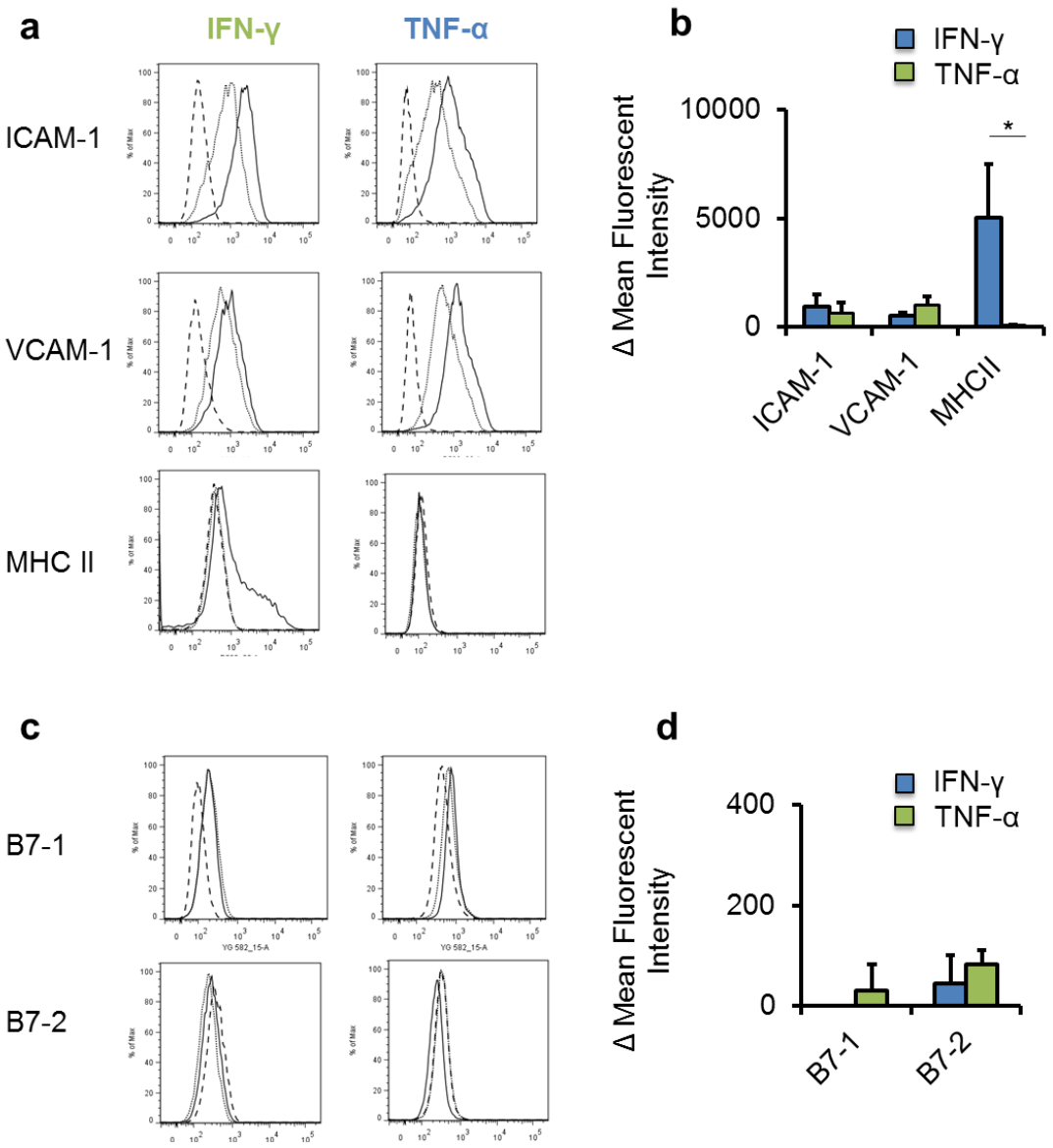
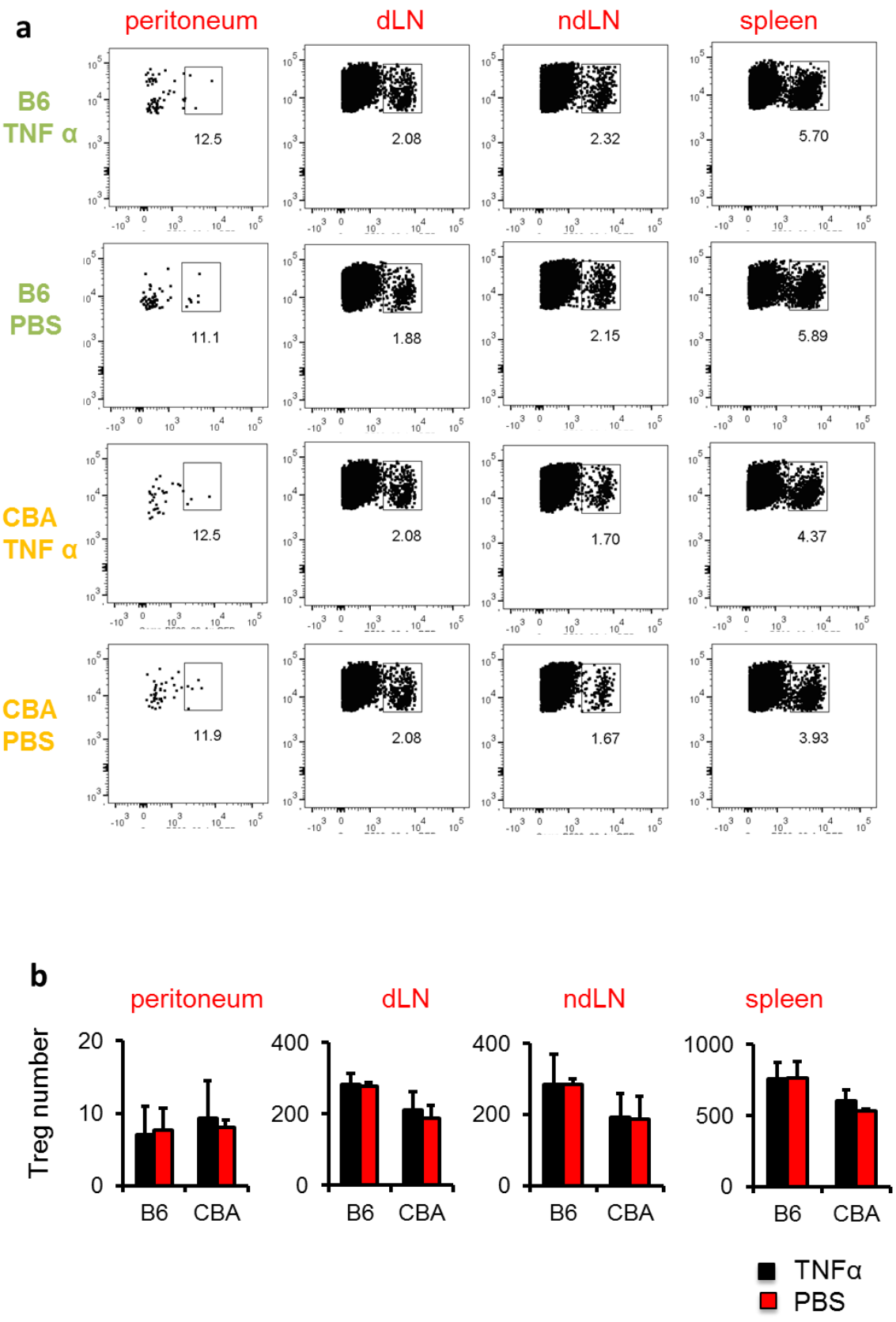



**Figure 19 Expression of adhesion and co-stimulatory molecules by the endothelium.**

*Murine primary microvascular endothelial cells (ECs) were stimulated with either IFN-γ (600U/ml for 48 hours) or TNFα (10ng/ml for 48 hours) or cultured in medium alone. In panel **a**. histograms indicate the expression of VCAM-1, ICAM-1 and MHC class II molecules by endothelial cells measured by flow cytometry. Dotted lines indicate untreated ECs whereas dashed lines indicate isotype antibody controls. Solid line histograms indicate cytokine-treated ECs. Panel **c** shows the expression of the co-stimulatory molecules CD80 (B7-1) and CD86.In panel **b** and **d**, the average delta mean fluorescence intensity (ΔMFI = MFI of stimulated ECs – MFI of unstimulated ECs) measured in triplicate samples from 2 experiments of identical design is shown.*

To address the contribution of these molecules in the recruitment of Tregs in our model we repeated the above experiments using TNF- α treatment of ECs instead of IFN-γ. Previously, it has been shown that TNF-α treatment is unable to activate the promoter of CIITA that is necessary for IFN-γ induced MHC upregulation (Nikcevich et al. 1999). Furthermore, TNF-α treatment of endothelial cells showed marked upregulation of adhesion molecules such as VCAM-1 and ICAM-1 with no induction of Class II MHC on the murine ECs (Figure 19). Thus, to address the contribution of these molecules in the recruitment of Tregs in our model, experiments were carried out where recruitment of GFP tagged Tregs in the peritoneum of syngeneic mice pre-treated with 20ng TNF-α were analysed. As shown in Figure 20, pre-treatment of mice with TNF-α did not increase migration of Tregs, while the recruitment of endogenous monocytes was increased in the peritoneum and draining lymph node. These data further support the non-redundant role of IFN-γ in sustaining Treg trafficking to inflammatory sites.


a
peritoneum
dLN
ndLN
spleen
B6 TNF α
12.5
2.08
2.32
5.70
B6 PBS
11.1
1.88
2.15
5.89
CBA TNF α
12.5
2.08
1.70
4.37
CBA PBS
11.9
2.08
1.67
3.93
b
peritoneum
dLN
ndLN
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20
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B6
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TNFα
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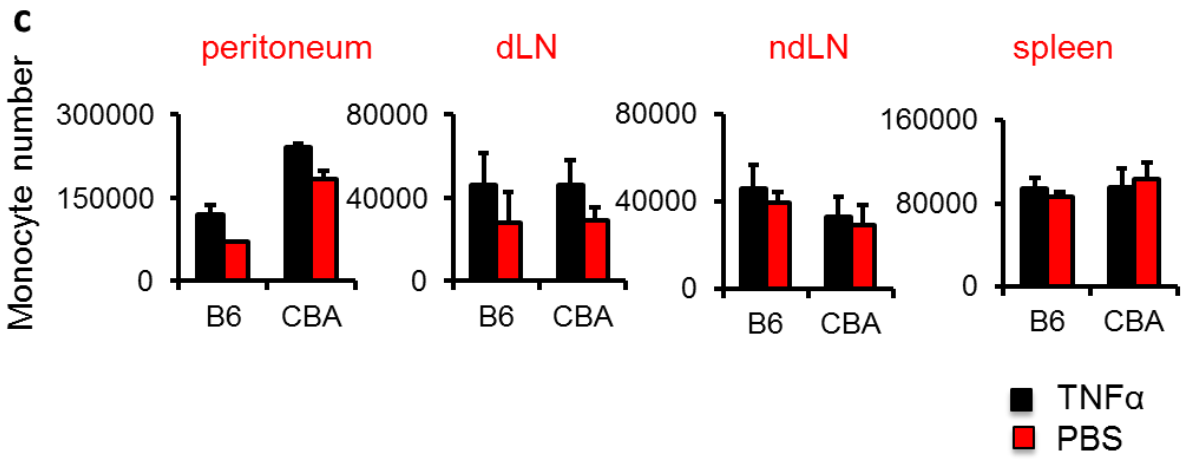



**Figure 20 TNF$\alpha$ does not enhance Treg recruitment to target tissue.**

*Total CD4⁺ T cells from Foxp3-GFP reporter mice ($10^7$/mouse) were injected intravenously into syngeneic C57BL/6 or irrelevant CBA/Ca mice that had received an intraperitoneal injection of 20 ng TNF-α(Peprotech) or control saline 48 h earlier. The presence of GFP⁺ Tregs in lavage, dLN, ndLN and spleen was analysed by flow cytometry. Representative dot plots are shown in panel **a**. The mean number of Treg cells (in the total CD4⁺ population) present in the peritoneal lavage and lymphoid organs is shown in panel **b**. The recruitment of endogenous monocytes in lavage, dLN, ndLN and spleen was analysed by CD68 staining. The mean number of monocytes detected in the peritoneal cavity and lymphoid organs of at least three recipients is shown in panel **c**. Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. (n=3, N=4) *P<0.05.*

Treg function has been shown to be dependent on IFN-γ signalling. Furthermore, Tregs themselves are also known to produce low levels of IFN-γ (Koch et al. 2009). Therefore, we sought to investigate whether the requirement for IFN-γ is in part due to its ability to facilitate Treg localisation to target tissue by enhancing antigen presentation. To distinguish between the contribution of IFN-γ by either target tissue or Treg cells, we injected purified CD4+ T cells from either Interferon gamma receptor knockout (IFN-γRKO, labelled with PKH26) into syngeneic wild type mice or purified CD4+ T cells from Foxp3-GFP reporter mice into IFN-γRKO syngeneic recipients, in both cases following IP treatment with IFN-γ.(Figure 21) Recruitment of Tregs from IFN-γRKO mice to the peritoneum and draining lymph nodes was similar to that of Tregs from WT mice. In contrast, recruitment of WT Tregs into the peritoneum and dLN of IFN-γRKO mice was significantly reduced suggesting that IFN-γ acts through the endothelium via MHC Class II upregulation and self-antigen presentation in sustaining efficient recruitment of Tregs.

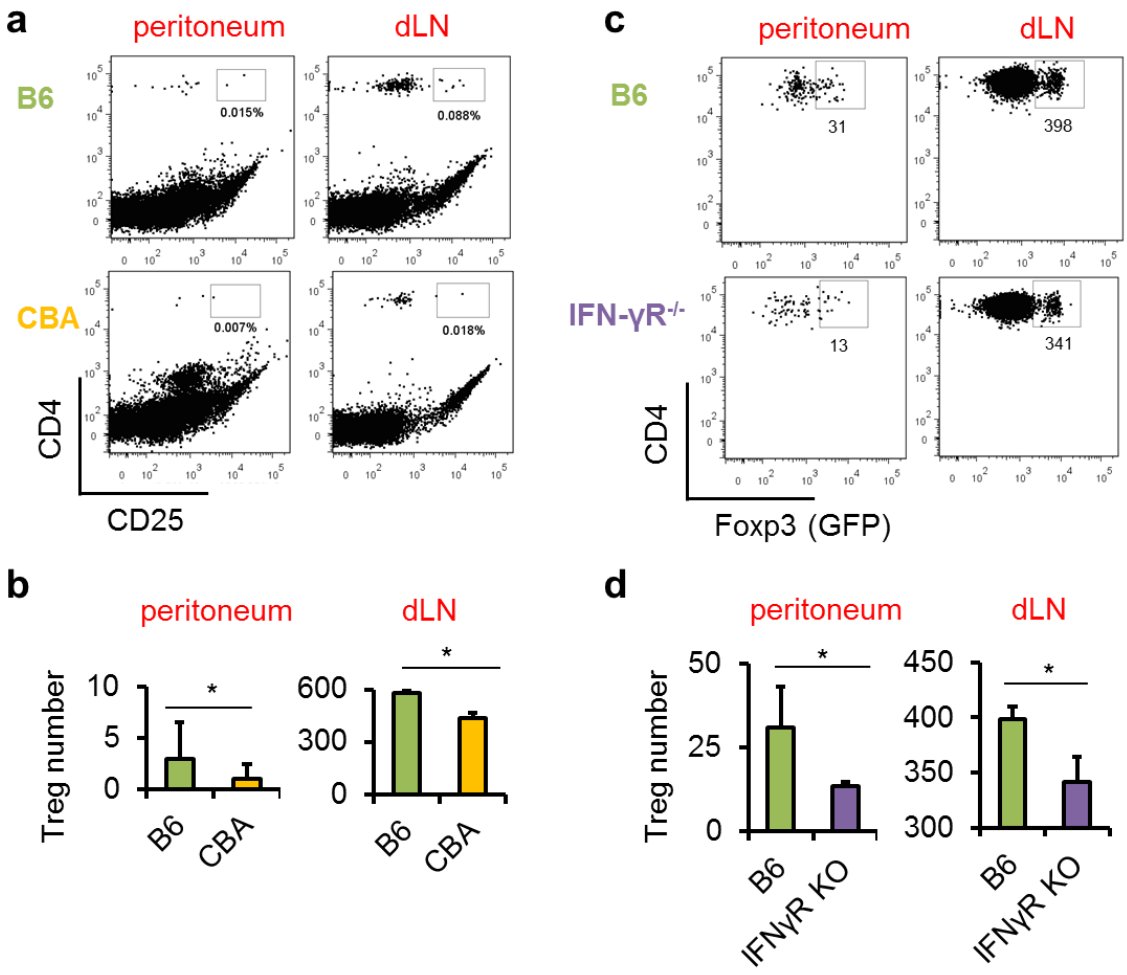



**Figure 21 Treg accumulation requires target tissue responsiveness to IFN-γ stimulation**

*(**a**,**b**) Total CD4$^+$ T cells from IFN-γ receptor KO mice (10$^7$/mouse) were labelled with 2 μM PKH26 and injected intravenously into syngeneic WT H2-B (C57BL/6) or irrelevant H2-K (CBA/Ca) mice that had received an intraperitoneal injection of 600 U IFN-γ 72 h earlier. The presence of PKH26$^+$, CD4$^+$CD25$^+$ Tregs in the peritoneal lavage and dLN was analysed by flow cytometer 16 h later. In panel **a**, representative dot plots of IFN-γR KO Treg cells detected in the peritoneal lavage and dLN are shown. The mean number of Treg cells (in the total CD4$^+$ population) present in the peritoneal cavity and dLN is shown in panel **b**. (n=3, N=4) *P<0.05. Total CD4 T cells from Foxp3-GFP reporter mice (10$^7$/mouse) were injected intravenously into WT C57BL/6 or IFN-γ receptor KO mice that had received an intraperitoneal injection of 600 U IFN-γ72 h earlier. The presence of PKH26$^+$, CD4$^+$CD25$^+$ Tregs in the peritoneal lavage and dLN was analysed by flow cytometry 16 h later. Tregs were gated on CD4$^+$GFP$^+$ population. Representative dot plots are shown in panel **c**. The mean number of Treg cells (in the total CD4$^+$ population) present in the peritoneal cavity and dLN is shown in panel **d**. Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. (n=3, N=2) *P<0.05.*

Previous studies have shown that CXCR3 is required for efficient Treg recruitment to some inflammatory sites (Muller et al. 2007; Santodomingo-Garzon et al. 2009). In addition, IFN-γ is known to induce CXCR3 ligands such as CXCL10 (A D Luster and Ravetch 1987a). Therefore we analysed CXCR3 expression on Tregs and their migration to CXCL10 *in vitro*. A relatively small fraction of Tregs expressed CXCR3, and did not migrate efficiently in response to CXCL10 as compared with the CCR7 ligands CCL19 and CCL21 (Figure 22). These data highlight that the diminished production of CXCL10 by IFN-γRKO recipients is unlikely to significantly impact the recruitment of Tregs to target tissue.

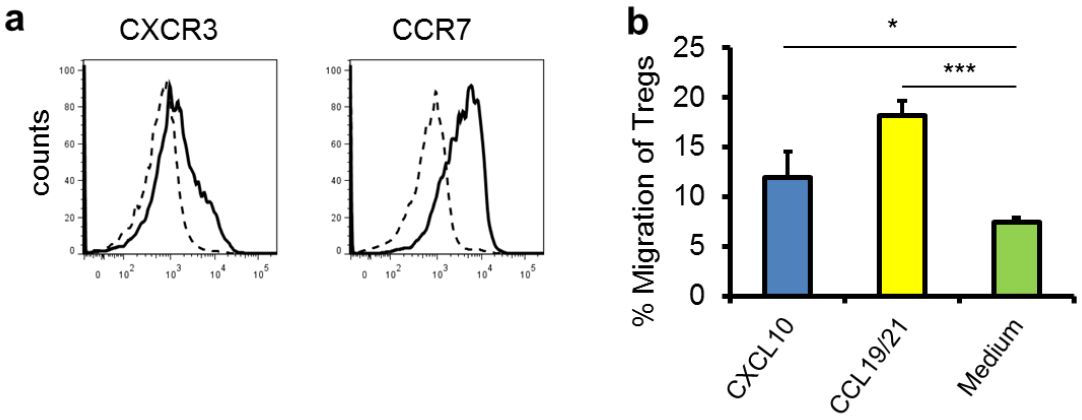



**Figure 22 In-vitro expanded H2-D-allospecific Tregs express low CXCR3 and reduced migration to CXCL10 *in vitro*.**

*The expression of CXCR3 and CCR7 by GFP$^+$ Treg cells was analysed by flow cytometry (**a**) and their migration in response to their respective ligands CXCL10(300 ng ml$^{-1}$) and CCL19/21 (200 ng ml$^{-1}$) was assessed by a 6hr in vitro chemokinesis transwell assay (10$^6$/well) (**b**). The number of migrated cells in the bottom of the transwell was evaluated by hemocytometric counting from 3 replicate wells (n=3). Results were expressed as average percentage of migrated cells. Data are representative of three independent experiments of identical design (N=3). Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. *P<0.05, ***P<0.001*

#### 4.1.3.4 Cognate recognition of alloantigen presented by the endothelium induces Treg recruitment.

Previously, cognate recognition of antigen displayed by the endothelium has been shown to play a crucial role in the recruitment of Teffs into non-lymphoid tissue both *in vitro* (Greening et al. 2003; Marelli-Berg et al. 1999b) and *in vivo* (Marelli-Berg et al. 2004b; Savinov et al. 2001; Savinov et al. 2003). Recent studies have shown that recruitment of Teffs is facilitated by both perivascular DCs and endothelial cells (Calderon et al. 2011; Walch et al. 2013). Administration of IFN-γ (72 hours prior) via the intraperitoneal route was shown to induce Class II MHC expression in the vascular endothelium of the peritoneal membrane and in the dLN (Figure 23), and was mainly upregulated by CD11c+ cells in the dLN. However, CD11c+ cells were not detected in the peritoneal membranes. Thus, it is likely that the endothelium plays a key role in recruitment of Tregs in this model.

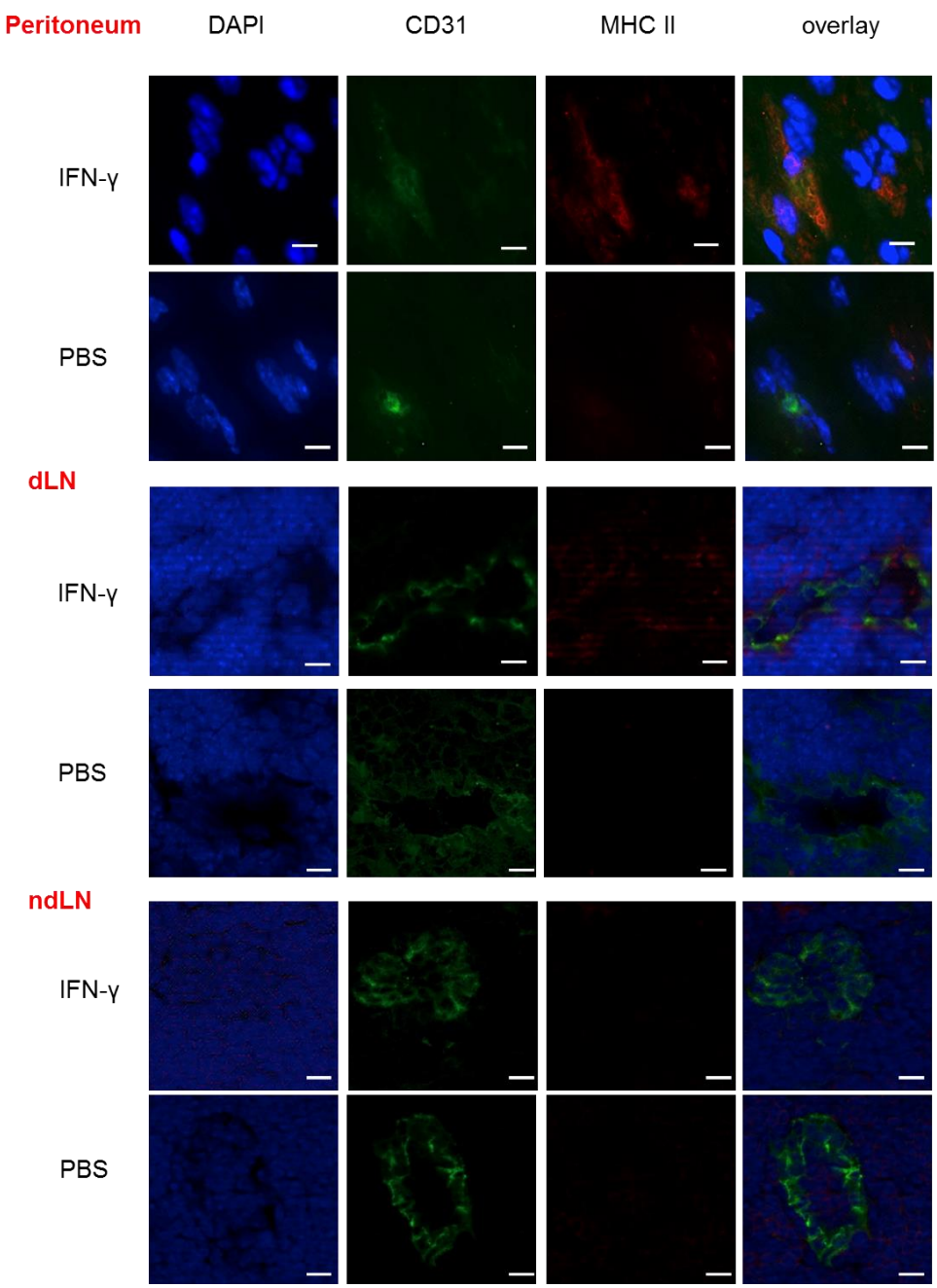



***Figure 23 IFN-γ treatment induces MHC class II expression in the peritoneum and Draining lymph nodes.***

*The peritoneal membrane, dLN and ndLNs from C57BL/6 mice that had received 600 U IFN-γ or control saline i.p. 72 h earlier were stained with anti-CD31 (green fluorescence) and anti-MHC II mAbs (red fluorescence) and analysed by wide-field fluorescence microscopy). Nuclei are highlighted by DAPI (blue). Representative pictures taken by wide field fluorescence microscopy are shown. Scale bar: 10 µM*

To directly visualise this phenomenon *in vivo*, intra vital microscopy experiments were carried out in which H2-D allospecific Tregs selected by *in vitro* culture were injected intravenously into allogeneic (BALB/c) or irrelevant (CBA/Ca) recipients that received intra-scrotal injections of IFN-γ 72 hours earlier. The labelled Tregs were observed within the microvascular bed of the cremaster muscle for up to 40 minutes. While no differences in rolling flux were observed in the groups, Tregs were seen to have higher T cell adhesion and transendothelial migration in the BALB/c blood vessels as compared to the CBA/Ca blood vessels (Figure 24).

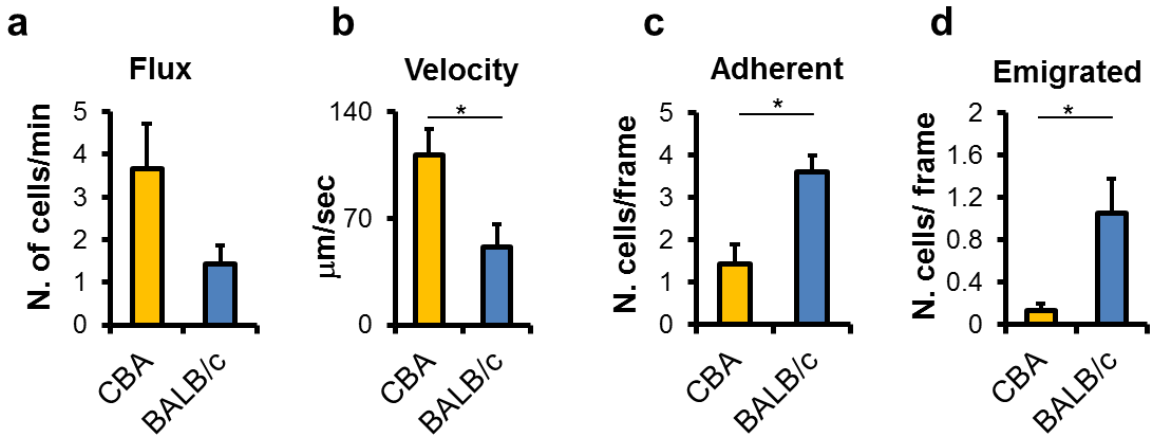



**Figure 24 Treg recruitment to non-lymphoid tissue is induced by cognate recognition of the endothelium *in vivo***

*Allogeneic male H2-D (BALB/C) and H2-K (CBA/Ca) mice were treated with intrascrotal administration of IFN-γ (1,200 U in 400 µl saline). After 72 h, fluorescently labelled (CFSE) Treg cells were injected intravenously after surgical exteriorization of the cremaster muscle and T-cell interactions with venular walls were visualized and quantified by intravital microscopy for up to 40 min. (**a**) rolling; (**b**) velocity; (**c**) firm adhesion; (**d**) extravasation. Results are from six mice per group, and significant differences between the H2-D in comparison with the H2-K strains of mice are shown by asterisk. Error bars represent standard deviations. Statistical significance was calculated using unpaired Student's t-test.*P<0.05 (N=2)*

No CD11c+ DCs were observed in the cremaster muscle through fluorescence microscopy. In addition, dissected and sectioned cremaster muscles visualized by fluorescence microscopy showed increased Treg influx in BALB/c cremaster muscles as compared to CBA/Ca controls (Figure 25).

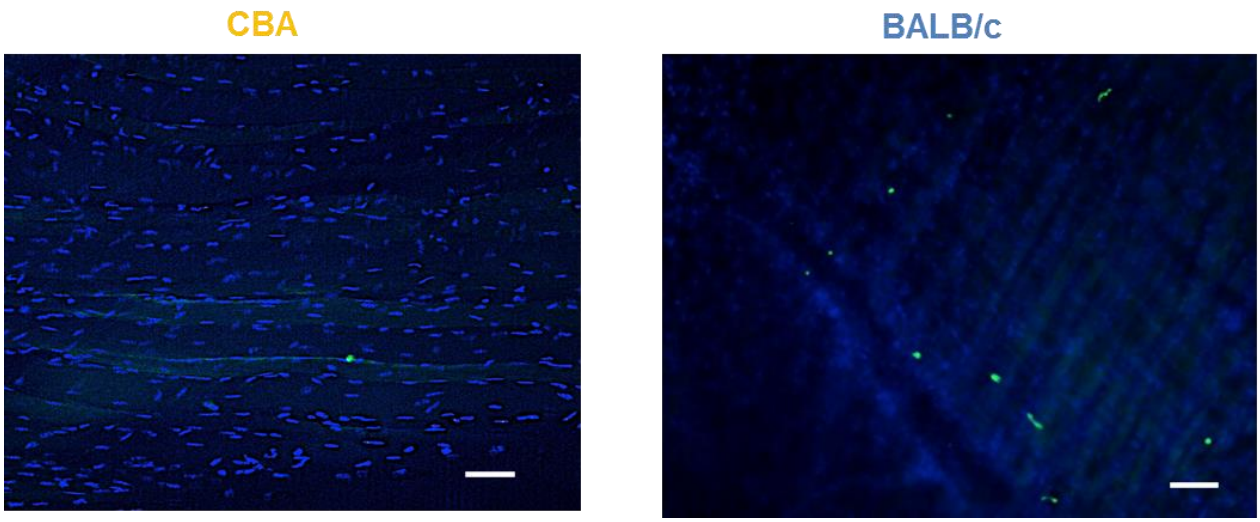



**Figure 25 H2-D-allospecific Tregs are recruited to IFN-γ treated allogeneic H2-D (Balb/c) cremaster muscle and not to H2-K (CBA/Ca) treated IFN-γ cremaster muscles.**

*Cremaster muscle sections from the previous experiment were fixed in 4% PFA and visualized by fluorescent microscopy indicating a large tissue influx of T cells in cremaster tissues from BALB/c but not control CBA/Ca mice. Nuclei are stained by DAPI (blue). Scale bar, 10 µM.*

#### 4.1.3.5 Antigen specific Treg recruitment requires PI3K P110δ activity

The results above indicated that TCR defined antigen specificity; via cognate antigen recognition displayed by endothelium facilitates recruitment of Tregs to sites of inflammation. To investigate the molecular mechanism underlying the antigen driven Treg trafficking, we sought to further characterise the molecular mechanism involved downstream of the TCR. The class I phosphoinositide 3-kinases (PI3K) have been described to have important functions in immune cells. In particular, the class I PI3K isoform p110δ is expressed at high levels in leukocytes and is a major isoform controlling a number of antigen-evoked immune responses. Loss of PI3K p110δ signalling has previously shown to result in diminished primary and secondary T cell responses. Genetic inactivation of PI3K p110δ in naïve T cells has been shown to result in their failure to differentiate into Th1 and Th2 subsets.

Additionally, in mice, loss of PI3K p110δ has been implicated in reduced Treg numbers and function. Although Treg cells deficient in p110δ have been shown to be responsive to IL-2 and only partially lose their suppressive ability *in vitro*, they are unable to maintain their suppressive potential *in vivo* (Patton et al. 2006). Previous studies have shown that Teffs bearing a functionally inactive variant of the P110δ subunit lost TCR-induced migration and failed to localise to antigenic tissue efficiently (Jarmin et al. 2008). Thus, we sought to investigate whether the loss of suppressive function in p110δ deficient Tegs observed *in vivo* could be explained partly through aberrant trafficking and localisation of the p110δ deficient Tregs to antigenic sites.

To investigate this hypothesis, we performed *in vitro* trans-endothelial migration experiments of Tregs with direct anti-H2-D (BALB/c) allospecificity through both H2-D (BALB/c, allospecific)- and H2-K (CBA/Ca, irrelevant)-expressing endothelial cell monolayers following pharmacological inhibition of p110δ activity. Tregs with direct H2-D (BALB/c) allospecificity were treated with either PI3K p110δ selective inhibitor IC87114 or PBS containing vehicle (1% dimethylsulphoxide) at 37$^{o}$C for 1 hour. Post treatment, the migration of both sets of Tregs was observed through IFN-γ pre-treated endothelial monolayers of H2-D (BALB/c) and H2-K (CBA/Ca) origin. The migration of Tregs through H2-D (BALB/c) endothelium was significantly impaired upon PI3K p110δ inhibition as compared to the vehicle controls, while the modest migration observed through irrelevant H2-K (CBA/Ca)-derived ECs remained unaffected by the p110δ inhibition (Figure 26).

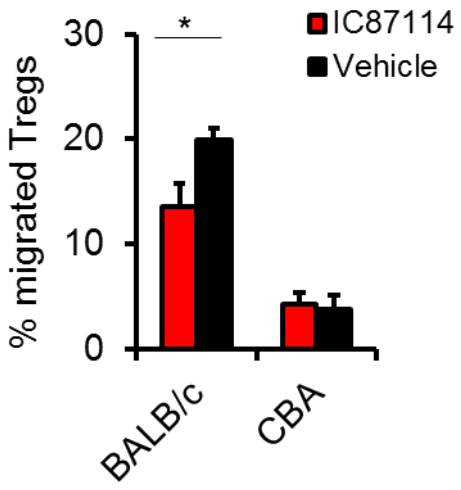



**Figure 26 PI3K p110δ activity is required for antigen-driven Treg TEM *in vitro*.**

*H2-D-allospecific Tregs were treated with 5 µM IC87114 or with vehicle control for 30 min at room temperature and subsequently washed before use. Vehicle control-treated (filled symbols) and inhibitor-treated (open symbols) T cells (5 × $10^5$/well) were seeded onto allogeneic H2-D (BALB/c) and irrelevant H2-K CBA/Ca mice-derived IFN-γ-treated EC monolayers. T-cell migration measured at 6 h is shown. Data are representative of at least three independent experiments of identical design. Error bars represent standard deviations. Statistical significance was calculated using an unpaired Student's t-test.*P<0.05.*

To evaluate the role of p110δ in Treg migration *in vivo*, we compared the recruitment of IC87114- and vehicle-treated Tregs with direct H2-D (BALB/c) allospecificity from circulation into the peritoneum of IFN-γ treated allogeneic H2-D (BALB/c) and irrelevant H2-K (CBA/Ca) mice. Equal numbers of IC87114-treated Tregs labelled with DDAO-SE and vehicle-treated Tregs labelled with CFSE were co-injected i.v into H2-D (BALB/c) and H2-K (CBA/Ca) recipients that had received an i.p injection of IFN-γ 48 hours earlier. Upon p110δ inhibition, Treg migration was unaffected in the CBA/Ca recipients while p110δ inhibition significantly reduced recruitment of inhibitor-treated Tregs in the peritoneal cavity and the draining lymph nodes of the H2-D (BALB/c) recipients (Figure 27). These results indicate that PI3k p110δ activation by the TCR contributes to the recruitment of Tregs by cognate endothelium in a manner similar to that operating in Teff migration.

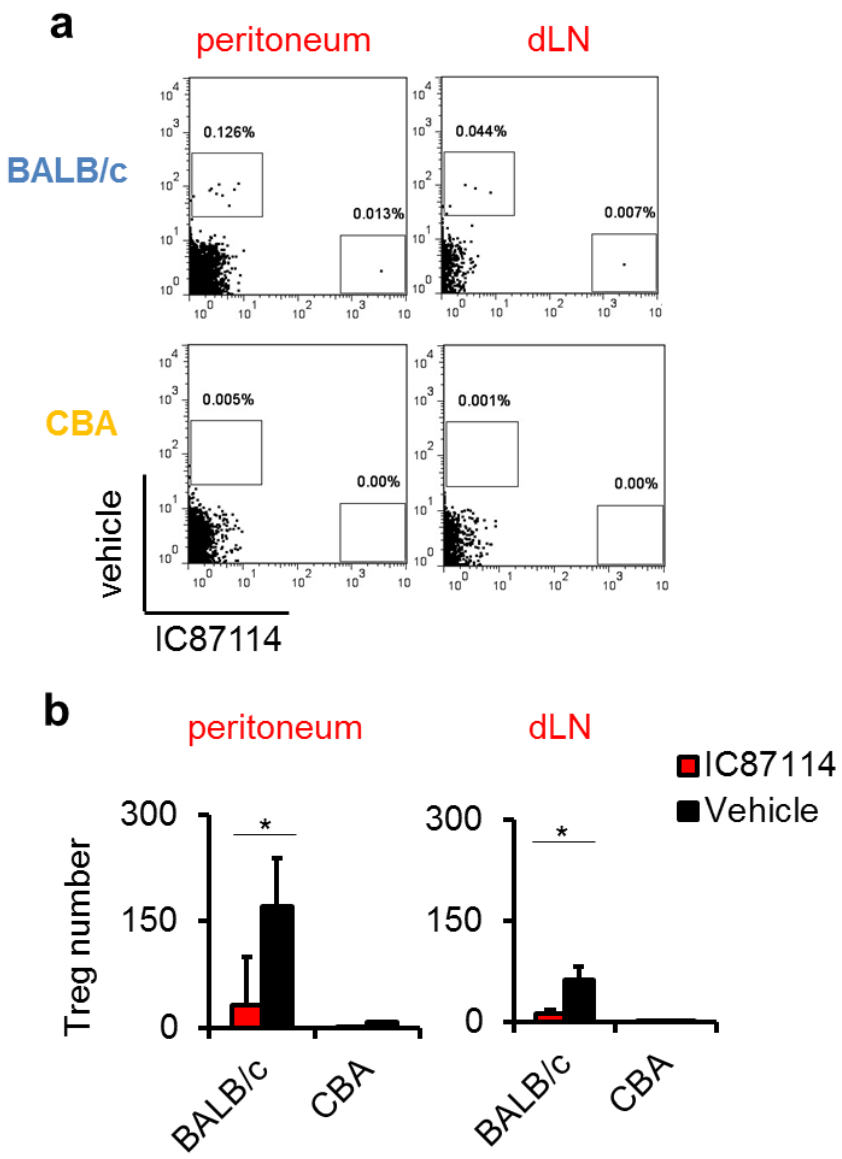



**Figure 27 PI3K p110δ activity is required for antigen-driven Treg recruitment *in vivo***

*H2-D-allospecific Tregs were treated with 5 µM IC87114 or with vehicle control for 30 min at room temperature and subsequently washed before use. CFSE-labelled vehicle-treated Tregs and DDAO SE-labelled IC87114 Tregs were co-injected ($10^7$/mouse) intravenously into H2-D (BALB/c) and H2-K (CBA/Ca) mice that had received intraperitoneal injections with 600 U IFN-γ 72Hr earlier. The presence of labelled T cells in the peritoneal cavity and dLNs was analysed after 24 h through flow cytometry. Representative dot plots from 3 experiments are shown in panel **a**. The mean number of Treg cells (in the total $CD4^+$ population) detected in the peritoneal cavity and dLNs is shown in panel **b**. Error bars represent standard deviations. Statistical significance was calculated using an unpaired Student's t-test. (n=3, N=3) *P<0.05.*

#### 4.1.3.6 Tregs can inhibit recruitment of Teffs into antigen rich target tissue

It is thought that similar to Teffs , naturally occurring Tregs require initial activation within the draining lymph nodes to enable them to migrate into sites of antigen rich tissue, and also utilise similar molecular mediators (PI3K P110δ) that direct their recruitment to antigen presenting endothelium (Jarmin et al. 2008; Q. Tang and Bluestone 2006). Thus, other mechanisms must exist to sustain Treg migration and maintain their preferential accumulation into target tissue allowing the establishment of optimal Treg:Teff ratios for termination of the T effector responses during the late stages of the immune response.

To address this issue we investigated whether Tregs infiltrating into target antigen-rich tissue were capable of affecting the recruitment of Teff cells. Teffs with the same allospecificity as the Tregs cells were generated by i.p injection of BALB/C splenocytes into C57BL/6 recipients for a period of 7 -10 days. The total lymphocytes of the recipients were then removed and purified for their CD4+ population. These were further expanded with inactivated BALB/c splenocytes for 7 days to generate an allospecific CD4 T cell population composed of a mixture of effector memory and central memory subsets (Figure 28).

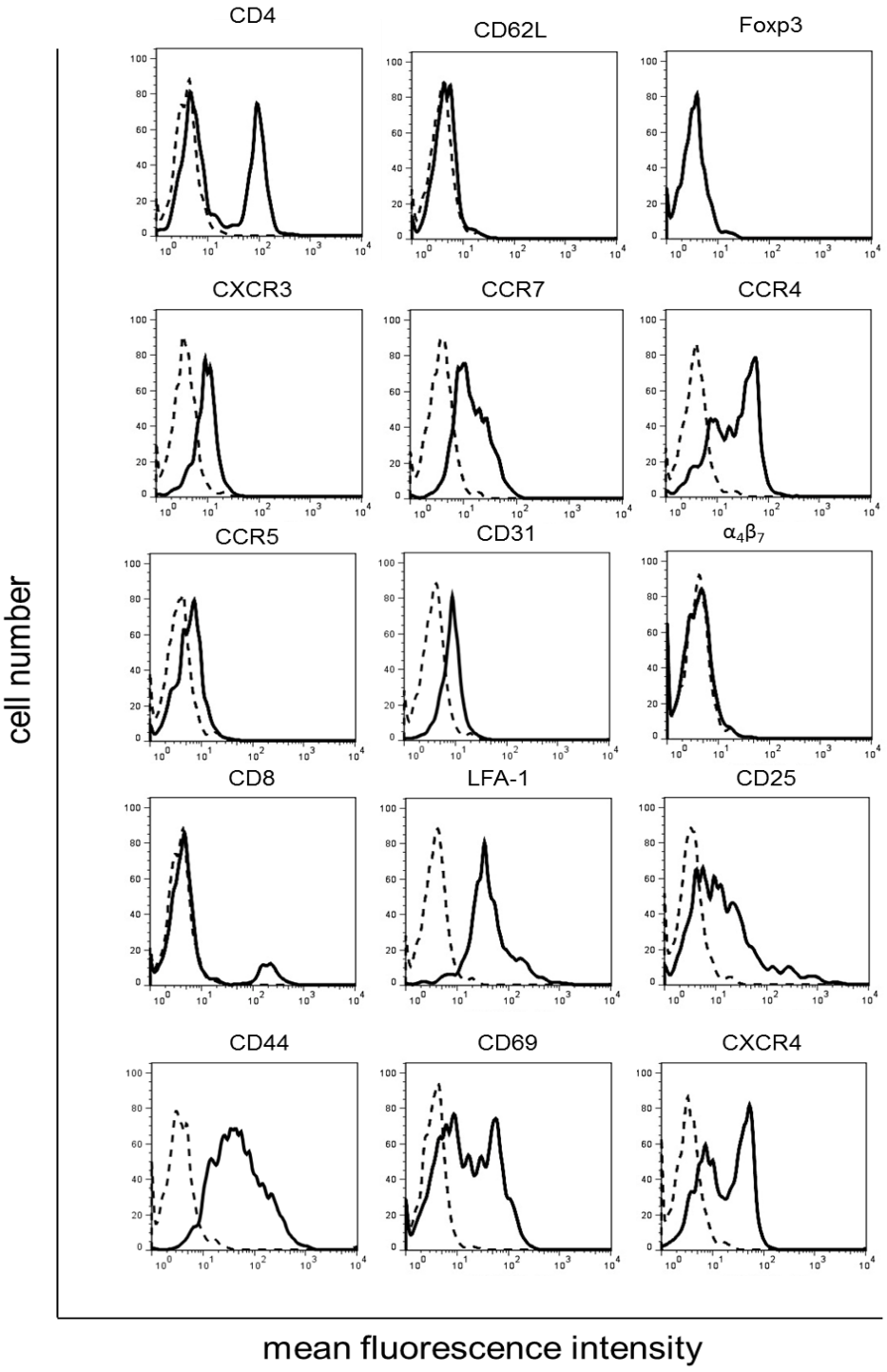



**Figure 28 Phenotype of allospecific conventional memory T cells.**

*H2-D -allospecific memory/effector T cells were generated by in vitro stimulation of naïve CD4+CD25- T cells from H2-B (C57BL/6) mice with H2-D (BALB/c)-derived) splenocytes. Expression of the surface molecules indicated above each histogram was analysed by flow cytometry. The dotted lines represent antibody isotype control staining.*

Time lapse imaging of both allospecific Tregs and Teff/cm, populations over CBA/Ca endothelial monolayers revealed no differences of intrinsic mobility between the two T cell groups (Figure 29).

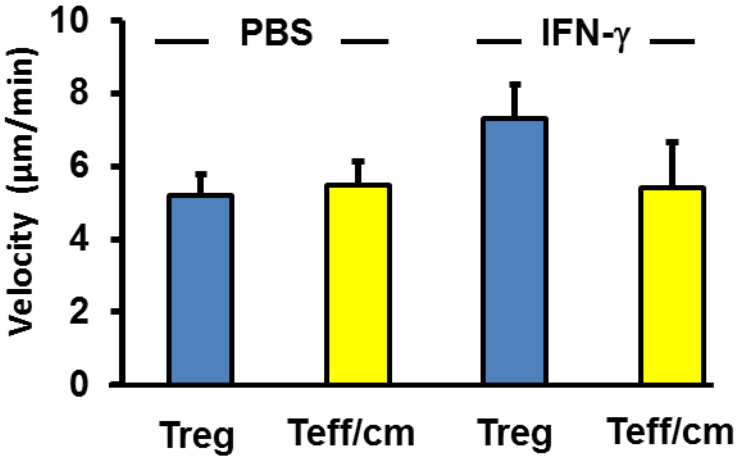



***Figure 29 Timelapse microscopy reveals no intrinsic differences between motitlity of Treg and Teff/cm cells.***

*H2-D-allospecific Treg or H2-D -allospecific conventional T cells were seeded onto confluent H2-K (CBA/Ca)-derived EC monolayers pre-treated with IFN-γ (300 U ml$^{-1}$) for 72 h. T-cell migration was monitored by timelapse microscopy. The crawling speed (µm min$^{-1}$) of T cells was measured using ImageJ analysis software. About 30–40 cells were tracked in each experimental condition. Error bars represent standard deviations. Statistical significance was calculated using an unpaired Student's t-test. N=2.*

Adoptively transferred H2-D allospecific T cells preferentially accumulated within the peritoneal cavity of the IFN-γ treated H2-D (BALB/c) recipients. (Figure 30) However, upon co-injection with equal number of H-2D specific Tregs, their localisation to the peritoneal cavity and draining lymph nodes was inhibited. Teff/cm populations were present within the non-draining lymph nodes and spleen of the co injected mice ruling out that Tregs induced apoptosis of the Teff/cm population. These results suggest that Tregs recruited into antigenic tissue can inhibit the further influx of antigen specific T effector cells.

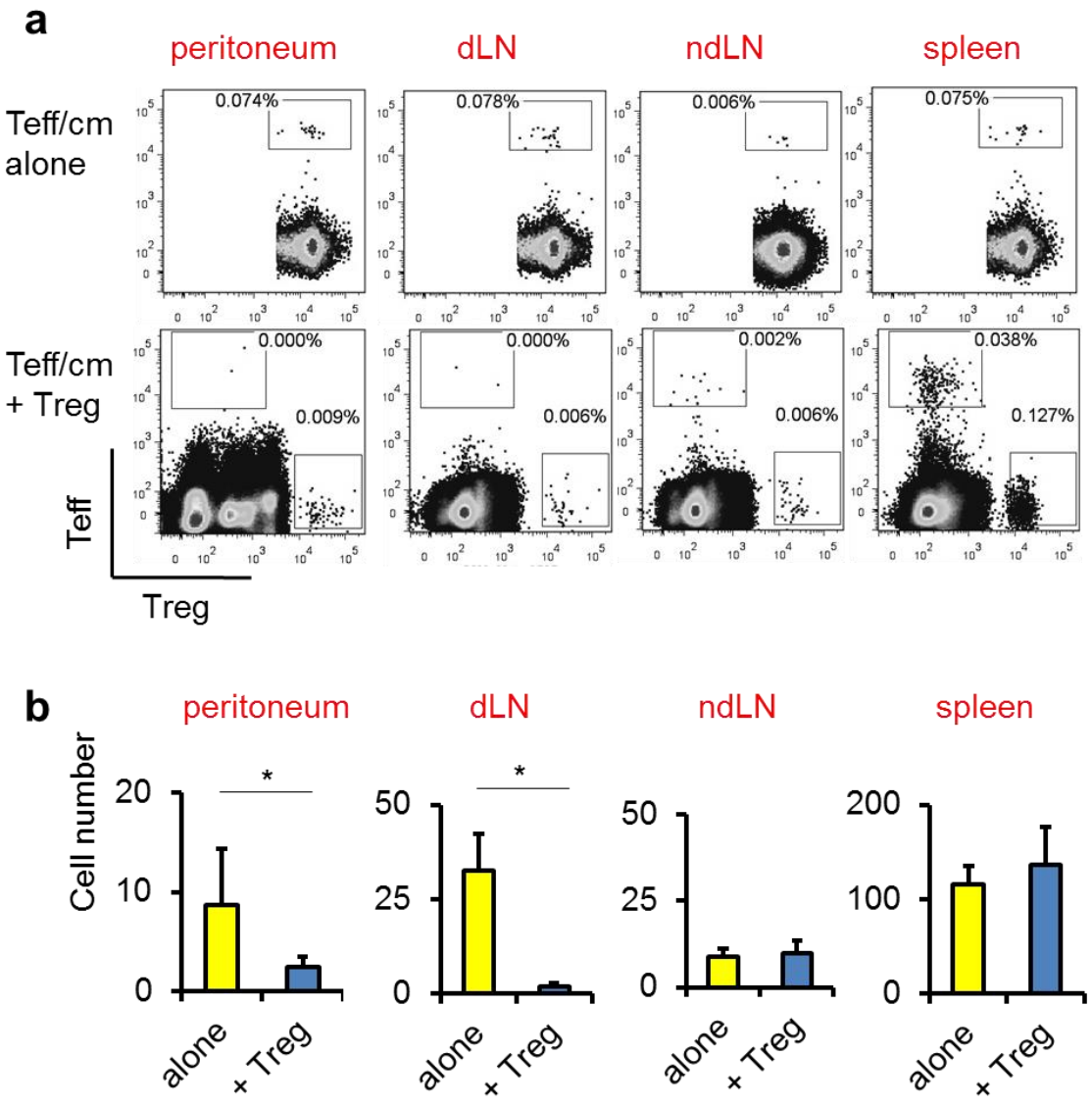



**Figure 30 Treg cells inhibit the recruitment of primed conventional T cell into target tissue.**

*H2-D-allospecific conventional T cells ($10^7$/mouse) were labelled with 1 µM DDAO SE and co-injected intravenously with CFSE-labelled H2-D-allospecific Tregs ($10^7$/mouse) into H2-D (BALB/C) mice that had received an intraperitoneal injection of 600 U IFN-γ 72 h earlier. The presence of DDAO SE+ Teff/cm and CFSE+ Tregs in the peritoneal cavity, dLN, ndLN and spleen was analysed 16 h later by flow cytometry. Representative dot plots and the mean number of labelled cells present in the peritoneal lavage are shown in panels **a** and **b**, respectively. Error bars represent standard deviations. Statistical significance was calculated using an unpaired Student's t-test. *P<0.05 (n=3, N=2).*

### 4.1.4 Discussion

Previous studies from our group have demonstrated that display of antigen by the endothelium in response to inflammatory stimuli such as IFN-γ enables the recruitment of T cells that express specific TCRs capable of recognizing those antigens (Marelli-Berg et al. 2004a). Antigen-driven T cell recruitment adds an additional level of specificity to selective migration to non-lymphoid inflammatory antigenic tissue. In this study, we have extended this function of the endothelium to the recruitment of Tregs. Furthermore, we showed that Treg trafficking to non-lymphoid inflammatory tissue is directed through the display of self-antigens. We also show that Tregs inhibit further recruitment of Teffs to the same site, thus favouring the establishment of the optimal Treg:Teff ratios necessary for regulation.

As we have mentioned previously, Tregs like Teffs constitute a heterogeneous pool that express homing and chemokine receptors depending on their target tissue and the type of inflammatory response (Gratz and Campbell 2014). However, Tregs have been shown to use combinations of homing molecules that can redundantly function to control their migration (Campbell and Koch 2011). Thus, we hypothesised the existence of additional mechanisms that would allow for the efficient recruitment of Tregs towards antigen bearing target tissue sites.

Our results here indicate a property of the target tissue site, namely the endothelium lining the microvascular vessels, which enables the efficient recruitment of Tregs. This concept can be illustrated in the studies observing Treg migration in allotransplantation. Despite the presence of alloreactive Treg in high frequency (>10%) (Lin et al. 2008), MHC mismatched vascularized tissue grafts are rejected acutely via direct allorecognition (Issa et al. 2013). The explanation that allospecific Tregs are not in sufficient numbers relative to the responding allospecific conventional T cells does not fully explain the lack of regulation of the alloresponses compared to the efficient control of autoimmunity. In addition, adoptive transfer of a large number of polyclonal Tregs is relatively inefficient as compared to Tregs with selected specificity for the alloantigen (Issa et al. 2013; Tang et al. 2012). These observations can be explained by the absence of cognate endothelium within the graft sites thereby causing inefficient Treg recruitment and regulation of alloresponses. Assuming that Tregs would promptly recognize self –MHC peptide complexes on autologous endothelium, they would be able to rapidly migrate into target tissue to suppress, for instance, autoimmune responses. In transplantation, only a fraction of Tregs would cross-react to allogeneic endothelium and gain access to the allograft sites in sufficient numbers to regulate alloresponses. Moreover, unlike conventional T cell activation, which is restricted topographically

to the lymph nodes, Tregs activation can occur in non-lymphoid target tissue and subsequently induce migration towards draining lymph nodes (N. Zhang et al. 2009). Thus the absence of cognate endothelium in allografts might result in delayed and inefficient recruitment and subsequent activation. In line with this, the recent study by (Fan et al. 2010) analysing the dynamics of alloresponses *in vivo* demonstrated that rapid invasion of effector T cells followed by delayed arrival of Tregs were ineffective in controlling tissue damage. Similarly, in therapeutic settings, autologous Tregs are not as efficient as allospecific Treg in preventing graft rejection (Issa et al. 2013; Tang et al. 2012).

Naturally occurring Tregs produce little or no IFN-γ on their own following activation (Koch et al. 2009). However our results show that Treg recruitment is kick-started and strictly dependent on IFN-γ mediated MHC Class II molecule expression on the endothelium. Thus, it is likely that IFN-γ produced by other sources such as activated T cells would initiate their recruitment. This highlights the sequential coordination of migratory events by Teffs and Tregs during a physiologic immune response which orchestrates the synchronised development of effector and regulatory elements. In the first steps, IFN-γ produced through activation or recruitment of conventional T cells would subsequently induce endothelial display of MHC Class II thereby allowing the entry of Tregs. Treg recruitment would then dampen the T cell mediated inflammation and likely prevent further Teff recruitment and activation thus resolving the inflammation.

This scenario is indirectly supported by the observations here, which show abrogation of Teff recruitment when both Tregs and Teff are simultaneously recruited to the target tissue. In contrast, both populations arrived and co-localised in the spleen and draining lymph nodes of IFN-γ treated recipients suggesting that Tregs must be activated as a consequence of local production of IFN-γ( by conventional cells) for this effect to occur.

It is unclear how Tregs prevent further recruitment of conventional Teffs. Given that migrating Tregs are first engaged by cognate endothelium in our model, it is possible that they can modulate the extravasation cascade. Previously, the report by (James et al. 2003) has shown that anergic T cells can inhibit recruitment of other T cells to target tissue *in vivo* by inhibiting chemokines via the activity of dipeptidyl peptidase (CD26) (James et al. 2003). This mechanism might also be functional in Tregs. Tregs have also been shown to prevent stable conjugate formation between APCs and Teff cells in the LN *in vivo* (C. E. Tadokoro et al. 2006). To date, no reports have highlighted Treg mediated inhibition of Teff recruitment via a direct action of the Tregs on the endothelium. However, it is likely that Tregs may indirectly reduce endothelial

recruitment of Teffs by inhibiting the cytokine production of Teffs or through production of immunoregulatory cytokines such as TGFβ and IL-10 (N. Zhang et al. 2009).

# 4.2 Part 2: CD28 and CTLA-4 regulate Treg migration

## 4.2.1 Introduction

While TCR-induced signalling is instrumental for regulating migration of T cells during immunity, signals provided by co-stimulatory receptors also impact migration. Two of the best characterised co-stimulatory receptors are CD28 and CTLA-4, which bind to the B7 family co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2). Historically, studies have implicated their role in several aspects of T cell biology ranging from T cell development in the thymus to T cell activation, proliferation, and cytokine secretion. Although both receptors share considerable homology and similar ligand specificity, their impact on T cell functions are known to be quite distinct from each other (reviewed in M.-L. Alegre et al. 2001b; Sansom 2000). A simplistic overview of the body of studies up to date indicates that CD28 acts as a positive regulator and CTLA-4 as an opposing inhibitory regulator of T cell functions. The interplay between positive and inhibitory co-stimulation involves a complex network of signalling pathways, the integration of which greatly impacts the outcome of the ensuing immune response.

Both CD28 and CTLA-4 receptors are known to engage pathways that regulate adhesion and cytoskeletal rearrangements and were therefore predicted to have a role in regulating T cell migration. In a study by (Mirenda et al. 2007), antibody-mediated CD28 stimulation induced adoptively transferred memory CD8+ T cells to migrate to extra-lymphoid tissue, with enhanced infiltration into kidney, liver, spleen, gut and heart independently of TCR-derived signals and homing receptor expression. Interestingly, they also observed that CTLA-4 mediated signals induced an opposite effect by preventing T cell migration. CD28 and CTLA-4 were therefore proposed to positively and negatively control the entry of primed T cells into non lymphoid tissue, respectively by regulating T cell motility. Since conventional T cells transiently express CTLA-4 only after activation/priming (Freeman et al. 1992), the functional role of CTLA-4 on the migration of in this T cell population is limited to the timeframe of CTLA -4 expression.

Tregs play a vital role in maintaining immune homeostasis. An imbalance in their number or function is thought to contribute to a variety of inflammatory and autoimmune conditions (Dejaco et al. 2006). Crucial to their function, is their ability to effectively localise into the target tissue where suppression and tolerance are to be achieved (Burrell et al. 2012).

Unlike conventional T cells, Tregs express both CD28 and CTLA-4 receptors constitutively. A recent study observing the spatiotemporal expression of CTLA-4 at the Treg-APC immunological synapse (IS) has shown a very different regulation of the effect of CTLA-4 signals than those observed in Teffs (Yokosuka 2010). While CTLA-4 in Teffs prevents CD28 mediated recruitment of PKC-θ and CARMA1 scaffolding protein inhibiting activation and proliferation, PKC-θ recruitment to the IS is largely absent in Tregs. Instead, CTLA-4 recruits protein kinase C-η (PKC-η) member of the protein kinase family which positively regulates Treg function (Kong et al. 2014). Likewise, while CTLA-4 mediated recruitment of phosphatases such as SHP-1, SHP-2 and PP2A in Teffs (Chuang et al. 2000; Marengere et al. 1996) have been shown to be crucial for its inhibitory function, these are rarely recruited along with CTLA-4 to the Treg IS (Yokosuka 2010). Thus, differential integration of these and other signalling pathways in Tregs could favour migration distinctive from that seen in Teffs.

Little is known about the interplay of CD28 and CTLA-4 signals and the effect of concomitant delivery engagement of both receptors in the regulation of Treg migration. In a recent report from (Lu et al. 2012), Tregs were shown to differ from conventional T cells in resisting CTLA-4-mediated reversal of TCR stop-signal. In their study, CTLA-4 signalling reduced the contact times of DO11.10 x $CD4^+CD25^-$ Tconvs, but not DO11.10 x $CD4^+CD25^+$ Tregs, with OVA peptide presenting DCs in lymph nodes. Additionally, they observed that concentrations of anti-CTLA-4 that showed reversal of anti-CD3 stop-signal on Foxp3-negative cells had no effect on Foxp3-positive cells.

Tregs and Teffs also display distinct metabolic programmes to support their survival and differentiation (Michalek et al. 2011c). While Teffs are highly glycolytic, Tregs rely more on oxidative phosphorylation of fatty acids as their main source of energy.

A key metabolism regulating kinase; the mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) pathways are generally thought to play important and opposing roles in cell fate decision by regulating T cell metabolic responses in both Teffs and Tregs (Michalek et al. 2011c). In Teffs, T cell activation stimulates the mammalian target of rapamycin complex (mTOR) to upregulate glycolysis and diminishes lipid oxidation (Michalek and Rathmell 2010). In addition, selective signalling from the two different components of mTOR-mTORC1 and mTORC2 can direct T cell differentiation in different ways (Delgoffe et al. 2011). This study demonstrated that differentiation into the Th1 and Th17 subsets of helper T cells was selectively regulated by signalling from mTOR complex 1 (mTORC1), while differentiation into the Th2 subset was regulated by signalling from mTOR complex 2 (mTORC2). In contrast, inhibition of mTOR has been shown to favour the development of iTregs. For example, in the study by (Esposito et al.

2010), rapamycin-mediated inhibiton of EAE was associated with the disappearance of CD4(+)CD45RB(high) effector T (T(eff)) cells and selective expansion of T(reg) cells bearing the CD4(+)CD45RB(low)FoxP3(+)CD25(+)CD103(+). In addition, the study by (Battaglia et al. 2005) demonstrated that inhibiting mTOR by rapamycin treatment selectively expanded the naturally occurring CD4+CD25+FoxP3+ Treg cells *in vitro*.

However, Tregs also display a requirement for signalling through the mTOR catalytic subunit mTORC1 for engaging lipid oxidation and also for their functional fitness. In the recent study (H. Zeng et al. 2013c), disruption of mTORC1 through Treg-specific deletion of the essential mTORC1 component raptor led to a profound loss of Treg-cell suppressive activity *in vivo*. They concluded that mTORC1 signalling maintains Treg suppressive activity by inducing cholesterol and lipid metabolism and by upregulating the Treg receptors CTLA-4 and ICOS. In addition, they also demonstrated that mTORC1 maintains Treg function partly through inhibition of mTORC2.

Most of these and other studies of the metabolic properties of Teff and Treg have focused on how they impact function and differentiation. Recently, a study from our group (R. Haas et al. 2015) has demonstrated that the glycolytic pathway is engaged during Teff cell migration. In their study, inhibiting Teffs basal or chemokine induced aerobic glycolysis through the glycolysis inhibitor 2-deoxyglucose (2-DG) or via rapamycin-mediated inhibition of mTOR resulted in decreased chemotaxis *in vitro* and *in vivo*. Yet, little is known about which pathways are essential for Treg motility and migration.

Emerging evidence from recent discoveries suggest that energy metabolism can also indirectly affect T cell homing pathways by regulating differential expression of adhesion and chemokine receptors. In the recent report by (Sinclair et al. 2008), loss of CD62L and CCR7 associated with activation of naïve T cells was shown to be regulated by mTOR signalling and upstream PI3k p110δ signalling. This CD62L and CCR7 loss prevents effector T cells re-entering secondary lymphoid organs and facilitates their redirection to peripheral tissues. This paper also highlighted that the pathways that regulate T cell metabolic switches are also closely integrated with those that are known to affect T cell migration. One example is the PI3K-Akt-mTOR pathway. CD28 recruitment of PI3K and subsequently downstream Akt activation has been shown to be crucial for recruitment of primed Teffs to antigenic tissue (Mirenda et al. 2007); in addition PI3K-Akt activation is also required for CD28-induced glucose transporter GLUT1 expression, and glycolysis upregulation (Jacobs et al. 2008). We hypothesized that CTLA-4 signals, by blocking CD28-induced Akt activation, might also prevent the increase in glucose utilization. This could potentially imply a role for CTLA-4 in regulating energy metabolism and migration. Should this be the case,

differential expression and activation of downstream pathways by CTLA-4 on Tregs might underlay their unique metabolic phenotype or differential use of metabolic pathways.

### 4.2.2 Aims

The study by (Mirenda et al. 2007) has implicated both CD28 and CTLA-4 co-receptors in regulating migration of primed T cells to antigenic sites. In addition, their study highlighted that this regulation was independent of TCR-derived signals and homing receptor expression. Given the ample evidence to suggest that Tregs and Tconv display differences in their co-receptor expression, downstream signalling and metabolic pathways, we hypothesized that differential expression of these receptors by Tregs may underlay differences in the trafficking patterns of Teff and Treg cells.

In this part of the study, the overall aim was to investigate the effect of CD28- and CTLA-4-induced signals, delivered either separately or in conjunction, on Treg migration and to establish if their affects, if any, were TCR- and/or homing receptor- dependent. Secondly, we sought to identify the metabolic pathways that are engaged during Treg motility and migration and assess whether CD28 and CTLA-4 signals impacted Treg migration by altering such metabolic pathways. To address these issues we conducted the following experiments:

1. Assessment of *in vitro* and *in vivo* Treg migration in response to antibody mediated triggering of CD28, CTLA-4 and both co-receptors.
2. Characterisation of CD28 and CTLA-4 signalling to investigate dependency on TCR signalling and to identify any differences brought about in the expression of receptors that are known to modulate motility (i.e chemokine receptors, adhesion molecules).
3. Analysis of Treg motility following selective inhibition of metabolic pathways using predefined metabolic inhibitors, so as to identify those that are essential for motility.
4. Analysis of the effect of CD28 and CTLA-4 signalling on metabolic pathways essential for Treg migration.

## 4.2.3 Results

### 4.2.3.1 The anti CD28 (clone 37.51/37.52) and anti CTLA-4 (clone UC10-4F10-11 ) antibodies bind specifically to the extracellular domains of CD28 and CTLA-4.

Both CD28 and CTLA-4 co-stimulatory receptors share considerable homology and bind to the same ligands on APCs. The stimulating anti- mouse CD28 (clone 37.51/37.52) is a well-established antibody which induces CD28 signals.

In the study by Schneider et al 2006, an anti-CTLA-4 antibody (clone UC10-4F10-11) was used to induce signalling in T cells that could reverse the TCR induced stop-signal. In another study, Olsson et al reported that ligation of CTLA-4 using this same antibody suppressed CD28-mediated activation of transcription factors NF-κB and AP-1 in T cell blasts. Thus we selected this antibody to induce signalling via the CTLA-4 receptor. Additionally, we tested the reactivity of this antibody towards the CD28 co-receptor. To achieve this, we performed a flow cytometry based iso-control. Here we co-stained T cells with increasing concentrations of unlabelled anti-CTLA-4 antibody with a fixed concentration of PE conjugated anti-CD28 antibody. As a control, we co-stained cells with increasing concentrations of unlabelled anti-CD28 antibody with a fixed concentration of PE conjugated anti-CD28 antibody. As shown in Figure 31, increasing concentration of unlabelled anti-CTLA-4 antibody had no effect on the anti-CD28 PE staining of the T cells. In the case of the control, increasing unlabelled anti-CD28 antibody concentrations diminished anti-CD28 PE staining indicating competition between the two antibodies for the same binding sites.

Together with above mentioned reports indicating the anti-CTLA-4 (UC10-4F10-11) antibody acts as a signal inducing antibody, these results suggest that this murine anti-CTLA4 antibody is capable of selectively inducing signalling via the CTLA-4 receptor and does not bear any cross reactivity with the epitope of the murine anti-CD28 antibody.

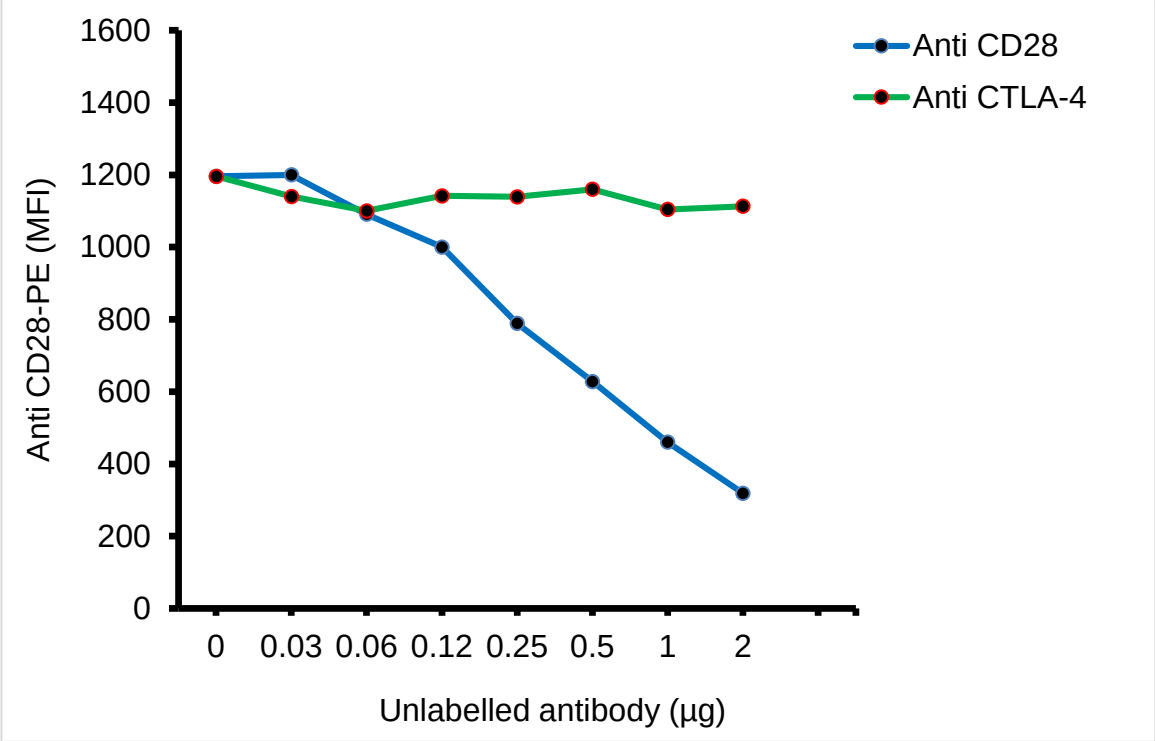



**Figure 31 Anti CTLA-4 antibody (clone UC10-4F10-11) is does not bear cross reactivity to CD28**

*An iso-control experiment was performed to test cross reactivity of the anti CTLA-4 antibody to CD28. Foxp3+ Tregs isolated from Foxp3-GFP C57BL6 mice were stained with a fixed concentration (1ug) of PE conjugated anti CD28 and increasing concentrations of either unlabelled anti CTLA-4 or, as a control, unlabelled anti CD28 antibody. The data plots indicate mean fluorescent intensity of the PE Anti-CD28 staining of Foxp3+ Tregs on the Y axis and increasing concentration of unlabelled antibody on the X axis. N=1.*

### 4.2.3.2 CD28 and CTLA-4 co-receptors regulate migration of Tregs differentially.

In conventional T cells, both CD28 and CTLA-4 co-receptors have been shown to regulate T cell migration and motility. Within the lymph nodes, CD28 activation promoted egression and migration of memory T cells towards the non-lymphoid tissue bearing the specific antigen. Furthermore CTLA-4 signalling antagonized the pro-migratory effect of CD28 and prevents Teff localisation to antigen-rich tissue. CTLA-4 engagement by APCs during T cell-APC interaction was also shown to reverse the TCR induced stop signal, thereby promoting detachment from the APC. Although both co-receptors share the same ligand specificity, CTLA-4 binds to its receptors with much higher affinity and competes with CD28 for ligands. Thus, through reversal of the TCR induced stop signal, CTLA-4 can outcompete and inhibit CD28 pro-migratory signalling by facilitating disengagement. However less is known regarding the role of the CD28 and CTLA-4 signalling on the migration of Tregs, which express both CD28 and CTLA-4 co-receptors constitutively (Figure 32).

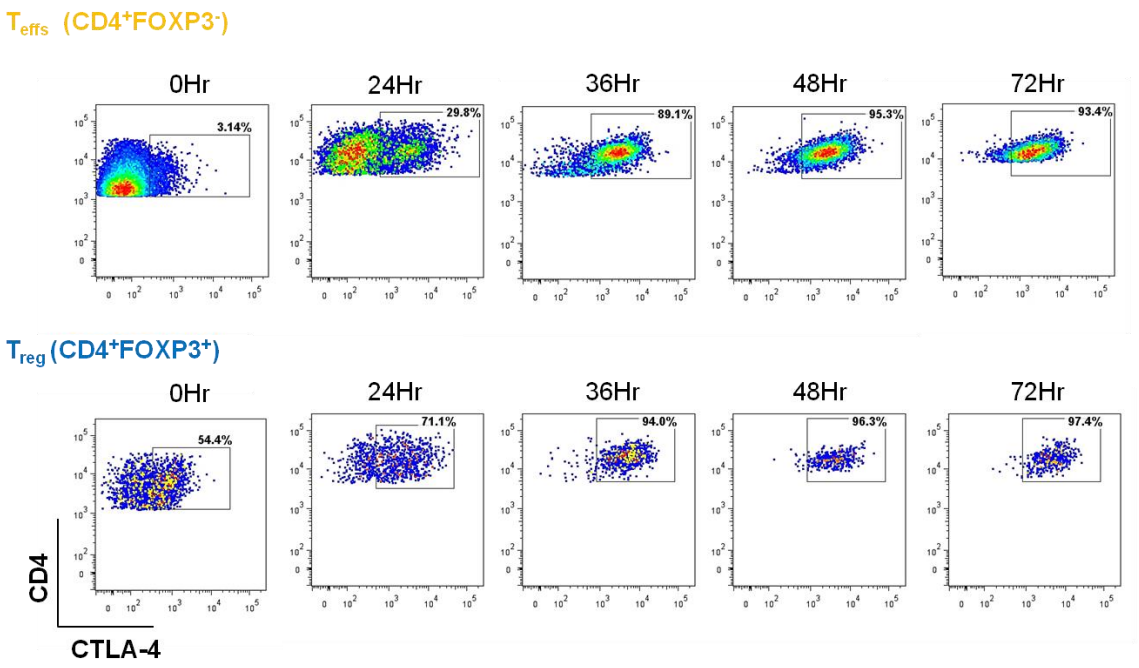



**Figure 32 CD4+Foxp3+ Tregs expressed CTLA-4 constitutively while CD4+ Foxp3- Teffs express CTLA-4 only after activation.**

*CD4+CD25-FOXP3- T cells and CD4+CD25+FOXP3+ Tregs were isolated from C57BL6 mice spleens and lymph nodes and purified through cell sorting. The T cells were then subjected to activation using plate bound anti-CD3 (1ug/ml) and anti CD28 (5ug/ml) for a period of 4 days at 37°C with 5% $CO_2$. T cell populations were sampled at indicated time points and stained for both Foxp3 and CTLA-4 expression using the method described earlier. Dot plots indicate CD4+ T cells gated on either Foxp3+ or Foxp3- T cell populations as analyzed by flow cytometry. n=3, N=3*

Tregs might display a migratory pattern different to Teffs as a result of the concomitant signalling of both receptors. To address this hypothesis, we observed the trans-endothelial migration of *in vitro* expanded Tregs following antibody-mediated stimulation of CD28 and CTLA-4 receptors. CD4+Foxp3+ Tregs isolated via flow activated cell sorting from Foxp3-eGFP reporter mice were expanded *ex vivo* through co-culture with sub-lethally irradiated immature bone marrow dendritic cells and IL-2. On day 7 of co-culture, Tregs were removed from culture, subjected to antibody-mediated CD28 and/or CTLA-4 ligation and seeded onto IFN-γ activated endothelial cell monolayers in a transwell assay to observe trans-endothelial migration. Our results indicated that, while CTLA-4 stimulation did not inhibit Treg migration *per se*, CTLA-4-induced inhibitory signalling prevented CD28-induced enhancement of migration through the endothelium. These results suggest that CTLA-4 acts by inhibiting CD28-induced migration in Tregs (Figure 33).

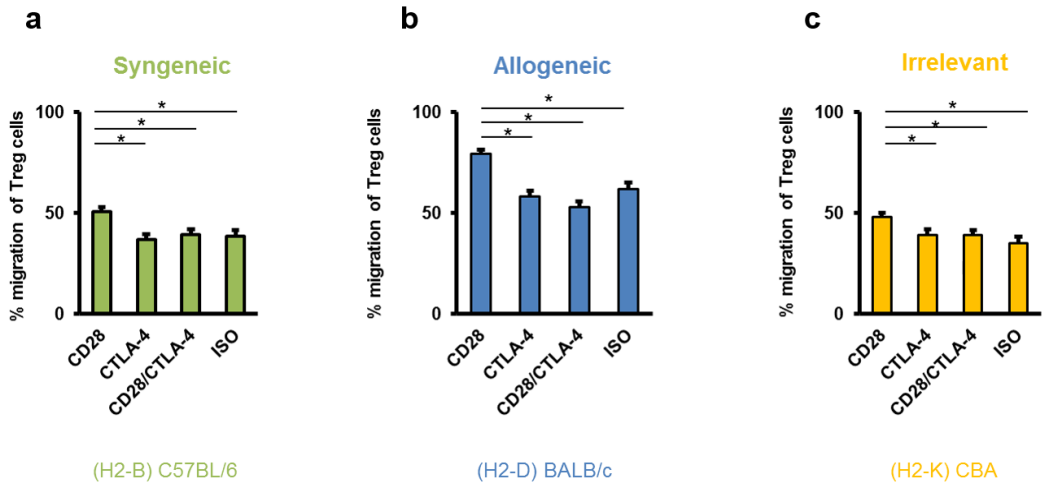



**Figure 33 CTLA-4 activation abolishes the pro-migratory activity induced in Treg cells by CD28.**

*CD4+CD25+Foxp3+ Tregs were isolated from Foxp3-GFP C57BL6 mice and expanded through co-culture with sub-lethally irradiated immature bone marrow-derived allogeneic (H2-D) dendritic cells. Following 7 days of co-culture, expanded allospecific Tregs were treated with CD28 and/or CTLA-4 antibody-mediated ligation. As a control, Tregs were treated with an isotype-matched control antibody and secondary antibodies. Tregs were then placed over IFN-γ-treated (**a**) syngeneic, (**b**) allogeneic or (**c**) irrelevant endothelial cell monolayers grown on a 3-μm pore polycarbonate transwell and incubated for 24 hours at 37°C with 5% $CO_2$. The number of migrated cells was evaluated by hemocytometric counting. Results are expressed as a percentage of migrated cells after 24 hours. Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. (n=3, N=4) *P<0.05.*

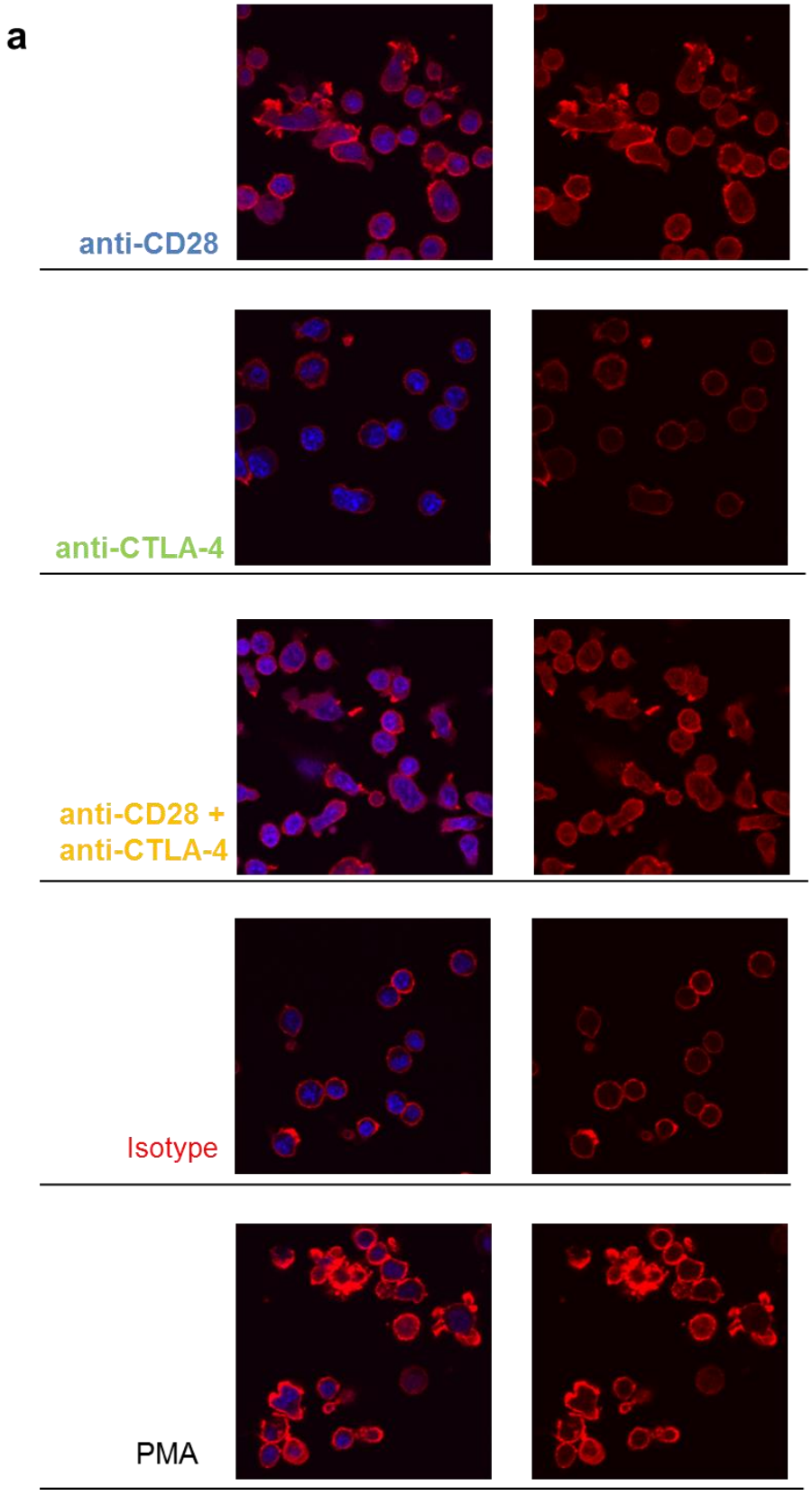
#### 4.2.3.3 CD28- and CTLA-4-mediated regulation of Treg migration occurs independently of the TCR

While CD28 signals have been shown to augment the TCR signalling pathway, there is considerable evidence to suggest that CD28 signals can affect pathways independent of the TCR. In one study, CD28 ligation by recombinant B7 molecules on primary human CD4+ T cells without any TCR stimulus were shown to upregulate the survival factor Bcl-xl and the inflammatory IL-8 and BAFF genes, in a NF-κB-dependent manner (Marinari et al. 2004). In terms of T cell cytoskeletal rearrangements, CD28 induction of F actin reorganisation by the Ras homologue Cdc42 occurs independently of ZAP-70 [z-chain (TCR)-associated protein kinase of 70 kDa] activation (Salazar-Fontana et al. 2003). Additionally, TCR independent signalling by CD28 also activates the Rho GTPases Vav1 (Michel et al. 2000).

In order to address whether CD28/CTLA-4 regulation of Treg motility can occur independently of TCR triggering, we performed *in vitro* trans-endothelial migrations assays to observe the migration of Tregs in contexts where co-stimulatory signals would be delivered with/without TCR engagement. Tregs isolated from H2-B (C57BL/6) mice were expanded *in vitro* against H2-D alloantigens (BALB/c) to generate Tregs selected for their specificity for H2-D alloantigens, as described in Materials and Methods. Following antibody-mediated CD28 and/or CTLA-4 crosslinking, Treg migration through endothelial monolayers formed from IFN-γ activated endothelial cells isolated from allogeneic H2-D, (BALB/c), syngeneic H2-B (C57BL/6) and irrelevant H2-K (CBA/Ca) backgrounds was monitored. Due to their weak affinity towards self-antigens, these Tregs are likely to have low affinity reactions with the self-antigen displayed by the syngeneic (H2-B) endothelium in addition to the strong allo-antigens reactivity against the allogeneic (H2-D) endothelium, while no TCR engagement would occur against antigens displayed by the endothelium of irrelevant origin. Thus, each endothelial variant represents a different level of TCR engagement. CD28 induced pro-migratory signals leading to enhanced transendothelial migration irrespective of the endothelial origin, which were abrogated by CTLA-4 stimulation. This suggests that CD28 induced migration and CTLA-4 induced inhibition of this event can occur in the absence of signals provided by the TCR [Figure 33 (b-c)].

In addition to these experiments, we observed the effects of CD28 and CTLA-4 stimulation on F-actin reorganisation in Tregs using confocal microscopy. Tregs were plated onto anti-CD28 and/or anti-CTLA-4 antibody-coated tissue culture plates and images were taken by confocal laser scanning microscopy following 5-10 minutes of incubation (Figure 34). Rapid aggregation of F-

actin towards the polarised edges can be visualised in the case of CD28 triggering. Although CTLA-4 triggering had little effect on its own, it inhibited CD28-induced actin rearrangement and polarisation. Phorbol myristate acetate (PMA) treatment served as a positive control showing F-actin upregulation with little directionality and polarisation. These experiments provide further evidence to suggest that TCR-independent signalling by CD28/CTLA-4 regulates Treg migration, as no TCR/CD3 stimulation was present.


a
anti-CD28
anti-CTLA-4
anti-CD28 +
anti-CTLA-4
Isotype
PMA

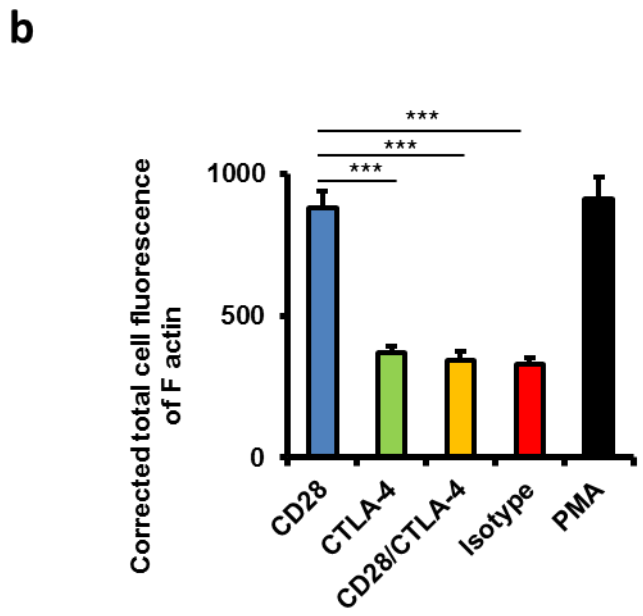


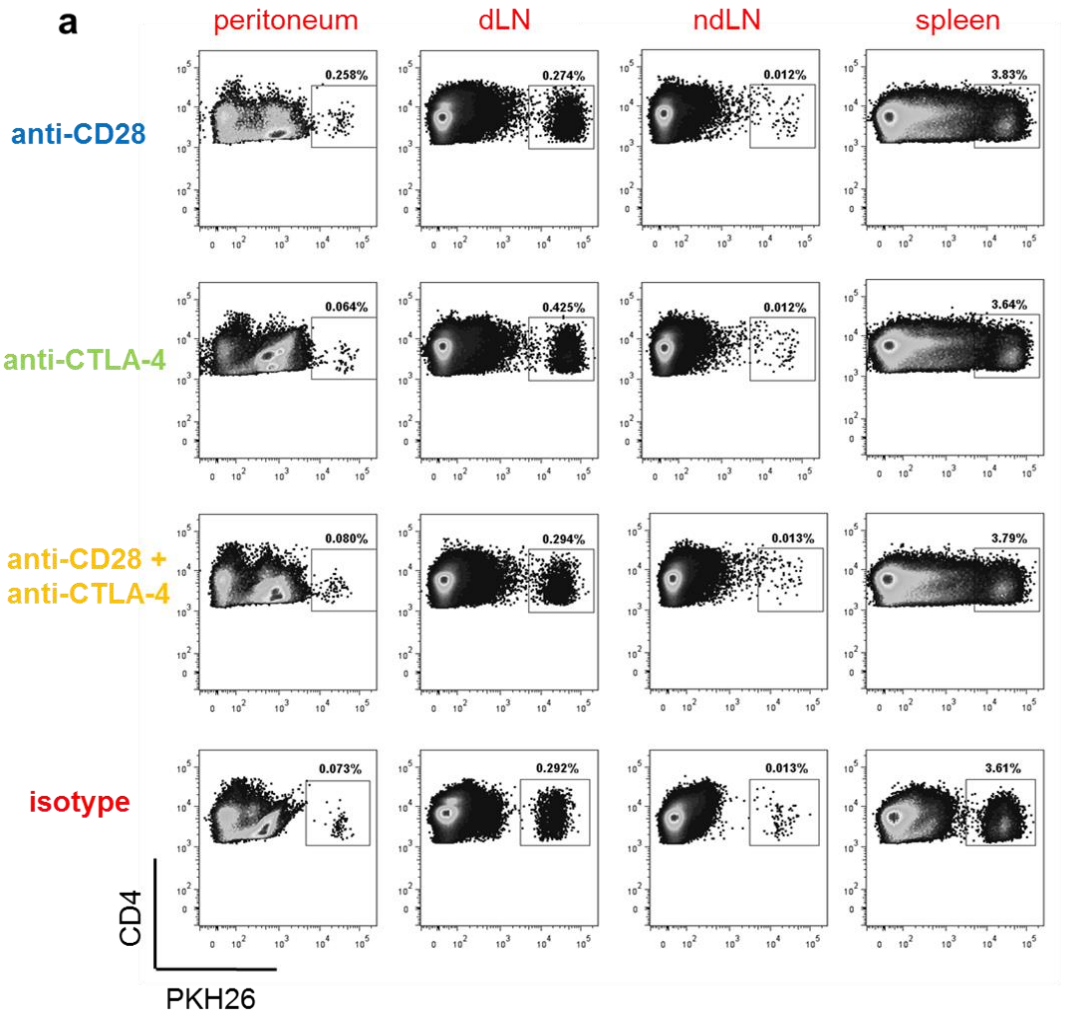

**Figure 34 CTLA-4 inhibits CD28 induced actin reorganisation and polarisation.**

*CD4+CD25+Foxp3+ Tregs were isolated from Foxp3-GFP C57BL6 mice and expanded through co-culture with sub-lethally irradiated immature bone marrow-derived allogeneic (H2-D) dendritic cells. Tregs were harvested after seven days and added to wells of a tissue culture plate containing glass coverslips coated with anti CD28 and/or anti CTLA-4 antibodies. As controls, an isotype-matched control antibody was used for coating and PMA was added in the medium of some wells as a positive control for F actin up regulation. Cells were quickly spun down at low speeds to facilitate attachment, and subsequently fixed, pemeabilised and stained for F actin (Red) and DNA (Blue). The coverslips were mounted and imaged using a confocal microscope. (**a**)Images represent maximum projections of a Z scan of ten 1um slices. CD28 stimulation increased F actin and polarisation of F actin (arrows), while CTLA-4 did not. CTLA-4 triggering together with CD28 prevented F actin upregulation and polarisation. (**b**) Bar graph represents the mean of the corrected total cell fluorescence of F actin from 15-20 individually observed cells using Image J. Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. (n=3, N=4) *** p<0.005*

Following *in vitro* trans-endothelial migration assays, we carried out similar experiments *in vivo* using the intra-peritoneal model of T lymphocyte recruitment. CD4+CD25 Tregs isolated through immunomagnetic selection from H2-B background mice were co-cultured with sub lethally irradiated allogeneic (H2-D) bmDCs and IL-2 to selectively expand Tregs crossreactive to H2-D alloantigens.

To establish whether CD28 and CTLA-4 co-stimulation can regulate Treg migration into non-lymphoid tissue *in vivo*, prior to adoptive transfer, allospecific (H2-D) Tregs were treated with CD28, CTLA-4 or both crosslinking antibodies and their migration into the peritoneal cavity of either allogeneic H2-D (BALB/c) or irrelevant H2-K (CBA/Ca) donor mice given IFN-γ i.p. injection 48 hours earlier was observed 24 hours later. Thus antigen presentation and TCR engagement would only occur in H2-D background recipients.

These *in vivo* experiments recapitulated the *in vitro* findings in that greater number of Tregs accumulated in the peritoneal cavity of mice when the Tregs were pre-treated with CD28 stimulation (Figure 35). Fewer Tregs were recovered from the peritoneal lavage when CTLA-4 stimulation was given together with CD28 stimulus while CTLA-4 triggering on its own did not have a significant effect. Interestingly, similar results were also obtained when using H2-K, CBA/Ca irrelevant) as recipient mice (Figure 36). This experiment further suggested that CD28 and CTLA-4 regulate trafficking of Tregs into target non lymphoid tissue independently of the TCR signal.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dLN
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spleen
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3.83%
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0.425%
0.012%
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0.073%
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ndLN
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Treg number
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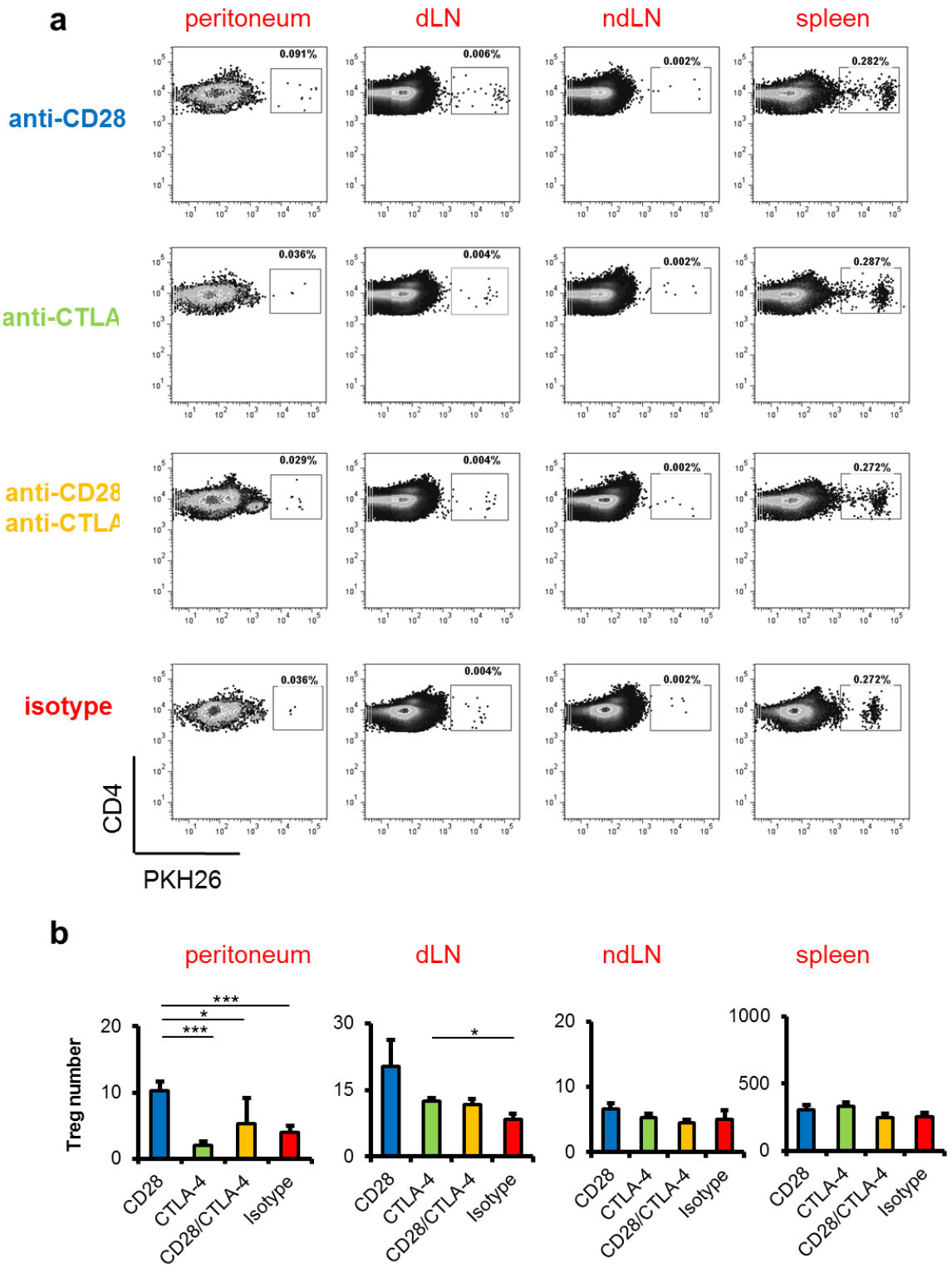
CD28
CTLA-4
CD28/CTLA-4
Isotype

**Figure 35 CTLA-4 prevents CD28-induced Treg migration *in vivo*.**

*In vitro expanded allospecific Tregs were treated with CD28 and/or CTLA-4 antibody-mediated ligation. Tregs were then labelled with PKH26 and injected intravenously into BALB/c (allogeneic) mice that were given intra-peritoneal injections of IFN-γ 48 hours earlier. Equal number of Tregs treated with corresponding isotype control and secondary antibodies were labelled with CFSE and co-injected. 24 hours later, cells were recovered from the indicated tissues and analysed using flow cytometry. (**a**)Dot plots shown represents percentage of Treg cells in the recovered CD4+ donor population. (**b**) Bar graphs indicate absolute number of labelled cells recovered. Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. (n=4, N=3) * p<0.05*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Treg number
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CD28
CTLA-4
CD28/CTLA-4
Isotype

**Figure 36 CD28 and CTLA-4 regulate Treg migration independently of the TCR signals induced by antigen recognition *in vivo*.**

*Tregs isolated from H2-B (C57BL/6) mice and expanded by in vitro co-culture with immature H2-D (BALB/c) derived bmDCs to generate H2-D allospecific Tregs. These allospecific Tregs were then treated with CD28 and/or CTLA-4 antibody-mediated ligation, labelled with PKH26 and injected intravenously into H2-K (CBA/Ca, irrelevant) mice that were given intra-peritoneal injections of IFN-γ 48 hours earlier. As before, equal number of Tregs treated with an isotype control and secondary antibodies were labelled with CFSE and co-injected. Cells were then recovered from indicated tissues 24 hours later and analysed using flow cytometry.(**a**)Dot plots shown represents percentage of Treg cells in the recovered CD4+ donor populations.(**b**) Bar graphs indicate absolute number of labelled cells recovered . Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. (n=4, N=3) * p<0.05*

#### 4.2.3.4 Glycolytic pathways are essential for Treg migration

In view of the above findings we sought to investigate other signalling pathways and mechanisms through which CD28 and CTLA-4 co-receptors might be able to exert their effects on Treg migration. Previously, CD28-mediated induction of PI3K signalling was shown to be necessary for effective localisation to antigenic sites following antigen rechallenge (Mirenda et al. 2007). In conventional T cells, both CD28 and CTLA-4 have been shown to activate the PI3K-Akt pathway (Helga Schneider et al. 2008). As mentioned earlier in Section 1.3.4.1, the PI3k-Akt and subsequent downstream mTOR pathways initiated by CD28 are known to upregulate glucose metabolism. With this in mind and our own recent evidence suggesting that aerobic glycolysis is necessary for T cell migration (R. Haas et al. 2015); we set out to investigate whether metabolic changes induced by CD28/CTLA-4 could be responsible for the regulation of Treg migration by these receptors.

First we sought to identify the metabolic pathways utilized by Tregs cells for migration. Unlike conventional and effector T cells, Tregs are known to utilize AMP kinase activity and preferentially use lipid oxidation for their energy requirements (Michalek et al. 2011c) during differentiation and development. Thus, we first tested the hypothesis that Tregs may use lipid oxidation as a means to provide energy for migration. H2-D allospecific Tregs were assessed for their migration through H2-B (syngeneic) endothelial monolayers in an *in vitro* trans-migration assays where the Tregs were pre-treated with Etomoxir, an inhibitor of fatty acid oxidation. Etomoxir blocks the activity of the enzyme carnitine palmitoyltransferase 1A (CPT1-A) which shuttles fatty acids into the inner membrane of the mitochondria for oxidation. No differences in Treg migration following treatment with Etomoxir were observed (Figure 37). Similarly, following treatment with Etomoxir, H2-D allospecific Tregs that were injected *in vivo* into IFN-γ i.p injected H2-B recipient mice did not reveal any differences in migration to the peritoneal cavity as compared to untreated Tregs (Figure 38).

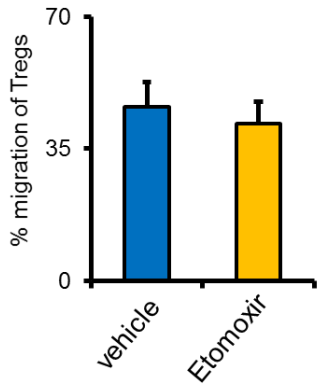



**Figure 37 Blockade of Fatty acid oxidation does not inhibit Treg migration *in vitro.***

*In vitro expanded Tregs, removed after 7 days of co-culture were treated with the CPT-A inhibitor etomoxir for 4 hours before being placed over IFN-γ-treated syngeneic endothelial cell monolayers grown on a 3-µm pore polycarbonate transwell and incubated for 24 hours at 37°C with 5% $CO_2$ . Tregs treated with the vehicle only were used as a control. The migrating cells at the bottom were determined through hemocytrometric counting. Results are expressed as a percentage of migrating cells after 24 hours. Error bars represent standard deviation. Statistical significance was calculated with unpaired Student's t-test. (n=4, N=3)*

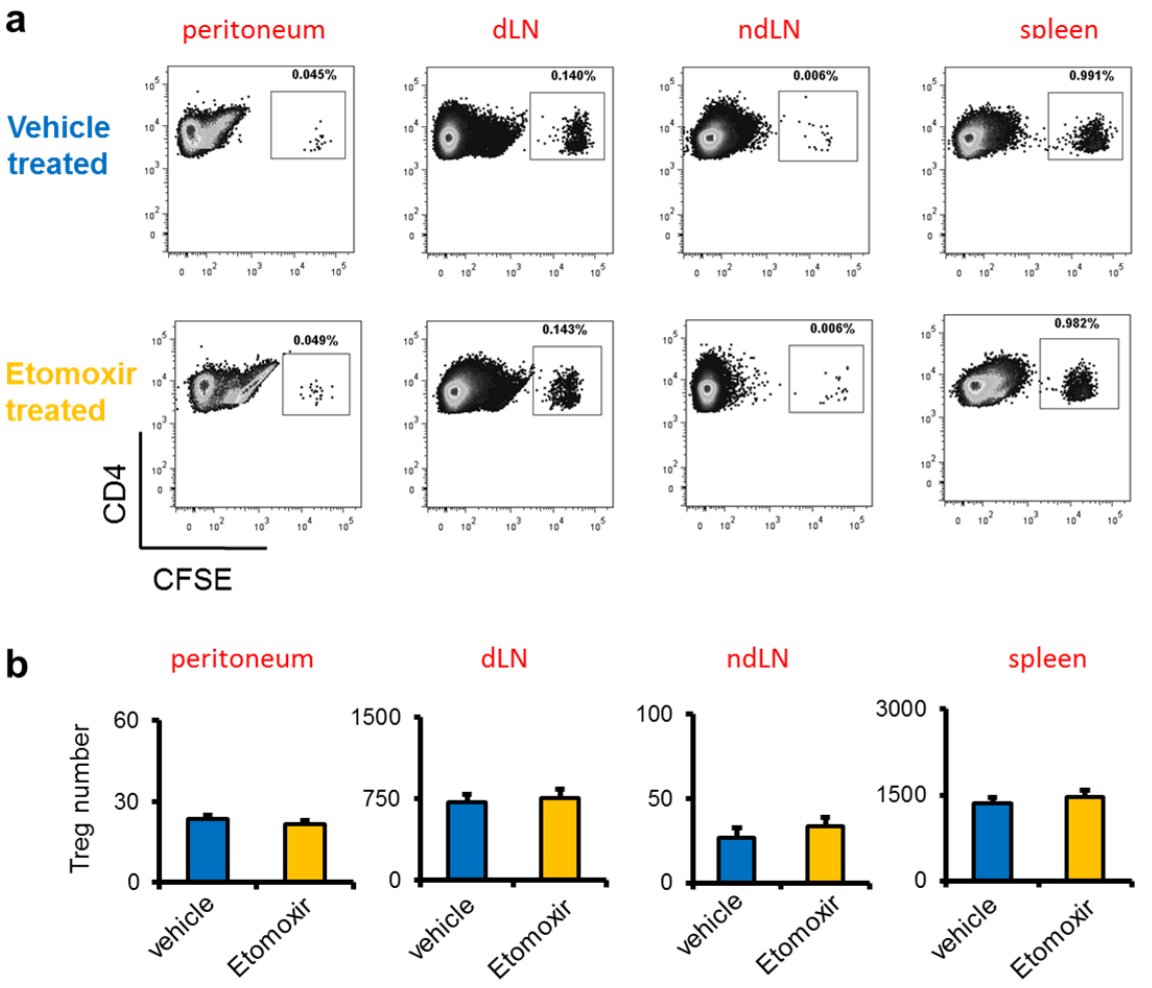



**Figure 38 Blockade of Fatty acid oxidation does not inhibit Treg migration *in vivo*.**

*In vitro expanded Tregs, removed after 7 days of co-culture were treated with the CPT1-A inhibitor Etomoxir for 4 hours before being labelled with PKH26 and intravenously injected into C57BL/6 (syngeneic) mice that had received an intraperitoneal injection of IFN-γ 48-72 hours earlier. Equal number of vehicle treated, CFSE labelled Tregs were co-injected as a control. Cells were recovered from tissues indicated after 24 hours and analysed using flow cytometry.(**a**)Dot plots show percentage of Treg cells in the recovered in CD4+ donor populations from one representative(n=4). (**b**) Bar graphs indicate average of absolute number of labelled cells recovered .Error bars represent standard deviation (n=4)*

While oxidation of fatty acids has been shown to be the dominant metabolic pathway utilised by Tregs under steady state, it is possible that glucose metabolism in Tregs is retained for other highly energy-dependent processes such as migration. We therefore tested the possibility that, like conventional T cells, Tregs utilise glycolysis for migration by inhibiting glycolysis using the glucose analogue 2-deoxyglucose (2-DG). Migration of Tregs was inhibited by the glycolysis inhibitor 2-DG both *in vitro* (Figure 39) and *in vivo* (Figure 40).

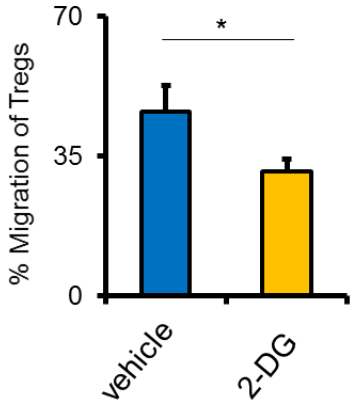



**Figure 39 Blocking Glycolysis abrogates Treg migration *in vitro.***

*In vitro expanded Tregs, harvested after 7 days of co-culture were treated with the glycolysis inhibitor 2-DG for 4 hours before being placed over IFN-γ-treated syngeneic endothelial cell monolayers grown on a 3-µm pore polycarbonate transwell and incubated for 24 hours at 37°C with 5% $CO_2$ . Tregs treated with a vehicle only were used as a control. The migrating cells at the bottom were determined through hemocytrometric counting. Results are expressed as a percentage of migrated cells after 24 hours. Error bars represent standard deviation. Statistical significance was calculated with unpaired Student's t-test. (n=4, N=3) * P>0.05*

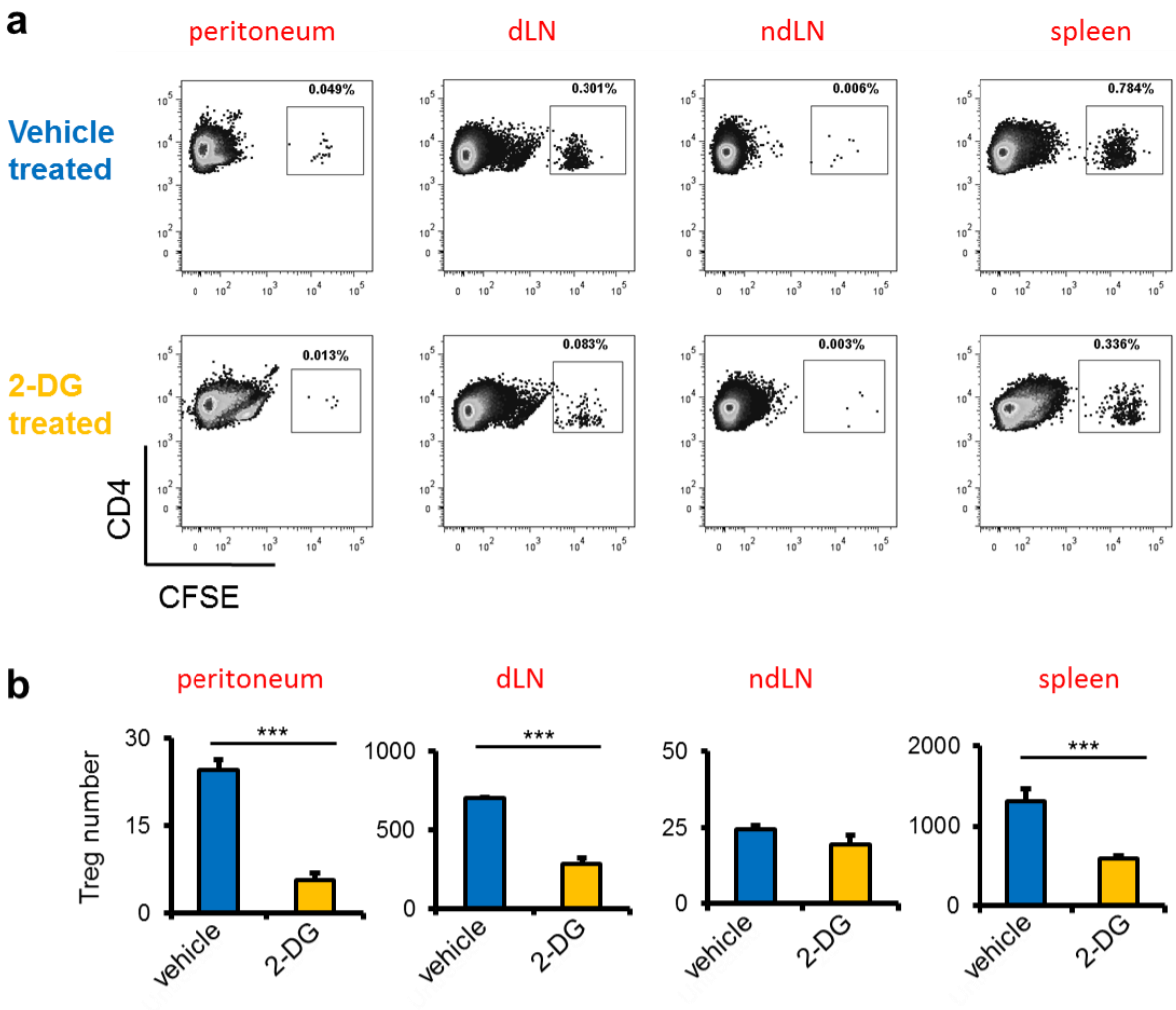



**Figure 40 Blocking Glycolysis abrogates Treg migration *in vivo.***

*In vitro expanded Tregs, harvested after 7 days of co-culture were treated with the glycolysis inhibitor 2-DG for 4 hours before being labelled with PKH26 and intravenously injected into C57BL/6 (syngeneic) mice that had received an intraperitoneal injection of IFN-γ 48-72 hours earlier. Equal numbers of vehicle-treated, CFSE-labelled Tregs were co-injected as a control. Cells were recovered from tissues indicated after 24 hours and analysed using flow cytometry. (**a**)Dot plots shows percentage of Treg cells in the recovered in CD4+ donor populations from one representative. (**b**) Bar graphs indicate average of absolute number of labelled cells recovered. Error bars represent standard deviation. Statistical significance was calculated with unpaired Student's t-test. (n=4, N=3) * p<0.05*

In line with these experiments, activation of glycolysis using the electron transfer chain Complex I inhibitor, metformin, which stimulates glycolysis via AMPK, increased Treg migration *in vitro* (Figure 41) and *in vivo* (Figure 42).

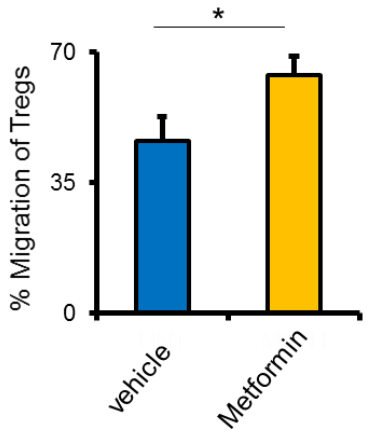



**Figure 41 The anti-diabetic drug metformin increases Treg migration *in vitro.***

*In vitro expanded Tregs, harvested after 7 days of co-culture were treated with metformin for 4 hours before being placed over IFN-γ-treated syngeneic endothelial cell monolayers grown on a 3-µm pore polycarbonate transwell and incubated for 24 hours at 37°C with 5% $CO_2$. Tregs treated with a vehicle only were used as a control. The migrating cells at the bottom were determined through hemocytrometric counting. Results are expressed as a percentage of migrated cells after 24 hours. Error bars represent standard deviation. Statistical significance was calculated with unpaired Student's t-test. (n=4, N=3) * P>0.05*

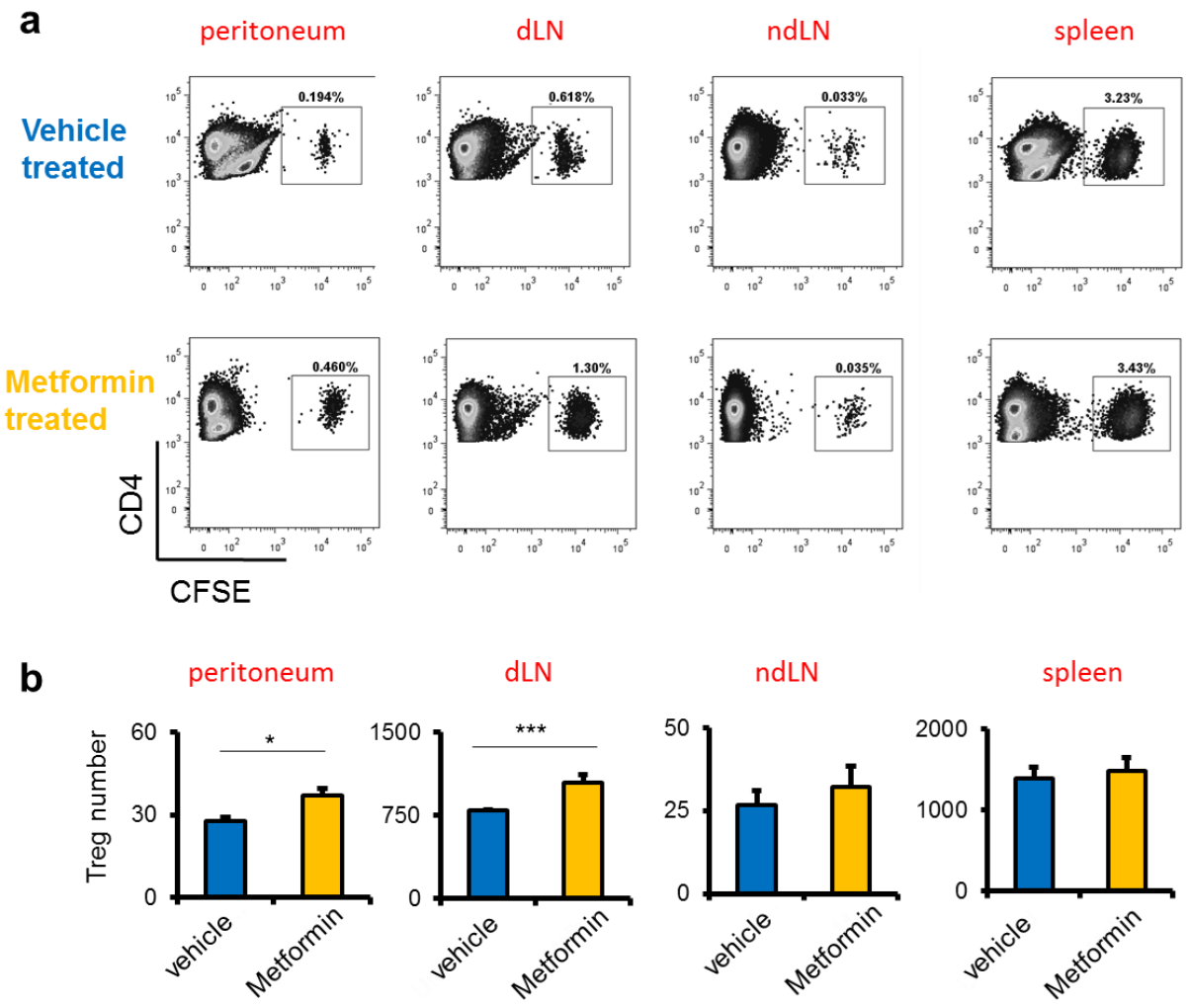



**Figure 42 The anti-diabetic drug metformin increases Treg migration *in vivo*.**

*In vitro expanded Tregs, harvested after 7 days of co-culture were treated with metformin for 4 hours before being labelled with PKH26 and intravenously injected into C57BL/6 (syngeneic) mice that had received an intraperitoneal injection of IFN-γ 48-72 hours earlier. Equal number of vehicle treated, CFSE labelled Tregs were co-injected as a control. Cells were recovered from tissues indicated after 24 hours and analysed using flow cytometry. (**a**)Dot plots shows percentage of Treg cells in the recovered in CD4+ donor populations from one representative. (**b**) Bar graphs indicate average of absolute number of labelled cells recovered. Error bars represent standard deviation. Statistical significance was calculated with unpaired Student's t-test. (n=4, N=3) * p<0.05*

Prolonged use of metabolic inhibitors can significantly impact cell survival and can thus influence migration studies. Therefore, we measured cell apoptosis after treatment with the metabolic inhibitors using an annexin V/propium iodide staining assay. Exposure to the various metabolic inhibitors, at the concentrations and durations used, did not affect the surface molecule phenotype (Figure 43), or survival of the Treg population (Figure 44).

Collectively, these results suggest that Tregs utilise glycolysis as a means of energy production for migration rather than the dominant fatty acid oxidation pathway used for energy during a resting state.

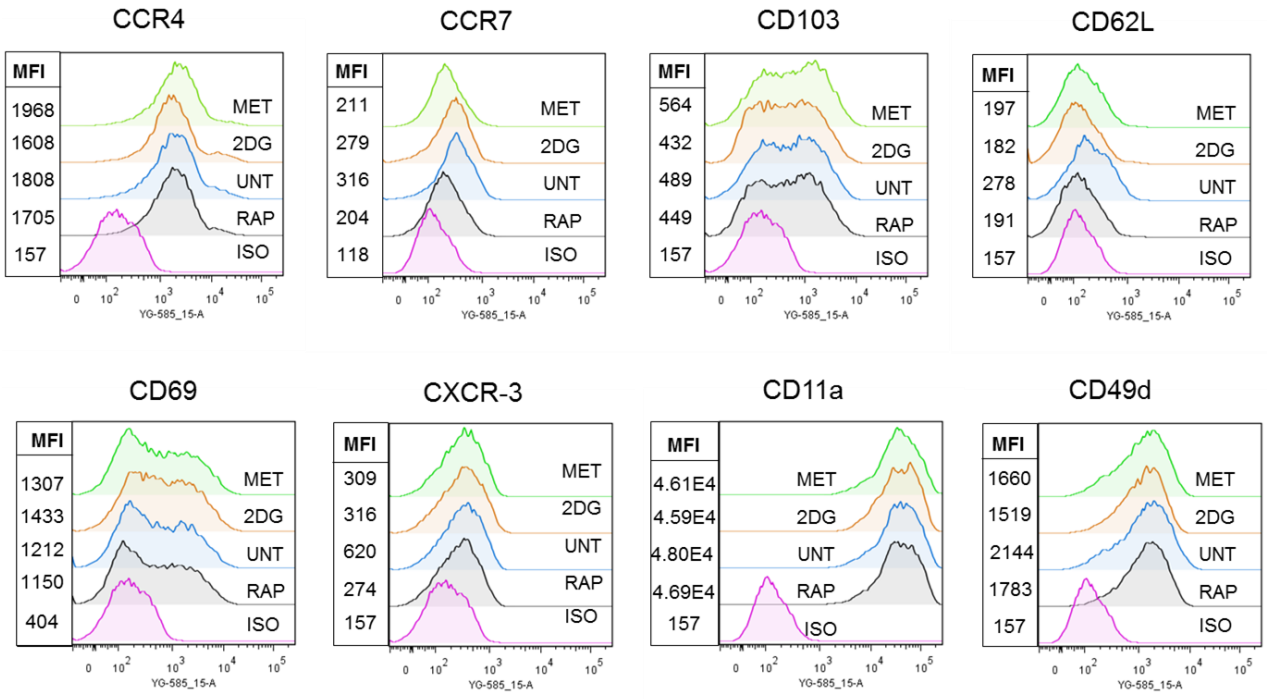



**Figure 43 Flow cytometric characterisation of Tregs treated with various metabolic inhibitors.**

*In vitro expanded Tregs, harvested after 7 days of co-culture were treated with the indicated metabolic inhibitors for a period of 4-6 hours before being analyzed using flow cytometry. Histograms represent staining while the adjacent numerical values represent the mean fluorescence intensity of staining. Isotype–matched control for the antibodies used is denoted in the bottom of each plot (pink histograms). n=3, N=2*

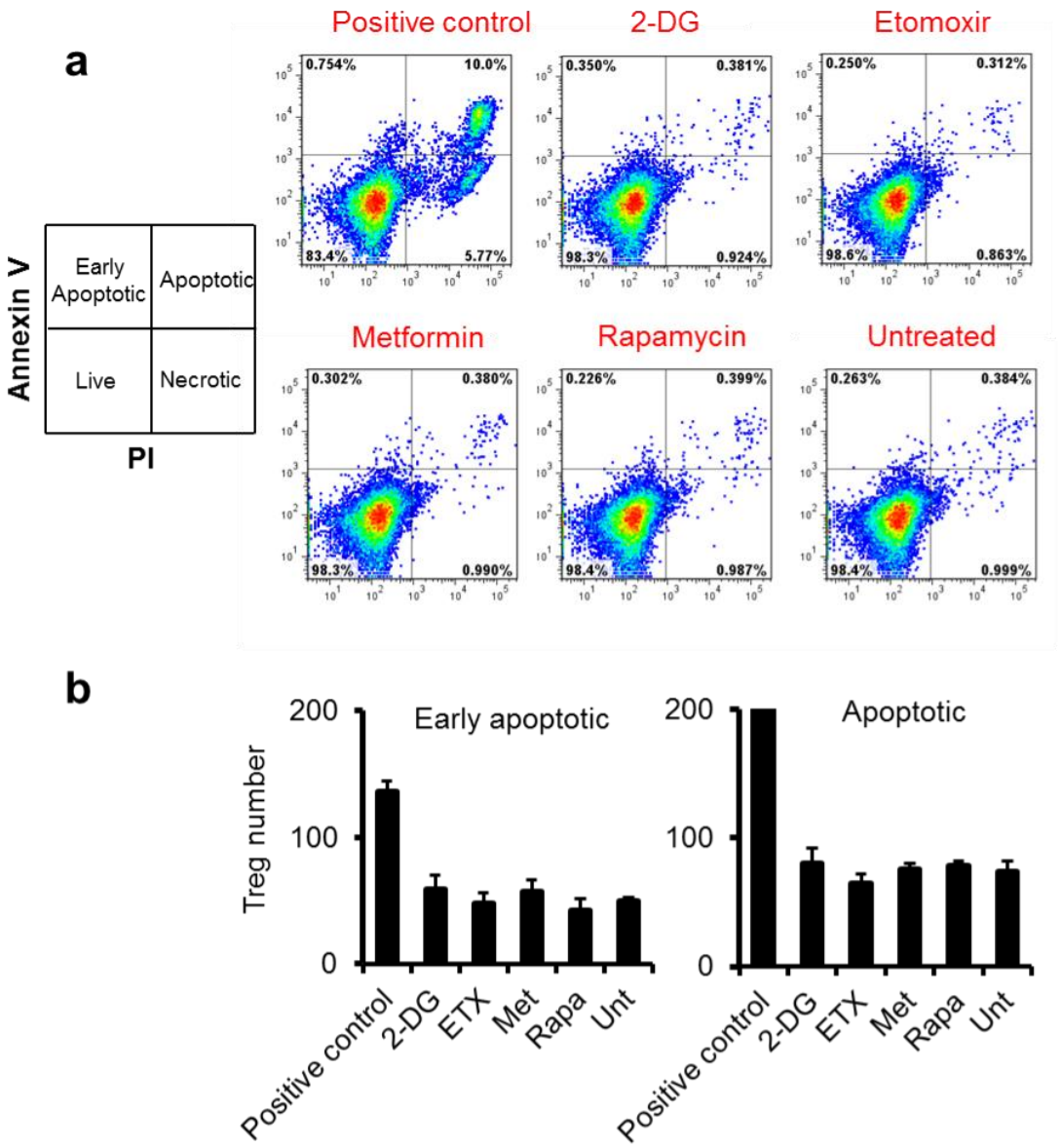



**Figure 44 Metabolic inhibitors did not induce apoptosis in Tregs**

*Apoptosis of Tregs treated with the various metabolic inhibitors/drugs indicated was measured using an annexin V/propium iodide live/dead cell distinguishing assay. Following treatment with the metabolic inhibitors, Tregs were analysed using flow cytometry and divided into live (Annexin V -ve, PI -ve), necrotic (Annexin V -ve, PI +ve), early apoptotic (Annexin +ve, PI -ve) and apoptotic (Annexin +ve, PI +ve) cell populations. Untreated Tregs and Tregs subjected to heat induced apoptosis were used as negative and positive controls respectively. (**a**)The quadrant dot plots are representative of one data set. (**b**) The bar graphs represent cumulative data showing the means of the percentages of apoptotic and early apoptotic Treg cells. (n=4, N=3).Error bars are indicative of standard deviations.*

#### 4.2.3.5 CD28/CTLA4 regulate glycolysis in Tregs

The central hypothesis of this study is that CD28 and CTLA-4 may regulate Treg migration via signaling pathways that affect Treg metabolism. Therefore, we first investigated the effects of CD28 and CTLA-4 signals on the extracellular acidification rate (ECAR), which quantifies proton production as a proxy for lactate production, and thus reflects overall glycolytic flux; and the oxygen consumption rate (OCR), a measure of mitochondrial respiration, in real time via the use of the Seahorse analyzer. CD4+Foxp3+ CD25+ Tregs isolated from the spleen and lymph nodes of Foxp3-GFP reporter mice mouse, through flow activated cell sorting (FACS), were co-cultured with sub-lethally irradiated immature bmDCs and IL-2 for 7 days. Following incubation, expanded Treg populations were plated into the XF analyser for real-time analysis of ECAR and OCR in response to CD28 and or CTLA-4 ligation. Antibody mediated triggering of the CD28 receptor on Tregs doubled the ECAR upon glucose addition compared to the cells treated with an isotype control highlighting an increase in the overall ECAR following CD28 stimulation (Figure 45). Interestingly, triggering the CTLA-4 receptor did not have an effect on the ECAR on its own but when activated during CD28 triggering CTLA-4 signals prevented CD28-induced ECAR upregulation. The OCR did not seem to differ in any of the conditions tested (Figure 46).

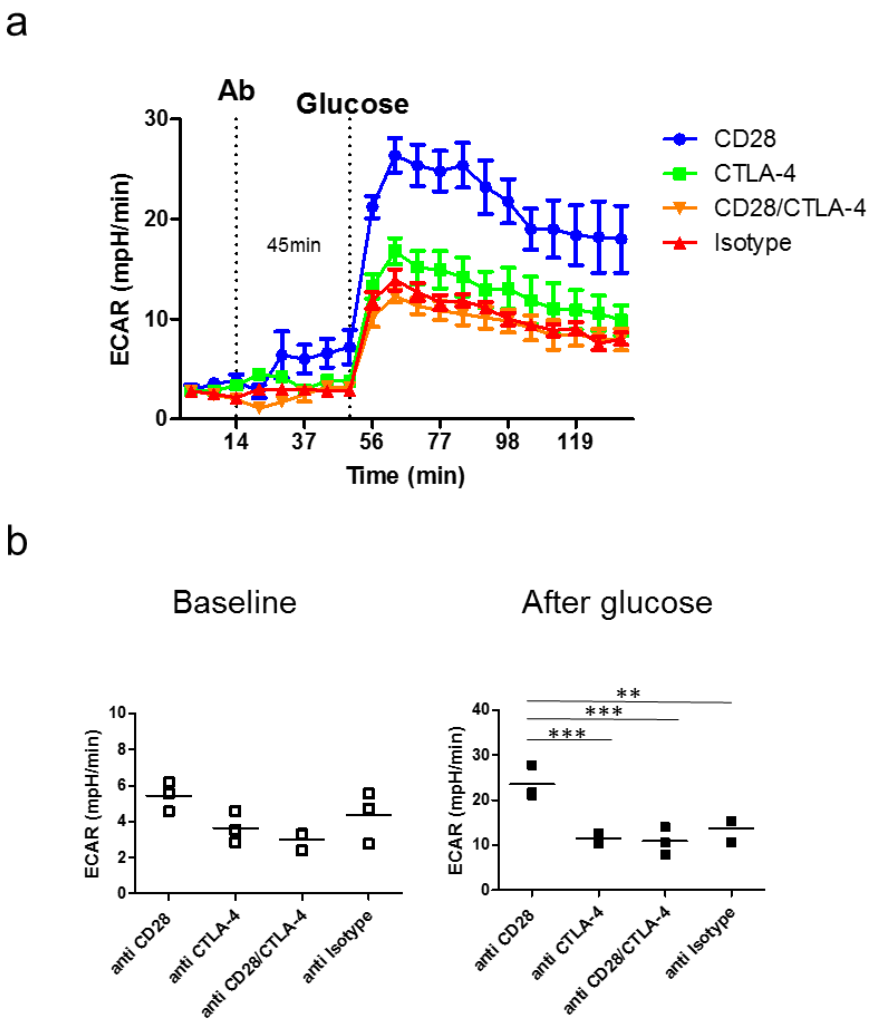



**Figure 45 CTLA-4 inhibited CD28 induced upregulation of ECAR in Foxp3+ve Tregs**

*In vitro expanded Tregs, harvested after 7 days were first rested in serum free, unbuffered XF assay medium (Seahorse biosciences) for 1 hour. Cells were subsequently seeded (6X10$^5$/well) into the seahorse XF24 cell plates for analysis. Real time analysis of ECAR was performed using the XF analyser. Wells were injected first with anti CD28 and/or anti CTLA-4 antibodies along with an appropriate secondary antibody for crosslinking. Isotype-matched and secondary antibodies were used as controls. A second injection followed at the time point indicated (dashed line) introducing D-glucose into the wells. (**a**) The line data plot shows real time analysis of ECAR. (**b**) Cumulative data here indicates the mean from three experiments (n=5, N=3) at (i) baseline and (ii) after addition of glucose. Statistical test involved using a one way ANOVA with Bonferroni correction *p>0.05, **p>0.005, ***p>0.0005*

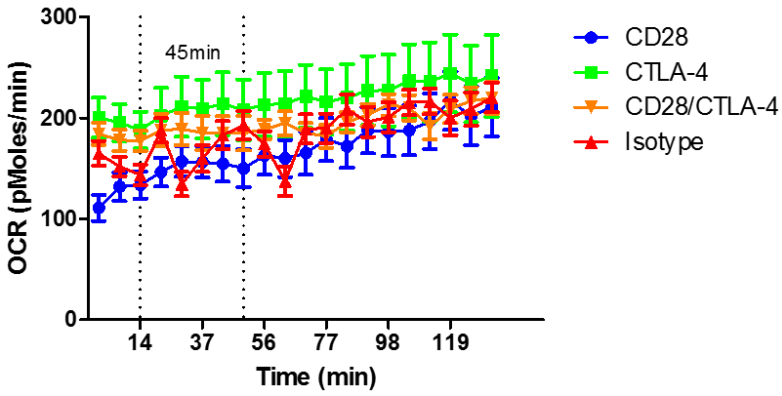



**Figure 46 The OCR of Foxp3 +ve Tregs does not change in response to CD28/CTLA-4 stimulation**

*In vitro expanded Tregs, harvested after 7 days were first rested in serum free, unbuffered XF assay medium (Seahorse biosciences) for 1 hour following which they were seeded (6X10$^5$/well) into the seahorse XF24 cell plates for analysis. Real time analysis of OCR was performed using the XF analyser. Wells were injected first with anti CD28 and/or anti CTLA-4 antibodies along with an appropriate secondary antibody for crosslinking. Isotype-matched and secondary antibodies were used as controls. A second injection followed at the time point indicated (dashed line) introducing D-glucose into the wells. Statistical test involved using a one-way ANOVA with Bonferroni correction.*

CD28 signaling in conventional T cells was previously shown to increase the expression of surface glucose transporter Glut1 (Jacobs et al. 2008). We therefore measured glucose uptake by Tregs in response to CD28 and/or CTLA-4 signaling by using the glucose uptake reporter 6NBDG. 6NBDG does not undergo phosphorylation by the hexokinases and thus accumulates within the cells in its fluorescent form. CD4+ CD25+ Tregs isolated from the spleen and lymph nodes of C57BL/6 mice, through flow activated cell sorting (FACS), were co-cultured with sub lethally irradiated immature bmDCs and IL-2 for 7 days. Following incubations, expanded Tregs were removed from culture and subjected to glucose starvation for 1 hour. Tregs were then cultured in 6NBDG-containing, glucose-free, serum-free medium following antibody-mediated CD28 and/or CTLA-4 crosslinking. 6NBDG uptake was enhanced in response to CD28 stimulation (Figure 47). As expected, CTLA-4 stimulation on its own did not affect 6NBDG uptake, but when present along with CD28 stimulation it inhibited the CD28-induced enhancement of 6NBDG uptake. These results suggest that CD28 signaling increases glycolysis, while CTLA-4 can prevent CD28-induced increase of glycolysis.

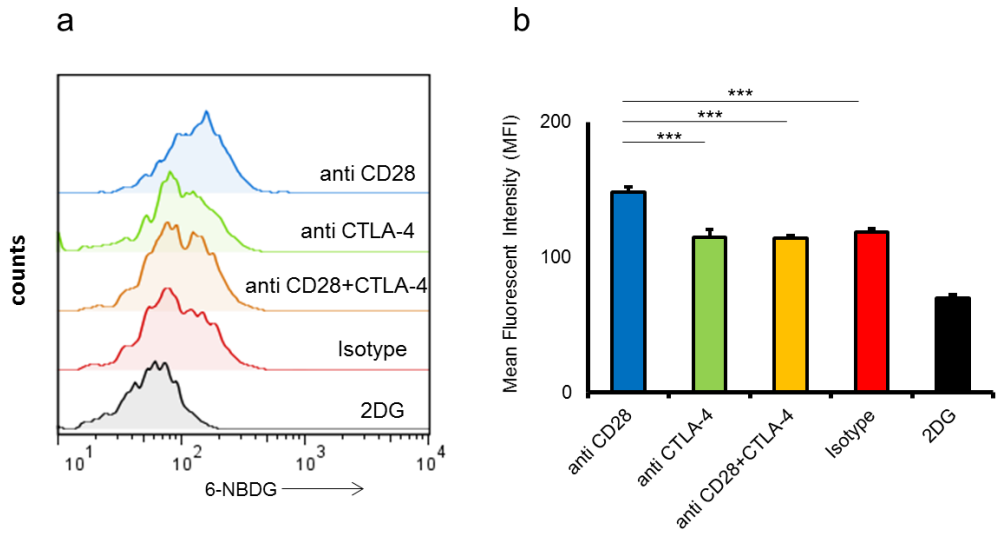



**Figure 47 CTLA-4 inhibits CD28-induced glucose uptake in Tregs**

*In vitro expanded Tregs, harvested after 7 days were first rested in glucose free and serum free, T cell medium for 1 hour. T cells underwent antibody stimulation of CD28 and/or CTLA-4 as previously described. Tregs were then re-suspended in medium containing the glucose uptake indicator 6-NBDG for 10 minutes after which they were placed on ice and analyzed immediately by flow cytometry. Non-fluorescent glucose analog 2-DG was used as a negative control for staining. (**a**) The histograms are representative of all conditions in one representative experiment. (**b**) The bar graph represents the average MFI from 3 different experiments. (n=3, N=3). Error bars represent standard deviation. Statistical significance was calculated with unpaired Student's t-test. ***P>0.005*

**CD28/CTLA-4 regulates Treg migration through modulation of glucose metabolism**

To test whether CD28/CTLA-4-induced changes to Treg glycolysis affected their migration, we compared Treg migration in the presence or absence of glucose in the surrounding medium following activation of CD28, CLTA-4 or both receptors in trans-endothelial migration assays. As expected, CD28 stimulation induced an increase in migration of Tregs in glucose-sufficient medium. In contrast, Treg migration was not modified by CD28 stimulation in the absence of glucose (Figure 48), suggesting that glucose uptake is necessary for the effects of CD28 signals on Treg motility. The results were not influenced by any cell death associated with glucose deprivation as baseline levels of migration remained similar in both glucose sufficient and deficient medium (Figure 48) and as shown by trypan blue mediated live/dead cell staining performed at the end of the assay (Figure 49).

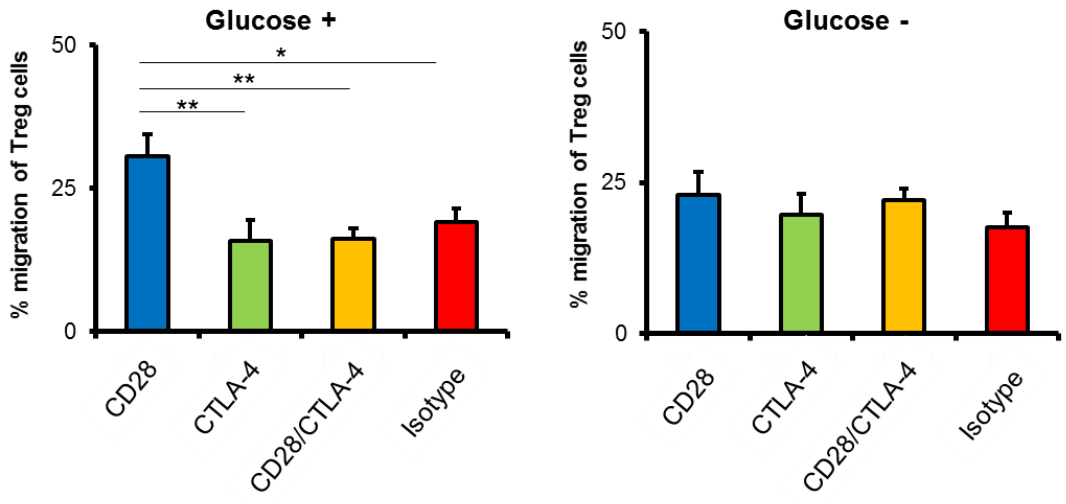



**Figure 48 Extracellular Glucose is essential for CD28- induced Treg migration *in vitro.***

*In vitro expanded Tregs, harvested after 7 days of co-culture were treated with CD28 and/or CTLA-4 stimulation before being placed over IFN-γ-treated syngeneic endothelial cell monolayers grown on a 3-µm pore polycarbonate transwell and incubated for 24 hours at 37°C with 5% $CO_2$. Tregs treated with the isotype-matched and secondary antibodies were used as a control. In these assays, Tregs were re-suspended in either serum-free or serum-free & glucose-free medium. Migrated cells in the bottom chamber were enumerated by hemocytometric counting. Results are expressed as a percentage of migrated cells after 24 hours. Error bars represent standard error of means. Statistical significance was calculated with unpaired Student's t-test. * P>0.05, (n=3, N=4)*

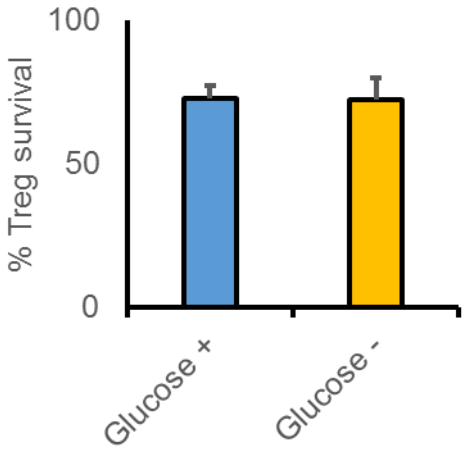



**Figure 49 Tregs display similar survival in glucose free medium**

*In vitro expanded Tregs, harvested after 7 days of co-culture with immature bmDCs were incubated for 24 hours at 37°C with 5% $CO_2$ in either serum-free or serum-free, glucose-free medium. Live/dead cell discrimination of Tregs from both media was then assessed through trypan blue staining and hemocytometric counting. Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. (n=3, N=3)*

Next, we sought to investigate whether this effect could be observed in a physiological environment *in vivo*, using a previously described tissue infiltration model measuring infiltration of i.p. injected T cells into the peritoneal membrane (Mirenda et al. 2007). Tregs were first subjected to antibody-mediated CD28 and CTLA-4 stimulation following which they were labeled with the *in vivo* tracking dye PKH26. Cells were then injected i.p. into recipient mice that were also given an i.p injection of 6NBDG simultaneously to visualize Treg glucose uptake in parallel with Treg migration. This approach overcomes the barrier posed by the rapid loss of fluorescence associated with 6NBDG injected via other routes that would otherwise make it impossible to track T cells and their glucose uptake when injected by a different route. CD28 stimulation led to an increased number of Tregs infiltrating the peritoneal membrane, which was accompanied by enhanced 6NBDG uptake by the infiltrating Treg cells. Both of these effects were prevented by CTLA-4 co-ligation (Figure 50). These results suggest that Treg cells, which acquire enhanced motility *in vivo* following CD28 stimulation, are also likely to enhance glucose consumption *in vivo*.

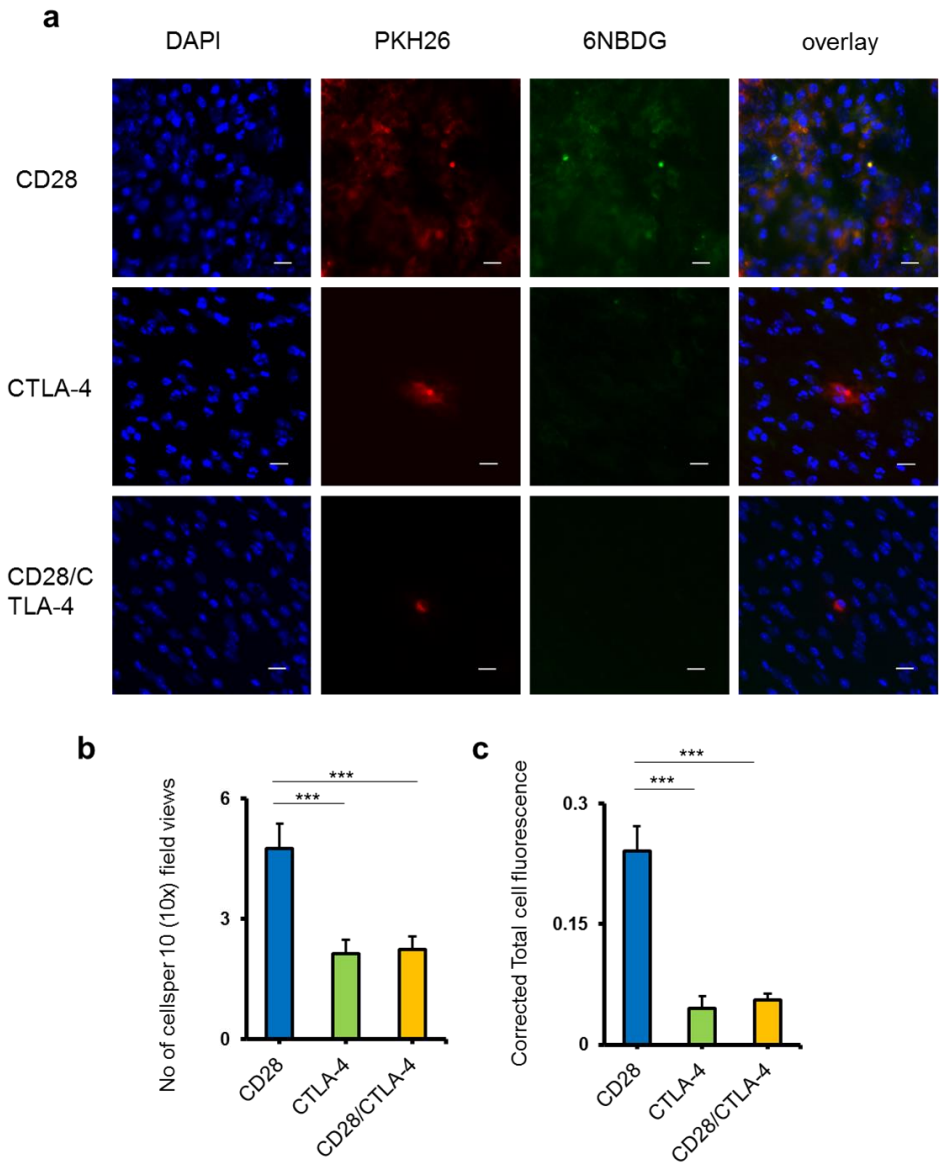



**Figure 50 CTLA-4 inhibition of CD28-induced glucose uptake prevents Treg migration *in vivo***

*In vitro expanded Tregs were treated with CD28 and/or CTLA-4 antibody-mediated ligation. Tregs were then labelled with PKH26 and injected via the intra-peritoneal route into C57BL/6 mice that were given intra-peritoneal injections of IFN-γ 48 hours earlier. A co-injection of 6NBDG was given immediately afterwards and the mice were sacrificed within 1 hour. The peritoneal membranes were removed and imaged using a fluorescence microscope to determine the number of infiltrating cells (**b**) and (**c**) 6NBDG (green) uptake by the infiltrating cells. DAPI was used as a nuclear stain. The images (**a**) are representative of the each condition where green and red can be seen together in tandem (yellow). (**b**) Bar graphs represent cumulative data indicating number of cells counted manually in 10 10x fields of view. (**c**) Bar graphs represent cumulative data showing the mean CTFC of 6NBDG from 10-12 cells from one 10x field of view. Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. *** p<0.0005 (n=4, N=2)*

We further explored the link between co-stimulation, migration and metabolic regulation by assessing the metabolic activity and migration of CTLA-4 KO Tregs. Mice deficient in CTLA-4 display uncontrolled T cell proliferation similar to lymphoproliferative disorder. Tregs were isolated from 4 week-old CTLA-4 KO (H2-B background) mice and expanded *in vitro* by co-culture with immature bmDCs from BALB/c (H2-D) mice to selectively expand H2-D allospecific CTLA-4 KO Tregs. H2-D allospecific Tregs from age-matched WT mice were used for comparison. A comparative analysis of ECAR between the WT and CTLA-4 KO Tregs, determined via the seahorse analyser, revealed that the glycolytic activity of CTLA-4-deficient cells was constitutively higher compared to that of their WT counterpart (Figure 51). Surprisingly, mitochondrial respiration was also significantly more elevated in CTLA-4 KO Tregs. These results suggest a hyper-metabolic signature displayed by CTLA-4 KO Tregs that is associated with their proliferation. Addition of a recombinant B7-1 (CD80) molecule in the Seahorse increased the glycolytic response of CTLA-4 Tregs, presumably due to dominant CD28 signalling, while their OCR remained unchanged. In contrast, the ECAR of CTLA-4-competent Treg cells remained unchanged, likely due to simultaneous activation of CD28 and CTLA-4 signalling by the B7-1 molecules.

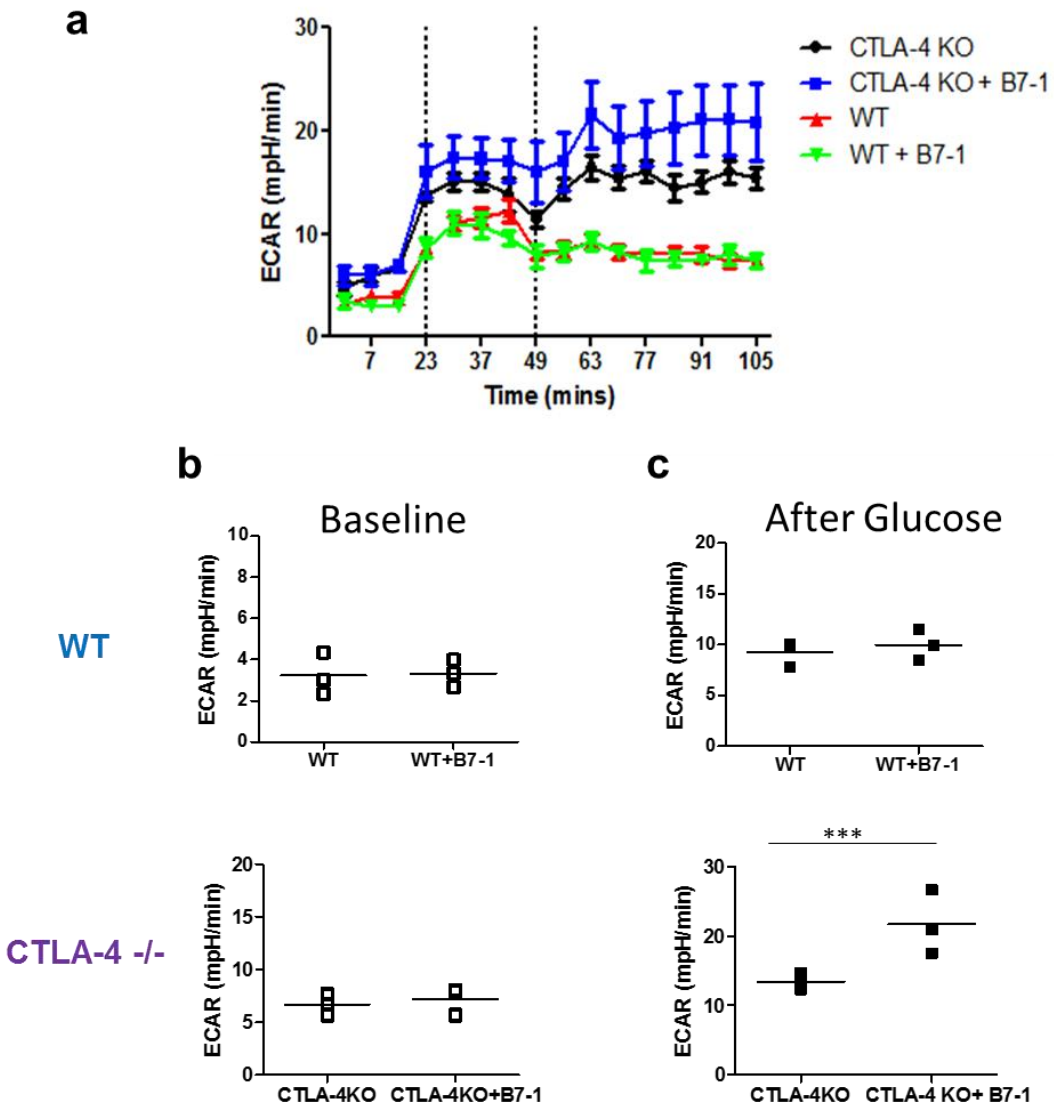



**Figure 51 CTLA-4KO Tregs display inherently higher ECAR compared to WT Tregs**

*CTLA-4 KO Tregs and WT Tregs isolated from lymph nodes and spleens of age matched CTLA-4KO or WT mice by immunomagnetic seperation were co-cultured with immature bmDCS and IL-2 to facilitate in vitro expansion. Expanded Tregs from both CTLA-4KO and WT mice were then analysed for their metabolic profiles using the Seahorse XF analyser. The plot depicts real time analysis of ECAR observed in CTLA-4KO Tregs using the XF analyser. Cumulative data here indicates the mean from three experiments at (**b**) baseline and (**c**) after addition of glucose. Statistical test involved using a one way ANOVA with Bonferroni correction *P>0.05 n=3, N=4*

In parallel, we tested H2-D allospecific CTLA-4 KO Treg migration through IFN-γ-activated syngeneic (H2-B) endothelium in an *in vitro* trans-endothelial migration assay following stimulation with B7-1 recombinant protein. Here, CTLA-4 KO Tregs displayed enhanced migration through the endothelium when subjected with B7-1 stimulation while CTLA-4-competent H2-D allospecific WT Tregs did not respond to any such stimulation (Figure 52). These results further suggest that CTLA-4 signals inhibit CD28 induced pro-migratory signals and associated glycolysis.

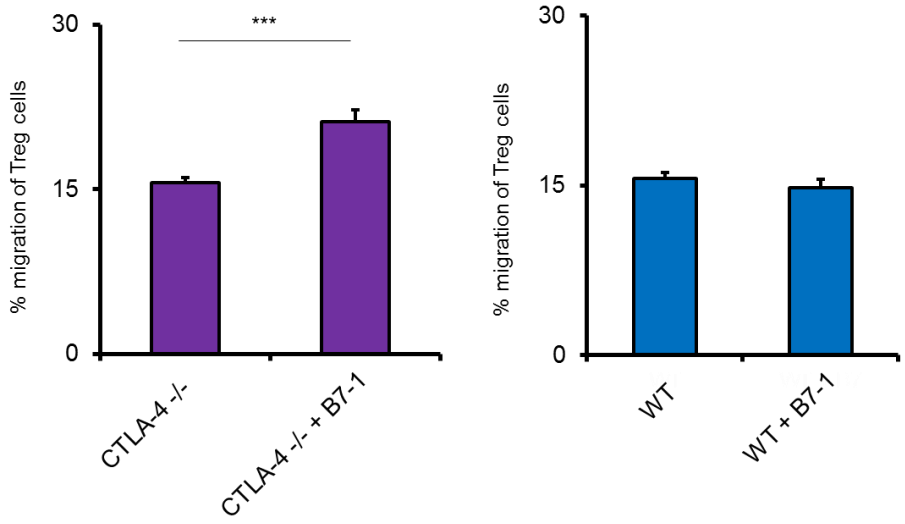



**Figure 52 CTLA-4KO Tregs increase migration in response to B7-1 stimulation.**

*CTLA-4 KO Tregs were isolated and expanded as described in the legend to Figure 44. Tregs were then placed over IFN-γ-treated allogeneic endothelial cell monolayers grown on a 3-µm pore polycarbonate transwell and incubated for 24 hours at 37°C with 5% $CO_2$. The number of migrated cells was evaluated by hemocytometric counting. Results were expressed as average percentage of migrated cells at 24 hours after migration. Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test * p< 0.05; ** p< 0.01 , n=3,N=4*

**CD28/CTLA-4 regulation of Treg migration is dependent on PI3K-Akt but independent of mTORC1**

In most T lymphocytes, the mTORC1 complex of the mTOR kinase couples upstream PI3K/Akt signalling with the control of glucose uptake and glycolysis (Chi 2012; Powell and Delgoffe 2010; Rao et al. 2010). Conversely, Tregs exhibit metabolic characteristics that are more akin to that of long lived CD8 memory T cells. CD8 memory T cells have higher mitochondrial mass than CD8 effectors and they use primarily fatty acid oxidation to meet their energy demands. Indeed CD4 Tregs also have a higher mitochondrial mass than that of CD4 effectors (Figure 53) suggesting an increased mitochondrial oxidative capacity.

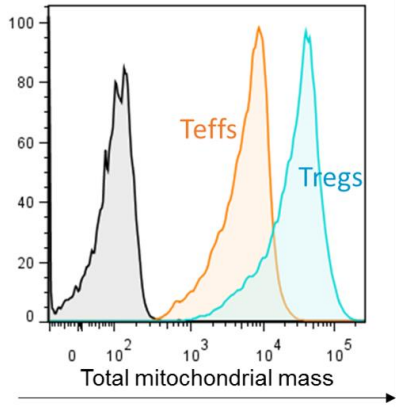



**Figure 53 CD4+CD25+ Tregs display higher mitochondrial mass than CD4+CD25- Teffs**

*Purified CD4+CD25+ Treg and CD4+CD25- Teff cells populations, isolated from lymph nodes of C57BL/6 mice by immunomagnetic separation were stained with mitotracker green dye and analysed using flow cytometry to indicate the total mitochondrial mass within the cells. These CD4+CD25+ Treg populations displayed high levels of Foxp3 expression (<95%). The histogram represents mitochondrial uptake of the mitotracker green dye in the Treg cells from 3 experiments. (n=3, N=3)*

Inhibiting mTORC1 with rapamycin favours the development of both CD8 memory T cells and CD4 Tregs after infection. However, Tregs still maintain mTORC1 activity for non-proliferative functions such as their ability to supress the activation of T cells (H. Zeng et al. 2013c). In (Mirenda et al. 2007) study, PI3K activation via CD28 was shown to be necessary for enhancement of memory CD8 T cell migration *in vivo.* In their study, abrogation of CD28 recruitment of PI3K through a point mutation in the cytoplasmic domain ($CD28^{Y170F}$) resulted in the loss of CD28-induced memory T cell migration. We therefore sought to investigate which components of this

PI3K-Akt-mTOR axis are essential for co-stimulation regulated Treg metabolism and subsequently migration.

We started by investigating the role of CD28-induction of the PI3K pathway on the metabolism and migration of Tregs isolated from CD28$^{Y170F}$ transgenic mice. The CD28 $^{Y170F}$ transgenic mice carry a point mutation in the cytoplasmic tail of CD28, which prevents recruitment and subsequent activation of PI3K in all T cells (Okkenhaug et al. 2001). These mice have normal Treg thymic output. However, they display fewer Tregs in the periphery and secondary lymphoid tissue (Figure 54).

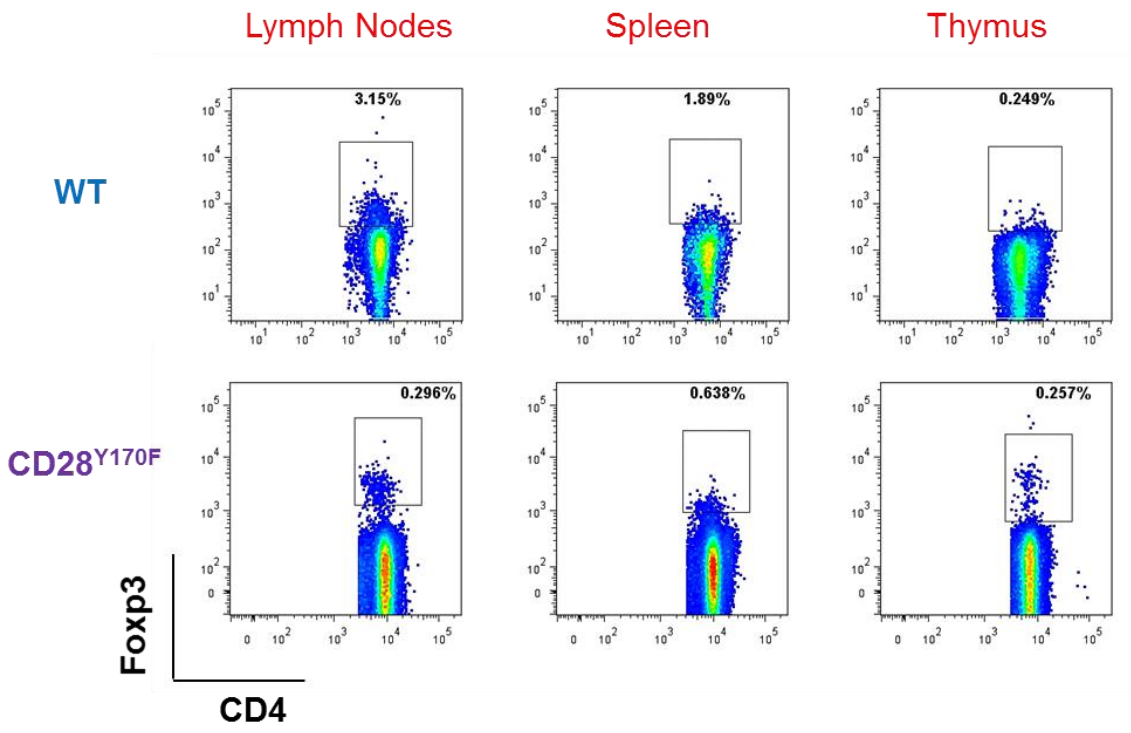



**Figure 54 CD28Y170F mice display lower numbers of Tregs in the periphery than WT mice**

*Tregs from the indicated tissues from CD28$^{Y170F}$ and corresponding age matched WT mice were identified by CD4+Foxp3+ staining and flow cytometry. The dot plots are representative from 3 individual mice.*

This may be a consequence of poor induction of the survival factor BCL-xl resulting from the point mutation in the cytoplasmic tail of CD28. In the next set of experiments we investigated migratory and metabolic features of CD28$^{Y170F}$ Tregs. First, we compared migration of Tregs isolated and

expanded from both WT and CD28 $^{Y170F}$ mice using *in vitro* trans-endothelial migration assay. Similarly to what was reported for CD28$^{Y170F}$ Teff, Tregs from CD28 $^{Y170F}$ mice failed to display the enhancement in extravasation through endothelial monolayers when subjected to CD28 stimulation observed in the WT Tregs (Figure 55).

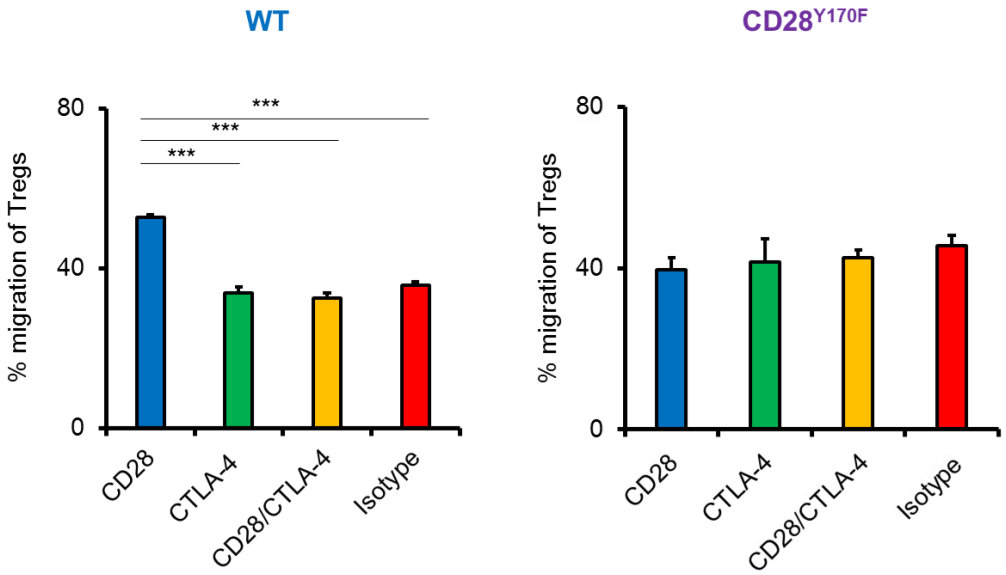
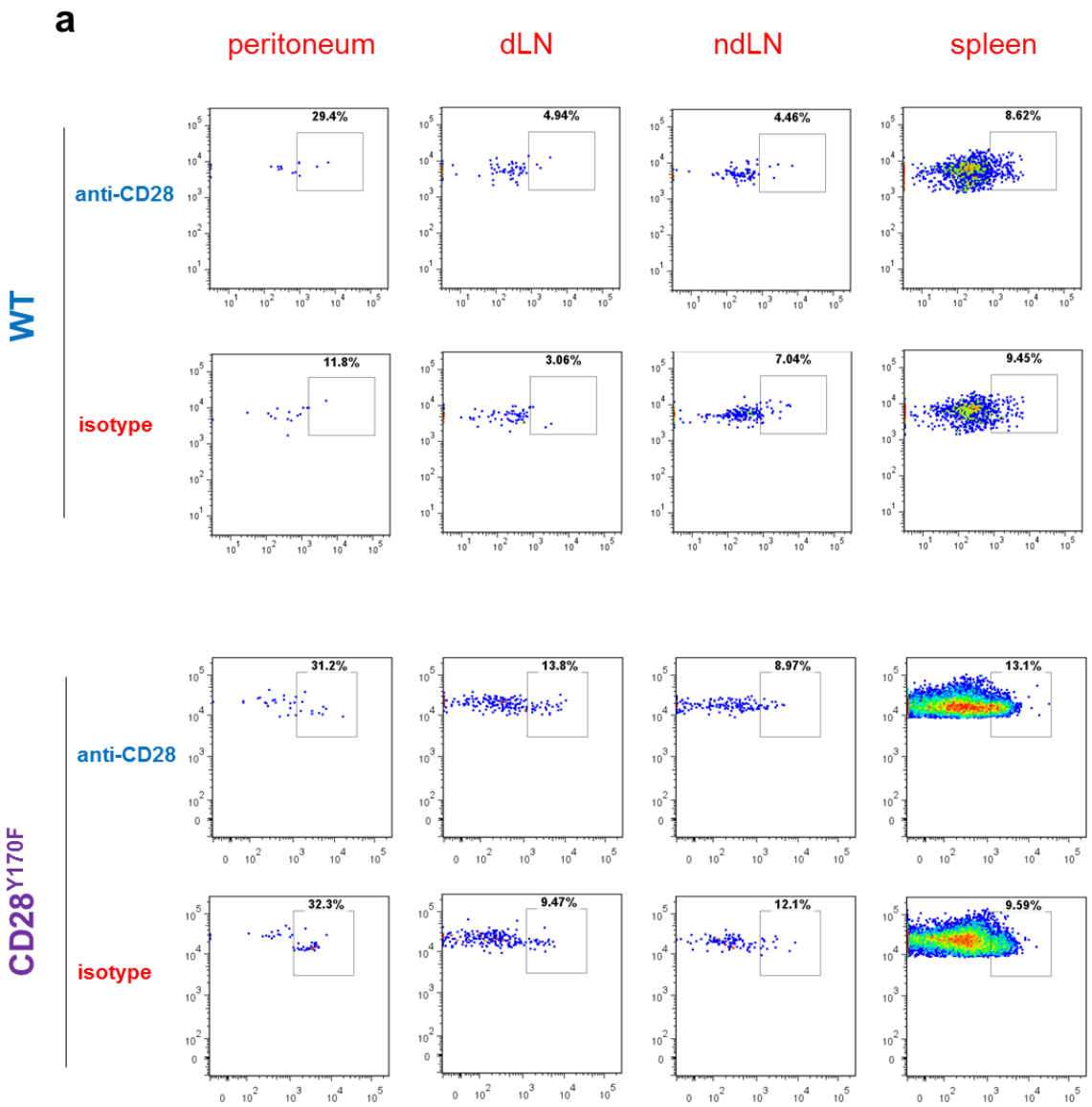



**Figure 55 CD28 stimulation fails to enhance trans-endothelial migration of CD28$^{Y170F}$ Tregs *in vitro.***

*CD4+CD25+ Tregs isolated from CD28$^{Y170F}$ or WT mice by immunomagnetic selection were expanded in vitro through co-culture as described in Figure legend 44. These Tregs were stimulated with CD28 and/or CTLA-4 antibody-mediated ligation. As a control, Tregs were treated with an isotype-matched control and secondary antibodies. Trans-endothelial migration of these Tregs was then assessed through IFN-γ-treated endothelial cell monolayers in a transwell assay. The number of migrated cells was evaluated by hemocytometric counting. Results were expressed as average percentage of migrated cells after 24 hours. Error bars represent standard deviation.Statistical significance was calculated with unpaired Student's t-test; *** p< 0.005 n=4, N=2*

Second, we compared CD28 $^{Y170F}$ Treg migration with that of WT Tregs *in vivo* using the intra-peritoneal recruitment model. Due to the fewer numbers of CD28 $^{Y170F}$ Tregs, we modified our *in vitro* Treg expansion protocol by co-culturing purified CD4 T cells from CD28 $^{Y170F}$ with immature allogeneic dendritic cells for a period of 2 weeks to selectively induce proliferation amongst the Treg populations. We then observed their migration *in vivo* by identifying recovered cells in the peritoneal lavage that were both labelled (i.e adoptively transferred) and stained positively for

the Treg nuclear transcription factor Foxp3. For adequate comparison, WT Tregs were also generated in a similar fashion from age-matched controls. Similar to the results obtained *in vitro*, Tregs from CD28 $^{Y170F}$ mice failed to upregulate their migratory activity upon CD28 stimulation when compared to WT Tregs *in vivo* (Figure 56). These results confirm the pivotal role of PI3K-mediated signals in CD28 induced Treg migration.



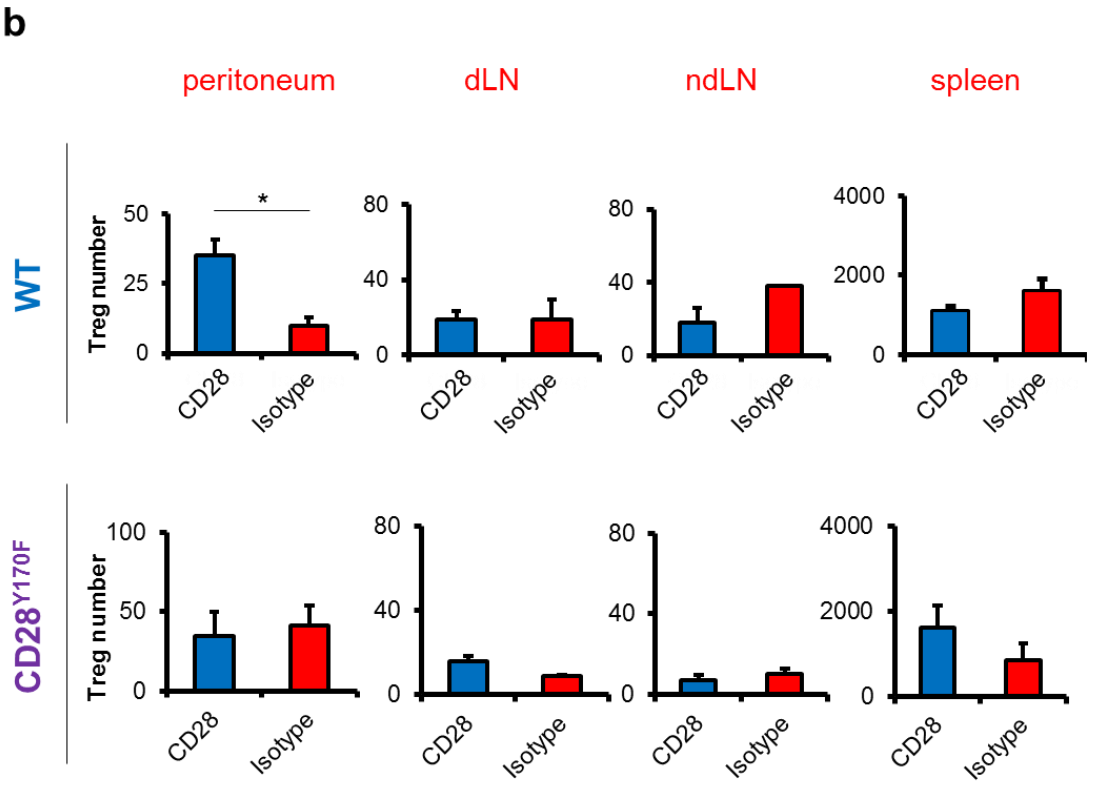



**Figure 56 CD28 stimulation fails to enhance migration of CD28$^{Y170F}$ Tregs *in vivo.***

*Enriched CD4+CD25+ Tregs from CD28$^{Y170F}$ mice were expanded through co-culture with sub-lethally irradiated immature bmDCS and IL-2. T cells were then labelled with PKH26 and injected intravenously into mice that were given intra-peritoneal injections of IFN-γ 48 hours earlier. Equal number of T cells treated with corresponding isotype-matched control and secondary antibodies were labelled with CFSE and co-injected. 24 hours later, cells were recovered from the indicated tissues, stained for Foxp3 and analysed using flow cytometry. In a parallel experiment, CD4+ CD25+ Tregs from WT mice were used to observe CD28 induced migration as a control. (**a**)Dot plots shown represents percentage of Treg cells in the recovered CD4+ donor (PKH26 labelled) population. (**b**) Bar graphs indicate absolute number of labelled cells recovered in 4 mice (n=4). Error bars represent standard deviations. * p<0.05*

Further, since CD28 co-stimulation induces glucose uptake and glycolysis via the PI3K-Akt signalling pathway, we predicted that CD28 $^{Y170F}$ Tregs would be less efficient at inducing glycolysis upon CD28 triggering. To test this hypothesis, we analysed the ECAR/OCR rates of the CD28 $^{Y170F}$ Tregs and WT Tregs before and after CD28 stimulation. Remarkably, the baseline ECAR of the CD28 $^{Y170F}$ Tregs was significantly lower to that of WT Tregs (Figure 57). Perhaps this occurred due to inefficient CD28 signalling in their development and proliferation during the *in vitro* expansion. In addition unlike WT Tregs, CD28 $^{Y170F}$ Tregs were unable to upregulate the ECAR following CD28 stimulation.

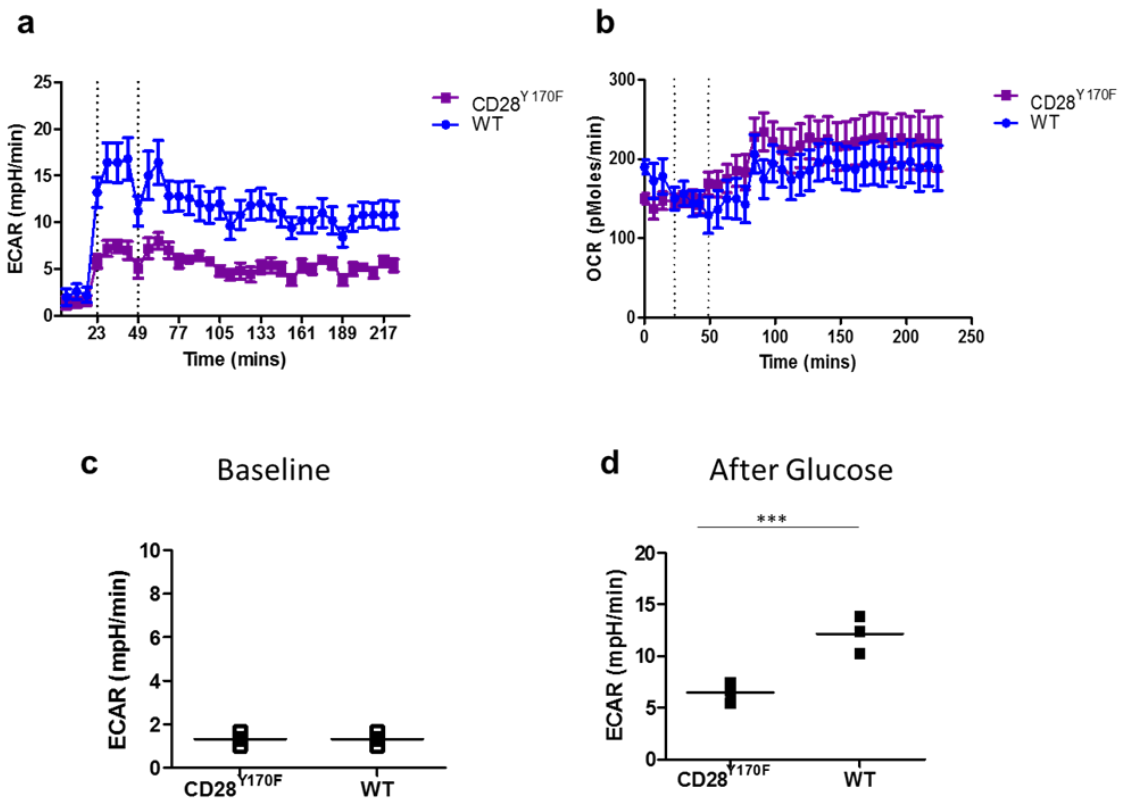



**Figure 57 CD28$^{Y170F}$ Tregs display inherently lower ECAR compared to WT Tregs**

*CD28$^{Y170F}$ Tregs and WT Tregs isolated from lymph nodes and spleens of age matched CD28$^{Y170F}$ or WT mice by immunomagnetic separation were co-cultured with immature bmDCS and IL-2 to facilitate in vitro expansion. Expanded Tregs from both CD28$^{Y170F}$ and WT mice were then analysed for their metabolic profiles using the Seahorse XF analyser. The plot depicts real time analysis of (**a**) ECAR and (**b**) OCR observed in CD28$^{Y170F}$ Tregs using the XF analyser. Cumulative data here indicates the mean ECAR from three experiments at (**c**) baseline and (**d**) after addition of glucose. Statistical test involved using a one way ANOVA with Bonferroni correction ***P>0.005 n=3, N=4*

Lastly, we tested the contribution of mTORC1 in CD28-induced Treg migration. To this aim, we monitored migration of Tregs following a short treatment with the mTORC1 inhibitor rapamycin. *In vitro* trans-endothelial migration assays of Tregs treated with rapamycin did not reveal any differences compared to the untreated Tregs in their migration (Figure 58).

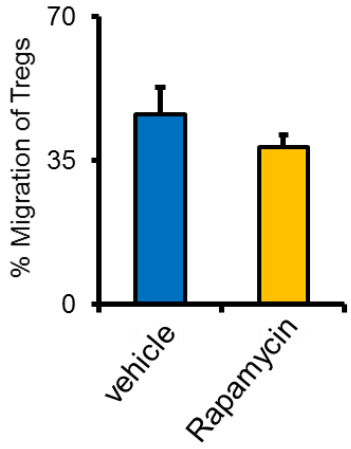



**Figure 58 *In vitro* migration of Tregs is supported by an mTORC1 independent mechanism.**

*In vitro expanded Tregs, harvested after 7 days of co-culture were treated with the mTORC1 inhibitor rapamycin (dose) for 4 hours before being placed over IFN-γ-treated syngeneic endothelial cell monolayers grown on a 3-µm pore polycarbonate transwell and incubated for 24 hours at 37°C with 5% $CO_2$ . Tregs treated with a vehicle only were used as a control. Migrated cells in the bottom chamber were quantified by hemocytrometric counting. Results are expressed as a percentage of migrated cells after 24 hours. Error bars represent standard error of means.* P>0.05*

These results are contrary to what has been observed in CD4 effector cells as seen by (R. Haas et al. 2015) study which showed that rapamycin mediated glycolysis inhibition in Teffs reduces their migration . While this dose did not affect the migration of Tregs it did impact proliferation as was seen by staining for the proliferating nuclear cell antigen (PCNA) (Figure 59).

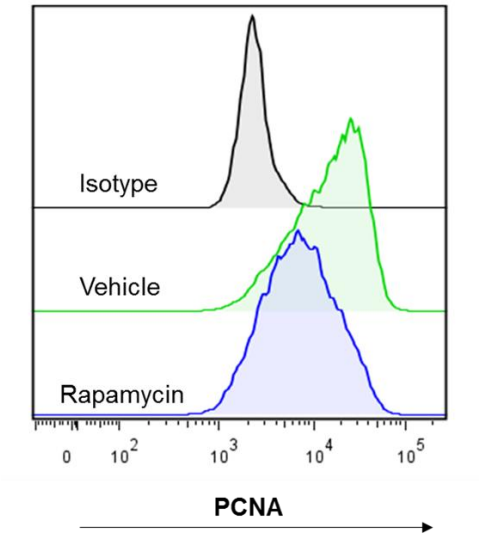



**Figure 59 Inhibition of mTORC1 in Tregs with rapamycin can disrupt their proliferation *in vitro.***

*In vitro expanded Tregs were treated with rapamycin for 4-6 hours following which they were stained for the proliferating cell nuclear antigen (PCNA).The histograms represent PCNA staining of the Treg cells by Flow cytometry. (n=3, N=3)*

*In vivo* Tregs treated with rapamycin did not exhibit any differences in migration in the peritoneal model of recruitment when compared to untreated Tregs (Figure 60). Additionally, CD28-induced migration of Tregs that was observed earlier remained similar when Tregs were pre-treated with rapamycin before adoptive transfer (Figure 60). Collectively these results imply that mTORC1 activity is dispensable in Treg migration. Furthermore, these results suggest that CD28/CTLA-4 regulation of Treg metabolism and migration occur via signalling molecules other than mTORC1.

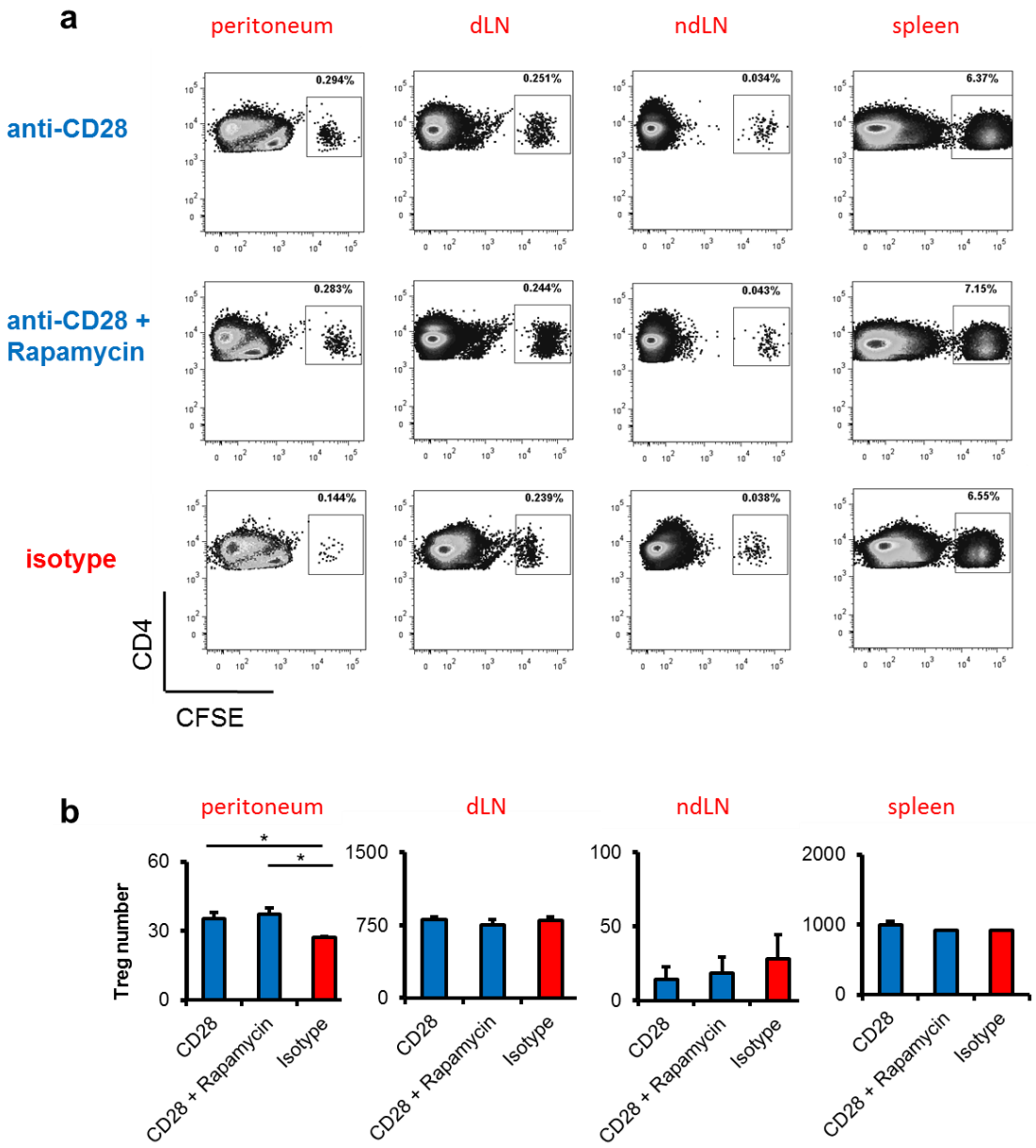



**Figure 60 CD28 induced *in vivo* migration of Tregs can occur independently of mTORC1 activity.**

*In vitro expanded Tregs, harvested after 7 days of co-culture were treated with CD28 antibody-mediated ligation along with the mTORC1 inhibitor rapamycin for 4 hours before being labelled with PKH26 and intravenously injected into C57BL/6 (syngeneic) mice that had received an intraperitoneal injection of IFN-γ 48-72 hours earlier. As a control, Tregs were treated with an isotype-matched control antibody and secondary antibodies were labelled with CFSE and co-injected. Cells were then recovered from tissues indicated after 24 hours and analysed using flow cytometry. (**a**)Dot plots shows percentage of Treg cells in the recovered in CD4+ donor populations from one representative. (**b**) Bar graphs indicate average of absolute number of labelled cells recovered (n=4). Error bars represent standard deviation. Statistical significance was calculated with unpaired Student's t-test. * p<0.05γ*

**CTLA-4 inhibits glycolysis induced by CD28, disrupting its ability to induce migration.**

We next interrogated the molecular mechanisms by which CTLA-4 disrupts CD28-induced upregulation of glycolysis and migration. To this aim, we performed western blots to observe changes in expression of key enzymes along the glycolytic and lipid oxidation pathways following CD28 and/or CTLA-4 stimulation in Tregs. We analysed the expression of key glycolytic enzymes such as the rate-limiting enzymes Hexokinase and its isoenzyme Glucokinase, Aldolase, Enolase-1, as well as an enzyme required for fatty acid oxidation carnitine palmitoyltransferase 1A (CPT1-A) (Figure 61). Stimulation of Tregs with CD28 stimulation resulted in upregulation of enzymes involved in glycolysis. Specifically, CD28 stimulation greatly increased expression of the hexokinases, Hexokinase I and Glucokinase (Hexokinase IV or GCK).

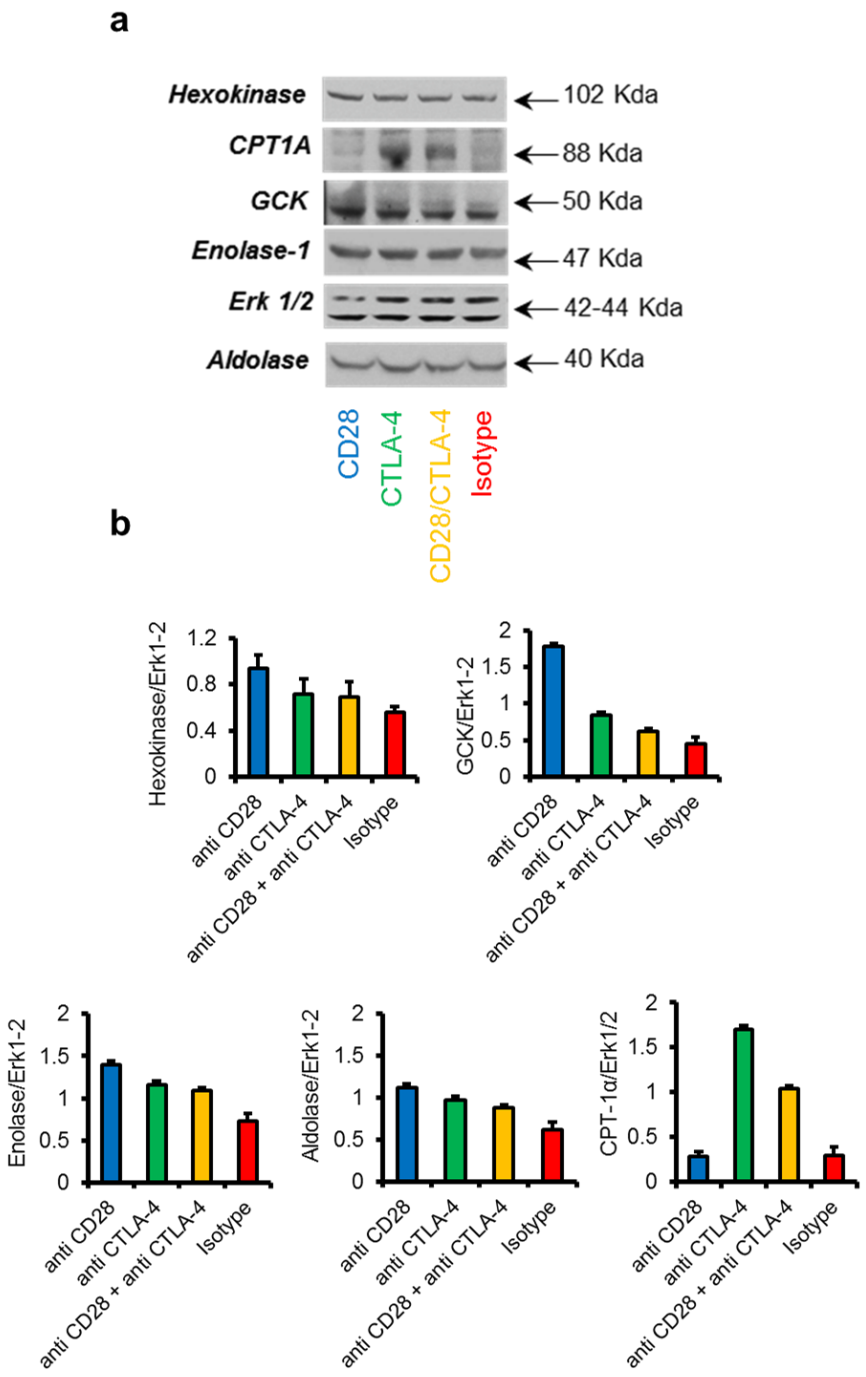



**Figure 61 CTLA-4 inhibits CD28 induced glycolysis and engages FAO in Tregs.**

*In vitro expanded Tregs harvested after 5 days of co-culture with immature bmDCs were treated with CD28 and/or CTLA-4 antibody-mediated ligation. As a control, Tregs were treated with an isotype-matched control antibody and secondary antibodies. (**a**) Cells were then recovered and analysed using western blotting as shown in the figure. (**b**) Bar graphs represent cumulative data from the three sets of blotting gels (n=4, N=3). Error bars represent standard deviation. Statistical significance was calculated with unpaired Student's t-test. n=3, N=3, ***p<0.0005.*

Hexokinases phosphorylate glucose to glucose-6 phosphate, an enzymatic reaction relevant to all pathways of glucose utilization, including glycolysis and the pentose phosphate pathway. Interestingly, the hexokinase isoenzyme GCK was not known to be expressed by Tregs.

Concomitant triggering of CTLA-4 reduced CD28-induced Hexokinase I and GCK expression. Strikingly, while not affecting the expression of glycolytic enzymes, CTLA-4 signalling greatly induced expression of CPT1-A, the rate-limiting enzyme for fatty acid oxidation. These results suggest a novel role of CTLA-4 inhibition of CD28 signalling. Collectively, the results imply that CTLA-4 signalling not only inhibits the glycolytic pathways that fuel CD28 induced Treg migration, but also reverts the Tregs back towards fatty acid oxidation, the predominant metabolic pathway of Tregs observed under steady state conditions.

### 4.2.4 Discussion

In this part of the study, we have investigated the effect of CD28 and CTLA-4 stimulation on the migration of Tregs. We show that glycolysis is an essential process for Treg migration *in vivo* and *in vitro* and CD28-mediated signalling induces glucose uptake and glycolysis in Tregs, thus enhancing Treg migration. Furthermore, our results demonstrate that CTLA-4 signalling in Tregs counteracts CD28 induced glycolysis thereby inhibiting Treg migration.

Our results revealed differences to those studies examining conventional T cells alone. In (Mirenda et al. 2007) study, CTLA-4 signals to T6 cell clones, that display constitutive expression of weakly expressed CTLA-4, were shown to inhibit migration. Here, we show that although Tregs do indeed express CTLA-4 constitutively, CTLA-4 on its own does not affect Treg migration. Instead we observed a more dynamic role for CTLA-4 acting through disruption of the pro-migratory signals brought through CD28 signalling.

Importantly, our studies reveal a direct, active role of CTLA-4 in the regulation of Treg metabolism and migration. As we have previously mentioned CTLA-4 can act through several different mechanisms to elicit its inhibitory role. For example, CTLA-4 can out compete CD28 for ligands and physically displace ligands off the APC surfaces (Hou et al. 2015a; Soskic et al. 2014). Although it is possible that this mechanism might contribute to the inhibitory effect on CD28 induced enhancement of Treg migration and metabolism, in our studies, which have been conducted by triggering CD28 and CTLA-4 signals by antibody ligation, CTLA-4 signals directly inhibit CD28-induced events, including cytoskeletal rearrangements and glycolysis.Furthermore, our results indicate that CTLA-4 not only passively prevent CD28 effects,which would be compatible with a competition mechanism, but also upregulates CPT1a on its own. This suggests that CTLA-4 signalling activity on Tregs affects transcriptional and/or post-transcriptional events.

In the previous chapter, we showed that cognate recognition of antigen-presenting endothelium by the Treg TCRs facilitated their extravasation and recruitment to antigen-rich tissue. The present study suggests that CD28 can act independently of the TCR. Although debated extensively, there is evidence from independent studies to suggest that TCR-independent signalling by the co-stimulatory receptor CD28 exists. In one study, F actin based cytoskeletal rearrangement via CD28 stimulation occurs independently of the TCR signals. In this context, it is important here to note that these results apply to CD28 and CTLA-4 regulation of migration in an *in vitro* activated cohort of Tregs, conventional naïve T cells did not seem to respond well to CD28 stimulation alone (FMB, unpublished observations). Moreover, there is no evidence suggesting

accumulation of naïve T cells in the lymphoid tissue of CD28KO, B7-1/2KO and CD28$^{Y170F}$ mice. As the majority of freshly isolated naïve Tregs are not truly naïve (due to their self-reactivity) it will be important to investigate whether freshly isolated Tregs are susceptible to CD28/CTLA-4-regulation of migration.

In a physiological context, these results imply that APCs such as DCs would be able to modulate Treg motility to tissues independently of antigen presentation. Such interactions may occur when co-stimulation is provided without TCR triggering at the end of an immune response or during resting immune state where all antigen has been removed. This would effectively enable Treg recruitment to antigenic sites even at the very late stages of the immune response thus, mediating tolerance in a timely manner.

We subsequently concentrated on the metabolic regulation of migration of Tregs via co-stimulatory CD28 and CTLA-4 receptors. Fatty acid oxidation and the AMPK pathways are believed to be the dominant energy production pathways during development and differentiation of Tregs (Michalek et al. 2011c). In addition, our observations pinpoint glycolysis and not fatty acid oxidation to sustain migration in Tregs.

In addition to this novel finding, we show that the anti-diabetic drug metformin can also increase Treg migration. Although the exact mechanism of action of metformin is unclear, there is evidence that metformin can activate the AMPK pathways which can induce both FAO and glycolysis. Given that the inhibitor of FAO did not reveal any differences in the migration of Tregs, we concluded that glycolysis played a key role in Treg migration.

Based on these observations, we tested the possibility that CD28/CTLA-4-mediated regulation of Tregs could occur via modulation of the glycolytic pathway. Given that both co-receptors are known to be associated with the PI3K-Akt pathway, which can regulate both metabolism and migration, we focused our investigation on the role of PI3K in these events. Crucially, we hypothesized that CD28 mediated induction of both glycolysis and migration would effectively require the action of the PI3K-Akt signalling pathway. Evidence suggesting this was first provided by the study by (Frauwirth et al. 2002), which implicated PI3K in CD28-mediated control of glucose metabolism. To test this hypothesis we have utilized Tregs from the CD28$^{Y170F}$ mice and shown that these Tregs are unable to display CD28 enhancement of migration both *in vitro* and *in vivo*. Furthermore these Tregs displayed poor upregulation of glycolysis in response to CD28 signals as compared to WT tregs. Collectively these results imply that CD28 stimulation of Tregs induces both glycolysis and motility, a feature that is dependent on the PI3K pathway.

Interestingly our results also indicate that the activation of downstream mTORC1 was dispensable for migration. This implies that the downstream target of CD28 induced PI3K-Akt pathway may differ to those that are linked to metabolic control and subsequently migration.

Our study also shows that CTLA-4 inhibits the CD28-mediated effects on Treg migration by abrogating the signals that couple glycolysis to migration.

In addition, a broad analysis of enzymatic control of key metabolic pathways also revealed that CTLA-4 may act not only as a counterbalance to CD28 signalling, but also may be required to restore the dominant FAO essential for other processes such as survival and proliferation. CPT1 A is a key enzyme involved in FAO. We have shown that CTLA-4 signalling induced upregulation of CPT1A in Tregs. Additionally CTLA-4 signalling disrupted CD28 mediated upregulation of glycolytic enzymes, particularly the hexokinases, Hexokinase I and GCK. Thus, these results imply that the constitutively expressed CTLA-4 on Tregs acts by facilitating a shift from glycolysis towards FAO thereby restricting motility and migration induced by the CD28 co-stimulatory receptor.

# 4.3 Part 3: The inhibitory co-receptor CD31 regulates T cell migration

## 4.3.1 Introduction

CD31 is an immunoglobulin-like receptor expressed by platelets, endothelial cells and on all hematopoietic cells including T and B lymphocytes. Homophilic interactions of CD31 expressed at high density at the lateral borders of endothelial cells with CD31 expressed on leukocytes have been shown to facilitate TEM *in vitro* and *in vivo* (reviewed in Marelli-Berg et al. 2013).

Historically, CD31 was regarded as a marker of endothelial cell lineage and its functional role in adaptive immunity has not been given much insight (Marelli-Berg et al. 2013). Recent studies have established the involvement of CD31 in the regulation of T cell homeostasis, effector function and trafficking. One of the reasons why CD31 was not studied in the context of regulating T cell migration was the observations that CD31-deficient mice display a very mild phenotype under steady state conditions with a normal number of T cells (Gordon S. Duncan et al. 1999b), and that T cells which undergo differentiation and acquire a memory phenotype were believed to not display CD31 (Demeure et al. 1996). However, under situations of immunological stress CD31 defciency has been reported to have very different results. For example, in models of EAE and CIA, CD31 deficiency has shown to enhance disease (Graesser et al. 2002; Tada et al. 2003; Wong et al. 2005). Several other observations of models of disease where CD31 deficient mice have much severe disease outcome are given in the Table 3. Secondly, CD31 loss on memory T cells has been reported to be a consequence of enzymatic shedding rather than transcriptional downregulation. In one study, CD31 activity after shedding could be restored using a peptide containing the CD31 amino acid residues 551-574 (Fornasa et al. 2010). Furthermore, human memory T cells have also been shown to acquire CD31 expression after TEM as a result of membrane transfer from ECs *in vitro* (Brezinschek et al. 1999).

Given that CD31 is expressed by both T cells and DCs and can engage in homophilic interactions, it is likely that CD31 signalling between APCs and T cells plays a role in conventional antigen presentation and T cell priming. The recent study by (Ma et al. 2012) has analysed this role of CD31 mediated interaction in T cell activation where CD31 deficient mice were shown to display a trend towards accelerated and pronounced allograft rejection. Their results were attributed to

enhanced proliferation of CD31-deficient T cells following immunization with diminished Treg suppression. Interestingly, their experiments also revealed enhanced activation induced cell death (AICD) in the CD31-deficient T cells. Here, the CD31 signals were shown to induce the activity of extracellular –signal –regulated kinase (Erk) activity, which is associated with pro- survival pathways, independently of the TCR signals. Thus, while CD31 signals regulate T cell activation, they also play a role in promoting T cell survival after activation. Therefore, the severe exacerbation observed in the models of autoimmunity, tumour and transplant rejection in CD31-deficient mice cannot be fully explained by CD31 mediated effects on T cell activation alone.

In addition to its engagement during interactions with antigen presenting DCs, CD31 triggering on T cells can also occur following interaction with other CD31-expressing cells. Among those that express CD31 abundantly are platelets and ECs. Platelets are known for their regulatory role in both non-specific inflammatory responses as well as in T cell-dependent adaptive immune responses (Semple et al. 2011).

Most importantly, frequent CD31 interactions between ECs and T cells are bound to occur during lymphocyte recirculation and have only recently been thoroughly investigated. Given that T cell interactions with antigen presenting endothelium are crucial for T cell recruitment and subsequent activation in antigen rich tissue and that CD31 engagement modulates the efficiency of TCR signals (Ma et al. 2012), recent studies have tried to elucidate the role of CD31 in antigen-dependent T cell recruitment.

A recent study by (Manes and Pober 2011) indicates that blockade of CD31 prevents migration of human effector memory T cells induced by antigen presenting ECs. Furthermore, the study by (Ma et al. 2012) further expanded upon the relative contribution of CD31 mediated interactions in the regulation of T cell trafficking by separately assessing the effect of CD31 deficiency in the T cells and endothelium. Here, CD31 interactions between T cell and endothelium were shown to facilitate the recirculation of memory T cells and their inflammation induced extravasation to antigen rich sites confirming the previous study by (Manes and Pober 2011). Loss of CD31 on T cells exclusively displayed inhibition of antigen specific recruitment to antigenic sites. In addition, the authors also indicate that selective loss of CD31 from the endothelium inhibits the recruitment of both antigen specific memory T cells during inflammatory conditions as well as naïve T cell recruitment to secondary lymphoid tissue during rest. While the latter observation may not be visible in the CD31 deficient mice displaying normal colonisation of lymphoid tissues due to compensatory mechanisms in TEM, such as CD99, JAMs and ICAMs interactions, the

former could account for the observations of exasperated disease severity in the various disease models in CD31 deficient mice described before.

Thus, one possible explanation for endothelial CD31s role in regulating memory T cell recruitment could be on account of compromised endothelial permeability (reviewed in Marelli-Berg et al. 2013). Indeed, TCR interaction with antigen loaded MHC by the ECs is known to transiently increase permeability through reorganisation of the endothelial cytoskeleton via means of rapid RhoA activation (Boulday et al. 2004). This transient effect could enable the migration of antigen specific T cells through vascular endothelium. Additionally, CD31 may stabilize the adherens junctions through its previously described dephosphorylation of β-catenin and enhancing anchorage of the vascular endothelial (VE)-cadherin complex thus behaving as a molecular 'gatekeeper' (Biswas et al. 2006). However, the overall explanation here suffers from a crucial drawback i.e. the observation that CD31 deficiency is not associated with any other cardinal signs of vascular leakage in CD31 deficient mice. Thus, while loss of CD31 from the endothelial junctions may partially account for enhanced memory T cell extravasation, other mechanisms may exist through which CD31 regulates effector and memory T cell extravasation into the antigen rich non-lymphoid tissue.

Within T cells, the Ig like CD31 receptor bears a distinctive feature in its cytoplasmic domain highlighting its role as a negative co-inhibitory receptor. Two immunoreceptor tyrosine inhibitory motif (ITIM) domains in its cytoplasmic domain are phosphorylated following TCR stimulation, and these have been shown to recruit protein-tyrosine phosphatases (PTPs), such as the Src homology 2 (SH2)-domain-containing protein SHP-2 as wells as SHP-1 leading to inhibition of TCR signalling (P. J. Newman 1999; D. K. Newman et al. 2001). However, the effect of recruitment of phosphatases on other signalling processes, particularly those that are crucial for T cell recruitment, such as chemokinesis have not been previously investigated.

### 4.3.2 Aims

Given that inhibition of TCR signalling by CD31 and vascular leakiness in the CD31 deficient endothelium cannot entirely account for the enhanced disease severity reported in various models of disease in the CD31 deficient mice, we predict the existence of another mechanism for this co-inhibitory receptor in regulating T cell recruitment. As mentioned before, CD31 homophilic interactions between T cells and ECs are crucial for recruitment of antigen specific T cells to antigen rich tissue. Loss of CD31 from either ECs or T cells has been shown to inhibit antigen specific recruitment. In this part, we further investigate the role of CD31 on T cells in influencing their migration. Here we hypothesise that the recruitment of phosphatases via the cytoplasmic ITIM upon phosphorylation may affect processes such as chemokine-dependent recruitment of T cells into tissues. Furthermore, given that CD31 deficient mice display no obvious phenotype during resting immune conditions we predict a differential role for this molecule in activated and naïve T cells. To test these hypotheses we carried out the following experiments.

1. Analysis of *in vitro* and *in vivo* chemokinesis of T cells from CD31-deficient mice compared to those obtained from WT mice.
2. Assessment of the impact of CD31-mediated regulation of T cell migration on both naïve and activated (primed) T cells.
3. Identification of signalling pathways that may be differentially regulated by CD31 in both cell types that could account for the phenotypes observed in CD31-deficient mice under resting and inflammatory states.

## 4.3.3 Results

### 4.3.3.1 CD31 regulates Chemokine –Induced T cell Migration *in vitro*

While CD31-deficient mice exhibit enhanced disease severity in models of T cell mediated inflammation such as autoimmune encephalomyelitis and collagen induced arthritis, these mice retain healthy secondary lymphoid architecture through their lifespan. In addition, WT and CD31 deficient naïve T cells adoptively transferred into WT recipients localise within the spleen at comparable levels. This suggests a differential effect of CD31 signals between naïve and activated T cells.

To test this hypothesis, we set up transwell based migration assays to observe the chemokinesis of both naïve and memory T cells from CD31-/- and WT mice. The chemokine ligands for CCR7 and CXCR3, characteristically expressed by naïve and activated T cells respectively were used to asses chemokinetic responses by both CD31-/- and WT T cells (Figure 62). While chemokine responses of naïve T cells from both WT and CD31-/- mice to the CCR7 ligands CCL19 and CCL21, were comparable, activated or memory T cells (HY specific T cell lines) from CD31-/- mice showed enhanced chemokine migration to the CXCR3 ligand CXCL10 compared to their WT counterpart.

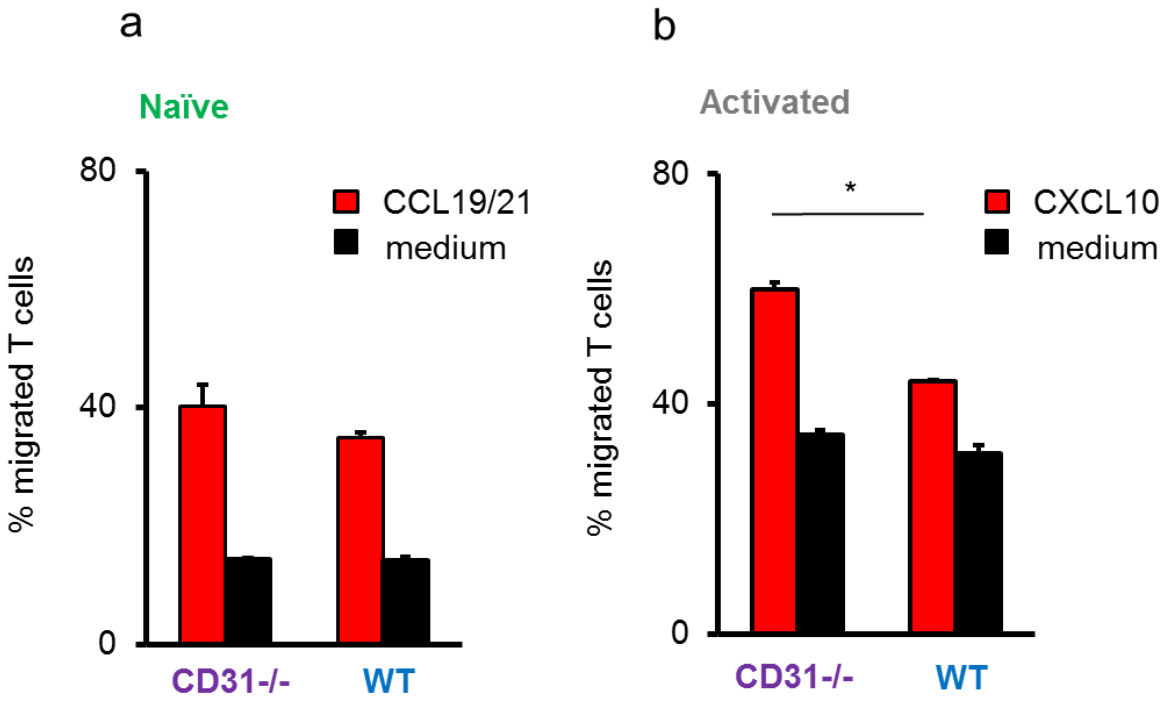



**Figure 62 CD31-deficient activated T cells display enhanced responses to CXCL10 chemokine *in vitro*.**

*Naïve and activated WT and CD31−/− T cell migration to the chemokines CCL19/21 and CXCL10, was assessed over 6 hours using transwell-based chemokinesis assays. The number of migrated cells in the bottom chamber was determined through hemocytometric counting of samples from each of the triplicate wells. Results are expressed as a percentage of migrated cells. The average percentage migration from four independent experiments is shown (n=3, N=4). Error bars indicate standard deviation. Statistical significance was calculated with unpaired Student's t-test.*p<0.01*

Additionally, flow cytometric analysis of Chemokine receptors CXCR3 and CCR7 revealed no significant difference in surface receptor expression between T cells from CD31-/- and WT mice (Figure 63).

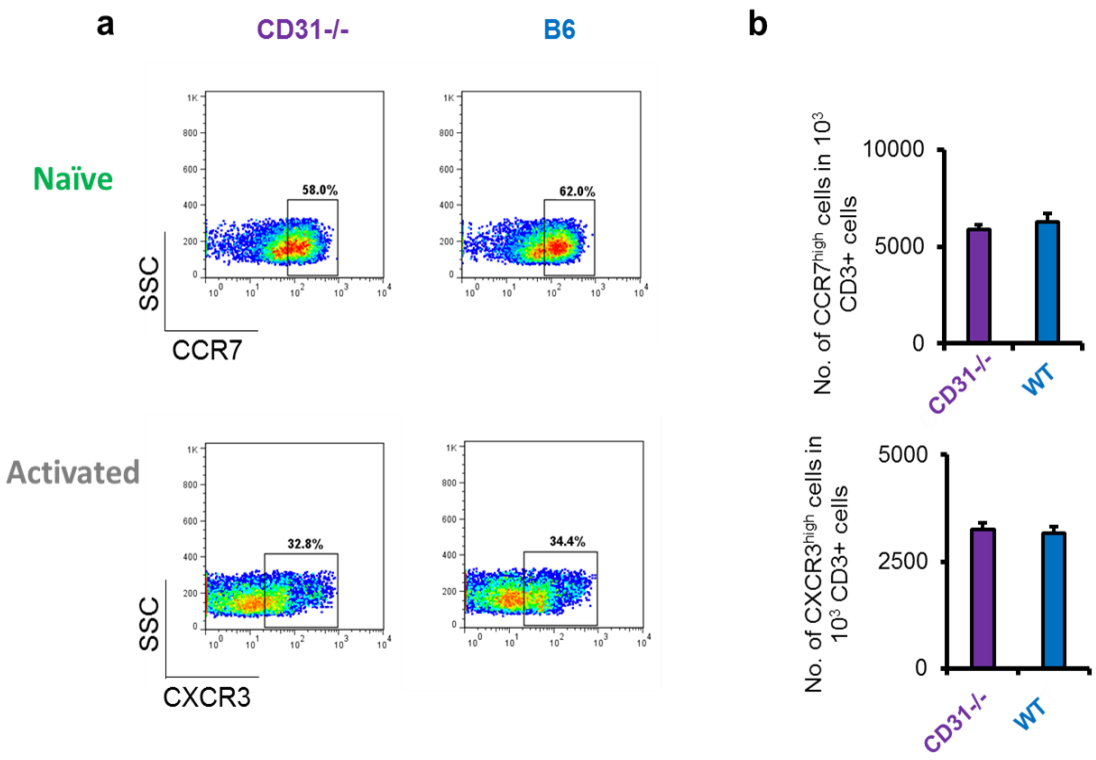



**Figure 63 Phenotype of WT and CD31-deficient naïve and activated T cells.**

*(**a**) These representative dot plots here show WT and CD31−/− CCR7$^{high}$ and CXCR3$^{high}$ T cells within the naïve and activated (anti-CD3 plus anti-CD28, 7 days) T cell pools respectively. (**b**) Bar graphs represent cumulative data from lymph nodes and spleen of 3 mice (n=3) indicating the absolute number of CCR7$^{high}$ and CXCR3$^{high}$ T cells within a given CD3 T cell population from both WT and CD31−/− mice. Cells analysed were first gated on CD3+ T cells.*

Finally, enhanced chemokine recruitment of CD31-/-activated T cells was not restricted to the CXCR3 ligand CXCL10 as similar enhanced chemokinesis effect was also seen with the CXCR4 ligand CXCL12 (Figure 64).

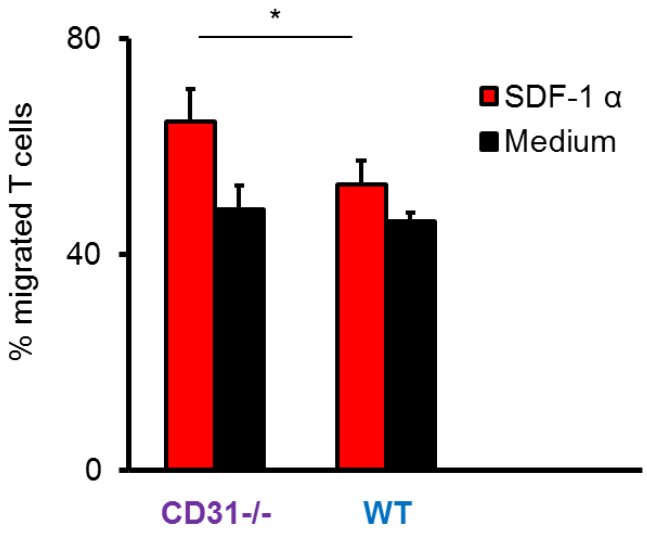



**Figure 64 CD31-deficient activated T cells display enhanced responses to CXCL12 *in vitro.***

*Activated WT and CD31−/− T cell migration in response to the chemokine CXCL12 (100 ng/ml) was assessed over 6 hours using a transwell-based chemokinesis assays. The numbers of migrated cells in the bottom chambers were determined through hemocytometric counting of samples from each of the triplicate wells. Results are expressed as a percentage of migrated T cells . (n=3, N=3). Error bars indicate standard deviation. Statistical significance was calculated with unpaired Student's t-test. *p<0.01*

#### *4.3.3.2* CD31 regulates Chemokine –Induced T cell Migration *in vivo*

To validate if CD31 negatively regulated chemokinesis *in vivo*, HY-specific WT or CD31-/- effector memory T cells were labelled with PKH26 and injected intravenously into WT recipients that had previously been injected with 1200ng of CXCL10 chemokine intra-peritoneally. To rule out any interference by antigen-induced migration of the adoptively transferred T cells, recipient mice of syngeneic specificity (H2-B) were used in these *in vivo* migration experiments. Localisation of labelled T cells within the peritoneal lavage was assessed after 16 hours by performing flow cytometric analysis. Analysis of PHK26 positively labelled T cells within the antibody labelled CD3+ positive population revealed a marked increase in CD31-/- T cell recruitment to the peritoneal cavity as compared to WT T cells (Figure 65). The proportion of CD4 and CD8 T cells within the migrated lymphocyte pool remained comparable between WT and CD31-/- donor T cells suggesting that both subsets were equally affected by CD31 signalling.

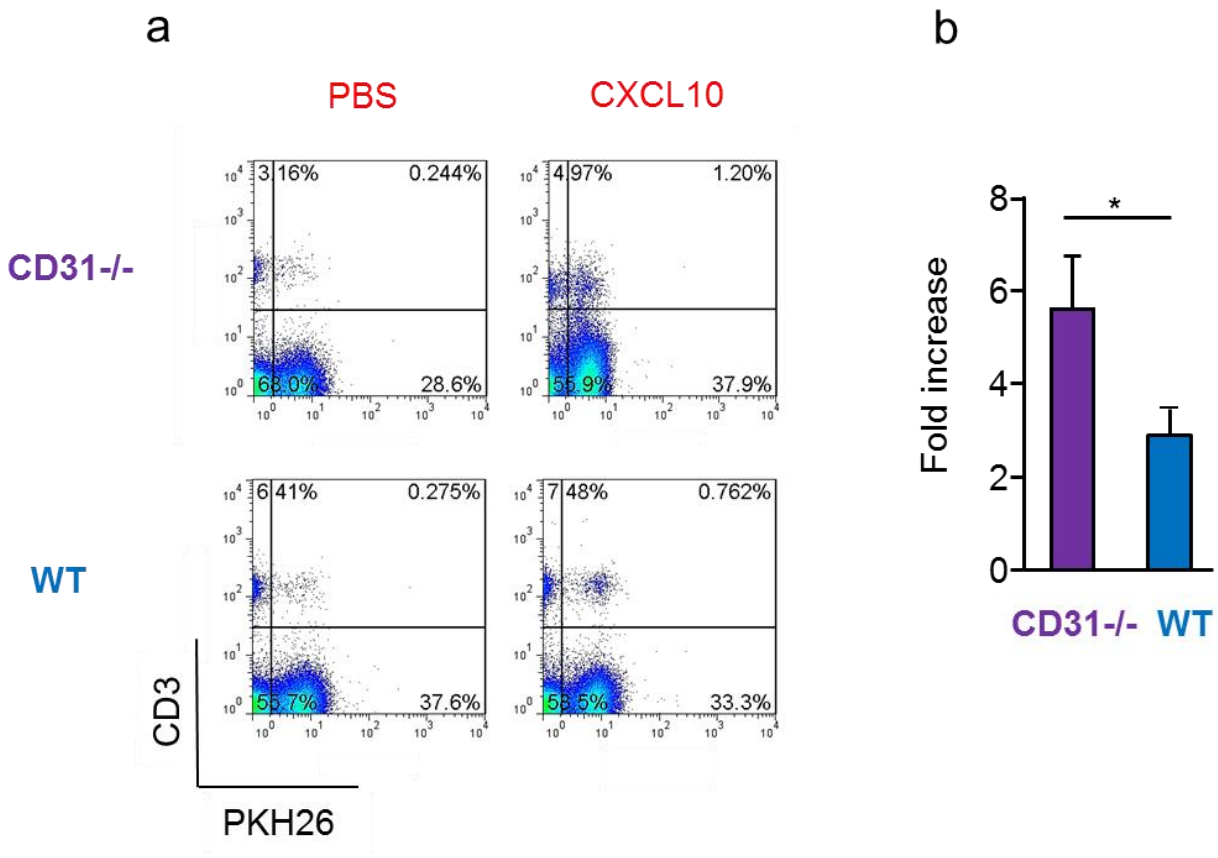



**Figure 65 CD31-deficient activated T cells display enhanced responses to CXCL10 chemokine *in vivo*.**

*Effector memory HY-specific WT or CD31−/− T cells were labelled with PKH26 and injected intravenously into syngeneic female mice that had received an intraperitoneal injection of 1.2 µg CXCL10 30 minutes earlier. Mice were sacrificed 16 hours later, and the presence of PKH26-labeled T cells in the peritoneal lavage was analysed by flow cytometry. Donor populations within a CD3 staining gate were analysed. Representative dot plots from 3 different experiments are depicted in panel (**a**). The average fold-increase (T cells in chemokine-treated animals/T cells in PBS-treated animals of PKH26 (FL-2)-labeled T cells gated in the CD3⁺ T cell population retrieved from at least five animals/group in 3 independent experiments of similar design is shown in panel (**b**). (n=5, N=3).Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. *p<0.04.*

A phenotypic analysis of receptor surface expression between HY specific T cells generated from both CD31-/- and WT mice via flow cytometric analysis revealed no significant differences (Figure 66). Collectively, these data suggest that CD31 induced signals negatively regulate extravasation of memory T cells in response to chemokine in non-lymphoid tissue.

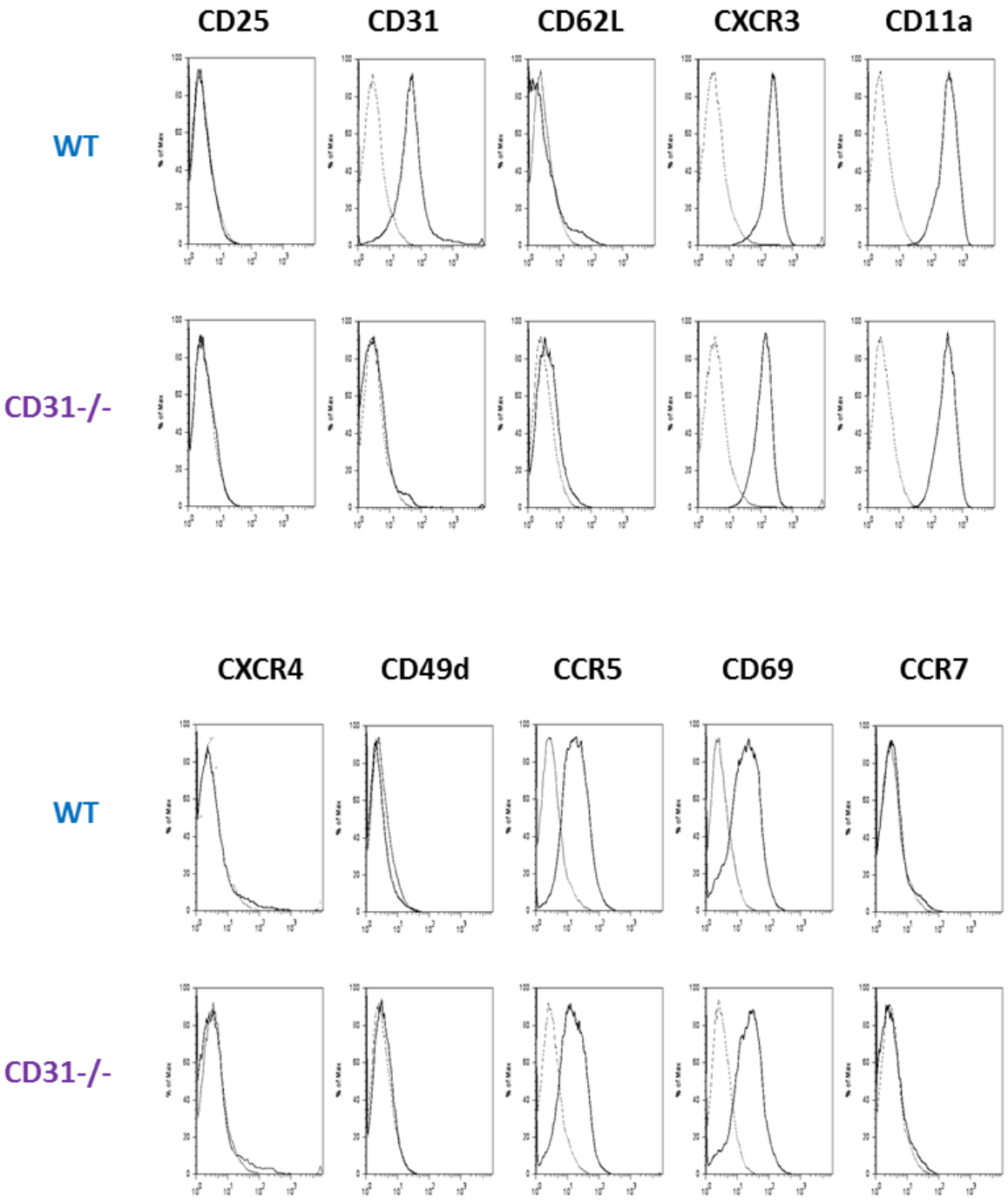



**Figure 66 Phenotype of HY-specific WT and CD31−/− T cells.**

*Histograms represent the expression of the molecules indicated above by WT and CD31−/− HY-specific T cells was assessed at the time of injection (i.e., 7–10 days following re-stimulation in vitro) by flow cytometry. Staining with an isotype-matched control antibody is indicated by the dashed lines profiles.(N=3)*

#### 4.3.3.3 CD31-mediated signals interfere with chemokine induced PI3K-Akt signalling pathways

PI3K kinase activation and subsequent downstream phosphorylation of Akt/PKB has been shown to play a pivotal role in both chemotaxis and cell polarisation mediated via numerous chemokine receptors (Ward and Marelli-Berg 2009). As CD31 receptor signalling has been shown to recruit phosphatases via the ITIM domains in the cytoplasmic tail of the receptor, we sought to investigate whether the increased chemotactic ability observed selectively by the memory T cells from CD31-/- mice could be correlated to enhanced Akt/PKB phosphorylation. Both naïve and antibody-activated T cells were exposed to 200ng/ml CCL19/21 and 300ng/ml CXCL10 chemokines, respectively, for 2 minutes. Using an antibody that exclusively recognises phosphorylation of Akt/PKB at the serine residue 473 (p-Akt473), activation of Akt following chemokine stimulation was measured in T cells from WT and CD31-/- mice by flow cytometric analysis (Figure 67).

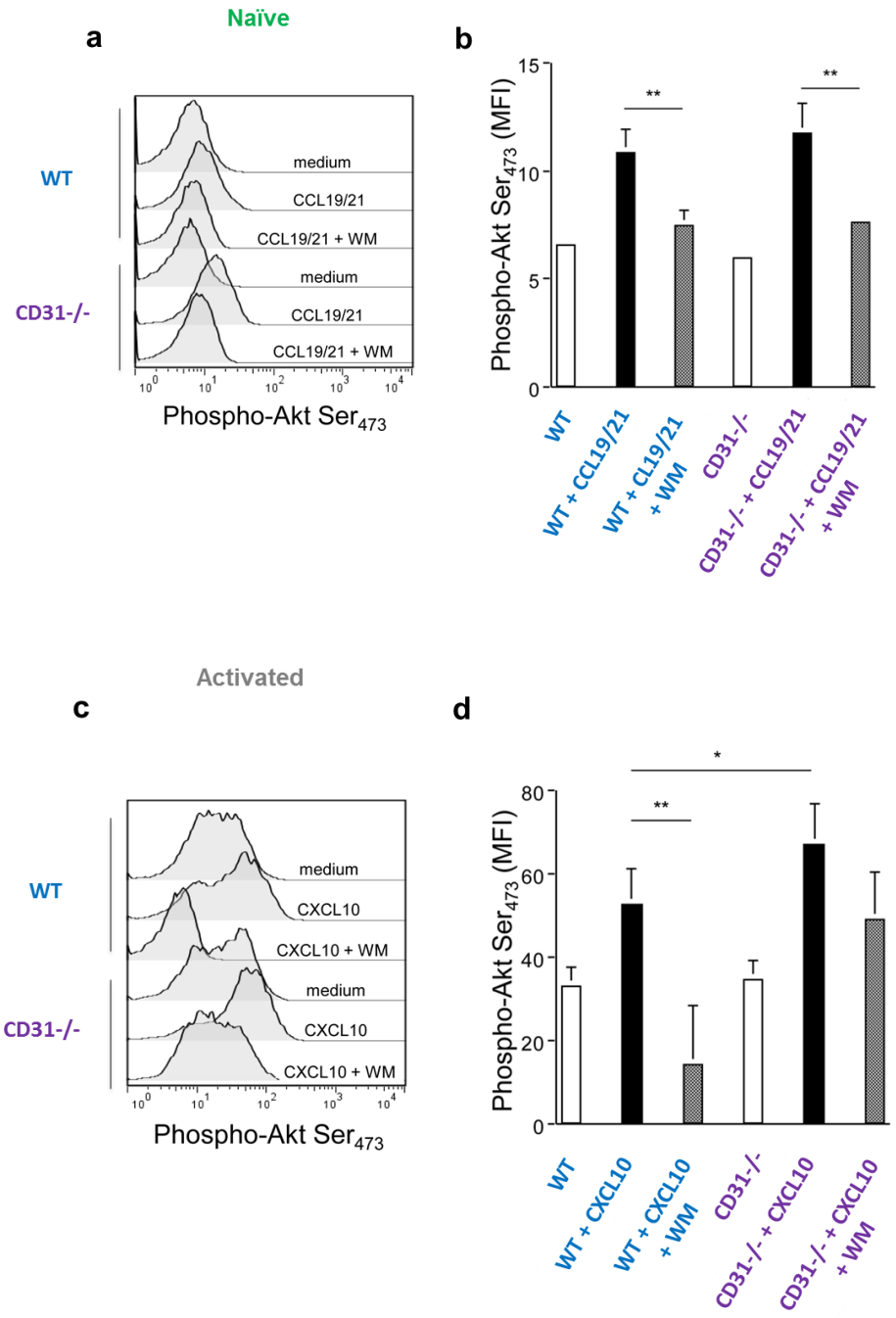



**Figure 67 CD31 inhibits chemokine-induced Akt phosphorylation in activated T cells.**

*Naïve and activated T cells were exposed to CCL19/21 and CXCL10, respectively, for 2 minutes. Phosphorylation of Akt at serine 473 was assessed by antibody staining and flow cytometry. In panels **a** and **c**, representative histograms of the experimental conditions indicated beside each profile are shown. Panel **b** and **d** indicate cumulative data of the mean fluorescence intensity (MFI) indicative of Akt phosphorylation obtained in the various conditions indicated in at least 4 independent experiments of identical design.(n=3,N=4). Statistical significance was calculated with unpaired Student's t-test. *p<0.05 **p<0.02.*

Phospho-Akt (S473) levels in naïve T cells stimulated with CCL19/21 chemokines from WT and CD31-/- were comparable. In contrast, phospho-Akt (S473) levels were significantly higher in activated CD31-/- T cells exposed to CXCL10 chemokine as compared to their WT counterparts. In addition, phospho-Akt (S473) levels in activated CD31-/- T cells exposed to CXCL10 chemokine remained significantly higher than in the activated WT T cells following pre-treatment with a suboptimal dose of the PI3K inhibitor Wortmannin. The data was further supported by the observation that the same suboptimal dose of Wortmannin significantly inhibited the migration of activated WT T cells to CXCL10 in *in vitro* chemokinesis assays, while CD31-/- T cells were inhibited significantly less. Phospho-Akt (S473) levels in naïve T cells from both CD31-/- and WT mice exposed to CCL19/21 were inhibited by Wortmannin equally. Similarly, chemokinesis of naïve T cells to CCL19/21 following Wortmannin treatment was slightly and comparably inhibited in WT and CD31-/- T cells (Figure 68). These results are consistent with previous findings, which indicate naïve T cell homing and migration to secondary lymphoid tissue to be relatively independent of PI3K signalling and largely mediated by activation of DOCK2 (dedicator of cytokinesis 2) (C. Nombela-Arrieta et al. 2004). Overall, these data show that CD31 signals attenuate chemokine-induced signalling by interfering with Akt phosphorylation in activated T cells.

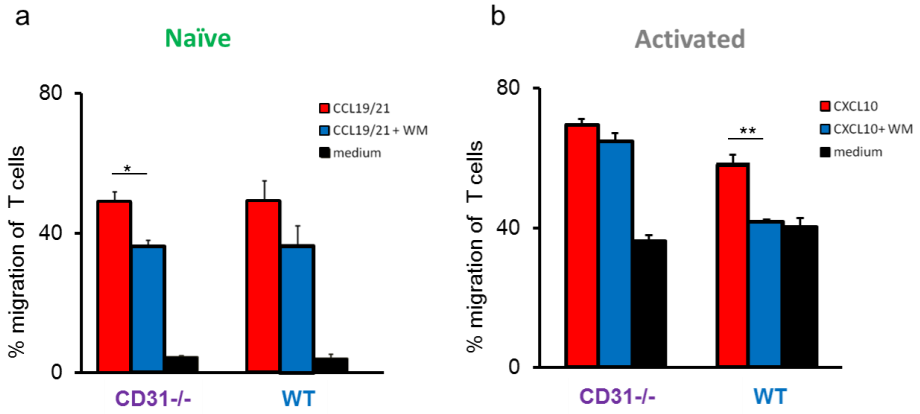



**Figure 68 CD31-deficient activated T cell chemokinesis is partially resistant to PI3K inhibition.**

*Naïve (**a**) and activated (**b**) WT and CD31−/−T cell migration in response to either CCL19/21 (naïve) or CXCL10 (activated) was assessed using a transwell-based chemokinesis assays. Some T cells were pre-incubated with the PI3K inhibitor Wortmannin (10 µM) for 30 minutes at RT. The number of migrated cells in the bottom chamber was determined through hemocytometric counting of each of the triplicate wells after 6Hrs. Results are expressed as a percentage. (n=3, N=3) Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test.**p<0.01, *p<0.05*

#### 4.3.3.4 Differential cellular segregation of CD31 molecules in naïve and activated T cells.

To further investigate the molecular basis of the different effects of CD31-mediated regulation of chemokine-induced signals in naïve and memory T cells, we analysed the segregation of the CD31 molecules within these cell types using confocal microscopy. In these experiments, naïve and antibody activated T cells from WT mice were stained for CD31 along with LFA-1, a surface integrin known to be expressed at low levels and homogenously dispersed on the membrane in naïve T cells and upregulated and clustered in activated T cells. Using confocal microscopy, naïve T cells exhibited uniform distribution of CD31 on the membrane surface (Figure 69). In contrast, in activated T cells the CD31 molecules, albeit downregulated, appeared to be present predominantly in large clusters.

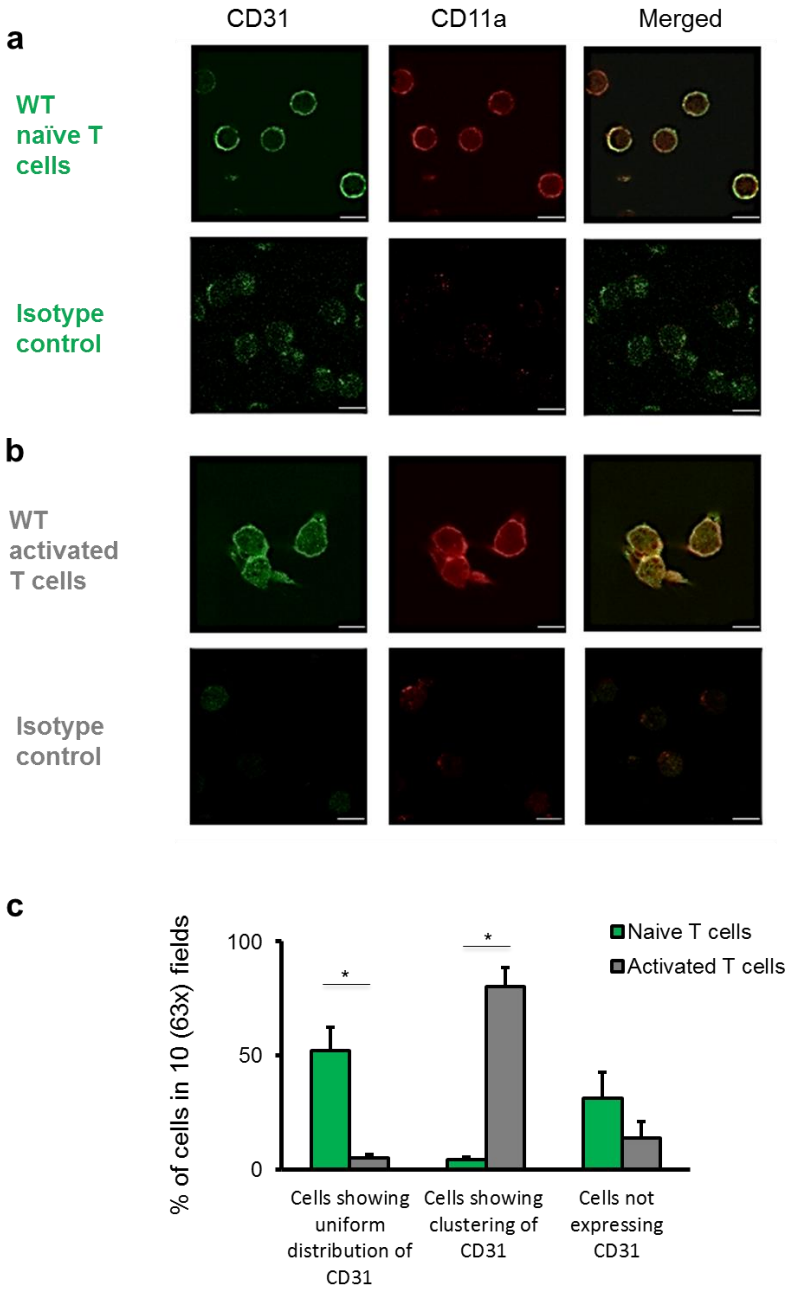



**Figure 69 CD31 molecules segregate differently in naïve and activated T lymphocytes.**

*Confocal images of naive WT T cells stained with rabbit-anti-mouse CD31 (green fluorescence) and rat anti- mouse LFA-1 (red fluorescence) followed by incubation with secondary antibodies Alexa Fluor 488-conjugated donkey anti-rabbit IgG and Alexa Fluor 647-conjugated goat anti-rat IgG, respectively, are shown in panel **a**. Added scale bar = 6 µm. Panel **b**: WT activated T cells generated via anti-CD3 and anti-CD28 treatment over 7 days were allowed to rest for 24 hours in low serum and then fixed. LFA-1 and CD31 expression was visualized as described above. Scale bar = 6 µm. The average CD31 distribution/expression from at least four 63× magnified fields obtained in three independent experiments of identical design is shown in panel **c** (n=4, N=3) Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. *p<0.001*

Confocal microscopy analysis of CD31 molecules on activated T cells migrating in response to CXCL10 chemokine revealed CD31 aggregation towards the polarised leading edge (lamellipodium) of the activated T cell (Figure 70). Chemokine receptors have previously been shown to segregate towards the lamellipodia of the migrating T cells. Thus, confocal microscopic analysis suggests that CD31 molecules in activated T cells are ideally segregated for interfering with chemokine receptor signalling. In this context, co-localisation of CD31 molecules and phosphatases to the leading edge in migrating granulocytes has been previously reported (Y. Wu et al. 2005).

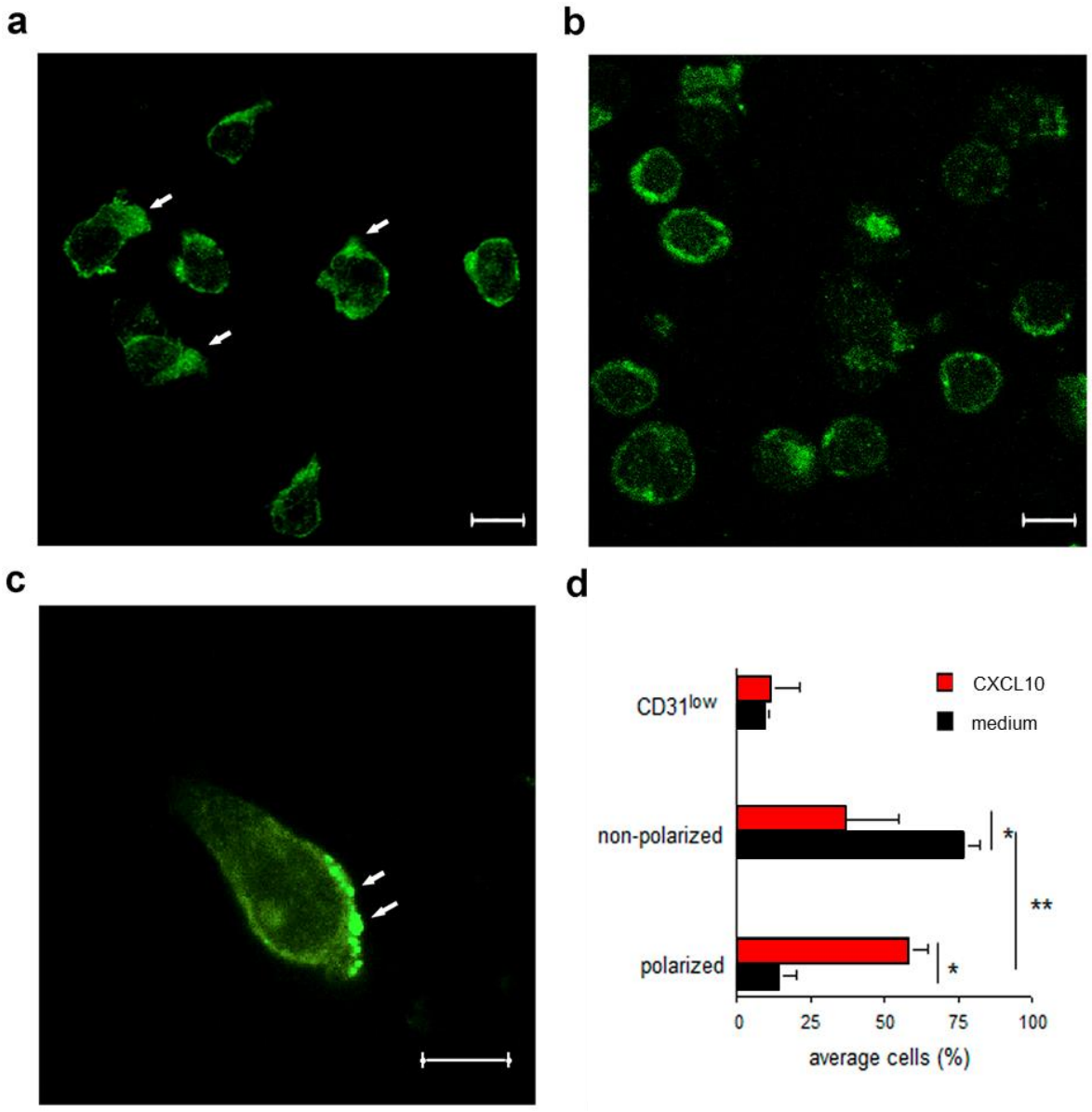



**Figure 70 Polarization of CD31 molecules in activated T cells following exposure to chemokines.**

*Activated WT T cells were allowed to migrate through transwells in response to the chemokine CXCL-10 (**a**) or incubated in medium alone (**b**) and then fixed for analysis. Cells were not permeabilised to allow surface staining only. Confocal images of T cells stained with rabbit-anti-mouse CD31 followed by incubation with Alexa Fluor 488-conjugated donkey anti-rabbit IgG are shown in panels **a** and **b**. A higher magnification of a migrating lymphocyte further depicting CD31 molecule polarization is shown in panel **c**. Confocal z stacks series were acquired using a step size of 0.5 µm. Added scale bar = 6 µm. The average CD31 distribution/expression from at least four 63× magnified fields obtained in three independent experiments of identical design is shown in panel **d** (n=4, N=3). Error bars represent standard deviations.Statistical significance was calculated with unpaired Student's t-test. *p<0.001*

#### 4.3.3.5 Mechanisms of CD31 Triggering During Chemokinesis

During trans-endothelial migration homophilic interactions most likely occur between CD31 molecules expressed on EC and T cells. However such cell:cell contact is unlikely to take place during migration through a transwell. Thus the observations described above do not explain the mechanism of CD31 triggering during chemokinesis. Previously, dynamic CD31 cis-membrane interactions (i.e. clustering within the same cell membrane, homophilic) have been described to induce signalling events in CD31-transfected human embryonic kidney and erythroleukemia cells (T. Zhao and Newman 2001). We hypothesised that CD31 molecules segregating in compact clusters on the memory T cell surface might elicit similar effects.

We therefore sought to investigate whether interfering with this molecular segregation could enhance T cells response to chemokines in the absence of intercellular CD31 engagement. As the CD31 domain responsible for cis-interactions has not been identified, we decided to disrupt CD31 oligomerization by steric interference. WT naïve and activated T cells were 'rested' in serum-free medium overnight and then treated with an anti-CD31 antibody known to inhibit CD31 homophilic interactions without inducing signalling, in order to prevent cis-membrane re-clustering. An isotype-matched antibody was used as a control. Naïve and activated T cells were then exposed to CCL19/21 and CXCL10, respectively, in a transwell-based chemokinesis assay. Anti-CD31-treated activated T cells displayed significantly enhanced chemokinesis, while antibody pre-treatment did not affect migration of naïve T cells. Together with the earlier observation that CD31 molecules segregate in compact clusters on activated T cell surface these data are consistent with the hypothesis that CD31 interactions on the same cell membrane are required to inhibit chemokinesis (Figure 71).

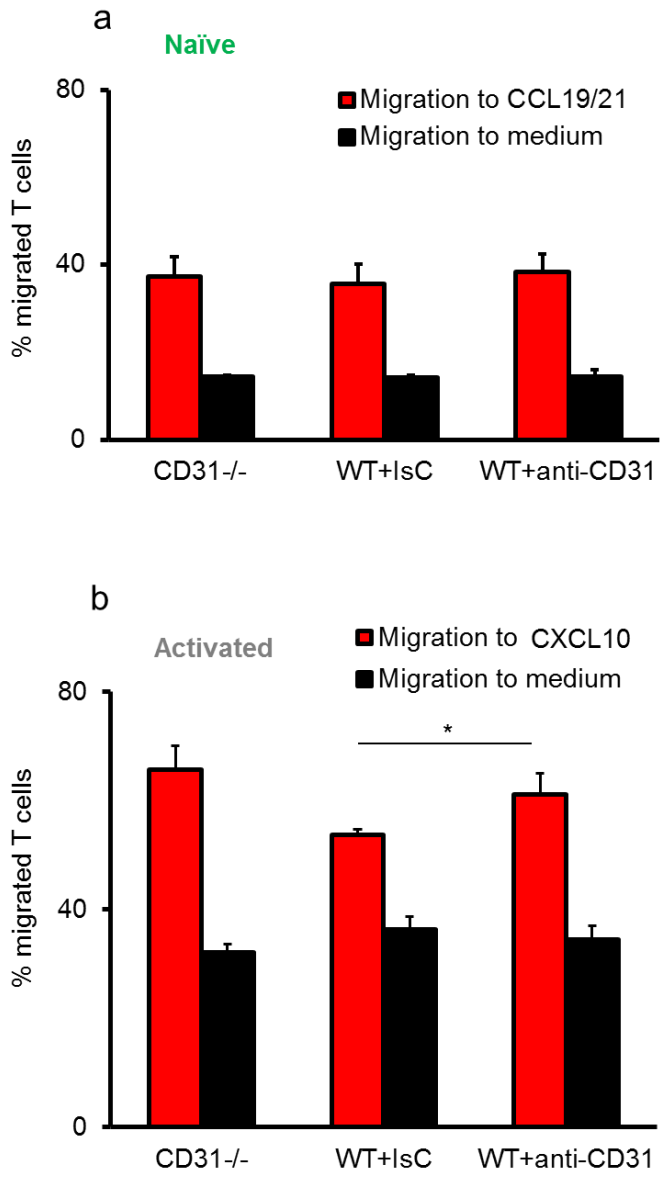



**Figure 71 Antibody-mediated CD31 'immobilization' enhances activated T cell chemokinesis.**

*Naïve (**a**) and activated (**b**) WT T cells were incubated overnight in RPMI 0.5% FCS. Some T cells were pre-incubated with an anti-CD31 mAb at saturating concentrations (5 µg/ml) for 30 minutes at RT. Migration in response to CXCL10 or medium through a transwell was assessed over 6 hours. Percentage migration was calculated by dividing the number of cells harvested from the bottom chamber following 6 hours incubation at 37°C by the original number of cells plated onto the transwell. The average percentage migration from three independent experiments is shown. Error bars represent standard deviations. Statistical significance was calculated with unpaired Student's t-test. *p<0.05, n=4, N=3*

### 4.3.4 Discussion

In this part of the study, we show that the Ig like CD31 receptor expressed on T cells attenuates T cell chemokinesis, a previously unknown function for this receptor. Furthermore, we show that CD31-mediated regulation of T cell chemokinesis is restricted to primed T cells where this molecule segregates in membrane clusters rather than in naïve (unprimed) T cells where CD31 is distributed homogenously on the membrane surface.

In the previous chapter, we showed that the CD28 and CTLA-4 co-receptor mediated regulation of migration occurs in activated Teffs and Tregs. Here we show that another inhibitory co-receptor which targets TCR signalling can also modulate T cell migration.

The results here indicate that in naïve T cells, despite having higher expression of CD31, the homogenously distribution of this receptor prevents its ability to interfere with chemokine-induced Akt phosphorylation and thus, does not affect T cell responses to the chemokines CCL19 and CCL21 that are expressed in the secondary lymphoid organs. Consistently, naïve T cell migration to secondary lymphoid tissue is known to be largely PI3K-Akt independent and largely mediated via DOCK2 activation (C. Nombela-Arrieta et al. 2004). In line with this observation, naïve T cell numbers and distribution remain unaffected by deletion of CD31.

Following activation, the spatial distribution of the CD31 molecules on primed T cells favours molecular interactions on the same cell membrane. This mechanism induces CD31 signalling in polarised migrating T cells. These observations were supported by the observation that blockade of CD31 interactions in the same membrane prevented modulation of T cell chemokinesis to inflammatory chemokine CXCL10. Previously, such dynamic homophilic cis CD31 interactions have been described to occur in human embryonic kidney and erythroleukemia cells transfected with CD31 encoding vector. However our results do not exclude the possibility of CD31 interaction with other known or unknown ligands on the T cells that could initiate signalling. For example, putative heterotypic ligands for CD31 previously described include the neutrophil specific antigen CD177 (NB1) and the ADP-ribosyl cyclase CD38 (Privratsky et al. 2010). Nonetheless, these results indicate that segregation of CD31 molecules in membrane clustering allows for signalling that modulates primed T cell chemokinesis.

A key feature observed in these results was the increased phosphorylation of the PI3K-Akt pathway and partial resistance to PI3K inhibition in the CD31-deficient primed T cells. As mentioned previously, the CD31 receptor is known to recruit phosphatases via the ITIM domains

located in its cytoplasmic tail (D. K. Newman et al. 2001; Newton-Nash and Newman 1999). Indeed, another phosphatase SHIP-1 was also shown to be recruited through these motifs (Pumphrey et al. 1999). Given that silencing of SHIP-1 has been shown to increase basal phosphorylation of protein kinase B (PKB)/Akt and its substrate GSK3β, as well as an increase in basal levels of polymerized actin following chemokine stimulation of primary activated human T cells (Stephanie J. Harris et al. 2011), it is likely that CD31 mediated recruitment of phosphatases following its engagement is essential for its ability to modulate chemokinesis.

In a physiological context, the distinct effects of CD31 signalling on naïve and activated T cell responsiveness to chemokines are likely to be related to the differences in their trafficking and proliferative potential. In naïve T cells, trafficking to the chemokines CCL19 and CCL21 expressed constitutively in the SLO facilitates their continuous circulation through the secondary lymphoid tissue (Marelli-Berg et al. 2008). Here, the trafficking is uncoupled with any inflammation and thus remains unregulated by means of CD31 signalling. Once naïve T cells encounter antigen presenting DCs, CD31- mediated interactions induce signalling that prevents excessive expansion of T cells following activation. Antigen stimulation then initiates reprogramming towards memory T cells that respond towards inflammation induced chemokines such as CXCL10 enabling them to access non lymphoid tissue where they engage in the immune response (Marelli-Berg et al. 2008). CD31 signalling here may prevent excessive infiltration of the memory T cells through modulation of their chemotactic responses thereby reducing tissue damage associated with excessive T cell infiltration.

Together with previous findings indicating its role as a regulator of T cell activation and survival following priming, CD31-mediated regulation of memory T cell chemokinesis defines this molecule as a unique multifunctional immunoregulatory receptor.

# Chapter 5 Discussion

To establish an effective immune response to an invading pathogen, the immune system must be able to specifically recognize and mount a response to it, while simultaneously limiting or preventing any non-specific responses that may be directed towards self-antigens. To achieve this, the adaptive immune system employs a series of carefully regulated processes that enable the selective development and maturation of only those lymphocytes that can recognize foreign antigens in the thymus (central tolerance). However, some lymphocytes with self-antigen specificity escape these selective processes and other measures to limit responses to self-antigens are required (peripheral tolerance). Naturally occurring regulatory T cells (referred to hereafter as Tregs), provide a key contribution in the maintenance of peripheral tolerance. Removal of deletion of this subset from the T cell pool results in autoimmune and inflammatory conditions occurring through unchecked self-reactivity thus highlighting their pivotal role in maintaining a state of tolerance to self-antigens (Sakaguchi 2005). Treg function involves their localization to both the site of T cell priming (draining LNs) (Ding et al. 2012) and the antigen-rich non-lymphoid tissue, site of the effector T cell response (Nguyen et al. 2007; Rudensky and Campbell 2006).

Treg trafficking to these sites is a key event that has only partially been investigated.
In my project, I have shown that the TCR-mediated regulation of Treg migration is similar to that of conventional T cells. This suggests that both T cell subsets are susceptible to this mechanism. However, constitutive expression of CTLA-4 by Tregs allows for CTLA-4-mediated inhibition of CD28 signalling against CD28 to impact their migratory ability differently than that of conventional T cells. Therefore, the differential expression of CD28 and CTLA-4 receptors might be partially responsible for endowing Tregs with their distinctive migratory patterns.

# 5.1 Recognition of antigen-presenting endothelium promotes Treg cell recruitment and circulation

In order for the immune system to engage a specific response to antigen, antigen specific T cell populations must be able to effectively migrate and locate to the tissues where the antigen is localised. Previously, reports have highlighted that although CD4+ T cells recruitment is largely regulated by Ag-nonspecific mechanisms, such as via the action of homing and chemokine receptors, Ag display by EC can facilitate this process (Marelli-Berg et al. 1999b; Marelli-Berg et al. 2001a; Marelli-Berg et al. 2004b). Additionally, the display of antigenic peptides from the underlying tissue promotes the selective recruitment of antigen-specific T cells (Marelli-Berg and Jarmin 2004a). In Part 1 of Chapter 4 we tested whether cognate recognition of the endothelium could also enable recruitment of naturally occurring regulatory CD4 T cell populations. Our results show that, due to their weak affinities towards self-antigens, regulatory T cells are endowed with the ability to be recruited to non-lymphoid inflammatory tissues upon upregulation of MHC class II molecules by the endothelium and through the cognate recognition of self-derived peptides. This effect underpins their timely and efficient recruitment to establish an optimal Treg:Teff ratio required for an effective immune response while preventing any non-specific tissue damage. Thus, these results highlight the role of the endothelium as an important element in immune homeostasis.

It is important here to point out that, even in the absence of any inflammation, Tregs are still recruited to the non-lymphoid tissue (Sather et al. 2007). However their numbers are significantly increased upon challenges such as autoimmunity, infection or even injury.

The study by (Sather et al. 2007) previously shown that Tregs can be found distributed throughout non-lymphoid tissues even during steady state (i.e absence of any overt inflammatory response). Furthermore, the study by (Rosenblum et al. 2011) and (Malchow et al. 2013) indicated that Tregs in these tissues are primed to attenuate subsequent autoimmune reactions in response to self-antigens. These ‘tissue resident’ Tregs may be derived partly from nTregs that are constantly circulating between lymphoid and non-lymphoid tissue, a process carefully regulated in part by the endothelium lining the microvasculature of both sites. However, it is also important to remember that ‘tissue residents’ Tregs are likely to include iTregs, the migration of which may be distinct from that of nTregs and similar to that of Teffs. Furthermore, iTregs may also have variable CTLA-4 expression, the impact of which may reflect differences in their migration.

Together with previous findings, the results presented in my thesis also highlight a mechanism whereby the endothelium at the lymphoid tissue sites facilitates the redistribution of the recirculating Tregs towards the non-lymphoid tissue.

## 5.2 CTLA-4 signalling inhibits CD28-induced migration of Tregs

How Tregs switch from their constant recirculation to become permanent tissue-resident Tregs remains an unexplored aspect of Treg biology. It is known that, upon activation, Tregs upregulate tissue selective integrins enabling them to be retained in peripheral tissues. For example, activated Tregs upregulate $\alpha_E\beta_7$ which interacts with E-cadherin expressed on tissue epithelial cells facilitating their localisation within peripheral tissue sites (Lehmann et al. 2002). Our results are consistent with an additional antigen-specific mechanism for Treg tissue retention involving the interaction of Treg CTLA-4 molecules with B7 ligands expressed by tissue-resident APCs. Although, the levels of CTLA-4 expression on Tregs in tissues have never been assessed, it is well know that Tregs need to be activated to enter non-lymphoid tissue (Corthay 2009). Thus, we hypothesize that Treg CTLA-4 upregulation following activation facilitates their retention within the peripheral non-lymphoid tissue.

In Chapter 5, we also show that CD28-induced antigen-independent migration in Tregs could be inhibited through signalling via the constitutively expressed CTLA-4 receptor in this subset. In light of these results, it is important to envisage where such inhibitory signalling would occur in physiological settings. Although constitutively expressed, CTLA-4 is rapidly upregulated on Treg surface following TCR induced activation, just as it happens in conventional T cells (Mead 2005). Thus, Tregs can receive two distinct levels of CTLA-4 signalling; one high and one low depending upon the level of expression of the receptor on the surface. These differential levels of expression could also result in the differences that are observed between 'tissue resident' and 'actively circulating' Tregs.

While actively circulating back and forth between lymphoid and non-lymphoid tissue, CD28-induced pro migratory signals would enable and enhance Treg migration. As such, lower levels of CTLA-4 would allow CD28-induced signalling to remain uninhibited thus favouring motility. Following activation and proliferation, Tregs display higher levels of CTLA-4. Here, the strong CTLA-4 signals induced by its ligands through tissue APCs would prevent CD28 induced motility. This would prevent Tregs from re-entering the circulation ensuring that the cells are retained within the local microenvironment. Retention through this mechanism could also explain the maintenance of continuous tolerance that is achieved by permanently residing Tregs in the non-lymphoid tissue.

It has recently been argued that CTLA-4 signalling is relatively modest and therefore unlikely to be the main mechanism of its inhibitory function (Walker and Sansom). As mentioned previously, the mechanism through which CTLA-4 brings forth its inhibitory signalling continues to be a controversial topic and therefore highlights the conflicting nature of the literature. Recently, the study by (Wakamatsu et al. 2013) noticed very little change in gene expression of Tregs following CTLA-4 stimulation, lasting for only a few hours. Similarly, comparative analysis of gene expression profiling of CTLA-4 deficient and WT T cells were described as 'minimal' in the study by (Friedline 2009) 2009. Another independent study by (Corse and Allison 2012) noted two-fold level of difference in only 10 independent genes when comparing CTLA-4 expressing cells with CTLA-4 deficient T cells leading the authors to claim 'no obvious signature of active negative regulation' in CTLA-4-bearing T cells (Walker and Sansom 2015).

In our opinion, the studies showing little change in gene expression did not clearly discriminate between Tregs that display high and low CTLA-4 surface levels. It is likely that these observations were affected by the presence of CTLA-4$^{low}$ Tregs, which are the largest Treg population in steady state conditions. Furthermore, these studies do not totally account for the inherent differences of CTLA-4 signalling between Tcon and Tregs. Recently, the study from (Stumpf et al. 2014) has shown that the onset of EAE is similar in mice bearing a point mutation in the cytoplasmic YVKM motif of CTLA-4, where the tyrosine residue at position 201 was replaced with valine (Y201V) to prevent phosphorylation, and wild type littermates. However, these mice developed more severe EAE as a direct result of impaired Treg suppressive activity in these mice. This observation supports the notion that Tregs are distinct from Tcon in their ability to be susceptible to inhibitory CTLA-4 signalling and is in support of our findings which show that CTLA-4 inhibitory signalling is indeed operational in Tregs.

Of note, to ensure CTLA-4-induced signalling in our study, we have utilized the same anti-CTLA-4 antibody that has been extensively studied and also reported in the studies by (Schneider et al. 2005) and (Wells et al. 2001) to initiate signalling upon crosslinking.

## 5.3 Treg metabolism and migration

One of the hallmark metabolic feature of Tregs, that sets them apart from Teffs, is their predominant utilization of the energy efficient metabolic pathways such as the fatty acid oxidation (FAO) and the AMPK pathways rather than aerobic glycolysis (Michalek et al. 2011c). Classically, studies have identified this T cell subset to metabolically resemble CD8 memory T cells more than Teffs (K. Araki et al. 2010; Chi 2012; Peter et al. 2010; van der Windt et al. 2012). However, our results suggest that Tregs meet their energy requirements for migration through glycolysis rather than FAO. This aspect of Treg motility has been previously overlooked and is therefore a novel finding.

Interestingly, while observing regulation of migration of Tregs via the TCR and all of the co-stimulatory receptors in this study, we have consistently identified a non-redundant role for the PI3K-Akt signalling pathway, which is crucial for both migration and metabolism. In terms of CD28 induced migration, recruitment of PI3 Kinase via the cytoplasmic tail is a crucial component in the regulation of migration of Tregs by this co-receptor. In addition, our results also indicate that CD28 induced PI3K-Akt signalling is necessary for glycolysis. In light of these novel findings, we can review earlier studies that have investigated PI3K kinase signalling initiated by CD28 from a different perspective.

For example, the study by (Okkenhaug et al. 2001) showed that the $CD28^{Y170F}$ mutant T cells that are unable to recruit PI3K were still capable of maintaining IL-2 production and proliferated to levels comparable to WT T cells. However, these T cells were prone to survival defects that correlated with reduced upregulation of pro-survival protein BCL-xl from the BCL-2 family of proteins. Recently, (Vander Heiden et al. 2002), et al have identified BCL-xl to facilitate the switch from glycolytic to oxidative mitochondrial metabolism in mammalian cells. Thus, it is possible that the defective survival observed in $CD28^{Y170F}$ T cells may have been associated with disengagement or dysregulation of mitochondrial metabolism. Indeed, PI3K induced activation of both BCL- xl and Akt are described to maintain survival through independent mechanisms (Plas et al. 2001). While Akt-mediated survival is dependent on promoting glycolysis and maintaining a physiologic mitochondrial potential, BCL- xl maintains mitochondrial integrity in the face of reduced mitochondrial potential that develops as the cells exhibit low glycolytic rates under conditions of growth factor deprivation (Plas et al. 2001). Together, these results suggest that poor survival of $CD28^{Y170F}$ T cells may be due to inability to maintain mitochondrial metabolism, on account of

their failure to upregulate BCL-xl. In contrast, their proliferation, which is glycolysis-dependent, remains unaffected as TCR- induced PI3K-akt signalling would be sufficient for glycolysis induction.

An interesting observation in our results was the utilization of the Hexokinase IV (Glucokinase or GCK) isoenzyme of the hexokinase family, rather than the more conventional hexokinase I, in pathways that regulate Treg motility. CD28-induced Treg motility and CTLA-4 mediated inhibition of CD28 signals in Treg migration directly correlated with the upregulation and downregulation of the GCK enzyme respectively. This selective utilization of GCK in Tregs is an interesting finding that could potentially reflect their metabolic signature and distribution. Very little is known with regard to GCK and T cell metabolism. Unlike other hexokinases, GCK is less susceptible to inhibition by the end products of glycolysis (Bell et al. 2002; Matschinsky 1996). Thus, the utilization of this enzyme may give Tregs an advantage over Tconv cells to maintain glycolysis and motility in environments where excessive proliferation and recruitment of Tconv has produced higher quantities of glycolytic end products, which are known to inhibit Tconv (R. Haas et al. 2015). In such environments, migration of Tconv - which utilize other hexokinase isoenzymes - would be inhibited while Tregs would continue to migrate. This may act as a safeguard to prevent excessive accumulation of Tregs in the tissue thus avoiding unchecked immunosuppression. The fewer number of Tregs could then continue to recirculate within the non-lymphoid tissue reaching several different sites that require suppression. This is supported by the observations by (R. Haas et al. 2015) who have demonstrated that conventional T cells are actively inhibited in migration by glycolysis products such as lactate while Treg migration remains unaffected. Furthermore, it is likely that within these environments, the suppressive function and proliferation of Tregs would remain intact as these are fuelled primarily through fatty acid oxidation.

Interestingly, in a recent study by (Hagiwara et al. 2012), the mTORC2 complex has been shown to activate GCK and Akt phosphorylation at Ser473 thereby inducing glycolysis in hepatocytes. Given that our results indicated CD28-induced Treg migration to be relatively independent of the mTORC1 and largely dependent on GCK, perhaps Tregs might be actively utilizing mTORC2 signalling for migration while restricting mTORC1 signalling for proliferative and functional fitness. This aspect needs further exploration.

Our results also open up the possibility that the GCK enzyme in Tregs may regulate Treg migration through actively integrating with cytoskeletal components. In cancer cells, higher glycolytic activity has been shown to increase cell motility and cytoskeletal remodelling (Shiraishi et al. 2015). In addition, in MSV-MDCK-INV tumor cells, the formation of targeted membrane extensions or protrusions has been shown to involve the translocation of glycolytic enzymes to

the Pseudopod (Jia et al. 2005). In endothelial cells, glycolysis has been observed to be the central metabolic pathway that assists in the coordinated motility associated with vascular vessel sprouting and angiogenesis (De Bock et al. 2013). It is possible that translocation of active glycolytic enzymes, such as GCK in Tregs, to membrane protrusion sites is necessary to facilitate cytoskeletal reorganisation for Treg motility (Murata et al. 1997; Toyoda et al. 1994).

The actin binding protein cofilin has been previously shown to associate with the glycolysis enzyme Triose-phosphate Isomerase (TPI) to increase activity of the Na,K-ATPase in HeLa and rat skeletal muscle cells through the action of Rho signalling pathways (J. Jung et al. 2002). Cofilin is a key regulator of actin dynamics that is important for T cell migration. A similar kind of association between potential actin regulators and GCK in T cells could provide the direct link between GCK activity and actin reorganisation that facilitates Treg migration (Samstag et al. 2013). Translocation of the GCK enzyme to membrane sites has not been described in T cells. However, hexokinase translocation from cytosol to periphery for migration has been described in neutrophils following activation. In rat hepatocytes, rapid translocation of GCK from the nucleus to cytosol actin filaments in the cytoplasm has been described previously to provide a source for localised glycolysis-derived energy thought necessary for cytoskeletal affiliated cellular functions.

# 5.4 Clinical implications of this study

## 5.4.1 Manipulation of co-stimulatory molecules

Similar to conventional T cells, Tregs also require TCR and co-stimulatory signalling to become fully active. Although some co-stimulatory pathways are known to differentially regulate Treg and Tconv cells, no single pathway is known to regulate one cell type exclusively (Riley et al. 2009). Thus, administration of agents that can selectively increase Treg function without affecting Tconv activity has proven to be difficult. A typical example is the CD28 superagonist, TGN1412, which was hypothesized to increase Treg activity thus restoring tolerance and improving transplant success (Attarwala 2010). However, healthy volunteers who were administered the TGN1412 in a phase I clinical trial suffered from near fatal multi organ failure associated with massive cytokine storm (Suntharalingam et al. 2006). Although the exact pathogenic mechanism of this event remains unclear, it is possible that Tconv cells here were also activated resulting in abnormally high cytokine production. Thus, a major challenge to the field of immunotherapy is the identification of pathways that are selectively utilised by Tregs and Tconv.

Furthermore, current therapies that provide immunosuppression rely on the administration of relatively non-specific molecular mediators that may broadly affect a variety of cell types (J. I. Duncan 1994; Eynott et al. 2003; Javier et al. 1997). In our study, we have identified metabolic pathways that may be selectively targeted to restrict the function of either Tconv or that of Tregs. In particular, the identification of GCK pathway for Treg migration opens the possibility of pharmacological exploitation that enables selectively targeting of Treg migration and, ultimately, their function.

Additionally, both CTLA-4 and CD28 co-stimulatory pathways are being investigated in clinical trials for the development of several immunotherapies. Fusion proteins of CTLA4 and antibodies (CTLA4-Ig) have recently undergone testing in Phase 2 clinical trials for rheumatoid arthritis (Westhovens et al. 2004). The CTLA-4-Ig drug Belatacept is currently being investigated as an alternative to conventional immunosuppression and has undergone extensive testing in clinical trials of renal transplantation (Kirk et al. 2014). CTLA-4 antagonists such as Ipilimumab are being devised as an effective treatment to increase immune activity to combat a variety of cancers including prostate, lung and skin cancer (Prieto et al. 2012; Singh et al. 2013; Tomasini et al. 2012). However, the effect of targeting these pathways on Treg migration has not been

investigated previously. Our results suggest that these pathways may indeed affect Treg re-circulation and localisation. Therefore, development of new therapies through these pathways should also take into account their effect on Tregs.

Finally, there are broader implications of my observations on the biology of other members of the B7 family of co-stimulatory receptors, such as ICOS and PD1. Targeting additional B7 co-receptor members such as PD-1 may prove to be valuable in improving the efficacy of immunotherapy-based therapeutic strategies. For example, the combined blockade of CTLA-4 and PD-1 was thought to provide more potent anti-tumor activity than by blocking a single co-receptor alone and has revealed promising results in clinical trials for a variety of cancers including melanoma (Callahan et al. 2015). However, the effect of other co-stimulatory receptors and the effect of combining the action of several co-stimulatory receptors on the migration of Tregs has not been studied previously. Further studies are required to address this.

### 5.4.2 Treg metabolism in transplantation and disease

In our experiments, we have highlighted metabolic pathways in Tregs that are likely to regulate their migration in a manner not previously observed in conventional T cells. The GCK induced activity correlated with pro –migratory signals associated with CD28 signalling. Inhibition with CTLA-4 reduced the overall pro-migratory CD28 signalling as well as GCK activity. These results suggest a potential link between regulation of Treg migration and GCK activity. Experimental results from other members of our research team have not identified the involvement of this hexokinase isoenzyme in migration of conventional T cells (Haas and Marelli-Berg, unpublished observations) data not shown). Thus, inhibition of GCK may serve as a valuable tool that could potentially and exclusively disengage Tregs from establishing pro-migratory signals. This might be useful in preventing Treg exit from graft sites while ensuring their retention within the grafts to achieve long-term immune tolerance.

Previously, T cell energy metabolism has been described as a crucial regulator of T cell fate differentiation in transplantation (Chi 2012; Priyadharshini and Turka 2015). For several years, the mTOR inhibitor rapamycin has been utilized to provide immunosuppression to favour graft survival in several transplantation settings. Inhibition of mTOR by rapamycin has been shown to induce the development of regulatory T cells while preventing Teff activation. Presently, the modulation of T cell metabolism is currently being investigated as an attractive alternative to

conventional immunosuppression in transplantation (H. Liu et al. 2014). Furthermore, PI-103, a dual inhibitor of Class IA phosphatidylinositide 3-kinase and mTOR is being proposed as the next step in development of novel immunosuppressant strategies for transplantation (Y.-J. Zhang et al. 2011). Interestingly, these strategies target the mTORC1 pathway of the mTOR complex. Our data suggest that Treg migration is independent of mTORC1 activity. It is possible that the mTORC2 pathway may be the crucial pathway involved in regulating Treg migration. Previously, (Hagiwara et al. 2012) have demonstrated that mTORC2 activates glycolysis partly through GCK. Thus, we hypothesize that inhibiting hexokinases such as GCK would prove to be a valuable addition to these immunosuppressive treatments. Administering a regimen of conventional immunosuppressant such as rapamycin followed with GCK hexokinase inhibitors may be a novel and efficient strategy that may not only promote Treg development but also favour Treg retention within the graft sites to rapidly achieve tolerance. The use of selective GCK or mTORC2 inhibitors in transplantation is an unexplored area that needs further investigation. Unfortunately such inhibitors have not yet been developed.

In contrast, the administration of GCK activating agents may promote Treg migration that may have therapeutic potential in the treatment of cancer. Here, facilitation of Treg exit and withdrawal from tumour sites would allow for uninhibited Tcon cell activity favouring stronger tumour clearing responses. In hepatocytes, GCK activity is regulated by the endogenous inhibitor GK regulatory protein (GKRP). Recently, two potent small-molecule GK–GKRP disruptors (AMG-1694 and AMG-3969) have been described as GCK activators for the treatment of type II diabetes mellitus (Lloyd et al. 2013). Thus, these may also be utilized to develop stronger anti-cancer treatments.

### 5.4.3 CD31 as potent inhibitory receptor for T cell migration

Together with previous findings indicating its role as a regulator of T cell activation and survival following priming, CD31 mediated regulation of memory T cell chemokinesis defines this molecule as a unique multifunctional immunoregulatory receptor. In terms of a therapeutic context, the activity of CD31 is open to possibility of pharmacological exploitation for several therapeutic purposes. Earlier, the CD31 derived peptide (residues 551-554) which can bind to truncated CD31 on memory T cells and activate signalling, was shown to inhibit activation of T cells and macrophages and not only delay the onset of atherosclerosis but also reduce its complications such as abdominal aortic aneurysms in mice (Fornasa et al. 2012). In another study, this treatment was effective in preventing lethal GVHD (Y. Chen et al. 1997). Given that in humans $CD31^{low}$ T cells accumulate with age, display enhanced proliferative capacity and are linked with enhanced susceptibility to autoimmune diseases (Gomez et al. 2003; Kilpatrick et al. 2008; Kimmig et al. 2002), the CD31 peptide may serve as an effective therapy in this context.

## 5.5 Future Work

In this section, I briefly discuss potential ideas for the continuation of this study.

First, studies are required to verify the involvement of the GCK hexokinase in the functional fitness of both Treg and Teff subset of T cells. Additionally, these studies need to carefully establish the impact of selective disruption of GCK on the migratory patterns of these T cell subsets. Given the absence of any selective Hexokinase IV (GCK) inhibitor, shRNA knockdown methodology could be employed to selectively disrupt GCK activity. Based on our data, one would predict that Treg migration would be significantly inhibited following GCK knockdown. In contrast, Teffs, which rely heavily on other hexokinases for their migration and function, would not be affected. By carrying out *in vitro* and *in vivo* migration assay experiments of GCK knockdown Tregs and Teffs, we would then be able to specifically address the relative contribution of this hexokinase in the motility of each subset. Conventional suppression assays could be used to identify any significant impairment of function of GCK-knockdown Tregs. Given that Tregs primarily utilize FAO and the AMPK pathway for functional fitness and glycolysis for migration, the effect of GCK knockdown on the Treg suppression is likely to be minimal.

Additionally, selectively disrupting GCK without inhibiting the activity of other hexokinases could minimizing impact of inhibiting other glycolysis activity which might be required for functions other than migration. This possibility could be tested by comparing suppression of GCK knockdown Tregs with that of Tregs treated with a pan-hexokinase inhibitor such as mannoheptulose (Sweet et al. 1996). Very little is known regarding GCK activity in T cells let alone Tregs. Thus, parallel studies observing the knockdown of GCK in Teffs would also be carried out to fully characterise the activity of this hexokinase in Teff function.

Second, our results suggest that Treg migration is largely independent of mTORC1 activity. As mentioned previously, GCK activity is induced by mTORC2 and Akt activity in hepatocytes (Hagiwara et al. 2012). As a consequence, Treg migration could be likely regulated by mTORC2- rather than mTORC1-dependent pathways. This can be addressed by selective genetic deletion (either by shRNA knockdown or Treg-specific knockout mice) of the Rictor or Raptor component proteins (from the mTORC2 and mTORC1 complexes respectively) in Tregs and compare their function and migration.

Lastly, a direct association between GCK activity and migration of Tregs needs to be addressed. For this, confocal microscope analysis and immunoprecipitation experiments may indicate the localisation of GCK with components of the cytoskeleton such as actin in Tregs following migration-inducing signals such as those provided by CD28. These experiments would further the evidence of the involvement of this hexokinase in the regulation of Treg motility.

Our results have described CTLA-4 as a crucial inhibitor of Treg migratory responses that facilitates their retention in tissue sites. To verify this hypothesis and to confirm the presence of active inhibitory signalling from the CTLA-4 receptor, we would employ the use of Tregs bearing the point mutation at position 201 in the intracellular YVKM motif that has been described previously (Stumpf et al. 2014). The YVKM motif is believed to be the key motif involved in the recruitment of phosphatases and other signalling proteins. The point mutation (replacing tyrosine with valine) in this motif renders it unable to undergo phosphorylation thus disengaging recruitment of the signalling proteins (Stumpf et al. 2014). Thus, we hypothesize that Tregs bearing this point mutation in the cytoplasmic domain would be less efficient at promoting Treg tissue localisation and, as a consequence, their function.

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